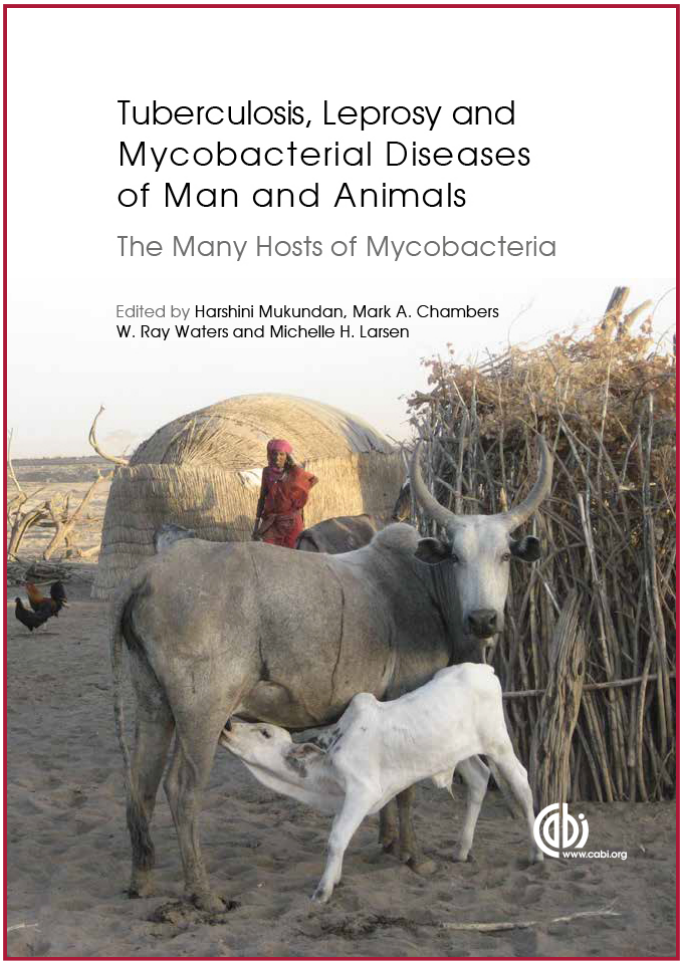
Tuberculosis, Leprosy and Mycobacterial Diseases of Man and Animals

The Many Hosts of Mycobacteria



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Immunopathogenesis of Mycobacterium bovis Infection of Cattle

Tuberculosis, Leprosy and Mycobacterial Diseases of Man and Animals

The Many Hosts of Mycobacteria

Edited by

Harshini Mukundan

Los Alamos National Laboratory (LANL), USA

Mark A. Chambers

University of Surrey, UK;
Animal and Plant Health Agency (APHA), UK

W. Ray Waters

United States Department of Agriculture (USDA), USA

and

Michelle H. Larsen

Albert Einstein College of Medicine (AECOM), Yeshiva University, USA

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CABI
Nosworthy Way
Wallingford
Oxfordshire OX10 8DE
UK

Tel: +44 (0)1491 832111
Fax: +44 (0)1491 833508
E-mail: info@cabi.org
Website: www.cabi.org

CABI
745 Atlantic Avenue
8th Floor
Boston, MA 02111
USA

Tel: +1 (617) 682 9015
E-mail: cabi-nao@cabi.org

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Introduction – The Many Hosts of Mycobacteria: An Interdisciplinary Approach to Understanding Mycobacterial Diseases

Christine Sizemore,* Karen Lacourciere and Tina Parker
National Institutes of Health, Bethesda, USA

Mycobacteria have been associated with human and animal disease for millennia. In particular, tuberculosis (TB) continues to cause significant human morbidity and mortality worldwide. The discovery in 1882 of the tubercle Bacillus, *Mycobacterium tuberculosis*, by the German physician and microbiologist Robert Koch was met with great enthusiasm as it defined the infectious nature of the disease. By 1915, a collaboration between the physician Albert Calmette and the veterinarian Camille Guérin resulted in the development of an attenuated strain of the bovine tubercle Bacillus, *Mycobacterium bovis*, that later became the basis of the Bacille Calmette–Guérin (BCG) vaccine, which is one of the most widely used childhood vaccines in the world. This discovery is a wonderful example of how, for centuries, collaborations among multiple scientific disciplines have positively impacted the control of infectious disease.

Since 2007, scientists from the United States Department of Agriculture's Agricultural Research Service (USDA, ARS), the Albert Einstein College of Medicine (AECOM) and the National Institute of Allergy and Infectious Diseases (NIAID) of the National Institutes of Health (NIH) have been convening the workshop *Many Hosts of Mycobacteria*. The workshop was founded on a principle of cross-disciplinary inclusion and the belief that by bringing together all members of the mycobacterial research community we could achieve a better understanding of mycobacteria and the diseases caused by them, and thus contribute to knowledge and the development of products to improve global health.

When researching a human infectious disease, experimental animal models are often employed to create or test hypotheses. However, the study of natural infections in animals, and the information that can be gained from them, is often overlooked. The knowledge that can be obtained by determining mechanisms that drive host specificity for related pathogens, as well as understanding disease transmission and differences in disease progression and presentation for the same pathogens in different hosts, can enhance our understanding of human disease. Focusing only on human disease also neglects the role of wildlife, livestock and other peri-domestic animals in transmission of infectious diseases. By bringing together a multidisciplinary team of leading scientists studying mycobacterial infections in humans and animals, *Many Hosts*

* E-mail: csizemore@niaid.nih.gov

of Mycobacteria created a venue for sharing knowledge about the spectrum of mycobacterial diseases, exploring host–pathogen variability, and understanding what the commonalities and differences in disease presentation and host specificity teach us.

Promoting discussion among experts in scientific disciplines that traditionally do not interact has resulted in the critical evaluation of mycobacterial infections in natural and artificial hosts and the identification of areas of mutual interest and collaboration. Participants in the workshop span from basic scientists to clinicians, animal modellers and product developers to individuals with zoological and wildlife expertise to cover the breadth of known species of mycobacteria and the various hosts that harbour them.

Many Hosts of Mycobacteria was initially driven by the TB research community's need to better understand and interpret the results of studies of candidate vaccines against human TB that were tested in a variety of animal species – cattle, non-human primates, guinea pigs and mice. Different animal models are useful for understanding particular aspects of human TB disease, but not all of these animals are practical models for many research needs because of size or cost. Initial discussions focused on differences in the host immune responses, but quickly evolved to include perspectives on how the molecular and pathophysiologic differences between various mycobacterial pathogens can manifest with a distinct host or tissue specificity and can be exploited in experimental models. As the workshop progressed, it became clear that these interdisciplinary discussions were providing unique insights that could not be gained from studying individual mycobacteria or hosts alone. By comparing the similarities and differences between each of the mycobacterial pathogens and hosts, the participants gained a better understanding of all mycobacteria. The concept of 'comparative mycobacteriology' was born as a framework to evaluate disease pathology and promises to contribute to different aspects of infectious disease research, which could ultimately improve vaccines, diagnostics and therapeutics for mycobacterial diseases such as TB and leprosy.

Although the greatest need for development of interventions for mycobacterial diseases is to improve human health, there are also many important applications for animals. Cattle are often infected from wildlife reservoirs such as deer, badgers or possums. Once an infection in livestock is discovered, the entire herd is typically culled, leading to considerable economic losses. In resource-limited settings where families are dependent on cattle for food and income, infected animals are frequently not culled and may lead to infection of humans through the consumption of meat or milk. A vaccine for cattle or the wildlife reservoirs of mycobacteria may minimize these infections. Zoo animals such as elephants can contract TB from their handlers. Colonies of non-human primates that are used in research are also affected by *M. tuberculosis* infection resulting in the removal and loss of valuable animals and research. A diagnostic that allows earlier detection and isolation to prevent transmission may be able to help protect these important animals.

The *Many Hosts of Mycobacteria* workshop has fostered an interdisciplinary approach and unique collaborations that benefit multiple scientific communities. The sharing of ideas and results across disciplines has allowed for enhancement of research in *M. tuberculosis*, *M. bovis*, *M. leprae* and other non-tuberculous mycobacteria. Most of all, it has increased the knowledge of researchers who are developing vaccines, diagnostics and drugs to prevent, diagnose and treat some of the oldest pathogens of man. This book serves to share the lessons learned from these workshops not only within the mycobacterial community, but also to a wider audience, as this approach may benefit other fields of research.

About the Cover

Mycobacterial diseases affect humans, livestock and wildlife in all regions of the world. Pathogenic mycobacteria can infect a wide variety of species, passing between them in complex webs of infection. We chose the cover photograph to illustrate vividly the intimate human–animal interface in a region of the

world (Sub-Saharan Africa) with a relatively high prevalence of bovine and human tuberculosis, leprosy and Buruli ulcer. The intent of the image is to capture the One Health essence of mycobacterial disease research. The suckling calf in the image demonstrates the role of ingestion of non-pasteurized milk in *Mycobacterium bovis* transmission. The indigenous Zebu cattle and Ethiopian tribesman portray genetic and socio-economic factors affecting control measures in diverse populations. In the background, chickens (likely to be harbouring *M. avium*) represent the confounding role of non-tuberculous mycobacteria for development of improved vaccines and diagnostic tests. In addition, iconic wildlife species (e.g. lions, elephants and rhinoceros) are native to Sub-Saharan Africa and survival of these species is hindered by chronic and debilitating infections with tuberculous mycobacteria species. (Photograph taken by Rea Tschopp (Wildlife Veterinarian/Epidemiologist at the Armauer Hansen Research Institute in Addis Ababa, Ethiopia).)

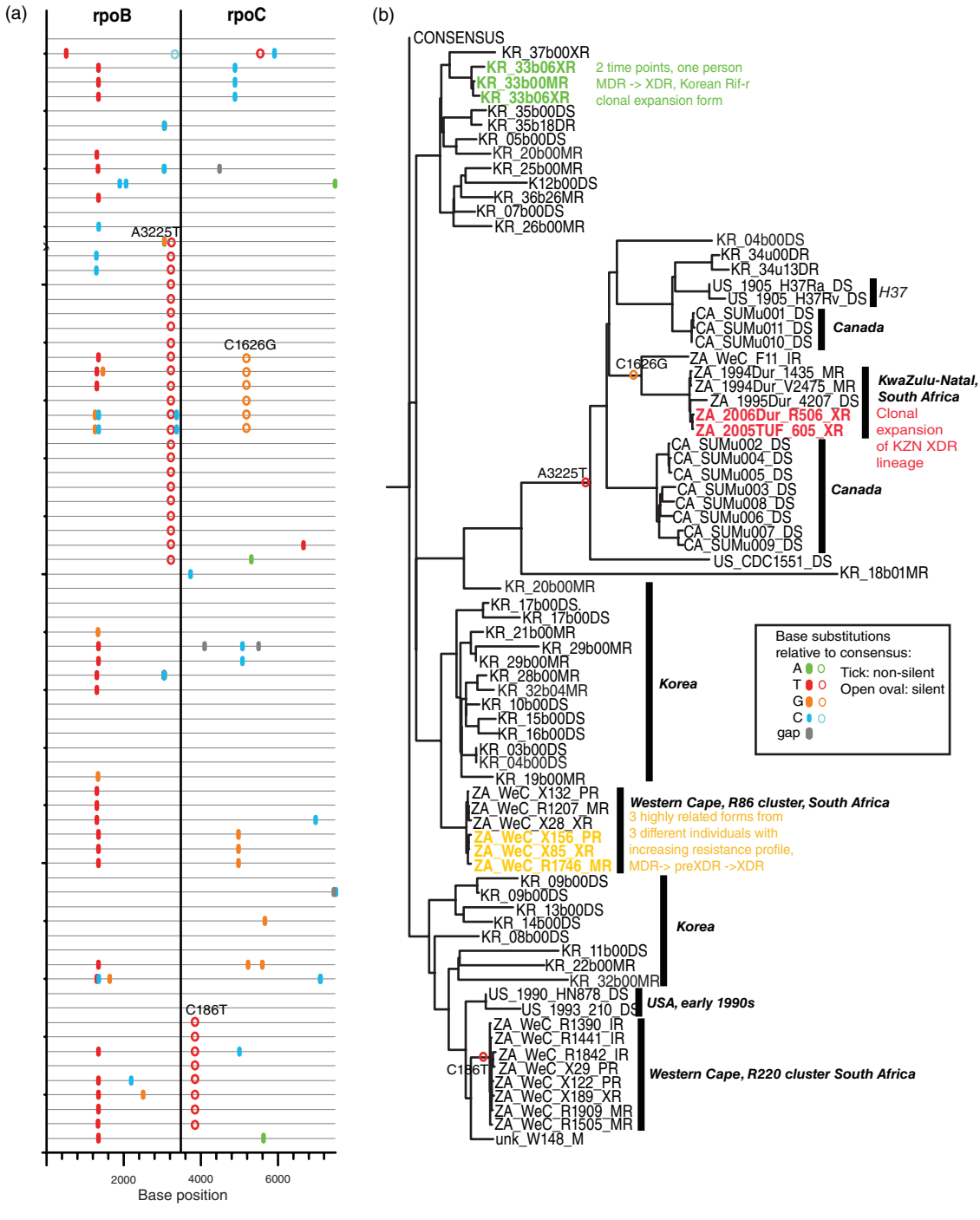

Plate 1. Mutational patterns associated with Rif resistance from sequences with known drug sensitivity, organized according to phylogenetic relationships.

