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Cutting Edge

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Cutting Edge: DNA Sensing via the STING Adaptor in Myeloid Dendritic Cells Induces Potent Tolerogenic Responses

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Cytosolic DNA sensing via the stimulator of IFN genes (STING) adaptor incites autoimmunity by inducing type I IFN (IFN-αβ). In this study, we show that DNA is also sensed via STING to suppress immunity by inducing IDO. STING gene ablation abolished IFN-αβ and IDO induction by dendritic cells (DCs) after DNA nanoparticle (DNP) treatment. Marginal zone macrophages, some DCs, and myeloid cells ingested DNPs, but CD11b$^+$ DCs were the only cells to express IFN-β, whereas CD11b$^+$ non-DCs were major IL-1β producers. STING ablation also abolished DNP-induced regulatory responses by DCs and regulatory T cells, and hallmark regulatory responses to apoptotic cells were also abrogated. Moreover, systemic cyclic diguanylate monophosphate treatment to activate STING induced selective IFN-β expression by CD11b$^+$ DCs and suppressed Th1 responses to immunization. Thus, previously unrecognized functional diversity among physiologic innate immune cells regarding DNA sensing via STING is pivotal in driving immune responses to DNA. *The Journal of Immunology*, **2013, 191: 3509–3513.**

Innate immune cells sense pathogen-specific molecules, and rapid production of IFN-αβ is a common host signature of microbial sensing. Pathogen DNA is sensed by TLR9 in endosomes and by cytosolic sensors, including DAI, IFI16/P202, DDX41, and cyclic GMP-AMP synthase (1–5). Cytosolic DNA sensors activate the stimulator of IFN genes (STING) adaptor to induce IFN-αβ (6). Mice lacking the DNA nucleases Trex-1 or DNase II developed lethal hyper-inflammatory and autoimmune syndromes due to sustained DNA sensing via STING that incited constitutive IFN-αβ expression (7, 8). These findings revealed a critical need to regulate DNA sensing in the absence of infection, and they suggested that defective cellular DNA processing at sites of infection, inflammation, or tissue remodeling where cells die may lower self-tolerance thresholds due to sustained STING activation driving constitutive IFN-αβ production.

Previously, we reported that nanoparticle cargo DNA was sensed in murine lymphoid tissues to induce IFN-αβ, which stimulated IDO enzyme activity in DCs to activate regulatory T cells (Tregs), suppress T cell responses, and protect mice from Ag-induced arthritis, although cargo DNA sensing to induce IDO was not TLR9-dependent (9). In this study, we show that nanoparticle cargo DNA is sensed selectively by myeloid DCs via the STING/IFN-αβ pathway to induce IDO in DCs, which activate Tregs to promote dominant T cell regulation.

Materials and Methods

Mice

Mice were bred under specific pathogen-free conditions, and procedures were approved by the Institutional Animal Care and Use Committee at Georgia Regents University. IFNAR knockout (KO), STING KO, CD11c diphtheria toxin receptor (CD11c^{DTR}), and CD169^{DTR} transgenic mice were described (10–12). To deplete DCs or marginal zone (MZ) macrophages (Mϕs), CD11c^{DTR} or CD169^{DTR} mice, respectively, were treated with DT (i.p., 10 μg/kg) 24 and 6 h before DNA nanoparticle (DNP) treatment as described (12, 13).

