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Differentiation of Antigen-Specific T Cells with Limited Functional Capacity during *Mycobacterium tuberculosis* Infection

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Despite the generation of *Mycobacterium tuberculosis*-specific T cell immune responses during the course of infection, only 5 to 10% of exposed individuals develop active disease, while others develop a latent infection. This phenomenon suggests defective *M. tuberculosis*-specific immunity, which necessitates more careful characterization of *M. tuberculosis*-specific T cell responses. Here, we longitudinally analyzed the phenotypes and functions of *M. tuberculosis*-specific T cells. In contrast to the functional exhaustion of T cells observed after chronic infection, *M. tuberculosis*-specific $CD8^+$ T cells differentiated into either effector ($CD127^{lo}$ $CD62L^{lo}$) or effector memory ($CD127^{hi}$ $CD62L^{lo}$) cells, but not central memory cells ($CD127^{hi}$ $CD62L^{hi}$), with low programmed death 1 (PD-1) expression, even in the presence of high levels of bacteria. Additionally, *M. tuberculosis*-specific $CD8^+$ and $CD4^+$ T cells produced substantial levels of tumor necrosis factor alpha (TNF-α) and gamma interferon (IFN-γ), but not interleukin 2 (IL-2), upon *in vitro* restimulation. Among *M. tuberculosis*-specific $CD8^+$ T cells, $CD127^{hi}$ effector memory cells displayed slower ongoing turnover but greater survival potential. In addition, these cells produced more IFN-γ and TNF-α and displayed lytic activity upon antigen stimulation. However, the effector function of *M. tuberculosis*-specific $CD8^+$ $CD127^{hi}$ effector memory T cells was inferior to that of canonical $CD8^+$ $CD127^{hi}$ memory T cells generated after acute lymphocytic choriomeningitis virus infection. Collectively, our data demonstrate that *M. tuberculosis*-specific T cells can differentiate into memory T cells during the course of *M. tuberculosis* infection independent of the bacterial burden but with limited functionality. These results provide a framework for further understanding the mechanisms of *M. tuberculosis* infection that can be used to develop more effective vaccines.

Mycobacterium tuberculosis is one of the most prevalent human pathogens, infecting 9.3 million people each year and leading to 1.3 million deaths (1). Because *M. tuberculosis* is a global health concern, the immune response against mycobacterial infection and the defensive mechanisms capable of mediating vaccine-induced protection have been studied intensively. *M. tuberculosis* is an intracellular pathogen that infects phagocytic antigen-presenting cells (APCs) in the lung. In these cells, *M. tuberculosis* survives in modified phagosomes and uses multiple mechanisms to evade both innate and adaptive host immunity, including perturbation of phagosome maturation, resistance to innate microbicidal activity and cytokine-mediated host defenses, and inhibition of antigen presentation (2). It has been well documented that a major protective immune response against *M. tuberculosis* is cell-mediated immunity (3, 4). In particular, T helper 1-type $CD4^+$ T cells are pivotal for controlling the pathogenesis of *M. tuberculosis* (5–10). In contrast to $CD4^+$ T cells, the role of $CD8^+$ T cells in defense against the bacterium remains unclear. Many studies have shown that the kinetics of $CD8^+$ and $CD4^+$ T cell responses in the lung are identical and that similar changes in activation/memory phenotypes occur in pulmonary $CD8^+$ and $CD4^+$ T cells (11). β_2-Microglobulin- or TAP1-deficient mice (12–14) and $CD8^+$ T cell-depleted mice (10, 12) are more susceptible to *M. tuberculosis* infection than wild-type mice, indicating an essential role for $CD8^+$ T cells in clearing the pathogen. In contrast, other studies have shown that $CD8^+$ T cell depletion does not affect the bacterial load in the lungs of mice suffering from an acute *M. tuberculosis* infection (15). Therefore, the roles of $CD4^+$ and $CD8^+$ T cells in controlling *M. tuberculosis* infection are still being elucidated.

Memory T cells are important for mediating more effective immune responses to pathogens. During most infections, 90 to 95% of antigen-specific $CD8^+$ T cells that are generated by clonal expansion die after antigen clearance, and the surviving cells become the long-lived memory T cell population (16, 17). However, the surviving cells are not simply preformed memory $CD8^+$ T cells constituting a minor population within the effector pool. These effector populations are identified by their expression of interleukin 7 receptor alpha (IL-7Rα [CD127]) (18) and L-selectin (CD62L) (19); $CD127^{hi}$ $CD62L^{lo}$ subsets are classified as effector memory cells, and $CD127^{hi}$ $CD62L^{hi}$ subsets are classified as central memory cells. Studies have shown that $CD127^{hi}$ $CD62L^{lo}$ effector memory cells are more efficient producers of gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α) than $CD127^{hi}$ $CD62L^{hi}$ central memory cells, whereas interleukin 2 (IL-2) production is a property of central memory cells (19, 20). The development of memory T cells correlates inversely with repeated encounters with antigen (20, 21). Acute infections with viruses, such as influenza and vaccinia viruses, induce the differentiation of functional memory T cells in the absence of antigens

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(22–24). In contrast, persisting viruses cause latent infection with periodic reactivation, such as with Epstein-Barr virus (25, 26), inducing functional T cells; smoldering infection with pockets of ongoing viral replication, inducing T cells with certain impaired effector functions, such as with cytomegalovirus (27–29); or chronic infection with viremia, inducing dysfunctional or exhausted T cells, as reported for a lymphocytic choriomeningitis virus (LCMV) model (30–32), human immunodeficiency virus (HIV) (33, 34), and hepatitis C virus (HCV) (35, 36).

