The Autoimmune Diabetes Locus *Idd9* Regulates Development of Type 1 Diabetes by Affecting the Homing of Islet-Specific T Cells

Hanspeter Waldner, Raymond A. Sobel, Nichole Price and Vijay K. Kuchroo

*J Immunol* 2006; 176:5455-5462; doi: 10.4049/jimmunol.176.9.5455

http://www.jimmunol.org/content/176/9/5455

**References**

This article cites 34 articles, 15 of which you can access for free at:
http://www.jimmunol.org/content/176/9/5455.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

---

*The Journal of Immunology* is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852

Copyright © 2006 by The American Association of Immunologists. All rights reserved.

Print ISSN: 0022-1767 Online ISSN: 1550-6606.
The Autoimmune Diabetes Locus Idd9 Regulates Development of Type 1 Diabetes by Affecting the Homing of Islet-Specific T Cells

Hanspeter Waldner, Raymond A. Sobel, Nichole Price, and Vijay K. Kuchroo

Several genetic insulin-dependent diabetes (Idd) intervals that confer resistance to autoimmune diabetes have been identified in mice and humans, but the mechanisms by which they protect against development of diabetes have not been elucidated. To determine the effect of Idd9 on the function of islet-specific T cells, we established novel BDC-Idd9 mice that harbor BDC2.5 TCR transgenic T cells containing the Idd9 of diabetes-resistant B10 mice. We show that the development and functional responses of islet-specific T cells from BDC-Idd9 mice are not defective compared with those from BDC mice, which contain the Idd9 of diabetes-susceptible NOD mice. Upon transfer, BDC T cells rapidly induced severe insulitis and diabetes in NOD.scid mice, whereas those from BDC-Idd9 mice mediated a milder insulitis and induced diabetes with a significantly delayed onset. BDC and BDC-Idd9 T cells expanded comparably in recipient mice. However, BDC-Idd9 T cells accumulated in splenic periarteriolar lymphatic sheaths, whereas BDC T cells were mainly found in pancreatic lymph node and pancreata of recipients, indicating that the transferred T cells differed in their homing. We provide evidence that the migration pattern of transferred BDC and BDC-Idd9 T cells at least partly depends on their differential chemotaxis toward the CCR7 ligand CCL19. Taken together, our data show that the Idd9 locus regulates development of type 1 diabetes by affecting the homing of islet-specific T cells. The Journal of Immunology, 2006, 176: 5455–5462.

The type 1 diabetes or insulin-dependent diabetes mellitus is a slowly progressive autoimmune disease, which is characterized by inflammatory cell infiltrates in the pancreas leading to destruction of insulin-producing islet β cells. With numerous studies in the animal model of insulin-dependent diabetes mellitus, the NOD mouse has revealed that autoreactive CD4+ and CD8+ T cells play crucial roles in the pathogenesis of this disease (1–3). Mice that are transgenic for the rearranged TCR genes from the diabeticogenic T cell clone BDC2.5 isolated from a NOD mouse developed diabetes that was more aggressive than in nontransgenic NOD mice (4). In this model, activation of islet-specific T cells in pancreatic lymph nodes (PLN) results in their homing to the pancreas and the initiation of β cell destruction. Overt diabetes usually develops when the majority of β cells have been destroyed and glucose regulation is lost. Progression to spontaneous diabetes depends on the recruitment of effector lymphocytes into the pancreas; however, the function of these autoreactive T cells can be regulated by several mechanisms in the peripheral immune compartment including immune deviation associated with IL-4 production and different populations of immunoregulatory cells (5–10).

