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Activation of the STING Adaptor Attenuates Experimental Autoimmune Encephalitis

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Cytosolic DNA sensing activates the stimulator of IFN genes (STING) adaptor to induce IFN type I (IFN- $\alpha\beta$) production. Constitutive DNA sensing to induce sustained STING activation incites tolerance breakdown, leading to autoimmunity. In this study, we show that systemic treatments with DNA nanoparticles (DNPs) induced potent immune regulatory responses via STING signaling that suppressed experimental autoimmune encephalitis (EAE) when administered to mice after immunization with myelin oligodendrocyte glycoprotein (MOG), at EAE onset, or at peak disease severity. DNP treatments attenuated infiltration of effector T cells into the CNS and suppressed innate and adaptive immune responses to myelin oligodendrocyte glycoprotein immunization in spleen. Therapeutic responses were not observed in mice treated with cargo DNA or cationic polymers alone, indicating that DNP uptake and cargo DNA sensing by cells with regulatory functions was essential for therapeutic responses to manifest. Intact STING and IFN- $\alpha\beta$ receptor genes, but not IFN- γ receptor genes, were essential for therapeutic responses to DNPs to manifest. Treatments with cyclic diguanylate monophosphate to activate STING also delayed EAE onset and reduced disease severity. Therapeutic responses to DNPs were critically dependent on IDO enzyme activity in hematopoietic cells. Thus, DNPs and cyclic diguanylate monophosphate attenuate EAE by inducing dominant T cell regulatory responses via the STING/IFN- $\alpha\beta$ /IDO pathway that suppress CNS-specific autoimmunity. These findings reveal dichotomous roles for the STING/IFN- $\alpha\beta$ pathway in either stimulating or suppressing autoimmunity and identify STING-activating reagents as a novel class of immune modulatory drugs. *The Journal of Immunology*, 2014, 192: 5571–5578.

Self-tolerance is an active and constitutive process that prevents autoimmunity. Recent studies on mice with defective DNA repair enzyme expression emphasize the potential for DNA to incite lethal autoimmunity by stimulating cytosolic DNA sensors that activate STING to induce IFN- $\alpha\beta$ production (1, 2). Multiple sclerosis (MS) is a chronic demyelinating autoimmune disease in which neuronal tissues are progressively targeted (3) due to loss of tolerance to CNS Ags such as myelin basic protein and myelin oligodendrocyte glycoprotein (MOG).

IDO is a natural immunomodulatory enzyme that attenuates autoimmunity in murine disease models, including experimental

autoimmune encephalitis (EAE; a model of MS), autoimmune rheumatoid arthritis, type I diabetes, systemic lupus erythematosus, and inflammatory bowel disease (4). In these syndromes IDO ablation accelerated disease onset and enhanced disease severity. Moreover, the immunomodulatory properties of soluble forms of CTLA4 (5, 6) and CD83 (7) depend, in part, on their ability to induce IDO-dependent regulatory phenotypes in dendritic cells (DCs), which promote de novo regulatory T cell (Treg) generation, activate resting Tregs, and suppress effector T cell responses (8, 9). Moreover, some immune stimulatory reagents (adjuvants) coinduce IDO, and this property of TLR9 ligands (CpGs) inhibited type I diabetes progression in NOD female mice (10–12). Diametric responses to immune adjuvants underscore the need to evaluate innate immune responses to inflammatory stimuli to discern underlying pathways that induce dominant stimulatory or regulatory responses by T cells (13). Sustained IFN- $\alpha\beta$ production is a key feature of chronic immune activation at sites of persistent infections such as HIV-1 (14). Sustained IFN- $\alpha\beta$ release, especially by plasmacytoid DCs, correlates strongly with risk of autoimmune syndromes such as systemic lupus erythematosus (15). IFN- $\alpha\beta$ and IFN type II (IFN- γ) have well-documented immune stimulatory properties, but IFNs are also potent IDO inducers, providing a rationale for increased IDO-mediated T cell regulation, particularly at sites of chronic inflammation.

Previously, we reported that small populations of DCs coexpressing the B cell marker CD19 upregulated IDO selectively (among DCs) in response to systemic treatments with soluble CTLA4 (CTLA4Ig), TLR9 ligands (CpGs), and DNA nanoparticles (DNPs) containing the cationic polymer polyethylenimine (PEI) and cargo DNA (12, 16, 17). IDO induction in CD19⁺ DCs was mediated by IFN- $\alpha\beta$, not IFN- γ , and by CD19⁺ DCs expressing IDO-stimulated Foxp3-lineage CD4 T cells (Tregs) to acquire

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Abbreviations used in this article: B6, C57BL/6; c-diGMP, cyclic diguanylate monophosphate; DC, dendritic cell; DNP, DNA nanoparticle; EAE, experimental autoimmune encephalitis; GFAP, glial fibrillary acidic protein; KO, knockout; MOG, myelin oligodendrocyte glycoprotein; MS, multiple sclerosis; IMT, 1-methyl-D-tryptophan; PEI, polyethylenimine; STING, stimulator of IFN genes; Treg, regulatory T cell; WT, wild-type.

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regulatory phenotypes that suppressed Th1 responses (8, 17). Tolerogenic responses to DNPs were dependent on cargo DNA sensing by small populations of myeloid DCs to activate STING and induced selective IFN- $\alpha\beta$ release that stimulated CD19⁺ DCs to express IDO (17, 18). In the present study, we tested the hypothesis that STING activation to induce IDO via IFN- $\alpha\beta$ signaling following systemic DNP treatment inhibits EAE progression and reduces disease severity.

