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**INNATE AND ADAPTIVE IMMUNITY TO *BORDETELLA PERTUSSIS* AND
*FRANCISELLA TULARENSIS***

A Dissertation in
Immunology and Infectious Diseases
by
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ABSTRACT

Respiratory infections by bacteria are the cause of significant deaths throughout the world. By understanding how different bacteria interact with the innate and adaptive immune systems of the hosts they infect, we can gain insight in how to better treat, vaccinate, and prevent the diseases they cause. In this dissertation the focus will be on two different bacterial pathogens which are often focused on in terms of respiratory infection, *Bordetella pertussis* and *Francisella tularensis*. Chapter 2 investigates the role of the inflammasome signaling complex and IL-1 signaling during interactions with *B. pertussis* and shows that the IL-1 response in the host, while required, does not require signaling through the inflammasome in the mouse model. In Chapter 3 we describe the development of a novel tool for studying CD8 T cell responses to *F. tularensis* LVS following vaccination by intranasal inhalation. Because *Francisella* can infect the respiratory tract as well as naturally infect through the intradermal route, which is the route by which humans are vaccinated, Chapter 4 reviews the currently limited immune responses generated locally within the skin. By understanding these various aspects of innate and adaptive immunity to each pathogen, we begin to develop an understanding of how both arms of the immune system interact to protect hosts during both primary and secondary challenges.

TABLE OF CONTENTS

List of Figures	vi
List of Tables	vii
Abbreviations	viii
Chapter 1 Introduction: The Host-Pathogen Interaction of <i>Bordetella pertussis</i> and <i>Francisella tularensis</i>	1
1.1 <i>Bordetella pertussis</i>	2
1.1.1 <i>Bordetella pertussis</i> Host-Pathogen Interactions.....	2
1.1.2 <i>B. pertussis</i> Immunity	3
1.2 <i>Francisella tularensis</i>	5
1.2.1 <i>Francisella tularensis</i> Host-Pathogen Interactions.....	5
1.2.2 <i>F. tularensis</i> Immunity.....	6
1.3 References.....	8
Chapter 2 Caspase-1-independent interleukin-1 β is required for clearance of <i>Bordetella pertussis</i> infections and whole-cell vaccine-mediated immunity	12
2.1 Abstract	13
2.2 Introduction.....	14
2.3 Materials and Methods.....	17
2.4 Results.....	21
2.5 Discussion	26
2.6 Author Contributions	37
2.7 References.....	38
Chapter 3 Development of a Novel <i>Francisella tularensis</i> Live Vaccine Strain Expressing Ovalbumin Reveals Memory and Effector CD8 T Cell Responses During Intranasal Vaccination and Re-challenge.....	43
3.1 Abstract	44
3.2 Introduction.....	45
3.3 Materials and Methods.....	48
3.4 Results.....	53
3.5 Discussion	59
3.6 Author Contributions	73
3.7 References.....	74
Chapter 4 Review of Intradermal Immune Responses to <i>Francisella tularensis</i> Live Vaccine Strain.....	79
4.1 Overview	80
4.2 Vector-borne Intradermal Infection	80

4.3 Intra-dermal Immunity Models for LVS	81
4.4 Future Perspective	85
4.5 References	86
Chapter 5 Summary and Discussion	91
5.1 References	95

LIST OF FIGURES

Figure 1.1. Bacterial clearance is mediated by IL-1 β and IL-1R but not IL-1 α	31
Figure 1.2. IL-1 signaling limits lung inflammation during <i>B. pertussis</i> infection	32
Figure 1.3. IL-1 is not required for recruitment of immune cells to the lung	33
Figure 1.4. IL-1 β production is caspase-1 dependent in vitro but independent in vivo	34
Figure 1.5. Adaptive immune responses are IL-1 dependent but Caspase-1 independent	35
Figure 1.S1. Lung pathology scoring of <i>B. pertussis</i> infection	36
Figure 2.1 Development of a stable LVS-OVA expression construct in LVS	62
Figure 2.2 OT-I cells expand during infection with LVS-OVA.....	63
Figure 2.3 OT-I cell kinetics through LVS-OVA infection	64
Figure 2.4 Memory markers through LVS-OVA infection	65
Figure 2.5 Intracellular cytokine staining of OT-I cells	66
Figure 2.6 Memory T cell response upon re-challenge with LVS-OVA.....	67
Figure S2.1 Stability of pKK214-vgrG-OVA without selection in macrophages	70
Figure S2.2 Stability of pKK214 without selection in liquid culture	71
Figure S2.3 Intracellular cytokine staining of endogenous CD4 T cells	72
Figure 3.1 IL-1 is required for survival of OT-I T cells	94

LIST OF TABLES

Table 1.1 Summary of bacterial strains, plasmids, primers, and synthetic DNA used in chapter 3	68
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ABBREVIATIONS

ACT	Adenylate cyclase toxin
AIM2	Absent in melanoma 2
ANOVA	Analysis of variance
aP	Acellular pertussis vaccine
ATP	Adenosine triphosphate
BG	Bordet-Gengou
BMDC	Bone-marrow derived dendritic cells
BMDM	Bone-marrow derived macrophages
cAMP	Cyclic adenosine monophosphate
CD	Cluster of differentiation
CFSE	Carboxyfluorescein succinimidyl ester
CFU	Colony forming unit
CpG	Unmethylated Cytosine triphosphate - phosphodiester -guanine triphosphate oligodeoxynucleotides
DC	Dendritic cell
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FPI	<i>Francisella</i> pathogenicity island
FSC	Forward scatter
GFP	Green fluorescent protein
GTP	Guanine triphosphate
H&E	Hematoxylin and eosin stain
HBSS	Hanks buffered saline solution
HRP	Horseradish peroxidase
i.d.	intra-dermal
IFN	interferon
Ig	Immunoglobulin
IGEPAL	octylphenoxypolyethoxyethanol
IL	Interleukin
IL-1R	Interleukin-1 receptor
i.n.	intra-nasal
i.p.	intra-peritoneal
kDa	kilodalton
LPS	Lipopolysaccharide
LVS	<i>Francisella</i> Live vaccine strain
M-CSF	Macrophage colony stimulating factor
MHA	Mueller-Hinton Agar
MLN	Mediastinal lymph node
MMP	Metalloproteinase

MOI	Multiplicity of infection
NF- κ B	Nuclear factor kappa B
NK	Natural killer cell
NLR	NOD-like receptor
NOD	Nucleotide-binding oligomerization domain
NLRP3	NLR family, pyrin domain containing 3
OT	Ovalbumin transgenic
OVA	Ovalbumin
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PTX	Pertussis toxin
PVDF	Polyvinylidene fluoride
RBC	Red blood cells
RIPA	Radioimmunoprecipitation assay buffer
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute media
SSC	Side scatter
TCR	T cell receptor
Th	T helper subset
TLR	Toll-like receptor
TNF	Tumor necrosis factor
wP	Whole-cell pertussis vaccine
WT	Wild-type

Chapter 1

Introduction to *Bordetella pertussis* and *Francisella tularensis*

1.1 *Bordetella pertussis*

1.1.1 *Bordetella pertussis* Host-Pathogen Interactions

Bordetella pertussis, first isolated in 1906 by Bordet and Gengou as the causative agent of a disease characterized by a distinct coughing fit, continues to cause significant disease to this day, despite the availability of vaccines [1]. The host-range of *B. pertussis* is limited to humans, though close relatives of the genus are known to infect a broad range of mammals including sheep, pigs, and mice. Because *B. pertussis* can infect mice and closely related members of the genus naturally infect mice, mouse models represent a useful tool for studying the immune interactions this pathogen has with its host [2].

As with many other pathogens, *B. pertussis* has a number of virulence factors it produces to modulate the host immune response. A number of toxins are produced, including the potent pertussis toxin (PTX), which is secreted by a type IV secretion system. This multi-subunit toxin enters the host cell cytosol and ribosylates the α -subunit of heterotrimeric G-proteins, affecting multiple downstream signaling pathways [3]. As a potent toxin that causes many of the disease symptoms during infection, the acellular pertussis vaccine contains PTX as one of the components, and has been shown to be sufficient as a single-component vaccine [4]. As an important virulence factor, PTX has been associated with modulating a number of host inflammatory pathways. Initially during infection, PTX appears to be important in protecting bacteria from alveolar macrophages [5]. Another important role for PTX is in the inhibition of neutrophil recruitment to the infected airways through inhibition of chemokine expression in resident lung cells [3,6]. The host immune response is ultimately able to overcome the effects of PTX through production of multiple cytokines including TNF α , IFN γ , IL-17, and IL-1 [6–9].

Another virulence factor produced by *B. pertussis* is adenylate cyclase toxin (ACT), which is a pore-forming toxin with enzymatic activity responsible for increasing cAMP levels in host cells [3]. Because of changes in cAMP, ACT can disrupt cytoskeletal rearrangements by the GTPase RhoA, inhibiting complement-mediated phagocytosis and micropinocytosis [10]. Through its pore-forming activity, ACT has also been shown to activate the caspase-1 inflammasome through NLRP3 in dendritic cells, as a result of an influx of extracellular K⁺ ions [11].

Because *B. pertussis* ACT was shown to activate caspase-1 *in vitro* and the IL-1R was important for clearance of *B. pertussis* from the respiratory tract of mice, it was suggested that the inflammasome was important for the generation of IL-1 β *in vivo* and that this led to protective Th17 responses during infection [11]. It was also shown that IL-1R signaling was required for overcoming the effects of PTX and for generation of immunity following vaccination with the acellular pertussis vaccine, which contains aluminum hydroxide (alum) adjuvant that acts through the NLRP3-containing inflammasome [9,12]. This led to the work in chapter 2 investigating the initiation of the IL-1 response *in vivo*, which we learned did not require the caspase-1 inflammasome but did require the component IL-1 β [13]. We also showed that similar to acellular vaccines, the whole-cell vaccine requires IL-1R signaling but that this also can be achieved in a caspase-1-independent fashion [13].

1.1.2 *Bordetella pertussis* Immunity

Though there are vaccines for *Bordetella pertussis* in developed countries, the disease has recently begun to re-emerge. Many factors have been suggested to contribute to this re-emergence including changes in the pathogen as a result of vaccine-mediated selection pressures, waning immunity, and changes from whole killed bacterial vaccines (wP) to acellular component

vaccines (aP) [14,15]. The wP vaccine, introduced in the 1950s, was effective in protecting people from *B. pertussis* and responsible for dramatic reductions in disease incidence [16,17]. Due to side-effects, the wP vaccine was replaced by safer acellular vaccines containing two or more *B. pertussis* antigens such as PTX, ACT, filamentous hemagglutinin (FHA), fimbriae, and pertactin [15]. While this acellular vaccine effectively protects children from severe disease, the incidence of *B. pertussis* has increased in adolescents and adults [18]. New research also shows that the aP vaccine fails to prevent transient infection and transmission by *B. pertussis* in non-human primates [19]. These problems highlight that we poorly understand the different immune responses each vaccine provides against *B. pertussis*.

While aP-vaccines and wP-vaccines both generate antibody responses and protect against severe disease, much of the research lately has been in studying the differences each vaccine has on generation of T cell mediated memory responses [15,19]. Renewed interest in the different innate immune responses to these different vaccines has led to experiments testing different adjuvants and new, attenuated vaccines which would skew the balance of Th1/Th2/Th17 responses [20]. Vaccination with wP vaccine, for example, has been shown to induce stronger Th1 and Th17 responses than aP vaccination, which induces a Th2/Th17 response [21]. A new live-attenuated *B. pertussis* vaccine, BPZE1, has also been developed and shown to generate Th1/Th17 responses and more rapid protection as compared with aP vaccine [22]. Another approach being taken is inclusion of CpG oligodeoxynucleotide adjuvants with acellular vaccine components, which skews immune responses towards Th1/Th17 production [21,23]. The generation of protective Th17 cells was also shown to require IL-1 β , which highlights the importance of this innate pro-inflammatory cytokine in directing T cell responses.

1.2 *Francisella tularensis*

1.2.1 *Francisella tularensis* Host-Pathogen Interactions

In contrast to *B. pertussis*, which is primarily an extracellular bacteria, *F. tularensis* strains are generally considered to replicate intracellularly. Within the *Francisella tularensis* species, there are four primary subspecies. Two of these subspecies, *F. tularensis mediasiatica* and *novicida* do not cause disease in humans [24]. Two other subspecies, *F. tularensis tularensis* (type A strains) and *F. tularensis holarctica* (type B strains) are able to infect humans following exposure to a very small number of bacteria (<15 bacteria), though in general type B strains are considered less virulent in humans and provide the basis for the live-attenuated vaccine strain, LVS [24]. Because of the high levels of mortality during aerosol exposure in untreated individuals, *F. tularensis* has been developed as a bioweapon in the past [25]. After recent anthrax attacks in the United States in 2001, there was renewed interest in developing a vaccine for *F. tularensis* [25].

Though able to infect a number of different cell types, the primary cells infected *in vivo* include antigen-presenting cells and phagocytes such as macrophages and dendritic cells [26–30]. Following entry into phagocytic cells through a unique looping phagocytosis mechanism, the bacteria escapes the phagosomal compartment and enters the cytosol where it begins its replicative cycle [31,32]. Though TLR2 is required for recognition of *F. tularensis*, the bacteria possess a number of immunosuppressive virulence factors which prevent cytokine production in the early days of infection, many of which are present on a duplicated, highly conserved AT-rich *Francisella* pathogenicity island (FPI) containing 16-19 genes [26,33,34]. The regulation of these genes occurs through the master regulator MglA and many of these genes are associated with the type VI secretion system [34]. Included within this FPI is *vgrG*, a secreted effector of

the type VI secretion system that is required for full virulence of *F. tularensis* and is used later in this thesis for tagging with ovalbumin epitopes [35,36].

Though *F. tularensis* delays production of inflammatory cytokines, eventually this is overcome by the immune system. LVS has been shown to both suppress and activate TLR2 and the AIM2-containing inflammasome, which leads to production of mature IL-1 β and IL-18, which are required for protection from *F. tularensis* LVS [37–39]. While IL-18 appears to be required for the production of early IFN γ responses, the role for IL-1 β appears to be involved in production of anti-*Francisella* LPS natural IgMs later in the infection [37]. Whether IL-1 β is involved in other aspects of adaptive immunity to *F. tularensis* remains an open question.

1.2.2 *Francisella tularensis* Immunity

Relative to other intracellular pathogens, the known immune correlates of protection are poorly described. Additionally, because the bacteria can be introduced through multiple different routes, have different outcomes in different strains of mice, and multiple strains of *F. tularensis* exist, there are many conflicting claims for which immune mediators are required for protection following infection [40]. Here we will focus on the immunology associated with infection by the *F. tularensis* live vaccine strain (LVS) and virulent type A strains of *F. tularensis* introduced by the intranasal route, which is of concern for weaponized bacteria. Later, in chapter 4, we focus on what is known about the dermal route of exposure to these bacteria.

Following introduction of the bacteria into the respiratory tract, alveolar macrophages take up bacteria [30]. The most well characterized cytokine involved in protection to *Francisella* is IFN γ , which activates phagocytic cells and increases reactive oxygen species (ROS) production to limit replication of *F. tularensis* [41,42]. LVS, as an attenuated strain, is more

susceptible to killing by ROS than the type A strain SchuS4, which possesses enzymes that degrade ROS [42]. Initially, IFN γ is primarily produced by natural killer (NK) cells, which are recruited by 72hr post-inoculation [43]. Additional immune mediators such as TNF α , reactive nitrogen intermediates, TLR2, and the inflammasome have been identified [40]. The role of type I interferon, such as IFN β , remains complicated, as it has been implicated in both the inhibiting gamma delta T cell production of IL-17A and also important for activating the inflammasome during infection [44,45]. Neutrophils seem to be involved with much of the pathology associated with intranasal *F. tularensis* infection and required for survival of intravenous and intradermal infection, but they do not seem to be required for protection from infections [46,47].

Many intracellular pathogens require cell-mediated immunity to kill infected host cells. T cells are associated with protection to challenges with virulent *F. tularensis* through prevention of dissemination of bacteria from the respiratory tract, though this is still poorly understood [48]. IFN γ production is required for protection of primary and secondary challenges with LVS and virulent *F. tularensis* [49]. Though IL-17 protects against primary infection with LVS, it does not seem to be required for protection against LVS secondary challenge or primary challenge with virulent *F. tularensis* [50]. Understanding how different T cells develop during primary infection and provide memory responses to secondary challenge is focused on in chapter 3. Advances in our understanding of how T cell memory develops against *F. tularensis* is likely required for successful vaccines against tularemia.

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Chapter 2

Caspase-1-Independent Interleukin-1 β Is Required for Clearance of *Bordetella pertussis* Infections and Whole-Cell Vaccine-Mediated Immunity

2.1 ABSTRACT

Whooping cough remains a significant disease worldwide and its re-emergence in highly vaccinated populations has been attributed to a combination of imperfect vaccines and evolution of the pathogen. The focus of this study was to examine the role of IL-1 α/β and the inflammasome in generation of the interleukin-1 (IL-1) response, which is required for the clearance of *Bordetella pertussis*. We show that IL-1 β but not IL-1 α is required for mediating the clearance of *B. pertussis* from the lungs of mice. We further found that IL-1 β and IL-1R deficient mice, compared to wild-type, have similar but more persistent levels of inflammation, characterized by immune cell infiltration, with significantly increased IFN γ and a normal IL-17A response during *B. pertussis* infection. Contrary to expectations, the cleavage of precursor IL-1 β to its mature form did not require caspase-1 during primary infections within the lung despite being required by bone marrow-derived macrophages exposed to live bacteria. We also found that the caspase-1 inflammasome was not required for protective immunity against a *B. pertussis* challenge following vaccination with heat-killed whole cell *B. pertussis*, despite IL-1R signaling being required. These findings demonstrate that caspase-1-independent host factors are involved in the processing of protective IL-1 β responses that are critical for bacterial clearance and vaccine-mediated immunity.

