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**E2f1** Mutation Induces Early Onset of Diabetes and Sjögren’s Syndrome in Nonobese Diabetic Mice

Mohammad Abdus Salam,* Khairul Matin,† Naoko Matsumoto,* Yuzo Tsuha,* Nobuhiro Hanada,‡ and Hidenobu Senpuku*‡

*E2f1* is an important regulator of T cell proliferation, differentiation, and apoptosis that controls the transcription of a group of genes that are normally regulated at the G1 to S phase transition in the cell cycle. Insulin-dependent diabetes mellitus (IDDM) and Sjögren’s syndrome (SS) are highly regulated autoimmune diseases that develop spontaneously in NOD mice. The aim of the present in vivo study was to explore the functional importance of the E2f1 molecule in IDDM and SS, in the context of whole animal physiology and pathophysiology, using *E2f1*-deficient NOD mice. For the experiment, we produced NOD mice homozygous for a nonfunctional *E2f1* allele onto a NOD background. *E2f1*-deficient NOD mice developed an early and increased onset of diabetes as compared with their littermates. These mice also exhibited a defect in T lymphocyte development, leading to excessive stimulation of spleen cells and thymocytes in vitro. Furthermore, *E2f1*-deficient mice showed a profound decrease of immunoregulatory CD4+CD25+ T cells, while the spleen cells of NOD mice lacking *E2f1* showed a significant increase of the proinflammatory cytokine IFN-γ following antigenic stimulation in vitro. Consistent with these observations, *E2f1* homozygous mutant NOD mice were highly predisposed to the development of IDDM and SS. The Journal of Immunology, 2004, 173: 4908–4918.

The nonobese diabetic mouse strain remains the best available model for the study of insulin-dependent type 1 diabetes mellitus (IDDM) (1–3). These mice develop a complex autoimmunity that is controlled by multiple genes, including those syntenic with genetic loci that control susceptibility in humans (2–4). IDDM and Sjögren’s syndrome (SS) are chronic multisystem and autoimmune disorders (5–8) that spontaneously develop in NOD mice at 4–6 mo of age (9). The development of IDDM is characterized by the infiltration of T lymphocytes, dendritic cells, and monocytes into the islets of the pancreas, as well as the terminal destruction of β cells (2, 5, 9–13). SS, on the other hand, is a systemic autoimmune disease characterized by hyposialivation, along with oral and ocular dryness, and is accompanied by clinical observation of a progressive loss of salivary and lacrimal function that is related to the presence of perivascular and periductal T lymphocyte infiltration (14–16) as well as systemic production of autoantibodies to ribonucleoprotein (17). In recent reports, it has been clearly shown that both the CD4 and CD8 subsets of T cells play a crucial role in the development of IDDM in NOD mice (18, 19), which also develop lymphocytic inflammation in their salivary glands (sialoadenitis) and lacrimal glands (dacryoadenitis) (20, 21). These findings led to the notion that recruitment of a threshold frequency of autoreactive T cells into the pancreatic islets and salivary glands may be required for progression to the destruction of β cell salivary gland tissue.

In autoimmune diabetes, the pathogenic T cells are a Th1 type that produce primarily the proinflammatory cytokine IFN-γ, and Th2 cells appear to mediate protection against diabetes development via secretion of IL-4 and IL-10 (22–25). Several studies have demonstrated that the autoimmune response in NOD mice is the result of a loss of immunoregulation, with a dominance of pathogenic Th1 cells over Th2 cells (23–26). Various islet autoantigens, including glucatime acid decarboxylase 65, heat shock protein 60, and insulin, have been implicated in the initiation of diabetes, while Th1 responses to these autoantigens have been detected in NOD mice (27–29). In contrast, it has been reported that some NOD-derived CD4 T cell clones can induce IDDM when in either a Th1 or Th2 mode (30, 31) and, in a subsequent study, the other articles showed IFN-γ signaling is dispensable for IDDM development in NOD mice (32–34). Therefore, the concept that skewing toward a Th1 vs a Th2 cytokine production profile, respectively, promotes or inhibits the diabeticogenic potential of NOD T cells is now a crumbling dogma.

Members of the E2F transcription factor family (*E2f1*--*E2f5*) are important regulators of cell proliferation, differentiation, and apoptosis. The most characteristic member of the E2F family is *E2f1* (34–36), which controls the initiation of DNA synthesis and subsequent transition of cells from the G1-G0 to S phase of the cell cycle (37, 38). The E2f1 molecule is activated through association with another transcription factor, DP1. Binding of the phosphorylated forms of the retinoblastoma (Rb) tumor suppressor protein

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*Abbreviations used in this paper: IDDM, insulin-dependent diabetes mellitus; SS, Sjögren’s syndrome; Rb, retinoblastoma; SSLP, simple sequence length polymorphism.

