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Induction of autoimmune diabetes in non-obese diabetic mice requires interleukin-21-dependent activation of autoreactive CD8$^+$ T cells

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Summary

Non-obese diabetic (NOD) mice lacking interleukin (IL)-21 or IL-21 receptor do not develop autoimmune type 1 diabetes (T1D). We have shown recently that IL-21 may promote activation of autoreactive CD8$^+$ T cells by increasing their antigen responsiveness. To investigate the role of IL-21 in activating diabetogenic CD8$^+$ T cells in the NOD mouse, we generated IL-21-deficient NOD mice expressing the highly pathogenic major histocompatibility complex (MHC) class-I-restricted 8.3 transgenic T cell receptor (TCR). IL-21 deficiency protected 8.3-NOD mice completely from T1D. CD8$^+$ T cells from the 8.3-NOD.*Il21*$^{-/-}$ mice showed decreased antigen-induced proliferation but displayed robust antigen-specific cytolytic activity and production of effector cytokines. IL-21-deficient 8.3 T cells underwent efficient homeostatic proliferation, and previous antigen stimulation enabled these cells to cause diabetes in NOD.Scid recipients. The 8.3 T cells that developed in an IL-21-deficient environment showed impaired antigen-specific proliferation *in vivo* even in IL-21-sufficient mice. These cells also showed impaired IL-2 production and *Il2* gene transcription following antigen stimulation. However, IL-2 addition failed to reverse their impaired proliferation completely. These findings indicate that IL-21 is required for efficient initial activation of autoreactive CD8$^+$ T cells but is dispensable for the activated cells to develop effector functions and cause disease. Hence, therapeutic targeting of IL-21 in T1D may inhibit activation of naive autoreactive CD8$^+$ T cells, but may have to be combined with other strategies to inhibit already activated cells.

Keywords: CD8+ T cells, IL-2, IL-21, NOD mouse, type 1 diabetes

Introduction

Non-obese diabetic (NOD) mice develop spontaneously autoimmune insulin-dependent type 1 diabetes (T1D), which shares many disease characteristics with human T1D. Susceptibility or resistance to T1D is determined genetically by several insulin-dependent diabetes (*Idd*) loci. The *Idd3* locus encompasses a 650 kb region on chromosome 3 and contains genes encoding interleukin (IL)-2 and IL-21 [1,2]. In the NOD mouse, polymorphisms at the *Il2* gene promoter and decreased transcription and stability of IL-2 mRNA are implicated in reduced IL-2 production, which has been correlated with reduced frequency and functions of CD4$^+$CD25$^+$ regulatory T cells (T_{regs}) [1,3,4]. The ability of the C57BL/6-derived *Idd3* locus to protect NOD mice from insulitis and diabetes has been correlated with reduced IL-21 mRNA and protein levels [1,5,6]. The importance of IL-21 in T1D pathogenesis is demonstrated by the failure of NOD mice lacking IL-21 or IL-21 receptor alpha chain (IL-21Rα) to develop T1D [7–11]. How IL-21 promotes pathogenesis of T1D is not yet clear.

IL-21 is produced mainly by natural killer (NK) T cells and CD4$^+$ T cells [12,13]. All CD4$^+$ T helper subsets can produce varying amounts of IL-21, depending on the context of stimulation and the cytokine milieu [14,15]. IL-21 acts as an autocrine growth factor that shifts the balance away from T_{regs} towards the T helper type 17 (Th17) lineage, promoting inflammation and immune response [16,17]. In psoriasis and multiple sclerosis Th17 cells, driven partly by IL-21, play a significant role in promoting

tissue damage [18–20]. Early studies in NOD mice lacking IL-21Rα have also implicated IL-21 in T1D pathogenesis via Th17 cells [8,15]. However, the role of Th17 cells in the pathogenesis of T1D remains controversial. In fact, Th17 cells produced in the gut have been shown to exert a protective effect in T1D [21–25].

$CD8^+$ T lymphocytes play a key role in the pathogenesis of autoimmune diseases by causing damage to target organs [26]. Two recent studies have implicated IL-21 in T1D pathogenesis via promoting expansion and survival of $CD8^+$ T cells [9,11]. Studies on the role of IL-21 in viral infections showed that IL-21 signalling is indispensable for robust primary and secondary $CD8^+$ T cell responses to chronic viral infections [27–31]. These studies suggested that IL-21 may also be needed for the efficient activation of autoreactive $CD8^+$ T cells. This possibility is supported by our recent finding that IL-21, in synergy with IL-15, enables naive autoreactive $CD8^+$ T cells to respond to weak TCR agonists and induce disease in an engineered model of T1D [32]. In the present study, we have examined the role of IL-21 in activating autoreactive $CD8^+$ T cells in the NOD mouse expressing the transgenic 8.3 T cell receptor (TCR) [33]. Our findings indicate that IL-21 is required for the initial activation of autoreactive $CD8^+$ T cells, but is dispensable for sustaining their effector functions and their ability to induce disease.

Materials and methods

Mice

NOD mice (NOD/ShiLtJ) and 8.3 TCR transgenic NOD mice [NOD.Cg-Tg(TcraTcrbNY8.3)1Pesa/DvsJ; for brevity, 8.3-NOD] were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). *Il21*$^{-/-}$ mice generated in a 129/SvEvBrd × C57Bl/6/J background (Lexicon Genetics Inc., The Woodlands, TX, USA) were obtained from MMRRC (Mutant Mouse Regional Resource Centre, Jackson Laboratory), back-crossed to NOD mice for 10 generations and back-crossed further to 8.3-NOD mice for two generations. At the fifth back-cross, mice were genotyped for known *Idd* loci and were selected for further breeding. The progeny of the 11th back-cross were intercrossed to generate NOD.*Il21*$^{-/-}$, NOD.*Il21*$^{+/-}$ and NOD.*Il21*$^{+/+}$ littermates. Mice were housed in micro-isolated sterile cages under specific pathogen-free (SPF) conditions. All experimental protocols were approved by the institutional ethical committee.

Antibodies and reagents

Antibodies against mouse CD3ε, CD4, CD8α, TCRVβ8.3, tumour necrosis factor (TNF)-α and interferon (IFN)-γ, conjugated to fluorochromes or biotin, and fluorescent streptavidin conjugates were purchased from BD Pharmingen Biosciences (Palo Alto, CA, USA) or eBioscience (San Diego, CA, USA). Major histocompatibility complex (MHC) class-I H-2k^d-restricted cognate antigenic peptides islet-specific glucose-6-phosphatase catalytic subunit-related protein ($IGRP_{206–214}$) (VYLKTNVFL) and its mimotopes NRP (KYNKANWFL; agonist), NRP-V7 (KYNKANVFL; super agonist) and TUM (KYQAVTTTL; non-agonist) were custom synthesized by Genscript (Piscataway, NJ, USA).

