

Article

Haploinsufficiency of interferon regulatory factor 4 strongly protects against autoimmune diabetes in NOD mice

Satoru Akazawa¹, Masakazu Kobayashi², Genpei Kuriya³, Ichiro Horie¹, Liping Yu⁴, Hironori Yamasaki², Minoru Okita⁵, Yuji Nagayama⁶, Toshifumi Matsuyama⁷, Masoud Akbari⁸, Katsuyuki Yui⁸, Atsushi Kawakami¹ and Norio Abiru¹

1. Department of Endocrinology and Metabolism, Unit of Translational Medicine, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan

2. Center for Health and Community Medicine, Nagasaki University, Nagasaki, Japan

3. Department of Endocrinology and Metabolism, National Hospital Organization Nagasaki Medical Center, Nagasaki, Japan

4. Barbara Davis Center for Diabetes, University of Colorado Health Sciences Center, Aurora, CO, USA

5. Department of Locomotive Rehabilitation Science, Unit of Rehabilitation Sciences, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan

6. Department of Molecular Medicine, Atomic Bomb Disease Institute, Nagasaki University,
Nagasaki, Japan

7. Division of Cytokine Signaling, Department of Molecular Microbiology and Immunology,
Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan

8. Division of Immunology, Department of Molecular Microbiology and Immunology,
Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan

Corresponding author: N. Abiru, Department of Endocrinology and Metabolism, Unit of
Translational Medicine, Nagasaki University Graduate School of Biomedical Sciences,
Nagasaki, Japan, 1-7-1 Sakamoto, Nagasaki 852-8501, Japan

email abirun@nagasaki-u.ac.jp

Received: 2 March 2015 / Accepted: 21 July 2015

Abstract

Aims/hypothesis: Interferon regulatory factor (IRF)4 plays a critical role in lymphoid development and the regulation of immune responses. Genetic deletion of IRF4 has been shown to suppress autoimmune disease in several mouse models, but its role in autoimmune diabetes in NOD mice remains unknown.

Methods: To address the role of IRF4 in the pathogenesis of autoimmune diabetes in NOD mice, we generated IRF4-knockout NOD mice and investigated the impact of the genetic deletion of IRF4 on diabetes, insulinitis and insulin autoantibody; the effector function of T cells in vivo and in vitro; and the proportion of dendritic cell subsets.

Results: Heterozygous IRF4-deficient NOD mice maintained the number and phenotype of T cells at levels similar to NOD mice. However, diabetes and autoantibody production were completely suppressed in both heterozygous and homozygous IRF4-deficient NOD mice. The level of insulinitis was strongly suppressed in both heterozygous and homozygous IRF4-deficient mice, with minimal insulinitis observed in heterozygous mice. An adoptive transfer study revealed that IRF4 deficiency conferred disease resistance in a gene-dose-dependent manner in recipient NOD/severe combined immunodeficiency mice. Furthermore, the proportion of migratory dendritic cells in lymph nodes was reduced in

heterozygous and homozygous IRF4-deficient NOD mice in an IRF4 dose-dependent manner.

These results suggest that the levels of IRF4 in T cells and dendritic cells are important for the pathogenesis of diabetes in NOD mice.

Conclusions/interpretation: Haploinsufficiency of IRF4 halted disease development in NOD mice. Our findings suggest that an IRF4-targeted strategy might be useful for modulating autoimmunity in type 1 diabetes.

Keywords

Autoimmunity, Dendritic cells, Interferon regulatory factor 4, NOD mice, T cells, Type 1 diabetes

Abbreviations

DC	Dendritic cell
FOXP3	Forkhead box protein P3
IAA	Insulin autoantibody
IRF	Interferon regulatory factor
mDC	Migratory dendritic cell
MFI	Mean fluorescent intensity

PE	Phycoerythrin
SCID	Severe combined immunodeficiency
SPC	Splenocyte
TCR	T cell receptor
Th	Helper T cell
Treg	Regulatory T cell

Introduction

Type 1 diabetes is an autoimmune disease that results from the autoreactive destruction of insulin-producing beta cells following mononuclear cell infiltration [1]. Studies of NOD mice (a well-characterised animal model of type 1 diabetes) have demonstrated that the beta cell destruction occurs as a consequence of a breakdown in immune regulation, i.e., activation of the innate immune system, expansion of autoreactive CD4⁺ T cells, CD8⁺ T cells, and autoantibody-producing B cells [2, 3]. However, the precise mechanisms that control this autoimmune process are still not clearly understood.

Interferon regulatory factor (IRF)4 is a transcriptional factor of the IRF family that regulates innate and adaptive immune responses [4]. In contrast to other IRFs, IRF4 is preferentially expressed in lymphocytes and its expression is not inducible by interferons, but rather by antigen receptor-mediated stimulation such as by plant lectins, CD3 or IgM crosslinking [5].

A series of reports have documented that IRF4 plays pleiotropic roles in lymphoid development and its immune response [6, 7]. At the molecular level, studies have suggested that IRF4, together with BATF (basic leucine zipper transcription factor ATF-like) and the activator protein-1 family member JUN, acts as a 'pioneer transcriptional factor' that prepares chromatin accessibility for the subsequent lineage-specific transcriptional factor and drives

gene expression for the differentiation of effector T cells [8-10]. It has been documented that IRF4 is necessary for the differentiation of diverse effector CD4⁺ T cell subsets, including helper T cell (Th) types 2 [11-13], Th9 [14] and Th17 [6, 15, 16], follicular Th cells [17], the sustained effector function of cytotoxic T cells [18-20], the regulation of effector regulatory T cells (Tregs) [21], the series of developmental stages of B cells [17, 22-26] and the development and function of dendritic cells (DCs) [27-30].

