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MINIREVIEW

Fighting Persistence: How Chronic Infections with *Mycobacterium tuberculosis* Evade T Cell-Mediated Clearance and New Strategies To Defeat Them

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ABSTRACT Chronic bacterial infections are caused by pathogens that persist within their hosts and avoid clearance by the immune system. Treatment and/or detection of such pathogens is difficult, and the resulting pathologies are often deleterious or fatal. There is an urgent need to develop protective vaccines and host-directed therapies that synergize with antibiotics to prevent pathogen persistence and infection-associated pathologies. However, many persistent pathogens, such as *Mycobacterium tuberculosis*, actively target the very host pathways activated by vaccination. These immune evasion tactics blunt the effectiveness of immunization strategies and are impeding progress to control these infections throughout the world. Therefore, it is essential that *M. tuberculosis* immune evasion-related pathogen virulence strategies are considered to maximize the effectiveness of potential new treatments. In this review, we focus on how *Mycobacterium tuberculosis* infects antigen-presenting cells and evades effective immune clearance by the adaptive response through (i) manipulating antigen presentation, (ii) repressing T cell-activating costimulatory molecules, and (iii) inducing ligands that drive T cell exhaustion. In this context, we will examine the challenges that bacterial virulence strategies pose to developing new vaccines. We will then discuss new approaches that will help dissect *M. tuberculosis* immune evasion mechanisms and devise strategies to bypass them to promote long-term protection and prevent disease progression.

KEYWORDS immune evasion, intracellular pathogens, *Mycobacterium tuberculosis*, T cell, vaccine development

The adaptive immune response has evolved to detect and destroy invading bacterial pathogens while simultaneously protecting the tissues of the host from damage (1). During an acute infection, adaptive responses synergize with innate pathways to drive bacterial clearance (2). A subset of pathogens, like *Mycobacterium tuberculosis*, cause persistent infections by employing virulence mechanisms that evade immune detection and inhibit adaptive responses (3, 4). This results in *M. tuberculosis* infections causing the most deaths by infectious disease each year (5). To prevent the ongoing epidemic, it is essential to develop an effective vaccine that protects against lung disease. A major challenge to *M. tuberculosis* vaccine development, however, is accounting for bacterial immune evasion tactics. *M. tuberculosis* effectively modulates adaptive responses from within the intracellular niche in antigen-presenting cells (APCs) that prevent T cell responses from sterilizing the infection (3, 6). Current vaccination methods activate the very processes that *M. tuberculosis* targets. Thus, it is essential to understand how *M. tuberculosis* suppresses adaptive responses to develop new approaches that bypass *M. tuberculosis*-mediated immune evasion. In this review, we highlight a subset of mechanisms used by *Mycobacterium* spp. to inhibit T cell

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responses from within APCs and draw similarities with other persistent bacterial pathogens. We will then discuss new approaches that might allow a full understanding of the *M. tuberculosis*-mediated immune evasion that is needed to develop vaccines that overcome pathogen virulence and protect against *M. tuberculosis*.

OVERVIEW OF *M. TUBERCULOSIS* IMMUNE CELL INTERACTIONS

M. tuberculosis is a facultative intracellular pathogen that resides inside APCs, including a variety of macrophage and dendritic cell (DC) subsets (7, 8). Upon inhalation of *M. tuberculosis*-containing aerosol droplets, *M. tuberculosis* efficiently targets alveolar macrophages that line the alveoli (8). Ideally, the initial infection by *M. tuberculosis* would stimulate inflammation in alveolar macrophages to activate protective adaptive immune responses that quickly respond to the lung and eradicate the infection. However, alveolar macrophages do not robustly detect or respond to *M. tuberculosis* infection, which results in a blunted inflammatory response and delays adaptive immune activation over 2 weeks (8–10). This delay is unlike other lung infections such as those caused by influenza virus or respiratory syncytial virus (11). These viral infections develop a robust pathogen-specific T cell response within 1 week, suggesting that *M. tuberculosis* actively uses the alveolar macrophages to avoid rapid adaptive immune activation and detection. Eventually, *M. tuberculosis*-infected alveolar macrophages migrate from the alveoli into the interstitial space through the direct activity of the specialized type VII secretion system ESX-1 (8). In the interstitial space, inflammatory macrophages and dendritic cell populations are infected by *M. tuberculosis*, triggering robust inflammation that causes the onset of adaptive immunity. *M. tuberculosis* antigens are trafficked to the draining lymph nodes by dendritic cells, where they activate *M. tuberculosis*-specific T cells that are required to protect against disease (12, 13).

For T cells to be activated during an *M. tuberculosis* infection, they must receive two distinct signals in the lung draining lymph node (14). Signal one is dependent on the antigen specificity of the T cell receptor (TCR) which detects pathogen-derived peptides loaded into major histocompatibility complex class I or II (MHC-I or MHC-II, respectively) (14). These peptide-MHC complexes are then presented on the surface of APCs to naive T cells. The second signal, also known as costimulation, is delivered to the T cell through the ligation of inflammation-induced molecules such as CD80, CD86, or CD40 on the surface of the APC (15, 16). Binding of distinct costimulatory molecule by T cells can skew their function, enhancing or inhibiting *M. tuberculosis* control (6, 17). In addition to signals one and two, a third signal, driven by stimulatory cytokines, enhances the activation of T cells, in particular, $CD8^+$ T cells (reviewed in reference 4). Following their activation in the lymph node, T cells then traffic to the lung environment in search of infected cells to eradicate (13). In the lungs, direct contact of both $CD4^+$ T cells and $CD8^+$ T cells with cells harboring *M. tuberculosis* can partially control disease, yet they are insufficient to sterilize the infection (4, 13, 18). The reasons T cells fail to fully control *M. tuberculosis* infection are complex. *M. tuberculosis* actively prevents effective detection by T cells and drives T cell exhaustion that limits the protective potential of T cells (3, 7). It is also possible that *M. tuberculosis* evolved to use T cell responses to help drive transmission. Unlike viruses like influenza virus, which actively evade immunity by mutating antigens to prevent detection, *M. tuberculosis* does not evolve rapidly, and T cell antigens are known to be hyperconserved, with few mutations across lineages (19, 20). This means that the antigens activating the *M. tuberculosis* T cell responses are very conserved across the human population. This has led some to hypothesize that *M. tuberculosis* actively stimulates robust T cell responses to drive tissue damage and subsequent transmission. Together, these data suggest that T cells are essential to protect against *M. tuberculosis* infection, yet their role in disease progression needs to be more carefully understood.

