Plasmacytoid Precursor Dendritic Cells From NOD Mice Exhibit Impaired Function

Are They a Component of Diabetes Pathogenesis?

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OBJECTIVE—Plasmacytoid precursor dendritic cell facilitating cells (p-preDC FCs) play a critical role in facilitation of syngeneic and allogeneic hematopoietic stem cell (HSC) engraftment. Here, we evaluated the phenotype and function of CD8\(^+\)/TCR\(^-\) FCs from NOD mice.

RESEARCH DESIGN AND METHODS—The phenotype of CD8\(^+\)/TCR\(^-\) FCs was analyzed by flow cytometry using sorted FCs from NOD, NOR, or B6 mice. The function of NOD FCs was evaluated by colony-forming cell (CFC) assay in vitro and syngeneic or allogeneic HSC transplantation in vivo.

RESULTS—We report for the first time that NOD FCs are functionally impaired. They fail to facilitate engraftment of syngeneic and allogeneic HSCs in vivo and do not enhance HSC clonogenicity in vitro. NOD FCs contain subpopulations similar to those previously described in B6 FCs, including p-preDC, CD19\(^+\), NK1.1\(^+\)/DX5\(^+\), and myeloid cells. However, the CD19\(^+\) and NK1.1\(^+\)/DX5\(^+\) subpopulations are significantly decreased in number in NOD FCs compared with disease-resistant controls.

CONCLUSIONS—These data demonstrate that NOD FCs exhibit significantly impaired function that is reversible, since FL restored production of functional FCs in NOD mice and suggest that FL plays an important role in the regulation and development of FC function. FCs may therefore be linked to diabetes pathogenesis and prevention.

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diabetes pathogenesis and prevention and may provide a novel cell-based approach to restore self-tolerance and regulation in treatment of type 1 diabetes.

RESEARCH DESIGN AND METHODS

Mice. Four- to six-week-old NOD mice (male and female; Taconic Laboratories, Germantown, NY), female nonobese resistant (NOR) mice, male C57BL/6 mice, and female C57BL/10SnJ mice (Jackson Laboratory, Bar Harbor, ME) were used. Animals were housed in the barrier facility at the Institute for Cellular Therapeutics (Louisville, KY) and were cared for according to National Institutes of Health animal care guidelines.

Antibodies. All monoclonal antibodies (mAbs) used in this study were purchased from BD Biosciences (San Diego, CA). c-Kit 5Sca-1 Lin (HSC) sorting experiments used the following mAbs: stem cell antigen-1 (Sca-1) phycoerythrin (PE), c-kit allophycocyanin (APC), and the lineage panel consisting of the following: CD8α fluorescein isothiocyanate (FITC), Mac-1 FITC, B220 FITC, Gr-1 FITC, CD45R B220 PerCP, Gr-1 APC, and CD19 APC. CD8α/TCR FITC, NK1.1 PE, and DX5 PE. FITC, NK1.1 FITC, DX5 FITC, B220 PerCP, Gr-1 APC, and CD19 APC, as previously described (15).

Statistical analysis. Experimental data were evaluated for significant differences using Student’s t test; P < 0.05 was considered significant. Graft survival was calculated according to the Kaplan-Meier method (4).

RESULTS

NOD FCs exhibit specific and significant differences in subpopulations compared with normal controls. In normal mice, the CD8+/TCR− FC (FC total) population is heterogeneous, with the dominant subpopulation phenotypically resembling p-preDC (B220+/CD11c+/CD11b−) (4). Smaller percentages of B-cell (CD19+), NK cell (NK1.1+DX5+), granulocyte (Gr-1+), and monocyte (CD14+) subpopulations are also present in FC total from normal mice (4). We found that NOD and NOR FCs are comprised of similar distinct heterogeneous subpopulations (Fig. 1A and C) and show a heterogeneous morpho-