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3. Abbreviations used in this paper: IDDM, insulin-dependent diabetes mellitus; SS, Sjögren’s syndrome; Rb, retinoblastoma; SSLP, simple sequence length polymorphism.
(39) may inactivate the E2f1-DP1 complex. The decision by individual cells to proliferate, remain quiescent, or die is crucial to all organisms. Dysregulation of the normal functions involved with this decision can result in failure of important reparative, inflammatory, or other adaptive responses and can lead to aberrant proliferation characteristics of late-onset autoimmune diseases due to the deposition of T lymphocytes in different glands and salivary gland dysplasia by destruction of acinar cells (40–43). Several recent studies demonstrated that a mutation of the E2f1 gene in mice causes enhanced T lymphocyte proliferation, leading to testicular atrophy, splenomegaly, salivary gland dysplasia, and other systemic and organ-specific autoimmunity (40–45). However, it is uncertain whether the function of E2f1 has an influence in the development of IDDM and SS. To address this issue, we generated E2f1–/– backcross mice used for the intercross. Laboratory (Bar Harbor, ME). They were housed in polypropylene mouse cages with pine chips and maintained in accordance with guidelines established by the National Institute of Infectious Diseases. The E2f1–/– allele was functionally disrupted by insertion of a neomycin-resistant gene (neo) that had been backcrossed from the original chimeric stock and mixed with the 129SV and C57BL/6 genome (42) onto the NOD inbred background. Heterozygous carriers of the E2f1+/− allele were used as breeders and each backcross generation was identified by typing DNA taken from the tail by PCR genotyping for the microsatellite markers shown in Table I which were linked to the indicated Idd loci. In the N5 backcross generation, E2f1+/− heterozygotes, shown by these PCR analyses to be fixed as homozygous for the NOD allele at the indicated linkage markers of Idd susceptibility loci, were intercrossed to generate E2f1+/+, E2f1−/−, and E2f1+/− NOD mice. A separate genomic PCR assay to detect the wild-type allele (392 bp) or mutant E2f1 allele (271 bp) was designed using an L31 intron primer (5′-GCTGGAATGGTGTCAGCACAGCG-3′) and the E2f1 wild-type exon L26 primer (5′-TCCAAAGATCATTACGTTGGTCT-3′) (42) or the neo gene primer. E2f1−/− was also genotyped using GGATATGATTCTTGGACTTCT (5′-GAGCGGCGATACCGTAAAGCACGAGG-3′) and (5′-GCTGGAATGGTGTCAGCACAGCG-3′) primers (46). PCR (25 μl) were amplified using a PTC-200 (MJ Research) for 39 cycles (94°C, 1 min; 56°C, 1 min; 72°C, 1 min) and then analyzed on a 3% agarose gel by electrophoresis.

Materials and Methods

Generation of E2f1-deficient NOD mice

NOD/LtJ and E2f1−/− mice were originally purchased from The Jackson Laboratory (Bar Harbor, ME). They were housed in polypropylene mouse cages with pine chips and maintained in accordance with guidelines established by the National Institute of Infectious Diseases. The E2f1−/− allele was functionally disrupted by insertion of a neomycin-resistant gene (neo) that had been backcrossed from the original chimeric stock and mixed with the 129SV and C57BL/6 genome (42) onto the NOD inbred background. Heterozygous carriers of the E2f1+/− allele were used as breeders and each backcross generation was identified by typing DNA taken from the tail by PCR genotyping for the microsatellite markers shown in Table I which were linked to the indicated Idd loci. In the N5 backcross generation, E2f1+/− heterozygotes, shown by these PCR analyses to be fixed as homozygous for the NOD allele at the indicated linkage markers of Idd susceptibility loci, were intercrossed to generate E2f1+/+, E2f1−/−, and E2f1+/− NOD mice. A separate genomic PCR assay to detect the wild-type allele (392 bp) or mutant E2f1 allele (271 bp) was designed using an L31 intron primer (5′-GCTGGAATGGTGTCAGCACAGCG-3′) and the E2f1 wild-type exon L26 primer (5′-TCCAAAGATCATTACGTTGGTCT-3′) (42) or the neo gene primer. E2f1−/− was also genotyped using GGATATGATTCTTGGACTTCT (5′-GAGCGGCGATACCGTAAAGCACGAGG-3′) and (5′-GCTGGAATGGTGTCAGCACAGCG-3′) primers (46). PCR (25 μl) were amplified using a PTC-200 (MJ Research) for 39 cycles (94°C, 1 min; 56°C, 1 min; 72°C, 1 min) and then analyzed on a 3% agarose gel by electrophoresis.