Although most *M. tuberculosis* infections are asymptomatic and latent, approximately 1 in 10 latent infections eventually progresses to active disease. However, validated markers that indicate tuberculosis (TB) onset and correlate with protection against active disease have yet to be identified. In addition, the functional and phenotypic changes in *M. tuberculosis*-specific T cell immune responses in the lung during the course of infection have not been examined systematically. Therefore, in this study, we investigated longitudinally the differentiation and function of *M. tuberculosis*-specific T cells generated during *M. tuberculosis* infection. Our data provide fundamental insight that can be applied to the development of more effective vaccines against the bacterium.

MATERIALS AND METHODS

Mice. Specific-pathogen-free female C57BL/6 (B6) mice 5 to 6 weeks of age were purchased from Japan SLC, Inc., and maintained under barrier conditions in a biosafety level 3 (BSL-3) biohazard animal room at the Medical Research Center of Yonsei University College of Medicine. All experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the College of Medicine, Yonsei University, and performed in accordance with the IACUC guidelines for the ethical use of animals.

Infection and counts. Mice were exposed to a predetermined dose of *M. tuberculosis* Erdman for 60 min in the inhalation chamber of an airborne-infection apparatus (Glas-Col, Terre Haute, IN, USA). The bacteria were counted 1 day after exposure, and approximately 200 to 300 viable *M. tuberculosis* bacteria were found to have been delivered to the lung. To enumerate the viable bacteria, the mice were euthanized with CO_2. The lung and spleen were homogenized in 0.04% Tween 80 in phosphate-buffered saline (PBS), and the tissue homogenates were serially diluted in PBS and plated in duplicate onto Middlebrook 7H11 agar (Difco). The colonies were counted after 3 to 4 weeks of incubation at 37°C, and the results are presented as the mean $\log_{10}$ CFU per organ. For viral infections, mice were infected intraperitoneally (i.p.) with 2×10^5 PFU of LCMV Armstrong (Arm) 53b, a triple-plaque-purified isolate of LCMV ARM CA1371 (37), which was obtained from Rafi Ahmed (Emory Vaccine Center, Atlanta, GA, USA).

Histopathology. For histopathological examination, the lungs were excised from all animals, stored in 10% formalin, and then embedded in paraffin at different times. From the paraffin-embedded tissue blocks, 2- to 3-μm sections were prepared and stained with hematoxylin and eosin (H&E). The H&E-stained lung sections were photographed using a microscope (Olympus Co.), and the level of inflammation in the lung was analyzed using the Image J program (National Institutes of Health, Bethesda, MD, USA).

Cell isolation. Lymphocytes from the lung and spleen were isolated as described previously (38). Briefly, each lung was perfused with ice-cold PBS before collection for lymphocyte isolation, chopped into small pieces, and incubated in RPMI supplemented with 1.3 mM EDTA at 37°C on a shaking incubator for 30 min. After the incubation, the small fragments were incubated again in complete medium solution containing 0.1% collagenase type II (Worthington Biochemical, Lakewood, NJ, USA) for 1 h. The lung lymphocytes were isolated by density gradient centrifugation by underlaying with 1× Percoll solution (Sigma-Aldrich, St. Louis, MO, USA).

In vitro stimulation assay. For $CD8^+$ T cell stimulations, 1.5×10^6 cells were incubated with ESAT-6_{17-25} (AIQGNVTSI), $Mtb32_{93-102}$ (GAP INSATAM), $TB10.4_{3-11}$ (QIMYNYPAM), $Ag85A_{2-11}$ (SRPGLPVEYL), and $Pst3S_{285-293}$ (ISGVGNDLV) peptides at 0.2 μg/ml for 6 h in the presence of brefeldin A and monensin.

For $CD4^+$ T cell stimulations, the cells were incubated with a mixture of ESAT-6, Mtb32, TB10.4, Ag85A, and PstS3 peptides (1 μg/ml of each peptide). A total of 10, 33, 11, 29, and 33 peptides spanning the mature forms of ESAT-6, Mtb32, TB10.4, Ag85A, and PstS3, respectively, of *M. tuberculosis* were synthesized as 17- to 20-mer peptides that overlap by 10 amino acids.

For LCMV-specific T cell stimulation, lymphocytes were stimulated with 0.2 μg/ml of LCMV GP_{33-41} peptide to induce a $CD8^+$ T cell response and with 1.0 μg/ml of GP_{66-80} peptide to induce a $CD4^+$ T cell response. All peptides were synthesized by GenScript.

