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Characterization of the Protective Role of Th17 Cells in an Fc Receptor-Targeted Vaccine Strategy Against Fracisella tularensis Infection

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CHARACTERIZATION OF THE PROTECTIVE ROLE OF Th17 CELLS IN AN Fc RECEPTOR-TARGETED VACCINE STRATEGY AGAINST *Francisella tularensis* INFECTION

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Abstract

Using a murine model, we have previously showed that targeting an inactivated form of *F.tularensis* (iFt) bacteria to Fcγ receptors by utilizing an IgG2a, anti-LPS monoclonal antibody (mAb) provides 100% protection against a lethal dose with the live vaccine strain (LVS). The binding of anti-LPS monoclonal antibodies and iFt create an immune complex (mAb-iFt) allowing the Fc-region of the antibody to target Fc receptors expressed on antigen presenting cells, leading to a more robust and effective processing and presentation of the bacterial antigens. Although the resulting protective response generated relies on both humoral and cell-mediated immunity, in our study we focus specifically on a subpopulation of T helper (TH) lymphocytes by using C57BL/6 wild-type mice as an *in vivo* model for immunization and *F. tularensis* infection. Mice were immunized using fixed bacteria (iFt) or immune complexes (mAb-iFt) followed by a lethal dose of *F. tularensis* live vaccine strain (LVS) administered intranasal. Our data suggests that Th17 cells (characterized as $CD3^+/CD4^+/IL17A^+/ROR\gamma T^+$ cells), a T-lymphocyte subpopulation involved in pathology during autoimmunity but also protection against infectious diseases, was significantly increased in mAb-iFt immunized mice compared to mice immunized with iFt alone. Furthermore, the increased levels of IL17A, a cytokine predominantly secreted by Th17 cells, in the mAb-iFt immunized groups suggests a protective role played by this T lymphocyte subpopulation in this *F.tularensis* vaccine model.

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1. Introduction

1.1 Immunity and Vaccination

There are two branches of the immune system constantly working together as a defense against foreign pathogens. The innate immune system is considered the first line of defense. It includes physical barriers such as mucous membranes, the skin, and cellular components such as a system of complement proteins and phagocytes.

The adaptive immune system works in a more specific manner, and exhibits a brief delay in activation at the expense of a more targeted response (Owen and Punt 2013). This adaptive branch primarily consists of cell-mediated and humoral components. Should innate immune response not eliminate a pathogen, the adaptive immune system is activated. This branch consists of natural killer cells, T lymphocytes, and B-lymphocytes (responsible for antibody production). The adaptive immune system has high sensitivity to small molecular differences between host self-antigens and pathogenic non-self antigens.

Recognition of pathogens and foreign molecules is fundamental to the function of the immune system. In innate immunity, this recognition occurs through patterns via phagocytic cells such as macrophages, dendritic cells, and neutrophils. These immune cells find molecular patterns known as PAMPs (pathogen associated molecular patterns). A number of PAMPs can be recognized via receptors of the innate immune system, notably molecular structures such as bacterial lipopolysaccharides (LPSs) on the surface of gram-negative bacteria or viral double-stranded DNA. Foreign PAMP signals are recognized by a variety of receptors known as PRRs (or pattern recognition receptors)

expressed in a number of forms. For example, a class of transmembrane PRRs termed toll-like receptors (TLRs) are responsible for the recognition of several PAMPs. LPS molecular patterns are recognized via TLR4 and bacteria flagella are recognized via TLR5. These associations set off a cascade of intracellular mechanisms resulting in the induction of the adaptive immune response and release of pro-inflammatory proteins (Owen and Punt 2013).

PRRs allow early categorization of which sort of pathogen the immune system is dealing with, and are therefore powerful tools in the tailoring of a specific response to a particular pathogen (Owen and Punt 2013). Once the innate immune cell receptors notice PAMP signals, various cellular components are employed to engulf and process the pathogen. The immune response becomes progressively more engaged as the pathogen and its antigens are identified by host cells. Once identified, the adaptive immune branch generally arrives with a primary response to an initial insult from a pathogen.

In large part, antibody activity defines the role of adaptive, humoral B-lymphocyte immunity. This humoral B-lymphocyte response is employed for antibodies to bind in a very specific manner to pathogens. The functional roles of antibodies can be summarized into several categories. Agglutination is the clumping of foreign cells by attachment of antibodies. Neutralization is the activity of antibodies to attach to the surface of a target cell or even the secreted products of a pathogen to prevent attachment to a host cell. Perhaps most relevant to this study is the use of antibodies as a beacon and activator of other immune cells. Attachment of antibodies to foreign invaders marks them for numerous cellular mechanisms including phagocytic uptake and complement

activation. IgG is the most abundant form of antibody found within healthy individuals constituting roughly 80% of all antibody production. Immunoglobulin G (IgG) antibodies opsonize cells as targets for phagocytosis and activate the complement pathway. (PubMed Health 2016). Recognition via this arm of the immune response comes in the form of B-cell receptors on the surface of B-lymphocytes or in the solubilized form of antibodies via Fab regions. The cell-mediated T-lymphocyte response does not have a secreted form of receptor and relies on T-cell receptors for recognition. T-lymphocytes have a multitude of roles; there exists cytotoxic, helper, and regulatory T cell groups. Activation and recruitment of various immune cells and proteins summarize the activity of helper T cells, while activated cytotoxic T cells produce perforin to directly destroy pathogens or virally infected cells. Regulator T cells serve the role of suppressing the immune response (PubMed Health 2016).

Generation of vast lymphocyte receptor diversity occurs during development and maturation of T and B lymphocytes in primary and peripheral lymphoid organs (Owen and Punt 2013). Specificity of target is key for the activation of the adaptive immune response. Once the pathogen has been eliminated from the host system following primary infection, these adaptive T cells and B cells will store a population of clonally selected lymphocytes employed as defense (Santori 2015). This concept of immunological memory allows for a quicker and more efficient response in the event of subsequent infection. The deliberate exposure of a weakened or inactivated form of a pathogen primes the immune system for a recall response to eliminate an otherwise more virulent

and harmful form of similar pathogen. This intentional priming for a recall response is the core concept of vaccination.

Because vaccination models use a form of the pathogen itself to elicit an immune response, there is always a risk of infection becoming more widespread than intended. Alternatively, if the pathogen's virulence is significantly neutralized the immune system may evoke the adaptive immune system to generate sufficient levels of memory cells. In these instances, subsequent infection will remain harmful, as they are not eliminated effectively. In order to maximize the generation of protective immune components through vaccination, chemical or biological agents known as adjuvants are sometimes employed in vaccine development in order to boost the immunogenicity of a particular vaccine (Lee and Nguyen 2015).

1.2 Role of T lymphocytes in Immunity

Groups of cells that mature in the thymus, known as T cells, are generally characterized by immunological roles and respective surface receptors. A large majority of these thymic developing cells have alpha and beta glycoprotein chains on their surface, therefore termed αβ T cells. In contrast, a minute number of γδ T cells have distinctive gamma and delta glycoprotein surface chain receptors. Regardless, αβ T cells can be divided into several effector subgroups (Owen and Punt 2013). αβ T cells will be the topic of discussion throughout.

As previously mentioned, phagocytic cells are an essential component in the clearance of foreign pathogens. Professional antigen presenting cells (APCs) play the role of engulfing and digesting foreign invaders, ultimately leading to display of their

constitutive peptide fragments via major histocompatibility complex proteins. The cascade of intracellular events continues as antigens are recognized by T-cell receptors, which activates that particular T cell. T cells are restricted in activation based on major histocompatibility complex type I and II proteins (Rock et. al. 2016). The distinction of MHC I proteins and MHC II proteins, in short, is that class I proteins derive antigens from the cytoplasm while class II proteins present exogenous antigens that originate extracellularly. They also vary based on the subset of mature T cell involved. T cells go through two distinct selection processes during maturation. These rigorous processes of positive and negative selection provide mature T cells with both MHC restriction and self-tolerance (Owen and Punt 2013). One result is that cytotoxic CD8 T cells can only be activated via MHC class I proteins, while CD4 T helper cells specifically recognize MHC II presented antigens.

T cells have a clear division in roles amongst the subgroups of cytotoxic CD8 T cells and helper CD4 T cells. Cytotoxic T cells eliminate tumor and infected host cells, favoring endogenous MHC I presentation. Helper T cells guide the behavior of other immune cells, fulfilling a critical role in many immune pathways. Through secretion of small proteins known as cytokines, helper T cells activate and recruit other cell types such as B cells, cytotoxic T cells, and innate immune cells such as macrophages. They also direct the immune response away from responses that would otherwise favor certain pathogens. Major subsets of helper T cells have also been established including Th1, Th2, Th17, Treg, and TFH cells. There are polarizing cytokines, which induce the expression of each subset of T helper cells. Each subset also has unique master gene regulators, or

transcription factors. Likewise a set of effector cytokines, expressed following differentiation, accompany each subset. There is also significant cross regulation of T helper cells, notably in pairing Th1/Th2 subsets and Th17/Treg subsets, where the upregulation of one leads to the downregulation of the other (Owen and Punt 2013).

1.3 Th17 Cells in Health and Disease

A specialized T helper cell subset known as Th17 (separate from Th1 and Th2) cells has been identified as the primary source of IL-17 production. Much like IL-12 and IL-4 are polarizing in for naïve CD4+ lymphocytes towards Th1 and Th2 lineages, respectively, TGF-β is known to polarize naïve T helper cells towards the Th17 lineage in the presence of IL-6 (Jin and Dong 2013). This distinction is further demonstrated in the requirement for a novel set of transcription factors which show no overlap with the transcription factors required for Th1 or Th2 cells, including signal transducer and activator of transcription 3 (Stat3) and retinoic acid receptor related orphan receptor γ (RORγ) (Jin and Dong 2013). Th17 cells reside mainly at mucosal sites such as in the gastrointestinal tract and the airways. Although they were originally discovered as a perpetrator of autoimmune disease, they are critical to mucosal homeostasis and contribute to protection from bacterial and fungal pathogens (Stadhouders et al. 2017).

