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IL-10 Impacts Autoimmune Diabetes Via a CD8\(^+\) T Cell Pathway Circumventing the Requirement for CD4\(^+\) T and B Lymphocytes

Balaji Balasa, Joanna D. Davies, Jae Lee, Augusta Good, Brian T. Yeung, and Nora Sarvetnick

IL-10 is essential for an early phase of diabetes in nonobese diabetic (NOD) mice, but later becomes protective against its development. The mechanism by which IL-10 mediates the pathway to diabetes in these mice is unknown. Herein, we dissected the cellular and costimulation requirements for diabetes in transgenic (tg) NOD mice that expressed IL-10 in their pancreatic islets (IL-10-NOD mice). We found that IL-10 alone did not cause diabetes because the offspring (IL-10-NOD-\(scid\) mice) from backcrosses of IL-10-NOD mice with NOD-\(scid\) mice had no diabetes. Moreover, these IL-10-NOD-\(scid\) mice were free of lymphocytic infiltration. Treatment of IL-10-NOD mice with depleting anti-CD4 mAb or control mAb had no effect on diabetes. Surprisingly, depletion of CD8\(^+\) T cells by treatment with the corresponding mAb inhibited diabetes without attenuating insulitis, demonstrating a critical role for CD8\(^+\) T cells in the disease process. Interestingly, B cell-deficient IL-10-NOD mice readily developed diabetes with kinetics and incidence similar to those observed in wild-type mice, demonstrating that B lymphocytes as APCs were not required in the disease process. Administration of anti-CD40 ligand (CD40L) mAb did not prevent disease, indicating that CD40/CD40L costimulation is not required for diabetes in IL-10-NOD mice. Immunization of IL-10-NOD mice with CFA or heat-shock protein 65, known to block diabetes in NOD mice, had no effect on their diabetes. We demonstrate that IL-10 contributes early to the pathology of diabetes via a CD8\(^+\) T cell pathway, eliminating the requirement for B lymphocytes and CD40/CD40L costimulation. Our findings provide a mechanism for the participation of IL-10 in the early development of diabetes.


T
he nonobese diabetic (NOD)\(^3\) mouse, an animal model for human insulin-dependent diabetes mellitus (IDDM) (1, 2), spontaneously develops clinical diabetes at 4 to 7 mo of age, when the destruction of β cells causes glucose levels to rise (3). Diabetes in NOD mice is a T cell-dependent disease (4) and requires an interplay between CD4\(^+\) and CD8\(^+\) T cells (5, 6). A crucial role for B lymphocytes (7, 8) and macrophages (9, 10) as APCs has also been documented.

Cytokines produced by T cells and APCs play a pivotal role in autoimmune diabetes. In that context, the role of Th2 cytokine IL-10 in the pathogenesis of this disease has recently been examined. IL-10 has exhibited paradoxical effects on diabetes of the NOD mouse. Accordingly, treatment of young NOD mice with recombinant IL-10 (11, 12) and an adoptive transfer of islet-specific T cells transduced with IL-10 cDNA (13) prevented diabetes. These findings demonstrated that IL-10 is an immunosuppressive factor in IDDM of the NOD mouse. In contrast, anti-IL-10 mAb treatment of young NOD mice prevented insulitis (14). BALB/c mice expressing IL-10 transgene in their insulin-producing β cells (IL-10-BALB/c mice) of the pancreas did not develop diabetes, but their offspring (IL-10-NOD mice) from backcrosses to NOD mice became diabetic at an accelerated rate (15). Similarly, NOD mice expressing IL-10 transgene in glucagon-producing α cells of the pancreas developed accelerated diabetes (16). These findings demonstrated that IL-10 is an immunostimulatory factor in IDDM of the NOD mouse.

To determine how the IL-10 participates in these pathogenic anti-islet inflammatory responses, we performed genetic, T cell-depletion, and Ab-blocking studies. First, we verified the role of autoreactive lymphocytes by backcrossing IL-10-BALB/c mice with NOD-\(scid\) mice. Next, to identify the role of CD4\(^+\) and CD8\(^+\) T cells, we depleted IL-10-NOD mice of these cells with mAbs. To identify the role of B cells, we bred the IL-10-NOD mice with NOD-\(\mu\)MT (B cell-deficient) mice. Finally, to examine the contributions of CD40/CD40 ligand (CD40L) as costimulator of diabetes in the IL-10-NOD mice, we blocked its activity with blocking anti-CD40L mAb. The results revealed that IL-10 does not cause dysfunction of β cells leading to diabetes, and rather modulates the disease process through CD8\(^+\) T cells without the participation of B cells and the established CD40-CD40L costimulation pathway.

