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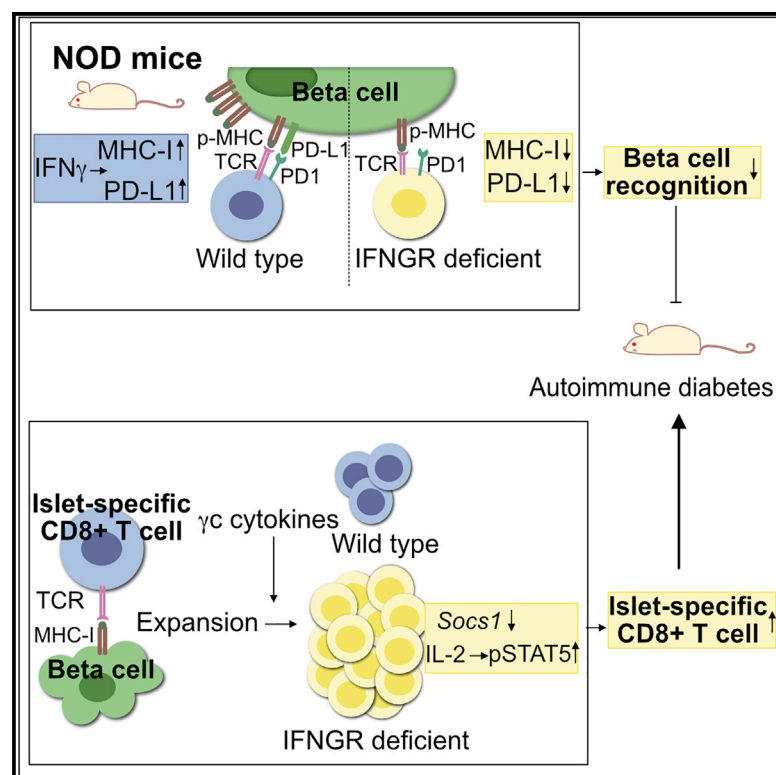
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Interferons limit autoantigen-specific CD8⁺ T-cell expansion in the non-obese diabetic mouse

Graphical abstract



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In brief

Jhala et al. find a 10-fold increase in the number of islet antigen-specific CD8⁺ T cells in NOD mice lacking IFN γ receptors. These cells have increased proliferative responses to IL-2. This study helps us understand the mechanistic basis of the lack of therapeutic benefits from deficiency of IFN γ in autoimmune diabetes.

Highlights

- NOD mice deficient in all three interferon receptors were generated using CRISPR
- 10-fold increased islet-specific CD8⁺ T cells in IFNGR-mutant mice
- T cells from IFNGR-mutant mice have increased proliferative response to IL-2
- Diabetes occurs in interferon-receptor-mutant mice despite reduced islet infiltration



Article

Interferons limit autoantigen-specific CD8⁺ T-cell expansion in the non-obese diabetic mouse

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SUMMARY

Interferon gamma (IFN γ) is a proinflammatory cytokine implicated in autoimmune diseases. However, deficiency or neutralization of IFN γ is ineffective in reducing disease. We characterize islet antigen-specific T cells in non-obese diabetic (NOD) mice lacking all three IFN receptor genes. Diabetes is minimally affected, but at 125 days of age, antigen-specific CD8⁺ T cells, quantified using major histocompatibility complex class I tetramers, are present in 10-fold greater numbers in *Ifngr*-mutant NOD mice. T cells from *Ifngr*-mutant mice have increased proliferative responses to interleukin-2 (IL-2). They also have reduced phosphorylated STAT1 and its target gene, suppressor of cytokine signaling 1 (SOCS-1). IFN γ controls the expansion of antigen-specific CD8⁺ T cells by mechanisms which include increased SOCS-1 expression that regulates IL-2 signaling. The expanded CD8⁺ T cells are likely to contribute to normal diabetes progression despite reduced inflammation in *Ifngr*-mutant mice.

INTRODUCTION

Interferon gamma (IFN γ), mainly produced by T lymphocytes and natural killer (NK) cells, plays a central role in many immune responses against infection and cancer but also in pathological immune responses in immunoinflammatory and autoimmune diseases. IFN γ is at the center of the Th1/Th2/Th17 immune response paradigm, in which naive CD4⁺ T cells differentiate into polarized subtypes (Harrington et al., 2005; Stevens et al., 1988) although the subtypes may also be considered a continuum rather than a polarization (Kelso, 1995; Kiner et al., 2021). The Th1 response is characterized by production of IFN γ as well as interleukin-2 (IL-2) and results in activation of macrophages and CD8⁺ T cells. Assays for IFN γ in peripheral blood exposed to antigens are used for diagnostic assessment of tuberculosis (Bastian and Coulter, 2017). IFN γ itself is used to activate macrophages in individuals with chronic granulomatous

disease to activate free radical production (Yu et al., 2020). Despite expectations, inhibition of IFN γ alone has so far not found a place in therapy for immunoinflammatory diseases, whereas effective drugs, such as calcineurin inhibitors and recently introduced Janus kinase (JAK) inhibitors, affect the production or action of many other cytokines as well as IFN γ . The record of anti-IFN γ or IFN γ gene deletion in protecting from immunopathology is quite modest, although they continue to be in clinical development, and one, emapalumab, has been approved for treatment of hematophagocytic lymphohistiocytosis (Locatelli et al., 2020). However, several diseases thought to be mediated by a Th1 response become worse with IFN γ neutralization or gene deletion (Bettelli et al., 2004; Geboes et al., 2007; Vandenhaute et al., 2019; Willenborg et al., 1999). This is potentially associated with IFN γ 's ability to inhibit T cell proliferation (Driver et al., 2017), which is poorly understood at the molecular level.



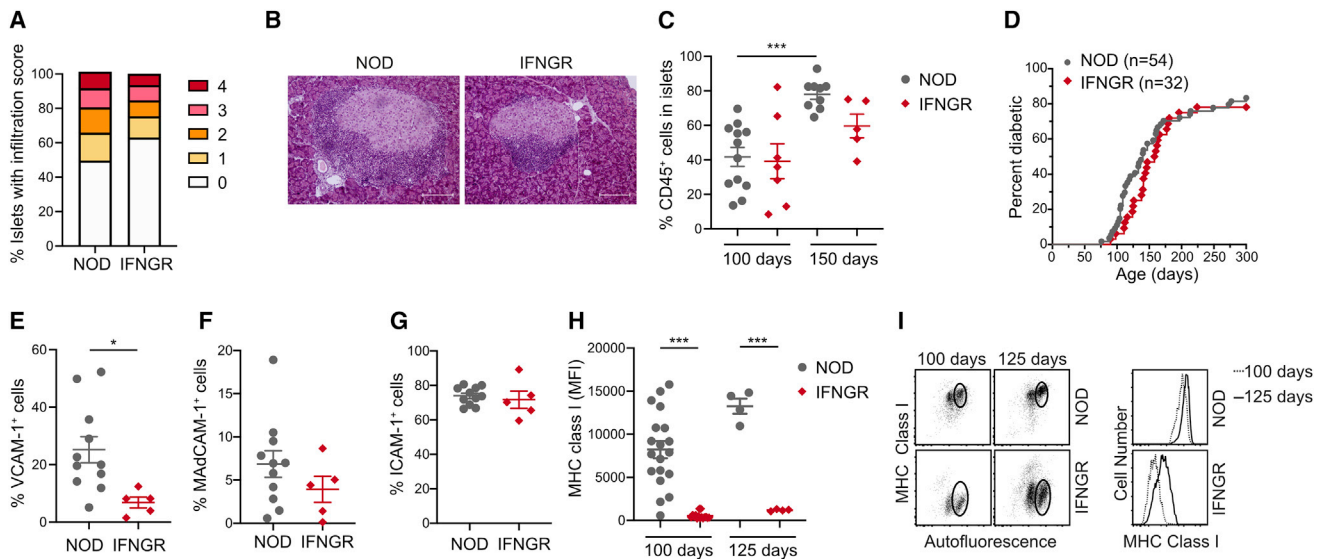


Figure 1. Normal insulinitis and diabetes despite reduced IFN γ -regulated inflammation

(A) Pooled insulinitis scores of pancreas sections from 100-day-old female wild-type NOD (NOD; $n = 10$) or *Ifngr1*-mutant (IFNGR; $n = 10$) mice. Groups were not significantly different. One-way ANOVA with Bonferroni's post-test.

(B) Representative photos of insulinitis. Magnification, $\times 200$; scale bar, 100 μm .

(C) The proportion of CD45 $^{+}$ cells in islets from female wild-type NOD or *Ifngr1*-mutant mice measured by flow cytometry at 100 or 150 days of age. Mean $\pm$ SEM is shown for individual mice. *** $p = 0.0004$ for NOD mice, one-way ANOVA with Sidak's post-test.

(D) Cumulative diabetes incidence of age-matched female cohorts of wild-type NOD or *Ifngr1*-mutant mice, calculated as follows: number of diabetic mice / (number of diabetic mice + number of mice still being monitored by 300 days of age). Curves were not significantly different using a log rank test.

(E–G) The proportion of MECA-32 $^{+}$ endothelial cells expressing cell adhesion molecules in islets isolated from female wild-type NOD or *Ifngr1*-mutant mice at 120 days of age, measured by flow cytometry. Data show mean $\pm$ SEM for individual mice; (E) VCAM-1 $p = 0.02$, (F) MAdCAM-1 $p = 0.26$, (G) ICAM-1 $p = 0.59$, unpaired t test.

(H) MHC class I on autofluorescent β cells from islets isolated from female mice at 100 and 125 days of age. Data show mean $\pm$ SEM for individual mice. *** $p < 0.0001$, one-way ANOVA with Sidak's post-test.

(I) Representative plots of data presented in (H). Dot plots (left) show islet cells from NOD and *Ifngr1*-mutant mice at 100 and 125 days of age, with autofluorescent β cells circled. Histograms (right) show MHC class I expression on β cells from NOD and *Ifngr1*-mutant mice at 100 and 125 days of age.

An example of this paradox is type 1 diabetes (T1D), an immunoinflammatory disease caused by loss of immune tolerance to pancreatic β cells that results in autoimmunity associated with a Th1 multi-cytokine profile (Arif et al., 2004; Trembleau et al., 1995). T1D is regarded as a T cell-mediated disease in which IFN- γ plays a major role. IFNs mediate histopathological hallmarks of T1D, such as increased expression of major histocompatibility complex (MHC) class I proteins on the surface of β cells (Campbell et al., 1985; Kay et al., 1991; Thomas et al., 1998), associated with STAT1 phosphorylation, increased expression of adhesion molecules on high endothelial venules adjacent to inflamed islets, and production of chemoattractants (Calderon et al., 2011; Thomas et al., 2009). All of these features increase the ability of CD8 $^{+}$ cytotoxic T cells to target β cells and would be expected to increase the rate of disease progression.

However, disruption of the genes encoding individual IFNs or their receptors does not prevent autoimmune diabetes from developing in the non-obese diabetic (NOD) mouse (Hultgren et al., 1996; Kanagawa et al., 2000; Quah et al., 2014; Serreze et al., 2000). In contrast, targeting the downstream IFN receptor signaling pathways by disrupting *Stat1* expression (Kim et al., 2007), overexpressing suppressor of cytokine signaling-1 (SOCS-1) in β cells (Chong et al., 2004; Flodström-Tullberg

et al., 2003), or treating with JAK1/JAK2 inhibitors (Ge et al., 2020; Trivedi et al., 2017) prevents diabetes in NOD mice, but all of these interventions target a wider group of molecules and pathways than IFNs alone.

Our aim in this study was to understand the mechanistic basis of the lack of therapeutic benefits from deficiency of IFN- γ by studying autoantigen-specific T cells in T1D. We did this by investigating redundancy among IFNs by genetically ablating expression of all three IFN receptors directly on the NOD background using CRISPR technology, but diabetes progressed even in triple IFN-receptor-deficient mice.

