FUNCTIONS AND REGULATION OF CD8 T CELLS DURING MURINE INFECTION WITH *MYCOBACTERIUM TUBERCULOSIS*

by

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ABSTRACT

Mycobacterium tuberculosis (*Mtb*), causing tuberculosis (TB), is estimated to infect one third of the human population, and kills about 1.8 million people per year. CD8 T cells are an essential component of adaptive immunity against *Mtb*. *Mtb*-specific CD8 T cells in both mice and non-human primates have been found to exhibit two distinct functional phenotypes: either cytokine secreting or cytolytic activity. In this study, we established an in vitro culture system to expand and differentiate cells and then examined the effects of specific cytokines on priming of CD8 T cells in both vitro and vivo. We hypothesize that these cytokines affect the priming of *Mtb*-specific CD8 T cell responses. In addition, we optimized the immunohistochemical staining for CD8 on granulomas from infected non-human primates, which will enable us to better characterize the distribution and functions of CD8 T cells within granulomas. Finally, we determined whether IL-21 plays a role in bacterial control and immune responses in mice infected by *Mtb*. In summary, our study will contribute to a better understanding in the roles of CD8 T cells against *Mtb* and mechanisms by which they are modulated during the infection, which may aid the development of protective therapies and vaccines.

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PREFACE

This thesis is to recognize the four years' lessons I have learned and to appreciate the support from my family and friends.

1.0 INTRODUCTION

Mycobacterium tuberculosis (*Mtb*), causing tuberculosis (TB), is estimated to infect one third of the human population, and kills about 1.8 million people per year (1). Approximately ninety percent of infected people contain the bacilli in granulomas and are asymptomatic (2). The importance of CD8 T cells in control of *Mtb* had been difficult to define (3). A recent study has showed that depletion of CD8 T cells resulted in elevated bacterial loads and increased pathology in *Mtb*-infected rhesus macaques immunized with Bacillus Calmette-Guérin (BCG) (4). Data from our laboratory demonstrate that depleting CD8 T cells prior to infection exacerbates disease in macaques (Lin, et al, unpublished). **It is still unclear what specific factors contribute to the priming and maintenance of the *Mtb*-specific CD8 T cells.** Cytotoxic activities of *Mtb*-specific CD8 T cells were impaired in the lung of CD4-T-cell deficient mice (12). Several viral infection models indicated that CD4 T cells were crucial for maintaining CD8 T cell survival and effector functions, via IL-21 signaling (5-7). Cytokines related to inflammation, such as IL-10, are involved in the control of TB via antigen-presenting cell regulation, or by altering CD8 T cell responses directly (8). Thus, **we hypothesize that the function of *Mtb*-specific CD8 T cells is shaped by inflammation-related cytokines.**

1.1 EPIDEMIOLOGY OF TUBERCULOSIS

Approximately one third of the world population is infected with *Mtb*, and an estimated1.8 million people die due to TB annually (1). This is made worse with the emergence of HIV (13) and the increased reports of multi-drug resistant strains of *Mtb* (14).The bacillus is transmitted primarily via the aerosol pathway. Ninety percent of infected individuals will become latent TB patients without obvious symptoms, while the other ten percent will develop active and contagious TB (2). An attenuated strain of *Mycobacterium bovis,* BCG, is the only vaccine available for the prevention of pulmonary TB, and although it is widely used, particularly in developing countries, its efficacy in adults is still controversial (15).

1.2 IMMUNE RESPONSES AGAINST *MTB* INFECTION

Once inhaled into the lung, the bacillus is first phagocytized in alveolar macrophages and induces production of chemokines and pro-inflammatory cytokines, which recruit dendritic cells (DC) to the site of infection. DCs take up *Mtb* or *Mtb* antigens and migrate to the draining lymph nodes, where they prime T cells. Primed T cells then migrate back to the lung, and form granulomas that contain *Mtb* infected and uninfected macrophages. Successful containment of *Mtb* inside the granulomas leads to asymptomatic infection (2). CD4 T cells are essential during *Mtb* infection, as demonstrated by increased bacterial burdens and shortened survival time in both CD4-/- and MHC-II-/- infected mice (16), in SIV-infected non-human primates, and HIV-

infected humans (13, 17). *Mtb*-specific CD8 T cells are present in *Mtb* infected humans (18) and mice (19, 20). Both In vivo depletion and adoptive transfer of CD8 T cell indicated that CD8 T cells conferred protection to Mtb infection in mice (19-21). Mice deficient in TAP, a molecule essential for the assembly of peptide/MHC class I/β2-m complexes (22), was compared with CD1d-/- mice. TAP-/- mice, but not CD1d-/- mice, showed impaired resistance to *Mtb* infection (23), confirming the importance of class-I MHC restricted T cells in the control of *Mtb*. The reduced resistance in CD8α-/- mice (24, 25) also confirmed that class I MHC restricted CD8 T cells were critical in *Mtb* infection. A recent study established the essential role of CD8 T cells by demonstrating that depletion of CD8 T cells in BCG-immunized rhesus macaques led to increased pathology and bacterial burden in lungs and lymph nodes after *Mtb* infection (4), which suggests that CD8 T cells may play a more essential role in human *Mtb* infection. Unpublished data from our lab demonstrate that depletion of CD8 T cells in Cynomolgus macaques prior to infection with M. tuberculosis prevented control of early infection and exacerbated disease (Lin, et al, in preparation).

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1.2.1 CD4 T CELLS

CD4 T cells are the essential part of immune protection against Mtb infection in both human and mice. HIV-infected patients have a higher chance of getting reactivation of TB. In the murine model, mice depleted of CD4 T cells have a shorter survival post Mtb infection, compared with the wild type mice. It has been shown that early production of IFN-γ by CD4 T cells is crucial for effective immune responses

against Mtb in mice (16). In addition, our lab has recently published a paper demonstrating that depleting CD4 T cells in Cynomolgus macaques caused higher bacterial loads at monkey lungs and lymph nodes at early stage of infection. More interestingly, depleting CD4 T cells had significant impact on CD8 T cells, including increased levels of IFN-γ production and reduced percentages of CXCR3+ CD8 T cell populations, a type of CD8 T cells involved in T cell recruitment and maturation and as well expressing/co-expressing more cytokines than CXCR3- CD8 T cells. (47)

1.2.2 B CELLS

The role of B cells during Mtb infection is controversial, from being protective to unrelated and even harmful. Our lab recently published a paper describing clusters of B cells formed in the lymphocyte cell region of granulomas from Mtb-infected non-human primates, suggesting that B cells may play a role in the forming of granulomas and containing the bacterium. It was also proposed that B cells may modulate functions of Mb-containing macrophages via antibody-FcR recognition. (48)

1.2.3 ANIMAL MODELS OF TUBERCULOSIS

The most prevailing animal model for tuberculosis research is the mouse. Different mouse strains have been used for studying tuberculosis, including C57BL/6, C3H and DBA/2, with C57BL/6 being the most common. Similar to humans, mice are able to contain Mtb infection for at least one year. However, mice develop a higher bacterial load in lungs compared with humans. In addition, 90% of infected humans exhibit

clinically latent infection, which is absent in mice. In contrast, infected mice develop a chronic infection and eventually die of progressive inflammation. In terms of granulomas, murine granulomas do not exhibit organized structures and caseous necrosis as humans do. With all these disadvantages, mice are still widely used in the research of tuberculosis for reasons including low cost, convenient housing, reagents specific for mice and various genetic knockout strains.

In additional to the mouse model, there are also rabbit, fish, guinea pig and non-human primate models. Of all these models, the responses to Mtb infection in non-human primates are of most similar to that of humans in terms of disease progression, pathology, clinical symptoms and structures of granulomas. Many immunological reagents are available for studying this model because of research on humans as well as related with HIV. However, this model also has several limiting factors, such as expensive cost for purchasing and housing as well as non-consistent immune responses against infection among the monkeys due to genetic variance.

1.2.4 CD8 T CELLS

CD8 T cells are able to produce cytokines, such as IFN-γ, TNF and IL-2, and cytotoxic molecules, such as perforin and granzyme B. One of the most important functions for IFN-γ is to activate macrophages to kill *Mtb* through reactive nitrogen intermediates (RNI) (26). *Mtb*-infected IFN-γ-/- mice exhibited reduced control of bacterial loads and severely shortened survival (27). TNF is also critical to the maximal killing of *Mtb* by macrophages in synergy with IFN-γ (28), as well as maintenance of granulomas in

mice (29), although granuloma formation is maintained in TNF-neutralized macaques (49). Both TNF-α-/- mice and TNF-neutralized macaques were impaired in the control of *Mtb* infection (30, 49). Killing of infected macrophages by T cells utilize several cytotoxic pathways, such as the ligation of CD95/CD95L (ligand), and degranulation of molecules, including perforin and granzyme B. Perforin is a pore-forming molecule, which opens up holes on the cellular surface and allows entry of cytolytic molecules such as granzyme B into target cells to induce apoptosis. T cells express CD95L which binds to CD95 on infected macrophages and induce their apoptosis, causing reduced viability of intracellular *Mtb* (31, 32). In one study, CD95-/- mice have been demonstrated to exhibit reduced control of bacterial load compared with WT mice, suggesting a role for the CD95/CD95L in the control of infection, while perforin-/- mice had a comparable level of bacterial control as WT mice in this study (24). However, another study reported reduced survival time of perforin-/- mice compared with WT mice following *Mtb* infection (25), suggesting perforin may also play a role in controlling *Mtb*. In addition, CD8 T cells in primates express granulysin which confers an additional cytolytic function against *Mtb* or *Mtb* infected cells (33).Levels of granulysin in CD8 T cells from patients infected by multi-drug resistant *Mtb* were lower than that in healthy volunteers, suggesting granulysin of CD8 T cells may play an important role in the control of infection. A transgenic mouse strain expressing granulysin displayed a reduced level of bacterial load with enhanced CD8 T cell cytotoxicity (34). The absence of granulysin in mice may impede our investigations using the murine model to fully understand contributions of CD8 T cells to the control of infection in humans.

