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STING, nanoparticles, autoimmune disease and cancer: a novel paradigm for immunotherapy?

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Abstract

DNA has potent immunogenic properties that are useful to enhance vaccine efficacy. DNA also incites hyper-inflammation and autoimmunity if DNA sensing is not regulated. Paradoxically, DNA regulates immunity and autoimmunity when administered systemically as DNA nanoparticles (DNPs). DNPs regulated immunity via cytosolic DNA sensors that activate the signaling adaptor Stimulator of Interferon Genes (STING). In this review, we describe how DNA sensing to activate STING promotes regulatory responses and discuss the biological and clinical implications of these responses for understanding disease progression and designing better therapies for patients with chronic inflammatory diseases such as autoimmune syndromes or cancer.

Keywords

DNA; STING; nanoparticles; immunity; tolerance; autoimmunity; cancer; immunotherapy

Introduction: *DNA sensors and immune responses to DNA*

DNA is present in all cells as it contains the genetic information needed to synthesize RNA and proteins that mediate metabolic processes in living cells. Evolution has equipped mammals with an array of sensors that recognize pathogen-associated molecular patterns (PAMPs), including an array of DNA sensors that recognize pathogen DNA in endosomal and cytosolic compartments of cells. PAMP sensors provide early warnings of infection and stimulate pro-inflammatory (immunogenic) responses by innate and adaptive immune cells. The DNA sensor Toll-like receptor-9 (TLR9) binds strongly to un-methylated cytosyl-guanyl dimers (CpG) in DNA, a hallmark of prokaryotic (bacterial) and mitochondrial DNA, though TLR9 also binds weakly to CpG motifs in nuclear DNA. TLR9 is expressed in endosomes of certain innate immune cells such as some macrophage (MΦ), dendritic cell (DC) and B cell subsets in mice and humans. Hence TLR9 is ideally positioned to sense invading or ingested pathogens and activate the signaling adaptor MyD88, which then stimulates downstream immunogenic responses such as type I interferons (IFN-I) and NFκB that drive protective host immunity mediated by innate and adaptive immune cells.

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Mammalian cells also express cytosolic DNA sensors that activate the adaptor Stimulator of Interferon Genes (STING, also known as TMEM173, MITA, MPYS, ERIS, TEMEM). Like the TLR9/MyD88 pathway, STING interacts with downstream adaptors TBK1, IRF3 and STAT6 to induce IFN-I and NFκB pro-inflammatory responses when DNA is sensed (Fig. 1). Cytosolic DNA sensors were discovered because some viruses and intracellular pathogens that stimulate DNA-dependent host immunity were not sensed by TLR9. Recent reviews describing cytosolic DNA sensors that activate STING describe current understanding of the molecular basis of cytosolic DNA recognition and STING signaling¹⁻⁹. Hence, these topics are not covered in detail here and key points are summarized briefly to provide relevant context only (next section).

The focus of this review is to describe recent evidence that cytosolic DNA sensing via STING not only incites immunity but also *suppresses (or regulates)* immunity (Fig. 1). Like immune stimulation, immune regulation is an active set of processes and is not simply a consequence of attenuating immunogenic signaling downstream of DNA sensors^{3, 10, 11}. We discuss the implications of this particular perspective on immune responses to DNA, which challenges the prevailing paradigm that DNA sensing incites immunogenic responses; a view predicated in large part on the common assumption that pro-inflammatory cytokine production by cultured cells following DNA exposure is a surrogate parameter for induction of protective immunity *in vivo*. This view is potentially misleading because immune regulatory processes that promote tolerance may predominate in local tissue microenvironments, even though pro-inflammatory cytokine production is also detected at these sites in response to inflammatory insults. Hence, immunogenic or regulatory responses to DNA can be discerned only in whole organisms (i.e. *in vivo*) using readouts that evaluate immunologic parameters such as delayed type hyper-sensitivity, antibody production, allograft rejection and disease progression. The realization that the immune system plays critical roles in tumor development and therapy provides other approaches to measure immunological responses *in vivo*, though definitive assays to detect tumor-specific immunity requires more sophisticated measurements than assessing tumor growth. Assays to detect cytokine and chemokine responses to inflammatory insults *in vivo*, and studies using cultured cells to assess cytokine production in response to defined insults are still valuable for elucidating the potential to activate innate immunity but these approaches alone provide little information about physiologic responses to DNA *in vivo* since increased pro-inflammatory cytokine production is commonly observed when immune regulatory or stimulatory responses predominate.

In Section 1, we provide a brief overview of immunogenic responses to DNA following cytosolic DNA sensing to provide context for describing emerging evidence that DNA is also sensed to incite regulatory responses via STING. In Sections 2-4, we consider biological and clinical implications of this emerging new perspective on immune responses to DNA.