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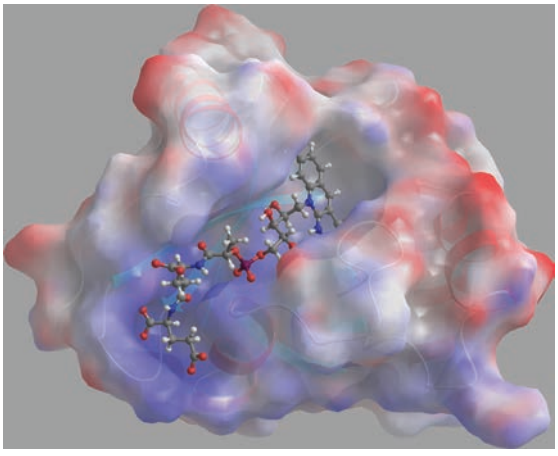

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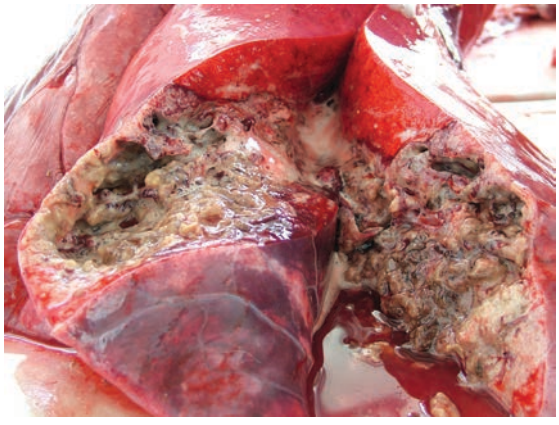

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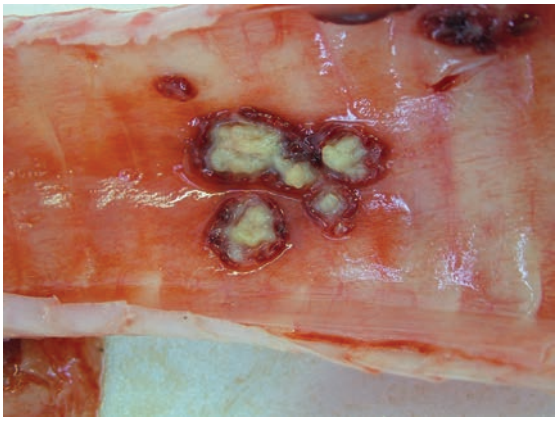

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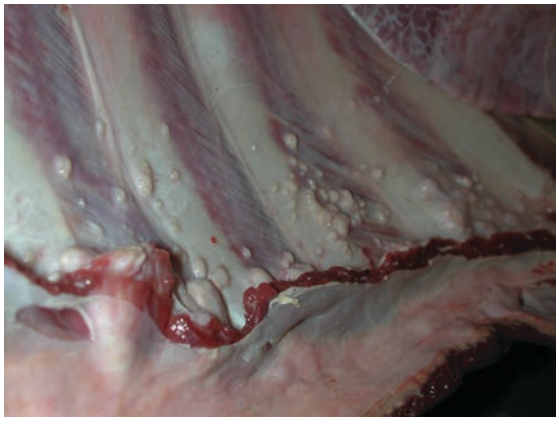

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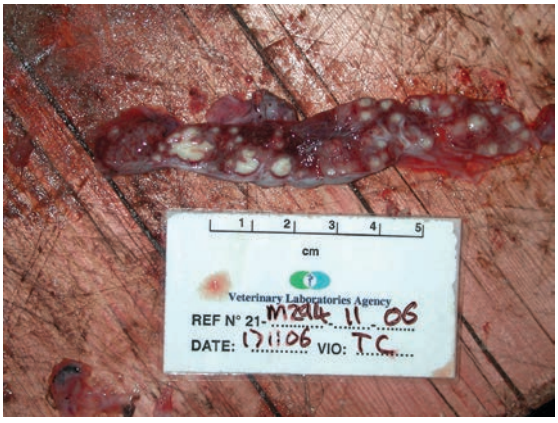

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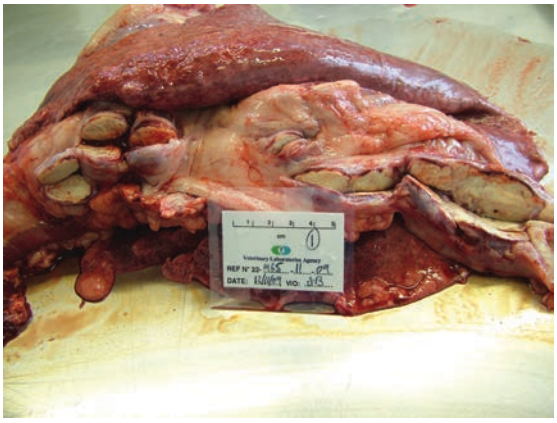

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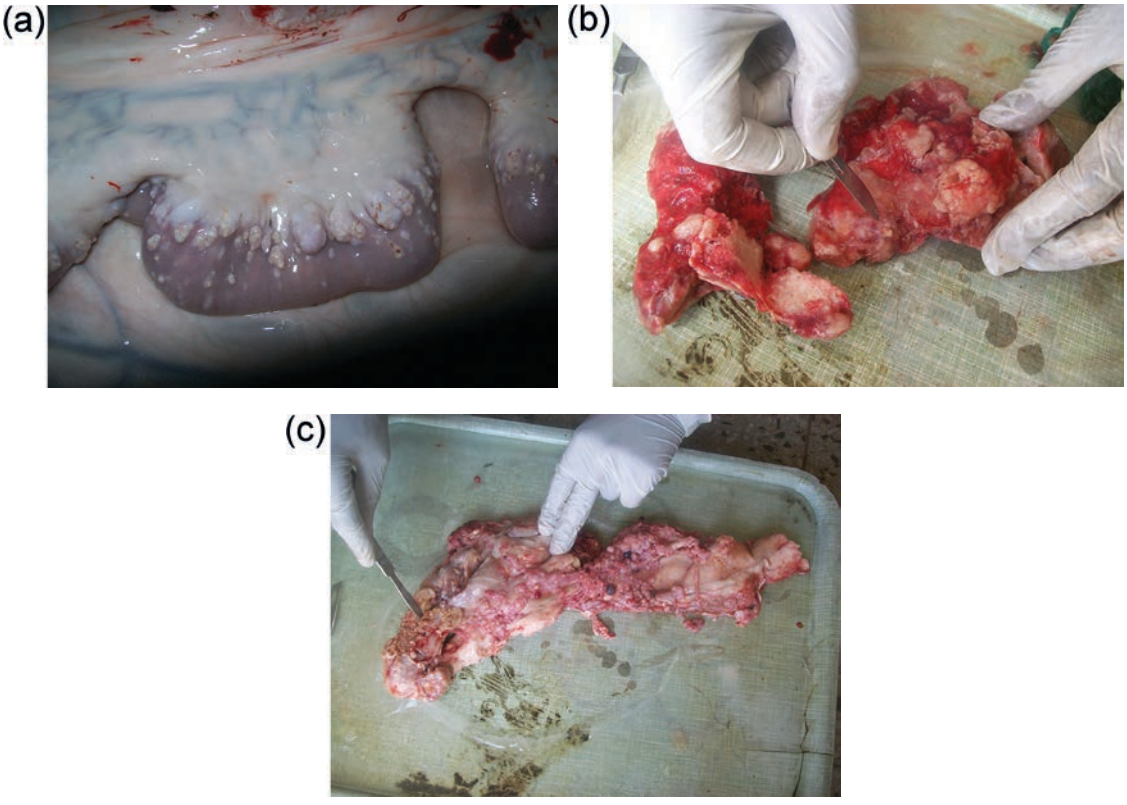

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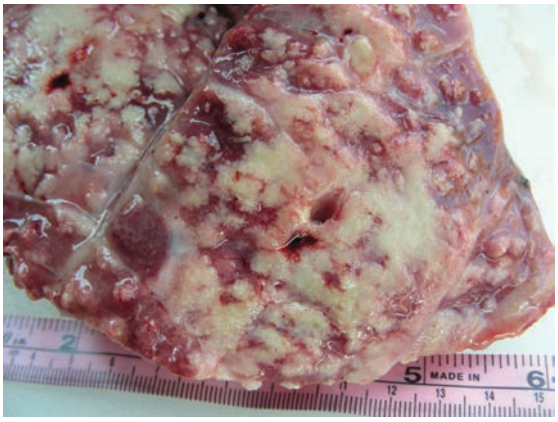

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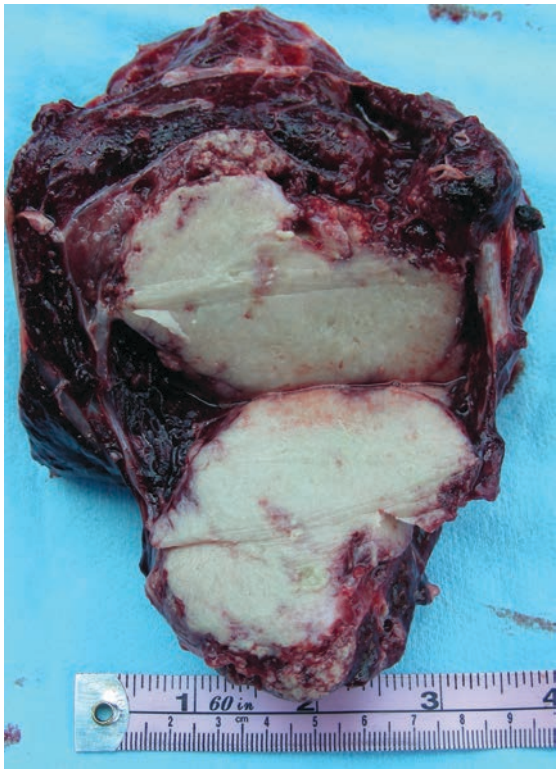

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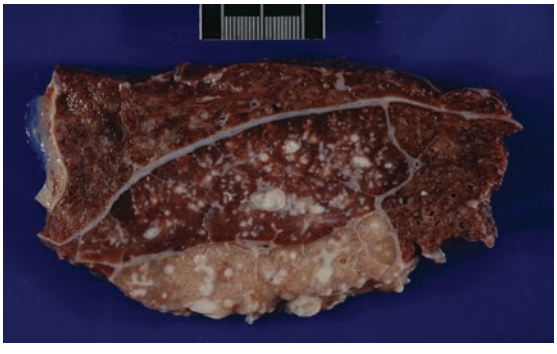

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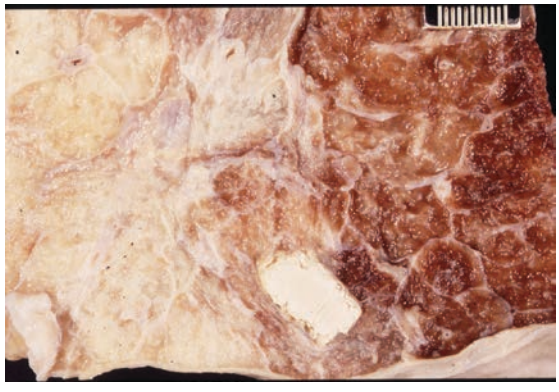

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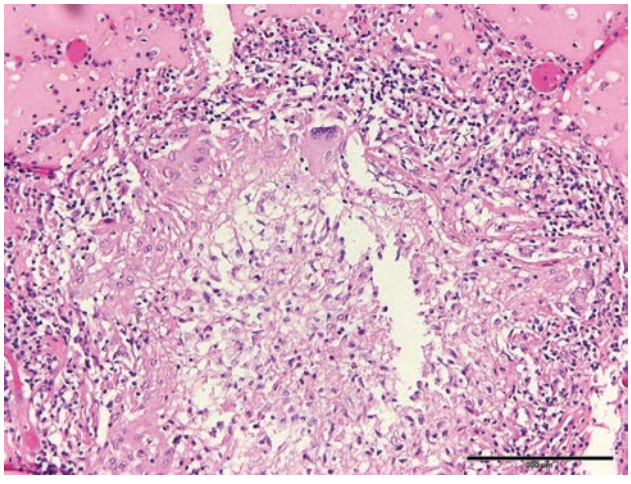

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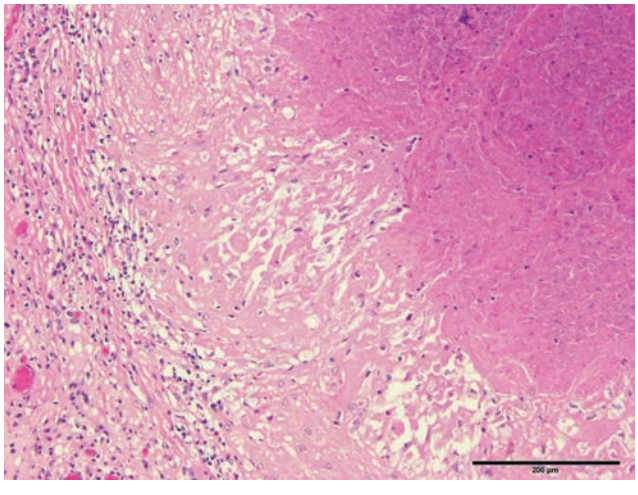

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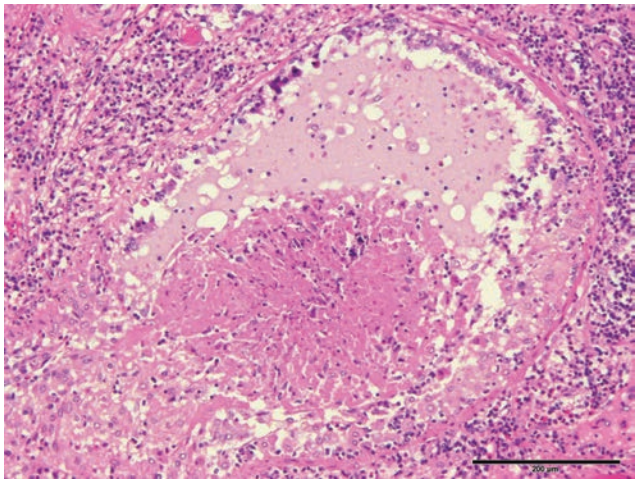

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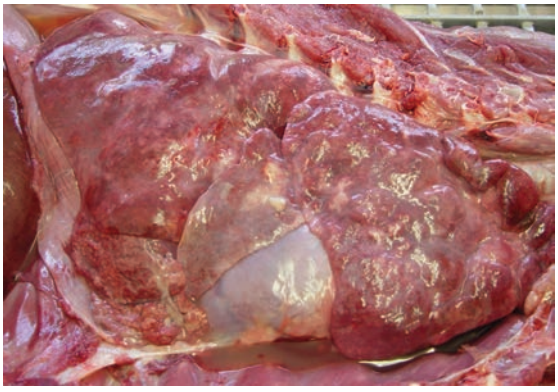

