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Regulation of Diabetes Development by Regulatory T Cells in Pancreatic Islet Antigen-Specific TCR Transgenic Nonobese Diabetic Mice

Osami Kanagawa, Ana Militech, and Barbala A. Vaupel

Nonobese diabetic (NOD) mice carrying a transgenic TCR from an islet Ag-specific CD4 T cell clone, BDC2.5, do not develop diabetes. In contrast, the same transgenic NOD mice on the SCID background develop diabetes within 4 wk after birth. Using a newly developed mAb specific for the BDC2.5 TCR, we examined the interaction between diabetogenic T cells and regulatory T cells in NOD.BDC transgenic mice. CD4 T cells from NOD.BDC mice, expressing high levels of the clonotype, transfer diabetes to NOD.SCID recipients. In contrast, CD4 T cells expressing low levels due to the expression of both transgenic and endogenous TCR α-chains inhibit diabetes transfer. The clonotype-low CD4 T cells appear late in the ontogeny in the thymus and peripheral lymphoid organs, coinciding with resistance to cyclophosphamide-induced diabetes. These results demonstrate that diabetic processes in NOD.BDC mice are regulated by a balance between diabetogenic T cells and regulatory T cells. In the absence of specific manipulation, regulatory T cell function seems to be dominant and mice remain diabetes free. Understanding of mechanisms by which regulatory T cells inhibit diabetogenic processes would provide means to prevent diabetes development in high-risk human populations. The Journal of Immunology, 2002, 168: 6159–6164.

Insulin-dependent diabetes mellitus (IDDM) is caused by an autoimmune destruction of pancreatic β cells (1). The nonobese diabetic (NOD) mouse develops spontaneous IDDM that shares many of the key features of the human disease (2) and is an excellent small animal model to dissect the pathogenesis of IDDM. The disease is primarily mediated by T cells recognizing pancreatic β cell Ags (3–5). However, in the initial phase of the disease, macrophages, B cells, and T cells infiltrate the pancreatic islets without destroying the β cells (2). After a gradual increase in the cellular infiltrate, termed insulitis, the progressive destruction of β cells takes place, leading to a complete loss of insulin production and dysregulation of glucose metabolism. Although the course of human IDDM development cannot be studied systematically, there are ample observations suggesting that similar progression of immune destruction of pancreatic β cells takes place in human IDDM (1).

Susceptibility to the development of IDDM has a strong linkage to certain class II MHC genes both in humans and mouse (6, 7). However, environmental factors also play an important role in the development of IDDM. This is most clearly evident from the concordance of disease development in identical twin studies (8), which is <50%. Although there are no known factors for the regulation of diabetes development in humans, numerous factors that include infection with bacteria and virus and external manipulations, such as immunization with CFA and cyclophosphamide (CY) injection, influence diabetes development in NOD mice (9, 10).

Recently, it has been suggested that T cells bearing a unique surface marker and/or expressing a unique surface receptor play an important role in down-regulating autoimmune responses (11–13). These are either CD25+ CD4+ regulatory T cells (11) or nonpoly- morphic TCR (Vα14 for mouse and Vα24 for humans) positive NK T cells (13, 14). Both cell types, under certain experimental conditions, are capable of regulating a diabetogenic process (13, 15). However, the role of these T cells in the natural course of diabetes development as well as in the protection of diabetes by external manipulations remains unclear. Studies of the precise cell-cell interactions and the effect of environmental factors during diabetes development/protection has been difficult due to the heterogeneity of the cell types present in vivo.

Transgenic mice carrying TCR genes from an islet Ag-specific CD4 T cell clone (BDC2.5) were established by Mathis and Benoist in the B6 × SJL F2 background (16) and backcrossed to NOD mice (from here on referred to as NOD.BDC mice). In the early stages of backcrossing, the transgene-positive mice showed high penetrance and an accelerated development of diabetes (16). However, after extensive backcrossing to NOD mice, the incidence of diabetes in NOD.BDC transgenic mice decreased drastically. Mathis and Benoist’s group demonstrated that genetic elements derived from B6 mice were responsible for the early development of diabetes in BDC TCR transgenic mice (17). T cells bearing BDC 2.5 TCR were still diabetogenic, since mice carrying a monoclonal T cell population (NOD.BDC mice on the NOD.SCID background) developed diabetes very rapidly (18). Furthermore, several external factors, including CY, induced diabetes in NOD.BDC mice more rapidly than in normal NOD mice (19). The diabetogenic T cells seem to be suppressed by cells present in NOD.BDC but not in NOD.BDC.SCID mice. Thus, the NOD.BDC mouse serves as an excellent model to examine the interactions between defined diabetogenic T cells and other cell types for the regulation of diabetes development in vivo.
The major obstacle in examining the cellular interaction in normal NOD.BDC mice has been the lack of an appropriate reagent that can distinguish diabetogenic BDC2.5 TCR-bearing T cells from other T cells. To overcome this problem, we generated a B cell hybridoma that produces an Ab specific to the BDC2.5 TCR. Using this mAb, we examined the interactions between diabetogenic T cells and regulatory T cells in NOD.BDC mice.

