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CD4\(^+\) T Cell-Dependent IFN-\(\gamma\) Production by CD8\(^+\) Effector T Cells in *Mycobacterium tuberculosis* Infection

Tyler D. Bold* and Joel D. Ernst*†‡

Both CD4\(^+\) and CD8\(^+\) T cells contribute to immunity to tuberculosis, and both can produce the essential effector cytokine IFN-\(\gamma\). However, the precise role and relative contribution of each cell type to in vivo IFN-\(\gamma\) production are incompletely understood. To identify and quantitate the cells that produce IFN-\(\gamma\) at the site of *Mycobacterium tuberculosis* infection in mice, we used direct intracellular cytokine staining ex vivo without restimulation. We found that CD4\(^+\) and CD8\(^+\) cells were predominantly responsible for production of this cytokine in vivo, and we observed a remarkable linear correlation between the fraction of CD4\(^+\) cells and the fraction of CD8\(^+\) cells producing IFN-\(\gamma\) in the lungs. In the absence of CD4\(^+\) cells, a reduced fraction of CD8\(^+\) cells was actively producing IFN-\(\gamma\) in vivo, suggesting that CD4\(^+\) effector cells are continually required for optimal IFN-\(\gamma\) production by CD8\(^+\) effector cells. Accordingly, when infected mice were treated i.v. with an MHC-II-restricted *M. tuberculosis* epitope peptide to stimulate CD4\(^+\) cells in vivo, we observed rapid activation of both CD4\(^+\) and CD8\(^+\) cells in the lungs. Indirect activation of CD8\(^+\) cells was dependent on the presence of CD4\(^+\) cells but independent of IFN-\(\gamma\) responsiveness of the CD8\(^+\) cells. These data provide evidence that CD4\(^+\) cell deficiency impairs IFN-\(\gamma\) production by CD8\(^+\) effector cells and that ongoing cross-talk between distinct effector T cell types in the lungs may contribute to a protective immune response against *M. tuberculosis*. Conversely, defects in these interactions may contribute to susceptibility to tuberculosis and other infections. *The Journal of Immunology*, 2012, 189: 2530–2536.

In humans and mice, adaptive immune responses to *Mycobacterium tuberculosis* involve CD4\(^+\) and CD8\(^+\) T cells (1, 2) and the essential cytokine IFN-\(\gamma\) (3–5). There is no consensus on the relative contribution and significance of CD8\(^+\) cells in tuberculosis, with some studies supporting their importance (6–8) and others indicating that they are dispensable (9–11). In contrast, CD4\(^+\) cells are clearly essential for host defense against *M. tuberculosis*. HIV infection is a major risk factor for development of active disease; in HIV-infected people, the risk for active tuberculosis is proportional to the number of peripheral blood CD4\(^+\) T cells (12). Furthermore, *M. tuberculosis*-infected mice that are deficient in, or depleted of, CD4\(^+\) cells have drastically reduced survival compared with wild-type mice or those lacking CD8\(^+\) cells (1). However, the specific contributions of CD4\(^+\) cells to immunity to tuberculosis are not fully understood, and it is not known precisely why this cell type is so critical for control of infection (13).

Although it is widely believed that CD4\(^+\) cells are an essential source of IFN-\(\gamma\) in tuberculosis, this is unlikely to be their sole contribution to host defense. Notably, one recent study demonstrated that effector CD4\(^+\) T cells specific for the *M. tuberculosis* Ag ESAT-6 can provide protection to *M. tuberculosis*-infected mice without producing IFN-\(\gamma\) themselves, highlighting the significance of other, undefined CD4\(^+\) cell effector functions (14). Conversely, production of this cytokine is unlikely to be exclusive to CD4\(^+\) cells. Although the extent to which other cells contribute to IFN-\(\gamma\) in vivo remains unclear, numerous other cell types, including CD8\(^+\) cells, \(\gamma\)\(\delta\) T cells, NK cells, NKT cells, and even cells of myeloid lineage, are capable of IFN-\(\gamma\) production during *M. tuberculosis* infection (15–18). Consistent with these observations, mice depleted of CD4\(^+\) cells retain some IFN-\(\gamma\) expression and survive infection longer than do mice deficient in IFN-\(\gamma\) (19, 20), indicating that other sources of this protective cytokine exist in vivo. These results suggest the possibility of nonclassical modes of IFN-\(\gamma\) production and indicate that the in vivo function of CD4\(^+\) effector cells in tuberculosis warrants further study.

