Innate and Adaptive Cellular Immune Responses to *Mycobacterium tuberculosis* Infection

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Host resistance to *Mycobacterium tuberculosis* (*Mtb*) infection requires the coordinated efforts of innate and adaptive immune cells. Diverse pulmonary myeloid cell populations respond to *Mtb* with unique contributions to both host-protective and potentially detrimental inflammation. Although multiple cell types of the adaptive immune system respond to *Mtb* infection, CD4 T cells are the principal antigen-specific cells responsible for containment of *Mtb* infection, but they can also be major contributors to disease during *Mtb* infection in several different settings. Here, we will discuss the role of different myeloid populations as well as the dual nature of CD4 T cells in *Mtb* infection with a primary focus on data generated using in vivo cellular immunological studies in experimental animal models and in humans when available.

Unlike many less-virulent microorganisms that are readily cleared by phagocytes or soluble innate effector molecules, *Mycobacterium tuberculosis* (*Mtb*) establishes a chronic infection primarily in the lungs. The activation of bacilli-laden macrophages by effector T-helper 1 (Th1) cells is the key cell-to-cell interaction in immunological control of *Mtb* infection. Although this classic paradigm is essentially correct, it is now clear that the diversity of myeloid cells and CD4 T cells responding to *Mtb* infection is far more complex. Multiple populations of innate immune cells with distinct functions cooperate for control of *Mtb* infection. CD4 T cells are indeed critical for host resistance, but the mechanisms of CD4 T-cell-dependent con-

trol are poorly understood. Moreover, CD4 T cells can also play a major role in driving tissue damage during tuberculosis. Here, we will review the current knowledge of the functional heterogeneity of myeloid cells, and the role of CD4 T cells in both host protection and immunopathology during *Mtb* infection with a focus on data generated from single-cell analysis of in vivo studies.

ESTABLISHMENT OF INFECTION

Infection with *Mtb* occurs via the aerosol route, and consequently, lung resident myeloid cells are the primary cells initiating “first contact” with the bacilli. Alveolar macrophages (AMs)

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are long-lived, specialized innate immune cells that reside in pulmonary alveoli and ingest the inhaled bacteria, and therefore, AMs are critical in setting the stage for the subsequent immune response against *Mtb* (Murphy et al. 2008; Guillems et al. 2013a). Lung resident myeloid cells, in particular AMs, have been recognized to play a dual role in *Mtb* control. Whereas they can contribute to host resistance, they are also key to establishment of infection in the first place.

Role of Alveolar Macrophages in Early Events of *Mtb* Infection

Situated at an important barrier site, AMs perform critical sentinel tasks to both preserve proper lung function and avoid collateral damage from exposure to harmless antigens. This is achieved by their great capacity for phagocytosis while being able to maintain a relatively low-cellular activation state and low-migratory potential (Guillems et al. 2013b). Phagocytosis of *Mtb* is facilitated by binding to complement receptors, mannose receptor (MR), surfactant molecules, and DC-SIGN (dendritic cell-specific intracellular adhesion molecule-3-grabbing nonintegrin) (Berrington and Hawn 2007; Jo 2008). In addition, AMs express a large array of pattern recognition receptors (PRR), including Toll-like receptors (TLRs), C-type lectin receptors (CLRs), and Nod-like receptors (NLRs), all of which have been shown to participate in *Mtb* recognition. Among the TLRs, TLR-2, -4, and -9 are of particular importance in sensing *Mtb*, with NOD2, Mincle, Dectin-1, MR, and DC-SIGN contributing to PRR-driven macrophage activation (Jo et al. 2007; Jo 2008; Reiling et al. 2008; Kleinnijenhuis et al. 2011). Once activation has been sufficiently elicited, AMs in murine experimental systems as well as TB patients can produce nitric oxide and reactive oxygen species, two antimycobacterial effector molecules shown to be able to kill *Mtb* (Nicholson et al. 1996; Jo et al. 2007). Therefore, it is not clear why AMs are not able to eliminate the bacilli before infection is established.

Macrophage depletion studies around the time of aerosol challenge, however, revealed that lung-resident AMs and not CCR2-depen-

dent myeloid cells, such as inflammatory monocytes/macrophages (IMs), play an important role in establishment of infection and initial growth of bacteria (Leemans et al. 2001; 2005; Samstein et al. 2013). Moreover, elegant studies using adoptive transfer approaches of *Mtb*-infected AMs exposed an important early role for these cells not only in establishment of infection, but also in influencing initiation and priming of T-cell responses 2–3 wk later (Divangahi et al. 2010). When *Mtb*-infected AMs that had a predisposition for apoptotic cell death caused by Alox5 deficiency were adoptively transferred into naïve wild-type mice, a greater CD4 and CD8 T-cell response was observed compared with transfer of wild-type AMs. This data highlights the importance of AMs in early stages of infection and their ability to set the stage for the ensuing immune response to *Mtb* infection, including adaptive immunity.

Spread of *Mtb* from Macrophages to Other Myeloid Cells

The cellular events that immediately follow infection of AMs in the airways are not well understood. Once engulfed by the macrophage, *Mtb* potently inhibits macrophage activation and becomes highly resistant to clearance. Virulent *Mtb* manipulates the response of infected cells to avoid detection and elimination through a variety of immune evasion strategies, including inhibition of phago-lysosome fusion and detoxification of nitrogen and oxygen radicals and dormancy (Flynn and Chan 2003; Pieters 2008; Gengenbacher and Kaufmann 2012; Mariotti et al. 2013). When the cell-intrinsic response to *Mtb* proves inadequate and/or the bacilli replicate to sufficient numbers within AMs, the infected cells burst. Release of bacteria from infected cells allows for infection of neighboring cells, and the cell death modalities of infected macrophages play an important role in dissemination of *Mtb* infection (Keane et al. 1997; Chen et al. 2006; Lee et al. 2009). Apoptotic cell death is associated with bacterial containment, enhanced antigen-cross presentation by dendritic cells (DCs), and efferocytosis, all processes important for controlling infection

(Chen et al. 2006; Behar et al. 2010; Divangahi et al. 2010; Martin et al. 2012, 2014). In contrast, death of macrophages resulting in cytolysis (e.g., necrosis) allows the bacilli to spread and disseminate (Fratazzi et al. 1999; Divangahi et al. 2009; Lee et al. 2011). Importantly, for replicating bacteria to cause cytolysis of the cell, the bacilli need to reach a particular threshold termed burst-size, estimated to be 25 bacteria per cell (Lee et al. 2006; Repasy et al. 2013), which may take several days to achieve given the slow replication time of *Mtb*. During this time, it is possible that the infection goes largely undetected by other host cells. It is not clear what signals trigger the recruitment of circulating myeloid effector cells, but the cell death outcome of infected AMs is likely a critical determinant.

