Cross-Reactive Antigen Is Required to Prevent Erosion of Established T Cell Memory and Tumor Immunity: A Heterologous Bacterial Model of Attrition

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*J Immunol* 2002; 169:1197-1206; doi: 10.4049/jimmunol.169.3.1197

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Cross-Reactive Antigen Is Required to Prevent Erosion of Established T Cell Memory and Tumor Immunity: A Heterologous Bacterial Model of Attrition

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Induction and maintenance of T cell memory is critical for the control of intracellular pathogens and tumors. Memory T cells seem to require few “maintenance signals,” though often such studies are done in the absence of competing immune challenges. Conversely, although attrition of CD8+ T cell memory has been characterized in heterologous viral models, this is not the case for bacterial infections. In this study, we demonstrate attrition of T cell responses to the intracellular pathogen Listeria monocytogenes (LM) following an immune challenge with a second intracellular bacterium, Mycobacterium bovis. Mice immunized with either LM or recombinant LM (expressing OVA; LM-OVA), develop a potent T cell memory (LM) following an immune challenge with a second intracellular bacterium, Mycobacterium bovis bacterial infections. In this study, we demonstrate attrition of T cell responses to the intracellular pathogen Listeria monocytogenes (LM) following an immune challenge with a second intracellular bacterium, Mycobacterium bovis (bacillus Calmette-Guérin, BCG). Mice immunized with either LM or recombinant LM (expressing OVA; LM-OVA), develop a potent T cell memory response. This is reflected by peptide-specific CTL, IFN-γ production, and frequency of IFN-γ-secreting T cells to native or recombinant LM Ags. However, when the LM-infected mice are subsequently challenged with BCG, there is a marked reduction in the LM-specific T cell responses. These reductions are directly attributable to the effects on CD4+ and CD8+ T cells and the data are consistent with a loss of LM-specific T cells, not anergy. Attrition of the Ag (OVA)-specific T cell response is prevented when LM-OVA-immunized mice are challenged with a subsequent heterologous pathogen (BCG) expressing OVA, demonstrating memory T cell dependence on Ag. Although the reduction of the LM-specific T cell response did not impair protection against a subsequent LM rechallenge, for the first time, we show that T cell attrition can result in the reduction of Ag-specific antitumor (B16-OVA) immunity previously established with LM-OVA immunization. The Journal of Immunology, 2002, 169: 1197–1206.

The importance of T cell memory for the control of intracellular pathogens and tumor control is well documented (1–4), yet the role of Ag in the maintenance of T cell memory remains controversial (5–8). Although memory cells can be maintained or will even proliferate in the absence of Ag and MHC (5, 9, 10), in the face of immune challenge with distinct pathogens, attrition of an otherwise stable memory has been documented (11, 12). However, the factors responsible for establishing the equilibrium between these competing requirements remain unclear. If the role of Ag in maintenance of memory is complex, the dependence on Ag to determine the pool of memory cells during the initial infection is less confounding. It has been shown that the generation of memory is governed by the initial burst size of the effector response (13, 14) which in turn is defined by the Ag burst during the initial phase of infection (15, 16). In the Listeria monocytogenes (LM) bacterial model, it was found that although Ag was essential to the continued expansion of the memory pool in the early phase of infection, effector cells could proliferate in the absence of Ag (17). However, as noted by the authors, even this Ag-independent proliferation of effector cells did not contribute to the expansion of the memory pool, suggesting that not all “T cell burst activity” translates to memory enhancement. Following challenge with lymphocytic choriomeningitis virus, 40–60% of CD8+ T cells appear to be virus specific, yet <5% of these cells survive following pathogen clearance (13). With the resolution of the virus infection, a new homeostasis of the memory pool is established but virus-specific CD8+ T cells can remain as high as 10% of the memory pool (12, 13). However, as indicated above, this otherwise stable memory T cell pool is subject to Ag-dependent attrition and a single secondary viral infection can reduce Ag-specific CD8+ T cells by 2- to 5-fold, depending on the viruses involved (12).

In this study, we use two intracellular bacteria, LM and bacillus Calmette-Guérin (BCG), and we contrast the attrition of T cells previously observed in viral models (11, 12) with that in our model of heterologous bacterial infections. In addition, we examine two types of immune challenge where host protection is in part dependent on Ag-specific T cell memory and ask what the in vivo consequences of attrition of this memory might be?

Following i.v. infection, LM grow rapidly, reaching maximum burdens within 72 h, after which the bacteria are equally rapidly cleared from an immune competent mouse and are generally undetectable in the spleen by day 7 (1, 17, 18). Initial control of LM is primarily mediated by innate immunity (19), but the prevention of chronic infection and sterile immunity are dependent on LM-specific CD8+ T cells (20). Several well-characterized CD8+ epitopes have been described previously (21) and this work focuses on a dominant and protective peptide, LLO01–90, (22), derived from a major virulence factor listeriolysin O (LLO), which...
is essential for the release of LM from the endosome (23). In mice with an i.v. administration of BCG, the bacterial burden peaks at about 2–3 wk after infection, then declines to a plateau where it remains chronically (24, 25). Since the reduction of BCG in the spleen is not evident until ~3 wk after infection, this results in sustained inflammation and splenomegaly (24, 25).

As in the viral models, here we show that a secondary heterologous infection induces an Ag-specific T cell attrition with respect to a primary immunization. A novel observation with these results relates to the in vivo consequence of this attrition process. Although the BCG-induced reduction of LM-specific T cell response did not compromise host immunity to subsequent LM rechallenge, attrition of CD8+ T cell memory resulted in the lost of tumor immunity.

