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Defects in the Differentiation and Function of Bone Marrow-Derived Dendritic Cells in Non-Obese Diabetic Mice

Due to their high immunostimulatory ability as well as the critical role they play in the maintenance of self-tolerance, dendritic cells have been implicated in the pathogenesis of autoimmune diseases. The non-obese diabetic (NOD) mouse is an animal model of autoimmune type 1 diabetes, in which pancreatic beta cells are selectivly destroyed mainly by T cell-mediated immune responses. To elucidate initiation mechanisms of beta cell-specific autoimmunity, we attempted to generate bone marrow-derived dendritic cells from NOD mice. However, our results showed low proliferative response of NOD bone marrow cells and some defects in the differentiation into the myeloid dendritic cells. NOD dendritic cells showed lower expressions of MHC class II, B7-1, B7-2 and CD40, compared with C57BL/6 dendritic cells. In mixed lymphocyte reactions, stimulatory activities of NOD dendritic cells were also weak. Treatment with LPS, INF-γ and anti-CD40 stimulated NOD dendritic cells to produce IL-12p70. The amount of IL-12, however, appeared to be lower than that of C57BL/6. Results of the present study indicated that there may be some defects in the development of NOD dendritic cells in the bone marrow, which might have an impact on the break-down of self tolerance.

Key Words: *Diabetes Mellitus; Dendritic Cells; Mice, Inbred NOD; Autoimmunity*

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INTRODUCTION

The non-obese diabetic (NOD) mouse is a well-established model of autoimmune type 1 diabetes (1). Although the precise mechanism is unknown, destruction of pancreatic beta cells seems to be initiated and maintained by sensitized and activated auto-reactive T cells. Indeed, T cells reactive with a number of pancreatic beta cell proteins, e.g., glutamic acid decarboxylase (GAD), have been demonstrated (2, 3). Including a role of antigen presenting cells, however, the initiation mechanism responsible for triggering T cell-mediated autoimmunity is still unknown.

Dendritic cells are believed to play a key role in the initiation of primary immune responses in vivo (4-7). Due to their remarkably high ability to stimulate immune responses, it has been proposed to use dendritic cells for immunotherapy of tumors and viral infections (8-11). Dendritic cells have also been implicated in autoimmune diseases. In NOD mice, dendritic cells have been suggested as a crucial antigen presenting cell (APC) due to their peri-insular accumulation in the initiation stage of insulitis (12, 13). Adoptive transfer of dendritic cells also showed their potential capacity to induce autoimmune responses (14, 15).

This study was initiated to investigate whether adoptive transfer of dendritic cells presenting an autoantigen such as GAD could induce or accelerate insulitis in young NOD mice. To generate bone marrow-derived dendritic cells from NOD mice, bone marrow cells were cultured in the presence of granulocyte-macrophage-colony stimulating factor (GM-CSF) following the same method that was previously used for the culture of C57BL/6 bone marrow-derived dendritic cells in our laboratory. Unfortunately, we could not obtain mature dendritic cells. NOD bone marrow cells seemed to have some defects in differentiation into myeloid dendritic cells. Since dendritic cells are of key importance in the control of immune response, we examined various aspects of bone marrow-derived dendritic cells of NOD mice in comparison with those of C57BL/6 mice.

METHODS AND MATERIALS

Mice

NOD mice were purchased from the Jackson Labo-

ratory (Bar Harbor, ME, U.S.A.). C57BL/6 and BALB/c mice were purchased from Daehan Laboratory Animal Research Center (Eumsung, Korea). All mice were purchased at 5 wk of age, and maintained under specific pathogen-free conditions until the experiments.

