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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

Dean K. Smith,* Renu Dudani,* Joao A. Pedras-Vasconcelos,* Yvan Chapdelaine,* Henk van Faassen,* and Subash Sad3*†

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 (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⁺ 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.

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 1 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 loss 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 (2%), Tween 80 (0.05%), and albumin-dextrose supplement (ADC, 10%; Difco, Detroit, MI). At mid-log phase (AD50 = 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 containing glycerol (0.5%) and oleic acid-albumin-dextrose supplement (OADC, 10%; Difco).

BCG-OVA is a previously described recombinant strain (26) engineered with a partial sequence of the OVA gene (codons 230–359), downstream of the Ag 85B secretion signal (27), under the control of HSP60 promoter (28). Codons 230–359 of OVA gene encode the SIINFEKL epitope (OVA257-264) and its flanking sequences (29). Single colonies were used to inoculate liquid cultures and stocks were grown and prepared as above.

A Listeriolysin-positive, streptomycin-resistant strain of LM (10403S) was kindly provided by Dr. W. Conlan (Institute for Biological Sciences, National Research Council of Canada, Ottawa, Ontario, Canada). The bacteria were grown in brain-heart infusion (BHI) medium (Difco) supplemented with 50 μg/ml streptomycin (Sigma-Aldrich Canada, Oakville, Ontario, Canada). At mid-log phase (AD50 = 1.0), bacteria were harvested and frozen in 20% glycerol and stored at –80°C. CFU were determined by performing serial dilutions in 0.9% NaCl, which were spread on BHI-streptomycin agar plates.

OVA-expressing LM-OVA, as described previously (30), was grown to AD50 = 0.4, and aliquots were stored in 20% glycerol at –80°C. CFU were determined by plating 10-fold dilution on BHI agar.

Mice and immunizations

Female BALB/c and 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) in accordance with the guidelines of the Canadian Council on Animal Care. For immunizations with LM and LM-OVA, frozen stocks were thawed and diluted in 0.9% NaCl. With the exception of the LM rechallenge and tumor study, mice were inoculated with 5 × 107 LM or LM-OVA CFU suspended in 200 μl of 0.9% NaCl via the lateral tail vein (i.v.). The LM rechallenge dose was 1 × 106 CFU i.v. and the mice in the tumor study received 1 × 105 CFU i.v. of LM-OVA. Age-matched control mice were inoculated with 200 μl PBS. For immunization with BCG and BCG-OVA, frozen BCG-OVA aliquots were thawed, washed once, and resuspended in PBS-T at appropriate dilution. Mice were inoculated with 1 × 106 CFU of i.v. BCG (except where indicated) or BCG-OVA suspended in 200 μl of PBS-T. Age-matched control mice were inoculated with 200 μl PBS-T.

Cell lines

P815 and EL4 cells were obtained from American Type Culture Collection (Manassas, VA) and maintained in RPMI 1640 medium (Life Technologies, Grand Island, NY) supplemented with 8% FBS (HyClone, Logan, UT) and referred to hereafter as R8. PHem3.3 cells expressing the peptide 91–99 of LLO (LLD91-99) were kindly provided by Dr. M. Bevan (University of Washington, Seattle, WA). EG7 cells, a subclone of EL4 stably transfected with the gene encoding OVA (31), were obtained from American Type Culture Collection. B16-OVA cells expressing the OVA gene were obtained from Dr. E. Lord (University of Rochester, Rochester, NY), 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 striainers (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 × 105 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 LLO91-99 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.

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 resuspended 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 10-fold wells were prepared as described above. One hundred microliter aliquots of these suspensions were prepared for spleen cell suspensions. These dilutions were spread on Middlebrook 7H10 solid medium as above. 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 deter-

Fluorescein isothiocyanate (FITC)-labeled 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 × 104/well using feeder cells from unimmunized mice. 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 (1 μg/ml PBS). Plates were washed once with PBS and 1.0 × 103 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 (Amsham 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 titered such that 30 × 104, 3 × 105, or 0.3 × 106 spleen cells were incubated with 5 × 105 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 LLO91-99 for C57BL/6, H-2b, pHem3.3 cells were used for CTL against OVA257-264. The total number of spleen cells in each flask was normalized to 0.3 × 106 cells by adding the required number of syngeneic spleen cells from unimmunized mice as feeder cells. Cultures contained 0.1 μg/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, Canada).
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 × (cpm experimental – cpm spontaneous)/(cpm total – cpm spontaneous)). Error bars on figures indicate SD.

T cell purifications and culture conditions

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 two to three mice.

