Immune-evasion Strategies of Mycobacteria and their Implications for the Protective Immune Response

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Abstract

Mycobacteria are intracellular pathogens that have macrophages as their main host cells. However, macrophages are also the primary line of defence against invading microorganisms. To survive in the intracellular compartment, virulent mycobacteria have developed several strategies to modulate the activation and the effector functions of macrophages. Despite this, antigen-specific T cells develop during infection. While T-cell responses are critical for protection they can also contribute to the success of mycobacteria as human pathogens, as immunopathology associated with these responses facilitates transmission. Here, we provide a brief overview of different immune-evasion strategies of mycobacteria and their impact on the protective immune response. This understanding will further our knowledge on host–pathogen interactions and may provide critical insights for the development of novel host-specific therapies.

Introduction

Mycobacteria are one of the most important and well-adapted group of human pathogens. Among diseases caused by mycobacteria, tuberculosis, caused by *Mycobacterium tuberculosis*, is undeniably one of the oldest recorded human diseases, and still one of the biggest killers. While mortality has decreased significantly since the 1990s (WHO, 2015), in 2014 alone, 9.6 million people were estimated to fall ill with tuberculosis and 1.5 million died from the disease (WHO, 2015). After tuberculosis, leprosy and Buruli ulcer, caused by

Mycobacterium leprae and *Mycobacterium ulcerans*, respectively, are the second and third most common mycobacterial diseases in humans (Walsh *et al.*, 2010). Although leprosy was declared eliminated by the end of 2000 in most countries by the World Health Organization (WHO), incidence remains fairly stable, suggesting active transmission (Schreuder *et al.*, 2016; Walsh *et al.*, 2010). On the other hand, Buruli ulcer is a re-emerging disease mainly due to environmental factors including deforestation, artificial topographic alterations (such dams and irrigation systems), and increasing populations engaged in basic manual agriculture in wetlands (Merritt *et al.*, 2010; van Ravensway *et al.*, 2012).

The outcome of mycobacterial infections depends on their interaction with the host immune system. These interactions can result in control of infection, establishment of latent infection or development of disease (Casanova and Abel, 2002). Specifically for *M. tuberculosis*, genetic analyses suggest that this pathogen and humans coevolved for thousands of years (Comas *et al.*, 2013). During this time, *M. tuberculosis* has developed multiple and redundant strategies to manipulate macrophage activation and establish an intracellular niche to prevent elimination. Despite this, antigen-specific T cells are activated upon exposure to mycobacteria, and these cells are an essential component of the protective response (Cooper, 2009). However, the inflammatory environment generated during infection can have important consequences for T-cell function and control of infection. In some cases this environment may even promote T-cell-mediated immunopathology, which facilitates transmission of infection. Therefore, immune evasion by mycobacteria is a rather sophisticated process, wherein the highly adapted pathogen uses multiple and redundant strategies to manipulate the host response and establish a suitable environment for proliferation, while promoting the immune-mediated damage required for transmission.

Here, we discuss some of the strategies used by mycobacteria to evade the host immune system and how these strategies impact the protective response.

Mycobacteria use redundant strategies to inhibit the microbicidal activity of myeloid cells

Upon infection, resident macrophages and dendritic cells (DCs) are likely to be the first cells to interact with mycobacteria. Owing the success of mycobacteria as pathogens, it is not surprising that some of the most important adaptations that pathogenic mycobacteria have acquired throughout evolution are directed at modulating the activation and the effector functions of these cells. For example, one of the first mycobacterial virulence traits identified and studied in detail, was the ability of *M. tuberculosis* to inhibit the fusion of mycobacteria-containing phagosomes with acidified lysosomes (Armstrong and Hart, 1971). A process similar to phagosome–lysosome fusion is autophagy. Autophagy is controlled by autophagy-related genes (Atg) and relies on the formation of an autophagosome which engulfs components of the cytoplasm and delivers them to degradation in the lysosome (Mizushima *et al.*, 2011). It is now well established that *M. tuberculosis* can also interfere with autophagy (Romagnoli *et al.*, 2012; Shin *et al.*, 2010). Although phagocytosis and autophagy are independent processes, both phagosomes and autophagosomes mature through acidification and acquisition of proteolytic enzymes that degrade intracellular material, including mycobacteria. These processes are not only important to control intracellular pathogen growth, but also affect acquired immune responses. Indeed, proteins and peptides degraded in the phagolysosome or autophagolysosome can then be loaded in major

histocompatibility complex (MHC) molecules thereby inducing T-cell activation (Vyas *et al.*, 2008). Here we discuss some of the mechanisms wherein mycobacteria interfere with phagosome–lysosome fusion and autophagy to prevent rapid elimination and establish an intracellular niche to thrive.

The highly characteristic cell wall (Brennan and Nikaido, 1995) and capsular layer (Sani *et al.*, 2010) of mycobacteria is composed of unique glycolipids, glucans and proteins that engage several host pathogen recognition receptors (PRRs) thereby shaping the response of myeloid cells in the infection site (Awuh and Flo, 2016). Phagocytosis of *M. tuberculosis* has been shown to be mediated through multiple receptors expressed by macrophages, such as Fcγ receptors, complement receptor type 3 and lectin receptors (Schäfer *et al.*, 2009). Fcγ receptors and complement receptor type 3 promote phagocytosis of the bacteria coated with IgG antibodies and complement protein fragment C3bi, respectively (Caron and Hall, 1998).

During primary infection, phagocytosis of *M. tuberculosis* appears to be mostly through non-opsonic mechanisms (Schäfer *et al.*, 2009). This occurs likely because serum and complement components are limited in the alveolar space when the first interaction between *M. tuberculosis* and macrophages occurs (Schäfer *et al.*, 2009; Schluger, 2001). On the other hand, opsonized uptake of *M. tuberculosis* may be more important at later stages of the infection (Schäfer *et al.*, 2009). The relevance of these mechanisms is highlighted by the fact that phagocytosis of opsonized mycobacteria does not appear to inhibit phagosome–lysosome fusion (Kang *et al.*, 2005) whereas phagocytosis of non-opsonized mycobacteria does (Hmama *et al.*, 2015; Kang *et al.*, 2005). For example, phagocytosis of mycobacteria through mannose receptor (MR) binding of the mannose-capped lipoarabinomannan (ManLAM), one of the most prominent molecular patterns found in the cell wall of pathogenic mycobacteria (Brennan and Nikaido, 1995), results in phagosome–lysosome fusion arrest (Kang *et al.*, 2005). Interestingly, however, ManLAM has also been shown to interact with other PRRs, namely the dendritic cell-specific intracellular adhesion molecule 3 grabbing nonintegrin (DC-SIGN) and, more recently, Dectin-2 expressed by DCs (Yonekawa *et al.*, 2014). While the engagement of ManLAM with Dectin-2, in what regards phagosome–lysosome fusion, has not been yet determined, engagement of ManLAM with DC-SIGN does not induce phagosome–lysosome arrest (Kang *et al.*, 2005).

The effect of ManLAM on phagocytic cells is however not limited to phagosome-lysosome arrest but it also stimulates the production of cytokines, co-stimulatory molecules and MHC-II expression, important for macrophage function and stimulation of acquired responses (Kallenius *et al.*, 2016). Indeed, stimulation of bone marrow-derived DCs with ManLAM induces the production of IL-10, IL-12p40, IL-6 and TNF-α, up-regulation of the co-stimulatory molecules CD80 and CD86 and MHC-II, in a Dectin-2-dependent manner (Yonekawa *et al.*, 2014). Importantly, mice deficient in Dectin-2 are more susceptible to disease after *M. avium* pulmonary infection, showing that recognition of ManLAM by Dectin-2 is important to control infection (Yonekawa *et al.*, 2014). On the other hand, a recent study compared the virulence *M. tuberculosis* and *M. bovis* BCG with isogenic strains lacking the *CapA* locus, which is involved in the biosynthesis of the mannose cap of LAM (Afonso-Barroso *et al.*, 2013). This study showed that mycobacteria lacking the mannose cap of LAM were not less virulent than the isogenic strains that have ManLAM (Afonso-Barroso *et al.*, 2013). Thus, taken together, these data suggest that during infection the effects of ManLAM may be redundant and in the absence of this molecular pattern other

ligands are engaged by the same receptors in myeloid cells. In this regard, several molecular patterns have been identified to interact with DC-SIGN (Appelmelk *et al.*, 2008; Driessen *et al.*, 2009; Geijtenbeek *et al.*, 2003; Pitarque *et al.*, 2005). An example is α-glucan that has been shown to induce the production of IL-10 upon lipopolysaccharide (LPS) stimulation of DCs, without altering IL-12p40 production (Geurtsen *et al.*, 2009). In the context of the whole bacteria however, it has been shown that stimulation of monocyte-derived DCs with *M. bovis* BCG, grown in conditions that are permissive to the maintenance of the capsular layer (rich in α-glucan), produced significantly lower levels of IL-12 and TNF-α, when compared to *M. bovis* BCG that did not have the α-glucan-rich capsular layer (Sani *et al.*, 2010). A recent study identified PPE10 protein, secreted by Esx-5, as the main responsible for maintaining the mycobacterial capsular integrity (Ates *et al.*, 2016). Interestingly, *M. marinum* deficient in Esx-5 or PPE10 were less virulent that the complemented strains in zebrafish embryos (Ates *et al.*, 2016), highlighting the importance of the capsular layer, and α-glucan, in the immune response and control of infection.