Generation of dendritic cells from bone barrow

Dendritic cells were generated from bone marrow cultures following Dr. Inaba's method (16) with modification. Bone marrow cells were obtained from femurs and tibias of female mice at 6-8 wk of age. Red blood cells were lysed with Tris-buffered ammonium chloride (90 mL of 0.16 M NH_4Cl, 10 mL of 0.17 M Tris, pH 7.2). Lymphocytes and I-A positive cells were eliminated by treating with a cocktail of monoclonal antibodies [53-6.72 (anti-CD8, ATCC TIB105), GK1.5 (anti-CD4, ATCC TIB207), RA3-3A1/6.1 (anti-B220, ATCC TIB46), B21-2 (anti-I-$A^{b,d}$, ATCC TIB229) or M5/114.15.2 (anti-I-A&I-E, ATCC TIB120)] and complement (guinea pig serum; Serotec, Oxford, UK) for 40 min at 37℃. After washing, the bone marrow cells (1×10^6 cells/well) were plated in 24 well culture plates (Costar, Cambridge, MA, U.S.A.) in 1 mL of RPMI 1640 containing 10% FCS, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, 10 mM HEPES (Gibco BRL, Grand Island, NY, U.S.A.), 50 μM 2-mercaptoethanol and 20 ng/mL recombimant murine GM-CSF (Peprotech, London, UK) in the presence or absence of 20 ng/mL recombinant murine IL-4 (Endogen, Woburn, MA, U.S.A.). The culture medium was fed every 2 days by replacing 75% of medium. Non-adherent cells were collected on day 7 and day 8 of culture, in NOD and in C57BL/6 mice, respectively. After 24-48 hr of subculture, the non-adherent cells which had acquired typical dendritic cell morphology on phase contrast microscope were used as the source of dendritic cells in subsequent experiments.

Flow cytomeric analysis

For flow cytometry, $1\text{-}5\times10^5$ dendritic cells were incubated for 30 min at 4℃ using the following FITC-conjugated monoclonal antibodies (PharMingen, San Diego, CA, U.S.A.): anti-CD80 (B7-1; 16-10A1), anti-CD86 (B7-2; GL1), anti-CD40 (3/23), and anti-I-A^k (10-3.6 that has been reported to react with NOD H-2^{g7} (18)). Dendritic cells were also stained with hybridoma culture supernatant anti-I-$A^{b,d}$ (B21-2, ATCC TIB229) and anti-CD11c (N418, ATCC HB224), in which PE-conjugated goat anti-rat IgG (Serotec, Oxford, UK) was used as a secondary antibody. After washing, stained cells were fixed with 1% para-formaldehyde, and analyzed by FACS Vantage (Beckton Dickinson, U.S.A.). Bindings of 10-3.6 and M5/114.5.2 monoclonal antibody to NOD MHC class II molecules were confirmed by flow cytometric analysis of NOD spleen cells stained with those antibodies.

Endocytosis

To investigate endocytic capacity of bone marrow-derived dendritic cells, 1×10^6 cells were incubated at 37℃ or 0℃ for 1 hr with 1 mg/mL FITC-dextran (42,000 Da, Sigma, St. Louis, MD, U.S.A.). After incubation, cells were washed twice with cold HBSS, stained for the surface MHC class II molecules as described above. Double stained dendritic cells were analyzed by flow cytometry.

Mixed lymphocyte reaction (MLR)

Various numbers of irradiated (3,000 Rad, Gamma cell 3000, Elan, Nordion, Canada) bone marrow-derived dendritic cells (1×10, 1×10^2, 1×10^3, 1×10^4, and 1×10^5/well) were co-cultured with allogeneic T cells (1×10^5/well) in 96-well culture plates. T cells were obtained from spleen cells of BALB/c mice by passing through nylon wool column. After 3 days, T cell proliferation was measured by using a BrdU incorporation assay kit (Amersham, Arlington Height, IL, U.S.A.). Briefly, cells were pulsed with 10 μM/well of BrdU labeling solution for 24 hr. BrdU incorporated cells were fixed and incubated with 100 μL of peroxidase-conjugated anti-BrdU solution for 2 hr at room temperature. After adding 100 μL of 3,3'5,5'-tetra-methylbenzidine (TMB) substrate solution, the reaction was stopped by adding 25 μL of 1 M sulphuric acid, and the absorbance was read with a ELISA reader (E max; Molecular Device, U.S.A.) at 450 nm.