Flow cytometry

Expression of cell surface markers was evaluated by flow cytometry using fluorescence activated cell sorter (FACS-)Canto flow cytometer (Becton Dickinson Flow Cytometry Systems, San Jose, CA, USA) and the data were analysed using FlowJo software (Tree Star Inc., Ashland, OR, USA).

Cell proliferation

Total lymph node cells (2×10^5 cells) or purified $CD8^+$ T cells ($2{\cdot}5 \times 10^4$ cells) were cultured in 96-well culture plates with the indicated peptides using irradiated splenocytes as antigen-presenting cells (APCs) (1×10^5 cells) or with anti-CD3/CD28-coated beads for 72 h. Cell proliferation was measured by [^{3}H]-thymidine incorporation [34].

To measure antigen-induced proliferation *in vivo*, 8.3 $CD8^+$ T cells were labelled with carboxyfluorescein diacetate succinimidyl ester (CFSE), as described previously [35], and injected intravenously. Bone marrow-derived dendritic cells (BMDCs) cultured with granulocyte–macrophage colony-stimulating factor (GM-CSF) and IL-4 were pulsed with $IGRP_{206–214}$ or the control peptide TUM for 1 h at 37°C, washed, resuspended in phosphate-buffered saline (PBS) and injected subcutaneously in hind footpads. Donor cells recovered from the draining inguinal lymph node were evaluated to measure proliferation.

Cytotoxic T lymphocyte (CTL) assay and enzyme-linked immunosorbent assay (ELISA)

CTL activity was measured using RMA-S-K^d target cells loaded with the cognate peptide, as described previously [1,32]. The amount of IL-2 in the culture supernatants was determined by sandwich ELISA using antibody pairs purchased from BD Pharmingen Biosciences (Palo Alto, CA, USA).

Monitoring T1D and insulitis

Onset of T1D was monitored by measuring urine glucose levels using Keto-Diastix (Bayer, Canada). Animals with two consecutive readings of >3 were considered diabetic. At the time of euthanasia, pancreatic tissues were processed for histopathology analysis. At least three non-overlapping (200 μm apart) 5-μm sections were evaluated for insulitis [32].

Statistical analysis

Cumulative incidence of T1D was analysed using Prism software (GraphPad Software Inc., La Jolla, CA, USA). For diabetes incidence, significance was calculated using log-rank (Mantel–Cox) test. For all other parameters, statistical significance was calculated by Student's *t*-test.

Results

Ablation of the *Il21* gene protects female 8.3-NOD mice from T1D and insulitis

The 8.3-NOD mouse expresses a highly pathogenic, MHC class I-restricted, transgenic 8.3 TCR specific to a peptide derived from the $IGRP_{206–214}$ [33,36]. In these mice, the 8.3 TCR transgenic $CD8^+$ T cells (8.3 T cells) infiltrate pancreatic islets from 3 weeks of age [33]. Female 8.3-NOD mice develop T1D at 2–3 months of age compared to 4–6 months required for overt diabetes in non-TCR transgenic NOD mice [37]. Disease penetrance in our NOD colony is greater than 90% in 8.3-NOD females and about 50% in males (Fig. 1a,b). Genetic ablation of the *Il21* gene abrogated completely T1D incidence in female and male 8.3-NOD mice (Fig. 1a). Strikingly, a partial reduction in IL-21 availability was sufficient to reduce T1D incidence by 50–60% in $Il21^{+/-}$ females expressing either the 8.3 TCR or a polyclonal TCR repertoire (Fig. 1a,c), although *Il21* gene heterozygosity did not diminish T1D incidence in male 8.3-NOD mice (Fig. 1b). IL-21 deficiency completely prevented mononuclear cell infiltration of pancreatic islets in 8.3-NOD mice (Fig. 1d). These results show that the highly diabetogenic 8.3 TCR transgenic $CD8^+$ T cells require IL-21 to induce insulitis and cause diabetes, and that a partial reduction in IL-21 availability is sufficient to attenuate their pathogenic potential.

IL-21-deficient 8.3 TCR transgenic $CD8^+$ T cells show impaired antigen-induced proliferation

Several reports have shown that IL-21 is required for sustaining the expansion of antigen-specific T cells during chronic viral infections [27–31]. Therefore, we evaluated the ability of IL-21-deficient 8.3 T cells to proliferate in response to cognate $IGRP_{206–214}$ peptide or to its mimotope NRP. As shown in Fig. 2a–c, IL-21-deficient cells showed significantly reduced proliferation to TCR ligands or to anti-CD3/CD28 cross-linking, but responded similarly to PMA and ionomycin. These cells also showed comparable levels of proliferation to stimulatory combinations of cytokines, IL-7 or IL-15 along with IL-21, although the magnitude of this response was low compared to antigen-induced proliferation (Fig. 2d).

An earlier report suggested a role for IL-21 in T cell homeostasis in the NOD mouse [2]. However, we did not observe any difference in total T cell numbers or the frequency and numbers of 8.3 T cells in 8.3-NOD.$Il21^{-/-}$ mice (Fig. 3a,b). To evaluate the impact of IL-21 deficiency on homeostatic expansion of $CD8^+$ T cells, we injected CFSE-labelled splenocytes from 8.3-NOD or 8.3-NOD.$Il21^{-/-}$ mice into NOD.*Scid* or NOD.*Scid*.$Il21^{-/-}$ recipients. As shown in Fig. 3c, expansion of $CD8^+$ T cells from IL-21-deficient or wild-type donors was comparable in NOD.*Scid* and NOD.*Scid*.$Il21^{-/-}$ recipients, suggesting that IL-21 is dispensable for homeostatic expansion of $CD8^+$ T cells. Collectively, the above results indicate that $CD8^+$ T cells that develop in IL-21-deficient mice proliferate to a lesser extent following TCR stimulation, and that this does not arise from a general proliferation defect, as these cells undergo efficient cytokine-driven homeostatic expansion *in vivo*.

Antigen-stimulated IL-21-deficient 8.3 T cells display efficient CTL activity and cytokine production, and mediate islet destruction following homeostatic expansion

Next we addressed the consequence of IL-21 deficiency on antigen-induced effector functions of $CD8^+$ T cells. As shown in Fig. 4a, IL-21-deficient 8.3 T cells displayed normal antigen-specific cytolytic activity as they lysed target cells pulsed with NRP-V7 peptide efficiently (Fig. 4a). IL-21-deficient 8.3 T cells also showed efficient IFN-γ and TNF-α production following antigen stimulation (Fig. 4b). Hence, even though $CD8^+$ T cells from 8.3-NOD.$Il21^{-/-}$ mice show reduced proliferation to the cognate antigen, their ability to become cytolytic effector cells upon antigen stimulation was not compromised.