RESULTS

Diabetes in *Ifngr1*-mutant NOD mice despite reduced islet inflammation

We used CRISPR-Cas9 gene editing to introduce mutations within the first exons of *Ifnar1*, *Ifngr1*, and *Ifnlr1* in NOD mice. These receptor mutations led to premature stop codons that ablate IFN-receptor signaling for all three IFNs (Figures S1A–S1C; Table S1). We initially characterized the *Ifngr1*-mutant NOD mice. There was no significant difference in insulinitis (Figures 1A and 1B) or frequency of CD45 $^{+}$ islet-infiltrating

immune cells (Figures 1C and S2A–S2C) between *Ifngr1*-mutant and control NOD mice (Figures 1A–1C). Consistent with this, there was no difference in the rate of development of diabetes in female mutant and control mice, with approximately 80% of both groups developing diabetes by 250 days of age (Figure 1D). As expected, there were changes in IFN-regulated gene expression in islets when insulinitis was established. Endothelial cells from islets identified by the marker MECA-32 were analyzed by flow cytometry for the vascular adhesion molecules VCAM-1, MadCAM-1, and ICAM-1 (Figures 1E–1G). VCAM-1 expression was significantly reduced on islet endothelial cells from *Ifngr1*-mutant mice. Islet cell MHC class I expression was markedly reduced on β and non- β cells (Figures 1H, 1I, and S1D), consistent with previous findings that MHC class I expression on islet cells undergoing autoimmune attack is regulated by IFN γ (Thomas et al., 1998).

Increased antigen-specific CD8⁺ T cells in *Ifngr1*-mutant mice

Measurement of islet autoantigen-specific CD8⁺ T cells in peripheral lymphoid tissues and blood is a good biomarker of progression of insulinitis (Chee et al., 2014). Strikingly, CD8⁺ T cells that bind to MHC class I tetramers for the 206–214 peptide of the islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP_{206–214}) and Insulin_{B15–23} were increased 5- to 10-fold in *Ifngr1*-mutant mice at 125 days of age (Figures 2A–2C and S2D). When insulinitis began in *Ifngr1*-mutant mice, the number of CD8⁺ T cells rapidly increased, and the number of IGRP-specific and insulin-specific T cells in peripheral lymphoid organs was greater than in wild-type NOD mice for any given percentage of CD45⁺ cells in islets (Figures 2D and 2E). This occurred in female and male *Ifngr1*-mutant mice (males not shown). This indicated much greater polyclonal expansion of antigen-specific T cells and may also reflect a change in islet retention of T cells.

We further characterized the phenotype and clonality of IGRP-specific CD8⁺ T cells. The proportion of effector memory CD8⁺ T cells (CD44^{hi}IL-7R^{hi}) in peripheral lymphoid tissues and islets was also increased in mutant mice (Figures 2F and 2G). Bulk T cell receptor (TCR β) sequencing of IGRP tetramer-binding and non-binding CD8⁺ T cells from peripheral lymphoid tissue was performed. The distribution and abundance of unique TCR β clonotypes were different between IGRP tetramer binding and non-binding CD8⁺ T cell populations (represented by different Rényi diversity profiles). As expected, IGRP tetramer binding CD8⁺ T cell populations were less diverse, with oligoclonal expansion of few TCR β clones (Figures 2H and 2I). Importantly, clonal expansion of IGRP tetramer binding CD8⁺ T cells in *Ifngr1*-mutant mice was dominated by a smaller number of TCR β clones compared with control NOD mice (Figure 2I). In the absence of IFN γ signaling in NOD mice, there was an accumulation of clonally restricted, effector memory islet autoantigen-specific CD8⁺ T cells in peripheral lymphoid tissue.

There was no comparable increase in the number of CD4⁺ T cells specific for the islet autoantigens proinsulin (Figures 2J–2L) and chromogranin A (data not shown), although significantly higher percentages of proinsulin and chromogranin-specific CD4⁺ T cells were CD44^{hi} in mutant mice. Nor was there a significant difference in the total CD4:CD8 ratio (data not shown)

between *Ifngr1*-mutant and wild-type mice. CD4⁺FoxP3⁺ regulatory T (Treg) cells were increased in mutant mice (see below). These data suggest that, in wild-type NOD mice, IFN γ receptor signaling restricts the expansion of islet autoantigen-specific CD8⁺ T cells.

Increased proliferation of CD8⁺ T cells in response to IL-2 in *Ifngr1*-mutant NOD mice

We next explored the mechanism of increased antigen-specific CD8⁺ T cell expansion in the absence of IFN γ signaling. T cells from islets of *Ifngr1*-mutant NOD mice were cultured with IL-2, and the resulting T cells were enumerated (Figures 3A–3C). There were significantly more IGRP tetramer-binding cells cultured from islets of *Ifngr1*-mutant mice than wild-type NOD mice. Islets were also isolated from wild-type NOD mice and cultured *in vitro* with IL-2 or IL-2 plus an anti-IFN γ monoclonal antibody (Figures 3D–3F). Expansion of CD8⁺ T cell numbers by IL-2 was significantly increased when islets were co-cultured with an anti-IFN γ antibody (Figure 3E) compared with IL-2 alone. IGRP-specific T cells were also increased (Figure 3D), although the increase was not significant because of a single outlier with high numbers of IGRP-specific T cells in the culture with IL-2 alone. Anti-IFN γ on its own without IL-2 did not stimulate T cell expansion (data not shown).

The IL-2 receptor is composed of a high-affinity ligand-binding α chain, a β chain with lower affinity, and the common γ chain (γ C), which is non-ligand binding but drives much of the signal transduction and is shared with other members of this cytokine family. The γ C chain initiates signal transduction by activation of members of the JAK family of kinases, which then phosphorylate members of the signal transduction and activation of transcription (STAT) family, especially STAT5 in the case of IL-2 (Ross and Cantrell, 2018). Expansion of IGRP tetramer-binding CD8⁺ T cells over a 2-week period from approximately 100 days of age was observed *in vivo* in *Ifngr1*-mutant mice, but this was virtually completely inhibited by treatment with the JAK1/JAK2 inhibitor LN3103801 (Figure 3G). Absence of IFN γ by genetic deficiency or antibody neutralization caused an increased response to IL-2 that appeared to be mediated through the JAK-STAT pathway.

Spleen cells from *Ifngr1*-mutant mice were also tested for responsiveness to IL-2 (Figures 4A–4C). They were cultured first with anti-CD3 and anti-CD28 for 3 days and then washed and cultured with 5 or 50 U/mL IL-2. Both concentrations of IL-2 increased the number of activated CD8⁺ T cells from *Ifngr1*-mutant mice more than cells from wild-type mice (Figure 4A). Unphosphorylated STAT5 (data not shown) and phosphorylated STAT5 levels were increased in cells from *Ifngr1*-mutant mice compared with wild-type mice after culture with IL-2 (Figures 4B and 4C) or IL-15 (Figure 4D). Phosphorylated STAT1 was consistently reduced in CD8⁺ T cells from *Ifngr1*-mutant mice (Figure 4E). Consistent with an increase in responses to common γ chain cytokines, there was an increase in the proportion of CD8⁺ T cells expressing CD25, a known STAT5-responsive gene (Figure 4F).

Therefore, the absence of IFN γ signaling increased CD8⁺ T cell sensitivity to IL-2 in T cells that were isolated from a site of pathology but also in peripheral T cells. IL-2 sensitivity in

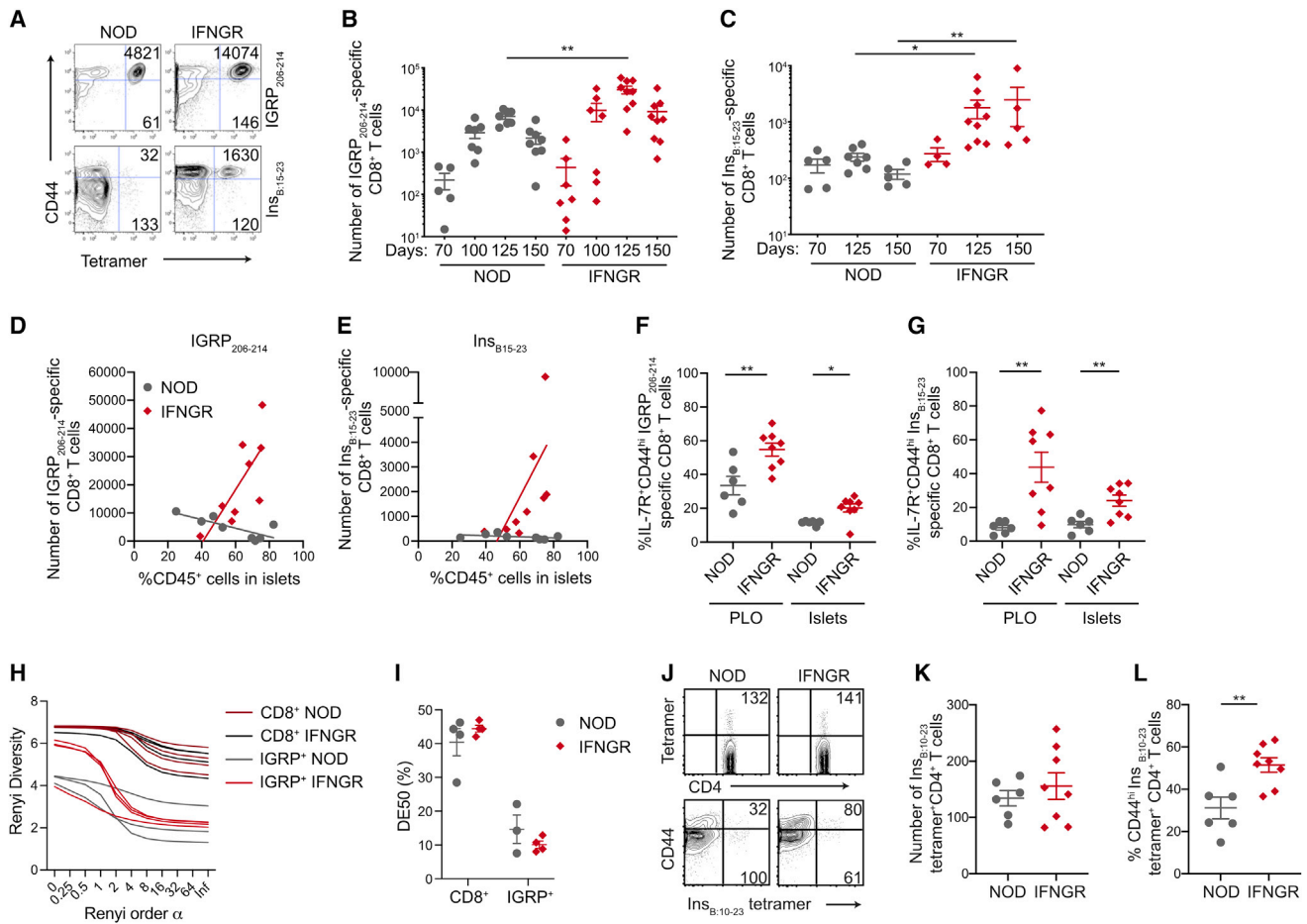


Figure 2. Increased β cell antigen-specific T cells in *Ifngr1*-mutant mice

Peripheral lymphoid organs were harvested from wild-type or *Ifngr1*-mutant female NOD mice at 70, 100, 125, and 150 days of age, and the peripheral lymphoid organs were analyzed by tetramer enrichment.

(A) Representative plots of tetramer staining in the two genotypes at 125 days of age.

(B and C) Absolute numbers of (B) IGRP₂₀₆₋₂₁₄-specific and (C) Insulin_{B15-23}-specific CD8⁺ T cells. Data show mean ± SEM of individual mice. **p < 0.0001 NOD versus IFNGR 125 days (B); *p = 0.036 NOD versus IFNGR 125 days and **p = 0.0009 NOD versus IFNGR 150 days; one-way ANOVA with Sidak's post-test. (D and E) Correlation between the proportion of CD45⁺ cells in the islets and the number of tetramer⁺ cells in 125-day-old mice. IGRP₂₀₆₋₂₁₄: NOD r = 0.83, p = 0.008; IFNGR r = -0.71, p = 0.057 (D); Insulin_{B15-23}: NOD r = 0.81, p = 0.01; IFNGR r = -0.33, p = 0.42 (E), Spearman's correlation.

(F and G) The proportion of effector memory CD44^{hi} IL-7R^{hi} CD8⁺ T cells recognizing (F) IGRP₂₀₆₋₂₁₄ or (G) Insulin_{B15-23} in the peripheral lymphoid organs (PLOs) and islets of 140-day-old wild-type NOD or IFNGR mice. Pooled data show mean ± SEM of individual mice. **p < 0.005, *p = 0.01, unpaired t test.

(H) Rényi diversity profiles of TCRβ clonotypes derived from TCRβ sequencing of sorted CD8⁺CD44⁺ (CD8⁺) and IGRP-specific CD8⁺ (IGRP⁺) T cells from wild-type or *Ifngr1*-mutant mice. Each colored curve represents the diversity profile of TCRβ clonotypes from respective populations. The scale of Rényi order α corresponds to different calculated diversity metrics. As α approaches infinity, more weight is given to abundant TCRβ clones; therefore, the steeper the slope of each curve, the lower the diversity of the repertoire.