Initially the predominant myeloid cell within the airways, eventually the proportion of AMs among myeloid cells decreases as newly recruited myeloid effector cells arrive in the lung within the first 2 wk after low-dose aerosol challenge (Wolf et al. 2007; Garcia-Romo et al. 2013).

HETEROGENEITY OF INNATE EFFECTOR CELLS

Macrophages and DCs are by far the most studied innate effector cells in murine models of tuberculosis. Most of our knowledge regarding the antimycobacterial myeloid response to *Mtb* infection in the lungs, however, has been extrapolated from in vitro studies using bone-marrow-derived macrophages and DCs. Nonetheless, immunohistochemical techniques clearly established that macrophages are the primary cell type laden with *Mtb* bacilli and present in pulmonary tuberculous granulomas. Although DC responses and functions have been more examined in vivo, most studies have used CD11c as a single definitive marker to identify conventional DCs (cDCs) in the lungs.

Nonetheless, DCs have been shown to be critically important to generate *Mtb*-specific T-cell responses, findings consistent with their known function in priming naïve T cells (Banchereau and Steinman 1998; Tian et al. 2005; Khader et al. 2006).

Identifying Myeloid Subset Diversity In Vivo

Recent advances in multicolor flow cytometry have revealed that pulmonary myeloid effector cells are vastly heterogeneous, with lung resident pulmonary DCs and macrophage subsets in addition to a plethora of effector populations, such as neutrophils, IMs, inflammatory monocyte-derived dendritic cells (mDCs), plasmacytoid DCs and cDCs being recruited to the site of infection (de Heer et al. 2005; Mayer-Barber et al. 2011; Guillems et al. 2013b). Based on CD11c and CD11b expression, most pulmonary myeloid effector cell types, including granulocyte receptor (Gr-1)-expressing neutrophils, have been reported to harbor live bacteria (Wolf et al. 2007). These diverse myeloid cells express a number of effector molecules and cytokines, such as inducible nitric oxide synthase (iNOS), tumor necrosis factor- α (TNF- α), interleukin-12 (IL-12)/23p40, IL-1 α , and IL-1 β , that are each critical for control of the infection (Cooper et al. 1997, 2007, 2011; Yamada et al. 2000; Saunders et al. 2004; Sköld and Behar 2008; Mayer-Barber et al. 2010, 2011). Indeed, in the lungs of *Mtb*-infected mice, at least five different myeloid cell types express CD11c, and most of them in addition CD11b, a surface molecule often considered as a pan-myeloid marker (Gonzalez-Juarrero and Orme 2001; Mayer-Barber et al. 2011; Guillems et al. 2013b). Even more concerning then is that natural killer (NK) cells, which can up-regulate both CD11b and CD11c on activation, can comprise up to 40% of all CD11b-expressing cells in the lungs of *Mtb*-infected mice (Mayer-Barber et al. 2011). These studies highlight the importance of using sufficient numbers of flow cytometric parameters together with CD68, a macrophage/DC-restricted molecule (Rabinowitz and Gordon 1991), to accurately identify desired populations of interest.

Conventional Dendritic Cells

Arguably, the most in vivo work has been performed regarding the role of CD11c-expressing cells, presumably cDCs, which are required for priming of naïve CD4 and CD8 T-cell responses

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against *Mtb* (Gonzalez-Juarrero and Orme 2001; Tian et al. 2005; Khader 2006; Wolf et al. 2007; Samstein et al. 2013). Pulmonary cDCs (CD11b⁺/dim, Ly6C^{neg}) express high levels of CD11c and various degrees of CD103 after *Mtb* infection (Mayer-Barber et al. 2011). CD103⁺ DCs are present exclusively in the lung airways and parenchyma after *Mtb* infection and are considered lung resident DCs, with migratory capacity to the draining lymph node (Geurts-vanKessel et al. 2008; Geissmann et al. 2010; Williams et al. 2013b; Leepiyasakulchai et al. 2013; Anderson et al. 2014). cDCs and CD103⁺ cDCs represent a functionally and phenotypically distinct pulmonary DC subset after *Mtb* infection and produce predominantly IL-12/23p40 (Mayer-Barber et al. 2011; Leepiyasakulchai et al. 2013). They express all the classical DC surface molecules (high levels of major histocompatibility complex class II (MHC-II), CD80, and CD86) as well as additional markers associated with antigen presentation and antigen-presenting cell (APC)-T-cell interactions, such as CD40, CD70, DEC-205, CD83, and PD-L2 (Mayer-Barber et al. 2011; KD Mayer-Barber, unpubl.).