Adoptive transfer of polyclonal $CD8^+$ T cells from $Il21ra^{-/-}$ NOD donors, along with IL-21Rα-deficient $CD4^+$ T cells, failed to induce T1D in NOD.*Scid* recipients [9,11], suggesting that homeostatic expansion alone is insufficient to elicit the pathogenic potential of IL-21-deficient diabetogenic $CD8^+$ T cells. However, the failure of $Il21ra^{-/-}$ to develop T1D could be reversed by the transfer of wild-type DCs [11]. These reports indicated that inefficient activation may underlie the inability of 8.3 T cells to cause disease in 8.3-NOD. $Il21^{-/-}$ mice. Given that IL-21 deficiency did not diminish the ability of 8.3 T cells to develop effector functions upon antigen stimulation (Fig. 4a,b) and to undergo homeostatic expansion (Fig. 3), we investigated whether previous antigen stimulation would enable 8.3 T cells to induce T1D in NOD.*Scid* mice. To this end, we stimulated IL-21-deficient and control 8.3 $CD8^+$ T cells with the cognate peptide $IGRP_{208–214}$ for 2 days before adoptive transfer to NOD.*Scid* recipients. NOD.*Scid* mice lack both NK T cells and $CD4^+$ T cells, the major producers of IL-21 [15], and hence IL-21 is unlikely to be available to the activated donor cells. As shown in Fig. 4c, IL-21-deficient 8.3 $CD8^+$ T cells stimulated by cognate antigen *in vitro* induced T1D in all NOD.*Scid* recipients within 10 days after adoptive trans-

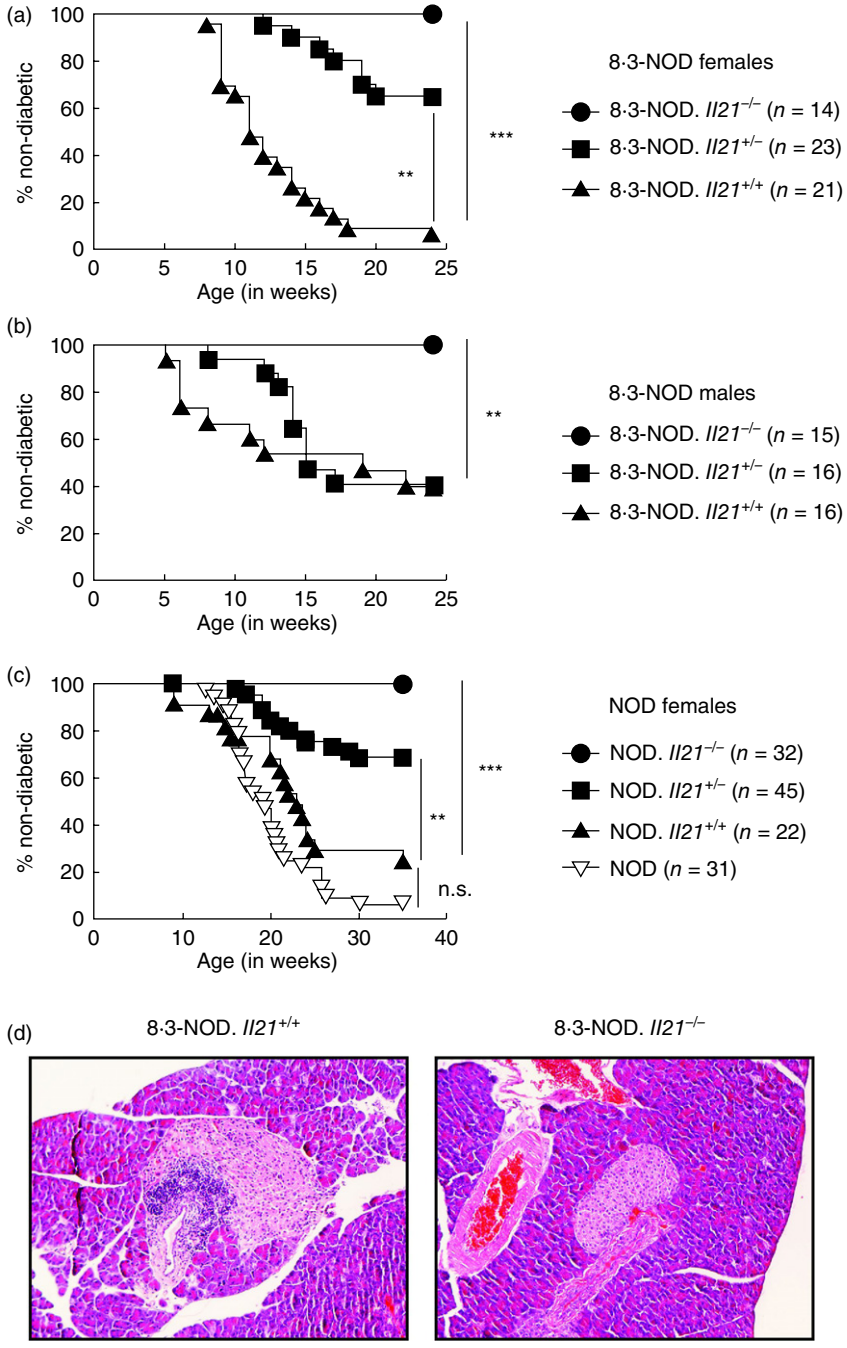
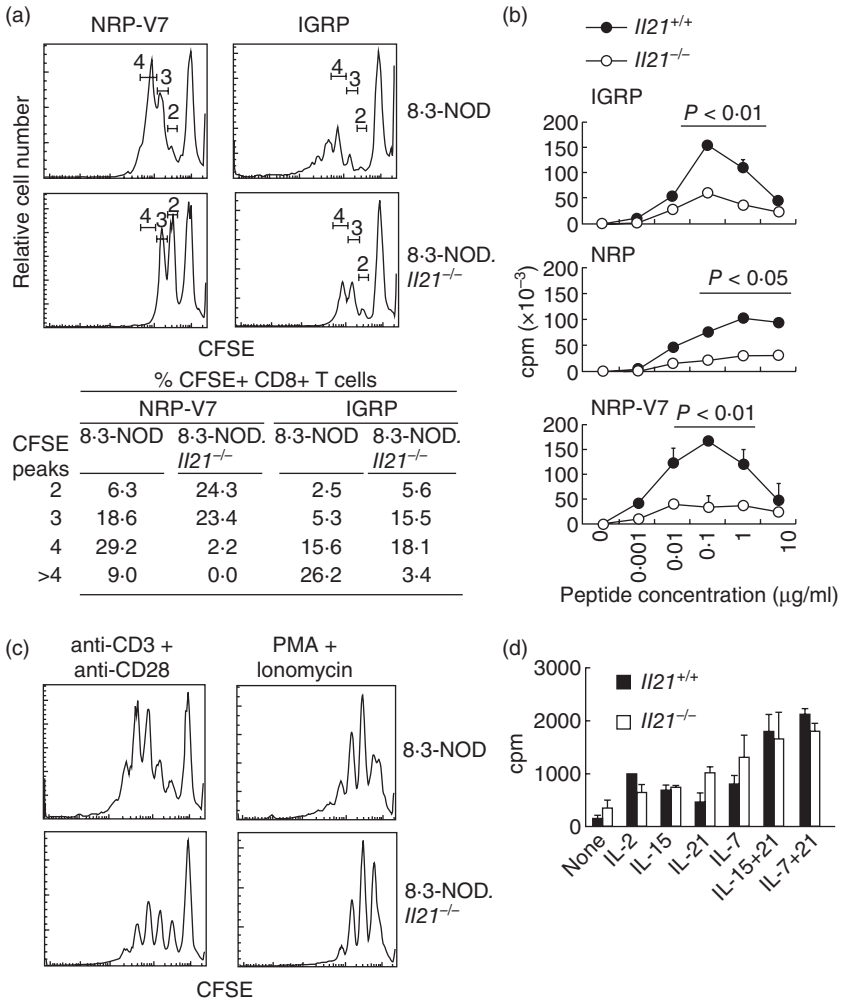