Diabetes in NOD mice is a polygenic disease. At least 20 genetic loci (known as insulin-dependent diabetes (Idd) loci) on different chromosomes that predispose to this disease have been identified by genome-wide linkage analyses. These susceptibility loci include the NOD MHC (H-2d) and genetic intervals that lie outside of the MHC locus (11–13). Congenic mouse strains that carry the NOD genome except for a defined introgressed Idd interval derived from diabetes-resistant mice such as C57BL/6 and C57BL/10 have been developed (14–16). These Idd congenic strains facilitate besides the identification of polymorphic alleles, the functional analysis of Idd loci and the cellular mechanisms by which they confer resistance to the development of diabetes. For example, the Idd3 locus has been identified and mapped to a 0.15 cM interval, which contains the variant candidate gene Il2 (17). Recently, a novel congenic NOD strain, NOD.B10 Idd9, which contains ~48 cM of a genetic interval (Idd9) from chromosome 4 of diabetes-resistant C57BL/10 mice has been developed and characterized (16). This study demonstrated that compared with diabetes-susceptible NOD mice, NOD.B10 Idd9 mice are remarkably resistant to the development of spontaneous diabetes but not to insulitis. The main mechanism underlying the Idd9-mediated resistance to diabetes was attributed to inflammatory cells in the islets, which express CD30 and produced the anti-inflammatory cytokine IL-4.

We hypothesized that the resistance to diabetes conferred by the B10 Idd9 interval is due to its effects on the function of diabetogenic CD4+ T cells. We intercrossed BDC2.5 TCR transgenic (hereafter referred to as BDC mice) and NOD.B10 Idd9 congenic mice to generate BDC-Idd9 mice. These novel mice express the B10-derived Idd9 in all of their tissues including their CD4+
BDC2.5 TCR T cells. Therefore, they provide a tool for dissecting the effects of Idd9 on the function of islet-specific CD4+ T cells.

In this study, we report that the Idd9 did not affect the development and activation status of islet-specific T cells in the BDC-Idd9 mice. BDC-Idd9 T cells were not defective in their responses to a BDC2.5 mimic peptide in vitro or to β cell Ag in recipient mice. We demonstrate that islet-specific T cells containing the B10-derived Idd9 significantly delayed the onset of diabetes in NOD.scid mice when compared with onset in mice that received cells from BDC mice. Finally, in adoptive transfer experiments, we show that BDC-Idd9 T cells transferred into recipients had impaired capacities to home in to PLN and infiltrate the pancreas but accumulated in splenic periarteriolar lymphatic sheaths (PALS) in comparison to BDC T cells. This different homing pattern was associated with a differential chemotactic reactivity of BDC and BDC-Idd9 T cells toward the CCR7 ligand CCL19. Taken together, these results indicate that Idd9 affects the homing of islet-specific T cells and thereby regulates the infiltration of pancreatic islets and induction of diabetes.

Materials and Methods

Mice

NOD.B10 Idd9R28 (16) NOD.scid and NOD mice were obtained from Taconic Farms. BDC2.5 TCR transgenic NOD mice (4) were originally obtained from D. Betenbaugh and C. Benoist (Joslin Diabetes Center, Boston, MA) and maintained by breeding with NOD mice in our animal facility. BDC2.5 TCR transgenic NOD mice that contained the B10 Idd9 interval (termed BDC-Idd9 mice) were generated by crossing male BDC2.5 TCR transgenic NOD mice with female NOD.B10 Idd9R28 mice. F1 hybrids were transgenic for BDC2.5 TCR and were subsequently crossed with NOD.B10 Idd9R28 mice. Transgenic F1 hybrids were screened for the homzygous presence of the B10 Idd9 genetic interval by PCR using nine microsatellite markers that differentiate NOD and B10 genomic segments between the markers D4Mit31 and D4Mit42 (15). BDC-Idd9 transgenic founders were selected and maintained by subsequent breeding with NOD.B10 Idd9R28 mice. Mice were identified for the transgenic BDC2.5 TCR by screening for the expression of TCRVbeta4 on CD4+ PBL by flow cytometry. Female mice were used for experiments when they were 4–6 wk of age. Mice were housed at the Partners Research Building (Cambridge, MA) under specific pathogen-free and viral Ab-free conditions in a temperature-controlled room (21°C, 40–50% humidity) in a 12-h light-dark cycle. Female mice were used for experiments when they were 4–6 wk of age. Mice were housed at the Partners Research Building (Cambridge, MA) under specific pathogen-free and viral Ab-free conditions in accordance with the guidelines of Harvard Medical School.

