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Highlights

- STING gain-of-function in LTi cells prevents lymph node development in mice
- STING gain-of-function impacts all types of ILCs, but especially ILC3s
- Humans with STING gain-of-function mutations have fewer ILCs

In Brief

Bennion et al. report that a STING gain-of-function mutation prevents the development of lymph nodes and ILCs in mice. Humans with this mutation also have fewer ILCs. In mice, expression of STING gain-of-function in lymphoid tissue inducer (LTi) cells is sufficient to prevent development of lymph nodes.

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STING Gain-of-Function Disrupts Lymph Node Organogenesis and Innate Lymphoid Cell Development in Mice

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SUMMARY

STING gain-of-function causes autoimmunity and immunodeficiency in mice and STING-associated vasculopathy with onset in infancy (SAVI) in humans. Here, we report that STING gain-of-function in mice prevents development of lymph nodes and Peyer’s patches. We show that the absence of secondary lymphoid organs is associated with diminished numbers of innate lymphoid cells (ILCs), including lymphoid tissue inducer (LTi) cells. Although wild-type (WT) α4β7+ progenitors differentiate efficiently into LTi cells, STING gain-of-function progenitors do not. Furthermore, STING gain-of-function impairs development of all types of ILCs. Patients with STING gain-of-function mutations have fewer ILCs, although they still have lymph nodes. In mice, expression of the STING mutant in RORγT-positive lineages prevents development of lymph nodes and reduces numbers of LTi cells. RORγT lineage-specific expression of STING gain-of-function also causes lung disease. Since RORγT is expressed exclusively in LTi cells during fetal development, our findings suggest that STING gain-of-function prevents lymph node organogenesis by reducing LTi cell numbers in mice.

INTRODUCTION

Stimulator of interferon genes (STING) is a cytosolic sensor of cyclic dinucleotides that are produced by the host (e.g., cGAMP) or bacteria (e.g., c-di-GMP, c-di-AMP, cGAMP) (Ablasser et al., 2013; Burdette et al., 2011; Sun et al., 2013; Whiteley et al., 2019). Gain-of-function mutations in STING cause a systemic autoinflammatory disease known as STING-associated vasculopathy with onset in infancy (SAVI) (Liu et al., 2014). We previously generated heterozygous STING N153S mice that have a SAVI-associated mutation (Warner et al., 2017). We previously generated heterozygous STING N153S mice that have a SAVI-associated mutation (Warner et al., 2017). STING N153S mice can only be studied as heterozygous animals since homozygous expression of STING N153S causes early embryonic lethality (Warner et al., 2017). Similar to humans with SAVI, heterozygous STING N153S mice develop systemic inflammation and lung disease as well as T cell cytopenia (Luksch et al., 2019; Warner et al., 2017). However, unlike humans with SAVI, STING N153S mutant mice develop severe combined immunodeficiency (Bennion et al., 2019). The mechanisms of immunodeficiency associated with STING gain-of-function are incompletely understood.

During infection with γ-herpesvirus-68 (γHV68), heterozygous STING N153S mice fail to adequately generate antigen-specific CD8+ T cells and virus-specific immunoglobulin G (IgG) (Bennion et al., 2019). Indeed, STING N153S animals exhibit greater viral burden than Rag1−/− animals, which completely lack B cells and T cells (Bennion et al., 2019). In addition to defects in adaptive immunity, STING N153S causes an innate immunodeficiency (Bennion et al., 2019). Although STING gain-of-function
absence of LNs and Peyer’s patches. We hypothesized that STING N153S may result in a deficiency of committed ILC progenitors, leading to reduced numbers of mature ILCs. However, when we quantitated intestinal ILCs in 6- to 7-week-old STING N153S mice and WT littermate control animals (Figure 1I), we found fewer ILC1s and NK cells, as well as ILC3s, and an increased frequency of ILC2s (Figures 1J–1K). Upon quantitation of absolute numbers of ILCs, all types of ILCs were reduced in the STING N153S small intestine (38-fold fewer ILC1s and NK cells, 2.9-fold fewer ILC2s, and 28-fold fewer ILC3s than in WT; p < 0.005 for ILC2s and p < 0.0001 for ILC1s and ILC3s) (Figures 1I–1K). Thus, STING N153S has a large effect on development and/or survival of all three groups of ILCs in the adult small intestine. LTi-like ILC3s, defined by expression of CCR6 (Klose et al., 2013; Rankin et al., 2013; Sawa et al., 2010; Vorarbourg et al., 2010), exhibited a 41-fold reduction in STING N153S mice compared to WT littermates (Figure 1L). In the STING N153S spleen, ILC numbers also were globally reduced. To distinguish between NK cells and ILC1s, we performed EOMES staining, which confirmed that there were fewer ILC1s (1.7-fold reduction, p < 0.05) and NK cells in STING N153S mice (30.2-fold reduction, p < 0.0001) (Figures S1C–S1E).

We hypothesized that STING N153S may result in a deficiency of committed ILC progenitors, leading to reduced numbers of mature ILCs. However, STING N153S had no effect on the number of alpha-lymphoid progenitor (αLP) cells, which are precursors to all types of ILCs. Furthermore, there was no difference in the numbers of common helper-like innate lymphoid cell progenitors (CHILPs) 1 or CHILP2 cells (Figures S1F–S1J), which are precursors to specific subsets of ILCs (Constantinides et al., 2014; Klose et al., 2014). NK cells have distinct progenitors known as refined NK-cell progenitors (Fathman et al., 2011).
Figure 1. Absence of Lymph Nodes and Peyer’s Patches in STING N153S Mice

(A and B) Representative photographs of WT (left panels) and STING N153S (right panels) animals 15 min after unilateral, subcutaneous footpad injection of Evans Blue dye. Inguinal lymph nodes (LNs) are shown in (A), and retroperitoneal LNs are shown in (B). Discernible LNs are marked by black arrows.

(C) Representative H&E staining of serial skin sections and inguinal fat pads of WT and STING N153S mice. Images are representative of 20 sections per mouse from three mice per genotype from two independent experiments. Scale bar: 200 μm.

(D) Representative H&E staining of serial sections from the small intestines of WT and STING N153S mice. n = 3 mice from two independent experiments. Scale bar: 200 μm.

(E and F) Total number of discernible cervical, inguinal, brachial, axillary, and mesenteric LNs (E) and Peyer’s patches (F). Data represent the mean of six STING N153S and six WT littermate control mice.

(G) IgA levels in the serum and stool of STING N153S and WT littermate animals were quantitated by ELISA. Data represent the mean of eight samples per genotype. Dashed line denotes the limit of detection.

(H–L) Flow cytometric analysis of intestinal leukocytes of 6- to 7-week-old STING N153S mice and WT littermate control animals. (H) Representative FACS dot plots of intestinal leukocytes, indicating the gating strategy for ILC2, ILC3, and LTi-like CCR6^-ILC3 populations. Numbers (red text) indicate the percent of CD45^Lin^- cells in each gate (lineage markers: CD19, CD5, and CD3). Percent and total number of ILC1 and NK cells (NKp46^-GATA3^ RORgamma^-) (I), ILC2s (J), ILC3s (K), and LTi-like CCR6^-ILC3s (L).

Data represent the mean from n = 6 animals per genotype. All data were pooled from at least two independent experiments. Results were analyzed by Mann-Whitney U test. *p < 0.05; **p < 0.01; ***p < 0.001.
STING N153S diminished the numbers of refined NK-cell progenitors (3.7-fold reduction, p < 0.001) in the bone marrow of STING N153S animals (Figures S1K–S1M). Thus, STING N153S reduces the number of refined NK-cell progenitors, but not other ILC progenitor cells in the bone marrow. Collectively, these results suggest that STING N153S preferentially impacts mature ILCs.

