Double deficiency in IL-17 and IFN-γ signaling significantly suppresses the development of diabetes in the NOD mouse

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Abstract

Aims/hypothesis T helper type (Th) 17 cells have been shown to play important roles in mouse models of several autoimmune diseases that had been classified as Th1 diseases. In the NOD mouse, the relevance of Th1 and Th17 are controversial, because single cytokine-deficient NOD mice develop diabetes similarly to wild-type NOD mice.

Methods We studied the impact of IL-17/IFN-γ receptor double deficiency in NOD mice on the development of insulitis/diabetes compared to the IL-17 single-deficient mice and wild-type mice.

Results The IL-17 single-deficient NOD mice showed a delayed onset of diabetes and reduced severity of insulitis, but the cumulative incidence of long-life diabetes in the IL-17-deficient mice was similar to that in wild-type mice. The IL-17/IFN-γ receptor double-deficient NOD mice showed an apparent decline of longstanding diabetes onset, but not of insulitis compared to that in the IL-17 single-deficient mice. We also found that double-deficient NOD mice had severe lymphopenic phenotype and preferential increase in Tregs among $CD4^+$ T cells compared to the IL-17 single-deficient mice and wild-type NOD mice. Adoptive transfer study with $CD4^+CD25^-$ T cells from young nondiabetic IL-17 single-deficient NOD mice, but not that from older mice, showed significantly delayed disease onset in immune-deficient hosts compared to that from the corresponding wild-type mice.

Conclusions/interpretation These results indicate that IL-17/Th17 participates in the development of insulitis and that both IL-17 and IFN-γ signaling could synergistically contribute to the development of diabetes in NOD mice.

Keywords IL-17, IFN-γ, NOD mice, type 1 diabetes, Th17, lymphopenia

Abbreviations

Th	T helper type
Scid	severe combined immunodeficiency
Tregs	regulatory T cells

IAAs insulin autoantibodies

SPCs splenocytes

Introduction

Type 1 diabetes results from the autoimmune destruction of pancreatic β-cells mediated by CD4+ and CD8+ T cells [1]. T helper type (Th) 1 cells have been believed to play a key role in the pathogenesis of type 1 diabetes in the nonobese diabetic (NOD) mouse, because pancreatic islet-infiltrating mononuclear cells and diabetogenic T-cell clones derived from NOD islets show strong expression of T helper type (Th) 1 cytokines [2-5]. However, an unresolved issue remains as to why the elimination of Th1 cytokines or their signaling molecules, including IFN-γ, IL-12 and IFN-γ receptor does not to reduce the incidence of diabetes in NOD mice [6-10].

Th17 cells that produce IL-17 have been shown to play important roles in mouse models of several autoimmune diseases including experimental autoimmune encephalomyelitis, rheumatoid arthritis and autoimmune thyroiditis and others that had previously been thought to be Th1-dominant [11-16]. In the context of NOD mice, there are data suggesting that Th17 cells play a pathogenic role in type 1 diabetes development [17]. The administration of GAD peptide inserted into immunoglobulin molecules inhibited diabetes development, dependent on the induction of splenic IFN-γ that inhibited IL-17 production [18], and treatment with neutralizing anti-IL-17 antibodies prevented the development of diabetes in NOD mice [19]. However, similarly to INF-γ knockout in NOD mice, an IL-17 knockdown NOD line created by directly introducing a short hairpin RNA construct does not alter diabetes susceptibility [20].

The differentiations of naïve $CD4^+$ T cells to effector or regulatory T cells are more plastic than previously thought [21]. Th17 cells have been shown to convert to Th1 cells in a transfer model of colitis [22]. In type 1 diabetes, diabetogenic BDC2.5 $CD4^+$ T cells polarized in vitro to the Th17 cell phenotype convert to Th1-like cells after adoptive transfer into NOD-Scid mice, ultimately causing β-cell destruction and diabetes[23, 24]. Thus, it is possible that the conversion of Th17 cells to Th1 cells or Th1/Th17 cells co-expressing Th1 and Th17 cytokines in the pancreatic islets might counteract the disease inhibition by the elimination of a single cytokine gene from NOD mice.

To address this issue we produced NOD mice genetically deficient in both IL-17 and IFN-γ receptor, and we evaluated the insulitis/diabetes development in comparison to IL-17 single-deficient

NOD background mice.

Materials and methods

Mice

NOD and NOD-Scid mice were purchased from Clea Japan (Tokyo Japan). IL-17-deficient NOD mice were generated as described (originally on a 129/Sv x C57BL/6 genetic background) [25]. IFN-γ receptor (IFN-γR)-deficient NOD mice were obtained from Dr. Osami Kanagawa (Laboratory for Autoimmune Regulation, RIKEN Research Center for Allergy and Immunology, Yokohama, Japan). All animal experiments described in this study were conducted with the approval of the institutional Animal Experimentation Committee, and were conducted in accordance with the Guidelines for Animal Experimentation of Nagasaki University.

Establishment of IL-17 single-deficient and IL-17/IFN-γ receptor double-deficient NOD mice

IL-17-deficient mice were backcrossed with NOD mice for eight successive generations. An analysis of the microsatellite markers of the diabetes susceptibility (*Idd1-14*) loci by PCR of the tail DNA as described [26] showed that the mice were homozygous for all of the NOD alleles (namely, *IL-17*$^{-/-}$ NOD mice). IFN-γ receptor (IFN-γR)-deficient NOD mice were crossed with *IL-17*$^{-/-}$ NOD mice, and the resulting F1, IL-17$^{+/-}$/IFN-γR$^{+/-}$ NOD mice were intercrossed to produce IL-17 single-deficient (IL-17$^{-/-}$/IFN-γR$^{+/+}$), IL-17/IFN-γ receptor double-deficient (IL-17$^{-/-}$/IFN-γR$^{-/-}$), and wild-type (*wt*) (IL-17$^{+/+}$/IFN-γR$^{+/+}$) NOD littermate mice. Only female mice were used for the present study. These mice were selected by PCR analysis of tail DNA as described[25, 26]. Tail DNA was extracted with the REDExtract-N-Amp Tissue PCR kit (Sigma, St. Louis, MO).