Recently, CD4\(^+\) cells were shown to play an important role in the long-term development and function of CD8\(^+\) cell responses in *M. tuberculosis* and other infections, and they are required for optimal CD8\(^+\) memory cell responses (21–27). Less is known about the short-term influence of CD4\(^+\) effector cells on CD8\(^+\) effector cells at the site of infection, and the determinants governing effector T cell function in peripheral tissues are not well understood. In assays involving ex vivo restimulation, both CD4\(^+\) and CD8\(^+\) T cells are capable of IFN-\(\gamma\) production; however, of the two, CD4\(^+\) T cells appear to have a greater overall capacity for production of the cytokine (19). To study the in vivo function of CD4\(^+\) and CD8\(^+\) effector cells, assays that can quantitate in vivo cytokine production, rather than assess cell capabilities, are required. For this purpose, we and other investigators have used direct intracellular cytokine-staining techniques without ex vivo restimulation (28–35).

In this study, we used this technique to determine the relative contribution of various cell types to IFN-\(\gamma\) production at the site of infection in the lungs. This approach also provided a method to examine whether CD4\(^+\) T cells play an important role in potentiating the optimal response of CD8\(^+\) T cells in vivo. We observed that CD4\(^+\) effector T cells in the lungs positively and immediately influence the effector function of CD8\(^+\) T cells. These findings
reveal an interaction that expands the range of activities of CD4+ T cells during M. tuberculosis infection and identify an activation mechanism for CD8+ effector T cells in vivo that is independent of Ag recognition.

Materials and Methods

Mice

C57BL/6, IFN-γ−/−, IFN-γR1−/−, and MHC-II−/− (B6.129S2-H2d(M1)Ifnγt1) mice for aerosol M. tuberculosis infection experiments were either bred in the New York University School of Medicine Skirball animal facility or purchased from Taconic Farms. All animal experiments were done in accordance with procedures approved by the New York University School of Medicine Institutional Animal Care and Use Committee and in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health under the Assurance of Compliance Number A3435-01.

Aerosol infection of mice

Mice at 8–12 wk of age were infected with ~100 CFU M. tuberculosis H37Rv via the aerosol route using an Inhalation Exposure Unit (Glas-Col), as previously described (36). To verify inoculum size, three to five infected mice were euthanized 24 h postinfection, and lungs were homogenized and plated on Middlebrook 7H11 medium supplemented with 10% v/v albumin, dextrose, catalase enrichment. To determine bacterial population size at time points postinfection, lungs were homogenized, diluted in PBS + dextrose catalase enrichment. To determine bacterial population size at time points postinfection, lungs were homogenized, diluted in PBS + Tween-80 (0.5%), and added to 7H11 plates. Plates were incubated at 37°C for 3 wk, and single colonies were counted.

Tissue processing and flow cytometry

To isolate cells from infected tissues for flow cytometry, mice were euthanized with CO2 followed by cervical dislocation. Tissues were removed and mechanically disrupted using a gentleMACS dissociator (Miltenyi Biotec) in the manufacturer-recommended HEPS buffer. Lung suspensions were incubated in Collagenase D and DNase at 37°C with 5% CO2 for 30 min, and cells were isolated by forcing suspensions through a 70-μm cell strainer. RBCs were removed by ACK lysis, and live cells were counted by trypan blue exclusion. Cell suspensions were stained using the following fluorescently labeled Abs from BioLegend, BD Pharmingen, or eBioscience: anti-CD8 FITC, anti-CD3 PE, anti-CD4 (L3T4) FITC or Pacific Blue, anti-ΔX5-FITC, anti-IFN-γ (XM1G1.2) allophycocyanin, and rat IgG1 allophycocyanin isotype control. Flow cytometry was performed using a FACSCalibur or LSR II (BD Biosciences) at the New York University Cancer Institute Flow Cytometry and Cell Sorting facility. Analysis of flow cytometry data was performed using FlowJo software.

