Myron Christodoulides *Editor*

Vaccines for Neglected Pathogens: Strategies, Achievements and Challenges

Focus on Leprosy, Leishmaniasis, Melioidosis and Tuberculosis

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Springer

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ISBN 978-3-031-24354-7 ISBN 978-3-031-24355-4 (eBook)
https://doi.org/10.1007/978-3-031-24355-4

The Jenner Institute

This Springer imprint is published by the registered company Springer Nature Switzerland AG
The registered company address is: Gewerbestrasse 11, 6330 Cham, Switzerland

Contents

Chapter 1
Introduction

Myron Christodoulides

1.1 Neglected Tropical Diseases and the VALIDATE Pathogens

Neglected Tropical Diseases (NTDs) are caused by a diversity of pathogens including viruses, bacteria, parasites, fungi, and toxins, which affect 2–3 billion people globally who live in the least developed countries (LDC) and low-to-middle income countries (LMIC). The World Health Organization classifies around 24 NTDs (Table 1.1) that are prevalent mainly in tropical and sub-tropical areas and these conditions impact enormously on personal and population health, with debilitating social and economic consequences to communities and countries. The WHO has launched a roadmap for NTDs entitled 'Ending the neglect to attain the Sustainable Development Goals: a road map for neglected tropical diseases 2021–2030', with relevant documents available at https://www.who.int/teams/control-of-neglected-tropical-diseases/ending-ntds-together-towards-2030.

Despite the global efforts to understand and control these NTDs, their burden is still one major factor (mingling amongst the complex inter-related issues of health, social and economic inequalities, poverty, lack of clean water and malnutrition, war and displacement, colonial legacies and tenacious autocratic, oligarchic, totalitarian and/or theocratic forms of government, low educational attainment, high childhood mortality and low life expectancy) that contributes to keeping countries firmly entrenched in the Development Assistance Committee (DAC) list for Official Development Assistance (ODA) (https://www.oecd.org/dac/financing-sustainable-development/development-finance-standards/daclist.htm).

These pathogens, particularly the eukaryotes and prokaryotes, are complex organisms that are difficult to treat and challenging to vaccine developers seeking

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M. Christodoulides (ed.), *Vaccines for Neglected Pathogens: Strategies, Achievements and Challenges*, https://doi.org/10.1007/978-3-031-24355-4_1

Table 1.1 The World Health Organization list of NTDs and their causative agents

Disease (alphabetical order)	Organism	Pathogen-type
Buruli ulcer	*Mycobacterium ulcerans*	Bacterium
Chagas disease	*Trypanosoma cruzi*	Parasite
Chikungunya	Chikungunya virus (CHIKV)	Virus
Chromoblastomycosis	Major: *Fonsecaea pedrosoi; Phialophora verrucose; Cladophialophora carrionii*	Fungi
	Less frequent: *Rhinocladiella aquaspersa; Exophiala dermatitidis*	Fungi
	Others *Exophiala jeanselmei, E. spinifera; Fonsecaea* (*monophora, nubica, pedrosoi*); *Phialophora richardsiae; Catenulostroma chromoblastomycosum; Aureobasidium pullulans; Rhytidhysteron* sp.; *Chaetomium funicola*	Fungi
Deep mycoses	Primary systemic organisms—*Coccidioides immitis; Histoplasma capsulatum; Blastomyces dermatitidis; Paracoccidioides brasiliensis*	Fungi
	Opportunistic pathogens include *Cryptococcus neoformans; Candida* spp.*; Aspergillus* spp.*; Penicillium marneffei;* the Zygomycetes (*Rhizopus, Rhizomucor, Absidia, Mucor); Trichosporon beigelii; Fusarium* spp.	
Dengue	Dengue virus	Virus
Dracunculiasis	*Dracunculus medinensis*	Parasite (Guinea worm)
Echinococcosis	*Echinococcus granulosus* (cystic echinococcosis (CE) = hydatid disease)	Parasite tapeworms
	Echinococcus multilocularis (Alveolar Echinococcosis (AE) disease)	
Foodborne trematodiases	Clonorchiasis—*Clonorchis sinensis*	Parasite worms
	Opisthorchiasis—*Opisthorchis viverrini, O. felineus*	
	Fascioliasis—*Fasciola hepatica, F. gigantica*	
Human African trypanosomiasis (gambiense) ‘sleeping sickness’	*Trypanosoma brucei gambiense*	Parasite
Human African trypanosomiasis (rhodesiense)	*Trypanosoma brucei rhodesiense*	Parasite
Leishmaniasis (cutaneous)	*Leishmania (Viannia) braziliensis, L. mexicana, L. (Leishmania) amazonensis, L. (Viannia) guyanensis* (New World)	Parasite
	L. major, L. aethiopica, L. tropica (Old World)	
	L. (Viannia) panamensis (New World)	

Table 1.1 (continued)

Disease (alphabetical order)	Organism	Pathogen-type
Leishmaniasis (visceral)	*Leishmania donovani* complex, *L. donovani sensu stricto* (East Africa and Indian subcontinent)	Parasite
	L. infantum (Europe, North Africa and South America)	
Leprosy	*Mycobacterium leprae*	Bacteria
Lymphatic filariasis	*Wuchereria bancrofti* (90% of cases); *Brugia malayi, B. timori*	Parasite filarial worms
Fungal mycetoma (eumycetoma)	*Madurella mycetomatis* (most prevalent causative agent worldwide)	Fungi
Bacterial mycetoma (actinomycetoma)	*Actinomadura madurae*, *A. pelletieri; Streptomyces somaliensis; Nocardia brasiliensis, N. asteroides*	Bacterium
Onchocerciasis (river blindness)	*Onchocerca volvulus*	Parasite worm
Rabies	Rabies lyssavirus (RABV)	Virus
Scabies and other ectoparasitoses (burrowing parasites)	*Sarcoptes scabiei var hominis*	Mite
	Ixodes spp.	Tick
	Pediculus spp.	Louse
Schistosomiasis (bilharzia)	*Schistosoma mansoni, S. haematobium, or S. japonicum*	Parasite worms
Soil-transmitted helminthiases—ascariasis, trichuriasis, ancylostomiasis, necatoriasis, strongyloidiasis and toxocariasis	*Ascaris lumbricoides* (roundworms); *Trichocephalus trichiurus* (whipworms); *Toxocara canis* (canine roundworms); *Ancylostoma duodenale* (hookworms); *Strongyloides stercoralis* (threadworms); *Necator americanus* (hookworms)	Parasite worms
Snakebite envenoming	600 venomous snakes globally; 5.4 million snake bites annually; 1.8–2.7 million cases of envenomings; ~80,000–140,000 deaths annually; ~340,000–420,000 amputations and other permanent disabilities annually[a]	Snake
Taeniasis (tapeworm infection)/ cysticercosis (larval cyst)	*Taenia solium*	Parasite pork tapeworm
Trachoma	*Chlamydia trachomatis*	Bacterium
Yaws	*Treponema pallidum pertenue*	Bacterium

Taxonomies drawn from data available in PubChem and NCBI Taxonomy Browser

[a] Data from WHO factsheet (https://www.who.int/news-room/fact-sheets/detail/snakebite-envenoming)

prophylactic solutions. In this book, our focus is the pathogens that are central to the VAccine deveLopment for complex Intracellular negLecteD pAThogEns (VALIDATE) network of global researchers. This network's members seek to develop vaccines against *Mycobacterium leprae* (causing leprosy), *Leishmania* spp. (causing leishmaniasis), *Mycobacterium tuberculosis* (causing tuberculosis) and *Burkholderia pseudomallei* (causing melioidosis). Leprosy and tuberculosis are diseases of antiquity, and one must marvel at the resilience of mycobacterial pathogens. The earliest evidence of human tuberculosis comes from ancient Egypt circa 3700 BC [1, 2] and of animal tuberculosis circa 2000 BC in an Indian elephant [2]. Humans hypothetically obtained *Mycobacteria* from the first domesticated cattle and goat herds, occurring circa 8000–6000 BC in the north-eastern basin of the Mediterranean and the Middle East (today Iraq, Iran, Israel-Palestine, Syria) [2]. And how old is leprosy? Clinical descriptions of leprosy are available from India circa 600 BC and the disease may have been established already in China during the first millennium BC. The earliest osteological evidence of leprosy comes from 200 BC in skeletons of 4 adult males in Ptolemaic Egypt [2, 3]. The history of leishmaniasis is arguably more fascinating from a paleoparasitological perspective, and reviewed exhaustively by Steverding [4]. *Leishmania*-like species have been documented in extinct sand fly species preserved in 20-million- and 100-million-year-old fossil ambers and the genus *Leishmania* is believed to have evolved in the Mesozoic era, 252–66 million years ago. Descriptions of leishmaniasis lesions date back to the 7th Century BC Assyria and remarkably a paleoparasitological study of 42 Egyptian mummies dating from 2050 to 1650 BC found *Leishmania donovani* mitochondrial DNA in 4 specimens, suggesting that visceral leishmaniasis was present in ancient Egypt [5]. The George Ebers Papyrus (https://digi.ub.uni-heidelberg.de/diglit/ebers1875ga), dating from 1555 BC in Egypt, but most certainly reflecting circumstances dating back to 3000 BC [6], also mentions what may be a description of cutaneous leishmaniasis. By comparison, melioidosis [variously called Whitmore's disease, Nightcliff gardener's disease (referring to Nightcliff, a northern suburb of Darwin, Australia, where melioidosis is endemic), pseudoglanders, or the 'Vietnam time bomb' [which refers to American soldiers that had been infected with *B. pseudomallei* during the war and suffered no ill effects at the time, but then developed fatal disease many years later] appears to be a more recent infection, and was first recognised in Rangoon, Myanmar (Burma), in 1911 by Whitmore and Krishnaswami [7, 8]. Its relatively recent appearance is perhaps a consequence of human contact with *B. pseudomallei* contaminated soil and water in environments, recently settled.

Following the WHO's classification, only leishmaniasis and leprosy figure as NTDs, but all four pathogens share a distinguishing feature of an intracellular stage of their life cycle within human cells. A strong argument can be made for categorising melioidosis as a NTD of global importance, since it is difficult to treat and cases number ~165,000 annually with 89,000 deaths (https://www.validate-network.org/pathogens/melioidosis). The WHO defines a NTD as a disease that is 'almost absent from the global health agenda', 'has very limited resources' and is 'overlooked by global funding agencies'—melioidosis satisfies all their criteria.

Analysis of the data within the 2021 G-FINDER Neglected Disease Report (from the global health think tank Policy Cures) Research) (https://www.policycuresresearch.org/about-us/) allows comparison of the expenditure on research and development into these diseases (Table 1.2). Although funding for tuberculosis research has fallen marginally in relative terms over the past few years (down by 33 million US$ from 2019), it was still 684 million US$ in 2020, which is the second most funded disease behind HIV/AIDS (1368 million US$ in 2020) and just ahead of malaria (618 million US$ in 2020). This sum is twice as much as the total funding that all other NTDs receive (328 million US$ in 2020, Table 1.2). Neither the G-FINDER report, which covers funding from basic research right through to post-registration studies of new products, nor a systematic review on NTD funding between 2000 and 2017 [9] have data on the investments made in melioidosis

Table 1.2 Research and development funding into NTDs 2020 (in USD, millions)

		Basic research	Drugs	Vaccines	Biologics	Diagnostics	Microbicides	Vector control products	Unspecified	Total
VALIDATE pathogens	Leishmaniasis	20.72	21.44	2.47	0.03	0.12			0.03	44.81
	Leprosy	6.29	1.13	0.43	0.03	0.21			0.06	8.17
	Melioidosis[a]									
	Tuberculosis	195.51	341.87	72.94	0.33	65.85			7.46	683.97
Kinetoplastid diseases	Chagas' disease	7.99	22.36	2.13	–	2.46		0.02	<0.01	34.95
	Human African trypanosomiasis (sleeping sickness)	18.88	13	0.06	–	0.88		–	–	32.82
	Multiple kinetoplastid diseases	2.32	31.27	–	–	–		0.09	<0.01	33.69
Helminth infections	Schistosomiasis (bilharzia)	10.78	4.4	3.56	–	2.51		0.51	–	21.77
	Onchocerciasis (river blindness)	1.17	10.14	0.71		1.29		0.02	–	13.32
	Lymphatic filariasis (elephantiasis)	7.21	3.15			0.88		0.02	1.18	12.43
	Tapeworm (taeniasis/cysticercosis)	3.68	1.5			1.48		–	–	6.66
	Hookworm (ancylostomiasis and necatoriasis)	2.18	0.78	1.25					–	4.21
	Whipworm (trichuriasis)	2.72	0.3						–	3.02
	Strongyloidiasis and other intestinal roundworms	2.27	0.48	–		0.02			–	2.77
	Roundworm (ascariasis)	1.63	0.15						–	1.78
	Multiple helminth infections	6.64	3.11	–		0.73		–	0.01	10.49
Other NTDs	Buruli ulcer	0.86	1.01	0.02		0.29			0.27	2.46
	Dengue	28.62	23.55		3.94	8.18		10.09	0.78	75.16
	Mycetoma	0.4	0.32			–			–	0.72
	Scabies	0.66	0.21			–			0.31	1.18
	Snakebite envenoming	0.42	7.13		5.92	0.84			0.89	15.2
	Trachoma			1.91		–			–	1.91

Data are drawn from the 'Neglected disease research and development: new perspectives' G-FINDER report (2021)

[a]No data available for melioidosis in this report—no reported funding. Grey shading denotes that category was not included in G-FINDER. Criteria used to classify a NTD for the report were (1) the disease disproportionately affects people in the LDC and LMIC, (2) there is a need for new products, i.e. there is no existing product (vaccine, biological, etc.) or improved and/or additional products are required, and (3) there is a market failure, i.e. insufficient commercial market to attract private industrial investment. Policy Cures Research, G-FINDER data portal, https://gfinderdata.policycuresresearch.org

research and development. A recent review from Savelkoel et al. [10] cites the most recent figure for global investment for melioidosis non-biodefence research and development to have been less than 4 million US$ in 2016 [11], and it is unlikely to be any greater today. Thus, the total funding on the VALIDATE-specific diseases of leishmaniasis, leprosy and melioidosis can be estimated as ~57 million US$ in 2020, which is a mere 8% of the funding that was received for tuberculosis. Thus, tuberculosis certainly is not neglected in terms of funding, and rightly so given the global burden of disease. In 2020, an estimated 5.6 million men, 3.3 million women and 1.1 million children fell ill with tuberculosis, with 1.5 million people dying. Only deaths from COVID-19 surpassed tuberculosis as the leading infectious killer in 2020. However, with the continual rollout of COVID-19 vaccines globally, tuberculosis is set to return as the leading cause of death from any infectious disease, even above HIV/AIDS-related illnesses (680,000 in 2020) (https://repository.gheli.harvard.edu/repository/12559/).

Analysis of funding over the past decade (2011–2020) shows that it has been concentrated largely in tuberculosis research and development (Fig. 1.1, Table 1.3), which accounted for 65% of all funding. This was followed by the kinetoplastid diseases at 15%, with only 1% provided for leprosy, and no data available for melioidosis. What is clear from the data is that funding has been remarkably consistent over the years for each of the disease or disease groups (Fig. 1.1). Analysis of published research into the four VALIDATE NTDs demonstrates that tuberculosis predominates with over 33,000 articles published just in the past decade, followed by

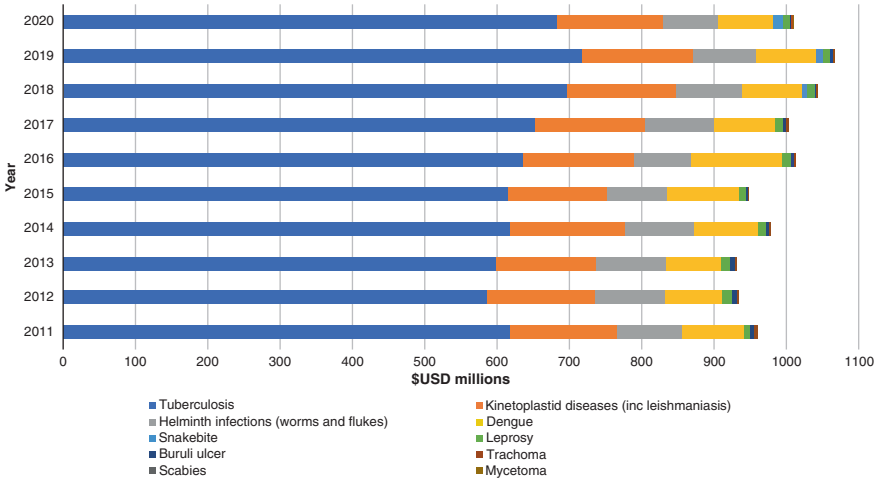

Fig. 1.1 Cumulative spending on NTD research and development for the period 2011–2020. Data are collated from the 'Neglected disease research and development: new perspectives' G-FINDER report (2021). Policy Cures Research, G-FINDER data portal, https://gfinderdata.policycuresresearch.org

Table 1.3 Cumulative funding for NTD research and development from 2011 to 2020

Disease	Funding ($USD millions)	% of total funding
Tuberculosis	6424	64.97
Kinetoplastid diseases (inc leishmaniasis)	1484	15.01
Helminth infections (worms and flukes)	898	9.08
Dengue	872	8.82
Snakebite	34.9	0.35
Leprosy	108	1.09
Buruli ulcer	40.3	0.41
Trachoma	23.2	0.23
Scabies	1.2	0.01
Mycetoma	2.2	0.02
Total	**9887.8**	**100**

Data are drawn from the 'Neglected disease research and development: new perspectives' G-FINDER report (2021). Policy Cures Research, G-FINDER data portal, https://gfinderdata.policycuresresearch.org

Table 1.4 Number of articles in PubMed for each of the VALIDATE NTD pathogens

Pathogen	Search term	Total number of articles and year range	Total number of articles in last decade (2012–2022)
Mycobacterium tuberculosis	*'Mycobacterium tuberculosis'*	85,332 (1925–2022)	33,365
Mycobacterium leprae	*'Mycobacterium leprae'*	7555 (1932–2022)	1449
Mycobacterium ulcerans	*'Mycobacterium ulcerans'*	1344 (1951–2022)	580
Leishmania	*'Leishmania'*	30,101 (1904–2022)	12,245
Burkholderia pseudomallei	*'Burkholderia pseudomallei'*	3234 (1948–2022)	1671
Malaria	*'Malaria'*	106,103 (1828–2022)	41,901
Human immunodeficiency virus	'HIV'	398,629 (1954–2022)	153,353
SARS-CoV2	'COVID'	330,343 (2020–Feb. 2023)	330,343

over 12,000 articles on *Leishmania* (all forms), with less than 2000 articles each for leprosy and melioidosis (Table 1.4). A paltry 1344 articles have been published on *Mycobacterium ulcerans* over the past 70 years (Table 1.4). To put this literature into perspective, in the past decade over 41,000 articles have been published into malaria, and over 153,000 into HIV and of course, these are dwarfed by the output on COVID-19, which stands at an astonishing 330,343 articles published from 2020!

1.2 Book Synopsis

The overall focus of the book is vaccines for NTDs caused by the organisms studied by the VALIDATE network. The book begins with an introductory chapter on the creation of the VALIDATE network and its expansion into a global network of academic and clinical investigators, public health scientists, administrators, and policy makers. Diversity of the network is its strongest feature, and enables scientific collaboration between individuals from LDC, LMIC and developed countries. Indeed, a strength of the book is that many of the chapters are written by researchers from LDC/LMIC countries, where these diseases are endemic.

The introductory chapter is followed by contributions that cover mycobacterial diseases. There are two chapters on leprosy, the first providing a current perspective on Hansen's disease and the second discussing the challenges that *Mycobacterium leprae* presents to vaccine developers. Next, we have chapters that focus on *Mycobacterium tuberculosis* and cover correlates of protection for tuberculosis, animal models, and Bacillus Calmette-Guérin (BCG) vaccine and fermentation strategies for BCG vaccine development. The final chapter in this part focuses on Buruli ulcer, which is caused by *Mycobacterium ulcerans.* Our next section explores leishmaniasis, with chapters that describe the plethora of diseases caused by *Leishmania* spp. and the development of canine *Leishmania* vaccines (CVL) and efforts to develop human *Leishmania* vaccines (HVL). A chapter on the human challenge model for *Leishmania* research explains how studying human infection under controlled conditions provides a meaningful model to test vaccines. The book closes with a chapter on *Burkholderia pseudomallei* (melioidosis) vaccine development.

Acknowledgements This book could not have been possible without the contributions of many: first, I express my gratitude to all authors, all of whom contributed their articles with enthusiasm and showed patience with my editing; to the staff at Springer Publishing for commissioning this book; and to the VALIDATE steering committee for funding publication of this *e-book*, to make the content open access and freely available to scientific communities and the lay public interested in these particular diseases of global importance.

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Chapter 2
The VALIDATE Network: Accelerating Vaccine Development for Tuberculosis, Leishmaniasis, Melioidosis and Leprosy

Samantha Vermaak, Samantha Sampson, and Helen McShane

Abstract Established in June 2017, VALIDATE is an international network of researchers working to accelerate vaccine development for four neglected intracellular pathogens that cause significant mortality and morbidity globally: *Mycobacterium tuberculosis*, *Leishmania* spp., *Burkholderia pseudomallei* and *Mycobacterium leprae*. In 5 years, VALIDATE has grown to have more than 550 members from over 250 institutes across 72 countries and has had several successes and important outputs. This chapter discusses VALIDATE's origins, achievements and future direction.

Keywords Vaccine development · Tuberculosis · Leishmaniasis · Melioidosis Leprosy · NTDs · Research network · Interdisciplinary

2.1 Introduction

VALIDATE ("VAccine deveLopment for complex Intracellular negLecteD pAThogEns") is an international network of researchers working to accelerate vaccine development for four intracellular pathogens—*Mycobacterium tuberculosis* (causing tuberculosis (TB)), *Leishmania* spp. (causing leishmaniasis), *Burkholderia pseudomallei* (causing melioidosis) and *Mycobacterium leprae* (causing leprosy). These pathogens cause significant mortality and morbidity globally, disproportionately affecting low- and middle-income countries (LMICs), and are often neglected due to the poor predicted commercial return on investment for new tools to control these pathogens and marginalisation of affected populations.

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M. Christodoulides (ed.), *Vaccines for Neglected Pathogens: Strategies, Achievements and Challenges*, https://doi.org/10.1007/978-3-031-24355-4_2

The VALIDATE Network was set up in June 2017 in response to a UK Medical Research Council Global Challenges Research Fund grant call for 'Networks to address vaccine R&D challenges primarily relevant to the health or prosperity of Low- and Middle-Income Countries (LMIC)'. The VALIDATE Consortium application, consisting of 32 world-leading researchers from 22 institutes in 8 countries, led by Prof Helen McShane of the University of Oxford, was successfully awarded £1.6M, and VALIDATE was born. Fast-forward 5 years to June 2022, global interest in VALIDATE has grown its membership to more than 550 members from over 250 institutes across 72 countries (44 LMIC) (Fig. 2.1).

The vision for VALIDATE encompasses three main aims:

1. To create an engaged and interactive community.
2. To accelerate vaccine development for our focus neglected pathogens.
3. To help early-career researcher members progress their careers.

VALIDATE addresses these aims by increasing information dissemination across: (1) our focus pathogens; (2) species (vaccine target, reservoir and model species; with a One Health perspective); (3) continents, countries and research groups; and (4) disciplines. Membership is free and open to anyone working on any research area that will feed into accelerating vaccine development for the four focus pathogens, as well as interested members of the public. An underlying principle of VALIDATE is that by working together, we can advance research more quickly and effectively.

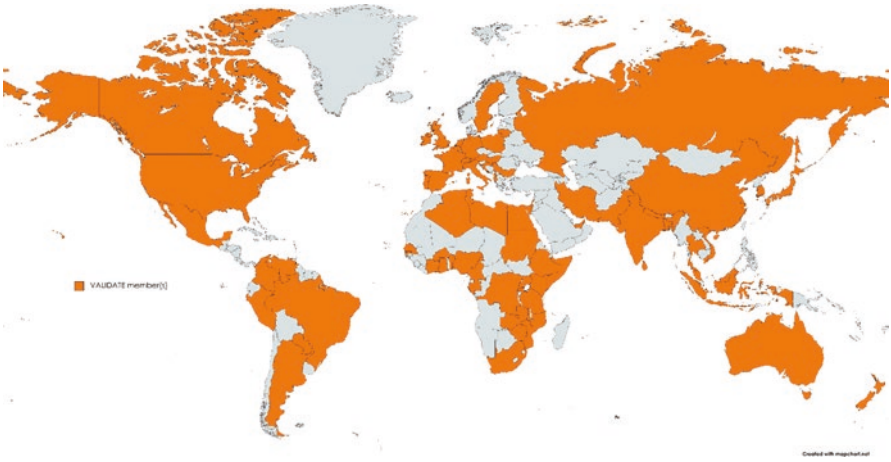

Fig. 2.1 VALIDATE has members based in 72 countries worldwide. (Map created with MapChart (https://www.mapchart.net), which is licensed under a Creative Commons Attribution-ShareAlike 4.0 International License. Creative Commons License (CC BY-SA))

2.2 VALIDATE's Focus Pathogens

VALIDATE provides a unique opportunity to bring together individuals working on four complex neglected intracellular pathogens. These four pathogens were chosen because they share a common lifestyle as pathogens of macrophages, induce similar end-stage pathologies (e.g. granulomatous inflammation, tissue remodelling) and alter host immune and metabolic responses. By exploiting their synergies, similarities and differences, we aim to expedite vaccine development for each pathogen.

2.2.1 Tuberculosis (TB)

TB is primarily a disease of the respiratory system (although it can also affect other parts of the body) caused predominantly by bacteria from the *Mycobacterium tuberculosis* (*M. tb*) complex. It affects humans, cattle and many wildlife species including badgers, white-tailed deer, possums, African buffalo, lions, rhinoceros and elephants. In 2020, TB caused disease in 10 million people, with 1.5 million people killed [1]. One quarter of the world's population is latently infected with TB, with a 10% lifetime risk of progression to active disease. This risk is higher in those with concurrent HIV infection, despite widespread use of antiretroviral therapy. TB is the second leading cause of death from an infectious disease (besides COVID-19; prior to 2020, TB was the leading infectious killer for many years) [1]. The current TB vaccine, Bacillus Calmette-Guérin (BCG), was first developed 100 years ago and has highly variable efficacy, particularly against lung disease in LMICs [2]. Ending the TB epidemic by 2030 is one of the United Nations' Sustainable Development Goals, but while infection rates were dropping gradually year on year (prior to the COVID-19 outbreak, during which they have increased), an efficacious vaccine will be necessary if this goal is to be achieved [3]. Of increasing urgency is the fact that antibiotic-resistant TB strains are on the rise. An effective TB vaccine would reduce the global need for antibiotic treatment, reducing antimicrobial resistance, and be preventative against both drug-sensitive and drug-resistant strains of *M. tb*. Additionally, TB treatment is long (6–24 months depending on drug sensitivity) and expensive and has a significant impact on the economic development of afflicted countries and their inhabitants. The average total cost to a patient with TB in an LMIC of US $538-1268 is equivalent to a year's wages [4], while almost one in two TB-affected households face costs higher than 20% of their household income [1].

Bovine TB, caused by the very closely related *Mycobacterium bovis*, is a zoonotic pathogen with substantial impacts on animal and human health, as well as economic productivity, where 'much of our understanding of transmission mechanisms, diagnostics, control, and multi-host infection systems remains opaque' [5]. It is estimated that >50 million cattle are infected worldwide, costing US$3 billion annually [6]. Currently, the BCG vaccine cannot be used in cattle due to the inability of routine diagnostic tests to differentiate between infected and vaccinated animals.

Wildlife act as a disease reservoir, with both cattle and many wildlife species being destroyed in unsuccessful attempts to control the disease. Alternative control measures, including a vaccine, are urgently needed [7].

2.2.2 Leishmaniasis

Leishmaniases are caused by around 20 protozoan *Leishmania* species transmitted by sandfly bites and affect both humans and dogs as well as around 68 other mammalian species [8]. Three main forms of leishmaniasis occur—visceral, cutaneous and mucocutaneous. Ninety-eight countries are considered endemic for leishmaniasis, with over 1 billion people at risk and around 12 million people infected at any one time [8]. A small proportion of those infected go on to develop disease with 700,000–1,000,000 human cases of leishmaniasis occurring annually, causing 20,000–40,000 deaths per year as well as disfigurement and disability [9, 10]. Poverty increases the risk for leishmaniasis, and the WHO classes leishmaniasis as a neglected tropical disease (NTD). Leishmaniasis has economic implications for countries and individuals affected due to treatment costs, income loss due to illness and death of a family's wage earner [11]. In India, treatment costs were found to be 1.2–1.4 times annual per capita income [12].

Canine leishmaniasis is a potential zoonotic infection that results in thousands of dogs being culled every year. Diagnostics in dogs have limited sensitivity. While some therapeutic vaccines exist, there is no efficacious preventative vaccine [8].

2.2.3 Melioidosis

Melioidosis is caused by the bacterium *Burkholderia pseudomallei* and is mainly found in the tropical climates (in the soil) of Southeast Asia and Northern Australia. Cases are also found in the Indian subcontinent, Sri Lanka and China, with sporadic cases found in Central/South America and Africa. It is likely the disease is underreported due to its non-specific symptoms and lack of awareness [13]. There are an estimated 165,000 cases annually and 89,000 deaths; in some areas melioidosis has a death rate of over 40% [14]. A 2019 review set the global burden of melioidosis at 4.6 million DALYs (84.3 per 100,000 people) and recommended that WHO add the disease to their NTD list [15]. Melioidosis infection often requires intensive care treatment, and drug therapy takes 3–6 months and involves antibiotics to which resistance can develop. Both these factors can inflict a heavy financial burden on sufferers [15]. *B. pseudomallei* has been recognised as a potential bioterrorist threat, as prior bioweapon release has utilised closely related diseases causing significant mortality and sickness and because *B. pseudomallei* is readily found in soil [16].

2.2.4 Leprosy (Hansen's Disease)

Other members of the *Mycobacterium* family aside from *M. tb* also cause significant disease in humans. *M. leprae* causes leprosy, with ~200,000 new cases annually in 118 countries: 79% of cases occur in 3 countries, namely, Brazil, India and Indonesia. Leprosy can be treated with antibiotics, but, if untreated, it can cause nerve damage, blindness and limb disfiguration and loss [17]. In many parts of the world, suffering from leprosy also leads to stigma and discrimination. Leprosy is a listed WHO NTD, with the 2021–2030 Global Strategy calling for an effective vaccine as a key part of the roadmap to elimination [18]. As *M. leprae* is closely related to *M. tb*, as well as to other pathogenic mycobacteria, research findings can have useful implications for other species of *Mycobacteria*, and vaccines may provide cross-protection, depending on the antigen(s) used.

These four intracellular pathogens are adept at evading the host immune response, and the precise nature of protective immunity is, yet, ill-defined. *Mycobacterium tuberculosis* and *Mycobacterium leprae* have co-existed with humans for millenia, and their persistence as pathogens of global importance is testament to their ability both to evade host immunity and to cause disease. For all these pathogens, development of efficacious vaccines is challenging. However, vaccines remain one of the most important public health resources. They were estimated to save two to three million lives each year—a figure taken before the COVID-19 pandemic's global vaccination programme—and save millions more people from the long-term consequences of diseases such as pneumonia, measles and polio [19]. The only human infectious disease ever eradicated, smallpox, was eliminated via a global vaccination programme; eradication of smallpox is estimated to have saved 150–200 million lives since 1977 [20]. Vaccines are the most cost-effective way to control infectious diseases, with significant health, economic and community benefits [21, 22]. The Global Alliance for Vaccines and Immunization (GAVI) estimates that from 2011 to 2030, immunisation could prevent, just in the 94 lowest-income countries, illness costs of $1510 billion [23]. Yet, none of VALIDATE's target pathogens have universally efficacious vaccines; VALIDATE and its members want to change this.

2.3 VALIDATE: Outputs and Successes

2.3.1 Pump-Priming Funding

VALIDATE has held five rounds of pump-priming funding, where members could apply to receive up to £50,000 for a 12-month project. Projects in rounds 1–4 had to be collaborative, with involvement from members from at least 2 different institutes. Projects in round 5 had to focus on tuberculosis and be led by an early-career researcher (ECR) member. Applications were competitively reviewed by the VALIDATE Network Management Board (NMB). The projects had to be

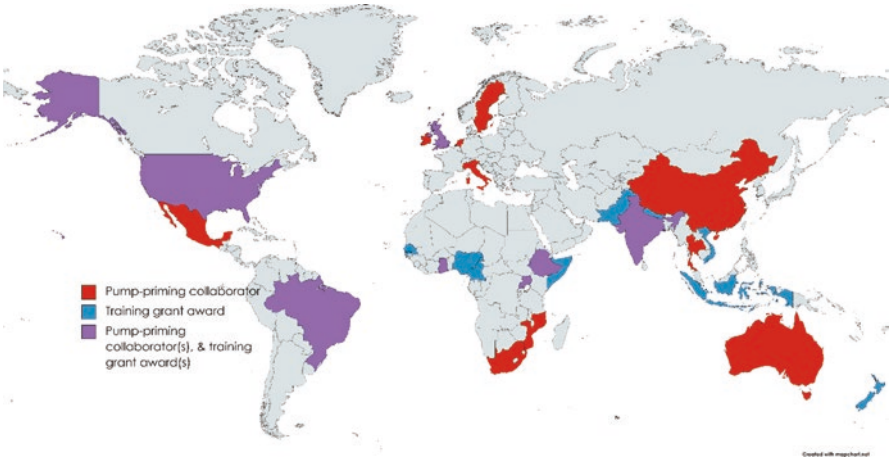

Fig. 2.2 VALIDATE-funded pump-priming projects and training grants. (Map created with MapChart (https://www.mapchart.net), which is licensed under a Creative Commons Attribution-ShareAlike 4.0 International License. Creative Commons License (CC BY-SA))

scientifically excellent and advance vaccine development for VALIDATE's focus pathogens and/or produce pilot data for future external grant applications.

Thirty projects have been funded, involving members from 17 countries (Fig. 2.2). Eighteen (60%) of the projects were led by an ECR, with the expectation that this will be beneficial to their careers and provide data which could support future follow-on grant applications to other funding bodies. Seven (23%) were led by LMIC members, with 16 (53%) involving an LMIC member (as lead or collaborator). Fourteen (47%) projects were led by members identifying as female. Twelve of the 13 projects running in 2019 were significantly impacted by the COVID-19 pandemic and laboratory shutdowns across the world and required no-cost extensions and/or scope alterations. Project details and outcomes can be found at www.validate-network.org/funded-validate-projects.

2.3.2 Fellowships

VALIDATE awarded two Fellowships in 2018, providing salary and consumables to two ECR post-doctoral members, outlined below. These awards were originally for 2 years but were extended into 2021 when the COVID-19 pandemic affected the Fellows' projects. The VALIDATE Fellows joined the VALIDATE NMB for the term of their Fellowship, which they felt offered them invaluable insight into what fundable grant applications look like, what reviewers look for and how to review grant applications, as well as invaluable exposure to senior scientists and their discussions.

2.3.2.1 Dr. Jomien Mouton, Stellenbosch University (South Africa): Identification of Latency Associated Antigens and Biosignatures Associated with *Mycobacterium tuberculosis*

Dr. Mouton's Fellowship enabled her to secure full employment at Stellenbosch University, something which is not usual for post-doctoral researchers, as well as a South African National Research Foundation (NRF) Y rating. This allowed her to become the primary supervisor for students and to apply for funding calls, including being a co-investigator for a successful application to acquire an Amnis Imagestream Imaging Flow Cytometer as the first of its kind in Africa (ZAR12,000,000). She was also a co-investigator on a further VALIDATE pump-priming project, with a new collaborator found via VALIDATE. Her Fellowship supported her through a period of maternity leave, which would otherwise have been unpaid, and enabled her to establish three new international collaborations and publish four papers (with an additional two in preparation)—including her first senior author paper. She says, 'Receiving the VALIDATE Fellowship has had a tremendous impact on my research and career. Apart from being appointed as a researcher and being able to supervise my own students, I have had the opportunity to serve on the VALIDATE Management Board and expand my network by meeting new people at the annual meeting. The funding I have received as part of my Fellowship has made research possible that has not yet been done in South Africa, to our knowledge. This has a large impact on capacity development and knowledge transfer in scarce skills and allows us to be able to use this data to apply for follow-on funding'.

2.3.2.2 Dr. Rachel Tanner, University of Oxford (UK): Characterising the BCG-Induced Antibody Response to Inform the Design of Improved Vaccines Against *M. tuberculosis, M. leprae* and *M. bovis*

Dr. Tanner's Fellowship enabled her to form eight new collaborations (one of which was with an industrial partner) and publish two publications with two further manuscripts in preparation. She was elected a Research Fellow at Wolfson College, University of Oxford, and presented work from this Fellowship at interview. She joined the British Society of Immunology (BSI) Vaccine Affinity Group (BSI-VAG) committee, which came about because of networking during the VALIDATE 2019 Annual Meeting. Dr. Tanner also participated in a number of outreach efforts, including having her video clips used in the YouTube 'Life in a Day' movie directed by Ridley Scott and Kevin McDonald that premiered at Sundance Film Festival, taking part in the BSI's 'A day in the life of a vaccine researcher' blog, and being interviewed for a 500 Women Scientists article, as well as co-presenting a 'VALIDATE for Schools' virtual talk. In 2019, Dr. Tanner was awarded the UK 'Women of the Future' Award for Science. She says, 'This award has had a significant impact on my research, allowing me the freedom to pursue my own interests and expand my skills and expertise… [it] has also been invaluable to applications

for follow-on grants, as I am able to demonstrate increasing independence and a track record of securing funding, as well as providing preliminary data for the basis of these proposals'.

In early 2022 VALIDATE awarded two further 2-year Fellowships to ECRs based in South Africa and Mexico:

- Dr. Nastassja Kriel, Stellenbosch University—Identifying persister *Mycobacterium tuberculosis* biosignatures.
- Dr. Cristian Segura-Cerda, CIATEJ—Evaluation of the efficacy of BCGΔBCG1419c vaccination plus a booster of EsxG/EsxH-derived peptides to prevent tuberculosis progression caused by *Mycobacterium tuberculosis* strains prevalent in Latin America.

Details on all four Fellowships can be found at www.validate-network.org/funded-validate-projects.

2.3.3 Training Grants

Post-doctoral ECR members could apply for training grants of up to £3000 (increased to £3500 in 2022) to participate in a training opportunity that would contribute to their career development. This included opportunities such as attending a course or workshop, participating in a laboratory exchange visit (to gain knowledge and expertise that they could take back to their home institute) or (for LMIC members) presenting at a conference.

VALIDATE has awarded 34 training grants to members in 17 countries, as well as funding 3 LMIC-student places on the Jenner Institute's 2019 Vaccinology in Africa course and 2 LMIC places on University of Cape Town's 2019 Flow Cytometry workshop. A total of 59% (20) of training grant awardees were female, and 56% (19) were based in LMICs. Four awardees withdrew after award, and, unfortunately, the COVID-19 pandemic stopped 10 from attending their training. Training grants are listed at www.validate-network.org/funded-validate-projects.

One example of a successful training grant was Dr. Eduardo Ramos Sanchez, a post-doctoral researcher studying leishmaniasis at the University of Sao Paulo (USP), Brazil. He had a 1-month laboratory exchange to the McShane group at Oxford University, UK, where he was trained on the mycobacterial growth inhibition assay (MGIA) and other assays used in this research group, and the visit provided opportunities for both sides to consider future TB-leishmaniasis collaborative work. On returning to Brazil, Dr. Ramos Sanchez's home laboratory has since obtained a licence to work with mycobacteria and can now work with two different experimental models.

Other examples include Dr. Shraddha Siwakoti (B.P. Koirala Institute of Health Sciences (BPKIHS), Nepal) who visited the Cooper laboratory at University of Leicester, UK, to discuss the tools and activities required to set up a new immunology laboratory at her home institute; Dr. Isadora Lima (FIOCRUZ, Brazil) visited

the University of Surrey and Public Health England, UK, to learn laser capture microdissection, which she now teaches as a short course at her home institute and has developed protocols for use in her home laboratory; and Farah Isse Mumin (Red Sea University, Somalia) who was funded to attend a VALIDATE co-hosted vaccinology workshop that improved his undergraduate teaching and connected him to researchers in nearby Ethiopia whom he has since visited.

2.3.4 Mentoring

VALIDATE has held 5 mentoring calls, setting up 24 mentee-mentor relationships (18 for LMIC ECRs) pairing VALIDATE post-doctoral ECR and senior Principal Investigator members. These mentoring pairings are for 12 months, providing career and research support and guidance for our ECR members. Mentoring can be an invaluable career advancement tool, and these are a few of the comments received from VALIDATE's mentees:

> *[mentor]* encouraged me to attend meetings and conferences... to expand my network and discuss my research. Over the last year I have taken on the role of President of the Researcher Association to support and represent contract researchers across *[institute]* at various levels (academic board, research strategy committee, REF committee) - this position has increased my visibility and collegiate skills. I have also submitted two grants with *[mentor]*'s encouragement. I have found the whole experience hugely beneficial and would highly recommend to others to seek a mentor at every stage in their career…I only wish I had sought a mentor sooner.
>
> As an early career researcher, having a mentor was of great value to expand my viewpoints and consider new ways of approaching my research... He is guiding me through the development of my post doc, not only by giving me training in techniques… but also providing new inputs to our project... Having the opportunity to be in touch with a researcher in my area, but from a different country and culture, gave me a completely new perspective and broadened my horizons.
>
> *[mentor]* helped me to identify some of the things I need to do to help push my career forward. Since then, I started pushing for opportunities that can help boost my profile, and extend my network. She supported me with a number of grant applications where references are required.
>
> I believe that the mentoring meeting helped give me the confidence to apply for and be successful in achieving a more senior post of senior lecturer at *[institute]* as well as to establish a number of new collaborative relationships with staff there. This led to the submission of a Newton Institutional Links application, on which I was lead applicant.

2.3.5 ECR Career Development Network

VALIDATE recognises the challenges faced by post-doctoral and early PI researchers trying to progress their careers. As part of VALIDATE's aim to encourage and support career progression amongst members, especially ECRs, and to help address inequality in scientific career progression, we created a 2-year-long ECR Career

Development Network (ECDN) programme for members identifying as female. We ran an open call for members to join the ECDN and received 13 applicants from 11 countries (7 from LMIC members). Starting in February 2022, with a kick-off meeting (virtual due to the COVID-19 pandemic), the network aims to form an engaged and connected sub-network of ECRs wanting to develop their careers and to help support and accelerate their careers by providing them with peer support, networking opportunities, group mentoring, accountability groups, one-to-one coaching, introductions to senior scientist role models and monthly virtual talks and workshops. VALIDATE also funded the ECDN members' attendance at the 2022 Global Forum on TB Vaccines.

2.3.6 Information Dissemination

VALIDATE hosts a website (www.validate-network.org) that aims to be a 'one-stop-shop' for members where all relevant funding calls, training and job opportunities, events, publications and news are highlighted so that members can easily keep up to date and hear about useful opportunities. The VALIDATE Twitter account (@NetworkVALIDATE) also helps members keep up to date on news in the field and has >1000 followers. VALIDATE members receive a quarterly newsletter, and connecting with each other is promoted via the VALIDATE LinkedIn profile as well as a searchable members' directory on the VALIDATE website. Video content, including recordings of public talks, is available via VALIDATE's YouTube channel, while the VALIDATE Microsoft Teams Hub contains recordings of members-only events as well as shared standard operating procedures (SOPs) and protocols for use by members. VALIDATE runs a multitude of communications activities, both on our website and via national media, celebrating World Days such as World NTD day and events like the BCG vaccine centenary in 2021 and highlighting and celebrating members' research.

2.3.7 Events

VALIDATE hosted in-person annual meetings in 2017, 2018 and 2019, with a virtual meeting in 2021 (due to the COVID-19 pandemic). Twenty-six travel scholarships were awarded to LMIC members to support their attendance in the in-person meetings (Fig. 2.3).

Thirteen virtual seminars highlighting 20 researchers' work, including our industry partners, have been streamed online, with recordings available to members. VALIDATE has also hosted a number of in-person and virtual ECR workshops for career support and progression (see www.validate-network.org/workshops), a

Fig. 2.3 VALIDATE members at the 2019 Annual Meeting

co-hosted Global Challenges Research Fund (GCRF) Vaccinology Workshop (2018, Bangkok), a virtual Collaboration Workshop for members in 2020 and a Melioidosis Vaccine Symposium 'Moving from animal models to man' in 2019.

All the VALIDATE events have helped boost dissemination of information between our members, including unpublished research results, helped stimulate and nurture new collaborations, as well as provided training and support for our ECR members via the ECR workshops.

A particular success story arose from initial discussions on the lack of melioidosis vaccine candidates in clinical trials at the 2018 Annual Meeting, a situation the TB vaccine field was at in 2001. We held a Melioidosis Symposium in 2019 because of those discussions, where melioidosis vaccine researchers and experienced TB vaccine clinical trial researchers were brought together to discuss the roadmap to first-in-man melioidosis vaccine clinical trials and the lessons learned in the TB field when moving vaccine candidates from animals to humans. A new UK-USA-Thailand collaboration was formed, linking lab-based vaccine developers with clinical trial researchers and facilities, and this collaboration was successfully awarded £3.2M MRC Biomedical Catalyst: Developmental Pathway Funding Scheme (DPFS) funding (plus further Defense Threat Reduction Agency (DTRA) funding) for the first human clinical trial of a melioidosis vaccine. VALIDATE has, therefore, directly contributed to the development of a candidate melioidosis vaccine.

2.3.8 Outreach

VALIDATE initiated a BCG100 Programme in 2021, celebrating the centenary of the first use of BCG, including two keynote talks by four world-leading scientists in the TB vaccine field that were live-streamed (with recordings available via the VALIDATE YouTube channel) so that all members, as well as members of the public, could attend. One hundred and fifty people attended the first talk ('BCG Then and Now'), with 78 (including 42 non-members) from 22 countries (15 LMIC) attending the second ('TB and the use of BCG in Animals: Does it matter to people?'). As part of an Enriching Engagement award from Wellcome, VALIDATE created 3 computer games aiming to engage and educate 11–14-year-olds around the creation of the BCG vaccine, the challenges of vaccine delivery and the workings of the human immune system. The games are free for use by teachers and researchers to promote discussion and learning in the classroom and at outreach events via www.validate-network.org/bcg-adventures. Within the first 6 months of release, >27,000 people from 99 countries have engaged with the games (Fig. 2.4).

A 'VALIDATE for Schools' programme has seen 3 online talks by 5 of our researchers (2 senior, 3 post-doctoral, based in the UK and South Africa) reach over 300 school children and engage them in discussions around vaccines, immunology and careers in Science, Technology, Engineering, Mathematics and Medicine (STEMM). Given during the COVID pandemic, these talks saw the children asking lots of questions, and VALIDATE received excellent feedback from their teachers. VALIDATE has also organised and coordinated work experience visits by school students to the Jenner Institute laboratories at the University of Oxford.

A video 'explainer' about leprosy was made in collaboration with VALIDATE member Dr. Hua Wang and has had 3700 views via the VALIDATE YouTube

Fig. 2.4 BCG adventure games

channel (www.youtube.com/channel/UC4jnwrHLkvK-xJjYJYpnYog). A similar video was made for leishmaniasis as part of World NTD Day 2022.

In 2018, the VALIDATE Directors and Network Manager met with two UK Members of Parliament to discuss the importance of vaccines and of the UN General Assembly 2018 High Level Meeting on TB. This led to one of the MPs contacting the International Development Minister to urge high-level UK attendance. Prof McShane was also interviewed as part of research for the 2021 'Advances in vaccine technologies' UK Parliament POSTnote. Additionally, welcome addresses were given at the VALIDATE 2021 Annual Meeting (held online across 3 days in 3 different time zones to maximise the ability of members to attend) by The Newton Fund India, the UK High Commission in South Africa and the British Ambassador to Colombia.

2.3.9 Data Sharing

From 2018 VALIDATE had a dedicated bioinformatician available to support members and a data-sharing portal to facilitate members making comparisons of similar data sets, both published and unpublished, across species/country/research group/trials, aiming to find lessons to learn from the data already available through our members and advance the field. Interestingly, neither of these resources were much utilised by VALIDATE members so they were discontinued in 2020, although available data sets are listed on the VALIDATE website (www.validate-network.org/data-sharing).

2.3.10 Equipment Exchange

To capacity build, and save on landfill, VALIDATE runs an equipment exchange scheme where we help coordinate donations of surplus equipment from one member laboratory to another member laboratory who can use it. LMIC members get priority on donated items, which have ranged from large laboratory equipment all the way down to 51 laboratory coats. In 2018, for example, a Bactec MGIT machine was donated to researchers at the MRC/Uganda Virus Research Institute and London School of Hygiene & Tropical Medicine Uganda Research Institute by Animal and Plant Health Agency (APHA)/University of Oxford. VALIDATE coordinated the donation and shipment, contributing 50% of the shipping costs. Now in Uganda, the MGIT has greatly improved the unit's capacity to perform mycobacterial culture for TB diagnosis and experimental assays such as the MGIA. This has led to several new collaboration opportunities for the Uganda team, including participating in a multi-centre TB vaccine trial. Additionally, members of staff and postgraduate students are trained to use the machine and can use it for their research projects.

2.4 The Future of VALIDATE

While the true economic and human cost of the COVID-19 pandemic has yet to be accurately determined, it has severely impacted the public health landscape. The pandemic is exerting lasting effects on treatment and prevention programmes for communicable diseases, such as those caused by VALIDATE's focus pathogens, particularly in LMICs [24].The pandemic, however, has also highlighted the relief that effective vaccines can bring and has emphasised the need for continued vaccine development efforts.

VALIDATE's original Medical Research Council funding ends on 31 March 2023. Excitingly, VALIDATE was awarded a further $1.5M award from the Bill and Melinda Gates Foundation (BMGF), which enables VALIDATE to continue through to 2024. This funding has also led to a new partnership with the Collaboration for TB Vaccine Discovery (CTVD), with interactions including joint grant calls for both CTVD and VALIDATE members.

The BMGF funding is solely for TB research, so the VALIDATE Management Team are actively seeking matched funding to continue to facilitate research to accelerate vaccine development for our other three focus pathogens. The COVID-19 pandemic has shown us all how crucial vaccines are in the fight against infectious disease, and, as VALIDATE's support in accelerating the first human trial of a melioidosis vaccine shows, we can advance vaccine development more quickly when researchers working on pathogens that have similarities in their infectious behaviour learn from each other's experience and research findings. It is important, therefore, that VALIDATE's remit of increasing communication and information dissemination across our four focus pathogens continues, until we have efficacious vaccines for these neglected infectious diseases.

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Part I
Leprosy and Buruli Ulcer

Chapter 3
A Current Perspective on Leprosy (Hansen's Disease)

Khushboo Borah Slater

Abstract Leprosy, also known as Hansen's disease, is an ancient chronic infectious disease that remains a major problem in the world today, infecting over 200,000 people each year, particularly affecting resource-limited and the most disadvantaged sections of society in under-developed countries of the world. *Mycobacterium leprae*, a slow-growing mycobacterium, causes leprosy in humans. Leprosy causes nerve damage and permanent disabilities including blindness and paralysis. People affected by leprosy face stigma and discrimination in society. Although multidrug therapy is available, millions of people are still affected by leprosy, so new vaccine, drug and disease management approaches are urgently needed for control, prevention and treatment of this disease. This chapter is a general review of leprosy, the current treatment and prevention measures and challenges that need to be addressed for complete eradication of this disease.

Keywords *Mycobacterium leprae* · Leprosy · Vaccine · Diagnosis Immunity · Treatments

3.1 Introduction

Leprosy, also known as Hansen's disease, is an ancient chronic human infectious disease that remains a major public health problem in many developing countries. Leprosy is caused by the pathogen *Mycobacterium leprae*, first discovered over a century ago by the Norwegian scientist Gerhard-Henrik Armauer Hansen. *M. leprae* is a slow-growing mycobacterium and an obligate intracellular pathogen, which can survive out of the human host for up to 45 days [1–3]. The genome sequence analysis of *M. leprae* revealed massive gene decay and reductive evolution with

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M. Christodoulides (ed.), *Vaccines for Neglected Pathogens: Strategies, Achievements and Challenges*, https://doi.org/10.1007/978-3-031-24355-4_3

functional genes accounting for less than half of the genome and pseudogenes accounting for the remaining more than half of the genome [3]. The genome size of *M. leprae* is 3.27 megabase which is extremely reduced in comparison to its related pathogen, *M. tuberculosis* (4.41 megabase) [3]. Gene deletions include those primarily involved in energy metabolism (a curtailed respiratory chain resulting in no ATP formation from NADH oxidation), microaerophilic and anaerobic respiratory chains, catabolism of carbon and nitrogen compounds and regulatory pathways [2–4]. The disease incubation period averages over 5 years, and the symptoms may take between 1 and 20 years to occur [5]. Leprosy affects the nerves, skin, respiratory tract, bones, testes and eyes, and can cause permanent deformity and disability if left untreated [6–8].

Historically, the first cases of leprosy were described in parts of China, India and Egypt: around third BC in China and around 600 BC in an Indian treaty Sushruta Samhita. There are hypotheses about the origin of this disease in Africa or in Asia and then its spread towards the European and American continents through human migrations [9, 10]. The global number of cases reported between 1960 and 1980 was 10–12 million. The number of cases declined drastically to 5.5 million in 1991 with the approval and use of multidrug therapy (MDT) since 1981. The number of cases further declined to 265,661 in 2006. Between 2006 and 2013, the number of cases was largely stable (215,656 in 2013) with a very slow reduction in case numbers [6]. Over the past 8 years, around 200,000 leprosy cases are being registered every year. According to the World Health Organisation (WHO) (https://www.who.int/news-room/fact-sheets/detail/leprosy), there were 202,256 new leprosy cases registered in 2019. The prevalence of leprosy differs from one region to another. For instance, the highest prevalence of this infection is in South-east Asia accounting for over 70% of the total cases globally as per records in 2013. India, Brazil and Indonesia are three countries with the highest number of new cases annually [11]. Bloke et al. [11] correctly predicted the unlikely elimination of future leprosy incidences in these high-endemic countries by 2020 (WHO's previous target for global leprosy elimination by 2020) using the current methods for passive and active case detection and current MDT [11]. There are gender and age-related distributions; the proportion of women in the detected cases were lower than men (for instance, 0.5% in Pakistan, 56% in South Sudan); the proportion of children in detected cases were 0.6% in Argentina and Mexico and 39.5% in the Federated States of Micronesia [9]. Gender inequalities in physical appearance and social stigma are some of the reasons for gender-related leprosy distributions [12]. The sociocultural outcomes of the disease have been found to affect women more than men, such as more social and family rejections and restrictions.

3.2 Disease Transmission and Infection

M. leprae transmission occurs through air droplets and close contact with infected individuals. Lepromatous leprosy patients are usually most contagious due to the large number of bacilli (up to seven billion) in their infected tissues [9]. The main dissemination and entry route for the leprosy bacilli have been found to be the upper respiratory tract, as determined with experimental mouse models [3, 9, 13, 14]. A summary of leprosy transmission routes, infection and symptoms is shown in Fig. 3.1. Identification of leprosy transmission is hindered because clinical manifestations of the infection can usually take up to 10–15 years in the close contacts [15]. People living within leprosy-endemic communities are exposed to *M. leprae*, but few develop diseases, likely because most of the population develop protective immunity. Ramaprasad et al. [15] demonstrated the subclinical transmission of *M. leprae* involving transient infection of nose detected by PCR and a consequent mucosal immune response measured by salivary anti-*M. leprae* IgA (sMLIgA) tests.

The distinct phases in the immunology of leprosy are not completely understood. At the site of infection such as in the nasal mucosa, *M. leprae* encounters

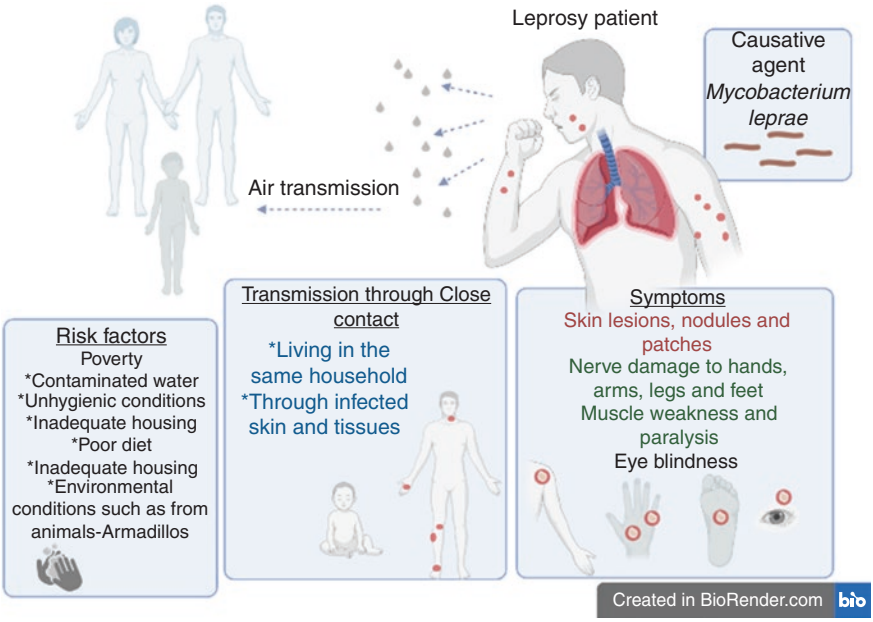

Fig. 3.1 Overview of leprosy transmission routes, infection and symptoms. *Mycobacterium leprae* is the causative agent of leprosy. The symptoms are nodules, lumps, bumps, lesions and patches on the skin, blindness, nerve damage, muscle weakness and paralysis to the hands, arms, legs and feet. The main route is transmission through air droplets from infected individuals and through contact with infected skin and tissues. Transmission can occur through close contact, such as living with a leprosy patient in the same household. The main factors are poverty, inadequate housing and unhygienic conditions, poor diet and contaminated water

macrophages or dendritic cells as the first contacts in the absence of an adaptive immune response. The bacilli are taken up by these immune cells with concomitant production of cytokines and chemokines and stimulation of cell-mediated Th1 or Th2 immune responses [4]. *M. leprae*-infected dendritic cells present phenolic glycolipid-1 (PGL-1) antigen on their cell surface with subsequent antibody responses and T cell stimulations. Monocytes and dendritic cells in the tuberculoid lesions express Toll-*like* receptors (TLRs) that recognize *M. leprae* antigens and induce Th-1 type cell-mediated immune responses for proinflammatory cytokine production and granuloma formation [4, 16]. In macrophages, the bacilli are challenged with antimicrobial peptides and reactive oxygen and nitrogen species to eliminate the infection. Schwann cells (both myelinated and nonmyelinated) are one of the primary targets of *M. leprae* infection [4, 17, 18]. *M. leprae* adheres to the Schwann cell surface through binding of the PGL-1 to laminin-2 of the axon unit, followed by ingestion of the bacilli by the Schwann cells [19]. *M. leprae* can proliferate in the Schwann cells, and it uses host cell nutrients to synthesize its biomass [2, 18]. Schwann cells are also able to present *M. leprae* antigens to T cells, which may be responsible for the inflammatory responses and consequent nerve damage [4].

Several genetic factors have been identified to be involved in specific host immune responses to *M. leprae* and in heterogeneity of the clinical manifestations of the disease [20–23]. Mi et al. [21] provided a comprehensive review of the cell type-specific immunological and genetic factors associated with various clinical forms of the disease: genes *VDR, OPA1, SLC7A2, RAB32, SLC29A3, LRRK2, IRGM, CTSB, DEFB1, PARK2, PACRG* and *TNF* are associated with macrophage-specific immune responses; *TLR1, TLR2, NOD2, HLA, MICA* and *MICB* are associated with dendritic cell responses; *FLG* is associated with keratinocytes; *IL23R, IL12B, TNFSF15, TYK2, SOCS1, IL18R1* and *LTA* are associated with T cells [21]. A genome-wide association analysis identified single nucleotide polymorphisms in C13orf31, LRRK2, NOD2 and RIPK2 genes strongly associated with multibacillary leprosy and that variants of genes in the NOD2-mediated signalling pathway are involved in susceptibility to *M. leprae* infection [24]. Distinct cytokine expression was found to be associated with the multibacillary and resistant form of the disease. Yamamura et al. [25] demonstrated a predominant expression of interleukin (IL)-4, IL-5 and IL-10 in multibacillary leprosy and the expression of IL-2 and interferon gamma (IFN)-γ in the lesions analysed from 16 patients with lepromatous and tuberculoid leprosy.

M. leprae infection induces host cell metabolic reprogramming. Formation of lipid droplets and accumulation of oxidised phospholipids, cholesterol and fatty acids that results in “foamy cells” is a characteristic of infected cells such as that observed in the infected skin lesions, macrophages and Schwann cells [26]. Foamy cell formation is also a characteristic of the related *M. tuberculosis* infection [27]. In addition to up-regulation of lipid metabolism genes in infected host cells, a decrease in mitochondrial ATP formation with a concomitant rise in glycolytic activity has been reported. Infected Schwann cells showed increased glucose uptake and pentose phosphate pathway activity during *M. leprae* infection [17]. These changes in host cell metabolism are linked to innate immune responses during infection. In

infected Schwann cells and macrophages, lipid droplet formation regulates the production of Prostaglandin E2 (PGE2) immunomodulator that controls regulatory T cell (Treg) and macrophage bactericidal activity [26]. Inhibition of lipid droplet formation has been shown to down-regulate production of the anti-inflammatory cytokine IL-10 and cause a switch from an anti-inflammatory to a pro-inflammatory phenotype [28, 29]. *M. leprae* infection induces an elevated expression of indoleamine 2,3-dioxygenase (IDO-1) in macrophages and dendritic cells of lepromatous lesions that increases tryptophan degradation and suppression of innate and adaptive immunity, which in turn decelerates immune signalling to eradicate the infection and supports the survival of *M. leprae* in the host environment [26, 30]. Accumulation of iron in infected host macrophages has been hypothesised to benefit the survival of *M. leprae*. The increase in iron metabolism is linked to the non-responsiveness of macrophages to IFN-γ signalling and promotion to an anti-inflammatory M2-like phenotype [26].

3.3 Disease Classification and Pathophysiology

In infected individuals, the bacillus is generally found in the macrophages, keratinocytes and histiocytes of the skin causing dermatological conditions and in the Schwann cells of peripheral nerves causing axonal dysfunction and demyelination [3, 7]. Infection of Schwann cells leads to their de-differentiation and reprogramming, which consequently leads to degeneration and deformation of the peripheral nerves. White et al. [7] reviewed the various clinical manifestations of the disease and provided a summary of the various clinical forms of leprosy and their classifications. The different clinical forms of leprosy are likely due to the genetic and biological variability between infected individuals. According to the WHO, different forms of leprosy are classified based on the symptoms such as the presence of bacilli in the skin smears and visible lesions [7, 8]. Infection is classified as "paucibacillary" with 1–5 skin patches and no apparent bacteria in skin smears. Individuals with more than five skin patches and visible bacteria in the skin smears are classified as "multibacillary" [7]. A study by Pardillo et al. [31] compared the practice of counting lesions to assign treatment regimens in 264 untreated leprosy patients. This study found misclassification, where 38–51% of patients assigned as paucibacillary cases (according to the WHO classification) had multibacillary infection and were therefore at risk of under-treatment and developing drug resistance [31]. The classifications based on the immune responses to infection are tuberculoid (TT), borderline tuberculoid (BT), borderline borderline (BB), borderline lepromatous (BL) and lepromatous (LL), with the last one being the most severe form of the disease, causing extensive nerve damage and physical disability [7, 32, 33] (Fig. 3.2). An alternative WHO classification categorises TT and BT types as paucibacillary and BB, BL and LL as multibacillary [34].

The clinical manifestations of TT are skin lesions (large hypochromic macules, large thickened infiltrated plaques) and nerve damage (usually around skin lesions)

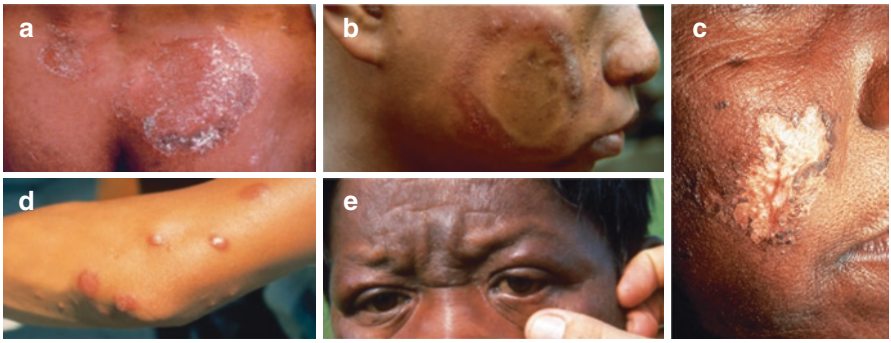

Fig. 3.2 Classification of different forms of leprosy. (**a**) Tuberculoid (TT) leprosy: this shows a leprosy lesion on the lower back that has healed spontaneously and remains only a thin scar with a complete loss of sensation. (**b**) Borderline tuberculoid (BT) leprosy: this shows an annular lesion on the cheek of a boy with BT. (**c**) Borderline borderline (BB): this shows a scarred lesion on the right cheek. (**d**) Borderline lepromatous (BL) leprosy: this shows a few raised and erythematous lesions on the arm of a patient. (**e**) Lepromatous leprosy: this leprosy patient has marked loss of the eyebrows and eyelashes and thickening of the facial skin. (The images are from the Wellcome Collection with 4.0 International (CC BY 4.0) licence)

and sensory impairment affecting mainly the hands and feet [9]. LL patients present with multiple lepromas on the skin, (most frequently on the face, earlobes, fingers and toes) and peripheral nerve damage with hypertrophy, sensory and motor impairment [9, 35]. BT patients present with several large asymmetrical and hypoaesthetic skin lesions; BB patients present with non-anaesthetic annular lesions, and BL patients present with more than ten bilateral and non-anaesthetic lepromas and annular lesions [9]. The clinical manifestations associated with various forms of leprosy are summarised in Table 3.1. The immunological responses correlated with tuberculoid TT and BT are Th1 cell-mediated responses involving IL-2 and IFN-γ signalling and formation of delineated granulomas to arrest bacterial growth [34]. A Th2 immune response with IL-10 and IL-4 signalling that impedes granuloma formation, allowing bacterial replication and causing infiltration of skin and nerves and severe clinical manifestations, is associated with BL and LL disease types [34, 36].