Materials and Methods

Bacterial strains

*Mycobacterium bovis* BCG (Aventis Pasteur) was kindly provided by R. North (Trudeau Institute, Saranac Lake, NY) and cultured at 37°C under constant shaking in 7H9 medium containing glycerol (0.2%), Tween 80 (0.05%), and albumin-dextrose supplement (ADC, 10%; Difco, Detroit, MI). At mid-log phase (A600 = 1.0), bacteria were harvested and frozen at −80°C (in 20% glycerol). CFU were determined by plating serial dilutions in PBS-0.025% Tween 80 (PBS-T) on Middlebrook 7H10 solid medium (in 20% glycerol). CFU were determined by plating serial dilutions (28). Codons 230–359 of the Ag 85B secretion signal (27), under the control of HSP60 promoter (30) and used at the concentrations indicated.

Assessment of bacterial burden in spleen

Single-cell suspensions from infected mice were teased as above in RPMI 1640. With LM-immunized mice, an aliquot of the suspension was lysed with water for 30 s and then evaluated for the numbers of viable bacteria. CFU were determined by plating 100-μl aliquots of serial 10-fold dilutions in 0.9% saline on appropriate plates as above. For spleen cells from BCG- and BCG-OVA-immunized mice, the cells were not lysed and the 30-μl spots were prepared on BHI-streptomycin agar plates. Once the plates were incubated at 37°C for 3 days, these dilutions were spread on Middlebrook 7H10 solid medium as before. Plates were incubated for 24 h for LM and for 21–30 days for BCG or BCG-OVA at 37°C, and colonies were counted visually. Error bars on figures indicate SD.

Assessment of T cell responses

The concentration of IFN-γ in the 72-h culture supernatants was determined with a one-step sandwich ELISA (32). Error bars on figures indicate SD. Enumeration of IFN-γ-secreting cells was done by ELISPOT assay (21). 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 to mimic the heterologous antigen. These cultures were established in R8-A or R8-A plus Ag (peptide at 10 μg/ml or OVA at 100 μg/ml) supplemented with IL-2 (1 ng/ml, unless otherwise indicated) and incubated for 48 h at 37°C in 5% CO2. The cells were then lysed with H2O, the plates were washed (PBS-T), and incubated with the biotinylated secondary Ab (4°C overnight) followed by avidin-peroxidase conjugate (room temperature, 2 h). Spots were revealed using diaminobenzidine. For plate-bound anti-CD3 stimulation, flat-bottom 96-well plates were incubated overnight at 4°C with 10 μg/ml anti-CD3 Ab (H-129.19) in PBS. Plates were washed once with PBS and 1.0 × 10^5 CD4+ or CD8+ T cells (see below) were added in 200 μl R8-A/well.

Frequency of IFN-γ-secreting CD8+ T cells was also evaluated in the liver. Briefly, single-cell suspensions were made as described above for spleens. Cells were reconstituted in 40% Percoll (Amersham Pharmacia, Uppsala, Sweden) and layered onto 70% Percoll. Cells were isolated from the interface after centrifugation at 600 × g for 30 min.

Cytotoxicity assays

Single-cell suspensions from pooled spleens from immunized mice were resuspended in R8-A as described above. These preparations were titrated such that 30 × 10^5, 3 × 10^6, or 0.3 × 10^6 spleen cells were incubated with 5 × 10^4 irradiated (10,000 rad) appropriate Ag-bearing target cells in 10 ml R8-A: for BALB/c, H-2d, pHem3.3 cells were used for CTL against LLOδ9-99; for C57BL/6, H-2b, EG7 cells were used for CTL against OVA257-264. The total number of spleen cells in each flask was normalized to 5 × 10^5 cells by adding the required number of syngeneic spleen cells from unimmunized mice as feeder cells. Cultures contained 0.1 ng/ml IL-2 (except where indicated) and were placed in 25-cm2 tissue culture flasks (Falcon; BD Labware) kept upright. After 5 days (37°C, 8% CO2), cells were harvested from the flasks, washed, counted, and used as effectors in a 51Cr release CTL assay against appropriate targets (30). In some experiments, spleen cells from LM-injected mice were tested directly ex vivo for their lytic activity, rather than after restimulation with Ag-bearing targets for 5 days. Following the assay, 50 μl/well of cell-free supernatant was collected and mixed with 200 μl OptiPhase HiSafe 3 ( Fisher, Ontario, pHem3.3, EG7, and B16-OVA cells were all cultured in R8 supplemented with 400 μg/ml G418 (Rose Scientific, Edmonton, Alberta, Canada).

Spleen cell cultures

Single-cell suspensions were prepared by teasing the pooled spleens (n = 2–3) between the frosted ends of two sterile glass slides in RPMI 1640. Cells were subsequently passed through Falcon 2360 cell strainers (BD Labware, Franklin Lakes, NJ), centrifuged, and resuspended in R8 supplemented with 50 μg/ml gentamicin (Life Technologies), hereafter referred to as R8-A. Cultures were established at a density of 5.0 × 10^6 cells/well in the absence or the presence of Ag, in 96-well round-bottom tissue culture plates and maintained at 37°C in 5% CO2.