(I) TCRβ repertoire diversity represented as diversity evenness 50 (DE₅₀) values. Each dot represents a population of sorted cells, with mean ± SD for 3 NOD and 4 IFNGR mice.

(J–L) The proportion of CD44⁺ Insulin_{B10-23}-specific CD8⁺ T cells in PLOs of 140-day-old mice.

(J) Representative plots and pooled data showing (K) the number of Insulin_{B10-23}-specific CD8⁺ T cells and (L) the proportion of CD44⁺ Insulin_{B10-23}-specific CD8⁺ T cells. Female mice were analyzed (A–D and J–L), and both sexes were analyzed (F–I). Data show mean ± SEM for individual mice. **p = 0.005, unpaired t test.

Ifngr1-mutant NOD mice was associated with reduced pSTAT1 and increased pSTAT5.

Role of Treg cells and the PD-1 pathway in *Ifngr1*-mutant mice

The rapid increase in antigen-specific CD8⁺ T cells and onset of diabetes in *Ifngr1*-mutant mice raised the possibility that normal

regulatory control of CD8⁺ T cell numbers and function had been compromised in these NOD mice. One possibility was that reduced Treg cells could promote clonal expansion of antigen-specific CD8⁺ T cells and target cell destruction. However, there were more, not fewer, CD4⁺ CD25⁺ FoxP3⁺ Treg cells in *Ifngr1*-mutant mice (Figure 5A). Similarly, the proportion of Helios⁺CD4⁺ Foxp3⁺ thymus-derived natural Treg cells was

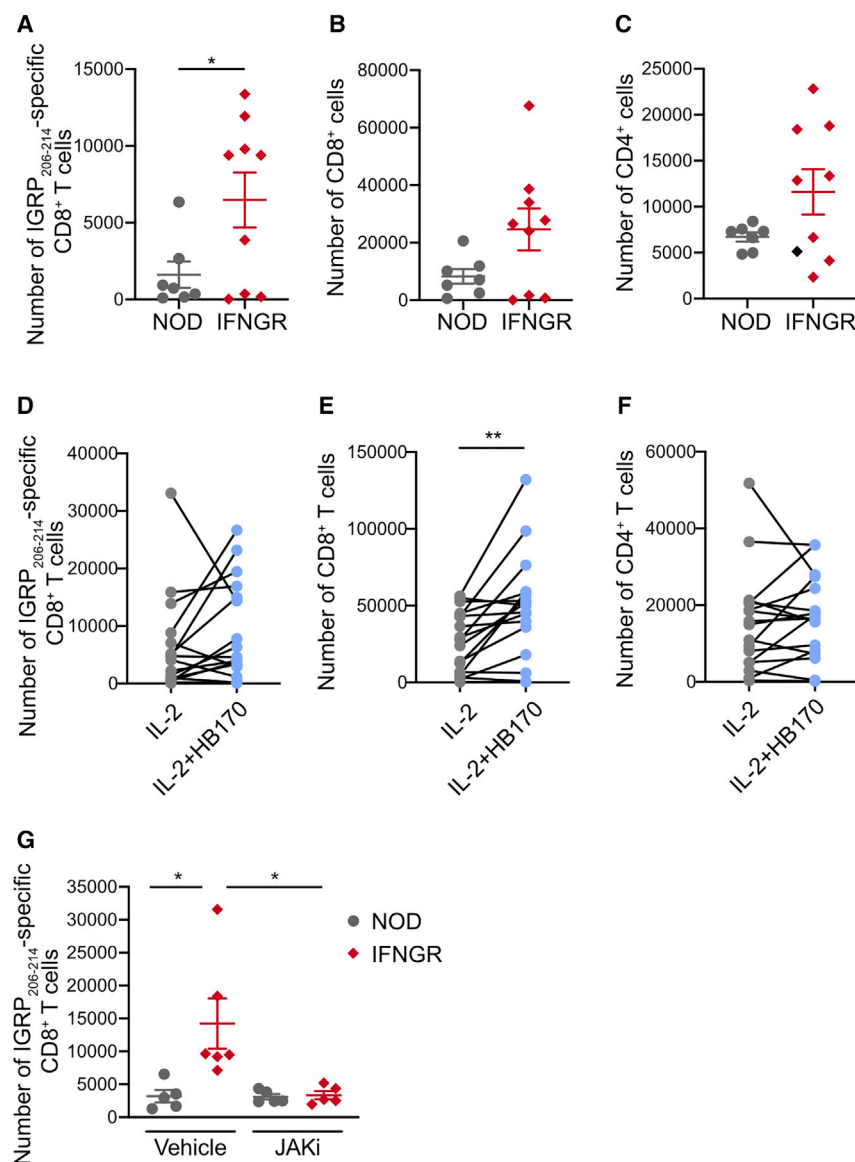


Figure 3. Expansion of IGRP-specific T cells in *Ifngr1*-mutant islets is IL-2 dependent

(A–C) The number of (A) IGRP₂₀₆₋₂₁₄-specific CD8⁺ T cells, (B) CD8⁺ T cells, and (C) CD4⁺ T cells after culture of wild-type NOD or *Ifngr1*-mutant islets for 5 days with IL-2. Data show mean ± SEM for individual 100- to 120-day-old mice. *p = 0.04, unpaired t test.

(D–F) The number of (D) IGRP₂₀₆₋₂₁₄-specific CD8⁺ T cells, (E) CD8⁺ T cells, and (F) CD4⁺ T cells after culture of wild-type NOD islets for 5 days with IL-2 in the presence of an anti-IFN γ Ab (HB170) or isotype control. Data show paired samples for islets isolated from individual mice. **p = 0.002, paired t test.

(G) The number of IGRP₂₀₆₋₂₁₄-specific CD8⁺ T cells in the PLOs of wild-type NOD or *Ifngr1*-mutant NOD mice after 2 weeks of treatment with vehicle or JAK inhibitor (LN3103801). Data show mean ± SEM of individual mice from 3 independent experiments.

Female and male mice were analyzed (A–G). *p = 0.016 (vehicle treated), *p = 0.018 (IFNGR).

at this time point (Figure 1C). Islet endothelial cells also had undetectable PD-L1 expression in *Ifngr1*-mutant NOD mice (Figure 5D). This indicated that T cells from *Ifngr1*-mutant NOD mice were free of the inhibition mediated by islet cell PD-L1. Because PD-L1 was undetectable or very reduced, we expected that PD-L1 checkpoint inhibition would have little effect on diabetes development. Administration of an anti-PD-L1 blocking antibody results in rapid onset of autoimmune diabetes in wild-type NOD mice (Ansari et al., 2003). As expected, autoimmune diabetes was not accelerated in *Ifngr1*-mutant mice given an anti-PD-L1 antibody (Ab) (Figure 5E). Lack of PD-L1 expression may be in part responsible for the increased clonal expansion *Ifngr1*-mutant NOD mice display. Administration of anti-PD-L1 Ab to wild-type

higher in *Ifngr1*-mutant mice (Figures 5B and S2E). These data are consistent with increased signaling through CD25, and increased Treg cell numbers have also been seen previously (Driver et al., 2017).

A second possibility for the increase in antigen-specific CD8⁺ T cells is inhibition of the PD-1 pathway. PD-L1 is the ligand for the T cell immune checkpoint receptor PD-1 and is upregulated on β cells by IFN γ or type I IFNs (Colli et al., 2018; Osum et al., 2018) and is also involved in controlling development of autoreactive T cells (Freeman et al., 2000). Without this interaction, antigen-specific T cells are increased, as is their functional capacity (Ansari et al., 2003; Pauken et al., 2013). β Cells from wild-type NOD mice at 120–150 days of age showed increased expression of PD-L1, whereas β cells from age-matched *Ifngr1*-mutant NOD mice did not have detectable PD-L1 (Figure 5C), even though CD45⁺ immune cells were present in their islets

NOD mice caused increased proliferation in islet-infiltrating CD8⁺ T cells (Figure 5F). However, inhibition of the PD-L1 pathway *in vitro* did not augment wild-type NOD islet T cell proliferation in response to IL-2 (data not shown), suggesting that the sensitivity to IL-2 seen in *Ifngr1*-mutant NOD mice was not due to the absence of PD-L1. Wild-type NOD mice transplanted with *Ifngr1*-mutant bone marrow displayed increased IGRP tetramer-binding T cells despite having β cells able to increase PD-L1 normally in response to IFN γ (Figures 5G and 5H). Consistent with this, 2 of 5 wild-type recipients transplanted with *Ifngr1*-mutant bone marrow developed diabetes after anti-PDL1 Ab administration, whereas *Ifngr1*-mutant recipients of wild-type bone marrow did not develop diabetes (Figures 5I and 5J). Lack of PD-L1 expression in the islets is unlikely to be the only reason for increased CD8⁺ T cell clonal expansion in *Ifngr1*-mutant NOD mice but may contribute.

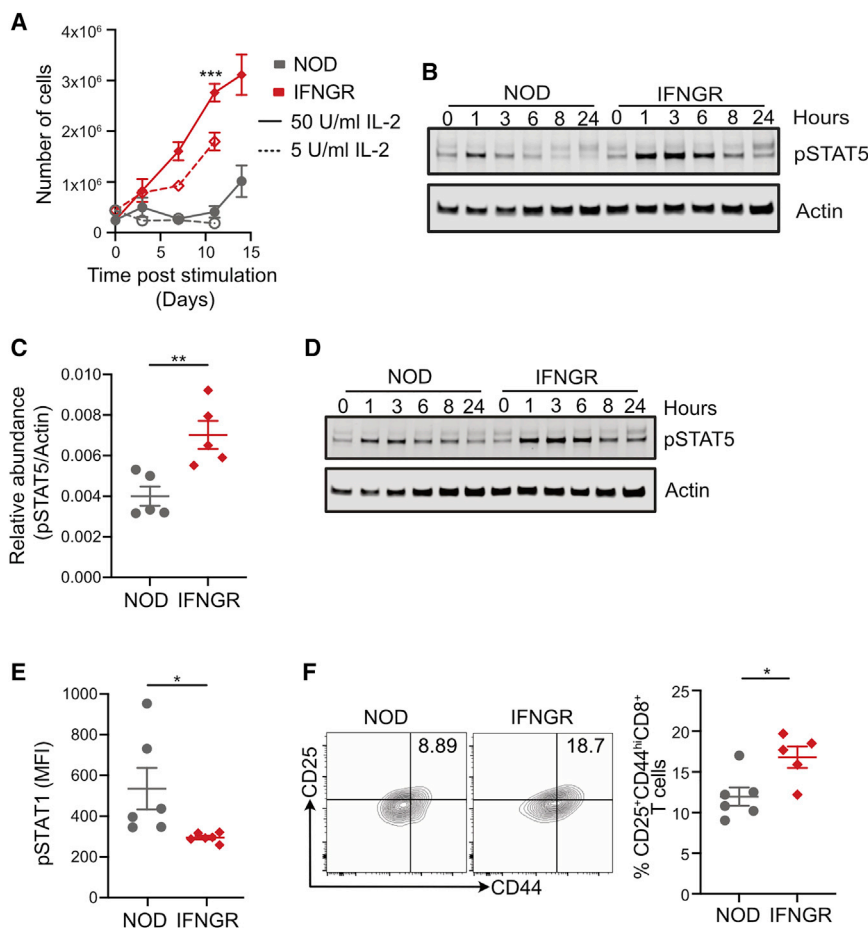


Figure 4. Expansion of *Ifngr1*-mutant CD8+ T cells in response to IL-2 is associated with increased pSTAT5 signaling

(A) Splenocytes from 70- to 100-day-old wild-type NOD or *Ifngr1*-mutant female and male mice were activated for 3 days with anti-CD3 and anti-CD28 and then plated at the same density in medium containing 5 U/mL or 50 U/mL IL-2. The number of cells was counted over a 2-week period. Data show mean $\pm$ SEM of 5–6 mice from 2 independent experiments. *** p = 0.0001, day 11 (NOD versus IFNGR, 50 U/mL IL-2), unpaired t test. (B and C) Western blot of activated splenocytes cultured with 5 U/mL IL-2 for 0–24 h as indicated. (B) Representative data. (C) Pooled quantified data from the 3-h time point, showing mean $\pm$ SEM for individual mice from 5 mice from 2 independent experiments; ** p = 0.0069, unpaired t test. (D) Western blot of activated splenocytes cultured with 10 ng/mL IL-15 for 0–24 h as indicated. Data are representative of 2 independent experiments. (E) Mean fluorescence intensity (MFI) of pSTAT1 on CD8⁺ T cells after 7 days of culture with IL-2. Data show mean $\pm$ SEM of individual mice from 2 independent experiments. * p = 0.04, unpaired t test. (F) Representative plots and pooled data of the proportion of CD25⁺CD44^{hi}CD8⁺ T cells in the islets of female mice. Data show mean $\pm$ SEM. * p = 0.0203, unpaired t test.