Detection of IFN-γ-producing cells by direct intracellular cytokine staining

To detect intracellular IFN-γ produced by cells in vivo, a protocol was developed based on a previous study (35). However, in contrast to this study, optimal detection of IFN-γ-producing cells from the lungs of mice infected with M. tuberculosis did not require treatment of mice with i.v. brefeldin A or inclusion of brefeldin A in tissue-processing buffers (Supplemental Fig. 1). Instead, after euthanasia, tissues were rapidly placed on ice, and all cell-isolation steps with the exception of collagenase/DNase digestion (37°C for 30 min) were carried out quickly and on ice. Cells were stained for surface markers at 4°C for 30 min, followed by permeabilization and fixation with Cytofix/Cytoperm (BD Biosciences) at 4°C for 30 min. Finally, fixed cells were stained with anti-IFN-γ or a rat IgG1 isotype control at 4°C for 30 min. Flow cytometry dot plot gates for IFN-γ+ cells were set based on comparison with isotype control and unpermeabilized cells stained for IFN-γ.

CD4+ T cell depletion

Mice were treated i.p. with 500 μg mAb GK1.5, which depletes CD4+ T cells, or a rat IgG2b isotype control (LTF-2). Efficiency of CD4+ T cell depletion after GK1.5 treatment was determined to be 96.2% by flow cytometry of cell suspensions from lungs, spleen, and blood. In mice treated with LTF-2 isotype control, no differences were observed in CD4+ T cell number or bacterial burden compared with untreated mice.

Systemic treatment of mice with synthetic peptides

Mice were treated i.v. with peptides containing the following epitopes: for CD4+ T cells, Ag85B peptide 25 (FQDAYNAAGGHNA VF) and for CD8+ T cells, Mb32A109--318 (GAPINSATAM). Peptides were synthesized by EZBiolab to a purity > 95%, and they were initially dissolved and stored in DMSO at 10 mg/ml. One hundred micromgrams of stock peptide was diluted in 100 μl sterile PBS and administered via tail vein or retro-orbital sinus, and tissues were harvested for intracellular cytokine staining from 2 to 6 h after injection.

Results

Identifying cells stimulated in vivo to produce IFN-γ

To determine the relative contribution of various cell types capable of producing IFN-γ, we used direct intracellular cytokine staining without ex vivo restimulation to identify which cells are stimulated to produce this crucial cytokine in the lungs of mice infected with M. tuberculosis. We first identified a postinfection time point when IFN-γ protein is abundant in the lungs by assaying IFN-γ in lung tissue homogenates throughout the course of infection. IFN-γ protein was first detected 21 d postinfection, increasing to a peak at day 28 and decreasing to a lower level by days 42–49 (Fig. 1A). These results were consistent with our previous observations that, because of a delay of 10–12 d in the initiation of adaptive immunity postinfection, effector CD4+ T cells with specificity for an M. tuberculosis Ag are first recruited to the lungs beginning on day 17 postinfection. Likewise, IFN-γ mRNA is first detectable on day 17 and reaches a peak on day 28 postinfection (37). Based on these findings, in subsequent experiments we chose to characterize the IFN-γ responses of lung cells on day 28 postinfection.

To identify the cells actively producing IFN-γ in the lungs during infection with M. tuberculosis, we isolated cells from infected mice and stained them for intracellular IFN-γ without restimulation. IFN-γ+ cells were readily detectable, making up 2–5% of the total leukocyte population (Fig. 1B); the staining procedure was specific for intracellular IFN-γ and did not include surface-bound IFN-γ, as determined by staining of nonpermeabilized cells (Supplemental Fig. 1). In previous studies in which in vivo IFN-γ production was examined, mice were treated with brefeldin A, and cells were processed in the presence of brefeldin A to prevent secretion of intracellular cytokines (34, 35). However, we found that if tissues were processed expediently and on ice, this treatment was not required and did not increase the detection of IFN-γ-producing cells (Supplemental Fig. 1). We also verified the specificity of the XM1G1.2 Ab for IFN-γ–producing cells by comparing cells from the lungs of infected C57BL/6 mice with those from infected ifng−/− mice and from uninfected C57BL/6 mice. For all cell types examined, with the exception of CD11b+, Gr-1+ neutrophils, IFN-γ staining was highly specific for cells from infected IFN-γ–replete C57BL/6 mice (Supplemental Fig. 2). Although IFN-γ production by neutrophils has been described in a variety of contexts (38–42), we determined that the XM1G1.2 Ab bound nonspecifically to intracellular neutrophil components, raising questions about the significance of these previous reports. Therefore, we gated this cell type out of subsequent analyses. Analysis of the cells producing IFN-γ protein in the lungs of infected mice showed that 90% were CD3+ T cells; of these, 65% were CD4+ T cells, and 35% were CD8+ T cells (Fig. 1C, 1D). These data confirm results obtained in other studies that used ex vivo restimulation and indicate that IFN-γ production during M. tuberculosis infection is mostly accounted for by CD4+ T cells, with a smaller, but significant, contribution from CD8+ T cells (43). The CD3− component of the IFN-γ+ cell population was largely accounted for by Dx5+ NK cells. Although most IFN-γ+ cells at day 28 postinfection were CD4+ T cells, only a small percentage of all CD4+ T cells in the lungs was activated in vivo to produce this effector cytokine. This was also true for CD8+ T cells and Dx5+ NK cells.
Direct relationship of CD4+ and CD8+ T cell activation in vivo