While T cells are required for protection against tuberculosis (TB), how T cells mechanistically contribute to protection remains unclear. T cells can protect by controlling antimicrobial resistance pathways which directly restrict bacterial growth or by

regulating disease tolerance, the ability to withstand an infection and the subsequent tissue damage (1, 21). Given that T cells are unable to provide sterilizing immunity against *M. tuberculosis*, it is likely that many antimicrobial mechanisms activated by T cells are ineffective against *M. tuberculosis* or are actively inhibited by the pathogen. Evidence also suggests that dysregulated T cell responses, such as increased gamma interferon (IFN-γ) production or altered mitochondrial dynamics, can drive failed disease tolerance (22, 23). Together, these studies suggest that an important role of T cells during *M. tuberculosis* infection is to promote disease tolerance against infection. Given the modest protection provided by even the best new vaccine candidates, the question of whether a T cell-mediated vaccine could activate antimicrobial resistance mechanisms remains uncertain. Evidence from the nonhuman primate model suggests that an ongoing *M. tuberculosis* infection can eliminate subsequent infections with new *M. tuberculosis* strains, but the mechanisms of this protection and the harnessing of these mechanisms for a vaccine remain unknown (24). However, a recent study examining the protective response in nonhuman primates immunized with *Mycobacterium bovis* BCG by the intravenous route suggests that this is a possibility (25). In this study, the authors found sterilizing immunity that was driven by a unique $CD4^+$ T cell subpopulation that must now be further investigated for their protective potential.

Many current vaccine strategies for *M. tuberculosis* are aimed at augmenting T cell responses that are required to control *M. tuberculosis*. Most of these protective pathways were identified because hosts lacking single immune genes were more susceptible to *M. tuberculosis* infection than are otherwise healthy hosts. However, in many healthy individuals infected with fully virulent *M. tuberculosis*, these responses are inadequate on their own (4, 7, 26). A possible reason that current approaches are not yielding the progress hoped is that they do not account for *M. tuberculosis* immune evasion tactics. *M. tuberculosis* has developed distinct mechanisms that delay, block, and distract host immune mechanisms that might be the best equipped to eradicate the disease. Without fully understanding how *M. tuberculosis* avoids normally effective immune mechanisms, the challenge to overcome *M. tuberculosis* virulence strategies and develop fully protective vaccines remains.

MECHANISMS OF *M. TUBERCULOSIS* IMMUNE EVASION

***M. tuberculosis* inhibits antigen processing and presentation to prevent T cell-mediated clearance.** The ability of T cells to recognize their cognate antigens presented in MHC molecules is essential to activate T cell responses that detect and destroy infected cells in the lung environment. However, *M. tuberculosis* infection delays the activation of antigen-specific T cell responses, skews the antigen specificity of T cells, and prevents the effective detection of infected cells (13, 27, 28) (Fig. 1). *M. tuberculosis*-infected mice elicit a muted antigen-specific $CD4^+$ T cell response compared to that with BCG-infected mice as a result of suboptimal *M. tuberculosis* antigen presentation independent of antigen levels, suggesting that *M. tuberculosis* interferes with optimal immune activation (29). Additionally, *M. tuberculosis* is able to evade direct killing by T cells through metabolic functions such as *de novo* tryptophan synthesis that bypasses the induction of indoleamine 2,3 dioxygenase (IDO) and tryptophan restriction in host cells (30). Thus, *M. tuberculosis* effectively manipulates the activation and effector functions of *M. tuberculosis*-specific CD4 and $CD8^+$ T cells by modulating antigen processing and presentation in MHC molecules that contribute to pathogen persistence in the lungs.

Inhibition of phagolysosome fusion and maturation. *M. tuberculosis*-specific T cell responses are delayed in their activation, which allows uninhibited *M. tuberculosis* growth over the first weeks of infection (8, 13, 26). This delay is due to several factors, including *M. tuberculosis*-mediated inhibition of antigen processing and loading of antigens into the MHC. For all APCs, fusion of the phagocytosed particles in a phagosome with the lysosome is a general mechanism to kill the pathogen and provide pathogen-derived peptides that are then loaded into MHC-II molecules (31, 32). *M. tuberculosis* efficiently prevents lysosome fusion, growing instead in a modified phago-

