TITLE: Virally activated CD8 T cells home to BCG-induced granulomas, but enhance anti-mycobacterial protection only in immunodeficient mice.

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RUNNING TITLE: Virus activated CD8 T cells access chronic granulomas

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ABSTRACT

The effect of secondary infections on CD4 T cell-regulated chronic granulomatous inflammation is not well understood. Here, we have investigated the effect of an acute viral infection on the cellular composition and bacterial protection in Mycobacterium bovis strain bacille Calmette Guérin (BCG)-induced granulomas using an immunocompetent and a partially immunodeficient murine model. Acute LCMV co-infection of C57BL/6 mice led to substantial accumulation of IFNγ-producing LCMV-specific T cells in liver granulomas and increased local IFNγ. Despite traffic of activated T cells that resulted in a CD8 T cell dominated granuloma, BCG liver organ load was unaltered from control levels. In OT-1 TCR transgenic mice, OVA immunization or LCMV co-infection of BCG-infected mice induced CD8 T cell dominated granulomas containing large numbers of non-BCG specific activated T cells. The higher baseline BCG organ load in this CD8 TCR transgenic allowed us to demonstrate that OVA immunization and LCMV co-infection increased anti-BCG protection. Bacterial load remained substantially higher than in mice with a more complete TCR repertoire. Overall, this study suggests that peripherally activated CD8 T cells can be recruited to chronic inflammatory sites, but their contribution to protective immunity is limited to conditions of underlying immunodeficiency.
INTRODUCTION

Immune responses to chronic infections by intracellular pathogens include delayed type hypersensitivity reactions such as granulomatous inflammation. Granuloma formation around infected macrophage prevents dissemination of the pathogen and protects surrounding healthy tissue from inflammatory infiltrates. Immunopathologies can arise from long-term deposition of extracellular matrix around the granuloma leading to fibrosis and organ damage. CD4 T cells are essential for regulating the formation and ongoing function of granulomas induced in response to many infectious agents, and in the absence of these T cells, chronic infectious diseases, including tuberculosis and histoplasmosis, become widely disseminated. (6, 29, 36).

Very little research has been directed at understanding what effect, if any, secondary viral infections may have upon granuloma function. Specifically, we questioned what the role of virally-activated CD8 T cells might be in augmenting or disrupting granuloma function in the presence or absence of immune deficiency. Over the course of a lifetime, the opportunities for co-infection with mycobacterium and an unrelated viral infection are quite widespread (14). We used a mycobacterial model of Mycobacterium bovis strain bacille Calmette Guérin (BCG) i.p. infection of C57BL/6 mice to model chronic granuloma formation in the liver. At a stage when the infection is chronic, the mice were co-infected with LCMV strain Armstrong to induce a dramatic virus-specific immune response. Our studies were designed to answer three questions. Do CD8 T cells induced by viral infection or systemic immunization gain access to BCG-induced granulomas? Does viral infection interfere with granulomatous protection against chronic BCG infection and lead to reactivation of disease? If activated CD8 T cells have access to BCG-induced granulomas, do they provide better protection against BCG or interfere with anti-BCG protective responses? Our data describes a significant accumulation of LCMV-specific IFNγ producing CD8 T cells in co-infected liver
granulomas, but without accompanying augmentation or diminution of protective function in immunocompetent hosts, and with limited protection in immunodeficient ones. These results are discussed in relation to both antigen-specific and non-antigen specific requirements for T cell function in granuloma formation.
MATERIALS AND METHODS

Animals. In these studies we used C57BL/6 mice (Jackson Laboratories, Bar Harbor, MN) and a TCR transgenic strain, OT-1, specific for SIINFEKL (OVA257-264) peptide in association with MHC class I H-2Kb (gift of K. Hogquist, University of Minnesota-Minneapolis). For adoptive transfer, we used P14 TCR–transgenic mice expressing a Vα2/Vβ8.1 heterodimer specific for lymphocytic choriomeningitis virus (LCMV) glycoprotein peptide (gp) p33 (KAVYNFATM) and H-2D\(^b\) (37) on a CD45.1 background. Animals were housed in animal facilities at the UW Medical School that has AAALAC accreditation and meets PHS policy. All experiments involving mice were reviewed and approved by the appropriate institutional review committees.

Mycobacterium infections. BCG (substrain Pasteur; Staten Serum Institute) was grown in Middlebrook 7H9 (Difco Laboratories, Detroit, MI) 0.05% Tween-80 with 10% Oleic Acid Dextrose Catalase (OADC)(Difco) supplement and stored in frozen aliquots at -70°C. For infections, ampoules were thawed and the inoculum diluted in saline plus 0.05% Tween 80 and briefly exposed to sonic oscillation in order to obtain a single cell suspension. Mice were infected by i.p. injection with 1 x 10\(^7\) BCG in 100 µl (18) in order to maximize the production of liver granulomas. The dose injected is not lethal and induces a disease that is partially cleared with time. Infection was verified by histology of liver tissue samples.

Co-infections with LCMV and vaccinia virus-ova (VV-ova). LCMV-Armstrong (1) was used in these studies. Five weeks after BCG infection, C57BL/6 or OT-1 mice were infected with LCMV (2 x 10\(^5\) PFU/mouse i.p.). Nine days later, mice were sacrificed and their organs isolated for study by histology and flow cytometry. VV-ova (39) infection of C57BL/6 or OT-1 mice was initiated 5 weeks by i.p. injection of 1 x 10\(^8\) pfu/mouse. These infections were analyzed seven days after VV-ova infection.
**Histology.** Small pieces of liver were fixed in 10% formalin, prior to being imbedded in paraffin for thin sectioning (8-10 µm). H&E staining and Ziehl-Neelsen staining for acid-fast bacteria were done by the UW Department of Pathology’s Histopathology Service.

**Organ load.** Bacterial colony formation was determined by plating serial dilutions of liver homogenates on Middlebrook 7H10 agar plates (Difco) supplemented with 10% OADC and 10 µg/ml cycloheximide. Colonies were counted after 3 weeks incubation at 37°C. Data is presented as individual mouse values after a log 10 transformation. ANOVA was used to determine statistical significance.