IRF4 is thought to play a crucial role in the pathogenesis of autoimmune diseases, since the genetic deletion of IRF4 has been shown to abrogate several autoimmune diseases in mouse models, including experimental autoimmune encephalomyelitis [15], experimental colitis [31] and lupus nephritis [32]. Previous studies have suggested that a lack of IRF4 confers disease resistance due to failure of the specific differentiation of CD4⁺ T cells, especially Th17 cells, in models of autoimmune diseases including MRL/lpr mice and NOD mice [15, 32-34].

However, it is still unclear whether one or more specific Th subsets, such as Th17 cells, play a main role in autoimmune pathogenesis in type 1 diabetes, and we and others have reported that a lack of IL-17 does not alter diabetes susceptibility in NOD mice [35, 36].

Studies have indicated that IRF4 is essential for sustaining the differentiation of CD8⁺ T cells by regulating transcriptional factors that are crucial for effector generation (T-bet and

Blimp-1) [18, 19]. Furthermore, the expression of IRF4, which is regulated by antigen–T cell receptor (TCR) affinity, has also been reported to be required for the metabolic function and migratory behaviour of activated CD8⁺ T cells in a dose-dependent manner [20]. We therefore hypothesised that the dosage of IRF4 expression regulates autoimmune pathogenesis in NOD mice, mediated by autoreactive CD4⁺ and CD8⁺ T cells.

To this end, we generated heterozygous and homozygous IRF4-deficient NOD mice and evaluated the influence of the genetic modification of IRF4 on the development of autoimmune diabetes, and on the effector function of CD4⁺ and CD8⁺ T cells in vivo and in vitro.

Methods

Mice NOD mice and NOD/severe combined immunodeficiency (SCID) mice were purchased from Clea Japan (Tokyo, Japan). IRF4-deficient mice had been previously generated [37].

These mice were maintained by breeding at the Laboratory Animal Center for Animal Research at Nagasaki University under specific pathogen-free conditions. Only female mice were used in this study.

Establishment of IRF4-deficient NOD mice IRF4-deficient mice were backcrossed with NOD mice for 15 successive generations. These mice were selected by a PCR analysis of tail

DNA, as previously described [37, 38]. An analysis of the microsatellite markers of the diabetes susceptibility (Idd1-14) loci by PCR of the tail DNA, as previously described [38], showed that the mice were homozygous for all of the NOD alleles. Genotyping of chromosome 13 confirmed that the NOD/B6/129 polymorphic markers D13Mit80 (8.7 cM from IRF4 loci), D13Mit218 (21.8 cM), D13Mit163 (25.4 cM), D13Mit84 (25.7 cM) and D13Mit61 (Idd14) (41.0 cM) were all derived from the NOD background.

Homozygous IRF4-deficient (*Irf4^{-/-}*), heterozygous IRF4-deficient (*Irf4^{+/-}*) and wild-type (WT) NOD mice were established by intercrossing heterozygotes of the 15th generation. All animal experiments described in this study were approved by the institutional animal experimentation committee and were conducted in accordance with the Guidelines for Animal Experimentation.

Monitoring for spontaneous diabetes Mouse blood glucose levels were monitored for spontaneous diabetes using the OneTouch Ultra blood glucose monitoring system (Johnson & Johnson, Tokyo, Japan). Monitoring was conducted weekly when the mice were 12–20 weeks old and then every other week from 20 to 50 weeks of age. Monitoring was terminated when the mice became moribund. Mice with blood glucose levels of more than 13.9 mmol/l for two consecutive measurements were considered diabetic.

Measurement of serum IgG and insulin autoantibodies We performed an ELISA to measure IgG in the serum of mice at 4 weeks of age using the Mouse IgG ELISA Quantitation Set (Bethyl Laboratories, Montgomery, TX, USA). We evaluated the levels of insulin autoantibody (IAA) in serum using a 96-well filtration plate micro IAA assay, as previously described [39].

Histology Pancreatic sections from 18-week-old mice were histologically analysed by staining paraffin-embedded samples with hematoxylin and eosin. A minimum of 30 islets from each mouse were evaluated under a microscope by two different observers. The severity of insulinitis was scored as follows: 0, no lymphocytic infiltration; 1, lymphocytic infiltration occupying <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 >75% of the total islet cell area, or small retracted islets.

Adoptive transfer experiments CD4⁺CD25⁻ T cells or CD8⁺ T cells were purified from the spleens of 10- to 12-week-old prediabetic *Irf4*^{-/-}, *Irf4*^{+/-} and WT NOD mice using magnetic bead cell sorting (Miltenyi Biotech, Auburn, CA, USA). The purity of the CD4⁺CD25⁻ and CD8⁺ T cells was at least 92% and 98%, respectively, as confirmed by flow cytometry

analysis. Purified CD4⁺CD25⁻ (1.0×10^7) and CD8⁺ (0.5×10^7) T cells were combined and i.p. injected into 10-week-old NOD/SCID mice, and the mice were then monitored for blood glucose twice weekly after the adoptive transfer.

Flow cytometric analysis Single-cell suspensions were prepared from mouse spleens, pancreatic lymph nodes and inguinal lymph nodes. Red blood cells were lysed in ammonium chloride buffer. For surface staining, cells were stained for 20 min with the corresponding fluorescent-labelled or biotin-conjugated antibodies against surface molecules: CD3e (145-2C11), CD4 (GK1.5), CD8 (53-6.7), B220 (RA3-6B2), CD44 (IM7), CD62L (MEL-14), I-A^{g7} (I-A^k, 10-3.6) and CD11c (N418).