**STING N153S Mouse Spleens Have B Cell Zones and Express Chemokines Known to Regulate Splenic Organization**

STING N153S mice have splenomegaly and exhibit histological abnormalities of the spleen (Luksch et al., 2019; Warner et al., 2017). To more carefully evaluate the spleens of STING N153S animals, we performed histological staining and flow cytometry. Consistent with prior reports (Luksch et al., 2019; Warner et al., 2017), histological analysis of STING N153S and WT littermate spleens revealed disorganized architecture (Figure S2A). However, unlike IκBα mutant mice that lack LN (Mooster et al., 2015), we found that the mucosal adressor cell adhesion molecule (MADCAM-1)-expressing marginal sinus remained intact in the STING N153S spleen (Figure S2B). Immunofluorescent staining of CD3 revealed that T cell zones were absent in STING N153S spleens (Figure S2C), a finding that may reflect a large reduction in T cell numbers (Warner et al., 2017). Flow cytometric analysis of STING N153S and WT littermate splenocytes revealed no difference in the number of follicular dendritic cells (FDCs), a 5-fold increase in fibroblastic reticular cells (FRCs) in STING N153S spleens, and no difference in the numbers of WT and STING N153S endothelial cells (Figures S2D–S2G). Furthermore, we confirmed expression of the chemokines CCL19 and CCL21, which organize T cell zones (Dieu et al., 1998; Gunn et al., 1998; Luther et al., 2000), as well as the B-cell-attracting chemokine CXCL13, which is produced by FDCs (Figure S2H) (Cyster et al., 2000). Thus, STING N153S does not create a deficiency of splenic FDCs or FRCs, which regulate lymphoid tissue organization.

**STING N153S Mice Have Fewer LTI Cells in Fetal Tissues**

Because adult STING N153S mice have fewer ILCs and LTI-like cells, we hypothesized that STING N153S fetuses would also have fewer LTI cells, which are required for LN organogenesis. Indeed, in STING N153S fetuses, we observed a reduction in the percent and number of gut LTI cells (~4.5-fold reduction, p < 0.0001) (Figures 2A and 2B). CD4 is used as a surrogate marker for LTI cells in embryonic LN anlagen, since most CD4+ cells in developing LN represent CD3+ RORγ+ TIL cells (Eberl et al., 2004; Kelly and Scollier, 1992). Histological analysis of cervical LN anlagen from embryonic day 18.5 (E18.5) STING N153S and WT littermate fetuses revealed diminished CD4 staining intensity, suggesting a reduced accumulation of LTI cells (Figures 2C and 2D). There was no difference in the intensity of VCA-1 staining, a marker of lymphoid tissue organizer (LTo) cells, or in the two-dimensional size of the LN anlagen (Figures 2E and 2F). Thus, the STING N153S mutation reduces the accumulation of LTI cells in the fetal gut and LN anlagen. LTI cells activate noncanonical NF-κB signaling in LTo cells by engaging the lymphotxin-β receptor (LTβR), leading to signaling events required for LN development (Mooster et al., 2015; Onder et al., 2013; Rennert et al., 1998). Therefore, we set out to test whether non-canonical NF-κB signaling downstream of the LTβR is intact in STING N153S cells. Mouse embryonic fibroblasts (MEFs) have previously been used to study LTβR signaling (Dejardin et al., 2002; Mooster et al., 2015), so we generated MEFs and treated them with a monoclonal antibody (mAb) agonist of LTβR or isotype control mAb, as described previously (Dejardin et al., 2002). SDS-PAGE and western blot of IκBα and RelB revealed no differences in NF-κB activation downstream of LTβR (Figure S2I). Treatment of WT and STING N153S MEFs with the LTβR agonist antibody caused a similar upregulation of genes that contribute to lymphoid tissue organogenesis, including Vcam1, Icam1, Madcam1, and Cxcl10 (Figures S2J–S2M), which are upregulated downstream of LTβR signaling (Caponio et al., 2007; Dejardin et al., 2002; Hoffmann et al., 2003; Mooster et al., 2015). Thus, LTβR-mediated noncanonical NF-κB signaling remains intact in STING N153S MEFs, suggesting that LTβR signaling is not dysregulated by the mutation.

**Single-Cell RNA Sequencing of α4β7+ Cells from the Fetal Liver Suggests Fewer Mature LTI Cells in STING N153S Fetuses**

To confirm that α4β7+ cells from WT and STING N153S fetal livers (Figure 3A) express STING as well as transcription factors known to impact ILC differentiation, we performed fluorescence-activated cell sorting sequencing (FACS-seq), a type of single-cell RNA sequencing. We detected STING (Tmem173) gene expression in at least a subset of α4β7+ cells (Figure 3B) and additionally confirmed that there was no difference in expression of IκBα and Tox, transcription factors required for LN development (Figures 3C and 3D) (Aliahmad et al., 2010; Cupedo and Mebius, 2005). However, α4β7+ cells from STING N153S fetuses expressed higher levels of Tcf7, a gene highly expressed in early lymphoid progenitors (Figure 3E) (Yang et al., 2015). This may suggest that there are fewer mature α4β7+ cells in STING N153S fetal livers than in the WT control livers. Using flow cytometry, we confirmed similar expression levels of Id2, Tox, and NFIL3, all of which are involved in α4β7+ progenitor cell differentiation into mature LTI cells (Figures 3F–3I) (Aliahmad et al., 2010; Cherrier et al., 2012; Geiger et al., 2014; Yokota et al., 1999). Additionally, FACS-seq did not reveal differences in the expression of type-1-IFN-stimulated genes (Figure S3A). Finally, no other genes known to be involved in LTI cell differentiation and function were appreciably affected (Figure S3B) (Chea et al., 2016). Thus, our FACS-seq and flow cytometric analyses confirmed that STING is expressed in at least a subset of α4β7+ cells, but without impacting the expression of most key transcription factors involved in LN and ILC development.

**STING N153S Impairs Generation of α4β7+ Progenitors into LTI Cells and ILCs**

Increased expression of Tcf7 may reflect the differentiation status of α4β7+ cells in the fetal liver (Yang et al., 2015), consistent with what we observed in the fetal gut (Figure 2B). To begin to determine whether STING N153S impacts the differentiation of ILCs, we assessed the capacity of WT and STING N153S α4β7+ cells to differentiate into mature ILCs for 6 or
14 days in an OP9 stromal cell culture system in the presence of interleukin-7 (IL-7) and stem cell factor (SCF) (Cherrier et al., 2012). On day 6, only ~5% of Lin−CD19−CD3+ STING N153S cells were LTi cells, compared with ~50% in the WT cell culture (Figures 4A, top panels, and 4B). We also detected a higher percentage of NK cells and ILC2s in STING N153S cell cultures compared with WT controls (Figures 4A, bottom panels, and 4B). Cell-intrinsic nucleic acid sensing may direct T cells into

Figure 2. STING N153S Reduces Numbers of LTI Cells in Fetal Tissues as Well as Their Accumulation in Developing LN Anlagen

In (A) and (B), leukocytes were harvested from the fetal gut on E16.5–E18.5 and analyzed by flow cytometry. In (C)–(F), frozen sections of cervical LN anlagen of E18.5 fetuses were analyzed by widefield fluorescence microscopy.

(A) Representative FACS dot plots of Lin−CD45+CD127+ cKIT+ cells isolated from the fetal gut. Numbers indicate the percent of events in each gate.

