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-α/β). In this study, we show that DNA is also sensed via STING to suppress immunity by inducing IDO. STING gene ablation abolished IFN-α/β 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-α/β 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-α/β (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-α/β 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-α/β production.

Previously, we reported that nanoparticle cargo DNA was sensed in murine lymphoid tissues to induce IFN-α/β, 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-α/β 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 (CD11cDTR), and CD169DTR transgenic mice were described (10–12). To deplete DCs or marginal zone (MZ) macrophages (Mφs), CD11cDTR or CD169DTR 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, Swannanoa, NC) 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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equal volumes of DNA and YoYo-1 in saline and vortexing. The ratio of YoYo-1 to denonucleotide 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 FACS Diva (BD Biosciences) or FlowJo (Tree Star, Ashland, OR) software.

**DC and T reg suppression assays**

Assays were performed as described (15, 16). In brief, MACs-enriched splenic DCs (or Treg) 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 relevant expression was calculated as 2-ΔΔCt(ΔCtbase) where ΔCt is the early linear phase of amplification; relevant expression was calculated as 2-ΔΔCt(ΔCtbase) where ΔCt is the early linear phase of amplification.

**In vivo T cell suppression assays**

B6 mice harboring marked (Thy1.1) OVA-specific OT-2 T cells were immunized with Act-mtOVA 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′-TACCAGTGTCAACGTCACAC-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-ΔΔCt(ΔCtbase) - Ct 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 (CpGfree 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 CD11cDTR mice expressing human DTR under control of CD11c promoters to deplete DCs (Table I). DT-mediated depletion of MZ (CD169+) MΦs in CD169DTR 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 CpGfree 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 CpGfree 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). Significant, ~10% of myeloid DCs (R3, CD8α+) and ~15% of other DCs (R4, CD8α+), 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 (CD8α+) DCs expressed the immune-activating marker CD11b (20), but CD8α+ 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.
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 Ms 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.

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

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

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

**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 Ms (D) were analyzed to detect cargo DNA (YoYo-1⁺). Data are representative of experiments using more than three mice.

**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 × 10⁻⁶, respectively). nd, not done.

**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
(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 DNP.

c-diGMP inhibits Ag-specific Th1 responses

DNP 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 by c-diGMP were abrogated in STING-deficient mice. 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 by c-diGMP were abrogated in STING-deficient mice.

**FIGURE 3.** STING mediates regulatory responses to DNP and dying cells. B6 (A, C) or STING KO (B, C) mice were treated with DNP (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 [3H]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 MACS-purified splenic DCs (CD11c+), and quantitative RT-PCR was used to detect cytokine gene transcripts (D). Data are cytokine/β-actin transcript ratios (×10^−5) 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.
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 CD11c+ 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 has no financial conflicts of interest.

References

FACS-sorted DCs from DNP-treated mice

- **A** sorted CD19^+ DCs
  - CPM vs. No. of sorted DCs (x10^-3)
  - no 1MT: ○
  - +1MT: ●

- **B** sorted CD19^- DCs
  - CPM vs. No. of sorted DCs (x10^-3)
  - no 1MT: ○
  - +1MT: ●

- **C**
  - Gated Lymphocytes
    - 0.1%
    - non-immunized
  - Gated CD4^+ Thy1.1^+
    - 7.2%
    - OVA-immunized
    - 1.1%
    - OVA-immunized + c-diGMP
    - 8.2%
    - 0.3%

CD4 → IFN-γ
Supplemental Figure Legends

Supplemental Figure S1. Discrete subsets of MZ MΦs and DCs ingest DNPs rapidly. A. B6 mice were treated with CpG-free DNA/Rh-PEI (i/v, 3hr, red) and spleen sections were stained to detect cells expressing CD169 (MOMA1), CD11c and CD11b (green). Arrows highlight coincident staining (yellow). Original magnification, x100; scale bars, 200µm. BC. Total splenocytes from untreated B6 mice (B) or (C) B6 mice treated with DNPs containing YoYo-1 labeled cargo DNA (CpG-free, i/v, 3hrs) were stained with anti-CD11c mAb and analyzed by flow cytometry to detect cargo DNA. DE. Cells from YoYo-1+ DNP-treated mice were stained with CD11c and CD11b (as in Fig. 1) mAbs and gated YoYo-1+ CD11b+CD8αneg (D, R3) or CD11bnegCD8α+ (E, R4) DCs were analyzed further. Numbers indicate the relative proportions of gated YoYo-1+ DCs cells in each quadrant. Data are representative of experiments performed using 3 or more mice.

Supplemental Figure S2. Systemic DNPs or c-diGMP treatments suppress T cell responses. AB. B6 mice were treated with DNPs (i/v, 24hrs) and graded numbers of FACS-sorted CD19+ (A) or CD19neg (B) splenic DCs were cultured with OT-1 CD8 T responder T cells and OVA peptide (pOVA) +/-1MT. T cell proliferation was assessed by measuring 3H-Thy incorporation after 72hrs. C. B6 mice harboring marked (Thy1.1) OT-2 T cells were immunized with Act-mOVA splenocytes +/- c-diGMP to activate STING as depicted in Fig. 4A. After 120hrs. spleen cells were stained with anti-Thy1.1 and anti-CD4 mAbs and to detect donor OT-2 cells and OT-2 cells expressing intracellular IFNγ (Th1) and were subjected to FACS analyses. Total OT-2 T cells and Th1 (IFNγ+) cells were calculated and used to compile data shown in Fig. 4B (n=6/group); analogous procedures were used to compile data reported for LN cells in Fig. 4C.