Multiple Mechanisms Compensate to Enhance Tumor-Protective CD8+ T Cell Response in the Long-Term Despite Poor CD8+ T Cell Priming Initially: Comparison Between an Acute Versus a Chronic Intracellular Bacterium Expressing a Model Antigen

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Multiple Mechanisms Compensate to Enhance Tumor-Protective CD8+ T Cell Response in the Long-Term Despite Poor CD8+ T Cell Priming Initially: Comparison Between an Acute Versus a Chronic Intracellular Bacterium Expressing a Model Antigen

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We evaluated CD8+ T cell responses against the dominant CTL epitope, OVA257–264, expressed by an acute (Listeria monocytogenes (LM) OVA) vs a chronic pathogen (Mycobacterium bovis bacillus Calmette-Guérin (BCG) OVA) to reveal the influence on CD8+ T cell memory and consequent protection against a challenge with OVA-expressing tumor cells. Infection with lower doses of both pathogens resulted in stronger bacterial growth but weaker T cell memory indicating that memory correlates with pathogen dose but not with bacterial expansion. The CD8+ T cell response induced by LM-OVA was helper T cell-independent and was characterized by a rapid effector response followed by a rapid, but massive, attrition. In contrast, BCG-OVA induced a delayed and weak response that was compensated for by a longer effector phase and reduced attrition. This response was partly dependent on CD4+ T cells. CD8+ T cell response induced by BCG-OVA, but not LM-OVA, was highly dependent on pathogen persistence to compensate for the weak initial CD8+ T cell priming. Despite a stronger initial T cell response with LM-OVA, BCG-OVA provided more effective tumor (B16OVA) control at both local and distal sites due to the induction of a persistently activated acquired, and a more potent innate, immunity. The Journal of Immunology, 2002, 168: 5737–5745.

Activation of CD4+ and CD8+ T cells requires differential compartmentalization of Ag within the APC (1). Protein Ags from the extracellular milieu are pinocytosed by APCs and processed within endosomes. The peptides generated are presented in the context of MHC class II molecules to stimulate CD4+ T cells, but not CD8+ T cells, aiding Ab and cell-mediated responses (2). In contrast, CD8+ T cells are stimulated when peptides from endogenously derived Ags (intracellular bacteria, viruses, or tumors) are presented on MHC class I molecules (1, 3). CD8+ T cells possess the unique ability to mediate specific cytotoxicity (by perforin and Fas-dependent pathways) toward infected cells and tumors (4–6).

After the initiation of an immune response, the vast majority (>95%) of activated T cells die by apoptosis but a small portion of those T cells survive (<5%) for extended periods (7). These long-lived memory T cells possess the unique ability to respond rapidly and specifically to Ags (8). Although the presence of Ag does not appear to be crucial for maintenance of memory cells, as memory CD8+ T cells survive even in the absence of Ag (9), antigenic persistence (in lymphocytic choriomeningitis virus infection) is required for extravasation and maintenance of rapid effector function (10, 11).

Listeria monocytogenes (LM)3 is a facultative intracellular pathogen whose growth in primary infections is controlled during the first week of infection mainly by innate immunity (12, 13). LM induces potent CD4+ and CD8+ T cell responses that facilitate protection against secondary infection (14, 15). LM has been shown to induce potent CD8+ T cell activation due to egression of the bacterium into cytosol mediated via the secretion of the enzyme listeriolysin (16, 17). The intracellular pathogen, Mycobacterium bovis bacillus Calmette-Guérin (BCG), in contrast to LM, induces a chronic infection in the host resulting in a potent immune activation (18–20). Mycobacteria induce the activation of both CD4+ as well as CD8+ T cells (19–23) which seem to play a role in curtailing the expansion of the pathogen (23–25). Mycobacteria have been shown to reside within permeable phagosomes, allowing passage of proteins (Ags) across the phagosomal membrane for presentation via MHC class I molecules (26).

In contrast to LM, where the virulence factors and the frequencies of memory T cells have been extensively evaluated (14, 15), the nature of CD8+ T cell memory induced during mycobacterial infections, although addressed previously (23, 27), has remained unclear. Further, the influence of pathogen persistence on the development and maintenance of CD8+ T cell responses has not been addressed in a chronic bacterial infection. Therefore, we evaluated CD8+ T cell response to the CTL epitope OVA257–264 expressed by LM and BCG. Although both the pathogens differed in

3 Abbreviations used in this paper: LM, L. monocytogenes; BCG, M. bovis bacillus Calmette-Guérin; BHI, brain heart infusion; CD62L, CD62 ligand.
their relative persistence in vivo, they induced potent CD8^+ T cell response to OVA_{257–264} in the long term. However, qualitative and quantitative differences were noted in the response induced by the two recombinant bacteria resulting in differential protection against a challenge with OVA-expressing tumor cells.

