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Impaired Activation of Islet-Reactive CD4 T Cells in Pancreatic Lymph Nodes of B Cell-Deficient Nonobese Diabetic Mice

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Despite the impressive protection of B cell-deficient (\(\mu MT^{-/-}\)) nonobese diabetic (NOD) mice from spontaneous diabetes, existence of mild pancreatic islet inflammation in these mice indicates that initial autoimmune targeting of \(\beta\) cells has occurred. Furthermore, \(\mu MT^{-/-}\) NOD mice are shown to harbor a latent repertoire of diabetogenic T cells, as evidenced by their susceptibility to cyclophosphamide-induced diabetes. The quiescence of this pool of islet-reactive T cells may be a consequence of impaired activation of T lymphocytes in B cell-deficient NOD mice. In this regard, in vitro anti-CD3-mediated stimulation demonstrates cyclophosphamide-induced diabetes. The quiescence of this pool of islet-reactive T cells may be a consequence of impaired activation of T lymphocytes in B cell-deficient NOD mice. In this regard, in vitro anti-CD3-mediated stimulation demonstrates impaired activation of lymph node CD4 T cells in \(\mu MT^{-/-}\) NOD mice as compared with that of wild-type counterparts, a deficiency that is correlated with an exaggerated CD4 T cell:APC ratio in lymph nodes of \(\mu MT^{-/-}\) NOD mice. This feature points to an insufficient availability of APC costimulation on a per T cell basis, resulting in impaired CD4 T cell activation in lymph nodes of \(\mu MT^{-/-}\) NOD mice. In accordance with these findings, an islet-reactive CD4 T cell clonotype undergoes suboptimal activation in pancreatic lymph nodes of \(\mu MT^{-/-}\) NOD recipients. Overall, the present study indicates that B cells in the pancreatic lymph node microenvironment are critical in overcoming a checkpoint involving the provision of optimal costimulation to islet-reactive NOD CD4 T cells. The Journal of Immunology, 2001, 167: 4351–4357.

The nonobese diabetic (NOD) mouse is a widely utilized model for study of the immune pathogenesis of type I diabetes mellitus (1), wherein T lymphocytes are the requisite effector population mediating destruction of islet \(\beta\) cells (2, 3). Numerous studies have been focused on identification of the Ag(s) and APC population(s) responsible for the activation of islet-reactive T lymphocytes. Importantly, it has become clear that B cell-deficient NOD mice are protected from spontaneous diabetes, suggesting that B lymphocytes are critical APCs in NOD diabetes (4–7). By virtue of their Ag receptor specificity, B lymphocytes efficiently process and present a high density of antigenic epitopes, making them a likely candidate APC population for presentation of islet autoantigens in vivo (8–15). In this regard, we and others have demonstrated a requirement for B lymphocytes as critical APCs in the development of fulminant insulitis and spontaneous diabetes in NOD mice (16–18).

Given the importance of cognate T/B interaction to the pathogenesis of diabetes, we reasoned that diabetes resistance in B cell-deficient (\(\mu MT^{-/-}\)) NOD mice may be the result of inefficient activation of diabetogenic T cells. The present study demonstrates impaired activation of islet-reactive CD4 T cells in the pancreatic lymph node of \(\mu MT^{-/-}\) NOD mice, suggesting the critical role of B lymphocytes in the lymph node microenvironment for the effective provision of costimulatory signals to diabetogenic T cells.

Materials and Methods

Mice

NOD/LtJ, NOD/scid, NOD.NONThy-1.1, BALB/c, C57BL/6 (B6), and \(\mu MT^{-/-}\) B6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME), BDC2.5 NOD and TCR Cu \(^{+/-}\) NOD mice were generously provided by C. Benoist and D. Mathis (Joslin Diabetes Center, Harvard University, Boston, MA). \(\mu MT^{-/-}\) NOD mice (N10 backcross) were generated as previously described (18). All mice were housed under specific pathogen-free barrier conditions at the University of Pennsylvania; mice on the NOD background were monitored weekly for the development of spontaneous diabetes. There has not been a single \(\mu MT^{+/-}\) NOD mouse that has become diabetic spontaneously in our colony, with some mice being monitored to an age of 50 wk. Blood glucose levels were measured with Accu-Chek Advantage test strips (Boehringer Mannheim, Indianapolis, IN); diabetes was defined as readings of >250 mg/dl on 2 consecutive days.