2.2 Introduction

One of the critical initial mediators of inflammation is interleukin-1 (IL-1), which has pleiotropic effects in the body. Initially discovered as an immune component involved in induction of fever responses, the role of this cytokine has expanded to include chemokine induction, vascular permeability, expression of adhesion molecules, T cell differentiation, and has further been implicated in a number of pathologic conditions [1]–[6]. The requirement for IL-1 during infections has been studied in multiple infection models including *Shigella flexneri*, *Salmonella enterica*, *Staphylococcus aureus*, *Candida albicans*, influenza and *Bordetella pertussis* [7]–[12]. IL-1 mediated protection against extracellular pathogens often occurs through the production of IL-6, which polarizes CD4⁺ T cells to become IL-17-secreting Th17 cells, which can recruit neutrophils to the site of infection and mediate bacterial killing [13], [14]. For example, *B. pertussis* infection leads to the recruitment of specific Th17 cells that were recently shown to be dependent upon IL-1R signaling [10], [15], [16].

The most well characterized members of the IL-1 family include IL-1 α , IL-1 β , IL-18, and IL-33. The latter three cytokines are processed into their active form by the caspase-1 containing inflammasome complex [11], [17]. The IL-1 α and IL-1 β molecules both bind the IL-1R and exert similar downstream activities; however, their tissue localization and methods of activation differ, suggesting they may have distinct roles in immunity [1].

The induction of IL-1 is tightly regulated and requires two activation signals to produce the active IL-1 α and IL-1 β . During infection, expression of IL-1 β is often induced downstream of the Toll-like receptors through translocation of the transcription factor NF- κ B [18]. Following expression of the precursor proteins in the cytosol, a second signal is often required to cleave the proteins into their active mature forms [19], [20]. In the case of IL-1 α , a calcium-activated

cysteine protease, calpain, cleaves the precursor into its active form. Additionally, the IL-1 α precursor has been shown to translocate to the nucleus to regulate expression of IL-6, IL-8, TNF α and IFN γ or can be released from necrotic cells as a danger signal [21]–[24]. The processing of IL-1 β is regulated by the inflammasome, a complex of proteins including an adapter protein, ASC, and Nod-like receptors (NLRs) that, upon stimulation, assembles and activates pro-caspase-1 [17], [25]. Following activation, the cysteine protease caspase-1 cleaves the 31 kDa pro-IL-1 β into its active 17 kDa form [26]. Recently, NLRP3 was shown to be a key component of the inflammasome, which is activated by the pore-forming *Bordetella pertussis* adenylate cyclase toxin through K⁺ efflux. Because the IL-1R signaling pathway is required for clearance of *B. pertussis* and rapid vaccine-mediated immunity, it was suggested that NLRP3-inflammasome activation of IL-1 β would be required for controlling infection [10], [27].

Given that *B. pertussis* infections are highly transmissible, affecting 50 million people worldwide every year and re-emerging in many countries as a result of adaptation, and the limitations of the current generation of vaccines, it is critical to understand how the immune system processes IL-1 during infection by this particular respiratory pathogen [28]–[30]. In this study we focus on the factors upstream of the IL-1R during *B. pertussis* infection. Here we have found that IL-1 β was required for clearance of *B. pertussis* and could be processed into its active form in a caspase-1 independent manner in the murine lung. Our vaccination results also show that IL-1 related signaling is important for protective immunity to *B. pertussis* and can be initiated independently from caspase-1, which has been shown to be activated by the current alum adjuvant in acellular pertussis vaccines. Future studies looking at alternative cleavage mechanisms will help to identify how IL-1 β is processed during infection. Increasing our understanding of the regulation

and function of IL-1 signaling against respiratory pathogens has broad implications for control of disease and improvement in vaccine design.

2.3 MATERIALS AND METHODS

Mouse Strains

C57BL/6J, IL-1R^{-/-} (B6.129S7-*Il1r1*^{tm1Imx}/J), and IL-18^{-/-} (B6.129P2-*Il18*^{tm1Aki}/J) mice were purchased from The Jackson Laboratories (Bar Harbor, ME). Caspase-1^{-/-}11^{-/-} mice were obtained from Dr. Richard A. Flavell (Yale School of Medicine). IL-1 α (B6.129-*Il1a*^{tm1Yiw}) and IL-1 β -deficient mice (B6.129-*Il1b*^{tm1Yiw}) on a C57BL/6J background were provided by Dr. Lloyd S. Miller (University of California, Los Angeles) and originally produced by Dr. Yoichiro Iwakura [31]. All mice were maintained in specific pathogen-free conditions at the Pennsylvania State University animal care facilities.

Bacterial Strains and Cell Lines

Bordetella pertussis infections were performed using the streptomycin-resistant derivative of Tohama I strain 536 [32]. Bacteria were grown on Bordet-Gengou (BG) agar (HiMedia) supplemented with 15% defibrinated sheep blood (Hema Resources & Supply, Inc.) and 20 μ g/mL streptomycin (Sigma-Aldrich) or shaking overnight in Stainer-Scholte broth supplemented with 500 μ g/mL heptakis (Sigma-Aldrich) at 37°C [33].

Bone marrow-derived macrophages (BMDMs) were generated from bone marrow isolated from the femur and tibia as previously described, with minor modifications [34], [35]. Briefly, bones were crushed in a 70 μ m nylon cell strainer (BD Biosciences), pelleted and resuspended before seeding plates. Cells were grown on bacterial grade petri dishes in 10 mL DMEM containing 2 mM GlutaMAX, 1 mM HEPES (Life Technologies), 10% FCS (Gibco, HyClone) and 20% M-CSF containing L929 cell supernatant. An additional 5 mL of medium was added at day 3, and

cells were collected on day 6 with 1 mM PBS-EDTA and seeded into 24-well tissue culture-treated plates overnight.

Mouse Infection

Mice were lightly anesthetized with isoflurane (Abbott Laboratories) and bacteria (5×10^5 CFU) were delivered into the respiratory tract in a 50 μ L droplet of sterile phosphate-buffered saline (PBS) via the external nares. Mice were euthanized by carbon dioxide inhalation and dissected in a biosafety cabinet with aseptic technique. Serial dilutions of the inoculum were plated on BG-blood agar containing 20 μ g/mL streptomycin incubated for 4 days at 37°C to confirm the infection dose and lungs were homogenized in ice-cold sterile PBS at 30 minutes post-inoculation for day 0. Similarly, at indicated time points post-inoculation, lungs were excised and homogenized in 1 mL of sterile PBS. Aliquots were taken from this homogenate and either centrifuged and stored at -20°C for ELISAs and immunoblots or serially diluted ten-fold in sterile PBS with 100 μ L plated for CFU counts to determine bacterial colonization with a limit of detection of 10 bacteria per lung.

Flow Cytometry

Mouse lungs were collected from euthanized mice in 5 mL DMEM with 10% FCS and passed through a 70 μ m cell strainer using a 3 mL syringe plunger. Cells were pelleted and resuspended in 2 mL 0.84% ammonium chloride to lyse red blood cells. Cells were blocked with anti-CD16/32 (93) and stained with fluorophore-conjugated CD45 (30-F11), CD3 (17A2), CD4 (GK1.5), CD8a (53-6.7), CD11b (M1/70), Ly6G (1A8), and F4/80 (BM8) from BioLegend and BD Biosciences, fixed in 2% paraformaldehyde, and analyzed on a BD LSR II flow cytometer.

All analyzed cell populations were gated on FSC-A/FSC-H to exclude doublets and on CD45⁺ cells. Analysis was performed with FlowJo (Tree Star, Inc.).

Histology

Mouse lungs were inflated with approximately 2 mL formalin intratracheally, collected in 5 mL formalin, embedded in paraffin wax and sectioned in 5 μ m slices, and stained with hematoxylin and eosin (H&E) at the Huck Microscopy & Histology Core Facilities at The Pennsylvania State University. Two blinded, independent scorings were performed on histology sections based on previously established criteria including peribronchiolar inflammation, with percent bronchioles and degree of inflammation scored separately, perivascular inflammation, and intra-alveolar inflammation [36]. Images were collected with an Olympus BX51 scope with ProGres software.

Cell culture infections

Bone marrow-derived macrophages were seeded in 24-well or 6-well tissue culture treated plates (Costar) and allowed to adhere overnight at 37°C in 5% CO₂. Assay solutions were freshly prepared. Cells were washed and indicated wells were primed with 100 ng/mL *E. coli* 011:B4 LPS (Sigma-Aldrich) for three hours. After three hours, all wells were aspirated and replenished with fresh media, media containing 5 mM ATP (MPBiomedicals), or media containing live *B. pertussis* diluted from overnight cultures. Prior to collection of supernatant, plates were centrifuged at 500 \times g for 5 minutes.

ELISAs and Immunoblots

Mouse lungs were collected in ice-cold PBS, homogenized, centrifuged, and supernatant was removed and frozen at -20°C until analysis. Cell culture supernatants were collected by spinning down plates at 500 \times g for 5 minutes and carefully pipetted into fresh plates or cell lysates were

lysed in RIPA buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 1% IGEPAL, 0.5% sodium deoxycholate) and stored at -20°C . For measuring IL-1 β and IL-10, OptEIA kits (BD Biosciences) were used according to manufacturer recommendations. IL-17 and IFN γ were measured by paired capture/detection antibodies (R&D Systems) or the DuoSet ELISA Development System (R&D Systems). Standard curves were generated with a 4-parameter logistic curve-fit and optical density (450 nm–570 nm) was interpolated to determine concentration using Gen 5 software and a Biotek Epoch Plate Reader.

For immunoblotting, lung homogenates or cell culture lysates were diluted in 2X Laemmli sample buffer (BioRad). Cell culture supernatants were concentrated by a methanol/chloroform precipitation method described previously [10]. Samples were boiled 5 minutes, run on a 12% TGX pre-cast gel and transferred to a polyvinylidene difluoride (PVDF) membrane with the Trans-Blot Turbo mixed molecular weight program (BioRad) and blocked in 5% bovine serum albumin (Sigma-Aldrich) prior to detection with Abcam anti-IL-1 β (ab9722) and secondary goat anti-rabbit-HRP (Jackson ImmunoResearch) utilizing the Immobilon chemiluminescence detection reagent (Millipore). Images were taken with the ChemiDoc XRS+ System (BioRad).

Vaccination

Heat-killed bacteria vaccine was generated from overnight cultures of *B. pertussis* strain 536 following growth in Stainer-Scholte medium. Bacteria were washed in PBS and diluted to 10^9 bacteria/mL and incubated at 65°C for 30 minutes to heat-kill bacteria which was confirmed by plating on BG-blood agar. Vaccine was stored at -20°C before use. Vaccination was performed by intraperitoneal injection of 200 μL 28 and 14 days prior to the challenge infection (5×10^5 CFU).

Statistical Analyses

Significant differences between groups were determined using Student's t-test, one-way ANOVA with Dunnett's or Tukey's post-hoc test or the nonparametric Kruskal Wallis test with Dunn's post-hoc test for significance using GraphPad Prism with p values <0.05 considered significant.

Ethics Statement

All animal experiments were carried out by following recommendations and approval from the Pennsylvania State University Animal Care and Use Committee (protocols 39724 & 40029) with great care taken to minimize suffering of animals.

2.4 RESULTS

IL-1 β is required for efficient clearance of *B. pertussis*

Respiratory tract infection by *B. pertussis* induces the production of critical inflammatory mediators including IL-1 [37]. The importance of IL-1 during *B. pertussis* infections to date has been ascribed to promoting development of IL-17 producing CD4⁺ T helper (Th17) cells and the recruitment and activation of neutrophils and macrophages [27], [38], [39].

While both IL-1 α and IL-1 β are produced in the lungs during *B. pertussis* infections, the roles of these specific molecules in the clearance of the bacteria has not been determined [10], [27], [40]. To define the roles of each subtype of IL-1, we inoculated *B. pertussis* by the intranasal route into wild-type mice and mice lacking IL-1 α , IL-1 β , or IL-1R. At days 3, 7, 14, and 28 post-inoculation, bacterial CFUs were determined from excised lungs. Early during the infection, no significant differences in bacterial load were observed between wild-type and the

knockout mice ([Figure 1](#)). However, by day 14 post-inoculation, the bacterial burden recovered from IL-1 α , IL-1 β , and IL-1R deficient mice was significantly higher than that of wild-type mice (3.9-fold, 8.7-fold, and 4.0-fold higher than wild-type, respectively). On day 28 post-inoculation, the difference between wild-type and IL-1 α deficient mice was no longer apparent; however, both the IL-1 β (>220-fold higher than wild-type) and IL-1R deficient mice (>280-fold higher than wild-type) remained very significantly colonized ($p < 0.01$) by *B. pertussis* ([Figure 1](#)). These data suggest that IL-1R-mediated signaling requires IL-1 β but not IL-1 α to efficiently clear *B. pertussis* infection.

Increased inflammation in IL-1 deficient mice

Given the critical role that IL-1 plays in initiating inflammation during infection we hypothesized that IL-1 deficient mice would have significantly higher bacterial-mediated tissue pathology in the lungs. Hematoxylin and eosin (H&E) stained sections of lungs of wild-type, IL-1 α deficient, IL-1 β deficient, and IL-1R deficient mice were analyzed in a blinded fashion ([Figure 2A–L](#)). On day seven post-inoculation, all examined mice showed pronounced bronchopneumonia ([Figure 2E–H](#)). By day 28, wild-type and IL-1 α deficient lungs showed reduced alveolar exudate and fewer inflamed bronchioles suggesting resolution of inflammation consistent with the lower bacterial numbers in these mice. However, the majority of bronchioles of both IL-1 β and IL-1R deficient mice remained inflamed with diffuse bronchopneumonia ([Figure 2I–L, S1](#)). Particularly, the intra-alveolar inflammation in the lungs of IL-1 β deficient and IL-1R deficient mice was markedly higher than that of wild-type mice. This suggests that IL-1R mediated signaling is not required for initiation and establishment of inflammation in the lung but may be required for subsequent resolution of inflammation.

A recent report examining the contribution of Th1 and Th17 cells in adaptive immunity to *B. pertussis* showed involvement of both T helper subsets in protective immunity following primary infection and whole-cell vaccination [41]. To determine whether these responses were dependent on IL-1R signaling, as was suggested in previous publications, we measured lung cytokine responses during infection [10], [42]. Our data show that primary infection induces significantly higher amounts of IFN γ in IL-1 β and IL-1R deficient mice than in wild-type mice on day 14 post-inoculation. We did not detect any significant difference in the amount of IL-17A produced by wild-type, IL-1 β deficient and IL-1R deficient mice (Figure 2M–N). It should be noted, however, that a trend (IL-1 β ^{-/-} p=0.08, IL-1R^{-/-} p=0.4) toward lower IL-17A was observed in the IL-1 deficient mice (Figure 2M). These findings demonstrate that primary infection is capable of producing potent pro-inflammatory responses independent of IL-1R signaling but that these responses are not sufficient for normal clearance of *B. pertussis*.

IL-1 is not required for recruitment of immune cells to lung

During *B. pertussis* infection, initial inflammatory events lead to the recruitment of macrophage and dendritic cell populations followed by a peak of neutrophils approximately seven days post-inoculation, when bacterial numbers are also highest [37]. At this time point bacterial numbers begin to decline and macrophages and neutrophils present in the lungs begin to be replaced by CD4⁺ T cells as the adaptive immune response develops and spurs the production of IgA and IgG which are involved in clearance of *B. pertussis* [37]. To investigate whether IL-1 is required for the differences in cells present during infection, we inoculated mice with *B. pertussis* and collected the total lung homogenate and analyzed T cell subsets, neutrophils, and macrophages by flow cytometry. CD8⁺ T cells comprised 30–40% of total immune cells and were not increased by *B. pertussis* infection. In contrast, CD4⁺ T cells increased in proportion

from approximately 20% to approximately 60% of total immune cells in wild type, IL-1 β -deficient and IL-1R-deficient mice (Figure 3A, B). Except for day 7 post-inoculation in the IL-1 β -deficient mice, no significant defects were observed between mouse strains in the proportion of neutrophils during infection (Figure 3C). By day 28, the proportions of neutrophils in infected wild-type, IL-1 β deficient, and IL-1R deficient mice were similar to those of uninfected mice (Figure 3C). Despite harboring more bacteria in the lungs throughout the infection, similar proportions of macrophages are present in the lungs of IL-1 β and IL-1R deficient mice until 28 days post-inoculation when a higher proportion of CD11b⁺F4/80⁺ macrophages were recovered from the lungs of IL-1 β and IL-1R deficient mice (Figure 3D) positively correlated with CFUs ($r^2 = 0.445, 0.842$ respectively). A likely explanation for this persistence of macrophages is persistent infection in the IL-1-deficient mice. This also suggests that IL-1 signaling may contribute to the bacterial killing ability of macrophages during *B. pertussis* infection.

Caspase-1 independent IL-1 β mediates clearance of *B. pertussis*

Previous data has shown that the NLRP3/caspase-1-containing inflammasome is activated by the *B. pertussis* adenylate cyclase toxin through formation of a pore in the host cell membrane and efflux of K⁺ ions [10]. Given that IL-1 β mediates the efficient clearance of *B. pertussis* from the lungs, we also expected the inflammasome to be required for control of the primary infection [10]. To examine the direct role for caspase-1-dependent IL-1 β secretion in response to live *B. pertussis*, we generated BMDMs from wild-type and caspase-1 deficient mice and exposed each to *B. pertussis* at an MOI of 10 for 24 hours. Consistent with previous publications, *in vitro* LPS/TLR4 stimulation alone was insufficient for inflammasome-dependent secretion of IL-1 β but required an additional signal such as ATP (Figure 4A, B), which causes an efflux of intracellular K⁺ and activation of the NLRP3/caspase-1 inflammasome [43], [44].

Infection of caspase-1 deficient BMDMs with live *B. pertussis* induced release of IL-1 β into the culture supernatant, although at significantly lower levels than wild-type cells ([Figure 4A](#)). These supernatants were precipitated from wild-type and caspase-1 deficient BMDMs infected with *B. pertussis*, blotted, and probed with anti-IL-1 β antibody to determine whether active IL-1 β was secreted. Only precursor IL-1 β was observed in caspase-1 deficient macrophages suggesting that caspase-1 is still required for cleavage and secretion of mature IL-1 β in *in vitro* culture conditions ([Figure 4B](#)).

