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Prolonged Antigen Presentation, APC-, and CD8⁺ T Cell Turnover during Mycobacterial Infection: Comparison with Listeria monocytogenes

Henk van Faassen, Renu Dudani, Lakshmi Krishnan, and Subash Sad

We expressed the CTL epitope of OVA (OVA\textsubscript{257–264}) in an acute (Listeria monocytogenes (LM)-OVA) and a chronic intracellular pathogen (Mycobacterium bovis (BCG)-OVA), to evaluate the kinetics of Ag presentation. LM-OVA proliferated rapidly in vivo, resulting in profound LM-OVA expansion within the first 24 h of infection, culminating in the generation of a potent CD8⁺ T cell response, which peaked on day 7 but underwent a rapid attrition subsequently. In contrast, BCG-OVA exhibited reduced growth in vivo, resulting in a delayed CD8⁺ T cell response that increased progressively with time. Relative to LM-OVA, BCG-OVA induced persistently increased numbers of apoptotic (annexin V⁺) CD8⁺ T cells. Ag presentation in vivo was evaluated by transferring Thy1.2⁺ carboxyfluorescein-labeled OT1 transgenic CD8⁺ T cells into infected Thy1.1⁺ congenic recipient mice. LM-OVA induced rapid Ag presentation that was profound in magnitude, with most of the transferred cells getting activated within 4 days and resulting in a massive accumulation of activated donor CD8⁺ T cells. In contrast, Ag presentation induced by BCG-OVA was delayed, weaker in magnitude, which peaked around the second week of infection and declined to a low level subsequently. Increasing the dose of BCG-OVA while enhancing the magnitude of Ag presentation did not change the kinetics. Furthermore, a higher dose of BCG-OVA also accelerated the attrition of OVA\textsubscript{257–264}⁺ specific CD8⁺ T cells. Relative to LM-OVA, the dendritic cells in BCG-OVA-infected mice were apoptotic for prolonged periods, suggesting that the rapid death of APCs may limit the magnitude of Ag presentation during chronic stages of mycobacterial infection. The Journal of Immunology, 2004, 172: 3491–3500.

When peptides from endogenously derived Ags (intracellular bacteria, viruses, or tumors) are presented on MHC class I molecules (1, 2), CD8⁺ T cells are stimulated. After differentiation, effector CD8⁺ T cells possess the unique ability to mediate specific cytotoxicity (by perforin and Fas-dependent pathways) toward infected cells and tumors (3–5). However, the vast majority (>95%) of Ag-specific T cells activated at the onset of the immune response die by apoptosis and only a small portion of those T cells survive (<5%) for extended periods (6, 7). These long-lived memory T cells possess the unique ability to respond rapidly and specifically to Ags (6–8). Although it has been suggested that (in the context of viral infections) the presence of Ag is not critical for the maintenance and survival of CD8⁺ T cell memory (9), Ag persistence is required for extravasation and induction of rapid recall effector functions (10–12).

Analyzing Ag presentation in vivo is challenging and the approaches used that are based on activation of CD8⁺ T cell hybridomas by APCs in vitro have largely been indirect (13–16). However, the ability of such APCs to prime naive T cells in vivo is unclear. Using more direct approaches wherein Ag presentation was evaluated in vivo with untouched APCs, it was reported that Ag presentation during viral infections occurs only within the first 24 h of infection (17–19). After phagocytosis, Ag presentation during acute viral infections ensues rapidly and the cells generated continue to divide uninterrupted for at least up to seven divisions (20). In contrast, the duration of Ag presentation during chronic infections is not clear.

Listeria monocytogenes (LM)³ and mycobacteria are the most widely studied intracellular bacteria (21). The growth of LM in primary infections is controlled during the first week of infection mainly by innate immunity (22–24). LM egress into the cytosol of the infected phagocytic cell (25) and induce an acute infection (<7 days) in an immunocompetent host, resulting in the development of a potent and protective CD8⁺ T cell response (22–24, 26). In contrast, mycobacteria reside within permeable phagosomes of APCs, allowing passage of proteins (Ags) across the phagosomal membrane for presentation via MHC class I molecules (27) and cause a chronic infection (>6 mo) in the host (21, 27, 28). The cellular immune responses induced during mycobacterial infections have been controversial with reports of potent immune activation vs immune suppression (29–39). We have previously reported that recombinant Mycobacterium bovis (BCG) expressing OVA induces the development of a potent and protective CD8⁺ T cell response (38). However, the nature of the T cell response generated, and the duration of Ag presentation against such a pathogen has remained elusive. Although it is assumed that mycobacteria may induce persistent Ag presentation, this has remained unresolved because direct assessment of Ag presentation in vivo is tedious.

In this report, we evaluate the initiation, duration, and the extent of Ag presentation during infection with the two recombinant intracellular bacteria, LM and BCG (both expressing the same model

³ Abbreviations used in this paper: LM, Listeria monocytogenes; BCG, Mycobacterium bovis; CD62L, CD62 ligand.
Ag, OVA). Using an adoptive transfer model wherein OVA 257-264-specific OT-1 TCR transgenic CD8+ T cells (expressing Thy1.2) are transferred into congenic B6.PL (expressing Thy1.1) recipients that are infected with recombinant LM and BCG (LM-OVA and BCG-OVA), we show that the kinetics and the duration of Ag presentation are pathogen dependent, and that the extent of Ag presentation is modulated during the various stages of mycobacterial infection.

