Deficiency of the innate immune adaptor STING promotes autoreactive T cell expansion in NOD mice

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Abstract

Aims/hypothesis Stimulator of IFN genes (STING) is a central hub for cytosolic nucleic acid sensing and its activation results in upregulation of type I IFN production in innate immune cells. A type I IFN gene signature seen before the onset of type 1 diabetes has been suggested as a driver of disease initiation both in humans and in the NOD mouse model. A possible source of type I IFN is through activation of the STING pathway. Recent studies suggest that STING also has antiproliferative and proapoptotic functions in T cells that are independent of IFN. To investigate whether STING is involved in autoimmune diabetes, we examined the impact of genetic deletion of STING in NOD mice.

Methods CRISPR/Cas9 gene editing was used to generate STING-deficient NOD mice. Quantitative real-time PCR was used to assess the level of type I IFN-regulated genes in islets from wild-type and STING-deficient NOD mice. The number of islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP)₂₀₆₋₂₁₄-specific CD8⁺ T cells was determined by magnetic bead-based MHC tetramer enrichment and flow cytometry. The incidence of spontaneous diabetes and diabetes after adoptive transfer of T cells was determined.

Results STING deficiency partially attenuated the type I IFN gene signature in islets but did not suppress insulinitis. STING-deficient NOD mice accumulated an increased number of IGRP₂₀₆₋₂₁₄-specific CD8⁺ T cells (2878 ± 642 cells in NOD.STING^{-/-} mice and 728.8 ± 196 cells in wild-type NOD mice) in peripheral lymphoid tissue, associated with a higher incidence of spontaneous diabetes (95.5% in NOD.STING^{-/-} mice and 86.2% in wild-type NOD mice). Splenocytes from STING-deficient mice rapidly induced diabetes after adoptive transfer into irradiated NOD recipients (median survival 75 days for NOD recipients of NOD.STING^{-/-} mouse splenocytes and 121 days for NOD recipients of NOD mouse splenocytes).

Conclusions/interpretation Data suggest that sensing of endogenous nucleic acids through the STING pathway may be partially responsible for the type I IFN gene signature but not autoimmunity in NOD mice. Our results show that the STING pathway may play an unexpected intrinsic role in suppressing the number of diabetogenic T cells.

Keywords CD8⁺ T cells · NOD mice · STING · Type 1 diabetes · Type 1 interferon

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Abbreviations

BMDM	Bone marrow-derived macrophage
CFSE	Carboxyfluorescein succinimidyl ester
cGAMP	Cyclic GMP-AMP
cGAS	Cyclic GMP-AMP synthase
DMXAA	5,6-Dimethylxanthenone-4-acetic acid
FOXP3	Forkhead box P3
IAA	Insulin autoantibodies
IGRP	Islet-specific glucose-6-phosphatase catalytic subunit-related protein
ILN	Inguinal lymph node

Research in context

What is already known about this subject?

- Activation of stimulator of IFN genes (STING), a hub for nucleic acid sensing, results in type I IFN production
- Type I IFN is implicated in pathogenesis of type 1 diabetes
- STING has antiproliferative and proapoptotic functions in T cells that are independent of IFN

What is the key question?

- Is activation of the STING pathway involved in diabetes pathogenesis in NOD mice?

What are the new findings?

- The type I IFN gene signature was partially attenuated by loss of STING in NOD mice
- Loss of STING did not attenuate development of autoimmunity in NOD mice
- STING-deficient NOD mice accumulated an increased number of islet-specific glucose 6-phosphatase catalytic subunit-specific CD8⁺ T cells that were able to rapidly induce diabetes after adoptive transfer

How might this impact on clinical practice in the foreseeable future?

- Our data suggest that systemic immune modulation using STING antagonism could trigger autoimmunity in susceptible individuals

IRF3	IFN regulatory factor 3
Ly-6G	Lymphocyte antigen 6 complex locus G6D
MDA5	Melanoma differentiation associated gene 5
PD-1	Programmed cell death protein-1
PDCA-1	Plasmacytoid dendritic cell antigen-1
PLN	Pancreatic lymph node
SAVI	STING-associated vasculopathy with onset infancy
SLE	Systemic lupus erythematosus
STING	Stimulator of IFN genes
TLR	Toll-like receptor
TREX-1	Three-prime repair exonuclease-1
TIGIT	T cell immunoreceptor with Ig and ITIM domains
TIM-3	T cell immunoglobulin and mucin domain containing protein 3

Introduction

Type 1 diabetes results from destruction of pancreatic beta cells by autoreactive cytotoxic T cells specific for beta cell antigens. Environmental factors have been suggested to play a pivotal role in triggering of islet autoimmunity in individuals who are genetically more susceptible to developing type 1 diabetes [1, 2]. Despite many agents being proposed, the trigger for type 1 diabetes remains unclear because the disease process starts a long time before diagnosis. Viral infections have been suggested as exogenous triggers involved in the initiation of autoimmunity in susceptible individuals [3]. Virus infection can provoke a bystander immune response via pattern-recognition receptors recognising virus-derived foreign DNA as a pathogen-

associated molecular pattern [4]. However, recent advances indicate that host-derived DNA can act as a danger-associated molecular pattern and also elicit a self-inflammatory response [5, 6]. In both cases, intracellular sensing of nucleic acid is required to stimulate these immune responses.