Inflammatory Monocytes and Macrophages

IMs are characterized by their high Ly6C and little CD11c expression (within CD68⁺, CD11b⁺ myeloid cells) and are recruited to the lungs after low-dose aerosol infection (Mayer-Barber et al. 2011). They do express MHC-II as well as costimulatory markers, such as CD80 and CD86, albeit to a lesser extent when compared with CD11c⁺ DC subsets (Mayer-Barber et al. 2011). In addition, they display phenotypic markers corresponding to the IMs described in nonlymphoid tissue (e.g., 7/4, F4/80, CD14 high, and CD115 low) (Dunay et al. 2008; Sköld and Behar 2008; Varol et al. 2009; Geissmann et al. 2010; Mayer-Barber et al. 2011). They were found to be multifunctional and produce predominantly IL-1 α , IL-1 β , and TNF- α , pro-inflammatory cytokines important for bacterial control (Mayer-Barber et al. 2011). They also share functional properties, e.g., IL-10 and iNOS production, similar to myeloid-derived

suppressor cells (MDSCs) that have been described under unresolved pathological conditions such as chronic infections, inflammation, and cancer (Biswas and Mantovani 2010). IMs are very responsive to regulatory cytokines *in vivo*, in particular they preferentially express high levels of IL-10 and IFN- γ receptor, and IFN- γ from CD4 T cells is able to directly modulate their inflammatory cytokine production in the lungs of *Mtb*-infected mice (Mayer-Barber et al. 2011). Although IFN- γ is important for induction of iNOS, it potently suppresses IL-1 α , IL-1 β , and IL-10 expression by pulmonary IMs. In addition to adaptive interferon (IFN), innate-derived type I IFNs are also able to potently limit proinflammatory IL-1 cytokine production by IMs while inducing anti-inflammatory IL-10 production, reflecting perhaps an important checkpoint in limiting excessive inflammation and/or immune evasion strategies triggered by *Mtb* itself (Stanley et al. 2007; Mayer-Barber et al. 2011). Based on a large array of phenotypic markers assessed by single-cell analysis, IMs seem to represent a single homogeneous cell population (Mayer-Barber et al. 2011). Interestingly, use of an intravascular staining technique using flow-cytometry to examine the distribution of IMs in the lung parenchyma and pulmonary blood vasculature revealed that IMs are primarily located inside the lung-associated vasculature and that only a small fraction of IMs were actually present inside the lung tissue itself (Mayer-Barber et al. 2011; Anderson et al. 2014).

Despite their uniform appearance, IMs can thus be separated into two subsets based on their localization within the lung, and future studies need to determine the functional role of tissue resident IMs, perhaps representing true macrophages versus vasculature-associated monocytes.

Inflammatory Dendritic Cells

Inflammatory mDCs comprise the most functionally diverse pulmonary innate effector cell type in response to *Mtb* infection. They are identified based on their high CD11c, CD13, and high Ly6C, 7/4 and TLR2 expression (with-

in CD68⁺, CD11b⁺ myeloid cells) (Mayer-Barber et al. 2011). They also express high levels of MHC-II, CD80, and CD86 and are primarily located in the lung parenchyma, rather than the lung vasculature (Mayer-Barber et al. 2011; Anderson et al. 2014), suggesting that these cells have potent antigen-presentation capacity. Inflammatory mDCs are highly polyfunctional and are able to coexpress IL-1 α , IL-1 β , IL-10, TNF- α , and iNOS at the single-cell level in vivo, where they are recruited to the lungs between 2 and 3 wk after *Mtb* infection. Based on their functional profile, they resemble an iNOS and TNF- α -producing DC subset that has been previously implicated in murine resistance to intracellular bacteria, parasites, or viruses as well as human psoriasis (Serbina et al. 2003; Lowes et al. 2005; Lin et al. 2008; De Trez et al. 2009). Similar to IMs, their effector functions are directly regulated during infection by cell-intrinsic type I IFN receptor signaling (Mayer-Barber et al. 2011). Whereas IL-1 α , IL-1 β , and iNOS expression are negatively regulated, type I IFNs induce anti-inflammatory IL-10 production in mDCs. However, in contrast to IMs, CD4 T-cell-derived IFN- γ is unable to limit the proinflammatory cytokine production by mDCs in vivo. Unraveling the functional heterogeneity of inflammatory mDCs, their antimicrobial properties, and whether these diverse characteristics can be attributed to multiple functionally distinct subsets within mDCs will be important in our understanding of their role in tuberculosis control and pathogenesis.

Neutrophils

Although neutrophils are the cell type predominantly infected in the airways of active tuberculosis patients, their role in host resistance against *Mtb* in experimental models remains poorly understood and controversial at best (Eum 2010; Lowe et al. 2012). The short-lived nature of these cells has made it difficult to isolate them and perform functional studies in vitro. Gr-1⁺ staining was thought to be specific for neutrophils, but the Gr-1 monoclonal antibody (mAb) recognizes both Ly6C and Ly6G, antigens expressed also by monocytes/macrophages and

DCs. Therefore, its use for accurately identifying neutrophils in vivo has proven problematic, and the use of a Ly6G-specific mAb instead of Gr-1 has made it possible to more accurately distinguish these cells in vivo. Along these lines, neutrophil depletion studies targeting Gr-1 must be interpreted with some caution, because Ly6C-expressing monocytes, mDCs, as well as effector CD4 and CD8 T cells, express Ly6C. Studies in which neutrophils were experimentally recruited to the airways before infection showed a decrease in bacterial loads, whereas depletion of neutrophils shortly after infection increases bacterial burden (Pedrosa et al. 2000; Sugawara et al. 2004; Blomgran and Ernst 2011). Therefore, neutrophils can contribute to host resistance. Potential antimycobacterial effector functions of neutrophils involve TNF- α , reactive oxygen species, antimicrobial peptides, extracellular traps (NETs), efferocytosis of infected myeloid cells, and boosting the ability of DCs to prime T-cell responses (Blomgran and Ernst 2011; Blomgran et al. 2012; Lowe et al. 2012). In contrast, neutrophils have also been suggested to adapt a regulatory IL-10 producing, anti-inflammatory phenotype that limits antimycobacterial control (Zhang et al. 2009). Moreover, there is mounting evidence including association studies in humans that neutrophils during later stages of infection could mediate pathological changes and disease progression (Berry et al. 2010; Dorhoi et al. 2014). It is possible that neutrophils directly contribute to mortality by promoting necrotic lung pathology, liquefaction of granulomas, and collapse of lung functions. In this context, IFN- γ has been suggested to regulate neutrophil recruitment and function (Nandi and Behar 2011), perhaps via inhibition of IL-1 and IL-17 (Desvignes and Ernst 2009; Mayer-Barber et al. 2011), and depletion of Ly6G-expressing cells via administration of 1A8 mAb has extended the survival of susceptible IFN- γ , CARD9, and microRNA-223-deficient animals (Dorhoi et al. 2010, 2013; Nandi and Behar 2011). Indeed, the influx of a large number of GR-1⁺ and Ly6G⁺ cells into the lungs could be considered a general hallmark of highly susceptible mice that undergo cachexia as well as uncontrolled bacterial replication, regardless of