Monitoring for spontaneous diabetes

Blood glucose levels were monitored using the One-touch Ultra (Johnson & Johnson, Tokyo). Mice with blood glucose levels over 13.9 mmol/l in two consecutive measurements were considered

diabetic.

Measurement of insulin autoantibodies

Mice were bled at 8, 12 and 16 weeks of age, and serum samples were obtained and stored at −20°C until the antibody assay. The levels of insulin autoantibodies (IAAs) were evaluated by a 96-well filtration plate micro-IAA assay, as described [27]. The index value of 0.01 was selected as the cutoff limit at the 100th percentile of 50 Balb/c and C57BL/6 mouse samples.

Histology

Pancreatic sections were histologically analysed by fixing the tissue specimens in 10% formalin and staining the paraffin-embedded samples with hematoxylin and eosin. A minimum of 30 islets from each mouse were examined microscopically by two different observers for the presence of insulitis. The severity of insulitis was scored as follows: 0, no lymphocytic infiltration; 1, lymphocytic infiltration occupying less than 25% of the total islet cell area; 2, lymphocytic infiltration occupying 25%–50% of the total islet cell area; 3, lymphocytic infiltration occupying 50%–75% of the total islet cell area; 4, lymphocytic infiltration occupying more than 75% of the total islet cell area, or small retracted islets.

Adoptive transfer experiments

Donor $CD4^+CD25^-$ T cells were purified from the spleens of 10- or 18-week-old prediabetic mice, and $CD4^+$ T cells were purified from 15- to 22-week-old newly diabetic mice, using magnetic bead cell sorting (Miltenyi Biotech, Bergisch-Gladbach, Germany). Purified $CD4^+CD25^-$ T cells or $CD4^+$ T cells were adoptively transferred into 8- to 10-week old NOD-Scid mice, and the recipient mice were monitored for blood glucose biweekly after the adoptive transfer.

Flow cytometric analysis

Single cell suspensions of splenocytes (SPCs) were prepared from spleens of NOD mice at 10 weeks of age. Red cells were lysed in ammonium chloride buffer. For surface staining, cells were

stained for 20 min with the corresponding fluorescently labeled antibodies against surface molecules: CD3e (145-2C11), CD4 (GK1.5), CD8 (53-6.7), B220 (RA3-6B2), CD44 (IM7), CD62L (MEL-14) (all from eBioscience, San Diego, CA). For the intracellular cytokine staining, the prepared SPCs were stimulated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) and 500 ng/ml ionomycin (both from Sigma) in the presence of 2 μM monensin for 5 h. Thereafter, the cells were stained with APC-Cy5 conjugated anti-CD4, followed by intracellular IFN-γ and IL-17 staining with PE-Cy7-conjugated anti-IL-17 (eBio17B7) and PerCP-Cy5.5-conjugated anti-INF-γ (XMG1.2) antibodies (all from eBioscience). Alternatively, the cells were resuspended with PBS and stained with FITC-conjugated anti-CD4 and PE-conjugated anti-CD25 (PC61) (BD Biosciences, San Diego, CA), followed by intracellular Foxp3 staining with PE-Cy5-conjugated anti-FoxP3 (FJK-16s; Foxp3 staining kit, eBioscience). All cells were analysed on a FACSCanto II flow cytometry system using FACS Diva software (BD Biosciences).

Statistics analysis

Group differences were analysed by Mann-Whitney's U test or Student's *t*-test and differences between the Kaplan-Meier survival curves were estimated by the log-rank test using Dr. SPSS II software for Windows. The χ^2 test was used to compare the incidence of diabetes at each week of age. *P*-values less than 0.05 were considered significant. The severity of the insulitis was analysed by a Ridit analysis, and *t*-levels higher than 1.96 or lower than −1.96 were considered significant.

Results

Diabetes and insulitis in the IL-17-deficient and IL-17/IFN-γ receptor-deficient NOD mice

In our colony, ~75% of the female and 30%–40% of the male NOD mice usually develop diabetes by 48 weeks. A life-table analysis revealed that the onset of spontaneous diabetes in the IL-17$^{-/-}$/IFN-γR$^{+/+}$ NOD mice was significantly delayed compared to the onset in *wt* NOD littermate mice (p<0.05 by the log-rank test). The weekly incidence of diabetes in the IL-17$^{-/-}$/IFN-γR$^{+/+}$ NOD mice was also significantly lower from 15 to 24 weeks of age compared to that in *wt* NOD mice

(p<0.05 by χ^2 test). However, the cumulative incidence of diabetes at 50 weeks of age in the IL-17$^{-/-}$/IFN-γR$^{+/+}$ NOD mice was similar to that in the *wt* mice (IL-17$^{-/-}$/IFN-γR$^{+/+}$; 80.0 % vs. *wt*; 85.7%) (Fig. 1a). As for the IL-17/IFN-γ receptor double-deficient NOD mice, the onset of diabetes was significantly suppressed compared to those in the IL-17$^{-/-}$/IFN-γR$^{+/+}$ NOD mice and in the *wt* NOD mice (IL-17$^{-/-}$/IFN-γR$^{-/-}$ vs. IL-17$^{-/-}$/IFN-γR$^{+/+}$ and vs. *wt*, p=0.01 by the log-rank test). The weekly incidence of diabetes in the IL-17$^{-/-}$/IFN-γR$^{-/-}$ NOD mice was significantly lower from 26 to 50 weeks of age compared to that in the IL-17$^{-/-}$/IFN-γR$^{+/+}$ (p<0.05 by χ^2 test) and from 16 until 50 weeks of age compared to that in *wt* NOD mice (p<0.05 by χ^2 test). The cumulative incidence of diabetes at 50 weeks of age in the IL-17$^{-/-}$/IFN-γR$^{-/-}$ NOD mice was 43.8%, and disease suppression was maintained throughout the entire lifespan (Fig. 1a).

