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Transfusion of Nonobese Diabetic Mice with Allogeneic Newborn Blood Ameliorates Autoimmune Diabetes and Modifies the Expression of Selected Immune Response Genes

Sundararajan Jayaraman,* Tejas Patel,* Vasu Patel,* Shahnaz Ajani,* Rebecca Garza,* Arathi Jayaraman,* Sung Kwon,* Rajvir Singh,* Damiano Rondelli,† Bellur S. Prabhakar,‡ and Mark Holterman*

Although allogeneic bone marrow transplantation has been shown to prevent autoimmunity diabetes in heavily irradiated nonobese diabetic (NOD) mice, a similar procedure is not suitable for the treatment of patients with type 1 diabetes because of associated severe side effects. Therefore, we evaluated whether newborn blood (NBB), equivalent to human umbilical cord blood, could be used for diabetes prevention without recipient preconditioning. To test this hypothesis, unconditioned, prediabetic female NOD mice were given a single injection of whole NBB derived from the allogeneic diabetes-resistant mouse strain C57BL/6. Transfusion of allogeneic NBB but not adult blood prevented diabetes incidence in a majority of treated mice for a prolonged period of time. This was accompanied by the release of insulin in response to a challenge with glucose. Invasive cellular infiltration of islets was also substantially reduced in these mice. Although NBB transfusion induced a low level of hematopoietic microchimerism, it did not strictly correlate with amelioration of diabetes. Induction of genes implicated in diabetes, such as Il18, Tnfa, and Inos but not Il4, Il17 or Ifng, was repressed in splenocytes derived from protected mice. Notably, expression of the transcription factor Tbet/Tbx21 but not Gata3 or Rorgt was upregulated in protected mice. These data indicate that allogeneic NBB transfusion can prevent diabetes in NOD mice associated with modulation of selected cytokine genes implicated in diabetes manifestation. The data presented in this study provide the proof of principle for the utility of allogeneic umbilical cord blood transfusion to treat patients with autoimmune diabetes. The Journal of Immunology, 2010, 184: 3008–3015.

The NOD mouse model is not only useful for understanding the mechanisms of autoimmunity diabetes but also for answering clinical questions that are not possible to address in humans (2). The mouse newborn blood (NBB) provides a useful model for human umbilical cord blood at birth for a number of reasons. Whereas the frequency of Sca-1+ HSCs is similar in both NBB and bone marrow, only the NBB can repopulate the immune system of irradiated hosts without GVHD when reconstituted across MHC barriers and result in generalized immunosuppression (8).

Human umbilical cord blood at birth represents a particularly desirable source of transplantable cells over bone marrow because it contains large numbers of HSCs, progenitor cells, and naive T cells with reduced alloreactivity and a limited capacity to cause GVHD (9). Although infusion of autologous T cells from cryopreserved cord blood units has been used in diabetic patients as a source rich in T regulatory cells, its efficacy to ameliorate autoimmune diabetes has not yet been elucidated (10). The diabetogenic potential of bone marrow cells was indicated by the ability of bone marrow to transfer diabetes from diabetic to nondiabetic HLA-identical siblings (11). This is in line with studies in the NOD mouse model showing that the bone marrow retains the stemness to propagate diabetes (4, 7, 12). Although autologous umbilical cord blood had palliative effects (10), it is not an ideal choice for HSCs to rest the immune system in patients with T1D because like bone marrow, umbilical cord blood from patients with T1D may have progenitors of autoreactive T lymphocytes that can perpetuate diabetes. On the other hand, HSCs derived from umbilical cord blood of allogeneic, nondiabetic individuals may provide better protection against diabetes because they lack diabetogenic T lymphocytes. This concept needs to be evaluated in a preclinical model prior to the application of this promising strategy to treat patients with T1D.

