Original Article

Bone Marrow Is a Preferential Homing Site for Autoreactive T-Cells in Type 1 Diabetes

Ruobing Li, Nicolas Perez, Subha Karumuthil-Melethil, and Chenthamarakshan Vasu

OBJECTIVE—The pancreatic microenvironment is considered to be the primary location of autoreactive T-cells in type 1 diabetes. Diabetogenic T-cells have also been detected in the spleens of NOD mice. However, it is not known whether bone marrow also contains T-cells specific for self-antigens in hosts with autoimmunity. In this study, we investigated whether autoreactive diabetogenic T-cells are present in the bone marrow of NOD mice.

RESEARCH DESIGN AND METHODS—Bone marrow and splenic T-cells of female NOD mice were purified and tested for their cytokine secretion and proliferation in response to stimulation with immunodominant peptides of pancreatic β-cells. The diabetogenic nature and homing properties of purified bone marrow T-cells were compared with those of splenic T-cells in NOD-*Scid* and wild-type mice.

RESULTS—The bone marrow T-cells from both hyperglycemic and young euglycemic mice demonstrated profoundly higher proliferation and cytokine production in response to stimulation with β-cell antigens than T-cells from spleen. Bone marrow T-cells showed rapid expansion and aggressive infiltration into pancreatic islets in NOD-*Scid* mice and induced hyperglycemia earlier than splenic T-cells. Adoptive transfer of bone marrow T-cells resulted in their trafficking predominantly to bone marrow and pancreatic lymph nodes.

CONCLUSIONS—Our study demonstrates that a large number of diabetogenic T-cells are present in the bone marrow of female NOD mice and that these autoreactive T-cells can be detected long before clinical onset of the disease. ***Diabetes*** **56:2251–2259, 2007**

Type 1 diabetes is caused by autoreactive T-cell–mediated destruction of insulin-producing pancreatic islet β-cells. T-cells reactive against β-cell antigens can be detected in the pancreatic microenvironment as early as 4 weeks of age in NOD mice, and this number gradually increases and peaks at the hyperglycemic stage (1–3). Although the pancreas and pancreatic lymph nodes have been considered the major sites of priming and expansion of β-cell antigen–reactive T-cells (4,5), these autoreactive T-cells have also been found in other secondary lymphoid organs (SLOs), such as the spleen and peripheral lymph nodes (6,7). Whereas splenic T-cells from NOD mice are capable of inducing diabetes in syngeneic immune-deficient mice, peripheral lymph node T-cells are less effective in transferring the disease. This suggests the presence of a higher frequency of autoreactive T-cells in the spleen (8,9). Recent studies have shown the importance of pancreatic and gut-associated lymph nodes in priming diabetogenic β-cell–reactive T-cells (4,10,11). However, the role of an important central lymphoid organ, bone marrow, in priming and/or maintaining autoreactive T-cells is not known.

It is well established that T-cell precursors that originate in bone marrow migrate to the thymus for further development. Recent studies have shown that mature T-cells often traffic from the periphery to bone marrow and vice versa (12,13). Significant numbers of mature $CD4^+$ and $CD8^+$ T-cells have been detected in bone marrow throughout the life of both humans and animals (14,15). Interestingly, recent studies have demonstrated that bone marrow not only hosts a large number of memory T-cells but can also initiate primary immune response to foreign antigens (16,17).

In light of reports that bone marrow provides a conducive environment for the long-term maintenance of memory lymphocytes against foreign antigens (18–21), we investigated whether self-antigen–specific T-cells accumulate in the bone marrow of mice with autoimmune disease. Because T-cells reactive against pancreatic β-cells play an important role in the pathogenesis of type 1 diabetes, we focused our attention on a spontaneous model of type 1 diabetes. Moreover, because infiltration of autoreactive T-cells in pancreatic islets occurs long before hyperglycemia is detected, it is possible that a portion of these self-antigen–specific T-cells trafficks into bone marrow.

Although recent studies have shown that β-cell antigen–specific T-cells in the periphery can be quantified to predict type 1 diabetes (22,23), additional approaches to detect significant numbers of autoreactive T-cells in accessible anatomical locations during early insulitis will be extremely useful for initiating therapy at a prehyperglycemic stage. In this study, we report that bone marrow is a preferential homing site for β-cell antigen–reactive diabetogenic T-cells in female NOD mice and that these T-cells can be detected in significant numbers several weeks before the clinical stage of the disease. Further, bone marrow T-cells from female NOD mice appeared to be

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Received for publication 16 April 2007 and accepted in revised form 17 June 2007.

Published ahead of print at http://diabetes.diabetesjournals.org on 27 June 2007. DOI: 10.2337/db07-0502.

Additional information for this article can be found in an online appendix at http://dx.doi.org/10.2337/db07-0502.

APC, antigen-presenting cell; CFSE, carboxyfluorescein succinimidyl ester; FACS, fluorescence-activated cell sorting; IFN-γ, γ interferon; IL, interleukin; MHC, major histocompatibility complex; SLO, secondary lymphoid organ; TCR, T-cell receptor.