The above described data highlight the complex interaction between mycobacteria and phagocytic cells during infection. Specifically for ManLAM, its recognition through MR results in phagosome-lysosome arrest (Kang *et al.*, 2005), but its recognition through other PRRs may result in a different response leading to the production of cytokines, including TNF-α, IL-6, IL-12, IL-10 (Kallenius *et al.*, 2016) that have multiple roles during infection (Domingo-Gonzalez *et al.*, 2016; Torrado and Cooper, 2013). When these data are taken together with other data showing that (i) several mycobacterial molecular patterns are recognized by host cells through multiple PRRs, including Toll-like receptors (TLR), NOD-like receptors, Dectin-1 and Mincle; and (ii) engagement of these receptors leads to the production cytokines important in macrophage function and in the infection process (Awuh and Flo, 2016; Killick *et al.*, 2013; Mortaz *et al.*, 2015; Rajaram *et al.*, 2014); it is plausible to conclude that most of the molecular patterns of mycobacteria have redundant effects on phagocytic cells (Killick *et al.*, 2013). Because of these redundant functions, in some cases absence of one receptor in the host, or one molecular pattern in the pathogen, does not translate in altered bacterial burdens or pathology to the host. The data described above for ManLAM are an example. On the host side, absence of TLR-2 or TLR-9 does not impact the ability of mice to control *M. tuberculosis* infection; however, mice deficient in both TLR-2 and TLR-9 revealed to be more susceptible to *M. tuberculosis* infection (Bafica *et al.*, 2005).

Together, these data suggest not only that there is some degree of redundancy in the effects of mycobacterial molecular patterns, but also that the targeting of specific PRRs is critical for the pathogen to establish an intracellular niche and survive in the infected host.

In addition to molecular patterns recognized by cell-surface receptors during phagocytosis, *M. tuberculosis* has also been shown to secrete effector molecules that interfere with intracellular vesicle trafficking. Two of the most well characterized effectors that interfere with phagosome–lysosome fusion are PtpA (Bach *et al.*, 2008) and SapM (Vergne *et al.*, 2005). The mechanisms by which these proteins interfere with phagosome–lysosome fusion have been recently reviewed (Hmama *et al.*, 2015).

Other important mycobacterial proteins that trigger phagosome-lysosome arrest include TB10.4 (encoded by *esxH*) and TB9.8 (encoded by *esxG*), which are secreted by the mycobacterial Esx-3 type VII secretion system (Mehra *et al.*, 2013). The TB10.4/TB9.8 heterodimer is able to interact with the hepatocyte growth factor-regulated tyrosine

kinase substrate (Hgs/Hrs), a component of the Endosomal Sorting Complex Required for Transport (ESCRT) (Mehra *et al.*, 2013). The ESCRT transport system is composed by four protein complexes that are sequentially recruited to the endosomal membrane, promoting the delivery of phagocytosed mycobacteria to the lysosome, thus restricting intracellular bacterial growth (Mehra *et al.*, 2013). The heterodimer was shown to directly target Hrs, inhibiting delivery of bacteria to lysosomes (Mehra *et al.*, 2013). It is worth noting that TB10.4/TB9.8 are highly antigenic, and a high frequency of T cells specific for these antigens has been shown to correlate with protection against *M. tuberculosis* infection (Hervas-Stubbs *et al.*, 2006). In fact, the introduction of the *M. tuberculosis* Esx-3 locus into an *M. smegmatis* strain lacking the endogenous Esx-3 region generates highly protective immunity against *M. tuberculosis* (Sweeney *et al.*, 2011). There is also a role for Esx-3 of *M. tuberculosis* and *M. smegmatis* in iron and zinc acquisition (Serafini *et al.*, 2009; Siegrist *et al.*, 2014).

The Esx-1 type VII secretion system also secretes other highly antigenic T-cell proteins that interfere with intracellular vesicle trafficking, such as the early secretory antigen target 6 kDa (ESAT-6, encoded by *esxA*) and the culture filtrate antigen 10 kDa (CFP-10, encode by *esxB*) (Tan *et al.*, 2006). However, a more important biological role of these proteins in mycobacterial pathogenesis is their phagosomal permeabilization properties implicated in rupture and translocation of *M. tuberculosis* and *M. leprae* from the phagosome into the cytosol (van der Wel *et al.*, 2007), activation of autophagy (Watson *et al.*, 2012) as well as the inflammasome (Mishra *et al.*, 2013). The importance of these proteins in infection is highlighted by several studies showing that, mutations affecting the synthesis or secretion of ESAT-6/CFP10 attenuate the virulence of mycobacteria in murine models of infection (Hsu *et al.*, 2003; Lewis *et al.*, 2003; Pym *et al.*, 2002, 2003).

Together, these data show that mycobacteria use redundant mechanisms to prevent macrophage activation and functions, highlighting the importance of these strategies to prevent rapid elimination.

Initial studies on macrophages infected with *M. tuberculosis* or *M. bovis* BCG suggested that autophagy induction could overcome phagososme–lysosome arrest imposed by mycobacteria (Gutierrez *et al.*, 2004). This and following studies, established autophagy as an important antimycobacterial defence mechanism, not only by overcoming phagosome-lysosome arrest, but also by promoting the generation of antimicrobial peptides with activity against mycobacteria (Ponpuak *et al.*, 2010). The critical role of autophagy during *M. tuberculosis* infection is underlined by the acute susceptibility of mice conditionally deficient for Atg5 in phagocytic cells (Castillo *et al.*, 2012; Kimmey *et al.*, 2015; Watson *et al.*, 2012), a phenotype similar to that of IFN-γ-deficient mice (Cooper *et al.*, 1993; Flynn *et al.*, 1993; Nandi and Behar, 2011). However, more recent data showed that deficiency in other Atg genes important for autophagy did not impact control of *M. tuberculosis* infection (Kimmey *et al.*, 2015), suggesting that Atg5 participates in unique functions not provided by other essential Atg proteins. Indeed, Atg5 deficiency in alveolar macrophages or DCs did not significantly impact control of infection; as opposed to Atg5 deficiency in neutrophils, which resulted in uncontrolled recruitment of these cells to the infection site (Kimmey *et al.*, 2015). The role of neutrophils in the susceptibility of Atg5 deficient mice was determined by antibody depletion studies that reverted the acute susceptible phenotype of Atg5 deficient mice (Kimmey *et al.*, 2015).

Although the data discussed above suggest autophagy-independent roles of Atg5 in

controlling resistance to *M. tuberculosis* infection, several factors produced by *M. tuberculosis* have been identified to interfere with the autophagic process. The ε-aminoacetyltransferase Eis, for instance, has been shown to acetylate Lys-55 in a JNK-specific phosphatase and is critical to activate the autophagy regulator Beclin 1 (Kim *et al.*, 2012). Similar to phagosome–lysosome fusion, the Esx-1 secretion system has also been implicated in inhibiting autophagy in human DCs (Romagnoli *et al.*, 2012). Likewise, the PE_PGRS47 protein has been recently shown to inhibit autophagy with impact on antigen presentation, but without affecting MHC-II expression (Saini *et al.*, 2016). This protein was identified in a screening of random genomic DNA fragments of *M. tuberculosis* capable of inhibiting antigen processing and loading into MHC-II (Saini *et al.*, 2016). Compared to the wild-type or PE_PGRS47 complemented strains, macrophages infected with the mutant PE_PGRS47 strain showed an increase in microtubule-associated light chain 3 (LC3), a central component of autophagosome formation, and p62, which is recruited to newly formed autophagosomes (Saini *et al.*, 2016). These findings thus suggest that PE_PGRS47 prevents the formation of autophagosomes containing intracellular *M. tuberculosis*. Moreover, *in vivo* experiments show that mice infected with the PE_PGRS47 mutant strain of *M. tuberculosis* were more resistant to infection, maintaining lower bacterial burdens throughout chronic infection when compared to mice infected with the wild-type or complemented strains (Saini *et al.*, 2016). Interestingly, while there were no differences in the frequency of antigen-specific CD4 T cells at day 9 post infection (when T cells are primed in the lymph nodes, discussed below), there were more antigen-specific CD4 T cells at day 24 post infection in mice infected with the *M. tuberculosis* strain deficient in PE_PGRS47 than in mice infected with the wild-type or complemented strains (Saini *et al.*, 2016). These data show that by interfering with intracellular vesicle trafficking, *M. tuberculosis* impairs antigen-processing and subsequent loading into MHC-II molecules.