1. Immunogenic responses to DNA via STING

1.1. Cytosolic DNA sensors that activate STING

An surprising number of cytosolic DNA sensors that activate STING have been identified to date in mammalian cells including cGAS, DAI, IFI16, DDX41, DNA-PKcs, Mre11; reviewed in ⁶. Many immune and non-immune cells express STING, suggesting that the ability to sense cytosolic DNA is a feature of many cell types. In contrast, TLR9 expression in mice and humans is restricted to some innate immune cells such as discrete macrophage (MΦ), dendritic cell (DC) and B cell subsets. As most functional studies on cytosolic DNA sensing have employed cultured cells it is unclear if all physiologic cells that express STING can also sense cytosolic DNA. Moreover, the significance of having an array of cytosolic DNA sensors, which may be functionally redundant, is currently unclear.

STING does not sense DNA directly but interacts with cytosolic DNA sensors ⁶, though critical DNA-sensor and sensor-STING interactions that drive responses to DNA have not yet been fully defined. The cytosolic DNA sensors listed above are responsive to double stranded DNA (dsDNA) but structural requirements for DNA recognition (e.g. length, base composition, stranded-ness) are not fully understood. These are important points to resolve as RNA:DNA hybrids and single-stranded DNA generated by some viral pathogens, as well as synthetic DNA and DNA oligonucleotides, may be sensed by some DNA sensors. These issues will likely have important implications for (i) understanding innate immune responses to pathogens and, (ii) engineering reagents that activate STING in particular cell types to elicit immunogenic or immune regulatory responses of therapeutic benefit to patients.

1.2 cyclic-GMP-AMP synthase (cGAS) and cyclic dinucleotides (CDNs)

To date, the enzyme cyclic-GMP-AMP (cGAMP) synthase (cGAS) is the best understood cytosolic DNA sensor due to a spate of high profile reports describing the enzymology and structure of cGAS in the last two years ^{1, 4, 7, 12, 13}. DNA binding induces cGAS to synthesize the cyclic dinucleotide (CDN) c[G(2',5')pA(3',5')p] (2'3' c-GAMP), which has an unusual (non-canonical) 2'-5' phosphodiester linkage rather than the canonical 3'-5' linkages found in CDNs synthesized by pathogenic organisms such as listeria ¹⁴. In eukaryotic cells 2'3' cGAMP made by cGAS functions as a secondary messenger by binding and activating STING located on the endoplasmic reticulum. This finding raises an intriguing parallel with microbial infections because listeria releases a natural bacterial CDN called cyclic diguanylate monophosphate (cdiGMP) into the cytosol of infected macrophages, and cdiGMP is sensed to activate STING ¹⁴⁻¹⁶. Thus, 2'3' cGAMP and cdiGMP represent archetypal eukaryotic and prokaryotic CDNs, respectively, and both CDNs serve as secondary signaling molecules to activate STING. Due to their ability to trigger IFN-I production via STING CDNs are under scrutiny as potential vaccine adjuvants ^{2, 17, 18}. However, why would a microbial organism such as listeria, which resists host immunity to cause chronic infections in immunocompetent hosts, evolve a strategy to actively trigger a potent host immunogenic response? This point hints at underlying complexity in the role of STING in provoking adaptive immunity. We discuss some key considerations about using CDNs as immune adjuvants in clinical settings below following a description of regulatory responses to DNA and CDNs via STING that attenuate immunity and autoimmunity in mice.

When activated by cytosolic DNA sensors or CDNs generated by cGAS, STING interacts with the signaling adaptor TBK1 to trigger at least two distinct pathways strongly associated with immunogenic responses, one via IRF3 to induce IFN-I (IFN β in particular), and another that engages IKKs to activate NF κ B, which promotes production of pro-inflammatory cytokines such as TNF α , IL-6, and IL-12. Due to the primary focus in the field on elucidating host responses to microbial infections, most studies on cytosolic DNA sensing evaluated immunogenic responses elicited via STING in response to infection by DNA viruses such as herpes simplex virus 1 or other intracellular pathogens such as listeria¹⁹. Host defense may be the primary reason why mammals evolved an array of cytosolic DNA sensors that activate STING. However, the immune stimulatory and regulatory attributes of DNA hint at a pivotal role for DNA sensors in inciting or inhibiting immunity in response to particular inflammatory insults.

