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CD4+ T cell dependent IFN-γ production by CD8+ effector T cells in *M. tuberculosis* infection

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Abstract

Both $CD4^+$ and $CD8^+$ T cells contribute to immunity to tuberculosis, and both can produce the essential effector cytokine IFN-γ. However, the precise role and relative contribution of each cell type to in vivo IFN-γ production are incompletely understood. To identify and quantitate the cells that produce IFN-γ at the site of *M. 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-γ in the lungs. In the absence of $CD4^+$ cells, a reduced fraction of $CD8^+$ cells were actively producing IFN-γ in vivo, suggesting that $CD4^+$ effector cells are continually required for optimal IFN-γ production by $CD8^+$ effector cells. Accordingly, when infected mice were treated intravenously 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-γ responsiveness of the $CD8^+$ cells. These data provide evidence that $CD4^+$ cell deficiency impairs IFN-γ production by $CD8^+$ effector cells and that ongoing crosstalk 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 TB and other infections.

INTRODUCTION

In humans and mice, adaptive immune responses to *Mycobacterium tuberculosis* involve $CD4^+$ and $CD8^+$ T cells (1, 2) and the essential cytokine IFN-γ (3–5). There is currently 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). On the other hand, $CD4^+$ cells are clearly essential for host defense against *M. tuberculosis*. HIV infection is a major risk factor for development of active disease, and in HIV-infected people, the risk of 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 when compared to wild type mice or those lacking $CD8^+$ cells (1). However, the specific

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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).

While it is widely believed that $CD4^+$ cells are an essential source of IFN-γ 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* antigen ESAT-6 can provide protection to *M. tuberculosis*-infected mice without producing IFN-γ 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-γ in vivo remains unclear, numerous other cell types, including $CD8^+$ cells, γδ T cells, NK cells, NKT cells and even cells of myeloid lineage, are capable of IFN-γ production during *M. tuberculosis* infection (15–18). Consistent with these observations, mice depleted of $CD4^+$ cells retain some IFN-γ expression and survive infection longer than mice deficient in IFN-γ (19, 20), indicating that other sources of this protective cytokine exist in vivo. These results suggest the possibility of non-classical modes of IFN-γ production and indicate that the in vivo function of $CD4^+$ effector cells in tuberculosis warrants further study.

Recently, $CD4^+$ cells have been shown to play an important role in the long-term development and function of $CD8^+$ cell responses in *M. tuberculosis* and other infections, and 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-γ production, but of the two, $CD4^+$ T cells appear to have a greater overall capacity for production of the cytokine (19). To study 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 others have employed direct intracellular cytokine staining techniques without ex vivo restimulation (28–35).

Here we have used this technique to determine the relative contribution of various cell types to IFN-γ 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 antigen recognition.

MATERIALS AND METHODS

Mice

C57BL/6, IFN-$\gamma^{-/-}$, IFN-γR1$^{-/-}$, and MHC-II$^{-/-}$ (B6.129S2-*H2d*$^{lAb1\text{-}Ea}$/J) 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, Inc. All animal experiments were done in accordance with procedures approved by the NYU 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 weeks of age were infected with ~100 CFU of *M. tuberculosis* H37Rv via the aerosol route using an Inhalation Exposure Unit (Glas-Col) as previously described (36). To

verify inoculum size, 3–5 infected mice were euthanized 24 hours after infection 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 post-infection, lungs were homogenized, diluted in PBS + Tween-80 (0.5%), and added to 7H11 plates. Plates were incubated at 37° C for 3 weeks and single colonies were counted.

Tissue processing and flow cytometry

To isolate cells from infected tissues for flow cytometry, mice were euthanized with CO_2 followed by cervical dislocation. Tissues were removed and mechanically disrupted by using a gentleMACS™ dissociator (Miltenyi Biotec) in the manufacturer-recommended HEPES buffer. Lung suspensions were incubated in Collagenase D and DNase at 37° C with 5% CO_2 for 30 minutes and cells were isolated by forcing suspensions through a 70 mM cell strainer. RBCs were removed by ACK lysis and live cells counted by trypan blue exclusion. Cell suspensions were stained using the following fluorescently-labeled antibodies (Biolegend, BD Pharmingen, or eBioscience): anti-CD8 FITC, anti-CD3 PE, anti-CD4 (L3T4) FITC or Pacific Blue, anti-DX5-FITC, anti-IFN-γ (XMG1.2) APC, and rat IgG1 APC isotype control. Flow cytometry was performed using a FACSCalibur or LSR II (BD Biosciences) at the NYU 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). In contrast to this study, however, 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 (Figure S1). Instead, after euthanasia, tissues were rapidly placed on ice and all cell isolation steps except collagenase/DNase digestion (37° C for 30 minutes) and ACK lysis (room temperature for 5 minutes) were carried out quickly and on ice. Cells were stained for surface markers at 4° C for 30 minutes followed by permeabilization and fixation with Cytofix/Cytoperm (BD Biosciences) at 4° C for 20 minutes. Finally, fixed cells were stained with anti-IFN-γ or a rat IgG1 isotype control at 4° C for 30 minutes. 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 with an intra-peritoneal dose of 500 μg of either monoclonal antibody 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 when compared to untreated mice.