There is evidence of a protective role in vaccine induced long lived Th17 memory cells. Lindenstrøm and colleagues (2012) explored the capacity of a cationic liposome-induced adjuvant's role in provoking a combined Th1 and Th17 response. They were able to characterize a Th17 response two years post vaccination using a *Mycobacterium*

tuberculosis challenge in this cationic adjuvant formulation (CAF) module. It was demonstrated that a long-lived memory Th17 population is inducible. Furthermore, this Th17 cell population maintained antigen specificity (Lindenstrøm et al. 2012). It is proposed that a similar population is inducible following FcR-targeted intranasal vaccination. These readily available cells having the immunological benefit of providing tightly regulated pro-inflammation and polarizing a Th1-like response in a short period.

A mechanism the *Francisella tularensis* pathogen seems to utilize is the suppression effect on Th1 responses observed in the presence of the cytokine IL-10 (Slight et al. 2013). IL-10 demonstrates a well-studied immunosuppressive capability. It has also shown significant involvement in both the protection and pathogenicity of *Francisella tularensis* infection. Th17 cells hold an interesting dynamic, as IL-10 is found to be expressed in a subset of non-pathogenic Th17 cells to prevent over-inflammation. In IL-$10^{-/-}$ mice, inflammation generated by IL-17 in pulmonary infected mice leads to increased mortality following *Francisella tularensis* live vaccine strain infection (Slight et al. 2013). This result indicates that IL-17 activity can mediate protection via inflammation, but must be tightly regulated via anti-inflammatory cytokines to avoid host pathology. It was further demonstrated that the role of IL-10 varies based on the route of infection of LVS. Interestingly, cutaneous LVS infection has shown that knocking out IL-10 suppresses the potential pathogenic effects of IL-17, while pulmonary LVS infection has shown a requirement of IL-10 to limit otherwise harmful inflammation (Slight et al. 2013). Regardless of these findings, in hosts facing a

SchuS4 challenge the presence of IL-10 appeared to play a less significant role in mouse models as similar survival kinetics were observed (Metzger et al. 2013).

Th17 cells have also been shown to overcome the pathogens suppressive affect on Th1 cells, specifically through the production of effector cytokine IFN-γ and production of the polarizing cytokine IL-12. (Lin et al. 2009). To further explain the role of effector cytokines released via Th17 cells, IL-17R$^{-/-}$ mouse models displayed inefficient clearing of bacteria from lung, increased bacterial dissemination into the spleen, and a general reduction in survival when faced with another gram-negative bacterium, *Klebsiella pneumonia* (Curtis and Way 2009).

There is a seemingly unestablished middle ground of IL-17 activity. IL17A (the predominant subtype of IL-17), has a primary role of mediating promotion of pro-inflammatory cytokines and chemokines, characterized by accumulation of neutrophils and macrophages (Slight et al. 2013). A pathogenic effect of Th17 cells is also observed in several major autoimmune diseases such as multiple sclerosis and rheumatoid arthritis. The role of Th17 cells in MS has been shown using AEA models in mice to induce central nervous system inflammation and lesion formation (Jin and Dong 2013). Further studies of multiple sclerosis models have shown subsets of Th17 cells which contribute to pathogenic and nonpathogenic effects. “Pathogenic” Th17 cells upregulate several chemokine ligands, and do not express IL-10. “Nonpathogenic” Th17 cells, on the other hand, produce IL-10. Nonetheless, these subsets still share the role of IL17A secretion and polarization via TGF-β and IL-6 (Sharma et al. 2013).

These subsets are dependent on the differentiation signals they receive. TGF-β1 plays a role in nonpathogenic Th17 cell polarization, while TGF-β3 plays a role in the said pathogenic Th17 subset polarization (Sharma et al. 2013). The role of IL-23 dependency in the Th17 cell type is unclear. Some researchers have reported IL-23 to induce switching of non-pathogenic Th17 cells to pathogenic (Lee 2013) while other have found it to be required for expansion and maintenance (Stadhousers et. al. 2017).

IL-6 is another cytokine with a defined role in the Th17- Treg dynamic (Tanaka et al. 2014). The IL-6 cytokine is considered pro-inflammatory and significant in the polarization of naïve CD4 T cells to Th17 cells rather than Treg cells when TGF-β is also present. In this way, IL-6 effective inhibits Treg differentiation. This dynamic is delicate in the development of autoimmune and chronic inflammatory diseases (Tanaka et al. 2014).

1.4 *F. tularensis* Pathogenesis and Anti-Bacterial Immune Responses

Francisella tularensis is defined as a gram-negative intracellular bacterium, capable of infecting humans through several routes of infection and causing tularemia. Some common routes of infection include insect bites from infected organisms such as horse and deer flies, and skin contact with infected animals. Most concerning to the public, however, is inhalation of a contaminated aerosol (Celli and Zahrt 2013). Transmission via this route of infection give *F. tularensis* organisms an unsettling potential for use as a bio-weapon. This pneumonic route is the most potent route of administration, leading *F. tularensis* infection to take on the name “lawnmower disease”.

Mortality by pulmonary *Francisella tularensis* infection generally occurs via septic shock and widespread inflammation (Sharma et al. 2011).

Several forms of the disease are commonly studied in the lab setting, including a more virulent Type A SchuS4 strain developed from the tularensis subspecies. This strain of the pathogen is known to cause infection with a dosage as low as roughly 10 colony forming units, and an alarming mortality rate of roughly 60% if left untreated (Celli and Zahrt 2013). Another subspecies of the disease, holarctica (Type B), displays milder disease symptoms in humans. This subspecies has been used for the development of an attenuated live vaccine strain (LVS), which retains its lethality in mice. The potential weaponization of this bacterium, the lack of an approved vaccine, and the various routes of infection has led the Center for Disease Control and Prevention to classify *F. tularensis* as a category A bioterrorism threat (Celli and Zahrt 2013).

As many intracellular pathogens do, *F. tularensis* displays an ability to survive the various mechanisms of phagocytes which generally cause pathogen death. Particularly, this bacteria survives macrophage uptake and induction of toxic reactive oxygen species (ROS) and reactive nitrogen species (RNS). The pathogen shows an ability to escape the phagosome to replicate in the cytoplasm of the phagocyte, eventually leading to apoptosis and escape from the cell. Analysis of the *Francisella tularensis* genome has revealed the presence of several genes believed to be involved in the phagocytic survival and eventual escape from phagocytes. For example, the gene encoding for IgLC. The IgLC protein has demonstrated ability to inhibit phagosome maturation (Steiner et al. 2014). Another protein coded by the "*F. tularensis* pathogenicity island" is the katG enzyme, which

demonstrates an ability to detoxify hydrogen peroxide and reactive nitrogen species (Steiner et al. 2014). This is evident in mice infected with a mutant strain lacking a functional katG gene, which have shown survival to LVS challenge, but not the insult of the SchuS4 strain (Steiner et al. 2014).

A characteristic of macrophages infected with *F. tularensis* is the expression of prostaglandin E2 (PGE2) (Hunt et al. 2012). PGE2 is a great ally to many pathogens such as *Salmonella typhi*, *Mycobacterium tuberculosis*, and *Legionella pneumophila* due to immunosuppresion. In regards to *F. tularensis*, it has been shown that FT induced-PGE2 has the effect of directly acting on CD4 T helper cells to downregulate the production of IFN-γ (Hunt et al. 2012). More indirectly, macrophages that produce FT induced-PGE2 also secrete a soluble factor (FTMØSN). This soluble factor has the effect of producing IL-10 and through MARCH1 expression and an additional unknown intermediary step, downregulate class II expression and induce ubiquitination on other macrophages. Lack of MHC class II expression equates to less CD4 T cell activation. This result limits the ability of cells to participate in an adaptive immune response (Hunt 2012). Nonpathogenic Th17 cells also produce IL-10, so there must be a tolerable extent produced in protected groups over the course of infection.

Research by Woolard and colleagues (2007) has established the role of these PGE2 molecules to push the immune system towards a Th2-like response. This is a bad thing for host defense. T helper type 1 responses are important for the production of pro-inflammatory cytokines, mainly IFN-γ, that are critical in immune response against intracellular pathogens.

The Th1 cytokine interferon-gamma (IFN-γ) has a known role in the immune response directed towards limiting intracellular bacterial infection. Secretion of this cytokine occurs primarily from the CD4+ Th1 cell population. In an IFN- γ dependent manner, proliferation of the pathogen once it is inside the phagocyte and eventual apoptosis of the cell (basis of the disease's virulence) is hindered (Steiner et al. 2014). Upregulation of Th1 cytokines, particularly IFN-γ, should therefore demonstrate the effect of lessening *F. tularensis* pathogenicity.

In contrast to the effects of FT induced- PGE2; is the IL-17 cytokine, which is primarily produced by Th17 cells. IL-17 has shown the ability to upregulate IL-12 production in both DCs and macrophages. Additionally IL-17 induces IFN-γ production in macrophages and supports effective clearance of LVS. (Lin et al. 2009).

Defense against *F. tularensis* is multi-faceted. *F. tularensis* uptake by macrophages occurs via looping phagocytosis after recognition by mannose receptors or recognition by FcRs following opsonization (Steiner et al. 2014). This FcR uptake can be used against *F. tularensis* in generating a primary response in a vaccine strategy. FcR targeting of the pathogen to APCs has shown a pathway responsible for the generation of *F. tularensis*-specific, gamma interferon (IFN-γ)-secreting, effector memory (EM) CD4+ T cells during infection. Furthermore, this pathway is shown to be adjuvant-independent (Bitsaktsis et al. 2014).

1.5 Vaccination Models and FcγR-targeting

It is critical for vaccine models to remain immunogenic to generate pathogen-specific, long-lived memory lymphocytes. Vaccines should also not cause harmful side effects to the host due to a heightened, often inflammatory, immune response. Various vaccination strategies are available each having a unique set of benefits and potential issues. For example, live, attenuated pathogens are regarded as one of the most effective in eliciting an immune response, but at the cost of possible reversion back to virulence while in the host. For example, multiple vaccination strategies have been employed against poliomyelitis caused by the poliovirus. The Sabin oral polio vaccine (OPV) contains a live, attenuated form of the virus. This OPV is favored for the poliomyelitis eradication program due to its ability to induce a systematic and mucosal immune response (Baicus 2012). Major risks arise using OPV vaccination in the appearance of Vaccine-Associated Paralytic Poliomyelitis cases (VAPP) and the emergence of Vaccine Derived Polioviruses strains. Reversion to neurovirulence can occur when the OPV strains undergo genetic variation by point mutation during replication in the small intestine. Such cases were reported during first dosage at a rate of 1 in 750,000 (Baicus 2012). Alternatively, the Salk formalin-inactivated polio vaccine (IPV) poses no risk of vaccine related disease. This IPV strategy is used to phase out OPV use (Baicus 2012).

