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Suppression Of Inflammation Of Cytokine Following Induced Francisella Tularensis Infection

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SUPPRESSION OF INFLAMMATION OF CYTOKINE FOLLOWING INDUCED FRANCISELLA TULARENSIS INFECTION

Master's Program in Biological Science

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Dedication

To my sweet friends and family

SUPPRESSION OF INFLAMMATION OF CYTOKINE FOLLOWING INDUCED FRANCISELLA TULARENSIS INFECTION

by

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THESIS

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Abstract

Francisella tularensis is intracellular bacteria which is the causative agent of the disease Tularemia. Highly virulent in both humans and animals, it takes only as few as 10 microorganisms to cause a lethal infection. The bacteria can enter via direct or indirect routes causing the activations of the host innate inflammatory response to ensue. The bacteria invade host dendritic cells and neutrophils but predominately macrophages. This causes a mass inflammatory response resulting in the cytokine storm. Activation of Natural Killer T (NKT) cells has been shown to suppress inflammation in *in vivo* studies. Development and optimization of an *in vitro* co-culture assay were used to isolate NKT cell bulk purified populations from other cell types. Bulk purified NKT cells directly suppressed the production of inflammatory cytokines from *Francisella tularensis*-infected macrophages. This was attributed to a cell contact dependent mechanism that seemed in involve CD40/CD40L interactions. The bulk NKT cell population contains two subsets described by their antigen recognition and effector mechanism. Further separations showed that the type I NKT subset inhibited Il-6 secretion and suppressed inflammation in a dose dependent manner *in vitro*. Further studies are needed to fully characterize the inhibitory properties of type I and type II NKT cell subsets in *Francisella tularensis* immunity.

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Chapter 1. Introduction

1.1 Francisella tularensis and Tularemia Disease

Francisella tularensis (*F. tularensis*) is a zoonotic disease and the causative agent for the disease tularemia. The CDC has classified *F. tularensis* as a category A gram-negative coccobacilli. The bacterial colonies can be sustained in various environments. It takes less than 10 microorganisms to cause a severe lethal infection. The bacteria can live in an aqueous environment and still retain its full virulent pathogenicity.

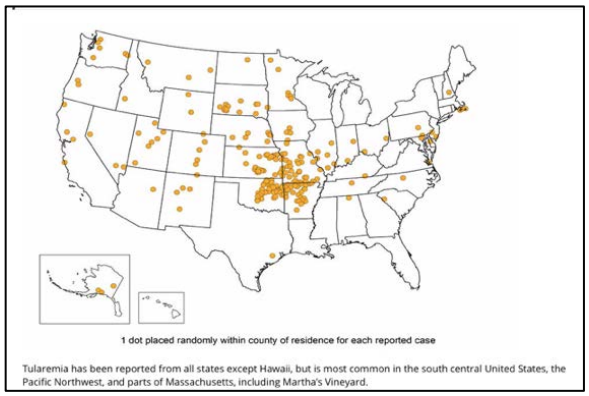

Figure1. Reported cases of Tularemia in the USA [1]

It was first isolated from ground squirrels in 1911 in Tulare County, California (USA), and from a human tularemia case in 1914 in Ohio (USA). The name "*Francisella tularensis*" was coined in 1959 to honor Dr. Edward Francis, who greatly contributed to improve the knowledge on human tularemia. [24]

There are four strains of *Francisella* that have been categorized into two forms: Type A and Type B. *Francisella tularensis subsp. tularensis* is a Type A strain that is native to North America and transmitted mainly through ticks and rabbits with high virulence that affects humans and animals. The Type B strain is commonly found throughout Europe and Asia. It is typically transmitted through mosquitos and is not considered as virulent as the Type A Strain. The less virulent strains are more *F. tularensis holartica, F. tularensis novicia,* and *F. tularensis medistatica*. An attenuated strain of *F. tularensis holartica* was live vaccine strain (LVS) used for research purposes. [1]

Tularemia is a disease that has a high lethality rate if not properly diagnosed, up to 35%. The disease is contact dependent from a direct or indirect vector that is an infected carrier. The person can also contract disease by

aerosolizing or eating/drinking contaminated water and uncooked meat. [2] Currently, tularemia is not known to be transferable from human to another human.

Table1. Characterizes F. tularensis Sub -strains Virulence in Humans and Animals

BSL	Strains	Type	Virulence Humans	Virulence Animals
3	*F. Tularensis tularensis*	A	+++	++
2	*F. t. holartica (LVS) **	B	+	++
1	*F. t. novicida*	B	-	+++
1	*F. t. mediasiatica*	B	-	+++

There are six different forms of tularemia disease:

1. Ulceroglandular is considered by the CDC to be the most common form of Tularemia. This form of the disease is the result from a direct bite from an infected vector. At the site of contact, the ulcer will form along with swelling in the lymphatic system pending the site of bacterial contact.

2. Glandular is similar in form to Ulceroglandular without the bacteria-induced ulcer at the site of contact. This form will have swelling in the lymphatic areas commonly in the groin or armpit. Often contact with this form is due to handling infected carcasses of animals.

3. Oculoglandular is the bacteria entering through the mucous membranes through the eyes. Contact is through direct route of a person touching an infected animal and transferring the bacteria by touching their face. Inflammation, irritation and ulcer in the eye will occur.

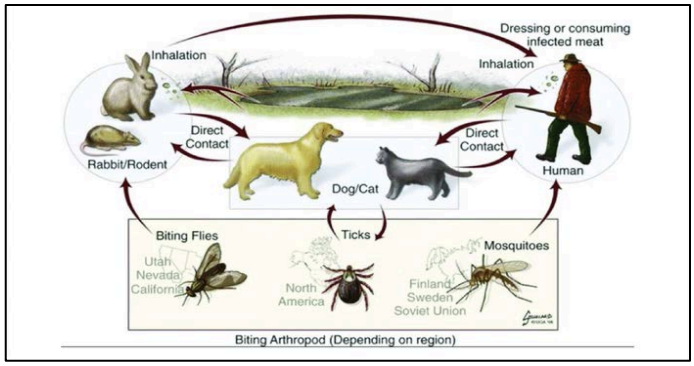

Figure 2. | Sylvatic cycle of Francisella tularensis, illustrating the transmission cycles and the relevant biting insects depending on the region (Art by Brad Gilleland, UGA College of Veterinary Medicine. © 2004 -2019 University of Georgia Research Foundation, Inc.).[3]

4. Unlike the other forms of tularemia of direct infection from an infected vector, Oropharyngeal is caused by eating or drinking contaminated water or uncooked meats. It will have symptoms that consist of swelling, inflammation, irritation, sore throat, and swelling of the lymphatic systems.

5. Typhoidal tularemia is the rarest case of the disease because the difficulty in pinpointing physical manifestations. The disease comprises of any of the common tularemia symptoms without manifesting any one form.

