Mode of Adjuvant Action of the Nasally Delivered Cytokine Interleukin 1 Alpha

by

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Dissertation submitted in partial fulfillment of
the requirements for the degree of Doctor of Philosophy in the Department of Pathology in the Graduate School
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ABSTRACT

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Abstract

Although monophosphoryl lipid A was recently approved by the Food and Drug Administration, more vaccine adjuvants are needed to meet the demand for vaccines against new, emerging, and re-emerging diseases. Additionally, characterizing the mechanisms of action of potent vaccine adjuvants is important for moving toward more rational vaccine design based on the careful selection of antigens and adjuvants to stimulate only the desired immune responses. Two experimental vaccine adjuvants, compound 48/80 (C48/80) and IL-1, were evaluated in these studies. The safety and efficacy of the mast cell activator C48/80 was evaluated when used as an adjuvant delivered intradermally (ID) with recombinant anthrax protective antigen (rPA) in comparison with two well-known adjuvants. Mice were vaccinated in the ear pinnae with rPA or rPA + C48/80, CpG oligodeoxynucleotides (CpG), or cholera toxin (CT). All adjuvants induced similar increases in serum anti-rPA IgG and lethal toxin-neutralizing antibodies. C48/80 induced balanced cytokine production (Th1/Th2/Th17) by antigen-restimulated splenocytes, minimal injection site inflammation, and no antigen-specific IgE. Our data demonstrate that C48/80 is a safe and effective adjuvant, when used by the intradermal route, to induce protective antibody and balanced Th1/Th2/Th17 responses. Histological analysis demonstrated that vaccination with C48/80 reduced the number of resident mast cells and induced an injection-site neutrophil influx within 24 hours. Nonetheless, rPA + C48/80 significantly increased antigen-specific IgG titers in mast cell-deficient mice compared to antigen alone, suggesting that C48/80 has mast cell-dependent and mast cell-independent mechanisms of action.

IL-1α and β have been shown to have strong mucosal adjuvant activities, but little is known about their mechanism of action. Bone marrow chimeric mice were intranasally vaccinated with *Bacillus anthracis* lethal factor (LF) with or without 4 µg IL-1α or a control adjuvant (cholera toxin) to determine if IL-1R1 expression on stromal cells or hematopoietic cells was sufficient for the maximal adjuvant activity of nasally delivered IL-1α. IL-1α was not active in IL-1R1-deficient (*Il1r1*$^{-/-}$) mice given *Il1r1*$^{-/-}$ bone marrow, demonstrating that the adjuvant activity of IL-1 was due to the presence of IL-1R1 and not contaminants. Cytokine and chemokine responses induced by vaccination with IL-1α were predominantly derived from the stromal cell compartment and included G-CSF, IL-6, IL-13, MCP-1, and KC. Nasal vaccination of *Il1r1*$^{-/-}$ mice given wild-type bone marrow (WT→KO) and WT→WT mice with LF + IL-1α induced maximal adaptive immune responses, while vaccination of wild-type mice given *Il1r1*$^{-/-}$ bone marrow (KO→WT) mice resulted in significantly decreased production of LF-specific serum IgG, IgG subclasses, lethal toxin-neutralizing antibodies, and mucosal IgA compared to WT→KO and WT→WT mice ($p < 0.05$). Our results suggest that IL-1R1 expression in the hematopoietic compartment is sufficient for the maximal induction of antigen-specific adaptive immunity after nasal vaccination adjuvanted with IL-1α and that while stromal cells are required for maximal adjuvant-induced cytokine production, the adjuvant-induced stromal cell cytokine responses are not required for effective induction of adaptive immunity.

Dedication

This dissertation is dedicated to my loving husband, Marshall Thompson, and both of our families. Without your support, this would never have been possible.

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1. Introduction

1.1 Vaccines

1.1.1 A Brief History and Overview

The practice of inoculation against disease may have begun as early as the 10th century in China, but records show that the Chinese had at least four methods of inoculation against smallpox by 1695, and variolation was practiced as early as the 16th century by Brahmins in India [1]. Despite the known link between variolation and protection against smallpox, the practice did not catch on in Europe or the Americas until the 18th century. Although Edward Jenner is generally credited with developing the first vaccine, a cattle breeder in England named Benjamin Jesty is the first known individual to have vaccinated his family against a smallpox outbreak using material taken from cattle infected with cowpox in 1774 [2]. However, Edward Jenner was the first scientist to initiate a vaccination program and publish his findings in 1796 and 1798.

Since the time of Jenner, immunization programs have led to the eradication of smallpox, the near eradication of polio, and the control of several other infectious diseases, including measles, mumps, rubella, and diphtheria. The effort to create vaccines against several new and emerging diseases is currently under way. Unfortunately, not all diseases can be successfully targeted for eradication, as many infectious organisms have multiple host species. These diseases may instead be targeted for elimination, defined by the CDC as “reduction to zero of the incidence of infection caused by a specific agent in a defined geographical area as a result of deliberate efforts; continued measures to prevent re-establishment of transmission are

required," or control, defined as "the reduction of disease incidence, prevalence, morbidity or mortality to a locally acceptable level as a result of deliberate efforts; continued intervention measures are required to maintain the reduction" [3]. Examples of these include measles and diarrheal diseases, respectively.

Vaccines are useful measures for infectious disease control and elimination. Though some vaccines are mainly beneficial to the individual receiving them (tetanus), many others are beneficial to the community as a whole by the induction of "herd immunity". Herd immunity is beneficial for protection against diseases requiring person to person transmission. For example, vaccinating just a portion of a population can reduce the number of susceptible individuals a disease can be transmitted to, thus resulting in a fall of the disease reproduction rate [4]. This herd immunity requires ongoing vaccination of the population and can wane in the event that vaccination stops. The importance of herd immunity is also apparent when one considers groups that refuse vaccination, such as populations refusing on religious grounds [5, 6] and more recently out of fears of vaccine-induced autism [7]. These groups have recently seen increased rates of vaccine-preventable diseases, including measles [8] and pertussis [9], and have been at the root of many disease outbreaks [8].

1.1.2 Vaccine antigens

Immunity is established through vaccination by stimulating the immune system to produce adequate levels of antibody and a population of memory cells that will respond rapidly to infection with the target organism and provide protection. Vaccines may use several different types of antigen, including inactivated organisms, live attenuated organisms, protein subunits, or polysaccharides.

1.1.2.1 Inactivated organisms

Killed organisms maintain their antigens while being unable to replicate in the host. Vaccines using inactivated organisms are therefore able to induce antibody to a number of different antigens and induce a primarily CD4+ T helper cell response [10]. Unfortunately, because they are unable to replicate in the host, they generally induce less potent immune responses than attenuated organisms and generally require the addition of adjuvants (e.g., aluminum salts) and/or multiple immunizations [11]. Several examples of this type of antigen exist on the market today, including the trivalent inactivated influenza vaccine, the whooping cough (*Bordetella pertussis*) vaccine, and the Salk inactivated polio vaccine.

1.1.2.2 Attenuated organisms

Attenuated organisms are able to actively replicate in the host but are unable to cause significant disease. These vaccines tend to induce long-lasting immunity and may only require one initial immunization with no subsequent boosts, as living microbes confront the host with larger and more sustained doses of antigen [12]. Currently used examples of systemically administered attenuated organism vaccines include the yellow fever vaccine and the smallpox vaccine. However, attenuated organisms are also popular for use in mucosally administered vaccines, including the oral Sabin polio vaccine and the more recently FDA approved nasal FluMist® vaccine. The immune system is able to respond to these vaccines in much the same way as to natural infection, and in the case of attenuated viruses, the immune system can generate cytotoxic T cell (CTL) memory, as the attenuated viruses are able to replicate intracellularly [10, 11].

The active replication is also effective at inducing long-lasting immunity. Investigation into the long-term immunity in humans induced to the DryVax® smallpox vaccine has demonstrated stable maintenance of memory B cell populations lasting greater than 50 years [13]. Another study investigating both antiviral antibody and antiviral T cell responses showed stable persistence of antibody for 1-75 years after vaccination, although there was a more drastic decline in T cell responses, with a half-life between 8-15 years [14]. In response to replicating antigens, extensive homing of antibody-secreting cells to the bone marrow can be detected by week 2 or 3 of the primary response, whereas non-replicating antigens generally require a secondary response [15].

Unfortunately, there are drawbacks to the use of attenuated organisms in vaccines. These vaccines are unsafe for use in immunocompromised individuals, as even an attenuated strain may cause severe reactions [16]. This has been documented with vaccination against herpes varicella zoster [17]. Additionally, attenuated organisms have the ability to mutate or revert back to a virulent form [18].

1.1.2.3 Subunit vaccines

The use of individual protective antigens from organisms has benefits over the use of whole organisms. Whole organisms have multiple antigens that can be recognized by the immune system, which may interfere with the ability of the immune system to recognize the protective antigens [12]. The multiple epitopes presented also increase the risk of inducing hypersensitivity [12]. By contrast, subunit vaccines are generally safe and well tolerated by both humans and animals [19], and they present only a limited number of antigenic epitopes. Subunit vaccines comprise a number of

types of antigens, including recombinant proteins, DNA vaccines, and virus-like particles (VLPs) [20, 21]. Bacterial exotoxins, such as the diphtheria and tetanus toxins, are commonly used in vaccine preparations. The toxins must first be inactivated, a process that currently uses formaldehyde to cross-link and inactivate the proteins, forming a toxoid. Protective antibodies are generated against the toxoids, and upon subsequent challenge, the toxins are neutralized by antibody binding and are then phagocytosed [22]. Toxin subunits, such as *Bacillus anthracis* protective antigen, can also be immunogenic and non-toxic when administered without one of the other toxin subunits (e.g., lethal factor and edema factor) [23, 24]. Although protein subunits are safer than whole-killed or live attenuated organisms, they are not generally as immunogenic and require the addition of adjuvants to vaccines to stimulate protective immunity [25]. Advancing the development of molecular vaccines was recently put forth as one of the top initiatives in vaccine development by the United States-European Commission Task Force on Biotechnology Research, which cited the many "favorable characteristics of subunit vaccines, such as simplicity of design, ease of production and potential agility in the face of emerging infectious threats" as grounds for facilitating and supporting their development [19]. Current subunit vaccines include the toxoid-based diphtheria and tetanus vaccines, the acellular pertussis vaccine, and vaccines using VLPs, such as the hepatitis B vaccine [11, 26] and the recently approved HPV vaccine, Gardasil® [21].

1.1.2.4 Polysaccharide vaccines

Polysaccharide antigens have the ability to induce B cell activation in the absence of T cell help and subsequent production of IgM and IgG1antibodies in mice [27]. In the absence of a protein carrier, these antigens are generally unable to induce

memory B cell development [28]. However, Obukhanych et al. have demonstrated the ability of polysaccharide antigens to induce a memory B cell population with similar antigen responsiveness to naïve B cells; unfortunately, secondary activation of these cells is negatively regulated by antigen-specific IgG [29]. Vaccines against *Streptococcus pneumoniae*, *Neisseria meningitidis*, *Haemophilus influenzae* type B, and *Salmonella typhi* are based on their capsular polysaccharides [28, 30]. Although effective in adults, inducing protective anticapsular antibodies that persisted for 5-10 years, these vaccines were less effective in children, for whom only 20% had detectable antibodies after 12 months, and were not at all immunogenic in infants less than 2 years of age [28]. Recently, however, vaccines with protein carrier-conjugated polysaccharide antigens have been developed [28, 30, 31]. These vaccines stimulate T cell-dependent B cell responses and are much better at inducing long-lasting memory responses to antigen [28]. For *H. influenzae* type b, the conjugate vaccines have proven more effective, especially in children under two years of age [28, 32]. In instances where target pathogens have multiple serotypes (e.g., meningococci and streptococci), however, the use of conjugate vaccines has been more restricted due to the expense and distribution of different serotypes geographically [28].

1.1.3 Considerations in vaccine development

Several factors are required for a successful vaccine: effectiveness, availability, stability, cost per dose, and safety [12, 33]. Vaccines must stimulate protective levels of immunity at relevant sites, which last over a sustained period of time [12]. Vaccines requiring frequent boosts throughout the lifetime of an individual are not feasible. Cost is also a largely prohibitive factor in creating new vaccines, as the estimated cost of

developing a new vaccine is over 500 million dollars [33]. General production costs must also be considered, as vaccine antigens may be expensive to produce and cost is prohibitive to the application of vaccines in developing countries [33]. The cost-effectiveness of vaccines varies based on factors such as the cost per dose, induction of herd immunity, and the likelihood/cost of adverse events from a disease in the absence of vaccination [34]. Some vaccines are therefore much more cost effective than others, but even this can depend on the population receiving the vaccine, with those at high risk receiving more benefit than those that are unlikely to contract infection or have increased morbidity/mortality as a result of infection [34]. Vaccines that are cost effective in developed countries may not be cost effective in developing nations and vice versa. The use of adjuvants in vaccines to boost immune responses with lower doses of antigen and fewer boosts, which may reduce overall costs, will therefore be of great benefit.

1.1.4 Mucosally administered vaccines

Currently, there are several mucosally administered vaccines licensed for use in humans. These include the oral polio vaccine (OPV), *Salmonella typhi* Ty21a oral vaccine, the killed whole cell *V. cholerae* plus recombinant cholera toxin B subunit (Dukoral®), the live attenuated *V. cholerae* O1 strain CVD 103-HgR oral vaccine (Orochol®), and a cold-adapted influenza nasal vaccine (FluMist)®. Vaccines administered by a systemic route are also in use for many of these diseases. Several studies have compared the use of OPV to the use of the inactivated polio vaccine (IPV), and most have concluded that both have advantages and drawbacks [35]. Although OPV has the advantage of stimulating mucosal immunity to polio virus, it also carries the risk of inducing paralysis with the administration of the first dose. IPV, however, does

not carry the risk of inducing paralysis, but it is unable to stimulate mucosal immunity [35, 36]. Both oral cholera vaccines have proven to be as effective as the inactivated whole cell parenteral vaccine in several clinical trials [37]. The cold-adapted influenza vaccine is also able to induce greater mucosal IgA responses than the trivalent influenza vaccine (TIV), despite inducing lower serum IgG responses [38]. Both vaccines have been shown to reduce viral shedding and to have similar efficacy in preventing laboratory-confirmed influenza, with FluMist® providing 85% effectiveness compared to the 71% effectiveness of TIV [38]. Although TIV is effective in most adults, systemic immune responses are not induced in individuals that have not been previously primed, including children [38]. In this group, FluMist® has been shown to be effective after a single dose [38]. Additionally, FluMist® has been shown to be more effective than TIV at preventing seasonal influenza infection in children less than 59 months of age [39]. Nonetheless, in a healthy adult population, FluMist® has been shown to be less effective than TIV [40].

It is evident from the currently licensed examples that mucosally administered vaccines are able to elicit the mucosal arm of the immune system in the response to immunization. Unfortunately, mucosal vaccination strategies have many challenges to overcome. Immunizing antigens delivered at mucosal surfaces face the same barriers as invading pathogens, including epithelial tight junctions and mucosal secretions that serve to wash away potential pathogens. Therefore, greater amounts of and/or stronger immunogens are required to induce immune responses following vaccination [41]. Although live attenuated organisms are potent stimulators of immunity when delivered mucosally, there are drawbacks to their use as vaccine antigens (as discussed above).

Unfortunately, they are the current standard in mucosal vaccines. Dukoral® is the only subunit-based mucosal vaccine on the market, but it is not currently licensed for use in the US. Vaccine adjuvants will likely be required to induce potent immune responses to subunit vaccine antigens delivered systemically and locally. The work described herein provides new information that contributes to the information necessary to develop new subunit vaccines that will be safe for use in humans.

1.2 Mucosal Immune System

1.2.1 Mucosal-associated lymphoid tissue (MALT)

Mucosal surfaces consist of inductive sites, where antigens encounter primarily immature immune cells and are processed for presentation, and effector sites, where primed immune cells (e.g., mature T and B cells) are deployed to interact with pathogens and combat infection (e.g., cytotoxic functions of T cells and antibody secretion by plasma cells). MALT is important tissue for the induction of mucosal immunity, characterized by the presence of organized lymphoid tissue underlying a follicle-associated epithelium (FAE) containing microfold cells (M cells) [42-44]. Foreign antigens encountered at mucosal surfaces are transported, processed, and presented at inductive sites throughout the body, which are connected to effector sites by the common mucosal immune system [44], where immune effector cells are deployed [44]. The effector sites of MALT are present in all mucosal tissues and comprise disseminated/nonorganized lymphoid tissue [45, 46]. Peyer's patches in the distal ileum and nasopharynx-associated lymphoid tissue (NALT) are two of the main inductive sites of MALT [44]. NALT was first recognized in rodents and is thought to be analogous to the Waldeyer's ring in humans [44], though recently, follicles formed by lymphoid

aggregates have been identified in the nasal mucosa of children [44]. NALT is comprised of FAE with M cells, high endothelial venules, T and B cell-enriched areas, and antigen-presenting cells (APCs) [44], and it has been found to be a potent inductive site for the mucosal immune system [44].

1.2.2 Mucosal immune responses

1.2.2.1 Innate immune responses

The immune response to an antigen can be characterized in two main branches, the innate immune system and the adaptive immune system. The innate immune system is the first responder to infection and can be induced when local cell populations encounter specific pathogen associated molecular patterns (PAMPs), such as polysaccharides or double-stranded RNA. The local cell populations, including epithelial cells and resident DCs, recognize PAMPs through pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs) [47]. Ligation of these receptors leads to the production of cytokines and chemokines [47], which can activate APCs and attract inflammatory cell populations, such as neutrophils, which can phagocytose microorganisms/particles or degranulate and release molecules such as defensins [48].

Pathogens encountered at mucosal surfaces can interact with the epithelium and DCs or be transported across the mucosal barrier through M cells, where they are able to interact with APCs and lymphocytes on the basolateral side. Mucosal epithelial cells responding to the presence of pathogens through TLR signaling produce cytokines and chemokines that act on cells underlying the mucosal surface, such as DCs [49], which can then extend projections into the lumen where they can directly interact with antigen [50]. It is unclear as to whether DCs need TLR signals to extend projections into the

lumen. Studies have focused on the presence of TLRs on gut epithelium and have not directly tested the requirement for TLR expression on DCs [50]. However, evidence suggests that DCs do not require TLR ligation to extend projections across the mucosal barrier, as lamina propria DCs do not express TLR4 [51], but LPS alone can still induce DC extension across the lamina propria [50]. It is likely that they instead respond to CX3CL1 (murine neurotactin), as mice deficient in CX3CR1 are unable to extend dendrites into the lumen [52]. It has also been demonstrated that DCs can extend projections through stratified and pseudostratified epithelia that lack tight junctions, such as those present in the oral cavity, pharynx, esophagus, urethra, and vagina [53]. Despite these findings, the involvement of DC projections in the response to the mucosal delivery of vaccines and vaccine adjuvants has not been evaluated.

M cells present in the epithelium contain a large invagination on their basolateral side where immune cells, such as lymphocytes and DCs, can associate with them. M cells promote uptake of foreign particles and deliver them via transepithelial transport to the basal side of the epithelium where they interact with the associated lymphocytes and DCs [42, 54]. After DC interaction with pathogen components (which may interact with TLRs or C-type lectin receptors) or inflammatory cytokines, DCs up-regulate the expression of co-stimulatory (CD80/86) and MHC class II molecules, as well as chemokine receptors, such as CCR7, which allow DCs to migrate through lymphatic vessels and home to lymph nodes [53, 55]. DCs in the subepithelial dome regions of Peyer's patches are known migrate to interfollicular T cell zones to present antigen for local immune responses and to local draining lymph nodes to induce a systemic immune response [49].

Lymphocyte homing Lymphocytes activated in the local lymphoid tissues (local draining lymph nodes or local MALT) by DCs migrate to the mucosa by up-regulating homing receptors on their surface that are imprinted upon them by the activating DCs [49]. Although induction of immune responses at one site of the mucosal immune system results in effector function at other, more distant mucosal sites, generating the term “common mucosal immune system”, compartmentalization has also been recognized [56-58]. Some cells up-regulate tissue specific homing receptors, like $\alpha_4\beta_7$-integrin which interacts with MADCAM1, expressed primarily by venules in the small and large intestines. This compartmentalization helps direct immune responders to the site of original antigen encounter [49]. Another example, the CCR9 ligand, CCL25, is expressed constitutively in the small intestine. Some homing receptors direct cells to more generalized areas in the common mucosal immune system [49]. For example, the ligand CCL28, recognized by CCR10 on IgA-secreting B cells, is expressed by cells in the stomach, colon, salivary glands, and mammary glands [15]. Nasal immunization has been shown to induce the up-regulation of CCR10 and $\alpha_4\beta_1$-integrin on IgA-secreting B cells which allows them to migrate to respiratory and genito-urinary tracts that express the corresponding ligands, CCL28 and VCAM1 [44]. Orally induced IgA-secreting B cells express both CCR9 and CCR10 as well as $\alpha_4\beta_7$- and $\alpha_4\beta_1$-integrins, which direct them to areas expressing the ligands CCL25 and/or CCL28 with MADCAM1 and/or VCAM1, such as the small intestine [44]. These studies have highlighted the importance of carefully selecting the mucosal route of immunization to induce mucosal immune responses in the desired locations. Although nasal immunization is able to elicit immune responses in the respiratory and genital tracts, it is a poor inducer of gut immune

responses. Conversely, oral immunization stimulates potent gut immune responses and is a poor inducer of respiratory immunity, while rectal or vaginal immunization primarily stimulates immune responses only local to the site of vaccination [59-61].

1.2.2.2 Adaptive immune responses

The adaptive immune system is primed to respond to an antigen by cells of the innate immune system, such as DCs. Once activated, cells of the adaptive immune system aid in the defense against the primary infection, but they are also able to respond quickly upon re-infection. Mucosal surfaces are common points of entry for many microorganisms and thus are sites of vigorous immunological activity.

Mucosal IgA IgA is found in both monomeric and polymeric forms [41]. Although IgA is most commonly observed in mucosal secretions as dimeric IgA, larger polymers are also found [62]. Secretory IgA (SIgA) is the predominant Ig class in external secretions [43], with an average of 3 g being secreted per day by an average adult [63]. It performs a variety of beneficial functions at mucosal surfaces. In the lumen, SIgA binds antigen, inhibits bacterial or viral adherence to epithelial cells and their subsequent uptake, and neutralizes biologically active antigens such as toxins [41, 43, 49, 64]. In addition, SIgA is resistant to the endogenous proteases produced at many mucosal sites [43, 49]. IgA can also act during transport through the epithelium by neutralizing intracellular pathogens or within the lamina propria where it has been shown to mediate antibody-dependent cell-mediated cytotoxicity (ADCC), degranulate eosinophils, and inhibit complement activation [41, 43, 49]. The induction of IgA by vaccination with OPV has been shown to correlate with the efficacy of the vaccine [65].

Interestingly, IgA deficiency is recognized as the most common immunodeficiency in humans, with estimates of occurrence at up to 1 in 400 individuals [66]. Despite the benefits of inducing IgA, most of these individuals are asymptomatic, the reasons for which have not been fully ascertained [65, 66]. IgA deficiency has been linked with many immune disorders, including recurrent sinopulmonary infections, gastrointestinal diseases, such as celiac disease, autoimmune disorders including arthritis and Crohn's disease, and an increase in allergy [66, 67]. Thus, antigen-specific SIgA provides protective mechanisms not mediated by IgG or SIgM and therefore has the potential to be extremely beneficial in the immune response to pathogens encountered on mucosal surfaces.

Mucosal T cell responses T cells are present at mucosal inductive sites and mucosal effector sites. CD4+ T helper cell responses are generally categorized as Th1, Th2, or Th17 and have been correlated with protection against obligate intracellular pathogens, humoral immunity, and protection against extracellular bacteria (especially those that colonize mucosal surfaces), respectively [68]. In the mouse NALT, mRNA characterization has demonstrated that CD4+ T cells have a dominant cytokine profile of Th0 cells and CD4+ T cells isolated from the NALT of naïve mice have also been shown to be Th0 cells [44]. This indicates that CD4+ T cells in the murine NALT are able to differentiate to Th1, Th2, or Th17 cells after contact with nasally administered antigen. The development of Th2 CD4+ T cells induces T helper function that promotes the generation of IgA-producing B cells at the site of antigen encounter (the NALT) and at more distant mucosal sites, such as the genito-urinary and respiratory tracts [44]. The development of a Th1 CD4+ T cell population, however, helps induce CD8+ CTLs.

CD8+ T cells are direct mediators of cellular immunity, with the ability to directly lyse antigen-expressing target cells [69]. In the gut mucosa, CD8+ CTLs have been shown to provide protection against rotavirus infection and to mediate rotavirus clearance in an infection model [43]. Although important in clearance, CTLs may not be critical for preventing re-infection in the airway mucosa [43].

1.2.2.4 Immune response to mucosal vaccination

Vaccination at mucosal sites can induce the production of both systemic and mucosal IgG and IgA [24, 43, 49, 70]. By contrast, systemic routes of immunization, such as intramuscular, induce primarily systemic immune responses, with poor generation of mucosal immunoglobulins (Ig) [71]. Intranasal vaccination can induce antigen-specific IgA responses in the salivary glands, the upper and lower respiratory tracts, the male and female genital tracts, and the small and large intestines [43, 49] while maintaining the ability to induce systemic antibody responses and CTL responses [24, 70, 72, 73]. Staats et al. and Porgador et al. have previously demonstrated that intranasal immunization is more effective than subcutaneous immunization for the induction of peptide-specific responses [73, 74]. Following immunization with 1, 10, or 100 µg HIV peptide, only mice immunized IN with adjuvant (e.g., CT or IL-1α) significantly increased peptide-specific lysis compared to mice immunized IN with antigen alone [73]. Mice immunized s.c. with peptide plus CFA/IFA did not have significantly or numerically increased peptide-specific lysis compared to naïve animals. However, although s.c. immunization was able to increase the percent of tetramer-positive CD8+ T cells to 1%, IN immunization with peptide + CT or IL-1α increased the percentage to 3% and 2.5%, respectively [73]. Roediger et al. have also reported this

phenomenon following vaccination with a VSV expressing *M. tuberculosis* Ag85B [75]. Although systemic immunization was able to induce significant increases in lung and splenic CD8+ T cells at two weeks after immunization, only IN immunization significantly increased the number of CD8+ T cells in the airway lumen and did so at one week after vaccination. IN immunization was also able to increase the number of IFNγ+ T cells (CD4+ or CD8+) in both the airway lumen and the lung, while systemic immunization only enhanced their production in the spleen. These differences were reflected in the ability of the vaccinated mice to survive a pulmonary *M. tuberculosis* challenge. Two weeks after vaccination with either formulation, only IN immunization significantly decreased the log CFU/lung compared to naïve challenged animals. Together, these studies suggest that mucosal immunization more potently stimulates the production of T cell responses in both mucosal and systemic compartments than systemic routes of immunization. Nonetheless, stimulating T cell responses required either codelivery of a vaccine adjuvant or a viral vector delivery platform. The ability of mucosal immunization to induce the production of mucosal Ig, CD4+ T cells, and CTLs as well as systemic immune responses makes it an ideal vaccine route for targeting pathogens generally encountered on mucosal surfaces.

1.3 Vaccine adjuvants

1.3.1 Overview

A vaccine adjuvant is defined as any substance that, when incorporated into a vaccine formulation, acts generally to accelerate, prolong, or enhance the quality of specific immune responses to vaccine antigens. Adjuvants act in several ways to do this: increasing the biological or immunologic half-life of vaccine antigens, improving

antigen delivery to APCs, as well as antigen processing and presentation by the APCs, and by inducing the production of immunomodulatory cytokines [26, 76].

Aluminum-based adjuvants are currently the only vaccine adjuvants approved for use worldwide [25], although others, such as MF59 (oil-in-water emulsion; Novartis) and MPL (monophosphoryl lipid A; GlaxoSmithKline) [77], have been approved for use in the European Union or the European Union and the US, respectively. Until very recently, aluminum-based adjuvants were the only vaccine adjuvants licensed for use in the US. MPL was approved for use as part of an adjuvant system (AS04; GlaxoSmithKline) in the HPV vaccine Cervarix in 2009 [78]. In addition to MPL, many promising candidates, including other TLR ligands (CpG oligonucleotides), saponin derivatives, and cytokines are currently being evaluated for future use [25].

1.3.2 Safe mucosal vaccine adjuvants are needed

There are currently no approved mucosal adjuvants or mucosally delivered subunit vaccines. Although mucosal surfaces are ideal targets for vaccination, they also present several problems, including natural defense mechanisms at mucosal surfaces (e.g., clearance of vaccine components by mucus and ciliary action) and the difficulty of getting components across the mucosal barrier [49]. Vaccine adjuvants, such as cholera toxin (CT), may activate epithelial cells to induce inflammation or increase tight junction permeability [79]. Unfortunately, CT, which is one of the most potent and most widely used mucosal vaccine adjuvants for research, is not safe for use in humans. When administered orally, amounts as small as 5 μg can cause diarrhea in humans [80]. Although it has the ability to activate several cell types and induce potent mucosal immune responses [81-83], CT also induces proinflammatory responses in the brain and

redirects co-administered vaccine proteins into the olfactory nerves and the olfactory bulb [84, 85]. CT adjuvant effects include increases in antigen-specific IgE following oral or nasal delivery [81, 86, 87], and the use of CT in vaccines has also been directly linked to immediate hypersensitivity reactions leading to death after antigen challenge [88]. Another common experimental mucosal vaccine adjuvant, *Escherichia coli* heat-labile toxin (LT), has also been associated with negative side effects in humans, as both native and mutant LT (mLT) used as adjuvants were recently associated with the development of Bell's palsy following intranasal delivery in humans [89-91]. It is therefore evident that these toxin adjuvants exhibit safety concerns that will likely prevent their use in nasally delivered vaccines for humans.

1.3.3 Cytokines

Using non-foreign proteins, such as the immune system's own cytokines and chemokines, is one manner in which the immune response to vaccination can be manipulated more safely. An extremely large number of cytokines have been evaluated for their ability to enhance antigen-specific immune responses following systemic and mucosal vaccination in a variety of animal models. A smaller number of studies have evaluated the ability of nasally delivered cytokines to augment immune responses, and only a few cytokines have been characterized in depth following nasal delivery.

1.3.3.1 IL-12

Serum antibody production IL-12 is commonly known as a Th1 cytokine. It induces NK, T, and B cells to produce IFNγ, and it is the primary cytokine involved in Th1 differentiation [92]. When used as a nasal vaccine adjuvant, IL-12 has been shown by several groups to enhance serum antibody production, including IgM,

IgG, and IgA, to a variety of antigens. However, IL-12 has been used at a variety of doses and dosing schedules with a variety of outcomes. For instance, Bradney et al. delivered 0.1 μg IL-12 + 10 μg HIV peptide to mice on days 0, 7, 14, and 28 but failed to see an increase in anti-peptide IgG geometric mean titer (GMT) compared to serum anti-HIV peptide IgG titers in mice immunized with peptide alone [72]. By contrast, Baca-Estrada et al. delivered 0.5 μg IL-12 on days 0 and 21 with 0.8 μg herpesvirus type 1 glycoprotein D (gD)-containing liposomes and noted a significant increase in anti-gD serum IgG compared to immunization with gD-containing liposomes alone (~1:75,000 vs. undetectable) [93]. The use of IL-12 in this nasal vaccine formulation also significantly increased serum bovine herpesvirus neutralizing antibody titers (approx. 1:650 vs. undetectable in the absence of IL-12) [93]. Using a more complex dosing schedule that would likely not be relevant for human vaccine design, Boyaka et al. demonstrated increases in serum anti-TT IgG and IgA following vaccination with 1 μg liposome-complexed IL-12 delivered on days 0, 3, 7, 10, 14, and 17 with 20 μg TT delivered on days 0, 7, and 14 (approx. IgG titers of 1:2,097,152 vs. 1:4,096 and IgA titers of 1:2,048 vs. 1:32, respectively) [94]. In fact, the majority of studies delivering recombinant IL-12 (rIL-12) have used doses of 1 μg, albeit with different total amounts and dosing schedules. Several studies have also examined the ability of IL-12 incorporated into a plasmid to induce immune responses [95]. Although it is difficult to compare doses between studies using plasmid IL-12 and studies delivering rIL-12, all forms of IL-12 have consistently been shown to increase antigen-specific IgG2a and decrease antigen-specific IgG1 antibody production compared to antigen delivered alone [95-97], indicating that its coadministration with vaccine antigens induces a Th1 bias in

the immune response. This is unsurprising given its in vivo role in immune response development.

Very few studies delivering IL-12 with antigen have compared the IL-12-induced antibody response to the response induced by more common vaccine adjuvants, such as CT. Unfortunately, the few studies that have included CT as a control adjuvant did not observe significant increases in the antigen-specific antibody titer when using IL-12 as compared to antigen alone [72, 95, 98]. Although Okada et al. reported significant increases in anti-HIV IgG2a following IL-12 + antigen immunization, they only compared the antigen + IL-12-induced anti-HIV total IgG responses to those induced by antigen + 10 µg CT; neither group induced a significant increase in anti-HIV IgG when compared to serum antibody titers measured in mice immunized with antigen alone [95]. Albu et al. also compared the antigen-specific IgG response induced by vaccination with antigen + IL-12 or CTB [98], and they also failed to detect an increase in antigen-specific antibody with either adjuvant compared to antigen alone. It is therefore difficult to determine the significance of IL-12-induced antigen-specific serum antibody production with respect to other well-known adjuvants.

IL -12 has also frequently been used in combination with other adjuvants. Although Bradney et al. failed to observe adjuvant activity when IL-12 was administered nasally to mice with an HIV peptide antigen, they did observe an increase in serum anti-peptide IgG when mice were nasally immunized with HIV peptide combined with 0.1 µg rIL-12 mixed with IL-1 and IL-18 [10]. Similarly, Marinaro et al. demonstrated that 0.1 µg rIL-12 incorporated into a vaccine already containing 1 µg CT could enhance anti-TT IgG2a production and inhibit anti-TT IgE [17]. These observations suggest that low dose

rIL-12 may be useful to enhance the adjuvant activity of other adjuvants while simultaneously enhancing IgG2a/Th1 responses.

Mucosal antibody production Several studies have demonstrated the ability of rIL-12 to induce mucosal IgA production to a variety of codelivered antigens. However, unlike many of the studies that examined serum antibody production, a number of studies examining mucosal antibody production with rIL-12 have used alternate dosing schedules, with IL-12 delivery given both with the vaccine and then at various time points following vaccination. For instance, Boyaka et al. delivered 1 µg liposome-complexed IL-12 to mice on days 0, 3, 7, 10, 14, and 17 with an antigen delivery schedule of 0, 7, and 14 [94]. Using this schedule, they induced the production of fecal (1:512), vaginal (1:23), and salivary (1:64) anti-TT IgA that was significantly increased when compared to anti-TT IgA responses induced by immunization with TT alone (~1:4). They also noted significant IL-12-dependent increases in IgA AFCs in the lungs, CLNs, and lamina propria lymphocytes (19, 15, and 934 vs. 1, 1, and 23 AFC/10^6 cells, respectively). Similarly, Lynch et al. delivered 1 µg IL-12 on days 0, 1, 2, and 3 with pneumococcal polysaccharide (PPS3) delivery on day 0. Using this dosing schedule, they noted a significant increase in BAL anti-PPS3 IgA, but this was reported only as an OD increase at an undefined BAL dilution [97]. Interestingly, using a similar dosing schedule to Lynch et al., with antigen delivery on day 0 and IL-12 delivery on days 0, 1, 2, and 3, Arulanandam et al. observed a significant decrease in fecal anti-OVA IgA production; this was also reported only as a change in OD at an undefined fecal extract dilution [96]. Baca-Estrada et al. used a more typical dosing schedule of 0 and 21 days with antigen-containing liposomes and free IL-12; although absolute

increases in lung antigen-specific IgA were noted (Ag alone, 1:66; Ag + free IL-12, 1:577), the increases were not significant due to large amounts of animal to animal variation [93]. Unfortunately, the different methods of measuring the immune response between the studies makes it difficult to compare their results because Boyaka et al. and Baca-Estrada et al. were the only groups to calculate endpoint titers for mucosal antibody responses following vaccination with antigen + IL-12 only. It is important to note that comparing antigen-specific immune responses between studies is sometimes difficult given that few use the same procedures for measuring and calculating the induced responses. For instance, ELISA values measured using a single sample dilution and reporting OD values are not as sensitive as endpoint titers, and it is therefore difficult to compare ELISA results between studies reporting endpoint titers and those reporting OD. Nonetheless, it does appear that IL-12 has the potential to increase mucosal IgA production, but more studies need to be carried out to confirm this, including studies comparing IL-12 to more well-known mucosal adjuvants.

Cell-mediated immunity As a Th1-associated cytokine, IL-12 has been evaluated for its ability to induce CD8+ cytotoxic T lymphocytes as well as delayed-type hypersensitivity reactions. The groups that have examined the ability of IL-12 to induce these responses have used three different forms of IL-12, including recombinant protein [73], plasmid expressed [95, 99], and bacterium expressed [100]. Using 0.1 μg rIL-12 as an adjuvant coadministered with an HIV peptide immunogen, no significant increase in peptide-specific lysis was observed compared to mice immunized with antigen alone at a variety of antigen doses at an E:T ratio of 25:1 [73]. In this same study, CT used as a positive control adjuvant induced ~40-60% peptide-specific lysis. Staats et al. also failed

to see an increase in the number of peptide-specific IFNγ-secreting CD8+ T cells. By contrast, when Bermudez-Humaran et al. immunized mice with a recombinant lactococci cosecreting the HPV E7 protein and IL-12, cell-mediated cytotoxicity was significantly increased compared to cytotoxic T lymphocyte (CTL) responses induced by vaccination with a recombinant lactococci secreting only E7 (~15% vs. 5%, respectively) at an E:T ratio of 30:1 [100]. Using the same formulation, they also observed significant increases in the numbers of antigen-specific IFNγ-secreting CD4+ and CD8+ T cells over those induced by antigen alone (CD8: 200 vs. 100 per 10^4 cells; CD4: 225 vs. 50 per 10^4 cells). Considering that the % lysis reported by Staats et al. following immunization with peptide alone was near 15%, it is possible that the differences between that study and Bermudez-Humaran's lay in the type of antigen (protein vs. peptide) or the potency of the antigen.

Codelivering 10 µg of a plasmid expressing IL-12 and 2 µg each of two HIV protein-expressing plasmids, Xin et al. demonstrated significantly increased antigen-specific lysis compared to that induced by immunization with antigen alone at an E:T ratio of 20:1 (~45% vs. <20%, respectively) following only one vaccination [99]. When Okada et al. included 2 µg of pIL-12 in a vaccine formulation with liposomes and plasmid-expressed antigen delivered three times, they also observed an increase in antigen-specific lysis at an E:T ratio of 25:1 when compared to antigen plus liposomes [95]. However, they did not compare the ability of IL-12 to increase specific lysis in the absence of liposomes. It is interesting to note that although Staats et al. did not observe a significant increase in antigen-specific lysis following three immunizations with antigen + IL-12, adding an additional boost with antigen alone (~10% vs. <5%, respectively)

resulted in a significant increase in antigen specific lysis (at an E:T ratio of 20:1) compared to antigen alone delivered four times. Given that a total of 0.3 µg IL-12 was delivered over the three immunizations, it is likely that Staats et al. also delivered the lowest dose of IL-12 of the studies reported here. Had the IL-12 dose been optimized and increased into the ranges used by other studies to induce serum antibody production, it is possible that a stronger effect of IL-12 on specific lysis would have been seen. However, three of the four studies discussed here did observe significant increases compared to antigen alone (or plus liposomes) when IL-12 was added to the vaccine formulation.

Protection against challenge The ability of IL-12 to augment vaccine-induced immune responses and enhance protection against infectious challenge has been evaluated using both viral and bacterial challenge models. Two weeks following intranasal immunizations on days 0, 7, and 14 with a tetanus toxin (TT) vaccine, Boyaka et al. challenged mice with 100 MLDs (minimum lethal doses) of TT. All mice that received the vaccine formulation with TT + liposome-complexed IL-12 (1 µg delivered on days 0, 3, 7, 10, 14, and 17) survived the challenge; none of the mice receiving TT alone survived [94]. Following one vaccination with a pneumococcal conjugate vaccine (day 0) and 1 µg IL-12 on days 0, 1, 2, and 3, Lynch et al. challenged mice with 5×10^4 CFU type 3 pneumococcal bacteria. Although the vaccination regimen did not induce complete protection against challenge, nearly 80% of mice that received the vaccine and IL-12 survived the challenge, compared to 40% survival of those that received the vaccine only [97]. Using a different approach, Parker et al. evaluated the ability of vaccination with IL-12-expressing HSV-1 to protect against systemic challenge with 100

LD_{90} of a highly pathogenic HSV strain [101]. Mice immunized with as few as 100 pfu HSV/IL-12 had significantly greater survival than sham-immunized mice (0/29). At this dose, 4/19 mice survived compared to 0/19 mice immunized with the parent virus. As the immunization dose increased, so did survival following challenge, with 92% and 100% of mice immunized with 10,000 and 100,000 pfu surviving challenge, compared to 30% and 80% of mice immunized with the parent strain. Although only a few studies have evaluated the ability of IL-12 to induce protective immunity, it does appear to be effective in this regard.

1.3.3.2 IL-2

Serum antibody production IL-2 is involved in T cell proliferation and the induction of T regulatory responses [102]. As such, it has typically been investigated for its ability to induce CTLs (see below), but its ability to induce serum antibody production has been examined by a small number of groups. In two separate studies, Xin et al. demonstrated that a single administration of plasmid-incorporated IL-2 to mice induced significant decreases in serum anti-HIV IgG1 and increases in serum anti-HIV IgG2a (16 and 10 fold, respectively, compared to antigen alone) following codelivery with two other plasmids expressing HIV proteins [99, 103]. Steidler et al. took a different approach and engineered a recombinant *L. lactis* that co-expressed IL-2 and tetanus toxin fragment C (TTFC) [104]. Following nasal inoculation on days 0, 14, and 28 with 10^9 bacteria, the IL-2-expressing bacterium induced significant increases in serum anti-TTFC IgG on days 24, 35, and 80 post-primary immunization (approx. 10-, 10-, and 5-fold higher than TTFC-expressing bacteria alone).

Macaques have also been used to examine the effectiveness of IL-2 as a nasal vaccine adjuvant [105]. Following four nasal immunizations with 1.5 mg DNA containing a mutated SIVmac239 genome and IL-2, macaques were challenged rectally and serum antibody responses were then monitored. At 6-8 weeks after the challenge, animals immunized with IL-2 had significantly greater gp120- and gp41-specific IgG (~1:50,000) than those given no incorporated cytokine (~1:1,000) or incorporated IL-12 (~1:5,000); by 12-16 weeks, animals receiving IL-2 also had increased anti-SIV IgG compared to the other immunization groups (~1:50,000 vs. ~1:8,000) [105]. This study also evaluated the ability of IL-2 to augment virus-neutralizing antibodies and demonstrated a nearly 50-fold increase from ~1:100 in antigen-alone immunized macaques to ~1:50,000 in macaques immunized with the vaccine formulated with IL-2. Although this study used a slightly different approach, examining antibody titers after challenge, the results indicate that IL-2 is effective at inducing serum antigen-specific and neutralizing antibody production in non-human primates and demonstrates the efficacy of IL-2 used as a nasal vaccine adjuvant.

Mucosal antibody production Of the numerous studies that have examined intranasal delivery of IL-2, only a few have examined mucosal antibody production. Two such studies were performed in mice by Abraham et al. [106] and Ferko et al. [107] using very different delivery methods. While Abraham et al. used a liposomal formulation to deliver 25 µg levan (a bacterial polysaccharide) and 0.02-0.2 µg IL-2, Ferko et al. delivered 2 x 10^5 PFU of an IL-2-expressing influenza virus (*ca* Sing-IL-2). Unlike many studies involving other nasally delivered cytokine adjuvants, Abraham et al. noted an increase in the total number of pulmonary antibody-secreting plasma cells

(ASCs) (from 3,712 to ~7,480 per set of lungs) in addition to an increase in the number of levan-specific ASCs (undetectable to ~330 per set of lungs), of which 90% were anti-levan IgA ASCs. The amount of anti-levan IgA in lung lavages was also significantly increased at both 0.02 and 0.2 µg IL-2, but these increases were only presented as significant increases in OD at a 1/3 dilution. Following delivery of an influenza virus expressing IL-2 (*ca* Sing-IL-2), Ferko et al. also demonstrated a 3.5-fold increase in the production of nasal virus-specific IgA (1:50 for flu alone vs. 1:200 for flu + IL-2) and a 2-fold increase in vaginal virus-specific IgA compared to flu alone (1:200 and 1:400, respectively).

In addition to the studies in mice, one group has also examined the impact of IL-2 incorporation into an avian influenza virus vaccine in chickens [108]. In this study, chickens were immunized twice (days 0 and 14) with approximately 105 EID_{50} H5N2 with or without 50 µg IL-2. In the lung and trachea, the total number of IgA and IgG ASCs increased significantly at weeks 3, 5, and 7 following vaccination with virus + IL-2 compared to vaccination with virus alone. However, this study did not examine the impact of vaccination on virus-specific ASCs or IgA, and the number of IgA-secreting cells was counted in fixed tissue sections instead of in an ELISPOT assay. In contrast to many of the studies using a mouse model, however, Xiawen et al. compared the adjuvant activity of IL-2 to an equivalent dose of CpG. CpG and IL-2 induced statistically equivalent amounts of IgA-secreting cells at all time points examined. Although IL-2 induction of mucosal IgA responses has not been often examined, studies thus far are encouraging.

Cell-mediated immunity Using an influenza virus expressing IL-2, Ferko et al. induced a twofold increase in the number of antigen-specific IFN-γ-secreting cells in both the spleen and lymph nodes of immunized mice 10 days after the primary immunization and a nearly fourfold increase in the mediastinal lymph nodes 4 weeks after the primary immunization compared to IFNγ responses induced by immunization with virus alone [107]. In addition, two studies by Xin et al. demonstrated significantly increased target cell lysis following delivery of 10 µg pIL-2 at E:T ratios of both 20:1 and 80:1 (increases of ~40% and ~30% compared to lysis induced by immunization with plasmid-expressed antigen alone) [99, 103]. They also demonstrated significant increases in DTH following codelivery of 1 or 10 µg pIL-2 compared to antigen alone (14.2 and 16.7 vs. 11.1 x 10^{-2} mm, respectively).

Protection against challenge The ability of IL-2 to induce protective immune responses when intranasally codelivered with a vaccine has been evaluated in both mouse and monkey models. Using a mouse model of influenza infection, Ferko et al. evaluated the ability of immunization with an IL-2-expressing influenza virus to protect mice against challenge with 2 x 10^5 pfu of the WT virus [107]. The addition of IL-2 to the vaccine provided 100% protection, while immunization with virus alone provided only 57% protection. The IL-2-expressing virus also decreased the viral titer in the lungs from 620 (WT) to <86 $TCID_{50}$/g tissue. Similarly, in a bacterial challenge model, Abraham et al. increased survival following challenge with 5 x 10^7 cfu *P. aeruginosa* from ~10% in those receiving antigen-containing liposomes to 45% in those receiving liposomes containing antigen and IL-2 [106].

In a macaque model of SIV infection, Bertley et al. used CD4+ T cell counts as a marker of disease progression following rectal challenge with 10 50% animal infectious doses (ID_{50}) of pathogenic SHIV89.6P virus [105]. Four nasal immunizations with 1.5 mg DNA containing a mutated SIVmac239 genome and IL-2 did not significantly increase the CD4+ T cell count compared to those receiving the DNA vaccine alone. The addition of IL-2 did, however, provide greater protection than the vaccine formulation that included IL-12, as 5/5 animals immunized with antigen and IL-2 survived the challenge, while only 1/5 animals immunized with antigen and IL-12 survived. Interestingly, 4/5 animals receiving the DNA vaccine alone and 1/5 of the non-immunized animals survived; two of the macaques receiving the DNA vaccine alone seroconverted, and viremia was never detected. Although the DNA vaccine alone was effective at protecting some of the animals, IL-2 does appear to have provided some level of increased protection in this model as well.