1.2.5 FACTORS INFLUENCING CD8 T CELLS

Priming of CD8 T cells involves antigen presenting cells (APCs) and CD4 T cells. APCs, particularly DCs, activate naïve CD8 T cells through TCR/MHC class I-peptide complexes and CD28-CD80/CD86 interactions. Pro-inflammatory and anti-inflammatory cytokines, such IL-12 and IL-10, modulate activation and differentiation of CD8 T cells (35). *Mtb*-infected IL-12p40-/- mice lose their capacity to control *Mtb*, with marked reduction of lymphocyte activation and accumulation in lungs (36). Reports on *Mtb*-infected IL-10-/- mice are controversial. IL-10-/- mice were first observed to have no difference in control of *Mtb* infection compared to WT mice (37). However, a recent study reported IL-10-/- mice exhibited better protection against *Mtb* with decreased bacterial load, accelerated recruitment of T lymphocytes to the lung, and enhanced early Th1 responses (38). IL-10 blocks phagosome maturation and subsequently facilitates intracellular pathogen survival and growth within macrophages. IL-10 is also able to block antigen presentation by down-regulating MHC molecule expressions on macrophages (39). IL-10 inhibits migration of *Mtb*-infected DCs from lungs to lymph nodes, and activated T cells from lymph nodes back to lungs. IL-10R is expressed on a variety of cell, including APCs and CD8 T cells. Effects and mechanisms of IL-10 signaling on the functions of CD8 T cells during *Mtb* infection are not known (8). Additional cytokine candidates mediating CD8 T cell responses include IL-6 and IL-1. IL-6 is produced by a variety of cells, including T cells, B cells and monocytes, and promotes differentiation of cytotoxic T cells, possibly through augmenting the expression of IL-2 receptor and production of

IL-2 (40). IL-1 has been demonstrated to be required for IL-2 production and proliferation of helper-independent CD8 cytotoxic T lymphocytes (CTLs) (41).

CD4 T cells can shape activation and maintenance of primary and/or secondary CD8 T cell responses. However, depending on various disease models, the requirement and specific mechanisms of CD4 T cells on CD8 T cells vary. Modulations of CD8 T cells by CD4 T cells could occur through several pathways, including enhancing DC activation of CD8 T cells via CD40-CD40L interactions and secreting cytokines, such as IL-2 and IL-21 (42). Our lab had previous demonstrated that CD4 T cells were required for development of cytolytic activity in CD8 T cells in mice during Mtb infection (12). Our lab also showed that CD40 was required for optimal resistance to *Mtb* infection in mice (43).

IL-21 is an IL-2 family cytokine dominantly produced by CD4 T cells and critically associated with survival and cytolytic activities of Ag-specific CD8 T cells in viral infections (5-7). Sustained expansion of Ag-specific CD8 T cells also depends on IL-2 signaling in non-lymphoid tissues (44, 45). *Mtb*-infected CD4-/- mice have been demonstrated with impaired CD8 T cell cytolytic activity and diminished IL-2 production in lungs, implying possible effects of IL-2 and IL-21 signaling on CD8 T cells during *Mtb* infection (12).

2.0 PRIMING OF DUAL-FUNCTIONAL CD8 T CELLS DURING *MTB* INFECTION IN MICE

2.1 INTRODUCTION

Previous literature found that Th1-type immune responses, such as IFN-γ and TNF are critical for the control of Mtb in mice. In addition, the cytolytic activity of the immune responses is significant for protecting mice against Mtb. Our lab had published a paper in 2009 indicating that Mtb-specific CD8 T cells in mice expressed distinct functional phenotypes: either IFNγ-secreting or CD107 positive, a cytolytic marker. However, cells expressing both IFNγ and CD107 are rarely observed. Similar results were also observed in our non-human primates infected by Mtb (Lin et al, in preparation). We will define CD8 T cells expressing both CD107 and IFN-γ as dual-functional CD8 T cells.

Einarsdottir (PhD Thesis 2010) showed that a significant portion of naive CD8 T cells were primed into multifunctional cells when adoptively transferred into infected mice, especially at week 3 post-infection, compared with cells transferred in mice prior to infection. In addition, pro-inflammatory and anti-inflammatory cytokine profiles exhibited dynamic changes in both lungs and lymph nodes during the course of infection. Thus, we propose that the priming of CD8 T cells during Mtb infection is influenced by the cytokine environment. Our studies will evaluate effects of specific

cytokines, such as IL-10 and IL-6, on the priming of CD8 T cell effector functions during *Mtb* infection.

The aim of this study is to identify potential cytokines influencing the functional development of CD8 T cells first in vitro then in the vivo environment. We started with setting up an in vitro culture that primes naïve OT-I cells within specific priming environments. We then manipulated levels of specific cytokines to identify their role in the priming of CD8 T cells. Finally, we are using the mouse model to evaluate the effects of the identified cytokines on functional development of CD8 T cells in vivo.

2.2 MATERIALS AND METHODS

2.2.1 Mice

C57BL/6 RAG1-/- background OTI (CD45.2) and B6.SJL-Ptprc Pepc/BoyJ (CD45.1) breeding pairs were obtained from Jackson Laboratories, while C57BL/6 RAG1-/- background OTII (CD45.2) mice were ordered directly from the same company. The mice were kept under specific pathogen-free conditions in a BSL3 facility. For the in vivo cell adoptive transfer experiments, MACS column-isolated OT-I and OT-II cells from mouse spleens were adoptively transferred in PBS to recipient mice by tail-vein injection at $5X10^3$ cells per cell type per mouse.

2.2.2 *Mtb* infection of mice

Mice were infected with Mtb Erdman strain at a low bacterial inoculum, using a nose-only exposure aerosolizer. After 24 hours, we crushed whole lungs from a single mouse and plated the homogenates on 7H10 media agar plates. The plates were incubated for 21 days under the environment of 37-degree room temperature with 5% CO_2. Then we read colony forming units (CFU) by counting the number of bacterial colonies on the plates.

2.2.3 In vitro cell culture

OT-I or OT-II mice spleens were used to isolate OT-I or OT-II cells by the MACS cell separation technology. $5X10^4$ of both cell types are mixed with $2.5X10^4$ bone-marrow derived dendritic cells primed with specific OT-I (ova 257-264) or OT-II (ova 323-339) epitopes. Lung or lymph nodes from Mtb-infected mice were prepared for single-cell suspensions by being crushed through a 40 um cell strainer with a 5 ml syringe plunger. We separated the single-cell suspensions into either supernatants or cell suspensions by centrifugation. Then we added the supernatants to the in-vitro cell culture to mimic the tissue environment of infected mice in vivo. The cultures were incubated for 5 days at 37-degree room temperature with 5% CO_2.

2.2.4 Cytokine analysis

Bio-Rad kits were used to examine cytokine levels within supernatants of infected mice at different stages of infection. We selected the following cytokines for examination: IL-1beta, IL-6, IL-10, IL-13, IL-12p70, and TNF. The plates were read on a Luminex 100 IS Bio-Plex system machine.

2.2.5 Flow cytometry

We examined the proliferation and functional properties of OT-I cells within the in-vitro cell culture. After 5 day incubation, the cell cultures were re-suspended and incubated in T cell medium containing 3um monensin, anti-CD107a and CD107b fluorescein isothiocyanate antibody, and OT-I and OT-II specific peptides (4ug/ml) for ova-specific stimulation, for 5 hours at 37-degree room temperature with 5% CO_2.. Then cells were washed in FACS wash buffers and stained with anti-CD4 alexa fluor 700, anti-CD8 pacific blue, and anti-CD45.2 antibodies in FACS buffer added with 20% mouse serum for 15 min. Then, cells were treated for intracellular cytokine staining by first being permeabilized in permeabilization buffer and then stained with anti-IFNγ-phycoerythrin-Cy7, anti-IL-2 Peridinin-chlorophyll-Cy5.5and anti-TNF allophycocyanin-Cy7 antibodies. Fort the proliferation study, we treated OT-I cells with CFSE prior to start of the culture and interpreted the containment of CFSE as color FITC. An LSRII was used to read the samples and data were analyzed using FlowJo software. All antibodies were purchased from BD Biosciences.

2.2.6 STATISTICAL ANALYSIS

Graphpad Prism 5.0 was used for the statistical analysis. Comparison between two groups of parametric data sets was performed using Student's test. Nonparametric data was analyzed using the Mann-Whitney test. Studies including more than two groups of parametric or nonparametric data sets were compared with ANOVA or the Kruskal-Wallis test. A statistically significant comparison with P values less than 0.05 are indicated

2.3 RESULTS:

2.3.1 Expansion of OT-I cells in vitro

We cultured naïve OT-I cells in vitro together with tissue supernatants harvested from lungs and lymph nodes of infected mice. We distinguished OT-I cells from other cells in the culture using the congenic marker CD45.2, while mice used for deriving dendritic cells and providing supernatants expressed CD45.1. After 5 days incubation, we counted total live cells and calculated numbers of CD45.2+ OT-I cells via ratio of CD45.2+/live cells in the flow cytometry gating. OT-I cells were significantly expanded when cultured with either lung or lymph node supernatants, especially with infected supernatants (Figure 2.1). Thus, we knew that OT-I cells were able to proliferate in the in-vitro culture system and the expansion of naïve OT-I cells was enhanced by

supernatants from infected tissues. Similar results were observed when we used CFSE to track proliferation of OT-I cells under the same in vitro conditions. We did not see much difference in the ability to prime OT-I cells using supernatants from infected lung or lymph node.

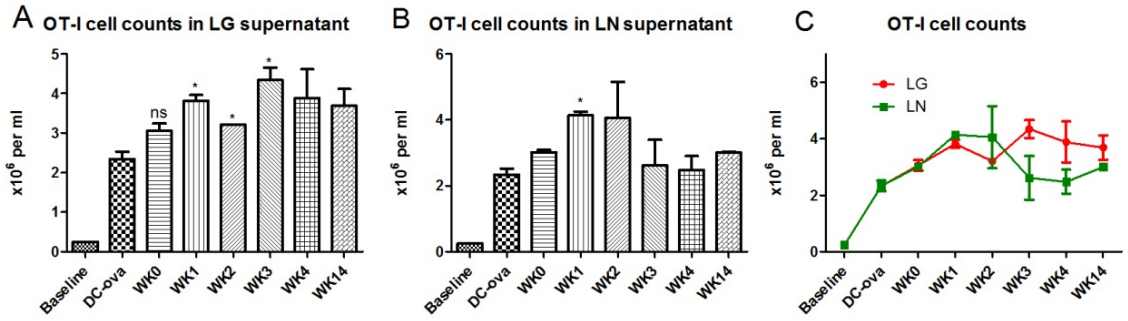

Figure 2.1 Expansion of OT-I cells in vitro.

5X104 OT-I cells were cultured for 5 days and number of OT-I cells were calculated by the total live cell count and the percentage of OT-I cells of live cells based on flow cytometry data.