Genotyping for Idd9

Genomic DNA was isolated by digesting samples of tail tissues with proteinase K, which was followed by a phenol/chloroform extraction. Amplification was performed in 50 μl of final volume containing 1.5 mM MgCl2, 200 μM each dNTP, 1.25 U Taq polymerase, and 2 μM each primer using a PTC-100 model thermal cycler (MJ Research). Primers (Invitrogen Life Technologies) were designed according to published DNA sequences of the following microsatellite markers for Idd9: D4Mit31, D4Mit42, D4Mit59, D4Mit72, D4Mit76, D4Mit204, D4Mit258, and D4Mit310. Amplifications were done with a hot start and run for 35 cycles at 94°C for 30 s, 55°C for 30 s, 72°C for 45 s with a final extension of 10 min at 72°C. PCR products were electrophoresed on 3–4% MetaPhor gels (Cambrex).

Flow cytometry

Single-cell suspension of spleens, thymi, and PLN were prepared by staining the tissues through cell strainers. Cells were lysed by hypotonic shock. Thymocytes or spleen cells (1 × 10^6/sample) were washed in 50 μl of FACS buffer (PBS/0.1% Na3VO4/1% FCS) and stained with FITC-, PE-, or allophycocyanin-conjugated Abs for 20–30 min at 4°C. Cells were subsequently washed twice in FACS buffer before they were analyzed on a FACSCalibur flow cytometer using CellQuest software (BD Biosciences). At least 30,000 live cells per sample were analyzed by gating on characteristic forward and side scatter profiles. PBL were isolated from tail vein blood, depleted of RBC and stained as above. Fluorochrome-conjugated Abs were purchased from BD Pharmingen and included anti-CD4 (RM4-5), CD8 (53-6.7), TCR Vbeta4 (KT4), CD19 (1D3), CD25 (PC61), CD62 ligand (CD62L, MEL-14), and CD69 (H1.2F3). Anti-mouse CCR7 mAb (4B12) was purchased from eBioscience.

Peptide Ag

The BDC2.5 mimic peptide p79 (AVRPLWVRME) (18) was synthesized by Synpep to >95% purity as determined by HPLC.

Stimulation of T cells

Spleen cells from BDC and BDC-Idd9 mice were cultured in triplicates of 96-well plates in DMEM (BioWhittaker) supplemented with 10% heat-inactivated FCS, 2 mM l-glutamine, 10 mM HEPES, and 2 mM 2-ME (DMEM complete) in the presence of different concentrations of BDC2.5 mimic peptide p79 and incubated at 37°C for 2 days. Proliferation of responding cells was determined by incorporated thymidine after adding 1 μCi of [3H]thymidine to each well during the last 12 h of culture.

ELISA

The concentration of cytokines was determined in culture supernatants of BDC and BDC-Idd9 spleen cells stimulated with the BDC2.5 mimic peptide p79. Supernatants of these cultures were assayed for cytokine production after 40 h by quantitative capture ELISA according to the manufacturer’s guidelines. Assays were developed with TMB Microwell Peroxidase Substrate (Kirkegaard & Perry Laboratories) and were analyzed at 450 nm.

CFSE labeling and adoptive T cell transfer

T cells were isolated from spleen of nondiabetic BDC and BDC-Idd9 mice by using CD3 T cell enrichment columns (R&D Systems). FACS staining determined that CD3-enriched cells from both BDC and BDC-Idd9 mice contained comparable numbers of CD4+ Vβ4+ (BDC2.5 TCR) T cells. Cells were resuspended in PBS at 5 × 10^6 cells/ml and incubated with 2.5 μM CFSE (Molecular Probes) at room temperature for 5–10 min. Cells were subsequently quenched in equal volume of PBS and washed twice with PBS. For diabetes experiments, purified T cells were left unstained and 3–5 × 10^6 T cells in equal numbers were injected i.v. into female NOD.scid mice (6–8 wk-old). For T cell migration studies, cells from spleen and PLN were isolated from recipients 4 days after T cell transfer and analyzed by flow cytometry, as described.

Analysis of diabetes

Glucose concentration in urine of mice was determined using Glucostix (Bayer) twice a week. Animals were classified as diabetic when urine glucose was >250 mg/dl. Diabetic mice also exhibited polyuria and weight loss.

