The STING Ligand 3’3’-cGAMP Effectively Elicits Mucosal and Systemic Immunity Following Sublingual Immunization

THESIS

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Abstract

Anthrax is caused by *Bacillus anthracis*, a zoonotic bacterial pathogen affecting humans and livestock worldwide. The currently available vaccine for humans, anthrax vaccine adsorbed (AVA), has a cumbersome immunization schedule and is recommended only for certain high risk adults. The development effective vaccines will depend on selection of appropriate routes of vaccination and appropriate adjuvants. Sublingual immunization is a form of mucosal immunization in which vaccines are applied topically to the floor of the oral cavity. Sublingual vaccines induce antibody production both systemically and at local mucosal sites adjacent to and distant from the sublingual tissues. Stimulator of Interferon Gamma Genes (STING) is a DNA sensor present on the endoplasmic reticulum of mammalian cells. STING has been found to be essential to Type I interferon responses to a variety of pathogens and STING ligands have been shown to act as adjuvants for intranasal and systemic vaccines. 3’3’ cyclic GMP-AMP (3’3’-cGAMP) is a STING ligand of bacterial origin. In this work, mice were immunized by sublingual application of protective antigen (PA) of *Bacillus anthracis* alone or combined with one of the following experimental adjuvants: cholera toxin (CT), CpG ODN (CpG), or 3’3’-cGAMP. 3’3’-cGAMP effectively elicited systemic IgG and IgA antibody production and these antibodies effectively protected against lethal toxin of *Bacillus anthracis*. 3’3’-cGAMP also induces secretory IgA (sIgA) production in the saliva and elicited balanced
Th1/Th2/Th17 responses. 3’3’-cGAMP significantly increased splenic and bone marrow antibody secreting cells. 3’3’-cGAMP is a promising adjuvant for sublingual immunization, though further studies elucidating both safety and efficacy are warranted.
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Chapter 1: Introduction

*Bacillus anthracis*

Anthrax is a bacterial disease of humans and livestock caused by *Bacillus anthracis* a gram positive, spore forming, non-motile, rod shaped, facultative anaerobe (1–3). Infection with *Bacillus anthracis* occurs following ingestion or inhalation of spores or following contact of spores with broken skin and clinical signs vary depending on the organ system affected (1–3). Anthrax spores are able to persist for decades in the environment due to their resistance to heat, cold, dessication, ultraviolet light, and gamma radiation (3). Humans may also be infected through contact with infected animals or their products, including wool and animal hides (3). Anthrax is found worldwide and is enzootic or endemic in Central and South America, southern and eastern Europe, Asia, Africa, the Caribbean, and the Middle East (3). In the United States, anthrax occurs sporadically in livestock and wildlife in Western and Great Plains states (3).

Though the majority of naturally occurring anthrax cases are cutaneous, highest mortality is associated with inhalational infection which accounts for 5% of historical cases in the United States and has a case fatality rate of up to 89% (1, 3). Inhalational infection initially causes flu-like symptoms followed by development of hypotension and pulmonary edema, and culminating in death (1–3). *Bacillus anthracis* has been used
historically and in modern times as an agent of bioterrorism and is classified as a category A bioterrorism agent because it is associated with a high risk to human health (1, 3). Historically, anthrax has been used as a bioweapon against livestock during World War I and World War II (3). Release of anthrax spores, whether intentionally, as in the United States in 2001, or accidentally, as in the Soviet city of Sverdlosk in 1979, is associated with high mortality (1, 3).

When anthrax spores are aerosolized, they are inhaled and settle in the respiratory tract based on size, with larger particles (≥ 5 microns) remaining in the upper respiratory tract while small particles (≤ 5 microns) are transmitted deeper into the lymph nodes of the lower respiratory tract (1, 3). Spores may remain dormant within tissues for weeks to months, but are eventually taken up by alveolar macrophages, at which time spores germinate (3). Systemic signs occur as spores within pulmonary lymph nodes release toxins, leading to hemorrhagic necrosis and mediastinitis followed by bacteremia and further dissemination (3). Clinical signs progress from flu-like symptoms including sore throat and muscle aches to respiratory signs such as dyspnea and cyanosis, followed by shock and hypotension as infections disseminate throughout the body, a process which can occur in as little as two days (3). Dormant spores are resistant to antimicrobial agents, which are the mainstay of treatment, and must be administered for long periods of time until spores have had a chance to germinate (3).
The major virulence factors of *B. anthracis* are the anti-phagocytic capsule γ-linked-poly-D-glutamic acid (γDGA) and the exotoxins lethal toxin (LeTx) and edema toxin (EdTx) (1, 3). These exotoxins consist of two components – a shared binding subunit, protective antigen (PA), and either the enzymatic subunit edema factor (EF) in the case of EdTx or lethal factor (LF) in the case of LeTx (1, 2). PA binds the anthrax toxin receptor, which leads to calcium dependent cleavage of PA by the enzyme furin, followed by formation of a heptameric prepore on the cell membrane (2–4). This prepore is eventually converted into a pore by endosomal acidification and it is through this pore that LF and EF enter the cytosol (4). EdTx causes edema by increasing host cyclic adenosine monophosphate (cAMP), leading to upregulation of the anthrax toxin receptor and disruption of interstitial fluid balance (3). LeTx inactivates mitogen-activated protein kinase kinases (MAPKK), leading to alterations in cytokine production and local tissue necrosis (3). PA is the major immunogen and, because it is a component of both LeTx and EdTx, vaccination against PA provides protection from both anthrax exotoxins (1, 2).

Because of its high risk to human health, anthrax is best controlled by prevention. Prevention strategies include industrial hygiene measures, restrictions on importation of high risk animal products such as wool from endemic areas, and vaccination of both humans and animals (3). Currently, the only available FDA licensed vaccine against anthrax in humans is the PA-based vaccine anthrax vaccine adsorbed (AVA), which has been available since 1970 (1–3). AVA is made up of cell free culture supernatant adsorbed to the adjuvant aluminum hydroxide (1, 2). Administration of this vaccine is
cumbersome as it requires a priming series of six subcutaneous or intramuscular immunizations over a period of 18 months followed by annual boosters (1–3). AVA is capable of inducing significant increases in anti-PA serum IgG titers and has been shown to effectively prevent inhalational anthrax in rhesus macaques and in workers in US goat hair processing mills (3). The duration of protection is unknown in humans, but evidence exists for one to three years of protective immunity and studies in rhesus macaques show protection for up to 4 years following three intramuscular doses of AVA (3). Though AVA is associated with development of protective systemic immunity, parenteral vaccines such as AVA are incapable of inducing immune responses in mucosal compartments. Immunization via a mucosal route could induce protective immunity in the form of secretory IgA (sIgA) directly in the respiratory tract, the site of exposure for inhalational Bacillus anthracis, while also providing adequate systemic immunity (1).

**Mucosal Immunization with a Focus on Sublingual Immunization**

**Mucosal immune system and mucosal immunity**

The mucosal surfaces of the body, including the gastrointestinal, urogenital, and respiratory tracts, are in direct contact with the environment and so act as the primary portals of entry for most pathogens (5–7). The mucosal immune system is functionally and anatomically independent from the systemic immune system and has evolved in mammals to confer protection from pathogens at these points of entry through a variety of mechanisms (5, 8–11). Bacteria that make up the normal flora live on mucosal surfaces
and outcompete and prevent colonization by pathogenic bacteria (5). The epithelial cells making up the mucosal surface provide both a direct barrier to entry of pathogens and contain goblet cells, which secrete mucus that forms a protective coating over the mucosal surface (5). Within the upper respiratory tract, ciliated epithelial cells help capture pathogens and move them away from the lungs via the mucociliary escalator (5). Antimicrobial compounds are secreted by specialized cells within the mucosal epithelium, such as Paneth cells within the gastrointestinal tract (GIT) (5). Homeostasis is also maintained by intraepithelial T cells and innate lymphoid cells (5).

Perhaps most importantly, sIgA at mucosal sites prevents colonization of pathogens, both by neutralizing soluble molecules such as microbial toxins and by preventing pathogens from adhering to mucosal surfaces (7, 9, 12). Class switching from IgM to IgA in mucosal B cells is dependent on the cytokines TGFβ and IL-10 (7). IgA may be membrane bound or secreted, but the majority of IgA is present as dimers or oligomers of sIgA in both humans and mice (12). sIgA is secreted by plasma cells and, because it is unable to activate the complement cascade, it neutralizes antibodies in a non-inflammatory manner (12). sIgA reaches mucosal surfaces via the polymeric Ig Receptor (PIgR) found on epithelial cells which binds IgM and IgA (5, 12). PIgR is a protein that transports IgA across the epithelial cell surface to the mucosal surface, where sIga can exert its effects (12). Following sublingual immunization, IgA responses may enhanced when neutrophil populations decrease because neutrophils negatively regulate B cell IgA
production (13). sIgA also contributes to the formation of a biofilm by the microbiota and
downregulates production of inflammatory epitopes by this normal flora (12).

