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Cytosolic DNA Sensing via the Stimulator of Interferon Genes (STING) Adaptor: The Yin and Yang of Immune Responses to DNA

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Summary

DNA is immunogenic and many cells express cytosolic DNA sensors that activate the Stimulator of Interferon Genes (STING) adaptor to trigger interferon type I (IFN-β) release, a potent immune activator. DNA sensing to induce IFN-β triggers host immunity to pathogens but constitutive DNA sensing can induce sustained IFN-β release which incites autoimmunity. Here we focus on cytosolic DNA sensing via the STING/IFN-β pathway which regulates immune responses. Recent studies reveal that cytosolic DNA sensing via the STING/IFN-β pathway induces indoleamine 2,3 dioxygenase (IDO), which catabolizes tryptophan to suppress effector and helper T-cell responses and activate Foxp3-lineage $CD4^+$ regulatory T (Treg) cells. During homeostasis, and in some inflammatory settings, specialized innate immune cells in the spleen and lymph nodes may ingest and sense cytosolic DNA to reinforce tolerance that prevents autoimmunity. However, malignancies and pathogens may exploit DNA-induced regulatory responses to suppress natural and vaccine-induced immunity to malignant and infected cells. In this review we discuss the biologic significance of regulatory responses to DNA and novel approaches to exploit DNA-induced responses immune for therapeutic benefit. The ability of DNA to drive tolerogenic or immunogenic responses highlights the need to evaluate immune responses to DNA in physiologic settings relevant to disease progression or therapy.

Keywords

STING; DNA sensors; tolerance; autoimmunity

Introduction

The immune adjuvant properties of DNA are well known and are exploited to enhance vaccine responses. Recent reports describe a surprisingly large array of cytosolic DNA sensors, many of which activate the Stimulator of Interferon Genes (STING, aka MITA,

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ERIS, MPYS, TMEM173) to induce IFN-β in a broad range of cell types (reviewed in [1–6]. IFN-β is a potent immune cell activator, inciting host defense against many pathogens. As most mammalian cells express cytosolic DNA sensors, DNA sensing may have wider biological significance than signaling pathogen presence. Moreover, cytosolic DNA sensing to activate the STING/IFN-β pathway has been shown to incite lethal hyper-inflammatory and autoimmune syndromes in mice with defective DNA-catabolizing enzymes [7, 8]. Thus microbial DNA sensing signals danger but immunogenic DNA is inherently dangerous and responses to DNA must be regulated - even under sterile homeostatic conditions - to avoid inciting *horror autotoxicus*.

Several reviews describe the recent rapid progress in elucidating cytosolic DNA sensors that induce immunogenic responses to infections or vaccines, and that provoke spontaneous hyper-immunity via the STING/IFN-β pathway [1–6]. However, this focused perspective neglects immune regulatory responses mediated by some interferon-stimulated genes (ISGs). For example, IFN-β has been shown to induce indoleamine 2,3 dioxygenase (IDO), an enzyme that regulates T-cell responses and activates Foxp3-lineage $CD4^+$ regulatory T (Treg) cells in settings of inflammation (reviewed in [9]). Recent studies also highlight unanticipated roles for IFN-β in attenuating host immunity to lymphocytic choriomeningitis virus infection [10, 11] and *Listeria monocytogenes* vaccination [12], though downstream regulatory mechanisms were not defined. Here we focus on immune regulatory responses to cytosolic DNA sensing via the STING/IFN-β pathway in physiologic settings, consider the potential biologic significance of such responses, and discuss novel opportunities to manipulate these responses for therapeutic benefit