DNPs and cyclic diguanylate monophosphate

DNPs were prepared by mixing polyethylenimine (PEI) or rhodamine-conjugated PEI (Rh-PEI; VWR International, Suwanee, GA) with CpG^{free} pGiant (InvivoGen, San Diego, CA) (9). Mice were injected i.v. with 30 μg pDNA (N/P ratio of 10:1) or cyclic diguanylate monophosphate (c-diGMP). YoYo-1–labeled DNA (Invitrogen, Carlsbad, CA) was prepared by adding

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Abbreviations used in this article: c-diGMP, cyclic diguanylate monophosphate; DC, dendritic cell; DNP, DNA nanoparticle; DT, diphtheria toxin; DTR, diphtheria toxin receptor; KO, knockout; Mϕ, macrophage; 1MT, 1-methyl-D-tryptophan; MZ, marginal zone; PEI, polyethylenimine; Rh-PEI, rhodamine conjugated polyethylenimine; STING, stimulator of IFN genes; Treg, regulatory T cell.

equal volumes of DNA and YoYo-1 in saline and vortexing. The ratio of YoYo-1 to deoxynucleotide pairs was 1:200; for 30 μg DNA, 2.2 μl 100 μM YoYo-1 was used.

Immunofluorescence

Fixed frozen sections (7 μm) were incubated with CD11c (N418), CD11b (M1-70) (BioLegend, San Diego, CA), and DyLight 488–conjugated goat anti-hamster or goat anti-rat Abs (Jackson ImmunoResearch Laboratories, West Grove, PA) as described (9).

Cytokines

IFN-αβ bioactivity was assessed using a viral interference assay as described (9). Spleen IL-1β was measured by ELISA (eBioscience, San Diego, CA).

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 (14).

Flow cytometry

Spleen cells were stained with CD11c and CD11b mAbs (BioLegend) and sorted using a MoFlo cell sorter into RNA protection reagent (Omega Bio-Tek, Norcross, GA). For analysis, spleen cells were incubated with mAbs and analyzed on an LSR II cytometer (BD Biosciences). Data were analyzed using FACSDiva (BD Biosciences) or FlowJo (Tree Star, Ashland, OR) software. mAbs were from eBioscience or BioLegend.

DC and Treg suppression assays

Assays were performed as described (15, 16). In brief, MACS-enriched splenic DCs (or Tregs) were cultured with responder OT-1 T cells and OVA peptide (or responder A1 T cells, APCs, and H-Y peptide) for 72 h and [^{3}H]thymidine incorporation was measured. 1-Methyl-D-tryptophan (1MT; 100 μM) was added to parallel DC cultures to block IDO-mediated suppression.

In vivo T cell suppression assays

B6 mice harboring marked (Thy1.1) OVA-specific OT-2 T cells were immunized with Act-mOVA splenocytes (10^6/mouse, i.v. or s.c.), and OT-2 expansion and Th1 differentiation (intracellular IFN-γ) were assessed after 120 h in spleen or inguinal lymph nodes, respectively (9). c-diGMP was administered (100 μg, i/v) 6 h before and 48 h after OVA immunization.

Quantitative RT-PCR

RNA was purified using HP total RNA kits (Omega Bio-Tek), reverse-transcribed using a random hexamer cDNA RT kit (Clontech, Mountain View, CA), and quantitative RT-PCR was performed using an iQ5 system and SsoFast EvaGreen supermix (Bio-Rad, Hercules, CA). Primers for murine β-actin were (forward) 5′-TACGGATGTCAACGTCACAC-3′ and (reverse) 5-AAGAGCTATGAGCTGCCTGA-3′. Validated quantitative PCR primer sets for IFN-β1 and IL-1β were purchased (RealTimePrimers.com and Qiagen, Germantown, MD). Threshold cycle (Ct) values were set in the early linear phase of amplification; relevant expression was calculated as $2^{Ct(\beta\text{-actin}) - Ct(\text{target gene})}$.

Statistical analysis

The unpaired Student *t* test (Graphpad Prism) was used for statistical analyses.