Although other studies analyzed IFN-γ production by CD4+ and CD8+ T cells during M. tuberculosis infection, most of these used intracellular cytokine-staining assays based on ex vivo restimulation (23, 44, 45), which provides information about the functional capabilities of cells but not their functional activity in vivo. Therefore, we examined the relationship of CD4+ and CD8+ T cell activation in vivo in the lungs of infected mice and observed a linear correlation between the percentage of CD4+ T cells producing IFN-γ and the percentage of CD8+ T cells producing IFN-γ on day 28 postinfection (Fig. 2A, 2B). Similar correlations were observed on days 21 and 35 postinfection (data not shown).

We hypothesized that CD4+ T cells may be required for optimal activation of CD8+ effector T cells at the site of M. tuberculosis infection. To determine whether there is a causal relationship between CD4+ T cells and the production of IFN-γ by CD8+ T cells in vivo, we compared the percentage of CD8+ T cells producing IFN-γ in the lungs of CD4+ T cell-deficient MHC-II–/– mice infected with M. tuberculosis with that in wild-type C57BL/6 mice. We observed that the percentage of CD8+ T cells producing IFN-γ in the lungs of wild-type mice was 2.1-fold higher (p = 0.03) than in MHC-II–/– mice (Fig. 2C), suggesting that CD4+ T cells promote IFN-γ production by CD8+ T cells in vivo.

Acute influence of CD4+ effector T cells on IFN-γ production by CD8+ effector T cells

Because of the correlation between IFN-γ-producing CD4+ and CD8+ T cells in the lungs, we hypothesized that the influence of CD4+ T cells is exerted in an active and ongoing manner at the site of infection. We tested this by acutely depleting CD4+ T cells from the lungs of C57BL/6 mice. Compared with mice treated with the CD4+ T cell-depleting GK1.5 Ab 24 h prior to lung cell harvest, mice treated with an isotype control Ab demonstrated 2- to 4-fold higher percentages of CD8+ T cells producing IFN-γ (Fig. 3). This indicates that CD4+ T cells are continually required for optimal CD8+ effector function during M. tuberculosis infection.

Activation of CD4+ effector T cells in vivo induces IFN-γ production by CD8+ effector T cells

The observation that depletion of CD4+ T cells reduced the frequency of CD8+ T cells producing IFN-γ prompted us to determine whether acutely and selectively increasing the activation of CD4+ T cells could increase the frequency of IFN-γ+ CD8+ T cells. We and other investigators recently reported that CD4+ effector T cells are suboptimally activated in the lungs of infected mice and that this is due, in part, to the limited availability of an immunodominant Ag, Ag85B (31, 46). This Ag deficit could be overcome by administration of a synthetic peptide corresponding to a well-characterized I-Aβ–restricted CD4+ T cell epitope from Ag85B (aa 240–254), termed “peptide 25.” Intravenous administration of peptide 25 to mice infected with M. tuberculosis caused rapid activation of the effector CD4+ T cells already recruited to the lungs that are specific for this Ag and increased the frequency of IFN-γ+ CD4+ T cells. When CD4+ effector T cells in the lungs of infected mice were activated by peptide 25 injection, we also observed increased IFN-γ+ CD8+ T cells (Fig. 4A). This effect of the peptide 25-dependent increase was specific; when we injected it into M. tuberculosis-infected mice that had not developed CD4+ effector T cells responsive to peptide 25 [because of infection with a strain from which the gene encoding Ag85B was deleted (37, 46)], there was no increase in the frequency of IFN-γ+ CD4+ T cells in the lungs (Supplemental Fig. 3). Consistent with our observation of a direct relationship between CD4+ and CD8+ effector T cell activation in the lungs, this demonstrates that activation of CD4+ effector T cells induces IFN-γ production by CD8+ effector T cells at the site of M. tuberculosis infection.