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their genetic make-up (Eruslanov et al. 2005; Lyadova et al. 2010). It is possible, however, that during these late stages of lethal *Mtb* infection, the Ly6G and Gr-1 expressing population might not reflect true neutrophils (Antonelli et al. 2010; Lyadova et al. 2010). Moreover, these cells share characteristics of immature myeloid-derived suppressor cells, and future studies have to determine their role in tuberculosis pathogenesis (Obregón-Henao et al. 2013; Tsiganov et al. 2014).

PRIMING OF *Mtb*-SPECIFIC T-CELL RESPONSES

HIV infection induced CD4 T-cell depletion, and mice or nonhuman primates experimentally depleted of CD4 T cells are highly susceptible to *Mtb* infection (Müller et al. 1987; Orme 1988; Leveton et al. 1989; Flory et al. 1992; Lin et al. 2012; Pawlowski et al. 2012; Yao et al. 2014). Indeed, it is well established that T helper cells are critical for containment of *Mtb*.

Delay in T-Cell Priming Following *Mtb* Exposure

The initiation of the CD4 T-cell response to *Mtb* is notoriously slow, as CD4 T cells first arrive in the lungs of infected mice several weeks after exposure. This lag time between the establishment of infection and the arrival of T cells at the site of infection likely contributes to the inability of the host to clear the organism by allowing the bacteria to increase significantly in number before adaptive immune cells can mediate their protective effects.

More recently, the use of *Mtb*-specific T-cell receptor (TCR) transgenic (Tg) CD4 T cells has allowed the visualization of the earliest events of CD4 T-cell priming in *Mtb* infection. This is typically performed by adoptively transferring congenically marked TCR Tg CD4 T cells into naïve mice that are then infected with *Mtb*, thereby dramatically increasing the precursor frequency of antigen-specific CD4 T cells so that they can be visualized in vivo without the need for expansion. Using CD4 T cells that are specific for Ag85b (Wolf et al. 2008) or ESAT-6 (Gallegos et al. 2008; Reiley et al. 2008), it was

definitively shown that initial recognition of *Mtb* by naïve CD4 T cells (read out by the first appearance of the activation marker CD69) occurs in the lung-draining lymph nodes. Interestingly, delivery of bacilli and subsequent T-cell priming is not accelerated by increased bacterial inoculum size, but once the bacteria reach the lymph node, the CD4 T-cell response is directly proportional to the number of viable organisms present in the lymph node (Wolf et al. 2008). The mechanisms leading to the delay in *Mtb*-specific T-cell priming are not known, but there is evidence that infected myeloid cells, not free bacteria or soluble antigens, must traffic to the lung-associated lymph nodes to initiate the T-cell response.

Role of Dendritic Cells in Priming of CD4 T Cells during *Mtb* Infection

Mtb-infected bone marrow-derived dendritic cells but not macrophages instilled into the trachea of mice can migrate into the lymph node and prime CD4 T cells (Bhatt et al. 2004). Indeed, dendritic cells are well understood to be essential in the induction of T-cell responses, and transient depletion of CD11c⁺ cells impairs CD4 T-cell priming during intravenous *Mtb* infection (Tian et al. 2005). Moreover, autocrine production of IL-12p40 homodimers has also been shown to play a major role in the migration of instilled bone marrow-derived dendritic cells from the lungs to the lymph nodes following *Mtb* infection (Khader 2006). Dissemination of viable bacilli from the lung to secondary lymphoid organs always precedes the first evidence of adaptive T-cell response against *Mtb* between days 9 and 12, indicating that T-cell priming requires bacteria in the lymph node (Chackerian et al. 2002). *Plt/plt* mice that are deficient in the CCR7 ligands, CCL19, and CCL21, develop high levels of bacteria in their lungs, but very few bacteria are found in the draining lymph nodes, indicating that activated myeloid cells are essential for bacteria to arrive in the lymph node (Wolf et al. 2007). Because most innate effector cell types are infected with *Mtb*, it is possible that multiple cell types contribute either directly or indirectly to the delivery of bacteria to the

lymph node. For example, depletion of neutrophils decreases the migration of dendritic cells to the lymph node and delays the priming of CD4 T-cell responses (Blomgran and Ernst 2011). Moreover, infection of mice with a *nuoG* mutant *Mtb* that cannot suppress neutrophil apoptosis leads to more rapid trafficking of bacteria to the lymph nodes and CD4 T-cell priming (Blomgran et al. 2012). Therefore, it has been suggested that apoptotic neutrophils play an important role in facilitating activation of *Mtb*-specific CD4 T cells by modulating the migratory capacity of dendritic cells.

Cooperation between Migratory CCR2⁺ Monocytes and Conventional Dendritic Cells in Priming T Cells during *Mtb* Infection

A recent study has shown that depletion of CCR2⁺ cells with injection of diphtheria toxin into CCR2-DTR mice dramatically reduces the appearance of bacteria in the lymph node and delays CD4 T-cell priming (Samstein et al. 2013). Adoptive transfer of wild-type (WT) CCR2⁺ cells into MHC-II KO mice depleted of endogenous CCR2⁺ monocytes, completely restored trafficking of bacteria to the lymph node but not CD4 T-cell priming. These data indicate that CCR2⁺ myeloid cells are required for delivering viable bacilli to the lymph node but themselves do not present antigen to CD4 T cells. In contrast, depletion of conventional dendritic cells using zbtb46-DTR mice had no impact on bacterial trafficking to the lymph node, but dramatically decreased CD4 T-cell priming. Therefore, it is likely that CCR2⁺ myeloid cells are essential for trafficking bacteria to the lymph node where conventional dendritic cells then uptake bacteria and directly present antigen to prime naïve CD4 T-cell responses. Ultimately, it is very likely that a complex orchestration of diverse myeloid effector cells acting together is required for optimal priming of *Mtb*-specific CD4 T cells.