6. Pneumonic tularemia is the most serious form of the disease. It is caused by inhaling aerosolized bacteria. The bacteria in the lungs will induce troubled breathing, cough, severe chest pain. It can cause secondary infections in the lungs and if left untreated can become septic spreading throughout the blood stream. [4]

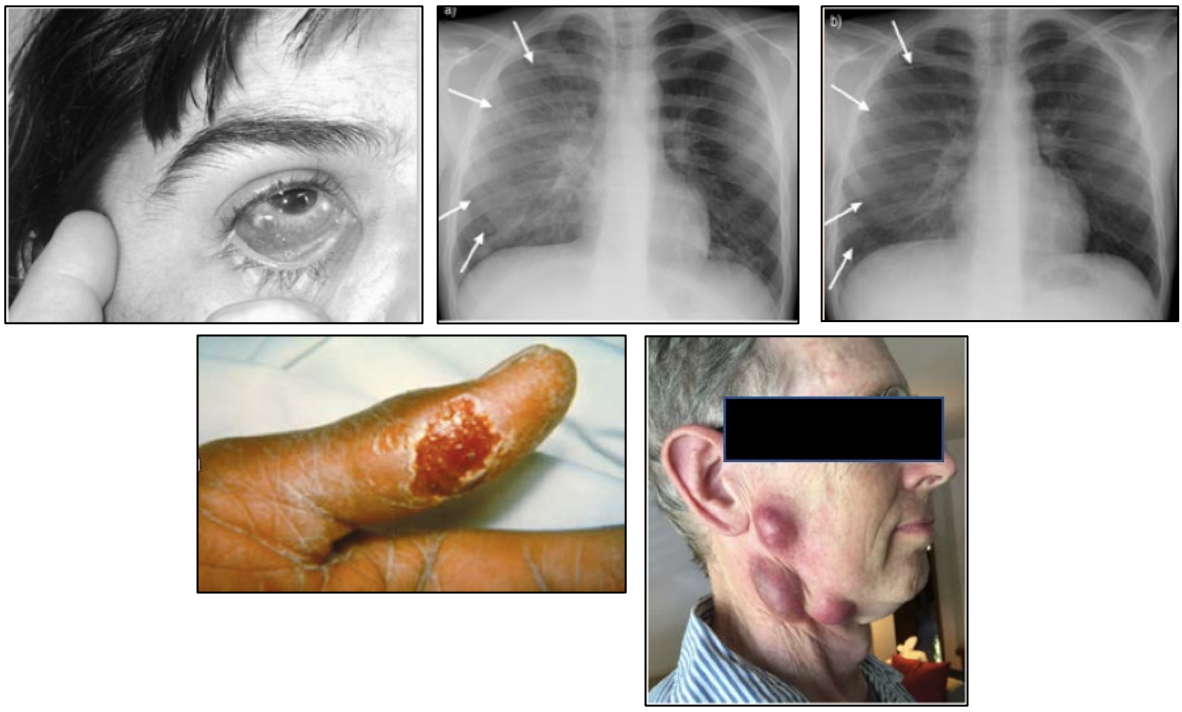

Figure. 3-7 The images are the clinical manifestations of different routes of exposure to Tularemia. Oculoglandular formation of an ulcer in the eye [5] Ulceroglandular from a direct bite from an infected vector [2] Lungs that are infected with Pneumonic Tularemia [6], Glandular tularemia causing a lymphatic infection [7]

Incubation times for symptoms to manifest is 3-5 days following exposure. [4] Lethality rates for tularemia that have lymphatic inflammation are 30-60% lethal. [8] Currently treatment for tularemia disease is limited to administration of antibiotics, particularly gentamicin and streptomycin. New approaches to tularemia treatments have been studied and characterized in different ways to avoid relapse or resistant replication of the bacteria. Quinolones (ciprofloxacin) have demonstrated to achieve adequate blood levels with high intracellular penetration after oral administration to be used as a potential additional drug. [9] The ketolide compounds (telithromycin and cethromycin) are a subclass of macrolide antibiotics which have been designed to address the problem of macrolide resistance in respiratory pathogens such as *Streptococcus pneumoniae*. Telithromycin

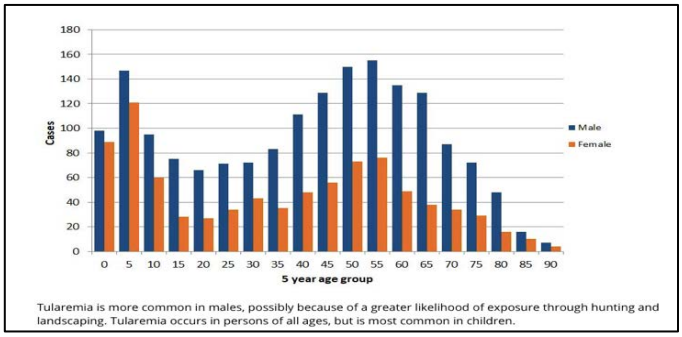

Figure 8. All Reported Cases of Male to Female Age Range of Exposure to Tularemia until 2017 ([1])

was more effective *in vitro* against French isolates of *F. tularensis* subsp. *holarctica* than erythromycin. [10] The symptoms of the disease can be easily misdiagnosed if not ascertained early on. Depending on the route of exposure physical manifestations can vary. In general, all forms of tularemia have symptoms of a high fever of 104 °C. [1] Other symptoms of tularemia are flu-like symptoms that consist of malaise, coughing, fatigue, and secondary infection in the lungs such as pneumonia. If left untreated, tularemia can cause inflammation in the lungs and the heart, including the pericardia sac around the heart. In severe cases, tularemia can cause meningitis and bone infections (osteomyelitis). The documented cases are more likely to be male, the premise is that there are more males likely to be exposed because of hunting and outdoorsman who are most likely to be exposed by touching a contaminated animal or by drinking contaminated water.[4]

Biological Weapon

During World War II in the 1960s *F. tularensis* was kept and studied as a biological weapon of war. The United States Department of Biodefense, as well the US Allies, studied alternating the genetics of *F. tularensis* to be used as a bioweapon. The United Stated later destroyed its *F. tularensis* stocks in 1973 and discontinued all state-sponsored biological warfare programs. The Soviet Union continued to work on bioweapons up until the early 1990s. *F. tularensis* was categorized as a bioweapon because of its ability to survive in various types of environments, including water at low temperatures, while retaining its fully virulent integrity. During the 1970s, an expert from the World Health Organization approximated, “if 50 kg of virulent *F. tularensis* were dispersed as an aerosol over a metropolitan area with a population of 5 million, there would be an estimated 250,000 incapacitating casualties, including 19,000

deaths." By aerosolizing the bacteria, it would result in inhalation the of organism and cause pneumonic respiratory tularemia, the most severe form of the disease. [1, 8, 11]

1.2 Mechanism.

Once *F. tularensis* enters the host, it infects a set of host cells such as macrophages, dendritic cells (DC), hepatocytes, neutrophils and type II alveolar lung epithelial cells, with the primary host cell being macrophages. The bacterial survival in the host depends on entry into the host cell, replication and proliferation. (11) Indeed, mutant bacteria unable to escape the phagosome into the cytosol, where it replicates, are rapidly cleared from the host. In order for the bacteria to enter the cell, it will bind to different immune receptors, that play a functional role in the uptake of the bacteria, the mannose receptor (CD206), complement receptor (CD11b), scavenger receptor- A (SR-A), and Fcγ receptor (CD16/32), all of which activate phagocytosis. [12],[13] Of these, Fcγ is the main receptor which plays a critical role in the uptake the bacteria. [12]

After successful completion of the uptake of bacteria, the bacteria residing within the phagosome, now referred to as the Francisella-containing phagosome (FCP). [11]Upon maturation, the FCP, acquires surface markers for early to late endosomes such as EEA-1, LAMP-1 and LAMP-2 and CD63. [14] However, the FCP will not fuse with the lysosome instead the bacteria will seek to disrupt the phagosomal membrane to escape into the host cell cytosol. [15]The transcription factor MglA is a master transcription factor which regulates genes responsible for phagosomal escape. It is a critical component for intracellular survival proliferation. [14] The bacteria complete its phagosomal escape utilizing a Type-VI secretion system that aids in finalizing the disruption of the membrane. [16]