Another possibility is that there are fewer exhausted T cells in *Ifngr1*-mutant mice to reinvigorate with checkpoint inhibitor treatment. The level of expression of the exhaustion markers PD-1, TIM-3, and T-cell immunoreceptor with immunoglobulin and ITIM domain (TIGIT) was similar in islet T cells of wild-type and *Ifngr1*-deficient mice (Figure 5K), suggesting that this is not the case.

Reduced *Socs1* expression in islet T cells of *Ifngr1*-mutant and triple-mutant mice

We hypothesized that IFN γ -mediated inhibition of JAK-STAT signaling downstream of IL-2 is due to increased expression of SOCS-1. SOCS-1 is known to inhibit γ c signaling and, therefore, to inhibit signaling by cytokines, including IL-2, -4, -7, -13, -15, and -21 (Cornish et al., 2003; Davey et al., 2005; Gagnon et al., 2007). SOCS-1 is also induced in many cell types by IFN γ (Sakamoto et al., 1998), but a link between IFN γ action and reduced IL-2 signaling has not been made previously. *Socs1* transcript expression was increased in NOD islets cultured *in vitro* with IFN γ , and this was prevented when IFN γ signaling was deficient (Figure 6A). Consistent with this, when islets were isolated from 120-day-old NOD mice, when it is known that IFN γ is expressed in the islets, levels of *Socs1* gene expression were much lower in *Ifngr1*-mutant NOD mice

than in wild-type NOD mice, whereas *Socs3* and *Ptpn2* gene expression was not different (Figure 6B). We examined *Socs1* transcript expression in islet-infiltrating T cells in *Ifngr1*-mutant mice using a reporter of *Socs1* promoter activity called NOD.SOCS1/hCD4 (Chong et al., 2003). In these mice, SOCS1 promoter activity is reported with human CD4 (hCD4), which is detected on the cell surface. NOD.SOCS1/hCD4 mice were bred with *Ifngr1*-mutant mice. Expression of hCD4 was examined by flow cytometry in the islets of wild-type and *Ifngr1*-mutant reporter mice (Figure 6C). Islet-infiltrating CD45⁺ cells from *Ifngr1*-mutant reporter mice had significantly reduced hCD4 expression, indicating reduced *Socs1* promoter activity. We also examined *Socs1*, *Socs3*, and *Ptpn2* transcript expression in T cells derived from islets of *Ifngr1*-mutant NOD mice by culture in IL-2 for 5 days (Figure 6D) and found that *Socs1* transcript expression was reduced. These studies show that SOCS-1 is consistently reduced in *Ifngr1*-mutant mice immediately after islet isolation and after culture with IL-2.

In contrast, another negative regulator of cytokine signaling, *Ptpn2*, which is induced in T cells in response to TCR signaling (Twohig et al., 2019), was not different in activated T cells from wild-type NOD and *Ifngr1*-mutant mice. Increased IL-2 signaling is known to reduce pro-apoptotic factors in T-cells (Newton and Strasser, 2000). Hence, we looked for expression of Bcl-2-like protein 11 (BIM) and Bcl-2 associated X protein (BAX) in mutant mice and found that these molecules were expressed at lower levels in activated T cells (Figures 6E and 6F).

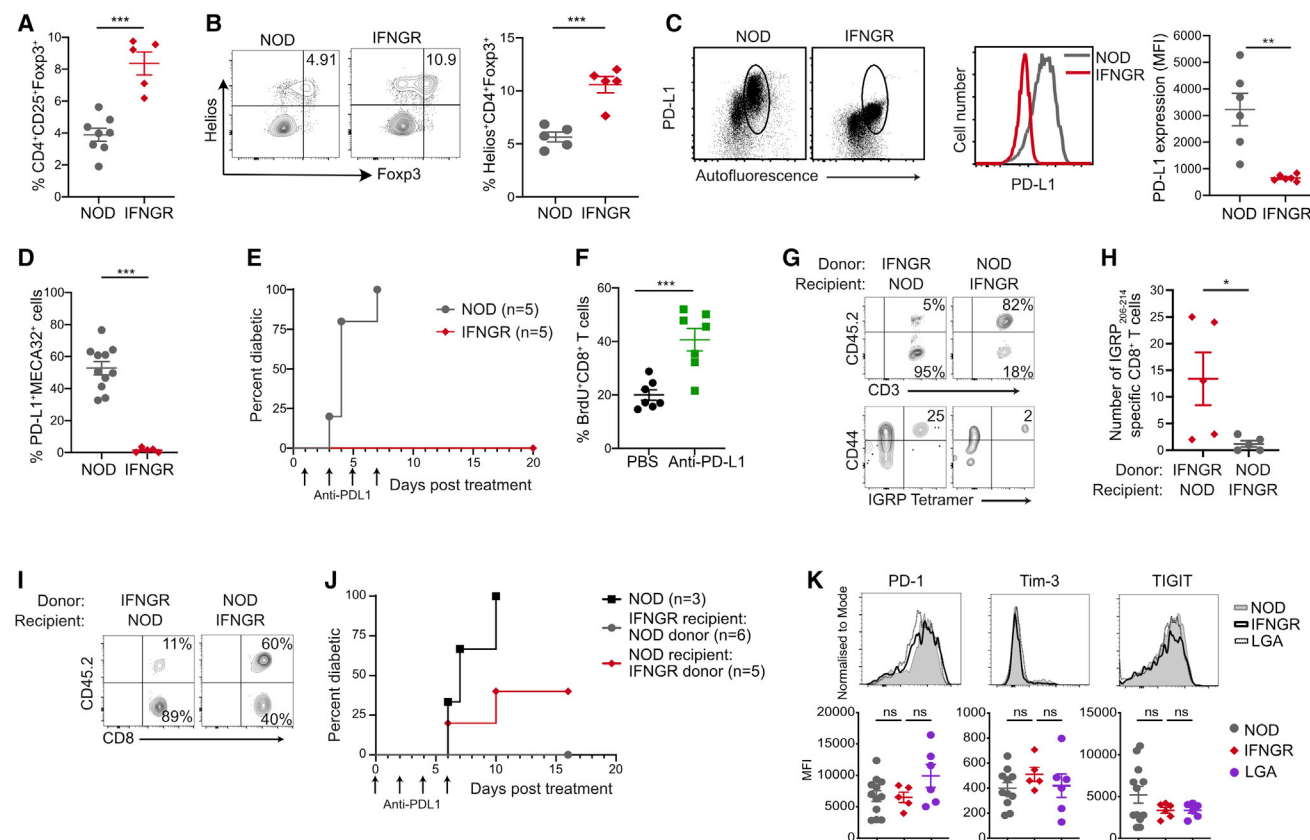


Figure 5. Role of Treg cells and the PD-1 pathway in *Ifngr1*-mutant mice

(A) The proportion of T cells in the spleens of 150-day old female wild-type or *Ifngr1*-mutant NOD mice that are CD4⁺CD25⁺Foxp3⁺, measured by flow cytometry. Mean $\pm$ SEM for individual mice is shown. ***p = 0.0001, unpaired t test.

(B) Representative plots and pooled data of the proportion of Helios⁺CD4⁺Foxp3⁺ T cells in the spleens of 150-day-old female wild-type or *Ifngr1*-mutant NOD mice, measured by flow cytometry. Mean $\pm$ SEM for individual mice is shown. ***p = 0.0005, unpaired t test.

(C and D) Expression of PD-L1 in islets isolated from wild-type NOD and *Ifngr1*-mutant female mice at 120–150 days of age and analyzed by flow cytometry by gating on (C) autofluorescent β cells (circled) or (D) MECA-32⁺ endothelial cells. Shown in (C) are representative plots for β cells and pooled data showing mean $\pm$ SEM of individual mice, **p = 0.018. Shown in (D) are endothelial cells; ***p < 0.0001. Statistical analysis used unpaired t tests.

(E) Cumulative diabetes incidence of 100-day-old female NOD or *Ifngr1*-mutant mice treated with an anti-PD-L1 Ab on days 1, 3, 5, and 7. p = 0.0002, log rank (Mantel-Cox) test.

(F) The proportion of BrdU⁺CD8⁺ T cells in the islets of wild-type female NOD mice on day 7 after one injection with 250 μ g anti-PD-L1 or PBS. Data show mean $\pm$ SEM of individual mice from 2 independent experiments. ***p = 0.0008, unpaired t test.

(G and H) Donor chimerism (G, top panel) and enumeration of IGRP₂₀₆₋₂₁₄-specific CD8⁺ T cells (G, bottom panel, and H) from bone marrow chimeras 15 weeks after transfer of T cell-depleted bone marrow from 6–7-week-old female *Ifngr1*-mutant or NOD.CD45.2 donors into lethally irradiated female NOD.CD45.2 or *Ifngr1*-mutant recipients. Shown in (G) are representative plots and in (H) pooled data showing mean $\pm$ SEM of individual mice from one experiment; *p = 0.03, unpaired t test.

(I and J) Bone marrow chimeras 8 weeks after transfer of T cell-depleted bone marrow from 6- to 7-week-old female *Ifngr1*-mutant or NOD.CD45.2 donors into lethally irradiated female NOD.CD45.2 or *Ifngr1*-mutant recipients. Shown in (I) are representative plots showing donor chimerism and in (J) cumulative diabetes incidence after injection of anti-PD-L1 on days 0, 2, 4, and 6. Unmanipulated female NOD mice (n = 3) were included as controls. p = 0.0018, log rank (Mantel-Cox) test.

(K) Representative histograms and pooled data of the MFI of PD-1, Tim-3, and TIGIT expression in CD8⁺ T cells from wild-type, *Ifngr1*-mutant, or triple-mutant (labeled LGA for lambda, gamma, alpha) female mice. Data show mean $\pm$ SEM of individual mice. ns, no significant difference.

Reduced insulinitis and delayed diabetes in triple-mutant mice

Redundancy between IFNs is a possible explanation for why deficiency of IFN γ or its receptor or the type 1 IFN receptor do not protect from diabetes in NOD mice. We therefore produced NOD mice with deleterious mutations (i.e., premature stop codons) in the receptors for the three classes of IFNs (Table S1).

At 100 days of age, triple IFN receptor-mutant mice had significantly reduced insulinitis compared with wild-type and *Ifngr1*-mutant NOD mice (Figures 7A and 7B); most had insulinitis histology scores of zero. At 120 days, the small number of CD45⁺ leukocytes isolated from the islets included myeloid cells and a limited lymphoid infiltrate (Figures 7C and 7D). There was no difference in the proportions of B cells, conventional dendritic cells (cDCs), plasmacytoid dendritic cells