2.3.2 Function development of OT-I cells in vitro

In the same experiment described above, we examined expression of CD107, IFN-γ, IL-2 and TNF by OT-I cells. Based on Figure 2.2, it is obvious that lung and lymph node supernatants caused dramatic difference in the priming of OT-I cell function. First, CD107 and IL-2 are the two major functions of OT-I cells cultured with lung supernatants, while lymph node supernatants induced higher responses for all of the four functions. Second, percentages of OT-I cells expressing CD107 or IL-2 are higher in later stage of infected lungs while all four markers peaked when cultured with supernatants from early stage infected lymph nodes. Thus, both tissue type and time of

infection impact the priming of effector functions in OT-I cells in vitro. It also suggests that this in vitro model could be used for identifying potential factors influencing priming of CD8 T cells during Mtb infection. In addition, we also compared results of OT-I cell priming with supernatants, tissue homogenates or cell suspensions from the infected tissues, and the functions primed by supernatants were consistently equivalent or better than the functions primed by the other two tissues. Thus, we performed all of our experiments with supernatants only.

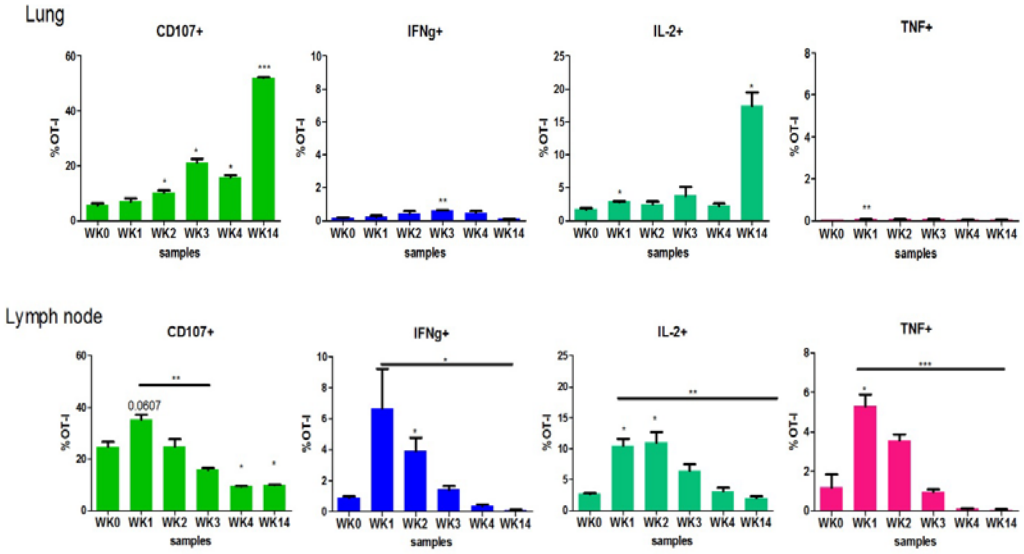

Figure 2.2 Percentages of OT-I cells expressing each of the four functional markers: CD107, IL-2 and IFN-γ. 5X10^4 OT-I cells were cultured for 5 days as described in the above session. Percentages of OT-I cells expressing each of the functional phenotype were analyzed by flow cytometry.

We assessed co-expression of CD107 and IFN-γ in these OT-I cells. OT-I cells in this in vitro system did not exhibit identical functional phenotypes (fewer CD107+ IFN-γ+ cells) as we observed for in vivo adoptive transfer experiment, which might be

due to lack of factors present in vivo, such as CD4 (OT-II) cells in our in vitro culture. Instead, we observed a majority of OT-I cells expressed the CD107+ IFN-γ- phenotype. (Figure 2.3)

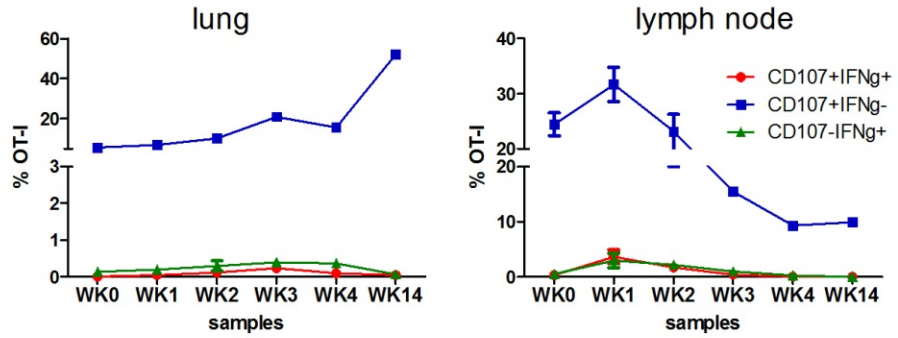

Figure 2.3 Percentage of OT-I cells expressing CD107 and/or IFN-γ. 5X10^4 OT-I cells were cultured for 5 days as performed in the previous figure. Percentages of OT-I cells expressing these functional phenotype were analyzed by flow cytometry.

2.3.3 Cytokine profiles within infected lungs and lymph nodes

Cytokine profiles within lungs and lymph nodes harvested from various stages of infection were examined. The overall profiles are quite different between the two sample types and the level of individual cytokine varies during different stages of infection (Figure 2.4). By comparing the tissue cytokine profiles and outcome of OT-I cell functional development in vitro, we identified several interesting cytokine candidates potentially affecting the priming of OT-I cells during Mtb infection. First, levels of IL-1beta and IL-12 increased significantly within lung supernatants at later stages of infection, where priming of OT-I cells was improved in the previous experiment. Second, TNF levels peaked in both the late stage of lung infection and

early stage of lymph node infection, where OT-I effector functions also peaked in vitro. Third, IL-6 and IL-10 levels peaked at week 1 lymph node supernatants. Fourth, the IL-10 level in lung started high at week 0 and declined to the lowest level at week 3. Based on these data, we started to examine effects of these selected cytokines on the priming of OT-I cells in vitro.

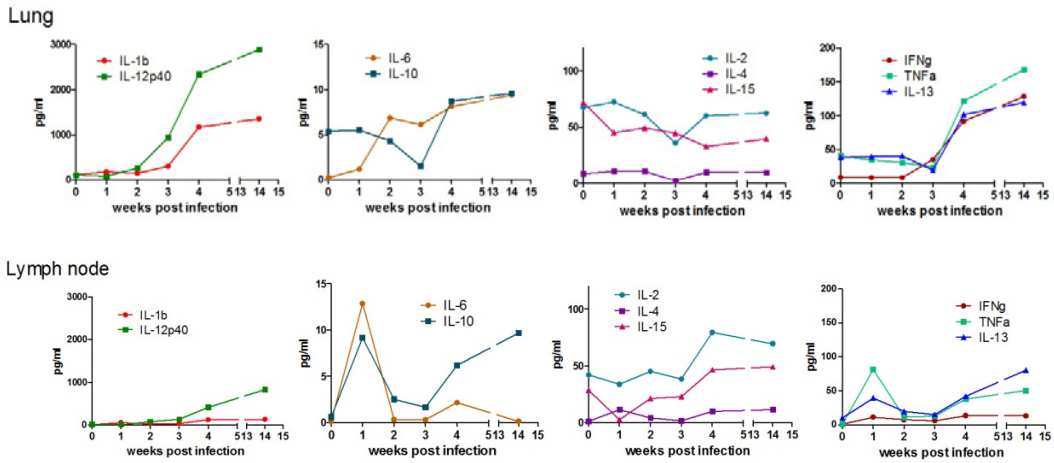

Figure 2.4 Cytokine profiles within lungs and lymph nodes from mice infected by Mtb. Organs were homogenized into single-cell suspensions on a 1 organ: 1ml ratio.

2.3.4 Effects of CD4+ OT-II cells on the priming of OT-I cells in vitro

CD4 T helper cells are considered as important factors for functional development of CD8 T cells. In *Mtb*-infected CD4 T cell deficient mice, the cytolytic activity of CD8 T cells is readily detected in the lymph node, but greatly impaired in the lung, while IFN-γ expression of CD8 T cells were comparable to WT infected mice (12). These observations indicate a critical role of CD4 T cells in the maintenance of CD8 T

cell functions, but specific mechanisms are not known. In a preliminary experiment in vivo (T. Einarsdottir, PhD Thesis, 2010), OT-I and OT-II cells were adoptively transferred together into infected mice and a significant portion of OT-I cells exhibited CD107+ IFN-γ+ phenotype. In the previous in vitro experiments, we did not include OT-II cells in the culture to avoid complexity during analysis. Here, we examined whether OT-II played a role in affecting OT-I cell priming in our in vitro model. The same numbers of OT-II and OT-I cells were added into the culture identical to the previous settings. OT-I cells exhibited overall improvement in expression of CD107 and IFN-γ when added together with OT-II cells. Particularly interesting, the percentage of CD107+ IFN-γ+ OT-I cells was increased. (Figure 2.5) This suggests that OT-II cells influence priming of effector functions of OT-I cells within our in vitro model. Thus, we decided to add OT-II cells into our in vitro culture for all the following experiments.

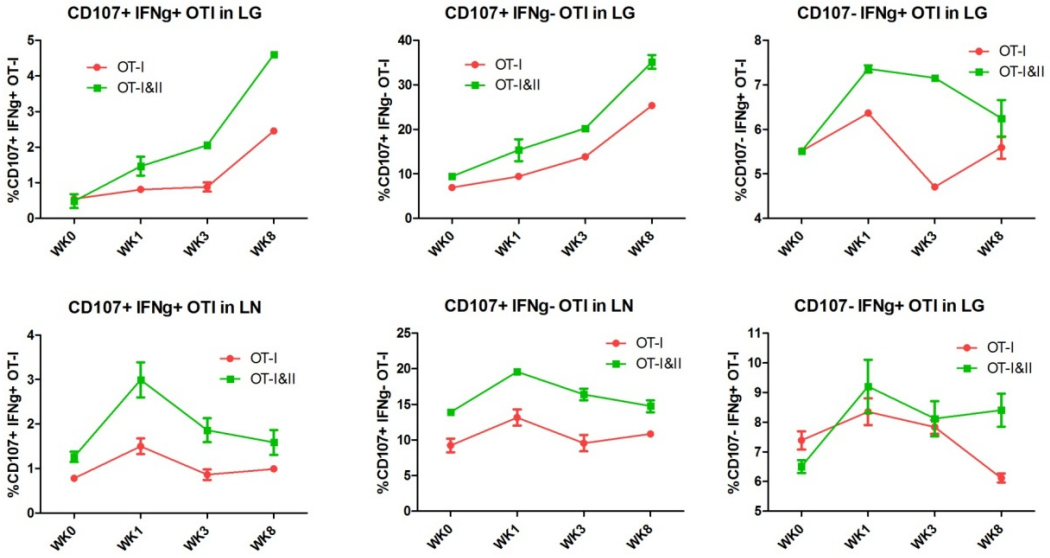

Figure 2.5 Comparison of functions of OT-I cells cultured with or without OT-II cells. Naïve OT-II cells were added together with OT-I cell into the culture, with everything else the same as in the previous experiments. Functional phenotypes of OT-I cells were analyzed by flow cytometry as above. We observed overall statistically significant differences between OT-I cells cultured with OT-II and ones cultured alone.