1.3.3.3 GM-CSF

Serum antibody production As a stimulating factor, GM-CSF induces the proliferation of macrophage, erythroid, granulocyte, eosinophil, megakaryocyte, and multipotent progenitors [109]. However, its receptor is also expressed on monocytes, macrophages, granulocytes, and lymphocytes, and GM-CSF is required for the in vitro differentiation of DCs [109]. Several studies have demonstrated that GM-CSF is able to increase antigen-specific serum antibody production following intranasal vaccination. Okada et al. delivered 2 µg of a DNA vaccine with 2 µg of plasmid GM-CSF (pGM-CSF) and liposomes on days 0, 7, and 21 [95]. Using this procedure, the use of pGM-CSF significantly increased anti-HIV serum IgG production on day 28, with a four-fold

increase in serum titer compared to antigen plus liposomes alone (1:16,000 vs. 1:4,000). This group also compared the ability of CT to augment immune responses when coadministered with the DNA vaccine in the absence of the liposomes and found no significant increase compared to antigen plus liposomes. Unfortunately, because CT was not administered with the liposome formulation, it is impossible to compare the adjuvant activities of CT and GM-CSF in this study. Okada did demonstrate, however, that pGM-CSF had a superior capacity to increase anti-HIV IgG compared to an identical dose of pIL-12 [95]. Using a different approach, Kim et al. evaluated the ability of GM-CSF to augment immune responses when expressed by an adenoviral vector [110]. In this study, mice were immunized four times on a tri-weekly basis with 1×10^8 pfu of adenovirus expressing amyloid β (aβ) with or without 1×10^8 pfu of an adenoviral vector expressing GM-CSF. Mice immunized with the GM-CSF-expressing adenovirus had increased anti-aβ serum IgG, IgG1, and IgG2b (~20 µg/ml) compared to mice receiving the aβ-expressing adenovirus alone (negligible). They also reported small (<5 µg/ml) increases in anti-aβ IgG2a and no increases in anti-aβ IgM compared to the aβ-expressing adenovirus alone. In contrast to these two studies, which both used vectors expressing GM-CSF, Bradney et al. delivered 4 µg rGM-CSF with 10 µg of an HIV peptide on days 0, 7, 14, and 28. GM-CSF significantly increased serum anti-peptide IgG responses (1:10,000) compared to antigen alone (<1:10), and the increase was comparable to that induced by 1 µg CT (1:10,000). Taken together, these mouse studies indicate that GM-CSF is an effective adjuvant for inducing antigen-specific serum immune responses when delivered nasally.

One group evaluated the ability of GM-CSF to augment neutralizing antibody titers [111]. However, following immunization with 5 x 10^5 pfu of an attenuated vesicular stomatitis virus (VSV) expressing GM-CSF, no increase in VSV-neutralizing antibody titers was seen compared to a similar virus expressing EGFP (3,413 vs. 2,560, respectively). However, as this is the only study known to us that has examined neutralizing antibody production following intranasal immunization with GM-CSF, it is difficult to say whether a different delivery method (e.g., recombinant protein) would result in neutralizing antibody production.

Mucosal antibody production Okada et al. delivered 2 µg of a DNA vaccine with 2 µg of pGM-CSF and liposomes on days 0, 7, and 21 [95]. Using this procedure, they demonstrated a significant increase in anti-HIV fecal IgA production on day 28 compared to fecal IgA responses induced by immunization with antigen plus liposomes (1:16,000 vs. 1:5,700). When they compared the ability of CT to augment fecal IgA production after immunization with DNA vaccine in the absence of the liposomes, they found no significant increase compared to antigen plus liposomes. Unfortunately, because CT was not administered with the liposome formulation, it is impossible to compare the adjuvant activities of CT and GM-CSF in this study.

Cell-mediated immunity Following two intranasal administrations of 0.2 µg DNA vaccine and 0.2 µg pGM-CSF, Okada et al. observed no significant increase in antigen-specific footpad swelling compared to footpad swelling in mice immunized with DNA vaccine alone (15.8 vs. 14.3 x 10^{-2} mm) [95]. This study also evaluated the ability of pGM-CSF to enhance antigen-specific target cell lysis when added to a vaccine formulation containing pIL-12 + antigen. The addition of pGM-CSF significantly

increased specific lysis at E:T ratios of 25:1 and 5:1 (~50% and 28%, respectively) compared to the lytic responses measured after immunization with the pIL-12 + antigen formulation (~40% and 18%, respectively). Unfortunately, the ability of pGM-CSF by itself to increase immune responses above those induced by antigen alone was not evaluated. Staats et al. determined that rGM-CSF did not provide adjuvant activity for the induction of peptide-specific CTL because there was no increase in peptide-specific CTL with the use of rGM-CSF versus CTL responses induced by immunization with antigen alone [73]. Despite the lack of effect on antigen-specific CTL responses, rGM-CSF significantly increased the number of peptide-specific IFNγ-secreting cells as compared to responses induced by immunization with antigen alone (~150 vs. ~10 per 10^6 splenocytes, respectively) as well as the number of tetramer-positive CD8+ T cells (~1.5% vs. <0.5%, respectively) [73]. Interestingly, similar to the results reported for the addition of rIL-12 to the vaccine formulation, boosting all groups with antigen alone on day 42 significantly increased the effect of rGM-CSF on the induction of target cell lysis (~25%), increasing it to levels equal to those induced by CT (~30%) and IL-1α (~25%) (<5% by antigen alone). Ramsburg et al. also evaluated the ability of GM-CSF to increase CD8+ T cell responses [111]. Unlike the other studies, however, they created an attenuated vesicular stomatitis virus (VSV) expressing GM-CSF. Compared to a virus expressing EGFP, GM-CSF increased the number of VSV tetramer-positive CD8+ T cells in the lungs (28.2% and 16.3%, respectively) 30 days post-infection. They also noted that VSV-GMCSF1 increased the ability of those T cells to expand after a booster immunization compared to VSV-EGFP.

Protection against challenge At least two studies have evaluated the ability of GM-CSF to increase protection against challenge. Similar to the work described by Ramsburg et al. with a GM-CSF-expressing VSV, Parker et al. constructed a GM-CSF-expressing HSV-1 and evaluated its ability to protect against challenge with 1 x 10^6 pfu HSV [101]. The expression of GM-CSF by the virus induced HSV-1-specific immune responses that significantly increased survival compared to sham-immunized animals when mice were immunized with 10,000 or 100,000 pfu (22/38 and 39/39 surviving, respectively); immunization with 10,000 or 100,000 pfu of HSV-1 vaccine lacking GM-CSF induced survival of 12/39 and 34/40 mice, respectively. Compared to IL-12 (as discussed above), GM-CSF expression required higher immunization doses to induce protection. Nambiar et al. used a similar model, and immunized mice with 5 x 10^5 cfu BCG expressing GM-CSF and evaluated the bacterial load after an aerosol challenge with *M. tuberculosis* (unknown dose) [112]. Immunization with GM-CSF/BCG significantly decreased (~1 log reduction) the bacterial load in the spleen compared to immunization with non-cytokine secreting BCG when mice were challenged at 4 or 12 weeks following vaccination; lung CFU were only decreased when mice were challenged at 4 weeks after immunization (~1.5 log reduction vs. ~0.5 log reduction at 12 weeks).

1.3.3.4 IL-1

Serum antibody production IL-1 is a potent proinflammatory cytokine with a wide range of effects on the host immune system. These effects include the up- and down-regulation of many genes, including cytokine and chemokine receptors, cytokines and chemokines, and adhesion molecules, resulting in the trafficking of cell populations (e.g., neutrophils) into areas of inflammation [113]. IL-1 has also been

shown to play a role in Th17 differentiation [114]. Staats et al. was the first to use IL-1 as an adjuvant in nasally administered vaccines [115]. IL-1 has since been used with a variety of codelivered antigens in three animal models: mice [72, 73, 115, 116], rabbits [116], and monkeys [117]. In their first study, 4 μg of either IL-1α or IL-1β was codelivered with 50 μg TT or 100 μg OVA and compared the induced immune responses to those induced by 1 μg CT plus antigen. Both IL-1 proteins induced serum antigen-specific IgG equivalent to that induced by immunization with CT + antigen. Staats et al. also evaluated the ability of CT and IL-1 to enhance serum antibody production when delivered with all three immunizations or only with the first immunization. Compared to TT delivered without adjuvant, IL-1 increased serum anti-TT IgG at least 2 log after delivery with all three immunizations and at least 1.5 log when delivered only with the first immunization [115]. When OVA was used as the antigen and the adjuvants were delivered with all three immunizations, both IL-1α and IL-1β increased serum anti-OVA IgG to approximately 1:100,000 compared to <1:1,000 for antigen alone and 1:10,000 for OVA + CT [115]. When IL-1 was only delivered with the first OVA immunization, it induced anti-OVA IgG titers of 1:10,000, while OVA alone and OVA + CT (delivered only with the first immunization) induced titers <1:1,000. In general, the anti-OVA IgG subclass profiles were similar between the mouse strains and adjuvants (i.e., IL-1α, IL-1β, and CT). However, when OVA was used as the antigen, IL-1 delivered with all three immunizations induced greater anti-OVA IgG1, IgG2a, and IgG2b responses than CT delivered with all three immunizations. In addition to protein antigens, Bradney et al. have also evaluated the ability of IL-1 to augment serum antigen-specific immune responses to an HIV peptide antigen [72]. Following three immunizations, codelivery of

4 µg IL-1α increased anti-peptide IgG production to ~1:50,000, compared to ~1:10,000 for antigen + CT and <1:10 for antigen alone. In a recently published study, Gwinn et al. evaluated the ability of IL-1β to induce serum antibody responses to immunization with TT or pneumococcal surface protein A (PspA) [116]. When 5 µg IL-1 was nasally codelivered with either PspA or TT, it induced serum antigen-specific IgG production equivalent to that induced by a subcutaneous injection of PspA with alum or an intranasal immunization with TT + CT. One other group has also evaluated the ability of nasally delivered IL-1 to augment antigen-specific serum antibody production [118]. Following immunization on days 0 and 28 with 1 µg rHA plus 1 µg of either IL-1α or IL-1β, Kayamuro et al. demonstrated significantly increased serum anti-HA IgG when compared to anti-HA IgG titers induced by immunization with rHA alone, though these responses were only reported as ODs at an undefined serum dilution. However, they also compared the IL-1-induced immune response to that induced by 1 µg CT. At a serum dilution of 1:1,000, CT-induced anti-HA IgG was approximately 1.2 OD_{450}, while IL-1α at 0.1 – 1 µg increased anti-HA IgG to 0.7-1.1 OD_{450}. In the absence of adjuvant, HA-induced responses were < 0.2 OD_{450}. Although it is difficult to compare the magnitude of the responses between their study and ours, it is evident from the inclusion of CT by both groups that IL-1 increased antigen-specific serum immune responses to levels equivalent to those induced by CT.

Gwinn et al. have also evaluated the ability of nasally delivered IL-1 to augment immune responses in rabbits [116]. Unfortunately, the antigen-specific immune responses induced in the rabbits were highly variable and did not reflect a dose-responsive relationship with IL-1. When TT + IL-1 was delivered intramuscularly, the

serum anti-TT IgG titer was significantly increased compared to the anti-TT IgG titer induced by intranasal delivery of TT + IL-1 (1:16,777,226 and 1:1,048,576, respectively).

Mucosal antibody Within the studies discussed above, the ability of IL-1 to induce mucosal antibody production was also evaluated. In the first study by Staats et al., 4 μg IL-1α or IL-1β was codelivered with OVA or TT and compared to 1 μg CT plus OVA or TT as well as antigen alone [115]. Staats et al. compared the ability of the adjuvants to augment mucosal immune responses to the codelivered antigen when delivered only with the first immunization or with all three immunizations. Regardless of the codelivered antigen (TT or OVA), IL-1 was able to induce vaginal antigen-specific IgA when it was codelivered with antigen all three times (1:50-1:100), whereas CT was unable to induce vaginal anti-OVA IgA. Conversely, CT was able to induce vaginal IgA production following vaccination with TT. In another experiment, following three immunizations with TT + IL-1β, doses from 0.2 to 5 μg IL-1 were able to induce significantly increased vaginal anti-TT IgA (1:512-1:1,448), and the response was similar to that induced by 1 μg CT (1:1,048). Kayamuro et al. have also demonstrated the ability of IL-1α and IL-1β to induce mucosal antibody production [118]. Following immunizations on days 0 and 28, they reported increased nasal wash anti-HA IgA following vaccination with HA + CT (1 μg) or either IL-1 (0.2-1 μg) at dilutions of 1:6 and 1:30 (OD_{450} ~ 1.2 and 0.6, respectively) compared to vaccination with HA alone (OD_{450} <0.2). They also demonstrated significantly increased anti-HA IgA in saliva, vaginal, and fecal samples following immunization with HA + IL-1α or IL-1β as compared to immunization with HA alone, but these were reported in OD_{450} at undefined sample dilutions. Although the different reporting methods make it difficult to directly compare

the studies, IL-1 was shown by all studies described to increase mucosal immune responses to the co-delivered antigen in mice. Despite the promising results in mice, IL-1 has not yet been shown to significantly enhance mucosal antigen-specific antibody production in rabbits. Although the ability of IL-1 to induce antigen-specific vaginal IgA production in rabbits has been evaluated, IL-1-adjuvanted vaccines did not induce IgA in rabbits under the conditions tested [116]. This may reflect issues with current mucosal vaccine delivery techniques. Regardless, it is unclear whether IL-1 would have the ability to elicit mucosal IgA in humans.

Cell-mediated immunity The ability of IL-1 to augment cell-mediated immune responses has also been evaluated. In two studies, Staats et al. and Gwinn et al. examined DTH following three intranasal immunizations, and IL-1 significantly increased the antigen-specific ear swelling response to injected antigen over that induced by vaccination with antigen alone [115, 116]. Compared to CT, IL-1 induced significantly increased ear swelling following immunization with OVA [115]. However, when codelivered with TT, CT and IL-1 have been shown to induce similar degrees of ear swelling in both BALB/c and C57BL/6 mice [115].

Staats et al. also evaluated increases in antigen-specific lymphoproliferative responses following immunization with TT plus adjuvant, using [H^3]thymidine incorporation in T cells following incubation with the vaccine antigen [115]. IL-1α, IL-1β, and CT increased TT-specific lymphoproliferation to nearly 12,000 counts per minute (cpm) in C57BL/6 and 16,000-22,000 cpm in BALB/c mice, while TT alone induced little lymphoproliferation. In addition, IL-1α has been shown to significantly increase epitope-specific CTLs based on lysis of target cells following four immunizations with 100 µg HIV

peptide and 4 µg IL-1 [73]. Peptide-specific lysis at an E:T ratio of 25:1 was increased to 45% with the use of IL-1 as an adjuvant as compared to ~15% in mice immunized with antigen alone; this increase was similar to that induced by CT (~55%). In the same study, the number of antigen-specific IFNγ-secreting CD8+ T cells was also increased with the use of IL-1 compared to mice immunized with antigen alone (~150 per 10^6 vs. ~10 per 10^6, respectively), while CT showed a similar increase to ~200 per 10^6. The % of tetramer-positive CD8+ T cells was also increased (<0.5% vs. 2.5% for antigen alone and antigen + IL-1, respectively).

In contrast to the results reported by the Staats group, Kayamuro et al. evaluated the ability of IL-1α and IL-1β to induce HA tetramer-positive CD8+ T cells and peptide-specific IFNγ-secreting CD8+ T cells and failed to observe significant increases [118]. Although IL-1α did slightly increase the fluorescence intensity of tetramer-positive cells (~1.75 vs. ~1.1 for HA alone), only CT significantly increased this response (~2.3). Similarly, HA + IL-1α or HA + IL-1β did not significantly increase the number of IFNγ-secreting CD8+ T cells compared to HA alone (35, 10, and 5 per 10^6 splenocytes for IL-1α, IL-1β, and HA, respectively), although in this instance, neither did immunization with HA + CT (~25/10^6 splenocytes) [118]. However, the variation in the response induced by IL-1α was relatively large. All of these studies indicate that IL-1 has the ability to augment cell-mediated immunity, though the magnitude of the induced response may differ based on the antigen or antigen dose used.

Protection against challenge IL-1β has been evaluated for its ability to provide adjuvant activity and enhance vaccine-induced survival following challenge with either 1.125 x 10^5 cfu *S. pneumoniae* or 10 LD_{50} TT [116]. Intranasal immunization with

PspA and IL-1 significantly increased survival following IV challenge on day 72 to 68% compared to 0% survival in mice immunized with PspA alone. Protection provided by IL-1β adjuvanted nasal immunization was equal to that induced by subcutaneous administration of PspA + alum (68%). Similarly, intranasal immunization with 0.2 – 5 µg IL-1β + TT provided 100% protection following a TT challenge while immunization with TT alone provided only 10% protection. Kayamuro et al. also evaluated the ability of IL-1 to enhance vaccine-induced protection against challenge [118]. They intranasally administered 256 hemagglutinating units of influenza virus 14 days after the second of two immunizations (on days 0 and 28) and evaluated the ability of 1 µg HA alone, HA + 1 µg CT, or HA + 1 µg IL-1α or IL-1β to protect against morbidity. While coadministration of CT with HA provided100% survival, immunization with HA alone provided only 10% survival. The use of IL-1 as a nasal vaccine adjuvant provided significant protective immunity, as 80-85% of mice survived infection, which was not significantly different from the protection induced by vaccination with HA + CT.

1.3.3.5 The future of cytokines as vaccine adjuvants

It is evident from the described studies that more work is required before many of these cytokine adjuvants can be said definitively to be potent nasal vaccine adjuvants for inducing any of the responses discussed above. It is important to always consider the methods of antigen and adjuvant delivery (e.g., recombinant proteins, plasmids) as well as the types of antigen being used when evaluating any adjuvants for their effectiveness. Nonetheless, cytokines have so far shown promising results when given nasally with a variety of vaccine antigens and have the ability to induce many different types of immune responses. As such, cytokines may be ideal for inclusion in vaccines in which only

certain immune responses are desired. In the future, more studies need to evaluate cytokine adjuvants with respect to other well-known vaccine adjuvants, such as CT or TLR ligands, to give study results more meaning with respect to the already published literature.

1.3.4 Vaccine adjuvant mechanisms of action

Before new vaccine adjuvants are used in humans, it is important to understand their mechanisms of action. Knowledge of both the primary adjuvant mechanisms of action and any additional side effects is a crucial aspect for the safe use of any drug or compound in humans. This has been recently highlighted by FDA-issued warnings for several widely used medications following the development of long-term unforeseen side effects, including Zicam™ and Vioxx™ [119, 120]. The mechanisms of action of most vaccine adjuvants are not well known, and there is still a great amount of confusion surrounding the mechanisms of action of some of the most studied adjuvants, including alum. Much work has been done to determine the modes of action of vaccine adjuvants, but for most, their stimulatory effects and their adjuvant effects have not been separated. Insight into the mechanism of action of vaccine adjuvants may lead away from the current empirical design of vaccines and to a more rational approach focused on tailoring adjuvants to each vaccine [121].

1.3.4.1 Current understanding of adjuvant mechanisms

Alum Aluminum salt-containing adjuvants, generally referred to as alum, are the longest- and most widely used vaccine adjuvants. Alum adjuvants are also the vaccine adjuvants with one of the least characterized mechanisms of action. Alum's function has been proposed to involve three components: 1) creating a depot effect in tissue to

prolong antigen exposure, 2) formation of particulate antigens to facilitate uptake by APCs, and 3) activation of complement and stimulation of macrophages [122, 123]. These possible mechanisms of action, however, have been challenged, as antigen does not need to be adsorbed to alum for it to maintain its adjuvant effects and antigen is frequently released by the "depot" rapidly [123, 124]. Aluminum salts have been shown to activate macrophages to present antigens and induce Th2-biased responses [122, 123]. Alum enhances the activation of naïve T cells and the maturation of DCs [125] and can activate caspase-1 [126]. With the addition of LPS or OVA as antigen, alum significantly increases the release of IL-1β from PBMCs and DCs, as well as the production of IL-4, -5, -6, -10, -18 and IFNγ [125, 126]. The antibody-inducing adjuvant effects of alum have also been shown to be independent of MyD88 [127], although its stimulation of IL-6 production is reliant on MyD88 [126]. Although many of these properties were originally believed to be linked to activation of the inflammasome via Nalp3 activation of caspase-1, it is still unclear whether caspase-1 is required for alum to enhance antigen-specific immune responses [128-130]. A number of studies have demonstrated abrogated or decreased responses to vaccination with alum in caspase-1- or NALP3-deficient mice compared to those induced in WT mice [131], but other groups have observed only slightly decreased or unchanged antigen-specific immune responses [132]. It is possible that the use of different antigens, antigen doses or routes of immunization is the cause of the discrepancy. Nonetheless, the mechanism of alum's adjuvant activity is still unclear [133].

Cholera toxin Cholera toxin has been characterized as both a systemic vaccine adjuvant and as a mucosal vaccine adjuvant. Systemically, injection of CT

induces the production of IgE and a skewed Th2 response [24, 86]. One study investigated the ability of CT to influence the location of APCs. When conjugated to OVA and given i.v., CT was shown to induce the formation of a band of OVA+ CD11c+ cells in the marginal zone of the spleen, in a manner dependent on the B subunit of CT (CTB) binding to the GM1 ganglioside receptor [134]. The same study demonstrated that whole CT, but not CTB alone, was able to induce increases in splenic T cell zone CD86+CD11c+ cells [134].

Mucosally applied CT stimulates the production of mucosal IgA [135-137] and antigen-specific IgE [87], and *in vitro* studies have demonstrated that CTB stimulates the production of IgA in a TGF-β-mediated manner [82]. Similarly, at three and seven days following nasal vaccination of mice with CTB, IL-2, -4, -6, -15, -18, and IFNγ mRNAs are increased in the NALT cell population [137, 138]. CT stimulates an increase in the number of CTLs [73] and increases mucosal permeability [79]. As a nasal adjuvant, it increases the number of IgG and IgA antibody-secreting cells in the CLNs [139] and stimulates local DCs in the NALT, but not CLN DCs, to present antigen to T cells after intranasal immunization [140]. Additionally, when delivered orally to mice, Shreedhar et al. have demonstrated that CT induces the migration of DCs from the subepithelial dome (SED) region into Peyer's patches [141], and Anosova et al. have demonstrated that both WT CT and mutant CT given orally to mice induce DC migration into the follicle-associated epithelium [142]. In vitro, stimulation of macrophages with CT enhances their APC capabilities in a manner dependent on CT-induced macrophage production of IL-1α [143], and stimulation of isolated NALT from mice with CTB has also been shown to result in IL-1 production [137].

CT is a known inducer of IL-17 production [144, 145], and IL-17 was recently implicated in the ability of CT to enhance mucosal antigen-specific IgA production [146]. Interestingly, oral immunization of *Il17a*$^{-/-}$ mice with OVA + CT resulted in significantly decreased serum anti-OVA IgG1 and fecal anti-OVA IgA production when compared to WT mice, but intranasal immunization did not. However, the mucosal response to intranasal immunization was only evaluated in bronchoalveolar lavage (BAL) samples. Although *Il17a*$^{-/-}$ /- mice produced little BAL anti-OVA IgA, the titers observed in WT mice varied considerably, and the differences were not significant between the two groups. BAL anti-OVA IgA titers in WT mice vaccinated with OVA + CT were also not significantly increased when compared to those induced by vaccination with OVA alone. Without normalizing mucosal antigen-specific antibody responses, it is difficult to say if the large variation in BAL IgA titers was due to animal-to-animal variation or technical inconsistencies with sample collection.

Emulsion adjuvants The mode of action of emulsion adjuvants was initially characterized in 1967 as a depot effect [147]. However, their mechanism of action is currently considered largely unknown [129, 130]. Depending on the type of emulsion, it either may (water in oil) or may not (oil in water) associate with and retain the antigen at the injection site to sustain its release over a long period of time [147, 148]. These adjuvants, which contain surfactants, have more recently been shown to recruit and interact with APCs at the site of injection and to increase antigen presentation to T cells [148, 149]. Surfactant-containing adjuvants have also been shown to induce both apoptosis and necrosis [150], the latter of which has been shown to induce DC maturation and cross-presentation of antigens to CD8+ T cells [150, 151]. Local tissue

damage at the site of injection may therefore be a mechanism of action for emulsion adjuvants.

MF59 is an oil-in-water emulsion that has been evaluated in human clinical trials and has been shown to have a good safety profile [130]. Like the other emulsion adjuvants, its mechanism of action is unclear. MF59 has been shown to target monocytes, macrophages, and granulocytes, causing a number of effects including increased antigen uptake, release of chemoattractants, and cell differentiation [152], but it does not extend the presence of antigen at the site of injection [153] or change antigen biodistribution following intramuscular delivery [154]. Although Mosca et al. recently reported that alum, MF59, and CpG activated a core set of immune response genes following injection and that MF59 more strongly induced these factors [155], the importance of these findings to MF59's mechanism of action is unclear. Dupius et al. demonstrated that although MF59 increases the production of several chemokines following coinjection with antigen, CCR2-deficient mice had only partial decreases in monocyte influx following coinjection with antigen when compared to WT mice [156]. However, they did not evaluate the effect of CCR2 deficiency on antigen-specific adaptive immune responses.

Monophosphoryl lipid A (MPL) MPL is a TLR4 ligand, which is safer for use in humans than the polysaccharide from which it is derived (LPS) [157]. However, one study demonstrated its ability to also signal through TLR2 to induce NF-κB activity and the production of IL-10, TNF, and IL-12p40 by PBMCs [158]. Cytokine production and NF-κB activity were decreased following the addition of anti-TLR2 or anti-TLR4 antibodies to the cell culture. This report has recently been contended by Didierlaurent

et al. in a study demonstrating no impact on TNF production or NF-κB activity in PBMCs incubated with anti-TLR2 antibody; the ability of peptidoglycan to activate PBMCs was inhibited under the same culture conditions [159]. Given that Martin et al. used a fivefold-higher dose of anti-TLR2 antibody than Didierlaurent et al. and did not include other control TLR agonists, it is difficult to say whether their results reflect antibody cross-reactivity or an inability to completely abolish TLR4 signaling.

Although the adjuvant activity of MPL is known to be TLR dependent, its mechanism of action is still primarily characterized by the responses that it induces and not those that are required for MPL-induced antigen-specific immunity. In vitro studies of MPL demonstrate that it induces the up-regulation of CD80 and CD86 on human monocytes and stimulates the production of TNF-α, IL-10, IL-12 and the activation of NF-κB [158]. Other in vitro work shows that MPL increases IL-1β mRNA (pro-IL1β) but cannot induce IL-1β release or increase caspase-1 activity [160]. It has been proposed that this is due to an active anti-inflammatory function, as MPL has been shown to signal through only through the TRIF (Toll-interleukin 1 receptor domain-containing adapter inducing interferon-β) signaling pathway and not through the MyD88 signaling pathway [161]. MPL's adjuvant activity has also been linked with its ability to activate APCs [162]. In the same study that contested the ability of MPL to activate TLR2, Didierlaurent et al. also demonstrated the ability of MPL to enhance antigen uptake by BMDCs and subsequent T cell proliferation alone or in combination with alum (AS04 adjuvant) [159] when compared to the effects of antigen alone or antigen delivered with alum.

Mast cell activators Mast cells were long believed to be mediators of allergic responses and unimportant in the generation of immune responses to pathogens.

However, in a study published in 1996, Malaviya et al. demonstrated that mast cell-deficient mice were less efficient at clearing a bacterial injection into the intraperitoneal cavity than WT mice or mast cell-reconstituted mice [163]. Additionally, they demonstrated that bacterial clearance correlated with neutrophil influx and was directly related to TNF production. Learning from these results, the same group evaluated the ability of three mast cell activators to augment innate and adaptive immune responses [164], and a new class of vaccine adjuvants has arisen from these results. The mast cell activator compound 48/80 (C48/80) has been used since at least the early 1980s to increase histamine levels [165], but McLachlan et al. was the first group to evaluate its efficacy as a vaccine adjuvant [164]. They also demonstrated that mast cell-deficient mice reconstituted with TNF-/- mast cells had significantly decreased serum antigen-specific IgG production compared to mast cell-deficient mice reconstituted with WT mast cells following vaccination with antigen plus C48/80, but the difference between the two groups decreased over time. These results suggest that C48/80 also has a mast cell-independent mechanism of adjuvant activity.

1.4 Remaining questions

Vaccine design has come a long way since Jenner first vaccinated one boy against smallpox. However, many of the current challenges posed by new, emerging, and re-emerging diseases will require more complex solutions than those needed to combat smallpox, and these will only be identified using a rational approach to vaccine design. Vaccine adjuvants must be considered as part of the solution, but it is important that their mechanism of action be understood. Although there is information on the

mechanism of action of several vaccine adjuvants, as described above, most have not been characterized in depth [166].

Many important questions remain to be answered. What specific cell types are being activated by the most potent adjuvants, and is it possible that just one responding cell type is required for adjuvant activity? What early immune responses are important for adjuvant-induced antibody class switching? Or for the induction of CD8+ T cells? Are the inflammatory activities of many adjuvants required for their ability to enhance adaptive immune responses? Understanding these issues will help us select or design adjuvants that target these specific processes, which could potentially avoid inducing other immune responses that may inhibit vaccine-induced immunity against particular diseases or that may be potentially harmful. Mucosal adjuvants pose other challenges, including the role of the mucosal barrier. Do these adjuvants stimulate the mucosal epithelium, or are the adjuvants simply being taken across the mucosal barrier by M cells, DC projections, or non-specific protein transporters? Do these adjuvants need to stimulate the mucosal epithelium?

Answers to these questions will greatly improve the process of vaccine design. For example, if a mucosal vaccine adjuvant is working primarily by increasing mucosal permeability and activating mucosal stromal cells to produce immunomodulatory cytokines and chemokines, then its direct application at a mucosal surface would be advantageous for a mucosally administered vaccine. These adjuvants may also benefit from the inclusion of bioadhesives in the vaccine formulation to increase the time of contact of adjuvant and epithelium [43, 167]. If an adjuvant stimulates DC maturation and antigen presentation on the basal side of the mucosal stromal layer, then this

adjuvant may be better used if it were either delivered across the mucosal barrier (e.g., systemically) or delivered with another adjuvant that acts to increase mucosal permeability.

2. The mast cell activator compound 48/80 is safe and effective when used as an adjuvant for intradermal immunization with Bacillus anthracis protective antigen[1]

2.1 Introduction

Immunization programs have led to the eradication of smallpox, the near eradication of polio, and the control of other infectious diseases, including measles, mumps, rubella, and diphtheria. While there are many success stories, HIV, pandemic Influenza, and a variety of emerging infectious diseases (West Nile Virus, Dengue, etc.) provide active reminders that safe and effective vaccines continue to be needed to combat infectious diseases.

The addition of adjuvants to vaccines may increase vaccine potency by enhancing the magnitude of antibody or cellular responses induced, reducing the time to seroprotection or selectively inducing CD4+ Th1, Th2, Th17, or CD8+ T cell responses [123, 168]. The mechanism of action of adjuvants varies depending on the adjuvant used, but the end result is thought to include the activation and migration of dendritic cells as well as the expression of antigen-presenting molecules, providing superior induction of antigen-specific T and B cell responses [123, 168]. Despite increasing information on the mechanism of action of adjuvants, there are currently very few vaccine adjuvants licensed for human use. It has recently been reported that the mast cell activator compound 48/80 (C48/80) is an effective adjuvant for the induction of

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[1] Reprinted from Vaccine, volume 27, Afton L. McGowen, Laura P. Hale, Christopher P. Shelburne, Soman N. Abraham, Herman F. Staats, The mast cell activator compound 48/80 is safe and effective when used as an adjuvant for intradermal immunization with Bacillus anthracis protective antigen, 3544-3552, 2009, with permission from Elsevier.

anthrax lethal toxin-neutralizing antibody responses when delivered intranasally or via the footpad to mice with anthrax protective antigen (PA; 5 μg or 0.5 μg, respectively) [164]. The adjuvant activity of C48/80 was associated with its ability to induce dendritic cell migration via a mechanism that required mast cells and mast cell-derived TNF but did not involve activation of TLRs 2, 3, 4, 5, 7, 8, or 9 or require MyD88 [164]. Despite the demonstration that C48/80 provided effective adjuvant activity when delivered by the nasal route, no information regarding the adjuvant activity of C48/80 when delivered by the intradermal route is available. Additionally, studies by Heib et al. [169] and Fang et al. [170] have demonstrated that mice lacking mast cells have lower responses to immunization. Following transcutaneous immunization adjuvanted with imiquimod, Heib et al. observed decreased CTL responses in mast cell-deficient mice compared to WT mice. Similarly, adjuvanting with CTA1-DD, Fang et al. demonstrated decreased serum antigen-specific IgG production and antigen-specific plasma cells in mast cell-deficient mice compared to WT mice following nasal vaccination. The prevalence of mast cells in the dermis [171] suggests that the use of mast cell-activating adjuvants in intradermal vaccines could further increase the potency of this immunization route.

Safety is an important issue that must be evaluated for any new adjuvant [123, 172]. For example, although Complete Freund's Adjuvant (CFA) and Incomplete Freund's Adjuvant (IFA) are potent adjuvants that have been used for many years in research settings, they often induce excessive injection site inflammation when used in humans and are therefore too toxic for routine human use [173-175]. Additionally, some adjuvants induce undesirable immune responses, such as antigen-specific IgE, that

could sensitize the host to allergic or anaphylactic responses upon antigen challenge [163, 176-180].

Along with the search for new adjuvants, alternate routes of vaccine administration are being increasingly explored as mechanisms for creating a more robust and well-rounded immune response. The use of intradermal immunization has shown promise for rabies and influenza [181, 182]. Many studies using the intradermal (i.d.) route of vaccination have demonstrated the ability to use much smaller doses of antigen than traditional intramuscular (i.m.) vaccination while still achieving long-lasting, protective immunity. For example, Lodmell et al. have demonstrated that a rabies DNA vaccine given one time i.d. in the ear of dogs protected 100% against rabies virus challenge one year later, while 100% of dogs given the same vaccine i.m. developed rabies at challenge [183]. When administered i.d., the human influenza vaccine has been shown to induce equivalent, if not stronger, immune responses than the traditional i.m. injection when given at one fifth the dosage [182, 184, 185]. Similarly, a study comparing i.d. immunization to i.m. immunization against Hepatitis B virus in dialysis patients has demonstrated increased efficacy using the i.d. route [186].

In the current study, the adjuvant activity and safety profile of C48/80 were evaluated when administered intradermally into the ear pinnae of mice with *Bacillus anthracis* protective antigen. CpG, a TLR9 ligand adjuvant known to induce Th1 type responses [187, 188] that has a history of use in humans [189-192], and cholera toxin (CT), a known Th2 adjuvant [81, 86], were used as control adjuvants.

2.2 Materials and Methods

2.2.1 Mice

Female C3H/HeN and C57BL/6 mice were obtained from the Charles River/National Cancer Institute. Mice were housed in filter top cages and provided food and water ad libitum. All procedures were approved by the Duke University Institutional Animal Care and Use Committee. Mast cell-deficient *Kit*$^{W\text{-}sh}$/HNihrJaeBsmJ mice (SASH) were obtained in house from Dr. Laura Hale (available from Jackson Laboratory as strain # 005051) [193].

2.2.2 Vaccination

Mice were immunized i.d. in the dorsal side of the left ear pinnae with 10 µl of vaccine (diluted in PBS) delivered with a Gastight syringe using a 31-gauge needle (Hamilton Co., Reno, Nev.). Mice were anesthetized with ketamine-xylazine prior to immunization and ear tagged in the right ear following immunization. Mice were divided into groups of five. All mice, except naïve mice, received 0.5 µg of rPA (List Biologicals) as immunogen, either with or without adjuvant. Adjuvants included 3, 10, 30, or 90 µg C48/80 (Sigma), 0.1 or 1.0 µg CT (List Biologicals), and 1 or 10 µg CpG DNA (CpG ODN 1826; Invivogen). CT and CpG doses were similar to those used intradermally by other groups [178, 194, 195]. Mice were immunized on days 0 and +21. Serum samples were collected on days +35 and +42. To examine the adjuvant effects of mast cell products, C3H/HeN mice were immunized as above with 0.5 µg of rPA with or without adjuvant. Adjuvants included 30 or 60 µg C48/80, 2 µg chymase, 20, 60, or 120 µg histamine, 20, 60, or 120 ng TNFα, or 2 µg chymase, 60 µg histamine, and 60 ng TNFα. Lysates obtained from freeze-thawed cell lines were also examined for adjuvant

activity: 100 or 1,000 MC/9 (American Type Culture Collection (ATCC)) or bone marrow-derived mast cells; 1,000 or 10,000 J774A (ATCC) or MODEK (obtained from Dr. Peter Ernst) cells. The n per group for each experiment is listed in the Appendix.

2.2.3 Ear Swelling Assay

Ear thickness measurements were taken of the left ear immediately prior to and 24 hours post-vaccination with a dial thickness gauge (Mitutoyo, model no. 7326). The results are expressed as "vaccine-induced ear swelling", obtained by subtracting the ear thickness prior to immunization from the ear thickness 24 hours post-immunization. Ear swelling is expressed in units of millimeters.

2.2.4 Sample Collection

Blood samples were collected from anesthetized mice by orbital sinus or maxillary venipuncture. Samples were collected into 1.5 ml centrifuge tubes, allowed to clot and centrifuged at 13,000 rpm at 4℃ for 25 minu tes in a Heraeus Biofuge fresco centrifuge. The serum was transferred to a new tube and stored at -20℃ until tested.

2.2.5 Ex-vivo Restimulation of Spleen Cells

Mice were euthanized on day +42 using CO_2 overdose, their spleens were immediately harvested, and a single cell suspension of spleen cells was prepared. Splenocyte restimulation was done as previously described [115] with the following exception: 2.5×10^6 cells per well were plated in 250 µl into 48-well plates. 250 µl of either T cell media or a solution of 2 µg/ml rPA in media (to yield a final concentration of 1 µg/ml) was then added to the cells. The plates were incubated at 37℃ for 60 hours. Supernatants were harvested to 96-well deep well plates and stored at -80℃ until

analyzed. Thawed samples were tested for the presence of cytokines (IL-2, -4, -10, -17, IFNγ) using a bead-based multiplex fluorescent immunoassay from R&D (Minneapolis, MN). Values less than the low value of the standard curve were assigned a value of ½ the low standard. Data shown are the mean antigen-specific cytokine production for each group (i.e., LF-induced cytokine production – unstimulated cell cytokine production).

2.2.6 Lethal Toxin-Neutralization Assay

This procedure was performed as outlined by Staats et al. [196] with the following exceptions. Serum collected from mice on day +42 post-immunization was used to measure the titer of anthrax lethal toxin neutralizing antibodies in an anthrax macrophage toxicity assay. The amount of toxin used was fourfold greater than the dose required for killing 100% of the cells. Serum samples were first diluted 1:64 in media and then serially diluted 1:2. rPA and LF were added at concentrations of 0.75 µg/ml and 0.375 µg/ml, respectively, for final concentrations of 0.1875 µg/ml. Seventy-five percent neutralization titers (NT_{75}) were calculated by plotting percent neutralization versus serum dilution and using linear regression to calculate the dilution at which 75% of the cells were viable. Samples with an NT_{75} less than 1:128 were below our tested range and were assigned a value of one less than the lowest dilution for graphical representation and statistical evaluation.

2.2.7 Enzyme-linked Immunosorbent Assay

ELISAs were performed as outlined in Bradney et al. [72] and Nordone et al. [167] except that ELISA plates (384 well) were coated with rPA at 2 µg/ml in CBC buffer.

The log2 endpoint titers were used for statistical analysis. Endpoint titers below the tested range were assigned a value of one less than the lowest dilution for graphical representation and statistical evaluation.

2.2.8 IgE ELISA

ELISA plates (384 well) were coated with 15 µl purified anti-mouse IgE (clone R35-72; BD Pharmingen Cat. # 02111D) at 5 µg/ml in CBC buffer. After overnight incubation, non-specific binding was blocked by adding 30 µl/well dry milk in CBC buffer and incubated for at least 2 hours. Plates were washed in ELISA wash buffer (PBS, 0.1% Kathon, 0.05% Tween20) and diluted samples (1:16) were plated in complete sample diluent (10% 10X PBS, 1% W/V bovine serum albumin, 1% W/V non-fat dry milk, 5% normal goat serum, 0.05% Tween20, 0.5% Kathon, dH_20) for overnight incubation. Plates were washed and biotinylated rPA (15 µl/well) was added at 2 µg/ml diluted in secondary antibody diluent (% 10X PBS, 1% W/V bovine serum albumin, 5% normal goat serum, 0.05% Tween20, 0.5% Kathon, dH_20) and incubated for at least 2 hours at room temperature. Plates were washed, streptavidin-AP diluted in secondary antibody diluent was added (15 µl/well), and plates were incubated for at least 2 hours. Plates were washed with ELISA wash buffer and 15 µl Attophos substrate (Promega) was added to each well and incubated for 15 minutes before reading at 440/560 nm. Endpoint titers below the tested range were assigned a value of one less than the lowest dilution for graphical representation and statistical evaluation.

2.2.9 Histology

Vaccinations were performed as described above. Mice were euthanized 4 and 24 hours after vaccination, and ears were removed and fixed in 10% formalin prior to paraffin embedding. Sections were cut at a thickness of 5 μm and stained with hematoxylin and eosin (H&E) or Toluidine Blue. Sections were evaluated by a pathologist blinded to the treatment groups. Mast cells were counted at 40X in at least 14 (0.16 mm^2) fields per ear section. Degranulating mast cells were defined as having at least three granules evident outside the cell, while hypogranulated mast cells were defined as having granules reduced in density throughout the entire cell and a clearly visible nucleus. The numbers of degranulating and hypogranulated mast cells were added to yield the number of activated mast cells. The percent of activated mast cells was determined by dividing the number of activated mast cells by the total number of mast cells counted in each ear section.

2.2.10 Statistics

Paired two sample t-tests were used to calculate significance between serum IgG1 and IgG2a (within groups). p values < 0.05 were considered significant. Analysis of variance tests (ANOVAs) for multiple comparisons (Bonferroni) were performed in GraphPad Prism 5 for serum IgG and IgG subclasses, serum IgE, ear swelling data, and total mast cell numbers. Antigen-specific spleen cell restimulation cytokine data were log transformed and compared using ANOVA with a Bonferroni post-test. Any antigen-specific cytokine production value equal to 0 was assigned a value of 0.01 prior to the log transformation to prevent loss of data.

2.3 Results

2.3.1 Compound 48/80 is an effective adjuvant when administered by the intradermal route with anthrax protective antigen.

To evaluate the adjuvant activity of C48/80 (a mast cell activator) when delivered by the intradermal route, female C3H/HeN mice were intradermally vaccinated with 0.5 μg rPA alone or combined with C48/80 (3, 10, 30, or 90 μg) on days 0 and 21. Control adjuvants included CpG (1 or 10 μg) due to its Th1-polarizing activity [187, 188] and CT (0.1 or 1 μg) due to its well-known Th2-polarizing effects [81, 86]. Immunization with rPA alone induced moderate serum anti-rPA IgG titers by day +42 (Figure 1). The use of 3 μg C48/80 as an adjuvant did not induce the production of significantly greater amounts of anti-PA IgG than PA alone. Increasing the dose of C48/80 to 10, 30, or 90 μg augmented the induction of anti-rPA IgG antibodies and increased GMTs 53-, 177-, and 813-fold (1:2,522,926, 1:8,388,608, and 1:38,543,920, respectively) relative to rPA alone on day +42 ($p < 0.001$). The serum day +42 anti-rPA IgG titers induced by PA + 30 or 90 μg C48/80 were not significantly different than those induced by PA + CpG (1 or 10 μg; 1:11,068,835 and 1:67,108,864, respectively) or PA + CT (0.1 or 1 μg; 1:25,429,504 and 1:25,429,504, respectively) (Figure 1). Therefore, C48/80 provided adjuvant activity comparable to that provided by CpG or CT when delivered intradermally.

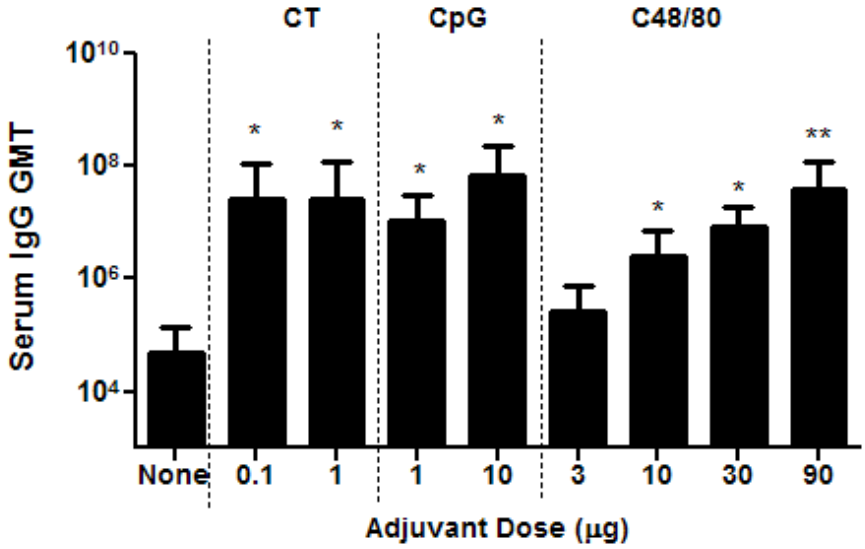

Figure 1. Intradermal vaccination with adjuvant significantly increases serum anti-rPA IgG geometric mean titers.

Mice were intradermally immunized in the ear pinna with 0.5 µg rPA with or without adjuvant on days 0 and +21. Serum samples taken on day +42 were tested by ELISA. Bars represent the geometric mean titers for each group on day +42 for all replicates, with error bars representing the 95% confidence level (CL). 90 µg C48/80, CT, and CpG groups have an n = 5. * Significantly greater than rPA alone and rPA + 3 µg C48/80. ** Significantly greater than rPA alone, rPA + 3 µg C48/80, and rPA + 10 µg C48/80.

Antigen-specific serum IgG subclasses reflect the subset of CD4+ T helper cells that are induced by vaccination, with IgG1 and IgG2a corresponding with Th2 and Th1 responses, respectively [124]. It is still unclear what effects Th17 CD4+ T cells have on B cell class switching and IgG subclass profiles [197]. To determine how C48/80 influenced the antigen-specific IgG subclass responses, day +42 serum samples were tested for rPA-specific IgG1, IgG2a, IgG2b, and IgG3. IgG1 titers were significantly higher than the IgG2a, IgG2b, and IgG3 titers in groups immunized with rPA alone ($p < 0.001$) or rPA plus C48/80 for all doses tested ($p < 0.001$). Mice vaccinated with rPA

plus 1 μg or 10 μg CpG had anti-PA IgG2a titers that were greater than the anti-PA IgG1 titers, although only 1 μg CpG induced a significant increase ($p < 0.05$). Mice vaccinated with rPA plus 1 μg CT also had similar levels of IgG1 and IgG2a. IgG1 titers were 12-fold greater than IgG2a titers in mice vaccinated with rPA plus 0.1 μg CT ($p < 0.05$) (Figure 2). These data demonstrate that C48/80 and CT induced Th2-biased IgG antibody responses while CpG induced a Th1-biased response.