Manipulating pathogenic genomes via DNA vaccines is a field of study that has been broadly expanded in recent years. Cloning a gene of interest into a plasmid vector has shown promise in achieving an improved immune response in cellular studies (Lee et al. 2012). Using this recombinant DNA approach, it is possible to target desired antigens

to specific APCs (Lee et al. 2012). Inactivation or fixation of a pathogen is another commonly used technique in vaccine development. A setback in this form of vaccination is the low immunogenicity elicited in response, protection from live attenuated vaccines typically outlasts that of a killed or inactivated vaccine (Lee et al. 2012). Adjuvants may be employed to promote the immune response. However, the only vaccine adjuvant approved for commercial usage is the use of aluminum salts, or Alum. These aluminum salts are poor inducers of T cell responses (Lee and Nguyen 2015). Proper vaccine development is tailored to obtain a desired type of antigen-specific immune response.

A novel approach to vaccine development is to utilize Fc-gamma receptors on APCs to enhance the protective immune response. Various FcγRs are located on the surface of APCs for antibody detection. FcγRs vary with proposed molecular mechanisms that include both activating and inhibitory functions, along with distinct IgG subclass binding affinities and cellular distribution. FcγRI is a receptor containing an intracellular tyrosine activation molecule (ITAM). It is also a high affinity receptor capable of binding monomeric and multimeric IgG (Poel et al. 2011). FcγRIIA and FcγRIII are also receptors containing an ITAM. Both FcγRI and FcγRIII are constitutively expressed on a number of cell types including APCs. Inversely, FcγRIIB contains an intracellular tyrosine inhibitory molecule (ITAM) expressed on B cells. While FcγRIIB inhibits B lymphocyte activation, it does not prevent it, and enhancement of mucosal immunity is reported via FcγR targeting (Rawool et al. 2008). Using an intranasal immunization model in mice which targets inactivated *F. tularensis* organisms to Fcγ receptors via mouse IgG2a anti-LPS mAb has shown clear benefits. Improved

uptake and presentation of iFt immunogen by APCs. Increased recruitment and activation of dendritic cells in lungs. Enhanced *F. tularensis*-specific cytokine and antibody response and generation of effector memory CD4 T cells are among the most notable of these benefits. There is also a general increased protection against *F. tularensis* infection (Babadjanova et al. 2015).

It was previously demonstrated that the FcR-targeted immunogen vaccination method exhibits 100% protection against an otherwise lethal dosage of FT LVS challenge (Iglesias et al. 2012). A goal of the current study is to further establish the usage of FcR-targeting as a viable option in vaccine development. It is proposed here that in secondary immune tissues such as lung and spleen, there will be a clear cytokine profile of Th17 cells in protected groups. It is also expected that flow cytometry techniques will reveal unique surface and intracellular markers of Th17 lymphocytes. Determining the presence of surface markers of CD3 and CD4 will indicate the presence of T cells and T helper cells, respectively. Confirming the intracellular presence of IL17A and the transcription factor RORγT will give a unique characterization as Th17 cells. In addition, we aim to see a distinctive cytokine profile of non-pathogenic Th17 cells. The anticipated cytokine profile will show a TGF-β1 and IL-6 polarized cell population among protected groups. Although the role of IL-23 is not entirely clear in pathogenic Th17 polarization, we predict IL-23 to demonstrate expansion and maintenance of a protective T cell subpopulation. It is proposed that immune tissue samples from protected groups will show an increased presence of effector cytokines IL-6, IL-12, and IL-17A post-infection.

In IC protected groups, significantly greater populations of Th17 cells are expected in lung tissue samples during early stages of pulmonary infection.

Previous literature has demonstrated the effectiveness of the Fcγ-receptor targeting vaccination strategy (Babadjanova et al. 2015). In the current study, further investigation via ELISAs performed on tissue samples post immunization and challenge showed distinct Th17 cytokine profiles. Tissue samples isolated from protected mice also show evidence of positively identified Th17 cells in the early stages post infection. Flow cytometry analysis gave qualitative and quantitative data for the presence of these cells potentially being trafficked throughout the body of protected mice. The role of Th17 cells in health and disease is not entirely clear. Our studies point towards a protective role of Th17 cells over the course of bacterial infection.

2. Materials & Methods:

2.1 Murine Model

C57BL/6 wild-type mice were purchased from Jackson Laboratories (Bar Harbor, ME). These mice were housed and cared for in the rodent facilities in McNulty Hall, Seton Hall University. The mice in which were utilized for experiments in this project were 6-10 weeks of age. All protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC).

2.2 *F. tularensis* Bacteria

The live vaccine strain of *F. tularensis* (FT LVS) was provided by Dr. Edmund Gosselin (Albany Medical College, Albany, NY). The organisms were grown at 37^{o}C to a concentration of 2.6x10^{9} CFU/mL, using Mueller-Hinton broth with a 2% IsovitaleX^{TM} (Fisher Scientific) supplement. Live *F. tularensis* samples were stored at -80^{o}C in liquid nitrogen.

2.3 Inactivation of Bacteria & Immune Complex Generation

Once FT LVS organisms were grown to a concentration of 2.6x10^{9} CFU/mL in Mueller-Hinton broth supplemented with 2% IsovitaleX^{TM} (Fisher Scientific), 25 mL of bacteria were centrifuged and resuspended in 25 mL of 2% paraformaldehyde and sterile PBS solution for fixation. Samples were incubated at room temperature with shaking for 90 minutes. The inactivated bacteria were then centrifuged and washed three times with sterile PBS. To confirm fixation, 100 uL of inactivated *F. tularensis* (*iFt*) at 2.6x10^{8} CFU) were plated on nutrient rich chocolate blood agar plates for seven days. Aliquoted samples were stored in PBS at -20^{o}C. These inactivated samples were used as an

immunogen for one vaccination protocol. These inactivated organisms were also used to generate immune complexes (ICs) for a separate vaccination model in these experiments.

For generation of mAb-iFt immune complexes, $1x10^9$ iFt bacterial organisms were mixed with IgG2a anti-iFt LPS mAb in a PBS solution to a concentration of 1µg/mL. IC samples were then incubated overnight with shaking at 4^oC. iFt and mAb-iFt IC samples were then administrated intranasally (i.n.) following incubation.

2.4 Timeline for Immunization

In groups of three, C57BL/6 wild-type mice were immunized i.n. on day 0 and followed up with a booster dosage on day 21. Inactivated LVS FT (iFt) was used to immunize one group. The mAb-iFt immune complex (IC) was used to immunize another. A control group of mice utilized PBS in place of the iFt and IC immunogens. Mice were then challenged with a $1x10^4$ CFU dosage of FT LVS on day 35. Following infection, mice were sacrificed via CO_2 overexposure on day 2 and day 5 as tissue collection of spleen and lung samples occurred.

2.5 Dose Titrations of Wild-Type C57BL/6 Mice

Dose titrations were utilized to determine the lethal dose (LD_{100}) for wild-type mice. These mice were separated into 3 groups of mice with 5 mice in each group. *F. tularensis* bacteria were administered intranasal (i.n.) with $5x10^3$ CFU FT LVS, $1x10^4$ CFU FT LVS, or $2x10^4$ CFU FT LVS. Survival for both groups of mice was monitored for 14 days post-infection. The dosage that eliminated about 100% of the experimental group of mice by day 11 or 12 was utilized as the LD_{100} for immunization and challenge studies.

2.6 Flow Cytometry Screening

Samples of spleen and lung tissue were gathered pre-infection, on day 2 post infection, and on day 5 post infection. For lung samples, an enzyme solution was required to break down tissue. This digestion buffer contained RPMI (Life Technologies, 02. mg/mL DNaseI (Sigma), 0.4 mg/mL Collagenase D (sigma), and 1 M $MgCl_2$. A 30 minute incubation followed at 37ºC before digested tissue samples were forced through a cell strainer and the obtained suspension was washed and resuspended in RPMI containing 2% FBS. Cell suspensions were then layered on 5 mLs of Lympholyte (Cedarlane Laboratories- Burlington, NC), and spun for 30 minutes at RT. The resulting interface containing immune cells was obtained from each sample and added to RPMI with 2% FBS. 200,000 isolated immune cells from both spleen and digested lung samples were stained for surface markers CD3 and CD4. Intracellularly, these immune cells were stained for markers of IL17A and RORγT. Samples were stained with PerCp/Cy5.5 anti-CD3 (BD Biosciences), FITC anti-CD4 (BD Biosciences), PE anti-IL17A (BD Biosciences), and APC anti- RORγT (BD Biosciences).

2.7 Enzyme Linked Immunosorbent Assays (ELISAs)

The mice utilized in these experiments were separated into three groups with three mice in each group. Samples of spleen and lung tissue were gathered on day 2 post infection and on day 6 post infection. These samples were homogenized and spun down by use of a centrifuge, and supernatant was collected and stored at -20ºC. 96-well plates were coated overnight with cytokine specific primary antibody according to vendor instructions for each assay. Following the assay procedure, the 96-well plate was read

using a SpectraMax M5 plate reader at wavelengths of 450 nm and 570 nm for absorbance values to quantify attached proteins and background in each sample, respectively. A standard of each cytokine was provided and serially diluted to establish a curve to convert optical density to sample concentration. The assays employed consisted of IL-23 (BD Biosciences), IL-12 (BD Biosciences), IL-17A (BD Biosciences), IL-6 (BD Biosciences), and TGF-β1 (Invitrogen).

2.8 Statistical Analysis

The Log-Rank (Mantel-Cox) test was used for survival curves using the GraphPad Prizm 4 software. The flow cytometry data was analyzed using the FlowJo software.