Antibodies and flow cytometry. To detect the expression of surface molecules on T cells, 1.5×10^6 cells were stained with anti-CD4 (RM4-5), anti-CD8 (53-6.7), anti-CD44 (IM7), anti-CD62 L (MEL-14), anti-PD-1 (RMP1-30), and anti-CD127 (A7R34) antibodies. To measure the proliferation capability and apoptotic signal *ex vivo*, cells were stained with antibodies against Ki-67 (B56) and Bcl-2, respectively. D^bMtb32_{93-102}, $K^bTB10.4_{3-11}$, and D^bGP33_{33-41} monomers conjugated to streptavidin-allophycocyanin (Invitrogen, Life Technologies, Carlsbad, CA, USA) were used to detect *M. tuberculosis*- and LCMV-specific $CD8^+$ T cells. In the case of $K^bTB10.4_{3-11}$ staining, FASER-allophycocyanin activator (Miltenyi Biotec, Germany) was used to enhance the signal of $TB10.4^+$ cells. For intracellular staining, antibodies against CD107a/b, TNF-α (MP6XT22), IL-2 (JES6-5H4), and IFN-γ (XMG1.2) were used in combination with anti-CD8 and anti-CD4 antibodies. The antibodies were purchased from eBioscience (San Diego, CA, USA), Biolegend (San Diego, CA, USA), and BD Biosciences (San Jose, CA, USA). All samples were acquired on FACSCalibur and FACSCantoII flow cytometers (BD Biosciences) and analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

Statistical analysis. Statistical significance between groups was determined by the two-tailed unpaired Student's *t* test using Prism software version 5.02 (GraphPad, La Jolla, CA, USA).

RESULTS

Differentiation of *M. tuberculosis*-specific $CD8^+$ T cells into memory cells during *M. tuberculosis* infection. To evaluate the host immune response to *M. tuberculosis* infection, we infected mice via aerosol exposure and then examined the numbers of viable bacilli over time. At 4 weeks following infection with 200 to 300 CFU of the virulent Erdman strain of *M. tuberculosis*, the bacterial burden in the lungs and spleen peaked. This level was maintained even in the late phase of infection (Fig. 1A). Histopathological changes in the lung tissues were observed from day 15 postinfection and rapidly increased from day 30 postinfection (see Fig. S1 in the supplemental material).

We next analyzed the immune response of *M. tuberculosis*-specific $CD8^+$ T cells longitudinally by tetramer staining for D^b-$Mtb32_{93-102}$- and $K^bTB10.4_{3-11}$-specific $CD8^+$ T cells. The frequency of *M. tuberculosis*-specific $CD8^+$ T cells increased progressively and appeared to be maintained for 5 to 9 weeks postinfection (Fig. 1B and C). All tetramer-positive $CD8^+$ T cells expressed CD44, indicating that the cells were activated by T cell receptor (TCR) stimulation. The frequency of *M. tuberculosis*-specific $CD8^+$ T cells in the spleen showed a similar pattern of kinetics, even though the frequency was much lower in the spleen than in the lung (see Fig. S2 in the supplemental material).

To more carefully investigate *M. tuberculosis*-specific $CD8^+$ T

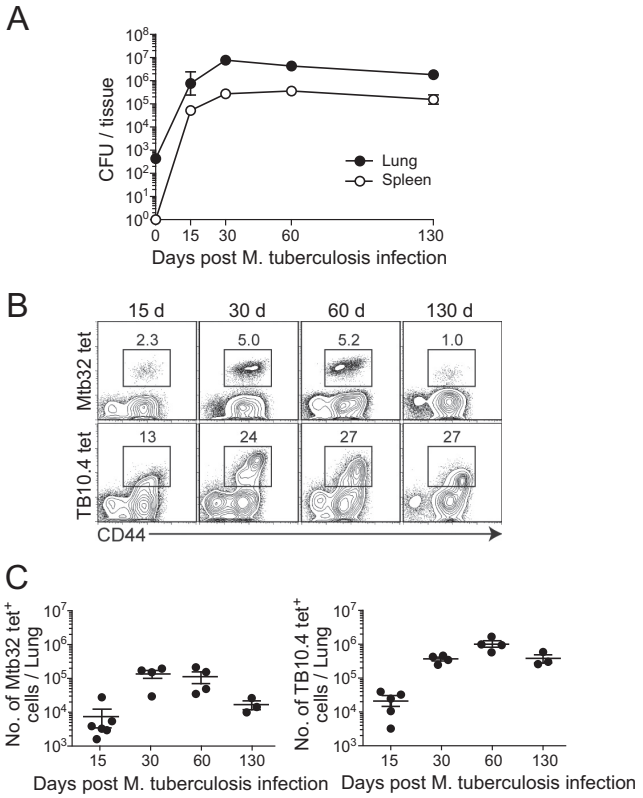

FIG 1 Kinetics of *M. tuberculosis*-specific CD8$^+$ T cells in the lung during *M. tuberculosis* infection. Lung lymphocytes were isolated at the indicated time points after initial infection with *M. tuberculosis*. (A) Kinetics of *M. tuberculosis* Erdman growth in the lungs of C57BL/6 mice infected aerogenically with 300 CFU bacilli. The bacterial burden in the lungs was assessed at the indicated times. (B) $D^bMtb32_{93\text{-}102}$- and $K^bTB10.4_{3\text{-}11}$-specific CD8$^+$ T cells were identified by tetramer staining. The numbers in the plots represent the percentages of tetramer-positive cells among CD8$^+$ T cells. d, day(s). (C) The absolute numbers of D^b-$Mtb32_{93\text{-}102}$- and $K^bTB10.4_{4\text{-}11}$-specific CD8$^+$ T cells in the lung were calculated. The graphs show the means ± standard errors of the mean (SEM). The data are representative of at least two independent experiments ($n = 3$ or 4 at each time point).