Cytokines, reagents, and additional Ags

Recombinant mouse IL-2 and GM-CSF were obtained from ID Labs (London, Ontario, Canada). OVA was purchased from Sigma-Aldrich, prepared in R8, and stored at −80°C. Peptides LLOδ9-99 and OVA257-264 were kindly provide by Dr. G. Willick (National Research Council, Ottawa, Ontario, Canada) and were synthesized in the peptide synthesis facility of our institute. Sonicated LM (LM-Ag) was prepared as previously described (30) and used at the concentrations indicated.
To purify CD4\(^+\) T cells by positive selection, 0.5–1 × 10\(^8\) cells were pelleted and resuspended in 1 ml R8-A. Dynabeads mouse CD4 (Dynal, Lake Success NY) were added to the resuspended cell pellet at a ratio of four beads/cell and incubated at 4°C for 30 min in a rotating platform, after which the CD4\(^+\) T cells were removed using a Dynal MPC-1 magnet according to the manufacturer’s instructions. Detachment of Dynabeads from CD4\(^+\) T cells was accomplished using Detachabead mouse CD4 (Dynal), as per the manufacturer’s instructions: 1 U of Detachabead was added per 10\(^6\) target cells, and the suspension was incubated for 60 min at ambient temperature in a rotating platform. Purified CD4\(^+\) T cells were then washed/magnetically separated from the detached Dynabeads as before. The resulting purified T cells were >95% pure as determined by follow-up analysis with PE-conjugated rat anti-mouse CD4 (YTS 191.1; Cedarlane Laboratories, Hornsby, Ontario, Canada), and counts were determined by using MicroBeta Trilux apparatus (model 1450, Wallac, Ontario, Canada). The percent cytotoxicity was calculated using the formula: 100 \times \frac{(cpm \text{ experimental} - cpm \text{ spontaneous})}{(cpm \text{ total} - cpm \text{ spontaneous})}. Error bars on figures indicate SD.

**T cell purifications and culture conditions**

In some experiments, the above CD4-depleted splenocytes were pelleted, resuspended in 0.5–1 ml R8-A, and used to purify CD8\(^-\) T cells. CELLection Biotin Binder Dynabeads precoated, as per the manufacturer’s instructions (Dynal), with biotin-conjugated rat anti-mouse CD8\(\beta\) (mAb 53.5.8; BD PharMingen, Ontario, Canada) were added to the resuspended cell pellet at a ratio of 5 beads/cell and incubated for 15–20 min at 4°C in a rotating platform. Magnetic isolation of the CD8\(^-\) T cells was done as described above for CD4\(^+\) T cells. Dynabead detachment was done using the CELLection Biotin binder kit Releasing Buffer (DNase; 188 U/10\(^5\) Dynabeads) 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 CD8a (YTS169.4; Cedarlane Laboratories). Analysis was performed using EPICS XL flow cytometer and EXPO software (Beckman Coulter, Fullerton, CA).

**Accessory cell preparation**

Bone marrows from BALB/c mice were flushed from the femurs and tibias of one to three normal mice, and single-cell suspensions were made by passing them through Falcon 2360 cell strainers (BD Labware). Cells were resuspended at 1 × 10\(^8\) cells/ml in R8-A. Medium was supplemented with 5 ng/ml recombinant murine GM-CSF, and cells were placed in a Falcon 353111 tissue culture flask (BD Labware) and cultured for 6–8 days. Non-adherent cells were removed at days 2 and 4 of culture, and fresh R8 plus GM-CSF was added. On the day of the experiment, nonadherent cells were harvested (>80% CD11c\(^+\)), washed in R8, counted, and placed in culture with purified CD4\(^+\) or CD8\(^-\) T cells at 5.0 × 10\(^5\) cells/well.

Adherent splenocyte fractions from unimmunized and day 30 BCG (10\(^6\) CFU)-immunized BALB/c were obtained by incubating spleens cells with R8-A in a Falcon 3003 tissue culture dish (BD Labware) for 90 min at 37°C in 5% CO\(_2\). Nonadherent cells were discarded, and the adherent cell fraction was harvested by incubating cells with cold PBS for 10 min at 37°C. Detached cells were harvested, counted, and used at 5 × 10\(^4\) cells/well.

**Tumor model**

C57BL/6 mice were immunized with LM-OVA and challenged with BCG as described. B16-OVA melanoma cells were grown in R8, washed three times with PBS to remove serum, detached, centrifuged, and resuspended at 1 × 10\(^7\) cells/ml in PBS containing 0.1% mouse serum. One × 10\(^6\) B16-OVA cells were implanted into the shaven lower dorsal region of the mice. From day 5 onward, detectable solid tumor size was measured using calipers and tumor size (expressed in square millimeters) was calculated by multiplication of diametrically perpendicular measurement.

**Results**

**Attrition of LM-specific CTL and IFN-γ responses following challenge with BCG**

BALB/c mice were first immunized with LM, which induced a potent CTL and IFN-γ response to the dominant LM peptide LLO\(_{91-99}\). However, when LM-immunized mice were infected on day 30 with either 10\(^5\) or 10\(^6\) CFU of BCG, a dose-dependant attrition in the response to LM was evident at day 60 (Fig. 1). At 10\(^6\) CFU of BCG/mouse, there was a reduction in the LLO\(_{91-99}\) specific CD8\(^-\)-mediated CTL lysis in excess of 10-fold, when 5 day restimulated bulk spleen cultures were assayed (Fig. 1A). This can be seen by contrasting E:T ratios for equivalent percent cytotoxicity in Fig. 1 (e.g., –2.5 vs 25 for LM and LM plus BCG10\(^6\), respectively). This reduction in CTL activity was independent of

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**FIGURE 1.** Attrition of LM-specific CD8\(^-\) T cell response following subsequent challenge with BCG. BALB/c mice were injected with LM and then challenged with either 10\(^5\) or 10\(^6\) CFU of BCG on day 30 or maintained as LM-positive control mice. Spleen cells were harvested on day 60, restimulated with pHem cells, and then used in a chromium release CTL assay (A). Alternatively, spleens from infected mice were incubated with LLO\(_{91-99}\) peptide (10 μg/ml, B), or LM Ag (1 μg/ml, C) for 72 h, and the supernatant was assayed for IFN-γ production by ELISA. These are representative data of assays conducted for at least three experiments, and each experimental group consisted of two to three mice.
the presence or absence of IL-2 (0.1 ng/ml) during the restimulations, although the maximum cytotoxicity was enhanced by the presence of IL-2 (data not shown).

