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Comparison of a T Cell Clone and of T Cells from a TCR Transgenic Mouse: TCR Transgenic T Cells Specific for Self-Antigen Are Atypical

Cathleen M. Dobbs and Kathryn Haskins

It has been widely assumed that T cells from TCR-transgenic (Tg) mice better represent the behavior of T cells from normal mice than do in vitro cultures of T cell clones. We have found that autoreactive T cells arising in the presumably more physiological environment of the BDC-2.5 TCR-Tg mouse, despite being apparently "naive" in surface phenotype, are highly activated functionally and do not resemble CD4+ T cells from a spontaneously diabetic nonobese diabetic (NOD) mouse or the NOD-derived, diabetogenic CD4+ T cell clone of origin, BDC-2.5. Our results suggest that autoreactive T cells cloned from the spontaneously diabetic NOD mouse more closely resemble effector T cells arising during the natural disease process. The Journal of Immunology, 2001, 166: 2495–2504.

The nonobese diabetic (NOD) mouse spontaneously develops autoimmune diabetes and thus provides an informative experimental model of human insulin-dependent diabetes mellitus (1, 2). A central role of T cells in the pathogenesis of autoimmune diabetes has been demonstrated in the NOD mouse model. Islet-reactive T cell clones generated from NOD mice have provided a valuable means to characterize the properties that render a T cell diabetogenic (e.g., Ag specificity, cytokine production profile) and to investigate the roles of distinct T cell subsets in the pathogenesis of insulin-dependent diabetes mellitus (3, 4).

BDC-2.5 is an islet Ag-specific, diabetogenic CD4+ T cell clone derived by our laboratory from the spleen and lymph nodes of a newly diabetic female NOD mouse (5, 6). The BDC-2.5 T cell clone expresses a Vα1 Vβ4 TCR (7) and reacts with a β granule membrane Ag in the context of NOD MHC class II (6, 8). BDC-2.5 is of the Th1 phenotype in that it produces the cytokines IL-2, IFN-γ, and TNF, but not IL-4, upon culture with islet cells and NOD APC(9). The BDC-2.5 T cell clone has been extensively characterized with respect to pathogenicity; it rapidly induces extensive insulitis and hyperglycemia in NOD or NOD/Lt scid/scid (NOD-scid) mice 7–14 days of age, but cannot transfer disease to NOD recipients over 3 wk old, or to adult NOD-scid mice (9–11). In young NOD recipients, the cellular constituents of the pancreatic infiltrate are similar to those in spontaneous disease, consisting of CD4+ T cells, CD8+ T cells, B cells, and macrophages (12).

Advances in transgenic (Tg) mouse technology have made possible the generation of αβ TCR-Tg mice expressing an essentially monoclonal immune system comprised of T cells of a single defined specificity (13). This technology has been applied to autoimmune disease through the development of TCR-Tg mice with T cells specific for self peptide (14–17). The rearranged TCR genes of the BDC-2.5 T cell clone have been expressed in Tg mice (15), which were subsequently crossed onto the NOD and NOD-scid genetic backgrounds. Although the majority (>95%) of peripheral CD4+ T cells in the BDC-2.5 TCR transgene-positive/NOD (2.5 Tg/NOD) mice express the β TCR of the BDC-2.5 T cell clone, ~25–65% of the CD4+ T cells express an endogenous α TCR (15). Diabetes occurs spontaneously in only 10–15% of the 2.5 TCR Tg/NOD mice (18). In contrast, the 2.5 TCR Tg/NOD-scid mice have a monoclonal peripheral T cell repertoire, consisting of CD4+ T cells that exclusively express the BDC-2.5 TCR and their diabetes incidence is 100% by 3–5 wk of age (19) (C.M.D. and K.H., unpublished data).

T cells from TCR Tg mice are commonly thought to provide a more physiological model for normal T cell behavior than long-term, in vitro-cultured T cell clones (20). Accordingly, the functional activity and pathogenicity of T cells from TCR Tg mice is widely believed to be more representative of the behavior of T cells from normal mice than is that of in vitro T cell clones. One theory behind this reasoning is that the T cell that develops in the TCR-Tg mouse is “naive” with respect to memory for Ag. This question becomes more complex when the TCR in question is specific for a self peptide. To test these assumptions, and to investigate whether T cells arising in the presumably more physiological environment of the maturing mouse are different from T cell clones cultured long-term in vitro, we compared the in vivo activity and in vitro characteristics of the in vitro-cultured BDC-2.5 T cell clone to that of T cells from 2.5 TCR Tg/NOD-scid mice. We chose the 2.5 TCR Tg/NOD-scid mice for our study because they have a monoclonal T cell repertoire; all of the T cells from the 2.5 TCR Tg/NOD-scid mice are CD4+ and, unlike those from the 2.5 TCR Tg/NOD mice, express exclusively both the α and β TCR of the BDC-2.5 T cell clone. Our results indicate that there are fundamental differences in the phenotype and in vivo function of the BDC-2.5 T cell clone compared with that of T cells from the 2.5 TCR Tg/NOD-scid mice. We provide evidence to...
suggest that in vitro-cultured T cells cloned from non-Tg autoimmune-prone mice may better reflect the behavior of self-reactive T cells that develop during spontaneous autoimmune disease than the same T cells generated in a TCR-Tg mouse.