Materials and Methods

Mice

NOD/\(shi\) mice were part of the rodent breeding colony at The Scripps Research Institute (La Jolla, CA). IL-10-BALB/c mice expressing IL-10 transgene in their islets under the control of human insulin promoter (17) were backcrossed to NOD/\(shi\) NOD-\(scid\) mice to generate IL-10-NOD or IL-10-NOD-\(scid\) mice, respectively. These mice were typed for the transgene’s presence by PCR (17). The \(scid\) mutation in IL-10-NOD-\(scid\) mice was verified by flow cytometry and an immunodiffusion test. We randomly...
used N2-N5 backcross generation mice in all the experiments described in this manuscript. We have selected the mice that are IL-10 transgene r+ve and 1-14/1-14. Note that the mice were not typed for additional information on MHC class I and class II loci. The kinetics of disease in N7 backcross generation mice were similar to those observed in N2-N5 backcross mice (B. Balasa and N. Sarvetnick, unpublished observations).

B cell-deficient (μMT) NOD mice, carrying their genome-targeted disrupted region of the membrane exon of the Ig μ-chain gene (μMT) (−/−), were generated by backcrossing C57BL/6-μMT mice onto the NOD background for eight generations, were kindly provided by Drs. Diane Mathis and Christopher Benoist (Illkirch, France). The IL-10-BALB/c mice were backcrossed to NOD-μMT mice to generate experimental animals (mutant −/− as well as +/− and +/+ control littersmates). The μMT allele was detected by PCR using genomic DNAs from tails as described (www@jax.org). B cell-deficient IL-10-NOD mice (IL-10-NOD-μMT) (−/) were further screened by typing their PBLs with two-color flow cytometric using FITC-conjugated anti-B220 mAb and phycoerythrin-conjugated anti-CD3 mAb (PharMingen, San Diego, CA).

Antibodies

B cell hybridomas secreting mAbs against mouse CD40L (MR1, hamster IgG) were purchased from American Type Culture Collection (ATCC, Manassas, VA). mAbs, generated in the form of ascites fluid, were purified by affinity chromatography on a HiTrap protein G column (Pharmacia Biotech, Piscataway, NJ). The rat IgG2b mAb to mouse CD4 (YTS 191.1.2) and CD8 (YTS 169.4.2.1) molecules were purified from hybridoma cell culture supernatants (18).

Ab treatment of mice

Depletion of T cells.

Groups of 1-wk-old pups from IL-10-NOD mice were injected i.p., on alternate days (days 7, 9, and 11), with isotype-matched 500 μg of depleting anti-CD4 (rat IgG2b) or anti-CD8 (rat IgG2b) mAb or control rat IgG in PBS. When test animals were assessed at 5 wk of age, this procedure regularly resulted in >90% depletion of appropriate T cell subsets as determined by FACS analysis (data not shown). This mAb treatment schedule (three injections of 500 μg doses on alternate days) was repeated on 5-wk-old nondiabetic mice because at 3- to 4-wk posttreatment the T cell subset population bounces back. Control mice were injected with PBS or rat IgG (Sigma, St. Louis, MO) only. The results were pooled from two to three separate experiments. To confirm the efficacy of depleting anti-CD4 mAb, we similarly treated female NOD/shi mice.

Treatment of mice with anti-CD40L mAb.

Groups of IL-10-NOD mice were injected i.p., starting at 2 to 3 days of age, with 200 μg of the indicated mAb dose and then at 3-day intervals for 30 days. Survived animals then received three additional 200-μg doses when they were 6 wk of age. We used the same batch of hamster anti-CD40L mAb that was used earlier to prevent diabetes in NOD mice. The current studies were performed in parallel with our previous studies (19). Before its use, the functional activity of anti-CD40L mAb was confirmed by its binding to CD40L on anti-CD3 mAb-stimulated splenocytes by flow cytometry (data not shown). Control mice were injected with hamster IgG. The results were pooled from two to three separate experiments.

CFA or heat-shock protein 65 (hsp65) immunizations.

Groups of mice were immunized with 50 μl of CFA-PBS or IFA-PBS (1:1, v/v) emulsion containing 50 μg of hsp65 following the protocol described earlier (20, 21). Control mice were injected with PBS only. In a separate set of experiments, the injections were repeated at 3 wk of age. The results are representative of two or more separate experiments. As a positive control, 1-wk-old female NOD/shi mice were treated with CFA or hsp65 as above.