Reactions or inflammatory responses to *M. leprae* that occur during the disease or during treatments are the causes of nerve and skin damage. Reactions are categorised into type 1 reactions, type 2 reactions and diffuse lepromatous leprosy [8, 10, 16, 37, 38] (Table 3.2). Type 1 or reversal reactions are associated with BT, BB and BL forms of the disease driven by the cell-mediated immune response to *M. leprae* [8, 39]. Patients with type 1 reactions suffer pain in lesions, nerve damage such as lagophthalmos, the loss of ability to close the eyelids, inflammatory eye conditions leading to blindness and nerve injury in the feet leading to disability [4, 7, 8]. There is an increase in the levels of proinflammatory cytokines such as tumour necrosis factor (TNF)-α, IL-1, IL-2 and IFN-γ. Type 1 reactions typically occur at the onset or after the completion of MDT treatment. An analysis of patient records from India between 1983 and 1998 revealed widespread disease and multibacillary cases as the main risk factors for

Table 3.1 Classification based on clinical manifestations [7, 8]

Classification	Clinical manifestations			Number of bacilli
	Skin	Nerve	Systemic	
Tuberculoid (TT)	– Skin lesions (large hypochromic macules, large thickened infiltrated plaques)	– Nerve damage (usually around skin lesions)	–	Paucibacillary (1–5 lesions/ skin patches)
		– Sensory impairment affecting mainly the hands and feet		
Borderline tuberculoid (BT)	Several large asymmetrical and hypoaesthetic skin lesions	– Pain or tenderness in nerves, with or without loss of nerve function	–	Paucibacillary (1–5 lesions/ skin patches)
Borderline borderline (BB)	Non-anaesthetic annular lesions	– Nerve damage in the hands, feet or face	–	Multibacillary (>5 lesions/skin patches)
Borderline lepromatous (BL)	More than 10 bilateral and non-anaesthetic lepromas and annular lesions		Fever, malaise, lymphadenitis, uveitis, neuritis, arthritis, dactylitis, orchitis	Multibacillary (>5 lesions/skin patches)
Lepromatous (LL)	– Multiple lepromas on the skin (most frequently on the face, earlobes, fingers and toes)	Peripheral nerve damage with hypertrophy, sensory and motor impairment		Multibacillary (>5 lesions/skin patches)

Table 3.2 Classification of leprosy reactions

Reaction type	Leprosy forms	Reaction manifestations	References
Type 1 or reversal	BT, BB, BL	– Driven by the cell-mediated immune response to *M. leprae*	[4, 7, 8]
		– Increase in proinflammatory cytokines	
		– Pain in lesions	
		– Nerve damage such as lagophthalmos	
		– Inflammatory eye conditions leading to blindness	
		– Nerve injury in the feet leading to disability	
Type 2 or erythema nodosum leprosum (ENL)	BL, LL	– Nerve, eyes, lymph nodes, skin inflammation	[4, 7, 41]
		– Painful subcutaneous nodules	
Lucio phenomenon or "pretty leprosy"	LL	– Diffuse skin infiltration, soft wrinkles, painful irregular lesions	[38, 43]

Type 1 reactions, with the incidences typically recorded after 6–12 months of MDT [40]. Type 2 reaction, also known as erythema nodosum leprosum (ENL), is a cellular dysfunction characterised by acute fever and acute nerve, eye, lymph node and skin (painful subcutaneous nodules) inflammation and is primarily associated with BL and LL forms of the disease [4, 7, 41]. The levels of C3 immune complexes in serum and its complement activation product C3d in plasma were higher in ENL patients, showing a hyper-catabolism of C3 as a feature of ENL that could be useful for early diagnosis [42]. ENL can occur during different periods of the disease and during MDT treatment, with some patients experiencing Type 2 reactions after being cured. Kumar et al. [40] found the occurrence of ENL reactions during the second or third year of MDT treatment in patients in Chandigarh, India, and identified LL and a bacterial index ≥ 3 as risk factors for ENL. Interestingly, Kumar et al. [40] identified the female gender as the common risk factor for both Type 1 and ENL reactions. Diffuse lepromatous leprosy is a non-nodular form of lepromatous leprosy characterised by diffuse skin infiltration and soft wrinkles [38, 43]. This clinical form is also termed as "pretty leprosy". Lucio phenomenon is a thrombotic reaction associated with the diffuse lepromatous leprosy characterised by the presence of nodules and diffuse infiltration and pure diffuse leprosy. Patients with the Lucio phenomenon suffer from fever, anaemia, hepatosplenomegaly, lymphadenopathy and painful irregular lesions [38, 43].

3.4 Diagnosis and Treatment

Leprosy can be cured with timely diagnosis and MDT treatment [44]. Untreated, it can cause progressive and permanent damage to the skin, nerves, limbs and eyes. According to the WHO, a case of leprosy was identified as hypopigmented or reddish skin lesions, thickening of peripheral nerves and loss of sensation and skin-smear positive for acid fast bacilli. Skin lesions as the dermatological condition are the indicators in 90% of leprosy cases [9]. A clinical review by Moschella et al. [45] discussed the limitations of using only skin lesions as a diagnostic sign. In multibacillary infections presenting with reduced hypopigmentation and reduced lesions, following the diagnostic sign of skin lesions resulted in non-identification of around 30% of leprosy cases. Moschella et al. [45] proposed a combination of diagnostic signs including the presence of thickened nerve and hypopigmented or erythematous skin lesions with and without sensory loss, or impaired nerve function, to improve diagnostic efficacy. Certain leprosy cases can show solely neuronal symptoms without skin lesions termed as neuritic leprosy, and a nerve biopsy is a 100% confirmatory diagnosis for this. Lepromin is a widely used skin antigen test used to measure the ability of an individual to develop a granulomatous response to a mixture of antigens derived from *M. leprae* from different sources [4, 39]. However, lepromin test is not 100% leprosy-specific, as individuals without any contact with *M. leprae* can also show a positive lepromin reaction [4]. Skin lesions are used for smear tests and biopsies, which are gold standard laboratory diagnostic tests for leprosy. The smear test is rapid and effective for earlobe smears, but this test is usually negative for paucibacillary and tuberculoid

leprosy [9]. Microscopic examination using Ziehl-Neelsen staining for acid-fast bacilli is a standard diagnostic technique for samples such as tissue fluid smears and skin biopsies. Slit-skin smear is a commonly used procedure for assessing acid-fast bacilli in infected skin lesions during and after treatment [4].

Skin biopsies used to detect multibacillary and relapse infections are highly specific with almost 100% diagnostic accuracy, but the sensitivity of this test is around 50%. The reduced sensitivity is caused by the technicalities of the test including sample handling, staining and interpretation [45]. Other diagnostic tests include histological examination, serological anti-PGL-1 antigen, skin, fluorescent leprosy antibody (FLA)-abs and PCR tests. The PGL-1 serological test is highly sensitive and specific for patients with multibacillary disease, but not for paucibacillary cases. This test cannot predict who amongst the close contacts will develop the disease. PCR is a highly sensitive molecular diagnostic test with 100% specificity and sensitivity between 34% and 74% for tuberculoid forms of the disease and 80% in lepromatous diseases [15, 46, 47]. Several gene targets including Pra-36 KDa, Pra-18 KDa, RLEP, Ag85B, 16SRNA, *folP*, *rpoB* and *gyrA* are used [9, 48]. PCR was useful in confirming cases with atypical clinical and histopathological features. However, PCR diagnosis is limited when it comes to detecting paucibacillary cases and cannot distinguish between live and dead bacteria [15]. Clinical diagnosis also includes examination of patient's medical history, e.g. if they lived in nations with endemic leprosy.

Dapsone, the first drug discovered for treating leprosy in 1941, was a breakthrough for leprosy cure. Clofazimine and rifampicin (discovered later than dapsone) were the other two effective antibiotics for leprosy treatment. MDT was first approved in 1981 by the WHO and consists of these three first-line antibiotics. The recommended duration for MDT treatment for multibacillary (LL, BL and BB disease forms) is 12–24 months and for paucibacillary cases is 6 months. This treatment regime has been proven to produce effective clinical responses and low rates of relapses [9, 38, 49, 50]. Dapsone at a dose of 100 mg daily and rifampicin at 600 mg monthly are prescribed for paucibacillary cases in adults, and clofazimine at 50 mg daily along with dapsone (100 mg daily) and rifampicin (600 mg daily) are prescribed for multibacillary cases [38, 50]. Fluoroquinolones are the second-line antibiotics administered to patients showing intolerance, resistance such as rifampicin-resistant leprosy and clinical failures to first-line antibiotics [9]. A combination of ofloxacin (400 mg/day), minocycline (100 mg/day), clofazimine (50 mg/day) or clarithromycin (500 mg/day) is used to treat rifampicin-resistant cases [38]. Rifampicin- and ofloxacin-resistant cases are treated with a combination of minocycline (100 mg/day), clarithromycin (500 mg/day) and clofazimine (50 mg/day) [38].

Leprosy relapses are also treated with standard MDT [9]. There are geographical variations in the relapse rate of the infection. The risk of relapse is very low for both paucibacillary and multibacillary patients who have completed MDT [51, 52]. The risk of relapses is higher in patients with irregular and inadequate therapy, in patients with failure to respond to therapies and in patients with persistent *M. leprae* infection and co-infections such as human immunodeficiency virus (HIV). Relapse cases are identified by the reappearance of positive acid-fast bacilli, appearance of active lesions and increase in bacterial index after the patient has been negative [51].

Techniques such as measuring bacterial viability through cultivation of *M. leprae* in mouse foot pads and through staining microscopy and PCR analysis are used to monitor relapses during treatment. Immunological tests such as anti-PGL1 and anti-35 kD antibodies and PGL-1 serum antigen ELISA are used to monitor treatment and to detect early infection and relapse cases [51]. Cell-mediated immunological response such as Th1 and Th2 levels can aid in identifying the type of relapses, e.g. the relapse of BL/LL patients to TT/BT is associated with an elevated Th1 response (increased IFN-γ and IL-2 cytokines and IgG2 antibodies), while relapse of TT/BT to BL/LL is associated with an elevated Th2 response (increases in IL-4, IL-5, IL-6 and IL-10 cytokines and IgG1 antibodies) [51].

Early detection and treatment of nerve damage are critical to preventing deformity in leprosy patients. Patients should be assessed routinely during and after MDT treatment for peripheral nerve function and damage [50, 53]. Steroids have been used to treat neuropathy in leprosy since the 1970s [53]. Corticosteroids are administered to treat Type 1 and 2 leprosy reactions with a treatment duration of more than 12 weeks [9]. Prednisolone at a dose of 40–60 mg daily is recommended for treating sensory loss and muscle weakness in Type 1 reactions. For treating Type 2 reactions, thalidomide is administered at a dose of 400 mg daily [50, 54, 55]. It is important to monitor the response of patients to steroid treatments, and alternative therapies would be required to overcome the non-responsiveness and any adverse effects. In some cases, intolerance and adverse side effects are observed with corticosteroid treatments. A case study by Biosca et al. [55] investigated the use of methotrexate at a low dose of 5–7.5 mg per week as an alternative to corticosteroid treatment, which had adverse effects (such as insulin-dependent diabetes, hypertension, heart failure, depression, polyphagia, centripetal obesity and facial plethora) on a 58-year-old patient suffering from borderline lepromatous leprosy. Methotrexate improved Type 1 reaction treatment in this patient; skin lesions were reduced, and bacterial index was reduced from 5+ to 1+ [55].

Prevention of leprosy infection requires appropriate monitoring and management of the transmission process and tracing contacts of both symptomatic and asymptomatic individuals. There are several risk factors for close contacts of patients to acquire leprosy such as genetic relationship (children, parents, siblings) and physical distance (living under the same roof and next-door neighbours). A cohort study of 1037 newly diagnosed patients and their 21,870 contacts identified age of the contact and disease classification of the patient as two other factors, in addition to genetic relationship and physical distance associated with the risk of the contact in acquiring leprosy [56]. Contacts with older ages and those that have been in close contact with paucibacillary (2–5 lesions) and multibacillary patients are at a higher risk in acquiring the disease [56]. Moet et al. [56] recommended contact surveys to be extended beyond the household contacts to neighbours and consanguineous relatives of patients with paucibacillary (2–5 lesions) and multibacillary leprosy. There is a need to develop better diagnostic approaches to detect early-stage infection in contacts. MDT alone is insufficient to prevent new cases of leprosy or relapse. Follow-up post-treatment is important to evaluate the efficacy of MDT. According to Smith et al. [44], symptomatic contacts should be given the MDT regimen, and a combination of chemoprophylaxis and rifampicin was

recommended for asymptomatic contacts. Rifampicin used at a single dose for contacts in a randomized control trial provided a protective efficacy of ~60% [57]. Administration of immunoprophylaxis with Bacillus Calmette-Guérin (BCG) vaccination along with single dose of rifampicin had a greater protective efficacy of ~80% [57].

3.5 Challenges and Future Direction

3.5.1 The WHO Roadmap

According to the current WHO strategy to end leprosy by 2030, the following roadmap and targets are outlined: 120 countries with zero new autochthonous cases; 70% reduction in annual number of new cases detected; 90% reduction in rate per million population of new cases with grade 2 disability; 90% reduction in rate per million children of new child cases with leprosy [5, 58]. To achieve these targets, integrated efforts and national and international partnerships are required. For example, the WHO has listed political commitment for resources, engaging stakeholders; improving healthcare systems, surveillance and data management; and monitoring transmission, treatment and drug reactions, research and innovation for developing vaccines, preventative chemotherapy and management of leprosy reactions, neuritis and disabilities. It is important to develop interventions to monitor and reduce discrimination and stigma associated with leprosy-affected individuals [5, 58].

To improve diagnosis, treatment, management and monitoring of leprosy, a government and public/private sector intervention is required to meet the political, health and financial requirements to tackle the endemic. Interventions such as school surveys and epidemiological mapping can provide an alternative for systematic contact tracing [44, 57]. Leprosy education in schools such as signs and symptoms of the disease could provide a way of increasing social and disease awareness in endemic regions. Screening of school children with the support of healthcare systems could enhance case detection in families and communities [59, 60].

Poverty and socio-economic status are major risk factors for leprosy, and complete elimination of leprosy will require addressing these factors in affected communities [9, 22, 61]. There are multiple social determinants such as undernutrition and poverty in the transmission of leprosy. Poverty and leprosy are linked; disease transmission is higher at an individual level, such as living in a crowded household, and at community level, such as living in an endemic area with high population [36, 49, 62]. People living in poor conditions with inadequate housing, contaminated water, insufficient diet and co-infections or other diseases affecting the immune system are at a high risk of acquiring leprosy [22]. Implementation of poverty reduction programmes such as identification of factors responsible for poverty and inequality will need to be incorporated into public health approaches to eliminate leprosy [36]. Measurement of detection rates provides information about the known

prevalence of the disease and helps to implement control measures. Between 2005 and 2007, Penna et al. [63] used spatial span statistics to demonstrate that detection rates varied significantly in the north, north-east and central-west endemic regions of Brazil. The study concluded a time-dependent behaviour of case detection across the three regions, which may be attributed to the ease of access to primary healthcare [63]. Identification of such spatial distribution of leprosy prevalence will help to implement control measures. Chemoprophylaxis and immunoprophylaxis of "contacts" of leprosy patients could be used routinely in referral centres to break any transmission chain [10, 57, 64].

The services for diagnosis, treatment, care, rehabilitation and management for leprosy reactions need to be made easily and equally accessible to patients [10]. The main challenge for under-developed countries is that patients suffer delays in detection, treatment and care. Nerve damage can occur any time before, during or after treatment, and the degree of damage reflects the delay between the onset of symptoms and diagnosis, which can occur over many years [10]. A survey conducted by Raffe et al. [65] amongst patients in Nepal revealed a delay of up to 24 months from detection to receiving drugs such as corticosteroids for treating leprosy reactions. Raffe et al. [65] found inconsistencies in drug availabilities and treatment follow-ups. Research on epidemiology and clinical trials would need to adopt standardized tools, so that the outcomes from various research studies can be compared. Genetic studies on heterogenous populations could identify risk factors for the development of disease and neuropathy across different endemic regions of the world [53]. Identification of biomarkers of the disease would lead to rapid diagnosis and early treatment to prevent nerve damage and deformity [66].

3.5.2 Stigma and Discrimination

Stigma and discrimination are two major problems faced by leprosy patients. "The biggest disease today is not leprosy or tuberculosis, but rather the feeling of being unwanted" (Mother Teresa). The stigma associated with leprosy is the perception of physical deformity that leads to social discrimination and reduced opportunities [67]. The stigma and disabilities in individuals with leprosy patients lead to many devastating problems such as loss of employment, community rejection and in some cases forced isolation [36]. In endemic nations such as Brazil and India, leprosy-affected individuals are isolated into communities because of the lack of housing and employment opportunities [66, 68–70]. There is a need to place laws against discrimination to fight the stigma of leprosy and to adopt measures such as introducing patients with leprosy into the community [36]. A way to remove stigma and change perception is to eliminate the fear and prejudice about deformity through education about the disease in communities. An understanding and knowledge of leprosy will reduce misconceptions around the cause, transmission and treatment of the disease [67]. The effects of stigma and discrimination are greater in women patients than in men [67, 71]. Social awareness, health programmes and future

research are needed to identify the factors contributing to gender inequalities and to improve women's compliance with anti-leprosy therapy [12]. Alves et al. [72] reviewed the importance of having knowledge, information and training about leprosy in education and in healthcare settings to improve patient care. In particular, the involvement of dermatologists in training sessions for healthcare professionals working at different levels and in education such as undergraduate courses is essential to raise awareness about this neglected disease and to remove social stigma and discrimination of affected individuals. The role of dermatologists is important for leprosy patient treatment and care as they can identify and treat deformities and skin lesions and assure patients about the treatment and cure [73]. Programmes that involve training of dermatologists to assess neurological damage, sensory testing, use of monofilaments and physiotherapy has been suggested for improving leprosy treatment and patient care [50]. The current COVID-19 pandemic had adverse effects on other infectious diseases including leprosy in terms of care, disease control, treatment and management. Leprosy patients may suffer from elevated reactions because of COVID-19 infection. In addition, leprosy treatment may interfere with inflammatory responses and make leprosy patients more susceptible to contracting COVID-19 [74].

3.5.3 Alternative Therapies

These are needed to overcome the limitations of current regimens [44]. There are commonly associated side effects with the current MDT such as haemolytic anaemia, hepatotoxicity, gastrointestinal symptoms, headaches, dizziness, renal failure and pigmentation [38, 53, 75]. New drugs such as ofloxacin and minocycline have reduced adverse effects and have enhanced bactericidal activity in mice and in humans [53]. Multidrug-resistant strains exhibiting resistance to dapsone, rifampicin and in some cases fluoroquinolone have emerged and added to the threat of leprosy. Dapsone resistance in *M. leprae* is due to a mutation in the dihydropteroate synthase, *folP1* gene; rifampicin resistance is due to mutations in the sub-unit B of the RNA polymerase *rpoB* gene; quinolone resistance is due to mutation in the DNA gyrase *gyrA* gene [9]. There are adverse effects also with steroid therapy such as bruising, muscle weakness, peripheral neuropathy, teratogenicity, drowsiness, mood disorder and insulin resistance [38].

3.5.4 Animal and Ex Vivo Models

The inability to culture *M. leprae* in vitro has limited research on this pathogen [2]. There is no suitable animal model, and disease pathogenesis in mice is different to that in humans, and the use of armadillos is not practical for drug and vaccine testing [10]. There have been some efforts to identify the physiology and metabolism of *M. leprae*

during its intracellular growth in human host cells [2, 18]. Borah et al. [18] used isotopic tracing in a *M. leprae*-Schwann cell model and demonstrated that *M. leprae* used host glucose pools to synthesize amino acids during infection. Although the metabolic profile of the pathogen could be predicted in this ex vivo model system, the intracellular metabolic fluxes of the pathogen and the vulnerable metabolic nodes that can be targeted for novel drug development could not be measured.

The inability to culture *M. leprae* in vitro has also hampered investigations on drug resistance and screening for new drugs. Mouse foot pads have been the only feasible method to measure drug susceptibility of a *M. leprae* strain [9]. However, using mouse foot pads for *M. leprae* cultivation is lengthy and technically challenging and is therefore of limited use for studying drug resistance and for compound screening. New antibiotic therapy such as bedaquiline (newly approved for tuberculosis treatment) has shown similar anti-leprosy efficacy as rifampicin in mouse models, but clinical trials on leprosy patients are outstanding.

3.5.5 Diagnostics

PCR as a diagnostic tool is one of the most reliable and robust techniques. However, PCR primer targets, amplicon sizes and primers need to be standardized across various diagnostic settings and reference centres to achieve comparable epidemiological data across endemic regions of the world. Real-time PCR has been used for diagnostic purposes and holds greater promise for sensitive detection than conventional PCR [64]. However, the equipment and reagents needed for this technique are expensive, and the availability of appropriate laboratory facilities such as storage of RNA at −80 °C for analysis will need to be considered. Recent research efforts have made breakthroughs in developing sensitive and efficient techniques such as nanotechnology-based biosensors and imaging. However, we need to consider the applicability of such tools across various reference centres and laboratories in endemic regions and if they are cost effective.

3.5.6 M. leprae *Vaccine*

There is no specific vaccine against *M. leprae*, and this makes disease prevention a major challenge. There are several bottlenecks for leprosy vaccine research, including our incomplete knowledge about the immunological processes that are responsible for pathogenesis and nerve damage. Identification of *M. leprae* antigens is required to develop a leprosy-specific vaccine. The available information about the *M. leprae* genome enables the engineering of antigens that can be expressed in fast-growing bacteria and their follow-on assessment for vaccine development. Young et al. [76] used such a strategy and constructed a *M. leprae* recombinant DNA library using bacteriophage λgt11 to drive recombinant DNA expression in

Escherichia coli. Antigens were isolated from the recombinant DNA library using monoclonal antibodies that recognized the *M. leprae* epitopes produced in *E. coli* [76]. In addition to identification of suitable antigens, extensive research will be required to identify the complex immunoregulatory mechanisms to avoid any immune reactions that can elicit nerve injury from exposure to antigens. The currently used preventative strategy that includes using BCG vaccine needs reviewing, and more research is needed to improve the efficacy of this vaccine. Multiple trials have highlighted ambiguities in the efficacy of BCG vaccine to protect against leprosy [1, 75]. For example, a low protection of 20% was reported in Myanmar, and a high protection rate of 80% was reported in Uganda [1].

Vaccine development for leprosy has been severely hampered by the limited research on pathogen biology, mainly due to an inability to cultivate *M. leprae* in vitro. Recently, Borah et al. [2] provided a mixture of nutrients that could be used a starting point in the formulation of an axenic growth medium for *M. leprae*. This study used genome-scale modelling to investigate in vitro growth of the pathogen and nutritional requirements through interrogation of RNA-seq data of the pathogen isolated from mouse foot pads [2]. The usefulness of this media is yet to be tested experimentally.

3.6 Conclusions

Leprosy remains an endemic disease despite the availability of MDT therapy. To eradicate leprosy completely, we need to develop alternative therapies to overcome the problems of drug resistance and drug-associated side effects. New interventions are required to tackle the current limitations in disease diagnosis, treatment, management and care. Acceleration of research focused on the pathogen's biology, and the nature of the host's cellular immune response is needed to devise therapeutics such as new vaccines for disease prevention and management.

Conflict of Interest The author declares no conflicts of interest.

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Chapter 4
Leprosy Vaccines: Developments for Prevention and Treatment

Hua Wang

Abstract Over 200,000 new leprosy cases are reported globally every year. A vaccine for leprosy can eliminate the debilitating, biblical, and stigmatised disease in the twenty-first century. Since the 1940s, many clinical studies have consistently shown that the BCG vaccine offers some level of protection but ranging between 18% and 90%. Throughout this time, different versions of BCG and new developments have resulted in new leprosy vaccine candidates and prevention strategies. Examples are the vaccine and drug combinatory therapy that has shown promise in decreasing transmission and the subunit vaccine candidate, LepVax, which has been shown to reduce bacterial count and delay nerve function impairment in animal models and safe in healthy adults in early studies. The WHO officially recommended the BCG vaccine as a leprosy vaccine in 2018, a century later after it was first used as a tuberculosis vaccine in 1921. However, a better leprosy vaccine and prevention strategy is still needed because we do not exactly know how *Mycobacterium leprae* spreads and causes neurological damage in leprosy patients. The history and latest developments in leprosy vaccines are explored in this chapter.

Keywords *Mycobacterium leprae* · BCG · Vaccine · LepVax · Drug treatments

4.1 Introduction

Leprosy is an age-old infectious disease that continues to be endemic in some regions of the Americas, Africa, and South-east Asia [1]. It is caused by the bacterium called *Mycobacterium leprae*, discovered by Gerhard Armauer Hansen in 1874 [2]. Hence, leprosy is also called Hansen's disease. Leprosy primarily affects the skin and peripheral nerves. Every year, over 200,000 new leprosy cases are reported globally [1]. In 2019, India, Brazil, and Indonesia accounted for 79% of the

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M. Christodoulides (ed.), *Vaccines for Neglected Pathogens: Strategies, Achievements and Challenges*, https://doi.org/10.1007/978-3-031-24355-4_4

Fig. 4.1 The hand of a leprosy patient (right) with terminal phalanges examined by a health worker (left) in Bhutan. (Image source: Wellcome Collection. The Leprosy Mission International. Attribution 4.0 International (CC BY 4.0))

202,166 newly registered leprosy cases [1]. A third of the diagnosed patients experience disabilities because of nerve damage (Fig. 4.1). Consequently, leprosy is the leading infectious cause of disability worldwide [3, 4], and an estimated three to four million people are living with disabilities caused by leprosy [5].

Multi-drug therapy (MDT)[1] introduced by the World Health Organization (WHO) in 1981 remains highly effective to cure leprosy, but early diagnosis and treatment are paramount to preventing permanent nerve damage that can progressively lead to deformity and disability. Alarmingly, cases of drug resistance and disease relapses have been reported [6–8]. There have been many leprosy vaccine candidates and a leprosy vaccine does exist: in 2018, the WHO recommended one dose of the Bacille Calmette-Guérin (BCG) vaccine for healthy neonates at the earliest opportunity to reduce the risk of leprosy in countries or settings where it is common [9] (Fig. 4.2). However, meta-data analyses of clinical trials found that the BCG vaccine has variable protection ranging from 18% to 90% against leprosy [10–12].

[1] Rifampicin, clofazimine, and dapsone.

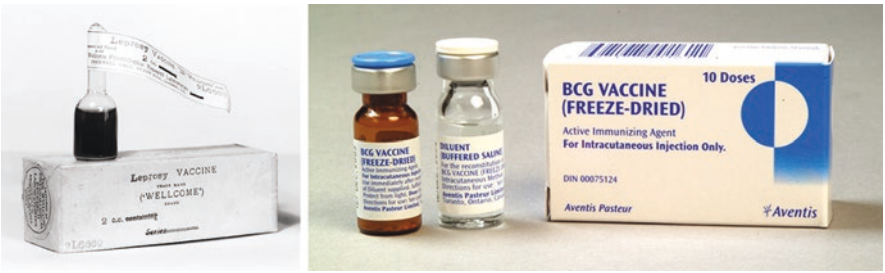

Fig. 4.2 Photographs of vaccines. **Left**: a leprosy vaccine of unknown composition produced by the Wellcome Physiological Research Laboratories in London, United Kingdom, circa 1978. (Image source: Wellcome Collection. Attribution 4.0 International (CC BY 4.0)). **Right:** a BCG vaccine to prevent tuberculosis, manufactured by Aventis Pasteur Canada in 2002. (Image source: Sanofi Pasteur Canada Archives)

In the next decade, the WHO Global Leprosy Strategy, 2021–2030, boldly aims 'towards zero leprosy' [5], focusing on interrupting transmission and achieving zero autochthonous cases. To ultimately bring leprosy to zero, an effective leprosy vaccine is essential and pivotal as part of the global strategic effort to eradicate the debilitating disease in the twenty-first century. In this chapter, we highlight the leprosy vaccine successes and investigate current leprosy vaccine developments and strategies.

4.2 The BCG Vaccine Has Variable Protection Against Leprosy

A misconception is that there is no leprosy vaccine. Studies show that the BCG vaccine used to prevent tuberculosis caused by *M. tuberculosis*, a bacterium closely related to *M. leprae*, offers more protection against leprosy than against tuberculosis [12, 13]!

The BCG vaccine is live attenuated *M. bovis* BCG strain. It was originally developed by Jean-Marie Camille Guérin and Léon Charles Albert Calmette in the early 1900s using attenuated *M. bovis*, a bacterium more closely related to *M. tuberculosis*, as an experimental vaccine to protect cattle from bovine tuberculosis [14]. In 1921, BCG was administered for the first time to a newborn baby in Paris to prevent human tuberculosis [15]. Now, BCG is one of the most widely used vaccines worldwide. In 1987, the Brazilian Ministry of Health recommended BCG vaccination or repeat vaccination of contacts to reduce the incidence of leprosy [16]. However, it was only in 2018 that leprosy was included in the WHO BCG vaccine program. Why did it take so long?

BCG vaccination against leprosy was first suggested by J. M. M. Fernandez in 1939 [17], who reported lepromin[2] conversation among children following BCG administration. It was postulated that BCG may confer some protection against leprosy due to possible common antigens between *M. bovis* BCG and *M. leprae*. The finding initiated five early small-scale trials in the 1950s in Brazil [18], India [19], Argentina [20], Venezuela [21], and Japan [22]. The trials showed that BCG vaccine has partial or wide protection (26–96%) against leprosy, but they had inadequate controls to draw any definitive conclusion. Furthermore, because leprosy has a long incubation period, on average of 5 or more years before the disease manifests in a clinically diagnosable form [5], long follow-ups and large-scale trials are needed to provide the necessary robust data. A plethora of clinical trials and community surveys then followed from the 1960s to the 2000s in Uganda [23–25], New Guinea [26, 27], India [11, 28–33], Myanmar (Burma) [34–38], Malawi [39–41], Kenya [42], Venezuela [43], Vietnam [44, 45], Brazil [46–53], and Indonesia [54]. Interestingly, the trial data in BCG protection were heterogeneous but showed protection wherever they were studied. To make sense of the heterogeneity, Setia et al. [10], Zodpey [11], and Merle et al. [12] carried out meta-data analyses and found that BCG protection against leprosy remained variable, between 18% and 90%. While the extrema are wide and with no definitive reasons for the heterogeneity, the authors agreed the trials consistently showed that BCG protects against leprosy. The authors commented that the variability between studies was due to several factors: study population (genetics, household contact, geography), environmental bacteria (cross-reaction), BCG dose number, *M. bovis* BCG diversity of sub-strains (genotype, phenotype, and vaccine manufacturer), nutrition, economic background, study bias, publication bias, and data collection/methodology.[3] These are ongoing factors to consider and to address for future studies.

In 2013, the WHO published new recommendations for manufacturing and evaluating BCG vaccine (for tuberculosis) [55]. In 2018, the WHO officially included leprosy in the single-dose BCG vaccination recommendation [9]. The inclusion of leprosy for BCG vaccination has huge implications for public health and research moving forward. It recognises that the BCG vaccine is important to prevent both tuberculosis and leprosy.

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[2] Lepromin is a skin test to classify the type of leprosy. It is carried out by an intradermal injection of inactivated *M. leprae* extract to check if the body responds to the bacterial antigens. An early reaction within 48 h (Fernández reaction) of erythema and induration indicates tuberculoid leprosy. A late reaction at 3 weeks (Mitsuda reaction) of nodule and indurated lesion indicates borderline tuberculoid leprosy. A lepromatous leprosy patient will not have a positive reaction.

[3] Numerous classifications have been used over the years to recognise leprosy as a disease that can be characterised on a spectrum due to the different immune responses. There are two classification systems that are commonly used, the Ridley and Jopling classification and the WHO classification. The Ridley and Jopling classification of leprosy was proposed in 1966 and is based on clinical and histopathologic observations: polar tuberculoid (TT), borderline tuberculoid (BT), borderline (BB), borderline lepromatous (BL), subpolar lepromatous (LLs), and polar lepromatous (LLp). In 1982, the WHO simplified the classification with paucibacillary (PB) leprosy that correlates with TT and BT and multibacillary (MB) leprosy that correlates with BB, BL, and LL.

Generally, a single dose of BCG showed higher protection against leprosy in young individuals. The BCG protection wanes over time but can last for 10–30 years [12, 13].

4.3 The Recombinant BCG Vaccines to Improve Efficacy Against Leprosy

Strategies to increase BCG vaccine immunogenicity include mixed vaccine with the addition of killed *M. leprae* or killed *M. vaccae* (an environmental mycobacterium) and recombinant BCG (rBCG) that expresses foreign molecules.

In three large clinical trial studies in Venezuela [56], Malawi [57], and India [58] comparing the efficacy between BCG and BCG + killed *M. leprae*, no significant difference in protection was found in Venezuela (56% vs. 54%) after 5-year follow-up and in Malawi (49% vs. 49%) after 6- to 9-year follow-up. However, an improvement was found in the Indian study (34% vs. 64%), after 4- to 7-year follow-up.

There are contradictory conjectures and a lack of studies on the premise that presensitisation to environmental mycobacteria may improve, diminish, or mask BCG immunogenicity [59–65]. In a small population vaccination trial of children in close contact with leprosy in Vietnam [66], BCG + killed *M. vaccae* was found to have a modest improvement in protection at 66%, compared to BCG (58%) and *M. vaccae* alone (55%). Further studies are needed but killed *M. leprae* is a scare material. *M. leprae* cannot be cultured with an artificial growth medium and is therefore difficult to isolate in large quantities for experimental studies. Currently, *M. leprae* cultivation requires animals such as mice [67–69] or armadillos [70–72], which is costly, with months of maintenance and growth time required to isolate sufficient bacterial samples.

The rBCG was first introduced by Stover et al. in 1991 [73] and enabled the expression of foreign antigens in BCG. In essence, BCG is immunogenic and is used as a vector to elicit specific immune responses guided by the foreign antigen. Since then, a repertoire of antigenic rBCG candidates have shown promise, in improving immunogenicity not only against tuberculosis [74] but also against viruses (respiratory syncytial virus [75, 76], human metapneumovirus [77], measles [78], human immunodeficiency virus type 1 [79, 80]); bladder cancer [81, 82]; the protozoa parasites *Leishmania* [83], *Plasmodium* spp. [84, 85], and *Toxoplasma gondii* [86]; and the bacteria *Streptococcus pneumoniae* [87], *Borrelia burgdorferi* [88], and *Bordetella pertussis* [89–91].

Several rBCG candidates have been developed for leprosy. Ohara et al. [92, 93] first constructed the rBCG/85A vaccine with *M. leprae* antigen Ag85A and then the rBCG/BA51 vaccine with *M. leprae* antigen Ag85 and *M. tuberculosis* major protein MBP51. They found that a repeat immunisation in C57BL/6 mice with rBCG/85A vaccine drastically inhibited the multiplication of *M. leprae* in the mouse footpads compared to control and BCG. This was improved with the rBCG/BA51

vaccine with one-dose immunisation inhibiting multiplication of *M. leprae* in the mouse footpads, compared to control and BCG in C57BL/6 and BALB/c mice. Furthermore, *M. leprae* lysate stimulated a higher level of interferon-γ (IFN-γ) production in spleen cells from rBCG/BA51 immunised C57BL/6 mice than BCG and rBCG/85A, an indication of improved host immune defence against *M. leprae*.

Makino et al. [94–96] constructed the rBCG-SM vaccine secreting *M. leprae* major membrane protein II (MMP-II). MMP-II is an antigen that can stimulate dendritic cells (DC) to produce interleukin (IL)-12 p70 and activate T cells to produce IFN-γ during the pro-inflammatory response important for adaptive and innate immunity. In the initial in vitro and ex vivo studies, the rBCG-SM-infected DC stimulated BCG-vaccinated donor naïve and memory type $CD4^+$ and $CD8^+$ T cells, to produce significantly higher levels of IFN-γ than the rBCG-vector and killed rBCG-SM. A similar outcome was found for IFN-γ production by splenic T cells of C57BL/6 mice infected with rBCG-SM. This was also later confirmed by Maeda et al. [97]. Furthermore, Makino et al. [95] found that rBCG-SM-infected DC increased intracellular production of perforin in $CD8^+$ T cells. Perforin is a pore-forming cytolytic protein produced by cytotoxic T cells that allows passive diffusion of pro-apoptotic proteases to enter target cells to control infection [98]. In a subsequent study, rBCG-SM-stimulated macrophages induced granulocyte-macrophage colony-stimulating factor (GM-CSF) cytokine production and inhibited the production of IL-10 [96]. The T cell activation was found to be dependent on GM-CSF production. IL-10 can block the reactivation of memory T cells. Therefore, the inhibition can potentially benefit anti-mycobacterial immune responses. This has been found in IL-10-deficient mice with a decreased bacterial burden [99].

Tabouret et al. [100] designed the rBCG::PGL-1 vaccine to study the role of PGL-1 in the pathogenesis of leprosy. PGL-1 is a species-specific phenolic glycolipid 1 from *M. leprae* with virulence, protective, and immunomodulatory properties. They found that rBCG::PGL-1 enhanced invasion via the complement receptor 3 (CR3) of human monocyte-derived macrophages, increased uptake by DCs, and impaired inflammatory responses. Recently, Doz-Deblauwe et al. [101] found that rBCG::PGL-1 enhanced CR3-mediated non-opsonic phagocytosis in polymorphonuclear neutrophils and DCs and activated Syk-calcineurin/nuclear factor of activated T cells signalling to rewire host cytokine responses to *M. leprae*. Although no *M. leprae* infection challenge was carried out, the insights on the PGL-1 could help rBCG vaccine development, by considering immune responses during leprosy pathogenesis and the mechanisms of nerve damage causation.

Horwitz et al. [102] designed the rBCG30 vaccine to overexpress *M. tuberculosis* 30 kDa major secretory protein antigen 85B, which they found to offer better protection than BCG against *M. tuberculosis* and *M. bovis* challenge in animal models. Gillis et al. [103] further evaluated rBCG30 and found that it could stimulate $CD4^+$ and $CD8^+$ in cytokine responses from BCG-immunised BALB/c mice and needed boosting with purified *M. tuberculosis* 30 kDa antigen 85B to reduce *M. leprae* burden in mouse footpads.

Now, there is only one rBCG vaccine in clinical trials, the VPM1002 vaccine. The clinical trial evaluations are in phases II and III for tuberculosis (ClinicalTrials.gov Identifier: NCT03152903, NCT04351685), in phases I and II for recurrent non-muscle invasive bladder cancer (ClinicalTrials.gov Identifier: NCT02371447), and in phase III for SARS-CoV-2 infection[4] (ClinicalTrials.gov Identifier: NCT04439045, NCT04387409). The VPM1002 vaccine has not been evaluated as a vaccine candidate for leprosy. The VPM1002 is a genetically modified BCG that has the urease C encoding gene replaced by the listeriolysin O encoding gene from *Listeria monocytogenes* [115–117]. Urease C neutralises phagosomes that contribute to mycobacteria survival, whereas listeriolysin O forms transmembrane β-barrel pores in the phagolysosome membrane. Therefore, VPM1002 can effectively release mycobacterial antigens into the cytosol to trigger immunogenic responses. The VPM1002 system can potentially be used and further modified as a leprosy vaccine. Now that BCG is more widely recognised as a vaccine for leprosy, this offers promise for rBCGs such as VPM1002, rBCG/85A, rBCG-SM, rBCG::PGL-b, and rBCG::PGL-1 and the tuberculosis rBCGs as leprosy vaccine candidates in clinical studies.

4.4 The Cross-Reactivity and Subunit Leprosy Vaccines

Other leprosy vaccine candidates besides the *M. bovis* BCG and rBCGs include (1) non-pathogenic or closely related *M. leprae* mycobacterium species to induce cross-reactivity such as the ICRC (Indian Cancer Research Centre bacilli), *M. vaccae*, *M. duvalii*, *M. welchii* (*M. w*) or *M. indicus pranii* (MIP) [118],[5] and *M. habana* and (2) recombinant protein subunits, such as the LEP-F1 + GLA-SE (LepVax), to induce target-specific immune responses. *M. vaccae*, as previously discussed, is like BCG in leprosy protection. *M. duvalii* is an early vaccine candidate proposed in 1974 [119] that showed some cross-reactivity. However, Shepard et al. in 1976

[4] The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is the causative agent of the 'coronavirus disease 2019' (COVID-19) outbreak. Before an effective COVID-19 vaccine was made available for the public, existing approved vaccines were assessed for COVID-19 mitigation. The BCG vaccine has been found to train the immune system to fight off infections caused by viruses and therefore an attractive candidate as a COVID-19 vaccine [104–109]. However, there is no direct evidence that BCG provides protection against COVID-19 in humans [110–113]. In mice and hamster studies, BCG vaccination provided no protection against SARS-CoV-2 [114]. The phase III clinical trial is a small population group evaluation to determine if BCG can mitigate COVID-19, before moving to phase IV clinical trial with a large population group to assess safety and efficacy. Clinical trials to assess the efficacy of BCG vaccination against COVID-19 are being performed around the world (ClinicalTrials.gov).

[5] Previously *M. welchii* (*M. w*). It was renamed to MIP in 2009 after its lineage and to avoid confusion with *M. tuberculosis*-W Beijing.

[120] later found that *M. duvalii* and *M. duvalii* + BCG offered less protection and no change in protection, respectively, when compared to BCG in mice footpad immunisation studies.

The *M. habana* vaccine was reported by Singh et al. [121, 122] to reduce *M. leprae* counts better than BCG in mice footpad immunisation studies. Furthermore, Singh et al. [123] found that *M. habana* induced a positive Mitsuda reaction in monkeys. Additionally, Chaturvedi et al. [124] identified *M. habana* proteins in the cell wall and cell membrane fractions that were recognised by leprosy antisera, and the 65 kDa protein [125] and 23 kDa proteins [126] were found to induce cell-mediated immune responses. The latest study identified two additional *M. habana* proteins, an enoyl-coenzyme A hydratase and antigen 85B, both recognised by leprosy antisera [127]. These proteins can be used in vaccine studies and as serodiagnosis tools. However, the *M. habana* efficacy as a leprosy vaccine remains uncertain. A small vaccination study of 31 lepromatous leprosy patients and 36 household contacts found positive lepromin reaction only after 15 weeks, but also had systemic side effects [128]. It is a short time frame to draw a conclusion considering that leprosy has a long incubation period. Therefore, further studies are required to understand the efficacy and the safety profile.

The ICRC vaccine is a gamma-radiation inactivated group of leprosy-derived cultivable slow-growing mycobacteria belonging to the *M. avium* complex isolated in 1958 from a leprosy patient [129–131]. Early immunological studies from 1974 to 1978 all demonstrated reactivity [132–134]. Bhide et al. [135] reported in 1978 that ICRC offered protection against *M. leprae* infection in the mouse footpad model. This led to small trials by Deo et al. [136] and Bhatki et al. in the early 1980s [137] that continued to show promising outcomes. ICRC resulted in negative to positive lepromin conversion in 58% of lepromatous leprosy patients and 91% of borderline lepromatous patients. Chaturvedi et al. [138] reported that ICRC has a dose-dependent lepromin conversion at eighth week (high dose and 1/30th dose resulted in 79% and 46% lepromin conversion, respectively) and resulted in >90% lepromin conversion in healthy subjects from household contacts of leprosy patients and non-contacts in a general population in Bombay at the end of 1 year; patients remained stable up to 3 years; and no nerve toxicity was reported, as hypersensitivity to *M. leprae* antigens can lead to nerve damage. In a large-scale comparative study in India, Gupte et al. [58] reported 66% protection by ICRC versus 34% protection by BCG after 4–7-year follow-up. Interestingly in the same comparative study, BCG combined with killed *M. leprae* offered 64% protection, similar to ICRC. A recent ICRC formula evaluation found that ICRC candidate strain C-44 is coated with human immunoglobulin G that may play a role in the immune responses [139].

The MIP vaccine was developed in the National Institute of Immunology, India, and showed promising early initial outcomes. Chaudhuri et al. [140] and Talwar

et al. [141] reported that 20 of the 32 patients had negative to positive lepromin reaction conversion after 4–6 weeks from a single administration and remained stable after 6–11 months. However, in the large-scale comparative study in India reported by Gupte et al. [58], MIP only offered 26% protection compared to 66% protection by ICRC, 34% protection by BCG, and 64% protection by BCG + killed *M. leprae*, after 4–7-year follow-up. In a double-blind immunoprophylactic trial conducted in an endemic area of Kanpur Dehat, Uttar Pradesh, Sharma et al. [142] showed that the low MIP protection was attributable to a decrease in protection over time and offered greater protection for contacts. They found that MIP had protective efficacy of 69%, 59%, and 39% at, 3-, 6-, and 9-year follow-up, respectively, for household contacts after the initial vaccination. Similarly, the protective efficacy was 68%, 60%, and 28% at 3-, 6-, and 9-year follow-up, respectively, for both patients and contacts after the initial vaccination. The MIP vaccine was less effective for patients: the protective efficacy was 43%, 31%, and 3% at 3-, 6-, and 9-year follow-up, respectively. However, smaller studies have found that MDT and MIP as immunotherapy for multibacillary leprosy patients could shorten recovery time, reduce bacterial load, clear granuloma, and reduce neuritis [143–147]. The MIP vaccine has received approval by the Drugs Controller General of India and the US Food and Drug Administration [148]. In 2017, the Indian Council for Medical Research launched a vaccine programme to eradicate leprosy in leprosy endemic districts [149–151]. The patients, family members, and contacts will receive two doses of autoclaved MIP at 6 months intervals. Studies are ongoing to evaluate the efficacy of MDT and MIP immunotherapy.

LepVax is the latest vaccine candidate moving in the clinical trial pipeline [152] (Fig. 4.3). LepVax is a defined subunit vaccine containing a chimeric recombinant protein (LEP-F1) consisting of a tandem linkage *of M. leprae* antigens ML2531, ML2380, ML2055, and ML2028 and a synthetic Toll-*like* receptor 4 (TLR4) agonist glucopyranosyl lipid adjuvant-stable emulsion (GLA-SE). In the *M. leprae* mouse challenge studies, LepVax raised an immune response not affected by prior BCG immunisation. Additionally, immunised mice infected with *M. leprae* had significantly fewer bacteria recovered in the mouse footpad experiments, compared to unimmunised control mice. After 12 months, the bacterial burden in immunised mice was approximately 85% lower than mice immunised with GLA-SE adjuvant formulation alone. Importantly, LepVax immunisation delayed motor nerve function impairment in *M. leprae*-infected nine-band armadillos, demonstrated as post-exposure immunoprophylaxis. LepVax dosage, safety, and immunogenicity parameters were evaluated in the phase 1a clinical trial on 24 healthy adult volunteers in the United States [153]. The study outcome published in 2020 concluded that LepVax was safe and immunogenic and LepVax will start phase 1b/2a clinical trial in 2022 to carry out the same evaluation in leprosy endemic regions (ClinicalTrials.gov Identifier: NCT03947437).

Fig. 4.3 A vial of LepVax (LEP-F1). The vaccine development is a partnership between the American Leprosy Missions and the Infectious Disease Research Institute (now Access to Advanced Health Institute) in Seattle, Washington, that started in 2002. (Image source: American Leprosy Missions)

4.5 Vaccine and Drug Combinatory Therapy

The combination of immunotherapy and chemotherapy can shorten leprosy treatment time and potentially improve the treatment outcome. When the WHO recommended MDT for leprosy in 1981, patients were required to be on the regimen for at least 2 years.[6] An early evaluation of the MIP vaccine candidate by Talwar et al. [154] found there was more rapid bacterial clearance in vaccinated patients who were also receiving MDT. Zaheer et al. [155] investigated if chemotherapy in combination with immunotherapy, i.e. MDT + MIP, could reduce the treatment time by

[6] The latest WHO 2018 'Guidelines for the Diagnosis, Treatment and Prevention of Leprosy' recommends 'a 3-drug regimen of rifampicin, dapsone and clofazimine for all leprosy patients, with a duration of treatment of 6 months for PB leprosy and 12 months for MB leprosy'.

inducing cell-mediated immune responses. They reported that MIP helped overall in the treatment; 13 of 31 BL and LL patients or multibacillary leprosy patients who received MDT and MIP were bacteriologically negative within 2 years, compared to 5 of 25 controls. The vaccinated patients had either upgraded in disease spectrum or were completely cleared of granuloma. Furthermore, 80% of vaccinated BL and LL patients had lepromin conversions, compared to 14% of the controls.

Sharma et al. [156] also reported a faster bacterial clearance for patients receiving both MDT and MIP within 2 or 3 years. They found that 90% of the vaccinated patients had negative to positive lepromin conversions compared to 38% of the placebo group, and the patients released from treatment had no incidence of relapses in a 5-year follow-up. They concluded that the addition of MIP to MDT could reduce treatment time from 4–5 years to 2–3 years. Kaur et al. [145] and Kamal et al. [144] have similarly reported that MDT + MIP improves treatment outcomes. Due to the long incubation period of leprosy, long-term follow-up is needed for the safety and efficacy of shortening the treatment time.

Katoch et al. [157] reported a comparative study between MIP and BCG with MDT. They found that the patient groups receiving MDT and MIP, or BCG, had no detectable viable bacilli in the local and distal sites by mouse footpad analysis, whereas viable bacilli were detected in the patients on MDT alone within 2 years. Additionally, patients receiving MDT and MIP, or BCG, also had accelerated granuloma clearance. As with the previous studies, they also concluded that the addition of immunotherapy to achieve negative bacteriology could reduce treatment time by about 40% and found no relapses in the 10–12 years post-treatment follow-up. Interestingly, MIP did perform slightly better than BCG in bacterial and granuloma clearance. In contrast, Narang et al. [147] found that although MIP or BCG improved clinical outcomes, BCG performed better than MIP. However, immunisation by BCG on its own of close contacts of leprosy patients has been reported to precipitate PB leprosy on potentially asymptomatic infected or previously exposed individuals [158–160].

The addition of immunotherapy to patients under MDT generally shows positive clinical outcomes. What about close contacts of leprosy patients and transmission? It has been shown that a single dose of rifampicin (one of the drugs in the leprosy MDT) to close contacts of patients is 57% effective at preventing leprosy within 2 years, but with no effect after 2 years [161, 162]. Richardus et al. [163, 164] investigated whether chemoprophylaxis with rifampicin and immunoprophylaxis with BCG on contacts of leprosy patients could reduce transmission. Although they found a 42% reduction in PB leprosy cases of close contacts of leprosy patients in the first year, they noted that it was not statistically significant, due to low patient cases. Thus, more studies are needed to understand the clinical benefits of the combination of MIP or BCG with MDT on reducing transmission.

The Leprosy Post-Exposure Prophylaxis (LPEP) programme (Fig. 4.4), funded and coordinated by Novartis Foundation, launched in 2015, and ended in 2018, was established to explore contact tracing and to evaluate single-dose rifampicin post-exposure prophylaxis (SDR-PEP) to reduce and curb transmission in Brazil, Cambodia, India, Indonesia, Myanmar, Nepal, Sri Lanka, and Tanzania [165–167]. The programme outcome varied in countries that showed an increase in the number

Fig. 4.4 Health education in Nepal about leprosy and SDR-PEP for the contacts of a leprosy patient (household contacts and neighbours) to get their consent before screening and SDR-PEP administration in the community. (Photograph: Tom Bradley/Netherlands Leprosy Relief)

of detected cases in the first year but followed by a reduction in cases, indicating a reduction in leprosy incidence. Furthermore, a 2040 projection model indicates that LPEP could have a huge impact in interrupting *M. leprae* transmission. Future programmes to include immunotherapy may demonstrate greater impact.

Overall, the studies indicate that a combination of chemotherapy and immunotherapy is a powerful therapeutic intervention to treat leprosy patients and potentially as a control strategy to reduce transmission.

4.6 Conclusion and Vaccine Outlook

The current BCG vaccine for leprosy offers only partial protection. Leprosy is not eliminated, despite early 'elimination' declaration by WHO defined as 'the reduction of prevalence to a level below one case per 10,000 population' [168]. This has drawn major criticism, because it changed public perception and shifted away the resources and financial support needed to carry out fundamental and long-term epidemiological studies [169–172]. *M. leprae* remains a bacterium that requires animals for cultivation. We still do not exactly know how *M. leprae* transmission occurs, how it induces immune responses, and what is the mechanism underlying the nerve damage.

Table 4.1 A list of leprosy vaccine candidates and treatment and transmission reduction strategies

Candidates	Purpose
BCG	Vaccine with live attenuated bacteria
BCG + *M. leprae*	Vaccines with live attenuated and killed bacteria to improve immunogenicity
BCG + *M. vaccae*	
BCG + *M. duvalii*	
rBCG/85A	Vaccines with live attenuated bacteria expressing recombinant proteins to improve immunogenicity
rBCG-SM	
rBCG::PGL-1	
rBCG::PGL-b	
rBCG30	
VPM1002	
ICRC	Vaccines with environmental bacteria for cross-reactivity
M. vaccae	
M. duvalii	
M. welchii or *M. indicus pranii* (MIP)	
M. habana	
LepVax, LEP-F1	Vaccine with subunit proteins
MDT + MIP	Treatment consists of vaccine and drugs to reduce treatment time
MDT + BCG	
Rifampicin	Prevention for close contacts

The recognition that BCG is a leprosy vaccine by the WHO is a critical admission that can help push current vaccine research forwards and support social changes. Historically, leprosy sufferers are stigmatised and discriminated against by their community [5, 173]. Unfortunately, stigma and discrimination are still happening today. According to the WHO, there are 127 discriminatory laws in 22 countries based on leprosy [5]. A widely recognised leprosy vaccine that is already in use can change the dialogues within communities and perceptions about the disease. Table 4.1 summarises the leprosy vaccines and strategies to reduce treatment time and transmission discussed in this chapter. The development of rBCGs, killed related mycobacteria, and subunit recombinant vaccine candidates is showing promise in clinical trials for the future, with an improved and effective leprosy vaccine as immunoprophylaxis, a supplement to chemotherapy, and post-exposure immunoprophylaxis.

Acknowledgements H.W. gratefully acknowledges the support from the University of Glasgow pump-prime funds to develop an independent research career and the research funding support from The Royal Society (Research Grant: RGS\R2\192167) and The Academy of Medical Sciences (Springboard: SBF007\100061). The Springboard scheme is supported by the Wellcome Trust; the British Heart Foundation; the Government Department of Business, Energy, and Industrial Strategy; and Diabetes UK.

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Chapter 5
Current Progress and Prospects for a Buruli Ulcer Vaccine

Justice Boakye-Appiah, Belinda Hall, Rajko Reljic, and Rachel E. Simmonds

Abstract Buruli ulcer (BU), one of the skin-related neglected tropical diseases (skin NTDs), is a necrotizing and disabling cutaneous disease caused by subcutaneous infection with *Mycobacterium ulcerans.* Leading on from the World Health Organization's (WHO) establishment of a global BU initiative in 1998, >67,000 cases of BU have been reported from over 32 countries, mostly from West Africa and Australia. While treatment is currently in the transition period from rifampicin plus streptomycin (injection) to an all-oral regimen, it cannot hope to eradicate this opportunistic environmental pathogen. *M. ulcerans* is genetically very similar to related pathogenic organisms *M. marinum*, *M. leprae* and *M. tuberculosis.* However, *M. ulcerans* carries a unique megaplasmid, pMUM001, encoding the biosynthetic machinery responsible for production of a lipid-like exotoxin virulence factor, mycolactone. This diffusible compound causes the substantial divergence in BU's pathogenic aetiology from other mycobacterial infections. Hence, mycolactone is cytotoxic and immunosuppressive and causes vascular dysfunction in infected skin. A major recent advance in our understanding of BU pathogenesis has been agreement on the mycolactone's mechanism of action in host cells, targeting the Sec61 translocon during a major step in secretory and membrane protein biogenesis. While vaccine development for all mycobacteria has been challenging, mycolactone production likely presents a particular challenge in the development of a BU vaccine. The live-attenuated vaccine BCG is known to provide only partial and transient protection in humans but provides a convenient baseline in mouse preclinical stud-

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M. Christodoulides (ed.), *Vaccines for Neglected Pathogens: Strategies, Achievements and Challenges*, https://doi.org/10.1007/978-3-031-24355-4_5

ies where it can delay, but not prevent, disease progression. No experimental vaccine strategy has yet conferred greater protection than BCG. However, there is now the prospect of developing a vaccine against mycolactone itself, which may provide hope for the future.

Keywords *Mycobacterium ulcerans* · Buruli ulcer · Vaccine · Mycolactone · Whole cell · Subunit

5.1 Buruli Ulcer

The neglected tropical disease (NTD) Buruli ulcer (BU) is caused by subcutaneous infection with *Mycobacterium ulcerans*, resulting in necrosis of subcutaneous fatty tissue and the formation of ulcers with undermined edges which can extend to 15% of body surface area [1]. Much of its obscurity may be attributed to the fact that it predominantly affects the poor [2, 3], usually in remote rural areas with limited access to health services [4, 5]. Hence, while BU is considered a rare disease on a global scale, its impact on endemic communities should not be underestimated. BU is associated with social stigma [6] and presents a large financial [7, 8] and psychological [9] burden to patients and their care-givers, especially since most patients are young teenagers. A major global intervention came in 1998 when the WHO launched its Global BU Initiative (GBUI). This served as a forum for disease control and research efforts. Its success is clear from the global decrease in BU prevalence since 2010 [4], although this does not take into account under-reporting in countries without effective national control programmes [5]. Moreover, it brought about much of the research described in this chapter, and its success has underpinned the WHO's most recent integrated approach to control all skin NTDs [10].

5.1.1 Epidemiology and Transmission

Infections that were most likely BU were first described by Sir Albert Cook at the turn of the twentieth century [11]. However, it was not until 1948 that the causative organism was identified by Peter MacCallum [12], due to the fortuitous breakdown of an incubator. To date, a total of >67,000 cases of BU have been reported worldwide in 32 countries including Japan, Papua New Guinea and Central and South America. At present, the highest prevalence of BU is in West Africa although there has recently been a worrying increase in cases in Australia's state of Victoria [13]. The disease burden is difficult to objectively assess in many endemic countries (especially those that are lower-middle-income countries or least developed countries) due to the remote location of affected communities and lack of credible health system data [5] (Fig. 5.1).

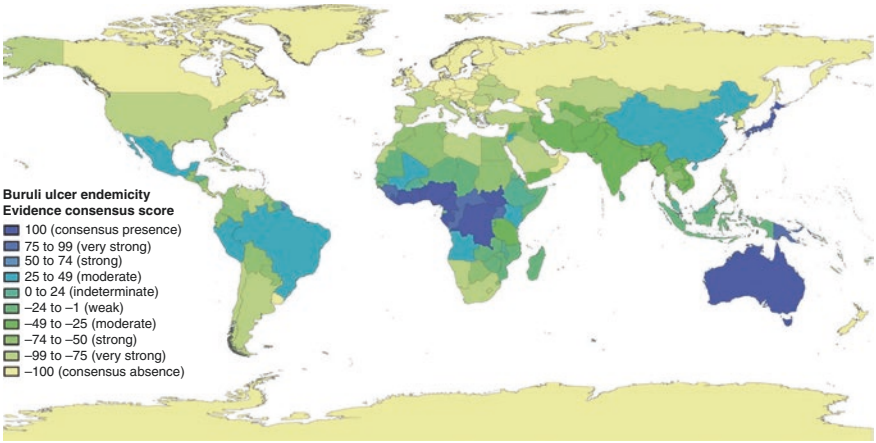

Fig. 5.1 Evidence consensus for Buruli ulcer presence and absence worldwide. These findings were based on a comprehensive systematic review of peer-reviewed evidence in the scientific literature as well as surveillance and laboratory data from country programmes. (From Simpson et al. [5])

The exact mode of transmission of *M. ulcerans* is unknown; however it seems almost certain that this opportunistic environmental pathogen enters the body by mechanical transfer. No incidences of person-to-person transmission have been reported, with the notable exception of a case involving a human bite [14]. Cases linked to other types of minor trauma, such as abrasions and even snake bite [15, 16], suggest that skin surface contamination may be important. Insects have been implicated in both Australia and West Africa, but this topic remains controversial and may vary between different environments (recently reviewed in [17–19]).

5.1.2 Clinical Presentations and Current Treatments

BU presents clinically as painless skin lesion(s) in one of five forms, nodule, papule, plaque or oedema and ulcers (Fig. 5.2), and, in some cases, bone involvement can result in osteomyelitis [20]. Nodules/papules are the first sign of (localised) infection, and the WHO has categorised more advanced lesions according to severity, with Category I including single small ulcers <5 cm diameter; Category II including larger ulcers of 5–15 cm, as well as plaques and oedema; and Category III including large ulcers >15 cm, multiple ulcers or ulcers that have spread to include particularly sensitive sites such as the eyes, bones, joints or genitals [20]. The more serious manifestations are much more common in African countries than Australia, most likely due to differences in health infrastructure.

Hence, the most common presentation of BU is a necrotising skin ulcer [1, 20]. Typically, the edges of these ulcers are ‘undermined’ due to subcutaneous necrosis,

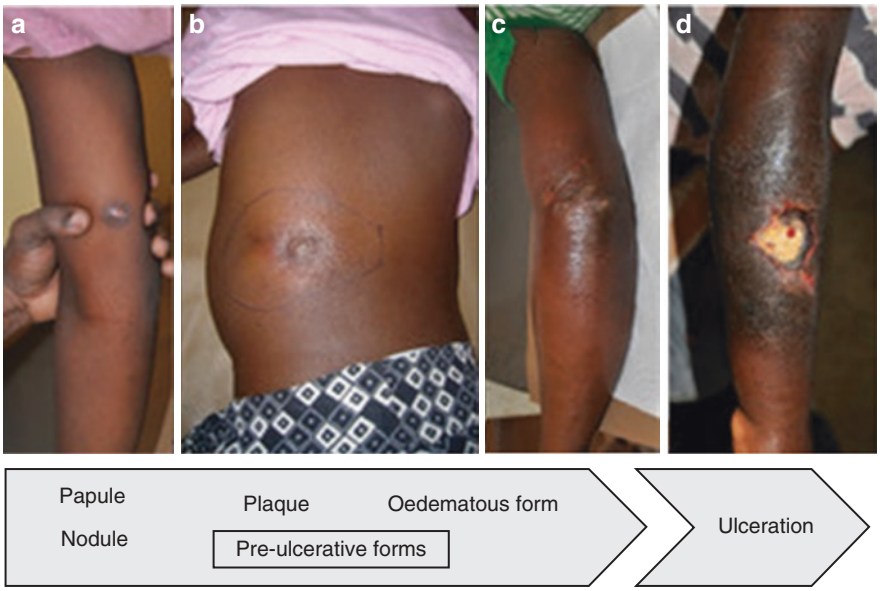

Fig. 5.2 Clinical presentations of Buruli ulcer. BU can present clinically through a spectrum including a papule or nodule a painless palpable lump under the skin; (**a**) or plaque an area of tough necrotic skin which can be of any size, but the epidermis remains intact; (**b**), which can also be found with or without oedema (**c**). If the epidermis breaks down, ulcers of varying sizes can form, but due to the characteristic undermined edges (**d**), the true lesion size is often much larger than the ulcerated region. Images by kind courtesy of Prof Richard Phillips. (Kumasi Centre for Collaborative Research, Ghana)

meaning that ulcers are frequently larger than the area of dermal breakdown. Remarkably, given the extensive tissue loss that can occur, BU patients are usually otherwise well, rarely experiencing the severe pain that might be expected based on the physical appearance of the lesions.

Until 2004, the only medical intervention available was radical surgery, either in the form of wide excision and debridement some 10 cm beyond the extent of affected tissue, or even limb amputation [21]. Although *M. ulcerans* was known to be sensitive to a range of antimycobacterial antibiotics from an early stage [22–25], a key success of the WHO GBUI was the testing [26] and introduction [20] of effective antibiotic regimens. Initially, a combination of rifampicin and streptomycin (for 8 weeks) was used [27–29]. To tackle the poor compliance and ototoxicity from injectable streptomycin [30], this is now transitioning to an all-oral combination including clarithromycin [31]. While antibiotic therapy can cause so-called paradoxical reactions, where lesions can appear to worsen or appear in new locations, this should not be confused as treatment failure [1, 32]. Fortunately, antimicrobial resistance has not yet been reported in Buruli ulcer, which supports its classification as an opportunistic environmental pathogen and argues against ‘re-seeding’ of environmental niches from patient lesions.

Since antibiotics were introduced, there has been a significant reduction in surgical intervention for BU [33, 34]. Indeed, antibiotic treatment of BU at an early nodule/papule stage can result in healing before ulceration [35]. Therefore, surgery is now usually reserved for patients with severe disease [36], although clinical decision-making varies from clinic to clinic [37]. With or without surgery, BU comes with a high burden of disability and deformity due to the extensive tissue damage caused and the risk of contractures [20]. Careful wound management and physiotherapy are critical to minimise these risks. Consequently, improved diagnostic tools and public health measures aimed at early detection of BU are now a key goal of the WHO.

5.2 *Mycobacterium ulcerans*

The closest genetic relative of *M. ulcerans* is *M. marinum*, another pathogenic mycobacterium that causes 'fish tank granuloma', to which its genome is 98% identical [38, 39]. Despite this phylogenetic similarity, major changes in the *M. ulcerans* genome have altered its interaction with the host [38, 39]. First, 'reductive evolution' has occurred with pseudogene accumulation and gene deletion due to the accumulation of single nucleotide polymorphisms (SNPs). Second, two different insertion sequences (IS*2404* and IS*2606* [40]) have proliferated throughout the genome leading to disruption and loss of virulence regions. These include the well-characterised Early Secreted Antigenic Target 6 kDa (ESAT-6) secretion system 1 (ESX-1) that allows other mycobacteria to escape the phagosome [41, 42]. Third, it has acquired a plasmid, pMUM, which carries the only virulence genes identified to date [43]. These genes encode the polyketide synthases and accessory proteins that manufacture mycolactone. Notably, there are two lineages of *M. ulcerans*, which may explain some of the divergence between findings in Africa and Australia [44], including subtle differences in mycolactone structure and function [45].

5.2.1 Mycolactone

The identification of mycolactone [46], and the subsequent understanding of its effects on host cells and tissues, has been critical to the understanding of BU pathogenesis [47]. Mycolactone is a lipid-like molecule with a 12-membered lactone ring that can vary in the hydroxylation and methylation pattern on the longer polyketide side chain. The most potent congener found in most African strains is known as mycolactone A/B (Fig. 5.3a). Purified mycolactone can replicate the ulceration caused by *M. ulcerans* [46, 49], and strains that cannot produce it lose their virulence [50]. To date, the best characterised consequences of mycolactone exposure are cytopathic/cytotoxic effects and immune suppression, although vascular dysfunction has also recently been described [51, 52]. All of these have now been

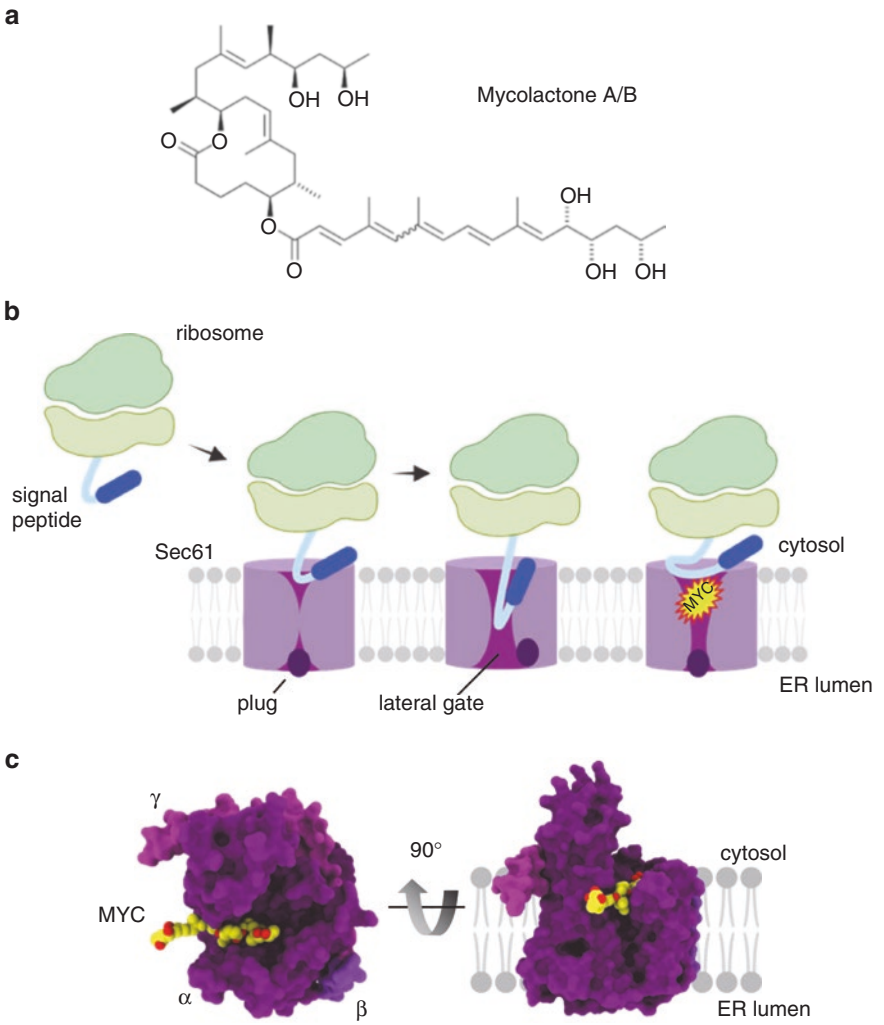

Fig. 5.3 Structure and function of mycolactone, the *M. ulcerans* virulence exotoxin. (**a**) The chemical structure of mycolactone. (**b**) Sec61-dependent co-translational translocation of proteins into the ER involves recognition of a signal peptide or signal anchor by the signal recognition particle and its receptor (not shown), which transfers it to Sec61. This results in reorganization of the translocon and movement of the Sec61α plug domain, opening the central pore and allowing transit of the translating protein into the ER. Mycolactone binds Sec61α, preventing the signal peptide from accessing its binding site at the lateral gate. Although the lateral gate is open, the plug remains closed, and the translocon is locked in an inactive state. (**c**) The structure of inhibited Sec61, with mycolactone bound inside the lateral gate of Sec61α. Dark purple, Sec61α; light purple, Sec61β; pink. Sec61γ; yellow/red, mycolactone (from PDB:6Z3T). Two views are shown, looking down from the cytosol towards the ER and from the side, as in (**a**). (Adapted from [48])

shown to be dependent on activity of mycolactone against the normal function of the Sec61 translocon [53–57], which is the main entry point to the canonical secretory pathway of secreted proteins, type I and type II transmembrane proteins and multi-pass membrane proteins [58, 59] (Fig. 5.3b). Indeed, the structure of mycolactone bound to Sec61 has recently been solved (Fig. 5.3c) [57].