The erosion of the LLO01-99-specific CTL lysis at the higher BCG dose correlated with a 5-fold decrease in LLO01-99 peptide-induced CD8\(^+\) IFN-\(\gamma\) (Fig. 1C) and a 7-fold reduction of IFN-\(\gamma\), when spleen cells were stimulated with LM-Ag (Fig. 1B). This latter response is suggestive of CD4\(^+\) T cells, given the requirement for processing before MHC class II presentation for soluble Ags (34). At the 10\(^5\) BCG dose, the reductions were less dramatic but still evident, and the remainder of the experiments were conducted at the 10\(^6\) CFU of BCG/mouse (see Discussion). The lower apparent reduction in the IFN-\(\gamma\)-levels in comparison to the reduced CTL response may simply reflect the higher background associated with the ELISA in contrast to that seen in the CTL assays. The attrition of the CTL and IFN-\(\gamma\) responses toward LM Ags did not progress, but was stable over a long period of time, as the data shown in Fig. 1 was reproducible at days 90 and 170 (data not shown). Although this phenomenon was highly reproducible, occasionally the LM-positive control CTL cultures would reach saturation during the restimulations and titering of the effector cells would be required to reveal the differences between LM and LM plus BCG groups (see Materials and Methods).

The loss of Ag-specific IFN-\(\gamma\) production in LM\(^+\) BCG-infected mice was directly attributable to effects on CD4\(^+\) and CD8\(^+\) T cells
To exclude the possibility of inhibitory effects mediated by APCs and/or other cell types in LM plus BCG-infected spleens, purified CD4\(^+\) and CD8\(^+\) T cells were incubated with bone marrow-derived dendritic cells and either the LM-Ag or LLO01-99 peptide, respectively (Fig. 2A). Here again, the same trend in terms of reduced IFN-\(\gamma\)-production with the LM plus BCG group was clear. However, the reduction evident with the purified T cells was \(~2\)-to \(~3\)-fold (Fig. 2A) compared with 5- to 7-fold (Fig. 1, B and C) for the bulk spleen cultures. The reduction in the magnitude of the differences is at least in part attributable to the normalization of CD4\(^+\) and CD8\(^+\) T cell numbers following purification. Previously we had observed that while the total cell number (including T cells) are substantially increased in the enlarged spleens of mice infected with 10\(^6\) CFU of BCG, T cell percentages were reduced due to the disproportionate expansion of accessory cells (30, 64). Since this is not the case with LM-infected mice, the absolute numbers of T cells in bulk spleen cell cultures obtained from LM plus BCG-infected mice are about half of those seen in their LM-infected counterparts.

BCG infection enhances T cell activation and APC function
We next considered the possibility that the T cells were exhibiting a generalized anergy rather than an attrition of the LM Ag-specific response. When purified T cells were obtained from either LM- or LM plus BCG-infected mice and incubated with plate-bound anti-CD3 Ab, IFN-\(\gamma\)-levels from T cells obtained from the LM plus BCG-infected groups were in excess of that produced by the LM controls (Fig. 2B). This result excludes a generalized suppression of T cell responses induced by BCG. On the contrary, T cells from the LM plus BCG-infected mice were more responsive. This heightened responsiveness to polyclonal stimulation with anti-CD3 Ab in the LM plus BCG-infected mice correlates positively with the sustained CD4\(^{Ag4\text{high}}\) expression that we have observed with CD4\(^+\) and CD8\(^+\) T cells from BCG-infected mice (64).

An additional consideration is that the enhanced IFN-\(\gamma\) levels seen in the purified T cells from LM plus BCG infected mice, in response to anti-CD3 stimulation, also includes cytokines produced by BCG-specific T cells which are being chronically stimulated following BCG challenge (26, 64). With the data presented here, the striking contrast between the LM-specific and polyclonal T cell responses is strongly supportive of deletion of LM-specific T cells as a mechanism to account for the reduced recall to LM Ags.

To explore the role of APC and accessory cell function in LM plus BCG-infected spleens, purified CD4\(^+\) and CD8\(^+\) T cells from LM-immunized mice were cultured with adherent cells obtained from either day 30 BCG-infected or unimmunized mice (Fig. 3A). The enhanced IFN-\(\gamma\) levels observed with CD4\(^+\) T cells stimulated with LM Ag, or the similar levels seen with CD8\(^+\) T cells stimulated with LLO01-99 peptide, indicated that the adherent APCs from BCG-infected mice were better than or equal to the adherent APCs obtained from unimmunized mice, with no apparent evidence of suppression of the IFN-\(\gamma\) response.

**FIGURE 2.** IFN-\(\gamma\) production from purified CD4\(^+\) and CD8\(^+\) T cells obtained from LM plus BCG-infected mice stimulated LM Ag or anti-CD3. Purified CD4\(^+\) and CD8\(^+\) T cells (1 \times 10\(^5\)/well) from LM control, or LM plus BCG (10\(^6\) CFU)-challenged mice (as in Fig. 1), were obtained on day 60 and cultured under various conditions for 72 h. A. CD4\(^+\) or CD8\(^+\) cells from either LM- or LM plus BCG-treated mice were cultured with either LM Ag (1.0 \mu g/ml) or LLO01-99 peptide (10.0 \mu g/ml), respectively, in the presence of syngeneic bone marrow-derived dendritic cells (1 \times 10\(^3\)/well). B. Purified CD4\(^+\) and CD8\(^+\) T cells from LM- or LM plus BCG-challenged mice were examined with respect to their IFN-\(\gamma\) response to plate-bound anti-CD3. IFN-\(\gamma\) levels were determined by ELISA. These data are representative of two experiments, and each experimental group consisted of two to three mice.