PROTECTIVE CD4 T-CELL RESPONSES DURING *Mtb* INFECTION

Although a few key pathways have been elucidated, much is unknown about the mechanisms

of CD4 T-cell-dependent control. Moreover, it should be emphasized that the main protective outcome of the T-cell response against *Mtb* is the containment of bacterial growth as the bacilli are not cleared.

Th1 Immunity Is Required for Host Resistance to *Mtb*

Early studies found that mice with defects in IFN- γ expression are extremely susceptible to *Mtb* infection (Cooper et al. 1993; Flynn et al. 1993). A recent study found that RAG KO mice reconstituted with a mixture of IFN- γ KO CD4 T cells + WT CD4-depleted splenocytes died earlier than RAG KO mice reconstituted with WT CD4 T cells + WT CD4-depleted splenocytes, supporting the hypothesis that IFN- γ production by CD4 T cells themselves is important for survival of *Mtb* infection (Green et al. 2013). Moreover, mice deficient in the Th1-polarizing cytokine IL-12 (Cooper et al. 1997) or, albeit to a lesser extent, the Th1 lineage-specifying transcription factor Tbet (Sullivan et al. 2005) succumb early following *Mtb* exposure. Therefore, CD4 T cells polarized toward the Th1 phenotype are likely critical for host resistance to *Mtb* infection, and there is evidence in humans that the IL-12/IFN- γ axis is also critical for control of *Mtb* infection. Individuals with inborn errors in IL-12/23p40, IL-12R β 1, IL-12R β 2, IFN γ R1, IFN γ R2, or STAT1 are highly susceptible to even avirulent nontuberculosis mycobacteria (Filipe-Santos et al. 2006; Vosse et al. 2013). Moreover, individuals who develop neutralizing autoantibodies against IFN- γ become very susceptible to both tuberculosis and opportunistic nontuberculous mycobacterial infections (Browne and Holland 2010). Indeed, given the severity of disease in its absence, it may appear that IFN- γ is the most important T-cell-derived protective effector molecule described to date in *Mtb* infection.

IFN- γ -Independent Mechanisms of Protection

Despite the critical role of IFN- γ in host resistance to *Mtb* infection, it is not the only mechanism of CD4 T-cell-dependent control. In fact,

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in *Mtb*-infected individuals IFN- γ levels positively correlate with the severity of pulmonary disease, fever, and weight loss (Tsao et al. 2002). MHC-II-deficient mice eventually develop normal levels of IFN- γ in their lungs (Caruso et al. 1999), and depletion of CD4 T cells at late time points following *Mtb* infection has no effect on IFN- γ message levels in the lung (Scanga et al. 2000), yet in both settings, loss of CD4 T cells leads to the death of the host. Reconstitution of RAG KO mice with T cells showed that TNF- α KO CD4 T cells are not as protective as WT CD4 T cells against *Mtb* infection, but the effect was minor, indicating that TNF- α production does not account for the IFN- γ independent effects of CD4 T cells against *Mtb* (Saunders et al. 2004). In a CD4 T cell:macrophage coculture system, it was found that primed CD4 T cells suppressed the growth of *Mtb* in IFN- γ R KO WT macrophages almost as well as WT macrophages (Cowley and Elkins 2003). Importantly, adoptive transfer of in vitro Th1 polarized ESAT-6-specific TCR Tg CD4 T cells into WT mice leads to dramatically decreased bacterial loads (Gallegos et al. 2008), but a comparison of WT and IFN- γ TNF- α double-KO TCR Tg cells found that the majority of the host protective effect mediated by the adoptively transferred effector cells was independent of these two cytokines (Gallegos et al. 2011). Finally, whereas increasing numbers of *Mtb*-specific CD4 T cells through vaccination or through adoptive transfer of TCR Tg T cells leads to enhanced bacterial control, injection of recombinant IFN- γ into WT *Mtb*-infected mice is not protective (Moreira et al. 2002). Therefore, there is clear evidence that CD4 T cells can induce the control of *Mtb* infection in vitro and in vivo via mechanisms independent of both IFN- γ and TNF- α .

IL-17 is another major inflammatory cytokine that can be produced by subsets of *Mtb*-specific CD4 T cells. In humans, Ag-specific Th17 responses can be detected in individuals that are BCG (*Bacillus Calmette–Guérin*)-immunized and were found to be recalled by boosting with an Ag85a-expressing viral vector (Tameris et al. 2013), but Th17 responses are almost absent in individuals with active tuberculosis (Perreau et al. 2013). Interestingly, in mice, a

similar phenomenon is observed. Ag-specific Th17 cells are readily generated by immunization with complete Freund's adjuvant which contains dead *Mtb* (Shenderov et al. 2013) but are relatively rare during *Mtb* infection when compared with Th1 responses. In fact, it appears that Th17 cells do not have a major role in control of *Mtb* infection. Mice deficient in the Th17-promoting cytokine IL-23p19 completely lack IL-17-producing CD4 T cells but display only small increases in bacterial burden at very late time point postinfection (Khader et al. 2005; 2011). Likewise, mice deficient in the IL-17 receptor display normal control of bacterial growth after a typical 100 colony-forming units (CFU) aerosol exposure to *Mtb* (Aujla et al. 2007; Khader et al. 2011). However, there is evidence that vaccine-elicited Th17 cells may contribute to protection by driving the expression of Th1-cell-recruiting chemokines (Khader et al. 2007).