Mycolactone has a cytopathic effect on cultured mammalian cells characterized by cytoskeletal rearrangement, followed by rounding up and detachment from tissue culture plates [50, 60, 61]. It is also cytotoxic and induces apoptosis several days after exposure [50, 62–64] as well as cell cycle arrest in G_0/G_1 phase [46, 65]. We now know that the pathway to apoptosis involves changes in intracellular Ca^{2+} gradients [66, 67], the so-called integrated stress response [64, 68], and autophagy [69]. Cells carrying mutations in the gene encoding the major Sec61 subunit, Sec61α, are highly resistant to the cytopathic and cytotoxic effects of mycolactone and can proliferate in its presence [54, 57, 64, 67].

Mycolactone's immunosuppressive effects are wide-ranging, which is unsurprising considering its inhibition of Sec61 and the consequent loss of secretory proteins (most cytokines and chemokines) and receptors (constitutive and induced), which normally act in elegant concert to mediate both innate and adaptive immune responses [48, 70]. Mycolactone has been shown to strongly suppress innate immunity by limiting phagocytosis [71] and inflammatory responses by monocytes, macrophages and dendritic cells [53, 71–74]. It limits adaptive immunity by suppressing both antigen presentation by dendritic cells and T cell activation [75–77]. Specific evidence demonstrated Sec61-dependent effects on TNF, IL-6 and Cox-2 production, antigen processing mediated by invariant chain during MHC class II processing and T cell activation [53, 54, 78]. A notable exception here is the recent discovery that mycolactone can induce the production of the cytokine IL-1β, by acting as the 'second signal' during inflammasome activation [48, 79]. This observation is entirely in line with Sec61 inhibition by mycolactone, since IL-1β does not use the canonical secretory pathway for its production.

Yet, there are no drugs described that can counteract the effect of mycolactone on the Sec61 translocon. Indeed, other inhibitors of Sec61 recapitulate the effects of mycolactone [80, 81] (R Simmonds, unpublished observation), and so this is not a viable treatment option. However, inhibitors of apoptosis such as Z-VAD-FMK, or genetic deletion of Bim, are able to at least delay cytotoxicity, both *in vitro* and *in vivo* [63, 82].

5.2.2 Immune Response to M. ulcerans *Infection*

The immunosuppressive properties of mycolactone described above are thought to explain the histopathology of BU lesions. Here, the lesions display coagulative necrosis, with clusters of extracellular acid-fast bacilli visible at the base of the subcutaneous tissue, and epidermal hyperplasia [3]. The cellular infiltrate of immune cells, normally expected in a microbial infection, is reduced and limited to

the periphery of the lesion. In their elegant work, Ruf and Pluschke have shown that, in both humans and pigs, the infiltrating leukocytes are restricted to a 'belt' outside the necrotic core of the early ulcerative lesions [83, 84]. This contains T cells, CD68-positive macrophages and neutrophils, as well as clusters of B cells [83]. However, the immune cells are not able to access the necrotic core containing *M. ulcerans*, which contains neutrophilic debris and stains strongly and diffusely for apoptotic markers [83, 84]. Notably this picture changes remarkably during antibiotic therapy [85–87], which is presumed to be a result of a drop in mycolactone production.

Despite this, there is considerable evidence that both human and animal hosts can mount an immune response to *M. ulcerans* [88]. Critically, spontaneous recovery from BU without treatment reported in both humans [89–92] and animal models [93, 94] shows that the immune system can contain the infection in some circumstances. Both T cell [95–99] and serological [100–102] responses to *M. ulcerans* antigens have been demonstrated in the blood of BU patients. Moreover, their household contacts also display similar responses although they had never experienced clinical disease [100, 102–104]. Experimentally, IFN-γ protects against *M. ulcerans* infection in mice [105], and similarly a human genome SNP in the *IFNG* gene increases susceptibility to BU [106]. Such genetic studies in BU patients have identified a range of disease-modifying SNPs in genes involved in the cellular response to infection, including *iNOS*, the inducible nitric oxide synthase that generates bactericidal NO in macrophages [106]. Although the intramacrophage stage of *M. ulcerans* infection is thought to be transient [71], SNPs in genes involved in this response also impact BU, including in *PARK2*, *NOD2* and *ATG16L1* [106–108].

5.3 Vaccine Candidates

Notwithstanding the obvious serious sequelae of infection, the motivation for a BU vaccine also encompasses the origins of the infection from the environment. It is now clear that there are certain environments where *M. ulcerans* is highly prevalent, especially those disturbed by human activity, such as mining or agricultural land use [109, 110]. Unfortunately those living in such environments are at high risk of developing BU [111], even if they adhere to risk-reducing guidance [112–115]. Therefore, a vaccine may be the only realistic hope of BU eradication.

Although studies aimed at developing a vaccine against *M. ulcerans* infection date back to the 1950s and the work of the Australian microbiologist Frank Fenner [116, 117] (https://www.science.org.au/learning/general-audience/history/interviews-australian-scientists/professor-frank-fenner), there is still currently no effective vaccine that provides long-term protection from BU [118]. Early attention focussed on the Bacillus Calmette-Guérin (BCG) strain of *M. bovis* that is primarily known as the vaccine for *M. tuberculosis* [119]. Indeed, most countries endemic for BU have a current national

BCG vaccination policy for all citizens (www.bcgatlas.org), although Australia now only vaccinates special groups.

5.3.1 Human Studies with BCG

In two early randomised controlled trials using BCG in Uganda, there was evidence that BCG did confer some protection against BU even though this was thought to be short-lived [120, 121]. However, it should be noted that these studies were confounded by many factors. For example, in the first randomised trial with Rwandan refugees [120], participants were selected based on their tuberculin skin test (TST) negativity, which ruled out TB and latent TB infection, but almost certainly included both BCG-vaccinated and unvaccinated individuals (as TST in response to BCG wanes dramatically over time [122]). Moreover, this trial could not be fully completed, as the participants were lost to follow-up due to relocation of refugees. The second [121] was more successful in that the trial aims were fully achieved, but the outcomes were similar, in that partial and short-term protection was observed. Thus, an overall efficacy of BCG vaccination of 47% was reported, which declined sharply after 12 months, and was also notably highly variable depending on the immune status of participants on the outset. In that study, included participants had a broad spectrum of immune status, including those with known previous BU disease, presence or absence of BCG scar and even individuals with latent TB infection [121].

Since then, multiple other clinical studies found no evidence that BCG confers any long-term protection. For example, an observational study by Phillips et al. [123] found no association between BCG (presence of scar) and BU disease incidence amongst participants recruited from Congo, Ghana and Togo, replicating results from Benin [124]. For further reading on these studies, we refer the readers to two excellent recent reviews on the subject [125, 126].

The poor efficacy of BCG from these human studies is most likely due to insufficient immune cross-reactivity with *M. ulcerans* and suboptimal performance of BCG in countries with high exposure to non-tuberculous mycobacteria (NTM). In the case of cross-protection, this is likely the result of the divergent pathophysiologies of the infections they cause despite a high degree of genetic homology between different mycobacterial species. Thus, similarly to *M. ulcerans*, BCG vaccine offers only partial protection against *M. leprae* in human clinical trials [127, 128]. Furthermore, while multiple other environmental and non-environmental factors are undoubtedly involved, it is well known that BCG efficacy against TB is drastically reduced in geographical settings with high burden of non-pathogenic mycobacteria due to immunological interference (reviewed in [129]). In other words, the reasons for failure of BCG to impart better, longer-lasting protection against BU disease may be the same as those that also undermine its efficacy against TB.

5.3.2 *Mouse Studies of BU Vaccine Candidates*

Development of BU vaccines that offer improved protection over BCG has frequently involved *in vivo* models of *M. ulcerans* infection [130]. The mouse hind footpad model of *M. ulcerans* infection was originally developed by Fenner [116] and continually refined over many decades, predominantly in the BALB/c and C57BL/6 strains of mice. Today, it is the gold standard for studying treatment interventions and new vaccine candidates against BU disease. *M. ulcerans* bacteria are injected subcutaneously into the footpad, and (depending on the injected dose and mouse strain) the initial signs of swelling may appear over the metatarsal area approximately 2–5 weeks later. If untreated, the swelling progresses and then extends into the leg, finally leading to onset of ulceration. These stages of experimental pathogenesis of *M. ulcerans* infection in mouse footpads can be graded according to their physical appearance, according to a process originally proposed by Stanford [23] and later refined by Converse [34] in line with modern animal welfare legislation (Fig. 5.4). This allows for experimental humane points to be achieved without causing undue suffering to animals, usually before the point of ulceration. Notably, the analgesic effects of mycolactone [131, 132] mean that the animals do not experience inflammatory, hypoxic or tissue pressure pain even at the more severe grades. Objective measures of the intervention can also be taken in terms of physical parameters (footpad diameter), enumeration of bacteria in footpad via either culturable bacilli or quantification of bacterial DNA and measurement of inflammatory markers in blood or tissue.

An alternative model involving subcutaneous injection of *M. ulcerans* into the central portion of the tail has also been described [71]. The main outcome measure

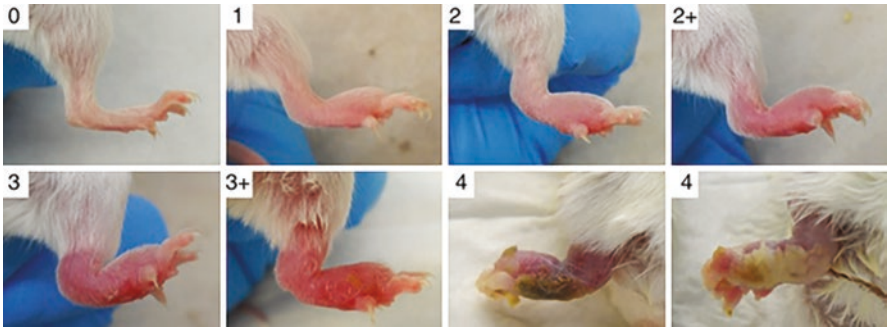

Fig. 5.4 Pathogenesis after subcutaneous *M. ulcerans* injection in the mouse (BALB/c) footpad. Subcutaneous (s.c.) injection of *M. ulcerans* into the healthy mouse footpad (*0*) leads to progressive swelling and inflammation. (*1*) Grade 1, slight swelling; (*2* and *2+*) Grade 2, swelling with inflammation; (*3* and *3+*) Grade 3, swelling with inflammation of the leg; (*4*) Grade 4, swelling with inflammation and possible ulceration [130]. For most vaccine studies, the human endpoint is when cage bedding is observed sticking to the sole of the foot, indicating ulceration. Despite the dramatic appearance, the analgesic effects of mycolactone mean that the mice do not display signs of pain nor lose mobility

here was time to ulceration in days (60–70 days in unvaccinated C57BL/6 and BALB/c mice, respectively) [133, 134]. A low-dose infection model using a recombinant bioluminescent strain of *M. ulcerans* allowing for bacteria enumeration in live animals [135, 136] has also been used [137].

Here, we have categorised the various vaccine candidates tested in mice under two broad arms: whole bacteria and subunit vaccines, including those based on mycolactone (Table 5.1). Despite its lack of efficacy in human clinical trials, BCG has proven useful as a baseline to compare the efficacy of other vaccine candidates (recently reviewed in [125, 126]), as it provides short-lived but measurable protection against mouse footpad infections. This has been reproducible since Fenner's first attempts at a vaccine in the 1950s [116, 117] and is seen even when BCG booster approaches are used [148]. These studies showed that vaccine-mediated protection from *M. ulcerans* infection may be Th1-mediated, via sustained levels of IFN-γ and TNF and the absence of IL-4, IL-10 and IL-17 [139].

5.3.2.1 Whole Bacteria Vaccines

In concert with the earliest BCG studies in mice, several reports have attempted to use *M. marinum* as a vaccine against *M. ulcerans* infection. Early attempts showed increased efficacy over BCG, but these were also still short-lived and waned with time [116, 117]. More recently, there has been some interest in overexpressing antigens in BCG and *M. marinum* and using these recombinant strains as whole bacteria vaccines. By this design, *M. ulcerans*-specific antigens were presented in a vaccine which lacked the virulent and immunomodulatory potential of mycolactone. These studies have focused on antigens that are known to be immunodominant in *M. tuberculosis* including EsxH, the *M. ulcerans* ortholog of *M. tuberculosis* TB10.4 antigen and proteins of the Ag85 complex. The latter is made up of Ag85A, Ag85B and Ag85C and is known to be secreted from BCG and to elicit strong Th1 responses [149]. Each of these 30–32 kDa proteins is highly conserved between different species of mycobacteria, being involved in the synthesis of cord factor and the organisation of mycolic acids in the bacterial cell wall. Notably Ag85A induced measurable, but relatively weak, IFN-γ responses during whole blood restimulations of BU patients and their household contacts [103].

Hart et al. [142] used recombinant *M. marinum* expressing *M. ulcerans* Ag85A (MU-Ag85A). Although this did not seem to delay the onset of ulceration (the experimental endpoint), it did significantly reduce the bacterial load of the challenged footpads. Hart et al. applied this same technology to generate BCG expressing *M. ulcerans* Ag85B with and without a fusion with EsxH. Mice challenged with *M. ulcerans* following a single subcutaneous vaccination with BCG MU-Ag85B-EsxH [144] or BCG MU-Ag85B [143] displayed significantly less bacterial burden at 6 and 12 weeks post-infection, reduced histopathological tissue damage and significantly delayed (but not prevented) onset in ulceration compared to vaccination with BCG.

Table 5.1 Summary of vaccine candidates tested in the mouse model of *M. ulcerans* infection

Type	Candidate	Description	Approach(es)	Outcome	Reference
Whole bacteria	BCG	Well-characterised attenuated strain of *M. bovis*	Live vaccine	Confers only transient protection, even when booster regimens are undertaken	Reviewed in [125, 126]
	M. marinum	The closest genetic relative of *M. ulcerans*, lacking the pMUM plasmid	Live vaccine	Greater protection than BCG	[117]
	M. ulcerans	Virulent strains of *M. ulcerans*	Live vaccine	Some protection with low inoculum, no protection when high inoculum given	[117, 138]
			Formalin-killed	No protection	[138]
			Dewaxed bacteria (by treating with organic solvents)	Complete protection (at 28 days) for dewaxed MU with increased serum IgG against MU	[138]
		Mycolactone-negative strain of *M. ulcerans* (Mu_5114), which has lost MUP038 required for mycolactone synthesis	Live vaccine	Some protection, but less than BCG for live vaccine	[138, 139]

Immunodominant proteins of mycobacteria	Ag85 complex	The Ag85 complex is secreted from BCG and elicits strong Th1 responses. *M. ulcerans* and BCG Ag-85A have 84% aa homology. MU-Ag85A solicited measurable but relatively weak IFN-γ responses in whole blood restimulation of BU patients [103]	BCG-Ag85A DNA vaccine	Reduced cfus in footpads >20-fold	[140]
			MTB-Ag85B DNA vaccine	No improvement over BCG	[134]
			MU-Ag85A DNA vaccine/ recombinant protein/ DNA prime-protein boost	DNA prime-protein boost most successful, but protection on par with BCG	[141]
			BCG, *M. smegmatis* or MM overexpressing MU-Ag85	Slowed development of footpad swelling (delaying euthanasia), enhanced over BCG alone	[142, 143]
	Ag85 EsxH complex	EsxH is the *M. ulcerans* homologue of TB10.4, an immunodominant antigen of *M. tuberculosis*	BCG overexpressing MU-Ag85B-EsxH (single dose)	Greater protection than BCG alone and BCG-MU-Ag85B	[144]
	Hsp18 (MUL_2232)	An immunodominant cell wall antigen of *M. leprae* and has been identified in humans with BU	Recombinant protein/ prime with vesicular stomatitis virus vector-protein boost	Slight reduction in cfu (with prime boost), but no protection despite a strong humoral and CMI response	[133, 145, 146]
	Hsp65	A well-conserved immunodominant antigen present in all mycobacteria. *M. ulcerans* and *M. leprae* Hsp65 have 96% amino acid homology. Sera of BU patients recognises Hsp65 [102]	ML_Hsp65	Minimal protection, much reduced compared to BCG	[134]
	MUL_3720	Highly expressed 21 kDa protein of MU of unknown function	Recombinant protein/prime with vesicular stomatitis virus vector-protein boost	No protection despite a strong humoral and CMI response	[133, 146]

(continued)

Table 5.1 (continued)

Type	Candidate	Description	Approach(es)	Outcome	Reference
Mycolactone production (polyketide synthase domains)	ACP-1	The MDa-sized, mycolactone-synthesising polyketide synthase enzymes are associated with the *M. ulcerans* cell wall. Several individual domains of these proteins have been tested, as each solicited responses in whole blood restimulation of BU patients [103]. Sera of BU patients recognise ER, ATp and KR A [102]	DNA vaccine-protein boost	No protection	[147]
	ACP-2		DNA vaccine-protein boost	No protection	[147]
	ACP-3		DNA vaccine-protein boost	No protection	[147]
	ATac-1		DNA vaccine-protein boost	No protection	[147]
	ATac-2		DNA vaccine-protein boost	No protection	[147]
	ATp		DNA vaccine-protein boost	Less or equivalent to BCG	[147]
	ER		DNA vaccine-protein boost	Reduced cfus without any clinical effect	[137, 147]
	KR A		DNA vaccine-protein boost	No protection	[147]
	KS		DNA vaccine-protein boost	No protection	[147]

Polyketide synthase domains*: ACP-1, ACP-2 and ACP-3* acyl carrier protein type 1, 2 and 3, *ATac-1 and ATac-2* type 1 and type 2 acyltransferase with acetate specificity, *ATp* acyltransferase with propionate specificity, *ER* enoylreductase, *KR A* ketoreductase A, *KS* load module ketosynthase domain. Bacteria: *BCG M. bovis* Bacillus Calmette-Guérin strain, *ML M. leprae*, *MM M. marinum*, *MTB M. tuberculosis*, *MU M. ulcerans*. Other: *CMI* cell-mediating immunity, *cfu* colony-forming units, *Hsp* heat shock protein

Others have attempted vaccines using various doses and strains of *M. ulcerans* itself. Once again, Fenner paved the way and found that low, but not high, doses of *M. ulcerans* (1615E) provided protection against footpad infections [117]. Though not explained, this may have been due to the immunomodulatory action of mycolactone. In an attempt to bypass this, Fraga et al. [139] used a mycolactone-deficient strain of *M. ulcerans* (5114) that had lost the MUP038 gene involved in mycolactone biosynthesis [150]. This strain delayed the onset of footpad swelling post-challenge similarly to BCG. Finally, an interesting approach was taken by Watanabe et al. [138], who inactivated and dewaxed *M. ulcerans* by organic solvent treatments, prior to using it as a vaccine in mice. This candidate conferred complete protection against swelling at 28 days post-challenge, though the authors did not investigate if this protection was long-lasting.

5.3.2.2 Subunit Vaccines for BU

An alternative approach has been the use of acellular/subunit vaccines formulated with adjuvants and delivered as proteins or DNA. Tanghe et al. [140] demonstrated that a DNA vaccine based on BCG-Ag85A was able to confer partial protection (like BCG) against *M. ulcerans* infection in mice, as measured by reduced bacterial load. This was further improved on with MU-Ag85A, particularly when used as a DNA-prime protein-boost regimen, with a 100-fold reduction of bacterial load compared to unvaccinated mice [141]. These experiments also demonstrated that the protective immune responses were localised and Th1-mediated, with strong roles for IL-2 and IFN-γ. However, while this vaccine delayed the onset of footpad ulceration, it was less effective than BCG, a finding later replicated by Roupie et al. [147].

Other immunodominant antigens of *M. ulcerans* that have been investigated as vaccine candidates include MUL_2232 (also known as Hsp18, homologous to an immunodominant cell wall antigen of *M. leprae* that is reactive with the sera of patients with BU [100]) and MUL_3720 (a highly expressed 21 kDa protein with unknown function [151, 152]). However, despite their strong induction of IgG antibodies, they failed to provide any protection in either the footpad or tail infection models [133, 145]. No further improvement was reported when vesicular stomatitis virus-based RNA replicon particles encoding these proteins were used [146]. Prior to this, Coutanceau et al. [134] had tried a DNA vaccine using *M. leprae* Hsp65 antigen, but this did not confer any protection despite inducing strong IgG antibody responses. These studies give credence to the thinking that T cell responses, rather than antibodies, may have a more significant role in *M. ulcerans* immunity.

Moreover, different domains of the three large mycolactone polyketide synthases mlsA1, mlsA2 and mlsB encoded by pMUM001 and found associated with the *M. ulcerans* cell wall [150] have been investigated as vaccine candidates. These included the acyl carrier protein type 1, 2 and 3 (ACP-1, ACP-2 and ACP-3), type 1 and type 2 acyltransferases (acetate) (ATac-1 and ATac-2), acyltransferase (propionate) (ATp), enoylreductase (ER), ketoreductase A (KR-A) and the load module ketosynthase domain (KS). Many of these domains have been shown to induce

humoral or cellular responses, supporting their immunogenicity. Of these, ER, ATp and KR-A have been shown to discriminate serological responses between BU patients and controls in non-endemic regions [102]. Other domains, particularly ER and KS, were able to successfully induce IFN-γ and IL-5 during whole blood restimulations of BU patients and their household contacts [103].

Unfortunately, vaccine trials using this strategy have been disappointing. Roupie et al. [147] used a DNA prime/protein boost protocol and found that the antibody and cellular (IL-2 and IFN-γ) immune responses to these antigens varied, with ATp providing the strongest response amongst the nine domains in line with, or better than, the MU-Ag85A control. However, this did not significantly extend the time for mice to display 4 mm footpad swelling or reduce bacterial numbers in infected feet. More recently, an approach that involved electrostatically coupling the ER domain to the Toll-Like receptor 2 (TLR-2) agonist adjuvant R_4Pam_2Cys was tested [137]. In this low-dose challenge tail model, this vaccine provided reduced protection compared to BCG and was associated with ER-specific serum IgG titres and IL-2/IL-4 in the draining lymph nodes.

With limited success so far with both whole bacteria and subunit protein candidates, it has been postulated that a vaccine design based on mycolactone could provide the much sought-after protection against BU. Evidence that such a toxin-blocking vaccine might be fruitful comes from the successful generation of mycolactone-neutralising antibodies using a truncated and non-cytotoxic mycolactone derivative. This compound (PG-203) lacking the so-called 'Southern' chain and conjugated to BSA via a diethylene glycol-based linker, it elicited protein-based immune responses as determined by ELISA and other neutralisation assays [153]. The vaccine potential of mycolactone has also been demonstrated using *in vitro* display methods comprising both phage and yeast [154].

5.4 Prospects

So, what are the prospects of a BU vaccine in the future? Based on the available evidence with BCG, a BU-specific vaccine is needed. While none of the promising preclinical candidates described here fully meet the criteria to be advanced to human studies, these partial successes strongly suggest that, with further improvements, such a vaccine may yet be achievable.

To that end, we would like to conclude this review with a preliminary report from our own attempts of developing a subunit-based vaccine against BU. Using our expertise from BCG-boost subunit vaccines studies for TB [155–157], we have recently developed several formulations that were tested in the mouse footpad model of *M. ulcerans* infection. These formulations contain individual or combinations of *M. ulcerans* antigens, as well as mycolactone itself, mixed with different types of adjuvants and delivery systems. While the data are yet to be published, we were very encouraged to observe that one of these formulations, which we have termed 'BuruliVac', was particularly effective in preventing swelling and ulceration

of the mouse footpad and completely prevented footpad swelling in all experimental animals. This was corroborated by absence of C-reactive protein and other inflammatory markers in the tissue (Boakye-Appiah and Reljic, unpublished).

These ongoing proof-of-principle vaccine studies demonstrate that it is feasible to prevent *M. ulcerans* infection in this experimental model and that future efforts should be concentrated on further optimising and advancing such second-generation vaccine candidates against BU. Recent developments in vaccination strategies that allow specific targeting of skin resident memory T cells may be of value here [158]. However, it should also be noted that unlike BCG, a new BU-specific vaccine will come with a significant caveat, in that its clinical development and eventual licensure will depend on it being able to attract sufficient interest from pharmaceutical industry. BU, despite being the most significant mycobacterial disease after TB and leprosy, is an NTD that affects a relatively small proportion of population, mostly in the endemic areas in Western Africa. Vaccine development is an extremely costly undertaking for the pharmaceutical industry, amounting to hundreds of millions of US dollars. This investment can only be recouped by selling enough doses and over a prolonged period. The battle to develop a BU vaccine will therefore be fought on two separate fronts, in research laboratories and in the commercial arena. We, the scientific community, have the responsibility to ensure that if it comes to that second battle, we have something to fight with, a vaccine that has a real chance to eradicate the terrible affliction that is BU.

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Part II
Tuberculosis

Chapter 6
Correlates of Protection from Tuberculosis

Marco Polo Peralta Alvarez, Julia L. Marshall, and Rachel Tanner

Abstract Multiple immunological mechanisms interact to protect against *Mycobacterium tuberculosis* (*M.tb*) infection and/or tuberculosis (TB) disease. However, development of a much-needed new and effective TB vaccine is hindered by the lack of validated correlates of protection. The identification of correlates of protection would facilitate the rational design, optimisation and evaluation of TB vaccine candidates. In this chapter, we discuss what is currently known about protective immunity against *M.tb* and potential correlates of protection that have been proposed to date, both including and also looking beyond the central role of IFN-γ producing CD4+ T cells to consider innate and humoral immune parameters. Approaches to identifying and validating correlates of protection will also be reviewed.

Keywords *Mycobacterium tuberculosis* · Correlates of protection · T cell Immunity · Infection models

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M. Christodoulides (ed.), *Vaccines for Neglected Pathogens: Strategies, Achievements and Challenges*, https://doi.org/10.1007/978-3-031-24355-4_6

6.1 Introduction

One of the major barriers hampering the development of a new and efficacious TB vaccine is the lack of validated immune correlates of protection, defined as 'a statistical relation between an immune marker and protection' [1]. Such correlates can be subdivided into mechanistic (causally responsible for protection) or non-mechanistic (significantly correlated with, but not causally responsible for, protection). Correlates of protection, principally antibody levels, have been instrumental in developing vaccines against a range of infectious diseases including those caused by *Haemophilus influenzae* type b (Hib), pneumococci, *Clostridium tetani*, *Corynebacterium diphtheriae* and several viruses. An immune correlate of protection would similarly be invaluable in rational TB vaccine design, through, for example, facilitating the identification of protective antigens, as well as optimisation of the vaccine delivery system, adjuvant, dose and regimen. It would also provide an early indication of vaccine efficacy, thus expediting clinical TB vaccine trials that currently require very large sample sizes and long follow-up periods to accrue a sufficient number of 'cases' meeting clinical endpoint criteria.

However, the field is caught in a dilemma whereby potential correlates of protective immunity can only be validated in clinical trials when a highly effective vaccine is developed, yet the design and evaluation of vaccine candidates are extremely difficult in the absence of a validated correlate. A further challenge is the complexity of clinical manifestations, as correlates of protection from *Mycobacterium tuberculosis (M.tb)* infection may differ from those of progression to active TB disease (ATB), reactivation or reinfection; and vaccine-induced protection may differ from natural protection from infection, between host compartments, populations or different antigens or vaccine platforms.

Our understanding of which components of the immune response are necessary, and/or sufficient, to achieve protection from TB is incomplete. It is established that following inhalation of *M.tb* in aerosols, bacteria are phagocytosed primarily by dendritic cells (DCs) and macrophages. The latter are the main cellular reservoir where *M.tb* resides and replicates by blocking phagolysosomal fusion. Infected DCs traffic to the local lung-draining mediastinal lymph nodes (LNs) where they present antigen in association with MHC class II. The precise location where *M.tb* is phagocytosed is thought to play a role in the establishment of potentially protective primary immune responses. DCs sampling the mucosal tissue have faster antigen trafficking to LNs compared to those sampling the lung parenchyma, affecting the activation and proliferation of antigen-specific CD4+ T cells [2] (Fig. 6.1). After activation, CD4+ T cells are primed as T-helper 1 (Th1) cells, expand and migrate to the lung tissue where they become a primary source of IFN-γ and TNF-α production during the acute stage of infection, which stimulates macrophages to kill intracellular mycobacteria by activating downstream pathways including inducible nitric oxide synthase (iNOS). As such, most TB vaccine studies and clinical trials to date have focused on the T-helper 1 (Th1) response—the frequency of IFN-γ producing CD4+ T cells—as the main immunological read-out. Indeed, several studies have confirmed the necessity for CD4+ T cells and IFN-γ in protective immunity.

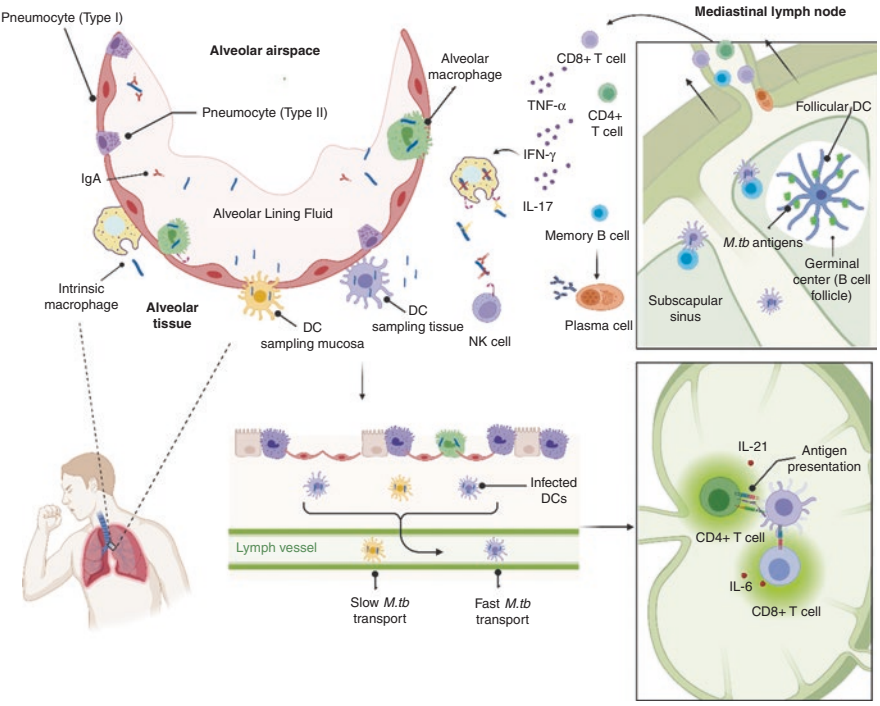

Fig. 6.1 Immune response to *M.tb* infection. Alveolar macrophages and DCs phagocytose aerosolised *M.tb* that reaches the lung. Antigen location may lead to different speeds of DC trafficking through the lymph vessels. Upon arrival at the LNs, DCs present *M.tb* antigen which activates specific T cells. Days after the initial infection, adaptive immune cells arrive at the site of infection and secrete IFN-γ, TNF-α and IL-17 amongst other cytokines which stimulate macrophages to kill intracellular mycobacteria. Activated specific B cells secrete antibodies that may increase opsonization, phagocytosis and other potentially protective responses mediated by T cells and NK cells. *DC* dendritic cell, *LN* lymph node, *NK* natural killer. (Created with BioRender.com)

However, other evidence suggests that these parameters alone are not sufficient for, and do not necessarily correlate with, protection (see Sect. 6.3.1.1). An ever-expanding literature supports a role for other cellular subsets, cytokines and immune mechanisms in protection, offering new candidate correlates of protection.

6.2 Innate Immunity

6.2.1 Early Clearance and a Role for Innate Immunity

M.tb case-contact studies demonstrate that up to 55% of people exposed to *M.tb* do not develop a traceable adaptive immune response, as detected by interferon gamma release assay (IGRA) or tuberculin skin test (TST) [3, 4]. Persistent IGRA-negative *M.tb* contacts have low risk of progression to TB disease, suggesting that *M.tb* is

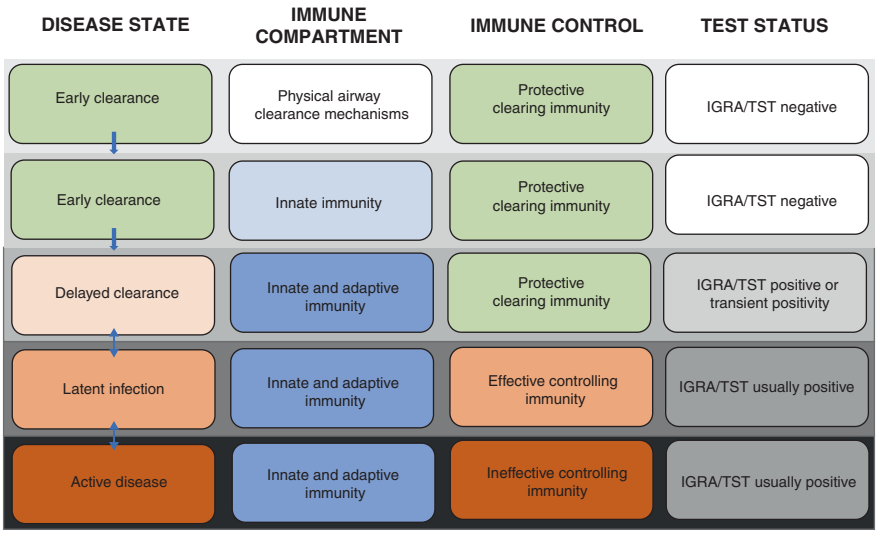

Fig. 6.2 Hypothesized relationship between *M.tb* clearance and immune mechanisms following natural exposure in humans. *IGRA* interferon gamma release assay, *TST* tuberculin skin test. (Created with BioRender.com)

cleared in these individuals [3, 5, 6]. This 'early clearance' could be evidence of protective innate immune mechanisms independent of an adaptive immune response [7]. However, this phenomenon has not been proven, and an early efficient protective adaptive immune response could simply result in rapid elimination and IGRA reversion [8, 9]. Alternatively, persistently IGRA-negative individuals may have developed an adaptive response, and infection may even persist but is never detected on systemic IGRA, due to T cell anergy, IFN-γ-independent mechanisms or because the response is restricted to the local lung mucosa [6, 10–12] (Fig. 6.2).

Despite this uncertainty, as early clearance is a model of protective immunity against *M.tb*, further investigation into the underlying immune mechanisms is warranted, as it could lead to new insights into correlates of protection. In addition to this putative protective role independent of adaptive immunity, evidence suggests that the innate immune response is crucial in defining adaptive immune polarisation, for example, to a Th1 or Th2 response [13–15]. This further highlights the central role the innate immune response may play in protection against *M.tb* infection and TB disease.

6.2.2 Mononuclear Phagocytes

Mononuclear phagocytes (monocytes, DCs and resident macrophages) are likely key in the development of a protective immune response against *M.tb* infection [16, 17]. Human monocyte and DC primary immunodeficiency syndromes are

characterised by disseminated mycobacterial infection after intradermal (ID) BCG vaccination [18]. DCs are professional antigen-presenting cells connecting the innate and adaptive immune response [19]. Macrophages are professional phagocytes, likely amongst the first cells to encounter *M.tb* on infection, but also the main niche in which the obligate intracellular mycobacteria resides [14, 20].

Delayed DC migration to the draining LNs and dysfunctional DC-mediated antigen presentation increase risk of overwhelming *M.tb* infection in mice, likely due to the delay in the development of an effective adaptive immune response [21–23]. Further, DC depletion in mice delays *M.tb*-specific CD4+ T cell activation and results in overwhelming *M.tb* infection [24]. In humans, CXCL10+ $CD14^{dim}$ monocytes are associated with BCG growth inhibition in a functional *ex vivo* mycobacterial growth inhibition assay (MGIA) [25]. CXCL10 is a chemokine facilitating homing to the infection site, regulated by IFN-γ production [26]. Genetic inability to produce IFN-γ in humans is characterised by mycobacterial susceptibility and associated with disseminated BCG disease [27, 28]. Conversely, systemic monocytosis and a high monocyte to lymphocyte (ML) ratio are associated with poorly controlled TB disease and increased risk of developing TB disease [29–33]. It may be that only certain monocyte effector subtypes are protective or, alternatively, that chronic but not acute monocytosis reduces protective efficacy.

6.2.3 Natural Killer Cells

Human natural killer (NK) cells can directly recognise *M.tb* infected cells *in vitro* and induce apoptosis within 24 hours via perforin and granzyme proteases [34, 35]. They also directly lyse infected cells via antibody-dependent cell-mediated cytotoxicity (ADCC). Circulating cytotoxic NK cells are associated with reduced risk of *M.tb* infection in healthy TB contacts [36]. Additionally, IFN-γ+ TNF-α+ NK cells derived from historically BCG-vaccinated adults improve BCG growth inhibition in the MGIA [37, 38]. A low level of systemic cytotoxic NK cells increases the risk of progression to active TB disease. While NK cells alone do not mediate protection in *M.tb* challenge mouse models, depletion of both NK cells and T cells increases susceptibility to TB, compared with depletion of T cells alone, and adoptive transfer of NK cells reduces *M.tb* burden [39].

6.2.4 Neutrophils

Neutrophils have the potential to be key players in early defence against *M.tb* infection. They can migrate to the site of infection within minutes, are professional phagocytes and release potent anti-microbial oxidative granules which kill *M.tb in vitro* [40–42]. Depletion or enhanced recruitment of neutrophils in mice leads to increased or decreased mycobacterial burden, respectively, and a high neutrophil

count is protective against *M.tb* infection in contacts of TB patients [43–45]. However, this protective effect may only be upon initial exposure, as neutrophilia is a hallmark of active TB disease in humans and uncontrolled *M.tb* infection in susceptible mice [46–50].

6.2.5 Donor Unrestricted T Cells

Donor unrestricted T cells (DURTs) are innate CD3+ T cells which respond to MHC-independent non-peptide epitopes [51]. They include mucosal-associated invariant T (MAIT) cells and the CD1-restricted natural killer T (NKT) cells and γδ T cells [52, 53]. *In vitro*, DURTs can directly recognise and kill *M.tb* infected cells, and they have diverse effector functions and are enriched in the human lung [54–57]. They could therefore be important in early immune protection, acting as the bridge between the innate and adaptive response. However their protective potential in *M.tb* is unclear [58, 59]. The MR1 polymorphism rs1052632 is associated with increased susceptibility to TB in a Vietnamese cohort [60], and evidence for protection in mouse and human models for DURTs is contradictory [57, 61–65]. Research into DURTS has been constrained by difficulty in defining these cells and a lack of animal models for some cell types such as group 1 CD1-restricted T cells [54–56, 66]. With the advent of tetramer technology, many of these barriers may be overcome.

6.2.6 Trained Innate Immunity

Traditionally, innate immunity has been viewed as a static non-specific immune system without capacity for memory. However, it is now known that NK cells mature into long-lived effector subclasses upon pathogen exposure [67]. Furthermore, epigenetic reprogramming of bone marrow-derived monocytes, termed training, has been demonstrated following BCG exposure, affecting the subsequent monocyte response to homologous and heterologous pathogens [68]. Trained monocytes have been associated with protection from *M.tb* challenge following BCG vaccination in mice [69]. Training has also been associated with early clearance in BCG-exposed but uninfected adults [25]. Understanding the plasticity of the innate immune response following *M.tb* exposure, and its subsequent effect on the adaptive immune system, would enable further targets for vaccine development.

While the evidence for a role of the innate immune system in protection against *M.tb* is compelling, further research is required to fully elucidate these mechanisms and to understand the impact on any subsequent protective adaptive immune response.

6.3 Conventional T Cells

6.3.1 CD4+ T Cells

6.3.1.1 IFN-γ Producing CD4+ T Cells

A large body of evidence indicates that IFN-γ producing CD4+ T cells are necessary for protection against TB. Mice and non-human primates (NHPs) deficient in CD4+ T cells during acute or chronic *M.tb* infection have increased bacterial burden and mortality compared with control animals [70–73]. Furthermore, the adoptive transfer of CD4+ T cells from immunised mice protects non-immunised mice against *M.tb* challenge [74]. The increased risk of TB disease due to decreased CD4+ T cell number and function associated with HIV infection in humans, and SIV infection in NHPs, provides further evidence of a critical role for this cell type [75–77]. Risk of reactivation increases as CD4+ T cell levels decrease, and these patients are more likely to present with disseminated disease [78, 79]. IFN-γ knockout (KO) mice succumb to rapid and fatal TB disease [80, 81], and partial or complete IFN-γ receptor deficiency in humans leads to disseminated *M.tb* infection and BCG-osis [82, 83]. However, it should be noted that clinical deterioration in CD4+ T cell-deficient mice cannot be attributed to sustained loss of IFN-γ, as other cell types also produce this cytokine [70, 71]. IFN-γ-independent mechanisms of CD4+ T cell-mediated control of *M.tb* infection have also been demonstrated [84].

Despite the clear importance of IFN-γ producing CD4+ T cells in the immune response to *M.tb*, several key findings challenge the idea that this measure represents a correlate of protection. The magnitude of purified protein derivative (PPD)-specific IFN-γ production following BCG vaccination does not correlate with protection against *M.tb* challenge in mice [85, 86]. Furthermore, studies of TB vaccine candidates have demonstrated the induction of potent antigen-specific IFN-γ producing CD4+ T cell responses, but this did not translate into improved protection over BCG alone [87, 88]. Kagina et al. assessed the frequency and extended cytokine profile of specific T cells in a study of 5662 BCG-vaccinated infants with a 2-year follow-up to identify those who developed TB and those who did not develop TB (divided into 2 groups of protected infants according to household contact status) [89]. There were no differences between groups in CD4+ T cell IFN-γ production or any of the other T cell properties [89]. Interestingly, in a post hoc correlate analysis in the same population, the BCG antigen-specific IFN-γ ELISpot response was associated with reduced risk of TB disease. The main effect appeared to be in the first 6–12 months of follow-up, suggesting an early protective effect in infancy [90]. Such contrasting findings may result from differing time-points of sample collection, the IFN-γ assay used, sample type or case definitions.

In studies comparing patients with ATB and latently *M.tb*-infected (LTBI) individuals or uninfected household contacts (both considered to have some degree of protection), findings regarding the role of IFN-γ producing CD4+ T cells are conflicting. Some have suggested that *M.tb*-specific Th1 cells and IFN-γ production are

depressed during active TB disease [91, 92], while others report the converse [93, 94]. In such situations it is difficult to disentangle cause from effect, as high levels of IFN-γ may be driven by antigen load in acute infection, and patients with chronic TB may exhibit signs of Th1 inhibition as a secondary process. In a study comparing TB patients of diverse disease severity, antigen-specific IFN-γ CD4+ T cell responses correlated with the activity of *M.tb* infection but not the severity of TB disease [95, 96].

6.3.1.2 Polyfunctional CD4+ T Cells

Polyfunctional T cells are defined as those that simultaneously co-produce two or more proinflammatory cytokines. Polyfunctional CD4+ T cells secreting IFN-γ, TNF-α and IL-2 have been shown to correlate with protection from *Leishmania* and have been associated with slower progression to AIDS in HIV-infected individuals [97, 98], but their role in *M.tb* infection remains unclear. Individuals with LTBI or ATB patients following therapy have been reported to have higher frequencies of polyfunctional CD4+ T cells than those with ATB, although the converse has also been suggested [99–102]. As previously noted regarding IFN-γ producing CD4+ T cells, it is not possible to discern whether frequency of this cellular subset plays a causal role in control of *M.tb* or simply reflects the underlying bacterial burden.

The BCG vaccine and a range of TB vaccine candidates including live mycobacterial vaccines, those using viral vectors and recombinant antigen vaccines have been shown to induce polyfunctional CD4+ T cells [103]. However, the strongest evidence supporting a role for the cellular subset as a correlate of protective immunity comes from studies in which two or more distinct vaccine candidates eliciting a range of protective responses are compared. rBCG-XB has been shown to induce stronger HspX-specific polyfunctional T cell responses than BCG, which was associated with superior protection [104], and delivery of Ag85B:CpG with polypropylene sulphide nanoparticles (NP) induced more polyfunctional Ag85B-specific CD4+ T cells than the same vaccine without NP, which correlated with superior protection in the lung [105]. In a study of BCG and four different TB vaccine candidates, levels of vaccine-induced protection also correlated with the magnitude and quality of polyfunctional CD4+ T cells [106].

Conversely, other preclinical studies do not support such an association [103]. In humans, in the 2-year follow-up study of BCG-vaccinated infants previously described, there was no association between the polyfunctional cytokine profile of induced T cells and protective efficacy [89]. Furthermore, boosting BCG or VPM1002 with MVA85A elicited superior PPD- and Ag85A-specific polyfunctional T cells, but this did not translate into improved protection obtained with either BCG or VPM1002 alone [88, 107]. It is possible that such discrepancies result from differences in vaccination protocols or methods of measuring polyfunctional T cells or that dual-cytokine producing T cells are a better correlate than those producing three cytokines [103]. A comprehensive review by Lewinsohn et al. concluded that polyfunctional CD4+ T cells are not sufficient and may not even be necessary to mediate protection [103].

6.3.1.3 Th17 Cells

Following exposure to *M.tb*, innate myeloid cells induce the production of cytokines such as IL-23 and IL-1β which drive the differentiation and polarisation of naïve CD4+ T cells towards Th17 cells. Th17 cells are the primary producers of IL-17 during TB, but they can also produce other cytokines including IL-22, IL-21, TNF-α and GM-CSF. While these cells have an important role in the protective immune response to rapidly growing extracellular bacteria, their contribution to protection against intracellular bacteria such as *M.tb* is less well-characterised [108]. Memory Th17 cells are present in the blood of people who have been exposed to mycobacteria, and the magnitude of the IL-17 response has been shown to correlate with the clinical outcome of *M.tb* infection [109, 110]. It appears that Th17 cells are particularly important in the early stages of infection and play a role in granuloma formation and induction of chemokines leading to recruitment of neutrophils and circulating CD4+ T cells [111]. Excessive IL-17 production can lead to tissue damage, and thus Th17 cells have been implicated in TB pathology [112]. During the chronic phase of infection, a balance must be achieved with Th1 and Th17 responses to control bacterial growth but limit immunopathology.

Following BCG vaccination, IL-17 has been shown to drive Th1 responses by downregulating IL-10 and upregulating IL-12 production by DCs [113]. Depletion of IL-17 during *M.tb* challenge reduces chemokine expression and accumulation of IFN-γ producing CD4+ T cells in the lung [114]. Interestingly, IL-17 KO mice are unable to control infection by the hypervirulent *M.tb* strain HN878, although they do survive infection with less pathogenic strains [115]. In the 2-year follow-up study of BCG-vaccinated infants by Kagina et al., frequencies of BCG-specific Th17 cells did not correlate with protection against TB [89]. However, polyfunctional Th17 cells were a correlate of local protective immunity following mucosal BCG vaccination in NHPs [116]. Further studies are required to assess whether Th17 cells, or IL-17 production by other cells, represent a correlate of protection.

6.3.2 CD8+ T Cells

CD8+ T cells are activated by presentation of antigen in association with MHC class I, and *M.tb* contains several MHC class I restricted immunodominant antigens that are recognised by human populations [117]. Similar to CD4+ T cells, cytokine-producing CD8+ T cells primarily secrete IFN-γ, TNF-α and IL-2 which have critical functions in *M.tb* infection as described [118]. CD8+ T cells also have cytolytic functions and secrete granzymes, granulysin and perforin. Although their role in protection from TB is less well-defined than CD4+ T cells, antigen-specific CD8+ T cells are induced during *M.tb* infection and are capable of recognising *M.tb*-infected macrophages—particularly those that are heavily infected [119, 120]. Studies have also shown that cytotoxic T cells (CTLs) are capable of killing *M.tb*-infected cells [121]. While some murine studies support a role for CD8+ T cells in

the containment of infection, others do not [122–124]. The involvement of CD8+ T cells may depend on the phase of infection, and it has been suggested that mice may not be the most appropriate model for evaluating the relevance of this cell type in humans as they lack some important immune features relating directly to CD8+ T cell function and specificity [125]. While CD8+ T cells provide less protection than an equivalent number of CD4+ T cells, adoptive transfer experiments show that CD8+ T cells can mediate protection against TB, even in the absence of CD4+ T cells. In the more closely related NHP, CD8+ T cell depletion leads to a significant decrease in immunity against *M.tb* in previously infected and treated animals upon reinfection [126].

BCG vaccination has been shown to elicit CD8+ T cell responses, and activation of CD8+ T cells following vaccination can protect against *M.tb* challenge in mice [127]. Furthermore, depletion of CD8+ T cells compromises BCG vaccine-induced immune control of *M.tb* replication in NHPs [126]. Several TB vaccine candidates aim to elicit potent CD8+ T cell responses, including VPM1002 for which cross-priming of CD8+ T cells has been proposed as a major mechanism underlying the superior protection conferred over BCG [128] and the RhCMV/TB vaccine which stimulates HLA-E restricted CD8+ T cells and is extremely protective in NHPs [129]. While the induction of CD8+ T cells may prove beneficial, their capacity for mediating protection could be highly dependent on the vaccine antigen(s) selected [130].

6.4 Humoral Immunity

For most successful prophylactic vaccines, the induction of broadly neutralizing antibodies against exposed and stable immunodominant pathogen epitopes is sufficient to achieve protection [131, 132]. However, for a complex intracellular pathogen such as *M.tb*, the protective immune response has been traditionally considered to be almost entirely cell-mediated, with humoral immunity largely overlooked [133].

6.4.1 B Cells

Memory B cells constitute a key element of central immune memory, particularly against pathogens with a long incubation period [132]. The rationale for historically neglecting the biological relevance of humoral immunity to the control of *M.tb* predominantly derives from early B cell KO mouse models, where the absence of humoral responses did not affect the course of *M.tb* infection [134–136]. Conversely, adoptive transfer of B cells did reverse the increased lung immunopathology in B cell KO *M.tb*-infected mice, and depletion of CD20+ plasmablasts induced increased bacterial burden in the granulomas of NHPs [137, 138]. Differences in experimental design and implementation (dose, route of delivery, phase of infection and

Mycobacterium strain) may be plausible explanations for such inconsistencies [139]. Furthermore, B cell subsets of mice and humans hold considerable phenotypic and functional discrepancies, limiting the ability to translate preclinical studies in this context [140]. Treatment of rheumatoid arthritis patients with the monoclonal anti-CD20 antibody rituximab results in B cell depletion, but this does not affect risk of reactivation or new ATB [141].

The Tyk2 gene of the Janus Kinase (JAK) family is essential for effective IL-23 intracellular signalling and the maintenance of mitochondrial respiration in primary pro-B cells [142, 143]. Interestingly, recent large human cohort studies have found that genetic variants of Tyk2, found in approximately 1 in 600 Europeans, are associated with an increased susceptibility to *M.tb* infection [142]. Furthermore, IL-23 is required for long-term control of *M.tb* and B cell follicle formation in the infected lung [144]. Whether Tyk2 variations have a negative impact on the establishment of specific humoral responses against *M.tb* remains to be determined.

In humans, two studies have reported long-lasting PPD-specific memory B cells that persist for decades after BCG vaccination, but their functionality remains poorly defined [145, 146]. The utilization of memory B cell and plasmablast subsets as a predictive tool for disease stratification and as potential correlates of protection has been previously discussed [147]. However, while the frequency of plasmablasts may correlate with the presence of numerous circulating *M.tb* antigens, heterogeneous plasmablast dynamics and the inability to detect *M.tb* can hinder evaluation of which subjects are *M.tb* resisters, individuals with LTBI, ATB patients or potentially even individuals who have achieved sterilizing immunity [148]. Mucosal vaccination with the attenuated *M.tb* vaccine candidate MtbΔ*sigH* confers significant protection against a lethal TB challenge, and this is strongly associated with levels of inducible bronchus-associated lymphoid tissue (iBALT) in the lung, suggesting an important role for B cells [149].

6.4.2 Antibodies

Most efforts to characterize the antibody response to *M.tb* have relied on non-high-throughput detection methods in serum or plasma against different *M.tb* antigen mixtures, such as PPD, and lack standardized methods for quantification [150]. This may limit the reliability of results, particularly when comparing data sets from different studies [151]. Furthermore, broad quantification of antigen-specific antibodies of a single isotype might be too simplistic a measure, as antibody titre does not consider the quality of the response based on biochemical and functional properties such as Fc receptor binding, affinity, opsonizing ability and influence on mycobacterial growth restriction. Antibodies could contribute to protection directly through increasing phagocytosis and phagolysosome formation in professional phagocytes, blocking uptake by non-professional phagocytes and/or indirectly enhancing T cell-mediated immunity [133].

Monoclonal antibodies against lipoarabinomannan (LAM) and arabinomannan (AM) have been linked to prolonged survival during *M.tb* infection in mice, suggesting potential functional relevance for infection control [152, 153]. Intriguingly, Kumagai et al. demonstrated that *M.tb* infection influenced host protein, including antibody, glycosylation in mice, and the glycosylation patterns of antibodies may affect their function [154, 155]. In humans, individuals with LTBI harbour low-magnitude antibody responses with a distinct constant domain (Fc) glycosylation when compared to ATB patients [156]. In the same study, antibodies from LTBI individuals drove enhanced phagolysosomal maturation, inflammasome activation and macrophage killing of intracellular *M.tb* [156]. Moreover, healthy individuals highly exposed to *M.tb* who tested negative by IGRA and TST, so-called 'resisters', display enhanced antibody avidity and distinct *M.tb*-specific IgG Fc profiles [157, 158]. Some antibodies isolated from exposed but uninfected healthcare workers mediated protection against *M.tb* challenge when transferred to mice [159]. Taken together, the growing body of literature indicates that the relevance of the antibody response in protection against TB may be greater than previously appreciated [160, 161].

However, little is known about the antibody responses induced by BCG or other TB vaccine candidates as few studies have measured this parameter [162]. An early study by de Vallière et al. reported significant induction of LAM-specific IgG following both primary and secondary BCG vaccination in healthy volunteers. Incubation with post-BCG vaccination serum significantly increased BCG internalization by macrophages and mycobacterial growth inhibition *in vitro*, an effect that was reversible by preabsorption of IgG [163]. Consistent results from an independent study have shown opsonization mediated by BCG vaccine-induced IgG follows a dose-response biological gradient [146]. In addition, in BCG-vaccinated infants, an association has been reported between vaccine-induced Ag85A-specific IgG antibodies and a reduced risk of TB disease [90]. The M72/AS01$_E$ vaccine candidate has been shown to be a potent inducer of antibodies which were sustained throughout the 3-year follow-up period, although it remains to be determined whether these contribute to the ~50% efficacy observed against ATB in *M.tb*-infected individuals [164].

The recent finding that intravenous (IV) BCG confers superior protection from TB compared with other routes of immunisation in NHPs has brought to light the relevance of TB vaccine delivery routes [165]. Among other immune parameters, titres of IgG and IgA against *M.tb* whole cell lysate were significantly higher in serum and bronchoalveolar lavage (BAL) fluid following BCG administered by the IV route compared with aerosol or intradermal (ID) vaccination [165, 166]. Notably, this response was characterized by robust IgM secretion, which correlated with reduced bacterial burden and prevention of *M.tb* infection, indicating the relevance of isotype [166]. Secretory IgA may be of particular importance in protecting against pulmonary infection at the mucosal surface by blocking mycobacterial entrance and/or modulating proinflammatory responses [167–170]. A recent NHP study demonstrated that mucosal BCG vaccination by endobronchial installation prevented *M.tb* infection and TB disease in NHP, and IgA was a correlate of local protective immunity [116].

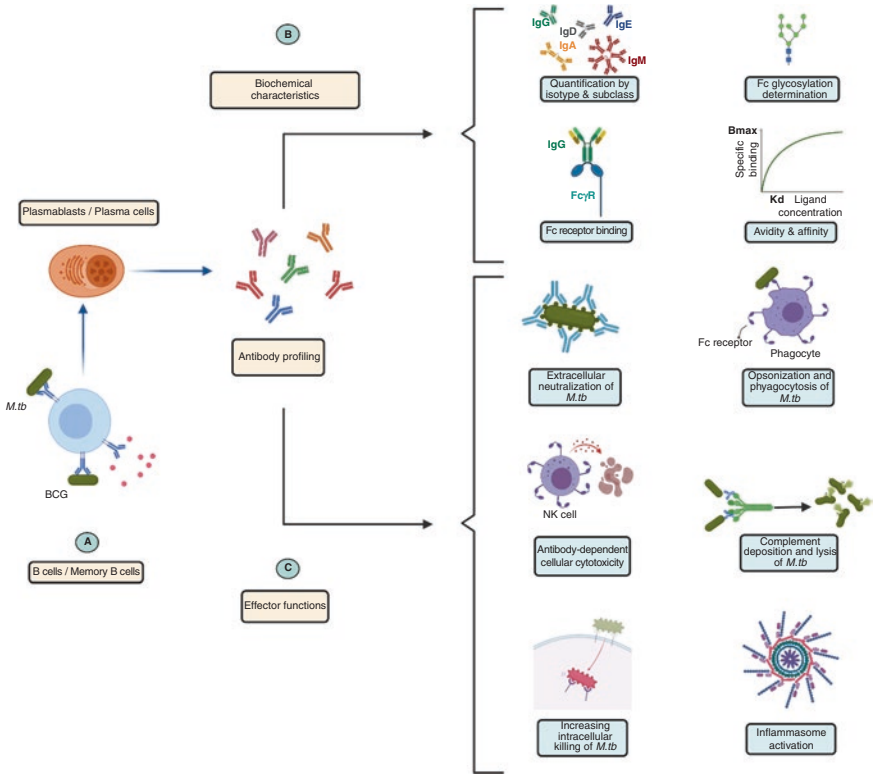

Fig. 6.3 Potential mechanisms of humoral immunity against *M.tb*. Circulating frequencies of memory B cells and plasmablasts (**a**), biochemical characteristics of antibodies such as glycosylation and avidity (**b**) and antibody effector functions such as ADCP and ADCC (**c**) may represent potential humoral correlates of protection, although further study is required. *ADCP* antibody dependent cellular phagocytosis, *ADCC* antibody dependent cellular cytotoxicity (Created with BioRender.com)

Characterization of the biochemical and functional features of *M.tb*-specific antibodies in preclinical and clinical studies may provide valuable insights regarding their relevance as a potential correlate of *in vivo* protection against TB (Fig. 6.3).

6.5 Approaches to Identifying Correlates of Protection

There are several methods for characterizing potential correlates of protection from TB, including comparing immune responses between protected and unprotected animals or individuals, conducting human experimental medicine studies and using tractable *ex vivo* functional assays.

6.5.1 Preclinical Models

Preclinical studies are pivotal to (a) assess the safety of a vaccine candidate and (b) evaluate vaccine efficacy and identify potential correlates of protection that can then be validated in humans [132]. In addition to expediting TB vaccine development, correlates or surrogates of protection would be valuable from a 3Rs (Replacement, Reduction and Refinement) perspective as they would allow the estimation of vaccine efficacy without the need to infect animals with virulent *M.tb*—a procedure of 'Moderate' severity under EU legislation and UK Animal Scientific Procedures Act (ASPA) licensure [171]. However, surrogates of protection derived from preclinical models have uncertain predictive value, thus hindering candidate prioritization for progression to clinical trials [172]. The typical animal models utilized for TB vaccine evaluation are mice, guinea pigs, cattle and NHPs [173, 174]. Additionally, novel humanized animal models have been introduced as a 3Rs approach [175].

By comparing responses between groups in vaccine studies and by cross-sectional analysis of responses from those animals in which vaccination was successful (protected) compared to animals in which vaccination failed (unprotected), it is possible to identify potential correlates of protection. Preclinical studies have the advantage that one can challenge vaccinated animals with virulent *M.tb*, which is unethical in humans, and that the exact timing, dose and route of infection can be manipulated. They also allow access to relevant tissue sites that would not be possible in humans. Each model has a unique set of advantages and disadvantages based on its ethical, monetary and logistical costs, susceptibility to *M.tb* infection and extent to which it can reflect human physiology and the clinical spectrum of disease.

6.5.1.1 Murine Models

Of all the preclinical models for TB vaccine evaluation, mice are the most extensively utilized due to relatively low cost, short generation time, standardization due to inbreeding and abundance of commercial reagents [175]. However, key features of TB lesions in humans such as necrosis and hypoxia are lacking in the most widely used mouse strains, and the immune system bears discrepancies with humans in both innate and adaptive features [140]. Furthermore, the most commonly utilized mouse strains, BALB/c and C57BL/6, exhibit different sensitivities to *M.tb* infection underlined by the constitutive expression of IL-10 [176]. There is some inconsistency in how 'protection' is defined in murine, and other preclinical, models compared with humans. While in humans vaccine efficacy is based on prevention of *M.tb* infection or TB disease using clinical endpoints, in preclinical models it is based on an improvement in a disease-related readout such as bacterial load, pathology score or long-term survival. As such, a vaccine may be considered 'protective' in preclinical assessment even in the presence of measurable bacteria or pathology or if some animals do not survive [172]. A further limitation is the differences between artificial aerosol *M.tb*

challenge and natural transmission in humans [172]. Nevertheless, murine models have provided critical insights into the immune response to *M.tb* and allowed the generation of hypotheses about which parameters are associated with protection, as detailed in the preceding sections. Notably, murine studies identified the importance of IFN-γ in the control of *M.tb* and the potential association between polyfunctional CD4+ T cells and protection from *M.tb* challenge [81, 106].

6.5.1.2 Guinea Pigs

Guinea pigs are more susceptible to TB than mice and are generally considered to follow a more representative process of human infection with strong initial immunity which is eventually associated with tissue damage, leading to extensive caseation and tissue necrosis and ultimately death [177]. Due to such parallels with features of human TB and high reproducibility, the guinea pig model is the most commonly used system to further evaluate vaccine candidates which appear promising in the mouse. However, BCG vaccination confers stronger protection in guinea pigs than mice, which may limit ability to detect incremental improvements conferred by vaccine candidates [173]. To overcome the issue of unnatural *M.tb* challenge models, a natural exposure system has been developed in guinea pigs whereby they breathe the extracted air from a ward of TB patients and thus receive multiple low-dose aerosol exposures to clinical strains [178]. However, use of guinea pigs entails a higher ethical, economic and logistical expense than mice, the commonly available strain is outbred, and 100% of guinea pigs develop active disease compared with ~10% of humans. While a number of studies report safety and efficacy outcomes of candidate TB vaccine studies in guinea pigs, evaluation of immune responses and therefore identification of potential correlates of protection are limited by a lack of reagents. However, the recent emergence of new technologies, reagents and assays provides promise for the utility of this model in the future [179].

6.5.1.3 Cattle

The adaptive immune system of cattle is similar to that of humans, with several aspects making them more representative of the human response to *M.tb* infection than rodents including less reliance on antigen-specific IFN-γ activation of macrophages, a more active role for cytotoxic cells and the presence of genes encoding cytokines not found in mice such as IL-26 [180, 181]. Furthermore, cattle are a natural target species of TB infection, and *M. bovis* infection offers a wide spectrum of TB disease that resembles that found in humans. Antigen-specific expression of IFN-γ and IL-2 following BCG vaccination in calves has been shown to correlate with protection from *M. bovis* challenge [182], and a study of the viral booster vaccines MVA85A and Ad85A identified antigen-specific IFN-γ memory responses by cultured ELISpot and *in vitro* IL-17 production as correlates of protection following *M. bovis* challenge [183].

6.5.1.4 Non-human Primates (NHPs)

The immune response in humans and NHPs is very similar due to their close evolutionary relationship. Together with susceptibility to pulmonary infection with strains of *M.tb* that are pathogenic to humans, and similarities in the spectrum of TB disease exhibited, this makes NHPs the most attractive model for preclinical TB vaccine evaluation and the most relevant for identification of immune correlates of protection [175]. However, NHPs incur significant ethical and monetary costs, as well as variability across individuals, which may limit their widespread use [175]. Furthermore, different subspecies of macaques differ in their susceptibility to *M.tb* infection and response to BCG vaccination [184, 185], although corresponding variations in CD4+ and CD8+ T cell and myeloid DC subsets could bring to light useful immune parameters for the identification of correlates of protection [186].

Importantly, as previously described, recent NHP studies have demonstrated superior protection, and in some cases sterilizing immunity, from TB following BCG vaccination administered by different routes [116, 165, 187]. Pulmonary mucosal BCG vaccination has been shown to prevent infection following repeated limiting-dose *M.tb* challenge, and polyfunctional Th17 cells, IL-10 and IgA were identified as correlates of local protective immunity [116]. Antigen-specific Th1/Th17 cells in the lung were also associated with the degree of *M.tb* control in granulomas in a study of *M.tb*-infected macaques [188]. IV BCG vaccination confers superior protection compared with delivery by ID or aerosol routes or as an intratracheal mucosal boost [165, 187]. Regardless of whether such a strategy could ever be deployable in humans, this finding provides a key opportunity for the identification of immune correlates and mechanisms of vaccine-mediated protection against TB. Superior protection in IV BCG-vaccinated NHPs was associated with greater induction of multifunctional CD4+ T cell producing IFN-γ and TNF-α [187] and with higher numbers of antigen-responsive CD4+ and CD8+ T cells in the blood, spleen, BAL and lung lymph nodes, as well as a higher frequency of antigen-responsive T cells across all lung parenchymal tissues [165]. Further insight into the exact mechanisms of protection will require additional cross-sectional analysis of responses in studies where a greater range of protection is elicited within an intervention group.

