Analysis of the expression of candidate genes for type 1 diabetes susceptibility in T cells

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Abstract. Type 1 diabetes is characterized by T-cell–mediated autoimmune destruction of pancreatic β-cells. Currently, approximately 50 type 1 diabetes susceptibility genes or chromosomal regions have been identified. However, the functions of type 1 diabetes susceptibility genes in T cells are elusive. In this study, we evaluated the correlation between type 1 diabetes susceptibility genes and T-cell signaling. The expression levels of 22 candidate type 1 diabetes susceptibility genes in T cells from nonobese diabetic (NOD), control C57BL/6 (B6), and NOD-control F1 hybrid mice were analyzed in response to 2 key immunoregulatory cytokines: interleukin-2 (IL-2) and transforming growth factor β (TGF-β). Exogenous gene expression studies were also performed in EL4 and Jurkat E6.1 T-cell lines. Significant differences in the expression of Clec16a, Dlk1, Il2, Ptpn22, Rnls, and Zac1 (also known as Plagl1) were observed in T cells derived from the 3 strains of mice, and TGF-β differentially influenced the expression of Cita4, Foxp3, Il2, Ptpn22, Sh2b3, and Zac1. We found that TGF-β induced Zac1 expression in both primary T cells and EL4 cells and that exogenous expression of Zac1 and ZAC1 in T-cell lines altered the expression of Il2 and DLK1, respectively. The results of our study indicate the possibility that additional genetic pathways underlying type 1 diabetes susceptibility, including those involving Clec16a, Dlk1, Rnls, Sh2b3, and Zac1 under IL-2 and TGF-β signaling in T cells, may be shared between human and NOD mice.

Key words: Type 1 diabetes, Genetic susceptibility genes, CD4+ T cell, Nonobese diabetic (NOD) mice, Transforming growth factor β
sis and to identify the missing link between non-MHC type 1 diabetes susceptibility genes and T-cell signaling, we analyzed the regulation of candidate type 1 diabetes susceptibility genes and other genes characteristic of the transcriptional signature of Tregs [21, 22] in T cells from NOD, control C57BL/6 (B6), and their hybrid F1 mice.

**Materials and Methods**

**Mice**

NOD/Shi and B6 mice were purchased from CLEA Japan, Inc. B6 mice were used as a nondiabetic control strain. Two reciprocal outcrosses [23] were performed as follows. Female NOD mice were crossed with male B6 mice, and male NOD mice were crossed with female B6 mice to obtain NOD × B6 F1 (NODB6F1) and B6 × NOD F1 (B6NODF1) mice, respectively. Male NOD, B6, and reciprocal F1 mice at 8–12 weeks of age were used in this study. In the present study, we attempted to examine the genetic components underlying differences in gene expression patterns that were genuine and, to the extent possible, unaffected by environmental factors (e.g., hyperglycemia). Hyperglycemia is known to alter gene expression patterns [24]. Male NOD mice do not develop diabetes mellitus by 8–12 weeks of age [25] (http://www.clea-japan.com/), but develop insulin (93.3% (14/15) at 9 weeks of age (http://www.clea-japan.com/). The development of diabetes is dependent on homozygosity for the NOD mouse’s unique MHC region and their F1 mice do not develop diabetes or insulin [26]. Thus, by using male NOD mice, we attempted to eliminate or at least reduce the possibility of hyperglycemia affecting gene expression patterns in the present study. The experimental designs were approved by the Ethics Committee of the Osaka University Graduate School of Medicine. The guidelines in “Principles of laboratory animal care” (NIH publication no. 85–23, revised 1985; http://grants1.nih.gov/grants/olaw/references/phspol.htm) were also followed.