Materials and Methods

Bacterial strains

BCG-OVA is a previously described recombinant strain (38) engineered with a partial sequence of the OVA gene (codons 230–359), downstream of the Ag 85B secretion signal (40), under the control of HSP60 promoter (29). Codons 230–359 of the OVA gene encode the SHINEFLK epitope (OVA 257-264) and its flanking sequences (41). Single colonies were used to inoculate liquid cultures, which were incubated at 7°C under constant shaking in 7H9 medium containing glycerol (0.2%), Tween 80 (0.05%), and ampinbioxo-a-sulfate supplement (ADAC, 10%; Difco, Detroit, MI). At mid-log phase (OD 600 = 1.0), bacteria were harvested and frozen at –80°C (in 20% glycerol). CFU were determined by plating serial dilutions in PBS with 0.025% Tween 80 (PBS-T) on a Middlebrook 7H10 solid medium (in 20% glycerol). CFU were determined by plating serial dilutions in PBS and stained in binding buffer with Annexin V FITC for 15 min at room temperature. Spots were revealed using di-amino benzidine.

Assessment of T cell responses

Enumeration of IFN-γ-secreting cells was done by ELISPOT assay (38). Briefly, spleen cells were incubated in anti-IFN-γ Ab coated ELISPOT plates, varying the number of spleen cells from immunized mice to achieve a final cell density of 5 × 10^6/well using feeder cells from unimmunized mice. The spots were counted visually.

T cell purifications and culture conditions

To purify CD8+ T cells by positive selection, 0.5–1 × 10^6 cells were pelleted and resuspended in 0.5–1 ml of R8-A. CELLlection Biotin Binder Dynabeads precoated as per manufacturer’s instructions (DyNa Biotech, Great Neck, NY), with biotin-conjugated rat anti-mouse CD8β mAb (53.5.8; BD Biosciences), were added to the resuspended cell pellet at a ratio of five beads per cell, and incubated for 15–20 min at 4°C in a rotating platform. CD8β T cells were separated by magnetic isolation. Dynabead detachment was done using the CELLection Biotin Binder Kit Releasing Buffer (DNAse; 188 U/10^6 Dynabeads) in a 37°C shaker for 15 min, followed by two to three rounds of washing/magnetic separation. This protocol resulted in >95% pure CD8+ T cells as determined by follow-up analysis with PE-conjugated rat anti-mouse CD8α (BD Biosciences). Analysis was performed using EPICS XL flow cytometer and EXPO software (Beckman Coulter).

In vivo Ag presentation

Spleen cells were obtained from donor OT-1 transgenic mice, and stained with CFSE (42). Briefly, spleen cell suspensions were prepared and RBC was removed by ammonium chloride treatment. Spleen cells were resuspended in PBS (20 × 10^7/ml) and an equal volume of CFSE (5 μM in PBS) was added. After 8 min at room temperature, an equal volume of PBS was added for quenching. After 1–2 min at 4°C, cells were washed with HBSS. CD8+ T cells were purified as described above and 5 × 10^4 purified CD8+ T cells were injected into recipient congenic B6.PL mice (Thy1.1+) via the lateral tail vein in 200 μl of HBSS. Recipient mice were preinjected with PBS, LM, LM-OVA, or BCG-OVA. At different time intervals, recipient mice received OT-1 CD8+ T cells. Four days after the transfer of donor OT-1 CD8+ T cells, spleens were removed from the recipient mice and spleen cells were evaluated for the presence of donor (Thy1.2+) CD8+ T cells and the reduction in CFSE intensity.

Flow cytometry

For evaluation of CD8+ T cell activation in normal mice, aliquots (1 × 10^7) were washed and incubated in 50 μl of RPMI 1640 plus 1% FBS with the following Abs on ice: anti-mouse CD62L-PE, anti-mouse CD44-FITC, and anti-mouse CD8-Cy. After 30 min, cells were washed and fixed in 1% formaldehyde in PBS and acquired on an EPICS XL flow cytometer. In vivo Ag presentation in vivo, aliquots (20 × 10^7) of spleen cells from recipient mice were incubated in 200 μl of RPMI 1640 plus 1% FBS with the following Abs on ice: anti-mouse Thy1.2-FITC, anti-mouse CD8-Cy. After 30 min on ice, cells were washed, fixed, and 100,000 CD8+ events were acquired. For evaluation of the fate and phenotype of donor OVA 257-264-specific CD8+ T cells in vivo, aliquots (10 × 10^6) of spleen cells were incubated in 200 μl of PBS plus 1% BSA (PBS-BSA) with anti-CD11b/32 at 4°C. Cells were then washed with PBS-BSA and incubated for 30 min with streptavidin-Cy5 and PE-H-2K<sup>OVA</sup> 257-264 tetramer at room temperature. Cells were washed with PBS and fixed in 0.5% formaldehyde and acquired on an EPICS XL Flow Cytometer. For evaluation of apoptotic OVA 257-264-specific CD8+ T cells, 10 × 10^6 spleen cells were washed in PBS and...
incubated (in 200 μl of binding buffer) at 4°C with anti-CD16/32 for 10 min followed by incubation with anti-CD86CyC and PE-H-2KbOVA257–264 at room temperature. After 30 min, 2.9 ml of binding buffer containing 10 μl of AnnexinV-FITC were added. After 15 min at room temperature, cells were washed and acquired on an EPICS XL Flow Cytometer.