We next compared the severity of the insulitis at 12 and 18 weeks of age in the IL-17$^{-/-}$/IFN-γR$^{-/-}$, IL-17$^{-/-}$/IFN-γR$^{+/+}$ and *wt* NOD mice. The severity of insulitis was significantly attenuated in the IL-17$^{-/-}$/IFN-γR$^{-/-}$ and IL-17$^{-/-}$/IFN-γR$^{+/+}$ NOD mice compared to that in the *wt* mice (by Ridit analysis). However, there were no significant differences between the IL-17$^{-/-}$/IFN-γR$^{-/-}$ and IL-17$^{-/-}$/IFN-γR$^{+/+}$ NOD mice at 12 or 18 weeks of age (Fig. 1b,c).

IAAs levels in the IL-17 deficient and IL-17/IFN-γ receptor-deficient NOD mice

We then determined the levels of IAAs in the IL-17$^{-/-}$/IFN-γR$^{-/-}$, IL-17$^{-/-}$/IFN-γR$^{+/+}$ and *wt* NOD mice at 8, 12, and 16 week of ages. Despite the suppression of insulitis/diabetes development in the IL-17$^{-/-}$/IFN-γR$^{-/-}$ and IL-17$^{-/-}$/IFN-γR$^{+/+}$ mice, the serum levels of IAAs and the percentage of mice positive for IAAs did not significantly differ between the IL-17$^{-/-}$/IFN-γR$^{-/-}$ and *wt* NOD mice or between the IL-17$^{-/-}$/IFN-γR$^{+/+}$ and *wt* mice, at all ages (data not shown).

Adoptive transfer of the effector CD4$^+$ T cells from IL-17$^{-/-}$ NOD mice into NOD-Scid mice

Our aforementioned data suggest that IL-17 may play an important role in the development of insulitis. However, it is possible that IL-17 is associated with diabetes development only in younger

NOD mice and not in elder mice, since disease inhibition by IL-17 single-deficiency was not maintained past 24 weeks of age. We therefore compared the diabetogenicity of $CD4^+$ effector T cells from IL-$17^{-/-}$ NOD and *wt* NOD mice at different ages to adoptively transfer diabetes into NOD-SCID mice.

The adoptive transfer of purified $CD4^+CD25^-$ effector T cells from 10-week-old nondiabetic IL-$17^{-/-}$ mice demonstrated significantly delayed disease onset compared to those from the corresponding *wt* NOD mice (Fig. 2a). No significant differences in disease development between IL-$17^{-/-}$ and *wt* NOD mice were observed following adoptive transfer with $CD4^+CD25^-$ T cells from 18-week-old nondiabetic mice (Fig. 2b), or with $CD4^+$ T cells from 15- to 22-week-old newly diabetic mice (Fig. 2c).

Flow cytometric analysis for $CD4^+$ T cells in the IL-17 deficient and IL-17/IFN-γ receptor-deficient NOD mice

We firstly counted lymphocyte numbers and found that 10-week-old IL-$17^{-/-}$/IFN-γ$R^{-/-}$ NOD mice had significantly reduced numbers of lymphocytes including $CD3^+$, $CD4^+$ and $CD8^+$ T cells and B cells in the spleen and the numbers were almost half or less than half of those in the IL-$17^{-/-}$/IFN-γ$R^{+/+}$ or *wt* NOD mice. The percentages of $CD4^+$ T cells, $CD8^+$ T cells, and B cells in the SPCs were not significantly different among three groups (Table 1). As for the $CD4^+$ T cell fraction, the production of cytokines was evaluated by intracellular cytokine staining after stimulation with PMA and ionomycin for 5 h. The percentages of IFN-γ-producing cells did not significantly differ among the three groups, although the number in the IL-$17^{-/-}$/IFN-γ$R^{-/-}$ mice tended to be decreased compared to the other groups (3.22 ± 1.37 % vs. 4.42 ± 0.90 % in IL-$17^{-/-}$/IFN-γ$R^{+/+}$ mice, p=0.11 vs. 4.19 ± 0.66 % in *wt* mice, p=0.11, respectively) (Fig. 3a-c,d). IL-17-producing cells among the $CD4^+$ T cells were observed only in *wt* mice (0.34 ± 0.11 %) as expected, and double-positive cells with IL-17 and IFN-γ were not observed in any of the groups (Fig. 3a-c,e).

We next determined the activation markers including CD44 and CD62L on $CD4^+$ T cells and regulatory T cells (Tregs) population without stimulation. No significantly differences were found in

level of activation markers on the $CD4^+$ T cells among three groups (Fig. 4 a-c,g). As for Tregs, the percentage of $Foxp3^+$ $CD25^+$ cells to $CD4^+$ T cells was significantly higher in the IL-$17^{-/-}$/IFN-$\gamma R^{-/-}$ mice than in the IL-$17^{-/-}$/IFN-$\gamma R^{+/+}$ mice (6.8 ± 0.96% vs. 4.7 ± 0.54%, $p<0.01$) and in the *wt* mice (vs. 5.4 ± 0.32%, $p<0.05$) (Fig. 4 d-f,h). The preferential increase of Tregs could be systemic phenotype since higher percentage of $CD25^+$ cells to $CD4^+$ T cells were observed in mesenteric lymph nodes or pancreatic draining lymph nodes (data not shown).

