The Role of Multifunctional T Cells in IL-10 deficient CBA/J Mice Infected with *Mycobacterium tuberculosis*

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Background

Abstract

Most humans infected with *Mycobacterium tuberculosis* (*M.tb*) can exhibit asymptomatic control of the infection however, 10% of humans can reactivate infection to develop contagious tuberculosis (TB). IL-10 is an immunosuppressive cytokine that has been hypothesized to play a role in the reactivation of *M.tb* infection. Previous studies have shown that CBA/J IL10 knockout mice can better control *M.tb* infection compared to wild type CBA/J mice. This has been noted by a measured decrease in the pulmonary bacterial load throughout infection, an increase in survival compared to *M.tb*-infected CBA/J mice, and enhanced early T_H1 mediated immune responses. Multifunctional $CD4^+$ T cells, T cells that produce IFN-γ, TNF-α, and IL-2, have recently been linked to control of *M.tb* infection. The goal of this project was to analyze multifunctional T cells in the CBA/J mouse strain, a strain that is naturally susceptible to *M.tb*, as well as in CBA/J IL-10 knockout mice. The mediastinal lymph node of CBA/J mice and CBA/J IL10 knockout mice was analyzed early in infection for the presence of multifunctional $CD4^+$ T cells. Our hypothesis was the IL-10 knockout mice would show higher numbers of T cells as well as more T cells with the ability to produce multiple pro-inflammatory cytokines at early time points in infection. It was found that the CBA/J IL10 knockout mice possessed more $CD4^+$ T cells with the ability to produce one, two, or three of the protective cytokines. Therefore, the presence of multifunctional CD4 T cells may be linked with improved control of *M.tb* infection, a finding that could lead to further therapeutic development.

Introduction

Tuberculosis Prevalence in the World

Nearly one-third of the world's population is infected with *Mycobacterium tuberculosis (M.tb).* According to the CDC, in 2010, there were 8.8 million people who developed tuberculosis and 1.5 million TB-related deaths worldwide (1). In 2010, 5.7 million new and recurrent TB cases were being treated (2). Officially classified as a "disease of poverty," by the World Health Organization (WHO), 95% of TB deaths are in the developing world (2). In the 97 countries that count for this percentage of TB deaths, it expected that financing TB care and control would amount to US$ 4.4 billion dollars in 2012 (2). TB is currently the leading cause of death for people who are infected with human immunodeficiency virus (HIV) (1). In the African region, where TB is the most prevalent, 44% of TB patients also test HIV-positive, and the WHO considers TB and HIV to be global co-epidemics.

TB remains a global epidemic, and while there have been decreasing absolute numbers of infected individuals since 2006 (2), infection is estimated to occur at the rate of one person per second, leading research efforts on *M.tb* to achieve an incredible importance (3).

Reactivation of Latent M.tb Infection

M.tb is a slow-growing facultative, intracellular pathogen transmitted primarily by the respiratory route, leading to pulmonary infection in 85% of humans who develop the disease (4). However, infection does not usually lead to active disease. The pathogen has evolved to avoid destruction by both innate and adaptive immunity and to induce lung pathology, exemplified by a classically defined granuloma (5). The granuloma succeeds in allowing for containment rather than elimination of the pathogen, and can usually allow for the infected individual to remain

asymptomatic and noninfectious for their entire life (5). However, about 5-10% of the infected individuals revert from clinical latency of *M.tb* and develop reactivated tuberculosis (TB).

The mouse as a model for M. tb susceptibility

Mouse tuberculosis is a model of tuberculosis for susceptible humans, classified by their inability to resolve infection and prevent disease (5). The immunology of mouse TB is similar in important ways to human TB and is currently used to investigate key questions about the disease (5). Resistance and susceptibility to *M.tb* infection can be modeled in mice, with the CBA/J mouse modeling relative susceptibility to *M.tb* due to its increased pulmonary bacterial load and decreased survival when compared to the resistant C57BL/6 mouse strain (7). The CBA/J mouse can be characterized by its increased production of interleukin-10 (IL-10) during late stages of chronic TB infection (7). IL-10 is a potent immunosuppressive cytokine that affects innate and acquired immune responses, and in *M.tb*, is thought to promote disease progression. One of the targets of IL-10 via macrophages is Th1-type $CD4^+$ immunity, specifically the inhibition of pro-inflammatory cytokines interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF), and interleukin-2 (IL-2), leading to reduced protective immunity in *M.tb* infection (8). It is important to note that IL-10 has been identified as a correlate of susceptibility for tuberculosis in humans (9). Due to the fact that the absence of IL-10 leads to improved control of *M.tb* infection, a CBA/J IL-10 deficient mouse provides a model of protection from TB susceptibility.

The Importance of IL-10 Early in M.tb Infection

The CBA/J IL-$10^{-/-}$ mouse shows a very strong protected phenotype at late time points in *M.tb* infection when compared to the CBA/J mouse (unpublished data). This has been

demonstrated through decreased bacterial load in the lungs of CBA/J IL-$10^{-/-}$ mice beginning as early as day 60 post infection (unpublished data). Additionally, the IL-10 knockout shows improved granuloma formation in comparison with the CBA/J granuloma, which is generally observed to be a macrophage dominated and disorganized lesion (8). In the absence of IL-10, CBA/J mice form compact, leukocyte-rich, mature granulomas. These granulomas are characterized by necrotic centers and cuffing of $CD4^{+}$ T cells as well as the presence of collagen, an indication of fibrosis (unpublished observation).

Although the CBA/J $IL10^{-/-}$ mouse demonstrates control of *M.tb* infection at late time points, it is of interest to discover the earliest possible period of time post infection that IL-10 absence affects the immune response. IL-10 is thought to inhibit host antimicrobial mechanisms early in infection and it is known that in the absence of IL-10 there are enhanced levels of IFN-γ in the lung early during infection, which can lead to increased *M.tb* killing controlled by an IFNγ-mediated activation of macrophages via reactive oxygen and nitrogen intermediates (10). IL-10 also blocks phagosome maturation, allowing for *M.tb* growth and survival, and may have a role in blocking antigen presentation through downregulation of major histocompatibility molecules (10). In recent studies, our lab has discovered that blocking IL-10 receptor for the first 21 days of infection leads to decreased bacterial load in the CBA/J mouse as determined at day 120 of infection (unpublished observation).

Th1 Immunity and Multifunctional T cells

Anti-*M.tb* immunity in mice is considered to be Th1 mediated, mediated predominantly by CD4 Th1 cells with the assistance of CD8 T cells (4). Past studies have indicated that the protective role of CD4 and CD8 T cells is based on their ability to secrete key Th1 cytokines to activate macrophages at infection sites (5). While it has been shown that Th1-type $CD4^{+}$ and

cytokines are necessary for *M.tb* protection, they do not serve as a correlate of protection against *M.tb* (12). Interferon-gamma (IFN-γ), is a cytokine critical in protection against bacterial intracellular pathogens, but neither in humans nor in mice does the frequency of IFN-γ cells correspond to protection (12). IFN-γ, produced by both CD4 and CD8 T cells, is necessary but not sufficient for protection against *M.tb*. TNF, secreted by macrophages, dendritic cells, and T cells, is also believed to play complex and important roles in immune and pathologic response in tuberculosis through macrophage activation in synergy with IFN-γ (4).

Recently, there have been studies showing the importance of T cells with complex phenotypic and polyfunctional cytokine-secreting profiles, including a group that co-expresses IFN-γ, TNF, and IL-2 (13). In humans, vaccination with modified Vaccinia virus Ankara expressing Ag85A (MVA85A), a dominant *M.tb* antigen, induces T cells with this complex, triple-cytokine producing profile (13). Additionally, multifunctional T cells secreting IFN-γ, TNF, and IL-2 have been shown to correlate with protection from other parasitic infections in mice, such as *Leishmania major* (14). It is thought that combined analysis of different cytokines coexpressed by multifunctional T cells can differentiate between TB patients and patients with latent TB, invoking the idea that quality rather than quantity of *Mtb*-specific T cell response generates protection and long term memory (12).