Fig. 1. Interleukin (IL)-21 is required for the development of type 1 diabetes (T1D) in non-obese diabetic (NOD) mice expressing the 8.3 transgenic T cell receptor (TCR). (a) *Il21*$^{-/-}$, *Il21*$^{+/-}$ and *Il21*$^{+/+}$ female 8.3-NOD littermates, expressing the major histocompatibility complex (MHC) class-I-restricted 8.3 transgenic TCR, were monitored for T1D development by monitoring urine glucose. Diabetic mice were tested over 2 consecutive days. Cumulative incidence of T1D is shown. $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$ (log-rank test). (b) Incidence of T1D in *Il21*$^{-/-}$, *Il21*$^{+/-}$ and *Il21*$^{+/+}$ 8.3-NOD males. (c) T1D incidence in non-TCR transgenic *Il21*$^{-/-}$, *Il21*$^{+/-}$ and *Il21*$^{+/+}$ female NOD mice. (d) Pancreatic sections from representative *Il21*$^{-/-}$ and *Il21*$^{+/+}$ 8.3-NOD mice were assessed for cellular infiltration in the islets.

fer, as in the case of wild-type donor cells. Even though the proportion of CD8+ T cells in the lymph nodes was reduced substantially in recipients of IL-21-deficient donor cells compared to recipients of wild-type cells (Fig. 4d), both groups of mice showed a similar level of islet infiltration (Fig. 4e) and developed T1D (Fig. 4c). To determine whether IL-21 produced by donor cells is sufficient for T1D induction, we transferred splenocytes adoptively from diabetic NOD mice to NOD.*Scid* and NOD.*Scid.Il21*$^{-/-}$ recipients. As shown in Fig. 4f, both groups of recipient mice developed T1D between 30 and 50 days after cell transfer, suggesting that IL-21 available from donor cells is sufficient for activated diabetogenic cells to induce disease. In addition, antigen-stimulated 8.3 T cells from IL-21-deficient mice caused diabetes in NOD.*Scid.Il21*$^{-/-}$ mice within 10 days (Fig. 4c). Collectively, the above results indicate that IL-21 is required for efficient activation of diabetogenic CD8+ T cells by antigen, but is dispensable during subsequent stages of islet destruction. Hence, the inability of 8.3-NOD.*Il21*$^{-/-}$ to develop T1D is related most probably to the defective activation of 8.3 T cells by the endogenous autoantigen IGRP.



% CFSE+ CD8+ T cells

CFSE peaks	NRP-V7 8·3-NOD	NRP-V7 8·3-NOD. *Il21*$^{-/-}$	IGRP 8·3-NOD	IGRP 8·3-NOD. *Il21*$^{-/-}$
2	6·3	24·3	2·5	5·6
3	18·6	23·4	5·3	15·5
4	29·2	2·2	15·6	18·1
>4	9·0	0·0	26·2	3·4

Fig. 2. Antigen-induced proliferation is reduced in interleukin (IL)-21-deficient 8.3 T cell receptor (TCR) transgenic CD8$^+$ T cells. (a) Total lymph node cells from IL-21-deficient 8.3 TCR transgenic non-obese diabetic (NOD) mice and IL-21-sufficient littermate controls were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE) and stimulated with 1 μg/ml of islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP)$_{206–214}$ or the super agonist NRP-A7. Cell proliferation was evaluated by flow cytometry on gated CD8$^+$ T cells. The numbers above the CFSE peaks indicate the number of cell division cycle. The proportions of cells corresponding to each CFSE peak are indicated in the table. Representative data from two similar experiments are shown. (b) Purified CD8$^+$ T cells from IL-21-deficient and control 8.3-NOD mice were stimulated with the cognate peptide IGRP$_{206–214}$ or the mimotopes NRP or NRP-V7 in the presence of irradiated NOD splenocytes as antigen-presenting cells (APC) and cell proliferation was measured by [^{3}H]-thymidine incorporation. (c) Proliferative response of CFSE-labelled total lymph node cells or purified CD8$^+$ T cells to anti-CD3/anti-CD28 beads or phorbol myristate acetate (PMA)/ionomycin. (d) Proliferation of purified CD8$^+$ T cells to cytokine stimulation for 3 days. Representative data from two or three similar experiments are shown for CFSE assays, and mean values of at least three experiments are shown for thymidine incorporation.

8.3 T cells from IL-21-deficient mice undergo limited antigen-driven expansion *in vivo* even in wild-type NOD mice

As activation of naive T cells occurs first in draining lymph nodes, we investigated whether diabetogenic CD8$^+$ T cells from 8.3-NOD.*Il21*$^{-/-}$ mice would respond to cognate antigens in draining lymph nodes. We injected CFSE-labelled *Il21*$^{+/+}$ or *Il21*$^{-/-}$ 8.3 CD8$^+$ T cells into NOD mice, followed by wild-type BMDCs pulsed with cognate peptide or a control peptide into one of the hind footpads. The draining and the non-draining inguinal lymph nodes were analysed to evaluate proliferation of donor 8.3 T cells. As shown in Fig. 5, wild-type and IL-21-deficient donor 8.3 T cells proliferated in the draining lymph nodes of mice injected with IGRP-loaded DCs, but not in mice injected with the control TUM peptide-loaded DCs or in non-draining lymph nodes. Even though IL-21-deficient 8.3 T cells divided to a comparable extent as control cells in terms of the number of cell division cycles in the draining lymph nodes of IGRP-loaded DCs, their proliferation was less robust compared to wild-type 8.3 cells, as deduced from the proportion of CFSE^{lo} population (32% *versus* 7·3%, Fig. 5). These results show that CD8$^+$ T cells generated in an IL-21-free environment display decreased antigen-driven expansion.