phology with Wright-Giemsa staining under light microscopy (Fig. 1B and D). p-preDC FCs represent the major CD8+/TCR− FC subpopulation in all strains (female and male NOD mice, female NOR mice, and male B6 mice) examined (Fig. 1E). The B220+/CD11c+ FC population in female and male NOD mice is significantly increased compared with control NOR or B6 mice (Fig. 1F; P < 0.05). The B220+/CD11c+ cell subset is significantly decreased compared with NOR mice (Fig. 1F; P < 0.007). As previously shown, the dominant cell population in CD19+ FC is pre-B-cells (B220+/CD11c−/intracytoplasmic IgM+) (4). Of the female NOD FCs, 14% were CD19+, which is significantly decreased compared with NOR and B6 mice (Fig. 1F, P < 0.05). Approximately 0.27% of NOD FCs are CD19+/CD11c−/B220− cells (Fig. 1G), which is not significantly different from the control strain. DCs with a similar phenotype from normal LN and spleen have been shown to function as p-preDC (16). Of the female NOD FC total, B220+/NK1.1+DX5+ and B220+/Gr-1+ populations were significantly decreased compared with B6 FCs (Fig. 1K and J). The B220+/CD14+ population was not significantly different from all strains examined (Fig. 1L).

FCs produce cytokines and upregulate activation markers after stimulation. We evaluated whether NOD FCs resemble NOR FCs in response to CpG-ODN stimulation. CD86 was upregulated on NOR FCs, whereas CD80 and class II expression was similar in the absence of CpG stimulation (Fig. 2A and B). However, whereas CD86 was upregulated on NOR FCs, CD80 expression was markedly decreased with stimulation (Fig. 2A and B). After CpG-ODN stimulation, the majority of NOR FCs were in a more activated state compared with NOD FCs, as evidenced by their dendritic morphology (Fig. 2C, right panel). In contrast, NOD FCs did not exhibit a mature morphology after CpG treatment (Fig. 2C, left panel).

We also examined chemokine and cytokine production by NOD and NOR p-preDC FCs after CpG-ODN stimulation. In the presence of CpG-ODN, p-preDC FCs produced more MIP-1α/CCL3, RANTES/CCL5, interferon-γ-induced protein (IP)-10, IL-6, and TNF-α compared with the levels of those in absence of stimulation (Fig. 2D). Notably, p-preDC FCs from NOR mice produced higher amounts of IL-6 (5×), RANTES/CCL5 (3.5×), MIP-1α/CCL3 (2.1×), and TNF-α (1.9×) compared with NOD p-preDC FCs (Fig. 2D). In addition, we found that NOR p-preDC FCs produce GM-CSF more efficiently in response to CpG-ODN stimulation, whereas NOD p-preDC FCs do not (Fig. 2D). Taken together, these data demonstrate that NOD p-preDC FCs are impaired in their ability to produce chemokines and cytokines after CpG-ODN stimulation.

NOD CD8+/TCR− FC function is significantly impaired in vivo. We next examined the ability of NOD FCs to facilitate HSC engraftment using a syngeneic model (13,14). NOD recipients were ablatively conditioned with 950 cGy TBI and reconstituted with 500 HSCs ± 30,000 FCs sorted from NOD donors. Only 4 of 13 (31%) recipients of HSCs plus FCs and 4 of 17 (24%) recipients of HSCs engrafted survived up to 130 days (Fig. 3B). In striking contrast with normal controls (4), NOD FCs did not improve HSC engraftment in NOD recipients, as evidenced by the similar engraftment of HSCs with FCs compared with the HSCs alone (P = 0.579).

We then examined the function of NOR FCs. NOR mice are MHC-congenic to NOD mice, but do not develop diabetes (Fig. 3A) (5). Five (31%) of 16 recipients of HSCs alone engrafted and survived up to 130 days. In contrast,
70% (7 of 10) of recipients of HSCs plus FCs engrafted long term with survival over 130 days (Fig. 3C). Therefore, NOR FCs significantly enhance engraftment of HSCs in limiting numbers of HSCs ($P = 0.029$).