IL-12 ELISA

Bone marrow-derived dendritic cells were stimulated with 1 μg/mL lipopolysaccharide (LPS) (*E. coli* 026:B6; Sigma, St. Louis, MO, U.S.A.), 5 μg/mL anti-CD40 (MH 40.3; PharMingen, San Diego, CA, U.S.A.), 5 μg/mL anti-I-A^k (10-3.6; PharMingen, San Diego, CA, U.S.A.), hybridoma culture supernatant anti-I-A & I-E (M5/114.15.2, ATCC TIB120) and 3 ng/mL recombinant murine IFN-γ (R&D, Minneapolis, MN, U.S.A.) for 40 hr. IL-12p70 production was determined by a mouse IL-12p70 ELISA kit (Endogen, Woburn, MA, U.S.A.). Briefly, 50 μL of standards or culture supernatants of dendritic cells was incubated in anti-mouse IL-12p70 precoated strip well plates at room temperature for 2 hr. After 50 μL of prepared biotinylated antibody was added, the plates were incubated at room temperature for 1 hr, and then incubated with 100 μL of Streptavidin-HRP at

room temperature for 30 min. TMB substrate solution was added and the plates were developed in the dark at room temperature for 30 min. The reaction was stopped by adding 100 μL of stop solution, and the absorbance was read at 450 nm using a ELISA reader (E max; Molecular Device, U.S.A.). The relationship between absorbances and IL-12p70 concentrations was linear between 0 and 333.3 pg/mL, with correlation coefficients >0.998, consistently.

RESULTS

Characteristics of NOD dendritic cells cultured with GM-CSF

To generate mature dendritic cells, bone marrow cells of female NOD mice were cultured in the presence of GM-CSF. As we previously observed in C57BL/6 (18), loosely adherent aggregates of growing dendritic cells were recognized at day 4 of culture. Dendritic cells began to float at day 7 in NOD, while it was at day 8 in C57BL/6. After 24-48 hr of subculture, most of the non-adherent cells showed the veil- or sheet-like processes. No morphologic difference between NOD and C57BL/6 dendritic cells was noted. To examine expression of surface molecules, flow cytometry was done. In both NOD and C57BL/6 dendritic cells, T cell markers such as CD4, CD8, and CD3 and B cell markers such as B220 were negative (data not shown). Most of C57BL/6 dendritic cells highly expressed MHC class II molecules, co-stimulatory molecules (B7-1, B7-2) and CD40 (Fig. 1). In contrast, NOD dendritic cells showed negative or low expression of MHC class II molecules as well as B7-1, B7-2 and CD40 (Fig. 1A). Meanwhile, NOD spleen cells stained with 10-3.6 monoclonal antibody showed similar fluorescence intensity to C57BL/6 spleen cells stained with B21-2 monoclonal antibody (data not shown).

To investigate capacity of antigen-uptake, endocytosis of FITC-dextran by the dendritic cells was measured by flow cytometry. Most of NOD dendritic cells showed characteristics of immature dendritic cells which have endocytic activities and do not express surface MHC class II molecules (Fig. 1B). In contrast, most of C57BL/6 dendritic cells showed both endocytic activity and MHC class II expression on their surface (manuscript in preparation).

Yields of NOD dendritic cells

Growing cell aggregates were observed from day 2 of bone marrow culture in both NOD and C57BL/6. Compared with C57BL/6 cultures, NOD cultures consistently showed smaller size and lower number of the cell aggregates (data not shown). Table 1 shows yields of dendritic cells. Numbers of collected NOD dendritic cells were around 30% of the yield of C57BL/6. In C57BL/6 mice, yields of dendritic cells were improved by adding IL-4 into the bone marrow cultures (manuscript in preparation). In NOD mice, however, no such effects of IL-4 on the bone marrow cell proliferation was observed (Table 1).

