STING is required in conventional dendritic cells for DNA vaccine induction of type I T helper cell-dependent antibody responses

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Abstract

STING is required in conventional dendritic cells for DNA vaccine induction of type I T helper cell-dependent antibody responses

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DNA vaccines elicit antibody, T helper cell, $CD8^+$ T cell, and cytotoxic T lymphocyte (CTL) responses. Currently, little is known about the mechanism that DNA vaccines employ to induce adaptive immune responses. Studies have demonstrated that *stimulator of interferon genes* (*STING*) and conventional dendritic cells (cDCs) play critical roles in DNA vaccine-induced antibody and T cell responses. *STING* activation by double stranded DNA (dsDNA)-sensing proteins initiates the production of type I interferons (IFNs); however, the link between *STING* and cDCs remains unclear. In this study, I investigated the role of *STING* within cDCs on DNA vaccine induction of antibody and T cell responses. *STING* knockout (*STING*$^{-/-}$) and conditional knockout mice that lacked *STING* in cDCs (*cDC STING cKO*) were immunized intramuscularly with a DNA vaccine that expressed influenza A nucleoprotein (pNP). Both *STING*$^{-/-}$ and *cDC STING cKO* mice had significantly lower type I T helper (Th1) antibody (anti-NP IgG_{2C}) responses as well as lower frequencies of Th1-associated T cells (NP-specific IFN-γ^+CD4^+ T cells) post-immunization than wild-type (WT) and *cDC STING littermate control* mice. By contrast, all mice had similar Th2-type (NP-specific IgG_1) antibody concentrations. Moreover, *STING*$^{-/-}$ mice developed significantly lower polyfunctional effector $CD8^+$ T cells than WT, *cDC STING cKO*, and *cDC STING littermate control* mice. These findings suggest that *STING* within cDCs mediates DNA vaccine induction of Th1 responses, including IFN-γ^+CD4^+ T cell and Th1-type IgG_{2C} antibody responses. The induction of $CD8^+$ effector cell

responses also requires *STING*, but not within cDCs. These findings provide new insight into the mechanism through which DNA vaccines induce Th1 responses.

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1 Introduction to DNA vaccines

1.1 History of DNA vaccines

Infectious diseases such as influenza A, *Yersinia pestis* (plague), and variola virus (small pox) have caused morbidities and death in humans and other animals alike [1]. The rise of globalization and global warming as well as changes in animal agriculture have increased the threat of the emergence and dissemination of pathogens that can result in global pandemics [1]. Until recently, most U.S. Federal Drug Administration (FDA)-approved vaccines were either protein-based vaccines, including subunit vaccines and virus-like particles, or whole pathogen-based vaccines, including live-attenuated pathogens or killed whole pathogens [2]. To produce whole pathogen-based vaccines, a pathogen is grown in large quantities and killed or inactivated by chemical means [2]. Since whole pathogen-based vaccines are made of pathogens that have their genomes intact, the potential exists for the pathogen to revert to its wild-type (WT) form, thus producing a replication-competent pathogen that can induce illness and potentially infect others [3]. Despite the potential safety concerns associated with whole pathogen-based vaccines, several have been approved for use in humans by the FDA, including the MMR (measles, mumps, and rubella) and hepatitis A/B vaccines [2]. By contrast, protein-based vaccines eliminate the safety concerns associated with whole pathogen vaccines by producing or isolating antigenic protein(s), which are essential for host cell entry or pathogenesis, while removing the genetic elements of the pathogen [2-3]. Both whole pathogen and protein-based vaccines can induce antibody (Ab) responses and in some cases cytotoxic T lymphocytes (CTL) responses, which provide immunity in vaccinated individuals [2-3]. Fortunately, multiple types of FDA-approved vaccines provide protection against a range of pathogens (Figure 1) [4-6].

Although these vaccine strategies have saved countless lives, they are not easily adaptable to combat novel infectious diseases since they rely on the isolation of pathogens or their proteins, which increases manufacturing lead times [7]. For example, the annual influenza A vaccine can take five to six months to produce because growing the influenza A virus, inactivating it, and conducting quality controls increase production times [2,4,8].

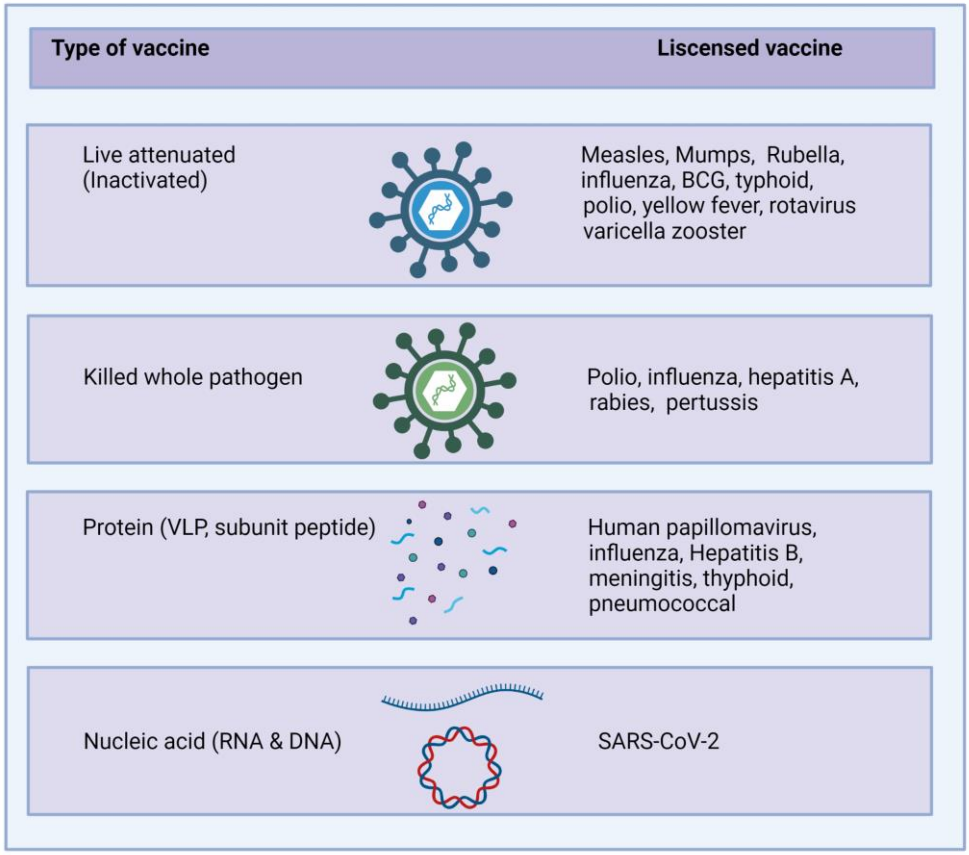

Figure 1: Different types of vaccines

Schematic representation of the different types of vaccines approved for clinical use against various pathogens, namely live-attenuated, whole pathogen, protein-based, and nucleic acid-based vaccines. Created with Biorender.com

In contrast, nucleic acid-based vaccines, including RNA- and DNA-based vaccines, have been demonstrated to significantly decrease vaccine lead times as well as to be a viable option for combating the emergence of novel infectious diseases. Nucleic acid vaccines are also hypothesized to be safer than whole pathogen-based vaccines since they do not contain the genes necessary for pathogen replication. Instead, nucleic acid vaccines encode and target only the protein or proteins that are essential for pathogen survival, including those required for host cell entry and pathogenesis [4-6, 9-12]. DNA vaccines as plasmids and RNA vaccines as messenger RNAs (mRNAs) have the potential to combat emerging and novel infectious diseases, as is evident from the numerous DNA and RNA vaccines that were developed to combat SARS-CoV-2, many of which were approved for clinical use [6, 9-12]. Research into the efficacy of using nucleic acid as a vaccine began in the 1990s, when initial studies demonstrated that DNA vaccines can induce protective immunity *in vivo* against infectious diseases and cancers [9-12]. Moreover, DNA and RNA vaccines can be used to deliver immune-modulating adjuvants, such as *interleukin* (*IL*) *12*, *IL-23*, and *granulocyte-macrophage colony-stimulating factor* (*GM-CSF*), which increase the potency of nucleic acid vaccines [9-12]. Crucially, RNA and DNA vaccines can be produced rapidly in response to an emerging infectious disease, induce protective immune responses, and provide lasting immunity [6, 9-12]. In order to induce robust immune responses nucleic acid vaccines must be optimized for expression in host cells.

1.2 DNA vaccine optimization

DNA vaccines rely on a backbone of bacteria-derived plasmids; thus, these plasmids possess an origin of replication, an antibiotic resistance gene (selectable marker), and multiple cloning sites (Figure 2). To maximize gene expression in mammalian host cells, bacterial promoters upstream of the gene of interest (GOI) are replaced with promoters recognized by mammalian host cell transcription factors [9, 15-20]. Early studies used the promoter from the oncogenic simian vacuolating virus 40 (SV40) to increase protein expression in mammalian cells [21-23]. The use of SV40 was replaced by the promoter derived from cytomegalovirus (CMV) [9,15-20]. The CMV promoter has been increasingly used in DNA vaccines because it is highly constitutively expressed in a range of mammalian cells; induces the expression of vaccine-encoded proteins readily; and does not suffer from transcriptional read-throughs as might be expected from a strong promoter [9, 15-20].

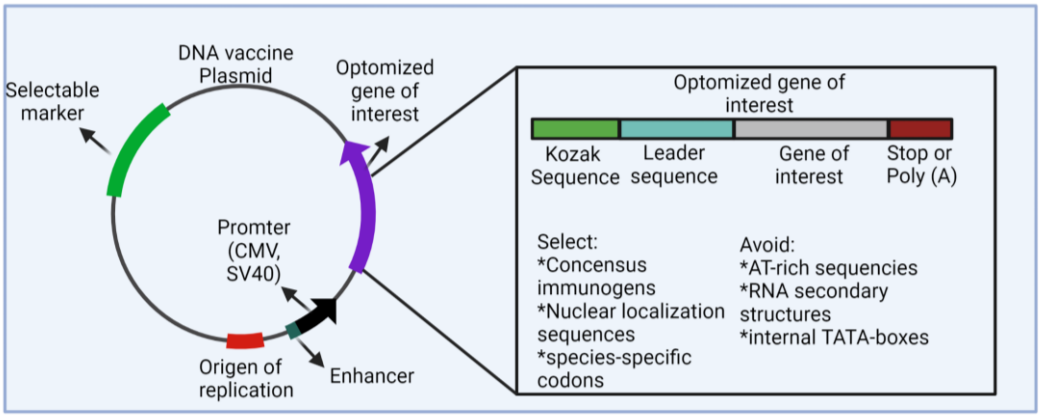

Figure 2: DNA vaccine optimization

Efforts have been made to optimize DNA vaccines to increase antigen expression and vaccine immunogenicity, including the optimization of transcriptional elements in the plasmid backbone (enhancer sequences), codon optimization for increasing protein (antigen) expression, and the use of strong promoters (CMV). Strides have been made in limiting potential RNA secondary structures that might decrease antigen expression in cells. Besides optimizing DNA vaccine plasmid construction, vaccine-encoded adjuvants such as IL-12 and IL-23 as well as technologies for increasing DNA vaccine transfection efficiencies have been employed to increase immunogenicity and antigen expression. Created with Biorender.com

Furthermore, researchers have attempted to use cell-specific promoters to target specific immune cell types at the site of vaccination, including myocytes (muscle cells); however, this approach has been demonstrated to greatly reduce gene expression and subsequent DNA vaccine immunogenicity [9, 15-20].

Crucially, the addition of a poly (A) signal or a termination site (stop codon) immediately after the GOI is critical for proper protein expression and structure [9, 23-25]. Although it is not required, many DNA vaccines include enhancer elements either upstream or downstream of the target antigen's open reading frame (ORF) [9, 20-31]. Many enhancer elements are derived from viruses, but their use may be limited as studies have suggested that some might have oncogenic properties, which is considered a safety concern when seeking clinical approval for a vaccine [9, 20-31].

To increase DNA vaccine protein expression and immunogenicity, it is vital to optimize the stability and expression of DNA vaccine-encoded mRNA. RNA optimization results in more efficient translation and thus protein expression [26-28]. The removal of elements of DNA that can induce mRNA secondary structures can limit the nuclear export of mRNA as well as translation [26-28]. Furthermore, the inclusion of leader sequences increases protein expression by stabilizing DNA vaccine-encoded mRNA [29-31]. Studies have demonstrated increased mRNA stability and protein expression in DNA vaccines that included the signal sequence from HIV-1, HIV envelope leader, or leader sequences derived from *Mycobacterium tuberculosis* [29-31]. Lastly, the proteins encoded by DNA vaccines must be codon-optimized for expression in mammalian cells to increase protein expression and DNA vaccine immunogenicity [20].

1.3 Advantages and disadvantage of DNA vaccines

DNA vaccines possess several advantages over traditional protein-based and whole pathogen-based vaccines. First, the construction and assembly of DNA vaccines is simple. For example, a GOI from a pathogen can be made synthetically or through PCR expansion and easily inserted into a DNA vaccine backbone to produce new DNA vaccines rapidly. Second, since DNA vaccines are nonreplicating, nonliving, and nontransmitting, they do not entail the risk of pathogen reversion and subsequent secondary infections, which vaccines such as live-attenuated vaccines entail [32]. Third, unlike protein-based vaccines that predominantly induce the generation of antigen (Ag)-specific Ab responses, DNA vaccines can trigger Ag-specific Ab and T cell responses [2,6,9, 33]. Fourth, safety studies have demonstrated that DNA vaccines

do not induce adverse reactions or toxicity in clinical trials and that repeated exposure of naked DNA does not elicit anti-DNA Abs, which in theory could promote the development of autoimmune diseases [34-37]. Fifth, unlike mRNA, protein-based, and live-attenuated vaccines, DNA vaccines can be stored at temperatures above 4^{o}C and continue to be immunogenic [38]. Sixth, DNA vaccines can be made to co-express genetically encoded adjuvants or be co-delivered with plasmids that express such adjuvants to increase vaccine immunogenicity, including IL-12, IL-23, and GM-CSF [9-12, 39-41]. Lastly, DNA vaccines can be used to combat infectious diseases, allergens, and cancers [9, 32-37].

Despite DNA vaccines being studied for decades, barriers have restricted their approval for clinical use. For example, although DNA vaccines can induce Ag-specific B cell and T cell responses, they elicit low immunogenicity responses in humans [42-44]. Moreover, safety concerns exist that DNA vaccines could promote the generation of anti-DNA Abs and subsequent autoimmune disease progression, as studies have suggested that DNA vaccines combined with adjuvants can induce anti-DNA Abs and autoimmune disease-like symptoms; however, these findings are limited [45-46]. Lastly, a potential risk exists that DNA vaccines could integrate into the host's genome, which could result in insertional mutations as well as increase the risk of cancer if the vaccine DNA integrated into tumor suppression genes [47]. Despite this risk, recent studies have demonstrated that integration events are rare and can be further mitigated by reducing bacterial impurities that may be present after plasmid isolation [48-51].

1.4 DNA vaccine mechanism of action and immunogenicity

DNA vaccines can elicit Ab, CTL, and effector CD8^{+} T cell responses post-vaccination (Figure 3). This section presents an overview of the mechanisms required to induce DNA vaccine immunogenicity.

1.4.1 DNA vaccine-induced antibody responses

Similar to traditional vaccines (e.g., protein-based and live-attenuated vaccines), DNA vaccines can elicit Ag-specific Ab responses. Currently, DNA vaccines are delivered through an array of routes, including epidermal (ED), intradermal (ID), and intramuscular (IM). Upon DNA vaccination, cells at the vaccination site internalize vaccine DNA, from where it is translocated to the nucleus by an unknown mechanism for transcription and subsequent antigen expression in the cytoplasm [9,52-53].

Studies have found that antigen-presenting cells (APCs) are for the induction of Ab responses, the most crucial of which are dendritic cells (DCs), although skin-resident DC-like Langerhans cells (LCs) are the primary APCs required when ED immunization is used [9, 25, 52-57]. Several mechanisms exist through which APCs obtain vaccine-encoded antigens. APCs at the vaccination site can be directly transfected by vaccine DNA, resulting in antigen expression and antigen loading onto major histocompatibility complex-II (MHC-II), which is then presented on the surface of the APCs and to T cells [9, 25, 52-57]. Due to the low abundance of APCs in the muscles, epidermis, and dermis, most vaccine DNA is internalized by somatic cells at the site of vaccination, including myocytes (muscle cells) and keratinocytes (skin cells) [9, 25, 52-57]. Transfected somatic cells express DNA vaccine-encoded antigens that can be phagocytosed and processed by APCs, resulting in antigen-loaded MHC-II on the surface of APCs [9, 25, 52-57]. Moreover, studies have found that some DNA vaccine-transfected somatic cells undergo apoptosis, which results in apoptotic bodies that possess vaccine DNA and vaccine-encoded antigens [58-60]. These apoptotic bodies are phagocytosed by APCs, where DNA vaccine-encoded antigens are processed and loaded onto MHC-II [58-61].

Cells at the vaccination site produce pro-inflammatory factors, including cytokines and chemokines, which result in continued APC recruitment to the site as well as APC activation/maturation [52-61]. Moreover, APCs express Ag-specific pattern recognition receptors (PRRs), which can recognize the pathogen-associated molecular patterns (PAMPs) and damage-associated molecular molecules (DAMPs) activated after DNA vaccination, resulting in the expression of pro-inflammatory molecules, the upregulation of co-stimulatory molecules such as CD80, and ultimately APC activation/maturation [62-64].

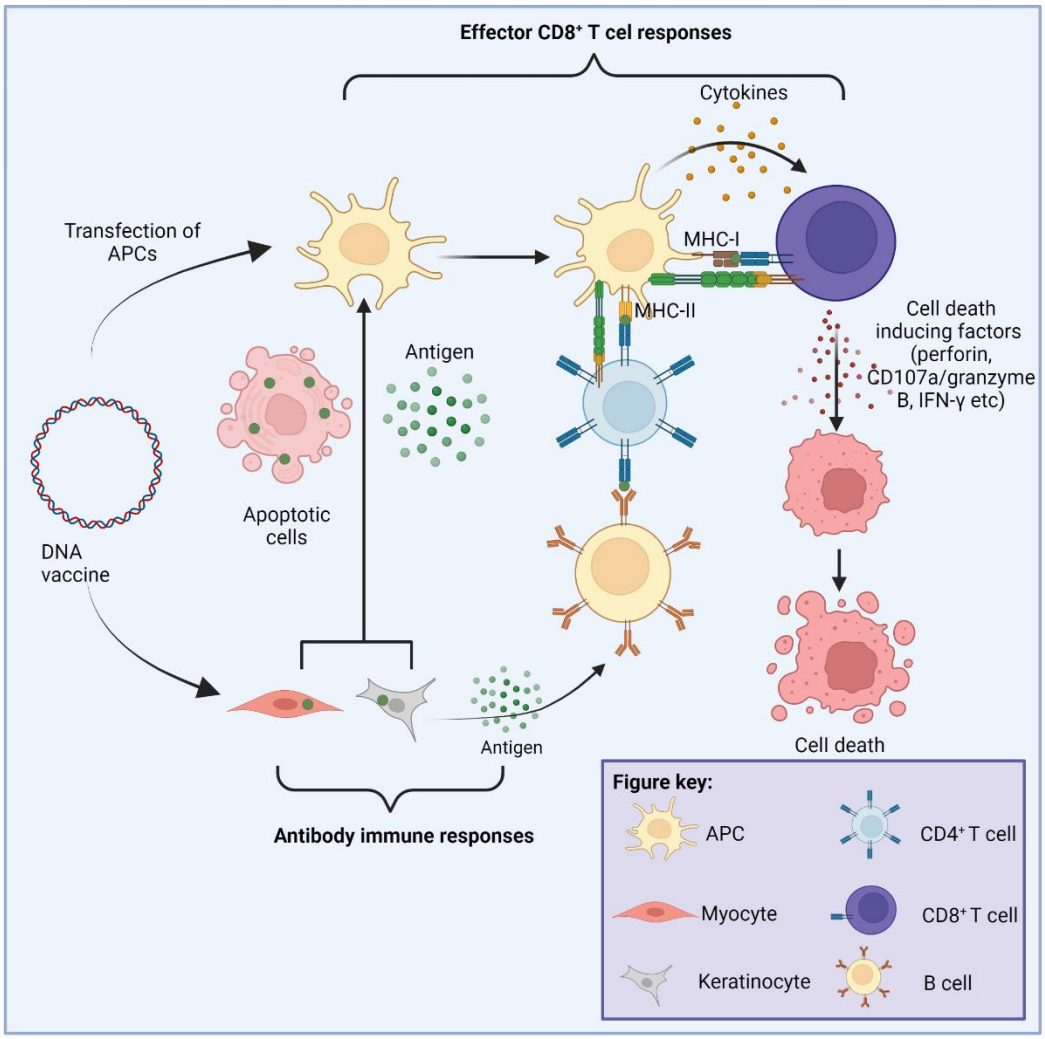

Figure 3: Introduction to DNA vaccine immunogenicity

DNA vaccines can induce antibody (Ab), cytotoxic T lymphocyte (CTL), and effector $CD8^+$ T cell responses. Vaccine DNA transfects antigen-presenting cells (APCs) as well as somatic cells found at the vaccination site, including keratinocytes (skin cells) and myocytes (muscle cells), resulting in the expression of vaccine-encoded antigens. Moreover, DNA vaccine-transfected somatic cells express antigens and some somatic cells undergo apoptosis, resulting in the formation of apoptotic bodies that contain vaccine-derived peptides, proteins, and vaccine DNA. Apoptotic bodies and exogenous antigens can be phagocytosed by APCs (dendritic or Langerhans cells), which then process vaccine-encoded antigen/peptides and present them to $CD4^+$ T and $CD8^+$ T cells through MHC-II and MHC-I, respectively. This results in the induction of Ab responses by B cells, CTL responses, and effector $CD8^+$ T cell responses. Created with Biorender.com

After APCs are exposed to vaccine-encoded antigens and become activated, they migrate to the draining lymph nodes (LNs) and secondary lymphoid organs (SLOs) where they generate Ab responses [52-61]. In the LNs and SLOs, APCs present DNA-encoded antigens on MHC-II to naïve $CD4^+$ helper-T cells, resulting in their differentiation into T-helper 1 (Th1), T-helper 2 (Th2), and T follicular helper-T cells (Tfh) [54-57, 62-66]. The pro-inflammatory environment induced by vaccine DNA as well as by infection greatly influences the differentiation of naïve $CD4^+$ T cells into Th1 $CD4^+$T, Th2 $CD4^+$ T, and Tfh T cells as well as their proportions [65-73]. For example, studies have demonstrated that the production of type I interferons (IFNs) biases the generation of Th1 T cells, whereas the production of IL-4 biases the generation of Th2 T cells [69-73]. Differentiated helper-T cells interact with B cells, resulting in the production of Abs, including immunoglobulin A (IgA), IgG, and the short-lived Ab IgM [67-71]. Both Th1 $CD4^+$T and Th2 $CD4^+$ T cells influence the generation of Ag-specific IgG responses. Th1 $CD4^+$T cells promote B cells to undergo IgG class-switching by secreting interferon gamma (IFN-γ), which results in the production of the Th1-associated Ab IgG_2, a subclass of IgG, whereas Th2 $CD4^+$T cells promote the production of the Th2-associated Ab IgG_1 by secreting IL-4 [54-58, 65-69]. The IgG_2:IgG_1 ratio is suggestive of either a Th1 biasing, Th2 biasing, or a balanced Th1/Th2 response post-vaccination. A higher IgG_2:IgG_1 ratio suggests that Th1 biasing is occurring while a lower ratio indicates that Th2 biasing is occurring. Although DNA vaccines can induce both IgG_2 (Th1) and IgG_1 (Th2) Ab responses, the induction and biasing of these responses are influenced by the route of immunization as well as the presence or absence of Th1-/Th2-polarizing adjuvants [13-19, 24-25, 35-44, 65-69]. Physiologically, Th1-associated IgG_2 is produced in response to bacteria, viruses, and cancers, whereas Th2-associated IgG_1 is produced in response to allergens and intestinal helminths [64-69]. Since DNA vaccines induce IgG_2 (Th1) and IgG_1 (Th2) Ab responses, they are of great interest in combating infectious diseases, cancers, and allergies.

1.4.2 DNA vaccine-induced effector $CD8^+$ T cell responses

DNA vaccines, unlike protein-based vaccines, can elicit robust CTL and effector $CD8^+$ T cell responses that are critical for recognizing and killing intracellular pathogens and cancer cells [16-21, 32-37, 63]. Both $CD4^+$ and $CD8^+$ T cells can possess cytotoxic effector functions, but effector $CD8^+$ T cells are the primary cell type dedicated to killing infected and cancerous cells [74-76]. Ideally, DNA vaccination

results in effector $CD8^+$ T cells with multiple effector functions (polyfunctional $CD8^+$ T cells), which provide them with multiple avenues for killing infected and cancer cells. IFN-γ, tumor necrosis factor-α (TNF-α), granzyme B, and IL-2 (Figure 4) are common effector functions used to assess polyfunctional $CD8^+$ T cells.

Similar to DNA vaccine induction of Ab responses, vaccine-encoded antigens must be obtained by APCs for the induction of effector $CD8^+$ T cell responses [52-57]. To induce an effector $CD8^+$ T cell response, APCs must load and display DNA vaccine-encoded antigen peptides on MHC-I [9, 52-57]. APCs can be directly transfected by vaccine DNA, resulting in the expression of vaccine-encoded antigens that are then processed and loaded onto MHC-I [9, 52-57]. Moreover, transfected somatic cells can express DNA-encoded antigens and display antigen peptide-loaded MHC-I complexes on their cell surface; however, some somatic cells undergo apoptosis, resulting in apoptotic bodies that possess DNA vaccine-encoded antigens as well as antigen-loaded MHC-I, which can be phagocytosed by APCs [9, 52-57]. Phagocytosed apoptotic bodies can then be processed by APCs, resulting in antigen presentation on MHC-I (cross-presentation) as well as MHC-II [9, 52-57]. The acquisition of antigens as well as exposure to pro-inflammatory cytokines result in APC activation/maturation, which in turn results in the upregulation of co-stimulatory proteins, such as CD80 and CD86 [62-64].