To determine whether inflammasome activation is required for the generation of mature IL-1 β in the lungs during primary infection, wild-type and caspase-1 deficient were intranasally inoculated with *B. pertussis* (5×10^5 CFU) and lungs were homogenized and serially diluted on BG-blood agar. Surprisingly, bacterial numbers were similar in wild-type and caspase-1 deficient mice, indicating that caspase-1 is not required for clearance of *B. pertussis* from the lungs ([Figure 4C](#)). Since caspase-1 is also required for the processing of IL-18 [7], these data suggest that IL-18 is also not required for clearance of a primary *B. pertussis* infection. This was confirmed by inoculation of IL-18 deficient mice, which controlled and cleared *B. pertussis* as efficiently as wild-type mice (data not shown). To determine whether mature IL-1 β was generated at the site of infection, murine lung homogenates harvested on day 7, at the peak of infection and IL-1 expression, were probed for mature and pro-IL-1 β by immunoblotting. Interestingly, the mature form of IL-1 β was observed in wild-type and caspase-1 deficient mice ([Figure 4D](#)). Together these data indicate that during *B. pertussis* infections of the lung, caspase-1-dependent activation of IL-1 β is not necessary and suggests the involvement of other enzymes.

Adaptive immune responses are IL-1 dependent but caspase-1 independent

Previously we have shown IL-1R-mediated signaling to be important for the rapid clearance of *B. pertussis* from the lungs of mice given an acellular *B. pertussis* vaccine [27]. A recent study also showed that the current acellular vaccine acts through promoting a NLRP3-independent IL-1 response that generates robust Th1 and Th17 responses, a phenomenon also observed during primary infection [41]. However, the mechanism behind whole-cell vaccination is not known. To determine whether protective immunity is dependent on any alternate caspase-1-dependent inflammasomes, we vaccinated animals by administering two doses of heat-killed *B. pertussis* antigens intraperitoneally 28 and 14 days prior to a challenge inoculation. All unvaccinated animals had approximately 10^6 CFU of *B. pertussis* three days after challenge. Vaccination allowed both wild type and caspase-1 deficient mice to completely clear *B. pertussis* infection within seven days, indicating that caspase-1 is not required for the generation of protective immune responses to a whole cell vaccine. Vaccinated IL-1R deficient mice failed to clear *B. pertussis* and still had approximately 10,000 CFU in their lungs, indicating that IL-1R signaling is required for the efficient generation and/or function of protective immunity (Figure 5). Neither IL-1 β nor IL-1R was required for the generation of antibodies specific for *B. pertussis* (data not shown) suggesting a role in inflammation and/or cellular responses. These findings suggest that the sterilizing memory response requires IL-1R-mediated signaling but not caspase-1.

2.5 DISCUSSION

Experimental infections of mice lacking inflammasome components have shown that the inflammasome has an essential role in generating protective immune responses against multiple pathogens ranging from viruses to bacteria to fungi [45], [46]. Depending on the pathogen, one or more specific NLRs are recruited to the inflammasome complex which leads to activation of

caspase-1 and secretion of mature IL-1 β and IL-18 [47]. Previous work has suggested that infection with *B. pertussis* leads to activation of the NLRP3/caspase-1 inflammasome and this activation depends on the pore-forming activity of the adenylate cyclase toxin [10]. These observations were demonstrated *in vitro* with bone marrow-derived dendritic cells (BMDCs) and in this study in bone marrow-derived macrophages (BMDMs) [10]. These results are consistent with numerous studies indicating that inflammasome activation is required for mature IL-1 β in *in vitro* models. In contrast, infection models utilizing *Mycobacterium tuberculosis* and *Pseudomonas aeruginosa* have shown that while IL-1R signaling is required to control infection, caspase-1 deficient mice are capable of generating mature IL-1 β and clearing the infections, stressing the importance of using animal models for host-pathogen interaction studies [48]–[50]. Similarly, our studies have shown that mature IL-1 β could be detected in caspase-1 deficient mice and these mice are capable of clearing *B. pertussis* infection. Interestingly, infections in caspase-1/11 deficient mice showed no defect in bacterial clearance, suggesting that the protective IL-1 β response in the lung can arise independently from these inflammasomes.

Host IL-1 signaling generally leads to robust Th1 and Th17 responses which have an important role in activating phagocytes and multiple other inflammatory pathways. In this study, we examined the upstream processing and signaling that generates this protective response to *B. pertussis*, observing no role for IL-1 α but a significant role for IL-1 β and its downstream IL-1R-mediated signaling. We also determined that IL-18, another cytokine that is classically caspase-1 dependent, is not required for clearance of primary *B. pertussis* infections (data not shown). Further, while previous studies showed a defect in bacterial clearance in IL-17A and IL-17R knockout mice, we observed no significant defect in the production of this cytokine in the lungs of IL-1 β /IL-1R deficient mice during a primary infection, suggesting these cytokines are not

sufficient for *B. pertussis* clearance in the absence of IL-1R signaling [10], [41], [42]. These findings are significant because recent work has shown that protective Th17 responses require IL-1R signaling when alum is used as an acellular vaccine adjuvant but do not require IL-1R during a natural infection [41].

The effect of IL-1 during infection has been attributed to its role in activating the recruitment of immune cells to local sites of infection through the upregulation of chemokines and cytokines. However, histological examination shows that IL-1 β /IL-1R deficient mice are able to recruit inflammatory cells to the same extent as that of wild type mice. In addition, the persistence of diffuse inflammation in IL-1 deficient mouse lungs is correlated with the bacterial burden. This finding is similar to previous work using another IL-1R deficient mouse strain (B6; 129S1-*Il1r1*^{tm1Roml/J}) in which *B. pertussis* caused death but also highlights that there may be significant mutation and strain-specific effects of IL-1 signaling [27]. Seven days post-inoculation we also observed a reduced percentage of neutrophils in IL-1 β deficient mice, but this was not observed in IL-1R deficient mice. This may suggest the early defect in neutrophil recruitment in IL-1 β deficient mice may impact bacterial clearance during the latter part of the time-course and resolution of inflammation. However, we observed a similar amount of neutrophil recruitment in IL-1R deficient mice compared to wild-type, suggesting neutrophil recruitment may not impact bacterial clearance in the latter portion of the infection. We are currently investigating the early neutrophil defect in the infection process. These data would suggest that while IL-1 signaling is not required for recruitment of cells, the activation of robust bacterial killing mechanisms during primary infections does require IL-1 β . This cytokine most likely plays a role in overcoming the effects of pertussis toxin and adenylate cyclase toxin, which

have been shown to inhibit neutrophil and macrophage antimicrobial defenses [27], [37], [51], [52].

During the immune response to pathogens, inflammatory sites are often dominated by immune cells with diverse sets of secreted enzymes that have been shown to be involved in cleaving proteins or the extracellular matrix [53], [54]. Because caspase-1 is not required for the maturation of IL-1 β in the lungs of infected mice, alternative host-associated factors are likely responsible. Known enzymes include the serine proteinase 3 (PR3), neutrophil elastase (NE), cathepsin G, matrix metalloproteases (MMPs), stromelysin-1, gelatinases A and B, and caspase-8, which have all been shown to be capable of cleaving pro-IL-1 β into a biologically active form *in vitro* or in the mouse model [20]. Indeed, during arthritis-induced sterile inflammation, caspase-1-independent processing of IL-1 β leads to disease-associated inflammation as a result of neutrophil recruitment and proteinase 3, while *P. aeruginosa* infections of the cornea require neutrophil elastase [49], [55]. Therefore, we speculate that neutrophil or macrophage-derived enzymes present during the peak of *B. pertussis* infections, when IL-1 β expression and immune cell recruitment also peak, could lead to the processing of IL-1 β in the lung, but the specific mechanism remains to be examined.

Another important outcome of infection is the generation of a protective adaptive immune response. Natural immunity following infection has been shown to be more protective than the current acellular vaccines, so understanding which molecular signals generate this enhanced protection is important for improving future vaccines [28], [29]. Here we have demonstrated that the caspase-1 inflammasome is not required to generate a protective immune response following vaccination using heat-killed *B. pertussis* as a vaccine though IL-1 signaling is required [27]. This is consistent with a recent publication showing that the acellular vaccine

protects NLRP3 deficient mice from challenge infections [41]. The latter study does not rule out the requirement for caspase-1 in the generation of protective immunity as other NLRs may be involved. However, no other NLRs have been shown to be involved in recognizing *B. pertussis* thus far. Our work, on the other hand, provides definitive proof that caspase-1 and thus inflammasome-dependent IL-1 is not required for generating immunity from a heat-killed vaccine. Taken together, these data suggest that protective *B. pertussis* vaccines could function through activation of the IL-1R in an inflammasome-independent manner during the recall response that acts on antigen-specific Th1 and Th17 cells, similar to the response seen during a primary infection.

Overall, our work shows that the IL-1 β /IL-1R axis is required, independent from inflammasome activity, to resolve *B. pertussis* infections in the mouse model. Interestingly, we have also observed that a closely related bacterium *B. bronchiseptica*, which shares over 90% of its genome with *B. pertussis* and is consistently more virulent in mouse models, is cleared normally in IL-1R or caspase-1 deficient mice (unpublished data). IL-1R signaling was not required for the control of *B. parapertussis* infection, suggesting some unique aspect of *B. pertussis* results in a particular need for the IL-1R signal [27]. We propose that inflammasome-independent IL-1 signaling, in addition to robust Th1, Th17, and antibody responses, synergize to overcome the effects of *B. pertussis* pertussis toxin and adenylate cyclase toxin that impair the phagocytic capacity of macrophages in the lung. These findings highlight the need to further understand how bacterial virulence factors interact with and can be overcome by the host immune response during infections.

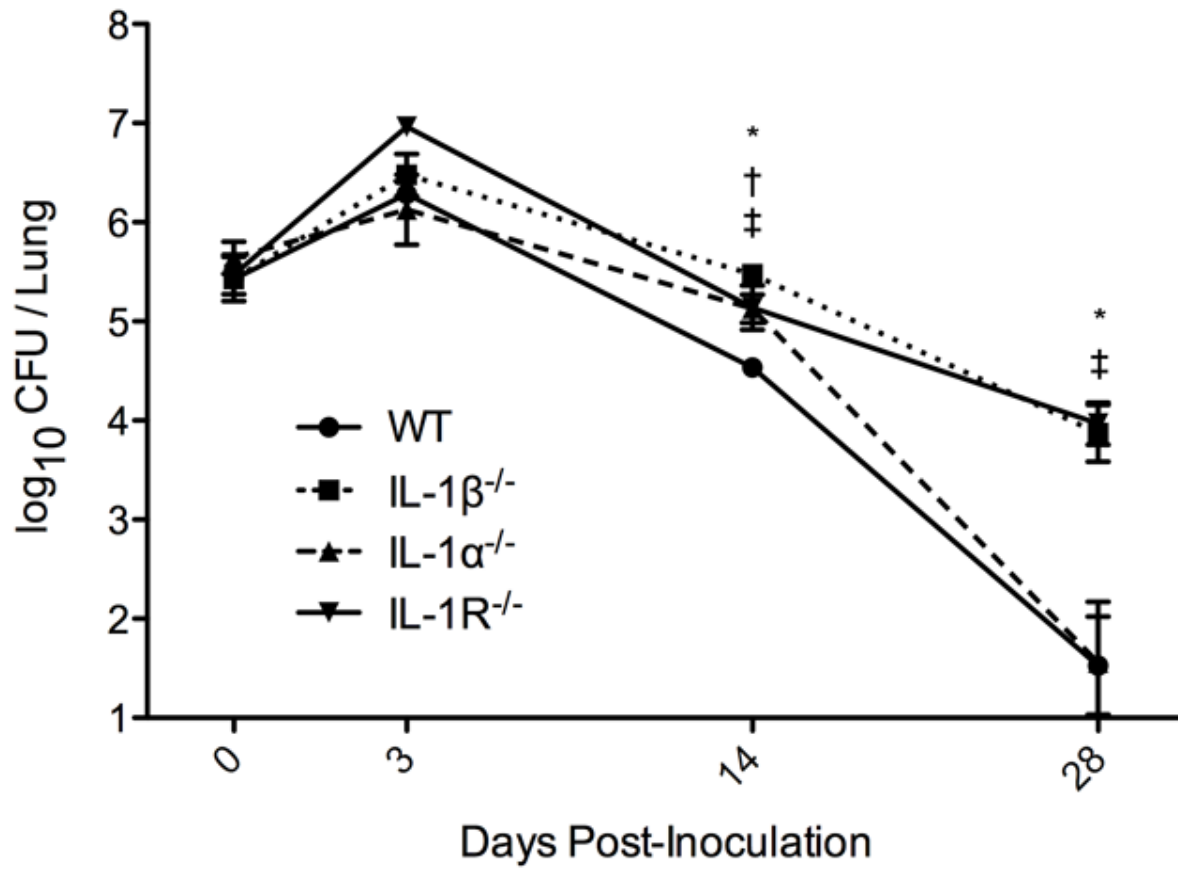


Figure 1.1. Bacterial clearance is mediated by IL-1β and IL-1R but not IL-1α.

Groups of 5–8 mice were inoculated with *B. pertussis* (5×10^5 CFU) suspended in a 50 μL droplet of PBS and euthanized at indicated time points to determine bacterial CFUs in lung homogenates. The mean \pm standard error was graphed for each group at the specified time-point and is representative of three independent experiments. Significance was calculated using one-way ANOVA with Dunnet's multiple comparison post-hoc analysis. * $p < 0.05$, ** $p < 0.01$; WT vs IL-1β^{-/-} (*), vs IL-1α^{-/-} (†), vs IL-1R^{-/-} (‡).

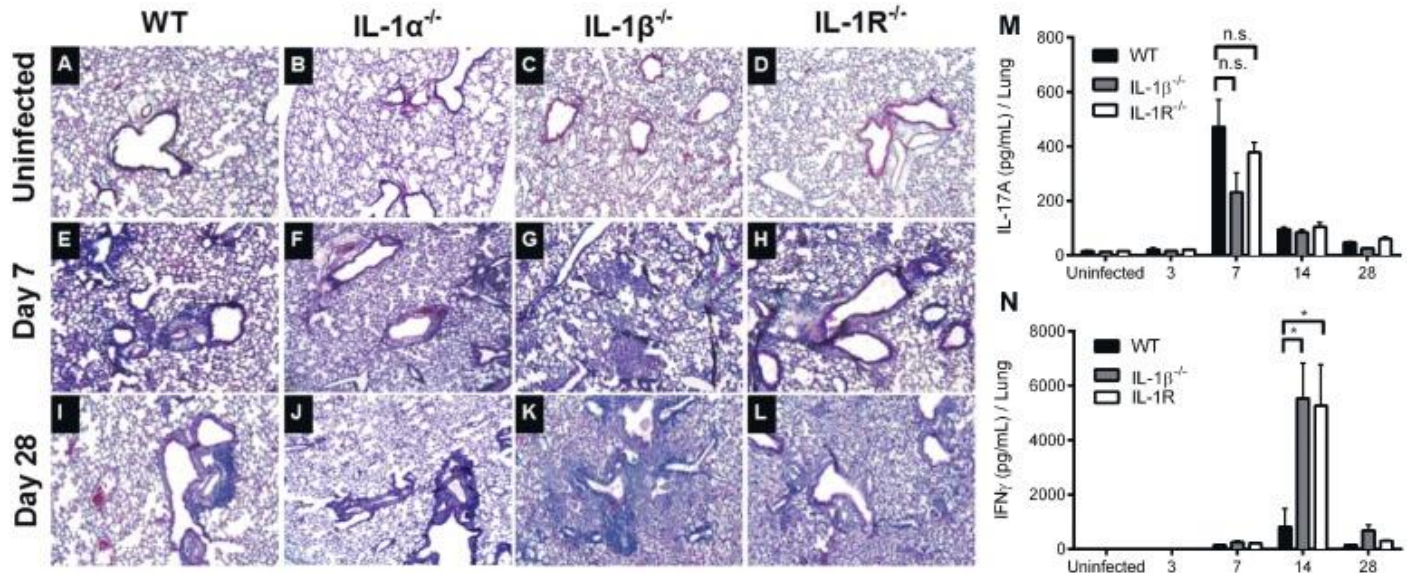


Figure 1.2. IL-1 signaling limits lung inflammation during *B. pertussis* infection.

Groups of four mice were either (A–D) uninfected or inoculated with *B. pertussis* and euthanized at (E–H) 7 days or (I–L) 28 days post-inoculation. Automated H&E staining was performed on 5 μ m paraffin-embedded sections. Images were taken and are representative of 10–12 fields per group. Lung homogenate cytokine measurements (M–N) were measured by ELISA from uninfected mice or on days 3, 7, 14, or 28 post-inoculation (n=4–8). ELISA significance was determined by one-way ANOVA with Dunnett's post-hoc test. *p<0.05 represents a significant difference from WT mice.