Since there were no differences in the frequency of CD4 T cell at day 9 post infection (Saini *et al.*, 2016), the data discussed above also suggest that inhibition of autophagy may not impact T-cell priming. In this regard, several studies have shown that mycobacteria can modulate antigen presentation through different mechanisms, including inhibition of MHC-II expression or antigen-processing and loading (Hmama *et al.*, 2015). However, these mechanisms may not fully explain the delayed T-cell priming during mycobacterial infection (discussed below) but may rather be more important for T-cell function in the infection site. A recent study took advantage of MHC-II mixed bone-marrow chimeras to compare the bacterial burdens in myeloid cells that were capable (MHC-II-expressing), or not (MHC-II deficient), of interacting with CD4 T cells (Srivastava and Ernst, 2013). Results showed that myeloid cells expressing MHC-II had reduced bacterial burdens when compared to myeloid cells that did not express MHC-II (Srivastava and Ernst, 2013). Therefore, direct recognition of infected cells by CD4 T cells appears to be required for controlling intracellular *M. tuberculosis* in the infection site. Accordingly, depletion of CD4 T cells resulted in increased numbers of bacteria in MHC-II-expressing myeloid cells but not MHC-II deficient cells (Srivastava and Ernst, 2013). These data suggest that, by interfering with autophagy and phagosome–lysosome fusion, *M. tuberculosis* may prevent the interaction between infected macrophages and antigen-specific CD4 T cells at the infection site, thereby limiting the ability of CD4 T cells to activate *M. tuberculosis*-infected macrophages.

Collectively, these studies show that during the first interaction with myeloid cells in the infection site different mycobacterial molecular patterns are recognized by distinct host

receptors, resulting in signals leading to phagosome-lysosome arrest, but also the production of pro- and anti-inflammatory cytokines and chemokines with important roles during infection (Domingo-Gonzalez *et al.*, 2016; Torrado and Cooper, 2013). The different and redundant strategies used by mycobacteria to inhibit phagosome–lysosome fusion and autophagy highlight the key role of these strategies in the early establishment of an intracellular niche for the pathogen to proliferate (Fig. 7.1). During the course of infection, the balance between these signals may play key roles in determining the early control of infection and induction of T-cell responses (Fig. 7.1).

Mycobacteria modulate the microenvironment to subvert infected-cell death pathways and recruit permissive macrophages

Although mycobacteria are well equipped to prevent and escape phagosome–lysosome fusion and autophagy, macrophages can still activate apoptotic cell death programmes

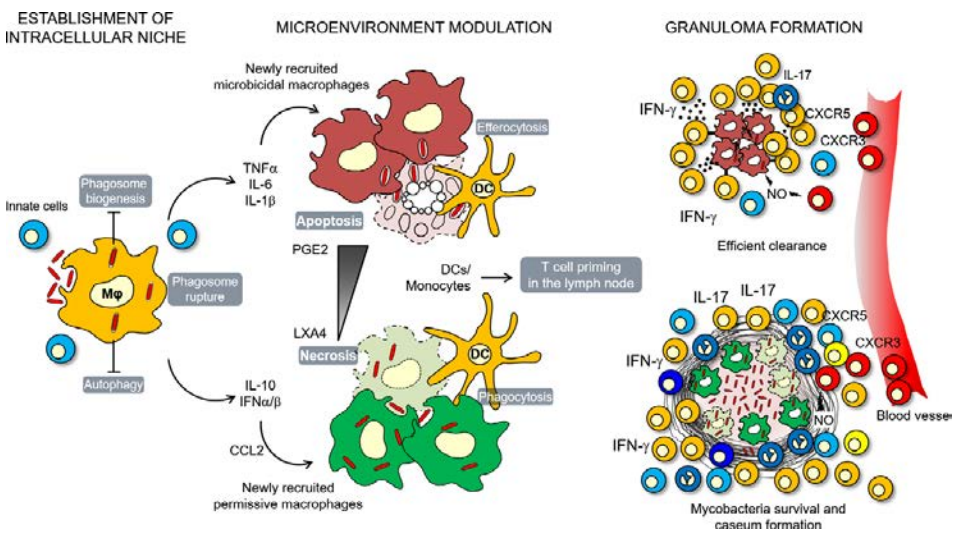

Figure 7.1 Progression of the immune response to *M. tuberculosis* infection. Early interaction with phagocytes through PRRs results in the phagocytosis of the bacteria. Virulent *M. tuberculosis* interferes with intracellular vesicle trafficking and escapes the phagosome to prevent early elimination and establish an intracellular niche. At the same time macrophages, and other inflammatory cells in the site of infection, produce inflammatory mediators that can impact the cell death modality of the infected-cell and induce the recruitment of other inflammatory cells. High levels of IFN-αβ induce the recruitment of CCR2-expressing monocytes with limited ability to control bacterial growth. As infected cells die newly recruited macrophages and monocytes efferocytose or phagocytose the bacteria and a granulomatous reaction begins to form. After the onset of acquired immunity, T cells begin to accumulate and a granuloma is formed. The efficient interaction between T cells and macrophages together with a balanced production of cytokines and chemokines results in a non-damaging inflammatory environment and control of infection. On the other hand, phagocytosis of the bacteria by permissive macrophages with a weak capacity to interact with T cells results in progression of infection. As the granuloma coalesces, the inflammatory environment generated may promote the constant accumulation of T cells contributing to immunopathological consequences to the host.

to prevent further microbial intracellular replication and increase exposure of microbial antigens. Apoptosis is a tightly regulated process of cellular deconstruction that minimizes inflammation and pathology by containing the dismembered cellular contents of dying cells within membrane-bound vesicles, called apoptotic bodies (Taylor *et al.*, 2008). These apoptotic bodies emit signals to be engulfed and digested by phagocytes in a process called efferocytosis (Martin *et al.*, 2014). On the other hand, necrosis is a more inflammatory process when compared to apoptosis, as it is defined by loss of membrane integrity with the release of intracellular contents to the extracellular space (Moraco and Kornfeld, 2014).

A growing body of evidence points to the ability of *M. tuberculosis* to inhibit apoptotic and promote necrotic cell death of macrophages as an important determinant of virulence and pathogenesis (Behar *et al.*, 2010; Chen *et al.*, 2008; Chen *et al.*, 2006; Divangahi *et al.*, 2009; Divangahi *et al.*, 2010). Early studies suggested apoptosis, but not necrosis, of the mycobacterial-infected cells to have direct bactericidal effects on intracellular bacterial viability. However, more recent data suggest that apoptosis itself is not intrinsically bactericidal but instead requires efferocytosis (Martin *et al.*, 2012).