**FIGURE 3.** Spleen cells from BCG-infected mice do not suppress cytokine production by LM-specific T cells. CD4\(^+\) or CD8\(^+\) cells (1 \times 10\(^5\)/well) obtained from LM-immunized mice were cultured with adherent APCs (1 \times 10\(^4\)/well) derived from either nonimmunized control mice or day 30 BCG (10\(^6\) CFU)-challenged mice (A). IFN-\(\gamma\) response with CD8\(^+\) T cells (1 \times 10\(^5\)/well) obtained from day 60 LM-immunized mice were incubated with LLO01-99 peptide (10 \mu g/ml) in the presence of varying densities of APCs within bulk spleen cells derived from either nonimmunized control or day 30 BCG (10\(^6\) CFU)-challenged mice (B). Representative data from two experiments are shown, and each experimental group consisted of two to three mice.
To extend these observations beyond adherent cells, CD8+ T cells obtained from day 60 LM-immunized mice were incubated with varying densities of whole spleen cells derived from either day 30 BCG-infected mice or unimmunized controls (Fig. 3B). Here again, spleen cells from BCG-infected mice enhanced the IFN-γ response in the presence of LLO91–99 peptide. These results are consistent with our earlier work in which the superior APC function of BCG-infected spleens was demonstrated, due in part to the elevated number of MHC class II+ cells (64). Thus, although BCG challenge appears to induce the loss of the established LM-specific T cells, the APCs within the BCG-infected spleens enhance IFN-γ production from the LM-specific T cells when the appropriate cognate Ag is present.

Reduced frequency of LM-specific T cells and ex vivo CTL activity following BCG infection

To more directly assess the in vivo status of the mice in this infection model, we determined the frequency of IFN-γ-producing cells via ELISPOT and also performed direct ex vivo CTL assays (Fig. 4). When bulk spleen cells were incubated with LLO91–99 peptide for 48 h, we observed a large decrease in the frequency of IFN-γ-producing cells in the LM plus BCG group, with or without the presence of IL-2 (Fig. 4A). Since IL-2 improved the signal on the membranes of the 96-well ELISPOT plates and did not abrogate the attrition phenomenon seen in these ELISPOTs or the 5-day restimulation CTL assays, all other reported ELISPOT data were determined in the presence of 1 ng/ml IL-2. Efforts to measure CTL activity directly from LM-immunized mice ex vivo had been unsuccessful beyond 7 days after LM injection (data not shown). Therefore, to detect LM-specific CTL directly, 60 days or more after LM immunization, it was reasoned that the mice would require a LM rechallenge to “boost” their response. As expected, the LM in vivo boosting increased the absolute numbers of IFN-γ-positive cells, yet a 50-fold reduction in the frequency of IFN-γ-secreting cells in the LM plus BCG-infected mice was still observed (Fig. 4B). In addition to the 6-fold increase in the absolute numbers that resulted from the in vivo boosting (Fig. 4, A vs B), the other important difference between the pre- and postrechallenge response was the requirement of exogenous LLO91–99 peptide. In Fig. 4A, in the absence of LLO91–99 peptide, there were few (if any) IFN-γ ELISPOTs, reflecting the high specificity of the LLO91–99 peptide (and therefore CD8+ T cell) response. Whereas in Fig. 4B, the ELISPOT numbers represented were essentially equivalent in the presence or absence of exogenous LLO91–99 peptide. This result is not surprising given that the spleen cells have been primed in vivo with LM (including the immunodominant LLO91–99 peptide). Although the ELISPOTs represented in Fig. 4B could also reflect NK cell activity unrelated to any LM peptide, the specificity of the target lysis shown in Fig. 4C does not support this view. Small but highly specific levels of CTL lysis were observed in LM-positive control mice, with near background killing evident in the LM plus BCG-infected group; again reflecting a 10-fold reduction as in Fig. 1A. Fig. 4D represents ELISA data for a replicate 96-well tissue culture plate matched to the ELISPOT data in Fig. 4B. Although the frequency of IFN-γ-positive cells was not affected by LLO91–99 peptide (Fig. 4B), exogenous peptide did enhance the levels of IFN-γ produced (Fig. 4D). From a technical perspective, the ELISPOT was clearly more sensitive than the ex vivo assay, since the former did not require in vivo boosting. However, both assays again demonstrated a substantial decline in the LM-specific T cell response in the LM plus BCG-infected mice.

Given that the extent of the Ag-specific erosion of T cell responses observed in this model of attrition is dependent on the percentage of CD8+ T cells in the assay (Fig. 2A), we undertook ELISPOT comparisons with bulk spleen and purified CD8+ T cells from LM and LM plus BCG-infected groups (Fig. 5). ELISPOT analysis of bulk spleen indicated a dramatic 60- to 80-fold reduction in the frequency of LLO91–99-specific IFN-γ-secreting T cells in the LM plus BCG-infected group (Fig. 5A). Whereas when CD8+ T cell numbers are normalized, this fold reduction decreased to 8- to 10-fold (Fig. 5B).

To determine how generalized this reduction of Ag-specific T cell was, unenriched T cell populations from spleen and liver of

![FIGURE 4](http://www.jimmunol.org/)

**FIGURE 4.** Loss of LLO peptide-specific IFN-γ-producing T cells and reduced ex vivo CTL function in BCG-challenged mice. A, Spleen cells from BALB/c mice, as in Fig. 2, were harvested at day 70 and assayed for LLO91–99 peptide (10 μg/ml)-specific IFN-γ-producing cells via ELISPOT (≥1 ng/ml IL-2). B, ELISPOT results for mice as in A that were rechallenged at day 70 with LM and assayed at 72 h after rechallenge (+IL-2; data in the absence of exogenous peptide are shown (see Results). C, Ex vivo CTL data from LM rechallenged mice as in A, B. Corresponding IFN-γ levels for 72-h cultures shown in B from a separate 96-well culture plate, with and without LLO91–99 peptide (no IL-2 added). Representative data for three experiments are shown, and each experimental group consisted of two to three mice.