Results
Doubling times of LM-OVA and BCG-OVA
C57BL/6 mice were infected with LM-OVA and BCG-OVA and the bacterial burden was evaluated at various time intervals. For LM-OVA, there was a strong increase in bacterial burden initially within the first 24 h, which was followed by a rapid reduction, and by day 5 no bacteria were detectable in the spleens (Fig. 1A). When fold-increase over a 24-h period was calculated (Fig. 1B), there was an ~100-fold increase in LM-OVA burden within the first 24 h of infection. In contrast to LM-OVA, BCG-OVA infection was slow and chronic which resulted in an ~13-fold increase in the presence or absence of kanamycin because the plasmid carrying the gene for OVA also expresses the kanamycin resistance gene (38), and any discordance in the number of colonies in the absence or presence of kanamycin would be indicative of antigenic loss variants of BCG-OVA. As is evident from Fig. 1C, for most of the time points tested, no significant differences were noted in the number of BCG-OVA colonies grown in the absence or presence of kanamycin. However, at day 70, only 50% of the colonies expressed kanamycin resistance suggesting that antigenic loss variants may develop in the long-term. When fold-increase in BCG-OVA burden over a 24-h period was calculated (Fig. 1D), BCG-OVA burden increased at a remarkably constant rate during the first 2 wk of infection, doubling approximately every 3–4 days. Subsequently, the BCG-OVA burden declined gradually.

Based on the measurement of bacterial burden in vivo, it is difficult to determine the actual doubling time of the bacteria because some bacteria could be proliferating and others could be dying simultaneously. Therefore, we evaluated the doubling time of the two bacteria in vitro. Doubling of bacteria was enumerated by determining the average time interval taken by the bacteria to cause doubling of the OD as well as CFU. By this analysis, the average doubling time of LM-OVA was 46.6 min (0.77 h), whereas BCG-OVA doubled every 29.5 h (Fig. 2).

Effect of cell generation during LM-OVA and BCG-OVA infections
We evaluated the activation of CD8+ T cells during LM-OVA and BCG-OVA infections kinetically. We chose to determine the expression of CD62L and CD44 because CD44 is expressed on memory CD8+ T cells (43) irrespective of their activation status (6, 7, 44), and down-regulation of CD62L is an indicator of recent activation (7, 44, 45). Additionally, various investigators have recently shown that the differential expression of CD62L vs CD44 on CD8+ T cells discriminates between resting memory (CD44high/CD62Lhigh) and effector memory (CD44high/CD62Llow) CD8+ T cells (45–48). LM-OVA infection resulted in a rapid down-regulation of CD62L expression in CD8+ T cells, which was quite pronounced at day 5 (Fig. 3A, lower right quadrant) with ~17% of CD8+ T cells exhibiting a CD62Llow/CD44high phenotype. The numbers of such CD62Llow/CD8+ T cells declined at later time intervals and the percentage of CD62Llow/CD8+ T cells was restored to levels comparable to uninfected controls. In contrast, BCG-OVA infection resulted in a delayed, but progressive, increase in the numbers of CD62Llow/CD8+ T cells (Fig. 3A). When the relative percentages of effector memory (CD44high/CD62Llow) vs resting memory (CD44high/CD62Lhigh) CD8+ T cells were plotted kinetically during LM-OVA and BCG-OVA infections (Fig. 3, B and C), it was clear that LM-OVA induced a profound effector memory CD8+ T cell response initially (day 5) which was short-lived (Fig. 3B). In contrast, BCG-OVA induced a delayed effector memory CD8+ T cell response. In contrast, when the relative percentages of resting memory CD8+ T cells (CD44high/CD62Lhigh) were evaluated, LM-OVA induced more resting memory CD8+ T cells in comparison to BCG-OVA, which failed to induce any significant increase in resting memory CD8+ T cells (Fig. 3C). We also measured the frequency of CD8+ T cells against OVA257–264 by ELISPOT assay at various time intervals (Fig. 3D). During infection with LM-OVA, CD8+ T cell frequency peaked around day 10, which dropped considerably at subsequent time points. In contrast, during infection with BCG-OVA, the kinetics of induction and maintenance of CD8+ T cell frequency was quite different. On day 10 of BCG-OVA infection, CD8+ T cell frequency was low, which increased significantly at
was detected on day 5 after infection. At subsequent time intervals, the numbers of annexin V⁺ CD8⁺ T cells declined to background levels. In contrast, BCG-OVA infection induced a slow and progressive increase in the numbers of annexin V⁺ CD8⁺ T cells, and the numbers of such cells were maintained even in the long-term. Fig. 4B gives an overall picture of the dynamic changes in annexin V⁺ CD8⁺ T cells during LM-OVA and BCG-OVA infections.

Ag presentation during LM-OVA and BCG-OVA infections

We first tested Ag presentation in an in vitro model where OVA257–264-specific CD8⁺ T cells (OT-1 transgenic cells) were incubated with dendritic cells that were preincubated with LM-OVA or BCG-OVA for 4 h. As is evident in Fig. 5A, both LM-OVA and BCG-OVA induced the stimulation of OT-1 CD8⁺ T cells, indicating processing and presentation of OVA257–264. However, presentation by LM-OVA was more efficient. The amount of OVA generated during the 4 h in vitro culture could be influenced by the differential proliferation rates of the two pathogens.