Development of the Adaptive Immune Response in Mediastinal Lymph Node in Mtb infection

In the host cell, *M.tb* modulates early innate immune responses and delays the onset of adaptive immune response (4). This results in a delay in activation of $CD4^+$ T cells in the local lung-draining mediastinal lymph node (11). In mice, the earliest antigen-specific $CD4^+$ T cell response in the MLN is noted 12 days post aerosol infection (15).

In further characterization of the development of adaptive immunity in *Mtb* infection, it has been found that activation of T cells is the limiting step in the development of the adaptive immune response and after proliferation in the lymph node, $CD4^+$ effector T cells move rapidly to the lungs (11). Therefore, in order to gauge the earliest possible T cell response to *Mtb*, it is necessary to analyze the T cell populations within the MLN during the first few weeks of infection.

Multifunctional T cells in the Mediastinal Lymph Node (MLN) Early in M.tb Infection

In this study, we considered the CBA/J IL-10-deficient mouse to be a model of protective TB. Considering the importance of IL-10 early in infection, we analyzed the numbers of T cells and cytokine profile of those T cells present in the lymph node during early time points post infection in both CBA/J and CBA/J $IL10^{-/-}$ mice. We specifically looked for the presence of T cells producing IFN-γ, TNF, and IL-2 in any combination. **We proposed a two-pronged hypothesis that in the CBA/J $IL10^{-/-}$ mice, (A) there would be more T cells as well as more activated T cells present in the MLN at early time points post infection and (B) there would be more multifunctional, cytokine producing T cells in the MLN.** Using flow cytometry, we analyzed the T cells present in the MLN from days 10 to 31 of *M.tb* infected CBA/J and CBA/J $IL10^{-/-}$ mice, staining both for activation surface markers and intracellular cytokines.

Materials and Methods

Tissue Culture Media: 1 bottle 500 ml Dulbecco's Modification of Eagle's Media (Cellgro/Fisher MT15-017-CV), containing 10 ml minimal essential amino acids (Sigma M7145), 5 ml HEPES buffer (Sigma H0887), 5 ml L-glutamine (Sigma G7513), 5 ml Penicillin/Streptomycin (Sigma P0781), 660 µl 2-mercaptoethanol (50mM), and 45 ml heat inactivated fetal bovine serum (Atlas F-0500-D, Lot A20530B).

Gey's Solution: 4.15g NH_4Cl (8mM), 0.5g $KHCO_3$ (5mM), 500 ml bottled LPS free H_20, sterile filtered.

10X PBS: 2.56g $NaH_2PO_4 \cdot H_20$, 22.49g $Na_2HPO4 \cdot 7H_20$, 87.66g NaCl, QS to 1 liter with ddH_20.

10X PBST: 2.56g $NaH_2PO_4 \cdot H_20$, 22.49g $Na_2HPO4 \cdot 7H_20$, 87.66g NaCl, 5 ml Tween 20, QS to 1 liter with ddH_20.

Deficient RPMI (dRPMI): 10.10g RPMI-1640 (Cellgro 90-022-PB), 10.42 ml HEPES (Sigma H0887), QS to 1 liter in ddH_20. pH to 7.4, sterile filter and add 1g/L of sodium azide.

Aerosol: *M.tb* Erdman (ATCC no. 35801) was obtained from American Type Culture Collection. Stocks were grown according to published methods. Mice were infected with *M.tb* Erdman using an inhalation exposure system (Glas-Col) calibrated to deliver 50–100 CFU to the lungs of each mouse.

<u>Mice</u>: CBA/J mice (National Cancer Institute, Frederick, MD) were crossed with C57BL/6 IL-10-/- mice (Jackson laboratories, Bar Harbor, Maine) for eight generations. At each cross progeny were earpunched and DNA was screened for the presence of a neomycin cassette at the *il10* gene locus and IL-10+/- mice were selected for further breeding. At the eighth generation, heterozygotes were crossed and IL-10-deficient homozygote CBA/J mice were selected. A homozygous breeder colony of CBA/J IL-10-/- mice was maintained thereafter.

Specific pathogen-free, age/sex-matched CBA/J IL-10-/- and CBA/J wild-type (NCI) were maintained in ventilated cages inside a biosafety level 3 (BSL3) facility and provided with sterile food and water *ad libitum*. All protocols were approved by The Ohio State University's Institutional Laboratory Animal Care and Use Committee.

At specific time points, mice were euthanized by CO2 asphyxiation in accordance with Institutional Animal Care and Use Committee protocol.

<u>Single Cell Suspension</u>

The mediastinal lymph node from CBA/J and CBA/J $IL10^{-/-}$ mice were gently passed through a 70 µm cell strainer (BD Falcon) to form a single cell suspension. Cell suspensions were equalized to 5 ml using tissue culture media (TCM) and centrifuged for 7 min at 1,200 rpm at 4°C. The supernatant was poured off and the cell pellet was resuspended. Gey's Solution (2 ml) was added for 3 min at room temperature to lyse the red blood cells. The reaction was quenched by adding 8 ml TCM. The cell suspension was centrifuged for 7 min at 1,200 rpm at 4°C. The supernatant was discarded and the cell pellet was resuspended in 1 ml of TCM.

Cells were added to Trypan blue in a 1:10 dilution in order to count viable cells.

The cells were counted using a hemacytometer and adjusted to equal concentrations at each

experimental time point.

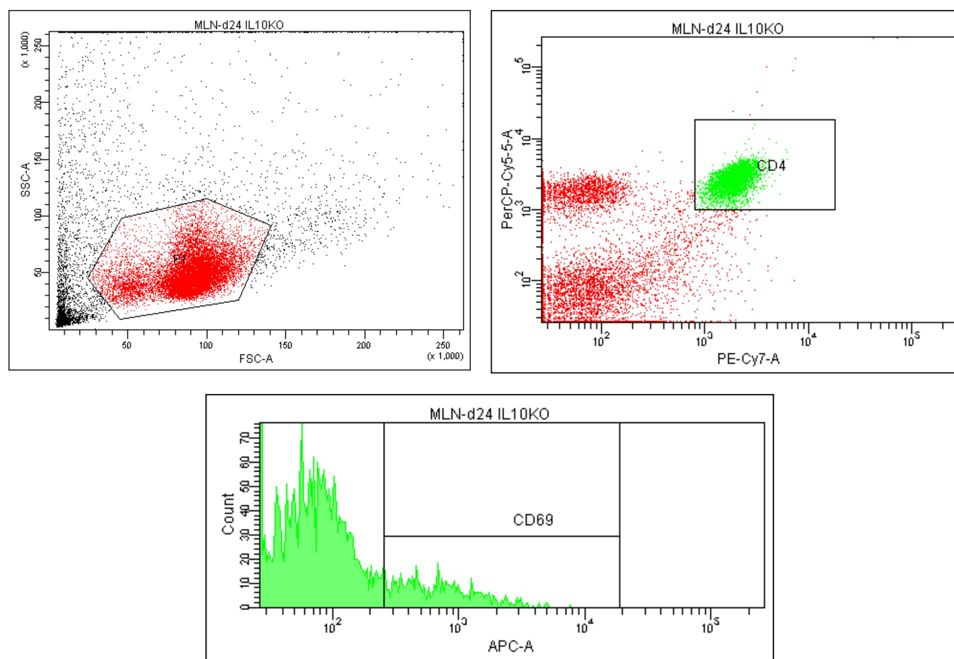
Intracellular Flow Cytometry

From the single cell suspension, 500 μl of cells were plated in a 24 well plate. The cells were crosslinked for 4 hours at 37ºC with 250 μl of anti-CD3, anti-CD28, and BD GolgiStop™ Protein Transport Inhibitor (at concentrations noted in Table 1). The cells were then fixed in equal volume of dRPMI. Then, 200 μl of cells were transferred to a 96 well plate, centrifuged for 7 min at 1,200 rpm at 4°C, the supernatant was decanted, and the cells were resuspended in 100 μl dRPMI. The cells were spun and the supernatant decanted a second time, but resuspended in BD Fc Block at 12.5 μg/mL (BD Biosciences) and incubated for 20 minutes at at 4°C. The fluorochrome-conjugated anti-cytokine antibodies for CD3, CD4, and CD8 were added to the cells (at concentrations that can be noted in Table 2) and incubated for another 20 minutes at 4°C. The cells were permeabilized with a Cytofix/Cytoperm fixation/permeabilization solution kit from BD Biosciences and cytokines IFN-γ, TNF, and IL-2 were stained using fluorochrome conjugated antibodies (at concentrations that can be noted in Table 3).