Materials and Methods

Mice and induction of EAE

Mice were bred under specific pathogen-free conditions at Georgia Regents University or purchased from Taconic Farms, and procedures were approved by the Institutional Animal Care and Use Committee. IDO1-knockout (KO), IFNAR-KO, IFN- γ R-KO and STING-KO mice were described previously (19–21). To induce EAE, mice aged 8–12 wk were immunized s.c. at two sites on the rear flank with 100 μ g myelin oligo-dendrocyte glycoprotein peptide (MOG_{35–55}, MEVGWYRSPFSRVVH-LYRNGK; Bio Basic Canada) emulsified in CFA (Difco Laboratories, Detroit, MI) containing 4 mg/ml *Mycobacterium tuberculosis* H37Ra (Difco Laboratories). Pertussis toxin (200 ng; Sigma-Aldrich) was given i.p. on days 0 and 2 after immunization. Clinical symptoms of EAE were scored on a scale from 0 to 5 as described by Das Sarma et al. (22), with slight modifications as follows: 0, no clinical signs; 0.5, partial limp tail; 1, full limp tail or waddling gait; 1.5, limp tail and waddling gait/lumbar weakness; 2, partial paralysis of one hindlimb; 2.5, paralysis of one hindlimb or partial paralysis of both hindlimbs; 3, paralysis of one hindlimb and partial paralysis of the other hindlimb; 3.5, paralysis of both hindlimbs or partial paralysis of both hindlimbs and weakness of the upper limb; 4, ascending paralysis, that is, complete paralysis of both hindlimbs and weakness of the upper limb; 4.5, three paralyzed limbs; 5, four paralyzed limbs/moribund or dead. Some mice were given drinking water containing the IDO inhibitor 1-methyl-D-tryptophan (1MT; 2 mg/ml) and Nutrasweet (to increase palatability) as described (23).

DNP and cyclic diguanylate monophosphate treatments

DNPs were prepared by mixing 7 μ l PEI (150 mM) with 21 μ g CpG^{free} LacZ pDNA (InvivoGen, San Diego, CA) in 200 μ l 5% glucose solution (N/P = 16.7) as described (17). Chemically synthesized cyclic diguanylate monophosphate (c-diGMP) was dissolved in PBS at 500 μ g/ml. DNPs and c-diGMP (100 μ g/dose) were injected i.v.

Immunohistochemistry

After sacrifice, mice were perfused (PBS then paraformaldehyde at 4%) and lumbar spinal cord tissues were harvested, left in paraformaldehyde (3 d) and embedded in paraffin. Five-micrometer sections were deparaffinized, rehydrated, submerged in Target retrieval solution (20 min; Dako, catalog no. S-1699) and incubated in 3% H₂O₂ (5 min). Nonspecific biotin/avidin (Vector Laboratories, Burlingame, CA) and Fc/Fab staining was blocked using kits from Jackson ImmunoResearch Laboratories (catalog no. 015.000.008). Mouse on Mouse kits (Vector Laboratories) were used to dilute primary mouse anti-mouse/human IDO mAb (E7, 1:50; Santa Cruz Biotechnology, catalog no. 365086), neurons (NeuN rabbit polyclonal, 1:500; Millipore, catalog no. ABN78), microglia (IBA rabbit, 1:500; Wako Chemicals USA, catalog no. 019-19741), and astrocytes (glial fibrillary acidic protein [GFAP] rabbit monoclonal, 1:500; Cell Signaling Technology, catalog no. 123895). After 60 min, sections were incubated with biotinylated anti-mouse IgG Ab from the Mouse on Mouse kit and Alexa Fluor 488-conjugated AffiniPure F(ab')₂ fragment donkey anti-rabbit IgG (H+L) (Jackson ImmunoResearch Laboratories, catalog no. 711-546-152) for 45 min. After washing, streptavidin conjugated with Alexa Fluor 555 (1:400; Invitrogen/Molecular Probes, catalog no. S32355) was applied for 15 min. Slides were mounted in FluorSave reagent (Calbiochem, catalog no. 345789) and analyzed by confocal microscopy (Zeiss LSM 510).

Cytokines and Proliferative Responses to MOG

Splenocytes (10⁶) were cultured in RPMI 1640 in triplicate in 96-well U-bottom plates in the presence or absence of 20 μ g/ml MOG peptide for 72 h. Cytokine levels in culture supernatants were analyzed using multiplex kits from Bio-Rad or eBioscience. For proliferation assays, cells were pulsed with 0.5 μ Ci [³H]thymidine for the last 16 h of incubation and mean thymidine incorporation in triplicate wells was measured.

IDO enzyme activity

Cell-free spleen homogenates were added to IDO enzyme cocktails, and kynurenine generated after 2 h was measured by HPLC as described (24).

Flow cytometry

Single-cell suspensions of spleen and CNS (pooled brain and spinal cord) prepared as described (25) were stimulated in medium containing 20 mg/ml MOG peptide (37°C, 5% CO₂, 18 h); 1 μ l/ml brefeldin (BD Biosciences) was added for the last 4 h of culture. After staining the surface marker CD4 (BD Pharmingen, clone RM4-5), cells were fixed and permeabilized (Foxp3/transcription factor fixation/permeabilization buffer; eBioscience), followed by staining with mAbs to mouse Foxp3 (eBioscience, clone FJK-16S), IL-17A (eBioscience, clone eBio17B7), and IFN- γ (BD Pharmingen, clone XMGI.2). Flow cytometric analyses were performed using a LSRII cytometer (BD Biosciences), and data generated were analyzed using FACS Diva (BD Biosciences) or FlowJo (Tree Star, Ashland, OR) software.

Radiation chimeric mice

Mice were irradiated (900 rad) and after 24 h mice received $\sim 10^7$ nucleated bone marrow cells i.v. harvested from the long bones of donor mice and were allowed to recover for 10 wk before use in EAE induction experiments.

Statistical analysis

EAE scores were evaluated with two-way ANOVA. The unpaired Student *t* test was used for statistical evaluations of cytokine and cell frequency measurements. Two-tailed *p* values <0.05 were considered significant. GraphPad Prism was used to perform all data analyses.