IL-22 is often associated with Th17 cells and is found in the bronchiolar lavage fluid of tuberculosis patients at higher levels than IL-17 (Scriba et al. 2008; Matthews et al. 2011). Interestingly, IL-22 is predominantly produced by a subset of CD4 T cells distinct from the IL-17-producing cells in both humans (Scriba et al. 2008) and mice (Behrends et al. 2013). However, Th22 cells are unlikely to play a major role in host defense against *Mtb* as IL-22 blockade (Khader et al. 2007; Wilson et al. 2010) of WT mice has no apparent effect on control of *Mtb* infection, and IL-22-deficient mice also display normal bacterial loads (Khader et al. 2007) and long-term survival of *Mtb* infection (Behrends et al. 2013).

Role of CD4 T-Cell Migration

It was recently found that CD4 T cells must directly recognize MHC-II on *Mtb*-infected cells to suppress intracellular growth of the bacilli. This study used bone marrow chimeric mice reconstituted with congenically marked WT and MHC-II KO bone marrow, to evaluate the role of direct recognition of infected cells by CD4 T cells in bacterial control by myeloid cells (Srivastava and Ernst 2013). It was found that similar frequencies of WT and MHC-II KO cells

were infected in the lung, but the KO cells contained higher numbers of bacilli in each infected cell. Moreover, depletion of CD4 T cells resulted in identical frequency distributions of bacteria/infected cell between the WT and KO macrophages. This clearly shows that for CD4 T cells to induce myeloid cells to limit replication of ingested bacteria, they must directly interact with the infected cells and recognize antigen. Therefore, the ability of CD4 T cells to enter the lung and interact with infected cells is another critical aspect of CD4 T-cell-dependent control of *Mtb* infection.

A recent study used an intravascular staining technique (Anderson et al. 2014) to examine the distribution of pulmonary CD4 T cells among the airways, the lung parenchyma, the lung-associated blood vasculature, and the peripheral circulating blood of *Mtb*-infected mice (Sakai et al. 2014). Surprisingly, it was found that Ag-specific CD4 T cells are present in similar numbers in both the lung parenchyma and blood vasculature. Interestingly, the parenchymal *Mtb*-specific CD4 T cells expressed high levels of the activation marker CD69 and PD-1, whereas the intravascular effector CD4 T cells expressed high levels of KLRG1, indicating a terminally differentiated phenotype. The intravascular *Mtb*-specific CD4 T cells expressed higher levels of Tbet and produced much higher levels of IFN- γ in vivo and on peptide restimulation in vitro. Moreover, adoptively transferred parenchymal CD4 T cells were able to rapidly migrate back into the lungs of infection matched mice, whereas intravascular donor CD4 T cells entered lung tissue poorly and were instead retained in the blood vasculature. Importantly, it was found that purified parenchymal CD4 T cells were able to protect *Mtb*-infected T-cell-deficient recipient mice much better than equal numbers of intravascular T cells. Therefore, *Mtb*-specific CD4 T cells dramatically vary in their ability to enter the lung parenchyma, and control of *Mtb* infection correlates with the ability of the CD4 T cell to enter the lung parenchyma rather than secrete high levels of IFN- γ . The mechanisms that regulate CD4 T-cell differentiation into a parenchyma-homing phenotype, however, are not understood.

PATHOGENIC CD4 T-CELL RESPONSES DURING *Mtb* INFECTION

Immune-mediated damage accounts for a large amount of the pathology of tuberculosis. Although the critical role for CD4 T cells in host resistance to *Mtb* is clear, there is also evidence that these cells can contribute to immunopathology associated with mycobacterial infections in both humans and experimental animal models.

Pathology Associated with Therapeutic Vaccination during *Mtb* Infection

In the late 1800s, Robert Koch performed experiments to promote the diagnostic and therapeutic value of preparations containing killed *Mtb* or bacterial extracts. He found that injection of naïve guinea pigs with dead bacilli had little effect on the animals, even when high doses were administered. However, when he inoculated *Mtb*-infected guinea pigs with preparations of dead bacteria, the animals succumbed, and the time to death depended on the dose he gave (Koch 1891). Some people may have even suffered lethal reactions on inoculations with Koch's intended remedy (Virchow 1891). The exacerbation of tuberculosis on therapeutic vaccination is now referred to as the Koch phenomenon, and has been observed in several animal models. For example, repeated injections of nonvirulent BCG into *Mtb*-infected mice leads to severe tissue damage (Turner et al. 2000a; Moreira et al. 2002). Although the mechanisms of the Koch reaction are not well understood, one study indicated that the induction of Th17 cells may promote disease in the model of repeated BCG injection-induced tuberculosis exacerbation (Cruz et al. 2010).

Interestingly, in the 1950s and 1960s, a large number of studies by Yamamura and colleagues explored the observation that in rabbits, prophylactic vaccination followed by multiple rounds of boosting with avirulent mycobacteria results in a dramatic increase in pulmonary cavities following a challenge with virulent mycobacteria. Prophylactic immunization of guinea pigs (Turner et al. 2000b) or mice (Taylor et al.

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2003) with certain protein antigens or DNA vaccine constructs can predispose to necrotic lung damage when those animals are later challenged with *Mtb*. Although the role of CD4 T cells themselves has not formally been tested, this is consistent with observations in CD4 T-cell-deficient HIV/*Mtb* coinfecting individuals, where the pathology of tuberculosis is altered.