The NOD mouse model is not only useful for understanding the mechanisms of autoimmunity diabetes but also for answering clinical questions that are not possible to address in humans (2). The mouse newborn blood (NBB) provides a useful model for human umbilical cord blood at birth for a number of reasons. Whereas the frequency of Sca-1+ HSCs is similar in both NBB and bone marrow, only the NBB can repopulate the immune system of irradiated hosts without
inducing GVHD across allogeneic barriers (13–15). In contrast to adult blood, NBB contains mostly immature T cells (CD4+ plus CD8+) and a few single-positive, TCR<sup>+</sup>CD4<sup>+</sup> and TCR<sup>+</sup>CD8<sup>+</sup> cells (13, 14). Therefore, mouse NBB appears to be an ideal source of somatic HSCs that can be used for diabetes treatment. To test this possibility, we developed a clinically relevant model in which unconditioned diabetes-prone female NOD mice were transfüsed at prediabetic stage with NBB derived from diabetes-resistant C57BL/6 mice. The data indicate that allogeneic NBB transfusion could prevent diabetes by halting the progression of inflammatory responses in islets and reducing the inflammatory cytokine expression. Within the limitations of the extrapolation of data obtained from animal models to clinical settings, these data suggest that allogeneic NBB transfusion may provide a potentially useful clinical strategy to treat patients with T1D.

Materials and Methods

Mice

Female NOD/Ltj (H-2<sup>b</sup>) and timed pregnant C57BL/6J (H-2<sup>b</sup>) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in filter-top cages in temperature-controlled and light-cycled rooms. The animal protocol was approved by the University of Illinois at Chicago Biomedical Resources Center, and experiments were conducted according to the principles outlined by the Animal Welfare Act and the National Institutes of Health guidelines for the care and use of animals in biomedical research.

Blood collection

Neonatal blood was collected in the anticoagulant Alsever’s solution by a cardiac puncture from newborn mice up to 5 of age. The volume of blood obtained from a single newborn mouse varied between 10 and 50 μL. Blood from 8–13 newborn mice from the same litter was pooled per experiment. Adult blood was collected in Alsever’s solution from the tail vein.

Flow cytometry

After RBC lysis, nucleated cells were stained with mAbs conjugated with FITC (CD3, CD8, CD25, DX5, and K<sup>+</sup>), PE (CD4, CD11c, CD44, CD45R, CD45RA, CD62L, CD69, and NK1.1), or allophycocyanin (Sca-1, CD3, and mouse plasmacytoid dendritic cell Ag-1 [mPDCA-1]). All Abs were obtained from eBioscience (San Diego, CA) except CD4, CD34, CD44, CD45R, and K<sup>+</sup> Abs, which were purchased from BD Pharmingen (San Diego, CA), and anti-mPDCA-1 Ab was obtained from Miltenyi Biotec (Auburn, CA). Isotype-matched normal mouse Ig conjugated with fluorochromes was used to determine the background staining of cells. Data were acquired on a BD LSR (BD Biosciences, San Jose, CA) or a CyAn ADP flow cytometer (DakoCytomation, Carpinteria, CA). Off-line color compensation and data analysis were done using the FlowJo6.4.1 software (Tree Star, Ashland, OR).

Treatment of mice and diabetes monitoring

A small aliquot of whole NBB or adult peripheral blood was treated with RBC lysis solution (eBioscience), and the total numbers of nucleated cells were counted in a hemocytometer. Known numbers of nucleated cells were stained to assess the frequency of Sca-<sup>1</sup> cells, and this information was used to calculate the total numbers of Sca-<sup>1</sup> cells present in the NBB preparation. For injection into NOD recipients, whole blood was centrifuged, and the numbers of nucleated cells were adjusted by resuspending in sterile HBSS (Invitrogen, Carlsbad, CA). Unconditioned 12-wk-old female NOD/Ltj mice were injected with newborn or adult C57BL/6-derived whole blood through a tail vein.

Diabetes was monitored by determining glucose levels at weekly intervals in the tail vein blood using a glucometer (Accu-Chek Advantage, Roche Diagnostics, Indianapolis, IN). Mice were considered diabetic when the blood glucose levels reached >250 mg/dl for two consecutive readings within the same week of the initial diagnosis. For the glucose tolerance test, mice were fasted for 4 h, and blood was drawn from the tail vein at 0, 15, 45, and 60 min after an i.p. injection of dextrose (2 g/kg body weight). Insulin levels in sera were measured using an Ultra Sensitive Mouse Insulin ELISA kit obtained from Crystal Chem (Downers Grove, IL; sensitivity range: 0.1–12.8 ng/ml).

Determination of chimerism

To determine chimerism by flow cytometry, tail vein blood was collected in Alsever’s solution at various time intervals after NBB transfusion. After RBC lysis, nucleated cells were stained with an FITC-conjugated Ab against K<sup>+</sup>, the MHC class I Ag expressed by donor C57BL/6 cells. The cells were then analyzed by flow cytometry. At the end of the experiment, mice were killed, and an aliquot of splenocytes pooled from three to five mice from the same group was stained with FITC-conjugated anti-K<sup>+</sup> Ab and assessed by flow cytometry.