Once activated, APCs migrate to draining LNs and SLOs where they interact with naïve $CD8^+$ T cells to induce effector $CD8^+$ T cell responses. In the LN/SLO cognate, T cell receptors (TCRs) on naïve $CD8^+$ T cells can bind to peptide-loaded MHC-I on the surface of APCs, resulting in the binding of co-stimulatory proteins on the surface of the APCs and naïve $CD8^+$ T cells [62-64,73-78]. Once bound, APCs release cytokines that program $CD8^+$ T cell effector functions. Studies have highlighted that type I IFNs, IL-6, tumor necrosis factor-α (TNF-α), and IL-2 are critical cytokines required for the development of polyfunctional $CD8^+$ T cells as well as effector memory $CD8^+$ T cells [62-64, 73-78]. For example, studies have found that mice that do not respond to type I IFNs, namely interferon-alpha/beta receptor (IFNAR) knockout mice, do not effectively clear multiple viruses, including influenza A and Zika virus, and that the $CD8^+$ T cells in these mice undergo suboptimal proliferation and possess reduced effector functions compared with WT mice [79-82].

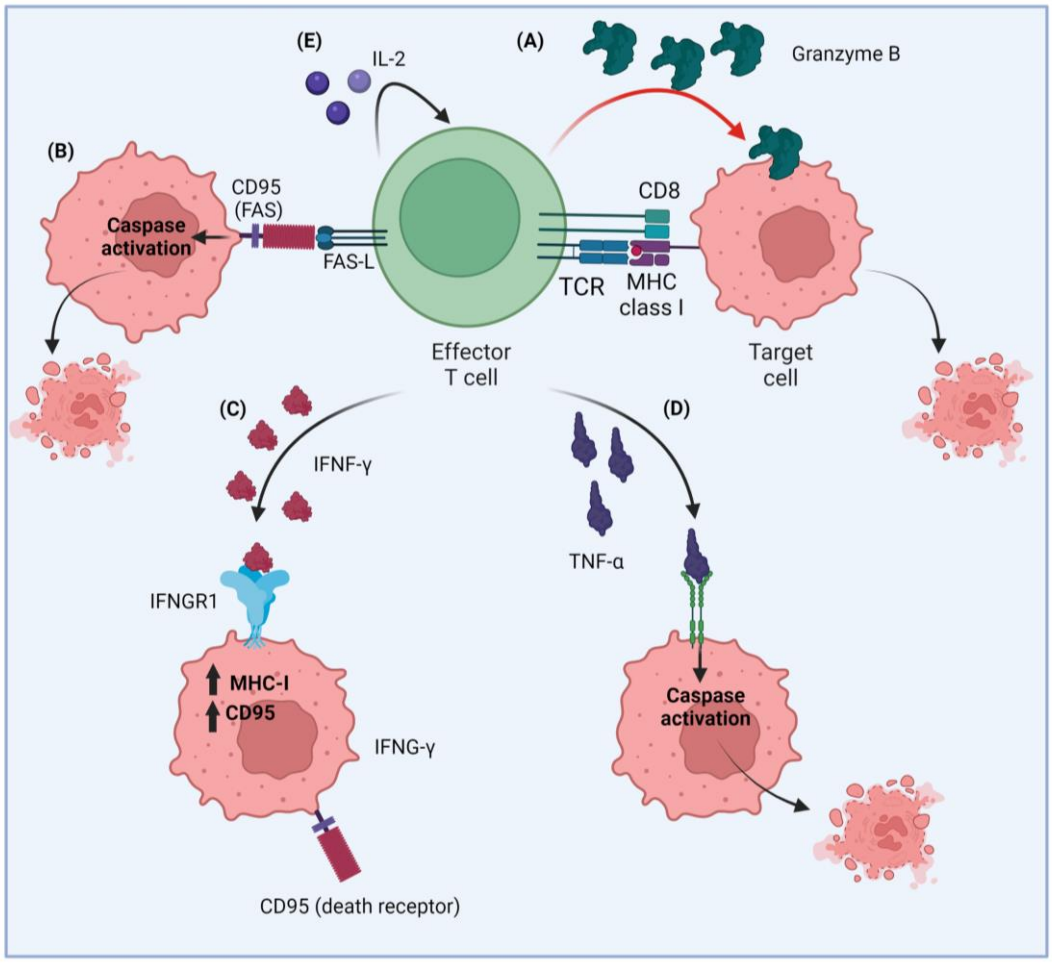
(E)
IL-2
(A)
Granzyme B
(B)
CD95
(FAS)
Caspase
activation
FAS-L
CD8
TCR
MHC
class I
Effector
T cell
Target
cell
(C)
IFNF-γ
(D)
TNF-α
IFNGR1
MHC-I
CD95
IFNG-γ
Caspase
activation
CD95 (death receptor)

Figure 4: Introduction to polyfunctional effector CD8$^+$ T cells

Effector CD8$^+$ T cells are one of the primary cell types that sense, bind, and kill cancer cells as well as intracellularly infected host cells. DNA vaccines induce the potent effector CD8$^+$ T cell responses required to clear pathogen infections or cancerous cells. Ideally, vaccine DNA will induce the generation of effector CD8$^+$ T cells that possess multiple effector functions (polyfunctional effector CD8$^+$ T cells), which grant effector CD8$^+$ T cells multiple routes for killing target cells. DNA vaccine studies quantify CD8$^+$ T cell polyfunctionality by measuring expression of different effector functions, including IFN-γ, IL-2, granzyme B, and TNF-α. Upon engaging a target cell, effector CD8$^+$ T cells can directly kill it by releasing (A) cytolytic factors, such as granzyme B and perforin, (B) and/or bind to FAS (CD95) to induce caspase activation in the target cell, resulting in apoptosis. Effector CD8$^+$ T cells can also indirectly kill target cells by secreting (C) IFN-γ and (D) TNF-α, which bind to IFN gamma receptor (IFNGR) and tumor necrosis factor receptor 1 (TNFR1), respectively. The activation of IFNG1 by IFN-γ induces upregulation of CD95, which can be recognized by FAS-L on effector CD8$^+$ cells, resulting in apoptosis through caspase activation. Moreover, the activation of TNFR1 on the target cell by secreted TNF-α induces caspase activation and apoptosis of the target cell. (E) IL-2 is an important cytokine that triggers metabolic and transcriptional changes that lead to increased survival, proliferation, and effector differentiation. Created with Biorender.com

Although multiple cytokines have been identified as playing a crucial role in programming $CD8^+$ T cell effector functions and memory, the exact cell(s) required to produce these cytokines has not been fully characterized. This is because studies have indicated that plasmacytoid DCs (pDCs), a DC subset that produce large quantities of IFN-α, can aid in programming $CD8^+$ T cell effector functions and pathogen clearance [83-84, 173]. Moreover, some DNA vaccine studies have suggested that somatic cells can program $CD8^+$ T cell effector functions by presenting antigens to $CD8^+$ T cells through MHC-I, and that these somatic cells produce pro-inflammatory cytokines, including type I IFNs, IL-6, and TNF-α [124]. Most evidence suggests that antigen presentation by professional APCs is the main mechanism through which effector $CD8^+$ T cells are programmed [85-87]. Taken together, DNA vaccine induction of polyfunctional $CD8^+$ T cell responses requires type I IFNs, other pro-inflammatory factors, APCs, and to a lesser extent somatic cells.

1.5 DNA vaccine delivery

The site of DNA vaccination can have an effect on vaccine induction of Th1-associated (IgG_2) Ab responses, Th2-associated (IgG_1) Ab responses, and effector $CD8^+$ T cell responses. DNA vaccines can be delivered through epidermal (ED), intradermal (ID), and intramuscular (IM) immunization, which are described in the following three subsections. The immune microenvironments at these vaccination sites influence DNA vaccine induction of Ab and effector $CD8^+$ T cell responses.

1.5.1 Epidermal

Multiple methods exist for delivering vaccine DNA into the epidermis, including gene gun (GG) immunization and microneedle (MN) immunization [88]. In GG immunization, biodegradable adhesives are used to adhere vaccine DNA to gold microparticles [88]. These microparticles are then coated onto the inside of tubing to make cartridges that can be fired by a GG [88]. Using compressed gas, DNA vaccine containing these gold microparticles is fired from the GG into the epidermis of the recipient, whereas MN immunization uses patches of small needles to deliver a vaccine into the ED layer of the skin [88].

GG and MN immunization deliver vaccine DNA to the cells of the epidermis, which includes keratinocytes and LCs and results in their transfection (Figure 5) [88-91]. Post-vaccination, DNA vaccine-transfected cells will express vaccine-encoded antigens. LCs were found to be the APCs of the epidermis

that are required to induce Ab, CTL, and effector $CD8^+$ T cell responses through tracking and characterizing green fluorescent protein (GFP) positive cells in the LN post-DNA vaccination [88-91]. A study indicated that LCs account for 3–5% of all nucleated cells of the epidermis [88]. LCs were originally defined by the presence of the surface protein langerin (CD207), but studies of the epidermis and dermis have found that a subset of cDCs can expresses langerin [88,92]. LCs, like cDCs, express CD11c and MHC-II, migrate via CCR7, and present antigens to initiate an adaptive immune response, but LCs also express CD11b and other macrophage markers [88]. Studies have also found that LCs arise from a macrophage precursor but acquire DC-like properties and are also radio-resistant, suggesting that they are of an embryotic origin [88,93-94]. Due to the functional similarities between LCs and cDCs, LCs are often described as a DC-like cell type.

Upon ED DNA vaccine delivery, transfected keratinocytes and LCs express vaccine-encoded antigens. Furthermore, nontransfected LCs can phagocytose and process keratinocyte-derived apoptotic bodies that possess vaccine DNA, encoded antigens, and MHC-I complexes, resulting in antigen cross-presentation by LCs [88, 90-91,95]. Once LCs acquire DNA vaccine-encoded antigens, antigen-loaded MHC-I, and/or vaccine DNA, they undergo activation/maturation and migrate to the skin-draining LNs [88-95]. There, LCs present antigens to naïve $CD8^+$ T cells and naïve $CD4^+$ T cells in an MHC-I- and MHC-II-restricted manner, respectively, resulting in Ab production and effector $CD8^+$ T cell responses [88-95]. Studies have found that the Th2-polarizing cytokines thymic stromal lymphopoietin (TSLP), IL-4, IL-5, and IL-13 are produced locally in the skin as well as in the spleen post-ED vaccination, resulting in Ag-specific Th2-associated IgG_1 Ab production and minimal IgG_2 Ab production, which indicates that ED vaccination favors a Th2-biased response in mice [90-91,95, 95-97].

Additionally, ED DNA vaccination induces CTL and effector $CD8^+$ T cell responses [90-97]. Although these responses are often associated with Th1 responses, studies have found that Th2 $CD4^+$ T cells can aid in programming $CD8^+$ T cell effector functions; however, the mechanism has not been fully characterized [98-99]. Moreover, DNA vaccine studies that utilize ED immunization have co-delivered vaccine-encoded IL-12, a cytokine required for Th1 polarization, to increase CTL responses and IgG_2 production [100-103]. ED delivery of DNA vaccines is of great interest since it can induce Ab, CTL, and effector $CD8^+$ T cell responses without the use of needles.

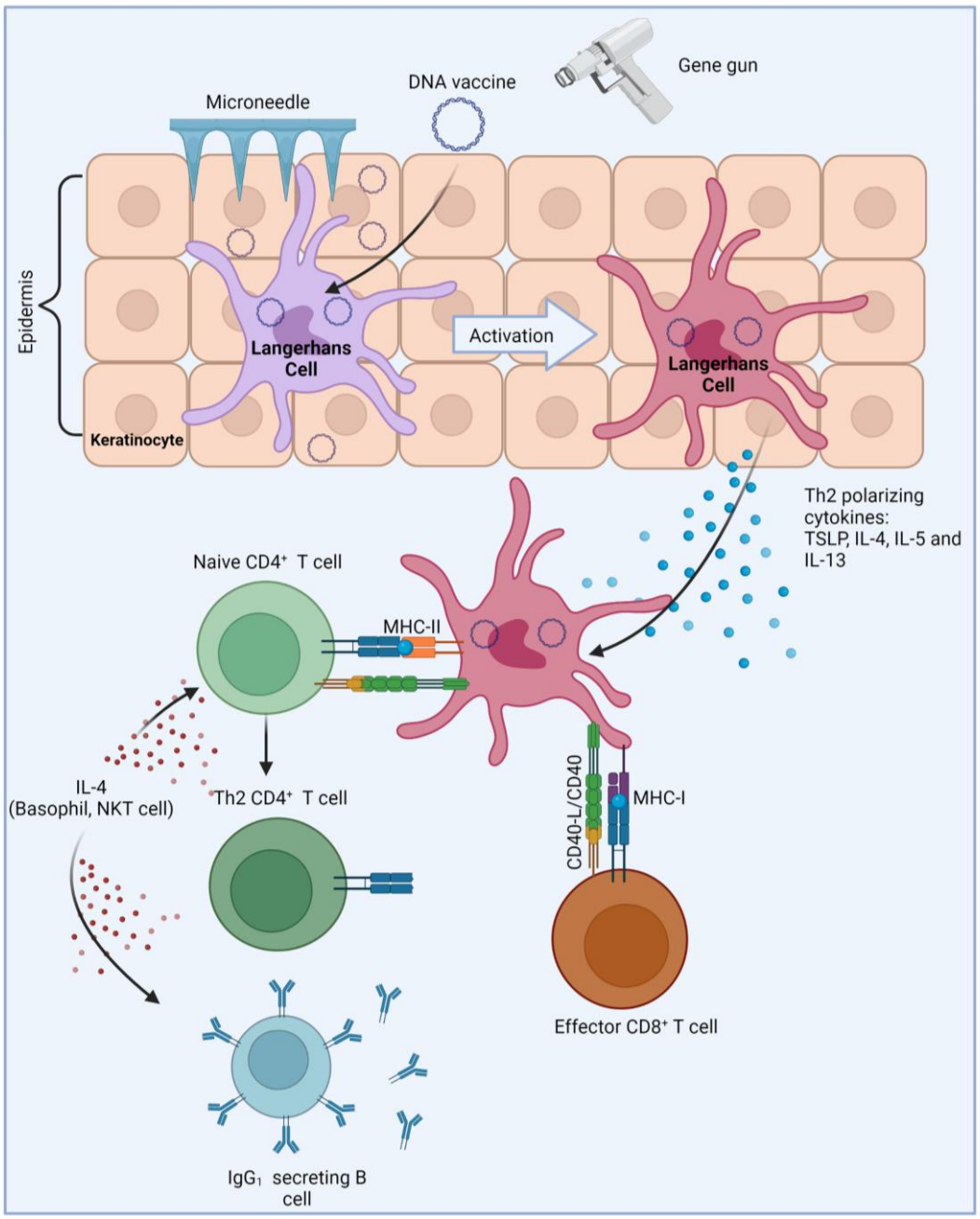
Gene gun
DNA vaccine
Microneedle
Epidermis
Langerhans Cell
Activation
Langerhans Cell
Keratinocyte
Th2 polarizing cytokines: TSLP, IL-4, IL-5 and IL-13
Naive CD4⁺ T cell
MHC-II
IL-4 (Basophil, NKT cell)
Th2 CD4⁺ T cell
CD40-L/CD40
MHC-I
Effector CD8⁺ T cell
IgG1 secreting B cell

Figure 5: Epidermal DNA vaccine immunization

Epidermal DNA immunization is a one of several routes of immunization that can be used to induce immune responses. Microneedle (MN) patches and gene guns (GGs) are two technologies used to transfect keratinocytes (somatic cells) as well as Langerhans cells (LCs), the primary APC of the epidermis. Unlike conventional dendritic cells (DCs), LCs are more akin to monocytes but acquire DC-like characteristics, including the expression of MHC-II and CD11c. Post-DNA vaccination transfected keratinocytes and LCs release Th2-polarizing cytokines, such as TSLP, IL-4, and IL-5, resulting in LC activation and migration to the skin-draining lymph nodes (LNs). Once in the LNs, LCs present vaccine-encoded peptides on MHC-II and MHC-I to $CD4^+$ T and $CD8^+$ T cells, respectively. Studies in mice have found that following epidermal DNA vaccination, Th2-polarizing cytokines such as IL-4 are elevated in the spleen. Furthermore, this route of immunization has been found to preferentially induce B cells to produce IgG_1, which is suggestive of a Th2-polarized immune response. Moreover, LCs program effector $CD8^+$ T cell functions. Created with Biorender.com

1.5.2 Intradermal

The dermis is another delivery site for DNA vaccines. It lies inferior to the epidermis and comprises fibroblasts, a type of somatic cell and immune cell, including DCs, $CD4^+$ T cells, $CD8^+$ T cells, macrophages, and mast cells [104]. In ID immunization, a needle is often used to inject vaccine DNA into the dermis, although Bioject Medical Technologies Inc developed a needle-free system for ID immunizations . Upon ID DNA vaccination, vaccine DNA transfects cells in the dermis; however, the cell type and proportion of cells that are transfected by vaccine DNA have not been well characterized. Although cells passively endocytose vaccine DNA, electroporation (EP) or lipid carriers can be used to increase cell transfection efficiencies in ID as well as IM immunizations [105-107]. EP involves an electrical field being applied to the vaccination site which permeabilizes cell membranes while simultaneously shuttles the negatively charged DNA into the cell.

ID vaccination, like other forms of DNA vaccination, requires APCs – specifically DCs – to induce adaptive Ab, CTL, and effector $CD8^+$ T cell responses [108-110]. DCs of the dermis are a heterogeneous group of cells that can change their surface markers in response to inflammation and other immune insults. Dermal DCs have been divided into two subgroups, namely dermal cDC1s and dermal cDC2s [88, 111]. Dermal cDC1s in both murine and humans are defined by the expression of XCR1 and CD8 and in mice CD103 [112-113]. Dermal cDC1s have a high turnover rate, readily migrate to the skin-draining LNs, and comprise approximately 20–40% of resident skin-draining lymph cells [111, 114]. Conversely, dermal cDC2s are defined by the expression of interferon regulator factor IRF4 [111].

Studies have found that murine dermal DC1s possess an increased capacity to present apoptotic cell-derived antigens for cross-priming to $CD8^+$ T cells compared with dermal cDC2s, and also that mice that lack cDC1s fail to mount $CD8^+$ T cell responses against subcutaneous infections with West Nile virus [115-118]. Moreover, dermal cDC1s induce Th1 responses by producing IL-12, which results in naïve $CD4^+$ differentiation into Th1 $CD4^+$ T cells that subsequently secrete IFN-γ, which promotes B cells to secrete IgG_2 Abs [111, 119-120].

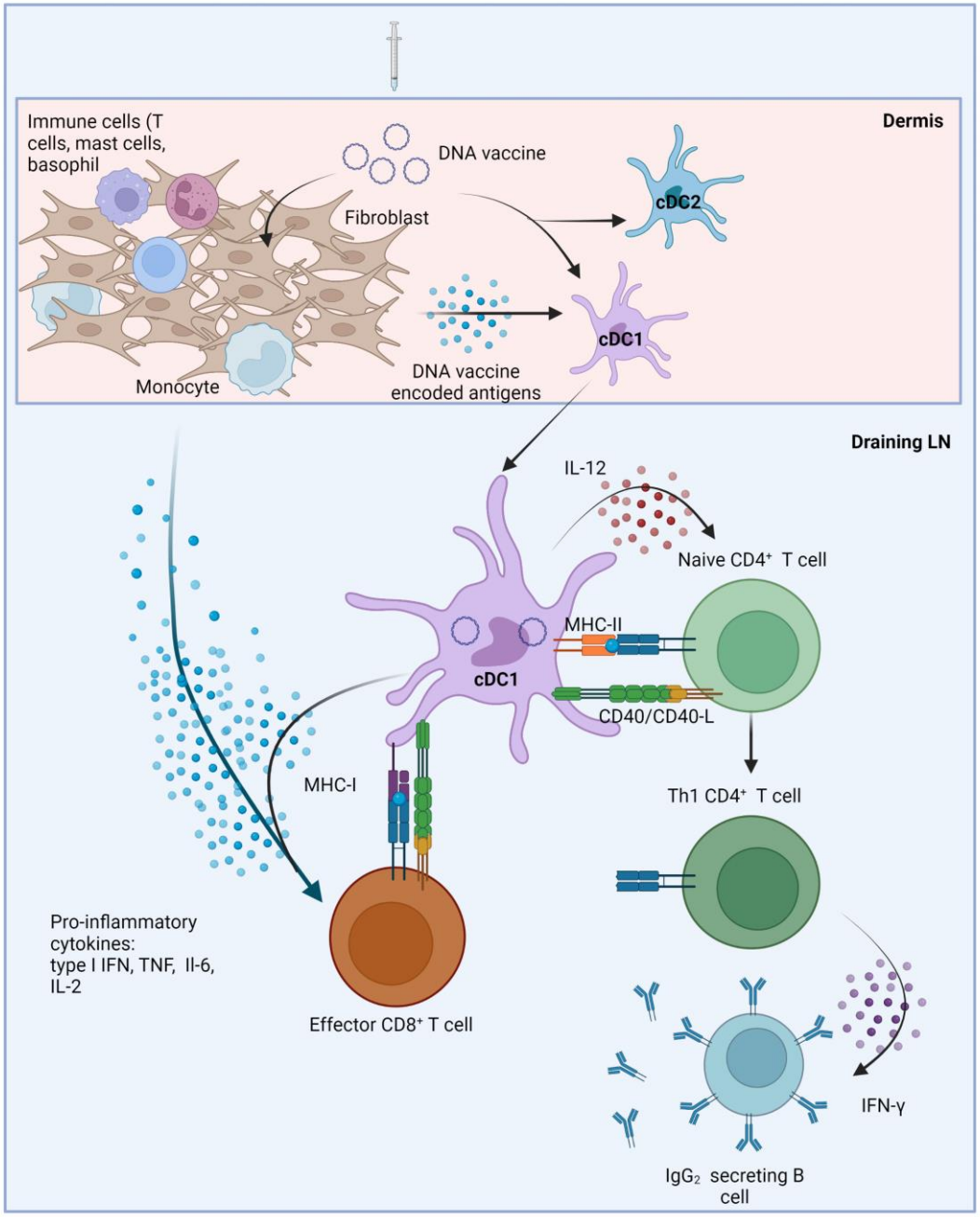
Dermis
Immune cells (T cells, mast cells, basophil
DNA vaccine
Fibroblast
cDC2
cDC1
Monocyte
DNA vaccine encoded antigens
Draining LN
IL-12
Naive CD4⁺ T cell
MHC-II
cDC1
CD40/CD40-L
MHC-I
Th1 CD4⁺ T cell
Pro-inflammatory cytokines: type I IFN, TNF, Il-6, IL-2
Effector CD8⁺ T cell
IFN-γ
IgG2 secreting B cell

Figure 6: Intradermal DNA vaccine immunization

Intradermal (ID) DNA immunization can be used to generate DNA vaccine induction of immune responses. DNA is loaded into a syringe and then injected into the dermis, where cells such as fibroblasts, conventional DCs (cDCs), T cells, and mast cells are transfected with vaccine DNA. This results in the production of pro-inflammatory cytokines, including IL-6 and type I IFNs. cDCs are required to induce adaptive immune responses post-DNA vaccination. They can acquire DNA vaccine-encoded antigens exogenously, through apoptotic bodies, or through being directly transfected. Studies have subdivided cDCs in the dermis into dermal cDC1s and dermal cDC2s. Dermal cDC1s have increased cross-presentation capabilities compared with cDC2s. Once dermal cDCs have obtained vaccine-encoded antigens, they present antigen peptides on MHC-I and MHC-II as well as migrate to the skin-draining LNs. Once in the LNs, they present peptide-loaded MHC-I and MHC-II complexes to naïve $CD8^+$ T cells and naïve $CD4^+$ T cells, respectively. Post-ID immunization preferentially induces the production of Ag-specific IgG_2 Abs, which suggests a Th1 response. Studies have reported elevated IFN-γ-secreting T cells post-ID immunization, further indicating that ID induces a Th1-biased response. Furthermore, dermal cDCs program effector $CD8^+$ T cell responses. Created with Biorender.com

As previously mentioned, Th1 $CD4^+$ T cells help to program effector $CD8^+$ T cell responses [111, 119-120]. Murine dermal cDC2s were found to polarize Th2 responses. Studies have suggested that dermal cDC2s can produce Th2-polarizing factor, TSLP, and IL-13, which drive Th2 T cell differentiation as well as IgG_1 and IgE production [121-122].

Once dermal DCs obtain DNA vaccine-encoded antigens and antigen-loaded MHC-I, they are activated by pro-inflammatory factors released by somatic and immune cells and migrate to the skin-draining LNs. There, they present antigens to $CD4^+$ T and $CD8^+$ cells in an MHC-II- and MHC-I-restricted fashion, respectively, resulting in the induction of Ab, CTL, and effector $CD8^+$ T cell responses (Figure 6) [108-110]. Although the dermis possesses Th1-polarizing dermal cDC1s and Th2-polarizing dermal cDC2s, DNA vaccine studies that have used ID immunization have demonstrated that ID immunization preferentially induces skewed Th1 responses, as evidenced by the preferential production of Ag-specific IgG_2 Abs [100, 105-107, 119, 123].

1.5.3 Intramuscular

IM immunization is the most common route of immunization for clinically approved vaccines, including those against SARS-CoV-2 (mRNA/DNA), influenza A (attenuated pathogen), and *Clostridium tetani* (protein-based). Myocytes (muscle cells) account for the vast majority of cells found in the skeletal muscular space, but there are also immune cells present in this space at a steady state. Studies have characterized such immune cells using flow cytometry and demonstrated that $CD8^+$ cDCs and monocytes are the primary types present in muscle [124-132]. Independent of vaccine type, $CD8^+$ cDCs were found to be the sole DC subset able to present exogenous Ag on MHC-I (cross-presentation) and MHCI-II, resulting in the induction of Ab, CTL, and effector $CD8^+$ T cell responses [96-97, 124-132].