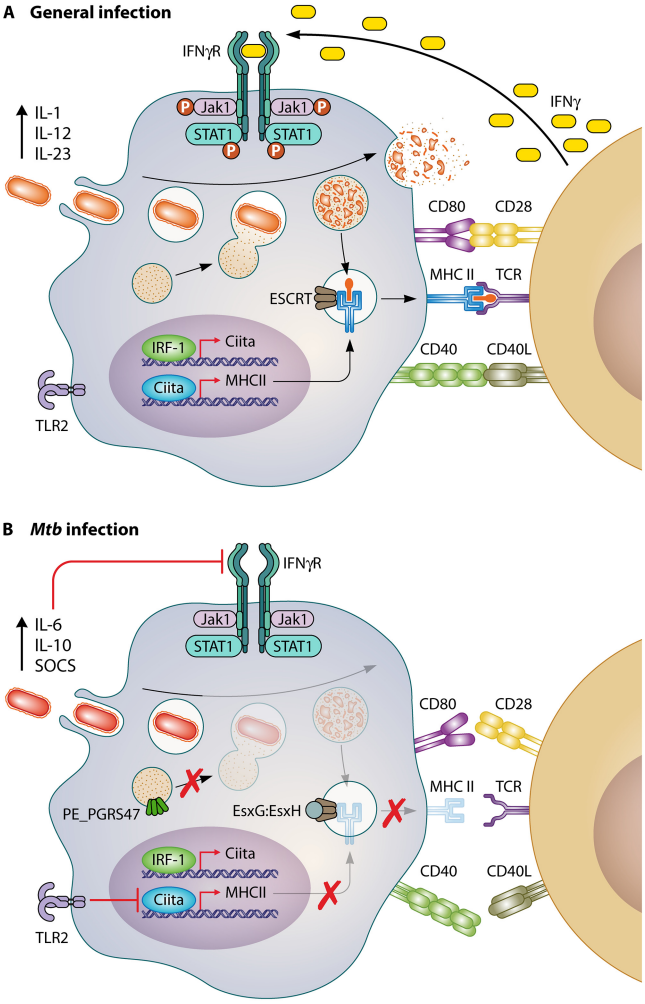

FIG 1 *M. tuberculosis* inhibits effective antigen presentation. (A) Schematic of a normal response to phagocytosed bacteria. Antigen-presenting cells phagocytose a pathogen. Fusion of the phagolysosome causes degradation and production of pathogen-specific peptides. Pathogen-derived peptides then bind to the MHC-II complex and are trafficked to the surface of the cell. There, T cells recognize the presented antigen leading to increased immune cell recruitment, cytokine production, and antibody memory. (B) A schematic of *M. tuberculosis* (*Mtb*)-mediated evasion of antigen presentation. The APC engulfs *M. tuberculosis*, and the expression of PE_PGRS47 and EsxG-EsxH inhibits phagolysosome fusion by preventing the degradation of *M. tuberculosis* antigens. Innate immune detection of lipoproteins results in the activation of TLR2 by *M. tuberculosis*, which inhibits the induction of CIITA and MHC-II through unknown mechanisms. Together, these *M. tuberculosis*-mediated alterations prevent the effective expression of MHC molecules and prevents effective clearance by *M. tuberculosis*-specific T cells.

some compartment that is protected against bacterial degradation (33). This inhibition results in inefficient MHC-II loading of *M. tuberculosis*-derived peptides and prevents efficient $CD4^+$ T cell detection. One way that *M. tuberculosis* blocks lysosome fusion is through the manipulation of the host endosomal sorting complex required for transport (ESCRT) (34–36). ESCRT sorts ubiquitin-labeled surface receptors into intraluminal vesicles of multivesicular bodies to be degraded in the lysosome and loaded into MHC-II molecules (36). *M. tuberculosis* secretes EsxH and EsxG via the ESX-3 type VII secretion system. These effectors then dimerize and inhibit phagosome maturation through the interactions with the ESCRT pathway (34, 35). EsxG-EsxH-dependent ESCRT inhibition limits the activation of *M. tuberculosis*-specific $CD4^+$ T cells in both macrophages and dendritic cells, suggesting that these virulence components are key to the delay in T cell activation during infection. In the absence of EsxH, T cells are more effectively activated and better control infection. In contrast, *M. tuberculosis* strains overexpressing EsxG-EsxH further limit the activation of $CD4^+$ T cells. Therefore, EsxH

impairs phagolysosome fusion in an ESCRT-dependent manner, resulting in the suboptimal antigen processing and presentation of *M. tuberculosis* proteins that are needed to activate $CD4^+$ T cells.

M. tuberculosis employs multiple effectors to modulate other host phagosomal fusion pathways. Maturation of the phagosome depends on the recruitment and dissociation of multiple membrane markers, including phosphatidylinositol-3-phosphate (PI3P), the acidifying proton pumps V-ATPase and Hv1, and multiple Rab-GTPases, each of which is disrupted by *M. tuberculosis* (37–42). The SecA2-dependent secreted factors SapM and PknG inhibit both phagosome and autophagosome maturation by inhibiting PI3P phosphorylation, Rab5 dissociation, and Rab7 recruitment (43). Additionally, the secreted protein tyrosine phosphatase PtpA specifically binds and inhibits V-ATPase trafficking to the phagosome (44). It is currently unknown how *M. tuberculosis* inhibits Hv1 localization to the phagosome. Another recent study identified that the *M. tuberculosis* protein PE_PGRS47 plays an important role in modulating antigen presentation by inhibiting lysosome function (45). Rather than directly preventing lysosome fusion with the phagosome, PE_PGRS47 manipulates the host autophagy pathway. Autophagy is a highly conserved mechanism used by cells to remove unnecessary, damaged components and plays a role in controlling intracellular pathogens (46). Fusion of autophagosomes with the lysosome allows foreign antigens to be efficiently loaded and presented on MHC-II molecules (47). PE_PGRS47 limits MHC-II antigen presentation by preventing effective autophagosome-lysosome fusion. PE_PGRS47 deletion mutants show increased autophagic vesicles, in addition to more acidified phagosomes and increased lysosome fusion (45). The inhibition of autophagosome-lysosome fusion by PE_PGRS47 has functional consequences on the activation of $CD4^+$ T cells. Infection of mice with PE_PGRS47-deficient strains resulted in significantly more activated $CD4^+$ T cells due to the increase in *M. tuberculosis* antigens loaded into MHC-II molecules. This directly shows that pathogen-mediated inhibition of lysosome fusion is an effective immune evasion tactic to avoid rapid $CD4^+$ T cell responses. While *M. tuberculosis* possesses numerous pathways to inhibit host phagolysosome fusion/maturation, it also expresses at least one failsafe mechanism to neutralize acidification that the bacteria do experience. A comparative lipidomics study between *M. tuberculosis* and BCG identified an *M. tuberculosis*-exclusive robustly produced extracellular lipid, 1-tuberculosinyladenosine (1-TbAd), which was shown to possess acid-neutralizing properties, result in protected growth at low pH, and induce phagosomal swelling in infected human macrophages (48, 49).

When viewed through this lens, it is not surprising how prevalent the inhibition of phagolysosome maturation and lysosome fusion is among successful pathogens. For example, *Chlamydia trachomatis*, *Salmonella enterica* serovar Typhimurium, and *Brucella abortus* all inhibit lysosome fusion by producing specific virulence factors (50–52). While it remains to be directly tested for these pathogens, evading lysosome fusion might not only protect bacterial viability but also hinder $CD4^+$ T cell activation, similar to what occurs during *M. tuberculosis* infection. In addition, given the importance of lysosome inhibition to virulence, several *M. tuberculosis* factors may contribute in parallel to ensure success. It is essential to identify these redundant mechanisms in *M. tuberculosis* to delineate strategies that overcome lysosome inhibition and drive a more rapid and robust activation of protective $CD4^+$ T cells.