Stimulator of IFN genes (STING) is a central hub for cytosolic nucleic acid sensing in innate immunity and plays an important role in viral and tumour immunity [7]. STING is widely expressed in haematopoietic, endothelial and epithelial cells. A range of reports has focused on the function of STING in antigen-presenting cells during virus infection and tumour development [8]. In the cells responsible for innate immunity, pathogen- or tumour-delivered cytosolic nucleic acids stimulate a cyclic GMP-AMP synthase (cGAS)–cyclic GMP-AMP (cGAMP)–STING signalling cascade that activates both NF- κ B and IFN regulatory factor 3 (IRF3) transcription pathways to induce expression of type I IFN [9]. Sensing of aberrant cytosolic DNA due to loss of function of DNA degradation enzymes, such as three-prime repair exonuclease-1 (TREX-1), can cause type I IFN-mediated autoinflammatory syndromes such as Aicardi–Gouttieres syndrome [10]. Gain-of-function mutations in STING can exacerbate inflammation, causing systemic vasculitis with lupus-like syndrome in humans that is characterised by increased type I IFNs and inflammatory cytokines (STING-associated vasculopathy with onset infancy [SAVI]) [11]. These reports suggest that dysregulated type I IFN production via the cGAS–cGAMP–STING pathway can contribute to the development of autoimmunity.

Recently, we generated NOD mice that were deficient for granzyme A, a trypsin-like serine protease that activates nucleases involved in the degradation of nucleic acids in the

cytoplasm. These mice exhibited an increased diabetes incidence associated with accumulation of cytosolic DNA and increased type I IFN responses [12]. Transient elevation of a type I IFN-induced gene signature is a feature of early type 1 diabetes. An elevated type I IFN gene signature precedes autoimmunity in peripheral blood mononuclear cells of children genetically at risk of type 1 diabetes [13] and an increased type I IFN gene signature is evident in the islets of NOD mice at 3–4 weeks of age, before the infiltration of T cells [14, 15]. Type I IFN has therefore been postulated to be causative in type 1 diabetes pathogenesis by stimulating the immune system, beta cells and their milieu for initiation of islet autoimmunity. We hypothesise that the STING pathway is involved in sensing endogenous aberrant DNA, thereby increasing type I IFN production and type I IFN responses.

Although the role of STING in adaptive immunity is not fully elucidated, recent advances suggest that STING also has antiproliferative and proapoptotic functions in T cells that are T cell intrinsic and IFN independent [16–20]. A constitutively active STING mutation decreased proliferation and reduced T cell viability due to a disturbance of Ca^{2+} influx and increased endoplasmic reticulum stress upon T cell receptor stimulation in an IFN-independent manner [18, 19]. This recently described role for STING suggests that it may affect the progression of autoimmune diabetes where autoreactive T cells play a dominant role. To elucidate directly whether and how STING is involved in the pathogenesis of type 1 diabetes, we used CRISPR/Cas9 gene editing to generate STING-deficient NOD mice.

Methods

Ethics statement All mice were maintained at St Vincent's Institute, and all experiments were approved by the St Vincent's Health animal ethics committee (AEC approval numbers 017/17 and 018/17).

Mice A previously described method for CRISPR/Cas9 gene editing was adapted to target *Sting1* (also known as *Tmem173*), the gene that encodes STING, in the NOD mouse strain [21]. NOD/ShiLtJ (The Jackson Laboratory, USA; <https://www.jax.org/strain/001289>) embryos were co-injected with Cas9 RNA and sgRNAs that were designed to delete the genomic region encompassing exons 3, 4 and 5 (sgRNA no. 1 5'GGATGACTAGTCAGGACCTT3', sgRNA no. 2 5'TACTTGCGGTTGATCTTACC3'). G_0 offspring were screened for gene mutations by amplifying the genomic regions encompassed by the sgRNAs using gene-specific primers. DNA sequencing of the PCR amplicons revealed a 962 bp deletion (em1: Chr18: 35,738,680–35,739,641) and 956 bp deletion (em2: Chr18: 35,738,683–35,739,638). Mice with either of these mutations

were backcrossed to NOD/ShiLtJ mice, and subsequent offspring were intercrossed to establish two STING-deficient NOD mouse lines: NOD.*Sting1*^{em1/em1} and NOD.*Sting1*^{em2/em2} (henceforth, both lines referred to as NOD.STING^{−/−}). NOD.Cg-Tg(TcraTcrbNY8.3)^{1Pesa} transgenic NOD mice (NOD8.3) [22] were crossed with NOD.STING^{−/−} mice to produce STING-deficient NOD8.3 mice (NOD8.3/STING^{−/−}). Genotyping of *Sting1* alleles and the 8.3 TCR transgene was performed using DNA isolated from tail biopsies and standard PCR/gel electrophoresis methods. *Sting1* alleles were detected using two different primer combinations: primer set 1 for simultaneous detection of the deletion alleles and wild-type allele (forward GGGCGTCTCCTTGA GGTGTA; reverse AACTGTATGGCAGACATGGA AATAC) and primer set 2 for detection of the wild-type allele (forward CCTCCAAAACACTGCTGACA; reverse CACCCTTCACACAGACAGGA).