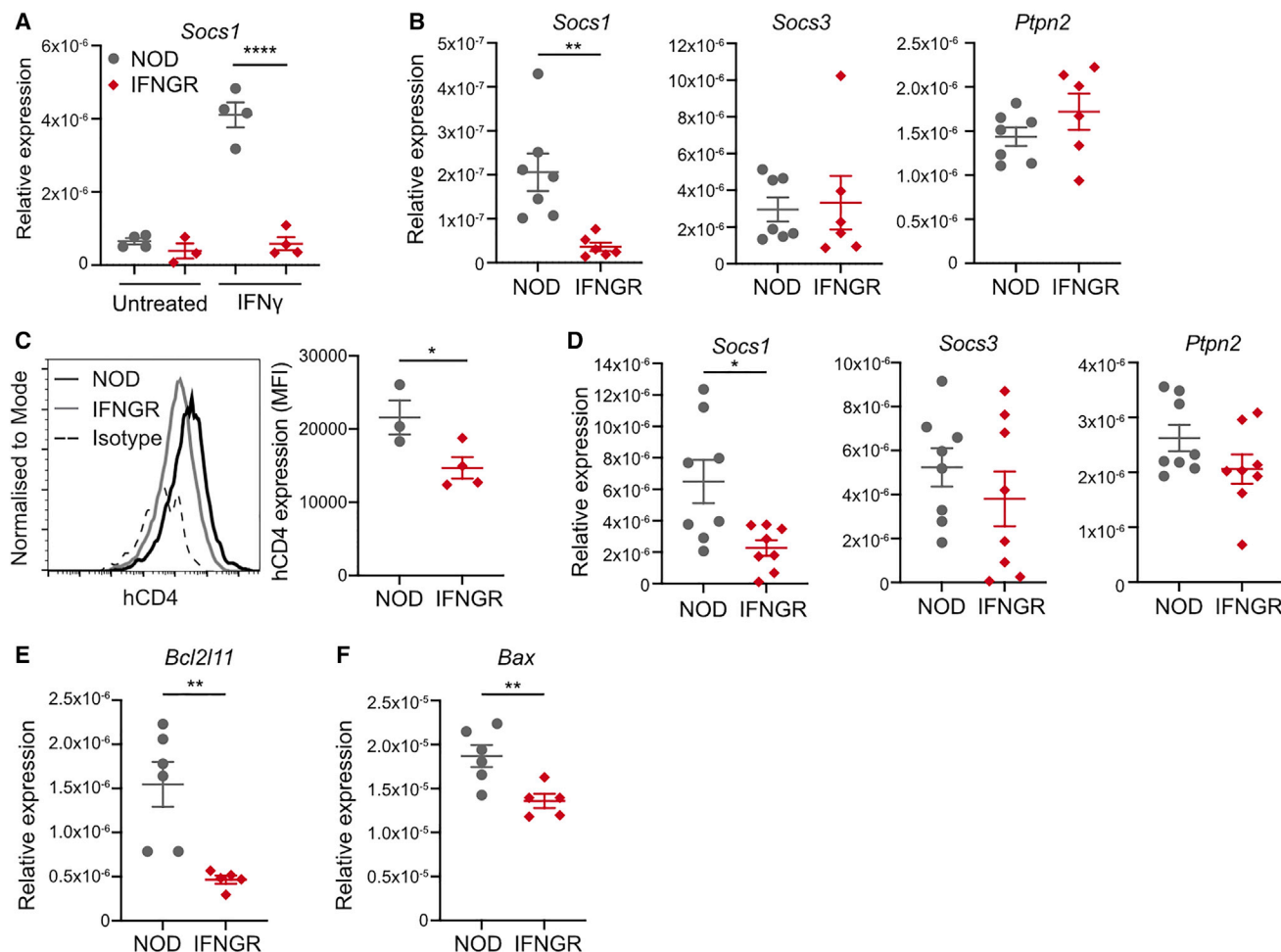


Figure 6. Reduced *Socs1* expression in islet T cells of *Ifngr1*-mutant mice

(A) Expression of *Socs1* in the islets from wild-type NOD or *Ifngr1*-mutant female and male mice cultured for 4 h with 100 U/mL IFN γ . Data show mean $\pm$ SEM of individual mice from 2 independent experiments. ****p < 0.0001, one-way ANOVA.

(B) Expression of *Socs1*, *Socs3*, and *Ptpn2* relative to *Rn18s* (calculated as $2^{(-\Delta\Delta CT)}$) in whole islets isolated from 90- to 120-day-old female wild-type NOD or *Ifngr1*-mutant mice. Data show mean $\pm$ SEM of individual mice from 2 independent experiments. **p = 0.004, unpaired t test.

(C) Representative histograms and MFI of the hCD4 reporter for SOCS-1, measured by flow cytometry on islet-infiltrating CD45⁺ cells isolated from female wild-type NOD or *Ifngr1*-mutant NOD mice. Data show mean $\pm$ SEM of individual mice from 3–4 individual mice. *p = 0.04, unpaired t test.

(D) Expression of *Socs1*, *Socs3*, and *Ptpn2* relative to *Rn18s* (calculated as $2^{(-\Delta\Delta CT)}$) in islet T cells after 5 days of culture in IL-2. Islets were isolated from 140-day-old female wild-type NOD or *Ifngr1*-mutant mice. Data show mean $\pm$ SEM of individual mice from 2 independent experiments. *p = 0.01, unpaired t test.

(E and F) Expression of (E) *Bcl2l11*, **p = 0.004 and (F) *Bax*, **p = 0.009 in T cells after culture for 7 days with 50 U/mL IL-2. Data show mean $\pm$ SEM of individual mice from 2 independent experiments.

(pDCs), or macrophages in the spleens of wild-type, *Ifngr1*, or triple-mutant mice (Figures S2F and S2G). Insulinitis, although increased by 150 days of age, was still reduced in triple-mutant mice compared with wild-type NOD mice, which all had advanced insulinitis (Figure 7A). In fact, of the five triple-mutant mice analyzed at 150 days of age, three had no histological evidence of immune cells around the islets, whereas the other two had insulinitis comparable with wild-type NOD mice (Figure 7B).

Ifnlr1 mutant mice had insulinitis scores similar to wild-type and *Ifngr1*-mutant NOD mice (Figures 7E and 7F). The proportion of CD45⁺ cells in islets of 100-day-old female single mutant mice was similar to wild-type NOD mice (Figure 7C). Unaffected onset

and progression of insulinitis in single *Ifngr1*, *Ifnar1*, or *Ifnlr1*-deficient mice compared with triple-mutant mice suggests that more than one IFN receptor can facilitate entry of T cells into islets.

Remarkably, despite the clear delay in insulinitis development, the cumulative diabetes incidence in *Ifnlr1*/*Ifngr1*/*Ifnar1* triple-mutant mice was the same as in wild-type NOD mice and only slightly delayed in females and slightly reduced in males (Figure 7G). This result indicates that progression to diabetes from initiation of insulinitis was faster in triple-mutant mice, taking approximately 30–40 days from onset of insulinitis (approximately day 120) to diabetes (approximately day 150–160) compared with approximately 70 days in wild-type mice. This rapid

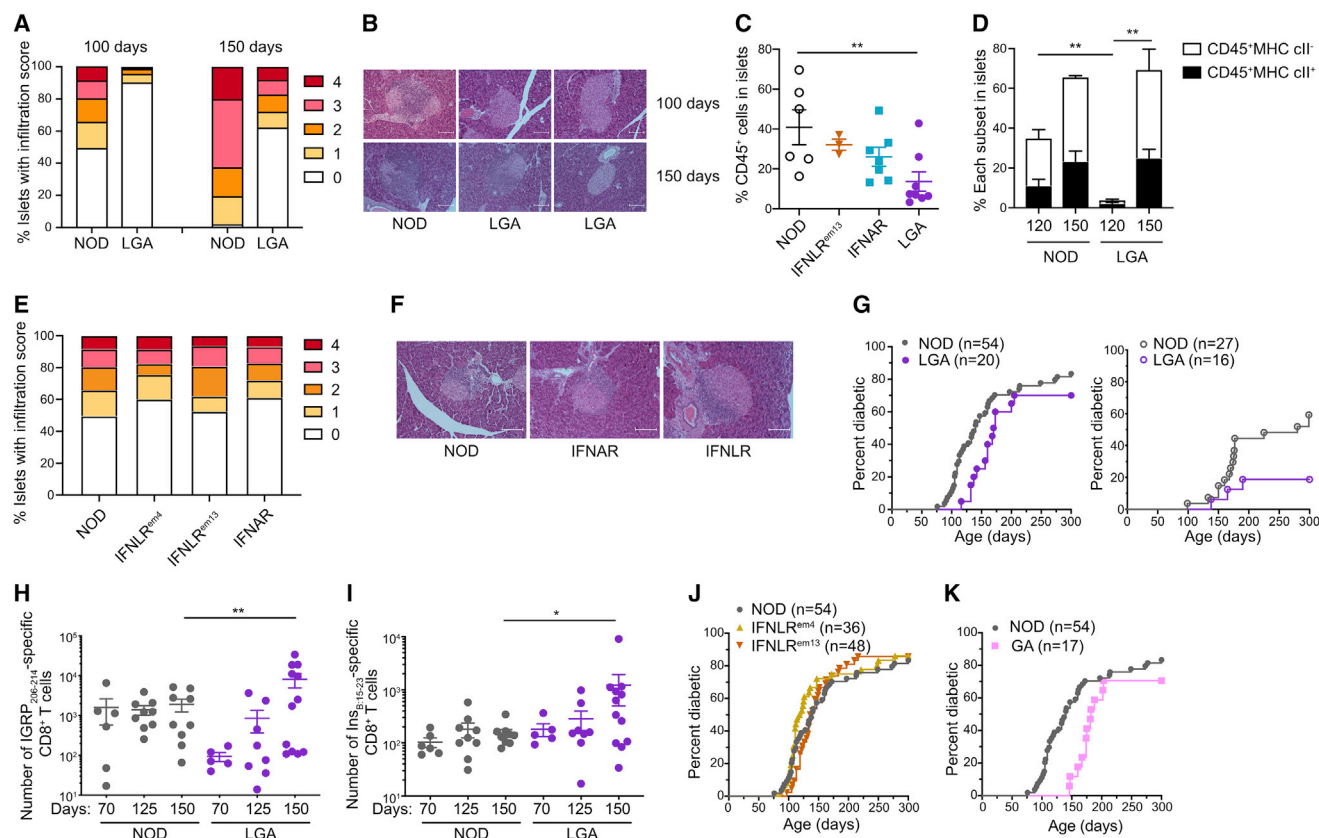


Figure 7. Reduced insulinitis with increased antigen-specific CD8⁺ T cells in triple IFN receptor-deficient NOD mice

(A) Pooled insulinitis scores of pancreas sections from female wild-type NOD (100 days old, $n = 10$; 150 days old, $n = 3$) or triple-mutant mice (labeled LGA; 100 days old, $n = 10$, 150 days old, $n = 5$). $p = 0.036$, 100 days NOD versus LGA; $p = 0.009$, 150 days NOD versus LGA; one-way ANOVA with Bonferroni's post-test.

(B) Representative photos of insulinitis. Magnification, $\times 200$; scale bar, $100 \mu\text{m}$.

(C) The proportion of CD45⁺ cells in islets from female wild-type NOD and single- or triple-mutant mice measured by flow cytometry at 100 days of age. Mean $\pm$ SEM is shown for individual mice. $**p = 0.006$, NOD versus LGA, one-way ANOVA with Sidak's post-test.

(D) The proportion of islet cells that are CD45⁺MHC class II⁺ or CD45⁺MHC class II⁻ in female wild-type NOD and triple-mutant mice measured by flow cytometry at 120 and 150 days of age. $**p < 0.005$, two-way ANOVA with Tukey's multiple comparisons test.

(E) Pooled insulinitis scores of pancreas sections from 100-day-old female wild-type NOD ($n = 10$) or *Ifnlr1*^{em4}-mutant (labeled IFNLR^{em4}, $n = 8$) and *Ifnlr1*^{em4}-mutant (labeled IFNLR^{em13}, $n = 9$) and *Ifnar1*-mutant (labeled IFNAR, $n = 9$) mice. Groups were not significantly different. One-way ANOVA with Bonferroni's post-test. Wild-type NOD mice are the same mice in (A) and (E) and in Figure 1A.

(F) Representative photos of insulinitis. Magnification, $\times 200$; scale bar, $100 \mu\text{m}$.

(G) Cumulative diabetes incidence of age-matched female (left, $p = 0.07$) and male (right, $p = 0.017$) cohorts of wild-type NOD or triple-mutant mice. Statistics were calculated by log rank (Mantel-Cox) test.

(H and I) Absolute numbers of (H) IGRP₂₀₆₋₂₁₅-specific ($**p = 0.005$ at 150 days) and (I) Ins_{B15-23}-specific CD8⁺ T cells ($*p = 0.04$ at 150 days). Data show mean $\pm$ SEM of individual female and male mice. Statistical analysis by one-way ANOVA with Sidak's post-test.

(J and K) Cumulative diabetes incidence of age-matched female cohorts of wild-type NOD, (J) *Ifnlr1*^{em4}-mutant (labeled IFNLR^{em4}), and *Ifnlr1*^{em4}-mutant (labeled IFNLR^{em13}) or (K) *Ifnlg1/Ifnar1* double-mutant mice (labeled GA). Diabetes incidence was calculated as follows: number of diabetic mice / (number of diabetic mice + number of mice still being monitored by 300 days of age). Curves were not significantly different using a log rank test with multiple comparisons. For clarity, the diabetes incidence curves for the single-, double-, and triple-mutant mouse lines are shown separately with the same wild-type NOD cohort.

progression was associated with increased numbers of autoantigen tetramer-binding CD8⁺ T cells (Figures 7H and 7I) similar to that seen in single *Ifnlg1*-mutant mice (Figures 2B and 2C).

As might be expected based on their degree of insulinitis, the two *Ifnlr1*-mutant NOD lines also had diabetes incidence curves similar to wild-type NOD mice (Figure 7J). Notably, *Ifnlg1/Ifnar1* double-mutant NOD mice also exhibited a delayed onset and cumulative incidence similar to triple-mutant NOD mice (Figure 7K), as also reported by others (Carrero et al., 2018), indicating that the lack of type III IFN receptor signaling does not contribute

to the delayed diabetes onset observed in triple-mutant NOD mice.