Inhibition of MHC surface expression. Once *M. tuberculosis*-specific T cells are activated in the draining lymph node, they must then traffic to the lung and identify *M. tuberculosis*-infected cells to control pathogen growth and contain disease (13). For both $CD4^+$ and $CD8^+$ T cells, this is known to require direct contact with the MHC on infected cells within the lung environment (4, 18). On resting macrophages, the surface expression of MHC-II is moderate, which limits the ability of macrophages to stimulate $CD4^+$ T cells directly (53). However, the addition of cytokines such as IFN-γ results in the robust upregulation of MHC-II by the class II transactivator (CIITA) (53, 54). Intracellular pathogens have evolved mechanisms to directly inhibit the induction of CIITA and

prevent MHC-II upregulation. *Chlamydia trachomatis*, for example, directly degrades the transcription factors USF-1 and RFX-5 that are required for IFN-γ-dependent upregulation of MHC-II (55, 56). In contrast, *M. tuberculosis* infection does not appear to actively target MHC-II upregulation but rather uses innate immune detection of *M. tuberculosis* against the host. Several studies have found that mycobacterial lipoproteins and other cell envelope components inhibit MHC-II upregulation by acting as Toll-like receptor 2 (TLR2) agonists (3, 57). The 19-kDa lipoprotein of *M. tuberculosis* limits MHC-I expression and prevents IFN-γ-induced HLA-DR (an MHC-II surface receptor), the FcγR1 (a high-affinity IgG receptor), and CIITA expression (58–60). *M. tuberculosis* also inhibits macrophage responses to IFN-γ by inducing cytokines like interleukin 6 (IL-6) which can inhibit Th1 differentiation while inducing the suppressor of cytokine signaling (SOCS) (61, 62). SOCS inhibits STAT1 phosphorylation, thus limiting antigen presentation. These findings are similar to those observed during *Brucella abortus* infection, which also induces IL-6 secretion to inhibit IFN-γ-mediated induction of interferon regulatory factor 1 (IRF-1) and CIITA (63). By stimulating innate responses that are hard wired into APCs, *M. tuberculosis* effectively prevents the subsequent induction of MHC molecules that would help T cell-mediated clearance. Data suggest that *M. tuberculosis* stimulates TLR2, TLR4, and TLR9, yet how these innate receptors directly or indirectly prevent the upregulation of surface MHC molecules remains to be fully understood (64). Additionally, how to overcome the initial TLR activation following *M. tuberculosis* infection to strongly upregulate MHC molecules is an outstanding question that must be addressed in the future.

Inhibition of direct T cell detection. Even if infected APCs induced robust surface expression of MHC molecules, the question remains of whether T cells are capable of detecting *M. tuberculosis*-infected cells. Work from several groups suggests that *M. tuberculosis* might use decoy antigens to drive dominant T cell responses against proteins that are subsequently downregulated in *M. tuberculosis* as persistence begins (27, 28, 65, 66). This allows *M. tuberculosis* to evade protective T cell responses by eliminating the expression of the antigens for which the majority of T cells are specific. This evasion strategy results in a T cell repertoire that is focused on nonprotective antigens that do not improve disease outcome. Similarly, recent studies from the Behar group suggest an inability of *M. tuberculosis*-specific $CD8^+$ cells to directly detect infected macrophages, which may explain a limited role for $CD8^+$ cells *in vivo* (28). It has been a conundrum as to why $CD8^+$ T cells do not play a more central role in the protective response against *M. tuberculosis*. *M. tuberculosis* secretes antigens directly into the cytosol, and $CD8^+$ T cells are well equipped to eliminate cells infected with intracellular pathogens, yet the loss of $CD8^+$ T cells results in a minimal change in *M. tuberculosis* disease progression (4). Interestingly, this mirrors findings for other intracellular pathogens like *Chlamydia trachomatis*, where $CD8^+$ T cells play a minimal role in protection but contribute greatly to the immunopathology that occurs during chronic infections (67, 68).

In recent studies, *M. tuberculosis*-infected macrophages were detected by $CD4^+$ T cells specific for ESAT-6 and Ag85b and restricted *M. tuberculosis* growth (28). Yet, polyclonal $CD4^+$ T cells isolated from the infected lungs of mice did not effectively detect infected macrophages (27). In contrast, $CD8^+$ T cells specific for the immunodominant antigen TB10.4 could not detect *M. tuberculosis* in macrophages, while polyclonal $CD8^+$ T cells could (27, 28). These findings raise the possibility that *M. tuberculosis* carefully controls what antigens are available for presentation in infected cells to dictate the T cell repertoire that is activated. Clearly, this virulence strategy allows for the effective evasion of protective T cell responses, yet how *M. tuberculosis* controls antigen availability and prevents effective detection by T cells on macrophages remains unknown. In these reports, there were no obvious problems with antigen processing or antigen presentation (32, 64). However, it was noted that infection with BCG results in a greater capacity of T cells to recognize infected macrophages, suggesting that the RD1 locus may play a role in masking infected macrophages. Any