IgG2b levels were also increased by vaccination. All adjuvant groups induced significantly higher titers of IgG2b than PA alone (1:323; $p < 0.001$). Vaccination with PA + 10, 30, or 90 μg of C48/80 increased IgG2b titers to 1:7,061, 1:217,904, and 1:24,834, respectively. Mice vaccinated with PA + 0.1 or 1 μg CT induced IgG2b titers of 1:456,419 and 1:330,281, respectively. PA + 10 μg CpG induced the greatest increase in IgG2b with a titer of 1:2,097,152, while PA + 1 μg CpG induced a titer of 1:301,124. IgG3 levels were nearly nonexistent in the rPA alone group and very low in all mice vaccinated with C48/80, 0.1 μg CT, or 1 μg CpG as adjuvants (GMT < 1:100). Groups vaccinated with 1 μg CT and 10 μg CpG developed anti-rPA IgG3 GMTs of 1:5,161 and 1:21,619, respectively, which were significantly increased over all other groups ($p < 0.01$ and $p < 0.001$, respectively).

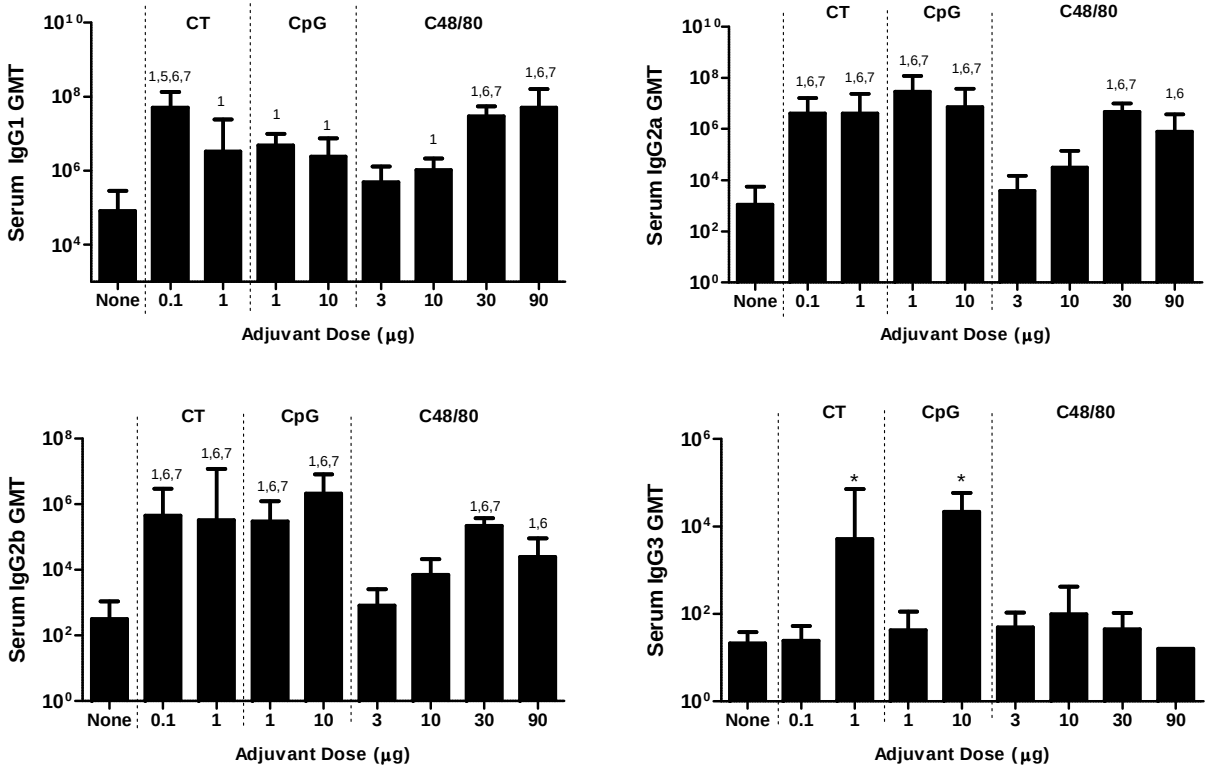

Figure 2. Intradermal immunization with the adjuvants CT, CpG, or C48/80 differentially affects serum anti-rPA IgG subclass profiles.

Serum anti-rPA IgG1, IgG2a, IgG2b, and IgG3 geometric mean titers after intradermal immunization in the ear pinna with 0.5 µg rPA in PBS with or without adjuvant on days 0 and +21. Bars represent the geometric mean titers for each group on day +42 for all replicates, with error bars representing the 95% CL. Serum samples were tested by ELISA. 1 Significantly greater than rPA alone. 5 Significantly greater than 10 µg CpG. 6 Significantly greater than PA + 3 µg C48/80. 7 Significantly greater than PA + 10 µg C48/80. * Significantly greater than all other groups.

2.3.2 Compound 48/80 augments the induction of lethal toxin-neutralizing antibodies after intradermal immunization with anthrax protective antigen.

Induction of antigen-specific IgG measured by ELISA does not always correlate with protective antibody responses [198, 199]. It has been shown, however, that anthrax lethal toxin (LeTx)-neutralizing antibody responses correlate with survival [200, 201]. Therefore, we tested day +42 serum from C3H/HeN mice immunized i.d. with rPA plus or minus adjuvant for its ability to neutralize LeTx using a macrophage protection assay. Of the twelve serum samples tested from mice vaccinated with 0.5 μg rPA alone, only one had a detectable level of LeTx-neutralizing antibodies (Figure 3). C48/80 induced LeTx-neutralizing antibodies in a dose-dependent fashion, with 30 and 90 μg C48/80 augmenting significantly increased LeTx-neutralizing antibody titers (NT_{75}) of 1:1,062 ($p < 0.001$) and 1:1,318 ($p < 0.001$), respectively. PA + 0.1 or 1.0 μg CT induced an NT_{75} of 1:624 and 1:1,886, respectively, while PA + 1.0 or 10 μg CpG induced titers of 1:1,751 and 1:2,494, respectively. The adjuvant groups 30 μg C48/80, 0.1 μg CT, 1 μg CT, 1 μg CpG, and 10 μg CpG induced NT_{75}s that were significantly greater than rPA alone and rPA + 10 μg C48/80 ($p < 0.05$), but they were not significantly different from each other. These data demonstrate that intradermal immunization with any of the three tested adjuvants induced significant increases in LeTx-neutralizing antibodies over antigen alone that were of sufficient magnitudes to protect mice from LeTx challenge, as previous work has demonstrated that an NT_{50} of 1:1,250 is sufficient to protect animals from LeTx challenge [196, 202]. However, their ability to induce LeTx-neutralizing antibodies relative to ELISA-binding antibodies varied. Relative to the serum anti-PA IgG titers at day 42, PA + 30 μg C48/80 and PA + 1 μg CpG induced the greatest

proportions of neutralizing antibodies (i.e., higher NT_{75} with lower serum ELISA titer) of all adjuvants tested (7,899 and 6,321 IgG/NT_{75}, respectively). Although C48/80 did not induce the highest serum IgG titer in our study, it induced functional antibodies at an efficiency 1.7-fold greater than 1 µg CT and almost 3.5-fold greater than that of 10 µg CpG. PA + 90 µg C48/80, 10 µg CpG, or 0.1 µg CT induced neutralizing antibodies at similar rates, which were the worst of the adjuvants tested (29,244, 26,908, and 40,732 IgG/NT_{75}, respectively).

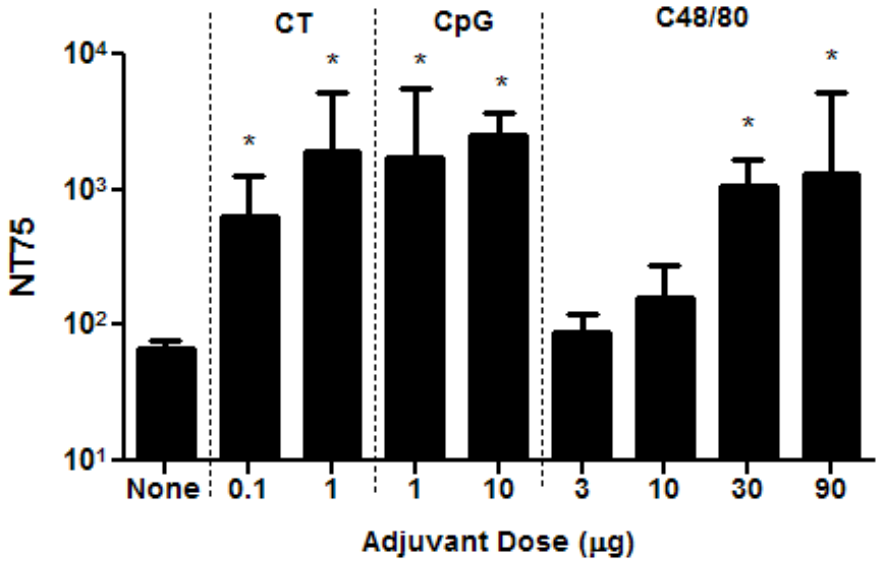

Figure 3. Intradermal immunization with C48/80 increases the serum lethal toxin neutralization titer 75 similar to CT or CpG.

Mice were intradermally immunized in the ear pinna with 0.5 µg rPA with or without adjuvant on days 0 and +21. Bars represent the geometric mean titers for each group on day +42 for all replicates, with error bars representing the 95% CL. * Significantly greater than rPA alone, rPA + 3 µg C48/80, and rPA + 10 µg C48/80.

2.3.3 Compound 48/80 does not induce PA-specific IgE.

Adjuvant-induced, antigen-specific IgE responses are considered a safety hazard for vaccines. For example, given orally as an adjuvant, CT can induce the production of antigen-specific IgE resulting in anaphylaxis upon antigen challenge [88]. We therefore monitored rPA-specific IgE responses induced by intradermal vaccination of C3H/HeN mice with rPA alone or combined with adjuvant (Figure 4). PA + 10 or 30 μg C48/80 or 1 μg CpG did not significantly induce the production of IgE as anti-PA IgE geometric mean titers were lower than the limit of sensitivity of our assay (1:16). Immunization with PA + 90 μg C48/80 or 10 μg CpG did induce significant increases in anti-PA IgE (1:18 and 1:23, respectively; $p < 0.05$). Our positive control Th2 adjuvant, CT (0.1 μg and 1 μg), induced anti-PA IgE titers that were significantly greater than all other groups ($p < 0.01$; 1:128 and 1:91, respectively). These results demonstrate that C48/80 provides effective adjuvant activity while inducing IgE at levels less than CpG, an adjuvant used by others to drive established Th2 responses toward Th1 [203].

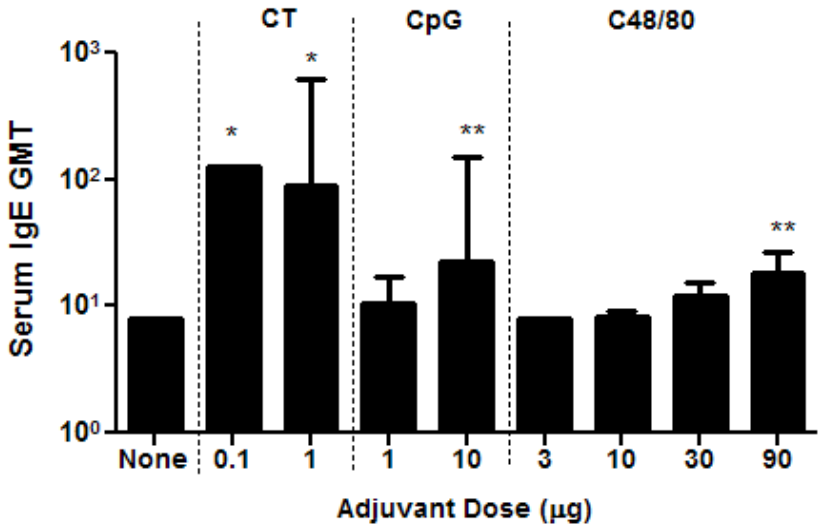

Figure 4. Intradermal immunization with C48/80 increases serum anti-rPA IgE to levels similar to the Th1-biased adjuvant CpG.

Serum anti-rPA IgE geometric mean titers after intradermal immunization in the ear pinna with 0.5 µg rPA in PBS with or without adjuvant on days 0 and +21. Lines represent the geometric mean titers for each group on day +42 for all replicates. Serum samples were tested by ELISA. * Significantly greater than all other groups. ** Significantly greater than rPA alone, rPA + 3 µg C48/80, and rPA + 10 µg C48/80.

2.3.4. Compound 48/80 induces splenocyte production of Th1, Th2, and Th17 cytokines when used as an adjuvant for intradermally administered vaccines.

Adjuvants are useful tools for directing the immune response toward the desired $CD4^+$ T helper cell response to combat different types of pathogens [204]. To evaluate the ability of C48/80 to influence antigen-specific T cell responses induced by vaccination, spleens harvested at the end of each experiment were restimulated with rPA and supernatants were evaluated for the presence of Th1 (IFNγ), Th2 (IL-4 and IL-5), and Th17 (IL-6 and IL-17) cytokines (Table 1). Antigen restimulation of splenocytes from naïve mice or mice immunized with rPA alone did not result in significant cytokine production upon spleen cell restimulation when compared to media-treated splenocytes

from naïve mice. Vaccination with the highest dose of each adjuvant tested (30 or 90 µg C48/80, 1 µg CT, or 10 µg CpG), resulted in the production of statistically equivalent amounts of IL-4, -5, -6, -17, and IFNγ between groups, which were significantly greater than the amounts produced by naïve mice, mice vaccinated with rPA alone, and mice vaccinated with PA + 3 µg C48/80 ($p < 0.05$). IL-5 production induced by PA + 30 µg C48/80 or 1 µg CT was also significantly greater than 1.0 µg CpG-induced IL-5 production ($p < 0.05$). The highest concentrations of IL-4, IL-5, IL-6, and IL-17 in this study were produced by splenocytes from mice immunized with 1.0 µg CT as adjuvant. CT-induced IL-4 production was significantly greater than that induced by PA alone and all of the low-dose adjuvant groups ($p < 0.05$). PA + 30 µg C48/80 induced the lowest absolute production of the Th17-related cytokines, IL-6 and IL-17, but the levels were significantly increased over PA alone ($p < 0.001$ and 0.05, respectively) and were not significantly less than the levels induced by 90 µg C48/80, 1.0 µg CT, or 10 µg CpG. These data indicate that C48/80 induces an antigen-specific cytokine response between that induced by a known Th1 adjuvant (CpG) and a known Th2 adjuvant (CT), when used as an adjuvant for intradermally administered vaccines.

Table 1. Antigen-specific cytokine production after 60 hr splenocyte restimulation with 1 µg rPA

Group	Treatment	Cytokine Production (pg/ml)				
		IL-4	IL-5	IL-6	IL-17	IFNγ
a	Naïve	0 ± 0	1 ± 2	2 ± 3	2 ± 6	11 ± 15
b	PA alone	0 ± 0	9 ± 17	2 ± 3	0 ± 1	26 ± 46
c	PA + 3 µg C48/80	1 ± 3	11 ± 20	3 ± 5	2 ± 5	68 ± 170
d	PA + 10 µg C48/80	1 ± 2	59 ± 52[a,b]	16 ± 14[a]	6 ± 11	230 ± 226
e	PA + 30 µg C48/80	10 ± 10[a,b,c,d,g,i]	258 ± 149[a,b,c,i]	52 ± 17[a,b,c]	37 ± 29a,[b,c,d]	1,184 ± 475[a,b,c]
f	PA + 90 µg C48/80	38 ± 12[a,b,c,d,g,i]	911 ± 389[a,b,c]	89 ± 10[a,b,c]	54 ± 39[a,b,c]	865 ± 663[a,b,c]
g	PA + 0.1 µg CT	3 ± 6	68 ± 69[a,b]	64 ± 40[a,b]	65 ± 72[b]	878 ± 681[b]
h	PA + 1.0 µg CT	51 ± 30[a,b,c,d,g,i]	513 ± 592[a,b,c,i]	213 ± 116[a,b,c,d]	657 ± 443[a,b,c,d]	1,503 ± 330[a,b,c]
i	PA + 1.0 µg CpG	0 ± 0	12 ± 24	99 ± 20[a,b,c]	73 ± 42[a,b,c,d]	3,321 ± 624[a,b,c]
j	PA + 10 µg CpG	13 ± 6[a,b,c,d,g,i]	78 ± 36[a,b,c]	112 ± 61[a,b,c]	138 ± 116[a,b,c,d]	3,686 ± 421[a,b,c]

Superscript letters indicate treatment groups that are significantly different ($p < 0.05$).

2.3.5 Compound 48/80 induces minimal injection site swelling.

Injection site reactions are a common adverse event observed after vaccination [205]. We therefore monitored vaccine-induced ear swelling 24 hours after vaccination as a measure of adverse events. Mice immunized with rPA alone had a vaccine-specific ear swelling thickness of 0.0046 ± 0.0097 mm at 24 hours after immunization (Figure 5). Immunization with PA + 0.1 or 1 μg CT, 1 μg CpG, or 30 or 90 μg C48/80 induced significant levels of ear swelling. The swelling induced by 30 or 90 μg C48/80 (0.0427 ± 0.018 and 0.0666 ± 0.057 mm) was significantly increased vs. mice immunized with rPA alone or PA + 3 or 10 μg C48/80 ($p < 0.001$). The amount of swelling induced by 30 μg C48/80 was similar to that induced by 10 μg CpG (0.0406 ± 0.024 mm), which was not significantly greater than rPA alone ($p > 0.05$). However, 1.0 μg CT induced over twice as much ear swelling (0.0955 ± 0.025 mm) as the other groups tested (rPA alone, 30 μg C48/80, and 10 μg CpG; $p < 0.001$). Interestingly, the low doses of CT and CpG induced greater amounts of swelling than any other group (0.2276 ± 0.049 and 0.1519 ± 0.022, respectively; $p < 0.01$). Although 90 μg C48/80 induced significantly less swelling than the low doses of CT or CpG, necrosis occurred at the injection site and resulted in a loss of tissue prior to the second injection (data not shown). However, no adverse reactions were noted when using 30 μg C48/80. Our results demonstrate that C48/80 does not induce excessive injection site swelling when used as an adjuvant for intradermally administered vaccines.

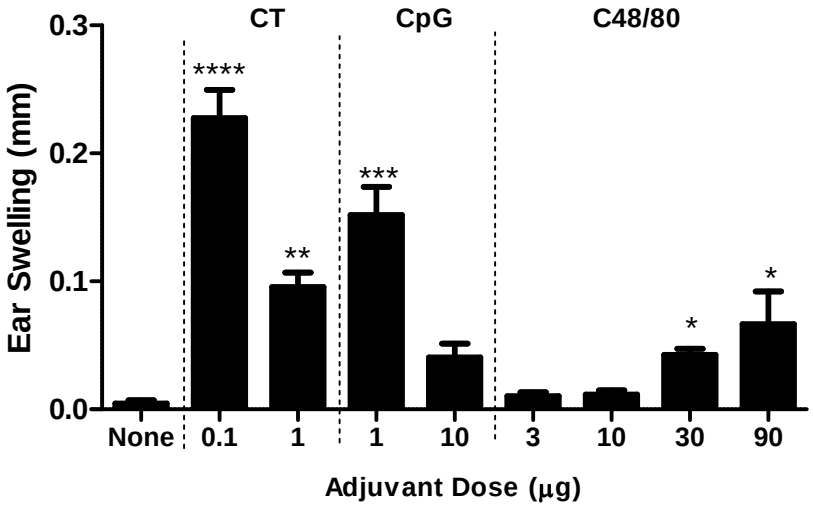

Figure 5. Intradermal immunization with rPA + C48/80 induces minimal ear swelling.

Ear swelling in mm 24 hours after intradermal immunization in the ear pinna with 0.5 µg rPA in PBS with or without adjuvant on day 0. Bars represent the mean swelling for each group for all replicates. Measurements were made using a dial thickness gauge. Error bars represent standard deviation. * Significantly greater than rPA alone, rPA + 3 µg C48/80, and rPA + 10 µg C48/80. ** Significantly greater than rPA alone, rPA + 3 µg C48/80, rPA + 10 µg C48/80, rPA + 30 g C48/80, and rPA + 10 g CpG. *** Significantly greater than rPA alone, rPA + 1 µg CT, rPA + 10 µg CpG, rPA + 3 µg C48/80, rPA + 10 µg C48/80, rPA + 30 µg C48/80, and rPA + 90 µg C48/80. **** Significantly greater than all other groups.

2.3.6 Compound 48/80 induced cellular influx into the injection site.

It was also of interest to assess cellular infiltrates at the injection site at 24 hours as an additional indicator of injection site inflammation. Using the anti-rPA serum IgG titers and serum LeTx-neutralization titers, we chose the dose of each adjuvant that induced the greatest serum IgG titer without causing ear necrosis: 30 µg C48/80, 1 µg CT, 10 µg CpG. Mice were vaccinated ID in the ear pinnae at time 0 and ears were harvested 4 and 24 hours later. Ear sections taken from mice immunized with PA alone showed little inflammation (Figures 6B/G) compared to unvaccinated ears (Figures

6A/F). Vaccination with PA plus 30 µg C48/80 induced edema by 4 hours that persisted through 24 hours, at which point cellular infiltrate was present in all sections examined. The cellular infiltrate contained primarily neutrophils (Figure 6H). Edema was also present in ears from mice vaccinated with rPA plus 10 µg CpG at both 4 and 24 hours (Figures 6D/I) with both mononuclear cells and neutrophils present at 24 hours (Figure 6I). Ears taken from mice vaccinated with rPA plus 1 µg CT showed less edema than that induced by the other two adjuvants and minimal cellular infiltrate (Figures 6E/J). Separation of the ear tissue was visible in all ear sections taken from mice vaccinated with CT (Figure 6E), a histologic indicator of the massive swelling noted grossly. Sections were also stained with Toluidine Blue to highlight the mast cells in the ear tissue. Fully granulated mast cells are evident in sections from naïve mice or mice immunized with PA alone (Figures 6K and 6L). However, mast cells could not be identified in most of the ear tissue examined from mice vaccinated with C48/80 (Figure 6M).

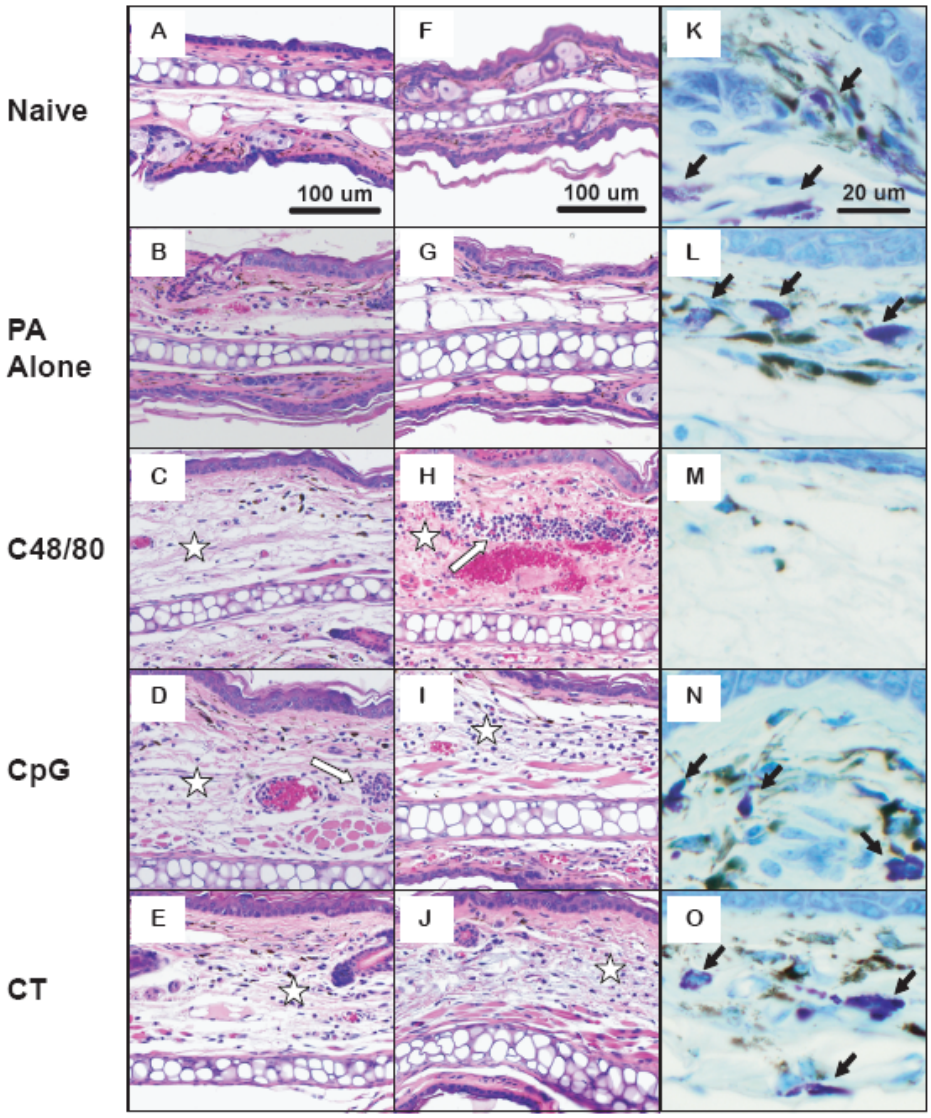

Figure 6. Intradermal administration of C48/80 is associated with mast cell degranulation.

Histological changes in the ear pinna following intradermal vaccination. Mice were immunized with 0.5 µg rPA in PBS with or without the indicated adjuvant. Hematoxylin and eosin-stained sections are shown 4 (A-E) or (F-J) hours after immunization. Stars indicate areas of prominent edema and white arrows indicate inflammatory infiltrates. Toluidine Blue-stained sections (K-O) show that mast cells (black arrows) are readily detected following immunization with PA alone or with 1 µg CT or 10 µg CpG adjuvants, but not following immunization with 30 µg c48/80.

The total number of mast cells in the tissue at four hours after vaccination decreased from 49 ± 12 in ears vaccinated with PA alone to 20 ± 11 MC/mm^2 in ears vaccinated with PA + C48/80 ($p < 0.05$). Mast cells were almost totally absent in the regions with maximal edema and infiltrate (Figure 7a). A similar picture was seen at 24 hours after vaccination, with 51 ± 12 MC/mm^2 in ears vaccinated with PA alone and 16 ± 4 MC/mm^2 in ears vaccinated with PA + C48/80 ($p < 0.01$) (Figure 7b). The total number of mast cells present in the ears of mice vaccinated with either PA + CT or PA + CpG did not differ from the number present in the ears of mice vaccinated with PA alone (Figure 7). Although the percentage of activated mast cells (defined as degranulating + hypogranulated mast cells) did not differ between vaccination groups (data not shown), the decrease in mast cell number induced by C48/80 was likely due to their activation. The histological examination therefore suggests that although C48/80 and CpG are inducing measurable ear swelling, they are encouraging the migration of neutrophils or neutrophils and monocytes to the site of injection, respectively, and that activation of mast cells by C48/80 results in their absence from the sections. Although this analysis did not determine what immune parameter induced the ear swelling, we are confident that an innate immune mechanism is responsible because ear swelling measurements were taken just 24 hours after the initial vaccination, which is not sufficient time to induce antigen-specific immune responses such as antibody production or a cellular immune response.

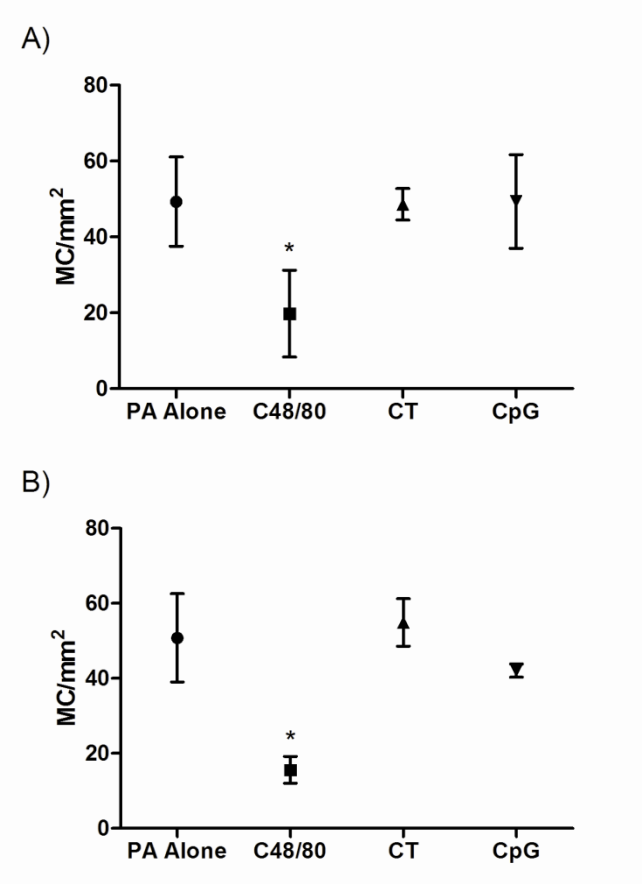

Figure 7. Intradermal administration of C48/80 decreases the number of mast cells in the ear pinna.

Total number of mast cells present per mm^2 in ear sections taken at 4 (a) or 24 (b) hours after intradermal vaccination in the ear pinnae. Sections were stained with Toluidine Blue, and mast cells were counted in a minimum of 14 (0.16 mm^2) fields at 40x. Error bars represent standard deviation. * $p < 0.05$ against all other groups.

2.3.7 The mast cell products chymase, histamine, and TNFα do not provide adjuvant activity when delivered i.d.

Because C48/80 is a known mast cell activator and was shown to induce the degranulation of mast cells following i.d. vaccination (Figure 7), it was of interest to determine if mast cell granule contents could themselves have adjuvant activity. C3H/HeN mice were vaccinated i.d. with 0.5 µg rPA with or without 30 or 60 µg C48/80,

2 µg chymase, 20 or 60 µg histamine, 20 or 60 ng TNFα, or 2 µg chymase, 60 µg histamine, and 60 ng TNFα (Figure 8a). Similar to previous experiments, mice vaccinated with either dose of C48/80 had significantly elevated serum anti-PA IgG titers when compared to mice vaccinated with rPA alone ($p < 0.001$). However, none of the remaining adjuvanted groups had significantly elevated serum IgG, and in fact, 20 µg histamine significantly decreased serum anti-PA IgG titers when compared to PA alone ($p < 0.01$).

To determine if a mast cell product other than the three tested previously could mediate the mast cell-dependent adjuvant activity of C48/80, C3H/HeN mice were vaccinated i.d. with 0.5 µg rPA with or without 30 µg C48/80, 120 ng TNF, 120 µg histamine, or the lysates of mast cells (MC/9 cell line or BMMCs), epithelial cells, or macrophages (Figure 8b). We increased the doses of TNF and histamine to 120 ng and 120 µg, respectively, to determine if the previously used doses were too low. Although the use of C48/80 as an adjuvant induced significantly increased anti-PA IgG compared to four of the adjuvanted groups, it did not significantly increase the anti-PA IgG titer over rPA alone. No other group provided significant adjuvant activity. These data demonstrate that these mast cell granule contents and mast cell lysates do not provide adjuvant activity at the doses used.

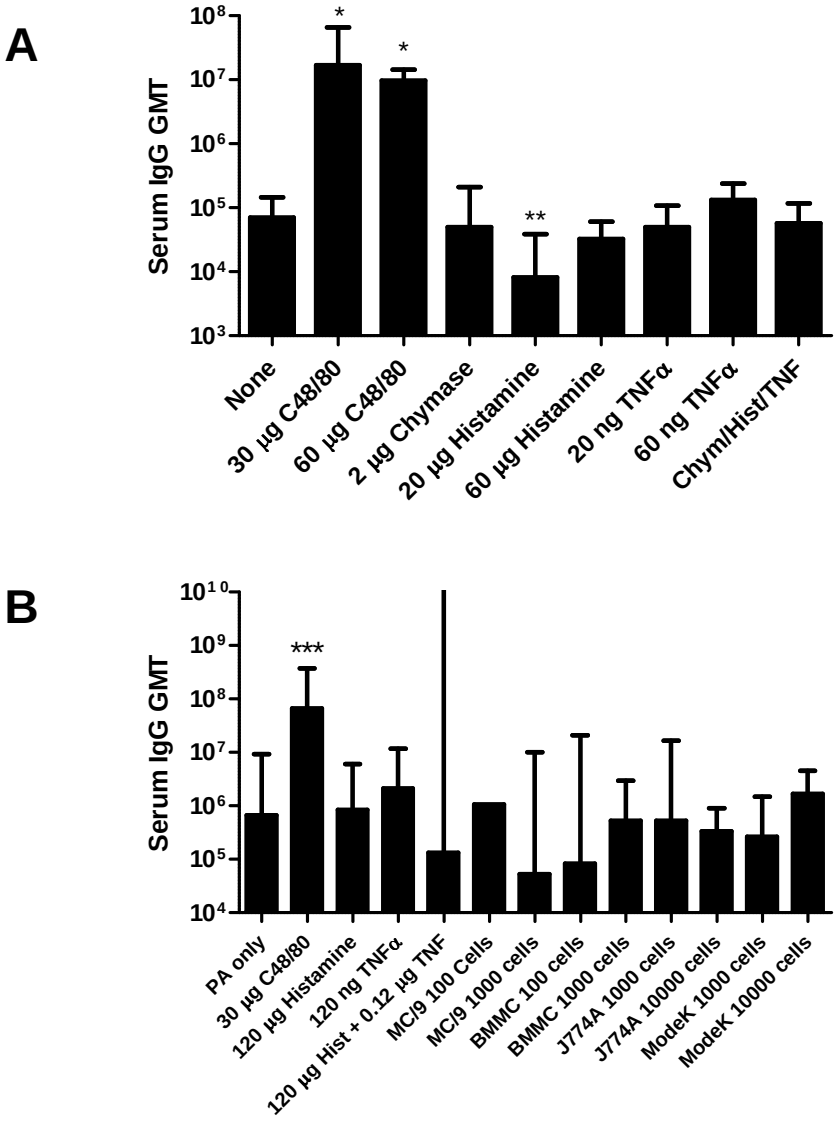

Figure 8. Intradermal administration with mast cell products does not increase serum anti-rPA IgG geometric mean titers.

Mice were intradermally immunized in the ear pinna with 0.5 µg rPA with or without adjuvant on days 0 and +21. Serum samples taken on days +35 (a) or +42 (b) were tested by ELISA. Bars represent the geometric mean titers for each group on day +42 for all replicates, with error bars representing the 95% confidence level (CL). * Significantly increased over rPA alone. ** Significantly decreased vs. rPA alone. *** Significantly increased over Hist + TNF, MC/9 100, BMMC 1000, and MODEK 1000.

2.3.8 C48/80 has adjuvant activity in the absence of mast cells.

Prior experiments in our lab have demonstrated that C48/80 can induce cytokine production by cells other than mast cells (data not shown). To determine if C48/80 could provide adjuvant activity in the absence of mast cells, we immunized WT C57BL/6 and SASH W^{sh}/W^{sh} mice, which are known to be mast cell deficient, i.d. in the ear pinnae with 0.5 µg rPA with or without 10 or 30 µg C48/80 or 1 µg CpG (Figure 9). Interestingly, SASH mice had significantly decreased serum anti-PA IgG following vaccination with rPA alone compared to WT mice (Mann-Whitney; $p = 0.037$), and this trend continued in the adjuvanted groups, though to a lesser extent. SASH mice had more varied responses to vaccination than did the WT mice. In contrast to the results originally seen in WT C3H/HeN mice, WT C57BL/6 mice responded poorly to vaccination with the same doses (an approximately 850-fold difference), which among other possible problems, may reflect strain-specific differences in the response to either the antigen or adjuvant dose delivered intradermally. A dose response study in C57BL/6 mice is necessary to evaluate this possibility. Despite these differences, SASH mice vaccinated with rPA + 30 µg C48/80 had significantly increased serum anti-PA IgG compared to mice vaccinated with rPA alone ($p < 0.05$). Interestingly, CpG was unable to provide significant adjuvant activity in SASH mice, although it was impaired similarly to C48/80 in WT C57BL/6 mice when compared to WT C3H/HeN mice. Although these data appear to indicate that C48/80 can provide adjuvant activity when delivered i.d. in the absence of mast cells, studies with optimal doses of antigen and adjuvant are required. However, in mast cell-deficient mice, rPA + C48/80 significantly increased serum anti-rPA IgG

titers compared to rPA alone, suggesting that C48/80 can provide significant adjuvant activity in the absence of mast cells.

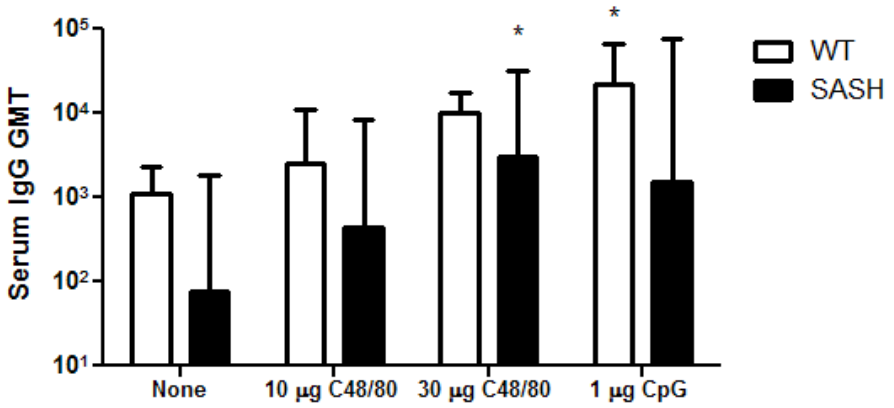

Figure 9. C48/80 has adjuvant activity in the absence of mast cells.

Serum anti-rPA IgG geometric mean titers after intradermal immunization in the ear pinna with 0.5 µg rPA with or without adjuvant on days 0 and +21. Serum samples taken on day +42 were tested by ELISA. Bars represent the geometric mean titers for each group on day +42 for all replicates, with error bars representing the 95% confidence level (CL). * Significantly greater than PA alone within the same mouse strain ($p < 0.05$).

2.4 Discussion

In this study, we have demonstrated that C48/80 provides adjuvant activity when co-administered with *Bacillus anthracis* protective antigen by the intradermal route. Vaccination using C48/80 as an adjuvant was associated with increases in serum IgG, serum LeTx-neutralizing antibodies, and antigen-specific Th1/Th2/Th17 responses. Unlike CT, C48/80 did not induce significantly increased levels of antigen-specific IgE compared to rPA alone until its highest tested dose was reached, and this dose did not provide significantly better adjuvant activity than the lower dose (30 µg). In addition, C48/80 encouraged the migration of inflammatory cells into the ear pinna while inducing

less injection site inflammation than CT. C48/80 was the only adjuvant to activate mast cells.

This study is the first to demonstrate that an intradermally administered mast cell activator is able to provide vaccine adjuvant activity and confirms our recent observation that mast cell activators provide effective adjuvant activity [164]. Others have previously documented that mast cells are able to regulate adaptive immune responses in the skin. For example, Mazzoni et al. demonstrated that the application of some mast cell activators in addition to vaccination with OVA and LPS suppressed the ability of DCs to induce Th1 CD4+ T cells [206]. Although their study involved the use of a second adjuvant (LPS) and did not focus on the induction of humoral immunity, they did demonstrate the ability of C48/80 to influence the development of T cell responses, much like we saw in our study. However, we noted an increase in both Th1 and Th2 cytokines. Although their study did not focus on the ability of mast cell activators to provide adjuvant activity or induce humoral immunity, it did demonstrate the important role that mast cells play in shaping the adaptive immune response. Our study builds on this study to demonstrate the potency of mast cell activators as vaccine adjuvants when delivered intradermally.

Our results confirm the observations of others [183, 185, 207-211] that intradermal immunization is an effective route of immunization that requires reduced antigen doses for the induction of protective immunization. Others have utilized rPA as an immunogen by the intramuscular [200, 212] or nasal [24, 136] routes and used a total of 30-60 or 15-120 μg of antigen, respectively, with three immunizations. Although all four studies demonstrated the induction of LeTx-neutralizing antibodies, the

intradermal route used in our study required 15-120-fold less antigen. After just two immunizations, totaling 1 μg of antigen, we induced LeTx-neutralizing antibody titers (NT_{50}) in excess of 1:1,900. By contrast, Boyaka et al. [24] and Matyas et al. [212] both induced LeTx-neutralizing titers of ~1:1,000, but they required two nasal immunizations totaling 20 μg of antigen and three intramuscular immunizations for a total of 30 μg of rPA, respectively. By comparison, our results demonstrate the ability of intradermal immunization to induce potent immune responses using reduced antigen doses.

Adjuvants are useful tools for directing the immune response toward the desired CD4+ T helper cell response to combat different types of pathogens [204]. With regard to our study, vaccine-induced Th2 responses are likely to be the most effective in combating *B. anthracis*, as antibody-mediated immune responses have been shown by others to provide protection against anthrax lethal toxin challenge [196, 213] and *B. anthracis* spore challenge [214, 215]. PA + 30 μg C48/80 was second only to CT in IL-5 production, yet it produced much greater levels of IFNγ than it did IL-4, -5, -6, or -17 and reduced the IgG1/IgG2a ratio to 1.1. Although mast cells are generally considered to play a role in the induction of Th2 effector immune responses [216], connective tissue mast cells, such as those found in the mouse ear pinna, have the ability to produce the Th1 cytokine IFNγ [216]. It is therefore possible that C48/80 is acting through the connective tissue mast cells to stimulate an environment favorable for the development of Th1 immune responses. Of the three adjuvants tested, C48/80 induced the most balanced cytokine profile.

Although adjuvants are useful for increasing and directing the immune response to vaccine antigens, several well-known adjuvants have been shown to induce strong

inflammatory reactions. For instance, complete Freund's adjuvant (CFA) induces fibrosis at the site of injection [217] and incomplete Freund's adjuvant has been associated with injection site inflammation when used in humans [218, 219]. In addition to inflammatory reactions, the induction of antigen-specific IgE is also an important consideration in vaccine safety, as it has the potential to induce anaphylactic reactions [88, 220, 221]. To monitor these potential side-effects, we measured adjuvant-induced injection site inflammation (i.e., ear swelling) and antigen-specific serum IgE. In addition to its role as a strong Th2 adjuvant, CT is known to be a potent inducer of vaccine antigen-specific IgE when given as a mucosal or intradermal adjuvant, causing such serious reactions as anaphylaxis upon antigen challenge [81, 86, 88, 222]. As expected, CT induced the greatest amounts of both injection site swelling and antigen-specific IgE. In agreement with our previous study [164], vaccination with 30 μg C48/80 did not induce antigen-specific IgE, and in fact, it induced similar injection site swelling as 10 μg CpG, which has been safely used in several clinical trials [174, 189-192]. Although mast cells are thought to be involved in the IgE-mediated allergic response [173], it has been shown that IgE-mediated anaphylaxis also occurs in mast cell-deficient mice [223], demonstrating that mast cells are not required for the induction of antigen-specific IgE. Our results confirm our previous observations that the mast cell activator C48/80 does not induce antigen-specific IgE at doses required to stimulate potent antigen-specific immunity.

Histological evaluation was necessary to more closely examine the injection site reactions. The large neutrophil influxes seen when mice were vaccinated i.d. with rPA plus C48/80 were likely due to the mast cell-activating capacity of C48/80, as several

studies have demonstrated the ability of mast cells to rapidly recruit neutrophils [163, 224, 225]. In a study by Malayvia et al., it was demonstrated that neutrophils migrated to the bladder of mice after *E. coli* challenge in a mast cell-dependent fashion [226]. The reduction in the number of mast cells observed in ear tissues is presumably due to C48/80-induced mast cell degranulation. Until recently, neutrophils were considered to only play a role in the innate immune response, where they acted as important mediators of several processes, including bacterial clearance in infection [224, 227]. However, it is becoming increasingly apparent that neutrophils do play a role in inducing the adaptive immune response [228, 229]. Two groups have demonstrated that neutrophil depletion alters the balance of Th1/Th2 cytokines in response to infection in favor of Th2 [230, 231]. It has also been shown that neutrophils can interact with and modulate the maturation of dendritic cells [232, 233], as well as migrate to lymph nodes [234] and prime naïve T cells in vivo [228]. Such evidence suggests that recruiting neutrophils to the site of vaccination may be beneficial for driving immune responses.

C48/80 has safely been used in human subjects, and our data are consistent with the few studies that have applied C48/80 cutaneously or intradermally to humans for allergy studies, in which it did not induce any serious long-term side effects [235-239]. In one of these studies, researchers intradermally injected 700 µg of C48/80 into human subjects and no significant side effects were noted beyond the traditional wheal response [239]. Unfortunately, injection of 90 µg C48/80 in the mouse ear did induce necrosis and tissue loss. Comparatively, however, the concentration given to the mice was approximately 9,000 µg/ml, whereas the wheal response in humans was induced at 700 µg/ml, indicating that 90 µg was an excessive dose of adjuvant. This demonstrates

that judicious application of mast cell activators appears to have no significant adverse effects and may provide a novel class of compounds for use as vaccine adjuvants.

It is interesting that C48/80 was able to significantly increase serum antigen-specific IgG over that induced by PA alone in the absence of mast cells. Although the doses of antigen and adjuvant were not tested prior to the experiment in C57BL/6 mice and did induce variable responses in the mice, PA + 30 µg C48/80 was still able to induce significantly increased anti-PA IgG when compared to PA alone in SASH mice. It is possible that a significant difference could be seen between WT and SASH mice vaccinated with rPA + C48/80 with more strain-appropriate doses of antigen and adjuvant. These data may indicate that C48/80 has a mast cell-independent mechanism of action. Given that tissue necrosis was seen at the high dose of C48/80, it is possible that C48/80 induces some non-apoptotic cell death with the subsequent release of inflammatory factors, such as IL-1 [240]. However, our data demonstrate that injection site swelling was minimal, and no signs of tissue damage were evident in the histological studies. Future studies of C48/80's mechanism of action could use immunohistochemistry to evaluate markers of apoptosis or necrosis or evaluate tissue histology over a longer time period. A previous study by McLachlan et al. [164] demonstrated that i.d. immunization in the footpad of W/W^v mice with 0.5 µg rPA + 32 µg C48/80 resulted in decreased titers compared to WT mice; anti-rPA IgG titers in W/W^v mice were still increased compared to rPA alone. However, it is unclear whether rPA alone-induced anti-PA IgG titers were decreased in W/W^v mice compared to WT mice, as that data was not included. In this study, we demonstrated a significant decrease in anti-PA IgG titer in W^{sh}/W^{sh} mice compared to WT mice following vaccination with PA

alone that persisted in the three adjuvanted groups, including rPA + CpG. It is therefore possible that mast cell deficiency affects the quality of the immune response to intradermally administered antigen regardless of the adjuvant used. Heib et al. [169] and Fang et al. [170] have both observed significant decreases in antigen-specific immune responses in mast cell-deficient mice compared to WT mice following immunization by two different routes with different antigen-adjuvant combinations. Unfortunately, it is difficult to draw a strong conclusion from the in vivo study reported by Fang et al. because their method of calculating endpoint titers was not indicated and their titers were low (<1:300). Nonetheless, when taken together, all of these studies indicate that mast cells are important mediators of immune responses following vaccination with a variety of antigen-adjuvant combinations. It was also demonstrated by McLachlan et al. [241] that intradermal delivery of C48/80 or 1 x 10^5 *E. coli* induced lymph node hypertrophy in a mast cell-dependent manner, which is in keeping with the above hypothesis that mast cell deficiency may result in a depressed immune response to an intradermal antigen challenge.