Materials and Methods

Mice

The BDC 2.5 TCR transgenic mice on the NOD background (NOD.BDC, 13 generations backcrossed to NOD) and NOD.SCID background (NOD.BDC.SCID) were established in our mouse colony at Washington University Medical School. Normal NOD and NOD.SCID mice were obtained from The Jackson Laboratory (Bar Harbor, ME) and maintained in our mouse colony at Washington University Medical School.

Production of hybridoma-producing anti-TCR Ab

A hybridoma cell line producing Ab specific for the BDC2.5 TCR was established by the method described previously (20). Briefly, BALB/c mice were immunized with BDC2.5 T cell line (1 x 10^7) i.p. four times at 2-wk intervals. Four weeks after the last immunization, mice were boosted with the same number of T cells i.v. and 3 days later the spleen cells were fused to a myeloma cell line. Hybridoma supernatants were tested for the capacity to stimulate BDC 2.5 T cells to produce IL-3. One hybridoma cell line, aBDC, producing an IgG2a Ab was established and cloned twice under limiting dilution conditions. The Ab was purified and biotinylated for the experiments.

Immunofluorescence analysis

Surface immunofluorescence analysis of thymocytes, lymph node cells, and T cell clones was performed as described previously (21). In brief, T cell clones were incubated with abDC Ab for 30 min at 4°C, washed, and reincubated with FITC-conjugated goat anti-mouse IgG Ab. For two-color surface immunofluorescence, cells were incubated with rat Ab specific for Thy1 (AT83), CD4 (GK1.5), or CD8 (3.155) for 30 min at 4°C, followed by incubation with FITC-conjugated goat anti-rat IgG. The cells were counterstained with none, biotinylated anti-Vß4 Ab (KT4), or biotinylated aBDC Ab followed by avidin-PE. For three-color staining, cells were incubated with PE-anti-CD4, FITC-anti-CD8, and biotin-labeled aBDC Ab for 30 min at 4°C, washed, and further incubated with streptavidin-RED613 (Life Technologies, Rockville, MD). For the analysis of CD25 expression, cells were stained with FITC-anti-CD4, PE-anti-CD25 (7D4), and biotinylated aBDC Ab followed by streptavidin-RED613. Stained cells were analyzed by a FACS-Calibur analyzer using the CellQuest program (BD Biosciences, Mountain View, CA). PE- or FITC-labeled anti-CD4, FITC-labeled anti-CD8, and PE-labeled anti-CD25 Abs were purchased from BD Pharmingen (San Diego, CA). Other Abs were purified and biotinylated in our laboratory.

T cell proliferation assay

Lymph node cells were treated with anti-CD8 Ab (3.155; rat IgM Ab) plus complement. Aliquots of cells were stained with biotinylated anti-CD4 Ab followed by avidin-PE. CD4-positive T cell clones were sorted based on their clonotype expression using a FACSVantage sorter (BD Biosciences). Pancreatic ß cells were prepared from B10.BR mice by the methods described previously. Cells (2.5 x 10^7) irradiated NOD spleen cells and indated NOD mouse (20 wk old) lung cells were culutred with irradiated (2000 rad) pancreatic ß cells in a final volume of 200 ml 5% FCS/DMEM in flat-bottom microtiter plates. After 72 h of incubation with a 6-h pulse with [3H]thymidine, cultures were harvested for counting.

CY-induced diabetes

Mice received 200 mg/kg CY on days 0 and 14 and were monitored for the development of diabetes. Mice with high blood glucose on day 14 did not receive a second CY injection.

Cell transfer

Transfer of diabetes by spleen cells was conducted by the method described previously. In brief, NOD.SCID mice receive either nonfractionated spleen cells, cells sorted based on their surface phenotype, or a mixture of two different populations of cells. For an enrichment of cell populations based on their expression of CD4 and clonotype, cells were stained with anti-CD4 Ab and aBDC Ab as described above and sorted for the clonotype-high and clonotype-intermediate CD4-positive populations using a FACSVantage cell sorter. Mice were monitored for the development of diabetes for 5 wk.