In IM DNA immunization, vaccine DNA is injected into the muscle using a syringe. Although cells at the vaccination site can passively endocytose vaccine DNA, this process is not particularly efficient. To increase transfection efficiencies, EP can be employed or vaccine DNA can be formulated with lipid carriers to increase transfection efficiencies [105-107, 133-134]. Upon IM immunization, myocytes become transfected with vaccine DNA and to a lesser degree tissue-resident $CD8^+$ cDCs, and the monocytes become transfected [128-132].

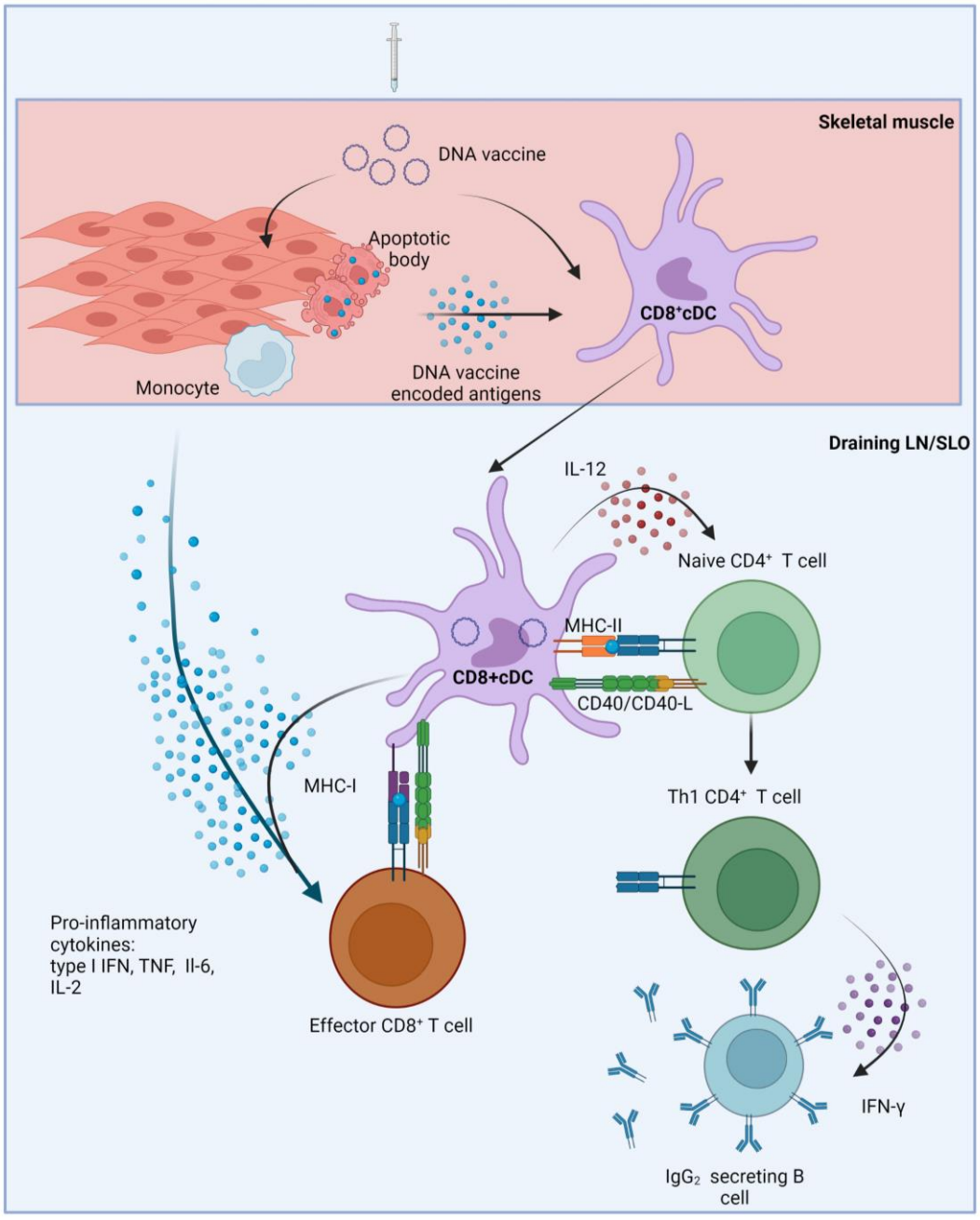
Skeletal muscle
DNA vaccine
Apoptotic body
CD8⁺cDC
Monocyte
DNA vaccine encoded antigens
Draining LN/SLO
IL-12
Naive CD4⁺ T cell
MHC-II
CD8+cDC
CD40/CD40-L
MHC-I
Th1 CD4⁺ T cell
Pro-inflammatory cytokines: type I IFN, TNF, Il-6, IL-2
Effector CD8⁺ T cell
IFN-γ
IgG2 secreting B cell

Figure 7: Intramuscular DNA vaccine immunization

Intramuscular (IM) immunization is the most commonly used route for clinically approved vaccines as well as a common route for DNA vaccine immunization. Upon intramuscular DNA immunization, cells in the muscle are transfected with vaccine DNA, including myocytes (muscle cells) and cDCs, resulting in the secretion of pro-inflammatory cytokines. Studies have found that migratory $CD8^+$ cDCs are required to mediate DNA vaccine immunogenicity. Nontransfected $CD8^+$ cDCs can endocytose exogenous antigens and/or acquire antigens from transfected myocyte-derived apoptotic bodies. Once $CD8^+$ cDCs display vaccine-encoded antigens on HMC-I and MHC-II and have been exposed to pro-inflammatory cytokines, they are activated and migrate to LNs or SLOs. There, they present antigen-loaded MHC-I and MCH-II to naïve $CD8^+$ T and naïve $CD4^+$ T cells, respectively. $CD8^+$ cDCs promote T cells to secrete IFN-γ, resulting in a Th1-biased response, as evidenced by the preferential secretion of IgG_2 over IgG_1 by B cells. Moreover, $CD8^+$ cDCs prime effector $CD8^+$ T cell responses with the help of pro-inflammatory cytokines, including type I IFNs. Created with Biorender.com

Nontransfected cDCs can obtain DNA vaccine-encoded antigens through phagocytosis and processing of myocyte-derived apoptotic bodies [52-57, 128-132]. Transfected myocytes and cDCs as well as nontransfected cells produce pro-inflammatory factors in response to the presence of both DAMPs and PAMPs, resulting in the activation/maturation and migration of cDCs to the draining LNs or SLOs [128-132].

In the LNs/SLOs, cDCs display antigen peptides to naïve $CD4^+$ T and naïve $CD8^+$ T cells in an MHC-II- and MHC-I-restricted manner [128-132]. Studies have found that IM DNA vaccination results in a biased Th1 response, as evidenced by preferential IgG_2 production and the generation of IFN-γ-secreting T cells, which promote B cell class-switching from IgG to IgG_2 [158-159]. Unfortunately, cDC subsets in skeletal muscle have not been defined. Presumably, similar to the dermis, they can be divided using cDC1 and cDC2 cell markers, but further studies are required to properly define these subsets found at steady state in skeletal muscle. Furthermore, $CD8^+$ cDCs coordinate effector $CD8^+$ T cell responses by presenting antigen-loaded MHC-I to naïve $CD8^+$ T cells (Figure 7) [128-132]. Taken together, studies have demonstrated that ED, ID, and IM DNA immunization can elicit DNA vaccine-induced protective immune responses, making these routes of immunization the most commonly used for DNA vaccination.

1.6 DNA vaccine induction of Th1 and Th2 responses

DNA vaccines can induce both Th1 and Th2 responses. Research has identified that cDCs are the APCs required to induce Th1 and Th2 responses [135-136]. Both are heavily regulated responses that have been studied for decades, but the underlying mechanisms that govern DNA vaccine-induced Th1 and Th2 responses have not been well characterized. The following subsections describe each type of response in more detail.

1.6.1 Th1 responses

Th1 responses occur in response to intracellular pathogens, cancers, as well as DNA vaccines (Figure 8). Much of what is known about the induction of Th1 responses comes from decades of research using intracellular pathogens. Although there many cDC subsets present in LN and non-LN tissues, strides have been made in simplifying the phenotypic characterization of Th1-inducing cDCs. The cDCs that drive Th1 responses are called cDC1s [135-138]. Murine tissue-resident cDC1s express *MHC-II*, *CDllc*, and *CD8*

as well as the transcription factors *IRF8* and *basic leucine zipper ATF-like transcription factor 3* (*BATF3*), while migratory cDC1s also express *CD103* [135-138]. Moreover, human cDC1s are characterized as $CD64^{-}HLA\text{-}DR^{+}CD141^{+}$ cells [135-138]. Like all cDCs, cDC1s can present antigens via MHC-I and MCH-II, but functionality studies have indicated that cDC1s possess a superior ability to cross-present antigens to $CD8^{+}$ T cells compared with their Th2-inducing cDC counterpart cDC2s [118,139].

Furthermore, cDCs such as cDC1s possess multiple PRRs that can be activated by multiple stimuli, including foreign RNA, foreign DNA, foreign protein, and polysaccharides [140-141]. The activation of cDC1 PRRs results in cDC1 activation and the production of pro-inflammatory cytokines, including IL-6, TNF-α, IL-1, IL-12, and type I IFNs, which promote cDC maturation [135-138,140-142]. Studies have suggested that somatic cells and pDCs can also aid in cDC1 maturation by producing pro-inflammatory cytokines, such as type I IFNs [142- 144]. The production of type I IFNs has been demonstrated to be critical for cDC1 activation/maturation and the subsequent induction of Th1 responses [135-138, 140-145]. Interferon-α/β receptor (IFNAR) activation by type I IFNs in cDCs triggers nuclear factor-kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) activation, resulting in the upregulation of MHC-I, MHC-II, and the co-stimulatory proteins CD80 and CD86 [135-138, 140-145].

Mature cDC1s migrate to LNs/SLOs where cDC1-bearing cognate MHCI-II/peptide complexes and co-stimulatory proteins can bind to naïve $CD4^{+}$ T cells, resulting in the activation of several signaling pathways [136, 140-145]. The binding of cDC1, MHC-II, and naïve $CD4^{+}$T cell TCRs as well as the binding of co-stimulatory proteins on both cells result in the release of Th1-polarizing cytokines by cDC1s, including IFN-γ, IL-12, IL-18, and IL-23, which promote naïve T cell differentiation into Th1 $CD4^{+}$ T cells [136, 145-151].

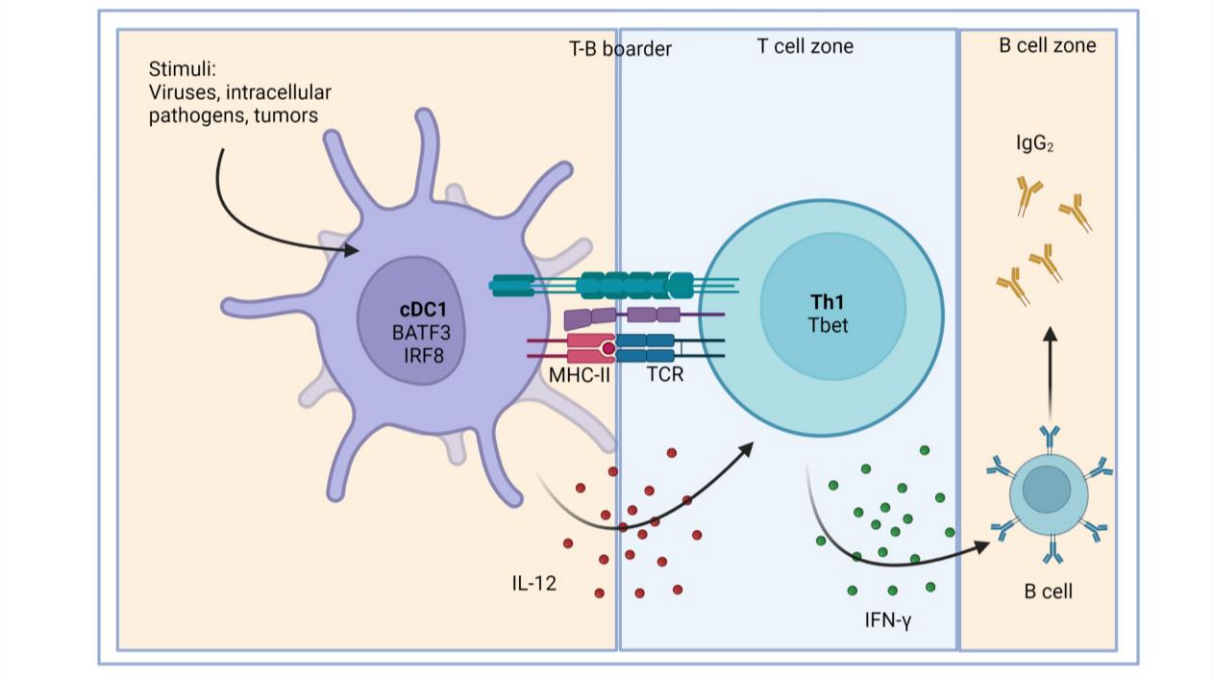

Figure 8: Induction of Th1 responses

The induction of a Th1 response is associated with intracellular pathogen infections and anti-cancer responses, but DNA vaccines can also induce Th1 responses. Strides have been made in simplifying the characterization of Th1-inducing cDCs by creating the cDC subclass of cDC1s. cDCs are classified by the expression of *CD8*, *BATF3*, and *IRF8.* cDCs can be activated through PRR activation by DAMPs and PAMPs as well as by pro-inflammatory cytokines produced by other cells. Activated cDC1s display foreign antigens on MHC-I and MHC-II and present antigen peptides to naïve $CD8^+$ T and naïve $CD4^+$ T cells, respectively. When cDC1s engage naïve $CD4^+$ T cells, they secrete Th1-polarizing cytokines such as IL-12 and IL-23. Th1-polarizing cytokines induce the differentiation of naïve $CD4^+$ T cells into Th1 $CD4^+$ T cells, which are classified by the expression of *Tbet*. Th1 $CD4^+$ T cells produce IFN-γ in concert with other immune cells, driving B cell class switching to IgG_2 secretion. Moreover, cDC1 and Th1 $CD4^+$ T cells prime effector $CD8^+$ T cell responses by producing pro-inflammatory cytokines, such as IL-6 and type I IFNs. Created with Biorender.com

The most studied Th1-polarizing cytokine is IL-12, which is critical for naïve $CD4^+$ T cell differentiation into Th1 $CD4^+$ T cells. IL-12 is a heterodimer composed of the subunits p35 and p40 and is secreted by mature cDC1s [136, 146-148]. Secreted IL-12 binds the IL-12 receptor (IL-12R) complex, which is composed of IL-12Rβ1 and IL-12β2 on naïve $CD4^+$ T cells [136, 152-154]. The recognition of IL-12 by the IL-12R complex results in the activation of the JAK-STAT signaling pathway [136, 152-154].IL-12 activation of JAK-STAT activates Jak2 and Tyk2 kinase and the transcription factors STAT1, STAT3, and STAT4 [136, 152-154]. STAT1 activation results in the expression of the transcription factor *T-box expressed in T cells* (*Tbet*), which is a transcription factor used to classify Th1 $CD4^+$ T cells [136,152-155]. Although Tbet expression is used to classify Th1 $CD4^+$ T cells, fully differentiated Th1 cells also express other transcription factors, including *NF-κB*, *nuclear factor of activated T cells* (*NFAT*), and *activating transcription factor 2* (*ATF-2*) [136, 152-155].

A hallmark of Th1 responses is IgG_2 production by B cells. Studies have demonstrated that the secretion of IFN-γ promotes class-switch recombinase in B cells to make IgG_2 Abs [156-159]. Although there are multiple sources of IFN-γ, studies have suggested that IFN-γ secretion by $CD4^+$ T cells is a critical source of IFN-γ required for IgG_2 production [156-159]. Another hallmark of Th1 responses is the generation of potent effector $CD8^+$ T cell responses. In the LNs/SLOs, cDC1s present antigen-loaded MHC-I to cognate naïve $CD8^+$ T cells' TCR [136]. The binding of MHC-I to $CD8^+$ T cells' TCR as well as the binding of stimulatory proteins result in the secretion of pro-inflammatory cytokines that program $CD8^+$ T cell effector functions, including IL-2, IL-6, IFN-γ, and type I IFNs [62-64, 73-78,136]. A study indicated that Th1 $CD4^+$ T cells can help to program effector $CD8^+$ T cells by providing additional pro-inflammatory cytokines [98]. Although the mechanism through which Th1 responses are induced by pathogens and cancer has been thoroughly studied, little is known about the mechanism that drives DNA vaccine induction of Th1 responses, including the PRRs and innate immune pathways required for DNA vaccine induction of Th1 responses.

1.6.2 Th2 responses

The induction of Th2 responses mainly occurs in response to allergens and helminth infections, but DNA vaccines can also induce Th2 responses (Figure 9) [67-69, 95-97]. Similar to Th1 T cell

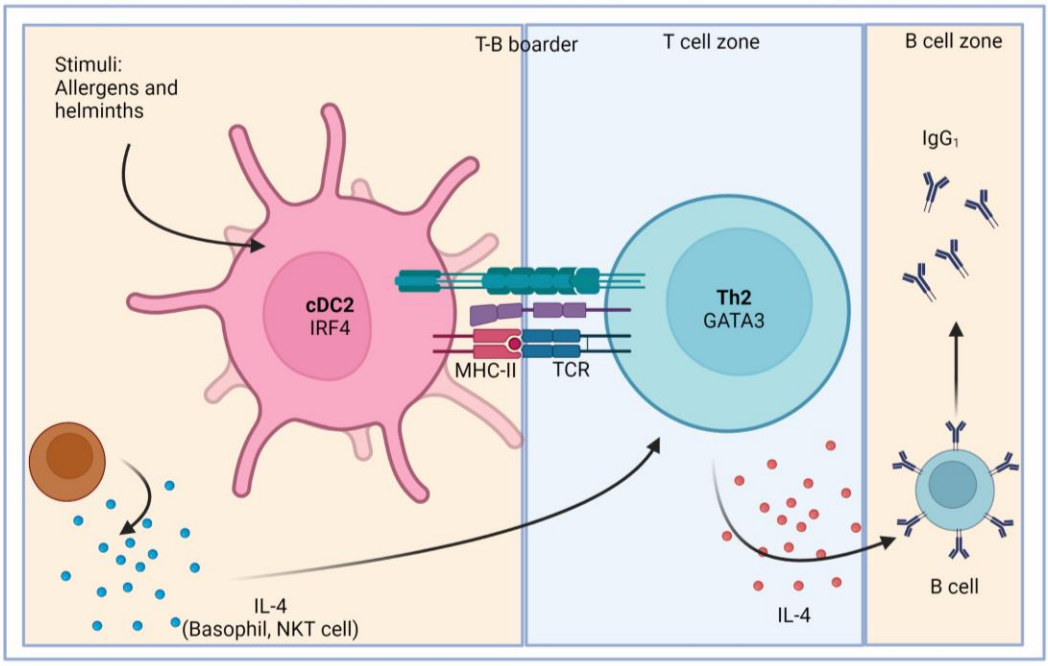

Figure 9: Induction of Th2 responses

The induction of a Th2 response is associated with helminth infections and allergens, but DNA vaccination can induce Th2 responses. Although the exact mechanisms that induce Th2 responses have not been well characterized, cDC2s – a cDC subset that expresses *IRF4* – are known to be the primary cDC that drives Th2 responses. cDC2s display antigens on MHC-I and MHC-II to naïve $CD8^+$ T and naïve $CD4^+$ T cells, respectively. The production of IL-4 is required to drive naïve $CD4^+$ T cell differentiation into Th2 $CD4^+$ T cells, although the cells required to produce IL-4 are not currently known. Once differentiated, Th2 $CD4^+$ T cells characteristically express *GATA3* and produce IL-4, which promote B cells to undergo class switching to IgG_1 as well as IgE production. cDC2s possess inferior cross-presentation capabilities but can present antigen peptides on MHC-I to naïve $CD8^+$ T cells as well as prime $CD8^+$ T cell effector functions. Created with Biorender.com

responses, cDC1s are required for the induction of adaptive immune responses, but Th2 responses require cDC2s for the induction of Th2 responses [160-162]. cDC2s are defined by the expression of *IRF4* [163]. cDC2s can process antigens and display them on MHC-II and MHC-I, but they possess inferior cross-presentation abilities compared with cDC1s [118,139]. Unfortunately, the exact mechanism that promotes Th2 responses has not been well characterized though IL-4 production is required for induction of Th2 responses [90-91, 95, 164].

Moreover, studies have suggested that cDC2s are activated by and can produce the Th2-polarizing cytokines TSLP, IL-5, and IL-13, which promote cDC2 activation and migration to the draining LNs and SLOs [90-91,95,164]. In the draining LNs and SLOs, cDC2s display antigens on MHC-II to naïve $CD4^+$ T cells and present antigens on MHC-I to naïve $CD8^+$ T cells [90-91, 95, 164]. Studies have found a central role for IL-4 in driving the differentiation of naïve $CD4^+$ T cells into Th2 $CD4^+$ T cells. Evidence suggests that cDC2s do not produce IL-4 while other immune cells such as basophils, innate lymphoid cell-2s (ICL2s), and natural killer T cells (NKT) do produce it [90-91, 95, 164].

Independent of the source, IL-4 binds to the IL-4 receptor (IL-4R) on naïve $CD4^+$ T cells, which promotes Th2 differentiation through the phosphorylation of the transcription factor STAT6 [164]. Activated STAT6 induces the expression of the transcription factor *GATA binding protein 3* (*GATA3*), which defines cDC2s [164]. GATA3 subsequently drives IL-4 production [164], which in turn promotes B cells to undergo class switching from IgG to IgG_1 and IgE, although the exact mechanism has not been defined [121-122, 165-167]. Moreover, studies have indicated that Th2 $CD4^+$ T cells can aid in programming $CD8^+$ T cell effector functions, but the mechanism has also not been well defined [98-99]. Notably, although ED DNA vaccine delivery is known to induce a Th2-biased response (IgG_1 Ab response), it induces robust effector $CD8^+$ T cell responses, possibly because LCs, and not cDC2s, are the main APCs that mediate DNA vaccine induction of adaptive immune responses; nevertheless, more studies are required to gain an enhanced understanding of this finding [90-91, 95-97].

1.7 Concluding remarks

DNA vaccines are an exciting technology used to combat infectious diseases and cancers. Unlike traditionally used protein-based vaccines, DNA vaccines can elicit Ab, CTL, and effector $CD8^+$ T cell responses. Moreover, they can induce both Th1 and Th2 responses, as suggested by the production of IgG_2 and IgG_1, respectively. Unfortunately, the PRRs and innate immune signaling pathways required to mediate DNA vaccine induction of adaptive responses have not been fully defined. The following chapters aim to define the relationship between innate immune signaling pathways and DNA vaccine induction of adaptive responses. They also aim to provide new insights into the mechanisms through which the innate immune pathways mediate vaccine immunogenicity.

2 DNA vaccines require *STING* for the induction of Th1-associated antibody responses and $CD8^+$ T cell polyfunctionality

2.1 Introduction to innate immune signaling and DNA vaccine immunogenicity

Although early studies revealed that DNA vaccines can induce Ab, CTL, and effector $CD8^+$ T cell responses, researchers had little understanding of how or whether the activation of the innate immune system by DNA vaccines influences downstream Ab and effector $CD8^+$ T cell responses. Since vaccine DNA is exposed to the cytoplasm of transfected cells, it was hypothesized that innate immune dsDNA-sensing pathways might be required to mediate DNA vaccine immunogenicity. The first innate immune pathway to be investigated for its role in mediating DNA vaccine immunogenicity was the *toll-like receptor 9 (TLR9)* pathway [177]. TLR9 is an endosomal dsDNA-sensing protein that, upon binding dsDNA, activates the adaptor protein *myeloid differentiation primary responses protein 88* (*MYD88*), which results in the phosphorylation and translocation of *interferon regulatory factor 3/7* (*IRF3/7*) into the nucleus. Once in the nucleus, phosphorylated IRN3/7 induces expression type I IFN [168] (Figure 10). Moreover, *TLR9* activation results in the activation of the transcription factor *nuclear factor Kappa B* (*NF-κB*), which induces the production of pro-inflammatory cytokines, including IL-6 and IL-12 [168]. TLR9 has been extensively studied for recognizing the PAMP oligodeoxynucleotide CpG (CpG ODN) motifs [169-172]. Both bacterial and viral DNA can contain CpG motifs, but DNA in mammalian cells lacks these motifs. These motifs have been demonstrated to activate a wide range of immune cells, including DCs, natural killer (NK) cells, and B cells [173-175].

Since DNA vaccines are mostly commonly propagated and isolated from bacteria such as *E. coli*, they can possess CpG motifs; thus, researchers hypothesized that DNA vaccines require *TRL9* to mediate DNA vaccine-induced Ab and effector $CD8^+$ T cell responses. This hypothesis was bolstered by a report that DNA vaccines had increased immunogenicity in mice when co-administered with exogenous CpG ODN [176]. A 2004 study vaccinated $TLR9^{-/-}$ and WT ($TLR9^{+/+}$) mice using ID immunization twice with a DNA vaccine containing a CpG motif that expressed glycoprotein D (gD) derived from bovine herpesvirus type I [177].