The presence of *F. tularensis* in the cytosol is recognized by several receptors, the innate pattern-recognition receptors (PRRs), Toll-receptors (TLRs), and Nod-like receptors (NLRs). [11] The PPRs recognize Pathogen-associated molecular patterns (PAMPs) and Danger-associated molecular patterns (DAMPs) that trigger initiation of the innate immune response in the host. [17] This activation is initiated by the inflammasome and result in production of the Proinflammatory cytokines, IL-18 and IL-1β. [18] Once these pro-inflammatory cytokines are activated, a cascade of signaling events leads to the production of the pro-inflammatory cytokines IFN-γ, IL-6, and TNF-α, the main effector cytokines of the innate immune system. [19] Continuing production of the inflammatory cytokines causes an overproduction of pro-inflammatory, and anti-inflammatory cytokines, resulting in a dysregulated cytokine response, referred to as a cytokine storm. This severe inflammatory response results in pneumonia, hypovolemia, edema, and in many cases, it will result in death. [20]

1.3 Type I and Type II NKT Cell

NKT cells are a relatively small subset of immune cells [[21] that share properties of both innate Natural Killer (NK) cells and adaptive T cells. NKT cells are activated within 24 hours of infection responding to infectious diseases by recognizing lipids presented by the MHC-like protein CD1d. Collectively and individually, NKT cells respond to activation through a wide variety of effector mechanism that result in activation or inhibition of immune cells, as necessary. Two subsets of NKT cells have described that are generally considered to have inflammatory (type I) and regulatory (type II) properties. However, there is still a vast amount of knowledge that is unknown regarding the type I and type II NKT cell subpopulations.
More specifically NKT cells have been suggested to contribute to a large range of autoimmune disease and the murine model that would include type I diabetes, multiple sclerosis, rheumatoid arthritis, systemic lupus erythematosus, autoimmune hepatitis, and sarcoidosis. [22]

CD1 family members are surface immune receptor proteins that share functional and structural characteristic of the classic MHC Class I and Class II proteins. All CD1 proteins contain three extracellular domains $\alpha 1$, $\alpha 2$, $\alpha 3$, a transmembrane domain and a C-terminal domain and pair with $\beta 2$-microglobulin. NKT cells specifically bind to the CD1d member of the family. While MHC proteins present degraded proteins to T cells, CD1d presents lipids.

While NKT cells do utilize a T cell receptor (TCR), they have a severely limited diversity of sequences, with a large majority possessing the TCRα chain rearrangement Vα14/Jα18. This arrangement of T cell receptor genes is specifically expressed by type I NKT cells while type II NKT cells express a slightly wider diversity of T cell receptor genes. Both subsets also express the NK cell marker NK1.1 (CD161). The recognition and activation of type I NKT cells relies on the recognition of the lipid α-galactosylceramide (α-GalCer) presented by CD1d. This specific lipid has been identified in both human and animal host as the recognition ligand for type I NKT activation. Indeed, these type I NKT cells can be distinguished from type II NKT cells by their ability to bind to tetramers composed of the CD1d protein presenting α-GalCer while type II NKT cells do not bind this reagent.

The activation that occurs with the NKT cells by the α-GalCer lipid has a robust response that is marked by upregulation of surface proteins and cytokine production. With this activation, individual NKT cells have been shown to secrete both Th1 and Th2 cytokines, IFNγ and IL-4,

respectively. [21] In general, type II NKT cells show inhibition for the pro-inflammatory functions of the type I NKT cells, as well as the dendritic cell and T cell populations. [23] It is, therefore, important to define the parameters and characteristics of these two cell types in *Francisella* immunity.

1.4 Preliminary Data

Figure 9a.

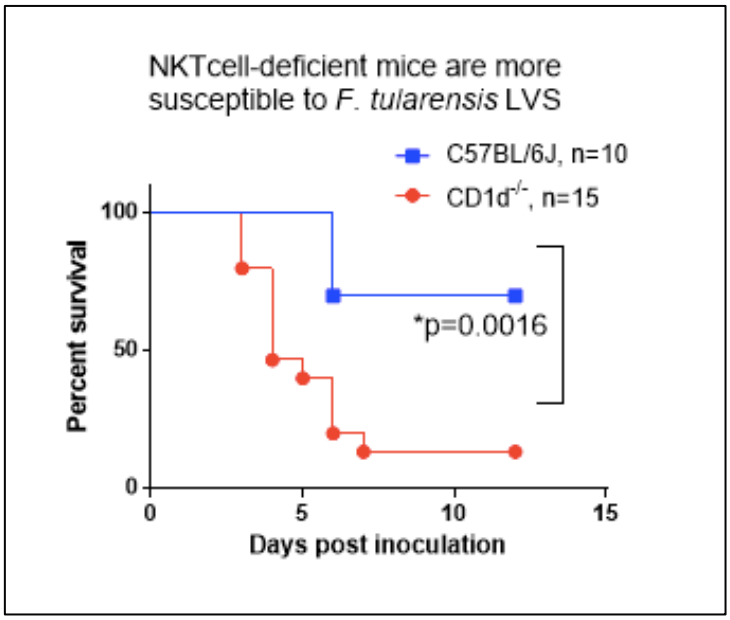

Figure 9a. NKT cell sufficient (C57BL/6J) and deficient (CD1d-/-) mice were infected with *Francisella tularensis* LVS and monitored over the subsequent 14 days. Mice deficient in NKT cells demonstrated a much poorer survival compared with mice possessing NKT cells. Mantel Cox Test, P-Value <0.002

Figure 9b.

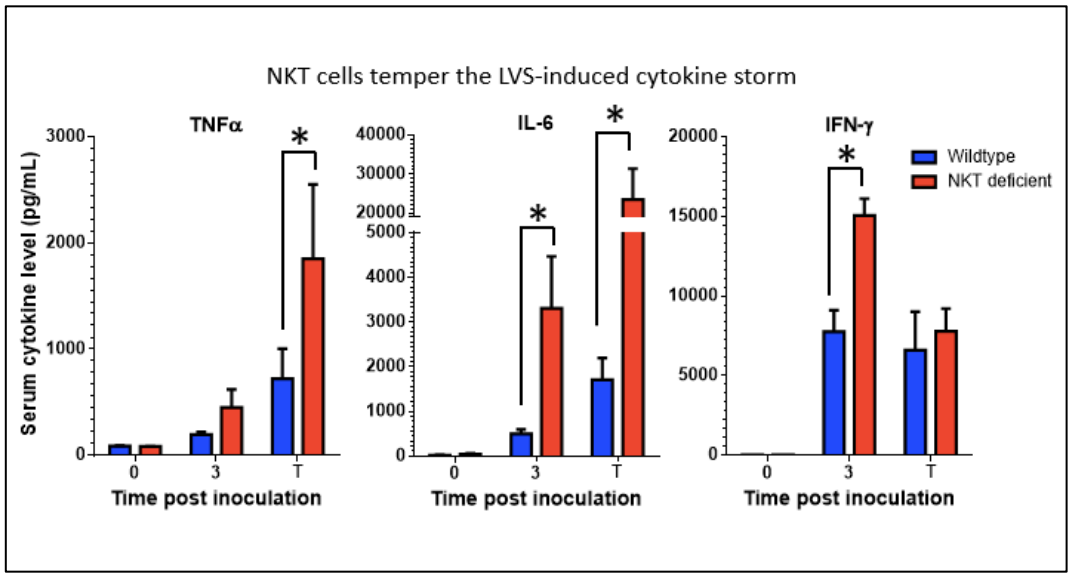

Figure 9b. Serum cytokine levels of TNFα, Il-6, IFN-γ from LVS-inoculated wildtype and NKT cell deficient mice. The presence of NKT cells controlled the levels of the cytokine storm. T-test, P-Value <0.05

Mice sufficient and deficient in NKT cells were infected with *F. tularensis* and their survival and cytokine response was compared. Preliminary data suggest that mice lacking NKT cells are more sensitive to the infection (Figure 9a) and produce higher cytokine levels (Figure 9b) than mice having NKT cells. The wildtype mice that contain NKT cells showed lower levels of the cytokine secretion, as opposed to the NKT deficient mice which were lacking the NKT cells. These data suggest a regulatory role for NKT cells in controlling the *F. tularensis*-directed immune response. Due to their effector mechanism, activated NKT cells may attack and destroy the bacterial infection while also preventing an overactive cytokine response culminating in reduced lethality.