Although peptide 25 is described as an MHC class II-restricted epitope, presented to CD4+ T cells by mouse I-Aβ, we further verified that activation of CD8+ T cells after peptide 25 treatment was a consequence of CD4+ T cell activation and not the result of
presentation of a cryptic MHC I-restricted epitope in peptide 25. When CD4+ T cell-deficient MHC-II-/- mice were treated with peptide 25, we observed no increase in CD8+ T cell activation (Fig. 4B). The failure of CD8+ T cells to respond to peptide 25 injection in MHC-II-/- mice was not due to impaired function of CD8+ T cells as a result of CD4+ T cell deficiency, because CD8+ cells were capable of being activated by injection of Mtb32A309-318, a well-characterized MHC I-restricted epitope from *M. tuberculosis* (Fig. 4B). These results suggest that IFN-γ production by CD8+ effector T cells in *M. tuberculosis* infection is acutely influenced by the activation status of CD4+ effector T cells and may be at least partially independent of TCR Ag recognition.

**CD4+ T cell-dependent activation of CD8+ T cells does not require IFN-γ responsiveness**

To better understand the mechanism by which CD4+ effector T cells influence activation of CD8+ T cells, we investigated whether the influence of CD4+ T cells on CD8+ T cells requires that the CD8+ T cells respond to IFN-γ produced by activated CD4+ T cells. When we infected IFN-γR1-/- mice and treated them with i.v. peptide 25, as with C57BL6 mice, we observed increased activation of both CD4+ and CD8+ T cells (Fig. 5), indicating that the influence of CD4+ T cells on CD8+ T cells does not require the ability of CD8+ T cells to respond to IFN-γ. Compared with wild-type mice, IFN-γR1-/- mice demonstrated significantly larger fold increases in the activation of both CD4+ and CD8+ T cells after injection of peptide 25 (Fig. 5), suggesting that T cells that develop in the absence of IFN-γ signals may be more capable of activation and effector cytokine production.

**Discussion**

In this study, we re-evaluated the essential role of CD4+ effector T cells in immunity to tuberculosis, focusing on in vivo production of IFN-γ, the consequences of CD4+ T cell deficiency, and their interaction with CD8+ effector cells in the lungs. One possible explanation for the great significance of CD4+ T cells in tuberculosis is that *M. tuberculosis* is classically understood to survive within the phagosomes of cells it infects. This property argues for a predominant role of the endocytic pathway for Ag presentation by MHC II and activation of CD4+ cells. However, recent studies revealed that *M. tuberculosis* and its Ags are ca-
In this context, IFN-γ provides protection to mice in secondary infection (7, 45). In transferred into IFN-γ, the lung bacterial burden by a greater amount when they were compared with those in infected mice not receiving peptide. Graphs depict the fraction of IFN-γ+ among CD3+ CD4+ (left panel) or CD3+, CD8+ (right panel) T cells in the lungs. Data are representative of two independent experiments. *p < 0.05.

FIGURE 5. Indirect activation of CD8+ T cells with peptide 25 is independent of IFN-γ signaling. C57BL/6 or IFN-γR−/− mice infected with M. tuberculosis were injected i.v. with Ag85B peptide 25 to activate CD4+ T cells. Two hours after injection, the frequency of IFN-γ+ T cells was compared with those in infected mice not receiving peptide. Depletion or activation of CD4+ effector T cells in the lungs exerted an effect on IFN-γ production by CD8+ T cells within a matter of hours, implying that ongoing cross-talk between the cell types influences CD8+ effector T cell function on a short time scale. Our studies further illustrate the usefulness of direct intracellular cytokine staining without ex vivo restimulation for studying immune responses in vivo, because the effect of CD4+ T cells on CD8+ T cell production of IFN-γ would not have been identified if we had used polyclonal ex vivo restimulation.