DISCUSSION

Transgenic BALB/c mice that overexpress IFN γ in their β cells (Sarvetnick et al., 1988) and, later, IFN α (Stewart et al., 1993) illustrate the pro-inflammatory and pro-autoimmune effect of IFNs that allow autoimmune diabetes to develop in a strain not genetically pre-disposed to it. Similarly, humans with

gain-of-function STAT1 mutations have been observed to develop autoimmune diabetes (Chaimowitz et al., 2020; Toubiana et al., 2016). If additional IFN γ caused diabetes, it was logical to think of IFN γ as a therapeutic target in mice and humans that were genetically programmed for diabetes, and the way to confirm this was to genetically disrupt its action. However, several groups have shown that NOD mice backcrossed with mice deficient in IFN γ or its receptors, or expressing a dominant-negative receptor, surprisingly developed diabetes normally (Hultgren et al., 1996; Kanagawa et al., 2000; Serreze et al., 2000; Thomas et al., 1998). This occurred despite loss of β cell upregulation of MHC class I expression, one of the pathological hallmarks of T1D in humans and NOD mice, known to be IFN γ mediated (Thomas et al., 1998).

We have now shown that, despite lack of IFN γ -mediated gene expression changes on APCs, endothelial cells, and β cells, *lfng1*-mutant NOD mice developed much greater numbers of antigen-specific CD8 $^{+}$ T cells, showing that a major effect of IFN γ in immune responses is curtailment of clonal expansion. This was also observed in NOD mice with mutations in the genes for the receptors of all three IFN receptors, although there was a delay in progression to diabetes in mice lacking both IFN γ and IFN α receptors. This confirms that unchanged progression to diabetes in single-knockout mice is not due to redundancy between IFNs. We propose that increased expansion of autoantigen-specific CD8 $^{+}$ T cells was likely responsible for equal progression to diabetes in *lfng1*-mutant NOD mice compared with the wild type; i.e., that T cell number compensated for the reductions in pro-inflammatory effects of IFN γ . These increased T cells developed, and were able to destroy β cells, despite reduced β cell MHC class I expression, reduced chemokine production, and reduced adhesion molecule expression on islet endothelial cells and β cells, and increased Treg cells. Antigen-specific CD8 $^{+}$ T cells were present in 10-fold higher numbers after onset of insulinitis in all *lfng1*-mutant NOD mice studied. This expansion appeared to apply primarily to antigen-experienced CD8 $^{+}$ T cells; i.e., cells involved in the autoimmune disease process. There was no general expansion of all CD8 $^{+}$ T cells. Increases were found in CD8 $^{+}$ T cells specific for proinsulin and IGRP. T cells specific for other autoantigens may also have been increased. Although IGRP-specific T cells are well recognized as a dominant pathogenic subpopulation of CD8 $^{+}$ T cells in NOD mice (Chee et al., 2014; Trudeau et al., 2003), it is unusual to see the prominence of proinsulin-specific CD8 $^{+}$ T cells we observed. They are low-affinity cells that are usually present in low numbers and without markers of antigen experience (Wong et al., 1999, 2009). Our data suggest that T cells in *lfng1*-mutant mice expanded because of increased sensitivity to γ c cytokines rather than increased stimulation through the TCR. In islets from NOD mice incubated with anti-IFN γ Ab, T cells responded more to IL-2. Similarly, splenic T cells, initially activated through the TCR and CD28, expanded to a greater extent in response to IL-2 in *lfng1*-mutant mice than in the wild type. In contrast it is unlikely that the observed increased expansion of T cells was due to stimulation through the TcR because MHC class I protein, was reduced in *lfng1* mutant mice. γ c cytokines increase proliferation and reduce apoptosis after T cell activation and expansion when T cell growth factors

are limiting (Newton and Strasser, 2000), and we observed reduced expression of pro-apoptotic molecules in T cells from our *lfng1*-mutant mice.

There are several potential mechanisms for enhanced lymphocyte expansion and function, and these mechanisms have been the focus of intense investigation because of their potential in tumor or viral immunotherapy. One possibility is the checkpoint pathway that involves PD-1 on T cells and its ligands on epithelial and antigen-presenting cells. PD-L1 is IFN γ regulated, and our data confirm low PD-L1 expression on β cells from infiltrated islets from *lfng1*-mutant mice compared with wild-type mice. Administration of an anti-PD-L1 Ab resulted in significant islet T cell proliferation, as expected. Data from groups studying blockade of this pathway in melanoma models show that this increase in proliferation is usually short lived (Huang et al., 2017; Pauken et al., 2016). Therefore, it is plausible that the PD-1 pathway may be partly responsible for the increased antigen-specific CD8 $^{+}$ T cells. But increased antigen-specific CD8 $^{+}$ T cells are not a feature of transgenic NOD mice with SOCS-1 expressed in beta cells under control of the insulin promoter (RIP-SOCS1) NOD mice, in which PD-L1 expression on β cells is reduced (Chee et al., 2014) in the context of a genetically normal T cell compartment. Also, blockade of PD-L1 in cultures of islet-derived T cells stimulated with IL-2 are less potent in stimulating proliferation compared with neutralization of IFN γ (data not shown), suggesting that IFN γ -regulated pathways other than PD-1 are involved in responses to IL-2. Our data support a model where increased expansion of antigen-specific CD8 $^{+}$ T cells from *lfng1*-mutant mice was due to more than one pathway, including IL-2 sensitivity and lack of PD-L1 inhibition and possibly others. We suggest that another such pathway, associated with IL-2 sensitivity, may be mediated by reduced levels of SOCS-1.

IL-2 signals via a receptor complex composed of CD25, CD122, and CD132 through the JAK-STAT pathway. Signaling through this complex is inhibited by SOCS-1.

SOCS-1 was discovered as a negative feedback inhibitor of the cytokines that induce it, like IFN γ (Endo et al., 1997; Naka et al., 1997; Starr et al., 1997). The kinase inhibitory region of SOCS-1 acts as a pseudo-substrate for JAK1, JAK2, and Tyk2 (Liau et al., 2018). JAK inhibitors act similarly as SOCS-1, but they bind in the ATP-binding pocket of JAKs (Liau et al., 2018). Mice with SOCS1 deficiency, as well as being hyper-responsive to IFN γ , have T cells that are hyper-responsive to cytokines that signal through the γ c receptor (Cornish et al., 2003; Davey et al., 2005; Gagnon et al., 2007). Our data suggest that the pro-inflammatory cytokine IFN γ not only limits signaling through its own receptor by stimulating SOCS-1 expression to prevent overshoot of the immune response, but increased SOCS-1 may also limit the immune response much more broadly. We suggest IFN γ -induced SOCS-1 inhibits expansion of T cells by IL-2 and that this is in part responsible for the increased numbers of CD8 $^{+}$ T cells we have observed in *lfng1*-mutant NOD mice.

Support for this model comes from the suppression of expanded T cell numbers in *lfng1* mutant NOD mice that were treated with a JAK1/JAK2 inhibitor with mechanism of action that closely mimics SOCS-1. These data are consistent with IFN γ acting as a principal regulator of STAT1 activation and

SOCS1 expression. *lfrgr1* mutant mice are not a complete knockout of SOCS-1 and do not have the same phenotype as IFN γ /SOCS1 double knockouts (Alexander et al., 1999) but indicate that reduced SOCS1 in T cells may regulate their number and function. IFN γ /SOCS1 double knockouts were generated because SOCS1 KO mice die when young from liver damage and other inflammatory effects and these are mediated in part by IFN γ . This allowed the mice to survive and T cells from these mice to be tested for responses to γ c cytokines. IFN γ /SOCS-1 double-knockout mice die younger than IFN γ knockout mice (Metcalfe et al., 2002). Analysis of pathological changes in IFN γ knockout mice revealed an increased lymphocyte count in peripheral blood and the peritoneal cavity and an increased spleen size compared with wild-type littermates. However, IFN γ knockout mice were not tested in these studies for hypersensitivity to γ c cytokines.

Humans with rare inactivating mutations in the IFN γ receptor can, at times, develop overactivity of the immune response that resembles the disease hemophagocytic lymphohistiocytosis (Humblet-Baron et al., 2019). This appears to be consistent with the notion that IFN γ has important roles in limiting, terminating, or contracting immune responses. These exaggerated responses may compensate for lack of IFN γ and allow these individuals to deal successfully with almost all infections. Recently there has been interest in autoimmune diabetes in individuals heterozygous for STAT1 gain-of-function mutations (Toubiana et al., 2016). The exact mechanistic basis of this is not well understood. It might be expected that these individuals have a phenotype that resembles IFN γ transgenic mice, including increased MHC class I expression and increased PD-L1 on β cells and other epithelial cells and upregulated endothelial cell adhesion molecule expression. These features and IL-2 sensitivity and levels of SOCS1 in immune cells have not been measured in affected individuals. Diabetes in one such patient was reversed by the JAK1/JAK2 inhibitor ruxolitinib (Chaimowitz et al., 2020).

The many pathways downstream of IFN γ indicate its role as a “master regulator” of inflammation (Zha et al., 2017) but, surprisingly, as a negative as well as positive regulator. Sensitivity to IL-2 resulting in greater expansion of CD8 $^{+}$ T cells, which we observed in *lfrgr1*-mutant T cells, is a phenotype that may be explained at least in part by low SOCS1 levels in T cells. Our data illustrate that, during a normal immune and inflammatory response, IFN γ promotes feedback that affects other cytokine pathways to prevent the usually undesired outcome of an unrestrained immune response. In some diseases, this regulation could be counterproductive, and reducing it may be beneficial. IFN γ -mediated regulation of CD8 $^{+}$ T cell numbers in wild-type mice does not prevent progression of diabetes in NOD mice, but it clearly slows it. It is unclear whether such endogenous processes could be enhanced to fully prevent progression or induce reversal of disease. Therapeutic use of JAK inhibitors, drugs that mimic the action of SOCS-1, are an attempt to do so pharmacologically. Conversely, heightened sensitivity to IL-2 may be useful to enhance immune responses in other settings.

Limitations of the study

It is likely that several biochemical pathways account for inhibition of the immune system by IFN γ , as discussed above. We

had only limited success in weighing the relative contributions of these pathways. Also, these studies focused on mouse and not human T1D, and it is unclear how directly applicable they are to human diabetes.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.celrep.2022.110747>.

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AUTHOR CONTRIBUTIONS

G.J., T.G., S.A., C.S., P.M.T., E.G.P., L.M., E.B., N.P., and D.J.D.G. designed and conducted experiments and collected and analyzed data. B.K., T.C.B., J.C., T.W.H.K., and H.E.T. designed the study, analyzed data, and wrote the manuscript. L.B. and I.S. provided reagents and wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
CD3 (500A2) V500	BD Biosciences	560771 (RRID:AB_1937314)
CD8 (53-6.7) APC	BioLegend	100712 (RRID:AB_312751)
CD8 (53-6.7) PerCPy5.5	BD Biosciences	551162 (RRID:AB_394081)
CD4 (GK1.5) APC-cy7	BioLegend	100414 (RRID:AB_312699)
CD4 (GK1.5) FITC	BD Biosciences	553729 (RRID:AB_395013)
CD45 (30-F11) PerCP-Cy5.5	BioLegend	103132 (RRID:AB_893340)
CD45.2 (104) APC	BioLegend	109814 (RRID:AB_389211)
CD45RA (14.8)	BD Biosciences	553380 (RRID:AB_394822)
CD44 (1M7) PE-Cy7	BioLegend	103030 (RRID:AB_830787)
PD-1 (29f.1A12) BV605	BioLegend	135220 (RRID:AB_2562616)
TIM-3 (215,008) AF488	RD Systems	FAB1529G (RRID:NA)
TIGIT (VSIg9) PE/Dazzle	BioLegend	142110 (RRID:AB_2566573)
CD11b (M1/70) eFluor450	eBioscience	48011282 (RRID:AB_1582236)
CD11c (N418) eFluor450	eBioscience	48011482 (RRID:AB_1548654)
B220 (RA3-6B2) eFluor450	eBioscience	48045282 (RRID:AB_1548761)
B220 (RA3-6B2) PE	BioLegend	103208 (RRID:AB_312993)
Ly6G (RB6-8C5) eFluor450	eBioscience	48-5931-82 (RRID:AB_1548788)
F4/80 (BM8) eFluor450	eBioscience	48-4801-82 (RRID:AB_1548747)
PD-L1 (M1H5) APC	eBioscience	17-5982-82 (RRID:AB_2688092)
TCR-beta (H57-585) FITC	eBioscience	11596182 (RRID:AB_465323)
MECA-32 (MECA-32) BV786	BD Biosciences	743076 (RRID:AB_2741269)
CD16/CD32 (2.4G2)	BD Biosciences	553142 (RRID:AB_394656)
MAdCAM-1 (MECA-367) PE	BioLegend	120709 (RRID:AB_2629559)
VCAM-1 (429) PE-cy7	BioLegend	105719 (RRID:AB_2214047)
CD25 (3C7) APC	BioLegend	101910 (RRID:AB_2280288)
FoxP3 (FJK-16s) PE	eBioscience	12-5773-82 (RRID:AB_465936)
Helios (22F6) FITC	eBioscience	11988382 (RRID:AB_11041115)
Helios (22F6) eFluor450	eBioscience	48-9883-42 (RRID:AB_2574136)
pY701 pSTAT1 (14/P-STAT1)	BD Biosciences	612132 (RRID:AB_399503)
Tyr694 pSTAT5	Cell signaling technologies	9351 (RRID:AB_2315255)
Beta-Actin (8H10D10)	Cell signaling technologies	3700 (RRID:AB_2242334)
MHC-1 (SF1-1.1) Biotin	BD Biosciences	553564 (RRID:AB_394922)
CD127 (A7R34) BV785	BioLegend	135037 (RRID:AB_2565269)
Streptavidin APC	BioLegend	405207 (RRID:NA)
CD3 (145-2C11)	BD Biosciences	553057 (RRID:AB_394590)
CD28 (NA/LE 37.51)	BD Biosciences	553294 (RRID:AB_394763)
anti-IFNgamma (HB170)	Walter and Eliza Hall antibody facility	NA
PD-L1 (M1H5)	Bioceros	NA
Chemicals, peptides, and recombinant proteins		
IL-2	Peptotech	200-02
IL-15	Peptotech	200-15
IFN γ	Biolegend	575306
IFN- α	PBL-Assay Science	12105-1
IFN λ 2	R&D systems	4635-ML/CF