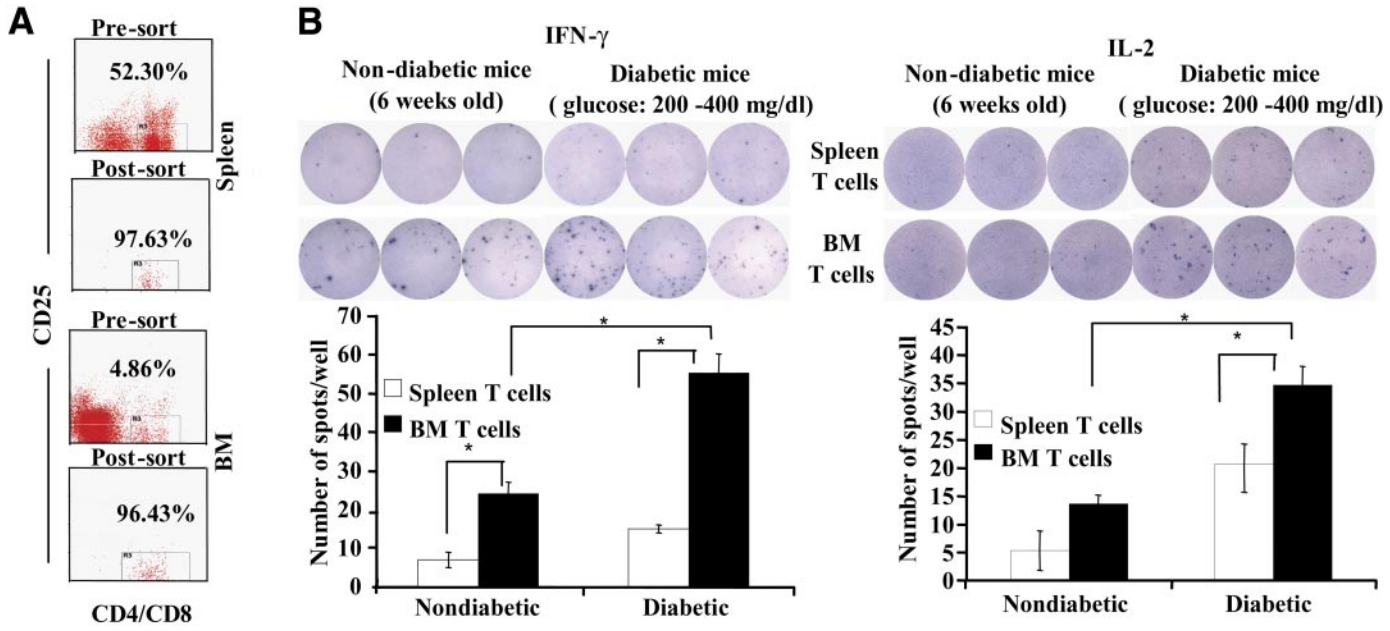

FIG. 1. Bone marrow (BM) in NOD mice houses T-cells specific for pancreatic β-cell antigens. EliSpot assay was performed to examine self-antigen–specific T-cells. *A*: Spleen and bone marrow cells from euglycemic (6-week-old) and hyperglycemic (12- to 15-week-old, glucose levels 200–400 mg/dl) NOD mice were stained with a mixture of fluorescein isothiocyanate–labeled anti-CD4 and CD8 antibodies and phycoerthrin-labeled anti-CD25 antibody, and the CD25$^-$ fraction of T-cells was sorted by FACS. *B*: EliSpot assay was carried out using anti–IFN-γ– and anti–IL-2 antibody–coated plates and purified T-cells in the presence of APCs and β-cell peptide mixture for 16 h. At the end of the assay, EliSpot plates were scanned and read using an EliSpot reader. Representative triplicate wells where 2 × 10^5 T-cells/well were used are shown. Each bar in the bar diagrams represents the mean ± SD of the number of spots using T-cells from three pooled samples (each pooled sample contained cells from four to five mice) in triplicate. The assay was repeated three times. *Statistically significant at $P < 0.05$.

immigrants from the periphery and showed the ability to traffic preferentially to both bone marrow and pancreatic lymph nodes.

RESEARCH DESIGN AND METHODS

Wild-type female NOD/LtJ, NOD.BDC2.5 TCR-Tg (T-cell receptor transgenic), and NOD-*Scid* mice were purchased from The Jackson Laboratory. Glucose levels in the tail vein blood samples of wild-type and NOD-*Scid* mice were monitored with Ascensia Microfill blood glucose test strips and glucose meter (Bayer).

Antibodies and peptide antigens. Fluorochrome-labeled antibodies for T-cell markers were purchased from Caltag Laboratories, eBioscience, and BD Pharmingen. Major histocompatibility complex (MHC) II–restricted immunodominant β-cell antigen peptides, namely insulin B$_{(9–23)}$, GAD65$_{(206–220)}$, GAD65$_{(524–543)}$, IA-2β$_{(755–777)}$, and IGRP$_{(123–145)}$, and BDC2.5 peptide (YVRPLWVRME), described in earlier studies (24–30), were custom synthesized (Genescript) and used in this study.

Flowcytometry and high-speed sorting. Single-cell suspensions of spleen, bone marrow, peripheral lymph nodes (auxiliary, brachial, inguinal, and lumbar), and pancreatic lymph nodes were tested separately. For blocking the Fc receptor, cells were incubated with anti-CD16/CD32 antibody for 15 min. Subsequently, these cells were stained with fluorochrome-labeled antibodies, washed, and analyzed using an LSR Cytometer (BD Biosciences). Data were analyzed using CellQuest Pro, WinMdi, or Weasel applications. Splenocytes and bone marrow cells from nondiabetic (glucose <100 mg/dl) and diabetic (glucose >200 mg/dl for at least 2 consecutive weeks) mice were stained with fluorescein isothiocyanate–labeled anti-mouse CD4 and CD8 antibody mixture and phycoerthrin-labeled anti-mouse CD25 antibody, and CD25$^-$ T-cells were isolated by fluorescence-activated cell sorting (FACS) using a MoFlo high-speed sorter.

CFSE staining and T-cell proliferation assay. Cells were stained with 5 μmol/l CFSE (carboxyfluorescein succinimidyl ester) (1 × 10^7 cells/ml in PBS) for 15 min at room temperature. For proliferation assay, CFSE-labeled cells were incubated (5 × 10^5 cells/well) in 96-well flat-bottom plates in RPMI-1640 medium supplemented with 2% mouse serum with or without an equal molar mixture of immunodominant peptides (10 μg/ml). CD11c$^+$ dendritic cells (5 × 10^4 cells/well) enriched from spleens of young (4–6 weeks old) NOD mice (using a magnetic cell-sorting system) were used as antigen-presenting cells (APCs). CFSE dilution in CD4$^+$ T-cells was tested by FACS on day 5.

Cytokine analysis. Cytokine-secreting cells were detected using EliSpot assay kits (eBiosciences). Polyvinylidine fluoride membrane–based 96-well plates (Millipore) were coated with anti–interleukin (IL)-2 or –γ-interferon (IFN-γ) antibody and incubated with varying numbers (1 × 10^3 to 2 × 10^5) of T-cells enriched from bone marrow and spleen in the presence or absence of peptide-pulsed dendritic cells (5 × 10^4 cells/well) for 16 h at 37°C. Plates were developed following the manufacturer's instructions and read using an EliSpot reader at Cellular Technologies.

Adoptive transfer of T-cells. Purified BDC2.5 TCR-Tg CD4$^+$ T-cells or total T-cells from wild-type mice were labeled with CFSE and injected intravenously (2 × 10^6 cells/mouse) into prehyperglycemic wild-type mice. An adoptive transfer experiment was also carried out to test the diabetogenic potential of T-cells from bone marrow and spleen. Purified T-cells were injected intravenously (2 × 10^5 cells/mouse) into 7- to 8-week-old NOD-*Scid* mice, and the mice were monitored for glucose levels every 7 days until the first set of spleen T-cell recipients showed hyperglycemia (glucose >250 mg/dl). Sets of control mice and T-cell–recipient euglycemic mice were killed at weeks 1, 7, and 13, and lymphoid and pancreatic tissues were characterized by FACS or histochemical staining.

Histochemical analysis of pancreatic tissues. Pancreata were fixed in 10% formaldehyde, and 5-μm paraffin sections were cut and stained with hematoxylin and eosin. Stained sections were examined in a blinded fashion using a grading system in which 0 = no evidence of infiltration, 1 = peri-islet infiltration, 2 = 25% infiltration of each islet, 3 = >25–50% infiltration of each islet, 4 = >50% infiltration of each islet, and 5 = complete loss or only remnants of islets seen. Approximately 100 islets were examined for every group.