**Flow cytometry and antibodies.** Isolation of bulk granulomas and splenocytes has been described previously (16, 32, 41). Briefly, for granulomas livers were homogenized with a tissue blender, and liver granulomas allowed to settle by virtue of their higher density. After decanting the supernatant, settled granulomas were washed in RPMI1640. The granuloma pellet was digested with 5 mg/ml Type I collagenase (Catalogue #0130, Sigma Chemical Co., St. Louis, MO) at 37°C for 40 min with shaking. Granulomas were disrupted using a syringe, followed by removal of any tissue debris by filtering through gauze layers and washing in RPMI. The live leukocyte count was determined by Trypan-blue staining. Splenocytes or granuloma cell suspensions were incubated for 30 minutes at 4°C with different labeled antibodies at saturation, then washed and analyzed. Unlabelled 2.4G2 anti-Fc receptor antibody (50µg/ml) was used to block binding of labeled antibodies to Fc receptors. Cell surface staining on 10-20,000 events was measured using a FACSCalibur flow cytometer (BD Biosciences; San Jose, CA), and data was analyzed using either CellQuest (Macintosh version 3.0, BD Biosciences) or FlowJo (Macintosh version 6.2.1; Tree Star., Ashland, OR) software. Fluorochrome-labeled antibodies were purchased from Pharmingen (San Diego, CA) or Sigma (St. Louis, MO).
Construction of the MHCI D\textsuperscript{b} tetramers that contain the LCMV CTL epitope peptides nucleoprotein 396-404 (np)396–404 or gp33–41 has been described previously (34). OVA SIINFEKL peptide specific MHCI k\textsuperscript{b} tetramers were purchased from Beckman Coulter (Fullerton, CA). For detection of LCMV-specific or SIINFEKL-specific CD8 T cells, single cell suspensions from spleen or granuloma were surface stained with anti-CD8 antibodies and fluorochrome-labeled MHC I tetramer for 1 h at 4°C, followed by four-color flow cytometry.

**Flow cytometric detection of Intracellular IFN\textsubscript{\gamma}, TNF, and activated caspase 3.**

Single cell suspensions of spleen or granuloma cells were cultured in cRPMI 1640-10% FBS plus 1:1000 dilution of Golgistop (Pharmingen). Cells were activated with either 5 µg/ml anti-CD3 antibody (145-2C11) or 0.2 µg/ml LCMV np396-404 peptide for 4-6 hours at 37°C, 5% CO\textsubscript{2}. Cells were washed once with FACS staining buffer and surface stained for 30 min. at 4°C with the indicated antibodies. After washing, cells were permeabilized for 20 min at room temperature in Cytofix/Cytoperm (Pharmingen) followed by washing three times with FACS staining buffer + 0.1% saponin and staining with either 4 µg/ml anti-IFN\textsubscript{\gamma}, 4 µg/ml anti-TNF, or 1:10 dilution of FITC-conjugated rabbit anti-caspase 3 (cat# 559341) (Pharmingen) and unlabeled 2.4G2 at 4°C for 30 min. Cells were then washed three times with FACS staining buffer + 0.1% saponin, fixed, and analyzed by four-color flow cytometry. For measurement of intracellular IFN\textsubscript{\gamma} in response to mycobacterial PPD, Golgistop addition was delayed for 12 hours to allow processing of the complex protein mixture. Subsequent steps were the same as above.

**Measurement of secreted cytokines.** Samples for cytokine analysis were collected from 1 x 10\textsuperscript{6} liver granuloma cells or spleen cells (live by trypan blue exclusion) seeded into 96-well plates in 0.2 ml cRPMI and stimulated with 5 µg/ml αCD3 antibody. After 72
hours, cell culture supernatants were harvested and stored at -70°C until testing. ELISA measurement of secreted IFNγ was previously described (23). Measurement of secreted TNF used matched reagents from Pharmingen adapted to fluorescence detection using advice from the manufacturer. All data are expressed as the amount of secreted cytokine per 1 x 10^6 cells. Confirmatory data was obtained as a custom multiplex cytokine analysis from Linco Research (St. Charles, MO).

**Real-time PCR.** Total RNA was isolated from 10^6 splenocytes or granuloma infiltrating cells using Trizol (Invitrogen; Carlsbad, CA) according to the manufacturer’s instructions. cDNA was prepared using MMLV reverse transcriptase (Gibco-BRL; Gaithersburg, MD) and used as a template for realtime quantitative PCR using primers for IFNγ and TNF (Biosource International; Camarillo, CA) and β-actin (19). Amplification of primer specific products by a Cepheid Smart Cycler (Sunnyvale, CA) was detected using SYBR Green I fluorescence (Molecular Probes; Eugene, OR). Relative signal intensities for cytokines and β-actin were calculated by the comparative Ct (cycle threshold) method using a dilution series of a strongly positive cDNA, and reported as an adjusted cytokine level by dividing each cytokine value by the β-actin value for the same sample.

**Immunofluorescence.** C57BL/6 (CD45.2+) BCG infected mice were intravenously transferred with 200,000 LCMV-specific P14 transgenic (CD45.1+) T cells prior to LCMV superinfection. Nine days later, mice were perfused with PBS under deep anesthesia. Liver sections were post-fixed overnight in 25% sucrose/3% paraformaldehyde in PBS before freezing in Tissue-Tek OCT compound (Sakura Finetek, Torrance, CA) on dry ice. Sections (5µm) were costained with antibodies to CD8-APC and CD45.1-FITC (1:200 in PBS/1% BSA), and imaged with a Bio-Rad MRC 1024 confocal laser scanning microscope (Bio-Rad, Hercules, CA) at 400x magnification. Digital data were exported into Adobe Photoshop (version 8.0) for further analysis and presentation. Staining with
CD8 APC and CD45.1 FITC allowed us to visualize the accumulation of double positive cells within the granulomatous lesions after LCMV superinfection. For staining with LCMV np396-specific MHC I tetramer, livers were harvested from BCG and BCG+LCMV infected mice as above without transfer of P14 T cells. Frozen sections were co-stained with LCMV np396-specific MHC I tetramer-APC and CD8b-FITC (1:100 and 1:50 respectively in PBS) to visualize accumulation of double positive cells within granulomatous lesions.
RESULTS