Therefore, we evaluated the ability of control LM and BCG (not expressing OVA) for their ability to process and present a fixed amount of exogenous OVA. Because exogenous OVA does not gain access to the MHC class I processing pathway, activation of OT-1 CD8⁺ T cells by these control vectors is indicative of presentation of exogenous OVA. As is indicated in Fig. 5B, both LM as well as BCG were able to traffic exogenous OVA through the MHC class I processing pathway. Incubation of APCs with OVA in the absence of pathogens did not induce MHC class I presentation indicating that exogenous protein Ags do not gain access to MHC class I pathway. Interestingly, BCG exhibited only a moderately reduced (∼2.7-fold) potency to process and present OVA, relative to LM. Thus, the phagosomal localization of BCG does not appear to be a major deterrent to processing Ags through the MHC class I pathway.

To evaluate Ag presentation in vivo, we then addressed the onset, duration, and the extent of Ag presentation during LM-OVA and BCG-OVA infections. For this we used an adoptive transfer model, wherein recipient mice (B6.PL), which express Thy1.1, are preinjected with PBS, LM, LM-OVA, BCG, and BCG-OVA. At various time intervals (days 0, 6, 17, 30, and 60), recipient mice are injected with CFSE-labeled CD8⁺ T cells from OT-1 transgenic mice (expressing Thy1.2 and the TCR for OVA257–264). After 4 days of OT-1 transfer, spleens from the recipient mice are analyzed for the numbers of donor (Thy1.2⁺) CD8⁺ T cells and their intensity of CFSE. Transferred OT-1 CD8⁺ T cells did not proliferate in PBS-injected recipient mice at all the time points tested (Fig. 6). Infection of recipient mice with LM induced a marginal reduction in CFSE expression of donor OT-1 CD8⁺ T cells during the first few days, but at later time intervals, CFSE expression of

FIGURE 3. The phenotype of CD8⁺ T cells during LM-OVA and BCG-OVA infections. C57BL/6 mice were infected with LM-OVA and BCG-OVA as described in Fig. 1. At various time intervals, spleens were removed and the expression of CD62L vs CD44 on gated CD8⁺ T cells was evaluated (A). Numbers in the panels indicate the percentages of cells within each quadrant. The changes in the percentages of effector (B) and resting (C) memory CD8⁺ T cells during LM-OVA and BCG-OVA infections were plotted. The frequency of IFN-γ-secreting OVA257–264-specific CD8⁺ T cells was enumerated at various time intervals by ELISPOT assay (D). Number of spots per 1 × 10⁶ spleen cells is indicated.

Kinetics of CD8⁺ T cell apoptosis during LM-OVA and BCG-OVA infections

Because annexin V binds to cells that are committed to undergo apoptosis (49), we evaluated the binding of CD8⁺ T cells to annexin V during the various stages of LM-OVA and BCG-OVA infection (Fig. 4A). After LM-OVA infection, there was a rapid enhancement in the numbers of annexin V⁺ CD8⁺ T cells, which day 21 and decreased slowly at subsequent time intervals. Thus, relative to peak frequencies, there was a lesser drop in frequency during BCG-OVA infection in comparison to that during LM-OVA infection. Further, the drop in CD8⁺ T cell frequency during LM-OVA infection was rapid (occurring within 7 days of peak frequency) in comparison to BCG-OVA infection. Thus, despite the induction of a more potent initial CD8⁺ T cell response by LM-OVA, rapid attrition of the response generated resulted in similar frequencies between LM-OVA and BCG-OVA at later time intervals.

FIGURE 4. Apoptosis during LM-OVA and BCG-OVA infections. C57BL/6 mice were infected with LM-OVA and BCG-OVA as described in Fig. 1. At various time intervals, spleens were removed and spleen cells stained with anti-CD8 Ab and annexin V (A). Numbers in the panels indicate the percentages of cells within the gate. The change in the numbers of annexin V⁺ CD8⁺ T cells during LM-OVA and BCG-OVA infection was plotted (B).
FIGURE 5. Ag presentation by LM and BCG in vitro. Dendritic cells (JAWS, H-2b, $5 \times 10^5$/ml) in RPMI 1640 + 8% FBS were incubated with a different multiplicity of infection (MOI) of LM-OVA and BCG-OVA (A). In another experiment (B), APCs were incubated with 10 MOI of control LM or BCG (not expressing OVA) in the presence or absence of soluble OVA (1 $\mu$g/ml). After 1 h, 10 $\mu$g/ml gentamicin were added to remove extracellular bacteria. At 4 h, cells were layered on mouse lymphocyte and centrifuged to remove dead organisms and cells. Live viable cells were then incubated ($5 \times 10^5$/well) with OT-1 CD8$^+$ T cells ($5 \times 10^5$/well) in the presence of 50 $\mu$g/ml gentamicin. Supernatants were collected at 24 h and the production of IFN-$\gamma$ was measured by ELISA.