Results and Discussion

DCs sense nanoparticle cargo DNA via STING

Treatment (i.v.) with DNPs containing PEI and plasmid DNA lacking TLR9 ligands (CpG^{free} pDNA) stimulated IFN-αβ and IL-1β (by 3 h) and IDO activity by 24 h (Table I). DNPs did not induce IFN-αβ or IDO but still induced IL-1β in STING-deficient (STING KO) mice (Table I). Moreover, IL-1β and IFN-αβ expression peaked ~3 and ~6 h after treatment in DNP-treated B6 mice, respectively (data not shown), indicating that IFN-αβ and IL-1β were induced via distinct pathways. IFN-αβ and IDO induction was also abolished in DT-treated $CD11c^{DTR}$ mice expressing human DTR under control of CD11c promoters to deplete DCs (Table I). DT-mediated depletion of MZ ($CD169^+$) Mϕs in $CD169^{DTR}$ mice did not block IFN-αβ or IDO induction (Table I). Systemic treatment with c-diGMP (200 μg, i.v.), a microbial second messenger sensed by STING (17), induced IFN-αβ, IDO, and, to a lower extent, IL-β, and these responses were STING-dependent (Table I). Thus, cargo DNA was sensed via STING to induce IDO, and DCs mediated this response, whereas MZ Mϕs that mediated regulatory responses to apoptotic cells via IDO (12, 13) were not required to induce IDO after DNP treatment.

Discrete populations of MZ DCs ingest DNPs rapidly

Rh-PEI containing CpG^{free} cargo DNA was used to identify cells that ingested DNPs rapidly. After brief treatment with Rh-DNPs (i.v., 3 h), Rh-PEI staining was concentrated in splenic MZ (Fig. 1A), and Rh staining associated strongly with the MZ Mϕ marker CD169 and to lesser extents with MZ DCs (CD11c) and myeloid (CD11b) cells (Supplemental Fig. 1A). Cultured cells ingest DNPs containing PEI by non-specific endocytosis (18), although uptake mechanisms in physiologic cells remain poorly defined.

To identify cells containing ingested cargo DNA, B6 mice were treated with DNPs containing PEI and CpG^{free} cargo DNA labeled with a fluorescent dye (YoYo-1). After 3 h, small numbers of DCs ($CD11c^+$) and non-DCs contained YoYo-1^+ DNA relative to untreated mice (Supplemental Fig. 1B, 1C). Myeloid cells were analyzed to detect cargo DNA (Fig. 1B, 1C, R1–R4), which was present in ~10% of myeloid DCs (R3, $CD8\alpha^-$) and ~15% of other DCs (R4, $CD8\alpha^+$), whereas no cargo DNA was detected in Mϕs (R1, $F4/80^+$) or plasmacytoid DCs (R2, $B220^+$). Surprisingly, MZ Mϕs ($CD169^+$, $F4/80^-$) that ingested DNPs rapidly contained no detectable cargo DNA (Fig. 1D), suggesting that cargo DNA was degraded soon after DNP uptake. Most gated YoYo-1^+ DCs expressed the integrin CD103, a marker of regulatory DCs (19) and F4/80 (Supplemental Fig. 1D, 1E). Some myeloid ($CD8\alpha^-$) DCs expressed the immune-activating marker 33D1 (20), but $CD8\alpha^+$ DCs did not (Supplemental Fig. 1D, 1E). Thus, cargo DNA accumulated rapidly and selectively in small populations of DCs and non-DCs located in splenic MZ.

$CD11b^+$ DCs respond selectively to nanoparticle cargo DNA and c-diGMP

To identify cells that sensed and responded rapidly to cargo DNA, B6 mice were treated with DNPs (3 h) and IFN-β1 transcription was assessed in FACS-sorted (MoFlo) splenocytes expressing CD11c and/or CD11b. IFN-β1 was selected, as STING activates IFN regulatory factor 3, a potent IFN-β inducer (21), and the FACS sorting strategy is shown in Fig. 2C (R1–R4). IFN-β1 transcription was elevated only in sorted myeloid $CD11b^+$ DCs relative to basal levels in untreated mice (Fig. 2A, black bars), relative to basal levels in untreated mice (Fig. 2A, dotted line). Selective IFN-β1 induction in $CD11b^+$ DCs was also observed in mice treated with c-diGMP (Fig. 2A, gray bars); as expected, DNPs did not induce IFN-β1 in STING KO mice (Fig. 2A, white bars). Cell type–specific responses to DNPs and c-diGMP also manifested when IL-1β transcription was assessed but myeloid non-DCs were the major source of IL-1β (Fig. 2B). Thus, $CD11b^+$ DCs ingested and sensed DNPs and c-diGMP via STING to stimulate IFN-β. In contrast, non-DC myeloid cells sensed these reagents to induce IL-1β but not IFN-αβ