Statistical analysis. Means ± SD and statistical significance (P value) were calculated using Microsoft Excel or SSPS. $P \leq 0.05$ was considered significant.

RESULTS

Bone marrow houses a large number of T-cells specific for β-cell antigens. To test for the presence of T-cells specific for self-antigens, EliSpot assay was carried out using flow-sorted CD25$^-$ T-cells (Fig. 1*A*). CD25$^+$ T-cells were excluded to avoid the discrepancy in the results because bone marrow contains a significantly higher ratio of CD25$^+$ to CD25$^-$ T-cells compared with spleen (online appendix Fig. 1 [available at http://dx.doi.

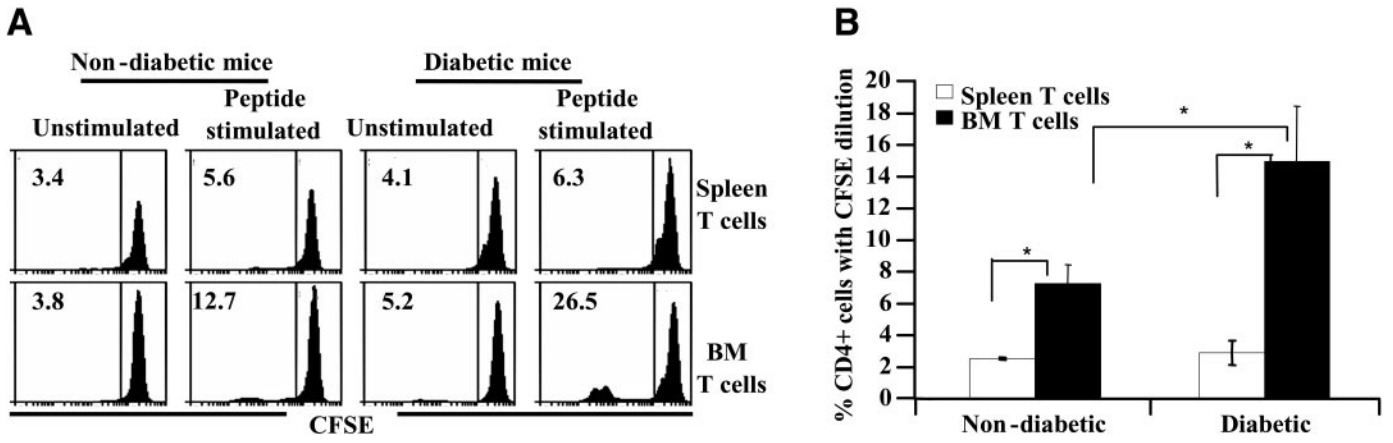

FIG. 2. Bone marrow (BM) T-cells significantly proliferated following stimulation with β-cell antigens. CFSE dilution assay was conducted to examine bone marrow and splenic T-cell proliferation in response to β-cell antigen peptides. Splenic and bone marrow cells from euglycemic (6-week-old) and hyperglycemic (12- to 15-week-old; glucose levels 200–400 mg/dl) NOD mice were stained with a mixture of fluorescein isothiocyanate–labeled anti-CD4 and CD8 antibodies and phycoerthrin-labeled anti-CD25 antibody, and the $CD25^-$ fraction of T-cells was sorted by FACS. Proliferation assay was carried out by incubating CFSE-labeled T-cells (5×10^5 cells/well) in the presence of APCs (5×10^4 cells/well) and the peptide mixture (10 μg/ml). After 5 days, cells were stained with phycoerthrin-labeled anti-CD4 antibody and examined for CFSE dilution in the $CD4^+$ population. Representative histograms are shown. Each bar in the bar diagram represents the mean ± SD of $CD4^+$ T-cells with CFSE dilution using T-cells from three pooled samples (each pooled sample contained cells from three to four mice) in triplicate. The assay was repeated three times. *Statistically significant at $P < 0.05$.

org/10.2337/db07-0502]). Specifically, $CD25^-$ T-cells were incubated in the presence of purified splenic dendritic cells as APCs and the peptide mixture for 16 h so that only antigen-experienced T-cells can produce detectable levels of IFN-γ and IL-2 within this short period. As seen in Fig. 1*B*, bone marrow T-cells from 6-week-old NOD mice demonstrated a significant number ($P = 0.032$) of T-cells responding to peptide mix compared with T-cells from the spleen. Specifically in 6-week-old NOD mice, the frequency of detectable IFN-γ–producing cells in response to peptide stimulation was ~25 per 2×10^5 bone marrow T-cells compared with <10 per 2×10^5 splenic T-cells. This difference was significantly high ($P = 0.007$) in hyperglycemic mice. Whereas <20 T-cells for 2×10^5 splenic T-cells produced detectable levels of IFN-γ following exposure to self-peptides, >65 IFN-γ–producing cells were detected per 2×10^5 bone marrow T-cells (Fig. 1*B*, *left*). In addition, examination of IL-2–producing T-cells in spleen and bone marrow showed a trend similar to that of IFN-γ–producing cells (Fig. 1*B*, *right*). Both bone marrow and splenic T-cells depleted of $CD4^+CD25^+$ T-cells failed to produce significant amounts of IL-4, IL-10, and transforming growth factor-β1 (not shown).

Bone marrow T-cells profoundly proliferate following exposure to β-cell antigens. To further examine the self-antigen–specific response of T-cells, proliferation assay was carried out using the CFSE dilution method. CFSE-labeled purified bone marrow and splenic T-cells were cultured in the presence of dendritic cells and the β-cell antigen peptides for 5 days and tested for $CD4^+$ T-cells with CFSE dilution. $CD4^+$ T-cells from the bone marrow of both 6-week-old and hyperglycemic NOD mice showed a significantly higher ability to proliferate against β-cell antigens than splenic T-cells ($P = 0.023$ and $P = 0.0037$, respectively) (Fig. 2*A* and *B*). Bone marrow $CD4^+$ T-cells from hyperglycemic mice showed a significantly higher number of $CD4^+$ T-cells with CFSE dilution than those from 6-week-old NOD mice ($P = 0.0061$). However, splenic $CD4^+$ T-cells from 6-week-old euglycemic and hyperglycemic (>12-week-old) mice did not show a major difference in antigen-induced proliferation. Euglycemic mice of similar age as the hyperglycemic mice that were used in this experiment also showed a similar antigen-specific T-cell response (not shown).

Bone marrow T-cells are more potent in inducing hyperglycemia than splenic T-cells. Because bone marrow demonstrated a profoundly higher number of self-antigen–specific T-cells than spleen, we examined whether these T-cells are diabetogenic and compared them with the known diabetogenic nature of splenic T-cells (5,7–9). Purified bone marrow and splenic T-cells from prehyperglycemic (10- to 12-week-old) NOD mice were transferred intravenously into 8-week-old NOD-*Scid* mice (2×10^5 cells/mouse), and the mice were monitored for blood glucose levels. As observed in Fig. 3, 80% of the mice that received bone marrow T-cells turned hyperglycemic within 5–9 weeks post–adoptive transfer, whereas mice that received splenic T-cells began to show hyperglycemia only at week 13. Pancreatic tissues from these mice were tested for signs of insulitis.