**Routes/modes of delivery of mucosal vaccines**

Mucosal immunization routes include intranasal, sublingual, and oral immunization as
well as vaccines administered directly into the genital tract. Mucosal immunization
induces a variety of benefits not associated with parenteral immunization (7). Systemic
vaccines, such as those administered via intramuscular or subcutaneous injection, are
unable to elicit mucosal antibody responses, but do elicit systemic antibody responses
comprised primarily of IgG with little IgA (1, 7, 12). Mucosal vaccines, however, are
able to elicit both local mucosal responses mediated by sIgA and systemic immune
responses mediated by IgG at similar magnitudes to those induced by systemic vaccines
(1, 5, 7, 8, 10, 14, 15). Mucosal vaccines are capable of eliciting both humoral and cell
mediated immunity with long-term B and T cell memory responses (7). Antigen
presenting cells (APCs), primarily dendritic cells (DCs), are present beneath the mucosal
epithelium (5, 8). These APCs present antigens to B and T cells in inductive sites,
including Peyer’s patches in the intestinal tract or draining lymph nodes, such as the
cervical lymph nodes following uptake of antigen from the sublingual mucosa (16). In
these inductive sites, T and B cells acquire mucosal homing receptors or receptor ligands
which facilitate mucosal immune responses both at the site of antigen uptake and in
distant effector sites, with the strongest responses occurring adjacent to the vaccination
site (7).
Induction of tissue homing responses depends on the site of immunization and the antigen and adjuvants used. In general, intranasal vaccination is associated with responses in the respiratory, gastrointestinal, and genital tracts (7, 14). Sublingual immunization is capable of producing responses in the respiratory and gastrointestinal tracts, and oral immunization is associated with gastrointestinal, salivary gland, and mammary mucosal immune responses (7, 14). Rectal and intravaginal immunizations produce only local responses in the rectum and genital tracts, respectively. (7). Tissue homing is determined by chemokine receptor and integrin expression within the cell. These homing receptors or their ligands are acquired within inductive sites such as Nasal Associated Lymphoid Tissue (NALT) for intranasal immunization or cervical lymph nodes (CLNs) for sublingual immunization (5, 7). For example, α4β7 integrin attaches to mucosal addressin cell adhesion molecule 1 (MADCAM1) in the intestinal mucosa (7). This integrin may be expressed by IgA+ B cells and mucosal CD4+ and CD8+ T cells (7). Lymphocytes expressing the CCR9 integrin are attracted to CCL25 producing epithelium in the gut (7). CCR10 expressing B cells are attracted to CCL28, which is produced by mucosal cells in the gastrointestinal and genital tracts (7).

Besides their ability to elicit robust immune responses in both the mucosal and systemic compartments, there are practical and safety benefits associated with mucosal vaccination. Because these vaccines are administered without needles, they can be given by non-medical personnel and are not associated with spread of blood-borne pathogens.
(6, 7). They are also appropriate for mass vaccination and may have higher rates of patient compliance versus injectable vaccines (7). A variety of challenges are also associated with mucosal immunization. These include selection of adjuvants and routes that are safe and that effectively induce immune responses in appropriate effector sites. Each mucosal route has benefits and drawbacks which must be carefully considered during vaccine development.

Orally administered vaccines were some of the first mucosal vaccines developed and this route has led to successful protection against GIT pathogens such as cholera and rotavirus (7, 14, 15, 17). Oral vaccines are easily administered without specialized equipment. Vaccines via this route are associated with protection in the GIT, mammary glands, and salivary glands (7, 14). However, because of their frequent exposure to antigens, tolerance is readily elicited at mucosal surfaces and this is especially true in the GIT (7). Under normal conditions, this tolerance prevents inflammatory responses to innocuous antigens such as those associated with food or with the normal gut microflora (7). Careful selection of an appropriate adjuvant can help shift the immune response away from T regulatory (Treg) predominant responses associated with tolerance and towards effector responses (7). Another drawback of the oral route is degradation of antigens by the harsh gut environment, which means that oral vaccines must contain higher doses of antigen and adjuvant than those administered via other mucosal routes (7, 14, 15). Oral immunization with certain adjuvants, such as cholera toxin (CT), is also associated with diarrhea (7).
Successful intranasal vaccines have been developed in humans and animals, including commercially licensed vaccines against influenza and *Bordetella bronchiseptica* (7). Vaccination via the intranasal route elicits protection in the respiratory, gastrointestinal, and urogenital tracts (7, 18). Intranasal vaccination requires lower doses of antigen and adjuvant than oral immunization because antigen is not degraded as it is in the stomach (7, 8, 14, 19). However, intranasal immunization may require special delivery devices such as nebulizers (7, 8). Furthermore, use of bacterial toxins as adjuvants for the intranasal route is associated with redirection of antigen and adjuvant to the central nervous system via the olfactory nerves, leading to Bell’s palsy in human clinical trials (20–22).

Because of the side effects associated with oral and intranasal vaccines, other mucosal routes are being explored. Rectal and intravaginal vaccines are less favorable because of low compliance rates, but are being investigated as potential sites for immunization against genital pathogens including human immunodeficiency virus (HIV) (7, 23). Sublingual immunization has been successfully used to experimentally immunize mice and swine. This route presents many of the benefits of intranasal immunization with fewer safety concerns. Sublingual vaccines are applied topically to the sublingual mucosa on the floor of the oral cavity, and this route is well established for drug delivery in humans and animals. Examples include nitroglycerin for the treatment of angina in humans, anesthetic administration in cattle, horses, and dogs, and allergen specific
immune therapy (ASIT) in humans and dogs (9, 24–29). Unlike parenteral ASIT, sublingual ASIT is not associated with anaphylactic reactions and can be administered safely at home by patients or pet owners (8, 29).

The sublingual mucosa comprises the tissues in the floor of the oral cavity, ventral to the tongue and medial to the mandibles. It is made up of a pluristratified epithelium that overlies a thin lamina propria (8, 14, 30). In mice, capillaries, mononuclear cells, and fibroblasts are present within the lamina propria with additional mononuclear cells present beneath the lamina propria, but no organized lymphoid tissue is present (8, 14, 30). The majority of the antigen presenting cells (APCs) in the sublingual tissues are dendritic cells or macrophages that are MHC class II+ CD11b+, MHC class II+ CD11c+, or MHC class II+ F4/80+ (8, 14, 30). Langerins+ cells resembling epidermal Langerhans cells are also present (8, 14, 30). Following immunization with cholera toxin, these cells increase in population within two hours and return to baseline population by six hours (14). Following exposure to an antigen, APCs travel to their inductive sites in the CLNs, which include the mandibular, accessory mandibular, and superficial parotid lymph nodes in mice (16).

Because of this route’s utility in ASIT and drug administration, interest has increased in sublingual immunization in recent years (7, 8). Studies in mice show similar induction of mucosal responses to intranasal vaccines and similar magnitude and patterns of systemic responses to intramuscular immunization (7, 8, 10, 14, 15, 17). Comparison of oral,
pharyngeal, and sublingually delivered vaccines clarify that sublingual vaccines are absorbed directly through the oral mucosa rather than being swallowed with the same vaccine doses yielding inadequate immune responses in animals vaccinated via the oropharynx, but successful induction of antibody responses in animals immunized sublingually (14, 15, 17). Sublingual immunization effectively elicits mucosal IgA, systemic IgG, cytotoxic CD8+ T cell, and CD4+ T cell responses (7–10, 14, 17). Unlike intranasal immunization, no special equipment is necessary for administration of sublingual vaccines (8, 14). This route is also associated with fewer safety concerns, with no evidence of transport of antigen or adjuvant across the olfactory nerves and little IgE induction (7, 8, 15, 21, 31, 32). Adjuvants such as bacterial toxins that are associated with side effects during intranasal immunization may therefore be safe when administered via the sublingual route (14, 15, 33). Lower antigen doses may be used for the sublingual route versus the oral route because tolerance is induced less readily (8, 15, 17).

Responses to sublingual immunization may be seen both locally in the nasal passages and saliva and at distant sites, including the urogenital, respiratory, and gastrointestinal tracts (14). In mice, immune responses have been shown to be protective against human papilloma virus and *Chlamydia muridarum* following sublingual immunization (10, 15). Preliminary studies in swine show protection from influenza challenge and induction of antibodies against *Bacillus subtilis* following sublingual immunization (34, 35). In a small proof of concept study, women were immunized sublingually with a commercially available vaccine against human papilloma virus that is adjuvanted with the particulate
alum (36). Sublingual immunization with this vaccine was considered ineffective because it had few effects on serum IgG and no mucosal antibodies were produced; however, alum is a large particle that is not easily absorbed through the mucosal epithelium, which may explain the lack of response to immunization (36).