Table I. DCs sense DNA via STING to induce IFN-αβ and IDO

Mice (*n*)	DNPs (c-diGMP)	IFN-αβ (U/μl) (Serum Bioactivity, 24 h)	IL-1β (ng/ml) (Spleen, 3 h)	IDO Activity (pmol/h/mg) (Kyn ex vivo, 24 h)
B6 (9)	−	<0.1	0.3 ± 0.1	9.6 ± 1.4
B6 (10)	+	2.9 ± 0.4***	5.2 ± 0.9**	25.9 ± 2.5***
STING KO (5)	−	<0.1	0.3 ± 0.1	6.4 ± 1.6
STING KO (3)	+	<0.1	3.3 ± 0.4**	7.6 ± 2.3
CD11cDTR + DT (1)	−	<0.1	nt	5.1
CD11cDTR + DT (2)	+	0.2 ± 0.1#	nt	7.3 ± 2.1
CD169DTR + DT (2)	−	<0.1	nt	5.7 ± 0.5
CD169DTR + DT (3)	+	2.0 ± 0.3*	nt	26.3 ± 4.4*
B6 (4)	c-diGMP (200 μg i.v.)	7.8 ± 0.8***	0.9 ± 0.2*	16.3 ± 3.3*
STING KO (2)	c-diGMP (200 μg i.v.)	<0.1	0.2 ± 0.04	6.5 ± 0.3 (3)

*$p < 0.05$, **$p < 0.01$, ***$p < 0.0001$; #not significant.
nt, Not tested.

via a STING-independent pathway (Table I). Functional diversity regarding DNA responsiveness among physiologic innate immune cells has not been described previously. Our findings suggest that splenic CD11b$^+$ DCs may occupy a distinct functional niche, potentially acting as sentinels to detect blood-borne sources of DNA such as microbes and apoptotic cells. Consistent with this notion, intravital analyses of lymph nodes revealed that CD11b$^+$ DCs were the major cell type that ingested immunizing Ags rapidly (22). Their counterparts in splenic MZ may also ingest and sense DNA selectively to induce rapid regulatory responses via IDO that lower the risk of autoimmunity. Hence, functional dichotomy among innate immune cells may be pivotal in elaborating diametric responses to DNA that incite or suppress immunity. Disparate DNA sensitivity may arise due to differential ability to ingest, degrade, or respond to DNA among innate immune cells. Thus, most CD169$^+$ MZ Mϕs and some CD8α$^+$ DCs ingested DNPs but did not respond, suggesting that cargo DNA was degraded rapidly or did not enter the cytosolic compartment, or that cytosolic DNA sensing via STING is defective in these cell types.