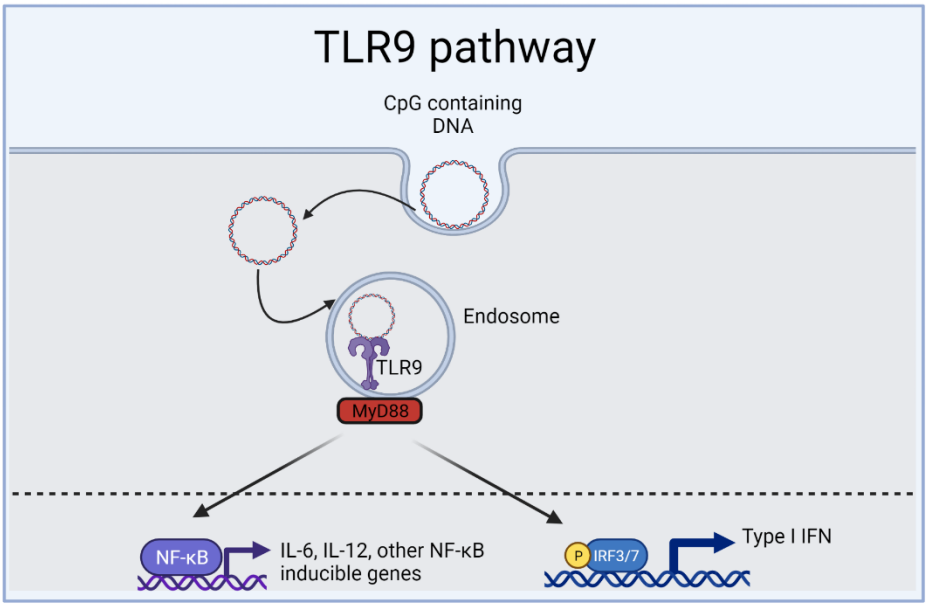

Figure 10: The toll-like receptor 9 pathway

Early experiments hypothesized that the TLR9 recognition of CpG motifs in DNA vaccine was required to mediate DNA vaccine immunogenicity. The GpG motif containing dsDNA is taken up by host cells through endocytosis, where it then enters the cell. Endosomes containing TLR9 encircle dsDNA where TLR9 can recognize and bind dsDNA. Upon TLR9 activation, MyD88 is activated, resulting in NF-κB and IRF7 translocation into the nucleus where they initiate the production of NF-κB-stimulated genes (IL-6, IL-12, and IFN-α) as well as IRF-7-stimulated genes (type I IFNs), respectively. Created with Biorender.com

TLR9$^{-/-}$ and WT mice generated similar anti-gD IgG titers post-vaccination. Moreover, the study found that TLR9 was dispensable for DNA vaccine induction of CTL responses as TLR9$^{-/-}$ mice exhibited similar numbers of IFN-γ secreting splenocyte cells compared with the WT mice after splenocytes were stimulated with glycoprotein D peptides.

This study found that TLR9 influenced DNA vaccine induction of Th1 and Th2 responses by measuring Ag-specific Th1-assocated IgG_2 and Ag-specific Th2-associated IgG1 concentrations, finding that TLR9 was dispensable for DNA vaccine induction of Th1 and Th2 responses [177]. Interestingly, it was found that type I IFNs were similar between *TLR9*$^{-/-}$ KO mice and WT mice, which indicates that DNA vaccines induce type I IFN production in a TLR9-independent manner [177-178].

To investigate whether type I IFNs are required to mediate DNA vaccine immunogenicity, another study IM vaccinated interferon-alpha/beta receptor knockout (IFNAR$^{-/-}$) and WT (IFNAR$^{+/+}$) mice with 10 μg of a DNA vaccine that expressed β-galactosidase (LacZ) [179]. DNA vaccinated IFNAR$^{-/-}$ mice developed significantly lower concentrations of Ag-specific IgG and IgG_{2a} but not IgG_1 compared with WT mice, suggesting that type I IFNs are required for DNA vaccine induction of Th1-associated IgG_2 Ab responses but not for Th2-associated Th2 IgG_1 responses. Additionally, IFNAR$^{-/-}$ mice had significantly lower frequencies of Ag-specific IFN-γ$^+$ CD4$^+$ T cells compared with the WT mice. Studies have also demonstrated that IFN-γ secretion by CD4$^+$ T cells is critical for B cells to undergo class-switching from IgG secretion to IgG_2 secretion [158-159], which indicates a critical role for type I IFNs in driving DNA vaccine induction of Th1-associated IgG_2 Ab responses. Moreover, DNA-vaccinated IFNAR$^{-/-}$ mice had significantly reduced frequencies of Ag-specific IFN-γ$^+$ CD8$^+$ T cells, suggesting that type I IFNs are required for DNA vaccine induction of effector CD8$^+$ T cell responses [179].

Although DNA vaccines had been demonstrated to require type I IFNs to induce IgG_2 Ab and effector CD8$^+$ T cell responses, it remained unclear whether any innate immune proteins are required upstream of type I IFNs to mediate DNA vaccine immunogenicity. Shortly after *TLR9* was found to be dispensable for DNA vaccine immunogenicity, *Z-DNA binding protein 1* (*ZBP1*, renamed *DAI*) was discovered to bind cytoplasmic dsDNA and induce the production of type I IFNs (Figure 11) [180-183]. ZBP1 was demonstrated to bind dsDNA and subsequently activate *TANK binding kinase 1 (TBK1)* [180-

183]. Activated TBK1 then phosphorylates IRF3/7, resulting in the production of type I IFNs [180-183]. Since type I IFNs had been demonstrated to be vital for DNA vaccine induction of Th1 responses, researchers hypothesized that ZBP1 and TBK1 are required to mediate DNA vaccine induction of Th1 Ab and effector $CD8^+$ T cell responses [180].

Furthermore, studies have demonstrated that DNA-vaccinated *TBK1*$^{-/-}$ mice generate significantly lower frequencies of IFN-γ^+ $CD4^+$ T cells and significantly lower IgG_{2a} titers compared with WT mice. Additionally, $CD8^+$ T cells from *TBK1*$^{-/-}$ mice were found to have diminished effector functions compared with WT mice as they failed to lyse a comparable percentage of LacZ peptide-pulsed cells *in vitro* [180]. Using bone marrow chimeras, it was demonstrated that *TBK1* must be present in hematopoietic cells but not in nonhematopoietic cells, which suggests that *TBK1* is required in an immune cell for DNA vaccine immunogenicity. Interestingly, DNA-vaccinated WT and *ZPB1*$^{-/-}$ mice exhibited similar Ag-specific Ab and effector $CD8^+$ T cell responses. The data from these studies indicate that *TBK1* is required upstream of type I IFN production for mediating DNA vaccine induction of Th1-associated IgG_2 responses and effector $CD8^+$ cell responses. Moreover, the data indicate that DNA vaccines may require another cytosolic dsDNA-sensing innate immune protein upstream of *TBK1* to mediate vaccine immunogenicity.

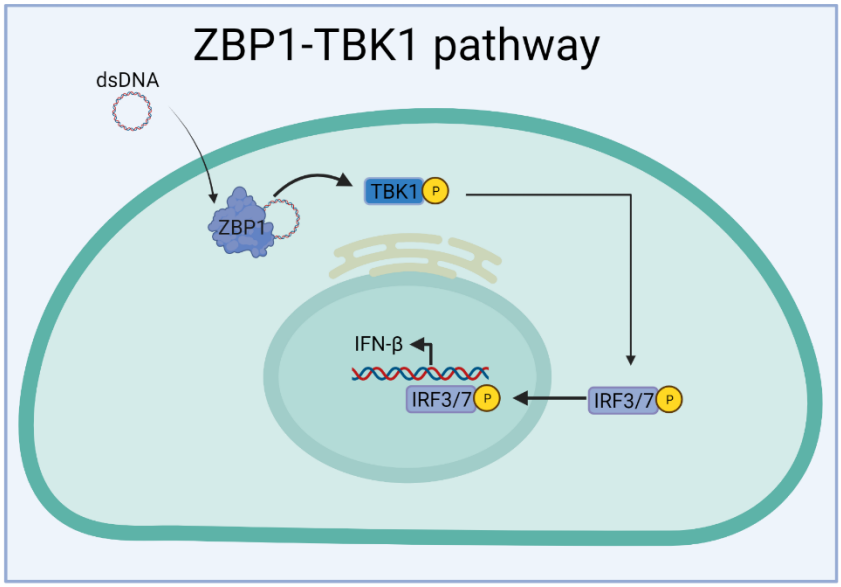

Figure 11: The ZBP1-TBK1 pathway

Studies have assessed whether ZBP1 and/or TBK1 are required to mediate DNA vaccine immunogenicity. ZBP1 is a cytoplasmic dsDNA-sensing protein. At the time of these experiments, it was known that when ZBP1 binds to dsDNA and becomes activated, it activates TBK1 through an unknown mechanism. Phosphorylated TBK1 induces IRF3/7 activation and translocation into the nucleus, which results in the production of type I IFNs. Subsequent studies have found that TBK1 is activated by STING, but the mechanism through which ZBP1 activates STING remains unknown. Created with Biorender.com

In 2013, the innate immune cytosolic dsDNA-sensing protein 2'3' cyclic GMP-AMP (cGAS) was discovered [184]. *cGAS* binds to cytoplasmic dsDNA and upon doing so converts GTP and ATP into the secondary messenger cGAMP [184-187]. As cGAMP accumulates in the cytoplasm, it binds to the protein *stimulator of interferon gene (STING)*, which is a dimeric protein located on the endoplasmic reticulum, resulting in STING phosphorylation [188-190]. Once phosphorylated, *STING* recruits and phosphorylates TBK1, resulting in the production of type I IFNs (Figure 12).

Since cGAS can bind to dsDNA and STING is upstream of TBK1, researchers hypothesized that cGAS and STING are required to coordinate DNA vaccine induction of Ab and effector $CD8^+$ T cell responses. In 2016, this hypothesis was tested in WT, *cGAS*$^{-/-}$, and *STING*$^{-/-}$ mice [191]. The mice were IM vaccinated two or three times with 10 μg of a DNA vaccine that encoded influenza A hemagglutinin (HA), and then Ag-specific Ab titers and effector $CD8^+$ T cell responses were measured. The study found that the WT and *cGAS*$^{-/-}$ mice generated similar titers of HA-specific IgG, IgG_{2C}, and IgG_1 as well as similar numbers of IgG Ab-secreting cells (ASCs), which suggested that *cGAS* is dispensable for DNA vaccine induction of Ab responses. cGAS was also found to be dispensable for DNA vaccine induction of effector $CD8^+$ T cells as the WT and *cGAS*$^{-/-}$ mice developed similar numbers of IFN-γ-secreting $CD8^+$ cells. Although *cGAS* was found to be dispensable for DNA vaccine immunogenicity, the study found that *STING* is required for DNA vaccine induction of Ab and effector $CD8^+$ T cell responses. STING$^{-/-}$ mice generated significantly lower IgG and IgG_{2C} titers, suggesting that *STING* is required for DNA vaccine induction of Th1-associated IgG_2 responses. Moreover, DNA vaccinated *STING*$^{-/-}$ mice generated significantly fewer IFN-γ secreting $CD8^+$ cells compared with the WT mice, indicating that *STING* is required for DNA vaccine induction of effector $CD8^+$ T cell responses. Phosphorylated STING induces the activation of the NF-κB pathway, which results in the induction of pro-inflammatory cytokines that can aid in effector $CD8^+$ T cell programming, including IL-6 and TNF, thus this study evaluated whether *STING* is required for the production of pro-inflammatory cytokines and found that *STING*$^{-/-}$ mice had lower concentrations of IL-6, TNF-α, and IFN-β compared with WT mice post-vaccination, suggesting that DNA vaccines require *STING* for the induction of pro-inflammatory cytokines and subsequent effector $CD8^+$ T cell responses [191-193].

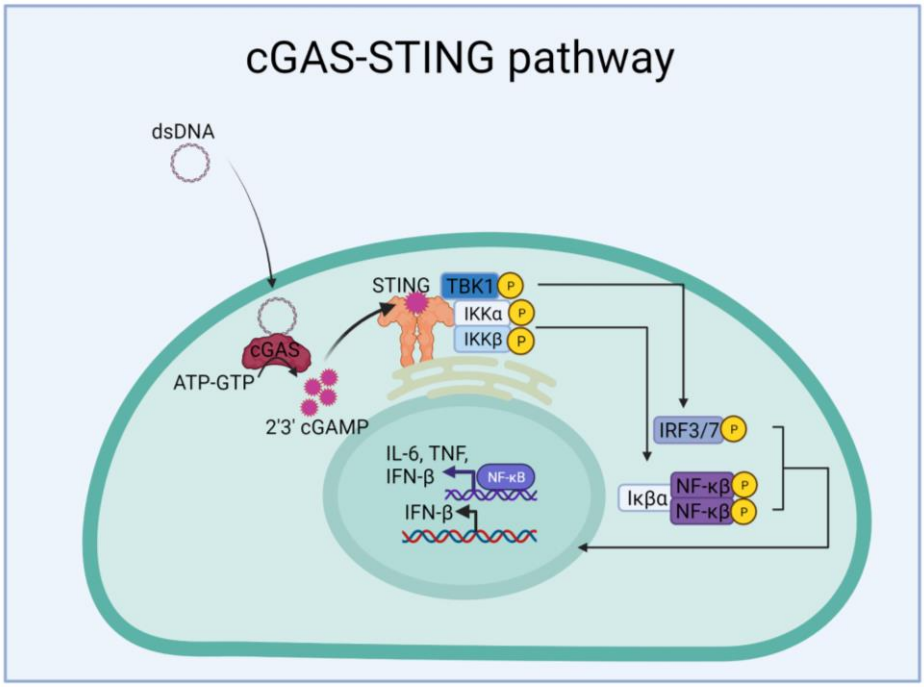

Figure 12: The cGAS-STING pathway

Double-stranded DNA enters a cell's cytoplasm where it is recognized and bound by cGAS, resulting in cGAS activation. cGAS converts ATP-GTP into the secondary messenger cGAMP upon activation. As cGAMP accumulates within a cell, it is recognized by dimeric STING on the endoplasmic reticulum, resulting in STING activation. STING activation results in the phosphorylation of TBK1 and IKKα/IKKβ (Iκβα). Activated TBK1 and Iκβα subsequently phosphorylate IRF3/7 and NF-κB, respectively. IRF3/7 and NF-κB translocate into the nucleus where they activate IFN-β and NF-κB genes (IL-6, TNF, and IFN-β). Created with Biorender.com

Taken together, the aforementioned studies indicate that the cytosolic dsDNA-sensing proteins TLR9 and cGAS are dispensable for DNA vaccine immunogenicity, but that *STING* and subsequent type I IFN production are required for DNA vaccine induction of Ab responses, specifically Th1-associated IgG_2 responses and effector $CD8^+$ T cell responses. Interestingly, later studies have found that *STING* can be activated by many cytosolic dsDNA-sensing proteins, including cGAS, *interferon gamma inducible protein 16 (IFI16)*, *ZBP1, and DEAD-Box Helicase 41 (DDX41)*, although the mechanisms have not been fully characterized (Figure 13) [194-197]. Therefore, I further investigated the mechanism through which *STING* governs the downstream DNA vaccine induction of Th1- and Th2-biased Ab and effector $CD8^+$ T cell responses.

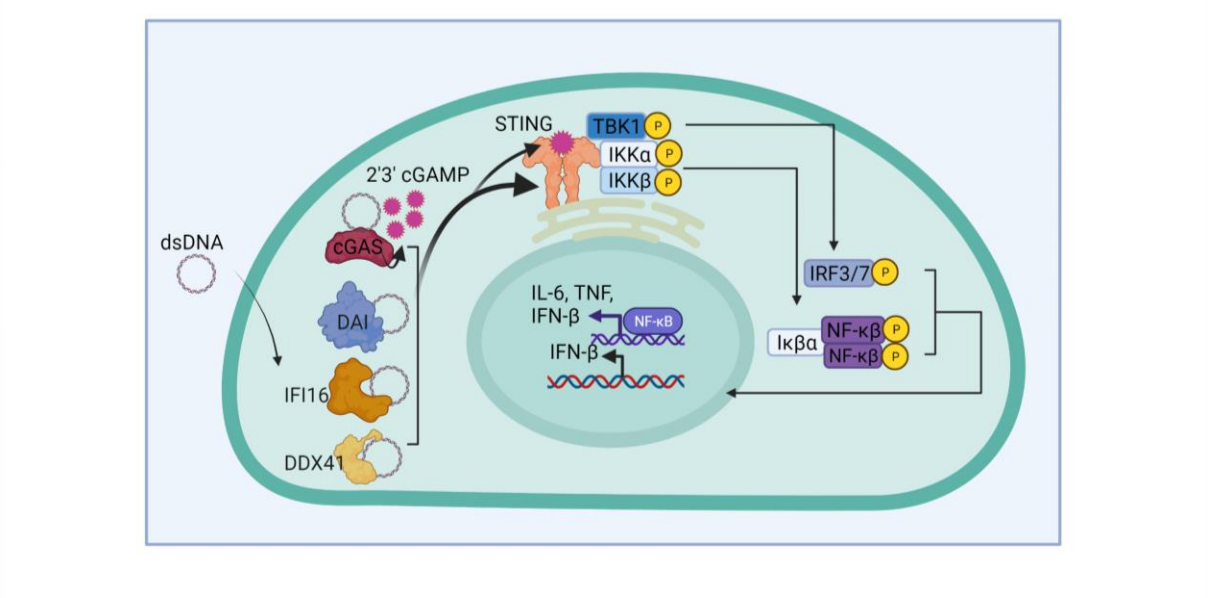

Figure 13 Alternative STING activation

Although cGAS has been well characterized in terms of its role in activating STING through cGAMP, evidence suggests that STING can be activated by other dsDNA-sensing proteins, including DAI (ZBP1), IFI16, and DDX41, although the exact mechanism is not well understood. Created with Biorender.com

2.2 Results

2.2.1 STING, but not cGAS, is required to mediate DNA vaccine induction of Th1-associated IgG_{2C} antibody responses

Since DNA vaccines can be introduced into various sites of vaccination including the epidermis and muscle which consist DC-like LCs and DCs, respectively, I wished to assess whether *STING* is required independently of the modality of vaccination as well as independently of the vaccinating antigen. Although studies have demonstrated that *STING* but not cGAS is required to mediate DNA vaccine-induced IgG_2 Ab and effector $CD8^+$ T cell responses, I hypothesized that *STING,* but not cGAS, is required for the induction of Th1-biased Ab responses independent of the antigen used. To test this hypothesis, WT, *cGAS*$^{-/-}$, and *STING*$^{-/-}$ mice were vaccinated by IM delivery and electroporation (IM/EP) with 10 µg of a codon-optimized DNA vaccine expressing influenza A nucleoprotein (pNP; Figure 14). Sera were collected 14, 21, and 28 days post-vaccination to analyze the induction of NP-specific IgG Ab responses. *STING*$^{-/-}$ mice generated significantly lower anti-NP IgG Ab concentrations at 21 and 28 days post-vaccination compared with WT and *cGAS*$^{-/-}$ mice (Figure 15a), a result that confirmed previous studies that *STING,* but not *cGAS*, plays a role in DNA vaccine immunogenicity [191]. The lower IgG concentrations in *STING*$^{-/-}$ mice led me to investigate whether both Th1 (IgG_{2C}) and Th2 (IgG_1) Ab responses were impacted by *STING* or *cGAS*. An analysis of IgG_{2C} (figure 15b) and IgG_1 (figure 15c) concentrations as well as the IgG_{2C}:IgG_1 ratio (Figure 15d) in vaccinated WT, *cGAS*$^{-/-}$, and *STING*$^{-/-}$ mice revealed that *STING*$^{-/-}$ mice developed significantly lower Th1-associated anti-NP IgG_{2C} than WT and *cGAS*$^{-/-}$ mice, but they had comparable levels of Th2-associated NP-specific IgG_1 Ab concentrations.

To test whether the modality of DNA vaccination affects the requirement for *STING* to mediate vaccine induction of Th1-associated IgG_{2C} responses, I vaccinated WT, *cGAS*$^{-/-}$, and *STING*$^{-/-}$ mice with 1 µg of pNP via GG immunization. Vaccine DNA was delivered into the skin of the abdomen by removing fur prior to vaccination (Figure 16). I observed a similar outcome to that of IM/EP-vaccinated *cGAS*$^{-/-}$, *STING*$^{-/-}$, and WT mice (Figure 17). Taken together, these data are consistent with previous findings that have indicated that *STING* is required to induce Ab responses, but they further indicated that *STING* is necessary for generating Th1-associated IgG_{2C} Ab but not Th2-associated IgG_1 responses.

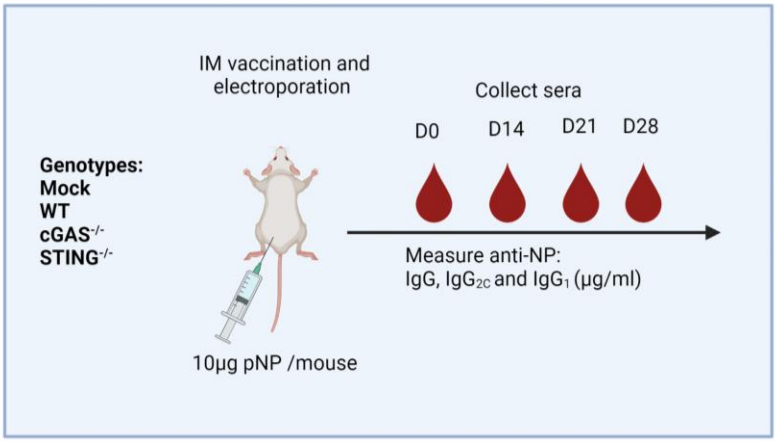

Figure 14: Experimental design for evaluating IM-/EP-induced antibody responses

WT, *cGAS*$^{-/-}$, and *STING*$^{-/-}$ mice were IM/EP vaccinated with 10 µg of pNP split between the interior tibiales. Then, each muscle was electroporated (see Materials and methods [Chapter 4]). Sera were collected prior to vaccination (D0) as well as 14 (D14), 21 (D21), and 28 days (D28) post-vaccination. At each timepoint, anti-NP IgG, IgG_{2C}, and IgG_1 concentrations were calculated (µg/mL). Created with Biorender.com

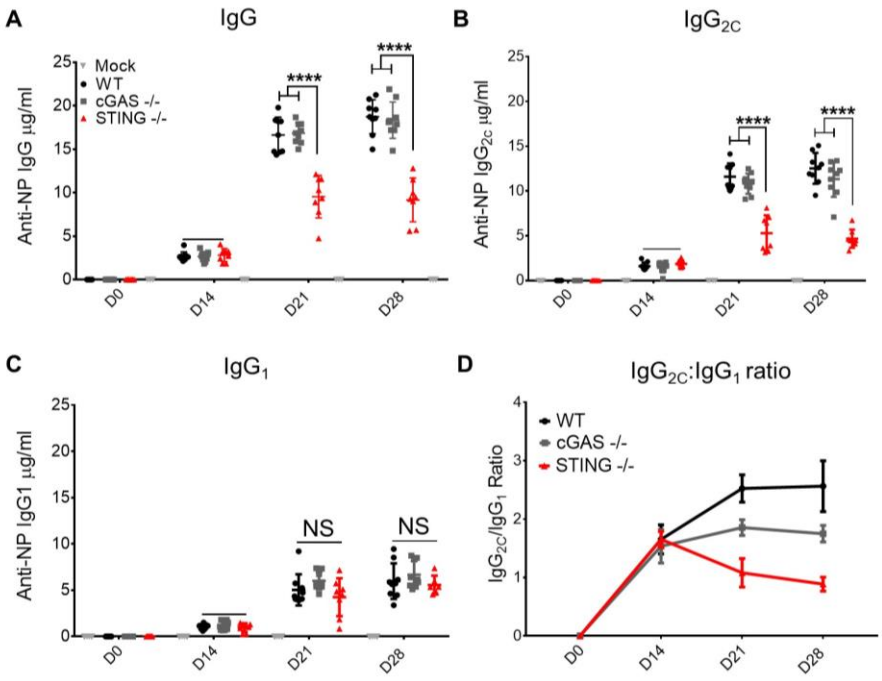

Figure 15: *STING* is required for DNA vaccine induction of IgG_{2C} (Th1) antibody responses

WT, *cGAS*$^{-/-}$, and *STING*$^{-/-}$ mice were IM/EP vaccinated with a DNA vaccine (pNP) expressing influenza nucleoprotein (pNP). Sera were collected prior to vaccination (D0) and then at 14 (D14), 21 (D21), and 28 days (D28) post-vaccination. The concentrations of anti-NP (**A**) IgG, (**B**) IgG_{2C}, and (**C**) IgG_1 antibody responses were measured using ELISA. Each ELISA plate used goat anti-mouse IgG and known concentrations of mouse IgG, IgG_{2C}, or IgG_1 to create a standard curve to back-calculate antibody titers (µg/mL). (**D**) The Th1:Th2 ratio was calculated as IgG_{2C}:IgG_1, where a higher ratio was indicative of a Th1 response. Three independent experiments consisting of three to nine mice were performed; the data presented are the average ±SD of three to nine mice/genotype. At each timepoint, a one-way ANOVA was conducted; ****$p < 0.0001$, NS = nonsignificant.