One key mechanism wherein virulent *M. tuberculosis* prevents apoptosis and promotes necrotic death of infected macrophages is by regulating eicosanoid production (Chen *et al.*, 2008). Mechanistically, avirulent *M. tuberculosis* H37Ra strain has been shown to stimulate the production of prostaglandin E_2 (PGE_2), which is required for plasma membrane protection by regulating synaptotagmin 7, the calcium sensor involved in the lysosome-mediated repair mechanism (Divangahi *et al.*, 2009). On the other hand, virulent *M. tuberculosis* H37Rv and Erdman strains stimulate the production of lipoxins, such as lipoxin A4 (LXA4), which in turn down-regulate cyclooxygenase (COX)2 mRNA, thereby reducing PGE2 production and preventing its membrane protective effects, resulting in necrotic death of the infected cell (Chen *et al.*, 2008; Divangahi *et al.*, 2009). The relevance of these pathways is demonstrated by the observation that Alox5-deficient macrophages, which cannot synthesize LXA4, undergo more apoptosis and are better able to control *M. tuberculosis* H37Rv growth when compared to wild-type macrophages. On the other hand, PGE2-deficient macrophages undergo more necrosis and do not to control intracellular growth of either virulent or avirulent *M. tuberculosis* (Divangahi *et al.*, 2009). Therefore, apoptosis appears to be the fate of *M. tuberculosis*-infected cells if mitochondrial and plasma membrane integrity is protected by PGE2, whereas necrosis occurs if LXA4 predominates. How *M. tuberculosis* induces LXA4 production is not yet determined. Likely, the Esx-1 secretion system-induced rupture of the mycobacteria vacuole (van der Wel *et al.*, 2007), as well as the pore forming activity of ESAT-6 (Smith *et al.*, 2008), are important in the process. Another candidate is CpnT, a NAD^+-glycohydrolase which depletes cellular NAD^+ pools, resulting in host necrotic cell death (Danilchanka *et al.*, 2014; Sun *et al.*, 2015).

The ability of *M. tuberculosis* to prevent apoptosis of its host cells can also affect the T-cell response (Divangahi *et al.*, 2010). Indeed, when alveolar macrophages from *M. tuberculosis*-infected wild-type or Alox5-deficient mice were transferred into wild-type mice, the Alox5-deficient macrophages were able to stimulate pulmonary and systemic CD4 and CD8 T-cell responses, in contrast to wild-type macrophages (Divangahi *et al.*, 2010). These enhanced T-cell responses were not directly induced by the transferred macrophages. Instead, the antigen cargo contained within the apoptotic vesicles of Alox5-deficient macrophages was phagocytosed by DCs that, in turn, stimulated T-cell responses (Divangahi *et al.*, 2010).

The role of lipid mediators can also directly influence T-cell responses. For example, PGE2 has been shown to directly promote the differentiation and proinflammatory functions of human and murine Th17 cells (Boniface *et al.*, 2009; Gopal *et al.*, 2012). In this regard, IL-17 and Th17 cells are an important component of the protective immune response to highly virulent strains of *M. tuberculosis* (Gopal *et al.*, 2014), but uncontrolled Th17 responses can result in important immunopathological consequences to the host (Cruz *et al.*, 2010; Torrado and Cooper, 2010). More recently, IL-17 was found to promote the intracellular growth of *Mycobacterium bovis* BCG and *M. tuberculosis* by inhibiting the intrinsic apoptotic pathway (Cruz *et al.*, 2015a). This study showed that IL-17 induces a down-regulation of the apoptosis master regulator p53 resulting in a reduction in the percentage of caspase-3-positive macrophages, as well as an increase in the ratio of Bcl2/Bax proteins (Cruz *et al.*, 2015a). Together, these data suggest that during the early stages of infection the balance between eicosanoids plays a key role in determining the cell death modality of the infected cell; as the infection progresses the inflammatory environment generated in the infection site may alter this balance (Fig. 7.1). Future research is required to address these issues.

In addition to regulating the infected-cell death modality, PGE2 has been recently shown to regulate the production of type I interferons (IFN-α/β) (Mayer-Barber *et al.*, 2014). IFN-α/β has been shown to have a largely detrimental role in tuberculosis (Berry *et al.*, 2010; Manca *et al.*, 2001; Ordway *et al.*, 2007), with more virulent strains (such as Beijing strains) inducing the highest production of IFN-α/β (Carmona *et al.*, 2013; Manca *et al.*, 2001). The induction of IFN-α/β is likely associated with the phagosome membrane rupture mediated by the Esx-1 secretion system (van der Wel *et al.*, 2007) triggering a cytosolic DNA sensor and activating the Sting/Tbk1/Irf3 axis (Manzanillo *et al.*, 2012). This pathway is also activated by multiple PRRs, including TLRs (Barber, 2011). In this regard, *M. tuberculosis* Beijing strains have been shown to induce IFN-α/β through a TLR-4-dependent pathway (Carmona *et al.*, 2013).

The detrimental role of IFN-α/β during mycobacterial infections is complex and likely involves different mechanisms. One of these mechanisms is associated with the recruitment of permissive macrophages to the infection site. Indeed, induction of IFN-α/β production by intranasal challenge of *M. tuberculosis*-infected mice with polyinosinic-polycytidylic acid condensed with poly-l-lysine and carboxymethylcellulose (Poly-ICLC) results in elevated bacterial burdens associated with increased accumulation of a $CD11b^{+}Gr1^{int}$ myeloid population recruited via the CCL2-CCR2 pathway (Antonelli *et al.*, 2010). In Poly-ICLC treated mice, $CD11b^{+}Gr1^{int}$ cells displayed decreased expression of MHC-II and elevated bacterial levels when compared to the same subset of cells purified from infected, untreated controls (Antonelli *et al.*, 2010).

In another recent paper, it was shown that both *M. tuberculosis* and *M. marinum* actively promote the recruitment of permissive macrophages by using cell-surface-associated phthiocerol dimycoceroserate (PDIM) lipids through a host CCR2-mediated pathway (Cambier *et al.*, 2014). Interestingly, these PDIM appear to mask underlying mycobacterial molecular patterns. In the absence of PDIM, these molecular patterns signal a TLR-dependent recruitment of macrophages that produce nitric oxide and control intracellular *M. tuberculosis* and *M. marinum* growth (Cambier *et al.*, 2014). Whether or not this CCR2 pathway is also activated by IFN-α/β, was not determined. This intricate immune evasion strategy also provides an explanation for the observation that *M. tuberculosis* initiates infection in the lower airways, a relatively sterile environment. Indeed, in the upper airways the normal flora

provides the TLR stimulation required to recruit microbicidal macrophages that are capable of controlling *M. tuberculosis* infection (Cambier *et al.*, 2014).

The other detrimental role of IFN-α/β is by suppressing IFN-γ-dependent macrophage activation (Teles *et al.*, 2013). This role of IFN-α/β is particularly evident in leprosy, which presents in two distinct clinical manifestations: a disseminated form (lepromatous) or a self-healing form (tuberculoid). Self-healing tuberculoid lesions were shown to be associated with IFN-γ and its downstream vitamin D-dependent antimicrobial genes, whereas disseminated and progressive lepromatous lesions were associated with an IFN-α/β gene programme, including IL-10 (Teles *et al.*, 2013). Importantly, IFN-γ-induced antimicrobial activity against *M. leprae* in monocytes was abrogated by IFN-α/β or IL-10 (Teles *et al.*, 2013). Recently, IFN-α/β was also shown to induce IL-10 production, which in turn inhibits the production of TNF-α, IL-12, and IL-1β and impairs the ability of IFN-γ-activated macrophages to control *M. tuberculosis* (McNab *et al.*, 2014). Taking into account that tuberculosis also consists of a spectrum of disease, even though not as distinct as leprosy, it is possible that IFN-α/β responses are involved in defining this spectrum.

Despite its largely detrimental role, IFN-α/β may also have protective functions during *M. tuberculosis* infection. Indeed, mice lacking both IFN-α/β and IFN-γ signalling are more susceptible to infection, when compared to single deficient mice (Desvignes *et al.*, 2012; Moreira-Teixeira *et al.*, 2016). In this model, the function of IFN-α/β receptor signalling was suggested to limit the early recruitment of permissive macrophages (Desvignes *et al.*, 2012). In accordance with this protective role is the observation that IFN-α/β may promote expression of the inducible Nitric Oxide Synthase (iNOS, encoded by *Nos2*) during the early stages of *M. tuberculosis* infection (Moreira-Teixeira *et al.*, 2016). Therefore, low amounts of IFN-α/β may be protective by activating macrophages to restrict intracellular mycobacterial growth; however, production of too much IFN-α/β results in the recruitment of large numbers of permissive macrophages, preventing control of infection.

Collectively, these data show that mycobacteria modulate the inflammatory environment of the infection site to interfere with the cell death modality of its host cells while promoting the recruitment of permissive macrophages to the infection site (Fig. 7.1).

Delayed T-cell priming during mycobacterial infections

Antigen-specific T cells are an essential component of the protective response to mycobacteria (Cooper, 2009). During tuberculosis, T-cell effector responses are dominated by Th1 (defined by IFN-γ production) and, to a lesser extent, Th17 (defined by IL-17 production) phenotypes, as well as CD8 T cells. These populations play key roles in the protective and pathological responses (Behar, 2013; Cooper, 2009; Lin and Flynn, 2015; Torrado and Cooper, 2010; Torrado *et al.*, 2011).