1.5 Hypothesis

F. tularensis induces inflammation through cytokine storm signaling to cause disease. Our preliminary data suggested that at least one population of NKT cells can suppress the cytokine storm after infection. Based on previous experimentation in the lab, we hypothesized that type I NKT cells will be able to directly suppress inflammation through a cell contact dependent mechanism.

Chapter 2. Specific Aims

Francisella tularensis is an emerging pathogen stimulating an overactive pro-inflammatory response from the immune system leading to death of the host. The lethality of the bacteria takes as little as ten cells to cause a lethal infection. *F. tularensis* is a gram-negative coccobacilius bacteria. It has been classified as a potential biological weapon. Despite having a low bacterial load, it can cause a high lethality rate if the bacteria is contracted by hosts due to its stimulation of high inflammation in the host, otherwise known as a cytokine storm. The cytokine storm induces over-activation of immune cells resulting in host cell damage and in many cases, can even lead to death. Therefore, control of the excessive inflammation is critical to allowing infected individuals to recover. NKT cells are a subset of white blood cells with regulatory functions that could potentially control this lethal response. Ultimately, these investigations will lead to a better understanding of the role of NKT cells in this and other pro-inflammatory infections.

Aim 1. The Role of NKT Cells in F. tularensis Mediated Immunity

Through an *in vitro* assay I established and characterized the independent roles of the NKT cells induced by *F. tularensis*-mediated infected. The NKT cells have the capacity, whether in bulk purified or individual subsets, to suppress inflammation. Isolating the NKT cell populations and culturing them with infected macrophages, I quantified the secreted inflammatory cytokines and chemokines. By fixation and neutralizing surface protein interactions we gained insight into the suppressive mechanism. If these molecules are involved in mediating the suppression, we should see a reversal of inhibition in the presence of the antibody.

Aim 2. The Independent Role of Type I and Type II NKT Cells

Bulk NKT cells were separated into the type I and type II NKT cells. With the isolation of the cell populations, I investigated their ability to inhibit IL-6 production. By independent co-culture of type I and type II NKT cells with infected macrophages we determined their relative ability to suppress IL-6 production.

Chapter 3 - Methods and Materials

Cell Line

Murine Macrophage Cells are derived from C57BL/6 adherent Bone Marrow Derived Macrophages that has been immortalized by the retrovirus J2.

Origin: Mouse

Purchased from BEI Resources: NR-9456

Preservation and Culture Information

Growth Media: DMEM with additives

Serum Media: Fetal Bovine Serum purchased from CORNING USDA Approved Origin Ref: 35-010-CV

Cultured in a T75 flask passaging every 2-3 days and supplementing the media with 21ml of Cell Growth DMEM Media with Ciprofloxacin until 80% of confluency is observed.

Bacteria Culture

Francisella tularensis subsp. *holarctica* CDC LVS (BEI Resources, catalog #NR-646) is a categorized risk group 2 bacteria.

Culturing bacteria is first inoculated on Mueller-Hinton plates. Necessary supplements used in growth media includes IsovitaleX 1% and Defibrinated Sheep Blood 5% with an incubation period of 48 hours at 37°C. Post incubation period the bacteria will be aliquoted, diluted and prepared for long term storage using a 15% glycerol/PBS solution. Bacteria must be stored at -80°C until ready to use.

In Vitro Assay

Day 1: Using the C57BL/6 cells they will be plated in a 96 well round bottom plate at 2e5/ml at 200ul/well, with a 24-hour incubation period for the cells to adhere.

Day 2: After cell adherence *F. tularensis* (LVS) is used at 1e10 CFU/ml at 100ul/well is used to infect the macrophages for 2 hours. Post two-hour infection two rounds of liquid gentamicin (15750060 Thermo Fischer) is treated in two rounds a high dose and low dose. Ratio used 1:1 Gentamicin is used within this assay to treat and exterminate any external bacteria from being internalized which will cause activation of the pathways that we are not interested in. High dose

treatment 100ul/well, with an incubation period of 96min and low dose 100ul/ well with an incubation period of 24hrs at 37°C.

Male Mice Spleens are used and grounded with 1x sterile PBS all supernatant is collected and centrifuged at 1500 RPM for 10 minutes

RBC lysis buffer is used to lyse primarily all red blood cells 30 secs which is neutralized with a FBS based media, after centrifuging 1500 RPM for 10 minutes. Cells are washed with supplemented media and centrifuged. The cell suspension will continue to be labeled with filter purified antibodies (refer to antibody section). Total incubation period of 90 min for the antibodies to bind to the cells and the lipids, the cells are prepared to process through the Beckman Fluorescent Activated Cell Sorter. The target population for the sorting is NKT type I and type II. After completion of the sorting the both type I and type II NKT subsets will be added into the In Vitro Assay at 1e5/well. They will be left for 24 hours in an incubator at 37°C.

Day 3: At the collection time point all supernatant including the controls and the treatments will be collected and transferred to a 96 well plate and stored in the -80°C until ready to use. Following this assay an ELISA will be used to quantify how much IL-6 is being produced

Fixed NKT Cell Protocol

The fixed NKT protocol is designed to stop and inhibit the secretion of the NKT cell production. This will allow for only surface proteins to remain active.

(100ul) 0.15% Glutareldahyde incubate for 30 seconds

Add 100ul of 0.2M Lysine

Wash 2x with Media No Ciprofloxacin

Buffers

1. Cell Growth Media with Ciprofloxacin – Total Volume 500ml

- 10% Fetal Bovine Serum (CORNING USDA Approved Origin Ref: 35-010-CV)
- 1% 1 M Hepes in 0.65% NaCl (Lonza Catalog No: 17-737E)
- 1% L-Glutamine 200mM (100x solution) purchased from GE Lifescience/Hyclone
- 1 % Amphotericin B (Thermo Fischer 15290018)
- Completed with (DMEM) Dulbecco’s Modification of Eagle’s Medium with 4.5 g/L glucose, L-glutamine & Sodium Pyruvate (Corning 10-013-CM)

- 500ul of Ciprofloxacin 10 mg/1ml (Aeros 449620250)

2. Cell Growth Media without Ciprofloxacin- Total Volume 500ml

- 10% Fetal Bovine Serum (CORNING USDA Approved Origin Ref: 35-010-CV)
- 1% 1 M Hepes in 0.65% NaCl (Lonza Catalog No: 17-737E)
- 1% L-Glutamine 200mM (100x solution) purchased from GE Lifescience/Hyclone
- 1 % Amphotericin B (Thermo Fischer 15290018)
- Completed with (DMEM) Dulbecco's Modification of Eagle's Medium with 4.5 g/L glucose, L-glutamine & Sodium Pyruvate (Corning 10-013-CM

3. Cell Sorting Media – Total Volume 500ml

- 5% Fetal Bovine Serum (CORNING USDA Approved Origin Ref: 35-010-CV)
- 1% Penicillin/Streptomycin
- Completes with 470 ml of Media with no phenol red (Corning)