Histology and immunohistochemistry

Spleen and pancreas were removed from recipient mice at indicated time points and fixed in 10% PBS formalin. Paraffin-embedded sections from each pancreas and spleen were stained with H&E and scored for histologic disease by a blinded observer. At least 20 islets per pancreas in at least two different sections of each organ were examined for the presence of mononuclear cell infiltrates. Peri-insulitis refers to the presence of few mononuclear cells outside of predominantly intact islets. Severe insulitis refers to the presence of cellular infiltrates with destruction of the entire islet. To determine T cell infiltrates, paraffin sections of pancreas and spleen were immunostained with anti-CD3 mAb and CD3+ cells were enumerated, as previously described (19).

Chemotaxis assay

Spleen cells (5 × 10^6 cells/ml) from BDC and BDC-Idd9 mice in 100 μl of DMEM complete were transferred to the upper chamber of 5 μm Transwells (Costar; Corning) in duplicates containing 500 μl of DMEM complete supplemented with 100 ng/ml each CCL19 (PeproTech), CCL21 (R&D Systems), or without chemokine in the lower chamber. Cells that had migrated to the lower chambers were collected after an incubation period of 2–3 h at 37°C. They were stained with mAbs specific for CD4 and TCR Vβ4, and double-positive cells were counted by flow cytometry.

Statistical analysis

Statistical significance of differences in data was determined by Student’s t test (two-tailed, unpaired). The Kaplan-Meier analysis was used to calculate diabetes incidence, and the log-rank test was used to determine its significance. A value of p < 0.05 was considered statistically significant.
Results

Normal development and selection of islet-specific T cells containing the B10-derived Idd9 interval

Thymi and spleens from BDC-Idd9 and BDC mice had comparable total numbers of cells: thymus, $2.0 \times 10^8 \pm 7.2 \times 10^7$ vs $2.0 \times 10^8 \pm 7.4 \times 10^7$ (n = 9 mice) and spleen, $2.9 \pm 1.2 \times 10^7$ vs $3.5 \pm 1.1 \times 10^7$ (n = 6 mice), respectively. Thymocytes in both lines were skewed toward the CD4$^+$ T cell population, which was expected because the transgenic BDC2.5 TCR was originally isolated from an MHC class II-restricted CD4$^+$ T cell clone. There were no apparent differences in the relative proportions of CD4$^+$/CD8$^-$, CD4$^+$/CD8$^-$, CD4$^-/CD8^+$, and CD4$^-$/CD8$^-$ thymocytes in BDC-Idd9 and BDC mice as determined by flow cytometry (Fig. 1A). In the spleen of both lines, the majority of T cells were CD4$^+$ and $>90\%$ of them expressed the transgenic TCR $\gamma$6 at similar levels. Flow cytometric analysis of the expression of the T cell activation markers CD25, CD62L, and CD69 demonstrated similar profiles between spleen cells from BDC and BDC-Idd9 mice, indicating that most islet-specific T cells in both transgenic lines were in a resting state (Fig. 1B). These data show that the Idd9 locus did not have an apparent effect on the development, selection or activation state of islet-specific transgenic T cells.

Effect of Idd9 on functional responses of islet-specific T cells

Because the Idd9 interval contains candidate genes that contribute to activation of cells (16), we determined whether there were differences between activation-induced proliferation and cytokine responses of BDC and BDC-Idd9 T cells.

We first analyzed T cell proliferation following Ag-specific T cell stimulation in splenocytes from BDC and BDC-Idd9 mice. As the natural ligand of the BDC2.5 TCR is unknown we used the recently identified BDC2.5 mimic peptide p79 to stimulate splenocytes from the two transgenic lines (18). T cells from both lines proliferated vigorously in response to the p79 mimic peptide. There was no significant difference in the proliferative response to p79 between BDC and BDC-Idd9 T cells except at the highest Ag concentration (1 $\mu$g/ml; $p < 0.05$) (Fig. 2A). We next determined cytokine production in the supernatants of p79-stimulated T cell cultures. Both BDC and BDC-Idd9 splenic T cells secreted IL-2 following stimulation with p79 but IL-2 concentrations were significantly reduced in supernatants of stimulated BDC cultures at each Ag concentration compared with those of BDC-Idd9 cultures ($p < 0.05$) (Fig. 2B). Significant amounts of IFN-γ and IL-4 were not detected in supernatants from either transgenic line confirming