Materials and Methods

Preparation of immunogens

For generating BCG-OVA, we obtained M. tuberculosis DNA and the BCG-Escherichia coli shuttle vector pMV261 from Dr. A. Laszlo (Health Canada, Ottawa, Ontario, Canada) and Dr. W. R. Jacobs (Howard Hughes Medical Institute, Bronx, NY), respectively. Cloning was performed in E. coli strain HB101 using ampicillin (100 μg/ml) with pUC vector and kanamycin (25 μg/ml) using pMV261. BCG (Pasteur strain) was grown on Middlebrook 7H10 solid medium (Difco, Detroit, MI) containing 10% oleic acid-albumin-dextrose supplemented with 0.5% glycerol, or in liquid culture Middlebrook 7H9 containing 10% ADC supplement (Difco), 0.2% glycerol, 0.05% Tween 80. BCG selection medium contained 15 μg/ml kanamycin. For storage, mid-log phase cultures (OD600 = 0.7–1.0) were frozen in 20% glycerol at −70°C. The numbers of CFU were determined by plating serial dilutions on solid medium without antibiotics. A partial sequence of the OVA gene (230–359) was cloned in the pMV261 vector, downstream of the Ag85B secretion signal (26), and under the control of the HSP60 promoter (22). The secretion signal was amplified by PCR on a M. tuberculosis DNA template, and cloned in MscI and BamHI restriction sites of the pMV261 vector. Codons 230–359 of the OVA gene, which encode the SIINFEKL epitope and its 5′–3′ flanking sequences (29), were cloned by RT-PCR using total RNA from EG7 cells as a template, with the SNAP RNA isolation kit (Invitrogen, Carlsbad, CA) and the Titan one tube RT-PCR kit (Boehringer-Mannheim, Indianapolis, IN). The resulting PCR product was inserted downstream of the secretion signal using the BamHI and HindIII restriction sites to generate the plasmid pMO230. The insert was verified by sequencing. Plasmid DNA (5 μg) was introduced in BCG by electroporation (30, 31) and plated on solid medium containing kanamycin (15 μg/ml). Single colonies were used to inoculate liquid cultures. BCG containing pMO230 is referred to as BCG-OVA.

For generation of OVA-expressing LM (LM-OVA), the plasmid pJD-OVA was replicated in E. coli strain HB101. Plasmid DNA was introduced in LM strain 10403S by electroporation (32). Chromosomal integration was selected by several passages at 42°C on brain heart infusion (BHI) agar with erythromycin (5 μg/ml) at 37°C in BHI liquid culture without erythromycin, and again on BHI agar with erythromycin (1 μg/ml). The loss of β-galactosidase activity was determined by growing the bacteria in the presence of 5-bromo-4-chloro-3-indolyl β-D-galactoside. The tLM-OVA was grown at OD600 = 0.4 and aliquots were stored in 20% glycerol at −70°C. CFU were determined by plating 10-fold dilution on BHI agar.

Mice and immunizations

Female C57BL/6 mice, 6–8 wk of age, were obtained from Charles River Breeding Laboratories (St. Constant, Canada). Mice were maintained in the animal facility at the Institute for Biological Sciences (National Research Council of Canada, Ottawa, Canada) in accordance with the guidelines of the Canadian Council on Animal Care. For immunization with BCG-OVA, frozen BCG-OVA aliquots were thawed, washed once, and resuspended in PBS with 0.025% Tween 80 at appropriate dilution. Mice were inoculated with 1 × 10^7 or 1 × 10^8 BCG-OVA suspended in 200 μl of PBS (with 0.025% Tween 80) via the lateral tail vein. Age-matched control mice were inoculated with 200 μl PBS (with 0.025% Tween 80). For immunization with LM-OVA, frozen stock was thawed and diluted in 0.9% NaCl. Mice were inoculated with 1 × 10^7 or 1 × 10^8 LM-OVA suspended in 200 μl of 0.9% NaCl, via the lateral tail vein.

Cytokine and recombinant mouse IL-2 was obtained from ID Laboratories (London, Ontario, Canada). Sodium chromate (NaCrO_4, 250–500 mCi/mg Cr) was obtained from Amersham (Oakville, Ontario, Canada). Anti-mouse CD8-Cy, anti-mouse CD44-FITC (Pgp1), anti-mouse CD62 ligand (CD62L)-biotin (Mel14), and streptavidin-PE were obtained from BD PharMingen (Ontario, Canada).