1. Immunogenic DNA: *a danger signal and a potentially dangerous adjuvant*

DNA sensing alerts hosts to the presence of dangerous pathogens and DNA is used widely as a vaccine adjuvant to drive immunity. Until recently, DNA sensing in mammals was considered an exclusive attribute of specialized immune cells, such as plasmacytoid dendritic cells (pDCs) and some B cells, all expressing Toll-Like receptor-9 (TLR9), which senses prokaryotic DNA. TLR9 binds unmethylated CpG dimers in DNA to induce IFN-type I and this response elicits host immunity to microbial infections due to the immunogenic effects of ISGs, including an array of pro-inflammatory cytokines. Thus TLR9 detects danger (pathogens) and elicits responses that eliminate them. As detailed in several recent reviews, cytosolic DNA sensors extend the scope of this ‘defense against danger’ paradigm due to their number and broad distribution in a wide range of immune and stromal cell types [1–6]. Several cytosolic DNA sensors, including cyclic GMP-AMP synthase (cGAS) have been shown to activate STING, which interacts with TANK binding kinase (TBK1) and interferon response factor-3 (IRF3) to induce IFN-β (Figure 1). Cyclic dinucleotides (CDNs), such as cyclic diguanyl monophosphate (cdiGMP), have also been shown to activate STING to induce IFN-β, and some microbial organisms such as *Listeria* produce CDNs, which are sensed via STING to alert hosts to the presence of microbial infections [13–16]. In mammalian cells, the nucleotidyltransferase cyclic GMP-AMP synthase (cGAS) synthesizes the CDN cG[2’–5’]pA[3’–5’]p (2’3’-cGAMP) when cytosolic DNA is sensed; the [2’–5’] phosphodiester linkage is unique to 2’3’-cGAMP and differentiates it from microbial CDNs (reviewed in [17]). Thus microbial cdiGMP (3’–5’

linked) and endogenous 2'3'-cGAMP made by cGAS are distinct CDN isotypes that both activate STING to trigger IFN-β release. CDNs generated by cGAS activate UNC51-like kinase (ULK1/ATG1), which inactivates STING to prevent sustained signaling during autophagy [18] These developments raise key unresolved questions regarding (i) optimal DNA isoforms that activate cGAS and other cytosolic DNA sensors, (ii) cell-type specificity of functional DNA sensing activity and (iii) STING mutations and regulatory mechanisms that affect DNA and CDN sensing to stimulate IFN-β, especially in humans [19].

Immunogenic DNA is also dangerous, as shown by studies with mice lacking the DNA repair enzymes DNAseII or Trex-1; these mice developed lethal hyper-inflammatory or autoimmune syndromes due to sustained cytosolic DNA sensing via STING, which induced chronic IFN-β production [7, 8]. These studies provide striking demonstrations of the inherent potential to induce life-threatening autotoxicity, in this case due to innate DNA immunogenicity. A key issue is the source of immunogenic DNA in sterile tissues in the absence of inflammatory stimuli. Dying cells are the obvious source as cells die constitutively, even in healthy tissues, due to finite cell longevity and mechanical or metabolic stress associated with normal tissue function or tissue remodeling. However, it is unclear how DNA from dead or dying cells accesses the cytoplasm of other cells that can sense cytosolic DNA to activate the STING/IFN-β pathway. Inflammatory insults such as infections, tumor growth and tissue wounding, which enhance cell death, amplify opportunities to sense DNA and induce immunity, but also lower the tolerance barriers that prevent autoimmunity. Degrading DNA [7] and attenuating STING signaling are two ways to suppress chronic DNA sensing in sterile tissues, but another way to prevent autotoxicity may be to stimulate regulatory ISGs, for example the tryptophan catabolizing enzyme indoleamine 2,3 dioxygenase (IDO), which reinforce tolerogenic processes in homeostatic and inflammatory settings. The crucial need to allow immunity to infections to manifest on one hand, while maintaining self-tolerance on the other, suggests that cytosolic DNA sensing may incite both immunogenic and tolerogenic responses to 'foreign' and 'self' DNA. From an immunologic perspective, the key point is that DNA is an inherently 'dangerous' biomolecule and responses to DNA must be finely tuned to match particular physiologic circumstances.