To determine chimerism by RT-PCR, splenocytes were lysed in TRIzol (Invitrogen), and total RNA was isolated. The first-strand cDNA was synthesized using the T-primed First-Strand Kit (Amersham Biosciences, Piscataway, NJ). PCR was performed with 1.0 μL cDNA in final volume of 25 μL containing 0.4 μM concentrations each of K<sup>+</sup> (16) and β2-microglobulin (17) primer sets and Amplitaq Gold PCR Master Mix (Applied Biosystems, Foster City, CA). Amplicons were electrophoresed on a 1% agarose gel in the presence of ethidium bromide, visualized under UV light, and imaged using a CCD camera (Alpha Innotech Gel Documentation System, Alpha Innotech, Santa Clara, CA).

Assessment of gene expression by real-time RT-PCR

Spleen cells (5 × 10<sup>6</sup> ml) were stimulated with 100 ng PMA and 1 μg ionomycin in complete RPMI 1640 medium supplemented with antibiotics and 10% FBS (18). After overnight culture, cells were lysed in TRIzol (Invitrogen), and total RNA was extracted, treated with DNase using TURBO DNA-free kit (Applied Biosystems), and reverse transcribed to cDNA using High-Capacity cDNA Reverse Transcription kit (Applied Biosystems). Real-time quantitative RT-PCR was performed on an ABI Prism 7500 Real-Time PCR system (Applied Biosystems) using 1 μl cDNA equivalent to 100 ng RNA and 2× SYBR Premix Ex Taq (Perfect Real-Time) reagent (Takara-Clontech, Mountain View, CA). Primer set for mouse Gapdh was designed using the Primer Express version 2.0 software (Applied Biosciences): forward 5’TGG TGA AGG TCG GTG TAC 3’- and reverse 5’CCA TGT AGT TGA GGT CAATG AGG 3’- primer sets for H-2K<sup>b</sup>, H-2D<sup>b</sup>, Bf1q, Traa, Inos, Thx21, Gata3, and Roog were designed according to sequence databases (19–22). All primers were synthesized at Integrated DNA Technologies (Coralville, IA). Relative quantitation for all assays used Gapdh as the normalizer. Quantitation of gene expression in comparison with Gapdh was calculated using the comparative threshold cycle method (23). Sizes of amplicons and the absence of primer-dimer were verified by electrophoresis of PCR products on a 4% low-melt agarose gel.

Determination of lymphokine secretion

Culture supernatant was obtained from splenocytes after overnight activation with PMA and ionomycin, as described above. The amounts of lymphokines were determined using ELISA kits obtained from eBioscience.

Histology and confocal microscopy

Pancreata were fixed in 10% buffered formalin, and 6-μm sections were stained with H&E and observed under a light microscope (Olympus BX51, Olympus, Melville, NY). At least three sections were analyzed per pancreas by three different investigators. The numbers of islets per section were counted, and an islet score was assigned as follows: 1, no to mild peri-insulitis; 2, moderate infiltration (>50%); and 3, severe (>50%) and destructive infiltration.

Hydrated sections were blocked with 10% FBS, incubated with 1:100 concentration of guinea pig anti-insulin Ab (Zymed Laboratories, South San Francisco, CA) followed by incubation with 1:1000 diluted tetramethyl rhodamine iso-thiocyanate–rabbit antisera against guinea pig Ig (Sigma-Aldrich, St. Louis, MO). Sections were counterstained with Hoechst and mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Confocal images were obtained using a Zeiss LSM510 laser-scanning microscope and processed using the Zeiss LSM Image browser (4.0 version; Zeiss, Oberkochen, Germany).

Statistics

Statistical analysis was performed by using an unpaired two-tailed Student t test (Prism, GraphPad, San Diego, CA).