2.3.5 Examining effects of cytokines on priming of OT-I cell in vitro

Based on previous in vivo data, OT-I cells expressed the most CD107+ IFN-γ+ phenotypes at week 3 post infection. Thus we examined cytokine effects in supernatants from week 3 infected mice. Either recombinant cytokine(s) or neutralizing antibody was supplemented into the culture at day 0. We setup 7 treatment groups: IL-10, IL-1beta, IL-6, IL-12, TNF, combinations of IL-1b, IL-12 and TNF, and anti-IL-10, as shown in the table below. The selection of these specific cytokines was based on the cytokine profiles we examined in the previous session (Figure 2.4).

Amounts of recombinant cytokines added were determined according to their maximal levels measured in Luminex. The concentration of anti-IL-10 neutralizing antibody was based on the manufacturer's protocol. After 5 days of incubation, we examined CD107, IFN-γ, IL-2 and TNF levels of OT-I cells and found anti-IL-10 treatment promoted both CD107 and IFN-γ expressions in OT-I cells. In addition, the expression of CD107 was enhanced by the addition of IL-12 and TNF. (Figure 2.6) However, we only observed an increase in the percentage of CD107+ IFN-γ- OT-I cells by these treatments, but not the dual functional CD107+ IFN-γ+ phenotype (Figure 2.7).

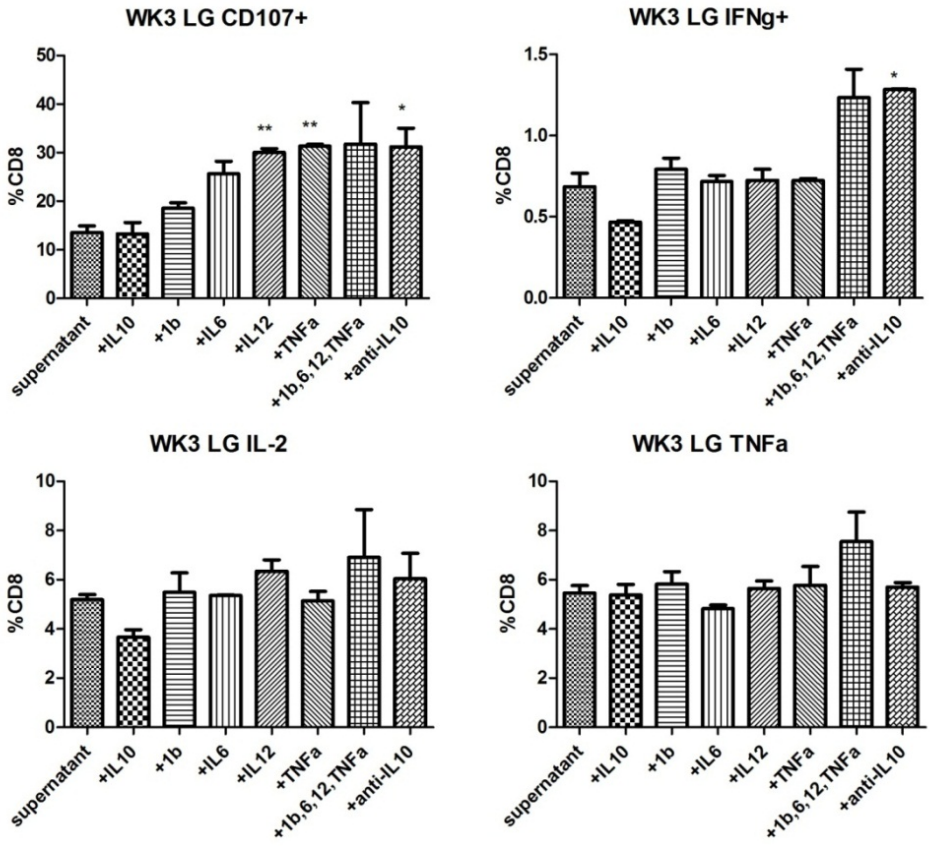

Figure 2.6 Effects of cytokines on the functional development of OT-I cells in presence of OT-II cells in week 3 lung. Recombinant cytokines or neutralizing antibody was added to the in vitro culture of OT-I and OT-II cells, supplemented by supernatants from week 3 infected mice. Levels of cytokines added were determined by the peak level of the corresponding cytokines as measured in the previous experiment. The percentages of OT-I cells expressing each functional phenotype were analyzed by flow cytometry. The stars on each bar indicate statistically significant differences of the treatment group with non-treated control with supernatant only.

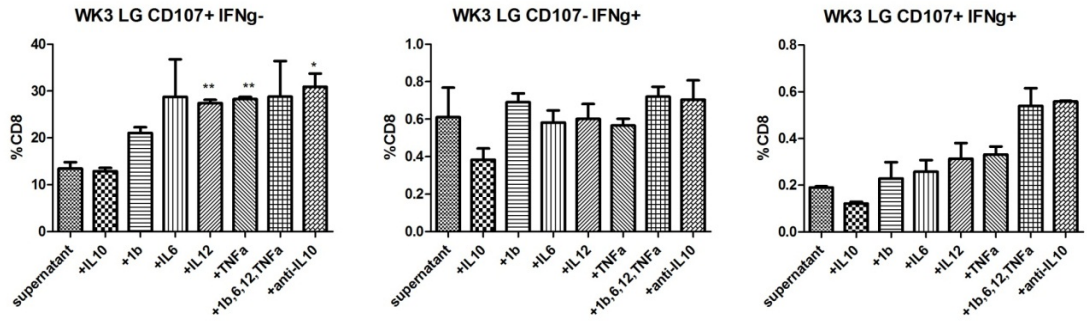

Figure 2.7 Percentages of OT-I cells expressing either CD107 or IFN-γ or both when being cultured together with OT-II cells within week 3 supernatants. The percentages of OT-I cells expressing these functional phenotypes were analyzed by flow cytometry. The stars on each bar indicate statistically significant differences of the treatment group with non-treated control with supernatant only.

Since we observed a peak of IL-6 together with IL-10 levels in the week 1 lymph node supernatants (Figure 2.4), we examined effects of IL-6 and IL-10 on priming of OT-I cells in vitro in the context of week 1 lymph node supernatants. Anti-IL-10 antibody treatment promoted expression of CD107 and IL-2 on OT-I cells. Although the results did not exhibit an enhancement in the proportion of CD107+ IFN-γ+ cells in all of our treatment groups as the previous observation in vivo, it may be due to the drastic differences between the vitro and vivo environments. But we still identified several potential cytokines playing a role in OT-I cell priming, particularly IL-10. To verify its effects in vivo, we need to perform an in vivo experiment.

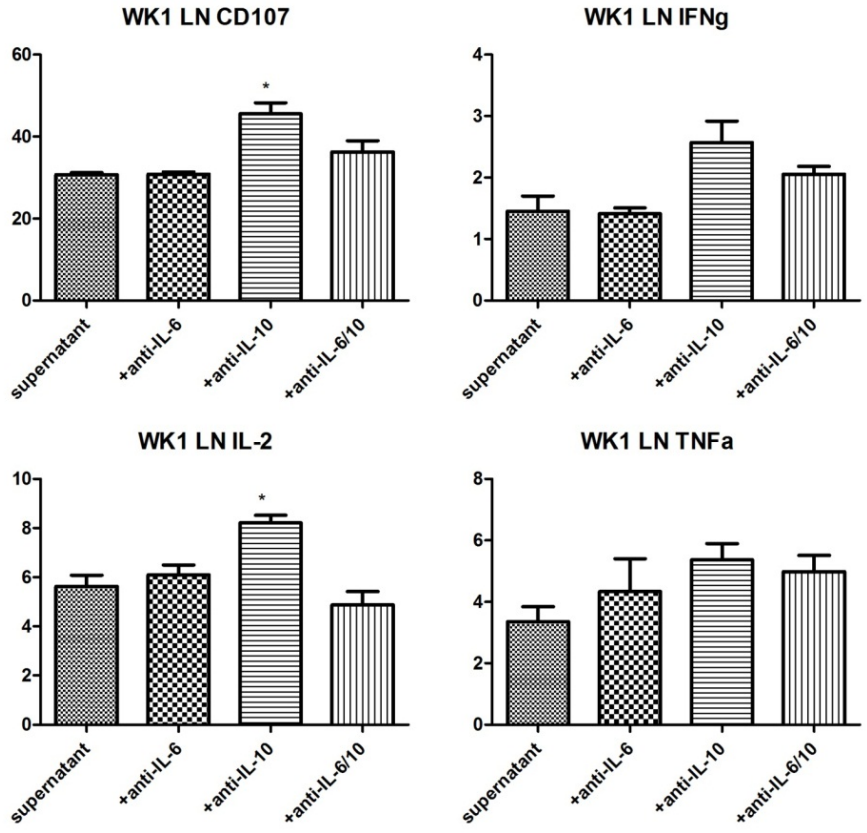

Figure 2.8 Effects of cytokines on the functional development of OT-I cells in presence of OT-II cells in week1 LN. Recombinant cytokines or neutralizing antibody was added to the in the vitro culture of OT-I and OT-II cells, supplemented by supernatants from week 1 infected mice. Levels of cytokines added were determined by the peak level of the corresponding cytokines as measured in the previous experiment. The percentages of OT-I cells expressing each functional phenotype were analyzed by flow cytometry. The stars on each bar indicate statistically significant differences of the treatment group with non-treated control with supernatant only.

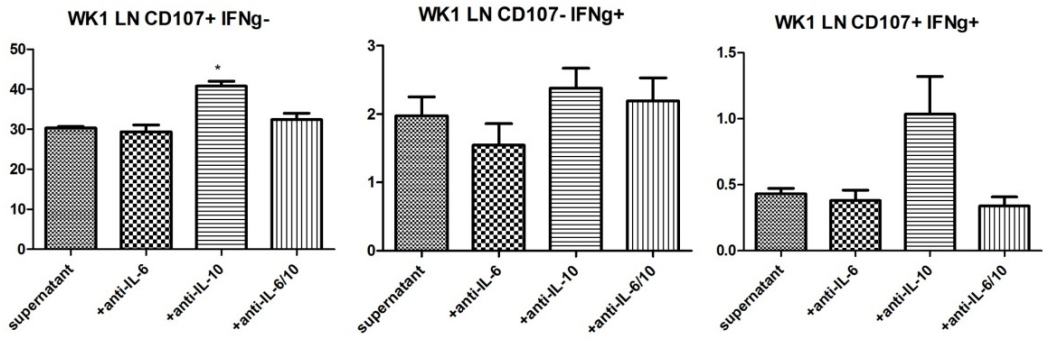

Figure 2.9 Percentages of OT-I cells expressing either CD107 or IFN-γ or both when being cultured together with OT-II cells within week 1 supernatant. The percentages of OT-I cells expressing these functional phenotypes were analyzed by flow cytometry. The stars on each bar indicate statistically significant differences of the treatment group with non-treated control with supernatant only.