cells at the infection site, the phenotypes of both $D^bMtb32_{93\text{-}102}$- and $K^bTB10.4_{3\text{-}11}$-specific CD8$^+$ T cells in the lung were characterized by staining with canonical markers commonly used to discriminate between effector and memory T cells (Fig. 2A and B; see Fig. S2A and B in the supplemental material). *M. tuberculosis*-specific CD8$^+$ T cells rapidly downregulated both CD127 and CD62L expression by day 15 postinfection. Interestingly, the expression of CD127 on *M. tuberculosis*-specific CD8$^+$ T cells seemed to be restored progressively, unlike CD62L expression. These results indicate that a substantial population of *M. tuberculosis*-specific CD8$^+$ T cells at the infection site differentiated from an effector (CD127^{lo} CD62L^{lo}) to an effector memory (CD127^{hi} CD62L^{lo}) phenotype over the course of infection. Meanwhile, the expression of programmed death 1 (PD-1) on *M. tuberculosis*-specific CD8$^+$ T cells was induced temporarily by day 15 postinfection, as its expression was downregulated thereafter (Fig. 2C and D; see Fig. S3C in the supplemental material). Taken together, these results demonstrate that *M. tuberculosis*-specific CD8$^+$ T cells differentiate into an effector memory, but not central memory, phenotype even after the late phase of *M. tuberculosis* infection.

Functional capacity of *M. tuberculosis*-specific T cells during *M. tuberculosis* infection. Although large numbers of *M. tuberculosis*-specific CD8$^+$ T cells were generated postinfection, the bacterial growth was not controlled. Therefore, we assessed whether *M. tuberculosis*-specific CD8$^+$ T cells are capable of producing effector cytokines at the single-cell level. To address this, we performed a kinetic analysis of CD8$^+$ T cell function. Interestingly, upon *in vitro* restimulation with the $Mtb32_{93\text{-}102}$, $TB10.4_{3\text{-}11}$, and $ESAT\text{-}6_{17\text{-}25}$ peptides, we observed a substantial increase in TNF-α and IFN-γ coproducers among the IFN-γ-producing CD8$^+$ T cell population at day 30 postinfection compared with the number of coproducers at day 15 postinfection (Fig. 3A and B). In the case of $Mtb32_{93\text{-}102}$ stimulation, the percentage of dual-cytokine producers among IFN-γ-producing CD8$^+$ T cells gradually decreased after day 30. In contrast, TB10.4- and ESAT-6-specific CD8$^+$ T cells continued to produce both TNF-α and IFN-γ until a late time point.

Next, we assessed the functionality of CD4$^+$ and CD8$^+$ T cells specific to various *M. tuberculosis* antigens in the late phase of infection. We investigated the capacity of *M. tuberculosis*-specific CD8$^+$ and CD4$^+$ T cells to produce IL-2 and IFN-γ at day 100 postinfection. Interestingly, a very limited population of IFN-γ-producing CD8$^+$ T cells produced IL-2 upon stimulation with various *M. tuberculosis* peptides (Fig. 3C, left). The percentage of IFN-γ-producing CD8$^+$ T cells varied according to the specific antigenic peptide (Fig. 3D), but the percentage of IL-2 and IFN-γ producers among them was below 15% for various *M. tuberculosis* antigen-specific CD8$^+$ T cells (Fig. 3E). This phenomenon was also observed in the spleen (see Fig. S4 in the supplemental material). To analyze the functionality of CD4$^+$ T cells specific to *M. tuberculosis*, the cells were stimulated with a pool of the various *M. tuberculosis* antigens (Fig. 3C, right). Whereas *M. tuberculosis*-specific CD8$^+$ T cells responded to the different antigens, CD4$^+$ T cells primarily responded to ESAT-6 and produced a substantial level of IFN-γ (Fig. 3D). These results suggest that ESAT-6 contains the dominant CD4$^+$-specific epitope. Similar to *M. tuberculosis*-specific CD8$^+$ T cells, ESAT-6-specific CD4$^+$ T cells could produce only a limited amount of IL-2 (Fig. 3E). These data demonstrate that *M. tuberculosis*-specific CD8$^+$ and CD4$^+$ T cells can produce both IFN-γ and TNF-α upon antigen stimulation but are very limited in their capacity to produce IL-2.