1.3. Chronic DNA sensing incites hyper-inflammation and autoimmunity via STING

A different clinical perspective on cytosolic DNA sensing to activate STING emerged from studies on mice engineered to ablate two different DNA repair endonucleases, Trex-1 and DNaseII^{20, 21}. Mice lacking intact Trex-1 or DNaseII genes developed spontaneous and lethal hyper-inflammatory and autoimmune syndromes. Hyper-immune syndromes in these mice were driven by chronic STING activation to induce constitutive IFN-I production since ablating STING or IFN-I receptors (IFNAR) in mice lacking Trex-1 or DNaseII alleviated disease severity and lethality. Moreover, DNA-induced IFN-I production by cells derived from Trex-1-deficient mice was abolished by ablating cGAS DNA sensing, revealing that cGAS was the obligate cytosolic DNA sensor that incited hyper-immune responses in mice lacking Trex-1²². It is not clear if cGAS is the obligate STING-activating DNA sensor in all tissue cells or if other cytosolic DNA sensors can compensate for the loss of cGAS functions. Alternatively, some DNA sensors may function in a cell-type specific manner. As mice lacking Trex-1 or DNaseII functions developed radically different hyper-immune phenotypes, suggesting that these two DNA repair endonucleases may have distinct, non-redundant roles in regulating DNA-induced autoimmunity. These findings raise key questions about the source of DNA that is sensed to incite chronic STING activation in aseptically tissues. Enhanced DNA sensing may occur because of increased cell death due to loss of DNA repair functions. Alternatively, loss of DNA repair functions may impair the ability of cells that ingest dying tissue cells to degrade DNA and prevent DNA sensing to activate STING. Cells die continuously in aseptically tissues; even during homeostasis, tissues undergo continuous re-modeling. Moreover, environmental insults increase mechanical or metabolic stress that wounds tissues and incites local inflammation. These responses are likely to enhance cell death rates and consequently, increase access to immune stimulatory DNA that may be sensed to activate STING when DNA repair enzyme functions are defective (Fig. 1). Phagocytic M Φ s and DCs in tissues (particularly in inflamed tissues) are potential cells that ingest dying cells, and some DNA may escape degradation and be transferred into the cell cytosolic compartment to activate STING. If so, this process may be a component of the sentinel functions of M Φ s and DCs, which are specialized to detect tissue damage and respond by activating immune cells (the 'danger' response). Some non-immune cell types may also ingest DNA to activate STING. These points notwithstanding, the findings from Trex-1 and DNaseII deficient mice reinforce the prevailing paradigm that

DNA is a potent immune adjuvant by demonstrating that DNA incites hyper-inflammation and lethal autoimmunity when DNA repair functions are defective.

2. Immune regulatory responses to DNA via STING

Evidence that DNA regulates immunity is more difficult to find because regulation is more difficult to measure. TLR9 ablation in MRL^{lpr} mice prone to developing a spontaneous autoimmune syndrome that resembles systemic lupus erythematosus (SLE) in humans led to *accelerated* disease onset, indicating an unexpected regulatory role of TLR9 signaling in autoimmune disease progression in this model²³. Increased titers of anti-DNA specific antibodies are an early and hallmark feature of many lupus-like syndromes; in effect, DNA behaves like an autoantigen in these syndromes. TLR9 signaling to induce IDO-dependent regulatory phenotypes in specialized DC subsets that inhibit T cell immunity was also indicated in studies using synthetic oligonucleotides containing un-methylated CpG dimers as TLR9 ligands in mice^{24, 25}. A hint that STING signaling may regulate host immunity to an infection emerged from a study to assess the role of STING in protective host immune responses to listeria infection in mice²⁶. STING or IRF3 ablation led to *increased* protective immunity, indicating that STING/IRF3 signaling attenuated host immunity, though the regulatory mechanism was not defined in this study.

A number of mechanisms regulate immunity in settings of chronic inflammatory diseases. Here we focus exclusively on DNA-induced regulation of immunity mediated by cells induced to express indoleamine 2,3 dioxygenase (IDO), an intracellular enzyme responsive to IFN-I and IFN-II that regulates host immunity to pathogens and tumors, and inhibits autoimmunity and alloimmunity²⁷. We justify this selective approach because DNA-mediated regulation via STING has been linked to the IDO pathway. Other mechanisms that mediate regulatory responses to DNA may well be discovered as this phenomenon is explored further.

2.1 DNA nanoparticles (DNPs): tools to evaluate regulatory responses to DNA

DNA nanoparticles (DNPs) are used widely as vehicles for gene transfer and DNP-mediated gene transduction (using cargo DNA) or gene knockdown (using cargo siRNA) offers promising approaches to treat a range of clinical diseases; reviewed in²⁸. From a clinical perspective, it is important that DNPs elicit minimal inflammatory or other potentially toxic responses when and the choice of polymer and cargo DNA are therefore key factors that must be evaluated for optimal efficacy and minimal toxicities. Despite this, most studies focused on assessing cytokine responses to DNPs cultured cells, while few studies have fully evaluated responses to DNPs in physiologic settings. Moreover as discussed below, the route of DNP delivery is a crucial factor driving diametric immune responses to DNPs and other defined insults.