Assessment of diabetes

E2f1+/+, E2f1−/−, and E2f1+/− NOD mice were monitored weekly for clinical onset of diabetes which was determined by the presence of glucose in the urine and blood. Urine was tested with Ames reagent strips (Uristix; Bayer Medical, Newbury, U.K.) and confirmed positive by blood glucose measurements. All positive animals eventually displayed weight loss that progressed to death unless killed earlier.

Collection of saliva

At 25–30 wk old, NOD/LtJ, E2f1−/−, N5 (NOD.E2f1−/−), and (NOD × B10.D2F1) mice were injected with a mixture of isoproterenol (0.20 μg/ml) and pilocarpine (0.05 μg/ml) in PBS (1). After 48 h of restimulation, saliva was collected from the mouth into 1.5-ml microfuge tubes using a microspipette for 15 min. Total volume and weight were measured and calculated per body weight, and the samples were then stored at −80°C for further analyses.

Histological examination

Nondiabetic mutant and wild-type E2f1 allele NOD mice were killed at 12–16 wk of age after blood serum had been collected. The pancreas and submandibular salivary glands were snap-frozen in OCT compound, cut into 6-μm serial sections (Microm, Zeiss, Germany), and stained with H&E for histological observation of mononuclear cell infiltration. Histological observations and photomicrography were performed using an Olympus BX50WI microscope (Olympus, Tokyo, Japan).

T cell stimulation assay and cytokine detection

Spleen cells, depleted of RBC, were cultivated at 1 × 10^7/ml in a 24-well plate in culture medium (RPMI 1640 supplemented with glutamax, 10% FBS, antibiotics, 10 mM HEPES, and 5 × 10^-3 M 2-ME) in the presence of soluble anti-CD3 Ab (145-2C11) at 1 μg/ml for 3 days. After washing, the cells were restimulated in culture medium without stimulation for 2 days at 37°C. Dead cells were removed using a Ficoll-Hypaque gradient and replaced at 0.5 × 10^6 cells/ml cultured medium in a 24-well plate previously coated with anti-CD3 Ab at 1 μg/ml in PBS (1). After 48 h of restimulation, the supernatant was removed and analyzed for the cytokines IFN-γ and IL-4 by ELISA using commercial kits from Endogen (Cambridge, MA).

Cell cycle analysis

Thymus cells and spleen cells from 6-wk-old wild-type and mutant E2f1 allele NOD mice were separately primed in vitro and cultivated at 1 × 10^6 cells/ml in culture medium in a 24-well plate previously coated with anti-CD3 Ab at 1 μg/ml in PBS (1).

Table II. Frequency of homozygosity in N5 intercross generation mice

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>Number</th>
<th>% of Total Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOD.E2f1+/+</td>
<td>33</td>
<td>24.44 (n = 135)</td>
</tr>
<tr>
<td>NOD.E2f1−/−</td>
<td>69</td>
<td>51.12 (n = 135)</td>
</tr>
</tbody>
</table>

* DNA samples from the N5 intercross generation were typed by genomic PCR and the results demonstrated that NOD.E2f1−/−, NOD.E2f1+/−, and NOD.E2f1+/+ mice were produced at a 1:1:2 ratio.
10^5/ml in 24-well plates in culture medium (RPMI 1640 supplemented with glutamax, 10% FBS, antibiotics, 10 mM HEPES, and 5 x 10^{-5} M 2-ME) in the presence of soluble anti-CD3 Ab at 1 μg/ml for 4 h. During the final 45 min of culture, the cells were labeled with 10 μM BrdU. The cell cycle positions and active DNA synthetic activities of the cells were determined by analyzing the correlated expression of total DNA and incorporated BrdU levels. BrdU-incorporated cells were stained with FITC-anti-BrdU Ab and the total DNA content was stained with 7-aminoactinomycin D using a BrdU Flow kit (BD Pharmingen, San Diego, CA), after which they were analyzed with FACSscan CellQuest software (BD Biosciences, San Jose, CA).