Results

Phenotypic characterization of NBB from C57B6 mice

Previous studies indicated that the frequency of Sca-1<sup>+</sup> cells in FcII-coll pirified mononuclear cells obtained from adult bone marrow and NBB of B10.D2 mice ranged from 12–17%, whereas only <1% of the adult PBMCs expressed this determinant (13, 14). In contrast to bone marrow and adult blood, the NBB contained large numbers (40%) of immature, double-positive (CD4<sup>+</sup>CD8<sup>+</sup>) cells with only 3% of TCR-bearing mature cells (13, 14). To confirm and extend
These findings, blood was collected from newborn mice (n = 50) of the diabetes-resistant C57BL/6 strain up to 5 d of age in the anticogulant Alsever’s solution. After RBC lysis, nucleated cells were stained with various Abs and analyzed by flow cytometry. A typical cytogram shown in Fig. 1 indicated that three populations, designated as B, C, and D, could be distinguished in NBB based on forward angle and side-scatter properties. This is in contrast to the commonly observed single population of cells displaying high forward light scatter in RBC-lysed adult peripheral blood (not shown). Although the proportion of cells in gate D varies between experiments (7–15% of total cells, gate A), only the cells in gate D expressed all of the determinants analyzed. The frequency of cells expressing the HSC marker Sca-1 represented ~15% of cells in gate D, which is comparable to those reported in Ficoll-purified mononuclear cells from neonatal B10.D2 mice (13, 14). In contrast, adult PBMCs from C57BL/6 mice contained <1% Sca-1+ cells (data not shown), consistent with previous observations in B10.D2 mice (13, 14).

As reported in B10.D2 mice, we also found that the NBB of (CD4+CD8+) T cells with a low frequency of CD3+ mature T cells expressed all of the determinants analyzed. The frequency of cells expressing CD69, CD45R, and CD45RA. Interestingly, the naturally occurring T regulatory cells of 14.9% in five different experiments) as well as CD69, CD45R, and CD45RA. Notably, we found that a majority (58%) of cells in gate D were positive for CD44, a determinant ubiquitously expressed by a number of cell types (24) including the mesenchymal stem cells (25). In addition, a significant fraction (52%) of cells displaying low forward and high side-scatter properties (gate B) also expressed CD44 (Table I, line 4). Further analysis revealed that a significant fraction (40%) of cells in gate D displayed CD62L, expressed by naive T lymphocytes (Fig. 1). Only a minor fraction, ~10% of cells in gate D, expressed the MHC class I determinant Kb (range: 4–14.9% in five different experiments) as well as CD69, CD45R, and CD45RA. Interestingly, the naturally occurring T regulatory cells of the phenotype CD4+CD25+ and NK1.1+ cells represented <10% of cells in gate D. Dendritic cells expressing CD11c, Dex-5, and mPDCA determinants also constituted a small fraction of NBB. Finally, only a minor fraction of cells expressed CD34, a marker expressed by human but not mouse HSC (26).

Further analysis showed that 3–5% of Sca-1+ cells coexpressed MHC class I, CD44, CD62L, CD3, CD4, CD8, CD25, and DX5 (Table I, lines 3, 5, 7, 9, 11, 13, 15, and 17), consistent with the expression of Sca-1 in a variety of cell types (26). However, a modest proportion of cells expressing CD69, CD45R, CD45RA, NK1.1, and CD11c coexpressed the Sca-1 determinant (Table I, lines 19, 21, 25, and 27). Taken together, these data indicate that, like B10.D2 mice (13, 14), the NBB of diabetes-resistant C57BL/6 mice contained immature T cells (CD4+ plus CD8+, CD62L+) and more Sca-1+ cells than adult blood among mononuclear cells. Interestingly, CD44 appears to be ubiquitously expressed in the NBB without a large representation of suppressor cells such as T regulatory cells (CD4+CD25+) and NK1.1 cells.