Assessment of diabetes

Starting at 5 wk of age, the mice were treated for diabetes by weekly or bi-weekly measurements of blood glucose (BG) levels using a one-step Bayer Glucometer Elite (Bayer, Elkhart, IN). Animals were considered diabetic when BG levels were >300 mg/dl. Control mice are transgene negative littersmates. The IL-10-NOD-μMT mice (n = 13) were obtained upon screening a total of 92 pups belonging to ten different litters. The wild-type IL-10-NOD mice (n = 12) were obtained upon a screening of 47 pups belonging to five different litters.

Histologic analysis

Lymphocytic infiltration of the islets was evaluated on hematoxylin and eosin (HE)-stained paraffin sections of pancreas taken at several levels throughout the organ (19). Paraffin-embedded pancreatic tissue was stained with an immunoperoxidase method using polyclonal Abs to porcine insulin and synthetic glucagon, followed by a biotinylated secondary Ab and an avidin-biotin complex as described earlier (19).

Lymphocyte proliferation assays

Splenocytes from age-matched individual transgenic (tg) or non-tg mice were cultured at 8 × 105 cells/well in 200 μl of serum-free HL-1 medium supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 × 10−3 M 1-glutamine, and 3 × 10−3 M 2-ME in 96-well flat bottom microculture in the presence of indicated Ags for 5 days. To identify the nature of proliferating cells, we added nondepleting anti-CD4 (YTS 177; IgG2a) or anti-CD8 (YTS 105; IgG2a) mAb (kindly provided by Dr. Joanna D. Davies, The Scripps Research Institute, La Jolla, CA) or control rat IgG (50 μg/ml) to the cultures. The cultures were pulsed with 1 μCi of [3H]TdR/well during the last 18 h of the assay and were later harvested as described (19). [3H]TdR uptake was measured in a β scintillation counter. The results were expressed as a stimulation index: [(mean cpm with Ag)/(mean cpm without Ag)]. OVA (Sigma) and porcine insulin (Calbiochem, La Jolla, CA) were purchased. Stimulation index values >3 over the background values were considered significant.

Statistical analysis

The significance of the data was done by student’s t test (paired or unpaired) and Logrank test using Statview software (Abacus Concepts, Berkeley, CA) as required. A value of p < 0.05 was taken as the level of significance.

Results

Transgenic expression of IL-10 does not cause clinical diabetes in immunodeficient IL-10-NOD-μMT mice

BALB/c mice expressing IL-10 transgene in their insulin-producing β cells (IL-10-BALB/c mice) of the pancreas did not develop diabetes. However, their offspring (IL-10-NOD mice) from backcrosses to NOD mice became diabetic at an accelerated rate (15). It is possible that the expression of IL-10 transgene may compromise β cell function. In addition, it was argued that localized production of IL-10 in β cells caused the endothelial damage and then necrosis of microcapillaries that affected many islets (22). To test the hypothesis that IL-10-accelerated diabetes is an autoimmune phenomenon, we backcrossed the diabetes-prone IL-10-NOD mice to NOD-μMT mice and then assessed the susceptibility of their offspring to diabetes. As shown in Table I, IL-10-NOD-μMT mice (n = 13; 0% incidence; p < 0.0001) did not develop diabetes over
a 12-wk period. The wild-type mice (IL-10-NOD mice) (n = 12; 92% incidence) readily developed accelerated diabetes. Additionally, the pancreatic islets from these mice were completely free of lymphocytic (T and B cell) infiltration (see Fig. 4a). These results demonstrate that IL-10 alone does not cause diabetes in NOD mice that are immunologically compromised.

Expression of IL-10 transgene in islets did not inhibit CD4+ T cell responses to islet Ags, and CFA or hsp65 treatments did not block accelerated diabetes