Cell lines

EL4 (H-2^d) was obtained from American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 medium (Life Technologies, Grand Island, NY) supplemented with 2-mercaptoethanol (2-ME) and 8% FBS (HyClone Laboratories, Logan, UT) and 10 μg/ml gentamycin (Life Technologies). EG7 cells, subclones of EL4 stably transfected with the gene encoding OVA (33), were obtained from American Type Culture Collection and cultured in RPMI plus 8% FBS, additionally containing 400 μg/ml G418 (Rose Scientific, Edmonton, Alberta, Canada). B16OVA cells, expressing the gene for OVA, were obtained from Dr. E. Lord (University of Rochester, Rochester, NY) and cultured in RPMI plus 8% FBS, additionally containing 400 μg/ml G418.

Assessment of bacterial burden in spleen

Spleens from infected mice were harvested and single cell suspensions were prepared by twizeezing the spleens between the frosted ends of two sterile glass slides in RPMI medium (Life Technologies, Burlington, Ontario, Canada). Spleens were lysed with water for 10 s and the suspension was evaluated for the numbers of viable bacteria. CFU were determined by plating serial 10-fold dilutions on appropriate plates. For spleen cells from LM-OVA-immunized mice, dilutions were prepared in 0.9% saline and 100 μl samples were spread on BHI-streptomycin agar plates. For spleen cells from BCG-OVA-immunized mice, dilutions were prepared in PBS-T (0.025% Tween 80) and 100 μl samples were spread on Middlebrook 7H10 solid medium containing glycerol (0.5%) and oleic acid-albumin-dextrose supplement (10%, Difco). Plates were incubated for 24 h for LM-OVA and for 21–30 days for BCG-OVA at 37°C and colonies were counted visually.

Purification of CD8^+ T cells

CELLection Biotin Binder Dynabeads, precoated per manufacturer’s instructions (Dynal Biotech, Lake Success, NY) with biotin-conjugated rat anti-mouse CD8α/μ mAb (53.5.8; BD PharMingen), 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. Magnetic isolation of the CD8β^+ T cells was done using a Dynal Biotech M-PC-1 magnet according to the manufacturer’s instructions. Dynabead detachment was done using the CELLection Biotin Binder kit Releasing Buffer (DNase; 188 U/10^7 Dynabeads; Dynal Biotech) in a 37°C shaker for 30–60 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 (YTS169.4; Cedarlane Laboratories, Hornby, Ontario, Canada).

Assessment of T cell responses

Enumeration of IFN-γ-secreting cells was done by ELISPOT assay (34). Briefly, spleen cells were incubated in anti-IFN-γ Ab-coated ELISPOT plates in various numbers (in a final cell density of 5 × 10^5/well using feeder cells) in the presence of IL-2 (1 ng/ml) and medium or OVA_{257–264} (10 μg/ml). Incubation lasted either overnight or for 48 h. The plates were subsequently blocked, incubated with the biotinylated secondary Ab (4°C, overnight) followed by avidin-peroxidase conjugate (room temperature, 2 h). Spots were revealed using diaminobenzidine.

Cytotoxicity assays

Single cell suspensions from spleens of immunized mice were prepared as described above. After washing, 3 × 10^5, 5 × 10^5, and 0.3 × 10^6 spleen cells from immunized mice were incubated with 5 × 10^5 irradiated (10,000 rad) Ag-bearing target cells (EG7 cells) in 10 ml of RPMI plus 8% FBS. The total number of spleen cells in each flask was normalized to 30 × 10^6 cells by adding the required number of spleen cells from unimmunized mice as feeder cells. Cultures contained 0.1% ml/gl IL-2 and were placed in 25-cm² tissue culture flasks (Falcon; BD Biosciences, Franklin Lakes, NJ), kept upright. In some experiments, purified CD8^+ T cells from various infection groups (3 × 10^7/flask) were incubated with 27 × 10^6 normal spleen cells (from unimmunized mice) in the presence of IL-2 and irradiated EG7 cells as described above. After 5 days (37°C, 8% CO₂), cells were harvested from the flasks, washed, counted and used as effectors in a standard ^51Cr-release CTL assay.

For preparation of targets for CTL assay, EL4 cells were incubated with medium or with OVA peptide 257–264 (SINIFKEL). For labeling, 5 × 10^5 target cells (EL4 and EL4-OVA_{257–264}) were incubated with 50 μl of Cr (100 μCi) and 25 μl RPMI plus 8% FBS medium. After 45 min, targets were washed twice, and various ratios of effectors and targets were cocultured for 4 h in 96-well round bottom tissue culture plates, and the supernatants were collected and radioactivity was detected by gamma-counting. The percent cytotoxicity was calculated using the formula: 100 × ((cpm experimental – cpm spontaneous)/(cpm total – cpm spontaneous)).