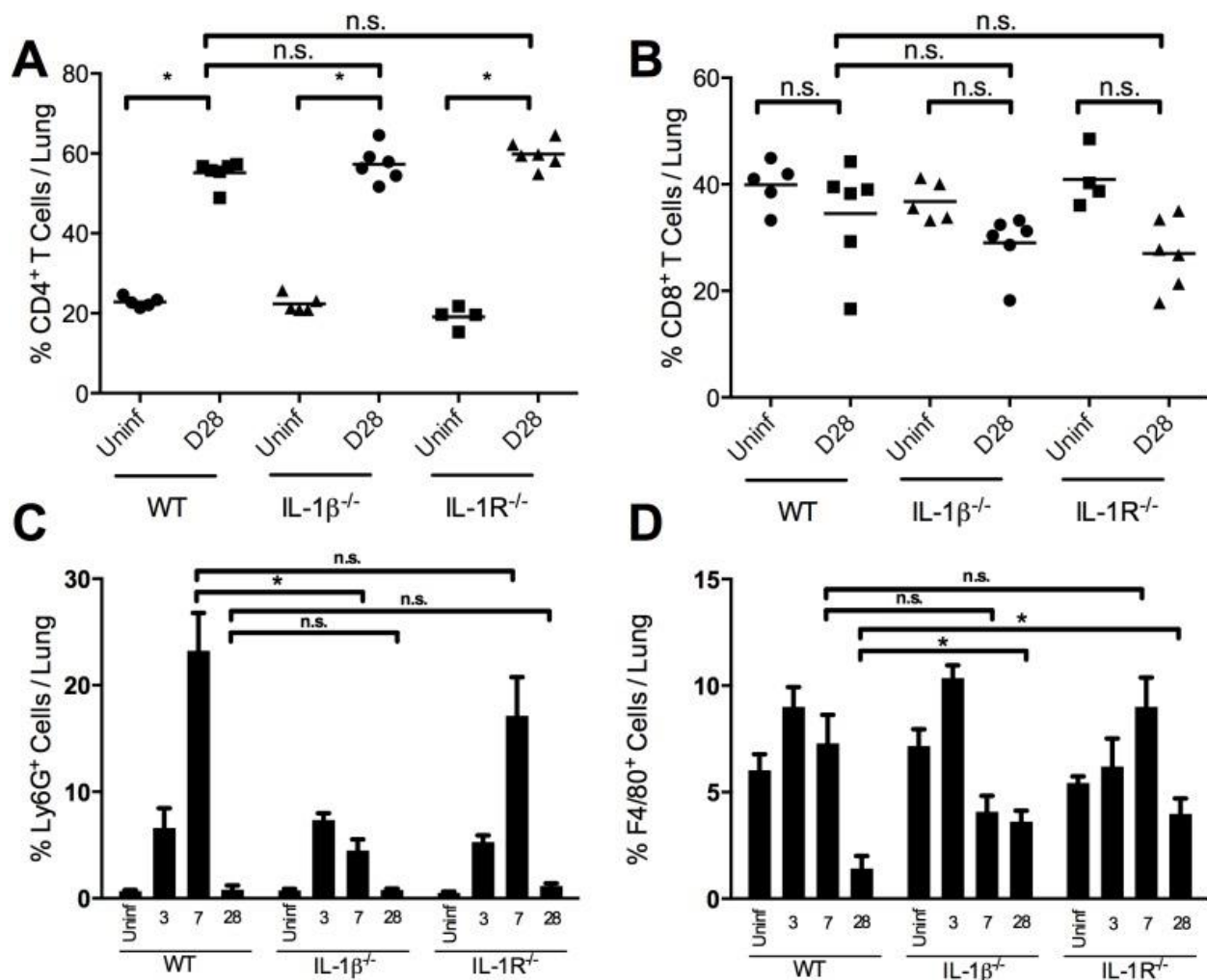


Figure 1.3. IL-1 is not required for recruitment of immune cells to the lung.

Lungs were excised and made into a single cell suspension for flow cytometry at the indicated days post-inoculation. Cells were stained with anti-CD45 to identify hematopoietic cells and anti-CD11b, CD3, CD4, CD8, Ly6G, and F4/80 for further subsets. Gating for T cells subsets was performed on CD45⁺CD3⁺ cells with quadrant gating to identify either (A) CD4⁺ or (B) CD8⁺ single-positive T cells. Gating on CD45⁺CD11b⁺ cells, (C) Ly6G⁺ neutrophils or (D) F4/80⁺ macrophages were identified. Data was collected with a BD LSRII flow cytometer. Bars indicate the mean percentage of each group (n=6) with (*) indicating p<0.05 using the Kruskal-Wallis test with Dunn's post-hoc test for multiple comparisons.

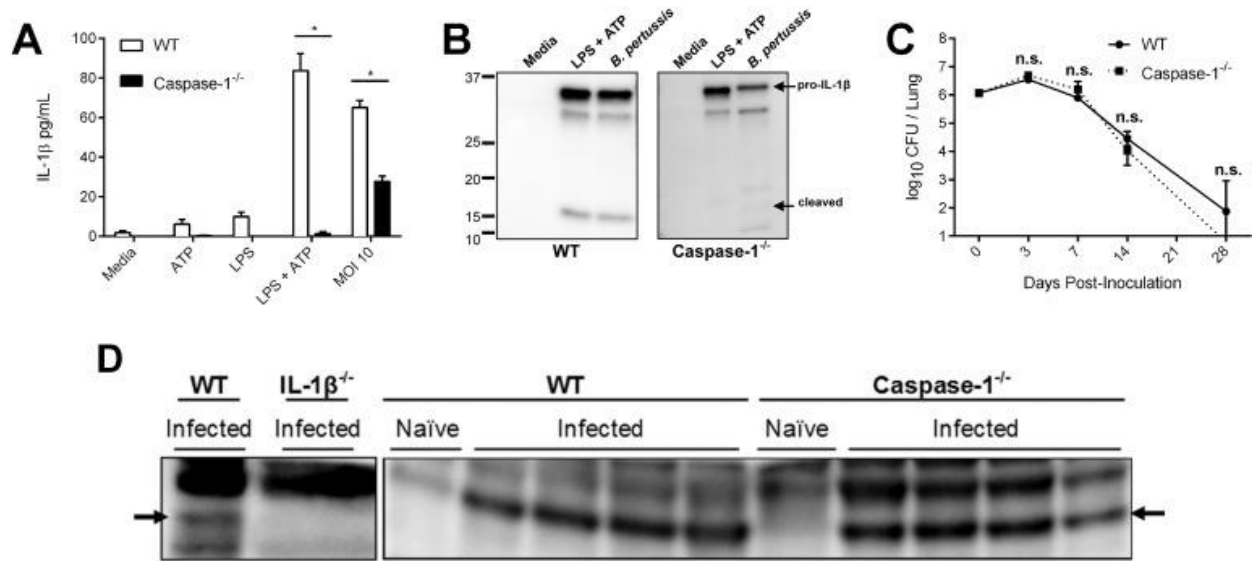


Figure 1.4. IL-1 β production is caspase-1 dependent in vitro but independent in vivo.

To determine if *in vitro* IL-1 β processing is dependent upon caspase-1, bone marrow-derived macrophages were (A) primed 3 h prior to inoculation and were washed and treated with fresh media, ATP (5 mM), or live *B. pertussis* (MOI 10) or (B) seeded at 10^6 cells/well and primed with LPS or washed and treated with media, ATP (5 mM), or live *B. pertussis* (MOI 100) for 6 hours in FBS-free DMEM, and supernatant was collected. The requirement for caspase-1 during infection and the ability to produce IL-1 β in response to live *B. pertussis* was assessed by (C) inoculating groups of four mice per time point with *B. pertussis* (5×10^5 CFU) and lungs were dissected for determining bacterial CFUs \pm standard error. (D) To identify whether cleaved IL-1 β was produced in caspase-1 deficient mice, lung homogenates from day 3 post-inoculation were run on 12% gels and probed with anti-IL-1 β . Data is representative of at least two independent experiments.

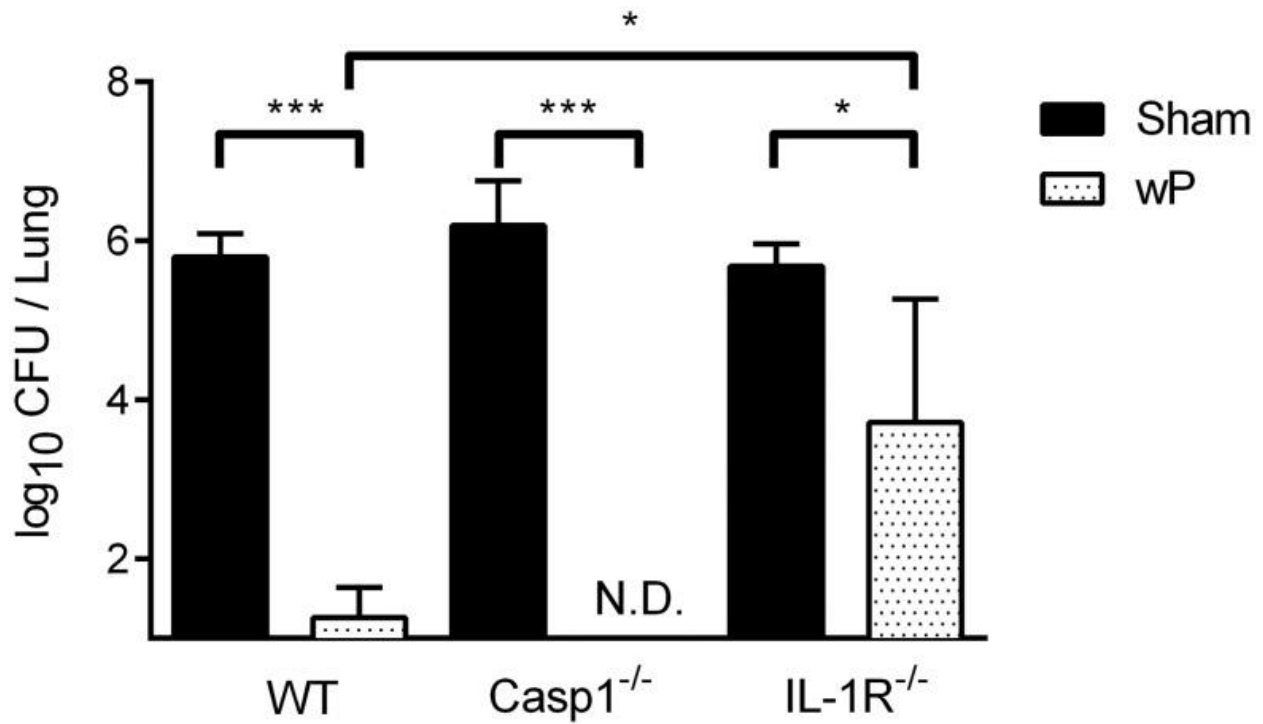


Figure 1.5. Adaptive immune responses are IL-1 dependent but Caspase-1 independent.

Groups of four mice were either sham vaccinated with PBS or vaccinated with heat-killed *B. pertussis* (wP) by administering 200 μ L *i.p.* at 28 and 14 days prior to challenge inoculation with 5×10^5 CFU. Lungs were excised at seven days post-challenge and to determine bacterial CFUs. Bar graphs represent the mean CFU per lung \pm standard deviation. Significance was determined by one-way ANOVA and Tukey's multiple comparison post-hoc test with * $p < 0.05$, *** $p < 0.001$. Data is representative of two independent experiments.

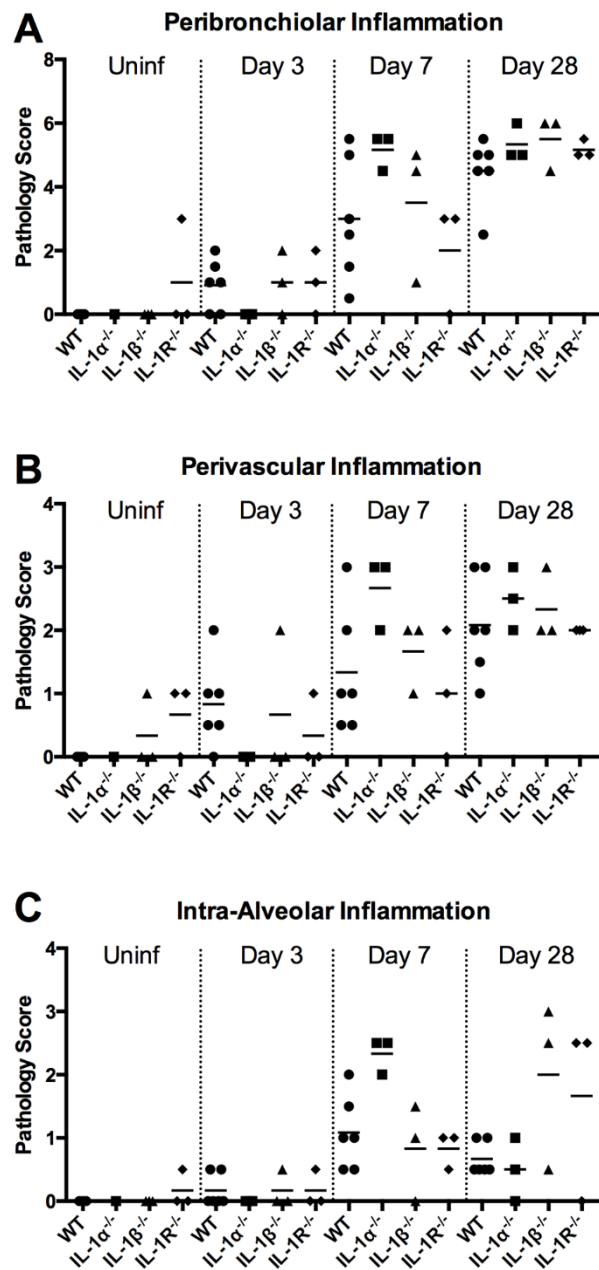


Figure 1.S1. **Lung pathology scoring of *B. pertussis* infection.** Groups of mice (n=3–6) were inoculated and lungs were collected for histology. (A) Peribronchiolar (degree and % lung affected combined), (B) perivascular, and (C) intra-alveolar inflammation were scored (0–4) and averaged from two blinded, independent scorings. Bars indicate mean score of each group.

2.6 AUTHOR CONTRIBUTIONS

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Conceived and designed the experiments: DEP SJM ETH GSK. Performed the experiments: DEP SJM. Analyzed the data: DEP SJM. Contributed reagents/materials/analysis tools: GSK ETH. Wrote the paper: DEP.

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Chapter 3

Development of a Novel *Francisella tularensis* Live Vaccine Strain Expressing Ovalbumin Reveals Memory and Effector CD8 T Cell Responses During Intranasal Vaccination and Re-challenge

3.1 ABSTRACT

Studying the development of pathogen-specific memory and effector T cells has remained a problem for establishing the protective immune mechanisms for *Francisella tularensis* infection. We have developed a novel tool for studying CD8 T cell responses to the *Francisella tularensis* Live Vaccine Strain (LVS) using a codon-optimized fragment of chicken ovalbumin (OVA), a model antigen, tethered to *Francisella* vgrG, a protein secreted by the type VI secretion system. We have shown that *Francisella*-specific CD8 T cells expand after the first week of infection and persist within the local lung environment and spleen for ten weeks beyond clearance of the bacterium. Kinetics of CD8 effector (T_{eff}) and central (T_{cm}) memory cells were also examined in lung, draining lymph node, and spleen throughout a primary infection and re-challenge with a lethal dose of LVS-OVA. We also show that *Francisella*-specific CD8 T cells produce IFN γ but not IL-17A during primary or recall responses. Together, our data show that LVS-OVA can be used to study multiple aspects of *Francisella*-specific CD8 T cell responses, which will enable us to develop more protective vaccines for tularemia.

3.2 INTRODUCTION

Francisella tularensis is a Gram-negative coccobacilli which invades phagocytic cells, escapes the endosomal compartment, and replicates within the host cell cytosol [1,2]. During the early days of infection, very few cytokines are elicited while the bacteria spread systemically throughout the host. When infected with the highly virulent strains of *F. tularensis*, this leads to rapid death of infected mice [3]. A closely related strain, *F. tularensis holarctica* was used to generate an attenuated live vaccine strain (LVS), but the exact changes which cause the attenuation are poorly characterized [4–6].

One of the primary challenges in the *Francisella* field is development of a protective vaccine for the highly virulent strains of *Francisella tularensis tularensis* which have been categorized as potential bioweapons due to their high levels of mortality following aerosol exposure. Many limitations for the development of a protective vaccine exist for *Francisella*, including a live-attenuated vaccine that has poorly understood attenuation, a risk of reversion to virulence of the vaccine, and the lack of tools to study *Francisella*-specific cell-mediated immune responses [3,6,7]. Few correlates of protective immunity are known to be protective against challenge with highly virulent *F. tularensis*, primarily the cytokine IFN γ with others (TNF α , IL-12, IL-6) providing varying protection depending on the route of vaccination or to challenge with LVS [8–13]. The contribution of T cells to *Francisella* immunity has primarily focused on the primary response, as it has previously been challenging to study pathogen-specific cellular immunity. An interesting subset of CD4⁻CD8⁻ double-negative T cells have been shown to produce IL-17A and IFN γ in response to intranasal LVS in the lungs, but whether they are involved in memory has not been evaluated [14].

While it is known that CD4, CD8, and CD4⁻CD8⁻ double-negative T cell responses arise during vaccination with LVS, their localization and effector functions remain poorly characterized and whether they are essential for protection is uncertain [14–17]. Further, the differences in protection to challenge is highly dependent upon the route of vaccination (intranasal or intradermal), suggesting that T cells are endowed with different properties by these two different routes. It has been suggested that differences in the initial phagocytic cell subsets infected by intradermal and pulmonary infection lead to differences in the T cell responses during challenge, given that they may have differences in their antigen presentation capabilities, activation, or localization [18,19]. To date, studying the pathogen-specific cells that arise during vaccination has been hampered by the lack of tools to study them. The only *Francisella*-specific epitope described is the glycoprotein Tul4₈₆₋₉₉ epitope (RLQWQAPEGSKCHD) and a MHC-II tetramer has been generated for this epitope by the NIH Tetramer Facility, but has not been shown to stain antigen-specific cells [20,21]. Further, no TCR-transgenic mice have been made which recognize *Francisella*-specific epitopes, preventing detailed analysis of the T cell responses which arise during infection. By expressing a fragment of chicken ovalbumin (OVA) protein in LVS, we are now able to utilize the TCR-transgenic OT-I and OT-II mouse strains, which following adoptive transfer into a congenic wild-type mice can be used to study CD8 and CD4-specific responses, respectively [22,23].

Here we report the development of a *F. tularensis* LVS which expresses a fragment of the OVA protein containing epitopes specific for both OT-I and OT-II TCR transgenic T cells, termed LVS-OVA. We further examined the proliferative response of antigen-specific CD4 and CD8 T cells during vaccination with LVS-OVA by the intranasal and intradermal responses and

during recall responses following intranasal challenge. This novel tool will allow further studies into the cell-mediated immune response to *Francisella* vaccines.

3.3 MATERIALS AND METHODS

Mouse Strains. C57BL/6J (Thy1.2⁺), C57BL/6J Thy1.1⁺ congenic mice (B6.PL-Thy1^a/CyJ), OT-I (C57BL/6-Tg(TcraTcrb)1100Mjb/J), and OT-II (B6.Cg-Tg(TcraTcrb)425Cbn/J) mice were purchased from The Jackson Laboratories (Bar Harbor, ME). Unless otherwise indicated, experiments were performed using heterozygous wild-type Thy1.1⁺/Thy1.2⁺ recipient mice with OT-I and OT-II (Thy1.1⁻/Thy1.2⁺) donor splenocytes.