It has earlier been shown that young NOD mice exhibit spontaneous T cell reactivity to islet Ags such as glutamic acid decarboxylase 65 (GAD65) and hsp65 (23–25). Similarly, we have also observed a spontaneous T cell reactivity of NOD splenocytes against GAD65 and hsp65 in vitro (19, 26). Because IL-10-NOD mice developed accelerated diabetes, we examined whether these mice exhibit an increased T cell reactivity to islet Ags (GAD65 and hsp65) in lymphocyte proliferation assays. The results are shown in Table II. The splenocytes from individual transgenic IL-10-NOD mice (n = 7) proliferated in vitro against GAD65 and hsp65 at a higher intensity than those cells from age-matched non-tg littermates (n = 7). However, the responses to hsp65 (p = 0.06) and to GAD65 (p = 0.42) between the groups were not statistically significant. The responses between GAD65 and hsp65 within each group were also found not statistically significant (for transgene +ve mice: p = 0.28) (for transgene -ve mice: p = 0.755). Lymphocytes from neither group of mice proliferated in response to porcine insulin (mean stimulation index <2.0). Lymphocyte proliferative responses to islet Ags were abrogated by the addition of anti-CD4 but not anti-CD8 mAb (not shown). These data suggest that expression of IL-10 transgene in pancreatic islets of NOD mice did not enhance the CD4+ T cell auto-reactivity against GAD65 and hsp65.

CFA or hsp65 immunizations protected NOD mice from diabetes by inducing regulatory (Th2) CD4+ T cells (20, 25, 27). To determine whether these treatments would similarly protect IL-10-NOD mice from accelerated diabetes, we immunized 46 1-wk-old pups (of which 15 mice were transgene +ve) with CFA (Fig. 1A) and 33 mice (of which 7 mice were transgene +ve) with hsp65 (Fig. 1B). Control mice were injected with PBS only (Fig. 1A, n = 10; Fig. 1B, n = 12). The mice were monitored for diabetes by measuring their BG levels at weekly intervals. Surprisingly, neither of these treatments protected IL-10-NOD mice from accelerated diabetes. The incidence of diabetes in CFA-treated mice (11 of 12; 91.7% incidence) did not differ significantly from those of PBS-treated mice (9 of 10; 90% incidence) (p = 0.6246). Additionally, the incidence of diabetes in hsp65-treated mice (5 of 7; 71.4% incidence) did not differ significantly from those of PBS-treated mice (11 of 12; 91.7% incidence) (p = 0.6425). In a separate set of experiments, injection of two doses of CFA or hsp65 at 1 wk and 3 wk of age did not block diabetes in IL-10-NOD mice (data not shown). As a positive control, we treated young (1-wk-old) NOD mice either with CFA or hsp65. The mice were monitored for a period of 28 wk. Both CFA (n = 8; 6 of 8 mice) or hsp65 (n = 8; 6 of 8 mice) treatments effectively blocked insulitis and diabetes, demonstrating the effectiveness of these treatments in modulation of the disease (data not shown). In agreement with the established findings in the literature, histologic examination of the pancreata from protected NOD mice showed peri-insulitis but not insulitis (not shown). Taken together, CFA and hsp65 treatments failed to block accelerated diabetes in IL-10-NOD mice.

Expression of IL-10 transgene in islets circumvents the requirement for B lymphocytes and CD40-CD40L costimulation pathways in the process of diabetogenesis

Recent studies employing NOD-μMT (B cell-deficient) mice (7, 28) and B cell-depleted (anti-IgM-treated) NOD mice (8) have demonstrated that B cells are necessary as APCs for the initiation of insulitis and diabetes. Because IL-10 has immunostimulatory effects on B cells (29), we hypothesized that B cells play a role in the initiation and perpetuation of accelerated diabetes in IL-10-NOD mice. To test this hypothesis, we backcrossed the IL-10-BALB/c mice with NOD-μMT mice to generate IL-10 +ve B cell-deficient (−/−) (n = 16), heterozygous (+/−) (n = 17), and wild-type (+/+) (n = 13) mice. The results

Table II. In vitro T cell reactivity to islet antigens in transgenic and non-transgenic IL-10-NOD mice