LU were calculated from all the dilutions of various experimental groups. A LU is defined as the number of effector cells per 10^6 spleen cells that yield 50% specific lysis of a population of 2.5 × 10^6 target cells.
Tumor models

Two tumor models (solid tumor and metastasis) were established to decipher the relative protective potential of CD8+ T cells induced by various vectors. Both the tumor models involved B16 melanoma tumor cells expressing the gene for OVA (B16OVA). In the s.c. model, 1 × 10^6 B16OVA cells (in PBS) were injected in the shaven lower dorsal region. From day 5 onward, detectable solid tumor size was measured using calipers. Tumor size, expressed in millimeters squared, was obtained by multiplication of diametrically perpendicular measurements. In the metastasis model, 5 × 10^5 B16OVA tumor cells were injected i.v. and 15 days later, lungs were removed and the number of black tumor foci were counted visually under a dissection microscope.

Flow cytometry

Spleen cell suspensions were prepared as described above. Aliquots (1 × 10^6) were washed and incubated in 50 μl RPMI plus 1% FBS with the following Abs on ice: anti-mouse CD62L-biotin, anti-mouse CD44-FITC, and anti-mouse CD8-Cy. After 30 min, cells were washed and incubated with streptavidin-PE for an additional 30 min as described above. Cells were subsequently washed and fixed in 1% formaldehyde in PBS and acquired on an EPICS XL flow cytometer (Beckman Coulter, Hialeah, FL) and analysis was done using EXPo software (Beckman Coulter).

Results

Infection with lower pathogen dose results in higher pathogen growth but reduced CD8+ T cell memory

C57BL/6 mice were infected with either 10^2 or 10^4 LM-OVA or BCG-OVA and, at various time intervals, spleens were removed and the number of viable bacteria was enumerated (Fig. 1A). As expected, infection with a higher dose resulted in a higher bacterial burden for both LM-OVA and BCG-OVA. For LM-OVA, there was a rapid increase in the bacterial replication initially, peaking around day 3. By day 7, no bacteria were detectable in either the high- or low-dose infected mice. In contrast, infection with BCG-OVA resulted in a progressive expansion in the numbers of bacteria that peaked around day 21 after infection. We also evaluated the relative increase in the bacterial growth in relation to the input dose. Bacterial burdens at peak time periods (day 3 for LM-OVA and day 21 for BCG-OVA) were divided by the bacterial burdens at day 1 to give an indication of the relative increase in pathogen growth (Fig. 1B). For LM-OVA, low-dose infection (10^2) resulted in an ~7-fold expansion in bacterial numbers compared with only 1.85-fold expansion after infection with 10^5 bacteria. Similarly with BCG-OVA, the 10^2 dose resulted in an ~98-fold increase in bacterial numbers in comparison to 15-fold expansion obtained after infection with higher dose.

Differential CD8+ T cell response induced by LM-OVA vs BCG-OVA

During infection with LM-OVA, CD8+ T cell frequencies peaked around day 7 with higher infection dose resulting in higher frequencies. At subsequent intervals, the frequencies dropped considerably for both the infection doses, but the difference in frequencies between low and high infection dose was maintained (Fig. 2A). In contrast, during infection with BCG-OVA, the kinetics of induction and maintenance of CD8+ T cell frequencies were quite different from that induced by LM-OVA. On day 7 of BCG-OVA infection, CD8+ T cell frequency was measurable only in the high-dose (10^5) infected mice. At day 21, the CD8+ T cell frequency in the low-dose infected mice was detectable and the frequency in the high-dose infected mice increased significantly. At
day 50, the frequencies for both the high- and low-dose infected mice did not change appreciably. At day 150, the frequencies for both the low- and high-dose infected mice declined (~3–6 fold). Thus, relative to peak frequencies, there was a lesser drop in the frequency during BCG-OVA infection in comparison to that during LM-OVA infection (Fig. 2A). Further, the drop in the 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 CD8+ T cell response (day 7) by LM-OVA, rapid attrition of the response generated resulted in similar frequencies between LM-OVA and BCG-OVA at later time intervals. We measured cytolytic activity of CD8+ T cells (in Ag-restimulated cultures) from mice infected with LM-OVA or BCG-OVA. The kinetics of induction and maintenance of this function of CD8+ T cells appeared to correlate with the ELISPOT results described above. LM-OVA induced a more potent cytolytic activity initially followed by a profound attrition subsequently (Fig. 2B). In contrast, BCG-OVA induced modest cytolytic activity initially followed by a stronger response at later intervals.