2.3.6 Examining effects of IL-10 on priming of OT-I cell in vivo

Mice (CD45.1) were infected with Mtb-ova, an Mtb strain that expressed both OT-I and OT-II specific epitopes. At week 0 and week 3, the same amount of OT-I and OT-II cells (CD45.2) were adoptively transferred into the mice. Mice were treated with anti-IL-10R antibody in PBS or pure PBS across the infection. Lungs and lymph nodes were harvested 3 weeks after cell transfer. Functions of OT-I cells were examined. This experiment is ongoing.

2.4 DISCUSSION

The functions of Mtb-specific CD8 T cells in both mice and non-human primates share similar phenotypes: either cells exhibiting cytolytic activities or cells secreting Th1 cytokines such as IFN-γ. In a previous experiment done in our lab, naïve OT-I cells were adoptively transferred together with OT-II cells at different time points during infection. The proportion of OT-I cells expressing both IFN-γ and CD107 was significantly increased, especially in the cells transferred at week 3 of infection. This suggests that priming dual functional CD8 T cells is possible, though the priming factors leading to this phenotype are unknown. Thus, in this study we proposed an in vitro priming culture of OT-I cells, aiming to mimic the in vivo priming process, identify and test potential cytokine factors that may play a role in the CD8 T cell priming.

We started by establishing an in vitro culture system to expand and differentiate naïve OT-I cells. By supplementing tissue supernatants from various time points of infection into the culture, we found that different environment of priming caused significant difference in the functional development of OT-I cells. In addition, we compared the priming of OT-I cells under the environment of tissue homogenates, supernatants and cell suspensions (separated by spinning), and found that functions induced within supernatants were mostly equivalent or enhanced compared with that induced within tissue homogenates. It suggested that soluble factors within supernatants were playing a major role in priming the OT-I cells in our culture. As shown in many studies, cytokines were the third

signal of CD8 T cell activation, specifically during functional differentiation. Thus, we then moved to examine effects of specific cytokines within supernatants on OT-I priming in vitro.

We started by characterizing both pro-inflammatory and anti-inflammatory cytokines within the supernatants by Luminex. As expected, the profiles within lungs and lymph nodes were quite distinct. In addition, profiles underwent dynamic changes from the early to the late stages of infection. Based on the previous priming results and the corresponding cytokine profiles, we were able to identify several cytokine candidates which might influence OT-I priming. For example, the level of IL-10 declined to the lowest point within lung supernatants at week 3, when it was shown in vivo that transferred OT-I were primed into the most dual functional phenotype. Thus, we examined effects of the cytokines on priming in vitro by adding recombinant cytokines or neutralizing antibodies into the culture. It turned out that IL-10 seemed to be the most effective candidate since depletion of IL-10 led to significant changes in function and percentage of dual functional phenotype in the OT-I cells.

Lastly in the vitro culture, we tested whether OT-II cells could further promote the priming of OT-I cells. As stated above, our previous in vivo adoptive transfer experiment was performed by transferring both OT-I and OT-II together into the mice. It is likely that OT-II played an important role in helping the development of OT-I cells, especially the dual functional phenotype. Thus, we further improved our in vitro culture by adding the same amount of OT-II cells as that of OT-I cells, and found that the presence of OT-II cells did significantly

promoted cytokine production of OT-I cells and more importantly the proportion of OT-I cells exhibiting the dual functional phenotype.

Our current goal was to verify the priming of dual functional OT-I cells in vivo and further improve the priming by modulating specific cytokines during the process of infection. We performed a preliminary experiment in which OT-I and OT-II were together transferred to mice at week 0 and week 3 of infection (by Mtb-ova), and we harvested the lungs and lymph nodes three weeks after each transfer. In the meantime, the mice were treated with anti-IL-10R antibodies to block the signaling of IL-10 throughout the infection. We did not observe significant differences in single or dual functional phenotypes of OT-I cells between the treated and control groups at either time points. It suggests that blocking IL-1O activity alone did not lead to significant changes in the priming of OT-I cells in vivo.

For the future direction, we are hoping to further move on with the current progress. One of the efforts is to repeat the anti-IL10R blocking experiment in vivo in order to test the consistency of the previous experiment. In addition, as suggested by the in vitro priming data that multiple treatments led to changes in OT-I priming, we could try combine several cytokine/neutralizing antibody treatments together for the in vivo experiments. What's more, the specific role of IL-10 directly on CD8 T cells is largely unknown due to the lack of IL-10R KO mouse model. Once this model becomes available, we could examine the direct role of IL-10 on OT-I/CD8 T cells during Mtb infection of mice by adoptively transferring the receptor-KO cells into WT infected mice.

For the long term goal, we are aiming to define the optimal immune responses of CD8 T cells during Mtb infection. One way to confirm if dual functional CD8 T cells are more potent than other functional CD8 T cells is by transferring dual functional OT-I cells that have been activated in vitro into mice infected by Mtb-ova. Although OT-I cells provide a useful tool to study antigen-specific CD8 T cells, we will need to further prove our findings on normal CD8 T cells. To start with, we will replace OT-I with CD8 T cells and ova-pulsed DCs with Mtb-infected DCs in the in vitro culture, which is supplemented with various treatments suggested by the OT-I system. Then, we will repeat the same in vivo experiments as above, but using normal CD8 T cells as the transferred cells.

Our ultimate goal is to improve efficacy of TB vaccines, particularly BCG. One of the most obvious defects of the BCG vaccine is its low efficacy in inducing strong CD8 T cell responses. Our current data suggest that modulating cytokine levels may influence the functional development of CD8 T cells during Mtb infection. In addition, we revealed that the time to deliver the cytokine treatments could also influence the outcome of CD8 priming. Thus, we will continue to contributing to the induction of optimal CD8 responses against Mtb infection in humans.

3.0 PILOT STUDY: TESTING THE ROLE OF IL-21 DURING *MTB* INFECTION IN MICE

3.1 INTRODUCTIONS

IL-21, an IL-2 family cytokine member, is produced by multiple cells, including CD4 T cells and NKT cells (50). It was previously shown in the literature that it had impact on the functional development of CD8 T cells (5-7). However, its role in Mtb infection was not known. In this study, we examined overall levels of IL-21 during Mtb infection in mice and determined its role by comparing bacterial load and immunological responses between wild mice and IL-21R knockout mice after Mtb infection.

Recent studies report that IL-21 is critical to the survival of activated CD8 T cells and maintenance of their effector functions during viral infections (5-7). There are no such studies carried out in the field of TB yet. We will fill in this gap by examining the role of IL-21 signaling on *Mtb*-specific CD8 T cells.

3.2 MATERIALS AND METHODS

3.2.1 Mice

For this pilot experiment, we ordered 4 IL-21R KO mice and 5 C57/BL6 mice as background control (1 control for Day 1 CFU). They were kept in the BSL3 pathogen-free environment.

All the mice were infected together at a low-dose aerosol manner, as described in the project 1.

3.2.2 Flow cytometry

We had two time points of tissue harvest: week 4 and week 8. At each of the point, 2 knockout and 2 control mice were harvested for their lungs and lymph nodes. As described in project 1, the tissues were separately prepared into single-cell suspensions for staining. The cells were first stained by MHC-I-GAP-APC tetramer and MHC-II-ESAT6-PE tetramer for 30 min to bind GAP-specific CD8 and ESAT6-specific CD4 T cells. Then cells were stimulated by 4ug/ml ESAT6 and 4ug/ml GAP peptides together with anti-CD107a-FITC and anti-CD107b-FITC for 5 hours, similarly as described as in project 1. After the incubation, we followed the same protocol in project 1 to surface stain the cells with anti-CD4 AF400 and anti-CD8 Pacific blue, and then to stain intracellular molecules with anti-IFN-γ-PE-Cy7, anti-TNFa-APC-Cy7 and anti-IL-2-PerCP-Cy5.5. Finally the cells were fixed by 2% PFA, read in LSRII and analyzed by flowjo.

3.2.3 ELISA

We purchased a ready-to-go ELISA kit from eBioscience company to measure levels of IL-21 in lungs and lymph nodes supernatants harvested from different stages of infection of mice.

3.2.4 Statistics

Graphpad Prism 5.0 was used for the statistical analysis. Comparison between two groups of parametric data sets will utilize Student's test. Nonparametric data will be analyzed using the Mann-Whitney test. Studies including more than two groups of parametric or nonparametric data sets will be applied with ANOVA or the Kruskal-Wallis test. A statistically significant comparison is marked by a P value less than 0.05.

3.3 RESULTS

3.3.1 Levels of IL-21 in lungs and lymph nodes from early to chronic infection of mice

IL-21 levels changed differently in lung and lymph node as the disease was progressing, with IL-21 level gradually declining in lung while overall increasing in lymph node.

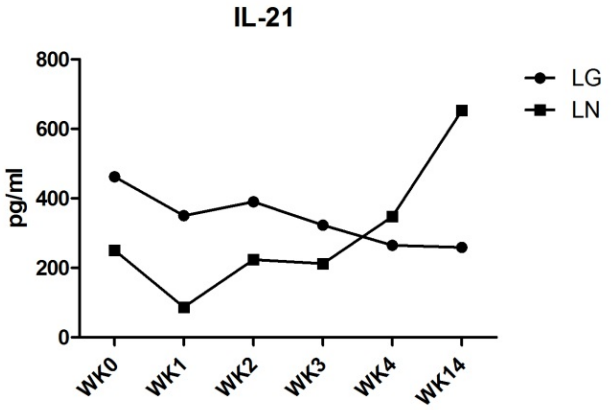

Figure 3.1 Level of IL-21 at lungs and lymph nodes from mice infected by Mtb at week 0 (before infection), 1, 2, 3, 4 and 14. Each organ was ground into 1ml single-cell suspension.

3.3.2 Bacterial load in lungs and lymph nodes at week 4 (top) and week 8

We did not observe significant difference in CFU between the knockout and wild type mice at either lungs or lymph nodes at either time point. It might be due to the small size of our animal group. However, there does not seem to be a significant effect of a lack of IL-21 signaling on control of Mtb infection in mice.