Preparation of Surface Stains for Flow Cytometry

The cells remaining from the single cell suspension were added to an equal volume of dRPMI, plated at 200 μl per well into 96 well plate, and centrifuged for 7 min at 1,200 rpm at 4°C. The supernatant was decanted and the cells were resuspended in 100 μl dRPMI. The cells were spun and the supernatant decanted a second time, but the cells were resuspended in BD Fc Block (BD Biosciences) and incubated for 20 minutes at at 4°C. The surface stains for anti-CD3, anti-CD4, and anti-CD8 were added to the cells (at concentrations that can be noted in Table 2) and

incubated for another 20 minutes at at 4°C. The cells were then washed twice in dRPMI, resuspended in 200 µl of dRPMI and read using a LSRII flow cytometer and analyzed with FACSDiva Sofware (BD Biosciences). Lymphocytes were gated according to their forward and side scatter profiles and the $CD4^+$ and $CD8^+$ T cells were gated based on the presence of CD4 or CD8 and CD3. The populations of $CD4^+$ and $CD8^+$ T cells were further analyzed using surface marker and cytokine expression.



Flow Cytometry Gating

This is representative of the gating using in the flow cytometry analysis on BD FACSDiva. The top left image shows a forward and side scatter profile and gating of lymphocytes. The top right image shows $CD4^+$ T cells, a population of cells with CD4 expression (PE-Cy7-A) and CD3 expression (PerCP-CY5.5-A). The bottom image shows $CD4^+$ T cells that are expressing a level of CD69 (APC-A) that exceeds the isotype control.

Data Analysis and Statistics: Flow cytometry data was converted from percentages to absolute numbers using a percentage hierarchy developed in FACSDiva Softare and a spreadsheet in Microsoft Excel (Microsoft Office, Redmond, WA). Statistical significance was determined using the Prism 4 software (GraphPad Software, San Diego, CA). The unpaired two-tailed Student *t* test was used for comparisons of CBA/J and CBA/J IL10$^{-/-}$ mice.

Table 1. Antigens Used in Crosslinking

Antigen Used	Company	Catalog Number	Concentration
Anti-CD3	eBioscience	16-0031-86	10μg/mL
Anti-CD28	eBioscience	16-0281-86	1 μg/mL
Golgi Stop™	BD Biosciences	554724	as directed in kit

Table 2. Surface Stains

Surface Marker	Company	Catalog Number	Concentration
PerCP-Cy5.5 anti-CD3e	BD Biosciences	551163	12.5 μg/mL
PE-Cy7 anti-CD4	BD Biosciences	552775	12.5 μg/mL
Allophycocyanin-Cy7 anti-CD8	BD Biosciences	557654	12.5 μg/mL
Allophycocyanin anti-CD69	BD Biosciences	560689	12.5 μg/mL
FITC anti-CD25	BD Biosciences	553072	12.5 μg/mL

Table 3. Intracellular Stains

Cytokine	Company	Catalog Number	Concentration
PE-Cy7 anti-IFNγ	BD Biosciences	554413	12.5 μg/mL
PerCP-Cy5.5 anti-TNF	BD Biosciences	560659	12.5 μg/mL
PE anti-IL-2	BD Biosciences	554428	12.5 μg/mL

Results

1. Analysis of T Cell Number in MLN in CBA/J (WT) and CBA/J $IL10^{-/-}$ Mice

The mediastinal lymph node (MLN) was collected from CBA/J (wild type) and CBA/J $IL10^{-/-}$ (knockout) mice at days 10, 15, 18, 24, and 31 post infection. The MLN was made into a single cell suspension and the cells were divided among surface flow cytometry, and intracellular flow cytometry. The phenotypic surface markers CD3, CD4, and CD8 were tagged with fluorescently labeled antibodies and analyzed via flow cytometry to determine total numbers of T cells present in the MLN. The data of CBA/J mice reflects a group of 4 mice and the data for the CBA/J $IL\text{-}10^{-/-}$ mouse reflects a group of 5 mice analyzed individually. These experiments were repeated twice to assess experimental variability. In both CBA/J and the CBA/J $IL10^{-/-}$ mice, the total number of T cells increased as the infection progressed (Figure 1). By day 31, the CBA/J $IL10^{-/-}$ mice averaged $9.0x10^7$ T cells in the lymph node, while in the CBA/J mice had only about 50% of the number of T cells present in the knockout mice, $4.5x10^7$ T cells at day 31 This result did not achieve significance due to large experimental variation, most likely associated with MLN collection methods.

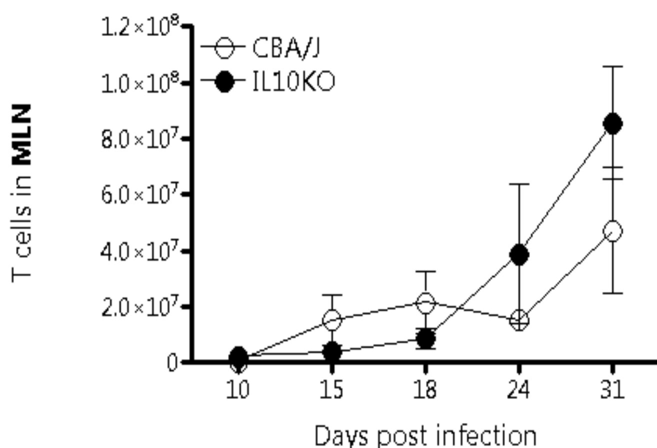


Figure 1. T cells in the MLN of CBA/J and CBA/J $IL10^{-/-}$ mice
Mediastinal lymph node cells from CBA/J and CBA/J $IL10^{-/-}$ mice were analyzed by flow cytometry. Cells were gated according to their forward and side scatter profiles and expression of CD3. Data represents 4-5 mice per group per time point and is representative of 2 experiments. Statistical analysis was performed via the unpaired Student's *t* test.

The proportions and total numbers of $CD4^+$ T cells were subsequently analyzed. Throughout the time points measured, the $CD4^+$ T cells of both the wild type and knockout mice rose from slightly more than 20% at day 10 to 35% of the total T cell population within the MLN by day 31 (Figure 2a). The populations of $CD4^+$ T cells (Figure 2b) remained equivalent in both mouse strains. The populations of both wild type and knockout mice $CD4^+$ T cells increased throughout the experiment, showing 150% growth in number from day 24 to 31. The percentage of $CD8^+$ T cells did not differ significantly throughout the experiment. The $CD8^+$ T cells accounted for 20% of the total T cells in the CBA/J mice at day 10 post infection and 25% of the T cells present in the MLN of CBA/J $IL10^{-/-}$ mice at the same time point (Figure 3a). However, by day 31 post infection, the $CD8^+$ T cells only accounted for 20% of the total T cells present in both mouse strains. The $CD8^+$ T cells (Figure 3b) present in the MLN consistently remained under $5x10^6$ throughout the experiment, until the final time point of day 31. At day 31, the knockout $CD8^+$ T cell population rose to $1.25x10^7$ while the wild type mice averagely achieved $7.5x10^6$ $CD8^+$ T cells in the MLN. From analysis of $CD8^+$ T cell percentages and absolute numbers, there were no significant differences between the wild type and the knockout mice.