**Mucosal Adjuvants**

Appropriate adjuvant selection is critical to the development of safe, effective vaccines. Adjuvants enhance the magnitude, quality, and longevity of antigenic responses and can confer the ability to produce antibodies to multiple epitopes of the same antigen (epitope spread) (7, 19, 37). Adjuvants are especially important for antigens like PA of *Bacillus anthracis*, which may not be immunogenic enough to elicit protective immune responses alone, and for pathogens in which development of live attenuated vaccines is impossible or unsafe (1, 7, 19, 37, 38). Adjuvants can assist in the development of innate immune and T cell responses and can be used to polarize T helper cell responses, potentially yielding vaccines that are more effective for certain pathogens or in specific populations (7, 38). The ideal mucosal adjuvant should be: 1) dosage sparing, allowing for fewer vaccine doses to elicit an effective response, 2) dose sparing, with less antigen needed to elicit a response, and 3) should broaden the immune response (19, 37, 38). Because tolerance is easily elicited at mucosal sites to prevent harmful inflammatory responses to innocuous substances such as food or normal flora, mucosal adjuvants must be able to shift immune responses away from Treg predominant, tolerance promoting responses and
towards effector T cell responses (5, 7). Effective mucosal adjuvants must also be able to cross the mucosal barrier (7, 19, 39).

**Adjuvants for mucosal vaccines**

A variety of adjuvants exist, but only some are safe and effective enough for potential use in commercial sublingual vaccines. Pattern recognition receptors (PRRs) in the innate immune system recognize molecules common to a variety of pathogens, especially foreign genetic material, which acts as a signal of cell damage or of invasion by pathogens (38, 40, 41). PRRs include: 1) toll-like receptors (TLRs) which sense endogenous and exogenous RNA and DNA, 2) RNA helicase retinoic acid inducible gene I (RIG-I) which detects 5’ triphosphorylated RNA, and 3) melanoma differentiation associated gene 5 (MDA5), which detects double-stranded RNA molecules (dsRNAs) (19, 38, 40, 42, 43). Many adjuvants act as ligands of PRRs, including exogenous pathogen-associated molecular patterns (PAMPs) and endogenous damage-associated molecular patterns (DAMPs) (7, 38, 40). Potential mucosal adjuvants include bacterial enterotoxins such as cholera toxin (CT), toll-like receptor ligands, mucoadhesive molecules, particles, saponins, oil-in-water or water-in-oil emulsions, and cytokines (7, 38).

Aluminum salts (alum) include aluminum hydroxyphosphate, aluminum phosphate, and aluminum hydroxide based adjuvants (37, 38). These are approved for use in human vaccines (36–38). Alum is a particulate adjuvant and vaccine antigens may be adsorbed
onto alum particles (37). Alum recruits and is readily internalized by APCs and can facilitate activation of the innate immune system via DC activation (37). Alum had been thought to act in part by forming depots of antigen in the tissue, but this mechanism is no longer thought to be the primary way through which alum acts as an adjuvant (38). Alum elicits production of DAMPs, including dsDNA and uric acid, through its triggering of necrotic cell death (38). Parenteral vaccination with alum primarily leads to a mixed Th1 and Th2 response in humans (37, 38). Unfortunately, adjuvants made up of large particles such as alum may be inappropriate for sublingual immunization because of difficulty being absorbed through the sublingual mucosa. This is evidenced by the ineffectiveness of an alum-based vaccine to elicit effective immune responses against HPV in women when administered sublingually (36).

Bacterial toxins such as cholera toxin (CT) or the heat labile toxin I (LT-I) from *E. coli* act as powerful mucosal adjuvants and elicit strong, broad based immune responses. CT skews responses towards Th2, Th17, and cytotoxic T lymphocyte (CTL) responses, whereas heat labile toxin is associated with a mixed Th1 and Th2 response (6, 7). These adjuvants induce both mucosal and systemic IgA production and enhance antigen presentation by APCs (6, 11). These toxins are complexes composed of an A subunit which contains an A1 portion and an A2 chain connecting to a pentamer of B subunits (7). These B subunits bind gangliosides, especially GM1, expressed ubiquitously on the membrane of most nucleated cells, allowing uptake into the cell (7). ADP-ribosylation of membrane bound guanine nucleotide binding protein subunit GSα is mediated by the A1
portion, leading to increased cytoplasmic cAMP and downstream effects that promote balanced Th1, Th2, and Th17 immune responses (7, 44). However, these toxins are also associated with side effects such as diarrhea when administered orally and facial nerve paralysis when administered intranasally and these severe side effects make bacterial enterotoxins inappropriate for human use (6, 7, 20, 21). In an attempt to improve safety, enterotoxins have been engineered with mutations in the A1-A2 subunit (6, 7, 45). Despite its complete lack of enzymatic activity, LTK63, a detoxified version of heat-labile toxin, has been associated with Bell’s palsy (7, 21). The isolated B subunits of CT and heat-labile toxin have also been explored as adjuvants with improved safety profiles versus holotoxins. However, these subunit adjuvants may be less effective than holotoxins when administered via the oral route (6).

Because of their effectiveness as mucosal adjuvants, bacterial toxins are the most widely studied adjuvant for sublingual immunization. LT-I and LTK63 are associated with effective sIgA production in the salivary gland and genital tract of sublingually immunized mice, with increases in splenic IgM and IgA antibody secreting cells (ASCs), and increases in CD4+ and CD8+ T cells in the CLNs (15). The mutant enterotoxin LTK63 was less potent than LT (15). CT has also successfully been used as a sublingual adjuvant in a variety of mouse experiments. These models have shown successful induction of sIgA in the genital tract, respiratory tract, and salivary gland following immunization against HPV (10, 15). Sublingual immunization of mice against influenza using CT as an adjuvant led to induction of Th1 and Th17 responses, production of sIgA
in the respiratory tract, and successful protection against challenge (30, 46). Similar results were seen with sublingual immunization against *Helicobacter pylori* in mice, but sIgA was produced in the GIT (17). Sublingual immunization with bacterial toxin adjuvants has also been successful in swine. Yucatan miniature pigs immunized with CT against H1N1 had reduced viral shedding and fewer lung lesions on histopathology, though hemagglutination inhibition titers were not as high as those in animals vaccinated with a conventional intramuscular vaccine (34). Five-week old piglets immunized against tetanus toxoid adjuvanted with heat-labile toxin successfully produced neutralizing sIgA in the urogenital tract and saliva, IgG in the serum and saliva, and a balanced Th1 and Th2 response (33). Neonatal mice sublingually immunized with ovalbumin and CT produced a Th2 predominant immune response characterized by elevations in IgG2b, IL-4, IL-5, and IL-6 (32).

A variety of TLR agonists have been used experimentally as adjuvants. These are typically synthetic or bacterially derived PAMPs (7, 38, 42). Promising adjuvants include TLR3 ligands such as the double stranded RNA (dsRNA) Poly I:C which induces DC activation and production of IL-12 and IFN-γ through activation of the RIG-I-like receptor (RLR) MDA5 and through TLR3 binding (38, 40, 47, 48). These ligands stimulate type I interferon (IFNβ) production, which activates DCs and natural killer (NK) cells, and has effects on T cells leading to enhanced T and B cell supported immunity, including increased magnitude of Th1 and CD8+ T cell responses (38, 40).
Another promising group of adjuvants that act as TLR ligands are oligodeoxynucleotides containing CpG motifs (CpG ODNs) (38, 49). These dinucleotides contain unmethylated CG and are rare in eukaryotic DNA but commonly seen in prokaryotic DNA (42). These PAMPs act as ligands for TLR9, which is located within the endolysosomal compartment of a variety of cells (42, 43). TLR9 signals through MyD88, toll-like interleukin-1-resistance (TIR) domain-containing adaptor-inducing interferon β (TRIF) adaptor molecules and lead to the induction of NF-κβ (7, 37, 42, 49). These molecules may be synthetic or of bacterial origin and strongly polarize towards Th1 responses (6, 38). TLR9 is located primarily on plasmacytoid DCs (pDCs) and B cells in humans, but is also expressed on myeloid cells in mice (38, 42). TLR-9 stimulation leads to production of cytokines IL-6, IL-12, and CXCR3, the antibody IgM, and production of type I IFN (42). CpG ODNs are associated with increased mucosal immune responses following intranasal or intravaginal immunization against herpes simplex virus in mice and following oral immunization of mice against salmonella (42). In orally immunized piglets, CpG was associated with increased pseudorabies specific serum IgG and mucosal IgA titers (42). In neonatal mice, sublingual immunization with CpG and OVA led to enhanced IgG2a responses and increased splenic IFN-γ (32). CpG ODNs are FDA approved for use in humans and act as successful adjuvants in a variety of populations (19, 37, 42). CpG ODNs are generally considered safe when used as vaccine adjuvants; however, their use has been associated with induction of autoimmune myocarditis and arthritis in mice (42). One participant in a human clinical trial of a hepatitis B vaccine using CpG ODN as an adjuvant developed Wegener’s granulomatosis, an autoimmune
inflammation of the vasculature (42). CpG ODNs have also been associated with elevations in local adverse events and flu-like symptoms in human clinical vaccine trials (42).