![FIGURE 5](http://www.jimmunol.org/)

**FIGURE 5.** Decreased frequency of IFN-γ-producing CD8+ T cells in the spleens of BCG-challenged mice. ELISPOT results for day 60 LM and LM plus BCG mice as in Fig. 4A (+IL-2) for whole spleen (A) and purified CD8+ T cells (B). Representative data for two experiments are shown, and each experimental group consisted of three mice.
in this attrition study, we found that our previous results with LLO_{91-99} peptide in BALB/c mice were recapitulated in C57BL/6 mice. In the LM-OVA plus BCG-infected group, the response to OVA_{257-264} was reduced as measured by CTL activity and ELISPOTs for both whole spleen and purified CD8^{+} T cells (Fig. 7). Fig. 7A illustrates a dramatic 60-fold reduction in OVA_{257-264}-specific CTL cytotoxicity with the bulk spleen cells from LM plus BCG-infected mice, representing the largest fold reduction seen with these assays. In these experiments, the ELISPOT data indicated a 40- and 35-fold reduction in the frequency of OVA_{257-264}-specific cells for bulk spleen cells and purified CD8^{+} T cell responses, respectively, in the LM-OVA plus BCG-infected group (Fig. 7, C and E, respectively). However, in these same panels it can be seen that if BCG-OVA was the secondary challenge pathogen, then the attrition of OVA-specific responses was prevented and even boosted with purified CD8^{+} T cells (Fig. 7E). In evaluating these results, an important consideration is the growth of BCG in vivo, as this relates to the dose of BCG, which was demonstrated to be relevant to the attrition process (Fig. 1). However, as shown in Fig. 7B, CFU for BCG and BCG-OVA were found to be comparable at day 30 after BCG with only a slight reduction in the latter, despite the pre-established response to OVA with LM-OVA immunization. To monitor CD4^{+} T cells, intact OVA protein was incubated with bulk spleen cells and it was found that challenge with BCG essentially abrogated the response to this Ag (Fig. 7D). In comparison to the response seen with OVA_{257-264} (Fig. 7, C and E), the smaller 16-fold reduction in OVA-specific CD4^{+} T cells (Fig. 7D) is likely reflective of the limit of detection of the ELISPOT assay and the low number of precursors responsive to the intact OVA Ag. Taken together, these data imply that the presence or absence of Ag at the time of subsequent infections is critical for the maintenance of T cell memory.

In vivo consequences of Ag-specific T cell attrition

Given the magnitude of reduction in T cell memory described here, it raised the question of what the biological relevance might be? This was first considered with respect to LM rechallenge in the BALB/c model and the attrition of LM-specific T cell responses was found to have no impact on the ability of LM plus BCG-infected mice to control LM on rechallenge. This was true even...
following repeated BCG challenges (three times) to LM-immunized mice and over a period of 1 year (data not shown). Since the stimulation of innate immunity by BCG in a chronic manner has been shown to induce nonspecific protection against LM (25, 30, 35), the results obtained with LM plus BCG plus LM infection were interpreted to be due to numerous inflammatory APCs associated with BCG infection, which could compensate for reduced memory.

We then considered models in which the non-Ag-specific responses typically associated with infectious agents could be minimized in an Ag-specific immune challenge. It was hypothesized that challenge with a tumor that expressed an Ag that cross-reacted with the primary immunization would reduce the role of nonspecific immunity and perhaps reveal evidence of T cell attrition in vivo. Such a model would also permit us to consider whether the apparent attrition observed in the spleen of LM plus BCG-infected mice was reflective of a systemic reduction of Ag-specific T cells or simply due to increased trafficking induced by mycobacterial Ags.

B16-OVA is a virulent transgenic melanoma cell line derived from C57BL/6 mice that have been engineered to express OVA and is used here as a model of a solid s.c. tumor (36). Immunization of mice with LM-OVA or BCG-OVA (26) results in the control of the growth of B16-OVA in these mice, and this protection is dependent on the OVA-specific T cell responses. C57BL/6 mice were immunized with LM-OVA and challenged with BCG at day 30 or left as a positive control for tumor protection. After an additional 30 days, these mice were implanted with B16-OVA and tumor growth was monitored. Two additional controls were established: one group was comprised of unimmunized mice, while a second group received BCG only, 30 days before implantation with B16-OVA. These latter two groups served as positive controls for the tumor growth and were used to examine the nonspecific antitumor effects of BCG, respectively. As can be seen in Fig. 8, although prior immunization with LM-OVA resulted in control of tumor growth, BCG challenge before tumor implantation resulted in an abrogation of tumor immunity. BCG challenge without prior immunization with LM-OVA had no significant effect on B16-OVA growth. These results clearly demonstrated that the LM-OVA plus BCG infection induced a systemic reduction of OVA-specific T cell function and that there can indeed be substantial in vivo impact on host protection with this type of immune challenge.

**Discussion**

Since a host can potentially be challenged with multiple pathogens, understanding how an ongoing immune response to an infection alters responses generated to previously encountered pathogens is important. From the host’s perspective, there is a clear selective advantage for both the maintenance of memory against life-threatening pathogens, while also having a capacity, and therefore the “immune space,” to respond to new challenges. Hence, a balance must be struck between the acquisition of new memory and the maintenance of previously generated memory (12). Although the details of mechanisms involved remain to be determined, three principle conclusions can be drawn from our results with this model of bacterially induced attrition. First, we confirm that as in the viral model (11, 12), heterologous pathogen infection can result in the reduction of an Ag-specific memory T cell response to the primary infection. Second, this loss is a function of the absence of cross-reactive Ag during the latter infection. Third, it is clear that there can be negative consequence in terms of host protection against certain types of challenge due to the attrition of an Ag-specific response.