We tested the nature of CD8+ T cell response induced by LM-OVA vs BCG-OVA at day 30 after infection with 10^4 LM-OVA or BCG-OVA. Infection of mice with BCG-OVA, but not LM-OVA resulted in splenic enlargement due to the presence of higher numbers of splenocytes (Fig. 3A). When CD8+ T cells were evaluated for cell size (by forward scatter), CD8+ T cells from BCG-OVA-infected mice had a higher proportion of large cells in comparison to the CD8+ T cells from LM-OVA-infected mice (Fig. 3B). Three-color staining of CD8+ T cells revealed that LM-OVA-infected mice had a higher percentage of resting memory (CD44highCD62Lhigh) CD8+ T cells, whereas BCG-OVA-infected mice, in contrast to LM-OVA, exhibited a higher proportion of effector memory (CD44highCD62Llow) CD8+ T cells (Fig. 3C).

We also tested whether antigenic restimulation was required for the cytolytic activity of CD8+ T cells. When CD8+ T cells were tested ex vivo, no CTL activity was detectable in any group of mice (data not shown). When cells were cultured for 5 days in the absence of exogenous peptide, CD8+ T cells from BCG-OVA-infected mice exhibited a higher level of peptide-specific cytolytic activity than LM-OVA (Fig. 3). Induction of this cytolytic activity required the addition of exogenous IL-2 during the 5-day culture period. When cells were cultured in the presence of exogenous peptide, specific cytolytic activity in both the groups increased; however, CD8+ T cells from LM-OVA-infected mice exhibited higher cytolytic activity than BCG-OVA. Thus, when fold-stimulation was compared without and with exogenous peptide stimulation, CD8+ T cells from BCG-OVA-infected mice exhibited a 7.5-fold increase in specific cytolytic activity in comparison to a 200-fold increase in the case of LM-OVA.

**Differential protection mediated by LM-OVA vs BCG-OVA**

We determined whether the induction of a CD8+ T cell response by LM-OVA or BCG-OVA would influence subsequent protection against OVA-expressing tumors. We used B16OVA tumor cells because these tumor cells can grow s.c. under the skin as a solid tumor (Fig. 4) as well as in the lungs as metastatic foci (Figs. 5 and 6). Fig. 4 illustrates the s.c. tumor progression in various groups of mice preinfected (120 days previously) with 10^2 (A) or 10^4 (B) dose of the pathogen, respectively. At low dose of the pathogen, LM-OVA immunization failed to show any detectable influence on tumor progression (Fig. 4, A and C). In contrast, BCG-OVA infection resulted in a delay in the onset with four of five mice having no detectable tumors within the first nine days of tumor challenge (Fig. 4, A and C). At later time intervals all mice developed tumors. When mice were preimmunized with a higher dose of the pathogen (10^6), both LM-OVA as well as BCG-OVA induced a significant degree of protection (Fig. 4B) and this was...
particularly so for BCG-OVA. Immunization with control LM or BCG (not expressing OVA) failed to influence tumor growth, indicating that stimulation of systemic innate immunity, which is particularly relevant for BCG as a chronic pathogen, does not influence tumor protection at a distal site.

We also measured protection against the same tumor cells (B16OVA) in the metastasis model where tumor cells proliferate in the lungs following i.v. injection. Tumor cells were injected into mice at day 30 or 150 following preimmunization with various immunogens or PBS (Fig. 5, A and B). Foci were enumerated 2 wk after tumor challenge and the dotted line in the figure indicates a threshold (≥250) beyond which accurate counting of individual foci is not possible. PBS-injected control mice challenged with B16OVA tumor cells harbored 250–500 tumor foci. At a low dose (10²) of the pathogen, LM-OVA preimmunization induced only a modest reduction in the number of tumor foci in the lungs when tumor challenge was given on day 30, and failed to induce any protection when tumor challenge was given on day 150. In contrast, infection with low dose (10²) BCG-OVA induced a stronger protection at both day 30 as well as day 150. This level of protection mediated by BCG-OVA was partly due to the induction of Ag-independent activation of innate immunity as control BCG, that does not express OVA, also induced a partial reduction in the number of tumor foci. When mice were immunized with a higher dose (10⁴), LM-OVA also induced a significant reduction in the number of tumor foci at both day 30 and 150. Fig. 6 gives a visual overview of the black tumor foci in the lungs of various experimental groups described above.