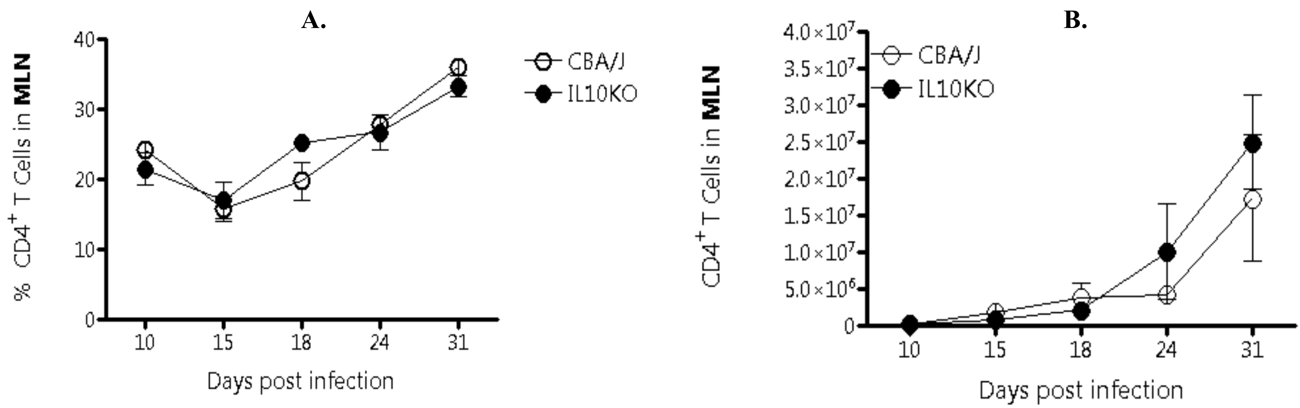


Figure 2. CD4$^+$ T cells in MLN of CBA/J and CBA/J IL10$^{-/-}$ mice. The cell numbers are expressed in (A) percentage and (B) absolute numbers of T cells present.

Mediastinal lymph node cells from CBA/J and CBA/J IL10$^{-/-}$ mice were analyzed by flow cytometry. Cells were gated according to their forward and side scatter profiles and expression of both CD3 and CD4. Data represents 4-5 mice per group per time point and is representative of 2 experiments. Statistical analysis was performed via the unpaired Student's *t* test.

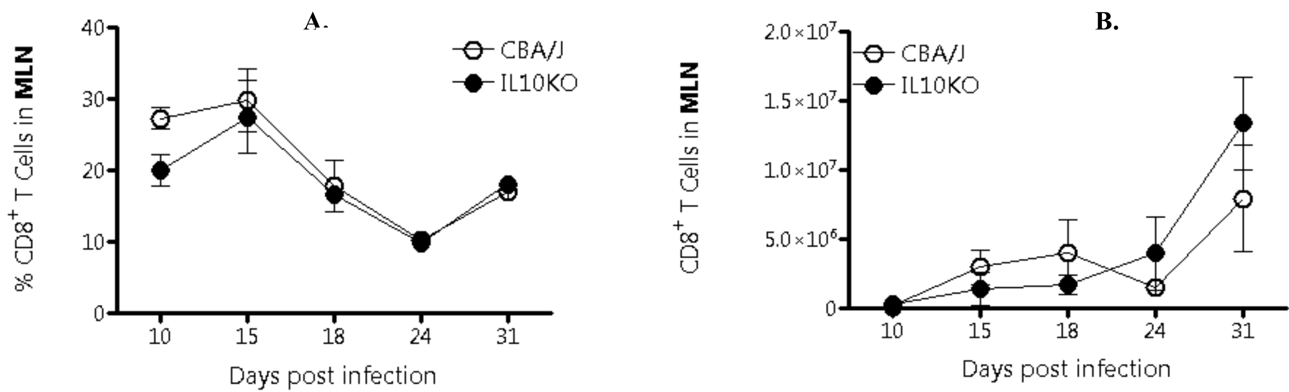


Figure 3. CD8$^+$ T cells in MLN of CBA/J and CBA/J IL10$^{-/-}$ mice. The cell numbers are expressed in (A) absolute numbers of T cells present and (B) percentage.

Mediastinal lymph node cells from CBA/J and CBA/J IL10$^{-/-}$ mice were analyzed by flow cytometry. Cells were gated according to their forward and side scatter profiles and expression of both CD3 and CD8. Data represents 4-5 mice per group per time point and is representative of 2 experiments. Statistical analysis was performed via the unpaired Student's *t* test.

2. T Cell Activation, measured by CD69 and CD25 Surface Markers on T Cells, in the MLN

CD69, an early T cell activation marker, was determined on T cells. In percentage analysis, day 10 post infection showed an equivalent activation of $CD4^+$ T cells in both strains of 33.4% for the wild type mice and 34.7% for the knockout mice (Figure 4a). From day 15 through the end of the experiment, both the wild type and knockout mice show a decrease in activated $CD4^+$ T cells to 8.2% for the wild type mice and 10.2% for the IL-10 knockout. Among $CD4^+$ T cells, both CBA/J and CBA/J $IL10^{-/-}$ mice showed increasing numbers of activated cells in the time points measured post infection (Figures 4b). While the CBA/J $IL10^{-/-}$ mice had a trend for more CD69 expression on days 24 (average of $1.3x10^5$ cells) and 31 (average of $2.5x10^6$ cells) when compared to CBA/J mice on the same days ($5.2x10^5$ and $1.4x10^6$ cells), it was not a significant difference. The percentages of $CD8^+$ T cells in both the wild type and the knockout MLN show an overall decrease from day 10 to 31 (Figure 5a).The $CD8^+$ T cells of both mouse strains show no discernable pattern of activation throughout the experiment, largely remaining at low levels, below $2.5x10^5$, days 10-31 (Figure 5b).

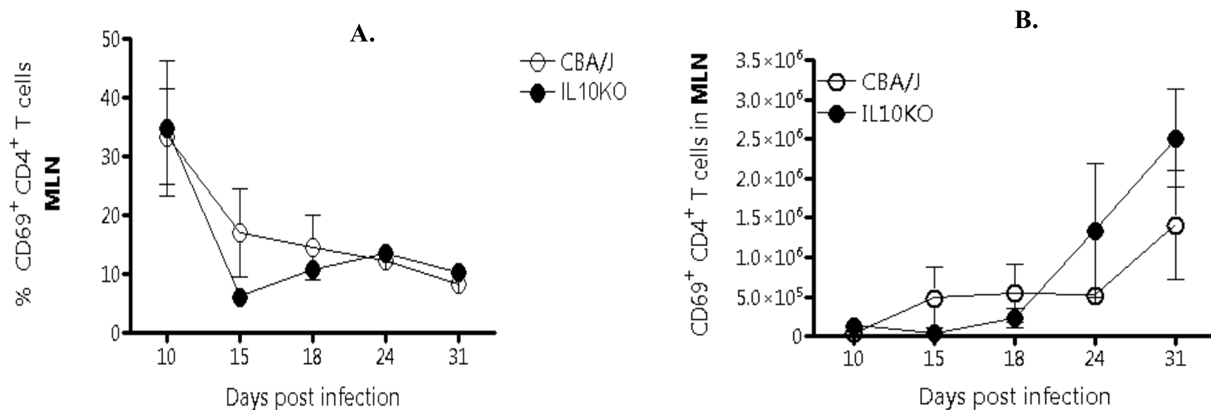


Figure 4. $CD69^+$ $CD4^+$T cells in MLN of CBA/J and CBA/J $IL10^{-/-}$ mice. In (A) the $CD4^+$ T cells with the CD69 activation marker are expressed in percentage and (B) shows the absolute numbers. Mediastinal lymph node cells from CBA/J and CBA/J $IL10^{-/-}$ mice were analyzed by flow cytometry. Cells were gated according to their forward and side scatter profiles and expression of CD3, CD4, and CD69. Data represents 4-5 mice per group per time point and is representative of 2 experiments. Statistical analysis was performed via the unpaired Student's *t* test.