Materials and Methods

Mice

All mice were bred and maintained under pathogen-free conditions at the Center for Laboratory Animal Care at the University of Colorado Health Sciences Center (Denver, CO). NOD-scid mice (21) were originally obtained from the breeding colony at The Barbara Davis Center for Childhood Diabetes (Denver, CO) or purchased from The Jackson Laboratory (Bar Harbor, ME). Six-week-old DO11.10 TCR-Tg mice (22) on the BALB/c background were purchased from The Jackson Laboratory and were used as T cell donors for the tyrosine phosphorylation experiments. Diabetic NOD mice were used as donors and NOD-scid mice were used as recipients in adoptive transfer experiments. BDC-2.5 TCR Tg/NOD (2.5 TCR Tg/NOD) mice (15), were obtained from J. Katz (Washington University, St. Louis, MO) and were crossed to NOD-scid mice. BDC-2.5 TCR Tg mice on the NOD-scid background (2.5 TCR Tg/NOD-scid mice) have a monoclonal peripheral lymphocyte repertoire, consisting only of CD4+ T cells that express the TCR of the BDC-2.5 T cell clone (Vα1/Vβ4) and of no other CD4+ T cells. CD8+ T cells, or B cells (19). 2.5 TCR Tg/NOD-scid mice were typed using PCR of tail DNA to identify the presence of the BDC-2.5 transgene (7) followed by PCR amplification of the DNA-dependent protein kinase catalytic subunit gene with subsequent Alu digestion to identify transgene-positive mice homozygous for the scid mutation (23). Alternatively, 2.5 TCR Tg/NOD-scid mice were typed by flow cytometric analysis of PBLs to identify mice with a peripheral pool of lymphocytes expressing CD3, CD4, and Vβ4 but not CD8 or B220. One-hundred percent of 2.5 TCR Tg/NOD-scid mice develop diabetes between 3–5 wk of age. Prediabetic and diabetic 2.5 TCR Tg/NOD-scid mice were used as lymphoid cell donors in adoptive transfer experiments.

Spleen cell and thymocyte preparation

Single-cell suspensions of spleen cells and thymocytes from 2.5 TCR Tg/NOD-scid mice or NOD mice were prepared in HBSS using glass homogenizers. For adoptive transfer experiments, mononuclear cells were counted and mononuclear cell viability was determined using a hemocytometer and phase contrast microscopy. For phenotypic analyses, RBC were removed from spleen cell preparations by incubation in ACK lysis buffer (150 mM NH4Cl, 10 mM KHCO3, 0.1 mM EDTA, pH 7.2) before determining mononuclear cell counts and viability.

Culture of T cell clones

The BDC-2.5 T cell clone was derived from the spleen and lymph nodes of a newly diabetic female NOD mouse (6). The BDC-2.5 T cell clone (5–10×106) was maintained by stimulation every 2 wk with β cell granule membrane protein from β cell tumors (0.625 μg/ml) as a source of Ag (8), γ-irradiated (3500 rad from a 60Co source) NOD spleen cells (1.25–10×106) as a source of APC, and supernatant from PHA-stimulated EL-4 cells as a source of IL-2 (equivalent to 7 U/ml IL-2) in complete medium (CM), which was prepared by supplementing DMEM (Life Technologies, Gaithersburg, MD) culture medium with 44 mM sodium bicarbonate, 0.55 mM l-arginine, 0.27 mM l-asparagine, 1.5 mM l-glutamine, 1 mM sodium pyruvate, 50 mM L-gentamicin sulfate, 50 μM 2-ME, 10 mM HEPES, and 10% FBS. T cell clones were incubated at 37°C with 10% CO2. Four days following antigenic restimulation, cloned BDC-2.5 T cells for adoptive transfer experiments and phenotypic analyses were expanded by a 4-day culture in CM containing EL-4 supernatant equivalent to 14 U/ml IL-2.

The CD8+ CTL-2 cell line (American Type Culture Collection, Manassas, VA) was maintained by passage every 3–5 days in CM containing EL-4 supernatant equivalent to 17.5 U/ml IL-2 (24). CTL-2 cells were used after 4–5 days in culture for perforin analysis.

Adoptive transfer

Adult (6–15 wk old) NOD-scid mice received i.v. injections of the BDC-2.5 T cell clone (106 cells/injection) once every 3 wk for 3 wk, or a single i.v. injection of spleen cells or thymocytes from a prediabetic or diabetic 2.5 TCR Tg/NOD-scid mouse (1–3×106 cells/injection), or a diabetic NOD mouse (2×106 cells/injection).

Diabetes

Beginning at 2 wk of age, 2.5 TCR Tg/NOD-scid mice were monitored daily for elevated urine glucose using Diatix (Miles Laboratories, Elkhart, IN). Beginning 5–7 days after adoptive transfer, recipient NOD-scid mice were monitored daily for elevated urine glucose. Mice exhibiting glucosuria were tested for elevated blood glucose using a Precision Q.I.D. glucometer (Medsense, Walltham, MA). Mice with blood glucose levels >15 mM were considered overtly diabetic.

Histology

Recipient NOD-scid mice were sacrificed upon the development of diabetes. Nondiabetic mice were sacrificed 4 wk following the first T cell injection. Pancreata were removed for histological analysis as described previously (6, 10). Briefly, pancreata were fixed in 10% neutral buffered formalin and embedded in paraffin. Tissue sections were stained with hematoxylin and eosin to determine the extent of mononuclear cell infiltration, or with aldehyde fuchsin, a dye that selectively stains the disulfide bond in insulin, to determine the extent of islet β cell degranulation.

Quantification of IFN-γ and IL-4 in pancreatic homogenates

Cytokine concentrations were determined essentially according to a protocol successfully used by Arreaza et al. (25) to detect IL-4 and IFN-γ in pancreatic homogenates. Briefly, pancreata were isolated, snap frozen on dry ice, and stored at −70°C until processing and analysis. Pancreata were thawed and homogenized in PBS containing the following protease inhibitors at the indicated concentrations: 4 μg/ml aprotinin, 1 μg/ml leupeptin, 1.4 μg/ml pepstatin, and 40 μg/ml PMSF. Samples were centrifuged at 14,000 rpm for 5 min at 4°C to remove cell debris. The supernatants were assayed by ELISA for IFN-γ and IL-4 concentrations using purified and biotinylated Ab pairs, and according to a protocol, from BD PharMingen (San Diego, CA). An aliquot of each sample was assayed for total protein using the Pierce BCA flow cytometry kit (Pierce, Rockford, IL). The amount of cytokine in each sample was then normalized relative to the amount of total protein. The values are shown as picograms of cytokine per nanogram of total protein.