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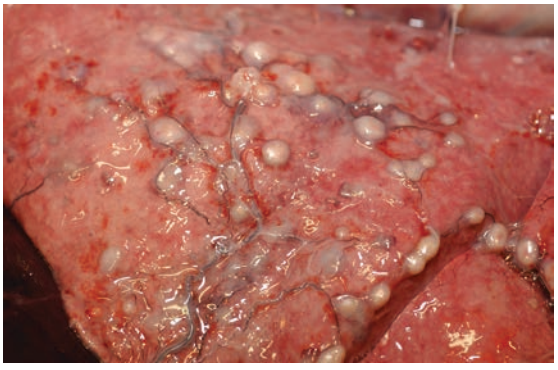

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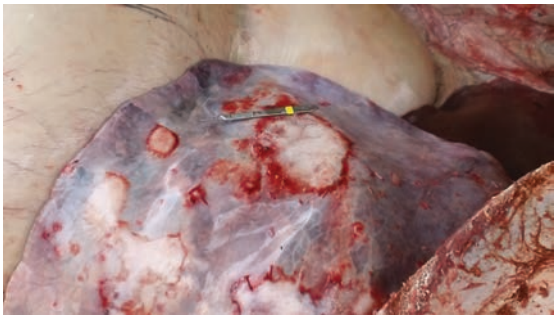

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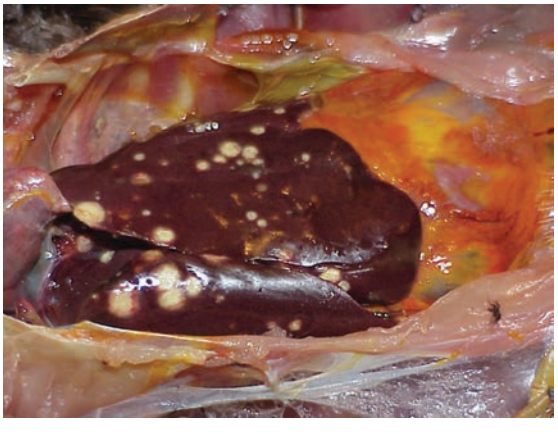

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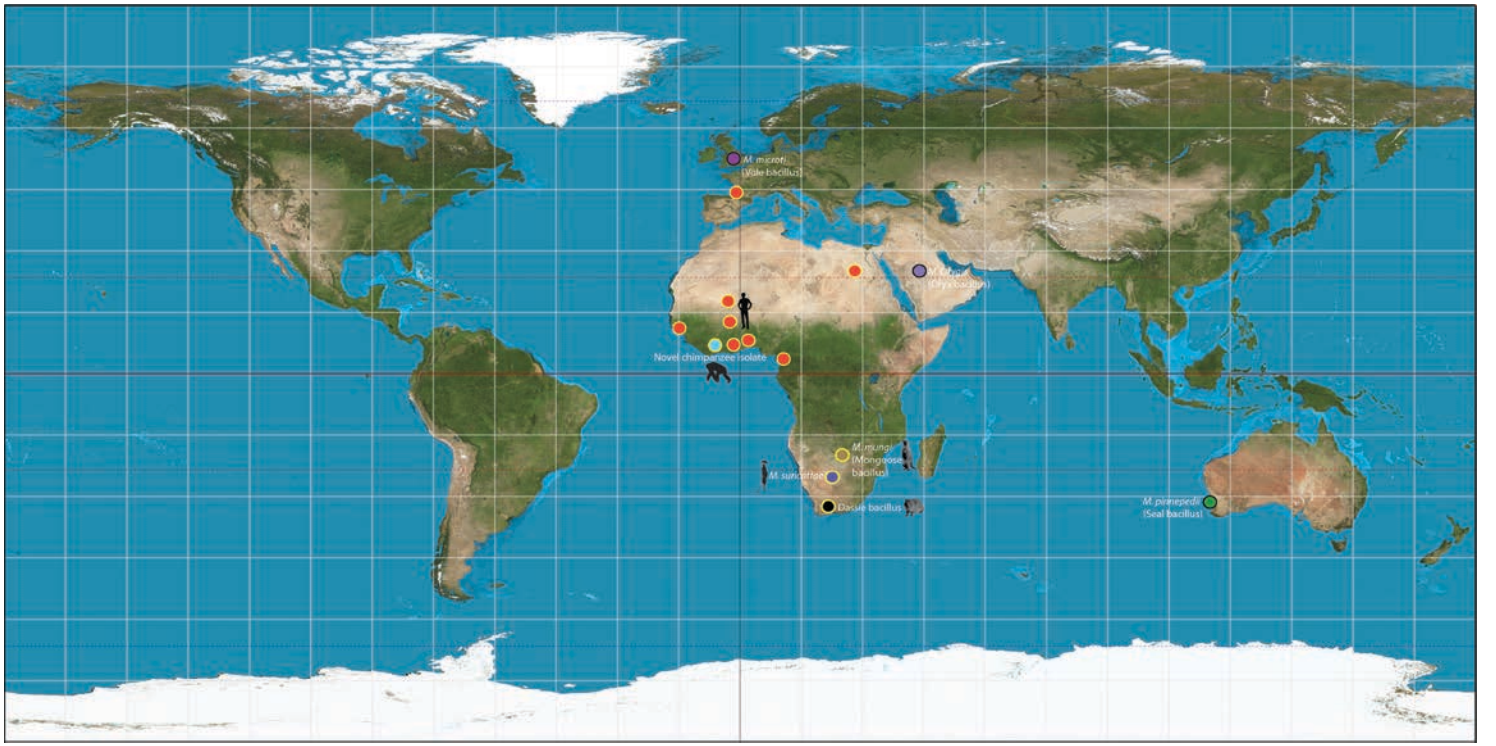

Plate 20. Origin of lineage six wildlife-associated TB strains in southern Africa (circles with yellow boundary) and human-associated *M. africanum* in northwest Africa (orange circles with yellow boundary). Other wildlife-adapted TB strains identified in the phylogenetic schematic (circles with black boundary) are included for comparison.

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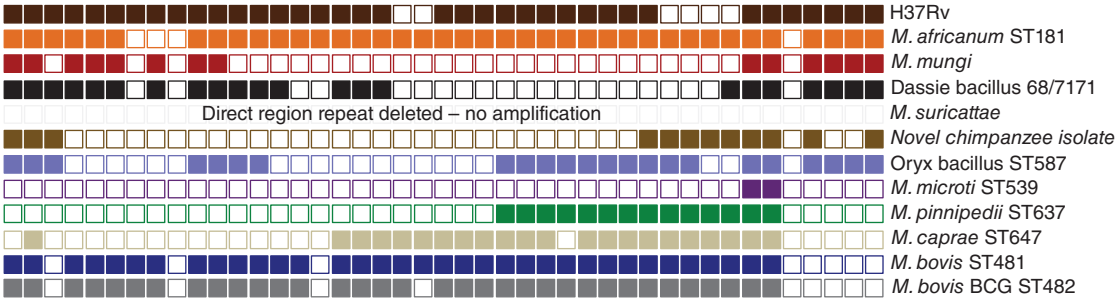

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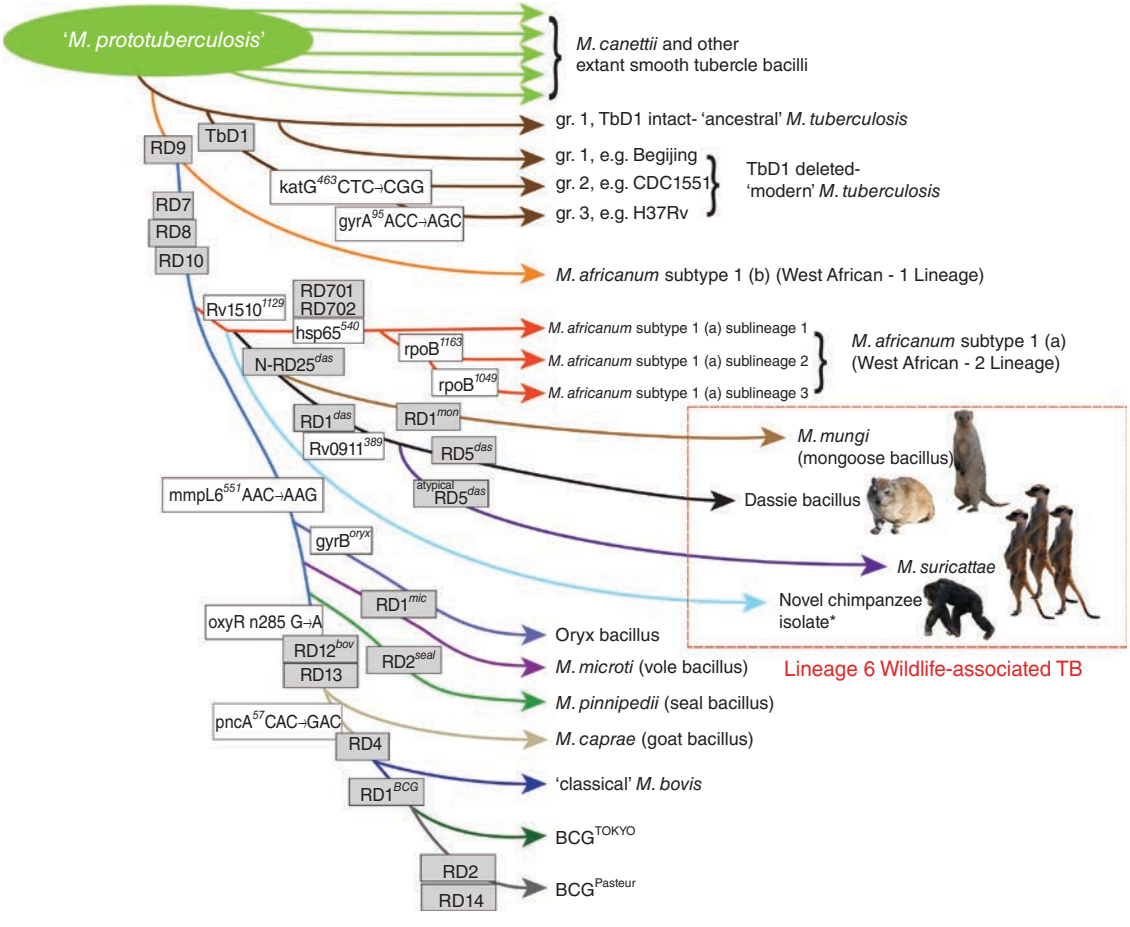

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8 Immunopathogenesis of *Mycobacterium bovis* Infection of Cattle

W. Ray Waters,[1]* Jayne C. Hope,[2] Carly A. Hamilton,[2] Mitchell V. Palmer,[1] James McNair,[3] Robin A. Skuce,[3,4] Adrian R. Allen,[3] Bryce M. Buddle,[5] Bernardo Villarreal-Ramos[6] and H. Martin Vordermeier[6]

[1]National Animal Disease Center, Ames, USA; [2]University of Edinburgh, Edinburgh, UK; [3]Agrifood and Biosciences Institute, Belfast, UK; [4]Queens University, Belfast, UK; [5]Hopkirk Research Institute, Palmerston North, New Zealand; [6]Animal and Plant Health Agency, Addlestone, UK

Introduction

Infection with the tubercle bacillus, and the ensuing immunopathogenesis, is a quintessential example of the complex and ancient interplay between host and pathogen (reviewed in Cooper and Torrado, 2012; Reece and Kaufmann, 2012). Upon entry into the host, *Mycobacterium tuberculosis* has a unique ability to delay onset of adaptive immune responses in mice or humans (reviewed in Ottenhoff, 2012). Likewise, adaptive responses are delayed with *M. bovis* infection of cattle (Vordermeier *et al*., 2009a; Waters *et al*., 2009). This delay in the adaptive response is likely to give the pathogen both a foothold for infection (a critical mass of tubercle bacilli) and a defined niche to dictate the ensuing response (Cooper, 2009). The tubercle bacillus is also armed with a multitude of immune evasion tactics enabling intracellular survival and persistence. Examples given by Ottenhoff (2012) include:

1. Inhibition of important host defence mechanisms such as phagosome maturation and phagolysosome fusion, autophagy, antigen processing and presentation, apoptosis and interferon (IFN)-γ receptor signalling.
2. Capacity to escape from the hostile phagosome to the cytoplasm.
3. Mechanisms to inhibit and scavenge toxic oxygen and nitrogen intermediates.

Once infection is established, primarily lymphocytes and macrophages are recruited to the site of infection in humans and cattle, forming granulomas. Granulomas are dynamic structures with continual movement of host cells into and out of the structure, orchestrated by the host as well as by the pathogen (Ramakrishnan, 2012). With humans, various stages of tuberculosis are recognized including active (either primary or post-primary), latent and reactivation or secondary disease (Barry *et al*., 2009; Young *et al*., 2009; Hunter, 2011). Primary disease occurs when immunocompetent individuals are first infected with *M. tuberculosis*. With this form, the bacillus typically spreads from the initial site of infection to regional lymph nodes (and potentially elsewhere) until immunity develops and the infection is controlled. Only 5–10% of individuals infected

* E-mail: ray.waters@ars.usda.gov

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with *M. tuberculosis* develop clinical manifestations of primary tuberculosis within 2 years after exposure. The majority of infected individuals develop latent tuberculosis, defined as having evidence of *M. tuberculosis* infection by tuberculin skin test or IFNγ release assay (IGRA) without clinical signs of disease (Lin and Flynn, 2010). Latent infection is a dynamic equilibrium in which the host controls but does not clear the bacillus (Behr and Waters, 2013). With *M. bovis* infection of cattle, the disease is generally considered slowly progressive, without clear delineation of the various stages associated with tuberculosis of humans (Pollock *et al*., 2006). As with human tuberculosis (originally described in Bayle, 1810; Laënnec, 1837), the disease in cattle may be defined microscopically according to the degree of fibrosis, necrosis, caseation and mineralization (Wangoo *et al*., 2005). Thus, there are many similarities in the immunopathogenesis of bovine and human tuberculosis, and a few distinct differences.