2. Active regulation of immune responses to DNA: *the flip side of ISGs*

Some ISGs stimulate immunity whereas other ISGs, such as IDO, have been shown to suppress immunity. A recent comprehensive survey of responses to 14 human DNA and RNA viruses identified a central role for cGAS in triggering ISG responses [20], indicating that cytosolic DNA sensing is pivotal in elaborating host responses to DNA and RNA virus infections. It is unclear why cGAS is responsive to RNA viruses, though cGAS may sense retroviral cDNA or RNA:DNA hybrids. This point notwithstanding, IFN-β is released following STING activation by cytosolic DNA sensors such as cGAS, and IFN-β is a potent activator of innate (e.g APCs) and adaptive (T/B cells) immune cells. However, activated immune cells may drive dominant immunogenic or tolerogenic responses, contingent on other factors in affected microenvironments that shape downstream responses to (i) insults driving immune responses and (ii) other ISGs responsive to IFN-β [21]. To illustrate this paradigm with a specific example, oligonucleotides containing unmethylated CpG dimers

(CpGs) ligate TLR9 and are widely regarded as immune stimulator adjuvants. However, when CpGs were administered systemically (by intravenous injection) to mice, antigen-specific Th1 or Th2 effector responses elicited in vivo were suppressed in spleens or lungs in a CpG dose-dependent manner [22–26]. Consistent with the widely known immune adjuvant properties of TLR ligands, low CpG doses (25 μg) enhanced splenic Th1 responses. In striking contrast, higher CpG doses (100 μg) suppressed splenic Th1 responses due to IFN-αβ-mediated IDO induction in a subset of DCs expressing the B-cell marker CD19, which activated Treg cells [22–24]. Thus IFN-αβ signaling is the pivotal driver of both stimulatory (Th1) and regulatory (Treg) responses to TLR9 ligands, and IDO is the critical ISG driving dose-dependent immune regulatory outcomes following TLR9 ligation in vivo. As TLR9-sensing induces IFN-αβ release at high and low doses, it is unclear why IDO induction was dose-dependent, although one potential explanation is that there are lower local IFN-αβ signaling thresholds for inducing immunogenic responses than IFN-αβ signaling thresholds for inducing $CD19^+$ DCs to express IDO. IDO is not the only ISG that regulates immunity and IFN-αβ signaling may synergize with regulatory cytokines (e.g. TGF-β, IL-10) to drive dominant regulatory outcomes in some inflammatory settings. For example, systemic exposure to apoptotic cells, which drives tolerogenic responses, was shown to stimulate the release of regulatory (TGF-β, IL-10) and pro-inflammatory (IL-6, TNF-α, IL-12) cytokines in spleens of mice [27]. However, administering IDO inhibitor at the same time enhanced pro-inflammatory but reduced regulatory cytokine production and drove effector T-cell responses [27], indicating that the balance of pro-inflammatory and regulatory cytokines, and not the release of specific cytokines per se, is the critical factor influencing immune outcomes. The key lesson from these studies is that cytosolic DNA sensing to activate STING and drive IFN-β release may have tolerogenic or immunogenic consequences in physiologic settings of inflammation which are relevant to clinical disease, including autoimmune syndromes, cancer and chronic infections. Consistent with this paradigm, IFN-αβ and IFN-γ was shown to suppress MOG-induced EAE (experimental autoimmune encephalomyelitis), a model of multiple sclerosis (MS) [28] Moreover, lupus-prone MRL^{lpr} mice, a model of human systemic lupus erythematosus (SLE), lacking TLR9 genes exhibited accelerated onset of lupus symptoms and more severe pathology compared with MRL^{lpr} mice with intact TLR9 genes [29]. These observations emphasize the critical importance of evaluating immune responses to DNA rigorously in physiologic settings relevant to disease progression or therapy, since extrapolations based on responses to DNA by cultured cells may reflect cell-type specific responses to DNA but may nevertheless be misleading with regard to dominant responses to DNA that manifest in vivo.