*Virus activated CD8 T cells accumulate in BCG-induced granulomas.* To address the question of whether CD8 T cells activated by viral infection have access to BCG-induced granulomas, we co-infected mice with 2x10⁵ pfu LCMV or 1x10⁶ VV-ova i.p. 5 weeks after infection with BCG. At this stage BCG infection is chronic and granuloma formation is well established. Mice were sacrificed 9 days after LCMV or 7 days after VV-ova to study the composition and phenotype of granuloma infiltrating cells. Granuloma cell preparations are made free of contaminating liver parenchymal cells in order to exclusively study cells at the inflammatory site. Seven days is ample time for T cell homing since granuloma formation can be initiated de novo after one week in an adoptive transfer model (23) and is past the peak of both LCMV-specific and VV-ova specific T cell accumulation in the spleen (5, 39). *Figure 1A* shows staining patterns of splenocytes from naïve C57BL/6 mice for comparison. In BCG-infected mice the CD8:CD4 T cell ratio was 1:2 in splenocytes and close to 1:1 in granuloma infiltrating cells in agreement with previous experiments by ourselves and others (*Figure 1B*, left hand panels and (23)). On the ninth day after LCMV co-infection, the ratio of CD8:CD4 T cells was 3:1 in the spleen and CD8 T cells were dominant among granuloma infiltrating cells (16:1) (*Figure 1B*, right hand panels). Directly addressing our question, staining with LCMV epitope-specific MHC I tetramer showed that both LCMV np396-404- and LCMV gp33-41- (data not shown) specific T cells expanded in the spleen as expected, and importantly, both populations were present in a larger proportion among granuloma infiltrating cells. For class I D<sup>b</sup>/np396-404 tetramer+CD8+ cells, the relative fractions averaged 4.9±0.6% vs 7.2±0.8% (p≤0.01 by ANOVA) of gated lymphocytes in spleen and granuloma respectively (*Figure 1C*, right plots, bottom panels)(average±SEM; 2.0±0.1% vs 4.4±0.8% in spleen and granuloma for class I D<sup>b</sup>/gp33-41 tetramer+CD8+ cells; p≤0.001 by ANOVA -data not shown). The mean frequency in total cells is shown
in Figure 1D left. Thus, after LCMV co-infection, the proportion of virus specific cells accumulated in chronic granulomatous lesions to a greater extent than in spleen despite the absence of LCMV in the granuloma. To a somewhat lesser extent, VV-ova co-infection of BCG-infected mice also increased the CD8 ratio in the spleen (1:1). A greater increase in the proportion of CD8 T cells was observed in the granuloma (12:1). Additionally, OVA-responsive T cells detected by class I K\(^6\)/OVA257-264 tetramer were enriched among granuloma infiltrating cells (Figure 1C) (0.5±0.2% vs 2.3±0.3% of gated lymphocytes in spleen and granuloma respectively; p≤0.01 by ANOVA). The mean frequency in total cells of class I K\(^6\)/OVA257-264 tetramer positive CD8 T cells is shown in Figure 1D, right. Immunofluorescence was also used to demonstrate the accumulation of LCMV specific T cells in the BCG-induced liver granuloma after LCMV superinfection for 9 days. LCMV-specific np396 MHC tetramer-APC and CD8-FITC were used to co-stain frozen liver sections. We were able to clearly visualize CD8+ LCMV np396 tetramer+ cells within the granulomatous lesions after LCMV superinfection (Figure 1E, top right panel). We also used a P14 adoptive transfer model to demonstrate the presence of P14 transgenic T cells specific for LCMV gp33 in the granuloma. C57BL/6 (CD45.2+) BCG infected mice were transferred with 200,000 LCMV-specific P14 transgenic (CD45.1+) T cells prior to LCMV superinfection. Nine days later, mice were perfused and liver sections fixed for preparation of frozen liver sections. Staining with CD8-APC and CD45.1-FITC allowed us to visualize the accumulation of double positive cells within the granulomatous lesions after LCMV superinfection (Figure 1E, bottom right panel). These images clearly show the presence of LCMV-specific CD8 T cells in the BCG-induced granuloma after LCMV superinfection and are in complete agreement with our FACS data. Together, this data indicates that virus-activated CD8 T cells can accumulate in BCG-induced liver granulomas.
**Virus specific T cells in the granuloma have a highly activated phenotype.** Next, we examined the activation state of the granuloma-infiltrating virus-specific CD8 T cells. Our data showed that almost all these cells expressed an activated phenotype. **Figure 2A** shows that after LCMV co-infection, among the CD8-gated granuloma cells, class I D\(^b\)/np396-404 tetramer positive cells were 98% CD44+ and 78% LFA-1+. Similar data were seen for class I D\(^b\)/gp33-41 tetramer positive cells (data not shown). Mean frequency in total granuloma is shown in **Figure 2C**. Thus, LCMV-specific T cells with a highly activated phenotype locate to the site of chronic BCG infection and the granuloma repertoire reflects the systemic activated T cell repertoire.

The greater ratio of CD8:CD4 T cells in the LCMV-co-infected granuloma might result from both increased CD8 T cell recruitment and/or increased apoptosis of CD4 T cells. Therefore, we compared caspase 3 expression on CD4 T cells found in BCG-induced granulomas and BCG-induced granulomas altered by LCMV co-infection. Our data showed that the increased proportion of CD8 T cells were clearly the result of higher recruitment as we found equivalent levels of caspase 3 expression by granuloma infiltrating CD4 T cells between BCG alone and BCG+LCMV infections (**Figure 2B, D**).

**The accumulation of LCMV-specific T cells in the granuloma increases IFN\(\gamma\) cytokine levels, but not TNF levels, and does not alter the mycobacterial antigen (PPD) response.** We examined whether the accumulation of virally activated T cells in the granuloma effected expression of protective Th1 cytokines TNF and IFN\(\gamma\). We measured IFN\(\gamma\) mRNA, secretion and intracellular levels in the granuloma. Direct ex vivo measurement of IFN\(\gamma\) mRNA levels from isolated granuloma cells was done by real time quantitative PCR and normalized to \(\beta\)-actin mRNA levels measured in the same samples. Normalized IFN\(\gamma\) mRNA levels were twice as high in LCMV co-infected samples (**Figure 3A** left) in the experiment shown. As a confirmation, we assessed the
recall IFN$_\gamma$ response of isolated cells by measuring cytokine production using ELISA assay of supernatants taken from three day in vitro cultures of granuloma infiltrating cells. IFN$_\gamma$ production by unstimulated cells or in response to mycobacterial purified protein derivative (PPD) was essentially unaltered between control BCG-induced granuloma cells and BCG+LCMV granuloma cells, while $\alpha$CD3-elicited production of IFN$_\gamma$ was 8-fold higher by BCG+LCMV granuloma cells (Figure 3A right). The greater response to $\alpha$CD3 by BCG+LCMV cultures was likely the result of the recruited CD8 T cells in the granulomas (Figure 1C) which was also supported by intracellular IFN$_\gamma$ staining (see below).