Diabetes, insulinitis and insulin autoantibodies Female and male mice were monitored for spontaneous diabetes for 300 days. Mice with two consecutive blood glucose measurements of more than 15 mmol/l were diagnosed diabetic. For histology, tissues were fixed with 10% vol./vol. neutral buffered formalin. Pancreatic sections from three levels (200 μm apart) of paraffin-embedded samples were analysed by staining with H&E. Two independent investigators scored the severity of insulinitis in a blinded manner as follows: 0, no lymphocytic infiltration; 1, peri-islet infiltration; 2, extensive peri-islet infiltration; 3, intra-islet infiltration; and 4, extensive intra-islet infiltration or total beta cell loss. Insulinitis scores were calculated by the method previously described [23]. Insulin autoantibodies (IAA) were measured by non-competitive ELISA assay using 96-well plates as previously described [24]. Each sample was tested with and without plate-bound insulin in duplicate and the absorbance value at 450 nm was calculated by subtracting the absorbance of the sample without plate-bound insulin from the absorbance of the sample with plate-bound insulin.

Islet isolation Islets were isolated using collagenase P (Roche, Basel, Switzerland) and Histopaque-1077 density gradients (Sigma-Aldrich, USA) as previously described [25].

Adoptive transfer of carboxyfluorescein succinimidyl ester-labelled 8.3 T cells CD8⁺ T cells isolated from NOD8.3 or NOD8.3/STING^{−/−} mice were labelled with carboxyfluorescein succinimidyl ester (CFSE) and suspended at a concentration of 2.5×10^7 cells/ml in PBS as previously described [23]. CFSE-labelled T cells (0.5×10^7 cells) were intravenously injected into the tail veins of recipient mice. The pancreatic lymph nodes (PLNs) and islets were harvested from the recipient mice 5 days after the transfer. Proliferation of CFSE-labelled cells was assessed by flow cytometry as described below.

To obtain dendritic cells, spleen was digested with collagenase (1 mg/ml) and DNase (0.02 mg/ml) and then treated with EDTA. Non-dendritic cells were magnetically depleted then dendritic cells were enriched using anti-CD11c (Miltenyi Biotec, Germany). CFSE-labelled CD8⁺ T cells (1×10^5 cells/well) were incubated with islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP)₂₀₆₋₂₁₄ peptide (1 μ mol/l)-coated dendritic cells (1×10^4 cells/well) in quadruplicate. Proliferation of CFSE-labelled T cells was measured by flow cytometry after 3 days of culture.

Adoptive transfer into irradiated NOD mice NOD mice, aged 8–10 weeks, received 650 rad-irradiation twice, and 20×10^6 splenocytes from non-diabetic 12- to 15-week-old NOD or NOD.STING^{-/-} donors were transferred into the irradiated mice intravenously. The recipient mice were monitored for diabetes until 150 days after the transfer.

Real-time PCR Bone marrow cells were cultured in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin, 292 mg/ml L-glutamine (Gibco, USA) and 20% L929 conditioned medium. After incubation overnight, semi-adherent cells were collected in fresh complete DMEM and incubated for 4 days to obtain a population of differentiated macrophages. Bone marrow-derived macrophages (BMDMs; 1.5×10^5) generated from NOD and NOD.STING^{-/-} mice were stimulated with STING agonist (5,6-dimethylxanthenone-4-acetic acid [DMXAA], 50 ng/ μ l) for 2 h. Total RNA was extracted using ISOLATE RNA Micro Kit (BIOLINE, Luckenwalde, Germany) and cDNA was synthesised using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Quantitative real-time PCR analysis was performed using a LightCycler 480 (Roche) and TaqMan gene expression assays for *Mx1* (probe ID: Mm00487796_m1), *Ifit1* (probe ID: Mm00515153_m1), *Oas1a* (probe ID: Mm00836412_m1), *Oas1b* (probe ID: Mm00449297_m1), *Isg15* (probe ID: Mm01705338_s1), *Cxcl10* (probe ID: Mm00445235_m1), *Ifnb1* (probe ID: Mm00439552_s1) and *Actb* (probe ID: Mm002619580_g1) (Applied Biosystems, Foster City, CA, USA). Threshold cycle (C_t) values were normalised to the C_t values of actin in individual samples and expressed as ΔC_t . Relative expression levels were then calculated as $2^{-\Delta C_t}$.

Flow cytometry Single-cell suspensions were prepared from spleen, PLN and inguinal lymph node (ILN) by mechanical disruption using the flat end of a syringe plunger. Disrupted cells were passed through a 70 μ m filter-mesh and treated with ammonium chloride buffer to lyse red blood cells. For surface staining, cells were stained with the corresponding fluorescence-labelled antibodies. The following antibodies