Media with no phenol red is used due to the color altering the laser reading on the Beckman Cell Sorter Machine

4. Enriched media at 15% FBS – Total Volume 250ml (exact volumes are used)

- 37.5 ml Fetal Bovine Serum (CORNING USDA Approved Origin Ref: 35-010-CV)
- 2.5 ml 1 M Hepes in 0.65% NaCl (Lonza Catalog No: 17-737E)
- 2.5 ml Non-Essential Amino Acids
- 2.5 ml Amphotericin B (Thermo Fischer 15290018)
- 2.5 ml Penicillin/Streptomycin
- 200ml DMEM) Dulbecco's Modification of Eagle's Medium with 4.5 g/L glucose, L-glutamine & Sodium Pyruvate (Corning 10-013-CM)

5. Neutralizing Media – 250 Volume (exact volumes are used)

- 37.5 ml Fetal Bovine Serum (CORNING USDA Approved Origin Ref: 35-010-CV)

- 200ml (DMEM) Dulbecco's Modification of Eagle's Medium with 4.5 g/L glucose, L-glutamine & Sodium Pyruvate (Corning 10-013-CM)

6. RBC Lysis Buffer

- Ammonium (Sigma-Aldrich 294993)
- Chloride (Sigma-Aldrich S9888)
- Potassium buffer in Ix sterile PBS

Cell Sorter

Beckman Cell MoFlo Astrios Cell Sorter - Fluorescence Activated Cell Sorting. Labeling and preparing the cells is key to a successful separation. Individually labeled cells are exposed one at a time in a liquid stream onto a laser beam. The light that is emitted from the laser is collected by a set of PMT's after passing through several filters which will assign a physical charge to the cell. The stream overview has an influx of 100um stream, the amplitude of approximately 35,000 with a pressure maximum of 24 PSI. After preparing the labeled cells from the splenocytes. The particle enters the stream, which will trigger the laser. The progression of the particle will progress down the stream, the cell will enter the last droplet before breaking from the stream. At this point the stream is charged with magnetic plates on the outside the stream flow. Before the cell can retain a charge the drop delay which is the point of cell analysis will "interrogate" whether the targeted cell in every three drops contains the population. Adjusting the events per seconds one out three droplets will contain the cell needed at the drop breakoff point. The droplets that contain the target particle will separate from the stream and will retain the charge. Based on the physical charge of the droplet the cell will be pulled and deflected into the assigned conical tube. With the use of six spleens consistently after replicated studies the approximate yield for the NKT cell type I population is 3e5 cells/ml and NKT cells type II population is 3e5 cells/ml.

Mice Protocol

All animal procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals following OLAW guidance and animal protocol (A-201208-1), with approved by the Institutional Animal Care and Use Committee. Per established protocol four to seven male mice from the C57BL/6 lines at the age between six to eight weeks were euthanized

and their spleens were extracted and placed in Media without Ciprofloxacin on ice. With only approximately a twelve to sixteen-hour timespan can the spleens be kept on ice in the 2°C without losing the integrity of the tissue.

ELISA

Mouse IL-6 Uncoated ELISA Kit

Enzyme linked immunosorbent assay for the quantitative detection of Mouse IL-6

(Thermo Fischer Scientific Invitrogen Ref 88-7064-88)

All reagents and antibodies were used in accordance with protocol provided by the kit.

Antibodies

- mCD1d- PBS 57- PE (Bioscience 50-4317-U500)
  - Purpose of this antibody is the Lipid will bind to the NKT cell
- Empty Tetramer APC mCD1D unloaded – APC (Bioscience 20-4317-U500)
  - Non-Specific, No lipid present incapable of binding to target cell population
- Anti-Mouse CD16 / CD32 "FC Shield" (Bioscience 70-0161-M001)
  - Fc Shield is used for the detection of NK Cells. The Purpose is to block the FC receptors CD16 and CD32 from binding.
- BV510 Rat Antibody B220/CD45R (BD Bioscience 563103)
  - Used for labeling all B cells
- Anti-Mouse NK1.1 PerCP – Cy5.5 (TONBO Sciences 65-594-U100)
  - Labels NK and NKT cells
- Anti-Mouse CD3 violet Fluor 450 (TONBO Sciences 75-0032-U100)
  - T cell markers for activation

Antibodies are used in In Vitro Assay to label non-targeted cells and the targeted subset NKT cells populations. Antibodies are sterilized and incubated with the cells 90mins before being sorted with the Beckman Cell Sorter

3.1 Experimental Design

The overall strategy of the experimental design is to culture purified NKT cell subsets with infected macrophages. The protocol has been optimized and designed for the efficacy of this procedure. The initial setup is key to the long-term success of the experiment. Using the C57BL/6 macrophages after 80% confluency and morphology is correct. The macrophages will be plated at 2e5/ml in a round bottom 96 well culture plate. This number has been previously optimized to allow for the infection to be maintained throughout the three-day procedure. The number of cells is kept consistent to avoid any overgrowth of cell proliferation after the cells have phagocytized by the bacteria from infection. The plated cells are incubated for approximately 12-14 hours to allow the cells to adhere to the plate. *F. tularensis LVS* is used to infect the cells at an MOI of 30 with the concentration of 3e7 in 200ul/well. The bacteria will be incubated and co-cultured with the macrophages for 2 hours. This allows enough time for the macrophages to undergo phagocytosis and initiate cytokine secretions.

After the infection period the media is removed and a series of gentamicin treatments is added to kill extracellular bacteria and prevent overgrowth. The treatment includes a high and low dose. The high dose of gentamicin is incubated for 1 hour. The media is replaced with a low dose of gentamicin which is present throughout the co-culture period to prevent shedding of the bacteria.

During this time, male C57BL/6 mice splenocytes have been collected and are kept in suspension within cell growth media without ciprofloxacin on ice until ready to use. For this, the spleens are dissected and are ground into a single cell suspension and red blood cells lysed using RBC lysis buffers. The addition of the neutralizing media stops the lysis; the cells are ready for labeling. (Please see “Antibody” section in methods) The antibodies will be sterilized by filtration before being added to the splenocytes and incubated at 4°C for 90min. The buffer used

for the labeling of the splenocytes is the Cell Sorting Media. This media is used purposely with no phenol red to reduce refraction because of the color engaging with the laser. At this point, the FACS sorter is setup to isolate and separate the NKT type I and type II cell populations.

Table 2. Gating Strategy

The gating strategy defines a series of populations for the isolation and separation of the NKT subset populations is setup in the following way.

All Lymphocytes Entire Cell Population Identified →	Non-B Cell Populations Identified →	T cell Population Identified ↴
NKT Cells Identified →	NKT Type I and Type II Separated and Collected	

The cells are collected in Enriched media with 15% FBS to help stabilize the cells after the interrogation by the cell sorter. Once the NKT cell type I and type II are collected they will be added to the *in vitro* assay at 1e5/ml at 100ul/per well. The assay will be incubated for 24 hours and all supernatant is collected. The mouse IL-6 ELISA will be used to quantify how much pro-inflammatory marker is being secreted in the presence of the NKT type I and type II cells.

Chapter 4. Results

In the *in vitro* assay we designed, C57BL/6 macrophages infected with LVS alone secrete a large amount of the inflammatory cytokines IL-6 and IL-1β while uninfected macrophages do not produce much of either cytokine (Figure 10). Addition of a purified total NKT cell population to the infected macrophages showed a drastic suppression of both IL-6 and IL-1β. This demonstrates that bulk purified NKT cells can directly suppress inflammation. The data was analyzed with Graphpad Prism Software 9th Version using t-test resulting in p-value = 0.01.