8.3 TCR transgenic CD8$^+$ T cells from IL-21-deficient mice show reduced TCR-induced IL-2 production

Next we examined the mechanisms underlying decreased antigen-specific proliferation of diabetogenic CD8$^+$ T cells from *Il21*$^{-/-}$ mice. The gene coding for IL-2, the key autocrine growth factor for T cells, is subject to epigenetic control in CD8$^+$ T cells and resides within the *Idd3* locus that also harbours the *Il21* gene [38–44]. This consideration raised the possibility that reduced antigen responsiveness of 8.3 T cells from 8.3-NOD.*Il21*$^{-/-}$ mice may arise from perturbation of the *Il2* gene by ablation of the adjacently located *Il21* gene. To interrogate this possibility, we measured the amount of IL-2 produced in cultures of IL-21-deficient and control 8.3 T cells. As shown in Fig. 6a, IL-2 production following IGRP peptide stimulation was reduced significantly in IL-21 deficient 8.3 T cells compared to control cells. This reduction was associated with

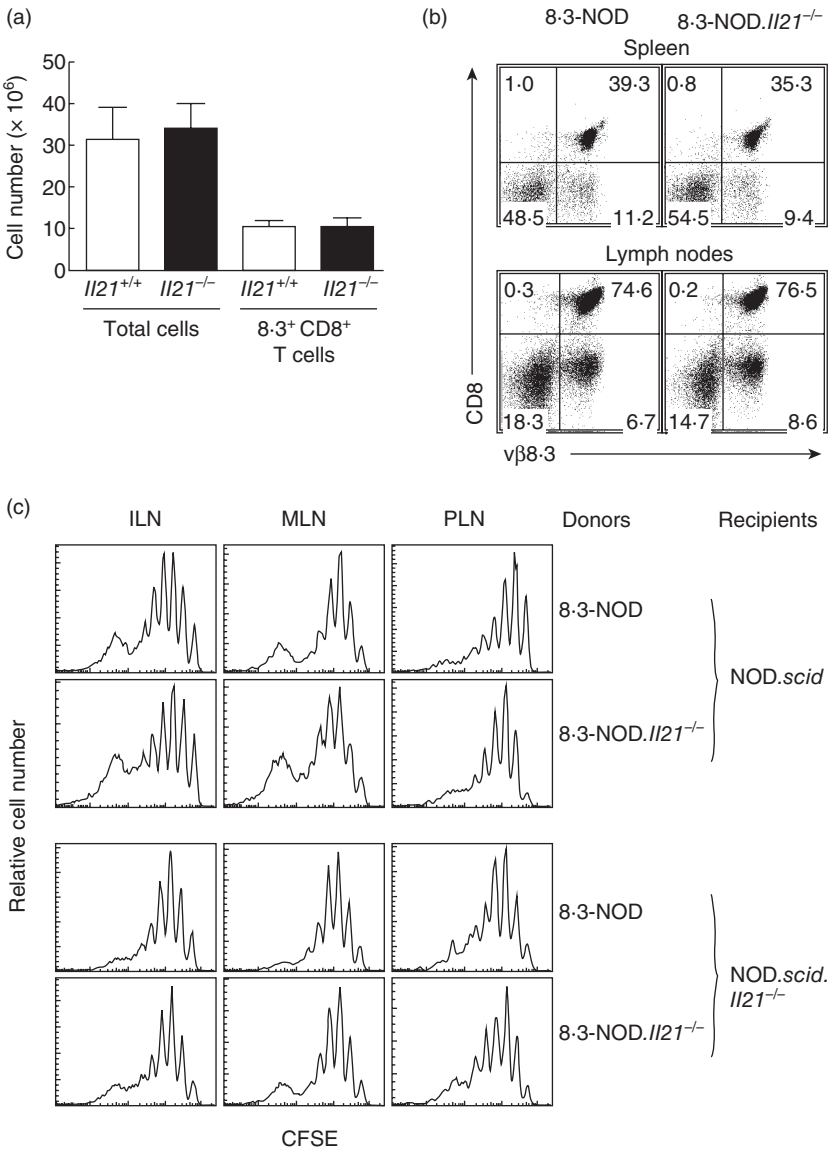


Fig. 3. Interleukin (IL)-21 deficiency does not compromise the T cell numbers in 8.3-non-obese diabetic (NOD) mice or homeostatic proliferation of 8.3 T cells. (a) Total and Vβ8.3$^+$CD8$^+$ T cell numbers in the spleens of 8.3-NOD.*Il21*$^{-/-}$ and control mice. Data from four mice per group are shown. The absolute number of Vβ8.3$^+$CD8$^+$ T cells was calculated from their frequency in individual mice. (b) Representative profile of Vb8.3$^+$CD8$^+$ T cells in the spleen and lymph nodes of 8.3-NOD.*Il21*$^{-/-}$ and control mice. (c) Total splenocytes from IL-21-deficient 8.3 T cell receptor (TCR) transgenic NOD mice and IL-21-sufficient littermate controls were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE) and transferred adoptively to NOD.*Scid* or NOD.*Scid.Il21*$^{-/-}$ recipients (5×10^6 cells/recipient). Five days after cell transfer, single-cell suspensions from inguinal (ILN), mesenteric (MLN) and pancreatic (PLN) lymph nodes were stained for CD8 marker. Proliferation of donor CD8$^+$ T cells was evaluated from the dilution of CFSE in gated CD8$^+$ T cells. Representative data from two similar experiments with at least four mice per group are shown.

decreased *Il2* gene transcription (Fig. 6b). Interestingly, 8.3 TCR transgenic CD8$^+$ T cells lacking one functional allele of the *Il21* gene also showed significantly reduced levels of *Il2* transcripts (Fig. 6b). Next, we added exogenous IL-2 to cultures of 8.3 T cells stimulated with antigen. As shown in Fig. 6c, exogenous IL-2 augmented antigen-induced proliferation in both wild-type and IL-21-deficient 8.3 T cells, yet the latter showed a significantly reduced response compared to wild-type cells. Addition of IL-7 or IL-15 did not augment proliferation of 8.3 T cells in response to antigen whereas, paradoxically, exogenous IL-21 inhibited proliferation of 8.3 T cells from both wild-type and IL-21-deficient mice (Fig. 6c). These results suggest that impaired IL-2 production, and possibly an IL-2-independent defect, may contribute to the reduced antigen-induced proliferation of 8.3 CD8$^+$ T cells in NOD.*Il21*$^{-/-}$ mice.