Figure 3B shows that splenocytes isolated from LCMV-co-infected mice and pulsed with LCMV np396-404 peptide had high numbers of CD8 T cells staining positive for intracellular IFN$_\gamma$, whereas control spleens had very low numbers to no CD8 IFN$_\gamma$ positive cells whether stimulated with LCMV np396-404 peptide or left unstimulated. As expected, granuloma-infiltrating cells from control BCG-infected mice had a baseline measurement of IFN$_\gamma$ that was higher than in splenocytes for either unstimulated conditions or for LCMV np396-404 stimulation. Granuloma cells from LCMV co-infected animals had substantially higher levels of CD8 IFN$_\gamma$+ T cells after LCMV np396-404 stimulation. This indicates that the activated CD8 LCMV tetramer positive cells that accumulate in BCG-induced granulomas are functioning effector cells that respond specifically to virus epitopes. IFN$_\gamma$-specific staining in response to $\alpha$CD3 stimulation showed that the LCMV np396-404 specific response was approximately 1/3 of the total CD8 response in LCMV co-infected splenocytes or granuloma cells consistent with the immunodominance of this epitope (45).

Interestingly, IFN$_\gamma$+ staining on CD8 negative lymphocytes was also greater in LCMV co-infected animals (compare control spleen vs LCMV spleen & control
granuloma vs LCMV granuloma; bottom row, Figure 3B top). Such IFNγ producing lymphocytes are likely to be CD4 cells arising in response to LCMV or could represent bystander activation of CD4 T cells reactive to mycobacterial antigens. To distinguish between LCMV-specific and BCG-specific IFNγ production by granuloma lymphocytes, we measured intracellular IFNγ produced in response to mycobacterial PPD under conditions allowing protein processing and presentation since PPD is a complex mixture of proteins. Figure 3C (top panels) shows that CD8 production of IFNγ did not arise from cells responding to PPD since the CD8 PPD response was no greater than the media response for both BCG control infection and BCG+LCMV infection. In the control BCG infection, granuloma CD4 T cells responding to PPD with IFNγ production represented approximately 6-7% of the total cells in the lymphocyte gate (5.9% vs 13.1% CD4+IFNγ+ for media vs PPD response) (Figure 3C, bottom panels). αCD3 stimulation did not yield any additional CD4 responsive cells under these conditions. Likewise, in the BCG+LCMV infection, although the baseline of CD4+ IFNγ+ T cells was greater (Figure 3B), the PPD responsive cells constituted approximately 5-6% of the total. The balance of the responsive cells in the LCMV co-infection (33.1% responding to αCD3 vs 19.4% to PPD and unstimulated) were most likely responding to LCMV CD4 epitopes (49). While bystander activation of mycobacterial specific T cells cannot be completely excluded in this co-infection model, they probably comprised a small fraction of the total activated T cells in the granuloma. Collectively, the LCMV co-infected BCG granuloma had a higher level of IFNγ associated with recruited virus-specific CD8 T cells and a slight increase in CD4 T cells. BCG-specific CD4 T cell responses were not affected (Figure 3A, 3C bottom plots, middle). Because both the ELISA (Figure 3, right) and the intracellular staining (Figure 3B, C) assays depend on reactivation, a more conservative estimate may be in the PCR data indicating a doubling of the local IFNγ in the lesions.
Although IFN-γ levels were increased by LCMV co-infection, in vitro TNF production by total granuloma infiltrating cells in response to PPD, LCMV or αCD3 was not increased as measured by ELISA (Figure 4A). Likewise, intracellular staining for TNF production following LCMV co-infection of BCG-infected mice was largely unaltered from mice infected with BCG alone (Figure 4B). We examined whether the large fraction of LCMV-specific CD8 T cells present after LCMV co-infection contributed to TNF production. At day 9 after LCMV infection alone, only a very small population of CD8 cells in the spleen stained with TNF-specific antibodies, even after αCD3 stimulation. This fraction did not appreciably change in BCG-infected spleen or in BCG-induced granuloma infiltrating cells (1.2%, 1.5%, and 1.7% respectively). Likewise TNF+CD8-cells were present in essentially equal fractions (6.4%, 4.9%, and 8.4% respectively) and were not altered by recall challenge with αCD3 (Figure 4B), PPD, or LCMV (data not shown). Similar data were obtained when intracellular staining was examined for CD4 cell populations (data not shown). While the fraction of CD8 negative cells in total granuloma-infiltrating cells which stained with TNF-specific antibodies decreased from 23.6% to 8.4% with LCMV co-infection (Figure 4B, bottom plots) the TNF positive proportion of the CD8 negative cells was roughly equivalent. Surface expression of Mac-1 indicated that the major source of TNF in the chronic granuloma was macrophage (data not shown).

**Accumulation of virally activated T cells in granuloma does not affect organ load.**

Microscopic examination of thin liver sections showed that the BCG-induced granulomas found in the control infection remained well-formed after LCMV co-infection (Figure 5A, top panels). Large numbers of inflammatory cells were also present outside of the liver granulomas, but the number of acid fast bacilli visible in sections stained by the Ziehl Neelsen method remained at the low levels characteristic of controlled infections (Figure
Plating liver organ homogenates for colony forming units also showed no difference in organ load between control infections of BCG alone and BCG+LCMV co-infection or BCG+VV-ova co-infection (Figure 5B). Organ load measured after VV-ova co-infection trended downward, but there was no statistical difference between the three groups, and likely lacks biological significance. These experiments established that CD8 T cells activated by LCMV or VV-Ova are recruited to the granuloma and are able to enhance production of protective IFNγ, yet have no fundamental effect upon protection against BCG in this short-term interval.