Bacterial Strains and Cell Culture. Cloning was performed using *Escherichia coli* DH5 α grown at 37°C in LB broth or LB agar containing tetracycline or kanamycin (10 μ g/mL or 50 μ g/mL, respectively). *Francisella tularensis* Live Vaccine Strain (LVS, from ????) was grown shaking in BHI broth at 37°C or on modified Mueller-Hinton II agar supplemented with hemoglobin (ThermoScientific) and IsoVitalex (BD Biosciences) at 37°C in 5% CO₂ with tetracycline or kanamycin (2 μ g/mL or 10 μ g/mL, respectively). For allelic exchange, Km^R LVS primary recombinants were struck out on plates containing 5% sucrose and resulting colonies were screened for secondary recombination by plating single colonies on antibiotic-free, ampicillin, or kanamycin plates as previously described [24].

Mid-log phase liquid cultures of LVS were frozen at -80°C directly in BHI and enumerated regularly to calculate appropriate inoculation doses for mouse inoculation.

For immunoblots, liquid cultures (OD₆₀₀ ~0.10) were pelleted and resuspended in bacterial lysis buffer followed by sonication. Sonicate was mixed with 5X Laemmli buffer (BioRad) before running on 12% pre-cast Criterion TGX gels (BioRad). Protein was transferred to PVDF membrane using the Trans-Blot Turbo mixed molecular weight program. Membranes were then blocked in 5% BSA/TBS-T for 1h at room temperature, probed with anti-6xHis-Biotin (1:5000, Rockland #600-406-382) in 5% BSA/TBS-T for 1h at room temperature, washed, and probed

with a secondary streptactin-HRP (1:50000, Jackson ImmunoResearch) for 1h at room temperature. Blots were developed using Millipore Immobilon Chemiluminescent Substrate and images were collected on a BioRad ChemiDoc XRS+.

Bone marrow-derived macrophages (BMDMs) were generated as previously described [25]. For infections of BMDMs with LVS-OVA, cells were seeded overnight on tissue culture treated plates and allowed to adhere at 37°C in 5% CO₂. Cells were washed and media containing bacteria at an MOI of 100 was added before centrifugation at 300xg for 10 minutes and incubated for 1h. Media containing gentamicin (10ug/mL) was added to cells and incubated 1h before washing with antibiotic-free media. At indicated time-points post-inoculation, cells were washed and lysed in PBS containing 0.01% sodium deoxycholate by 5min at 37°C and vigorous pipetting. Lysates were serially diluted in PBS and plated on modified Mueller-Hinton agar or plates containing 5ug/mL tetracycline.

Plasmids.

Plasmid pKK214 containing GFP was obtained from Dr. Thomas Kawula(??) and the GFP gene was removed by restriction digestion and replaced with a cloning site containing XbaI restriction sites. LVS genomic DNA was used to amplify the promoter for the bacterioferritin gene (*bfrp*) by PCR and to introduce flanking XbaI and BamHI restriction sites. The complete *vgrG* gene was amplified with primers introducing a BamHI site and replacing the stop codon with an XhoI site. To generate the codon-optimized ovalbumin epitope tag (containing OVA amino acids 239-345, a 6xHis tag, stop codon, and flanking XhoI and XbaI sites), the LVS codon frequency table from the Codon Usage DB (<http://www.kazusa.or.jp/codon/cgi->

bin/showcodon.cgi?species=376619) was imported into OPTIMIZER

(genomes.urv.es/OPTIMIZER) using the standard setting of “one amino acid – one codon”[26].

The codon-optimized sequence was submitted for synthesis to Integrated DNA Technologies as a gBlock Gene Fragment and confirmed by sequencing using the pCR4 cloning vector

(Invitrogen). The bacterioferritin promoter, *vgrG*, and OVA₂₃₉₋₃₄₅-6xHis fragments were ligated with T4 DNA ligase (NEB) and cloned into XbaI-digested pKK214 to generate pKK214-*vgrG*-

OVA or pKK214-OVA_{gB}. For chromosomal integration into LVS, the plasmid pMP815

containing *blaB* flanking regions was obtained from Dr. Martin Pavelka and digested with ApaI

and Sall. Primers containing ApaI and Sall sites were used to amplify *bfrp-vgrG-OVA* from

pKK214-*vgrG*-OVA before digestion and ligation into pMP815 to generate pMP815-*vgrG*-

OVA. Electroporation was used to introduce plasmids to *E. coli* and LVS. A summary of

primers, plasmids, and strains used can be found in Table 1.

Mouse Infection and Adoptive Transfer

Mouse inoculation was performed by thawing enumerated frozen bacterial stocks and serially

diluting in PBS to 1000 CFU or 10000 CFU for vaccination and challenge experiments,

respectively. Mice were lightly anesthetized with isoflurane and a 50uL droplet was administered

to the external nares and inhaled or injected intradermally at the base of a single ear. Mice were

ethanized by CO₂ inhalation and dissected aseptically in a biosafety cabinet.

For adoptive transfer of OT-I and OT-II splenocytes (Thy1.2⁺), sex-matched spleens were

aseptically collected in RPMI 1640 containing 10% FBS, 1mM L-glutamine, 1mM sodium

pyruvate, 1x non-essential amino acids, 10mM HEPES, 55μM beta-mercaptoethanol and

penicillin/streptomycin. Spleens were passed through a 70μm mesh filter by crushing with a

3mL syringe plunger and pelleted. Red blood cells were lysed with sterile 0.84% ammonium

chloride/EDTA/Sodium bicarbonate and washed with complete media. For experiments with CFSE-labelled splenocytes, cells were washed with plain RPMI 1640 before resuspension at a concentration of 10^7 cells/mL in RPMI 1640 containing 5uM CFSE. Staining was quenched by addition of 10% FBS and cells were washed twice with complete RPMI. Cells were mixed at a 1:1 ratio in RPMI 1640 and a total of 10^7 cells in 500uL (5×10^6 OT-I/OT-II cells each) were transferred to Thy1.1⁺/Thy1.2⁺ or Thy1.1^{+/+} recipients by intraperitoneal injection 24h before bacterial inoculation.

Flow Cytometry and Intracellular Cytokine Staining

Mice were euthanized and cold PBS was used to perfuse tissues by cardiac injection. The caudal mediastinal lymph node (MLN) and spleen were collected in HBSS with 1.3mM EDTA and crushed with a 3mL syringe plunger through a 70µm nylon filter. Lungs were collected, minced and collected in HBSS with 1.3mM EDTA, shaken 30 min. at 37°C, pelleted, and resuspended in complete RPMI containing 200U/mL collagenase type I (Worthington Biochemical Corporation) and shaken at 37°C for 1h before passing through a 70µm nylon filter. Spleen and lung cells were treated with RBC lysis solution and washed with PBS/2%FBS. Cells were stained with CD4-AF700 (RM4-5), CD8a-PerCP-Cy5.5 (53-6.7), Thy1.1-AF488 or -PE (OX-7), Thy1.2-BV510 (53-2.1), CD44-APC-Cy7 (IM7), CD62L-PE-Cy7 (MEL-14), KLRG1-APC 2F1 IFNγ-PE (XMG1.2), IL-17A-PE-Cy7 (TC11-18H10) purchased from BioLegend 30 min on ice, washed, and fixed in PBS/1% PFA prior to running on a BD LSR Fortessa. Analysis was performed using FlowJo v10.0.8 software (company). Graphs and statistical analyses were generated using GraphPad Prism 6.

Intracellular cytokines were analyzed by stimulating $\sim 2 \times 10^6$ cells in 96-well plates at 37°C and 5% CO₂ incubated in the presence of 1xBrefeldin A (BioLegend) alone (unstimulated) or

1xBrefeldinA and 50ng/mL phorbol 12-myristate 13-acetate (PMA) (Sigma) / 5ng/mL Calcium Ionophore A23187 (Sigma #C7522) for 4h in complete RPMI. Cells were stained for extracellular markers as above, fixed, and followed by permeabilization with 0.1% saponin in PBS/2%FBS. Antibodies for intracellular cytokines were incubated 30min in permeabilization buffer and washed 3x with permeabilization buffer and 3x with PBS/2%FBS and analyzed immediately.

3.4 RESULTS

Generation of LVS-OVA

We sought to constitutively express a fragment of the chicken ovalbumin protein, OVA₂₃₉₋₃₄₅, in the *F. tularensis* LVS strain. The bacterioferritin promoter, which has been shown to be highly active in *Francisella*, was ligated with a codon-optimized sequence of OVA₂₃₉₋₃₄₅ (OVA) directly to the bacterioferritin promoter or to the C-terminus of full-length vgrG, generating pKK214-vgrG-OVA (Figure 1A). After electroporation of both pKK214-OVAgB and pKK214-vgrG-OVA constructs into LVS, bacterial lysates were grown and sonicated in lysis buffer for immunoblot analysis of the expression of each construct. While vgrG-OVA₂₃₉₋₃₄₅ was expressed at very high levels, OVA₂₃₉₋₃₄₅ alone was not detected, suggesting this product is degraded or poorly expressed (Figure 1B). Despite previous work showing that pKK214 plasmids are stably maintained without selection [27], we assessed the stability of the pKK214-vgrG-OVA plasmid without selection in an *in vitro* macrophage infection assay (Figure S1) and during infection of mice and observed no significant loss of plasmid by plating on plates with or without tetracycline (Figure 3C). We further serially passaged LVS-OVA in liquid culture over 9 days without selection and also observed no loss of plasmid (Figure S2). We also attempted to generate a chromosomally integrated LVS-OVA through allelic exchange into the *blaB* locus using the pMP815 system and were able to observe expression of vgrG-OVA in primary recombinants; however, we were unable to cure these bacteria of the integration plasmid, suggesting insertion into regions outside the *blaB* locus, which precluded the use of this LVS-OVA strain (Figure 1B). These data show that our pKK214-vgrG-OVA expression construct is highly expressed in LVS-OVA and that the plasmid is robustly maintained without antibiotic selection for multiple rounds of replication *in vivo*, which is useful for *in vivo* experiments.

Antigen-specific proliferation of CD8⁺ OT-I cells

To test the ability of LVS-OVA to activate antigen-specific OT-I and OT-II T cells, we utilized an adoptive transfer model into wild-type Thy1.1⁺/Thy1.2⁺ congenic recipient mice. OT-I and OT-II splenocytes were collected and stained with CFSE, a long-lasting stain that can be used to detect dividing cells by dilution of the CFSE fluorescence [28,29]. Upon TCR stimulation with cognate antigen and co-stimulatory signals, OT-I and OT-II T cells will begin to divide in response to LVS-OVA. One day prior to infection with LVS-OVA, 10⁷ splenocytes (5x10⁶ each of OT-I and OT-II splenocytes) were transferred to wild-type congenic mice by intraperitoneal injection. At days 5, 7, and 9 post-inoculation with LVS-pKK214 (vector control) and LVS-OVA, the lung draining mediastinal lymph nodes (MLN), the lungs, and spleens were collected and analyzed by flow cytometry to identify CFSE⁺ OT-I (CD8⁺) and OT-II (CD4⁺) donor cells. As expected, LVS-OVA lead to robust proliferation of antigen-specific OT-I cells at approximately day 7 post-inoculation while LVS-MCS4 was unable to stimulate cells to expand (Figure 2A-C). By day 9 post-inoculation, OT-I T cells represented the majority of the CD8 T cells present in the lung (~60%) and a large proportion of the spleen (~40%), but was not as abundant in the MLN, suggesting that upon activation in the MLN, OT-I cells migrate to the periphery, secondary lymphoid organs, and lungs (Figure 2C). Additionally, by day 9, OT-I⁺CFSE⁺ cells were mostly undetectable, suggesting that transferred OT-I⁺ cells had undergone at least 5 rounds of cell division (Figure 2B). Interestingly, CFSE-labelled OT-II cells did not appear to undergo cell division in any of the tissues analyzed during these time points, suggesting that LVS-OVA is unable to stimulate the OT-II T cells (Figure 2A-C). This failure of OT-II cells to proliferate suggests the CD4 OVA₃₂₃₋₃₃₉ epitope (ISQAVHAAHAEINEAGR) is not properly presented or is poorly immunogenic in this model, as endogenous CD4 T cells

expand during the infection (supplemental data, Figure X). Together, these data show that LVS-OVA specifically activates OT-I T cells and can be used to study CD8 T cell responses to *Francisella*.

OVA-specific CD8 T cell response during infection

Previously, tracking the kinetics of *Francisella*-specific T cell responses has been limited by a lack of tools to identify the specific cells from the total population of T cells. Utilizing adoptive transfer of OVA-specific T cells, we measured the proportion and number of *Francisella*-specific CD8⁺ OT-I T cells in the lungs, MLN, and spleen during infection with LVS-OVA and their contraction following bacterial clearance (Figure 3A-F). We observed a pattern similar to other pathogens where the memory T cell response peaks and rapidly contracts as pathogen is cleared (Figure 1D, 3D-F). In the lungs and MLN, the contraction is rapid, as cells have significantly decreased from day 7 at all later time points (Figure 3A-B, D-E). In the spleen, however, OT-I cells only decrease significantly from day 14 to later time-points (Figure 3C, F). These data suggest that *Francisella*-specific CD8 T cells respond differently in different tissues, possibly as a result of differences in antigen exposure, chemokines, cytokines, or other cell types, which may result in different cell function during re-challenge.

***Francisella*-specific memory T cell development**

In addition to being able to track the number of *Francisella*-specific CD8 T cells, we analyzed the memory markers CD62L and CD44 to assess the kinetics of *Francisella*-specific CD62L⁻CD44⁺ effector memory T cells (T_{eff}) and CD62L⁺CD44⁺ central memory T cells (T_{cm}) in the lung, MLN, and spleen over time [7,30,31]. In the lungs, effector memory OT-I cells remain increased in frequency through pathogen clearance but decrease in total numbers after the peak of infection and eventually decrease in frequency and number after pathogen clearance

(Figure 4A,B). The lung draining MLN, however, has a similar frequency of effector OT-I cells, except at day 28, suggesting these cells do not accumulate in the lymph node (Figure 4A,B). In the spleen, OT-I effector memory cells peak at day 14 and contract in frequency and total number in later time-points, but there is an increase in the frequency at day 28, suggesting effector memory cells may transiently accumulate in the spleen following pathogen clearance (Figure 4A,B).

Effector memory cells (T_{eff}), which are described as $CD62L^{\text{lo}}CD44^{\text{hi}}$, are generally considered the subset which is responsible for producing rapid cytokine and cytolytic responses in infected tissues [32]. Central memory cells (T_{cm}), which expresses both CD62L and CD44, are not generally considered to produce effector cytokines and migrate to proliferate in secondary lymphoid organs. The primary role of T_{cm} cells is generally considered to be their ability to rapidly expand into a new population of effector cells upon re-exposure to antigen [33,34]. We observe this subset of cells to continually expand in frequency in the lungs of mice following clearance of LVS-OVA even 71 days post-inoculation (Figure 4C) though the total number of central memory cells is only significantly increased from other time-points at day 71 (Figure 4D). In the lung draining MLN, OT-I central memory cells represent a larger percentage of the population after the peak of infection but decreases in numbers after pathogen clearance (Figure 4C, D). Similar to the lung, the frequency of central memory OT-I cells increases during pathogen clearance and at day 71, compared to day 28 (Figure 4C, D). The total number of central memory cells is also lower at day 28 compared to later time-points, suggesting that central memory cells may accumulate or proliferate in the spleen after LVS-OVA has been cleared but that this occurs over many weeks (Figure 4C, D). These data show that central

memory cells reside in both the initial and secondary sites of infection and may have different properties upon re-challenge.

***Francisella*-specific CD8 IFN γ and IL-17A Responses**

One of the primary effector functions of CD8 T cells is the secretion of IFN γ to activate phagocytic cells to kill intracellular pathogens, including *Francisella* [19,35–37]. Additionally, the role of IL-17A in primary and secondary challenge with *Francisella* has been difficult to establish [37–40]. To study these two inflammatory cytokines in *Francisella*-specific CD8 and CD4 T cells, we isolated cells from the lung, MLN, and spleen and performed intracellular cytokine staining on ex vivo stimulated cells using PMA/calcium ionophore. While CD8 T cells generally do not secrete IL-17A, IFN γ is produced in multiple infection models [41,42] and both cytokines were assessed in both the CD4 and CD8 T cell populations at various time-points following inoculation with LVS-OVA. As expected, we observed that CD8⁺ OT-I cells produce IFN γ during infection with LVS-OVA but the response peaked earlier in the lungs and lung draining MLN than in the spleen, where IFN γ -producing cells peaked around day 14 (Figure 5A). We did not observe any significant stimulation of IL-17A⁺ OT-I cells at any time-point during infection (Figure 5B). While CD4⁺ OT-II T cells did not specifically respond to LVS-OVA, the endogenous CD4 response generated IFN γ ⁺ cells at all time-points in the lung but significant IL-17A⁺ cells were only observed at day 7 (Figure S3). These results show that LVS-OVA is able to generate IFN γ ⁺ CD8 T cells by the intranasal vaccination route, but whether these cells produce other important effectors such as perforin and granzymes remains to be examined.

Recall response of OT-I Cells to LVS

One of the great advantages to using antigen-specific adoptive transfer models is the ability to identify the memory cells which are responding to re-exposure to antigen rather than new naïve cells which may be more abundant in various tissues. After 30 days post-inoculation with LVS-OVA, we challenged vaccinated mice with a lethal dose of LVS-OVA intranasally to determine how CD8⁺ OT-I cells respond upon re-exposure to *Francisella*. In the lungs we observe an increase in T_{cm} frequency in the lungs and spleen but not in the MLN, but the total number increases significantly in each of these tissues after challenge (Figure 6A,B). Because T_{eff} cells are considered to be the main contributor to effector function, one would expect they would respond primarily in the lung during re-challenge. Consistent with this, we observe a significant increase in the number of T_{eff} cells in the lung of re-challenged mice (Figure 6D), though their frequency is lower than before challenge (Figure 6C). Interestingly, we did not observe a significant increase in T_{eff} cells in either the lung draining MLN or spleen, suggesting that lung-homing T_{eff} cells may be sufficient to control the spread of *Francisella* to systemic organs (Figure 6C,D). We also observed that IFN γ ⁺ but not IL-17A⁺ OT-I T cells are present in the lungs, MLN, and spleen following re-challenge (Figure 6E,F), consistent with data suggesting that IFN γ is required for protection to secondary challenge to *Francisella* [37,43].