**Differential regulation of CD8⁺ T cell response during LM-OVA vs BCG-OVA infections**

We evaluated whether the CD8⁺ T cell response induced by LM-OVA and BCG-OVA differs in helper T cell dependence. Control or CD4⁺ T cell-deficient C57BL/6 mice were infected with 10⁵ LM-OVA or BCG-OVA. On day 30, spleens were removed and the effects on CD8⁺ T cell response were evaluated. Absence of CD4⁺ T cells in LM-OVA-infected mice did not influence CD8⁺ T cell response toward OVA₂₅₇₋₂₆₄ (Fig. 7, A and B). In contrast to LM-OVA, absence of CD4⁺ T cells in BCG-OVA-infected mice resulted in a reduction in CD8⁺ T cell response. Reduction in this CD8⁺ T cell response in BCG-OVA-infected mice occurred despite an increase in the bacterial burden (Fig. 7C).

**Differential requirement for pathogen persistence during acute vs chronic bacterial infection**

We evaluated whether pathogen persistence influences CD8⁺ T cell responses. C57BL/6 mice were infected with LM-OVA and at the peak of bacterial growth (48 h), mice were injected with either PBS or ampicillin daily until day 15. At various time intervals, spleens were harvested from control or ampicillin-treated mice and the effects on bacterial burden and CD8⁺ T cell frequency were evaluated. Administration of antibiotics resulted in a rapid clearance of LM-OVA (Fig. 8A). However, there was no difference in CD8⁺ T cell frequency (measured at day 23) between control and antibiotic-treated mice (Fig. 8B). Similarly, no difference in cytolytic activity (expressed in LU) was noted between control vs antibiotic-treated mice (Fig. 8C). Even at day 7, no difference in CD8⁺ T cell frequency was noted (data not shown). When mice...
were challenged with B16OVA tumor cells on day 30, pretreatment with antibiotics did not influence protection (Fig. 8D). These results suggest that during LM-OVA infection, T cell responses are mainly elicited during the initial, but not later, phase of the pathogen growth.

We then evaluated whether the CD8\(^+\) T cell response induced by the chronic pathogen, BCG-OVA, depended on the persistence of the pathogen. Antibiotic treatment (day 21–50) reduced the bacterial persistence to undetectable levels (Fig. 9A), and resulted in a significant decline in CD8\(^+\) T cell frequency (A) and cytolytic activity (B) against OVA\(_{257-264}\) was evaluated. For infection with BCG-OVA, the effect of CD4-deficiency on bacterial burden was also evaluated at day 30 (C).

The precise quantitation of OVA expressed by LM-OVA vs BCG-OVA is difficult due to the relative difference in the growth of the two bacteria, with LM-OVA doubling in <20 min, and BCG-OVA doubling in >24 h. However, when bacterial lysates were evaluated by Western blots, both LM-OVA and BCG-OVA expressed very low levels of OVA (<0.01% of total protein). Thus, as a consequence of the differential doubling times, LM-OVA infection would result in much higher initial Ag expression than BCG-OVA (by antibiotic treatment) after the peak growth phase of bacteria, to decipher the relative influence of these two bacteria on CD8\(^+\) T cell response. OVA was used as a model Ag because the CTL epitope, OVA\(_{257-264}\), has been well characterized, and protection mediated by OVA\(_{257-264}\)-specific CD8\(^+\) T cells could be tested in vivo against a challenge with OVA-expressing tumor cells.

During LM-OVA infection, CD8\(^+\) T cell memory was maintained long after the elimination of pathogen. Further, removal of LM-OVA (by antibiotic treatment) after the peak growth phase of the bacterium did not influence the generation of protective memory CD8\(^+\) T cells. This result is in concordance with a recent report where priming during LM infection was shown to occur within, but not after, the first 24 h of infection (36), supporting the notion that maintenance of T cell memory is Ag-independent (9).

Discussion

Generation of T cell memory is important for long-term efficacy of vaccines. The manner in which 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 (11, 35). We evaluated CD8\(^+\) T cell response to OVA, which was expressed by two different intracellular bacteria, LM and BCG, to decipher the relative influence of these two bacteria on CD8\(^+\) T cell response. OVA was used as a model Ag because the CTL epitope, OVA\(_{257-264}\), has been well characterized, and protection mediated by OVA\(_{257-264}\)-specific CD8\(^+\) T cells could be tested in vivo against a challenge with OVA-expressing tumor cells.

The precise quantitation of OVA expressed by LM-OVA vs BCG-OVA is difficult due to the relative difference in the growth of the two bacteria, with LM-OVA doubling in <20 min, and BCG-OVA doubling in >24 h. However, when bacterial lysates were evaluated by Western blots, both LM-OVA and BCG-OVA expressed very low levels of OVA (<0.01% of total protein). Thus, as a consequence of the differential doubling times, LM-OVA infection would result in much higher initial Ag expression and, hence, T cell priming, in comparison to BCG-OVA.