(B) Total number α4+7RORγ+ LTI cells in the fetal gut on E16.5–E18.5. Data represent the mean of n = 20–23 fetuses per genotype pooled from three independent experiments.

(C)–(F) Cervical LN anlagen sections were stained with DAPI and with antibodies against VCAM-1 (left panel) and CD4 (middle panel). Scale bar: 40 μm.

(D) Quantitation of CD4 staining intensity relative to the size of LN anlagen, defined as the total area of VCAM-1 and CD4 staining.

(E) Quantitation of VCAM-1 staining intensity relative to the size of LN anlagen.

(F) Quantitation of the size of LN anlagen, based on merged VCAM-1 and CD4 staining of WT and STING N153S cervical LN anlagen on E18.5. Quantitation is from n = 7–9 cervical LN anlagen per genotype from two independent experiments. FACS data were analyzed by unpaired t test, and immunofluorescence data were analyzed by Mann-Whitney U test. *p < 0.05; ****p < 0.0001.
a T helper 2-type lineage (Imanishi et al., 2014), and an increase in ILC2 and NK cell frequency may suggest that STING gain-of-function biases ILC progenitors into helper ILC lineages. On day 6, we observed ~6.5-fold fewer STING N153S ILC1s and ~2.6-fold fewer ILC2s compared with WT controls. However, there was a much larger effect on the number of ILC3s (~21-fold) and LTi cells (~145-fold) in STING N153S cultures (Figures 4C–4G). Since STING N153S impairs the differentiation of multiple ILC subsets after 6 days in the OP9 stromal cell system, we reasoned that a longer experiment (14 days) may elucidate whether this reflects a delay in differentiation. In contrast to what was observed on day 6, we found no difference in the numbers of WT and STING N153S ILC1s and ILC2s on day 14. However, the numbers of ILC3s and LTi cells were still reduced in STING N153S cultures compared with WT controls (~10-fold and ~8.6-fold, p < 0.0001 and p < 0.001) (Figure S4). Thus, STING N153S delays the differentiation of ILC1s, ILC2s, ILC3s, and LTi cells in a cell culture system but has its largest effect on ILC3s and LTi cells (Figure 4H). This result is consistent with our in vivo findings demonstrating a preferential effect of STING N153S on ILC3s and LTi cells in mice (Figures 1H–1L and 4H).

STING gain-of-function mutations cause apoptosis of T cells (Gulen et al., 2017; Wu et al., 2019), so we hypothesized that STING N153S may similarly cause apoptosis of ILCs. However, we observed no difference in the percent or number of annexin V-positive or annexin V-negative α4β7+CD127+ cells, which represent a mixture of mature LTi and LTi progenitor cells (Figures S5A–S5C). STING gain-of-function mutations also reduce the proliferative capacity of T cells (Cerboni et al., 2017), but bromodeoxyuridine (BrdU) labeling in vivo did not reveal any differences in the percent or number of proliferating LTi cells (Figure S5D). We reasoned that dead or dying cells may be difficult to detect in vivo, so we also tested for apoptosis of ILCs in the OP9 co-culture system. We observed a small increase in the frequency of total dead cells in the STING N153S culture when compared to WT samples (Figures S5E–S5G). Thus, STING N153S may produce subtle effects on apoptosis of ILCs, although we only detected this effect in cell culture and not in cells freshly isolated from mice.

Human SAVI Patients Have Diminished Frequencies of ILCs in Peripheral Blood

To test whether STING gain-of-function affects ILCs in humans with SAVI, we performed flow cytometric analysis of peripheral blood mononuclear cells (PBMCs). In healthy control subjects, circulating ILCs represent approximately 0.1% of total CD45+ blood mononuclear cells (PBMCs). In healthy donor and SAVI patients were being treated with a JAK1/2 inhibitor, and one patient was being treated with mycophenolate mofetil (MMF) (Figure 5A). Obtaining samples from patients with untreated disease was not possible for ethical reasons. Frequencies of

In (A)–(H), fetal livers were harvested on E14.5 from WT and heterozygous STING N153S animals. (A) Illustration depicting α4β7+ fetal liver cells and their key transcription factors. (B–E) α4β7+ cells were single-cell sorted by FACS into 96 well plates, followed by RNA sequencing (FACS-seq). Violin plots of FACS-seq results demonstrating expression levels of Tmem173 (STING) (B), Id2 (C), Tox (D), and Tcf7 (E) in α4β7+ progenitor cells. Data represent the mean number of counts per gene from 48 cells per genotype performed as a screening experiment. (F) Total number of α4β7+ cells (Lin−CD45+ cK−IT−CD127+α4β7+) in WT and STING N153S fetal livers. (G–I) Mean fluorescence intensity (MFI) of the transcription factors Id2 (G), Tox (H), and Nfil3 (I) in α4β7+ cells from the fetal livers of WT and STING N153S mice. Data in (E)–(I) represent the mean of n = 20–24 (F), n = 13 (G), n = 6–9 (H), and n = 5–11 (I). Data in (F)–(I) were pooled from at least two independent experiments and analyzed by unpaired t test. *p < 0.05; **p < 0.01.
Lin-/CD7+ cells were similar in healthy control and JAK-inhibitor-treated SAVI patients (Figure 5B). However, patients with STING gain-of-function exhibited a 3-fold reduction in the frequency of circulating total ILCs and ILC2s (Figures 5C–5E). Thus, STING gain-of-function reduces the numbers of circulating ILCs, consistent with a prior report that numbers of circulating NK cells are reduced in patients with STING gain-of-function mutations (Liu et al., 2014). Since SAVI patients have LNs, which we confirmed histologically (Figures S6A and S6B), our results indicate that the effects on lymphoid organogenesis only occur in mice. This may suggest species-specific differences in the effects of STING gain-of-function in LTi cells. Studies of LTi cells in this rare patient population were not possible.

**Cell-Type-Specific Expression of STING Gain-of-Function in RORγT+ Lineages Causes T Cell Cytopenia and Lung Disease**

Next, we set out to examine whether the expression of STING N153S in mature LTi cells might be sufficient to reduce the number of LTi cells and prevent LN development in mice. We generated transgenic LoxP-STOP-LoxP STING N153S (floxed-STOP STING N153S) mice where Cre-mediated recombination leads to expression of the STING N153S mutant (Figure 6A). RORγT is expressed specifically in LTi cells in the developing fetus (Sun et al., 2000). In adult mice, RORγT is expressed in LTi-like cells, ILC3s, Th17 cells, and T cells at the double-positive (DP) stage of development (Sawa et al., 2010; Sun et al., 2000; Takeda et al., 2009). We crossed the floxed-stop STING N153S mice with RORγT+ mice to generate transgenic mice expressing STING N153S in LTi cells. As seen in Figure 6B, the number of LTi cells in the lungs of STING N153S mice is significantly reduced compared to WT mice (Figure 6B). These results suggest that STING gain-of-function in LTi cells is sufficient to cause T cell cytopenia and lung disease.

**Figure 4.** STING N153S Fetal Liver α4β7+ Progenitor Cells Do Not Differentiate into LTi Cells after 6 Days in an OP9 Cell Culture System

α4β7+ progenitor cells from the fetal liver were co-cultured with OP9 stromal cells, SCF, and IL-7. Cells were allowed to differentiate for 6 days and analyzed by FACS.

(A) Representative FACS plots of adult WT (left panels) and STING N153S (right panels) CD45+CD3−CD19− cells. Cell frequencies within each gate are denoted in red, and cell population names are labeled in blue.