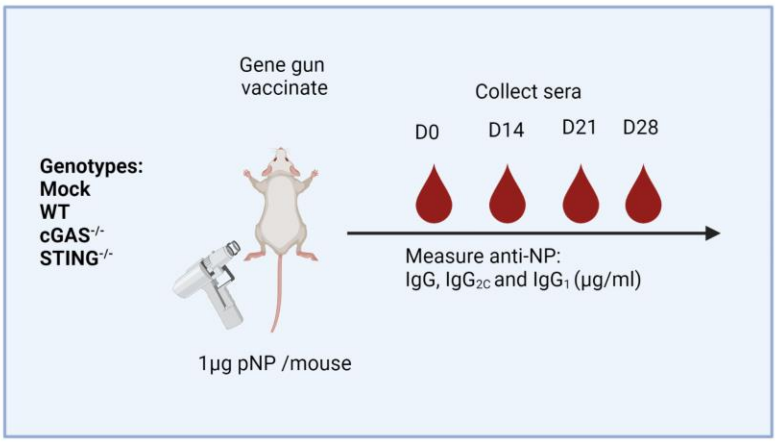

Figure 16: Experimental design for assessing GG-induced antibody responses

WT, *cGAS*$^{-/-}$, and *STING*$^{-/-}$ mice were gene gun (GG) vaccinated with 1 µg of pNP. Abdominal skin was exposed by shaving the mice prior to vaccination and epidermal immunization was conducted using a GG. Sera were collected (D0) prior to vaccination as well as 14 (D14), 21 (D21), and 28 days (D28) post-vaccination. At each timepoint, anti-NP IgG, IgG_{2C}, and IgG_1 concentrations were calculated (µg/mL). Created with Biorender.com

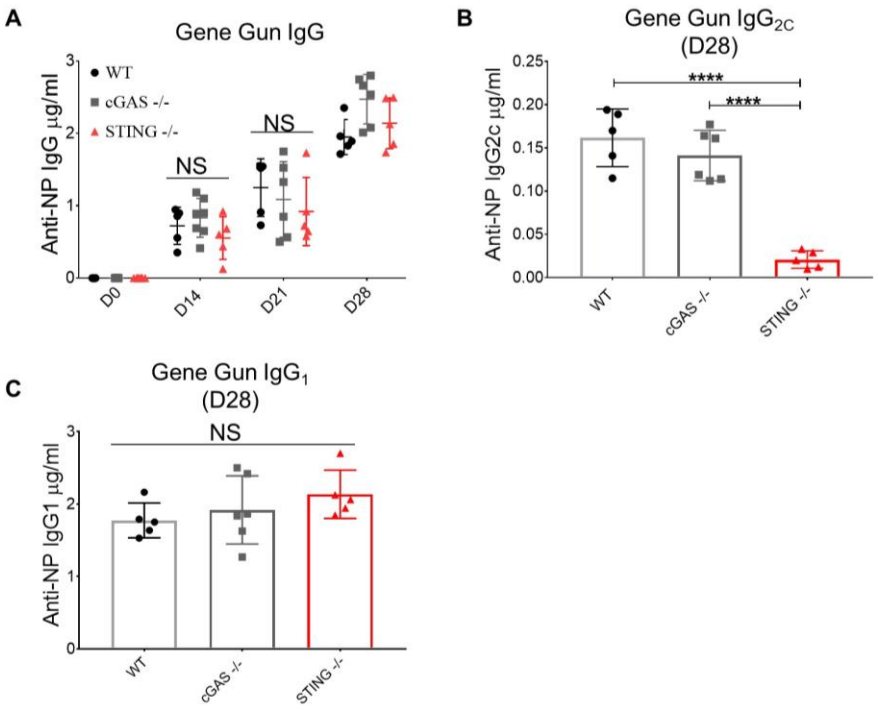

Figure 17: *STING* is required for DNA vaccine induction of IgG_{2C} antibody responses independent of the vaccine delivery method

WT, *cGAS*$^{-/-}$, and *STING*$^{-/-}$ mice were vaccinated with pNP using a gene gun to deliver vaccine DNA into the dermis. Sera were collected prior to vaccination (D0) as well as 14 (D14), 21 (D21), and 28 days (D28) post-vaccination. (**A**) The concentration of anti-NP IgG was measured, although the overall responses were lower than those in IM/EP vaccinated mice. Anti-NP (**B**) IgG_1 and (**C**) IgG_{2C} titers were measured 28 days post-vaccination. Three independent experiments were performed, which consisted of 5–10 mice; the data presented are the average ± SD of five to six mice/genotype. A one-way ANOVA was employed to compare groups; *$p < 0.05$, **$p < 0.01$, and NS = nonsignificant.

2.2.2 STING is required for DNA vaccine induction of polyfunctional CD8$^+$ T cell responses

A hallmark of DNA vaccines is their ability to induce Th1 responses that mediate the induction of CD8$^+$ T cell responses, including CTLs [14-16, 198-199]. To determine whether *STING* or *cGAS* plays a role in DNA vaccine induction of CD8$^+$ T cell responses, I vaccinated WT, *cGAS*$^{-/-}$, and *STING*$^{-/-}$ mice with pNP by IM/EP vaccine delivery (Figure 18). The mice were sacrificed 21 days post-vaccination and the frequencies of antigen-specific CD8$^+$ T cells were measured using tetramers specific for $NP_{366-374}$, an immunodominant NP-specific CD8$^+$ T cell epitope, through flow cytometry (Materials and methods [Chapter 4], Figure 32). The frequencies of $NP_{366-374}$ tetramer-binding CD8$^+$ T cells were not statistically different between WT, *cGAS*$^{-/-}$, and *STING*$^{-/-}$ mice (Figure 19a), indicating that *cGAS* and *STING* are not required for DNA vaccine induction of antigen-specific CD8$^+$ T cells. Next, to determine whether *STING* impacts the effector functions of CD8$^+$ T cells, I measured the frequencies of polyfunctional CD8$^+$ T cells expressing one or more cytokines (IL-2, TNF-α, and IFN-γ) and the cytolytic markers CD107a/granzyme B following stimulation with $NP_{366-374}$ using intracellular cytokine staining (ICS) followed by flow cytometry (Materials and methods [Chapter 4], Figure 34). To compare CD8$^+$ T cell effector functions, a polyfunctionality index score was calculated for each mouse. The polyfunctional index enumerates cellular polyfunctionality as a one-dimensional value, where a greater value is assigned to CD8$^+$ T cells that express more functions [200]. CD8$^+$ T cells in *STING*$^{-/-}$ mice exhibited significantly lower polyfunctional index scores compared with those in WT and *cGAS*$^{-/-}$ mice (Figure 19b, c). This finding suggests that *STING* and *cGAS* are not required for the generation of antigen-specific CD8$^+$ T cells, but that *STING* is required for the induction of polyfunctional CD8$^+$ T cell responses.

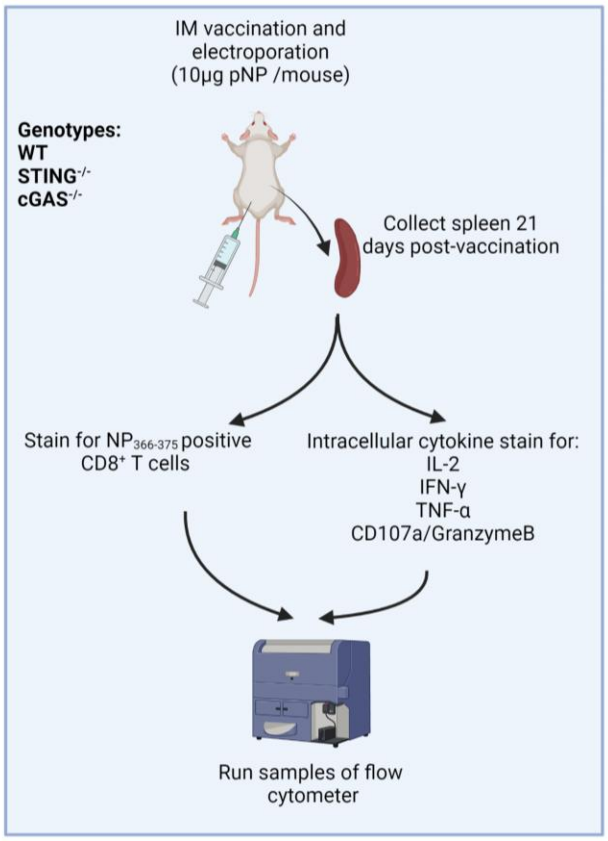

Figure 18: Experimental design for evaluating DNA vaccine induction of polyfunctional effector CD8⁺ T cell responses

WT, *STING*$^{-/-}$, *cDC LitC*, and *cDC STING cKO* mice were IM/EP vaccinated with 10 µg of pNP. The mice were sacrificed 21 days post-vaccination, spleens were collected, and single-cell suspensions were produced. Splenocytes were either stimulated with the CD57Bl/6 CD8⁺ T cell immunodominant peptide $NP_{366-347}$ to evaluate polyfunctionality or stained to quantify the frequencies of Ag-specific tetramer-positive ($NP_{366-374}$) CD8⁺ T cells through flow cytometry (see Materials and methods [Chapter 4]). Created with Biorender.com

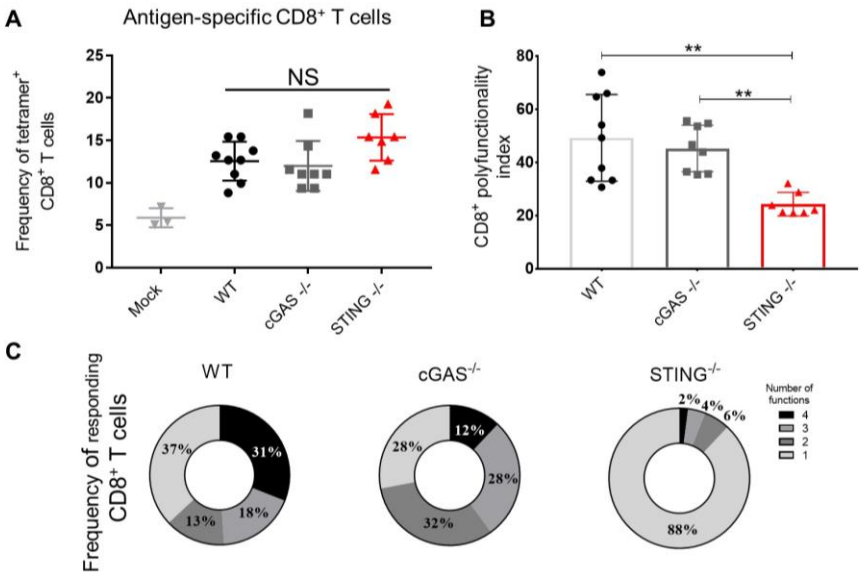

Figure 19: *STING* is required for DNA vaccine induction of antigen-specific CD8⁺ T cell polyfunctionality

WT, *cGAS*$^{-/-}$, and *STING*$^{-/-}$ mice were IM/EP vaccinated with pNP. The mice were sacrificed 21 days post-vaccination and splenocytes were isolated and made into single-cell suspensions. (**A**) The frequency of tetramer-positive cells within CD8⁺ T cells was measured for each genotype using an MHC-I tetramer that presented the NP immunodominant peptide ($NP_{366-374}$) for C57bl/6. CD8⁺ T cells were gated as outlined in Supplemental Figure 3b. (**B**) Polyfunctional scores were determined by stimulating splenocytes overnight with 1 µg/mL of $NP_{366-375}$ followed by ICS staining for IL-2, TNF-α, IFN-γ, and CD107a/granzyme B and flow cytometry analysis, as demonstrated in Supplemental Figure 4. Polyfunctionality scores were calculated as described in Chapter 4 and by Larsen et al. [43]. (**C**) Pie charts showing the relative average proportion of CD8⁺ T cells producing one to four immune factors (IL-2, TNF-α, IFN-γ, and/or CD107a/granzyme B) after $NP_{366-374}$ stimulation. Three independent experiments consisting of three to nine mice were performed; the presented data are the average ± SD of three to nine mice/genotype. Groups were compared using a one-way ANOVA; ****$p < 0.0001$, NS = nonsignificant.

2.3 Discussion

The results of my experiments confirmed that *cGAS* is not required for DNA vaccine-induced Ab and $CD8^+$ T cell responses. Additionally, the results suggested a critical role for *STING* in coordinating DNA vaccine induction of Th1-associated IgG_2 Ab responses, which has been described in previous studies [180, 191]. Moreover, the results indicated that *STING* is not required for DNA vaccination induction of Ag-specific $CD8^+$ T cells but is required for the programming of polyfunctional $CD8^+$ T cell effector functions. Previous studies have demonstrated that DNA vaccines require TBK1 in hematopoietic cells to induce Ab and T cell responses [28]. Given that *STING* is immediately upstream of *TBK1*, I attempted to identify in which hematopoietic cell *STING* is required for mediating DNA vaccine induction of Ab and effector $CD8^+$ T cells, as described in the following chapter.

3 *STING* is required within conventional dendritic cells to coordinate DNA vaccine induction of Th1-associated antibody responses, but is dispensable within conventional dendritic cells for vaccine-induced polyfunctional $CD8^+$ T cell cells

3.1 Introduction to dendritic cells and DNA vaccines

DNA vaccine induction of Ab and effector $CD8^+$ T cell responses is greatly influenced by the site of vaccination (Section 1.5). As outlined in Chapter 1, IM and ID DNA vaccine delivery relies on $CD8^+$ cDCs to induce Th1 responses, including vaccine-induced IgG_2 production and effector $CD8^+$ T cell responses. Numerous studies have attempted to exploit the requirement of DCs to increase DNA vaccine immunogenicity by directly targeting cDCs [201-204]. Some studies have targeted cDCs by developing DNA vaccines that encode fusion proteins that consist of the target antigen fused to ligands recognized by DCs or fused to DC-targeting Abs [201-204]. For example, a DNA vaccine was developed to express *X-C* motif chemokine ligand 1 (XCL1) fused to influenza a HA (pXCL1-HA) [202]. XCl1 is recognized by X-C motif chemokine receptor 1 (XCR1), which is expressed on cDCs, and the study demonstrated that pXCL1-HA induced significantly higher titers of anti-HA Th1-associated IgG_2 Abs compared with mice vaccinated with a pan-immune (NK cells, B cells, cDC macrophages, T cells, and other immune cells) targeting DNA vaccine [202]. The study observed an increased IgG_2 response induced by pXCL1-HA in both IM and ID vaccinated mice, further suggesting a critical role of cDCs in mediating DNA vaccine Th1-associated IgG_2 responses independent of the modality of vaccination. Moreover, the aforementioned studies have indicated that cDC-targeting DNA vaccines increase effector $CD8^+$ T cell responses. Taken together, cDCs have been demonstrated to be required for DNA vaccine induction of Ab and effector $CD8^+$ T cell responses. Therefore I set out to investigate if *STING* is required within cDCs to mediate DNA vaccine induction of immune responses.

3.2 Results

3.2.1 *STING* expression within conventional dendritic cells is not required for DNA vaccine induction of $CD8^+$ T cell responses

Given that cDCs are required to drive DNA vaccine-induced Th1 responses and previous reports have indicated that *TBK1* (a protein immediately downstream of *STING*) is required within hematopoietic cells to mediate DNA vaccine immunogenicity, I hypothesized that *STING* is required within cDCs to mediate DNA vaccine-induced Th1-associated IgG_2 Ab as well as $CD8^+$ responses [52-57, 180]. To test this hypothesis, I developed conditional knockout mice that had *STING* selectively absent in cDCs.

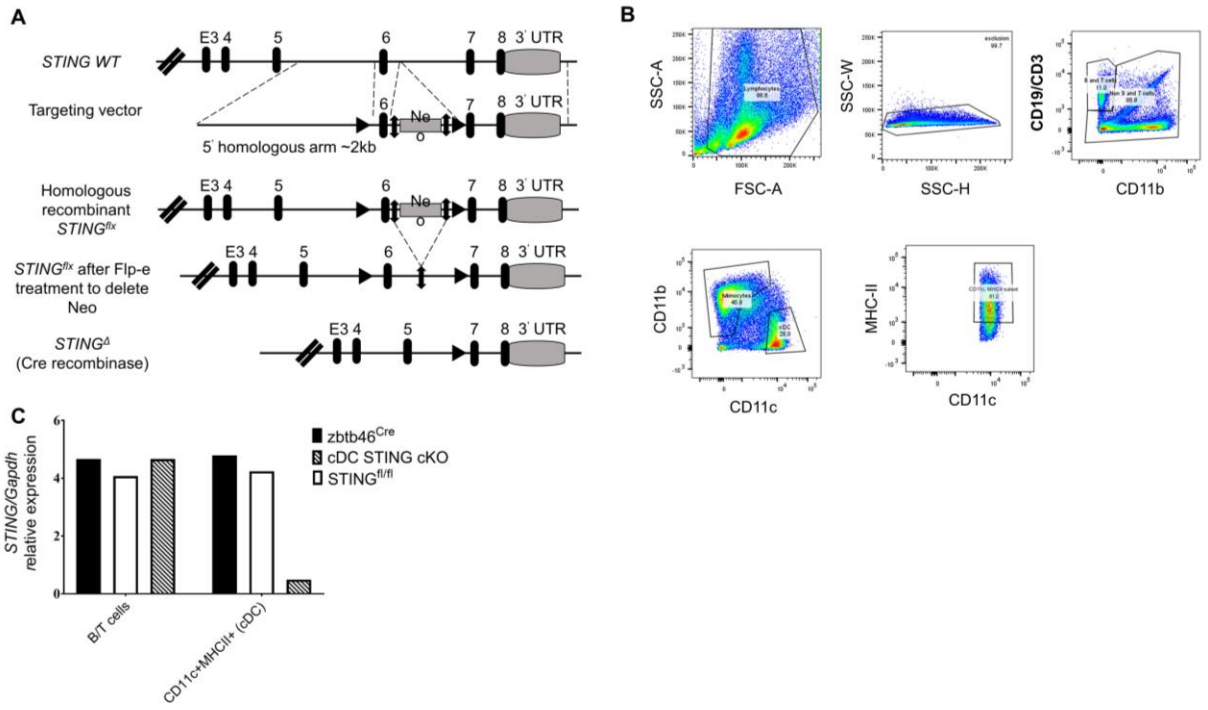

Figure 20: Validation of *zbtb46^{Cre} x STING$^{fl/fl}$ (cDC STING cKO)* mice

(**A**) Generation of a conditional allele using a sequence replacement strategy to knock out the *STING* gene. The construct contained LoxP sites that flank exon 6, a 2kb 5' homologous, and a Neomycin (Neo) cassette flanked by FRT sites for selective deletions. The Neo element allowed for positive selection in embryonic stem cells. After homologous recombination of the conditional KO construct, the Neo was excised FLPe administration. The *STING* gene was normally expressed until the Cre-mediated deletion of exon 6. This recombination created a frameshift mutation that resulted in a nonfunctioning *STING* gene. Gating strategies were used to isolate individual immune cells to measure relative *STING* expression with a BD FACS ARIA III cell sorter. (**B**) Lymphocytes were identified by excluding doublets using forward and side light scatter, then B/T cells were isolated ($CD19^{+}CD3^{+}$), and then non-B/T cells were further gated to isolate cDCs ($CD11c^{+}MHC\text{-}II^{hi}$). (**C**) qRT-PCR analysis of *STING* exon 6 to verify deletion under Cre control. Splenocytes from *zbtb46^{Cre}*, *STING$^{fl/fl}$*, and *cDC STING cKO* mice were isolated through flow cytometry as described above, RNA was extracted and made into cDNA, and then the relative expression of *STING* was measured.

To make conditional KO (cKO) mice, *STING*$^{fl/fl}$ mice on a C57BL/6 background were crossed with *zbtb46*^{Cre} mice on a C57BL/6 background, which resulted in *zbtb46*^{cre} *x STING*$^{fl/fl}$ mice (cDC STING cKO) (Materials and methods [Chapter 4], Figure 20a,b). *Zinc finger and BTB domain containing 46 (zbtb46)* is a gene that is only expressed in cDCs ($CD8^+$ and $CD8^-$), not in pDCs [205]. I verified that *STING* was absent in cDCs by isolating the cells through flow cytometric sorting (BD FACS ARIA) from *zbtb46*^{cre}, *STING*$^{fl/fl}$, and *cDC STING cKO (zbtb46*^{cre}*x STING*$^{fl/fl}$*)* mice and measured the relative levels of *STING* (Figure 20c).

First, I tested whether *STING* is required within cDCs to mediate DNA vaccine induction of $CD8^+$ T cell responses. WT, *STING*$^{-/-}$, *cDC STING cKO*, and *cDC STING littermate control* (*cDC STING LitC*) mice were vaccinated (to control for potential deleterious effects on *STING* due to the introduction of flox sites) through the IM/EP delivery of 10 µg of pNP (Figure 21). The mice were sacrificed 21 days post-vaccination and splenocytes were isolated to measure Ag-specific $CD8^+$ T cell responses using $NP_{366\text{-}374}$ tetramer staining (Figure 22a) as well as polyfunctional $CD8^+$ T cell responses using ICS and flow cytometry (Figure 22b, c). WT, *STING*$^{-/-}$, *cDC STING cKO*, and *cDC STING LitC* mice exhibited similar frequencies of tetramer-binding $CD8^+$ T cells (Figure 22a). In addition, WT, *cDC STING cKO*, and *cDC STING LitC* mice developed similar levels of polyfunctional $CD8^+$ T cell responses but *STING*$^{-/-}$ mice developed significantly lower polyfunctional effector $CD8^+$ T cell responses (Figure 3b, c). Together, these results indicate that while *STING* is required for the induction of polyfunctional $CD8^+$ T cell responses but it is not required within cDCs.

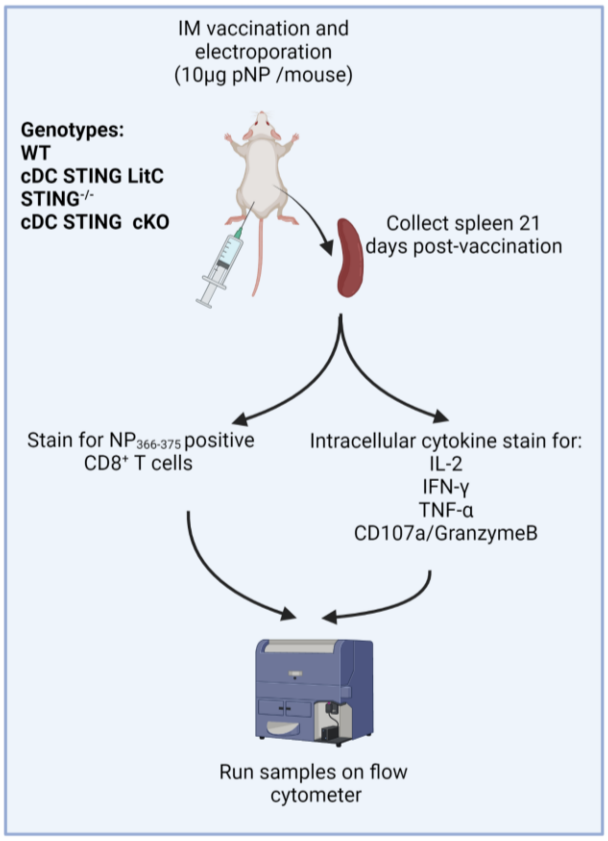

Figure 21: Experimental design for evaluating CD8⁺ polyfunctionality in *cDC STING cKO* mice

WT, *STING*$^{-/-}$*, cDC LitC,* and *cDC STING cKO* mice were IM/EP vaccinated with 10 µg of pNP. Mice were sacrificed 21 days post-vaccination, spleens were collected, and single-cell suspensions were produced. Splenocytes were either stimulated with the CD57Bl/6 CD8⁺ T cell immunodominant peptide $NP_{366-347}$ to evaluate CD8⁺ T cell polyfunctionality or stained to quantify the frequencies of Ag-specific tetramer-positive ($NP_{366-374}$) CD8⁺ T cells through flow cytometry (see Materials and methods [Chapter 4]). Created with Biorender.com

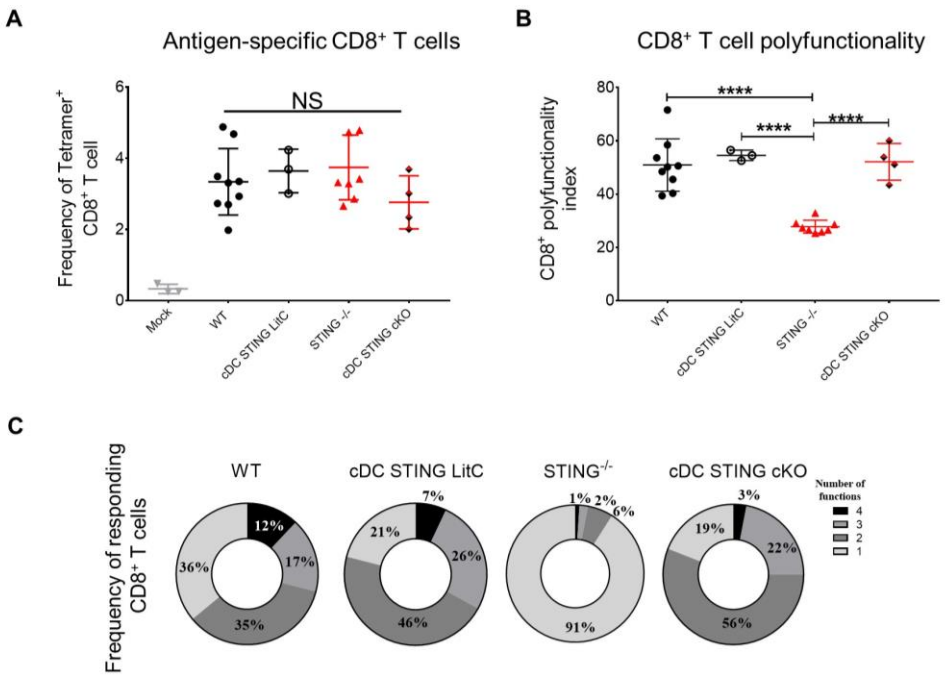

Figure 22: *STING* is not required within conventional dendritic cells for mediating DNA vaccine induction of CD8⁺ T cell responses

WT, *STING$^{-/-}$, zbtb46^{Cre} x STING$^{fl/fl}$* (*cDC STING cKO),* and *cDC STING littermate control* (*cDC STING LitC*) mice were IM/EP vaccinated with pNP. Mice were sacrificed 21 days post-vaccination and splenocytes were collected. (**A**) Tetramer-positive cells within CD8⁺ T cells were measured for each genotype using a NP immunodominant peptide ($NP_{366-374}$) containing tetramer, as described above and demonstrated in Supplemental Figure 3c. (**B**) Polyfunctional scores were determined by stimulating splenocytes overnight with 1 µg/mL of $NP_{366-374}$; staining for IL-2, TNF-α, IFN-γ, and CD107a/granzyme B; and analyzing them using flow cytometry and Boolean gating, as demonstrated in Supplemental Figure 4. The CD8⁺ T cells' polyfunctionality index scores are presented. (**C**) Pie charts depicting the relative average proportion of responding CD8⁺ T cells that produced at least one immune function (IL-2, TNF-α, IFN-γ, and/or CD107a/granzyme B) after $NP_{366-374}$ stimulation. Three independent experiments were performed consisting of three to nine mice; the data presented are the average ± SD of three to nine mice/genotype. Groups were compared using a one-way ANOVA; ****p < 0.0001, NS

3.2.2 STING is not required within cDCs to induce pro-inflammatory cytokines post-DNA vaccination

Given that I observed that *STING* was required for programming $CD8^+$ T cell responses but was not required within cDCs, I wondered whether differences existed in the pro-inflammatory cytokines produced by *STING*$^{-/-}$ and *cDC STING cKO* mice. The production of pro-inflammatory cytokines influences the generation of Th1 or Th2 responses as well as $CD4^+$ and $CD8^+$ T cell effector functions (191, 205-208). To determine whether *STING* within cDCs influences the induction of pro-inflammatory cytokines, I vaccinated WT, *STING*$^{-/-}$, *cDC STING cKO,* and *cDC STING LitC* mice with pNP through IM/EP delivery. Serum concentrations of pro-inflammatory cytokines were measured prior to vaccination at 6 and 24 hours post-vaccination (Figure 23). A control group of mice were mock-treated by IM injection of saline followed by IM/EP to control for the induction of pro-inflammatory cytokines induced by the vaccination method. Consistent with previous studies, I found that sera from *STING*$^{-/-}$ mice had significantly lower levels of TNF-α (Figure 24a), IL-6 (Figure 24b), and IFN-β (Figure 24c) compared with sera from WT, *cDC STING cKO,* and *cDC STING LitC* mice. *STING*$^{-/-}$ mice also had lower levels of IL-2 (Figure 24c) compared with sera from WT, *cDC STING cKO,* and *cDC STING LitC* mice, but these differences did not reach statistical significance. These findings suggested that *STING* mediates the induction of pro-inflammatory cytokines after DNA vaccination, but not within cDCs.