Bone marrow T-cells show aggressive infiltration into islets compared with splenic T-cells. To examine the kinetics of T-cell infiltration in the islets of bone marrow and splenic T-cell recipients, purified bone marrow and splenic T-cells from prehyperglycemic (10- to 12-week-old) NOD mice were transferred intravenously into 8-week-old NOD-*Scid* mice (2×10^5 cells/mouse) as described above. Sets of mice from each group were killed at week(s) 1, 3, and 13 posttransfer and pancreatic sections examined for insulitis. While pancreatic islets of bone marrow T-cell recipients showed lymphocyte infiltration as early as day 7 after the adoptive transfer, islets of splenic T-cell recipients had no detectable infiltration, even after 21 days (Fig. 4*A* and *B*). In mice that received bone marrow T-cells, >80% of their islets showed significant infiltration of T-cells (grades 1–4 insulitis), whereas in mice that received splenic T-cells, islets appeared to be infiltration free (grade 0). Because ~80% of the bone marrow T-cell recipient mice became hyperglycemic by week 13 posttransfer, the rest of the mice (euglycemic) along with euglycemic mice from other groups were tested for insulitis. Mice that received bone marrow T-cells

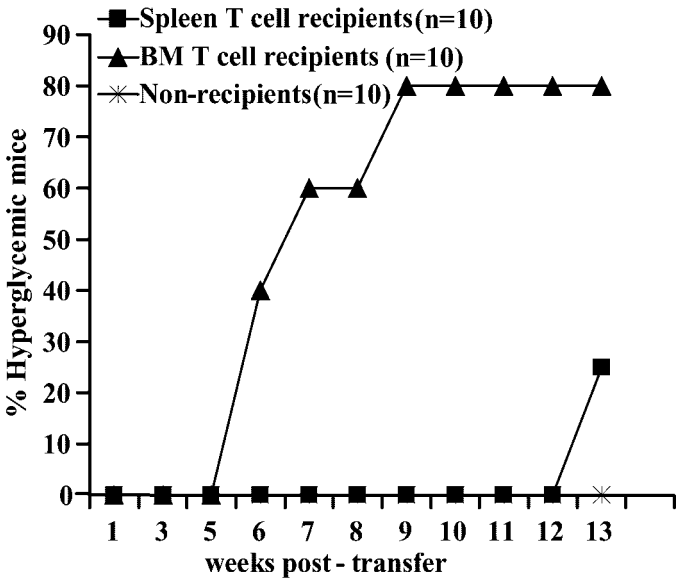

FIG. 3. Bone marrow (BM) T-cells induce rapid hyperglycemia in NOD-*Scid* mice. FACS-sorted T-cells from prehyperglycemic NOD mice (10–12 weeks old; euglycemic) were intravenously transferred into NOD-*Scid* mice (2×10^5 cells/mouse; $n = 10$/group), and glucose levels were monitored every week. Mice that showed glucose levels >250 mg/dl for 2 consecutive weeks were considered hyperglycemic.

showed glucose levels between 100 and 130 mg/dl. These mice showed a significantly higher number (~80%) of islets with severe cellular infiltration and destruction (grades 3–5 insulitis) compared with the mice that received splenic T-cells (~25% islets with grades 3–5 infiltration/destruction) (Fig. 4*B*). Spleen T-cell recipients had glucose levels between 80 and 100 mg/dl.

Bone marrow and splenic T-cells undergo lymphopenia-driven expansion differently. Because bone marrow T-cells showed the ability to induce hyperglycemia more rapidly than splenic T-cells, adoptively transferred cells were tested for their dynamics and trafficking properties in NOD-*Scid* mice. For this purpose, NOD-*Scid* mice were injected intravenously with bone marrow or splenic T-cells from prehyperglycemic mice. Sets of these mice were killed 1, 3, and 13 weeks later and their spleen and lymph nodes tested for $CD4^+$ and $CD8^+$ T-cells by FACS. Mice that received splenic and bone marrow T-cells showed different abilities to expand in the lymphopenic condition than NOD-*Scid* mice. Both $CD4^+$ and $CD8^+$ T-cells from the donor bone marrow expanded more rapidly than $CD4^+$ and $CD8^+$ splenic T-cells in the NOD-*Scid* mice (Fig. 5). Specifically, while ~13% of spleen and ~47% of lymph node cells of bone marrow T-cell recipients were $CD4^+$ T-cells by 3 weeks posttransfer, splenic $CD4^+$ T-cells expanded to only <4 and 14%, respectively. By week 13, >50% of spleen and lymph node cells of bone marrow T-cell–recipient mice were $CD4^+$ T-cells, whereas $CD8^+$ T-cells constituted only ~8% of splenic and 12% of the lymph node cells. Intriguingly, bone marrow $CD8^+$ T-cells demonstrated a rapid increase in the pancreatic lymph nodes but not in the peripheral lymph nodes and spleen ($P < 0.032$). Furthermore, the increase in the $CD4^+$ T-cell numbers in the pancreatic lymph nodes of bone marrow T-cell recipients was several folds higher than that present in those of the splenic T-cell recipients ($P = 0.0002$). These results show that T-cells from bone marrow and spleen have different properties in their ability to proliferate under lymphopenia conditions. This difference in the expansion property may be directly or indirectly contributing to the ability of bone marrow T-cells to accumulate in the islets and destroy β-cells rapidly in comparison with splenic T-cells.

NOD bone marrow T-cells home primarily to bone marrow and pancreatic lymph nodes. To determine whether the trafficking properties of bone marrow and splenic T-cells are different, CFSE-labeled T-cells from prehyperglycemic NOD mice were adoptively transferred into mice of the same age-group. The recipient mice were