For intracellular IRF4 staining of T cells, splenocytes (SPCs) were stimulated with plate-bound anti-CD3 (BD Biosciences/BD Pharmingen, San Diego, CA, USA) and soluble anti-CD28 (37.51) (2 µg/ml) (eBioscience, San Diego, CA, USA) for 36 h and then stained with phycoerythrin (PE)-conjugated anti-IRF4 (3E4) antibodies. For the intracellular cytokine staining, SPCs were stimulated with 50 ng/ml phorbol 12-myristate 13-acetate (PMA) and 500 ng/ml ionomycin (both from Sigma, St Louis, MO, USA) in the presence of 2 µM monensin for 5 h. Thereafter, the cells were stained with allophycocyanin-conjugated anti-CD4, followed by intracellular staining with PE-Cy7-conjugated anti-IL-17 (eBio17B7)

and PerCP-Cy5.5-conjugated anti-IFN- γ (XMG1.2) antibodies (all from eBioscience). For intracellular IRF4 staining of DCs, cells were directly stained without culture.

For intracellular staining of granzyme B and perforin, purified CD8⁺ T cells were activated with plate-bound anti-CD3 and anti-CD28 for 48 h, followed by incubation with recombinant mouse IL-2 (100 U/ml; eBioscience) for 72 h. Before intracellular staining, the activated CD8⁺ T cells were re-stimulated with PMA and ionomycin in the presence of monensin during the final 5 h. Thereafter, the cells were stained with PE-conjugated anti-granzyme B (16G6) and FITC-conjugated anti-perforin (eBioOMAK-D) antibodies. For intracellular forkhead box protein P3 (FOXP3) staining, cells were stained with FITC-conjugated anti-CD4 and PE-conjugated anti-CD25 (PC61), followed by intracellular FOXP3 staining with PE-Cy5-conjugated anti-FOXP3 (FJK-16s). All cells were analysed on a FACSCanto II flow cytometry system using FACSDiva software (BD Biosciences).

Statistical analysis Data are expressed as means (SD). Group differences were analysed using Student's *t* test. Differences between Kaplan–Meier survival curves were evaluated by the logrank test, using SPSS II software for Windows (SPSS Inc., Chicago, IL, USA). *p* values of less than 0.05 were considered significant.

Results

IRF4 expression on T cells and DCs, lymphocyte numbers and IgG production We first assessed the expression of IRF4 in CD4⁺ and CD8⁺ T cells after TCR stimulation and in DCs without stimulation. T cells and DCs derived from *Irf4*^{-/-} NOD mice were used as negative controls. The expression levels of IRF4 in T cells and DCs derived from *Irf4*^{+/-} NOD mice were almost half those from WT NOD mice (mean fluorescent intensity [MFI] of CD4⁺ T cells: 3221±71 in WT vs 1806±209 in *Irf4*^{+/-}; CD8⁺ T cells: 2255±234 in WT vs 1271±192 in *Irf4*^{+/-}; DCs: 1014±83 in WT vs 720±70 in *Irf4*^{+/-}; all p<0.01) (Fig. 1a–c).

Since IRF4 knockout mice have been reported to have lymphadenopathy [37], we assessed the cell numbers of lymphocyte subsets in SPCs and pancreatic lymph nodes. Flow cytometric analysis revealed that, compared with WT NOD mice of the same age, 12-week-old *Irf4*^{-/-} NOD mice had significantly increased numbers of lymphocytes, including CD3⁺, CD4⁺ and CD8⁺ T cells and B cells, but a slightly increased proportion of CD4⁺ T cells and a decreased proportion of CD8⁺ T cells (Table 1). In contrast, *Irf4*^{+/-} NOD mice showed normal lymphocyte subset distribution and cellularity.

We also found that IRF4 deficiency lowered the serum concentrations of IgG in a gene-dose-dependent manner in NOD mice (p<0.01) (Fig. 1d).

Diabetes, insulinitis and IAA levels in IRF4-deficient NOD mice To study the role of IRF4 in

the pathogenesis of autoimmune diabetes in NOD mice, we compared the incidence of diabetes from 12 to 50 weeks of age, the level of insulinitis at 18 weeks and the level of IAA at 12 weeks among female *Irf4*^{-/-}, *Irf4*^{+/-} and WT NOD mice. We found that *Irf4*^{-/-} NOD mice were completely protected from the development of insulinitis/diabetes, whereas littermate WT NOD mice showed the normal development of insulinitis/diabetes as usually observed in our colony. Importantly, heterozygous IRF4 deficiency completely suppressed the development of diabetes, although minimal insulinitis was observed in a small fraction of mice (Fig. 2a,b). IAA expression was absent in both *Irf4*^{+/-} and *Irf4*^{-/-} NOD mice (Fig. 2c). All of the *Irf4*^{-/-} NOD mice, but not the *Irf4*^{+/-} mice, became moribund after 40 weeks of age for unknown reasons. This type of early death is also sometimes observed in *Irf4*^{-/-} non-NOD mice, and it is unlikely that their deaths are associated with autoimmunity.