**Transfusion of allogeneic NBB prevents diabetes in NOD mice**

Because NBB contains Sca-1+ HSCs and produces minimal GVHD in irradiated allogeneic adult mice (13, 14), we reasoned that the NBB derived from diabetes-resistant mice could be used to substitute the mutated HSCs implicated in autoimmune diseases (27). To test this, 12-wk-old female prediabetic NOD mice (H-2k, Mls-1b) were given a single i.v. injection of whole NBB derived from diabetes-resistant B6 mice (H-2b, Mls-1b). In five different experiments, each mouse was given whole NBB containing 25–50 × 10^6 nucleated cells. The numbers of Sca-1+ cells present in 25–50 × 10^6 nucleated cells ranged from 0.125–0.5 × 10^6 Sca-1+ cells, as assessed by flow cytometry (see Materials and Methods for details). In contrast to previous studies of allogeneic bone marrow and HSC transplantations (3–8), no recipient preconditioning was used. Cumulative data from five experiments shown in Fig. 2 indicate that 80% of untreated female NOD mice became diabetic (nonfasting blood glucose: >250 mg/dl) by 28 wk of age. Transfusion of allogeneic NBB prevented the onset of diabetes significantly (untreated versus NBB transfused: p = 0.006). Only 30% of treated mice showed aberrant glycemic control in contrast to 80% of untreated NOD female mice. Protection against diabetes was not reversible as late as 38 wk of age (data not shown). No apparent GVHD, as indicated by weight loss, anemia, diarrhea, and general appearance, was observed in protected mice. In contrast to NBB, adult C57BL/6 PBMC containing similar numbers of Sca-1+ cells (25 × 10^6 mononuclear cells containing ~0.125 × 10^6 Sca-1+ cells) as NBB cells failed to prevent diabetes in NOD mice (untreated versus adult blood transfused: p = 0.54, NS).

To determine whether NBB transfusion can reverse overt diabetes, newly diagnosed diabetic mice (blood glucose levels ranging from 250–400 mg/dl) at 16 wk of age were given a single i.v. injection of 25 × 10^6 nucleated allogeneic NBB cells. Although diabetes was not fully reverted, four out of five treated mice maintained the same level of hyperglycemia and body weight and survived longer than...
untreated, diabetic littermates until the end of the observation period, 32 wk of age (data not shown). Taken together, these data indicate that although transfusion of allogeneic NBB at the prediabetic stage prevented diabetes, it could only delay the progression of diabetes in newly diagnosed diabetic mice.

**Restoration of normoglycemia and insulin release response in protected mice**

I.p. glucose tolerance test performed at 28–32 wk of age (16 wk after NBB infusion) revealed that treated nondiabetic mice displayed normal blood glucose clearance as untreated nondiabetic mice (Fig. 3A). In contrast, untreated diabetic mice and those treated with allogeneic NBB but remained diabetic displayed hyperglycemia, which did not change after glucose challenge. Serum insulin levels were increased by ∼3-fold in response to a glucose challenge. In contrast, increases in insulin levels were barely observed in untreated diabetic mice and in NBB-treated diabetic mice. These results indicate that allogeneic NBB transfusion can restore glycemic control by preserving the insulin-producing β cell function.

**Reduction of invasive insulitis by allogeneic NBB transfusion**

In the NOD mouse system, peri-insulitis, cellular infiltration at the periphery of islets, is seen at 3 to 4 wk of age (28), and in our model, NBB transfusion was given at 12 wk of age, when the pathologic process was already under way. Pancreata of untreated nondiabetic NOD mice displayed little or no cellular infiltration (grade 1, Fig. 4A) at 28 wk of age. As expected, islets of untreated diabetic mice displayed moderate (grade 2, Fig. 4B) and invasive cellular infiltration (grade 3, Fig. 4C). Whereas little or no apparent loss of insulin-producing β cells was found in pancreata of treated nondiabetic mice with grade 1 or grade 2 pathology scores (Fig. 4A, 4B, lower panel), β cells without invasive cellular infiltration could not be identified in the pancreata of overtly diabetic mice (Fig. 4C, lower panel). A majority (>70%) of islets in untreated diabetic mice (n = 3) had the highest level of cellular infiltration (grade 3, Fig. 4D). Interestingly, in mice that were treated with NBB and remained diabetic, 48% of islets displayed severe and 43% moderate cellular infiltration. Significantly, only 25% of islets examined from protected mice had severe cellular infiltration, whereas 46% had no or minimal infiltration. Although the frequency of pancreata with grade 1 pathology score in mice that were treated but remained diabetic was similar to those of treated nondiabetic mice, pancreata of overtly diabetic mice had severe (grade 3) cellular infiltration. These data indicate that amelioration of diabetes following transfusion with MHC- and minor histocompatibility Ag-mismatched NBB cells is due to the blockade of transition from mild to overt and invasive insulitis.