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Age (wk)</th>
<th>IL-10 tg</th>
<th>Diabetes</th>
<th>In Vitro Lymphocyte Proliferative Response (SI ± SD) to</th>
<th>GAD65</th>
<th>hsp65</th>
<th>Insulin</th>
</tr>
</thead>
<tbody>
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<td></td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>1</td>
<td>5</td>
<td>+</td>
<td>+</td>
<td>3432</td>
<td>2.64 ± 0.30</td>
<td>6.89 ± 1.56</td>
<td>0.91 ± 0.11</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>-</td>
<td></td>
<td>4242</td>
<td>5.09 ± 1.12</td>
<td>3.60 ± 0.49</td>
<td>1.40 ± 0.47</td>
</tr>
<tr>
<td>3</td>
<td>6</td>
<td>+</td>
<td>+</td>
<td>6666</td>
<td>2.83 ± 0.60</td>
<td>3.51 ± 0.45</td>
<td>1.28 ± 0.87</td>
</tr>
<tr>
<td>4</td>
<td>6</td>
<td>-</td>
<td></td>
<td>8205</td>
<td>4.53 ± 0.79</td>
<td>4.23 ± 0.56</td>
<td>1.43 ± 0.31</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>+</td>
<td></td>
<td>6560</td>
<td>8.48 ± 0.88</td>
<td>2.66 ± 0.51</td>
<td>1.92 ± 0.28</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>+</td>
<td></td>
<td>6774</td>
<td>5.66 ± 0.55</td>
<td>3.22 ± 0.19</td>
<td>1.36 ± 0.39</td>
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<tr>
<td>7</td>
<td>8</td>
<td>+</td>
<td></td>
<td>5643</td>
<td>1.28 ± 0.33</td>
<td>4.99 ± 0.86</td>
<td>ND</td>
</tr>
<tr>
<td>Mean SI</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4.36 ± 2.38</td>
<td>4.16 ± 1.42</td>
<td>1.38 ± 0.32</td>
</tr>
<tr>
<td>1</td>
<td>5</td>
<td>-</td>
<td>-</td>
<td>6386</td>
<td>3.54 ± 0.35</td>
<td>2.79 ± 0.51</td>
<td>0.79 ± 0.23</td>
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<tr>
<td>2</td>
<td>6</td>
<td>-</td>
<td>-</td>
<td>6666</td>
<td>3.98 ± 0.23</td>
<td>2.00 ± 0.20</td>
<td>0.72 ± 0.29</td>
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<tr>
<td>3</td>
<td>6</td>
<td>-</td>
<td>-</td>
<td>1757</td>
<td>1.28 ± 0.16</td>
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<td>1.25 ± 0.02</td>
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<tr>
<td>4</td>
<td>6</td>
<td>-</td>
<td></td>
<td>4110</td>
<td>2.06 ± 0.69</td>
<td>4.76 ± 1.13</td>
<td>1.61 ± 1.01</td>
</tr>
<tr>
<td>5</td>
<td>6</td>
<td>-</td>
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<td>4107</td>
<td>3.50 ± 0.33</td>
<td>3.14 ± 1.39</td>
<td>0.86 ± 0.10</td>
</tr>
<tr>
<td>6</td>
<td>6</td>
<td>-</td>
<td></td>
<td>1570</td>
<td>3.05 ± 0.29</td>
<td>2.63 ± 0.53</td>
<td>1.02 ± 0.23</td>
</tr>
<tr>
<td>Mean SI</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.76 ± 1.01</td>
<td>2.83 ± 1.00</td>
<td>0.89 ± 0.34</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(p = 0.42)</td>
<td>(p = 0.06)</td>
<td>(p = 0.11)</td>
</tr>
</tbody>
</table>

**Note:** In vitro proliferative responses of splenocytes from individual mice against GAD65 (40 μg/ml), hsp65 (20 μg/ml), and porcine insulin (40 μg/ml). The IL-10-NOD mice (N2 and N3 backcross generations) were generated by backcrossing of IL-10-BALB/c mice with NOD-μMT mice for 2 to 3 generations. p values were calculated by unpaired student’s t test. The p values <0.05 are considered significant. SI, stimulation index. ND, not determined.

* Bgr. cpm represent the cpm obtained with unstimulated lymphocyte cultures.
Surprisingly, the IL-10-NOD-\(\mu\)MT mice (\(-/-\)) readily developed accelerated diabetes (14 of 16 mice; 87.5% incidence). The incidence and kinetics of disease in these IL-10-NOD-\(\mu\)MT mice were not statistically significant from those of heterozygous (13 of 17 mice; 76.5% incidence) and wild-type (10 of 13 mice; 76.9% incidence) littermate controls (\(p = 0.4786\) vs wild-type mice; \(p = 0.1953\) vs heterozygous mice). As expected, non-tg \(\mu\)MT (\(-/-\)) mice did not develop diabetes (not shown). Therefore, the B lymphocytes as APCs are not required for the initiation of accelerated diabetes in IL-10-NOD mice.