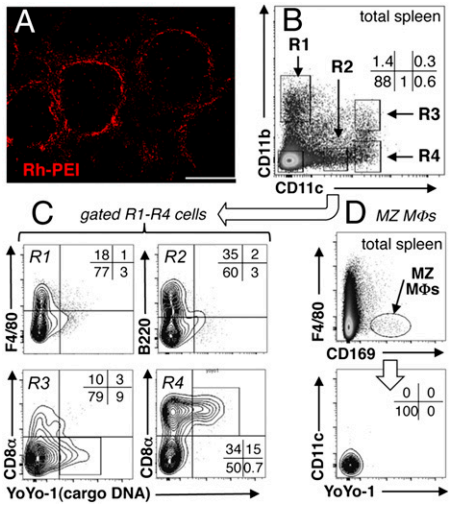

FIGURE 1. Identifying spleen cells that ingest DNPs. (**A**) B6 mice were treated with rhodamine (red)-PEI DNPs (Rh-DNPs, i.v.) and after 3 h spleen sections were examined to detect stained cells. Original magnification, ×100; scale bar, 200 μm. (**B**–**D**) Nanoparticles containing YoYo-1–labeled cargo pDNA were injected into B6 mice (i.v., 3 h), and myeloid (CD11c$^+$, CD11b$^+$) cells (B, C) and CD169$^+$ MZ Mϕs (D) were analyzed to detect cargo DNA (YoYo-1$^+$). Data are representative of experiments using more than three mice.

STING mediates dominant regulatory responses to DNPs

DCs and Tregs acquired potent T cell regulatory phenotypes soon after DNP treatment (9). To test whether intact STING was required for regulatory responses to DNPs, splenic DCs or Tregs from DNP-treated mice were cultured with responder T cells as described (9). Splenic DCs from DNP-treated STING KO mice stimulated robust OVA-specific

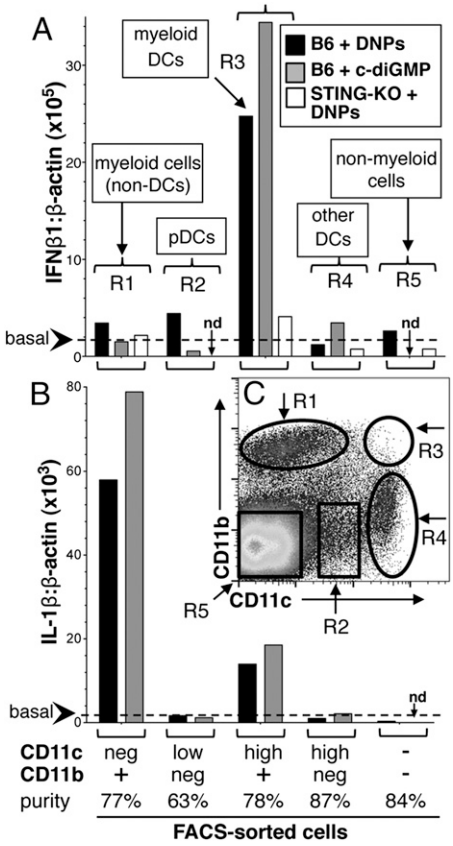

FIGURE 2. Nanoparticle cargo DNA and c-diGMP induce selective IFN-β1 expression by myeloid DCs. B6 mice were treated with DNPs or c-diGMP (i.v., 3 h). Splenocytes from treated mice were stained with CD11c and CD11b mAbs and cells were FACS-sorted using gates shown in (**C**) (R1-R5). (**A** and **B**) Quantitative RT-PCR analysis was performed on RNA from sorted cells to detect IFN-β1, IL-1β, and β-actin transcripts. Data show IFN-β1/β-actin (A) and IL-1β/β-actin (B) ratios for each sorted cell population and are representative of experiments using two or more mice. Dotted lines indicate basal IFN-β1/β-actin or IL-1β/β-actin ratios in RNA from untreated mice (<2 × 10^5 and <2 × 10^3, respectively). nd, not done.

(OT-1) T cell responses in the presence of OVA peptide (Fig. 3B, filled symbols), and adding the IDO-specific inhibitor 1MT did not enhance responses (Fig. 3B, open symbols). As expected, DCs from DNP-treated B6 mice suppressed OT-1 responses via IDO (Fig. 3A). Moreover, FACS-sorted $CD19^+$ DCs but not conventional ($CD19^-$) DCs from DNP-treated B6 mice mediated suppression via IDO (Supplemental Fig. 2A, 2B). Splenic Tregs from DNP-treated STING KO mice did not suppress male (H-Y)–specific (A1) T cell responses elicited ex vivo (Fig. 3C, open symbols); as expected, Tregs from DNP-treated B6 mice suppressed A1 T cell proliferation (Fig. 3C, filled symbols). Thus, cargo DNA sensing via STING was essential for DCs and Tregs to acquire potent regulatory phenotypes after DNP treatment.