One of the most intriguing aspects of the host response against mycobacteria is the delayed priming of the T-cell response. As priming and initiation of acquired immune responses are difficult to address in humans, due to limitations in sampling and determining time of exposure, the aerosol mouse model of infection has been used to address this issue (Gallegos *et al.*, 2008; Reiley *et al.*, 2008; Wolf *et al.*, 2008). Using this model, the bacteria is deposited in the lung lower airways where *M. tuberculosis* grows in an apparent uncontrolled manner, until day 21 post infection (Cooper and Khader, 2008; Khader *et al.*, 2007). Notwithstanding, several studies have shown that antigen-specific T cells only become

activated after day 7–10 of infection and that T-cell priming occurs in the lung draining mediastinal lymph nodes, but not in the lung (Gallegos *et al.*, 2008; Reiley *et al.*, 2008; Wolf *et al.*, 2008). This slow onset in the acquired response is in marked contrast to the kinetics typically observed for other aerogenic infections, such as influenza (Flynn *et al.*, 1998) and *Francisella tularensis* (Baron *et al.*, 2007), or with other intracellular bacteria, such as *Listeria monocytogenes* (Kursar *et al.*, 2002). In these infections, T-cell priming and expression of T-cell immunity in the infection site occurs as early as 3–7 days post infection and results in a rapid control of viral or bacterial proliferation (Baron *et al.*, 2007; Flynn *et al.*, 1998; Kursar *et al.*, 2002).

The slow pace at which the T-cell response is initiated could be, at least partially, explained by the reduced antigen production, associated with the slow proliferation rate of *M. tuberculosis.* However, during *in vitro* growth *M. tuberculosis* rapidly secretes T-cell antigenic proteins to the culture medium (Andersen *et al.*, 1992; Andersen *et al.*, 1991). Therefore, if the slow mycobacterial growth accounted for the insufficient antigen for CD4 T-cell activation, then the time required to initiate the immune response should be reduced by increasing the dose of infection. However, increasing the dose of infection by a factor of 10 does not significantly enhance T-cell priming (Reiley *et al.*, 2008; Wolf *et al.*, 2008). On the other hand, the observations that (i) T-cell priming in the draining mediastinal lymph node after aerosol challenge coincides with the arrival of viable bacteria to this location (Reiley *et al.*, 2008; Wolf *et al.*, 2008); and that (ii) the delay in the control of *M. tuberculosis* infection in the lungs after aerosol infection contrasts with the immediate control in the liver upon intravenous challenge, despite the fact that in both cases control of infection was associated with the accumulation of IFN-γ-producing CD4 T cells (Cooper, 2009; Cooper *et al.*, 1997a); suggest that dissemination of the bacteria to the draining lymph node is likely essential to T-cell priming. This hypothesis is also consistent with the observations that the lung environment is not optimal for T-cell priming, as myeloid cells isolated from lungs of *M. tuberculosis*-infected mice induce poor T-cell cytokine responses, when compared to the same populations isolated from the lymph nodes (Wolf *et al.*, 2007). Therefore to initiate T-cell responses, *M. tuberculosis*-infected myeloid cells need to migrate to the lymph node.

Still the question remains on why these cells do not migrate quicker to the draining lymph nodes. While there is still no definitive answer to this question, as referred above it is known that increasing the infection dose does not significantly enhance this response (Reiley *et al.*, 2008; Wolf *et al.*, 2008). As such, the bacteria itself does not appear to inhibit the migration of the infected cells; instead, primary infected cells are likely non-migratory cells. Therefore, despite the ability of *M. tuberculosis* to interfere with myeloid cell mechanisms required for T-cell activation, such as MHC-II expression (Hmama *et al.*, 2015), the delay in T-cell priming is largely attributed to the microenvironment of the lung and the transport of *M. tuberculosis*, or its antigens, to the draining lymph nodes.

In this regard, the transport of *M. tuberculosis* from the lung to the draining lymph nodes has been recently shown to be made by Ly6C monocytes, in a CCR2-dependent way (Samstein *et al.*, 2013). It is interesting to note, however, that these cells are not lung resident but migrate from the bone-marrow to the infection site in response to CCR2-binding chemokines, CCL2 and CCL7, where they presumably differentiate into macrophages or DCs (Serbina and Pamer, 2006). How these cells uptake *M. tuberculosis*, or its antigens, is still not known. The function of Ly6C monocytes in T-cell priming appears to be limited to shuttling the bacteria, or its antigens, to the draining lymph nodes as they do not prime

T cells directly (Samstein *et al.*, 2013). Perhaps this is the result of *M. tuberculosis'* ability to interfere with the differentiation of monocytes. Indeed, it has been suggested that *M. tuberculosis* infection impairs the differentiation of monocytes into DCs, inducing instead, the generation of macrophage-like cells that fail to up-regulate co-stimulatory and MHC-II molecules (Remoli *et al.*, 2011).

As T-cell priming is delayed, one could speculate that if primed T cells were already in the lung and ready to respond, control of infection would be more efficient. Indeed, mice infected with *M. tuberculosis* and subsequently treated with antibiotics to generate memory-immune mice (Cooper *et al.*, 1997a) or vaccinated with BCG (Cruz *et al.*, 2015b) control *M. tuberculosis* faster than naive mice; however, the kinetics of the protective response is still slow and control of infection is only observed at day 14 post infection (Cooper *et al.*, 1997a; Cruz *et al.*, 2015b). One possible caveat of these experiments is that the memory T-cell response generated by live bacteria does not populate the lung. To overcome this, transgenic CD4 T cells primed *in vitro* in Th1 differentiating conditions (to promote the development of IFN-γ-producing T cell) were transferred into naive mice before aerosol *M. tuberculosis* infection (Gallegos *et al.*, 2008). These transferred effector cells were shown to populate the lung and produce IFN-γ and, as expected, provided enhanced protection (Gallegos *et al.*, 2008); however, this enhanced protection was only detected after day 7 of infection (Gallegos *et al.*, 2008). Thus, delayed T-cell priming is not the only issue in preventing the rapid control of infection, but the ability of the effector T cells to sense antigen in the lung is also critical (Srivastava and Ernst, 2013). The establishment of an intracellular niche, described above, is therefore important for *M. tuberculosis* to shelter for the T-cell response.

The impact of the inflammatory environment in accumulating T cells

As T cells accumulate in the infected organ, bacterial proliferation is controlled (Cooper and Khader, 2008; Khader *et al.*, 2007; Winslow *et al.*, 2003). In the tuberculosis mouse model, transition from the exponential increase in bacterial numbers to a plateau phase occurs at day 21 of infection, and correlates with the accumulation of IFN-γ-producing CD4 T cells (Cooper and Khader, 2008; Khader *et al.*, 2007). The critical role of CD4 T cells and IFN-γ in the control of mycobacterial infections has been demonstrated in different animal models as well as in humans, particularly in those with acquired immunodeficiencies affecting CD4 T-cell immunity, such as HIV infection, which is one of the most important risk factors for development of active tuberculosis (Geldmacher *et al.*, 2012). Therefore, understanding the mechanisms that govern T-cell migration and accumulation within the infected tissue are important.

After priming, T cells exit the draining lymph nodes, enter into the peripheral blood circulation, and migrate to the lung tissue. Although the mechanisms that orchestrate the T-cell egress from the blood stream into the lung tissue are not completely understood, recent papers have identified key chemokine receptors that are important for T-cell migration into the lung tissue. One of these studies used intravascular flow cytometry, as previously described (Anderson *et al.*, 2014), to distinguish between *M. tuberculosis*-specific T cells that are in the lung vasculature or lung parenchyma (Sakai *et al.*, 2014). This approach identified two major subsets of CD4 T cells based on their location and phenotype. The CD4 T-cell population found mostly associated with the lung vasculature produced the

highest amounts of IFN-γ and expressed killer cell lectin-like receptor subfamily G member 1 (KLRG1), CX3CR1 and high levels of the Th1 transcription factor T-bet. On the other hand, the lung parenchyma was enriched with CD4 T cells that produced lower amounts of IFN-γ, expressed CXCR3 and intermediate levels of T-bet, but did not express KLRG1 (Sakai *et al.*, 2014). To determine their protective role during infection, each population was purified and adoptively transferred into T-cell-deficient naive mice that were subsequently challenged with *M. tuberculosis* through the aerosol route. This approach showed that the population better able to induce control of infection produced lower levels of IFN-γ, but was more capable of migrating to the lung parenchyma (Sakai *et al.*, 2014).