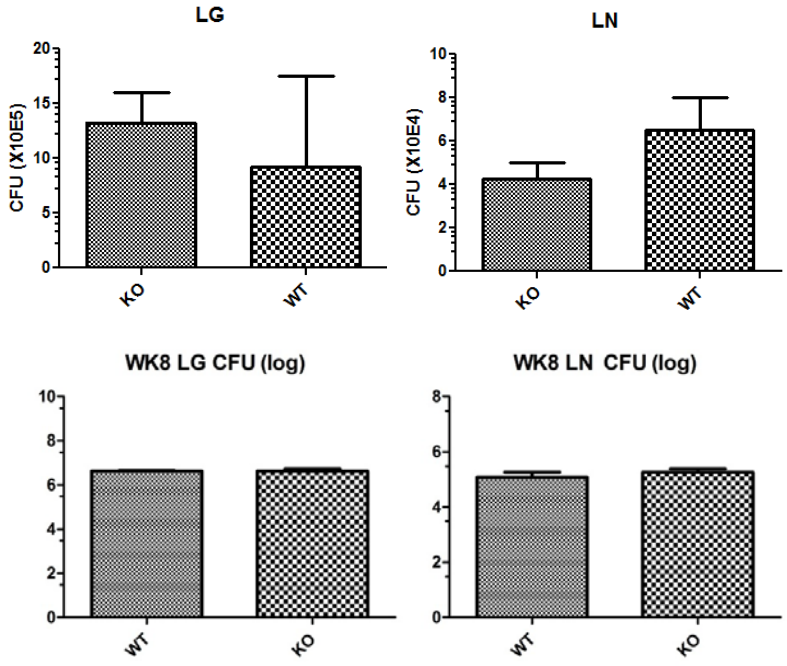

Figure 3.2 Colonies of forming units of the bacteria on agar plates. Infected tissues from week 4 (top) and week 8 (bottom) mice were plated on the plates for 3 weeks incubated at 37 degrees.

3.3.3 Immune responses of T cells at week 4

We compared expressions of four functional markers (CD107, IFN-γ, IL-2, TNF) in CD4 and ESAT6-specific CD4 T cells between knockout and wild type mice in

both lungs and lymph nodes. The CD4 T cells within lungs of knockout mice had lower levels of IFN-γ, IL-2 and TNF than the wild type mice. No difference was found in the lymph nodes. We also examined the same functions for ESAT6-specific CD4 T cells in both lungs and lymph nodes, but there was no difference between KO and WT.

Percentages of cells expressing either CD107 or IFN-γ or both in CD4 and ESAT6-specific CD4 T cells from KO lungs were lower than in WT mice lungs. Again, there was no difference between KO and WT for both cell types in the lymph nodes.

Since we were particularly interested in the CD107+ IFN-γ+ dual functional phenotype, we examined whether this cell type was affected if IL-21R was missing. The percentage of this type in CD4 T cells declined due to IL-21R KO. In addition, similar trend was observed in ESAT6-specific CD4 T cells, though not significant likely due to the small size of this experiment.

For CD8 T cells and GAP-specific CD8 T cells, we examined and compared their functions as what we did for the CD4, but no differences were observed between WT and IL-21R knockout mice.

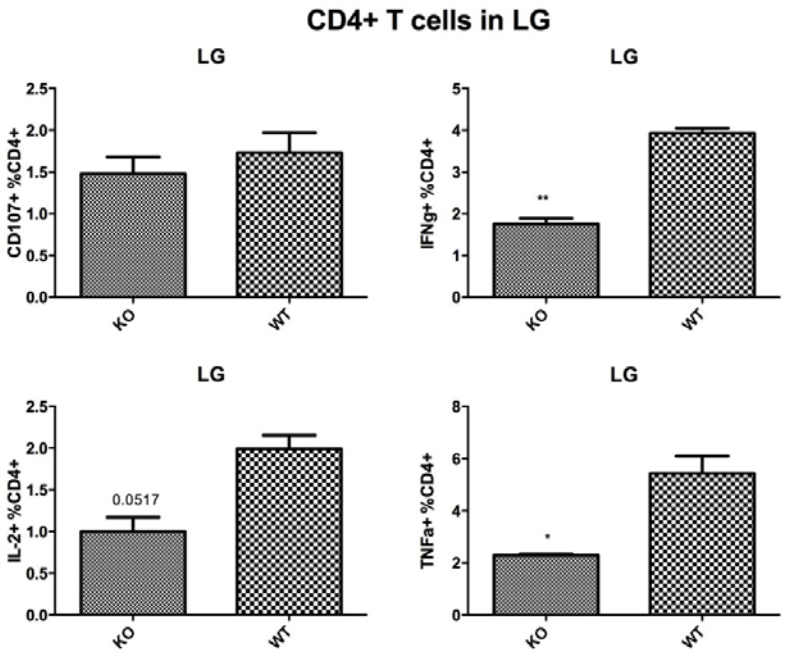

Figure 3.3 Comparison in percentages of CD4 T cells expressing CD107, IFN-γ, IL-2 or TNF in lung between KO and WT mice at week 4

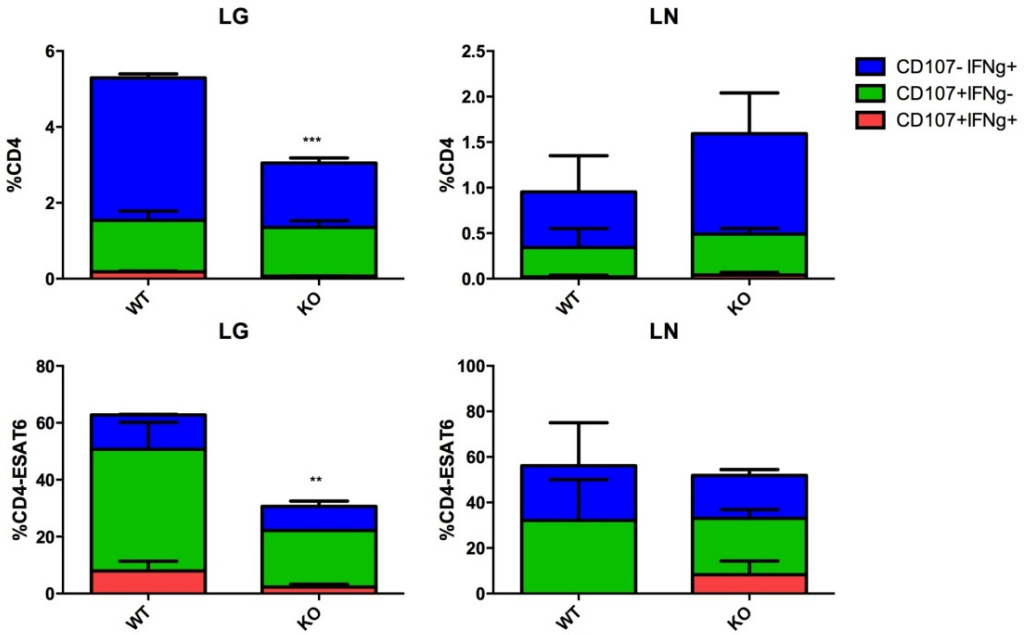

Figure 3.4 Comparison in percentages of functional CD4 T cells expressing CD107 or IFN-γ or both in lungs and lymph nodes between KO and WT mice at week 4

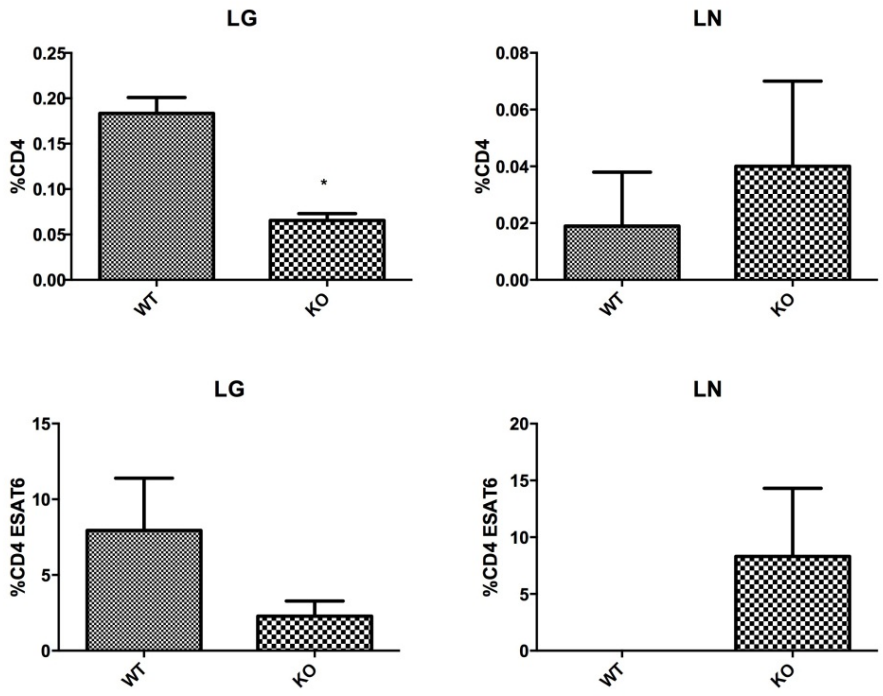

Figure 3.5 Comparison in dual functional CD4 T cells expressing both CD107 and IFN-γ in lungs and lymph nodes between KO and WT mice at week 4.

3.3.4 Immune responses of T cells at week 8

Similarly as in WK4, we examined immune responses of all cell types in lungs and lymph nodes from WK8 mice. Surprisingly, we found the absence of IL-21R caused enhanced percentages of CD4 and ESAT-6 specific CD4 T cells that expressed either CD107 or IFN-γ or both within both lungs and lymph nodes. No other difference was observed between the KO and WT.