(clone, catalogue number) were from Biolegend, USA: CD45 (30F11,103132), CD3e (145-2C11, 100341), CD4 (GK1.5,100408), CD8 (53-6.7,100722), CD19 (6D5,115529), CD25 (3C7,101910), plasmacytoid dendritic cell antigen-1 (PDCA-1; 927,127023), CD44 (IM7,103030), CD62L (MEL-14,104408), CD40 (1C10,102805), CD80 (16-10A1,104733), programmed cell death protein-1 (PD-1; 29F.1A12,135220) and T cell immunoreceptor with Ig and ITIM domains (TIGIT; VSIG9,142110). CD45R (RA3-6B2,48-0452-82), CD11c (N418,48-0114-82), CD11b (M1/70,48-0112-82) and lymphocyte antigen 6 complex locus G6D (Ly-6G; RB6-8C5,48-5931-82) antibodies were purchased from eBiosciences, USA. CD86 (GL1,563055), MHC I (SF1-1.1,553564), MHC II (OX-6, 554928) were from BD Biosciences, USA. T cell immunoglobulin and mucin domain containing protein 3 (TIM-3; 215008,FAB1529G) was obtained from R&D systems, USA. For intracellular staining of STING and forkhead box P3 (FOXP3), cells were fixed with Foxp3/Transcription Factor Staining Buffer Set (eBioscience, USA). For intracellular cytokine staining, cells were stimulated with 50 ng/ml phorbol 12-myristate 13-acetate and 500 ng/ml ionomycin (Sigma, St Louis, MO, USA) in the presence of Golgi Plug (BD Biosciences, USA) for 4 h and then fixed with Cytofix/Cytoperm solution (BD Biosciences). The Cytofix/Cytoperm solution was also used for staining granzyme B on CFSE-labelled 8.3 T cells without re-stimulation of the cells. After the fixation and permeabilisation, cells were stained for intracellular antigens of interest; STING (clone 41), FOXP3 (FJK-16S), IFN γ (XMG1.2) and granzyme B (NGZB). Beta cells were identified by their autofluorescence as previously described [25]. IGRP-specific CD8⁺ T cells were assessed using H2-Kd tetramer with magnetic bead-based enrichment as described previously [24]. Briefly, cells from peripheral lymphoid organs (spleen, ILNs, and mesenteric lymph nodes) were stained with phycoerythrin-conjugated IGRP₂₀₆₋₂₁₄ (VYLKTNVFL) H2-Kd tetramer (ImmunoID, Parkville, Australia) and phycoerythrin-conjugated I-A^{g7}-INS_{B10-23} (HLVERLYLVCGGEG) tetramer (NIH tetramer core, Georgia, USA). Cells were stained with anti-phycoerythrin microbeads (Miltenyi Biotec) and separated using the AutoMACS pro separator (Miltenyi Biotec). Subsequently, the cells were stained for surface molecules and analysed by flow cytometry. H2-Kd-TUM (KYQAVTTTL) tetramers were used to determine background staining. Data were collected on a BD LSRFortessa (BD Biosciences) and analysed using FlowJo analysis software version 10.7.1 (FlowJo, Ashland, OR, USA).

Statistics Statistical analysis for group differences was performed using the unpaired Student's *t* test or a two-way ANOVA and statistical analysis on Kaplan–Meier estimates were performed using the logrank test by GraphPad Prism

software version 8 (GraphPad, USA). All data shown as bar graphs are represented as mean $\pm$ SD. $p < 0.05$ was considered significant.

Results

Generation of STING-deficient NOD mice To determine whether STING is involved in the pathogenesis of type 1 diabetes, we used CRISPR/Cas9 gene editing in NOD mice to disrupt *Sting1*, the gene encoding STING. PCR-based genotyping (Fig. 1a) and sequence analysis (not shown) were used to identify and establish NOD.*STING*^{−/−} mice that contained genomic deletions encompassing exon 3, 4 and 5 of *Sting1*.

We confirmed the lack of STING protein in NOD.*STING*^{−/−} mouse spleen cells by flow cytometry (Fig. 1b). To verify functional deletion of STING, we stimulated BMDMs generated from NOD and NOD.*STING*^{−/−} mice with a STING agonist (DMXAA) that triggers activation of the cGAS–cGAMP–STING pathway to upregulate *Ifnb1* and *Cxcl10* [26]. Expression of *Ifnb1* and *Cxcl10* was upregulated in BMDMs derived from wild-type NOD mice after treatment with DMXAA but was not detected in NOD.*STING*^{−/−} BMDMs (Fig. 1c,d).

STING deficiency partially attenuates expression of type I IFN-regulated genes in islets A type I IFN signature has been observed in the early phase of islet autoimmunity in human type 1 diabetes and in NOD mice [13–15, 27, 28]. STING is a central hub in nucleic acid sensing resulting in production of type I IFN, therefore, we examined the expression of type I IFN-regulated genes in islets of NOD.*STING*^{−/−} mice. If DNA sensing via STING is important in driving the type I IFN gene signature in NOD mice, we would expect the gene signature to be reduced in NOD.*STING*^{−/−} mice. We detected a lower expression of *Oas1a*, *Oas1b* and *Isg15* in islets isolated from 20-day-old NOD.*STING*^{−/−} mice compared with wild-type NOD mice, while other IFN-regulated genes, including *Ifit1*, *Mx1* and *Cxcl10*, were unaffected by loss of STING (Fig. 2a–f). Gene expression was similar when comparing islets from wild-type and *STING*^{−/−} mice aged 100 days, with the exception of *Mx1*, which was slightly reduced in the NOD.*STING*^{−/−} islets (Fig. 2g–i). These data suggest that STING-dependent nucleic acid sensing may be partly involved in increasing type I IFN gene expression in the islets of young NOD mice. However, gene expression was not completely abolished, as in NOD mice lacking *Ifnar1* [15], indicating the presence of other stimulators of type I IFN in islets.