**SlIFNFEKL-specific activated CD8 T cells accumulate in BCG-induced granulomas after OVA antigen activation or LCMV bystander activation.** It is possible that increases in protection cannot be detected in animals in which BCG infection is controlled. OVA-specific TCR transgenic OT-1 mice are significantly immunocompromised because of greatly reduced levels of CD4 T cells and non-transgenic CD8 T cells (10). Since preliminary experiments indicated that OVA-specific TCR transgenic OT-1 mice infected with BCG had much poorer protection at six weeks, we treated BCG infected OT-1 mice with either OVA immunization to activate the OT-1 T cells or with LCMV co-infection. Baseline stainings of splenocytes from an uninfected OT-1 mouse are shown in Figure 6A. Consistent with expression of the OT-1 transgene, the CD8 to CD4 T cell ratio in control BCG-infected OT-1 mice was greater than during infection of C57BL/6 mice (Figure 6B, left column). CD8 T cells dominated in the granuloma, also (Figure 6B, lower left plot). After either OVA immunization or LCMV co-infection of BCG infected OT-1 mice, CD8 T cell abundance increased in both the spleen and granuloma (Figure 6B, middle and right columns) consistent with our hypothesis that peripherally activated T cells preferentially home to the granulomatus inflammatory site. Similar data were seen with VV-ova infection (data not shown). The antigen specificity of CD8 T cells accumulating in OT-1 after co-infection was examined using
class I K\(^{b}\)/OVA257-264 tetramer and class I D\(^{b}\)/np396-404 tetramer (Figure 6C). Although OVA immunization increased the total fraction of CD8 T cells in granuloma infiltrating cells compared both to spleen or to control granuloma cells, the ratio of transgenic cells to non-transgenic cells in the granuloma was lower. This agrees with previous data from our lab in which we observed that infection of AND mice transgenic for pigeon cytochrome C (PCC)-specific TCR resulted in the accumulation of non-PCC specific CD4 T cells in granuloma infiltrating cells (23). Interestingly, while LCMV co-infection of OT-1 mice also altered the CD8 to CD4 T cell ratio and promoted the accumulation of more CD8 T cells in the granuloma, tetramer staining for LCMV np396-404 was negative in both spleen and granuloma cell preparations (Figure 6C, lower panel). The detection of a CD8+ SIINFEKL-tetramer negative population in the granuloma suggests that a LCMV specific expansion occurs with an altered immunodominance (Figure 6C, upper panel, lower right). There was no measurable accumulation of LCMV-specific cells in the spleen either (CD8+ class I K\(^{b}\)/OVA257-264 negative) (Figure 6C, upper panel, upper right).

The expression of LFA-1 on gated CD8 T cells from the OT-1 infection is shown in Figure 6D. In all three conditions, class I K\(^{b}\)/OVA257-264 tetramer-specific cells were more activated in the granuloma than in the spleen suggesting bystander activation during both control BCG infection and BCG+LCMV co-infection. Consistent with the C57BL/6 data, the appearance of both LFA-1+ class I K\(^{b}\)/OVA257-264 tetramer negative populations (LCMV-specific) and LFA-1+ class I K\(^{b}\)/OVA257-264 tetramer positive populations (OVA specific) in the BCG induced granuloma suggests that highly activated, non-BCG specific T cells preferentially accumulate in BCG-induced granuloma in OT-1 mice. Mean frequency in total cells is shown in Figure 6E.

**Accumulation of SIINFEKL-specific activated CD8 T cells in BCG-induced granulomas decreases BCG load after OVA antigen activation.** Equivalent liver
granuloma formation in OT-1 mice was observed for BCG infection, BCG+VV-ova, BCG+LCMV (Figure 7A), and BCG+OVA immunization (data not shown). Overall, bacterial burden in liver from OT-1 infected mice was three logs higher than in C57BL/6 mice (Figure 7B) and closer to values seen in Rag-/- mice (data not shown). Unlike C57BL/6, in OT-1 mice liver bacterial burden was improved after viral co-infection (VV-ova or LCMV) or OVA immunization (log10 cfu/liver 6.4 for BCG control, 5.8 for BCG+OVA immunization, 5.1 for BCG+VV-ova co-infection, and 5.5 for BCG+LCMV co-infection). The higher baseline number of bacteria seen in the OT-1 was likely due to the limited CD4 T cell repertoire available and the inability of the transgenic and non-transgenic CD8 T cells to provide adequate protective function. Granuloma cells in OT-1 infections produced lower levels of IFNγ relative to C57BL/6 granuloma cells measured by ELISA from cell culture supernatants after three days in vitro stimulation with αCD3. Although OVA immunization and LCMV co-infection clearly enhanced the ability of OT-1 splenocytes to produce IFNγ, IFNγ producing cells localizing to the OT-1 granuloma were not sufficiently numerous to reach the level of C57BL/6 granuloma cell IFNγ production (Figure 7C, right) or to exert anti-mycobacterial protection comparable to C57BL/6 (compare Figure 7B to Figure 5B). Overall, the one log increased protection (Figure 7B) correlates with increased numbers of CD8 T cells in the granuloma (Figure 6B) and increased IFNγ levels (Figure 7C), underscoring its biological relevance.
DISCUSSION

Granulomatous hypersensitivity lesions are common during the chronic phase of a variety of infectious and autoimmune diseases, and individuals affected with granulomatous diseases are likely to experience concurrent acute viral infections. We were interested in access of virally activated T cells to granulomatous lesions. While clearly we must be cautious in generalizing our findings to other granuloma and virus models, our data clearly show that virus specific CD8 T cells can infiltrate granulomas.

The role of CD4 T cells in anti-mycobacterial control has long been studied. However, several recent studies have underscored the additional requirement for CD8 T cell function for control of M. tuberculosis infection both during primary immunity following vaccination (12) and during latency (48), and a major pathway of “cross-priming” to MHC-1 and CD1 restricted T cells has been identified (17, 42). Vaccination of mice with a TB DNA vaccine cocktail requires the presence of CD8 T cells for efficacy (12). Antibody depletion studies suggest that primary protective mechanisms induced by the vaccine cocktail involves the induction of activated CD8 cells making cytokines, including IFNγ and TNF, which can provide anti-mycobacterial protection in the absence of CD4 T cells. Similarly, depletion of T cell subsets during latency implicated IFNγ-producing CD8 T cells in continued control of bacterial replication at later timepoints in the lung (48). Given the importance of mycobacterial-specific CD8 T cells to antimycobacterial function, we designed this study to measure the effect upon bacterial control of the trafficking of non BCG-specific CD8s induced by non-related viral infection. Would virally specific CD8 T cells arrive at the site of mycobacterial infection, and if so would they diminish or augment bacterial control? Virally induced CD8 T cells might not be appropriately differentiated to act against macrophage infected by mycobacteria. Mouse hepatitis virus infection of CNS suggests that different CD8 T cell effector
mechanisms are responsible for control of coronavirus replication in different CNS cell types (4). In contrast, a clinical study associated acute hepatitis B co-infection with elimination of both hepatitis B and underlying chronic hepatitis C infection possibly via bystander mechanisms, such as the secretion of cytokines (20). Many virally induced CD8 T cells secrete IFNγ in large amounts, and this cytokine is key to control of mycobacterial replication. For this study, our primary model was LCMV co-infection of C57BL/6 mice chronically infected with BCG. LCMV co-infections offer the advantage of a superbly characterized infection course and technically advanced analysis reagents.