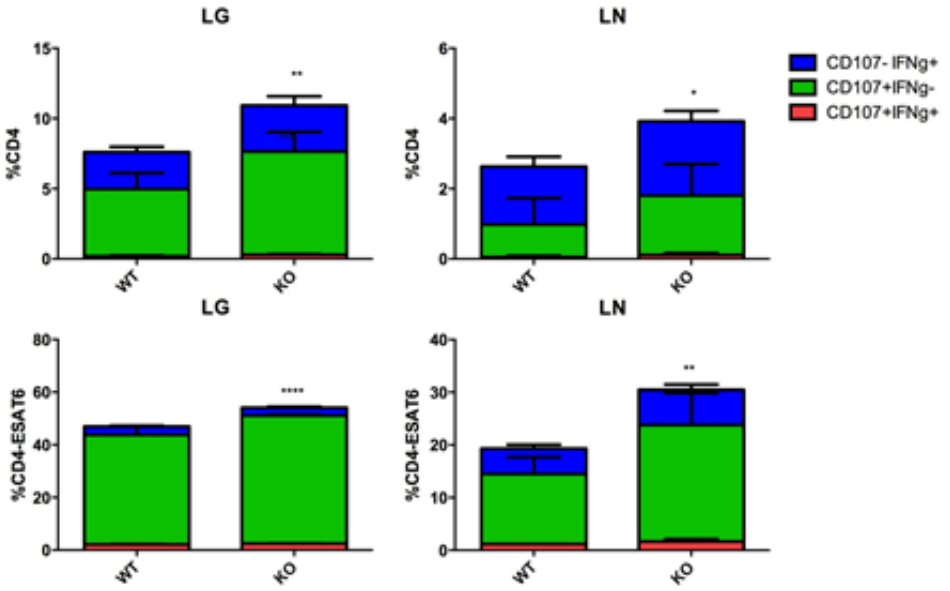

Figure 3.6 Comparison in percentages of functional CD4 T cells expressing CD107 or IFN-γ or both in lungs between KO and WT mice at week 8

3.4 DISCUSSION

IL-21R is the newest discovered member of the IL-2R family that shares a common gamma chain. It exists on a variety of immune cells, including CD4, CD8, B, DC, macrophages and NK cells. Its expression on T cells is a biphasic stage: first upregulated by the activation via TCR and then enhanced by the signaling through IL-21-IL-21R signaling. IL-21 is produced by a collection of cells, mostly by activated CD4 T cells such as Th1, Th2 and Th17 cells. Effects of IL-21 vary depending on its cell targets. For example, IL-21 promotes production of immunoglobulin from activated B cells but leads to apoptosis for unstimulated B

cells. It is also shown that IL-21 could promote cytolytic activities in NK cells and CD8 T cells. Thus, IL-21 has been used as an anti-tumor protein to suppress tumor in both mouse models and human clinical trials.

In this study, we assessed effects of IL-21 on control of TB in mice and on immune responses of both CD4 and CD8 T cells. In the previous literatures, IL-21 has been found critical for the differentiation of Th17 cells because it induces expressions of IL-23R, essential for stable differentiation of Th17 cells (51). In addition, IL-21 was also shown to inhibit development of Th17 cells. However, the role of IL-21 on Th1 or Th2 cells is controversial. Some reports indicated IL-21 has no impact on the CD4 cells. Some showed IL-21 promotes functions of CD4, while others suggested it reduced IFN-γ production. In our study, we found that IL-21R KO caused impaired Th1 responses (reduced IFN-γ, IL-2 and TNF production) at week 4 in the lung. It is possible that Treg cell activities were inhibited by IL-21, which in turn promoted functions of Th1 cells. But it is unclear if IL-21 has a direct role on these CD4 T cells. However, the absence of IL-21R enhanced CD107 expressions on CD4 T cells at week 8 at both lungs and lymph nodes. Although cytolytic CD4 T cells were found in both Mtb-infected humans and mice, it is unclear how important this phenotype of CD4 T cells is against Mtb. Our data may reveal an unknown role of IL-21 which influences the cytolytic activity of CD4 T cells.

For CD8 T cells, although we did not find any difference in immune responses between the KO and WT mice, it is also likely that knocking out IL-21 alone could not cause significant changes to CD8 T cells, but when knocked out

together with other cytokines. Other studies had shown that IL-21 and synergized with either IL-15 or IL-7 to promote cytokine secreting and cytolytic functions of CD8 T cells (52, 53). Our group had examined the effects of knocking out IL-15 gene on the control of TB in mice, and there was no significant change in both immune responses development following Mtb infection (54). It is unknown whether IL-21 and IL-15 ate playing synergic or redundant roles on CD8 T cells during Mtb infection. Thus, we propose that there may be an impact on CD8 T cell responses if both IL-21 and IL-15 were knocked out, which requires a double-knockout strain of mice with IL-21 and IL-15 deleted.

Lastly, we have found no difference in the CFU between the KO and WT, indicating deleting signaling of IL-21 alone does not have impact on the control of bacteria in mice. In the future when a mouse strain with both IL-15 and IL-21 signaling blocked, it is likely to test whether the new KO strain will exhibit different pathology and immune responses against the Mtb compared with the WT strain.

4.0 OPTIMIZATION OF CD8 STAINING IHC PROTOCOL ON NON-HUMAN PRIMATES GRANOLOMAS

4.1 INTRODUCTION

Although the mouse model is very popular in the study of TB, the non-human primate model is of essential importance to the research of Mtb, since this model shares far more similarities with Mtb-infected humans compared with the murine model. The critical role of CD8 T cells against TB is not revealed in mice, but in the non-human primate model. A preliminary study from our lab showed that depletion of CD8 T cells in Mtb-infected monkeys using anti-CD8 neutralizing antibodies caused severe development of the disease (Lin, et al, in preparation). As a result, studying CD8 T cells in the non-human primate model is a necessary step when we translate the data from the mouse model to the real TB in humans. There are a variety of tools to study CD8 T cells in non-human primates and the most important ones include flow, ELISPOT and IHC.

Immunohistochemistry (IHC) is a powerful technique to directly visualize distribution and functions of effector cells in the granulomas. However, the staining for CD8 in non-human primate granulomas has been a problem as a result of consistent weak fluorescent signals with high background, regardless of primary antibody (we used anti-human antibodies) or staining protocol used.

A new technique termed Polymer-TSA staining that efficiently amplifies weak fluorescent signals in many studies became available. In this study we

introduced this method to our non-human primate model of Mtb to optimize CD8 staining IHC protocol.

4.2 METHODS AND MATERIALS

4.2.1 IHC staining with Polymer-TSA (Tyramide Signal Amplification) technique

Tissues or animals were first perfused to exclude blood, followed by fixation using formalin in order to crosslink proteins and reduce solubility. The fixed tissues were embedded in paraffin to maintain the natural shape and architecture of samples during long-term storage and sectioning for IHC. The next step is to section the tissues into 4-5um slices with a microtome and then mount the sections onto glass slides with an adhesive. Then xylene was used to remove paraffin, followed by boiling in buffer (EDTA, Tris, pH 9.0) for 7 minutes to remove formalin. The boiled slides were cooled in the buffer for 30 min. Then blocking was done by 1% BSA in 1XPBS for 1 hour. Primary antibody (1:100, anti-CD8a, clone 1A5) was added to slides and incubated for 1 hour in room temperature. After the binding, H_2O_2 at 0.1% in 1XPBS was added to reduce interference by tissue peroxidases. Slides were rinsed with 1XPBS for 6 times to completely remove unbound antibodies or antibodies bound weakly to non-specific sites. Rabbit anti-mouse polymer conjugated with HRP was added, 30min, RT. Slides were then washed with 1XPBS 3 times with 5 min each time.

TSA at 1:50 dilution was added for 5 min, which will transform the chromogenic signal of HRP into fluorescent signal of Cy3. The tissue was sealed with Prolong Gold Mounting Medium with DAPI to prevent solubilization of precipitated colors and fluorophore photobleaching.

4.3 RESULTS

4.3.1 Testing CD8 IHC staining with the polymer technique

The first image is from a tissue slide not treated with TSA, thus exhibiting chromogenic signal. This revealed good staining of CD8 T cells and indicated that CD8 T cells are located within the lymphocyte cuff in the peripheral region of the granuloma (Figure 4.1). We added TSA to the protocol and transformed the chromogenic signal into fluorescent signal (Cy3) (Figure 4.2).

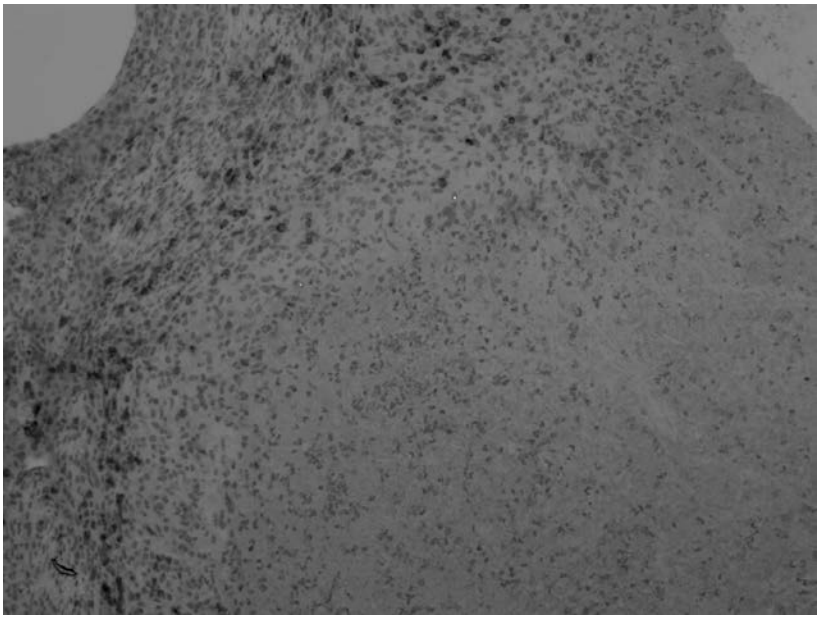

Figure 4.1 non-human primate granuloma with CD8 (dark) stained with polymer

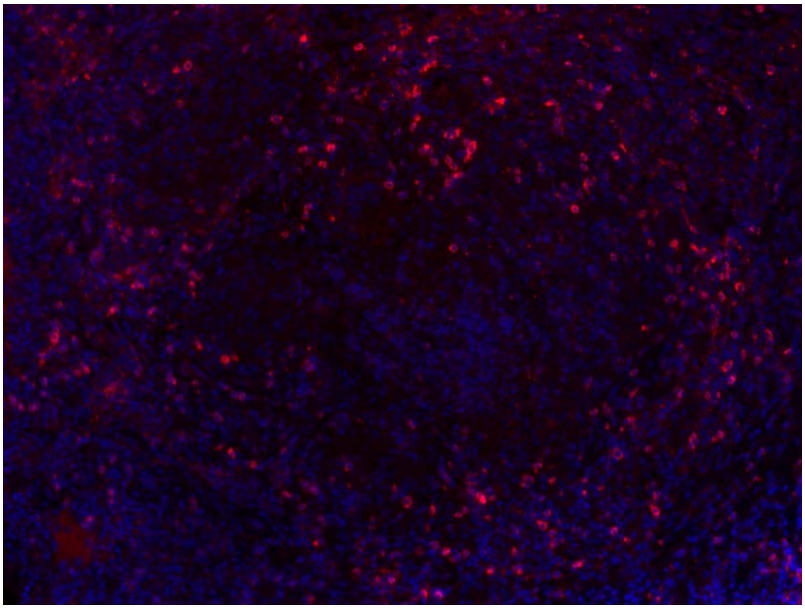

Figure 4.2 A non-human primate granuloma with CD8 (red) stained with Cy3 signal transmitted from the chromogenic signal by the TSA.