Histology

From each pancreas, 5–10 pairs of serial sections stained with H&E and aldehyde fuchsin (which stains islet \(\beta\) cells dark blue) were cut at 50-\(\mu\)m intervals (to avoid multiple assessments of the same islet), and examined for the presence of mononuclear cell infiltration. A total of 40–100 islets per animal was graded (in a blinded fashion) as follows: 0 = no inflammation (islet is completely free of mononuclear cell infiltration); 1 = peri-insulitic (poles of mononuclear cell infiltration directly adjacent to or involving <50% of the islet area); 2 = insulitic (>50% of the islet area is disrupted, or completely surrounded, by mononuclear cell infiltrate).

Cyclophosphamide treatment

Mice were injected i.p. with 200 mg/kg cyclophosphamide (CyP; Cytoxan, Mead Johnson, Princeton, NJ) dissolved in PBS. Two weeks following the initial treatment, mice remaining nondiabetic were given a second injection and followed for additional 4 wk. Mice were monitored every 3 days for the development of diabetes, as described above.
Adoptive transfer of diabetes

A total of 20–25 million cells harvested and pooled from spleen and lymph nodes of donor μMT<sup>−/−</sup> NOD mice was injected into the tail vein of recipient TCR α<sup>−/−</sup> NOD mice. Diabetes was monitored by weekly blood glucose measurements for 25 wk after adoptive transfer.

CFSE labeling of lymphocytes

Lymphocytes were labeled with CFSE (Molecular Probes, Eugene, OR), as previously described (19). Briefly, splenocytes or lymph node cells were resuspended at a concentration of 10<sup>6</sup> cells/ml in serum-free IMDM (Life Technologies/BRL, Gaithersburg, MD) at 37°C. An equal volume of a 1/350 dilution of the CFSE stock (5 mM in DMSO) was added. CFSE labeling was quenched by adding an equal volume of heat-inactivated FCS (HI-FCS), whereupon cells were washed twice and resuspended in IMDM containing 10% HI-FCS.

In vitro T cell stimulations

CFSE-labeled splenocytes or pooled lymph node cells (inguinal, axillary, and cervical) were plated in 24-well plates at a density of 1 × 10<sup>6</sup> total cells in 1 ml media containing 10% HI-FCS with the designated amount of anti-CD3 (145-2C11) and 2 μg/ml anti-CD28 (37.51) mAbs. Maximal division occurred upon activation with a dose of 2 μg/ml anti-CD3 mAb. All cells were incubated for 65–70 h at 37°C in 7% CO2. After incubation, the cultured cells were harvested and stained with allophycocyanin-conjugated anti-CD4 (RM-4; BD Pharmingen, Torrey Pines, CA) to allow the identification of CFSE-labeled CD4 T cells using flow cytometry. Ten thousand CD<sup>+</sup> events were collected within a live cell gate, which included blasts and cells, as determined by forward and side scatter.

Flow cytometry

A total of 1 × 10<sup>6</sup> cells was surface stained in 96-well plates with different mAbs: 53-6.7 FITC (anti-CD8α), M1/70 PE (anti-CD11b), RA3-6B2 biotin (anti-CD45R/B220), RM-4 allophycocyanin (anti-CD4), 10-3.6 (anti-I<sub>A</sub><sup>+</sup>), AF6-120.1 PE (anti-I<sub>A</sub><sup>+</sup>), KT4 biotin (anti-V<sub>B</sub>4 TCR), OX-7 PE (anti-CD90.1/Thy-1.1), 30-H12 biotin (anti-CD90.2/Thy-1.2) (BD Pharmingen). Biotin-conjugated mAbs were subsequently stained with streptavidin-RED670 (Life Technologies). All samples were analyzed on a FACScalibur flow cytometer (Becton Dickinson, Mountain View, CA) using CellQuest software. Subsequent analysis was performed using FlowJo software.