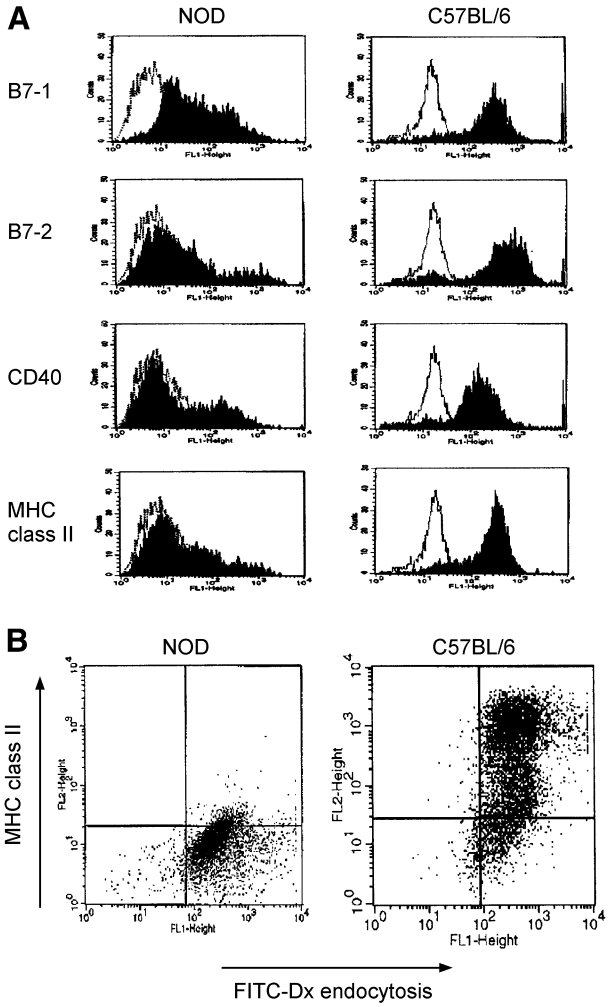

Fig. 1. Characteristics of NOD dendritic cells. Dendritic cells were generated from bone marrow cells of female NOD and C57BL/6 mice using GM-CSF. **A:** Expressions of surface molecules are examined by flow cytometry. To stain surface MHC class II molecules, anti-I-A^k (10-3.6) and anti- I-$A^{b,d}$ (B21-2) was used for NOD and C57BL/6, respectively. **B:** Two-color flow cytometric analysis of dendritic cells for endocytosis and surface MHC class II expression. Dendritic cells were incubated at 37℃ for 1 hr with FITC-dextran, and then stained with anti-MHC class II monoclonal antibodies as described in materials and methods. Control cells incubated at 0℃ were negative for FITC staining.

Table 1. Yields of dendritic cells

Mice	Culture condition	No. of harvested cells*
C57BL/6	GM-CSF alone	$3.80\pm0.89\times10^6$/well
NOD	GM-CSF alone	$1.28\pm0.25\times10^6$/well
	GM-CSF+IL-4	$1.35\pm0.07\times10^6$/well

Values are mean of three independent experiments±SD.
*Number of initial bone marrow cells was 1×10^7/well.

Effect of IL-4 on the expression of surface molecules of NOD dendritic cells

To investigate whether IL-4 can induce maturation of NOD dendritic cells, dendritic cells were generated in the presence of both GM-CSF and IL-4. As Fig. 2 shows, expressions of B7-1, B7-2 and CD40 were significantly increased by IL-4. Expression of MHC class II was also increased by IL-4. Compared to other surface molecules, however, increases in the MHC class II expression seemed to be relatively lower (Fig. 2B). We repeatedly examined the effect of IL-4 on the expression of MHC class II. In some experiments (as shown in Fig. 2B), IL-4 considerably increased expression of MHC class II. Sometimes, however, positive cell percents for MHC class II were as low as 10-20%, while those for CD80, CD86 and CD40 were consistently over 80%.

Further stimulation with LPS did not increase the expression of MHC class II in both dendritic cells that were cultured in the presence of GM-CSF and IL-4 (Fig. 2) and that were cultured in the presence of GM-CSF (data not shown). In addition, treatment with IFN-γ also did not increase the expression of MHC class II in NOD dendritic cells (data not shown).