Systemic treatment with DNPs suppressed immunity and autoimmunity in mice²⁹⁻³¹. DNP cargo DNA was sensed to activate STING and induce IFN-I production, just as described for immunogenic responses. However, cargo DNA sensing stimulated IDO enzyme activity in a discrete subset of splenic DCs specialized to activate Foxp3-lineage regulatory CD4 T cells (Tregs) and suppress effector T cell responses when induced to express IDO; reviewed in³².

IDO regulates T cell immunity by catabolizing the essential amino acid tryptophan (Trp) to induce downstream responses to Trp depletion, which is sensed by GCN2-kinase, and release of Trp catabolites (known as kynurenines), some of which are aryl hydrocarbon receptor (AhR) ligands^{33, 34}. Immune regulatory responses to DNPs were mediated by cargo DNA (not PEI) and were dependent on STING and IFN-I receptor signaling. Regulatory responses were not TLR9 dependent as regulation still manifested in TLR9-deficient mice, and when cargo DNA lacked CpG motifs recognized by TLR9. The use of CpG-free cargo DNA also abolished copious IFN γ production by Natural Killer (NK) cells, due to TLR9-dependent NK cell activation in mice treated with DNPs containing bacterial plasmid DNA³⁰. Hence the removal of CpG motifs from cargo DNA provides a straightforward way to avoid stimulating IFN γ release in response to DNPs, which may mediate toxic effects in some clinical settings.

Discrete M Φ and DC populations in splenic marginal zone ingested DNP cargo DNA rapidly. However IFN β transcription, the signature response to STING activation, was induced exclusively in myeloid (CD11b⁺) DCs, while other myeloid cells expressed IL-1 β ²⁹. These findings revealed unexpected complexity and functional diversity (division of labor) in innate immune cells that responded to DNPs that were not evident from studies using cultured cells. These findings are also inconsistent with the paradigm that DNA and DNPs are immunogenic^{28, 35}. In part, these disparities arise because *systemic* administration of DNPs was essential to incite regulatory responses³⁰, while other delivery routes are commonly used to incite immunogenic responses to DNPs or vaccines containing DNA or CDNs as adjuvants. Nevertheless as mentioned above, one study described elevated pro-inflammatory cytokines (IFN γ , IL-12) and anti-tumor responses in tumor-bearing mice following systemic DNP treatments³⁵. It is unclear why systemic DNP treatments elicited potent regulatory responses via IDO but slowed tumor growth in the latter study. One potential explanation is that DNPs incite distinct local responses in lymphoid tissues and tumor microenvironments. These points aside, immune responses to DNPs identify STING as an immune inhibitory pathway due to the ability of some innate immune cells to ingest DNPs and induce IDO-dependent regulatory phenotypes in DCs following cytosolic DNA sensing to activate STING/IFN-I signaling. Regulatory responses to DNPs may point to underlying processes that overcome the immunogenic properties of DNA produced naturally, such as during microbial infections and by cells that die in aseptic tissues (Fig. 1).

2.2. Dying cells: a natural source of immune regulatory DNA?

As discussed above, DNA may provoke autoimmunity in physiologic settings if released in excessive amounts by dying tissue cells. Paradoxically, it has long been known that apoptotic cells regulate rather than stimulate immunity³⁶. Regulatory responses to apoptotic cells were shown to be IDO-dependent as IDO ablation accelerated development of anti-DNA antibodies in lupus-prone MRL^{lpr} mice or IDO1-deficient mice treated with apoptotic cells³⁷. Moreover, pre-treatment with apoptotic cells induced robust tolerance to skin allografts applied one week later³⁸, and IDO inhibitors applied at the time of initial exposure to apoptotic cells abolish this tolerogenic response (T.L.M., unpublished data). Rapid production of regulatory cytokines (IL-10, TGF β) is a hallmark of tolerogenic responses to apoptotic cells³⁷. When administered to STING-deficient mice, apoptotic cells

did not stimulate IL-10, TGF β or IDO but instead stimulated production of the pro-inflammatory cytokine IL-6²⁹. These findings suggested that some spleen cells are specialized 'sentinel cells' that ingest apoptotic cells and transfer cellular DNA to cytosol to activate STING, which then induces IDO in tolerogenic DCs via IFN-I signaling (Fig. 1). Candidates for sentinel cells that mediate these pivotal processes include marginal zone M Φ s,³⁷ and CD11b⁺ DCs²⁹, which responded selectively to apoptotic cells or DNP cargo DNA, respectively. Further studies will be required to test these hypotheses but these tolerogenic pathways may be critical for lowering the risk of inciting autoimmunity at inflammatory lesions where large numbers of cells die. Though difficult to model *ex vivo*, interactions between dying cells and neighboring cells that ingest and sense DNA to activate STING are likely to be critical factors driving innate immune responses that can incite tolerogenic or immunogenic responses to DNA. These processes will likely have profound implications for understanding the origins of inflammatory diseases that develop and progress over long periods, such as chronic infections, autoimmune diseases and cancer, as well as the prospects for developing better treatments for these diseases.