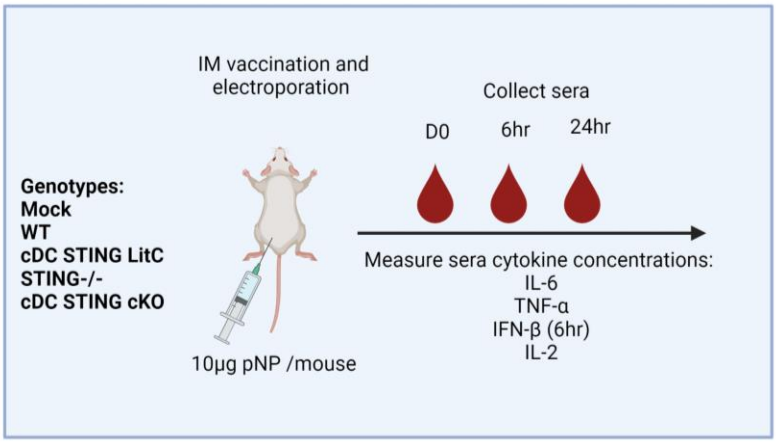

Figure 23: Experimental design for measuring DNA vaccine-induced pro-inflammatory cytokines

WT, *STING*$^{-/-}$, *cDC LitC,* and *cDC STING cKO* mice were IM/EP vaccinated with 10 μg of pNP and another group of mice were mock-vaccinated to gauge the effect IM/EP on the induction of pro-inflammatory cytokines. All mice were bled prior to vaccination and IM/EP to gain baseline concentrations of IL-6, TNF-α, IFN-β (6 h), and IL-2. Mice were also bled 6 h and 24 h post-vaccination to measure pro-inflammatory cytokines. Created with Biorender.com

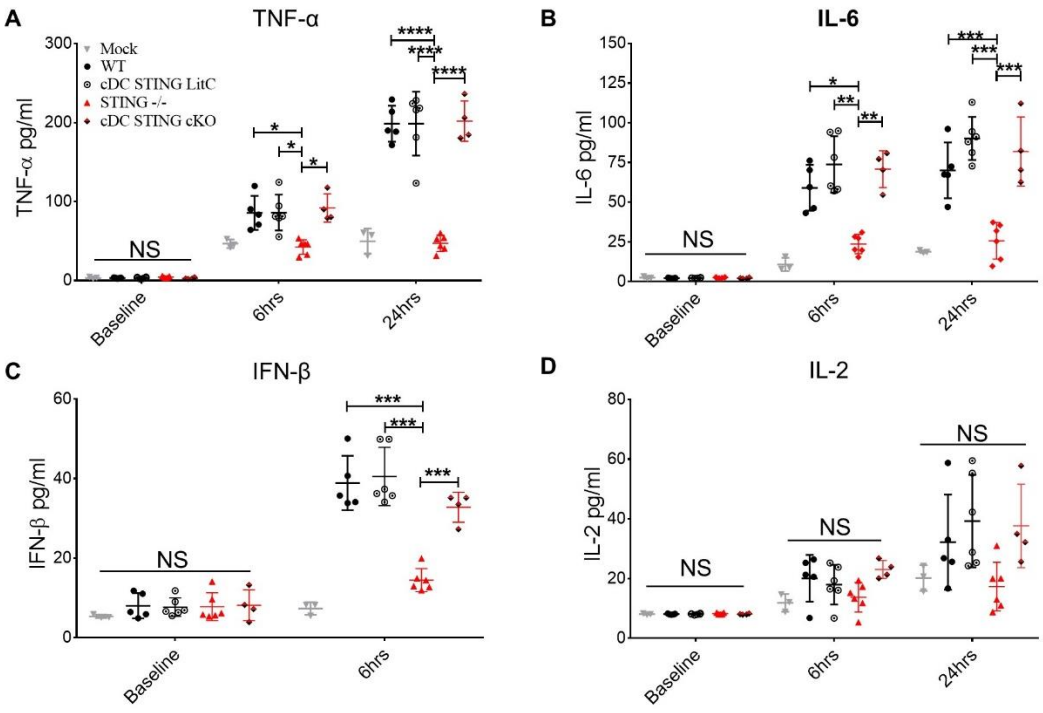

Figure 24: STING is not required within cDCs for the induction of innate pro-inflammatory cytokines

WT, *STING$^{-/-}$*, *cDC STING cKO*, and *cDC STING LitC* mice were IM/EP vaccinated with pNP. Sera were collected prior to vaccination (baseline) as well as 6 h and/or 24 h post-vaccination, and sera concentrations of (**A**) TNF-α, (**B**) IL-6, **(C)** IFN-β, and (**D**) IL-2 were calculated. Three independent experiments were conducted consisting of three to six mice; the data presented are the averages ± SD of three to six mice/genotype. At each timepoint, a one-way ANOVA was performed; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, NS = nonsignificant.

3.2.3 *STING* is required within cDCs for DNA vaccine induction of Th1-associated IgG_{2C} antibody responses

Since my findings indicated that *STING* is required for mediating optimal IgG response, specifically Th1-associated IgG_{2C} Abs, I next investigated whether *STING* within cDCs was necessary for generating these responses. I vaccinated WT, *STING*$^{-/-}$, *cDC STING cKO*, and *cDC STING LitC* mice with pNP through IM/EP delivery. IgG, IgG_1, and IgG_{2C} Ab responses were measured using ELISA prior to vaccination (D0) as well as 14, 21, and 28 days post-vaccination (Figure 25). *STING*$^{-/-}$ and *cDC STING cKO* mice developed significantly lower NP-specific IgG (Figure 26a) and IgG_{2C} (Figure 26b) 21 and 28 days post-vaccination, but exhibited comparable IgG_1 (Figure 26c) responses compared with WT and *cDC STING LitC* mice. Consequently, *STING*$^{-/-}$ and *cDC STING cKO* mice also had lower Th1/Th2 ratios (IgG_{2C}:IgG_1) than control mice (Figure 26d), indicating a lower Th1 response. Together, these results indicated that *STING* within cDCs is required to induce Th1 (IgG_{2C}) Ab responses. To determine whether the lower IgG_{2C} Ab responses observed in *STING*$^{-/-}$ and *cDC STING cKO* mice were due to a reduction in the number of antibody secreting cells (ASCs, Figure 27), the numbers of NP-specific IgG (Figure 28a), IgG_{2C} (Figure 28b), and IgG_1 (Figure 28c) ASCs were quantified 21 days post-vaccination using a B-cell ELIspot assay. *STING*$^{-/-}$ and *cDC STING cKO* mice were found to have developed comparable numbers of IgG_1 ASCs but significantly fewer IgG and IgG_{2C} ASCs than the control mice. This further supported my findings that *STING* is required within cDCs to induce IgG_{2C}-secreting B cells following DNA vaccination.

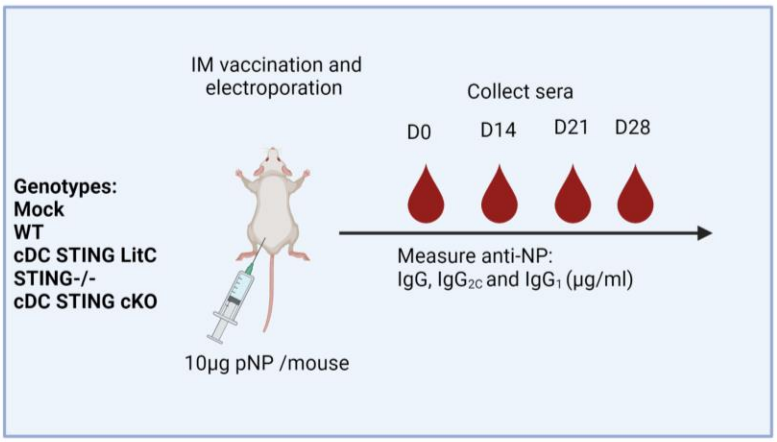

Figure 25: Experimental design for evaluating DNA vaccine-induced antibody responses in *cDC STING cKO* mice

WT, *STING*$^{-/-}$*, cDC LitC,* and *cDC STING cKO* mice were IM/EP vaccinated with 10 µg of pNP. Sera were collected prior to vaccination (D0) as well as 14 (D14), 21 (D21), and 28 days (D28) post-vaccination. At each timepoint, the concentrations of anti-NP IgG, IgG_{2C}, and IgG_1 were calculated. Created with Biorender.com

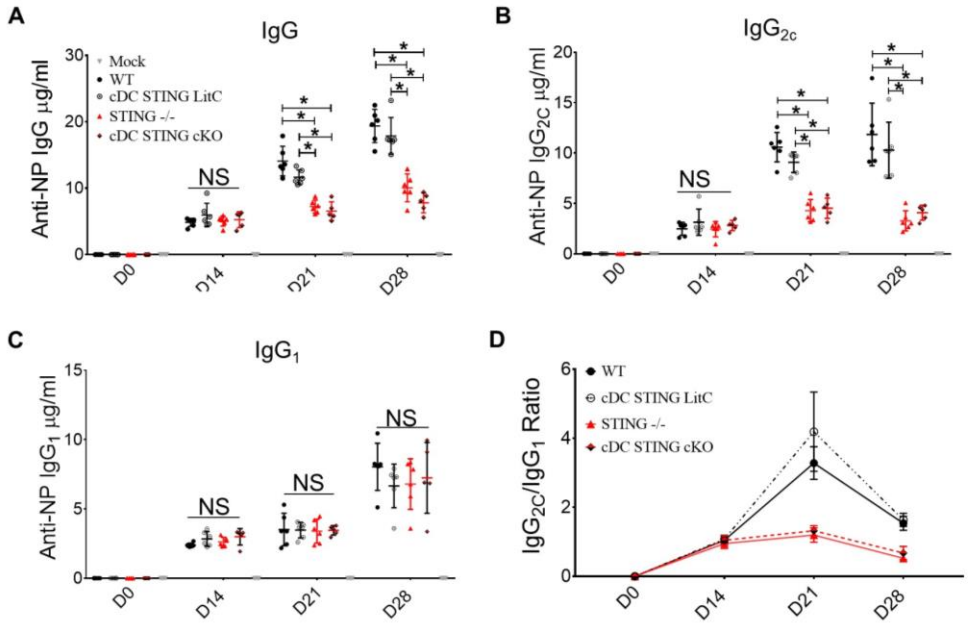

Figure 26: *STING* is required within conventional dendritic cells for DNA vaccine induction of IgG_{2c} antibody responses

WT, *STING*$^{-/-}$, *cDC STING cKO*, and *cDC STING LitC* mice were IM/EP vaccinated with pNP. Sera were collected prior to vaccination (D0) as well as 14 (D14), 21 (D21), and 28 days (D28) post-vaccination. Anti-NP (**A**) IgG, (**B**) IgG_{2C}, and (**C**) IgG_1 antibody responses were measured using ELISA as described above. (**D**) The Th1:Th2 ratio was calculated as the ratio of IgG_{2C}:IgG_1. A Th1:Th2 score >1 indicated a predominant Th1 response, with higher scores indicating stronger Th1 responses. Three independent experiments were performed consisting of three to seven mice; the data presented are the average ± SD of three to seven mice/genotype. A one-way ANOVA was employed to compare groups; *$p < 0.05$, NS = nonsignificant.

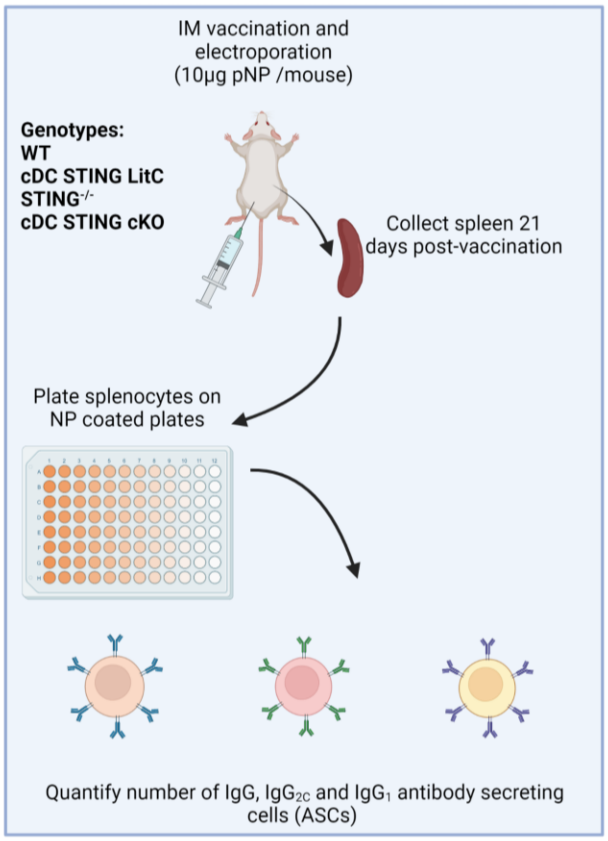

Figure 27: Experimental design for evaluating DNA vaccine-induced antibody-secreting cells in cDC STING cKO mice

WT, *STING$^{-/-}$, cDC LitC,* and *cDC STING cKO* mice were IM/EP vaccinated with 10 µg of pNP. At 21 days post-vaccination, the mice were sacrificed and spleens were collected and made into a single-cell suspension. Splenocytes were then added to ELISpot plates coated with recombinant NP and incubated overnight. The following day, the numbers of anti-NP IgG, IgG_{2C}, and IgG_1 antibody-secreting cells were evaluated. Created with Biorender.com

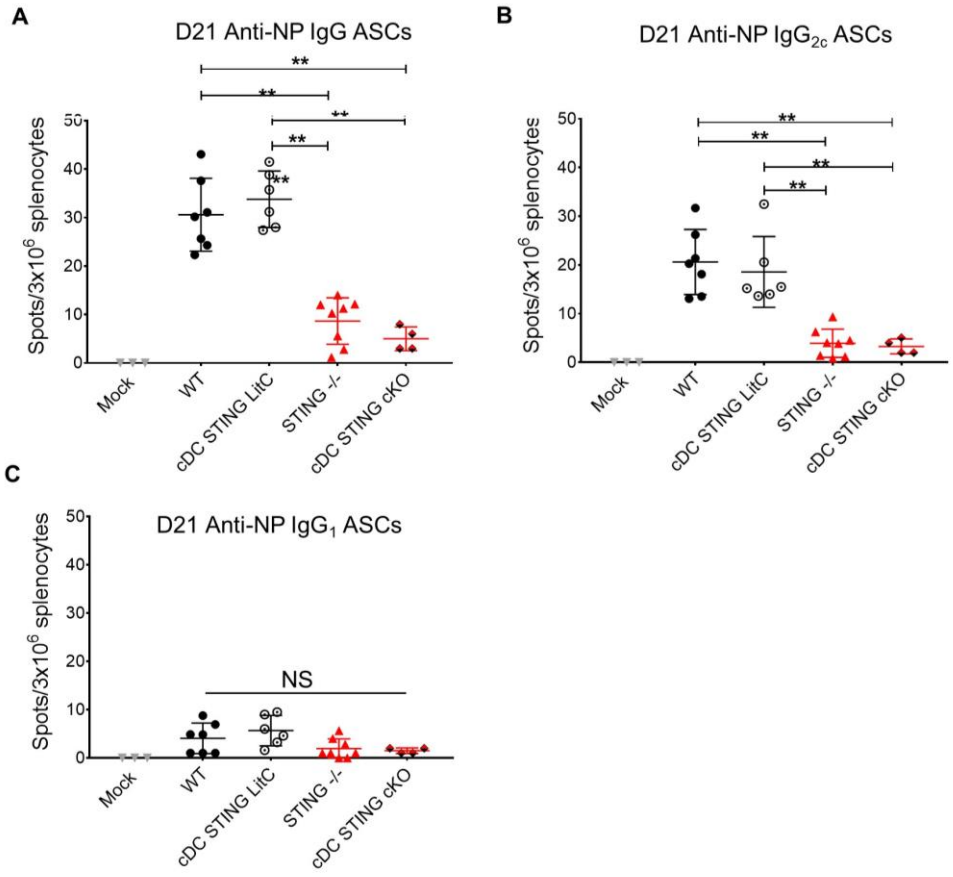

Figure 28: *STING* is required within cDCs for DNA vaccine induction of IgG_{2C} (Th1) antibody-secreting cells

WT, *STING$^{-/-}$, cDC STING cKO*, and *cDC STING LitC* mice were IM/EP vaccinated with pNP. Sera were collected prior to vaccination (D0) as well as 14 (D14), 21 (D21), and 28 days (D28) post-vaccination. Anti-NP antibody-secreting cells (ASCs) in the mice were measured 21 days post-vaccination using a B cell ELIspot assay. (**A**) IgG, (**B**) IgG_{2C}, and (**C**) IgG_1 ASCs are presented. Three independent experiments were performed consisting of three to seven mice; the data presented are the average ± SD of three to seven mice/genotype. A one-way ANOVA was employed to compare groups; **$p < 0.01$, NS = nonsignificant.

3.2.4 *STING* is required for DNA vaccine induction of antigen-specific Th1 CD4$^+$ T cell responses

Since I saw a reduction in IgG_{2C} Ab responses and CD4$^+$ T cells help drive different Ab responses I wanted to determine if *STING* within cDCs is required for DNA vaccine induction of CD4$^+$ T cell responses. I IM/EP-vaccinated WT, *STING*$^{-/-}$, *cDC STING cKO*, and *cDC STING LitC* mice with pNP and measured the frequencies of NP-specific CD4$^+$ T cells binding the NP-specific CD4$^+$ T cell epitope, $NP_{311-325}$, using tetramer staining and flow cytometry (Materials and methods [Chapter 4], Figure 33) 21 days post-vaccination (Figure 29). Both *STING*$^{-/-}$ and *cDC STING cKO* mice exhibited a trend toward lower frequencies of Ag-specific CD4$^+$ T cells, but these differences were not significantly different from the control groups (Figure 30a). To determine the impact of *STING* within cDCs on the induction of Th1 CD4$^+$ T cell responses, I evaluated cytokines produced by splenocytes stimulated with a C57Bl/6 CD4$^+$ T cell NP immunodominant peptide $NP_{311-325}$. Splenocytes were isolated 21 days post-vaccination and stimulated overnight with 1 µg/mL $NP_{311-325}$; then, concentrations of Th1 (IFN-γ, TNF-α, IL-2, and IL-6) and Th2 (IL-4) cytokines were analyzed in the supernatants using a flow-based cytokine detection kit [208]. Overall, I observed no significant differences in the production of TNF-α, IL-2, IL-4, and IL-6 (Figure 31). I also measured IL-10 (a marker of Tregs (17)) and IL-17a cytokines but observed no differences between the groups, which suggested that *STING* may have a limited impact on the induction of Tregs or Th17 T cells following DNA vaccination (Figure 31d, f). More studies are required to determine whether *STING* impacts these and other T cell subsets. However, *STING*$^{-/-}$ and *cDC STING cKO* splenocytes produced significantly less IFN-γ compared with WT and *cDC STING LitC* mice (Figure 30), suggesting a role for *STING* within cDCs in the induction of Th1-/IFN-γ-producing CD4$^+$ T cell responses. To further investigate this role, CD4$^+$ T cells from *STING*$^{-/-}$ and *cDC STING cKO* mice were stimulated in vitro with the NP CD4$^+$ T cell epitope $NP_{311-325}$, stained them for IFN-γ using ICS, and then analyzed them using flow cytometry (Figure 30c, d). The *STING*$^{-/-}$ and *cDC STING cKO* mice developed significantly lower frequencies of IFN-γ$^+$CD4$^+$ T cells compared with WT and *cDC STING LitC* mice (Figure 31c, d), further indicating that *STING* is required within cDCs for DNA vaccine induction of Th1 CD4$^+$ T cell responses.

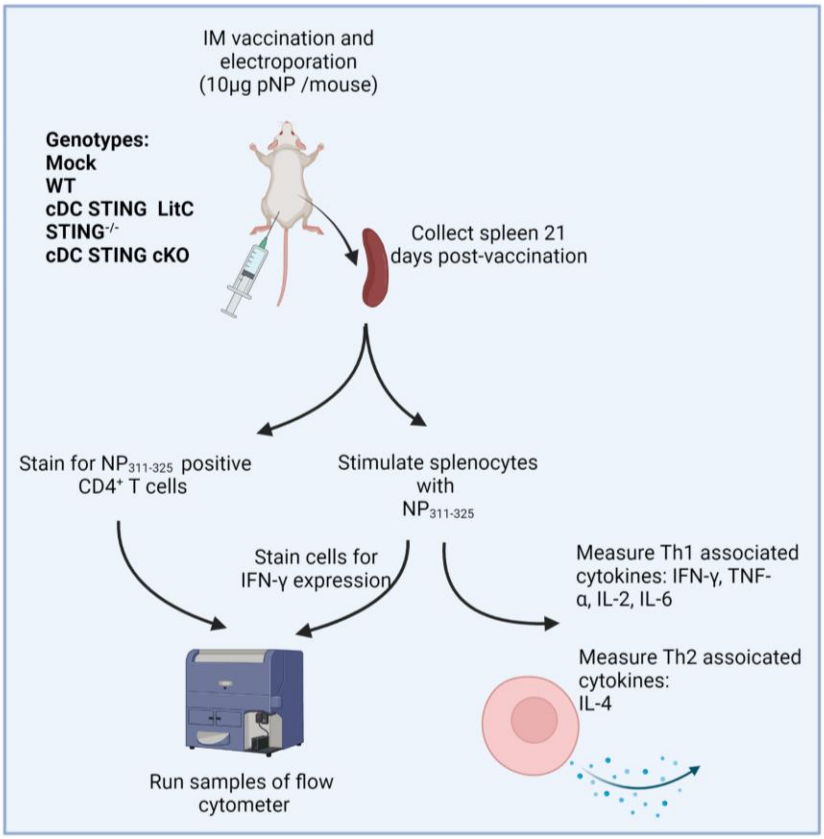

Figure 29: Experimental design for evaluating DNA vaccine induction of Th1 and Th2 cytokines in cDC STING cKO mice

WT, *STING$^{-/-}$, cDC LitC,* and *cDC STING cKO* mice were IM/EP vaccinated with 10 µg of pNP. The mice were sacrificed 21 days post-vaccination, spleens were collected, and single-cell suspensions were produced. Splenocytes were stimulated with the CD57Bl/6 CD4$^+$ T cell immunodominant peptide $NP_{325\text{-}325}$ (1 µg/mL) overnight and ICS stained for IFN-γ, and then supernatants were collected to measure the concentrations of Th1-associated cytokines (IFN-γ, TNF-α, IL-2, and IL-6) and Th2-associated cytokine (IL-4). Additionally, splenocytes were stained with the tetramer $NP_{311\text{-}325}$ to measure the frequencies of tetramer-positive CD4$^+$ T cells. Created with Biorender.com

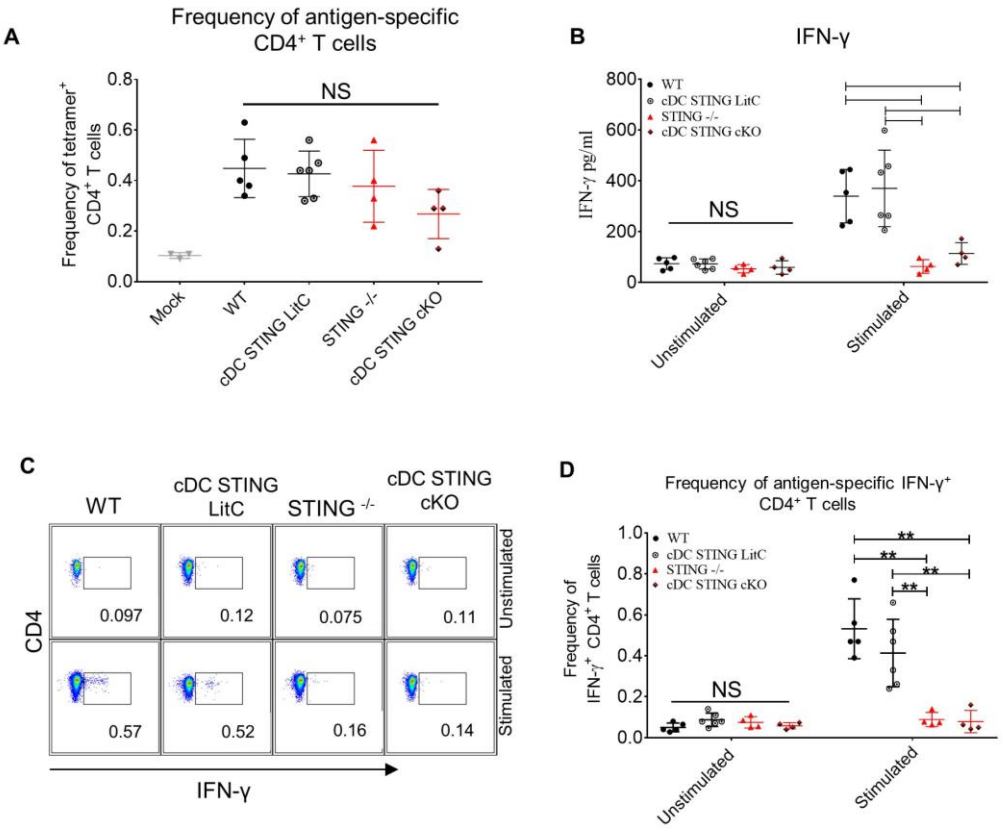

Figure 30: DNA vaccines require *STING* within cDCs to induce vaccine-generated IFN-γ$^+$CD4$^+$ T cells and IFN-γ production

WT, *STING$^{-/-}$*, *cDC STING cKO*, and *cDC STING LitC* mice were IM/EP-vaccinated with pNP. The mice were sacrificed 21 days post-vaccination and splenocytes were isolated. (**A**) The frequency of tetramer-positive cells within CD4$^+$ T cells was measured for each genotype using a MHC-II tetramer that presented the NP immunodominant peptide ($NP_{311-325}$) for C57bl/6. Additionally, splenocytes were stimulated with $NP_{311-325}$ to evaluate the cytokines that they produced. (**B)** IFN-γ production by CD4$^+$ T cells was measured by stimulating splenocytes with $NP_{311-325}$ peptide in vitro and analyzing the supernatants. The production of IL-2, IL-4, IL-6, IL-10, and TNF-α was also measured (Supplemental Figure 7). (**C, D**) Frequencies of IFN-γ$^+$CD4$^+$ T cells were measured using flow cytometry. **(C**) Gating scheme for the detection of IFN-γ$^+$CD4$^+$ T cells using flow cytometry. (**D**) Frequencies of IFN-γ$^+$CD4$^+$ T cells. Three independent experiments were performed consisting of three to six mice; the data presented are the average ± SD of three to six mice/genotype. Two separate one-way ANOVAs were employed to compare genotypes within unstimulated and stimulated groups; *p < 0.05, **p < 0.01, NS = nonsignificant.