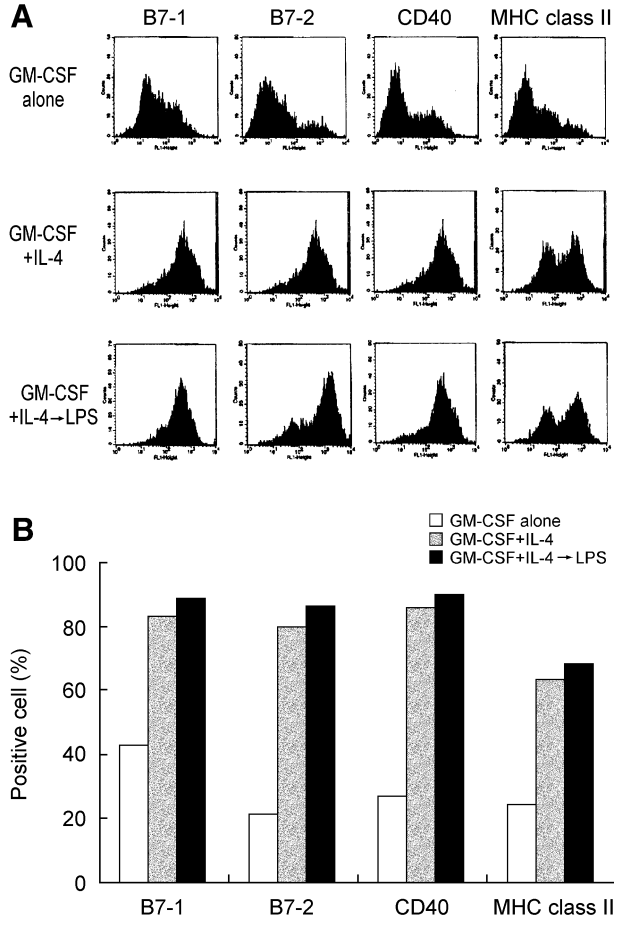

Fig. 2. Effect of IL-4 on the maturation of dendritic cells. Dendritic cells were generated from bone marrow cells of female NOD mice in the presence of GM-CSF alone or in the presence of both GM-CSF and IL-4. Some of the harvested dendritic cells were further stimulated with LPS for 1 or 2 days. Three independent experiments were performed, and representative results are shown in this figure. **A:** Expression of surface molecules are examined by flow cytometry. **B:** Positive percents of each group of dendritic cells for each surface molecule are shown as a graph.

Stimulatory effect of NOD dendritic cells in MLR

Stimulatory effects of NOD dendritic cells on T cells were tested in MLR. While C57BL/6 dendritic cells induced significant T cell proliferation response from the ratio of 1:100 (stimulator : responder), NOD dendritic cells induced strong T cell proliferation from the ratio of 1:10 (Fig. 3). When irradiated spleen cells were used as stimulators, T cell proliferation was observed at the ratio of 1:1 (data not shown).

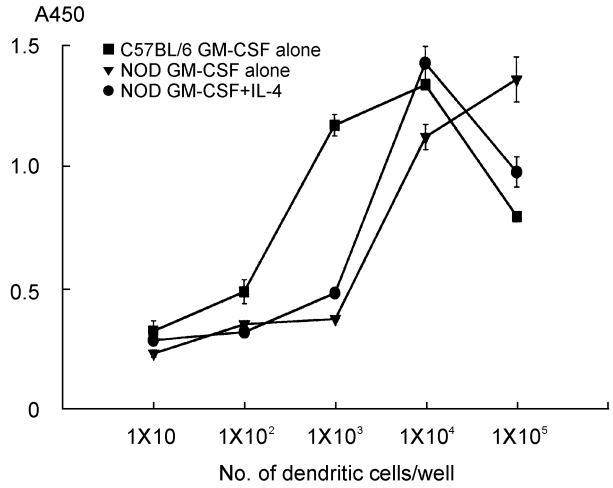

Fig. 3. Allostimulatory activities of NOD dendritic cells in mixed lymphocyte reaction. Irradiated dendritic cells generated under various culture conditions (1×10, 1×10^2, 1×10^3, 1×10^4, and 1×10^5/well) were used as allogeneic stimulators, and T cells (1×10^5/well) obtained from BALB/c mice were used as responders. Proliferation of responder T cells was measured by a BrdU uptake assay.

IL-12 production by NOD dendritic cells

IL-12 production by dendritic cells was assayed by IL-12p70 ELISA. LPS showed a strong stimulatory effect on dendritic cells to produce IL-12, and IFN-γ alone seemed to have only a minor stimulatory effect on both NOD and C57BL/6 mice (Fig. 4). Treatment with both IFN-γ and LPS appeared to have a synergistic effect in both NOD and C57BL/6 mice (Fig. 4). However, amount of IL-12 produced by NOD dendritic cells seemed to be lower than that of C57BL/6 dendritic cells (Fig. 4). Besides LPS, treatment with monoclonal antibodies against CD40 or MHC class II stimulated IL-12 production in C57BL/6 dendritic cells (Fig. 4) as determined by RT-PCR in our previous experiments (18). In NOD dendritic cells, treatment with an anti-CD40 monoclonal antibody induced IL-12 production, whereas treatment with an anti-MHC class II monoclonal antibody did not (Fig. 4).