Different functional capacities of effector and effector memory subsets during *M. tuberculosis* infection. We found that *M. tuberculosis*-specific CD8$^+$ T cells did not differentiate into central memory cells. Instead, they maintained an effector or effector memory phenotype, namely, CD127^{lo} CD62L^{lo} or CD127^{hi} CD62L^{lo}, respectively. These cells produced IFN-γ and TNF-α abundantly but generated only limited quantities of IL-2. To compare the functional properties of these two CD8$^+$ T cell populations during *M. tuberculosis* infection, Mtb32-specific CD8$^+$ T cells isolated from *M. tuberculosis*-infected mice at day 60 postinfection were examined for the expression of CD127, along with various functional markers (e.g., Bcl-2, Ki-67, and CD107a/b) and cytokine production. As a control representing a typical memory CD8$^+$ T cell, we used LCMV glycoprotein (GP) 33-41 epitope-specific CD8$^+$ T cells isolated from LCMV Arm-infected mice at day 60 postinfection, which is when LCMV-specific CD8$^+$ T cells differentiate into effector or central memory T cells. First, we examined the ongoing turnover status of *M. tuberculosis*-specific CD8$^+$ T cells by measuring *ex vivo* the expression of Ki-67 (a

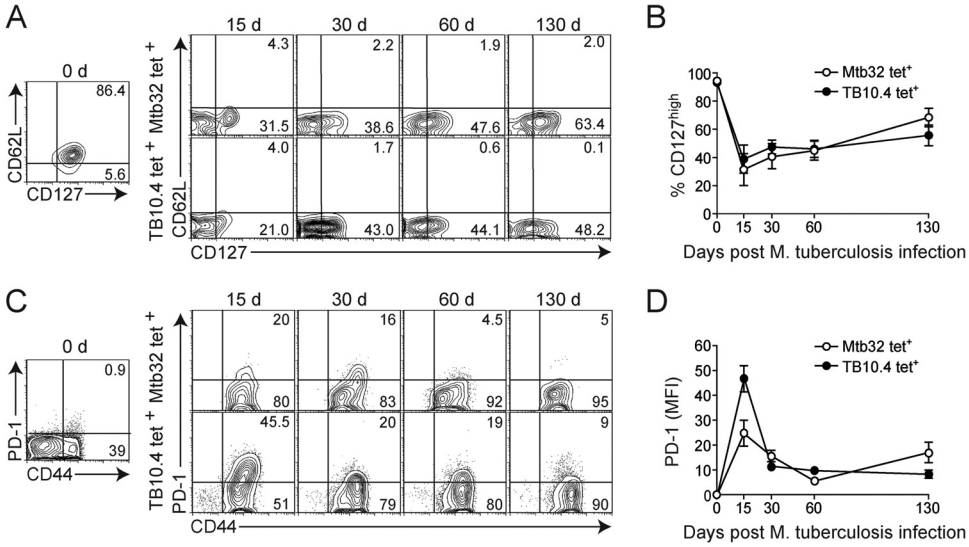

FIG 2 Phenotypic changes in *M. tuberculosis*-specific CD8$^+$ T cells in the lung during *M. tuberculosis* infection. Lung lymphocytes were isolated at the indicated time points after initial infection with *M. tuberculosis*. (A) After gating for CD8$^+$ T cells, the coexpression of CD127 and CD62L was analyzed for D^bMtb32_{93-102} (top) and $K^bTB10.4_{4-11}$ (bottom) tetramer-positive cells. The plot at day 0 represents naive CD8$^+$ T cells. The numbers in the plots indicate the percentages of CD127$^+$ CD62L$^+$ (central memory) and CD127$^+$ CD62L$^-$ (effector memory) cells. (B) Summary of the kinetic analysis in panel A for the percentages of CD127^{hi} tetramer-positive cells at the indicated time points after initial *M. tuberculosis* infection. (C) After gating for CD8$^+$ T cells, the coexpression of CD44 and PD-1 was analyzed for D^bMtb32_{93-102} (top) and $K^bTB10.4_{3-11}$ (bottom) tetramer-positive cells. The plot at day 0 represents naive CD8$^+$ T cells. (D) Summary of the mean fluorescence intensities (MFI) of PD-1 on the tetramer-positive CD8$^+$ T cells at the indicated time points after initial *M. tuberculosis* infection. The graphs show the means ± SEM. The data are representative of at least two independent experiments ($n = 3$ or 4 at each time point).

marker of recently proliferated cells). Among *M. tuberculosis*-specific CD8$^+$ T cells, CD127^{lo} effector T cells expressed a significantly higher level of Ki-67 than CD127^{hi} effector memory T cells (Fig. 4A). As expected, LCMV-specific memory T cells did not express Ki-67. Next, we examined the survival ability of these cells by measuring the expression level of Bcl-2, a marker of anti-apoptosis. The level of Bcl-2 in CD127^{hi} effector memory cells was significantly higher than that in CD127^{lo} effector cells (Fig. 4A).