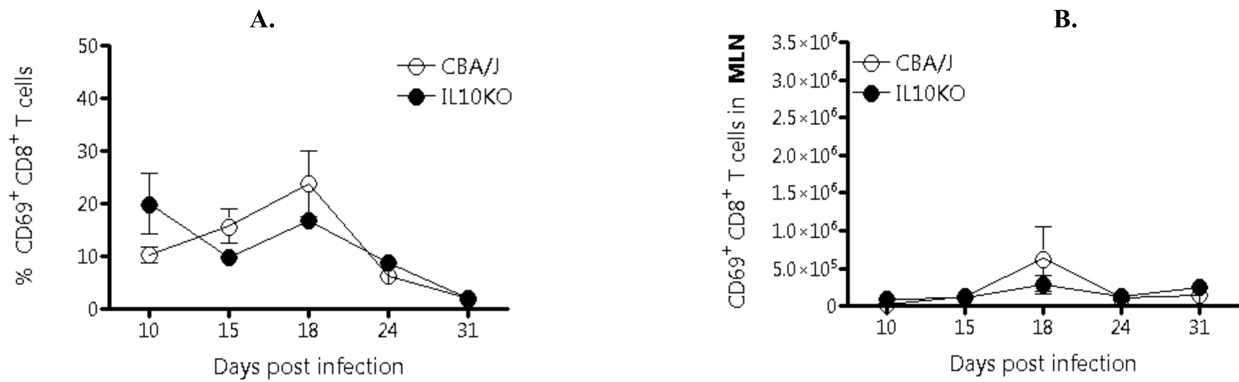


Figure 5. $CD69^+$ $CD8^+$ T cells in MLN of CBA/J and CBA/J $IL10^{-/-}$ mice. In (A) the $CD8^+$ T cells with the CD69 activation marker are expressed in absolute numbers and (B) shows the percentage. Mediastinal lymph node cells from CBA/J and CBA/J $IL10^{-/-}$ mice were analyzed by flow cytometry. Cells were gated according to their forward and side scatter profiles and expression of CD3, CD8, and CD69. Data represents 4-5 mice per group per time point and is representative of 2 experiments. Statistical analysis was performed via the unpaired Student's *t* test

CD25, the receptor for IL-2, a cytokine causing T cell proliferation was also determined. Both $CD4^+$ and $CD8^+$ T cells were found to have consistently low expression of CD25 throughout the early time points of the experiment (Figures 6a and 6b). In the $CD4^+$ T cell population, the $CD25^+$ cells increased equivalently in both mouse strains at day 24. The IL-10 knockout mice MLN contained $1x10^5$ more $CD25^+$ $CD4^+$ T cells by day 31, however this was not a significant increase. The $CD25^+$ $CD8^+$ T cells remained at very low levels throughout the entire experiment in both mouse strains.

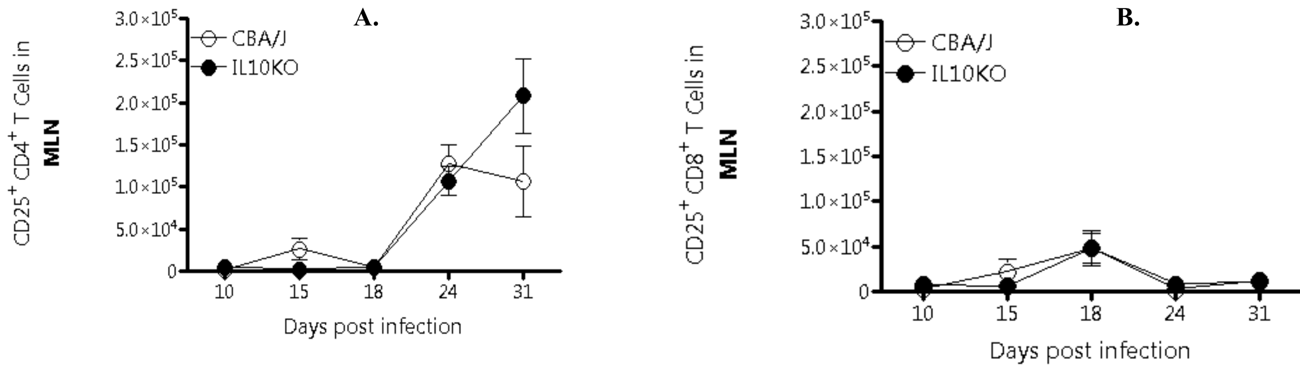


Figure 6. $CD25^+$ T cells in the MLN of CBA/J and CBA/J $IL10^{-/-}$ mice. (A) Shows the CD4 T cells that are expressing CD25 and (B) shows the CD8 T cells expressing CD25.

Mediastinal lymph node cells from CBA/J and CBA/J $IL10^{-/-}$ mice were analyzed by flow cytometry. Cells were gated according to their forward and side scatter profiles and expression of CD3, CD4 (in 6A) or CD8 (in 6B), and CD25. Data represents 4-5 mice per group per time point and is representative of 2 experiments. Statistical analysis was performed via the unpaired Student's *t* test

3. CBA/J $IL\text{-}10^{-/-}$ mice show higher levels of $CD4^+$ T cell cytokine production in the mediastinal lymph node.

The MLN cells used for intracellular flow cytometry were analyzed for multifunctionality, or capability of producing any combination of the pro-inflammatory cytokines IFN-γ, TNF-α, and IL-2. Cells used for intracellular flow cytometry were labeled on the surface for the phenotypic markers CD3, CD4 and CD8 and then were permeabilized and stained intracellularly for the cytokines of interest.

Single Positive T cells

In the population of $CD4^+$ T cells that could produce IFN-γ, the IL-10 knockout mice showed a consistent increase throughout the experiment. The CBA/J $CD4^+$ T cells positive for IFN-γ also increased throughout the experiment, but in a more stepwise manner (Figure 8a). By day 31, the IL-10 knockout mice MLN contained an average of $1.0x10^5$ more IFN-γ positive cells ($2.4x10^5$ cells in the knockout compared to $1.4x10^5$ cells in the wild type). The $CD8^+$ T cells that stained positive for IFN-γ production remained equivalent in both mouse strains in the

early time points of the experiment. However, after day 24, there was a split in the numbers of $CD8^+$ T cells producing IFN-γ, with the knockout mice containing $1.5x10^5$ more IFN-γ $CD8^+$ T cells than the wild type. By day 31, the T cells capable of producing IFN-γ in the knockout mice were continuing to increase while the wild type mouse showed a plateau (Figure 7A) or decrease (Figure 7B) in the IFN-γ^+ T cell populations.