Systemic treatment of mice with synthetic peptides

Mice were intravenously treated with peptides containing the following epitopes: for $CD4^+$ T cells, Ag85B peptide 25 (FQDAYNAAGGHNAVF); for $CD8^+$ T cells, $Mtb32A_{309–318}$ (GAPINSATAM). Peptides were synthesized by EZBiolab to a purity of >95% and initially dissolved and stored in DMSO at 10 mg/ml. 100 μg 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 2–6 hours 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 post-infection 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 days after infection, rising to a peak at day 28, and decreasing to a lower level by day 42 to 49 (Figure 1A). These results were consistent with our previous observations that, because of a delay of 10–12 days in the initiation of adaptive immunity after infection, effector $CD4^+$ T cells with specificity for an *M. tuberculosis* antigen are first recruited to the lungs beginning on day 17 post-infection. Likewise, IFN-γ mRNA is first detectable on day 17 and reaches a peak on day 28 post-infection (37). Based on these findings, for subsequent experiments we chose to characterize the IFN-γ responses of lung cells on day 28 post-infection.

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 (Figure 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 (Figure S1). In previous studies where in vivo IFN-γ production was examined, mice were treated with brefeldin A and cells 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 detection of IFN-γ producing cells (Figure S1). We also verified the specificity of the XMG1.2 antibody for IFN-γ producing cells by comparing cells from the lungs of infected C57BL/6 mice to those from infected *ifng*$^{-/-}$ 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 (Figure S2). Although IFN-γ production by neutrophils has been described in a variety of contexts (38–42), we determined that the XMG1.2 monoclonal antibody bound non-specifically 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 $CD4^+$ and 35% were $CD8^+$ T cells (Figure 1C and 1D). These data confirm results obtained in other studies that employed 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 post-infection were $CD4^+$ T cells, only a small percentage of all $CD4^+$ T cells in the lungs were 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

While other studies have analyzed IFN-γ production by $CD4^+$ and $CD8^+$ T cells during *M. tuberculosis* infection, most of these employed 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. We therefore 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 post-infection (Figure 2A and 2B). Similar correlations were observed on days 21 and 35 post-infection (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 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* to 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 (Figure 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. When compared to mice treated with the $CD4^+$ T cell-depleting GK1.5 antibody 24 hours prior to lung cell harvest mice treated with an isotype control antibody demonstrated 2- to 4-fold higher percentages of $CD8^+$ T cells producing IFN-γ (Figure 3A and 3B). 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 activation of $CD4^+$ T cells could increase the frequency of IFN-γ^+ CD8 T cells. We and others 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 antigen, Ag85B (31, 46). This antigen deficit could be overcome by administration of a synthetic peptide corresponding to a well-characterized I-A^b-restricted $CD4^+$ T cell epitope from Ag85B (amino acids 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 antigen, 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 (Figure 3A and 3B). This effect of peptide 25-dependent increase was specific, as when we injected it into *M. tuberculosis*-infected mice that had not developed CD4 effector T cells responsive to peptide 25 (due to infection with a strain from which the gene encoding Ag85B was deleted (37, 46)), there was no increase in the frequency of IFN-γ^+ $CD4^-$ cells in the lungs (Figure S3). 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-A^b, 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 (Figure 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,