**Lack of correlation between microchimerism and protection against diabetes**

Previous studies showed an association between significant level of chimerism and protection against diabetes following transplantation of NOD mice with allogeneic bone marrow or purified Sca-1^+ HSCs performed under cytoreductive conditions (3–8, 12). One study suggested that <1% chimerism was sufficient for protection against diabetes following allogeneic bone marrow transplantation (29). Therefore, it was of interest to determine whether protection against diabetes following allogeneic NBB transfusion in unirradiated NOD mice was associated with significant level of chimerism. Data shown in Fig. 5A indicate the presence of donor-derived H-2K^b-expressing

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**Table I. Phenotype of NBB from C57BL/6 mice**

<table>
<thead>
<tr>
<th>Phenotype Gate</th>
<th>Gate A</th>
<th>Gate B</th>
<th>Gate C</th>
<th>Gate D</th>
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<tr>
<td>1 Total Sca-1^-</td>
<td>2.4</td>
<td>0.6</td>
<td>0.02</td>
<td>17.8</td>
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<tr>
<td>2 Total K^-</td>
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<td>0.6</td>
<td>0.2</td>
<td>14.9</td>
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<tr>
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<tr>
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<td>7 CD62L^- + Sca-1^-</td>
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<tr>
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<tr>
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<tr>
<td>27 CD11c^- + Sca-1^-</td>
<td>1.9</td>
<td>0.4</td>
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Phenotypic characterization of NBB derived from C57BL/6 mice by flow cytometry. Cells were stained either with single Ab or double stained with anti-Sca-1 and indicated Abs. Data indicate total and double-positive cells among variously gated populations shown in Fig. 1. Data are from a representative of five different experiments (n = 50 newborn pups).

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**FIGURE 2.** Amelioration of diabetes in NOD mice by allogeneic NBB transfusion. Incidence of diabetes (>250 mg/dl) in untreated NOD mice and those treated with allogeneic NBB or adult blood is shown. Arrow indicates the time of injection of cells. Statistical significance: untreated versus NBB transfused, p = 0.006; untreated versus adult blood transfused, p = 0.54, NS.

**FIGURE 3.** Allogeneic NBB transfusion preserved the β cell function. A. I.p. glucose tolerance test was performed at 28–32 wk of age. Blood glucose levels in various groups of mice challenged with glucose are shown. Values indicate mean ± SD per group. Numbers of mice tested are indicated in parentheses. B. Corresponding insulin levels of these mice are shown. Mean values ± SD per group are indicated.
leukocytes in the peripheral blood of NOD recipients as detected by flow cytometry 5 wk after NBB transfusion. This microchimerism (<5% of allogeneic leukocytes) was transient and not detected at later time points in the peripheral blood. To ascertain whether a correlation exists between protection afforded by NBB transfusion and the level of chimerism, mice were killed between 30 and 38 wk of age, and their spleens were analyzed for the expression of donor-derived H-2Kb by flow cytometry. Data shown in Fig. 5B indicate that the level of microchimerism found in spleenocytes of mice that were protected from diabetes and those that remained diabetic after NBB transfusion did not differ. To further analyze the correlation between microchimerism and protection against diabetes in NBB-transfused mice, spleenocytes were analyzed for the expression of donor-derived H-2Kb gene by RT-PCR. The data shown in Fig. 5C indicate that out of seven mice tested, three (N-2, N-6, and N-7) were strongly chimeric, whereas one (N-3) weakly expressed the B6-derived MHC class I gene (Fig. 5C). Only two (N-2 and N-7) of these chimeric mice were protected from diabetes. Although chimerism could not be detected in another mouse (N-1) by RT-PCR, it did not develop diabetes. Induction of transient microchimerism in non-preconditioned mice is similar to blood transfusion-associated microchimerism in humans (30). Although microchimerism (>5%) (3–8, 12) and microchimerism (<1%) (29) induced by bone marrow transplantation or purified HSCs in myeloablated recipients have been implicated in diabetes protection, NBB transfusion in unconditioned NOD mice resulted in microchimerism that did not strictly correlate with protection against diabetes.