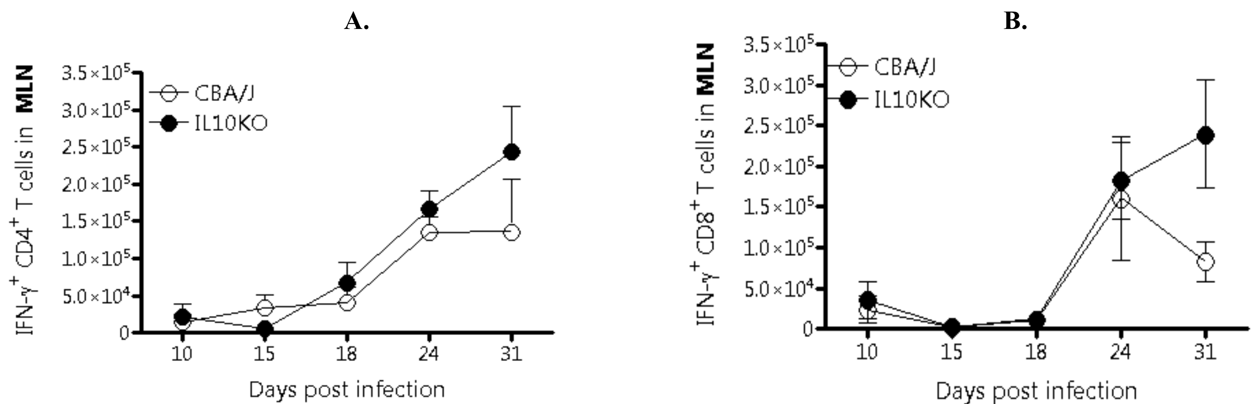


Figure 7. IFN- $^+$ T cells in the MLN of CBA/J and CBA/J $IL10^{-/-}$ mice. (A) Shows the CD4 T cells that are expressing IFN- and (B) shows the CD8 T cells expressing IFN- .
Mediastinal lymph node cells from CBA/J and CBA/J $IL10^{-/-}$ mice were analyzed by flow cytometry. Cells were gated according to their forward and side scatter profiles and expression of CD3, CD4 (in 7A) or CD8 (in 7B), and IFN-γ. Data represents 4-5 mice per group per time point and is representative of 2 experiments. Statistical analysis was performed via the unpaired Student's *t* test

The second cytokine analyzed was tumor necrosis factor-α (TNF). Of the three cytokines analyzed (IFN-γ, TNF, and IL-2), at the conclusion of the experiment, TNF was expressed by the largest number of cells. Until day 18, there were very few $CD4^+$ T cells producing TNF in the MLN in both mice strains. After day 18, both the wild type and the knockout mice showed a marked increase in TNF^+ $CD4^+$ T cells and day 31 showed the knockout mice with $1.25x10^6$ more $CD4^+$ T cells that are expressing TNF (Figure 8a). Compared to the $CD4^+$ T cells, the $CD8^+$

T cells in both strains of mice produce very little TNF throughout the entire experiment, remaining at $5.0x10^5$ cells or less from day 10 to day 31 (Figure 8b)

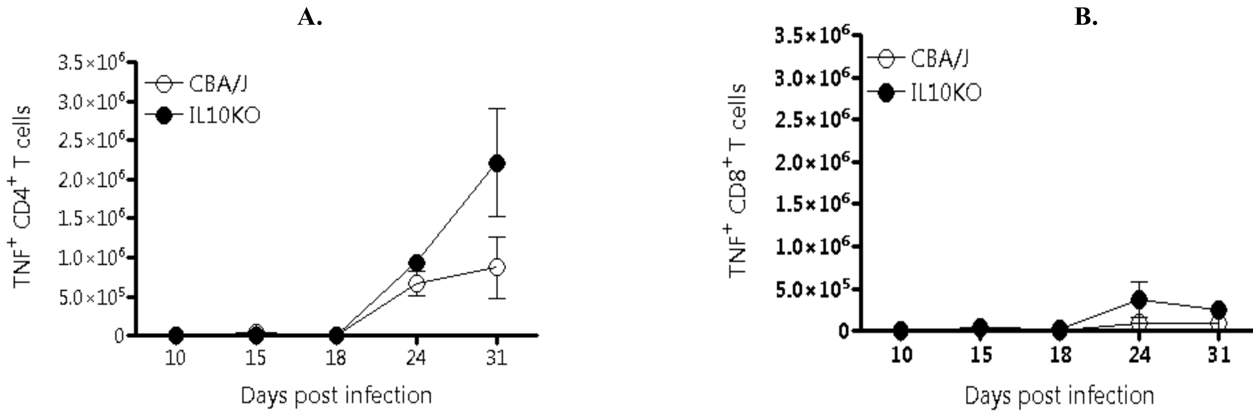


Figure 8. TNF+ T cells in the MLN of CBA/J and CBA/J IL10-/- mice. (A) Shows the CD4 T cells that are expressing TNF and (B) shows the CD8 T cells expressing TNF.

Mediastinal lymph node cells from CBA/J and CBA/J IL10-/- mice were analyzed by flow cytometry. Cells were gated according to their forward and side scatter profiles and expression of CD3, CD4 (in 8A) or CD8 (in 8B), and TNF. Data represents 4-5 mice per group per time point and is representative of 2 experiments. Statistical analysis was performed via the unpaired Student's *t* test

The third cytokine that was analyzed in this experiment was IL-2. The numbers of cells staining positively for IL-2 varied extensively throughout the experiment for both $CD4^+$ and $CD8^+$ T cells. For $CD4^+$ T cells, there were no significant differences between the wild type and the knockout mice during the course of the experiment. Both strains of mice peaked in number of CD4 T cells producing IL-2 at day 24, with $3.75x10^5$ $IL\text{-}2^+$ $CD4^+$ T cells in the wild type mice

and 3.0x10^5 IL-2^+ CD4^+ T cells in the knockout mice (Figure 9a). Compared to the CD4^+ T cells expressing IL-2, relatively few CD8^+ T cells stained positively for IL-2. Throughout the experiment, both wild time and knockout mice MLN contained IL-2^+ CD8^+ T cells in numbers less than or equal to 5.0x10^4 (Figure 9b).

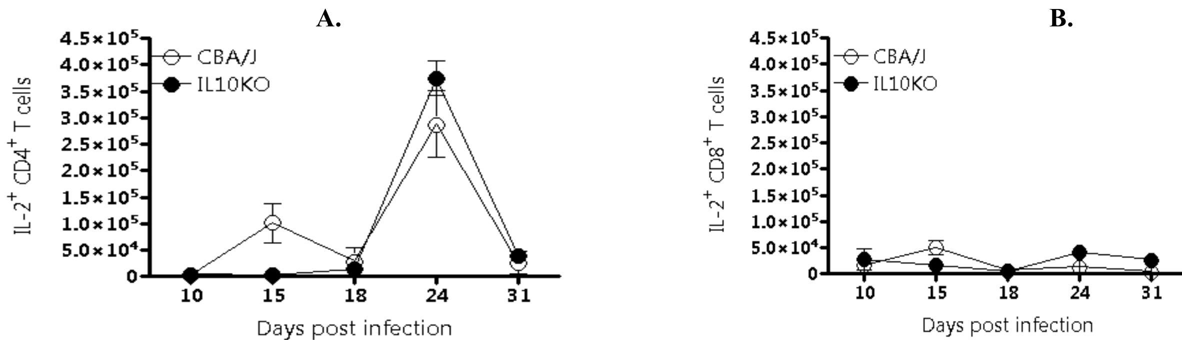


Figure 9. IL-2^+ T cells in the MLN of CBA/J and CBA/J IL$10^{-/-}$ mice. (A) Shows the CD4 T cells that are expressing IL-2 and (B) shows the CD8 T cells expressing IL-2.

Mediastinal lymph node cells from CBA/J and CBA/J IL$10^{-/-}$ mice were analyzed by flow cytometry. Cells were gated according to their forward and side scatter profiles and expression of CD3, CD4 (in 9A) or CD8 (in 9B), and IL-2. Data represents 4-5 mice per group per time point and is representative of 2 experiments. Statistical analysis was performed via the unpaired Student's *t* test

Double Positive T cells

The T cells in the MLN were also analyzed for the ability to produce two or three cytokines. These T cells are considered multifunctional, and were the T cells of greatest interest in this study. The first double positive T cells analyzed were expressers of IFN-γ and TNF. In both the wild type and the knockout, the number of IFN-γ and TNF expressing CD4^+ T cells remained constant until day 18, increased to about 1x10^5 at day 24 and then the wild type mice showed a slight decrease in the cell population to 5.4x10^4 cells and the knockout mice more than doubled the number of IFN-γ^+ TNF$^+$ CD4^+ T cells to 2.7x10^5 cells. Compared to the CD4^+ T cells, the CD8^+ T cells that were double positive for IFN-γ and TNF remained at low levels. The number of cells of both mice strains remained below 1x10^5 throughout the entire experiment.