Immunofluorescent staining and flow cytometric analysis

Immunofluorescent staining and flow cytometric analysis were used to assess the expression of adhesion molecules and activation markers on the BDC-2.5 T cell clone and splenic T cells from diabetic 2.5 TCR Tg/NOD-scid mice. Cells were stained with the following panel of anti-mouse mAbs purchased from BD PharMingen: 1) FITC-coupled anti-CD62 ligand (CD62L) (L-selectin; clone MEL-14), anti-CD11a (LFA-1; clone m714), anti-α, β integrin (CD49d, also very late Ag-4a and lymphocyte Peyer’s patch adhesion molecule (LPAM)-1; clone MFR4A.B), anti-CD44 (Pgp-1; clone IM7), anti-CD25 (IL-2 receptor α-chain; clone 7D4), anti-CD45RB (clone UCHL-1), and anti-CD69 (very early activation Ag; clone HI.2F3); and 2) PE-coupled anti-CD4 (L3T4; clone GK-1.5) or anti-Vβ4 (clone KT4). For the assessment of LPAM-1 expression, cells were stained with purified anti-LPAM-1 (α, β; clone DATK32; BD PharMingen), biotinylated rabbit anti-rat IgG (Vector, Burlingame, CA), and streptavidin-FITC (BD PharMingen) followed by PE-coupled anti-CD4. For the assessment of β, integrin expression, cells were stained with biotinylated anti-β, integrin mAb (LPAM-1P; clone M293; BD PharMingen) and streptavidin-FITC (BD PharMingen) followed by PE-coupled anti-CD4.

For the assessment of intracellular perforin expression, 2.5×106 cells/sample were first stained with PE-coupled anti-TCR-β (clone H5-5797; BD PharMingen). The next, the cells were stained for intracellular perforin using the Cytofix/Cytoperm kit from BD PharMingen and according to the manufacturer’s instructions. The cells were washed with 10 μl of ice in the formaldehyde-containing Cytofix/Cytoperm solution. The cells were then washed twice in the saponin-containing Perm/Wash solution and were incubated for 30 min on ice in 5 μl Perm/Wash solution containing 1 μg anti-mouse perforin mAb (clone KM585; Kamiya Biomedical, Seattle, WA). The cells were washed three times in Perm/Wash solution and were then incubated for 30 min on ice in 50 μl Perm/Wash solution containing 1 μg anti-rat IgG (Rockland, Gilbertsville, PA). Finally, the cells were washed three times in Perm/Wash solution and were resuspended in saponin-free staining buffer before flow cytometric analysis.

Irreverent purified, biotinylated, or FITC- and PE-coupled rat or hamster isotype control Abs were included in all experiments. Cells were analyzed for surface fluorescence and size using an EPICS flow cytometer (Coultier Electronics, Hialeah, FL) or a FACS Calibur (BD Immunocytometry Systems, San Jose, CA). A total of 10,000 cells were analyzed for each determination. The change in the mean fluorescence intensity (AMFI) for the
FITC-conjugated anti-activation marker Ab staining of CD4+ T cells relative to the background mean fluorescence intensity for the FITC-conjugated isotype control Ab staining was determined for each sample. Low surface expression was defined as a ΔMFI of <0.5 but >2. High surface expression was defined as a ΔMFI of >50.

Preparation of naive and Ag-activated CD4+ T cells from DO11.10 TCR-Tg mice

Following the removal of RBC by incubation in ACK lysis buffer and the subsequent removal of macrophages by adherence to plastic, naive CD4+ T cells were purified from the spleens of DO11.10 TCR-Tg mice by immunomagnetic depletion of CD8+ T cells and B cells using cellect-plus mouse CD4 immunocolumns (Cytovax Biotechnologies, Edmonton, Alberta, Canada). Ag-activated CD4+ T cells were generated by culturing RBC-depleted spleen cells (1.3 × 10^6/ml) from DO11.10 TCR-Tg mice with OVA (1 mg/ml) for 4 days in CM at 37°C with 10% CO2. CD4+ T cells were purified from the in vitro cultures using immunomagnetic depletion using cellect-plus mouse CD4 immunocolumns (Cytovax Biotechnologies).

Analysis of tyrosine phosphorylation

The tyrosine phosphorylation pattern of the BDC-2.5 T cell clone, splenic T cells from diabetic 2.5 TCR Tg/NOD-scid mice, and control naive and Ag-activated DO11.10 TCR Tg T cells was analyzed based on previously described methods (26, 27). Specifically, 1,000,000 cloned BDC-2.5 T cells, 1,000,000 CD4+ T cells from the spleens of diabetic 2.5 TCR Tg/NOD-scid mice, 1,000,000 CD4+ T cells from the spleens of unmanipulated DO11.10 TCR-Tg mice, or 1,000,000 CD4+ T cells from in vitro cultured DO11.10 TCR-Tg spleen cells and OVA were lysed for 30 min on ice in 0.2 ml reconstituted protease inhibitor cocktail tablet (Complet-eMini, EDTA-free; Boehringer Mannheim, Indianapolis, IN) supplemented with 100 μg/ml phenylmethylsulfonyl fluoride, 92 μg/ml sodium orthovanadate, 84 μg/ml sodium fluoride, and 1% Triton X-100. Samples were centrifuged at 10,000 × g for 10 min at 4°C to remove nuclear debris. A total of 50 μl of 4% SDS gel-loading buffer was added to each sample and the samples were boiled for 5 min at 100°C. Lysates (50 μl/sample) were resolved by electrophoresis on 10% polyacrylamide/SDS gels and were transferred to nitrocellulose membranes. Blots were blocked for 1 h at room temperature in wash buffer (1.9 M NaCl, 250 mM Tris (pH 6.8), 0.1% Tween 20) containing 5% BSA. The primary anti-phosphotyrosine Ab clone 4G10 (Upstate Biotechnology, Lake Placid, NY) was added at 1 μg/ml in wash buffer containing 1% BSA and blots were incubated overnight at 4°C. Blots were washed twice (30 min/wash) in wash buffer, the secondary sheep anti-mouse Ig-HRP Ab (Amersham Pharmacia Biotech, Piscataway, NJ) was added at 1:10,000 in wash buffer containing 0.1% BSA, and blots were incubated for 45 min at room temperature. Blots were washed twice (30 min/wash) in wash buffer and bands were visualized using the ECL system (Amersham Pharmacia Biotech).

Anti-adhesion molecule mAb treatment

Spleen cells from diabetic 2.5 TCR Tg/NOD-scid mice (1–2 × 10^7) were incubated for 30 min on ice with 100 μg control rat IgG (Sigma, St. Louis, MO) or with purified anti-CD62L in sterile PBS and injected i.v. into adult NOD-scid mice. In addition, 100 μg control rat IgG or purified anti-CD62L was administered i.v. to recipient mice every 2–3 days postsplenocyte cell transfer.