Modulation of expression of selected genes by NBB transfusion
To further gain insights into the mechanisms of protection mediated by NBB transfusion, induction of cytokine genes implicated in diabetes was analyzed. In five separate experiments, spleens from three to five cured and diabetic mice were simultaneously harvested between 16 and 26 wk after NBB transfusion (at 28–38 wk of age). Splenocytes from each group were pooled and stimulated overnight to five cured and diabetic mice were simultaneously harvested between 16 and 26 wk after NBB transfusion (at 28–38 wk of age). Representative results shown in Fig. 6B indicate that splenocytes of untreated prediabetic mice activated with PMA and ionomycin expressed lower levels of Il18 and Tnfa and higher levels of Ifng and Tbx21 in comparison with other genes simultaneously analyzed. Although the levels of gene expression in prediabetic mice cannot be directly compared with those of NBB-treated nondiabetic mice due to age differences, the pattern of gene expression was similar in both groups. Thus, the levels of Il18 and Tnfa mRNA were lower, whereas the expression of Tbx21 was higher in both prediabetic and cured mice when compared with NBB-treated diabetic mice (Fig. 6A, 6B). We also observed lower expression of Il18 and Tnfa and increased levels of Tbx21 mRNA in

**FIGURE 5.** Relationship between microchimerism and protection against diabetes. A. The presence of H-2Kb–expressing cells was determined by flow cytometry in the peripheral blood of untreated NOD mice and in those transfused with indicated numbers of mononuclear cells containing NBB at various time points. Statistical significance between groups is shown (n = 8–10 mice per group). B. Microchimerism was analyzed in spleenocytes after 14–18 wk of NBB transfusion by flow cytometry. Data are shown for NBB transfused but remained diabetic and those protected from diabetes after NBB transfusion (n = 10 per group). C. RT-PCR analysis of spleenocytes obtained after 18 wk of NBB transfusion. B6, spleen cells from C57BL/6 positive control; N-1 through N-7, spleen cells from NOD mice transfused with C57BL/6 NBB; NOD, spleen cells from NOD mice. Amplicon sizes of Kappa and β2-microglobulin are shown.
a small group of untreated NOD mice (n = 3) that did not develop diabetes at 32 wk of age (data not shown).

The amount of lymphokines present in the supernatant of splenocytes stimulated with PMA and ionomycin was determined by ELISA. The data shown in Fig. 6C indicate that the splenocytes from NBB-transfused and protected mice produced less TNF-α protein, consistent with the downregulation of Tnfa mRNA. The secretion of IL-17 and IFN-γ proteins by splenocytes of protected mice was slightly enhanced. Collectively, these data indicate that NBB transfusion led to the prevention of diabetes accompanied by selective reduction in the expression of cytokines implicated in diabetes, such as Il18 and Tnfa, with a concomitant upregulation of Tbx21 mRNA.

**Discussion**

We have demonstrated in this study that transfusion of unconditioned, prediabetic female NOD mice with allogeneic NBB but not adult blood prevented the onset of diabetes. However, NBB given after the disease manifestation delayed but did not revert diabetes. Abrogation of autoimmune diabetes was accompanied by the blockade of progression from prediabetes to overt diabetes and preservation of β cell function. Although low levels of microchimerism were induced by NBB transfusion, they poorly correlated with the level of protection against diabetes. Importantly, selective downregulation of inflammatory cytokine genes implicated in diabetes, such as Il18, Tnfa, and Inos, and upregulation of the transcription factor Tbx21 were consistently seen in NOD mice protected from diabetes by NBB transfusion.

Although transfusion of newly diagnosed, pre conditioned patients with T1D with autologous CD34+ cells mobilized from the bone marrow had palliative effects (40), the long-term benefits of this treatment procedure remain obscure. Because transplantation of bone marrow from a diabetic sibling into another HLA-identical nondiabetic sibling transferred diabetes (11), the progenitors present in the transferred autologous cord blood (10) or CD34+ cells mobilized from the bone marrow (40) are likely to reinstate the diabetogenic phenotype. Numerous studies demonstrated that transplantation of syngeneic T cell-depleted HSCs (7) or bone marrow (4, 5, 12) transferred diabetes into heavily irradiated NOD mice. These data are consistent with the notion that transplantation of nondiabetic, allogeneic but not autologous HSCs containing progenitors of diabetogenic T lymphocytes is a better option for treatment of patients with T1D. However, validation of allogeneic but not syngeneic NBB is potent in ameliorating spontaneous autoimmune diabetes in NOD mice will assure that allogeneic rather than autologous umbilical cord blood is a better option for treatment of patients with T1D. Nevertheless, the preclinical model described in this study provides the proof of principle for the notion that transplantation of neonatal whole blood derived from diabetes-resistant mice can provide robust protection against autoimmune diabetes without recipient preconditioning. In addition, our data shed light on the possible mechanisms involved in the abrogation of autoimmune diabetes by somatic stem cell therapy.