Aerosol and intratracheal inoculation are routinely used for experimental biology purposes to infect cattle with virulent *M. bovis*, each resulting primarily in a respiratory tract infection including lungs and lung-associated lymph nodes (reviewed in Vordermeier, 2010). Disease severity is dose- and time-dependent, closely mimicking natural infection of cattle (Buddle *et al*., 1994; Palmer *et al*., 2002). Recently, unique insights into *M. bovis* transmission have been gained through 'in contact' studies in which sentinel cattle are exposed to *M. bovis*-infected cattle in a model of natural infection (Khatri *et al*., 2012). With each of these routes of exposure, experimental approaches permit disease confirmation through post-mortem examination with laboratory analysis by histopathology and bacterial culture, defining the relationship between dose and route of infection, immune response and the pathogenesis of infection (Waters *et al*., 2012). The bovine infection model also provides significant opportunities to investigate the basis of genetic susceptibility and impacts of co-infection on pathogenesis and diagnostic techniques (Kao *et al*., 2007; Flynn *et al*., 2009; Claridge *et al*., 2012). Finally, access to naturally infected cattle provides a unique opportunity to evaluate vaccine and ante-mortem testing strategies, particularly as animals are often available for post-mortem inspection for infection confirmation as well as for gross and microscopic assessment of lesions. Thus, intervention strategies may be directly assessed, as opposed to being inferred based upon immunologic and clinical parameters.

Pathogenesis

The caseonecrotic granuloma is one of the hallmarks of tuberculosis. With the aim of gaining an understanding of the temporal evolution of the granuloma, researchers have performed sequential analysis of granulomas in experimentally infected cattle (Cassidy *et al*., 1998; Palmer *et al*., 1999). Microscopic granulomas can be seen as early as 7–15 days after experimental infection with 10^5 colony-forming units of *M. bovis* (Palmer *et al*., 2007). Early lesions, categorized as Stage I (initial, Fig. 8.1) granulomas (Wangoo *et al*., 2005; Palmer *et al*., 2007) are composed of accumulations of epithelioid macrophages with low numbers of lymphocytes, neutrophils and Langhan's multinucleated giant cells. Importantly, necrosis is absent in Stage I granulomas. Between 21 days and 60 days after experimental infection there is a steady progression through granuloma stages. Stage I granulomas are followed by Stage II (solid, Fig. 8.1) granulomas that are similar to Stage I granulomas but have central infiltrates of neutrophils and lymphocytes and a thin fibrous capsule. Central necrosis may be present. Stage III (necrotic, Fig. 8.1) granulomas exhibit complete fibrous encapsulation and significant central necrosis. Stage IV (necrotic and mineralized, Fig. 8.1) granulomas are characterized by multiple coalescing caseonecrotic granulomas with multicentric necrosis, dystrophic mineralization and thick fibrous encapsulation. By 60 days after experimental infection, granulomas of all four stages may be present on the same microscopic section of tissue. In fact, granulomas with morphology typical of Stage I may be seen in proximity to granulomas of more advanced stages, and are sometimes referred to as satellite granulomas. Acid-fast bacilli can be present in all stages

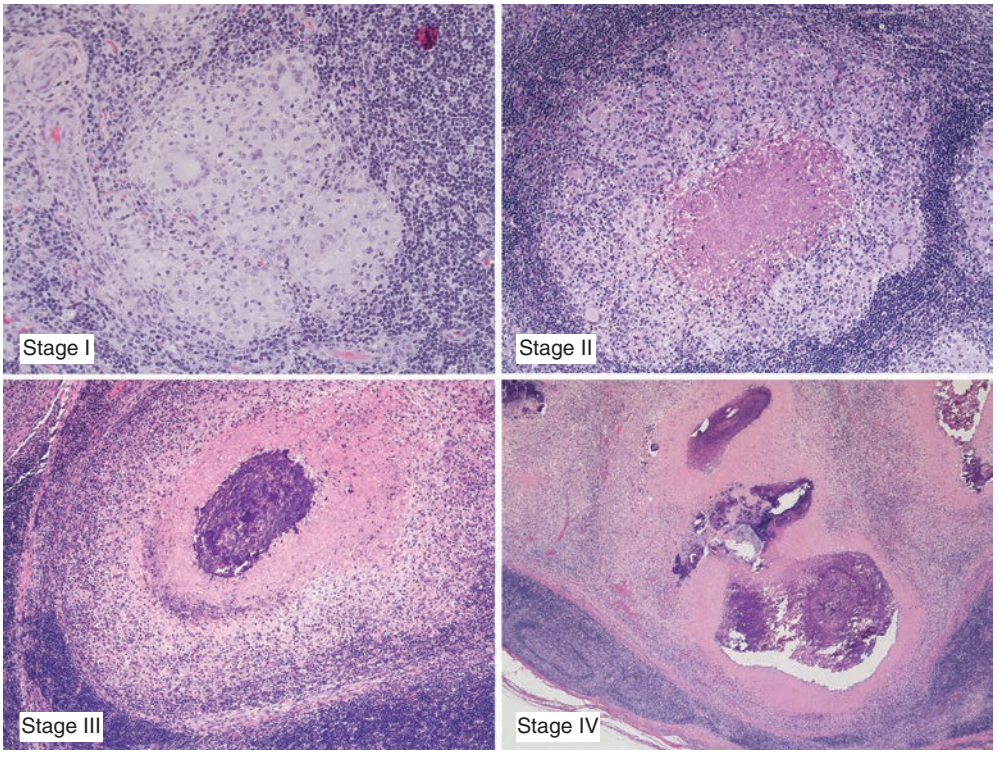

Fig. 8.1. Microscopic tuberculous lesions are staged (I–IV) based on adaptations of the criteria described by Wangoo *et al*. (2005). Stage I (initial) granulomas are characterized by accumulations of epithelioid macrophages with low numbers of lymphocytes and neutrophils. Multinucleated giant cells may be present but necrosis is absent. Acid-fast bacilli are often absent or present in low numbers within macrophages or multinucleated giant cells. Stage II (solid) granulomas are characterized by accumulations of epithelioid macrophages surrounded by a thin connective tissue capsule. Infiltrates of neutrophils and lymphocytes may be present as well as multinucleated giant cells. Necrosis when present is minimal. Stage III (necrotic) granulomas are characterized by complete fibrous encapsulation. Necrotic cores are surrounded by a zone of epithelioid macrophages admixed with multinucleated giant cells and lymphocytes. Stage IV (necrotic and mineralized) granulomas are characterized by thick fibrous capsules, irregular multicentric granulomas with multiple necrotic cores. Necrotic cores contain foci of dystrophic mineralization. Epithelioid macrophages and multinucleated giant cells surround necrotic areas and there may be moderate to dense infiltratres of lymphocytes. Acid-fast bacilli are often present in moderate numbers primarily located within the caseum of the necrotic core. Magnifications: Stage I, 20×; Stage II, 10×; Stage III, 10× and Stage IV, 4×.

but have been shown to be most numerous in Stage IV granulomas (Palmer *et al*., 2007).

T-cell immunity is considered essential in clearance of mycobacterial infections. As such, $CD3^+$ $CD4^+$ T cells are the predominant lymphocyte subtype in granulomas of all stages. Lower numbers of $CD8^+$ T cells, γδ T cells, as well as B cells, can be found in Stages I–III. Stage IV granulomas may contain dense clusters of B cells in the periphery or outside the capsule. γδ T cells have been described in both early- (Palmer *et al*., 1999) and late-stage granulomas (Wangoo *et al*., 2005).

It has been shown that the dose of infection when administered within a relatively low dose range (i.e. 1–1000 colony-forming units, CFU) does not necessarily correlate to the level of pathology once infection takes place (Johnson *et al*., 2007). However, in this same study it was shown that the proportion of

cells expressing IFNγ was directly affected by the infection dose. Thus, the nature of the immune response observed at the site of infection may vary according to the initial bacterial load; the higher the infectious dose, the higher the proportion of IFNγ positive cells in the granuloma.

In another study, Aranday-Cortes *et al*. (2013) determined that the presence of certain cytokines and chemokines was related to the stage of progression of the granuloma. Thus, IL-17A and CXCL9 were found to be transcribed to a greater extent in type I as compared to type IV granulomas. CXCL10 was also found to be transcribed to a greater extent in type I as compared to type IV granulomas; however, this was not a linear relationship, as type II and type III granulomas expressed lower CXCL10 than type IV granulomas. These results indicate that there may be characteristic responses in the development of the granuloma that could inform whether a lesion is progressing or is being contained. However, the data also indicate that the immune response during tuberculosis is complex and may not necessarily follow a linear pattern of progression.

An intended goal of vaccination is to elicit a more rapid and targeted immune response upon subsequent exposure to the pathogen, thereby limiting granuloma development. Johnson *et al*. (2006) found that granulomas in cattle vaccinated with bacille Calmette-Guerin (BCG) subsequently challenged with *M. bovis* were reduced in number and size as compared to granulomas found in non-vaccinated cattle challenged with *M. bovis*. Importantly, the percentage area stained for lymphocytes (i.e. primarily $CD3^+$ and γδ TCR^+ cells) and macrophages (i.e. $CD68^+$ cells) was reduced in BCG-vaccinated as compared to non-vaccinated animals after challenge with virulent *M. bovis*. Additionally, staining for B cells (i.e. $CD79^+$ cells) and IFNγ clustered more intensely in the medullary regions of lymph nodes of vaccinated animals as compared to those of controls. Thus, the priming of cattle with BCG affects the subsequent immune response to the pathogen at the site of infection.

It is likely that the classification of the TB granuloma into stages of development as well as determining the immune response within and in the surrounding areas of lesions will inform future studies in vaccine and diagnostic developments. Dean *et al*. (2014) recently used the classification of the TB granuloma as a tool to determine the level of vaccine protection against *M. bovis*. Future studies aimed at finding correlates between peripheral and local immune responses will permit the development of tools to assist discrimination between vaccinated and infected animals, as well as defining responses favourable for controlling infection. This in turn will inform vaccine and diagnostic development.

Innate Immune Responses and Initiation of Adaptive Immune Responses

Dendritic cells and macrophages

Dendritic cells (DC) are a heterogeneous population of professional antigen-presenting cells (APC) which are essential mediators of immunity (Banchereau and Steinman, 1998) and tolerance (Steinman *et al*., 2003). They originate from stem cell precursors in the bone marrow which give rise to circulating myeloid or lymphoid precursors within the blood that enter tissues and reside as immature DC. These cells act as sentinels and respond to infection, inflammatory signals or tissue damage by migrating away from the periphery towards lymph nodes where immune responses may be induced. During migration DC undergo maturation characterized by an increased expression of costimulatory molecules, adhesion molecules, reduced endocytosis and redistribution of intracellular MHC II to the cell surface. Thus DC are primed to effectively stimulate naïve or memory T-cell responses within the lymph nodes.

In cattle there is evidence (as in other species) for considerable heterogeneity in DC populations: both functional and phenotypic divergence has been reported, and cells of both myeloid and lymphoid origin are present (Howard and Hope, 2000; Miyazawa *et al*., 2006; Reid *et al*., 2011). Subpopulations of DC draining the skin (McKeever *et al*., 1991; Howard *et al*., 1997; Brooke *et al*., 1998) and mucosal

surfaces have been described which have differential capacity not only to stimulate T-cell responses (Howard *et al*., 1997) but which also display divergent capacities to interact with mycobacteria. Monocyte-derived DC (MoDC) infected with *M. bovis* or BCG induced effective memory $CD4^+$ and $CD8^+$ T-cell responses (Hope *et al*., 2000). However, assessment of *ex vivo* populations of DC isolated from afferent lymphatic vessels revealed that only a subset could uptake and present antigens from BCG or *M. bovis* (Hope *et al*., 2012). This could impact vaccine efficacy or the induction of protective immunity to infection.

Macrophages are a diverse population of specialized phagocytic cells which are essential for host defence, homeostasis and wound repair. They are derived from bone marrow precursors and circulating blood monocytes which differentiate into resident macrophages or DC upon tissue entry (Verschoor *et al*., 2012). Macrophages are considered the major host cell for mycobacteria *in vivo* and, due to the respiratory route of entry, alveolar macrophages have a pivotal role during mycobacterial immune responses. Macrophages express an array of cell surface receptors that recognize mycobacteria including toll-like receptors (TLRs) (Means *et al*., 1999), the macrophage mannose receptor that identifies mannosylated glycoproteins, Fc receptors which can bind opsonized cells, and complement receptors (Ernst, 1998). The early interactions of mycobacteria with DC in the respiratory tract also involve TLRs including TLR2, 4 and 9 but, binding to dendritic cell specific intracellular adhesion molecule 3, grabbing non-integrin (DC-SIGN) is particularly important for uptake of *M. tuberculosis* (Means *et al*., 1999; Tsuji *et al*., 2000; Von Meyenn *et al*., 2006). The downstream events triggered by binding and uptake lead to divergent responses in macrophages when compared with DC with respect to bacterial killing/survival, cytokine and cell surface molecule expression, antigen presentation and trafficking, all of which can directly influence the outcome of infectious challenge.

In vitro bovine monocyte-derived macrophages and MoDC were equally phagocytic for *M. bovis* or BCG, yet higher numbers of bacilli were retained within MoDC compared with macrophages, reflecting preferential survival of mycobacteria within DC. In addition, DC infected with *M. bovis* or BCG up-regulated CD80, CD40 and MHC II, which are required for antigen presentation and stimulation of T-cell responses. Coupled with the ability of DC to migrate towards lymph nodes this could indicate that infection of DC would prime T-cell immune responses to infection. Different cytokine profiles have also been reported for macrophages and DC in response to mycobacterial exposure: typically, MoDC secrete high levels of IL-12 associated with induction of IFNγ by T cells and NK cells (Denis *et al*., 2007) whereas macrophage responses are dominated by TNFα, IL-1β and IL-10 (Hope *et al*., 2004). In addition, infected MoDC and macrophages have been shown to express inflammatory chemokines (Widdison *et al*., 2011; Siddiqui and Hope, 2013) to attract NK cells and granulocytes *in vitro*. The expression profiles of key chemokines were significantly different between MoDC and macrophages (Siddiqui and Hope, unpublished observations), suggesting the potential chemoattraction of different subsets of lymphocytes. *In vivo* chemokine gradients produced by *M. bovis*-infected APC would contribute to inflammatory influx into the tissues and granuloma formation.