(B) Average frequencies of ILC and α4β7+ cell populations as a fraction of total CD45+CD3−CD19− cells. Cell frequencies within each gate are denoted in red, and cell population names are labeled in blue.

(C–G) Percent and number of ILC1 (C), NK cells (D), ILC2 (E), ILC3 (F), and LTi-like cells (G).

(H) Graphical summary of the fold reduction in numbers of ILCs by STING N153S compared to WT. Data represent the mean of 6–10 replicates per group pooled from at least two independent experiments. Results were analyzed by unpaired t test. *p < 0.05; **p < 0.01; ****p < 0.0001.

**Figure 5.** Quantitation of ILC and ILC Progenitor Cells in the Blood of Human SAVI Patients

(A) Gating strategy and representative FACS plots of peripheral blood ILC1 and ILC2 populations gated on the CD45+ live lymphocytes.

(B–E) Percent of total ILC & NK cells (Lin CD7+) (B), total ILCs (CD56+CD127+CD94−NKG2A+CD16−CD2−) (C), ILC1 (CD117−CRTH2+) (D), and ILC2 (CD117+CRTH2+) (E) as a frequency of CD45 live lymphocytes. Black dots (12 donors, left columns) denote healthy donors, purple dots (4 patients, middle columns) denote SAVI patients on JAK inhibitors (ruxolitinib), and yellow dots (1 patient, right columns) denote a SAVI patient on mycophenolate mofetil (MMF). Results were analyzed using unpaired t test. **p < 0.01.
to transgenic RORγ-T-Cre animals and confirmed excision of the STOP cassette in DP but not double-negative (DN) thymocytes (Figure 6B). We previously found that αβ T cells drive STING N153S-associated lung disease (Luksch et al., 2019), but we did not examine whether the expression of the mutant in T cells was sufficient to cause disease. Here, we found that STING N153S expression induced by RORγ-T-Cre was sufficient to cause perivascular lung inflammation in 13-week-old mice (Figure 6C), suggesting that the expression of STING N153S in T cells may be necessary and sufficient for certain features of disease in STING N153S mice. Flow cytometric analysis of thymocytes from 3-to-4-week-old RORγ-T-Cre-positive floxed-stop STING N153S mice and floxed-stop STING N153S control animals were harvested, and single-cell suspensions were prepared for FACS analysis. (D) Representative FACS plots of CD4 and CD8 expression gated on CD45+CD19−NK1.1− cells. Percent and number of DN (E), DP (F), CD4+ (G), and CD8+ (H) cells. Data represent the mean of five mice per group pooled from two independent experiments. Percent and number of CD3+ (I), CD4+ (J), CD8+ (K), and NK1.1+ (L) cells. Data represent the mean of 10 mice per group pooled from at least two independent experiments. Results were analyzed by unpaired t test. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

Figure 6. RORγ-T-Cre-Mediated Cell-Type-Specific Expression of STING N153S Diminishes Numbers of Single-Positive T Cells in the Thymus and Spleen

(A) Schematic of the floxed-STOP STING N153S construct. Mice with Cre-dependent expression of the STING N153S gene in the ROSA locus (floxed-stop STING N153S mice) were generated using transcription activator-like effector nucleases (TALENs).

(B) PCR amplification of transcriptional stop containing ROSA homology arms from double-negative (DN; CD8−CD4−) and double-positive (DP; CD8+CD4+) thymocyte DNA from an adult RORγ-T-Cre+ floxed-STOP STING N153S mouse.

(C) Images of H&E staining on lungs of 13-week-old RORγ-T-Cre+ floxed-STOP STING N153S mice (right panel) and floxed-STOP STING N153S control animals (left panel). n = 3 mice per genotype.

(D–H) Thymocytes from 3-to-4-week-old RORγ-T-Cre+ floxed-STOP STING N153S mice and floxed-STOP STING N153S control animals were harvested, and single-cell suspensions were prepared for FACS analysis. (D) Representative FACS plots of CD4 and CD8 expression gated on CD45+CD19−NK1.1− cells. Percent and number of DN (E), DP (F), CD4+ (G), and CD8+ (H) cells. Data represent the mean of five mice per group pooled from two independent experiments. Percent and number of CD3+ (I), CD4+ (J), CD8+ (K), and NK1.1+ (L) cells. Data represent the mean of 10 mice per group pooled from at least two independent experiments. Results were analyzed by unpaired t test. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

STING N153S Expression in RORγ-T+ Fetal LTI Cells Is Sufficient to Interfere with LN and Peyer's Patch Development

RORγT expression during fetal development is restricted to LTI cells (Sun et al., 2000), which act in concert with LTo cells to
initiate LN and Peyer’s patch organogenesis (van de Pavert and Mebius, 2010). We found that expression of STING N153S, specifically in RORγT+ LTi cells, was sufficient to prevent LN and Peyer’s patch development based on visual assessment and Evans blue staining of LNs (Figures 7A and 7B). However, we still observed very small mesenteric LNs and occasionally a single unilateral inguinal, brachial, or axillary LN in some animals that express the STING N153S mutant in LTi cells (Figure 7B). The sporadic presence of a residual LN despite the expression of STING N153S in LTi cells might suggest additional effects of STING N153S on nonhematopoietic cells. Alternatively, sporadic residual LNs might result from the incomplete excision of the floxed-STOP by Cre, which would be expected to occur in a small subset of LTi cells.

Postnatally, RORγT is expressed in mature ILC3s, including LTi-like cells (Sawa et al., 2010; Sun et al., 2000; Takatori et al., 2009). In the adult gut, we found that there was a similar percentage and number of CD45+ cells in WT and STING N153S animals. Cell-type-specific expression of STING N153S in RORγT-positive lineages caused a ~95-fold reduction in the number of ILC3s and a ~91-fold reduction in the number of LTi-like cells (Figures 7C–7I). There also was a corresponding increase in the percent of ILC1s and ILC2s, but no difference in the total number of these cell types (Figures 7J–7M). Collectively, these results demonstrate that the expression of STING N153S in DP T cells, ILC3s, and LTi-like cells is sufficient to reduce their numbers. Furthermore, our results suggest that STING gain-of-function signaling can impact the differentiation of progenitor cells as well as the lifespan of mature T cells and ILCs. Finally, expression of STING N153S in fetal LTi cells is sufficient to prevent the development of LNs and Peyer’s patches, revealing a deleterious role of STING gain-of-function during lymphoid tissue organogenesis in mice.

**DISCUSSION**

We discovered that the STING N153S gain-of-function mutation disrupts LN and Peyer’s patch organogenesis and interferes with LTi cell differentiation. Furthermore, we demonstrated that expression of STING N153S in RORγT-positive lineages is sufficient to interfere with the development of LNs and Peyer’s patches. Thus, STING gain-of-function dysregulates lymphoid tissue organogenesis in mice by interfering...
with the development of LTi cells and by reducing the numbers of mature LTi cells.

An effect of STING gain-of-function in lymphoid tissue organogenesis was unexpected, especially since other pattern recognition receptors have not been implicated in lymphoid tissue organogenesis. Furthermore, the impact of STING signaling in ILCs is incompletely understood (Cannesse et al., 2018; Marcus et al., 2018). Since CRISPR/Cas9 can sometimes produce off-target effects (Cradick et al., 2013; Frock et al., 2015; Fu et al., 2013; Hsu et al., 2013; Pattanayak et al., 2013; Wang et al., 2015), one potential question regarding the LN deficiency phenotype is whether a second mutation due to an off-target effect may be responsible for this particular defect. However, we corroborated the LN deficiency phenotype in independently generated STING N153S mice, and a similar defect was observed but not studied in another STING gain-of-function mouse that has a mutation in the neighboring amino acid (STING V154M mice) (Bouis et al., 2019). LN deficiency also occurred in our heterozygous transgenic mouse that expresses the STING N153S cDNA from the ROSA locus. It is unlikely that an off-target mutation elsewhere in the genome could explain the universal absence of LNs and Peyer’s patches in four independently generated heterozygous mouse models.