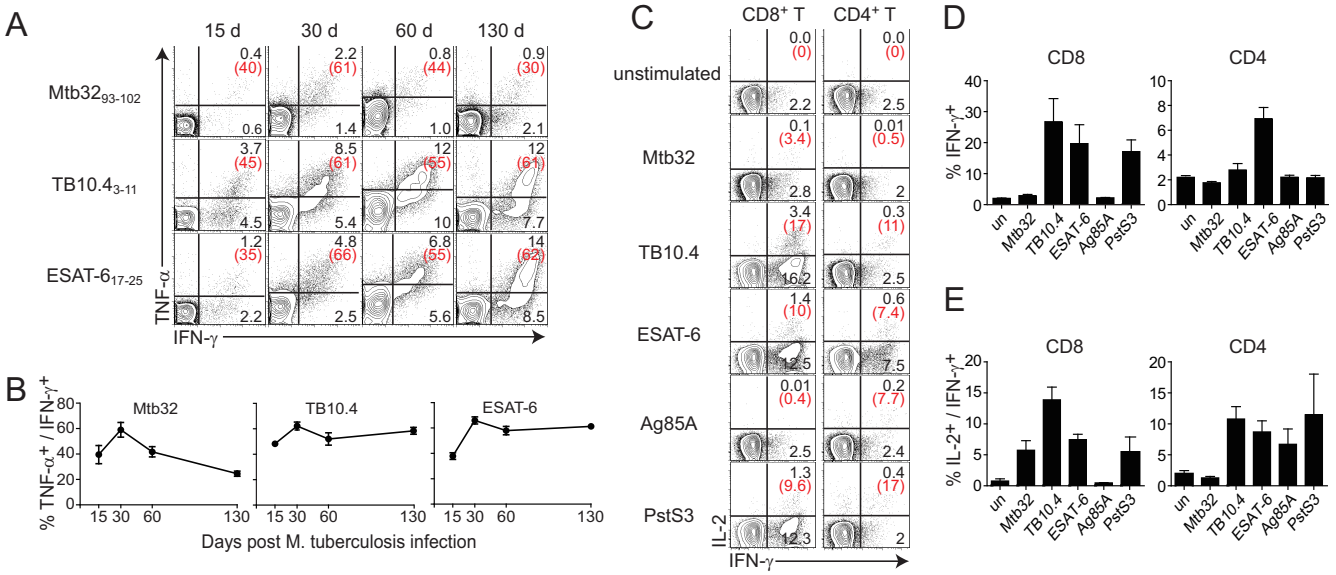

FIG 3 Functional changes in *M. tuberculosis*-specific CD8$^+$ T cells in the lung during *M. tuberculosis* infection. Lung lymphocytes were isolated at the indicated time points after initial infection with *M. tuberculosis* and then restimulated *in vitro* with $Mtb32_{93-102}$, $TB10.4_{3-11}$, or $ESAT6_{17-25}$ peptide. (A) Representative data showing the percentages of cytokine-producing cells among the CD8$^+$ T cells. The numbers in red depict the percentages of IFN-γ and TNF-α coproducers among IFN-γ-producing cells. (B) Summary showing the frequencies of TNF-α-producing cells among the IFN-γ$^+$ CD8$^+$ T cells. (C) Representative data showing the percentages of IL-2-producing cells among the IFN-γ$^+$ CD8$^+$ or IFN-γ$^+$ CD4$^+$ T cells. At day 100 postinfection, CD8$^+$ T cells were stimulated with Mtb32, TB10.4, ESAT-6, Ag85A, or PstS3 peptide, while CD4$^+$ T cells were stimulated with a pool containing all the peptides (see Materials and Methods). The numbers in red indicate the percentages of IFN-γ and IL-2 coproducers among the IFN-γ-producing cells. (D) Summary showing the total percentages of IFN-γ-producing cells according to peptide stimulation. (E) Summary of the percentages of IL-2-coproducing cells among the total IFN-γ$^+$ population according to peptide stimulation. The graphs show the means ± SEM. The data are representative of at least two independent experiments ($n = 3$ or 4 at each time point).

