Development and Function of Diabetogenic T-cells in B-cell–Deficient Nonobese Diabetic Mice

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Insulin-dependent diabetes (type 1 diabetes) in the NOD mouse is a T-cell–mediated autoimmune disease. However, B-cells may also play a critical role in disease pathogenesis, as genetically B-cell–deficient NOD mice (NOD.μMT) have been shown to be protected from type 1 diabetes and to display reduced responses to certain islet autoantigens. To examine the requirements for B-cells in the development of type 1 diabetes, we generated a B-cell–naïve T-cell repertoire by transplantation of NOD fetal thymuses (FTs) into NOD.scid recipients. Surprisingly, these FT-derived NOD T-cells were diabetogenic in 36% of NOD.scid recipients, despite the absence of B-cells. In addition, T-cells isolated from NOD.μMT mice were diabetogenic in 22% of NOD.scid recipients. Together, these results indicate that B-cells are not an absolute requirement for the generation or effector function of an islet-reactive T-cell repertoire in NOD mice. We suggest that conditions favoring rapid lymphocyte expansion can reveal autoreactive T-cell activity and precipitate disease in genetically susceptible individuals. Diabetes 50:763–770, 2001

Type 1 diabetes in the NOD mouse is characterized by the infiltration of T-cells and antigen (Ag)-presenting cells (APCs) into the pancreatic islets of Langerhan (insulitis), resulting in the eventual destruction of insulin-producing pancreatic β-islet T-cells (1). Diabetes pathogenesis in NOD mice is T-cell–dependent; adoptive transfer of splenic T-cells from NOD donors into irradiated NOD or NOD mice congenic for the scid mutation (NOD.scid) causes type 1 diabetes in the recipients (2,3). It has been shown that B-cells are not required at the effector stage of NOD type 1 diabetes, as T-cells from diabetic NOD donors transfer type 1 diabetes to B-cell–depleted NOD neonates (4). However, recent evidence suggests that B-cells play an important role in the development of autoreactivity in NOD type 1 diabetes; NOD mice rendered B-cell deficient by antibody treatment are protected from the development of insulin and diabetes (5–8). Using a genetic approach, several groups have shown that NOD mice homozygous for a germine mutation that disables production of membrane-bound Igμ (μMT), resulting in maturational arrest of B-cell development, affords protection against type 1 diabetes (6,8). However, the mechanism, or mechanisms, by which B-cells affect type 1 diabetes pathogenesis is unclear. B-cells could influence autoimmune disease through autoantibody production or as APCs involved in the selection or activation of autoreactive T-cells. High titers of anti-insulin and anti-GAD autoantibodies are found in prediabetic NOD mice and diabetic humans (9–11), but in contrast to T-cells, autoantibodies fail to transfer disease in NOD mouse models. When activated, B-cells become competent APCs (12) that may capture and present isle T-cell Ags to T-cells (13,14). In addition, Ag presentation by B-cells in the thymus could affect selection of the T-cell repertoire (15). Thus, there are several mechanisms by which B-cells could affect susceptibility to and onset of type 1 diabetes.

We present an independent approach to analyze the requirement(s) for B-cells in the development and function of an islet-reactive T-cell repertoire in NOD mice. To study the function of an NOD T-cell repertoire in the absence of B-cells, we transplanted NOD.NON-Thy1a (NOD.Thy1a) fetal thymuses (FTs) into NOD.scid mice, resulting in reconstitution of the recipients with NOD-derived Thy1a–marked T-cells. Surprisingly, NOD.scid mice reconstituted with NOD FT-derived T-cells developed type 1 diabetes. Parallel experiments using donor T-cells from genetically B-cell–deficient NOD.μMT mice showed that T-cells from these mice were also able to transfer diabetes to a low frequency of NOD.scid recipients. Rather, our results indicate that B-cells are not absolutely required for the development of an autoimmune repertoire or for the effector function against isle T-cells. These results indicate that autoreactive T-cells generated in B-cell–deficient NOD animals can become pathogenic under conditions of T-cell reconstitution and expansion in lymphoid-deficient hosts.