We previously found that STING N153S is sufficient to cause an immunodeficiency during viral infection that is more severe than that of STING goldenticket mice, which lack functional STING signaling. The immunodeficiency of STING N153S mice is also more severe than that of Rag1−/− animals, which lack adaptive immunity (Bennon et al., 2019). Severe immunodeficiency in STING N153S mice distinguishes the animal model from the disease associated with the analogous STING N154S mutation in humans. In contrast to what we observed in mice, patients with the STING N154S mutation have LNs (Liu et al., 2014). In mice, the deficiency of ILCs, LNs, and Peyer’s patches likely contributes to the severe immunodeficiency phenotype, including IgA deficiency and a failure to produce virus-specific IgG. We previously found that STING N153S dysregulates virus-specific CD8+ T cell responses after intranasal inoculation with γHV68, leading to reduced γHV68-specific CD8+ T cell responses in the lung. This might result from diminished antigen presentation as a consequence of LN deficiency (Banchereau and Steinman, 1998). Finally, since CD4+ T cells in the spleen help B cells to undergo antibody class switching (Victoria and Nussenzweig, 2012), the absence of T cell zones in the spleen may explain the impaired ability of STING N153S animals to produce virus-specific IgG (Bennon et al., 2019).

Development of LNs requires LTi cells, which represent a subset of ILC3s (Arts and Spits, 2015; Eberli et al., 2004). We found that there were fewer CD4+ LTi cells in STING N153S fetuses compared to WT littermate fetuses, and this defect corresponded with reduced CD4 staining in LN anlagen on E18.5. Although RORγ+T lineage-restricted expression of STING N153S blocked LN development, we cannot exclude contributions of STING N153S expression in other cell types. For example, STING is also highly expressed in lymphatic endothelial cells and may be expressed in LTo stromal cells (Heng et al., 2008).

An open question is whether alterations in the quantity or quality of LTi cells may explain the LN deficiency in STING N153S mice. LN anlagen develop as LTo on LTi cells ligates the LTIR on stromal LTo cells (Füterer et al., 1998; Rennert et al., 1996; van de Pavert and Melibius, 2010). LTIR stimulation induces noncanonical NF-κB signaling, which upregulates adhesion molecules and chemokines to recruit more LTi cells as well as T and B cells to the developing LN (Cupedo and Melibius, 2005; Dejardin et al., 2002; Ngo et al., 1999). Studies of LN development in mice deficient in noncanonical NF-κB signaling have suggested that LTi cell accumulation must exceed a threshold level of cells for LN anlagen to develop and persist postnatally (Kim et al., 2000; Onder et al., 2013). Our results are consistent with the hypothesis that diminished numbers of LTi cells, below a threshold, can cause LN deficiency. However, mice deficient for the transcription factor Nfil3 also have reduced numbers of LTi cells in the fetus, although Nfil3−/− mice still develop LNs (Xu et al., 2015). This may indicate that LTi cells are a heterogeneous population with only a subset of cells contributing to LN development. In STING N153S mice, an alternative explanation might be that the LTi cells, although reduced in number, also are somehow unfit in their capacity to promote LN development. An answer to this question will likely require more extensive mechanistic phenotyping and subset analysis of LTi cells.

We found that SAVI patients have lower frequencies of circulating ILCs in the blood. However, humans with STING gain-of-function mutations have LNs (Liu et al., 2014). These species-specific differences may reflect STING expression levels in ILC subsets or, alternatively, differential effects of the STING mutant in human and mouse cells. To better explain species-specific effects of STING gain-of-function on LN development, we would like to assess LTi-like cells in SAVI patient samples. However, this was not possible, in part because ILC3s and LTi cells are not readily detectable in circulation (Shikhaghaie et al., 2017). Future studies examining LTi and ILC3 cells from tonsils or intestinal tissues from SAVI patients may help to further define species-specific effects on these cell types.

Although we uncovered an immunological mechanism of LN deficiency caused by STING gain-of-function, the molecular mechanisms that underlie STING-N153S-mediated impairment of ILCs remain elusive. STING gain-of-function mutations cause pro-apoptotic and anti-proliferative effects in T cells (Cerboni et al., 2017; Gui et al., 2019; Wu et al., 2019), and we observed subtle effects on apoptosis in a cell culture differentiation system of ILCs, but not in freshly isolated ILCs. If STING gain-of-function induces apoptosis in ILCs, the effect appears to be subtle. Alternatively, STING N153S may cause ILC cytopenia via an alternative mechanism that remains to be identified.

Our group and others continue to pursue the molecular and cellular effects of STING gain-of-function mutations (Cerboni et al., 2017; Gui et al., 2019; Wu et al., 2019). Definitive reversal of the physiological and immunological effects of STING gain-of-function, which are established as type I IFN independent in mice (Bouis et al., 2019; Luksch et al., 2019), will likely require characterization of pathways that are less well understood, as well as genetic approaches that rigorously confirm mechanism under physiological conditions. This ongoing work may lead to a greater understanding of WT and mutant STING biology, in
addition to insights regarding fundamental mechanisms of LN development and ILC differentiation and survival in humans and in mice.

**STAR METHODS**

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**SUPPLEMENTAL INFORMATION**

Supplemental Information can be found online at https://doi.org/10.1016/j.celrep.2020.107771.

**ACKNOWLEDGMENTS**

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**REFERENCES**


Mombartaerts, P., Iacomini, J., Johnson, R.S., Herrup, K., Tonegawa, S., and Paty, a heterozygous S32I mutation in I


## STAR Methods

### Key Resources Table

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RESOURCE AVAILABILITY

Lead Contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Jonathan J. Miner (jonathan.miner@wustl.edu).

Materials Availability
All unique reagents generated in this study are available from the Lead Contact with a completed Materials Transfer Agreement. Commercially available reagents are indicated in the Key Resources Table.

Data and Code Availability
The dataset generated by the single-cell FACs-Sequencing of α4β7+ fetal liver (E14.5) progenitor cells during this study is available at Mendeley DOI: https://dx.doi.org/10.17632/9nck2z26tf.1

EXPERIMENTAL MODEL AND SUBJECT DETAILS

In vivo animal models
STING N153S mice were generated by our lab and published previously (Warner et al., 2017). All animals were housed in specific pathogen free facilities at Washington University in St. Louis. All STING N153S expressing animals were heterozygous and aged matched, co-housed littermate control animals were used for all experiments. Both sexes were used in all experiments and animals were randomly assigned to experimental groups. Floxed-STOP STING N153S mice were generated by and obtained from the Hope Center Transgenic Vectors Core at Washington University in Saint Louis. TALENs genome editing for the creation of transgenic mice has been described previously (Meyer et al., 2010). Briefly, to generate mice that conditionally expressed the STING N153S protein a targeting vector specific to the Rosa26 locus was assembled. This vector included a Rosa26 homology arm, a CAG promoter region, a transcriptional stop sequence flanked by loxp sequences, the STING N153S cDNA sequence, and a second Rosa26 homology arm. The targeting vector and TALENs were injected into C57BL/6J single-cell embryos obtained from superovulated C57BL/6J female mice mated to male C57BL/6J animals. Modified embryos were transferred into pseudo-pregnant female recipient mice. PCR assays and DNA sequencing confirmed targeted insertion of the vector into the Rosa26 locus. Expression of the mutant STING N153S protein was obtained by crossing heterozygous floxed-STOP STING N153S mice to RORγT-Cre-expressing animals. All other mouse strains were obtained as indicated in the Key Resources Table. Power analysis was conducted for Institutional Animal Care and Use Committee-approved in vivo studies in order to determine the number of animals needed per experimental group.
least two independent experiments were conducted to replicate findings. No outliers were excluded from analyses. The age and number of animals used for each experiment is listed in the figure legends.