Results

DNP treatment slows EAE onset and reduces disease severity

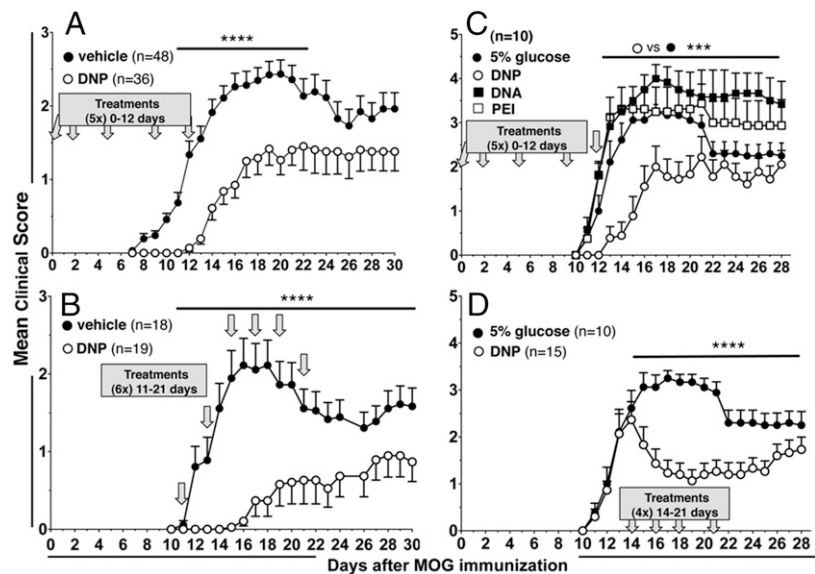
C57BL/6 (B6) mice were immunized with MOG and treated with pertussis toxin to induce EAE (see *Materials and Methods*). The effects of systemic DNP treatment on EAE progression were evaluated after administering DNPs (PEI/CpG^{free} pDNA) i.v. to groups of mice starting at the time of MOG immunization (day 0) and on days 2, 5, 9, and 12 (Fig. 1A, gray arrows). Control mice were given vehicle (5% glucose) and all mice were monitored to detect EAE onset and score clinical disease severity (see *Materials and Methods*). As expected, EAE symptoms began to manifest in vehicle-treated mice 8–12 d after MOG immunization (partial/full limp tail, clinical score 0.5–1), and peak disease severity occurred 14–24 d after immunization (mean clinical score >2), with minor relapse thereafter (Fig. 1A, filled symbols). DNP treatment delayed disease onset by ~ 4 d and reduced peak disease severity (mean clinical score ~ 1.5) significantly relative to vehicle-treated mice (Fig. 1A, open symbols).

Next, we tested whether administering DNPs later, when EAE first started to manifest, attenuated EAE. DNPs were administered i.v. from day 11 and every other day until day 21 (Fig. 1B, gray arrows). DNP treatment prevented disease onset by ~ 7 d, and subsequent disease severity was reduced significantly (mean clinical score <1) relative to outcomes in vehicle-treated mice (Fig. 1B, open and filled symbols, respectively). Delayed vehicle treatment had no impact on disease progression, peak disease severity, or minor relapse at late times (Fig. 1B, filled symbols) relative to mice given vehicle earlier (Fig. 1A). In contrast, treatments with PEI or cargo DNA alone induced no therapeutic responses in MOG-immunized mice relative to mice treated with vehicle (5% glucose, Fig. 1C). DNP treatments administered to MOG-immunized mice at peak EAE disease (starting on day 14 and given every other day until day 21) also induced significant therapeutic responses (Fig. 1D), indicating that regulatory responses to DNPs overcame established CNS-specific autoimmunity.

DNP treatment inhibits IDO expression in neurons during EAE

DNPs induce rapid IDO upregulation in mucosal and lymphoid tissues (18). To evaluate whether DNPs induced IDO in CNS

FIGURE 1. DNPs delay EAE onset and reduce disease severity. **(A)** B6 mice were injected i.v. with freshly prepared DNPs (21 μ g CpG^{free} pDNA, 7 μ l 150 mM PEI in 200 μ l 5% glucose) or vehicle (5% glucose) on days 0, 2, 5, 9, and 12 (5 \times , gray arrows) after MOG immunization. **(B)** As in (A), except that DNPs were administered later (from day 11) and then every other day until day 21 (6 \times). **(C)** B6 mice were treated with DNPs or PEI, cargo DNA, or vehicle (5% glucose) only from days 0 to 12 (5 \times). **(D)** B6 mice were treated with DNPs starting at peak disease from days 14 to 21 (5 \times). Mice were monitored daily for EAE onset and scored to assess disease severity using the scheme described in *Materials and Methods*. Clinical scores were compiled from one (C), four (A), and two (B, D) experiments and are expressed as mean scores \pm SEM at each time point. Data were analyzed by two-way ANOVA. *** p < 0.0012, **** p < 0.0001.



tissues, untreated (naive) and MOG-immunized mice treated with vehicle or DNPs three times (days 11, 13, and 15) were sacrificed on day 16, and CNS tissues were stained to detect IDO and costained to detect neuronal (NeuN)-, astrocyte (GFAP)-, or microglia/monocyte (Iba1)-specific markers (Fig. 2A). As expected, IDO expression was not detected and few astrocytes and microglia were present in CNS tissues from naive mice (Fig. 2A, upper panels). In contrast, most neurons (NeuN⁺) from CNS tissues of vehicle-treated, MOG-immunized mice expressed IDO, and expanded cohorts of microglia/infiltrating monocytes and activated astrocytes were present, but these cells did not express IDO (Fig. 2A, center panels); expanded cohorts of activated glial cells are a prominent feature of EAE, which correlates with increased neuropathology (26). However, neurons did not express IDO in CNS tissues from DNP-treated, MOG-immunized mice, and numbers of microglia/infiltrating monocytes and activated astrocytes in CNS tissues were markedly reduced (Fig. 2A, lower panels) relative to MOG-immunized mice, and they were comparable to numbers in naive mice. Thus, MOG immunization stimulated neurons to express IDO, whereas DNP treatment to induce IDO in non-CNS tissues (17) paradoxically suppressed MOG-induced IDO expression in neurons. These findings suggest that IDO induction in lymphoid tissues inhibits CNS infiltration of effector cells that incite neurons to express IDO.

DNP treatment reduces effector T cell/Treg ratios in CNS tissues during EAE

Absolute numbers of cells in CNS tissues were reduced significantly (~2-fold) in MOG-immunized mice treated with DNPs (3 \times , days 11, 13, and 15 and harvested on day 16) relative to vehicle-treated controls (Fig. 2B). Consistent with this finding, numbers of CD4 T cells expressing intracellular IFN- γ or IL-17 (Th1, Th17) after stimulating cells from CNS tissues with MOG peptide ex vivo (18 h) were reduced by DNP treatment (Fig. 2C, 2D, ~2- to 3-fold), but the relative proportions of Tregs and MOG-specific Th1 and Th17 T cells among infiltrating CD4 T cells were unaffected by DNP treatment (Supplemental Fig. 1A). Because DNPs suppressed IDO expression in CNS tissues from MOG-immunized mice (Fig. 2A), we evaluated whether DNPs induced IDO activity elsewhere in MOG-immunized mice by measuring levels of serum kynurenine, a tryptophan catabolite secreted by cells expressing IDO. Serum kynurenine increased significantly (Fig. 2F, ~2-fold) in DNP-treated, MOG-immunized mice relative to levels in vehicle-treated, MOG-

immunized mice, indicating that systemic DNP treatment stimulated IDO activity in non-CNS tissues of MOG-immunized mice.