as $CD8^+$ cells were capable of being activated by injection of $Mtb32A_{309–318}$, a well-characterized MHC I-restricted epitope from *M. tuberculosis* (Figure 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 T cell receptor antigen 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-$\gamma R1^{-/-}$ mice and treated them with intravenous peptide 25, as with C57BL6 mice, we observed increased activation of both $CD4^+$ and $CD8^+$ T cells (Figure 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. When compared to wild type mice, IFN-$\gamma R1^{-/-}$ mice demonstrated significantly larger fold-increases in the activation of both $CD4^+$ and $CD8^+$ T cells after injection of peptide 25 (Figure 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 reevaluated the essential role of $CD4^+$ effector T cells in immunity to TB, 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 TB is that *M. tuberculosis* is classically understood to survive within the phagosome of cells it infects. This property argues for a predominant role of the endocytic pathway for antigen presentation by MHC II and activation of $CD4^+$ cells. However, recent studies have revealed that *M. tuberculosis* and its antigens are capable of escaping the phagosome to enter the cytosolic compartment, and peptide epitopes are available for presentation by MHC I (47, 48). Concordantly, *M. tuberculosis* induces robust antigen specific $CD8^+$ T cell responses to several MHC I-restricted epitopes (49–51), and antigen specific $CD8^+$ T cells are capable of providing protection to mice in secondary infection (7, 45). In this, IFN-γ and perforin production are among the required effector functions (52, 53). Although both $CD4^+$ and CD8 cells are important components of immunity to TB, the unique mechanisms by which these cells control *M. tuberculosis* infection remain incompletely defined (13). Deficiency in $CD4^+$ cells greatly increases susceptibility to mycobacterial disease, as evidenced by the high rates of active disease in HIV/TB coinfection, yet the precise reason for this phenomenon is unclear. Mice deficient in $CD8^+$ T cells also succumb to infection earlier than wild type mice, yet they are less susceptible than mice deficient in $CD4^+$ T cells (1). This indicates that, although $CD8^+$ T cells are required for optimal anti-mycobacterial immunity, in the absence of $CD4^+$ cells, CD8 cells are not sufficient to control infection.

The major new finding in this study is that $CD4^+$ T cells actively influence CD8 T cells in a peripheral nonlymphoid tissue (the lungs) and increase $CD8^+$ T cell production of IFN-γ during infection with *M. tuberculosis*. Prior studies of $CD4^+$ T cell influences on $CD8^+$ T cells have focused on establishment and/or maintenance of $CD8^+$ T cell memory in the context of infection, while our studies reveal an additional effect that is acute and ongoing during infection. This effect of $CD4^+$ T cells does not involve $CD8^+$ T cell development, is not attributable to epitope cross-reactivity, does not require IFN-γ responsiveness of the $CD8^+$ T cells, and accounts for approximately 60% of the IFN-γ-producing CD8 T cells in the lungs of *M. tuberculosis*-infected mice. Our findings may provide a partial explanation for the recent finding that *M. tuberculosis* antigen-specific $CD4^+$ Th1 effector cells exhibit

in vivo antimycobacterial activity without producing IFN-γ themselves (14). In that study, adoptive transfer of ESAT-6-specific IFN-$\gamma^{-/-}$ $CD4^+$ Th1 effector cells reduced the lung bacterial burden by a greater amount when they were transferred into IFN-$\gamma^{+/+}$ recipients than IFN-$\gamma^{-/-}$ recipients. Our finding that $CD8^+$ T cells need not be IFN-γ-responsive themselves in order to respond to $CD4^+$ T cells with increased production of IFN-γ suggest that at least some of the greater benefit of transferring antigen-specific $CD4^+$ T cells into IFN-$\gamma^{+/+}$ versus IFN-$\gamma^{-/-}$ mice is attributable to activation of recipient $CD8^+$ T cells to produce IFN-γ in vivo.