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Tetramer- IGRP: ²⁰⁶⁻²¹⁴ H2-K ^d -VYLKTNVFL-PE	NIH tetramer core (https://tetramer.yerkes.emory.edu/)	NA
Tetramer-InsB: ¹⁵⁻²³ H2-K ^d -LYLVCGGEG-APC	NIH tetramer core	NA
Tetramer-Ins B: ¹⁰⁻²³ I-Ag ⁷ -HLVERLYLVCGGEG-PE	NIH tetramer core	NA
Tetramer-BDC2.5 mimotope I-Ag ⁷ -AHHPIWARMADA-APC	NIH tetramer core	NA
JAK1/JAK2-inhibitor (LN3103801)	Eli Lilly	NA
Collagenase-P	Roche	11213865001
Histopaque-1077	Sigma-Aldrich	10771
5-Bromo-2'-deoxyuridine (BrdU)	Sigma-Aldrich	B5002
CMRL-1066 culture medium	Gibco	11530037
RPMI-1640 culture medium	Gibco	11875119

Critical commercial assays

Foxp3/transcription factor staining kit	eBioscience	00-5523
BrDU Staining Kit	BD Pharmingen	552598
anti-PE beads	Miltenyi Biotec	130-048-801
anti-APC beads	Miltenyi Biotec	130-097-143
ISOLATE RNA micro kit	Bioline	BIO-52075
High-Capacity cDNA RT kit	Applied Biosystems	4368814

Deposited data

Bulk T cell receptor (TCR β) sequencing data	Gene Expression Omnibus (GEO)	Accession number: GSE199264
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Experimental models: Organisms/strains

NOD/Lt	BioResources Center, St. Vincents Institute	
NOD CD45.2(NOD.C-(Ptpcr-D1Mit262)/WehiJ)	BioResources Center, St. Vincents Institute	Steptoe RJ et al., 2004 (PMID: 14987741)
IFNGR	This paper	
IFNAR	This paper	
IFNLR	This paper	
LGA	This paper	
NOD.SOCS1/hCD4	BioResources Center, St. Vincents Institute	Chong MM et al., 2003 (PMID: 12705851)

Oligonucleotides

See Table S2	NA
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Software and algorithms

Flowjo v10	BD Biosciences	www.flowjo.com
Graphpad Prism v9	Graph Pad Software	www.graphpad.com/scientific-software/prism/
R (version 4.1)	NA	https://www.r-project.org
MixCR	Bolotin DA et al. (2015) PMID 25924071	https://github.com/milaboratory/mixcr/releases/tag/v3.0.13
MIGEC	Shugay M. et al. (2014) PMID 24793455	https://github.com/mikessh/migec/releases

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead Contact, Thomas Kay (tkay@svi.edu.au).

Materials availability

All unique mouse strains generated in this study are available upon request with a completed material transfer agreement.

Data and code availability

- Bulk T cell receptor (TCR β) sequencing data have been deposited in the Gene Expression Omnibus (GEO) under the accession number GSE199264 and are publicly available as at the date of publication.
- This paper does not report original code.
- Any additional information required to reanalyse the data reported in this paper is available from the [lead contact](#) upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice

Mutant NOD mice were generated by the Australian Phenomics Facility (Monash University, Clayton, Australia) as approved by the Monash University animal ethics committee. Selected mutant NOD mice were subsequently bred and maintained, along with wild-type NOD/Lt mice, NOD CD45.2 NOD.C-(Ptpcr-D1Mit262)/WehiJ (Steptoe RJ et al., 2004; PMID 14987741) and NOD.SOCS1/hCD4 mice (Chong MM et al., 2003; PMID: 12705851) at St Vincent's Institute. CRISPR/Cas9 gene editing was used to target *lfnr1*, *lfnr1* and *lfnr1* in the non-obese diabetic (NOD) mouse strain. Single guide RNAs (sgRNA) were designed within the first exon of each gene using the CRISPR Design Tool (<http://crispr.mit.edu>) for *lfnr1* (sgRNA 5'GCTCGCTGCTGCTGGGCGCGG-3'), *lfnr1* (sgRNA 5'CTGTCTGCGAAGGTCGGGAG-3'), and *lfnr1* (sgRNA 5'GAGTAGGGGCGCCACCGGT-3'). A pair of complementary oligonucleotides for each sgRNA were annealed and cloned into vector pX330-U6-Chimeric_BB-CBh-hSpCas9 (Addgene plasmid # 42,230) and verified by sequencing. Vectors containing the cloned sgRNA sequences were used to generate PCR products (Pfx50 DNA polymerase, Invitrogen) that were used as templates for *in vitro* transcription using the MEGAshortscript T7 Transcription Kit (Life Technologies). The sgRNAs were purified using the MEGAclear Transcription Clean-Up Kit (Life Technologies). Embryos were generating by mating superovulated NOD/ShiLtJArc females to NOD/ShiLtJArc males. The three sgRNAs and Cas9 mRNA (15 ng/ μ L, 30 ng/ μ L, respectively) were mixed in 1x Tris-EDTA nuclease free water and co-injected into either the cytoplasm or pronucleus using a Femtojet (Eppendorf). Injected embryos were transplanted into oviducts of pseudopregnant (C57BL/6xCBA) F1 recipient females to generate G0 offspring that were screened for mutations by sequencing PCR-derived amplicons for the first exon of each gene. Gene-targeted sequencing of G0, G1 and wild-type NOD mice using next-generation sequencing was also performed. Briefly, PCR was used to generate amplicons encompassing the three sgRNA target sites in each mouse. Amplicons for each gene were then pooled for individual mice. The pooled amplicons representing one mouse were uniquely bar-coded using Nextera XT indices (Illumina) and then purified using AMPure XP beads (Illumina). Purified bar-coded amplicons for each mouse were quantified using LabChip GX (PerkinElmer), normalized and then the mouse-specific amplicon bar-coded libraries were all pooled together. The pooled libraries were then sequenced using MiSeq, 2 $\times$ 250bp v2 chemistry, nano flow cell (Illumina). Sequences were separated based on mouse-specific bar codes, aligned to the NCBI37/mm9 genomic reference, and individual sequence mutations visualized using the Integrative Genomics Viewer ([Thorvaldsdóttir et al., 2013](#)).

G0 mice were selected for breeding based on chosen gene mutations and intercrossed to establish single-, double- or triple-mutant NOD mice. To genotype mice for individual mutations, DNA was isolated from tail biopsies and one of the following methods was used depending on the mutation to be genotyped: high-resolution melt (LightCycler 480 High Resolution Melting Master, Roche Diagnostics) or mutation-specific Taqman probe assays (LightCycler 480 Probes Master Mix, Roche Diagnostics) using a LightCycler 480II (Roche Diagnostics), or PCR-amplicon size detected by gel electrophoresis. Both male and female mutant mice and age matched controls between ages of 10–22 weeks were used for experiments unless indicated otherwise. All animal studies were conducted at St. Vincent's Institute and approved by the institutional animal ethics committee.

METHOD DETAILS

Diabetes and insulinitis

For diabetes monitoring mice were tested once a week starting at $\sim$ 80 days of age for elevated urinary glucose using Diastix reagent strips (Bayer Diagnostics). Mice with a positive glycosuria reading (>110 mmol/L) were confirmed diabetic the next day by a positive blood glucose reading (>15 mmol/L), using Advantage II Glucose Strips (Roche). Mice were tested by three different animal technicians over the 300-day time course and were blinded to the genotypes of the individual mice. For insulinitis scoring pancreata were prepared using Bouin's fixative and were paraffin embedded. Tissue sections (5 μ m) were stained with hematoxylin and eosin (H&E). At least three noncontiguous sections of each pancreas were microscopically examined by an investigator blinded to genotype and age and islets scored for infiltrating leukocytes as described ([Thomas et al., 1998](#)): islets scored as 0 (no infiltration), 1 (peri-islet infiltrate - leukocytes present at any point around islet), 2 ($>50\%$ peri-islet infiltrate), 3 (intra-islet infiltrate), or 4 ($>80\%$ intra-islet infiltrate). Between 20 and 65 islets/mouse with $n = 3$ –10 mice/genotype were scored and averaged to calculate insulinitis scores.

Islet isolation and culture

Islets of Langerhans were isolated using collagenase P (Roche, Basel, Switzerland) and Histopaque-1077 density gradients (Sigma-Aldrich) as previously described (Graham et al., 2012). Islets were cultured in CMRL-1066 medium (Life Technologies) containing penicillin/streptomycin, 2 mM glutamine and 10% FCS, with or without recombinant mouse IFN- γ (100 U/mL, BioLegend) or recombinant mouse IFN- λ 2 (100 U/mL, R&D systems) for 48 h at 37°C in 5% CO₂. Islets were made into single cell suspensions as previously described (Graham et al., 2012) and analyzed for MHC class I expression using flow cytometry. To expand islet-infiltrating T cells, islets were isolated as above and 50 hand-picked islets were placed in each well of a 48 well tissue culture plate with 1.5 mL of RPMI-1640 medium (Life Technologies) supplemented with antibiotics, 2 mM glutamine, nonessential amino acids, 50 μ M mercaptoethanol, 10% FCS and 10 U/mL rhIL-2 (Peprotech) and cultured with or without IFN- γ neutralizing antibody (1 μ g/mL, clone HB170) for 5 days at 37°C in 5% CO₂. Expanded T cells were stained with H2-K^d VYLKTNVFL tetramer to detect IGRP₂₀₆₋₂₁₄ specific CD8⁺ T cells and analyzed by flow cytometry as described below.

JAK inhibitor treatment

A fine suspension of the JAK1/JAK2 selective inhibitor LN3103801 (Eli Lilly) was prepared by sonicating the compound in vehicle (0.5% methyl cellulose +0.25% Tween-80) for 60 min. 95-100-day-old NOD or IFNGR mice were treated twice daily by oral gavage with vehicle or LN3103801 (3 mg/kg) for 2 weeks. At the end of the treatment period pooled spleen and non-draining lymph nodes were collected for further analysis.

BrdU injection

Bromodeoxyuridine (BrdU) (Sigma Aldrich) powder was dissolved in PBS (10 mg/mL). Mice were injected i.p. with 200 μ L (2 mg) of BrdU solution. Pooled spleen and non-draining lymph nodes were harvested 24 h after injection and BrdU incorporation was analyzed by flow cytometry.

Anti-PDL1 antibody treatment

Anti-PDL1 (M1H5) monoclonal antibody (mAb) was administered to 100-day old NOD or *Ifngr1-mutant* mice were injected with 500 μ g of anti-PD-L1 mAb intraperitoneally on day 1 followed by injection of 250 μ g of anti-PD-L1 mAb on days 3, 5 and 7. Mice were monitored for diabetes as described above. For T cell proliferation analysis, 7-8-week old NOD mice were injected with one dose of 250 μ g of anti-PD-L1 mAb on day 0. Mice were injected with BrdU on day 6 as described above. Islets were harvested on day 7 after injection and BrdU incorporation was analyzed by flow cytometry.