RESEARCH DESIGN AND METHODS

Mice used in these studies were maintained in a pathogen-free facility at the Hospital for Sick Children, Toronto, Ontario, Canada, in which the incidence of diabetes was 80–85% for NOD females and 10–15% for NOD males at 30 weeks of age. NOD.Thy1a–congenic mice were obtained from Jackson Laboratories (Bar Harbor, ME) and bred in our facility. These mice were maintained by brother-sister mating at N8 backcross to NOD. The incidence of diabetes in our NOD.Thy1a colony was 60–75% for females and 5–10% for males at 30 weeks of age. B-cell–deficient NOD.μMT mice were generated as described (8) and used at the N10 backcross to NOD. NOD.scid mice (Jackson Laboratories) were at the N10 backcross to NOD.
FT transplant. Embryonic day 15 (coitus plug = day 1) NOD.Thy1 + fetal thymuses were isolated, and four to six lobes were transplanted under the left kidney capsule of 3- to 4-week-old NOD.scid recipients (RESEARCH DESIGN AND METHODS). Two weeks posttransplant, the NOD.scid FT recipients were examined for FT-derived T-cell reconstitution in the peripheral blood by flow cytometry using FITC-conjugated anti-Thy1 + mAb (19XE5). Dead cells were excluded by PI staining. A: A kidney from a transplanted NOD.scid recipient 4 weeks posttransplant, showing the NOD.Thy1 + thymus tissue under the kidney (×). B: A representative FACS experiment showing the percentage of T-cells (Thy1++) in the PBL of a 4-week-old NOD.Thy1 mouse and a NOD.scid recipient of NOD.Thy1 FT. Compared with the PBL of NOD.Thy1 control, the NOD.scid FT recipient was successfully reconstituted with Thy1 + FT-derived T-cells. C: Generation of NOD FT-derived B-cell-naive T-cells in NOD.scid recipients. The experimental protocol for transplantation of NOD.Thy1 + FT into primary NOD.scid recipients followed by adoptive transfer of FT-derived T-cells into secondary NOD.scid recipients can be found in RESEARCH DESIGN AND METHODS.
immature T-cell precursors were depleted using anti-Thy1.2 (53-2.1) mAb. Ten to twenty million Thy1.1+ FT-T-cells were injected intraperitoneally into each NOD.scid recipient (secondary recipients). Twenty million splenic T-cells from 6-week-old NOD.μMT mice were used to reconstitute NOD. scid/lpr (RT-PCR). For flow cytometry, 1 × 10^6 NOD. scid/lpr T-cells were purified by immunodepletion using M1/70, RA3-6B2, 10-2.16, and magnetic beads, were transferred into NOD.scid recipients as positive controls. NOD.scid recipients injected with phosphate-buffered saline (PBS) served as negative controls. All NOD.scid recipients were monitored for glycosuria weekly using Tes-Tape (Eli Lilly, Toronto, ON) for 22 weeks postadoptive transfer. Recipients were scored as diabetic if they were positive for glycosuria by Tes-Tape on 2 consecutive days. Recipients that remained euglycemic at 22 weeks postadoptive transfer were killed, and their pancreata were isolated for histological examination.

Analysis of FT–T-cell recipients for the presence of mature B-cells. NOD.scid recipients of FT–T-cells were assessed for the presence of B-cells using flow cytometry and reverse transcriptase–polymerase chain reaction (RT-PCR). For flow cytometry, 1 × 10^6 LN and spleen cells were stained with FITC-conjugated anti-B220 mAb (RA3-6B2) and biotinylated anti-CD19 mAb (IC3; Pharmingen). Stained cells were analyzed on the FACScalibur. Ten thousand live (PI-excluding) cells were collected for each recipient. For RT-PCR analysis, RNA was prepared from spleen cells using Trizol (Gibco BRL, Burlington, ON, Canada). RNA isolated from 1 × 10^6 cells was used for the synthesis of cDNA using Superscript II reverse transcriptase (Life Technologies, Burlington, ON, Canada), and NOD spleen RNA was used in mock (minus RT) control reactions. A PCR strategy was used to detect V(D)J recombination at the Ig heavy chain (Igh) locus (16). A degenerate Ig Vh 5’-primer (VHALL) and an Ig Cα 3’-primer (Cα2A) were used to amplify the cDNA, as previously described (16). PCR products of 700 bp reflected transcrip- tion of the rearranged Igh locus. PCR products were separated on 0.8% agarose gels and transferred to nylon membranes (ZetaProbe; BioRad Laboratories, Hercules, CA). Southern blot hybridization analysis was performed using an Igα constant region probe (17). Images were collected on a phos- phor-imaging screen (Molecular Dynamics, Sunnyvale, CA) and analyzed by Image-Quant software (Molecular Dynamics). β-actin PCR controls were performed simultaneously for each cDNA sample. Assay sensitivity was determined by titrations of cDNA from 10 × 10^6 to 1 × 10^5 NOD spleen cells.