Our data on the relative contribution of various cell types to in vivo IFN-γ production support the notion that $CD4^+$ T cell deficiency increases susceptibility to *M. tuberculosis* because of the loss of a significant portion of the cells that produce this cytokine. While we observed that $CD4^+$ T cells are the most abundant cell type producing IFN-γ in the lungs of infected mice, we found that $CD8^+$ T cells make a substantial contribution as well. One recent study suggests that, in the absence of $CD4^+$ T cells, mice susceptible to *M. tuberculosis* can be partially protected by vaccine-induced activation of $CD8^+$ T cells (45). Therefore, it remains unclear why IFN-γ production by $CD8^+$ T cells, while necessary, is not sufficient for control of the infection in the absence of $CD4^+$ cells (52). One possible explanation suggests that $CD8^+$ T cells specifically target only certain cell types, and may be particularly important for activation of cells that do not express MHC II (54). As an alternative possibility, our data imply that in part, the increased susceptibility of $CD4^+$ T cell deficient mice may be the consequence of the requirement for $CD4^+$ T cell activity in optimal $CD8^+$ effector T cell function. However, in contrast to other studies showing a role for $CD4^+$ T cells in the development and maintenance of fully functional $CD8^+$ memory T cells over long time periods, we observed more immediate effects. 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 crosstalk between the cell types influences $CD8^+$ effector T cell function on a short time scale. Our studies further illustrate the utility of direct intracellular cytokine staining without ex vivo restimulation for studying immune responses in vivo, since the effect of $CD4^+$ T cells on $CD8^+$ T cell production of IFN-γ would not have been identified if we had employed 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 in order to be influenced by them. Interestingly, both $CD4^+$ and $CD8^+$ T cells were hypersensitive to activation in the context of IFN-γ receptor 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 and 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 in order to determine whether this phenomenon is important for control of infection with *M. tuberculosis*. Identification of an antigen independent pathway for activation of $CD8^+$ effector T cells could provide an important new avenue for pursuit of therapies against TB, especially in $CD4^+$ T cell-deficient individuals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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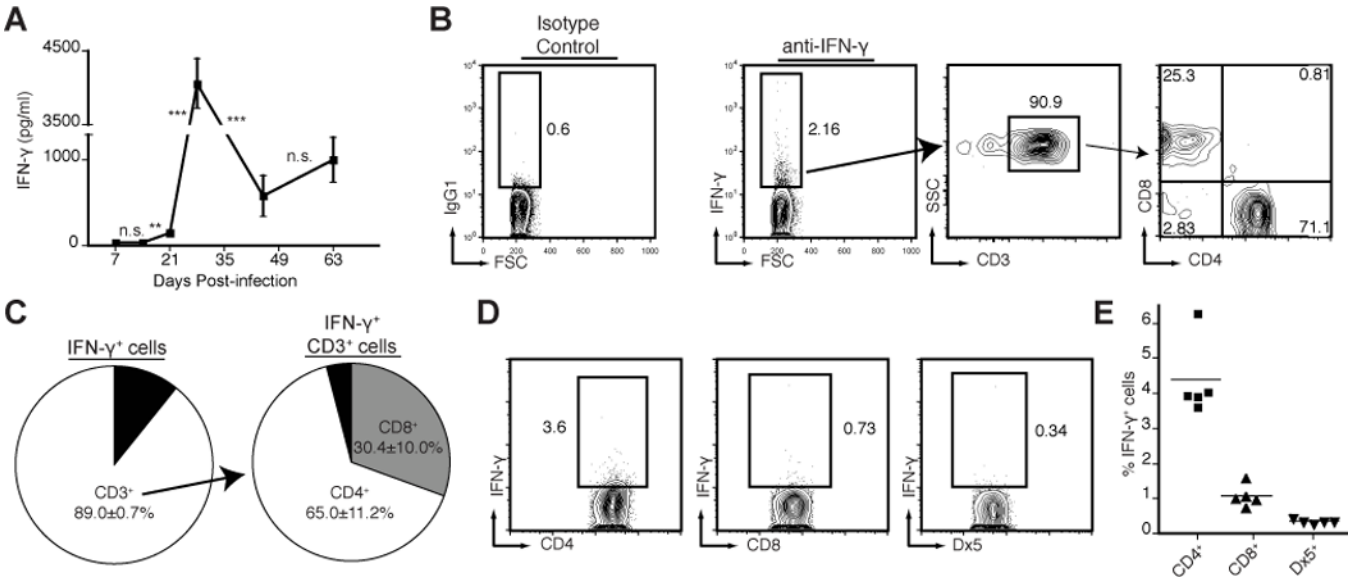

Figure 1. IFN-γ producing cells in the lungs of *M. tuberculosis* infected mice

(A) Concentrations of IFN-γ protein in lung homogenates during the initial nine weeks of infection, as measured by IFN-γ ELISA. Asterisks indicate statistical significance of differences in concentration of IFN-γ observed in n = 4 mice, between adjacent time points. ** $p<0.005$; *** $p<0.001$. (B) $CD3^+$ T cells account for 90% of the IFN-γ^+ cells in the lungs on day 28 post-infection as measured by direct intracellular cytokine staining followed by flow cytometry. IFN-γ-producing cells were identified relative to isotype control antibody staining. Flow cytometry plots show lung cells from a representative mouse. (C) Graphic representation of data from B, showing mean data from n=4 mice. (D) Frequency of IFN-γ producing cells within a given cell subset. Lung cells from day 28 post-infection were gated on relevant markers: $CD3^+$, $CD4^+$; $CD3^+$, $CD8^+$; or $CD3^-$, $DX5^+$. (E) Graphic representation of data from D, showing mean data from n=5 mice. All figures represent data from 2 or more experimental replicates. Data are representative of at least 2 independent experiments.