During LM-OVA infection, CD8\(^+\) T cell memory was maintained long after the elimination of pathogen. Further, removal of LM-OVA (by antibiotic treatment) after the peak growth phase of the bacterium did not influence the generation of protective memory CD8\(^+\) T cells. This result is in concordance with a recent report where priming during LM infection was shown to occur within, but not after, the first 24 h of infection (36), supporting the notion that maintenance of T cell memory is Ag-independent (9).
In contrast, removal of BCG-OVA (by antibiotics) after the peak growth phase of the bacterium resulted in a reduction in CD8\(^+\) T cell frequency and compromised protection against B16OVA tumor cells. When antibiotics were administered at a later phase of infection (day 70–100), no effects were noted on the frequency of OVA\(_{257-264}\)-specific CD8\(^+\) T cells (data not shown). Our results thus suggest that the requirement for antigenic persistence in the maintenance of CD8\(^+\) T cell memory can be pathogen- and time-dependent. Interestingly, during HIV infection, reduction of viral load by anti-retroviral therapy resulted in a decrease in HIV-specific CTL responses suggesting that HIV-specific CTLs depend on continued viral replication (37).

Saturation of CD8\(^+\) T cell frequency around day 21 despite continued BCG-OVA persistence in normal mice and a strong decline in CD8\(^+\) T cell frequency after antibiotic treatment implies a constant turnover of effector CD8\(^+\) T cells, as has been reported to occur during HIV infection (38). Infection of mice with BCG has been reported to induce profound apoptosis of T cells (39, 40). Production of high levels of IFN-\(\gamma\) during BCG infection induces apoptosis of effector T cells as infection of IFN-\(\gamma\)-deficient mice with BCG resulted in increased accumulation of CD44\(^{high}\)CD62L\(^{low}\) T cells (41). In addition to IFN-\(\gamma\), numerous other inflammatory cytokines and immuno-active compounds such as TNF-\(\alpha\), NO, and IL-6 are induced chronically by BCG (42–44). We have also noted that pre-existing CD8\(^+\) T cell memory to unrelated Ags is compromised in mice that are challenged with BCG (unpublished results). Thus, Ag presentation, effector generation, and cell death may occur continually during the initial phase of BCG infection. It is conceivable that “true memory” CD8\(^+\) T cells generated during BCG-OVA infection are revealed only when antibodies are used which mainly eliminates the generation of effectors while sparing Ag-independent memory CD8\(^+\) T cells. Hence, when memory CD8\(^+\) T cell responses are compared between LM-OVA and BCG-OVA following treatment with antibiotics, BCG-OVA exhibits a much weaker CD8\(^+\) T cell memory. Thus, BCG-OVA appears to induce a smaller “true memory” pool but a long-term effector phase. In contrast, LM-OVA induces a larger memory pool but a shorter effector phase due to its rapid initial proliferation and, hence, potent T cell priming relative to BCG-OVA.

Despite the continued BCG-OVA persistence after day 50, the OVA\(_{257-264}\)-specific CD8\(^+\) T cell response continued to decrease. The reasons for this are unclear and may involve mechanisms that interfere with Ag presentation. Human monocytes chronically infected with BCG in vitro fail to present mycobacterial, but not other, Ags, to CD4\(^+\) T cells (45). Alternatively, it is also possible that persistent activation results in anergy or deletion of specific T cells as has been reported in high-dose (10\(^6\)) viral infections (46, 47).

Our data indicates that the development of potent and protective CD8\(^+\) T cell memory, regardless of the bacteria used, correlates positively with the infection dose. These results are consistent with the other reports on viral infection models where viral dose correlates positively with T cell memory development (10, 11, 48). However, a 100-fold difference in the infection dose resulted in only a 5–10 fold difference in CD8\(^+\) T cell frequency. It is possible that the higher relative bacterial expansion with reduced infection doses could enhance T cell priming, resulting in less of a difference in T cell frequencies between low and high infection doses.

BCG-OVA-induced CD8\(^+\) T cells mediated stronger specific cytolytic activity in the absence of peptide restimulation. However, after restimulation with exogenous peptide, LM-OVA induced a more potent increase in specific cytotoxic response than BCG-OVA. This differential cytotoxicity without and with peptide restimulation can be explained when the state of CD8\(^+\) T cells is taken into account. BCG-infected mice in contrast to LM-OVA harbor a higher percentage of effector memory CD8\(^+\) T cells (CD44\(^{high}\)CD62L\(^{low}\)) in the spleen for longer periods of time. Resting memory CD8\(^+\) T cells have been reported to proliferate more than effectors in vitro (49). Furthermore, effectors would have an increased propensity for susceptibility to apoptosis in vitro. Due to differential pathogen persistence, the CD8\(^+\) T cells induced by LM-OVA may potentially enter into a resting state sooner than those induced by BCG-OVA.