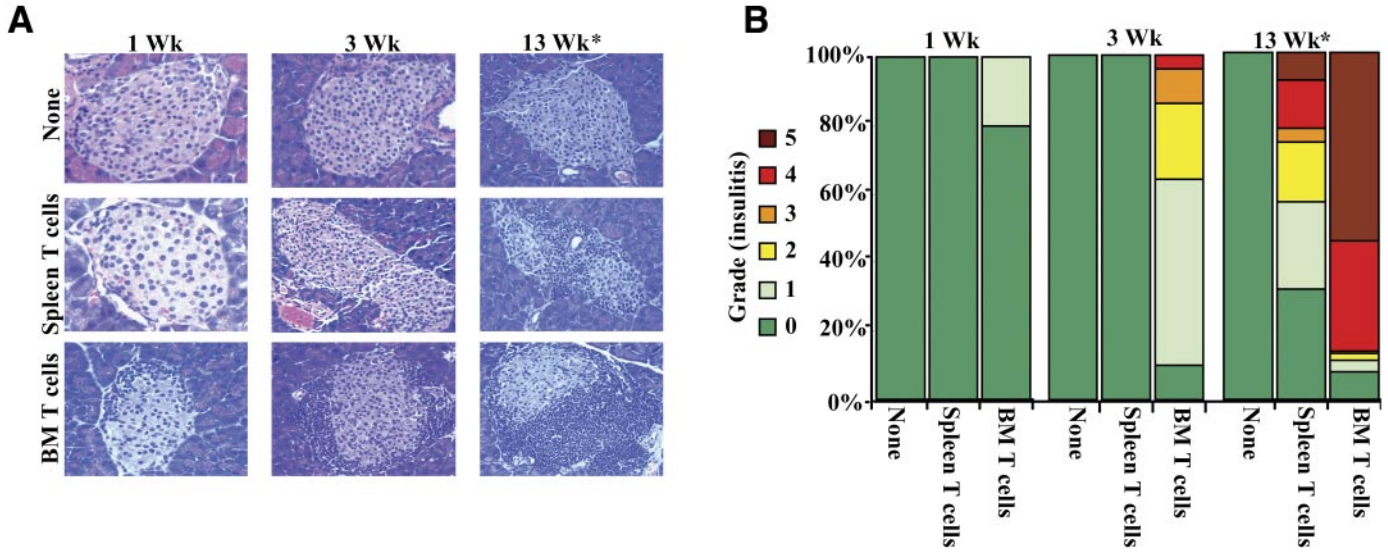

FIG. 4. Bone marrow (BM) T-cells show earlier and more profound infiltration into islets than splenic T-cells. FACS-sorted T-cells from 10- to 12-week-old euglycemic mice were transferred intravenously into NOD-*Scid* mice (2×10^5 cells/mouse), and glucose levels were monitored every week. Sets of three recipient mice per group were examined for insulitis at 1, 3, and 13 week(s) posttransfer. Hematoxylin and eosin–stained pancreatic sections were examined in a blinded fashion, and the severity of lymphocyte infiltration was scored as described in RESEARCH DESIGN AND METHODS. Representative islets are shown in *A*. The percentages of islets with different levels of lymphocyte infiltration are shown in *B*. One hundred islets were examined for each group. *Only the euglycemic mice (three per group) were examined at week 13, results shown here. The assay was repeated using the same number (three per group), with similar results (a total of six mice per group tested for each time point). (Please see http://dx.doi.org/10.2337/db07-0502 for a high-quality representation of this figure.)

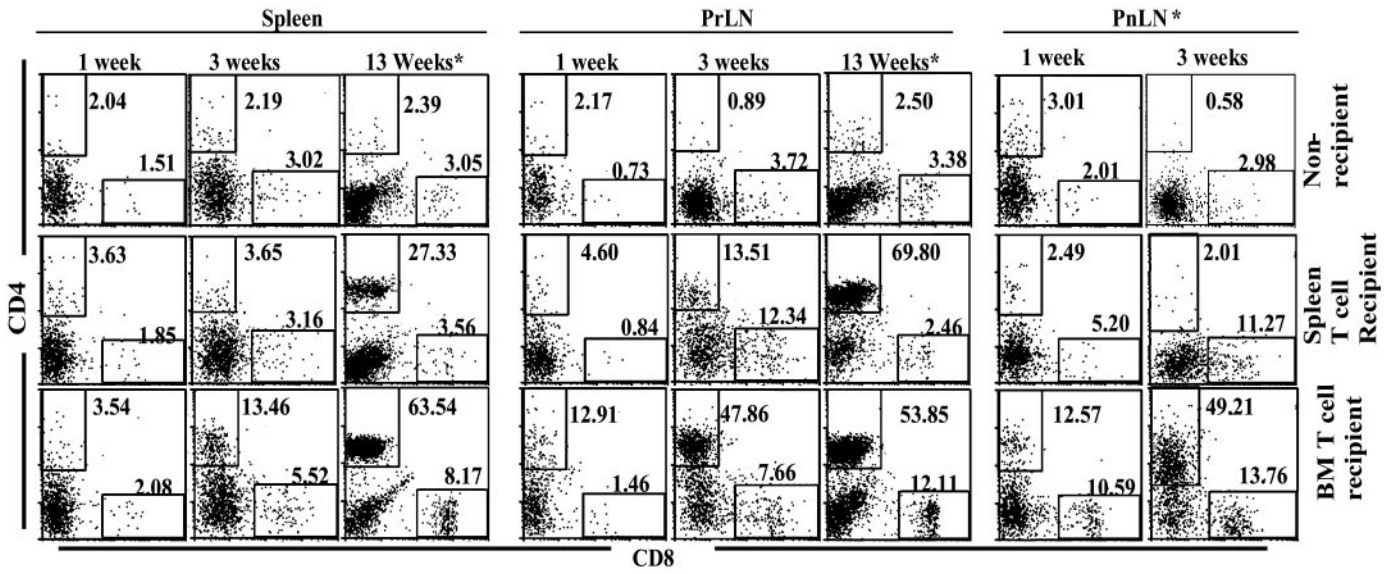

FIG. 5. Bone marrow (BM) T-cells expand more rapidly than splenic T-cells under lymphopenic condition. FACS-sorted bone marrow and splenic T-cells were transferred intrevenously in NOD-*Scid* mice (2×10^5 cells/mouse). Sets of three mice per group were killed 1, 3, and 13 week(s) posttransfer and splenic, peripheral (PrLN), and pancreatic (PnLN) lymph nodes tested for $CD4^+$ and $CD8^+$ T-cells by FACS. Dead cells and debris (gating on forward/side scatter graph) were excluded in the panels shown here. Percentages of cells positive for CD4 or CD8 are shown. Pancreatic lymph node cells were tested only from mice that were killed 1 and 3 week(s) posttransfer. *Only the euglycemic mice (three per group) were examined on week 13. The assay was repeated twice and carried out in triplicate for every mouse with similar results (at least six mice per group tested for each time point) and the representative values shown.

killed 24 h later and cells from different lymphoid organs examined for donor cells ($CFSE^+$) by FACS. As seen in Fig. 6, both $CD4^+$ and $CD8^+$ T-cells of donor origin trafficked into spleen, lymph nodes, and bone marrow within 24 h. Interestingly, mice that received bone marrow T-cells demonstrated a significantly higher number of donor T-cells in the bone marrow, as well as in the pancreatic lymph nodes, than mice that received splenic T-cells. In fact, a major part of the adoptively transferred donor T-cells migrated to pancreatic lymph nodes and bone marrow. Both $CD4^+$ and $CD8^+$ T-cells from the bone marrow migrated preferentially into pancreatic lymph nodes and bone marrow. Interestingly, while $CD4^+$ T-cells of bone marrow origin trafficked predominantly into pancreatic lymph nodes (46%) rather than into bone marrow (37%), $CD8^+$ T-cells showed a preference to bone marrow (48%) over pancreatic lymph nodes (24%). These observations suggest that the self-antigen specificity of bone marrow T-cells may be partially responsible for their preferential trafficking into the pancreatic microenvironment.