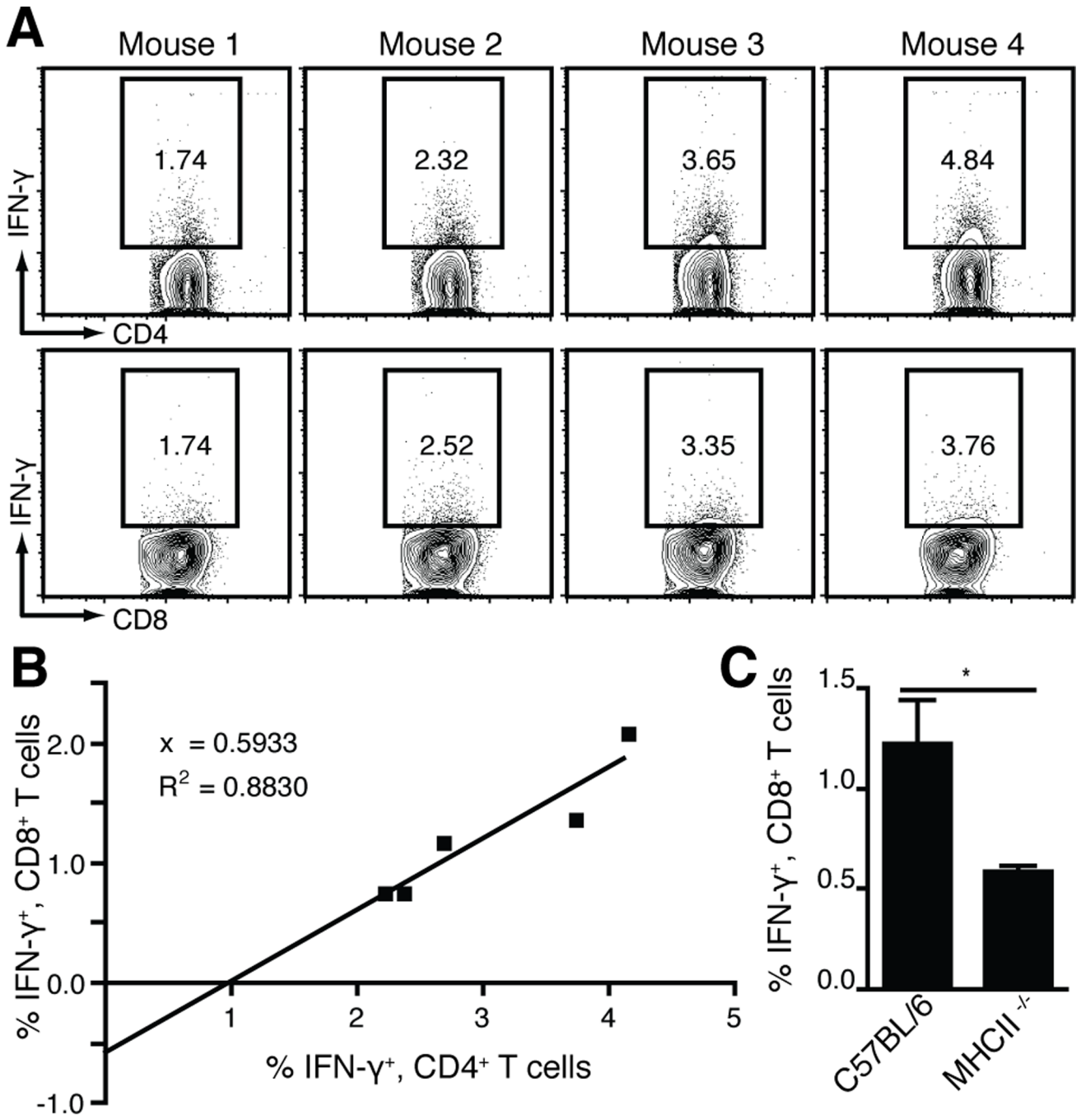

Figure 2. IFN-γ production by CD8$^+$ effector T cells is influenced by CD4$^+$ T cells

(A) IFN-γ production by CD4$^+$ or CD8$^+$ T cells from the lungs of individual mice on day 28 post-infection. Flow cytometry plots show CD3$^+$, CD4$^+$ (top row) or CD8$^+$ (bottom row) cells; values indicate the frequency of IFN-γ$^+$ cells within each population. (B) Linear correlation between the frequency of IFN-γ producing T cells in CD4$^+$ and CD8$^+$ subsets: graphic depiction of data in A, n=5 mice, with linear correlation performed with "x" indicating slope (p = 0.018) and "R^2" indicating goodness of fit for linear curve. Data shown in Panels (A) and (B) are from independent experiments. (C) Requirement of CD4$^+$ T cells for IFN-γ production by CD8$^+$ T cells. C57BL/6 or CD4$^+$ T cell deficient MHCII$^{-/-}$ mice were infected with *M. tuberculosis* and the frequency of IFN-γ$^+$ CD8$^+$ T cells in the lungs

was compared, n=4 mice. * $p<0.05$. Data are representative of at least 2 independent experiments.