Given that McLachlan et al. [241] demonstrated that lymph node hypertrophy was dependent upon mast cell TNF production, it was surprising that administration of TNF with rPA provided no adjuvant activity. Indeed, TNF has been used to successfully boost immune responses to *Mycobacterium tuberculosis* purified protein derivative (PPD) and antigen 85B in a s.c. recombinant BCG vaccine [242]. Histamine is also generally considered to be immunostimulatory, and its suppression has been shown to decrease proinflammatory cytokine production and cellular influx to a site of challenge [243, 244]. Although necrotic cell contents are generally immunostimulatory, it was also

interesting that freeze-thawed mast cell lysates did not provide adjuvant activity, either through the release of their granule contents or through the release of inflammatory mediators, such as IL-1 [240]. However, in-depth dose responses were not carried out prior to our experiment, and so it is possible that higher doses of any of the tested compounds would have adjuvant activity when delivered intradermally. Unfortunately, the cell lysates were not tested for histamine or TNF concentrations prior to their inclusion in the vaccine formulations, so it is unknown if these cells contained appreciable amounts of either, as many mast cell lines are thought to be immature [245]. However, in canines, cutaneous mast cells have been shown to contain 4.93 pg of histamine per cell [246], indicating that if our mast cells contained similar amounts, less histamine would have been delivered with the cell lysates than was given alone as an adjuvant (lysate range: 0.49 – 49 µg).

In summary, our findings demonstrate that the mast cell activator C48/80 is an effective adjuvant for intradermally delivered anthrax protective antigen. C48/80 induced enhanced antibody- and cell-mediated adaptive immune responses and LeTx-neutralizing antibodies while inducing undetectable antigen-specific IgE and resulting in minimal injection site inflammation. Our results suggest that mast cell activators represent a new class of adjuvants that may be safely administered with intradermally administered vaccines.

3. Maximal adjuvant activity of nasally delivered IL-1α is dependent upon hematopoietic cell expression of IL-1R1 and does not correlate with adjuvant-induced cytokine production

3.1 Introduction

3.1.1 IL-1: Production, signaling, and activity

Interleukin 1 (IL-1) was studied for many years in several fields, including immunology, inflammation, and cancer research [247], before it was identified in late 1984 as two separate proteins, one acidic (IL-1α) and one neutral (IL-1β). In vivo, IL-1α and IL-1β have different biological functions. IL-1α is constitutively expressed by most cell lines intracellularly, where it can act as an autocrine growth factor, and by several cell types as membrane-bound IL-1α, where it mediates inflammation via autocrine or juxtacrine mechanisms [240, 248]. IL-1α is initially synthesized as a precursor molecule that is cleaved to a mature molecule on the cell surface by calpain [240, 249]. Importantly, the precursor form of IL-1α is biologically active, and it is believed to mediate the inflammatory properties of necrotic cells [240]. Neither form is typically found in circulation [240, 249]. Unlike IL-1α, IL-1β is secreted by many cell types and its precursor is inactive [240]. By far, the most well-known mechanism of IL-1β precursor cleavage to the mature form is by caspase-1 in the cell cytoplasm, which is dependent upon activation of the inflammasome [240]. However, IL-1β can also be cleaved extracellularly by extracellular proteases, such as the neutrophilic proteinase-3, MMP-9, or mast cell chymase [240, 250]. Once cleaved, IL-1β mediates several inflammatory functions in vivo [113], though these will be discussed in the general context of IL-1 as

both IL-1α and IL-1β bind to the IL-1 receptor 1 (IL-1R1) and can mediate the same functions through that receptor, though with slightly different binding positions.

IL-1 is a potent proinflammatory cytokine with a wide range of effects on the host immune system. These effects include the up- and down-regulation of many genes, including cytokine and chemokine receptors, cytokines and chemokines, and adhesion molecules, resulting in the trafficking of cell populations (e.g., neutrophils) into areas of inflammation [113]. Though many of these effects have been known for more than a decade and were originally reviewed in depth by Charles Dinarello in 1996 [113], the involvement of IL-1 in the response to several pathogens and in the development of adaptive immune responses has been elucidated only in recent years. For instance, IL-1 has now been demonstrated to induce increases in intestinal tight junction permeability by up-regulating myosin L chain kinase mRNA [251] and to speed oral wound healing through inflammatory cell recruitment [252]. In the host response to infection, IL-1 has been shown to mediate the immune response to several pathogens, including *Staphylococcus aureus* and *Pseudomonas aeruginosa*, by recruiting neutrophils to the site of infection [253, 254], and to others, such as *Bacillus anthracis*, via mechanisms that may be dependent on IL-1-induced increases in macrophage killing of emerging vegetative bacilli [255]. Interestingly, constitutively expressed IL-1α has been shown to mediate IFNγ antiviral activity by acting on neighboring cells to activate NF-κB [256].

In addition to these functions, IL-1 has several effects on T cell activation and T helper cell polarization. In a recent paper by Ben-Sasson et al., IL-1 was shown to have direct effects on CD4+ T cells, including the enhancement of T cell cytokine production and antigen-driven expansion of in vitro-primed WT Th cells transferred to IL-1R1-/-

hosts [257]. Although the wider implications of these results are unclear because they were obtained using transgenic OT-II cells, they are in agreement with a previous study by Curtsinger et al., which demonstrated that IL-1 could induce naïve CD4+ T cell expansion in vitro with antigen and IL-2 [258]. In 2001, Nakae et al. demonstrated that IL-1β-/- mice had reduced sheep red blood cell-specific antibody production compared to WT mice, while IL-1α/β-/- mice produced normal levels of antibody to type I T-independent antigens, indicating that IL-1 is important in T cell-dependent antibody production [259]. More recently, Nambu et al. demonstrated that IL-1 is important for T cell priming and that T cell-derived IL-1 was required for dendritic cell (DC) activation of methyl BSA-specific T cell proliferation [260]. Following the discovery of the Th17 subset of T helper responses, IL-1 was also shown to play an important role in Th17 cell differentiation in both mice and humans [114, 261].

For IL-1 to transduce a signal, it must bind to IL-1R1 and induce a conformational change that allows the IL-1R1 accessory protein (IL-1R-AcP) to form a heterodimer with IL-1R1 [248]. Two intracellular signaling proteins, myeloid differentiation primary response gene 88 (MyD88) and interleukin-1 receptor activated protein kinase 4 (IRAK4) are then recruited to the receptor complex, and IRAK4 autophosphorylates. IRAK4 is then able to phosphorylate IRAKs 1 and 2, which associate with and oligomerize tumor necrosis factor-associated factor 6 (TRAF6) [248]. TRAF6 and IRAK1 are then able to associate with TGF-β-activated kinase 1 (TAK1) and TAK1-binding proteins (TAB) 1 and 2 on the membrane. Following phosphorylation, TAK-1 is able to activate IKKb, resulting in NF-κB release and translocation to the nucleus [240, 248]. Importantly, TAK1 is also

able to activate the mitogen-activated protein kinase (MAPK) p38 and JNK pathways, which mediate several of IL-1's functions, including IL-6 and IL-8 production [240, 248].

Nearly every cell type is able to respond to IL-1, but due to the inflammatory properties of IL-1, regulation of the IL-1 pathway is very tight and includes a decoy receptor (IL-1R2) and a receptor antagonist (IL-1Ra). The decoy receptor expresses a non-signaling cytoplasmic tail of only 29 amino acids and can also be found in a soluble form, where the rank of its binding affinities is IL-1β>IL-1α>IL-1Ra [113]; it thus serves as an effective mechanism for IL-1β neutralization in vivo, with an off rate of more than two hours [262]. IL-1R1 can also be found in a soluble form [113], though its purpose is less clear as it has been shown to both inhibit and increase inflammatory responses [240, 263]. In addition to these negative regulators of IL-1 function, IL-1 signaling increases the transcription of IL-1Ra and IL-1RII and decreases the transcription of IL-1R1. In this manner, the immune system attempts to limit the duration of inflammation. However, it is evident from the number of autoinflammatory diseases and the outcomes of severe sepsis that defects in IL-1 regulation do occur.

More recently, IL-1 has been implicated as an effector of the inflammasome, which has become a major topic of interest for its role in immune responses and autoinflammatory disorders [264-267]. The inflammasome is a multi-protein complex that is assembled in response to several different types of stimuli, including uric acid, bacterial toxins, and PAMPs, and its activation results in the production of several caspases and the release of proinflammatory cytokines, such as IL-1β and IL-18 [268, 269]. Recent studies have also identified a role for the inflammasome in alum's adjuvant mechanism of action [133]. Although inflammasome activation is beneficial in many

situations [269], the unchecked production of proinflammatory cytokines, such as those in the interleukin (IL)-1 family can be detrimental to the host [267, 269]. However, it is possible that the potent immune activation induced by the inflammasome could be mimicked in a vaccine setting by delivering a small amount of the proinflammatory cytokine IL-1 with the vaccine antigen. Such a strategy would be especially useful for mucosal vaccines, where higher antigen doses or more potent vaccine antigens (e.g., live, attenuated viruses) are required to overcome the body's natural barriers to infection (e.g., mucus).

3.1.2 Mucosal vaccine adjuvants

Mucosal vaccination has been shown to induce both systemic and mucosal antigen-specific humoral and cell-mediated immunity. Such a vaccination strategy is useful for pathogens usually encountered first at a mucosal surface, including polio, influenza, cholera, and inhalational anthrax, as mucosal vaccines elicit the mucosal arm of the immune system in the response to immunization [35, 37, 38]. However, there are drawbacks to using live attenuated organisms as vaccines, the current standard in mucosal vaccines, in immunocompromised individuals, and the requirement for cold-chain storage is an additional hindrance to their widespread use [16, 18, 270, 271]. Unfortunately, vaccines based on weaker antigens, such as peptides or toxin subunits, generally require an adjuvant to induce protective immunity [25], and there are currently very few licensed vaccine adjuvants worldwide. In addition, no adjuvants have been approved for mucosal use with subunit immunogens. Therefore, identifying and characterizing new vaccine adjuvants are top priorities for creating new and effective mucosally administered subunit vaccines.

Many currently used experimental mucosal vaccine adjuvants, such as cholera toxin, are not safe for use in humans due to strong induction of IgE specific for the vaccine antigen when orally [88] or nasally [87] delivered, redirection of vaccine antigens to the olfactory bulb [84], and other toxic side effects [85]. Although some of the enterotoxin-based adjuvants have been developed to lack their ADP-ribosylating activity or as only one subunit of the holotoxin (e.g., CTB), some studies have demonstrated that the CTB subunit is a potent inducer of mucosal tolerance [272]. Another recent study demonstrated that the ADP-ribosylating activity of the CTA subunit is required for it to induce potent mucosal immune responses; in the absence of the ADP-ribosylating activity, it induces mucosal tolerance to the co-delivered antigen [273]. Importantly, the absence of the ADP-ribosylating subunit does not prevent the redirection of the co-delivered vaccine antigen to the olfactory neuroepithelium [84, 274]. *Escherichia coli* heat-labile toxin (LT) has also been shown to have negative side effects in humans, as both native and mutant LT (mLT) used as adjuvants were recently associated with the development of Bell's palsy following the delivery of intranasal vaccines in humans [89-91]. Despite their potent mucosal adjuvant activity, these numerous adverse effects will likely prevent the use of CT or LT as mucosal vaccine adjuvants in humans.

As an alternative to toxin-based mucosal vaccine adjuvants, it has previously reported that IL-1α provides effective adjuvant activity when delivered nasally, enhancing the production of antigen-specific mucosal IgA, serum IgG [115], and serum LeTx-neutralizing antibodies [116], and it has been shown to be safe and well tolerated in cynomolgus macaques [117] and rabbits [116]. Although IL-1 is an effective mucosal vaccine adjuvant, little is known about its mechanism of action in this setting. As

discussed previously, IL-1 is a pleotropic cytokine, with the ability to induce the production of several cytokines [114, 252, 254, 275, 276] and the influx of specific cell populations into an area of inflammation [277]. Although several studies have examined the ability of vaccine adjuvants to induce serum cytokine and chemokine production as well as DC maturation [26, 278-280], it is unclear whether these responses are actually important in the development of antigen-specific adaptive immune responses. It is important to determine whether these responses are an adequate measure of adjuvant mechanism or potency or if they reflect adjuvant functions that are unrelated to the mechanism by which they induce potent antigen-specific immune responses. However, in the event that innate adjuvant responses are not required for adjuvant mechanisms of action, studies characterizing the early adjuvant-induced immune responses are still needed, as they would add to the general knowledge of adjuvant activities and potentially help differentiate activities that are important for developing adaptive immune responses from those that are not.

In addition to its ability to induce cytokine production, IL-1 induces the influx of specific cell populations into an area of inflammation [277]. It has been demonstrated that *Il1r1*$^{-/-}$ DCs produce lower amounts of cytokines in response to stimulation with LPS and LPS/anti-CD40 than WT DCs, although the activation marker profile is similar [281]. In addition, *Il1r1*$^{-/-}$ mice have Th2-skewed immune responses following *L*. *major* infection [282]. It is unclear whether any of these properties of IL-1 activity are related to the adjuvant mechanism of action of nasally delivered IL-1 or if the adjuvant activity of IL-1 is mediated through a particular cellular compartment (e.g., stromal or

hematopoietic cells), as it has effects on both hematopoietic [248] and non-hematopoietic [283-286] cells.

3.1.3 Role of the mucosal barrier in the response to vaccination

In general, the role of the mucosal barrier in the immune response to mucosal vaccination is understudied. However, it is understood that the mucosal epithelium does play a role in the immune response to pathogen invasion, both in its function as a barrier and via activation of cellular receptors (e.g., TLR ligands) and the subsequently activated pathways [287, 288]. To study the role of the mucosal barrier in other responses, the bone marrow chimeric mouse model has been used to examine the requirement for specific receptors (e.g., TLRs) on hematopoietic cells or stromal cells in response to various stimulants, such as flagellin or inhaled endotoxin. Models using external challenge routes with various antigens have also demonstrated a requirement for receptor presence in both compartments [289-291], though many of these studies have focused on innate immune responses or the ability to fight infection, not on the generation of adaptive immune responses. At least three studies have used this model to examine the airway immune response to LPS administration [289, 292, 293]. Interestingly, Andonegui et al. [292] used an i.p. injection of LPS to examine changes in the lung, while Hollingsworth et al. [293] and Noulin et al. [289] used inhalation models. Using the external challenge model to examine the requirement of MyD88 on hematopoietic or stromal cells, Noulin demonstrated that while WT hematopoietic cells were required for TNF and IL-12p40 production, WT stromal cells were required for LPS-induced bronchoconstriction, and WT cells were required in both compartments to mediate neutrophil influx. By contrast, Hollingsworth found that TLR4 was required on

only hematopoietic cells to mediate neutrophil and total cell influx equivalent to that seen in WT animals. In the injected model used by Andonegui, lung myeloperoxidase (a measure of neutrophil function) was decreased in animals expressing TLR4 on only their hematopoietic cells when compared to mice expressing TLR4 on both hematopoietic and stromal cells. Due to varied methods between the studies regarding doses (i.e., 12.5 or 50 µg or 7.49 µg/m^3) and method of challenge (i.e., i.p., nasal instillation in 50 µl saline, aerosolized), it is difficult to determine if those are the source of the discrepancy. However, Andonegui also demonstrated that P-selectin expression increased in lung tissue following LPS treatment, which resulted in a near immediate drop in the number of circulating leukocytes. Taken together, these studies demonstrate that the route of administration may affect which cellular compartment is required to be antigen/adjuvant responsive to induce an immune response (Table 2).

Table 2. The route of challenge influences which compartment mediates early immune responses.

Study	Route	Antigen (dose)	Response	Required compartment
Andonegui et al. [292]	i.p.	12.5 µg LPS	Myeloperoxidase	Stromal
			P-selectin expression	Stromal
			Decrease in circulating leukocytes	Stromal
			Decrease in leukocyte rolling flux	Either
Hollingsworth et al. [293]	Inhalation	50 µg LPS	Cellular influx	Hematopoietic
			Cytokine production	Hematopoietic
Noulin et al. [289]	Inhalation	7.49 µg/m^3 LPS	Cellular influx	Hematopoietic + Stromal
			Cytokine production	Hematopoietic

In another model examining the response to multiple TLR ligands, Chieppa et al. fed peptidoglycan, LPS, CpGs, poly(I:C), and flagellin to mice and examined the ability of DCs to extend projections across the epithelium into the bowel lumen [50]. Only antigens for which the TLR was expressed on the epithelial surface of the small bowel induced DC extensions: peptidoglycan (TLR2), LPS (TLR4), and CpGs (TLR9). Using a chimeric mouse model to generate mice expressing MyD88, TLR2, or TLR4 only on hematopoietic cells, Chieppa then demonstrated that DCs failed to create extensions into the lumen in response to fed antigen, including noninvasive and invasive *Salmonella*, indicating that epithelial cell activation in turn activated DCs to extend into the lumen of the small bowel. Similar to Chieppa et al.'s examination of the response to *Salmonella*, Schilling et al. [294], Minns et al. [287], and Sato and Iwasaki [295] investigated the requirement for various TLR ligands in the immune response to externally administered antigen. Using uropathogenic *E. coli*, Schilling demonstrated that TLR4 was required on both stromal cells and hematopoietic cells to mediate inflammatory cell influx to the bladder and a decrease in the CFU/g bladder tissue. However, unlike Schilling, both Minns and Sato examined the effects of TLR expression on the development of adaptive immune responses. Minns demonstrated that following oral infection with *Toxoplasma gondii*, TLR9 expression was required on both hematopoietic and stromal cells to induce intestinal inflammation and IFNγ+ CD4+ T cells (26.19% of lamina propria T cells in WT mice vs. 9.22% in TLR9-/- BM→WT mice and 3.82% in WT BM→TLR9-/- mice). Likewise, Sato demonstrated that MyD88 was required in both compartments for DCs to induce IFNγ+ CD4+ T cells following vaginal infection with HSV. Although, with the exception of the last two studies, most have

focused on innate immune responses to TLR ligand challenge or infection, it is evident that the bone marrow chimeric model has proved valuable in demonstrating that receptor expression for the administered ligands on only hematopoietic or stromal cells was not sufficient to generate immune responses that were comparable to those seen in WT animals. Together, these studies demonstrate that the mucosal stroma is important in the generation of immune responses to infection.

To our knowledge, only a handful of groups have used the bone marrow chimeric model to examine the mechanism of action of a vaccine adjuvant. For example, in a model using an injected vaccine, Sanders et al. examined the requirement for MyD88 on hematopoietic cells and stromal cells for OVA-specific antibody production in response to vaccination with OVA + flagellin [296]. Flagellin is known to signal through TLR5 and to also have TLR5-independent effects [297]. In MyD88-/- mice, Sanders demonstrated that no OVA-specific IgG or IgG1 was produced by day 14 after one immunization. By contrast, mice expressing MyD88 on either their hematopoietic or stromal cells produced OVA-specific IgG titers that were approximately 1/3 of those seen in WT mice, demonstrating that MyD88 expression in only one compartment was not sufficient for a full response to vaccination. They also demonstrated that at 90 min following vaccination, serum cytokine production was decreased in both groups, with different cellular compartments mediating the production of different cytokines/chemokines. For example, IL-12 and TNF production was primarily mediated by hematopoietic cells, KC production was primarily mediated by stromal cells, and both were required for IL-1α, IL-6, IP-10, and MCP-1 production. In a follow-up study [297], they i.p. vaccinated WT, MyD88-/-, and TLR5-/- mice with the same doses of OVA and flagellin two times (days 0

and 28) and examined OVA-specific IgG production on day 42. MyD88-/- mice had serum anti-OVA IgG titers that were approximately 50% of those seen in WT mice, while TLR5-/- mice had titers equivalent to those seen in WT mice. It is therefore possible that the initial requirement for MyD88 in both compartments seen on day 14 would have changed if mice in the original study had been vaccinated twice and OVA-specific IgG titers examined on day 42. In the follow-up study, with the exception of IL-18, serum cytokine production in MyD88-/- mice following vaccination was completely ablated and that in TLR5-/- mice was reduced for 6 of 12 cytokines examined (IL-1α, IL-6, IL-18, KC, G-CSF, MCP-1) and completely inhibited for the remaining 6 of 12 (TNF, IL-12p40, IL-17, Eotaxin, RANTES, MIP-1β). The authors concluded that serum cytokine/chemokine production was therefore not required in the induction of adaptive immune responses. However, as not all cytokine production was completely ablated in TLR5-/- mice, it is impossible to say if that is the case. In addition, the authors did not demonstrate that the reduction in antibody titer in MyD88-/- mice was not related to the ablation of serum cytokine production. Interestingly, in their original study [296], Sanders et al. also investigated flagellin-specific antibody production in IL-6-/- and TNFα-/- mice and demonstrated that flagellin-specific IgG production was impaired in both (~45% and ~20% compared to WT); they did not examine the impact of these deficiencies on the ability of flagellin to increase OVA-specific IgG titers. However, this model is complicated by the ability of flagellin to induce antigen-specific immune responses in a manner independent of TLR signaling.

3.1.4 Model, purpose, and outcome

It is evident from these studies that gaps exist in the scientific knowledge of the role of the mucosal barrier in the response to mucosal vaccination and in the role that adjuvant-induced cytokines and chemokines play in the development of antigen-specific adaptive immune responses. Elucidating the role of the mucosal barrier in the response to one potent mucosal vaccine adjuvant, IL-1α, may provide insight into the role of the mucosal barrier in the response to other mucosal vaccine adjuvants and help identify cell types important in the generation of immune responses to mucosally delivered vaccines. In addition, as IL-1 is known to be a potent inducer of cytokines and chemokines and can only signal through IL-1R1, it is an ideal adjuvant for studying the role of adjuvant-induced systemic and local cytokines and chemokines in generating an adaptive immune response to a co-administered vaccine antigen.

To examine the IL-1 adjuvant mechanism of action when delivered intranasally, we generated mice expressing IL-1R1 on only their stromal (radioresistant) cells or their hematopoietic cells using the bone marrow chimeric mouse model. We demonstrated that, when IL-1α was delivered nasally, serum cytokine production was dependent upon IL-1R1 expression only on stromal cells. By contrast, there was a dose-dependent requirement for IL-1R1 expression in the generation of antigen-specific adaptive immune responses: delivery of LF + 0.25 µg IL-1α required IL-1R1 on both hematopoietic cells and stromal cells, while IL-1R1 only on hematopoietic cells was sufficient for maximal adjuvant activity following delivery of LF + 4 µg IL-1α. As IL-1R1 expression only on stromal cells was sufficient for serum cytokine production but not for the generation of adaptive immune responses, it appears that these responses may not be related.

3.2 Materials and Methods

3.2.1 Mice

Female *Il1r1*$^{-/-}$ (B6.129S7-*Il1r1*^{tm1Imx}/J), CD45.1 congenic (B6.SJL-Ptprc^{a} Pepc^{b}/BoyJ), *Rag1*$^{-/-}$ (B6;129S7-*Rag1*^{tm1Mom}/J), C57BL/6, and GFP transgenic (C57BL/6-Tg(UBC-GFP)30Scha/J) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) at 4-6 weeks of age. Mast cell-deficient *Kit*$^{W\text{-}sh}$/HNihrJaeBsmJ mice (SASH) were obtained in house from Dr. Laura P. Hale (available from Jackson Laboratory as strain # 005051) [193]. Mice were housed in filter top cages and provided with food and water ad libitum. All procedures were approved by the Duke University Institutional Animal Care and Use Committee.

3.2.2 Mouse Irradiation and Bone Marrow Transfer

The protocol used was similar to those previously described [296, 298, 299]. Bone marrow was harvested the morning of the irradiation and transfer. Donor mice were sacrificed using CO2 euthanasia, and marrow was harvested by flushing the femurs, tibias, and humeri with cold media (RPMI 1640, 10% FBS, 1% Penstrep, 1% Hepes, 1% NEAA, 1% sodium pyruvate, 0.1% 2-ME). The harvested bone marrow was washed twice with sterile, cold PBS and resuspended to 1 x 10^7 cells/ml. Cells were stored on ice until transfer. Recipient mice were irradiated in an X-ray irradiator with 10.5 Gy of total body irradiation (Xrad 320, Orthovoltage). Recipients were anesthetized and transferred retro-orbitally with 5 x 10^6 donor cells. Mice were kept warm and were monitored through recovery from anesthesia. Mice were nasally administered 1 µg LPS (Invivogen *E. coli* 0111:B4) in 10 µl PBS three times after irradiation, on weeks 1, 3, and 5 to induce mucosal DC turnover and ensure that all DCs in the nasal cavity were of

donor origin. Mice were rested for a total of 8-10 weeks after irradiation, and chimerism was confirmed using flow cytometry for CD45.1 and CD45.2.

3.2.3 Flow Cytometry on Peripheral Blood Leukocytes

Whole blood samples were collected from mice into 2.5 – 5 ml 40 U/ml heparin and kept at room temperature until spun at 500 x g for 10 minutes. The supernatant was removed, and red blood cells were lysed in 10 ml ACK lysis buffer (0.15 M NH_4Cl, 1.0 mM $KHCO_3$, 0.1 mM Na_2EDTA in dH20, pH 9.5) for 4-8 minutes. Samples were centrifuged at 500 x g for 8 minutes and resuspended in 10 ml FACS buffer (PBS w/o calcium or magnesium, 0.5% FBS, 0.005% EDTA). Samples were centrifuged at 500 x g for 8 minutes. The supernatant was removed and samples were transferred to 96-well v-bottom plates and centrifuged at 400 x g for 2 minutes to pellet the cells. The plate was flicked to remove supernatant and then blotted prior to vortexing. Samples were washed in 200 µl FACS buffer, centrifuged, and the supernatant removed as before. Samples were blocked with unlabeled mouse IgG2a at 10 µg/ml in 100 µl FACS buffer for 30 minutes on ice. Plates were then washed two times. Specific antibodies were added to the wells in 100 µl (1:50 in FACS buffer) and incubated for 30 minutes on ice in the dark. PE mouse anti-mouse CD45.1 and APC mouse anti-mouse CD45.2 were purchased from BD Pharmingen. Plates were washed two times, and 200 µl 1% paraformaldehyde was added to fix cells prior to moving samples to FACS tubes. The samples were acquired on a four-color FACSCalibur and analyzed in FlowJo.

3.2.4 Nasal Vaccination

Mice were anesthetized with isoflurane prior to vaccination. Vaccines were administered intranasally in 10 µl (5 µl per nostril). Non-chimeric mice were vaccinated on days 0, 7, and 21. Chimeric mice were vaccinated on days 0, 7, 21, and 42. For each chimeric study replicate, mice were divided into groups of 4-8. Vaccine groups included PBS alone, rLF alone (LIST #172), rLF plus 0.25 or 4 µg IL-1α (R&D 400-ML), and rLF plus 1 µg CT (LIST #100B). For three of the four experiments, mice were vaccinated with 25 µg LF for the day 0, 7, and 21 immunizations and 50 µg LF on day 42. For the remaining experiment, mice were immunized with 50 µg LF each time. Doses were selected on the basis of dose response studies carried out in unirradiated WT and IL1R1KO mice (Figure 10). The results from the groups varied little, and the data were combined and analyzed together. Mice vaccinated with LF + CT served as a positive control to ensure that all mice had the ability to respond to vaccination equally with the production of antigen-specific antibodies. LF + CT-vaccinated mice responded similarly in all four chimeric groups. The n per group for each experiment is listed in Appendix B.

3.2.5 Sample Collection

Blood samples were collected from unanesthetized mice by submandibular lancet method [300]. Samples were collected into 1.5 ml centrifuge tubes and centrifuged at 13,000 rpm at 4℃ for 25 minutes. The serum supernatant was pipetted off into a new tube. Vaginal lavage samples were collected from anesthetized mice by pipetting 100 µl sterile PBS into the vaginal tract and pipetting in and out multiple times. Samples were centrifuged at 13,000 rpm at 4℃ for 25 minutes to remove cell debris.

Fecal samples were collected from each mouse and homogenized by vortexing for 20 minutes in a solution with fecal extraction buffer (PBS, 10% normal goat serum, 0.1% Kathon) at 1 ml buffer per 100 mg feces. Samples were centrifuged at 13,000 rpm at 4℃ for 25 minutes. The supernatant was then removed into a new tube. Nasal lavage samples were collected from euthanized mice. After removing the head from the body, the lower jaw was cut away and the nasal lavage samples were collected by injecting 1 ml of PBS posteriorly into the nasal cavity. Fluid exiting the nostrils was collected and spun at 13,000 rpm at 4℃ for 20 minutes. BAL samples were collected by inserting a gavage needle attached to a 1 ml syringe into the trachea and lavaging the lungs twice with 1 mL of PBS. All samples were stored at -20℃. Nasal cavity cells were harvested following nasal lavage by removing the top half of the skull using scissors to expose the nasal cavity. Cells were then scraped out of the cavity into 5 mL PBS using a flat-end spatula. Cells were centrifuged at 500 x g. Supernatants ("supe") were removed to a new tube, and the cells were resuspended in 200 µl PBS and transferred to a new tube.

3.2.6 Enzyme-linked Immunosorbent Assay

ELISAs were performed as outlined in Nordone et al. [167] except that ELISA plates were coated with rLF at 2 µg/ml in CBC buffer. The rLF used for coating plates was kindly provided by Greg Caldwell at The Burnham Institute for Medical Research (La Jolla, CA). Sample endpoint titers were calculated as the last dilution at which the sample RLU reading (560 nm) was three-fold greater than a similarly diluted naïve sample RLU reading (560 nm). The log2 endpoint titers were used for statistical analysis. Serum samples were plated at a starting dilution of 1:32. Vaginal and fecal samples were plated at a starting dilution of 1:4. Nasal lavage samples were plated

undiluted. Samples with undetectable titers were assigned a titer of one less than the starting plate dilution. Mucosal samples were normalized against the total amount of IgA or IgG present in the sample, as determined by plating samples on plates coated with either anti-IgA or anti-IgG at 5 µg/ml. A standard curve was prepared using a diluted standard. Total IgA and IgG were calculated using the sample RLU values falling within the linear range of the standard curve to calculate the total Ig concentration using the equation for the line. Normalized titers are expressed as titer/µg.

3.2.7 Avidity ELISA

Avidity ELISAs were performed as above with one exception [301]. After overnight incubation with samples, the plates were washed four times with ELISA wash buffer and then incubated for 15 minutes with ammonium thiocyanate (NH_4SCN; Sigma #431354) diluted in 15 mM phosphate buffer at concentrations of 0, 1, 2, and 3 M. Plates were then washed four times, and the secondary antibody was added. Sample titers were calculated as above for ELISA titers, and the titers at each NH_4SCN concentration were calculated as the percent of the titer at the 0 M concentration, e.g., (3 M titer/0 M titer)*100%. Due to the nature of the assay, only samples with measurable serum IgG titers were tested for antibody avidity.

3.2.8 Spleen Cell Restimulation

Mice were euthanized at the end date of the experiment using CO_2 overdose, their spleens were immediately harvested, and a single cell suspension of spleen cells was prepared. Briefly, spleens were crushed and forced through a 70-µm filter into 8 ml T cell Media (RPMI 1640, 10% FBS, 1% HEPES, 1% Pen/Strep, 0.1% 2-ME, 1% 1N

NaOH, 1% sodium pyruvate, 1% MEM non-essential amino acids, 2% MEM amino acids). Cells were transferred to 15 ml centrifuge tubes and centrifuged at 400 x g for 10 minutes. The supernatant was removed and cells were resuspended in 5 ml ACK lysing buffer and incubated for 5 minutes at room temperature. A total of 10 ml T cell media was added to each tube, and cells were centrifuged at 514 x g. The supernatant was removed, and cells were washed twice with 10 ml T cell media. Cells were resuspended in 5 ml T cell media and counted. The volume was adjusted to 1 x 10^7 cells/ml. Cells were plated in 250 µl media per well in 48-well plates (CoStar 353078). T cell media or 40 µg/ml rLF in media was then added to the cells (250 µl). The plates were incubated at 37℃ for 60 hr. Supernatants were harvested to 9 6-well deep-well plates and stored at -80℃. Thawed samples were tested for the presence of cytokines (IL-2, -4, -10, -17, IFNγ) using a multiplexed bead assay from R&D (Minneapolis, MN). Values less than the low value of the standard curve were assigned a value of ½ the low standard. Data shown are the mean antigen-specific cytokine production for each group (i.e., LF-induced cytokine production – unstimulated cell cytokine production).

3.2.9 Lethal Toxin-Neutralization Assay

Serum collected from mice post-immunization was used to measure the titer of anthrax lethal toxin neutralizing antibodies in an anthrax macrophage toxicity assay in the presence of two-fold excess toxin required for killing 100% of the cells. The serum was serially diluted 1:2, and 50 µl of the dilution was incubated with 50 µl PA (0.1875 µg/ml) and 100 µl LF (0.1875 µg/ml) for 1 hr at 37°C to allow neutralization to occur. Samples were then added to J774A cells and incubated at 37℃ for 4 hr. At the end of the incubation, 20 µl of CellTiter 96 Aqueous One Solution (Promega) was added, and

cells were incubated 2-4 hr at 37℃, until the color was fully developed. At the end of the incubation, the absorbance at 490 nm was measured. Samples with an NT_{50} less than 1:64 were below our tested range and were assigned a value of one less than the lowest log2 dilution (1:32) for graphical and statistical representation.

3.2.10 *Rag1*$^{-/-}$ mouse reconstitution

Rag1-/- mice were injected retro-orbitally with 5 x 10^7 splenocytes and 8 x 10^5 CLN cells in HBSS and were rested for 20 days. On day 20, peripheral blood was tested for reconstitution by flow cytometry using APC anti-mouse CD3, PE anti-mouse B220, and FITC anti-mouse IgM antibodies (1:200) using the above peripheral blood flow cytometry protocol. Mice were then nasally vaccinated as above on days 0, 7, 21, and 63.

3.2.11 Cytokine/chemokine detection

Serum, BAL, NL, and nasal cavity cells from WT mice were tested for the presence of cytokines and chemokines using a mouse 23-plex multiplex bead assay from Bio-Rad and mouse antibody duoset ELISAs (R and D Systems). Based on the profiles seen in WT mice, serum and NL samples from chimeric mice were evaluated using custom kits from Bio-Rad (serum: IL-6, Eotaxin, G-CSF, KC, and MCP-1; NL: IL-6, IL-10, IL-13, IFNγ, G-CSF, KC, MCP-1, and TNFα). Values less than the low value of the standard curve were assigned a value of ½ the low standard. Duosets included CCL19 (DY440), CCL20 (DY760), CCL21 (DY457), and CCL28 (DY533).

3.2.12 Trizol RNA extraction

Nasal cavity tissue was harvested at the indicated time points from sacrificed mice. Tissue samples were forced through 70-µm cell strainers into RPMI (5% FBS, 1% PenStrep, 1% HEPES) and centrifuged at 514 x g immediately. Media was removed from the pellet, and samples were resuspended in 1 ml Trizol reagent (Invitrogen 15596-026) and stored at -80ºC until mRNA was extracted per the manufacturer's protocol.

3.2.13 Illumina BeadChip Arrays

Mouse WG-6 v2 Expression BeadChips were purchased from Illumina, Inc. (San Diego, CA). Samples were spotted on the BeadChip arrays by Expression Analysis (Durham, NC).

3.2.14 Statistical Analysis

All analyses were done in GraphPad Prism v. 5.00. All antibody data were analyzed using ANOVA with Bonferroni's multiple comparison post-test. Samples with no detectable ELISA titers were assigned a value of one less than the lowest log2 dilution for statistical and graphical purposes. All cytokine data were log-transformed and analyzed using ANOVA with a Bonferroni's multiple comparison post-test. Any antigen-specific cytokine production value equal to 0 was assigned a value of 0.01 prior to the log transformation to prevent loss of data. A p value < 0.05 was considered significant. Array data were analyzed by Expression Analysis using a permutation analysis for differential expression (PADE). Expression between data sets was considered significantly different when $p < 0.05$ and the false discovery rate (FDR) for accumulated sets was less than 0.3.

3.3 Results

3.3.1 Antigen and adjuvant dose response studies

Before beginning studies in chimeric mice, it was first important to determine the most effective doses of antigen and adjuvant based on the production of serum immunoglobulin and serum lethal toxin (LeTx)-neutralizing antibodies. Several dose response studies were carried out to determine the optimal vaccine doses of rLF and rIL-1α. In all three studies, mice were vaccinated on days 0, 7, and 21. In the first study, WT mice were vaccinated with 6, 12.5, or 25 μg LF +/- 0.1 μg CT, 1 μg IL-1α, or 4 μg IL-1α (Figure 10A). Including CT or 4 μg IL-1α in the vaccine formulation significantly increased the serum anti-LF IgG titer for all three tested doses of LF ($p < 0.05$), while 1 μg IL-1α significantly increased serum anti-LF IgG titers in mice vaccinated with 12.5 μg LF ($p < 0.05$). LF, CT, and IL-1α were also tested in IL-1R1-/- and SJL mice to determine (1) if the adjuvant activity of IL-1α was solely dependent upon IL-1R1 and (2) if the doses chosen for each antigen/adjuvant were appropriate for use in other mouse strains on the C57BL/6 background. In the second experiment, IL-1R1-/- and WT C57BL/6 mice were vaccinated with 50 μg LF +/- 1 μg CT or 4 μg IL-1α (Figure 10B). In both strains of mice, 50 μg LF alone induced highly variable serum anti-LF IgG titers (IL-1R1-/- and WT range: 1:16 – 1:524,288; IL-1R1-/- and WT GMTs: 1: 12,417 and 1:169, respectively). Adding CT or IL-1α only significantly increased anti-LF IgG over LF alone in WT mice (1:16,780,000 and 1:9,636,000, respectively; $p < 0.001$). However, the addition of CT significantly increased the serum 50% LeTx-neutralization titer (NT_{50}) in both strains of mice compared to LF alone (IL-1R1-/-: 1:576; WT: 1:348; $p < 0.001$), while the addition of IL-1α significantly increased the serum NT_{50} over LF alone in WT

mice (1:274; $p < 0.001$). In the final experiment, SJL mice were vaccinated with 50 µg LF alone or LF + 0.1 µg CT or 0.25, 1, or 4 µg IL-1α (Figure 10C). Including any of the adjuvants in the vaccine formulation significantly increased the serum anti-LF IgG titer compared to LF alone-vaccinated mice ($p < 0.01$). There were no significant increases in serum NT_{50} for any of the vaccinated groups. Based on these experiments, 25 µg LF and 0.25 and 4 µg IL-1α were chosen for the innate and adaptive immune response studies.

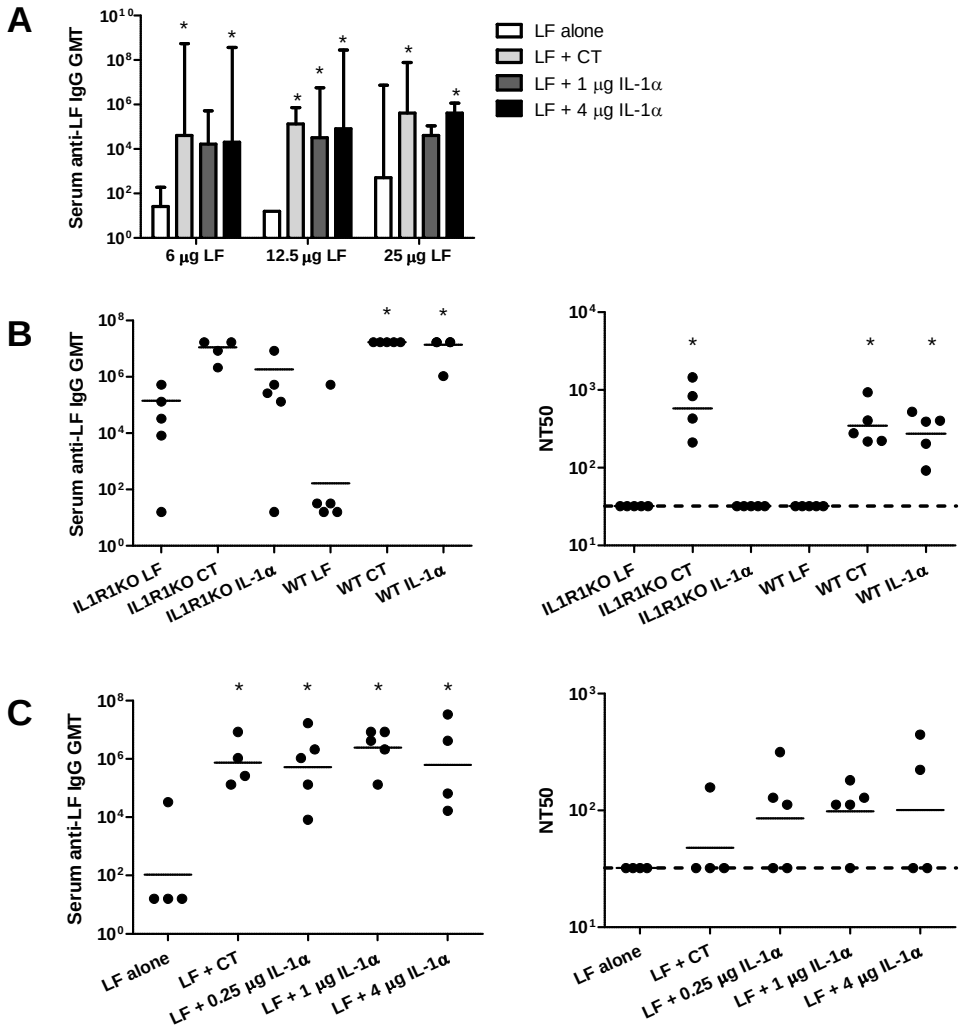

Figure 10. Serum anti-rLF IgG titers and NT_{50} following three dose response experiments.

Mice were nasally vaccinated on days 0, +7, and +21 with LF +/- adjuvant. (a) WT mice. Serum samples taken on day +42 were tested by ELISA for LF-specific serum IgG. Bars represent the geometric mean titers for each group, with error bars representing the 95% confidence level. (b) WT vs. IL-1R1-/- mice. Serum samples taken on day +42 were tested by ELISA and an anthrax lethal toxin-neutralization assay. Circles represent the titer for individual mice. Lines represent the group average geometric mean titer. (c) SJL mice. Serum samples taken on day +42 were tested by ELISA and an anthrax lethal toxin-neutralization assay. Circles represent the titer for individual mice. Lines represent the group average geometric mean titer. *: Significantly increased over LF alone.

3.3.2 Nasal vaccination with LF + IL-1α induces changes in gene expression in nasal cavity tissue

We examined the ability of LF or LF + adjuvant to induce gene expression changes in the nasal cavity, as they may reflect pathways through which the vaccine adjuvants mediate their activity. WT C57BL/6 mice were vaccinated with 25 µg LF, LF + 1 µg CT, or LF + 4 µg IL-1α, and nasal tissue was harvested at 3, 6, and 24 hours. The baseline for statistical significance was set at a p value of < 0.05 and a false discovery rate (FDR) of < 0.3. Compared to naïve mice, LF alone did not induce any significant changes in gene expression. LF + CT induced 1, 15, and 2 significant changes in gene expression at 3, 6, and 24 hours, respectively, compared to naïve and 0, 3, and 0, respectively, compared to LF alone-vaccinated mice (Table 3). Only 2 of the 21 gene expression changes induced by CT were gene up-regulation; 8 of the 15 changes at 6 hours compared to naïve were the down-regulation of olfactory receptors. Other changes at 6 hours included the down-regulation of Porcn, Stap2, Tst, Abca15, Atp7b, and Epc1 compared to naïve, and the up-regulation of Cebpa, Crem, and Nup54 compared to LF alone. At 3 hours, Mgat4b was up-regulated 24.34 fold compared to naïve, and at 24 hours, Rasl10a was down-regulated 5.2 fold compared to naïve. The functions of these genes are diverse and include copper transport (Atp7b), modulation of leptin gene expression (Cebpa), transcription factors (Crem), and modulation of the availability of serum glycoproteins (Mgat4b).

Table 3. Number of gene expression changes induced by vaccination

	3 Hours	6 Hours	24 Hours
	vs. Naive		
LF Alone	0	0	0
LF + IL-1α	395	476	550
LF + CT	1	15	2
	vs. LF Alone		
LF + IL-1α	538	565	545
LF + CT	0	3	0

Conversely, IL-1α induced a large number of significant gene expression changes in nasal cavity tissues: 395, 476, and 550 at 3, 6, and 24 hours, respectively, compared to naïve animals and 538, 565, and 545 at 3, 6, and 24 hours, respectively, compared to LF alone-vaccinated animals. LF + IL-1α resulted in the down-regulation of only 23 genes, and 22 of the 23 were only significantly down-regulated compared to naïve animals, not LF alone (Table 4). The Panther Classification system (www.pantherdb.com) was used to categorize the genes up-regulated by vaccination with LF + IL-1α or CT into significantly up- or down-regulated biological processes (Table 5). While IL-1α significantly altered 32 biological processes, CT only altered 2. A full list of the genes significantly up- or down-regulated by IL-1α compared to naïve animals can be found in Appendix A. Although both CT and IL-1α have been shown to be potent vaccine adjuvants [73, 115], these data demonstrate that they have very different actions on gene regulation at the site to which they are delivered and that local gene expression changes may not be a valid correlate of adjuvant activity.

Table 4. Genes down-regulated by IL-1α

	3 Hours	6 Hours	24 Hours
ATP7B	x	x	
CBFA2T2	x		
CERKL	x		
CPNE7	x		
GAL3ST3	x		
MYLC2PL	x		
OLFR535	x		
TMEM16E	x		
ELAC2	x		
TMEM143	x		
NADSYN1		x	
OLFR51		x	
OLFR711		x	
OLFR1391		x	
PTGER2		x	
RASGEF1B		x	
ABCA15			x
LTA			x
OLFR1443			x
RARB			x
TGM5			x
PLAC1L (vs. LF)		x	

Table 5. Biological processes in nasal tissue significantly up- or down-regulated by nasal vaccination compared to naïve animals

	p - value		
IL-1α	**3 Hours**	**6 Hours**	**24 Hours**
Apoptosis	1.94E-05	2.88E-14	
B-cell- and antibody-mediated immunity		1.74E-09	4.12E-05
Biological process unclassified	↓2.74E-10	↓9.93E-16	↓7.82E-14
Blood clotting	1.11E-02	2.54E-08	5.39E-09
Cell adhesion	5.73E-03	4.22E-06	
Cell adhesion-mediated signaling		1.10E-05	
Cell communication	1.01E-08	4.58E-13	5.76E-13
Cell motility	6.85E-04		
Cell proliferation and differentiation	1.27E-03	1.07E-04	2.14E-05
Cell surface receptor mediated signal transduction	1.10E-07	8.51E-07	1.33E-16
Cytokine and chemokine mediated signaling pathway	9.30E-21	3.78E-12	1.23E-31
Cytokine/chemokine mediated immunity	2.63E-15	1.38E-06	2.79E-11
Endocytosis		3.20E-03	
Granulocyte-mediated immunity	1.78E-06	1.50E-09	1.79E-11
Immunity and defense	1.02E-38	1.36E-45	1.32E-59
Induction of apoptosis			3.37E-04
Inhibition of apoptosis	4.01E-03		2.85E-09
Interferon-mediated immunity	4.21E-04		
Intracellular signaling cascade	5.65E-08	1.45E-02	3.05E-05
JAK-STAT cascade	9.08E-09	1.57E-02	8.01E-08
JNK cascade	3.15E-02		
Ligand-mediated signaling	2.60E-10	1.85E-07	4.07E-15
Macrophage-mediated immunity	9.12E-20	1.44E-22	5.64E-23
MAPKKK cascade	2.05E-02		
Natural killer cell mediated immunity		1.05E-10	9.37E-04
NF-kappaB cascade	2.55E-07		7.15E-03
Non-vertebrate process			1.18E-02
Other immune and defense	2.99E-05	1.96E-02	7.01E-03
Receptor mediated endocytosis		1.75E-05	4.57E-02
Receptor protein serine/threonine kinase signaling pathway			1.54E-02
Signal transduction	6.19E-12	7.08E-20	1.45E-23
Stress response	7.32E-04	1.44E-02	3.12E-04
CT			
Protein glycosylation	1.87E-02		
Transport		4.16E-02	

3.3.3 Nasal vaccination with IL-1α induces the acute production of serum cytokines and chemokines.

Innate immune responses help to shape the adaptive immune response [302-304], and adjuvant delivery is known to induce increased production of a variety of proinflammatory cytokines in the serum, including IL-6 and MCP-1, within 3 - 24 hours after adjuvant delivery [26, 278]. Although IL-1 is known to induce a variety of cytokines/chemokines in tissue culture and following systemic delivery, it is unclear what cytokine/chemokine profile is induced following intranasal delivery. Therefore, we evaluated the presence of cytokines in the serum of WT mice at 3, 6, and 24 hours after vaccination with LF ± 4 µg IL-1α (Table 6). In a panel of 23 cytokines and chemokines (BioRad), 3 were increased three hours after vaccination compared to naïve mice and mice vaccinated with LF alone: MCP-1 ($p < 0.001$), KC ($p < 0.001$), G-CSF ($p < 0.001$); IL-6, IL-9, and GM-CSF were significantly increased only compared to LF alone ($p < 0.05$). At 6 hours, IL-6, IL-9, and MCP-1 were no longer increased, but Eotaxin was increased compared to naïve mice ($p < 0.01$). Only G-CSF remained significantly increased in LF + IL-1α-vaccinated mice over naïve and LF alone at 24 hours after vaccination ($p < 0.05$). The biological significance of some increases that were significant compared to LF alone but not greatly increased (e.g., GM-CSF) is unclear and may be small.