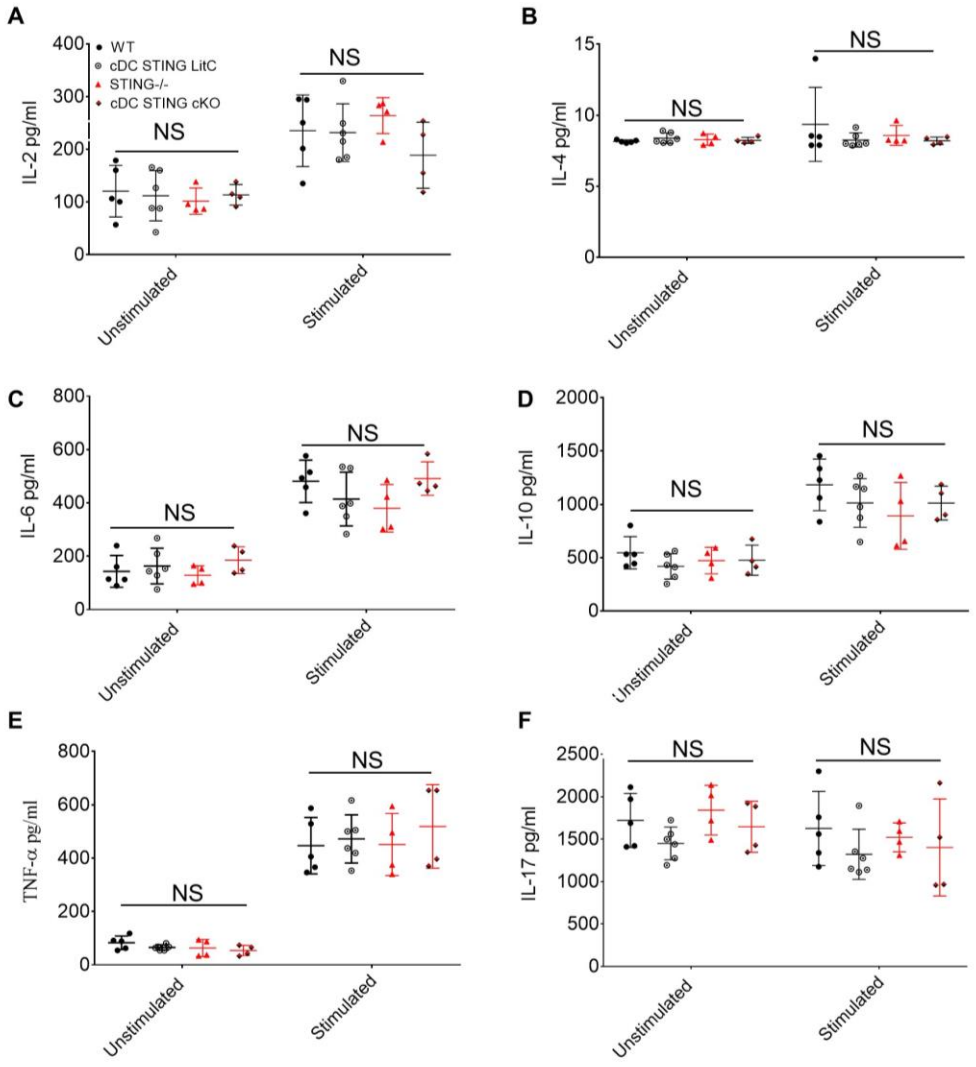

Figure 31: STING is not required within cDCs for CD4⁺ T cell production of some Th1-associated cytokines post-DNA vaccination

WT, *STING*$^{-/-}$, *cDC STING cKO*, and *cDC STING LitC* mice were IM/EP-vaccinated with pNP. The mice were sacrificed 21 days post-vaccination and spleens were made into single-cell suspensions. The cells were stimulated with $NP_{311-324}$ peptide, and the concentrations of (**A**) IL-2, (**B**) IL-4, (**C**) IL-6, (**D**) IL-10, (**E**) TNF-α, and (**F**) IL-17a were measured from the supernatants. Frequencies of IFN-γ⁺CD4⁺ T cells are presented. Three independent experiments were performed consisting of three to six mice; the data presented are the average ± SD of three to six mice/genotype. A one-way ANOVA was employed to compare groups for both stimulated and unstimulated conditions; NS = nonsignificant**.**

3.3 Discussion

A hallmark of DNA vaccines is their ability to induce a strong Th1-biased immune response [1-5]. However, the mechanisms that influence this Th1 bias are unclear. Here, I investigated the role of *cGAS* and *STING* in DNA vaccine induction of Th1- and Th2-associated Ab and T cell responses. Consistent with previous findings [191], I found that *STING*, but not *cGAS*, is required for DNA vaccine induction of IgG Ab responses. Strikingly, I further observed that *STING*, but not *cGAS*, is required for DNA vaccine induction of Ag-specific IgG_{2C} (Th1) Ab responses, but neither *STING n*or *cGAS* is required to induce Ag-specific IgG_1 (Th2) Ab responses (Figure 15). Additionally, I found that *STING* is required for DNA vaccine-induced IgG_{2C} responses independent of the modality of vaccine delivery since GG vaccinated *STING*$^{-/-}$ mice exhibited significantly lower IgG_{2C} concentrations compared with control mice, but generated normal concentrations of IgG_1 (Figure 17). The lack of a significant impact of *STING* on the total IgG responses following the GG delivery of DNA vaccines is likely due to the GG inducing predominantly IgG_1 responses, whereas IM induces primarily IgG_2 Ab responses [97], which is a finding consistent with my data that indicated that total IgG in GG vaccinated mice primarily consisted of IgG_1. These results provide the first evidence that *STING* is required for DNA vaccine induction of Th1-associated IgG_{2C} Ab, but not for Th2-associated IgG_1 Ab responses.

Moreover, Th1 responses play a role in programming $CD8^+$ T cell memory and effector functions (45), yet I found that neither *STING* nor *cGAS* was required for DNA vaccine induction of Ag-specific $CD8^+$ T cell responses. Instead, my results indicated that *STING*, but not *cGAS*, is required for DNA vaccine-induced polyfunctional $CD8^+$ T cell responses (Figure 19). My findings therefore provide new insight into the role of *STING* in mediating the ability of DNA vaccines to induce polyfunctional $CD8^+$ T cell responses and suggest a strong link to the induction of Th1 responses. Nevertheless, additional studies are still required to identify the precise mechanism underlying the ability of DNA vaccines to induce robust $CD8^+$ T cell responses.

Previous studies have highlighted a critical role of DCs in coordinating both Ab and $CD8^+$ T cell responses induced by DNA vaccines [205-208]. Using mice with *STING* selectively absent in cDCs (*cDC STING cKO*), this study surprisingly found that *STING* is not required within cDCs for DNA vaccine induction

of polyfunctional $CD8^+$ T cells (Figure 22). The discrepancy between my findings, namely that *STING* is required for the induction of polyfunctional $CD8^+$ T cell responses but not within cDCs, suggests that *STING* within other cell types likely mediates the ability of DNA vaccines to induce polyfunctional $CD8^+$ T cells. In support of this, I found that the production of pro-inflammatory cytokines (IL-6, TNF-α, and IFN-β) after DNA vaccination was significantly diminished in *STING*$^{-/-}$ mice but similar in control mice and mice that lacked *STING* in cDCs (Figure 24). Plasmacytoid dendritic cells (pDCs), a DC subset that still possess *STING* in *cDC STING cKO* mice, may potentially produce IFN-α/β post-DNA vaccination, which would subsequently aid in $CD8^+$ T cell effector programming, albeit in significantly lower quantities, and could also contribute to the induction of polyfunctional $CD8^+$ T cell responses [87, 173].Noteworthy, I found that *STING*$^{-/-}$ mice developed similar frequencies of Ag-specific $CD8^+$ T cells, even though they exhibited somewhat lower concentrations of IL-2 following DNA vaccination. The reason for this possible discrepancy is unclear, but it may be due to differences in the threshold of IL-2 production that are required to generate Ag-specific $CD8^+$ T cells responses versus facilitating their polyfunctionality. Unlike $CD4^+$ T cells, activated $CD8^+$ T cells can undergo maximum proliferation when exposed to even low concentrations of IL-2 [209]. Thus, it is possible that the somewhat lower amounts of IL-2 produced in *STING*$^{-/-}$ mice are still above the threshold required for the expansion of $CD8^+$ T cells, but is not sufficient for programming polyfunctional $CD8^+$ T cells, which rely on type I IFNs and other factors more significantly impacted by *STING*.

By contrast, I found that *STING* is required within cDCs for DNA vaccine induction of Th1-associated IgG_{2C} Ab responses (Figure 26), which suggests that *STING* within cDCs may play a key role in B cell IgG class-switching. Indeed, I found that DNA-vaccinated mice that lacked *STING* within cDCs developed lower frequencies of IFN-γ producing $CD4^+$ T cells, which are known to play a key role in promoting B cell IgG class-switching to IgG_{2C} production (Figure 28) [158-159]. These results are consistent with a previous study that demonstrated that $CD4^+$ T cells in *STING*$^{-/-}$ mice express significantly less IFN-γ than WT mice [210]; moreover, they extend these results by demonstrating, for the first time, that *STING* is specifically required within cDCs for DNA vaccine induction of Th1 IFN-γ-producing $CD4^+$ T cells (Figure 30). Additional studies are required to elucidate whether the absence of *STING* in cDCs results in atypical cDC and $CD4^+$ T cell interactions. For example, a reduction in Th1-polarizing cytokines (i.e., IL-12 and/or IL-23) produced by cDCs could adversely impact Th1 T cell polarization. Since *STING* is absent in both

$CD8^+$ and $CD8^-$ cDCs in *cDC STING cKO* mice, additional studies are also required to determine whether *STING* is required in both of these cDC subsets to mediate DNA vaccine-induced Th1 immune responses.

Taken together, these findings elucidate a clearer role for *STING* in mediating DNA vaccine induction of Ab and $CD8^+$ T cell responses. My results suggest that *STING* is not required for the induction of Ag-specific $CD8^+$ T cell responses but is required for inducing polyfunctional $CD8^+$ T cell responses. Although my studies have revealed that *STING* is dispensable within cDCs for DNA vaccine induction of polyfunctional $CD8^+$ T cells, *STING* is required within cDCs for the generation of Th1 (IgG_{2C}) Ab responses and Th1 IFN-γ-producing $CD4^+$ T cells. Moreover, these results provide new insight into the mechanisms whereby DNA vaccines induce Th1 responses. I propose that upon DNA vaccination, *STING* within cells other than cDCs triggers the production of pro-inflammatory cytokines (IL-6, TNF-α, and IFN-β) that are critical for the induction of polyfunctional $CD8^+$ T cell responses. Alternatively, *STING* may facilitate apoptosis in DNA transfected cells as well as cross-presentation to $CD8^+$ T cells by cDCs. Indeed, cross-presentation has been demonstrated to be a mechanism whereby IM-delivered DNA vaccines induce $CD8^+$ T cell responses (25), as a study found that *STING* activation can induce apoptosis [52].

The ability of DNA vaccines to induce Th1-polarized Ab, Ag-specific T cell, and polyfunctional CD8+ T cells responses is believed to be a key feature for the development of vaccines that are effective for a wide range of infectious diseases and cancers (Chapter 1). The findings reported here provide new insight into the underlying innate pathways required to mediate DNA vaccine immunogenicity and, specifically, the role of *STING* in the induction of Th1 responses. Together, the results have implications for the development of new DNA vaccine strategies and genetic adjuvants for enhancing or modulating DNA vaccine induction of Th1 immune responses. While my studies as well as previous studies have confirmed a requirement for *STING* to mediate DNA vaccine immunogenicity, whether RNA-based vaccines require *STING* remains unclear. Studies have demonstrated that *STING*$^{-/-}$ mice are susceptible to RNA virus infections, which suggests that *STING* may also be required to mediate RNA vaccine-induced immune responses [125]. However, additional studies have demonstrated that RNA viruses can induce mitochondrial damage, resulting in mitochondrial DNA release, which in turn activates *STING*, suggesting that *STING* may be indirectly required to mediate RNA vaccine-induced immune responses [124, 211].

Additional studies are still required to determine the host factors that influence RNA vaccine immunogenicity.

4 Material and methods

4.1 Mice

C57BL/6J (WT), *STING* $^{-/-}$, and *cGAS* $^{-/-}$ mice were bred and maintained in-house at the University of Washington. The *STING*$^{fl/fl}$ mice were a generous gift from Dr. Mohamed Oukka (University of Washington, Seattle, Washington). They were made by a commercial service (Biocytogen, Wakefield, MA) using a Cas9/sgRNA plasmid construct. In brief, a targeting vector was designed with a Neo cassette, flanked by FRT sites, and *STING* Exon 6 flanked by LoxP sites, was introduced into B6-derived embryonic stem (ES) cells. Targeted ES cells were introduced into host embryos, and cell embryos were surgically transferred into pseudo-pregnant (surrogate) mothers, resulting in F0 heterozygous floxed mice on the B6 background. Chimeric mice were crossed with B6J (JAX #000664) for six generations. *STING*$^{fl/fl}$ mice were bred with *zbtb46*^{Cre} mice (JAX #028538) to generate *zbtb46*^{Cre} x *STING*$^{f/fl}$ mice (*cDC STING cKO*). The *zbtb46*^{Cre} x *STING*$^{f/fl}$ genotyping was conducted using tail snips followed by PCR (Supplemental Table 1). *Zbtb46* is expressed by cDCs but not by pDCs [205]; thus, *cDC STING cKO* mice should have STING selectively absent in cDCs. I verified that cDCs in *cDC STING cKO* mice were missing *STING* through qPCR for the detection of *STING* (Figure 20). Three to five *STING*$^{fl/fl}$*, zbtb46*^{Cre}, and *cDC STING cKO* mice were sacrificed, and their spleens were collected, pooled, and made into single-cell suspensions by crushing through a 0.70 µm filter followed by a 20 mL wash with full RPMI media (RMPI 1640 supplemented with 10% FBS, nonessential amino acids, sodium pyruvate, pen/strep, and β-mercaptoethanol)**.** Red blood cells were lysed using an RBC lysis buffer (Thermo Fisher cat. no. 00-4300-54). Splenocytes were stained with Live/Dead fixable aqua dead (Thermo Fisher Scientific cat. no. L34957), PerCP-conjugated anti-mouse CD11c (Biolegend cat. no. 117326), BV605-conjugated anti-mouse CD19 (Biolegend cat. no. 115540), BV605-conjugated anti-mouse CD3 (Biolegend cat. no. 100237), APC-conjugated anti-mouse Ly-6G (Biolegend cat. no. 127614), PE-conjugated anti-mouse NK1.1 (Biolegend cat. no. 108708), APC-Cy7-conjugated anti-mouse CD11c, and Pacific blue-conjugated anti-mouse MHC-II (Biolegend cat. no. 107620). A BD Biosciences FACS ARIA III cell sorter was used to isolate cDCs ($CD11c^{+}MHCII^{hi}$) as well as B and T cells ($CD19^{+}CD3^{+}$). RNA was isolated (Qiagen RNeasy kit cat. no. 74004) and then converted into cDNA (Thermo Fisher cat. no. 4368814). The *STING* expression in each cell type was measured through qPCR using primer F: GGGAGCCGAAGACTGTACAT and primer R: CGCTGTTGGAATAAACCCGA. All mice were maintained by the University of Washington's Office of

Animal Welfare according to protocols approved by the Institutional Animal Care and Use Committee (IACUC).

4.2 DNA vaccination

A DNA vaccine that encodes a codon-optimized full-length nucleoprotein from A/Puerto Rico/8/1934 (pNP) was used to immunize the mice**.** The plasmid (plasmid nucleoprotein; pNP) employed for these studies was constructed as previously described [212]. Briefly, the influenza NP gene is under control of the human cytomegalovirus (CMV) immediate early promoter. This plasmid also includes the following additional elements for optimizing antigen expression: the hepatitis B virus (HBV) pre-S2 5' untranslated region (UTR), rabbit beta globin poly A, rat insulin intron A, the HBV env enhancer, and the CMV exon 1 and 2. Mice were injected with 10 µg of pNP in 50 µL of PBS split between both tibialis anterior and then electroporated using a BTX AgilePulse Waveform Electroporation System (cat. no. 47-0400N). BTX's AgilePulse voltage and pulse length settings used for IM vaccinations were as follows: Group 1 – pulse amplitude = 450 V, pulse width = 0.05 ms, pulse interval = 300 ms, group intervals = 500 ms, and two pulses; and Group 2 – pulse amplitude = 110 V, pulse width = 10 ms, pulse interval = 300 ms, group interval = 500 ms, and eight pulses. GG DNA vaccination was also used to vaccinate the mice with pNP. Specifically, mice were vaccinated with 1 µg of pNP using helium at 400 psi (Figure 16).

4.3 ELISA

Sera were collected at 14, 21, and 28 days post-vaccination to measure NP-specific IgG, IgG_1, and IgG_{2C} Ab titers, and sera samples were stored at −80°C until the time of assay. Microtiter plates were coated at 1 µg/mL of recombinant NP (Sino Biological, cat. no 11675-V08B) overnight at 4°C. Anti-NP IgG, IgG1, and IgG_{2C} concentrations (µg/ml) in the sera were calculated using purified mouse IgG (Southern Biotech cat. no. 0107-01), IgG_1 (Southern Biotech cat. no. 0102-01), or IgG_{2C} (Southern Biotech cat. no. 1078-01) to produce a standard curve. Purified mouse Abs were detected using 1 µg/mL of goat anti-mouse IgG-HRP (Southern Biotech cat. no. 1030-05), IgG_1-HRP (Southern Biotech cat. no. 1070-05), or IgG_{2C}–HRP (Sothern Biotech cat. no. 1078-05). O-phenylenediamine dihydrochloride substrate tablets (Thermo Fisher cat. no. 34006) were used to observe HRP-induced color changes. The HRP substrate reaction was terminated using 2N sulfuric acid after 2 minutes. Absorbance was measured at 450 nm and concentrations

of Abs were calculated using each plate's internal standard curve. Sera dilutions were optimized for each experiment and timepoint to ensure that the absorbance reading fell within each plate's internal standard curve. The data are representative of at least three independently preformed studies (n = 3–9 mice/genotype/study).

4.4 Analysis of antigen-specific T cell responses using tetramer staining and flow cytometry

Antigen (Ag)-specific $CD8^+$ T cells (Figure 32) and $CD4^+$ T cells (Figure 33) were measured by flow cytometry using PE- and/or APC-conjugated-$NP_{366-374}$ (H-2k(b)) and PE-conjugated $NP_{311-325}$ (I-A(b)) tetramers, respectively. The tetramers were obtained from the NIH Tetramer Core Facility (Atlanta, GA USA). Mice were sacrificed, spleens collected, and single-cell suspensions generated for each mouse as outlined above. A total of 1 x 10^6 splenocytes were stained and analyzed using flow cytometry (BD bioscience LSRII). To measure the frequencies of Ag-specific $CD4^+$ T cells, splenocytes were stained with the following Abs: Live/Dead fixable aqua dead (Thermo Fisher Scientific cat.no. L34957), BUV395-conjugated anti-mouse CD4 (BD cat. no. 565975), PerCP5.5-conjugated anti-mouse CD3 (Biolegend cat. no. 1002180), BV711-conjugated anti-mouse CD8 (Biolegend cat. no. 100748), PE-Cy7-conjugated anti-mouse CD44 (BD cat. no. 560569), and PE-conjugated $NP_{311-325}$. To measure the frequencies of Ag-specific $CD8^+$ cells, splenocytes were stained as follows: Live/Dead fixable aqua dead (Thermo Fisher Scientific cat. no. L34957), PerCP5.5-conjugated anti-mouse CD3 (Biolegend cat. no. 1002180), BUV395-conjugated anti-mouse CD4 (BD cat. no. 565975), BV711-conjugated anti-mouse CD8 (Biolegend cat. no. 100748), and PE-Cy7-conjugated anti-mouse CD44 (BD cat. no. 560569).

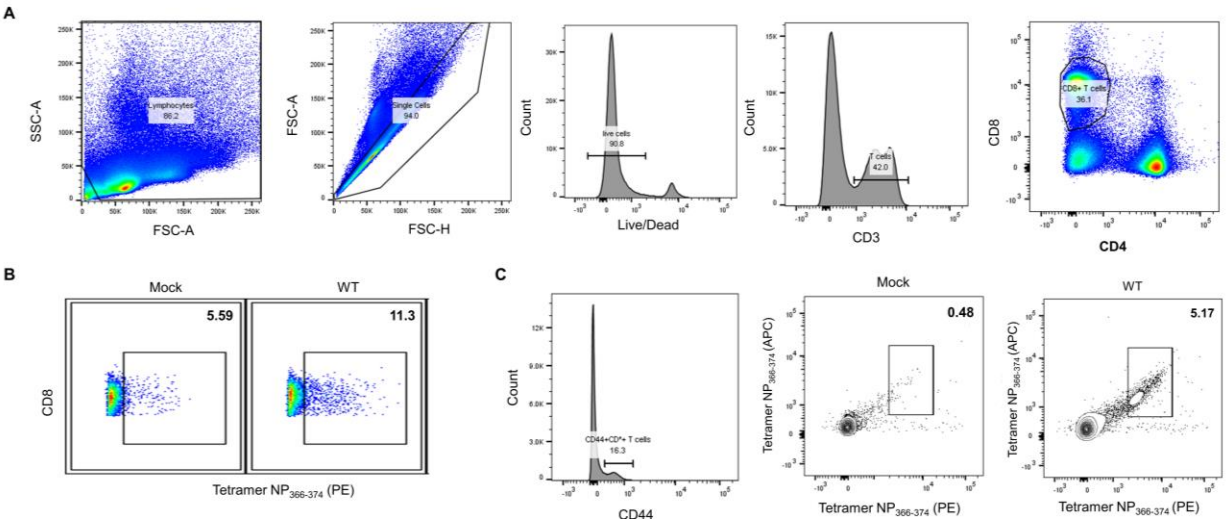

Figure 32: Gating strategies used for evaluating splenic tetramer binding CD8⁺ T cells

Representative gating strategies for splenocytes for identifying tetramer binding CD8⁺ T cells. **(A)** Lymphocytes were identified by excluding doublets using forward and side light scatter, removing dead cells with a live/dead viability dye, and then gating on CD3⁺ cells, followed by subsequent gating on T cell subset CD4⁺ and CD8⁺ cells. **(B)** CD8⁺ tetramer binding cells were identified using a PE-conjugated MHC-I tetramer ($NP_{366-374}$; Figure 19) or (**C**) CD8⁺ T cells were further gated for CD44⁺ expression and then tetramer binding CD8⁺ T cells were identified using a PE-conjugated MHC-I tetramer ($NP_{366-374}$) and a APC-conjugated MHC-I tetramer ($NP_{366-374}$; Figure 22).