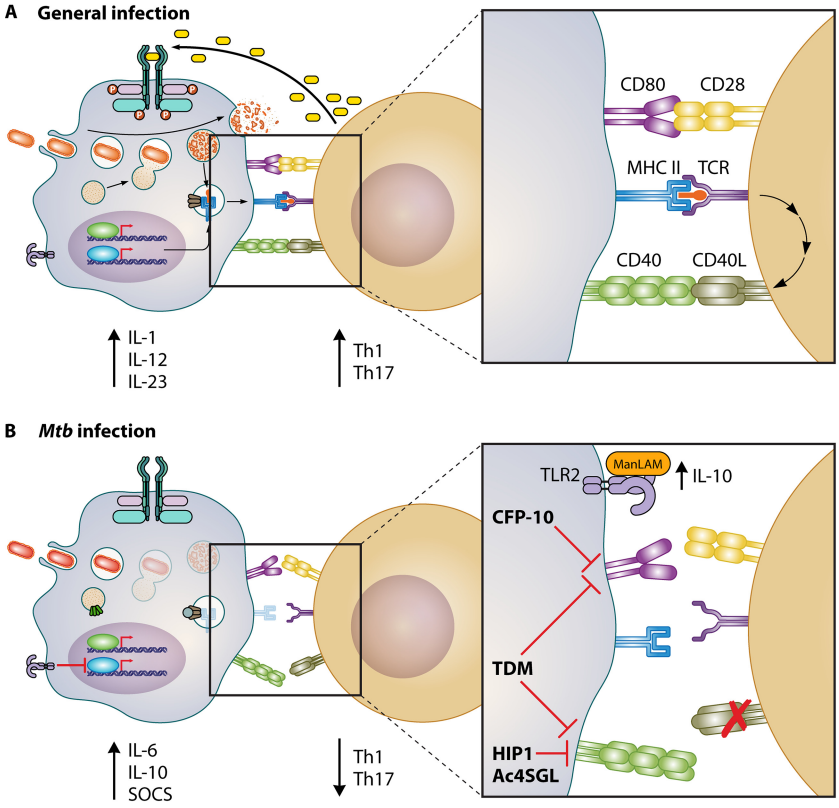

FIG 2 *M. tuberculosis* uses multiple effectors to manipulate costimulatory molecule activity and CD4$^+$ T cell polarization. (A) Schematic of the general response of costimulatory molecules in APCs during infection with an intracellular pathogen. APCs induce CD80/86:CD28 and/or CD40:CD40L binding, resulting in the proliferation of CD4$^+$ T cells and Th-1/Th-17 polarizing cytokine secretion from the APC. (B) Schematic of *M. tuberculosis*-mediated evasion of costimulatory. Infection of APCs with *M. tuberculosis* results in inhibition of costimulatory molecule expression. ManLAM from *M. tuberculosis* interacts with TLR2 to induce IL-10 secretion, repressing Th1 polarization. TDM inhibits the induction of CD80/86 and CD40, while Hip1 and Ac4SGL block robust activation of CD40. These virulence traits change the overall cytokine response and prevent the protective capacity of *M. tuberculosis*-specific T cells from eradicating the infection.

future vaccine must overcome the ability of *M. tuberculosis* to distract T cell responses away from protective antigens to allow the detection of infected cells.

***M. tuberculosis* modulates costimulatory molecules to skew T cell effector responses.** In addition to antigen-specific activation that is mediated by peptide-MHC complexes, effective T cell responses require efficient costimulation (16). Costimulation, or signal 2, occurs through the ligation of a variety of molecules on APCs with their cognate ligands on the surface of T cells (14, 16). While the interaction between CD28 on T cells and CD80/CD86 on APCs is most commonly studied, other molecules on APCs, including CD40 and OX40L, also play an important role. How each costimulatory molecule influences immune responses to distinct pathogens remains unclear, as costimulatory molecules can augment the MHC-TCR signal and influence the subsequent effector response. The absence of any costimulatory signal in the presence of antigen presentation results in T cell anergy, a state in which the T cell remains alive but is largely unresponsive to further stimulus (69). Given the importance of costimulation in shaping the subsequent host response, pathogens like *M. tuberculosis* directly manipulate these molecules to the benefit of the pathogen (Fig. 2). While our understanding of the mechanisms employed by *M. tuberculosis* to target distinct costimulatory pathways remains lagging, there is significant evidence that targeting distinct costimulation networks during immunization may improve *M. tuberculosis* infection outcomes.

A central aspect of costimulatory molecules is that their expression is increased on the surface of APCs following the activation of pathogen-associated molecular patterns and the subsequent cytokine response (15, 16). This regulation pattern ensures that T cell activation only occurs in the correct inflammatory context. Both type I and type II interferons as well as tumor necrosis factor alpha (TNF-α) feed back into APCs to drive the expression of CD40, OX40L, CD80, and CD86 to maximal levels (15, 70, 71). Thus, *M. tuberculosis* is well positioned to manipulate the induction of costimulatory molecules by carefully controlling the inflammatory response it induces during infection. Recent evidence suggests that while each costimulation marker plays an important role in driving a protective response against *M. tuberculosis*, these pathways are not fully functional during virulent *M. tuberculosis* infection (6).

Costimulation between CD80/CD86 and CD28 helps activate the Th1 effector responses during *M. tuberculosis* infection (72). Loss of CD28 results in muted Th1 induction and uncontrolled *M. tuberculosis* growth (72). In addition, the loss of CD40 results in severe susceptibility to *M. tuberculosis* infection due to the altered effector profile of $CD4^+$ T cells, while the loss of OX40L prevents the efficient induction of Th1 vaccine responses against *M. bovis* BCG (73, 74). Therefore, each distinct costimulatory molecule may help influence the overall host responses against *M. tuberculosis*. This is similar to other persistent pathogens like *Chlamydia trachomatis*, where a combination of costimulatory molecules appears to be required for effective protective immunity (75, 76). This highlights a supportive role for all costimulatory molecules during persistent infections that must be better understood.

Because costimulatory molecules play a critical role in determining the effector T cell response during *M. tuberculosis* infection, these pathways are actively manipulated during infection. Several reports suggest that during the chronic phase of *M. tuberculosis* infection, CD80, CD86, and CD40 are all downregulated in the lung, limiting the capacity of responding T cells to find infected cells and escape anergy (77). One mechanism driving the inhibition of costimulatory molecules is the presence of the surface lipid trehalose 6,6′-dimycolate (TDM) (78). Distinct macrophage populations, including alveolar and peritoneal macrophages, that were infected with wild-type *M. tuberculosis* did not express high levels of CD40, CD80, or CD86, while strains that were delipidated for TDM strongly induced the expression of these molecules. This broad inhibition is similar to the effect of VacA from *Helicobacter pylori*, which induces the dendritic cell transcription factor E2F1 to suppress activation (79, 80). It remains to be known if TDM directly induces E2F1 in a similar manner and should be pursued in the future. In addition to TDM, the mycobacterial cell wall component mannosylated lipoarabinomannan (ManLAM) binds TLR2, resulting in increased IL-10 production which prevents the robust differentiation of Th1 cells (81). Altogether, the inflammatory makeup of *M. tuberculosis* surface lipids directly impacts the magnitude of costimulatory molecule induction, thereby skewing T cells away from protective effector responses.