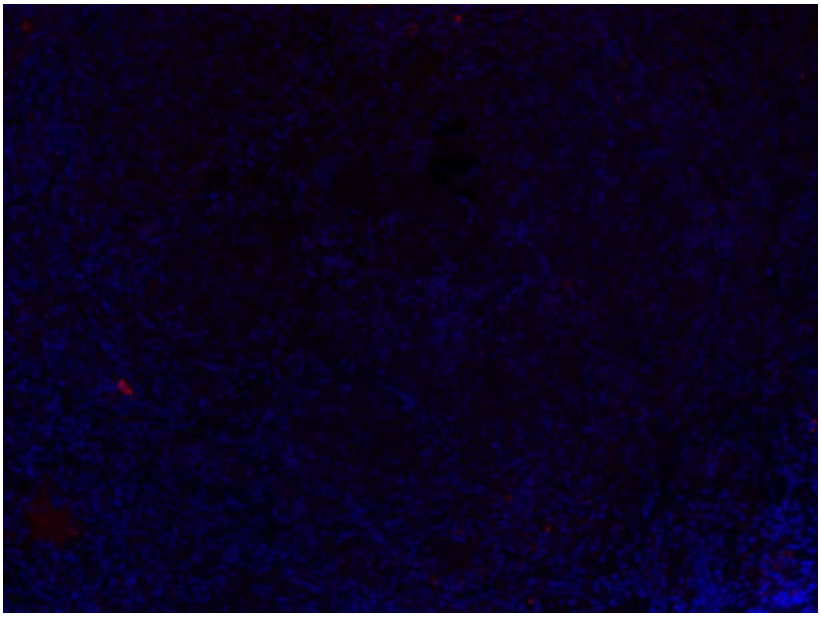

Figure 4.3 Isotype staining of an non-human primate granulomacoupled with the Figure 4.2

4.3.2 Co-staining CD8 and CD3 on monkey granulomas

Since not all CD8+ cells are CD3+ T cells, it is necessary to co-stain CD3 together with CD8 in the IHC to verify CD8+ T cells. The primary antibody for CD3 is from the rabbit and the secondary antibody is anti-rabbit antibody. Thus we were able to combine the staining protocols of the two markers. In the first image from a lung granuloma (Figure 4.4), we observed that both CD3 and CD8 were well stained at the peripheral region of granuloma. In the second image at higher power, we saw that most CD8+ cells are actually CD3+, which indicated the protocol was well established to stain both CD3 and CD8 in non-human primate granulomas.

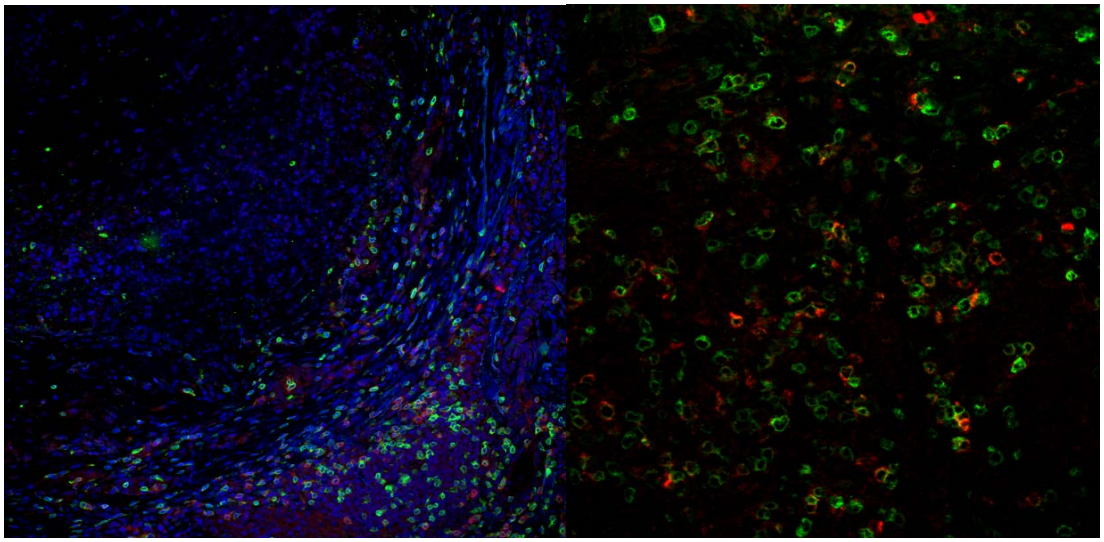

Figure 4.4 A non-human primate granuloma with CD8 (red), CD3 (green) and nuclei (blue). Left 20X and right 60X.

4.3.3 Testing staining of CD8 and granzyme B together

Besides distribution of CD8 T cells, we were also interested in observing functional markers of CD8 T cells in the granulomas. One of the markers is granzyme B, which is an important cytolytic molecule produced by CD8 T cells. Since the primary anti-granzyme B antibody we chose was from mouse, we used a direct conjugation kit to conjugate the primary antibody with anti-mouse AF488 antibody prior being added to the slide. In this image (Figure 4.5), some CD8+ cells were expressing granzyme B (green), suggesting efficacy of the co-staining protocol. In addition, many non-CD8 cells were also producing granzyme B, mostly likely neutrophils.

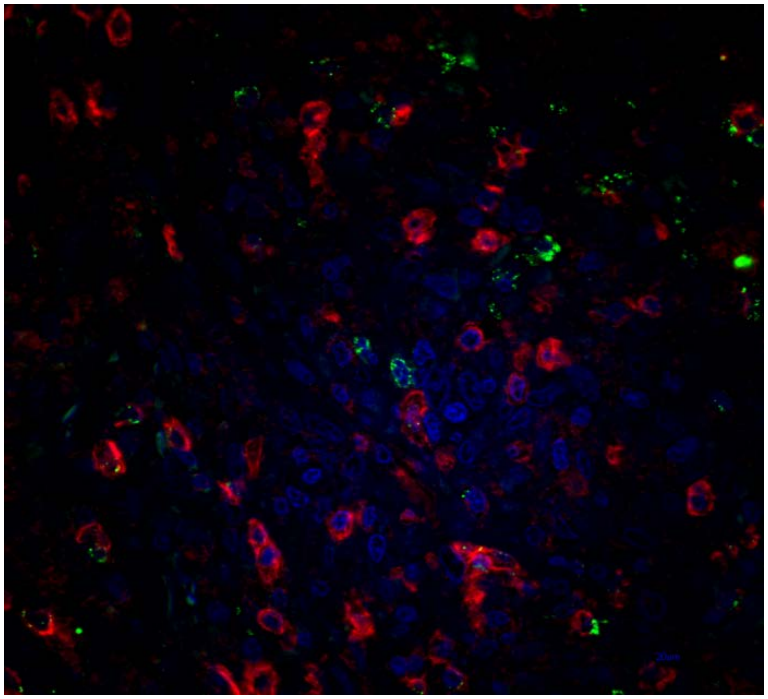

Figure 4.5 A non-human primate granuloma with CD8 (red), granzyme B (green) and nuclei (blue). (60X)

4.4 DISCUSSION

Although percentages of CD8 T cells as well as their functions were analyzable by flow cytometry, visualizing the cells within granulomas is still important because it enables us to better understand the distributions and spatial arrangement of CD8 T cells in the granuloma and provides snapshots of functional phenotypes of CD8 T cells. The IHC technique could also help us to track changes in the structures of granulomas as well as functions of specific immune cells during different stages of infection, in various types of granulomas as well as from monkeys receiving any treatment. For example, depleting CD4 T cells in monkeys infected by Mtb led to severe disease (47). Detection of CD8 T cells in granulomas will enable the comparison of numbers of CD8 T cells, proximity to CD4 T cells, and their functions within granulomas from the CD4 depleted and control monkeys. In addition, our lab has shown that depleting CD8 T cells also caused severe TB in monkeys (Lin, et al in preparation). With the CD8 staining technique, we can track the effect of depleting antibody on numbers and location of CD8 T cells, and further determine its impact on forming a structured granuloma.

In the previous several years, our lab had been trying to visualize CD8+ cells within the monkey granulomas. There were several successful trials, but then most staining failed after the initial anti-human CD8 primary antibody stock ran out. Although the same clone of antibody was order again from the same company, the staining never worked as it did in the first place. The key issue is the weak binding of the anti-human antibody to the monkey CD8 T cells. We tried

various amplification protocols, such as using indirect binding with biotin and increasing concentration of antibodies, but none of them worked well, and instead increased background staining. In addition to the single primary antibody, we also tried 6 other clones of anti-human CD8 primary antibodies, ran them through the various protocols, and none of them worked either. Thus, in order to break through this bottleneck, we need to apply a new signal amplification protocol to our current one.

The major goal of this study is to look for such a new amplification technique for staining CD8+ cells in monkey granulomas with anti-human CD8 antibody as the primary antibody. As shown in the results, we were able to establish a protocol to amplify the CD8 signal without substantially increasing background staining. This protocol has been verified multiple times on granulomas from different monkeys. In addition, the protocol was shown to work well with protocols for staining other markers, such as CD3 and granzyme B, suggesting it as a promising method for wider usage.

Due to time limit and sample availability, I was only able to work out the protocol of staining CD8 together with CD3 or granzyme B. I also tested staining CD4 and CD8 together, but the CD4 signal looked much weaker than being stained alone without CD8. The reason why CD8 staining is interfering with CD4 is not known. Thus, it leaves new questions for future investigation.

For the long term goal, we hope to apply this new technique of CD8 IHC staining to examine distributions, locations and interactions to other immune cells of CD8 T cells within various granulomas. For example, we are interested in

looking into the impact of CD4 T cells on CD8 T cells in monkeys depleted of CD4 T cells. We also have CD8-depleted monkeys and the IHC will help us track how this treatment influences the presence and functions of CD8 T cells within granulomas. What's more, we could compare the CD8 T cells between monkeys from various stages of infection or various types of granulomas. Overall, the visualization of CD8 T cells (together with the functions and co-localization with other cells) will greatly help us understand the role of CD8 T cells in the non-human primate model of TB and further facilitate the control of TB in humans.

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