MHC class I hyperexpression on beta cells is a hallmark of type 1 diabetes [29] and type I IFN can upregulate MHC class I expression on beta cells in vitro [15]. We observed equivalent expression of MHC class I on beta cells in NOD.*STING*^{−/−} mice compared with NOD mice at 120 days of age (Fig. 2m,n

Fig. 1 Genetic, protein and functional analysis confirm STING deficiency in NOD.*STING*^{−/−} mice. **(a)** Genomic DNA was analysed by PCR-based genotyping and agarose gel electrophoresis. Two sets of primers were used to detect the presence or absence of the genomic interval encompassing exon 3, 4 and 5 of *Sting1*. Black arrows indicate the PCR products of the wild-type allele and the white arrow indicates the mutant allele, in which the genomic interval has been deleted. The wild-type product is not efficiently amplified in the presence of the deletion allele for primer set 1, hence the design and use of primer set 2. **(b)** Spleen cells were fixed, permeabilised and stained with anti-STING antibody. Representative flow cytometry plots of NOD and NOD.*STING*^{−/−} cells are shown together with pooled data showing the proportion of STING-positive spleen cells. Data are mean $\pm$ SD for NOD ($n=5$), NOD.*STING*^{−/−} (em1, $n=3$; em2, $n=3$), *** $p < 0.0001$ for NOD.*STING*^{−/−} groups vs NOD (one-way ANOVA with Dunnett's post hoc test for multiple comparisons). **(c, d)** BMDMs were generated from wild-type or NOD.*STING*^{−/−} mice and stimulated with the STING agonist DMXAA. Relative expression levels ($2^{-\Delta C_t}$) of *Cxcl10* **(c)** and *Ifnb1* transcripts **(d)** were measured by quantitative real-time PCR. Data show mean $\pm$ SD for individual NOD ($n=2$) and NOD.*STING*^{−/−} ($n=3$) mice. NC, negative control; WT, wild-type

Fig. 2 A partial decrease in islet type I IFN gene signature does not affect antigen-presenting cell phenotypes in NOD.STING^{-/-} mice. (**a–l**) Total RNA was collected from the islets of NOD and NOD.STING^{-/-} mice at 20 days of age ($n=5$ mice/genotype) (**a–f**) and 100 days of age ($n=6$ mice/genotype) (**g–l**). Expression of type I IFN-regulated genes *Ifit1*, *Oas1a*, *Oas1b*, *Isg15*, *Mx1* and *Cxcl10* was evaluated by quantitative real-time PCR. Data show mean $\pm$ SD of ΔC_t values, with symbols showing individual mice, each performed in triplicate. For NOD.STING^{-/-} vs NOD: $*p=0.041$ for *Mx1* (**k**); $**p=0.0036$ for *Oas1a* (**b**), $p=0.0024$ for *Oas1b* (**c**) and $p=0.0017$ for *Isg15* (**d**) (Student's *t* test). (**m, n**) MHC class I expression on beta cells from 120-day-old NOD and NOD.STING^{-/-} mice as assessed by flow cytometry. Viable beta cells were identified by their high autofluorescence as described in the Methods. Representative histograms of MHC class I (**m**) and quantification of mean fluorescence intensity of MHC class I (**n**) on beta cells isolated from NOD ($n=6$) and NOD.STING^{-/-} ($n=6$) mice. Data show individual mice and the mean. No significant difference was noted

between the mouse groups (Student's *t* test). (**o, p**) Infiltrating leucocytes (PI⁺CD45⁺) from islets were analysed by flow cytometry for the expression of CD40, CD80, CD86 and MHC class II on conventional dendritic cells (CD3⁻CD19⁺PDCA-1⁻CD11c⁺). Representative histograms (**o**) and quantification of mean fluorescence intensity of CD40, CD80, CD86 and MHC class II (**p**) on cDC in islets of NOD ($n=5$) and NOD.STING^{-/-} ($n=6$) mice. Data show individual mice and the mean. No significant difference was noted between the mouse groups (two-way ANOVA with Sidak's multiple comparison test). (**q**) The proportion of CD45⁺ cells that were conventional dendritic cells (CD3⁻CD19⁺PDCA-1⁻CD11c⁺) and plasmacytoid dendritic cells (CD3⁻CD19⁺PDCA-1⁺CD11c⁺) in the islets of 120-day-old female NOD ($n=6$) and NOD.STING^{-/-} ($n=6$) mice. Data show mean and individual mice. No significant difference was noted between the mouse groups (two-way ANOVA). cDC, conventional dendritic cells; MFI, mean fluorescence intensity; pDC, plasmacytoid dendritic cells; PI, propidium iodide

and electronic supplementary material [ESM] Fig. 1a). This is consistent with our previous finding that IFN γ , and not type I IFN, primarily regulates MHC class I on beta cells in vivo in NOD mice [15, 30].

Conventional dendritic cells play a major role in presenting autoantigens to diabetogenic T cells, and local type I IFN production in inflamed islets could affect their maturation. No differences in the expression of CD40, CD80, CD86 and MHC class II on conventional dendritic cells were detected in NOD.STING^{-/-} and NOD mice at 120 days of age (Fig. 2o,p and ESM Fig. 1b) and there was no difference in the number of conventional or plasmacytoid dendritic cells (Fig. 2q). STING^{-/-} antigen-presenting cells efficiently stimulated islet

antigen-specific CD8⁺ T cells in vitro (ESM Fig. 2a). These data indicate that STING deficiency partially decreases the type I IFN signature in islets but does not attenuate antigen-presenting cell phenotype or function.

STING deficiency promotes spontaneous diabetes in NOD mice We next determined whether STING deficiency impacts spontaneous diabetes. Female NOD.STING^{-/-} mice (from the em2 line) had a significantly higher incidence of spontaneous diabetes than female wild-type NOD mice (Fig. 3a). The cumulative incidence of spontaneous diabetes at 300 days of age was 95.5% in the NOD.STING^{-/-} em1 line, 93.7% in the NOD.STING^{-/-} em2 line and 86.2% in wild-type NOD mice.