Diabetogenic T-cell receptor–expressing T-cells accumulate in bone marrow and pancreatic lymph nodes. To further examine the trafficking property of diabetogenic T-cells, $CD4^+$ T-cells from various lymphoid organs of NOD.BDC2.5 TCR-Tg mice were examined for the surface marker CD62L. $CD4^+$ T-cells from these transgenic mice homogenously express a diabetogenic Vβ4 T-cell receptor (TCR) specific for a mimitope, and autoreactive T-cells against this epitope have been detected in NOD mice. As observed in Fig. 7, while >60% of both bone marrow and pancreatic lymph node $CD4^+$ T-cells from 6-week-old mice showed memory phenotype (low CD62L expression), >90% of bone marrow and pancreatic lymph node T-cells from 20-week-old mice showed low CD62L expression. At each time point, cells expressing memory phenotype were lower in the spleen and peripheral lymph nodes than in the pancreatic lymph nodes and bone marrow. This observation shows that diabetogenic TCRs expressing memory T-cells are preferentially migrating to the pancreatic microenvironment and bone marrow.

Bone marrow T-cells with antigen specificity may be migrants from the periphery. To examine whether antigen-specific T-cells are expanded in bone marrow due to resident antigen presentation, CFSE-labeled BDC2.5 TCR-Tg $CD4^+$ T-cells were transferred intravenously into wild-type NOD mice of different age-groups, killed 5 days after the transfer, and tested for CFSE dilution in the donor T-cells. All age-groups of mice that received BDC2.5 peptide demonstrated proliferation of TCR-Tg cells in SLOs, as well as in bone marrow, demonstrating the ability of APCs in different organs to present antigen efficiently to these T-cells (Fig. 8). However, only the pancreatic lymph nodes, not other tissues, of mice that received control peptide showed a significant number of donor T-cells proliferating in the absence of exogenous cognate antigen. Further, older and hyperglycemic mice demonstrated significantly higher proliferation of donor T-cells, suggesting an increasing endogenous self-antigen presentation in the pancreatic microenvironment in NOD mice as they get older. However, TCR-Tg T-cells that trafficked into the bone marrow proliferated only in the presence of exogenously delivered cognate antigen, even in the hyperglycemic mice. This suggests that bone marrow does not support endogenous self-antigen presentation and the expansion of T-cells specific for β-cell antigens. Therefore, bone marrow–resident T-cells may have acquired self-antigen specificity in the pancreatic microenvironment before trafficking into the bone marrow. Moreover, a relatively higher number of β-cell–reactive T-cells in bone marrow than in spleen may be the result of preferential trafficking, gradual accumulation, and long-term homeostatic maintenance in this primary lymphoid organ.

DISCUSSION

Although the importance of bone marrow in hematopoiesis is well known, its function in T- and B-cell–mediated

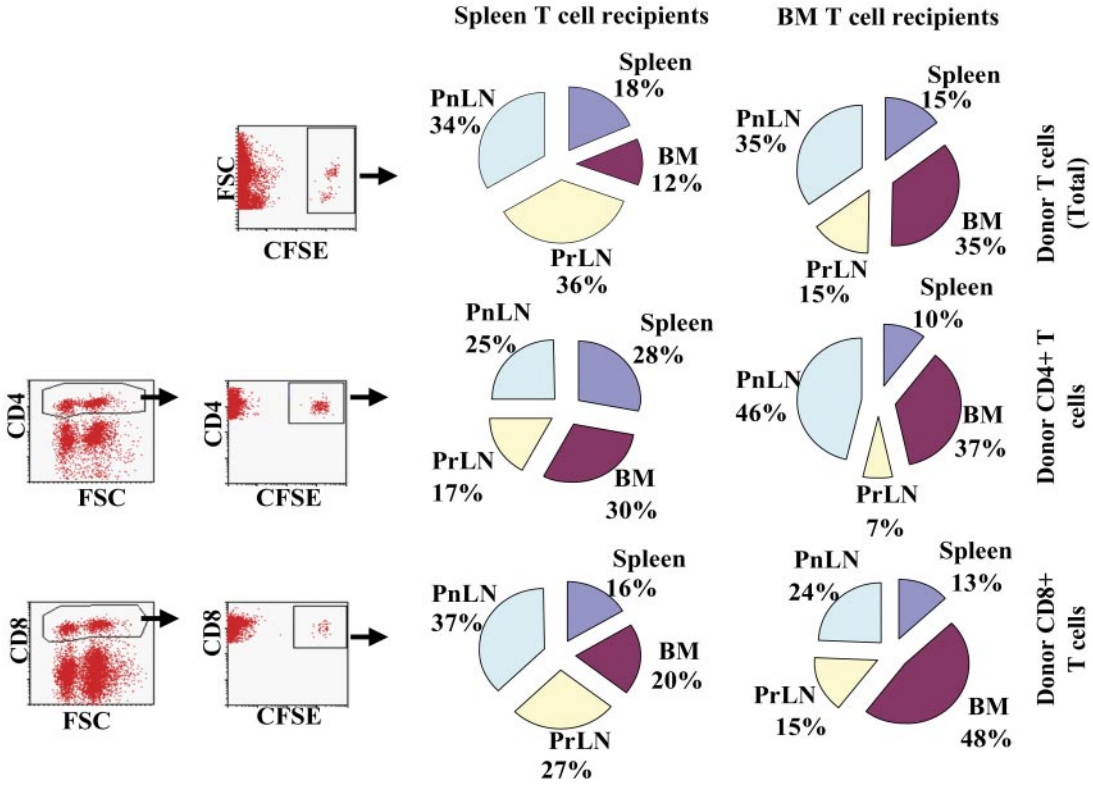

FIG. 6. Bone marrow (BM) T-cells traffic primarily into bone marrow and pancreatic lymph nodes (PnLN). Purified bone marrow and splenic T-cells from prehyperglycemic mice (10–12 weeks old) were transferred intravenously (2×10^6 cells/mouse) into prehyperglycemic mice. Recipient mice were killed 24 h later and examined for total and $CD4^+$ and $CD8^+$ $CFSE^+$ donor T-cells by FACS. Samples were gated for donor cells as shown on the left as scatter plots. Percentages of $CFSE^+$ cells among total cells (*top*), $CFSE^+CD4^+$ T-cells among total $CD4^+$ T-cells (*middle*), and $CFSE^+CD8^+$ T-cells among total $CD8^+$ cells (*lower*) were calculated. When CFSE-labeled splenic T-cells were adoptively transferred, the percentage of $CFSE^+$ T-cells in the spleen of a recipient was considered to be 1, and the relative values in other lymphoid organs were calculated. Similarly, when CFSE-labeled bone marrow T-cells were transferred, the percentage of $CFSE^+$ T-cells in the bone marrow of a recipient was considered to be 1. Pie diagrams represent relative values converted to relative percentages in which the total was 100%. Mean values from three recipients per group are shown. The assay was repeated twice using the same number of mice per group with similar results (a total of nine mice per group tested). PrLN, peripheral lymph nodes.