**Abs and chemicals**

The following mAbs were purchased from BD Pharmingen: FITC-conjugated anti-mouse anti-CD4 (H129.19), anti-CD8α (53-6.7), anti-CD25 (3C7), anti-CD25 (GTX12-095); PE-conjugated anti-mouse anti-CD8α (53-6.7), anti-CD45 (30-F11); PerCP-conjugated anti-CD4 (RM4-5), and nonconjugated anti-CD3e (145-2C11). BrdU flow kits were also purchased from BD Pharmingen. Alkaline phosphatase-conjugated goat anti-mouse IgG and IgM were purchased from Zymed Laboratories (San Francisco, CA). Culture medium RPMI 1640 (Sigma-Aldrich), HBSS (Invitrogen Life Technologies, Grand Island, NY), and FBS (ICN, Aurora, OH) were also purchased.

**Flow cytometry analyses of T cells**

Single-cell suspensions from thymus, spleen, and cervical lymph node specimens from 6-wk-old wild-type and mutant E2f1 allele NOD mice were obtained by teasing the tissues and forcing the resultant suspension through sterile nylon mesh. Cell numbers were determined using a hemacytometer and trypan blue exclusion to determine cell viability (>95% viable). For the measurement of CD4+CD8α, CD4-CD8α, CD4+CD25+, and CD8-CD25+ T cell, 10^6 cells were incubated with FITC-conjugated anti-CD4, PerCP-conjugated anti-CD4, and PE-conjugated anti-CD8 Abs, respectively, followed by PE- and FITC-conjugated anti-CD8 and anti-CD25 Abs, respectively. Fc block (BD Pharmingen) was used to prevent nonspecific binding. Three-color immunofluorescence analyses were performed for the detection of intracellular productions of IFN-γ and IL-4 by CD4+ and CD8α T cells after 4 h of culture in the presence of leukocyte activation kits (BD Pharmingen). Cells were analyzed using a FACSscan flow cytometer and the CellQuest program. Samples were gated on the forward light scatter and side light scatter and were used to identify viable lymphocytes and their ratios.

**Adoptive type 1 diabetes transfer experiments**

Spleen cells of 13 NOD.E2f1^−/− and 13 NOD.E2f1^−/+ female mice from 5- to 10-wk-old were isolated in RPMI 1640 medium supplemented with 10% FBS. RBC were depleted by a lysis with ACK buffer (0.155 M ammonium chloride, 0.1 M disodium EDTA, and 0.01 M potassium bicarbonate, pH 7.2). After washing, counting of the total cell numbers, and viability evaluation, splenocytes (5 x 10^6) were i.p. injected into 26 eight- to 10-wk-old NOD/SCID female mice, respectively. Beginning 1–2 wk after adoptive cell transfer, the recipient animals (NOD/SCID) were monitored twice a week for clinical onset of diabetes that was determined by the presence of glucose in the urine and blood. Urine was tested with Ames reagent strips and confirmed positive by blood glucose measurements. The
recipient animals were killed at the time they became diabetic and the pancreas and salivary glands were subjected for histological analyses.

Statistical analysis
A Kaplan-Meier cumulative survival test was used to compare the incidences of diabetes. Comparative analyses were performed by ANOVA. A $p < 0.05$ was considered to be statistically significant for two-tailed comparisons. All statistical analyses were performed using StatView for the Macintosh operating system.

Results
Establishment of the NOD.E2f1−/− mouse strain
Heterozygous carriers of the E2f1−/− allele from the backcross generation and all progeny of the N5 intercross generation were typed by PCR for the microsatellite markers shown in Table I, which were closely linked to the indicated Idd loci. The SSLP analyses identified mice homozygous for allelic variants characteristic of NOD mice at all of the Idd1–Idd15 linkage markers (Table I). Typing of these markers confirmed the homozygous presence of the NOD-derived genome at all identified Idd loci in the N5 progenitors used for the intercross (Table I). Genotyping by genomic PCR demonstrated that E2f1 mutant-type (NOD.E2f1−/−), heterozygous (NOD.E2f1+/−), and wild-type (NOD.E2f1+/+) animals were produced at the expected ratio of 1:2:1 (Table II). Homozygous E2f1−/− mice of the fifth (N5) backcross intercross generation were used to establish the NOD.E2f1−/− mouse strain, which was maintained by brother-sister mating. To discuss the potential for the autoimmune effects to be linked in NOD mice to allelic E2F-1 polymorphism, the original mouse strains (C57BL/6 and 129sv) to the knockout mouse were compared with NOD mice by SSLP analysis. As a result, there were no differences in the E2F-1 polymorphisms between NOD and the original strains.