2.3 Type I Interferon (IFN-I): a pivotal arbiter of immune responses to DNA

What are the pivotal factors that drive diametric immunogenic or regulatory responses to DNA? In addressing this question it is important to stress that immune cells must be activated to promote tolerance, as well as immunity; in other words, tolerance induction is an *active* immunologic process, not simply *passive* non-responsiveness to inflammatory and antigenic stimuli²⁷. Tissue inflammation activates many types of innate and adaptive immune cells and IFN-I and IFN-II (IFN γ) are common early signatures of inflammation that stimulate hundreds of downstream genes, known collectively as interferon-stimulated genes (ISGs). Thus, cytosolic DNA sensing has multiple consequences because IFN-I is downstream of STING. Due to the major focus in the field on host responses to microbial infections the traditional emphasis of most IFN-related research has been to identify ISGs that mediate host innate and adaptive immunity to suppress microbial infectivity and promote robust long-lasting protective immunity; of course, this is also the goal of prophylactic vaccination. Consequently, ISGs that suppress host immunity and promote tolerance have not been subjected to the same degree of intense scrutiny. As a recent illustration, a comprehensive screen to assess the effects of 350 human ISGs on the infectivity of 14 different viruses that infect humans identified 47 ISGs (including cGAS) that inhibited the infectivity of at least one virus³⁹; however in the same study, another 25 ISGs *enhanced* viral infectivity, a tacit reminder of the underlying complexity of host 'IFN signatures' to infection. This is an important point as excessive IFN-I correlates with higher risk of developing SLE in humans⁴⁰ but IFN-I has also been linked to robust immunosuppression mediated by HIV-1 and other pathogens that cause chronic infections^{41, 42}. These diametric effects of IFN-I imply that this family of closely related cytokines has evolved to play a pivotal role on determining immune outcomes.

IDO is an ISG since IFN-I and IFN-II are potent IDO inducers due to the presence of JAK-STAT signaling response elements (ISRE, GAS) in the promoter regions of all mammalian IDO genes sequenced to date. Consistent with this point, many infectious pathogens stimulate IDO, including influenza, listeria, leishmania and HIV-1^{41, 43-45}. M Φ s expressing

IDO mediate innate host defense to some intracellular pathogens (e.g. toxoplasma). Paradoxically, DCs and MΦs expressing IDO *regulate* T cell immunity in a range of clinically relevant settings, including infectious and autoimmune diseases and cancer; reviewed in ⁴⁶⁻⁴⁸. Thus, pathogens that cause chronic infections may stimulate host cells to express IDO in order to persist. ISGs like IDO that regulate host immunity are obvious evolutionary targets for pathogens to exploit in this way. The key point is that DNA sensing to activate STING may drive dominant immunogenic or regulatory responses to inflammation because the immunologic responses mediated by downstream ISGs depend critically on which cells and genes receive and respond to IFN-I (Fig. 1). Thus, IFN-I occupies a pivotal position in determining how the innate immune system elicits particular responses by adaptive immune cells to infections and other inflammatory insults.

3. Cytosolic DNA sensing in tumor microenvironments; *beneficial or detrimental?*

An emerging novel paradigm in cancer immunology is that STING/IFN-I signaling plays a critical role in eliciting clinical responses to cancer therapies (Fig. 2, green highlights). IFN-I signaling was essential for tumor antigen-specific cross-priming of tumor-specific CD8 T cells that mediated anti-tumor responses by CD8α⁺ DCs in melanoma bearing mice following vaccination ⁴⁹. Moreover, the cGAS/STING pathway was essential, while TLR9/MyD88 (or TRIF) signaling was dispensable, for DCs to sense tumor-derived DNA and prime effector CD8 T cell responses via IFN-I ^{50, 51}. These findings suggest that dying tumor cells are a major source of DNA that triggers IFN-I production via the cGAS/STING sensing pathway to prime effective anti-tumor immunity in response to standard therapies and immunotherapies ⁴⁶. However the tumor models used were highly immunogenic and were rejected spontaneously or were responsive to radiotherapy. Thus it is unclear if STING-mediated immunogenic responses will manifest in other tumor models in which local regulation may play more prominent roles. Moreover ISG signatures in tumor microenvironments may indicate unfavorable as well as favorable responses to DNA-damaging interventions such as radiotherapy ⁵², hinting at underlying complexity in endogenous responses to DNA.