### FIGURE 6. Ag presentation during LM-OVA and BCG-OVA infections. B6.PL recipient mice expressing Thy1.1 were injected with PBS, or $1 \times 10^8$ (i.v.) LM, LM-OVA, BCG, BCG-OVA as described in Fig. 1. At various time intervals (days 0, 6, 17, 30, 60) CFSE-labeled CD8$^+$ T cells from OT-1 transgenic mice (Thy1.2$^+$) were injected into recipient mice. After 4 days, spleens from recipient mice were removed and spleen cells were stained with anti-Thy1.2 and anti-CD8 Ab. The expression of CFSE was evaluated on gated Thy1.2$^+$ donor CD8$^+$ T cells. Numbers in the figure indicate the percentage of cells within the gate (dotted line).

Donor OT-1 CD8$^+$ T cells remained unchanged. LM-OVA infection resulted in a massive reduction in the CFSE intensity of donor OT-1 CD8$^+$ T cells within the first 4 days (indicating active proliferation of OT-1 CD8$^+$ T cells), with $\sim$85% of donor CD8$^+$ T cells having reduced CFSE expression (Fig. 6). When OT-1 donor CD8$^+$ T cells were transferred on day 6 after LM-OVA infection, a significant reduction in CFSE staining was still detectable ($\sim$19%), suggesting that Ag presentation was still occurring during this time period. Thus, Ag presentation during LM-OVA infection occurs rapidly, and interestingly, it persists for 6–10 days. In contrast, BCG-OVA infection induced a low level Ag presentation during the first 4 days, but at subsequent time intervals (days 6 and 17) Ag presentation during this infection increased substantially. This was followed by a gradual decrease thereafter, but persistent low-level Ag presentation occurred ($\sim$7%) even at day 60.

We also evaluated the numbers of donor Thy1.2$^+$ CD8$^+$ T cells in recipient mice infected with various pathogens described above. In PBS and LM injected mice, the numbers of donor Thy1.2$^+$ CD8$^+$ T cells always remained constant (2–4%) (Fig. 7). In LM-OVA infected mice, a massive increase in the numbers of donor Thy1.2$^+$ CD8$^+$ T cells occurred in the first 4 days with $\sim$30% of CD8$^+$ T cells in the recipient mice being of donor origin (Fig. 7). The numbers of donor Thy1.2$^+$ CD8$^+$ T cells in control BCG-infected mice were very low, particularly at later intervals. In contrast to LM-OVA, BCG-OVA infection did not induce significant accumulation of donor Thy1.2$^+$ CD8$^+$ T cells within the 4-day period at all the time intervals.

We evaluated the influence of BCG-OVA dose on the kinetics of Ag presentation. Infection of mice with a 100-fold higher dose of BCG-OVA increased the magnitude of Ag presentation, however, the kinetics did not change. Both doses of BCG-OVA induced the highest levels of Ag presentation during the second week of infection, and by day 30, a profound reduction in the level of Ag presentation was evident for both the doses (Fig. 8).

Because dendritic cells are considered to be the most potent APCs, we determined whether dendritic cells undergo persistent apoptosis during BCG-OVA infection. As is evident in Fig. 9, BCG-OVA induced a delayed but chronic apoptosis of dendritic cells. This is understandable considering the chronic expression of proinflammatory cytokines and mediators (e.g., TNF, IFN-$\gamma$, nitric oxide) by BCG (50). Because bystander dendritic cells have been shown to cross-present Ags that are expressed by dying dendritic cells (51), we determined whether such bystander transfer of Ag was occurring during BCG-OVA infection. To this end, we used TAP-deficient mice because it was reported previously that Ag presentation in vitro during mycobacterial infection is TAP-dependent. Normal and TAP-deficient recipient mice were infected with BCG-OVA and at day 10 CFSE-labeled donor OT-1 CD8$^+$ T cells were transferred, and the proliferation of donor cells was evaluated on day 25. In this experimental system, both the donor and recipient mice are Thy1.2$^+$, and donor cells are evaluated by measuring binding to H-2K$^d$OVA tetramers because no endogenous tetramer-positive cells are detectable in such an infection model unless OT-1 transgenic cells are injected. In normal recipients, all of the donor CD8$^+$ T cells had undergone extensive cycling, whereas very few of the donor cells had cycled in TAP-deficient recipients, indicating that Ag processing during mycobacterial infection is TAP-deficient even in vivo (Fig. 9C). Interestingly, when BCG-OVA-preinfected TAP-deficient mice were injected on day 10 with CFSE-labeled OT-1 CD8$^+$ T cells and T-depleted spleen cells from normal mice, extensive proliferation of donor OT-1 CD8$^+$ T cells was noted. This suggests that Ag is transferred from TAP-deficient APCs to normal bystander APCs, resulting in Ag presentation. How efficient this alternative pathway is remains unclear.

The decline in Ag presentation during BCG-OVA infection in the long-term despite the continued BCG-OVA persistence may be due to the suppressive host environment (host T cells, APCs, cytokines). We reasoned that if a rechallenge of such chronically infected mice with a fresh dose of BCG-OVA results in enhancement of cell cycling of donor OT-1 cells, then we could infer that the host environment was not suppressive for Ag presentation. Therefore, we reinjected BCG-OVA-infected mice on day 70 with BCG or BCG-OVA and CFSE-labeled OT-1 CD8$^+$ T cells. After BCG challenge only a low-level Ag presentation was noticeable.
However, reinfection of mice with BCG-OVA resulted in augmentation of Ag presentation with ~19% of donor CD8\(^+\) T cells reducing their CFSE expression (Fig. 10). These results suggest that the local environment in such chronically infected mice is not a deterrent to Ag presentation.