Early and increased incidence of diabetes in E2f1-deficient NOD mice
E2f1−/− mice were bred onto a NOD background and diabetes was assessed by weekly measurement of urinary glucose or blood glucose concentration. The incidence of diabetes was ~88% in female N5 mutant (NOD.E2f1−/−) (Fig. 1a) and 58% in male N5 mutant (NOD.E2f1−/−) mice (Fig. 1b), while 71% of the N5 wild-type (NOD.E2f1+/+) females and 25% of the N5 wild-type (NOD.E2f1+/+) males became diabetic by 32 wk of age (Fig. 1, $p < 0.05$ and $p < 0.001$, respectively). Disease onset was earlier and more severe in mutant NOD.E2f1−/− mice as compared with their wild-type littermates (Fig. 1). The incidence of diabetes in both heterozygote male and female N5 (NOD.E2f1+/−) mice was not significantly different as compared with the N5 wild-type (NOD.E2f1+/+) control mice (Fig. 1).

Low salivary flow rate in E2f1-deficient NOD mice
Salivary analyses of different mouse strains showed that the total amount of secreted saliva was significantly lower in both NOD and E2f1−/− as compared with the standard (NOD × B10.D2) F1 mice after stimulation by the secretagogue (Fig. 2). Our salivary analyses showed that the total amount of secreted saliva after stimulation by the secretagogue was significantly lower in N5 mutant (NOD.E2f1−/−) mice (380.0 ± 24.5 μg/100 g body weight) as
per genotype (p/H11569, littermates. The effect of genotype on the number of thymocytes per thymus was statistically significant. The number of thymocytes in mutant mice was increased 14.8 E2f1 mutant NOD.
molecule may help to maintain a normal nuclear structure and
Figure 5. Analyses of insulitis in NOD.E2f1−/− and NOD.E2f1+/+ group mice at 12–16 wk of age. The degree of mononuclear cell infiltration was graded as follows: grade 0, no infiltrating cells in the islets; grade 1, infiltrating cells adjacent to the islets but not in the islets; grade 2, infiltrating cells occupying <25–50% of the islets’ area; grade 3, infiltrating cells occupying 25–50% of the islets’ area; grade 4, infiltrating cells occupying >50% of the islets’ area. The assessment of insulitis was performed using H&E-stained pancreas sections. Results are expressed as the mean ± SD of the insulitis counts for six independent mice selected randomly. The asterisk denotes significantly different insulitis counts between two groups (p < 0.001).

compared with the standard NOD (620.0 ± 45.6 μg/100 g body weight, p < 0.01) and F1 (750.0 ± 21.5 μg/100 g body weight, p < 0.001) mice (Fig. 2).

Histological abnormalities of exocrine glands in E2f1-deficient NOD mice
Histological examinations of submandibular salivary gland and pancreas specimens from 12- to 16-wk-old E2f1-deficient N5 mutant prediabetic NOD mice revealed that large numbers of mononuclear inflammatory cells had infiltrated the salivary glands and pancreas islets with some evidence of acinar cell degeneration (Fig. 3, a and c). Abnormally large nuclei or nuclei doubled in number were observed in pancreas islets of N5 (NOD.E2f1−/−) mice (Fig. 3a). This morphology represents a more severe destructive cellular infiltration of the tissue and suggests that the E2f1 molecule may help to maintain a normal nuclear structure and possible DNA content within these organ tissues (40, 42). The histological examinations also revealed that severe insulitis in the mutant NOD.E2f1−/− group (grade 0, 20.9 ± 7.9%; grade 1, 14.8 ± 5.9%; grade 2, 16.1 ± 7.6%; grade 3, 20.9 ± 4.5%; and grade 4, 27.0 ± 5.7%) as compared with the wild NOD.E2f1+/+ group (grade 0, 26.3 ± 4.5%; grade 1, 28.3 ± 6.4%; grade 2, 16.9 ± 0.6%; grade 3, 10.1 ± 2.5%; and grade 4, 16.5 ± 2.21%; Fig. 4). In particular, there were large differences in grade 3 and grade 4 insulitis between these two groups. Furthermore, we analyzed whether the splenocytes of young E2f1-deficient NOD and standard NOD mice can induce lymphocytic infiltration in salivary glands and pancreas and development of diabetes by spleen T cells of NOD.E2f1−/− mice transferred into NOD.SCID mice with differing efficiencies. As expected, the majority (75%) of recipients injected with splenocytes from both E2f1-deficient NOD and NOD mice became diabetic by 10 wk after transfer (data not shown).