DNP treatment suppresses proinflammatory responses to MOG immunization in spleen

Systemic DNP treatment during MOG immunization (3 \times , days 11, 13, and 15) increased spleen cellularity (at day 16) relative to vehicle-treated MOG-immunized mice with EAE (Fig. 3A). Numbers (Fig. 3A) of Tregs and MOG-specific CD4⁺ T cells expressing intracellular IFN- γ and IL-17 also increased significantly (>2- to 3-fold), as did the relative proportions of Tregs and MOG-specific Th1 and Th17 cells among CD4 T cells in spleens of DNP-treated mice (Supplemental Fig. 1B). However, splenocyte proliferation, measured by thymidine incorporation ex vivo (72 h) in response to MOG peptide, was reduced substantially in DNP-treated mice (Fig. 3B). Consistent with this finding, MOG peptide-induced proinflammatory cytokine production by splenocytes from MOG-immunized mice (IL-17A/F, IL-6, IL-22, IFN- γ) was reduced significantly (60–95%) by DNP treatment (Fig. 3C). Thus, DNP treatment enhanced MOG-specific Th1 and Th17 T cell and Treg responses in spleen, but the overall effects of DNP treatment were to attenuate innate and adaptive immunity at this site.

IFN type I (IFN- $\alpha\beta$) but not type II (IFN- γ) signaling mediates therapeutic responses to DNPs

To evaluate IFN signaling requirements for therapeutic responses to DNPs to manifest, MOG-immunized mice with defective IFN type I (IFNAR-KO) or type II (IFN- γ R-KO) receptor genes were treated with DNPs (or vehicle) during EAE induction (days 0–12) or later, at the time of EAE onset (days 11–21). DNP-treated and vehicle-treated B6 (wild-type [WT]) mice (Fig. 4A, 4B) and vehicle-treated IFNAR-KO (Fig. 4C, 4E) and IFN- γ R-KO mice (Fig. 4D, 4F) were used as controls. DNPs had no significant therapeutic effects on EAE onset or peak disease severity when administered early (Fig. 4C) or later (Fig. 4E) to IFNAR-KO mice. Clinical scores at late stages of EAE progression (>20 d) were slightly worse in DNP-treated IFNAR-KO mice (>3) than for vehicle-treated controls (<3), suggesting that weak immune stimulatory effects of DNPs are masked by dominant regulatory responses mediated by IFN- $\alpha\beta$ receptors. In vehicle-treated IFNAR-KO mice (Fig. 4C), the time of EAE onset (days 8–12), attainment of peak EAE severity (days 14–16), and clinical scores at late stages of EAE (~2.5) were unaffected by loss of IFNAR

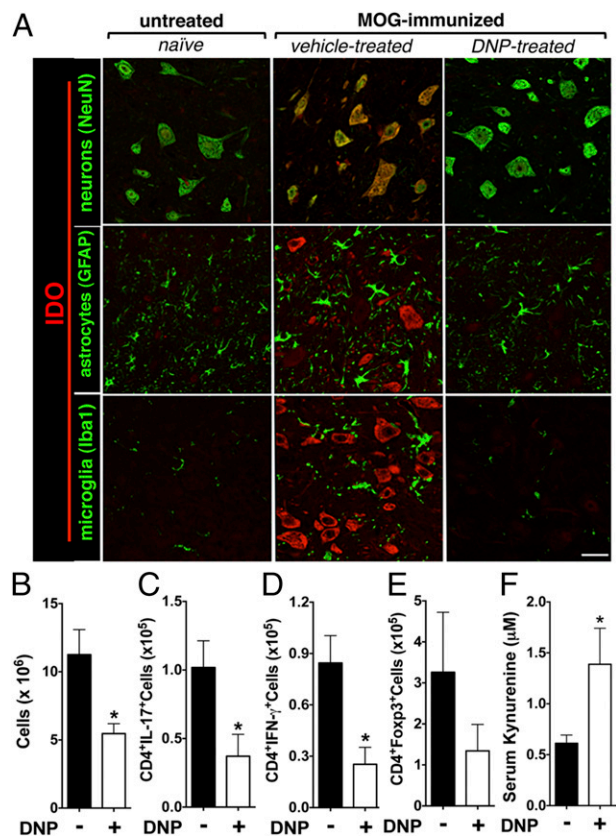


FIGURE 2. DNP treatment blocks IDO induction in neurons and reduces effector cell infiltration in CNS tissues. **(A)** Spinal cord tissue sections were prepared from mice 16 d after MOG immunization (or from untreated controls) with or without DNP treatment on days 11, 13, and 15 (3×). Tissues were stained to detect cells expressing IDO and specific markers expressed by neurons (NeuN), astrocytes (GFAP), or microglial cells (Iba1). Scale bar, 20 μm. **(B–E)** Total cell numbers **(B)** and numbers of Th17 (IL-17), Th1 (IFN-γ), and Tregs (Foxp3) in gated CD4⁺ T cells from CNS tissues **(C–E)** were assessed by flow cytometric analyses of pooled spinal cord and brains from individual mice stained to detect CD4⁺ T cells and intracellular IL-17, IFN-γ, and Foxp3 expression after stimulation ex vivo with MOG peptide (18 h) and added brefeldin A for the final 4 h. **(F)** HPLC analyses to detect kynurenine in serum samples from MOG-immunized mice (day 16) with or without DNP treatment as above. Data are representative of two experiments **(A)** or were compiled from three experiments **(B–F)**. Data are expressed as means ± SEM and were analyzed using the unpaired two-tailed Student *t* test. **p* < 0.05.

function relative to B6 controls (Fig. 4A). Thus, IFN-αβ signaling is essential for therapeutic responses to DNPs to manifest. In contrast, DNP treatment in IFN-γR-KO mice slowed EAE onset and reduced EAE severity significantly when DNPs were administered early (Fig. 4D) or late (Fig. 4F) relative to vehicle-treated controls. Consistent with previous studies on IFN-γR-KO mice (27), EAE onset (around days 12–14) and attainment of peak EAE severity (after day 20) were delayed, and peak clinical scores were higher (4, 5) in vehicle-treated IFN-γR-KO mice (Fig. 4D) relative to vehicle-treated B6 controls (Fig. 4B), especially when vehicle was given early (Fig. 4D). Thus, IFN-γ signaling is not required for therapeutic responses to DNPs, even though IFN-γ signaling regulates EAE progression in MOG-immunized mice (27), possibly by inducing IDO at specific phases of EAE progression (28).