Previous studies showed that both bone marrow and NBB contain a similar frequency (~15%) of Sca-1+ cells (13, 14) and comparably reconstitute the entire hematopoietic system in irradiated allogeneic mice (13–15). Therefore, it is likely that the allogeneic NBB-derived Sca-1+ HSCs may partially reset the immune system in unirradiated NOD mice and lead to the abrogation of diabetes. Differentiation of allogeneic, immature CD4+CD8+ neonatal T cells into mature T cells may also contribute to the altered immune system observed in NOD recipients. Because NBB contained large numbers of CD44+ cells, it may potentially differentiate into CD44+ mesenchymal stem cells and modestly suppress diabetes similar to those derived from the bone marrow (41). Other mechanisms include de novo generation of islets from pancreatic ductal cells (42), thwarting autoimmunity by inducing T cell tolerance (6), and boosting of the T regulatory cells implicated in the control of autoimmune diabetes in NOD mice (43, 44).

Importantly, our data indicate that amelioration of diabetes by allogeneic NBB transfusion is associated with selective suppression of cytokines implicated in diabetes such as Tnfa (31–34), Il18 (35), and Inos (36) but not Il4 (34, 45) or Il17 (46). The expression of Gata3 and Rorgt, respectively crucial for the transcription of IL-4 (38) and IL-17 (39), was also not reduced in protected mice. These data are consistent with the lack of involvement of Il4 and Il17 in diabetes suggested previously (47–49). The inducible expression levels of Tnfa appeared to be lower in protected mice as in prediabetic and untreated nondiabetic NOD mice. Inasmuch as TNF-α is implicated in diabetes (31–34), its reduction may minimize the β cell damage. In addition, the expression of Inos was diminished in protected mice. NO synthesized from L-arginine by inducible NO synthase in activated macrophages as well as by β cells exposed to inflammatory cytokines such as the β form of pro-IL-1 and TNF-α in vitro is considered to be toxic to β cells (50, 51). Although a similar toxic role for NO remains to be established in vivo, our data suggest...
that inducible NO synthase suppression following NBB transfusion may contribute to protection against spontaneous diabetes.

Our data also demonstrate that protection afforded by NBB transfusion accompanies notable expression of the transcription factor *Tbx21*, similar to prediabetic and untreated nondiabetic mice. The transcription factor *Tbx21* was originally described to be critical for polarization of Th1 cells without influencing the production of IFN-γ by CD8+ T cells (52). The absence of *Tbx21* in both innate and adaptive immune systems has been shown to prevent autoimmune diabetes in NOD mice, suggesting a pathogenic role for *Tbx21* in both innate and adaptive immune systems (37, 52, 53).

A low level of microchimerism is not unexpected in unirradiated mice, similar to that found after blood transfusion in humans (30). Although this may be related to the fact that only <10% of the NBB expressed MHC class Ag, H-2kβ and these cells may not significantly expand in unirradiated hosts. Alternatively, detection of CD45 variants expressed by C57BL/6-derived NBB may allow better determination of the level of chimerism in NOD recipients. Nevertheless, chimerism was detected in a majority (four out of seven) of NBB-transfused mice by RT-PCR, but it did not strictly correlate with the level of protection against diabetes. These data are consistent with previous studies indicating that induction of chimerism after bone marrow or HSC transfusion is highly variable in NOD mice (6–8, 12), and low levels of allogeneic but not syngeneic hematopoietic chimerism were sufficient to abrogate diabetes (7, 12, 29). Bone marrow transfusion-induced mixed chimerism has been suggested to reinitiate clonal deletion of autoreactive (β cell-reactive) T cells (6). Although it is difficult to track the diabeticogenic CD4+ T cells by tetramer staining without in vitro expansion that may introduce bias, tetramers that bind to diabeticogenic CD8+ T cells recognizing the IGRP 214–223 peptide (55) may allow ex vivo determination of the modulation of a subset of diabeticogenic T cells in protected mice.

In conclusion, the present data in the study demonstrated the utility of allogeneic NBB, functionally equivalent to umbilical cord blood, to ameliorate autoimmune diabetes in NOD mice. This model system may prove to be useful for the delineation of the mechanisms involved in abrogation of diabetes by allogeneic neonatal stem cell therapy in the absence of recipient preconditioning.

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