51Cr release assay for testing the function of Fas ligand (FasL)

The BDC-2.5 T cell clone and spleen cells from diabetic 2.5 TCR Tg/NOD-scid mice were activated for 48 h at 37°C in 10% CO2 in CM containing EL-4 supernatant (equivalent to 7 U/ml IL-2), 10 ng/ml PMA, and 1 μg/ml ionomycin before use as effector cells in the 51Cr release assay. K562-FasL effector cells, G10-K562-FasL effector cells, and L1210-Fas+ target cells (28–30) were a gift from R.C. Duke (Ceres Pharmaceuticals, Denver, CO). 51Cr release assays were performed essentially as previously described (28–30). Briefly, L1210-Fas+ target cells were labeled with 100 μCi 51Cr for 2 h at 37°C in 10% CO2. Target cells were washed twice with CM. Then, 5 × 10^3 viable target cells in a volume of 0.1 ml and 0.1 ml of viable effector cells, at a concentration necessary to give the desired E:T ratio, were added to each well of a 96-well V-bottom microtitre plate. Each E:T ratio was prepared in triplicate. Maximum release was determined by adding 5 × 10^3 target cells in a volume of 0.1 ml to 0.1 ml of 1% Triton X-100. Spontaneous release was determined in cultures containing 5 × 10^3 target cells in a volume of 0.2 ml of CM. Plates were incubated at 37°C in 10% CO2, for 16 h. Supernatant (0.1 ml) from each well was assayed for released 51Cr, which were expressed as cpm. The percent of specific release was calculated using the following formula: percent specific release = (cpm (sample) – cpm (spontaneous))/cpm (maximum) – cpm (spontaneous).

Results

The BDC-2.5 T cell clone and 2.5 TCR Tg/NOD-scid spleen cells differentially transfer diabetes to adult NOD-scid mice

Our first goal in the current study was to directly compare the in vivo activity of the BDC-2.5 T cell clone to that of T cells from 2.5 TCR Tg/NOD-scid mice. In a previous study (9), we described a model system for inducing diabetes in adult NOD-scid mice using multiple i.v. injections of islet-specific T cell clones. We demonstrated that the BDC-2.5 T cell clone could induce diabetes in adult NOD-scid recipients but did so only if CD8+ T cells from diabetic NOD donors were injected concomitantly (9). Herein, we used our previously described adult NOD-scid adoptive transfer system to examine differences in diabetogenicity between the BDC-2.5 T cell clone and T cells from 2.5 TCR Tg/NOD-scid mice. Fig. 1 shows the incidence and kinetics of diabetes in adult NOD-scid recipients of the BDC-2.5 T cell clone or of spleen cells from 2.5 TCR Tg/NOD-scid mice. As we previously reported, three separate i.v. injections (10^7 cells/injection) of the BDC-2.5 T cell clone alone over the course of 3 wk failed to induce diabetes in adult NOD-scid recipients (Fig. 1). In contrast, and in agreement with the findings of Kurrer et al. (19), a single i.v. injection of splenocytes from diabetic 2.5 TCR Tg/NOD-scid mice was sufficient to induce diabetes in adult NOD-scid recipients, and did so rapidly (Fig. 1). We also found that CD4+ T cells purified from the spleen of a diabetic 2.5 TCR Tg/NOD-scid mouse were able to rapidly (by day 7 posttransfusion) induce diabetes in an adult NOD-scid recipient, ruling out the possibility that macrophages contributed to the diabetogenicity of the Tg T cells (data not shown). The kinetics of diabetes induction in adult NOD-scid recipients of splenocytes (2 × 10^7 cells) from a diabetic NOD mouse is shown for comparison (Fig. 1). The ability of splenocytes from diabetic 2.5 TCR Tg/NOD-scid mice to readily transfer diabetes to adult NOD-scid mice and the failure of the BDC-2.5 T cell clone to do so suggested that, despite expressing the same TCR, the BDC-2.5 T cell developing in the Tg mouse was fundamentally different from the in vitro-cultured T cell clone.

Histological examination of hematoxylin and eosin-stained pancreatic sections from adult NOD-scid recipients of diabetic 2.5

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TCR Tg/NOD-scid spleen cells revealed a highly disorganized and widespread mononuclear cell infiltrate that not only destroyed the islet β cell mass but also invaded and destroyed the exocrine pancreas (Fig. 2D). In contrast, pancreatic sections from adult NOD-scid recipients of the BDC-2.5 T cell clone were devoid of any detectable mononuclear cell infiltration (Fig. 2C). The highly disorganized and widespread infiltrate observed in the pancreata from adult NOD-scid recipients of diabetic 2.5 TCR Tg/NOD-scid spleen cells was very different from the islet-restricted infiltrate observed in the pancreata from adult NOD-scid recipients of diabetic NOD spleen cells (Fig. 2B), which closely resembles that observed in spontaneously diabetic NOD mice. Aldehyde fuchsin staining demonstrated complete islet β cell degranulation in the pancreata of adult NOD-scid recipients of diabetic 2.5 TCR Tg/NOD-scid spleen cells. In contrast, no degranulation was observed in the pancreata of adult NOD-scid recipients of the BDC-2.5 T cell clone (data not shown).

The cytokine pattern of splenocytes from 2.5 TCR Tg/NOD-scid mice is not altered following adoptive transfer

One possible explanation for the difference in the ability of the BDC-2.5 T cell clone and splenocytes from 2.5 TCR Tg/NOD-scid mice to transfer diabetes to adult NOD-scid recipients was that the cell populations differed in their pattern of cytokine production. The BDC-2.5 T cell clone is of the Th1 phenotype in that it produces the cytokines IL-2, IFN-γ, and TNF, but not IL-4, upon culture with islet cells and NOD APC (9). T cells from 2.5 TCR Tg/NOD-scid mice similarly display a Th1 cytokine pattern when stimulated in vitro (data not shown). Because it is conceivable that a change in the cytokine pattern of the Tg cells from a Th1 pattern to a Th2 pattern following their adoptive transfer into adult NOD-scid recipients accounted for their ability to transfer diabetes (31), the expression of IFN-γ and IL-4 was examined in the pancreata of recipient mice before, and at 3, 6, and 9 days following, cell transfer. The amount of IFN-γ in the pancreata of adult NOD-scid mice increased progressively and significantly from day 0 before transfer until day 9, when 100% of the mice became diabetic (Fig. 3). In contrast, IL-4 was not detected in the pancreata of recipient mice at any timepoint examined (data not shown). Neither IFN-γ nor IL-4 was detected in the pancreata of adult NOD-scid recipients of the BDC-2.5 T cell clone at any time-point examined (data not shown). Thus, the ability of the 2.5 TCR Tg/NOD-scid spleen cells to transfer diabetes to adult NOD-scid mice did not appear to be due to a change in their cytokine production pattern from Th1 to Th2 posttransfer.