In this context, the chemokine receptor CXCR5 was also shown to have an important role in CD4 T-cell migration (Slight *et al.*, 2013). CXCL13 is the ligand for CXCR5 and during *M. tuberculosis* infection both CXCL13 and CXCR5 are required for the formation of ectopic lymphoid follicles containing B and T cells (Khader *et al.*, 2011; Slight *et al.*, 2013). These follicles have also been identified in tuberculosis patients (Ulrichs *et al.*, 2004) and their absence has been associated with uncontrolled disease (Ulrichs *et al.*, 2005). In mice, CXCR5 expression is required to control *M. tuberculosis* infection (Slight *et al.*, 2013). However, the enhanced susceptibility of CXCR5 was not associated with delayed CD4 T-cell priming, accumulation of CD4 T cells in the lung tissue, or altered cytokine production (Slight *et al.*, 2013). Instead, T cells from CXCR5-deficient mice locate in distinct perivascular cuffs, a phenotype also observed in wild-type mice that received CXCL13 (the ligand for CXCR5) neutralizing antibody (Slight *et al.*, 2013).

Together, these data suggest that the coordinated expression of CXCR3 and CXCR5 is critical for *M. tuberculosis*-specific cells to migrate to the lung parenchyma and into the *M. tuberculosis* lesion where they interact with infected macrophages. Nevertheless, whether these receptors mediate the extravasation across the lung blood vessel into the lung parenchyma or the migration within the parenchyma into the lesion is yet to be determined.

The mechanism underlying the accumulation of T-cell phenotypes associated with the lung vasculature or parenchyma is still not well understood. While we cannot exclude the possibility that these phenotypes are imprinted during priming, recent data suggest that the lung inflammatory environment generated during infection plays a role. In this regard, it has been previously shown that mice deficient in IL-27 signalling are more resistant to *M. tuberculosis* (Holscher *et al.*, 2005; Pearl *et al.*, 2004). More recently, we have found that the enhanced resistance of IL-27R-deficient mice is replicated in mice deficient in IL-27R only in the T-cell compartment (Torrado *et al.*, 2015). Therefore, we asked whether T cells differentiated differently in the absence or presence of IL-27-mediated signalling. Our data show that in IL-27R deficient mice, CD4 T cells were enriched in the lung parenchyma migratory populations, displaying reduced T-bet expression, CXCR3 and produced more IL-2 when compared to wild-type mice (Torrado *et al.*, 2015). Importantly, in competitive bone-marrow chimeras IL-27R-deficient CD4 T cells were more fit to survive within the lung lesion site, when compared to wild-type CD4 T cells (Torrado *et al.*, 2015).

Another important modulator of the T-cell response is nitric oxide produced by IFN-γ-activated macrophages in the lesion site (Cooper *et al.*, 2002). To test the hypothesis that the more activated cells within the lesion site were more sensitive to the inflammatory environment we resorted to the *M. avium* model wherein iNOS is not required to control infection and, indeed, it appears to benefit the host (Gomes *et al.*, 1999). Using this model, we also found an increased accumulation of CD4 T cells expressing high levels of T-bet in

iNOS deficient when compared to wild-type mice (Pearl *et al.*, 2012). Importantly, within the T-bet-expressing effector population, CD4 T cells expressing low levels of the early activation marker CD69 accumulate during chronic infection in higher numbers in iNOS deficient mice when compared to wild-type mice (Pearl *et al.*, 2012). These data suggest that the terminally differentiated population (based on the expression of high T-bet and reduced CD69) displays an enhanced sensitivity to the regulatory effects of nitric oxide (Pearl *et al.*, 2012). Therefore, while nitric oxide in the phagosome may restrain intracellular bacterial growth, it also regulates T-cell responses, preventing the accumulation of strongly activated and potentially pathological T cells at the site of infection (Fig. 7.1).

Since iNOS expression is required to control *M. tuberculosis*, the role of nitric oxide in this model is more difficult to address. To overcome this issue, a recent study used a genetically manipulated strain of *M. tuberculosis* that only proliferates in the presence of streptomycin (Mishra *et al.*, 2013). Without the confounding effect of bacterial proliferation, this study shows that iNOS deficient mice were still highly susceptibility to disease, displaying increased accumulation of neutrophils (Mishra *et al.*, 2013). This phenotype was associated with increased activation of the NLRP3 inflammasome, a process dependent on Esx-1-induced phagosome rupture, which resulted in elevated production of IL-1β (Mishra *et al.*, 2013). The protective role of nitric oxide was shown to be the inhibition of the inflammasome activation through protein nitrosilation (Mishra *et al.*, 2013). It is however important to note that, in the absence of IL-1R-mediated signalling mice are acutely susceptible to infection (Mayer-Barber *et al.*, 2010). As for the IFN-α/β discussed above, these data show that an adequate level of IL-1 is required to control infection but in excess, IL-1 causes important immunopathological consequences to the host.

Together, these data suggest that the protective capacity of CD4 T cells is defined by their ability to rapidly migrate to the lung parenchyma and interact with infected macrophages, rather than by producing high levels of IFN-γ. The protective function of IFN-γ is mediated by activating macrophages to control intracellular growth but also by restraining immunopathological responses (Nandi and Behar, 2011). The inflammatory environment generated in the lesion site also regulates T-cell responses. More research is required to discriminate between the pathological and protective inflammatory mediators and their impact in the control of infection.

The impact of chronic infection in T-cell function and granuloma formation

During chronic *M. tuberculosis* infection in mice, effector T-cell numbers remain relatively stable (Moguche *et al.*, 2015; Winslow *et al.*, 2003), suggesting that these cells are exposed to chronic antigen stimulation (Moguche *et al.*, 2015). Over the last decade, several papers have shown that T cells under repetitive exposure to their cognate peptide may display defects in their ability to proliferate and produce cytokines, a condition known as exhaustion (Barber *et al.*, 2006; Joshi *et al.*, 2007). In tuberculosis, CD4 T-cell activation (as measured by production of IFN-γ) is suboptimal in the lungs of infected animals, even at the peak of the response (Bold *et al.*, 2011; Egen *et al.*, 2011). As discussed above, mycobacteria can promote the recruitment of permissive macrophages that express low levels of MHC-II (Antonelli *et al.*, 2010; Cambier *et al.*, 2014), but can also interfere with the expression and loading of antigens in MHC-II molecules (Hmama *et al.*, 2015). Therefore, the suboptimal

activation of T cells may be associated with the reduced interaction with MHC-II molecules within the infection site (Srivastava and Ernst, 2013). Alternatively, T cells may develop an exhaustion phenotype. Indeed, the proliferative capacity of antigen-specific T cells reduces as the infection progresses (Reiley *et al.*, 2010), and this is a hallmark of exhausted T cells (Barber *et al.*, 2006).

One of the first papers to address T-cell exhaustion in tuberculosis determined the expression of two well-known markers of T-cell exhaustion in viral infections (Joshi *et al.*, 2007): Programmed Death 1 (PD-1) and KLRG1 (Reiley *et al.*, 2010). This study showed that, although during chronic *M. tuberculosis* infection T cells acquired the expression of either PD-1 or KLRG1, these are not markers of exhaustion during tuberculosis (Reiley *et al.*, 2010). Indeed, both populations produced IFN-γ and TNF-α upon restimulation, with the KLRG1 population producing the highest cytokine levels (Reiley *et al.*, 2010). Upon adoptive transfer into *M. tuberculosis* infected mice, the purified PD-1-expressing population maintained proliferation whereas the KLRG1-expressing population did not proliferate and their numbers declined over time (Reiley *et al.*, 2010). These data suggest that PD-1-expressing CD4 T cells represent a proliferative population, likely responsible for the maintenance of the antigen-specific T-cell pool throughout infection, while KLRG1-expressing CD4 T cells represent a terminally differentiated effector population (Reiley *et al.*, 2010). Recent data support these observations and show that when transferred into uninfected animals, PD-1-expressing CD4 T cells are better able to persist and to provide enhanced protection to *M. tuberculosis* infection when compared to terminally differentiated, KLRG1-expressing, CD4 T cells (Moguche *et al.*, 2015). Importantly, the PD-1-expressing CD4 T-cell population was shown to reside preferentially in the lung parenchyma, exhibit higher capacity to produce IL-2 and a small fraction expressed the chemokine receptor CXCR5 (Moguche *et al.*, 2015).