Discussion

NOD mice lacking IL-21 or IL-21Rα do not develop insulitis [7–10], indicating that IL-21 may play an important role in initiating the autoimmune response prior to mononuclear cell infiltration of the islets. In agreement with this prediction, in this study we have shown that autoreactive CD8$^+$ T cells bearing the aggressive 8.3 transgenic TCR also require IL-21 to initiate T1D. We have also shown that CD8$^+$ T cells from 8.3-NOD.*Il21*$^{-/-}$ mice proliferate poorly to antigen stimulation and that this defect results, at least partly, from reduced *Il2* gene expression.

Two recent studies have addressed the pathogenic mechanisms of IL-21 in T1D. Using the spontaneous NOD T1D model, McGuire *et al.* have shown that IL-21 secreted by a subset of CD4$^+$ helper cells that express CCR9 and infiltrate

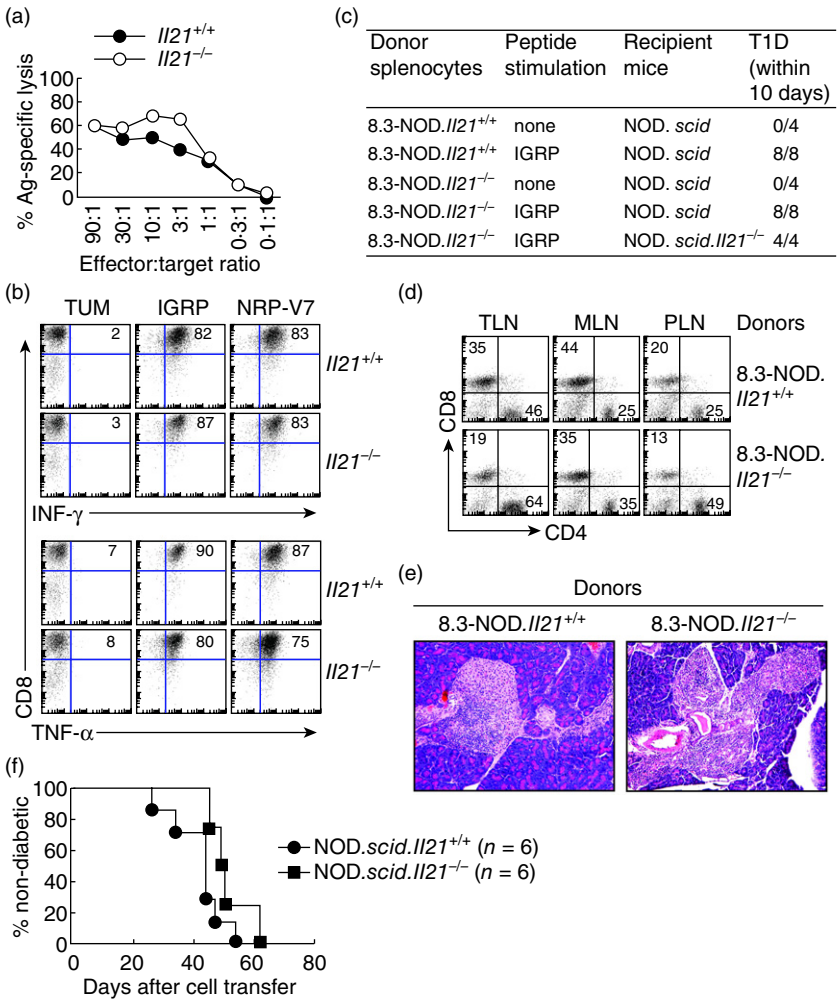


Fig. 4. Interleukin (IL)-21 deficiency does not compromise antigen-induced effector functions of 8.3 T cells or their ability to cause type 1 diabetes (T1D). (a) Total splenocytes from *Il21*$^{+/+}$ and *Il21*$^{-/-}$ 8.3-non-obese diabetic (NOD) females were activated with 1 μg/ml of the cognate peptide islet-specific glucose-6-phosphatase catalytic subunit-related protein (IGRP)$_{206–214}$. After 60 h, cells were stained with CD8 and Vβ8.3^{+} and equivalent numbers of Vβ8.3^{+}CD8^{+} T cells were compared in cytotoxic T lymphocyte (CTL) assay using RMA-S.K^{d} cells pulsed with NRP-V7 peptide as targets. Specific lysis was calculated as described in Methods. Both the effector cells did not lyse targets cells pulsed with the null peptide TUM (data not shown). (b) Splenocyte cultures stimulated with IGRP or NRP-V7 were stained for CD8 and intracellular interferon (IFN)-γ or tumour necrosis factor (TNF)-α and evaluated by flow cytometry. Data shown are representative of two independent experiments with similar results. (c–e) Splenocytes of IL-21-deficient and control 8.3-NOD mice were activated with IGRP peptide, transferred adoptively to NOD.*Scid* recipients and T1D onset was monitored (c). In these recipient mice, CD4 and CD8 T cell proportions in total (TLN) mesenteric (MLN) and pancreatic (PLN) lymph nodes (d) and insulitis (e) and were evaluated at the time of euthanasia on day 10. (f) Total splenocytes from diabetic non-T cell receptor (TCR) transgenic NOD mice were transferred adoptively to NOD.*Scid* and NOD.*Scid.Il21*$^{-/-}$ recipients, and T1D development was monitored.

the islets is needed for CD8^{+} T cell expansion and survival [9]. Van Belle and colleagues used a virus-induced T1D model that implicated IL-21 in facilitating DCs to transport antigens from pancreas to draining lymph nodes in order to activate CD4^{+} T cells, which then provide help to CD8^{+} T cells [11]. In the 8.3-NOD mouse model used in our study, the transgenic TCR allowed us to evaluate directly the antigen responsiveness of CD8^{+} T cells, revealing a fundamental defect in the ability of *Il21*$^{-/-}$ 8.3 T cells to undergo efficient antigen-induced proliferation. A similar defect in the expansion of viral antigen-specific CD8^{+} T cells has been shown to occur in *Il21*$^{-/-}$ and *Il21ra*$^{-/-}$ mice, which fail to clear chronic viral infection [27–29,45]. Even though these studies have shown that IL-21 acts directly on viral antigen-specific CD8^{+} T cells to sustain their expansion in a cell autonomous manner, the underlying mechanisms remain unclear. In *Il21*$^{-/-}$ mice, antigen-specific CD8^{+} T cells showed an elevated expression of the inhibitory receptor programmed death 1 (PD-1) 5 months after infection [27,28]. However, IL-21 deficiency did not affect PD-1 expression during primary or secondary responses following acute viral infection [31]. In another study, defective antigen-specific CD8^{+} T cell expansion in *Il21ra*$^{-/-}$ mice was correlated with elevated expression of TRAIL, a TNF-related apoptosis-inducing molecule implicated in activation-induced cell death [30]. In 8.3-NOD mice, CD8^{+} T cells bearing the transgenic TCR would constantly encounter the endogenous autoantigen, akin to chronic stimulation. However, we did not observe up-regulation of either PD-1 or TRAIL in freshly isolated 8.3 T cells from 8.3-NOD.*Il21*$^{-/-}$ mice, nor were these molecules modulated differentially upon antigen stimulation (data not shown).