Evidence for CD4 T-Cell-Mediated Immunopathology from Studies of HIV/*Mtb* Coinfection

As mentioned above, CD4 deficiency in HIV-coinfecting individuals leads to an inability to control *Mtb* infection, but it has also been recognized since the early days of the HIV epidemic that HIV coinfection alters the radiographic patterns of tuberculosis lung lesions. Consistent with poor bacterial control, CD4-deficient HIV coinfecting individuals with low CD4 T-cell counts are more likely to have miliary tuberculosis (large numbers of unusually small lesions visible on chest X-ray), lymphadenopathy caused by dissemination of the bacilli to the lung-draining lymph nodes and pleural effusion (Long et al. 1991; Batungwanayo et al. 1992; Mukadi et al. 1993; Pastores et al. 1993; Keiper et al. 1995; Awil et al. 1997; Jones et al. 1997; Perlman et al. 1997; Busi Rizzi et al. 2004; Geng et al. 2005; Chamie et al. 2010). In contrast, HIV-negative individuals are more likely to develop upper lung disease and, importantly, lung cavities. In fact, a large cohort study of HIV/tuberculosis coinfection found strong positive correlations between increasing numbers of CD4 T cells and the likelihood of cavitary tuberculosis (Chamie et al. 2010), indicating that CD4 T cells may contribute to cavity formation. HIV-infected individuals with extremely low numbers of CD4 T cells are even much more likely to have a completely normal chest X-ray. It has been suggested that cavitation is beneficial to *Mtb*. Individuals with cavitary tuberculosis are more infectious compared with individuals with active disease who do not display cavities (Rodrigo et al. 1997), indicating that cavities facilitate the transmission of *Mtb* from one host to the next, a critical step in the

life cycle of this obligate human pathogen. Consistent with the lack of cavities in HIV patients, CD4-deficient HIV patients are less likely to produce bacteria in their sputum. Moreover, a recent study found that human T-cell epitopes are hyperconserved in the tuberculosis genome (Comas et al. 2010), again supporting the hypothesis that *Mtb* benefits from T-cell recognition and may be under strong selective pressure to actually induce T-cell responses. Therefore, the phenotype of tuberculosis in CD4-depleted HIV-infected individuals, that is, the inability to control *Mtb* growth and lack of lung cavities, indicates that CD4 T-cell responses in HIV-negative individuals may be both required for host resistance and responsible for severe tissue immunopathology.

Immune Reconstitution Inflammatory Syndrome

Another scenario in which CD4 T-cell responses can mediate pathology during mycobacterial infection occurs in the context of CD4 T-cell reconstitution of lymphopenic HIV-infected individuals during therapy. Although the treatment of HIV infection with antiretroviral therapy (ART) usually leads to clinical improvement, some individuals experience a rapid deterioration in symptoms within the first few weeks of beginning ART, termed immune reconstitutive inflammatory syndrome (IRIS) (French 2012). This paradoxical worsening of disease occurs most frequently in individuals with severe T-cell deficiency and a concurrent microbial coinfection, which is often *Mtb*. The symptoms of the IRIS event depend on the site of infection and the particular microbe, but in *Mtb*-coinfecting individuals, the pathology often manifests as extreme lymphadenopathy, the worsening or formation of new pulmonary lesions, or meningitis. Some studies have found that HIV/*Mtb*-coinfecting individuals who develop IRIS display increased frequencies of *Mtb*-specific or -activated CD4 T cells at the time of the IRIS event, whereas those who do not develop IRIS lack this T-cell expansion at a similar time point posttreatment (Bourgarit et al. 2006; 2009; Meintjes et al. 2008). The mechanisms of

IRIS are not understood, but the prevailing hypothesis concerning the development of IRIS is that when CD4 T-cell numbers begin to recover as viral replication is suppressed with ART, the reconstituting T cells drive dysregulated inflammatory responses against the microbial coinfection leading to destructive tissue pathology.

A murine model of IRIS has been developed by reproducing the underlying immunological scenario preceding the development of immune reconstitution disease; that is, CD4 T-cell reconstitution of a mycobacterial-infected, T-cell-deficient host (Barber et al. 2010). In this model, TCR α KO mice are first inoculated with *Mycobacterium avium*, and after several months, the chronically infected mice are injected with CD4 T cells. Although it may be expected that CD4 T-cell reconstitution would lead to enhanced control of the established mycobacterial infection, instead, it leads to a rapid wasting disease, and most of the animals succumb to fatal immunopathology within a few weeks after the T-cell infusion (Barber et al. 2014, 2010). IFN- γ production by the reconstituting CD4 T cells, which is normally required for containment of mycobacterial infection, is a major mediator of the disease in this setting. Models of IRIS associated with other microbial infections (Roths et al. 1990; Roths and Sidman 1992, 1993; Bhagwat et al. 2006; Mutnal et al. 2013) have also found that in the setting of immune reconstitution, CD4 T cells can mediate severe immunopathology. Collectively, the data from studies of IRIS in HIV-coinfected individuals and the experimental animal model data strongly support the hypothesis that IRIS in humans is mediated by CD4 T cells and provide another example of CD4 T-cell-mediated pathology in mycobacterial infection.

Negative Regulation of T-Cell Responses during Tuberculosis

Most of the work on CD4 T-cell immunity and host resistance to *Mtb* infection has focused on the inductive signals that lead to the priming and differentiation of antigen-specific Th1 responses. Negative regulatory pathways play a major role in immune homeostasis, in control-

ling immune responses against self-antigens, tumors, and pathogens. Although bacterial evasion strategies certainly play a major role in the inability to the host to clear *Mtb* infection, inhibitory pathways may also contribute to the persistence of the bacilli. On the other hand, proper negative regulation of inflammatory responses is also important for preventing immunopathology. Several major negative regulatory pathways and cell types have been examined, primarily in the context of *Mtb* infection in mice, which serve to either limit control of the infection or prevent immune-mediated tissue damage.

IL-10 is an anti-inflammatory pleiotropic cytokine that, among other things, can suppress IL-1 and IL-12 production by dendritic cells and macrophages, so there has been much interest in the role of IL-10 in *Mtb* infection. During the past 15 years, multiple studies have examined and reexamined the role of IL-10 in resistance to *Mtb* infection. These studies have found that IL-10 either has a minor role in suppressing Th1 responses and limiting control of *Mtb* infection (Roach et al. 2001; Turner et al. 2002; Beamer et al. 2008; Redford et al. 2010) or has no discernible role at all (North 1998; Jung et al. 2003). Although the factors that contributed to the different outcomes observed in these studies are not clear, collectively, they show that this inhibitory cytokine is not a major factor leading to the inability of the host to control *Mtb* infection and may have a minor role in limiting immunopathology at very late stages of the infection (Higgins et al. 2009).