Figure 10.

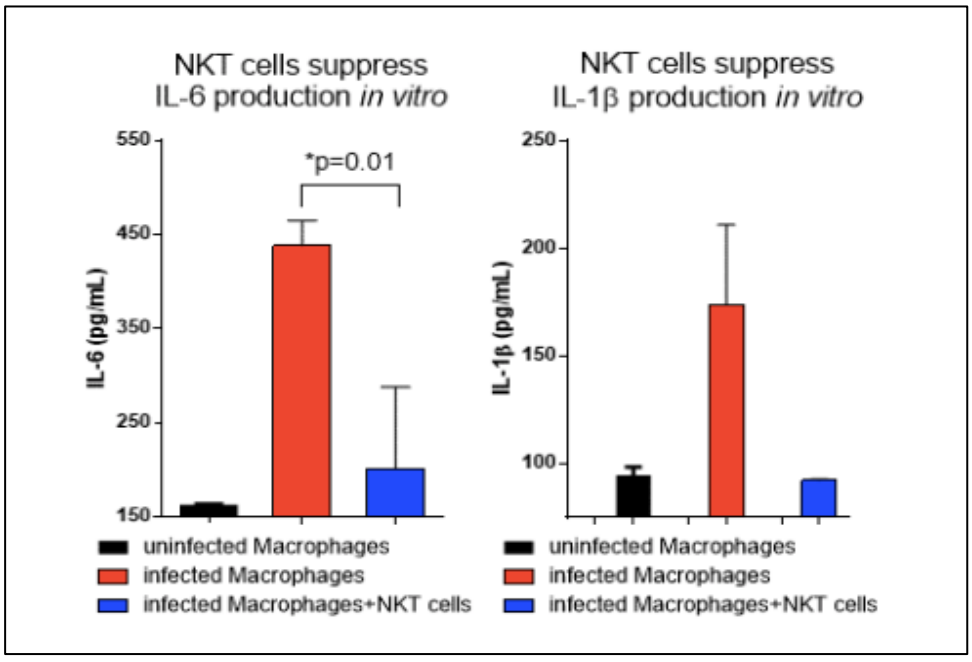

Figure 10. *In vitro* co-culture assay. Quantification of IL-6 and IL-1β in the supernatant by ELISA showed that NKT cells could suppress LVS-induced inflammation. P-Value =0.01 by T-test.

Figure 11.

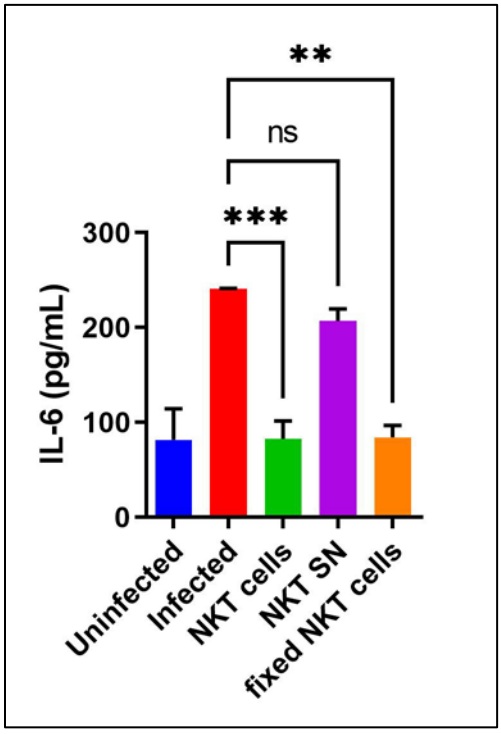

Figure 11. Supernatant transfer to freshly infected macrophages did not transfer the suppressive activity of the NKT cells. Glutaraldehyde fixation of surface effector molecules maintained NKT cell-ediated inhibition. **ANOVA was p-value = 0.0002 and *** Bonferroni post test showed p-value < 0.002 for ** and < 0.0005

Since total NKT cells could inhibit IL-6 and IL-1β, we sought to determine the mechanism resulting in this suppression (Figure 11). Culture supernatant from wells in which NKT cells demonstrated the ability to inhibit IL-6 were transferred to freshly infected macrophages. Addition of culture supernatant did not result in suppression of IL-6 in the recipient cultures. On the other hand, fixation of the NKT cells with glutaraldehyde to prevent secretion of cytokine while maintaining cell surface protein structure could inhibit IL-6 production. This led us to conclude that the suppressive mechanism of NKT cells was cell-contact dependent. The data was analyzed with Graphpad Prism Software 9th Version using an ANOVA and post-test Bonferroni. p-value < 0.002 for the ANOVA and < 0.005 for the post-test.

Figure 12.

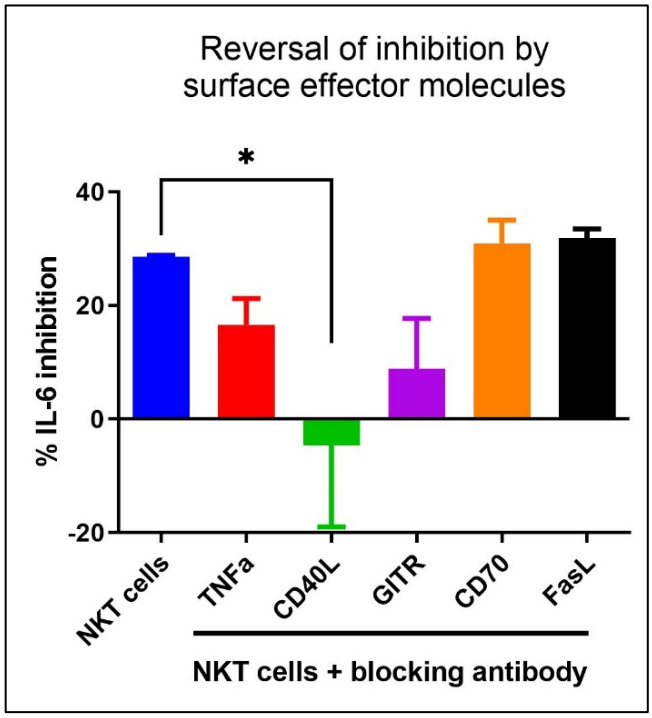

Figure 12. Addition of neutralizing antibodies demonstrate the role of surface proteins in NKT cell-mediated suppression of inflammation. Anti-CD40L demonstrated the most profound effect in preventing IL-6 inhibition by NKT cells with anti-GITR also showing some mild effect. ANOVA analysis showed an overall effect p-value of 0.016 and the Bonferroni post test showed a p-value of 0.0249 for CD40L.

Next, we sought to narrow the proteins potentially mitigating the suppression of IL-6. NKT cells express a number of NKT and T- cell surface receptors. Using neutralizing antibodies, we targeted known regulatory proteins on NKT cells to disrupt their interaction with ligands on the infected macrophage (Figure 12). With the addition of NKT cells this resulted in a 30% inhibition of the production of IL-6. The neutralization of the CD40/CD40L interaction during the NKT cell – macrophage interaction eliminated the ability of the NKT cells to inhibit IL-6 production. Secondly, neutralization of GITR also blocked the ability of NKT cells to inhibit IL-6 but only by about 50%. We can conclude that the CD40L is the primary surface protein used by NKT cells to inhibit IL-6.

Figure 13.