Histological examination of pancreata of adoptive transfer recipients. Pancreata from all recipients were evaluated by histological examination of sections as described (18). Insulitis was scored on a scale of 0–4, where 0 represents the absence of insulitis and 4 represents severe invasive insulitis. Immunohistochemistry of 5-μm serial sections was used to detect B220+ B-cells and Thy1.1+ or Thy1.2+ T-cells. Pancreatic sections were fixed with ethanol and blocked with 50 mg/ml bovine serum albumin (BSA). Pancreatic sections were incubated in 1% peroxide to inhibit endogenous peroxidase activity. The slides were washed with PBS + 1% BSA (PBS + BSA) before staining with biotinylated anti-B220 (RA3-6B2) mAb (Pharmingen), anti-Thy1.1, and anti-Thy1.2 (Pharmingen). Incubation with sections were rinsed with PBS + BSA before incubation with streptavidin- conjugated peroxidase followed by chromagen staining (Sigma Chemicals, St. Louis, MO). The slides were counterstained with hematoxylin and rinsed in PBS + BSA.

RESULTS

Generation of B-cell–deficient NOD mice by FT transplantation. FT transplantation was used to generate a B-cell–naive T-cell repertoire of NOD origin (19). Thy-1+ T-cells were removed from embryonic day 15 NOD.Thy1.2a mice harboring NOD.Tg(Prkca-Pepck-CD152)21P.P.L. Chiu (21) T-cells. Rare B-cell precursors in NOD matured in the absence of B-cells and was assessed for autoimmune effector function in a B-cell–deficient environment.

B-cell deficiency in NOD.scid FT–T-cell recipients. Interpretation of these experiments depended on verification of B-cell deficiency in NOD.scid recipients of NOD.μMT FT-derived and NOD.scid (21) T-cells. Rare B-cell precursors are present in day-15 FT, but their development has been found to require addition of exogenous cytokines (22). In addition, rare Ig− B-cells have been observed in aged CB-17.scid (23–25) and NOD.scid (21) mice, providing another potential source of B-cell contamination in the NOD FT-derived T-cell–reconstituted animals. We used two approaches to test for B-cells in FT-derived T-cell–reconstituted primary and secondary NOD.scid recipients (Fig. 1C). Two-color flow cytometric analysis of primary and secondary NOD.scid recipients of FT-derived T-cells was performed using antibodies to the B-cell markers B220 and CD19. B220− CD19+ cells were undetectable in the LNs and the spleens of both primary and secondary NOD.scid FT-T recipients. Indeed, these profiles were indistiguishable from age-matched unmanipulated NOD.scid mice (Fig. 2A). As an independent assessment, we used an RT-PCR approach followed by Southern blot hybridization to detect Igμ mRNA transcripts resulting from VDJμ recombination in the splenocytes of secondary NOD.scid FT-derived T-cell recipients (16). Titration experiments showed that this technique could detect Igμ transcripts in as few as 10 spleen cell equivalents from normal NOD animals (Fig. 2B, panel i). Under these experimental conditions, we could not detect full-length Ig VDJμ-containing transcripts in the splenocytes of NOD.scid FT–T-cell recipients (Fig. 2B, panel ii). Occasionally, we observed Igμ amplicons in NOD.scid mice (Fig. 2B, panel ii), which is consistent with reports that V(D)J recombination occurs with low frequency in scid mice (25,26). Together, the flow cytometry and PCR analyses strongly suggest that both primary and secondary NOD.scid mice harboring NOD FT-derived T-cells were indeed B-cell deficient.