3. DNA nanoparticles: *tools for elucidating regulatory responses to DNA in mice*

DNA nanoparticles (DNPs), which contain the cationic polymer polyethylenimine (PEI) and plasmid DNA (pDNA), are used as vehicles to transfer genes into cells and animals. DNPs are made by combining polymers and cargo DNA to form nanoparticles with specific surface electrostatic charge and size ranges, which may have profound effects on DNP processing in physiologic tissues. DNPs have been shown to provoke pro-inflammatory cytokine production and anti-tumor immunity in mouse models of lung and ovarian cancer [30, 31]. Unexpectedly, systemic (intravenous) treatment of mice with DNPs was shown to induce IDO enzyme activity in tissues, but sensing of cargo plasmid DNA to induce IFN-αβ

and IDO was not TLR9-dependent [32]. Moreover, IFN-αβ (but not IFN-γ) signaling was shown to induce IDO-dependent regulatory responses, which activated Treg cells to suppress helper/effector T cell responses.. In a different study, regulatory responses to DNPs were shown to be STING-dependent and systemic cdiGMP treatment to activate STING directly induced IDO [33]. These findings revealed that DNP cargo DNA enters the cytosolic compartment of cells to trigger potent regulatory responses via the STING/IFN-β/IDO pathway, and that this immunogenic response is capable of overcoming the immunogenic responses co-induced by DNPs. Systemic DNP or CDN administration is a key factor driving dominant immune regulatory outcomes, as intramuscular and sub-cutaneous cdiGMP injection in mice was shown to enhance humoral and cell-mediated immunity to vaccination [34]. However, it is unclear why systemic DNP treatments suppress Th1 responses to immunizing antigens [32, 33] but induce anti-tumor immunity in tumor-bearing mice [31]; distinct local responses to DNPs in lymphoid tissues and tumor microenvironments may offer a potential explanation.

4. Cytosolic DNA sensing by immune cells: *division of labor and functional dichotomy*

The type of cell that senses cytosolic DNA is likely to be a key factor influencing downstream immunological outcomes. Certain key questions remain unresolved: Which cell types ingest cellular DNA from dying cells or cargo DNA from DNPs by what mechanisms? Why do these cells fail to degrade all ingested DNA that end up in endosomes or lysozomes? –How is DNA transferred to the cytoplasm so that cytosolic DNA sensors can activate the STING/IFN-β pathway and induce downstream regulatory ISGs?

In Trex-1-deficient mice, non-hematopoietic (stromal) cells were shown to sense abnormal accumulations of ‘self’ DNA and trigger chronic immunogenicity, leading to autoimmunity [7]. In another study, a discrete subset of myeloid ($CD11b^+$) DCs was the only cell type in spleen that transcribed IFN-β1 genes after systemic DNP treatment, though other cell types ingested DNPs and contained cargo DNA [33]. Thus it may not be a coincidence that, in a recent study to examine antigen uptake in living lymphoid tissues using intra-vital techniques, $CD11b^+$ DCs were shown to ingest particulate antigens rapidly [35].

Other spleen cells have also been shown to ingest DNPs rapidly. Marginal zone macrophages (MZMs; $CD169^+$, $F4/80^{neg}$) in mouse spleen ingested DNPs rapidly and avidly, but unlike $CD11b^+$ DCs, no DNP cargo DNA was detected in MZMs [33], suggesting that MZMs ingest and degrade particulate material containing DNA such as chromatin, which resembles DNPs before DNA accesses the cytosol; this scenario is consistent with the ability of MZMs to remove blood-borne particulate materials in a way that does not incite autoimmunity [36]. Unlike MZMs, some splenic $CD8\alpha^+$ DCs and myeloid non-DCs ($CD11b^+CD11c^{neg}$) also ingested DNPs and retained cargo DNA but did not transcribe IFN-β1 genes [33], suggesting that cytosolic DNA sensing to activate the STING/IFN-β pathway may be defective in these cell types. Treating mice with cdiGMP elicited responses in the spleen that were remarkably similar to those induced by DNPs [33], reinforcing the conclusions that myeloid DCs are ‘first-responder cells’ and are specialized to sense cytosolic DNA and CDNs, and that the DNA sensing STING/IFN-β pathway may be functionally defective in other ‘non-responder’ cells. DNP and cdiGMP treatments were