To evaluate whether STING ablation modified responses to dying (apoptotic) cells, B6 and STING KO mice were treated with apoptotic thymocytes (10^7, i.v.) as described (12). STING ablation abolished hallmark TGF-β and IL-10 regulatory cytokine induction by apoptotic cells, and proinflammatory IL-6 was expressed instead (Fig. 3D); moreover, apoptotic cells did not induce IDO expression in spleens of STING KO mice (Fig. 3F). These outcomes suggest that DNA from dying cells is sensed via STING to induce regulatory responses via IDO analogous to regulatory responses induced by DNPs.

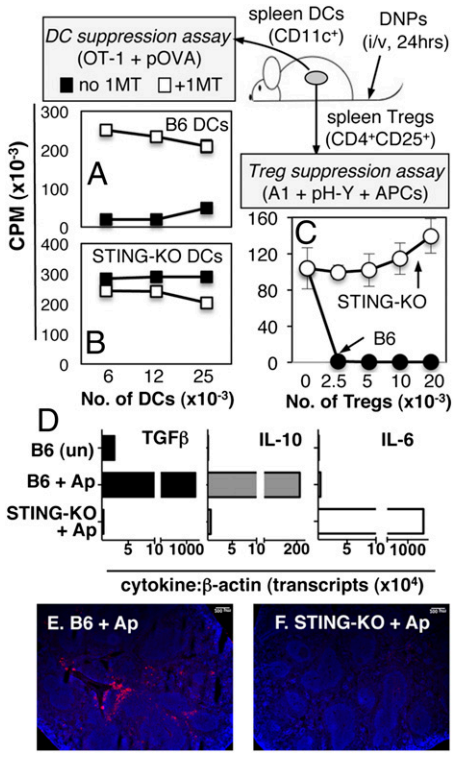

FIGURE 3. STING mediates regulatory responses to DNPs and dying cells. B6 (**A**, **C**) or STING KO (**B**, C) mice were treated with DNPs (i.v., 24 h). (A and B) Graded numbers of MACS-enriched DCs from treated mice were cultured with OT-1 T cells, OVA peptide (pOVA), with and without 1MT, and T cell proliferation was assessed by measuring [^{3}H]thymidine incorporation after 72 h. (C) Graded numbers of MACS-enriched Tregs from treated mice were cultured with H-Y–specific (A1) CD4 T cells, APCs (CBA), and H-Y peptide (pH-Y), and T cell proliferation was assessed as before. Data are the means (±1 SD) of triplicate cultures and are representative of at least two experiments. (**D**–**F**) B6 or STING KO mice were treated with apoptotic thymocytes (Ap, 10^7, i.v.). After 8 h, RNA was made from FACS-sorted splenic DCs ($CD11c^+$), and quantitative RT-PCR was used to detect cytokine gene transcripts (D). Data are cytokine/β-actin transcript ratios ($\times 10^{-4}$) for each RNA sample. (E and F) Immunofluorescence staining to detect IDO (red; Cy3, blue, DAPI counterstain; original magnification ×50) in spleens of mice treated (i.v.) with apoptotic cells for 24 h.