Studies examining the role of IL-21 in anti-viral responses concur that IL-21 exerts a cell autonomous effect on CD8^{+} T cells to sustain their proliferative potential [45]. These studies have shown normal or even elevated IFN-γ production by viral antigen-specific CD8^{+} and CD4^{+} T cells from *Il21*$^{-/-}$ and *Il21ra*$^{-/-}$-deficient mice, and normal IL-2 production by CD4^{+} T cells from virus-infected *Il21ra*$^{-/-}$ mice [28,29,31]. Some of these studies have shown reduced

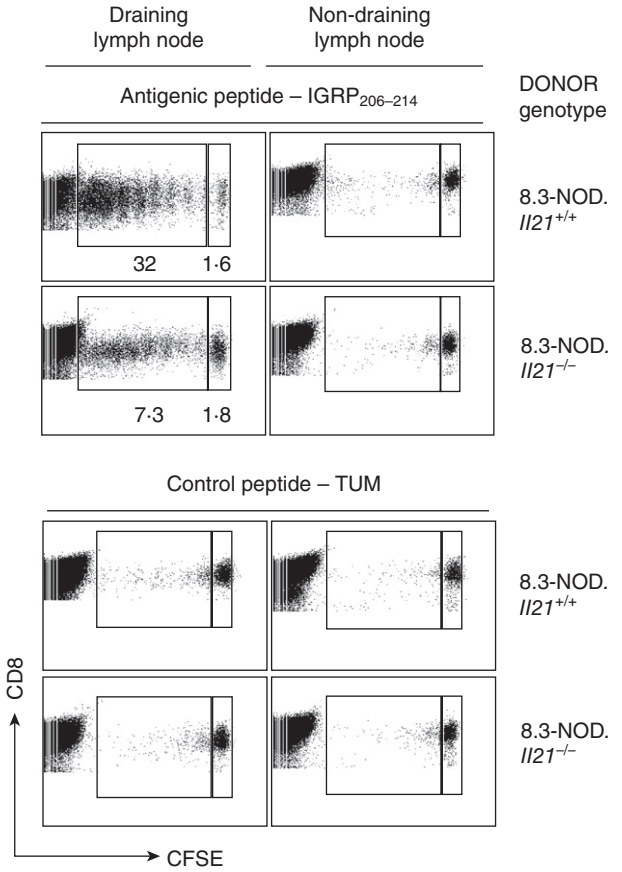


Fig. 5. CD8+ T cells from interleukin (IL)-21-deficient 8.3-non-obese diabetic (NOD) mice undergo less robust antigen-driven expansion *in vivo*. Total lymph node cells from IL-21-deficient and control 8.3-NOD mice were labelled with carboxyfluorescein diacetate succinimidyl ester (CFSE) and the equivalent of 1×10^5 CD8+ T cells were injected into 2- month-old NOD male mice. Two days later, 1×10^6 wild-type BMDCs loaded with the cognate islet-specific glucose-6-phosphatase catalytic subunit-related protein $(IGRP)_{206–214}$ or the control TUM peptide were injected into one of the hind footpads. After 2 days, draining and non-draining lymph nodes were harvested, stained for CD8 and CFSE fluorescence intensity on gated CD8+ T cells was evaluated. The CFSE-negative cells outside the boxed area represent CD8+ T cells of the recipient mice. Representative data from three independent experiments is shown.