**Cell culture and transfection**

Murine EL4 (EC85023105) and human Jurkat E6.1 (EC88042803) cells were purchased from the European Collection of Cell Cultures. EL4 is a murine thymoma cell line that retains many T-cell properties and is widely used for studying T-cell signaling and transcriptional regulation in T cells [27, 28]. Jurkat E6.1 is a human T-cell leukemia cell line that produces IL-2 and is also widely used for studying T-cell signaling [27, 29, 30]. EL4 cells were cultured in Iscove’s modified Eagle medium containing GlutaMax (Life Technologies, Gaithersburg, MD, USA), 5% FCS (Nichirei Bioscience Inc., Tokyo, Japan), and penicillin/streptomycin (Wako Pure Chemicals Industries Ltd., Osaka, Japan), as described previously [28], either in the presence or absence of 10 ng/mL recombinant human TGF-β1 (Peprotech, Rocky Hill, NJ, USA). Jurkat E6.1 cells were cultured in RPMI 1640 medium containing 10% FCS, 2 mmol/L L-glutamine, and penicillin/streptomycin. The pSG5.HA.mZac1 and pSG5.HA.hZAC1 expression vectors [31] were kindly provided by Prof. Shih-Ming Huang. Transfections of plasmids into EL4 and Jurkat E6.1 cells were performed with the Neon Transfection System (Life Technologies, Gaithersburg, MD, USA) according to the manufacturer’s protocols, with 1 pulse of 1080 V for 50 ms for EL4 cells and 3 pulses of 1380 V for 10 ms each for Jurkat E6.1 cells.

**CD4⁺ T cell preparation**

Lymph nodes (Cervical, axillary, brachial, inguinal, and mesenteric) and spleens were harvested from the mice, the tissues were gently minced in a cell strainer (BD Falcon, Franklin Lakes, NJ, USA), and red blood cells were lysed with RBC Lysis Buffer (BioLegend, San Diego, CA, USA), as described previously [32]. CD4⁺ T lymphocytes (T cells) were isolated from these cells using the CD4⁺ T Cell Isolation Kit, an MS Column, and a MidiMACSTM (Miltenyi Biotec, Auburn, CA, USA). The numbers of live T cells were counted using a Countess® Automated Cell Counter (Life Technologies, Gaithersburg, MD, USA) according to the manufacturer’s protocols.

**In vitro activation**

Primary T cells (1 mL at 10⁶ cells/well) were activated for 4 d with Dynabeads mouse CD3/CD28 T Cell Expander (Life Technologies, Gaithersburg, MD, USA) at a concentration of 1 bead/cell in RPMI 1640 medium containing 10% FCS, 2 mmol/L L-glutamine, penicillin/streptomycin, and 100 U/mL recombinant murine IL-2 (Wako Pure Chemicals Industries Ltd., Osaka, Japan), with or without 10 ng/mL recombinant human TGF-β1, as described previously, with modifications [18, 21, 28, 32].
**Gene expression analysis**

Complementary DNA templates of cells were prepared using the Ambion® Cells-to-CT™ Kit (Life Technologies, Gaithersburg, MD, USA). The transcript expression levels were measured relative to β-actin (Actb) expression using quantitative PCR (qPCR) in a StepOnePlus™ Real-Time PCR System (Life Technologies, Gaithersburg, MD, USA). All reactions were performed using the TaqMan® Universal Master Mix and in triplicate for each sample. The expression levels are given as dCT values, which were calculated by subtracting the cycle threshold value (cycle number at which fluorescence is first detected) for Actb from the cycle threshold value for each transcript using StepOne software (Life Technologies, Gaithersburg, MD, USA). The gene expression level ratio was also calculated using StepOne software. Information regarding the TaqMan® probes has been provided in Table 1. The Mm00494250_m1 TaqMan® probe was used to examine Zac1 expression levels in primary T cells and EL4 cells. The Mm00494251_m1 and Hs00414677_m1 TaqMan® probes were used to confirm the expression of murine Zac1 and human ZAC1, respectively.

**Statistical analysis**

Statistical analyses were performed using the IBM SPSS statistical program, version 20, and ANOVA was utilized to detect significant intergroup differences. Data are presented as mean ± SEM, and a *p* value less than 0.05 (one-way ANOVA, Tukey’s test) was considered statistically significant. An *f* value less than 0.05 (one-way ANOVA, Tukey’s test) was considered statistically significant in 3 compared groups.