2.9 Ethics Statement

All animals were handled in accordance with good animal practice defined by relevant national and/or local animal welfare bodies. Briefly, mouse anesthesia was performed via i.p. injections of a ketamine/xylazine cocktail, while mouse euthanasia was achieved via CO_2 administration followed by cervical dislocation.

3. Results:

3.1 Identifying the LD_{100} Dose of FT LVS in C57BL/6 Mice

For the purpose of our study, it was necessary to determine a lethal dosage of FT LVS in unimmunized wild-type C57BL/6 mice in order to define the lowest dose required to eliminate about 100% of the experimental population in the absence of any immunization protocol. The LD_{100} was identified as $1x10^4$ CFU (colony forming units) of *F. tularensis* live vaccine strain. The *F. tularensis* organisms were administered intranasal.

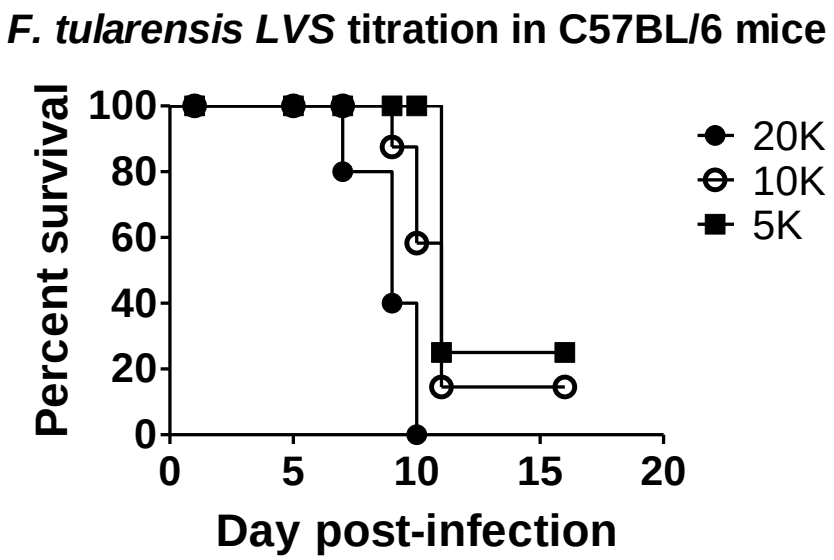


Figure 1: The Kaplan-Meier survival plot above illustrates dose titrations for C57BL/6 wild-type mice. Doses were titrated out to $2x10^4$ CFU FT LVS, $1x10^4$ CFU FT VLS, and $5x10^3$ CFU FT LVS. A majority of the population had succumbed to infection by day 11 when faced with $1x10^4$ CFU FT LVS challenge while a $5x10^3$ CFU FT LVS dose still showed complete survival on day 10. A $2x10^4$ CFU FT LVS dosage eliminated its entire population by day 10.

3.2 Fcγ Receptor Targeting Mediates Protection against Lethal *F. tularensis* LVS Challenge in C57BL/6 Mice

It has been previously demonstrated that intranasal administration of mAb-iFt ICs enhance protection against FT LVS challenge (Rawool et al. 2008). Improved protection is mediated by several cellular mechanisms using this Fc receptor-targeting model. Binding of iFt to antigen presenting cells is heightened, and rate of iFt internalization by APCs is increased (Iglesias et al. 2012). Mice were immunized on days 0 and 21 with PBS, $1x10^9$ iFt, or 1 µg/mL mAb-iFt and challenged on day 35 with $1x10^4$ CFU FT LVS. As a novel vaccination strategy, C57BL/6 wild-type mice were immunized with a complex of inactivated *F. tularensis* and IgG2a, anti-LPS monoclonal antibody. These mAb-iFt immunized mice showed complete protection from an otherwise lethal dosage of FT LVS ($1x10^4$ CFUs). Unimmunized mice succumbed to the infection within 10-14 days. The use of mAb-iFt ICs demonstrates a straightforward approach for targeting immunogens to FcR.

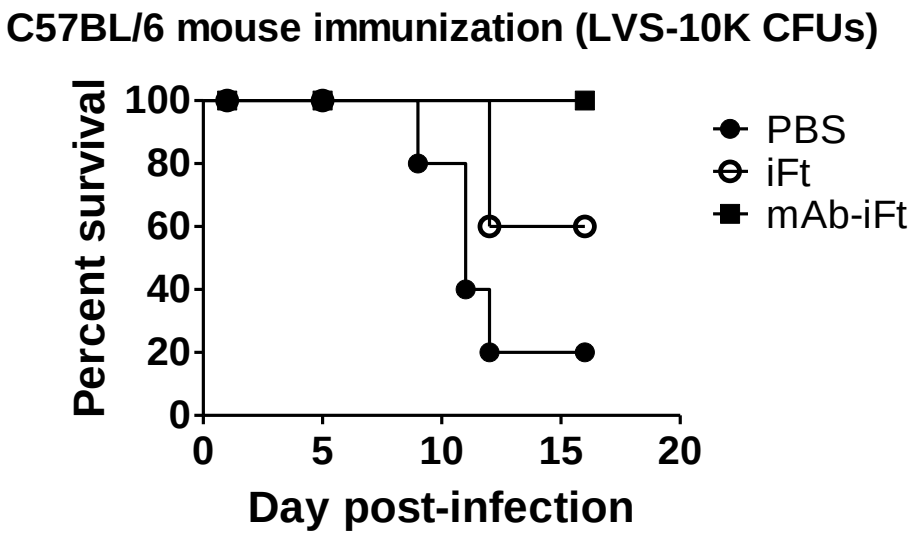


Figure 2: Kaplan-Meier survival plot resulting from immunization and challenge experiments of C57BL/6 wild-type mice. Wild-type C57BL/6 mice were immunized before facing challenge with $1x10^4$ CFU FT LVS. PBS mice represent the control group with no immunization protocol. The iFt label represents mice immunized with *F. tularensis* bacteria fixed with paraformaldehyde. The mAb-iFt label represents wild-type mice immunized with a complex of inactivated *F. tularensis* and IgG2a, anti-LPS monoclonal antibody. The results show that immunization of wild-type mice with mAb-iFt exhibits complete protection.

3.3 Fcγ Receptor Targeting Generates a Protective Th1 Response Against *F. tularensis* in Immunized Mice

IL-12 down-regulation due to the unique *F. tularensis* LPS structure attributes to the low endotoxic activity of the pathogen (Babadjanova et al. 2015). Pro-inflammatory cytokines such as IL-12 and IL-17 offer vital protective roles against intracellular infection. Recruitment, enhancement, and polarization of immune cells such as neutrophils, macrophages, and T cell subsets are among the immunological benefits of having these cytokines present. Importantly, IL-12 production facilitates the polarization of Th1 cells, which are responsible for downstream IFN-γ production. In the focus of this study, Th17 cells stimulate DCs and macrophages to produce IL-12 (Lin et al. 2012). Evoking a Th1-cell response is critical in defense against this intracellular pathogen. Even in mAb-i*Ft* immunized mice, IFN-$\gamma^{-/-}$ mice have shown a significant drop in survival rate in comparison to a wild-type control (Rawool et al. 2008). The efficiency of a Th1 immune response is vital against intracellular bacteria. Here we aim to show that Th1 responses are improved, inducted by IL-12 upregulation.

IL-12 Cytokine Concentrations

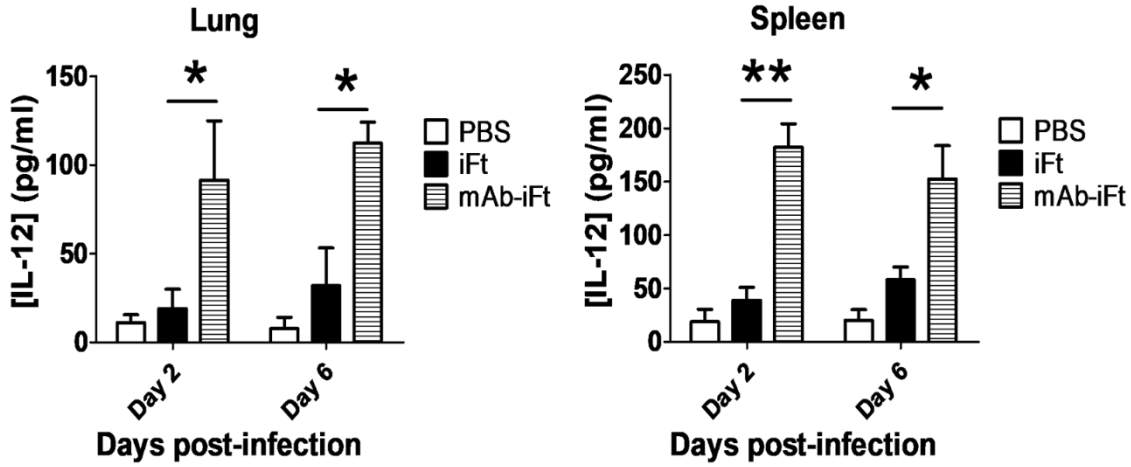


Figure 3: Data generated from ELISA analysis on lung and spleen samples at day 2 and day 6 post-infection time points. PBS mice represents the control group with no immunization protocol. The iFt group represents mice immunized with *F. tularensis* bacteria fixed with paraformaldehyde. The mAb-iFt label represents wild-type mice immunized with a complex of inactivated *F. tularensis* and IgG2a, anti-LPS monoclonal antibody. Figure shows IL-12 cytokine concentration in pg/mL, which contributes towards a Th1 response. Both time points show IC immunized groups having a greater concentration of this cytokine in lung and spleen samples. (*) P-value < 0.1; (**) P-value < 0.05; bars represent SD.