Adoptive transfer study with Treg-depleted effector T cells into NOD/SCID mice Type 1 diabetes has been considered to be a T cell-mediated disease. The disease resistance induced by IRF4 deficiency may be due to attenuation of the effector function of T cells in NOD mice. To assess the effector function of IRF4-deficient T cells, we compared the development of adoptively transferred diabetes in recipient NOD/SCID mice that received combined Treg-depleted CD4⁺CD25⁻ T cells (1.0×10^7) and CD8⁺ T cells (0.5×10^7) purified from SPCs of prediabetic *Irf4*^{-/-}, *Irf4*^{+/-} and WT NOD mice. Two-thirds (8/12) of the recipient mice

transferred with effector T cells from WT NOD mice rapidly developed diabetes, as expected, while the mice with *Irf4*^{-/-} T cells did not develop diabetes until 50 days post-transfer (Fig. 3, p<0.05). The 25% of mice (3/12) transferred with T cells from *Irf4*^{+/-} NOD mice developed diabetes, but with a significant reduction in diabetes incidence compared with mice transferred with WT T cells (Fig. 3, p<0.05).

To investigate the respective effector functions of IRF4-deficient CD4⁺ and CD8⁺ T cells, we conducted an adoptive transfer experiment with combined CD4⁺CD25⁻ and CD8⁺ T cell subsets alternately derived from *Irf4*^{-/-} and WT NOD mice. Diabetes development was suppressed to 11% (1/9) in mice transferred with *Irf4*^{-/-} CD4/*Irf4*^{+/+} CD8 subsets compared with mice with both subsets from WT NOD mice (Fig. 3, p<0.05). No diabetes was observed in mice with *Irf4*^{+/+} CD4/*Irf4*^{-/-} CD8 subsets (Fig. 3). Histological analysis revealed that the severity of insulinitis was not significantly different between recipient mice with *Irf4*^{-/-}CD4/*Irf4*^{+/+}CD8 and those with *Irf4*^{+/+}CD4/*Irf4*^{-/-}CD8 subsets (mean insulinitis score at 50 days after transfer: 2.02±1.66 in four mice with *Irf4*^{-/-}CD4/*Irf4*^{+/+}CD8 vs 2.14±0.77 in three mice with *Irf4*^{+/+}CD4/*Irf4*^{-/-}CD8, p=0.9). These results indicate that IRF4 plays a crucial role in effector functions in not only CD4⁺ T cells, but also CD8⁺ T cells in NOD mice.

Flow cytometric analysis for CD4⁺, CD8⁺ T cells and DCs in IRF4-deficient NOD mice

Because IRF4 affects the effector function of CD8⁺ as well of CD4⁺ T cells, we evaluated the phenotypes of each T subset. We first determined the expression of surface molecules, identifying memory and naive T cells, CD44 and CD62L, and the Treg population on CD4⁺ T cells without stimulation. We found that *Irf4*^{-/-} NOD mice did not maintain T cell homeostasis, and that the populations of memory and naive T cells were significantly reduced in *Irf4*^{-/-} compared with WT NOD mice. However, the populations of memory and naive T cells in *Irf4*^{+/-} mice were equivalent to those in WT NOD mice (Fig. 4a–c,g). With regard to Tregs, the percentages of CD4⁺CD25⁺FOXP3⁺ T cells were significantly reduced in *Irf4*^{-/-} and *Irf4*^{+/-} NOD mice in a gene-dose-dependent manner (9.7±0.8% in WT vs 7.8±0.4% in *Irf4*^{+/-} and 4.3±0.5% in *Irf4*^{-/-} NOD mice, p<0.01) (Fig. 4d–f,h).

We next evaluated cytokine expression in CD4⁺ T cells by intracellular staining after stimulation. As previously observed in *Irf4*^{-/-} C57BL/6 mice [15], the percentage of IL-17-producing CD4⁺ T cells was significantly reduced in stimulated SPCs derived from *Irf4*^{-/-} NOD mice, while the percentages of IL-17-positive cells from *Irf4*^{+/-} NOD mice were almost equal to those from WT NOD mice. In contrast, the percentages of IFN-γ-producing cells did not significantly differ among the three groups (Fig. 5a–c,g). We also assessed the effector molecules of cytotoxic T cells, granzyme B and perforin, in purified CD8⁺ T cells

after TCR stimulation followed by incubation with IL-2. The production of granzyme B and perforin were severely impaired in *Irf4*^{-/-}, but not in *Irf4*^{+/-}, CD8⁺ T cells compared with WT CD8⁺ T cells (Fig. 5d–f,h).

Because IRF4 also plays an important role in the development and function of dendritic subsets, we next evaluated DCs in NOD mice. The proportion of CD4⁺ DCs in the spleen was reduced in *Irf4*^{-/-} compared with WT NOD mice, as has been previously reported in *Irf4*^{-/-} non-NOD mice [27]. This reduction was not, however, significant in *Irf4*^{+/-} NOD mice (Fig. 6a–f,j). In the lymph nodes, the proportion of migratory (m)DC (MHC-II^{hi}CD11c^{int}) was reduced in *Irf4*^{-/-} compared with WT NOD mice, as has also been reported in *Irf4*^{-/-} non-NOD mice [29, 30]. Interestingly, the proportion of mDCs in the lymph nodes of *Irf4*^{+/-} NOD mice was intermediate between the other two strains, suggesting that the mDC proportion is dependent on the dose of the *Irf4* gene (Fig. 6g–i,k).

Discussion

In this study, we determined the impact of the genetic deletion of IRF4 in NOD mice on the pathogenesis of type 1 diabetes. We found that *Irf4*^{-/-} NOD mice were completely protected from spontaneous diabetes, insulinitis and autoantibody production, indicating that IRF4 is a crucial transcriptional factor in the development of autoimmune diabetes in NOD mice, as has

been reported for other autoimmune diseases [15, 31-33]. Compared with WT NOD mice, the spleens and lymph nodes of *Irf4*^{-/-} NOD mice had phenotypes of lymphadenopathy, including larger size, an increased number of T and B cells and dysregulated naive/memory CD4⁺ T cell populations. Severe hypogammaglobulinaemia was also observed in *Irf4*^{-/-} NOD mice. These phenotypes are consistent with a previous report of *Irf4*^{-/-} mice of non-NOD background [37].