STING mediates therapeutic responses to DNPs

DNPs regulate T cell responses via a STING-dependent pathway (18). To test whether STING was required to alleviate EAE,

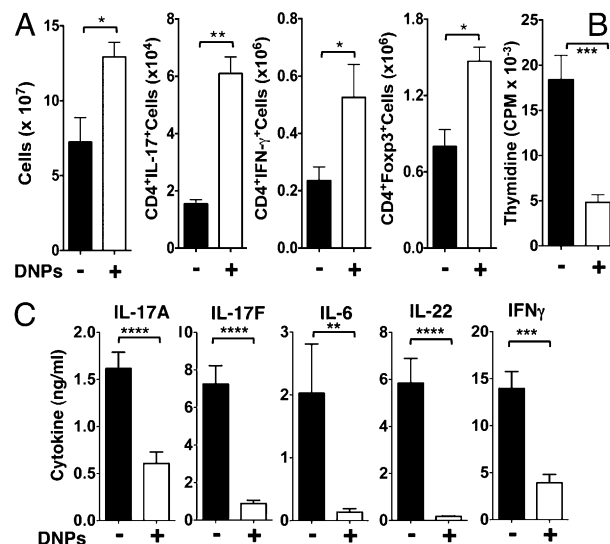


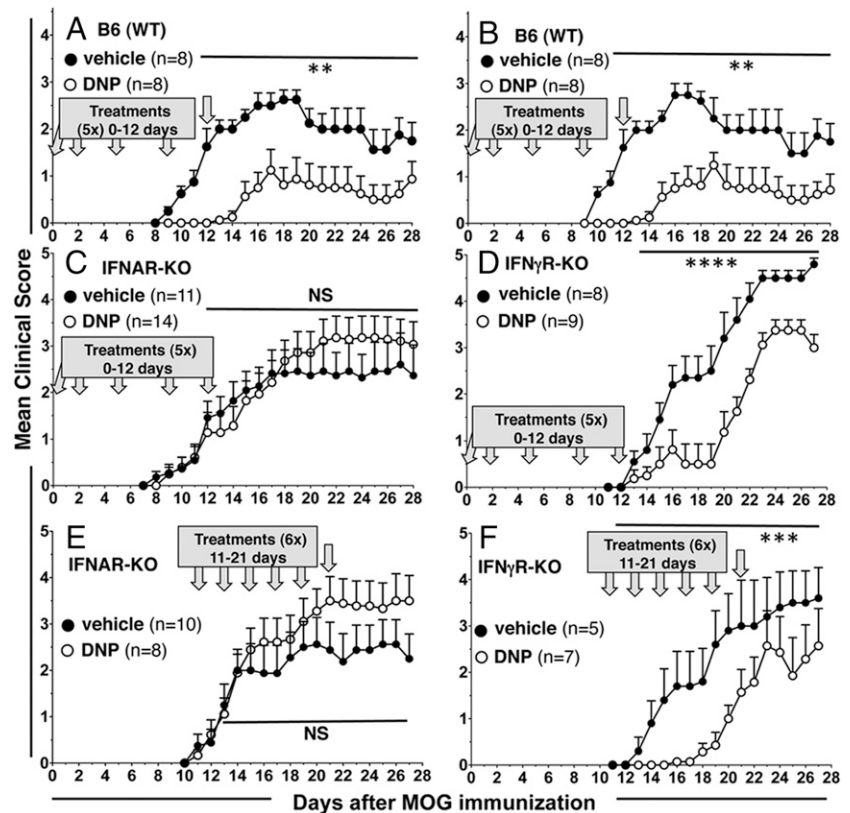
FIGURE 3. DNP treatment attenuates Th1/Th17 responses to MOG immunization elicited in spleen. **(A)** B6 mice were immunized with MOG and treated i.v. with DNPs or vehicle (3×, days 11, 13, and 15). Mice were sacrificed on day 16 and spleen cellularity and numbers of splenic CD4⁺ T cells expressing intracellular IL-17, IFN-γ, and Foxp3 were assessed by flow cytometric analyses after ex vivo culturing with MOG peptide (18 h) and brefeldin A (last 4 h). **(B)** and **(C)** Splenocytes from DNP-treated (i.v., 5×, days 0, 2, 5, 9, and 12) or vehicle-treated MOG-immunized mice were harvested (day 13) and stimulated ex vivo with MOG peptide (20 μg/ml, 72 h), T cell proliferation was assessed by thymidine incorporation **(B)**, and levels of IL-17A/F, IL-6, IL-22, and IFN-γ in culture supernatants were determined by multiplex analyses **(C)**. Data are representative of two experiments (*n* = 4) and are expressed as means ± SEM. Data were analyzed using the unpaired two-tailed Student *t* test. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001.

MOG-immunized, B6, and STING-deficient (STING-KO) mice were treated with DNPs or vehicle (Fig. 5A, 5B). STING-KO mice succumbed uniformly to EAE (Fig. 5B, filled symbols), although EAE onset was slower (days 14–16) and disease scores were lower (<2) than in B6 mice (Fig. 5A, filled symbols). Early DNP treatment (days 0–12) accelerated EAE onset and increased disease severity (>2) relative to vehicle-treated STING-KO mice (Fig. 5B, open symbols). Administering DNPs later (days 11–21) induced no therapeutic responses in STING-KO mice relative to vehicle treatments (Fig. 5C). Analyses of serum kynurenine levels in MOG-immunized mice revealed that DNP treatments before EAE onset (days 0–12) or at the time of EAE onset (day 11) did not induce IDO activity in STING-KO mice, although MOG immunization alone led to elevated IDO activity in STING-KO mice relative to control B6 mice (Table I). Thus, therapeutic responses to DNPs via IDO were STING-dependent and, as in mice lacking IFN-γ receptors, loss of STING signaling revealed cryptic immune stimulatory properties of DNPs normally masked by dominant regulatory responses in mice with intact STING genes.