Mycobacteria can persist within macrophages and DC by preventing phagosome maturation and evading lysosome fusion (Flynn and Chan, 2003; Nguyen and Pieters, 2005; Rohde *et al*., 2007) thereby evading intracellular destruction and reducing antigen presentation and subsequent T-cell responses. The production of nitric oxide (NO) is a key response contributing to the capacity of cells to control intracellular bacteria. In bovine studies, NO production was predominantly seen in macrophages (Denis and Buddle, 2008) and could be significantly enhanced by the addition of IFNγ (Piercy *et al*., 2007). The activation of macrophages with IFNγ promotes intracellular killing, at least in part through the induction of autophagy which has been shown to contribute not only to destruction of intracellular *M. tuberculosis* and BCG but also to increase antigen presentation to T cells (Gutierrez *et al*., 2004; Puleston and Simon, 2014).

Thus, early differential interactions of *M. bovis* with APC are central to the downstream events occurring post-infection. Further reciprocal stimulation of innate and adaptive cells

will contribute to the eventual outcome of exposure and determine whether disease or immunity ensues.

Natural killer cells

NK cells are large granular lymphocytes which are traditionally cells of the innate immune system but as essential drivers of the adaptive immune response, NK cells are found at the interface between innate and adaptive immunity. NK cells have two main effector functions: cytotoxicity of target cells through the release of preformed granules containing perforin and granulysin (Reefman *et al*., 2010) and cytokine production including IFNγ, TNFα, GM-CSF (Fehniger *et al*., 1999; Cooper *et al*., 2001; Boysen *et al*., 2006), IL-10 (Fehniger *et al*., 1999; Cooper *et al*., 2001) and IL-22 (Cella *et al*., 2009; Dhiman *et al*., 2009). NK cell function and maintenance of self-tolerance is determined by a complex interplay between activating and inhibitory NK cell receptors (Long *et al*., 2013). These germ line encoded receptors enable NK cells to distinguish cellular stress related ligands, MHC class I molecules and MHC class I-like molecules. In addition to key roles in innate defence, NK cells are located at the bridge between innate and adaptive immune responses through interactions with APC and have recently been shown to have features of adaptive immunity including 'memory'. Evidence for NK cell memory was first identified in mice (O'Leary *et al*., 2006; Sun *et al*., 2009) and human memory-like NK cells have been shown to migrate into tuberculous pleural fluid via a CXCR3 and CXCR4 axis (Fu *et al*., 2011, 2013).

Phenotypically distinct subsets of NK cells with divergent functions have been described across species. Human NK cells are defined as CD3- with variable expression of CD56 and CD16 (Cooper *et al*., 2001). Similarly, discrete subsets of NK cells also exist in mice which are differentiated based on their expression of CD27 and CD11b (Chiossone *et al*., 2009). NKp46 (CD335), a natural cytotoxicity receptor expressed exclusively by NK cells, is a pan species marker that identifies this heterogeneous cell population (Moretta *et al*., 2005; Walzer *et al*., 2007b). The development of a monoclonal antibody specific to this receptor has allowed for the characterization of NK cells in cattle (Storset *et al*., 2004). Bovine NK cells can be subdivided into two subsets based on their differential expression of the adhesion/costimulatory molecule, CD2 (Boysen *et al*., 2006). The NKp46+ CD2+ and NKp46+ $CD2^{-}$/low (referred to as $CD2^{-}$ herein) subsets are predominantly localized within the peripheral blood and lymph nodes/afferent lymph respectively (Boysen *et al*., 2006; Lund *et al*., 2013) and these subsets have divergent functions with the $CD2^{-}$ NK cells being more functionally active (Boysen *et al*., 2006, 2008). Within bovine peripheral blood, NK cells account for 0.5–10% of the total lymphocyte population with an increased prevalence in neonatal calves, particularly those aged between 8 and 120 days old (Kulberg *et al*., 2004; Graham *et al*., 2009).

NK cells are responsive during mycobacterial infection *in vivo*. Following infection of mice with *M. tuberculosis* or *M. bovis* BCG, increased numbers of activated NK cells are recruited to the lungs where they secrete IFNγ (Junqueira-Kipnis *et al*., 2003). During studies of active *M. tuberculosis* infection in humans, NK cells infiltrate the lungs where they are localized to granulomas (Portevin *et al*., 2012). Furthermore, the importance of NK cell- and γδ T cell-derived IFNγ during BCG immunization of infants has been recently defined, highlighting the significance of innate effector cells during anti-mycobacterial immune responses (Zufferey *et al*., 2013). The frequency, phenotype and function of NK cells during *M. bovis* or *M. bovis* BCG infection in cattle have not yet been fully defined.

Early interactions between populations of innate immune cells, particularly NK cells and DC, are fundamental in determining the nature of the adaptive immune response. Nevertheless, direct activation of NK cells has been reported, for example binding of *M. bovis* BCG to NK cells via NKp44 (Esin *et al*., 2008) or recognition of *M. bovis* via TLR2 expressed by human NK cells (Marcenaro *et al*., 2008). However, for the most part, NK cells require indirect activation through interplay with APC in order to become functionally mature (Lucas *et al*., 2007). In cattle, Hope *et al*. (2002) described interactions between NK cells and DC in the context of mycobacteria. An NK-like

population was shown to proliferate and produce IFNγ in response to *M. bovis* BCG infected DC (Hope *et al*., 2002). More recently it was shown that preferential stimulation of $CD2^-$ NK cells occurred following co-culture of bovine NK cells with *M. bovis*-infected DC (Siddiqui and Hope, 2013). This subset was effectively recruited in response to chemokines secreted by the DC resulting in significant expression of IFNγ which could potentiate Th1 bias and the development of protective immunity.

A central role for IL-12 in the cross-talk between DC and NK cells is evident from a number of studies. DC-derived IL-12 acting in synergy with IL-18 is essential to drive cytokine production by NK cells (Ferlazzo *et al*., 2004; Chaix *et al*., 2008). Bovine DC-secreted IL-12 in response to *M. bovis* or *M. bovis* BCG (Hope *et al*., 2004) and blockade of IL-12 secretion partially abrogated the capacity of NK cells to produce IFNγ in response to infected DC (Siddiqui and Hope, 2013). Importantly, the ability of DC to produce IL-12 depends on rapid IFNγ production by NK cells (Gerosa *et al*., 2002; Piccioli *et al*., 2002; Mailliard *et al*., 2003), resulting in a positive feedback loop involving secretion of IL-12 and IFNγ from mature DC and NK cells, respectively. This demonstrates the reciprocal nature of NK-DC interactions with effects on both cell populations being evident. DC that have been in contact with NK cells were shown to increase IL-12 (Van Elssen *et al*., 2010), CCR7 expression and, in cattle showed enhanced expression of MHC class II (Siddiqui and Hope, 2013). Thus NK cells may influence the adaptive immune response by inducing effective APC.

Bovine NK cells stimulated with IL-2, IL-15, IL-2/12 or IL-12/15 restrict the replication of *M. bovis* BCG within monocyte-derived and alveolar macrophages (Endsley *et al*., 2006). Additionally, NK cells proliferate and produce IFNγ in response to interactions with *M. bovis*-infected macrophages, which enhances the release of IL-12 and NO by activated macrophages (Denis *et al*., 2007). Thus exposure of macrophages to NK cells augments mycobacteria killing. Whether bovine NK cells exposed to *M. bovis*-infected DC are increased in their cytolytic potential for immature DC, as demonstrated for human NK cells (Ferlazzo *et al*., 2004), remains to be elucidated.

Protective immunity against *M. bovis* infection in cattle is driven by Th1 polarized immune responses which are characterized by IFNγ production (Buddle *et al*., 2005). Interactions between NK cells and DC *in vivo* were shown in murine models to mediate the induction of Th1 responses through the secretion of NK cell-derived IFNγ (Martín-Fontecha *et al*., 2004). Co-localization between NK cells, DC and T cells has been demonstrated in lymph nodes indicating a role for innate immune cell interactions in shaping T-cell responses *in vivo* (Bajenoff *et al*., 2006; Lucas *et al*., 2007; Walzer *et al*., 2007a). Further studies are essential to determine if bovine NK cells, DC and T cells co-localize *in vivo* and whether the reciprocal interactions between NK cells and DC can influence downstream responses of $CD4^+$ and $CD8^+$ T cells such that protective immunity may be induced.

Polar and apolar lipid fractions

The first point of contact between *M. bovis* and its host is likely to be interaction between innate immune cells such as macrophages or dendritic cells (DC) and bacterial surface molecules, many of which are lipids or contain lipid moieties. A number of *M. tuberculosis* lipid compounds have been described to interact with receptors on DC or macrophages such as lipomannan (LM), lipoarabinomannan (LAM), phosphatidylinositol mannosides (PIMs), and cord factors trehalose mono- and dimycolate (TMM and TDM) as well as the phthiocerol dimycocerosates (PDIMs) (see Kremer and Besra, 2005, for details on mycobacterial lipids). These surface-bound mycobacterial lipids interact with a variety of innate surface receptors including Toll- (Underhill *et al*., 1999) and C-type lectin receptors such as DC-SIGN (Ehlers, 2010) modulating innate immune responses. For example, it has been shown that monomycolyl glycerol (MMG) modulates host immunity resulting in hypervirulence (Reed *et al*., 2004). However, few data exist which describe the effects of *M. bovis*-derived lipids on bovine innate immune cells with only a few studies using non-target-species animal models or compounds not directly isolated from

M. bovis. This is therefore a field that invites more and detailed work.

In a recent study (Pirson *et al*., 2012), *M. bovis* polar and apolar lipid preparations were prepared and assessed for biological activity in bovine monocytes, monocyte-derived macrophages and monocyte-derived DCs (MoDC). The apolar lipid preparation contained TDM, TMM, PDIMMs, PGL and MMG; while the polar fraction contained, among other lipids, a range of PIMS, penta-acyl trehalose and glucose monomycolate (GMM). These lipid fractions, particularly the polar fractions, altered the cytokine production profile by both fresh and cultured bovine monocytes as well as MoDC inducing significant levels of IL-10, IL-12, MIP-1β, TNFα and IL-6. Further, BoLA class II, CD86 and CD1b expression decreased on all cell types tested after exposure to the polar lipid fraction. Polar lipids also significantly increased CD40 expression only on monocytes and monocyte-derived macrophages but had no effect on its expression on MoDC. Finally, *M. bovis* polar lipid-treated macrophages were less capable as APC than untreated macrophages, while similar treatment of MoDC had no effect on their capability to stimulate allogenic mixed lymphocyte reactions. These data demonstrated that *M. bovis* lipids modulate innate cell responses and in particular may hamper the ability of the host APCs to induce an appropriate immune response. As noted above, differential responses of macrophages and DC may also be relevant to the induction of downstream immune responses. Recent studies (Pirson and Vordermeier, unpublished data) indicate that *M. bovis* lipids also stimulate IFNγ production by NK cells.

Cell-mediated Immune Responses: Correlates of Protection versus Pathology

IFNγ and delayed type hypersensitivity (DTH) responses

A clear feature of the bovine immune response to *M. bovis* infection is an early and persistent production of IFNγ in response to various *M. bovis* antigens, detectable both *in vivo* and within *in vitro* re-stimulation assays (i.e. IGRAs) (Pollock *et al*., 2001). Peripheral IFNγ responses to complex and specific mycobacterial antigens are consistently detectable 2–3 weeks after experimental infection (Waters *et al*., 2010). As with *M. tuberculosis* infection of humans, CD4, CD8 and γδ T cells (as well as NK cells) contribute to the response (Pollock *et al*., 2001; Endsley *et al*., 2009); however, T helper 1 (Th1) CD4 T cells are generally considered the predominant cell type responsible for the robust IFNγ response (Hope *et al*., 2000; Walravens *et al*., 2002). Clearly, CD4 T cells and an intact Th1 response are essential for control of *M. bovis* infection in cattle. These responses, however, are not sufficient for clearance as *M. bovis* infection almost invariably results in persistent and progressive disease. Given the importance of Th1 cells in the adaptive response to tuberculosis, it is not surprising that IFNγ release assays (IGRAs) and DTH (i.e. skin test) responses are useful correlates to infection (reviewed by Schiller *et al*., 2010). The level of these responses, however, do not necessarily correlate with the severity of infection (Waters *et al*., 2012). Most effective tuberculosis vaccines also elicit specific IFNγ and DTH responses (Black *et al*., 2002), but not all vaccines that induce IFNγ and DTH responses are protective against tuberculosis and the amount of IFNγ produced in response to vaccination does not necessarily correlate with the level of protection afforded by the vaccine (Mittrücker *et al*., 2007; Abebe, 2012; Waters *et al*., 2012). Also, protection is not linked with maintenance of a DTH response (Whelan *et al*., 2011a). With that said, non-sensitizing vaccines (e.g. doses of BCG that do not elicit a DTH response) have proven ineffective in vaccine efficacy studies with cattle (Buddle *et al*., 2011). Evaluation of IFNγ responses after experimental infection may also be useful indicators of protection, or lack thereof, in vaccine efficacy studies. Responses with diagnostic potential, such as IFNγ responses, are generally inversely correlated with responses that predict vaccine efficacy, particularly to antigens not included within the vaccine. For instance, increasing ESAT-6/CFP10-specific IFNγ responses after challenge is a negative indicator of vaccine efficacy (e.g. with BCG or *M. bovis* ΔRD1 mutant vaccines) as these responses have been shown

to positively correlate with tuberculosis-associated pathology (Vordermeier *et al*., 2002; Dietrich *et al*., 2005; Waters *et al*., 2009). Thus, failed vaccine approaches resulting in increasing antigen burden and associated pathological changes evoke immune responses such as ESAT-6/CFP10-specific IFNγ responses, indicative of infection.