Our data in Figure 1 shows that CD8 T cells expanded by LCMV infection accumulated in BCG-induced liver granulomas. This was evident from both the frequency of LCMV-specific CD8 T cells among the total population (Figure 1D) and in calculation of the absolute numbers of T cells present (data not shown). We also demonstrated accumulation of LCMV-specific cells in the granuloma by immunofluorescence using both LCMV np396-specific tetramer staining and CD45.1 staining of transferred P14 cells (LCMV gp33 specific) (Figure 1E). While systemically activated CD8 T cells are reported to accumulate in the liver and undergo apoptosis (13), our data shows specific recruitment of activated cells to the granuloma and not accumulation of apoptotic cells in the liver. In granuloma cells caspase 3 expression levels were very similar on CD4 T cells from control BCG infection and BCG+LCMV infection (Figure 2B,D). For CD8 T cells, BCG+LCMV actually had five-fold less caspase3 staining on a fractional basis than did BCG infection alone due to the accumulation of activated CD8s lacking caspase3 expression (data not shown). Swain and colleagues found that systemic contraction of BCG-activated CD4 T cell populations in wild-type mice is associated with increased apoptosis and is dependent upon IFNγ- and NO-mediated mechanisms (11). Similar mechanisms were found to operate during
SEA-induced tolerance of CD4 T cells in TCR transgenic mice (7). This mechanism was not seen despite increases in IFNγ levels. Figure 2A shows that LCMV-specific T cells that accumulated at the site of chronic BCG infection had a highly activated phenotype. We saw previously that peripherally activated T cells lacking specificity for the inciting pathogen accumulate in liver granulomas (24, 43). Studies in a murine model of virally-induced demyelination have also suggested that CD8 T cells lacking specificity for myelin can home to brain inflammatory sites from the periphery and incite neuropathology if specifically activated (21). The CD4:CD8 T cell ratio in granuloma infiltrating cells is a consistent characteristic that varies between pathogenic agents (22, 23, 41, 51). Our data strongly suggest that the ratio largely reflects systemic activation. It remains to be distinguished whether virus-specific T cell accumulation in the granuloma reflects a systemic level of activation seen earlier or active accumulation. Mechanisms contributing to accumulation at chronic inflammatory sites are likely to encompass chemokines such as RANTES, MIP-1alpha, and MCP. Increased levels of these three chemokines, and RANTES most markedly, are produced in vitro by granuloma infiltrating cells in response to LCMV peptide after LCMV co-infection as assayed by multiplex analysis (data not shown). Enhanced proliferation of LCMV-specific CD8 T cells in the granuloma is unlikely to contribute substantially to their accumulation since granuloma cells are typically in a non-proliferative state and day 9 of superinfection is past the point of LCMV-specific T cell expansion. We tested our assumption using flow cytometric intracellular cell cycle analysis and saw no alteration in the extremely low numbers of cells in S phase in BCG+LCMV co-infection compared to BCG-induced granulomas (data not shown).

After either OVA immunization or LCMV co-infection of BCG infected OT-1 mice, CD8 T cells were more abundant in both the spleen and granuloma (Figure 6B). Despite
this, although OVA immunization increased the total fraction of CD8 T cells in granulomas compared to spleen and to control granuloma cells, the granuloma ratio of transgenic cells was lower than in the spleen. We previously observed that infection of AND mice transgenic for a PCC-specific TCR results in preferential accumulation of non-PCC specific CD4 T cells in granuloma infiltrating cells (23). In both AND and OT-1 mice, when the repertoire is limiting BCG-specific T cells in the granuloma are preferred, unlike in the C57BL/6 where the initial repertoire is broader. Transgenic OT-1 T cells may also be refractory to normal localization signals. Other studies have demonstrated that transgenic T cells are resistant to a well-characterized inhibitor of T cell activation, TCDD, when “supraphysiological” numbers of antigen-specific T cells are present in TCR transgenic mice (33).

LCMV co-infection of OT-1 mice alters the CD8:CD4 T cell ratio and promotes the accumulation of more CD8 T cells in the granuloma, yet tetramer staining for np396-404 was negative in both spleen and granuloma cell preparations. McGavern et al. found that LCMV infection of OT-1 mice does not lead to any detectable expansion of LCMV peptide-specific IFNγ production (30), and other reports have detailed altered LCMV immunodominance in genetically deficient mice (45-47). Over 70% of the anti-LCMV response against immunodominant epitopes is reported to come from three distinct TCR populations (44). OT-1 mice, may have alterations in the availability of specific Vβ families that abrogate normal immunodominance patterns. Alternatively, BCG infection and/or the extensive TCR transgene expression may bias the ability of dendritic cell populations to present antigen for expansion of the measured LCMV-specific populations. Crowe et al. found that differing APC populations are required for efficient presentation of various immunodominant influenza epitopes, leading to changing patterns of immunodominance following primary and secondary infections (9).
The co-infection model using OT-1 mice also demonstrated that highly activated SIINFEKL-specific T cells preferentially accumulated in BCG-induced granuloma vs. spleen (Figure 6D) suggesting bystander activation. Bystander activated OT-1 T cells can modulate the immunopathology of herpes simplex keratitis disease in the absence of HSV-specific T cell reactivity (2). Bystander activation can lead to complex outcomes, including non-reciprocal heterologous immunity (27), bystander recruitment without bystander activation (35), and attrition of bystander CD8 T cells (31). Our experiments in Figure 6 do not exclude the possibility of dual specificity of the OVA tetramer staining cells arising from imperfect allelic exclusion or low affinity recognition (3, 28).