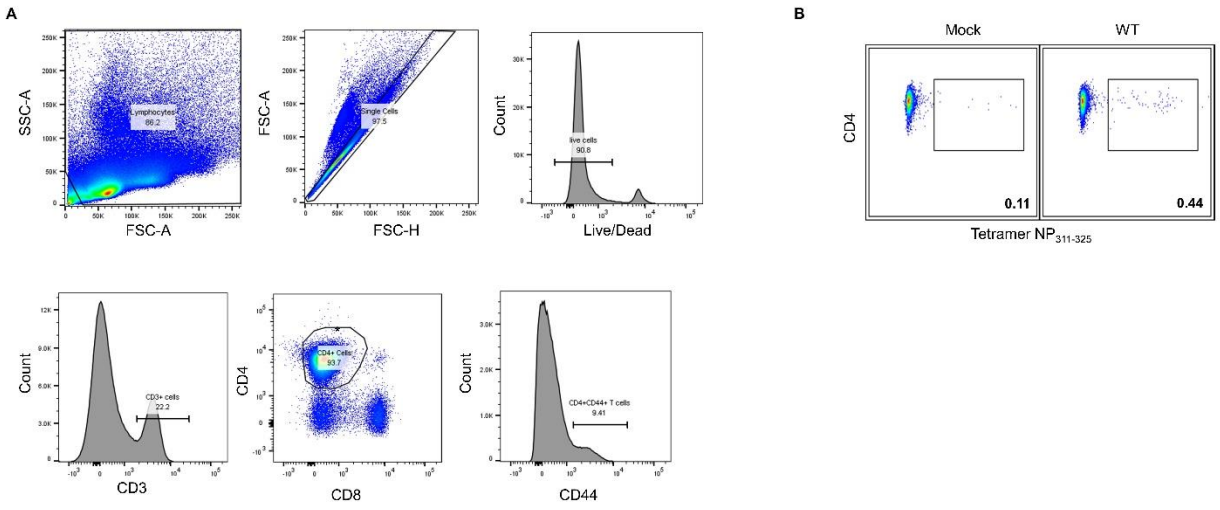

Figure 33: Gating strategies used for evaluating splenic tetramer binding CD4⁺ T cells

Representative gating strategy for splenocytes for identifying tetramer binding CD8⁺ T cells. **(A)** Lymphocytes were identified by excluding doublets using forward and side light scatter, removing dead cells with a live/dead viability dye, and then gating on CD3⁺ cells, followed by subsequent gating on T cell subset CD4⁺ and CD8⁺ cells and then gating for CD44⁺CD4⁺ T cells. **(B)** CD4⁺ Tetramer binding cells were identified using a PE-conjugated MHC-II tetramer ($NP_{311-325}$).

4.5 Analysis of polyfunctional $CD8^+$ T cell responses

Single-cell suspensions from spleens were collected 21 days post-vaccination as outlined above, and then 1 x 10^6 cells were plated in duplicate wells in a 96-well plate and rested for 24 hours (h) in full RPMI media (details above) at 37°C. To evaluate $CD8^+$ T cell polyfunctionality, cells were either stimulated with 1 µg/mL $NP_{366-374}$ or media only (unstimulated). After 1 hour, 1x brefeldin A (Biolegend cat.no. 420601) and FITC-conjugated CD107a/granzyme B (Biolegend cat.no. 121606) were added to the wells and incubated overnight (approx. 16 h). The following day, cells were stained for IL-2 (Biolegend cat.no. 503808), IFN-γ (Biolegend cat.no. 505818), FITC-conjugated anti-human/mouse granzyme B (Biolegend cat. No. 515403), and TNF-α (Biolegend cat.no 506308) using BD's Fixation/Permeabilization Solution Kit (cat.no. 554714) following the manufacturer's protocol. T cells were first gated using BV605-conjugated anti-mouse CD3 (Biolegend cat. No. 100237) and then $CD8^+$ T cells were gated using BV711-conjugated anti-mouse CD8 (Biolegend cat. No. 100748). The frequencies of $CD8^+$ T cells expressing IL-2, TNF-α, IFN-γ, and/or cytolytic markers CD107a/granzyme B were then measured using flow cytometry (BD Biosciences LSRII) and Boolean gating (Figure 34). The frequencies of mouse $CD8^+$ T cells in media were subtracted from their frequencies following NP peptide stimulation. These data were then used to calculate a polyfunctional index as previously described (43). $CD8^+$ T cell polyfunctionality is defined as the frequency of $CD8^+$ T cells expressing any three or more of the cytokines IFN-γ, IL-2, and TNF-α and/or co-expressing the cytolytic marker CD107a/granzyme B after stimulation with the immunodominant NP peptide $NP_{366-374}$, specific for C57BL/6 mice.

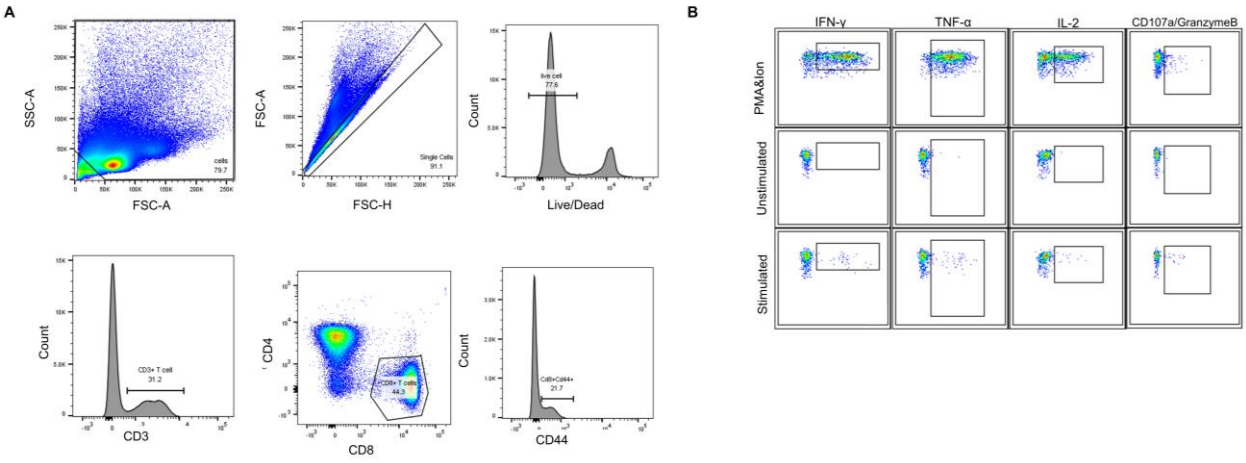

Figure 34: Gating strategies used for evaluating $CD8^+$ T cell polyfunctionality in $NP_{366-374}$-stimulated splenocytes

(**A**) Lymphocytes were identified by excluding doublets using forward and side light scatter, removing dead cells with a live/dead viability dye, and then gating on $CD3^+$ cells. Then, T cell subset $CD4^+$ and $CD8^+$ cells were identified followed by gating for $CD44^+CD8^+$ T cells. **(B)** Representative gating of IFN-γ^+, TNF-α^+, IL-2^+, and CD107a/granzyme B^+ CD8 T cells. PMA and Ionomycin were used to set initial gates for each immune function outlined above. Once the gates had been set, Boolean gating was used to evaluate polyfunctional $CD8^+$ T cells.

4.6 Analysis of Th1- and Th2-associated cytokines produced by peptide-stimulated splenocytes

To measure Th1 and Th2 cytokine expression, 1 x 10^6 cells were plated in duplicate, rested for 24 h in full media at 37°C, and then one well was stimulated overnight (approx. 16 h) with 1 µg/mL $NP_{311-325}$. Supernatants were collected and analyzed for IFN-γ, TNF-α, IL-2, IL-4, IL-6, IL-10, and IL-17a through flow cytometry (BD Bioscience LSRII) using BD's mouse Th1/Th2/Th17 Cytometric Bead Array Kit (cat. no. 560485) following the manufacturer's protocol.

4.7 Analysis of IFN-γ expression in peptide-stimulated $CD4^+$ T cells

To measure IFN-γ expressing $CD4^+$ T cells, mouse splenocytes were isolated as outlined above. A total of 1 x 10^6 cells were pated in duplicate in a 96-well plate and rested for 24 h. The cells were then stimulated with 1 µg/mL $NP_{311-325}$, the immunodominant NP peptide for C57BL/6 $CD4^+$ T cells, or remained in media only. After 1 hour, 1x brefeldin A (Biolegend cat. No. 420601) was added to the wells and the cells were incubated overnight (approx. 16 h). The following day, splenocytes were stained with PerCP5.5-conjugated anti-mouse CD3 (Biolegend cat. no. 1002180), BUV395-conjugated anti-mouse CD4 (BD cat. no. 565975), and IFN-γ (Biolegend cat. no. 505818) using BD's Fixation/Permeabilization Solution Kit (cat. no. 557414) following the manufacturer's protocol. The frequencies of $CD4^+$ T cells expressing IFN-γ was measured using flow cytometry (DB Bioscience LRSII).

4.8 B-cell ELISpot

Mouse splenocytes were used to enumerate NP-specific ASCs 21 days post-vaccination. ELISpot plates (Millipore Sigma cat. no. S2EM004M99) were coated with 10 µg/mL recombinant NP (Sino Biological, cat. no 11675-V08B) in PBS and then incubated overnight at 4°C. Mouse splenocytes were processed as outlined above. Then, 3 x 10^6 splenocytes in 100 µL of media were plated in NP-coated plates in duplicate and then incubated overnight (approx. 16 h) at 37°C. The following day, the cells were discarded and the wells washed five times with PBS. Following the final wash, 100 µL of anti-mouse IgG-HRP, IgG_1-HRP, or IgG_{2C}–HRP diluted at 1:2000 in PBS was added to each well. Spots were developed using the BD ELISpot AEC Substrate Set (BD cat. no. 551951), visualized using a CTL imager (Cellular Technologies), and enumerated using the ImageJ Cell Counter.

4.9 Cytokine analysis

Sera were collected from mice prior to vaccination (baseline) as well as at 6 h and 24 h post-vaccination. Sera from WT, *zbtb46*^{Cre} *x STING*$^{fl/fl}$ (*cDC STING cKO*), and *cDC STING LitC* mice were diluted at a ratio of 1:5 while sera from *STING*$^{-/-}$ mice were diluted at 1:2. Levels of IFN-γ, TNF, IL-2, IL-4, IL-6, IL-10, and IL-17a in diluted sera were then measured using flow cytometry (BD Bioscience LSRII) using BS's Mouse Th1/Th2/Th17 Cytometric Bead Array Kit (cat. no. 560485) following the manufacturer's protocol. IFN beta (IFN-β) was measured in sera diluted at 1:2 prior to vaccination and 6 h post-vaccination using a Mouse IFN-β ELISA Kit (PBL Assays cat. no. 42410-1).

4.10 Statistical analysis

All data are presented as the mean of individual mice ± SD. Statistical analyses were performed using one-way ANOVA followed by a Tukey's post-test and Student's *t* test using GraphPad Prism 7.

4.11 Graphical figures

Graphical figures were produced using Biorender.com.

5 Conclusions and future directions

5.1 Summary of conclusions

In this dissertation, I have highlighted the history of DNA vaccines, their development, and clinical significance; vaccine delivery; as well as the Th1 and Th2 responses induced by DNA vaccines (Chapter 1). This dissertation set out to examine the underlying mechanisms that govern DNA vaccine-induced Ab and effector $CD8^+$ T cell responses, a hallmark of DNA vaccines. To this end, I demonstrated that the innate dsDNA-sensing protein *cGAS* is dispensable for DNA vaccine immunogenicity independent of DNA vaccine delivery modality as WT mice and *cGAS*$^{-/-}$ developed similar Ag-specific antibody and effector $CD8^+$ responses. Moreover, I revealed a critical role for *STING* in coordinating DNA vaccine-induced IgG_2 (Th1) Ab responses, but not IgG_1 (Th2) Ab responses, independent of the route of DNA vaccination as DNA-vaccinated *STING*$^{-/-}$ mice generated significantly lower concentrations of Ag-specific IgG and Th1-associated IgG_{2C} but not Th2 associated IgG_1 (Th2) Ab responses post-DNA vaccination (Chapter 2). Additionally, I demonstrated that *STING* is required to mediate DNA vaccine immunogenicity independent of encoded vaccine antigens, as others have demonstrated a similar phenotype in DNA-vaccinated *cGAS*$^{-/-}$ and *STING*$^{-/-}$ mice [180, 191].

Furthermore, I demonstrated that cGAS is required for DNA vaccine induction of effector $CD8^+$ T cell responses as vaccine DNA induced similar polyfunctional $CD8^+$ T cells in *cGAS*$^{-/-}$ and WT mice, further indicating that *cGAS* is not required for DNA vaccine immunogenicity (Chapter 2). Moreover, I demonstrated that *STING* is required for DNA vaccine induction of effector $CD8^+$ T cell responses as *STING*$^{-/-}$ mice developed significantly lower polyfunctional $CD8^+$ T cells compared with WT mice. Since STING activation induces the production of type I IFNs, a crucial cytokine required for effector $CD8^+$ T cell functions and intracellular pathogen clearance, it is not surprising that DNA-vaccinated *STING*$^{-/-}$ mice developed significantly lower polyfunctional $CD8^+$ T cells. Although these initial studies in my dissertation confirmed observations made by previous studies, they did not provide a mechanism through which *STING* is required to mediate DNA vaccine induction of IgG_{2C} Ab and effector $CD8^+$ T cell responses.

To gain a more complete understanding of the mechanism by which *STING* governs DNA vaccine immunogenicity, I set out to develop conditional knockout mice (cKO) that had *STING* selectively missing in different immune cells. With input from Dr. Edward Clark and Dr. Deborah Fuller, we decided to focus

our attention on cDCs as they were demonstrated to be required for mediating DNA vaccine immunogenicity, including Ab and effector $CD8^+$ T cell responses. To make mice that had *STING* conditionally knocked-out in cDCs, we graciously received $STING^{fl/fl}$ mice from Dr. Mohamed Oukka from the University of Washington. We were able to cross these mice with $zbtb^{Cre}$ mice, which resulted in $STING^{fl/fl}$ *x* $zbtb^{Cre}$ (*cDC STING cKO)* mice. Once the first set of mice were bred, I validated *STING* as absent in cDCs by isolating cDCs from these mice and then performed qtPCR to measure relative *STING* expression (Chapter 3). Once I had validated that the *cDC STING cKO* mice lacked *STING* in cDCs, I investigated whether *STING* is required within cDCs to mediate DNA vaccine Ab and $CD8^+$ T cell responses.

Given that $STING^{-/-}$ mice developed significantly lower concentrations of Ag-specific Abs and lower effector $CD8^+$ T cells responses, I hypothesized that *STING* is required within cDCs to mediate these responses. I found that *STING* is not required within cDCs for DNA vaccine induction of Ag-specific effector $CD8^+$ T cells as WT, *cDC STING cKO*, *cDC STING littermate control* (*cDC STING LItC*), and $STING^{-/-}$ mice generated similar frequencies of $CD8^+$ T cells post-vaccination. Interestingly, *cDC STING cKO*, *cDC STING LitC*, and WT mice generated similar polyfunctional $CD8^+$ T cells while $STING^{-/-}$ developed significantly lower polyfunctional $CD8^+$ T cells. This result was surprising and highlighted that *STING* is required for DNA vaccine induction of $CD8^+$ T cells but not within cDCs. To gain an enhanced understanding of the role of *STING* in mediating DNA vaccine induction of polyfunctional $CD8^+$ T cell responses, I measured pro-inflammatory cytokines that are known to influence effector $CD8^+$ T cell programming. I found that $STING^{-/-}$ mice produced significantly lower concentrations of IL-6, TNF-α, and IFN-β post-vaccination. Although there was less IL-2 production in $STING^{-/-}$ mice, it was not significant; moreover, studies have highlighted that $CD8^+$ T cells require lower levels of IL-2 to undergo proliferation compared with $CD4^+$ T cells, which may explain why I did not observe a significant difference in the frequencies of Ag-specific $CD8^+$ T cells [209].

Future experiments are required to discover which cell types *STING* is required in to mediate DNA vaccine-induced effector $CD8^+$ T cell responses. It is possible that *STING* is required in nonhematopoietic or hematopoietic cells. A bone marrow chimera using $STING^{-/-}$ and WT mice could reveal whether

nonhematopoietic or hematopoietic cells require *STING* to mediate DNA vaccine induction of polyfunctional $CD8^+$ T cells. Such an experiment was conducted in the past with *TBK1*$^{-/-}$ mice and WT mice, the results of which indicated that TBK1 is required within hematopoietic cells for DNA vaccine immunogenicity [180]. Given that TBK1 is immediately downstream of *STING,* it is likely that *STING* is required within another type of hematopoietic cells, possibly pDCs, as these are known to produce large quantities of type I IFNs [173]. Moreover, additional studies are required to elucidate which cells are required for producing pro-inflammatory cytokines for DNA vaccine induction of effector $CD8^+$ T cell responses.

Additionally, I sought to investigate whether *STING* is required within cDCs to coordinate DNA vaccine induction of Ab responses, particularly Th1-associated IgG_{2C} Ab responses as *STING*$^{-/-}$ mice generated reduced concentrations of Ag-specific IgG_{2C} post-vaccination. I found that *STING* is required within cDCs for DNA vaccine induction of IgG and IgG_{2C} (Th1) but not IgG_1 responses as both *STING*$^{-/-}$ and *cDC STING cKO* mice generated significantly less Ag-specific IgG_{2C} than WT and *cDC STING LitC* mice. Since cDCs promote $CD4^+$ T cell differentiation, I also sought to investigate whether *STING* is required to generate Ag-specific CD4 T^+ cells, which may explain the reduction in IgG_{2C} Ab production by *STING*$^{-/-}$ mice and *cDC STING LitC* mice. I found that *STING* is not required for inducing Ag-specific $CD4^+$ T cells post-DNA vaccination as WT, *STING*$^{-/-}$, *cDC STING cKO*, *and cDC STING LitC* mice generated similar frequencies of Ag-specific $CD4^+$ T cells. Although *STING* is not required for the induction of Ag-specific $CD4^+$ T cells, the possibility still existed that *STING* is required within cDCs for the production of Th1- or Th2-polarizing cytokines, which can then include IgG_2 and IgG1 Ab production by B cells. I found that *STING* is required within cDCs for DNA vaccine induction of IFN-γ^+CD4^+ T cells and IFN-y secretion by $CD4^+$ T cells, which may explain why *cDC STING cKO* and *STING*$^{-/-}$ mice produce significantly fewer IgG_2 (Th1) Ab responses as IFN-γ promotes B cells to class-switch to IgG_{2C} [158-159].

Although I demonstrated that *STING* is required within cDCs for DNA vaccine induction of IFN-γ^+CD4^+ T cells, IFN-y secretion by $CD4^+$ T cells, and subsequent IgG_{2C} production, gaps still exist and addressing them could provide a clearer mechanism. It is possible that cDCs do not make other Th1-polarizing cytokines, including IL-12 and IL-23. Additionally, it is possible that a reduction occurs in Th1 ($Tbet^+$) T cells or T_fH ($PD\text{-}1^+Tbet^{+/-}$) T cells when *STING* is absent in cDCs, resulting in suboptimal IgG_{2C}

Ab responses. Future studies are required to gain better insights into the role of *STING* in cDCs in shaping Th1-associated IgG_2 responses. Collectively, the results from these experiments suggest that *STING* is required for DNA vaccine induction of polyfunctional $CD8^+$ T cell but not within cDCs, and also that *STING* is required within cDCs to mediate DNA vaccine induction of Th1-associated IgG_2 Ab responses (Figure 35). Some evidence suggests a critical role for *STING* in mediating mRNA vaccine induced immune responses [213]. Researchers found that mRNA vaccines formulated with ionizable lipids induced mitochondrial (mt) damage resulting in mtDNA release and subsequent STING activation [213]. Furthermore, these researchers found that mRNA vaccine induced mtDNA release and *STING* activation increased effector $CD8^+$ T cell responses, but these researchers did not look at antibody responses. Taken together, both lipid formulated mRNA vaccines and DNA vaccines require *STING* for the induction of robust adaptive immune responses. These results provide far-reaching insights into the role of *STING* in both effector $CD8^+$ T cell responses and Ab responses, which will influence future adjuvant and nucleic acid vaccine research and formulations.

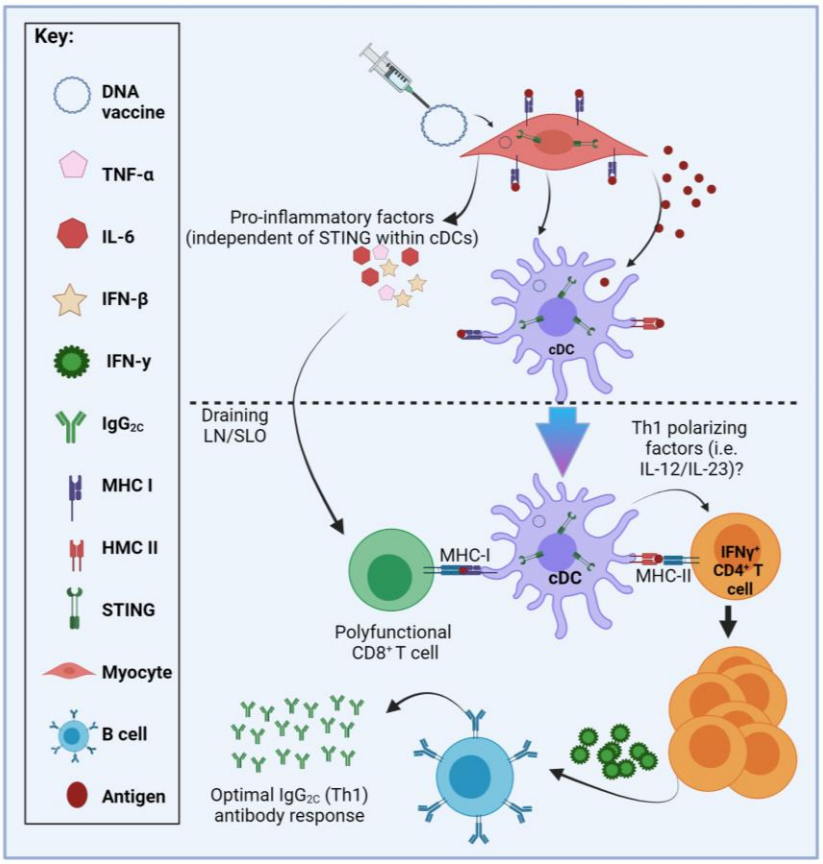

Figure 35: Model of *STING* in mediating DNA vaccine-induced IgG_2 (Th1) antibody responses and polyfunctional $CD8^+$ T cell responses

Upon IM/EP vaccination, myocytes are transfected with vaccine DNA, which activates *STING* in a cGAS-independent manner, promoting the release of the pro-inflammatory cytokines IL-2, IL-6, TNF-α, and IFN-β. cDCs obtain vaccine DNA either by direct transfection or through the phagocytosis of DNA vaccine containing apoptotic bodies. Activated cDCs then migrate to the LNs/SLOs where they express DNA vaccine-encoded antigen peptides on MCH-I and MHC-II. Activated cDCs present antigen-loaded MHC-II to $CD4^+$ T cells, which promotes the generation of IFN-γ^+CD4^+ T cells and IFN-γ secretion, in turn promoting IgG class-switching from IgG to IgG_{2C} in B cells. Additionally, cDCs present antigen-loaded MHC-I to $CD8^+$ T cells and promote the induction of polyfunctional $CD8^+$ T cells in a *STING*-dependent manner, but *STING* is not required within cDCs for the induction of polyfunctional $CD8^+$ T cells. This suggests that *STING* is required in other cell types to promote effector $CD8^+$ T cell programming. Created with Biorender.com

5.2 Clinical application of *STING* and DNA vaccines

STING is a critical protein that has been studied extensively for controlling pathogen infections and cancer cells as well as its role in mediating vaccine immunogenicity. Interest has been increasing in modulating STING activation to increase Th1 responses, including effector $CD8^+$ T cell and IgG_2 Ab responses [214-216]. For example, a research group designed a genetically encoded, constitutively active *STING* (*STING*^{V114M}) that they used as an adjuvant with an mRNA vaccine that targets cancer antigens, and they found that the addition of *STING*^{V114M} increased anti-cancer effector $CD8^+$ T cells [214]. The insights gained from the results of this dissertation can be used to help guide future *STING* adjuvant designs for use in future DNA vaccine trials. For example, the use of DNA vaccine-encoded *STING* adjuvants might induce more robust DNA vaccine-generated IgG_2 Ab responses and effector $CD8^+$ T cell responses required to combat cancers and pathogen infections. Future experiments will elucidate a more refined mechanism by which *STING* mediates DNA vaccine induction of Ab and effector $CD8^+$ T cell responses as well as drive better designs of *STING* adjuvants that can be used in concert with DNA vaccines.

Moreover, the gene *TMEM173* which encodes the protein STING is heterogeneous in the human population. Several mutations in the *TMEM173* allele have been identified in humans that can influence both human health and immune responses to nucleic acid vaccines. It was found that R232 in STING is the most common *TMEM127* allele found in humans and that *HAQ* is the second most common allele [217]. *HAQ* is a combination of 3 mutations found in STING which include R71H, G230A and R293Q [217]. The STING allele *HAQ* as well as *H232* have been identified as loss-of-function mutations [217]. *R232/R232* is the most common alleles in Europeans, *R232/HAQ* is the most common alleles in East Asians, and *Q293* is the most common allele in African populations [217]. It is estimated that ~30% of East Asians, and ~10% of Europeans possess a loss-of-function allele and studies have found that humans that possess *HAQ* or *H232* produce less IFN-α after vaccinia virus stimulation [217-218]. More research is required to gain a better understanding of the distribution of *TMEM127* alleles in different populations of humans. Since majority of DNA vaccine clinical trials do not result in approval for use in humans it is possible that many of the participants in these trials have partially or fully null *TMEM173* alleles which results in non-protective vaccine induced antibody and effector $CD8^+$ T cell responses. Future DNA vaccine clinical trials should screen participants' *TMEM173* alleles to identify if loss-of-function alleles correlate with suboptimal adaptive

responses post-DNA vaccination. Furthermore, given the prevalence of loss-of-function *TMEM173* alleles in the human population future DNA vaccine studies should consider including vaccine encoded *STING* as an adjuvant to increase DNA vaccine efficacy in participants that have both loss-of-function *TMEM173* alleles and functioning *TMEM173* alleles alike.

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