Table 6. Serum cytokine induction in WT mice after nasal vaccination with LF + IL-1α

	3 Hours			6 Hours			24 Hours		
	Naive	LF	LF + IL-1α	Naive	LF	LF + IL-1α	Naive	LF	LF + IL-1α
IL-6	279	282	870[b]	667	243	597	238	203	328
IL-9	387	305	535[b]	263	378	368	359	366	383
Eotaxin	957	902	1696	511	671	1205[a]	959	893	991
G-CSF	93	4437	39,773[ab]	201	2885	25,108[ab]	108	106	4,275[ab]
GM-CSF	97	80	134[b]	75	88	114[a]	90	85	106
KC	67	571	6,599[ab]	38	91	396[ab]	61	56	135
MCP-1	261	728	2,269[ab]	213	253	394	230	236	334

[a]Significantly increased over naïve at the same time point ($p < 0.05$)
[b]Significantly increased over LF at the same time point ($p < 0.05$)

Serum cytokine profiles may not reflect local mucosal cytokine production after nasal vaccination. To evaluate local mucosal cytokine production after nasal immunization, nasal lavage and BAL samples were collected and examined for the presence of 23 cytokines and chemokines at 3, 6, and 24 hours following vaccination with 25 µg LF alone, 4 µg IL-1α alone, or 1 µg CT alone (Tables 7 and 8). There was a large degree of variation in the BAL samples, which was likely due to the collection procedure but could also reflect variation in vaccine drainage from the nasal cavity to the lungs. LF and CT did not induce any significant increases in BAL cytokines at any of the time points examined. IL-1α induced significant increases in IL-9, IL-13, G-CSF, and MCP-1 at three hours compared to naïve ($p < 0.05$). No significant increases in cytokine production were observed at any other time point. Less variation in cytokine production was seen in nasal lavage samples. Vaccination with LF alone did not induce significant increases in any cytokine at 3 or 24 hours compared to naïve. However, IL-13 and KC were significantly increased at six hours compared to naïve. When CT was administered, significant increases were seen in IL-13 and KC production compared to naïve at 3 and 6 hours and in IL-6 at 6 and 24 hours ($p < 0.05$). The increases in IL-13 and KC induced by either LF alone or CT were small, however, and may not be biologically significant. By contrast, nasal administration of IL-1α induced significant increases in the concentration of a large number of cytokines in the nasal lavage compared to naïve and/or LF alone ($p < 0.05$): TNF and MCP-1 at 3 hours; IL-1β, IL-9, IL-10, IL-13, G-CSF, IFNγ, and MIP-1α at 3 and 6 hours; KC and MIP-1β at 3, 6, and 24 hours; IL-6 at 24 hours. These data indicate that IL-1α induces the measureable production of more cytokines at sites local to the site of vaccination than it does

systemically and that CT is a poor inducer of the examined cytokines/chemokines at the examined time points and locations. Importantly, IL-1α induced different profiles of cytokine/chemokine induction mucosally and systemically.

Table 7. Nasal lavage cytokine content (pg/ml) in WT mice after vaccination with LF or adjuvant alone

Nasal Lavage												
	3 Hours				6 Hours				24 Hours			
	Naive	LF	IL-1α	CT	Naive	LF	IL-1α	CT	Naive	LF	IL-1α	CT
IL-1β	3	2	22[ab]	2	2	2	16[ab]	2	2	2	4	2
IL-6	49	198	842	355	26	31	1,272	721	0	0	1,056[ab]	183[ab]
IL-9	15	4	123[ab]	18	5	27	80[a]	22	12	11	33	15
IL-10	1	1	37[ab]	1	1	1	19[ab]	1	1	1	1	1
IL-13	23	3.2	81[b]	35[b]	3.2	37[a]	82[a]	39[a]	35	45	55	19
G-CSF	120	221	3,144[a]	76	115	54	3,095[ab]	75	32	31	682	37
IFNγ	1	1	46[ab]	1	1	1	30[ab]	1	1	1	2	1
KC	14	38	468[ab]	98[a]	9	33[a]	549[ab]	47[a]	17	24	155[a]	13
MCP-1	5	5	44[ab]	5	5	5	15	4.5	5	5	15	5
MIP-1α	2	2	40[ab]	2	2	2	20[ab]	2	2	2	2	2
MIP-1β	1	1	49[ab]	1	1	1	28[ab]	1	1	1	6[ab]	1
TNFα	2.0	2.0	22[ab]	2	2	8	13	2	2	2	50	2

[a]Significantly increased over naïve at the same time point ($p < 0.05$)
[b]Significantly increased over LF at the same time point ($p < 0.05$)

Table 8. Bronchoalveolar lavage cytokine content (pg/ml) in WT mice after vaccination with LF or adjuvant alone

	BAL											
	3 Hours				6 Hours				24 Hours			
	Naive	LF	IL-1α	CT	Naive	LF	IL-1α	CT	Naive	LF	IL-1α	CT
IL-1β	6	4	11	3	3	2	5	4	2	2	4	4
IL-9	33	35	76[a]	44	30	28	20	47	36	35	45	39
IL-13	53	58	199[a]	124	49	64	38	111	84	80	98	63
G-CSF	5	6	176[ab]	2	5	2	31	2	2	2	60	371
MCP-1	5	12	130[a]	60	11	14	7	38	24	24	38	20
TNFα	12	37	245	156	40	50	28	132	110	114	140	82

[a]Significantly increased over naïve at the same time point ($p < 0.05$)
[b]Significantly increased over LF at the same time point ($p < 0.05$)

Several chemokines have also recently been implicated in adjuvant mechanisms of action or have been shown to be useful as adjuvants themselves [278, 279, 305, 306]. As the multiplex bead assays do not include the majority of these chemokines, we further examined the ability of IL-1α to induce the production of CCL19, CCL20, CCL21, and CCL28 in serum and mucosal tissues. No increases were seen in CCL19, CCL21, or CCL28 as a result of vaccination with IL-1α (data not shown). However, vaccination with LF plus either dose of IL-1α significantly increased CCL20 production in the nasal lavage, cell supernatants, and nasal cavity cells compared to naïve and LF alone-vaccinated animals ($p < 0.05$) (Figure 11). There was no significant increase in BAL CCL20 in mice vaccinated with LF + 4 µg IL-1α compared to naïve and LF alone (109.5 vs. 19.36 and 21.38 pg/ml, respectively). Therefore, IL-1α increases the production of CCL20 at sites local to the nasal cavity.

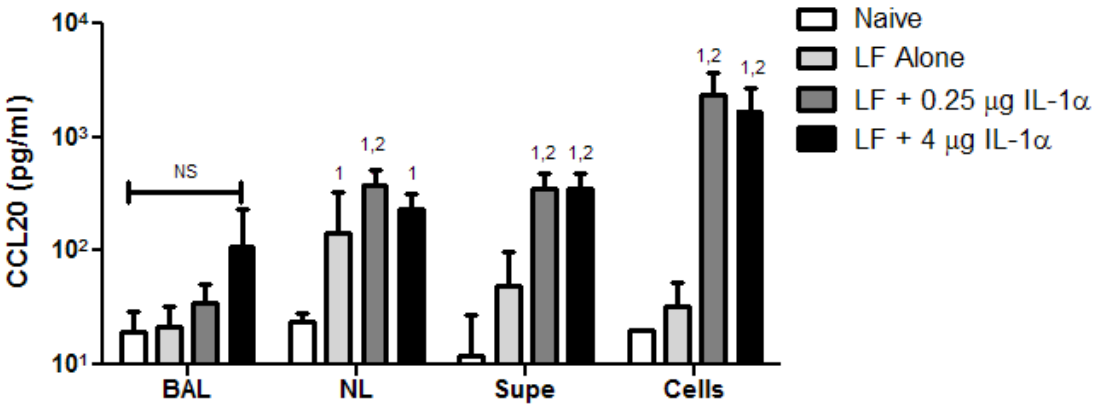

Figure 11. Vaccination with LF + IL-1α increased CCL20 production in the nasal cavity at three hours vaccination.

Bars represent mean CCL20 production (pg/ml) and error bars represent the 95% confidence level. 1: Significantly greater than Naïve ($p < 0.001$). 2: Significantly greater than LF Alone ($p < 0.05$).

3.3.4 Serum and nasal cytokine and chemokine production induced by vaccination with IL-1α is primarily mediated by stromal cells.

Many of the cytokines/chemokines induced by IL-1α can be produced by either hematopoietic or nonhematopoietic cell types, but it is unclear what cell types are responsible for their production following nasal delivery of IL-1α. To determine if the absence of IL-1R1 in either the stromal or bone marrow-derived cell compartment influenced the production of cytokines and chemokines after nasal immunization using IL-1α as the adjuvant, IL-1R1 mouse bone marrow (BM) chimeras were generated by transferring 5 x 10^6 BM cells from *Il1r1*$^{-/-}$ (KO) or CD45.1 congenic (WT) mice into lethally irradiated mice of the same strains to create four groups of BM chimeras: WT→WT, KO→KO, WT→KO, and KO→WT (donor→recipient). This allowed us to determine if the cytokine-inducing activity of IL-1α was dependent upon IL-1R1 expression in only the stromal (radioresistant) cell compartment, the hematopoietic cell compartment, or both compartments. At week 9, BM chimerism was confirmed by flow cytometry (Figure 12), and the mice were vaccinated in week 10.

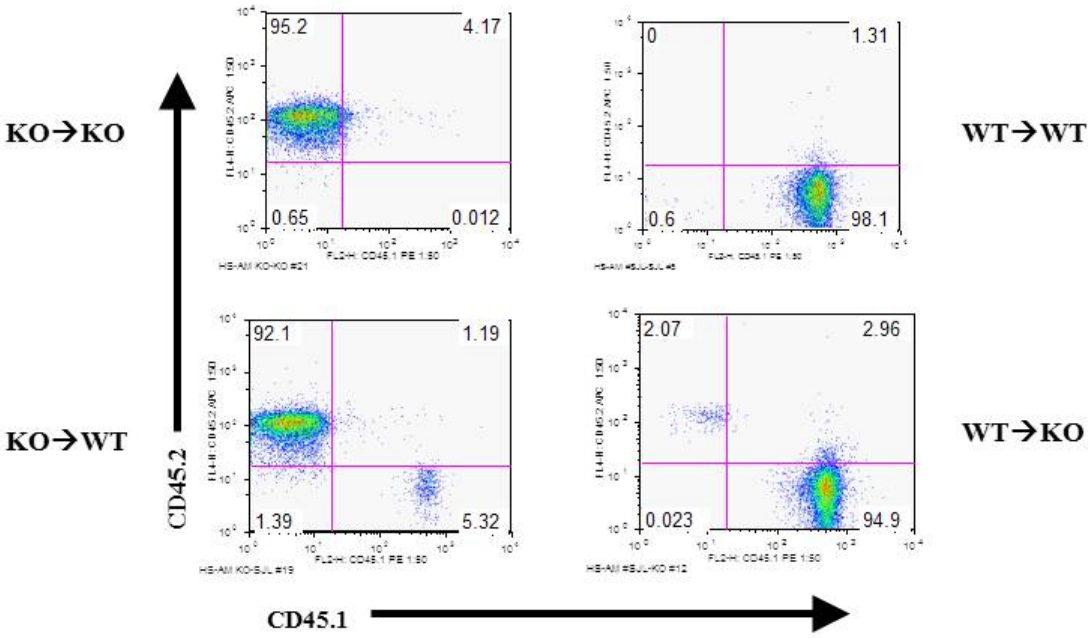

Figure 12. Representative plots of bone marrow chimerism 10 weeks after bone marrow transfer.

Based on the profiles seen in WT mice, serum samples were taken from chimeric mice at three hours after vaccination and tested for Eotaxin, MCP-1, KC, G-CSF, and IL-6 (Table 9). Vaccination with LF + CT did not induce the production of any of the five cytokines. Eotaxin was not increased in any of the vaccinated mice. KO→WT mice had significantly increased levels of the other four cytokines and chemokines that were equivalent to the levels induced in WT→WT mice. The other four cytokines and chemokines were significantly increased in KO→WT mice vaccinated with LF + IL-1α compared to LF alone-vaccinated mice ($p < 0.001$) and were equivalent to the levels induced in WT→WT mice ($p < 0.001$ compared to LF alone). Conversely, vaccination of WT→KO mice did not increase the production of those cytokines and the levels were

similar to those in LF alone-vaccinated WT→KO and LF + IL-1α -vaccinated KO→KO mice. Thus, IL-1α appears to be primarily acting on the stromal cell compartment to induce systemic cytokine production.

Local mucosal cytokine production after nasal vaccination was also investigated based on the profiles seen in WT mice (Tables 7 and 8). Because IL-1α induced a different profile of cytokine/chemokine production in WT nasal lavage samples than it did in the serum, nasal lavage samples from chimeric mice were examined for a larger panel of cytokines: IL-6, IL-10, IL-13, IFNγ, G-CSF, KC, MCP-1, and TNFα (Table 10). Due to limitations in the number of mice available, mice vaccinated with 4 μg IL-1α were only examined at 3 hours after vaccination and those vaccinated with 0.25 μg IL-1α were examined at only 3 and 24 hours after vaccination. Similar to the results seen in serum samples, increases in cytokine production were the lowest in WT→KO mice, while those in KO→WT mice were similar to WT→WT mice. No increases were seen in any group for IL-10, IFNγ, or TNFα. At three hours after vaccination, both doses of IL-1α induced significant increases in IL-6, KC, and MCP-1 in KO→WT and WT→WT mice when compared to naïve and WT→KO mice ($p < 0.05$). Vaccination of KO→WT and WT→WT mice with 0.25 μg IL-1α also induced significant increases in G-CSF at three hours when compared to naïve and WT→KO mice ($p < 0.05$). WT→KO mice vaccinated with the low dose of IL-1α had significant increases in IL-6 and KC compared to naïve mice ($p < 0.05$). WT→KO mice vaccinated with 4 μg IL-1α had significantly increased G-CSF and KC compared to naïve mice ($p < 0.05$). In KO→WT and WT→WT mice, the high dose of IL-1α also induced a significant increase in IL-13 when compared to WT→KO mice ($p < 0.05$). At 24 hours after vaccination, only G-CSF and IL-6 remained significantly

increased compared to naïve in WT→WT mice ($p < 0.05$), while only IL-6 was significantly increased in KO→WT mice ($p < 0.05$). These data indicate that cytokine/chemokine production following nasal vaccination with IL-1α was primarily dependent on IL-1R1 expression by stromal (radioresistant) cell populations, although IL-1R1$^{+/+}$ hematopoietic cells were able to mediate some KC and G-CSF production.

Table 9. Serum cytokine production (pg/ml) in IL-1R1 chimeric mice three hours after vaccination with LF or LF + IL-1α

	Naïve*	KO/KO		WT/KO		KO/WT		WT/WT	
		LF	LF + IL-1α	LF	LF + IL-1α	LF	LF + IL-1α	LF	LF + IL-1α
IL-6	2	5	4	6	9	6	257[a,b]	7	222[a,b]
Eotaxin	272	521	313	562	572	336	837	479	1128
G-CSF	82	45	70	124	175	104	13,439[a,b]	44	8,257[a,b]
KC	35	57	62	62	85	69	3,763[a,b]	71.27	1,939[a,b]
MCP-1	158	222	186	447	311	207	3,440[a,b]	262	2,831[a,b]

[a]Significantly greater than naïve and LF alone within the same chimeric group ($p < 0.001$)
[b]Significantly greater than KO/KO and WT/KO IL-1α ($p < 0.001$)
*Naïve animals were WT/WT

Table 10. Nasal lavage cytokine production (pg/ml) in IL-1R1 chimeric mice after vaccination with IL-1α alone

		3 Hours 0.25 μg IL-1α			3 Hours 4 μg IL-1α			24 Hours 0.25 μg IL-1α		
	Naïve*	WT/KO	KO/WT	WT/WT	WT/KO	KO/WT	WT/WT	WT/KO	KO/WT	WT/WT
IL-6	2	166[a]	503[ab]	449[ab]	74	595[ab]	852[ab]	34	465[ab]	639[ab]
IL-10	1	1	1	0	1	1	1	1	1	1
IL-13	13	17	56	53	10	61[b]	72[b]	13	40	54
IFNγ	1	1	2	2	1	2	3	1	2	2
G-CSF	41	150	723[ab]	639[ab]	656[a]	1,484[a]	1,896[a]	372	452	539[a]
KC	18	89[a]	439[ab]	371[ab]	130[a]	859[ab]	887[ab]	26	50	50
MCP-1	5	5	137[ab]	96[ab]	7	84[ab]	120[ab]	11	22	19
TNFα	2	1	2	2	2	3	4	2	1	1

[a]Significantly greater than naïve ($p < 0.05$)
[b]Significantly greater than WT/KO within the same time point/dose ($p < 0.05$)
*Naïve mice were WT/WT

Nasal lavage samples from chimeric mice vaccinated with either 0.25 or 4 µg IL-1α alone were also examined for CCL20 (Figure 13). Interestingly, only 0.25 µg IL-1α significantly increased CCL20 production in the NL. WT→WT mice vaccinated with 0.25 µg IL-1α induced significantly greater CCL20 production than the KO→WT and WT→KO mice (1,008 vs. 387 and 165 pg/ml, respectively; $p < 0.001$). KO→WT CCL20 production was also significantly greater than WT→KO ($p < 0.05$). At 24 hours after vaccination, the response followed the same trend, but there were no significant differences between groups. Unfortunately, due to sample limitations, only one naïve animal was included.

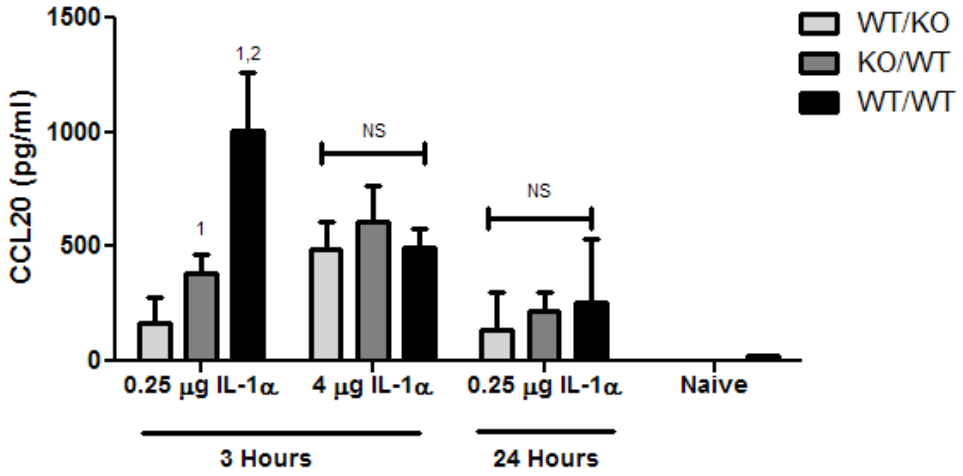

Figure 13. Vaccination with LF + 0.25 µg IL-1α increased CCL20 production in the nasal cavity at three hours post-vaccination.

Bars represent mean CCL20 production (pg/ml) and error bars represent the 95% confidence level. 1: Significantly greater than WT/KO ($p < 0.001$). 2: Significantly greater than KO/WT ($p < 0.05$).

3.3.5 IL-1R1 is required on bone marrow-derived cells for maximal serum antibody responses to nasal vaccination with LF + IL-1α in IL-1R1 chimeric mice

Although several studies have examined the ability of vaccine adjuvants to induce cytokines after vaccination, it is unclear whether adjuvant-induced cytokine production is related to antigen-specific adaptive immunity. To determine which IL-1-responsive cellular compartment is required to induce antigen-specific humoral responses, chimeric mice were vaccinated on days 0, 7, 21, and 42 with LF alone or LF + 1 µg CT or 0.25 or 4 µg IL-1α. On day 56 after the initial vaccination, serum samples were collected and tested for the presence of rLF-specific IgG (Figure 14). Mice vaccinated with LF alone produced little anti-LF IgG (<1:80). Mice vaccinated with LF + CT produced significantly greater amounts of anti-LF IgG than LF alone in all four chimeric groups ($p < 0.001$), which did not significantly differ from each other (range: 1:7,798,000–1:45,360,000). KO→KO mice vaccinated with LF + 4 µg IL-1α did not produce a significantly greater amount of anti-LF IgG than mice vaccinated with LF alone (<1:100). However, the other three chimeric groups had significantly increased serum anti-LF IgG titers ($p < 0.001$) when vaccinated with either dose of IL-1α. At the low dose of IL-1α, WT→WT mice produced significantly more IgG than WT→KO mice (1:319,557 vs. 1:1,095, respectively). WT→WT and KO→WT (1:26,008) did not differ significantly. Conversely, at the high dose, WT→WT mice produced significantly more anti-LF IgG than KO→WT mice (1:18,720,000 vs. 1:392,772, respectively), and WT→WT and WT→KO (1:2,731,000) mice did not differ significantly, indicating a dose-dependent requirement for IL-1 responsiveness in different compartments.

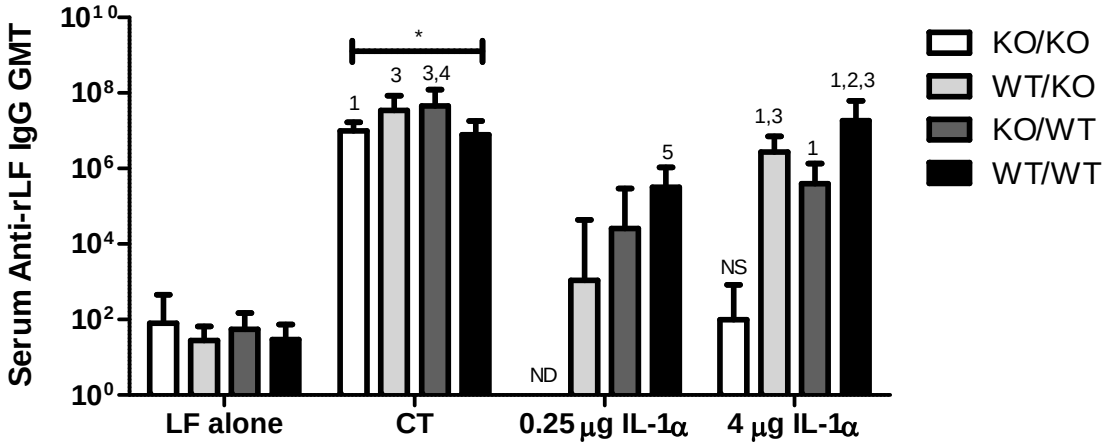

Figure 14. Serum anti-rLF IgG titers on day +56 after intranasal immunization with rLF + IL-1α were significantly impaired in KO→WT mice.

Mice were vaccinated on days 0, +7, +21 and +42 after a 10-week rest period following bone marrow reconstitution. Serum samples taken on day +56 were tested by ELISA. Bars represent the geometric mean titers for each group on day +56 for all replicates, with error bars representing the 95% confidence level. 1: Significantly greater than KO/KO 4 μg IL-1 ($p < .001$). 2: Significantly greater than KO/WT 4 μg IL-1 ($p < .01$). 3: Significantly greater than 0.25 μg IL-1 within the same chimeric group ($p < .001$). 4: Significantly greater than 4 μg IL-1 within the same chimeric group ($p < .01$). 5: Significantly greater than WT/KO 0.25 μg IL-1 ($p < .001$). *: CT groups do not differ significantly from each other. NS: Not significant over LF alone within the same chimeric group. ND: No data.

To further examine the response to vaccination, we examined serum IgG subclass production. Day 56 serum samples were tested for the presence of LF-specific IgG1, IgG2b, IgG2c, and IgG3 (Figure 15). KO→KO mice vaccinated with LF plus IL-1α did not produce significantly greater amounts of any anti-LF IgG subclass than LF alone. The anti-LF IgG1 and IgG2b responses were very similar to the anti-LF IgG responses. When vaccinated with the low dose of IL-1α, WT→WT mice produced significantly greater amounts of both IgG1 and IgG2b than WT→KO mice ($p < 0.01$) and greater anti-LF IgG2b than KO→WT mice ($p < 0.05$). WT→KO mice produced amounts of IgG1 and

IgG2b that were similar to mice vaccinated with LF alone ($p > 0.05$). KO→WT (and WT→WT did not differ significantly. At the high dose of IL-1α, WT→WT mice produced significantly more IgG1 and IgG2b than KO→WT mice ($p < 0.01$), and WT→KO mice produced significantly more IgG2b than KO→WT mice. Differences between the chimeric groups were more pronounced for anti-LF IgG2c and IgG3 production. None of the chimeric mice vaccinated with LF + 0.25 µg IL-1α produced a significantly greater amount of anti-LF IgG2c or IgG3 than LF alone-vaccinated mice. At the high dose of IL-1α, however, all three groups with at least one IL-1-responsive cellular compartment produced significantly greater amounts of IgG2c than LF alone-vaccinated mice. WT→KO and WT→WT mice produced significantly greater amounts of anti-LF IgG2c than KO→WT mice ($p < 0.001$). Only WT→WT and WT→KO mice vaccinated with LF + 4 µg IL-1α had significant increases in anti-LF IgG3 compared to LF alone-vaccinated mice ($p < 0.05$). These data support the dose-dependent requirement for IL-1α responsiveness seen in the serum IgG data, as decreased anti-LF IgG subclass production was seen in both groups expressing IL-1R1 in only one compartment when vaccinated with the low dose of IL-1α and only in KO→WT mice vaccinated with the high dose.

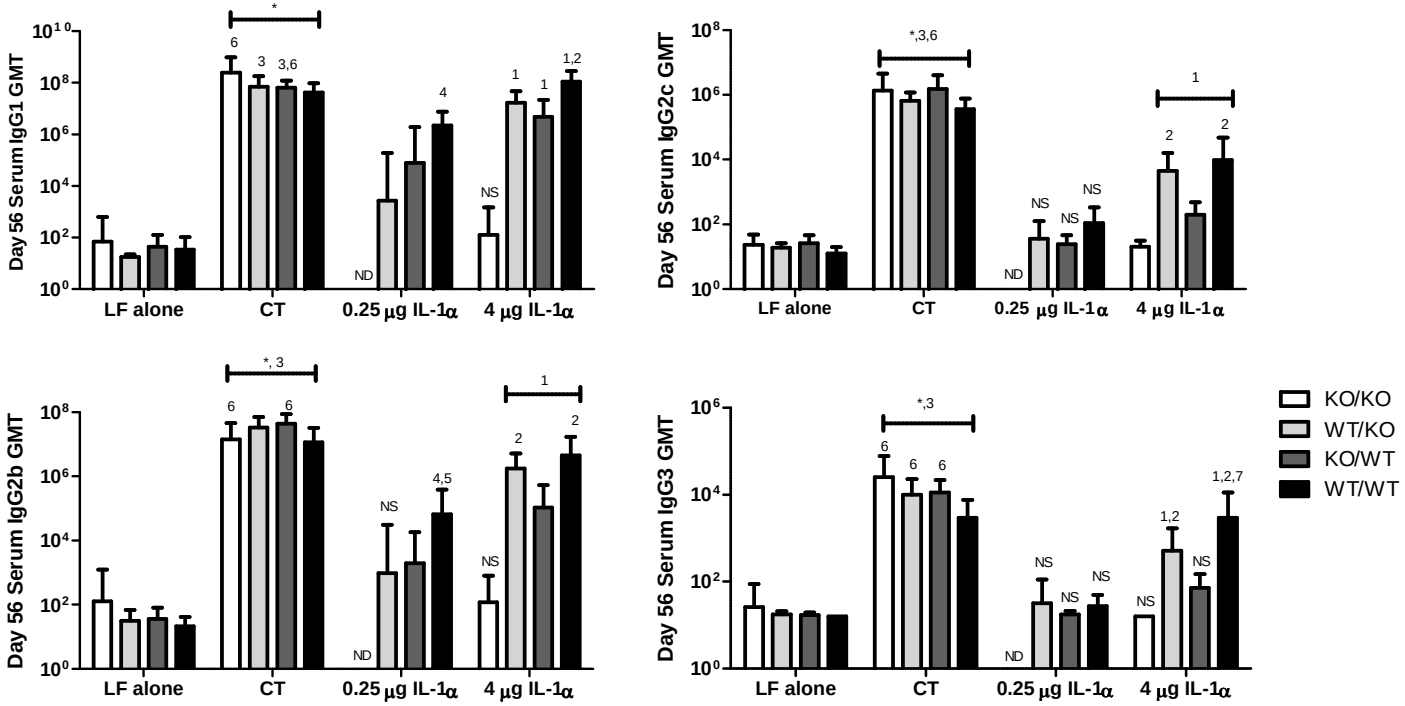

Figure 15. Serum anti-rLF IgG subclass titers on day +56 after intranasal immunization were significantly impaired in KO→WT mice vaccinated with LF + IL-1α.

Mice were vaccinated on days 0, +7, +21, and +42 after a 10-week rest period following bone marrow reconstitution. Serum samples taken on day +56 were tested by ELISA. Bars represent the geometric mean titers for each group on day +56 for all replicates, with error bars representing the 95% confidence level. 1 Significantly greater than KO/KO 4 µg IL-1 ($p < .001$). 2 Significantly greater than KO/WT 4 µg IL-1 ($p < 0.01$). 3 Significantly greater than 0.25 µg IL-1 within the same chimeric group ($p < .05$). 4 Significantly greater than WT/KO 0.25 µg IL-1 ($p < .001$). 5 Significantly greater than KO/WT 0.25 µg IL-1 ($p < 0.05$). 6 Significantly greater than 4 µg IL-1 within the same chimeric group. 7 Significantly greater than WT/KO 4 µg IL-1. NS Not significantly increased over LF alone within the same chimeric group. ND: No data. * CT groups do not differ significantly from each other.

The goal of vaccination is to induce protective immunity against the pathogen of interest, but serum anti-rLF IgG ELISA titers have been shown not to correlate with protection against an aerosolized *Bacillus anthracis* spore challenge [176]. To evaluate the induction of protective humoral immunity after nasal immunization of chimeric mice, we measured serum anthrax LeTx-neutralizing antibody titers (NTs), which have been shown to correlate with protection against an aerosolized *Bacillus anthracis* spore challenge [176]. Day 56 serum samples were tested in an anthrax LeTx-neutralization assay to determine the ability of the induced antibodies to neutralize lethal toxin (Figure 16). Serum from LF alone-vaccinated mice did not neutralize lethal toxin, while serum from LF + CT-vaccinated mice induced NT_{50}s ranging between 1:403 and 1:1,354 ($p < 0.001$ vs. LF alone). Again, KO→KO mice vaccinated with LF + 4 µg IL-1α did not respond to vaccination. WT→KO and WT→WT mice vaccinated with LF + 4 µg IL-1α produced significantly greater amounts of neutralizing antibodies than LF alone-vaccinated mice, with NT_{50}s of 1:221 and 1:300, respectively ($p < 0.001$). However, vaccination of KO→WT mice with LF + 4 µg IL-1α induced an NT_{50} of only 1:64, which was not significantly increased. None of the mice vaccinated with LF + 0.25 µg IL-1α produced significantly increased amounts of neutralizing antibody compared to LF alone-vaccinated mice. These data indicate that the low dose of IL-1α was not sufficient to induce protective immune responses in the chimeric mice. Additionally, these data support the serum antibody data, suggesting that only hematopoietic cells are required to be responsive to IL-1 to induce protective immune responses.

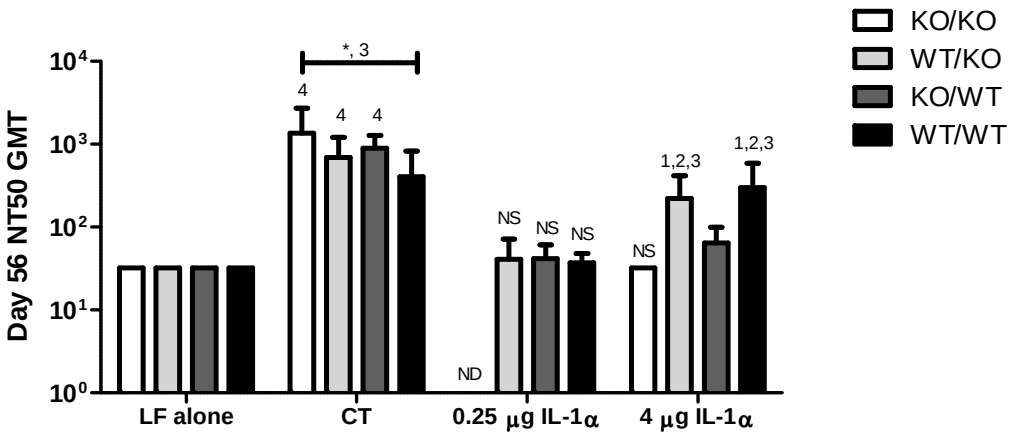

Figure 16. Serum NT_{50} on day +56 after intranasal immunization with rLF + IL-1α is impaired in KO→WT mice.

Mice were vaccinated on days 0, +7, +21 and +42 after a 10-week rest period following bone marrow reconstitution. Serum samples taken on day +56 were tested by ELISA. Bars represent the geometric mean titers for each group on day +56 for all replicates, with error bars representing the 95% confidence level. 1: Significantly greater than KO/KO 4 µg IL-1 ($p < .001$). 2: Significantly greater than KO/WT 4 µg IL-1 ($p < .001$). 3: Significantly greater than 0.25 µg IL-1 within the same chimeric group ($p < .001$). 4: Significantly greater than 4 µg IL-1 within the same chimeric group ($p < .001$). *: CT groups are not significantly different from each other. NS: Not significantly increased over LF alone within the same chimeric group. ND: No data.

Binding avidity of antibody for its specific antigen is an additional antibody property that has been shown to positively correlate with protective activity (e.g., virus or toxin neutralization) [307-309]. As such, avidity was measured using an NH_4SCN elution assay (Figure 17). Antibody titers declined in all vaccine groups and all chimeric groups with increasing concentrations of NH_4SCN. Mice vaccinated with CT had relatively high antibody avidity. At 3 M NH_4SCN, serum anti-LF IgG titers in CT-vaccinated mice were still above 40% of the 0 M titer at 3 M NH_4SCN in all three chimeric groups examined. These data indicate that the absence of IL-1R1 in one compartment did not affect the ability of the immune system to generate high avidity antibodies. Titers fell off the most

rapidly in mice vaccinated with LF + 0.25 µg IL-1α, with 1 M NH_4SCN causing an approximately 50% reduction in the serum anti-LF IgG titer in all three chimeric groups; at 3 M NH_4SCN, serum anti-LF IgG titers in all three chimeric groups had been reduced by 87-95%. There were no significant differences in antibody avidity between chimeric groups for LF + 0.25 µg IL-1α-vaccinated mice at any tested concentration of NH_4SCN. Similar to the development of neutralizing antibodies, this suggests that the low dose of IL-1α was not sufficient to induce high avidity antibodies. By contrast, antibodies in WT→WT and WT→KO mice vaccinated with LF + 4 µg IL-1α maintained >30% binding activity following incubation with 3 M NH_4SCN. However, KO→WT mice vaccinated with LF + 4 µg IL-1α had significantly greater reductions in titer at 1, 2, and 3 M NH_4SCN (reduced to 47%, 20%, and 12% of the 0 M titer, respectively) than WT→KO (70%, 40%, and 32%, respectively) and WT→WT (78%, 46%, and 36%, respectively) mice ($p < 0.05$). Therefore, the ability of IL-1α to induce the production of high avidity anti-LF antibodies was affected by the absence of IL-1R1 on hematopoietic cells.

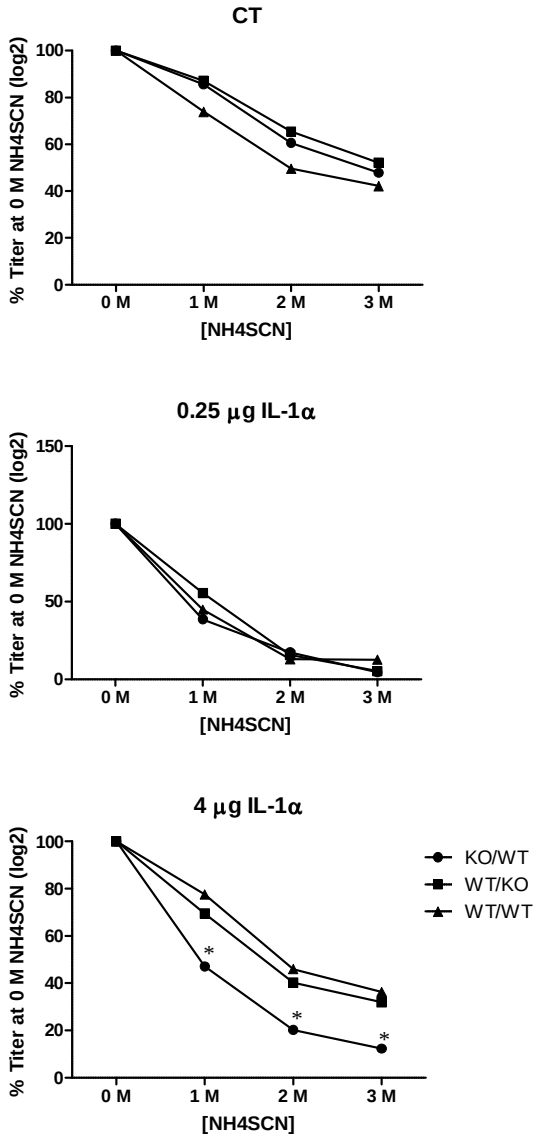

Figure 17. Antibody avidity on day +56 after immunization with rLF + IL-1α is impaired in KO→WT mice.

Mice were vaccinated on days 0, +7, +21 and +42 after a 10-week rest period following bone marrow reconstitution. Serum samples taken on day +56 were tested using an NH4SCN elution ELISA. Lines represent titers as a percentage of the titer at 0 M NH4SCN. *: Significantly less than WT/KO and WT/WT ($p < 0.01$).

Taken together, these results demonstrate that there is a dose-dependent requirement for IL-1-responsiveness in either the stromal or hematopoietic compartments, as IL-1R1 was required in only the hematopoietic compartment following vaccination with LF + 4 µg IL-1α to induce antigen-specific immune responses equivalent to those seen in mice expressing IL-1R1 in both compartments. By contrast, although the low dose of IL-1α was not sufficient to induce neutralizing or high avidity antibodies, IL-1R1 was required in both compartments following vaccination with LF + 0.25 µg IL-1α, as antigen-specific serum IgG production was decreased in both groups expressing IL-1R1 in only one compartment. It is also evident that serum and local cytokine and chemokine responses three hours after vaccination with LF + IL-1α do not correlate with the adaptive immune response.

3.3.6 Mucosal antibody responses to vaccination with LF + IL-1α are impaired in IL-1R1 KO→WT mice

One of the strengths of mucosal vaccination is the ability to induce antigen-specific mucosal IgA production, and when used as a nasal vaccine adjuvant, IL-1 has been shown to enhance antigen-specific mucosal antibody production [115, 116]. We therefore investigated mucosal antibody production in the chimeric mouse model to determine if the IL-1R1 expression requirement for mucosal antibody responses followed the requirement seen for serum antibody responses. Mice vaccinated with LF alone produced little anti-LF mucosal IgG or IgA (Figures 18 and 19). Mucosal antibody production was highly variable between animals for all groups, and fecal anti-LF IgA production was low in all groups (range of group averages: 0 - 7 titer/µg) (Figure 18). Nasal and vaginal anti-LF IgA production was detectable in all groups vaccinated with LF

+ CT (nasal: 23.8 – 41.5 titer/µg; vaginal: 36.2 - 77.4 titer/µg), though none were significantly increased compared to LF alone. KO→WT and WT→WT mice vaccinated with LF + 0.25 µg IL-1α had detectable vaginal anti-LF IgA (4.2 and 196.7 titer/µg, respectively), but the titers were not significantly increased compared to LF alone-vaccinated mice. WT→WT mice vaccinated with LF + 4 µg IL-1α had significantly increased vaginal anti-LF IgA compared to KO→KO, WT→KO, and KO→WT mice ($p < 0.01$) and mice vaccinated with LF + CT ($p < 0.05$). WT→KO and WT→WT mice had significantly increased anti-LF nasal IgA production compared to LF alone-vaccinated mice ($p < 0.05$) and KO→KO mice vaccinated with LF + IL-1α ($p < 0.01$). KO→WT mice vaccinated with the high dose of IL-1α also produced detectable vaginal and nasal anti-LF IgA (16.5 and 74.2 titer/µg, respectively), but only nasal anti-LF IgA was significantly increased over LF alone ($p < 0.05$). Although the titers were highly variable within groups, the general pattern of production was similar to that seen in the serum responses, with a dose-dependent requirement for IL-1 responsiveness in different compartments.

A large amount of variability was seen within vaccine/chimeric groups for anti-LF mucosal IgG production. Few significant differences were seen between groups, although there were absolute differences (Figure 19). Only WT/KO mice vaccinated with LF + CT produced a significantly greater amount of anti-LF nasal IgG than LF alone-vaccinated mice ($p < 0.05$). However, while all LF + CT-vaccinated chimeric groups produced similar amounts of anti-LF nasal IgG (range: 300 – 666 titer/mg), the LF + 4 µg IL-1α-vaccinated groups were varied, with no production seen in KO→KO and only 53 titer/µg in KO→WT mice (0% and 10% of the values in the LF + CT-vaccinated

chimeras, respectively). By contrast, WT→KO and WT→WT mice produced 543 and 293 titer/µg, respectively (81% and 98% of the values in the LF + CT-vaccinated chimeras, respectively). No nasal lavage data are available for LF + 0.25 µg IL-1α-vaccinated mice. The results were similar for fecal anti-LF IgG production, as there were absolute differences between groups but no significant differences. Anti-LF fecal IgG production ranged from 40 – 114 titer/µg in LF + CT-vaccinated mice. Vaccination of KO→KO mice with LF + 4 µg IL-1α did not induce fecal anti-LF IgG production. Vaccination of WT→KO, KO→WT, and WT→WT mice with LF + 0.25 µg IL-1α induced 629, 15, and 153 titer/µg anti-LF fecal IgG, respectively. Following the same trend, vaccination of WT→KO, KO→WT, and WT→WT mice with LF + 4 µg IL-1α induced 107, 7, and 390 titer/µg anti-LF fecal IgG, respectively. Interestingly, fecal IgG is the only adaptive immune response for which the low and high doses of IL-1α induced a similar pattern of immunoglobulin production. For vaginal anti-LF IgG production, only KO→WT mice vaccinated with LF + CT had a significant increase compared to LF alone-vaccinated mice ($p < 0.01$). These titers were also significantly increased compared to KO→WT mice vaccinated with either dose of IL-1α ($p < 0.05$). No other significant differences were seen between groups. Although there were few significant differences between groups due to variability, these data generally confirm the pattern seen in serum antibody responses. At the high dose of IL-1α, only IL-1R1+/+ hematopoietic cells correlated with maximal mucosal IgA and IgG production following vaccination.

Due to the variability in mucosal antibody production, the number of mice responding to vaccination with anti-rLF IgA or IgG production was also analyzed (Table 11). Including an adjuvant in the vaccine formulation increased the number of animals

per group with detectable anti-LF IgA or IgG production, and overall, the general pattern resembled that seen in the serum responses (Figs. 14-17). The number of mice producing anti-LF IgA or IgG varied little between mice vaccinated with LF + CT, and there was little variation among chimeric groups for fecal IgA induction by any vaccine formulation. Although there were no significant differences in the number of responders between chimeric groups vaccinated with LF + 0.25 µg IL-1α, only WT→WT mice had a significantly greater number of mice produce vaginal anti-LF IgG and IgA and fecal anti-LF IgG than LF alone-vaccinated mice ($p < 0.05$). Similarly, there were no significant differences between the chimeric groups in the number of responders to vaccination with LF + 4 µg IL-1α. However, with the exception of fecal anti-LF IgA and vaginal anti-LF IgG, KO→WT mice had fewer responders than did WT→KO and WT→WT mice. Although there was a large amount of variability in mucosal anti-LF Ig responses that reduced the significance of the differences between groups, both the number of responders and the magnitude to which they responded following vaccination with LF + IL-1α followed the general patterns seen for the serum adaptive immune responses.

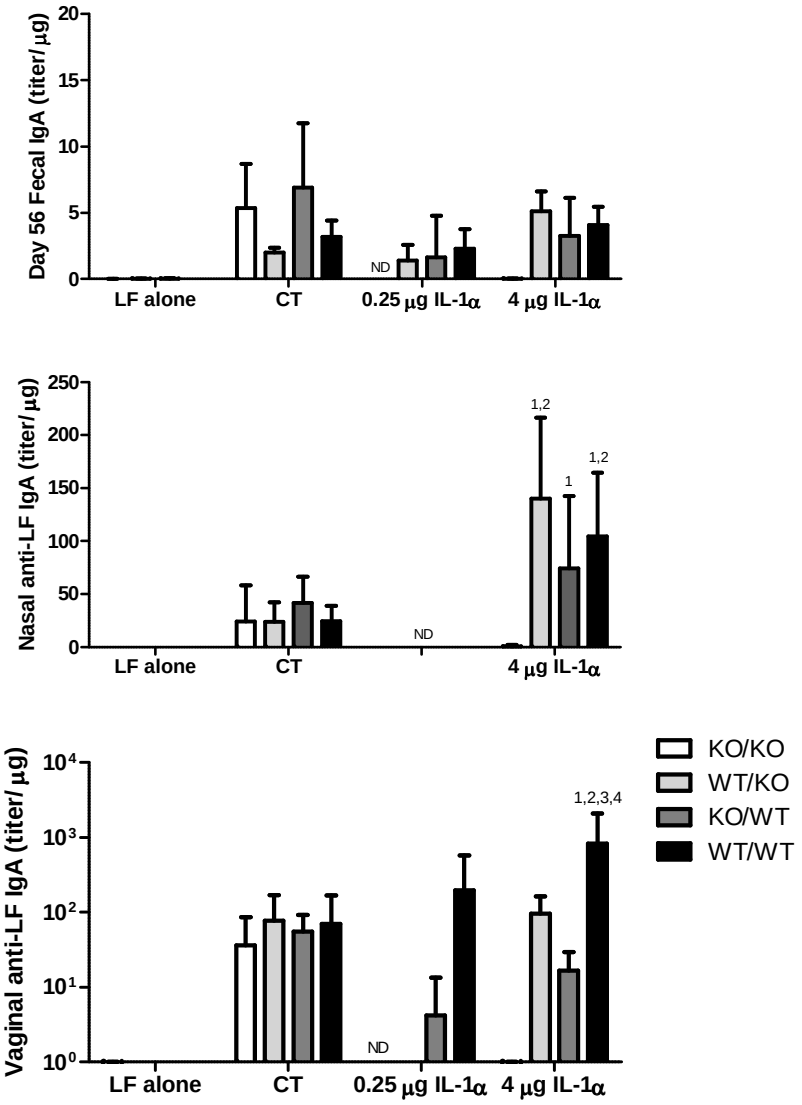

Figure 18. Normalized LF-specific mucosal IgA production on day +56 after intranasal immunization with rLF with or without adjuvant.