**STING ligands as new molecular adjuvant for mucosal vaccines**

The need for safe, effective adjuvants that stimulate mucosal immune responses has led to the examination of novel compounds and pathways. An example of this is Stimulator of interferon gamma genes (STING), a transmembrane protein that lies on the endoplasmic reticulum of macrophages, dendritic cells, and fibroblasts (19, 50). In vertebrates, this protein senses cytosolic cyclic dsDNA (CDNs) including both PAMPs and DAMPs leading to the activation of Ikβ kinase (IKK) and TANK-binding kinase 1 (TBK1), which then activate transcription factors of interferon regulatory factor 3 (IRF3) and NF-κβ, leading to production of IFNβ and pro-inflammatory cytokines (19, 41, 47, 48, 50, 51). This ability is TLR independent (19, 50). CDNs work as bacterial second messengers and assist with motility and development in a variety of bacterial species (19, 50). STING is able to directly detect prokaryotic CDNs and to detect dsDNA indirectly via the actions of cyclic GMP-AMP synthase (cGAS), which is essential for cellular responses to a variety of pathogens including retroviruses such as HIV (19, 43, 47, 50, 52). cGAS binds dsDNA regardless of sequence and can also bind synthetic DNA:RNA hybrids (43, 47, 50, 53). This binding leads to dimerization of cGAS and produces the second messenger 2’3’ cyclic GMP-AMP (2’3’cGAMP), which then binds to STING (43,
cGAS is essential for the activation of IRF3 and induction of IFNβ by cytosolic DNA (47, 50).

A variety of CDNs are available for use as potential adjuvants. Bis-(3’5’)-cyclic dimeric guanosine monophosphate (c-di-GMP) is a PAMP produced by a variety of bacteria including *Pseudomonas aeruginosa*, where it assists in biofilm formation, and by the protozoa *Dictyostelium*, where c-di-GMP assists with motility and proliferation (39, 41). In cultured murine and human DCs, c-di-GMP induces a broad array of cytokines and chemokines (39). When used as an adjuvant in systemic vaccines against MRSA, c-di-GMP is associated with more potent induction of humoral immunity than alum (19). C-di-GMP is associated with a balanced immune response when used as a mucosal adjuvant, including induction of local and distant sIgA in the respiratory and genital tracts and Th1 immune responses (19). C-di-GMP significantly boosts mucosal immune responses in the saliva and respiratory tract and induces Th1 responses following intranasal or sublingual immunization using H1N1 virosomes (19). Intranasal immunization with c-di-GMP in mice leads to induction of increased NK, NK T cell, B cell, and T cell responses in the lungs and reduced colonization with *Klebsiella pneumonia* and *Streptococcus pneumonia* following challenge (19). Immunization with c-di-GMP in ferrets as an adjuvant for H5N1 yields comparable protective responses as intramuscular immunization with TLR4 agonists (19). Intranasal immunization with c-di-GMP in mice leads to enhanced antigen uptake by APCs and epithelial cells *in vivo*, by altering the cytokine milieu and enhancing pinocytosis (55).
Bis-(3’5’)-cyclic dimeric adenosine monophosphate (c-di-AMP) and bis-(3’5’)-cyclic dimeric inosine monophosphate (c-di-IMP) are STING ligand PAMPs that have also been used in experimental immunization models (19, 39, 50). c-di-IMP induces a similar immune response to that produced by c-di-GMP (19). c-di-AMP is produced by *Listeria monocytogenes* and *Chlamydia trachomatis* and has immune modulatory effects (19, 39, 50). *In vitro*, c-di-AMP is more potent at activating murine DCs and macrophages than c-di-GMP, but this effect is not seen *in vivo* (19). C-di-AMP is associated with a balanced immune response when used as an intranasal adjuvant in mice, where it promotes Th1, Th2, and Th17 induction (19, 39).

Though c-di-AMP, c-di-GMP, and c-di-IMP are successful adjuvants in animal and *in vitro* experiments, cGAMP has a greater binding affinity for STING, inducing higher production of IFNβ and activation of IRF3 (41, 50, 56–58). 3’3’-cGAMP, or canonical cGAMP, is produced by prokaryotes and is able to activate both human PBMC-derived DCs and murine bone marrow-derived DCs *in vitro* (39, 58). 3’3’-cGAMP promotes a balanced immune response when used as an intranasal vaccine adjuvant with OVA (39). This includes enhanced IFN-γ, IL-2, IL-4, and IL-17, indicative of Th1, Th2, and Th17 responses, and production of sIgA in the respiratory tract (39). 3’3’-cGAMP produces a Th17 response of a lower magnitude than that induced by immunization with c-di-AMP following intranasal immunization, which may be associated with fewer of the adverse effects, like arthritis, associated with IL-17 in murine studies (39).
2’3’cGAMP is synthesized by mammalian cGAS and is able to diffuse through gap junctions to activate cells adjacent to this in which it is produced (41, 43, 59).

2’3’cGAMP induces enhanced antigen-specific IgG1 production and T cell activity, and it has a higher affinity for STING than 3’3’-cGAMP (39, 54). 2’3’cGAMP successfully induces OVA-specific antibodies and CD8+ T cells following intramuscular immunization in mice (47). Though 2’3’cGAMP has a higher binding affinity for STING than 3’3’-cGAMP, both induce comparable induction of IFNβ (56). This may be due to the presence of specific phosphodiesterases that degrade 2’3’cGAMP, leading to increased turnover within the cell (56). Single nucleotide polymorphisms among human STING alleles occurs in 1 – 20% of the population and some haplotypes have dramatically reduced responses to all CDNs except 2’3’cGAMP (60). Human STING is also refractory to the STING ligand 5,6-dimethylxanthenone-4-acetic acid (DMXAA), which has potent antitumor effects in mouse models but has been ineffective in human clinical trials (57).

**Study Aims**

In this study, we examined whether the STING ligand 3’3’-cGAMP is an effective adjuvant when delivered via sublingual immunization (Figure 1.) We compared 3’3’-cGAMP to CT, a bacterial enterotoxin that is known to be an effective mucosal adjuvant but that is too toxic for clinical use, and to CpG, a TLR9 agonist. To assess efficacy, we examined systemic and mucosal antibody production, induction of antibody secreting
cells in the spleen and bone marrow, and cytokine production. We also examined local responses in the tongue and sublingual tissues and effector site responses in the cervical lymph nodes, and performed a toxicity assay to ascertain the protective efficacy of antibodies produced. Finally, we analyzed the expression of gene mRNA to help elucidate the mechanism by which 3’3’-cGAMP may act as an adjuvant.

Figure 1: Schematic of hypothesis.

Figure 1: The cyclic dinucleotide and STING ligand 3’3’-cGAMP will successfully act as an adjuvant following sublingual immunization, leading to similar induction of serum and mucosal antibodies as the cyclic nucleotide cAMP, which is induced by cholera toxin administration.
Chapter 2: Materials and Methods

Animals

Female C57BL/6J mice (The Jackson Labs, Bar Harbor, ME) aged 9 – 12 weeks and specific pathogen free for Sendai virus, mouse hepatitis virus, minute mouse virus, mouse parvovirus, Theiler murine encephalitis virus, rotavirus, *Mycoplasma pulmonis*, and endo- and ectoparasites were used for all experiments. Mice were group housed in individually ventilated polycarbonate cages (Allentown Inc, Allentown NJ) with *ad libitum* access to triple filtered water and Teklad Irradiated Diet 7912 (Harlan Laboratories, Madison, WI). All procedures involving animals were approved by The Ohio State University’s Institutional Animal Care and Use Committee.

Sublingual Immunization

Mice were anesthetized via intraperitoneal injection of ketamine and xylazine diluted in sterile saline to reach a deep plane of anesthesia as assessed by toe pinch. Animals were placed in dorsal recumbency and immunized via sublingual application of 10µL phosphate buffered saline (PBS) containing either 10µg protective antigen of *Bacillus anthracis* (PA, BEI Resources, Manassas, VA), 10µg PA and 2µg cholera toxin (CT, List Biological Laboratories, Campbell, CA), or 10µg PA and 10µg CpG ODN (CpG). For the STING ligand group, animals were immunized sublingually with 10µg PA and 10µg
3’3’-cGAMP (InvivoGen, San Diego, CA) for a total volume of 13µL, due to the concentration of 3’3’-cGAMP.