Our adoptive transfer study with effector *Irf4*^{-/-} T cells showed the complete suppression of diabetes in recipient mice. These findings indicate that total elimination of IRF4 in T and B cells leads to their dysregulated function and diminishment of the intrinsic effector function of T cells that is associated with the development of diabetes in NOD mice. Our findings also revealed that: (1) total elimination of IRF4 abrogated the expression of effector molecules of CD8⁺ T cells after stimulation; and (2) deletion of IRF4 in CD8⁺ T cells protected recipient mice from rapid progression of diabetes. These results indicate that the expression of IRF4 is indispensable not only in CD4⁺ T cells, but also in CD8⁺ T cells, for the development of insulinitis/diabetes in NOD mice.

Of note, *Irf4*^{+/-} mice showed complete resistance against diabetes development in our study.

This is the first report to identify the importance of haploinsufficiency of IRF4 for disease resistance in autoimmune disease. This finding is highly critical from a clinical point of view,

as it suggests the importance of subtle differences in IRF4 expression due to gene polymorphisms such as *SPII* for disease development or activity in systemic lupus erythematosus [40]. Similar results have been demonstrated in studies of mitogen-inducible gene 6 protein in streptozotocin-induced diabetes and in our previous study of IL-17 in autoimmune thyroiditis in NOD-H2^{h4} mice [41, 42].

Currently, IRF4 is considered to be a key component that translates TCR affinity or the strength of TCR signalling into the functions of activated CD8⁺ T cells, including their differentiation and expansion in a dose-dependent manner [20, 43]. Interestingly, this gene-dose effect was evident in IRF4-haploinsufficient CD8⁺ T cells. When *Irf4*^{-/-}, *Irf4*^{+/-} and WT CD8⁺ T cells were transferred into the same mice and activated in vivo, clonal expansion was most robust in *Irf4*^{+/+} CD8⁺ T cells, followed by *ifr4*^{+/-} and by *Irf4*^{-/-} CD8⁺ T cells [20]. Consistent with these data, our adoptive transfer study showed that IRF4 deficiency in effector T cells conferred disease resistance in recipient mice in a gene-dose-dependent manner. These results suggest that the dosage of IRF4 affects the function of autoreactive CD8⁺ T cells, or possibly CD4⁺ T cells, which is associated with diabetes susceptibility in NOD mice.

Despite the partial deficit of diabetogenicity in *Irf4*^{+/-} effector T cells, *Irf4*^{+/-} NOD mice did

not develop diabetes, and the mechanisms behind this are not completely explained by defects in T cells. We therefore investigated the phenotype of DCs, since previous reports have demonstrated that lack of IRF4 impairs the development of CD8⁻CD11b⁺ DCs in the spleen [27, 28] and reduces the recruitment of mDCs to draining lymph nodes [29, 30]. Interestingly, haploinsufficiency of IRF4 did not affect the development of CD4⁺ DCs in the spleen, but did reduce the proportion of mDCs in lymph nodes. These results suggest that IRF4 deficiency impairs the migratory function of DCs to draining lymph nodes in a gene-dose-dependent manner, and that this deficit in DC function could play a role in the complete disease resistance of *Irf4*^{+/-} NOD mice.

Clinical intervention trials conducted with patients with recent-onset type 1 diabetes and using targeted immunotherapy to T and B cells have demonstrated transiently reserved residual insulin productivity [44-47], although the treatments did not appear to fundamentally alter the overall disease course. Our study of IRF4-deficient NOD mice clarifies the important role of IRF4 in regulating T cells, DCs and possibly other cells in the pathogenesis of autoimmune diabetes, and our results demonstrate that the partial elimination of IRF4 fully suppresses the development of insulinitis/diabetes in NOD mice. All of these findings indicate that IRF4 could be an effective therapeutic target in autoimmune diabetes.

Acknowledgements

We thank M. Motomura, Y. Ohyama, M. Tanaka, H. Kawachi and S. Chuganji (Nagasaki University, Nagasaki, Japan) for technical assistance.

Funding

This study was supported by research grants from the Japan Society for the Promotion of Science (Nos 23791036 and 24591334).

Duality of interest

The authors declare that there is no duality of interest related to this study.

Contribution statement

All authors contributed to the conception and design of the study; acquisition, analysis and interpretation of data; and drafting and editing of the manuscript. All of the authors approved the final version of the manuscript. SA, MK and NA had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

References

- [1] Atkinson MA, Maclaren NK (1994) The pathogenesis of insulin-dependent diabetes mellitus. *N Engl J Med* 331: 1428-1436
- [2] Bluestone JA, Herold K, Eisenbarth G (2010) Genetics, pathogenesis and clinical interventions in type 1 diabetes. *Nature* 464: 1293-1300