**SAVI patient samples**

ILCs were analyzed from blood samples drawn from a total of 5 patients with STING gain-of-function mutations (V155M, N154S, or R281Q). There were 2 males (2 years-old, V155M and 10 years-old, N154S) and 3 females (8 years-old, V155M, 24 years-old, V155M, and 10 years old, R281Q). Four patients were on a JAK1/2 inhibitor, and one patient was on mycophenolate mofetil. For ethical reasons, SAVI patients were not removed from medical treatments. Hilar lymph nodes were obtained from SAVI patients (1 male, 1 female, both age 14) who underwent lung transplantation. Written informed consent (parental consent, in case of minors) was obtained from all participants of the study. The study and protocols conform to the 1975 Declaration of Helsinki and were approved by the comité de protection des personnes Ile de France II and the French advisory committee on data processing in medical research.

**Cell lines**

OP9 cell lines were used in the co-culture of mouse primary bone marrow cells. OP9 cell lines were cultured in MEM medium supplemented with 20% FCS and 1% penicillin/streptomycin (OP9 medium) at 37°C in a humidified atmosphere at 5% CO2.

**METHOD DETAILS**

**Quantitation of lymph nodes**

Bilateral cervical, inguinal, brachial, and axillary lymph nodes as well as mesenteric lymph nodes from WT and STING N153S mice were dissected and the number of discernible nodes counted. A string of mesenteric lymph nodes was counted as one lymph node. For Evans Blue staining of lymphatics and lymph nodes, mice were anesthetized and 25 µL of 5% Evans Blue dye in PBS was injected into one forefoot and one hindfoot. Fifteen minutes after Evans Blue injection, mice were euthanized and dissected for lymph node visualization and quantitation (Harrell et al., 2008). Any sign of a lymph node, regardless of size, was counted. To quantitate Peyer’s patches, the small intestine was divided into proximal, middle, and distal segments, and quantitated visually. For adoptive transfer studies, splenocytes were isolated from adult WT mice and single cell suspensions were obtained after disruption of tissue through a 70-µm filter. After erythrocyte lysis in ACK buffer, splenocytes were washed and counted. 5 million bulk splenocytes were transferred into Rag1–/– STING N153S or Rag1–/– animals via intravenous injection. Mice were euthanized 3.5 weeks later, and Evans Blue staining was used to aid in quantitation of lymph nodes and Peyer’s patches.

**Inguinal fat pad and intestine histology**

Inguinal fat pads (and attached abdominal skin) were harvested at the bifurcation of the superficial epigastric vein (approximately 1 cm diameter). Small intestines were removed and flushed with PBS to remove any fecal matter. Tissues were fix in 4% paraformaldehyde for 24 hours and then embedded in paraffin. Inguinal fat pads were sequentially sectioned with 20 sectioned analyzed per mouse. Small intestines were serially sectioned with at least 48 sections analyzed per mouse.

**Quantitation of IgA**

IgA levels in the serum and stool were determined using a commercial mouse IgA ELISA kit (Immunology Consultants Laboratory catalog no. E-90A) according to the manufacturer’s protocol. All fecal samples were weighed and then suspended in sterile PBS at 100 µL per 10 mg, vortexed, and then spun down and supernatant transferred to new tube. Samples were diluted 1:300 and then analyzed.

**Splenic stromal cell analysis**

Isolation of follicular dendritic cells (FDC), fibroblastic reticular cells (FRC), and endothelial cells was performed as previously described (Sato et al., 2016). Briefly, spleens were harvested and digested using a cocktail of Collagenase D, DNase I, and
Dispase I. Digested spleens were filtered, washed, and then enriched for FDCs and FRCs by negatively selecting for CD45 −B220− Ter119− cells. Cells were washed and then stained with antibodies against CD45, CD19, CD31 (clone 390), PDPN (clone eBio8.1.1), CD54 (clone 3e2), and CD21/35 (clone 7e9).

**Gene expression analysis**

Total RNA from spleen homogenates or MEFs was isolated using the RNaseasy kit (QIAGEN) per the manufacturer’s protocol. TaqMan RNA-to-Ct 1-Step kit (Applied Biosystems) was used to measure mRNA expression. Primer and probe assays were obtained from Integrated DNA Technologies. ∆∆Ct values were calculated and then normalized to the housekeeping gene (GAPDH). Samples where the target gene did not amplify were assigned a CT value normalized to the housekeeping gene (GAPDH). Samples where the target gene did not amplify were assigned a CT value of 38 as the limit of detection.

**Noncanonical NF-κB signaling in MEFs**

For studies of noncanonical NF-κB signaling, primary MEFS were stimulated with 2 μg/ml of anti-LTαR antibody (clone eBio3C8) for 24 hours. Cells were then harvested and analyzed by western blot or qRT-PCR.

**SDS-PAGE and western blot**

Primary MEFS were lysed in RIPA buffer (CST, catalog no. 9806S) supplemented with a protease inhibitor (Thermo Fisher, catalog no. 78430) and phosphatase inhibitor (Thermo Fisher, catalog no. 88667). An equal amount of protein was loaded and separated on 10% SDS-PAGE gels (Bio-Rad), then transferred to polyvinylidene fluoride membranes (EMD Millipore). Primary antibody against RelB (clone C1E4), IκBα (clone L34A5), and GAPDH were stained and detected by the use of horseradish-peroxidase-conjugated secondary anti-rabbit antibody (Invitrogen, catalog no. 31460). All the blots were performed using PierceTM ECL Substrate (Thermo Fisher Scientific) and scanned with a ChemiDocTM Touch Imaging System (Bio-Rad).