We have earlier shown that blockade of CD40-CD40L costimulation by hamster anti-CD40L mAb effectively abrogated autoimmune insulitis and diabetes in NOD mice (19). Therefore, we similarly blocked this costimulatory pathway (using the same batch of anti-CD40L used in our earlier study) in IL-10-NOD mice. The treated mice were tested for diabetes. Figure 2B depicts the results of treating these mice with anti-CD40L mAb or control hamster IgG starting when the mice were 2 to 3 days old and injected at 3-day intervals for 30 days. Surprisingly, the diabetes developed in anti-CD40L-treated mice reaching a cumulative incidence of 86% (12 of 14) at 11 wk of age. These results did not differ (\(p = 0.3206\)) from those of control hamster IgG-treated mice (9 of 11; 82% incidence). The islets from these treated groups exhibited insulinitis (not shown). Thus, anti-CD40L mAb did not block accelerated diabetes of IL-10-NOD mice.

**Treatment of IL-10-NOD mice with depleting anti-CD8 mAb retards diabetes but not insulinis**

The islets of Langerhans in IL-10-NOD mice are infiltrated with many more CD4\(^+\) T cells and very few CD8\(^+\) T cells (15). The natural course of diabetes in the NOD mouse requires the participation of both CD4\(^+\) and CD8\(^+\) T cell as the depletion of CD4\(^+\) (30, 31) and CD8\(^+\) T cell subsets with corresponding mAb completely retarded insulinis. It is established that IL-10 possesses differential effects on T cell subsets via direct and indirect mechanisms (33–35).

In this study, we addressed whether treatment of IL-10-NOD mice with depleting rat IgG2b mAb to CD4 and CD8 molecules...
would block insulitis and diabetes. The results are shown in Figure 3A. Anti-CD4 mAb treatment did not block diabetes because IL-10-NOD mice (21 mice were treated, of which 9 mice were transgene+ve; n = 9) developed diabetes at 5 to 9 wk of age (89% incidence) closely resembling that of PBS (control)-treated mice (n = 8; 88% incidence) (p = 0.572). Similar results were obtained in control rat IgG-treated mice (data not shown). However, efficacy of the anti-CD4 mAb was verified by blocking diabetes in female NOD/shi mice because treated young NOD mice failed to develop insulitis and diabetes over a 32-wk period (data not shown). In contrast, depleting anti-CD8 mAb treatment completely prevented accelerated diabetes (16 mice were treated; 11 were transgene+ve; n = 11; 0% incidence) even at 17 wk of age (anti-CD4 group vs anti-CD8 group, p = 0.0003; anti-CD8 group vs PBS group, p = 0.0002).

Next, we examined whether blockade of diabetes in anti-CD8 mAb-treated IL-10-NOD mice was associated with obliteration of insulitis. We scored several HE-stained sections for lymphocytic infiltration of the islets from individual mice. The results are shown in Figure 3, B–D. Irrespective of the treatments employed, IL-10-NOD mice showed extensive lymphocytic infiltration in their islets. These findings demonstrate that anti-CD8 mAb treatment, as opposed to its effectiveness on insulitis in NOD mice (32), did not abolish lymphocytic infiltration into the islets of IL-10-NOD mice (Fig. 4). However, the islets from these mice contained significant numbers of insulin-positive β cells. On the other hand, HE sections of pancreata from anti-CD4 mAb- and PBS-treated mice showed extensive lymphocytic infiltration accompanied by complete or near to complete loss of insulin-positive β cells (Fig. 4).

Discussion

The results from this study demonstrated for the first time that expression of IL-10 transgene affects the disease process of NOD mice via the CD8+ T cell pathway without a requirement for B cells as APCs and the well-described CD40-CD40L costimulation pathway.

A critical role for CD8+ T cells in acceleration of diabetes in IL-10-NOD mice emerged from experiments showing that depleting anti-CD8 but not anti-CD4 mAb effectively abrogated the disease (Fig. 3A). However, anti-CD4 and anti-CD8 mAb-treated IL-10-NOD mice developed severe insulitis (Fig. 4A). It is apparent that the lymphocytic infiltrate in anti-CD8 mAb-treated mice may be nonpathogenic because these mice failed to develop diabetes. On the other hand, the lymphocytic infiltrate in anti-CD4 mAb-treated mice may be of pathogenic nature because these mice developed diabetes. These findings contrast with earlier reports demonstrating that similar treatments inhibited both insulitis and diabetes in NOD mice (30–32). Furthermore, abrogation of B cells as APCs in the disease process of IL-10-NOD mice (Fig. 2A) is in complete contrast to the earlier results showing that B cells are necessary for the initiation of disease in NOD mice (7, 8, 28). Thus, the differential effectiveness of depleting mAb to CD4 and CD8 molecules on diabetes and insulitis and the abrogation of a requirement for B cells as APCs in the disease process suggest a fundamental difference in the mechanistic processes of disease development in IL-10-NOD and NOD mice. In support of the current findings, elsewhere it was shown that IL-10 could prime the CD8+ T cells against P815 tumor cells in anti-CD4 mAb-treated mice (36). Certainly, the proinflammatory IL-10 plays a critical role during very early stages of autoimmune process via CD8+ T cell pathway. In that regard, CD8+ T cells are implicated in the disease process of IDDM patients (37–39).