3.5 DISCUSSION

Given the difficulties presented by the current vaccines for *Francisella*, understanding the development of the memory T cell response is critical for developing novel strategies to protect against this pathogen. By developing LVS-OVA, we are now able to track the development and phenotypes of *Francisella*-specific T cell responses through an infection, weeks beyond infection, and during re-challenge. Adoptive transfer into gene-knockout mice can begin to help us study the role of specific cytokines for the development of CD8 T cell responses to *Francisella* and OVA-expressing attenuated mutants of *Francisella* could help us to understand how different mutations affect the development failed or protective memory responses. We were able to show that our OVA-expression construct was well expressed in LVS and that it could be used in the absence of selective antibiotics *in vivo*. While we showed that LVS-OVA was able to stimulate the expansion of CD8⁺ OT-I T cells, we did not observe an expansion of CD4⁺ OT-II cells, so we limited our study to the development of CD8 memory and effector T cell phenotypes (Figure 1). This tool can be used in the future to study the interaction of attenuated mutants of *Francisella* strains and the highly virulent Type A strains with antigen-presenting and CD8 T cells.

We first sought to study the initial expansion stage of antigen-specific OT-I cells in the first days of infection in various tissues. We observed a delay in expansion as compared with another intracellular bacterium, *Listeria monocytogenes*, which displays rapid CD8 expansion at day 3 post-inoculation whereas during LVS-OVA infection the expansion occurs between days 7-9, suggesting that the stealth strategy employed by *Francisella* has a dramatic effect on the kinetics of T cell expansion[44]. Indeed, *Francisella* poorly activates inflammatory pathways until many days into an infection, where it is then found in multiple systemic organs[3,7,45].

Shortly after T cell expansion, however, *Francisella* begins to be controlled and eliminated. Our data suggest the peak of CD8 T cells is likely somewhere between day 9 and 14 and remains to be studied in greater detail (Figure 2). Of interest is how different routes of infection, adjuvant therapies, and the different cell types initially infected by *Francisella* may change the kinetics of T cell expansion [18,46–48].

Identification of correlates of protection is a major need for developing vaccines which preferentially induce responses that are protective but not overly damaging to the host. To date, studying the phenotypes of individual *Francisella*-specific T cells has not been possible but we have been able to show the kinetics of expansion and contraction of antigen-specific OT-I cells throughout infection with LVS-OVA and weeks beyond (Figure 3). We have also been able to begin dissecting the differences in tissue localization of these antigen-specific T cells and their individual memory and effector phenotypes (Figure 5, 6). It is already well appreciated that tissue localization of memory t cells can affect their protective ability and this will be expanded using LVS-OVA[49–52]. Determining whether these responses can be imitated or enhanced by intradermal vaccination, which remains more likely as a route of vaccination in humans, is an area of active research that will be pursued in future studies.

Development of this model is the first time that we have been able to track *Francisella*-specific T cell responses due to a lack of tool including pathogen-specific tetramers, though some *in vitro* models have been used to indirectly study the effector function of T cells in some tissues previously [19,53–55]. Given the wide range of tools available for studying OT-I memory cell responses in other infection models, LVS-OVA can be used to gain a broader understanding of intracellular bacteria and their interaction with the adaptive immune system. Future studies will incorporate challenge models using the highly virulent Type A strains of *Francisella* to

study whether there are apparent defects in the response of CD8 T cells and how these may be enhanced.

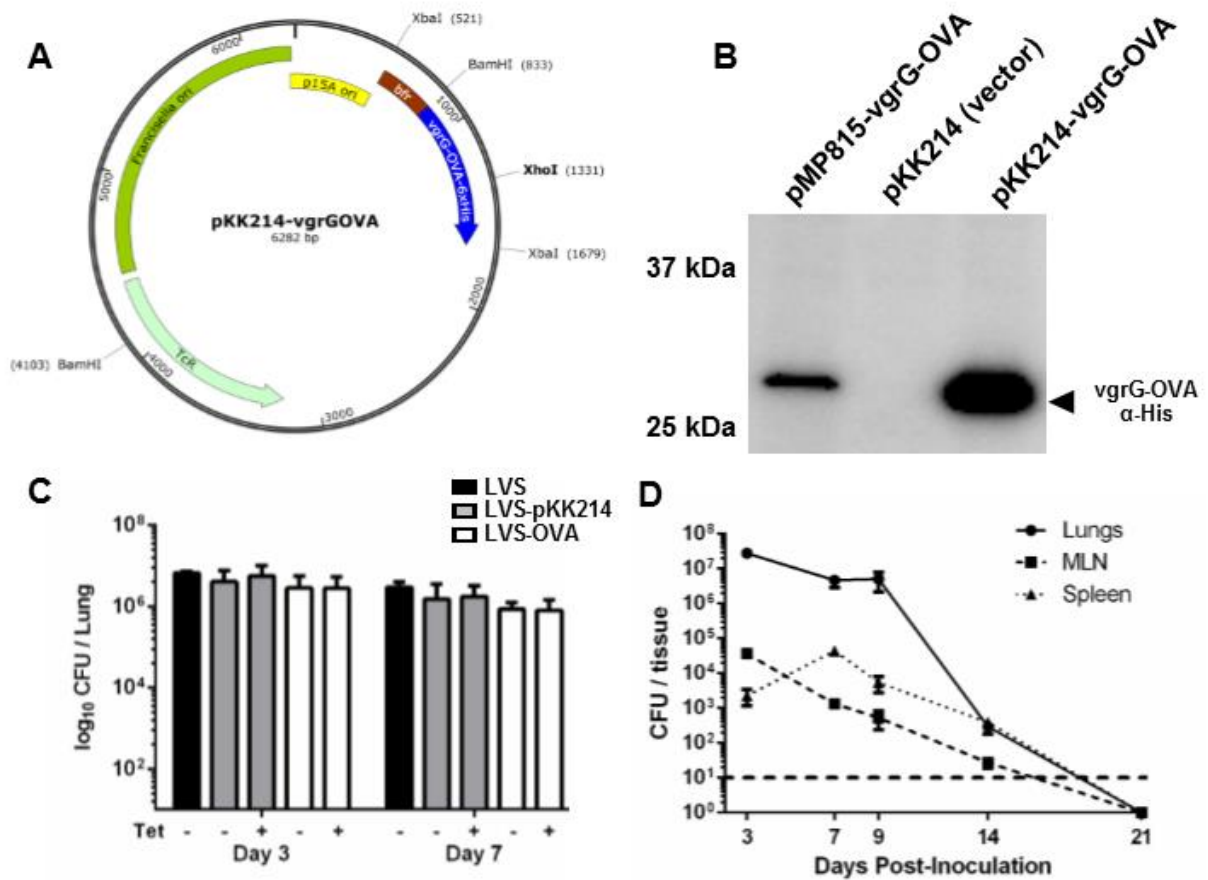


Figure 2.1. Development of a stable LVS-OVA expression construct in LVS. *Francisella tularensis* live vaccine strain was electroporated with (A) pKK214-vgrG-OVA and (B) shown to highly express vgrG-OVA by immunoblot using anti-6xHis antibody. The stability of this plasmid in the absence of antibiotic selection was determined by (C) intranasal inoculation with 1000cfu LVS-OVA and plating on either plain MHA or plates containing tetracycline. (D) Growth kinetics of the LVS-OVA strain in the lungs, MLN, and spleen were assessed at indicated time-points by serial dilution of tissue homogenates on MHA plates.

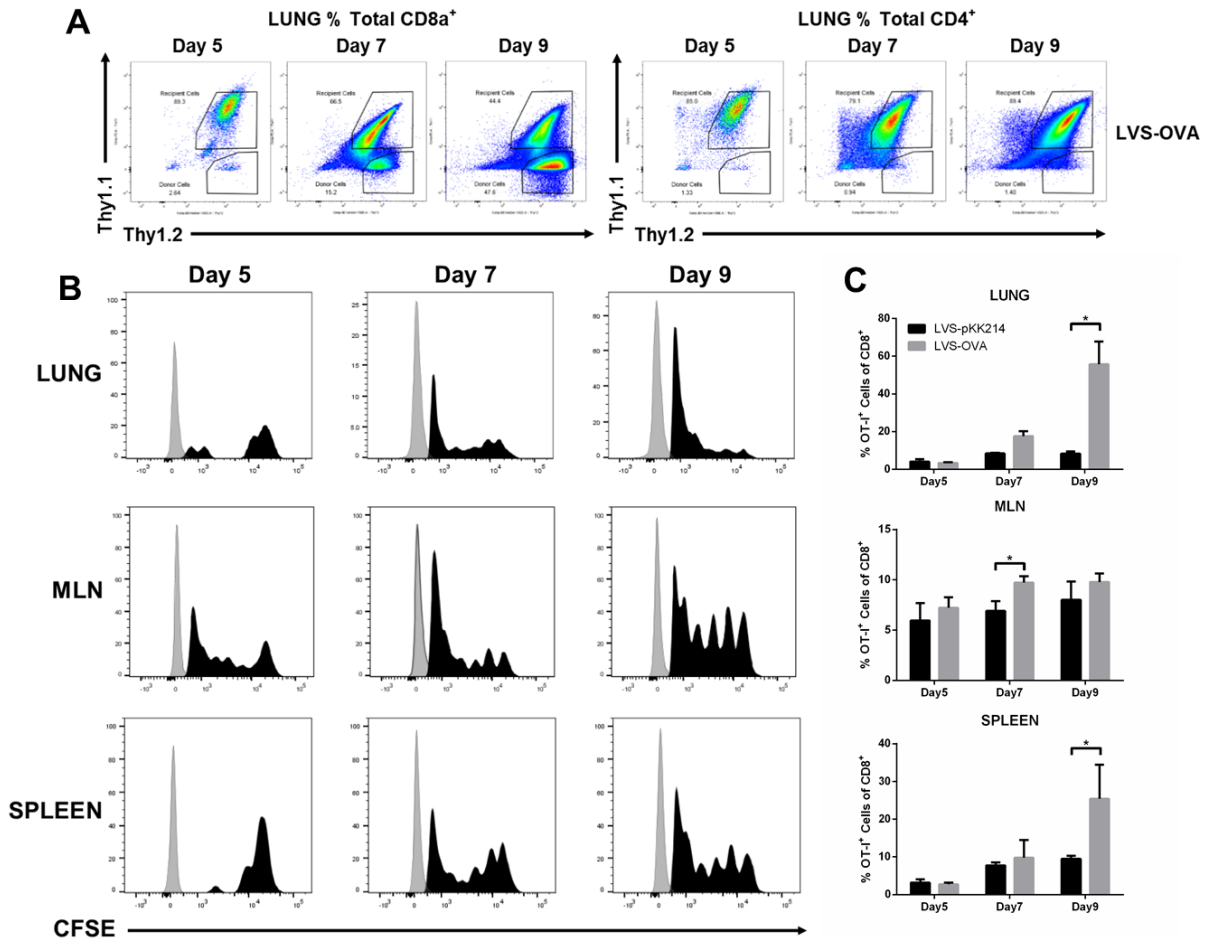


Figure 2.2. OT-I Cells Expand During Infection with LVS-OVA. After transfer of OT-I and OT-II splenocytes, mice were inoculated with 1000cfu LVS-OVA intranasally (i.n.) and tissues were collected at days 5, 7, and 9 post-inoculation. A representative dot plot (A) of the congenic markers Thy1.1/Thy1.2 on either CD8⁺ or CD4⁺ cells was used to differentiate donor OT-I or OT-II cells (Thy1.1⁺/Thy1.2⁺) and the endogenous CD8 and CD4 cells (Thy1.1⁻/Thy1.2⁺). (B) Within the CD8⁺OT-I gate, cells were divided into CFSE⁻ (gray) and CFSE⁺ (black) populations and shown as a histogram to show CFSE dilution as OT-I cells begin cell divisions. (C) Frequency of OT-I cells as a percent of the total CD8⁺ population during infection with control LVS-pKK214 (not expressing OVA) and LVS-OVA. Experiments were performed twice with (n=3)/time point. Statistical significance was determined by unpaired t-tests between control and LVS-OVA.

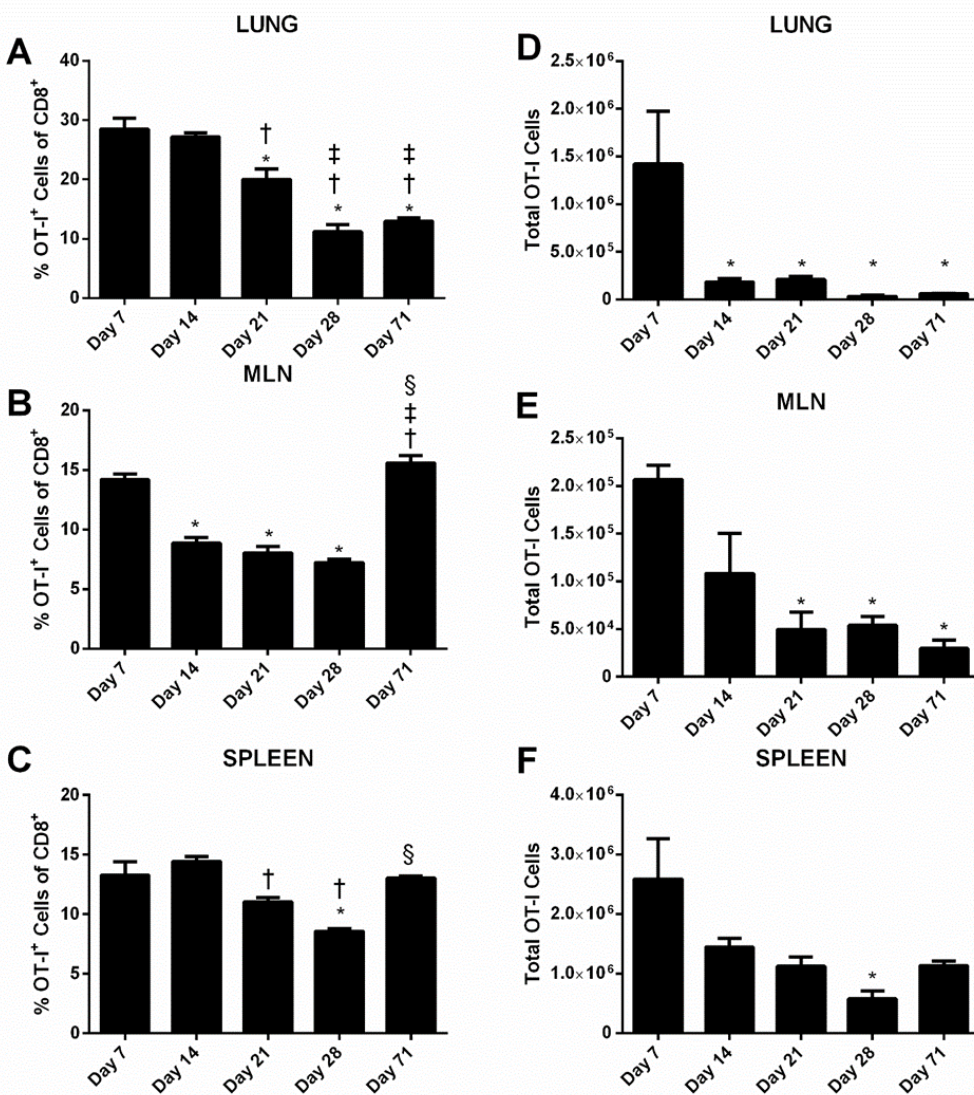


Figure 2.3. OT-I Cell Kinetics Through LVS-OVA infection. Mice (n=3) were inoculated with 1000cfu LVS-OVA 24h after transfer of OT-I and OT-II splenocytes and tissues were collected weekly until day71. The percent OT-I T cells in the CD8 population is shown for (A) lungs, (B) MLN, and (C) spleen. The total number of OT-I cells in each tissue was calculated for each time point in the (D) lungs, (E) MLN, and (F) spleen. Statistical significance was determined by one-way ANOVA followed by Tukey's multiple comparisons test with comparison between day 7 (*), day 14 (†), day 21 (‡), and day 28 (§).