Discussion

In the present study, we first determined the impact of the genetic deletion of IL-17, a potent proinflammatory cytokine, in the NOD mouse to investigate whether IL-17 is involved in the pathogenesis of type 1 diabetes. Our results showed that the severity of insulitis was attenuated in both the IL-17 single-deficient and IL-17/IFN-γR double-deficient NOD mice, with no significant difference between these two types of mice, indicating that IL-17 rather than IFN-γ signaling plays a key role in the build-up of the inflammatory infiltrate into islets in NOD mice, as is the case for numerous other autoimmune diseases (Fig. 1b,c)[11-16]. This result is also consistent with that obtained by Martin-Orozco et al. describing that *in vitro*-polarized Th17 cells derived from BDC2.5 TCR-Tg NOD mice transfer extensive insulitis, but does not produce diabetes into newborn NOD mice [23].

Regarding the development of diabetes, we found that the onset of diabetes was significantly delayed in the IL-17 single-deficient NOD mice, although they remained susceptible to long-life diabetes, as is consistent with the report by Joseph et al. [20] (Fig. 1a). In the different line of IL-17 single-deficient NOD mice (original IL-$17^{-/-}$ NOD mice) (n=47) and the *wt* littermate control mice (n=44), we observed the same delayed-onset result in *IL-17*$^{-/-}$ NOD mice (vs. Control, $p<0.05$ by the log-rank test) (Suppl. Fig. 1).

Previous studies have demonstrated that the phenotype of delayed onset in IFN-γR-deficient NOD mice is due to the presence of 129-derived genes closely linked to the knockout gene rather than due to a lack of the target gene [10, 28]. However, our mapping study with polymorphic markers on

chromosome 1 distinguishing NOD from the 129 alleles showed that the maximum interval of the 129-derived genes surrounding the IL-17a gene was less than 1 cM (Suppl. Fig. 2), and there are no identified iddm loci in this region, suggesting that the resistance to the development of insulitis and diabetes in the IL-17$^{-/-}$ NOD mice is attributable to the lack of IL-17 rather than to the influence of the 129-derived genes.

The phenotype of delayed onset in the IL-17 single-deficient NOD mice indicates that IL-17 might participate in the pathogenesis of the early phase of the development of diabetes. This hypothesis was verified by our adoptive transfer study, which showed the successful adoptive transfer of diabetes by CD4^{+}CD25^{-} effector T cells from younger nondiabetic *wt* mice but not by those from the IL-17 single-deficient NOD mice of the same age (Fig. 2a). However, such attenuation of diabetes development by IL-17-deficient diabetogenic T cells was no longer seen following a similar transfer of the same cells derived from older nondiabetic or diabetic mice (Fig. 2b,c). Taken together, the present results suggest that IL-17 participates in the pathogenesis of the early phase of the development of diabetes, but elimination of IL-17 could be readily dispensable in the late phase of diabetes.

In vitro-polarized Th17 cells derived from BDC2.5 TCR-Tg NOD mice do not transfer diabetes in newborn NOD mice but do transfer diabetes in immune-deficient hosts through conversion to Th1 cells or Th1/Th17 cells co-expressing Th1 and Th17 cytokines[23, 24]. We hypothesized that such a conversion of Th17 to Th1 cells or to Th1/Th17 cells may have compensated for the disease inhibition by IL-17 single deficiency in NOD mice in the present study. To test our hypothesis, we also evaluated the impact of the genetic deletion of both IL-17 and IFN-γ signaling in the NOD mice to determine whether such a double deficiency could clearly suppress disease. As hypothesised, IL-17/IFN-γR double deficiency significantly suppressed the longstanding incidence of diabetes compared to IL-17 single deficiency in NOD mice (Fig. 1a). These results indicated that Th1 and Th17 cytokines could synergistically contribute to the development of diabetes in NOD mice, since IFN-γR-deficient NOD mice exhibit minimal or no inhibition of disease[10, 28].

We fortuitously found that IL-17/IFN-γR double-deficient NOD mice had severe lymphopenic phenotype. Previous study demonstrated that wild-type NOD mice have mild lymphopenia compared

to nonautoimmune strain and as a result compensatory homeostatic expansion of T cells generate anti-islet autoimmunity resulting in the development of diabetes[29]. In contrast, NOD mice harboring C57BL/6-derived *Idd3* genetic interval (which encodes the IL-2 and IL-21 genes) (NOD.*Idd3* mice) are disease–resistance, are not lymphopenic. Recently it has been shown that naïve T cells from NOD mice exhibit a greater propensity to differentiate in to Th17 cells than those from NOD.*Idd3* mice and IL-21 signaling in antigen presenting cells plays a central role in such Th17 cell development[30].

Alternatively, several studies have demonstrated that diabetes susceptibility and protection in NOD mice are correlated with lymphopenia and homeostatic expansion under a variety of experimental conditions. Thymectomy at weaning or treatment with cyclophosphamide which causes lymphocyte apoptosis accelerate diabetes onset in NOD mice [31, 32]. In contrast, immunization with complete Freund's adjuvnt (CFA) increased T cell numbers and protects NOD mice from diabetes [29, 33]. Kanagawa et al have reported that IFN-γR deficiency abrogates to cyclophosphamide-induced acceleration of diabetes and CFA mediated protection in NOD mice[34, 35]. Thus, Th17 cell development and IFN-γ signaling might play a critical role of diabetes susceptibility mediated by lymphopenia-induced homeostatic expansion in NOD mice, although the precise kinetics and mechanisms of severe lymphopenia in these mice are still being elucidated.