Although the underlying mechanism and biological relevance to immune control of tuberculosis of this phenomenon is unclear, we determined that CD8+ T cells did not require the ability to sense IFN-γ produced by CD4+ effector T cells to be influenced by them. Interestingly, both CD4+ and CD8+ T cells were hypersensitive to activation in the context of IFN-γR deficiency. This result could reflect the inability of effector T cells in these mice to receive IFN-γ signals that result in death of a subset of this population in wild-type mice (55) or the absence of an IFN-γ-induced negative-feedback mechanism that limits the development of inflammatory T cell responses (56). Other candidates by which the CD4+ effector T cell influence on CD8+ T cells may be mediated include CD40–CD40L interactions between the cell types, required for CD4+ T cell help of naive CD8+ T cells (57, 58), and the cytokines IL-12, IL-15, and IL-18, all of which have been implicated in cytokine-driven IFN-γ production or activation of CD8+ T cells (38, 59). Moreover, a recent study revealed that these and other cytokines can act synergistically to promote IFN-γ production by CD8+ effector memory T cells (30). Future studies aim to precisely identify the mechanism by which CD4+ T cells potentiate the activation of CD8+ effector T cells to determine whether this phenomenon is important for control of infection with M. tuberculosis. Identification of an Ag-independent pathway for activation of CD8+ effector T cells could provide an important new avenue for pursuit of therapies against tuberculosis, especially in CD4+ T cell-deficient individuals.
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Disclosures
The authors have no financial conflicts of interest.

References


Figure S1. Validation of direct intracellular staining for IFN-γ producing cells. (A) Controls to verify the specificity of XMG1.2 antibody staining for intracellular IFN-γ included unpermeabilized cells as well as permeabilized cells treated with isotype control antibody. Flow cytometry plots show all leukocyte-gated lung cells from day 28 post-infection. Values indicate the frequency of cells in the population falling within the gate depicted. (B) Brefeldin A treatment of mice or cells during tissue harvest does not affect detection of intracellular IFN-γ. Flow cytometry plots show CD3⁺CD4⁺ cells from lungs on day 28 post-infection. Mice infected with *M. tuberculosis* were treated with 250 μg intravenous brefeldin A or left untreated. 6 hours after treatment, lungs were processed on ice in buffer alone or in buffer containing brefeldin A (20 μg/mL). (C) Graphical representation from data in B, from n=3 mice per treatment group. “n.s.”: not significant. Data are representative of at least 2 independent experiments.
Figure S2. XMG1.2 antibody stains neutrophils non-specifically. XMG1.2 antibody binding to lung cells after permeabilization was compared in 3 different circumstances: Infected C57BL/6 or ifng<sup>−/−</sup> mice or uninfected C57BL/6 mice. (A) XMG1.2 binds intracellular components of lung neutrophils from each of these three groups; however, among CD3<sup>+</sup> populations, only cells from <i>M. tuberculosis</i> infected C57BL/6 mice stained. Flow cytometry plots show a representative mouse from each group, with different cell types from the same mouse shown. Values indicate the frequency of cells binding XMG1.2 antibody in each cell subset. (B) Graphic depiction of data from A. Asterisks indicate statistical significance of differences in frequency of IFN-γ<sup>+</sup> cells detected among one cell type between different treatment groups, n=3 mice. * p<0.05; ** p<0.005. “n.s.”: not significant. Data are representative of at least 2 independent experiments.
Figure S3. Dependence of increased CD8+ T cell activation on CD4+ T cell activation after injection of a CD4+ T cell epitope peptide. During infection with H37Rv (top row), injection of Ag85B peptide 25 increased the frequency of IFN-γ+ production by both CD4+ and CD4- lymphocytes in vivo. In contrast, during infection with Ag85B-deficient M. tuberculosis (bottom row), treatment with peptide 25 did not alter the frequency of IFN-γ-producing cells among either cell type. C57BL/6 mice were infected with either M. tuberculosis H37Rv or an Ag85B-null strain. On day 21 post-infection, 4 mice from each infection group were injected intravenously with peptide 25 (100 μg), and 4 mice received a vehicle control. 6 hours later IFN-γ production by CD4+ and CD4- lymphocytes was assessed by direct intracellular cytokine staining and flow cytometry. ** p<0.005; *** p<0.0001; “n.s.”: not significant.