Fig. 3 STING deficiency increases the incidence of diabetes in NOD mice with equivalent severity of insulinitis. **(a)** Cumulative incidence of spontaneous diabetes in female wild-type NOD mice ($n=55$) compared with STING-deficient NOD mice (em1, $n=49$; em2, $n=54$); $p=0.045$ (logrank test with correction for multiple testing). **(b)** Cumulative incidence of diabetes after adoptive transfer of splenocytes from non-diabetic 12- to 15-week-old NOD or NOD.STING^{-/-} mice into 8- to 10-week-old recipient irradiated NOD mice ($n=20$ recipients of NOD cells and $n=23$ recipients of NOD.STING^{-/-} cells pooled from $n=3$ independent experiments); $p=0.0006$ (logrank test). **(c)** Representative H&E-stained sections of pancreas harvested from 100-day-old female NOD and NOD.STING^{-/-} mice. Arrows show insulinitis. Magnification $\times 100$; scale bar, 100 μm . **(d)** Combined insulinitis scores of female NOD and NOD.STING^{-/-} mice at 30, 100 and 150 days of age. The insulinitis score for each mouse was calculated as described in the Methods. Data show the scores of three levels of the pancreas from individual mice and mean of $n=5$ –15/group; no significant

difference between groups was noted (two-way ANOVA with Sidak's multiple comparison test). **(e)** The percentage of CD45⁺ cells in islets cells isolated from NOD and NOD.STING^{-/-} mice at 84 and 120 days of age as determined by flow cytometry. Data are presented for individual mice and as mean of $n=6$ /group performed in three independent experiments; no significant difference was noted between groups (two-way ANOVA with Sidak's multiple comparison test). **(f, g)** The proportion of CD3⁺CD45⁺ cells **(f)** and CD4⁺CD3⁺ and CD8⁺CD3⁺ T cells **(g)** in the islets of 120-day-old female NOD ($n=6$) and NOD.STING^{-/-} ($n=5$ or 6) mice. Data are presented as means and for individual mice pooled from three independent experiments. No significant difference was noted between groups (Student's t test). **(h)** Absorbance (at 450 nm) for IAA of serum from individual NOD ($n=15$) and NOD.STING^{-/-} ($n=10$) mice at 84 days of age measured by ELISA. Each sample was assayed in duplicate. Data are presented for individual mice and as mean. No significant difference was noted between groups (Student's t test)

Diabetes incidence was also greater in male mice, with an incidence of 69.2% in the NOD.STING^{-/-} em2 line and 51.2% in NOD mice ($p=0.02$, logrank test). To confirm whether STING deficiency enhances the autoimmune pathogenicity of immune cells, splenocytes from NOD and NOD.STING^{-/-} mice were adoptively transferred into irradiated wild-type NOD mice. STING-deficient splenocytes transferred diabetes earlier than wild-type NOD splenocytes (median survival 75 days for NOD recipients of

NOD.STING^{-/-} mouse splenocytes and 121 days for NOD recipients of NOD mouse splenocytes) (Fig. 3b).

We assessed the level of insulinitis and development of IAA. Despite the increased diabetes incidence, NOD.STING^{-/-} mice displayed a similar severity of insulinitis at 30, 100 and 150 days of age compared with wild-type NOD mice (Fig. 3c,d). Flow cytometric analysis confirmed that NOD.STING^{-/-} mice had a similar frequency of CD45⁺ cells in the islets at 84 and 120 days of age, and the proportion of

CD3⁺CD45⁺ cells, CD4⁺CD3⁺ and CD8⁺CD3⁺ cells was equivalent at 120 days of age, when compared with wild-type NOD mice (Fig. 3e–g). There was no significant difference in levels of IAA when comparing NOD and NOD.STING^{-/-} mice at 84 days of age (Fig. 3h), indicating normal B cell autoantibody-producing function.

STING deficiency promotes generation of autoreactive T cells in NOD mice Autoimmune diabetes in NOD mice results from autoreactive T cell-mediated destruction of beta cells. IGRP-specific CD8⁺ T cells comprise a large proportion of the antigen-specific T cells in NOD mice and have also been detected in human type 1 diabetes [31, 32]. A recent report suggested that STING has an intrinsic role in repression of T cell proliferation [18]. Therefore, we hypothesised that STING deficiency may promote diabetes development by promoting the expansion of autoreactive T cells. To test this hypothesis, we tracked the number of IGRP₂₀₆₋₂₁₄-specific CD8⁺ T cells in the peripheral lymphoid tissues in NOD mice using MHC tetramer enrichment [31]. NOD.STING^{-/-} mice had significantly increased numbers of IGRP₂₀₆₋₂₁₄ tetramer⁺ CD8⁺ T cells (2878 ± 642 cells in NOD.STING^{-/-} mice and 728.8 ± 196 cells in wild-type NOD mice) in their peripheral lymphoid tissues compared with wild-type NOD mice (Fig. 4a,b). The IGRP₂₀₆₋₂₁₄ tetramer⁺ CD8⁺ T cells in peripheral lymphoid tissues of NOD.STING^{-/-} mice had a memory phenotype with high levels of CD44 expression, while the number of the CD62L-high naive cells was equivalent to that of wild-type NOD mice (Fig. 4c,d). We saw no difference in the number of INS_{B10-23}-specific CD4⁺ T cells in NOD.STING^{-/-} and wild-type mouse tissues (Fig. 4e).