Prevention of TB in macaques has also been demonstrated following immunisation with the RhCMV/TB vaccine candidate, which was associated with greater induction and maintenance of high frequencies of highly effector-differentiated circulating and tissue-resident *M.tb*-specific CD4+ and CD8+ memory T cell responses compared with BCG [129]. Interestingly, there were no significant antibody responses to the nine TB antigens in the RhCMV vector inserts, suggesting that antibodies do not contribute to the protection observed [129]. However, IgG1 was the only subclass tested, and the potential relevance of different isotypes has been highlighted in Sect. 6.4.2 [166].

6.5.1.5 Novel Humanized Animal Models

While mammals are the most frequently used experimental animal models in TB vaccine development, mycobacterial infection of invertebrates (zebrafish, the fruit fly *Drosophila melanogaster* and the amoeba *Dictyostelium discoideum*) has provided novel insights, particularly with respect to elucidating the early events following mycobacterial infection. Such models offer advantages in terms of ethics, resources, costs and technological ease [175]. Zebrafish can develop granulomas after infection with *M. marinum* with some parallels to human *M.tb* infection, and the innate and adaptive immune responses share the same primary cellular components with humans [189]. However, a major limitation is that zebrafish do not have lungs, and additional evidence is required to establish the ability to translate these findings to human physiology during *M.tb* infection [175].

6.5.2 Clinical Studies

While preclinical studies have undoubtedly been central to advancing our understanding of the immune response to *M.tb* and generating hypotheses regarding potential correlates of protection, the immunopathogenesis of *M.tb* in humans is complex, highly heterogeneous between individuals and subject to biological and environmental influences that are impossible to model experimentally in animals. Thus, studies using clinical samples are key. There are two main approaches to identifying potential correlates of risk of, or protection from, TB using clinical samples. The first is to perform studies using samples from TB vaccine efficacy trials, comparing immune responses in individuals who remained healthy ('controls') with those who became *M.tb* infected or developed TB disease ('cases'). However, few TB vaccine efficacy trials have been conducted to date, which limits opportunities for such analysis. The other strategy is to perform observational studies comparing immune responses in (a) individuals with LTBI, who are considered to have some degree of protection, with ATB patients; (b) LTBI individuals who go on to develop ATB with those who do not; or (c) household contacts of ATB patients who remain uninfected with those who become *M.tb* infected or develop ATB. An overview of clinical studies and their main findings with respect to potential correlates of protection is summarized in Table 6.1.

Table 6.1 Clinical studies discussed in this chapter with findings of relevance to potential correlates of protection

Clinical study cohort	Study type/ intervention	Relevant findings with respect to potential correlates of protection	Reference
South African infants	Case-control; BCG vaccination	No differences between protected and unprotected infants in frequencies of BCG-specific CD4+ T cells, CD8+ T cells, γδ T cells or extended cytokine profiles	[89]
South African infants	Case-control; BCG vaccination	Two distinct clusters of infants with different myeloid and lymphoid activation and inflammatory gene expression patterns. Infants with the highest or lowest ML ratios at risk of developing TB disease	[190]
BCG-vaccinated South African infants	Post hoc analysis of Phase IIb MVA85A efficacy trial	BCG-specific IFN-γ secreting T cells and Ag85A-specific IgG associated with reduced risk of TB disease. Frequency of activated HLA-DR+ CD4+ T cells, which may be driven by CMV infection, associated with increased risk	[90, 192]
South African adults	BCG revaccination	Long-lived BCG-reactive NK cells induced	[195]
IGRA+ and IGRA− Indian adults	BCG revaccination	Anti-mycobacterial Th1/Th17 responses significantly boosted	[196]
M.tb-infected South African adolescents	Prospective cohort study	16-gene signature could predict TB progression a year prior Minimal role for granulocytes/whole blood components. Interferon response gene module over-represented	[197]
LTBI and ATB patients in intermediate- and high-burden settings	Cohort study	Gene signature for ATB dominated by a neutrophil-driven IFN-inducible gene profile, consisting of both IFN-γ and Type I IFN-αβ signalling	[49]
ATB patients in Africa	Prospective cohort study	4-gene signature predicting progression to ATB up to 2 years before onset of disease; confirms importance of Type I IFN pathway	[198]
LTBI and ATB patients from South Africa	Cohort study	Antibodies from LTBI individuals have unique Fc functional profiles, selective binding to FcγRIII and distinct glycosylation patterns and drive enhanced phagolysosomal maturation, inflammasome activation and macrophage killing of intracellular *M.tb*	[156]
Exposed but uninfected healthcare workers and LTBI Ugandan adults	Cohort study	Enhanced antibody avidity and distinct *M.tb*-specific IgG Fc profiles in resisters	[157]

ML ratio, ratio of monocytes to lymphocytes; NK, natural killer; LTBI, latently *M.tb* infected; ATB, active TB disease

6.5.2.1 Clinical TB Vaccine Trials

As discussed, a study of over 5500 South African infants attempted to identify correlates of protection against childhood TB disease after BCG vaccination at birth. Infants were followed for 2 years and classified as controls if they did not have a household TB contact and did not develop TB disease (community controls, $n = 55$) or as having a household TB contact in which case they were grouped into those who developed TB disease (cases, $n = 29$) and those who did not develop TB disease (household contact controls, $n = 55$) [89]. Unfortunately, no differences were identified between protected and unprotected infants in the frequencies of BCG-specific CD4+ T cells, CD8+ T cells, γδ T cells or the extended cytokine profiles of these cells [89]. A different study in BCG-vaccinated South African infants with a 2-year follow-up compared host responses in blood at 10 weeks of age between infants who developed ATB during the follow-up period and those who remained healthy [190]. Gene expression analysis failed to identify any correlates of protection, but two distinct clusters of infants were evident with different myeloid and lymphoid activation and inflammatory patterns. Cases from each cluster demonstrated distinct patterns of gene expression, suggesting that unique correlates of risk may not be found within clusters. Interestingly, infants with the highest or lowest ratios of monocytes to lymphocytes (ML ratio) were at risk of developing TB disease [190]. This association has also been reported in other studies [29, 191].

Although the candidate TB vaccine MVA85A did not confer statistically significant efficacy in a Phase IIb trial in BCG-vaccinated South African infants [88], the samples collected provide a rich resource for identification of potential correlates of protection. In a case-control correlates of risk analysis using samples from 53 infants who developed TB disease and 205 matched controls, BCG-specific IFN-γ secreting T cells were associated with reduced risk of TB disease [90]. This is inconsistent with the previously described findings of Kagina et al., which may be a result of different methods for measuring IFN-γ and the different time-points post-BCG vaccination at which this was done [89]. Levels of Ag85A-specific IgG were also associated with reduced risk of TB disease, while the frequency of activated HLA-DR+ CD4+ T cells was associated with increased risk as validated in an independent cohort of *M.tb*-infected adolescents [90]. A follow-up transcriptomics analysis found that cytomegalovirus (CMV) infection was a major driver of CD8+ T cell activation and that a CMV-specific IFN-γ response was associated with increased risk of developing TB disease [192]. In CMV-positive infants, NK cell signatures and cell frequencies were associated with lower risk of TB disease [192]. Interestingly, a potential role for NK cells in protection from TB has also been identified in a multi-cohort study using CyTOF for systems analysis of immune cell frequency combined with transcriptional analysis [193].

Promising results from more recent vaccine trials provide further opportunities for assessing potential correlates of protection. A Phase IIb efficacy trial of the M72/AS01$_E$ candidate vaccine reported 54% protection against ATB in *M.tb*-infected individuals, with an overall efficacy of 49.7% (90% CI, 12.1–71.2; 95% CI,

2.1–74.2) at 3 years [164], while BCG revaccination in South African adolescents reduced the rate of sustained QFT conversion by 45.4% [194]. An international consortium known as the 'TB Immune Correlate Program' has been formed to evaluate samples from these trials, although the relatively small number of participants that reached clinical endpoints (39 individuals developed ATB in the M72/AS01$_E$ trial, and 57 individuals had sustained QFT conversion in the BCG revaccination trial) may limit statistical power to identify correlates. BCG revaccination has been shown to induce long-lived BCG-reactive NK cell responses in South African adults [195] and to boost adaptive polyfunctional Th1/Th17 responses and innate effector cells in a different cohort of IGRA+ and IGRA− Indian adults [196].

6.5.2.2 Observational Studies

Observational studies do not include an intervention and are therefore less logistically complex than vaccine trials. Several such studies have taken a systems analysis approach to enable the unbiased simultaneous detection of multiple parameters—particularly through transcriptomics. In a prospective cohort study, Zak et al. defined a 16-gene signature that could predict TB progression a year prior to TB diagnosis. This signature was comparable in whole blood and peripheral blood mononuclear cells, which suggests a minimal role for granulocytes and other whole blood components. The only gene module that was over-represented in the risk signature was the interferon response [197]. Another transcriptomic study reporting a gene signature that could discriminate between LTBI and ATB also demonstrated the importance of the interferon pathway, as it was dominated by a neutrophil-driven IFN-inducible gene profile, consisting of both IFN-γ and Type I IFN-αβ signalling [49].

Additional studies across different countries have validated and refined the signatures for progression to ATB down to four transcripts and again confirm the importance of the Type I IFN pathway in TB disease risk [198, 199]. Scriba et al. showed that progression from LTBI to ATB is defined by sequential inflammatory processes triggered by a rise in Type I IFN and activation of the complement cascade, including a change in ML ratio, a rise in HLA-DR+ CD4+ T cells and a decline in naïve T cells [200]. One challenge to such longitudinal studies of progression to ATB is the relatively low rate of reactivation (5–10%) in LTBI individuals, necessitating large sample sizes and long follow-up periods. Furthermore, it is difficult to draw any inferences, as findings may represent features of subclinical or incipient TB disease rather than being a cause of TB susceptibility. Nevertheless, there is obvious merit in the ability to identify individuals at risk of progression to ATB.

In addition to transcriptomic studies, other high-throughput and unbiased systems approaches such as proteomics, metabolomics and systems serology are being applied to similar cohorts and could shed light on new protective immune pathways and parameters to progress to further study [156, 201].

6.5.3 Controlled Human Infection Models

Controlled human infection models (CHIMs) are a powerful tool to fast-track vaccine development and to characterise pathophysiology and have led to the licensure of vaccines against influenza, malaria and cholera [202–206]. Given the unclear predictive value of animal models, a *Mycobacteria* CHIM could expedite large-scale field trials and aid therapeutic development and the identification of correlates of protection. However, infecting humans with virulent *M.tb* would be unethical. *M.tb* clearance requires months of treatment with potentially toxic therapeutics, and volunteers may remain infectious during antibiotic therapy, with risk of community transmission [207, 208]. In addition, there is an unclear curative endpoint due to the lack of a definitive marker of infection. This leads to risk of treatment failure and the establishment of latency in volunteers [9, 209]. Instead, PPD and BCG have been used as surrogate models of *M.tb* infection in CHIMs. Both products are licenced for human use, as PPD is used for TST, while BCG is delivered as a vaccine. This facilitates their ease of procurement and delivery in CHIMs.

6.5.3.1 PPD CHIMs

While PPD cannot be used to test vaccine efficacy as it is not a live replicating organism and hence quantification of bacterial growth control is not possible, it contains antigens from *M.tb* not found in BCG and hence could provide adjunct information about immune mechanisms following *M.tb* antigen exposure.

6.5.3.2 BCG CHIMs

BCG is a live replicating *Mycobacterium*, which has been used safely as a licenced ID vaccination against TB in humans for over 100 years [210, 211]. BCG is less virulent than *M.tb* in humans as it lacks the region of difference 1 (RD1) locus which encodes the virulent ESX-1 secretion system [212, 213]. BCG does not cause disease in immunocompetent people, and therefore a BCG CHIM enables the study of an effective mycobacterial clearing immune response [214, 215]. While BCG elicits a similar CD4+ T cell-mediated immune response to that of *M.tb* [215–217], differences in immune responses between BCG and *M.tb* have been described in human *in vitro* and animal *in vivo* models [13, 218, 219]. In the absence of an *M.tb* CHIM, the BCG CHIM is a useful surrogate, designed to be used in conjunction with *M.tb* animal models and human TB epidemiological studies.

The first BCG CHIM utilised the ID route, which enables easy quantification of BCG growth control via skin punch biopsy [215]. Peak BCG growth following $1–4 \times 10^5$ colony forming units (CFU) ID injection of BCG Danish 1331 was demonstrated at 1–2 weeks, with 2 weeks providing the least variable endpoint while allowing time for the adaptive immune response to affect growth control. This safe,

feasible BCG CHIM model demonstrated superior mycobacterial growth control in historically BCG vaccinated compared with BCG-naïve participants and has been validated against a vaccine effect following virulent challenge in mice, NHPs and cattle [215]. This supports the utility of this model to assess vaccine efficacy in this *M.tb*-naïve UK adult population, where BCG vaccination is known to be protective [220].

The ID BCG CHIM has also been used to assess the efficacy of MVA85A, demonstrating no added benefit over historic BCG vaccination alone [221]. This finding corresponds to results from the Phase IIb efficacy trial in South African infants, supporting the validity of this model in assessing mycobacterial vaccine efficacy [88]. Importantly, both ID BCG CHIM studies reported an association between the PPD-specific IFN-γ ELISpot response at 2 weeks post-challenge and control of growth of mycobacteria isolated from a skin biopsy of the challenge site [88, 215]. This is consistent with the findings of the correlates of risk analysis using samples from the MVA85A efficacy trial where IFN-γ ELISpot was associated with lower risk of TB disease [90]. Transcriptional analysis of blood samples from human challenge studies also identified IFN-γ and IL-18 transcripts as correlates of *in vivo* mycobacterial growth control [222].

While ID BCG CHIMs show promise, the natural route of *M.tb* infection is via the airway. Vaccine efficacy varies depending on route of delivery, and protective immune mechanisms can be localised to the tissue infection site [165, 223]. Hence work is underway to develop an aerosol BCG CHIM. Three studies in the 1960s and 1970s delivered intrapulmonary BCG to healthy volunteers or lung cancer patients and demonstrated good safety profiles [224–226]. In 2019, a proof-of-concept safety study was published, which delivered up to 0.5 tuberculin units of PPD and 1×10^4 CFU BCG by local bronchoscopic instillation to mycobacteria-sensitised South African adults without any clinically significant adverse events [227]. However, bronchoscopic instillation is not the natural route of infection. To model naturally acquired *M.tb* infection, McShane et al. are developing an aerosol CHIM in which BCG is inhaled using an Omron NE-U22 micro air mesh nebuliser. Following aerosol inhalation, BCG is recovered at a defined time-point via bronchoscopic saline lavage (ClinicalTrials.gov NCT02709278) (Fig. 6.4). Work is ongoing to quantify the BCG in the airway, which could then be used as a marker of *in vivo* mycobacterial growth control, informing vaccine efficacy and providing opportunities for the identification of immune correlates of protection (H McShane, personal communication).

6.5.3.3 Other CHIM Models

One major drawback of the BCG CHIM is the inability to test efficacy of vaccine candidates based on the RD1 antigens, many of which are currently in the vaccine development pipeline [228, 229]. Furthermore, an *M.tb* CHIM would enable full elucidation of immune mechanisms, some of which may not occur following exposure to the less virulent BCG. Candidates for an *M.tb* CHIM could include the

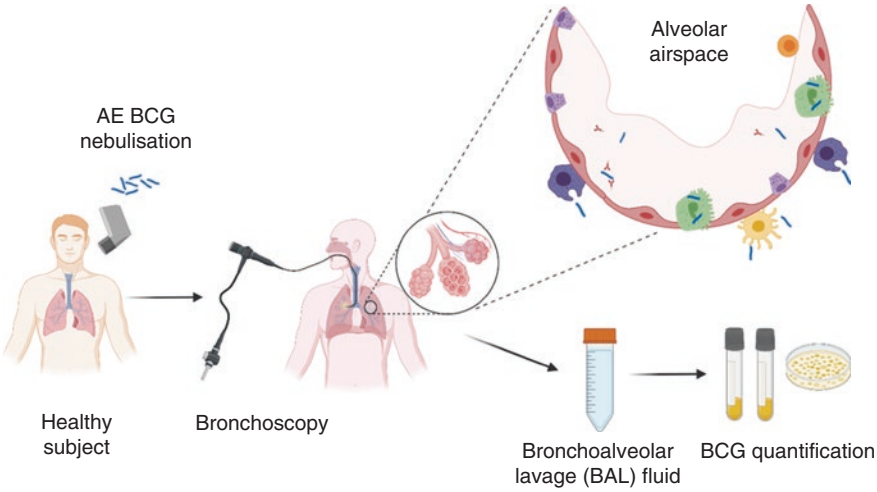

Fig. 6.4 Inhaled BCG controlled human infection model (CHIM). Healthy *M.tb*-naïve subjects receive BCG by the aerosol route using a nebuliser. BCG is recovered via bronchoscopic saline lavage at a defined time-point. *AE* aerosol. (Created with BioRender.com)

attenuated vaccine candidate MTBVAC [230]. More preferable would be a genetically modified but virulent strain that can be definitely cleared, such as the *M.tb* strain being developed by a consortium of bacterial geneticists with support from the Gates Foundation, which only survives in the presence of continuous antibiotic or other therapy (S Fortune, personal communication).

In the absence of an ethical virulent *M.tb* strain available for CHIM studies, the BCG CHIM promises to be a useful tool to fast-track vaccine candidate selection and to further our understanding of protective immune mechanisms.

6.5.4 Mycobacterial Growth Inhibition Assays

An alternative approach to identifying correlates of protection is the use of functional mycobacterial growth inhibition assays (MGIAs). Rather than measuring predefined individual immune parameters of unknown relevance, MGIAs are unbiased and take into account a range of immune mechanisms and their complex interactions in an *ex vivo* environment; as such they may represent a surrogate of protective efficacy in themselves [231]. They also provide a tractable model in which effector functions may be studied through such techniques as cell depletion or concentration or *in vitro* 'adoptive transfer'. MGIAs offer further advantages of being high-throughput, inexpensive and a 3Rs refinement to preclinical vaccine efficacy and correlates of protection studies, as they avoid the need for *in vivo M.tb* challenge [232].

While simple in principle—blood or cells are collected pre- and post-vaccination, and ability to inhibit growth of mycobacteria is measured following *in vitro*

inoculation—these assays are notoriously technically challenging and have historically suffered from a lack of reproducibility and transferability [233]. Several approaches have been described with varying degrees of success, including the addition of stimulated lymphocytes to infected monocytes [234], the co-culture of bone marrow-derived macrophages and splenocytes [235] and the use of luciferase-expressing reporter strains of mycobacteria [236]. These and other MGIAs have been comprehensively reviewed elsewhere [231]. More recently, a significant effort has been made by Tanner et al. to develop a simplified cross-species assay known as the 'direct MGIA'. This assay, adapted from a method originally developed for TB drug evaluation by Wallis et al. [237], has now been optimized and harmonized for use in humans, mice and NHPs [238–240]. Mycobacterial growth in the MGIA has been associated with TB disease state and treatment status [241], and ability to detect a BCG vaccine effect using this assay has been demonstrated across multiple studies [238, 239, 242–245]. Some degree of biological validation has been achieved through correlation with protection from *in vivo* mycobacterial challenge [239, 245] or protection from vaccine candidates of varying efficacy [246, 247].

Various MGIA studies have indicated a detrimental effect of depletion, or enhancing effect of enrichment, of CD4+ T cells and/or CD8+ T cells on control of mycobacterial growth [248–251]. A role for γδ T cells has also been proposed [249]. Notably, most published MGIA studies report no correlation between mycobacterial growth inhibition and IFN-γ responses, despite both being significantly enhanced following BCG vaccination [25, 236, 238, 242, 252]. However, in an *in vivo* challenge study in UK adults, control of mycobacterial growth at baseline in historically BCG-vaccinated volunteers was associated with the IFN-γ ELISpot response measured at 2 weeks post-challenge [245]. The authors suggest that this disparity is likely because the challenge study focused on associations with post-infection *in vivo* responses, which permits consideration of re-stimulated memory responses in historically vaccinated volunteers [245]. Since the MGIA models an infection, immune parameters induced by *in vivo* challenge and contributing to control of mycobacterial replication *in vivo* may reveal those driving control of mycobacterial growth in the MGIA. Interestingly, there was an association between MGIA control and frequencies of several subsets of polyfunctional CD4+ T cells including IFN-γ, TNF-α and IL-2 triple-positive cells [245], which was consistent with findings by Smith et al. [244]. However, others have reported no correlation between MGIA response and polyfunctional T cells, which may be due to the measurement of effector responses soon after vaccination [25, 247].

In an MGIA study comparing healthy volunteers, individuals with LTBI and patients with active TB disease, several immune parameters were associated with differential mycobacterial control including distinct monocyte subsets, B cell subsets and IgG1 responses [241]. IgG responses post-challenge have also been weakly associated with control of mycobacterial growth in BCG-vaccinated individuals [245]. Hierarchical cluster analysis of serum cytokine responses revealed correlations between high analyte levels and enhanced mycobacterial control for several cytokines including CXCL-10 and PDGF-BB [241]. Interestingly, Joosten et al. noted an association between control of mycobacterial growth and the presence of a

CXCL10-producing CD14^{dim} monocyte population, which was dependent on the presence of T cells. Thus, trained innate immunity may contribute to the superior control of mycobacterial growth they observed in individuals with recent exposure to *M.tb* and some BCG vaccinated individuals [25]. Finally, distinct transcriptomic profiles have been associated with good vs. poor mycobacterial control in the MGIA, with good controllers showing enrichment for gene sets associated with antigen processing/presentation and the IL-23 pathway and poor controllers showing enrichment for hypoxia-related pathways [245]. Such insights may be hypothesis-generating with respect to potential correlates of protection that can be taken forward to *in vivo* studies.

6.6 Conclusion

The lack of validated immune correlates of protection remains a major barrier to the development of new TB vaccines. While IFN-γ secreting CD4+ T cells are clearly a cornerstone of the immune response to TB, the fact that boosting with MVA85A failed to confer enhanced protection over BCG alone, despite generating a higher level of these cells, suggests that we need to look beyond this paradigm. Progress is now being made in understanding the role of previously under-explored immune parameters including unconventional T cells, Th17 cells, B cells and antibodies, although their exact contribution to protective immunity and potential as correlates of protection remain ill-defined. For such a complex pathogen, it is possible that a 'biosignature' comprising a combination of immune measures rather than any given single parameter will be necessary. Compartmentalization of immune responses may also be important to consider, particularly as local responses in the lung may correlate better with protection from initial infection than those in the periphery. Recent advances in the TB field are providing key opportunities for correlate of protection analysis. Notably, evidence of sterilizing immunity in preclinical studies, positive efficacy results in proof-of-concept clinical trials, advances in systems technologies and the development of *in vivo* CHIMs and *in vitro* MGIAs will provide the samples and tools to identify new correlates, with the aim of facilitating and expediting the design, optimization, prioritization and clinical assessment of candidate TB vaccines.

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Chapter 7
Animal Models of Tuberculosis

Huoming Li and Hao Li

Abstract Tuberculosis (TB) is an important zoonotic disease caused by infection with *Mycobacterium tuberculosis* (Mtb) complex and has a significant impact on public health. Animal models are suitable tools to mimic the clinical symptoms observed in human TB and provide an opportunity to understand immune responses to infection and the pathophysiology and pathogenesis of TB. In this chapter, we summarize the animal models that are used in Mtb research, including common models such as the mouse, rat, guinea pig, non-human primates, rabbit, cattle and zebrafish, as well as discuss some newly established animal models.

Keywords Animal models · *Mycobacterium tuberculosis* complex · Tuberculosis Zoonosis

7.1 Introduction

In 2020, an estimated ten million people fell ill with tuberculosis (TB) worldwide, and approximately 1.5 million people died from TB. TB is the second leading infectious killer after COVID-19 (above HIV/AIDS) [1]. In recent years, the global incidence of TB has increased further due to antibiotic abuse and the prevalence of HIV, both of which aggravate TB control [2]. Therefore, the development of anti-TB drugs and vaccines has become urgent, to control TB spread. With the development of modern molecular sequencing technology, more information about the biological characteristics of *Mycobacterium tuberculosis* (Mtb) complex has been determined and promoted further research on the pathogenesis of TB. In general, acquiring enough disease information is impossible simply by observing clinical symptoms, and the development of animal models has provided complementary tools for human disease research.

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M. Christodoulides (ed.), *Vaccines for Neglected Pathogens: Strategies, Achievements and Challenges*, https://doi.org/10.1007/978-3-031-24355-4_7

In recent years, the pathogenesis of Mtb infection and the host immune response mechanisms have been widely researched and elucidated by immunological techniques and animal models (Fig. 7.1). Since Robert Koch in 1882 first used guinea

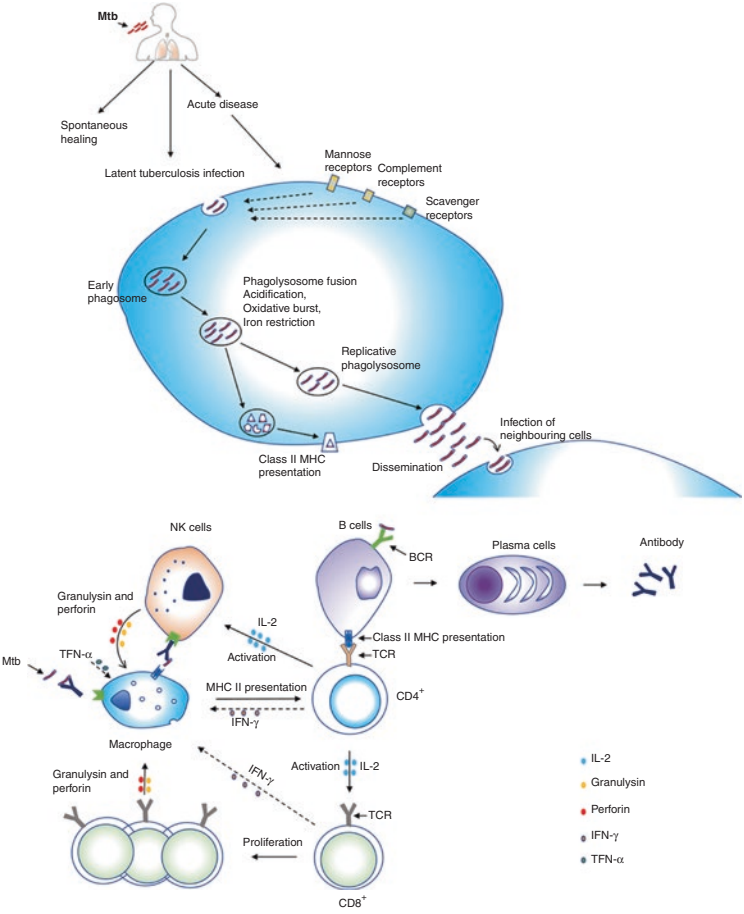

Fig. 7.1 (**a**) The intracellular transport process of Mtb. After Mtb infects the host, macrophages engulf the pathogen with the assistance of a variety of receptors, including complement receptor, mannose receptor and scavenger receptors. Then, common signaling pathways are activated leading to cell activation and cytokine production. Mtb is a classical intracellular pathogen and can persist in the early phagolysosome. After phagosomes mature, intracellular bactericidal substances such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) will kill some Mtb. Surviving Mtb then begin to replicate and multiply within the macrophage, until the cell ruptures and disseminates bacteria to the surrounding cells. (**b**) T cell-dependent regulation of anti-TB immune response in vivo. There are several ways that cellular and humoral immunity may cooperate to protect the host from TB infection. $CD4^+$ T cells can activate B cells to secrete antibody by major histocompatibility complex; $CD4^+$ T cells can produce cytokines, e.g. IL-2 that can activate Natural Killer (NK) cells and promote cytotoxic immune responses associated with antibodies. Conversely, specialized phagocytes process and present TB antigens to $CD4^+$ T cells, which results in increased proliferation of cytotoxic $CD8^+$ T cells and enhanced Mtb killing. Meanwhile, the different T cells produce interferon γ (IFN-γ) that synergizes with tumour necrosis factor α (TNF-α) to activate macrophages

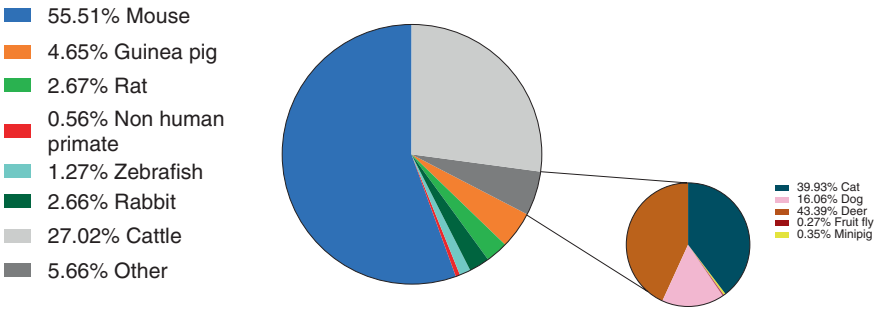

Fig. 7.2 Pie chart showing the different animal models used for TB research. A PubMed search was done using the following keywords: "mouse AND tuberculosis", "rat AND tuberculosis", "guinea pig AND tuberculosis", "non-human tuberculosis AND tuberculosis", "zebrafish AND tuberculosis", "cattle AND tuberculosis" and other animal models, including "cat tuberculosis", "deer tuberculosis", "minipig tuberculosis", "fruit fly tuberculosis" and "dog tuberculosis". The pie chart percentages were calculated as the proportion of each animal model to all the animal models

pigs to prove that Mtb caused TB, various animal models—including the mouse, rat, rabbit, guinea pig, zebrafish, non-human primate, cattle, etc.—have been used to study various aspects of TB [3] (Fig. 7.2). Indeed, animal models have contributed to the development of infection technologies such as aerosol instillation and bacterial growth control following infection through innate and adaptive immune responses, which have provided invaluable contributions to our understanding of TB (Fig. 7.1). Each animal model cannot mimic completely the symptoms of human TB, so in practical application, the characteristics of different animals are often complementary to each other to fulfill research aims. For example, studies with the mouse, which lacks some immunological features, often require using guinea pigs as the complementary animal model to test vaccine effectiveness [4, 5]. At present, the main problem for every experimental animal model is that they cannot fully reflect human symptomatic TB.

A clear understanding of the advantages and disadvantages of each animal model is necessary for experimental Mtb research. Mouse models are currently the most widely used laboratory animals, but they lack some characteristics of human TB including the formation of mature granulomas, central necrosis and cavities [6]. Guinea pigs are highly susceptible to Mtb and often need only a few bacilli to establish an infection in vivo [4]. Traditionally, rabbits were thought to be resistant to TB infection: however, recent reports suggest that rabbits can be used to establish models of chronic progressive granulomatous disease [7]. Although the rabbit model has been widely used, because some of the histopathological features in this animal closely resemble human TB, the lack of available commercial reagents has limited its use [7, 8]. By contrast, non-human primate cynomolgus monkeys are known to mimic TB symptoms in humans, including the formation of solid masses, necrosis and cavities [9]. The major advantage of this model is that it can establish latent TB infection and provide an opportunity to understand latency mechanisms. However, the high cost and hard management of this model limit its wide use in TB research. The use of animal models has greatly boosted TB research in recent years, but

animals are not humans, and even *Homo sapiens*, who are highly similar at the genetic level, cannot fully mimic every aspect of human TB [8]. Therefore, key to successful experimental research is to consider the characteristics of the various animal models available and make reasoned choices. In this review, the characteristics of various animal models are summarized, pointing out their advantages and disadvantages and the research progress that each one has made. Such information informs selection of the most appropriate models to be used for TB research.

7.2 Mouse (Order Rodentia, Family Muridae, *Mus musculus*)

Mouse models are very popular in TB research, and they have been used extensively in developing diagnostics and experimental drugs and vaccines. The first scientist to use mice was Robert Koch [10]. The mouse is often chosen as the research animal model because the genetic and immunological background of humans and mice shares many similarities. Some important discoveries on the pathogenesis of TB, e.g. the roles of adaptive immunity and the process of granulomatous formation, have been discovered with mouse models. In addition, many preclinical trials of new TB drugs and vaccines must first be done in a mouse model before moving into human clinical trials.

In animal experiments, Mtb (usually the reference strain H37Rv) first invade and multiply in the lungs before spreading to other tissues. When establishing the mouse animal models, different infection routes are used, including aerosol exposure, intranasal, intratracheal and intravenous (Table 7.1). The evaluation standards are usually determined by observing the bacterial load in the lungs, histopathological changes and the immune index of the mice. A low-dose infection mouse model, e.g. with the C57BL strain, was established by inoculating ~100–200 bacilli via aerosol exposure. In this chronic infection mouse model, Mtb infection produced a well-tolerated infection dominated by a Th1-type immune response. In contrast, a high-dose infection BALB/c mouse model was established by intratracheal injection and resulted in a persistent pathological process characterized by progressive lung consolidation, extensive lung fibrosis and the presence of many T cells and anti-inflammatory cytokines. This high infection dose led to a persistent high bacterial load and host mortality. In addition, the phenotype of the mouse infection model was also influenced by various factors including environment, nutrition, immune status, gut microbiota and *Mycobacteria* species. In addition, host genetic factors may have different effects on establishing the mouse TB model. For example, if the mouse models were deficient in superoxide genes, this may affect host killing of Mtb in vivo. Expression of the host *sst1* gene has been reported to exacerbate disease progression [28].

The Mtb genetic background may also play an essential role in infection. For example, the (3 R)-hydroxyacyl-ACP dehydratases—HadAB and HadBC—which

Table 7.1 Biological characteristics of various murine subspecies used to model pathological features of TB

Species	Strains	Routes	Doses	Pathology	References
CBA/2	H37Rv	a	Low	Weight loss; extensive granulomatous infiltrates; necrosis and fibrosis	[11]
C3HeB/FeJ	H37Rv; *M. bovis*	a; in	Low	Necrotic granulomas; caseous necrotic; highly loaded with bacilli; liquefactive necrosis	[12–14]
DBA/2 J	H37Rv	it	Low	Low bacilli burden in lungs; higher activated dendritic cells (DCs); increased expression of TNF-α, IFN-ϒ and iNOS	[15]
Hall Institute multi-coloured mice	H37Rv	in	Low	Lung consolidation	[16]
Melbourne University albino	H37Rv	in	Low	Lung consolidation	[16]
C57BL/6J	H37Rv; CDC 1551; Erdman	a; in; ip; iv; it	Low	Necrosis; less bacterial load; inflammatory lung lesions; small and diffuse lesions; less densely packed granulomas with mononuclear cells; cellular infiltration; expanding granulomas; high production of IFN-ϒ, TNF-α; early induction of IL-12	[17–19]
BALB/c	H37Rv; 950,100 Beijing strain; S093 Canetti; *M. bovis*	a; in; ip; iv; it.	High; Low	Necrosis; less bacterial load; high burden of bacteria cause death; rapid and massive pneumonia; low production of IFN-ϒ, TNF-α; low CD8 cytotoxic activity; low apoptosis	[20–27]

a aerosol, *in* intranasal, *ip* intraperitoneal, *iv* intravenous, *it* intratracheal

can affect Mtb growth, colony morphology and biofilm formation, can also significantly influence the virulence of Mtb in a mouse model [29]. Mtb genes also exert a major influence on disease outcome by regulating innate and adaptive immunity [30]. Inducing low-level IL-1β production promotes macrophage phagocytosis, making it easier to establish infection in mice [31]. The total immunoglobulin isolated from the exposed healthcare workers in a TB-specialized hospital was reported to inhibit Mtb growth in vivo in an aerosol infection mouse model [20]. Of course, the nutrition and feeding environment of the mouse could likely affect experimental data. It has been shown that a high-fat diet provided to C3HeB/FeJ strain made TB infection in this mouse more likely to develop into a progressive tuberculosis and also impaired the protective effect of BCG vaccination, which may be related to dysbiosis of the gut microbiota [32].

Humans have different susceptibilities to Mtb infection because of heterogeneous immune responses. Only about 5% of infected humans would eliminate all pathogens, 5%–10% would develop active TB, and 90% become latent TB infection (LTBI) [33]. Thanks to advances in genetics technologies, many mouse models can be used in TB research. Currently, genetic approaches also provide a powerful tool to develop different species with special genetic backgrounds, including inbred Mtb-susceptible strains of CBA, DBA/2, C3HeB/FeJ and 129/SvJ mice and Mtb-resistant strains of BALB/c and C57BL/6J mice, which would provide strong support for TB research [34]. For example, in the DBA/2 mouse infection model, expression of the *Trl1–4* gene can significantly affect DBA/2 mouse susceptibility to Mtb infection, which could allow further study of the regulatory network and immune cells involved and promote the development of new drugs and vaccines [34, 35]. The BALB/c mouse is the most widely used mouse for Mtb infection studies, particularly for identification of virulence factors and in the evaluation of vaccines [36–39]. The recent studies have established mouse models that are contributing significantly to TB research [40]. For example, humanized mice have been used to investigate post-chemotherapy relapse TB [40], and a new outbred mouse model named Diversity Outbred (DO) can provide potentially a novel vaccination model that can better reflect the TB outcomes observed in humans [41].

Mouse models have several advantages, including low cost, the availability of abundant commercial reagents and mature immunological evaluating indices. Mouse models also have important disadvantages: (1) the mouse is not a natural reservoir for Mtb, and the pathways and immune cells involved during infection maybe different; (2) the current common mouse model(s) cannot fully mimic the symptoms of human TB; (3) humanized mice can mimic aspects of human infection, but are expensive; (4) some TB symptoms cannot be fully replicated in mouse models. Despite these disadvantages, there is still an urgent need to develop novel mouse models.

7.3 Rat (Order Rodentia, Family Muridae, *Rattus norvegicus)*

The use of rats in TB research was first reported in 1923 by Gloyne and Page, but it was not until 1950 that the characteristics of the rat model were elaborated by Gray et al. [42, 43]. Earlier reports showed that rats are resistant to infection with human, bovine and avian strains of tubercle bacilli [16, 44]. However, later research has shown that if the bacteria can directly infect some target organs of the host, such as the lungs, liver or spleen, the result will be significant pathological changes in the rats. The rat model is very similar to the mouse model, and infection can be established through a variety of inoculation methods, including oral, subcutaneous, intraperitoneal, intranasal and aerosol [42]. Currently, several rat breeds, such as Wistar [45], nude [46], American cotton [47] and Lewis [48], have been used in TB research. The pathology following infection is influenced by the breed and route of

Table 7.2 The biological characteristics of rats as models for TB research

Species	Strains	Routes	Doses	Pathology	Reference
Lewis	H37Rv (TMC 102); RIRv (TMC 205)	iv	Various	Non-caseating tubercles; delayed-type tuberculin hypersensitivity	[50]
Nude	*M. tuberculosis* (ATCC 35812)	a	Low	Pulmonary granulomas with central necrosis; thick collagen fibres; multinucleated giant cells	[46]
Wistar	H37Rv (ATCC 27294)	a	Low	Granulomatous lesions; mild inflammatory; mononuclear cell infiltration	[45, 51]
Albino rats	*M. bovis*	it	Low	Abscess formation; fibrous adhesions; lesions with caseous central	[52]
American cotton rats	H37Rv	it; a	High; low	Necrotic granulomas; highly cellular granuloma without central necrosis; nodular lesions; calcification; grossly enlarged intrathoracic lymph node	[47]
Spontaneously diabetic Goto Kakizaki rats	*M. tuberculosis* (ATCC 358,121)	a	Low	Larger granulomas; no necrotic lesions; tumour growth factor (TGF)-β, IL-1β, IL-2, IL-18 and iNOS expressed strongly	[48]

a aerosol, *iv* intravenous, *it* intratracheal

infection: for example, pulmonary infection of American cotton rats produced necrotic granulomas, whilst the granulomas observed in Wistar and Lewis rats did not have necrotic lesions [49]. Previous studies have shown that all pathological changes and disease processes are dose-dependent in rat models. Even different subspecies of the same rat can have different immune responses: for example, the American cotton rat has two subspecies *S. fulviventer* and *S. hispidus*, and the infection mortality rate in *S. fulviventer* was significantly higher than that observed in *S. hispidus* [47]. Although there have not been many reports on the use of the rat model in TB research in recent years, the rat has some specific characteristics that make it still a promising TB research animal model (Table 7.2).

7.4 Rabbit (Order Lagomorpha, Family Leporidae, *Oryctolagus cuniculus*)

In nature, there are very few reported cases of Mtb infection in rabbits, and they mainly involve non-tuberculous mycobacteria (NTM) such as *Mycobacterium avium* [53]. There is only one reported case of a rabbit on a New Zealand farm infected with *M. bovis* [54]. Therefore, it seems plausible that rabbits might be

resistant to TB or have some sort of self-protection mechanisms against Mtb infection [55]. Interestingly, the rabbit was the first laboratory animal to be used for TB research, recorded in 1867 [56]. Generally, rabbits have been widely and successfully used as animal models for many years, both in the production of immunological reagents and as infection models [57, 58]. In recent years, the New Zealand white rabbit has become common in laboratory research [53], and because rabbit animal models closely reflect the symptoms of some human diseases, they are often used to explore the pathogenesis of diseases and to develop new vaccines and therapies [58].

Rabbits are relatively resistant to Mtb infection when compared with mice and guinea pigs. Furthermore, different infection routes, mycobacterial strains and doses may lead to different disease outcomes (Table 7.3). Rabbits infected with HN878, a hyper-virulent Mtb strain, can develop progressive cavitation that is similar to cavitation seen in humans with active TB [64]. However, rabbits infected with CDC1551, a hyper-immunogenic clinical Mtb isolate, became latently infected [59]. In terms of infection route, rabbits infected with Mtb HN878 by aerosol inoculation developed granulomas and lung cavitation [65, 66]. In addition, the rabbit model was resistant to mimicking natural infection with *M. bovis* but showed significant susceptibility through inhalation or bronchial inoculation [67]. Interestingly, different subspecies are also susceptible to different Mtb strains; thus, inbred strains of New Zealand white rabbits have higher susceptibility to Mtb than outbred strains [68].

Rabbits have several advantages in TB research: (1) due to their relative resistance to Mtb infection, latent TB infection can be studied in this model [59, 69]; (2) rabbits can develop similar pulmonary cavitation as seen in humans, so they can be used to study the factors of disease formation and the pathogenesis of bronchial infection [67]; (3) rabbits are large animals to allow facile collection of sufficient blood and tissue samples from a single animal without sample enrichment [53]; (4) rabbits are also suitable for pharmacological experiments with anti-TB drugs, including drug penetration, distribution and cell accumulation [70]; (5) rabbits have

Table 7.3 Biological characteristics of the rabbit as model for TB research

Species	Strains	Routes	Doses	Pathology	Reference
New Zealand white	H37Rv (ATCC36801); H37Rv (ATCC25177); Mtb CDC1551; Erdman strains; *M. smegmatis*; *M. bovis Ravenel*; *M. bovis* AF2122	Aerosol; intradermal; bore hole filled with medical gelatin sponges; inhalation; bronchial; subcutaneous; intratracheal; intraperitoneal	Different doses	Inflammatory cell infiltration or necrosis; abscesses; pulmonary granulomas with caseous necrosis; liquefaction in the centre; accumulation of lymphocytes, fibroblasts and macrophages; pulmonary cavitation; granuloma and lung cavity formation	[53, 59–63]

been used to study bovine para-TB infection [71]. However, there are some disadvantages principally that they require higher biocontainment, there is a lack of immunological reagents and often the clinical signs are not obvious. Regardless, rabbit models for TB research will become increasingly popular in the future as new reagents are developed and biosafety conditions are established.

7.5 Zebrafish (Order Cypriniformes, Family Cyprinidae, Subfamily *Danioninae*, *Danio rerio*)

The zebrafish was first used in TB research by the group of Ramakrishnan in 2002 [72]. It is a useful tool to demonstrate Mtb virulence and pathogen-vertebrate host interactions (Table 7.4). Their use has become common in recent years and has benefited from the development of genetic approaches and improved imaging techniques [78]. Zebrafish are vertebrate, but they do not have lungs and are not a natural Mtb reservoir, so they cannot present some symptoms of mammalian TB. *Mycobacterium marinum*, which shares 85% genome homology with Mtb, can establish an infection in zebrafish that shares some similarities to human and mouse infections. There are two established zebrafish infection models with their own characteristics: an embryonic-larval model and an adult model [49, 78]. The immune markers during TB infection in adult zebrafish are similar to those of mammals, such as humans and mice. However, the embryonic-larval model is preferred for studying the effects of innate immunity on Mtb infection, due to its lack of an adaptive immune system.

In nature, zebrafish infection by *M. marinum* via the gastrointestinal tract has been reported [79]. However, zebrafish can be infected in the laboratory with *M. marinum* via various routes, including intraperitoneal and intramuscular and via

Table 7.4 Biological characteristics of zebrafish as models for TB research

Zebrafish stage	Mtb strains	Routes of injection	Doses	Pathology	Reference
Embryonic-larval	*M. marinum*	Caudal vein; duct of Cuvier in embryos; hindbrain ventricle; muscle; notochord; otic vesicle; intravenous; yolk injection	Low	Macrophages adopt a distinctive epithelioid morphology; granulomas form; macrophage aggregates with pathological features	[73, 74]
Adult	*M. marinum*; *M. peregrinum*	Caudal vein; duct of Cuvier in embryos; hindbrain ventricle; muscle; notochord; otic vesicle; intravenous; intraperitoneal; intramuscular	Low	Necrotic (caseating) granulomas; most granulomas form a fibrotic and/or cellular cuff	[73, 75–77]

the caudal vein. Local injections via the notochord, muscle, hindbrain ventricle, otic vesicle and yolk have been developed in zebrafish to study macrophage and neutrophil chemotaxis. Latent, chronic and active TB states can be established in zebrafish and depend on the inoculation doses and the different *M. marinum* strains. Latent infection model can be established by low-dose inoculation, and chronic progressive disease and acute infection by high-dose inoculation [80]. Early injection of *M. marinum* into the yolk of zebrafish embryos can provide a method to achieve a systemic *Mycobacteria* infection [75]. In addition, the *M. marinum* yolk infection model can be useful for high-throughput applications since it can be automated using an injection robot [75, 81, 82].

Active TB in zebrafish is characterized by rapid lethal inflammation, whilst chronic disease shows swelling of the abdomen and haemorrhages and skin ulceration typical of TB [83]. Following infection, bacteria invading zebrafish are phagocytized by macrophages and form caseating granulomas similar to human TB [72, 84]. Most granulomas also contain a necrotic centre where bacteria settle and form a cuff of cells, separate from the surrounding tissue [76, 77].

Zebrafish have been used extensively to evaluate new drugs and vaccines and are a useful model for gene editing and real-time imaging techniques. Embryonic-larval and adult zebrafish have been used to investigate each life stage of TB infection. Only the innate immune system exists in embryonic-larval zebrafish, and the main functional immune cells in this life stage are macrophages and neutrophils (the most suitable cells for studying zebrafish innate immune responses) [85–87]. In addition, the embryonic-larval model can develop externally and remain transparent, thus proving to be useful for studying host-pathogen interactions with real-time imaging systems [78, 88]. The use of fluorescent reporter systems has enabled more molecular details to be discovered, such as immune cell types, subcellular structures, intracytoplasmic trafficking and immune-modulatory mechanisms [89]. The development of gene editing techniques has enabled researchers to artificially design models on the genetic level, such as gene knockouts, to better meet research needs [90–94]. The larval model has been used to evaluate the efficacy of TB drugs such as rifampicin, isoniazid, ethambutol and moxifloxacin [95]. In addition, the embryonic infection model has been used to investigate early pathogenesis mechanisms, e.g. p62, a ubiquitin-mediated receptor protein contributed to host resistance to Mtb infection [96], and to elucidate the underlying mechanisms of the isoniazid-induced hepatotoxicity [97].

The adult zebrafish is more suitable for studying the pathogenic mechanisms of TB based on the mature innate and adaptive immunity system. The most common route for injecting bacteria into adult zebrafish is intraperitoneal, which results in progressive or chronic infection symptoms. At 1-week post-infection, form within many tissues and organs, including fatty tissue, pancreas, liver, spleen, adipose tissue and gonads. The histology of zebrafish granulomas is similar to human TB with a caseating and necrotic core surrounded by leukocytes and epithelial cells [98]. Like human control of TB, zebrafish control Mtb infection with their adaptive immune system. However, since zebrafish lack lymph nodes, immune cells can only

develop and function in tissues or organs such as the spleen, kidney and thymus. The adaptive immune system begins to function at 4 weeks post-infection [76, 84].

The main advantage of zebrafish models is that granuloma formation can be reproduced, which is difficult in other animal models [99]. Other advantages include their small size, easy of breeding and their use for real-time imaging. However, zebrafish do have some disadvantages: for example, (1) they do not have lungs and cannot systematically reflect the symptoms of TB in mammals, limiting their use for studying underlying mechanisms of TB, and (2) blood samples for detecting immunological markers are difficult to collect [100].

7.6 Cattle (Order Artiodactyla, Family Bovidae, Subfamily Bovinae, *Bos taurus*)

A century has passed since *Mycobacterium bovis* was identified as the etiological agent of bovine TB [101]. Despite extensive research on the pathogenesis of *M. bovis* infection, the prevention, control and treatment of bovine TB still face great challenges. At present, BCG vaccination is the most effective way to prevent bovine TB. However, immune protection in cattle begins to decline 2 years after BCG vaccination, and this can be solved by repeating BCG vaccination [102–104]. Studies have shown also that BCG vaccine does not protect 6-month-old calves from bovine TB infection after pre-sensitization to environmental TB. This suggests that sensitivity to TB in the environment in human clinical trials is one of the main reasons for the wide variation in immune efficiency with BCG vaccination [105, 106]. Since calves are immune from birth, this model can be used to mimic vaccine immunity of human newborns in many developing countries. Despite decades of research and numerous new vaccines being tested, nothing superior to BCG has yet emerged. Cattle are a suitable model for simulating human diseases because of the similarity in disease type (Table 7.5). In addition, the outcrossing of almost all experimental cattle can serve as a link between vaccine testing in small animal models and subsequent human experiments.

Cattle with bovine TB show significant pathological changes and immune responses in various target organs that are comparably observed in human and mainly in the lungs, respiratory tracts and lymph nodes [101, 112]. Generally, cattle in-the-field become infected when they inhale or ingest pasture, water or fomites contaminated with *M. bovis* [113]. In experimental research, there are many routes by which TB can spread to cattle other than via the respiratory tract and include the intravenous [114], subcutaneous [115], oral [116], intranasal [117], intratracheal [118], intratonsillar [119] and aerosol routes [120]. More recently, some researchers have tried to establish the infection model upon exposure to infectious sources in laboratory animals, in order to mimic natural infection conditions [105, 107, 121]. In cattle, as in other animal models, the doses and routes of inoculation can significantly affect the outcome of infection. Unlike other animals, cattle rarely shed

Table 7.5 The biological characteristics of cattle as models for TB research

Species	Strains	Routes	Application	Reference
Cattle	*M. bovis*	Inhale or ingest pasture, water or fomites contaminated with *M. bovis*; intravenous; subcutaneous; oral; intranasal; intratracheal; intratonsillar; aerosol	DNA vaccines; recombinant protein vaccine	[107–109]
Neonatal calves	*M. bovis*	Subcutaneously; intratracheal; aerosol	Modified BCG; attenuated vaccine; *Mycobacterium microti*; inactivated vaccine; DNA vaccines	[105, 110, 111]

M. bovis, and establishing infection in other cattle usually requires large doses of bacteria [122]. Neill et al. fully elucidated, experimentally, the relationship between challenge doses and pathological changes in cattle [123].

Calmette and Guerin created the first attenuated TB vaccine in 1906—BCG vaccine—that can protect against artificial or natural TB infection. Since then, new anti-TB vaccines have been developed that inactivate some of BCG's own genes, such as auxotrophic mutants, to reduce skin test responses and develop more safe vaccines for immuno-deficient individuals [108]. A gene deletion vaccine based on *M. bovis* is also an option, whereby some virulence or metabolism-related genes were deleted and the vaccine showed a significantly increased IFN-γ response in a calf infection model [105]. Relatively safe inactivated vaccines and DNA vaccines have also been developed, but trials in calf models did not show superior immune performance over BCG [107, 109]. Thus, safer and effective vaccines need to be developed, and both neonatal and adult bovine models are important for validating vaccine efficacy.

The main advantage of using cattle is that they are the natural host for *M. bovis* and thus display the most complete pathogenic mechanisms and pathological changes. Promising vaccine candidates can be tested on natural hosts prior to clinical trials. Other advantages are that the pathology and immunology of bovine TB are very similar to human TB, and related commercial immunological reagents are readily available. In addition, cattle are suitable for screening anti-TB vaccines and drugs, and it is relatively easy to collect large numbers and volumes of blood samples. Conversely, the disadvantages of using cattle are high cost of the animals and the larger facilities required for experimental work.

7.7 Guinea Pigs (Order Rodentia, Family Caviidae, Cavia, *C. porcellus*)

Guinea pigs have been used for more than a century for studying TB [4]. This animal model has played a key role in elucidating the pathogenesis of TB. Guinea pigs are highly susceptible to Mtb and can be infected by aerosol inoculation with

Table 7.6 Biological characteristics of guinea pigs as models for TB research

Species	Strains	Routes	Doses	Pathology	Reference
Dunkin-Hartley strain	H37Rv strain (ATCC 25618); HN878; CDC1551/CSU93; Erdman-K01; Bacille Calmette-Guérin (BCG); *Mycobacterium bovis*; *Mycobacterium leprae*; *Mycobacterium ulcerans*	Subcutaneously; aerosol; intradermal; intrapleural	Low	Microgranulomas in the lungs, liver and spleen; granulomatous necrosis; fibrosis and mineralization of central necrotic cores; mixed inflammatory response; granulomatous lymphadenitis; granulomatous pancreatitis	[127–130]

ultra-low doses of bacteria [124]. At present, the guinea pig strain most used in the laboratory is the outbred Dunkin-Hartley strain, although inbred strains do exist [125, 126] (Table 7.6). Several well-characterized inoculation routes have been used to establish TB infection in guinea pigs, including the aerosol route and intratracheal and intranasal instillation [131]. The aerosol route is the most used. After infection with Mtb, symptoms similar to those of human TB appear at the lesion, such as central necrotic granuloma surrounded by lymphocytes, macrophages, multinucleated giant cells and fibrotic capsules [4]. However, cavitation is rarely observed in guinea pigs, which is a shortcoming of the model that may affect the testing of some anti-TB compounds [131, 132].

In order to clearly establish and characterize the symptoms of guinea pig infection with Mtb, the aerosol method was used initially to inoculate very low doses of bacteria [4]. The process of bacterial replication in the lungs and spleen of guinea pigs after infection was comprehensively described by Alsaadi and Smith in 1973 [133]. In the lungs, the logarithmic growth phase was reached after 16 days of infection, and after a few weeks of replication, the bacterial load reached its maximum and remained stable [133]. TB infection in guinea pigs shows dose-dependent characteristics, e.g. aerosol infection with 20–50 bacilli enables animals to reach humane end points more quickly than low-dose infection in non-sensitive animal models, and high-dose infection leads to earlier humane end points [134–137]. Importantly, high doses of infection are not consistent with the nature of natural transmission and are not particularly relevant to clinical trials.

The guinea pig model has been used widely to test new anti-TB drugs such as Capreomycin [138], PA-824 [139] and vaccines, such as the subunit vaccine composed of proteins Ag85b and ESAT6-CFP10 [137, 140–142]. Guinea pigs have some immunological features, such as the substantial similarities between the guinea pig and human CD1 systems [143, 144], which can be an advantage in testing for more types of vaccines, including glycolipid vaccines [136, 145]. The guinea pig model is also useful for identifying antigen targets for vaccine development. Testing of live attenuated TB vaccines has also been done with guinea pig models to provide a full profile of biosafety and potency prior to use in humans [142, 146].

Guinea pigs are considered an ideal model for testing anti-TB drugs because they mimic the pathological lesion changes observed in humans [147]. A significant advantage of the guinea pig model compared to the mouse model is that it can mimic the symptoms of latent infection and thus provides an opportunity for surrogate studies of the latency of human TB [148]. Disadvantages of guinea pigs as a model are the paucity of immunological tools, reagents and guinea pig strains. Nevertheless, the development of tools for guinea pig research is likely to increase their use in the future.

7.8 Non-Human Primates

Non-human primates (NHPs) have become increasingly popular in TB research, largely because NHPs provide excellent cellular and immunological insights into TB [49, 149] (Table 7.7). NHPs were first used in TB research in 1956, when Leno et al. used rhesus macaques to develop anti-TB drugs [150, 151]. In the past few decades, the application of NHPs in TB research has made great progress. For any animal model, the first consideration is whether the model is susceptible to the target pathogen. Previous studies have shown that both Old World and New World NHPs are susceptible to all Mtb strains tested [161]. Initially, rhesus macaques were used as animal models to study the pathogenesis of TB and to test new drugs and vaccines. Interest in using NHPs has increased with the emergence of human immunodeficiency virus (HIV) [162]. NHPs commonly used in the laboratory include rhesus macaques (Order Primates, Family Cercopithecidae, *Macaca mulatta*), cynomolgus macaques (*Macaca fascicularis*) and the common marmoset (*Callithrix jacchus*), all of which can recapitulate the full spectrum of outcomes of TB infection seen in humans. Indeed, different Mtb strains can produce different outcomes, ranging from rapidly progressive TB to latent TB. Many Mtb strains have been used in NHPs, including Mtb 5159, CDC1551, H37Rv and the Erdman strain. The latter is most used to study TB in NHPs [9, 163–167]. In addition, the routes, locations and doses of Mtb inoculation can affect infection outcomes [149]. For example, intrabronchial instillation can mimic all the outcomes of TB infection seen in humans, but it does not reflect the natural course of infection and bypasses the immune defences of the host's upper respiratory tract [9, 165]. The first reported infection route of Mtb in NHPs was intratracheal instillation [150, 151], and the most commonly used methods currently in the laboratory are intra-airway instillation and inhalation of aerosolized bacilli [168]. Furthermore, Capuano et al. refined the infection process by introducing the fibre-optic bronchoscope, which could precisely quantify bacterial inocula into targeted organs [9]. Regardless, the results of some infections can still vary, and individual genetic differences, sample preparation and delivery before challenge can all significantly influence outcomes [169]. Therefore, more specialized and advanced equipment are needed urgently to produce better data by NHP models.

Table 7.7 The biological characteristics of non-human primates (NHPs) as animals to study TB

Species	Strains	Routes	Doses	Pathology	References
Rhesus macaques	BCG; *M. tuberculosis* CDC1551; H37Rvs; Erdman; *M. tuberculosis* 5159	Aerosol; intratracheal; intravenous; intrabronchial	Low	Caseous granulomas and cavitary disease in the lungs; discrete granuloma; TB hilar lymphadenopathy	[150–157]
Cynomolgus macaques	Erdman	Aerosol; bronchoscopic instillation	High	Extensive bilateral TB pneumonia; abundant necrosis; ocular TB, meningitis and spondylitis	[9, 153, 158]
			Moderate	Less necrosis; more cellular; less extrapulmonary disease infiltration	
			Low	Granulomas with much smaller and densely cellular	
Common marmoset	*M. tuberculosis* CDC1551; Beijing strain; *M. africanum* N0091; *M. tuberculosis* K04; Erdman strain	Aerosol; intratracheal	Various	Cavitary TB; extensive extrapulmonary disease; solid cellular non-necrotizing lesions; marginal fibrosis; inflammation; hyperplasia; caseous lesions	[159, 160]

NHPs infected with different doses of the Erdman strain Mtb by intratracheal instillation produce a range of symptoms from active TB to latent TB [158]. This was proved by the development of dose-dependent TB in Mtb-infected macaques. High doses of bacteria caused monkeys to die of TB between 7 and 13 weeks post-infection, with pathological findings of extensive bilateral tuberculous pneumonia, abundant necrosis and extrapulmonary disease at the lesion sites [149]. Infection with moderate doses of bacteria led to a slower progression of disease in macaques, with the host dying between 14 and 24 months post-infection, with pathological findings of extensive pulmonary changes such as necrosis and extrapulmonary disease. Interestingly, cell infiltration in moderately infected macaques showed a tendency to increase compared to pathological changes after a high dose of infection. At low doses, the macaques developed a slower progression of the disease, and the host animals lived up to 19 months post-infection; their pathology showed smaller, denser granulomas at the site of the lesion, which were very similar to human TB [9, 149]. These pathological observations are extremely advantageous for studying human TB, especially with a low-dose infection model, and should provide a clearer understanding of the pathogenesis and pathological changes of human TB.

NHPs are generally considered to be very similar to humans, both anatomically, and pathologically and symptomatically for TB [168, 170]. The rhesus monkey is the first animal model of NHP, and its significant advantage is that it can perfectly recapitulate a series of symptoms of human TB, and the relevant commercial reagents and procedures are well developed [9, 149, 165]. Earlier studies showed that very low doses of Mtb could cause fatal TB in macaques [152, 171]. In NHP, there were also differences in susceptibility between macaque species. In rhesus monkeys, cynomolgus macaques and vervets, 100 colony forming units (CFU) of Erdman strains were inoculated into the three models by intrathecal injection [149, 164]. The infection time in vervet monkeys was faster than in rhesus monkeys, and the cynomolgus monkeys even showed resistance to Mtb infection [153]. However, different strains infected the same model differently [7, 128, 163]. For example, rhesus monkeys infected with the H37Rv strain showed subclinical symptoms [154], the attenuated strain CDC1551 led to latent TB, and Erdman strain showed progressive TB symptoms [155, 159]. Interestingly, the susceptibility of the same species of macaques from different geographical regions to the *Mycobacterium* strains also varies [172]. For example, rhesus macaques from India and China were inoculated with the same dose of Mtb, and whereas macaques from India were asymptomatic, the macaques from China showed progressive TB symptoms [154–156]. In 2013, a new NHP model, the common marmoset, was developed, which not only has the advantages of traditional NHP models but also has more prominent advantages in individual size of the monkey, their conditions for breeding and differences in their individual genetic backgrounds [159].

TB in NHPs and humans share similar symptoms [149]. After infection with Mtb, the bacteria enter the lungs and are taken up by professional phagocytes such as alveolar macrophages or dendritic cells [167]. After overcoming host innate and adaptive immune responses, the bacteria begin to replicate and spread in vivo. Every bacterium that survives in the body can form granulomas in the lungs. There is no uniform structure of TB granulomas, and in vivo granulomas are structurally and histopathologically diverse [165]. Lin et al. tracked the lesions in Mtb-infected cynomolgus macaques with F-fluorodeoxyglucose positron emission tomography/computed tomography imaging [167, 173] and showed that there were significant differences in lesions within individuals, and the final infection outcome could be predicted by the lesions [167]. Traditionally, the host can either control bacterial replication in vivo resulting in latent TB, or the bacteria start replicating and spreading to form active TB [149]. Latent infection carries the risk of reactivation and is a potential active TB outbreak point, and more research is needed to establish effective protocols for diagnosing and treating latent infections. The well-characterized cynomolgus monkey is the most suitable model for research into diagnostics and vaccines [174, 175]. Latent infection can remain stable in cynomolgus monkeys and reactivated when the host is immunosuppressed and thus mimic the symptoms of human latent TB [165].

Thus, the main advantages of NHPS are as follows: (1) similarity to humans; (2) rhesus macaque, cynomolgus macaque and common marmosets can reflect a series of similar outcomes to human TB; (3) cynomolgus macaques in particular present

symptoms like human TB, and rhesus macaques and common marmosets play an important role in simulating particular aspects of TB; (4) the common marmosets are small, easy to group and house and cost-effective.

7.9 Other Models

The biological characteristics of other animals used to study TB are shown in Table 7.8 and discussed below.

7.9.1 Cat (Order Carnivora, Family Felidae, Felis catus*)*

Recently, five Abyssinian cats from an Italian cat farm were diagnosed with TB interstitial pneumonia, drawing our attention to the possibility of using cats as experimental animal models for TB research [181]. One interesting finding was that infection of cats with *Bartonella henselae*, the etiological agent of cat scratch disease, showed TB-like symptoms such as caseous necrosis and Langhans giant cells [182, 183].

The main etiological agents of cat TB are *M. bovis*, *M. microti* and some non-TB *Mycobacteria*. The incidence of TB in cats has been low, thanks to specialized eradication programmes and the use of commercial feed for pets [184]. TB in cats is transmitted mainly through the digestive tract, skin and by inhalation, and surprisingly there are no specific clinical TB characteristics in cats [185]. Granulomatous inflammation, cell infiltration and numerous alveolar macrophages containing Mtb are present in lesions [186]. There are very few reports on the use of cats as a TB model animal, and further research is needed to determine if they have any advantages over the more commonly used animal models.

Table 7.8 Biological characteristics of other animals used to study TB

Species	Strains	Routes	Applications	Reference
Cat	*M. bovis*; *M. microti*; *non-tuberculous mycobacteria*	Digestive tract; skin; inhalation	Diagnostic	[176]
Dog	*M. bovis*	Bites from wildlife	Drug search; pathogen detection	[177]
Deer	*M. bovis*; *Mycobacterium avium* complex	Aerosol	Diagnostic; vaccine	[178]
Fruit fly	*M. marinum*	Abdomen injection	Testing interactions between the pathogen and the host	[179]
Minipig	W-Beijing (Mtb) HN878	Aerosol	Natural transmission	[180]

7.9.2 *Dog (Order Carnivora, Family Canidae,* **Canis familiaris***)*

Dogs are typical 'sniffer' animals with a very sensitive olfactory system, and they are used widely for drug searches, search-and-rescue and detecting pathogens [187] and pathological signals and conditions, e.g. malaria [188]. Some microbial infections produce special volatile organic compounds that can be sensed by sniffer animals [189]. Companion animals are rarely infected with TB, although there has been a report of a dog infected after being bitten by a wild animal infected with Mtb [177]. This raises the possibility that the frequent close contact between pets and humans may provide an opportunity for TB to spread inter-species. In dogs, the main symptoms of TB affect the lungs and regional lymph nodes. The first reported case of TB in a dog was published in 2016 [190]. The main pathological changes of a dog with cardiac tuberculoma were diffuse pneumonia, fibrinous pericarditis and large, yellow, semi-solid masses of caseous necrosis in the left and right atrium. Importantly, Mtb was isolated from the faeces of infected dogs, suggesting that faecal matter can act as a potential source of Mtb infection for animals and humans. Mtb can also be found in dog nasal secretions and urine. Dogs should be considered as a model animal to demonstrate the source and incidence of TB infection in humans, during studies of TB induced by *M. tuberculosis* and *M. bovis* infection.

7.9.3 *Deer (Order Artiodactyla, Family Cervidae, Genus* **cervus***)*

Deer are globally ubiquitous, and some subgroups feed in artificial facilities such as game parks, zoological parks and gardens, where they can come into close contact with humans [191]. Many deer farms have been established to provide antler, venison and deer by-products. The occurrence of TB has been detected early in deers, and both captive and wild deer herds are highly susceptible to *M. bovis*, which has stimulated research in TB in this animal [192]. This is a major advance in understanding the aetiology, pathogenesis and epidemiology of TB in natural hosts and has important implications for detecting and treating TB.

M. bovis is the primary *Mycobacterium* detected in deer, which can be infected through contact with animals that carry the pathogens, such as tubercule-infected cattle and brush-tailed possums [193]. Indeed, there is another explanation: farmed animals and wild populations may have been infected by inhaling or licking terminally ill possums [194, 195]! Farmed deer are valuable economically, and it is important to develop a suitable method to detect *M. bovis*. Initially, intradermal tests were used, but deer sensitivity to the presence of *M. bovis* in the environment produced false-positive data that severely compromised the accuracy of the tests [196]. Carter et al. established a comparative cervical test in 1985, which showed up to 90% sensitivity in experimentally infected herds [197]. Subsequently, in 1985,

Griffin and Cross developed a more sophisticated and accurate laboratory test that involved all aspects of immunological indicators in animals infected with TB [198]. In 2002, a more accurate ELISA-based antibody test was developed to compensate for the false-positives of previous tests [196].