How *M. tuberculosis* modulates the makeup of surface lipids to impact costimulatory signals remains unclear. One possible mechanism is mediated by the serine hydrolase Hip1. Hip1 was identified in a genome-wide transposon screen and subsequently found to be required for *M. tuberculosis* to persist long term in animals (82, 83). Later studies found that Hip1 is important for GroEL hydrolysis and that Hip1 inhibits the inflammatory response to *M. tuberculosis* (84–86). The loss of Hip1 results in the increased expression of costimulatory molecules, in particular, CD40, on infected APCs (17, 85). In addition, the loss of Hip1 increases the production of proinflammatory cytokines that drive both Th1 and Th17 responses against *M. tuberculosis* infection. This observation has important implications, as wild-type *M. tuberculosis* infections do not effectively activate Th17 responses, even though a balanced Th1/Th17 response provides improved overall protection against *M. tuberculosis*. To test this hypothesis directly, Rengarajan and colleagues examined how the presence or absence of Hip1 changes CD40 expression on *M. tuberculosis*-infected APCs to skew the effector T cell response (17). Their results clearly show that CD40 is required to activate a subset of $CD4^+$ T cells

to produce IL-17. Infecting cells with a Hip1 mutant or treating wild-type *M. tuberculosis*-infected dendritic cells with purified CD40L-trimer overcame Hip1-mediated CD40 repression and induced a more balanced Th1/Th17 $CD4^+$ T cell response. This balanced effector response of $CD4^+$ T cells improved the capacity of the host to restrict *M. tuberculosis* growth and prevent disease. These important studies not only show that *M. tuberculosis* modifies detection by the innate immune system to avoid robust costimulation but also provide a foundation for the premise that overcoming pathogen-mediated immune evasion is critical to develop therapies that effectively protect against *M. tuberculosis*.

Beyond Hip1, the mycobacterial secreted factor CFP-10 and the Ac_4SGL prevent the maximal expression of costimulatory molecules on APCs (87, 88). When cells express CFP-10, they are unable to induce CD80 expression following exogenous stimuli. How this inhibition occurs remains unknown (87). Similar to Hip1, Ac_4SGL inhibits CD40 surface expression but through a distinct mechanism that suppresses NF-κB activation (88). Thus, *M. tuberculosis* uses multiple mechanisms to prevent the efficient induction of costimulatory molecules on infected APCs. The consequences of these changes to costimulatory molecules remain to be investigated carefully *in vivo*. In the future, it will be important to identify the mechanisms *M. tuberculosis* employs to block costimulatory molecule expression and investigate the regulation of each costimulatory molecule to overcome pathogen-mediated inhibition and improve the overall balance of the host response against *M. tuberculosis*.

***M. tuberculosis* induces inhibitory ligands that repress T cell function.** While the primary immune response in most individuals adequately contains *M. tuberculosis* infection, the lack of sterilizing immunity presents a challenge (1, 4). As effector T cells respond to persistent antigen stimulation, they begin to upregulate inhibitory receptors, such as PD1, Tim3, and CTLA4 (3, 4, 22, 89, 90). These receptors prevent overexuberant inflammation and induce a phenotypic state known as exhaustion (16). While exhaustion is important to ensure that inflammatory damage does not occur, *M. tuberculosis* likely uses exhaustion to its advantage to prevent effective clearance. As T cells become exhausted, they show dysfunction in their ability to activate upon antigen stimulation, produce cytokines, and directly kill infected cells (16). Several studies have shown that PD1 and Tim3 are upregulated on *M. tuberculosis*-specific T cells as infection progresses to the chronic phase (22, 89). The induction of these inhibitory molecules reduces the production of proliferative cytokines like IL-2 and effector cytokines such as IFN-γ and TNF. A recent study by Jayaraman and colleagues showed that TB-specific T cells coexpress PD1 and Tim3 and that the removal of Tim3 activity improves T cell function and *M. tuberculosis* control (89). However, in other studies, genetic deletion of the inhibitory receptor PD1 resulted in the pathological production of IFN-γ that resulted in exacerbated TB disease and early lethality (22, 91). Together, these studies suggest that modulating inhibitory receptors may have pleotropic effects on the host response that must be understood more fully.

To date, most studies examining T cell exhaustion have focused on the expression of the inhibitory molecules on the T cells themselves but not on the ligands present on the *M. tuberculosis*-infected APCs. It is possible that *M. tuberculosis* actively exploits the induction of these ligands during infection to promote T cell dysfunction and prevent effective clearance. This prediction is not without support, as other chronic bacterial infections are known to target these pathways. For example, during genital infections with *Chlamydia trachomatis*, pathogen-specific $CD8^+$ T cells are incapable of contributing to the protective response. The inhibition of $CD8^+$ T cell function was found to be a direct result of *C. trachomatis* actively inducing the PD1 ligand PDL1 on infected cells (92). Reversing this virulence tactic resulted in more effective bacterial control and T cell function. In addition, *Salmonella enterica* serovar Typhimurium and *Helicobacter pylori* also manipulate PD-L1 levels to suppress T cell responses and drive persistent infections (93, 94). In agreement with studies on other pathogens, *M. tuberculosis*-infected APCs express significantly more inhibitory ligands that may drive T cell

dysfunction (95). However, it has yet to be examined whether *M. tuberculosis* is actively inducing these pathways or if PD-L1 induction is simply a by-product of persistent inflammation. Clearly, the role of T cell exhaustion is important in the balance between control and susceptibility to *M. tuberculosis*, yet much more study is needed to understand how inhibitory ligands are modulated during infection in the APC and which inhibitory molecules on T cells can be targeted without exacerbating disease.

NEW APPROACHES TO UNRAVEL *M. TUBERCULOSIS*-MEDIATED T CELL EVASION

As discussed above, *M. tuberculosis* evades immune clearance by carefully engaging the infected APCs to prevent elimination by T cells. Even though these immune evasion mechanisms are becoming clearer, several key questions remain. These include defining how distinct cell populations in the infected lung drive unique aspects of immune evasion during *M. tuberculosis* infection, identifying all *M. tuberculosis* genes that modulate immune detection and developing approaches that bypass *M. tuberculosis* virulence traits to more effectively activate protective T cell responses. In the second part of this review, we evaluate and discuss new tools and approaches that should be applied to address these outstanding questions in *M. tuberculosis*-mediated immune evasion.