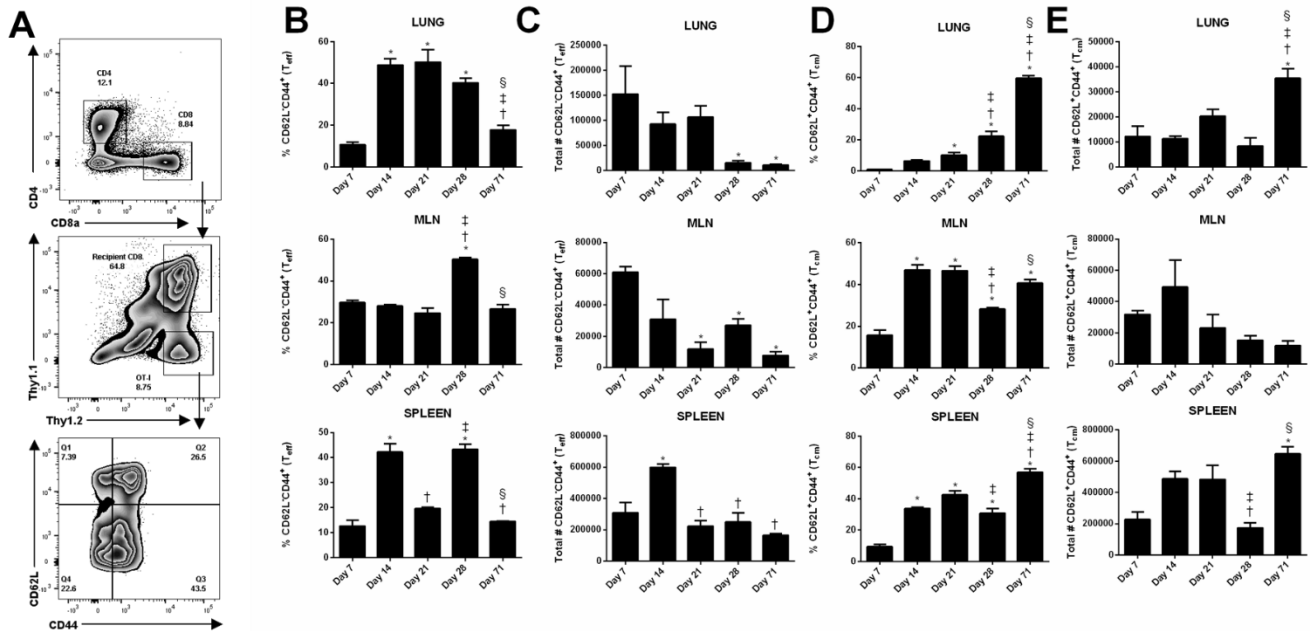


Figure 2.4. Memory markers through LVS-OVA infection. Mice were inoculated ($n=3$) with 1000cfu LVS-OVA intranasally (i.n.) 24h after transfer of OT-I and OT-II splenocytes and tissues were collected weekly to day 28. A representative dot plot showing the gating strategy for memory markers (A) is shown, gating single cells by CD4/CD8, followed by gating for Thy1 allele to identify transferred cells, followed by CD62L/CD44 gating. Memory subsets for CD8⁺ OT-I cells were gated on CD62L and CD44 to determine memory subsets CD62L⁺CD44⁺ (T effector memory, T_{eff}) and CD62L⁺CD44⁺ (T central memory, T_{cm}). The % OT-I cells of CD8 cells for (B) T_{cm}, (C) total number OT-I T_{cm}, (D) % OT-I T_{eff} and (E) total number of OT-I T_{eff} are shown. Statistical significance was determined by one-way ANOVA followed by Tukey's multiple comparisons test with comparison between day 7 (*), day 14 (†), day 21 (‡), and day 28 (§).

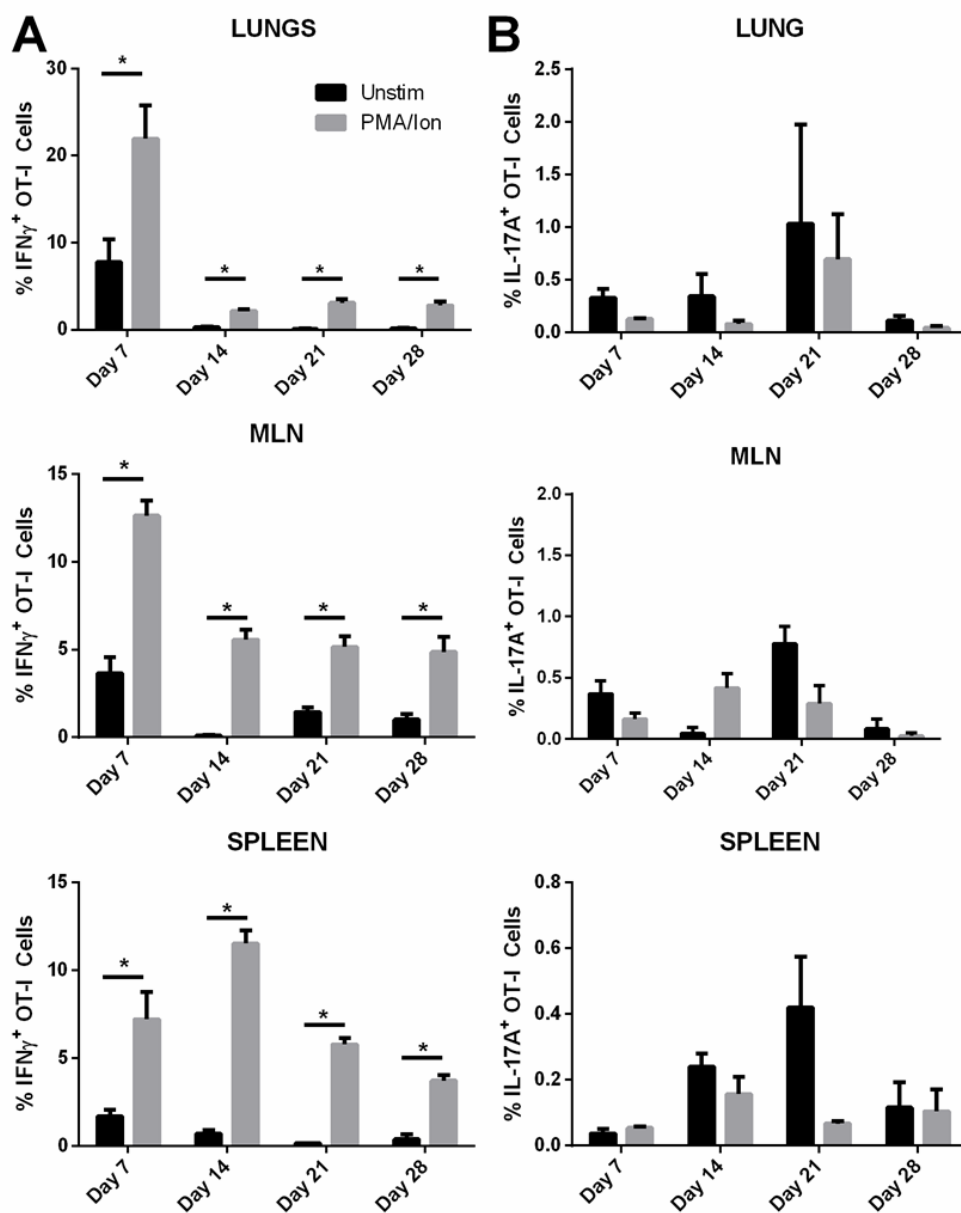


Figure 2.5. Intracellular cytokine staining of OT-I cells. Mice were inoculated (n=3) with 1000cfu LVS-OVA intranasally (i.n.) 24h after transfer of OT-I and OT-II splenocytes and tissues were collected weekly. Cells from the lungs, MLN, and spleen were incubated with either brefeldin A alone (Unstimulated) or brefeldin A plus PMA/calcium ionophore for 4h at 37°C. Cells were stained for extracellular markers to identify CD8⁺ OT-I cells followed by intracellular staining for (A) IFN γ and (B) IL-17A. Statistical significance was determined by unpaired t-tests between control and LVS-OVA followed by multiple comparison correction using the Holm-Sidak method.

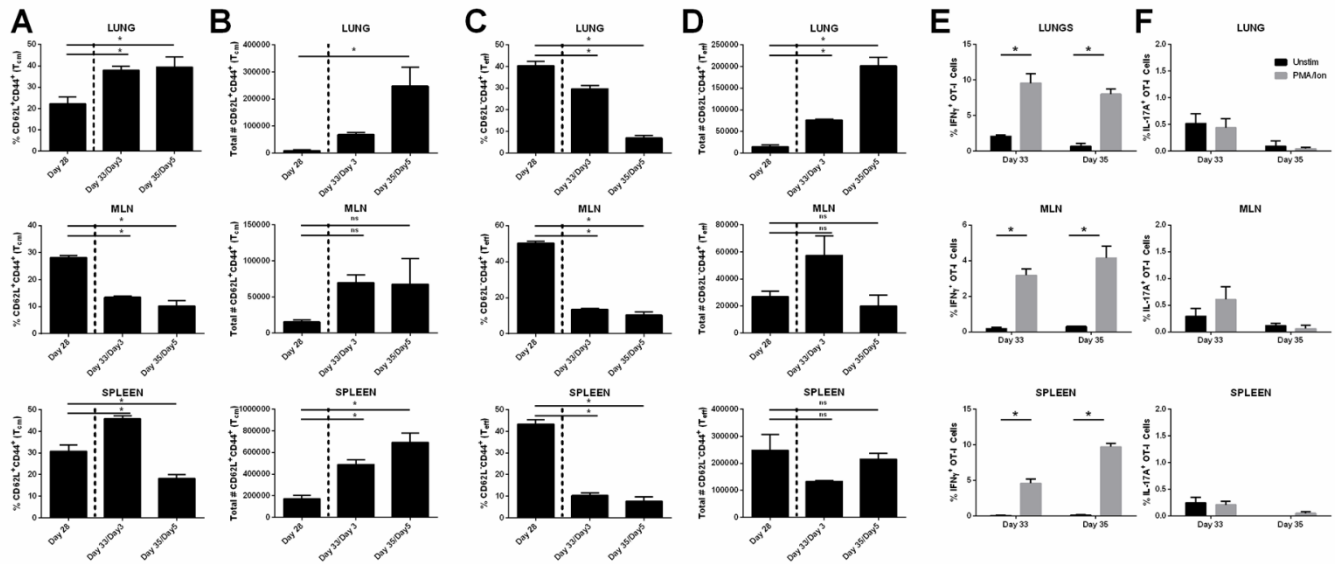


Figure 2.6. Memory T Cell Response Upon Re-Challenge with LVS-OVA. Mice (n=3) were vaccinated with 1000cfu LVS-OVA i.n. and were re-challenged at day 30 with 10000cfu LVS-OVA i.n. and tissues were collected 3 and 5 days post-challenge. OT-I T cells were stained for memory markers to identify the (A) %Tcm, (B) number Tcm, (C) %Teff, and (D) number Teff cells in the lungs, MLN, and spleen. Tissues were also stained for intracellular (E) IFN γ and (F) IL-17A in unstimulated and stimulated OT-I T cells. Statistical analysis was performed using one-way ANOVA for memory cells and unpaired t-test for comparing unstimulated to stimulated cells.

Strain or plasmid	Description	Reference or source
Strains		
<i>E. coli</i> strains		
DH5α	F- Φ 80 <i>lacZ</i> Δ M15 Δ (<i>lacZYA-argF</i>) U169 <i>recA1 endA1 hsdR17</i> (rK-, mK+) <i>phoA supE44</i> λ - <i>thi-1 gyrA96 relA1</i>	Life Technologies
<i>F. tularensis</i> strains		
LVS	<i>F. tularensis</i> subsp. <i>holarctica</i> live vaccine strain	
LVS-OVA	LVS carrying pKK214-vgrG-OVA	This study.
Plasmids		
pCR4-TOPO	Ap ^R , Km ^R , cloning vector	Invitrogen
pKK214	Tc ^R , <i>E. coli</i> - <i>F. tularensis</i> shuttle vector	Kuoppa K <i>et al.</i> (2001) From: ???
pMP815	Km ^R , <i>blaB</i> integration vector with <i>sacB</i>	LoVullo <i>et al.</i> 2009
pKK214-vgrG-OVA	Tc ^R , pKK214 containing <i>bfrp-vgrG-OVA-6xHis</i>	This study.
pKK214-OVAgB	Tc ^R , pKK215 containing <i>bfrp-OVA</i>	This study.
pMP815-vgrG-OVA	Km ^R , pMP815 containing <i>bfrp-vrgG-OVA-6xHis</i>	This study.
Primers and DNA		
Bfr_fwd (XbaI)	CAATACTGCATCTAGAGATCCATACCC ATGATGGTTAC	For pKK214
Bfr_fwd (ApaI)	TATGGGCCCCGATCCATACCCATGATGG TTAC	For pMP815
Bfr_rev (BamHI)	GCCGCGGGATCCTATTGTTACCTCCATT ATTTAAACTCTAATCA	For ligation with vgrG or OVA gBlock
vgrG_fwd (BamHI)	TATGGATCCATGTCAAAGCAGACCAT AT	For ligation with <i>bfrp</i>
vgrG_rev (XhoI)	GATCTCGAGTCCAACCATTGTTGCTGT AGA	For ligation with OVAgB
OVA gBlock	AGATATTCTAGACTCGAGGGATCCATG TCAATGTTAGTTTTATTACCAGATGAA GTTTCAGGTTTAGAACAATTAGAATCA ATTATTAATTTTGAAAAATTAAGTAA TGGACTTCATCAAATGTTATGGAAGAA AGAAAAATTAAGTTTATTTACCAAGA ATGAAAATGGAAGAAAAATATAATTTA ACTTCAGTTTTAATGGCTATGGGTATTA CTGATGTTTTTTCATCATCAGCTAATTT ATCAGGTATTTTCATCAGCTGAATCATT AAAAATTTCAAGCTGTTTCATGCTGC TCATGCTGAAATTAATGAAGCTGGTAG AGAAGTTGTTGGTTCAGCTCATCATCA	Integrated DNA Technologies

	TCATCACCATTA ACTCGAGTCTAGAGA GCTCTATAGA	
OAgB_fwd (BamHI)	TATGGATCCATGTCAATGTTAGTTTTAT TACC	For ligation with <i>bfrp</i>
OAgB_fwd (XhoI)	TATCTCGAGATGTCAATGTTAGTTTTAT TACC	For ligation with <i>vgrG</i>
OAgB_rev (XbaI)	ATATCTAGAGAGCTCTTAATGGTGATG ATGATG	For ligation with pKK214
OAgB_rev (SalI)	ATAGTCGACGAGCTCTTAATGGTGATG ATGATG	For ligation with pMP815

Table 1.1. Summary of bacterial strains, plasmids, primers, and synthetic DNA used in Chapter 3.

RAW Macrophage 24h Invasion Assay

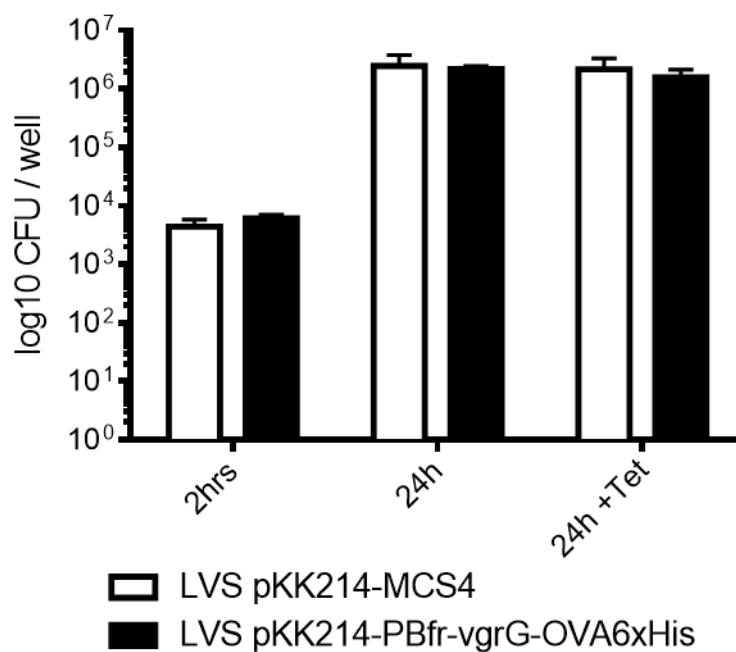


Figure S2.1. Stability of pKK214-vgrG-OVA without selection in macrophages. BMDMs were infected (MOI=100) with LVS containing the empty pKK214 vector (pKK214-MCS4) or pKK214-vgrG-OVA, treated with gentamicin after 1 hour, and incubated 24 hours without antibiotic. Cells were lysed and plated on plain and tetracycline-containing plates to determine if plasmid was maintained.

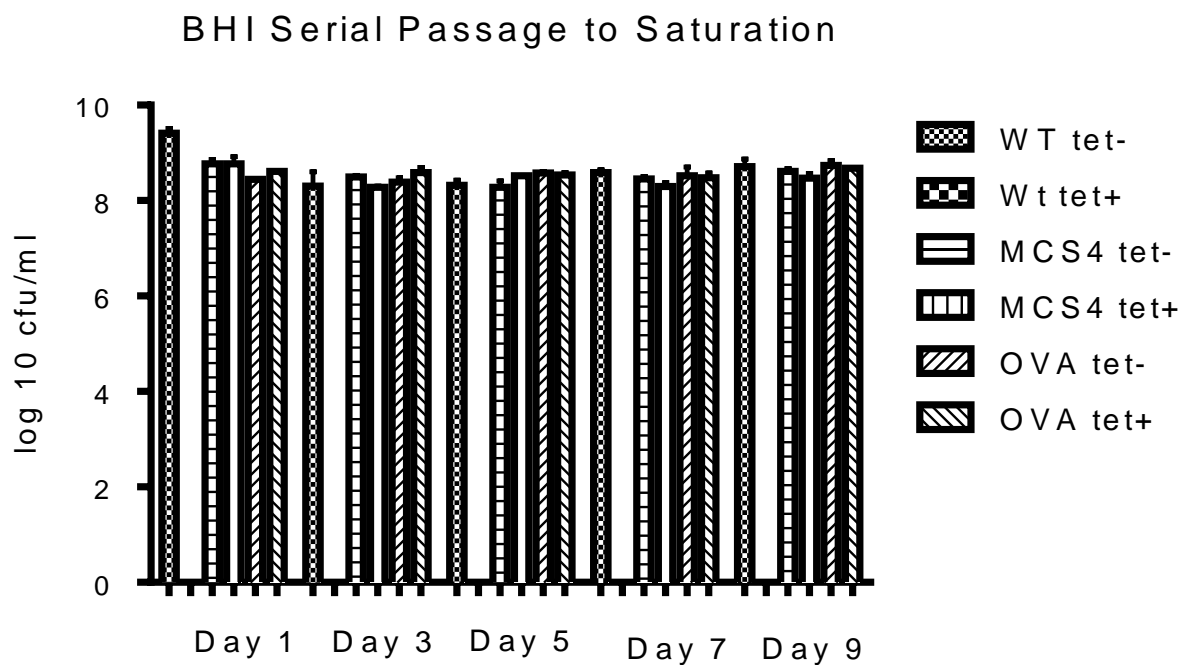


Figure S2.2. Stability of pKK214 without selection in liquid culture. LVS containing pKK214 or pKK214-vgrG-OVA were passaged from saturated overnight cultures without antibiotics and plated at the indicated time points on plain and tetracycline containing plates to determine if plasmid is maintained without selection.