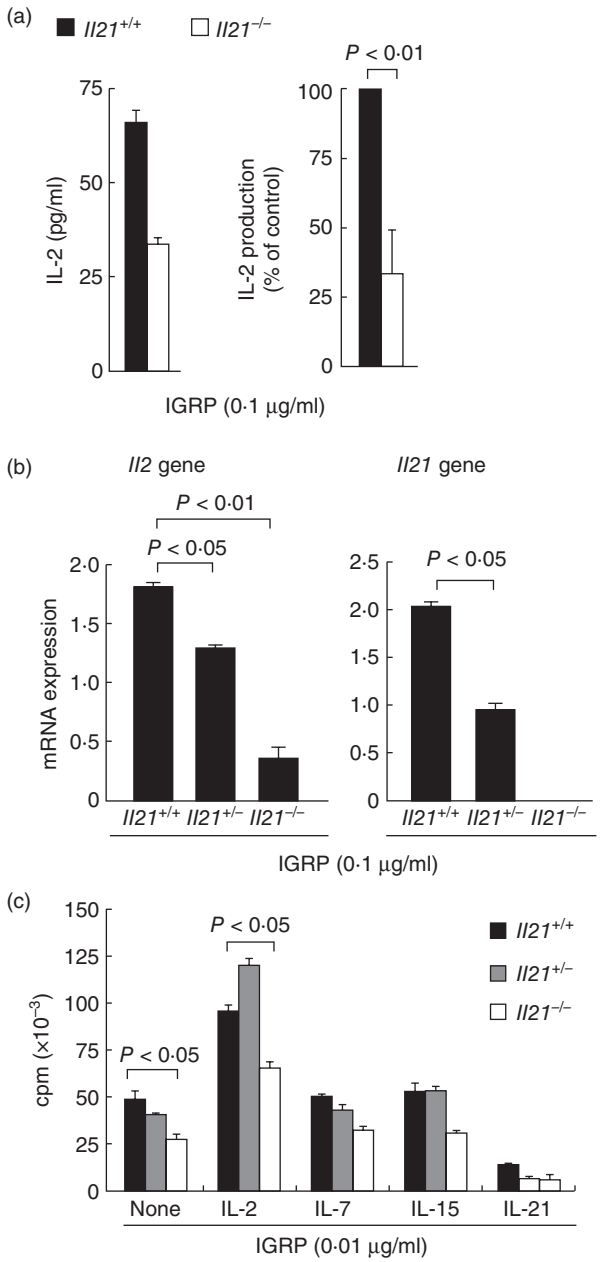


Fig. 6. Impaired antigen-induced proliferation of interleukin (IL)-21-deficient 8.3 T cells is associated with decreased IL-2 production. (a) Purified 8.3 cells from IL-21-sufficient and -deficient 8.3-non-obese diabetic (NOD) mice were activated with islet-specific glucose-6-phosphatase catalytic subunit-related protein $(IGRP)_{206–214}$ in the presence of irradiated antigen-presenting cells (APC), and IL-2 production in the culture supernatants was measured. Representative data from one of the three experiments (left panel) and normalized values from three independent experiments (right panel) are shown. (b) Expression of *Il2* and *Il21* genes was determined by quantitative polymerase chain reaction (qPCR) in IGRP-stimulated cells. (c) Purified 8.3 cells from IL-21-sufficient, heterozygote and -deficient NOD mice were activated with the cognate peptide $IGRP_{206–214}$ presented by irradiated wild-type APC in the presence of indicated cytokines and cell proliferation was measured. Cumulative data from three independent experiments are shown for (b) and (c).

IL-2 production by antigen-specific CD8+ T cells in *Il21*$^{-/-}$ mice. We have observed that 8.3 T cells from *Il21*$^{-/-}$ mice produced significantly less IL-2 following antigen stimulation and that this was associated with decreased *Il2* mRNA expression. At least one report has alluded to the possibility that introduction of the *Il21* knock-out allele might influence the expression of *Il2* gene, as these genes are located only 95 kb apart on chromosome 3 [30]. Even though daily administration of IL-21 to lymphocytic choriomeningitis (LCMV)-infected *Il21*$^{-/-}$ mice for more than a week reversed the defective IL-2 production in viral antigen-specific CD8+ T cells [28], this reversal does not rule out completely the

possible influence of the *Il21* knock-out allele on *Il2* gene expression, and further experiments are needed to resolve this issue.

The addition of exogenous IL-2 could not reverse completely the defective antigen-induced proliferation of 8.3 T cells from *Il21*$^{-/-}$ mice, suggesting that either IL-21-dependent autocrine IL-2 production is necessary to achieve maximal expansion of activated CD8$^+$ T cells, or IL-21 may also modulate the expression of molecules that influence T cell proliferation. We did not find any significant difference in the induction of CD25 between antigen-stimulated 8.3 T cells from *Il21*$^{-/-}$ and control 8.3-NOD mice (data not shown). Moreover, normal IFN-γ production and CTL activity of *Il21*$^{-/-}$ 8.3 T cells, suggesting that lack of IL-21 signalling does not impair TCR signalling pathways that promote effector functions. Consistent with this prediction, protein tyrosine phosphorylation and calcium flux response following TCR stimulation were not affected in *Il21*$^{-/-}$ 8.3 T cells (data not shown). In agreement with this, viral antigen-specific cells in control and IL-21 or IL-21Rα-deficient mice produced comparable levels of IFN-γ [28,30]. These considerations raise the possibility that an IL-21-sufficient environment is necessary for naive CD8$^+$ T cells to sustain full proliferation potential in response to antigen stimulation. This requirement may be dispensable when antigen stimulation is accompanied by potent activation of the innate immune system and induction of other inflammatory cytokines that could compensate for IL-21, and/or when the immune response is directed towards several strong immunodominant antigens. This notion is supported by the ability of *Il21*$^{-/-}$ and *Il21ra*$^{-/-}$ mice to clear acute viral infection and mount a memory response [31]. Conversely, productive CD8$^+$ T cell activation during persisting viral infection or to a limiting autoantigen may depend upon the continuous availability of IL-21, presumably from innate immune cells, in order to clear chronic infections or to cause autoimmune pathology.

Intriguingly, the addition of IL-21 alone during antigen stimulation of CD8$^+$ T cells inhibits proliferation (Fig. 6c). In an earlier report, immunization and concomitant stimulation of NK T cells resulted in increased expansion of antigen-specific CD8$^+$ T cells in *Il21*$^{-/-}$ mice than in control mice, suggesting that NK T-derived IL-21 may limit T cell expansion [46]. The basis for these incongruous observations, i.e. reduced proliferation of CD8$^+$ T cells from *Il21*$^{-/-}$ mice following antigen stimulation *versus* inhibition of antigen-induced proliferation in wild-type CD8$^+$ T cells upon simultaneous addition of IL-21, is unclear. Nevertheless, these observations suggest that IL-21 may modulate TCR responses either alone or along with other signal inputs. We have observed that IL-7 and IL-15, cytokines implicated in T cell homeostasis, prevent IL-21-mediated inhibition of CD8$^+$ T cell proliferation to antigen (data not shown). Similarly, a recent report showed that IL-21-induced signal transducer and activator of transcription-3 (STAT-3) activation could substitute for impaired co-receptor signalling via CD8-associated lymphocyte-specific protein tyrosine kinase (Lck) in human CD8$^+$ T cells [47]. Other studies have also suggested that STAT-5 activation by gamma chain cytokines may synergize with the TCR signalling machinery [48]. We have shown that IL-21 enhances IL-7-induced STAT-5 activation significantly [34]. Clearly, further investigation will reveal how IL-21 signalling modulates TCR signalling that promotes proliferation without affecting effector functions.

Notwithstanding the complexities of how IL-21 modulates the outcomes of TCR stimulation, its pathogenic role in T1D has been well established by many studies, including the present study [7–11]. Even a partial reduction in the amount of IL-21, as observed in NOD.*Il21*$^{+/-}$ mice, reduces the incidence of T1D in the female NOD and 8.3-NOD mice. These observations reinforce the notion that inflammatory cytokines available at the time of initiation of an autoimmune response could be a key trigger for stimulating potentially autoreactive CD8$^+$ T cells to become autoaggressive CTLs. This notion is supported further by our earlier findings that exposure of diabetogenic naive CD8$^+$ T cells to IL-15 and IL-21 enables their activation by weak agonists to cause T1D [32]. Recently we have shown that IL-15 deficiency and blockade of IL-15 signalling before the onset of insulitis protects NOD mice from T1D [49]. However, clinical diagnosis of T1D patients is usually made after most of the insulin-producing beta cells have been destroyed by the ongoing autoimmune response. Our findings indicate that IL-21 is crucial for the initial activation of autoreactive CD8$^+$ T cells but not for sustaining their pathogenic effector functions. Hence, combining therapies targeting IL-21 with blockade of IL-15 would be more effective in inhibiting autoreactive memory CD8$^+$ T cells and preserving the remaining functional islet mass, as well as in prolonging the survival of islet transplants.

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Disclosure

The authors declare no conflicts of interest.

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