**FIGURE 1.** Flow cytometry analysis of thymocytes and splenocytes in BDC and BDC-Idd9 mice. A, T cells from thymi and spleens of mice were stained with indicated Abs (FITC-conjugated anti-CD4, PE-conjugated anti-CD8, and anti-TCR $\gamma$6). Dot plots representing two-color flow cytometry analysis of BDC and BDC-Idd9 mice are shown. Values in quadrants represent percentage of live-gated cell populations. Values on top of each graph refer to total number of thymocytes (n = 9) or splenocytes (n = 6) ± SD. B, Expression of T cell activation markers on T cells from BDC-Idd9 and BDC mice. Live CD4$^+$ gated populations of spleen cells from BDC-Idd9 and BDC mice were assessed for the expression of CD25, CD62L, and CD69 (all PE-conjugated). Dotted lines show staining of isotype control Ab. Values in each histogram represent percentage of gated T cell populations for marker (M1).
that T cells from both lines had a naive phenotype. Taken together, these data demonstrate that T cells from BDC-Idd9 were not deficient in their proliferative or IL-2 response to BDC2.5 mimic peptide p79.

**Islet-specific T cells containing the B10 Idd9 interval delay induction of diabetes**

To directly determine the effect of Idd9 on the pathogenicity of islet-specific T cells, we transferred BDC and BDC-Idd9 T cells into NOD.scid mice, which develop neither spontaneous insulitis nor diabetes (20). Purified T cells from spleen of donor BDC-Idd9 and BDC mice that had been tested for the absence of urine glucose were used for the transfers. As shown in Fig. 1A, the spleens of these mice have equivalently high proportions of CD4+Vβ4+ islet-specific T cells. Within 2–3 wk following transfer, 70–80% of NOD.scid mice that had received T cells from BDC mice developed severe diabetes (Fig. 3). In contrast, recipients of T cells from BDC-Idd9 mice started to develop diabetes only 10 wk after transfer at an incidence of 25%. The diabetes incidence in these recipient mice reached a maximum of 50% by week 14 after T cell transfer. The time of diabetes onset between recipients of BDC and BDC-Idd9 T cells was significantly different as determined by Kaplan-Meier analysis (p = 0.001 by log-rank test). Both groups of recipient mice showed comparable severity of disease once they had developed diabetes (data not shown). Taken together, these data demonstrate that islet-specific T cells that contain the NOD-derived Idd9 induced diabetes in recipients rapidly, whereas those that contain the B10 Idd9 did so in a significantly delayed fashion.

**Impaired infiltration of pancreas and Langerhans islets by BDC-Idd9 T cells**

To determine whether the difference in diabetes onset in NOD.scid recipient mice was associated with the extent of infiltration and destruction of islets, we examined pancreata of recipients 10 days after T cell transfer by histology. Pancreata from recipients of T cells from BDC mice showed severe insulitis in 90% and peri-insulitis in 10% of the islets. In contrast, we found severe insulitis and peri-insulitis in only 39% and 52% of islets in recipients that had received BDC-Idd9 T cells, respectively (Fig. 4). Remarkably, the extent of insulitis and peri-insulitis detected even 30 days after the transfer of BDC-Idd9 T cells affected only 35 and 48% of the islets in recipients, respectively (data not shown).

To assess whether the extent of insulitis in NOD.scid recipient mice correlated with the infiltration of donor T cells, we examined pancreas sections of NOD.scid recipient mice 10 days after transfer of BDC and BDC-Idd9 T cells and determined the frequency of T cells by immunohistochemical detection of CD3+ cells. Pancreata of mice that had received BDC T cells contained significantly more CD3+ cells/mm² than pancreata of recipients of BDC-Idd9 T cells (p < 0.01) (Fig. 5A). Furthermore, T cells from BDC mice diffusely infiltrated the islets (Fig. 5C) whereas BDC-Idd9 T cells were localized more numerously in peri-insular areas of the pancreas in recipients (Fig. 5B). Thus, fewer numbers of T cells in the pancreas of NOD.scid mice that had received BDC-Idd9 donor T cells correlated with milder insulitis. Taken together, our data indicate that infiltration of the pancreas by BDC-Idd9 T cells was impaired in recipient mice.