T-cell effector and central memory (Tcm) subsets

Immunological memory develops naturally as a result of infection and memory T-cell responses are generally considered indispensable for vaccine-elicited protection against most intracellular infectious agents. However, the relationship between effector T-cell responses and long-lasting T-cell memory is not completely understood in humans (Todryk *et al*., 2009) or cattle (Waters *et al*., 2009, 2011). Vaccination strategies are often designed to generate memory cells capable of responding rapidly and efficiently upon subsequent infection. Pathogens and their derivatives are generally transported to lymphoid organs by APCs for initiation of T-cell responses. Initiation of this primary response may take days to weeks to develop, relying on exposure of naïve T cells to antigens in secondary lymphoid organs, expansion of antigen-specific T cells and homing of effector cells to the site of infection (Mackay *et al*., 1990). For instance, with both human and bovine tuberculosis, there is a 2–3 week delay in the response at the primary site of infection that is advantageous for the pathogen, enabling it to define the ensuing response (Ernst, 2012). After initial encounter with cognate antigen and subsequent proliferation/activation, the number of antigen-specific T cells increases up to ~10,000-fold (Hou *et al*., 1994; Murali-Krishna *et al*., 1998; Whitmire *et al*., 1998). In addition to clonal expansion, activated T cells differentiate and exhibit effector functions, expressing important mediators of pathogen control (e.g. IFNγ, TNF-α, IL-17 and cytotoxic granules). If the immune system successfully controls the infection, only a few memory cells remain as 90–95% of the antigen-specific T-cell population undergoes apoptosis (Wilkinson *et al*., 2009; Totté *et al*., 2010). Subsets of memory CD4 T cells in humans include: (i) central memory T cells (Tcm, $CD62L^{+}CCR7^{+}$) that preferentially localize to lymphoid tissues and exhibit great proliferation potential and IL-2 production; and (ii) effector memory T cells (Tem, $CD62L^{-}CCR7^{-}$) that preferentially localize to peripheral tissues and have immediate effector functions (Sallusto *et al*., 1999; Champagne *et al*., 2001; Woodland and Kohlmeier, 2009; Sallusto *et al*., 2010). Tem cells may remain blood associated, either circulating or contained within splenic red pulp or hepatic sinusoids. Upon restimulation, Tem cells undergo relatively little proliferation and secrete minimal IL-2 (Champagne *et al*., 2001; Sallusto *et al*., 2004, 2010). Short-term IFNγ responses to mycobacterial antigens measuring effector and Tem responses are utilized as a correlate to infection with tuberculosis tests for cattle and humans (e.g. IGRAs). Additionally, assessment of long-term IFNγ production (i.e. as a surrogate to Tcm responses) may be used to detect past *M. tuberculosis* infection in humans with negative IGRAs (Butera *et al*., 2009). Thus, measurement of effector and memory T responses may each provide diagnostic benefit.

Upon stimulation by mycobacterial antigens (e.g. rESAT-6:CFP10 or *M. bovis* PPD), bovine peripheral blood CD4, CD8 and γδ T cells from *M. bovis*-infected cattle proliferate and display an activated phenotype (i.e. ↑CD25, ↑CD26, ↑CD44) after 3–6 days in culture (Waters *et al*., 2003; Maue *et al*., 2005). Mycobacterial antigen-activated CD4 cells also decrease expression of CD62L and increase expression of CD45RO (associated with memory T cells) while decreasing expression of CD45R (associated with naïve T cells phenotype) (Maue *et al*., 2005). The variant splices A, B and C of CD45 receptor are not described for cattle (Bembridge *et al*., 1995). These findings demonstrate that cattle exhibit an expected T-cell effector phenotype upon antigen activation within short-term cultures. Additionally, with bovine tuberculosis vaccine efficacy studies, long-term (14 day cultures) T-cell responses (so-called cultured ELISPOT responses, Vordermeier *et al*., 2006) negatively correlate with mycobacterial burden (Waters *et al*., 2009) and tuberculosis-associated pathology and positively with vaccine-induced protection (Whelan *et al*., 2008; Vordermeier *et al*.,

2009b). With this assay, T-cell lines are generated via stimulation of PBMC with mycobacterial antigens including Ag85A, TB10.4 and *M. bovis* PPD. Effector T-cell responses wane over time and memory cells are sustained via addition of IL-2 and fresh medium. After 13 days of culture, cells are washed, transferred to plates containing autologous APCs, cultured overnight and the ensuing response measured by IFNγ ELISPOT. Studies with samples from humans have demonstrated that the responding cells within these long-term cultures (up to 14 days) are mainly Tcms and that this response, in contrast to effector responses, correlates with better infection outcomes (Todryk *et al*., 2009). With cattle, BCG vaccination of neonatal calves induces significant protection against *M. bovis* challenge at 12 months but not at 24 months after vaccination. This loss of efficacy correlates with a significant reduction in the numbers of antigen-specific IFNγ-secreting cells within long-term PBMC cultures (Thom *et al*., 2012). Tcms are likely to contribute to the long-term cultured ELISPOT response to BCG vaccination by cattle, although a lack of an anti-bovine CCR7 antibody has hindered this characterization. In the assessment of the migration pattern of γδ T cells, Vrieling *et al*. (2012) recently demonstrated that an anti-human CCR7 antibody cross-reacts with bovine CCR7 molecules. Recent studies have demonstrated that Tcm cells, with a minor contribution by Tem cells, are the primary cell type responding in long-term cultured IFNγ ELISPOT responses to *M. bovis* infection in cattle (Maggioli *et al*., 2012). Data on the response to vaccination and subsequent challenge to access the correlation between Tcm and Tem responses to protection/pathology are still lacking. However, these data demonstrate the potential for defining a protective signature elicited by vaccination to prioritize vaccine candidates for efficacy testing within calves.

IL-17 and IL-22

There is considerable interest in the role of IL-17 producing cells in the immune response to persistent infection (Cooper, 2010). Significant IL-17 responses are elicited by *M. tuberculosis* infection of mice and humans (Khader and Cooper, 2008; Jurado *et al*., 2012) and *M. bovis* infection of cattle (Vordermeier *et al*., 2009b). Early expression of IL-17 is required for rapid accumulation of protective memory cells in tuberculosis infection of mice (Khader *et al*., 2007); however, the absence of IL-17 during *M. tuberculosis* infection only modestly alters the inflammatory response (Khader *et al*., 2005; Umemura *et al*., 2007). γδ and other non-$CD4^+$ T cells are the primary producers of IL-17 during *M. tuberculosis* infection in mice. Early IL-17 produced by γδ T cells occurs prior to αβ T-cell priming, thus biasing the ensuing adaptive response (Lockhart *et al*., 2006). However, excessive IL-17 responses may be detrimental, resulting in a destructive influx of granulocytes as well as increased amounts of MIP2, TNF-α and IL-6 within affected lungs (Cruz *et al*., 2010). Thus, the timing and amount of IL-17 produced in response to tuberculosis is critical for the balance between control of the bacilli and detrimental inflammatory responses.

With cattle, stimulation of PBMCs with either *M. bovis* PPD or Ag85A elicits IL-17 mRNA expression in samples from *M. bovis*-infected or BCG plus viral-vectored Ag85A vaccinated cattle (Vordermeier *et al*., 2009b). With BCG/Ag85A-vaccinated cattle, IL-17 responses to Ag85A 10 weeks after vaccination and prior to challenge negatively correlate with tuberculosis-associated pathology at post mortem, indicating that vaccine-elicited IL-17 responses are protective (Vordermeier *et al*., 2009b). Likewise, Rizzi *et al*. (2012) recently demonstrated that IL-17 mRNA expression is increased in response to *M. bovis* PPD stimulation in cattle vaccinated with a BCG strain over-expressing Ag85B; and, post-vaccination/pre-challenge IL-17 responses negatively correlated with lesion severity after experimental infection (Rizzi *et al*., 2012). IL-17 responses to *M. bovis* PPD detected after challenge, however, do not correlate with protection as there is a ~20-fold increase in IL-17 gene expression detected in samples from non-vaccinated, vaccinated/protected and vaccinated/non-protected groups (Vordermeier *et al*., 2009b). Additionally, IL-17 mRNA expression (60 and 90 days after experimental infection) in response to *M. bovis* PPD correlates with the presence of gross tuberculous lesions, suggesting that IL-17 may prove useful as a

biomarker of infection (Blanco *et al*., 2011). Using laser capture microdissection followed by qPCR, Aranday-Cortes *et al*. (2013) demonstrated increased IL-17A and IL-22 expression within tuberculous granulomas as compared to non-affected tissues from experimentally infected cattle. Expression was greatest in early lesions with decreasing expression in more advanced lesions, perhaps suggestive of down-regulation of IL-17A and IL-22 associated with expression of other immune-modulating cytokines. Further studies with bovine tuberculosis should prove useful for delineating potential roles for IL-17 and other Th17 cytokines (e.g. IL-22, Bhuju *et al*., 2012) in protective and detrimental responses to *M. bovis* in cattle.

γδ T cells

Bridging innate and adaptive immune functions, γδ T cells play a critical role in the early immune response to various infections of cattle including leptospirosis, anaplasmosis, paratuberculosis, theileriosis and tuberculosis (reviewed in Guzman *et al*., 2012). The frequency of γδ T cells within the peripheral lymphocyte pool in humans and mice is ~5–10% (Kabelitz, 2011) whereas γδ T cells constitute up to 60% of the circulating lymphocyte population in neonatal calves and ~25% in adult cattle (Hein and Mackay, 1991; Jutila *et al*., 2008). Populations of bovine γδ T cells are commonly divided based upon their expression of Workshop Cluster 1 (WC1) (Mackay *et al*., 1989; Clevers *et al*., 1990; Morrison and Davis, 1991; Machugh *et al*., 1997), a transmembrane glycoprotein and member of the scavenger receptor cysteine rich (SCRC) superfamily, which includes CD163, CD5, CD6 and DMBT1 (Sarrias *et al*., 2004). The majority of bovine peripheral blood γδ T cells express WC1, and it is the $WC1^+$ subpopulation that has been shown to respond to *M. bovis* infection, although recent studies indicate that $WC1^-$ γδ T cells are also responsive (McGill *et al*., 2014).

In cattle, γδ T cells undergo dynamic changes in distribution after *M. bovis* infection, with a marked decrease in the circulating pool shortly after infection (Pollock *et al*., 1996; Cassidy *et al*., 1998). The initial decline of peripheral blood γδ T cells has been attributed to trafficking to the site of infection as circumstantially evidenced by the accumulation of γδ T cells within tuberculous lesions of cattle (Cassidy *et al*., 1998; Palmer *et al*., 2007). Following the initial decrease in circulation, percentages of $WC1^+$ γδ cells within the peripheral blood of *M. bovis*-infected cattle increase with a concomitant increase in CD25 expression, indicating an active response to infection (Pollock *et al*., 1996). Similarly, $WC1^+$ γδ T cells are among the first cells to accumulate at the site of DTH responses following PPD injection of *M. bovis*-infected cattle (Doherty *et al*., 1996). And, as with mice (Dieli *et al*., 2003), bovine γδ T cells rapidly infiltrate lungs as well as lung- and head-associated lymphoid structures 1 week after intranasal BCG vaccination (Price *et al*., 2010). These findings demonstrate that bovine γδ T cells traffic to sites of mycobacterial infection, *in vivo*.

With *in vitro* studies, γδ T cells from *M. bovis*-infected cattle proliferate and produce IFNγ in response to stimulation with complex *M. bovis* antigens – including PPDs, whole cell sonicates and culture filtrate proteins (Rhodes *et al*., 2001; Smyth *et al*., 2001) – as well as specific proteins such as Ag85, MPB83, hsp 16.1 and ESAT-6/CFP10 (Rhodes *et al*., 2001; Maue *et al*., 2005). Human and murine γδ T cells proliferate and secrete cytokine in response to both protein and nonprotein phosphoantigens of *M. tuberculosis* (Haregewoin *et al*., 1989; Born *et al*., 1990; Morita *et al*., 1995; Fournie and Bonneville, 1996). Welsh *et al*. (2002) demonstrated that γδ T cells from *M. bovis*-infected cattle respond to pyrophosphates, including isopentyl pyrophosphate (IPP) and monomethyl phosphate. γδ T cells from *M. bovis*-infected cattle also respond to mycolylarabinogalactan peptidoglycan (a complex mycobacterial cell wall component, Vesosky *et al*., 2004) as well as antigens within proteinase-K treated *M. bovis* whole cell sonicates (Welsh *et al*., 2002). Thus, it is evident that bovine γδ T cells respond to a variety of antigens upon *M. bovis* infection; however, the role of this response (i.e. protective versus detrimental) is unclear.

γδ T-cell deficient mice are able to temporarily control BCG (Ladel *et al*., 1995) and

low-dose *M. tuberculosis* infection (D'Souza *et al*., 1997), but exhibit a more severe inflammatory response as compared to control mice, suggesting a role for γδ T cells in granuloma formation and maintenance. In agreement, depletion of $WC1^+$ γδ T cells from SCID-bo mice prior to *M. bovis* infection significantly alters the architecture of the developing granuloma (Smith *et al*., 1999). In contrast, depletion of $WC1^+$ γδ T cells from *M. bovis*-infected cattle has no effect on disease severity or granuloma formation (Kennedy *et al*., 2002). Instead, these animals exhibit a reduction in early IFNγ production and an increased skewing towards a Th2 type response, suggesting a role for γδ T cell-derived cytokines in establishing Th1 immunity. Along these lines, incubation of $WC1^+$ γδ T cells with autologous *M. bovis*-infected dendritic cells induces enhanced expression of MHC II and CD25, as well as increased secretion of IFNγ by the γδ T cells (Price and Hope, 2009). Dendritic cells, in turn, produce increased levels of IL-12 when incubated with γδ T cells, suggesting that reciprocal interactions between dendritic cells and $WC1^+$ γδ T cells may occur *in vivo*. Together, these findings support the notion that early and specific γδ T-cell responses bias the immune response in favour of a Th1 response. Additional functions of γδ T cells in the immune response to mycobacteria include IL-17 production (Lockhart *et al*., 2006; Umemura *et al*., 2007; McGill *et al*., 2014) direct cytotoxicity (Stenger *et al*., 1998; Skinner *et al*., 2003) and – potentially – regulatory functions (Guzman *et al*., 2012). However, further studies are required to fully define these functions with *M. bovis* infection in cattle.

Cytotoxic T lymphocytes (CTLs)

Cytolysis of mycobacteria-infected cells can result in direct killing or release of bacilli for eventual killing via other mechanisms (reviewed by Flynn and Chan, 2001). $CD8^+$ T cells are the primary cell type performing CTL functions with mycobacterial infections; however, other lymphocyte populations such NK cells, $CD4^+$ T cells and γδ T cells also have cytolytic capacity (Stenger *et al*., 1998; Canaday *et al*., 2001). Primary effector functions of $CD8^+$ T cells are production of IFNγ necessary for macrophage activation and lysis of infected macrophages (Einarsdottir *et al*., 2009) and production of perforin and granulysin essential for CTL function via pore formation and antimicrobial functions, respectively. Additionally, IFNγ from $CD4^+$ T cells is required for effective $CD8^+$ T-cell responses (Green *et al*., 2013); thus, CTL functions are essential for the control of mycobacterial infections and $CD4^+$ T-cell responses are supportive of this response.