**Histologic Analysis of Sublingual Tissue**

For analysis of the immune response in the sublingual tissue and tongue, three animals per group were immunized once and euthanized two hours later via inhalational overdose of CO₂. Tissue collection involved removal of the mandible with attached soft tissues, decalcification with TBD decalcifier, and fixation of tissues in formalin. Tissues were cut into sagittal sections and stained using hematoxylin and eosin. Aperio Imagescope (Leica Biosystems Inc, Buffalo Grove, IL) was used for image capture and to measure the width of the epithelium of the sublingual tissues and the underlying lamina propria. The sublingual mucosa was divided into thirds by length and the ratio of the epithelium to the lamina propria was calculated every 100 µm and averaged for each third of the sublingual tissue.

**Flow Cytometric Analysis of Sublingual Tissue, Tongue, and Cervical Lymph Nodes**

Analyses of cell populations in the sublingual tissues and tongue were performed on three animals per group via flow cytometry two hours after a single immunization. The tongue was sharply dissected from the sublingual tissue at its base and the sublingual tissues were removed from the mandible via sharp dissection. Tissues were macerated with stainless steel operating scissors, then placed in a 0.5 g/mL solution of collagenase in RPMI 1640 medium containing 10 mM Hepes, 2 mM L-glutamine, 100 U/mL penicillin,
100 µg/mL streptomycin, 2 x 10^{-5} M 2-ME, 1 mM sodium pyruvate, and 10% fetal bovine serum (cRPMI) and incubated on a magnetic stirrer set to 1200 RPM at 37°C for 40 minutes. The resulting cell suspension was passed through a 70 µm nylon mesh filter, washed, and resuspended in FACS buffer solution (1x PBS, 1% BSA, and 0.01% NaN₃). Flow cytometry analysis was performed after extracellular staining with the following fluorophore-conjugated monoclonal antibodies: B220, CD11b (Miltenyi Biotec, Auburn, CA), Gr-1, F4/80 (Abd Serotec, Raleigh, NC), Ly6G, c-kit (Biolegend, san Diego, CA), CD19, CD3e (BD Biosciences, San Jose, CA). Labeled cells were analyzed using an Accuri C6 flow cytometer (BD biosciences, San Jose, CA).

Analysis of cell populations within the cervical lymph nodes (CLNs) was performed 4 hours following a single immunization. CLNs were removed via sharp dissection, pressed through a 70 µm nylon mesh filter, washed, and suspended in FACS buffer solution. Flow cytometry analysis was performed using an Accuri C6 flow cytometer after extracellular staining using the following fluorophore-conjugated monoclonal antibodies: IgG, IgA (Southern Biotech, Birmingham, AL), GL7, α4β7 (LPAM) (BD Biosciences, San Jose, CA), CCR9 (eBiosciences, San Diego, CA), and B220 (Miltenyi Biotec, Auburn, CA).
ELISA and ELISPOT Assays for PA-Specific Ab responses and Antibody Secreting Cells

Six animals per group were immunized at weekly intervals for three consecutive weeks (days 0, 7, and 14.) Blood samples were collected weekly via submandibular venipuncture prior to immunization. Vaginal wash samples were collected from anesthetized mice by instilling and gently aspirating PBS into the vaginal canal. Feces were collected at weekly intervals. Saliva was collected once on day 28, via pipette directly from the mouths of animals following IP administration of 0.025 µg pilocarpine. Saliva, vaginal wash, and blood samples were centrifuged for 5 minutes at 10,000 RPM and the supernatant was collected and stored at -80°C until analysis was performed. Fecal samples were suspended in 0.01% NaN3 and agitated for 20 minutes, followed by centrifugation and storage of supernatant as described for wash samples.

Animals were euthanized on day 35 for collection of tissues. The spleen was macerated, pressed through a 70-µm nylon mesh filter, then incubated for 5 minutes in ammonium-chloride-potassium lysing buffer solution (ACK). Cells were then washed and resuspended in cRPMI. Bone marrow was harvested from the femur and tibia by removing soft tissues and epiphyses followed by flushing the marrow canal with sterile PBS. Bone marrow cell suspensions were incubated in ACK buffer followed by cRPMI as for splenocytes. Nasal washes were collected by rinsing the nasal cavity with 1 mL of sterile PBS. Bronchoalveolar lavage (BAL) samples were collected by inserting a 22-Ga over the needle catheter into the trachea and gently instilling then aspirating 1.0 mL of
sterile PBS. Nasal wash and BAL samples were then processed and stored as for previous secretion samples.

Antigen-specific antibody levels in plasma and external secretions were assessed on microtiter plates coated with 5 µg/mL PA as previously described (13, 61). IgM, IgG, and IgA antibodies were detected using HRP-conjugated goat anti-mouse µ-, γ-, or α-H chain specific antisera (Southern Biotech, Birmingham, AL). IgG subclass responses were measured using biotin-conjugated rat anti-mouse IgG1, IgG2a, IgG2b, and IgG3 mAbs and HRP-conjugated streptavidin (BD Biosciences, San Jose, CA). Color was developed using ABTS substrate (Amresco Inc, Solon, OH) and absorbance was measured at 405 nm, for 0.1 s on a Perkin Elmer Victor 3V plate reader (Perkin Elmer Inc, Waltham, MA). Titers were expressed as the log₂ dilution yielding an OD₄₀₅ ≥ 0.1 above those obtained with non-immunized control samples.

The frequency of antibody secreting cells (ASCs) were detected using ELISPOT. Spleen and CLN samples were processed as previously described and suspended in cRPMI. 96-well nitrocellulose plates were coated with 5µg/mL of PA in cRPMI and incubated overnight at 37°C in a 5% CO₂ atmosphere. Spleen and CLN samples were added to plates at a rate of 1 x 10⁶ cells per well. Detection of ASCs were detected using goat anti-mouse µ-, γ-, or α-H chain specific antisera. Spots were visualized using TMB liquid horseradish peroxidase substrate (Moss Inc, Pasadena, MD). Spots were counted manually using light microscopy.
**Cytokine Analysis**

To analyze cytokine production following cell stimulation, CLN cells and splenocytes from animals immunized three times were collected and processed as previously described. 3 x 10^6 cells/mL were placed into a 96-well microtiter plate in cRPMI alone or with 10 µg/mL PA to stimulate cytokine production. Cells were cultured for 5 days at 37°C in a 5% CO₂ atmosphere. Supernatants were collected from each well and frozen at -80°C until analysis was performed. Cytokines were analyzed using the Bio-Plex Pro Mouse Cytokine Standard 23-Plex, Group I (Bio-Rad Laboratories Inc, Hercules, CA) per manufacturer’s instructions.

**Toxin Neutralization Assay**

Blood was collected at euthanasia (D35) from animals immunized three times as for ELISA analysis (n = 3 mice per group). Serum was collected from clotted blood and stored at -80°C until use. Raw 264 macrophages were cultured for 4 days at 37°C in a 5% CO₂ atmosphere in RPMI followed by transfer of cells to 96-well plates. Serum samples from each mouse were then added to the monolayer of macrophages in increasing concentrations and serum and cells were co-cultured with PA (0.5 µg/mL) and lethal factor (LF, 0.5 µg/mL, List Biological Laboratories, Inc, Campbell, CA). overnight. Macrophage viability was observed through addition of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenylterrazolium bromide (MTT, Sigma-Aldrich, St. Louis, MO), which accumulates within cells with functioning mitochondria and stains them purple. Toxin neutralizing
antibody titers were assessed by observing the lowest concentration of serum that protected macrophages from death caused by LeTx, as evidenced by MTT staining.

**Quantitative RT-PCR**

For *in vitro* analysis of type I interferon induction by 3’3’-cGAMP, CLNs and sublingual tissues were collected from two naïve mice. Cells were plated at 1 x 10^6 cells/mL/well in a 12 well culture plate and cultured overnight at 37°C in a 5% CO₂ atmosphere in crPMI alone or stimulated with 5µg/mL 3’3’-cGAMP or 5µg/mL poly I:C (InVivogen, San Diego, CA). Cells were then collected and RNA extraction was performed by incubating cells in chloroform followed by generation of cDNA using Superscript II reverse transcriptase (ThermoFisher Scientific, Waltham, MA). Real-time PCR was performed using a SYBR green system.

For analysis of type I interferon induction *in vivo*, three mice per group were immunized sublingually with either PBS, 10µg PA and 10µg 3’3’-cGAMP, or 10µg PA and 10µg poly I:C. Animals were euthanized 4 hours after immunization and the CLN and sublingual tissues were collected for real-time PCR as for *in vitro* CLN and sublingual tissue analysis.

**Statistical Analysis**

Results are expressed as mean with error bars indicating one standard deviation. Statistical significance was determined by ANOVA followed by Dunnet’s test for
significance versus the PA group. For multiplex analysis, Tukeys test was performed to assess significance. Tests were considered significant at probability of $p < 0.05$.