Expanding *ex vivo* approaches to reflect cell diversity in the lung environment. Within the infected lung, *M. tuberculosis* encounters a variety of cell types, including alveolar macrophages, interstitial macrophages, dendritic cells, and neutrophils (8). To understand how *M. tuberculosis* impedes T cell responses, it is essential to understand how each distinct host cell population interacts with *M. tuberculosis* and enables immune evasion. To date, however, most studies examining the mechanisms of *M. tuberculosis* pathogenesis employ *ex vivo* models that use primary bone marrow-derived macrophages (BMDMs), peritoneal macrophages, or immortalized cell lines such as RAW or Thp1 cells (7). While these approaches are certainly useful, recent studies suggest that an important aspect of *M. tuberculosis* disease is the progression of cellular interactions that occur in the complex lung environment (8–10). How *M. tuberculosis* infection of each cellular population plays into the overall immune evasion remains unknown. As described above, alveolar macrophages are the initial niche for *M. tuberculosis* following inhalation, and these cells are inherently different than BMDMs. Not only is the innate response distinct between alveolar macrophages and BMDMs, but elegant work by Russell and colleagues also showed that the metabolic differences between macrophage populations in the lungs drive disease progression (10). While interstitial macrophages use glycolysis for energy, alveolar macrophages rely on fatty acid oxidization, which results in a permissive nutrient pool for *M. tuberculosis* growth. It is likely that the permissive alveolar macrophage niche sequesters *M. tuberculosis* away from dendritic cells that are more capable of triggering the upregulation of key surface MHC and costimulatory molecules needed for rapid T cell activation (8). Yet, this has remained difficult to test due to a lack of *ex vivo* models that recapitulate the functions of alveolar macrophages. Effectively examining how *M. tuberculosis* manipulates distinct APC populations to evade T cell responses requires *ex vivo* systems that model distinct cell populations from the lung and/or methods to identify and isolate infected cells directly from the infected lung environment.

One reason for the reliance on BMDMs is the ease with which millions of cells can be isolated and manipulated without using large numbers of animals. In contrast, studying alveolar macrophages remains a challenge. These cells must be isolated directly from the bronchoalveolar lavage (BAL) fluid in the lungs, and from each mouse, only 10^5 cells can be isolated (96, 97). These low numbers and the time involved in isolation limit the scale and reproducibility of primary alveolar macrophage studies. Recently, Fejer et al. discovered an approach to differentiate fetal liver cells, the origin of alveolar macrophages, into alveolar macrophage-like cells called Max Planck Institute (MPI) cells that can be propagated *ex vivo* for over 100 generations (97). MPI cells have similar morphology and express similar surface markers to alveolar macrophages, differentiating them from bone marrow-derived macrophages. The innate response in

these cells was remarkably similar to alveolar macrophages isolated from BAL fluid. Following lipopolysaccharide (LPS) treatment or treatment with heat-killed *M. tuberculosis*, MPI cells showed a muted IL-10 response and a strong activation of IL-1a that mirrored that of alveolar macrophages but not BMDMs. In a more recent study, Woo et al. showed that MPI cells can phagocytose live *M. tuberculosis* cells, support *M. tuberculosis* replication, and activate innate responses, including cytokine production, autophagy, and lipid accumulation (98). Together, these studies suggest that MPI cells may be a useful model for the alveolar macrophages that play a critical role in *M. tuberculosis* pathogenesis and immune evasion. In the future, it will be important to understand how *M. tuberculosis* can target MPI cells and alveolar macrophages to alter their antigen presentation and costimulatory capacity during infection.

It is also important to consider how infected cells directly from the lung environment are manipulated by *M. tuberculosis*. Examining distinct cell types *in vivo* requires tools that allow the identification and isolation of all *M. tuberculosis*-infected cell populations. One successful approach is leveraging bacterial reporters that allow the sensitive detection of *M. tuberculosis*-infected cells while preserving virulence. These reporters range from standard fluorescent strains expressing bright and stable fluorescent proteins to metabolic reporters that discriminate between live and dead bacteria or indicate the activation of particular *M. tuberculosis* transcriptional responses (8–10, 99). By coupling these sensitive reporters to cell-sorting or whole-tissue-imaging approaches, it is now possible to directly examine how distinct cellular populations are manipulated by *M. tuberculosis* infection and how this impacts protective T cell responses.

Identifying new mechanisms used by *M. tuberculosis* to manipulate T cell responses. *M. tuberculosis* contains several genes that drive immune evasion mechanisms during infection. However, no global approaches have been employed to systematically define the repertoire of *M. tuberculosis* immune evasion genes. With proteomic and global genetic approaches now readily available, the time is ripe for those in this field to investigate how individual or groups of *M. tuberculosis* genes contribute to the evasion of T cell responses.

Global genetic approaches in *M. tuberculosis* were developed over 15 years ago with the seminal papers using transposon hybridization (TRASH) (100). TRASH uses a pooled library of transposon mutants made with the Mariner transposon. These global transposon experiments determine how the relative representation of each mutant changes between two distinct conditions. Recent advances in deep-sequencing technology (now termed transposon sequencing [Tn-seq]) have accelerated the speed and sensitivity of Tn-seq experiments, yet few studies have focused on immune-related phenotypes (101). Most published studies use *M. tuberculosis* genetic screens to focus on the pathogen itself by characterizing phenotypes such as physiology, growth, and survival (102–104). However, these bacterial genetic approaches have the power to disentangle exactly how *M. tuberculosis* inhibits APCs from effectively communicating with T cells. For example, while Hip1 is known to inhibit CD40 induction in APCs, it is likely that other *M. tuberculosis* genes also contribute to CD40 inhibition (17). By infecting an APC population with a transposon mutant library and isolating host cells that can or cannot induce robust CD40 expression, it is now possible to identify *M. tuberculosis* mutants that directly impact this important immune cell function. The applications of this approach regarding our understanding of *M. tuberculosis* factors that drive persistence are endless.

Of course, transposon approaches have their limitations. By definition, transposon libraries cannot assess the role of essential genes, reducing the percentage of the genome probed by a significant margin (102). To address this, recent developments in protein depletion and CRISPR interference (CRISPRi) approaches now allow the investigation into these essential genes (105–107). The recent tour de force study that created a library of barcoded hypomorphic alleles for essential genes throughout the *M. tuberculosis* genome will be an outstanding tool for immunologists to use in the future to probe even deeper into the *M. tuberculosis* genome to understand the evasion

of the adaptive immune response (107). Additionally, robust yeast two-hybrid screens have proven effective at uncovering unexpected interactions between *M. tuberculosis* proteins and host networks that influence the ability of APCs to signal to T cells. The interaction between EsxH and the ESCRT system described above was originally found in a yeast two-hybrid screen and suggests that this approach is useful to delineate specific interactions between *M. tuberculosis* and the host (34). The recent creation of an *M. tuberculosis*-host protein-protein interaction network to identify *M. tuberculosis* immune evasion and suppression strategies, such as the LpqN-CBL interaction, is a promising step forward in characterizing the *M. tuberculosis*-host interface (108).