We also found that double-deficienct NOD mice had preferential increase in Tregs among $CD4^+$ splenic T cells. Several recent findings highlight the plasticity within the $CD4^+$ Th cell population including Th1, Th2, Th17 and Tregs, and Th17 cells and Tregs are more plastic than Th1/Th2 cells [21]. Networks of cytokines are critical for determining $CD4^+$ T cell fates and effector cytokines [36]. For example, Thomas et al clearly demonstrated that systemic overproduction of IL-1β in 6- to 16-week-old NOD mice impairs Treg function and promotes the Treg to Th17 conversion [37]. A pair of studies showed that *ex vivo* or *in vitro*-generated Th17 cells can be converted to Th1 phenotype by combined IFN-γ and IL-12 signaling through epigenetic processes [38, 39]. As described above, *in vitro*-polarized Th17 cells are readily reprogrammed into other T cell lineages upon transfer into lymphopenic hosts [23, 40]. Of note, the treatment with cyclophosphamide not only fails to accelerate diabetes but also confer permanent protection of diabetes by the preferential generation of Tregs in the

IFN-γR deficient NOD mice [34]. It is possible that IFN-γR deficiency under the lymphopenic condition inhibits such a Th shift from Th1 to Th17 or from Tregs to another effector T-cell lineage, resulting in a preferential increase in Tregs population and disease-resistance and in our double-deficient NOD mice.

Thus, we here demonstrated that that IL-17/Th17 participates in the development of insulitis and that both IL-17 and IFN-γ signaling could synergistically contribute to the effector T cells/Tregs balance to effector T cells during homeostasis expansion and subsequent development of diabetes in NOD mice.

From the clinical point of view, the therapeutic efficacy of the inhibition of Th17 cells in some autoimmune diseases has been demonstrated [18, 19]. It was reported that children with new-onset type 1 diabetes have an increased proportion of memory $CD4^+$ cells which have increased IL-17 secretion, suggesting that upregulation of Th17 immunity is associated with human type 1 diabetes[41, 42]. This implies a novel potential therapeutic strategy for human type 1 diabetes based on the control of IL-17 immunity. However, the results presented in the present study indicate that a single blockade of an effector cytokine such as IL-17 or IFN-γ readily compensates for the Th shift from Tregs to effector Th lineage through the multiple networks of cytokines. The appropriate timing or therapeutic strategy to inhibit the Treg/Teff conversion — such as a combination blockade of multiple cytokines or transcriptional factors such as JAK-STAT pathway, Runx3, and IRF-4 — should be carefully considered with the goal of preventing or delaying the development of type 1 diabetes [36].

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Duality of interest

The authors are not aware of any duality of interest.

Contribution statement

All authors contributed to the conception and design of the study, acquisition, analysis and interpretation of data and drafting and editing the manuscript. All of the authors approved the final version of the manuscript. G Kuriya, M Kobayashi and N. Abiru had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Figure legends

Fig 1 Diabetes and insulitis in the IL-17 deficient and IL-17/IFN-γ receptor deficient NOD mice. (**a**) Incidence of diabetes in *wt* NOD mice (■, n=21), IL-17$^{-/-}$/IFN-γR$^{+/+}$ NOD mice (○, n=20) and IL-17$^{-/-}$/IFN-γR$^{-/-}$ NOD mice (▲, n=16). The χ^2 test was used to compare the incidence of diabetes at each week of age (*: p<0.05, IL-17$^{-/-}$/IFN-γR$^{+/+}$ vs. *wt*, †: p<0.05 IL-17$^{-/-}$/IFN-γR$^{-/-}$ vs. *wt*, ‡: p<0.05, IL-17$^{-/-}$/IFN-γR$^{+/+}$ vs. IL-17$^{-/-}$/IFN-γR$^{-/-}$). The severity of insulitis in 12- (**b**) or 18-week-old (**c**) *wt* NOD mice (n=5), IL-17$^{-/-}$/IFN-γR$^{+/+}$ NOD mice (n=5) and IL-17$^{-/-}$/IFN-γR$^{-/-}$ NOD mice (n=5). The severity of insulitis was scored as described in materials and methods. Levels of insulitis: 0; white bar fill, 1; dotted fill, 2; horizontal-stripes fill, 3; diagonal-stripes fill and 4; black bar fill. A T Ridit analysis was used, and T-levels of higher than 1.96 or lower than −1.96 were considered significant.

Fig 2 Adoptive transfer of effector CD4$^+$ T cells into 8- to 10-week-old NOD-SCID mice. (**a**) CD4$^+$CD25$^-$ T cells (1 x 10^7) from 10-week-old nondiabetic IL-17$^{-/-}$ NOD mice (○, n= 5) or *wt* NOD mice (■, n= 9) were transferred into recipient NOD-SCID mice. $^*p$=0.03 by the log-rank test. (**b**) CD4$^+$CD25$^-$ T cells (1 x 10^7) from 18-week-old nondiabetic IL-17$^{-/-}$ NOD mice (○, n= 10) or *wt* NOD mice (■, n= 6) were transferred into recipient NOD-SCID mice. p=0.84 by the log-rank test. (**c**) CD4$^+$ cells (5.5 x 10^6) from 15-to 22-week old freshly diabetic IL-17$^{-/-}$ NOD mice (○, n=10) or *wt* NOD mice (■, n=10) were transferred into recipient NOD-SCID mice. p=0.48 by the log-rank test.