Flow cytometry

Single cell suspensions of islets were stained with PerCp-Cy5.5-labeled anti-CD45 antibody (30-F11) (BD Pharmingen) and biotinylated anti-H2-K^d (BD Pharmingen; SF1-1.1) followed by streptavidin-allophycocyanin (APC) (BioLegend) or anti-PD-L1-APC (M1H5, eBioscience) antibodies. Beta cells were identified based on their autofluorescence as previously described (Darwiche et al., 2003). Single cell suspensions from spleen were prepared using standard methods. For flow cytometry analysis Tris-buffered ammonium chloride was used to lyse red blood cells. Viable cell counts were determined using the Trypan Blue exclusion method. The following antibodies were purchased from BD Pharmingen, BioLegend or eBioscience: anti-CD3-V500 (500 A2), anti-CD11c (N418), anti-B220 (RA3-6B2), anti-CD11b (M1/70), anti-F4/80 (BM8) conjugated to eFluor450 anti-CD4-PE or APC-Cy7 (GK1.5), anti-CD8 α -PerCpCy5.5 (53-6.7), anti CD44-PE-cy7 (1M7), anti-IL7R α (CD127)-Brilliant violet 785 (A7R34), anti-PDL1-APC (M1H5), anti-PD-1(CD279)-Brilliant violet 605 (29f.1A12), anti-TCR β -FITC (H57-585), anti-MECA-32-BV786 (MECA-32), anti-MAdCAM-1-PE (MECA-367), Anti-VCAM-1-PE-Cy7 (429), Anti-CD25-APC (PC61), Anti-Stat1 (pY701)-AF488 (4a) and Anti-FoxP3-PE (FJK-16S). Fc receptors were blocked before surface staining by incubating cells with anti-CD16/CD32 antibody (clone 2.4G2). Propidium iodide (PI, Calbiochem) was added immediately prior to flow cytometry analysis to exclude dead cells. FoxP3 was stained intracellularly using FoxP3/Transcription Factor Fixation/Permeabilization kit (eBiosciences). For detection of pSTAT-1 cells were fixed in 4% PFA for 10 min at room temperature, and permeabilized with ice-cold methanol for 30 min on ice, followed by staining with pSTAT1 antibody. Intracellular detection of BrdU was performed using the APC-BrdU FlowKit (BD Biosciences). Flow cytometry data were collected on an LSR-Fortessa cell analyzer with FACSDiva software (BD Biosciences) and analyzed using FlowJo software version 10 (Tree Star, Ashland, OR).

Tetramer staining and magnetic bead-based enrichment

The tetramer staining and magnetic bead-based enrichment method was previously described (Chee et al., 2014). All tetramers were obtained from the NIH tetramer core (Georgia, USA). Single cell suspensions from peripheral lymphoid organs (PLO), (pooled spleen and non-draining lymph nodes comprising of 2 inguinal and 4 mesenteric lymph nodes per sample) were stained with phycoerythrin (PE)-conjugated I-A^{g7}-INS_{B10-23} (HLVERLYLVCGGEG) tetramer or allophycocyanin (APC) -conjugated H-2^{Kd}-INS_{B15-23} (LYLVCGGEG) for 1 h on ice to enrich insulin-specific CD4⁺ or CD8⁺ T cells respectively. IGRP₂₀₆₋₂₁₄ specific CD8⁺ T cells (H2-K^d, VYLKTNVFL) were stained and enriched as previously described (Chee et al., 2014). Chromogranin specific CD4⁺ T cells were detected using APC conjugated I-A^{g7}-BDC2.5 mimotope (AHHPIWARMDA) tetramer. I-A^{g7}-CLIP (PVSKMRMATPLLMQA) tetramer or H-2^{Kd}-TUM (KYQAVYTTTL) were used as controls. Cells were then washed and stained with anti-PE or anti-APC microbeads

(Miltenyi Biotec) followed by magnetic separation using an AutoMACSpro (Miltenyi Biotec) according to manufacturer's instructions. The separated fractions were stained and analyzed by flow cytometry. Gating strategy for tetramer enrichment was as follows: single cells were gated on forward and side scatter, and dead cells excluded using propidium iodide. From the live cell population, CD3⁺ dump⁻ (dump = CD11c, CD11b, B220 and F4/80) cells were gated as the T cell population for analysis of tetramer binding CD4⁺ or CD8⁺ T cells.

In vitro culture and activation of spleen cells

Single cell suspensions of spleen were prepared by gently pressing the tissues through 70-micron nylon mesh. Cell suspensions were washed twice in in RPMI 1640 medium containing 10% FBS, antibiotics, 2 mM L-glutamine, and 50 μ M-mercaptoethanol (RPMI 1640 complete medium) and gently overlaid on Histopaque-1083 (Sigma-Aldrich) solution, followed by centrifugation to fractionate lymphocytes. Purified lymphocytes were washed twice in complete RPMI-1640 medium and rested for 1 h at 37°C in 5% CO₂. For activation of lymphocytes, wells of a 12-well tissue culture plate were coated with purified anti-CD3 (145-2C11, 2.5 μ g/mL, BD Biosciences) antibody in 2 mL PBS for 3–4 h at 37°C in 5% CO₂. Coated wells were washed once with PBS and 2 $\times$ 10⁶ purified lymphocytes were plated in 2 mL of complete RPMI-1640 medium containing 1 μ g/mL anti-CD28 (NA/LE, 37.51, BD Biosciences) antibody and cultured for 72 h at 37°C in 5% CO₂. Activated spleen cells were plated at a density of 0.25 $\times$ 10⁶ cells/well in 1 mL complete RPMI-1640 medium with 50 U/mL rhIL-2 (Peprotech) and cultured for 14 days. Cell counts were obtained at days 3, 7, 10 and 14 after activation. Media was changed and IL-2 supplemented at each time point.

Western blot analysis of pSTAT1 and pSTAT5

For measurement of pSTAT1 peripheral blood was collected from mice by orbital bleed into heparinized collection tubes. Whole blood was stimulated with either recombinant mouse IFN- α (100 U/mL, PBL Assay Science) or recombinant mouse IFN- γ (100 U/mL, Biolegend) for 30 min at 37°C. Red blood cells were lysed (155 mM NH₄Cl, 10 mM Tris-HCl, pH 7.5). For measurement of pSTAT5, splenocytes activated with anti-CD3/anti-CD28 antibodies were treated with 5 U/mL IL-2 (Peprotech) and 1 $\times$ 10⁵ cells were collected after 0.5, 1, 3, 6, 8 and 24 h timepoints. Cells were washed twice in cold PBS and lysed in 100 μ L RIPA buffer containing 1% protease inhibitors (Sigma, P2714-1BTL) and 0.25% endonuclease (Lucigen, OC7850K) as well as sodium orthovanadate (0.1 mM), sodium pyrophosphate (2.3 g/L) and sodium fluoride (4.2 g/L) as phosphatase inhibitors. Clarified lysates were mixed with NuPAGE LDS Sample Buffer (ThermoFisher) and 0.24 μ L beta-mercaptoethanol and heated for 5 min at 99°C. Total protein was resolved on a 4–12% Bolt BIS-Tris Gel (ThermoFisher). Samples were then transferred to a 45 μ M nitrocellulose membrane. Membrane was probed for Tyr694 pSTAT5 (Cell Signaling Technologies 9351), anti-p-STAT1 (pY701) (clone 14/P-STAT1, BD Biosciences) or anti- β -Actin (8H10D10, Cell Signaling Technology) and imaged using anti-mouse IgG (H + L) DyLight secondary antibodies (ThermoFisher) for pSTAT1 and beta-actin or anti-rabbit IgG (H + L) for pSTAT5 on a Licor Odyssey CLx Near-Infrared Fluorescence Imaging System.

RT-PCR

Total RNA was extracted from 1 $\times$ 10⁵ activated splenocytes, 80–100 whole islets or expanded T cells from the islets. Cells or tissues were washed twice in cold Phosphate Buffered Saline (PBS) and lysed in 250 μ L of RNA lysis buffer RLY (Bioline). RNA was isolated using ISOLATE RNA Micro kit (Bioline), and first strand cDNA was generated from 500 ng RNA using High-Capacity cDNA Reverse Transcription kits (Applied Biosystems) according to the manufacturers' instructions. cDNA was diluted (1:20) and Real-time PCR analysis was performed using LightCycler 480 (Roche). Taqman gene expression primers for murine SOCS1 (Socs1; Mm00782550_s1), SOCS3 (Socs3; Mm00545913_s1), Ptpn2 (*Ptpn2*; Mm00501226_m1), BIM (*Bcl2l1*; Mm00437795_m1), BAX (*Bax*; Mm00432050_m1) and β -actin (*Actb*; Mm00607939_s1) were purchased from Applied Biosystems. Threshold cycle (Ct) values were normalised to the Ct values of actin in individual samples and expressed as Δ Ct.

Bulk TCR β sequencing and TCR β repertoire diversity analysis

TCR β amplicon libraries were generated with 5' Rapid Amplification of cDNA Ends (5'RACE) technology previously optimised (Principe et al., 2020). RNA was extracted from sorted cell samples using the RNeasy Plus Micro Kit (QIAGEN). RNA was transcribed to cDNA with a TCR β constant region primer and a modified SMARTerIIA primer (Integrated DNA Technologies), with 9nt long unique molecular identifiers (UMI) added to individual TCR β cDNA sequences. TCR β locus was amplified by nested PCR with additional TCR β constant region primers, and a universal primer to SMARTerIIA, with the final PCR adding sequencing adaptors and barcodes to the TCR β libraries. Products after each PCR were purified using AMPure XP AvenCourt Beads (Beckman Coulter) and run on the Agilent TapeStation for quality control of DNA integrity. Paired-end (2 $\times$ 300 bp) high-throughput sequencing was performed using the Illumina MiSeq platform. Data processing, aggregation of UMIs and alignment of CDR3 sequences to the IMGT/V-QUEST reference genome were performed using repertoire analysis software based on MIGEC and MiXCR pipelines. Only sequences with UMIs were aligned.

A TCR β clone was defined by the CDR3 amino acid sequence in this study. Clones that were less than 8 or greater than 20 amino acids in length, included a stop codon or a frameshift were defined as non-functional and were excluded. The number of total TCR β clones and frequency of unique TCR β clones in samples was determined. As Rényi diversity profiles are sensitive to the total sample size, all samples were down sampled to 1000 CDR3s to generate Rényi diversity profiles. All α values were generated by using the Rényi function from the vegan package in R. Rényi entropy is represented by (Joshi et al., 2019):

$$H_{\alpha}(x) = \frac{1}{1 - \alpha} \log_2 \left(\sum_{i=1}^n \rho_i^{\alpha} \right)$$

α is a scale of values, ranging from 0 to infinity. $\alpha = 0$ corresponds to the richness of the repertoire (number of unique TCR β clones). $\alpha = 1$, and $\alpha = 2$ correspond to traditional diversity metrics, Shannon's entropy and Simpson's diversity metric respectively. Where the curve reaches a plateau or $\alpha =$ infinity, it indicates how abundant the most dominant clonotypes are in the sample in terms of diversity. The more abundant the most frequent clone is in the sample, the more clonal the repertoire therefore the lower the diversity. Importantly, the gradient of the slope in each curve increases as the distribution of the TCR β repertoire becomes more monoclonal. Diversity Evenness 50 (DE₅₀) values were also calculated as a ratio of the number of unique TCR β sequences that made up 50% of the repertoire, divided by the total number of unique TCR β sequences. High DE₅₀ values correspond to each clonotype evenly represented in terms of frequency. Low DE₅₀ values correspond to a TCR repertoire dominated by certain specific CDR3s (Hosoi et al., 2018).

QUANTIFICATION AND STATISTICAL ANALYSIS

All Statistical analyses were performed using GraphPad Prism 9 software (GraphPad, San Diego, USA). A two-tailed unpaired Student's t-test was used for comparisons between two groups. Multiple comparisons were performed using one-way ANOVA with either Tukeys or Bonferroni post-hoc test as indicated in figure legends. Differences between paired measurements were analyzed by paired ttest. Diabetes incidence curves were compared using Log -rank (Mantel-Cox) test. In all graphs, each symbol represents an individual sample, and the error bars represent the mean $\pm$ standard error of mean. *p < 0.05, **p < 0.01, ***p < 0.001.