Statistical tests were performed using GraphPad Prism 6 (GraphPad Software Inc, La Jolla, CA).
Chapter 3: Results

Antigen-specific serum antibody responses induced by 3’3’-cGAMP as a sublingual adjuvant

To assess whether 3’3’-cGAMP is an effective adjuvant when delivered sublingually, we first analyzed serum antibody responses. Serum collected from mice immunized once weekly for three weeks (D0, D7, D14) with PA, PA and CT, PA and CpG, or PA and 3’3’-cGAMP was analyzed via ELISA for the production of IgG and IgA in the serum (Figure 2A.) Serum IgG subclasses were also analyzed to determine whether antibody responses indicated preferential Th1 or Th2 responses (Figure 2B.) To further quantify this, a ratio of IgG2a, which is associated with Th1 responses, and IgG1, which is associated with Th2 responses, was calculated (Figure 2C.) Serum IgG and IgA responses were significantly increased for all adjuvant groups, indicating that 3’3’-cGAMP is an effective adjuvant for sublingual immunization. The serum IgG subclass profile following immunization with 3’3’-cGAMP was similar to that induced by CT, with elevations in IgG1, IgG2a, and IgG2b. This profile is indicative of a balanced Th1 and Th2 response. This was further confirmed by the ratio of IgG2a to IgG1, which approached one, indicating nearly equal Th1 and Th2 responses.
Figure 2: Sublingual immunization with 3’3’-cGAMP induces systemic PA-specific antibody titers of comparable character and magnitude to CT. Mice were immunized sublingually once per week for three weeks (D0, D7, D14) with unadjuvanted PA, PA+CT, PA+CpG, or PA+3’3’-cGAMP. Serum antibody responses were significantly increased for all adjuvanted groups with comparable systemic IgG and IgA elevations for all adjuvants studied. (A) Serum IgG subclass profiles for CT and 3’3’-cGAMP were of a comparable character and magnitude (B). 3’3’-cGAMP induces a mixed Th1/Th2 response as evidenced by
similar elevations in both IgG2a and IgG1. This is further supported by analysis of the ratio of IgG2a, which is associated with Th1 responses, to IgG1, which is associated with Th2 (C). The ratio of IgG2a:IgG1 approached 1.0 in 3'3'-cGAMP immunized mice, indicating a mixed T helper cell profile. n= 6 mice per group, data expressed as mean titer + SD. Asterisks indicate statistically significant differences from the non-adjuvanted group (p ≤ 0.05, Dunnett’s test.)

To show potential for induction of memory B cell responses, we next analyzed PA-specific antibody secreting cells (ASCs) in the bone marrow (Figure 3). Splenocytes and bone marrow cells from animals immunized three times as previously described were analyzed via ELISPOT for IgG and IgM ASC induction. 3’3’-cGAMP failed to induce significant increases in IgG ASCs in the bone marrow or spleen. However, only 3’3’-cGAMP induced significant increases in IgA ASCs in the spleen and bone marrow, indicating that 3’3’-cGAMP has the potential to induce memory IgA responses following sublingual immunization and that it may be more effective at inducing these responses than CT.
Figure 3: 3’3’-cGAMP induces significant increases in IgA antibody secreting cells in the spleen and bone marrow. Samples of the spleen and bone marrow of animals immunized weekly three times (D0,D7, D14) were analyzed via ELISPOT for IgG and IgM ASC induction. Sublingual immunization with CT led to significantly increased IgG producing ASCs in the spleen and bone marrow (A). Only immunization with 3’3’-cGAMP led to significantly increased IgA producing ASCs in spleen and bone marrow (B). n= 3 mice per group, data
expressed as mean titer + SD. Asterisks indicate statistically significant differences from the non-adjuvanted group (p $< 0.05$, Dunnett’s test.)

**3’3’-cGAMP as a sublingual adjuvant promotes antigen-specific mucosal sIgA**

*antibody responses*

After establishing 3’3’-cGAMP’s effectiveness as an adjuvant, we next examined its effectiveness for inducing mucosal antibody responses. sIgA was measured via ELISA in the feces, vaginal wash, saliva, and BALs of mice immunized three times with PA alone or in combination with CT, CpG, or 3’3’-cGAMP. 3’3’-cGAMP successfully induced sIgA production in the saliva at a magnitude comparable to that induced by CT and CpG, indicating success as a mucosal adjuvant in sites local to the sublingual tissue (Figure 4.) No adjuvant induced increases in sIgA in the BAL or nasal wash (data not shown) following sublingual immunization, which may be indicative of failure to induce mucosal antibody responses in the upper or lower respiratory tracts. All adjuvants failed to induce significant increases in sIgA in the vaginal wash, indicating that sublingual immunization with PA and the adjuvants CT, CpG, or 3’3’-cGAMP may not effectively induce mucosal homing receptors to the urogenital tract. However, anthrax is not typically associated with infections in the genital tract and so may not be the ideal antigen for investigating mucosal antibody responses in this location. Only CT induced a trend towards increased sIgA in the feces (p = 0.0597), indicating that neither CpG nor 3’3’-cGAMP seem to induce homing of B cells to the GIT.
Figure 4: 3’3’-cGAMP induces sIgA production preferentially in the saliva.

Figure 4: 3’3’-cGAMP induces mucosal PA-specific antibody responses preferentially in the saliva. Secretory IgA titers in the feces, vaginal wash, saliva, and BAL were collected following three sublingual immunizations at weekly
intervals (D0, D7, D14). 3’3’-cGAMP induces significant increases in sIgA versus non-adjuvanted controls in the saliva only. No adjuvants tested induced significant increases in fecal sIgA, but feces in the PA+CT immunized group had a trend toward higher sIgA (p = 0.0597). n= 5 - 7 mice per group, data expressed as mean titer + SD. Asterisks indicate statistically significant differences from the non-adjuvanted group (p < 0.05 per Dunnett’s test.) For nasal wash samples, no adjuvanted group significantly increased production of sIgA over control non-adjuvanted samples (data not shown.)

3’3’-cGAMP as a sublingual adjuvant promotes mixed T helper cytokine response

To analyze cytokine responses, cervical lymph nodes and spleens from mice immunized three times as previously described were harvested at euthanasia on D35 and cells were cultured with or without restimulation with PA. Culture supernatants were analyzed via cytokine multiplex assay for the presence of a variety of cytokines (Figure 5.) Immunization with 3’3’-cGAMP induced production of the Th1 cytokines IL-12 and IFN-γ (Figure 5A.) These Th1 cytokines are associated with protection against intracellular pathogens. 3’3’-cGAMP induced a unique profile of Th2 cytokines as compared to CT (Figure 5B – C.) 3’3’-cGAMP failed to induce significant elevations in the proinflammatory Th2 cytokines IL-4 and IL-13, which promote antibody class switching to IgE and mucous production. However, 3’3’-cGAMP did induce Th2 related cytokines, especially IL-6, which is associated with B cell maturation, and IL-10, which is an anti-inflammatory Th2 cytokine associated with immune regulatory effects.
Production of the Th-17 associated cytokine IL-17 was also measured and 3’3’-cGAMP also induced significant increases in this cytokine from PA-restimulated cells. This may be an important component of the effective local sIgA production seen following immunization with 3’3’-cGAMP as IL-17 is associated with supporting mucosal antibody responses. Finally, 3’3’-cGAMP also induced production of the pro-inflammatory cytokines IL-1β, CCL5, GM-CSF, and IL-3. These cytokines stimulate innate immune responses, including responses that support mucosal immunity and the production of sIgA (62, 63). Taken together, these cytokine profile results indicate that 3’3’-cGAMP induces a balanced Th1 and Th17 profile with a unique Th2 profile and that it induces cytokines necessary for stimulation of APCs.

Figure 5: 3’3’-cGAMP induces a mixed Th1, Th2, and Th17 cytokine response, with a unique Th2 cytokine profile.
Figure 5 (continued)
Figure 5: 3’3’-cGAMP induces mixed Th1, Th2, and Th17 response with a unique profile of Th2 related cytokines. CLNs and splenocytes from mice
immunized once weekly for three consecutive weeks (D0, D7, D14) were cultured for five days with or without re-stimulation with PA. Culture supernatants were then collected and analyzed via multiplex analysis for cytokine and chemokine production. Immunization with 3’3’-cGAMP induces the Th1 cytokine IL-12 in a comparable manner to CT and induces a more robust IFN-γ response than CT or CpG (A). 3’3’-cGAMP immunization also induces the Th2 cytokine IL-6, but does not induce the Th2 cytokines IL-4, IL-5, IL-13, and IL-9 which are associated with the development of allergic responses and apoptosis (B). 3’3’-cGAMP induces production of the anti-inflammatory cytokine IL-10 (C), but does not induce production of the Th17 cytokine IL-17 (D). 3’3’-cGAMP also induces production of proinflammatory cytokines IL-1α, RANTES, GM-CSF, and IL-13 (E). This pattern of cytokine responses is similar to that induced by CT. n= 6 mice per group, data expressed as mean titer ± SD. Asterisks indicate statistically significant differences from the samples that were not restimulated with PA(p ≤ 0.05, Tukeys test.)