**Idd9 affects migration of islet-specific T cells to secondary lymphoid organs**

Different mechanisms could be responsible for the impaired pancreatic infiltration of BDC-Idd9 compared with BDC T cells in NOD.scid recipients. As the Idd9 interval contains genes that contribute to cell activation, we hypothesized that the B10 Idd9 had an inhibiting effect on the migration of T cells in recipients. To test this hypothesis, we adoptively transferred CFSE-labeled T cells from nondiabetic BDC-Idd9 and BDC mice into NOD.scid or NOD mice. Flow cytometric analysis of donor T cells from BDC-Idd9 and BDC mice before transfers had revealed equivalent proportions of islet-specific T cells in both lines, most of which were in a resting state as indicated by their expression of T cell activation markers such as CD25, CD62L, and CD69 (Fig. 1B). Four days after T cell transfer, we identified both BDC-Idd9 and BDC CD4+ T cells in the spleens of NOD.scid mice by flow cytometry. Recipients that had received BDC-Idd9 T cells contained higher
proportions of CFSE+ CD4+ T cells in the spleen than recipients that had been injected with BDC T cells (0.6 vs 0.2%) (Fig. 6A). Immunohistochemical analysis of spleens at 10 days after T cell transfer revealed that most of the donor T cells accumulated in PALS of the recipient mice. Notably, splenic PALS in recipients of BDC-Idd9 T cells were strikingly larger than those of mice that had received BDC T cells (Fig. 6B). As Ag-specific priming of islet-reactive T cells presumably takes place in lymph nodes draining the pancreas (21), we also determined recruitment of BDC and BDC-Idd9 T cells to PLN in NOD recipients as described earlier. At 4 days after cell transfer we detected higher frequencies of CFSE+ CD4+ T cells from BDC than BDC-Idd9 donor mice in PLN of recipient mice (1.0 vs 0.6%) (Fig. 6A). CD4+ T cells that had migrated to the spleen and PLN by day 4 after transfer displayed similar distribution of CFSE fluorescence, indicating that BDC and BDC-Idd9 T cells had expanded comparably in the recipients (Fig. 6A). We also observed comparable dilution of CFSE fluorescence among transgenic donor CD4+ T cells in recipient mice at 8 days after T cell transfer (data not shown). Thus, the observed differences in T cell accumulation were not the result of different capacities to proliferate within the secondary lymphoid organs examined. Instead, these data demonstrate that the Idd9 locus affected the migration of T cells resulting in differential recruitment of BDC-Idd9 and BDC islet-specific T cells to spleen and PLN.

Expression of CCR7 and migration to CCR7 ligands by BDC and BDC-Idd9 T cells

A number of chemokine receptors and adhesion molecules are involved in T cell migration to secondary lymphoid organs and non-lymphoid tissues (22). For example, T cells that express high levels of CCR7 enter the lymph nodes, whereas T cells that lack or have down-regulated this receptor circulate into nonlymphoid tissues (23, 24). Furthermore, T cell migration from the red pulp to the T cell area of the spleen (PALS) is dependent on CCR7 because deficiency in CCR7 reduces T cell accumulation in these areas (23). As we observed different homing between BDC-Idd9 and BDC T cells, we hypothesized that this might be due to different surface expression of CCR7 in these T cells before adoptive T cell transfers. Flow cytometric analysis of resting T cells isolated from spleens of BDC and BDC-Idd9 mice revealed that the majority of them (68 and 76%, respectively) expressed CCR7.
positive selection or clonal deletion of BDC2.5 TCR transgenic T cells was not apparent because CD4⁺ T cells were efficiently selected at comparable proportions in the thymi and spleens of both BDC and BDC-Idd9 mice. The vast majority of peripheral CD4⁺ T cells from both lines expressed the transgenic TCR Vβ4 and had a naive activation state as determined by their surface expression of CD25, CD62L, and CD69 by flow cytometry.