To assess whether NOR FCs facilitate engraftment of NOD HSCs, 500 NOD HSCs plus 30,000 NOR FCs ($n = 15$) were transplanted into NOD recipients conditioned with 950 cGy. All recipients of HSCs alone expired before 130
days after transplantation (Fig. 3D). In striking contrast, the majority of (11 of 21) animals transplanted with NOD HSCs and NOR FCs failed to enhance colony formation for in vivo facilitation (Fig. 5B). Four of nine (44%) recipients of HSC plus CD8\(^+/\)TCR\(^−\) cells (4). In NOD mice, 1–1.5% of FCs express NK1.1\(^+\)DX5\(^+\). To test the contribution of the NK1.1\(^+\) DX5\(^+\) FC subpopulation to total FC function. Donor NK cells have the potential to promote HSC engraftment and suppress GVHD in allogeneic transplantation (17). Our previous data showed that \(~4–6%\) of FCs are NK1.1\(^+\)DX5\(^+\) cells (4). In NOD mice, 1–1.5% of FCs express NK1.1\(^+\)DX5\(^+\). To test the contribution of the NK1.1\(^+\) DX5\(^+\) FC subpopulation to FC function, HSCs, CD8\(^+/\)TCR\(^−\) FCs, and CD8\(^+/\)TCR\(^−\)/NK1.1\(^+\)DX5\(^+\) cells were sorted from the marrow of B6 donors; 58% (7/12) of recipients of HSC plus CD8\(^+/\)TCR\(^−\) FCs survived up to 110 days, and 42% (5/12) of HSC plus CD8\(^+/\)TCR\(^−\)/NK1.1\(^+\)DX5\(^+\) recipients survived over 110 days (Fig. 5C). Survival of both groups was significantly enhanced compared with the group that received HSCs alone (\(P = 0.009\)).

**FL-mobilized NOD FCs facilitate HSC engraftment in allogeneic recipients.** We previously reported that FL treatment of NOD mice restored production of defective mature myeloid DCs and plasmacytoid DCs in spleen and pancreatic lymph nodes and significantly increased T\(_{reg}\) in pancreatic lymph nodes (18). This was associated with a significant delay in diabetes progression. To test whether FL treatment can restore the function of NOD FCs, we evaluated the phenotype and function of FL-PB FCs. NOD mice were treated with FL for 10 days. FCs were sorted from peripheral blood, and sorted FCs were stained with B220, CD11c, CD19, NK1.1DX5\(^+\), and CD11b mAbs. There was a significant increase in B220\(^+/\)CD11c\(^+\)/CD11b\(^−\) DC and NK1.1\(^+\)DX5\(^+\) subpopulations in FL-PB FCs (Fig. 6A). The percentage of CD19\(^+\) FCs and p-preDC FCs remained at the same levels as untreated NOD bone marrow FCs (Fig. 6A).

We next evaluated whether FL treatment can restore the facilitating function of NOD FCs. FL mobilized NOD PB FCs were in a more activated state than untreated NOD bone marrow FCs, as evidenced by their dendritic morphology (Figs. 6B and 1B). To test function of FL-PB FCs, HSCs were sorted from bone marrow of untreated

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**FIG. 1.** Continued.
NOD mice and FCs from the PB of FL-treated NOD mice. Conditioned B10 recipients received 5,000 HSCs plus 30,000 FL-PB FCs. Control mice were transplanted with 5,000 HSCs plus 30,000 FCs from bone marrow of untreated NOD mice. FL-PB FCs significantly enhanced engraftment of HSCs, as evidenced by 63% of recipients (n = 8) that received HSC plus FL-PB FCs surviving 120 days (Fig. 6C). A total of 13% and 20% of recipients of
HSCs alone (n = 9) or HSCs plus FCs (n = 8) from untreated NOD mice survived over 120 days, respectively (Fig. 6C).

To confirm that recipients of HSCs plus FL-PB FCs exhibited durable engraftment and multi-lineage reconstitution, animals were followed for >4 months. Three-color flow cytometric analysis was performed. Recipients of HSCs alone showed the presence of cells of donor origin including DCs (CD11c), macrophage (Mac-1) and granulocytes (Gr-1), and NK cells (NK1.1DX5) and the presence of low levels of T-cells (CD8, CD4, αβ-TCR + γδ-TCR) and B-cells (B220) (Fig. 6D). In contrast, recipients of HSCs plus FL-PB FCs showed donor chimerism for multi-lineages, including T-cells, B-cells, NK cells, macrophages, and granulocytes (Fig. 6E).