There was no significant difference in the onset of diabetes between the recipients.

Thymus, thymocyte, and peripheral T cell abnormalities in NOD.E2f1−/− mice
The E2f1 molecule was found to play an important role in normal thymic development. The thymi of 5-wk-old N5 mutant-type NOD.E2f1−/− mice were noticeably enlarged compared with those of N5 wild-type littermates (NOD.E2f1+/+), and thymus weight per 100 g body weight in E2f1-deficient NOD mice was also increased (26.2 ± 2.3) compared with their littermates (18.7 ± 0.9; Fig. 5a, p < 0.001). This increased thymus size reflected a consistent increase in thymic cellularity, demonstrated by an ~55% increase in the number of thymocytes per thymus (Fig. 5b, p < 0.001). Several mechanisms might explain the increased number of thymocytes. To investigate the mechanisms of thymus and thymocyte abnormalities, the developmental profiles of thymocytes from N5 (NOD.E2f1−/−) mice and N5 wild-type (NOD.E2f1+/+) mice were compared. By monitoring the expression of CD4 and CD8 cell surface markers, the extent of thymocyte maturation was assessed. As thymocytes mature, they progress sequentially through double-negative (CD4−CD8−), double-positive (CD4+CD8−), and single-positive (CD4+CD8− or CD4−CD8+) stages. Upon staining with anti-CD4 and anti-CD8 Abs, thymi of 5-wk-old N5 (NOD.E2f1−/−) mice were consistently found to contain a higher fraction of mature thymocytes (CD4+ or CD8+) than their N5 wild-type (NOD.E2f1+/+) littermates (Fig. 6, d–f, p < 0.01). Furthermore, the total number of thymocytes per thymus as well as the absolute number of cells in all thymocyte populations were increased in N5 (NOD.E2f1−/−) mice (Figs. 5b and 6). There were also significantly low numbers of double-positive (CD4+CD8+) thymocytes in N5 (NOD.E2f1−/−) mice (72.9 ± 2.9%) as compared with the wild-type NOD.E2f1+/+ mice (79.0 ± 1.6%; Fig. 6, d and e, p < 0.01). Finding from flow

FIGURE 5. Thymus and thymocyte abnormalities in E2f1-deficient NOD mice. a, Thymus weights from 5-wk-old mutant and wild-type mice are expressed as the fraction of total body weight. The increase in thymus size of the thymi in mutant (NOD.E2f1−/−) mice as compared with wild-type was statistically significant. Data are expressed as mean ± SD of 20 mice per genotype (*, p < 0.001). b, The numbers of thymocytes harvested from the thymi of 5-wk-old mutant and wild-type mice are shown. The number of thymocytes in mutant mice was increased ~55% as compared with their wild-type littermates. The effect of genotype on the number of thymocytes per thymus was statistically significant. Data are expressed as the mean ± SD of 15 mice per genotype (*, p < 0.001).
cytometric analyses of spleen and lymph node T cells revealed that the percentages of mature T cells (CD4⁺ and CD8⁺) were significantly increased in N5 (NOD.E2f1⁻/⁻) mice than in wild-type N5 (NOD.E2f1⁺/⁺) mice (Fig. 6, a–c, g–h, and i, p < 0.01), and the absolute number of spleen T cells (CD4⁺ and CD8⁺) was also significantly increased in mutant NOD.E2f1⁻/⁻ mice as compared with wild-type NOD.E2f1⁺/⁺ mice (Fig. 6k). These results indicated that E2f1-deficient NOD mice also have an increased number of mature peripheral T cells. Increased cell numbers in the peripheral tissues may be due to increased proliferation, decreased cell death as well as or in place of alteration of cell migration from the thymus in E2f1-deficient NOD mice.