In terms of vaccines, BCG is currently the only commercially approved vaccine with very high protection efficacy in humans and animals. An effective vaccine can protect animal populations, but the problems that arise are also of concern. Vaccinating farmed animals with BCG, for example, may lead to a higher false-positive rate in skin reactions for later TB tests, affecting normal test results [199]. It has been reported that either continuous or booster doses of BCG greatly improved immunity to BCG for deer [200]. The development of a new TB vaccine may help prevent TB in domestic and farmed animals.

7.9.4 Minipig (Order Artiodactyla, Family Suidae, **Sus domesticus***)*

Use of the minipig model in TB research was first reported in 2010 by Gil et al. [201]. Minipigs infected with Mtb show similar symptoms of latent TB infection (LTBI) to those seen in humans. A characteristic feature of this model is the ability to generate a very strong Th1-type immune response to control infection, as well as the ability to induce a strong local response during fibrosis. In terms of local pulmonary structure, minipigs are very similar to humans, which will greatly benefit our understanding of LTBI mechanisms and allow us to establish more rational treatment and prevention approaches in the future.

7.9.5 Fruit Fly (Order Diptera, Family Drosophilidae, **Drosophila melanogaster***)*

D. melanogaster is an ideal model organism for studying innate immunity, and it has been widely used to elucidate the pathophysiological mechanisms of pathogen infections and associated immune responses. The *Mycobacterium* commonly used in fruit flies is *M. marinum*, which is usually injected into the abdomen under CO_2 anaesthesia using specialized equipment. The pathology of *Drosophila* infected with Mtb is characterized by extensive tissue lesions and low bacterial load, which is useful for studying pathogen-host interaction mechanisms [202]. *Drosophila* have obvious advantages of easy feeding, handling, strong fertility, short passage time, low cost and mature technology, which is conducive to effective cost control [203, 204]. However, their lack of an adaptive immune system is a disadvantage and limits their use to studying only innate immune responses.

7.10 Conclusions and Perspectives

TB has been known for millennia, and it has been a century since the BCG vaccine was developed and given to people in 1921, but the infection is still not completely controlled. The main reasons are the complex mechanisms of tuberculosis initiation and progression and the difficulties in developing effective vaccines and drugs for absolutely preventing and treating TB. Using animal models is crucial in TB research, especially in simulating TB symptoms and elucidating mechanisms of pathogenesis and pathology. Although a variety of animal models have been used and described in detail above, none has been able to recapitulate all the symptoms of TB in humans. The development of LTBI animal models is still a challenge. An ideal animal model of LTBI needs to adequately mimic the state of persistent infections in TB patients. Although animal models like mouse, guinea pigs, rabbit and non-human primates can mimic human LTBI to a certain extent, they still cannot fully replicate human infection. Therefore, it is necessary to modify the available latent animal models or develop new models for LTBI research. Recently, the application of new techniques in genetics, immunology and molecular biology has led to new experimental animal models for TB research, such as humanized mice. Other experimental animal models, such as transgenic mice and knockout animal models, are also needed for developing and testing vaccines and drug treatments. However, many of the TB animal models are limited by their high cost and considerations of biosafety and ethics. To improve the efficacy of animal models to evaluate vaccines and drugs, new advanced imaging technologies are also needed. In addition, mathematical modelling plays an important role in collecting and analysing the data from TB animal models, and these models need to be developed further. In studies, it is important to choose the right TB animal models for different research aims and projects, and using complementary models is encouraged. Although there maybe disadvantages of different animal models, it is undeniable that all experimental animal models have made outstanding contributions to understanding the pathogenesis of TB, and they will continue to be used to develop new TB vaccines, drugs and therapies.

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Chapter 8
BCG: Past, Present and Future Direction

Hazel Morrison and Helen McShane

Abstract Bacillus Calmette-Guérin (BCG) is a live-attenuated vaccine developed over 100 years ago and remains the only vaccine ever licensed in the fight against tuberculosis (TB). It is one of the most widely used vaccines in the world, having been administered to over four billion people, with another 100 million children vaccinated with BCG every year. Despite this, significant debate exists surrounding its efficacy against TB and its place in routine infant vaccination schedules. Severe side effects following BCG administration are rare but may be seen in those with immune system dysfunction. Safer vaccines for use in these individuals would be valuable.

BCG has been shown in some studies to have beneficial effects on mortality and morbidity beyond that attributable to reduction in TB alone. Understanding the immunological mechanisms underpinning these non-specific effects is increasing and appears in part to be due to the induction of trained innate immunity. New vaccines developed against TB will either need to be given as a booster following initial BCG vaccination or be shown to be non-inferior with regard to these off-target effects.

Despite its age, widespread usage, and intensive study, we are still learning how BCG exerts its effects and unpicking what these really are. Alternative routes of administration and recombinant forms of BCG offer promising strategies to further harness the potential of this intriguing vaccine.

Keywords Bacillus Calmette-Guérin · BCG · Vaccine · Tuberculosis · Efficacy
Trained immunity · Non-specific effects

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M. Christodoulides (ed.), *Vaccines for Neglected Pathogens: Strategies, Achievements and Challenges*, https://doi.org/10.1007/978-3-031-24355-4_8

8.1 History of BCG

Bacillus Calmette-Guérin (BCG) was named after the French scientists Albert Calmette and Camille Guérin, who developed it for use as a vaccine against tuberculosis (TB) in the early 1900s at the Pasteur Institute in Lille [1]. Calmette was a physician and bacteriologist, who gained initial notoriety for his work developing snake antivenom in Southeast Asia in the 1890s. On returning to France, he was struck by the levels of TB disease amongst the working classes in the crowded, industrial cities of northern France and began to focus his considerable talents on trying to control and prevent the disease. Camille Guérin was a skilled young veterinarian whose father had died from TB. He joined the Pasteur Institute in 1897 and quickly became head of the laboratory [2]. His partnership with Calmette was to prove pivotal in TB vaccine research.

Previous attempts to produce a vaccine against TB, such as by heat or chemical inactivation of tubercle bacilli, had proved ineffective. Use of a live vaccine appeared to be required. Work conducted by the Nobel laureate Emil von Behring in 1902 demonstrated that inoculation with human tubercle bacilli strains could protect cattle against bovine TB. However, potentially infectious viable bacilli were subsequently found to be excreted in milk. Use of a paratuberculosis bacillus isolated from tortoises was also tested in cattle to no avail [3].

Building on this knowledge, Calmette and Guérin began their search for a human vaccine. Whilst attempting to culture tubercle bacilli for experimental use, they found that using standard potato and glycerol culture medium resulted in the unwanted clumping of bacteria. They tested the addition of ox bile as a solution. Serendipitously, this was found not only to reduce clumping but also to result in reduced virulence on subsequent subculture [4].

In 1908, starting with a virulent strain of *Mycobacterium bovis* (*M. bovis*), the causative agent of TB in cattle, they began the culture process that would eventually lead to BCG. Utilising their potato, glycerol and ox bile medium, they created new subcultures every 3 weeks, a process also termed passaging. After 30 passages, they had created a strain that was no longer lethal to guinea pigs [3]. In 1913, a vaccination trial in cattle was planned, but this was interrupted by the First World War. Despite the difficulties in obtaining sufficient potatoes and ox bile during the German occupation of Lille, they managed to maintain their cultures. By 1919, their "bile bacillus" had been passaged 230 times and failed to produce TB disease when injected into rabbits, guinea pigs or cattle [4].

At this point, Calmette and Guérin considered the bacilli to be sufficiently attenuated (weakened) that it would not cause disease in humans but might instead stimulate enough of an immune response to confer immunity against TB. The opportunity for the first test in humans came in 1921, courtesy of a Dr. Weil-Hallé,

a physician at the Charité Hospital, Paris. He contacted Calmette about a healthy newborn infant whose mother had died from TB shortly after childbirth. On 18 July 1921, the infant became the first human to receive a dose of BCG [3]. Calmette mistakenly believed that the natural route of infection for *Mycobacterium tuberculosis* (*M. tb*) was via the gastrointestinal tract, and therefore BCG was initially given orally. No negative sequelae of the vaccination were seen and the child survived to live a TB free life [1].

By the end of 1924, over 600 infants had been vaccinated orally with BCG, with no significant safety concerns identified [3]. Mass production of BCG began and thousands of infants throughout Europe were vaccinated over the next 5 years. BCG was adopted by the Health Committee of the League of Nations, the predecessor of the World Health Organisation, in 1928. Despite this, BCG uptake was initially slow and highly variable between different countries. Scandinavian countries such as Sweden and Norway enthusiastically embraced the new vaccine, and it was here that the now routinely used intradermal route of administration was established [5]. Uptake was much lower in countries such as Great Britain, where articles expressing considerable scepticism about Calmette's efficacy statistics were published in the medical press [6] and in the USA, where concerns circulated about the potential of the bacilli to regain full virulence [7].

In 1930, the Lübeck disaster nearly brought the history of BCG to a premature end. Around 250 infants in the German city of Lübeck were vaccinated with oral BCG. Tragically, scores of these children went on to develop TB and 72 died from the disease. Confidence in the safety of BCG was profoundly damaged, and Calmette and Guérin found themselves under intense scrutiny. Following an investigation over nearly 2 years, it was ascertained that the BCG stock had been contaminated with a virulent human *M. tb* strain during preparation in the local TB laboratory. Two of the doctors concerned were sentenced to time in jail for their role in the disaster.

BCG and its creators were fully exonerated, but confidence had been undermined and BCG use declined in many European countries [8]. It was not until the Second World War brought with it a deadly resurgence in TB cases throughout Europe and Asia that BCG use really took off. The advent of lyophilised (freeze dried) BCG around this time helped to facilitate large-scale vaccination programmes.

Subsequently, BCG has become one of the most widely used vaccines in history, with billions of doses delivered worldwide. It has been included in the WHO global extended programme of immunisations (EPI) since 1974 [9]. Current vaccination policies differ by country, but BCG is still widely given at birth in many. In higher income countries, reductions in TB incidence mean that BCG vaccination is now often reserved for specific higher-risk groups, such as migrants and healthcare workers (Fig. 8.1).

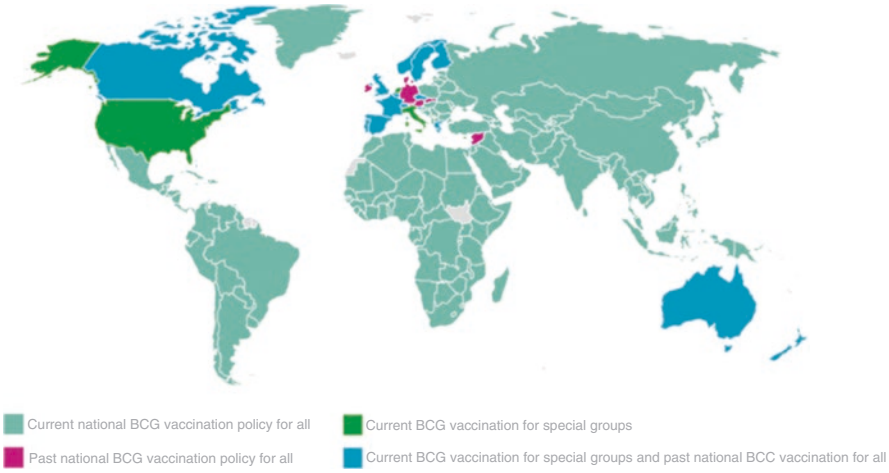

Fig. 8.1 Current and historical BCG vaccination strategies by country. (Reprinted with permission from www.bcgatlas.org, which contains an interactive version of this map with additional details including TB incidence and history of BCG vaccination practices for each country [10])

8.2 Evolution and Genetics of BCG Vaccines

From as early as 1924, seed stock from the original BCG was distributed from the Pasteur Institute in Lille to laboratories across the world. This enabled local production and distribution of the vaccine prior to the advent of lyophilisation techniques and archived seed lots. This has resulted in the existence of numerous "daughter" strains of BCG, which have each undergone additional in vitro evolution and thus contain distinct genetic variations and consequent morphological and phenotypic differences (Fig. 8.2) [11, 14].

The attenuation of BCG was achieved empirically by Calmette and Guérin. Subsequent genomic studies have identified the loss of a 9.5-kb DNA segment, the so-called region of difference 1 (RD1), as the major causative variation. RD1 is present in both virulent *M. bovis* and *M. tb* but absent from all sub-strains of BCG. This genetic locus codes for two key immunogenic antigens—early secreted antigenic target 6 (ESAT-6) and culture filtrate protein 10 (CFP-10), plus several parts of their secretion system (ESX-1) [15]. The ESX-1 secretion system is required for full virulence of *M. tb* and results in disruption of the phagolysosome within host infected macrophages [16]. It should be noted that other variations are also involved in BCG attenuation, as reintroduction of RD1 to BCG-Pasteur or BCG-Russia does not restore full virulence [17].

Numerous other genetic differences, including deletions and duplications of genomic regions, as well as single nucleotide polymorphisms (SNPs), exist between *M. tb* and BCG. Unlike RD1, these also vary between different BCG strains. For example, RD2 is present in BCG strains derived prior to 1927 ("early" strains) but lost from those propagated subsequently ("late" strains) [18]. Loss of RD2 is

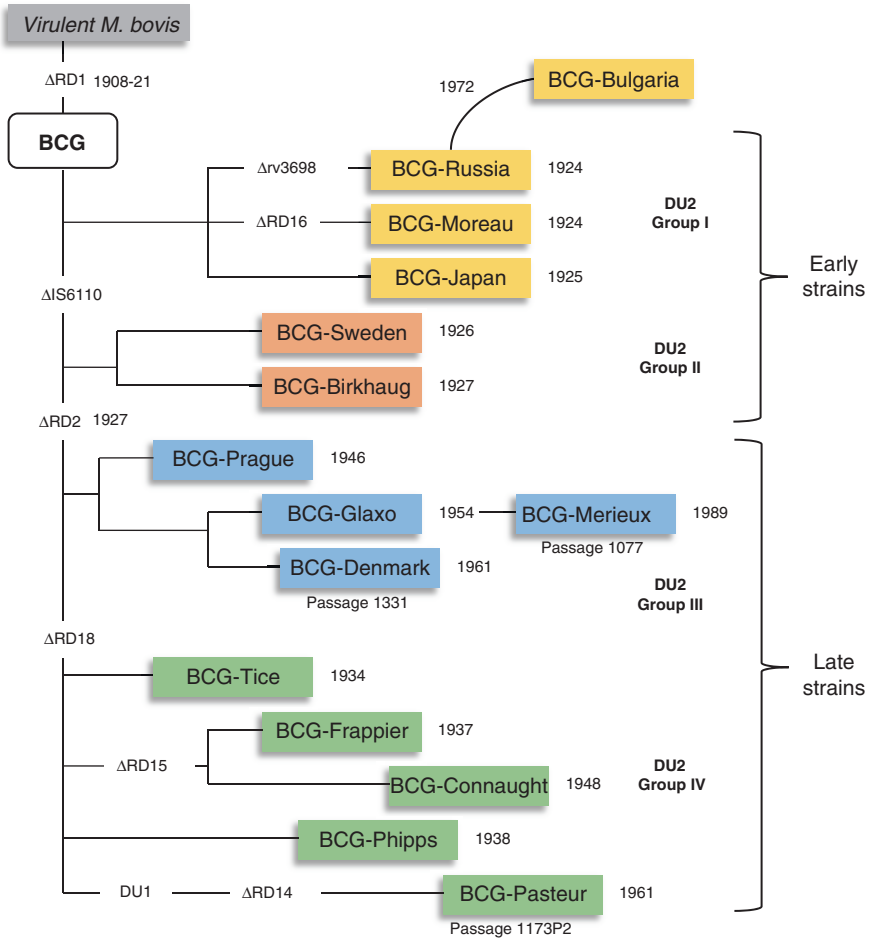

Fig. 8.2 Evolution timeline of BCG with selected key genetic variations [11–13]. Δ deletion, *RD* region of difference, *DU* duplication unit, *IS* insertion sequence

implicated in reduced virulence, via disruptions to host innate immune responses, and has been postulated by some to have caused an "over attenuation" of BCG [11]. Several other genomic polymorphisms are identifiable in late BCG strains, including point mutations in genes encoding for mycobacterium protein bovis (MPB) 83 and MPB 70. These antigenic proteins have been implicated in mycobacterial pathogenesis and are found in high levels in strains prior to 1927 but in only very small amounts in later strains [19].

As well as deletions, characteristic duplicated genome sections are seen across BCG strains. These are the so-called tandem duplications DU1 and DU2. DU1 occurs only in BCG-Pasteur. Four main forms of DU2 are seen across different BCG strains (Fig. 8.2). It is postulated that these tandem duplications may have

arisen due to the selective pressures of BCG growth on glycerol [11]. The impact these duplicated regions have on the immunogenicity and efficacy of BCG strains remains uncertain.

Today, the most used strains for BCG vaccine production worldwide include BCG-Bulgaria (Sofia SL222), BCG-Denmark (Danish 1331), BCG-Glaxo (Merieux 1007), BCG-Japan (Tokyo 172-1) and BCG-Pasteur (Pasteur 1173 P2) [12].

8.3 Immunogenicity of BCG

BCG is a whole cell, live-attenuated vaccine and therefore contains a wide variety of mycobacterial antigens, including proteins, lipids and polysaccharides, capable of stimulating an array of immune responses.

8.3.1 Innate Immune Responses

Following initial intradermal inoculation, host epidermal macrophages detect BCG via a number of pattern-recognition receptors (PRRs), including Toll-*like* receptors 2 and 4 (TLR2, TLR4) and the nucleotide-binding oligomerisation domain 2 (NOD2) receptor [20, 21]. These interact with a variety of BCG pathogen-associated molecular patterns (PAMPs) including mycobacterial cell wall components such as peptidoglycan, mycolic acids and mannosylated lipoarabinomannan [22, 23]. Activation of TLRs stimulates macrophage and dendritic cell (DC) maturation and the secretion of proinflammatory cytokines. Resident DCs phagocytose BCG and migrate to local draining lymph nodes to present BCG-derived antigens to $CD4^+$ T cells and thus initiate adaptive immune responses [24].

Punch biopsies of healthy adult volunteers following intradermal BCG injection have shown that live BCG persists at the site of inoculation for up to 4 weeks. Analysis of the early inflammatory milieu via creation of suction blisters demonstrates the predominant cell type to be $CD15^+$ neutrophils [25]. BCG-infected neutrophils exhibit synergistic co-operation with DCs to stimulate antigen-specific T-cell responses [26].

8.3.2 Adaptive Immune Response

Effective cellular immunity is known to be critical for adequate immunological control of *M. tb*, with those lacking key elements of T-cell immunity, for example, people living with HIV, at higher risk of TB disease [27]. BCG vaccination typically induces a T-helper type 1 (Th1)-dominated response, as characterised by production of cytokines such as tumour necrosis factor α (TNFα), interferon γ (IFNγ) and

interleukin-2 (IL-2) by $CD4^+$ T cells [28, 29]. BCG vaccination also induces modest increased expression of the cytotoxic markers, granulysin and perforin, by $CD8^+$ T cells [30].

Classically, the immune systems of infants demonstrate a bias towards Th2 regulatory responses following microbial exposure. Despite this, BCG has been shown to induce strong Th1 responses in infants [31]. This may, however, vary by geographical location, with UK infants exhibiting predominantly Th1-driven responses as compared to more Th2 polarised responses elicited in Malawian infants [32]. This observation was not repeated in a more recent study comparing Ugandan and UK infants, with Th1 responses predominating in both cohorts. In this study, no differences were seen in antigen-specific responses to purified protein derivative (PPD), regardless of maternal or infant mycobacterial exposure status [33]. Any true differences in immune responses to BCG vaccination and subsequent efficacy between infant cohorts are likely to be due to other causes, and further work will be needed to elicit these.

The humoral immune system has historically been considered of limited importance in protection against *M. tb*, but new evidence is emerging that may challenge this view [34]. Highly exposed household contacts of active TB patients who remain tuberculin skin test (TST) and IFNγ release assay (IGRA) negative, so-called resistors, have been shown to possess functionally distinct *M. tb*-specific antibodies [35]. Increased levels of antigen-specific IgG antibodies and long-lived memory B cells are induced following BCG vaccination and may result in opsonisation and subsequent enhanced phagocytosis of mycobacteria [34, 36].

8.3.3 Trained Immunity

Whilst immunological memory is classically thought of as the hallmark of the adaptive immune system, innate immune cells can be modified (or "reprogrammed") to elicit enhanced responses to subsequent homologous and heterologous stimuli, in a type of non-specific immune memory termed trained immunity. Epigenetic changes, such as histone modification and alterations in DNA methylation, are the main drivers of trained immunity [37]. Induction of distinct epigenetic and metabolic modifications in a variety of immune cells may explain the observed effects that BCG exerts on heterologous infections [38].

BCG has been shown to induce epigenetic reprogramming of monocytes, resulting in increased proinflammatory cytokine production in response to unrelated pathogens for at least 3 months post-vaccination [21]. In a mouse model of severe combined immunodeficiency, BCG vaccination leads to increased survival in mice following lethal challenge with *Candida albicans,* in part mediated by natural killer (NK) cells [39]. BCG induces trained immunity in human NK cells, with increases in proinflammatory cytokine production seen on re-stimulation with mycobacteria as well as unrelated bacterial and fungal pathogens [40].

Until recently, it was unclear what mechanisms underpinned the longevity of these trained immune responses seen in monocytes following BCG, despite the short life span of myeloid cells within the peripheral circulation. It has now been shown that BCG vaccination in humans induces a transcriptional shift within the haematopoietic stem cell compartment of the bone marrow, and this results in persistent epigenetic changes in peripheral monocytes for at least 3 months [41].

8.4 Efficacy of BCG Against TB

BCG vaccination is provided by 153 countries as part of their standard childhood vaccination programmes, with coverage exceeding 90% in around two thirds of these. Despite this, an estimated ten million new cases of TB disease occurred worldwide in 2020, resulting in around 1.5 million deaths [42]. This highlights the fact that good BCG vaccine coverage is clearly not sufficient to control the current TB pandemic.

Neonatally administered BCG has consistently been shown to offer good protection against TB meningitis and disseminated (miliary) TB in childhood [43, 44]. However, BCG efficacy against the most common form of TB disease, pulmonary TB, is highly variable [45, 46]. In the UK Medical Research Council (MRC) trial in the 1950s, BCG was shown to be over 70% effective in adolescents [47]. In contrast, little or no efficacy in any age group was seen in three large, randomised controlled trials (RCTs) in South India (the Chingleput trial), Brazil and Malawi [48–50]. Limited data on the duration of protection exists, although in populations where BCG does show a protective effect, this can be highly durable, lasting for at least 15 years in the UK MRC trial and up to 60 years in native Alaskans [51].

There is now evidence that BCG can protect against *M. tb* infection, as well as active TB disease, in some settings. This was previously impossible to determine, due to limitations in the tuberculin skin test (TST) as a diagnostic tool, with a positive TST seen due to mycobacterial infection and/or BCG vaccination. T cell-based IGRAs are unaffected by BCG status and therefore allow investigation of the effect that BCG may have on infection. Studies from outbreak settings have shown that BCG vaccination is associated with a reduction in risk of *M. tb* infection, as defined by positive IGRA, as well as lower rates of TB disease [52, 53].

8.4.1 Reasons for Variable BCG Efficacy

The underlying reasons for the wide variation in the protection afforded by BCG are still not fully understood (Table 8.1). Several potential explanations have been proposed. Strikingly, BCG efficacy seems to vary by latitude, with the observed protective effect decreasing nearer to the equator [54]. The most widely accepted hypothesis for this discrepancy relates to differences in exposure to non-tuberculous mycobacteria (NTM). Populations living in more tropical climates closer to the

Table 8.1 Factors known to influence efficacy of BCG protection against TB

Favours more protection	Evidence unclear	Favours lack of protection
Meningeal and miliary TB	BCG strain	Pulmonary TB in adults
Neonatal vaccination	BCG revaccination	
Tuberculin skin test negative		
Latitude >40°		

equator, particularly in rural areas, are likely to have experienced greater levels of NTM exposure, and this prior mycobacterial sensitisation may limit the protective effect of BCG. Two theories exist to explain this. The blocking hypothesis holds that immune responses elicited by prior NTM exposure prevent protective BCG vaccine effects from developing, potentially by preventing replication of live BCG at the site of inoculation [55, 56]. The masking hypothesis suggests that prior NTM sensitisation itself confers a level of protection against subsequent *M. tb* exposure that BCG is not able to improve upon [57]. BCG effectiveness in trials is increased if tuberculin skin test-positive individuals (a marker of prior mycobacterial sensitisation) are stringently excluded [45].

Co-infection with any of a variety of helminth species, such as *Ascaris* (*Ascaris lumbricoides*), whipworm (*Trichuris trichiura*) and hookworms (including *Ancylostoma duodenale*), prevalent in tropical regions, may be associated with reduced immunogenicity of BCG [46, 58]. Viral infections, such as cytomegalovirus (CMV) in the neonatal period may impair the development of BCG-specific immune responses [59].

Other proposed, but less well accepted, explanations for efficacy variations include underlying genetic or microbiome differences between host populations, variability in nutritional status, exposure to ultraviolet light and vitamin D levels, as well as differences in virulence levels of circulating *M. tb* strains [9, 60, 61]. Timing of BCG vaccination and circadian rhythms may have an impact on immunological responses, potentially influenced by circadian oscillations in macrophage and leukocyte functions. Morning BCG vaccination has been shown to induce stronger trained and adaptive immune responses in humans compared to evening vaccination, with early morning vaccination resulting in the highest levels of cytokine production [62].

Clear variations in both genotype and phenotype exist between different strains of BCG and in vitro immunological responses have been shown to differ between strains [63]. Despite these clear differences and long held assumptions that this may impact on vaccine efficacy, there is no consistent evidence that efficacy does differ significantly between strains [45].

8.4.2 BCG Revaccination

It remains unclear whether revaccination with BCG can improve protection. Several studies have previously found that additional doses of BCG do not increase efficacy or duration of protection against TB disease. In the late 1980s, the Karonga

Prevention Trial randomised nearly 50,000 individuals across a range of ages in rural Malawi with a visible BCG scar to receive a second dose of intradermal BCG or placebo. The latest 30-year follow-up has confirmed the original study findings that BCG revaccination does not provide additional protection against TB disease in this population. However, subgroup analysis suggests there may be modest benefit in those who are HIV-negative, particularly if the second vaccination occurred in childhood [50, 64].

In the BCG-REVAC study conducted in Brazil, no additional protection was seen from BCG revaccination offered to school aged children. Extended follow-up suggests that a second BCG dose could however offer increased protection in regions with expected lower prevalence of NTM exposure and with earlier age at revaccination [49, 65].

Studies looking at BCG revaccination have generally focused on TB disease as their primary end point. A recent RCT conducted in *M. tb* uninfected healthy South African adolescents has shown BCG revaccination to have a modest protective effect against sustained *M. tb* infection. The trial compared the ability of a novel protein-adjuvant subunit vaccine AERAS-404 (comprised of the H4 antigen and IC31 adjuvant) or BCG revaccination to prevent *M. tb* infection, as defined by positive *M. tb*-specific IGRA. Whilst neither intervention reached statistical significance for the primary end-point of preventing new initial *M. tb* infection, BCG revaccination provided statistically significant vaccine efficacy of 45% ($p = 0.03$) in reducing sustained IGRA conversion (believed to indicate established *M. tb* infection) [66]. A larger confirmatory trial of these findings is underway (clinicaltrials.gov NCT04152161). If this supports the initial findings, then BCG revaccination could represent a readily available, safe and cost-effective public health intervention to protect selected high-risk populations [66].

8.4.3 Measuring BCG Protection?

For most licensed vaccines in use today, validated immunological surrogate markers of clinical protection (termed correlates of protection) exist, for example, levels of vaccine-induced antibodies. No such marker exists for BCG, and whilst it is well established that $CD4^+$ T cells and key cytokines including TNFα and IFNγ are essential for controlling mycobacterial infection, it has not been shown that vaccine-induced increases in these immune responses correlate with increased protection [67]. Presence of a visible BCG vaccination scar has historically been taken to represent appropriate vaccine "take", and in some countries BCG revaccination was routinely recommended for those who did not develop a visible vaccine scar [9].

The proportion of infants undergoing BCG vaccination who go on to develop a visible scar at the sight of inoculation varies in different studies from as low as 52% and as high as 97%, with the 80–90% range most commonly seen [68, 69]. Scar formation may be influenced by a wide variety of factors including training level of

the provider and inoculation technique (in particular size of post-vaccination wheal), with scarring more likely to result from intradermal rather than subcutaneous vaccination [70]. Strain of BCG utilised may also be a factor, with BCG-Denmark shown to induce a higher proportion of BCG scars than BCG-Russia [71, 72].

BCG scarring does not appear to correlate in any meaningful way with evidence of BCG efficacy against TB. There is no association between scar size and protection against either TB or leprosy [73]. However, presence of a BCG scar may be associated with increased overall survival in high mortality settings [68]. In West Africa, BCG-vaccinated children with a visible BCG scar at 6 months of age had lower all-cause mortality in the following 12 months than those who did not develop a scar [74].

8.5 Efficacy of BCG Against Other Infections

8.5.1 BCG and Other Mycobacterial Infections

Multiple studies have shown that BCG provides greater protection against leprosy, caused by infection with *Mycobacterium leprae*, than it does against TB. Protection is afforded against both the tuberculoid and more severe lepromatous forms of the disease, with estimates of efficacy ranging from 20–80% [50, 75, 76]. BCG may also offer some protection against infection with *Mycobacterium ulcerans*, which causes Buruli ulcer disease [77], although this is not consistently seen across all studies [78].

8.5.2 BCG "Non-Specific" Immunity

In mouse models, BCG has been found to offer protection against a broad range of non-related infections including systemic candidiasis, disseminated schistosomiasis and listeriosis [39, 79, 80]. In humans, BCG vaccination of healthy volunteers leads to an increased production of monocyte-derived proinflammatory cytokines, including TNF-α and IL-1β, not only in response to mycobacterial stimuli but also to heterologous pathogens including *Candida albicans* and *Staphylococcus aureus* [21]. BCG has also been shown to protect against an experimental human viral infection, with reduced yellow fever vaccine viremia following BCG vaccination in healthy volunteers seen in correlation with an upregulation of IL-1β, a mediator of trained immunity [81].

Albert Calmette himself noted that, epidemiologically, BCG appeared to reduce childhood mortality to a greater extent than would have been anticipated by the effect on TB disease alone [3]. Observational studies have shown that BCG vaccination reduces all-cause infant mortality across a variety of settings [82, 83]. Combined

analysis of three RCTs conducted in West Africa showed that early BCG vaccination of low birthweight infants resulted in a reduction in all-cause mortality by 38% within the neonatal period and by 16% within the first year of life, with effects mainly attributable to reduced deaths from sepsis and respiratory tract infections [84]. However, the same effect was not seen in studies of children in Greenland or Denmark, where BCG vaccination resulted in no detectable reduction in morbidity from infectious diseases other than TB [85, 86]. A systematic review across a variety of settings found that BCG vaccination appeared to be associated with reduction in all-cause mortality, but the authors noted a high risk of bias in several of the published studies [83].

A more recent study conducted in Uganda expands upon these findings. Healthy infants of varying birthweights were randomly assigned to receive BCG on day of birth or at 6 weeks of age and followed up until 10 weeks of age, with investigators and clinicians blinded to allocation. Rates of physician-diagnosed non-tuberculous infectious disease were significantly lower in the early BCG versus delayed vaccination group in the first 6 weeks of life (98 presentations verses 129 presentations, respectively, hazard ratio of 0.71 [95% CI 0.53–0.95], $p = 0.23$). No difference between the groups was observed after the delayed group had also received BCG. Epigenetic differences in histone trimethylation at the TNF promotor region in peripheral blood mononuclear cells between the groups in the first 6 weeks of life provide evidence that induction of trained immunity may underpin the observed differences in infectious morbidity [87].

At the opposite end of the age spectrum, a recent double-blind RCT has shown that vaccination of elderly patients (aged 65 years and older) with BCG also results in a reduction in infections of the respiratory tract, in the year following BCG vaccination [88].

8.5.3 BCG and COVID-19 Disease

From the early stages of the coronavirus 2019 (COVID-19) pandemic, BCG was postulated as a possible tool in the fight against the disease. Background rationale included the known potential of BCG to boost trained immunity against heterologous infections, coupled with initial epidemiological data that appeared to suggest less severe COVID-19 outbreaks were seen in countries with a universal BCG vaccination policy [89, 90]. However, as more data has accrued, subsequent analysis with correction for confounding variables including large disparities in COVID-19 testing rates has shown no convincing epidemiological correlation between BCG vaccination policy and COVID-19 spread [91]. Multiple clinical trials have been registered looking at the question of BCG for protection against COVID-19 disease, including the BRACE trial of 10,000 healthcare workers across 5 countries (clinicaltrials.gov NCT04327206) [92]. Results are currently awaited from these studies.

8.6 BCG and Non-Communicable Diseases

8.6.1 BCG and Cancer

In 1929, a study carried out at the Johns Hopkins Hospital found a correlation between TB and a lower incidence of cancer at postmortem [93]. This led to the first notion that BCG might be harnessed as a cancer treatment. In 1935, Holmgren utilised intravenous BCG injection as a putative treatment for stomach cancers [94], but around this time, enthusiasm for BCG was severely dampened by the Lübeck disaster and subsequent concerns about BCG safety (see Sect. 8.1). Seminal studies in the 1950s demonstrated clear evidence of BCG-mediated inhibition of cancer tumorigenesis in mice, via the activation of the reticuloendothelial system [95].

Efforts were rekindled to utilise the potential of BCG immunotherapy against cancer in the 1960s and 1970s, with studies showing successful regression of melanoma metastatic to the bladder following intralesional BCG injection [96]. Today, BCG anticancer immunotherapy is most widely used against bladder cancer, with intravesical BCG utilised as a successful treatment for high-grade non-muscle-invasive bladder cancer and carcinoma in situ for many years [97–99].

Several other potential uses of BCG within oncology remain under investigation. BCG is utilised as an immunomodulatory adjuvant in a personalised anti-tumour vaccine undergoing phase III clinical testing against colon cancer (clinicaltrials.gov NCT02448173 [100]). Intra-pleural administration of BCG has been associated with possible survival benefit in several lung cancer studies, although high-quality evidence is lacking, and its potential role as an immunotherapeutic agent in malignant pleural mesothelioma is being investigated [101, 102].

As well as immunotherapy of established cancer, BCG may play a role in cancer prevention in some populations. In secondary analysis of long-term follow-up from a historical BCG vaccination trial, a single dose of intradermal BCG in childhood has been shown to be associated with a reduced incidence of lung cancer in American Indian and Alaskan Natives [103].

8.6.2 BCG and Allergic, Autoimmune and Inflammatory Diseases

Atopic and allergic asthma phenotypes are characterised by an inappropriate imbalance of Th1 verses Th2 immunological responses, with Th2 polarisation and elevated levels of IgE, IL-4 and IL-5 seen. BCG is known to typically induce strong Th1 responses and has been shown in mice to suppress allergen-induced airway inflammation [104]. In a small randomised controlled study, percutaneous BCG vaccination of adults with moderate to severe asthma resulted in improved lung function and reduced medication use. This was associated with suppression of

Th2-type cytokine responses in sputum [105]. However, observational studies looking at BCG vaccination and effect on atopy and asthma in humans from a wide range of settings have shown conflicting and inconsistent results [106, 107].

Studies in mice have shown beneficial effects of BCG against a range of other inflammatory and autoimmune conditions, including type 1 diabetes mellitus (T1DM) [108] and multiple sclerosis (MS). In a recent randomised controlled study of adult patients with established T1DM, two doses of BCG have been shown to reduce haemoglobin A1c (HbA1c) levels, a marker of long-term blood sugar control, to near normal levels for at least 5 years. Possible mechanisms for this observed effect include alteration in systemic glucose transport and epigenetic reprogramming of aberrant T regulatory (Treg) cells [109]. A larger, phase II study further exploring the effects of repeated BCG vaccination in T1DM is ongoing (clinicaltrials.gov NCT02081326).

MS is a chronic, neurodegenerative disease characterised by autoimmune central nervous system (CNS) demyelination. Intracutaneous BCG has been shown to reduce active CNS lesions on magnetic resonance scans in a small study of patients with relapsing and remitting MS [110] and to result in a decreased risk of progression to MS following a solitary first demyelinating episode [111]. Studies have suggested that BCG may also play a role in other neuroinflammatory conditions. In a mouse model of Alzheimer's disease (AD), BCG vaccination reduces neuroinflammation and reverses cognitive behavioural decline [112]. Human observational studies have shown that bladder cancer patients treated with BCG were significantly less likely to develop AD at any age than those who did not receive BCG [113, 114]. The mechanisms underlying the effects of BCG on neuroinflammation remain unknown and further research in this area is needed.

8.7 Safety and Adverse Effects of BCG

BCG is one of the most widely used vaccines in the world, with around 100 million children newly vaccinated each year [42]. Although considered to be a very safe vaccine, mild local and systemic side effects immediately after vaccination (so-called reactogenicity) are commonly seen, with BCG amongst the most reactogenic vaccines in use today. Reactogenicity is influenced by BCG strain (likely due to differences in residual virulence as a result of genetic variability between strains) with Danish and Pasteur known to be more reactogenic than Tokyo or Glaxo strains [115]. BCG is the only vaccine in modern usage that routinely induces local ulceration and heals with scar formation.

The recommended route of BCG vaccination is via intradermal injection. Following inoculation, a small area of erythema develops, with a raised papule seen several weeks later. Associated mild swelling of ipsilateral axillary lymph nodes may occur. Papule formation is followed by shallow ulceration and healing with scar formation. This may take as long at 3 months in infants. Inadvertent

Table 8.2 Adverse event associated with BCG vaccination

Common	Uncommon (<1 in 100)	Rare (<1 in 1000)
Local ulceration and scar formation	Prolonged, discharging ulceration	Injection site abscess
Regional (axillary) lymphadenopathy <1 cm	Regional (axillary) lymphadenopathy >1 cm	Suppurative regional lymphadenitis
	Headache	Osteitis/osteomyelitis
	Fever	Disseminated BCG infection
		Anaphylaxis

injection into the subcutaneous or intramuscular layers can increase the risk of more severe localised reactions, including local abscess formation and discharging infection of regional draining lymph nodes (suppurative lymphadenitis) [115, 116].

Serious adverse reactions following BCG vaccination are rare, affecting less than 1 in 200,000 individuals [9]. However, more severe local and disseminated side effects may be seen, in particular in immunocompromised infants (Table 8.2). This may be due to primary genetic disorders of the immune system, such as severe combined immunodeficiency (SCID) and chronic granulomatous disease (CGD), or more commonly acquired immunodeficiency, in particular HIV [117].

Spread of BCG infection to distant sites signifies the most serious BCG adverse reactions. BCG infections of the liver, lungs and bones (osteitis and osteomyelitis) have all been documented. Whilst they are most common in immunocompromised individuals, BCG osteitis has been documented in seemingly immunocompetent individuals. Disseminated BCG disease, termed BCGosis, represents haematogenous spread to more than one distant site. Diagnosis can be challenging, and molecular diagnostics are required to distinguish BCG from other mycobacterial infections including TB [118].

Due to the risks of disseminated infection, the WHO recommends BCG should not be given to HIV-infected infants. However, significant improvements in HIV treatment mean that most mothers living with HIV will be taking antiretroviral treatment, infants provided with prophylactic treatment and vertical transmission is near eliminated. Therefore, pragmatically many infants are vaccinated without waiting for HIV testing.

Following intravesical installation of BCG for bladder cancer, systemic symptoms including general malaise and fever are common and affect around a third of patients. Localised bladder irritation with cystitis is seen in over 60% of patients, with visible haematuria in around 20% [119]. Severe side effects are uncommon but may be difficult to diagnose as they can occur at distant sites and be temporally removed from BCG installation. BCG prostatitis, nephritis, osteomyelitis, mycobacterial pneumonia, infection of prosthetic valves and joints and disseminated BCG infections with septicaemia have all been described. Treatment cessation is required in around 8% of patients due to side effects [120].

8.8 BCG Future Directions

8.8.1 Recombinant BCG

Despite extensive study, we still do not fully understand the full range of effects that BCG exerts against a variety of infectious and non-infectious diseases. Targeted genetic modification of BCG may allow retention of its positive non-specific effects, whilst aiming to increase the protection it affords against *M. tb.*

VPM1002 (Vakzine Projekt Management, Serum Institute of India) is a novel, genetically modified live vaccine, formed by recombination of BCG with genes from *Listeria monocytogenes* that confer phagosomal disruption properties. This aims to improve access of VPM1002 mycobacterial antigens to the host cell cytosol and enhance presentation to T cells via MHC class I molecules. In phase I and II trials, VPM1002 has been shown to be safe and immunogenic, inducing responses in $CD4^+$ and $CD8^+$ T-cell populations thought to be necessary for protection against *M. tb*. A phase II/III trial is ongoing in India, assessing prevention of relapse following VPM1002 vaccination in recently treated TB patients (clinicaltrial.gov identifier NCT03152903). Preliminary efficacy results are awaited [121, 122].

8.8.2 Route of Administration

BCG was initially given as an oral vaccine. Use of parental vaccination was pioneered in Norway, and the intradermal route became the most widely used in most countries. In Brazil, oral vaccination with high doses of BCG Moreau was routinely employed until as late as 1974 [10]. Recently, increased interest has focused on whether changing the route of BCG administration might improve levels of protection.

The natural route of *M. tb* infection is via inhalation of infectious aerosol droplets into the lungs. Delivering a vaccine by aerosol inhalation would allow alignment of the route of vaccination with the route of infection, with potential advantages anticipated particularly in improved local immunogenicity. In non-human primates, local pulmonary mucosal BCG delivery affords greater protection against subsequent *M. tb* challenge than standard intradermal vaccination and may be the result of superior induction of polyfunctional T-helper 17 cells and increased IgA levels [123]. Clinical studies are ongoing looking at pulmonary and systemic immunological responses following aerosol BCG inhalation in healthy adult volunteers (clinicaltrials.gov NCT02709278, NCT03912207).

Animal models have shown that intravenous (IV) BCG may afford significantly greater protection than vaccination via other routes. Interest in BCG given by the IV route is not new [124] but has been rekindled in recent years. In a study of rhesus macaques, IV BCG resulted in significantly greater protection against subsequent *M. tb* challenge then either intradermal injection alone or intradermal injection

followed by intratracheal boost [125]. A larger study, also in rhesus macaques, showed nine out of ten animals vaccinated with IV BCG were highly protected against *M. tb* challenge at 6 months. No evidence of established *M. tb* infection, so-called sterilising immunity, was seen in six out of ten animals. This unexpected result correlated with the expansion of activated mycobacteria-specific lung tissue resident T cells in IV vaccinated animals [126].

Whilst IV BCG is unlikely to represent a widely deployable vaccine strategy in humans, particularly neonates in low- and middle-income countries, the ability to induce sterilising immunity in the non-human primate model paves the way for ongoing interrogation of the immunological mechanisms underpinning these findings. This may in turn advance the search for the illusive immune correlates of protection against TB. If identified, efforts could be focused on developing a vaccine designed to trigger the same protective mechanisms but without the need to be administered intravenously.

8.9 Conclusions

Despite over a century of use and associated research, BCG continues to be controversial. Outside of its established efficacy against infant disseminated TB, uncertainties remain regarding its ability to protect against TB disease more broadly. New evidence suggests it may yet afford some protection against *M. tb* infection, but further research is needed to confirm these findings in a range of settings.

BCG is known to exert non-specific effects that may impact a range of disease processes. Knowledge of the immunological mechanisms underpinning these is increasing, but we are still far from fully understanding the wide range of potential effects BCG may induce. Current research looking at BCG revaccination, alternative routes of delivery and recombinant BCG vaccines may provide additional clues as to the best way to harness its potential benefits. Furthering our understanding of this complex vaccine remains important, both in terms of optimising its own use and in its role as a benchmark against which new TB vaccines are measured.

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Chapter 9
The Role of Fermentation in BCG Manufacture: Challenges and Ways Forward

Kenneth Barry Walker and Joanna Bacon

Abstract The BCG vaccine has been in existence for a century and has been instrumental in the control of tuberculosis. The method for producing the vaccine has not changed in a very long time and consists of pellicle growth followed by ball-milling, which is a lengthy and variable approach. There has been increasing interest in the possibility of producing the BCG vaccine by growing it in bioreactors, which could address some of the issues around variation between batches, increase yield, and circumvent the challenges associated with supply and demand. There is evidence that fermentation would be a quicker, more reproducible method of production, and would deliver BCG to a higher yield in a form that would be easier to characterise. However, a change to the manufacturing process may require new evidence of bioequivalence and may attract a requirement for preclinical studies as well as clinical trials from Phase I, through to efficacy studies. This chapter describes the history of the BCG vaccine and the issues of the current production method. We discuss the potential benefits of BCG fermentation and the regulatory steps required for such a method of production to be implemented.

Keywords BCG · Fermentation · Vaccine · Regulations · Flow cytometry

9.1 The BCG Vaccine: History and Approval

Mycobacterium bovis BCG (Bacillus Calmette-Guérin) was generated over a century ago for protection against infection with *Mycobacterium tuberculosis* and is one the oldest vaccines still in use [1]. BCG originated from a virulent strain of

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M. Christodoulides (ed.), *Vaccines for Neglected Pathogens: Strategies, Achievements and Challenges*, https://doi.org/10.1007/978-3-031-24355-4_9

Mycobacterium bovis isolated from the milk of a cow suffering from tuberculous mastitis, collected during studies of bovine tuberculosis. Around 1901, this isolate was transferred to the Institute Pasteur in Lille, France, and used by Albert Calmette and Camille Guérin in their investigations of culturing mycobacteria and potential development of a vaccine against tuberculosis (TB). The organism was difficult to culture and prone to clumping, making controlled subculture, and organism management challenging. To optimise bacterial preparations for their experiments, Calmette added ox bile, a detergent, to the glycerol-soaked potato slices, on which the *M. bovis* was cultured. Within a few months, a distinct and attenuated isolate with unusual colony morphology appeared. Calmette and Guérin continued the serial *in vitro* passaging of this *M. bovis* strain for the next 13 years (1908–1921). During this time, experiments with diverse animal models, including guinea pigs, rabbits, dogs, cattle, horses, chickens and non-human primates, established both the safety and efficacy of BCG, thereby consolidating these observations in support of vaccine development. When administered at different doses and by different routes, BCG was well-tolerated and failed to produce tuberculous lesions. Moreover, BCG vaccination provided protection against challenges with virulent *M. tuberculosis* strains. The first human trial occurred in July 1921 [2, 3]. An infant was given three 2 mg doses (6 mg total; ~2.4×10^8 colony-forming units) by the oral route. There were no deleterious side effects, and, most importantly, the child did not develop TB, despite the fact the infant's mother had died of TB shortly after giving birth. Over the next year, additional newborns were vaccinated, and no ill effects were reported. For the first time, a safe and apparently effective vaccine was available for the prevention of human TB. These clinical studies predate the regulatory framework we are now familiar with, and in general, the acceptance and use of vaccines used to be a considerably more informal process. As early as 1924, cultures of BCG were distributed by the Institute Pasteur to laboratories around the world consolidating its position in the fight against TB. The production and application of BCG in public health measures varied from country to country. Because BCG is a live vaccine, it was necessary to transfer cultures to fresh medium every few weeks. Despite standardisation efforts, different passaging conditions were used in different production laboratories; the *in vitro* evolution of BCG was observed and has since continued to this day. Dozens of distinct daughter strains emerged, including four that are currently in major use: BCG-Pasteur (1173P2), BCG-Japan (Tokyo-172), BCG-Danish (Copenhagen-1331) and BCG-Glaxo (1077) [4, 5].

9.2 How Is BCG Vaccine Cultured and Manufactured: Fundamental Limitations?

Current production of BCG that is licensed for parenteral administration to humans and veterinary use is by growth of *M. bovis* BCG in liquid medium in stationary flasks and a pellicle forms at the liquid-air interface [6]. The bulk drug dry substance is prepared by harvesting of the wet pellicle and ball-milling it to generate a

suspension of live bacteria, which is then lyophilised. Ball-millers consist of a rotating drum, containing ceramic balls, which grind the BCG and are used for grinding materials such as coal, pigments and feldspar for the pottery industry. The complexity of multiple culturing and processing steps has been challenging to standardise [7]. Current production times are lengthy (~21 days) and labour-intensive, leaving production centres unable to effectively respond to changes in demand or shortages [8–10]. There has also been a reduction in the number of BCG manufacturers, over time, particularly in the World Health Organisation (WHO) pre-qualified supplier programme, which has placed BCG availability for global public health mass vaccination programmes, at risk. Even obtaining BCG for recent clinical trials of new vaccines against TB has been a fraught process. A global shortage of BCG lasting months, occurred in 2014–2015, when technical issues halted production at a single site. The pellicle production method also causes quality control issues, where bacterial aggregation means that reliably assessing cell titres is challenging and will often produce highly variable results [9, 11, 12].

Variation in BCG batches, between and even within production centres, has been highlighted as a key issue [13–16]. The WHO has noted a need for standardising the production and quality assessment of BCG to not only mitigate the variable efficacy seen but to also allow for easier evaluation in clinical trials of "new" and "old" BCG vaccines [8, 11, 17]. If a new vaccine were to include expression of heterologous proteins in recombinant BCG vaccines, this might be a good time to move to a method of production that is more reproducible and defined to achieve standardisation. The current approach leads to clumpy bacteria that must be disrupted by ball-milling, which subsequently results in an ill-defined proportion of dead bacteria that will vary between batches. This in turn, could be impacting the types of immune/protective responses and resulting vaccine efficacy. The slower growth rate of BCG, prepared in this way, has previously been associated with larger scars, post-vaccination, and the increased prevalence of positive PPD (purified protein derivative) skin responses via a Mantoux test, providing further support for the involvement of BCG phenotype as a variable that impacts vaccination outcomes [13]. The quantitative measure for the variations between batches has been limited to colony-forming units, which does not provide full information about culture viability or the proportion of dead bacteria. How much of an impact this has in the context of other factors that potentially affect vaccine efficacy (BCG strain, exposure to environmental mycobacteria and different host-immune profiles) is not known [18–20], but a more consistent and quantitative method of production would almost certainly provide a foundation for the assessment of some of these factors. The introduction of different culture methods and medium composition will inevitably impact the physiological state of the organism and subsequent immune/protective immune responses. Our recent study showed that mycobacterial pellicles produced less alpha glucan than planktonically grown bacilli, it followed, that pellicles and their extracted sugars showed a reduction in C3 deposition compared to planktonic bacteria [21]. A previous study explored the impact of growing *M. bovis* BCG in flasks, on the protective efficacy of BCG in mice, compared to pellicle-grown, ball-milled BCG. The two approaches resulted in a similar level of protection against *M.*

tuberculosis infection [22]. In a further study, inclusion of surfactant in the growth medium reduced encapsulation on BCG. Higher levels of encapsulation promoted a more potent immune response including higher interferon-γ responses, higher polysaccharide-specific capsule antibody, higher IL-17 responses in the spleen and more multifunctional CD4+ T cells [23]. These immune responses correlated well with a reduced bacterial burden in the lung and spleen. Although, as described here, the culturing method will impact the immune response and might alter protective efficacy of BCG, and once these changes are defined and well-established, a more reproducible and consistent approach could be adopted, particularly in controlled systems such as bioreactors or fermenters.

9.3 Could Fermentation Be an Approach for BCG Vaccine Production?

There has been increasing interest in the possibility of producing the BCG vaccine by growing it in bioreactors, which could address some of the issues around variation between batches and increase BCG yield. Post-genomic studies have adopted the use of fermentation of *M. bovis* BCG in chemostats under defined and controlled conditions, combined with the screening of transposon mutant libraries to determine the genetic requirements of adaptation to slow growth rate (69 h mean generation time (MGT)) or fast growth rate (23 h MGT) [24]. These studies were later extended to determine an inventory of macromolecules under these different growth rates, and the proportion of lipids in the mycobacterial cell was found to change at the different growth rates, possibly reflective of the amounts of storage lipids [25]. This, and related studies of *M. tuberculosis*, using defined culture conditions in fermenters, have demonstrated the benefits of controlled conditions during fermentation in reducing heterogeneity and an increase in culture to culture reproducibility [26–28]. There have been two key studies that have observed the impact of fermenter growth on vaccine protection in animals infected with *M. tuberculosis*. The first study, by Dietrich et al. [9], showed that bioreactor-grown *M. bovis* BCG exhibited similar protection in BALB/c mice compared to pellicle-grown ball-milled BCG [9]. It should be noted that pellicle growth in Sauton minimal medium was required for preparation of the inoculum in the Dietrich study [9], followed by fermentations in the same medium. In contrast, Pascoe et al. [29] reported that a more consistent inoculum could be generated through growth, in a few days, in highly aerated shaking flasks [29]. Curiously, they observed that *M. bovis* BCG would not grow in Sauton medium using this approach, or in Sauton medium in a fermenter. It is possible that for *M. bovis* BCG to grow in Sauton medium, it first needs to be passaged and adapt in a clumpy biofilm-like state in static cultures prior to growth in stirred or shaking cultures. This raises concerns about the impact of multiple ill-defined culturing steps on the reproducibility of the resulting biomass and the genetic stability of the strain. The second notable BCG vaccination study in animals from

Lesellier et al. [30] demonstrated consistent induction of protection when the efficacy of bioreactor-grown BCG (delivered via the oral cavity) was evaluated in TB-infected badgers [30]. BCG was grown reproducibly whilst aerated at a controlled oxygen level of 10% dissolved oxygen tension, in Middlebrook 7H9 medium, harvested in late exponential phase after 7 days of growth, at a titre of $4.64 \times 10^9 \pm 2.20 \times 10^8$ colony-forming units (cfu) mL^{-1}. This study neatly demonstrated the advantage of fermentation over conventional production, in obtaining a higher BCG yield, and therefore subsequent increases in the availability of doses of BCG for distribution.

Equally of relevance is the development of new technologies (besides cfu mL^{-1}) for improved BCG characterisation, to ensure consistent outcomes. Fluorescence-labelling and flow cytometry allow for rapid determination of viability and interrogation of population heterogeneity. This type of characterisation has limited application in clumpy, ill-defined cultures and benefits from defined fermentation growth, if only to reduce blockages in the flow cytometer. Dietrich et al. [9], used Fluorassure reagent to quantify viable bacteria in their bioreactor growth. The reagent is taken up by metabolically active cells and cleaved by esterases to become fluorescent, with subsequent intracellular accumulation. Similarly, and more recently, Gweon et al. [31] assessed a method to measure viability, using fluorescein diacetate, which is also cleaved by intracellular esterases, used flow cytometry, compared to viable counts, and showed a strong positive linear relationship between the two methods [31]. There was significantly reduced covariance between replicate batches of BCG using fluorescence as a measurement. Esterases can still be detected in dead cells, allowing for false positive results: therefore, inclusion of a second dye that simultaneously measures the dead population would help to negate these issues. Hendon-Dunn et al. [32, 33] developed a flow cytometry method, using the two dyes, Calcein Violet-AM (labels metabolically active bacteria) and Sytox Green (labels the DNA of dead/compromised bacteria), which allows for the proportions of live or dead bacteria to be quantitatively determined and for non-culturable bacteria to be captured. This flow cytometry method was applied for the characterisation of bioreactor growths during studies to optimise BCG fermentation at Public Health England, Porton Down (Fig. 9.1) [29]. These studies identified Roisin's medium as an optimal medium for fermentation, as there was comparable growth compared to Middlebrook 7H9. Roisin's medium has the advantage of being a defined medium for which quantitative substitutions can be made for individual medium components [24, 25, 34]. This creates an opportunity for further optimisation of the medium for BCG production by fermentation to determine the impact of larger scale fermentation on the cultivation of BCG and protection/virulence studies in animal models. Other conditions for consideration are aeration and the inclusion of detergent in culture. In any bioreactor culture, detergent is necessary for generating single cells or smaller clumps of bacilli in a more reproducible and homogenous manner. However, consideration needs to be given to the presence of detergent because of changes to the cell wall and capsular composition of BCG, which in turn will impact the efficacy of BCG vaccination [23].

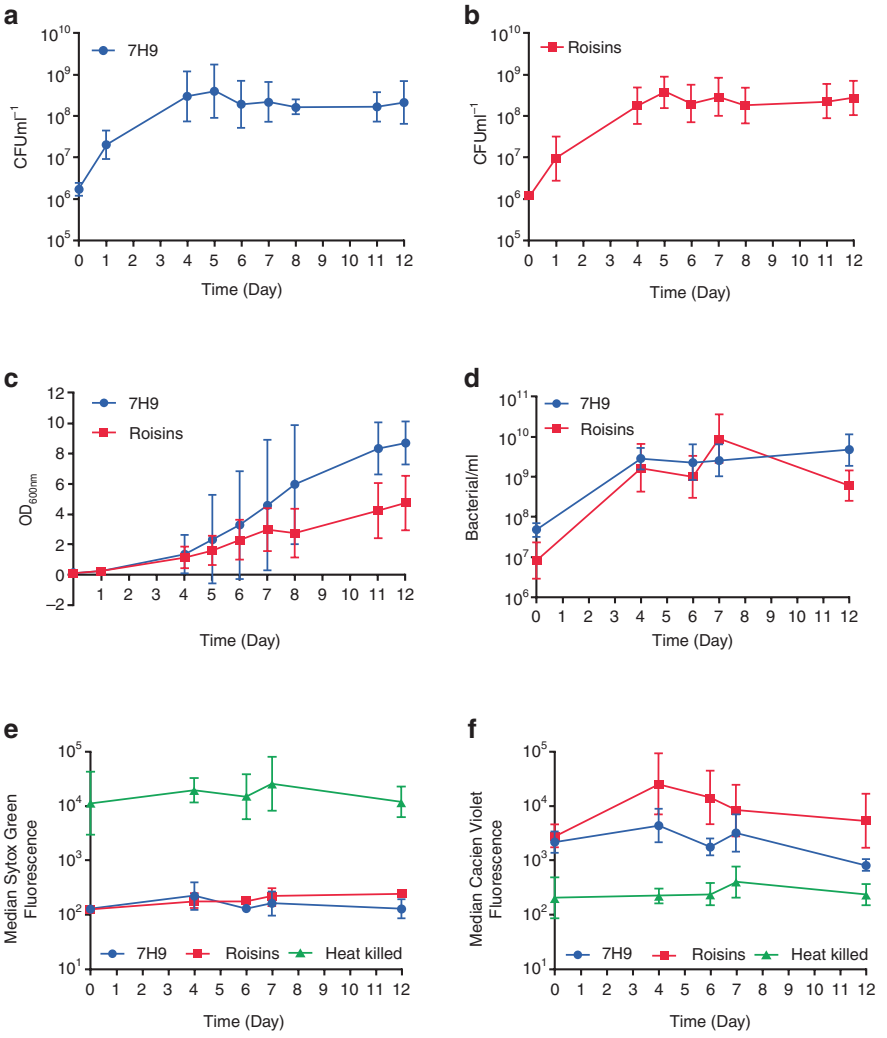

Fig. 9.1 The total viable counts of fermenter-grown *M. bovis* BCG in 800 mL of either Middlebrook 7H9 (**a**) or Roisin's minimal medium (**b**) over 12 days of culture in batch fermenters. The cultures were sampled daily for total viable counts (CFU mL^{-1}) (**a**, **b**) and turbidity (**c**). Samples were taken on days 0, 4, 6, 7 and 12 for total bacterial counts (bacteria/mL) determined using fluorogenic beads and flow cytometry (**d**). Samples were taken on the same days for analyses of cell death and metabolic state using flow cytometry and fluorescent dyes, Sytox Green (**e**) and Calcein Violet-AM (**f**), respectively. A heat-killed *M. bovis* BCG control was included. Data represent the mean average of three independent culture repeats ± standard error bars. (Source: Pharmaceutics 2020 Sep 22;12(9):900 doi: https://doi.org/10.3390/pharmaceutics12090900 [29])

9.4 BCG Product Characterisation

The Bill of Testing for the BCG vaccine, particularly, the batch release testing requirements, is limited compared to any of the more modern live vaccines in use or in clinical study (Table 9.1). This reflects the long history of the BCG vaccine and its passage from pre-National Regulatory Authority (NRA) regulations, into the International Committee of Harmonisation coordination of regulatory advice and regulations. In the last decade, improved technologies have provided additional avenues for BCG product characterisation, some of which have gained some traction with regulators and have been included in guidance documents (Table 9.2). Although progress has been made from both technology and assay development perspectives, hurdles remain in bringing some of these newer assays into the regulatory and batch release vaccine testing spaces. To do so, it requires significant investment and development time to ensure new approaches provide relevant information regarding the quality and functionality of the BCG vaccine. The International Committee of Harmonisation (ICH, www.ich.org) has developed a series of guidelines that provide the laboratory and data package framework and is required to

Table 9.1 European Union pharmacopeia; current BCG testing recommendations

Test identity	Methodology	Notes
Identity	Colony morphology, acid-fast staining of a smear of bacterial suspension on a slide	May include, molecular tests, e.g. PCR and/or targeted sequencing [40–42]
Sterility	Sterility testing—see "Requirements for Biological Substances and Requirements for Sterility of Biological Substances"	See ICH Quality Guidance Documents Q5A-E and Testing criteria for Biologics Q6B. www.ich.org
Absence of virulent mycobacteria	Guinea pig test $N \geq 6$ at 50× human dose. Observe after 6 weeks	No evidence of progressive TB; at least 2/3 survive in a 6-week observation period
Excess dermal reactivity	Guinea pig test, $N \geq 4$. 3 × tenfold dilution injection. Observe after 4 weeks	Skin reaction not significantly greater when compared to reference control BCG
Total bacteria	Particle counting, biomass determination, opacity determination	Greater than minimum yield as per product specification
Cultivable bacteria	Viable bacterial colony counting on solid media, alternative in vitro assays	Greater than minimal viability as per product specification
Stability	Viability of lyophilised vials of vaccines held at 37 °C for 4 weeks	Not less than 20% loss of viable bacteria
Consistency of manufacture	Assessment of test outcomes from at least ten vials	Within product specification for 10/10 vials
Moisture content of lyophilised vials (final form)	Test and outcome show equal or less moisture content than specification	

Table 9.2 Novel or recently included BCG vaccine characterisation tests

Test identity	Methodology	Notes
Identity	Whole genome sequencing, targeted PCR sequencing of specific genes, multiplex PCR	Not fully recognised by regulatory authorities [41]
Sterility	Molecular genetic screening for adventitious organisms	Not fully recognised by regulatory authorities, see also ICH Q4B Annex 8 (R1)
Absence of virulent mycobacteria	Molecular genetic screening for *M. tuberculosis*	Used by some developers and manufacturers [43]
Excess dermal reactivity	Cytokine production *in vitro*	Relevance, sensitivity and specificity, under assessment
Total bacteria	No alternative currently proposed	
Cultivable bacteria	Bioluminescence or calorimetric assays	Used by some developers and manufacturers. Relevance, sensitivity and specificity under assessment
Stability	Bioluminescence or calorimetric assays	Used by some developers and manufacturers. Relevance, sensitivity and specificity under assessment [44–46]
		See ICH Q5C
Consistency of manufacture	Using above technologies to determine consistency of product	

bring new assays into the regulatory environment (ICH Q2(R1); Q2(R2)/Q14 EWG; Q5C; Q5E). An aspect of BCG characterisation that remains unresolved are the assessment and measurement of potency that reflect clinical efficacy in clinical studies and in normal use. No agreed correlate of immunity has yet to be agreed, although some biomarkers of relevant biological activity have been studied for potential application [14]. The only accepted potency readouts for BCG, currently used by manufacturers and the NRA, are viable counts and relative viability against total bacterial numbers, using a variety of technologies [31, 35, 36]. This has been shown to have some association with clinical outcome, but no straightforward dose response curve has been determined, and relative (to a reference BCG vaccine) potency is difficult to quantitate. *In vivo* assessments (mouse protection studies, guinea pig protection studies) are complex, long-term and not amenable to manufacturing or batch release activities. A new assay that can determine *in vitro* bacterial inhibition has been studied extensively, measuring the reduction in bacterial replication by leucocytes *in vitro* culture. The assay has proven to be highly variable, and although much has been done to standardise protocols and measurement, there remains some way to go in showing sufficient specificity, reproducibility, and sensitivity, for routine use. Moreover, there is no clear evidence of validations of assay outcomes and clinical effects (a requirement of modern potency assays for biologicals) [37–39].

9.5 Regulatory Considerations

BCG is an authorised vaccine that is licensed for use, based on a range of evidence provided in the registration dossier, which include detail on the process of manufacture, in process control testing (and required outcomes), along with a full quality and Bill of Testing data package, for the product. As BCG has been used as a vaccine for a long period of time, the information contained in the regulatory documents accumulated prior to current regulatory frameworks and legislation came into place. Therefore, much of the data, quality control, and manufacturing information, that is, available for other current live vaccines, have not been produced for BCG. Existing product data for BCG has simply been incorporated into more recent legislative and operational frameworks, which presents challenges in the compatibility of the available data with regulatory requirements. Making significant changes in the manufacturing process, such as a switch to fermentation, may change the nature of the product, particularly with a live vaccine such as BCG [47]. Major redesigns of the manufacturing process are likely to trigger reviews by the NRA and a recommendation to consider fermented BCG as a new product, which would negate the existing licensure for use [48, 49]. A new product may require new evidence of bioequivalence and may attract a requirement for preclinical studies as well as clinical trials from Phase I through to efficacy studies [50]. The WHO recommendations to assure the quality, safety and efficacy of BCG vaccines (Annex 3 [51]) state that production of new BCG vaccine (i.e. a modified strain of BCG) is a prerequisite for initiation of clinical studies [52]. This places a large developmental burden on existing BCG manufacturers but may allow new products into the manufacturing space, as they will be undergoing testing to the current standards [53]. It should also be remembered that BCG is a major player in global health, and it remains a low-cost vaccine for both the user and consumer. The latter negatively impacts the economic pressure to change or improve the existing manufacturing processes. Because of these competing pressures, loss of manufacturing flexibility and the previously noted constraints of existing BCG availability, the WHO supported a discussion (Montreal, October 2015) on how to move to a more standardised and modern manufacturing process for BCG. Representatives from NRAs, manufacturers, and leading mycobacterial experts, attended. No clear way forward was agreed, which epitomises the complexities around establishing major change in BCG manufacturing processes, further restricting the options for the improvement of BCG manufacture and availability [8].

New live TB vaccines, including those based on BCG, will be travelling a conventional registration pathway and as such will suffer none of the manufacturing constraints described here. No new live TB vaccine has achieved approval and the time frame for such approvals remains unclear. Therefore, the concerns surrounding BCG manufacture, yield, and availability, remain significant considerations for all involved. What remains unclear is the degree of bioequivalence of BCG between differing manufacturing processes, as well as the functional comparisons of the new live TB vaccines. Comparisons of new vaccine candidates with BCG remain part of

the product development pathway and are incorporated into the preclinical and clinical studies designs already undertaken or planned. The effect of manufacturing on functional bioequivalence in terms of the regulatory Bill of Testing for release of BCG vaccines for human use is unknown. The WHO meeting supported the development of a study plan to directly compare classical pellicle production with bioreactor-grown BCG, in the battery of tests commonly used for the batch release of BCG, as well as a more exploratory preclinical efficacy study [22]. This study showed bioequivalence between these two manufacturing processes. Interestingly, in the exploratory preclinical efficacy studies, some minor differences were observed, the significance of which remain unclear. Further studies expanding on these observations are needed to inform the NRA, WHO and public health bodies, on the benefits and possible risks in changing manufacture of BCG, or to develop a strategy for BCG replacement, if deemed appropriate.