c-diGMP inhibits Ag-specific Th1 responses

DNPs suppressed Th1 responses by OVA-specific (OT-2) T cells to OVA immunization via IDO (9), and we hypothesized that STING activation mediates regulatory responses in this model. Because OT-2 T cell adoptive transfer is not feasible, in that STING KO mice do not have pure B6 backgrounds, we instead used c-diGMP to activate STING during OVA immunization and monitored the impact on responses by marked (Thy1.1) OT-2 T cells (Fig. 4A). Systemic c-diGMP treatment suppressed OT-2 clonal expansion and Th1 (IFN-γ^+, white bars) differentiation significantly relative to controls (Fig. 4A, Supplemental Fig. 2C). Moreover, c-diGMP treatment did not inhibit OVA-specific Th1 responses in IFN-αβ receptor–deficient (IFNAR KO) mice (Fig. 4C), indicating that the STING/IFN-αβ pathway mediated regulatory responses in this model. These outcomes contrast with reports that c-diGMP exhibited immune-activating (adjuvant) properties when administered to mice s.c. (23). Thus, the route of c-diGMP delivery to activate STING is a critical factor influencing immune outcomes.

In summary, to our knowledge the findings in this study show for the first time that selective nanoparticle cargo DNA uptake and DNA sensing via STING in discrete populations of myeloid DCs mediate potent Treg responses. Consistent with these findings, systemic c-diGMP treatment to activate STING also induced IDO and suppressed Th1 responses to OVA immunization. Moreover, regulatory responses induced

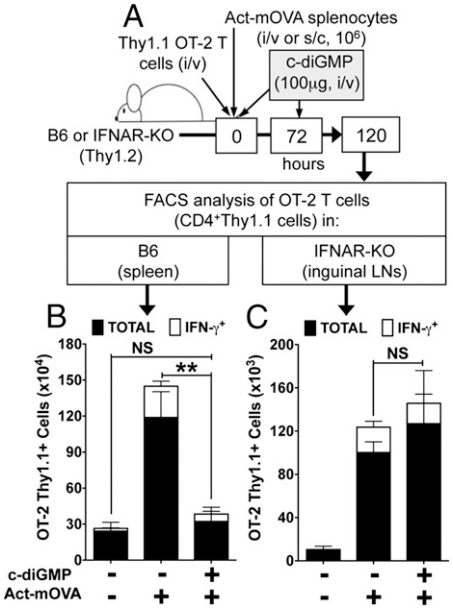

FIGURE 4. c-diGMP suppresses OVA-specific Th1 effector responses. (**A**) B6 or IFNAR KO (Thy1.2) mice harboring OVA-specific OT-2 CD4 T cells (Thy1.1) were immunized (i.v., B6 or s.c., IFNAR KO) with spleen cells from Act-mOVA mice and treated with c-diGMP (100 μg, i.v.) as indicated. One hundred twenty hours after OVA immunization, spleen (**B**) or inguinal (draining) lymph node (**C**) cells were analyzed to detect total OT-2 T cells ($CD4^+$Thy1.1, black bars) and OT-2 Th1 cells ($CD4^+$Thy1.1IFN-γ^+, white bars). Data are the mean numbers of OT-2 T cells in spleen or lymph nodes from two combined experiments ($n = 6$). $^{**}p < 0.004$.

by apoptotic cells were STING-dependent. These findings contradict the emerging paradigm that DNA sensing via the STING/IFN-αβ pathway incites immunity and autoimmunity (7, 8). We hypothesize that discrete populations of myeloid DCs in mouse lymphoid tissues function as sentinel cells that suppress proinflammatory and autoimmune responses to DNA by ingesting, sensing, and responding rapidly to cellular, microbial, or viral DNA entering the cytosolic compartment, as well as cyclic dinucleotides made by infectious pathogens such as *Listeria* (24). Furthermore, we hypothesize that IFN-αβ released selectively by $CD11b^+$ DCs in response to these stimuli induces selective IDO expression by MZ $CD19^+$ DCs in spleen, which activate Tregs to promote dominant regulatory responses. Thus, DNA sensing via STING inhibits or incites immunity, and different routes of DNA exposure, acute or chronic DNA exposure, and differential uptake, degradation, and sensing of DNA by specific innate immune or other cell types are pivotal factors that influence immune responses to DNA.

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Disclosures

A.L.M. and D.H.M. serve as consultants to NewLink Genetics Inc. and receive income from this source. The other author have no financial conflicts of interest.

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