**FIGURE 2.** Representative photomicrographs of hematoxylin and eosin-stained pancreatic tissue sections (original magnification, ×20) from adult (6 wk old) NOD-scid recipients that received the BDC-2.5 T cell clone or spleen cells from diabetic 2.5 TCR Tg/NOD-scid or NOD mice. A, Pancreatic tissue section from an uninjected adult NOD-scid mouse showing a healthy, uninfiltrated islet (closed arrow) and intact exocrine tissue. B, Pancreatic tissue section from an adult NOD-scid recipient of spleen cells from a diabetic NOD mouse showing an infiltrate that is restricted to an islet (closed arrow) and does not invade the exocrine tissue. C, Pancreatic tissue section from an adult NOD-scid recipient of the BDC-2.5 T cell clone showing two healthy, uninfiltrated islets (closed arrows). D, Pancreatic tissue section from an adult NOD-scid recipient of spleen cells from a diabetic 2.5 TCR Tg/NOD-scid mouse showing a widespread infiltrate that invades the islets and the exocrine pancreas.
mice using 10- to 12-week-old NOD-scid prediabetic 2.5 TCR Tg/NOD-scid mice. To investigate the functional activity of spleen cells and thymocytes from young, prediabetic 2.5 TCR Tg/NOD-scid mice, we conducted adoptive transfer experiments into adult NOD-scid mice using 10- to 12-day-old 2.5 TCR Tg/NOD-scid donors. Confirming our hypothesis, both spleen cells and thymocytes from young, prediabetic 2.5 TCR Tg/NOD-scid mice rapidly led to diabetes in adult NOD-scid recipients.

**Thymocytes and spleen cells from young, prediabetic 2.5 TCR Tg/NOD-scid mice transfer diabetes to adult NOD-scid mice**

The presence in diabetic 2.5 TCR Tg/NOD-scid mice of functionally activated T cells that did not express the typical activation markers led us to hypothesize that at no stage in their development would the TCR-Tg T cells be functionally naive. To investigate the functional activity of spleen cells and thymocytes from young, prediabetic 2.5 TCR Tg/NOD-scid mice, we conducted adoptive transfer experiments into adult NOD-scid mice using 10- to 12-day-old 2.5 TCR Tg/NOD-scid donors. Confirming our hypothesis, both spleen cells and thymocytes from young, prediabetic 2.5 TCR Tg/NOD-scid mice rapidly led to diabetes in adult NOD-scid recipients.

**LPAM-1 is expressed on the BDC-2.5 T cell clone but not on CD4+ T cells from the spleens of 2.5 TCR Tg/NOD-scid mice**

The ability of splenocytes from diabetic 2.5 TCR Tg/NOD-scid mice to readily transfer diabetes to adult NOD-scid mice and the failure of the BDC-2.5 T cell clone to do so might be explained by differences in migratory behavior following adoptive transfer. The ability of a T cell to migrate into a particular tissue is due, in part, to the ability of adhesion molecules expressed on the T cell to bind to vascular addressins expressed on endothelial cells in the tissue (33). Thus, we postulated that the BDC-2.5 T cell clone and T cells from the 2.5 TCR Tg/NOD-scid mouse would differ in their level or pattern of adhesion molecule expression. To test this hypothesis, we used immunofluorescent staining and flow cytometric analysis to compare adhesion molecule expression on the BDC-2.5 T cell clone to that on CD4+ spleen cells from diabetic 2.5 TCR Tg/NOD-scid mice.

**FIGURE 3.** Expression of IFN-γ in the pancreata of adult NOD-scid recipients of spleen cells from diabetic 2.5 TCR Tg/NOD-scid mice. NOD-scid mice (6-11 wk old) received a single i.v. injection of spleen cells from a diabetic 2.5 TCR Tg/NOD-scid mouse (2 × 10^6 cells/injection) on day 0. Mice were sacrificed before adoptive transfer, and at days 3, 6, and 9 postadoptive transfer, and the concentration of IFN-γ in the pancreas was determined by ELISA. Values are expressed as mean picograms of IFN-γ per nanogram of total pancreatic protein, and are shown for three to five mice per time point.

**FIGURE 4.** Activation marker expression on the BDC-2.5 T cell clone and CD4+ T cells from the spleens of diabetic 2.5 TCR Tg/NOD-scid mice. Subcultured BDC-2.5 T cell clones or spleen cells from diabetic 2.5 TCR Tg/NOD-scid mice were stained for the indicated markers and analyzed by flow cytometry. The mean fluorescence intensity (m.f.) for the FITC-conjugated anti-activation marker Ab staining of CD4+ T cells relative to the background mean fluorescence intensity for the FITC-conjugated isotype control Ab staining is indicated in the upper right quadrant of each plot. The data presented are representative of staining from at least three separate BDC-2.5 T cell clone subcultures or three individual 2.5 TCR Tg/NOD-scid mice.
culture (Fig. 6A). Specifically, the BDC-2.5 T cell clone expressed low but detectable levels of both chains (α4 and β7 integrin) of the LPAM-1 heterodimer (Fig. 6A), which regulates T cell migration to gut-associated lymphoid tissue (GALT) via its interaction with the mucosal vascular addressin, MAdCAM-1 (35, 36), but did not express CD62L, which regulates T cell migration into peripheral lymphoid organs via its interaction with the peripheral lymph node vascular addressin, PNAd (37). The mAb DATK32, which recognizes a conformational epitope of the LPAM-1 heterodimer comprised of both the α4 and β7 integrins (38), was used to confirm the expression of LPAM-1 on the BDC-2.5 T cell clone. LPAM-1 expression on the BDC-2.5 T cell clone is shown in Fig. 5B. In contrast to the BDC-2.5 T cell clone, CD4+ T cells from the spleens of diabetic 2.5 TCR Tg/NOD-scid mice expressed neither the α4 nor the β7 integrin but did express CD62L (Fig. 6A). In addition, the BDC-2.5 T cell clone expressed levels of CD11a that were ∼5-fold higher than those expressed on CD4+ T cells from the spleens of diabetic 2.5 TCR Tg/NOD-scid mice (Fig. 6A). These results extended and confirmed the results of the activation marker analysis. Specifically, the lack of expression of CD62L and the relatively higher level of expression of CD11a on the BDC-2.5 T cell clone is consistent with a memory phenotype, whereas the expression of CD62L, and the relatively lower level of expression of CD11a on CD4+ T cells from 2.5 TCR Tg/NOD-scid mice is consistent with the phenotype of a resting, naive T cell (32).