**Flow cytometric analysis of leukocytes**

For analysis of intestinal leukocytes, small intestines were harvested, flushed, and opened longitudinally, and Peyer’s patches were removed. Epithelial cells were removed via two rounds of gentle agitation with HBSS containing 10% FBS, 150 mM HEPES, and 5 mM EDTA. Tissues were washed with HBSS and digested with Collagenase IV (Sigma C5138) by shaking in a 37°C incubator for 40 minutes. Digested tissues were filtered through 100-μm filters and cellular debris removed via Percoll gradient for flow cytometric analysis. Single-cell suspensions were stained for CD45 (Invitrogen: clone 30-F11), NKP46 (Invitrogen: clone 29A1.4), CD4 (eBioscience: clone GK1.5), CCR6 (BD Biosciences: clone 140706), and lineage (Lin) markers, which consist of CD3ε (Biolegend: clone 145-2C11), CD5 (Biolegend: clone 53-7.3), CD19 (Biolegend: clone 6D5). Intracellular staining for RORγt (eBioscience: clone AFKJS-9) and GATA3 (BD Biosciences: clone L50-823) was performed according to eBioscience’s Intracellular Fixation and Permeabilization kit (Thermo Fischer Scientific: catalog no. 88-8824-00) before analysis on a Fortessa X-20. For analysis of bone marrow leukocytes, femurs and tibias of adult mice were collected and flushed with PBS for collection of bone marrow. Bone marrow was filtered, red blood cells lysed, and remaining cells were washed and resuspended for FACS analysis. Single-cell suspensions were stained with antibodies against lineage markers CD19, CD5, TER119 (Biolegend: clone TER119), B220 (Biolegend: clone RA3-6B2), CD11b (Biolegend: clone M1/70), CD11c (Biolegend: clone N418), NK1.1 (Biolegend: clone PK136), CD4 (Biolegend: clone Gk1.5), CD3ε, CD8α (Biolegend: clone S3-6.7), GR1 (Biolegend: clone RB6-8C5), Ly6G (Biolegend: clone 1A8), TCRγδ (Biolegend: clone GL3), CD45, CD127 (Biolegend: clone A7R34), FLT3 (Biolegend: clone A2F10), cKIT (Biolegend: clone ACK2), CD25 (Biolegend: clone 3C7), α4β7 (Biolegend: clone DATK32), PLZF (Invitrogen: clone Mags.21F7), ID2 (Invitrogen: clone ILCID2), CD27 (Invitrogen: clone LG.7F9), CD244 (Invitrogen: clone eBio244F4), CD122 (Invitrogen: clone TM-b1), and fixable live dead stain (Biolegend catalog no. 423105 or Thermo Fisher catalog no. L34965).

**Isolation of fetal gut and fetal liver cells**

Fetal livers were harvested at E13.5-14.5 and mechanically dissociated using a 1 mL pipette before being passed through a 70-μm filter. Cells were washed and resuspended for flow cytometric analysis. Fetal gut cells were isolated as described previously (Bando et al., 2015). Briefly, mouse fetuses were harvested at E16.5-18.5, and the small intestine was harvested. Intestinal tissue was minced and then digested in complete RPMI media and collagenase at 37°C for 25 minutes while shaking at 200 rpm. Following digestion, the tissue was dissociated in Gentlemacs tubes (Miltenyi Biotec catalog no. 130-096-334). Dissociated tissue was filtered through a 70-μm filter into a 50 mL conical tube and cell suspension was washed with cold FACS buffer (4% FBS in PBS). For in vivo BrdU labeling of fetal gut cells, 0.1 mg/gram of body weight BrdU was injected into a pregnant dam. Two-hours post BrdU injection, fetal livers were harvested as described above.

**LTi cell differentiation assays**

In vitro LTi cell differentiation was described previously (Cherrier et al., 2012). Briefly, one day prior to sorting, OP9 cells were plated into 24-well plates at a density of 3.0 × 10⁴ cells per well. Fetal livers were harvested on E13.5-E14.5, and single-cell suspensions were obtained. Negative selection was performed to remove CD3+, Ter119+, Gr-1+, and CD11c+ cells, and then CD3−CD19 B220 Gr1−CD45+ cKIT++CD127−α4β7+ cells were sorted and 1000 fetal liver progenitor cells were plated onto OP9
stromal cells along with 10 ng/µl of rSCF (Peprotech catalog no. 250-03) and rIL-7 (Peprotech catalog no. 217-17). A half-media change was performed on days 4 and 11. Cells were passaged onto new OP9 stromal cells on day 7. Analysis by flow cytometry was performed 6 or 14 days after sorting and co-culture.

**Single-cell RNA-seq**

E14.5 fetal liver cells were prepared according to the established protocols that have been previously described for LTi cell isolation. Single Lin^–^CD45^+^cKIT^mid^CD127^−^CD4^+^CD7^+^ cells were sorted directly into a 96-well plate (one cell per well) containing 2 µL of 10x lysis buffer (Takara catalog no. 635013) and 5% RNase inhibitor (Promega catalog no. PRN2611). After sorting, plates were immediately frozen at −80°C. Single-cell RNA sequencing of each well was performed at the Genome Technology Access Center (GTAC) at Washington University in St. Louis. Data are available at Mendeley DOI: https://dx.doi.org/10.17632/9nck2z26tf.1

**Flow cytometric analysis of human PBMCs**

Isolation of human PBMCs was performed using a Ficoll-Paque gradient. Cells were stained for the following markers: CD159a (NKG2A) (Miltenyi Biotec: VioBright FITC, clone REA110), CD294 (CRTH2) (BioLegend: PE-Dazzle594, clone BM16), CD127 (IL-7R) (Thermo Fisher: PE-Cy7, clone eBioRDR5), CD7 (BD Biosciences: Alexa Fluor 700, clone M-T701), CD94 (Miltenyi Biotec: APC-Vio770 clone REA113), CD117 (c-Kit) (BioLegend: BV605, clone 104D2), CD16 (BioLegend: BV605, clone 3G8), CD56 (BioLegend: BV785, clone 5.1H11), CD2 (BD Biosciences: BUV395, clone RPA-2.10), CD45 (BD Biosciences: BUV805, clone HI30), and lineage markers consisting of CD3 (BD Biosciences: BUV737, clone UCHT1), CD5 (BD Biosciences: BUV737, clone UCHT2), CD14 (BD Biosciences: BUV737, clone M5E2) and CD19 (BD Biosciences: BUV737, SJ25C1). Human serum IgG was used to block Fc receptors (Sigma-Aldrich). Surface membrane staining was performed using Brilliant Stain Buffer (BD Biosciences). Dead cells were excluded using the fixable viability dye eFluor506 (Thermo Fisher). Cells were fixed with 2% PFA prior to acquisition on a BD LSRFortessa (BD Biosciences) and subsequent analysis with FlowJo 10 (BD Biosciences).

**Histopathology of SAVI patient lymph nodes**

During lung transplantation, mediastinal lymph nodes from explanted lungs were retrieved and analyzed. Biopsies were fixed in 10% neutral buffered formalin, embedded in paraffin and stained with hematoxylin-eosin. Immunohistochemical staining was performed on an automated stainer (Bond Max; Leica Biosystems).

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Unless otherwise specified, all data were analyzed using GraphPad Prism software by Mann-Whitney or unpaired t test as specified in the figure legends. Flow cytometry data were analyzed using Cytobank or FlowJo v10.4.1.
Supplemental Information