It was initially surprising to us that the arrival of activated non-specific T cells capable of producing IFNγ did not materially improve or worsen granuloma function in the C57BL/6 model (Figure 3A and C, Figure 5). It might be that during chronic stage infection after control of BCG is established, additional effector cells have no effect. Earlier timepoints might reveal a positive or negative affect of LCMV activated cells on anti-BCG granuloma function. This suggests that short term, individuals with infectious diseases controlled by granulomatous inflammation can tolerate viral infections without adverse effect. In the OT-1 model, where baseline liver organ load was three logs higher, VV-ova co-infection was able to enhance control of liver organ load by more than a log, while LCMV co-infection led to a somewhat smaller decrease in organ load and had a higher variance. This data supports a role for CD8 T cells in protection when CD4 T cells are deficient. Macrophage tropic LCMV clone 13 variant decreases the response of murine macrophage to IFNγ in vitro, resulting in uncontrolled replication of Histoplasma (50, 52). Double infection with LCMV clone 13 and Histoplasma is also associated with reduced anti-fungal immunity mediated by CD8 T cells (53). A schistosome LCMV coinfection model demonstrated increased numbers of LCMV-
specific CD8 T cells in liver, enhanced hepatotoxicity, and increased morbidity (15). Yet other viral studies have strongly suggested that recruitment of bystander T cells can ameliorate chronic infections (20) and double infections with wild-type LCMV virus and virus expressing altered peptide ligands are less capable of inducing lethal immunopathology (25). Infectious interactions are not always predictable (27) and Jiang et al. found no killing of bystander bacteria when mice containing memory populations of antigen specific CD8 T cells were infected with mixed inoculums of epitope-specific and non-specific microbes (26). Swain and co-workers have found that influenza infection of the lung gives rise to complex heterogeneous CD4 T cell responses with a diverse spectrum of effector and memory phenotypes (40). Viral co-infection of the BCG-infected host is no doubt equally multi-faceted. Our data show that activated CD8 T cells irrespective of their specificity continuously home to BCG-induced granulomas and may be able to contribute to limited protection in immunodeficient conditions.

An interesting aspect of the unexpectedly high access of virus-activated CD8 T cells in the granuloma concerns stability and cellular traffic of these sites. Ramakrishanan reported that bacteria-infected macrophages have access to well-formed granulomas (8) and here we show that virus activated CD8 T cells can home to these sites. Previously, we have shown that GFP-expressing MOG-specific CD4 T cells home to BCG-induced granulomas (43). Granulomas can be induced in 2-3 days (38), but how long they survive and the dynamics of their formation is unknown. The data reported here argue that granulomas are either short-lived and continuously reformed or are dynamically restructured with an active cellular traffic in and out. The implications of a dynamic regenerating granuloma structure are very important for our understanding of nature of granulomatous diseases.
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REFERENCES


ABBREVIATIONS

lymphocytic choriomeningitis virus (LCMV)

Mycobacterium bovis strain bacille Calmette Guérin (BCG)

vaccinia virus-ova (VV-ova)

nucleoprotein 396-404 (np396-404)

glycoprotein 33-41 (gp33-41)

purified protein derivative (PPD)
FIGURE LEGENDS

Figure 1. LCMV–specific T cells can be found in BCG-induced chronic granulomas 9 days after acute LCMV infection. C57BL/6 mice were infected ip with BCG. At 5 weeks, groups were either held as control infections or co-infected with $2 \times 10^5$ PFU LCMV strain Armstrong. VV-ova co-infected mice received $1 \times 10^6$ pfu at 5 weeks. At 6 weeks of BCG infection, 9 day post LCMV or 7 day post VV-ova, mice were euthanized and their organs removed for analysis by flow cytometry. (A) Flow cytometric analysis of splenocytes isolated from naïve C57BL/6 mice as comparison. (B) Dot plots show lymphocyted-gated anti-CD4 and anti-CD8 antibody surface staining on splenocytes and granuloma infiltrating cells from the indicated groups. Numbers shown represent the fraction of the gated population shown in boxes. (C) Dot plots show lymphocyte-gated anti-CD8 antibody and specific tetramer reagent surface staining on splenocytes and granuloma infiltrating cells from the indicated test groups. Numbers shown represent the fraction of the gated population present in the upper right hand quadrant. Tetramer reagents used: SIINFEKL (MHC-I H-2 K$^b$/OVA257-264) (top) np396-404 (MHC-I H-2 D$^b$/LCMV np396-404) (bottom) Each group was composed of 3-4 mice. The experiment shown is representative of six with similar results. (D) Mean data are presented for the frequency in the total cell population of LCMV NP396-404 tetramer+ CD8+ (left) and OVA SIINFEKL tetramer+ CD8+ (right) cells in the indicated preparations. Error bars represents SEM. (E) Confocal images at 400x magnification from LCMV-superinfected BCG-infected liver granuloma (right panels) and BCG-infected liver granuloma (left panels) are shown. (Top row) Stainings show CD8-specific (FITC green) and LCMV np396 tetramer-specific (APC blue) T-cells in the liver granuloma 9 days after LCMV superinfection. (Bottom row) Stainings show CD8-specific (APC blue) and CD45.1-specific (FITC green) T-cells in the liver granuloma 9 days after P14 cell transfer and LCMV superinfection. Arrows point to double positive cells indicating accumulation of...
LCMV-specific cells. Liver autofluorescence was used to outline the lesion edges (dotted lines).

**Figure 2.** LCMV co-infection results in the accumulation of a highly activated LCMV-specific CD8 T cell population in BCG-induced granulomas and has no effect upon activated caspase 3 levels in CD4 T cells. The experimental groups shown are as described in Figure 1. (A) Dot plots show staining with anti-CD44 (left panels) or anti-LFA-1 (right panels) specific antibodies, and LCMV np396-404 tetramer on CD8 gated lymphocytes from granuloma infiltrating cells in BCG control infections or BCG+LCMV infections of C57BL/6 mice. The numbers shown represent the percentage of total cells contained in the indicated quadrants. (B) Dot plots show staining with anti-CD4 and anti-caspase 3 specific antibodies on lymphocyte-gated granuloma infiltrating cells. The numbers shown represent the CD4+ Caspase3+ percentage of the total CD4 population. (C) Mean data are presented for the frequency in the total cell population of LCMV NP396-404 tetramer+ CD8+ LFA-1+ (left) and LCMV NP396-404 tetramer+ CD8+ CD44+ (right) cells in the indicated preparations. Error bars represents SEM. (D) Mean data are presented for the frequency in the total cell population of Caspase 3+ CD4+ cells in the indicated preparations. Error bars represents SEM.