Despite a naive surface phenotype, 2.5 TCR Tg/NOD-scid T cells exhibit a tyrosine phosphorylation pattern that is consistent with an activated cell phenotype

There are distinct differences in tyrosine phosphorylation between resting naive and effector/memory CD4+ T cells as evidenced by anti-phosphotyrosine immunoblots of cell lysates from T cells from BALB/c mice (26). For example, resting naive cells do not display tyrosine phosphorylated proteins of 28, 47, and 50 kDa seen in effector/memory T cells (26, 27). As an additional measure of the functional status of the T cells in 2.5 TCR-Tg/NOD-scid mice, we assessed tyrosine phosphorylation in lysates prepared from T cells purified from the spleens of newly diabetic 2.5 TCR-Tg/NOD-scid mice and compared them to lysates prepared from culture (Fig. 6A). Specifically, the BDC-2.5 T cell clone expressed low but detectable levels of both chains (α4 and β7 integrin) of the LPAM-1 heterodimer (Fig. 6A), which regulates T cell migration to gut-associated lymphoid tissue (GALT) via its interaction with the mucosal vascular addressin, MAdCAM-1 (35, 36), but did not express CD62L, which regulates T cell migration into peripheral lymphoid organs via its interaction with the peripheral lymph node vascular addressin, PNAd (37). The mAb DATK32, which recognizes a conformational epitope of the LPAM-1 heterodimer comprised of both the α4 and β7 integrins (38), was used to confirm the expression of LPAM-1 on the BDC-2.5 T cell clone. LPAM-1 expression on the BDC-2.5 T cell clone is shown in Fig. 5B. In contrast to the BDC-2.5 T cell clone, CD4+ T cells from the spleens of diabetic 2.5 TCR Tg/NOD-scid mice expressed neither the α4 nor the β7 integrin but did express CD62L (Fig. 6A). In addition, the BDC-2.5 T cell clone expressed levels of CD11a that were ∼5-fold higher than those expressed on CD4+ T cells from the spleens of diabetic 2.5 TCR Tg/NOD-scid mice (Fig. 6A). These results extended and confirmed the results of the activation marker analysis. Specifically, the lack of expression of CD62L and the relatively higher level of expression of CD11a on the BDC-2.5 T cell clone is consistent with a memory phenotype, whereas the expression of CD62L, and the relatively lower level of expression of CD11a on CD4+ T cells from 2.5 TCR Tg/NOD-scid mice is consistent with the phenotype of a resting, naive T cell (32).
the BDC-2.5 T cell clone. The results are shown in Fig. 7. Also shown for comparison are the tyrosine phosphorylation patterns of lysates prepared from control naive and Ag-activated CD4\(^+\) T cells. Because OVA is not an endogenous murine Ag, CD4\(^+\) T cells purified from the spleens of unmanipulated DO11.10 TCR-Tg mice, which express the \(\alpha\) and \(\beta\) TCR of the MHC class II-restricted, OVA-specific DO11.10 T cell clone (22), were used as a source of naive T cells. CD4\(^+\) T cells purified from in vitro cultures of DO11.10 TCR-Tg spleen cells and OVA were used as a source of control Ag-activated T cells. Despite distinctly different surface phenotypes, the BDC-2.5 T cell clone and T cells from 2.5 TCR Tg/NOD-scid mice exhibited similar tyrosine phosphorylation patterns, showing tyrosine phosphorylated proteins of 28, 47, and 50 kDa. In addition, the 2.5 TCR Tg/NOD-scid T cells exhibited dramatically increased tyrosine phosphorylation compared with equivalent numbers of naive CD4\(^+\) T cells from the spleens of unmanipulated DO11.10 TCR-Tg mice. Thus, despite a naive surface phenotype, the CD4\(^+\) T cells from 2.5 TCR Tg/NOD-scid mice display an activated biochemical profile.

Anti-CD62L mAb treatment significantly delays the onset of diabetes in adult NOD-scid recipients of 2.5 TCR Tg/NOD-scid spleen cells

Based on our assessment of adhesion molecule expression, we hypothesized that the ability of 2.5 TCR Tg/NOD-scid spleen cells to transfer diabetes to adult NOD-scid recipients and the failure of the BDC-2.5 T cell clone to do so was due to the expression of CD62L on the Tg T cells. To test the functional significance of CD62L expression on CD4\(^+\) spleen cells from diabetic 2.5 TCR Tg/NOD-scid mice, spleen cell suspensions were incubated with the blocking anti-CD62L mAb MEL-14 (39) before adoptive transfer to adult NOD-scid recipients. Recipient NOD-scid mice were also treated with MEL-14. Treatment with the anti-CD62L mAb significantly delayed, but did not prevent, the onset of diabetes in adult NOD-scid recipients (Fig. 8). These results suggested that, although CD62L contributed to the ability of spleen cells from diabetic 2.5 TCR Tg/NOD-scid mice to transfer diabetes to adult NOD-scid recipients, it was not the sole mechanism responsible for the difference in in vivo activity between the Tg T cells and the BDC-2.5 T cell clone.