STING Gain-of-Function Disrupts

Lymph Node Organogenesis

and Innate Lymphoid Cell Development in Mice

Figure S1. Quantitation of lymph nodes after adoptive transfer and flow cytometric analysis of ILCs in WT and STING N153S animals. Related to Figure 1. (A) Representative photographs of Rag1−/− (left panel) and Rag1−/− STING N153S (right panel) animals following adoptive transfer of WT splenocytes. (B) Total number of discernible inguinal, brachial, axillary, and mesenteric lymph nodes and Peyer’s patches after adoptive transfer of WT splenocytes. Data represent the mean of 9 mice per genotype. Results were analyzed by Mann-Whitney test. (C-E) Flow cytometric analysis on splenocytes from WT and STING N153S littermate mice. (C) Representative FACS plots depicting ILC1 and NK cell populations gated on CD45+CD3−B220− splenocytes. Total number of ILC1s (D) and NK cells (E) in the spleens of STING N153S and WT mice. Data were collected from 8 mice per genotype. Results were analyzed by unpaired t test. (F-M) Bone marrow was collected from adult mice. Representative FACS plots of Lin−CD127+ bone marrow cells (F) from WT (top panels) and STING N153S (bottom panels) littermate mice. Lineage markers for bone marrow ILC progenitor stains include: TER119, B220, CD19, CD11b, CD11c, NK1.1, CD4, CD3ε, CD8α, GR1, Ly6G, and TCRγδ. Total number of bone marrow cells recovered (G). Total numbers of αLP cells (Lin−CD127+α4β7+FLT3+CD25−) (H), CHILP1 cells (Lin−CD127+α4β7+FLT3−CD25+ID2−PLZF−) (I), and CHILP2 cells (Lin−CD127+α4β7+FLT3−CD25+ID2−PLZF+) (J). Representative FACS plots (K) of WT (left panel) and STING N153S (right panel) CD3− CD19−NK1.1−CD11b−CD244+CD27+ cKit+CD127+ cells. Total numbers of Pre-NKp (L) and Refined NKp cells (M). n = 9 mice per genotype and data were analyzed by unpaired t test. All data were generated from at least 2 independent experiments. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.
Table S1. STING N153S lymph node deficiency in mice lacking downstream effectors of STING, the type I IFN receptor (IFNAR1), and cGAS. Related to Figure 1. STING N153S mice were crossed to *Ifnar1*^−/−*, *Irf3*^−/−*Irf7*^−/−*, or *cGas*^−/−* animals. Adult STING N153S animals deficient for the indicated genes were sacrificed and examined for visual evidence of mesenteric, inguinal, brachial, and axillary lymph nodes and Peyer’s patches. Quantitation of the number of discernible lymph nodes compared to littermate controls (e.g., control animals expressing WT STING). Number of Peyer’s patches are reported as the mean number observed per mouse. Data were collected from 3-6 mice per genotype in at least 2 independent experiments.

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Figure S2. Splenic organization, but not noncanonical NFκB signaling, is perturbed in STING N153S mice. Related to Figure 1. (A) H&E staining of paraffin embedded WT (left panel) and STING N153S (right panel) spleen sections. (B) MADCAM1 expression (green) in WT (left panel) and STING N153S (right panel) OCT embedded spleen sections (5µm). (C) B220 (red) and CD3 (green) expression in WT (left panel) and STING N153S (right panel). Scale bar equals 100 µm. Images are representative of 3 images collected from 3 mice per genotype. (D) Gating strategy for splenic stromal cell populations. (E-G) FACS quantitation of the percent and number of follicular dendritic cells (E), fibroblastic reticular cells (F) and endothelial cells (G). Data represent the mean of 6 mice per genotype. (H) mRNA was isolated from spleen homogenates, and gene expression was quantitated by qRT-PCR for Ccl19, Ccl21, and Cxcl13. Data represent the mean of 8 spleens per genotype. Data in (E-G) were analyzed by unpaired t test and data in (H) were analyzed by Mann-Whitney test. (I-M) STING N153S and WT littermate primary MEFs were stimulated with 2 µg/ml anti-LTβR antibody for 24 hours followed by SDS-PAGE, Western blot, or RNA isolation and qRT-PCR analysis. Representative Western blot (I) analysis of IκBα (top panel), RelB (middle panel, top band) and GAPDH from 3 independent generated primary MEF lines per genotype. qRT-PCR analysis of gene expression levels in primary MEFs (J-M). Data represent the mean of 6 samples per genotype and are reported as the fold change relative to control-treated MEFs. All data were pooled from at least 2 independent experiments. Results were analyzed by Mann-Whitney test. *, P < 0.05; **, P < 0.01; ****, P < 0.0001.
Figure S3. Single-cell RNA-seq analysis of type I IFN-stimulated gene and LTi associated gene expression in WT and STING N153S α4β7⁺ cells. Related to Figure 3. Fetal livers from WT and heterozygous STING N153S animals were harvested on E14.5, and α4β7⁺ cells underwent single-cell FACS sorting into 96-well plates, followed by RNA sequencing. (A) Indicated type I IFN-stimulated genes and (B) LTi cell-associated genes as measured by FACS-seq analysis of WT and STING N153S α4β7⁺ progenitor cells. Data represent the mean number of counts per gene from 48 cells per genotype performed as a single gene expression screen. Results were analyzed by Mann-Whitney test.
Figure S4. STING N153S fetal liver α4β7+ progenitor cells do not efficiently differentiate into LTI cells after 14 days in an OP9 cell culture system. Related to Figure 4. α4β7+ progenitor cells from the fetal liver were co-cultured with OP9 stromal cells, SCF, and IL-7. Cells were allowed to differentiate for 14 days and analyzed by FACS. (A) Representative FACS plots of adult WT (left panels) and STING N153S (right panels) CD45+CD3-CD19- cells. Cell frequencies within each gate are denoted in red and cell population names are labeled in blue. (B) Average frequencies of ILC and α4β7+ cell populations. (C-H) Percent and number of ILC1 (C), NK cells (D), ILC2 (E), ILC3 (F), LTI-like cells (G), and Lin-CD45+ cells (H). Data represent the mean of 8-15 replicates per group pooled from at least 2 independent experiments. Results were analyzed by unpaired t test. **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.
Figure S5. Apoptosis and proliferation in WT and STING N153S LTi and LTi progenitor cells. Related to Figure 4. (A-D) Flow cytometric analysis of fetal liver cells from E13.5-E14.5 STING N153S and WT fetuses. Representative dot plots gated on Lin (CD3, CD5, and CD19) CD45+cKIT<sup>+</sup>CD127<sup>+</sup>α4β7<sup>+</sup> cells (A). Percent and number of live annexin V<sup>+</sup>CD127<sup>+</sup>α4β7<sup>+</sup> cells (B). Percent and number of live annexin V<sup>-</sup>CD127<sup>+</sup>α4β7<sup>+</sup> cells (C). Percent and number of BrdU<sup>+</sup>CD127<sup>+</sup>α4β7<sup>+</sup>RORγT<sup>+</sup> (LTi) cells (D). Data represent the mean of 6-12 mice per group pooled from at least 2 independent experiments. (E-G) α4β7<sup>+</sup> progenitor cells from the fetal liver were co-cultured with OP9 stromal cells, SCF, and IL-7. Cells were allowed to differentiate for 6 days and analyzed by FACS. Representative dot plots gated on total cells (E). Percent of annexin V<sup>+</sup> live (F) and annexin V<sup>-</sup> dead (G) cells from total cells analyzed. Data represent the mean of 5-9 mice per group pooled from 2 independent experiments. Results were analyzed by unpaired t test. *, P < 0.05; **, P < 0.01; ***, P < 0.001.
Figure S6. SAVI patient lymph node histology. Related to Figure 5. Representative images of SAVI patient lymph nodes stained with (A) hematoxylin & eosin, or (B) anti-CD20.
Figure S7. Flow cytometric analysis of double negative T cells in the thymus as well as B cells and myeloid cells in the spleens of RORγt-Cre^+ floxed-STOP STING N153S mice. Related to Figure 6. (A-D) Percent and number of DN1 (A), DN2 (B), DN3 (C), and DN4 cells (D) in the thymus of floxed-STOP STING N153S and RORγt-Cre^+ floxed-STOP STING N153S mice. Flow cytometric analysis of splenocytes. (E) Percent and total number of CD45^+ cells. (F-H) Percent and number of B220^+ B cells (F), Ly6G^+ neutrophils (G) and CD11b^+ inflammatory monocytes (H). (I) Representative FACS plots of floxed-STOP STING N153S (left panels) and RORγt-Cre^+ floxed-STOP STING N153S (right panels) CD45^+Ly6G^- cells. (J) Percent and number of CD11c^+ dendritic cells. Data represent the mean of 5 mice per group pooled from 2 independent experiments. Results were analyzed by unpaired t test. *, P < 0.05; ****, P < 0.0001.