- [3] Anderson MS, Bluestone JA (2005) The NOD mouse: a model of immune dysregulation. *Annu Rev Immunol* 23: 447-485
- [4] Tamura T, Yanai H, Savitsky D, Taniguchi T (2008) The IRF family transcription factors in immunity and oncogenesis. *Annu Rev Immunol* 26: 535-584
- [5] Matsuyama T, Grossman A, Mittrucker HW, et al. (1995) Molecular cloning of LSIRF, a lymphoid-specific member of the interferon regulatory factor family that binds the interferon-stimulated response element (ISRE). *Nucleic Acids Res* 23: 2127-2136
- [6] Biswas PS, Bhagat G, Pernis AB (2010) IRF4 and its regulators: evolving insights into the pathogenesis of inflammatory arthritis? *Immunol Rev* 233: 79-96
- [7] Huber M, Lohoff M (2014) IRF4 at the crossroads of effector T cell fate decision. *Eur J Immunol* 44: 1886-1895
- [8] Glasmacher E, Agrawal S, Chang AB, et al. (2012) A genomic regulatory element that directs assembly and function of immune-specific AP-1-IRF complexes. *Science* 338: 975-980
- [9] Li P, Spolski R, Liao W, et al. (2012) BATF-JUN is critical for IRF4-mediated transcription in T cells. *Nature* 490: 543-546
- [10] Murphy TL, Tussiwand R, Murphy KM (2013) Specificity through cooperation: BATF-IRF interactions control immune-regulatory networks. *Nat Rev Immunol* 13: 499-509
- [11] Rengarajan J, Mowen KA, McBride KD, Smith ED, Singh H, Glimcher LH (2002) Interferon regulatory factor 4 (IRF4) interacts with NFATc2 to modulate interleukin 4 gene expression. *J Exp Med* 195: 1003-1012
- [12] Lohoff M, Mittrucker HW, Prechtel S, et al. (2002) Dysregulated T helper cell differentiation in the absence of interferon regulatory factor 4. *Proceedings of the National Academy of Sciences of the United States of America* 99: 11808-11812
- [13] Tominaga N, Ohkusu-Tsukada K, Udono H, Abe R, Matsuyama T, Yui K (2003) Development of Th1 and not Th2 immune responses in mice lacking IFN-regulatory factor-4. *International immunology* 15: 1-10
- [14] Staudt V, Bothur E, Klein M, et al. (2010) Interferon-regulatory factor 4 is essential for the developmental program of T helper 9 cells. *Immunity* 33: 192-202
- [15] Brustle A, Heink S, Huber M, et al. (2007) The development of inflammatory T(H)-17 cells requires interferon-regulatory factor 4. *Nat Immunol* 8: 958-966

- [16] Huber M, Brustle A, Reinhard K, et al. (2008) IRF4 is essential for IL-21-mediated induction, amplification, and stabilization of the Th17 phenotype. *Proceedings of the National Academy of Sciences of the United States of America* 105: 20846-20851
- [17] Bollig N, Brustle A, Kellner K, et al. (2012) Transcription factor IRF4 determines germinal center formation through follicular T-helper cell differentiation. *Proceedings of the National Academy of Sciences of the United States of America* 109: 8664-8669
- [18] Yao S, Buzo BF, Pham D, et al. (2013) Interferon regulatory factor 4 sustains CD8⁺ T cell expansion and effector differentiation. *Immunity* 39: 833-845
- [19] Raczkowski F, Ritter J, Heesch K, et al. (2013) The transcription factor interferon regulatory factor 4 is required for the generation of protective effector CD8⁺ T cells. *Proceedings of the National Academy of Sciences of the United States of America* 110: 15019-15024
- [20] Man K, Miasari M, Shi W, et al. (2013) The transcription factor IRF4 is essential for TCR affinity-mediated metabolic programming and clonal expansion of T cells. *Nat Immunol* 14: 1155-1165
- [21] Cretney E, Xin A, Shi W, et al. (2011) The transcription factors Blimp-1 and IRF4 jointly control the differentiation and function of effector regulatory T cells. *Nat Immunol* 12: 304-311
- [22] Ochiai K, Maienschein-Cline M, Simonetti G, et al. (2013) Transcriptional regulation of germinal center B and plasma cell fates by dynamical control of IRF4. *Immunity* 38: 918-929
- [23] Johnson K, Hashimshony T, Sawai CM, et al. (2008) Regulation of immunoglobulin light-chain recombination by the transcription factor IRF-4 and the attenuation of interleukin-7 signaling. *Immunity* 28: 335-345
- [24] Sciammas R, Shaffer AL, Schatz JH, Zhao H, Staudt LM, Singh H (2006) Graded expression of interferon regulatory factor-4 coordinates isotype switching with plasma cell differentiation. *Immunity* 25: 225-236
- [25] Klein U, Casola S, Cattoretti G, et al. (2006) Transcription factor IRF4 controls plasma cell differentiation and class-switch recombination. *Nat Immunol* 7: 773-782
- [26] Willis SN, Good-Jacobson KL, Curtis J, et al. (2014) Transcription factor IRF4 regulates germinal center cell formation through a B cell-intrinsic mechanism. *Journal of immunology* 192: 3200-3206