CD4\(^+\) T cells from the spleens of 2.5 TCR Tg/NOD-scid mice express perforin but the BDC-2.5 T cell clone does not

Perforin expression and perforin-dependent cytotoxic T lymphocyte (CTL) activity are normally associated with CD8\(^+\) and not CD4\(^+\) T cells. However, there are several accounts in the literature documenting the existence of perforin-dependent CD4\(^+\) CTL (40–44). Most relevant to this study are reports demonstrating that perforin-dependent CD4\(^+\) CTL arise only in the absence of CD8\(^+\) T cell responses (41, 42, 44). Because the BDC-2.5 T cell clone requires CD8\(^+\) T cells from diabetic NOD mice to transfer diabetes to adult NOD-scid mice (9), and because diabetes in the Tg NOD-scid mouse develops in the absence of CD8\(^+\) T cells (19), we hypothesized that CD4\(^+\) T cells from 2.5 TCR Tg/NOD-scid mice would express perforin. To test this hypothesis, we used immunofluorescent staining and flow cytometric analysis to examine the intracellular expression of perforin protein in the BDC-2.5 T cell clone and in T cells from diabetic 2.5 TCR Tg/NOD-scid mice. The murine CD8\(^+\) CTL clone CTLL-2 was used as a positive control for perforin expression (45, 46). Perforin was detected in T cells from the spleens of diabetic 2.5 TCR Tg/NOD-scid mice but was not detected in the BDC-2.5 T cell clone (Fig. 9). Thus, it may well be that the ability of CD4\(^+\) T cells from diabetic 2.5 TCR Tg/NOD-scid mice to transfer diabetes to adult NOD-scid recipients in the absence of CD8\(^+\) T cells from diabetic NOD mice is due to their acquisition of CD8\(^+\) T cell activity.

FIGURE 7. Tyrosine phosphorylation profile of cell lysates derived from the BDC-2.5 T cell clone and CD4\(^+\) T cells from the spleen of a diabetic 2.5 TCR Tg/NOD-scid mouse. Lysates were prepared from 10\(^6\) cloned BDC-2.5 T cells, 10\(^6\) 2.5 TCR Tg/NOD-scid CD4\(^+\) T cells, 10\(^6\) naive DO11.10 TCR Tg CD4\(^+\) T cells, and 10\(^6\) Ag-activated DO11.10 TCR Tg CD4\(^+\) T cells, were resolved on a 10% polyacrylamide/SDS gel, and were transferred to nitrocellulose. Blots were probed with the 4G10 anti-phosphotyrosine mAb and bands were visualized with sheep anti-mouse Ig-HRP and ECL. The arrows indicate proteins reported to be expressed in effector/memory cells but not in resting naive cells.

FIGURE 8. Anti-CD62L mAb-treatment delays the onset of diabetes in adult NOD-scid recipients of 2.5 TCR Tg/NOD-scid spleen cells. Eight-week-old NOD-scid mice received a single i.v. injection of 1 \(\times\) 10\(^7\) spleen cells from a 2.5 TCR Tg/NOD-scid mouse on day 0. Before adoptive transfer, the donor 2.5 TCR Tg/NOD-scid spleen cells were treated for 30 min at 4°C with 100 \(\mu\)g of control Ab (rat IgG) or with 100 \(\mu\)g anti-CD62L mAb (Mel-14). In addition, 100 \(\mu\)g of control Ab or anti-CD62L mAb was administered i.v. to recipient NOD-scid mice every other day beginning 2 days posttransfer. Recipient mice were monitored for elevated urine glucose. Those with elevated levels were tested for high blood glucose. Mice were considered overtly diabetic when their urine glucose was >2% and their blood glucose was >15 mM. The numbers in parenthesis indicate the final number of diabetics/total number of mice. Diabetes was significantly delayed in mice that received anti-CD62L treated cells as determined by the Mann-Whitney test (\(p < 0.05\)). The data presented are representative of results from two independent experiments.
The BDC-2.5 T cell clone is able to lyse Fas-transfected target cells but spleen cells from 2.5 TCR Tg/NOD-scid mice are not.

In a recent manuscript, Amrani et al. (47) demonstrated that CD4+ T cells from NOD mice Tg for the diabetogenic 4.1-TCR expressed perforin but killed islet β cells via FasL. Previously published data indicated that spleen cells from 2.5 TCR Tg/NOD-scid mice could efficiently lyse Fas-deficient islet cells (48), indicating that FasL expression by the 2.5 TCR Tg/NOD-scid T cells was not obligatory for β cell destruction. However, these studies did not rule out a contributory role for FasL in islet cell destruction. Therefore, it was important for us to determine whether the 2.5 TCR Tg/NOD-scid cells could kill via FasL. To investigate the expression of functional FasL on T cells from diabetic 2.5 TCR Tg/NOD-scid mice, spleen cells from diabetic 2.5 TCR Tg/NOD-scid mice were activated with PMA and ionomycin and were then tested for their ability to lyse target L1210 lymphoma cells expressing high levels of Fas (L1210-Fas). The BDC-2.5 T cell clone was also activated with PMA and ionomycin and tested for its ability to lyse the L1210-Fas target cells. Effector cells that do (G10-K562) or do not express FasL (K562) were included in the assay as positive and negative controls, respectively. The BDC-2.5 T cell clone was able to lyse the L1210-Fas target cells, and did so as efficiently as did the G10-K562 FasL+ control cells (Fig. 10). However, the 2.5 TCR Tg/NOD-scid spleen cells were unable to lyse the L1210-Fas target cells, even at a 50:1 effector-to-target cell ratio (Fig. 10 and data not shown). Because the L1210-Fas target cells are also susceptible to TNF-α-mediated lysis (49), this experiment did not rule out the possibility that the BDC-2.5 T cell clone kills in a TNF-dependent manner. However, it did demonstrate that the 2.5 TCR Tg/NOD-scid T cells do not kill via FasL or TNF-α.

Discussion

Although in vitro-cultured T cell clones generated from non-Tg autoimmune-prone mice have long been used to study the pathogenesis of autoimmune diseases (2, 3, 50), TCR-Tg mice and T cells from these mice are often assumed to provide more physiologically relevant autoimmune models to study effector and regulatory mechanisms of autoimmune disease. The increasing frequency in recent years with which TCR-Tg mice have been used as new relevant autoimmune models to study effector and regulatory mechanisms of autoimmune disease than do the same T cells generated in a TCR-Tg mouse.