**Results**

**Growth/survival rates of T cells**

Primary T cells were prepared from NOD, B6, and F1 hybrid mice and cultured for 4 d with anti-CD3/CD28 beads and IL-2 in the presence or absence of

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Full names of genes, gene symbols, and TaqMan® probes for the type 1 diabetes susceptibility genes analyzed in this study</th>
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<tr>
<td>Full name of the gene</td>
<td>Symbol</td>
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<tr>
<td>BTB and CNC homology 2</td>
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<tr>
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<td>Cblb</td>
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<tr>
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<td>Ctla4</td>
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<td>Delta-like 1 homolog (Drosophila)</td>
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<td>v-erb-b2 erythroblastic leukemia viral oncogene homolog 3</td>
<td>Erbb3</td>
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<tr>
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<td>Glis3</td>
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<tr>
<td>Peroxisome proliferator activated receptor gamma</td>
<td>Pparg</td>
</tr>
<tr>
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<td>Prkccq</td>
</tr>
<tr>
<td>Protein tyrosine phosphatase, non-receptor type 2</td>
<td>Ptpn2</td>
</tr>
<tr>
<td>Protein tyrosine phosphatase, non-receptor type 22 (lymphoid)</td>
<td>Ptpn22</td>
</tr>
<tr>
<td>Renalase, FAD-dependent amine oxidase</td>
<td>Rnls (C10orf59)</td>
</tr>
<tr>
<td>SH2B adaptor protein 3</td>
<td>Sh2b3 (Lnk)</td>
</tr>
<tr>
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</tr>
<tr>
<td>Vav 3 oncogene</td>
<td>Vav3</td>
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<tr>
<td>Zinc finger protein which regulates apoptosis and cell cycle arrest 1 (pleiomorphic adenoma gene-like 1)</td>
<td>Zac1 (Plagl1)</td>
</tr>
<tr>
<td>Beta-actin</td>
<td>Actb</td>
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Mm, probe for mouse gene; Hs, probe for human gene.
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The number of live T cells was counted before and after the culture period. Then, the growth/survival rates of T cells were calculated. The growth/survival rate of NOD T cells after 4 d of culture without TGF-β was significantly different from that of T cells from either B6 (p = 0.047) or F1 (p = 0.0026) mice in 12 independent experiments (Fig. 1a). In contrast, there was no significant difference in the growth/survival rates among B6, F1, and NOD T cells cultured with TGF-β. This resulted in significant differences in the growth/survival ratios of T cells from NOD mice compared to those from B6 or hybrid F1 mice, whereas the levels of Il2, Ptpn22, and Zac1 expression were significantly lower in T cells from NOD mice than in those from B6 mice. Clec16a expression was significantly higher in T cells from NOD mice than in those from B6 and F1 mice (p = 1.0 × 10^{-5} and p = 0.008, respectively; one-way ANOVA, Tukey’s test; Fig. 1b). The level of Il2 expression was significantly lower in T cells from NOD mice than in those from B6 mice (p = 6.9 × 10^{-3}; Fig. 2c). The level of Ptpn22 expression was significantly lower in T cells from NOD and F1 mice than in those from B6 mice (p = 3.1 × 10^{-6} and 0.0029, respectively; Fig. 2d). In contrast, the level of Zac1 expression was significantly lower in T cells from NOD mice than in those from B6 and F1 mice (p = 3.3 × 10^{-5} and 2.5 × 10^{-4}, respectively) but was comparable in both B6 and F1 T cells (Fig. 2e). Thus, the differ-

![Fig. 1](image-url) Comparison of the growth/survival rates of T cells from C57BL/6 (B6; n = 12), nonobese diabetic (NOD; n = 12), and NOD-B6 F1 hybrid mice (F1; n = 8) mice in response to transforming growth factor β (TGF-β). Data are expressed as mean ± SEM. (a) Growth/survival rates of T cells cultured without TGF-β (TGF-β (−); white bars) or with TGF-β (TGF-β (+); black bars). (b) Growth/survival ratios of TGF-β (+) T cells compared to TGF-β (−) T cells from B6 (white bar), F1 (gray bar), and NOD (black bar) mice. (c) Growth/survival ratios of TGF-β (+) T cells compared to TGF-β (−) T cells from B6NODF1 (n = 3; white bar) and NODB6F1 (n = 5; black bar) mice; *p < 0.05 and **p < 0.01.
ences in Zac1 expression were transmitted to F1 mice in a recessive manner.