The Idd9 locus contains several genes of the TNFR superfamily that are involved in cell activation and proliferation including Cd30, Cdl37, and Tnfr2 (16). Stimulation of spleen cells from both BDC and BDC-Idd9 mice with the BDC2.5 mimic peptide p79 resulted in comparable proliferative responses. Stimulated T cells secreted IL-2 but no detectable amounts of IFN-γ and IL-4, confirming their naive, undifferentiated phenotype. In agreement with a previous report that demonstrated reduced IL-2 production by NOD T cells in response to TCR-mediated stimulation (26), we detected lower concentrations of IL-2 in BDC than in BDC-Idd9 p79-stimulated T cell cultures.

We directly determined the diabetogenic function of BDC-Idd9 and BDC T cells by injecting them into NOD.scid mice, which are free from insulinitis and diabetes (27). BDC T cells rapidly induced overt diabetes in recipient mice within 3 wk after adoptive transfer. In striking contrast, T cells from BDC-Idd9 mice induced diabetes in a significantly delayed fashion starting at 10 wk after transfer only. Interestingly, once BDC-Idd9 T cells mediated diabetes, the incidence and severity of disease were not significantly different from that induced by BDC T cells, suggesting that once they entered the pancreas, the cells were equally diabetogenic.

Recent reports have shown that development of autoimmune diabetes can be controlled by regulatory CD4⁺ T cells, which express CD25 and/or CD62L (10, 28). Furthermore, IL-2 has been shown to be indispensable for the peripheral maintenance of CD4⁺CD25⁺ regulatory T cells in mice (29). As T cells from BDC-Idd9 mice secreted higher concentrations of IL-2 following p79 stimulation than those from BDC mice, we hypothesized that BDC and BDC-Idd9 mice harbored different frequencies of regulatory T cells. However, CD4⁺ T cells from BDC-Idd9 and BDC mice had comparable proportions of CD25⁺ (8 and 9%, respectively) and CD62L⁺ cells (77 and 72%, respectively). This argues against the likelihood that increased numbers of CD4⁺CD25⁺ or CD4⁺CD62L⁺ regulatory BDC-Idd9 T cells were responsible for the delayed onset of diabetes in recipient mice. As early as 10 days following transfer of BDC T cells, recipients showed a higher incidence of islet destruction than in recipients of BDC-Idd9 T cells. Furthermore, the extent of severe and peri-insulitis mediated by T cells from BDC-Idd9 mice did not change significantly over the examined period of 30 days after the adoptive cell transfer. Consistent with this finding, we detected 50% fewer BDC-Idd9 than BDC-derived donor T cells/mm² of pancreatic tissue as determined by immunohistochemistry, indicating that infiltration of the pancreas in recipient mice by BDC-Idd9 donor T cells was less efficient and was likely the major factor contributing to the later onset of disease. However, we cannot formally exclude the possibility that regulatory T cells from BDC-Idd9 mice that might be recruited to the pancreas have stronger regulatory functions than regulatory T cells from BDC mice, possibly due to higher IL-2 production following islet-Ag recognition.

To determine the cellular mechanism for the delayed diabetes onset and impaired pancreatic infiltration by BDC-Idd9 T cells, we conducted a series of adoptive transfers with CFSE-labeled T cells from BDC and BDC-Idd9 mice into NOD.scid or NOD recipient mice. We detected higher proportions and larger numbers of transferred BDC-Idd9 than BDC T cells in the spleens of recipient mice by flow cytometry and immunohistochemistry, respectively. The
immunohistochemical analysis of CD3+ cells in spleens of recipient mice further revealed a striking difference in the sizes of PALS among recipients of BDC and BDC-Idd9 T cells. Whereas recipients of BDC T cells had small splenic PALS, mice that had received BDC-Idd9 T cells had larger T cell areas.