also shown to induce comparable patterns of IL-1β transcription via a STING-independent pathway [33]; however myeloid non-DCs (not myeloid DCs) expressed the highest levels of IL-1β transcripts. Another recent report revealed that bacterial CDNs stimulate mucosal immunity in mice via a pathway dependent on STING and NFκB signaling but not IRF3 and IFN-αβ signaling to induce TNF-α [37]. In summary, responses to DNA by innate immune cells are surprisingly complex and functionally dichotomous, revealing tissue-, cell-type- and pathway-specific differences in how innate immune cells respond to DNA. The molecular basis of such complex physiologic responses to DNA are poorly understood but are critically important for elucidating pivotal pathways that control downstream immune responses to DNA.

5. Biologic significance of DNA-induced regulation: *good news and bad news?*

Cytosolic DNA sensing to induce regulation via STING may be biologically significant for several reasons. Regulatory responses to DNA may help maintain self-tolerance during homeostasis and inflammation, thereby reducing the risk of inciting autoimmunity. Apoptotic cells induce dominant tolerogenic responses that suppress autoimmunity via IDO, since IDO inhibition was shown to lead to a rapid increase in anti-DNA IgG titers in lupus-prone MRL^{lpr} mice, and rendered otherwise healthy (C57BL/6) mice susceptible to systemic autoimmune disease development in response to chronic exposure to dying cells [27, 38]. Moreover, MZMs have been shown to ingest dying cells and expressed IDO rapidly thereafter; MZM depletion abolished these tolerogenic responses to dying cells, identifying MZMs as key arbiters of regulatory responses to apoptotic cells.[27] However, the characteristic induction of regulatory cytokines (TGF-β, IL-10) and IDO by apoptotic cells was shown to be abolished in STING-deficient mice and pro-inflammatory IL-6 expression was induced instead, revealing that cytosolic DNA sensing to activate STING is required for tolerogenic responses to dying cells [33]. Similarly, microbial DNA sensing via STING in splenic or intestinal phagocytes that scavenge blood-borne (such as *Streptococcus*) or mucosal microbes to prevent sepsis or colitis may reinforce tolerance to protect tissues from immune-mediated damage [39, 40]

Conversely, DNA-induced regulatory responses may promote tumor progression. Tumor-associated inflammation inhibits anti-tumor immunity, and immune cells with regulatory phenotypes such as DCs, macrophages, monocyte-derived suppressor cells and Treg cells, are prominent features of tumor microenvironments; however, the actual molecular pathways that drive regulatory responses to tumor growth are poorly defined. A potential model to explain DNA-induced regulatory responses that drive tumor growth is one in which DNA from dying tumor cells is sensed via the STING/IFN-β pathway, which then induces regulatory ISGs such as IDO, which is expressed in many tumor microenvironments [41]. Interestingly, STING signaling has been shown to induce IFN-αβ-dependent, tumor-specific $CD8^+$ T-cell responses primed by $CD8\alpha^+$ DCs in tumor microenvironments, suggesting that cytosolic DNA sensing may promote effector T-cell responses [42, 43]. Key questions are whether DNA from dying tumor cells is sensed to activate STING and if IFN-αβ released promotes tolerogenic or immunogenic responses during tumor growth, and primes effector T-cell responses following immunotherapy. Similar considerations may be applicable to chronic infections such as leishmaniasis and murine leukemia virus in mice,

and HIV-1 in humans, all of which establish localized inflammation that suppresses host immunity and activates host Treg cells [44–46].