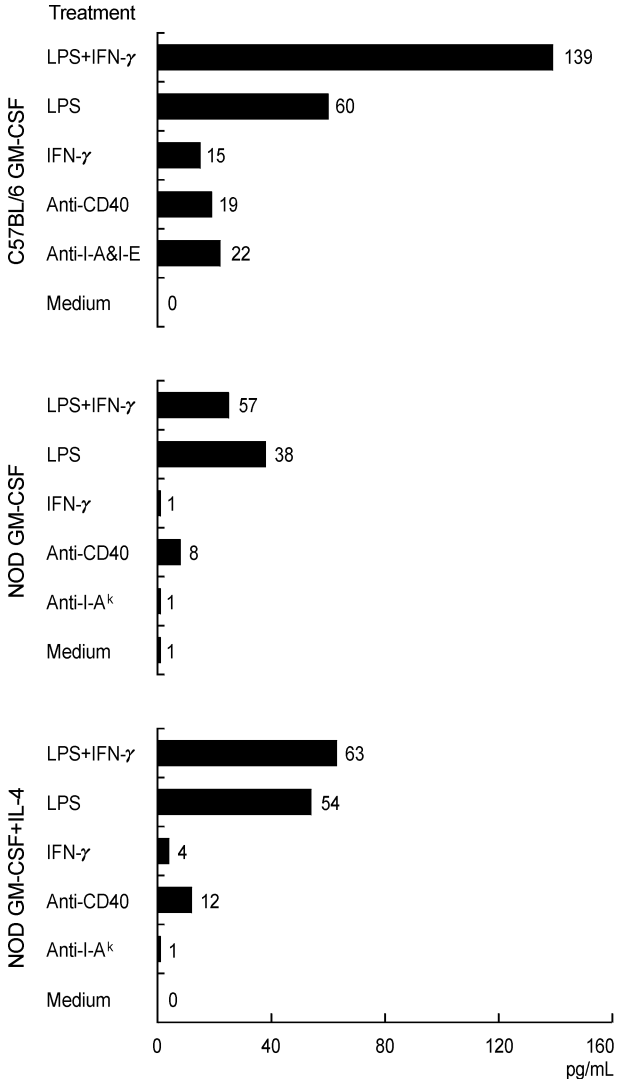

Fig. 4. Production of IL-12 by dendritic cells. Dendritic cells were incubated for 40 hr with various stimulators. IL-12p70 in the culture supernatants was measured by a ELISA kit.

DISCUSSION

Dendritic cells have been implicated in the development of autoimmune type 1 diabetes from two different viewpoints. As the most potent antigen presenting cells, it has been suggested that dendritic cells may play an important role in the initiation and/or progression of the autoimmune process (12, 14, 19). On the other hand, defects in the differentiation and function of antigen presenting cells have also been reported in both animal models and type 1 diabetes patients (20-24). In the present study, dendritic cells were generated from bone marrow cultures of NOD mice using GM-CSF. Immaturity of the NOD bone marrow-derived dendritic cells and poor proliferative responses of NOD bone marrow cells indicated that there might be some defects in the differentiation of dendritic cells in NOD mice.

Two subsets of dendritic cells have been described; lymphoid and myeloid dendritic cells (25). Myeloid dendritic cells are thought to induce immunity in naive T cells. They are GM-CSF-dependent and share precursors with macrophages. They are located in all non-hematopoietic tissues in immature form, but migrate to T cell zone of secondary lymphoid tissue following interaction with foreign antigens. In contrast, lymphoid dendritic cells are thought to induce tolerance in naive T cells. They are IL-3-dependent and share precursors with T and B cells. They are located in the thymic medulla and T cell zones of secondary lymphoid tissue. In this study, bone marrow cells were cultured in the presence of GM-CSF. Therefore, the bone marrow-derived dendritic cells of this study can be understood as myeloid dendritic cells.