**Gene expression levels after 4 d of culture with anti-CD3/CD28 beads, IL-2, and TGF-β**

The gene expression ratios of the 22 genes were compared in T cells from B6 and NOD mice after culture in medium containing TGF-β (Fig. 3a), with the expression level of each gene in the B6-derived T cells set to 1. The levels of Clec16a, Dlk1, Erbb3, Foxp3, Iksf1, Il2rb, and Rnls expression were significantly higher in NOD-derived T cells than in B6-derived T cells, whereas the levels of Glis3 and Ptpn22 expression were significantly lower in NOD-derived T cells than in B6-derived
T cells. Clec16a (3.8 ± 0.43-fold; \( p = 1.6 \times 10^{-6} \)), Dlk1 (3.5 ± 0.63-fold; \( p = 5.9 \times 10^{-8} \)), Pttn22 (0.49 ± 0.040-fold; \( p = 1.4 \times 10^{-11} \)), and Rnls (2.6 ± 0.28-fold; \( p = 7.1 \times 10^{-6} \)), which showed more than two-fold differences that were also statistically significant (\( p < 10^{-3} \); B6 vs. NOD by Student’s t-test), were further analyzed in F1 mice. Significant differences were observed in levels of Clec16a (Fig. 3b), Dlk1 (Fig. 3c), Pttn22 (Fig. 3d), and Rnls (Fig. 3e) expression. The level of Clec16a expression was significantly higher in NOD-derived T cells than in B6- and F1-derived T cells (\( p = 1.2 \times 10^{-6} \) and \( p = 0.018 \), respectively). The level of Clec16a expression was also significantly higher in F1-derived T cells than in B6-derived T cells (\( p = 0.019 \)), suggesting that phenotypic differences in Clec16a expression were transmitted to F1 hybrids in an additive manner (Fig. 3b). The level of Dlk1 expression was also significantly higher in NOD-derived T cells than in B6- and F1-derived T cells (\( p = 6.6 \times 10^{-3} \) and \( p = 0.031 \), respectively; Fig. 3c). In contrast, Pttn22 expression was significantly lower in NOD-derived T cells than in B6- and F1-derived T cells (\( p = 5.1 \times 10^{-9} \) and \( p = 8.2 \times 10^{-7} \), respectively). The level of Pttn22 expression in F1-derived T cells was intermediate to that observed in T cells from NOD and B6 mice, suggesting that the phenotypic difference in Pttn22 expression was transmitted to F1 hybrids in an additive manner (Fig. 3d). Furthermore, the level of Rnls expression was significantly higher in NOD-derived T cells than in B6 T cells (\( p = 2.4 \times 10^{-6} \)). F1-derived T cells showed an intermediate level of Rnls expression (Fig. 3e).