In this report, we explored the reasons why a CD4+ islet Ag-specific T cell clone, BDC-2.5, was unable to transfer diabetes to adult NOD-scid recipients but that T cells from TCR-Tg mice on the NOD-scid background bearing the TCR genes of the BDC-2.5 T cell clone (2.5 TCR Tg/NOD-scid) were able to do so very efficiently. We previously reported that the BDC-2.5 T cell clone, which was isolated from the spleen and lymph nodes of a diabetic NOD mouse, requires CD8+ T cells from a diabetic NOD mouse to transfer diabetes to adult NOD-scid mice (9). Thus, the activity of this clone is in keeping with studies demonstrating that both CD4+ and CD8+ T cells are required for the development of spontaneous diabetes in the NOD mouse (51) and for the transfer of diabetes to immunodeficient adult NOD recipients by spleen cells from diabetic NOD mice (52–54). Specifically, both CD4+ and CD8+ T cells have been shown to be absolutely required for the transfer of diabetes to γ-irradiated NOD (52) and NOD nude (53).

FIGURE 9. Perforin expression in the BDC-2.5 T cell clone and CD4+ T cells from spleens of diabetic 2.5 TCR Tg/NOD-scid mice. CD4+ CTLL-2 control cells, subcultured BDC-2.5 T cell clones, and spleen cells from diabetic 2.5 TCR Tg/NOD-scid mice were incubated with an Ab specific for TCR-β, fixed, permeabilized, and incubated with an Ab specific for perforin, as described in Materials and Methods. The data presented are representative of staining from two separate BDC-2.5 T cell clone subcultures or two individual 2.5 TCR Tg/NOD-scid mice.

FIGURE 10. Cytotoxic activity of the BDC-2.5 T cell clone and T cells from diabetic 2.5 TCR Tg/NOD-scid mice against L1210-Fas target cells. The BDC-2.5 T cells or T cells from the spleens of diabetic 2.5 TCR Tg/NOD-scid mice were incubated to cytotoxicity by incubation in PMA and ionomycin for 48 h before the cytotoxicity assay. K562-FasL+ control effector cells, G10-K562-FasL+ control effector cells, cloned BDC-2.5 T cells, or 2.5 TCR Tg/NOD-scid T cells were incubated with 51Cr-labeled L1210-Fas target cells at various E:T ratios for 16 h before harvest and determination of the percent of specific release. The data shown are for the 10:1 E:T ratio and represent the mean ± SEM for percent specific release determinations made on triplicate wells.
mice and for the efficient transfer of diabetes to adult NOD-scid mice (54), in that while CD4+ T cells alone were shown to be sufficient to transfer diabetes to adult NOD-scid mice, the transfer of CD4+ T cells alone was incomplete (only 58% diabetes incidence) and the onset of diabetes was prolonged (mean time to diabetes onset was 71.2 days).

Histologically, the islet-restricted infiltrate observed in the pancreata of adult NOD-scid recipients of the BDC-2.5 T cell clone plus CD8+ T cells from a diabetic NOD mice resembles that observed in the pancreata of spontaneously diabetic NOD mice and of diabetic adult NOD-scid recipients of diabetic NOD spleen cells (9, 21, 54, 55). The ability of spleen cells from diabetic 2.5 TCR Tg/NOD-scid mice to transfer diabetes to adult NOD-scid recipients without help from CD8+ T cells, coupled with a highly invasive infiltrate that does not resemble the islet-restricted infiltrate seen in NOD spontaneous diabetes, suggests that, unlike the in vitro-cultured cloned T cells, the Tg T cells differ fundamentally from the CD4+ T cells that mediate spontaneous diabetes.

Further support for this hypothesis comes from phenotypic analysis of the in vitro-propagated BDC-2.5 T cell clone. It has been demonstrated that the subset of CD4+ T cells in diabetic NOD mice that is capable of transferring disease expresses LPAM-1 (αβ2 integrin) (56–58), but lacks CD62L expression (59) and exhibits low CD45RB expression (60). We have demonstrated in this study that, like pathogenic CD4+ T cells, the BDC-2.5 T cell clone, which was derived from the spleen and lymph nodes of a diabetic NOD female mouse (5), expresses LPAM-1 and low levels of CD45RB but does not express CD62L.

It has been hypothesized that the GALT is an important site for the initial priming of diabeticogenic T cells (61). The expression of LPAM-1 is the hallmark of T cells that have arisen in the GALT (62). The expression of LPAM-1 by the BDC-2.5 T cell clone suggests that this clone represents a formerly naive, resting T cell that was induced in the GALT by β islet cell Ag to expand and to develop effector function. In contrast, the 2.5 TCR Tg/NOD-scid cells, as evidenced by their lack of LPAM-1 expression and by the ability of thymocytes and spleen cells from young prediabetic 2.5 TCR Tg/NOD-scid mice to rapidly transfer diabetes, do not appear to mature in the GALT as do other diabeticogenic T cells. Rather, they seem to emerge from the thymus as primed T cells. In fact, despite a naive surface phenotype, the CD4+ T cells from 2.5 TCR Tg/NOD-scid mice display an effector/memory-specific biochemical profile.

Although perforin expression and perforin-dependent CTL activity are normally associated with CD8+ and not CD4+ T cells, recent reports demonstrate that perforin-expressing CD4+ CTL can arise in the absence of CD8+ T cell responses (41, 42). Our data demonstrates that CD4+ T cells from 2.5 TCR Tg/NOD-scid mice express perforin but that the CD4+ BDC-2.5 T cell clone does not. In addition, consistent with the findings of Pakala et al. (48) for spleen cells from the 2.5 TCR Tg/NOD-scid mouse, and in contrast to the findings of Amrani et al. (47) for CD4+ T cells from the 4.1-NOD TCR Tg mouse, our data demonstrates that CD4+ T cells from the 2.5 TCR Tg/NOD-scid mouse do not kill via FasL or TNF-α. In contrast, our data demonstrates that the BDC-2.5 T cell clone does have the ability to kill via FasL and/or TNF-α, thus indicating a key difference in the effector mechanism used by the 2.5 TCR Tg/NOD-scid T cells and the parent BDC-2.5 T cell clone. Because the BDC-2.5 T cell clone requires CD8+ T cells from diabetic NOD mice to transfer diabetes to adult NOD-scid mice (9), and CD4+ T cells from diabetic 2.5 TCR Tg/NOD-scid mice do not, it is possible that the ability of Tg CD4+ T cells to induce disease in adult NOD-scid recipients in the absence of CD8+ T cells from diabetic NOD mice, plus the different in vitro effector mechanism exhibited by these cells, is due to their acquisition of CD8+ T cell activity.

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