9.6 Concluding Remarks

Considerable work has been done in refining and optimising fermentation technologies and approaches for BCG production, including the growth medium, the supplements, and the methods of BCG characterisation, all of which have been subject to review and assessment. The resulting published studies have informed potential manufacturers and vaccine developers of the approaches that are available and the issues of yield, comparability, and consistency of manufactured BCG characteristics. Despite much discussion at the highest levels, there seems to be no clear path to the improvement of current licensed BCG manufacturing processes though adoption of fermentation/bioreactor technology. There are multiple considerations, as noted here, ranging from regulatory constraints to the cost of changing the technology and the impacts of obtaining authorisation for a major change in the manufacturing procedures. There may be new approaches to these issues available with the development of a more flexible and pragmatic regulatory landscape, which has developed with the pressures and timelines of the COVID-19 vaccine development efforts, which have been so effective in delivering effective and safe vaccines in record time.

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Chapter 10
Progress in the Development of New Vaccines Against Tuberculosis

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Abstract Due to the shortcomings of currently available BCG vaccines, new strategies have been considered for the development of alternative vaccines against tuberculosis. Many candidate vaccines are in the pipeline with an aim to replace BCG or boost the effect of BCG for prophylaxis. In addition, therapeutic applications are also considered. In this chapter, the current advances and approaches are explored to develop pre- and postexposure vaccines for tuberculosis.

Keywords *M. tuberculosis* · Tuberculosis vaccines · Vaccine markers Tuberculosis markers · Adjuvants

10.1 Immunological Characteristics and Markers for *Mycobacterium tuberculosis*

Mycobacterium tuberculosis (Mtb) infection in humans has a complex pathology that creates challenges in finding targets of the immune reaction to the pathogen. First, Mtb bacilli enter the lungs in aerosol droplets. During the innate immune response, macrophages ingest the bacteria and recruit other immune cells [1]. Cells such as neutrophils, γδ T cells, NK cells, and $CD4^+$ T cells are activated to control the infection, leading to granuloma formation [2]. A small number of bacilli stay contained in the granuloma and may contribute to disease reactivation later in life [3]. In the majority of cases, $CD4^+$ T cells will control the bacteria from further

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M. Christodoulides (ed.), *Vaccines for Neglected Pathogens: Strategies, Achievements and Challenges*, https://doi.org/10.1007/978-3-031-24355-4_10

infection; however, in some cases, the infection can reactivate and develop into an acute, chronic, or extrapulmonary disease [2]. Mtb is efficient in evading the immune response and can be in the latent stage for years before symptoms are expressed [2]. Most people exposed to Mtb do not develop the clinical disease, for reasons that are currently not well understood [4].

The cytokines IL-8, IL-12, IL-17, IFN- γ, TNF- α, TGF-β, and memory T cells have been shown to be active in the immune response against tuberculosis (TB) [5]. While these markers are known to be involved in the immune response, Mtb does not have a known validated immune correlate of protection. Correlates of protection (COP), while complex, are quantitative or qualitative measurements that correlate to adequate levels of protection against a pathogen. These often correlate with the concentration of antibodies produced in response to the specific pathogens [6]. Without a COP, years of study are required to determine if markers are efficacious [4]. With a COP, vaccines could be optimized and explored based on their match with the proven immune response, leading to the licensure of vaccines [7]. Additionally, the COP could help explain some vaccine challenges. For example, the vaccine MVA85A produced a dismal response in phase 2, but in previous clinical trials, the vaccine produced high $CD4^+$ T-cell responses [8]. With a known COP, the vaccine's immune response could be matched with the standard.

In general, $CD4^+$ T cells play a predominant role in protective immunity against TB, but strategies that boost $CD8^+$ T-cell function have also been shown to enhance vaccine efficacy [8]. Depletion of $CD8^+$ T cells in a nonhuman primate model of TB led to reduced protection in immunized monkeys [8]. Similarly, $CD8^+$ T-cell depletion in Mtb-infected and then antibiotic-treated monkeys led to increased susceptibility to reinfection [8]. These studies indicate the importance of $CD8^+$ T cells in conferring immunity in vaccination or natural infection. Additionally, the human $CD8^+$ $CCR7^-$ $CD45RA^+$ effector memory T cells exhibit significant antimycobacterial activity [8].

10.2 Approaches for TB Vaccine Development

There are several approaches for the development of new vaccines against TB, based on our current understanding of immunity against Mtb and the status of the host. These approaches include preventive pre-exposure (for uninfected individuals/infants), preventive postexposure (for latent TB/adolescents and adults), and therapeutic (for active TB) [9, 10]. Pre-exposure vaccines aim to prevent the infection from being established by inducing a more robust protective or faster immune response than BCG [1]. Postexposure vaccines aim to induce either a robust and long-lasting response to prevent disease reactivation or eliminate latent TB by

inducing sterilizing immunity so that bacteria are unable to be reactivated later and lead to active TB [1]. The BCG vaccine follows the preventative pre-exposure approach. Vaccines can also be categorized in their biochemical forms: live attenuated, inactivated, adjuvanted protein subunit, and recombinant. Some vaccines have been created also as a boost to BCG [11, 12], and others have used differing modes of administration to boost immune responses [13].

There are currently at least 23 vaccine candidates in clinical trials in humans, as shown in Tables 10.1 and 10.2. Recent developments focus on their biochemical forms: live attenuated, inactivated, subunit, and recombinant vaccine types. Various antigens and adjuvant combinations are being tested in the case of subunit vaccines.

Table 10.1 Live attenuated, inactivated, and subunit/adjuvanted TB vaccines in clinical trials

Vaccine	Vaccine type	Adjuvant	Phase	NCT number or author
MTBVAC	Live attenuated		Two trials recruiting for phase 3	NCT03767946; NCT03152903
RUTI	Inactivated		Phase 2a not yet recruiting	NCT02711735
M. vaccae-based	Inactivated		Two phase 3 trials	NCT01977768; [13]
M. indicus pranii (MIP)	Inactivated		Two phase 3 trials	NCT00265226; NCT00341328
M. smegmatis	Inactivated		Phase 1	[14]
DAR-901	Inactivated		Phase 2a	NCT02712424
M72/AS01E	Subunit/ adjuvanted	AS01E	Phase 2b completed	NCT01755598
H4:IC31 (AERAS-404)	Subunit/ adjuvanted	IC31	Phase 2b	NCT02075203
H56:IC31	Subunit/ adjuvanted	IC31	Phase 2a ongoing; phase 2b recruiting	NCT03512249
ID93 + GLA-SE	Subunit/ adjuvanted	GLA-SE	Phase 2a	NCT02465216
GamTBvac	Subunit/ adjuvanted	CpG ODN + dextrans	Phase 2a	NCT03878004
AEC/BCO2	Subunit/ adjuvanted	BC02	Phase 1 recruitment completed; phase 2b	NCT03026972; NCT04239313
Mtb72F/AS02	Subunit/ adjuvanted	AS02	Phase 2b	NCT00397943

Table 10.2 Recombinant TB vaccines in clinical trials

Vaccine	Vector type	Phase	NCT number or author
VPM1002	Recombinant mycobacterial	Phase 2 completed, recruiting for phase 3; phase 2/3	NCT04351685; NCT03152903
Aeras-402	Recombinant mycobacterial	Two phase 2b trials	NCT02414828; NCT01198366
rBCG30	Recombinant mycobacterial	Phase 1; preclinical in mice	[15]
MVA85A/AERAS-485	Recombinant live viral vectored	Two-phase 2a trials completed	NCT00953927; NCT01151189
Ad35/AERAS-402	Recombinant live viral vectored	Phase 1 recruiting	NCT01683773
Ad5Ag85A	Recombinant live viral vectored	Phase 1 recruiting	NCT02337270
TB/FLU-04 L	Recombinant live viral vectored	Phase 2a	NCT02501421
ChAdOx1.85A	Recombinant live viral vectored	Phase 1	NCT04121494
ChAdOx1.85A + MVA85A	Recombinant live viral vectored	Phase 2	NCT03681860
ChAd3M72 + M72/AS01E	Recombinant live viral vectored	Phase 1	[16]

10.3 Live Attenuated TB Vaccines

Live attenuated vaccines contain whole Mtb in its weakened or altered form [17]. MTBVAC is the only live attenuated vaccine undergoing clinical trials in humans, and currently two trials are recruiting for the vaccine for phase 3 (NCT03767946; NCT03152903) (Table 10.1). MTBVAC, derived from the lineage 4 (L4) of Mtb complex, is designed as a replacement for BCG and as an immunotherapeutic agent [4]. It has been shown to be more attenuated than BCG [18]. In addition to the phase 3 clinical trials with MTBVAC, preclinical studies have been completed with attenuated Mtb belonging to strains L2 (MTBVAC-L2) and L3 (MTBVAC-L3). Vaccinations with MTBVAC, MTBVAC-L2, and MTBVAC-L3 have shown similar or superior protection compared to BCG in immunocompetent mice when the immunized mice were challenged with the three representative strains of Mtb. It appears that the three MTBVAC vaccine candidates could be combined into a polyvaccine to protect against the globally diverse strains of Mtb to provide worldwide protection against TB [18].

The MTBVAC vaccine has also been used as a vector to make a dual TB-human immunodeficiency virus (HIV) vaccine known as MTBVAC.HIVA^{2auxo} [19]. HIV clade-1 A immunogen HIVA was inserted into the parental strain MTBVAC to produce a recombinant strain, which provided similar protective efficacy to the parental MTBVAC strain against Mtb challenge in mice. MTBVAC.HIVA^{2auxo} also showed an increased safety profile in comparison with BCG and MTBVAC [19], showing promise for immunosuppressed individuals at risk of severe infection or death against the pathogen [20].

10.4 Inactivated TB Vaccines

Inactivated vaccines do not contain any infectious particles and are often considered safer than live vaccines, but their immunogenicity is much weaker and potentially may require multiple doses [20]. Inactivated vaccines have been used for both pre- and postexposure strategies and therapeutic applications [9]. Among the inactivated vaccines is RUTI, which contains detoxified and fragmented Mtb cells in liposomes (Table 10.1). Other inactivated TB vaccines currently in clinical trials include *M. vaccae*, *M. Indicus pranii* (MIP), and *M. obunese* (DAR-901) (Table 10.1).

RUTI, a phase 2b vaccine, has exhibited significant humoral and cellular immune responses against antigens expressed in actively growing and latent bacilli [21]. It has shown efficacy in controlling latent TB in experimental animals, i.e., mice, goats, guinea pigs, and mini pigs [22]. In addition to preventive/prophylactic applications, RUTI, MIP, and *M. vaccae* are being used as therapeutic vaccines to reduce drug treatment duration for patients with active TB [21, 23–25].

Additionally, RUTI has shown vaccine-induced inhibition in mycobacterial counts and a significant shift toward $Ly6C^-$ monocyte phenotype in the spleens of immunized mice [26]. Ly6C is an antigen on monocytes that, when elevated, can induce monocyte differentiation to macrophages, dendritic cells, tissue specific macrophages, and other cells [27]. Vaccination of mice with RUTI upregulated the expressions of $Ly6C^-$ related mRNA transcripts in splenocytes, producing a monocyte phenotype shift from $LyC6^+$ [26]. $Ly6C^-$ monocytes have been shown to have an anti-inflammatory role [28], whereas $Ly6C^+$ monocytes are pro-inflammatory [28]. Therefore, the RUTI vaccine may show a balanced immune response.

10.5 Subunit TB Vaccines with Adjuvants

Subunit vaccines contain selected parts of the pathogen to produce an appropriate immune response. Such vaccines are usually safer but generally induce less robust immune responses, as compared to attenuated and whole-cell inactivated vaccines [20]. Hence, to increase their efficacies, subunit vaccines are often administered with an appropriate adjuvant [1, 29].

A challenge for this type of vaccine is the required binding to the highly polymorphic major histocompatibility complex (MHC) molecules for antigen recognition by CD4+ T helper and CD8+ T cytotoxic cells [30, 31]. The MHC molecules in humans, also known as human leukocyte antigen (HLA), are divided into three groups, i.e., HLA class I, HLA class II, and HLA class III. HLA class I and HLA class II molecules have antigen presentation functions to CD4+ T helper and CD8+ T cytotoxic cells, respectively. HLA class I molecules are further divided into three categories known as HLA-A, HLA-B, and HLA-C. Similarly, HLA class II molecules are divided into three categories known as HLA-DP, HLA-DQ, and HLA-DR. Antigen−/peptide-based vaccines with differing MHC binding abilities or binding to multiple HLA molecules (promiscuous peptides) will widen the

coverage of the target population [32, 33]. The challenge is that MHC molecules are highly polymorphic and the frequency of MHC alleles differs in different populations. To identify appropriate antigens/peptides for vaccine development, databases have been designed based on the HLA allele/haplotype frequency in different populations, such as the Allele Frequency Net Database, which provides allele frequencies from 456 globally distributed populations in 90 countries [34]. Additional databases are available to determine compatibility of T helper lymphocyte and cytotoxic T lymphocyte epitopes to produce robust immune responses [35, 36]. Some databases include binding models based on an allele-specific quantitative structure-activity relationship, a model for human transporter associated with antigen processing, and B cell epitope prediction based on amino acid sequence [37].

Subunit/adjuvanted vaccines, noted as antigen/adjuvant in the literature, are an active area of research due to the wide variety of antigens/peptides and adjuvants (Tables 10.2 and 10.3). Mtb expresses around 4000 proteins [5], which leads to the possibility of selecting a large number of antigens as immune targets (Table 10.4). Common secreted antigenic proteins used in the development of subunit TB vaccines include ESAT-6, CFP10, MPT64, Ag85B, and Ag85A due to their different

Table 10.3 TB vaccine candidates undergoing preclinical studies

Vaccine	Vaccine type	Description	Author
Mtb H37Rv ΔleuD ΔpanCD strain	Live	The strain used as a TB infection model in *G. mellonella*	[38]
H56/CAF01	Subunit/ adjuvanted	Vaccination strategy of priming and mucosal pull immunization and analysis with the uptake of H56/CAF01	[39]
H64:CAF01	Subunit/ adjuvanted	Protein fusion vaccine	[4]
CysVac2/AdVax	Subunit/ adjuvanted	CysVac2 contains Ag85B and CysD; promotes generation of $CD4^+$ T cells	[40]
BCG-PSN	Subunit/ adjuvanted	BCG polysaccharide and nucleic acid injection	[41]
LT70	Subunit/ adjuvanted	Induced humoral and cell-mediated immunity and higher protective efficacy than BCG	[42]
rBCG:LTAK63	Recombinant	Subunit vaccine with LTAK63 adjuvant; lower levels of LTAK63 created a stronger immune response	[43]
rBCG(Δ)ais1/zmpl	Recombinant	Recombinant BCG	[2]
BCG-ZMPI	Recombinant	Recombinant live mycobacterial, lacking *zmp1* gene	[4]
FnBPA+ (pValac:ESAT-6)	Recombinant	BCG booster vaccine	[44]
M. smegmatis expressing Ag85B	Recombinant	Recombinant bacterium study to evaluate the immunogenicity	[45]
ChAdOx/MVA PPE15	Recombinant	Viral recombinant	[4]
CMV-6Ag	Recombinant	Contains ESAT-6, Ag85A, Ag85B, Rv3407; Rv1733; Rv2626, Rpf A, Rpf C, rpf D	[4]

Table 10.4 *M. tuberculosis* antigens and their functions

Antigen	Function	Components/category	Reference
ESAT-6	Product of Rv3875	Small 6 kDa protein involved in immune modulation	[46]
CFP10	Unknown. Exported protein cotranscribed with Rv3875|MT3989|MTV027.10	Cell wall and cell processes	[47]
MPT64	Transports proteins to bacterial surface	Transport protein	[48]
Ag85B	Function unknown	Abundant extracellular fibronectin-binding protein functioning as a mycolyltransferase involved in cell wall biosynthesis	[49]
Ag85A	Function unknown	Contains enzymatic mycolyltransferase activity that are involved in the biogenesis of cord factor and in the coupling of mycolic acids to arabinogalactan in cell walls	[50]
Rv1031	One of the components of the high-affinity ATP-driven potassium transport (or KDP) system, which catalyzes the hydrolysis of ATP coupled with the exchange of hydrogen and potassium ions	Cell wall and cell processes protein	[51]
Rv1198	Function unknown	Cell wall and cell processes protein	[52]
Rv2016	Function unknown	Conserved protein	[53]
Rv2031c	Stress protein induced by anoxia. Has a proposed role in maintenance of long-term viability during latent, asymptomatic infections, and a proposed role in replication during initial infection	Virulence, detoxification, adaptation	[54]
Rv3619c	Function unknown	Cell wall and cell processes protein	[55]
Rv3620c	Function unknown	Conserved protein.	[56]
PE35 (Rv3872)	Function unknown	Pe/ppe family	[57]
PPE39 (Rv2353c)	Function unknown	Pe/ppe family	[58]
PPE68 (Rv3873)	Function unknown	Pe/ppe family	[59]
Mtb9.8	Involved in activation of NF-kb pathway	Cell wall and cell processes	[60]
Mtb32A (Rv0125)	Function unknown; possibly hydrolyzes peptides and/or proteins (seems to cleave preferentially after serine residues)	Intermediary metabolism and respiration	[61]

(continued)

Table 10.4 (continued)

Antigen	Function	Components/category	Reference
Mtb39A (Rv1196)	Function unknown	Pe/ppe	[62]
MPT63 (Rv1926c)	Function unknown	Cell wall and cell processes; immunogenic protein product	[63, 64]
MPT83	Function unknown	Cell wall and cell processes protein	[65]
MPT64	Function unknown	Cell wall protein; immunogenic protein	[64]
Rv0569	Function unknown	Conserved protein	[66]
Rv660c	Function unknown	Conserved hypothetical	[67]
Rv0169	Unknown, but thought to be involved in host cell invasion (entry and survival inside macrophages)	Virulence, detoxification, adaptation	[68]
Rv3490	Involved in osmoregulatory trehalose biosynthesis	Virulence, detoxification, adaptation	[69]
Rv1085c	Unknown, but supposedly involved in virulence	Virulence, detoxification, adaptation	[70]
Rv0563	Possibly involved in adaptation. Hydrolyzes specific peptides and/or proteins	Virulence, detoxification, adaptation	[71]
Rv3497c	Unknown, but thought to be involved in host cell invasion. Predicted to be involved in lipid catabolism	Virulence, detoxification, adaptation	[72]
Rv1813	Modulation of host immune response	Conserved hypotheticals	[64]
Rv2608	Induces differential immune responses with a trend toward higher humoral immune responses	Pe/ppe family	[73, 74]

expression profiles in individuals actively infected with Mtb or individuals with latent TB [5, 75, 76]. Other proteins identified with high immunogenicity include Rv1031, Rv1198, Rv2016, Rv2031c [41, 77], Rv3619c [78], Rv3620c [79], PE35 [80], PPE39 [81], PPE68 [82], Mtb9.8, Mtb32A (Rv0125), Mtb39A (Rv1196) [83], MPT63 (Rv1926c) [84], MPT83 (Rv2873) [32], LppX [85], MPT64 (Rv1980c) [86, 87], and A60 complex [88]. Additionally, a new study showed that Rv0569 increased the release of Th1 cytokines IL-12p40, TNF-α, and IFN- γ [89]. Mtb antigens are expressed at different stages of infection, a challenge for creating a vaccine with adequate/appropriate immune responses against all the stages. Proteins expressed at different timelines include ESAT-6, which is always expressed, and Ag85B, which is expressed early in infection [39]. H56 is an example of a protein fusion and consists of Ag85B, ESAT-6, and Rv660c. This protein fusion stimulates immune responses to antigens expressed at different stages of Mtb infection. A vaccine with H56/CAF01 has shown activation of both innate and adaptive immunity in mice [90, 91]. Additionally, proteins Rv0169, Rv3490, Rv1085c, Rv0563, and

Table 10.5 Choice of adjuvants for *M. tuberculosis* vaccine antigens

Adjuvant	Components	Reference
AS01E	QS-21 (triterpene glycoside saponin); monophosphoryl lipid A	[93, 94]
IC31	KLK (antimicrobial polypeptide); ODN1a (phosphodiesterase backboned immunostimulatory oligodeoxynucleotide)	[95]
GLA-SE	Glucopyranosyl lipid	[96]
CpG ODN + dextrans	Dextran 500 kDa + dextran DAE 500 kDa with attached CpG oligodeoxynucleotides (TLR9 agonists)	[97]
BCO2	BCG-derived unmethylated cytosine-phosphate-guanine (CpG) DNA fragment; aluminum salt	[98]
AS02	AS03; 3-deacylated monophosphoryl lipid (MPL); QS-21	[99]
CAF01	Based on liposomes formed by N,N′-dimethyl-N,N′-dioctadecylammonium (DDA) with the synthetic mycobacterial immunomodulator α,α′-trehalose 6,6′-dibeheneate (TDB) inserted into the lipid bilayers	[100]

Rv3497c are expressed at different stages of the Mtb life cycle and could be promising for a multistage vaccine [92].

Aluminum-based adjuvants are extensively used in vaccines to drive a humoral immune response. Humoral immunity is not considered to have a major role in protection against Mtb, as it is an intracellular pathogen [2]. Therefore, novel adjuvants may be useful to induce protective Th1 responses [89]. Current adjuvants used in Mtb vaccines include IC31, GLA-SE, AS01E, QS21, CFA01, and others (Table 10.5) [5, 101].

The subunit vaccine H4/IC31 (AERAS-404, containing a fusion protein of antigens Ag85B and TB10.4 along with an adjuvant IC31) was studied in adolescents in a high-risk setting for TB to assess its efficacy in decreasing Mtb-specific immune response (an indicator of active TB) in individuals immunized with BCG in neonatal age [102]. The results were compared with BCG revaccination. All the participants had negative results for Mtb-specific immune response as determined by quantifying the concentration of IFN- γ secreted by using the QuantiFERON-TB Gold (QFT) In-Tube assay. QFT conversion, shown by the authors as having a higher risk of progression to TB, was determined in both H4/IC31 vaccinated group and BCG revaccinated group. BCG revaccination reduced the rate of QFT conversion (with an efficacy of 45% and 95% CI 6–68) compared to the H4/IC31 (efficacy of 31%, 95% CI 16–58) [102]. This study renewed interest in revaccination with BCG, which had previously been accepted as having no effect [103, 104].

In a phase 2b clinical trial, the subunit vaccine M72/AS01$_E$ (a fusion protein of Mtb32A [Rv0125] and Mtb39A [Rv1196] in the liposome based AS01$_E$) prevented pulmonary TB in adults already infected with Mtb (efficacy of 54%) [105]. When compared with six candidate TB vaccines, i.e., MVA85A, AERAS-402 (a replication-deficient Ad35 vaccine encoding a fusion protein of the Mtb antigens 85A, 85B, and TB10.4), H1/IC31 (a fusion protein of Ag85B-ESAT-6 [H1] formulated with the adjuvant IC31), M72/AS01$_E$, ID93 + GLA-SE (a 93 kDa fusion

protein of Rv3619, Rv1813, Rv3620, and Rv2608 in the adjuvant GLA-SE), and BCG, it was found that M72/AS01$_E$ induced higher memory Th1 cytokine-expressing CD4$^+$ T-cell memory responses [106]. The ability of M72/AS01E to induce the highest-memory CD4$^+$ T-cell response demonstrated that it was the best vaccine candidate, because the induction of these cells correlates with protective immunity in TB [106]. In a study reported by Tait et al., M72/AS01$_E$ vaccine had an efficacy of 50% against pulmonary tuberculosis in a phase 2B clinical trial after 3 years of follow-up, a result that represents the first subunit TB vaccine that had significant efficacy against clinical TB [107]. A meta-analysis including 7 studies that involved 4590 participants revealed that vaccine efficacy was 57% with significantly higher abundance of polyfunctional M72-specific CD4+ T cells in the vaccine groups versus the control group. Furthermore, the M72/AS01$_E$ vaccine against TB was safe [108].

10.6 Recombinant TB Vaccines

Recombinant vaccines are produced using techniques of genetic engineering and biotechnology. A piece of DNA encoding an antigenic protein of Mtb is inserted into an appropriate vector to transform either a bacterial, mammalian, or yeast cell. The recombinant vector produces the antigen in large quantities in those cells [109]. Because the recombinant protein can also be purified, the purified protein can be used as a subunit vaccine to stimulate the immune response and avoid some of the potential concerns of other types of vaccines, such as whole-cell vaccines [110].

Recombinant vaccines (Tables 10.2, and 10.3) can be classified based on the type of organism used to express Mtb antigens. These include live mycobacterial, live bacterial, and live viral vaccines. Bacteria such as *M. bovis* BCG, *M. vaccae*, and *M. smegmatis* have been used as live mycobacterial vectors [111–113]. *M. bovis* BCG is commonly used as an expression vector due to its stability, cost-effectiveness, and nonspecific immune stimulation [21, 114, 115]. *Lactobacillus lactis* has also been used as a vector in the recombinant bacterial vaccine Pnz8149-ag85a/NZ3900. In the preclinical stage, it was able to induce both cellular and antibody responses after mucosal immunization in mice [116]. Many other recombinant bacterial vaccine candidates are in preclinical stages of development and testing (Table 10.3).

Several live viral vector-based vaccines have also entered into clinical trials (Table 10.2). These vaccines have been designed using various viral vectors, i.e., vaccinia Ankara virus (MVA85A/AERAS-485), Adenoviruses (Ad35/AERAS-402, Ad5Ag85A, ChAdOx1.85A, and ChAd3M72), and influenza virus (TB/FLU-04 L) (Table 10.2). The MVA85A/AERAS-485 is in two phase 2a trials (Table 10.2). One other vaccine is in phase 2a, and five vaccines are currently being evaluated in phase 1 studies (Table 10.2). In general, live viral vector-based vaccines have advantages for safety and ease of production, as compared to whole-cell vaccines, but have the disadvantage of gene expression instability [20].

10.7 Recombinant Vaccine Candidates Based on *M. tuberculosis*-Specific Antigens

Many of the above-stated TB vaccine candidates undergoing clinical trials are based on cross-reactive antigens of Mtb. Immunization with these antigens will have the problem of false positivity in tuberculin skin test using purified protein derivative (PPD) of Mtb, as is seen with BCG. Furthermore, because of the cross-reactivity with environmental mycobacteria, these candidate vaccines may face the problem of masking or blocking effects. Hence, such vaccine candidates may not be recommended as booster vaccines in BCG pre-vaccinated individuals [117]. As per the masking hypothesis, early sensitization with environmental mycobacteria leads to some level of protection against TB, which masks the effect of vaccines given later in life due to the presence of cross-reactive antigens. The blocking hypothesis postulates that previous immune responses to cross-reactive antigens, because of sensitization due to exposure to environmental mycobacteria, prevent the taking of a new vaccine by efficient clearance of the new vaccine antigens by preexisting immune responses [117]. The use of Mtb-specific antigens as new TB vaccines may overcome the effects of blocking or masking [75]. Hence, to have TB vaccines better than BCG, researchers are exploring the possibilities of developing new subunit and/or recombinant vaccines based on Mtb-specific antigens [118].

In previous studies, three Mtb-specific regions, known as regions of differences (RDs), were identified using classical molecular and biochemical techniques, but the identification of all Mtb-specific regions was made possible with advances in whole genome sequencing and the comparative genome analysis of Mtb with other mycobacterial genomes [119]. These RDs are deleted/absent in all BCG sub-strains currently being used in different parts of the world [119]. The analysis of Mtb-specific regions for the expression of proteins based on the finding of open reading frames has suggested that these RDs can potentially encode several antigenic proteins [120]. To identify the proteins suitable as TB vaccine candidates, they were first tested for their ability to induce cellular immune responses in vitro with peripheral blood cells obtained from naturally infected animals and humans. Such experiments conducted with cells from humans and cattle identified six antigens that induced potent cellular immune responses, i.e., PE35, PPE68, ESAT-6, CFP10, Rv3619c, and Rv2346c [121, 122]. When tested in animal models of TB, all of these antigens were found to induce Th1 responses when given along with appropriate adjuvants and delivery systems such as DNA vaccine vectors and nonpathogenic mycobacteria, including BCG [123–127]. In animal models like mice and guinea pigs, immunizations with ESAT-6, CFP-10, and Rv3619c resulted in protection against challenges with *Mtb* [97, 128–130].

In humans, a recombinant subunit TB vaccine candidate, GamTBvac, has undergone phase 1 and phase 2 clinical trials. GamTBvac contains three Mtb antigens, i.e., ESAT-6, CFP10, and Ag85A, fused into two chimeric proteins with a dextran-binding domain from *Leuconostoc mesenteroides*. These fusions are formulated with the adjuvant containing dextran 500 kDa, diethylaminoethyl (DEAE)-dextran

500 kDa, and CpG oligodeoxynucleotides (ODN) [131]. In the phase 1 clinical trial, the safety and immunogenicity of GamTBvac were determined in healthy volunteers who were previously vaccinated with BCG. The GamTBvac achieved an acceptable safety profile and was well tolerated. Furthermore, immunization with GamTBvac resulted in a significant increase in the markers of cellular and humoral immunity, i.e., increased concentration of the protective Th1 cytokine IFN-γ and IgG antibodies. Furthermore, the immune responses were induced to all three antigens included in GamTBvac [131]. The phase 2 clinical trial with GamTBvac was a multicenter, double-blind, randomized, and placebo control study conducted in BCG vaccinated healthy volunteers without *Mtb* infection. The results showed that the vaccine confirmed an acceptable safety profile. Furthermore, GamTBvac induced antigen-specific interferon-gamma release, Th1 cytokine-expressing $CD4^+$ T cells, and IgG responses [132]. These results support further clinical testing of GamTBvac to demonstrate its ability to protect against clinical TB.

An additional recombinant bacterial vaccine in the preclinical stage and based on Mtb-specific ESAT-6 antigen is *L. lactis* FnBPA+ (pValac:ESAT-6). When delivered by the mucosal route in mice, this vaccine produced a systemic Th1 cell response (as indicated by significantly increased secretion of IFN-γ by spleen cells) and a significant increase in specific secretory immunoglobulin A production in colon tissue and fecal extracts of the immunized animals [133]. In a booster model in animals preimmunized with BCG, the pValac:ESAT-6 vaccine induced a significant increase in proinflammatory cytokines IL-17, IFN-γ, IL-6, and TNF-α from the spleen cells of immunized mice [44]. Another study was conducted on the same principles by fusing the genes of ESAT-6 and Ag85A and cloning them in the pValac vector to obtain the recombinant *L. lactis* $FnBPA^+$ (pValac:*e6ag85a*). When used for oral immunization in mice, this recombinant construct induced significant increases in the concentrations of IFN-γ, TNF-α, and IL-17 cytokines by stimulated spleen cells and significant production of antigen-specific sIgA in the colonic tissues of immunized mice [134]. These findings are novel and interesting because they represent the first successful step toward the development of vaccines for boosting the effect of BCG using the oral route for administration and by employing the recombinant techniques for the expression of an Mtb-specific antigen in the bacterium *L. lactis*.

10.8 Routes of Vaccine Delivery

BCG is administered intradermally and results in the induction of strong systemic responses but weak mucosal immune responses [135]. As Mtb is transmitted via the respiratory route, the same route may also be appropriate for the delivery of improved vaccines [136]. The effects of different routes of administration for TB vaccines are not readily discussed in the literature; however, some studies have shown that a route other than intradermal may have a better possibility of success in inducing appropriate immune responses and protection [137]. A comparative

analysis of the oral vs. intradermal administration of BCG was evaluated in a small-scale trial in humans, and the results showed that oral BCG produced a stronger mucosal immune response in bronchoalveolar lavage and secretary IgA in nasal washes and tears, whereas intradermal BCG produced a stronger immune response in the blood [138]**.** In another study, the comparison of intravenous, intradermal, and aerosol delivery of BCG vaccine in rhesus macaques showed that the intravenous administration of BCG was safe, induced significantly more antigen-responsive CD4+ and CD8+ T cells, and afforded better protection against challenge with the highly pathogenic Mtb Erdman strain [27]. Subunit vaccines can also be administered through the subcutaneous, intranasal, edible, and mucosal routes [135]. Nanoparticle-based vaccines have also been explored [2]. Edible-based strategies employ the use of antigens expressed in plants such as carrot, potato, tobacco, and *Lemna minor* (a species of aquatic freshwater common duckweed or lesser duckweed plant) to activate the immune response [2], and nanoparticle strategies use nanoparticles conjugated with antigens such as Ag85B to increase Th1 responses in lymphoid organs against Mtb [2]. However, an optimal route for Mtb vaccine delivery has yet to be identified. Therefore, more research is needed to identify the route that will induce maximum protection against TB.

10.9 Conclusion

BCG is currently the only available vaccine against TB in humans. However, BCG has many drawbacks, prompting concerted efforts to develop vaccines better than BCG. Among the approaches being used to develop new TB vaccines, whole-cell mycobacteria, subunit, and recombinant vaccine strategies are being explored. Proteins have been identified, and strategies to develop new vaccine candidates have been expanded. Differing routes of administration are another area of research for new vaccine development. The achievements in the field of developing improved vaccines against TB are quite encouraging with the hope that better TB vaccines for prophylactic and therapeutic applications in humans may become available in the coming years.

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Part III
Leishmaniasis

Chapter 11
Visceral and Tegumentary Leishmaniasis

Olayinka Osuolale

Abstract Visceral and tegumentary leishmaniasis are neglected tropical diseases caused by the protozoan parasite *Leishmania.* In this chapter, we discuss the causative organisms and the different clinical manifestations, their global and endemic distribution, and methods of vector and human-to-human transmission. We also explore current drug treatment regimens for both diseases and present a brief introduction to vaccine development.

Keywords Visceral · Tegumentary · Leishmaniasis · Neglected tropical disease Treatment · Drugs · Vaccine

11.1 Introduction

Leishmaniasis is a complex neglected tropical disease caused by protozoan parasites of the genus *Leishmania.* In this chapter, we describe the different clinical presentations of leishmaniasis, the global distribution of the disease complex, and current treatment regimens and briefly introduce the concept of vaccination to protect against infection and disease.

11.2 What Are Visceral Leishmaniasis and Tegumentary Leishmaniasis?

Visceral leishmaniasis (VL), also locally called dum-dum fever or kala-azar [1, 2] is a disease that affects the entire human system and is caused by a protozoan parasite transmitted through the bites of the *Phlebotomus papatasi* phlebotomine sandflies

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M. Christodoulides (ed.), *Vaccines for Neglected Pathogens: Strategies, Achievements and Challenges*, https://doi.org/10.1007/978-3-031-24355-4_11

[3]. It is caused by *Leishmania* species such as *Leishmania donovani* complex, *L. donovani* sensu stricto as the major protozoan in East Africa and the Indian subcontinent, and *L. infantum* in Europe, North Africa, and South America [1, 4, 5]. There exist two forms of VL with different characteristics of transmission: (1) anthropozoonotic VL occurs when the protozoan is transmitted from animal to vector to human, with humans serving as occasional hosts and dogs as the parasite's reservoir host, and (2) anthroponotic VL, in which the transmission cycle is from infected human to vector to human [6].

There are two forms of the *L. donovani* parasite in the transmission cycle: the promastigote flagellar form, which is peculiarly found in the gut of the phlebotomine arthropod vector, and the amastigote form, which develops in mammalian host cells [3, 7]. This transmission cycle is only made possible through the bite of female phlebotomine sandflies, which become infected when they ingest the amastigotes during a blood meal. Multiplication starts in the insect midgut, and amastigotes transform into small promastigotes that block the gut of the insect and are seen in the gullet, pharynx, and buccal cavity, from where they can be introduced into a new host via insect bite [1, 7, 8]. Inside the mammalian host, promastigotes are engulfed by dendritic cells and macrophages and transform into amastigotes by losing their flagella [3]. Through complex host-parasite interactions, they multiply and possibly survive in the phagolysosomes [9, 10]. The amastigotes escape dead macrophages and are engulfed by other viable macrophages and cause severe damage to the reticuloendothelial system [1], attacking the bone marrow, enlarging the liver and spleen and sometimes the lymph nodes [3].

Despite the fact that sandflies are the main vector for parasite and disease transmission, other routes of possible transmission have been reported, including via blood transfusion [11–13], organ transplantation [11], needle sharing [14], congenital [15], vertical, and sexual [12]. These routes of transmission are important as they can play a notable epidemiological role in sustaining and spreading the disease where the invertebrate vector is absent [16]. Mescouto-Borges and colleagues [15] reported two cases of congenital transmission of VL in the city of Palmas, Tocantins, Brazil. The presence of the parasite was detected with a polymerase chain reaction (PCR) test for the presence of *Leishmania* kDNA in bone marrow aspirates taken from the newborns. Sexual transmission of VL in humans was first reported in the UK, where no record of autochthonous leishmaniasis nor vector presence exists. This was reported in a woman who had not traveled out of the country but showed genital papule with intralesional *Leishmania* sp., and it was believed that she had been infected by her husband who had been diagnosed with VL many years before [17]. Although uncommon, there are also reports of genital lesions due to VL in human patients, including testicular infection detected in an immunocompromised boy with leukemia [18] and nodular ulcerative sore accompanied by intralesional *L. infantum* in the prepuce/foreskin of an adult man [19].

Clinical manifestation of VL ranges from asymptomatic to fully developed kala-azar [1]. Initially, it begins with symptoms such as fever, weakness, loss of appetite, and weight loss, which is followed later by anemia and enlargement of the lymph nodes, liver, and spleen [1, 3, 20, 21] that causes the archetypical protrusion/

swelling of the abdomen [1]. Other symptoms accompanying the disease condition include swelling of the face, malabsorption, diarrhea, bleeding of the mucous membranes, and nasal ulcers that cause breathing difficulties. There is also the possibility of secondary infection [1, 2]. VL is marked with a skin condition known as kala-azar, which means "black sickness," with the skin becoming earth-gray in color and presenting with diffused nodular lesions. Kala-azar is common [2].

Tegumentary leishmaniasis (TL) is a virulent, zoonotic, noncontagious disease affecting millions of people globally [1]. It is a NTD associated with poverty, and infection produces blisters or ulcers on the skin, which become difficult to heal and scar and sometimes extend to mucous membranes of the mouth, larynx, and nose [2]. Transmission to humans from wild and domesticated animals occurs via the bites of infected female phlebotomine sandflies, with *Lutzomyia* spp. as the commonest vector [1, 4, 6]. The *Leishmania* species responsible for TL are *Leishmania (Viannia) braziliensis*, *L. mexicana*, *L. (Leishmania) amazonensis*, and *L. (Viannia) guyanensis*, [7–11] as the main species in the New World [7, 8]; *L. major*, *L. aethiopica*, and *L. tropica* as the main species in the Old World [7, 8]; and *L. (Viannia) panamensis* in the New World [12, 13].

Four transmission cycle patterns have been described for TL, especially in Argentina; these are (1) transmission occurring in primary vegetation known as the wild cycle, (2) transmission associated with wild or secondary vegetation alterations described as possible peridomestic transmission, (3) peridomestic transmission in homes or settlements close to unused vegetation, (4) peridomestic transmission cycle occurring in rural or urban-rural links [14]. According to Kawa and Sabtoza [15], TL occurs in three primary ecological patterns, namely, the (1) sylvatic or rain forest where people actively involved in activities such as gathering are affected, (2) agricultural areas that have farmers affected in primary forests, and (3) peri-urban areas, where the inhabitants of the outskirts of cities are affected.

The transmission of *Leishmania* species that are responsible for TL begins when flagellated promastigotes are injected into humans through bites of infected female sandflies. Inside the human host and especially in the macrophage phagolysosomal compartment, these promastigotes transform into non-flagellated amastigotes characterized by their round shapes [16, 17]. Clinical manifestations of TL are often characterized by tendencies such as persistency, inactivity, and spread [18]. Symptoms range from self-healing cutaneous lesions to persistent sores/lesions and mucosal lesions throughout the skin that occur when parasites are spread through the blood and lymphatic systems [19–23]. Manifestation of symptoms is dependent on immunity of the individual and the *Leishmania* species involved [20].

11.3 The Global Distribution of VL and TL

Occurrence of VL is global and widely distributed on all continents, with the exception of Oceania [22]. The pattern of disease transmission has significantly changed from an initial predominantly rural distribution to the vector now invading

peri-urban and large urban areas [23, 24]. Regions of the world with predominant cases of VL include Africa, the Americas, and Southeast Asia [25]. Burza and colleagues [11] estimated new cases of the disease to be at ~700,000 to one million per annum, with well over 50,000 deaths. However, both figures are probably underestimates, as most cases of VL are either unidentified or not recorded [26, 27]. Most cases of VL are reported specifically in six countries, namely, Bangladesh, Nepal, Ethiopia, India, Brazil, and the Sudan [3, 25]. Leading factors contributing to increasing cases of VL include inadequate control measures, movement of people across continents, and co-infection of HIV with VL [28, 29]. Recently, the World Health Organization (WHO) [30] reported high burden cases of VL in 14 countries, including Bangladesh, Brazil, China, Ethiopia, Georgia, India, Kenya, Nepal, Paraguay, Somalia, South Sudan, Spain, the Sudan, and Uganda. However, there is currently a reduction in the number of reported cases of the disease, which has been attributed to a decline in cases in South Asia, where reported cases dropped from ~50,000 to 6746 during 2007 to 2016. Factors that accounted for this decline include improved living conditions, successful campaigns for elimination, and natural alternating trends of prevalence. This situation currently leaves Eastern Africa as the region with the highest burden of the disease globally with Ethiopia, the Sudan, Uganda, South Sudan, and Somalia recording the most observed number of cases. Bangladesh has now been replaced by Somalia in the top six countries with cases of VL [30]. Figures 11.1 and 11.2 show the status of endemic VL and the number of cases reported between 2005 and 2019 are also reported in Table 11.1.

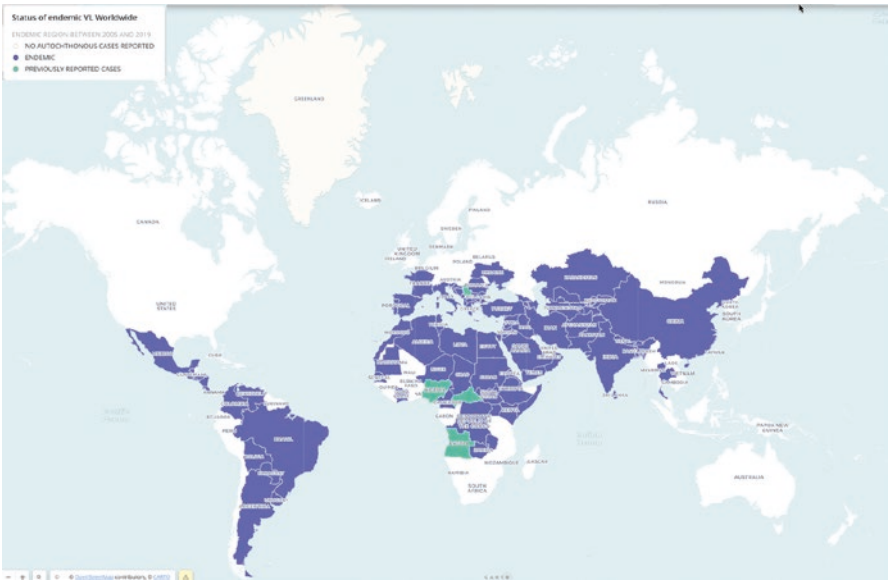

Fig. 11.1 Status of endemic VL between 2005 and 2019. (Map [31], data source [32])

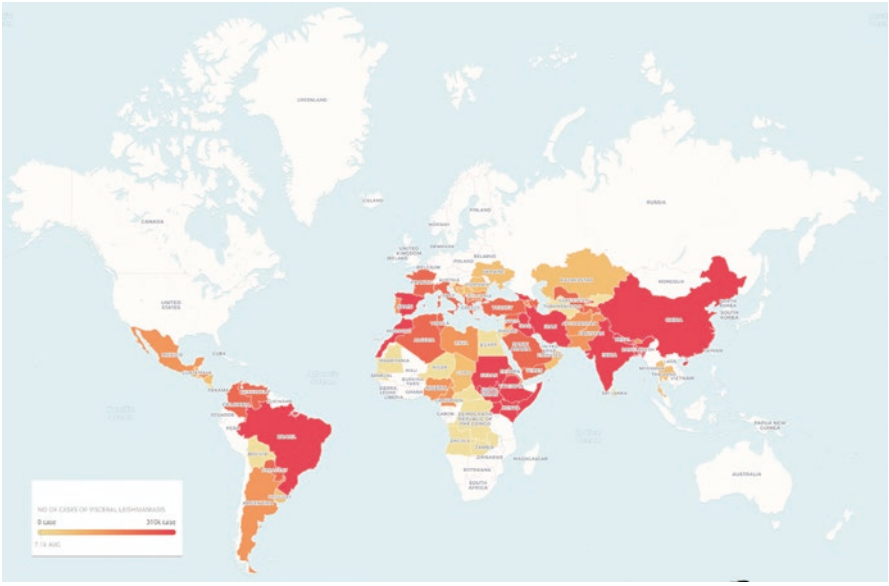

Fig. 11.2 The number of cases of VL reported between 2005 and 2019. (Map [33], data source [34])

A WHO report [35] on country-specific data on worldwide distribution of VL in 2016 recorded the following reported cases for various countries across continents and regions. Ethiopia and South Sudan recorded the highest numbers in Africa with 1593 and 4175 cases, respectively. In Southeast Asia, cases recorded included 255 for Bangladesh, 6249 for India and 242 for Nepal. In the Americas, Brazil, Paraguay, Colombia, and Venezuela reported figures of 3200, 64, 37, and 33, respectively. In the East Mediterranean, the Sudan recorded the highest number of VL cases with reported figures as 3810. European countries such as Georgia (60), Greece (57), Italy (49), Azerbaijan (44), and Uzbekistan (38), though having comparatively low figures, had the highest number of cases on the continent [35]

In Algeria, cases of VL reported from 48 provinces between 1998 and 2008 were 1562, an average of 142 cases annually, and an annual average incidence of 0.45 cases per 100,000 inhabitants, with 45 out of 48 provinces in the country reporting at least 1 case of the disease [36]. VL in Ethiopia occurs mostly in arid and semiarid regions, although recent reports suggest spread of the disease to previously non-endemic highland areas [37–40]. The estimated annual burden of the disease in this country is between 4500 and 5000 cases [37, 41, 42]. The Ministry of Health in Brazil in its 25-year notification on VL from 1990 to 2014 reported total cases of the disease at 78,444, with the northeastern region of the country accounting for ~67% of them. The annual mean number of cases in Brazil within this period was 3137 cases, an incidence of 2 cases per 100,000 inhabitants [43]. In Bangladesh, 45 out of the 64 districts in the country are endemic to VL [44]. Cases of the disease reported from 1998 to 2014 were 78,530 [45], with the disease usually affecting the

Table 11.1 Breakdown of cases of visceral leishmaniasis per country from 2005 to 2019

Country	Year															Total all years
	2019	2018	2017	2016	2015	2014	2013	2012	2011	2010	2009	2008	2007	2006	2005	
Afghanistan	20	23	14	9	8	12	16	24	21	11	nd	nd	nd	nd	nd	158
Albania	nd	45	22	15	15	33	54	53	52	60	62	75	108	117	136	847
Algeria	46	40	34	74	38	30	54	53	89	87	84	84	112	147	112	1084
Angola	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0
Argentina	9	2	9	11	8	11	6	24	15	21	18	19	17	2	0	172
Armenia	17	17	17	17	18	9	7	8	7	9	nd	14	9	5	3	157
Azerbaijan	61	40	51	44	28	6	14	22	15	32	16	35	32	23	24	443
Bangladesh	97	124	210	258	544	650	1103	1902	2874	3800	4293	4840	4932	9379	6892	41,898
Bhutan	1	3	1	nd	nd	nd	4	2	4	6	2	0	0	7	nd	30
Bolivia	1	0	0	0	0	0	0	0	0	0	nd	nd	nd	nd	nd	1
Bosnia and Herzegovina	nd	nd	0	0	0	2	1	0	0	1	1	0	2	0	1	8
Brazil	2529	3466	4103	3127	3223	3453	3253	2770	3894	3716	3693	3852	3604	3651	3597	51,931
Bulgaria	nd	1	0	3	5	12	13	2	3	4	6	2	6	6	12	75
Cameroon	28	nd	0	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	28
Central African Republic	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0
Chad	nd	2	0	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	2
China	166	180	190	321	514	292	120	218	293	402	539	529	382	294	335	4775
Colombia	11	16	29	37	21	31	13	9	11	34	54	33	54	44	66	463
Costa Rica	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0
Côte d'Ivoire	nd	nd	0	nd	nd	nd	nd	nd	nd	nd	nd	0	0	0	0	0
Croatia	0	0	nd	0	nd	nd	2	0	0	nd	1	4	4	6	7	24
Cyprus	0	2	1	1	0	nd	0	0	1	1	nd	0	0	2	nd	8

Democratic Republic of the Congo	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0
Djibouti	1	12	34	9	10	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	66
Egypt	0	0	0	0	0	0	0	0	0	nd	nd	1	nd	nd	nd	1
El Salvador	0	3	2	0	0	0	1	0	1	0	0	0	1	0	0	8
Eritrea	514	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	104	129	131	129	1007
Ethiopia	1360	1828	1490	1593	1990	2705	1732	2381	2032	1936	1083	1356	1579	2375	2585	28,025
France	nd	17	12	8	8	11	8	10	25	5	14	17	22	16	19	192
Gambia	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0
Georgia	47	52	38	49	64	55	117	106	123	141	169	171	182	174	160	1648
Greece	nd	51	83	57	64	82	75	47	41	28	28	30	48	35	48	717
Guatemala	1	4	2	2	2	0	1	0	2	0	1	1	nd	nd	nd	16
Honduras	3	8	8	7	6	2	3	0	7	7	3	3	4	9	7	77
India	2822	4360	5758	6249	8500	9241	13,851	20,572	33,155	28,382	24,213	33,598	44,533	39,173	32,803	307,210
Iran	72	67	60	61	59	26	81	94	90	91	94	125	153	139	133	1345
Iraq	170	259	172	183	427	362	575	1045	1167	1843	1549	1041	836	1434	2028	13,091
Israel	nd	1	nd	1	3	1	5	1	0	1	0	2	2	2	3	22
Italy	nd	72	nd	49	55	63	62	81	62	71	72	72	104	113	152	1028
Jordan	1	1	0	0	0	0	0	0	0	nd	nd	nd	nd	nd	nd	2
Kazakhstan	0	0	0	0	1	nd	0	0	2	nd	nd	0	0	2	0	5
Kenya	1247	891	950	692	894	880	181	457	406	nd	85	258	35	195	150	7321
Kyrgyzstan	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0
Lebanon	0	0	0	0	0	0	2	0	0	nd	0	nd	nd	nd	nd	2

(continued)

Table 11.1 (continued)

Country	Year															Total all years
	2019	2018	2017	2016	2015	2014	2013	2012	2011	2010	2009	2008	2007	2006	2005	
Libya	28	34	18	10	0	1	12	nd	nd	nd	3	3	2	nd	nd	111
Malta	0	2	5	0	nd	nd	0	3	2	nd	nd	nd	nd	nd	4	16
Mauritania	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0
Mexico	1	0	1	0	1	0	4	4	0	9	7	9	9	9	3	57
Monaco	nd	1	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	1
Montenegro	nd	nd	4	6	5	3	4	nd	nd	1	3	3	1	4	2	36
Morocco	91	106	106	92	81	85	111	113	107	139	134	163	160	170	114	1772
Nepal	185	208	244	237	217	311	325	575	886	708	824	1371	1433	1531	1463	10,518
Nicaragua	0	0	0	0	0	0	0	0	0	1	1	4	1	1	1	9
Niger	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0
Nigeria	nd	nd	0	0	0	0	0	57	0	0	0	0	1	0	2	60
Oman	0	0	nd	nd	1	0	nd	1	0	1	1	0	1	0	2	7
Pakistan	nd	nd	nd	nd	nd	7	7	14	10	nd	nd	nd	nd	nd	nd	38
Paraguay	22	19	34	64	92	118	107	76	114	144	82	54	70	66	21	1083
Portugal	nd	5	nd	nd	0	0	8	5	13	17	11	14	23	10	13	119
North Macedonia	nd	12	9	5	4	11	20	13	2	12	4	7	7	9	7	122
Romania	nd	0	1	1	1	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	3
Saudi Arabia	0	1	4	4	3	10	5	8	7	8	17	32	41	31	31	202
Senegal	0	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0
Serbia	nd	nd	0	0	0	nd	2	1	2	nd	nd	nd	nd	nd	nd	5
Slovenia	nd	0	0	0	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0
Somalia	293	413	857	734	1165	1045	936	394	290	nd	507	583	nd	nd	nd	7217
South Sudan	1013	1867	3567	4285	2840	7472	2364	5012 4353	11,862 10,468	9166	1907	582	758	1117	3141	40,079
Spain	nd	nd	196	176	nd	106	276	213	235	153	179	193	255	246	199	2427

Sri Lanka	nd	nd	nd	nd	0	0	nd	nd	nd	nd	nd	nd	nd	nd	nd	0
The Sudan	2563	2584	3894	3810	2829	3415	2389	5153	7418	6957	4880	3310	2788	1827	3713	57,530
Syrian Arab Republic	47	38	55	25	20	36	30	17	18	19	16	17	11	3	19	371
Tajikistan	15	32	23	25	26	47	63	46	25	40	53	15	14	19	14	457
Thailand	0	1	2	1	1	0	2	5	1	2	1	4	2	1	nd	23
Tunisia	27	19	23	17	30	44	38	37	55	36	55	63	100	121	120	785
Turkey	nd	nd	nd	23	23	22	33	13	30	36	23	13	41	20	32	309
Turkmenistan	0	0	0	1	0	0	0	0	0	nd	nd	nd	nd	nd	nd	1
Uganda	101	29	31	35	34	32	141	86	77	78	72	200	38	0	504	1458
Ukraine	0	nd	0	1	0	0	0	2	1	1	nd	2	3	3	1	14
Uruguay	3	1	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	4
Uzbekistan	41	34	49	38	34	42	37	38	28	25	26	13	5	1	6	417
Venezuela	23	43	40	33	37	9	7	9	15	15	15	18	17	41	58	380
Yemen	132	56	44	nd	5	15	nd	nd	0	nd	0	nd	nd	nd	nd	252
Zambia	nd	nd	0	0	0	0	0	0	0	nd	nd	nd	nd	nd	nd	0
Total per year	13,809	17,092	22,497	22,500	23,954	30,800	28,275	46,129	76,058	58,257	44,901	52,939	62,671	62,711	58,872	606,644

nd no data

poorest people living in remote rural areas in the country [46]. Reported cases of VL in Nepal are restricted mainly to 13 districts, which are located southeast of the Terai region in the country bordering the districts of Bihar state in India that has endemic disease [47]. Between 1980 and 2007, total reported cases in Nepal was 23,368 [47], with reported endemicity in poor rural areas [48]. From 1995 to 2010, reported cases of VL in Georgia was 1919 of which 1052 cases were from Tbilisi [49] where urban transmission appeared to be encouraged by the shape of the city, which is outstretched along banks of river Mtkvari, mostly in areas near forests and hills. Wild animals such as jackals and foxes frequently appear from here, facilitating synanthropic association with stray dogs and domesticated dogs [49], which are reservoirs of *Leishmania* parasite [50].

VL in India is usually a disease of the rural poor [51] and occurs generally in deprived/indigent communities living on the peripheries or suburbs of villages where more accessibility to sandfly vectors is provided [52, 53]. Most reported cases are from the state of Bihar [52]. Movement of the disease from southern parts of India occurred in the first 50 years of the C20th, with endemic reports in eastern states of Bengal, Assam, and Bihar [53]. Recent epidemiology of the disease in the country shifted from east to west, recording new foci in eastern Uttar Pradesh [54], Himachal Pradesh [55], and Uttarakhand [56], all of which have currently become endemic for the disease [57].

Regions prone to TL are Africa (especially in Tunisia, Morocco, and Ethiopia), Latin America (mostly in Colombia, Ecuador, Brazil, Venezuela, Bolivia, and Peru), the Middle East (largely in Afghanistan, Pakistan, Iran, Iraq, Syria, and Saudi Arabia), the Mediterranean Basin, and Central Asia [19, 21, 24] (Table 11.2). Approximately 95% of TL cases are reported in the Americas, Central Asia, the Mediterranean basin, and Middle East [25]. Cases of TL are mainly reported in countries such as Pakistan, Brazil, Peru, Saudi Arabia, Afghanistan, Bolivia, Tunisia, Syria, Algeria, Iran, Colombia [26], Argentina [5, 27], Costa Rica, and the Sudan [28], with an estimated one million people developing the disease annually [25, 26, 28]. Brazil accounts for 38.9% of the TL cases reported in the Americas, with cases reported in all states of the country, which shows adaptation of both parasites and vectors to human environments [29, 30]. From 1990 to 2013, the total number of cases of TL reported was 635,399, with an average incidence of 15.7 cases per 100,000 inhabitants [31]. Although, officially, cases reported annually in the country does not exceed 30,000 [28]. In the state of Amazonas, which has the highest burden of TL [32], the southern part shares more concentration of the disease with wide distribution between urban and rural areas [33]. In Panama, TL is regarded as a serious health issue and among the most ubiquitous parasitic zoonosis, with an estimate of 3000 new cases per year. There are ~60–100 cases per 100,000 inhabitants, although this number is likely to be underestimated by 50% [34], and infection is concentrated among the marginalized population [34, 35]. The disease is endemic in rural Bolivia [36] where the number of cases reported in 2006 was 33 new cases per 100,000 inhabitants [37]. TL is endemic in 7 of the country's 9 administrative departments, with 2909 cases of the disease reported in three provinces that make up the Department of La Paz [38]. Konate et al. [39] reported 2608 cases of TL from

Table 11.2 Breakdown of cases of cutaneous (tegumentary) leishmaniasis per country from 2005 to 2019

Country	Year															Total all years
	2019	2018	2017	2016	2015	2014	2013	2012	2011	2010	2009	2008	2007	2006	2005	
Afghanistan	55,225	38,407	32,065	34,912	29,392	19,065	23,621	33,894	31,293	32,145	32,937	24,585	30,319	19,689	12,752	450,301
Albania	nd	2	0	6	1	0	1	0	1	1	1	2	7	3	3	28
Algeria	10,293	10,847	13,106	10,678	7523	5423	6428	7418	11,742	10,173	10,666	8442	6764	14,379	30,227	164,109
Argentina	241	303	306	241	334	138	90	173	140	166	163	208	201	257	282	3243
Armenia	0	0	0	0	0	0	0	0	0	nd	nd	nd	nd	nd	nd	0
Azerbaijan	43	16	10	35	18	15	33	19	31	45	33	14	22	17	15	366
Belize	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0
Bhutan	1	0	2	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	3
Bolivia	2052	3127	2283	2222	2231	1683	2016	1767	1598	1440	1487	1650	3153	3152	2657	32,518
Bosnia and Herzegovina	nd	nd	0	0	0	0	0	0	0	nd	nd	nd	nd	nd	1	1
Brazil	15,484	16,432	17,528	12,690	19,395	20,418	18,226	23,547	21,395	22,397	21,989	20,123	21,530	22,397	26,685	300,236
Bulgaria	nd	0	nd	0	0	0	0	nd	nd	nd	nd	nd	nd	nd	nd	0
Burkina Faso	nd	615	712	1075	729	741	947	1134	1389	nd	nd	nd	nd	827	827	8996
Cameroon	nd	nd	51	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	51
Central African Republic	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0
Chad	82	46	8	nd	nd	nd	nd	nd	nd	nd	nd	nd	200	nd	nd	336
China	0	0	0	0	0	0	5	6	6	nd	nd	nd	nd	nd	nd	17
Colombia	5913	6362	7764	10,966	7541	11,586	9353	9757	9063	14,818	15,420	9595	13,331	16,241	18,043	165,753
Costa Rica	601	1247	2224	1148	1171	2150	1950	1453	1376	1143	2025	818	1807	1870	1676	22,659
Côte d'Ivoire	nd	nd	0	0	nd	nd	nd	0	0	0	0	0	0	0	1	1
Croatia	1	1	nd	0	4	nd	5	2	1	nd	nd	3	5	1	2	25
Cyprus	0	0	3	0	1	nd	0	5	0	nd	nd	0	0	4	nd	13

(continued)

Table 11.2 (continued)

Country	Year															Total all years
	2019	2018	2017	2016	2015	2014	2013	2012	2011	2010	2009	2008	2007	2006	2005	
Democratic Republic of the Congo	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0
Djibouti	0	0	0	0	0	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0
Dominican Republic	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0
Ecuador	1104	1237	1632	1197	1479	1175	873	1512	1385	1629	1735	1479	1185	1536	1925	21,083
Egypt	1811	1161	566	643	2243	1444	464	1260	864	318	174	471	nd	nd	nd	11,419
El Salvador	230	50	44	13	20	29	16	21	17	4	0	31	36	46	24	581
Eritrea	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0
Ethiopia	1665	882	1011	425	534	342	85	95	225	nd	nd	nd	nd	nd	nd	5264
France	nd	5	4	3 3	5	8	5	5	6	8	2	4	3	3	0	58
Gambia	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0
Georgia	0	0	0	0	1	0	5	6	6	5	3	11	1	2	5	45
Ghana	nd	nd	nd	nd	nd	nd	nd	nd	129	nd	nd	0	17	0	14	160
Greece	nd	0	1	2	2	1	1	3	2	2	7	4	6	0	2	33
Guatemala	1167	1044	775	835	562	258	664	572	549	410	519	494	287	602	1243	9981
Guinea	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0
Guinea-Bissau	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0
Guyana	19	27	21	396	132	64	4	7	15	15	9	14	6	6	7	742
Honduras	1985	1636	1854	2671	2040	1936	2074	1927	1736	1362	1502	1759	855	1300	1574	26,211
India	nd	nd	nd	nd	nd	72	172	146	139	nd	187	172	156	114	152	1310
Iran	8161	15,485	12,208	14,536	18,607	16,024	16,054	20,947	19,426	22,921	24,586	26,824	26,493	24,517	21,419	288,208
Iraq	7056	11,426	18,854	17,566	17,525	2691	1648	2486	2978	3113	2086	1250	655	1339	2435	93,108
Israel	nd	276	218	240	226	342	321	353	310	230	133	884	904	575	686	5698
Italy	nd	70	nd	47	76	80	95	26	24	22	22	23	22	36	73	616

Jordan	69	150	155	126	70	182	146	103	136	155	148	244	354	181	162	2381
Kazakhstan	55	39	131	185	24	14	14	12	24	4	9	0	0	1	7	519
Kenya	47	44	25	29	160	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	305
Kuwait	nd	4	1	7	0	2	14	4	7	12	8	nd	nd	nd	nd	59
Kyrgyzstan	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0
Lebanon	2	0	0	0	3	2	0	2	5	6	1	0	0	0	1	22
Libya	6744	2977	2815	2662	1632	516	505	1500	1327	2273	1691	1800	3884	7180	3819	41,325
Malawi	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0
Mali	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	55	55	86	77	273
Malta	0	4	1	0	nd	nd	0	0	1	11	nd	16	13	3	4	53
Mauritania	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0
Mexico	1014	576	842	447	479	418	970	567	342	456	387	284	443	431	861	8517
Monaco	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0
Montenegro	nd	nd	0	0	0	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0
Morocco	5455	11,834	6802	4903	2809	2555	2592	2877	4319	8707	6013	5128	3290	3361	3039	73,684
Namibia	nd	nd	0	0	0	0	nd	nd	nd	nd	nd	nd	nd	nd	nd	0
Nepal	16	19	4	1	0	0	0	0	0	0	0	1	0	0	0	41
Nicaragua	3321	3722	4343	5423	1925	1649	3035	1884	3146	3497	4047	5826	3719	2125	3521	51,183
Niger	nd	521	600	107	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	1228
Nigeria	nd	nd	55	nd	0	5	0	0	95	nd	nd	7	6	4	8	180
Oman	0	1	nd	nd	0	0	nd	2	0	3	4	7	6	4	8	35
Pakistan	53,574	19,361	8024	27,151	16,647	14,634	3717	6598	12,938	1387	3731	3631	4390	4151	10,441	190,375
Panama	920	1143	1164	1198	930	1581	1762	1811	3221	3221	1866	2109	2199	3774	1649	28,548
Paraguay	52	84	92	135	122	124	162	177	184	262	251	92	535	457	591	3320

(continued)

Table 11.2 (continued)

Country	Year															Total all years
	2019	2018	2017	2016	2015	2014	2013	2012	2011	2010	2009	2008	2007	2006	2005	
Peru	5349	6062	6631	7271	5459	6231	6948	6969	11,204	7612	6513	7650	10,183	8248	8067	110,397
Portugal	nd	nd	nd	0	0	0	1	1	2	nd	nd	nd	nd	nd	nd	4
North Macedonia	nd	0	0	0	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0
Saudi Arabia	1096	921	1007	1337	1490	2190	1988	1464	1951	4129	2549	2321	3286	3602	3883	33,214
Senegal	39	6	17	44	32	35	nd	nd	nd	nd	nd	9	10	8	5	205
Slovenia	nd	0	0	0	0	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	0
Spain	nd	nd	165	167	40	100	nd	18	10	10	10	16	6	6	16	564
Sri Lanka	nd	2189	980	883	1283	1367	nd	nd	nd	148	326	399	384	291	234	8484
The Sudan	nd	3299	4107	3011	3503	1053	336	206	111	752	nd	nd	nd	nd	nd	16,378
Suriname	130	118	132	255	241	390	382	594	nd	291	138	159	161	116	231	3338
Syrian Arab Republic	71,704	80,215	53,232	47,377	50,972	53,876	71,996	55,894	58,156	42,172	46,348	29,140	17,709	18,732	21,951	719,474
Tajikistan	55	40	28	60	83	128	86	26	13	3	22	38	13	nd	nd	595
Thailand	0	1	1	0	1	1	nd	nd	nd	nd	nd	nd	nd	nd	nd	4
Tunisia	7058	7467	4902	6065	6611	3368	4113	5376	5114	3811	1737	2750	2742	9030	15,373	85,517
Turkey	nd	1554	nd	1474	1986	1678	2268	1898	1803	2237	1696	1161	1499	1892	1926	23,072
Turkmenistan	105	212	182	52	49	55	59	158	81	nd	nd	89	101	69	73	1285
Ukraine	0	nd	0	0	0	0	0	2	2	0	nd	3	1	2	4	14
United States of America	nd	nd	nd	nd	nd	nd	nd	nd	nd	0	2	0	3	6	2	13
Uzbekistan	638	643	749	766	508	311	505	204	253	605	362	155	61	110	150	6020
Venezuela	2041	2612	2326	2057	2013	1661	1638	2104	1551	1952	2248	2392	2464	2553	2550	32,162
Yemen	4440	4763	4525	9120	4063	5000	3823	3629	2124	3234	1801	1090	1116	1975	2023	52,726
Total per year	277,058	261,285	217,288	234,857	214,927	184,811	192,216	202,621	213,966	199,317	197,594	165,432	166,598	177,311	203,406	3,108,687

nd no data

2006 to 2012 in the city of Ouagadougou, Burkina Faso. A recent evaluation of the disease in Ouagadougou from 2012 to 2016 by Sawadogo et al. [40] reported a total of 96 active cases across the years. Cases of TL have been reported in all regions of Colombia where a total of 102,010 cases occurred between 2007 and 2016. The Amazon region in the country recorded the highest incidence of TL cases, while the Andean region recorded the highest number of TL cases reported within this period [41].