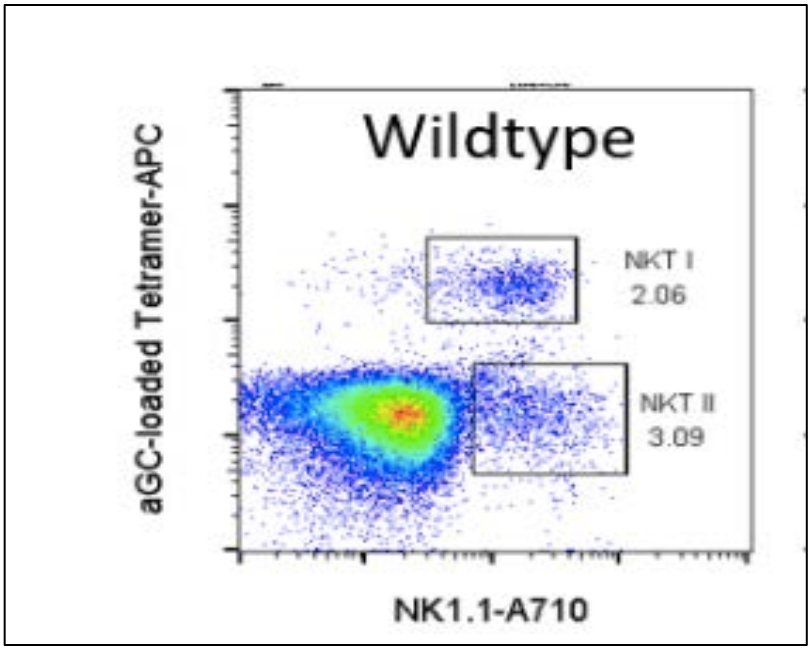

Figure 13. Wildtype mouse spleen stained for NK and T cell populations identify type I and type I NKT cell subsets.

Since the NKT cell population is comprised of two distinct subsets, we sought to determine the role of type I and type II NKT cells independently in the suppression of inflammation. Utilizing FACS sorting, we isolated and recovered these two subsets separately based on their flow cytometric staining (Figure 13).

Figure 14.

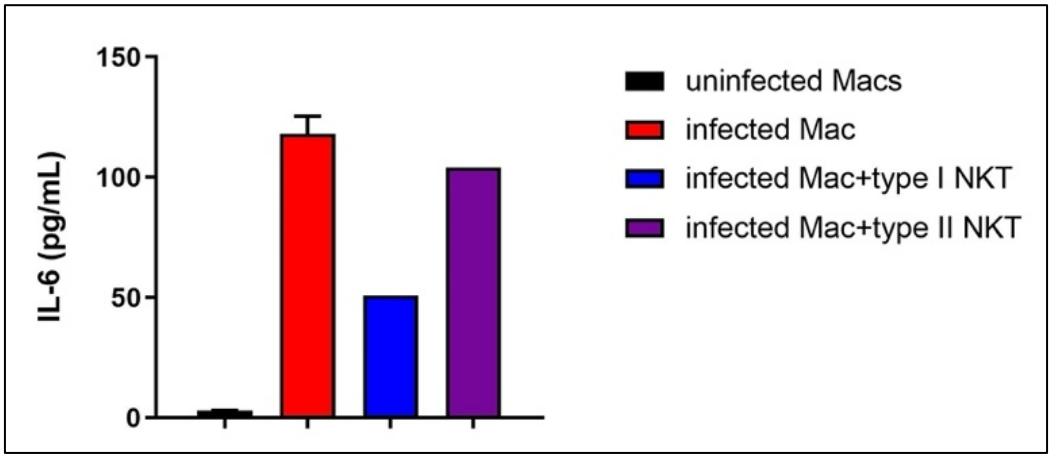

Figure 14. Co-culture of type I and type II NKT cells with LVS-infected macrophages revealed that type I NKT cells are able to inhibit IL-6.

These two populations were purified using the Beckman Coulter MoFlo Astrios cell sorter and independently cultured with LVS-infected macrophages (Figure 14). The inflammatory cytokine IL-6 secreted into the supernatant was measured by ELISA. Infection of macrophages resulted in high production of IL-6. Addition of type I NKT cells suppressed the IL-6 production by about 50% while addition of the type II NKT cells did not suppress the IL-6 production. Therefore, our results showed inhibition of IL-6 in the presence of the type I NKT cells but not type II NKT cells. This result reinforces our earlier result that NKT cells can directly suppress inflammation.

Figure 15.

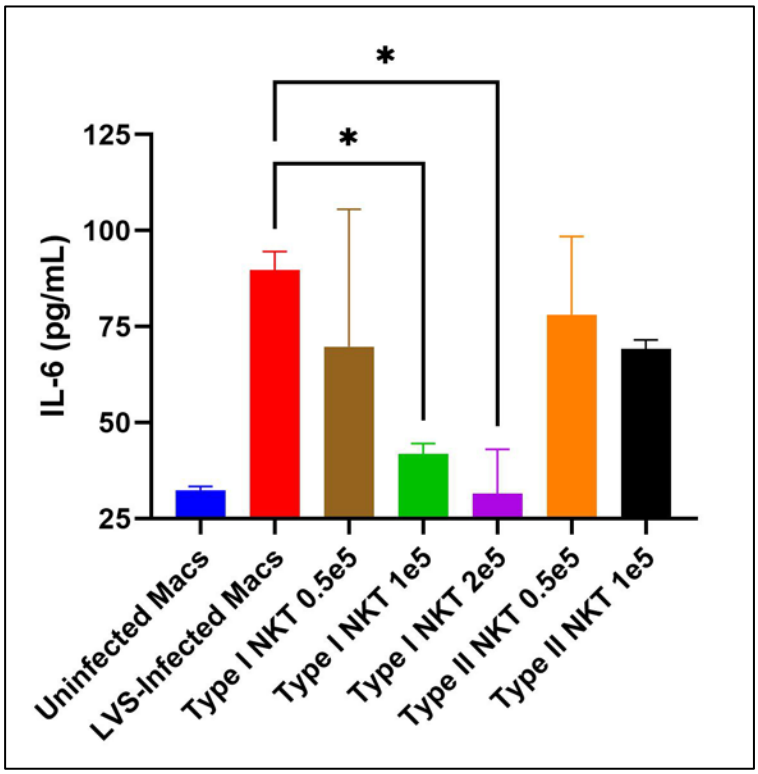

Figure 15. Dose titration of type I and type II NKT cells. Type I NKT cells demonstrated a dose-dependent inhibition of IL-6 secretion. Overall ANOVA p-value < 0.05 and individual p-values < 0.05 by Benjamini, Krieger and Yekutieli False Discovery.

To further demonstrate that this suppression of IL-6 production from infected macrophages was due to the purified NKT cells, we performed a titration of the type I and type II NKT cells (Figure 15). Increasing numbers, 0.5e5/well, 1e5/well, and 2e5/well of separately purified type I or type II NKT cells were added to infected macrophage cultures. The resultant titration showed a dose-dependent inhibition of IL-6 with the highest dose of 2e5/well showing the most suppression, indicating a direct correlation between the type I NKT cell number and inhibition of cytokine secretion. Alternatively, the addition of type II NKT cells did not show any inhibition, although, due to limited numbers of type II NKT cells recovered, we could not test 2e5/well dose.

Chapter 5. Discussion

We know that NKT cells do play a role in suppressing inflammation *in vivo*. To investigate the role of NKT cells in suppressing inflammation, we developed an *in vitro* co-culture assay in which purified NKT cells are cultured with infected macrophages. While this is a reductionist examination of the functioning on NKT cells, it is clear that NKT cells do not normally function in isolation. It is possible that this system lacks important factors provided by other immune cells or responses that may influence the functioning of the NKT cells *in vitro* compared with their functional role *in vivo*. However, this assay showed that NKT cells could directly suppress the production of inflammatory cytokines by macrophages in response to infection.