While these data show that PD-1-expressing CD4 T cells are required for protection against *M. tuberculosis* infection, mice deficient in PD-1 are extremely susceptible to infection, developing wasting disease associated with a massive accumulation of mycobacteria-specific T cells (Barber *et al.*, 2011; Lázár-Molnár *et al.*, 2010). On the other hand, KLRG1 deficient mice are more resistant to *M. tuberculosis* infection, associated with increased number of CD4 T cells (Cyktor *et al.*, 2013). Not only does this evidence the potential pathological role of the T-cell response, but also reveals that the PD-1 pathway is required to restrain pathological T-cell responses and prevent lethal disease.

Although PD-1 by itself may not be a marker of exhaustion, T cells express multiple inhibitory receptors during chronic *M. tuberculosis* infection, including TIM3, LAG-3, and 2B4 (Jayaraman *et al.*, 2016). Interestingly, T cells that coexpress TIM3 and PD1 produced less IFN-γ, IL-2 and TNF-α, but more IL-10 when compared with T cells expressing only TIM3, suggesting that T cells that coexpress multiple inhibitory receptors are more likely to be exhausted (Jayaraman *et al.*, 2016). Accordingly, TIM3 blockade resulted in increased frequencies of T cells producing IFN-γ, TNF-α and IL-2 and more importantly reduced bacterial burdens (Jayaraman *et al.*, 2016). These data show that T-cell exhaustion does develop during tuberculosis, and this may be an important mechanism to maintain chronic infection.

As T cells accumulate in the infection site, the granuloma begins to acquire a more intact structure, with a macrophage-rich centre surrounded by T and B cells. With time, some granulomata can undergo remodelling and accumulate necrotic material in the centre,

leading to the formation of caseum. The caseum can then undergo liquefaction resulting in the fusion with an adjacent airway, a process known as cavitation, which facilitates bacterial dissemination (Marakalala *et al.*, 2016; Ndlovu and Marakalala, 2016).

Classically, the granulomatous response has been associated with a protective response that contains infection and prevents spreading of the bacteria to other organs. The fact that granuloma formation associates with control of mycobacterial infections has been supported by the hypersusceptibility to *M. tuberculosis* infection of humans and mice under various immunocompromising conditions, such as TNF-α blockade therapies, deficiency in the myeloid differentiation primary response protein 88 (MyD88) or the IL-12/IFN-γ axis (Bean *et al.*, 1999; Chakravarty *et al.*, 2008; Cooper *et al.*, 1993, 1997b; Flynn *et al.*, 1993; Fremond *et al.*, 2004; Lawn *et al.*, 2002). As discussed above, infections by mycobacteria result in complex bacteria–host interactions wherein the bacteria can subvert phagocyte function. Therefore, it is possible that the hypersusceptibility to mycobacterial infections in immunodeficient individuals is a combination of a decreased microbicidal capacity of the infected macrophage and poorly formed granulomata. Furthermore, the role of granulomata in preventing spread of the disease to peripheral organs is also questionable. As discussed in the sections above, most of the *M. tuberculosis* modulatory functions are associated with the lung environment and we cannot exclude the possibility that the protective response in peripheral organs is more efficient in controlling bacterial growth, than it is in the lung. In support of this hypothesis is the fact that control of *M. tuberculosis* infection is more efficient after intravenous challenge than after aerosol challenge (Cooper, 2009; Cooper *et al.*, 1997a). Therefore, whether granulomata formation is host-protective or represents an evolutionary adaption wherein the bacteria promote the formation of these immunological structures to spread to new host is an area of intense research. In fact, these issues were the subject of a recent review (Ndlovu and Marakalala, 2016).

While the benefit of granulomata to the host is questionable, granuloma formation is likely critical for *M. tuberculosis* to spread to new hosts. Caseation followed by liquefaction of the granuloma can lead to rupture of the lesion and the release of infectious material into the airways. This material can then be aerosolized in cough droplets, thus facilitating bacterial transmission to new hosts (Cardona, 2011). However, not all granulomata evolve into caseating lesions. Indeed, these lesions can be highly heterogeneous in a single host, despite the fact that they are likely founded by a single bacterium (Lin *et al.*, 2014). Importantly, a substantial number of granulomata in animals with active disease and latent infection can become sterile after the onset of adaptive immunity (Lin *et al.*, 2014). Therefore, the local microenvironment of each granuloma determines control or progression of infection.

Despite heterogeneity in granulomata from a single host, a recent study used laser-capture microdissection, mass spectrometry, and confocal microscopy in human intact, caseous, and cavitary granulomata samples to design molecular maps of different regions of each granuloma type (Marakalala *et al.*, 2016). This study identified more alterations in protein and lipid signatures associated with different regions of the granuloma than between different granulomata (Marakalala *et al.*, 2016). Interestingly, the centres of caseous and cavitary granulomata (presumably more advanced lesions) were enriched in pro-inflammatory pathways with the presence of antimicrobial peptides, reactive oxygen species and pro-inflammatory eicosanoids (Marakalala *et al.*, 2016). In contrast, the most exterior tissue surrounding the granuloma displayed an anti-inflammatory signature (Marakalala *et al.*, 2016).

The impact of this structural organization of the granuloma in the T-cell response, and vice versa, is not yet determined. Future work will be critical to determine whether the anti-inflammatory rim of the granuloma is an evolutionary adaption of the host to prevent further immunopathological consequences or an intricate strategy of the pathogen to prevent the entrance of antigen-specific T cells or modulate their phenotype and function. Whatever the answer, understanding how T cells accumulate and organize within these structures will be critical to define T cell correlates of protection. As granulomata are essential for *M. tuberculosis* to spread to new hosts, T cells are also essential for the success of *M. tuberculosis* as a human pathogen. Indeed, *M. tuberculosis* epitopes recognized by T cells are evolutionarily conserved (Comas *et al.*, 2010; Coscolla *et al.*, 2015), suggesting that *M. tuberculosis* benefits from T-cell recognition.

Taken together with the data discussed above, pro-inflammatory eicosanoids appear to play a key role in the T-cell response but also in the progression of the granulomatous response. During the early stages of infection pro-inflammatory eicosanoids induce necrotic cell death of infected-macrophages, thus delaying T-cell responses and promoting phagocytosis of the bacteria by newly recruited macrophages. After the onset of acquired responses, these macrophages are likely defective in interacting with T cells and more permissive to intracellular growth. As these cells die, a necrotic centre begins to form promoting its fusion with the airways, facilitating transmission of infection (Fig. 7.1).

Mycobacterium ulcerans infection: an extreme among mycobacteria

Although *M. ulcerans* and *M. tuberculosis* display a high degree of genetic similarity (Demangel *et al.*, 2009), the phenotypic clinical manifestations associated to infection with these mycobacteria differ greatly. This is, at least in part, due to the fact that *M. ulcerans* produces mycolactone, a dermonecrotic exotoxin that is responsible for the extensive destruction of the subcutaneous tissue and adjacent epidermis observed in Buruli ulcer patients (George *et al.*, 1999). Early clinical manifestations of Buruli ulcer include the non-ulcerative nodules, plaques and oedemas. However, due to the indolent nature of the lesions, there is often a delay in health-care seeking, which allows non-ulcerative manifestations to progress to more severe ulcerative forms (Walsh *et al.*, 2008).

Mycolactone is a small hydrophobic macrolide produced by a group of polyketide synthases and polyketide-modifying enzymes, encoded by a giant virulence plasmid pMUM001 (Stinear *et al.*, 2004). This toxin has been shown to passively diffuse through the cell membrane (Snyder and Small, 2003) rather than via a transporter-mediated mechanism and concentrates in the cytoplasm (Snyder and Small, 2003).

Despite the unique ability of *M. ulcerans* to secrete a toxin, the mycobacterial nature of *M. ulcerans* plays an important role in the development of a cell mediated immune response. Like other pathogenic mycobacteria, *M. ulcerans* is an intracellular pathogen that elicits a persistent recruitment of inflammatory cells to the site of infection, namely neutrophils and macrophages (Oliveira *et al.*, 2005). Initial recognition of *M. ulcerans* appears to be mediated through TLR-2, TLR-4 and Dectin-1 that actively participate in the sensing and internalization of the pathogen (Lee *et al.*, 2009), contributing to an antimycobacterial response with the release of reactive oxygen species (Lee *et al.*, 2009), reactive nitrogen species (Torrado *et al.*, 2010) and inflammatory cytokines and chemokines (Torrado *et al.*, 2007). However, the

bacterial proliferation associated to the progressively increasing concentrations of mycolactone at the site of infection, eventually starts to hamper the potentially protective immune response mounted by the host.