Diabetes

Mice were monitored for the development of diabetes by measuring urine glucose with Diastix strips (Miles, Madison, WI) twice a week. Mice with glucosuria were tested for the blood glucose level and those showing >250 mg/dl of blood glucose two consecutive reading in a week were considered diabetic.

Results

Specificity of the aBDC Ab

The aBDC Ab only reacted with BDC 2.5 T cells but not with six other independent Ag-specific CD4 T cell clones derived from NOD mice (a representative result with an OVA-specific T cell clone is shown in Fig. 1A). Lymph node cells from NOD.BDC mice and control NOD mice were stained with anti-Thy1 Ab and counterstained with either anti-Vß4 Ab or aBDC Ab. As shown in Fig. 1B, T cells from NOD.BDC mice were stained uniformly with anti-Vß4 Ab, while only a fraction of T cells from normal NOD mice were positive with the same Ab. Staining of lymph node T cells from NOD.BDC mice with aBDC Ab was heterogeneous with high, low, and no staining populations. There was no aBDC Ab staining of T cells from normal NOD mice. Thus, aBDC Ab shows all of the characteristics of an anti-idiotypic Ab for BDC2.5 TCR.

Age-dependent phenotypical and functional change of T cells from NOD.BDC mice

Using aBDC Ab, the expression of BDC2.5 TCR was examined in both CD4 and CD8 single-positive T cell populations from young mice.
(8-wk-old) and old (25-wk-old) NOD.BDC mice. As shown in Fig. 2A, the T cell population from young NOD.BDC mice contains predominantly CD4-positive cells (41% CD4 and 7% CD8). Total number as well as percentage of CD8-positive T cells increased in old NOD.BDC mice (32% CD4 and 17% CD8). CD4 T cells from both young and old mice were stained uniformly with anti-V_{y4} Ab. However, staining with aBDC Ab showed age-dependent changes for the presence of clonotype-low and -negative populations (Fig. 2B). In young mice, very few T cells stained weakly with aBDC Ab, while there were significant numbers of clonotype-negative and -low populations of CD4-positive T cells from old mice. In contrast, CD8 T cells from both young and old mice stained uniformly with anti-V_{β4} and aBDC Abs. However, staining intensity with anti-V_{β4} Ab was significantly higher than that with aBDC Ab, similar to clonotype-low CD4 T cell populations in old mice. These clonotype-low T cells (both CD4 and CD8 populations) are very likely expressing both transgene-derived and endogenous TCR α-chains that associate with the transgenic β-chain.

It has been shown that BDC TCR transgenic mice on NOD background do not develop diabetes spontaneously; however, diabetes can be induced by a single injection of CY. When young (4- to 8-wk-old) and old (>25-wk-old) NOD.BDC mice were tested for the development of CY-induced diabetes, young mice became diabetic within 10 days after treatment, while old mice remained normoglycemic even after two injections of CY (Table I). All control NOD mice (12 wk old) developed diabetes after a second CY treatment. Thus, old NOD.BDC mice were more resistant to CY-induced diabetes than young NOD.BDC or normal NOD mice.

CD4-positive T cells from young and old NOD.BDC mice were tested for their reactivity to islet Ag in vitro. Lymph node cells from old mice exhibited significantly lower proliferative responses than those from young mice in vitro (Fig. 3). However idiotype-high CD4 T cells from old mice exhibited strong proliferation. In contrast, idiotype-intermediate CD4 T cell showed no proliferative response but exhibited a strong inhibitory effect on the proliferation of idiotype-high CD4 T cells (Fig. 3). Taken together, these results demonstrate a correlation between the appearance of clonotype-low/negative CD4 T cells, decreased islet Ag reactivity, and resistance to CY-induced diabetes development.

**FIGURE 2.** Age-dependent change in T cells expressing idiotypic determinant in BDC.NOD mice. A, Lymph node cells from old (25-wk-old) and young (8-wk-old) NOD.BDC mice were stained with FITC-conjugated anti-CD8 Ab and PE-conjugated anti-CD4 Ab. B, The same lymph node cells were stained with either anti-CD4 or anti-CD8 Ab followed by FITC-conjugated anti-rat Ab. Samples were counterstained with none (solid line), biotinylated anti-V_{β4} Ab (bold line) or biotinylated aBDC Ab (dotted line) followed by avidin-PE. Samples were analyzed by the methods described in Fig. 1 legend.