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. CellLection Biotin Binder Dynabeads precoated, as per the manufacturer’s instructions (Dynal), with biotin-conjugated rat anti-mouse CD8β.2 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 CD8β+ T cells was done as described above for CD4+ T cells. Dynabead detachment was done using the CellLection Biotin Kit Releasing Buffer (DNase; 188 U/10^8 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 CD8α (YTS 169.4; 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 × 10^7 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. Nonadherent 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 × 10^6 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 diometrically 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

![Image](http://www.jimmunol.org/Downloadedfromhttp://www.jimmunol.org/)
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 LLO\textsubscript{91–99}-specific CTL lysis at the higher BCG dose correlated with a 5-fold decrease in LLO\textsubscript{91–99} peptide-induced CD8\textsuperscript{+} 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\textsuperscript{+} T cells, given the requirement for processing before MHC class II presentation for soluble Ags (34). At the 10\textsuperscript{5} BCG dose, the reductions were less dramatic but still evident, and the remainder of the experiments were conducted at the 10\textsuperscript{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\textsuperscript{+} BCG-infected mice was directly attributable to effects on CD4\textsuperscript{+} and CD8\textsuperscript{+} 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\textsuperscript{+} and CD8\textsuperscript{+} T cells were incubated with bone marrow-derived dendritic cells and either the LM-Ag or LLO\textsubscript{91–99} 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\textsuperscript{+} and CD8\textsuperscript{+} 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\textsuperscript{9} 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\textsuperscript{+} expression that we have observed with CD4\textsuperscript{+} and CD8\textsuperscript{+} 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\textsuperscript{+} and CD8\textsuperscript{+} 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\textsuperscript{+} T cells stimulated with LM Ag, or the similar levels seen with CD8\textsuperscript{+} T cells stimulated with LLO\textsubscript{91–99} 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](http://www.jimmunol.org/)

**FIGURE 2.** IFN-\gamma production from purified CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells obtained from LM plus BCG-infected mice stimulated LM-Ag or anti-CD3. Purified CD4\textsuperscript{+} and CD8\textsuperscript{+} T cells (1 \times 10\textsuperscript{5}/well) from LM control, or LM plus BCG (10\textsuperscript{6} CFU)-challenged mice (as in Fig. 1), were obtained on day 60 and cultured under various conditions for 72 h. A, CD4\textsuperscript{+} or CD8\textsuperscript{+} T cells from either LM- or LM plus BCG-treated mice were cultured with either LM Ag (1.0 \textmu g/ml) or LLO\textsubscript{91–99} peptide (10.0 \textmu g/ml), respectively, in the presence of syngeneic bone marrow-derived dendritic cells (1 \times 10\textsuperscript{5}/well). B, Purified CD4\textsuperscript{+} and CD8\textsuperscript{+} 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.
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 cells was, unenriched T cell populations from spleen and liver of
LM or LM plus BCG-infected mice were contrasted. As was the case for purified CD8⁺ T cells in the spleen, an 8- to 10-fold reduction in IFN-γ LLO₉₁₋₉₉-specific T cells was observed in the liver of LM plus BCG-infected mice (Fig. 6). This suggests that the apparent loss of LM Ag-specific T cells is not restricted to the spleen and is potentially a more systemic phenomenon. Since this loss of Ag-specific T cell function is not restricted to the spleen, it implies that trafficking of Ag-specific T cells may not explain the differences observed in LM and LM plus BCG mice and this is further addressed in subsequent experiments.

Prevention of attrition when a cross-reactive Ag is introduced into LM and BCG

Having established this bacterial attrition model, we wanted to further examine the role of Ag in this process. To this end, recombinant LM and BCG were engineered to express OVA as a common model Ag (26). Since we wished to examine the peptide-specific CD8⁺ response to OVA, we selected the C57BL/6 mouse which presents the OVA-derived SIINFEKL peptide (OVA₂₅₇₋₂₆₄) in the context of H-2Kb. In a separate study we had demonstrated that both LM-OVA and BCG-OVA would induce a potent OVA₂₅₇₋₂₆₄ peptide-specific CTL and IFN-γ response to both OVA and OVA₂₅₇₋₂₆₄ (26). Using these recombinant bacteria in this attrition study, we found that our previous results with LLO₉₁₋₉₉ peptide in BALB/c mice were recapitulated in C57BL/6 mice. In the LM-OVA plus BCG-infected group, the response to OVA₂₅₇₋₂₆₄ 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 OVA₂₅₇₋₂₆₄-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 OVA₂₅₇₋₂₆₄-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 OVA₂₅₇₋₂₆₄ (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 could 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 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 106 CFU/mouse dose as a second bacterial challenge, yet even at the lower BCG dose at 105 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 10^6 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 recombinant 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-γ 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 carefully (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-γ production was enhanced. IFN-γ 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 immune-potent 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-α/β 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-γ 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-γ as the Ag levels decline. In our model, the BCG-induced IFN-γ 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-γ and CD44 were preferentially lost (12, 53). In addition to IFN-γ, numerous other inflammatory cytokines and immune-regulatory compounds such as TNF-α, 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


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