Among the deleterious effects of mycolactone, the toxin has been associated to structural alterations of actin- and tubulin-cytoskeleton, that disturbs the cell shape as well as multiple cytoskeleton-dependent cellular functions. In fact, mycolactone-induced cytoskeletal alterations can be mediated through the hyperactivation of the actin-cytoskeleton regulator Wiskott-Aldrich syndrome protein (WASP) (Guenin-Macé *et al.*, 2013), a nucleation promoting factor that activates the Arp2/3 complex in order to stimulate nucleation of branched actin filaments (Burianek and Soderling, 2013). By hijacking and disrupting WASP autoinhibition, mycolactone leads to uncontrolled activation of WASP and results in defective cell adhesion and migration due to the accumulation of small, highly branched actin filaments, rather than long microfilaments (Guenin-Macé *et al.*, 2013). Mycolactone also affects cytoskeleton-dependent functions with important consequences for cellular homeostasis. A recent proteomic study revealed several regulators and structural constituents of the cytoskeleton that are affected upon exposure to mycolactone (Gama *et al.*, 2014). For example, the cytoplasmic dynein 1 intermediate chain 2 (Dync1i2), a non-catalytic subunit of the microtubule-associated molecular motor dynein, was shown to be down-regulated in mycolactone-treated cells (Gama *et al.*, 2014). Several cellular processes dependent on cytoskeleton architecture and dynein-driven transport have been reported to be affected by mycolactone. Specifically, the toxin can affect the transport of vesicles and organelles throughout the cytoskeleton tracks, allowing the pathogen to subvert innate microbicidal defences. Indeed, increasing concentrations of mycolactone impair phagosome–lysosome fusion in IFN-γ activated macrophages, which results in a deficient control in bacterial proliferation (Torrado *et al.*, 2010), as well an accumulation of autophagosomes, given their inability to fuse with lysosomes (Gama *et al.*, 2014).

Mycolactone does not act only upon the cytoskeleton. Another well-known effect of mycolactone is the down-regulation of specific proteins implicated in host immunity, such as cytokines and chemokines (Baron *et al.*, 2016; Hall *et al.*, 2014; Simmonds *et al.*, 2009; Torrado *et al.*, 2007). Interestingly, however, the reduced cytokine production during *M. ulcerans* infection does not appear to result from decreased gene transcription or even protein translation, but rather a blockage in the process of protein export (Hall *et al.*, 2014; Simmonds *et al.*, 2009). Indeed, mycolactone has been shown to directly target the α subunit of the Sec61 translocon (Baron *et al.*, 2016) and inhibit the cotranslational translocation of nascent proteins into the endoplasmatic reticulum due to a conformational change in the Sec61 translocation complex, to a stabilized closed conformation (Hall *et al.*, 2014; McKenna *et al.*, 2016). The mycolactone-dependent blockade of Sec61 translocation ultimately results in the accumulation and degradation of newly synthesized ER-transiting proteins in the cytoplasm (Hall *et al.*, 2014; Simmonds *et al.*, 2009). Accordingly, it was recently shown that there is an excessive accumulation of ubiquitnated proteins in fibroblasts treated with mycolactone (Gama *et al.*, 2014).

Despite the impact of mycolactone on the innate response, an acquired immune response is mounted, with the accumulation and proliferation of antigen-specific T cells in the draining lymph node (Fraga *et al.*, 2011). However, similar to what is observed in *M. tuberculosis* infection, there is a delay in the generation of acquired immunity, taking up to 7 days post infection to develop a specific T-cell response. This delay has been attributed, at least in part,

to the ability of mycolactone to inhibit the maturation and migration of DCs to the draining lymph node (Coutanceau *et al.*, 2007). This delay allows exponential bacterial proliferation with the further build-up of mycolactone in the infected tissue, amplifying its deleterious effects. For example, the toxin has been shown to have a direct effect on T-cell responsiveness to stimulation by affecting the expression of the T-cell homing receptor CD62L, thus impairing their capacity to reach peripheral lymph nodes and respond to chemotactic signals (Guenin-Macé *et al.*, 2011). Moreover, mycolactone also induces the hyperactivation of the Src-family kinase, Lck, which results in a deregulation of intracellular signalling pathways involve in T-cell receptor activation (Boulkroun *et al.*, 2010). Ultimately, the uncontrolled proliferation of *M. ulcerans* leads to the complete destruction of the draining lymph node (Fraga *et al.*, 2011), either due to bacterial colonization as a result of dissemination of *M. ulcerans* via afferent lymphatic drainage or due to diffusion of mycolactone from the site of infection.

The end result of the effect of mycolactone is cell death. While *M. tuberculosis* can clearly manipulate the fate of the infected cell and determine the modality of cell death to its own advantage, *M. ulcerans* takes this trait to a whole new level. Indeed, the first identified function of mycolactone was the induction of cell death. It has been shown that mycolactone-treated cells present an arrest in the G0/G1-phase as early as 48 hours post incubation (Gama *et al.*, 2014; George *et al.*, 1999). Longer periods of incubation lead to an accumulation of the sub-G0/G1 population, compatible with apoptotic cells (Gama *et al.*, 2014; George *et al.*, 1999). Interestingly, the arrest of the G1 cell cycle progression has been shown to occur after drug-induced inhibition of actin assembly (Bohmer *et al.*, 1996; Huang *et al.*, 1998; Iwig *et al.*, 1995; Maness and Walsh, 1982; Reshetnikova *et al.*, 2000), further implicating the cytoskeleton in the mycolactone-mediated cytopathic effect. Additional evidence for the role of mycolactone as an apoptotic inducer are *in vivo* reports describing the presence of apoptotic cells in human Buruli ulcer lesions (Walsh *et al.*, 2005) and in infected lesions of experimental animal models (George *et al.*, 1999, 2000; Oliveira *et al.*, 2005; Silva-Gomes *et al.*, 2015). However, the continuous build-up of mycolactone during progressive *M. ulcerans* infection results in an overwhelming accumulation of apoptotic cells in the infected tissue. The inability of scavenger phagocytic cells to efficiently clear the tissue of these apoptotic cells eventually leads to secondary necrosis followed by autolysis. This post-apoptotic secondary necrosis is behind the progressively expanding necrotic areas observed in Buruli ulcer lesions (Silva *et al.*, 2009).

Despite the size of these necrotic lesions, with some exceeding 15 cm in diameter, Buruli ulcer is an indolent disease. Mycolactone is described to have an analgesic effect to annul the pain caused during *M. ulcerans* infection (Marion *et al.*, 2014). Hypoesthesia is achieved through the binding of mycolactone to type 2 angiotensin II receptors (AT2R), which triggers phospholipase A2 (PLA2) activation and arachidonic acid release (Marion *et al.*, 2014). Arachidonic acid is then metabolized by COX-1 into PGE2, leading to the activation of TRAAK channels and a consequent potassium-dependent hyperpolarization of neurons (Marion *et al.*, 2014). This strategy seems to provide an evolutionary advantage for *M. ulcerans*, in which the lack of pain could possibly allow an uncontrolled expansion of necrotic areas with numerous extracellular bacilli and the potential shedding of bacteria to the environment, perpetuating its infectious cycle.

Collectively, these data show that, like other mycobacteria, *M. ulcerans* is an intracellular pathogen and induces T-cell responses which are required to control infection. However,

the pathophysiology of Buruli ulcer is dominated by the effect of mycolactone, which makes *M. ulcerans* unique within the genus.

Conclusion

The outcome of mycobacterial infections is dependent on the complex interaction between the intracellular pathogen and the immune system. Through this interaction, mycobacteria have evolved to adapt to its host by developing intricate mechanisms to subvert the immune response. However, the immune response may also contribute decisively for the success of mycobacteria as human pathogens. In this regard, *M. tuberculosis* has developed several mechanisms to modulate the activation and the effector function of the phagocytic cell, likely to prevent its rapid elimination rather than to completely shut down the immune system. The initial manipulation of the phagocyte response then allows the bacteria to survive within the host cell, while an antigen-specific immune response is still being mounted. This response eventually culminates in the formation of an inflammatory granulomatous structure that prevents the spreading of the bacteria to peripheral organs. However, its formation and breakdown has also proven to be essential for the bacteria to spread to new hosts. Understanding this duality will be important for the development of host-specific therapies. As an extreme example of mycobacterial survival, the immune evasion mechanisms used by *M. ulcerans* mainly depend on the actions of the necrotizing toxin mycolactone.

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