To test the function of STING^{-/-} cells, CD8⁺ T cells were transferred from NOD8.3 or NOD8.3/STING^{-/-} mice, which have >90% CD8⁺ T cells that express the 8.3 TCR recognising IGRP₂₀₆₋₂₁₄. Equivalent levels of the effector molecule granzyme B were expressed in wild-type and STING^{-/-} CD8⁺ T cells (Fig. 4f). STING deficiency did not affect the proportion of T cell subsets including naive T cells, memory T cells and regulatory T cells. The total number of mononuclear cells in lymphoid tissues was also unaffected (ESM Fig. 2b–e). The proportion of IFN γ -producing CD4⁺ and CD8⁺ T cells in islets and the expression of exhaustion markers PD-1, TIGIT and TIM-3 in islet-infiltrating CD8⁺ T cells was the same for NOD.STING^{-/-} and NOD mice at 120 days of age (Fig. 4g,h). These data suggest that STING deficiency increases the number of autoreactive memory CD8⁺ T cells but does not affect their activation and effector function and does not affect the number of autoreactive CD4⁺ T cells.

Discussion

In individuals born with genetic susceptibility, autoimmune disease such as type 1 diabetes can be triggered by

environmental factors, such as viral and bacterial infection and early exposure to dietary constituents [1, 2]. Because expression of type I IFN-regulated genes is increased in the early phase of autoimmunity in NOD mice and individuals with type 1 diabetes [13–15], it has been suggested that type I IFN may change the islet microenvironment, allowing a break in tolerance in genetically susceptible individuals. The trigger for increased expression of type I IFN-regulated genes remains unknown. Using CRISPR/Cas9 gene editing, we generated NOD mice deficient in the nucleic acid adaptor protein STING to test the hypothesis that preventing sensing of endogenous nucleic acids would reduce the type I IFN gene signature in the islets of NOD mice.

We saw transient partial reduction in the type I IFN gene signature in the islets of STING-deficient mice around the onset of autoimmunity, suggesting that nucleic acid sensing via the cGAS–STING pathway may only partially account for the gene signature previously reported. Recent discoveries have revealed monogenic mutations that enhance endogenous nucleic acid sensing via pattern-recognition receptors and cause autoinflammatory syndromes as a result of constitutive type I IFN production and an inflammatory self-directed immune response [33]. In autoimmune disease, genetic susceptibility loci for systemic lupus erythematosus (SLE) were identified that encompass genes coding for the nucleic acid sensors Toll-like receptor (TLR) 7 and melanoma differentiation associated gene 5 (MDA5) [34, 35], and genes involved in the regulation of endogenous nucleic acid sensing such as DNase1, DNase1L3, RNase H2 and TREX1 [36–39]. Polymorphisms in *IFIH1*, the gene encoding the double-stranded RNA sensor MDA5, are associated with type 1 diabetes [40]. Haplo-deficiency of MDA5, a model for the type 1 diabetes-associated polymorphism, attenuated insulinitis and abolished diabetes in a virus-triggered model for autoimmune diabetes [41]. However, we identified no inhibitory effect of STING deficiency on the progression of autoimmune diabetes and immune homeostasis in NOD mice. These data suggest that sensing of endogenous nucleic acids via the cGAS–STING pathway is dispensable for the development of spontaneous autoimmune diabetes in NOD mice. The IFN gene signature was not completely abolished in the islets of STING-deficient mice, which suggests that other pathways for nucleic acid sensing, such as through TLR7, TLR9, myeloid differentiation primary response 88 (MyD88) or MDA5, might be relevant to the signature as their genetic deletion resulted in protection from autoimmune diabetes in NOD mice [41, 42]. Despite evidence for an increase in a type I IFN gene signature in humans and NOD mice, deficiency of receptors for type I IFN (IFNAR1) either resulted in no effect or partial protection from diabetes in female NOD mice depending on the animal facility [15, 43]. This is consistent with our current data suggesting that the type I IFN gene signature is not directly tied to insulinitis development. While

Fig. 4 Increased autoreactive T cells in NOD.STING^{-/-} mice. **(a–d)** IGRP₂₀₆₋₂₁₄ tetramer, CD8, CD44 and CD62L staining in peripheral lymphoid organs of 84-day-old NOD and NOD.STING^{-/-} mice. Representative staining of IGRP tetramer⁺CD8⁺ and CD44⁺IGRP tetramer⁺ cells are shown **(a)**, with quantification of IGRP₂₀₆₋₂₁₄ tetramer⁺ CD8⁺ T cells **(b)**, and CD44⁺ **(c)** and CD62L⁺ IGRP₂₀₆₋₂₁₄ tetramer⁺ CD8⁺ T cells **(d)**. Data show individual values and mean for NOD ($n=17$ –25) and NOD.STING^{-/-} ($n=10$ –28) mice. $**p=0.001$ and $***p=0.0006$ (Student's t test). **(e)** Quantification of INS_{B10-23} tetramer⁺CD4⁺ T cells in peripheral lymphoid organs of 84- to 120-day-old NOD and NOD.STING^{-/-} mice. Data show individual values and mean for NOD ($n=12$) and NOD.STING^{-/-} ($n=13$) mice. No significant difference was noted between groups (Student's t test). **(f)** Mean fluorescence intensity of granzyme B expression on CD8⁺ T cells from