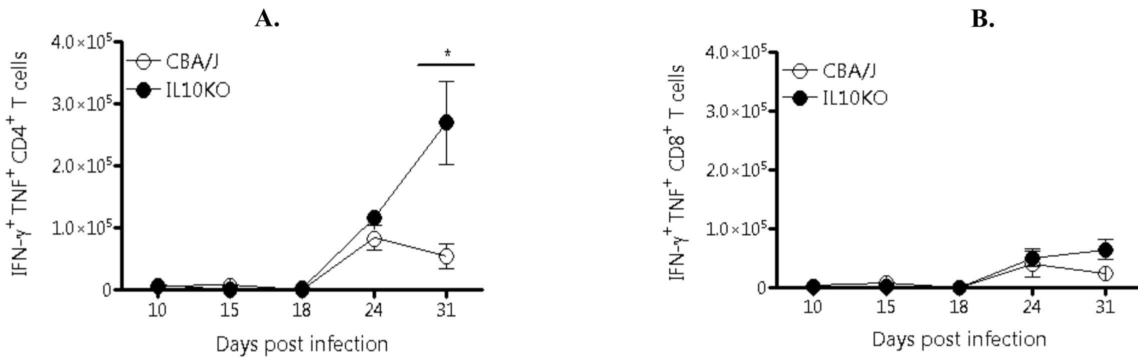


Figure 10. IFN $^{+}$ TNF^{+} T cells in the MLN of CBA/J and CBA/J IL10$^{-/-}$ mice. (A) Shows the CD4 T cells that are expressing IFN and TNF (B) shows the CD8 T cells expressing IFN and TNF.

Mediastinal lymph node cells from CBA/J and CBA/J IL10$^{-/-}$ mice were analyzed by flow cytometry. Cells were gated according to their forward and side scatter profiles and expression of CD3, CD4 (in 10A) or CD8 (in 10B), IFN-γ, and TNF. Data represents 4-5 mice per group per time point and is representative of 2 experiments. Statistical analysis was performed via the unpaired Student's *t* test

The second population of double positive cells in the MLN that were analyzed was IFN-γ^{+} IL-2^{+} T cells. Most likely due to the variability in IL-2 expression in the T cells, this double positive population showed variability throughout the time points as well. The CD4^{+} CBA/J T cells expressing both IFN-γ and IL-2 remained below $1x10^{4}$ cells for the entire experiment. However, the IL-10 knockout mice showed almost a 10 fold increase from averaging $1.9x10^{4}$ cells at day 18 to averaging $1.8x10^{5}$ cells at day 24 and maintained the difference at day 31. In the IFN-γ^{+} IL-2^{+} CD8^{+} T cell population, the cell numbers in both mouse strains remained at low levels, below $1x10^{4}$ cells, until day 24, when the IFN-γ^{+} IL-2^{+} CD8^{+} T cells in the knockout mice spiked at $3.7x10^{4}$. The corresponding cells from the CBA/J mice remained at $1.2x10^{4}$ for days 24 and 31.

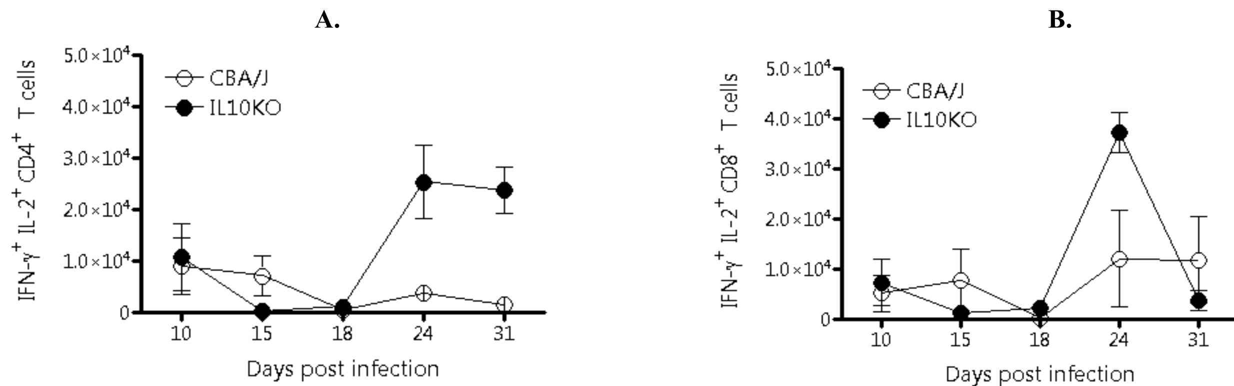


Figure 11. IFN $^{+}$ IL-2^{+} T cells in the MLN of CBA/J and CBA/J IL$10^{-/-}$ mice. (A) Shows the CD4 T cells that are expressing IFN and IL-2 (B) shows the CD8 T cells expressing IFN and IL-2.
Mediastinal lymph node cells from CBA/J and CBA/J IL$10^{-/-}$ mice were analyzed by flow cytometry. Cells were gated according to their forward and side scatter profiles and expression of CD3, CD4 (in 11A) or CD8 (in 11B), IFN-γ, and IL-2. Data represents 4-5 mice per group per time point and is representative of 2 experiments. Statistical analysis was performed via the unpaired Student's *t* test

The third and final population of double positive T cells that were analyzed was CD4^{+} and CD8^{+} T cells that expressed TNF and IL-2. As with the majority of the double positive cells, the TNF^{+} IL-2^{+} CD4^{+} T cells remained at very low levels until after day 18. After day 18, both mouse strains showed consistent increase in cell numbers of TNF^{+} IL-2^{+} CD4^{+} T cells. The knockout showed a greater number of cells at days 24 and 31 than the wild type mice; however the difference was not significant (Figure 12a). At days 24 and 31, the knockout mice averaged $1.5x10^{5}$ and $2.1x10^{5}$ cells while the wild type averaged $3.3x10^{4}$ and $1.4x10^{5}$ cells. The CD8^{+} T cells expressing TNF and IL-2 remained at low numbers throughout the experiment when

compared to CD4$^+$ T cells of the same cytokine profile. The cell numbers in the MLN for both mouse strains are at or below 5×10^4 throughout the entire experiment.

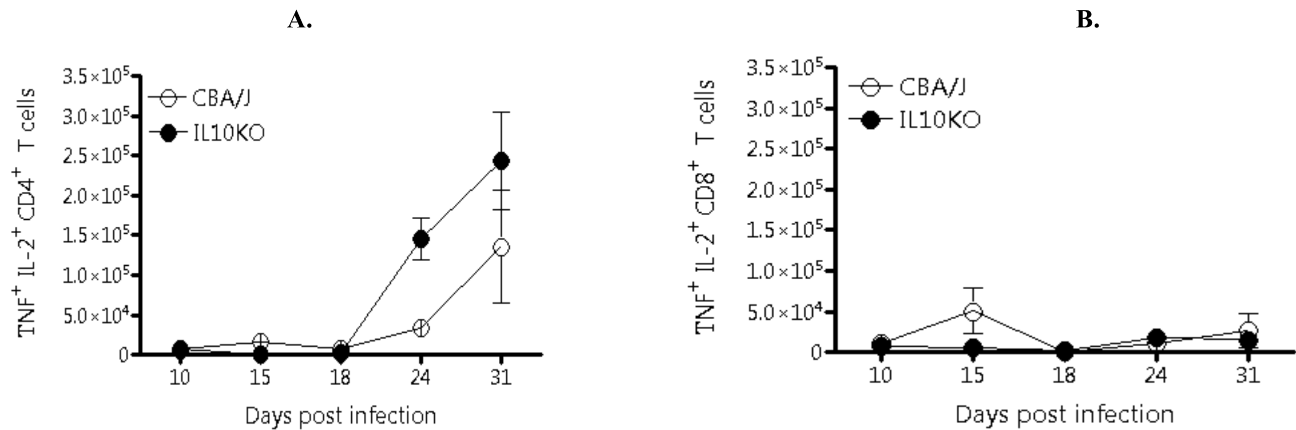


Figure 12. TNF$^+$ IL-2$^+$ T cells in the MLN of CBA/J and CBA/J IL10$^{-/-}$ mice. (A) Shows the CD4 T cells that are expressing TNF and IL-2 (B) shows the CD8 T cells expressing TNF and IL-2.