3.1. DNA sensing in tumor microenvironments: a potential source of tumor tolerance?

Counter to the paradigm described above, a pivotal role for STING in *protecting* mice from colorectal tumorigenesis driven by intestinal inflammation emerged from a recent study ⁵³. Potent immune regulation is also a hallmark of tumor microenvironments and is a major barrier to successful immunotherapy. The immune system restrains the ability of malignant cells to form tumors through molecular signals and cellular interactions collectively known as ‘cancer immunoediting’; reviewed in ⁵⁴. Malignancies that negotiate checkpoints in the cancer immunoediting process establish local microenvironments in which regulation predominates, allowing them to progress into overt tumors unimpeded by anti-tumor immunity (Fig. 2). By the time tumors are detected (clinical presentation) they are already ‘cocooned’ in tolerogenic tumor microenvironments that protects them from destruction by natural immunity and thwarts efforts to incite anti-tumor immunity using cancer vaccines. The key question is how do malignant cells initiate and sustain tolerogenic

microenvironments? One speculative answer is that local inflammation associated with malignant lesions that blocks tumor escape may eventually be subverted to permit tumor escape, possibly due to IFN-I mediated induction of IDO. If so, pre-malignant cells or other local cells that die may be a critical source of DNA, which may be sensed to activate STING/IFN-I signaling that (a) incites anti-tumor immunity or (b) induces IDO to suppress anti-tumor immunity at distinct stages in tumor development, therapy and relapse (Fig. 2, green and red highlights, respectively).

3.2 Implications for cancer immunotherapy

The realization that tumors present clinically with ‘mature’ tolerogenic microenvironments has profound implications for designing effective therapies. In particular, reagents that target common regulatory pathways found in tumor microenvironments (‘checkpoint blockade’) have led to some remarkable responses in pre-clinical studies; prompting the editors of *Science* magazine to cite cancer immunotherapy as the scientific breakthrough of 2013⁵⁵. Consequently, clinical trials are currently ongoing using cancer immunotherapy reagents such as monoclonal antibodies to block critical interactions between negative co-stimulatory molecules on effector T cells (e.g. CTLA4, PD-1) and their ligands (B7, PD-L, respectively) expressed by antigen presenting cells (APCs) or other cells in tumor microenvironments; reviewed in ⁵⁶. Abnormally elevated local IDO activity is also a common feature of mature tumor microenvironments, providing a rationale for using pharmacologic IDO inhibitors as a checkpoint blockade immunotherapy; reviewed in ⁴⁸. As illustrated by recent reports, cytosolic DNA sensing to activate STING is clearly an important factor to consider from the viewpoint of optimizing cancer immunotherapy. However, the ability of DNA to drive potent local regulatory responses via STING may be an equally important consideration to take into account, especially when considering how to design ‘prophylactic’ vaccines to impede tumor progression in humans with susceptible genetic profiles by interfering with the processes that initiate and sustain tolerogenic microenvironments that protect developing tumors.

4. STING activators in the clinic: to immunize, or to tolerize, that is the question

Reagents that activate STING have considerable potential as components of (a) prophylactic vaccines to induce protective immunity or (b) therapeutic vaccines to provoke immunity to established chronic infections or cancer. These applications are predicated on the assumption that STING activators are immunogenic and that regulatory responses to STING/IFN-I signaling will not attenuate or prevent immunogenic responses to STING activators. In this section we consider potential clinical implications of diametric responses to STING activating reagents for immunotherapy.

4.1 STING activators as immune adjuvants

As mentioned above, CDNs are under scrutiny as novel vaccine adjuvants due to their ability to STING/IFN-I signaling and promote immunogenic responses ^{2, 17}. Initial concerns about species specificity of responses to CDNs arose due to unfavorable outcomes from clinical trials to assess the efficacy of 5,6-dimethylxanthone-4-acetic acid (DMXAA), a

CDN analogue that binds and activates STING. DMXAA has vascular disrupting attributes that promoted impressive anti-tumor effects in mice ^{57, 58}, though it is not clear if the ability of DMXAA to activate STING is required for anti-tumor effects to manifest. Negative outcomes from clinical trials on DMXAA led to the realization that human STING (hSTING) genes exhibit polymorphisms that reduced responsiveness to canonical (microbial) CDNs such as cdiGMP and CDN analogues such as DMXAA ⁵⁹. This concern abated when it emerged that the non-canonical CDN isomer 2'3' cGAMP was the 'eukaryotic' second messenger generated by cGAS, as all common hSTING variants responded to 2'3' cGAMP, while none responded to DMXAA or another CDN analogue 10-carboxymethyl-9-acridanone, CMA ⁶⁰⁻⁶². Consequently, DMXAA and CMA are now regarded as a 'mouse ligands' for STING. Current research is focused on optimizing CDN delivery and bioavailability to generate effective immune responses to vaccines ². However, sustained exposure to CDNs attenuated STING signaling in isolated cells due to STING phosphorylation ¹¹, and the NOD-like receptor (NLRC3) attenuated DNA sensing to activate STING and inhibited responses to HSV-1 in mice ³. It is unclear if these cell-autonomous negative feedback control mechanisms also attenuate STING signaling in tissue cells from Trex-1 ²⁰ and DNaseII ²¹ deficient mice to slow progression of spontaneous hyper-immune syndromes.