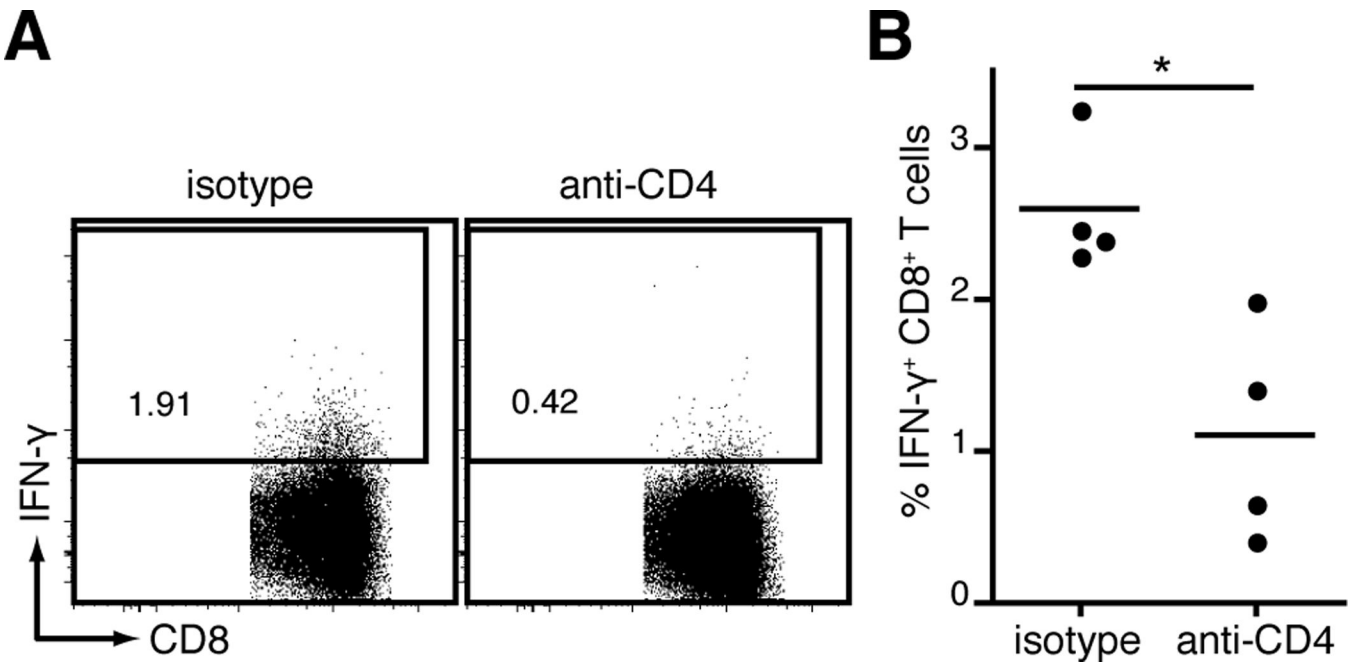

Figure 3. Production of IFN-γ by CD8$^+$ T cells requires continual presence of CD4$^+$ T cells

C57BL/6 mice infected with *M. tuberculosis* were treated with one intraperitoneal dose of monoclonal antibody: either anti-CD4$^+$ or isotype control. Cells were isolated from lungs 24 hours later. The frequency of CD8$^+$ T cells producing IFN-γ in each group was determined by flow cytometry. (A) Flow cytometry plots indicate CD3$^+$ CD8$^+$ cells from the lungs of a representative mouse from either treatment group. Values indicate the frequency of IFN-γ$^+$ cells within each subset 24 hours after treatment. (B) Graphic representation of data from A, indicating the frequency of IFN-γ$^+$ CD8$^+$ T cells in the lungs of n=4 mice. * $p<0.05$. Data are representative of 2 independent experiments.