**Gene expression differences with or without TGF-β**

Gene expression ratios of the 22 genes in T cells cultured with and without TGF-β were compared between B6 and NOD mice as follows. First, the gene expression levels in T cells from each strain cultured without TGF-β were set to 1. Then, the expression ratios of the genes in T cells cultured with TGF-β were compared with these levels (Fig. 4). The levels of Bach2, Cblb, Cdt101, Clec16a, Dlk1, Foxp3, Pttn22, Ubash3a, and Zac1 expression were significantly increased in T cells in the presence of TGF-β compared to those in T cells cultured without TGF-β in both strains. There was a greater than ten-fold increase in the levels of Cdt101 (17.87 ± 3.53-fold; \( p = 6.0 \times 10^{-5} \)) and Dlk1 (22.79 ± 8.45-fold; \( p = 0.0031 \)) expression in B6-derived T cells in the presence of TGF-β compared to that in the absence of TGF-β. In contrast, the level of Pparg expression significantly decreased in T cells in the presence of TGF-β in both strains (0.23 ± 0.041-fold; \( p = 4.9 \times 10^{-15} \) in B6-derived T cells). Significant differences in the TGF-β-induced alterations were observed in the expression of Clec16a, Ctl4a, Foxp3, Il2, Il2rb, Prkca, Pttn2, Sh2b3, Ubash3a, and Zac1. There were statistically significant differences in the TGF-β-induced alterations for Clec16a (Fig. 5a), Foxp3 (Fig. 5b), Il2 (Fig. 5c), Pttn22 (Fig. 5d), Sh2b3 (Fig. 5e), and Zac1 (Fig. 5f) among T cells from the 3 mouse strains. The level of Clec16a expression was significantly lower in NOD-derived T cells cultured with TGF-β than in those cultured without TGF-β (0.61 ± 0.059-fold; \( p = 1.2 \times 10^{-6} \)), whereas the levels of Clec16a expression were similar in T cells with and without TGF-β in both B6 and F1 mice (Fig. 5a). The level of Foxp3 expression was significantly higher in T cells treated with TGF-β than in those without TGF-β in B6, F1, and NOD mice (\( p = 1.1 \times 10^{-5} \), \( p = 2.1 \times 10^{-6} \), and \( p = 2.0 \times 10^{-6} \), respectively; Fig. 5b). Furthermore, the level of Foxp3 expression was significantly higher in NOD-derived T cells than in B6-derived T cells (\( p = 0.0075 \)). Interestingly, the level of Il2 expression was significantly lower in the presence of TGF-β than in the absence of TGF-β in B6-derived T cells (0.37 ± 0.048-fold; \( p = 7.6 \times 10^{-12} \)). However, the level of Il2 expression tended to be higher in the presence of TGF-β than in the absence of TGF-β in T cells from NOD mice (2.0 ± 0.52-fold). Thus, a differential expression response of Il2 to TGF-β was observed in B6 and NOD mice (\( p = 0.0097 \); Fig. 5c). Although the level of Pttn22 expression significantly increased in the presence of TGF-β in T cells from B6, F1, and NOD mice (\( p = 0.0066 \), \( p = 6.8 \times 10^{-4} \), and \( p = 0.012 \), respectively), a slight, but significant, difference in the increase of the Pttn22 gene expression ratio was observed in F1-derived T cells compared to that in B6-derived (\( p = 0.02 \)) and NOD-derived (\( p = 0.048 \)) T cells (Fig. 5d). The level of Sh2b3 expression significantly increased in the presence of TGF-β in B6 mice (\( p = 0.049 \)), whereas it significantly decreased in the presence of TGF-β in NOD mice (\( p = 6.0 \times 10^{-5} \); Fig. 5e). Finally, Zac1 expression significantly increased in the presence of TGF-β in B6, F1, and NOD mice (\( p = 0.00059 \), \( p = 4.3 \times 10^{-4} \), and \( p = 3.6 \times 10^{-5} \), respectively; Fig. 5f). A slight, but significant, differential expression response of Zac1 to TGF-β was observed (\( p = 0.043 \), B6 vs. F1).
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The level of Zac1 expression was significantly lower in NOD-derived T cells than in B6-derived T cells (Fig. 2e), and Zac1 expression was upregulated in the presence of TGF-β (Fig. 5f). Therefore, the expression of Zac1 in response to TGF-β was also evaluated in EL4 cells. The expression ratios of Zac1 were indeed significantly higher in EL4 cells after 3 d of culture with TGF-β (10 ng/mL) than in EL4 cells cultured without TGF-β (Fig. 6a). Furthermore, since Zac1 (Zinc finger protein which regulates apoptosis and cell cycle arrest 1) is a member of the subfamily of zinc finger transcription factors [31, 33, 34], EL4 cells were transfected with a Zac1 expression vector to determine whether any of the 22 genes selected in this study were regulated by Zac1 in EL4 cells. Of the 22 genes exam-
Fig. 4  Comparison of the expression ratios of 22 genes in the presence and absence of transforming growth factor β (TGF-β) in B6-derived (n=12) and NOD-derived (n=12) T cells. The gene expression levels in T cells from each strain cultured without TGF-β (TGF-β (−)) were set to 1; then, the expression ratios of the genes in T cells cultured with TGF-β (TGF-β (+)) were compared with these levels. White bars: B6-derived TGF-β (−) T cells; gray bars: B6-derived TGF-β (+) T cells; meshed bars: NOD-derived TGF-β (−) T cells; black bars: NOD-derived TGF-β (+) T cells. Data are expressed as mean ± SEM; *p < 0.05, **p < 0.01, and ***p < 0.001 vs. TGF-β (−) in each strain; #p < 0.05, ##p < 0.01, and ###p < 0.001 vs. indicated groups.