3.4 Recovery from *F. tularensis* Infection Relies on both Pro-inflammatory and Anti-inflammatory Cytokines

Successful resolution of *F. tularensis* infection depends on the balance between both pro-inflammatory and anti-inflammatory cytokines (Babadjanova et al. 2015). A rapid pro-inflammatory response is expected in protected groups of mAb-iFt immunized mice. Pro-inflammation is mediated by increased levels of cytokines such as IL-12 and IL-6 (Fig. 3 and Fig. 6). Although increased levels of the anti-inflammatory cytokine IL-10 have been reported to aid infectivity in unprotected groups (Babadjanova et al. 2015), it is demonstrated here that protected groups show an increase in IL-10. This observation suggests that a significant increase in the anti-inflammatory cytokine inhibits a harmful extent of inflammation. The non-pathogenic subset of Th17 cells is known to produce IL-10 in contrast to the cells found in inflammatory disease and autoimmune models (Sharma et al. 2013). Furthermore, in $IL10^{-/-}$ mice, inflammation generated by IL-17 in pulmonary infected mice leads to increased mortality following LVS infection (Slight et al. 2013). Suppressive immune responses are essential to regulate potentially hazardous pro-inflammation. Since protection is exhibited in mAb-iFt immunized subjects, pro-inflammatory responses have to be significantly reduced once the infection is resolved.

IL-10 Cytokine Concentrations

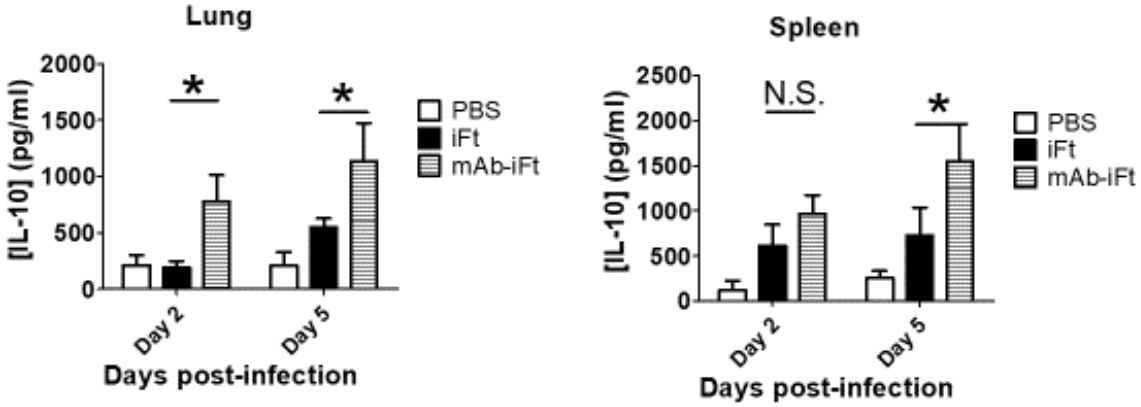


Figure 4: Data generated from ELISA analysis on lung and spleen samples at day 2 and day 5 post-infection time points. PBS mice represents the control group with no immunization protocol. iFt represents mice immunized with *F. tularensis* bacteria fixed with paraformaldehyde. The mAb-iFt label represents wild-type mice immunized with a complex of inactivated *F. tularensis* and IgG2a, anti-LPS monoclonal antibody. Figure shows IL-10 cytokine concentration in pg/mL. There is a significant increase of the cytokine in both early post infection and day 5 post-infection time points among IC immunized subjects across lung and spleen tissue samples. (*) P-value < 0.1; (**) P-value < 0.05; bars represent SD.

3.5 Generation of Th17 Cells is TGF-β/IL-6 Dependent

Enhanced T cell activation is a mechanism by which mAb-iFt ICs improve the immune response (Iglesias et al. 2012). Of the T cells induced, a subpopulation of T helper cells, Th17 cells, is analyzed in this study. Th17 cells have an impact on the dynamic of pro- and anti-inflammatory cytokine secretion throughout disease progression and immune response (Slight et. al. 2013). IL-12 and IL-10 concentrations are effected by Th17 cells, so it is of interest to determine the presence of polarizing cytokines of this T helper subset. IL-23, IL-6, and TGF-β1 cytokines are involved in Th17 polarization and effectiveness (Wu and Wang 2018). While IL-23 is more involved in maintenance and expansion of Th17 cells, IL-6 and TGF-β are sufficient to direct naïve CD4 cells to a Th17 lineage. Polarization via TGF-β1, opposed to TGF-β3, has a particular role in the delicate balance of polarizing non-pathogenic Th17 cells over nonpathogenic Th17 cells (Wu and Wang 2018).

Together with TGF-β, the IL-6 cytokine is critical for induction of transcription factor RORγT, the key transcription factor that guides Th17 differentiation (Stadhousers 2017). This IL-6 cytokine is also pleiotropic, acting to inhibit Treg differentiation and has a well-studied role in chronic inflammation (Tanaka et al. 2014). TGF-β1 and IL-6 concentrations are hypothesized to increase, demonstrating Th17 polarization. The pleiotropic IL-6 is also expected to show a dramatic rise following the asymptomatic early stages of infection in unprotected groups, signifying harmful over-inflammation.

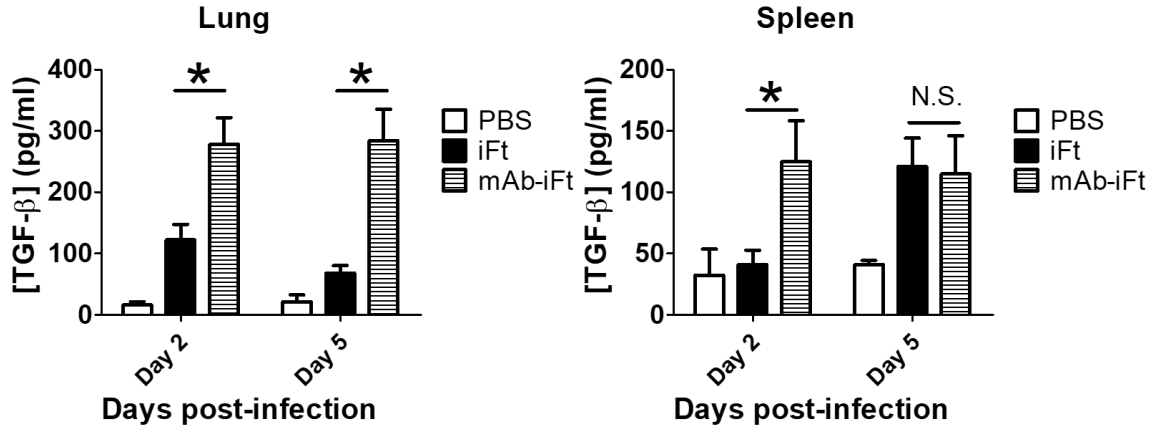


Figure 5: Data generated from ELISA analysis on lung and spleen samples at day 2 and day 6 post-infection time points. PBS represents the control group of mice with no immunization protocol. iFt represents mice immunized with *F. tularensis* bacteria fixed with paraformaldehyde. The mAb-iFt label represents wild-type mice immunized with a complex of inactivated *F. tularensis* and IgG2a, anti-LPS monoclonal antibody. It is shown that TGF-β1 cytokine concentration is greater in lung samples from IC immunized groups on both post infection time points. Spleen samples show a greater concentration in IC groups on day 2, as iFt groups show similar concentrations on day 5. An early response in both lung and spleen supernatant following pulmonary infection is reported. (*) P-value < 0.1; (**) P-value < 0.05; bars represent SD.

IL-6 Cytokine Concentrations

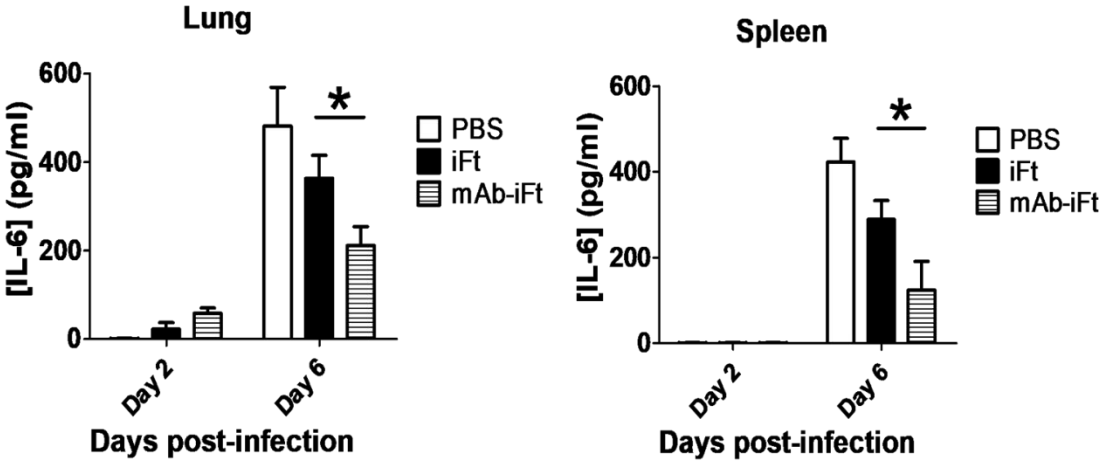


Figure 6: Data generated from ELISA analysis on lung and spleen samples at day 2 and day 6 post-infection time points of IL-6. PBS represents the control group of mice with no immunization protocol. iFt represents mice immunized with *F. tularensis* bacteria fixed with paraformaldehyde. The mAb-iFt label represents wild-type mice immunized with a complex of inactivated *F. tularensis* and IgG2a, anti-LPS monoclonal antibody. The figure shows a large increase in concentration in between days 2 and 6 in PBS control groups. There is little to no response in control and iFt immunized subjects 48 hours post-infection. As the infection progresses, however, a sharp increase in this pro-inflammatory IL-6 cytokine may correlate to its pathogenicity in PBS and iFt groups. (*) P-value < 0.1; (**) P-value < 0.05; bars represent SD.

3.7 Maintenance and Expansion of the Th17 Lineage via IL-23

Along with TGF-β1 and IL-6, the IL-23 cytokine is involved in the polarization and expansion of Th17 cells. The extent to which IL-23 affects Th17 cell lineage is unclear. Although incapable of solely polarizing naïve CD4 cells, IL-23 has an indispensable role in promoting Th17 cell expansion and maintenance (Mus et. al. 2010). IL-23 has also reported to be a key contributor to inducing and maintaining pathogenicity in Th17 cells observed in inflammatory disease and autoimmune models (Lee 2015). Our data shows that during the pulmonary infection utilized in this study, high levels of IL-23 concentration are reported in lung tissue of protected groups, but not in spleen tissue. This result indicates that IL-23 cytokines appear early in response to pathogen at the site of infection, but suggests these expansion and maintenance signals are not required in spleen tissue when the infection becomes systemic.