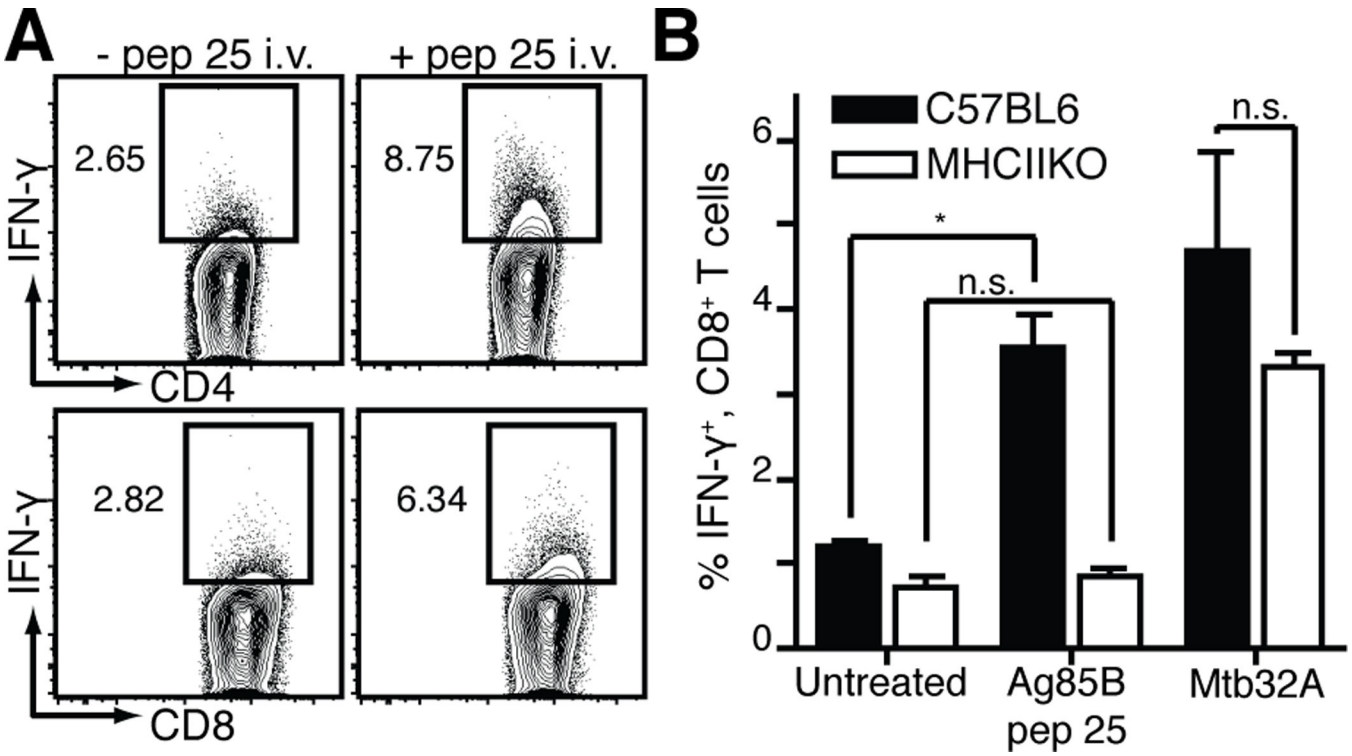

Figure 4. Activation of CD4$^+$ effector T cells activates CD8$^+$ effector T cells in vivo

(A) C57BL/6 mice infected with *M. tuberculosis* were injected intravenously with a synthetic peptide (100 μg) containing a known CD4$^+$ T cell epitope from *M. tuberculosis* Ag85B, peptide 25 (aa 240–254). 2 hours after injection, the frequency of IFN-γ$^+$ CD4$^+$ or CD8$^+$ T cells was compared to those in infected mice not receiving peptide. Flow cytometry plots show CD3$^+$CD4$^+$ (top row) or CD8$^+$ (bottom row) cells from the lungs of a representative mouse. Values indicate frequency of IFN-γ$^+$ cells in each subset. (B) CD8$^+$ T cell activation upon peptide 25 injection is not due to direct recognition of peptide 25 by CD8$^+$ T cells. Graph depicts the fraction of IFN-γ$^+$ CD8$^+$ T cells in the lungs of either C57BL/6 or MHCII$^{-/-}$ mice, either untreated or 2 hours after injection with 100 μg of either Ag85B peptide 25 (a CD4$^+$ T cell epitope) or Mtb32A$_{309–318}$ peptide (a CD8$^+$ T cell epitope). Asterisk indicates statistical significance of differences in frequency of IFN-γ$^+$ cells detected among one group of cells from n=4 mice of one genetic background between different treatment groups. * $p<0.05$; “n.s.”: not significant. Data are representative of 2 independent experiments.