Fig. 3 IFN-γ$^+$/IL-17$^+$ cells in CD4$^+$ splenocyte T cells in the IL-17 deficient and IL-17/IFN-γ receptor-deficient NOD mice. Splenocytes were prepared, stimulated with PMA and ionomycin for 5 h, stained for cell surface CD4 and intracellular IFN-γ and IL-17, and analysed with flow cytometry (**a-c**)**.** Representative staining of CD4$^+$ splenocytes for intracellular IFN-γ and/or IL-17. (**d**,**e**), Numeration of Th1 and Th17 cells, respectively. The data are means ± S.D. (n=5).

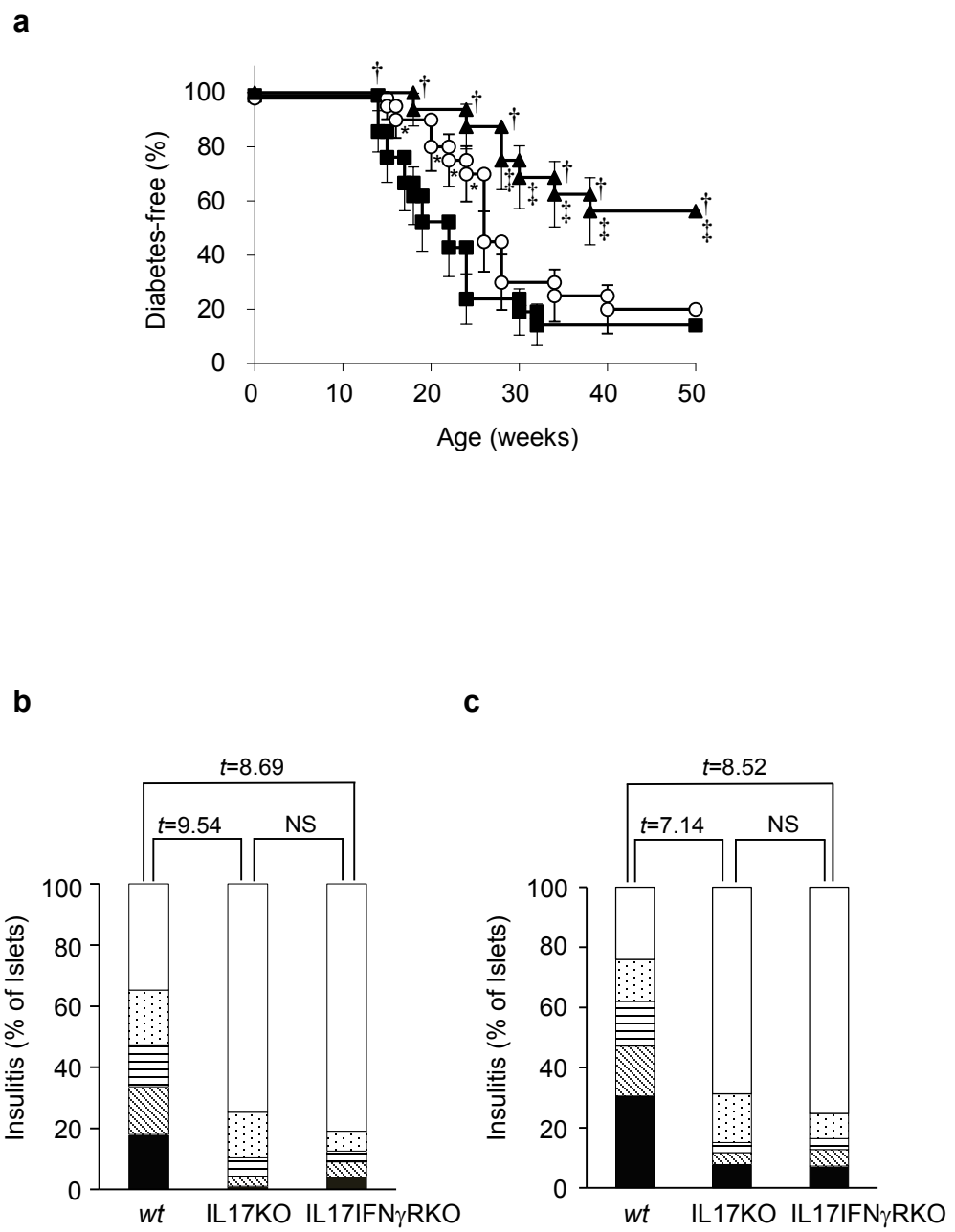
Fig. 4 Activation markers and Foxp3$^+$CD25$^+$ cells in CD4$^+$ splenocyte T cells. Unstimulated SPCs

were stained for cell-surface CD4, CD62L, CD44 and CD25 and intracellular Foxp3 and analysed with flow cytometry. Representative staining of $CD4^+$ SPCs for **(a-c)** CD62L and CD44 and **(d-f)** CD25 and Foxp3. Numeration of **(g)** each $CD44^{low}CD62L^{hi}$, $CD44^{hi}CD62L^{hi}$, $CD44^{hi}CD62L^{low}$ cells in *wt* NOD mice (black bar fill;), IL-$17^{-/-}$/IFN-$\gamma R^{+/+}$ NOD mice (solid grey fill) and IL-$17^{-/-}$/IFN-$\gamma R^{-/-}$ NOD mice (white bar fill) and **(h)** $CD25^+Foxp3^+$ cells. The data are means ± S.D. (n =5), and group differences were analysed by Mann-Whitney U test. *p<0.05, ***p<0.001