- [27] Suzuki S, Honma K, Matsuyama T, et al. (2004) Critical roles of interferon regulatory factor 4 in CD11b^{high}CD8α⁻ dendritic cell development. Proceedings of the National Academy of Sciences of the United States of America 101: 8981-8986
- [28] Tamura T, Taylor P, Yamaoka K, et al. (2005) IFN regulatory factor-4 and -8 govern dendritic cell subset development and their functional diversity. Journal of immunology 174: 2573-2581
- [29] Akbari M, Honma K, Kimura D, et al. (2014) IRF4 in dendritic cells inhibits IL-12 production and controls Th1 immune responses against *Leishmania major*. Journal of immunology 192: 2271-2279
- [30] Bajana S, Roach K, Turner S, Paul J, Kovats S (2012) IRF4 promotes cutaneous dendritic cell migration to lymph nodes during homeostasis and inflammation. Journal of immunology 189: 3368-3377
- [31] Mudter J, Amoussina L, Schenk M, et al. (2008) The transcription factor IFN regulatory factor-4 controls experimental colitis in mice via T cell-derived IL-6. The Journal of clinical investigation 118: 2415-2426
- [32] Lech M, Weidenbusch M, Kulkarni OP, et al. (2011) IRF4 deficiency abrogates lupus nephritis despite enhancing systemic cytokine production. J Am Soc Nephrol 22: 1443-1452
- [33] Biswas PS, Gupta S, Chang E, et al. (2010) Phosphorylation of IRF4 by ROCK2 regulates IL-17 and IL-21 production and the development of autoimmunity in mice. The Journal of clinical investigation 120: 3280-3295
- [34] Biswas PS, Gupta S, Chang E, Bhagat G, Pernis AB (2011) Aberrant ROCK activation promotes the development of type I diabetes in NOD mice. Cell Immunol 266: 111-115
- [35] Joseph J, Bittner S, Kaiser FM, Wiendl H, Kissler S (2012) IL-17 silencing does not protect nonobese diabetic mice from autoimmune diabetes. Journal of immunology 188: 216-221
- [36] Kuriya G, Uchida T, Akazawa S, et al. (2013) Double deficiency in IL-17 and IFN-γ signalling significantly suppresses the development of diabetes in the NOD mouse. Diabetologia 56: 1773-1780
- [37] Mittrucker HW, Matsuyama T, Grossman A, et al. (1997) Requirement for the transcription factor LSIRF/IRF4 for mature B and T lymphocyte function. Science 275: 540-543

- [38] Serreze DV, Chapman HD, Varnum DS, et al. (1996) B lymphocytes are essential for the initiation of T cell-mediated autoimmune diabetes: analysis of a new “speed congenic” stock of NOD.*Igμ^{null}* mice. *J Exp Med* 184: 2049-2053
- [39] Yu L, Robles DT, Abiru N, et al. (2000) Early expression of antiinsulin autoantibodies of humans and the NOD mouse: evidence for early determination of subsequent diabetes. *Proceedings of the National Academy of Sciences of the United States of America* 97: 1701-1706
- [40] Hikami K, Kawasaki A, Ito I, et al. (2011) Association of a functional polymorphism in the 3'-untranslated region of *SP11* with systemic lupus erythematosus. *Arthritis and rheumatism* 63: 755-763
- [41] Ahyi AN, Chang HC, Dent AL, Nutt SL, Kaplan MH (2009) IFN regulatory factor 4 regulates the expression of a subset of Th2 cytokines. *Journal of immunology* 183: 1598-1606
- [42] Horie I, Abiru N, Nagayama Y, et al. (2009) T helper type 17 immune response plays an indispensable role for development of iodine-induced autoimmune thyroiditis in nonobese diabetic-H2^{h4} mice. *Endocrinology* 150: 5135-5142
- [43] Nayar R, Schutten E, Bautista B, et al. (2014) Graded levels of IRF4 regulate CD8⁺ T cell differentiation and expansion, but not attrition, in response to acute virus infection. *Journal of immunology* 192: 5881-5893
- [44] Keymeulen B, Vandemeulebroucke E, Ziegler AG, et al. (2005) Insulin needs after CD3-antibody therapy in new-onset type 1 diabetes. *N Engl J Med* 352: 2598-2608
- [45] Keymeulen B, Walter M, Mathieu C, et al. (2010) Four-year metabolic outcome of a randomised controlled CD3-antibody trial in recent-onset type 1 diabetic patients depends on their age and baseline residual beta cell mass. *Diabetologia* 53: 614-623
- [46] Orban T, Bundy B, Becker DJ, et al. (2014) Costimulation modulation with abatacept in patients with recent-onset type 1 diabetes: follow-up 1 year after cessation of treatment. *Diabetes Care* 37: 1069-1075
- [47] Pescovitz MD, Greenbaum CJ, Bundy B, et al. (2014) B-lymphocyte depletion with rituximab and beta-cell function: two-year results. *Diabetes Care* 37: 453-459

Figure legends

Fig. 1 (a) Expression of IRF4 in CD4⁺ T cells, CD8⁺ T cells and DCs. SPCs from WT (red), *Irf4*^{+/-} (green) and *Irf4*^{-/-} (blue) NOD littermates were stimulated with anti-CD3/CD28 monoclonal antibodies and stained for CD4, CD8 and IRF4. IRF4 profiles of CD4⁺ and CD8⁺ cells are shown (left and centre). SPCs were stained for CD11c/I-A^{g7}, and IRF4 profiles of CD11c⁺I-A^{g7}⁺ cells are shown (right). MFI of IRF4 staining in (b) CD4⁺ and CD8⁺ T cells and (c) DCs derived from WT (black bars), *Irf4*^{+/-} (grey bars) and *Irf4*^{-/-} NOD littermates (white bars). Data are means±SD (*n*=3–5). (d) Serum IgG from female WT, *Irf4*^{+/-} and *Irf4*^{-/-} NOD littermates (*n*=10 per group) was evaluated with ELISA. ***p*<0.01

Fig. 2 Diabetes, insulinitis and IAAs in WT, *Irf4*^{+/-} and *Irf4*^{-/-} NOD mice. (a) Incidence of diabetes in WT (squares, *n*=40), *Irf4*^{+/-} (triangles, *n*=30) and *Irf4*^{-/-} (circles, *n*=40) NOD littermates. (b) Severity of insulinitis in WT, *Irf4*^{+/-} and *Irf4*^{-/-} NOD littermates at 18 weeks of age (*n*=5 per group). Insulinitis levels: 0 (white), 1 (light grey), 2 (dark grey), 3 (horizontal stripes) and 4 (black). (c) Levels of serum IAA in WT, *Irf4*^{+/-} and *Irf4*^{-/-} NOD littermates at 12 weeks of age (*n*=10 per group). The index value of 0.01 was applied as the cut-off limit. Each square/triangle/circle represents an individual animal. Small horizontal lines indicate the mean