**DISCUSSION**

CD8+/TCR− FCs play an important role in facilitating engraftment of syngeneic and allogeneic HSCs (1,14,19). CD8+/TCR− FCs (FC total) are heterogeneous, including the following phenotypic subpopulations: p-preDC, B-
cells, NK cells, and monocytes (4). The p-preDC FC subpopulation represents the majority of FCs and plays a critical role in this complex network (4). Removal of the p-preDC component from the FCs results in complete loss of facilitation, confirming that p-preDCs are the primary component in facilitation (4). However, p-preDC FCs facilitate HSC engraftment significantly less efficiently than total FCs (4), suggesting that other FC subpopulations expressing the B-cell and NK cell phenotypes may play a collaborative role in facilitation. FCs induce generation of T_{reg} in vitro and in vivo (20,21). More recently, T_{reg} have been shown to enhance engraftment of HSCs in syngeneic recipients (22). The fact that the FC maintains its tolerogenic function in vivo addresses one of the major concerns regarding DC-based therapies: how to avoid immune activation and maintain tolerogenicity after infusion in vivo (23). As such, FCs may offer a novel cell-based therapeutic approach to induce tolerance in the clinic for treatment of autoimmune disorders.

In the present study, we evaluated the function of FCs in the context of a mouse model for type 1 diabetes, a systemic autoimmune disease (5). We found that NOD FCs, as a heterogeneous population, share phenotypic characteristics similar to those previously described for wild-type FCs (4). However, the percentages of both CD19− and NK1.1−DX5− cells in NOD FCs were significantly decreased compared with NOR or B6 FCs. NOD FCs were functionally impaired and failed to facilitate engraftment of HSCs in both syngeneic and allogeneic recipients, as well as in an in vitro assay for FCs. Notably, FL treatment expanded PB FCs in NOD mice and restored the ability of NOD FCs from the PB to facilitate engraftment of allogeneic HSCs. We propose that the defective function of NOD FCs may be due to an abnormal activation status of the p-preDC FC subpopulation or the presence of impaired function of a collaborative subpopulation in FCs such as B-cells or NK cells. This hypothesis offers an attractive explanation for the mechanism by which FCs enhance HSC engraftment in vivo and induce tolerance.

The majority of FCs are B220+/CD11c+/CD11b− and share characteristic features of p-preDC, including morphology and phenotype, secretion of similar cytokines and chemokines, and maturation after stimulation with CpG-ODN (4). However, their distinct differences, including the fact that FCs produce IL-10 whereas p-preDC do not, may offer an understanding as to how p-preDC FCs remain tolerogenic in vivo. p-preDCs play an important role in regulating innate and adaptive immune responses. They selectively express toll-like receptors (TLR)-7 and TLR-9 and are specialized in rapid secretion of type 1 IFN after viral stimulation (24). p-preDC can promote the function of NK cells, B-cells, T-cells, and myeloid DCs through type 1 IFN during an antiviral immune response and differentiate into unique types of mature DCs, which directly regulate the function of T-cells and thus link innate and adaptive immune responses. We were the first to show that p-preDC have the potential to facilitate engraftment of HSCs (4). p-preDCs also induce tolerance to heart allografts (25). Several studies have demonstrated an association between the pathogenesis of autoimmune diseases (systemic lupus erythematosus, Sjogren’s Syndrome, and dermatomyositis) and defective function of IFN-α producing p-preDC (26–28). NOD mice exhibit an abnormal DC phenotype and function (10,11). We show here for the first time that FCs from NOD mice exhibit a functional defect in facilitating HSC engraftment in vivo and impaired function in vitro as well. However, the fact that FL treatment of NOD donors results in production of functional FCs implies that the defect is probably not cell intrinsic, but rather due to a lacking signal or activated state.

FL plays a critical role in the development of p-preDC in humans and mice (29,30). The ability of FL to promote p-preDC development in vivo was confirmed by experiments showing that administration of FL into human volunteers led to an increase in the number of PB p-preDCs in humans and that FL transgenic mice have increased numbers of p-preDCs, where FL-deficient mice have fewer p-preDCs (31). Our own work showed that treatment of prediabetic NOD mice with FL significantly decreased insulin and progression to diabetes and was associated with a significant increase in myeloid DCs, plasmacytoid DCs, and T_{reg} (18). When DCs from NOD mice bone marrow are treated with nuclear factor κB–specific ODN in vitro, administration of DCs into NOD mice can effectively prevent the onset of diabetes (32). FL is also a key cytokine for FC generation and expansion, as evidenced by FL–bone marrow culture and the mobilization of FC in peripheral blood (4). FL-mobilized PB FCs promote the establishment of donor chimerism and tolerance induction. In the present study, we showed that FL treatment can restore the function of NOD FCs, demon-
strating that FL can promote that development and function of FCs in NOD mice.