Analysis of cell division

Gates for each division peak were set using live gated cells; thereafter, live and dead cells were included in analysis, as previously described (20, 21), based on the property of CFSE-labeled cells to lose half of their fluorescence intensity with each round of division. Briefly, cell counts for each division peak, undivided cells (peak 0), and total gated CD4<sup>+</sup> cells were determined using FlowJo (http://www.flowjo.com) and exported into Microsoft Excel (http://www.microsoft.com) for analysis. The number of precursor cells that gave rise to daughter cells in each peak was determined by multiplying the normalized number of cells in a given peak, “N,” by the factor 1/2<sup>n</sup>, in which “n” is the division peak number. The total number of mitotic events that gave rise to the resulting division profile could then be closely approximated using the formula: Σ(N(2<sup>-n</sup> − 1)<sup>n</sup>). Precursor frequency calculations were performed to determine the percentage of cells from the original undivided pool that were recruited into the dividing pool (total number of dividing precursors/total number of precursor cells). Dose-response analysis was performed by setting as 100% mitosis, for each individual animal, the number of mitotic events achieved with the maximal dose of anti-CD3.

Selective depletion of lymphocyte populations

Specific depletion of lymphocyte populations was accomplished by negative selection of splenocytes or lymph node cells via MACS. Enriched populations of T cells were prepared by depleting with anti-B220 biotin (RA3-6B2) and anti-CD11b biotin (M1/70) mAbs, followed by streptavidin-conjugated MACS beads, which were then passed through columns using the VarioMACS system (Miltenyi Biotec, Sunnyvale, CA). All depletions yielded >95% efficiency in negative selection of the targeted population, as determined by flow cytometry.

In vivo tracking of islet-reactive CD4<sup>+</sup> T cell division

Splenocytes and lymph node cells were isolated from NOD (or Thy-1.1 NOD congenic) BDC2.5 TCR transgenic mice and enriched for T cells before CFSE labeling, as described above. A total of 10–20 × 10<sup>6</sup> of these CFSE-labeled T cells was injected by tail vein into wild-type (μMT<sup>−/−</sup> or μMT<sup>+/−</sup>) and μMT<sup>−/−</sup> NOD mice. For BDC2.5 T cell transfers, optimal activation has been shown to occur at about 85–90 h (22), and so recipient cells were harvested at this time point from spleen, nonpancreatic lymph nodes (pooled inguinal, axillary, and cervical), and pancreatic lymph nodes (typically three lymph nodes draining the pancreas were harvested using a dissecting microscope). Single cell suspensions were prepared and stained with anti-Thy-1.1 PE (OX-7) and/or anti-TCR V<sub>B</sub>4-bio (the BDC2.5 transgenic utilizes V<sub>B</sub>4 TCR) and anti-CD4 APC (RM-4.5) to allow for the identification of the transferred CD4<sup>+</sup> T cells using flow cytometry, as described above. A total of 5,000–10,000 CFSE<sup>+</sup>CD4<sup>+</sup>/V<sub>B</sub>4<sup>+</sup> or CD4<sup>+</sup>/Thy-1.1<sup>+</sup> events was collected within a live lymphoid gate including blasts and cells, as determined by forward and side scatter. Division history was subsequently analyzed, as described above. The normalized number of mitoses occurring in wild-type recipients was set as 100% mitotic activity for each experiment. Extent of division achieved by transferred cells in experimental animals was then calculated as a percentage of wild-type mitotic activity and averaged for five separate experiments.

Results and Discussion

Pancreatic islets of B cell-deficient NOD mice exhibit mild mononuclear cell infiltration

It is not clear whether protection of μMT<sup>−/−</sup> NOD mice from spontaneous diabetes is a result of arrested initial targeting of islet β cells or impaired progression of the anti-islet T cell response. Thus, to determine whether islet β cell targeting occurs in the absence of B lymphocytes, we undertook a systematic histological analysis of pancreata from μMT<sup>−/−</sup> NOD mice. We examined pancreata from a large cohort of μMT<sup>−/−</sup> NOD mice that had remained free from diabetes to a late age (n = 19, 30–50 wk old). In this cohort of animals, a total of 1492 individual islets was scored for the presence of islet inflammation. While 73% of these islets in aggregate were free of mononuclear cell infiltration, pancreata from all mice examined exhibited some degree of insulitis (Table I). There was wide variation in the percentage of islets affected among individual animals (mean = 25.8%; range = 2.8–66.7%). We next analyzed 396 islets from a cohort of younger μMT<sup>−/−</sup> NOD mice (n = 6, 10–12 wk old). In aggregate, 94% of these islets were free of infiltration. In contrast to the older mice, some younger mice were completely free of islets (mean = 6.1%; range = 0–10.8%). Overall, islet inflammation was significantly more prevalent in older than in younger μMT<sup>−/−</sup> NOD mice (p value = 0.01, Table I). The typical lesion in μMT<sup>−/−</sup> NOD mice consisted of a pole of mononuclear cells at the periphery of the islet (Fig. 1, A and B); only rarely (4.1% of islets in older mice) was the islet inflammation extensive enough to disrupt islet architecture (Fig. 1, C and D). As expected, the majority of islets in all control B cell-sufficient littermates (72% in older mice; 61% in younger mice) exhibited insulitis (Fig. 1, E and F). Although the islet inflammation seen in B cell-deficient NOD mice was of a benign nature, its existence indicated that the β cells in these mice were targeted by the immune system. However, it appears that in the absence of B lymphocytes, the anti-islet T cell response is not sufficiently activated to mediate diabetes. The nondestructive targeting of islets attests to a suboptimal capacity of non-B cell APCs (macrophages and dendritic cells) to activate islet-reactive T cells in B cell-deficient NOD mice.