6. Therapeutic implications: *using DNA to modulate autoimmunity*

DNP treatments have been shown to attenuate limb joint inflammation and cartilage destruction via an IDO-dependent mechanism in a murine model of antigen-induced arthritis [32]. DNP or cdiGMP treatments have also been shown to slow the onset and reduced the severity of MOG-induced EAE [47]. The therapeutic responses were shown to manifest when DNPs were applied either during MOG-immunization or later, when initial EAE symptoms were evident or after disease was fully established [47]. DNPs were shown to reduce effector T (Teff) cell infiltration into the central nervous system (CNS), attenuate pro-inflammatory cytokine production and antigen-specific T-cell responses in the spleen and increase Treg/Teff ratios. The therapeutic responses observed were dependent on cargo DNA sensing to activate STING and induce IDO via IFN type I (not type II) signaling, and cdiGMP treatments also attenuated EAE. Thus, regulatory responses induced by cargo DNA sensing by cytosolic DNA sensors or by CDNs to activate the STING/IFN-β pathway can be exploited to attenuate clinically relevant autoimmune syndromes. Recombinant IFN-β is a standard treatment for MS, although its mode of action is poorly defined and the recurrent interventions required to control MS induce increasingly severe side effects such as severe local pain, headaches and symptoms comparable with those induced by influenza infections [48], leading to therapy cessation in many cases. Moreover, another FDA-approved anti-MS drug, glatiramer acetate (Copaxone), has been shown to stimulate IDO-dependent regulatory responses that ameliorate EAE [49]. Potentially, administering DNPs or CDNs as STING activators to induce localized, endogenous IFN-β release, which promotes therapeutic regulatory responses in MS patients, may improve efficacy and avoid or reduce the toxic and pain-inducing side effects associated with exogenous IFN-β treatments.

Conclusions and Future Perspective

A large array of cytosolic DNA sensors is distributed over a wide range of cell types, and cytosolic DNA sensing to stimulate STING and induce IFN-β release activates immune cells and provides an early warning of danger in the form of infections. DNA sensing to activate the STING-IFN-β pathway also increases the risk of autoimmunity, particularly at sites of inflammation where increased cell death releases DNA. Here we discuss recent evidence that DNA elicits dominant tolerogenic responses via the STING-IFN-β pathway in some physiologic settings to reduce - not enhance - the risk of *horror autotoxicus*. Future perspectives based on this paradigm are to further elucidate molecular mechanisms and cellular pathways that mediate potent and dominant regulatory responses downstream of cytosolic DNA sensors, and to exploit this knowledge to develop improved treatments that prevent, slow or reverse hyper-immune syndromes.

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Abbreviations (non-standard)

STING	Stimulator of Interferon Genes
DC	dendritic cell
MZM	Marginal zone macrophage
DNP	DNA nanoparticle
CDN	cyclic dinucleotide
ISG	interferon-stimulated gens
cGAS	cyclic GMP-AMP synthase
ISG	interferon-stimulated gene