Mediastinal lymph node cells from CBA/J and CBA/J IL10$^{-/-}$ mice were analyzed by flow cytometry. Cells were gated according to their forward and side scatter profiles and expression of CD3, CD4 (in 12A) or CD8 (in 12B), TNF, and IL-2. Data represents 4-5 mice per group per time point and is representative of 2 experiments. Statistical analysis was performed via the unpaired Student's *t* test

Triple Positive T cells

The final cytokine profile that was analyzed was the T cells positive for IFN-γ, TNF, and IL-2.This measurement was only able to be taken accurately at day 24. The triple expresser CD4$^+$ T cells were more numerous in the IL-10 knockout mouse, averaging 1.0×10^5 compared to

the wild type mouse, which averaged 3.5×10^4. The triple expressing $CD8^+$ T cells were present in very low numbers for both IL-10 knockout mice and wild type mice. The IL-10 knockout mice averaged 1.2×10^3 cells and the wild type mice averaged 1.3×10^3 (Figure 13).

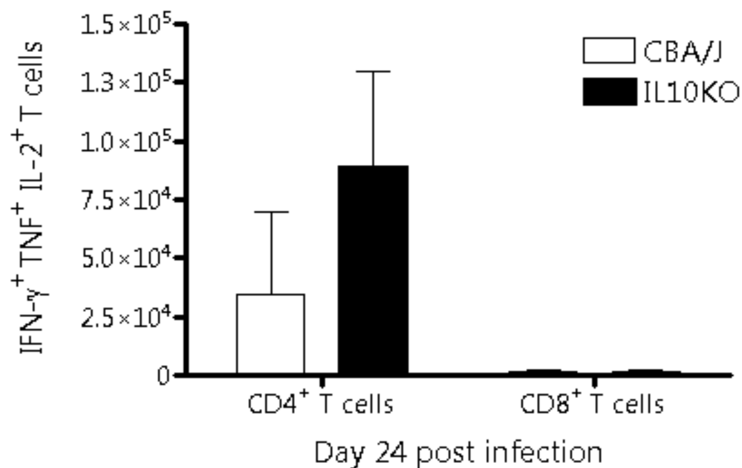


Figure 13. IFN $^+$ TNF$^+$ IL-2$^+$ T cells in the MLN of CBA/J and CBA/J IL10$^{-/-}$ mice at day 24 post infection. Figure 13 shows the CD4 T cells that are expressing IFN , TNF, and IL-2, and the CD8 T cells expressing IFN , TNF, and IL-2.

Mediastinal lymph node cells from CBA/J and CBA/J IL10$^{-/-}$ mice were analyzed by flow cytometry. Cells were gated according to their forward and side scatter profiles and expression of CD3, CD4 or CD8, IFN-γ, TNF, and IL-2. Data represents 4-5 mice per group per time point and is representative of 2 experiments. Statistical analysis was performed via the unpaired Student's *t* test

Discussion

Tuberculosis is a disease that affects millions of people in the world. One of the most puzzling aspects of TB is why some people develop active TB while others are able to contain the bacteria and keep the disease in a latent state. In studies using the CBA/J $IL10^{-/-}$ mouse model of protection from reactivation and the CBA/J model of susceptibility to reactivation, we have the ability to further explore the reactivation phenotype.

This project provides a preliminary analysis of the T cell profile within the mediastinal lymph node of CBA/J and CBA/J IL-10 deficient mice at early points in *M.tb* infection. We hypothesized that IL-10 deficient CBA/J mice would show increased numbers T cells in the MLN and that those T cells would have increased cytokine profiles. In this study, we show that while the numbers of total, $CD4^+$, and $CD8^+$ T cells in the MLN did not vary significantly throughout the experiment, the $CD4^+$ T cells in the IL-10 deficient mice showed greater ability to produce one, two, or three cytokines. In the knockout mice, $CD4^+$ T cells showed trends of greater production of T cells expressing single cytokines IFN-γ, TNF, and IL-2 and expressing double cytokines IFN-γ/TNF, TNF/IL-2, and IFN-γ /IL-2. Additionally, the knockout mice showed trends of increased T cells expressing IFN-γ, TNF, and IL-2.

The CBA/J $IL\text{-}10^{-/-}$ mice are protected in *M.tb* infection. Additionally, when there is early depletion of IL-10 in the CBA/J mouse, there is better control of infection, noted in both decreased bacterial burden in the lungs and organized, mature granuloma formation (unpublished data). Therefore, IL-10 is implicated in affecting early immune response to *M.tb*.

The lung-draining mediastinal lymph node is thought to be the site of *M.tb* adaptive response initiation (11). In accordance with the literature, a significant presence of antigen-specific T cells in the MLN isn't noted until 10-12 days post infection (15, 17). Throughout early *M.tb* infection, we observed increasing numbers of T cells and specifically $CD4^+$ T cells in the

MLN in the wild type and the $IL10^{-/-}$ mice, indicating the early proliferation of T cells. However, we did not observe increased numbers of $CD4^+$ T cells in the $IL10^{-/-}$ mice as has been previously noted (16). Based on our observations of T cell number and expression of early activation marker CD69, there was no acceleration of T cell activation in the MLN in the absence of IL-10.

Although we did not note increased acceleration or activation of T cells in $IL10^{-/-}$ mice, we did measure an increase in multifunctional T cells, $CD4^+$ T cells capable of producing one, two or three cytokines. Currently, there are contradictory views on the role of multifunctional T cells in relation to *M.tb* infection. Multifunctional T cells have been correlated with active *M.tb* infection in human peripheral blood mononuclear cells as well as with protection against *M.tb* infection in mice (12,14). These contradictory findings could be due to differences in experimental models. Our findings are in agreement with previous mouse models that suggest a greater number of multifunctional T cells in mice that are protected in *M.tb* infection (14). Multifunctional T cells have been studied in additional pathogens and past studies of elevated numbers of multifunctional T cells HIV and *Leishmania major* infection (12) support the idea that multifunctional T cells correlate with protection against intracellular pathogens.

While trends of increased cytokine production in the $IL10^{-/-}$ mice were observed in this experiment, statistical significance was often not achieved. This was most likely due to technical challenges in excising MLN at early time points as well as inconsistencies in MLN size among mice used. A repeat experiment with increased technique expertise could lead to significance in these trends. The multifunctional T cells needs to be further analyzed in *M.tb* infection to accurately assess if multifunctional T cells are responsible for control of infection. An adoptive cell transfer of multifunctional T cells into the CBA/J mouse could provide a further link to identify if multifunctional T cells could recover the protected phenotype.

In summary, we show that the absence of IL-10 does not lead to an early or enhanced T cell response in the MLN of CBA/J mice. We suggest that increased numbers of double and triple cytokine producing, multifunctional T cells are present in the $IL10^{-/-}$ mouse and that these cells may play a role in increased protection in *M.tb* infection.

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