**Fate of transferred donor cells**

We next evaluated the fate of transferred donor OT-1 cells over a prolonged period. Mice were injected with 1 \(\times\) 10\(^4\) OT-1 transgenic CD8\(^+\) T cells and PBS, LM-OVA, or BCG-OVA. Injection with such high numbers of transgenic cells ensures a minimal response by host CD8\(^+\) T cells due to competition for Ag presentation (52) (our unpublished results). At day 7, 20% of the CD8\(^+\) T cells in LM-OVA-infected mice were OVA\(_{257-264}\)-specific (Fig. 11A). In contrast, only 1.5% of CD8\(^+\) T cells in BCG-OVA-infected mice were OVA\(_{257-264}\)-specific. At day 30, the numbers of OVA\(_{257-264}\)-specific CD8\(^+\) T cells in LM-OVA-infected mice dropped to 7.3%, whereas the numbers of OVA\(_{257-264}\)-specific CD8\(^+\) T cells in BCG-OVA-infected mice increased modestly to 3.9%. This suggests that the Ag presentation and effector T cell generation during BCG-OVA infection was delayed, and weaker in magnitude. Annexin V staining of OVA\(_{257-264}\)-specific CD8\(^+\) T cells further indicated a massive apoptotic commitment of OVA\(_{257-264}\)-specific CD8\(^+\) T cells in LM-OVA-infected mice at day 7 (Fig. 11B and C). In contrast, apoptotic commitment of OVA\(_{257-264}\)-specific CD8\(^+\) T cells in BCG-OVA-infected mice increased gradually. We also evaluated the influence of BCG-OVA dose on the generation and maintenance of OVA\(_{257-264}\)-specific CD8\(^+\) T cells for prolonged periods. Mice were injected with 1 \(\times\) 10\(^4\) OT-1 transgenic CD8\(^+\) T cells and challenged with intermediate (10\(^4\)) or high (10\(^6\)) doses of BCG-OVA. At various time intervals, spleens were removed and the numbers of OVA\(_{257-264}\)-specific CD8\(^+\) T cells were evaluated by staining with OVA-tetramers and CD44. In the absence of any infection, transferred donor OT-1 transgenic CD8\(^+\) T cells comprise ~0.1% of all recipient CD8\(^+\) T cells (data not shown). As is evident in Fig. 12, a 10\(^4\) dose of BCG-OVA results in a gradual increase in the number of OVA\(_{257-264}\)-specific CD8\(^+\) T cells which peaks around the third week of infection and declines gradually. In contrast, injection with the high dose (10\(^6\)) of BCG-OVA results in a stronger increase in the numbers of OVA\(_{257-264}\)-specific CD8\(^+\) T cells initially; however, this is followed by an enhancement in attrition.

**Dependence on pathogen-persistence**

Prolonged Ag presentation during BCG-OVA infection (Fig. 6) and maintenance of CD8\(^+\) T cell frequency (Fig. 3D) despite the presence of higher numbers of apoptotic CD8\(^+\) T cells (Fig. 4) indicates that CD8\(^+\) T cells during this infection must be undergoing constant turnover. If this was the case, then removal of BCG-OVA by antibiotics should curtail the continued Ag presentation and reduce the frequency of CD8\(^+\) T cells. Because removal of BCG by antibiotics takes a long time, we injected BCG-OVA-infected mice daily with PBS or with the antibiotic mixture (600 \(\mu\)g of isoniazid and 300 \(\mu\)g of rifampicin) from either days 15 to 50 (Fig. 13A) or from days 60 to 90 (Fig. 13B). Injection of the antibiotics resulted in a reduction of BCG-OVA burden to below detection as expected. Administration of antibiotics from days 15 to 50 also resulted in a decrease in the frequency of OVA\(_{257-264}\)-specific CD8\(^+\) T cells (Fig. 13A). When antibiotics were used between days 60 and 90, the frequency of OVA\(_{257-264}\)-specific CD8\(^+\) T cells remained unaltered (Fig. 13B). These results suggest that during days 15–50, CD8\(^+\) T cells must be undergoing turnover, and this must be compensated for by continuous Ag presentation to maintain the frequency of OVA\(_{257-264}\)-specific CD8\(^+\) T cells. Because the degree of Ag presentation was reduced at day 60, and the numbers of apoptotic CD8\(^+\) T cells were also lower relative to earlier time periods, it is reasonable that the removal of BCG-OVA by antibiotics between days 60 and 90 did not make any difference in the frequency of OVA\(_{257-264}\)-specific CD8\(^+\) T cells. Thus, compared with viral, or acute bacterial infection models where the duration of Ag presentation is brief (<7 days), mycobacteria exhibit a prolonged phase of Ag presentation. However, despite such persistence of mycobacteria at high levels even beyond day 70, the degree of Ag presentation gets curtailed subsequently.

**Discussion**

The manner in which an Ag is presented to the immune system can have profound implications for protection as specific T cells have
to persist long-term in a functional state to mediate effective protection (10, 11, 47). Thus, evaluation of the onset and duration of Ag presentation, and the nature of T cell response generated by the immunogen, is essential. In this report, we evaluate the kinetics of the duration of Ag presentation and the CD8⁺ T cell response induced by two intracellular bacteria, LM and BCG, with different intracellular niches, growth characteristics, and survival. OVA was used as a model Ag which was expressed by both LM and BCG, so that Ag presentation could be evaluated to the same Ag expressed by two different intracellular bacteria.