In light of the controversy with respect to the role of Ag in the maintenance of T cell memory (5, 7, 8), our results suggest that during heterologous infections, the persistence of Ag is important for the survival of the memory. In this work, attrition was determined by functional assays such as Ag-specific CTL activity, IFN-γ secretion, the frequency of IFN-γ-expressing cells in vitro, and by monitoring the in vivo growth of tumor cells expressing an Ag that cross-reacts with a primary immunization. In the absence of direct tetramer staining for Ag-specific T cells, it is not possible for us to make definitive statements regarding the presence or absence of such cells. However, three lines of evidence suggest that the LLO- and OVA-specific T cells are likely to be deleted after BCG challenge. If significant numbers of Ag-specific T cells were present in LM plus BCG-infected mice and merely anergized, then the IL-2 and Ag used during the CTL restimulations and the ELISPOT assays would likely have contributed to their release from that state (37–39). Similarly, if anergy rather than deletion was responsible for the apparent attrition, then during the 3-day in vivo boosting with the LM, one might predict a trend toward an equalization of LM-specific T cell frequencies between LM and LM plus BCG groups, yet no such trend was apparent. Finally, our results indicating an enhanced IFN-γ response from purified T cells in the LM plus BCG-infected mice when stimulated with anti-CD3, but reduced LM-specific IFN-γ when stimulated with LM-Ags, argues against anergy and strongly supports deletion of LM-specific T cells. What can be said of the attrition process described here is that if there were Ag-specific T cells present in excess of what our assays indicated in the LM plus BCG groups, then these cells were of no apparent functional value.

The elegant work in the viral models that first demonstrated attrition and the role of Ag in this process (11, 12) found that a single infection would reduce the previously established CD8+ T cell response by 2- to 5-fold. Given the chronic inflammation induced by BCG (lasting >6 mo), the attrition model presented here differs from the viral models (11, 12), as the viruses were cleared by the time the T cell responses were evaluated. With the sustained inflammation in our bacterial attrition model, it is perhaps not surprising that the reduction of the LM-specific T cell response was more profound, with the estimate varying depending on the assay method (i.e., CTL vs ELISPOT, or whole spleen vs purified cells). The majority of the data presented here were with the BCG at 10^6 CFU/mouse dose as a second bacterial challenge, yet even at the lower BCG dose at 10^5 CFU/mouse, attrition was evident at a
Reduced level. The rational for using the $10^6$ dose is well supported from a number of perspectives. First, with infection models involving BCG, doses as high as $10^9$ CFU/mouse (and greater) are commonly reported, and when BCG is used therapeutically for the treatment of bladder cancer in humans, repeated doses well in excess of $10^6$ are routinely used (40). Furthermore, infections with the virulent counterpart, *Mycobacterium tuberculosis*, often result in a substantially higher bacterial burden in the lung (41) and may exert more impact on the attrition process than described here. Hence, even though BCG is an attenuated bacterium, it induces potent immune stimulation, which results in a profound attrition of pre-existing memory to unrelated Ags.

There is considerable controversy with respect to the influence of BCG on immune responses with some suggesting that BCG is a potent adjuvant for the induction of T cell response to recombiant Ags (28, 42), but, on the other hand, others have associated BCG with suppressive effects (43–49). Our previous observations (26) and results presented here support BCG as a potent inducer of CD8$^+$ T cell responses (IFN-$\gamma$ and CTL activity) for a prolonged period of time. In fact, the immune response to BCG in vivo is so profound that its characterization in vitro can be complex and easily misinterpreted as immunosuppressive, if not examined fully (64). Consistent with the role of BCG as a potent immune stimulator is the positive effect BCG has on the maturation of dendritic cells and other accessory cells (50, 51). Our results indicate that when adherent cells or whole spleen cells from BCG-infected mice were used as APCs, IFN-$\gamma$ production was enhanced. IFN-$\gamma$ production was also enhanced when purified CD4$^+$ and CD8$^+$ T cells obtained from LM plus BCG-infected mice were stimulated with anti-CD3. Taken together, these findings are consistent with the view of BCG as highly immunostimulatory, and this in turn is responsible for the degree of attrition of T cells to unrelated Ags.

Viewed in this light, the immune response to BCG is not different in kind but degree, when examining host responses to other bacteria, and BCG is likely among the more chronic and immunopotent of bacteria. As such, BCG at the doses used in this study may be an appropriate starting point to examine bacterial attrition for the reasons indicated above but clearly BCG is not representative of all bacteria. Although it is beyond the scope of this work, it would be useful to examine other bacteria in this heterologous infection model. Less chronic bacterial infections may likely have a diminished impact on established T cell responses, perhaps more comparable to that seen in the viral models. However, the complexities of selecting and ordering bacterial pathogens is illustrated by the reverse infection model involving BCG plus LM (30). In this case, the chronic and potent immune response induced by BCG was sufficient to prevent the growth of LM and the establishment of a measurable T cell response to the secondary challenge by LM.