Thymocyte and spleen cell proliferation in vitro in response to stimulation with mitogens was found to be distinguishable when cells isolated from 5-wk-old mutant (NOD.E2f1⁻/⁻) and wild-type (NOD.E2f1⁺/⁺) mice were compared (Fig. 7). Flow cytometric analyses of the cell cycle positions and active DNA synthetic activities of the cells revealed that the fractions of cells in the S phase in the thymus and spleen of N5 (NOD.E2f1⁻/⁻) mice were significantly increased (29.1 ± 3.6 and 11.5 ± 2.3, respectively) as compared with those in the wild-type N5 (NOD.E2f1⁺/⁺) mice.
(19.1 ± 4.9 and 4.1 ± 1.6, respectively; Fig. 7, b and d, p < 0.05 and p < 0.005, respectively), whereas cells in the G0–G1 phase in the thymus and spleen of N5 mutant (NOD.E2f1−/−) mice were significantly decreased (60.8 ± 6.4 and 77.9 ± 6.0, respectively) as compared with those in the wild-type N5 (NOD.E2f1+/+) mice (72.7 ± 6.8 and 91.2 ± 3.3, respectively; Fig. 7, b and d, p < 0.05 and p < 0.005, respectively). Furthermore, significant differences in cells in the G2 + M phase in the spleen were seen between the mutant-type NOD.E2f1−/− mice and wild-type NOD.E2f1+/+ mice (Fig. 7d, p < 0.05); however, there were no significant differences in the thymus between these two groups (Fig. 7b, p = 0.19).

CD4+CD25+ T cell subset reduced in number in E2f1-deficient NOD mice

Results of flow cytometric analyses of the CD4+CD25+ T cell subset revealed that a significant decrease of these cells in the spleen was observed in the 5-wk-old E2f1-deficient NOD mice compared with that in the 5-wk-old wild-type NOD.E2f1+/+ mice (Fig. 8, a and b, p < 0.01). Furthermore, the total number of the immunoregulatory CD4+CD25+ T cells in spleen were also decreased significantly in NOD.E2f1−/− (28.3 × 10⁵ cells/mouse) mice as compared with the wild-type NOD.E2f1+/+ (62.4 × 10⁵ cells/mouse) mice (p = 0.007, data not shown in figure). There was no significant difference in CD4+CD25+ T cells in the lymph nodes and thymus between these two groups of mice, although a slightly lower number of this subset was found in the mutant NOD.E2f1−/− mice as compared with the wild-type NOD.E2f1+/+ mice (Fig. 8, c–f). These results reflect that E2f1-deficient NOD mice are more susceptible to IDDM and SS than wild-type NOD.E2f1+/+ mice.

Th1 and Th2 cytokines increased in E2f1-deficient NOD mice

The roles of cytokines in autoimmune diabetes and SS have been extensively studied in NOD mice. Some cytokines, such as IFN-γ, are pathogenic, whereas others, like IL-4, are able to block the development of autoimmune diseases such as IDDM and SS. Spleen cells from E2f1-deficient NOD mice were stimulated with anti-CD3 mAb, and the supernatants were harvested to determine the levels of IL-4 and IFN-γ production. The production of IFN-γ by spleen T cells in 5-wk-old E2f1-deficient NOD mice was significantly increased (840.0 ± 216.7) as compared with the wild-type NOD.E2f1+/+ mice (443 ± 22.8; Fig. 9, a, p < 0.005). Furthermore, Th2 cytokine IL-4 production by spleen T cells in the mutant NOD.E2f1−/− mice was slightly increased (117.0 ± 18.2) as compared with the wild-type NOD.E2f1+/+ mice 98.0 ± 34.0); however, the difference was not significant (Fig. 9, p = 0.38).

Discussion

To investigate the functional role of the E2f1 molecule in IDDM and SS in NOD mice, we generated an E2f1-deficient NOD mouse (NOD.E2f1−/−) strain. Several studies have demonstrated that E2f1-deficient animals develop late-onset autoimmune features and are characterized by widespread inflammatory infiltrates, exocrine gland dysplasia, glomerular immunocomplex deposition, and hyperplastic thymic and peripheral T lymphocyte proliferation (40, 41, 43, 45–47). In the present study, our analyses of SSLP by PCR using microsatellite markers and neoR gene primers demonstrated that E2f1 homozygous mutant, heterozygous, and wild-type mice were produced at the expected ratio of 1:2:1 (Table II) (2, 41, 42).
The E2f-1 polymorphism was not found in the autoimmune strain (NOD) and nonautoimmune strains (C57BL/6 and 129sv). The SSLP analysis using the D2Mit286 marker as the Idd13 on chromosome 2 that is close to the E2F-1 gene did not show the polymorphism in NOD, NOD.E2f1−/−, 129sv, and C57BL/6 mice in our experiments. Our results also showed that the E2f1 mutation in NOD mice caused early and increased incidences of IDDM and SS (Fig. 1).