IL-23 Cytokine Concentrations

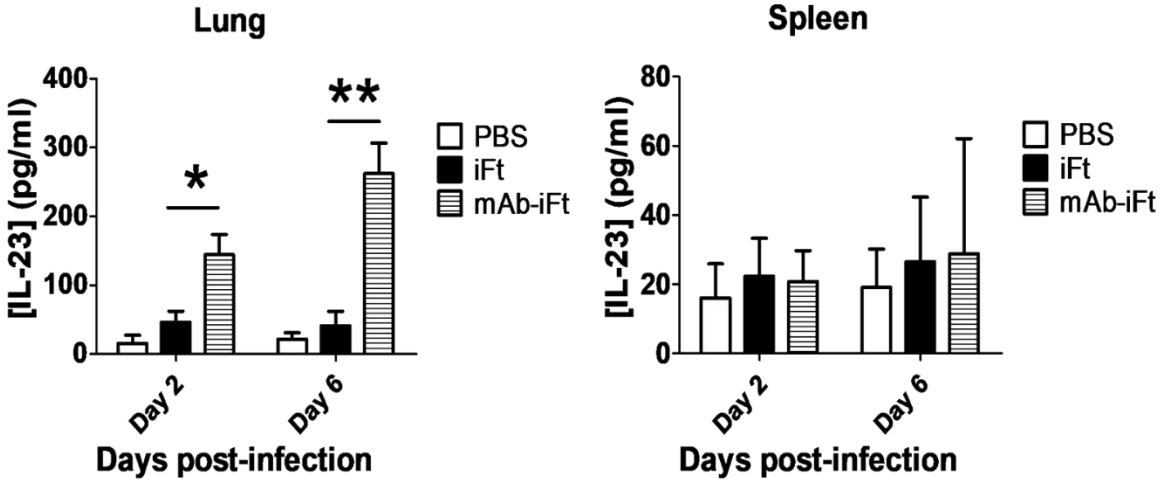


Figure 7: Data generated from ELISA analysis on lung and spleen samples at day 2 and day 6 post-infection time points. The above figure shows IL-23 concentration, a cytokine that expands the population of differentiated Th17 cells. PBS represents the control group of mice with no immunization protocol. iFt represents mice immunized with *F. tularensis* bacteria fixed with paraformaldehyde. The mAb-iFt label represents wild-type mice immunized with a complex of inactivated *F. tularensis* and IgG2a, anti-LPS monoclonal antibody. Cytokine concentration is greater in lung tissue samples from IC immunized groups. In the lung, there is a significant increase in IL-23 cytokine concentration observed in the mAb-i*Ft* group. Relative to other cytokines measured, the concentration of IL-23 is significantly lower in spleen tissue across all samples, never generating more than 60 pg/mL opposed to roughly 300 pg/mL concentration seen in day 6 post-infection IC lung tissue. (*) P-value < 0.1; (**) P-value < 0.05; bars represent SD.

3.8 Confirming the Presence of Th17 Cells via Flow Cytometry

It is demonstrated here that targeting the iFt immunogen to Fcγ receptors generates Th17 cells. ELISA data suggests that polarization occurs largely in the lung, more noticeably in the earlier stages of infection. As the disease becomes systemic, however, other immune organs are expected to become more engaged and flow cytometry data suggests trafficking patterns of the cell population. Surface markers of the CD3 and CD4 confirm cell lineage to be T helper cells. Intracellular markers for IL-17 and the transcription factor RORγT give a unique identity to lymphocytes as being Th17 lineage.

A significant increase of Th17 cells in mAb-i*Ft* protected groups at 48 hours post infection attributes to the importance of these cells early in pulmonary infection and their contribution to the balance of pro- and anti-inflammatory response. The cytokine profiles that result from the presence of Th17 cells influence the recruitment and activation of other immune cells. Additionally, the increased presence of $CD3^+/CD4^+$ T cells is indicative of increased internalization and presentation of antigen by APCs via the mAb-i*Ft* vaccination model. Early response also indicates that immunological memory is generated following mAb-i*Ft* immunization, allowing for recall response in subsequent challenge.

Day 2 Post Infection Lung Samples

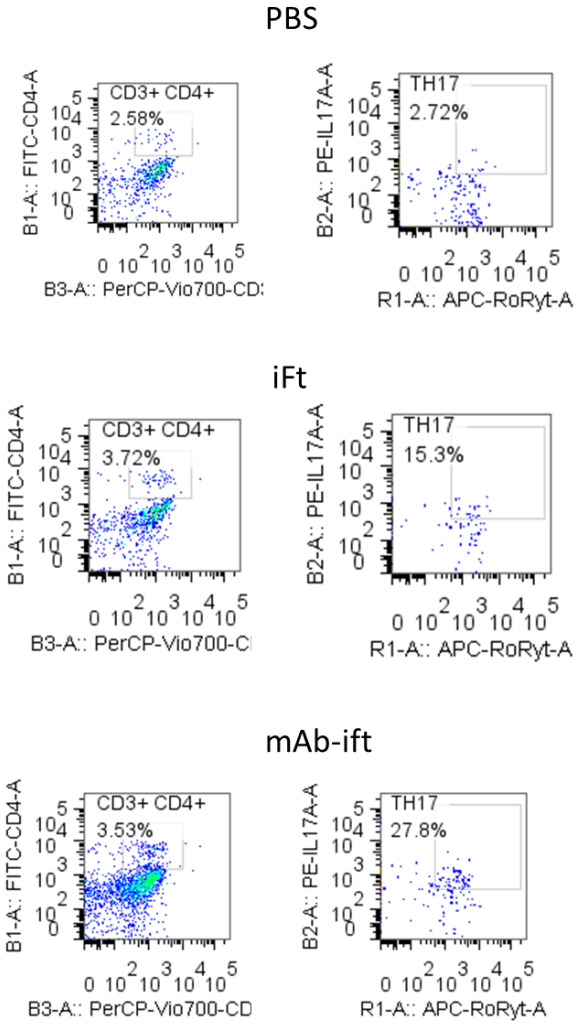


Figure 8: The figure above shows flow cytometry data generated from digested lung samples of mice treated with PBS, inactivated *Francisella tularensis* bacteria, and an immune complex consisting of monoclonal antibody and inactivated *Francisella tularensis* bacteria. The mice were challenged with 10K CFU FT LVS on day 35 following immunization on day 0 and a booster on day 21. The flow cytometry data shown above represents day 2 post-infection. PBS control samples shows from roughly

200,000 isolated lymphocytes, 2.7% of positively selected T helper cells are Th17 cells. iFt samples show 15.3% of positively selected T helper cells are Th17 cells. IC samples showed 27.8% of positively selected T helper cells are Th17 cells. There was no significant population of positively identified cells in pre-infection and day 6 post infection samples (data not shown).

Th17 Cells in Lung Post Infection

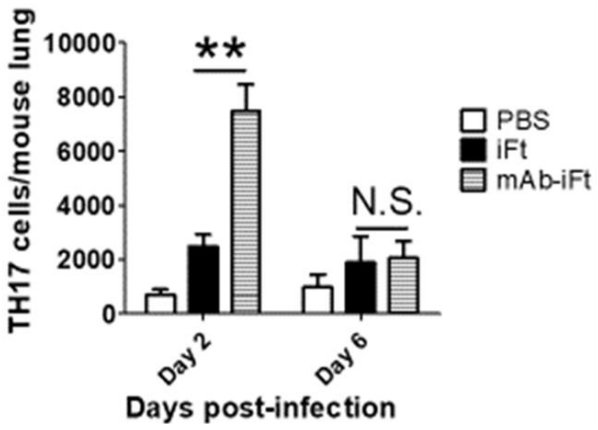
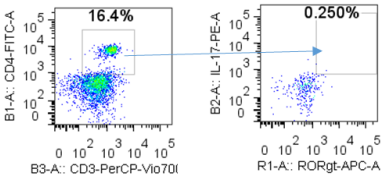


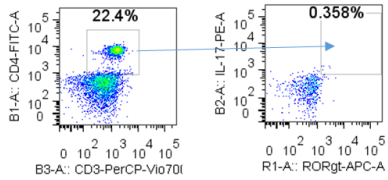
Figure 9: Positively identified Th17 cells from digested lung sample acquired at day 2 and day 5 post-infection of mice treated with PBS, inactivated *Francisella tularensis* bacteria, and an immune complex consisting of monoclonal antibody and inactivated *Francisella tularensis* bacteria. The mice were challenged with 10K CFU FT LVS on day 35 following immunization on day 0 and a booster on day 21. The number of positively identified Th17 cells in IC immunized groups 48 hours post infection compared to the control and iFt groups is roughly seven times and four times greater, respectively. Day 5 data showed the amount of Th17 cells in iFt and IC groups to be similar. (*) P-value < 0.1; (**) P-value < 0.05; bars represent SD.