Susceptibility of B cell-deficient NOD mice to CyP-induced diabetes

Since islet β cells in μMT<sup>−/−</sup> NOD mice demonstrated evidence of mild insulitis, we sought to determine whether a latent potential for progression to diabetes exists in these mice. CyP has been used to accelerate the onset of diabetes in NOD mice and is considered a reliable agent to convert a nonprogressive insulitis to a destructive state (23–27). Therefore, cohorts of μMT<sup>−/−</sup> and littermate
control (μMT+/+ or μMT+−/−) NOD mice that had reached an age of 30–50 wk without becoming diabetic were treated with CyP and followed for the development of diabetes. We also treated a cohort of younger (10- to 20-wk-old) μMT+/− (and control μMT+/+ or μMT+−/−) NOD mice to determine the functional significance of the mild islet inflammation seen in these mice. As expected, a majority of B cell-sufficient control NOD mice became diabetic within 4 wk of CyP treatment. Intriguingly, CyP also induced diabetes in a proportion of older μMT+−/− NOD mice (Table II). This finding demonstrates that B cell-deficient NOD mice indeed retain a latent potential for progression to diabetes. However, in line with their significantly milder degree of islet infiltration, younger μMT+−/− NOD mice were completely resistant to CyP-induced diabetes. Of note, the proportion of μMT+−/− NOD mice susceptible to CyP-induced diabetes (2/27) was comparable with the proportion of μMT+−/− NOD mice having >50% of their islets inflamed (3/25; Table I). The susceptibility of μMT+−/− NOD mice to CyP-induced diabetes suggested the existence of islet-reactive specificities in the peripheral immune repertoire of these mice. We confirmed that such islet-reactive specificities exist by performing adoptive transfer of μMT+−/− NOD lymphocytes into T cell-deficient TCRCα−/− NOD mice; two of three recipients subsequently

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**FIGURE 1.** Islet histology of B cell-deficient NOD mice. Serial pancreatic sections were stained with H&E (A, C, and E) and aldehyde fuchsins (B, D, and F). μMT+−/− NOD mice were consistently found to have mild islet inflammation (A and B) that was rarely invasive (C and D). Sections from control B cell-sufficient (μMT+/+) NOD mice (E and F) show typical destructive insulitis.

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**Table 1.** Islet histology in B cell-deficient NOD mice**

<table>
<thead>
<tr>
<th></th>
<th>% of Islets in Aggregate with Indicated Grade (total no. of islets)</th>
<th>% of Islets Inflamed/Mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>Peri-</td>
</tr>
<tr>
<td>NOD μMT+−/−</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30–50 wk (n = 19)</td>
<td>73.2 (1093)</td>
<td>22.7 (338)</td>
</tr>
<tr>
<td>10–12 wk (n = 6)</td>
<td>94.2 (373)</td>
<td>5.3 (21)</td>
</tr>
<tr>
<td>NOD μMT+−/− or +−/−</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30–50 wk (n = 4)</td>
<td>27.5 (14)</td>
<td>15.0 (6)</td>
</tr>
<tr>
<td>10–12 wk (n = 5)</td>
<td>39.0 (167)</td>
<td>22.8 (94)</td>
</tr>
</tbody>
</table>

*Normal, islets free of mononuclear cell inflammation; Peri-, mononuclear cell inflammation present immediately adjacent to, or invading into islets (as in Fig. 1, A and B); Invasive, mononuclear cell infiltrate completely surrounding islet, or invading into >50% of islet area (as in Fig. 1, C and D).

†Total number of islets is shown in parentheses.