**Effects of sublingual application of 3’3’-cGAMP on sublingual and tongue tissues**

Previous research has shown that populations of APCs including neutrophils and dendritic cells in the sublingual tissues increase within two to three hours of sublingual immunization, followed by a return to basal levels within six hours (13, 14). We analyzed changes in immune cell populations in the sublingual tissue and tongue two hours following a single sublingual immunization using histology and flow cytometry (Figure
6.) Immunization with 3’3’-cGAMP did not lead to obvious changes in morphology or influxes of immune cells in the sublingual tissues or tongue following immunization with CT or 3’3’-cGAMP (Figure 6A). Immunization with CpG led to obvious thickening of the sublingual epithelium. To further quantify this thickening, a ratio of the width of the epithelial layer compared to the width of the lamina propria was calculated by measuring these layers every 100 μm and the mean of each value was calculated for the rostral, middle, and caudal thirds of the tongue (Figure 6B.) This further confirmed the gross observation of epithelial thickening in the CpG immunized mice and showed that this thickening began in the middle portion of the sublingual tissue and extended rostrally to the tip of the sublingual tissues lying closest to the lingual surface of the incisors. This could indicate local inflammation associated with topical application of CpG to the sublingual tissues.

Flow cytometry was also performed on the sublingual tissues and tongue of mice two hours after a single sublingual immunization (Figure 7 A and B.) These results showed no significant changes in absolute or relative cell populations following immunization with 3’3’-cGAMP except for a small but significant increase in the relative percentage of mast cells in the tongue. Previous reports have shown that the sublingual mucosa contains a large population of dendritic cells (14), but the data from this experiment showed that the highest population of leukocytes in the sublingual tissue and tongue were mast cells, CD19+ B cells, and, in the case of mice immunized with 3’3’-cGAMP, T cells. When considered with the histologic data, this shows that 3’3’-cGAMP does not induce
significant inflammatory infiltrates in the sublingual tissue or tongue following sublingual immunization.

Figure 6: Sublingual immunization with 3'3'-cGAMP does not induce histologic changes in the sublingual tissues and tongue.
Figure 6: Sublingual immunization with CT or 3’3’-cGAMP does not induce histologic changes or changes in immune cell populations in the sublingual tissues. Previous research has shown that an influx of myeloid cells occurs within 3 hours of sublingual immunization and that this influx of cells downregulates sIgA(13). Histologic analysis of sublingual tissues 2 hours following a single immunization showed no obvious changes in morphology between nonadjuvanted, CT, and 3’3’-cGAMP groups (A). However, animals immunized with CpG had thickening of the sublingual epithelium. A ratio of epithelial width to width of the lamina propria indicated that this thickening occurred primarily in the middle and rostral third of the sublingual tissues (B). n = 2 mice per group for
histology, images shown are at low power and representative locations in middle third of sublingual tissue at 20x magnification.

Figure 7: Sublingual immunization with 3'3'-cGAMP does not induce changes in the cell populations of the sublingual tissues and tongue.

A. Sublingual Tissues

Continued
**Figure 7:** Flow cytometry analysis of cell populations in the tongue and sublingual tissues showed no significant changes in the absolute or relative frequencies of cell populations in the sublingual tissues for any group versus control (n = 3, Dunnett’s test.) Analysis of immune cell populations in the tongue showed a decreased absolute number of neutrophils in animals immunized with CT (B). This data also showed that the most immune abundant cells in the sublingual tissue and tongue are ckit+ mast cells, CD19+ B cells, and CD3ε+ T cells. Cytometry data expressed as mean titer + SD. Asterisks indicate statistically significant differences from the non-adjuvanted control (p≤0.05, Dunnett’s test.)
We next considered the effects of 3’3’-cGAMP on immune cell populations in the CLNs, which are the effector site following sublingual immunization. Flow cytometry was performed on CLNs 4 hours following a single sublingual immunization (Figure 8). 3’3’-cGAMP did not induce increases in the total number of B cells or in overall populations of IgG or IgA positive B cells in the cervical lymph nodes (Figure 8A). Corresponding with the fecal ELISA results, 3’3’-cGAMP also did not significantly increase gut homing B cell populations in the CLNs, though CT was associated with increases in CCR9+ gut homing B cells (Figure 8B.). 3’3’-cGAMP did not contribute to germinal center formation, but increases were seen in non-germinal center associated IgG+ B cells (Figure 8C.) Increases were also not seen in differentiated B cells following sublingual immunization with 3’3’-cGAMP (Figure 8D.)
Figure 8: Sublingual immunization with 3’3’-cGAMP does not induce germinal center formation or gut homing receptor induction in the cervical lymph nodes. CLNs were harvested 4 hours following a single sublingual immunization. Increases in total and IgG+ B cell populations were seen only in the CT group (A) and increases in gut homing receptors were seen only for animals immunized with CT (B). A trend towards increased germinal center IgG+ B cells was seen for animals immunized with CT. In contrast, animals immunized with CpG and 3’3’-cGAMP had increased numbers of IGG+ B cells that were not present in germinal centers (C). No adjuvant led to a statistically significant increase in differentiated IgG+ or IgA+ B cells (D). n= 3 mice per group, data expressed as mean titer +
SD. Asterisks indicate statistically significant differences from the non-adjuvanted group (p ≤ 0.05, Dunnet’s test.)

*Serum antibodies induced by 3’3’-cGAMP as a sublingual adjuvant provide protection against anthrax toxin*

To assess the ability of 3’3’-cGAMP to induce antibodies that are actually protective against infection, a macrophage toxicity assay was performed (Figure 9.) For this assay, serum samples collected from mice immunized three times as previously described was collected following euthanasia on D35. Cultured macrophages were exposed to this serum as well as LeTx (PA and LF.) The absence of PA-specific protective antibodies in the serum leads to cell death, which is then quantified via the addition of MTT, which is taken up by and stains only those cells with functioning mitochondria. The results of this assay showed that CT, CpG, and 3’3’-cGAMP were able to induce some protection against LeTx-induced toxicity, but that serum from 3’3’-cGAMP mice was able to protect cells at a lower concentration than CT or CpG. This is indicative of increased protective efficacy of PA-specific antibodies in animals immunized with 3’3’-cGAMP versus either CT or CpG.
Figure 9: Sublingual immunization with 3'3'-cGAMP increases serum levels of toxin-neutralizing antibodies. Serum was collected from mice immunized three times (D0, D7, D14) and euthanized on D35. Cultured RAW 264 macrophages were exposed to increasing concentrations of serum from immunized mice and to LeTx (PA + LF.) The minimum effective concentration of serum for protection of macrophages from the lethal effects of LeTx was assessed via MTT staining. Serum from 3’3’-cGAMP immunized mice was able to protect macrophages at a lower concentration than that seen with CT or CpG immunized mice, indicating a
higher concentration or higher efficacy of serum antibodies in animals immunized with 3’3’-cGAMP. N = 3 mice per group.

**3’3’-cGAMP as a sublingual adjuvant promotes mucosal and systemic immunity via induction of type-I IFN and IL-10 in sublingual tissues**

Following confirmation that 3’3’-cGAMP can act as a successful adjuvant, we next attempted to elucidate the mechanism through which 3’3’-cGAMP acts as an adjuvant during sublingual immunization. CLNs and sublingual tissue collected from naïve mice were culture *in vitro* without stimulation or with stimulation by Poly I:C, an adjuvant known to induce IFN-β production, or 3’3’-cGAMP (Figure 10.) Cells were analyzed via PCR for IFN-β, or type I interferon, CD40, IL-10, and TGF-β. Exposure to 3’3’-cGAMP led to significant increases in IFN-β in CLNs versus both unstimulated and Poly I:C stimulated cells. This indicates that 3’3’-cGAMP exerts its effects in part through the induction of type I interferon. To assess the effects of 3’3’-cGAMP *in vivo*, CLNs and sublingual tissues from mice immunized sublingually with PBS, PA and poly I:C, or PA and 3’3’-cGAMP were collected 4 hours after a single immunization were analyzed via PCR as described for *in vitro* studies (data not shown.) No significant differences were found for Poly I:C or 3’3’-cGAMP immunized mice for either tissue analyzed. This is likely because of inappropriate time point selection and further analysis of responses in the CLN and sublingual tissues is warranted at a variety of time points to help elucidate this mechanism *in vivo*. 