**Figure 3.** LCMV co-infection increases IFNγ mRNA levels. (A) The left-hand panel shows the relative expression level of IFNγ primer specific product in cDNAs prepared from granuloma total RNA in one experiment. The value derived from BCG control granuloma was arbitrarily set to one and the value for conA stimulated spleen cDNA is included for comparison. The right hand panel shows levels of secreted IFNγ measured by ELISA after 3 day in vitro culture in media, with mycobacterial PPD antigen, or with 5µg/ml αCD3. Error bars represent SEM. (B) Intracellular staining for IFNγ was
performed on splenocytes and granuloma infiltrating cells from either BCG alone infection or BCG+LCMV infection. Cells were cultured for 5 hours in the presence of Golgistop in media (top row), with 0.2 µg/ml LCMV np396-404 peptide (middle row), or with 5µg/ml αCD3 (bottom row). Dot plots show anti-CD8 and anti-IFNγ specific antibody staining on lymphocyte-gated cells from the indicated populations. Numbers are the percentage of gated cells in quadrants. (C) Intracellular staining for IFNγ was performed on granuloma infiltrating cells from either BCG alone infection (top rows) or BCG+LCMV infection (bottom rows). Cells were cultured overnight without Golgistop in media (left plots), with mycobacterial PPD (middle plots), or with 5µg/ml αCD3 (right plots). After Golgi-stop addition, cells were cultured for an additional 4-6 hrs before staining. Dot plots show anti-CD8 (top set) or anti-CD4 (bottom set) and anti-IFNγ specific antibody staining on lymphocyte gated cells from the indicated populations. Numbers are the percentage of gated cells in quadrants.

Figure 4. TNF levels are unchanged by LCMV co-infection. (A) The graph shows levels of secreted TNF measured by ELISA after 3 day in vitro culture in media, with 5 µl mycobacterial PPD antigen, with 1 µg/ml LCMV np396-404 peptide, or with 5µg/ml αCD3. One representative experiment of four with similar trends is shown. (B) Intracellular staining for TNF was performed on splenocytes from naïve C57BL/6 mice and from mice infected with LCMV virus for 9 days (top, left and right respectively) and on splenocytes and granuloma infiltrating cells from either BCG alone infection (lower left) or BCG+LCMV infection (lower right). Cells were cultured overnight in 5 µg/ml anti-CD3, followed by addition of Golgi-stop and an additional 4-6 hrs of incubation before staining for intracellular TNF. Dot plots represent CD8 and TNF specific antibody staining on lymphocyte gated cells. Numbers are the percentage of gated cells in quadrants.
Figure 5. Acute LCMV co-infection has no significant effect upon antitubercular protection in C57BL/6 mice at 6 weeks. (A) Pictures illustrate H&E stained (top panels) and Ziehl Neelsen stained (bottom panels) thin liver sections from the indicated groups at six weeks of infection. Microscopy was at 1000x times total magnification under oil. Arrowhead in the bottom right panel indicates an individual AFB. (B) Serial dilutions of liver homogenates were plated to determine the CFU/liver after BCG control infection, BCG+LCMV infection, or BCG+VVova infection. Dots represent values for individual mice and bars represent combined averages for the groups in three separate experiments. No statistical difference was seen between the three groups by ANOVA (n=12, 13, 7).

Figure 6. Enhanced accumulation of activated OVA-specific CD8 T cells can be found in BCG-induced chronic granulomas after OVA immunization of OT-1 mice or after acute LCMV infection of OT-1 mice. OT-1 mice were infected by i.p. injection with BCG. At 5 weeks, groups were either held as control infections, co-infected with 2x10^5 PFU LCMV strain Armstrong, or injected sq with 100µg OVA/CFA. At 6 weeks of BCG infection, 9 day post LCMV or 7 day post OVA/CFA, mice were euthanized and their organs removed for analysis by flow cytometry. (A) Dot plots show flow cytometric analysis of splenocytes isolated from naïve OT-1 mice. (B) Dot plots show lymphocyte-gated anti-CD4 and anti-CD8 antibody surface staining on splenocytes and granuloma infiltrating cells from the indicated test groups. Numbers shown represent the fraction of the gated population shown in boxes. (C) Dot plots show lymphocyte-gated anti-CD8 antibody and specific tetramer surface staining on splenocytes and granuloma infiltrating cells from the indicated test groups. Numbers shown are the fraction of the gated population present in the upper right hand quadrant. Tetramer reagents used; SIINFEKL (MHC-I H-2 K^b/OVA257-264); np396-404 (MHC-I H-2 D^b/LCMV np396-404). Each group
was composed of 2-3 mice. The LCMV data is representative of 3 similar experiments and similar results to the OVA immunization were found after co-infection at 5 weeks with recombinant VV-ova. (D) Dot plots show staining with anti-LFA-1 specific antibody and SIINFEKL tetramer on CD8 gated lymphocytes from granuloma infiltrating cells in BCG control infection, BCG+LCMV infection, or BCG+OVA imm of OT-1 mice. The numbers are the percentage of gated cells in quadrants. (E) Mean data are presented for the frequency in the total cell population of CD4+ (left), CD8+ (center left), LCMV NP396-404 tetramer+ CD8+ (center right), and LCMV NP396-404 tetramer+ CD8+ LFA-1+ (right) cells in the indicated preparations. Error bars represents SEM.

Figure 7. VV-ova or LCMV co-infection contributes to anti-mycobacterial protection in OT-1 mice at 6 weeks. (A) Pictures illustrate H&E stained (top panels) and Ziehl Neelsen stained (bottom panels) thin liver sections from the indicated groups of BCG infected OT-1 mice at six weeks of infection. Microscopy was at 1000x times total magnification under oil. Arrowheads in bottom panels indicate AFB. (B) Serial dilutions of liver homogenates were plated to determine the CFU/liver after BCG control infection (n=9), BCG+OVA imm (n=2), BCG+VV-ova infection (n=11), and BCG+LCMV infection (n=13) of OT-1 mice. Dots represent values for individual mice and bars represent combined averages for the groups in three separate experiments. The significance of the decrease in organ load from BCG infection alone was calculated by ANOVA (BCG+VV-ova p≤.0001; BCG+LCVM p<0.04). (C) The graph shows secreted IFNγ levels measured by ELISA after 3 day in vitro culture with 5 µg/ml αCD3 of splenocytes (left bars) or granuloma infiltrating cells (right bars) derived from C57BL/6 BCG alone, OT-1 BCG alone, OT-1 BCG+OVA immunization, and OT-1 BCG+LCMV. Error bars represents SEM.
**Figure A.**

![Graph showing cytokine production](image)

**Figure B.**

![Flow cytometry plots](image)

**Table:**

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<tr>
<th>Condition</th>
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**Legend:**
- media
- PPD
- LCMV 396 peptide
- αCD3

**Units:** pg/ml