We demonstrated here that CD8+ T cells are critical for accelerated diabetes, but the precise mechanisms by which the proinflammatory IL-10 accelerates disease in IL-10-NOD mice are currently not known. It is possible that IL-10 may act as a chemoattractant (40) and a differentiation factor (41–43) for CD8+ T cells.
T cells. The chemotactic activity of IL-10 should be tested by adoptive transfer of Ag-inexperienced CD8\(^+\) T cells from 3-wk-old NOD mice into IL-10\(^{-}\)-NOD-scid and IL-10\(^{-}\)-NOD-scid mice. We are currently testing this possibility. Alternatively, IL-10 may induce the differentiation of and/or cytotoxic potential in islet Ag-specific CD8\(^+\) T cells. Therefore, we are currently breeding

**FIGURE 4.** Histologic analysis of pancreata from IL-10-NOD-scid (a, e, i), PBS-treated (b, f, j), anti-CD4 mAb-treated (c, g, k), and anti-CD8 mAb-treated (d, h, l) IL-10-NOD mice. The sections were HE-stained. a, Pancreas from IL-10-NOD-scid mouse showing intact islets without any insulitis. b, Pancreas from PBS-treated IL-10-NOD mice showing severe insulitis. c, Pancreas from anti-CD4 mAb-treated IL-10-NOD mice showing severe insulitis. d, Pancreas from anti-CD8 mAb-treated IL-10-NOD mice showing severe insulitis. e, Pancreas from IL-10-NOD-scid mouse, immunostained for insulin using diaminobenzidine as a chromogen. Note the intact islets stained positively for insulin (brown-red color). f, Pancreas from PBS-treated IL-10-NOD mouse. Note the absence of staining for insulin. g, Pancreas from anti-CD4-treated IL-10-NOD mouse. Note that very few cells in the islets stained positively for insulin (brown-red color). h, Pancreas from anti-CD8 treated IL-10-NOD mouse. Note the presence of intact islet stained positively for insulin (brown-red color). i, Pancreas from IL-10-NOD-scid mouse immunostained for glucagon. Note the intact islets stained positively for glucagon (brown-red color). j, Pancreas from PBS-treated IL-10-NOD mouse immunostained for glucagon. Note the brown staining for glucagon as indicated by the arrow. k, Pancreas from anti-CD4-treated IL-10-NOD mouse immunostained for glucagon. Note the brown staining for glucagon as indicated by the arrow. l, Pancreas from anti-CD8-treated IL-10-NOD mouse immunostained for glucagon. Note the brown staining for glucagon as indicated by arrow.
IL-10-NOD-scid mice (N6 backcross) with TCR tg RAG-2-deficient 8.3-NOD mice expressing an islet-specific monoclonal CD8\(^+\) T cell repertoire (44).

In short, the early participation of IL-10 in the disease process provoked rapid pathogenic autoimmunity (15, 16) and anti-IL-10 mAb treatment abrogated insulitis in young NOD mice (14). In contrast, the later participation of IL-10 in the disease process protected NOD mice from destructive autoimmunity (11–13, 16). These paradoxes evidently relate to the period in which the immune system of the NOD mouse perceives high levels of IL-10. Presumably, as the current study shows, the presence of IL-10 during early stages of IDDM favors the generation of effector CD8\(^+\) T cells leading to acceleration of diabetes, whereas its presence during late stages of IDDM inhibits the generation of pathogenic CD4\(^+\) Th1 cells and concurrently promotes the development of CD4\(^+\) Th2 cells as shown elsewhere (12). Thus, our results demystify the biphasic requirement for the participation of IL-10 in the disease process.