Cyclic dinucleotides mediate therapeutic responses to EAE via STING

Based on studies using DNP-treated STING-KO mice, we hypothesized that systemic treatment with c-diGMP, which activates STING (29), also attenuates EAE. To test this hypothesis, MOG-immunized B6 mice were treated with chemically synthesized c-diGMP (100 μg i.p. on days 0 and 2). c-diGMP treatment delayed EAE onset by ~6 d, but it did not reduce peak EAE severity relative to vehicle-treated (PBS) mice (Fig. 5D, open and filled

FIGURE 4. Therapeutic responses to DNPs are dependent on IFN type I but not IFN type II signaling. **(A–D)** MOG-immunized B6 **(A, B; WT)**, IFNAR-KO **(C)**, or IFN- γ R-KO **(D)** mice were treated with DNPs (days 0–12, 5 \times) or vehicle (5% glucose). **(E and F)** As in **(C)** and **(D)**, except that treatments were administered to mice later (days 11–21, 6 \times). Mice were monitored daily for EAE onset and severity. Data were compiled from two or more experiments and are expressed as mean clinical scores \pm SEM at each time point. Data were analyzed by two-way ANOVA. ** p < 0.01, *** p < 0.001, **** p < 0.0001.



symbols, respectively). Similarly, c-diGMP treatment later (days 11 and 13) also slowed EAE progression (~ 4 d) but did not reduce peak disease severity relative to vehicle treatments (Fig 5E, open

and filled symbols). In contrast, combined early and late phase c-diGMP treatments (days 0, 2, 9, and 12) delayed EAE onset by ~ 15 d and reduced peak disease severity (~ 1.5) significantly

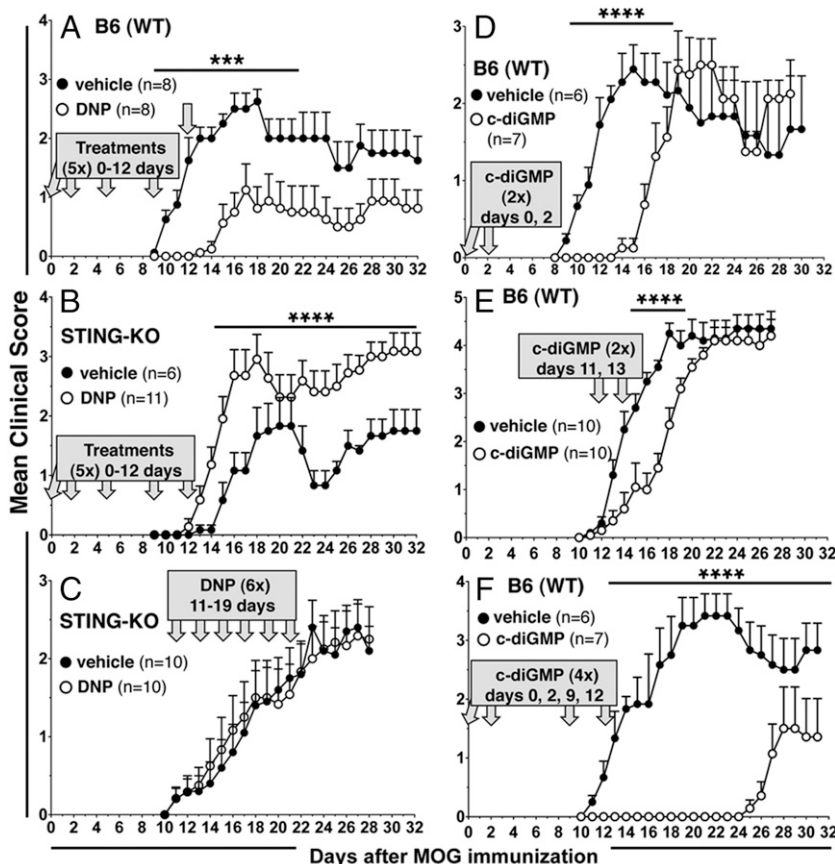


FIGURE 5. Therapeutic responses to DNPs are mediated by STING. **(A and B)** MOG-immunized B6 **(A, WT)** or STING-KO mice **(B)** were treated with DNPs (days 0–12, 5 \times) or vehicle (5% glucose). **(C)** As in **(B)**, except that STING-KO mice were treated later (days 11–21, 6 \times). **(D–F)** B6 mice were treated i.v. with c-diGMP (100 μ g) or vehicle (PBS) during MOG immunization **(D)**, days 0 and 2, at initial EAE onset **(E)**, days 11 and 13, or both phases **(F)**, days 0, 2, 9, and 12. Mice were monitored daily for EAE onset and severity. Data were compiled from one to two experiments and are shown as mean clinical scores \pm SEM at each time point. Data were analyzed by two-way ANOVA. *** p < 0.001, **** p < 0.0001.

Table I. IDO activity in MOG-immunized mice

Mice	MOG (n)	DNP Treatment	Serum Kynurenine (μ M)
B6	+ (4)	—	1.54 \pm 0.19
	+ (8)	+ Days 0–12	2.97 \pm 0.32*
	+ (5)	+ Day 11	3.47 \pm 0.42*
STING-KO	+ (4)	—	2.75 \pm 0.91
	+ (3)	+ Days 0–12	3.19 \pm 0.33 (ns)
	+ (5)	+ Day 11	3.76 \pm 0.27 (ns)

Mice were harvested on day 13.

* $p < 0.05$.

relative to vehicle-treated mice (Fig. 5E, open and filled symbols). Thus, STING activation after c-diGMP or cargo DNA sensing attenuates EAE progression and onset.

Therapeutic responses to STING-activating reagents are IDO-dependent

To test whether IDO is required for therapeutic responses to DNPs, B6 mice were treated with DNPs (5 \times , days 11–19). One group of DNP-treated mice was given the IDO-specific inhibitor 1MT on day 11 (1 mg i.p.) and then given drinking water containing 1MT (2 mg/ml) ad libitum until experimental endpoints. As expected, DNPs reduced EAE clinical symptoms significantly relative to vehicle treatments (Fig. 6A, open versus filled circles). 1MT treatment reduced therapeutic responses to DNPs significantly but not completely (Fig. 6A, open squares). To further test requirements for IDO function, IDO1-deficient (IDO1-KO) mice with pure B6 backgrounds were treated with DNPs (6 \times , days 11–21) or c-diGMP (2 \times , days 11 and 13). As shown in Fig. 6B, neither treatment induced therapeutic responses in IDO1-KO mice (open symbols). Rather, EAE severity was slightly higher at days 20–22 in mice treated with c-diGMP relative to vehicle-treated mice (Fig. 6B, open squares versus filled circles). Thus, functional IDO expression was essential for therapeutic responses to manifest following systemic treatments with DNPs or c-diGMP.