Incidence of insulitis and diabetes in NOD.scid T-cell recipients. NOD.scid recipients of normal NOD, NOD.μMT FT-derived, and NOD.μMT T-cells were followed for 22 weeks postadoptive transfer to assess diabetes onset. A total of 66% of NOD.scid recipients of 6-week-old NOD T-cells became diabetic within this observation period (Table 1, Fig. 3). Unexpectedly, 40% of the recipients of NOD.μMT T-cells and 22% of the recipients of NOD.μMT T-cells became diabetic during the observation period (Table 1, Fig. 3). These results suggest that B-cell–naive NOD T-cells created either by FT transplantation into NOD.scid recipients or by introgression of the μMT mutation onto the NOD background have the potential to cause diabetes in NOD.scid hosts.
Histological examination was performed on the pancreata of all NOD.scid T-cell recipients either at the time of diabetes onset or at 22 weeks post–T-cell transfer, whichever came first. Diabetic recipients of either NOD FT-derived or NOD.µMT T-cells displayed severe invasive insulitis (data not shown). Using immunohistochemical staining, the islet-infiltrating cells in these animals were of donor origin (Thy1a1), and neither Thy1b1 nor B2201 cells were detected in the lesions (data not shown). NOD.scid recipients of NOD FT-derived or NOD.µMT T-cells that remained euglycemic 22 weeks after adoptive transfer were also examined for pancreatic infiltration. Among these mice, 2 of 7 recipients of NOD.µMT T-cells and 5 of 14 recipients of NOD FT-derived T-cells had mean insulitis scores 1.5; this is suggestive of progression toward invasive insulitis (Fig. 4). Some of these animals might have become diabetic over a longer observation period after T-cell transfer. Altogether, 13 of 22 (59%) recipients of NOD FT-derived T-cells and 4 of 9 (44%) recipients of NOD.µMT T-cells demonstrated invasive insulitis and/or diabetes 22 weeks after adoptive transfer, despite the absence of B-cells.

**DISCUSSION**

Several groups have reported that B-cells are critical for the development of NOD insulitis and type 1 diabetes (6–8). A suggested mechanism of this effect is that NOD B-cells are uniquely capable of capturing and presenting islet Ags associated with NOD type 1 diabetes in the context of the MHC class II molecules (13,14,27). Although NOD.µMT mice are protected from type 1 diabetes, some animals became diabetic with the same kinetics as B-cell-sufficient NOD littermates, albeit with a much lower incidence (28). In this report, we present evidence that insulitis and diabetes can develop in the absence of NOD B-cells, and we suggest that under some conditions macrophages (Mfs) and dendritic cells (DCs) are sufficient to select an autoreactive T-cell repertoire and to precipitate autoimmune disease in NOD mice.

**TABLE 1**

Diabetes incidence in NOD.scid recipients of NOD, NOD.µMT, and NOD FT-derived T-cells

<table>
<thead>
<tr>
<th>T-cell donor</th>
<th>Incidence of diabetes</th>
<th>Time to diabetes average (days)</th>
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<tr>
<td>6-week-old NOD</td>
<td>67 (6 of 9)</td>
<td>114 (96–149)</td>
</tr>
<tr>
<td>6-week-old FT-derived</td>
<td>36 (8 of 22)</td>
<td>115 (94–182)</td>
</tr>
<tr>
<td>NOD.Thy1a</td>
<td>22 (2 of 9)</td>
<td>130 (126–134)</td>
</tr>
<tr>
<td>PBS-control</td>
<td>0 (0 of 6)</td>
<td></td>
</tr>
</tbody>
</table>