DMXAA and 2'3' cGAMP repolarized regulatory (M2) to stimulatory (M1) MΦs in mice to comparable extents, a key feature of DMXAA that primarily accounts for anti-tumor effects observed in mice ⁶³. DMXAA and 2'3' cGAMP treatment increased M1 MΦ markers (iNOS, IL-12p40) and decreased M2 MΦ markers (Arg-1, Fizz1) comparably. Intramuscular injection of OVA plus 2'3' cGAMP also stimulated OVA-specific antibody production and T cell responses, and these responses were abolished in mice lacking STING ¹³. The same study revealed that cells from cGAS-deficient mice, including fibroblasts, macrophages, and dendritic cells, did not produce IFN-I and other cytokines in response to DNA transfection or infection with the DNA virus Herpes Simplex Virus 1 (HSV1). Mice lacking cGAS or STING failed to clear HSV-1 infections and their survival was reduced dramatically, indicating that the cGAS/STING pathway is a critical pathway required to incite effective host immunity to HSV1. In another recent study, STING-mediated TNFα (not IFN-I) production was essential for mucosal adjuvant effects following cdiGMP treatment ⁶⁴. Collectively, these findings validate the use of DNA and CDNs as STING activators to potentiate immunity to vaccines.

On the other hand, use of STING activators risks provoking autoimmunity, and some individuals may be more susceptible to this potential toxicity than others. Defective DNA endonuclease functions in mice and humans have been associated with increased risk of developing SLE and thyroid autoimmunity ^{65, 66}. Moreover, recent studies identified mutant forms of hSTING driving enhanced pro-inflammatory responses in a new pediatric syndrome, deemed STING-associated vasculopathy with onset in infancy syndrome (SAVI), and in a familial inflammatory syndrome with lupus-like manifestations, indicating that gain-of-function mutations in STING can provoke spontaneous autoinflammatory diseases ^{67, 68}. In addition, human Trex1 polymorphisms correlated with higher risk of developing Aicardi-Goutieres syndrome (AGS), a rare lupus-like syndrome ⁶⁹. This provided the original impetus to study mice with defective Trex1 genes, which were prone

to lupus-like syndromes driven by excessive STING activation to induce constitutive IFN-I release²⁰. However, ablating IFN-I receptors (IFNAR) rescued mice lacking DNaseII from embryonic lethality due to excessive IFN-I production but IFNAR/DNaseII defective mice developed polyarthritis syndromes as adults that could be ameliorated using anti-TNF blockade therapies⁷⁰. Thus, even in the absence of IFN-I signaling, excessive DNA sensing to activate STING drove lethal pro-inflammatory responses leading to TNF-mediated arthritis.

4.2 STING activators as tolerogens to attenuate autoimmune syndromes

DNP treatments attenuated antigen-induced arthritis (AIA) and autoimmune encephalomyelitis (EAE, a model of multiple sclerosis) in mice^{30, 31}. Therapeutic responses to DNPs in the EAE model were abolished in mice lacking STING, IFNAR or IDO genes, indicating that regulatory responses to DNPs were critically dependent on STING/IFN-I signaling to stimulate IDO activity in DCs. Importantly, therapeutic responses manifested when DNPs were applied during MOG immunization to induce EAE, at the time of EAE onset, or when EAE was fully established. DNPs reduced pro-inflammatory cytokine production and antigen-specific T-cell responses in spleen and attenuated T cell infiltration into central nervous system (CNS) tissues. CDN (cdiGMP) therapy also attenuated EAE onset and severity. Thus, the STING/IFN- β pathway can be exploited therapeutically to attenuate clinically relevant autoimmune syndromes by systemic administration of STING activators. This administration route may preferentially deliver STING activators to sentinel myeloid cells in spleen specialized to promote tolerance to material from dying cells (Fig. 1); this may explain why the same reagents that activate STING can be delivered by different routes to incite or inhibit adaptive immunity.

An intriguing but unresolved question is whether STING activating reagents target the same downstream IFN-I responsive tolerizing pathways as recombinant IFN β , a standard treatment for multiple sclerosis (MS). The mode of action of IFN β in responsive MS patients and the reasons for differential responsiveness to IFN β are not well understood. Moreover, the recurrent IFN β treatments required to control MS also induce severe progressive toxicities such as severe local pain, headaches, leading to frequent therapy cessation⁷¹. Speculatively, the toxic effects of IFN β may arise from lack of local targeting to trigger selective downstream responses to IFN-I by tolerizing cells in spleen. If so, DNPs and CDNs may be more effective in targeting such cells to optimize beneficial regulatory responses and reduce toxic side effects caused by widespread responses to recombinant IFN β .