The importance of myeloid DCs on their ability to trigger B-cell growth and differentiation has been addressed (33). A recent study showed that the p-preDCs regulate B-cell function by producing IFN-α and IL-6, which thereby induces B-cell differentiation (34). Depletion of p-preDC from human blood mononuclear cells abrogates the secretion of immunoglobulins in response to influenza virus and affects the differentiation of activated B-cells into plasma cells through the secretion of IFN-αβ and IL-6 (34). Several experimental models in animals have shown that B-cells are involved in inducing T-cell tolerance in vivo (35,36). The role of B-cells in autoimmune diseases may occur through several mechanistic pathways that include self-reactive antibodies, secretion of inflammatory cytokines, participation in antigen presentation, and augmentation of T-cell activation (37). In NOD mice, the B-cell subpopulation (CD19+) within the total FC population is present at a much lower frequency.
compared with NOR and B6 controls. The function of the CD19/H11001 FC subpopulation remains elusive. Removal of this subpopulation from normal donors did not impair facilitation. It is formally possible that the CD19/H11001 FC subpopulation does not contribute to FC function or that there is redundancy in the system that is contributed from another FC collaborative subpopulation.

NOD FCs also contain significantly lower numbers of NK1.1/H11001/DX5/H11001 cells compared with B6 or NOR mice. It was unclear whether the failure of FC function was due to decreased numbers of the NK FC subpopulation. NK cells have been shown to play a major role in regulating early engraftment of allogeneic bone marrow cells, both by activation as well as inhibition of immune responses (38). Adoptive transfer of activated NK cells early after transplantation inhibits GVHD and promotes graft-versus-tumor (GVT) in the mouse model (39). Recently, various studies have investigated the interaction between NK cells and plasmacytoid DCs, suggesting that the cross-talk between NK cells and DCs leads to NK cell activation and DC maturation (40). NOD mice exhibit an abnormally low level of NK cell activity (7,41) and a defect in NK/T-cells (42). To evaluate whether NK FCs were involved in facilitation of HSC engraftment, we performed allogeneic HSC transplantation (B6 → C3H) using FCs depleted of the NK FC subpopulation. There was no difference in engraftment in mice that receive HSC plus FC total vs. FCs depleted of NK FCs, suggesting that NK FCs did not contribute to facilitation.

The interaction of DCs and Treg in the regulation of innate and adaptive immune responses has been reported (43). The consequences of DC interactions with Treg depend on the phenotype and maturation status of DCs. Mature DCs have the unique ability to promote natural Treg expansion, which limits immune responses to self-antigen (43,44), whereas immature DCs induce the generation of adaptive Treg, which control the immune response to non–self-antigen (45). Mice lacking CD28 or its ligands have decreased numbers of CD4/H11001/CD25/H11001 cells (46). These data suggest that both CD86 and CD80 are capable of delivering the signals that promote Treg generation. It has been shown that on the NOD mouse background, genetic ablation of CD86 results in only a subtle decrease in Treg numbers (20–30% reduction). The remaining Treg were largely dependent on CD80, as the additional injection of anti-CD80 blocking mAbs substantially decreased the number of CD4+/CD25+ cells (∼70% reduction). These data support a more important role for CD80 than CD86 in generation of Treg (47). Notably, we found that NOD FCs exhibit significantly impaired upregulation of CD86 after
stimulation with CpG. Similarly, and in contrast with FCs from diabetes-resistant donors, they failed to produce GM-CSF and produced significantly lower levels of IL-6 after CpG stimulation. Several groups have reported that NOD mice exhibited reduced Treg frequency (48,49), and their impaired suppressive function has been linked to diabetes pathogenesis (50). The fact that wild-type FCs can induce the generation of Treg, but only in the presence of CpG-ODN (21), and that they are impaired in function in diabetes-prone NOD mice suggests that FCs may also play a distinct role in diabetes pathogenesis.

In conclusion, our data reveal a novel defect in NOD FC function that is restored by treatment with FL. Our data suggest the critical role of FL in developing and maintaining the function of FCs. These findings may have clinical implications for the treatment of type 1 diabetes and possibly other autoimmune disease states.

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