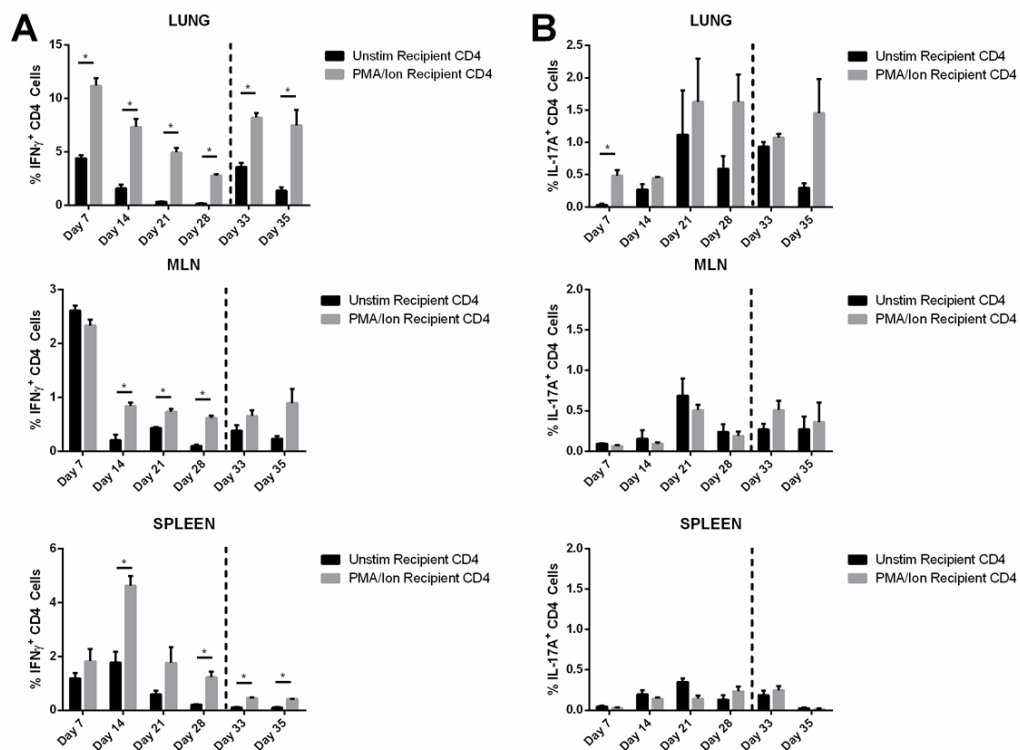


Figure S2.3. Intracellular cytokine staining of endogenous CD4 T cells. Endogenous Thy1.1+/Thy1.2+ CD4+ T cells were stained for (A) IFN γ and (B) IL-17A at the indicated time points. Dotted lines indicate re-challenge with a lethal dose of LVS. Statistical significance was determined by unpaired t-tests between control and LVS-OVA followed by multiple comparison correction using the Holm-Sidak method.

3.6 AUTHOR CONTRIBUTIONS

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Chapter 4

Review of Intradermal Immune Responses to *Francisella tularensis* Live Vaccine Strain

4.1 OVERVIEW

Francisella tularensis is a potential bioweapon which is highly infectious by multiple routes of exposure in a broad host-range of mammals including lagomorphs, rodents, non-human primates, and humans [1]. Because of its highly infectious nature and relatively high mortality rate in untreated individuals exposed to aerosolized bacteria, much of the immunology of the host-pathogen interaction has been studied in the context of the respiratory tract. While this is the most likely route of exposure for humans exposed to weaponized or accidentally aerosolized bacteria, *Francisella* can infect through the gastrointestinal tract by ingestion, handling of infected animals, and through bites of infected arthropods such as ticks and biting flies [2]. In this review, we will examine what is known about the immune response to intradermal infection rather than the more commonly studied intranasal infection.

4.2 VECTOR-BORNE INTRADERMAL INFECTION

Tabanids such as horse and deer flies transmit *F. tularensis* to humans through mechanical biting and seek multiple hosts as a part of their feeding strategy, which leads to multiple exposures with the potential for local outbreaks of tularemia [3–5]. Mosquitoes have also been shown to transmit *F. tularensis* to many small mammals but are poor long-term hosts for the bacterium [2,6]. Tick-borne *F. tularensis* has been linked to outbreaks and the bacteria has been shown to replicate well within these hosts, suggesting they are a major cause of transmission in nature [7–10]. While there are multiple vectors which transmit the bacteria intradermally, how the local immune response in the skin of these hosts interacts with the bacteria and directs the adaptive immune response is poorly understood. The clinical manifestations in humans most commonly associated with arthropod bites are ulceroglandular and glandular tularemia which present with or without an ulcer, respectively, dissemination to

lymphatics which results in enlarged local lymph nodes, and fever [6,11,12]. Our poor understanding of the dermal immune response is especially troubling given that vaccination with the *F. tularensis holarctica* live vaccine strain (LVS) is primarily administered by scarification of the skin, which is more likely to be used than intranasal vaccination [13–17].

4.3 INTRADERMAL IMMUNITY MODELS FOR LVS

Numerous species have been used to study various aspects of immunity to *F. tularensis* strains and vaccine candidates including rabbits, multiple strains of laboratory mouse, rats, guinea pigs, rhesus macaques, African green monkeys, and cynomolgus monkeys, and even humans [14,16–22]. For most animal models, however, very crude measures of their immune responses have been measured and mostly focus on their susceptibility to the different substrains of *F. tularensis*.

Rabbits

In cottontail rabbits (*Sylvilagus spp.*) for example, comparisons between type A and B strains were performed and showed that type A strains were highly virulent, killing animals between days 3-13 and causing microabscesses in livers and spleens whereas type B strains, which were rarely lethal, only experienced mild fever and lethargy with antibody responses peaking between day 14-21 [18]. While these data mirror experimental infections seen in mice, they differ somewhat from what is observed in humans, where disease is more severe with type A1b, followed by A1a and type B and not type A2 strains [23,24]. Rabbits have also been used to study attenuated SchuS4 strains by scarification (SchuS4 Δ *guaBA*, Δ *aroD*, and Δ *fipB* strains) and LVS with Δ *guaBA* and Δ *aroD* mutants protecting 27-36% of rabbits and prolonging time to death but LVS was unable to protect rabbits following aerosol SchuS4 challenge [25]. The only

local immune responses described in the skin show that injection of LVS at high doses leads to erythema and edema locally and that scarification, compared to intradermal and subcutaneous injection, lead to higher titers of F. tularensis-specific IgA [26].

Rodents

The most common animal model for studying *Francisella* immunology is the mouse, primarily Balb/c and C57BL/6 strains. The power of these animal models lies in the wide availability of gene knockouts of the immune system, which are useful for determining essential immune functions for protection to *F. tularensis*. While some differences between the mouse strains exist, many features of the immune response are shared, including the protective roles of IFN γ , TNF α , α/β T cells and others [27–31]. While much is known about the immune response to LVS and type A strains in the respiratory tract and systemic organs of infected mice, relatively little is understood about the local immune response in the skin of mice following intradermal vaccination and/or challenge with vaccine or virulent strains. Only a handful of studies have looked at the local skin immune response, but what has been shown is that both strains of mice upregulate IL-1 β , IL-6, IL-12p35, MIP-2 (CXCL2), KC (CXCL1), MCP-1 (CCL2), IP-10 (CXCL10), MIP-1 α (CCL3), MIP-1 β (CCL4), and RANTES (CCL5); Balb/c but not C57BL/6 upregulate IFN γ and IL-10; and C57BL/6 but not Balb/c upregulate IL-12p40, TNF α , and iNOS [32]. Another study has identified that Balb/c mice intradermally vaccinated with LVS express IL-12, TNF α , and IFN γ in the skin following a challenge with a lethal dose of LVS 5-6 weeks later, and that vaccinated mice prevent dissemination of challenge LVS to livers and spleens [19]. Interestingly, vaccination of Balb/c and C57BL/6 mice with LVS intradermally lead to different levels of protection against virulent *F. tularensis*, with Balb/c strains being protected against aerosol (type B) and intradermal (type A and B) infection while C57BL/6 were only

protected against type B strains and not type A (aerosol or dermal); neither strain was protected against aerosol challenge with a type A strain following dermal vaccination with LVS [33]. Additionally, neutrophils have been implicated in protection against primary intradermal LVS infection [34]. It has been suggested that different phagocytic cells disseminate LVS from the skin than those in the respiratory tract with interstitial macrophages and neutrophils containing LVS in the lungs compared with primarily alveolar macrophages when LVS is given intranasally, but the specific cell tropism in the skin has not been characterized [35]. Much of the focus to date has been on how the different routes of vaccination differ in their levels of protection provided following intranasal challenges and the differences the route of vaccination has on the subsequent immune response in the respiratory and systemic organs, but not the local immune response in the skin, which may have profound implications for improving future intradermal vaccines.

Non-Human Primates

Non-human primates have also been used to study immunity to *F. tularensis* including rhesus monkeys, African green monkeys, marmosets and cynomolgus monkeys [21,21,36–38]. While each of these primate models was susceptible to lethal respiratory infection by virulent *F. tularensis* the dermal immune response was poorly characterized beyond local replication of LVS in the skin and local lymph nodes and development of anti-*Francisella* antibodies, which were higher in respiratory vaccinated animals as compared with intradermal vaccination [37]. Similar to mouse models, dermal vaccination provided less protection than respiratory vaccination with LVS in monkeys and did not delay enlargement of trachea-bronchiolar lymph nodes, but it was able to prevent fatal septicemia as seen in unvaccinated animals when challenged with virulent SchuS4 [37].

Humans

Despite LVS being an experimental vaccine and virulent *F. tularensis* having a high mortality, humans have been involved in vaccination and challenge studies in the past. Prior to development of the LVS strain, the Foshay killed-bacteria vaccine was tested and shown to elicit weak antibody responses and failed to protect against local skin lesions in challenged individuals [39,40]. In unvaccinated individuals, intracutaneous inoculation lead to local skin lesions, antibody responses, regional adenopathy, and the majority of individuals required antibiotic treatment due to systemic effects including fever, anorexia and fatigue [16]. A second study of subjects vaccinated with Foshay vaccine and challenged cutaneously showed the majority experienced local lesions and a minority showed systemic disease requiring treatment by antibiotics, while another subset of subjects required removal or drainage of lymph nodes months after challenge [16]. Booster vaccination with Foshay vaccine 6-8 months after initial vaccination also failed to protect from local lesions, adenopathy, and systemic disease [16]. In the same study, individuals who were challenged with virulent *F. tularensis* and treated with antibiotics were re-challenged and showed no protection to skin lesion and mild protection of systemic disease in only a few individuals [16].

Initial studies comparing the LVS strain given by scarification to Foshay vaccine in humans showed increased protection from disease following low-dose aerosol challenge, but the protection was overcome by increasing challenge doses [15,17]. While high-dose aerosol vaccination with LVS was shown to be more protective than low-dose aerosol or scarification methods, the logistics and concern of aerosol vaccination led to scarification being adopted in the US [41]. More recently, LVS vaccine testing in humans was performed by scarification and subcutaneous inoculation, which were well tolerated at all doses with increasing doses

correlating with higher serum IgG, IgM, IgA and IFN γ responses [42]. While antibodies are useful indicators for exposure to *Francisella*, they are not yet correlated with protection in humans [43]. The LVS bacteria were not detected in any blood samples, suggesting it is restricted mainly to the skin in humans [42]. While microagglutination titers in this study correlated with the degree of reddening of the skin, IFN γ correlated with hardening of the injection site [42]. Considering the vast majority of LVS remain in the skin of vaccinated humans, understanding how the immune system is interacting with the infection could reveal ways to generate greater protection.

4.4 FUTURE PERSPECTIVE

The increasing importance of differences in the route by which vaccinations are given and their effect on challenge at distal mucosal sites has been appreciated in multiple pathogen models which have unsuccessful vaccines today, including chlamydia, HIV, tuberculosis and others [44–49]. Newer strategies for manipulating the development of protective immune responses include prime-boost vaccines, which may target different sites with different vaccine components and adjuvants to increase protection [50,51]. Understanding the basic immune response to *Francisella* in the skin may be required to understand why scarification fails to provide efficient protection against intranasal challenge. Future work studying the differences between the intradermal and intranasal routes of vaccination and which components of the immune response are correlated with protection will likely lead to better vaccines against the highly virulent *F. tularensis* strains.

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Chapter 5

Summary and Discussion

SUMMARY AND DISCUSSION

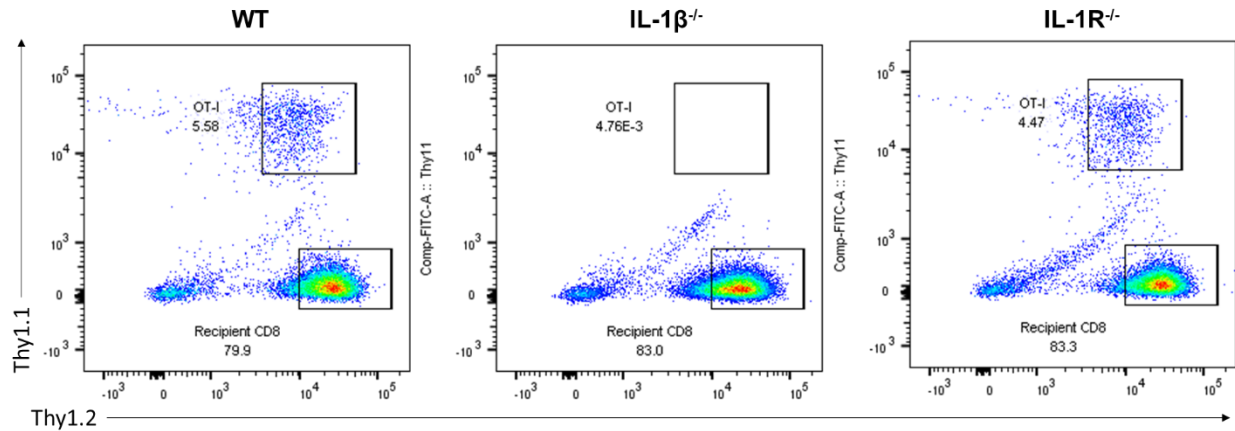
The work presented in this thesis focuses on how the innate immune system, through interactions with the bacteria *B. pertussis* and *F. tularensis*, can shape the adaptive immune response. The role of the inflammasome and IL-1 signaling was studied in the context of pertussis infection and it was found that while IL-1 β signaling was required for control of pertussis in primary infections and after vaccination, the inflammasome was not required for either infection. This was in contrast to the prevailing suggestion by Dunne et al. looking at a purely *in vitro* assay system [1].

The broader implication of the finding that IL-1 is important for both primary and secondary challenges to *B. pertussis* is that the original vaccine, which contains whole killed bacteria (wP), induced a potent IL-1 response. Unfortunately, the whole-cell vaccine also had side-effects which led to its replacement with an acellular vaccine containing purified proteins of *B. pertussis* and contains an adjuvant which primarily acts through activation of the NLRP3 containing inflammasome. Our finding that the whole-cell vaccine protects caspase-1 deficient mice suggests that the inflammasome-independent IL-1 response is sufficient for protecting mice after vaccination. This suggests that the acellular vaccine, which is known to be less protective than the whole-cell vaccine, likely has a sufficient IL-1 response. A more recent study has backed this up, showing that IL-1 generated by alum adjuvant in the current acellular pertussis vaccine is important for generating Th2 and Th17 cells and antibodies, but results in poor generation of Th1 cells [2]. Efforts to improve the acellular vaccine, therefore, will likely depend on generating responses beyond IL-1. Recent studies by Asokanathan et al. and Ross et al. have recently suggested the inclusion of CpG adjuvants in acellular pertussis vaccinations to generate more potent Th1 responses against *B. pertussis* [3,2].

The development of *Francisella* LVS which expressed OVA was also described and our initial experiments examined the pathogen-specific CD8 T cell response, which had previously been limited by a lack of tools. We found that CD8 memory T cells are found in the lungs, local lymph nodes, and spleen and that they persist to at least day 71. We were also able to show that central memory CD8 T cells are generated and recall rapidly upon re-exposure to a lethal dose of *Francisella*. We were also able to show that these CD8 T cells produced IFN γ but not IL-17A, similar to what many other infection models have shown. Intradermal vaccination has been shown to produce even greater IFN γ responses in the lung, but the stimulation methods used are different from those used in this study

Ongoing experiments using this tool aim to investigate the difference between intranasal vaccination, which was presented in this thesis, and intradermal vaccination, which is another major route by which animals humans are vaccinated. The intradermal route of vaccination is primarily understood in the context of how it protects or fails to protect mice upon challenge with virulent strains of *Francisella*. Using a novel OVA-expression plasmid, we hope to begin understanding the basic phenotypic differences of the CD8 T cell compartment following vaccination by intradermal and intranasal routes.

Another exciting, preliminary finding which overlaps with previous work looking at the role of IL-1 in adaptive immunity, but in the context of *Francisella* CD8 T cell responses, is that IL-1 receptor signaling on OT-I T cells appears to be required for the survival of transferred CD8 T cells after they have begun proliferation (Figure 5.1). Future experiments are required to confirm this finding but it would be the first observation of this phenotype. The role of different cytokines in the development of *Francisella*-specific CD8 T cell responses can be studied by



transfer of OT-I cells into different knockout mouse models, all made possible by the development of this novel LVS-OVA model.

Figure 3.1. IL-1 Is Required for Survival of OT-I T cells. OT-I (Thy1.1⁺/Thy1.2⁺) splenocytes were transferred into wild-type, IL-1 β ^{-/-} and IL-1R^{-/-} recipients (Thy1.2^{+/+}) 24h before inoculation with 1000cfu LVS-OVA intranasally. On day 14 post-inoculation, spleens were dissected and stained for Thy1 alleles.

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SELECTED PUBLICATIONS

1. **Place DE**, Williamson DR, Yuzefpolskiy Y, Katkere B, Kalia V, Kirimanjeswara GS. Caspase-1-independent interleukin-1 β is required for clearance of *Bordetella pertussis* infections and whole-cell vaccine-mediated immunity. *In prep.*
2. **Place DE** & Kirimanjeswara GS. Development of a Novel *Francisella tularensis* Live Vaccine Strain Expressing Ovalbumin Reveals Memory and Effector CD8 T Cell Responses During Intranasal Vaccination and Re-challenge. *In prep, Minireview Front. Immunol.*
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