Intuitively, the need for an attrition process to accommodate the generation of memory responses to new immune challenges is clear even if the details of the mechanisms involved are not. Apoptosis has been considered as a principal mechanism responsible for the attrition of effectors (12, 13, 52), allowing survival of a small percentage of cells as memory cells. In the viral attrition model, apoptosis of specific memory CD8$^+$ T cells appeared to be mainly due to the expression of IFN-$\alpha$B during subsequent viral infections (53). Although we have not addressed the mechanism(s) involved in BCG-induced attrition, BCG has been reported to induce profound apoptosis (45, 54), and IFN-$\gamma$ has been implicated in the induction of apoptosis of effector T cells during infections with BCG (55), LM (56), *Toxoplasma gondii* (57), and *Trypanosoma cruzi* (58). In these models, apoptosis appears to be induced by IFN-$\gamma$ as the Ag levels decline. In our model, the BCG-induced IFN-$\gamma$ response and subsequent apoptosis is occurring in the absence of LM Ag, which may account for the more profound attrition. Furthermore, in the viral attrition model, the authors interpreted their data as suggesting that the T cells expressing the highest level of IFN-$\gamma$ and CD44 were preferentially lost (12, 53). In addition to IFN-$\gamma$, numerous other inflammatory cytokines and immunomodulatory compounds such as TNF-$\alpha$, NO, and IL-6 are induced chronically by BCG (44, 51, 59). Given this view, we speculate that the absence of LM Ag and the cytokine-rich microenvironment of the LM plus BCG-infected spleen results in a form of “incomplete signaling” leading to the elimination of a portion of pre-existing memory T cells through apoptosis. Which cells within the memory T cell pool are eliminated in the absence of their cognate Ag may depend on a variety of factors, including the phase of cell cycle and/or the cell’s activation status, in addition to the balance between pro- and anti-apoptotic regulatory factors in the T cells, before their encounter with the secondary BCG infection.

Our results indicate that both CD4$^+$ as well as CD8$^+$ pre-existing memory T cells are susceptible to attrition. In the viral attrition model, it appears that only CD8$^+$ T cells are susceptible (60). The reason for this differential susceptibility of CD4$^+$ memory T cells during viral vs bacterial attrition model is not clear. Since viral infections are overwhelmingly biased toward CD8$^+$ T cell responses, it is possible that attrition during such infections affects CD8$^+$, but not CD4$^+$ T cells. Bacterial infections, on the other hand, due to cellular localization of bacteria, are not selectively biased toward CD8$^+$ T cell responses, which may cause attrition of CD4$^+$ T cells as well. Another possible explanation may relate to the chronicity of the pathogen since the viral infections used for evaluating attrition did not cause chronic infections (11, 12). It has been recently reported (61) that during an infection, CD4$^+$ T cells undergo limited proliferation whereas CD8$^+$ T cells undergo extensive clonal expansion. It is therefore possible that this differential proliferation and hence activation makes CD8$^+$ T cells selectively susceptible to attrition during acute infections. On the other hand, chronic pathogens like BCG might induce attrition in both subsets due to persistent activation of both CD4$^+$ as well as CD8$^+$ T cells.

In our model, even though there was a pronounced attrition of the LM-specific response, this had no apparent affect on the ability of mice to control LM on rechallenge (data not shown). This observation may not be so surprising since BCG induces a potent and long-term innate immune response in various lymphoid organs that is characterized by the increased accumulation of inflammatory cells that mediate nonspecific protection against a challenge with LM (25, 30, 35). Thus, chronic stimulation of innate immunity by BCG may compensate for the attrition of T cell memory in LM plus BCG-infected mice. Alternatively, or in conjunction with the enhanced innate function, the residual memory in LM plus BCG-infected mice (which is reduced but not eliminated) may be sufficient to control the rechallenge with LM. The other consideration is that there may be cross-reactive Ags shared between LM and BCG, although we are unaware of any specific shared epitopes.

The lack of an obvious impact with respect to LM rechallenge in LM plus BCG-infected mice is consistent with the results in the viral attrition model, where successive heterologous infections reduced CD8$^+$ T cell memory to the primary infection, but did not alter the ability to control the initial virus on rechallenge (11). Yet these observations stand in contrast to the reduction in immune responses following vaccinations in long-lived hosts, where periodic boosting of immune responses is needed to overcome the attrition of memory T cells and/or to reactivate “resting” memory
T cells. We therefore considered alternative models to evaluate the in vivo consequences of attrition.

The rechallenge model described above involves systemic administration of the pathogen and measurement of the consequent pathogen burden in spleen. To evaluate the consequences of attrition, we selected a model where memory T cells would need to extravasate to a distal site for mediating protection and more importantly where innate immune mechanisms would have a minimal influence in protection. The reduction of tumor immunity in vivo, which was associated with the reduction of OVA-specific T cell responses, strongly suggests that the Ag-dependent attrition, as measured by the various in vitro assays, is indeed reflective of the in vivo behavior of memory T cells and that this is not limited to the spleen. These results combined with the apparent Ag-specific attrition seen in the liver argue against a redistribution of Ag-specific T cells and suggest a functional loss of Ag-specific T cells in the host. Additional evidence in support of attrition vs trafficking of memory cells to account for the apparent loss of Ag-specific T cells is the stability of the phenomenon. As indicated in the results, at days 90 and 170 a comparable loss of Ag-specific T cell function was observed in the CTL and secretion IFN-γ assays. Even considering the chronic immune stimulation by BCG and the influence this might have on trafficking of Ag-specific T cells, we feel the weight of the evidence favors attrition.

To our knowledge, this is the first demonstration of an in vivo consequence associated with attrition models in mice and may have implications for vaccination in long-lived hosts. This raises important questions regarding immunization regimens that are aimed at boosting T cell memory to ongoing vaccines without considering the impact these regimens may have on pre-existing T cell memory. Our study may also bear relevance in antitumor immune responses, where BCG (62) or possibly other nonspecific immune stimulators (63) are used.

Acknowledgments

We are grateful to our National Research Council colleagues including Dr. L. Krishnan for her helpful discussions, Dr. W. Conlan for the LM strain and also for his insights, Dr. G. Willick for the peptides, Dr. L. Krishnan for her helpful discussions, Dr. W. Conlan for the LM specific CD8+ T cell memory determined by clonal burst size. Nature 369:652.