Pre-infection Spleen

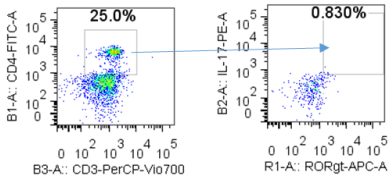
PBS



iFt

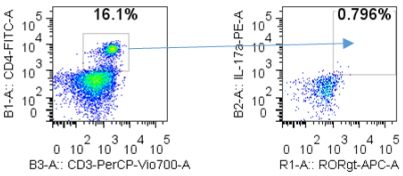


mAb-iFt

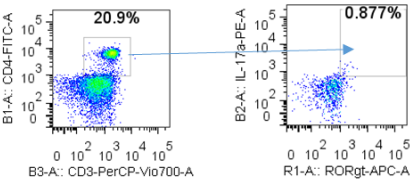


Day 2 Post Infection Spleen

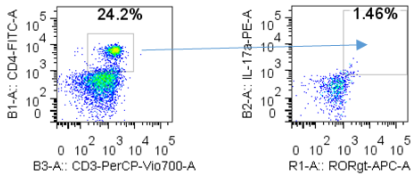
PBS



iFt



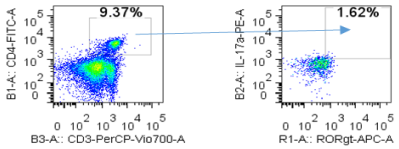
mAb-iFt



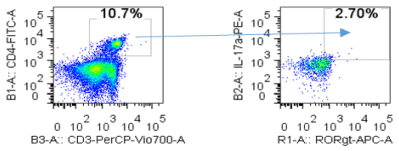
(A) (B)

Day 6 Post Infection Spleen

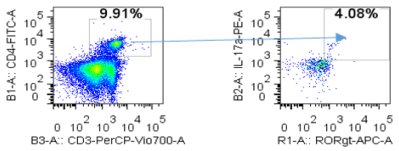
PBS



iFt



mAb-iFt



(C)

Figure 10: The figure above shows flow cytometry data generated from spleen samples of mice treated with PBS, inactivated *Francisella tularensis* bacteria, and an immune complex consisting of monoclonal antibody and inactivated *Francisella tularensis* bacteria. The mice were challenged with 10K CFU FT LVS on day 35 following immunization on day 0 and a booster on day 21. Images A, B, and C in the figure show pre-infection, day 2 post infection, and day 5 post infection lymphocytes, respectively. Top down on each image shows respective groups based on immunization protocol. PBS shows the control group with no inactivated pathogen present, the iFt data represents groups immunized with only inactivated FT LVS while the IC group represents the group immunized with immune complex of mAb-iFt. For pre-infection groups (A), PBS control samples showed of roughly 200,000 isolated immune cells, 0.25% of positively identified T helper cells are Th17 cells. iFt samples showed 0.358% of T helper immune cells to be Th17 cells. IC samples showed 0.83% Th17 cells. For day 2 post infection (B), PBS control samples 0.796% of positively identified T helper cells are Th17 cells. iFt samples showed 0.877% of T helper cells are Th17 cells. For IC groups 1.46% of cells are Th17 cells. In day 5 post infection groups (C), PBS control samples showed 1.62% Th17 cells. In iFt samples, 2.7% of T helper cells are found to be Th17 cells. In IC samples, the number of positively identified Th17 cells had risen to 4.08%.

Th17 Cells in Spleen Post Infection

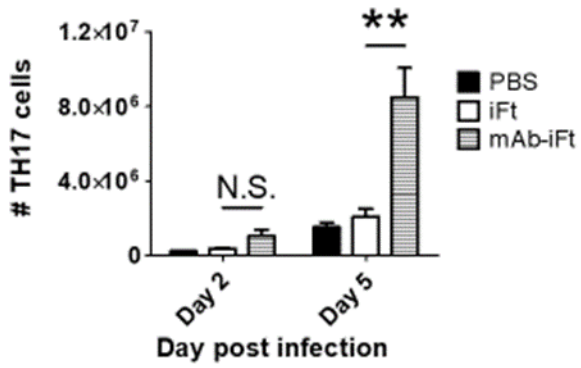


Figure 11: Positively identified Th17 from isolated lymphocytes in spleen tissue samples of mice treated with PBS, inactivated *F. tularensis* bacteria, and an immune complex consisting of monoclonal antibody and inactivated *F. tularensis* bacteria. There is a minor increase in Th17 cell population 48 hours post infection. By day 5 post-infection the number of positively identified cells is roughly four fold greater in IC immunized subjects in comparison to control and iFt immunization. The presence of a larger population is suggestive of a memory recall response in mAb-i*Ft* immunized subjects and a beneficial role of identified cells. (*) P-value < 0.1; (**) P-value < 0.05; bars represent SD.

The spleen sample result varies from what is observed in lung samples, where a significant increase of positively identified Th17 cells is reported 48 hours post infection before decreasing in day 5 samples. The route of infection in this experiment was intranasal, and a prominent response in the lung is observed early as the pathogen likely targets mucosal surfaces of the lungs and alveolar macrophages before becoming systemic over time.

3.9 IL-17A Secretion and Th17 Cell Response Contributes to the Generation of a Protective Th1 Response

IL-17A (also referred to as IL-17) has strong pro-inflammatory characteristics and is the primary effector cytokine of Th17 cells. IL-17A induces IL-12 production in dendritic cells. The IL-17A cytokine also induces macrophage production of IL-12 and IFN-γ, mediating critical Th1 responses (Lin et. al. 2009). IL-17A receptor is widely expressed among non-hematopoietic cells. The functions of this receptor include mediating the activation of transcription factor NF-κB via NF-κB activator 1 (Act1). The cytoplasmic domain of IL-17RA is described as directly interacting with Act1 in order to produce pro-inflammatory molecules. Additionally, treatment of fibroblasts and lung epithelial cell samples with IL-17A has shown a prominent response of promoting various immune responses such as granulopoeisis and neutrophil recruitment (Jin and Dong 2013). An increased concentration of this cytokine in IC groups further indicates the presence of Th17 cells and their role in protection against *F. tularensis* bacterial infection.

IL-17A Cytokine Concentrations

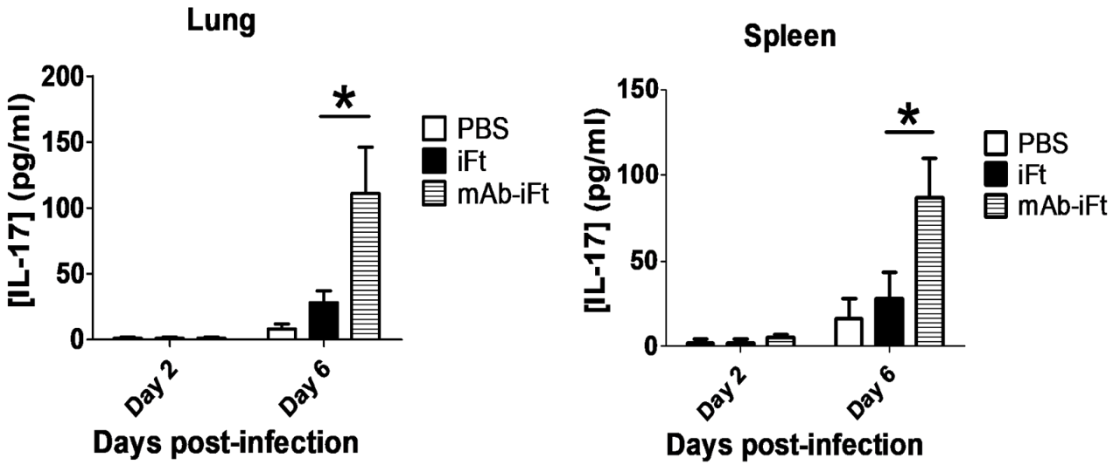


Figure 12: Data generated from ELISA analysis on lung and spleen samples at day 2 and day 6 post-infection time points. Figure 12 shows concentration of IL17A protein. Day 6 post infection showed a significantly greater concentration of this cytokine in IC immunized groups in lung and spleen samples. (*) P-value < 0.1; (**) P-value < 0.05; bars represent SD.

4. Discussion

Currently, there is no approved vaccine for *Francisella tularensis* bacterial infection, the causative agent of tularemia (Rawool et al. 2008). *F. tularensis* is considered by the Center for Disease Control to be a category A biological agent. Having high risk for use as a bioweapon through ease of aerosol spread and low infectious dose range, this pathogen poses as great a risk to public health as small pox and anthrax (Sharma et al. 2011). It has been established that in a murine model, a mucosal immunization protocol using an immune complex consisting of an inactivated form of *F. tularensis* live vaccine strain (FT LVS) and an IgG2A, anti-LPS monoclonal antibody for FcR targeting provides enhanced protection against FT LVS infection (Iglesias et al. 2012). The mechanism of FcγR-dependent uptake of antigen via APCs is expected here to result in robust T cell activation. This combats mechanisms employed by the *F. tularensis* pathogen such as MHC class II downregulation and an LPS component that promotes anti-inflammatory response in early stages. Furthermore, the current study investigates shifting the Th1/Th2 balance in favor of Th1 in mAb-iFt immunized samples via polarizing cytokines. It is proposed that a particular subset of $CD4^+$ T helper lymphocytes, known as Th17 cells, have a protective role in host defense against this intracellular bacterial pathogen using this novel vaccine strategy. The aim of the current study is to demonstrate that protected subjects have an ability to generate an early Th17 response to combat the immune evasion strategy of *F. tularensis* bacteria.

In order to determine the presence of this lymphocyte population, flow cytometry screening was utilized to determine unique surface and intracellular markers. A CD3

surface marker was used to determine the lineage of lymphocytes as T cells. To classify the cell population as T helper cells a CD4 surface marker was used. To define cells distinctly as Th17 T helper cells, intracellular markers for IL-17 and RORγT transcription factor were used. In order to generate a cytokine profile from inoculated and infected tissue samples, enzyme linked immunosorbent assays for various cytokines were utilized.

It was first essential to determine a proper lethal dosage of FT LVS in unimmunized wild type C57BL/6 mice. This dosage was identified as $1x10^4$ CFU administered i.n. (Figure 1). Once the dosage was defined, it was necessary to determine the effectiveness of Fcγ receptor targeting immunization strategy. During a 16-day observation period, the group of mAb-iFt immunized C57BL/6 mice exhibited a complete 100% protection against an otherwise lethal $1x10^4$ CFU dosage of FT LVS. Control mice and mice immunized with a paraformaldehyde treated form of the bacteria (iFt) showed mortality between days 10-14 (Figure 2).

Production of the IL-12 cytokine plays a significant role in overcoming the molecular mechanism *F. tularensis* has on suppressing the Th1 response. Promoting a Th1 response by inducing IL-12 ultimately results in production of IFN-γ, a beneficial immune response against intracellular bacterial infection, such as *F.tularensis*. As *F. tularensis* organisms infect host macrophages, IFN-γ limits intracellular replication. This cytokine also leads to secretion of inflammatory cytokines and chemoattractants (Steiner et. al. 2014). This current study demonstrates that in IC immunized groups, there is a robust IL-12 response in both tissue samples across both time points (Fig. 3). Th17 cells stimulate dendritic cells and macrophages to produce IL-12 (Lin et al. 2009). With IL-12

secretion being secondhand, it is difficult to pinpoint this increase to Th17 cells alone. However, it is evident that IL-12 production is largely beneficial to host immunity in protected subjects throughout the course of infection.