Table 1 Cell numbers of T cells ($CD4^+$, $CD8^+$) and B cells in the SPCs

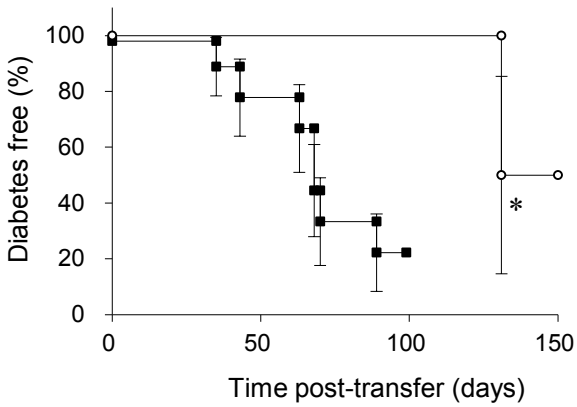
Mice	Total cell number ($\times 10^6$)	Cell number ($\times 10^6$) (% /SPCs)			
		$CD3^+$	$CD4^+$	$CD8^+$	$B220^+$
wt (n=5)	63.4±9.7	27.0±4.8 (42.7±5.1)	18.7±3.4 (29.4±3.5)	5.8±0.9 (9.2±1.4)	16.2±1.6 (25.7±1.8)
IL-$17^{-/-}$/IFN-$\gamma R^{+/+}$ (n=5)	63.4±8.3	24.7±3.0 (39.2±5.2)	16.2±2.6 (25.9±4.8)	5.4±0.5 (8.6±0.9)	15.4±1.1 (24.7±3.3)
IL-$17^{-/-}$/IFN-$\gamma R^{-/-}$ (n=5)	26.0±12.0*	12.2±6.3** (46.1±5.6)	8.4±4.3** (32.1±4.5)	2.4±1.4** (9.0±1.4)	6.5±2.8* (25.1±2.2)

The results are shown as means ± S.D. (*, P<0.001 **, P<0.005 *versus wt* or IL-$17^{-/-}$/IFN-$\gamma R^{+/+}$).



Kuriya et al. Fig. 1

a



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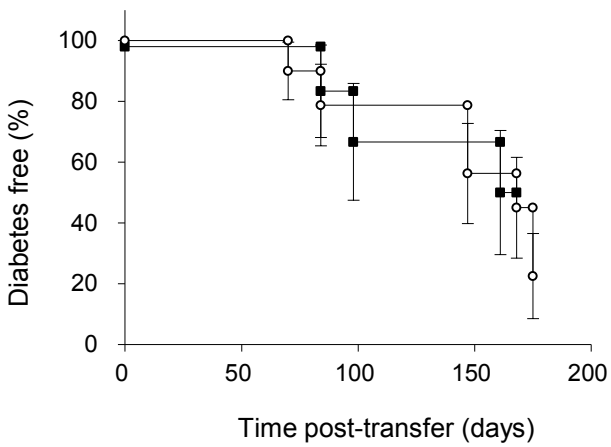