Altogether, the genetic toolbox to understand how *M. tuberculosis* directly contributes to immune evasion is now well developed and will inform our understanding of how to overcome *M. tuberculosis* virulence in the future.

Employing host genetic approaches to bypass *M. tuberculosis*-mediated immune evasion. In addition to understanding how *M. tuberculosis* drives immune evasion, it is important to clarify how host pathways that are targeted by *M. tuberculosis* become ineffective and devise strategies to overcome *M. tuberculosis*-mediated evasion. This requires the capability to modify host cells and define what host pathways are necessary and sufficient for immune evasion during *M. tuberculosis* infection. Much like bacterial genetics, global approaches to modify the host or integrate diversity into host models have exploded over the last decade (109). The application of CRISPR-Cas9 technologies to modulate gene expression in host cells is an exciting future area of research for the field of immune evasion. These approaches can be categorized into loss of function or gain of function. Loss-of-function CRISPR systems generally rely on catalytically active Cas9, or other editing enzymes, that result in double-strand breaks when targeted to the genome by a sequence-specific single guide RNA (sgRNA) (110, 111). By pooling thousands of sgRNAs, knockout libraries targeting all coding genes can be assembled and probed for immunological phenotypes during *M. tuberculosis* infection that are easily deconvoluted through deep sequencing using similar approaches to Tn-seq (112). The loss-of-function CRISPR approach will be essential to pin down the underlying host pathways that are targeted in APCs by *M. tuberculosis* to drive distinct immune evasion mechanisms observed during infection. Since many of the changes induced by *M. tuberculosis* alter the surface expression of MHC molecules and costimulatory molecules, it is straightforward to isolate distinct populations with differential expression of each immune molecule on the cell surface. By comparing the distribution of sgRNAs in a genome-wide library between wild-type *M. tuberculosis* and an *M. tuberculosis* deletion or overexpression mutant in immune evasion genes such as EsxH, the host immune pathways that result in reduced MHC-II function can be directly identified.

The loss-of-function CRISPR approaches will be key to understanding how *M. tuberculosis* evasion mechanisms target the APC to prevent effective T cell detection. However, they are less likely to identify host mechanisms that overcome *M. tuberculosis*-mediated inhibition and result in improved T cell detection. In contrast, gain-of-function genetic approaches that induce the expression of target host genes have the potential to identify critical host pathways that can overcome *M. tuberculosis* evasion tactics. Gain-of-function approaches using CRISPR allow the targeted induction of host genes driven by a catalytically dead Cas9 linked to transcriptional activators (113). Two such systems, the synergistic activator mediator (SAM) and the Calabrese system, robustly induce genes in an sgRNA-dependent manner independently of their normal expression patterns (110, 113). The ability to fine-tune gene expression broadly at a genome-wide level opens many experimental opportunities with regard to *M. tuberculosis*-mediated immune inhibition. As discussed above, $CD8^+$ T cells are unable to detect *M. tuberculosis*-infected macrophages, resulting in their inability to carry out their effector functions effectively. By infecting a genome-wide gain-of-function library of APCs with *M. tuberculosis*, it might be possible to identify host pathways that allow the effective detection of *M. tuberculosis* by $CD8^+$ T cells. These pathways, if effectively targeted, would be capable of overcoming the immune evasion tactics employed by *M.*

tuberculosis and could then be tested for improved disease control *in vivo*. We strongly believe gain-of-function genetic approaches will be key assets to identify new host targets that are capable of overcoming *M. tuberculosis* virulence.

Beyond experimental genetic variation induced with CRISPR, another compelling method to investigate *M. tuberculosis* immune evasion is leveraging the inherent diversity within the mouse population. Resources such as the Collaborative Cross (CC) and Diversity Outbred (DO) collections introduce genetic variability into the mouse populations that can then be examined directly during *M. tuberculosis* infection (reviewed in reference 114). By modeling the genetic diversity seen in a human population, insights into how *M. tuberculosis* evades immune detection and clearance in distinct environments can now be understood. These mouse resources are the product of eight founder parental strains that were intercrossed sequentially to introduce genomic loci from all eight founders in each derived mouse strain (115). A subset of these diverse strains was then interbred to homozygosity resulting in the CC panel of recombinant inbred mice. The CC panel models more diversity that is reflective of the human population with the advantage of recombinant lines that can be tested repeatedly and examined for the effectiveness of interventions. The CC panel has already been used to model the variability of BCG vaccination responses in mice, suggesting that the host genetic background plays a significant role in the efficacy of vaccine responses (116). It is not difficult to imagine that a subset of immune evasion strategies employed by *M. tuberculosis* may only be observable in hosts with a particular genetic background. This would be critical to understand if we are to develop immunizations that are broadly protective in a range of human hosts. The DO collection is similar to the CC panel, except each mouse is an outbred offspring of the intercrossed parental strains (117). While this allows modeling of more genetic variation, it comes with the caveat that each mouse is genetically unique, making follow-up studies challenging. Even so, the DO collection is already being used effectively to understand traits that drive *M. tuberculosis* disease progression in the mouse model (118, 119). We envision these resources as being central to fully delineating how *M. tuberculosis* can evade distinct aspects of the host response and in testing new approaches to overcome *M. tuberculosis* virulence in a diverse host population.

OUTLOOK

Our knowledge of *M. tuberculosis*-driven immune evasion has expanded rapidly over the last decade. Distinct mechanisms that *M. tuberculosis* employs to avoid robust activation and detection by the adaptive immune response are now being explored in great detail. These studies have uncovered a range of important immune pathways that are manipulated by *M. tuberculosis* to avoid robust T cell responses by impeding their activation and effector function. Even with this progress, there are still many unknowns of how *M. tuberculosis* evades protective host responses. In the next decade, the challenge will be to use these findings to devise immunization approaches that activate protective responses even in the face of *M. tuberculosis* evasion tactics. There are already promising new vaccine candidates in the pipeline that show encouraging results (3). However, preliminary evidence suggests that taking *M. tuberculosis* virulence traits into account may improve disease outcomes and should be considered as new vaccine formulations move through clinical trials. Some approaches described here may be useful to develop strategies that bypass immune evasion. Overall, it will be critical to ensure that new vaccines broadly protect the population while overcoming *M. tuberculosis* immune evasion to result in the most effective protective response.

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