The qualitative and quantitative differences in CD8⁺ T cell responses induced by LM-OVA vs BCG-OVA may be related to some or all of the following factors: their differential intracellular lifestyle (cytoplasmic vs phagosomal), the type of infection caused (acute vs chronic), and the relative differences in their doubling time (minutes vs days). However, although the phagosomal localization of BCG may be expected to restrain efficient MHC class I processing of Ags, such phagosomal membranes have been shown to be permeable to protein export (27). Indeed, both BCG as well as LM (not expressing OVA) do traffic exogenous OVA through the MHC class I processing pathway (53) (Fig. 5). However, although our in vitro experiments indicate that BCG-OVA is weaker than LM-OVA in Ag presentation, it is not clear whether this is due to the differential growth of the two pathogens during the 4-h incubation with APCs. The differential doubling times of LM-OVA vs BCG-OVA (46.6 min vs 29.5 h, respectively) may result in much higher initial Ag expression during LM-OVA infection. When bacterial lysates were evaluated by Western blots, both LM-OVA as well as BCG-OVA expressed very low levels of OVA (<0.01% of total protein). As a result of higher Ag load due to profound proliferation, LM-OVA infection would result in stronger T cell priming, in comparison to BCG-OVA. This is clearly reflected in our data where LM-OVA induced profound Ag presentation earlier, whereas BCG-OVA induced a slow but prolonged Ag presentation, which continued even at day 60 at a low level. As opposed to indirect approaches (13–16) that involve activation of CD8⁺ T cell hybridomas in vitro, and where the state of APCs can potentially get altered upon in vitro culture, and the sensitivity of detection may be low, we have used a more direct approach where Ag presentation has been evaluated in vivo with APCs that remain untouched. To our knowledge this is the first direct demonstration of the duration of Ag presentation in mycobacterial infection in vivo.

Despite the continued high BCG-OVA burden in vivo, the extent of Ag presentation decreased progressively. This may be due to multiple additive mechanisms. One explanation could be that the bacterial growth gets severely curtailed during later time intervals as the endogenous immune response develops strongly to counteract the proliferation of the pathogen. It is also conceivable that the generation and the continuous presence of activated Ag-specific CD8⁺ T cells also selects for Ag-loss variants. For this purpose, we evaluated the bacterial burden in the presence of kanamycin because the plasmid carrying the gene for OVA also expresses the kanamycin resistance gene (38). By this analysis, most of the time points tested did not reveal any discrepancy between the number of colonies with and without the plasmid; however, at day 70, only 50% of the colonies harbored the plasmid. However, it is worth noting that the profound decline in Ag presentation was evident even at day 30, a time point at which the majority of the bacteria still harbored the OVA plasmid, suggesting that antigenic loss may not be the reason for the decline in Ag presentation. The continuous turnover of APCs during chronic BCG-OVA infection might be an important mechanism that contributes toward a gradual decline in Ag presentation. Indeed, it was recently reported that during LM infection, host T cells are responsible for limiting the duration of Ag presentation (54). Considering the chronic inflammation that BCG induces (50), APCs that harbor BCG-OVA are expected to be eliminated continuously, either by mediators of innate immunity and/or by BCG-specific T cells. During initial stages, only the mediators of innate immunity may induce APC turnover, whereas at later stages, mediators of acquired immunity would also participate in APC destruction. It is noteworthy that the decline in Ag presentation becomes evident from the third week of infection, a time point when acquired immune responses peak. However, dendritic cells have been shown to cross-present Ag by picking up Ags from apoptotic APCs (51). Indeed, this detour pathway has recently been shown to be the major pathway in mycobacteria-infected dendritic cells (55). The length of time required for new APCs to replace the dying APCs is considered the possibility that the transferred OT-1 transgenic CD8⁺ T cells and the endogenous OVA-specific CD8⁺ T
cells compete for access to OVA257–264-presenting APCs. Indeed it has been previously reported that T cells compete for access to APCs (52). However, the peak frequency of OVA257–264-specific CD8+ T cells generated during BCG-OVA infection is ~105/spleen, and the transfer of high numbers (5 × 106) of OT-1 transgenic CD8+ T cells into the recipient mice might out-compete the local OVA257–264-specific CD8+ T cells for access to OVA257–264-presenting APCs.

It was previously reported that human monocytocytes chronically infected with BCG in vitro fail to present mycobacterial Ags, but not other Ags, to CD4+ T cells, suggesting that mycobacteria may actively sequester Ags from immune T cells that would allow replicating mycobacteria to persist in infected individuals (56). It is unclear whether Ags are also sequestered from CD8+ T cells.