‡Data are presented as the mean and range (in parentheses), for each group of mice, of the percentage of islets in each mouse that has either peri- or invasive inflammation. Statistical comparison was performed using an unpaired, two-tailed Student *t* test.

§Because no gender-specific differences were noted, equivalent proportions of males and females were included in all groups.

¶Statistical comparison of these two groups yielded *p* < 0.05.
became diabetic within 15 wk. These findings are in agreement with a recent study demonstrating the development of diabetes in NOD/scid mice after similar transfers of μMT−/− NOD T cells were performed (28).

Table II. CyP-induced diabetes in B cell-deficient NOD mice

<table>
<thead>
<tr>
<th>Mice</th>
<th>Fraction Diabetic</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10–20 wk</td>
</tr>
<tr>
<td>μMT−/− NOD</td>
<td>0/11</td>
</tr>
<tr>
<td>μMT+/− NOD</td>
<td>11/14</td>
</tr>
</tbody>
</table>

*Mice were injected with 200 mg/kg CyP and monitored for diabetes. Mice remaining euglycemic by day 14 after injection received a second treatment and were followed for 28 days more. Because no gender-specific differences were noted, equivalent proportions of males and females were included in all groups.

*b Age at beginning of treatment.

Impaired activation of μMT−/− NOD lymph node CD4 T cells in vitro

The existence of islet inflammation in the pancreata of μMT−/− NOD mice indicated early targeting of β cells by islet-reactive T cells. However, this population had failed to become sufficiently activated to cause spontaneous diabetes. A logical explanation for this failure is that NOD non-B cell APCs might be impaired in their ability to support the efficient activation of islet-reactive T lymphocytes. We have previously shown that in response to TCR/CD3-mediated stimulation, NOD CD4 T cells exhibit an impaired division capacity compared with nonautoimmune strain mice. Specifically, when CFSE-labeled NOD splenocytes were stimulated by anti-CD3/CD28 mAbs, CD4 T cells exhibited division arrest and failed to generate daughter cells in as advanced division peaks as those achieved by their nonautoimmune counterparts (20). Interestingly, when splenocytes were depleted of B cells in vitro, CD4 T cells failed to initiate division, even upon maximal stimulation by anti-CD3/CD28. This finding indicated that the observed CD4 T cell activation defect resided in the inability of NOD non-B cell APCs to provide optimal costimulatory signals. We therefore sought to determine whether the protection from diabetes seen in μMT−/− mice resulted from disruption of CD4 T cell division as a consequence of the reduced costimulatory capacity of NOD non-B cell APCs. Surprisingly, when CFSE-labeled μMT−/− NOD splenocytes were cultured with a maximally stimulatory dose of anti-CD3/CD28, CD4 T cells exhibited a division profile that was similar to wild-type NOD mice (Fig. 2A). Specifically, the division profile of both wild-type and μMT−/− NOD splenic CD4 T cells revealed a majority of daughter cells in divisions 1–4 and

**FIGURE 2.** Division of CD4 T cells in response to TCR/CD3-mediated stimulation of splenocytes in vitro. A, CFSE-labeled splenocytes from wild-type and μMT−/− NOD and C57BL/6 mice were cultured with 2 μg/ml anti-CD3 and 2 μg/ml anti-CD28 (——) or medium alone (———) for 70 h. B, Percentage of the maximal degree of mitosis achieved at different doses of anti-CD3 mAb. The average and SD at each dose are shown for NOD wild type (○), NOD μMT−/− (○), B6 wild type (●), and B6 μMT−/− (□) in three separate experiments.

**FIGURE 3.** Division of lymph node CD4 T cells in response to TCR/CD3-mediated stimulation in vitro. Unfractionated lymph node cells (pooled inguinal, axillary, and cervical) from NOD wild-type, NOD μMT−/−, B6 wild-type, and B6 μMT−/− mice were CFSE labeled and stimulated with either 2 μg/ml anti-CD3 and 2 μg/ml anti-CD28 (——) or medium alone (———) for 70 h. Data are representative of three separate experiments.
a comparable dose responsiveness to anti-CD3/CD28 (Fig. 2B). Similariy, a lack of B cells in the spleen of nonautoimmune B6 mice appeared to have a minimal impact on CD4 T cell mitotic activity, with μMT−/− B6 splenocytes demonstrating a dose responsiveness similar to wild-type counterpart B6 mice (Fig. 2B).