Fig. 3 Adoptive transfer of purified CD4⁺CD25⁻ T cells (1.0×10^7) and CD8⁺ T cells (0.5×10^7) from WT, *Irf4*^{+/-} and *Irf4*^{-/-} NOD mice into NOD/SCID mice. Recipient mice were divided into five groups according to the combination of transfer subsets: WT CD4⁺CD25⁻ T cells + WT CD8⁺ T cells ($n=12$, squares); *Irf4*^{+/-} CD4⁺CD25⁻ T cells + *Irf4*^{+/-} CD8⁺ T cells ($n=12$, triangles); *Irf4*^{-/-} CD4⁺CD25⁻ T cells + *Irf4*^{-/-} CD8⁺ T cells ($n=5$, circles); *Irf4*^{-/-} CD4⁺CD25⁻ T cells + WT CD8⁺ T cells ($n=9$, black diamonds); and WT CD4⁺CD25⁻ T cells + *Irf4*^{-/-} CD8⁺ T cells ($n=9$, white diamonds). Error bars: SE. * $p < 0.05$, ** $p < 0.01$ using the logrank test vs the subgroup that received both subsets from WT NOD mice

Fig. 4 Activation markers and FOXP3⁺CD25⁺ cells in CD4⁺ T cells. Representative staining of CD4⁺ SPCs for (a–c) CD44 and CD62L and (d–f) CD25 and FOXP3. Quantification of (g) CD44^{low}CD62L^{hi}, CD44^{hi}CD62L^{hi} and CD44^{hi}CD62L^{low} cells and (h) CD25⁺FOXP3⁺ cells in WT (black bars), *Irf4*^{+/-} (grey bars) and *Irf4*^{-/-} (white bars) NOD littermates. Data are means \pm SD ($n=5$). ** $p < 0.01$

Fig. 5 IFN- γ ⁺/IL-17⁺ cells in CD4⁺ T cells and effector molecules in CD8⁺ T cells. (a–c) Representative staining of CD4⁺ SPCs for intracellular IFN- γ and/or IL-17. (d–f) Purified CD8⁺ T cells were stimulated as mentioned in the Methods section. Representative data for the intracellular expression of granzyme B and perforin are shown. Quantification of (g)

IFN- γ - and IL-17-positive cells and **(h)** granzyme B- and perforin-positive cells in WT (black bars), *Irf4*^{+/-} (grey bars) and *Irf4*^{-/-} (white bars) NOD littermates. Data are means \pm SD ($n=5$).

* $p<0.05$, ** $p<0.01$

Fig. 6 DC subsets in spleen and peripheral lymph nodes. **(a–f)** SPCs were stained for CD11c, I-A^{g7}, CD4 and CD8. **(a–c)** CD11c/I-A^{g7} profiles and **(d–f)** CD4/CD8 profiles of DCs (CD11c⁺I-A^{g7+}). The numbers in the profiles indicate the proportions in the square **(a–c)** or in each quadrant **(d–f)**. **(g–i)** Cells from inguinal lymph nodes were stained for CD11c and I-A^{g7}, and the proportions (%) of mDCs (I-A^{g7hi}CD11c^{int}) are indicated. Populations of DC subsets in **(j)** spleen and **(k)** inguinal lymph nodes of WT (black bars), *Irf4*^{+/-} (grey bars) and *Irf4*^{-/-} (white bars) NOD littermates are summarised as means \pm SD ($n=3$). * $p<0.05$, ** $p<0.01$

Table 1 Number of T cells (CD4⁺, CD8⁺) and B cells in SPCs and pancreatic lymph nodes

Subgroup	Total cell number, ×10 ⁶	Cell number, ×10 ⁶ (% of total cells)			
		CD3 ⁺	CD4 ⁺	CD8 ⁺	B220 ⁺
SPCs					
WT	15.8±4.5	7.0±1.7 (44.4±2.4)	4.6±1.3 (28.9±1.6)	1.9±0.3 (12.5±1.5)	4.9±2.0 (30.3±4.4)
<i>Irf4</i> ^{+/-}	20.1±9.1	8.9±4.1 (43.9±2.9)	5.7±2.5 (28.6±3.0)	2.4±1.2 (11.8±0.7)	6.4±3.2 (31.6±3.7)
<i>Irf4</i> ^{-/-}	104.0±52.1**	46.4±23.7** (44.7±4.4)	35.1±17.3** (34.2±3.3*)	7.4±4.2* (6.9±1.6**)	33.3±13.0** (33.7±4.6)
Pancreatic lymph nodes					
WT	0.37±0.16	0.30±0.14 (81.9±3.5)	0.21±0.10 (57.6±5.9)	0.08±0.04 (21.0±1.8)	0.04±0.02 (10.3±3.6)
<i>Irf4</i> ^{+/-}	0.66±0.43	0.56±0.38 (84.4±5.1)	0.40±0.26 (60.5±4.5)	0.14±0.10 (21.0±1.6)	0.06±0.03 (10.6±2.8)
<i>Irf4</i> ^{-/-}	7.26±2.83**	6.20±2.34** (85.2±9.6)	4.95±1.81** (68.4±5.1*)	1.07±0.64** (14.4±6.5)	0.81±0.79 (11.2±7.6)

Data are means±SD

p*<0.05, *p*<0.01 vs WT