Data are % (n) or means (range).
There are multiple potential explanations for the difference observed in the NOD FT-derived and NOD.µMT T-cell transfer models compared with previously published results in NOD.µMT and anti-Igµ-treated NOD mice. Previously, gene-targeted disruption of the membrane-bound form of the IgM (µMT) B-cell receptor (6,8,28) or anti-Igµ treatment (5,7) was used to generate B-cell–deficient NOD mice. The µMT mutation was bred from a mixed 129/Sv × C57BL/6 background onto the NOD background. As a result, protective alleles at insulin-dependent diabetes loci (Idd) could have been transferred along with the µMT mutation, decreasing disease susceptibility in NOD.µMT mice. We do not favor this explanation, because the diabetes protection in NOD.µMT mice was demonstrated after the sixth backcross (N7) generation, and these mice were typed for microsatellite markers linked to 15 of the 19 known Idd susceptibility loci (8). The NOD.µMT animals used in the current study were products of three additional back-crosses (N10) and were typed for additional markers: D3Mit100 near the Idd10, 17, and 18 region and D10Mit87 near the IFNγR gene (data not shown) recently reported to be linked to a diabetes protection locus in 129/Sv mice (29). Diabetes incidence in NOD.µMT mice housed at the Jackson Laboratories from 1996 to the present is low, but detectable (3–5%) (D.V. Serreze, unpublished data); this is consistent with the idea that this mutation provides substantive but not absolute protection from diabetes. In independent studies, in vivo treatment with anti-Igµ Abs has suggested that removal of B-cells in the neonatal period protects NOD mice against type 1 diabetes. Furthermore, treatment of neonatal NOD mice with natural Ig alters diabetes development, although the mechanism of protection is unclear (30). In both instances, in vivo Ab treatment may provoke immunological modulations in addition to clearance or alteration of B-cell function. In contrast, our approach using NOD FT-derived T-cells did not require manipulation of the NOD genome or in vivo Ab treatments and thereby avoided effects independent of B-cell depletion.

T-cell responses to a variety of foreign Ags are poor in NOD.µMT homozygous mice compared with NOD.µMT/− and NOD controls, suggesting that the mutation causes global rather than autoantigen-specific effects on cellular immunity. For example, C57BL/6-µMT mice have reported defects in delayed-type hypersensitivity responses to in vivo challenges with pathogens such as Chlamydia (31), Mycobacteria (32), and Listeria (33). Furthermore, priming of NOD.µMT mice with keyhole limpet hemocyanin (KLH) generated lower recall responses compared with NOD T-cells (27), although T-cell responses to KLH were readily observed in C57BL/6-µMT mice (34). These data suggest that NOD.µMT T-cell responses to a broad array of antigens may be significantly different from the NOD parental strain. If this is indeed the case, the diabetes protection observed in NOD.µMT mice may reflect a general impairment in T-cell responses in addition to a specific requirement for B-cells in generation and function of diabeticogenic T-cells.

A fundamental feature of autoimmune diabetes susceptibility may be the extent and the kinetics of self-reactive T-cell proliferation and apoptosis in peripheral tissues. Indeed, NOD T-cells demonstrate enhanced proliferation (35) and resistance to apoptosis (36–38) compared with T-cells from nondiabetes-prone strains. Based on these findings, our adoptive transfer approach into NOD.scid recipients may favor the expansion of islet-reactive NOD FT-derived and NOD.µMT T-cells precipitating the development of type 1 diabetes. NOD.scid mice are excellent adoptive transfer recipients because of the absence of
mature lymphocytes, depressed natural killer cell cytolytic activity, and the absence of circulating complement (21). The paucity of mature lymphocytes in NOD.scid mice permits rapid expansion of donor T-cells. We have shown that FT-derived T-cells mature and disseminate in the lymphoid organs of primary and secondary NOD.scid recipients. Perhaps the T-cell expansion caused by repopulation of the NOD.scid host is permissive for expansion and activation of autoreactive T-cells. Consistent with this idea, we show that the autoreactive T-cells in NOD.μMT mice that rarely cause diabetes in situ have heightened pathogenic potential when transferred into the lymphopenic NOD.scid microenvironment.