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Figure 10: 3’3’-cGAMP exerts its effects through the actions of IFN-β. Exposure to 3’3’-cGAMP leads to increased IFNβ and IL-10 production in vitro. CLNs and sublingual tissues were collected from naïve mice and cultured overnight alone or with exposure to Poly I:C or 3’3’-cGAMP and cytokine production was assessed using rtPCR. Increased IFNβ and IL-10 production was seen in cells from the sublingual tissue and this pattern
was comparable to cytokine induction by poly I:C (A). This trend for IFNβ induction was also seen in the CLNs (B.) n = 2 mice per group.
Chapter 4: Discussion

Sublingual immunization presents the potential for effectively eliciting mucosal and systemic immune responses without the safety concerns associated with intranasal and oral immunization. Safe, effective sublingual vaccines depend on appropriate adjuvant selection. Here, we show that the STING ligand 3’3’-cGAMP promotes effective systemic and mucosal IgA responses similar in character to those induced by CT. 3’3’-cGAMP elicits a balanced Th1/Th2 cytokine response without eliciting potentially harmful inflammatory cytokine responses. Furthermore, the serum antibody responses induced by this STING ligand are protective against lethal toxin of *Bacillus anthracis*.

Appropriate adjuvant development and selection remains a challenge in mucosal vaccine development. Many of the adjuvants used in traditional systemic vaccines such as alum appear ineffective when used in sublingual vaccines (36). Bacterial enterotoxins are some of the most well studied mucosal adjuvants, and though they are highly effective at stimulating production of sIgA, they are also associated with adverse effects such as facial nerve paralysis and diarrhea after nasal or oral administration (6, 15, 20, 21). The TLR9 ligand CpG can act as an effective mucosal adjuvant for sublingual immunization, (19)but when used for sublingual immunization in mouse models, CpG does not elicit the high antibody responses observed with bacterial enterotoxins (32). This was confirmed in
the present study where CpG elicited limited serum and mucosal antibody responses, but was ineffective at eliciting significant cytokine responses or antigen-specific antibody secreting cells.

3’3’-cGAMP is a ligand for the recently described cytoplasmic DNA sensor STING (39, 59). STING detects CDNs alone or in concert with the cytoplasmic DNA receptor cGAS, which detects dsDNA and produces the non-canonical STING ligand 2’3’cGAMP (47, 48, 51). This second messenger can be detected even by STING alleles that do not respond to bacterial CDNs, which may be present in up to 20% of the population (19, 60). Further investigation into the ability of these polymorphic STING alleles to detect bacterial CDNs, which contain canonical linkages, is warranted – especially since this could impact the effectiveness of STING ligand-based adjuvants (19). STING activation leads to the production of IFN-β in an IRF3 dependent manner during viral infection, and here we have confirmed that IFN-β also plays a role in STING ligand related immune responses following sublingual immunization (50).

Mouse models using c-di-GMP for intranasal immunization have shown the potential for the use of CDNs as adjuvants. In mice, c-di-GMP immunization successfully induced NK, NKT, and T and B cell infiltration in the lungs and reduced colonization of Klebsiella following challenge (19). Various studies of c-di-GMP as a systemic or intranasal vaccine adjuvant have shown predominantly Th1-biased responses and that CDNs effectively elicit mucosal and systemic antibody responses (19). In the present
study, 3’3’-cGAMP elicited a balanced Th1/Th2/Th17 immune response following sublingual immunization with a unique Th2 related cytokine profile. While currently approved adjuvants such as alum successfully elicit Th2 biased responses, the ability to also induce a Th-1 response could yield improved protection against intracellular pathogens (37, 38). Furthermore, sublingual immunization with 3’3’-cGAMP was not associated with the production of the pro-inflammatory Th2 cytokines IL-4 and IL-13. These cytokines are associated with pro-inflammatory responses including IgE class switching and mucus production. This could be indicative of improved safety with STING ligand adjuvanted vaccines versus bacterial enterotoxins due to a lower risk of developing allergic responses.

Local cell responses in the sublingual tissue did not change at an early time point following immunization with CT or 3’3’-cGAMP. Animals immunized with CpG experienced thickening of the epithelial layer of the sublingual tissue. Further study of cell population characteristics and morphology of the sublingual tissue following CpG application are warranted to ascertain whether this difference was due to the adjuvant used or to the individual animals examined. Classically, the predominant APCs in the sublingual tissue are thought to be DCs along with mast cells and scattered T cells (14). In contrast with this information, we showed that mast cells, CD19+ B cells, and Cd3+ T cells made up the bulk of the immune cells present in both the tongue and the sublingual tissue. Population characteristics did not change significantly in either tissue two hours after the addition of any of the adjuvants studied, except in the case of cholera toxin.
where absolute numbers of neutrophils in the sublingual tissue and both absolute and relative populations of neutrophils in the tongue decreased. It is unknown whether this change is biologically relevant. CT is known to be a powerful mucosal adjuvant that readily induces sIgA following sublingual immunization. Previous studies from our lab have shown that sublingual immunization with EdTx induces an influx of neutrophils into the sublingual tissues and that these neutrophils negatively regulate mucosal IgA induction (13). The results of the present study seem to correspond with those reported previously in that CT did not induce an influx of neutrophils, but did induce significant increases in mucosal IgA production. This influx was also not seen with CpG or 3’3’-cGAMP, which may factor into their effectiveness as adjuvants. In the present study, tissues were examined at 2 hours. It is possible that efflux of neutrophils occurs rapidly following sublingual immunization with cholera toxin and that, given additional time, the neutrophil population would have returned to the level of those seen in mice immunized without adjuvants.

Besides eliciting local sIgA, bacterial enterotoxins such as CT are known to promote antibody responses in distant mucosal sites via the induction of B cell homing receptors in the CLNs. In the present study, only CT trended towards an increase in mucosal homing receptors to the gut. This corresponds with the sIgA analyses performed which showed that only CT seemed to promote increased production of sIgA in the feces, though these results did not reach significance. 3’3’-cGAMP elicited sIgA responses preferentially in the saliva. Following mucosal immunization, the most robust responses
tend to be in sites adjacent to the immunization site, which would be the salivary glands in the case of sublingual immunization. Responses in the salivary glands may be indicative of an immune response in the respiratory tract. However, since mice are obligate nasal breathers, this response may be more closely associated with immune responses in the GIT. No adjuvant induced significant increases in BAL or nasal wash samples in the present study. Future studies examining immune cell populations in the NALT, BALT, and respiratory tree following sublingual immunization with 3’3’-cGAMP may be warranted to determine which mucosal sites 3’3’-cGAMP most effectively targets following sublingual immunization.

In order to maintain homeostasis, the various components of the immune system must work together both on an organismal level and at the level of the cell itself. For example, cGAS has been shown to work synergistically with closely related OAS proteins to detect intracellular pathogens (43). In light of this, some studies have examined combinations of adjuvants to take advantage of multiple pathways for the induction of immune responses. CpG has been shown to work synergistically with 2’3’-cGAMP and 3’3’-cGAMP both in cultured human PBMCs and when administered parenterally in mice (49). Combination of 3’3’-cGAMP with additional PAMP or DAMP-based adjuvants may also provide a manner of inducing protective immune response in humans with refractory STING alleles – even if the STING ligand administered is ineffective, these patients could still mount a TLR mediated immune response, for example, while other patients could mount a more robust response by taking advantage of both pathways.
Despite their potential promise, further study is needed to examine the safety of STING ligands in a variety of populations, including young and elderly patients. Though strong inflammatory responses were not seen in the current study, inappropriate STING activation is associated with a variety of diseases and it is possible that strong activation using a vaccine adjuvant could elicit negative side effects in susceptible patients. Mutations in *TMEM173*, a candidate gene for encoding STING, have recently been associated with STING-associated vasculopathy with onset in infancy (SAVI), an autoimmune disease that causes vasculitis of small dermal vessels and interstitial lung disease (64). In this syndrome, STING is constitutively activated leading to excessive production of IFN-β in the T cells, monocytes, natural killer cells, alveolar type 2 pneumocytes, alveolar monocytes, bronchiolar epithelial cells, and dermal fibroblasts of affected patients (64). Safety and toxicity studies are necessary to ensure that STING ligands such as 3′3′-cGAMP are safe for use as adjuvants in a variety of human populations and that they are not associated with harmful type I IFN reactions.

This study shows that 3′3′-cGAMP is an effective sublingual adjuvant and is capable of eliciting both systemic IgG immune responses and local mucosal responses in the salivary glands. We also show that these antibody responses are protective against lethal toxin of *Bacillus anthracis in vitro*. STING ligand elicits a balanced Th1/Th2/Th17 response, which may be beneficial for successful vaccinations against intracellular and extracellular pathogens and for promotion of mucosal antibody responses. 3′3′-cGAMP
was not associated with strong sIgA responses in the gastrointestinal or urogenital tracts in the present study, but further work is needed to elucidate the ideal dose and dosing schedule for animals receiving 3’3’-cGAMP as an adjuvant.
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