Results of this study indicated defective maturation of myeloid dendritic cells in NOD mice. Similar findings, suggesting a correlation of dendritic cell defects with type 1 diabetes, were reported in humans; significantly decreased T cell stimulatory capacity and expressions of CD80 and CD86 were reported in monocyte-derived dendritic cells of type 1 diabetes patients and relatives (23, 24). In this study, immaturity of NOD dendritic cells, that had been cultured in the presence of GM-CSF, were indicated by low or no expression of MHC class II, co-stimulatory molecules and CD40 molecule and low capacity to stimulate T cells. Defective maturation of NOD dendritic cells was further indicated by the fact that stimulation with LPS could not increase the expression of MHC class II molecules. Adding IL-4 to the NOD bone marrow culture increased expression of

CD80, CD86 and CD40 to the levels of mature dendritic cells, but not MHC class II. These results indicated that bone marrow-derived NOD dendritic cells appeared to have some defects in the expression of MHC class II.

Low yield of NOD dendritic cells may represent defects in their bone marrow progenitor cells. Defects in the response of NOD bone marrow cells to the myeloid growth factors such as IL-3, GM-CSF and IL-5 in in vitro cultures were previously described (26). An association of diminished sensitivity of NOD hematopoietic precursors to CSF-1 with a quantitative inability to generate phenotypically and functionally mature macrophages was also demonstrated (20). In the present study, low proliferative response of NOD bone marrow cells to GM-CSF was observed and it was related with low yield of dendritic cells. Therefore, it is likely that there are some defects in the progenitors of myeloid dendritic cells in NOD mice.

Are there defects in NOD dendritic cells in vivo? Little is known about the function of dendritic cells in NOD mice. Adoptive transfer of dendritic cells isolated from the draining lymph nodes of the pancreas prevented diabetes in female NOD mice (27). Although exact mechanisms were unknown, it was suggested that transfer of those dendritic cells might regulate autoimmunity by inducing immune regulatory cells. Defects in dendritic cells, however, may be indirectly indicated by the low autologous mixed lymphocyte reaction (28), by decreased sensitivity of myeloid progenitor cells to GM-CSF (26) and a defect in bone marrow-derived antigen presenting cell function (20). Recently a reduced generation/maturation as well as dysfunction of splenic dendritic cells was reported in the biobreeding-diabetic prone rat (22), and similar impaired function of monocyte-derived dendritic cells was described in the preclinical phase human diabetes (23). An association of dendritic cell dysfunction with autoimmunity was also described in patients with premature ovarian failure (29).

In autoimmune type 1 diabetes, two subsets of dendritic cells may play different roles. Myeloid dendritic cells would be involved in the activation of various T cells including beta cell-reactive T cell clones, while lymphoid dendritic cells would be involved in the induction of deletion and/or anergy of self-reactive T cell clones. In a transgenic mouse model, adoptive transfer of bone marrow-derived dendritic cells obtained from C57BL/6 mice induced development of autoimmune diabetes (14). Adoptive transfer of bone marrow-derived dendritic cells presenting myelin basic protein led to experimental autoimmune encephalitis (15). These studies showed that mature dendritic cells presenting self-antigens could be potent inducers of autoreactive T cells. In NOD mice, presence of dendritic cells in the initiation of insulitis was demonstrated by immunohistochemistry (12). A role of infiltrating dendritic cells in triggering autoimmunity was further suggested by a report showing that infiltrating dendritic cells/macrophages but not B cells activated islet-specific T cells in transgenic NOD mice producing TNF-alpha by their islet cells (19). Therefore, mature dendritic cells seem to have the capacity to induce autoimmunity.

Then how can the immaturity of dendritic cells contribute to the development of autoimmunity? Defects in the suppressor cell activation were reported in NOD mice and diabetes patients (21, 28). Recent studies have shown that activation of immuno-regulatory T cells that were associated with Th2 cytokine profile inhibits destruction of beta cells in NOD mice (30, 31). Whether dendritic cells are involved in the activation of the suppressor/immuno-regulatory T cells remains to be investigated. In the present study, NOD dendritic cells appeared to be weak in both T cell stimulation and IL-12 production. As IL-12 is known to be one of the most important factors in determining direction of T cell differentiation toward Th1 or Th2 (32), whether low IL-12 production by dendritic cells is associated with autoimmunity of NOD mice should be further investigated. At the moment, how defects of dendritic cells exactly contribute to the breakdown of islet cell-specific self-tolerance in NOD mice is totally unknown. However, regarding that induction of both immuno-regulatory T cells and auto-reactive effector T cells requires initial T cell activation, defects in maturation and function of dendritic cells might have a profound impact on the development of autoimmune diabetes.

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