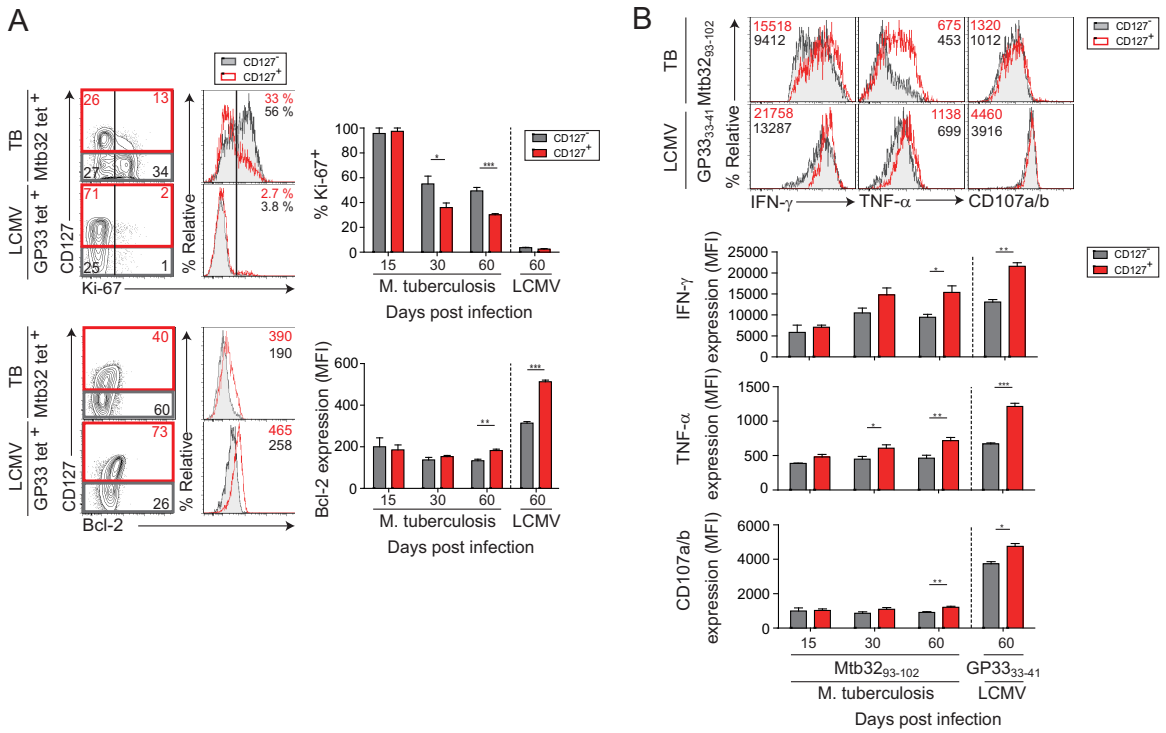

FIG 4 Different functional properties of effector and effector memory T cell subsets during *M. tuberculosis* infection. (A) Lung lymphocytes were isolated at day 60 after *M. tuberculosis* and LCMV Arm infection. $D^bMtb32_{93\text{-}102}$- and $D^bGP33_{33\text{-}44}$-specific $CD8^+$ T cells were identified by tetramer staining. Tetramer-positive cells were divided into effector (red boxes) and effector memory (gray boxes) populations based on CD127 expression. The levels of Ki-67 (top) and Bcl-2 (bottom) in each population are shown as histograms. The percentages of Ki-67^+ cells in $CD127^{hi}$ (red) and $CD127^{lo}$ (gray) cells are depicted at the top and summarized on the right. The MFI levels of Bcl-2 on $CD127^{hi}$ (red) and $CD127^{lo}$ (gray) populations are shown at the top. (B) Lung lymphocytes were restimulated *in vitro* with $Mtb32_{93\text{-}10}$ and $GP33_{33\text{-}41}$. After gating for $CD8^+$ T cells, IFN-γ^+ cells were divided into effector (gray bar) and effector memory (red bar) populations based on CD127 expression. The histogram represents the levels of IFN-γ, TNF-α, and CD107a/b expressed in IFN-γ^+ effector (gray lines) and effector memory (red lines) cells. The numbers in the plots represent the MFI (black for effector cells and red for effector memory cells), which is also summarized in bar graphs (below) as the means and SEM. The *P* values, determined by the Student *t* test, are indicated as follows: *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ ($n = 4$ per group).

LCMV-specific memory T cells, however, showed the highest level of Bcl-2 expression. Overall, the $CD127^{hi}$ effector memory population in *M. tuberculosis*-specific $CD8^+$ T cells displayed a lower ongoing turnover rate that was more sustainable than that of the $CD127^{lo}$ effector population.

We also compared the abilities of *M. tuberculosis*-specific $CD127^{hi}$ and $CD127^{lo}$ $CD8^+$ T cells to produce IFN-γ and TNF-α, as well as their capacities to acquire lytic activity upon antigen stimulation (Fig. 4B). Upon Mtb32 peptide stimulation, $CD127^{hi}$ effector memory $CD8^+$ T cells produced more IFN-γ and TNF-α. Furthermore, these cells showed a higher level of degranulation than $CD127^{lo}$ effector $CD8^+$ T cells. $CD8^+$ T cells specific for TB10.4-specific $CD127^{hi}$ and $CD127^{lo}$ $CD8^+$ T cells also showed similar response patterns (see Fig. S5 in the supplemental material). However, the effector function of *M. tuberculosis*-specific $CD8^+$ $CD127^{hi}$ effector memory T cells was inferior to that of LCMV-specific $CD8^+$ $CD127^{hi}$ memory T cells, suggesting the functional limitation of *M. tuberculosis*-specific memory $CD8^+$ T cells compared to typical memory $CD8^+$ T cells.

DISCUSSION

In this study, we characterized the longitudinal T cell immune response to *M. tuberculosis* infection. Although many studies have investigated this response, there is little information about its overall kinetics and the functional properties of *M. tuberculosis*-specific $CD8^+$ T cell populations during the course of infection. Here, we found that *M. tuberculosis*-specific $CD8^+$ T cells were capable of differentiating into a canonical effector memory state with a $CD127^{hi}$ $CD62L^{lo}$ $PD\text{-}1^{lo}$ phenotype. It has been generally known that effector memory $CD8^+$ T cells gradually convert into central memory $CD8^+$ T cells. However, this differentiation did not occur in the spleens or lungs of *M. tuberculosis*-infected mice even during the late phase of infection, which limited the cells' capacity to produce IL-2. In addition, when we compared the functional properties of the $CD127^{hi}$ $CD62L^{lo}$ effector memory population with those of the $CD127^{lo}$ $CD62L^{lo}$ effector population within *M. tuberculosis*-specific $CD8^+$ T cells, effector memory cells displayed slower ongoing turnover but greater survival potential and cytokine production than effector cells. In summary, our study provides an overview of *M. tuberculosis*-specific $CD8^+$ T cell immune responses at the site of *M. tuberculosis* infection over time.