<table>
<thead>
<tr>
<th>Mice</th>
<th>Day 14</th>
<th>Day 28</th>
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<tr>
<td>NOD.BDC (4–8 wk old)</td>
<td>10/10</td>
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<tr>
<td>NOD.BDC (&gt;25 wk old)</td>
<td>0/8</td>
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<tr>
<td>NOD (12 wk old)</td>
<td>2/5</td>
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*a Mice received 200 mg/kg CY (in 0.5 ml PBS) on days 0 and 14 i.p. and were monitored for the development of diabetes.

*b Number of diabetic mice/number of mice in the group.
Regulation of diabetes transfer by clonotype-low CD4 T cells

To test directly the function of clonotype-low CD4 T cells for the inhibition of diabetes development, we used a cell transfer model. As shown in Table II, total spleen cells from NOD.BDC (25-wk-old) mice did not inhibit diabetes efficiently (only 1 of 10 recipients became diabetic), whereas as few as 5 \times 10^5 sorted CD4 T cells with high expression of clonotype transferred diabetes to all mice tested. CD4 T cells from NOD.BDC mice expressing intermediate and no clonotype marker did not transfer diabetes. However, when clonotype-intermediate CD4 T cells were mixed with diabetogenic clonotype-high CD4 T cells for disease transfer, recipient mice remained diabetes free. There was no inhibition of diabetes by the same number of CD4-positive T cells from normal prediabetic NOD mice. Thus, it seems that clonotype-intermediate CD4 T cells have a potent inhibitory capacity over that of nonfractionated CD4-positive T cells from normal NOD mice. These clonotype-high and -intermediate CD4 T cells were analyzed for the expression of CD25, a marker for regulatory T cells (Fig. 5), although there is some difference between these two populations for the expression of CD25, the vast majority of CD4 T cells in both clonotype-high and clonotype-low populations were negative for the expression.

Generation of clonotype-low CD4 T cells in the thymus

It has been shown that the CD4^+ CD25^+ regulatory T cells are generated in the thymus in an age-dependent manner. Clonotype expression of the thymic CD4 single-positive T cells was examined using 8- and 25-wk-old NOD.BDC mice. As shown in Fig. 6, young NOD.BDC thymus contained very few CD8 single-positive T cells and a majority of CD4 single-positive T cells were of the clonotype-high phenotype (8% clonotype-low population). In contrast, old NOD.BDC thymus contained a significant number of CD8 single-positive populations and a significant number of CD4 single-positive cells with the clonotype-low phenotype (35%). These results demonstrate an age-dependent change in the NOD BDC thymus. Both CD8-positive T cells that are of the clonotype-low phenotype and clonotype-low CD4-positive single-positive T cells from normal NOD mice were assessed for CD25 expression on idiotype-high and -intermediate CD4 T cells from NOD.BDC mice. Lymph node cells from NOD.BDC mice (30 wk old) were stained with FITC-anti-CD4 and RED613-anti-idiotype and PE-anti-CD25 or control PE-antihuman CD4. Cells were gated for CD4 expression and idiotype expression (high and intermediate) and then analyzed for the expression of CD25 by a FACSCalibur analyzer using the CellQuest program.
cells were generated late in life and changed in the thymic T cell populations coincident with the appearance of the clonotype-low T cells in the peripheral lymphoid organs.

**Discussion**

We established a new mAb, aBDC, specific for the idiotypic determinant of the islet Ag-specific diabeticogenic BDC2.5 TCR. Using this mAb, we examined the T cell population in NOD.BDC TCR transgenic mice. Although BDC TCR-bearing T cells are diabeticogenic in the absence of other lymphocytes in the SCID background (18), normal NOD.BDC mice do not develop diabetes. Higher penetrance of diabetes development in this TCR transgenic mouse line reported initially was due to the mixed genetic background of the mouse used in the study (16). In fact, Gonzalez et al. (17) demonstrated that the genetic elements from B6 mice present in the original TCR transgenic mice are responsible for the development of the diabetes. Regardless, lack of diabetes development in NOD.BDC mice but not in NOD.BDC.SCID mice strongly suggested the presence of lymphocytes capable of inhibiting the diabeticogenic process.

In this study, we demonstrated that, in NOD.BDC mice, CD4 T cells expressing a high level of clonotype determinant are diabeticogenic but those expressing a low level of clonotype determinant are inhibitory. The results of our transfer experiments are the most direct demonstration of the function of these T cells. It should be noted that CD4 T cells from normal prediabetic NOD mice are also shown to inhibit diabetes transfer by spleen cells from diabetic mice (22). We confirmed this result (A. Suri, unpublished data), but clonotype-intermediate CD4 T cells used in this study were significantly more effective for the inhibition of diabetes transfer than the CD4 T cells from normal prediabetic NOD mice. In fact, $5 \times 10^5$ CD4 T cells from prediabetic NOD mice failed to exhibit any inhibitory functions in this study (Table II).