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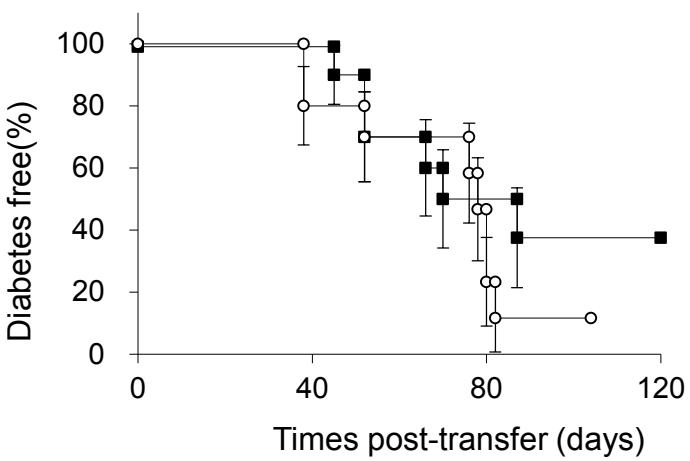
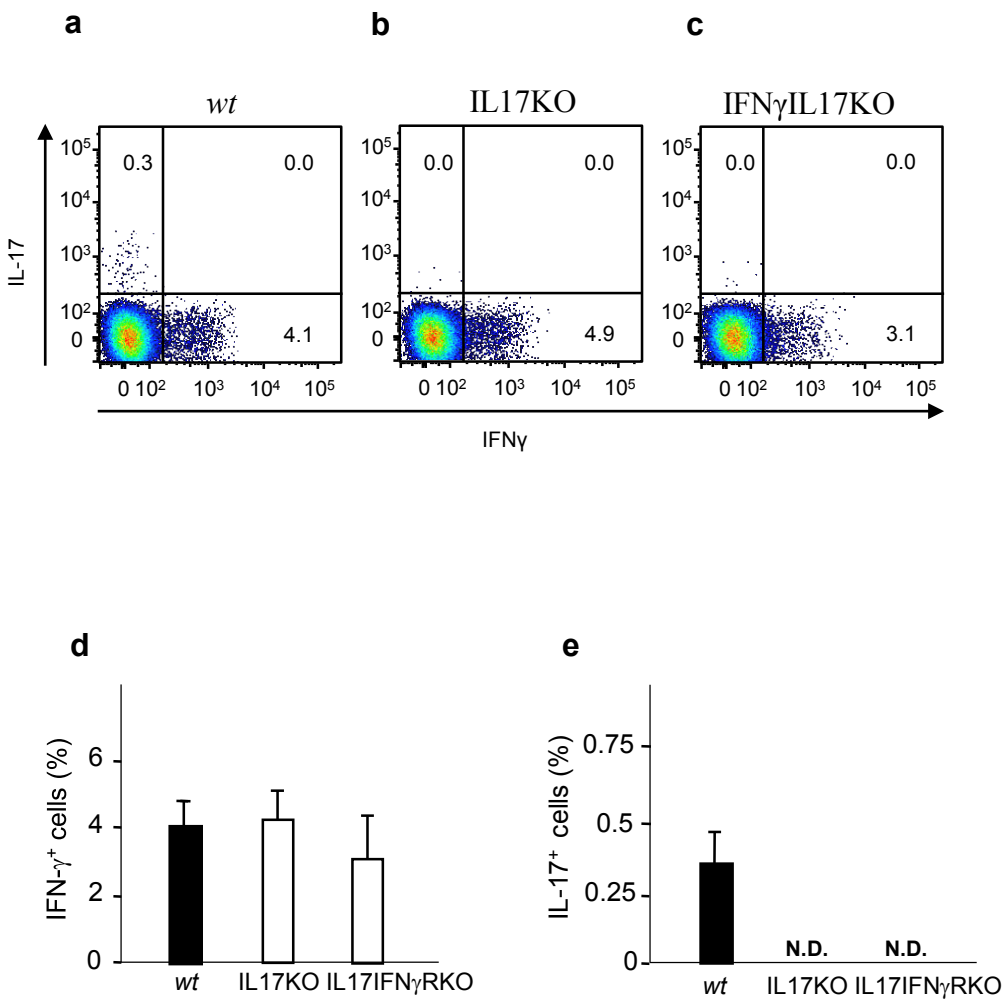
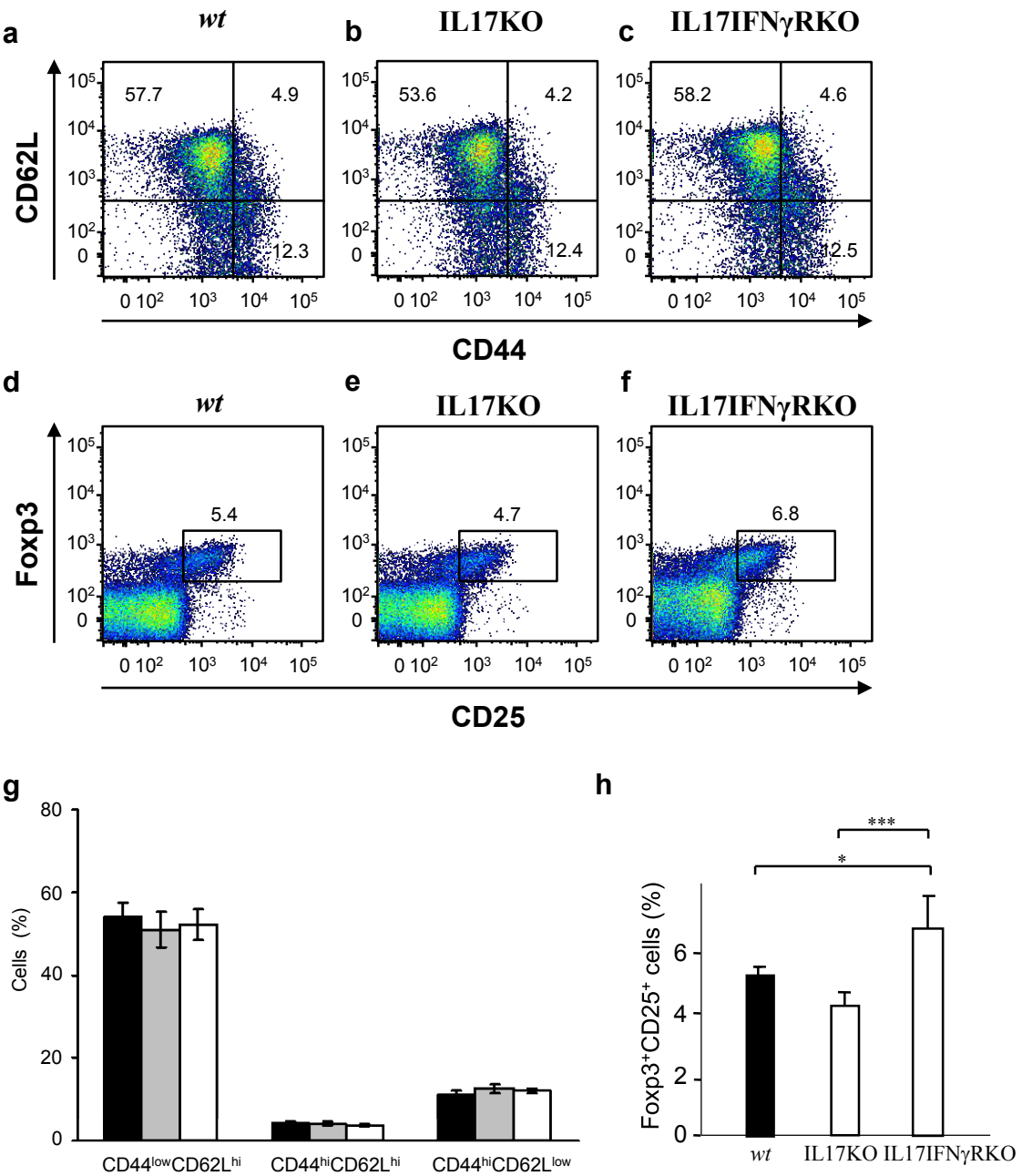


Kuriya et al. Fig. 2



Kuriya et al. Fig. 3



Kuriya et al. Fig. 4