As with the progressive decline in Ag presentation, the frequency of OVA257–264-specific CD8+ T cells also decreased gradually despite the presence of BCG-OVA. This may also be due to some of the mechanisms discussed above. Additionally, it is also conceivable that chronic Ag presentation causes T cell exhaustion as has been reported in persistent viral infections (57, 58). However, T cell exhaustion in viral models is strictly dependent on the persistence of high Ag levels (58). Indeed, infection of mice with a 100-fold higher dose of BCG-OVA resulted in an enhanced attrition of OVA257–264-specific CD8+ T cells, suggesting that this phenomena is dose related. The slow doubling time of BCG-OVA (~29.5 h) and very low OVA expression by BCG-OVA suggests that clonal exhaustion may not be the main reason behind the progressive drop in OVA257–264-specific CD8+ T cell frequency despite BCG-OVA persistence. We have previously reported that the OVA257–264-specific CD8+ T cells induced by low dose BCG-OVA are more protective than those induced by LM-OVA, indicating that OVA257–264-specific CD8+ T cells induced by BCG-OVA are not energized. Although BCG causes a chronic infection in the host, this infection model is different from typical chronic viral infection models because the doubling time of BCG-OVA is very long.

Chronic Ag presentation during BCG-OVA infection may result in a high turnover of effector CD8+ T cells. High numbers of effector (CD69hi,CD44hi) and apoptotic (annexin V+) CD8+ T cells were present for prolonged periods during BCG-OVA infection, and the numbers of such cells declined toward day 60. Consistent with this is the observation that daily treatment of mice with antibiotics between days 15 and 50, but not between days 60 and 90, results in a drop in OVA257–264-specific CD8+ T cell frequency. This is similar to the situation with HIV infection where treatment with the triple-combination antiretroviral therapy eliminated the viral load, but this also decreased the frequency of CTLs, consistent with the dependence of HIV-specific CTLs on continued viral replication (59, 60). In contrast, during acute infections, such as LM, reduction in the LM burden by antibiotics, after the first 24 h of infection does not influence the generation of CD8+ T cell memory (61). BCG has been reported to induce profound apoptosis of T cells (33). Production of high levels of IFN-γ during BCG infection induces apoptosis of effector T cells as infection of IFN-γ-deficient mice with BCG resulted in increased accumulation of CD44hi T cells (62). In addition to IFN-γ, numerous other inflammatory cytokines and immunomodulatory compounds such as TNF-α, NO, and IL-6 are induced chronically by BCG (50) which could contribute to apoptosis of effectors. To this end, we have previously reported that pre-existing memory CD8+ T cells to unrelated Ags are strongly deleted in mice that are challenged with BCG (63). Thus, Ag presentation, effector generation, and cell death may occur continually during the BCG infection and the net result of all these mechanisms may be a decreased accumulation of effectors. The dependence of CD8+ T cells on Ag persistence may be a feature of chronic infections that induce continual CD8+ T cell activation associated with the high turnover of effector CD8+ T cells, setting up a chronic circle of Ag presentation, effector T cell generation and death. In such a scenario, the need for antigenic persistence may vary with the extent and the rate at which effectors are getting deleted.

Effector CD8+ T cells may mediate a greater degree of protection due to the rapid extravasation of effectors to distal sites (10, 11, 47). We have also previously reported that OVA257–264-specific CD8+ T cells induced by BCG-OVA are more protective than those induced by LM-OVA (38). However, the survival of such effector T cells may be of limited duration as it has recently been shown that effector T cells expressing high levels of IFN-γ fail to survive long-term in vivo (64, 65). We have also noted that IFN-γ produced by OVA257–264-specific CD8+ T cells during in vitro stimulation is mainly by CD8+ T cells that are committed to undergo apoptosis (our unpublished observations), revealing a paradox that dying cells are the ones that are highly functional.

Recently it has been shown that during lymphocytic choriomeningitis virus (20), vaccinia virus (17), and HSV (18) infections, Ag presentation commences rapidly within the first 6 h of infection, which declines to background levels by 48 h. Furthermore, it was also demonstrated that the initial antigenic encounter triggers an instructive program wherein CD8+ T cells undergo multiple divisions without requiring further antigenic stimulation (19, 20).
C57BL/6 mice were infected with BCG-OVA (10^4). Mice were divided into control and antibiotic treatment groups and the bacterial burden and frequency of IFN-γ-secreting CD8^+ T cells were evaluated. Frequency per 1 × 10^6 spleen cells is indicated.

FIGURE 12. Increase in BCG-OVA dose accelerates the attrition of donor OVA_{257-264}^+ specific CD8^+ T cells. C57BL/6 mice were injected with 1 × 10^5 CD8^- OT-1 cells and infected the next day with 10^4 or 10^6 BCG-OVA. At various time intervals, spleens were removed from the recipient mice and the numbers of OVA_{257-264}^+ specific CD8^+ T cells were evaluated after staining with H-2Kb OVA_{257-264} tetramers and anti-CD8 and anti-CD44 Abs. Each time point involved evaluation of three mice per group. Numbers in the figure indicate the percentages within the gate.

LM-OVA, which causes an acute infection, we show that the duration of Ag presentation is not as brief as has been reported for viral infection models, as significant Ag presentation was detectable between days 6 and 10 of infection. In contrast, Ag presentation with BCG-OVA was progressive, as the degree of Ag presentation increased during the first 2 wk of infection, which was followed by a decline thereafter. Thus, our results reveal differences between different pathogens in the characteristics of CD8^+ T cell memory, which in turn is related to the initiation, extent, and the duration of Ag presentation. These results bear implications for designing future vaccines. The kinetics of Ag presentation need to be appropriately tailored for induction and maintenance of protective CD8^+ T cell responses. This is particularly relevant for tumor protection, where the nature of T cells and the ability to extravasate quickly can translate to host survival or death.

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