Fig. 5  Comparisons of gene expression ratios in T cells cultured with TGF-β (TGF-β (+); black bars) to those cultured without TGF-β (TGF-β (−); white bars) for B6 (n=12), NOD-B6 hybrid F1 (n=8), and NOD (n=12) mice. (a) Cda4 (f = 0.0015), (b) Foxp3 (f = 0.01), (c) Il2 (f = 0.013), (d) Ppm22 (f = 0.019), (e) Sh2b3 (f = 0.0064), (f) Zac1 (f = 0.029); *p < 0.05, **p < 0.01, and ***p < 0.001 vs. TGF-β (−) in each strain, or vs. indicated groups.
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ined, the level of Il2 expression in EL4 cells transfected with a Zac1-containing expression vector was significantly lower than that in EL4 cells transfected with the corresponding control vector (Fig. 6b). In EL4 cells, expression of Dlk1 or Pparg was not detected in the TaqMan® assays. However, Dlk1 and Pparg have been reported to be target genes of Zac1 [35, 36]; therefore, we tested these 2 genes in human Jurkat E6.1 T cells, in which the expression of both genes is detectable with human TaqMan® probes (Table 1). The level of DLK1 expression in Jurkat E6.1 cells transfected with a ZAC1-containing expression vector was significantly higher than that in cells transfected with the corresponding control vector (Fig. 6c). However, the level of PPARγ expression did not differ significantly in Jurkat E6.1 cells after transfection with either the ZAC1 expression vector or the control vector (data not shown).

Discussion

In the present study, significant differences in the growth/survival ratios of T cells, both in the presence and absence of TGF-β, were observed between NOD and B6 mice. Since TGF-β has been recently reported to play an important role in regulating the self-reactivity of peripheral T cells [20], this differential response might contribute to the development of type 1 diabetes in NOD mice. A transient pulse of TGF-β in islets during the priming phase of diabetes has been reported to be sufficient to inhibit disease onset by promoting the expansion of the intra-islet CD4+CD25+ T cell pool [37].

Autoimmune disease susceptibility alleles in NOD mice and resistance alleles on mouse chromosome 3 (Idd3) have previously been reported to be correlated with an approximately two-fold reduction in IL-2 production in T cells [38]. It has been also reported that the intra-islet Treg dysfunction that is secondary to defective IL-2 production is a cause of the development of diabetes in NOD mice [39]. In this study, a differential expression response of Il2 to TGF-β was observed between NOD-derived and B6-derived T cells. This difference might be related to the Idd3-linked destabilization of immune homeostasis in NOD mice.

We also found that the expression of Ctla4, Clec16a, Dlk1, Ptpn22, Rnls, and Sh2b3, which were originally identified in human type 1 diabetes association studies, differed among T cells from the 3 strains of mice. Recently, Winkler et al. [40] reported that the greatest diabetes discrimination could be obtained by screening for a combination of risk alleles for Ctla4, Clec16a, Ptpn22, and Sh2b3 in children with a high-risk HLA genotype. Alleles that confer susceptibility to type 1 diabetes at the renalase, FAD-dependent amine oxidase (RNLS)/10q23.31 (rs10509540) locus are associated with a younger age at diagnosis [41]. Taken together with our findings, these results suggest that these genes might confer susceptibility to type 1 dia-
betes in both humans and mice. Furthermore, NOD mice develop type 1 diabetes in an age-dependent manner [25] (http://www.clea-japan.com/). This may mean that aging plays an important role in the development of diabetes in NOD mice. Therefore, assessing the changes in gene expression profiles shown in this study in response to aging may yield interesting results.

The level of Zac1 expression was significantly lower in T cells from NOD mice than in those from B6 and F1 mice. Since Zac1 expression was induced by TGF-β and is known to induce cell cycle arrest [33], Zac1 might play a role in the significant differences observed in the growth/survival ratios of T cells between NOD and B6 mice, both in the presence and absence of TGF-β. Exogenous expression of Zac1 or Zac1 affected Il2 and Dlk1 expression, respectively. Taken together, these data suggest that Zac1 affects the expression of the type 1 diabetes susceptibility genes Il2 and Dlk1 in response to TGF-β in T cells.

In summary, we found different expression ratios of non-MHC genes in T cells from B6, F1, and NOD mice. The results of our study indicate the possibility that novel genetic pathways, including those involving Clec16a, Dlk1, Rnls, Sh2b3, and Zac1 under IL-2 and TGF-β signaling in T cells, are shared between humans and NOD mice, although there are still missing links. We hope that our findings will contribute to furthering the understanding of how type 1 diabetes susceptibility genes coordinately function in the immune system to maintain immune homeostasis [42, 43].

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Disclosure Statement

None of the authors have any potential conflicts of interest associated with this research.

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