Expert commentary

Recent discoveries of an array of cytosolic DNA sensors that activate STING has awakened intense interest in exploiting the *immunogenic* properties of DNA and other STING activating reagents to enhance the efficacy of vaccines designed to incite protective immunity and to treat patients with chronic infectious diseases or cancer. In the race to exploit these opportunities it will be important to assess if *regulatory* responses to DNA attenuate or abolish immunogenic responses to DNA or STING activators using assays that detect immune regulation *in vivo*. The key point is that immunologic responses to

environmental insults and therapeutic reagents are rarely simple (i.e. black or white), are often nuanced and complex (i.e. gray), and include elements that both excite and subdue immunity. The key lesson is that rigorous evaluations of immune responses using the most informative models are necessary. While studies based of simple readouts are informative, assumptions that they predict immune responses in more complex models may be misleading at best, - and at worst, potentially dangerous in clinical settings. These points notwithstanding, the field has uncovered unexpected complexity in innate immune DNA sensors that reveals previously unsuspected biological significance of DNA as both a potent adjuvant and a potential tolerogen.

Five-year view

Current rapid progress in defining cytosolic DNA sensing pathways that activate STING/IFN-I signaling provides unprecedented opportunities to develop novel vaccines to prevent infectious diseases and treat cancer patients. However, DNA sensing also has more subtle roles in disease progression that requires rigorous research to discern whether DNA incites autoimmunity and tumor-induced tolerance. While most research is focused on DNA from infectious microbes that incite host immune responses, future focus is likely to swing towards innate sources of DNA from dying cells as the source of inflammatory signals that drive hyper-immune (autoimmunity, allergy) or hypo-immune (cancer, chronic infections) syndromes. Research driven by these interests in the next five years is likely to bear fruit in the form of better understanding of the etiology of chronic inflammatory diseases, how to slow or prevent progression of these diseases before clinical presentation, and how to improve treatments for these syndromes after clinical presentation.

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Abbreviations

STING	Stimulator of Interferon Genes
DNP	DNA nanoparticle
PAMP	pathogen-associated molecular pattern
TLR9	Toll-like receptor-9
DC	dendritic cell
MΦ	macrophage
cGAMP	cyclic-GMP-AMP
cGAS	synthase
CDN	cyclic dinucleotide
IFN-I/IFNAR	interferon type I/receptor
ISG	interferon-stimulated gene
IDO	indoleamine 2,3 dioxygenase

GCN2	general control non-derepressible-2
AhR	aryl hydrocarbon receptor
EAE	experimental autoimmune encephalomyelitis

Key issues

- DNA is an immunogen but is also a tolerogen.
- The route and method of DNA delivery are critical factors driving particular immune responses in whole organisms.
- Evaluating responses to DNA by cultured cells is informative but has limited value in predicting immunological responses to DNA in whole organisms, and may even be misleading.
- Cytosolic DNA sensors that activate the adaptor STING are expressed by many cell types, greatly expanding the potential of DNA to be sensed to elicit immune responses by stimulating IFN-I production at sites of inflammation.
- Emerging evidence shows that the cytosolic DNA sensor cGAS is pivotal in delivering activation signals to STING via the second messenger and cyclic dinucleotide (CDN) 2'3'-cGAMP.
- Previous concerns that some CDNs were mouse specific abated when 2'3'-cGAMP was found to be a universal activator of polymorphic human STING genes.
- Incorporating DNA or CDNs in vaccines offers considerable potential to improve vaccine efficacy, provided that STING-mediated regulation does not attenuate adjuvant effects.
- DNA and CDNs also offer considerable potential to develop 'tolerogenic' vaccines to treat autoimmune syndromes.

Figure 1. DNA sensing to activate STING can incite or inhibit immunity

Environmental insults that cause tissue wounding and stress lead to cell death and release of cell DNA that may be sensed by sentinel cells or adjacent tissue cells to stimulate STING and activate immune cells. Downstream of STING IFN-I may incite (a) immunity or autoimmunity (green), or (b) regulatory responses that overcome (or attenuate) immunogenic responses and promote tolerance (red). cGAMP made by the DNA sensor cGAS or CDNs from external sources activate STING directly.

Figure 2. Divergent roles for DNA sensing pathways at different stages of tumor development and after therapy?

Tumor development begins when the immune system fails to restrain outgrowth of malignant cells leading to tumor escape (left). After clinical presentation, therapies such as anti-tumor vaccines combined with checkpoint blockade (cancer immunotherapy) may elicit clinical responses dependent in part on STING/IFN-I signaling to stimulate tumor-specific CD8 T cells via DC cross-priming (green highlights). The model posits that sensing of DNA from dying tumor cells to activate STING/IFN-I signaling may also promote tumor tolerance (red highlights) that (a) protects developing tumors before clinical presentation and (b) promotes relapse after therapy.