We considered the possibility that while the lack of B cells does not impact upon T cell division in the μMT−/− NOD spleen, B cells may be critical for optimal T cell activation in the lymph node microenvironment. In fact, previous studies have indicated that diabetogenic T cell activation occurs in pancreatic lymph nodes (22, 26, 29). The division profiles of CFSE-labeled lymph node cells from μMT−/− and wild-type mice were compared in response to anti-CD3/CD28-mediated stimulation in vitro. We found a striking impairment in the maximally stimulated proliferative profile of lymph node CD4 T cells from μMT−/− NOD mice compared with wild-type counterparts (Fig. 3). Using these maximally stimulated division profiles, the frequency of CD4 T cells in the starting pool that were recruited to divide was calculated. This analysis revealed that, compared with wild-type counterparts, very few lymph node CD4 T cells from μMT−/− NOD mice entered the division pool following 70 h of maximal stimulation by anti-CD3/CD28 (5.9 ± 1% for μMT−/− vs 50.5 ± 6% for wild type). Importantly, this degree of impairment was not observed in lymph node CD4 T cells from μMT−/− B6 mice (Fig. 3), indicating sufficient availability of non-B cell APC-mediated costimulation in these nonautoimmune mice. It is well established that T cell proliferation in response to soluble anti-CD3/CD28 mAb stimulation is an APC-dependent process, and that T cell:APC ratio is an important parameter influencing the degree of proliferation. Therefore, the suboptimal proliferative response of CD4 T cells from

FIGURE 4. Division of islet-reactive T cells in pancreatic lymph nodes. Purified T cells from BDC2.5 Thy-1.1 congenic NOD mice were CFSE labeled and injected into B cell-sufficient (μMT−/−) NOD (left) or μMT−/− NOD (right) recipients. Pancreatic (A) or nonpancreatic (B) lymph node cells were harvested 90 h later and analyzed by flow cytometry. Thy-1.1 vs CFSE profiles of live CD4+ Vβ4+ events are shown. Data are representative of five separate experiments.

Table III. Lymphoid and myeloid composition of spleen and lymph nodes

<table>
<thead>
<tr>
<th></th>
<th>CD11b</th>
<th>B220</th>
<th>CD4</th>
<th>CD8</th>
<th>CD4:APC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spleen</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NOD</td>
<td>4.6±1.1</td>
<td>52.6±8.8</td>
<td>26.5±4.8</td>
<td>8.1±2.5</td>
<td>0.5</td>
</tr>
<tr>
<td>B6</td>
<td>4.5±1.3</td>
<td>58.9±7.6</td>
<td>16.3±3.1</td>
<td>10.6±2.7</td>
<td>0.3</td>
</tr>
<tr>
<td>NOD μMT−/−</td>
<td>11.9±3.8</td>
<td>3.9±2.0</td>
<td>54.5±6.3</td>
<td>17.0±3.8</td>
<td>3.5</td>
</tr>
<tr>
<td>B6 μMT−/−</td>
<td>10.0±2.1</td>
<td>5.6±2.3</td>
<td>45.5±8.2</td>
<td>26.8±5.1</td>
<td>2.9</td>
</tr>
<tr>
<td>Lymph node</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NOD</td>
<td>1.4±0.2</td>
<td>34.0±8.9</td>
<td>46.5±8.1</td>
<td>15.9±2.1</td>
<td>1.4</td>
</tr>
<tr>
<td>B6</td>
<td>2.1±0.2</td>
<td>45.9±8.6</td>
<td>24.6±4.6</td>
<td>24.5±5.3</td>
<td>0.5</td>
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<tr>
<td>NOD μMT−/−</td>
<td>0.8±0.3</td>
<td>0.7±0.2</td>
<td>76.4±0.4</td>
<td>21.6±1.4</td>
<td>51.9</td>
</tr>
<tr>
<td>B6 μMT−/−</td>
<td>1.2±0.3</td>
<td>2.0±0.1</td>
<td>54.1±8.9</td>
<td>40.8±6.4</td>
<td>16.9</td>
</tr>
</tbody>
</table>

*a* Data are shown as percentages of total live lymphoid/myeloid-gated cells analyzed by four-color flow cytometry in four separate experiments.