Conversely, BDC T cells were found at higher proportions than BDC-Idd9 T cells in PLN of recipients 4 days after T cell transfer. We detected similar division of CD4+ T cells as determined by transferred CFSE-labeled T cells in PLN and spleen, indicating that the different frequencies of BDC and BDC-Idd9 T cells detected in the examined tissues was not due to differential proliferation or expansion in the recipient mice. Instead, our findings indicate that transferred T cells from BDC and BDC-Idd9 mice differed in their homing patterns in recipient mice. Taken together, these data show that T cells containing the B10-derived Idd9 locus were impaired in their migration to both PLN and pancreas, which correlated with significantly decreased numbers of pancreatic T cell infiltrates and milder insulitis in recipient mice.

Accumulation of T cells in secondary lymphatic organs is a migration dependent process that requires the interactions of adhesion molecules and chemokine receptors. CD62L and CCR7 are critical for the homing of naive T cells from blood into peripheral lymph nodes via high endothelial venules and splenic PALS (23, 30). The vast majority of CD4+ T cells from BDC and BDC-Idd9 mice expressed both receptors and at comparable levels. However, BDC-Idd9 T cells showed stronger chemotactic reactivity in vitro than BDC T cells toward CCL19, which is constitutively expressed by stromal cells in T cell areas of secondary lymphoid organs. We found stronger CCR7-mediated chemotaxis by BDC-Idd9 T cells, which may explain their increased accumulation in the splenic PALS in recipients compared with BDC T cells. In contrast, differential migration between BDC and BDC-Idd9 T cells to PLN may be considered unexpected, given their comparable expression of CCR7 and CD62L.

The vast majority of CD4+ T cells from BDC and BDC-Idd9 mice might diferentially express chemokine receptors such as CXCR3, CCR4 and CCR5, all of which have been shown to be involved in pancreatic T cell infiltration and development of insulitis or diabetes (31–33). Furthermore, expression of these surface molecules on T cells changes on Ag encounter in secondary lymphoid organs resulting in different T cell migration patterns (34). Naive BDC-Idd9 T cells were more sensitive to Ag stimulation than BDC T cells as judged by their IL-2 production following stimulation with the BDC2.5 mimic peptide p79. Thus, it is possible that upon encounter with islet Ag in recipient mice, BDC-Idd9 T cells have a higher state of activation than BDC T cells. Differences in the activation state between BDC-Idd9 and BDC T cells in vivo may result in their differential expression of chemokine receptors or adhesion molecules and may thereby contribute to differences in T cell migration patterns. Our present findings suggest that differential chemotactic reactivity to CCL19 between transferred BDC and BDC-Idd9 T cells is at least partly responsible for the observed T cell homing patterns in NOD.scid recipients. These results may help to identify new candidate genes within the Idd9 locus that contribute directly or indirectly to migration of islet-specific T cells and the subsequent development of autoimmune diabetes.

**Acknowledgments**

We thank Lea Cefalu and Christina Rossi for technical help and Drs. Diane Mathis and Christophe Benoist for their generous gift of BDC2.5 TCR transgenic mice. We also thank Drs. Linda Wicker and Christophe Benoist for reading the manuscript.

**FIGURE 7.** Expression of CCR7 and chemotaxis to CCR7 ligands by BDC and BDC-Idd9 T cells. A. CD4+ gated populations of spleen cells from BDC-Idd9 and BDC mice were assessed for the expression of CCR7 (filled histogram). The staining of the isotype control Ab (dotted histogram) is also shown. Values (inset) represent the percentage of CCR7+ cells and the mean fluorescent intensity (MFI) in live-gated CD4+ T cell population with marker (M1). B. Spleen cells from BDC and BDC-Idd9 mice were used in Transwell chemotaxis assays in presence of the CCR7 ligands, CCL19 and CCL21 (100 ng/ml), or of medium as control. Cells that migrated to lower chambers were stained with mAbs for CD4 and TCR Vβ4, and double-positive cells were counted by flow cytometry. Mean number of migrated transgenic T cells + SEM are shown and represented as histograms. One of three independent experiments with similar data is shown. *p < 0.05.
Disclosures
The authors have no financial conflict of interest.

References