Diabetes can be induced in young NOD.BDC mice with a single injection of CY as shown previously by Andre-Schmutz et al. (19). However, old NOD.BDC mice were totally resistant to the same treatment. The appearance of clonotype-intermediate CD4 T cells coincides with the resistance to CY-induced diabetes. Furthermore, there were significant numbers of clonotype-low CD4 T cells in the NOD.BDC mice that remained diabetes free after CY treatment. Thus, our results with NOD.BDC mice do not support the original speculation that CY induces diabetes in NOD mice by eliminating suppressor/regulatory T cells (10). However, our results are in agreement with recent demonstration that CY injection activates diabeticogenic BDC TCR-positive T cells via nonspecific activation of the inflammatory process (19). These findings need to be confirmed in normal NOD mice for the role of CY in the induction of diabetes.

Our results still do not explain why NOD.BDC mice failed to develop diabetes, since young mice have very few cells with regulatory T cell phenotypes. Analysis of the cells infiltrating the pancreas of NOD.BDC mice revealed that, even at the early stage of insulitis, both CD4 and CD8 T cells and B cells are present, similar to the previous report in normal NOD mice (2). Although we could not determine whether CD4 T cells infiltrating into islets are of the clonotype-high phenotype or not, it is possible that all T cells expressing islet Ag-specific TCR regardless of their functions can be recruited into the islet in this mouse line. Under such conditions, the number of regulatory T cell in the islets may be significantly higher than that in normal NOD mice and these T cells may functionally dominate to suppress diabeticogenic processes. Thus, the insulitis seen in this mouse may represent a protective process rather than a prediabetic process. This would partially explain the presence of extensive cellular infiltrate into islets without diabetes development in NOD.BDC mice. It is also possible that extensive cellular infiltrate without diabetes in the mice treated with CFA (9) may also represent a similar regulatory T cell-dominant state. Studies of cellular migration into the islet for both diabeticogenic and regulatory T cells and their interaction in vivo are necessary to fully understand the inhibition of the diabetic process in the NOD.BDC mouse model as well as in normal mice made resistant to diabetes by external manipulations.

Both CD4 and CD8 T cells with low clonotype expression develop only late in life in NOD.BDC mice. Since these T cells are stained with anti-V$\alpha$4 Ab with equal intensity as the clonotype-high CD4 T cell population, it is likely that these T cells express two TCR $\alpha$-chains (23), transgenic and an endogenous TCR $\alpha$-chains paired with the transgenic TCR $\beta$-chain. We do not know what regulates this age-dependent generation of T cells expressing two TCR $\alpha$-chains (23) and also the development of self-Ag-specific autoimmune T cells (24, 25). These results indicate that both CD4$^{+}$CD25$^{+}$ regulatory T cells and regulatory T cells in BDC.NOD mice are generated in the thymus. At present, we do not know what change in the thymus is responsible for the generation of T cells with regulatory functions. Thymic epithelial cells play an important role in the selection of immature thymocytes (26). It is possible that certain changes in the thymic epithelial cells may allow T cells expressing both transgenic and endogenous TCR $\alpha$-chains paired with the transgenic TCR $\beta$-chain to mature into either CD4 or CD8 single-positive T cells. However, this does not explain how these clonotype-low CD4 T cells acquire...
regulatory function. Further study of the relationship between age-dependent change in the thymic environment and the generation of T cells with unique surface phenotype and regulatory function is needed to understand the regulation of autoimmune diabetes as well as other organ-specific autoimmunity.

The exact relationship between CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells and regulatory T cells expressing low idiotypic determinant in NOD BDC mice is difficult to study. Studies of the expression of CD25 (Fig. 5) as well as a recently identified regulatory T cell marker, α<sub>2</sub> integrin (27, 28), in the CD4 T cells from the NOD.BDC mice showed no clear relationship between the expression of these markers and the expression of clonotype. Recent demonstration that some of the regulatory T cells also lack the CD25 marker (28) suggest that there may be no strict marker besides their functions for the regulatory T cells. Analysis of the clonotype-intermediate CD4 T cells with regulatory function identified in this study for their thymic selection, acquisition of regulatory functions, and change in the surface phenotype may provide useful information to clarify the issues concerning this interesting cell type.

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