Hematopoietic cells expressing IDO mediate therapeutic responses to DNPs

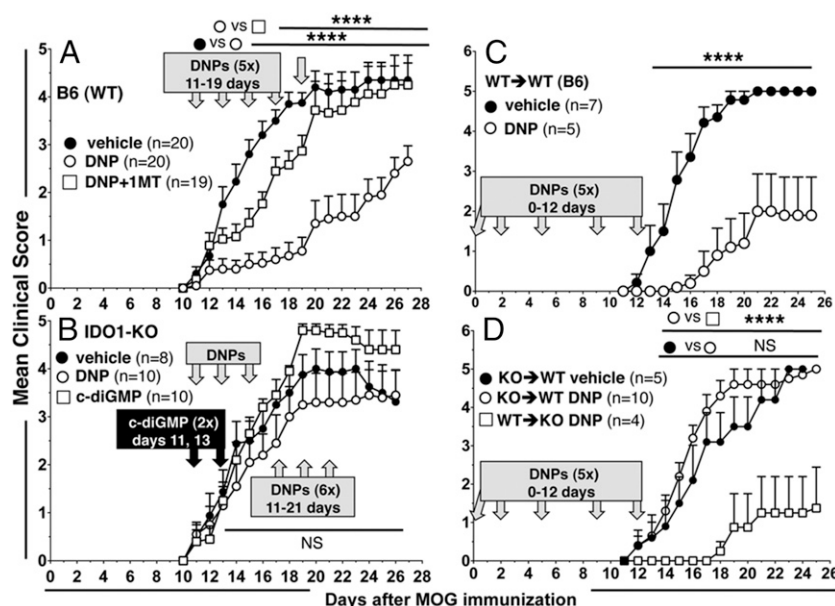
We hypothesized that IDO induced by DNPs in lymphoid tissues mediated therapeutic responses because MOG immunization

stimulated neurons to express IDO in CNS tissues (Fig. 2A), and IDO activity in CNS tissues generates neurotoxic tryptophan catabolites (30, 31). To test whether therapeutic responses to DNPs were dependent on IDO function in hematopoietic cells, B6 (WT) or IDO1-KO (KO) mice were lethally irradiated and reconstituted with bone marrow from WT or KO donors to create chimeric mice. This approach permits segregation of potential toxic effects of IDO in nonhematopoietic neurons from potential beneficial immune regulatory effects of IDO in hematopoietic cells such as DCs.

First, we monitored EAE induction and severity in irradiated B6 mice reconstituted with B6 bone marrow (WT \rightarrow WT). As shown in Fig. 6C (filled symbols), EAE manifested uniformly in MOG-immunized WT \rightarrow WT mice but EAE onset was delayed slightly (~12–14 d) and peak disease severity was higher in WT \rightarrow WT mice (5) relative to nonchimeric B6 mice (Fig. 1), suggesting that residual inflammation in radiation chimeras may exacerbate EAE susceptibility. Despite elevated EAE susceptibility, DNPs administered during MOG immunization (5 \times , days 0–12) delayed EAE onset (days 16–18) and reduced disease severity (~2) significantly in WT \rightarrow WT chimeric mice (Fig. 6C, open symbols). Thus, therapeutic responses to DNPs were unaffected by residual inflammation in chimeric mice.

Next, we evaluated EAE progression in B6 mice reconstituted with bone marrow from IDO1-KO (KO \rightarrow WT) mice. EAE onset (days 12–14) and disease severity (5) in vehicle-treated KO \rightarrow WT and WT \rightarrow WT mice were comparable, except for a slight delay in attaining peak disease severity in KO \rightarrow WT mice (Fig. 6D, filled circles). DNP treatment during EAE induction (5 \times , days 0–12) had no significant effect on EAE progression (days 12–14) or peak disease severity (5) in MOG-immunized KO \rightarrow WT chimeric mice (Fig. 6D, open circles), indicating that intact IDO1 gene function in hematopoietic cells was essential for therapeutic responses to DNPs to manifest. Whereas some hematopoietic cells from WT donors may remain viable in KO \rightarrow WT chimeric mice, these residual WT cells did not promote robust therapeutic responses to DNPs. In contrast, DNPs delayed EAE onset (days 18–20) and reduced peak disease severity (~1.5) significantly in WT \rightarrow KO chimeric mice with intact IDO1 genes in hematopoietic cells but not in nonhematopoietic cells (Fig. 6D, open squares). As therapeutic responses to DNPs were comparable in WT \rightarrow KO (Fig. 6D)

FIGURE 6. Therapeutic responses to STING-activating reagents are dependent on IDO function in hematopoietic cells. **(A)** MOG-immunized B6 mice were treated with DNPs (days 11–19, 5 \times), vehicle (5% glucose), or DNPs plus 1MT i.p. (500 μ l at 2 mg/ml) on day 11 and then provided in drinking water (2 mg/ml) from day 11 until experimental endpoints (day 27). **(B)** MOG-immunized IDO1-KO mice were treated with DNPs, vehicle, or c-diGMP as indicated. **(C and D)** Radiation chimeric mice were generated from B6 (WT) and IDO1-KO (KO) donors and recipients as described in *Materials and Methods* and were treated with DNPs or vehicle (days 0–12, 5 \times) 10 wk after bone marrow reconstitution. Mice were monitored and scored daily for EAE as before. Data were compiled from two pooled (A) or single experiments (B–D) and are expressed as mean clinical scores \pm SEM at each time point. Data were analyzed by two-way ANOVA. **** $p < 0.0001$.



and WT→WT (Fig. 6C) chimeric mice, these findings indicated that IDO1 gene function was required only in hematopoietic cells, not nonhematopoietic cells, for therapeutic responses to manifest.

Discussion

In this study we show that selective STING activation attenuates lethal EAE by stimulating IFN- $\alpha\beta$ release to induce IDO-dependent T cell regulatory responses. The cyclic dinucleotide c-diGMP and DNPs containing CpG^{free} cargo DNA triggered the STING/IFN- $\alpha\beta$ /IDO regulatory pathway to delay EAE onset and reduce disease severity substantially, even when treatments were given at the time of EAE onset or to mice with established EAE. Therapeutic responses were not induced by DNA or PEI alone and were dependent on cargo DNA sensing after DNP uptake by hematopoietic cells in peripheral lymphoid tissues to activate STING and induce regulatory responses via IFN- $\alpha\beta$ and IDO. Thus, selective STING activation is an effective strategy to induce potent regulatory responses that suppress CNS-specific autoimmunity.