To further investigate and derive a deeper understanding of the NKT cell activation, it was necessary to further identify the mechanism of activation within an *in vitr*o assay study. Fixation of cells prevents secretion of cytokines and is an accepted method for demonstrating reliance on cell surface receptors. Fixation of NKT cells prior to co-culture with infected macrophages did not affect the ability of bulk purified NKT cells from suppressing Il-6 production. We concluded thence that the suppressive activity is due to surface receptor interactions. However, it is possible that there remains a contribution by secreted cytokines that enhances the suppression of inflammation. Alternatively, it is possible that multiple mechanisms are utilized by different subsets of NKT cells and that only some of these are dependent on surface receptor interaction. Regardless, at least one of these potentially complementary mechanisms is dependent on surface receptor interactions. Through antibody blocking experiments, there is a large dependence on the CD40/CD40L pathway. Future experiments will

need to confirm its involvement as we have not seen an upregulation of CD40L on activated NKT cells *in vivo* (data not shown, CT).

NKT cells can be broadly categorized into two groups: type I or invariant NKT (iNKT) cells and type II NKT cells Both groups are activationally restricted by the MHC class I-like molecule called CD1d. CD1d is a member of a family of CD1 glycoprotein molecules expressed on various antigen-presenting cells (APCs) associated with β_2-microglobulin. In the absence of physiological ligands, alpha-galactosyl-ceramide (α-GalCer), a synthetic glycosphingolipid (GSL) derived from marine sponges, is the most potent known agonist of iNKT cells and an indispensable tool in studying the impact of NKT cell activation on microbial immunity. In contrast to iNKT cells, type II NKT cells are nonresponsive to α-GalCer. Since very little is known of the independent mode of action of the effect of the type I and type II NKT cells in infectious diseases, my results indicate an important role on microbial immunity. [26]

To further investigate and characterize the independent role of the Type I and Type II NKT cells, we isolated the NKT cell subset populations. Using the cell sorter and fluorescent labeling, I isolated the populations and cultured type I and type II NKT cells with infected macrophages. The results show that Type I NKT cells show significant inhibition of Il-6 and would suggest suppression of inflammation. While we did not see significant level of suppression with the type II NKT cells, it is possible that there is a synergistic effect between the Type I and Type II NKT cells. Further Research into understanding type II could possibly reveal this synergy.

To further understand the importance of type I and type II NKT cells in suppression of inflammation, I performed a titration of the type I NKT cells compared with type II NKT cells. Using three different concentrations 0.5e5/ml, 1e5/ml, and 2e5/ml, the increasing titer showed a

dose dependent suppression of the inflammatory marker IL-6. In comparison, the type II NKT cells showed very little suppression, confirming that within the isolated subset, there is no independent suppression of IL-6. As stated earlier, there remains a possibility that any activity of type II NKT cells is dependent on the presence of either type I NKT cells or some other interaction which is not provided in this isolation.

NKT cells can be activated in a TCR-dependent or independent pathway. The TCR-independent pathway is triggered by the simultaneous stimulation of IL-12 and IL-18. It is possible that the TCR-independent activation of type II NKT cells may play a larger role *in vivo* whereas *in vitro* these cytokines are not present (data not shown). It has been suggested that TLR activation of DC may upregulate type II NKT cell ligands presented by CD1d. It therefore seems feasible that like type I NKT cells, type II NKT cells are not limited to activation by TCR-engagement but can also be activated independently of TCR in a pro-inflammatory cytokine milieu. However, this needs to be directly addressed. Thus, type II NKT cells can be activated through the TCR by exogenous antigens, such as microbial lipids, or self-lipids that may be upregulated on CD1d in activated DC. Moreover, they can likely be activated indirectly by pathogen derived or endogenous TLR-ligands acting on DC, or by inflammatory cytokines independently of the TCR. It is likely that under most circumstances, both TCR-engagement and TCR-independent stimulatory signals contribute to type II NKT cell activation. [27]

Transcription factors play an important role in the development of MHC-restricted conventional T cells and their combinations guide the functional profile of these cells. The transcription factor promyelocytic leukemia zinc finger (PLZF), induced by TCR signaling by agonist self-ligands after positive selection, is crucial for the development of type I NKT cells. Type I NKT cells can be sub-grouped into distinct functional subsets based on the combinations

of transcription factors such as PLZF, T-bet and RORγt: NKT1 ($PLZF^{low}T\text{-}bet^{+}$), NKT2 ($PLZF^{hi}T\text{-}bet^{-}$) and NKT17 ($ROR\gamma t^{+}$) cells that secrete TH1-, TH2-, and TH17-like cytokine patterns, respectively, upon activation. Whether type II NKT cells follow similar developmental pathways and can be sub-grouped in similar functional subsets is not yet clearly understood. Studies have found that at least a subset of type II NKT cells have a constitutive production of Il-4 like type I NKT cells and these type II NKT cells could be identified as Il-4-reporter^{+} cells that did not bind the α-GalCer/CD1d-tetramer. [27] Interestingly, we observed the production of Il-4 specifically from co-culture wells containing type I NKT cells that were associated with suppression. However, direct addition of Il-4 to infected macrophages has yet to show any effect on the production of Il-6. It is possible that this NKT cell-produced Il-4 contributes to the suppression or biasing of the macrophage response that is dependent on cell-cell interactions.

5.1 Significance

This research has allowed us to establish the involvement of NKT cell subsets in the suppression of inflammation. To understand the mechanism of suppression, we sought to determine the subset of NKT cells responsible for suppression of inflammation and whether it is dependent on cell-cell contact or secreted effector molecules. My studies have shown the type I NKT cells directly suppress the ability of infected macrophages to produce IL-6. This suppression utilizes cell-cell contact dependent mechanism, in a bulk purified population. Having determined how NKT cells suppress inflammation induced by *F. tularensis* infection, future research will seek to specifically activate these suppressive NKT cell subsets to control inflammation. Since this future immunotherapeutic therapy would focus on controlling inflammation, it should be applicable to other inflammatory conditions as well. These findings will eventually lead to further development of treatment who have chronic inflammation and auto immune diseases. Overall it will lead to a person life span being elongated with a better quality of life in that duration of chronic illness.

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Appendix

List of publications and manuscripts

1. Ramos Muniz, M. G., Palfreeman, M., **Setzu, N**., Sanchez, M. **A**., Saenz Portillo, P., Garza, K. M., Gosselink, K. L., … Spencer, C. T. (2018). Obesity Exacerbates the Cytokine Storm Elicited by *Francisella tularensis* Infection of Females and Is Associated with Increased Mortality. *BioMed research international*, *2018*, 3412732. doi:10.1155/2018/3412732.
2. One manuscript in preparation.

Vita

Nicole R. Setzu was born and raised in El Paso, Texas October 25,1993. She graduated with a Bachelor of Science degree in Microbiology from The University of Texas at El Paso on December 2017 at El Paso, Texas. She immediately entered the Master of Science program at the University of Texas at El Paso and began August 2018. Nicole has been under the mentorship of Dr. Charles T. Spencer for the last eight years. Her research has focused on immunological responses and concepts of suppressing inflammation by Natural Killer T cells induced by *Francisella tularesnsis.*

Furthermore, she has one co-author manuscript and one more in preparation, she has received several fellowship awards and was nominated as an undergrad for Academic and Research Excellence Undergraduate Student in Microbiology. She has presented her research at national and international conferences. After, graduation she will continue her education in a doctoral program to continue to study research in Infectious Disease.

This Thesis Was Written by Nicole R. Setzu