ILNs, PLNs and islets of wild-type NOD8.3 and NOD8.3/STING^{-/-} mice on day 5 after adoptive transfer into NOD or NOD.STING^{-/-} recipient mice. No significant difference was noted between NOD8.3 and NOD8.3/STING^{-/-} in each tissue or recipient (two-way ANOVA). **(g)** The proportion of IFNγ-producing CD4⁺ and CD8⁺ T cells in islets from NOD ($n=5$) and NOD.STING^{-/-} ($n=4$) mice after stimulation with phorbol 12-myristate 13-acetate and ionomycin. Data are shown for individual mice and as mean. No significant difference was noted between groups (two-way ANOVA). **(h)** Mean fluorescence intensity of PD-1, TIGIT and TIM-3 on CD8⁺ T cells infiltrating into islets of 120-day-old NOD ($n=6$) and NOD.STING^{-/-} ($n=5$) mice. Data are shown for individual mice and as mean. No significant difference was noted between groups (two-way ANOVA). MFI, mean fluorescence intensity

diabetes in NOD mice is not dependent on virus infection, there is evidence for a possible viral aetiology in human type 1 diabetes [1, 2]. STING–cGAS signalling is required for clearance of DNA virus infections [44], and its deficiency in NOD mice is also likely to affect such infection models.

Contrary to our initial hypothesis, we observed a slightly increased diabetes incidence in STING-deficient NOD mice. A previous study also identified a paradoxical role for STING in controlling autoimmunity. Sharma et al observed an increase in SLE-like symptoms, including increased autoantibodies, a robust type I IFN gene signature and increased immune activation, in STING-deficient MRL.lpr mice [45]. This negative regulatory role of STING was independent of the transcription factor IRF3. Our findings are also consistent with a study showing reduced frequency of diabetes in NOD mice treated with cGAMP, although the activation of STING by cGAMP was not directly shown in that study [46]. Despite reduced frequency of diabetes, type I IFN was increased in the

serum, and inflammatory cytokines (TNF and IL-6) were increased in the spleen of cGAMP-treated NOD mice. These studies and ours indicate a possible role for STING in controlling autoimmunity that is independent of its DNA sensing function and of type I IFN production.

We detected an increase in autoreactive CD8⁺ T cells in STING-deficient NOD mice and a more potent capacity of STING-deficient T cells to transfer diabetes to immunodeficient mice. Although our data do not directly rule out a role for innate immune cells, the expansion of autoreactive CD8⁺ T cells is likely to be intrinsic to T cells as levels of MHC class I on beta cells, maturation markers on conventional dendritic cells, antigen presentation by dendritic cells and B cell number were equivalent in wild-type and NOD.STING mice. A previous study demonstrated a role for STING in promoting antibody responses [47]; however, there were normal levels of IAA in STING-deficient NOD mice. We did not directly observe increased capacity of early-phase proliferation of

STING-deficient T cells in our NOD.STING^{-/-} mice in vitro or of transferred STING-deficient NOD8.3 T cells (data not shown). Therefore, in NOD mice with a polyclonal repertoire of T cells and chronic inflammation, STING may restrain proliferation or promote apoptosis of autoreactive CD8⁺ T cells. While the mechanism remains unclear, others have also revealed antiproliferative and proapoptotic functions of STING in T cells [16–20]. One report documented decreased proliferation of CD4⁺ T cells transduced with a constitutive gain-of-function STING mutation [18]. Another recent report uncovered that a constitutively activating STING mutation reduced their viability due to a disturbance of Ca²⁺ influx and increased endoplasmic reticulum stress upon T cell receptor stimulation [19]. These previous findings are consistent with our data showing increased number of IGRP-specific cells and increased capacity of STING-deficient cells to transfer diabetes. However, we did not see any impact of STING deficiency on CD4⁺ T cells, with similar numbers of insulin-specific CD4⁺ T cells, similar IFN γ production and normal IAA, indicating normal interaction between CD4⁺ T cells and B cells. We are still gaining new insights into the role of STING in different cell types, including CD8⁺ T cells, and different disease settings. Our data suggest that STING, while primarily contributing to sensing self and non-self DNA, may contribute to autoimmunity by controlling autoreactive CD8⁺ T cell proliferation or apoptosis.

Current research is focused on targeting the cGAS–STING pathway for infectious diseases, cancer, autoinflammatory diseases and autoimmune diseases [48]. STING agonism has the potential to be used as an immunomodulatory therapy for cancer and a vaccine adjuvant for chronic viral or bacterial infections. Alternatively, STING antagonism has been suggested as therapy for autoinflammatory diseases such as SAVI, Aicardi-Goutieres Syndrome, SLE and metastatic cancer. Excessive STING agonism may induce apoptosis and suppress the expansion of tumour antigen-specific T cells, as previously shown [49]. Our findings of expanded autoreactive T cells and increased diabetes incidence in STING-deficient NOD mice suggest that systemic STING antagonism could trigger autoimmunity in individuals susceptible for T cell-driven autoimmune diseases such as type 1 diabetes.

In conclusion, we find that the innate immune adaptor STING is not primarily responsible for the type I IFN signature in islets during the early stages of pathogenesis but may have an intrinsic role to suppress the expansion of autoreactive T cells in NOD mice.

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Data availability All data generated or analysed during this study are included in this published article (and its supplementary information files).

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