Our findings appear contradictory to recent studies showing that constitutive DNA sensing to activate STING incited lethal autoimmunity in mice lacking DNA degrading enzymes (1, 2). Thus, increased access to DNA triggered sustained STING activation and constitutive IFN- $\alpha\beta$ release by stromal cells to incite progressive tolerance breakdown. Dominant regulatory responses induced by DNP cargo DNA and c-diGMP described in the present study may mimic physiologic responses to cellular DNA (chromatin) from dying (apoptotic) cells, which also promote potent regulatory responses via STING and IDO (17, 18, 21). Sustained STING activation in nonhematopoietic (stromal) tissues (1) or selective STING activation in hematopoietic cells may incite diametric immune outcomes because IDO is induced only in the second setting. Thus, the STING/IFN- $\alpha\beta$ pathway activates immune cells whereas other microenvironmental factors, such as the balance of pro/anti-inflammatory cytokines and metabolic effects of increased tryptophan catabolism by cells expressing IDO, determine immune outcomes (32).

Elevated IDO activity in CNS tissues may contribute to EAE pathogenesis owing to local catabolism of tryptophan, the substrate for serotonin synthesis and release of neurotoxic tryptophan catabolites (30, 31, 33). Another apparent paradox from the present study is that treatment with DNPs attenuated IDO expression in neurons of MOG-immunized mice with EAE, even though DNPs stimulate IDO activity in many tissues (17). Glial cells in inflamed CNS lesions expressed IDO in neuroinflammatory syndromes, including EAE, although neuronal IDO expression was not reported (30). In the present study, MOG immunization stimulated neurons but not glial cells to express IDO, and DNP treatments attenuated neuronal IDO expression induced by MOG immunization. Loss of neuronal IDO expression following DNP treatments may reflect reduced CNS infiltration by inflammatory cells expressing IFN- γ , a potent IDO inducer (30). Thus, DNP and c-diGMP treatments may inhibit neuronal IDO expression indirectly by suppressing MOG-specific responses and thereby reducing immune effector cell infiltration into CNS tissues. Similarly, vitamin D treatments also alleviated EAE by inducing IDO-dependent tolerogenic phenotypes in DCs that enhanced Treg functions (34).

DNPs enhanced spleen cellularity in MOG-immunized mice but reduced proinflammatory cytokine production substantially, even though more MOG-specific Th17 and Th1 effector cells were present in DNP-treated mice. Thus, regulatory responses to DNPs did not prevent T cell priming but were functionally dominant in the EAE model. In a previous study, systemic DNP treatments stimulated antitumor immunity and enhanced serum IFN- γ and IL-

12 levels (35). It is unclear how to reconcile these diametric effects of systemic DNP treatments in these disease models, but distinct features of inflammatory responses to local tumor growth and MOG immunization to induce EAE may be key factors as well as different DNP formulations. Our findings suggest that optimizing T cell regulatory responses induced by DNPs may improve therapeutic responses that attenuate EAE and other autoimmune syndromes, whereas antitumor effects of DNPs may be attenuated by their ability to induce IDO, a potent regulator of antitumor immunity (36).

CpG^{free} plasmid DNA was used as DNP cargo DNA in the present study to avoid IFN- γ production by TLR9-activated NK cells (17). As for other nanoparticle formulations used to deliver Ag-specific therapies to treat EAE (37), DNPs can be engineered to deliver defined autoantigens, and cargo DNA can transduce genes encoding autoantigens. DNPs containing biodegradable poly- β -amino ester derivatives as cationic polymers (38) instead of non-degradable PEI also induced potent and dominant regulatory responses in naive mice (H. Lemos, unpublished data), suggesting that biodegradable DNPs necessary for metronomic treatment regimens in clinical settings may possess immunomodulatory properties comparable with DNPs containing PEI.

Similar to DNPs, synthetic cyclic dinucleotides offer a versatile platform to develop STING-activating reagents as immunomodulatory drugs. Limited c-diGMP treatments slowed EAE onset and reduced disease severity substantially, suggesting that more aggressive treatments may be even more effective. Cyclic dinucleotides are produced by some microbial organisms such as *Listeria* and are sensed via STING at sites of infection to stimulate host defense (29). Mammalian cells synthesize cyclic guanosine-adenosine monophosphate when cytosolic DNA is sensed by the nucleotidyltransferase cyclic guanosine-adenosine monophosphate synthase, a key pathogen sensor that drives host immunity (39–41). Thus, cyclic dinucleotides from external or internal sources activate STING in mammalian cells. Subcutaneous c-diGMP administration enhanced Ag-specific immunity in mice, indicating that c-diGMP has immune stimulatory (adjuvant) properties in this setting (42). Thus, the route of c-diGMP administration is a critical determinant of immune responses, even though STING activation to induce IFN- $\alpha\beta$ may be the common pathway that incites or inhibits immunity. Genetic studies revealed that polymorphisms in human STING genes abrogate responsiveness to the vascular disrupting reagent 5,6-dimethylxanthine-4-acetic acid, suggesting that STING signaling is defective in some humans (43). However, a recent study revealed that responsiveness to cyclic dinucleotides is widespread among humans carrying an array of single nucleotide polymorphisms in human STING genes, although some single nucleotide polymorphisms correlated with defective IFN- β release following treatment with specific cyclic dinucleotides (44). Clearly, more research is needed to discern optimal reagents to manipulate immune responses in humans.

IFN- β is used to treat MS patients although the mode of action is not fully understood. IFN- β may recapitulate some downstream regulatory responses elicited by systemic DNP or c-diGMP treatments to activate STING and induce endogenous IFN- β release. However, IFN- β elicits multiple side effects that can be severe, causing some MS patients to discontinue treatment (30). Systemic administration of STING-activating reagents may offer improved ways to target immune regulatory pathways responsive to IFN- β while avoiding some detrimental responses to IFN- β , including immune stimulation.

In summary, DNPs and cyclic dinucleotides are two classes of STING-activating reagents that can be developed as immuno-

modulatory drugs to treat patients with hyperimmune syndromes such as autoimmune diseases. Diametric responses to STING activators highlight pivotal roles for the STING/IFN- β pathway in activating immune cells but they emphasize the importance of rigorous evaluation of innate and adaptive immune responses to these treatments to discern dominant immune outcomes in particular settings of inflammatory diseases and treatments.

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Disclosures

A.L.M. and D.H.M. are consultants and shareholders for NewLink Genetics, Inc., which licensed intellectual technology on manipulating the IDO pathway to modify immune responses. The other authors have no financial conflicts of interest.

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