Mice were vaccinated on days 0, +7, +21, and +42 after a 10-week rest period following bone marrow reconstitution. Bars represent the mean IgA titers on day +56 (vaginal) or day +66 or +82 (nasal lavage) with error bars representing the SD. 1: Significantly greater than LF alone within the same chimeric group ($p < 0.05$). 2: Significantly greater than KO/KO 4 μg IL-1 ($p < 0.05$). 3: Significantly greater than KO/WT and WT/KO 4 μg IL-1 ($p < 0.05$). 4: Significantly greater than CT within the same chimeric group. ND: No data.

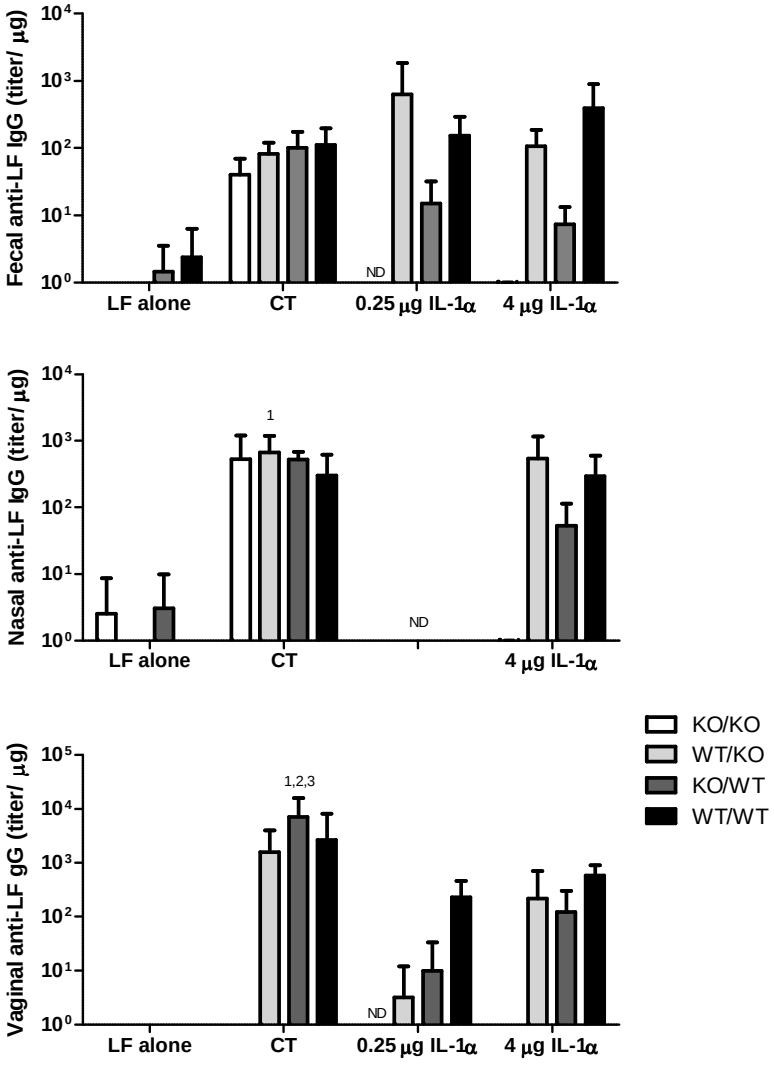

Figure 19. Normalized LF-specific mucosal IgG production on day +56 after intranasal immunization with rLF with or without adjuvant.

Mice were vaccinated on days 0, +7, +21, and +42 after a 10-week rest period following bone marrow reconstitution. Bars represent the mean IgA titers on day +56 (fecal and vaginal) or day +66 or +82 (nasal lavage) with error bars representing the SD. 1: Significantly greater than LF alone within the same chimeric group ($p < 0.05$). 2: Significantly greater than 0.25 µg IL-1 within the same chimeric group ($p < 0.05$). 3: Significantly greater than 4 µg IL-1 within the same chimeric group ($p < 0.05$). ND: No data.

Table 11. Percent responders to vaccination and total number of animals per group by chimeric group and vaccine formulation

		Vaginal IgG		Vaginal IgA		Fecal IgG		Fecal IgA		Nasal IgG		Nasal IgA	
		%	n	%	n	%	n	%	n	%	n	%	n
WT/KO		0%	10	0%	20	0%	20	5%	21	0%	10	0%	10
KO/WT	**LF Alone**	0%	9	0%	21	17%	18	22%	18	8%	12	0%	12
WT/WT		0%	8	0%	17	12%	17	0%	15	0%	6	0%	7
WT/KO		75%[1]	8	72%[1]	18	95%[1]	20	100%[1]	20	100%[1]	10	80%[1]	10
KO/WT	**LF + CT**	100%[1]	9	74%[1]	23	100%[1]	19	95%[1]	20	100%[1]	13	92%[1]	13
WT/WT		100%[1]	8	61%[1]	18	89%[1]	18	89%	18	78%	9	89%[1]	9
WT/KO		20%	5	0%	6	10%	10	60%[1]	10				
KO/WT	**LF + 0.25 µg IL-1α**	11%	9	18%	11	38%	13	69%[1]	13	ND		ND	
WT/WT		70%[1]	10	54%[1]	13	67%[1]	15	80%[1]	15				
WT/KO		57%	7	79%[1,2]	19	76%[1]	21	79%[1,2]	19	91%[1,2]	11	100%[1,2]	11
KO/WT	**LF + 4 µg IL-1α**	63%	8	48%[1]	25	54%	24	79%[1]	24	46%	13	54%	13
WT/WT		80%[1]	10	81%[1,2]	16	75%[1]	16	88%[1,2]	16	75%	8	88%[1,2]	8

1: Significantly greater than LF alone within the same chimeric group ($p < 0.05$); 2: Significantly greater than KO/KO LF + IL-1 ($p < 0.05$); ND: no data

3.3.7 Differential IL-1R1 expression affects vaccine-induced, antigen-specific splenocyte cytokine production in response to low-dose but not high-dose IL-1α used as a vaccine adjuvant

Given that the IL-1α-induced innate cytokine/chemokine response and IgG subclass profile were significantly altered in IL-1R1 chimeric mice, it was important to determine if antigen-specific cytokine responses were also impacted by the tissue distribution of IL-1R1. To examine the type of cytokine response generated by immunization, we restimulated splenocytes with LF and tested the supernatants for the presence of IL-2, IL-4, IL-10, IL-17, and IFNγ (Figure 20). Groups vaccinated with LF alone produced small amounts of each tested cytokine. LF + CT-vaccinated groups generally produced the highest levels of all of the tested cytokines, the amounts of which did not differ significantly between chimeric groups. Cells from KO→KO mice vaccinated with LF + 4 μg IL-1α did not produce significantly greater amounts of any of the five cytokines compared to cells from KO→KO LF alone-vaccinated mice. Cells from KO→WT mice vaccinated with any of the adjuvanted formulations and those from KO→KO mice vaccinated with LF + CT produced less IL-17 than did those from WT→KO or WT→WT mice, but only two groups significantly differed: WT→WT 0.25 μg IL-1α and KO→WT 0.25 μg IL-1α ($p < 0.05$).

Cells from WT→KO, KO→WT, and WT→WT mice vaccinated with LF + 4 μg IL-1α did not produce significantly different amounts of any of the five cytokines examined. However, cells from WT→WT mice in this vaccine group produced significantly greater amounts of IL-4 than LF alone ($p < 0.01$), while those from KO→WT and WT→KO mice did not; this difference was not reflected in cells from mice vaccinated with LF + CT, in

which cells from WT→KO, KO→WT, and WT→WT mice all produced significantly greater amounts of IL-4 than those from LF alone-vaccinated mice.

Cells from mice vaccinated with LF + 0.25 μg IL-1α generally produced lower amounts of each cytokine than did those from the other adjuvanted groups. Significant increases in cytokine production compared to cells from LF alone-vaccinated mice were only seen in cells from WT→KO mice for IL-17, cells from KO→WT mice for IFNγ, and WT→WT mice for IL-10, IL-17, and IFNγ. For cells from WT→WT mice, IL-10 production was significantly increased compared to cells from WT→KO mice ($p < 0.05$), and IL-17 production was significantly increased compared to cells from KO→WT mice ($p < 0.05$).

These data demonstrate that although the absence of IL-1R1 in one compartment does not affect splenocyte cytokine production in mice vaccinated with LF + 4 μg IL-1α, it does affect splenocyte cytokine production in mice vaccinated with LF + 0.25 μg IL-1α with a pattern similar to that seen for serum adaptive immune responses.

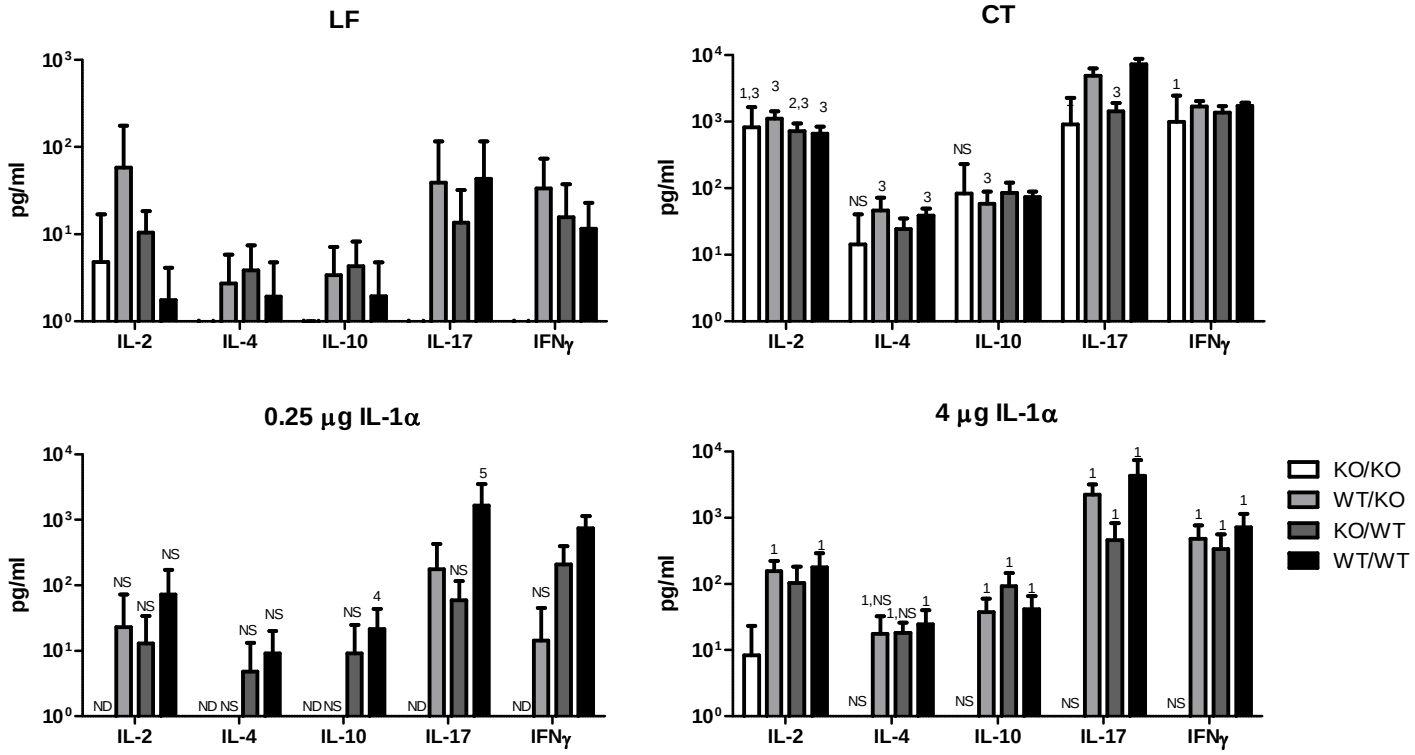

Figure 20. Cytokine production after splenocyte restimulation with rLF.

Splenocytes were removed after the last sample collection on day +56 after four vaccinations. Bars represent the mean cytokine production for each group for all replicates, with error bars representing the 95% confidence level. 1: Significantly increased over KO/KO LF + 4 µg IL-1. 2: Significantly increased over KO/WT LF+ 4 µg IL-1. 3: Significantly increased over LF + 0.25 µg IL-1 within the same chimeric group. 4: Significantly increased over WT/KO LF + 0.25 µg IL-1. 5: Significantly increased over KO/WT LF + 0.25 µg IL-1. NS: Not significantly increased over LF alone within the same chimeric group. ND: No Data.

3.3.8 Impact of CCR6 deficiency on IL-1α adjuvant activity

As CCL20 induction by LF + 0.25 μg IL-1α in chimeric mice correlated with the adaptive immune responses seen in the same vaccine groups, we investigated the impact of CCR6 deficiency on IL-1α nasal adjuvant activity. WT and CCR6-/- mice were vaccinated with LF alone, LF + 0.25 μg IL-1α, or LF + 4 μg IL-1α on days 0, 7, and 21. Due to a limited number of animals, no CT control was included; two doses of IL-1α were used to examine any possible dose effects associated with the requirement of CCR6 when vaccinating with IL-1α. Two additional groups of WT mice were also administered either anti-CCL20 antibody or isotype antibody i.p. six hours prior to the nasal vaccination. Overall, CCR6-/- mice had decreased responses to vaccination with any of the formulations and there was a large amount of variation in antibody titer within each group (Figure 21a). Although all groups vaccinated with either dose of IL-1α had absolute increases in serum anti-LF IgG endpoint titer over LF alone, only WT mice and WT mice given the isotype control antibody vaccinated with LF + 4 μg IL-1α had a significant increase (1:220,436 vs. 1:64 and 1:370,728 vs. 1:512, respectively; $p < 0.05$). Vaccination with LF + 0.25 μg IL-1α induced LeTx-neutralizing antibodies in only WT mice given anti-CCL20 antibody (1:108, NS) (Figure 21b). Vaccination with LF + 4 μg IL-1α induced LeTx-neutralizing antibodies in WT, WT + isotype, and WT + anti-CCL20 mice (1:77, 1:92, and 1:166, respectively), but only WT + isotype mice had a significant increase compared to LF alone ($p < 0.01$). Because serum anti-LF IgG production in CCR6-/- mice was 60-80% of that in WT mice, it is difficult to determine if the defect in serum LeTx-neutralizing antibody production in CCR6-/- mice was due to an overall defect in neutralizing antibody production in CCR6-/- mice or if CCR6 is required for IL-

1α nasal adjuvant activity. Because the serum immunoglobulin and LeTx-neutralizing antibody responses in chimeric mice followed trends similar to each other, it is likely that the lack of LeTx neutralization in CCR6-/- mice is related to the decreased antibody production in the strain compared to WT mice. However, because IL-1α was the only tested adjuvant, no straightforward conclusions can be drawn. Further studies with other adjuvants, such as CT, would help bring clarity to these data.

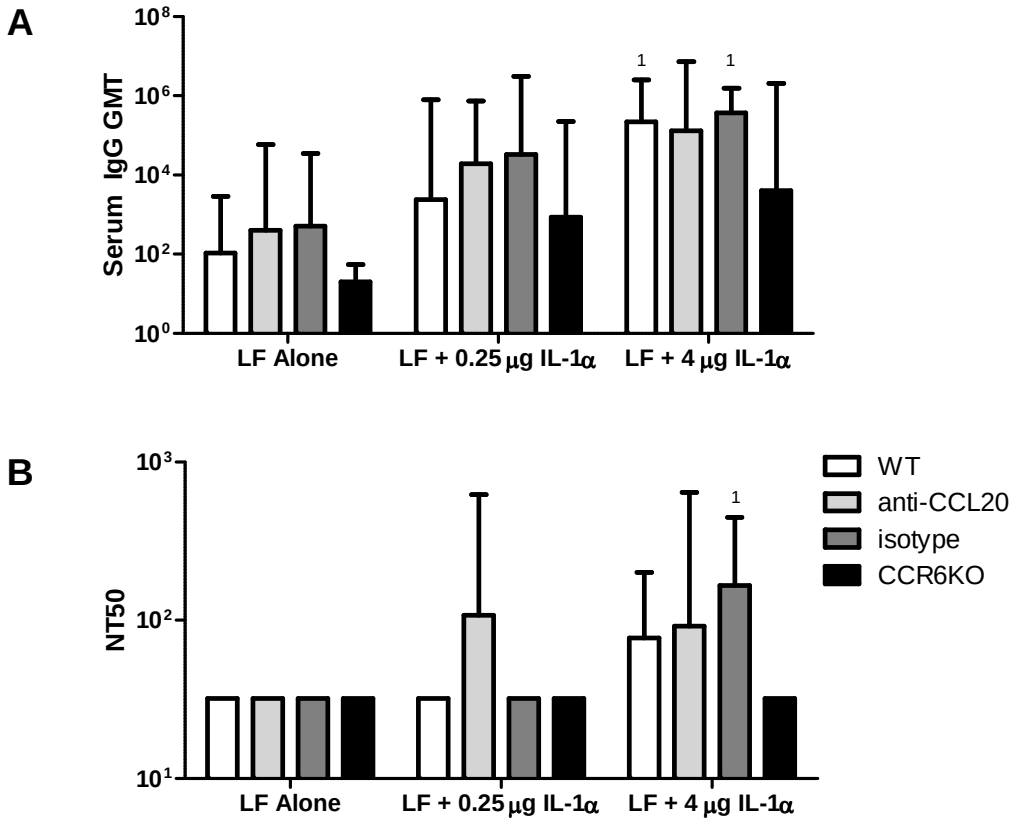

Figure 21. Adjuvant activity of IL-1α in CCR6KO mice, WT mice, and WT mice treated with anti-CCL20 or isotype antibody.

WT and CCR6KO mice were vaccinated on days 0, 7, and 21 with LF alone or LF + 0.25 or 4 μg IL-1α. Two groups of WT mice were administered either anti-CCL20 antibody or an isotype control i.p. six hours prior to nasal vaccination. Serum samples taken on day +42 were tested for the presence of anti-LF IgG (a) and lethal toxin-neutralizing antibodies (b). Bars represent the geometric mean titers for each group, with error bars representing the 95% confidence level. 1: Significantly greater than LF alone within the same mouse group ($p < 0.05$).

3.3.9 IL-1α adjuvant activity is not impaired in *Rag1*$^{-/-}$ mice adoptively transferred with *Il1r1*$^{-/-}$ splenocytes

Although there were some dose-dependent differences, the experiments in chimeric mice demonstrated that IL-1α adjuvant activity was the most depressed in mice with IL-1R1KO hematopoietic cells. As IL-1 and MyD88-dependent signaling have been shown to have direct effects on both T and B cells [119, 257, 310], we chose to evaluate the requirement for IL-1-responsive T and B cells in the response to vaccination using an adoptive transfer model. *Rag1-/-* mice were reconstituted with either WT or *Il1r1*$^{-/-}$ splenocytes and vaccinated with 25 µg LF alone, LF + 1 µg CT, or LF + 0.25 or 4 µg IL-1α on days 0, 7, 21, and 63. Adoptive transfer of splenocytes was confirmed by flow cytometry on day 60 after transfer (Table 12). Neutralizing antibodies were not produced after the first three vaccinations by some mice vaccinated with LF + adjuvant (Figure 22a), including the positive control, CT. Following the fourth vaccination, all WT mice vaccinated with LF + CT produced LeTx-neutralizing antibodies, but 2/4 WT→RAG and 1/4 IL1R1KO→RAG mice vaccinated with LF + CT did not produce neutralizing antibodies. Similarly, 4/4 WT mice vaccinated with LF + 0.25 µg IL-1α had detectable LeTx-neutralizing antibody titers, while 3/5 WT→RAG and 1/5 IL1R1KO→RAG mice did not. When vaccinated with LF + 4 µg IL-1α, 4/5 mice in each mouse group (WT, WT→RAG, IL1R1KO→RAG) had detectable LeTx-neutralizing antibody titers, and 4/5 IL1RKO→RAG mice had an NT_{50} >1:2,048 (Figure 22b). Only WT mice vaccinated with LF + CT and IL1R1KO→RAG mice vaccinated with LF + 4 µg IL-1α had a significantly increased NT_{50} compared to LF alone-vaccinated mice within the same mouse groups ($p < 0.05$). In addition, no significant differences in anti-LF IgG antibody titers were seen

between any of the groups vaccinated with LF + adjuvant and all groups were significantly increased over LF alone ($p < .01$) (Figure 22c). These data suggest that, although all positive control mice did not respond well to vaccination, the adjuvant activity of IL-1α was not inhibited in mice given *Il1r1*$^{-/-}$ T and B cells.

Table 12. Peripheral blood reconstitution with donor T and B cells

Donor	Mouse #	% gated	B220	CD3	IgM
WT	**21**	92.5	10.2	47.8	8.96
WT	**22**	58.9	11.4	50.9	6.72
WT	**23**	70.4	8.31	19	7.28
WT	**24**	12.1	2.54	3.35	1.98
WT	**25**	33.8	8.83	43.1	6.28
WT	**26**	75.2	12.5	45.4	8.45
WT	**27**	78.4	8.91	33.5	7.21
WT	**29**	34	15.3	71.2	15.7
WT	**30**	90.2	7.93	38.3	5.92
WT	**31**	90.1	6.38	34.1	5.58
WT	**32**	49.8	8.22	49	6.35
WT	**33**	87.9	8.76	32.2	5.74
WT	**34**	67	10.4	32.3	6.71
WT	**35**	31.7	5.6	54.6	4.36
WT	**36**	32.6	6.45	23.1	5.57
WT	**37**	84.4	10.5	34.8	9.75
WT	**38**	87.6	17.9	35.8	10.2
WT	**39**	16.3	5.58	34.5	4.66
WT	**40**	90.6	8.43	51	6.4
IL1R1KO	**41**	76.9	10.7	35.1	7.78
IL1R1KO	**44**	67.5	10.9	21.2	6.31
IL1R1KO	**45**	52.5	9.05	38.2	5.89
IL1R1KO	**46**	94.4	11.2	44.2	7.05
IL1R1KO	**48**	90.9	12	35.2	20.5
IL1R1KO	**49**	94.6	17.7	30.8	9.84
IL1R1KO	**50**	70.1	19.4	28.5	12.5
IL1R1KO	**51**	88.8	14.3	33.8	10.9
IL1R1KO	**52**	95.5	11.9	67	7.64
IL1R1KO	**53**	89.6	19.7	35.4	11.3
IL1R1KO	**54**	90.9	10.6	66.1	7.52
IL1R1KO	**55**	71.2	13.3	52	8.92
IL1R1KO	**56**	95.3	12.7	58	9.55
IL1R1KO	**57**	39.9	13.5	25.7	11.3
IL1R1KO	**58**	94.9	15.3	69.9	13.6
IL1R1KO	**59**	93.1	10.1	48.5	6.36
IL1R1KO	**60**	96.4	11.8	63.7	9.31
	RAG	46.2	37.3	13.2	10.4
	RAG	94.8	22.7	5.04	3.96
	WT	50.6	52.2	42.1	48.8

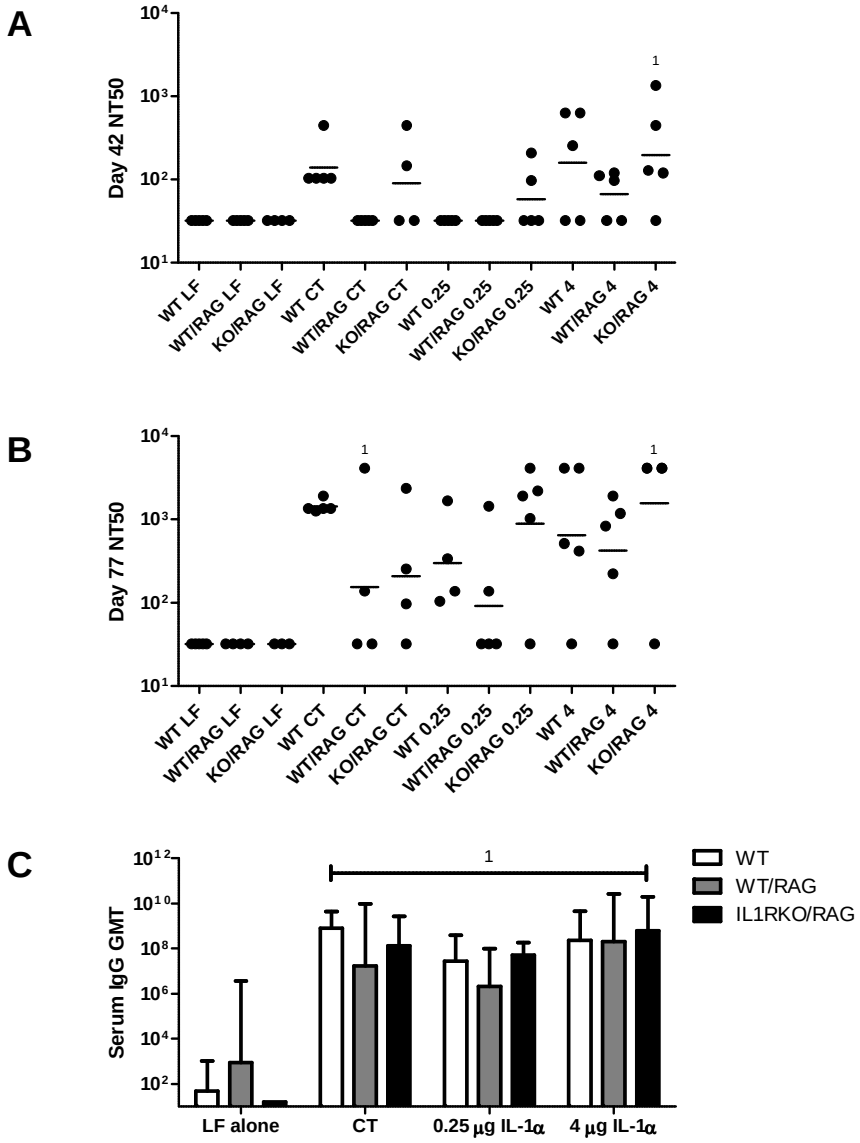

Figure 22. IL-1-responsive T and B cells are not required for the adjuvant activity of IL-1α.

Mice were vaccinated with LF alone or with 1 µg CT, 0.25 µg IL-1α, or 4 µg IL-1α on days 0, +7, +21, and +63 after a 20-day rest period following adoptive transfer of splenocytes. (a) Day 42 serum NT_{50}. (b) Day 77 NT_{50}. Lines represent the geometric mean titer for each group. (c) Day 77 serum anti-LF IgG. Bars represent the geometric mean titers for each group, with error bars representing the 95% confidence level. 1: Significantly greater than LF alone within the same mouse group ($p < 0.001$).

3.3.10 Mast cells are not required for the adjuvant activity of IL-1α

IL-1 acts on a variety of cell types, and it has been shown to induce mast cells to secrete IL-6 without causing degranulation [275]. In the previous studies, nasal lavage IL-6 production was slightly increased in WT→KO mice given 0.25 or 4 µg IL-1α nasally and hematopoietic cell expression of IL-1R1 correlated with maximal adjuvant activity. Additionally, mast cells have been reported to be required for the maximal adjuvant activities of imiquimod [169] and CTA1-DD [170]. Therefore, we chose to evaluate the requirement for mast cells in the nasal adjuvant activity of IL-1α. SASH mice have an inversion of the transcriptional regulatory elements upstream of the c-kit gene and do not produce mature mast cells. SASH and WT mice were vaccinated on days 0, 7, and 21 with LF alone, LF + 1 µg CT, or LF + 4 µg IL-1α. Mouse mast cell deficiency was not validated prior to or after the experiment. Two weeks after the final vaccination (day 35), both WT and SASH mice vaccinated with LF + CT or 4 µg IL-1α had significantly increased serum anti-LF IgG titers compared to LF alone-vaccinated mice ($p < 0.001$) (Figure 23a). WT and SASH mice vaccinated with LF + CT had serum anti-LF IgG GMTs of 1:38,540,000 and 1:29,210,000, respectively (NS). WT and SASH mice vaccinated with LF + 4 µg IL-1α had serum anti-LF IgG GMTs of 1:44,280,000 and 1:134,200,000, respectively (NS). Serum anti-LF IgG1 and IgG2c production was also similar between WT and SASH mice vaccinated with either LF + CT or LF + 4 µg IL-1α (Figure 23b,c). These data demonstrate that mast cells are not required for the adjuvant activity of nasally delivered IL-1α.

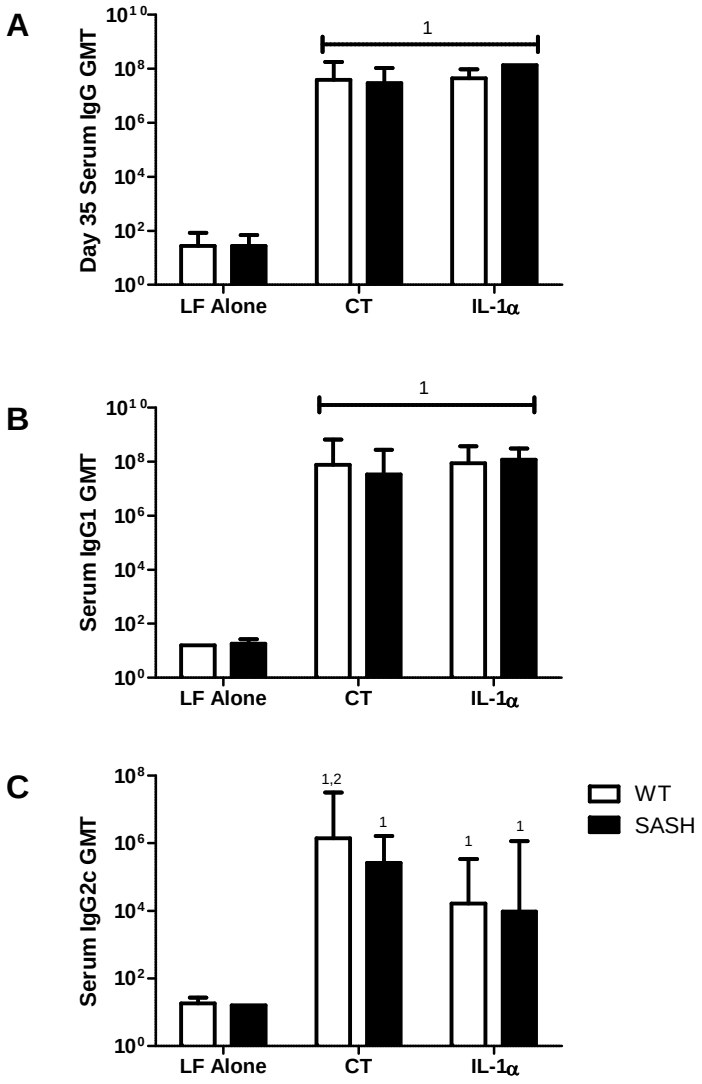

Figure 23. Mast cells are not required for the adjuvant activity of nasally delivered IL-1α.

Serum anti-rLF IgG (a), IgG1 (b), and IgG2c (c) titers on day +35 after intranasal immunization with rLF with or without adjuvant. Mice were vaccinated on days 0, +7, and +21. Serum samples taken on day +35 were tested by ELISA. Bars represent the geometric mean titers for each group on day +35, with error bars representing the 95% confidence level. 1: Significantly greater than LF alone within the same mouse group ($p < 0.001$). 2: Significantly greater than WT LF + 4 µg IL-1 ($p < 0.05$).

3.4 Discussion

In this study, we demonstrated that vaccination of IL-1R1 chimeric mice with LF + 4 μg IL-1α induced significantly increased serum anti-LF antibody production in mice expressing IL-1R1 in either the stromal or hematopoietic cellular compartments. However, the responses induced by vaccination were not equally increased across the three chimeric groups; IL-1R1 KO→WT mice produced significantly less antigen-specific serum IgG, serum IgG subclasses, and neutralizing antibodies than WT→KO and WT→WT mice. In addition, KO→WT mice had significantly lower antibody avidity than WT→KO and WT→WT mice. However, vaccination of IL-1R1 chimeric mice with LF + 0.25 μg IL-1α induced a different immune response profile across the three chimeric groups; WT→KO and KO→WT mice failed to induce significantly increased serum anti-LF IgG titers compared to mice immunized with LF alone, and the dose was not sufficient to induce neutralizing or high avidity antibodies. These data indicate a possible dose-dependent requirement of IL-1R1, though it appears that the maximal adjuvant activity of IL-1α after nasal immunization is primarily dependent upon IL-1-responsive hematopoietic cells because only this compartment was required at both tested doses. Conversely, IL-1α-induced innate cytokine and chemokine production required IL-1-responsive stromal cells, indicating that IL-1α-induced cytokine production was not required for IL-1α-induced anti-LF adaptive immune responses. In addition, although previous reports [115, 143] have suggested that CT may mediate its adjuvant activity by inducing IL-1, our data clearly show that CT maintains mucosal adjuvant activity in IL-1R1-/- mice, demonstrating that CT-induced IL-1 is not required for the mucosal adjuvant activity of CT.

Many studies have examined the early (0-48 hours) cytokine and chemokine profiles induced by specific adjuvants [26, 278-280], but few have compared these profiles with the adjuvant-induced adaptive immune response [296, 297]. Due to the ability of the chimeric model to assign adjuvant-induced responses to an adjuvant-responsive compartment, we examined the cytokine and chemokine profiles induced by nasal vaccination with IL-1α. Similar to two studies that have evaluated cytokine production in humans following a subcutaneous injection of IL-1β [311] or IL-1α or IL-1β [312], we noted the production of G-CSF and IL-6 (Tables 6-9). Other studies have examined early serum cytokine production following i.m. or s.c. administration of vaccine adjuvants to mice; for instance, MF59 has been shown to induce IL-5 and IL-6 [26], poly I:C has been shown to induce TNFα, IL-6, IL-10, MCP-1, IFNα, and IL-5 [76], and alum has been shown to induce IL-1β, IL-5, IL-6, IL-16, M-CSF, G-CSF, KC, IP-10, MIP-2, Eotaxin, and MCP-1 [313]. In addition, flagellin has been shown to induce the production of IL-6, IL-12(p40), TNFα, IFNα, IFNβ, and IFNγ following i.p. delivery [314]. These studies demonstrate that adjuvants induce varied profiles of cytokines and chemokines that are adjuvant specific. In keeping with the results in the literature, we demonstrated an adjuvant-specific profile of cytokine production following intranasal vaccination, as IL-1α induced the production of IL-6, G-CSF, KC, and MCP-1, while CT did not. However, similar to the other studies, which observed peak cytokine production at 3-6 hr, IL-1α-induced cytokine/chemokine production peaked at 3 hr after vaccination. It is noteworthy that the timing of sample collection therefore affects which cytokines are detected. Although IL-1α induced some cytokines/chemokines that were similar to the other adjuvants described, its profile was distinct.

In addition, an analysis of the mRNA expression data from the nasal cavities of mice vaccinated with LF alone, LF + CT, or LF + IL-1α indicated that IL-1α induced the up-regulation of a large number of genes, including cytokines and chemokines (e.g., IP-10, CCL2, CCL19, CXCL1), tight junction proteins (e.g., CLDN13, JAM2), and matrix metalloproteinases (e.g., MMP3, MMP12) (data not shown). Interestingly, both CT and IL-1α significantly down-regulated *Atp7b* and *Abca15* expression. *Atp7b* encodes a copper transport protein present at membrane tight junctions [315] that has been recently associated with cancer cell resistance to cisplatin [316]. Additionally, copper treatment of immune cells has been shown to induce tumor-associated macrophages to reprogram Treg cells to Th1 effector cells [317] and to increase proinflammatory cytokine production by rainbow trout macrophages [318]. Given the proinflammatory actions of copper and IL-1, it is possible that the down-regulation of *Atp7b* is related to the anti-inflammatory pathways induced by IL-1. *Abca15* is an ATP-binding cassette transporter that was only recently identified and currently has no associated function [319]. It is important to note that although CT is a very potent adjuvant, as evidenced here and in many other studies [24, 73], it was a poor inducer of changes in nasal cavity gene expression and the production of cytokines and chemokines in nasal lavage and BAL. Although CT was not examined for its ability to induce serum cytokines in the original 23-plex used to examine IL-1α-induced production in serum, serum from IL-1R1 chimeric mice vaccinated with LF + CT was examined for IL-6, KC, Eotaxin, MCP-1, and G-CSF but was found not to induce their production. More in-depth studies with an even larger panel of cytokines and chemokines could potentially identify a cytokine and/or chemokine that is required for the generation of adaptive immune responses following

vaccination with IL-1α or CT. Nonetheless, the vastly different profiles induced by these two adjuvants, which are known to be potent stimulators of adaptive immune responses, indicate that evaluating cytokine/chemokine production and mRNA expression changes is not a valid measure or correlate of adjuvanticity. Therefore, studies of adjuvant mechanism should also focus on areas of adaptive immune response generation, such as effects on affinity maturation, immune synapse formation, or B and T cell proliferative responses. Although innate immune system activation is related to the development of adaptive immune responses, identifying specific factors commonly induced by vaccine adjuvants and relating those factors to the potency of vaccine adjuvants may be improbable.

To our knowledge, no other group has directly investigated the adjuvant mechanism of action of IL-1, although some have indirectly addressed it while examining other mechanisms or the role of endogenous IL-1 in the immune response [257, 320]. Ueda et al. demonstrated that IL-1R1 was required on the nonhematopoietic, radioresistant cells for alum to induce granulopoiesis following reactive neutrophilia [320]. In addition, the effects of IL-1 on neutrophil migration are well known. However, although IL-1 does induce neutrophil influx when given as a vaccine adjuvant (unpublished observation), it is unlikely that they are involved in its mechanism of action. In our study, the neutrophil chemokine KC and the granulopoiesis cytokine G-CSF were produced primarily by the nonhematopoietic cells in the serum and nasal lavage. While G-CSF and KC remained increased in the nasal lavage of WT→KO mice, which did have anti-LF immune responses similar to WT→WT mice, their presence in KO→WT mice was not sufficient to mediate anti-LF adaptive immune responses equivalent to

those measured in WT→WT mice. Although neutrophils have been shown to be important in the clearance of many pathogens [321, 322], they were recently demonstrated to inhibit antigen presentation and antigen-specific immune responses following vaccination with several antigens (i.e., HEL, OVA, or lysteriolysin O) and adjuvants (i.e., IFA, CFA, alum) when comparing neutrophil-depleted or G-CSFR-/- mice to WT [323]. Therefore, it is unlikely that G-CSF, KC, or neutrophils play a large role in the nasal adjuvant activity of IL-1α.

Despite the significant induction of cytokines in serum and mucosal secretions after nasal immunization with IL-1α, our studies demonstrated that adjuvant-dependent early cytokine and chemokine production was not required for induction of adaptive immune responses induced by vaccination. Cytokines in the serum and local to the vaccination site (e.g., nasal cavity) were produced primarily by the IL-1R1-expressing stromal cell compartment, while the adaptive immune responses were only impaired in mice lacking IL-1R1 on their hematopoietic cells. Our results are in agreement with others that adjuvant-induced cytokine production was not required for induction of adaptive immunity. Sanders et al. recently demonstrated that, when vaccinating mice i.p. with flagellin, serum cytokine and chemokine induction and splenic DC maturation were largely impaired in TLR5-/- mice, though serum IgG responses to flagellin were not impaired [297]. However, in the same study, Sanders demonstrated that flagellin's immunogenicity as an antigen or an adjuvant (when delivered with OVA) is not restricted to MyD88, indicating that TLR signaling is not required for a flagellin-induced adaptive immune response to itself or co-delivered antigen. A recent study by Longhi et al. examined the ability of poly I:C to induce serum cytokine production when given i.p., and

although they demonstrated increased production of six cytokines (IL-6, IL-12(p40), TNFα, IFNα, IFNβ, IFNγ), only IFNα and IFNβ correlated with antigen-specific CD4+ T cell development [314]. In addition, CpG, which is known to be an effective Th1-inducing adjuvant [324], induced increased production of four of the same six cytokines (IL-6, TNFα, IL-12(p40), IFNγ) but induced weaker antigen-specific IFNγ+ CD4+ T cell responses. In our study, IL-1α also induced IL-6, but it did not induce the production of the other five cytokines examined by Longhi et al. Although unlike these studies we vaccinated mice i.n., all of these data suggest that many of the frequently used markers of innate immunity induction are not required for the induction of adaptive immune responses to the vaccine antigen.

Several other studies have also used the bone marrow chimeric mouse model to evaluate the innate and adaptive immune responses to many different proteins, TLR ligands, and viruses/bacteria. Many of those have demonstrated that the innate immune responses to externally delivered antigen are dependent upon both an antigen-responsive hematopoietic compartment and an antigen-responsive nonhematopoietic compartment. For example, Noulin et al. demonstrated that the innate immune response in the lung to inhaled endotoxin was dependent on MyD88 in both compartments, as MyD88 was required in the nonhematopoietic compartment for bronchoconstriction, in the hematopoietic compartment for cytokine production, and in both compartments for full recruitment of neutrophils [289]. By contrast, we found that IL-1R1 was required only on stromal cells to induce cytokine production equivalent to WT→WT following nasal delivery of IL-1α, which demonstrates that the innate

immunostimulatory effects of IL-1α are different from those of other previously examined experimental adjuvants.

Similar to the findings of Noulin et al., several studies examining the impact of stromal or hematopoietic cell receptor expression on the generation of adaptive immune responses have also demonstrated that they are dependent upon both an antigen/adjuvant-responsive hematopoietic compartment and an antigen/adjuvant-responsive nonhematopoietic compartment [296, 314, 325]. Two recent studies evaluated the requirements for TLR3 and MDA5 in poly I:C-induced antigen-specific IFNγ+ CD4+ T cell [314] and CD8+ T cell [325] responses when delivered i.p. to mice with HIV gag p24 or OVA, respectively. Unlike IL-1, which can only signal through IL-1R1, poly I:C signals through both MDA5 and TLR3, but it is unclear which receptor(s) mediate poly I:C-induced immune responses [325]. Longhi et al. demonstrated that both MDA5 and TLR3 were required on both stromal and hematopoietic cells for poly I:C to induce an increase in antigen-specific IFNγ+ CD4+ T cell responses [314]. While Longhi only investigated the impact of the two receptors together on the induction of CD4+ responses, Wang et al. used the bone marrow chimeric mouse model to demonstrate that MDA5 was required only on stromal cells for poly I:C to induce survival signals in CD8+ T cells [325]. However, Wang did show that TLR3 was required for the generation and expansion of antigen-specific CD8+ T cells. Taking these two studies together, poly I:C appears to require signaling capabilities in both compartments: TLR3 on hematopoietic cells and MDA5 on stromal cells. It is possible, however, that the use of different antigens and the different examined responses played a role in the study outcomes. Remarkably, all of these studies used different routes of

challenge/immunization but still demonstrated the requirement for antigen/adjuvant responsiveness in both compartments. However, as demonstrated by our results, only the hematopoietic compartment was required to be responsive to an external challenge with the high dose of IL-1α to induce anti-LF adaptive immune responses equivalent to those in WT→WT animals. Interestingly, when the dose was lowered to 0.25 μg IL-1α, IL-1R1 expression in only one compartment was not sufficient to induce a response equivalent to those seen in WT animals. As such, at the high dose, IL-1α must have been able to cross the mucosal epithelium and act on the IL-1R1+/+ hematopoietic cells.

As evidenced in the dose response studies (Figure 10), IL-1α is an effective adjuvant in WT animals at doses 16-fold less than those used in these experiments, and previous studies in our lab have lowered the dose to 20-fold less (0.20 μg) than the high dose used here (data not shown). It is therefore possible that such an excessive dose played a role in the results seen because the lower dose of adjuvant delivered nasally required adjuvant responsiveness in both compartments. KO→WT mice vaccinated with LF + 0.25 μg IL-1α generally responded better to vaccination than did WT→KO mice, which suggests that at a limiting dose, the role of the epithelium in the immune response becomes greater. Unfortunately, none of the chimeric groups vaccinated with the low dose of IL-1α produced significantly greater amounts of neutralizing antibodies than LF alone-vaccinated mice (Figure 14). Because WT→WT mice did not produce LeTx-neutralizing antibodies, it is difficult to draw conclusions regarding the requirement for IL-1R1 on hematopoietic or stromal cells when using the lowest dose that induces protection. Given that adaptive immune responses were decreased in KO→WT mice

administered either the high dose or the low dose of IL-1α, an adjuvant-responsive hematopoietic compartment appears to be required regardless of the challenge dose.

The difference between the effective doses in WT unirradiated mice given 0.25 µg IL-1α and WT→WT irradiated mice given the same dose is likely related to the effects of irradiation damage. Although the doses used in this study were picked based on a dose response study in WT mice, the irradiation damage to the chimeric animals appeared to alter the immune responsiveness of the mice. Responses to LF + adjuvant were approximately 10-fold lower in WT→WT irradiated animals than in WT unirradiated animals. Similarly, Sanders et al. previously indicated that irradiated/reconstituted mice have lower responses to flagellin than unirradiated animals [296]. Additionally, as it is known that more antigen is required when vaccinating mucosally to induce mucosal IgA production than serum IgG [24], it is not surprising that the mucosal antibody responses were so variable in the chimeras, especially given our lower serum responses compared to WT unirradiated mice. It is also important to note that because LF + CT-vaccinated mice produced similar amounts of serum antibodies, the differences seen between the chimeric groups when vaccinated with LF + 4 µg IL-1α were not simply due to the presence or absence of IL-1R1 in a specific compartment. Unfortunately, completing dose response studies in chimeric mice is both cost and time prohibitive, though this study demonstrates that such dose responses are important. Such distinct differences in the pattern of response are also important when considering the results of other bone marrow chimeric mouse studies and applying them to WT models of infection- or vaccine-induced immune responses; increasing or decreasing challenge doses in chimeras may have drastic effects on study outcomes. It is possible that such dose

effects played a role in the differences discussed above in the studies of Hollingsworth et al. [293] and Noulin et al. [289].

IL-1 has been shown to play a role in the development of Th immune responses, including increased Th2 responses in *Il1r1*-/- mice when compared to WT mice [282]. However, we did not see any significant differences in antigen-specific cytokine production between chimeric mice vaccinated with LF + CT or LF + IL-1α. Satoskar et al. previously demonstrated that IL-1R1-/- mice infected with *Leishmania major* or immunized with KLH produced greater amounts of antigen-specific IL-4 upon restimulation (fourfold and twofold greater than WT, respectively). By contrast, KO→KO mice did not have significantly decreased IL-4 production compared to WT→WT mice following vaccination with LF + CT (14.38 pg/ml [95% CI: -11 - 40] vs. 39.17 pg/ml [95% CI: 29 - 50], respectively; NS). In addition, they also detected twofold decreased IFNγ production, and we observed no significant decrease in IFN production in KO→WT mice compared to WT→WT mice (339 vs. 718 pg/ml, respectively). It is possible that this is a reflection of the route of delivery, as they delivered antigen subcutaneously and it has been shown that different routes of immunization induce different Th biases [326]. KLH-specific cytokine production was also only investigated on day seven following immunization, whereas we investigated LF-specific cytokine production following four immunizations.

IL-1 has also been shown to play a role in the development of the Th17 immune response in both mice and humans [114, 302]. In this study, only KO→WT mice vaccinated with LF + 0.25 μg IL-1α had significantly decreased antigen-specific IL-17 production by splenocytes following vaccination with any formulation compared to

WT→WT mice. As the nasal route of immunization has been associated with the development of Th17 immune responses regardless of the adjuvant used [326], it is possible that vaccinating nasally minimized the differences in IL-17 production between groups. Although there were no significant differences in the production of any of the five cytokines between groups vaccinated with LF + 4 μg IL-1α, groups vaccinated with LF + 0.25 μg IL-1α had more evident differences in cytokine production that closely mimicked their serum anti-LF immune responses, which again indicates that there is a dose-dependent requirement for IL-1R1 in different compartments.