At 5 wk old, N5 (NOD.E2f1−/−/−) mice displayed a significant enlargement of the thymus, spleen, and lymph nodes as compared with their littermates. Enlargement of these organs reflects an expansion in the population of mature CD4/CD8 single-positive thymocytes and peripheral T cells, which may have been due to a maturation stage-specific defect in the normal pathway of T cell apoptosis (40–42). Recently, apoptosis was shown to be an important event during normal thymocyte maturation (40, 41). The elimination of thymocytes bearing self-reactive T cell receptors occurs during negative selection and is believed to be mediated by TCR-stimulated apoptosis of CD4/CD8 double-positive thymocytes just before their conversion into mature thymocytes (40, 41, 48, 49). Those observations, taken together with our finding that NOD.E2f1−/− mice have a defect in TCR-stimulated apoptosis of CD4/CD8 double-positive thymocytes, raise the possibility that the E2f1 molecule is functionally active in the process of negative selection.

Recent studies have found that E2f1 functions primarily as a suppressor of cell proliferation (40, 41, 50). Consistent with these findings are observations that E2f1 DNA binding sites in the promoters of several cell cycle genes function primarily to repress transcription of these genes during G0 and G1 (51–53), presumably
by binding to the E2F1-Rb complex. Furthermore, several studies have suggested that repression of transcription the E2F1-Rb complex suppresses entry into the S phase (40, 41, 54). Thus, E2F1-deficient cells may be unable to tether Rb to DNA binding sites during the G0 or G1 phase, which may lead to an aberrant expression of particular cell cycle regulatory genes and inappropriate cell proliferation (40). A role of E2F1 in the suppression of thymocyte cell cycle progression might explain the excess proliferation and significantly different cell cycle distribution seen in the thymus and spleen T lymphocytes of 5-wk-old N5 (NOD.E2f1<sup>-/-</sup>) mice as compared with the wild-type NOD.E2f1<sup>+/+</sup> mice (Fig. 8). Moreover, a direct role of E2F1 as a suppressor of cell cycle progression may explain the additional phenotypes that we and other investigators observed in E2f1-deficient mice, such as testicular atrophy, hyposalivation or exocrine gland dysplasia, increased mortality, hair loss, and sporadic skin tumor formation (40–43). These results indicate that a deletion of the E2f1 molecule leads to enhanced T cell proliferation and suggest that excessive infiltration of T lymphocytes in pancreas islets and salivary glands caused severe IDDM and SS in E2f1-deficient NOD mice as compared with the wild-type NOD.E2f1<sup>+/+</sup> mice (Fig. 2). A role of apoptosis of epithelial cells in the effector phase of sialoadenitis in the NOD mouse was suggested after the study of some investigators (52–57), while other investigators did not find evidence for a role of apoptosis in the effector phase of sialoadenitis (58). In addition, in our experiments we did not find evidence for a role of apoptosis in the initiation phase of IDDM and SS in either group of mice. E2f1 deficiency may regulate proliferative activities of T cells and associate in the initiation phase rather than promotion of apoptosis. Therefore, the induction of autoreactive T cells was proposed as an important mechanism in the initiation phase of the disease in NOD mice.

CD4<sup>+</sup>CD25<sup>+</sup> immunoregulatory T cells represent a unique lineage of thymic-derived cells that potently suppress both in vitro and in vivo effector T cell functions. There is mounting evidence that CD4<sup>+</sup>CD25<sup>+</sup> T cells represent an important mechanism for the maintenance of self-tolerance (59–63), and they have been shown to prevent or limit experimentally induced organ-specific autoimmune diseases (1, 61, 64, 65). Several recent studies also demonstrated that removal of these cells from animals that normally do not develop autoimmune disease could result in autoimmunity (29, 66–67). In most mouse strains, this subset represents 7–10% of the CD4<sup>+</sup> T cell subset, while Salomon et al. (1) reported that these cells are reduced in number and represent only 5–6% of the CD4<sup>+</sup> T cells in NOD mice. In the present study, we found that both E2f1-deficient NOD and wild-type NOD mice have reduced numbers of CD4<sup>+</sup>CD25<sup>+</sup> T cell subsets, and that E2f1-deficient NOD mice may contribute to the development of autoimmune diseases by increasing T cell effector functions. We suggest that molecules such as E2F1, which can intervene in cell cycle control mechanisms, have a functional effect to restrict the development of early and increased onset of IDDM and SS in NOD mice. Furthermore, E2f1-deficient N5 (NOD.E2f1<sup>−/-</sup>) mice may be a useful model to study the functional role of E2f1 molecule and other E2F molecules in IDDM and SS, as well as other dry mouth-type diseases.

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