11.4 Current Treatment Regimens for VL and TL

In treating VL, considerations are on the following: the use of specific antileishmanial drugs and vigorous management of accompanying or secondary parasitic or bacterial infections, malnourishment, anemia, and reduced blood volume [3]. Treatment options available are insufficient and of unacceptable standards, owing to issues associated with efficacy, adverse effects, proliferating drug resistance, expense, and required hospitalization for treatment to be completed [58–60]. The display of drugs for treating VL is limited to antimonials and meglumine antimonite, paromomycin, oral miltefosine, and amphotericin B, the latter having two formulations in the form of free deoxycholate and lipid. Liposomal amphotericin B is the latest formulation of this drug [3, 24, 58, 61]. Efficacy rates reported for these drugs were above 90% with 93–95%, 85%, and 90% recorded in India, East Africa, and Ethiopia, respectively [24]. Pentavalent antimonials (Sb^v) were the first-line drug for the treatment of the disease [62]. In the mid-1990s, retrogression in efficacy of the drug was reported in Bihar where 39–69% of cases treated were only successful at doses of 20 mg/kg/day given for 30 days [63]. However, the drug remained effective in other endemic countries such as Bangladesh [64] and the Sudan [65]. In the Sudan, 95% or higher cure rate was achieved with Sb^v given as 30 days regimen [65]. Pentamidine became the second-line treatment for cases of VL, especially to prevent the problem posed by resistance to Sb^v in Bihar [66]. Its efficacy has also declined over the years, with 70% efficacy reported [66, 67]. In Bihar, patients who showed resistance to Sb^v demonstrated 83% possible cure and 73% absolute cure at posttreatment of 6 months [68]; while in the Sudan, a limited number of patients resistant to Sb^v showed resistance to pentamidine [69]. Its treatment toxicity, resistance, declining efficacy, and high cost led to its abandonment in India as well as being categorized as an unsuitable alternative to pentavalent antimonials [70].

Amphotericin B (AmB) was reintroduced in India for treating resistant VL [67], and it recorded high efficacy rate of >95% when used at a regimen of 0.75–1 mg/kg, given as 15–20 intravenous injections [67, 71]. AmB recorded similar efficacy in Uganda, and it is currently adopted as a second-line drug in East Africa [72]. However, a limitation of the drug is that it is unsuitable for use in interior remote areas, lacking or with inadequate, hospital facilities [62]. Overcoming the disadvantages of AmB led to lipid formulations of AmB [67] that include AmB colloidal dispersion [ABCD (Amphocil)], liposomal AmB (AmBisome), AmB lipid complex

[ABLC (Abelcet)] [73], and Fungisome [74]. All these formulae have been tested successfully in countries such as Kenya, Brazil, and India and from continental Europe. AmBisome has been used in Ethiopia [75] and the Sudan [67, 76] under basic field conditions: it also showed 89–100% efficacy in Bihar and 96% cure rate in northeastern India [77]. AmBisome monotherapy has shown treatment failures in the Sudan [78] and in Ethiopia where lack of efficacy was reported for patients co-infected with HIV [75]. Treatment regimen with AmBisome differs from one region to another: for example, in Southern Asia, 10 or 15 mg/kg AmBisome regimens can be used, and elsewhere it is 20 mg/kg [79]. Abelcet, another lipid formulation that has been used in India, has a cure rate was 90–100% [67]. However, it showed an efficacy of 33–42% when tried on HIV-co-infected patients in Europe [80, 81]. First usage of Amphocil was in Brazil where it was reported to have an efficacy rate of 90% and 100% at 10 and 14 mg/kg doses, respectively [82]. Different regimens of Amphocil used in clinical studies at doses of 7.5, 10, and 15 mg/kg produced 96–97% cure rates in India at posttreatment of 6 months [83]. A new AmB formulation, Amphomul, was safe and greatly effective on VL patients in a small study in India involving three varying short-course dosing plans [84]. Additionally, Fungisome at 14–21 mg/kg produced a cure rate of between 90.9 and 100% in India [85], while at 10 mg/kg, a 90% cure rate was recorded in patients with the disease [86].

The alkylphospholipid derivative, miltefosine, tested on patients aged 12 years in India showed 94% cure rate after 28 days [87]. In Northern Ethiopia, only one study on the drug was conducted, with a reported cure rate of 94% initially in HIV-negative patients and 78% initially in HIV-co-infected patients [88]. Limitations of this drug include its long half-life, which encourages resistance [89], VL relapse after treatment as reported in Nepalese patients [90], and post-kala-azar dermal leishmaniasis (PKDL) development in two patients in India reported after successful treatment of the disease with the drug [68]. Others limitations include reactions such as vomiting, anorexia, nausea, and diarrhea, all of which are usually brief and resolved as treatment continues [87], and teratogenic actions in animals that make it unsuitable for pregnant women [91].

In Kenya, first successful use of paromomycin (an aminoglycoside broad-spectrum antibiotic) in treating VL was carried out in the 1980s [92]. Cure rates of 94.6% were achieved in patients with the disease in India between 2003 and 2004, using a regimen of 15 mg/kg of paromomycin administered 21 days intramuscularly [93]. Short-course treatment with the drug produced cure rates of 84.3% and 92.8% in patients with VL in India, with doses of 11 mg/kg/day for 14 and 21 days, respectively [94]. Nonetheless, usage of paromomycin as a single treatment drug can pose problems such as relapse, treatment failure, and resistance development [66]. Sitamaquine, a primaquine analogue characterized by its significant antileishmanial activity and administered orally, was developed by the Walter Reed Army Institute in collaboration with GlaxoSmithKline as WR6026 [62]. Phase II trial of WR6026 on 120 VL patients in India at doses of 1.75–2 mg/kg/day for 28 days achieved a cure rate of 89–100% [66, 95]. In Kenya, a dose of 1 mg/kg/day for 28 days achieved a 50% cure rate [95]. In Brazil, a dose of 1 mg/kg/day did not

achieve any cure, whereas 4 days at a dose of 2 mg/kg/day resulted in an efficacy rate of 67%, but an increased dose of 2.5 mg/kg/day decreased efficacy [96]. Side effects of Sitamaquine include nephritis, headache, and abdominal pains, which occur mostly in patients that received higher doses [62]. Although the last 10 years have seen improvements in new drug development for VL, there still exists the need for more novel cures that are safe, effective, and easily transported to remote places across the globe [61].

The clinical manifestations of TL and the diameter and position of the sores/lesions are factors to be considered in treatment [42]. To prevent the disease from evolving to the severe and destructive mucosal form, it is important to treat the disease adequately and timely [43]. Drugs used for treating TL include sodium stibogluconate, systemic or intralesional pentavalent antimonials, meglumine antimonials (Glucantime®) [42], N-methylglucamine antimoniate (NMG) [44], and pentamidine [45]. First-choice drugs used in treating TL are the pentavalent antimonials (Sb^v), but failures are reported in various regions of the globe. Sb^v has two formulations, namely, sodium stibogluconate and meglumine antimoniate [10]. Sb^v prevents fatty acid oxidative and glycolytic pathways in amastigotes, although the mechanism of this action remains unknown [46]. Treatment of patients with a dose of 20 mg/kg/day for 20 and 30 days achieved a cure rate of 94.2% and 7% failure in Bolivia [47]. In Brazil, a dose of 5 mg/kg/day for 30 days had a cure rate of 86% in patients, with a reported failure at 16% [48]. Patients treated in Colombia with a dose of 20 mg Sb/kg/day for 10 and 20 days showed cure rates of 61% and 67%, respectively, with drug failure reported to be 39% [49]. Significant aftereffects of the drug include arthritis, muscle pain, cardiotoxicity, and nephrosis, with the latter two occurring primarily in older patients [10]. Pentamidine has been used to treat patients with *L. (V.) guyanensis* infection in French Guyana and Marseille, France, at a dose of 4 mg/kg on days 1 and 3, with treatment failures of 5% and 25% reported, respectively. Treatment failure corresponded to the commencement of treatment, 5% failure was observed when treatment was given within 1 month of infection, and 25% failure was observed when treated was commenced later [50]. In a treatment trial in Peru for *L. (V.) braziliensis* infection, 2 mg/kg every other day for seven injections recorded a 35% cure rate and a 58% failure in patients [51]. Clinical trials that involved local treatment with various formulations of paromomycin showed cure rates of 64% in Colombia [52] and 88.6% in Guatemala, although variation in the cure rates was likely attributed to the species of *Leishmania* predominant in a particular area [53]. TL was initially treated in 2005 with miltefosine in Colombia [54]. Treatment of *L. (V.) braziliensis* TL with oral miltefosine at a dose of 2.5 mg/kg/28 days and intravenous/hypodermal antimonial at a dose of 20 mg/kg/20 days was compared in Bolivia, with cure rates reported to be 88% and 94%, respectively [55]. Treatment trial with oral miltefosine in Colombia, where *L. (V.) panamensis* is the prevalent species, showed a cure rate of 91%, which was similarly reported for antimonials [56]. An efficacy rate of 53%, which is notably lower than antimonials, was reported in Guatemala where *L. (V.) braziliensis* and *L. (L.) mexicana* [56] predominate. In Brazil, miltefosine recorded a cure rate of 71.4% for *L. (V.) guyanensis* infection treatment [57].

Liposomal amphotericin B was evaluated in Brazil in an open clinical trial with doses ranging from 17 to 37 mg/kg, administered in 7–14 days. This regimen registered a cure rate of 70% after 3 months although a drop to 65% was recorded after 4 months of treatment owing to the one reported relapse. However, doses above 30 mg/kg achieved a final cure rate of 75% [58]. Concerning azoles, a 28-day administration of oral ketoconazole at 600 mg was assessed in 120 and 8 patients in Guatemala and Belize, respectively, and recorded 30% and 25% cure rate in patients having *L. (V.) braziliensis* infection and 89% and 100% cure rates in patients with *L. (L.) mexicana* infection. Patients with *L. (V.) panamensis* infection showed similar responses to ketoconazole and antimonials [59].

A more recent study in Brazil by Carvalho and colleagues [3] described the efficacy of systemic meglumine antimoniate against TL and proposed it as a future therapeutic drug for the disease. However, they suggested that improvements in drug delivery were necessary, to improve adherence to treatment, reduce side effects, and optimize cost-efficiency.

11.5 An Introduction to Vaccine Development for VL and TL

Considering the issues associated with drugs for treating VL, scientists continue to examine preventive vaccines for the disease [97, 98]. The possibility of developing a potent vaccine is helped by the knowledge that individuals who heal and recover from active infection are protected from reinfection [3]. Developing an efficient vaccine against VL depends on producing strong T-cell immunity [99]. Current research on preventing VL infection is directed at identifying novel preventive antigens that are capable of conferring immunity to uninfected persons [100]. Possible prophylactic vaccines to be considered should contain antigens that have the potential to activate cells in healthy persons not exposed to the parasites [101, 102]. Different experimental vaccines have been tested, especially in rodent and/or dog models [100].

In the first generation of vaccines, dead parasites were inoculated [103–105] in a process called leishmanization [105]. The killed parasites were either tested alone or combined with various adjuvants [100, 105]. Alum-precipitated, autoclaved *L. major* (ALM) administered together with Bacillus Calmette-Guerin (BCG) adjuvant showed promise as VL and post kala-azar dermal (PKDL) leishmaniasis vaccines [106]. When patients with persistent PFDL were given antimonial therapy combined with alum-precipitated autoclaved *L. major* (ALM)-BCG adjuvant, there was an improvement in cure rates, and the degree of relapse was lowered compared to treatment with antimonial alone [107]. Initial studies with this vaccine received recommendations for further evaluation for their prophylactic and therapeutic actions on VL and PKDL [108].

Second-generation vaccines include genetically modified parasites or recombinant proteins that were encoded by viruses expressing leishmanial genes, while third-generation vaccines include plasmid DNA-based vaccines encoding genes

containing eukaryotic promoter vectors [103, 104]. Recently, a third-generation vaccine that used simian adenovirus (ChAd63) was shown to efficiently evoke a broad range of CD8+ T-cell specific for *Leishmania* antigens. It contains two genes of *Leishmania donovani* encoding the KMP-11 and HASPB proteins [109]. Osman et al. [109] showed that intramuscular doses of 1×10^{10} and 7.5×10^{10} ChAd63-KH into mice effectively produced IFN-γ and activated dendritic cells and were safe. However, all of these experimental vaccines have not yet progressed to human trials [100].

Other vaccines developed using molecular approaches include polyprotein and heterologous prime boost vaccines. Q protein, Leish-111f, Leish-110f, and KSAC are multiphase or polyprotein compounds/products that have shown improved defense against experimental VL [98]. Q protein contains five genetically fused antigenic determinants (Lip2a, Lip2b, H2A, and P0 proteins) and was evaluated alongside BCG or CpG-ODN in mice and dogs [110, 111]. In dogs, 90% protection was recorded with Q protein + BCG along with a potent DTH reaction, while in cats, Q protein + CpG-ODN motifs induced permanent or lasting IgG production [110, 111]. Heterologous DNA-prime protein boost has also been used successfully against VL with antigens such as ORFF, cysteine proteinases, and GP63, although they remain untested in clinical trials [98]. Against *Leishmania infantum*, 60% immunity was obtained for dogs immunized with DNA-LACK primer/VV-LACK boost [112]. Similar levels of immunity were also reported in studies by Tewary et al. and Donji et al. [113, 114] with the murine intracutaneous model for VL.

The failure to develop TL vaccines stems from the lack of knowledge of memory responses and healing mechanisms produced following infections with *Leishmania* and how to evaluate these responses [115]. The availability of genome sequences has transformed vaccine development by enabling in silico identification of $CD4^+$ and/or $CD8^+$ T-cell epitopes [116, 117]. For example, Silva et al. identified $CD4^+$ and $CD8^+$ T-cell epitopes within the proteome of *L. (Viannia) braziliensis* using an in silico approach [118]. The first generation of TL vaccines were based on live attenuated or killed parasites [119]. TL patients in Venezuela who received immunotherapy together with monthly intradermal injections of a combination vaccine that contained autoclaved promastigotes form of *L. mexicana amazonensis* [MHOM/VE/84/MEL and active BCG] recorded varying cure rates from 91.2 to 98.7%, averaging at 95.7% [120]. First-generation TL vaccines are useful for developing countries because of their low cost of production [121], although maintaining consistent quality control could be a barrier [119]. Difficulties could be experienced when conditions for culture are standardized to produce the immunogen, with parasite subculturing leading to decreases in infectivity [122, 123].

Second-generation TL vaccines consist mainly of defined products to produce immune responses [119]. Crude or purified *Leishmania* have been used to generate immune responses. Currently explored *Leishmania* vaccines include antigenic parasite proteins produced in recombinant form [124]. A plethora of *Leishmania* proteins have been purified or expressed as recombinant proteins for evaluation as potential vaccines [119]. For example, receptors for C kinase (LACK) induced

resistance to *L. major* in immunized mice [125, 126]. Immunity against *L. major* infections has been achieved using the N-terminal region of H2B histone protein and the complete protein [127]. Vaccination of monkeys with Histone HI and Montanide ISA 720 adjuvant resulted in the reduction of lesions caused by *L. major* infection with increased self-healing [128]. GP63, a *Leishmania* parasite cell surface metalloprotease and a purified protein conferred strong immunity in mice against both *L. mexicana* and *L. major* infection, but immunity in monkeys was limited [129, 130].

Third-generation TL vaccines mainly consist of genetic immunization, and their stability offers practical advantages in tropical regions [119]. The gene encoding for GP63 protein was the first reported TL DNA vaccine, and it induced robust immunity in mice against *L. major* infection [131, 132]. Immunization of BALB/c mice with the iron superoxide dismutase protein of *L. donovani* reduced *L. amazonensis* parasite burden through induction of IFN-γ [133]. *L. infantum* H2A, H2B, H3, and H4 histone gene products and the A2, KMP11, and HSP70 proteins [134] were able to control *L. major* and *L. braziliensis* infections in BALB/c mice [135, 136]. Recently, Domínguez-Bernal et al. [137] reported that a HisAK70 DNA vaccine offered cross-immunity against *L. amazonensis* infection in BALB/c mice.

11.6 Conclusions

VL and TL remain major neglected tropical diseases reported globally. Their incidence is likely to increase with climate change and vector spread and population migration. Both diseases urgently need research into new safe and affordable drugs and effective prophylactic vaccines.

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Chapter 12
The Utility of a Controlled Human Infection Model for Developing Leishmaniasis Vaccines

Paul M. Kaye, Vivak Parkash, Alison M. Layton, and Charles J. N. Lacey

Abstract Controlled human infection models (CHIMs) are increasingly recognised as having an important role in the early development of vaccines for important human diseases, including those prevalent in low and middle-income countries. The leishmaniases are a group of clinically disparate parasitic diseases caused by multiple species of *Leishmania*. Widely heralded as potentially vaccine-preventable, progress in vaccine development for different forms of leishmaniasis has over past decades been slow, hampered by lack of funds, good experimental models and the challenges of progression through the normal clinical trial pathway. However, with a new generation of leishmaniasis vaccine candidates now progressing in clinical development, the value of a robust CHIM able to accelerate early-phase evaluation of new vaccine candidates has become increasingly apparent. Here, we briefly review the historic context of human infection studies in leishmaniasis and outline issues pertinent to the development of a new CHIM of sand fly-transmitted *Leishmania major* infection. Given the diversity and wide geographic distribution of the leishmaniases, we conclude with a discussion of future needs and challenges in the development of CHIMs for these important neglected diseases.

Keywords Controlled human infection · *Leishmania* · Sand fly · Vaccines Leishmanization

12.1 Introduction

The leishmaniases are poverty-related neglected diseases with a major impact on health worldwide [1–3]. Caused by infection with one of several species of *Leishmania* parasite, disease manifestations can be broadly classified as

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M. Christodoulides (ed.), *Vaccines for Neglected Pathogens: Strategies, Achievements and Challenges*, https://doi.org/10.1007/978-3-031-24355-4_12

tegumentary (affecting the skin and mucosa) or systemic (visceral leishmaniasis, VL, affecting the internal organs). With approximately 1 M reported cases of tegumentary leishmaniasis each year and 50,000–90,000 reported cases and up to 20,000 deaths from VL, the leishmaniases rank amongst the most important of the WHO's Neglected Tropical Diseases [4]. No vaccines are currently licensed for any form of human leishmaniasis, despite licensure of four vaccines for canine leishmaniosis [5], and, with a limited drug arsenal increasingly compromised by drug resistance [6], the need for new approaches to disease control, including vaccination, remains pressing.

The tegumentary leishmaniases represent a complex spectrum of diseases, including localised cutaneous leishmaniasis, disseminated cutaneous leishmaniasis, diffuse cutaneous leishmaniasis and mucocutaneous leishmaniasis. Diverse clinical forms and different geographies reflect the distribution of distinct parasite species belonging to two sub genera, *L. (Leishmania)* in the Old and New World and *L. (Viannia*) in the New World [2]. In contrast just two species, *L. donovani* and *L. infantum* (previously called. *L. chagasi* in the New World), are primarily responsible for VL, though other species are occasionally implicated [7, 8]. In addition, the variable presence of endosymbiotic *Leishmania* viruses [9], the increasing recognition of inter-species hybrids [10–12] and recent evidence that even single nucleotide polymorphisms within a species can contribute to diverse clinical outcomes [13] all add to the complexity of these diseases and may pose challenges for vaccine development.

Ninety percent of the VL burden lies in five countries (India, Bangladesh, Nepal, Sudan and Brazil). With the reduction in cases in South Asia, associated with a trinational elimination campaign that included use of single-dose AmBisome treatment and indoor residual spraying [14], Sudan may now harbour the greatest burden of VL of any single country, with a case fatality rate of between 1.1% and 4.8% [15], in part due to the ineffectiveness of AmBisome is East Africa and the reliance on sodium stibogluconate/paromomycin combination therapy [16]. HIV co-infection, which worsens the prognosis for VL patients in all regions, brings additional challenges for patient management [17–20]. Post-kala-azar dermal leishmaniasis (PKDL) is a severe and chronic form of tegumentary leishmaniasis that usually develops after treatment for VL caused by *L. donovani* but which can occur in the absence of previous VL or concomitant with VL therapy (para-kala-azar dermal leishmaniasis) [21]. PKDL is found in South Asia and East Africa, with several thousand cases estimated to occur each year. Many PKDL patients do not receive treatment and act as reservoirs for the transmission of VL [22, 23]. A lack of tools to prevent and/or treat PKDL is now a well-recognised challenge to VL elimination campaigns [24]. Like other forms of tegumentary leishmaniasis, PKDL significantly affects quality of life and can result in life-long stigmatisation [25], a feature of these diseases that is only recently beginning to be fully appreciated in terms of its impact on measurements of morbidity associated with leishmaniasis [26].

12.1.1 Leishmaniasis: A Vaccine-Preventable Disease

The leishmaniases are commonly accepted to be potentially vaccine-preventable diseases [27–29], and recent modelling highlights the potential benefits of vaccination as an additional tool to support VL elimination efforts in South Asia [30]. A recent and timely call for action to eliminate VL from East Africa expounds the potential benefits of therapeutic vaccination as an additional treatment option [31]. However, it makes no mention of the benefits of a prophylactic vaccine, perhaps reflecting perceptions of the challenge ahead.

Compared to many eukaryotic parasites, the *Leishmania* life cycle in the mammalian host is relatively simple. Infection is initiated by the introduction of metacyclic promastigotes into the skin during the bite of an infected phlebotomine sand fly. These highly motile parasites are rapidly engulfed by myeloid cells, including neutrophils, monocytes and resident dermal macrophages, the first of these often serving as an intermediate "shuttle" into the latter [32]. Within monocytes and macrophages, metacyclic parasites differentiate into non-motile intracellular amastigotes that reside within a parasitophorous vacuole that bears the hallmarks of a phagolysosome [33]. Intracellular amastigotes replicate and invade new host cells, though the precise mechanism involved in cell-cell spread remains unclear. Uncovering how *Leishmania* spreads cell to cell within tissues may open new approaches for vaccine design. Infected cells containing amastigotes disseminate locally or to more distant sites, though again how this occurs and whether it is a pathway amenable for therapeutic intervention are not entirely clear. Recent studies have also pointed to the presence of dormant or quiescent amastigotes [34], somewhat akin to "persisters" observed in tuberculosis [35]. Besides this diversity in metabolic activity within amastigotes, there is limited parasite diversity in the mammalian life cycle, with, for example, no formal mechanisms of antigenic variation or morphologically distinguishable transmissible form equivalent to the trypomastigote of *Trypanosoma cruzi* having been discovered to date. Thus, the amastigote represents a largely invariant target for the immune system. The longevity of these infections is therefore principally attributable to the ability of amastigotes to manipulate host cell function, directly affecting the infected myeloid cell or indirectly, through the release of virulence determinants in exosomes, affecting the function of other immune cells [36].

Despite this apparent simplicity in lifestyle, leishmaniasis has proved challenging from the perspective of vaccine development. First-generation prophylactic *Leishmania* vaccines composed of whole killed (autoclaved) promastigotes often adjuvanted with Bacillus Calmette-Guérin (BCG) were not efficacious [37]. Second-generation recombinant polyprotein vaccines adjuvanted with a variety of lipid-based adjuvants have entered early-phase clinical trials (often as therapeutic vaccines) but have not been progressed [38]. A third-generation adenovirus-vectored vaccine, ChAd63-KH, has progressed to Phase II as a therapeutic in PKDL patients [39, 40] and a genetically modified attenuated live vaccine (*L. major Cen*$^{-/-}$) is

approaching Good Manufacturing Practice (GMP) and likely to enter Phase I in 2023 [28, 41]. ChAd63-KH is based on a well-characterised simian adenovirus backbone (ChAd63), extensively tested in human volunteers and shown to have an excellent safety record [42]. ChAd-vectored vaccines induce potent $CD8^+$ and $CD4^+$ T-cell responses and antibodies in humans and are amenable to scalable manufacture at GMP, as evidenced by their use in the control of the COVID-19 pandemic [43]. ChAd63-KH encodes two *Leishmania* antigens, kinetoplastid membrane protein-11, a highly conserved protein in all *Leishmania* spp., and hydrophilic acylated surface protein B, engineered to increase strain coverage [44]. ChAd63-KH therefore fulfils many of the criteria for a pan-leishmaniasis vaccine. Likewise, the *L. major* $Cen^{-/-}$ vaccine has been shown to provide protection against both cutaneous and visceral leishmaniasis in rodent models, including after sand fly challenge [45]. Current approaches to *Leishmania* vaccine antigen discovery and vaccine development are further discussed elsewhere in this book (Chaps. 11, 13 and 14).

12.1.2 The Case for Controlled Human Infection

Controlled human infection models provide the unique opportunity of monitoring the course of infection from a defined starting point to a defined endpoint (be that clinical, microbiological or immunological) [46–49]. They uniquely offer delivery of important insights into the pathogenesis of disease (including characterisation of the incubation period), the identification of early correlates of protection or disease progression and a clearer understanding of the relationship between pathogen load, immunity and transmission. For the vaccine developer, they can play a central role in increasing efficiency and robustness of candidate selection and provide for important time and cost reductions. These attributes are of relevance where target disease incidence is low, and hence there is a need for large-scale efficacy trials under conditions of natural exposure. For example, a recent feasibility study on the development of a Nipah vaccine indicated that even in an epidemic setting in Bangladesh, Phase III trials might require decades and millions of doses for completion [50]. Similarly, in the case of VL, low incidence in even the most highly endemic regions would make conventional Phase III trials almost impossible [51].

The relative ease with which CHIMs can be conducted and their small scale also favours the application of newer more efficient adaptive clinical trials, particularly when multiple candidate vaccines are available for comparison, when a range of dosing schedules or formulations need to be evaluated or when strain/species-specific efficacy needs to be demonstrated. In the context of pandemic viral infections, the use of CHIMs can also extend to the rapid evaluation of new variant-specific vaccine candidates and for the understanding of the value of public health measures [52]. This may be increasingly applicable in the face of our changing understanding of *Leishmania* genetic diversity. CHIMs may also pose risks, however, including clinical risks associated with the nature of the infectious challenge and risks related to the ethical perception of experimentation on humans. These ethical and societal

concerns are of particular importance in developing CHIMs for endemic countries [53, 54]. The risk of oversimplification in a CHIM is also ever present, supporting a view that CHIMs for neglected diseases should also be established outside centres of excellence in the Global North, where confounding factors may be more matched to those where vaccines would be deployed.

12.2 Historical Perspective of Experimental Human Infections with *Leishmania*

12.2.1 The Early Years and Leishmanization: From Community to Mass Usage

In the tradition of tropical medicine at the time, once the parasites causing leishmaniasis had been identified, studies focused on identifying the mode of parasite transmission, leading to the first study involving the deliberate inoculation of parasites into human subjects in the early 1900s. The history of these early human infection studies, and their contribution to our understanding of leishmaniasis is described in detail elsewhere [47, 55, 56]. These scientifically led experimental studies were, however, predated by a local practice in endemic countries that sought to limit the disfigurement associated with leishmaniasis.

"Leishmanization" is the named coined for the deliberate intradermal inoculation of infectious material derived from an active cutaneous leishmaniasis lesion into a healthy person to promote immunity in that individual. The historic record is unclear when the process of leishmanization began, but it had likely been practiced across the Middle East for centuries, akin to variolation for smallpox. These practices, predating Jenner's adaptation of using a heterologous agent to provide protection, clearly demonstrated the ability of live infection to produce resistance to reinfection and represent a key evidence base supporting the argument that leishmaniasis is vaccine-preventable. Though widely practiced, leishmanization was discontinued as a public health tool due to safety concerns about excessive lesion development in the immunocompromised and poor standardisation, though many millions are likely to have benefited from its use. It provides the practical and theoretical basis for human infection models developed in the modern era.

12.2.2 Using Leishmanization for Vaccine Evaluation

In 1979, Greenblatt and colleagues reported on the results of inoculating 39 soldiers in a hyperendemic area of Israel with a previously frozen isolate of *Leishmania tropica major*. All developed lesions, with 39% (12/31 examined) having ulcerated lesions and 61% (19/31) having nodular lesions at 1 month postinoculation. Further

trials of this frozen isolate in 257 soldiers (151 males, 106 females) using needle and intradermal injector delivery methods showed more variable take rates, also dependent upon storage conditions and dose, though with no obvious differences due to the sex of the participants [57].

Khamesipour and colleagues updated this approach and further expounded the use of leishmanization as a means to evaluate vaccines [58]. In a key study conducted in Iran, 23 participants were first inoculated intradermally with 5×10^5 stationary phase *L. major* (MRHO/IR/75/ER), produced under GMP from a seed bank first used for leishmanization during the Iran-Iraq war. Overall take rate was 83% (19/23) with induration around the inoculation site of 17.4 ± 10.7 mm (mean, SD; $n = 18$ measured). This excellent take rate was achieved despite only 6% of the parasite inoculum being judged as viable based on motility. Ulceration was observed in 17 participants (7.97 ± 4.4 mm diameter; $n = 18$ measured) with a mean time to onset of 66 ± 23 days ($n = 19$). The remaining two participants developed ulcerated lesions on day 105 and 150, respectively. Self-healing occurred between day 75 and day 285 with a mean duration from onset to scar of 166 ± 67.7 days ($n = 19$). Two participants had a scar of 3 cm diameter, though, in general, scars were small (8.4 ± 6.2 mm; $n = 18$). Healing was associated with leishmanin skin test (LST) conversion in 100% (11/11) of participants tested, whereas 100% (3/3) of non-takes tested remained LST negative. After 18 months, 14 participants from the original study (11 takes and 3 non-takes) were subsequently rechallenged along with 5 new participants not previously exposed. Apart from one outlier that developed a very late ulcer (day 330), four of the five new participants developed lesions of similar size and magnitude as in the first study, whereas none of the 11 previous "takes" developed a lesion after rechallenge. Only one out of three "non-takes" developed a lesion on secondary challenge. These results are in line with an expectation that first exposure conferred protective immunity and the observation that not all exposed individuals develop disease. The authors concluded that this approach may be of value for rapid vaccine evaluation, given that a clinical readout could be obtained in approximately 2 months for most individuals. In addition, the immunity induced by the process of leishmanization itself would provide enhanced protection even in vaccine-naive individuals (i.e. those receiving a placebo), providing benefit to all participants in a clinical trial.

12.3 Ethical, Regulatory and Scientific Advances to Incorporate in a Modern-Day CHIM

The ethical and regulatory framework governing CHIMs is under constant review and subject to regional differences in interpretation and practice. However, some common guidelines are emerging regarding manufacturing principles and the conduct of such studies. There is also the recognition that CHIM studies may support regulatory approval of vaccines [59]. For leishmaniasis, further evolution of the model described by Khamesipour et al. has considered many of these new

recommendation and guidelines, resulting in a CHIM that places more emphasis on the importance of vector transmission, greater quality control in terms of the challenge agent and more restrictive clinical endpoints (see below).

12.3.1 The Importance of Vector Transmission

Virulence of *Leishmania* is now recognised to be attributable to a combination of both parasite and vector (including vector microbiota-related) factors [60]. Precisely how these various factors collectively ensure high infectivity of metacyclic promastigotes and, equally surprisingly, how they may even contribute to late stages of disease remain to be unravelled. Critically, it has been observed that when tested in experimental models, at least one candidate vaccine, a killed vaccine comprised of autoclaved *L. major* antigen (ALM) + CpG oligodeoxynucleotides that showed protection against needle challenge failed to show similar protection after sand fly challenge [61]. Whilst the door remains open for development of CHIMs that use needle challenge (with potentially greater ease of use and standardisation of infectious dose), the scientific case for employing vector transmission as part of a CHIM, as for malaria [62], is at present compelling.

12.3.2 Regulatory Standards and Other Risk Mitigation

Increased awareness of the potential for human adventitious agents to contaminate challenge agents during manufacture, notably those associated with spongiform encephalopathies and HIV, has led to an increasing emphasis on understanding the provenance of challenge agents used in CHIM studies. To circumvent deficiencies in the record related to most stocks of *Leishmania* available from repositories, the identification and characterisation of new challenge strains were deemed essential for a modern-day CHIM. As such, a new strain of *L. major* (*L. major* MHOM/IL/2019/MRC-02) was sourced directly from a patient who developed cutaneous leishmaniasis after exposure during a hiking trip in an endemic region of Israel. The adult male patient was screened as negative for HIV, HTLV-1, HBV and HCV and had presented with two lesions of approximately 1.5 cm diameter on the shin and a smaller lesion on the neck. He refused treatment, and the lesions all spontaneously resolved 3–4 months later leaving a scar. The patient has been followed up for over 2 years with no recurrence of leishmaniasis or any other unexpected clinical events of note. Parasites were isolated and frozen at passage one as a seed bank, after brief culture in media certified as free of agents causing spongiform encephalopathies. This seed stock was used directly to prepare pre-GMP research banks for quality control and further analysis and the final GMP clinical bank. Minimising the number of passages in vitro was deemed to be an important factor to help maintain virulence, although to some extent any loss of virulence may also be mitigated by vector transmission.

Further risk mitigation was achieved by confirmation of parasite species identity by next-generation sequencing, confirmation of the absence of *Leishmania* viruses (LRV1), evaluation for infectivity and drug sensitivity in pre-clinical models and effective development in sand fly vectors. Sand fly colonies used for these studies (and subsequently for the CHIM) were screened to eliminate any risks associated with sand fly transmitted viral diseases [63]. Prior to exposing participants to infective sand fly bites, volunteers were also evaluated to ensure the safety and reproducibility of the sand fly biting procedure, independently of infection (the FLYBITE study; ClinicalTrials.gov Identifier NCT03999970). Included within this study was an evaluation of the public perception of the proposed CHIM protocol [64, 65], emphasising the role of public participation and involvement in the design of studies of this nature.

To establish optimum conditions for a CHIM that could be beneficial for vaccine evaluation, participants from the UK were sought. Altruism was a major factor behind volunteers agreeing to participate in these studies. Unlike the previous studies of Khamesipour et al. [58], participants were unlikely to derive any direct benefit from the procedure itself, though arguably some protection might be afforded to them if they were to travel to a leishmaniasis-endemic country. Hence, alongside development of the model itself, risk mitigation for CHIMs might also include research that seeks to ensure that vaccines evaluated using CHIMs have both a route to market and can deliver public health good within the constraints of endemic country health service. Recent "vaccine agnostic" appraisals incorporating the ability of countries to pay for leishmaniasis vaccines and the manufacturing demand are encouraging that this principle can be met [66, 67]. Benefit directly accrued for the individual will, of course, increase, if CHIMs are conducted directly in endemic areas, where the "leishmanization" effect of the challenge would be more evident.

12.4 A *Leishmania major* CHIM Initiated by Sand Fly Bite

The LEISH_CHALLENGE study (ClinicalTrials.gov Identifier NCT04512742) was approved on 8 December 2020 by the UK Health Research Authority (IRAS ID 286420) with a positive ethical opinion provided by the South Central—Hampshire a Research Ethics Committee (REC reference 20/SC/0348). Key attributes of the CHIM are shown in Fig. 12.1.

Following an adaptive study design, up to 18 participants are scheduled to be enrolled and exposed (in cohorts of 6) to the bite of five *L. major* MRC-02-infected *Phlebotomus dubosqi*. Sand flies are placed in a watch-like biting chamber which participants wear for 30 min (details of the sand fly biting procedure are provided elsewhere [64]). Clinical and immunological follow-up is expected to be at least 28 days and until a lesion diameter of approximately 6 mm is achieved. At this time,

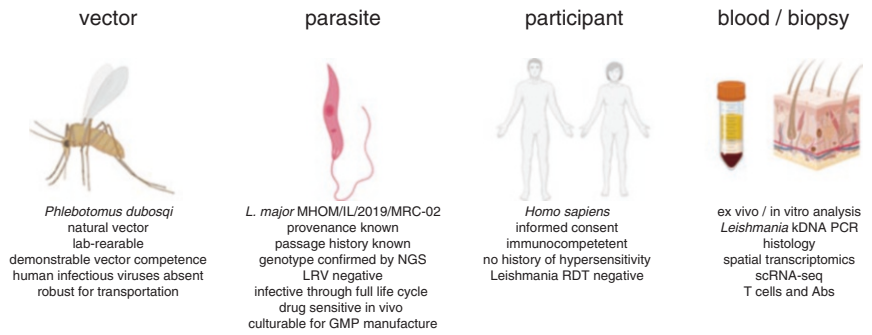

Fig. 12.1 Key components of the *Leishmania* CHIM. The figure summarises the key components of a recently developed CHIM for sand fly transmitted *Leishmania major* infection. For further details, see text. (Created with BioRender.com)

the lesion will be excised with a margin of normal looking skin under local anaesthesia, removing parasites and providing some therapeutic benefit. The biopsy site will be sutured and allowed to heal spontaneously. Recovered tissue will be used for parasitological confirmation of leishmaniasis and for immunological evaluation using conventional techniques to evaluate T-cell and antibody responses, as well as state of the art approaches in spatial and single-cell transcriptomics. If a secondary lesion develops, cryotherapy or other treatment options may be used. The first cohort of volunteers were enrolled and challenged in January 2022. Results from this study will be reported elsewhere.

One feature of this new CHIM that clearly sets it apart from the original model used by Khamesipour et al. [58] is the use of natural sand fly transmission. Apart from introducing elements from the sand fly that facilitate infectivity, the forced differentiation of the GMP parasite stock through the natural vector may serve to compensate for any loss of virulence that might occur during long-term frozen storage of parasites [57]. Another distinction is the early termination of the lesion by excision biopsy. This approach was adopted to minimise potential treatment-associated discomfort to participants whilst retaining a clinical window that should provide sufficient opportunity to evaluate the protective value of a vaccine in terms of (1) attack rate (i.e. the proportion of participants developing lesions), (2) rate of lesion development and (3) parasite load. Focus group studies of potential participants provided strong support for lesion excision as treatment [62]. A possible limitation of this approach may be that de-bulking parasites in this way may somehow limit the naturally acquired immunity induced by "leishmanization" [58]. However, this seems unlikely given that studies in the *L. major*-BALB/c mouse model suggest that low numbers of parasites may in fact have a greater ability to induce cell-mediated immune protection than that which occurs following full-blown infection [68] and the finding that two of three non-take participants in the Khamesipour study appeared to be protected against secondary challenge [58].

12.5 Accommodating Diversity: *Leishmania* CHIMs in Endemic Country Settings

12.5.1 CHIMs Using Other Leishmania Strains (Including Genetically Modified Strains)

L. major represents arguably the safest parasite species with which to conduct CHIM studies, given the focal nature of lesions and lack of reported disease reactivation following immune suppression. We have an incomplete understanding of the patterns of cross protection between species of *Leishmania* derived from rodent models and human epidemiology [27] and no evidence for or against cross protection based on human vaccine trials. In rodent models, *L. major* and *L. donovani* have been shown to induce cross species protection [69], and *L. major Cen*$^{-/-}$ cross protects against sand fly-transmitted visceral leishmaniasis due to *L. donovani* [45]. At least in the Old World, therefore, there is good reason to suspect that results from the use of a *L. major* CHIM might inform on the likely value of vaccines targeting VL or cutaneous leishmaniasis caused by *L. tropica*, as well as directly informing on vaccine protection against *L. major*.

In the New World and in the case of *L. aethiopica* in the Old World [70], patterns of disease are more challenging and diverse. At the present time, with our lack of complete understanding of the determinants or predictors of these complex manifestations and a paucity of effective treatments, it seems unlikely that the use of wild-type parasites of these species in a CHIM would be acceptable. Studies on cross protection in rodent models have been somewhat hampered by their failure to faithfully recapitulate these diverse clinical syndromes seen in humans. Nevertheless, a *L. donovani* fucose-mannose ligand vaccine cross-protected against *L. mexicana* (as well as *L. infantum*) [71] and a DNA vaccine encoding *L. amazonensis* P4 nuclease protected against both *L. amazonensis* and *L. major*, albeit with the need for different adjuvants [72]. This latter study suggests that if specific correlates of protection were available for different forms of leishmaniasis, a single-species CHIM could prove valuable in assessing how a vaccine containing conserved antigens might be tailored using different doses, routes or co-administered immune modulators to generate such species-selective responses.

CRSIPR-Cas9 allows rapid generation of unmarked strains defective in putative virulence factors or disabled in terms of replication competence [73–75]. One such strain, *L. major Cen*$^{-/-}$ [45] has been extensively assessed for its potential as a live attenuated vaccine for leishmaniasis, with impressive performance in animal models of cutaneous and visceral leishmaniasis, including those using sand fly challenge. There is clearly the potential to use this or other strains as future challenge agents, including a recently described *L. braziliensis Cen*$^{-/-}$ strain [76], though some hurdles remain. GMP manufacture and first-in-human studies of these genetically modified strains will need to be conducted to assess safety and growth characteristics in healthy subjects. Whilst there is sufficient replication in animals to promote long-lived immunity akin to leishmanization (at least in the case of the

L. major Cen$^{-/-}$ strain), lesion development in animals is muted or absent, and if this was recapitulated in humans, alternate clinical or parasitological endpoints would also be required for such strains to be used as challenge agents.

12.5.2 Sand Fly Diversity and Other Confounders

The intimate relationship between *Leishmania* and its vector is well-known, but only limited studies have directly addressed vector competence using transmission experiments and compared molecular mechanisms associated with vector-enhanced parasite infectivity. Laboratory-reared sand flies may also have their own specific and distinct microbiota, and how this may contribute to immune modulation is not known. Such considerations might be seen to argue for the development of CHIMs that are region-specific, each using naturally associated parasite-vector pairs. However, whilst this may provide the best scientific match, it is unclear at this time whether the effort would be justified. Few centres have current expertise to manage sand fly colonies suitable for the use in CHIMs. In the VL endemic state of Bihar, India, the Bill and Melinda Gates Foundation recently invested significantly to establish a suitably accredited facility for xenodiagnosis. It is unlikely and probably unnecessary for CHIMs to be established in every country with a diverse form of leishmaniasis, particularly when evidence of need is absent, and the flow of candidate vaccines limited. However, investment in regional centres, for example, covering South Asia, East Africa, the Middle East and South America, linked to sites where vaccine manufacture might take place, could make a major contribution to capacity building and the decolonisation of science.

Perhaps the more significant confounder in translating results from a CHIM conducted in the Global North is going to be in the target population. The presence of concurrent infections and differences in nutrition will have collective impact on immune health, either direct or indirect via resulting changes in the microbiota, that are not possible to mimic in populations in the Global North. Whilst baseline differences in skin structure due to ethnicity [77], age or gender may be evaluated by suitably-targeted recruitment into CHIM studies, the effects of, for example, long-term ultraviolet exposure on skin function would be harder to evaluate.

12.6 Prospects

Diversity is what makes leishmaniasis fascinating, yet challenging, from the perspective of vaccine development and the use of CHIM studies. Extending the argument that diversity matters, we would be faced with establishing CHIMs in every endemic setting where a potential vaccine might be used. More pragmatically, the view could be taken that efficacy of a vaccine or vaccines in a single-species CHIM model in the Global North would already dramatically reduce uncertainty about

vaccine potential and encourage early-phase clinical trials in different disease endemic countries linked to established correlates of protection (e.g. LST conversion or new biomarkers identified through CHIM studies). There is also precedent for CHIM studies sufficiently demonstrating efficacy to allow licensure of vaccines, and thus a larger-scale CHIM-based vaccination study could potentially result in vaccine approval [78]. Should endemic country CHIMs be required in the future, early appreciation of the challenges is essential, with an important case study from Malawi [79] paving the way for identifying the many issues needing to be considered.

Finally, in addition to vaccine development, many of the arguments for and against the use of CHIMs could also be extended to their use for the early-stage evaluation of new chemical entities or the re-purposing of immunotherapies for the treatment of leishmaniasis. Notably, the use of CHIMs could provide initial, more precise determination of pharmacokinetics and pharmacodynamics without the confounding factors associated with conducting such studies in patient populations. The development of CHIMs under such a "dual-purpose" banner may provide a greater incentive for funders than an argument based solely upon vaccine development.

Acknowledgements The authors acknowledge the contributions of the LEISH Challenge team (https://leishchallenge.org) to developing the ideas expressed in this chapter, especially Helen Ashwin, Charle Jaffe, Jovana Sadlova, Eli Schwartz, Barbora Vojtkova and Petr Volf. The development of a CHIM for leishmaniasis is funded by the UK Medical Research Council and the Department for International Development (grant No. MR/R014973/1).

PMK and CJNL are co-authors on a patent protecting the gene insert used for the development of the ChAd63-KH leishmaniasis vaccine.

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Chapter 13
Vaccines for Canine Leishmaniasis

Eduardo A. F. Coelho and Myron Christodoulides

Abstract Visceral leishmaniasis is a zoonotic disease in many countries and dogs are considered the main domestic reservoir of *Leishmania* parasites, and the presence of infected animals represents a potential risk for human disease. In this chapter, we review the state-of-the-art of canine visceral leishmaniasis (CanL) vaccines, discussing the properties and problems associated with the few currently licensed and discontinued vaccines and looking forward to the development of new, more effective vaccines. Reducing the incidence of CanL through vaccination will improve canine health and welfare and contribute to preventing human VL.

Keywords *Leishmania* · Canine · Visceral · Vaccine · Th1-immune response

13.1 Introduction

Leishmaniases are neglected tropical diseases (NTDs) that affect the poorest populations in the world, and around 380 million people distributed in 98 countries throughout Asia, Africa, the Middle East, and Central and South America are exposed to the risks of infection annually [1]. The disease complex has an annual total incidence of 1.5–2.0 million cases, split between approximately 1.0–1.5 million cases of tegumentary leishmaniasis (TL) and 0.5 million cases of visceral

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M. Christodoulides (ed.), *Vaccines for Neglected Pathogens: Strategies, Achievements and Challenges*, https://doi.org/10.1007/978-3-031-24355-4_13

leishmaniasis (VL) [1]. Risk factors for disease dissemination include poverty, population migration, malnutrition, poor hygiene, vector distribution and the status of host immunity [2]. Over 20 *Leishmania* parasite species are known, and they can be transmitted to mammals by approximately 70 different types of phlebotomine sandflies [3]. Based on the main clinical manifestations, leishmaniases are classified in two main groups: TL, which comprises the cutaneous, mucosal and cutaneous-diffuse forms of the disease, and VL, which is the most severe form of disease and may be fatal, if acute and not treated [4].

Human VL exists in zoonotic and anthroponotic forms, caused by *Leishmania infantum* and *L. donovani*, respectively [3]. The zoonotic form of VL can be found in the Mediterranean region and American continent, whereas the anthroponotic disease is found on the Asian and African continents, particularly India, Nepal, Bangladesh and East Africa (Somalia, the Sudan, South Sudan, Ethiopia). It has been suggested that, in endemic areas, there are five to ten times more cases of asymptomatic infection compared to clinically apparent VL in immunocompetent hosts [5]. The incubation period for symptomatic disease to develop can range from 10 days to 1 year, and clinical symptoms include fever, anorexia, weight loss, hepatosplenomegaly, haematological disorders, lymphadenopathy and pancytopenia [6]. Diagnosis of human VL is hampered by the variable sensitivity and/or specificity of the laboratory tests, whilst treatment is problematical due to toxicity of the drugs and their expense and the emergence of resistant parasite strains. More comprehensive information on human VL and TL epidemiology, global prevalence of diseases and their treatment is provided in Chap. 11 in this book.

In this chapter, we will focus on canine visceral leishmaniasis (CanL) and vaccine development. In countries where VL is zoonotic, dogs are considered the main domestic reservoirs of *L. infantum* parasites, and the presence of infected animals in these areas represents a potential risk for human disease. The canine disease shares similarity with human VL and provides a model to study the immunopathogenesis of *L. infantum* infection. This parasite species multiplies inside host macrophages in the liver, spleen, and bone marrow, and about 90% of infected dogs can remain asymptomatic or subclinical for years [7]. Since such animals usually do not progress to disease and remain asymptomatic, they contribute to the maintenance of the parasite's transmission cycle between sandflies and humans. In symptomatic cases, dogs develop cutaneous changes, such as alopecia, onychogryphosis and exfoliative dermatitis, which are associated with organic alterations, such as splenomegaly, lymphadenomegaly, renal azotemia and neurological disorders, amongst others [8, 9].

Control measures to prevent CanL are necessary given the high prevalence of canine infection compared to human disease. However, available control measures are not considered adequate to disrupt the spread of the disease. In this sense, health control and surveillance measures include (1) the use of chemical insecticides, (2) environmental management for vector population control and vector-human contact reduction, (3) canine serological surveys, (4) euthanasia of positive cases and timely diagnosis and (5) adequate treatment of human cases to prevent severe forms of disease and death. In addition, antileishmanial treatment for dogs and vaccine use

are strategies to reduce dog infectiousness and limit transmission of the parasite from canines to phlebotomine sand flies and to humans. However, distinct problems have limited the effectiveness of such control measures against CanL.

13.2 Diagnosis of CanL

The clinical diagnosis of CanL is facile in symptomatic dogs, due to the manifestation of cardinal signs and symptoms, but more difficult in asymptomatic animals and in those with few clinical signs known as oligosymptomatics [10]. For the latter in particular, laboratory tests are required. In order to provide sensitive and accurate diagnostic tests, parasitological, immunological and molecular tools have been developed to detect *L. infantum* in dogs. Classically, the diagnosis of CanL is performed with parasitological tests, where the direct demonstration of the parasites in samples collected from the animals is the strategy of choice. *Leishmania* amastigotes can be identified in stained smears of skin lesions, spleen, liver, bone marrow, and/or lymph node aspirates of the infected animals [11]. However, these examinations can yield false-negative results caused by the presence of low numbers of parasites in the smears and collected aspirates. Molecular techniques, such as polymerase chain reaction (PCR), have been used for diagnosis, and they are based on the *in vitro* amplification of specific-nucleotide sequences found in the parasites. CanL PCR tests show higher sensitivity and specificity to detect *Leishmania* contents compared to conventional parasitological diagnosis [12]. However, despite their high efficiency, PCR tests are expensive and require sophisticated equipment and trained professionals, which limits their use in field conditions, particularly in the least developed/low- to middle-income countries with endemic disease [13].

Serological diagnosis of CanL also has been done and is based on detecting IgG antibodies specific to *Leishmania* antigens. Laboratory tests such as enzyme-linked immunosorbent assay (ELISA), indirect fluorescent antibody test (IFAT) and direct agglutination test (DAT), amongst others, have been used for CanL diagnosis [14]. These tests are considered simpler and cheaper than parasitological methods, and sample collection is less invasive [9]. Soluble, crude and/or total antigenic extracts of *Leishmania* have been used for serodiagnosis of CanL: however, variable sensitivity and/or specificity has been observed, with false-positive results being found with sera from dogs infected by other pathogens, such as *Trypanosoma cruzi*, *Ehrlichia canis*, *Babesia canis* and *Toxoplasma gondii*, amongst others [13, 15]. In addition, asymptomatic dogs presenting low antileishmanial serology can be misdiagnosed as false negatives in serological trials [16]. New studies with modern biotechnological techniques have identified more refined and defined antigens to try and improve diagnosis of CanL, and a number of recombinant antigens have been evaluated [17]. However, further optimization is still needed to define the appropriate antigens to provide high-throughput performance for diagnosis of canine disease [18, 19].

The use of recombinant (r) parasite proteins has improved the sensitivity and specificity of the diagnostic tests. The rK39 kinesin-related protein presents immunodominant B-cell epitopes that are conserved amongst viscerotropic *Leishmania* species [20], and it has been evaluated for the serodiagnosis of CanL [21]. The rK39 antigen is suitable for detecting symptomatic disease cases, but lower sensitivity is found for detection of asymptomatic animals [22]. In Brazil, the rK39-based dipstick test has shown variable specificity when compared to conventional ELISA using parasite promastigote extracts [23]. Alternative kinesin-related proteins, such as rKLO8 and rK26, have been proposed also as capable of increasing diagnostic accuracy for CanL, and results combining such molecules have shown higher sensitivity and specificity [24]. A colloidal gold-based immunochromatographic test (ICT) based on detecting antibodies against a chimeric protein composed of B-cell epitopes from rK26 and rK39 proteins has been developed. This antigen showed potential application in screening surveys in exposed canine populations, with satisfactory diagnostic value when positive, negative and cross-reactive samples were evaluated [25].

A2 antigens were identified as a protein family of molecular weights between 45 and 100 kDa and are expressed in the amastigote stage of some *Leishmania* species, such as *L. donovani*, *L. infantum* and *L. amazonensis* [26]. The A2 gene product is composed of a variable number of repeated sequences of 10 amino acid residues [27]. Studies have shown that a rA2 antigen presents diagnostic efficacy for CanL [28, 29]. However, although ELISA assays have indicated that rA2 protein can distinguish between infected and non-infected dogs, A2 proteins have failed to distinguish between infected dogs and vaccinated and healthy animals, as well as animals infected with *Ehrlichia canis* and *Babesia canis*. This makes it difficult to use rA2 in serological trials [29, 30].

Ideally, immunological methods based on qualitative analysis using the naked eye could be developed that are user-friendly and have better field applications. Commercial kits for CanL identification have used biological fluids such as plasma, serum, whole blood or blood adsorbed onto filter paper. The specificity of the tests is high, as opposed to their sensitivity, which is variable and the main concern for clinical and epidemiological surveys [31, 32]. The use of combined antigens has been also evaluated as a method to increase the performance of CanL diagnostic tests. Magalhães et al. [33] used a mixture of three *L. infantum* recombinant protein antigens called Lci1, Lci12 and Lci13 for serodiagnosis of CanL, and results were promising. Another interesting approach is based on the use of multi-epitope chimeric proteins, in which specific B-cell epitopes are combined and assembled into a synthetic gene, leading to the production of a recombinant chimeric protein [34, 35], which can be used for lateral flow devices. Boarino et al. [36] produced a chimeric protein by fusing B-cell epitopes from K9, K39 and K26 antigens. ELISA assays showed sensitivity and specificity values of 96.0% and 99.0%, respectively, for CanL. Another chimeric protein composed of B-cell epitopes from the PQ10 and PQ20 proteins showed agreement from between 80.0% and 100% to detect asymptomatic CanL cases [19, 37].

Vale et al. [38] developed a chimeric protein based on the selection of specific B-cell epitopes of four *L. infantum* hypothetical proteins, which were previously shown to be antigenic in CanL or human VL. This chimeric protein had 100% sensitivity and 100% specificity for CanL, whilst a parasite antigenic preparation had sensitivity and specificity values of 26.0% and 96.4%, respectively. Thus, promising antigenic targets have been developed independently by several research groups, and these new candidates for serodiagnosis of CanL can help to identify infected dogs and to adopt effective control measures against the spread of disease.

13.3 Treatment of CanL

Therapeutic candidates have been developed for many years to try and treat CanL. However, their use requires administering veterinarians and problems associated with the parenteral administration of compounds include incomplete parasite clearance and undesirable toxic side effects [39, 40]. Treatment is also characterized by high rates of relapse, regardless of the antileishmanial drugs used and whether it is a single drug or combined drug therapy [41].

Miltefosine was originally developed as an anticancer agent in the 1990s and was first recorded for VL treatment in 2002 in India [42]. In 2016, the Brazilian Ministry of Health and the Ministry of Agriculture Livestock and Supply approved the registration of Milteforan® (Virbac, Brazil). Although there was a notable improvement in clinical symptoms when using this drug, it was not accompanied by parasitological clearance, suggesting that miltefosine treatment should not be recommended [43]. More recently, miltefosine treatment of *L. infantum*-infected dogs revealed clinical improvement of CanL with a reduction in parasite infectivity [44].

Allopurinol has parasitostatic activity, and its long-term administration maintains low parasite load, which contributes to preventing canine relapse [45]. Combination of allopurinol with miltefosine showed the most promise for CanL treatment [46]. However, induced resistance is also a problem associated with the use of allopurinol [47]. In most parts of the world, meglumine antimoniate is the most commonly used treatment for visceral leishmaniasis, and a combination of meglumine antimoniate and allopurinol is considered the most effective therapy for CanL [48]. However, treatment with the same human-used drugs is not recommended since it may induce parasite resistance and hamper human treatment [49]. Table 13.1 shows the drugs currently used to treat CanL.

The great challenges for CanL treatment are to identity a drug that (1) is not used to treat humans, (2) does not induce kidney damage or any other adverse effect, (3) controls parasite load, (4) interferes in the sandflies' life cycle and (5) blocks parasite transmission. In this context, other treatment options should be studied, such as immunotherapy, to improve CanL treatment efficacy. Immunotherapy involves using compounds that can modulate host immune responses, aiming to achieve prophylactic and/or therapeutic efficacy [58–60]. These agents exert their effects by

Table 13.1 Drugs used currently for treating CanL

Drug	Dose	Duration	Main side effects	Reference
Meglumine antimoniate	100 mg/kg	3–4 weeks	Nephrotoxicity	[50]
Sodium stibogluconate	50 mg/kg	3–4 weeks	Nephrotoxicity	[51]
Miltefosine	2 mg/kg	1 month	Digestive disorders	[43, 52]
Amphotericin B deoxycholate (AmBisome, liposomal)	0.5 mg/kg	2 months	Nephrotoxicity	[53]
Meglumine antimoniate + allopurinol	100 mg/kg + 10 mg/kg	2 months to 1 year	Nephrotoxicity and urolithiasis	[45]
Miltefosine + allopurinol	2 mg/kg + 10 mg/kg	1 month to 1 year	Digestive disorders	[54]
Allopurinol	20 mg/kg	Lifelong	Urolithiasis	[55]
Paromomycin	15–20 mg/kg	3 weeks	Nephrotoxicity	[56]

Adapted from Reguera et al. [57]

augmenting the host's natural defences, restoring any impaired effector functions and stimulating a protective response against infection that results in parasitism control [61, 62]. The different protocols used for immunotherapy or immunochemotherapy generally improve clinical signs, with a possibility to further reduce the parasite burden by activating the immune system against *Leishmania* infection. Taken together, these results showed that immunotherapy is a promising strategy for treating CanL. However, parasite clearance was not completed reached, irrespective of the treatment, and this is the strongest negative aspect of such studies. The search for new immunotherapeutic targets to improve these types of treatment is of great interest, given that the aims are to improve parasite control and develop approaches to blocking CanL transmission.

13.4 Immune Responses in CanL

Immunoprophylaxis has been considered as a strategy for preventing canine disease, and efforts have been made to deduce the immune response profile generated in CanL. Immune control of *L. infantum* infection requires a balance between inflammatory and regulatory actions, and protective immunity is dependent on the development of an antigen- and parasite-specific Th1-type cellular immune response, primed by the production of cytokines, such as IFN-γ, IL-12, GM-CSF and others, by T cells [63, 64]. Conversely, Th2-type cells and T regulatory cells act to promote disease, since they produce anti-inflammatory cytokines that lead to inhibition of the Th1-type response, thus contributing to the deactivation of infected phagocytes and, consequently, loss of control of the inflammatory process and/or the development of active disease [65].

In symptomatic CanL, immunological changes involving T cells include the absence of delayed-type hypersensitivity reactions to parasite antigens, the

reduction of T-cell numbers in the peripheral blood and a lower production of cytokines, such as IFN-γ and IL-2, by peripheral blood mononuclear cells (PBMC) of the infected animals [66]. For protection against infection, effector mechanisms associated with the activation of macrophages by IFN-γ, IL-12 and TNF-α cytokines to kill intracellular amastigotes via the L-arginine nitric oxide (NO) pathway have been described [67]. NO production and antileishmanial activity were detected in canine macrophage cell lines infected with *L. infantum* after incubation with these cytokines, as well as by macrophages from dogs immunized with killed parasite promastigotes [18]. The role of IL-12 cytokine in inducing the Th1-type response profile has also been described, and this molecule augments the production of IFN-γ by PBMC from dogs with experimental and natural symptomatic disease [68]. IL-12 was also detected in lymph node cells from dogs protected against *L. infantum* after immunization with DNA and vaccinia recombinant vectors expressing *Leishmania* homologue of activated C kinase protein [69]. In asymptomatic CanL, IL-12 and IFN-γ cytokines predominate and indicate that the Th1-type cellular profile is required for protection against active disease [70].

Regarding the humoral response, the production of antibodies specific to *Leishmania* antigens is found in dogs with symptomatic CanL [18]. Antibodies are required for parasite opsonization and phagocytosis by macrophages in infected animals [71]. In asymptomatic or subclinical infection, such processes occur at lower levels and can allow continued propagation of the parasites. As the parasite continues to propagate, there is an increase in the levels of circulating IgG antibodies, which can bind to *Leishmania* antigens leading to the formation of immune complexes, which trigger the production of IL-10 by infected macrophages [72]. There is concomitant hypergammaglobulinemia, followed by the deposition of the immune complexes that cause renal damage and, later on, renal failure, which is one of the most common causes of death in dogs caused by active disease [73]. Regarding antibody isotypes, IgG1 and IgG2 subclasses have been used as indicators for CanL, since a direct correlation between high levels of IgG1 anti-*Leishmania* antibodies and the appearance of clinical symptoms has been demonstrated in *L. infantum*-infected dogs. By contrast, IgG2 isotype antibodies are associated with asymptomatic infection and the predominance of a Th1-type response in dogs [74].

13.5 CanL Vaccines

The evidence of lifelong immunity against *Leishmania* infection has stimulated the development of prophylactic vaccination against leishmaniasis. With knowledge gained of the immune response mechanisms to protect against the parasite, an ideal CanL vaccine candidate should have the following attributes: it should (1) be safe and affordable to the hosts; (2) induce both $CD4^+$ and $CD8^+$ T-cell responses and long-term immunological memory, which could be boosted by natural infections, thereby reducing the number of repeat vaccine doses required for protection; and (3) be easy to produce and be stable at room temperature or at 4 °C, thus eliminating the

need for storage at −20 °C to −80 °C [75]. Advances in recombinant technology have enabled many parasite proteins to be tested with different immune adjuvants, as vaccine candidates against VL. These proteins have showed variable success in mammalian models, which was dependent on the vaccine formulation, the choice of adjuvant and the animal model used for testing [76–81].

13.5.1 Leish-Tec® Vaccine

An amastigote stage-specific gene family from *Leishmania* called A2 was identified in some parasite species. The A2 gene family encodes for at least seven members, and the proteins have molecular weights (*Mr*) ranging from 45 to 100 kDa. These proteins are composed mainly of a repetitive amino acid sequence, with each repeat encoding for a stretch of ten amino acid residues that share partial identity with the S antigens of the *Plasmodium falciparum* V1 strain, which causes malaria in man [82, 83]. The A2 protein has emerged as an effective vaccine candidate, and when used as a recombinant protein with saponin as adjuvant, it protected mice against *L. infantum* infection [84–86]. A2-induced protective immunity was associated with the production of high levels of IFN-γ cytokine that were produced by T cells and low levels of IL-10 cytokine. Immunity was also characterized by elevated levels of A2 protein- and parasite-specific IgG2a antibodies, which contributed to significant reductions in the parasite load in organs of vaccinated mice, compared to unvaccinated controls. Based on this success in the murine model, the A2 antigen was formulated with saponin to produce a CanL vaccine called Leish-Tec® (Ceva Laboratórios Ltda., Brazil). Leish-Tec® is the CanL vaccine currently licensed for use in Brazil, and it was shown to induce protective immunity in beagle dogs challenged intravenously with high doses of live *L. infantum* parasites, as well reducing the infectiousness of the animals to sandflies, when xenodiagnoses were performed [87, 88]. In addition, the vaccine did not seroconvert vaccinated animals, an important requirement for CanL vaccines, since euthanasia of seropositive dogs is recommended by the public health authorities in Brazil [89].

The study done between 2004 and 2008 in Porteirinha, Minas Gerais, an area of Brazil with endemic CanL, showed that the efficacy of Leish-Tec® in protecting exposed and vaccinated dogs was 71.4% (Confidence Interval (CI) 34.9–87.3%), when compared to unvaccinated and control animals [88]. Vaccination also led to reduced parasite transmission between dogs and humans. Moreover, if only vaccinated animals were considered, protection levels reached 96.4%, according to parasitological criteria adopted in the study. Vaccine efficacy amongst dogs that responded to vaccination with increased anti-A2 antibody levels was higher at 80.8% than the reported overall efficacy of 71.4%. In the study from Pereira et al. [90], seropositivity for anti-A2 IgG antibodies was detected in 98.0% of the vaccinated dogs, and this decreased to 81.13% at 6 months after vaccination but rising

again to 98.0% after a vaccination booster. The anti-A2 IgG2/IgG1 ratio in the dogs was found to be higher than 1.0, suggesting the predominance of a Th1-type response in Leish-Tec®-vaccinated dogs.

Xenodiagnosis is an important tool for evaluating the potential for transmission from a vaccinated animal, although it is limited by low sensitivity and specificity in field conditions. In Leish-Tec®-vaccinated dogs, vaccine efficacy remained significantly high at 58.1% (95% CI: 26.0–76.3%), and vaccination induced a 46.6% reduction in parasite transmission to the sandflies that were feeding on anti-A2 seropositive vaccinated dogs [87]. Only 5.4% of animals vaccinated with Leish-Tec® were infectious to sandflies, as compared to a positive rate of 36.6% amongst control and unvaccinated dogs. Therefore, Leish-Tec® induced an appreciable reduction in *Leishmania* transmission to vectors and was protective against *L. infantum* infection. Thus, this vaccine shows promising protective effects but needs to be further optimized to be more effective in dogs under field conditions. Finally, it should be noted that imperfect vaccines pose a threat because they are not completely sterilizing, and they allow more parasite virulent strains to survive and transmit. If the evolved parasite strains then infect unvaccinated dogs, they will be more virulent than the strains that circulated before the vaccine was used [91].

13.5.2 Leishmune® Vaccine

Leishmania nucleoside hydrolases (NH) proteins are associated with the synthesis of parasite DNA and replication and relevant for early infection of mammalian hosts [92]. The *L. donovani* NH antigen has a *Mr* of 36 kDa (NH36), and there is high homology of NH proteins from distinct *Leishmania* species. NH36 was specifically recognized by sera of VL patients [93], and when used as an immunogen in murine models, it protected mice against infection with *L. infantum* [94], *L. Mexicana* [95], *L. amazonensis* [96] and *L. major* [97]. It was also found to protect dogs against infection with *L. infantum* [98].

Leishmune® (Fort Dodge Saúde Animal, Brazil) was then developed as a CanL vaccine based on the NH36 antigen. It is considered a second-generation vaccine, and it was licensed for prophylaxis against CanL in Brazil between 2004 and 2013 [75]. In a study with 550 Leishmune®-vaccinated dogs that were exposed to an endemic area of VL, only 1.0% ($n = 5$) of the animals died of disease, and 1.2% ($n = 6$) were symptomatic [99]. By contrast, there were 39.0% deaths within the untreated control group, and 20.6% of the dogs were symptomatic. The vaccine was prophylactic against CanL and protected 98.0% of vaccinated dogs and also reduced the parasite burden accessible for transmission to sandflies. The anti-fucose mannose ligand (FML) antibody response induced by the vaccine was mainly of the IgG2 subtype. Up to October 2011, a total of 150,000 healthy dogs were vaccinated in Brazil, and it was also observed that Leishmune® formulated with twice the

concentration of saponin adjuvant had a therapeutic effect against naturally or experimentally acquired CanL [100].

Immunotherapy with saponin-enriched Leishmune® reduced not only CanL symptoms but also the rate of deaths and the parasite load in lymph nodes. Augmented immunochemotherapy using a combination of Leishmune®, allopurinol and amphotericin B promoted a sterile cure with negative PCR reactions for *Leishmania* DNA in dogs [101]. Leishmune® induced an immunological pattern characterized by enhanced levels of IFN-γ, NO and anti-*L. infantum* IgG2 antibody and an increased CD8+ T-cell response. The vaccine also induced early phenotypic changes in neutrophils and monocytes with increase in MHC II class and decrease in CD32+ and CD18+ activation markers, CD8+ T-cell activation and a selective pro-inflammatory response pattern. Sustained or increased proportions of CD4+ and CD21 B cells and increased proportions of CD8+ T cells and the diminished CD4+/CD25+ T cells have also been reported in dogs [102]. However, unlike the Leish-Tec® vaccine, Leishmune® induces a strong humoral response in vaccinated dogs, and this hampers the serological discrimination of infected animals. This fact made the public health authorities in Brazil discontinue Leishmune® as a prophylactic agent from 2014.