Similarly, other cytokines, such as TNF-\(\alpha\), also exhibited paradoxical effects on IDDM of the NOD mouse. Systemic injection of TNF-\(\alpha\)-accelerated (45) or retarded (46) diabetes in young and adult NOD mice, respectively. Additionally, anti-TNF-\(\alpha\) mAb treatment in young NOD mice prevented diabetes (46). It is possible that the early presence of TNF-\(\alpha\) may induce the generation of islet-specific CD8\(^+\) T cells to accelerate diabetes because TNF-\(\alpha\) modulates the generation of CD8\(^+\) CTLs (47). Conversely, higher levels of TNF-\(\alpha\) in adult NOD mice may abrogate priming of pathogenic CD4\(^+\) Th1 cells via inhibiting the Fc\(\gamma\)RII-dependent presentation of islet Ags by dendritic cells as suggested (48, 49). Therefore, individual cytokines can produce opposing effects depending upon the timing of their participation in the disease process.

Noticeably, IL-10-NOD mice did not respond to several of the treatments tested here. In that context, the failure of CFA or hsp65 treatments to protect IL-10-NOD mice (Fig. 1, A and B) contrasts with their effectiveness in protecting NOD mice from diabetes (20, 21, 25). Two possibilities can be entertained for these apparent discrepancies: 1) the treated IL-10-NOD mice may have developed diabetes before their immune system had an opportunity to shape a hsp65-specific regulatory CD4\(^+\) T cell repertoire of Th2 phenotype; or 2) the results may be related to the differential requirement for CD4\(^+\) T cells in regulating the autoimmune process of NOD and IL-10-NOD mice. In NOD mice, such protection is presumably mediated by regulatory CD4\(^+\) T cells (20), whereas in IL-10-NOD mice such protection may not be possible because of the inability of CD4\(^+\) T cells to mitigate the CD8\(^+\) T cell activity. Consequently, the hsp65-specific T cell reactivity may not participate in the IL-10-induced pathogenic autoimmunity tested here; thus, exactly which islet Ags drive the pathogenic islet-reactive T cells remains a daunting task.

Elimination of a requirement for CD40-CD40L costimulation in the disease process of IL-10-NOD mice is not expected (Fig. 2, A and B). However, further testing of costimulatory pathways (B7/CD82 and ICAM-1/LFA-1) by blocking mAb to B7-1 (n = 5; S/5), B7-2 (n = 9; S/9), B7-1 + B7-2 (n = 7; 6/7), ICAM-1-1 (n = 9; 8/9), and LFA-1 (n = 7; 6/7) molecules also failed to retard diabetes in IL-10-NOD mice (B. Balasa and N. Sarvetnick, unpublished data). Nevertheless, our results directly contrast with earlier findings that the blockade of these pathways with a corresponding mAb effectively abrogated diabetes in NOD mice (19, 50, 51). Consequently, a differential requirement for costimulation in the genesis of diabetes in NOD and IL-10-NOD mice has emerged. We hypothesize that these events may be related to the generation of very high affinity, relatively costimulation-independent effector CD8\(^+\) T cells in IL-10-NOD mice. Identification of MHC class I-restricted T cell epitopes of islet Ags would resolve this issue. In that context, very recent studies have showed that purified MHC class I and peptide complexes activate naive CD8\(^+\) T cells independently of the CD28/B7 and LFA-1/ICAM-1 costimulatory interactions (52). Therefore, several factors, such as the concentration of MHC-peptide complexes, TCR affinity, and the cytokine milieu in the islet environment during pathologic states may dictate the likelihood of bypassing costimulation.

Our findings suggest that there is more than one cellular pathway to \(\beta\) cell destruction, and that under the right (or wrong) circumstances, the microenvironment of the islet may help elicit a rapid and deadly CD8\(^+\) T cell response. Counterregulation of such T cell responses by host balancing factors is much less likely than for CD4\(^+\) T cell responses, thus negating circumvention of the disease. Because CD4\(^+\) T cells and B cells are unnecessary here, the disease process quickens dramatically. That is, the period of damage; release of \(\beta\) cell Ags; Ag capture, processing and presentation; priming of CD4\(^+\) cells; migration back to the islet, and subsequent diversification of the response all take time—in the NOD mouse an interval of several weeks (23, 24, 53, 54).

From the foregoing results, we conclude that IL-10-accelerated diabetes is a private interaction between CD8\(^+\) T cells and \(\beta\) cells as the well-described costimulation pathways and B lymphocytes are dispensable. Our findings shed light on the complex regulatory circuits between cytokines and T cell subsets that lead to spontaneous disease, demonstrating how sensitive this system is to perturbation of the islet microenvironment.

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