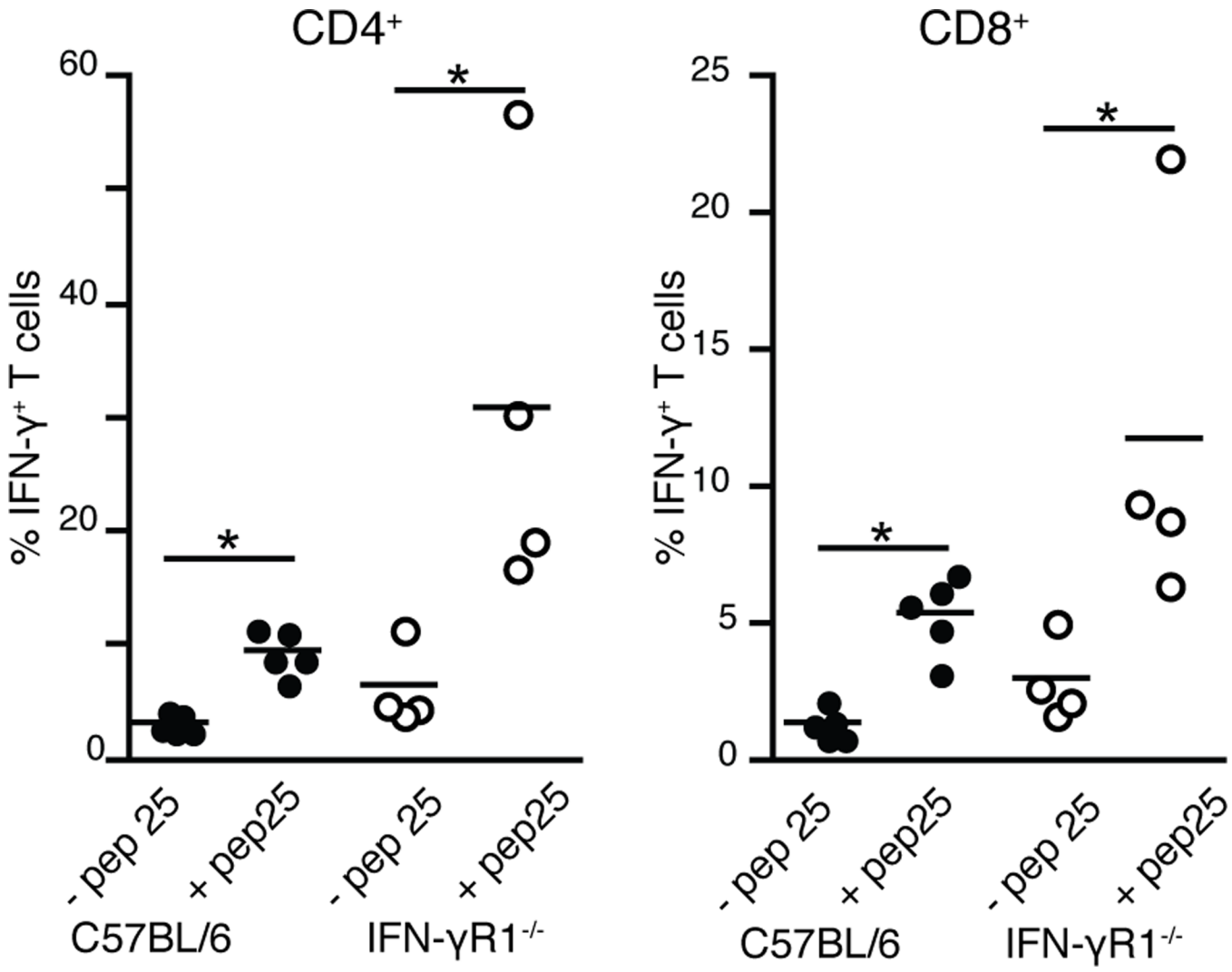

Figure 5. Indirect activation of CD8$^+$ T cells with peptide 25 is independent of IFN-γ signaling

C57BL/6 or IFN-γR$^{-/-}$ mice infected with *M. tuberculosis* were injected intravenously with Ag85B peptide 25 to activate CD4$^+$ T cells. 2 hours after injection, the frequency of IFN-γ$^+$ T cells was compared to those in infected mice not receiving peptide. Graphs depict the fraction of IFN-γ$^+$ among CD3$^+$, CD4$^+$ (left) or CD3$^+$, CD8$^+$ (right) T cells in the lungs. Asterisks indicate statistical significance of differences in frequency of IFN-γ$^+$ cells detected among one cell type between different treatment groups. * $p<0.05$; "n.s.": not significant. Data are representative of 2 independent experiments.