CTLs are also implicated in the host response to bovine tuberculosis. Antigen-specific CD8 T cells cause release of viable *M. bovis* from infected bovine macrophages, indicating CTL activity (Liebana *et al*., 2000). Activated $CD8^+$ T cells are detectable within the lymphocytic outer core of early-stage bovine tuberculous granulomas, indicating a potential role for these cells in the initial containment of the bacilli (Liebana *et al*., 2007). Bovine T cells also express a homologue of human granulysin, a potent antimicrobial protein stored in association with perforin in cytotoxic granules (Endsley *et al*., 2004). Additionally, antigenic stimulation of peripheral $CD4^+$ T cells from BCG-vaccinated cattle results in enhanced anti-mycobacterial activity against BCG-infected macrophages linked with increased perforin and granulysin transcription (Endsley *et al*., 2007). Expression of the bovine granulysin gene can be induced in $CD4^+$, $CD8^+$ and γδ T cells resulting in anti-mycobacterial activity (Endsley *et al*., 2004, 2007). Granulysin and granzyme A mRNA are detectable within granulomas of *M. bovis*-infected cattle (Endsley *et al*., 2004; Aranday-Cortes *et al*., 2012). Granzymes are a group of serine proteases released by $CD8^+$ T cells and NK cells in cytoplasmic granules along with perforin. Granulysin and perforin gene expression are also up-regulated in peripheral blood $CD4^+$ and $CD8^+$ T cells in both BCG- and *M. bovis* ΔRD1-vaccinated calves (protected) as compared with non-vaccinated (not protected) calves (Capinos Scherer *et al*., 2009). Thus CTLs are involved in the bovine immune response to *M. bovis* infection; however, further studies are required to determine their exact roles in protection and pathogenesis.

Polyfunctional T cells

Polyfunctional T cells simultaneously produce two or more cytokines in response to antigen, and higher frequencies of these cells are associated with control of chronic infections such as HIV, hepatitis C, leishmaniasis, malaria and tuberculosis (reviewed in Wilkinson and Wilkinson, 2010; Caccamo and Dieli, 2012). The majority of studies with human tuberculosis indicate that polyfunctional T-cell responses are associated with active disease (Sutherland *et al*., 2009; Wilkinson and Wilkinson, 2010); however, others indicate a protective role (Geluk *et al*., 2012). Few studies have been performed on the evaluation of polyfunctional T cells in cattle, largely due to the lack of necessary reagents. Whelan *et al*. (2011b) recently described the development of an assay to detect polyfunctional CD4 T cells in *M. bovis*-infected (natural infection) cattle. Bovine polyfunctional CD4 T cells exhibited a characteristic $CD44^{hi}$ $CD62L^{lo}$ $CD45RO^{+}$ TEM phenotype. Interestingly this study, as well as recent as yet unpublished results, suggests that polyfunctional $CD4^{+}$ T cells are associated with pathology rather than protection (Whelan *et al*., 2011b; Whelan, Villarreal-Ramos and Vordermeier, unpublished data). That a more diverse cytokine profile can be reflective of more severe disease has also recently been highlighted by the observation that cattle producing both IL-2 and IFNγ (measured by ELISA after *in vitro* stimulation) are more likely to present with visible pathology at post mortem than those that produce IFNγ only (Rhodes *et al*., 2014). Further studies are warranted to determine the exact role of polyfunctional T cells in the response to *M. bovis* infection as well as vaccination.

B Cells and Antibody Responses

Role for B cells?

Specific roles for B cells in the immune response to tuberculosis are generally considered supportive, rather than essential (reviewed by Maglione and Chan, 2009). Functions attributed to B cells in the response to tuberculosis include antigen presentation, APC regulation via Fc receptors, immune modulatory actions of immune complexes and antibody-dependent cytotoxicity. B cell aggregates are consistently detected in association with tuberculous lesions in mice, cattle, non-human primates, humans and other host species infected with *M. tuberculosis*-complex organisms. These tertiary structures contain naïve, memory and plasma cells as well as intermixed $CD4^{+}$ and $CD8^{+}$ T cells, follicular dendritic cells and mycobacteria-laden APCs (Ulrichs *et al*., 2004). Using immunohistochemistry, Aranday-Cortes *et al*. (2013) demonstrated the presence of B cells ($CD79a^{+}$ cells) within granulomas of tuberculous cattle. In that study, early granulomas (Stages I and II) displayed scattered B cells, whereas more advanced granulomas (Stages III and IV) showed satellite nests of $CD79a^{+}$ cells located peripherally and outside of the fibrous capsule. In mice, formation of B cell follicles within infected lung tissues is dependent upon IL-23 and CXCL13, and CXCL13 production is dependent upon IL-17A and IL-22 in this response (Khader *et al*., 2011). The presence of ectopic germinal centres indicates that the *M. tuberculosis* complex – and the ensuing inflammation – induces active B cell clusters that modulate the host response. Thus, these follicles provide at least a partial framework for coordinated immune control of mycobacterial growth in the affected tissues (Ulrichs *et al*., 2004).

Tuberculin boost

Several antibody-based tests have recently emerged for use in cattle, captive cervids, several wildlife reservoirs of *M. bovis* and various zoo species (most notably elephants). Intradermal tuberculin administration is known to significantly boost antibody responses in tuberculous cattle and cervids, but not in non-infected animals (Lyashchenko *et al*., 2004; Harrington *et al*., 2008). This phenomenon can be observed from 1 to 2 weeks to several months after tuberculin injection (Palmer *et al*., 2006; Chambers *et al*., 2009). The absence of seroconversion in non-infected animals and the short-lived antibody kinetics with features of an anamnestic response strongly suggest

that the tuberculin-boosted antibody response is due to memory B cells originally primed by mycobacterial infection that can be quickly activated upon re-stimulation by tuberculin administration.

Correlations to pathology

Cellular immune responses elicited by mycobacterial infections of cattle generally correlate with infection but not necessarily with the level of pathology (Waters *et al*., 2010). Inoculation of cattle with *M. tuberculosis*-complex strains that are attenuated in cattle (e.g. *M. tuberculosis* $H_{37}R_v$ or *M. bovis* Ravenel results in relatively robust cell-mediated immune responses and persistent colonization with minimal to no lesions. With *M. kansasii* inoculation, cell-mediated immune responses are elicited without detection of the organism or associated lesions. As expected, inoculation of cattle with virulent *M. bovis* (field strains such as 95-1315 or A2122/97) results in robust cell-mediated immune responses, persistent colonization and associated tuberculous lesions. Similar results were observed following the intratracheal inoculation of cattle with a virulent *M. bovis* strain (WAg201) and three attenuated *M. bovis* strains. One attenuated strain had a mutation in the *inh*A gene (WAg405, daughter strain of WAg201), a second strain (ATCC 35721) with a mutation in the principal sigma factor, *rpo*V gene and BCG (Wedlock *et al*., 1999). Extensive macroscopic lesions were found only in cattle inoculated with the virulent strain, while strong antigen-specific IFNγ and IL-2 were induced by all *M. bovis* strains. Interestingly, the virulent strain and two of the attenuated strains (WAg405 and ATCC 35721) elicited strong DTH responses to bovine PPD in the skin test in comparison to BCG and this correlated with rapid *in vitro* proliferation in unstimulated bovine alveolar macrophages and proinflammatory cytokine gene expression. Thus, regardless of the pathological and mycobacterial burden outcome, cell-mediated immune responses are elicited. In contrast, antibody responses generally correlate with levels of pathology associated with mycobacterial infections (Wedlock *et al*., 1999; Waters *et al*., 2010). For instance, mycobacterial-specific antibody is detectable relatively early after *M. tuberculosis* challenge of cattle, yet these responses wane over time, and this is likely to be coincident with the reduction of *M. tuberculosis* colonization. In contrast, with virulent *M. bovis* infection that leads to persistent infection and significant pathology, antibody responses persist, probably due to increasing antigen burden. With less virulent mycobacteria (e.g. *M. kansasii*), antibody responses are elicited and then wane, likely to be coincident with clearance of the pathogen. Regardless of disease expression, mycobacteria-specific antibody responses may be boosted by re-exposure to mycobacterial antigens (e.g. PPD for skin test) or other live mycobacteria, thereby potentially confounding interpretation of serologic tests.

Transmission of *Mycobacterium bovis* from Cattle to Cattle

In most countries with active control programmes, affected cattle herds contain low numbers of infected animals, suggestive of low rates of cattle-to-cattle transmission (Palmer and Waters, 2006). Entry of *M. bovis* into cattle herds may occur via cattle-to-cattle transmission through purchase of infected animals or contiguous spread (Goodchild and Clifton-Hadley, 2001), from wildlife reservoirs (de Lisle *et al*., 2002), or environmental exposure (Phillips *et al*., 2003). Transmission is generally by direct contact with tuberculosis-infected animals as the organism may occur in exhaled droplets, saliva, faeces, milk, urine, vaginal discharges, semen or exudate from tuberculous lesions (e.g. lymph nodes with draining tracts that communicate to the exterior). With *M. tuberculosis*, for example, airborne bacilli can be generated and carried in droplets ranging in size between 0.5 and 2 µm. Droplets of this size are significant in that they are readily dispersed and can remain suspended in air where they are dispersed (Segal-Maurer and Kalkut, 1994). To achieve airborne transmission, *M. bovis* must remain viable inside droplet nuclei. Gannon *et al*. (2007) found that 94%

viability was retained 10 min post-aerosolization, with a half-life of 1.5 h. Infection may also result from ingestion of infected feeds. Housing (e.g. cattle sheds, milking parlours) and crowding increases the contact of naïve animals with infected animals, enhancing the spread of this disease. Lesion distribution in tuberculous farmed cattle indicates aerosol infection, often as a result of exposure to small numbers of viable bacilli (Dean *et al*., 2005). In comparison, significantly larger numbers of bacilli are needed to initiate oral infection (Pollock *et al*., 2006).

M. bovis is also transmitted by indirect contact through contaminated feed and water, equipment or anything that mechanically transfers the organism between locations. Movement of infected animals resulting from transfer of ownership, sharing of breeding animals and fence-line contact with other herds is another common means of transferring the disease between herds and regions (Probst *et al*., 2010). This concept is also supported by Welby *et al*. (2012) where within-herd spread was a factor in herd level breakdowns, with trade in undetected infected animals posing a significant risk. Barlow *et al*. (1998) indicated that cattle movement is the likely cause of transmission in areas where *M. bovis*-infected reservoirs are absent. Also, cattle movements were more likely to be responsible for local breakdowns in isolated areas, rather than spread through wildlife (Bourne, 2007). Studies revealing social interactions also provide insight into the potential for cattle-to-cattle transmission. Sauter and Morris (1995) studying New Zealand cattle identified the herd dominance hierarchy and found that skin-test reactors were likely to be found in the top 50% within that hierarchy. The introduction and use of proximity data logging collars has improved social network tracing (Böhm *et al*., 2009), with up to 26 direct cattle-to-cattle interactions found per day. Cattle prominent in the social hierarchy are also likely to be more inquisitive and are therefore more likely to acquire infection from infectious herd cohorts (Böhm *et al*., 2009). Movement of cattle from TB endemic areas was seen to be a greater risk for TB breakdown compared to other variables (Gilbert *et al*., 2005). Specifically, Gopal *et al*. (2006) investigated herd breakdowns in north-east England and tracked 17 cases where skin-test reactors were traced to herds with the same genotype. Global trade agreements are increasingly being implemented to promote international trade of livestock, thereby increasing risks for inter-regional spread and distribution of TB over great distances.

A number of studies have examined nasal excretion as a source for transmission. Romero Tejeda *et al*. (2006) tested nasal exudates from Mexican cattle using PCR and found excretion from infected cattle. Menzies and Neill (2000) estimated that up to 20% of infected cattle can excrete *M. bovis*. McCorry *et al*. (2005) monitored nasal excretions from cattle infected under controlled conditions and identified two distinct periods of excretion, less than 30 days post-infection and greater than 80 days post-infection. However, transmission from infected cattle to naïve animals held under experimental conditions has not been a common finding; perhaps this was due to stringent airflow management for biological containment. Segal-Maurer and Kalkut (1994) reported that a single change of air reduced airborne *M. tuberculosis* by 67%. More recently, studies using an experimental model of bovine tuberculosis have demonstrated the complexity and unpredictability of cattle-to-cattle transmission (Khatri *et al*., 2012). Sentinel (non-infected) cattle were housed with *M. bovis*-infected cattle (naturally infected and from farms in England or Wales) in ten pens with six sentinels and four donors per pen. After 12 months of direct contact with donors, only 8 of 60 sentinels became infected, demonstrating a relatively low rate of transmission (13.3%) even under conditions of close and continued direct contact. Insights into disease transmission were obtained using molecular typing of *M. bovis* isolates obtained from donor and sentinel animals. Several patterns emerged. In two pens, transmission was likely to be due to an animal with extensive lesions, highlighting the highly infectious nature of animals with advanced disease. In another pen, the only donor with a spoligotype matching one of its infected receptor pen mates had a low pathology score and only a single visible lesion in a mediastinal lymph node, demonstrating that an animal with early-stage infection, and without visible lung pathology, can also be

infectious. In two other pens, the spoligotype pattern of isolates from the receptor cattle did not match spoligotype patterns from any of the lesions from donor animals, indicating either indirect contact via shared airspace (i.e. pen to pen transmission) or through transfer of *M. bovis* by husbandry procedures. Interestingly, in one of these two pens, the spoligotype of the *M. bovis* strain from one of the sentinel animals did not match a spoligotype from any of the donor animals in the study, potentially suggesting transmission from a donor with no visible lesions. This study clearly demonstrates the difficulties in determining the exact nature of transmission events with bovine tuberculosis herd breakdown events. Use of whole-genome sequencing may prove useful for the unravelling of these complex and seemingly unpredictable transmission scenarios (Biek *et al*., 2012; Godfray *et al*., 2013). Additionally, after an animal becomes infected, the period during which it may become infectious will vary (Goodchild and Clifton-Hadley, 2001) as well as the time period that it is detectable as 'infected' – diagnostically. Francis (1946) estimated this period to be between 30 and 50 days although Barlow *et al*. (1998) suggested that 6–20 months is more likely, especially when cattle are tested on a regular basis.