The anti-inflammatory cytokine IL-10 rises significantly in protected groups at later time points, suggesting that a potential over-inflammatory host response is being curbed. For the early 48-hour post-infection time point in lung this may be true as well, but there is no significant difference in spleen concentrations 48-hours post-infection. These results could also indicate that the infection is becoming resolved at the later time point, so greater suppression is necessary (Figure 4). Some IL-10 proteins are expected in control mice as a mechanism of *F. tularensis* to inhibit IFN-γ production downstream (Hunt et al. 2012). However, taken with the IL-17 data shown in figure 12, this IL-10 data may also suggest that more anti-inflammation signals are necessary when bacterial clearance is occurring. There is also research that states the non-pathogenic subset of Th17 cells secretes IL-10 opposed to the pathogenic subset that does not (Slight et al. 2013). Since the aim here is to characterize a protective role of Th17 cells, this IL-10 expression is an important distinction.

Polarization of Th17 cells from naïve T cells largely relies on signals from TGF-β and IL-6. With the pneumonic route of infection used in this procedure, it was expected that the largest population of protective cells would first appear in the lung. In agreement, it is seen in figure 5 and figure 6 that the lung displays greater concentrations of these cytokines at the earlier time point. This data suggests induction of Th17 cells from naïve T cells is occurring in the lung before being trafficked to other areas of the body. The

early response seen in these figures is also indicative of a recall response of quickly generating Th17 cells in mAb-iFt immunized groups. In spleen samples, it is worth noting that iFt immunized subjects show a dramatic increase in TGF-β1 concentration between day 2 and day 5, possibly contributing to a limited ability to fend of the disease in comparison to control subjects. Regardless, iFt groups show considerably less protection than mAb-iFt immunized groups.

In addition to polarizing naïve T cells into Th17 cells, the pleiotropic IL-6 cytokine is known to be pro-inflammatory, and induces the differentiation of $CD8^+$ T cells into cytotoxic T cells (Tanaka et al. 2014). In both lung and spleen tissues, there is a dramatic rise in concentration of IL-6 in PBS control groups between days 2 and 6 post infection (Fig.6). Alternatively, IC immunized groups show a more gradual and controlled rise in IL-6 concentration across these time points. Multiple aspects of IL-6 production are observed here. The controlled response in protected groups likely prevents a cytokine storm from harming the host as opposed to control mice where a rapid and robust concentration is reported day 6. Additionally, IL-6 polarizes Th17 cells in conjunction with TGF-β, particularly at the early stage in lung tissue.

IL-23 cytokine concentrations from lung and spleen tissues samples are represented in figure 7 at two post-infection time points of day 2 and day 6. IL-23 is a cytokine responsible for expanding and maintaining Th17 cells post-differentiation (Mus et. al. 2010). There is much more prevalent data generated from lung samples in this instance, possibly due to the fact of this being a pneumonic infection. A noticeable increase in the early time point of 48 hours post infection suggests a rapid immune

response in protected mice. Expansion of Th17 cells in the lung in response to pulmonary infection is expected. This increase can be correlated to Th17 cell expansion to combat the anti-inflammatory properties of FT LVS pulmonary infection and promote effective clearance of the bacteria. This sudden polarization of Th17 cells in protected mice further illustrates the memory recall response of the mAb-i*Ft* vaccine model. This also demonstrates that Th17 cell expansion is likely occurring in the lung rather than the spleen. As the concentration of IL-23 continues to rise in day 6 lung samples, the protein concentrations roughly remain level in iFt-immunized samples. IL-23 is described by Sharma et al. to be a contributor to the pathogenicity of Th17 cells, but here a clear increase in IL-23 is seen in protected groups. This data agrees more closely with research of Lin et al. (2009), who described the IL-23-Th17 cell pathway as critical for optimal induction of Th1 cell responses and protection against *F. tularensis* LVS. This group also determined that lung CFU was greater in IL-$23^{-/-}$ mice, and IL-17 production decreased in comparison to wild-type C57BL/6 mice following pulmonary FT LVS infection (Lin et. al. 2009).

In the earlier time point of 48-hours post intranasal infection there is a significant upregulation of positively identified Th17 cells in lung. Positively identified Th17 cells in isolated lymphocyte samples from digested lung tissue as shown by cell populations in figure 8 and figure 9. Being a pneumonic infection administered via intranasal, the lung is the likely early site of interaction between pathogen and host immune cells. As the infection progresses into day 6, the population of Th17 cells tapers off in lung, presumably as the host pulmonary tissue tries to regain normalcy and not prolong the

associated inflammation. This also exemplifies the trafficking of Th17 cells from the lung to other immune organs.

Inoculation and infection route plays a significant role in cellular response. Generally, the most virulent form of tularemia infection is inhalation resulting in a pneumonic infection with up to a 60% mortality rate (Roberts et al. 2018). It is likely that the robust 48-hour response in the lung is critical to the survival of our protected groups. Control PBS groups exemplify the asymptomatic inoculation of the disease as a relatively low number of Th17 cells that are recruited (Fig. 8 and Fig. 9).

The trafficking of Th17 cells is observed as the population of positively identified Th17 cells in spleen tissue rises almost simultaneously with the population decreasing in lung tissue. The presence of positively identified cells rises nearly four times in spleen tissue of IC immunized groups from pre-infection samples to day 5 post infection isolated lymphocyte samples (Fig. 10 and Fig. 11). The significantly larger number of Th17 cells day 5 post-infection suggests progression of the disease as the pulmonary infection spreads to other areas of the host.

The primary effector cytokine released by Th17 cells is known to be IL-17. ELISA data analysis reveals that the concentration of this cytokine is prevalent at the later time point post-infection in protected groups (Fig. 8). The delayed response could be in part due to IL-17A's role as a metaphorical clean-up crew, giving one last inflammatory push and signaling to phagocytes. IL-17A is a powerful pro-inflammatory mediator and recruiter of innate immune cells such as neutrophils in response to intracellular bacterial infection (Jin and Dong 2013). IL-17A and the IL-17 family of

cytokines as a whole are also linked as a driving force in chronic inflammation, which leads to tissue damage and autoimmune diseases such as rheumatoid arthritis and multiple sclerosis (Jin and Dong 2013). Perhaps the delayed response is the immune system pacing lightly as not to evoke an overly pro-inflammatory response in the host.

It is a concerted cellular effort to rid the host of tularemia infection. Th17 cells, as well as other T cell subsets, NK cells, and several other cell types coordinate a protective immune response against the bacterial pathogen. Although other cell types are involved in the immune response, it was our goal here to determine that Th17 cells do in fact hold a protective role. The early recall response seen in the lung and the associated cytokine profiles (Fig. 3, 5, 6, and 7, notably) possibly indicate the presence of a memory pool of Th17 cells induced by immunizations that occur on day 0 and day 21 before the day 35 infection. Additional study on this topic would include analyzing this potential memory pool of cells for distinctive surface marker separate of the Th17 cells observed in control groups. Furthermore, the use of IL-$17^{-/-}$ knockout mouse models could be an area of future study. Early post infection cytokine concentrations appear low for this effector cytokine, so it would be of interest to observe how host immunity is affected at this early stage. IL-17 is a potent promoter of neutrophil expansion and recruitment (Gaffen 2008). IL-17 induces IL-12 production in dendritic cells and promotes IL-12 and IFN-γ production in macrophages, mediating critical Th1 responses (Lin e. al. 2009). Additionally, IL-17 acts on various anti-microbial genes via a number of signaling pathways to upregulate molecules such as β-defensins, calgranulins and mucins (Gaffen 2008). As previously mentioned, the cytoplasmic domain of IL-17RA directly interacts

with Act1 in order to produce pro-inflammatory molecules through NF-κB activation as well (Jin 2013).

Alternatively, another gene target of IL-17 is CCL20, a chemokine with a known role in Rheumatoid arthritis pathology via activation of the MEK NF-κB signaling pathway. Even in infection, IL-17 can hold a detrimental role to host. In the case of *Candida albicans* infection in mice, alleviating the IL-17 response with IL-17 antibody treatment lightens pathogenic inflammation (Gaffen 2008). Therefore, aside from generating pro-inflammatory signals, a complete list of signaling targets for the IL-17 cytokine is far from being completed. Aside from observing pro-inflammatory cytokine concentrations, these proposed future experiments involving IL-17$^{-/-}$ knockout mice would include dose titration studies to analyze the extent of protection that IL-17 provides in *F. tularensis* infection.

In terms of advancing the vaccination model utilized in this experiment, the FcγR targeting method should be further investigated. The proposed mechanism of FcγR-dependent uptake of antigen via APCs results in robust T cell activation. This contests immune evasion tools employed by the *F. tularensis* pathogen such as MHC class II downregulation and an LPS component that promotes anti-inflammatory response in early stages. Furthermore, this mAb-iFt strategy shows success in combating intracellular bacteria by shifting the Th1/Th2 balance in favor of Th1 in mAb-iFt immunized samples via polarizing cytokines. Additional studies may benefit from using the more virulent type A *F. tularensis* SchuS4 strain. It is reported that protective mechanisms imposed by

IL-17 do not afford the same protection in the more virulent strain (Skyberg 2013). The FcγR targeting method should be further investigated in these studies.

A different approach to further understanding the mechanisms of Th17 cells could also be to utilize models displaying autoimmune or chronic inflammatory diseases. Isolation of lymphocytes from rheumatoid arthritis or multiple sclerosis murine models could be compared and contrasted to the T helper subpopulation seen in this study. Th17 cells are described as being subdivided into having pathogenic and nonpathogenic subsets, and this dynamic should be of interest moving forward. It has been shown in this study that protection to lethal *F. tularensis* infection is conferred by an FcγR-targeting vaccination strategy. In protected subjects, there is rapid growth in a population of positively identified Th17 cells following an otherwise lethal challenge. It is clear that in this intracellular bacteria challenge, Th17 cells offer a protective role.

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