Although Satoskar et al. observed increased Th2 cytokine production, serum antigen-specific IgG1 and IgG2a titers did not significantly differ between WT and IL-1R1-/- mice [282]. In the original characterization of IL-1R1-/- mice by Glaccum et al., serum antigen-specific IgG1 and IgM titers did not significantly differ from WT mice following immunization with thymus-dependent (TNP-KLH) or thymus-independent (DNP-Ficoll) antigens [327]. Our results following immunization with LF + CT agree with the data of both studies, as we did not observe any significant differences in anti-LF IgG subclass production between chimeric groups. However, we did demonstrate that IL-1R1 chimerism significantly impacted the ability of IL-1α to enhance anti-LF IgG subclass production, indicating that the addition of exogenous IL-1 may have effects not seen when comparing the effects of endogenous levels of IL-1 in WT and IL-1R1-/- mice.

It is worth noting, however, that DCs have been shown to extend across the mucosal epithelium into the small bowel and the trachea where they can sample antigen [328-330], but it is unclear if DCs have this ability in the nasal cavity. It is also unclear whether DCs primarily use this ability to traffic antigens across the mucosal barrier into

the lumen [331]. If DCs use this capability to capture antigen and traffic it to the local draining lymph node, it is possible that, in the WT→KO animals, DCs extending across the epithelium were activated by IL-1 to uptake antigen. Conversely, epithelium activation by IL-1 may have induced DCs to extend into the lumen and sample the coadministered LF in KO→WT animals; although immunization of KO→WT animals induced poor neutralization responses, serum anti-LF IgG was induced. If DCs can in fact extend projections across the nasal mucosa to sample antigen, then they likely played a role in the IL-1-induced LF-specific adaptive immune responses in one or both of these manners.

Chieppa et al. also demonstrated that an increase in CCL20 mRNA expression coincides with the DC extension response [50]. It was therefore interesting when CCL20 increased in the nasal lavage of mice given IL-1α (Figures 11 and 13) and corresponded to the adaptive immune responses in chimeric mice vaccinated with LF + 0.25 μg IL-1α, though it did not correlate with the adaptive immune responses after vaccination with LF + 4 μg IL-1α. However, as CCL20 is produced by both hematopoietic and nonhematopoietic cell types, this is not surprising and may reflect the ability of the high dose of IL-1α to cross the mucosal barrier and interact with hematopoietic cells in the nasal mucosa. IL-1α also induced a threefold increase in CCR6 expression in the nasal cavity at three hours after vaccination with LF + 4 μg IL-1α; unfortunately, due to a low sample number, this increase was not significant. Although anti-CCL20 antibody did not affect the production of serum anti-LF IgG in WT mice after vaccination with LF + 0.25 or 4 μg IL-1α, anti-LF and LeTx-neutralizing antibody production was impaired in CCR6-/- mice. Unfortunately, only IL-1α was included as an adjuvant. As such, it is impossible to

determine if the impaired immune responses were a global defect of CCR6-/- mice or if it was the result of a specific CCR6 requirement for IL-1α adjuvant activity. Adding to the difficulty of drawing conclusions from this study, CCR6-/- mice have not been used in any published studies examining antigen-specific IgG production. However, CCR6-/- mice have been shown to have impaired mucosal IgA responses to oral antigen challenge [332].

Although others have demonstrated that IL-1 can act directly on CD4+ T cells to enhance antigen-specific T cell proliferation and antibody production [257], we demonstrated that IL-1α maintained its nasal adjuvant activity in the absence of IL-1-resposive T and B cells in an adoptive transfer model. However, it is possible that DC and/or NK cell activation may play a role in the adjuvant activity of IL-1α. NK cells have been shown to express IL-1R1 [333] and to respond to IP-10 [334, 335], and IP-10 mRNA expression was increased by vaccination with IL-1α 11.3 fold compared to naïve in the nasal cavity (data not shown). In addition, Hall et al. demonstrated decreased IFNγ and increased IL-10 production by splenocytes of NK-depleted mice [336], while Yoshida et al. demonstrated decreased antibody production following immunization with HBsAg in NK cell-depleted mice that was restored following the transfer of Ag-pulsed DCs from non-NK cell-depleted mice [337]. Interestingly, Hall et al. demonstrated that only antigen-specific IgG2a and IgG2b production was inhibited following NK cell depletion, while antigen-specific IgG and IgG1 titers were not affected. These studies are in keeping with the results from our chimeric studies, in which we saw decreased anti-LF antibody production in mice with *Il1r1*$^{-/-}$ hematopoietic cells, especially with respect to LF-specific IgG2a and IgG2b production. It is therefore possible that NK-DC

interactions play a role in the adjuvant activity of IL-1, but experiments to examine the possible role of those interactions were out of the scope of the current study.

These results also indicate a possible role for T helper cells in IL-1α-adjuvanted immune responses. Although IL-1-responsive T and B cells were not required for IL-1 adjuvant activity, the possibility remains that IL-1 acts directly on other cells that then interact with T cells to increase T cell effector responses. In keeping with the above results from both this study and those of Hall et al. and Yoshida et al., two studies have demonstrated the importance of direct stimulation of DCs to induce the production of antigen-specific IgG2b and IgG2a/c [338, 339]. In the absence of TLR ligand-responsive DCs, inflammatory signals from other TLR ligand-responsive cell types induced DC maturation, but DCs matured in this manner were unable to induce IL-12p40+ CD4+ T cells [339] or IL-12p40 mRNA expression in the spleen [338]. Deficits in IL-12p40+ CD4+ T cells and IFNγ+ CD4+ T cells were also associated with decreased IFNγ production by NK cells [338, 340]. These studies demonstrate the cooperative nature between activated DCs, NK cells, CD4+ T helper cells, and antibody production by B cells. It is therefore possible that IL-1 adjuvant activity is dependent upon direct activation of DCs, which in turn activate NK cells and induce Th1-skewed CD4+ cells that prime B cells to undergo class switching and affinity maturation. Similar to the results in this study, the production of proinflammatory cytokines by non-DCs was able to partially compensate for the lack of TLR ligand nonresponsiveness, inducing DC maturation [339] and, in one study, the production of significantly greater amounts of IFNγ+ CD4+ T cells compared to *Myd88*$^{-/-}$ mice [340]. The latter study also demonstrated that the administration of IL-12 was unable to rescue IFNγ+ CD4+ T cell

induction to levels seen in WT mice, but it was able to significantly increase the survival of naive mice following challenge. Therefore, given that stromal cell cytokine production was not required or sufficient to induce adaptive immune responses in mice vaccinated with LF + IL-1, it is possible to surmise that such adjuvant-induced cytokine production is important for innate responses to infection and pathogen clearance.

In summary, this study demonstrated that IL-1-responsive hematopoietic cells are sufficient for the maximal induction of antigen-specific adaptive immunity following vaccination with the adjuvant IL-1α at a dose that also induces protective immunity. Although the stromal (radiation-resistant) cell compartment is able to respond with cytokine/chemokine production and partially compensate for the absence of IL-1R1 on hematopoietic cells, IL-1R1 expression on stromal cells only was not sufficient for the maximal adjuvant activity of IL-1α. Therefore, using adjuvant-induced innate immune responses as a biomarker of adjuvant activity or as an indicator of adjuvant mechanism may be inaccurate. In addition, dose effects on immune responses to challenge are important considerations when applying the results of chimeric models to WT models of infection- or vaccine-induced immune responses.

4. Future Directions

Although this work answered several questions with regard to IL-1α, many questions pertaining to IL-1 and other vaccine adjuvants remain to be answered. We demonstrated that the nasal adjuvant activity of IL-1α was primarily dependent upon IL-1R1-expressing hematopoietic cells. However, we only evaluated the humoral immune responses induced using LF + IL-1α, and our low dose was not optimal because it did not induce protective immunity. It is important to determine if the dose of IL-1α administered has a true, measurable impact on the adaptive immune responses in chimeric animals. It is also important to evaluate other adjuvants in the same manner. Although a few studies by others evaluating T cell responses were discussed, more studies are needed to fully evaluate adjuvants in comparison to each other. This includes evaluating the receptor requirements for both cell-mediated and humoral immune responses, as the method of inducing these responses may differ between adjuvants and cellular compartments.

This work and that of other studies indicate that many typically measured innate immune responses, such as cytokine/chemokine production, are not valid indicators of adjuvant activity. Adjuvants appear to induce adjuvant-specific cytokine/chemokine profiles, which are likely also dependent upon the route of delivery and the adjuvant dose. However, despite these actions, no study has provided convincing evidence that these responses can be linked to adjuvant activity. It is therefore imperative that future studies aim to identify early markers of the immune response that can be correlated to the adaptive immune response and the ability of an adjuvant to enhance a particular type of immune response (e.g., neutralizing antibodies or CTLs). Such studies could

provide potential methods for screening new and currently used adjuvants for the ability to induce particular immune responses in the absence of adverse effects.

Although we characterized a portion of IL-1α's mechanism of action when delivered nasally, this study did not identify a particular cell type or factor required for its adjuvant activity. Given the potent inflammatory activity of IL-1, it is unlikely that one cell type will be solely responsible. Instead, a network of factors may be at the heart of its adjuvanticity. However, many informative studies are still possible. As indicated, IL-1 has been found to have actions on both NK cells and DCs, and NK cell depletion in vivo has effects similar to those seen in the current study. Although many factors may be involved, this study suggests the mechanism outlined in Figure 24, in which IL-1 acts on DCs extending across the epithelium or on NK cells and DCs on the basal side of the epithelium. Activation of either cell or both by IL-1 may induce DC-NK cell crosstalk, resulting in an increase in DC stimulatory capacity and cytokine production by NK cells. It would be possible to examine their requirement in IL-1α adjuvant activity using a mixed bone marrow chimera model. Transferring mixed bone marrow from mice expressing the diphtheria toxin receptor on their NK cells and DCs and the bone marrow of *Il1r1*$^{-/-}$ mice to WT mice would create mice with an *Il1r1*$^{+/+}$ stromal cell population, *Il1r1*$^{-/-}$ DCs and NK cells, and *Il1r1*$^{+/+}$ cells for all remaining hematopoietic cells after toxin administration.

Alternatively, it is also possible to evaluate the impact of having only IL-1-nonresponsive DCs using a mouse strain developed by Hou et al. in which DCs are *Myd88*$^{-/-}$ and all other cells are *Myd88*$^{+/+}$ [338]. Unfortunately, both of these experiments can only evaluate the requirement for IL-1-responsive cell types and not the sufficiency

of those same cell types in IL-1α adjuvant activity. It may also be possible to nasally administer bone marrow-derived DCs to repopulate *Il1r1*$^{-/-}$ mice with *Il1r1*$^{+/+}$ DCs and evaluate their ability to rescue IL-1 α adjuvant activity using the procedures outlined by McCormick et al. [341]. However, this procedure may not result in proper DC localization in the nasal cavity, and the in vitro manipulation of DCs has the potential to activate the cells.

Additionally, it will also be important to determine how IL-1α crosses the mucosal epithelium in WT → *Il1r1*$^{-/-}$ mice. Although we originally stated that adjuvants acting primarily on hematopoietic cells may be more effective if delivered across the mucosal barrier (e.g., given systemically), IL-1α was not less effective when IL-1R1 was not expressed on stromal cells when it was given at the high dose. Several mechanisms are possible, including M cell-mediated transport and antigen sampling by *Il1r1*$^{+/+}$ DCs. Such a finding may identify a common mechanism of entry for other adjuvants that bind to cellular receptors.

Many important questions surrounding adjuvant mechanisms of action, including that of IL-1, remain, and although this study answered a few pertaining to IL-1, it also highlighted problems with current methods of correlating innate and adaptive immune responses. It is in that way that this study has contributed to the field of vaccinology. Looking forward, questions surrounding innate immune system activation and how it correlates with adaptive immune responses will be important for identifying more potent and safer vaccine adjuvants, which will be needed to meet the current challenges in developing vaccines for new, emerging, and re-emerging diseases.

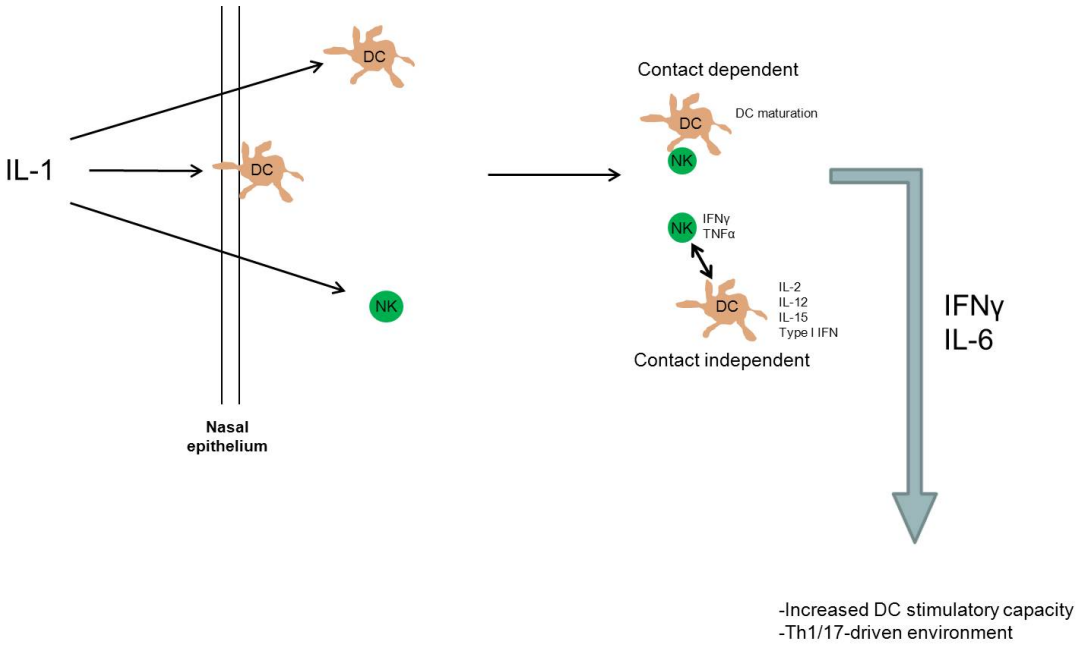

Figure 24. Proposed model of IL-1α adjuvant mechanism of action. IL-1 acts on IL-1-responsive DCs and NK cells to increase DC stimulatory capacity and antigen-specific immune responses.

Appendix A

Appendix Table 1. N per group for all mouse experiments

Figure	Response									
Figures 1-5		PA Alone	PA + 0.1 µg CT	PA + 1 µg CT	PA + 1 µg CpG	PA + 10 µg CpG	PA + 3 µg C48/80	PA + 10 µg C48/80	PA + 30 µg C48/80	PA + 90 µg C48/80
	IgG	15	5	5	5	5	10	15	15	5
	IgG1	15	5	3	5	5	10	15	15	5
	IgG2a	15	5	3	5	5	10	15	15	5
	IgG2b	15	5	3	5	5	10	14	15	5
	IgG3	15	5	3	5	5	10	14	15	5
	NT75	12	5	5	5	5	10	15	15	5
	IgE	15	5	4	5	4	10	15	20	5
	Ear Swelling	15	5	5	5	5	10	15	15	5
	IL-4	15	5	5	5	5	10	10	15	5
	IL-5	15	5	5	5	5	10	10	15	5
	IL-6	15	5	5	5	5	10	10	15	5
	IL-17	15	5	5	5	5	10	10	15	5
	IFNγ	15	5	5	5	5	10	10	15	5

Figure 8a		PA Alone	PA + 30 µg C48/80	PA + 60 µg C48/80	PA + 2 µg Chymase	PA + 20 µg Histamine	PA + 60 µg Histamine	PA + 20 ng TNFα	PA + 60 ng TNFα	PA + Chym/Hist/TNF
	Serum IgG	10	5	5	5	4	5	5	5	5

Figure	Response									
Figure 8b		PA Alone	PA + 30 µg C48/80	PA + 120 µg Histamine	PA + 120 ng TNFa	PA + 120 µg Hist + 0.12 µg TNF	PA + MC/9 100 Cells	PA + MC/9 1000 cells	PA + BMMC 100 cells	PA + BMMC 1000 cells
	Serum IgG	3	3	3	3	3	3	3	3	3
		PA + J774A 1000 cells	PA + J774A 10000 cells	PA + ModeK 1000 cells	PA + ModeK 10000 cells					
	Serum IgG	3	3	3	3					
Figure 9		PA Alone	PA + 10 µg C48/80	PA + 30 µg C48/80	PA + 1 µg CpG	PA Alone	PA + 10 µg C48/80	PA + 30 µg C48/80	PA + 1 µg CpG	
	Serum IgG	10	11	11	11	4	5	5	5	
Figure 10A		6 µg LF				12.5 µg LF				
		LF Alone	LF + CT	LF + 1 µg IL-1α	LF + 4 µg IL-1α	LF Alone	LF + CT	LF + 1 µg IL-1α	LF + 4µg IL-1α	
	IgG	3	3	3	3	3	3	3	3	
		25 µg LF								
		LF Alone	LF + CT	LF + 1 µg IL-1α	LF + 4µg IL-1α					
	IgG	3	3	3	3					
Figure 10B		IL-1R1-/- Mice			WT Mice					
		LF Alone	LF + CT	LF + 4 µg IL-1α	LF Alone	LF + CT	LF + 4 µg IL-1α			
	IgG	5	4	5	5	5	5			
	NT50	5	4	5	5	5	5			
Figure 10C										
		LF alone	LF + CT	LF + 0.25 µg IL-1α	LF + 1 µg IL-1α	LF + 4 µg IL-1α				
	IgG	4	4	5	5	4				
	NT50	4	4	5	5	4				
Figure 11			Naive	LF Alone	LF + 0.25 µg IL-1α	LF + 4 µg IL-1α				
	CCL20	BAL	4	5	10	10				
		Nasal Lavage	5	5	10	10				
		Cell Supe	4	5	10	10				
		Cell Lysate	5	5	9	9				

Figure Figures 14-20	Response	KO/KO			WT/KO			
		LF Alone	LF + CT	LF + 4 µg IL-1α	LF Alone	LF + CT	LF + 0.25 µg IL-1α	LF + 4 µg IL-1α
	IgG	7	8	11	19	20	10	21
	IgG1	7	8	11	19	20	10	21
	IgG2c	7	8	11	19	20	10	21
	IgG2b	7	8	11	19	20	10	20
	IgG3	7	8	11	19	20	10	20
	NT50	7	8	11	20	20	10	21
	NIgA	7	10	12	7	8	n.d.	10
	VIgA	7	7	11	20	19	6	19
	FIgA	7	8	11	20	20	10	19
	NIgG	7	8	11	10	10	n.d.	11
	VIgG	n.d.	n.d.	n.d.	10	8	5	7
	FIgG	7	8	11	20	26	15	27
	IL-2	5	5	5	12	15	3	15
	IL-4	5	5	5	12	15	3	15
	IL-10	5	5	5	12	15	3	15
	IL-17	5	5	5	12	15	3	15
	IFNg	5	5	5	12	15	3	15
		KO/WT			WT/WT			
		LF Alone	LF + CT	LF + 0.25 µg IL-1α	LF + 4 µg IL-1α	LF Alone	LF + CT	LF + 0.25 µg IL-1α	LF + 4 µg IL-1α
	IgG	21	23	12	24	18	19	14	19
	IgG1	21	23	13	25	18	19	14	19
	IgG2c	21	23	13	25	18	19	14	19
	IgG2b	21	22	13	25	18	19	14	19
	IgG3	21	22	12	24	18	19	14	19
	NT50	21	23	13	25	18	19	14	19
	NIgA	13	9	n.d.	11	11	13	n.d.	8
	VIgA	21	23	11	25	17	18	13	16
	FIgA	18	20	13	24	15	18	15	16
	NIgG	12	13	n.d.	13	7	9	n.d.	8
	VIgG	9	9	8	8	8	8	10	10
	FIgG	18	19	13	24	23	24	21	22
	IL-2	17	17	6	18	12	14	8	12
	IL-4	17	17	6	18	12	14	8	12
	IL-10	17	17	6	18	12	14	8	12
	IL-17	17	17	6	18	12	14	8	12
	IFNg	17	17	6	18	12	14	8	12

Figure	Response								
Figure 21		WT			WT + anti-CCL20				
		LF Alone	LF + 0.25 µg IL-1α	LF + 4 µg IL-1α	LF Alone	LF + 0.25 µg IL-1α	LF + 4 µg IL-1α		
	IgG	4	4	4	3	4	4		
	NT50	4	4	4	3	4	4		
		WT + Isotype Control			CCR6KO				
		LF Alone	LF + 0.25 µg IL-1α	LF + 4 µg IL-1α	LF Alone	LF + 0.25 µg IL-1α	LF + 4 µg IL-1α		
	IgG	4	3	4	3	4	3		
	NT50	4	3	4	3	4	3		
Figure 22		WT				WT Cells --> RAG			
		LF Alone	LF + CT	LF + 0.25 µg IL-1α	LF + 4 µg IL-1α	LF Alone	LF + CT	LF + 0.25 µg IL-1α	LF + 4 µg IL-1α
	IgG	5	5	4	5	4	4	5	5
	Day 42								
	NT50	5	5	4	5	5	4	5	5
	Day 77								
	NT50	5	4	3	5	4	4	4	5
		IL-1R1-/- Cells --> RAG							
		LF Alone	LF + CT	LF + 0.25 µg IL-1α	LF + 4 µg IL-1α				
	IgG	3	4	5	5				
	Day 42								
	NT50	5	5	5	5				
	Day 77								
	NT50	5	5	5	5				
Figure 23		WT			SASH				
		LF Alone	LF + CT	LF + 4 µg IL-1α	LF Alone	LF + CT	LF + 4 µg IL-1α		
	IgG	5	5	5	5	5	5		
	IgG1	5	5	5	5	5	5		
	IgG2c	5	5	5	5	5	5		

References

1. Plotkin, S.A., W.A. Orenstein, and P.A. Offit, *Vaccines*. Fifth ed, ed. T. Nguyen. 2008: Elsevier.

2. Pead, P.J., *Benjamin Jesty: new light in the dawn of vaccination.* The Lancet, 2003. **362**(9401): p. 2104-2109.

3. CDC, *MMWR: The Principles of Disease Elimination and Eradication*, C.f.D.C. Heath and Human Services, Editor. 1999. p. 23-27.

4. Garnett, G., *Role of Herd Immunity in Determining the Effect of Vaccines against Sexually Transmitted Disease.* The Journal of Infectious Diseases, 2005. **191**(s1): p. S97-S106.

5. CDC, *MMWR: Isolation of Wild Poliovirus Type 3 Among Members of a Religious Community Objecting to Vaccination -- Alberta, Canada, 1993*, C.f.D.C. Heath and Human Services, Editor. 1993. p. 337-339.

6. CDC, *MMWR: Measles Outbreak—Netherlands, April 1999–January 2000*, C.f.D.C. Heath and Human Services, Editor. 2000. p. 299-303.

7. CDC, *MMWR: Outbreak of Measles --- San Diego, California, January--February 2008*, C.f.D.C. Heath and Human Services, Editor. 2008. p. 203-206.

8. Dallaire, F., et al., *Long-Lasting Measles Outbreak Affecting Several Unrelated Networks of Unvaccinated Persons.* Journal of Infectious Diseases, 2009. **200**(10): p. 1602-1605.

9. Glanz, J.M., et al., *Parental Refusal of Pertussis Vaccination Is Associated With an Increased Risk of Pertussis Infection in Children.* Pediatrics, 2009. **123**(6): p. 1446-1451.

10. Welsh, R.M. and R.S. Fujinami, *Pathogenic epitopes, heterologous immunity and vaccine design.* Nat Rev Micro, 2007. **5**(7): p. 555.

11. Ulmer, J.B., U. Valley, and R. Rappuoli, *Vaccine manufacturing: challenges and solutions.* Nat Biotech, 2006. **24**(11): p. 1377.

12. Delves, P.J., et al., *Roitt's Essential Immunology*. Eleventh Edition ed. 2006, Malden, MA: Blackwell Publishing, Inc.

13. Crotty, S., et al., *Cutting Edge: Long-Term B Cell Memory in Humans after Smallpox Vaccination.* J Immunol, 2003. **171**(10): p. 4969-4973.

14. Hammarlund, E., et al., *Duration of antiviral immunity after smallpox vaccination.* Nat Med, 2003. **9**(9): p. 1131.

15. Cyster, J.G., *Homing of antibody secreting cells.* Immunological Reviews, 2003. **194**(1): p. 48-60.

16. Amorosa, V. and S. Isaacs, *Separate Worlds Set to Collide: Smallpox, Vaccinia Virus Vaccination, and Human Immunodeficiency Virus and Acquired Immunodeficiency Syndrome.* Clinical Infectious Diseases, 2003. **37**(3): p. 426-432.

17. Curtis, K., M. Connolly, and D. Northfelt, *Live, Attenuated Varicella Zoster Vaccination of an Immunocompromised Patient.* Journal of General Internal Medicine.

18. Shkap, V., et al., *Attenuated vaccines for tropical theileriosis, babesiosis and heartwater: the continuing necessity.* Trends in Parasitology, 2007. **23**(9): p. 420.

19. Gay, C.G. and T.L. Richie, *Advances in Immunology and Vaccine Discovery: Report of the United States-European Commission Workshop.* Vaccine, 2007. **25**(41): p. 7007.

20. Good, M.F. and D.L. Doolan, *Malaria Vaccine Design: Immunological Considerations.* Immunity, 2010. **33**(4): p. 555-566.

21. Shi, L., et al., *GARDASIL: Prophylactic Human Papillomavirus Vaccine Development - From Bench Top to Bed-side.* Clin Pharmacol Ther, 2007. **81**(2): p. 259-264.

22. Weingart, C.L., et al., *Neutralizing Antibodies to Adenylate Cyclase Toxin Promote Phagocytosis of Bordetella pertussis by Human Neutrophils.* Infect. Immun., 2000. **68**(12): p. 7152-7155.

23. Moayeri, M. and S.H. Leppla, *The roles of anthrax toxin in pathogenesis.* Current Opinion in Microbiology, 2004. **7**(1): p. 19.

24. Boyaka, P.N., et al., *Effective Mucosal Immunity to Anthrax: Neutralizing Antibodies and Th Cell Responses Following Nasal Immunization with Protective Antigen.* J Immunol, 2003. **170**(11): p. 5636-5643.

25. WHO. *Proceedings of the third global vaccine research forum*. 2002. Geneva: WHO.

26. Valensi, J.P., J.R. Carlson, and G.A. Van Nest, *Systemic cytokine profiles in BALB/c mice immunized with trivalent influenza vaccine containing MF59 oil emulsion and other advanced adjuvants.* J Immunol, 1994. **153**(9): p. 4029-4039.

27. Kovarik, J., et al., *Adjuvant effects of CpG oligodeoxynucleotides on responses against T-independent type 2 antigens.* Immunology, 2001. **102**(1): p. 67-76.

28. González-Fernández, Á., J. Faro, and C. Fernández, *Immune responses to polysaccharides: Lessons from humans and mice.* Vaccine, 2008. **26**(3): p. 292.

29. Obukhanych, T.V. and M.C. Nussenzweig, *T-independent type II immune responses generate memory B cells.* The Journal of Experimental Medicine, 2006. **203**(2): p. 305-310.

30. Fraser, A., et al., *Typhoid fever vaccines: Systematic review and meta-analysis of randomised controlled trials.* Vaccine, 2007. **25**(45): p. 7848.

31. Pace, D. and A.J. Pollard, *Meningococcal A, C, Y and W-135 polysaccharide-protein conjugate vaccines.* Arch Dis Child, 2007. **92**(10): p. 909-915.

32. WHO, *WHO position paper on Haemophilus influenzae type b conjugate vaccines. Replaces WHO position paper on Hib vaccines previously published in the Weekly Epidemiological Record.* Wkly Epidemiol Rec 2006. **81**: p. 445-452.

33. Rosenthal, K.S. and D.H. Zimmerman, *Vaccines: All Things Considered.* Clin. Vaccine Immunol., 2006. **13**(8): p. 821-829.

34. Ortega-Sanchez, I.R., et al., *Projected Cost-effectiveness of New Vaccines for Adolescents in the United States.* Pediatrics, 2008. **121**(Supplement_1): p. S63-78.

35. Mensi, C. and F. Pregliasco, *Poliomyelitis: Present Epidemiological Situation and Vaccination Problems.* Clin. Diagn. Lab. Immunol., 1998. **5**(3): p. 278-280.

36. Minor, P., *Vaccine-derived poliovirus (VDPV): Impact on poliomyelitis eradication.* Vaccine, 2009. **27**(20): p. 2649-2652.

37. Hill, D.R., L. Ford, and D.G. Lalloo, *Oral cholera vaccines: use in clinical practice.* The Lancet Infectious Diseases, 2006. **6**(6): p. 361.

38. Cox, R.J., K.A. Brokstad, and P. Ogra, *Influenza Virus: Immunity and Vaccination Strategies. Comparison of the Immune Response to Inactivated and Live, Attenuated Influenza Vaccines.* Scandinavian Journal of Immunology, 2004. **59**(1): p. 1-15.

39. Belshe, R.B., et al., *Live Attenuated versus Inactivated Influenza Vaccine in Infants and Young Children.* New England Journal of Medicine, 2007. **356**(7): p. 685-696.

40. Monto, A.S., et al., *Comparative Efficacy of Inactivated and Live Attenuated Influenza Vaccines.* New England Journal of Medicine, 2009. **361**(13): p. 1260-1267.

41. Mestecky, J., M.W. Russell, and C.O. Elson, *Perspectives on Mucosal Vaccines: Is Mucosal Tolerance a Barrier?* J Immunol, 2007. **179**(9): p. 5633-5638.

42. Neutra, M.R., N.J. Mantis, and J.-P. Kraehenbuhl, *Collaboration of epithelial cells with organized mucosal lymphoid tissues.* Nat Immunol, 2001. **2**(11): p. 1004.

43. Czerkinsky, C., et al., *Mucosal immunity and tolerance: relevance to vaccine development.* Immunological Reviews, 1999. **170**(1): p. 197-222.

44. Kiyono, H. and S. Fukuyama, *NALT- versus PEYER'S-patch-mediated mucosal immunity.* Nat Rev Immunol, 2004. **4**(9): p. 699.

45. Cesta, M.F., *Normal Structure, Function, and Histology of Mucosa-Associated Lymphoid Tissue.* Toxicologic Pathology, 2006. **34**(5): p. 599-608.

46. Chen, K. and A. Cerutti, *Vaccination Strategies to Promote Mucosal Antibody Responses.* Immunity, 2010. **33**(4): p. 479-491.

47. Kawai, T. and S. Akira, *The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors.* Nat Immunol, 2010. **11**(5): p. 373-384.

48. Borregaard, N., *Neutrophils, from Marrow to Microbes.* Immunity, 2010. **33**(5): p. 657-670.

49. Neutra, M.R. and P.A. Kozlowski, *Mucosal vaccines: the promise and the challenge.* Nat Rev Immunol, 2006. **6**(2): p. 148.

50. Chieppa, M., et al., *Dynamic imaging of dendritic cell extension into the small bowel lumen in response to epithelial cell TLR engagement.* J. Exp. Med., 2006. **203**(13): p. 2841-2852.

51. Uematsu, S., et al., *Detection of pathogenic intestinal bacteria by Toll-like receptor 5 on intestinal CD11c+ lamina propria cells.* Nat Immunol, 2006. **7**(8): p. 868-874.

52. Iwasaki, A., *Mucosal Dendritic Cells.* Annual Review of Immunology, 2007. **25**(1): p. 381-418.

53. Sabatté, J., et al., *Interplay of pathogens, cytokines and other stress signals in the regulation of dendritic cell function.* Cytokine & Growth Factor Reviews, 2007. **18**(1-2): p. 5.

54. Fujimura, Y., *Evidence of M cells as portals of entry for antigens in the nasopharyngeal lymphoid tissue of humans.* Virchows Archiv, 2000. **436**(6): p. 560.

55. Sozzani, S., *Dendritic cell trafficking: More than just chemokines.* Cytokine & Growth Factor Reviews, 2005. **16**(6): p. 581.

56. McDermott, M.R. and J. Bienenstock, *Evidence for a Common Mucosal Immunologic System: I. Migration of B Immunoblasts Into Intestinal, Respiratory, and Genital Tissues.* J Immunol, 1979. **122**(5): p. 1892-1898.

57. Pierce, N.F. and W.C. Cray, Jr., *Determinants of the localization, magnitude, and duration of a specific mucosal IgA plasma cell response in enterically immunized rats.* J Immunol, 1982. **128**(3): p. 1311-1315.

58. Parr, E.L. and M.B. Parr, *Immunoglobulin G, Plasma Cells, and Lymphocytes in the Murine Vagina after Vaginal or Parenteral Immunization with Attenuated Herpes Simplex Virus Type 2.* J. Virol., 1998. **72**(6): p. 5137-5145.

59. Brandtzaeg, P., *Mucosal Immunity: Induction, Dissemination, and Effector Functions.* Scandinavian Journal of Immunology, 2009. **70**(6): p. 505-515.

60. Kiyono, H. and S. Fukuyama, *NALT-versus Peyer's-patch-mediated mucosal immunity.* Nat Rev Immunol, 2004. **4**(9): p. 699 - 710.

61. Belyakov, I.M. and J.D. Ahlers, *What Role Does the Route of Immunization Play in the Generation of Protective Immunity against Mucosal Pathogens?* J Immunol, 2009. **183**(11): p. 6883-6892.

62. Macpherson, A.J., et al., *The immune geography of IgA induction and function.* Mucosal Immunol, 2008. **1**(1): p. 11-22.

63. Kaetzel, C.S., *The polymeric immunoglobulin receptor: bridging innate and adaptive immune responses at mucosal surfaces.* Immunological Reviews, 2005. **206**(1): p. 83-99.

64. Corthesy, B., *Roundtrip Ticket for Secretory IgA: Role in Mucosal Homeostasis?* J Immunol, 2007. **178**(1): p. 27-32.

65. Jenny M Woof, M.A.K., *The function of immunoglobulin A in immunity.* The Journal of Pathology, 2006. **208**(2): p. 270-282.

66. Cunningham-Rundles, C., *Physiology of IgA and IgA Deficiency.* Journal of Clinical Immunology, 2001. **21**(5): p. 303.

67. Ballow, M., *Primary immunodeficiency disorders: Antibody deficiency.* Journal of Allergy and Clinical Immunology, 2002. **109**(4): p. 581.

68. Peck, A. and E.D. Mellins, *Precarious Balance: Th17 Cells in Host Defense.* Infect. Immun., 2010. **78**(1): p. 32-38.

69. Shrikant, P., et al., *Regulating functional cell fates in CD8 T cells.* Immunologic Research, 2010. **46**(1): p. 12-22.

70. Rudin, A., et al., *Differential Kinetics and Distribution of Antibodies in Serum and Nasal and Vaginal Secretions after Nasal and Oral Vaccination of Humans.* Infect. Immun., 1998. **66**(7): p. 3390-3396.

71. Macpherson, A.J., et al., *The immune geography of IgA induction and function.* Mucosal Immunol. **1**(1): p. 11.

72. Bradney, C.P., et al., *Cytokines as Adjuvants for the Induction of Anti-Human Immunodeficiency Virus Peptide Immunoglobulin G (IgG) and IgA Antibodies in Serum and Mucosal Secretions after Nasal Immunization.* J. Virol., 2002. **76**(2): p. 517-524.

73. Staats, H.F., et al., *Cytokine Requirements for Induction of Systemic and Mucosal CTL After Nasal Immunization.* J Immunol, 2001. **167**(9): p. 5386-5394.

74. Porgador, A., et al., *Intranasal immunization with CTL epitope peptides from HIV-1 or ovalbumin and the mucosal adjuvant cholera toxin induces peptide- specific CTLs and protection against tumor development in vivo.* J Immunol, 1997. **158**(2): p. 834-841.

75. Roediger, E.K., et al., *Heterologous Boosting of Recombinant Adenoviral Prime Immunization With a Novel Vesicular Stomatitis Virus-vectored Tuberculosis Vaccine.* Mol Ther, 2008. **16**(6): p. 1161-1169.

76. Salem, M.L., et al., *The adjuvant effects of the toll-like receptor 3 ligand polyinosinic-cytidylic acid poly (I:C) on antigen-specific CD8+ T cell responses are partially dependent on NK cells with the induction of a beneficial cytokine milieu.* Vaccine, 2006. **24**(24): p. 5119.

77. Sesardic, D. and R. Dobbelaer, *European union regulatory developments for new vaccine adjuvants and delivery systems.* Vaccine, 2004. **22**(19): p. 2452.

78. FDA, *FDA Approves New Vaccine for Prevention of Cervical Cancer*. 2009, U.S. Food and Drug Administration.

79. Lycke, N., et al., *The adjuvant action of cholera toxin is associated with an increased intestinal permeability for luminal antigens.* Scandinavian Journal of Immunology, 1991. **33**(6): p. 691-698.

80. Levine, M.M., et al., *New knowledge on pathogenesis of bacterial enteric infections as applied to vaccine development.* Microbiological Reviews, 1983. **47**: p. 510-550.

81. Marinaro, M., et al., *Mucosal adjuvant effect of cholera toxin in mice results from induction of T helper 2 (Th2) cells and IL-4.* J Immunol, 1995. **155**(10): p. 4621-4629.

82. Kim, P.-H., et al., *Cholera Toxin and Cholera Toxin B Subunit Induce IgA Switching Through the Action of TGF-{beta}1.* J Immunol, 1998. **160**(3): p. 1198-1203.

83. Lycke, N., et al., *Cellular basis of immunomodulation by cholera toxin in vitro with possible association to the adjuvant function in vivo.* J Immunol, 1989. **142**(1): p. 20-27.

84. van Ginkel, F.W., et al., *Cutting Edge: The Mucosal Adjuvant Cholera Toxin Redirects Vaccine Proteins into Olfactory Tissues.* J Immunol, 2000. **165**(9): p. 4778-4782.

85. Armstrong, M., et al., *Proinflammatory Responses in the Murine Brain after Intranasal Delivery of Cholera Toxin: Implications for the Use of AB Toxins as Adjuvants in Intranasal Vaccines.* The Journal of Infectious Diseases, 2005. **192**(9): p. 1628-1633.

86. Bo Su, S., et al., *Cholera Toxin Prevents Th1-Mediated Autoimmune Disease by Inducing Immune Deviation.* J Immunol, 2004. **173**(2): p. 755-761.

87. Hodge, L.M., et al., *Immunoglobulin A (IgA) Responses and IgE-Associated Inflammation along the Respiratory Tract after Mucosal but Not Systemic Immunization.* Infect. Immun., 2001. **69**(4): p. 2328-2338.

88. Snider, D.P., et al., *Production of IgE antibody and allergic sensitization of intestinal and peripheral tissues after oral immunization with protein Ag and cholera toxin.* J Immunol, 1994. **153**(2): p. 647-657.

89. Couch, R.B., *Nasal Vaccination, Escherichia coli Enterotoxin, and Bell's Palsy.* New England Journal of Medicine, 2004. **350**(9): p. 860-861.

90. Lewis, D.J.M., et al., *Transient Facial Nerve Paralysis (Bell's Palsy) following Intranasal Delivery of a Genetically Detoxified Mutant of Escherichia coli Heat Labile Toxin.* PLoS ONE, 2009. **4**(9): p. e6999.

91. Mutsch, M., et al., *Use of the Inactivated Intranasal Influenza Vaccine and the Risk of Bell's Palsy in Switzerland.* N Engl J Med, 2004. **350**(9): p. 896-903.

92. Watford, W.T., et al., *The biology of IL-12: coordinating innate and adaptive immune responses.* Cytokine & Growth Factor Reviews, 2003. **14**(5): p. 361-368.

93. Baca-Estrada, M.E., M. Foldvari, and M. Snider, *Induction of Mucosal Immune Responses by Administration of Liposome-Antigen Formulations and Interleukin-12.* Journal of Interferon & Cytokine Research, 1999. **19**(5): p. 455-462.

94. Boyaka, P.N., et al., *IL-12 Is an Effective Adjuvant for Induction of Mucosal Immunity.* The Journal of Immunology, 1999. **162**(1): p. 122-128.

95. Okada, E., et al., *Intranasal immunization of a DNA vaccine with IL-12- and granulocyte- macrophage colony-stimulating factor (GM-CSF)-expressing plasmids in liposomes induces strong mucosal and cell-mediated immune responses against HIV-1 antigens.* The Journal of Immunology, 1997. **159**(7): p. 3638-3647.

96. Arulanandam, B.P. and D.W. Metzger, *Modulation of mucosal and systemic immunity by intranasal interleukin 12 delivery.* Vaccine, 1999. **17**(3): p. 252-260.

97. Lynch, J.M., D.E. Briles, and D.W. Metzger, *Increased Protection against Pneumococcal Disease by Mucosal Administration of Conjugate Vaccine plus Interleukin-12.* Infect. Immun., 2003. **71**(8): p. 4780-4788.

98. Albu, D.I., et al., *Intranasal Vaccination Using Interleukin-12 and Cholera Toxin Subunit B as Adjuvants To Enhance Mucosal and Systemic Immunity to Human Immunodeficiency Virus Type 1 Glycoproteins.* J. Virol., 2003. **77**(10): p. 5589-5597.

99. Xin, K.-Q., et al., *IL-15 expression plasmid enhances cell-mediated immunity induced by an HIV-1 DNA vaccine.* Vaccine, 1999. **17**(7-8): p. 858-866.

100. Bermúdez-Humarán, L.G., et al., *A Novel Mucosal Vaccine Based on Live Lactococci Expressing E7 Antigen and IL-12 Induces Systemic and Mucosal Immune Responses and Protects Mice against Human Papillomavirus Type 16-Induced Tumors.* The Journal of Immunology, 2005. **175**(11): p. 7297-7302.

101. Parker, J.N., et al., *Genetically engineered herpes simplex viruses that express IL-12 or GM-CSF as vaccine candidates.* Vaccine, 2006. **24**(10): p. 1644-1652.

102. Malek, T.R., *The Biology of Interleukin-2.* Annual Review of Immunology, 2008. **26**(1): p. 453-479.

103. Xin, K.Q., et al., *Intranasal administration of human immunodeficiency virus type-1 (HIV-1) DNA vaccine with interleukin-2 expression plasmid enhances cell-mediated immunity against HIV-1.* Immunology, 1998. **94**(3): p. 438-444.

104. Steidler, L., et al., *Mucosal Delivery of Murine Interleukin-2 (IL-2) and IL-6 by Recombinant Strains of Lactococcus lactis Coexpressing Antigen and Cytokine.* Infect. Immun., 1998. **66**(7): p. 3183-3189.

105. Bertley, F.M.N., et al., *Control of Simian/Human Immunodeficiency Virus Viremia and Disease Progression after IL-2-Augmented DNA-Modified Vaccinia Virus Ankara Nasal Vaccination in Nonhuman Primates.* The Journal of Immunology, 2004. **172**(6): p. 3745-3757.

106. Abraham, E. and S. Shah, *Intranasal immunization with liposomes containing IL-2 enhances bacterial polysaccharide antigen-specific pulmonary secretory antibody response.* The Journal of Immunology, 1992. **149**(11): p. 3719-3726.

107. Ferko, B., et al., *Live Attenuated Influenza Virus Expressing Human Interleukin-2 Reveals Increased Immunogenic Potential in Young and Aged Hosts.* J. Virol., 2006. **80**(23): p. 11621-11627.

108. Xiaowen, Z., et al., *Co-administration of inactivated avian influenza virus with CpG or rIL-2 strongly enhances the local immune response after intranasal immunization in chicken.* Vaccine, 2009. **27**(41): p. 5628-5632.

109. Shi, Y., et al., *Granulocyte-macrophage colony-stimulating factor (GM-CSF) and T-cell responses: what we do and don't know.* Cell Res, 2006. **16**(2): p. 126-133.

110. Kim, H.-D., et al., *Induction of a Th2 immune response by co-administration of recombinant adenovirus vectors encoding amyloid [beta]-protein and GM-CSF.* Vaccine, 2005. **23**(23): p. 2977-2986.

111. Ramsburg, E., et al., *A Vesicular Stomatitis Virus Recombinant Expressing Granulocyte-Macrophage Colony-Stimulating Factor Induces Enhanced T-Cell Responses and Is Highly Attenuated for Replication in Animals.* J. Virol., 2005. **79**(24): p. 15043-15053.

112. Nambiar, J.K., et al., *Modulation of pulmonary DC function by vaccine-encoded GM-CSF enhances protective immunity against Mycobacterium tuberculosis infection.* European Journal of Immunology, 2010. **40**(1): p. 153-161.

113. Dinarello, C.A., *Biologic basis for interleukin-1 in disease.* Blood, 1996. **87**(6): p. 2095-2147.

114. Chung, Y., et al., *Critical Regulation of Early Th17 Cell Differentiation by Interleukin-1 Signaling.* Immunity, 2009. **30**(4): p. 576-587.

115. Staats, H.F. and F.A. Ennis, Jr., *IL-1 Is an Effective Adjuvant for Mucosal and Systemic Immune Responses When Coadministered with Protein Immunogens.* J Immunol, 1999. **162**(10): p. 6141-6147.

116. Gwinn, W.M., et al., *Effective induction of protective systemic immunity with nasally administered vaccines adjuvanted with IL-1.* Vaccine, 2010. **28**(42): p. 6901-6914.

117. Egan, M.A., et al., *A comparative evaluation of nasal and parenteral vaccine adjuvants to elicit systemic and mucosal HIV-1 peptide-specific humoral immune responses in cynomolgus macaques.* Vaccine, 2004. **22**(27-28): p. 3774-3788.

118. Kayamuro, H., et al., *Interleukin-1 Family Cytokines as Mucosal Vaccine Adjuvants for Induction of Protective Immunity against Influenza Virus.* J. Virol., 2010. **84**(24): p. 12703-12712.

119. Barr, T.A., et al., *B Cell Intrinsic MyD88 Signals Drive IFN-{gamma} Production from T Cells and Control Switching to IgG2c.* J Immunol, 2009. **183**(2): p. 1005-1012.

120. Zwillich, T., *How Vioxx is changing US drug regulation.* The Lancet, 2005. **366**(9499): p. 1763-1764.

121. Schubert, C., *Boosting our best shot.* Nat Med, 2009. **15**(9): p. 984-988.

122. Hunter, R.L., *Overview of vaccine adjuvants: present and future.* Vaccine, 2002. **20**(Supplement 3): p. S7.

123. Guy, B., *The perfect mix: recent progress in adjuvant research.* Nat Rev Micro, 2007. **5**(7): p. 505.

124. McKee, A.S., M.W. Munks, and P. Marrack, *How Do Adjuvants Work? Important Considerations for New Generation Adjuvants.* Immunity, 2007. **27**(5): p. 687.

125. Sokolovska, A., S.L. Hem, and H. HogenEsch, *Activation of dendritic cells and induction of CD4+ T cell differentiation by aluminum-containing adjuvants.* Vaccine, 2007. **25**(23): p. 4575.

126. Li, H., S. Nookala, and F. Re, *Aluminum Hydroxide Adjuvants Activate Caspase-1 and Induce IL-1beta and IL-18 Release.* J Immunol, 2007. **178**(8): p. 5271-5276.

127. Schnare, M., et al., *Toll-like receptors control activation of adaptive immune responses.* Nat Immunol, 2001. **2**(10): p. 947.

128. De Gregorio, E., E. Tritto, and R. Rappuoli, *Alum adjuvanticity: Unraveling a century old mystery.* European Journal of Immunology, 2008. **38**(8): p. 2068-2071.

129. Lambrecht, B.N., et al., *Mechanism of action of clinically approved adjuvants.* Current Opinion in Immunology, 2009. **21**(1): p. 23-29.