immunity is not understood. Studies have demonstrated an important role for bone marrow in B-lymphopoiesis, as well as in the maintenance of antibody-secreting long-lived plasma cells (31,32). More recently, T-cells in bone marrow have attracted much interest, primarily because T-cells reactive to premalignant and cancer cells are found in the bone marrow of nonimmunized patients (33–35). Recent studies in mice have shown that bone marrow accumulates activated and memory T-cells after exposure to environmental antigens and promotes long-term persistence of antiviral memory cells (36,37). The majority of the earlier studies on bone marrow T-cells focused on antiviral and antitumor $CD8^+$ memory T-cells. Our observations, with respect to autoimmunity, demonstrate that bone marrow is a nest for diabetogenic T-cells in type 1 diabetic NOD mice.

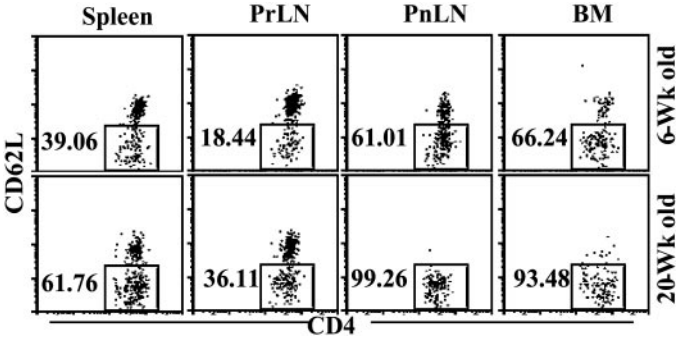

FIG. 7. Diabetogenic T-cells accumulate in the bone marrow and pancreatic lymph node (PnLN). Cells from various lymphoid organs of NOD.BDC2.5 TCR-Tg mice were tested for $CD4^+CD62L^{low/negative}$ memory T-cells by FACS. $CD4^+$ T-cells were gated for the panels shown here. Percentages of $CD62L^-$ cells are shown. Three mice for each age-group were tested, and the representative values are shown (a total of three mice per group tested). PrNL, peripheral lymph nodes.

Our results demonstrated that T-cells responsive to β-cell antigen peptides exist in the bone marrow and that these antigen-specific T-cells accumulate in the bone marrow in an age- and/or insulitis severity–dependent manner. Both EliSpot and CFSE dilution assays demonstrated that a significantly higher portion of bone marrow–resident T-cells, compared with splenic T-cells, is specific to β-cell antigens. In contrast to female NOD mice, neither diabetes-resistant female NOR mice nor male NOD mice of similar age groups (6 and 12–14 weeks old) had significant numbers of self-antigen–reactive T-cells in bone marrow or spleen (not shown). These observations further indicated that self-antigen–specific T-cells detected in bone marrow are indicative of the pathogenic T-cells present in the pancreatic microenvironment and the severity of insulitis.

Earlier studies demonstrated that pancreatic lymph nodes are necessary for priming and expansion of β-cell–reactive T-cells (4). Furthermore, T-cells with the ability to proliferate in response to stimulation with self-antigenic peptides and to induce/advance hyperglycemia were de-

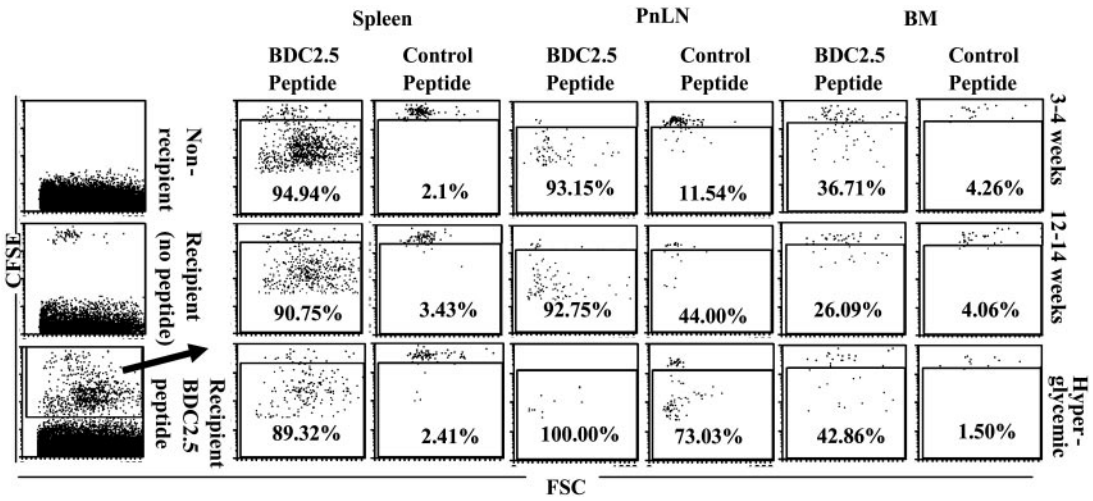

FIG. 8. Endogenous self-antigen presentation occurs in the pancreatic lymph nodes (PnLN) but not in the bone marrow. Purified naive $CD4^+$ T-cells from NOD-BDC2.5 TCR-Tg mice were labeled with CFSE and transferred intravenously (2×10^6 cells/mouse) into wild-type NOD mice of different age-groups. These mice were then injected intravenously with BDC2.5 or control ($Ova_{323–339}$) peptide (2 μg/mouse). After 5 days, mice were killed and spleen, pancreatic lymph nodes, and bone marrow cells examined for CFSE dilution in donor T-cells by FACS. $CFSE^+$ (donor) cells were gated (*far left* panel), and the percentage of cells among the gated population with CFSE dilution is shown. Representative values from the experiment using three mice per group and the cells from each mouse tested in triplicate are shown. In addition, this experiment was repeated twice using the same number ($n = 3$) of mice per group with similar results (a total of nine mice per group tested).

tected in the spleen (6,7–9,38). Therefore, in the current study, T-cells from the spleen and bone marrow of female NOD mice were compared. Earlier studies used much higher numbers of splenic T-cells ($\geq 5 \times 10^6$ cells/mouse) for examining whether hyperglycemia can be induced in immunodeficient mice. However, our current study shows that fewer cells (2×10^5 cells/ mouse) are needed for inducing diabetes in immunodeficient mice and that the bone marrow T-cells have a greater ability to induce diabetes. This suggests that at least a fraction of the β-cell antigen–specific T-cells is migrating to various lymphoid organs from the pancreatic microenvironment. Our observation that the proportion of autoreactive T-cells is significantly high in the bone marrow of female hyperglycemic NOD mice suggests that these diabetogenic T-cells in female NOD mice are migrants from the pancreatic microenvironment.