Foxp3⁺ regulatory CD4 T cells are essential in maintaining peripheral tolerance to self-antigens, but they also contribute to the inhibition of pathogen-specific immune responses (Josefowicz et al. 2012). During inflammatory conditions, Tregs (regulatory T cells) can functionally specialize to match the contemporaneous class of effector CD4 T-cell responses (Campbell and Koch 2011). In one of the earliest demonstrations of Treg specialization, it was found that Tregs up-regulate the Th1 lineage-specifying transcription factor Tbet during *Mtb* infection, and Tbet expression by Tregs is required for their normal expansion (Koch et al.

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2009). Moreover, it has been shown that Ag-specific Foxp3⁺ regulatory CD4 T cells are generated during *Mtb* infection (Shafiani et al. 2013) and that depletion of Tregs enhances Th1 priming, resulting in ~1 log lower bacterial loads (Scott-Browne et al. 2007). Conversely, injection of Ag85b TCR Tg Foxp3⁺ CD4 T cells delays T-cell priming, resulting in ~1 log increased bacterial loads (Shafiani et al. 2010). The role of Foxp3⁺ regulatory T cells may be even more pronounced during infection with hypervirulent strains of *Mtb* such as the W-Beijing strains (Shang et al. 2011). There is also evidence that the hyperinduction of Tregs during infections with W-Beijing strains of *Mtb* may overcome much of the protective effects of BCG vaccination (Ordway et al. 2011). Therefore, signals from regulatory T cells inhibit the priming of *Mtb*-specific CD4 T cells and may limit their function in the periphery, and they directly contribute to the inability of the host to clear *Mtb* infection.

Apart from anti-inflammatory cytokines and cell types described above, inhibitory receptors expressed by the activated T cells themselves are also critical in controlling T-cell function. Killer-cell lectin-like receptor G1 (KLRG1) is an inhibitory receptor widely expressed on natural killer (NK) cells, and when expressed on CD4 and CD8 T cells, it is often associated with a terminally differentiated or senescent phenotype (Akbar and Henson 2011). In *Mtb* infection, a subpopulation of T cells with high cytokine producing potential up-regulate KLRG1 (Reiley et al. 2010), and KLRG1-deficient mice display slightly increased T-cell responses and survive for extremely long periods following *Mtb* infection, albeit on a genetic background that is already capable of surviving for ~1 yr following infection (Cyktor et al. 2013).

Programmed death-1 (PD-1) is a cell surface inhibitory receptor that is highly expressed on activated CD4 and CD8 T cells. On binding to either of its ligands, PD-L1 or PD-L2, SHP-2 is recruited to an ITSM (immunoreceptor tyrosine-based switch motif) domain in the cytoplasmic tail of PD-1, which then blocks several components of the proximal TCR signal transduction machinery (Keir et al. 2008). PD-

1 is important for maintaining peripheral tolerance as older PD-1 knockout mice spontaneously develop autoimmune disease that varies in severity depending on the genetic background of the mouse. PD-1 also plays a major role in the regulation of immune responses to pathogens. The first evidence that PD-1 plays a role during infection was observed in chronic lymphocytic choriomeningitis virus infection in which it was found that blockade of PD-1/PD-L1 interactions boosts the function of the “exhausted” CD8 T cell and enhances viral control (Barber et al. 2006). It is now widely recognized that PD-1 is a major inhibitor of pathogen-specific T cells in mice, nonhuman primates, and humans. Moreover, PD-1 blockade also dramatically improves tumor-specific immunity and is one of the most promising new developments in cancer therapy (Topalian et al. 2012; Brahmer et al. 2012).

The major success of PD-1 immunotherapy in other model systems created much interest in the possibility of also targeting the PD-1 pathway in *Mtb* infection, and several recent studies have examined the expression of PD-1 on T cells in human tuberculosis and tested its role in murine models *Mtb* infection. Patients with active tuberculosis have higher numbers of PD-1-expressing T cells, and PD-1 blockade in vitro can enhance responses of cultured T cells to *Mtb* antigens (Jurado et al. 2008). Moreover, infection of PD-1 KO mice with BCG leads to enhanced T-cell responses and bacterial control rather than immunopathology (Sakai et al. 2010). However, rather than displaying enhanced protection, PD-1 knockout mice succumb rapidly on exposure to low doses of virulent *Mtb* (Lázár-Molnár et al. 2010; Barber et al. 2011; Tousif et al. 2011). It was found that PD-1 KO mice have greatly increased numbers of I-A^bESAT-6_{1–20} tetramer-binding CD4 T cells in their lungs and that transient depletion of CD4 T cells in the first month of infection prevented the severe tissue destruction and death of the PD-1 KO mice (Barber et al. 2011). Moreover, whereas injection of TCRαKO mice with WT CD4 T cells rescues them from early mortality, reconstituting TCRαKO with a mixture of WT and PD-1 KO CD4 T cells leads to the

rapid death of the animals. This indicates that in the absence of PD-1-mediated inhibition, CD4 T-cell responses are greatly enhanced, but this drives lethal immunopathology in *Mtb* infection instead of enhanced protection. Therefore, CD4 T-cell-mediated pathology in *Mtb* infection can also be revealed by removal of a single negative regulatory pathway. Thus, although CD4 T cells are clearly essential for containment for *Mtb* infection, these cells can also promote lung tissue destruction during *Mtb* monoinfection, during IRIS after antiretroviral therapy of HIV patients, and when certain negative regulatory pathways are perturbed.

CONCLUSIONS

With the advent of novel immunological tools and techniques for studying immune responses at the single-cell level in vivo, the classical paradigm of the T-cell macrophage interaction at the core of antituberculous immunity has now been expanded to include a large number of functionally diverse innate and adaptive effector cells with multiple cellular interactions. Whereas many of the effector functions of inflammatory myeloid cells as well as CD4 T cells are required for host protection, each of these cell types also has potential for mediating tissue damage, and a major goal should be to understand the factors that determine the balance between detrimental and protective cellular immunity. The dual nature of inflammation in *Mtb* infection underscores a major challenge of vaccine development as well as immunomodulatory host-directed treatment strategies for tuberculosis.

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