Transmission of *Mycobacterium bovis* from Wildlife to Cattle

The eradication of bovine TB from cattle herds in many countries has been frustrated by the existence of wildlife reservoirs of infection, proving strong evidence of transmission of *M. bovis* from wildlife to cattle. *M. bovis* has a wide host range infecting many wildlife species, but only a limited number including brushtail possums in New Zealand; badgers in the UK and Ireland; white-tailed deer in Michigan, USA; elk and bison in Canada; and wild boar in Spain and Portugal serve as maintenance hosts of *M. bovis* infection (de Lisle *et al*., 2002). Since the 1970s there has been increasing evidence that these maintenance hosts serve as an important source of infection for cattle, supported by *M. bovis* typing studies identifying common strains in wildlife and cattle in the same areas (Collins, 2011).

Studies in New Zealand have shown that a reduction of possum numbers by culling in a locality is followed by a reduction in the annual incidence of TB in cattle (Tweedle and Livingstone, 1994; Caley *et al*., 1999), and infection in livestock increased if infected possums returned (Ryan *et al*., 2006). A characteristic of wildlife as a source of infection was the extreme rarity of severely infected cattle and most herds had only one or two infected animals (Collins, 2011). Large-scale badger culling trials in the UK and Republic of Ireland have provided convincing evidence of transfer of infection from badgers to cattle. In the Randomized Badger Culling Trial (RBCT) undertaken in the UK, proactive culling resulted in a reduction in bovine TB incidence in cattle herds inside culled areas, but a temporary increase in adjacent areas. Reactive culling in response to herd breakdowns was associated with an increase of bovine TB in cattle, possibly as a result of social perturbations in badger social groups (reviewed by Wilson *et al*., 2011). Thus, non-selective culling may have positive and negative outcomes. Based on the results from the RBCT, Jenkins *et al*. (2008) suggested that the contribution of badgers to infection in cattle accounted for 50% in the experimental areas. Studies in the Republic of Ireland indicated that proactive culling had beneficial effects on the bovine TB incidence in cattle and there was no evidence that small-scale culling led to an increase in herd breakdowns (Griffin *et al*., 2005).

A reservoir of *M. bovis* infection in white-tailed deer in five counties in north-east Michigan, USA, has been a source of infection for cattle herds in this region. In a case-control study of farm risk factors for TB, Kaneene *et al*. (2002) found management practices to exclude deer from cattle areas were the most significant factors to reduce farm risk of TB. In Manitoba, Canada, an elk herd was implicated in an outbreak of *M. bovis* in 11 cattle herds surrounding Riding Mountain National Park, where co-mingling elk and cattle were feeding on the same hay bales and a unique strain of *M. bovis* was identified (Miller and Sweeney *et al*., 2013). In the regions of southern

Spain with highest cattle TB prevalence, wildlife species such as European wild boar and red deer showed a high prevalence of TB, which suggested that the disease was shared between domestic and wild hosts (Naranjo *et al*., 2008). In southern and eastern Africa, only four species are suspected to play a role as a maintenance host: buffalo, lechwe, and possibly greater kudu and common warthog. However, there has been no case of bovine spillback from wildlife to cattle confirmed, although indirect contacts between cattle and buffalo do occur at the periphery of several large conservation areas in southern Africa (de Garine-Wichatitsky *et al*., 2013).

Mechanisms to explain routes of transmission of *M. bovis* from wildlife to cattle include both direct and indirect contact. Direct aerosol spread between possums and cattle has been considered the principal route when cattle investigated terminally ill possums, with cattle attracted by the unusual behaviour of ill possums that do not show the avoidance behaviour of healthy animals (Sauter and Morris, 1995). In addition, terminally ill possums often have open draining lesions containing large numbers of organisms. In contrast, indirect contact between badgers and cattle has been considered the principal route for transmission of infection to cattle. Use of automated proximity loggers on badgers and cattle and at badger latrines located on pasture showed that direct contacts between badgers and cattle were very rare, while indirect contacts, visits to badger latrines by badgers and cattle were more common (Drewe *et al*., 2013). The detection of viable *M. bovis* at badger setts and latrines is strongly linked to the frequency of *M. bovis* excretion by infected badgers (Courtenay *et al*., 2006). Badger visits to farm buildings may also be important and badgers have been recorded defaecating and urinating in buildings, sometimes directly on to feed and regularly came within 2 m of housed cattle (Wilson *et al*., 2011). The transmission of infection between wildlife and cattle in Spain was considered most likely from indirect contact based on a study where camera traps were placed around at water spots, food bait stations and pasture on an extensive cattle farm also used for game hunting. Direct interactions between wildlife (deer and wild boar) and cattle were rare, while indirect interactions of wildlife followed by livestock were most frequent at water holes, suggesting water points as a hotspot for indirect interactions (Kukielka *et al*., 2013). Indirect contacts between white-tailed deer and cattle were considered the major route of transmission between the species and it has been demonstrated that experimentally infected deer transmitted *M. bovis* to cattle through sharing of feed in the absence of direct contact between the two groups of animals (Palmer *et al*., 2004).

Special Topics

Mycobacterium bovis-infected cattle with non-visible lesions: latency?

In contrast to *M. tuberculosis* infection in humans, the degree of latency resulting from *M. bovis* infection in cattle is unknown and controversial (Cassidy 2006; Alvarez *et al*., 2009). Most consider the disease as slowly progressive in cattle; however, given the high degree of genetic homology (~99.95%) between *M. bovis* and *M. tuberculosis* it is unclear why latency is a prominent disease stage in humans and has not been clearly defined in cattle. The difference may be host related as reactivation of latent *M. bovis* infection in humans has been demonstrated (Larsen *et al*., 2008) and *M. bovis* infection of Eurasian badgers results primarily in a prolonged non-clinical course of disease with minimal inflammation, suggestive of a latent state (Corner *et al*., 2011). Thus, there is potential for various disease outcomes with *M. bovis* infection that is host contingent.

Dormancy survival regulon (DosR) and enduring hypoxic response (EHR) genes are, in part, responsible for encoding dormancy related functions of *M. tuberculosis* (Lin and Ottenhoff, 2008), of which, many of these genes are conserved between *M. tuberculosis* and *M. bovis* (Alvarez *et al*., 2009). Responses to DosR and EHR antigens are detectable, albeit at low frequency (particularly with DosR antigens), in *M. bovis*-infected cattle (Jones *et al*., 2011). IL-1β responses to the EHR antigen Rv0188 are associated with responses

by infected animals exhibiting low or no pathology whereas responses to other EHR antigens are not correlated with disease status (Jones *et al*., 2011). While DosR and EHR antigens were identified with *in vitro* studies culturing *M. tuberculosis* under conditions mimicking latency (i.e. hypoxia and low levels of NO), responses to these antigens are not restricted to latent versus active *M. tuberculosis* infection of humans (Hinks *et al*., 2009; Schuck *et al*., 2009; Gideon *et al*., 2010). Thus, while *M. bovis* infection of cattle elicits responses to dormancy-associated antigens, it is still uncertain whether these responses are indicative of a latent stage of infection in cattle.

Latency is clinically defined as persistence of viable mycobacteria within a tuberculous lesion in a non-symptomatic host. On rare occasions, *M. bovis* is detected by culture or molecular techniques in tissues from animals without visible tuberculous lesions; however, it is uncertain if infected animals with no visible lesions would eventually develop progressive disease analogous to reactivation in human tuberculosis. Similarly, experimental infection of cattle with laboratory-adapted strains (i.e. *M. tuberculosis* H37Rv, *M. bovis* AN5 or *M. bovis* Ravenel) results in colonization with minimal to no lesions 4–5 months after challenge, despite vigorous cell-mediated immune responses (Whelan *et al*., 2010; Vordermeier, unpublished observations; Waters *et al*., unpublished observations). Using the clinical definition of latency, these animals would be classified as having latent infection; although, as with naturally infected cattle with no visible lesions, it is unknown if active disease would result spontaneously given time or be induced via immune suppression.

It has also been suggested that skin-test or IGRA-positive animals with no visible lesions upon post-mortem inspection may have latent infection (Alvarez *et al*., 2009). Recent studies have clearly demonstrated that IGRA-positive, skin-test negative animals are at increased risk to convert to skin-test positive status over a period of time and present with confirmed *M. bovis* infection upon post-mortem inspection (Cassidy, 2006). With this scenario, animals that are initially IGRA positive, skin-test negative and later confirmed as infected, could be classified as: (i) early-infected with progressive disease; or (ii) latently infected with the potential for subsequent progression to active disease. While IGRAs are a good correlate of infection (median test sensitivity, 87.6%; Schiller *et al*., 2010), the magnitude of the response is not indicative of the severity of lesions or duration of infection in cattle (Waters *et al*., 2012). Thus, animals that are potentially latently infected may have a similar magnitude of response as compared to animals with visible lesions (Waters *et al*., 2010). The possibility of latent *M. bovis* infection in cattle has a profound consequence for both evaluation and use of immune-based diagnostic tests, particularly given the pitfalls of current gold-standard tests. Even with thorough post-mortem examinations, extensive culture of tissues including lymph nodes/lungs with no visible lesions and histopathological assessment including *M. tuberculosis*-complex specific PCR, our ability to detect all infected animals is not 100%. This is likely to be due to the paucibacillary nature of the disease in certain animals, inability to sample all tissues and shortcomings of mycobacterial culture (e.g. harsh decontamination during processing). Fortunately, with more aggressive eradication/control programmes, slaughter surveillance and ante-mortem testing is used to identify tuberculosis-affected herds, of which aggressive measures such as whole-herd depopulation (stamping out) or stringent interpretation of ante-mortem tests may be applied to ensure removal of all infected animals, including those which may be 'latently' infected.

Host genetics: correlations to resistance

Patterns of infection at the population scale are determined largely by relative susceptibility. Animal-level risk factors separate broadly into genetic and non-genetic (environmental) risk factors that act jointly to influence susceptibility. It is biologically untenable that genetic variation in both host and pathogen does not play a role in the outcome of exposure to tuberculous bacteria. There is now compelling evidence from studies in humans,

mice, deer and rabbits that the outcome of infection with *M. tuberculosis*-complex bacteria has a significant genetic component (Allen *et al*., 2010). Species-level differences in susceptibility to bovine tuberculosis have been detected in cattle (Ameni *et al*., 2007); *Bos indicus* animals are more resistant than *B. taurus*. Recent developments suggest that it may be possible to increase the resistance to bovine tuberculosis by genetic selection.

In population-based studies, the proportion of the variance of any trait contributed by host genetic variation (heritability, h_2) can be estimated. Significant heritability is one of the key factors determining the potential success of breeding schemes in livestock production. Recent quantitative genetics studies have demonstrated significant heritability (21%) of susceptibility to bovine tuberculosis in Holstein cattle in Ireland (Bermingham *et al*., 2014) and in the UK (Brotherstone *et al*., 2010; Bermingham *et al*., 2011). For comparison, heritability for milk yield is estimated at around 28%, with substantial genetic gain achieved via selective breeding. Furthermore, field studies are likely to underestimate true heritability for infectious disease predisposition, due to unequal exposure to the pathogen and incomplete sensitivity of the diagnostic tests (Bishop and Woolliams, 2010). One of the significant read-outs of these large-scale quantitative genetics studies is that it should be possible to rank the bovine tuberculosis risk of individual sires (sire relative risk), based on the bovine tuberculosis status that follows in their progeny. Sire relative risk rankings for Holstein–Friesians in Ireland and the UK are currently being developed and are likely to overlap significantly. A recent Irish study also suggests that selective breeding towards bovine tuberculosis resistance should not have a negative impact on other desirable production traits (Bermingham *et al*., 2014).

Molecular genetics seeks to identify the individual genetic variants that explain the heritability of observed resistance/susceptibility phenotypes. Genome-Wide Association Studies (GWAS) have been used in the past in human genetics to find variation underpinning multiple traits, by examining genetic differences between panels of individuals with a phenotype of interest (cases) and individuals without the phenotype (controls). Can cattle exposed to tuberculosis be assembled into such case and control panels? Tuberculosis in cattle, as in all animal models and human studies, presents as a spectrum of infection outcomes or phenotypes (Barry *et al*., 2009; Young *et al*., 2009), a departure from the classical view that tuberculosis infection has a binary outcome. Extrapolation of this spectrum to cattle can clearly define animals as being susceptible (cases) or resistant (controls).

Having been able to classify cattle exposed to tuberculosis as cases or controls, we then must be able to index the differing genetic variation that underpins their phenotypic outcome. Due to the stunning achievements of the bovine genome sequencing project (Bovine Genome Sequencing and Analysis Consortium, 2009; Larkin, 2011) and associated genetic marker discovery (Bovine HapMap Consortium, 2009), cattle GWAS can now index variation at >700,000 genetic markers (SNPs) from across the whole 3 bn letter bovine genome. This development allows for an approach that does not require any *a priori* knowledge of the exact genes/loci causing particular phenotypes, thereby potentially identifying novel networks of genes involved in tuberculosis resistance, and pathways crucial to the host–pathogen interaction. The first of these bovine GWAS has just reported preliminary findings (BBSRC, 2011; Finlay *et al*., 2012; Bermingham *et al*., 2014). The study identified a number of genetic markers associated with the risk of acquiring bovine TB if exposed; some were associated with increased risk and others with reduced risk. Subject to further research and validation, these provisional results suggest that it might be possible to selectively breed cows that are more resistant to bovine tuberculosis.

Additionally, recent advances are taking the field of genetic epidemiology beyond the concept of associating individual genetic polymorphisms with phenotypes. Novel quantitative methodologies can now be employed to associate whole-genome variation with disease phenotypes (Daetwyler *et al*., 2008); such 'genomic selection' can then be used to predict the phenotypic outcome for any animal based on just its whole-genome genotype without the need to undertake lengthy and

costly trait recording or phenotyping exercises. This concept is an extension of genome-wide or genomic selection using SNP arrays (Meuwissen *et al*., 2001), which is now commonplace in most major advanced dairy cattle breeding programmes.

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USDA is an equal opportunity provider and employer. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

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