Because T helper 1-type $CD4^+$ T cells are crucial for protection against *M. tuberculosis*, the frequency of IFN-γ-producing $CD4^+$ T cells has been widely used as an indicator of this response. However, gauging the magnitude of T cell responses by a single parameter, such as IFN-γ production, may not reflect *M. tuberculosis*-specific immune responses and defense against the bacterium.

Recently, it was found that the polyfunctional profiles (IFN-γ^+ IL-2^+ TNF-α^+) of antigen-specific T cell responses are related to disease activity during *M. tuberculosis* infection (39, 40). Interestingly, the frequency of $CD4^+$ T cells producing TNF-α, but not IFN-γ and IL-2, was reported to be the strongest predictor of a diagnosis of active TB or latent infection (41). In our report, we found a very limited population of cells with an IFN-γ^+ IL-2^+ profile upon stimulation with various *M. tuberculosis* peptides, suggesting that this limitation only partially contributes to a persistent infection. This incomplete functional capacity of *M. tuberculosis*-specific T cells seems to be related to the cells' effector memory phenotype ($CD127^{hi}$ $CD62L^{lo}$), because the cells do not efficiently produce IL-2 compared with central memory T cells (19, 20).

Among the various inhibitory receptors, PD-1 is a phenotypic hallmark of functionally impaired T cells generated during chronic pathogen infection (38, 42–45). The interaction between PD-1 and its ligand, PD-L1, has been demonstrated to inhibit TCR signaling, causing the deterioration of T cell immune responses to human viruses, including HBV, HCV, and HIV (46–50). Interestingly, despite multiple studies of viral infection that demonstrate PD-1 as a reliable marker of exhaustion, previous studies reported the susceptibility phenotype of *PD-1* knockout (KO) mice during *M. tuberculosis* infection (51, 52). Reiley et al. showed that PD-1-expressing T cells proliferate and are functionally active rather than exhausted, thus representing a precursor population of antigen-specific T cells (53). Consistent with these reports, we observed that PD-1-expressing *M. tuberculosis*-specific $CD8^+$ T cells produce the effector cytokines IFN-γ and TNF-α and do not appear to be exhausted (Fig. 3A). Therefore, our results indicate that PD-1 is not a marker of chronically exhausted T cells during *M. tuberculosis* infection.

Even though the level of PD-1 has been reported to correlate with antigen presence (54), our study showed that PD-1 expression on *M. tuberculosis*-specific $CD8^+$ T cells was temporary, reaching a peak at day 15 postinfection and then decreasing progressively after day 30, when the bacterial burden had peaked. This observation could be caused by differences in the anatomical localization of *M. tuberculosis*-specific $CD8^+$ T cells and their potential responder cells, APCs, which present *M. tuberculosis* antigens. During the course of aerosol infection with *M. tuberculosis*, a granuloma consisting of various immune cells, including APCs, forms. This hallmark of TB develops in the lung after 4 to 5 weeks of infection. In general, bacteria are most commonly present in the central area of the granuloma (55). In contrast to APCs harboring *M. tuberculosis* or other, related antigens, *M. tuberculosis*-specific T cells rarely enter the central region of the granuloma and instead localize to peripheral regions (55). Therefore, the progressive loss of PD-1 expression by *M. tuberculosis*-specific $CD8^+$ T cells may be caused by their reduced opportunity to interact with APCs presenting *M. tuberculosis* antigens due to differences in their localization. Collectively, we showed that PD-1 expression on antigen-specific T cells does not always correlate with the pathogen titer, suggesting that PD-1 is not a marker for the bacterial burden in the case of *M. tuberculosis* infection.

The generation of different subsets of *M. tuberculosis*-specific $CD8^+$ T cells, namely, $CD127^{hi}$ $CD62L^{lo}$ effector memory or $CD127^{lo}$ $CD62L^{lo}$ effector populations, during *M. tuberculosis* infection may also be affected by differences in localization within the lung and/or frequency of interaction with APCs. Some *M. tuberculosis*-specific $CD8^+$ T cells may have little opportunity to contact antigens and subsequently differentiate into effector memory T cells, whereas other *M. tuberculosis*-specific $CD8^+$ T cells may contact antigens more frequently and remain as effector T cells. This hypothesis is supported by our observation that $CD127^{hi}$ effector memory cells displayed a slower ongoing turnover status than $CD127^{lo}$ effector cells because rapidly proliferating $CD8^+$ T cells, which express Ki-67, are dependent on antigen presentation.

Although the current vaccine against TB, *Mycobacterium bovis* bacillus Calmette-Guérin, is effective in protecting children from the disease, it does not induce consistent protection against adult TB. Meanwhile, the precise immune mechanisms of protection remain incompletely understood. Therefore, we need to understand the basic immunology underlying *M. tuberculosis* infection and to develop a TB vaccine with better efficacy. In this study, we have characterized the functional effector memory state of $CD8^+$ and $CD4^+$ T cells during *M. tuberculosis* infection and demonstrated that these functional effector memory populations increased upon infection. However, effector T cells may be physically restricted from contacting APCs by the granuloma, thereby delaying their differentiation. Overall, the results from this study provide information about the longitudinal immune response that can be applied to the development of better vaccines.

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AUTHOR CORRECTION

Differentiation of Antigen-Specific T Cells with Limited Functional Capacity during *Mycobacterium tuberculosis* Infection

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