Importantly, our observation that hyperglycemia is induced by bone marrow T-cells several weeks in advance compared with splenic T-cells indicates that bone marrow has a higher percentage of diabetogenic T-cells than spleen and/or that bone marrow–resident antigen-specific T-cells are functionally more potent or different from their peripheral counterparts. Our observations, in fact, suggest that whereas the frequencies of β-cell–reactive T-cells between bone marrow and splenic T-cells are significantly different, these T-cells are also functionally different. Bone marrow T-cells showed a better ability to expand in vivo in both pancreatic and nonpancreatic microenvironments in NOD-*Scid* mice. More importantly, bone marrow T-cells showed rapid infiltration into islets compared with splenic T-cells. Although it is presently not known why bone marrow T-cells rapidly expand in NOD-*Scid* mice and accumulate in the pancreatic microenvironment, it is conceivable that the memory phenotype of the bone marrow T-cells with self-antigen specificity may have some role in this phenomenon. Whereas endogenous self-antigen–driven proliferation of T-cells trafficking into the pancreatic microenvironment is one possibility, T-cells with β-cell antigen specificity expanded in other lymphoid organs could also be trafficking into the pancreatic microenvironment. This is in agreement with reports that antigen-specific T-cells can traffic preferentially into sites of inflammation and to a site where presentation of the specific antigen occurs (39,40).

Importantly, it is essential to note that $CD4^+CD25^+$ T-cells can suppress the effector T-cell function. In fact, the spleen and bone marrow showed different frequencies of $CD4^+CD25^+$ T-cells. Therefore, we anticipated that total T-cells from these tissues might respond differently to stimulation with self-antigens and influence the induction of diabetes. To exclude this possibility, we have used purified $CD25^-$ T-cells throughout the study. Hence, the differences in the abilities of the bone marrow and splenic T-cells to respond to antigen, to expand under lymphopenia conditions, and to induce insulitis are not due to the influence of these regulatory T-cells.

Intriguingly, examination of trafficking properties of T-cells in wild-type mice demonstrated that whereas bone marrow T-cells have their trafficking preference primarily to bone marrow and pancreatic lymph nodes, splenic T-cells have no specific trafficking preference. This suggests that β-cell antigen specificity of bone marrow T-cells could be partially contributing to their preferential trafficking to pancreatic lymph nodes. This has been further confirmed through examining diabetogenic TCR-expressing memory $CD4^+$ T-cells present in various anatomical regions of NOD.BDC2.5 TCR-Tg mice. Pancreatic lymph nodes and bone marrow of these mice demonstrated similar percentages of memory T-cells, which were significantly higher than those found in spleen and peripheral lymph nodes.

The presence of diabetogenic T-cells in the bone marrow suggests that these T-cells are immigrants from the peripheral organs, such as the pancreas and pancreatic lymph nodes. However, recent studies showing that bone marrow can support primary immune response against blood-born foreign antigens (17,21) and sustain memory T-cells for a long time (20,33,35) suggest that bone marrow can also act as an SLO. Therefore, it can be argued that bone marrow T-cells may have experienced self-antigens

in the bone marrow. Another possibility is that they experience self-antigen in the periphery but expand in the bone marrow, due to local antigen presentation. Therefore, we examined whether endogenous self-antigen presentation occurs in the bone marrow to induce autoreactive T-cells and/or expand antigen-experienced cells emigrated from the periphery. Wild-type NOD mice of different age-groups were adoptively transferred with purified $CD4^+$ T-cells from NOD.BDC2.5 TCR-Tg mice and examined for donor T-cell proliferation. Our results demonstrate that whereas bone marrow of all age-groups of mice, like SLOs, can support the presentation of exogenously delivered antigenic peptide, only the pancreatic lymph nodes, not the bone marrow or spleen, showed significant levels of endogenous self-antigen–induced proliferation of adoptively transferred T-cells. This indicates that self-antigen–specific T-cells found in bone marrow are most likely immigrating from the pancreatic microenvironment, and bone marrow primarily supports the homeostatic maintenance of these T-cells.

Although further studies are needed to understand the mechanism of bone marrow homing by self-antigen–specific T-cells and to carefully examine bone marrow $CD4^+$ and $CD8^+$ T-cells for their autoreactive and diabetogenic nature, our present study clearly demonstrates pathogenic T-cells in the bone marrow of NOD mice. Furthermore, our findings, although observed in a type 1 diabetes model, will have implications in other autoimmune conditions. In this context, it is important to note that recent studies have detected long-lived foreign antigen-specific memory $CD8^+$ T-cells and antibody-producing plasma cells in bone marrow (31,33,35). Our finding that T-cells against MHC II–restricted pancreatic β-cell antigen epitopes are present in large numbers in bone marrow compared with spleen strongly suggests that bone marrow acts as a preferential maintenance site for antigen-experienced lymphocytes. These findings warrant urgent additional studies to characterize bone marrow–residential T-cells of autoimmune patients and susceptible subjects with respect to their epitope specificities, memory phenotypes, and trafficking properties to realize the clinical significance of these T-cells.

In spite of the fact that T-cells specific to β-cell antigens are present in the peripheral blood, characterizing them for predicting hyperglycemia is hindered by their extremely low frequencies. In this context, a recent study demonstrating identical clonotypes of T-cells in both peripheral blood and pancreatic islets, albeit different in frequencies (22), holds promise in the early detection of diabetes in susceptible individuals. Although it is easier to obtain blood samples than bone marrow, the presence of a higher proportion of T-cells specific to β-cell antigens in bone marrow suggests that it can be considered a reliable alternative for predicting the disease using well-defined peptides and MHC tetramers. Therefore, further studies to analyze β-cell–specific T-cells from accessible anatomical sites including bone marrow of at-risk subjects would be important.

In light of our observations, it is also important to be vigilant while carrying out autologous and allogeneic bone marrow transplantation if the donor cells are from autoimmune-susceptible subjects in order to avoid adoptive transfer of the disease. Therefore, further understanding the characteristics of bone marrow–resident T-cells is important, not only to explore the possibility of using bone marrow T-cells for early detection of the disease, but also to realize the risk, if any, associated with bone marrow transplantation procedures.

ACKNOWLEDGMENTS

This work was supported by the Department of Surgery, University of Illinois at Chicago; National Institutes of Health Grant R21A1059745; and Juvenile Diabetes Research Foundation Grant 1-2005-27.

We thank Dr. Margalit Mokyr, University of Illinois at Chicago, for helpful comments on the manuscript.

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