B16OVA tumor cells were used to evaluate the protective influence of LM-OVA and BCG-OVA because protection against Ag-expressing B16 cells has been reported to be due to the induction of strong Ag-specific CD8\(^+\) T cell responses (50, 51). In addition to the role of CD8\(^+\) T cells, innate immunity also induces some protection against tumors (52). In the lungs, BCG-OVA exhibited stronger protection than LM-OVA. Because control BCG,
but not LM (neither expressing OVA), also had a moderate protective influence on tumors, the superior protection mediated by BCG-OVA (growing in the lungs) could partly be due to BCG-induced nonspecific inflammatory mediators released locally in a chronic manner. Enhancement of inflammation by BCG could result in increased bystander damage to cells in the lungs, including tumor cells, relative to that with LM. In fact, BCG when instilled directly into the bladder, has been used therapeutically against superficial bladder cancer (53, 54), and the protective mechanisms involved (nonspecific) might be similar to those operating in the lungs. Intravenous infection of mice with BCG stimulates innate immunity systemically (spleen, liver), resulting in nonspecific protection against other intracellular pathogens (55, 56). Thus, superior protection mediated by BCG-OVA against tumor cells in the lungs highlights the cooperativity between innate and acquired immunity. Interestingly, when the growth of tumor was measured at a distal site (s.c.), control BCG (not expressing OVA) failed to have any effect whereas BCG-OVA induced potent protection. Thus, innate immunity facilitates protection only at a local site where the pathogen is growing, whereas acquired immunity can mediate protection both at a local as well as a distal site. The reasons for this differential protection at local and distal sites by innate vs acquired immunity are unclear, but may involve differential trafficking/extravasation of the cells involved. At the distal site, BCG-OVA also induced stronger protection than LM-OVA. This is consistent with the notion that the state, rather than frequency, of T cells is important for protection against tumors (50). During lymphocytic choriomeningitis virus infection of mice, only activated cells extravasate efficiently to a peripheral site and provide protection, and continuous antigenic exposure was essential for keeping CD8+ T cells in an activated state (10, 11). Furthermore, priming of memory, but not effector, T cells by heat-killed bacteria was found to be ineffective in inducing long-term protection (35). It is conceivable that the stronger protection induced by BCG-OVA in the s.c. tumor model may be due to increased activated state of CD8+ T cells over a long period of time. This is particularly relevant when one considers Ag-specific control for tumors as compared with pathogens. On their own, tumor cells do not induce the activation/danger signals that a pathogen will readily induce (57, 58). Hence, the maintenance of Ag-specific T cells, in a signal 2-independent status, seems essential for effective tumor control.

We have previously reported that CD8+ T cells produce lower cytokine levels than CD4+ T cells, due to cytotoxicity of CD8+ T cells toward APCs by perforin and Fas-dependent pathways (59). This renders CD8+ T cells cytokine-dependent, particularly at low-dose immunizations (60). CD8+ T cell response in BCG-OVA-infected mice was partly CD4+ T cell-dependent whereas the absence of CD4+ T cells did not impair CD8+ T cell response in LM-OVA-infected mice. It is possible that the increase in the BCG-OVA burden (10-fold) in CD4+ T cell-deficient mice may enhance CD8+ T cell priming and partly compensate for the loss of CD4+ T cell help. These results are consistent with a report in which CD8+ T cell response was helper T cell-dependent during chronic, but not acute, viral infection (61). Recently, T cell priming in LM infection was reported to occur in the absence of CD4+ T cells (35), whereas the development of cytolytic CD8+ T cells during M. tuberculosis infection required Th cells (27).

Although CD8+ T cell responses have been characterized in acute bacterial, viral, and chronic viral infections (14, 48), the nature of CD8+ T cell response induced during a chronic bacterial infection, although addressed previously (23, 27), has remained unclear. Our results provide novel insights into the differences in the responses induced during acute vs chronic bacterial infections and the implications for tumor control with these live vectors. BCG has been used as a vaccine against tuberculosis with questionable efficacy (62). The potent generation and maintenance of protective T cell memory by BCG reported in this study suggest that the failure of the BCG vaccine may be due to reasons that do not involve inappropriate activation of T cells. Although BCG (in the absence of Ag) has been used in cancer therapy for a long time (53, 54), our study describes, for the first time, the potent protection by specific CD8+ T cells induced by BCG. Our results thus question the notion that BCG is a poor inducer of CD8+ T cell responses (63) and indicate that in comparison to LM, BCG is equally potent, if not better, in the long term.

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