Multiple independent lines of evidence support this reasoning in the context of autoimmune diabetes. The first comes from analyses of the Diabetes-Prone BioBreeding (DP-BB) rat and the related DR-BB strain. In DP-BB rats, a locus (iddm1) conferring severe T-cell lymphopenia (lyp) is required for spontaneous diabetes, but the lyp gene has not yet been identified (40,41). Interestingly, reconstitution of DP-BB animals with histocompatible T-cells from normal rats affords partial protection from diabetes, highlighting the direct contribution of T-cell lymphopenia to diabetes development (42,43). Although the nonlymphopenic DR-BB strain is diabetes resistant under normal conditions, interventions that produce lymphopenia, such as ionizing radiation and cyclophosphamide, induce type 1 diabetes in this strain (44,45). Recent studies demonstrate that the diabetogenic potential of the DR-BB T-cell repertoire is revealed after adoptive transfer of DR-BB T-cells into thymectomized and irradiated DP-BB recipients (46). Collectively, these studies demonstrate that the pathogenic potential of autoreactive T-cells in the BB rat is enabled when these cells are placed in a lymphopenic environment. Similar to the contribution of lyp to diabetes in the BB rat, diabetes development in NOD mice is dramatically accelerated after recovery from cyclophosphamide-induced lymphopenia (47,48). A particularly revealing example of the cyclophosphamide effect was reported in lethally irradiated NOD mice reconstituted with 50:50 mixed bone marrow isolated from MHC class II–deficient (NOD.I-A^(-/-)) and B-cell–deficient (NOD.μMT) mice (13). The resultant bone marrow chimerae were not spontaneously diabetic, a finding that the authors argue reflects the absence of MHC class II–expressing B-cells. However, these mice have insulinitis, and cyclophosphamide treatment caused rapid diabetes onset. Thus, the absence of MHC class II–expressing B-cells does not preclude the production and destructive function of an islet-reactive T-cell repertoire. Rather, these T-cells are generated in the absence of MHC class II–expressing B-cells and are pathogenic when conditions favor their expansion. We also report a high frequency of diabetes in 35-week-old NOD.μMT mice after cyclophosphamide treatment, underscoring the pathogenic potential of NOD T-cells that developed in the absence of B-cells. Considering the evidence from the BB rat and the cyclophosphamide-treated NOD mice, together with the data provided here, we suggest that conditions that favor extensive lymphocyte expansion can reveal autoreactive T-cells and precipitate disease in genetically susceptible individuals. This idea has important implications for broad T-cell–depleting therapies, which warn that the rebound effect of these agents may aggravate rather than alleviate autoimmune reactivity.

Previous reports suggest that NOD B-cells are uniquely capable of capturing and presenting specific islet Ags to islet-reactive NOD T-cells (13,14,27). However, we observed the development of type 1 diabetes in 36 and 22% of NOD.scid recipients of NOD FT-derived or NOD.μMT T-cells, respectively. Although these diabetes incidences are reduced compared with NOD.scid recipients of normal NOD T-cells, our results suggest that myeloid APCs, such as Mψs and DCs, are sufficient to select, activate, and sustain autoreactive T-cells. In our study, the absence of lymphocytes in the NOD.scid hosts may have enhanced the density of myeloid APCs and increased their effectiveness as stimulators of autoreactive T-cells. Mψs and mature DCs express high levels of costimulatory molecules (49,50) and are clearly implicated in type 1 diabetes pathogenesis. For example, the depletion of Mψs from NOD mice transgenic for a diabetogenic T-cell receptor protected ectopic islet grafts from destruction and blocked the capacity of the T-cell receptor transgenic T-cells to transfer disease into NOD.scid recipients (51). Mψs and DCs infiltrate NOD and NOD.scid islets before and independent of T- and B-cells (18,52–54) and produce proinflammatory mediators tumor necrosis factor-α and γ-interferon and nitric oxide that contribute to type 1 diabetes (55). Conversely, depletion of NOD Mψs prevents the onset of type 1 diabetes (51,52,56,57), and in other rodent models of type 1 diabetes, DCs and Mψs are necessary and sufficient to cause diabetes (58,59). Together, these data indicate that Mψs and DCs are also critical APCs for diabetes development and can suffice to select and activate an islet-reactive T-cell repertoire. However, the presence of B-cells likely contributes to a greater efficiency of T-cell activation and expansion, potentiating diabetes development. For these reasons, therapeutic strategies for the prevention of type 1 diabetes in susceptible individuals will likely be more efficacious when directed toward multiple types rather than B-cells alone.

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REFERENCES


DIABETOGENIC T-CELLS IN B-CELL-DEFICIENT MICE