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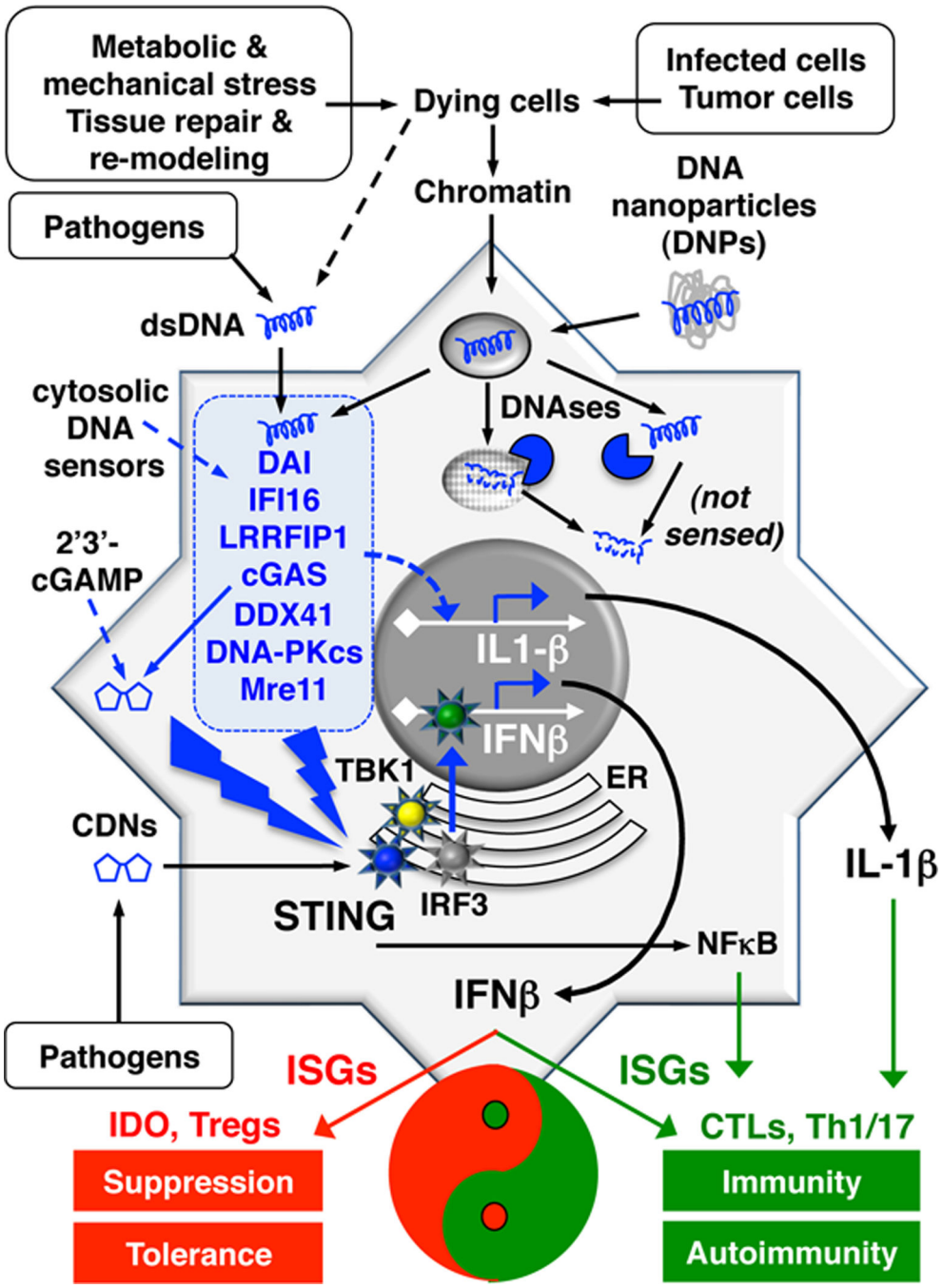

Figure 1. Cytosolic DNA sensing via STING stimulates or suppresses immunity

Various insults cause the release of host cellular DNA or microbial DNA, which is ingested by cells. Cytosolic DNA is sensed to trigger IFN-β release via STING, though some cells may express DNAses that degrade DNA, and CDNs of microbial origin, or generated by the cytosolic DNA sensor cGAS, activate STING directly (blue highlights). Downstream responses to DNA are mediated by ISGs that either stimulate immunity (e.g. pro-inflammatory cytokines) by driving cytotoxic and helper responses (highlighted green) or

promote tolerance (e.g. IDO) by activating Treg-cells that suppress effector responses (highlighted red).