13.5.3 CaniLeish® Vaccine

The first vaccine registered in Europe against CanL was LiESP/QA-21 vaccine (CaniLeish®, Virbac, France). It is composed of purified excreted-secreted proteins of *L. infantum* (LiESP) produced by means of a patented cell-free, serum-free culture system invented by the Institut de Recherche pour le Développement (IRD), and adjuvanted with QA-21, a highly purified fraction of the *Quillaja saponaria* saponin [103]. The vaccine protocol is three doses, with each dose containing 100 μg LiESP and 60 μg QA-21, given subcutaneously with 21-day intervals and with a booster dose after 1 year to complete the immunization schedule. Leish-Tec® and Leishmune® share similar vaccinations schedules.

In a randomized clinical trial of healthy beagle dogs vaccinated with CaniLeish® and later challenged with live *L. infantum*, the vaccine induced the development of a strong Th1-type immune response in the vaccinated dogs that was associated with an increase in anti-parasite IgG2 levels, which were protective against infection [104]. Similar results were found in a double-blinded controlled study under field conditions, where a 2-year follow-up showed that CaniLeish® prevented infection in 68.4% of vaccinated dogs, and, in those animals developing active CanL, disease progression was lower and generally less severe than that observed in unvaccinated dogs [105, 106]. In addition, the number of parasites isolated from sandfly midguts feeding on vaccinated dogs was significantly reduced compared to unvaccinated dogs, thereby preventing vector transmission of infection.

13.5.4 LetiFend® Vaccine

Studies have shown that an immune response directed against *Leishmania* internal antigens may play a role in controlling disease [107]. *L. infantum* ribosomal protein extracts have been shown to induce protection against experimental infection in mice [77, 108, 109]. These proteins were recognized by antibodies in sera from mice [110], dogs [111] and humans [112] developing VL. The antigenicity of *Leishmania* nucleosomal histones proteins has been shown in CanL, and antibodies in sera from dogs developing CanL recognized specific B-cell epitopes from parasite ribosomal proteins LiP2A, LiP2B and LiP0, and from histone protein H2A. Based on these observations, a chimeric protein called protein Q, composed of five B-cell epitopes derived from these four *Leishmania* proteins, was developed and licensed as LetiFend® (Laboratorios LETI, Spain) in 2016 [113].

The LetiFend® vaccination protocol consists of one dose, followed by annual boosters, and the product should only be administered to dogs aged 6 months or older. The LetiFend® pre-licensing phase III trial included 275 vaccinated dogs and 274 controls dogs, which were exposed to natural infection in two CanL endemic areas located in France and Spain during a 2-year period [113]. These were privately owned dogs of different breeds and ages and kept outdoors in 19 dog kennels. Measurements of the humoral responses to vaccine and SLA, detection of parasite in lymph nodes and clinical evaluation of the animals were done at pre-determined time points. A case of confirmed CanL was defined as any dog presenting clinical signals compatible with CanL, combined with a positive serology to *L. infantum* antigen and the presence of parasite in the collected samples. Results showed that 4.7% of vaccinated dogs and 10.2% of control dogs developed CanL, and this difference was statistically significant. Only two study sites were selected to perform the analysis of vaccine efficacy due to an unexpectedly low incidence of infection in some dog kennels. According to the results of this field study, LetiFend® showed 72% efficacy in preventing CanL in vaccinated dogs [113]. No adverse effects were observed after vaccine administration during either laboratory or field studies [113, 114]. Furthermore, vaccination with LetiFend® in this field trial did not appear to elicit false-positive results in *L. infantum* serological diagnostic tests [115].

In another study using a large-scale dog population of different breeds and ages over a 24-month period, vaccination with LetiFend® reduced clinical signs related to the progression of CanL [116]. Data from this study also established a direct relationship between disease severity and circulating immune complexes (CIC) levels. Vaccinated dogs presented significantly lower CIC levels than the control unvaccinated group, which correlated to a lower parasitic load. Moreover, because of vaccination, changes in the protein composition of CIC were detected, including a significant increase in complement system proteins in the vaccinated animals. It was hypothesized that the higher amount of these proteins could be related to activation of this major player in innate immunity, followed by elimination of extracellular parasites. The vaccine induced specific IgG2a isotype antibodies in the vaccinated animals, which correlated with reduction in organ parasitism.

Table 13.2 Side-by-side comparison of licensed CanL vaccines

Vaccine	Composition	Level of protection	Mammalian target	Reference
Leish-Tec®	A2 protein + saponin	96.4%	Dog	[88]
Leishmune®	NH36 protein + saponin	98.0%	Dog	[99]
CaniLeish®	*L. infantum* Excreted/secreted Protein + saponin QA-21	92.7%	Dog	[105, 106]
LetiFend®	Q chimeric Protein	98%	Dog	[113]

Adapted from Moreno [118]

An important confounding factor for CanL control is the possible interference of vaccination schedules in diagnosing *Leishmania* infection. The impact of vaccination on the ability of common diagnostic methods to detect infection must be assessed prior to licensure of any new CanL vaccines. Difficulties in diagnosing CanL have been reported for dogs vaccinated with Leishmune® and CaniLeish® in Brazil [117] and Europe [105]. The negative impact of CanL vaccination on diagnosis and control of *Leishmania* infection is expected to be higher whenever vaccines with only low to moderate efficacy are widely implemented in endemic areas. In such cases, a significant proportion of vaccinated and potentially infected dogs would be expected, which, if left undetected, could represent an important reservoir of the parasite, indirectly inducing a rise in the incidence of infection both in vaccinated and unvaccinated animals. Table 13.2 shows the main CanL vaccines currently available to protect against parasite infection.

13.5.5 New Unlicensed Vaccine Candidates for CanL

Biotechnological tools have been used to try and identify new candidates for inclusion in vaccines for mammalian VL. For example, an immunoproteomics approach has led to the identification of several antigenic *Leishmania* proteins that have been evaluated for their biological function, as well as their performance as diagnostic markers, vaccine candidates and/or as potential drug targets [119–121]. In a previous immunoproteomic study, several parasite proteins, including known and hypothetical antigens, which were expressed in the amastigote and promastigote extracts, were identified by antibodies in sera from dogs developing CanL, and some of them were individually evaluated in ELISA for diagnosis of CanL [122]. The use of such candidates as vaccines was also postulated, due to the existence of specific $CD4^+$ and $CD8^+$ T-cell epitopes in their amino acid sequences, as well as by conservation amongst *Leishmania* species, but not in other *Trypanosomatidae* or mammalian hosts [122].

In this context, one of the hypothetical proteins identified in the cited study, namely, LiHyp1, which belongs to the super-oxygenase family in *Leishmania*, was evaluated as a vaccine candidate against VL. Immunization of BALB/c mice with recombinant LiHyp1 protein plus saponin adjuvant induced a Th1 immune response in the vaccinated animals, which was primed by protein- and parasite-specific IFN-γ, IL-12 and

GM-CSF cytokine production, combined with the presence of low levels of IL-4 and IL-10 [123]. Animals subsequently infected with *Leishmania* parasites displayed significant reductions in the parasite load in their liver, spleen, bone marrow and draining lymph nodes, when compared to control unvaccinated groups. Protection was correlated with a parasite-specific and dependent IFN-γ production, mainly due to $CD4^+$ T cells, which proved to be the major source of this cytokine in these animals. Similar immune and protection profiles were found with other hypothetical proteins, such as LiHyD [124], LiHyT [125], LiHyp6 [126] and LiHyV [127].

The use of chimeric protein-based vaccines containing polypeptides could provide benefits in terms of a more robust protective response against *Leishmania* infection [128–130]. In a recent study, MHC class I and II molecule-specific peptide epitopes were predicted within the amino acid sequences of three *Leishmania* proteins—one hypothetical, prohibitin and small glutamine-rich tetratricopeptide repeat-containing protein—and the information used to produce a chimeric protein. Immunization of mice with this chimeric protein plus adjuvant significantly lowered the parasite burden in internal organs, accompanied by increased levels of IFN-γ, IL-2, IL-12 and GM-CSF cytokines and IgG2a isotype antibody. In addition, the $CD4^+$ and $CD8^+$ T-cell subtypes contributed to IFN-γ production in the protected animals [30].

A chimeric protein called ChimeraT, containing specific T-cell epitopes from prohibitin, eukaryotic initiation factor 5a, LiHyp1 and LiHyp2 proteins, was constructed and evaluated in mice as a vaccine with saponin adjuvant or incorporation into a liposome [131, 132]. Both vaccine formulations significantly reduced the parasite load in mouse internal organs and stimulated significantly higher levels of IFN-γ, IL-12 and GM-CSF cytokines by both $CD4^+$ and $CD8^+$ T cells, with correspondingly low levels of IL-4 and IL-10 production. In addition, antibodies were predominantly IgG2a isotype, and homologous antigen-stimulated spleen cells produced significant nitrite (as a proxy for nitric oxide).

Other vaccine candidates against leishmaniasis have been also tested against VL. The parasite surface antigen-2 (PSA-2) comprises three polypeptides with *Mr* ranging from 50.0 to 96.0 kDa [133]. The immunogen was mixed with *Corynebacterium parvum* bacteria as the adjuvant, and this mixture induced protection in mice against *Leishmania* challenge via a Th1-type immune response [134]. The kinetoplastid membrane protein-11 (KMP-11), which is a highly conserved protein in distinct *Leishmania* spp., was evaluated as a vaccine candidate in hamsters against *L. donovani* infection [135]. The NH36 antigen was also shown to be protective in BALB/c mice against infection with *L. infantum, L. mexicana* or *L. amazonensis*, demonstrating the potential of this antigen as a heterologous vaccine against different parasite species [94].

In a previous study, KSAC was shown to be immunogenic and effective in inducing protection in mice against *L. infantum*. This chimeric protein, which is composed of the *Leishmania* homolog receptors for activated C kinase (LACK), glycoprotein 63 kDa (gp63), thiol-specific antioxidant (TSA), hydrophilic acylated surface protein B (HASPB), sterol 24-*c*-methyltransferase (SMT) and KMP-11, A2 and CPB proteins, induced protective responses in the vaccinated animals, which were associated with high production of IFN-γ combined with low levels of IL-4 and a decreased antileishmanial IgG1 response [130]. Another chimeric protein,

Leish-111f, which is a combination of thiol-specific antioxidant (TSA), stress-inducible protein 1 (LmSTI-1) and the homolog of the eukaryotic translation initiation factor (eIF4A) proteins, when associated with immune adjuvants, was also able to protect BALB/c mice against *Leishmania* infection [136].

Another field that could be developed in relation to the discovery of new vaccine candidates against VL will be based on vector salivary proteins [137]. To date, evidence suggests that vector salivary molecules able to induce a Th1-type response in immunized animals could create a protective immunological environment at the bite site. This environment could have an impact when the parasites are injected, allowing the disease to be controlled and promoting a concomitant *Leishmania*-specific immunity [138]. The Th1-type immunological environment developed at the bite site to these antigens could promote a protective response against the parasite challenge. In this context, PdSP15, a 15 kDa salivary protein member of the family of small odorant binding proteins from *Phlebotomus duboscqi*, was evaluated as a vaccine candidate against leishmaniasis in a non-human primate [139]. Also, LJM19, an 11 kDa salivary protein with unknown function, and LJL143, a 38 kDa salivary protein with anticoagulant activity [140], both present in the saliva of *Lutzomyia longipalpis*, were shown to be protective against VL [141]. A summary of some of the single recombinant protein or polyprotein-based vaccine candidates for VL is shown in Table 13.3. Thus, distinct vaccine candidates have been identified and

Table 13.3 Recombinant proteins evaluated as vaccine candidates against visceral leishmaniasis

Candidate	Infective species	Model	Remarks	Reference
Aldolase	*L. donovani*	Hamster	Partial protection	[142]
Cysteine peptidases	*L. infantum*	Beagle dog	No protection	[143]
Cysteine proteinase III	*L. infantum*	BALB/c mice	Partial protection	[144]
Cyclophilin 1	*L. infantum*	BALB/c mice	High protection	[145]
dp72	*L. infantum*	BALB/c mice	Partial protection	[146]
eIF2	*L. donovani*	Hamster	65% protection	[147]
HASPB1	*L. donovani*	BALB/c mice	70–90% protection	[148]
LCR1	*L. infantum*	BALB/c mice	Partial protection	[149]
LdSir2HP	*L. donovani*	Hamster	High protection	[150]
Leish H1	*L. infantum*	BALB/c mice	High protection	[151]
L3 and L5	*L. infantum*	BALB/c mice	High protection	[152]
NH36	*L. infantum*	BALB/c mice	80% protection	[94]
ORFF	*L. donovani*	BALB/c mice	Partial protection	[153]
A2/CPA/CPB[a]	*L. infantum*	BALB/c mice	High protection	[154]
KSAC[a]	*L. infantum*	C57BL/6 mice	High protection	[130]
Leish-111f[a]	*L. infantum*	Beagle dog	No protection	[129]
NS protein[a]	*L. donovani*	BALB/c mice	High protection	[155]
Q protein[a]	*L. infantum*	Beagle dog	90% protection	[128]
8E/p21/SMT[a]	*L. donovani*	C57BL/6 mice	High protection	[156]

Adapted from Duarte et al. [137]
[a]Indicate polyprotein-based or chimeric vaccines

tested in animal models to develop new and effective vaccines to protect against *Leishmania* infection in both dogs and humans. Further studies are certainly necessary to develop these experimental vaccines for phase I studies.

13.6 Conclusions

CanL is a serious disease that afflicts canids, causing death if untreated. Infected dogs can be a focus of transmission via vector sandflies to other dogs and humans, in countries where VL is a zoonosis. Preventing new infections in dogs can help to stop the current increase of VL in humans. In this context, effective prophylactic measures to control CanL are imperative, and vaccines are probably the most economical way to control neglected tropical diseases. As described in this chapter, there are some licensed vaccines available for use in countries where CanL is prevalent. However, these vaccines are still considered to be suboptimal, and their efficacy data are unreliable, which is due mainly to the lack of study design standardization, methodological shortcomings and substantial differences found in the characteristics of the study dog populations. All these shortcomings preclude authoritative comparisons between the licensed vaccines. Additional studies are required to prove the efficacy of these vaccines and the new CanL vaccines that are in pre-clinical development.

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Chapter 14
Vaccine Development for Human Leishmaniasis

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Abstract The development of vaccines for human leishmaniasis is one of the most important approaches for effectively controlling and/or eradicating the several forms of the disease. Based on the knowledge obtained from the practice of leishmanization and its protective immune response, several strategies have been used to develop vaccines against *Leishmania* species, such as the use of whole killed and attenuated parasites, recombinant proteins, and DNA vaccines. An ideal vaccine should be safe, effective, and immunogenic. Although several candidates have achieved safety and some level of effectiveness, the current challenge in the development of prophylactic vaccines is to achieve long-lasting immune protection by generating a robust and irreversible Th1 adaptive immune response in the host, with rapid recruitment of memory and effectors T cells at key acute points of infection. However, despite all efforts over the years, due to the antigenic diversity of the parasite and the complexity of the host's immune response, human vaccine trials have been disappointing in mediating long-term immunity against sandfly-delivered infection. Therefore, more investments in this field should be carried out to translate preclinical findings from mice to humans through effective vaccine development strategies.

Keywords Human leishmaniasis · *Leishmania* · Vaccine development · Long-lasting immunity · Correlates of protection

14.1 History of Human Leishmaniasis Vaccines

Leishmaniasis has afflicted mankind from ancient to modern times. Even though *Leishmania* were only described as a new genus at the beginning of the C20th [1, 2], their presence has been reported in Egyptian mummies dated as early as

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M. Christodoulides (ed.), *Vaccines for Neglected Pathogens: Strategies, Achievements and Challenges*, https://doi.org/10.1007/978-3-031-24355-4_14

2050–1650 BCE [3, 4]. In fact, ancient societies had already observed a key fact from cutaneous leishmaniasis (CL) that healed individuals achieved lifelong protection from new infections [5]. Especially in endemic areas of Asia and Africa, this knowledge would be later applied as the rationale for the first attempt of active immunization against *Leishmania* parasites [6].

This practice, known as leishmanization, was based on inoculating exudates from active lesions into a hidden part of the body of healthy individuals, which would produce a single self-healing lesion and consequently induce a protective response against future infections [6]. This type of immunization was used in several countries for decades, especially in hyperendemic areas [7–9]. Large-scale vaccination trials were conducted in conflict areas during the 1970s and 1980s, including one in which almost two million soldiers and refugees in Iran were immunized with live virulent *L. major* harvested from culture media [10]. Although leishmanization is considered to this day the most effective control measure against CL, concerns regarding vaccine safety, the lack of standardization, and numerous adverse effects caused it to be discontinued in most countries that still adopted this method [11]. Taking into account these limitations, attention has shifted into new approaches aimed at developing a safe and effective *Leishmania* vaccine for humans (Table 14.1). This includes a refinement of the leishmanization method, which will be discussed later in this chapter.

First-generation vaccines against leishmaniasis focused on whole killed parasites. This method is very attractive, since they are quite simple to produce at low cost, which is a prerequisite for wide distribution in developing countries [29]. The first trials of a vaccine against leishmaniasis using dead parasites took place in Brazil in the 1940s, using a polyvalent vaccine of 18 strains of *Leishmania*, and these trials had conflicting results [29–31]. These studies were resumed in the 1970s by other Brazilian research groups, through the evaluation of a pentavalent preparation without adjuvant known as Leishvacin® [12–15]. Other efforts were made worldwide, associating different *Leishmania* preparations with adjuvants such as BCG and aluminum hydroxide [16–20]. However, despite showing promising

Table 14.1 Vaccines against leishmaniasis evaluated in human trials

Vaccine	Classification	Candidate	Adjuvant	Phase reached	Reference
Leishvacin	First generation	Pool of five *Leishmania* isolates	None	III	[12–15]
Autoclaved *Leishmania*	First generation	Killed *Leishmania* spp.	BCG	III	[16–20]
Leish-F1	Second generation	TSA, LmSTI1, and LeIF	MPL-SE	I	[21, 22]
Leish-F2	Second generation	TSA, LmSTI1 and LeIF	MPL-SE	II	[23, 24]
Leish-F3	Second generation	NH36 and SMT	MPL-SE and GLA-SE	I	[25, 26]
ChAd63-KH	Third generation	KMP-11 and HASPB	None	II	[27, 28]

results regarding their safety and immunogenicity, overall, these vaccines failed to provide satisfactory levels of protection [32].

Second-generation vaccines then began to exploit purified or recombinant proteins as vaccine antigens. Associated with different adjuvants responsible for optimizing their immunogenicity [33, 34], vaccines using this method have advantages such as purity and ease of large-scale production [35]. Some second-generation vaccines against leishmaniasis that have reached clinical trials include LEISH-F1, LEISH-F2, and LEISH-F3 [36]. LEISH-F1, one of the first second-generation vaccines tested in humans, is made up of the fusion of the TSA, LmSTI1, and LeIF proteins (Table 14.1), associated with the adjuvant MPL-SE. Several phase I trials have demonstrated the vaccine's immunogenicity and safety, in addition to its therapeutic efficacy in patients with cutaneous and mucocutaneous leishmaniasis [21, 22]. Based on the positive results of phase I, the same group reformulated the vaccine, now called LEISH-F2. This time, the aim was to achieve a protein more like its wild-type version, by excluding the histidine tail present in its recombinant predecessor. After having its safety and immunogenicity evaluated in phase I, the vaccine entered phase II to have its therapeutic effects evaluated on CL patients [23, 24]. LEISH-F3 is composed of NH36 and SMT proteins (Table 14.1) fused in tandem, formulated with the adjuvant GLA-SE. Phase I trials have demonstrated its safety and immunogenicity in a healthy population in the United States and Bangladesh [25, 26].

In order to optimize the specificity of protein-based vaccines, third-generation vaccines began to explore the potential of coding DNA in their composition [37]. The advantages of this type of approach include ease of production and administration, stability, and immunogenic potential [38, 39]. While many *Leishmania* genes have been evaluated for their vaccine efficacy, only one candidate has reached the clinical trial stage [36]. This vaccine uses the ChAd63 adenovirus as a vector for expression of the KH gene, constituted by the KMP-11 and HASPB antigens of *L. donovani* (Table 14.1). The results of phase I trials demonstrated the safety and immunogenicity of the vaccine, which is currently being evaluated for its therapeutic effect in patients with post-kala-azar dermal leishmaniasis (PKDL). Preliminary phase II results reported that the vaccine induced a potent cellular immune response and was responsible for the emergence of mild adverse effects [27, 28]. Despite promising results, the level of protection obtained by DNA vaccines is still limited, so more studies should be carried out to increase their effectiveness.

14.2 Strategies to Vaccine Design: Where Are Good Candidates to Be Found and How Do We Explore Their Potential?

Since the vaccine development field started to focus on immunogenic fractions instead of whole parasites, screening methods to search for these candidates became crucial. Therefore, genome sequencing of *Leishmania* spp. was a key step to understanding the molecular biology of these organisms [40–43]. Although different *Leishmania* species exhibit variable numbers of chromosomes and some

species-specific genes, their genomes display a high degree of genetic conservation [44, 45]. This aspect becomes especially attractive when we consider the design of a pan-*Leishmania* vaccine.

Among other approaches to discover novel vaccine candidates, bioinformatics has been widely explored for its potential to process large amounts of data that are deposited on different databases. This interdisciplinary field combines computational techniques with biological data, supporting a large area of studies [46]. Regarding vaccine design, several tools and algorithms can be applied to predict a number of important antigen features, such as transmembrane domains, subcellular localization, secondary and tertiary structures, HLA recognition, and B- and T-cell epitopes [47–52]. Such characteristics not only help to understand the function of these molecules but also contribute to the search for dominant and therefore increasingly promising epitopes, which should be recognized by the human immune system and hopefully can stimulate a protective response. Furthermore, given the processing and analytical capabilities inherent to bioinformatics, this approach substantially reduces the time required for the simultaneous screening of thousands of targets [53]. On the other hand, a major limitation to this method is that the output data quality is highly affected by the accuracy of the annotations and predictions made upon them [54, 55].

A different approach to antigen discovery is based on bacteriophage libraries. In 1985, it was demonstrated that an exogenous gene could be fused to the gene from a capsid protein of the phage M13, resulting in the expression of a hybrid protein on the viral surface [56]. This technique, known as phage display, made it possible to create phage libraries composed of billions of phages capable of expressing different exogenous peptide sequences on their surface. These sequences can then be selected through their affinity for different types of ligands, such as enzymes, antibodies, and cell surface receptors [57]. An important aspect of this technology is the link between genotype and phenotype, since it is possible to find the selected peptide sequence through the nucleotide sequence fused to the viral genome [58].

Libraries constructed by random peptide sequences are the most common type of library used in phage display selection, often helping to identify epitopes [59], many of which have been evaluated as candidates for a *Leishmania* vaccine in experimental models [60–65] (Table 14.2). The application of this method in vaccinology explores both the role of the bacteriophage as an immunogenic carrier of antigens as well as the identification of mimotopes. These are peptides that, despite having a different sequence from that of the native epitope, are able to interact with the paratope in an analogous way, often mimicking conformational epitopes [66, 67]. Besides the ease of large-scale production, relatively low cost, and safety, one of the main advantages of phage display is the possibility of selecting mimotopes, since it is estimated that approximately 90% of B-cell epitopes are discontinuous in nature [35, 68]. Furthermore, the use of phages as antigen carriers is capable of inducing both the cellular and humoral arms of immunity, which is fundamental in orchestrating an effective response against intra- and extracellular pathogens [59, 69].

Although having good candidates is important while developing a promising vaccine, it is only the first step in a very long process. A fundamental aspect for a

Table 14.2 Main phage display vaccine candidates against leishmaniasis

Vaccine presentation	Adjuvant	Protection	Experimental model	Main findings	Reference
Mimotopes anchored to M13 bacteriophage coat proteins	Saponin	*L. infantum* *L. amazonensis*	BALB/c mice	1. Specific Th1 immune response 2. Significant reduction of parasite burden in all organs evaluated	[60, 61]
Synthetic soluble peptides	Aluminum hydroxide	*L. infantum*	BALB/c mice	1. Significant protection (up to 98%) induction of mixed Th1/Th2 response	[62]
Synthetic soluble peptides	None	*L. major*	BALB/c mice	1. Up to 81.94% protection rate with peptide P2	[63]
Mimotopes anchored to M13 bacteriophage coat proteins	None	*L. infantum*	BALB/c mice	1. Specific Th1 immune response 2. Reduction of parasite burden (up to 65%) in all organs evaluated	[64]
Mimotopes anchored to M13 bacteriophage coat proteins	None	*L. amazonensis*	BALB/c mice	1. Induction of specific Th1 immune response 2. Significant reduction of parasite burden in all organs evaluated	[65]

successful vaccine is, in fact, how these candidates are explored. Despite peptide-based vaccines offer advantages like safety and ease for production, it is well-known that synthetic single peptides are poor immunogens and require some tweaks to be able to elicit a potent and hopefully long-lasting immune response [70]. Among commonly used approaches to overcome this issue and, in the right context, drive a protective immune response is the use of adjuvants, adenovirus vector, or chimeras [71].

Chimera vaccines are composed of multiple epitopes which can be repeated in tandem to enhance the immune response [72]. Several studies have demonstrated the potential of these vaccines against leishmaniasis in a murine model (Table 14.3), including polyproteins composed by conjugated antigens such as KSAC [82] and Q protein [73], T-cell epitopes for a specific protein [72, 74–81], and MHC I- and MHC II-specific epitopes from different proteins [83, 84]. Regardless of the specific target, multicomponent vaccines are especially interesting in the case of complex organisms such as *Leishmania* spp. that present an extensive antigen repertoire [76].

Table 14.3 Main chimera vaccine candidates against leishmaniasis

Candidate name	Composition	Adjuvant	Protection	Experimental model	Main findings	Reference
Q Protein	Lip2a, Lip2b, P0, and H2A proteins	None	*L. infantum*	Dogs	Clinical protection observed in anatomo-pathological and phenotypic levels. Early and intense long-lasting specific IgG2 antibody response	[73]
RCP (recombinant chimeric protein)	T-cell epitopes from LiHyp1, LiHyp6, LiHyV, and HRF proteins	Saponin	*L. infantum*	BALB/c mice	Significant reduction of parasite burden in all evaluated organs. Th1 cell-mediated immune response. Induction of predominantly IgG2a antibody isotype	[74]
F1F3 Chimera	T-cell epitopes from NH36 protein	Saponin	*L. amazonensis*	BALB/c mice	84% reduction of skin lesions size and 99.8% reduction of parasite load. Th1 cell-mediated immune response. Induction of higher titers of IgA, IgG, and IgG2a antibodies	[75]
Recombinant Chimeric Protein	T-cell epitopes from LiHyS, SGT, and PHB proteins	Saponin	*L. infantum*	BALB/c mice	Significant reduction of parasite burden in all evaluated organs. Th1 cell-mediated immune response. Induction of predominantly IgG2a antibody isotype	[76]
ChimeraT	T-cell epitopes from PHB, EIF5a, LiHyp1, and LiHyp2 proteins	Saponin	*L. infantum*	BALB/c mice	Significantly reduction of parasite load in distinct organs. Th1 cell-mediated immune response. Induction of predominantly IgG2a antibody isotype	[77, 78]
LiChimera	T-cell epitopes from Cpn60, Gcvl-2, Eno, CyP2, CyP40, and HyP proteins	AddaVax	*L. infantum*	BALB/c mice	Reduction of parasite burden in short- and long-term vaccinated mice. Cellular responses dominated by central and effector multifunctional CD4+ and CD8+ T memory cells	[79]
Chimera A and Chimera B	Chimera A: T-cell epitopes from H2A, LiP2a, LiP0, LACK, and CPC proteins chimera B: T-cell epitopes from CPA, CPB, PSA-50S, and A2 proteins	Saponin alone or in association with MPL-A	*L. infantum*	BALB/c mice	Reduction of parasite burden in the spleen. Th1 cell-mediated immune response. Generation of central and effector memory T cells	[72, 80]
ChimT	T-cell epitopes from LiHyp1, LiHyV, LiHyC, and LiHyG proteins	Saponin or MPL-A	*L. infantum*	BALB/c mice	Reduction of parasite burden in all evaluated organs. Th1 cell-mediated immune response. Induction of predominantly IgG2a antibody isotype	[81]

In addition to optimizing the chances of triggering an immunogenic response by recognizing at least one of its epitopes, vaccines composed of polyproteins demonstrate greater potential for mass application [85], especially when considering the genetic polymorphism of the mammalian immune system and the possible interactions of these antigens with different types of MHC [86]. Furthermore, the high level of conservation among the genomes of *Leishmania* spp. makes possible the development of a pan-effective vaccine against several species [87]. Despite these advantages, chimeric vaccines still need to be associated with adjuvants that are safe for use in humans and that together can stimulate the robust and long-lasting response associated with protection. This vaccine is yet to be developed.

Although whole parasite vaccines have the advantage of exhibiting the complete repertoire of antigens to the immune system [88], one of the biggest caveats about using attenuated organisms is the risk of reversion to virulence [89]. Particularly, older approaches such as maintaining the parasites in culture for long periods of time and exposure to chemical and physical attenuation did not ensure its safety. Random mutations and the return to a virulent state were often observed [90–92]. Fortunately, the use of attenuated strains gained a new momentum thanks to the progress made in genetic manipulation techniques. The discovery of the CRISPR-Cas9 system, for instance, proved to be of great importance for editing the genomes of several organisms, including different *Leishmania* species [93–95]. This system is based on two components: Cas9, an RNA-guided endonuclease, and a guide RNA sequence, which has the function of directing Cas9 to the complementary strand of the target DNA that will be cleaved [96]. Since genetic manipulation before CRISPR-Cas9 was largely based on homologous recombination with the use of antibiotics as selection markers [97–99], the development of this technology improved the ability to explore and edit the genome of a number of organisms. In addition to other possibilities, this method allows the precise deletion and insertion of genes in known locations, being able to introduce mutations, selection markers, and protein sequences of interest [94].

Several important genes for the survival of *Leishmania* spp. have been explored in vaccine development, such as those responsible for the expression of cysteine protease, biopterin transporter, p27, and centrin [100–109] (Table 14.4). Centrin is a constitutive protein of the eukaryotic cytoskeleton, responsible for the duplication and segregation of the centrosome. Deletion of the centrin encoding gene in *L. donovani* reduced the growth of the amastigote forms, although it did not interfere with the viability of the promastigotes [107, 110]. While multiplying inside macrophages, mutant amastigotes were unable to properly perform cell division, becoming multinucleated and entering a process of programmed cell death [107]. Immunization with this strain, called LdCEN$^{-/-}$, was able to provide protection against infection by *L. donovani* [108, 111, 112], *L. infantum* [113, 114], *L. mexicana* [115], and *L. braziliensis* [116] in mice, hamsters, and dogs. Immunity generated by vaccination was mediated by a CD4^{+} and CD8^{+} T-cell response, characterized by potent production of pro-inflammatory cytokines IL-12, IFN-ɣ, and IL-17 and reduction of IL-10 by macrophages [88, 116–118].

Table 14.4 Main attenuated live parasites vaccine candidates against leishmaniasis

Candidate name	Mutation target	Protection	Experimental model	Main findings	Reference
L. donovani BT1 null mutant	Biopterin	*L. donovani*	BALB/c mice	1. Reduction of parasite burden (65%) when compared to wild-type infection 2. Induction of protective immunity	[100]
L. mexicana cysteine proteinase-deficient mutant	Cysteine proteinase	*L. mexicana*	Hamster	1. Reduction of parasite burden 2. Reduction in the severity of lesions	[101]
L. donovani p27 gene knockout parasites (Ld27−/−)	p27 protein	*L. donovani* *L. braziliensis* *L. major*	BALB/c mice	1. Significantly lower parasite burden in the liver and spleen 2. Induction of protective immunity 3. No parasite survival beyond 20 weeks after infection	[102]
L. infantum KHARON1 null mutant	KHARON1 protein	*L. infantum*	BALB/c mice	1. Reduction of parasite burden 2. Unable to sustain infection in macrophages	[103]
L. infantum HSP70-II null mutant	Heat shock protein 70	*L. major*, *L. infantum* *L. braziliensis*	BALB/c mice, C57BL/6 mice	1. Reduction of parasite burden 2. Induction of long-term protection 3. Th1 cell-mediated immune response	[104–106]
L. donovani and *L. major* centrin deleted parasites (LdCEN−/−)	Centrin Protein	*L. donovani* *L. infantum* *L. mexicana* *L. braziliensis*	BALB/c mice, hamster, and dog	1. Reduction of parasite burden 2. Unable to sustain infection in macrophages 3. Protective immune response 4. Safe in immunocompromised mice	[107–118]

Despite all benefits, the use of this strain as a human vaccine raises concerns regarding its potential for visceralization, which can be fatal. Furthermore, the method used to obtain the centrin gene knockout required the insertion of an antibiotic resistance marker gene, an inadmissible feature from a human vaccine candidate. In light of these limitations, an attenuated *L. major* centrin gene deletion mutant ($LmCen^{-/-}$) was generated using the CRISPR-Cas technique. This

technology eliminates the need for resistance markers, which facilitates the approval of this strain as a vaccine by regulatory agencies and makes its evaluation possible in human clinical trials. Another relevant aspect for the safety of this strain is that *L. major* is a dermotropic species and its infection, unlike *L. donovani*, remains in the skin and does not cause visceral disease. Evaluation of LmCen$^{-/-}$ in a murine model was able to prevent the appearance of lesions after challenge by *L. major*, in addition to having reduced the parasite load within internal organs and induced a protective immune response analogous to leishmanization. Moreover, inoculation of LmCen$^{-/-}$ was unable to generate pathology in susceptible and immunodeficient mice, proving the safety of this vaccine [109].

The pursuit for knowledge and the advancement of new technologies have facilitated the search for increasingly promising vaccine candidates against leishmaniasis. The support of bioinformatics and genetic manipulation techniques has allowed the design and evaluation of different types of vaccines, whether composed of parasite fractions or those that exploited genetically modified whole parasites. Even though many candidates have been evaluated in preclinical trials, few had a chance to reach human clinical trials. There is still no vaccine available against human leishmaniasis. However, scientific efforts made in recent decades have brought us closer to achieving a safe, immunogenic, and effective human vaccine.

14.3 Immunological Insights into Vaccine Development

The host's immunity during leishmaniasis is complex and varies according to parasite or host species, parasite load and sandfly, or needle challenge. In general, a protective immune response during *Leishmania* spp. infection involves the cross talk between the innate immune response, including neutrophils, monocytes/macrophages and dendritic cells (DCs), and subsequent activation of a Th1 adaptive immune response. Both CD4$^+$ Th1 and antigen-specific CD8$^+$ T-cell activation result in the production of IFN-γ and TNF-α cytokines that upregulate inducible oxide nitric synthase (iNOS) and reactive oxygen species (ROS) expression by macrophages, important molecules that have been associated with disease control and parasite clearance [119–121].

The resolution of a primary *Leishmania* spp. infection in humans who recover from the cutaneous manifestation, but maintain chronic infection in the skin, leads to long-lasting immunity mediated by CD4$^+$ T cells. Healed patients establish a strong Th1 memory response with low number of parasites due to immune regulation mediated by IL-10, known as concomitant immunity, which confers resistance to a secondary infection, the same protection observed in the practice of leishmanization [122, 123]. Thus, from the knowledge of concomitant immunity comes the idea of developing vaccine-mediated immunity against different forms of leishmaniasis using several approaches, such as the use of attenuated live parasites, whole killed parasites, parasite protein, recombinant vaccines, and DNA vaccines, among others. However, despite all efforts in this field, human vaccine trials have been

disappointing in mediating long-term immunity when compared to leishmanization.

Healed humans and mice from experimental models of CL showed that upon antigen presentation, different populations of memory and effector $CD4^+$ T cells are generated. Central memory T (T_{CM}) cells ($Ly6C^-CD62L^+CCR7^+Ki\text{-}67^+$) reside in lymph nodes and can survive for life, regardless of persistent antigen presentation. During concomitant immunity, they have the capacity to transition into effector T (T_{EFF}) cells after the period of antigen presentation and activation by DCs. Moreover, they are important for the production of IFN-γ and TNF-α [119, 124]. Effector memory T (T_{EM}) cells ($Ly6C^-CD62L^-CCR7^-Ki\text{-}67^+$) can also produce these Th1 cytokines and are longer lived than T_{EFF} cells in the absence of antigen, but shorter lived than T_{CM} cells. They can be found in secondary lymphoid organs, blood, or periphery [119, 125]. Tissue resident memory (T_{RM}) cells ($Ly6C^-CD62L^-CCR7^-Ki\text{-}67^+$) are a non-circulatory population of memory T cells found at the distal site to the primary infection that respond quickly upon restimulation, producing IFN-γ and recruiting T_{EFF} cells [125, 126]. Along with T_{EFF} cells, T_{RM} cells are crucial in IFN-γ production at very acute time points of infection.

Regarding T_{EFF} cells, studies in experimental mouse models have demonstrated that the constant presence of the parasite in chronic subclinical infection is the key factor in Th1 concomitant immunity. Therefore, Peters et al. have shown that persistent antigen presentation is crucial for the maintenance of circulating T_{EFF} cells, short-lived $CD4^+$ T cells expressing $Ly6C^+CD44^+CD62L^-$ that are predominantly single producers of IFN-γ. These cells are rapidly recruited and responsible for IFN-γ production almost instantly after secondary challenge by sandfly bite, preventing the formation of a phagosomal pathogen niche and the development of the disease in mice [125, 127].

Several experimental vaccine formulations have been able to generate *Leishmania*-specific T_{CM} and T_{EM} cells and have successfully protected mice against needle challenge. However, although these memory T cells enhance Th1 response by cytokine production upon re-exposure to parasite antigen weeks to months after vaccination, the same vaccine formulations were ineffective in providing protection against sandfly bite-mediated challenge [127–131]. These observations highlight that the failure of *Leishmania* vaccines is not due to a lack of generating an appropriate Th1 memory response but due to a lack of generating T_{EFF} and T_{RM} cells, in addition to inflammatory conditions at the sandfly bite site that compromise the effector function of the memory response and should be considered when designing and testing vaccines. The human counterparts of T_{EFF} cells in mice are not characterized yet, and understanding how to best induce generation of T_{RM} and T_{EFF} cells in humans during vaccination against *Leishmania* infection is one of the major challenges that remains undefined [132].

Immune protection against sandfly bite, rather than just the needle challenge, is the other big issue that needs to be overcome in successful vaccine design. Vector transmission of *Leishmania* by female sandfly bite delivers into the skin a low number of promastigote parasites and active molecules present in the saliva, inducing a robust local inflammatory response associated with the recruitment of neutrophils

and monocytes. This specific inflammatory response in vector transmission has an important impact in the context of vaccination. Studies have shown that neutrophil recruitment is an important factor in impairing IFN-γ production by $CD4^+$ T cells and vaccine efficacy, due to suppression of T-cell activation by macrophages and DCs that are engaged in both antigen presentation and efferocytosis (i.e., clearance of apoptotic cells) of infected neutrophils [119, 132–134]. In addition, another important aspect that must be taken into account is the shortage of antigen availability during vector transmission when compared to needle challenge in many experimental models. The low number of parasites delivered by sandfly bite can hamper the development of a protective immune response, including T_{CM} activation in the draining lymph node. Despite the difficulty of maintaining sandfly colonies to reproduce the context of natural infection, efforts to replicate the low dose and inflammatory response conditions of vector transmission is an essential concern and should be used as the "gold standard" of preclinical research to interpret the effectiveness of protective immunity and vaccination [119, 123, 132].

14.4 Lessons from the COVID Era: What Have We Learned, and How Can We Translate It to *Leishmania* Vaccines?

Vaccine development is a lengthy process—a decade can easily pass by between the discovering phase and the start of clinical trials. In 2020, the SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2) pandemic shook the entire world, both for the speed with which it infected and killed millions of people and for the agility with which vaccines capable of containing the spread of the virus were developed. Coronaviruses are a group of large enveloped RNA viruses that usually cause mild disease in humans, the main reason why vaccination efforts were nonexistent up until recently [135]. This scenario dramatically shifted after the SARS-CoV and MERS-CoV (Middle Eastern respiratory syndrome coronavirus) outbreaks revealed a highly transmissible and pathogenic profile for these viruses [136].

Studies soon found a promising antigenic candidate for coronaviruses vaccines, a large surface protein responsible for receptor binding and cell invasion mechanisms known as "spike" protein [137]. Thankfully, due to the close relation between these pathogens, the discovery phase during vaccine design for SARS-CoV-2 could be significantly shortened and effective vaccines could be evaluated in clinical trials at an unprecedented speed. A pandemic like the one caused by SARS-CoV-2 justifies all the great scientific efforts and the number of financial investments made all over the world. It is also noteworthy that the success of different strategies explored during vaccine design brought attention not only to their advantages as a SARS-CoV-2 vaccine per se but more importantly to its capacity to be applied to vaccines against all types of etiologies, including leishmaniasis. Adenovirus (Ad) vector-based mRNA vaccines such as the ones developed by Johnson and Johnson and Oxford/AstraZeneca showed large potential as a platform for numerous infectious diseases. Aside from their high transduction efficiency and thermostability, Ad

vectors are especially attractive when we consider their ability to induce moderate levels of innate immunity, a key feature needed to activate adaptive immunity that is usually obtained only by the use of adjuvants [138]. This is one of many design approaches used in SARS-CoV-2 vaccines that we can draw experience from and that can be certainly translated to *Leishmania* vaccines.

A different kind of reflection provoked by the COVID-19 pandemic is what an effective vaccine looks like and what we should expect from it. Sterile immunity is often thought as the main goal for vaccination, despite being rather difficult to achieve. Admittedly, several vaccines including those against influenza, rotavirus, and the ones recently developed for SARS-CoV-2 fall under that category. However, the fact that these vaccines are unable to entirely block the infection does not mean they cannot prevent diseases or even reduce associated burden. We have witnessed first-hand COVID-19 vaccines significantly reducing hospitalization, morbidity, and mortality rates worldwide—while aided by important safety guidelines like social distancing and implementation of face mask obligation. Taking that into account, one can argue if we absolutely need to induce sterile immunity in a *Leishmania* vaccine, particularly since it is well-known that parasite persistence is required for long-life immunity. Furthermore, no vaccine alone can eradicate a complex multifactorial disease like leishmaniasis. Much like COVID-19, leishmaniasis control needs far more than an effective vaccine; it needs a One Health approach that encompasses vector control, reservoir vigilance, and environmental conservation programs.

14.5 Conclusions

The main concept of *Leishmania* long-lasting vaccination is to generate a robust and irreversible $CD4^+$ Th1 memory response and early IFN-γ-producing effector T-cell responsiveness at challenge site, which is crucial in preventing the establishment of a parasite niche, in addition to mediating parasite killing and infection control. Therefore, key points must be considered in vaccine evaluation: (a) cytokine production and cell differentiation by parasite-specific memory T cells (T_{CM} and T_{EM}), (b) persistent antigen presentation to maintain circulating IFN-γ-producing T_{EFF} cells required to mediate an optimal response, (c) induction of T_{RM} populations at the inoculated inflamed skin, and (d) to replicate the low-dose/high-inflammation conditions of experimental sandfly challenge as the "gold standard" of preclinical research. In conclusion, understanding of aspects related to the protective immune response in leishmaniasis has made important advances over the years and is crucial for translating preclinical findings from mice to humans through effective vaccine development strategies. The current prophylactic vaccine approach against all forms of leishmaniasis aims to obtain immune protection through a rapid recruitment of IFN-γ-producing T_{EFF} and T_{RM} cells in key acute times of *Leishmania* infection. This outcome should be able to occur even after natural sandfly challenge, preventing the development of the disease.

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Part IV
Melioidosis

Chapter 15
Vaccine Development Against Melioidosis

Michelle Galeas-Pena and Lisa A. Morici

Abstract Melioidosis in an infectious disease of humans and animals caused by the bacterium *Burkholderia pseudomallei*. Melioidosis is often considered a disease of the tropics, but recent data suggests that *B. pseudomallei* is distributed worldwide and the disease is likely largely underestimated. *B. pseudomallei* is inherently resistant to many antibiotics, which complicates treatment, particularly in low-resource countries. There is currently no licensed vaccine to prevent melioidosis. Fortunately, there has been significant progress over the last decade in our understanding of *B. pseudomallei* pathogenesis and host immunity. This has been paralleled by the discovery and testing of promising vaccine candidates against melioidosis. Collectively, these scientific advances spark optimism that licensure of a safe and effective vaccine is achievable.

Keywords Melioidosis · *Burkholderia pseudomallei* · Vaccines · Pathogenesis Host response

15.1 Melioidosis

Melioidosis, also known as Whitmore's disease, is caused by *Burkholderia pseudomallei*, a Gram-negative aerobic bacillus capable of infecting both animals and humans. The bacterium has an impressive collection of virulence factors encoded on two chromosomes, an unusual feature for most bacteria [1]. *B. pseudomallei* can cause a wide variety of disease manifestations, ranging from asymptomatic infection to life-threatening pneumonia or sepsis, which may be influenced by the route of infection and underlying human risk factors or immune status [2].

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M. Christodoulides (ed.), *Vaccines for Neglected Pathogens: Strategies, Achievements and Challenges*, https://doi.org/10.1007/978-3-031-24355-4_15

Melioidosis is most common in subtropical areas, with the highest incidence in Southeast Asia and Northern Australia. *B. pseudomallei* is also found in parts of Africa and has recently been detected in the US Virgin Islands and other Caribbean islands, including Puerto Rico [3]. While *B. pseudomallei* is not considered endemic in Europe or North America, four cases of melioidosis were recently reported in the USA. The four cases occurred in Kansas, Texas, Georgia, and Minnesota in individuals who had not traveled outside of the USA. Genetic analyses of the isolates linked the infection to a common source—a contaminated imported aromatherapy spray [4, 5]. There have been case reports of endemic melioidosis in Central and South America, pointing to a more global spread of *B. pseudomallei* than previously appreciated [6].

B. pseudomallei is a soil saprophyte, and routes of exposure may include inoculation, inhalation, or ingestion from a contaminated source. The incubation time to disease onset is 1–21 days from exposure with an average of 9 days, depending on the infectious dose and host factors. Melioidosis is considered an acute infection if the symptoms manifest for less than 2 months, whereas symptoms persisting longer than 2 months constitute a chronic infection. Latent infection with reactivation disease can also occur [7]. The outcomes of infection depend on several factors including *B. pseudomallei* strain, route of infection and dose, and host immune status. The most common host risk factor in development of severe melioidosis is diabetes mellitus [8], but other risk factors include chronic kidney, lung, and liver diseases and other conditions that impair the immune system [9]. Nonetheless, severe sickness and death can also occur in otherwise healthy individuals [10]. The case fatality rate percentage (CFR%) ranges from 8% in Europe up to 60% in Africa. In endemic areas, such as Australia, the CFR% is approximately 41% [11].

Melioidosis may result in a broad range of symptoms, such as pneumonia, fever, pain, cough, and headache. The disease often mimics the manifestations of more common pathogens leading to misdiagnosis and delayed treatment [2, 12]. Diagnosis based only on clinical manifestations is sometimes possible, but bacterial culture is the gold standard for proper diagnosis. Because of the nature of *Burkholderia* species, it may sometimes be dismissed as a contaminant, so appropriate training of clinical laboratory staff in endemic areas is important. In a presumed melioidosis case, clinical samples should be sent to laboratories with experience in identifying the bacterium. Common samples include blood, throat or wound swabs, and urine. Repeated sampling is needed as samples may initially test negative. *B. pseudomallei* grows slowly on most agar plates and may take a few days for visible detection. Antibiotic resistance profiles are helpful for identification, especially in settings with scarce resources. *B. pseudomallei* is resistant to gentamicin and polymyxin and sensitive to amoxicillin-clavulanic acid. This sensitivity pattern in a Gram-negative, oxidase-positive bacillus can be helpful for diagnosis. Molecular diagnostics are not common in most endemic areas. Proper clinical management must be initiated early in every suspect case. *B. pseudomallei* is susceptible to β-lactam antibiotics such as imipenem, meropenem, amoxicillin-clavulanic acid, and ceftazidime. In the eradication phase, trimethoprim/sulfamethoxazole is recommended [13].

Prevention may be achieved in endemic areas by avoiding high-risk activities and through additional protective measures for those at occupational risk. Often, this is difficult to achieve in poor countries with high *B. pseudomallei* endemicity. There is currently no licensed vaccine to prevent melioidosis. A vaccine could mitigate the public health burden of *B. pseudomallei* in areas of the world with high caseloads. In addition, *B. pseudomallei* has been classified as a Tier 1 select agent by the US Department of Health and Human Services and the US Department of Agriculture, due to its potential threat as a bioweapon. A vaccine to safeguard against *B. pseudomallei* misuse is therefore also highly desired.

15.2 Pathogenesis and Host Response

B. pseudomallei utilizes multiple virulence factors to invade human cells. This bacterium can enter mammalian cells via adherence using adhesion proteins such as fimbriae, type IV pili, flagellin [14–16], and components of the type V secretion system that work as autotransporters, such as BcaA, and the trimeric autotransporter adhesins BoaA and BoaB [17]. The type III secretion system proteins are used to invade and escape the endosome and are required for intracellular survival [18]. Type II, type III, and type VI secretion systems are required for the bacteria to exit the cells by membrane lysis [19, 20]. Autophagy is avoided by the effector protein BopA and translocator protein BipD [21–23]. Actin-based motility is mediated by BimA and leads to cell-to-cell fusion and the formation of multinucleated giant cells that are associated with granulomatous diseases, such as sarcoidosis and tuberculosis, complicating clinical diagnosis [24].

Immune responses to *B. pseudomallei* infection are triggered by pathogen-associated molecular patterns (PAMP) activation upon recognition of bacterial lipoproteins, lipopolysaccharide (LPS), and flagella by Toll-*like* receptor (TLR)2, TLR4, and TLR5, respectively, leading to a pro-inflammatory response mediated via NF-κB activation [25, 26]. Activation of complement pathway and destruction via the membrane attack complex is impaired by the presence of capsular polysaccharide (CPS), a major virulence determinant [27]. Activation of caspase 1 via the inflammasome leads to pyroptosis, releasing IL-1β and IL-18 [28, 29]. IL-18 promotes the induction of IFN-γ, helping to recruit additional macrophages. Macrophages that are activated via INF-γ produce nitric oxide that can kill sensitive strains [30]. However, some strains have developed resistance to oxidative stress by expressing superoxide dismutase C (sodC), alkyl hydroperoxide reductase C (ahpC), and a nonspecific DNA-binding protein known as dpsA [31, 32]. The release of IL-1β contributes to pathogenesis by recruiting neutrophils, which leads to tissue damage [33]. As commonly observed in other diseases, uncontrolled pro-inflammatory responses are associated with poor outcomes. An exacerbated release of IL-1β, IL-6, IL-12, TNF, and IFN-γ can cause severe melioidosis disease [27]. Both humoral and cell-mediated immune responses have been shown to protect against disease. Antibodies against LPS, O-polysaccharide (OPS), and Hcp-1 of

B. pseudomallei are associated with better outcomes in melioidosis [27, 34]. $CD4^+$ T cells that secrete IFN-γ are found in acute melioidosis patients and are thought to play a role in protection [35, 36]. However, further research is required to elucidate the innate or adaptive immune responses that are responsible for full protection against *B. pseudomallei*.

15.3 Vaccine Development

Over the last decade, several promising vaccine candidates have emerged using various approaches, and the most efficacious vaccines appear to be multivalent in nature. This is not entirely surprising, considering the sophisticated intracellular pathogenesis of *B. pseudomallei* that may require both arms of the immune response to target multiple antigens for complete protection. The various vaccine platforms include, but are not limited to, live-attenuated or inactivated whole-cell bacteria and multivalent subunit or conjugate vaccines. These are summarized below and listed in Table 15.1. The target bacterial antigens and their respective functions are described in Table 15.2.

15.3.1 Live-Attenuated Vaccines

A successful vaccine against *B. pseudomallei* will likely require both humoral and cellular immune responses for complete protection. Live-attenuated vaccines have long been considered the gold standard for achieving both arms of the immune response. Live-attenuated vaccines created by mutation of *tonB* and *hcp1*, genes belonging to a siderophore complex and the T6SS, respectively, induced robust immunity and significant protection in multiple studies [57, 58]. Deletion of *purM* created a highly attenuated strain (Bp82) that was avirulent in mice, Syrian hamsters, and immune incompetent mice [interferon (IFN)-$\gamma^{-/-}$, severe combined immunodeficiency (SCID)] [59]. Immunization of mice with Bp82 conferred 100% survival over 60 days against pulmonary melioidosis [60]. Deletion of *relA-spoT* produced a *B. pseudomallei* double mutant that displayed defects in stationary-phase survival and replication in murine macrophages and was attenuated in acute and chronic mouse models of melioidosis. Immunization of mice with the Δ*relA* Δ*spoT* live-attenuated strain resulted in full protection against infection with *B. pseudomallei* [39]. An *asd* mutant of *B. pseudomallei* 1026b was avirulent in BALB/c mice, and animals vaccinated with the mutant were protected against acute inhalation melioidosis [40]. Immunization with an *aroC* mutant also significantly protected mice against challenge [38]. Immunization with *B. thailandensis* strain E555, which possesses CPS, provided significant protection against *B. pseudomallei* challenge [42]. Collectively, these studies demonstrate the safety and protective efficacy of live-attenuated vaccines in animal models of melioidosis.

Table 15.1 Vaccine candidates developed and tested for *Burkholderia pseudomallei*. The vaccine studies described here are not prioritized in any way

Vaccine platform	Mouse strain	Immunization route	Antigen	Adjuvant	Challenge			Survival	Reference
					Route	Dose	Strain		
Whole bacteria	BALB/c	i.n.	Inactivated *B. pseudomallei*	CLDC	i.n.	$7{\cdot}5 \times 10^3$	1026b	100% at day 40	[37]
	C57Bl/6	i.p.	Live, attenuated (Δ*aroC*)	–	i.p.	6×10^3	A2	80% at day 150	
	C57Bl/6	s.c.	Live, attenuated (Δ*purM*)	–	i.n.	1×10^4	1026b	100% at day 60	[38]
	C57Bl/6	i.n.	Live, attenuated (Δ*relA–spoT*)	–	i.n.	1×10^3	576	100% at day 30	[39]
	BALB/c	i.n.	Live, attenuated (Δ*asd*)	–	i.n.	4×10^3	1026b	100% at day 80	[40]
	C57Bl/6	i.n.	Live, attenuated (Δ*tonB, hcp1)*	–	Aerosol	$1{\cdot}7 \times 10^3$	K96243	100% at day 27	[41]
	BALB/c	i.p.	*B. thailandensis* E555	–	i.p.	6×10^6	K96243	100% at day 35	[42]
Subunit	BALB/c	i.p.	Chronic antigens	Sigma adjuvant system	i.p.	7×10^4	K96243	30% at day 50	[43]
			Chronic + LolC					40% at day 50	
			Chronic + CPS					50% at day 50	
			CPS					10% at day 50	
Vectored	BALB/c	i.n.	Parainfluenza virus 5 + BatA	RECOMBITEK Lyme	i.n.	3×10^2	K96243	26% at day 25	[44]
	C57BL/6	i.n.	pVAX-hTPA-FliC	CFA	i.n.	2×10^2	1026b	53% at day 14	[45]

(continued)

Table 15.1 (continued)

Vaccine platform	Mouse strain	Immunization route	Antigen	Adjuvant	Challenge			Survival	Reference
					Route	Dose	Strain		
Glycoconjugate	C57BL/6	s.c.	CPS-Hcp1	Alhydrogel + CpG	Aerosol	$1{\cdot}6 \times 10^3$	K96243	100% at day 35	[46]
			CPS-TssM					80% at day 35	
			CPS-CRM197					67% at day 35	
	BALB/c	s.c.	OPS-BSA	Alhydrogel + CpG	i.p.	4×10^4	K96243	0% at day 14	[47]
			CPS-BSA			8×10^4		50% at day 35	
			CPS + BSA-LolC			8×10^4		70% at day 35	
			LolC			8×10^4		0% at day 35	
	BALB/c	i.p.	Synthetic CPS + TetHc	MPL/Sigma adj. system	i.p.	9×10^4-1×10^5	K96243	67% at day 35	[48]
	BALB/c	s.c.	OPS + *Campylobacter jejuni* AcrA	+/−Alum	i.n.	2×10^3	K96243	40% at day 12	[49]
								0% at day 21	
	BALB/c	i.p.	LPS-TetHc	–	i.p.	4×10^4	K96243	81% at day 29	[50]
			LPS only					62% at day 29	
			LPS and TetHc					75% at day 29	
	C57BL/6	s.c.	AuNP-Hcp1-LPS	Alhydrogel + Poly(I:C)	i.n.	1×10^5	K96243	10% at day 35	[51]
			AuNP-HA-LPS					20% at day 35	
			AuNP-FlgL-LPS					90% at day 35	
	C57BL/6	i.n.	AuNP-Combo2-LPS	CpG	i.n.	7.5×10^4 9×10^4	K96243	100% at day 35	[52]

Nano–/ microparticle	BALB/c	i.n. s.c.	OMVs	–	Aerosol	1×10^3	1026b	20% at day 14 60% at day 14	[53]
	BALB/c	s.c.	OMVs	_	i.p.	2×10^4	K93243	100% at day 21	[54]
	C57Bl/6	s.c.	OMVs	_	Aerosol	1.5×10^3	K93243	100% at day 30	[55]
	BALB/c	s.c.	Lysate/MP + Resiq/MP	–	i.p.	1×10^6	1026b	20% at day 14	[56]
			Lysate + Resiq/MP					60% at day 14	
			Lysate + Resiq/MP	Alum				100% at day 21	
								12% at day 26	

CPS capsular polysaccharide, *CLDC* cationic liposomes complexed with noncoding plasmid DNA, *CpG* cytosine-phosphate-guanine, *BSA* bovine serum albumin, *LPS* lipopolysaccharide, *OMV* outer membrane vesicles, *OPS* O-polysaccharide, *TetHc* Hc fragment of tetanus toxin, *i.n.* intranasal, *i.p.* intraperitoneal, *s.c.* subcutaneous, *MPL* monophosphoryl lipid A

Table 15.2 Antigens used in the vaccines covered in this chapter and their function

Protein	Full name	Function
AcrA	Multidrug efflux protein AcrA	Part of a complex of periplasmic membrane fusion protein family with a transmembrane transport function
ahpC	Alkyl hydroperoxidase reductase C	Resistance to oxidative stress
BatA	*Burkholderia* autotransporter A	Protein that exhibits lipolytic activity, contributing to virulence
BcaA	*Burkholderia* classic autotransporter A	Putative classic autotransporter of *Burkholderia* sp.
BimA	*Burkholderia* intracellular motility A	Mediates actin binding and assembly
BipD	*Burkholderia* invasion protein	It serves as a platform for the assembly of the translocon pore with *Burkholderia* invasion protein B (BipB) (a major translocon protein) and *Burkholderia* invasion protein C (BipC) (a minor translocon protein), allowing for the direct passage of effector proteins into the target host cell from the cytoplasm of *B. pseudomallei*
BoaA	*Burkholderia* oligomeric coiled-coil adhesin A	Reported to function as adhesins in vitro and to contribute to *B. pseudomallei* replication inside macrophage-like cells
BoaB	*Burkholderia* oligomeric coiled-coil adhesin B	Reported to function as adhesins in vitro and to contribute to *B. pseudomallei* replication inside macrophage-like cells
BopA	Effector protein BopA	Putative type III secretion system effector protein. Plays a role in mediating bacterial evasion from the host autophagic pathway
BopE	Guanine nucleotide exchange factor BopE	Part of the T3SS. Promotes invasion of epithelial cells via its guanine nucleotide exchange factor activity
BPSL1897	Uncharacterized protein	Putative membrane protein
BPSL2287	Iron-binding protein IscA	Part of an iron-sulfur cluster assembly
BPSL3369	Acetaldehyde dehydrogenase	Putative dehydrogenase
Buc18	*Burkholderia* collagen-like protein 8	Putative efflux pump
CPS	Capsular polysaccharide	Component of cell envelopes
dpsA	Nonspecific DNA-binding protein A	Resistance to organic oxidants
Fimbriae	Fimbriae	Fimbriae are long filamentous polymeric protein structures located at the surface of bacterial cells. They enable the bacteria to bind to specific receptor structures and thereby to colonize specific surfaces
Flagella	Flagella	Virulence factor used by bacteria for motility
Flagellin	Flagellin	Principal component of bacterial flagella
FlgL	Flagellar hook-associated protein	Flagellum hook. Relevant for bacterial motility

Table 15.2 (continued)

Protein	Full name	Function
FliC	Flagellin	Subunit protein which polymerizes to form the filaments of bacterial flagella
Hcp1	Hemolysin co-regulated protein 1	T6SS substrate and forms part of its secretion tube
LolC	Lipoprotein-releasing system transmembrane protein	Part of an ATP-dependent transport system responsible for the release of lipoproteins targeted to the outer membrane
LPS	Lipopolysaccharide	Major component of the outer membrane of gram-negative bacteria
OmpA	Outer membrane protein A	Acts as a porin with low permeability that allows slow penetration of small solutes. Plays a role in maintaining the position of the peptidoglycan cell wall in the periplasm
OPS	O-polysaccharide	O-antigen of LPS
relA	GTP pyrophosphokinase	Catalyzes the formation of pppGpp which is then hydrolyzed to form ppGpp; ppGpp represses biofilm formation
SodC	Superoxide dismutase C	Periplasmic superoxide dismutase that protects bacteria from oxidative stress
spoT	(P)ppGpp synthase/ hydrolase	Enzyme able to synthesize and degrade (p)ppGpp
T2SS	Type II secretion system	Secretion system used by bacteria to translocate proteins from the periplasm across the outer membrane
T3SS	Type III secretion system	Virulence factor that enables some bacteria to directly inject effector proteins into host cells, facilitating colonization
T4SS	Type IV secretion system	Secretion protein complex found in gram-negative bacteria, gram-positive bacteria, and archaea. It transports proteins and DNA across the cell membrane, allowing for bacterial conjugation
T6SS	Type VI secretion system	Secretion machinery extended through the bacterial membrane, used to transport substrates from the bacterial cytoplasm directly into a target cell or extracellular space
tonB		Siderophore transmembrane transporter activity. Energy transducer

15.3.2 Inactivated Whole-Cell Vaccines

Historically, whole-cell, inactivated vaccines have not provided the same level of protection as live vaccines in rodent models of melioidosis. This has prompted novel approaches to improve their protective efficacy. Incorporation of cationic liposomes complexed with noncoding plasmid DNA (CLDC) to a heat-killed *B. pseudomallei* vaccine conferred 100% survival of immunized mice that lasted greater than 40 days [37]. In another study, synthetic microparticles composed of acetylated dextran with encapsulated *B. pseudomallei* cell lysate induced significant protection against a lethal challenge in mice [56].

15.3.3 Subunit Vaccines

One of the vaccine targets that have been extensively studied is the CPS, a known protective antigen and virulence determinant for *B. pseudomallei*. The CPS is a homopolymer of unbranched 1 → 3 linked 2-*O*-acetyl-6-deoxy-β-D-manno-heptopyranose. Immunization with *B. pseudomallei* CPS linked to CRM197, a non-toxic mutant of diphtheria toxin, elicited IgG and opsonizing antibody responses. Immunization with a combination of CPS-CRM197 and recombinant Hcp1 protected 100% of mice following an otherwise lethal inhalation challenge with *B. pseudomallei* [46]. In another study, immunization with chemically synthesized CPS conjugated to tetanus toxoid induced serum IgM and IgG antibodies. While the CPS-specific antibody titers were considerably less than that induced by native CPS, 66% of BALB/c mice survived challenge with *B. pseudomallei* [48].

Immunization with lipopolysaccharide (LPS) conjugated to the Hc fragment of tetanus toxoid produced a better survival outcome (81%) compared to immunization with LPS alone (62%) [44]. *B. pseudomallei* O-polysaccharide (OPS II) conjugated to a carrier protein AcrA from *Campylobacter jejuni* elicited similar levels of protection to killed bacteria against a highly lethal challenge [49]. LPS glycoconjugates have also been successfully incorporated onto the surface of gold nanoparticles (AuNP). Mice immunized with AuNP-FlgL-LPS alone or with a protein combination (FlgL, Hcp1, and hemagglutinin) demonstrated up to 100% survival and reduced lung colonization following a lethal challenge with *B. pseudomallei* [51].

Other protein subunits have been identified as possible candidates due to their immunogenicity, such as FliC, BopE, AhpC, and several outer membrane proteins (OMP) [61–66]. A DNA vaccine composed of flagellin reduced bacterial burdens, lung inflammation, and pathology. Levels of plasma TNF-α, IFN-γ, and MCP1 cytokines were also reduced, signaling a reduction in systemic inflammation. Vaccinated mice displayed 53% survival compared to 15% survival in control mice [45]. Mice immunized with a single dose of parainfluenza virus 5 (PIV5) expressing BatA, a *Burkholderia* autotransporter protein, displayed 60% overall survival, with 78% of surviving mice clearing bacteria in the lungs and 44% clearing bacteria in the spleen [67]. A synthetic biology approach was used to predict the antigenicity and toxicity of a recombinant collagen-like 8 protein that is conserved among *Burkholderia* species [68]. Two formulations were created, one with a recombinant protein and another one using the β-barrel portion of the protein. Use of β-barrel peptides from Buc (Bucl8) adjuvanted with an oil-in-water nano-emulsion produced a strong Th2 response in C57BL/6 mice.

B. pseudomallei outer membrane vesicles (OMVs) contain numerous protective antigens associated with the bacterial outer membrane including OmpA, CPS, and LPS. *B. pseudomallei* OMV vaccines have demonstrated safety, immunogenicity, and protection against pneumonic and septicemic disease in mice and nonhuman primates [53, 54, 69]. When OMVs are produced under macrophage mimicking growth conditions, they contain proteins that are associated with intracellular

survival, conferring strong protection against inhalational melioidosis and eliciting both humoral and cellular immune responses [55]. Due to the sophisticated intracellular pathogenesis of *B. pseudomallei*, inclusion of antigens expressed during the chronic stage of infection may be important for obtaining complete vaccine protection. Three proteins, BPSL1897, BPSL2287, and BPSL3369, which were expressed in chronically infected mice, were combined with the surface protein OmpA (BPSL2765) to create a multicomponent subunit vaccine formulation. Mice immunized with the chronic antigens plus LolC or CPS displayed increased survival compared to mice immunized with only the individual subunits, LolC, or CPS [43].

15.4 Conclusions

In recent years, several promising vaccine candidates have emerged to combat melioidosis, which is considered an emerging and expanding infectious disease. Adjuvants are also being evaluated as part of vaccine development as they can drive different protective components of humoral and cellular immune responses. Going forward, it will be important to elucidate the immune mechanisms of protection to better inform vaccine design and to establish immune correlates of vaccine protection. Based on recent success in animal models, human clinical trials are currently being planned for more than one vaccine candidate. These include a phase 1 clinical trial of vaccine candidate CPS-CRM197/Hcp1 adjuvanted with alum/CpG that was developed at the University of Nevada, Reno, USA [46]. The trial will take place in Oxford in 36 healthy human subjects with a follow-up phase 1b planned in Ubon Ratchathani, Thailand. An OMV vaccine, developed at Tulane University, Louisiana, USA, is also being considered for phase 1 clinical trials planned in Australia [55]. For any vaccine, larger phase 2 and 3 clinical trials would be necessary to demonstrate vaccine safety and efficacy. Nonetheless, initiation of human vaccine trials for a disease once largely ignored sparks hope that licensure of human vaccines to prevent melioidosis may soon be realized.

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