130. Tritto, E., F. Mosca, and E. De Gregorio, *Mechanism of action of licensed vaccine adjuvants.* Vaccine, 2009. **27**(25-26): p. 3331-3334.

131. Eisenbarth, S.C., et al., *Crucial role for the Nalp3 inflammasome in the immunostimulatory properties of aluminium adjuvants.* Nature, 2008. **453**(7198): p. 1122-1126.

132. Franchi, L. and G. Núñez, *The Nlrp3 inflammasome is critical for aluminium hydroxide-mediated IL-1β secretion but dispensable for adjuvant activity.* European Journal of Immunology, 2008. **38**(8): p. 2085-2089.

133. Marrack, P., A.S. McKee, and M.W. Munks, *Towards an understanding of the adjuvant action of aluminium.* Nat Rev Immunol, 2009. **9**(4): p. 287-293.

134. Grdic, D., et al., *Splenic Marginal Zone Dendritic Cells Mediate the Cholera Toxin Adjuvant Effect: Dependence on the ADP-Ribosyltransferase Activity of the Holotoxin.* J Immunol, 2005. **175**(8): p. 5192-5202.

135. Hirano, T., et al., *Kinetics of mouse antibody and lymphocyte responses during intranasal vaccination with a lipooligosaccharide-based conjugate vaccine.* Immunology Letters, 2006. **107**(2): p. 131.

136. Sloat, B. and Z. Cui, *Nasal Immunization with Anthrax Protective Antigen Protein Adjuvanted with Polyriboinosinic–Polyribocytidylic Acid Induced Strong Mucosal and Systemic Immunities.* Pharmaceutical Research, 2006. **23**(6): p. 1217.

137. Matsuo, K., et al., *Induction of innate immunity by nasal influenza vaccine administered in combination with an adjuvant (cholera toxin).* Vaccine, 2000. **18**(24): p. 2713.

138. Matsuo, K., et al., *Cytokine mRNAs in the nasal-associated lymphoid tissue during influenza virus infection and nasal vaccination.* Vaccine, 2000. **18**(14): p. 1344.

139. Wu, H.Y., et al., *Development of antibody-secreting cells and antigen-specific T cells in cervical lymph nodes after intranasal immunization.* Infect. Immun., 1997. **65**(1): p. 227-235.

140. Porgador, A., et al., *Intranasal Immunization with Cytotoxic T-Lymphocyte Epitope Peptide and Mucosal Adjuvant Cholera Toxin: Selective Augmentation of Peptide-Presenting Dendritic Cells in Nasal Mucosa-Associated Lymphoid Tissue.* Infect. Immun., 1998. **66**(12): p. 5876-5881.

141. Shreedhar, V.K., B.L. Kelsall, and M.R. Neutra, *Cholera Toxin Induces Migration of Dendritic Cells from the Subepithelial Dome Region to T- and B-Cell Areas of Peyer's Patches.* Infect. Immun., 2003. **71**(1): p. 504-509.

142. Anosova, N.G., et al., *Cholera toxin, E. coli heat-labile toxin, and non-toxic derivatives induce dendritic cell migration into the follicle-associated epithelium of Peyer's patches.* Mucosal Immunol, 0000. **1**(1): p. 59-67.

143. Bromander, A., J. Holmgren, and N. Lycke, *Cholera toxin stimulates IL-1 production and enhances antigen presentation by macrophages in vitro.* J Immunol, 1991. **146**(9): p. 2908-2914.

144. Lee, J.-B., et al., *Intranasal Delivery of Cholera Toxin Induces Th17-Dominated T-Cell Response to Bystander Antigens.* PLoS ONE, 2009. **4**(4): p. e5190.

145. McGowen, A.L., et al., *The mast cell activator compound 48/80 is safe and effective when used as an adjuvant for intradermal immunization with Bacillus anthracis protective antigen.* Vaccine, 2009. **27**(27): p. 3544-3552.

146. Datta, S.K., et al., *Mucosal adjuvant activity of cholera toxin requires Th17 cells and protects against inhalation anthrax.* Proceedings of the National Academy of Sciences, 2010: p. -.

147. Herbert, W.J., *The mode of action of mineral-oil emulsion adjuvants on antibody production in mice.* Immunology, 1968. **14**: p. 301-318.

148. Jansen, T., et al., *Structure- and oil type-based efficacy of emulsion adjuvants.* Vaccine, 2006. **24**(26): p. 5400.

149. Dupuis, M., et al., *Dendritic Cells Internalize Vaccine Adjuvant after Intramuscular Injection.* Cellular Immunology, 1998. **186**(1): p. 18.

150. Yang, Y.-W., C.-A. Wu, and W.J.W. Morrow, *Cell death induced by vaccine adjuvants containing surfactants.* Vaccine, 2004. **22**(11-12): p. 1524.

151. Sauter, B., et al., *Consequences of Cell Death: Exposure to Necrotic Tumor Cells, but Not Primary Tissue Cells or Apoptotic Cells, Induces the Maturation of Immunostimulatory Dendritic Cells.* J. Exp. Med., 2000. **191**(3): p. 423-434.

152. Seubert, A., et al., *The Adjuvants Aluminum Hydroxide and MF59 Induce Monocyte and Granulocyte Chemoattractants and Enhance Monocyte Differentiation toward Dendritic Cells.* The Journal of Immunology, 2008. **180**(8): p. 5402-5412.

153. O'Hagan, D.T., A. Wack, and A. Podda, *MF59 Is a Safe and Potent Vaccine Adjuvant for Flu Vaccines in Humans: What Did We Learn During Its Development?* Clin Pharmacol Ther, 2007. **82**(6): p. 740.

154. Dupuis, M., D.M. McDonald, and G. Ott, *Distribution of adjuvant MF59 and antigen gD2 after intramuscular injection in mice.* Vaccine, 1999. **18**(5-6): p. 434-439.

155. Mosca, F., et al., *Molecular and cellular signatures of human vaccine adjuvants.* Proceedings of the National Academy of Sciences, 2008. **105**(30): p. 10501-10506.

156. Dupuis, M., et al., *Immunization with the adjuvant MF59 induces macrophage trafficking and apoptosis.* European Journal of Immunology, 2001. **31**(10): p. 2910-2918.

157. Vuopio-Varkila, J., et al., *Lipopolysaccharide-induced non-specific resistance to systemic Escherichia coli infection in mice.* J Med Microbiol, 1988. **25**(3): p. 197-203.

158. Martin, M., S.M. Michalek, and J. Katz, *Role of Innate Immune Factors in the Adjuvant Activity of Monophosphoryl Lipid A.* Infect. Immun., 2003. **71**(5): p. 2498-2507.

159. Didierlaurent, A.M., et al., *AS04, an Aluminum Salt- and TLR4 Agonist-Based Adjuvant System, Induces a Transient Localized Innate Immune Response Leading to Enhanced Adaptive Immunity.* The Journal of Immunology, 2009. **183**(10): p. 6186-6197.

160. Okemoto, K., et al., *A Potent Adjuvant Monophosphoryl Lipid A Triggers Various Immune Responses, but Not Secretion of IL-1{beta} or Activation of Caspase-1.* J Immunol, 2006. **176**(2): p. 1203-1208.

161. Mata-Haro, V., et al., *The Vaccine Adjuvant Monophosphoryl Lipid A as a TRIF-Biased Agonist of TLR4.* Science, 2007. **316**(5831): p. 1628-1632.

162. Freytag, L.C. and J.D. Clements, *Mucosal adjuvants.* Vaccine, 2005. **23**(15): p. 1804.

163. Malaviya, R., et al., *Mast cell modulation of neutrophil influx and bacterial clearance at sites of infection through TNF-[alpha].* Nature, 1996. **381**(6577): p. 77-80.

164. McLachlan, J.B., et al., *Mast cell activators: a new class of highly effective vaccine adjuvants.* Nat Med, 2008. **14**(5): p. 536-541.

165. Kérouac, R., et al., *Partial blockade of neurotensin-induced hypotension in rats by nephrectomy captopril and saralasin. Possible mechanisms.* Neuropeptides, 1983. **3**(4): p. 295-307.

166. Rappuoli, R., *Bridging the knowledge gaps in vaccine design.* Nat Biotech, 2007. **25**(12): p. 1361.

167. Nordone, S.K., et al., *Capric Acid and Hydroxypropylmethylcellulose Increase the Immunogenicity of Nasally Administered Peptide Vaccines.* AIDS Research and Human Retroviruses, 2006. **22**(6): p. 558-568.

168. McKee, A.S., M.W. Munks, and P. Marrack, *How Do Adjuvants Work? Important Considerations for New Generation Adjuvants.* 2007. **27**(5): p. 687-690.

169. Heib, V., et al., *Mast cells are crucial for early inflammation, migration of Langerhans cells, and CTL responses following topical application of TLR7 ligand in mice.* Blood, 2007. **110**(3): p. 946-953.

170. Fang, Y., et al., *Mast Cells Contribute to the Mucosal Adjuvant Effect of CTA1-DD after IgG-Complex Formation.* J Immunol, 2010. **185**(5): p. 2935-2941.

171. Gersch, C., et al., *Mast cells and macrophages in normal C57/BL/6 mice.* Histochemistry and Cell Biology, 2002. **118**(1): p. 41-49.

172. O'Hagan, D.T. and N.M. Valiante, *Recent advances in the discovery and delivery of vaccine adjuvants.* Nat Rev Drug Discov, 2003. **2**(9): p. 727-735.

173. Harissios Vliagoftis, A.D.B., *Rapidly changing perspectives about mast cells at mucosal surfaces*. 2005. p. 190-203.

174. Speiser, D.E., et al., *Rapid and strong human CD8+ T cell responses to vaccination with peptide, IFA, and CpG oligodeoxynucleotide 7909.* Journal of Clinical Investigation, 2005. **115**: p. 739-746.

175. Choi, I.H., et al., *Immunoglobulin E-dependent Active Fatal Anaphylaxis in Mast Cell-deficient Mice.* Journal of Experimental Medicine, 1998. **188**(9): p. 1587-1592.

176. Peachman, K.K., et al., *Correlation between Lethal Toxin-Neutralizing Antibody Titers and Protection from Intranasal Challenge with Bacillus anthracis Ames Strain Spores in Mice after Transcutaneous Immunization with Recombinant Anthrax Protective Antigen.* Infect Immun, 2006. **74**(1): p. 794-797.

177. Sawamura, D., et al., *Direct injection of plasmid DNA into the skin induces dermatitis by activation of monocytes through toll-like receptor 9.* the Journal of Gene Medicine, 2005. **7**(5): p. 664-671.

178. Wack, A., et al., *Combination adjuvants for the induction of potent, long-lasting antibody and T-cell responses to influenza vaccine in mice.* Vaccine, 2008. **26**(4): p. 552-561.

179. Yamada, H., K. Ishii, and D. Klinman, *Suppressive oligodeoxynucleotides inhibit CpG-induced inflammation of the mouse lung.* Crit Care Med, 2004. **32**(10): p. 2045-9.

180. Staats, H.F., et al., *In Vitro and In Vivo Characterization of Anthrax Anti-Protective Antigen and Anti-Lethal Factor Monoclonal Antibodies after Passive Transfer in a Mouse Lethal Toxin Challenge Model To Define Correlates of Immunity*. 2007. p. 5443-5452.

181. Kenney, R.T., et al., *Dose Sparing with Intradermal Injection of Influenza Vaccine*. 2004. p. 2295-2301.

182. Belshe, R.B., et al., *Serum Antibody Responses after Intradermal Vaccination against Influenza.* New England Journal of Medicine, 2004. **351**(22): p. 2286-2294.

183. Lodmell, D.L., et al., *One-time intradermal DNA vaccination in ear pinnae one year prior to infection protects dogs against rabies virus.* Vaccine, 2006. **24**(4): p. 412.

184. Alarcon, J.B., et al., *Preclinical Evaluation of Microneedle Technology for Intradermal Delivery of Influenza Vaccines.* Clin. Vaccine Immunol., 2007. **14**(4): p. 375-381.

185. Auewarakul, P., et al., *Antibody responses after dose-sparing intradermal influenza vaccination.* Vaccine, 2007. **25**(4): p. 659-663.

186. Fabrizi, F., et al., *Meta-analysis: intradermal vs. intramuscular vaccination against hepatitis B virus in patients with chronic kidney disease.* Alimentary Pharmacology & Therapeutics, 2006. **24**(3): p. 497-506.

187. Tengvall, S., et al., *CpG oligodeoxynucleotide augments HSV-2 glycoprotein D DNA vaccine efficacy to generate T helper 1 response and subsequent protection against primary genital herpes infection in mice.* Journal of Reproductive Immunology, 2005. **68**(1-2): p. 53.

188. Liu, L., et al., *CpG motif acts as a 'danger signal' and provides a T helper type 1-biased microenvironment for DNA vaccination.* Immunology, 2005. **115**(2): p. 223-230.

189. Halperin, S.A., et al., *A phase I study of the safety and immunogenicity of recombinant hepatitis B surface antigen co-administered with an immunostimulatory phosphorothioate oligonucleotide adjuvant.* Vaccine, 2003. **21**(19-20): p. 2461-2467.

190. Cooper, C.L., et al., *Safety and immunogenicity of CPG 7909 injection as an adjuvant to Fluarix influenza vaccine.* Vaccine, 2004. **22**(23-24): p. 3136-3143.

191. Cooper, C.L., et al., *CpG 7909 adjuvant improves hepatitis B virus vaccine seroprotection in antiretroviral-treated HIV-infected adults.* AIDS, 2005. **19**: p. 1473-1479.

192. Cooper, C.L., et al., *CPG 7909, an Immunostimulatory TLR9 Agonist Oligodeoxynucleotide, as Adjuvant to Engerix-B® HBV Vaccine in Healthy Adults: A Double-Blind Phase I/II Study.* Journal of Clinical Immunology, 2004. **24**(6): p. 693-701.

193. Tono, T., et al., *c-kit Gene was not transcribed in cultured mast cells of mast cell-deficient Wsh/Wsh mice that have a normal number of erythrocytes and a normal c-kit coding region.* Blood, 1992. **80**(6): p. 1448-1453.

194. Akhiani, A.A., L.A. Nilsson, and O. Ouchterlony, *Effect of cholera toxin on vaccine-induced immunity and infection in murine schistosomiasis mansoni.* Infect Immun, 1993. **61**(11): p. 4919-4924.

195. Uddowla, S., L.C. Freytag, and J.D. Clements, *Effect of adjuvants and route of immunizations on the immune response to recombinant plague antigens.* Vaccine, 2007. **25**(47): p. 7984-7993.

196. Staats, H.F., et al., *In Vitro and In Vivo Characterization of Anthrax Anti-Protective Antigen and Anti-Lethal Factor Monoclonal Antibodies after Passive Transfer in a Mouse Lethal Toxin Challenge Model To Define Correlates of Immunity.* Infect Immun, 2007. **75**(11): p. 5443-5452.

197. Takagi, R., et al., *B Cell Chemoattractant CXCL13 Is Preferentially Expressed by Human Th17 Cell Clones.* The Journal of Immunology, 2008. **181**(1): p. 186-189.

198. Duc, L.H., et al., *Immunization against anthrax using Bacillus subtilis spores expressing the anthrax protective antigen.* Vaccine, 2007. **25**(2): p. 346-355.

199. Berthold, I., et al., *Immunogenicity in mice of anthrax recombinant protective antigen in the presence of aluminum adjuvants.* Vaccine, 2005. **23**(16): p. 1993-1999.

200. Peachman, K.K., et al., *Correlation between Lethal Toxin-Neutralizing Antibody Titers and Protection from Intranasal Challenge with Bacillus anthracis Ames Strain Spores in Mice after Transcutaneous Immunization with Recombinant Anthrax Protective Antigen.* Infect. Immun., 2006. **74**(1): p. 794-797.

201. Staats, H.F., et al., *In Vitro and In Vivo Characterization of Anthrax Anti-Protective Antigen and Anti-Lethal Factor Monoclonal Antibodies after Passive Transfer in a Mouse Lethal Toxin Challenge Model To Define Correlates of Immunity.* Infect. Immun., 2007. **75**(11): p. 5443-5452.

202. Zeng, M., Q. Xu, and M.E. Pichichero, *Protection against anthrax by needle-free mucosal immunization with human anthrax vaccine.* Vaccine, 2007. **25**(18): p. 3588-3594.

203. Weeratna, R.D., et al., *CpG ODN can re-direct the Th bias of established Th2 immune responses in adult and young mice.* FEMS Immunology and Medical Microbiology, 2001. **32**(1): p. 65.

204. Korn, T., et al., *Th17 cells: Effector T cells with inflammatory properties.* Seminars in Immunology, 2007. **19**(6): p. 362-371.

205. Kimmel, S.R., *Vaccine adverse events: separating myth from reality.* American Family Physician, 2002. **66**(1): p. 2113-2120.

206. Mazzoni, A., et al., *Dendritic Cell Modulation by Mast Cells Controls the Th1/Th2 Balance in Responding T Cells.* J Immunol, 2006. **177**(6): p. 3577-3581.

207. Belshe, R.B., et al., *Serum Antibody Responses after Intradermal Vaccination against Influenza.* N Engl J Med, 2004. **351**(22): p. 2286-2294.

208. Kenney, R.T., et al., *Dose Sparing with Intradermal Injection of Influenza Vaccine.* N Engl J Med, 2004. **351**(22): p. 2295-2301.

209. La Montagne, J.R. and A.S. Fauci, *Intradermal Influenza Vaccination -- Can Less Be More?* N Engl J Med, 2004. **351**(22): p. 2330-2332.

210. Roukens, A.H., et al., *Intradermally Administered Yellow Fever Vaccine at Reduced Dose Induces a Protective Immune Response: A Randomized Controlled Non-Inferiority Trial.* PLoS ONE, 2008. **3**(4): p. e1993.

211. Sugimura, T., et al., *Improved antibody responses in infants less than 1 year old using intradermal influenza vaccination.* Vaccine, 2008. **26**(22): p. 2700-2705.

212. Matyas, G.R., et al., *Needle-Free Skin Patch Vaccination Method for Anthrax.* Infect. Immun., 2004. **72**(2): p. 1181-1183.

213. Cui, X., et al., *Late Treatment with a Protective Antigen Directed Monoclonal Antibody Improves Hemodynamic Function and Survival in a Lethal Toxin Infused Rat Model of Anthrax Sepsis.* The Journal of Infectious Diseases, 2005. **191**(3): p. 422-434.

214. Beedham, R.J., P.C.B. Turnbull, and E.D. Williamson, *Passive transfer of protection against Bacillus anthracis infection in a murine model.* Vaccine, 2001. **19**(31): p. 4409-4416.

215. Mohamed, N., et al., *A High-Affinity Monoclonal Antibody to Anthrax Protective Antigen Passively Protects Rabbits before and after Aerosolized Bacillus anthracis Spore Challenge.* Infect Immun, 2005. **73**(2): p. 795-802.

216. Kataoka, T.R., et al., *Involvement of connective tissue-type mast cells in Th1 immune responses via Stat4 expression.* Blood, 2005. **105**(3): p. 1016-1020.

217. Wiedemann, F., et al., *Histopathological studies on the local reactions induced by complete Freund's adjuvant (CFA), bacterial lipopolysaccharide (LPS), and synthetic lipopeptide (P3C) conjugates.* The Journal of Pathology, 1991. **164**(3): p. 265-271.

218. Alving, C.R., *Design and selection of vaccine adjuvants: animal models and human trials.* Vaccine, 2002. **20**(Supplement 3): p. S56-S64.

219. Barve, M., et al., *Induction of immune responses and clinical efficacy in a phase II trial of IDM-2101, a 10-epitope cytotoxic T-lymphocyte vaccine, in metastatic non-small-cell lung cancer.* J Clin Oncol, 2008. **26**(27): p. 4418-25.

220. Sugai, K., et al., *Dermal testing of vaccines for children at high risk of allergies.* Vaccine, 2007. **25**(17): p. 3454-3463.

221. Brotherton, J.M.L., et al., *Anaphylaxis following quadrivalent human papillomavirus vaccination.* CMAJ, 2008. **179**(6): p. 525-533.

222. Yamamoto, S., et al., *Mutants in the ADP-ribosyltransferase Cleft of Cholera Toxin Lack Diarrheagenicity but Retain Adjuvanticity.* The Journal of Experimental Medicine, 1997. **185**(7): p. 1203-1210.

223. Choi, I.H., et al., *Immunoglobulin E-dependent Active Fatal Anaphylaxis in Mast Cell-deficient Mice.* J. Exp. Med., 1998. **188**(9): p. 1587-1592.

224. Malaviya, R. and S. Abraham, *Role of mast cell leukotrienes in neutrophil recruitment and bacterial clearance in infectious peritonitis.* J Leukoc Biol, 2000. **67**(6): p. 841-846.

225. Echtenacher, B., D.N. Mannel, and L. Hultner, *Critical protective role of mast cells in a model of acute septic peritonitis.* Nature, 1996. **381**(6577): p. 75-77.

226. Malaviya, R., et al., *Contribution of mast cells to bacterial clearance and their proliferation during experimental cystitis induced by type 1 fimbriated E. coli.* Immunology Letters, 2004. **91**(2-3): p. 103-111.

227. Scapini, P., et al., *The neutrophil as a cellular source of chemokines.* Immunological Reviews, 2000. **177**(1): p. 195-203.

228. Beauvillain, C., et al., *Neutrophils efficiently cross-prime naive T cells in vivo.* Blood, 2007. **110**(8): p. 2965-2973.

229. Whale, T.A. and P.J. Griebel, *A sheep in wolf's clothes: Can neutrophils direct the immune response?* The Veterinary Journal, 2009. **180**(2): p. 169-177.

230. Tateda, K., et al., *Early Recruitment of Neutrophils Determines Subsequent T1/T2 Host Responses in a Murine Model of Legionella pneumophila Pneumonia.* The Journal of Immunology, 2001. **166**(5): p. 3355-3361.

231. Romani, L., et al., *An immunoregulatory role for neutrophils in CD4+ T helper subset selection in mice with candidiasis.* The Journal of Immunology, 1997. **158**(5): p. 2356-2362.

232. van Gisbergen, K.P.J.M., et al., *Neutrophils mediate immune modulation of dendritic cells through glycosylation-dependent interactions between Mac-1 and DC-SIGN.* The Journal of Experimental Medicine, 2005. **201**(8): p. 1281-1292.

233. Bennouna, S. and E.Y. Denkers, *Microbial Antigen Triggers Rapid Mobilization of TNF-{alpha} to the Surface of Mouse Neutrophils Transforming Them into Inducers of High-Level Dendritic Cell TNF-{alpha} Production.* The Journal of Immunology, 2005. **174**(8): p. 4845-4851.

234. Maletto, B.A., et al., *Presence of neutrophil-bearing antigen in lymphoid organs of immune mice.* Blood, 2006. **108**(9): p. 3094-3102.

235. Fadel, R., et al., *Short communication. Cetirizine inhibits bradykinin-induced cutaneous wheal and flare in atopic and healthy subjects.* Allergy, 2000. **55**(9): p. 888-891.

236. Gold, Y., A. Goldberg, and Y. Sivan, *Hyper-releasability of mast cells in family members of infants with sudden infant death syndrome and apparent life-threatening events.* The Journal of Pediatrics, 2000. **136**(4): p. 460-465.

237. Miloševic, D., et al., *Skin reactivity to vasomotor agents in non-eosinophilic and eosinophilic non-allergic rhinitis.* Journal of Laryngology & Otology, 2002. **116**(07): p. 519-522.

238. Rukwied, R., et al., *Sensitivity of human scalp skin to pruritic stimuli investigated by intradermal microdialysis in vivo.* Journal of the American Academy of Dermatology, 2002. **47**(2): p. 245-250.

239. Atkins, P., G.R. Green, and B. Zweiman, *Histologic studies of human skin test responses to ragweed, compound 48/80, and histamine.* Journal of Allergy and Clinical Immunology, 1973. **51**(5): p. 263-273.

240. Dinarello, C.A., *Immunological and Inflammatory Functions of the Interleukin-1 Family.* Annual Review of Immunology, 2009. **27**(1): p. 519-550.

241. McLachlan, J.B., et al., *Mast cell-derived tumor necrosis factor induces hypertrophy of draining lymph nodes during infection.* Nat Immunol, 2003. **4**(12): p. 1199-1205.

242. Shen, H., et al., *Novel recombinant BCG coexpressing Ag85B, ESAT-6 and mouse TNF-α induces significantly enhanced cellular immune and antibody responses in C57BL/6 mice.* Microbiology and Immunology, 2010. **54**(8): p. 435-441.

243. Beghdadi, W., et al., *Inhibition of histamine-mediated signaling confers significant protection against severe malaria in mouse models of disease.* The Journal of Experimental Medicine, 2008. **205**(2): p. 395-408.

244. Carlos, D., et al., *Histamine Plays an Essential Regulatory Role in Lung Inflammation and Protective Immunity in the Acute Phase of Mycobacterium tuberculosis Infection.* Infect. Immun., 2009. **77**(12): p. 5359-5368.

245. Moon, T.C., et al., *Advances in mast cell biology: new understanding of heterogeneity and function.* Mucosal Immunol, 2009. **3**(2): p. 111-128.

246. de Mora, F., et al., *Canine cutaneous mast cells dispersion and histamine secretory characterization.* Veterinary Immunology and Immunopathology, 1993. **39**(4): p. 421-429.

247. Dinarello, C.A., *The interleukin-1 family: 10 years of discovery.* FASEB J., 1994. **8**(15): p. 1314-1325.

248. Weber, A., P. Wasiliew, and M. Kracht, *Interleukin-1 (IL-1) Pathway.* Sci. Signal., 2010. **3**(105): p. cm1-.

249. Sims, J.E. and D.E. Smith, *The IL-1 family: regulators of immunity.* Nat Rev Immunol, 2010. **10**(2): p. 89-102.

250. Dinarello, C.A., *IL-1: Discoveries, controversies and future directions.* European Journal of Immunology, 2010. **40**(3): p. 599-606.

251. Al-Sadi, R., et al., *Mechanism of IL-1{beta}-Induced Increase in Intestinal Epithelial Tight Junction Permeability.* J Immunol, 2008. **180**(8): p. 5653-5661.

252. Graves, D.T., et al., *IL-1 Plays a Critical Role in Oral, But Not Dermal, Wound Healing.* J Immunol, 2001. **167**(9): p. 5316-5320.

253. Lin, T.-J., et al., *Pseudomonas aeruginosa Activates Human Mast Cells to Induce Neutrophil Transendothelial Migration Via Mast Cell-Derived IL-1{alpha} and {beta}.* J Immunol, 2002. **169**(8): p. 4522-4530.

254. Miller, L.S., et al., *MyD88 Mediates Neutrophil Recruitment Initiated by IL-1R but Not TLR2 Activation in Immunity against Staphylococcus aureus.* Immunity, 2006. **24**(1): p. 79.

255. Kang, T., et al., *Bacillus anthracis spores and lethal toxin induce IL-1β via functionally distinct signaling pathways.* European Journal of Immunology, 2008. **38**(6): p. 1574-1584.

256. Hurgin, V., et al., *Antiviral and immunoregulatory activities of IFN-{gamma} depend on constitutively expressed IL-1{alpha}.* Proceedings of the National Academy of Sciences, 2007. **104**(12): p. 5044-5049.

257. Ben-Sasson, S.Z., et al., *IL-1 acts directly on CD4 T cells to enhance their antigen-driven expansion and differentiation.* Proceedings of the National Academy of Sciences, 2009. **106**(17): p. 7119-7124.

258. Curtsinger, J.M., et al., *Inflammatory Cytokines Provide a Third Signal for Activation of Naive CD4+ and CD8+ T Cells.* J Immunol, 1999. **162**(6): p. 3256-3262.

259. Nakae, S., et al., *Mast Cells Enhance T Cell Activation: Importance of Mast Cell Costimulatory Molecules and Secreted TNF.* J Immunol, 2006. **176**(4): p. 2238-2248.

260. Nambu, A., S. Nakae, and Y. Iwakura, *IL-1β, but not IL-1α, is required for antigen-specific T cell activation and the induction of local inflammation in the delayed-type hypersensitivity responses.* International Immunology, 2006. **18**(5): p. 701-712.

261. Wilson, N.J., et al., *Development, cytokine profile and function of human interleukin 17-producing helper T cells.* Nat Immunol, 2007. **8**(9): p. 950-957.

262. Arend, W., et al., *Binding of IL-1 alpha, IL-1 beta, and IL-1 receptor antagonist by soluble IL-1 receptors and levels of soluble IL-1 receptors in synovial fluids.* J Immunol, 1994. **153**(10): p. 4766-4774.

263. Spörri, B., et al., *Soluble interleukin-1 receptor -- reverse signaling in innate immunoregulation.* Cytokine & Growth Factor Reviews, 2001. **12**(1): p. 27-32.

264. Cook, G.P., et al., *The NLRP3 inflammasome, a target for therapy in diverse disease states.* European Journal of Immunology, 2010. **40**(3): p. 631-634.

265. Pétrilli, V. and F. Martinon, *The inflammasome, autoinflammatory diseases, and gout.* Joint Bone Spine, 2007. **74**(6): p. 571-576.

266. Rodrigue-Gervais, I.G. and M. Saleh, *Genetics of inflammasome-associated disorders: A lesson in the guiding principals of inflammasome function.* European Journal of Immunology, 2010. **40**(3): p. 643-648.

267. Brydges, S.D., et al., *Inflammasome-Mediated Disease Animal Models Reveal Roles for Innate but Not Adaptive Immunity.* Immunity, 2009. **30**(6): p. 875-887.

268. Cassel, S.L., S. Joly, and F.S. Sutterwala, *The NLRP3 inflammasome: A sensor of immune danger signals.* Seminars in Immunology, 2009. **21**(4): p. 194-198.

269. Martinon, F., A. Mayor, and J.r. Tschopp, *The Inflammasomes: Guardians of the Body.* Annual Review of Immunology, 2009. **27**(1): p. 229-265.

270. Curtis, K., M. Connolly, and D. Northfelt, *Live, Attenuated Varicella Zoster Vaccination of an Immunocompromised Patient.* Journal of General Internal Medicine, 2008. **23**(5): p. 648-649.

271. Galhotra, A., et al., *Surveillance of cold chain system during intensified pulse polio programme - 2006 in Chandigarh.* Indian J Pediatr 2007. **74**: p. 751-753.

272. Sun, J.-B., C. Czerkinsky, and J. Holmgren, *Mucosally induced Immunological Tolerance, Regulatory T Cells and the Adjuvant Effect by Cholera Toxin B Subunit.* Scandinavian Journal of Immunology, 2010. **71**(1): p. 1-11.

273. Hasselberg, A., et al., *ADP-Ribosylation Controls the Outcome of Tolerance or Enhanced Priming Following Mucosal Immunization.* J Immunol, 2010. **184**(6): p. 2776-2784.

274. van Ginkel, F.W., et al., *Enterotoxin-Based Mucosal Adjuvants Alter Antigen Trafficking and Induce Inflammatory Responses in the Nasal Tract.* Infect. Immun., 2005. **73**(10): p. 6892-6902.

275. Kandere-Grzybowska, K., et al., *IL-1 Induces Vesicular Secretion of IL-6 without Degranulation from Human Mast Cells.* J Immunol, 2003. **171**(9): p. 4830-4836.

276. Wesa, A.K. and A. Galy, *IL-1{beta} induces dendritic cells to produce IL-12.* International Immunology, 2001. **13**(8): p. 1053-1061.

277. Rogers, H.W., et al., *Endogenous IL-1 is required for neutrophil recruitment and macrophage activation during murine listeriosis.* J Immunol, 1994. **153**(5): p. 2093-2101.

278. Seubert, A., et al., *The Adjuvants Aluminum Hydroxide and MF59 Induce Monocyte and Granulocyte Chemoattractants and Enhance Monocyte Differentiation toward Dendritic Cells.* J Immunol, 2008. **180**(8): p. 5402-5412.

279. Nijnik, A., et al., *Synthetic Cationic Peptide IDR-1002 Provides Protection against Bacterial Infections through Chemokine Induction and Enhanced Leukocyte Recruitment.* J Immunol, 2010. **184**(5): p. 2539-2550.

280. Korsholm, K.S., et al., *T-helper 1 and T-helper 2 adjuvants induce distinct differences in the magnitude, quality and kinetics of the early inflammatory response at the site of injection.* Immunology, 2010. **129**: p. 75-86.

281. Eriksson, U., et al., *Activation of Dendritic Cells through the Interleukin 1 Receptor 1 Is Critical for the Induction of Autoimmune Myocarditis.* The Journal of Experimental Medicine, 2003. **197**(3): p. 323-331.

282. Satoskar, A.R., et al., *Enhanced Th2-like responses in IL-1 type 1 receptor-deficient mice.* European Journal of Immunology, 1998. **28**(7): p. 2066-2074.

283. Awane, M., et al., *NF-{kappa}B-Inducing Kinase Is a Common Mediator of IL-17-, TNF-{alpha}-, and IL-1{beta}-Induced Chemokine Promoter Activation in Intestinal Epithelial Cells.* J Immunol, 1999. **162**(9): p. 5337-5344.

284. Cowland, J.B., et al., *Neutrophil Gelatinase-Associated Lipocalin Is Up-Regulated in Human Epithelial Cells by IL-1{beta}, but Not by TNF-{alpha}.* J Immunol, 2003. **171**(12): p. 6630-6639.

285. Elias, J. and V. Lentz, *IL-1 and tumor necrosis factor synergistically stimulate fibroblast IL- 6 production and stabilize IL-6 messenger RNA.* J Immunol, 1990. **145**(1): p. 161-166.

286. Yamamoto, T., et al., *Monocyte Chemoattractant Protein-1 Enhances Gene Expression and Synthesis of Matrix Metalloproteinase-1 in Human Fibroblasts by an Autocrine IL-1{alpha} Loop.* J Immunol, 2000. **164**(12): p. 6174-6179.

287. Minns, L.A., et al., *TLR9 Is Required for the Gut-Associated Lymphoid Tissue Response following Oral Infection of Toxoplasma gondii.* J Immunol, 2006. **176**(12): p. 7589-7597.

288. Rimoldi, M., et al., *Intestinal epithelial cells control dendritic cell function.* Annals of the New York Academy of Sciences, 2004. **1029**: p. 66-74.

289. Noulin, N., et al., *Both Hemopoietic and Resident Cells Are Required for MyD88-Dependent Pulmonary Inflammatory Response to Inhaled Endotoxin.* J Immunol, 2005. **175**(10): p. 6861-6869.

290. Patole, P.S., et al., *Toll-like receptor-4: Renal cells and bone marrow cells signal for neutrophil recruitment during pyelonephritis.* Kidney International, 2005. **68**(6): p. 2582-2587.

291. Sato, A. and A. Iwasaki, *From The Cover: Induction of antiviral immunity requires Toll-like receptor signaling in both stromal and dendritic cell compartments.* Proceedings of the National Academy of Sciences, 2004. **101**(46): p. 16274-16279.

292. Andonegui, G., et al., *Endothelium-derived Toll-like receptor-4 is the key molecule in LPS-induced neutrophil sequestration into lungs.* J. Clin. Invest., 2003. **111**(7): p. 1011-1020.

293. Hollingsworth, J.W., et al., *The Critical Role of Hematopoietic Cells in Lipopolysaccharide-induced Airway Inflammation.* American Journal of Respiratory and Critical Care Medicine, 2005. **171**(8): p. 806-813.

294. Schilling, J.D., et al., *Toll-like receptor 4 on stromal and hematopoietic cells mediates innate resistance to uropathogenic Escherichia coli.* PNAS, 2003. **100**(7): p. 4203-4208.

295. Sato, A. and A. Iwasaki, *From The Cover: Induction of antiviral immunity requires Toll-like receptor signaling in both stromal and dendritic cell compartments.* PNAS, 2004. **101**(46): p. 16274-16279.

296. Sanders, C.J., et al., *Both Radioresistant and Hemopoietic Cells Promote Innate and Adaptive Immune Responses to Flagellin.* J Immunol, 2008. **180**(11): p. 7184-7192.

297. Sanders, C.J., et al., *Induction of adaptive immunity by flagellin does not require robust activation of innate immunity.* European Journal of Immunology, 2009. **39**(2): p. 359-371.

298. Chakravarty, S. and M. Herkenham, *Toll-Like Receptor 4 on Nonhematopoietic Cells Sustains CNS Inflammation during Endotoxemia, Independent of Systemic Cytokines.* J. Neurosci., 2005. **25**(7): p. 1788-1796.

299. Patole, P.S., et al., *Toll-like receptor-4: Renal cells and bone marrow cells signal for neutrophil recruitment during pyelonephritis.* Kidney Int, 2005. **68**(6): p. 2582.

300. Golde, W.T., P. Gollobin, and L.L. Rodriguez, *A rapid, simple, and humane method for submandibular bleeding of mice using a lancet.* Lab Anim (NY), 2005. **34**(9): p. 39-43.

301. Ferreira, M.U. and A.M. Katzin, *The assessment of antibody affinity distribution by thiocyanate elution: a simple dose-response approach.* Journal of Immunological Methods, 1995. **187**(2): p. 297-305.

302. Benwell, R.K. and D.R. Lee, *Essential and synergistic roles of IL1 and IL6 in human Th17 differentiation directed by TLR ligand-activated dendritic cells.* Clinical Immunology, 2010. **134**(2): p. 178-187.

303. Joffre, O., et al., *Inflammatory signals in dendritic cell activation and the induction of adaptive immunity.* Immunological Reviews, 2009. **227**(1): p. 234-247.

304. Qin, H., et al., *TGF-{beta} Promotes Th17 Cell Development through Inhibition of SOCS3.* J Immunol, 2009. **183**(1): p. 97-105.

305. Kutzler, M.A., et al., *Plasmids encoding the mucosal chemokines CCL27 and CCL28 are effective adjuvants in eliciting antigen-specific immunity in vivo.* Gene Ther, 2009. **17**(1): p. 72-82.

306. Song, J.-H., et al., *CCR7-CCL19/CCL21-Regulated Dendritic Cells Are Responsible for Effectiveness of Sublingual Vaccination.* J Immunol, 2009. **182**(11): p. 6851-6860.

307. McCloskey, N., M.W. Turner, and D. Goldblatt, *Correlation between the avidity of mouse-human chimeric IgG subclass monoclonal antibodies measured by solid-phase elution ELISA and biospecific interaction analysis (BIA).* Journal of Immunological Methods, 1997. **205**(1): p. 67-72.

308. Park, Y.S., et al., *Enhancement of Antibody Responses to Bacillus anthracis Protective Antigen Domain IV by Use of Calreticulin as a Chimeric Molecular Adjuvant.* Infect. Immun., 2008. **76**(5): p. 1952-1959.

309. Rohatgi, S., et al., *Molecular Dissection of Antibody Responses against Pneumococcal Surface Protein A: Evidence for Diverse DH-Less Heavy Chain Gene Usage and Avidity Maturation.* Journal of Immunology, 2009. **182**(9): p. 5570-5585.

310. Rahman, A.H., et al., *MyD88 Plays a Critical T Cell-Intrinsic Role in Supporting CD8 T Cell Expansion during Acute Lymphocytic Choriomeningitis Virus Infection.* J Immunol, 2008. **181**(6): p. 3804-3810.

311. Elkordy, M., et al., *A phase I trial of recombinant human interleukin-1b(OCT-43) following high-dose chemotherapy and autologous bone marrow transplantation.* Bone Marrow Transplantation, 1997. **19**(4): p. 315-322.

312. Veltri, S. and J.W.S. II, *Interleukin 1 Trials in Cancer Patients: A Review of the Toxicity, Antitumor and Hematopoietic Effects.* Stem Cells, 1996. **14**(2): p. 164-176.

313. McKee, A.S., et al., *Alum Induces Innate Immune Responses through Macrophage and Mast Cell Sensors, But These Sensors Are Not Required for Alum to Act As an Adjuvant for Specific Immunity.* J Immunol, 2009. **183**(7): p. 4403-4414.

314. Longhi, M.P., et al., *Dendritic cells require a systemic type I interferon response to mature and induce CD4+ Th1 immunity with poly IC as adjuvant.* The Journal of Experimental Medicine, 2009. **206**(7): p. 1589-1602.

315. Hubbard, A.L. and L.T. Braiterman, *Could ATP7B Export Cu(I) at the Tight Junctions and the Apical Membrane?* Gastroenterology, 2008. **134**(4): p. 1255-1257.

316. Dmitriev, O.Y., *Mechanism of tumor resistance to cisplatin mediated by the copper transporter ATP7B.* Biochemistry and Cell Biology, 2011. **89**(2): p. 138-147.

317. Chatterjee, S., et al., *A Novel Copper Chelate Modulates Tumor Associated Macrophages to Promote Anti-Tumor Response of T Cells.* PLoS ONE, 2009. **4**(9): p. e7048.

318. Teles, M., et al., *Gene expression and TNF-alpha secretion profile in rainbow trout macrophages following exposures to copper and bacterial lipopolysaccharide.* Fish & Shellfish Immunology, 2011. **30**(1): p. 340-346.

319. Chen, Z.-q., et al., *Three ATP-binding cassette transporter genes, <i>Abca14</i>, <i>Abca15</i>, and <i>Abca16</i>, form a cluster on mouse Chromosome 7F3.* Mammalian Genome, 2004. **15**(5): p. 335-343.

320. Ueda, Y., et al., *IL-1R Type I-Dependent Hemopoietic Stem Cell Proliferation Is Necessary for Inflammatory Granulopoiesis and Reactive Neutrophilia.* J Immunol, 2009. **182**(10): p. 6477-6484.

321. Leendertse, M., et al., *Neutrophils Are Essential for Rapid Clearance of Enterococcus faecium in Mice.* Infect. Immun., 2009. **77**(1): p. 485-491.

322. van Faassen, H., et al., *Neutrophils Play an Important Role in Host Resistance to Respiratory Infection with Acinetobacter baumannii in Mice.* Infect. Immun., 2007. **75**(12): p. 5597-5608.

323. Yang, C.-W., et al., *Neutrophils Influence the Level of Antigen Presentation during the Immune Response to Protein Antigens in Adjuvants.* J Immunol, 2010. **185**(5): p. 2927-2934.

324. Li, W., et al., *Antigen-Specific CD4+ T Cells Produce Sufficient IFN-{gamma} to Mediate Robust Protective Immunity against Genital Chlamydia muridarum Infection.* J Immunol, 2008. **180**(5): p. 3375-3382.

325. Wang, Y., et al., *Cutting Edge: Polyinosinic:Polycytidylic Acid Boosts the Generation of Memory CD8 T Cells through Melanoma Differentiation-Associated Protein 5 Expressed in Stromal Cells.* J Immunol, 2010. **184**(6): p. 2751-2755.

326. Zygmunt, B.M., et al., *Intranasal Immunization Promotes Th17 Immune Responses.* J Immunol, 2009. **183**(11): p. 6933-6938.

327. Glaccum, M., et al., *Phenotypic and functional characterization of mice that lack the type I receptor for IL-1.* The Journal of Immunology, 1997. **159**(7): p. 3364-3371.

328. Hammad, H. and B.N. Lambrecht, *Dendritic cells and epithelial cells: linking innate and adaptive immunity in asthma.* Nat Rev Immunol, 2008. **8**(3): p. 193-204.

329. Arques, J.L., et al., *Salmonella Induces Flagellin- and MyD88-Dependent Migration of Bacteria-Capturing Dendritic Cells Into the Gut Lumen.* Gastroenterology, 2009. **137**(2): p. 579-587.e2.

330. Janot, L., et al., *Radioresistant cells expressing TLR5 control the respiratory epithelium's innate immune responses to flagellin.* European Journal of Immunology, 2009. **39**(6): p. 1587-1596.

331. Chen, D., et al., *Serum and Mucosal Immune Responses to an Inactivated Influenza Virus Vaccine Induced by Epidermal Powder Immunization.* J. Virol., 2001. **75**(17): p. 7956-7965.

332. Williams, I.R., *CCR6 and CCL20.* Annals of the New York Academy of Sciences, 2006. **1072**(Inflammatory Bowel Disease Genetics Barrier Function Immunologic Mechanisms and Microbial Pathways): p. 52-61.

333. Cooper, M.A., T.A. Fehniger, and M.A. Caligiuri, *The biology of human natural killer-cell subsets.* Trends in Immunology, 2001. **22**(11): p. 633-640.

334. Zeng, X., et al., *Interferon-Inducible Protein 10, but Not Monokine Induced by Gamma Interferon, Promotes Protective Type 1 Immunity in Murine Klebsiella pneumoniae Pneumonia.* Infect. Immun., 2005. **73**(12): p. 8226-8236.

335. Zeng, X., et al., *IP-10 Mediates Selective Mononuclear Cell Accumulation and Activation in Response to Intrapulmonary Transgenic Expression and During Adenovirus-Induced Pulmonary Inflammation.* Journal of Interferon & Cytokine Research, 2005. **25**(2): p. 103-112.

336. Hall, L.J., S. Clare, and G. Dougan, *NK Cells Influence Both Innate and Adaptive Immune Responses after Mucosal Immunization with Antigen and Mucosal Adjuvant.* J Immunol, 2010. **184**(8): p. 4327-4337.

337. Yoshida, O., et al., *Impaired dendritic cell functions because of depletion of natural killer cells disrupt antigen-specific immune responses in mice: restoration of adaptive immunity in natural killer-depleted mice by antigen-pulsed dendritic cell.* Clinical & Experimental Immunology, 2008. **152**(1): p. 174-181.

338. Hou, B., B. Reizis, and A.L. DeFranco, *Toll-like Receptors Activate Innate and Adaptive Immunity by using Dendritic Cell-Intrinsic and -Extrinsic Mechanisms.* Immunity, 2008. **29**(2): p. 272-282.

339. Sporri, R. and C. Reis e Sousa, *Inflammatory mediators are insufficient for full dendritic cell activation and promote expansion of CD4+ T cell populations lacking helper function.* Nat Immunol, 2005. **6**(2): p. 163-170.

340. Hou, B., et al., *Critical coordination of innate immune defense against Toxoplasma gondii by dendritic cells responding via their Toll-like receptors.* Proceedings of the National Academy of Sciences, 2011. **108**(1): p. 278-283.

341. McCormick, S., et al., *Mucosally Delivered Dendritic Cells Activate T Cells Independently of IL-12 and Endogenous APCs.* J Immunol, 2008. **181**(4): p. 2356-2367.

Biography

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Publications

Thompson, Afton L., Gregory D. Sempowski, Herman F. Staats. Maximal adjuvant activity of nasally delivered IL-1α is dependent upon IL-1R1 expression by hematopoietic cells and does not correlate with adjuvant-induced cytokine production. Journal of Immunology. Submitted.

Thompson, Afton L. and Herman F. Staats. Cytokines: the future of intranasal vaccine adjuvants. Clinical and Developmental Immunology. Submitted.

Wang, Sheena H., Afton L. Thompson, Anthony J. Hickey and Herman F. Staats. Dry powder vaccines for mucosal administration: critical factors for effective manufacturing and delivery. Current Topics in Microbiology and Immunology. Submitted.

Staats, Herman F., Jeffrey R. Fielhauer, Afton L. Thompson, Alice A. Tripp, Ashley Sobel, Massimo Maddaloni, Soman N. Abraham and David W. Pascual. Mucosal Targeting of a BoNT/A Subunit Vaccine Adjuvanted with a Mast Cell Activator 1 Enhances Induction of BoNT/A Neutralizing Antibodies in Rabbits. PLoS One 2011;6(1): e16532.

McGowen AL, Hale LP, Shelburne CP, Abraham SN, Staats HF. The mast cell activator compound 48/80 is safe and effective when used as an adjuvant for intradermal immunization with Bacillus anthracis protective antigen. Vaccine 2009;27(27):3544-52.

Rawson, P.D., A. McGowen, and C. Bartlett. “Temporal Variation in the Settlement of Blue Mussels (Mytilusedulis C. Linnaeus 1758 and M. trossulus Gould 1850) in Eastern Maine.” Journal of Shellfisheries Research, August 2004.