*b* Ratio is calculated as (CD4+ cells):(CD11b+ cells + B220+ cells).
lymph nodes of $\mu$MT$^{-/-}$ NOD mice may be attributable to a perturbation in the ratio of CD4 T cell:non-B cell APC. Importantly, compared with lymph nodes from wild-type NOD mice, the $\mu$MT$^{-/-}$ NOD lymph node contained a markedly greater CD4:APC ratio (1.4 for wild-type vs 51.9 for $\mu$MT$^{-/-}$; Table III). The skewed ratio of CD4:APC in $\mu$MT$^{-/-}$ NOD lymph nodes suggests that the impaired CD4 T cell activation profile may be a reflection of a reduced availability of APC costimulatory input on a per CD4 T cell basis. Interestingly, our data demonstrating a pronounced alteration in CD4:APC ratio may help to explain previously reported defects in the ability of CD4 T cells to support immune responses in B cell-deficient mice (30). By extension, an inability of islet-reactive CD4 T cells to become optimally activated in the pancreatic lymph nodes of $\mu$MT$^{-/-}$ NOD mice may result in their protection from spontaneous diabetes. In the next section, we directly assess this possibility in vivo.

Impaired activation of islet-reactive CD4 T cells in pancreatic lymph nodes of $\mu$MT$^{-/-}$ NOD mice

The BDC2.5 TCR transgene encodes an islet-specific CD4 T cell clonotype that has been shown to be preferentially activated in pancreatic lymph nodes (31). Following transfer to NOD mice, CFSE-labeled BDC2.5 CD4 T cells preferentially underwent several rounds of division upon homing to pancreatic lymph nodes, but not in other secondary lymphoid organs (22). We utilized this unique strategy to track the division history of adoptively transferred CFSE-labeled BDC2.5 T cells in both $\mu$MT$^{-/-}$ and wild-type NOD recipients. By day 4 following adoptive transfer, BDC2.5 CD4 T cells underwent up to eight rounds of division in the pancreatic lymph nodes of control B cell-sufficient NOD recipient mice (Fig. 4A). In the experimental group, we analyzed the division profile of adoptively transferred CFSE-labeled BDC2.5 T cells in pancreatic lymph nodes of $\mu$MT$^{-/-}$ NOD recipients. We standardized the extent of division in $\mu$MT$^{-/-}$ NOD mice by using the level of mitotic activity in wild-type mice in each experiment as the maximal level of division. We were therefore able to determine the degree to which the division potential of islet-reactive CD4 T cells was attained in the absence of B cells. This analysis revealed that, on the average, BDC2.5 CD4 T cells transferred into $\mu$MT$^{-/-}$ NOD recipients achieved approximately one-half (49.3%; $n = 9; p = 0.007$) of the maximal division capacity seen in B cell-sufficient controls (Fig. 4A). Importantly, transferred islet Ag-specific BDC2.5 cells failed to undergo division in either spleen or nonpancreatic lymph nodes (Fig. 4B). These findings directly demonstrate a significant impairment in the ability of B cell-deficient NOD pancreatic lymph nodes to support optimal activation of a bona fide islet-reactive CD4 T cell clonotype.

Previous studies have indicated that the NOD lymph node microenvironment may be a site of effective induction of tolerance in diabetogenic T cells (32–34). Lymph node dendritic cells, in particular those isolated from lymph nodes draining the pancreas, were shown to prevent development of diabetes when inoculated in the footpad of NOD mice, suggesting their capacity to induce tolerance in the islet-reactive T cell repertoire. Therefore, it is plausible that the reduced proliferation of islet-reactive T cells in the $\mu$MT$^{-/-}$ NOD pancreatic lymph nodes is related to the tolerogenic capacity of resident dendritic cells. In this context, the pancreatic lymph nodes of $\mu$MT$^{-/-}$ NOD mice may act as a privileged regional microenvironment that guards against the recruitment of sufficient islet-reactive T cells to effect $\beta$ cell destruction.

Overall, the above data demonstrate an impairment of CD4 T cell activation in lymph nodes of B cell-deficient NOD mice that may result from suboptimal delivery of costimulation from the non-B cell APC compartment of NOD mice. The present study demonstrates that although B cell-deficient NOD mice harbor anti-islet T cells, they fail in becoming activated to a level required for islet $\beta$ cell destruction. Indeed, we directly demonstrate deficient activation of islet-reactive CD4 T cells in the absence of B lymphocytes. Based on these results, we suggest that the indispensable role of B cells in NOD diabetesgenesis is their necessity for optimal activation of an islet-specific T cell response in the pancreatic lymph node microenvironment.

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References


