Immunotherapy of NOD Mice With Bone Marrow–Derived Dendritic Cells

Maryam Feili-Hariri, Xin Dong, Sean M. Alber, Simon C. Watkins, Russell D. Salter, and Penelope A. Morel

We evaluated two bone marrow–derived dendritic cell (DC) populations from NOD mice, the murine model for type 1 human diabetes. DCs derived from GM-CSF [granulocyte/macrophage colony-stimulating factor] + interleukin (IL)-4 cultures expressed high levels of major histocompatibility complex (MHC) class II, CD40, CD80, and CD86 molecules and were efficient stimulators of naive allogeneic T-cells. In contrast, DCs derived from GM-CSF cultures had low levels of MHC class II costimulation/activation molecules, were able to take up mannosylated bovine serum albumin more efficiently than GM + IL-4 DCs, and were poor T-cell stimulators. The two DC populations migrated to the spleen and pancreas after intravenous injection. To determine the ability of the two DC populations to modulate diabetes development, DCs were pulsed with a mixture of three islet antigen-derived peptides or with medium before injection into prediabetic NOD mice. Despite phenotypic and functional differences in vitro, both populations prevented in vivo diabetes development. Pulsing of the DCs with peptide in vitro did not significantly improve the ability of DCs to prevent disease, which suggests that DCs may process and present antigen to T-cells in vivo. In addition, we detected GAD65 peptide–specific IgG1 antibody responses in DC-treated mice. Overall, these results suggest that a Th2 response was generated in DC-treated mice. This response was optimal when using GM + IL-4 DCs, which suggests that the balance between regulatory Th2 and effector Th1 cells may have been altered in these mice. *Diabetes* 48:2300–2308, 1999

Dendritic cells (DCs) are known to be the most efficient antigen-presenting cells (APCs) and have the capacity to stimulate naive T-cells to initiate an immune response (1). DCs originate from lymphohematopoietic stem cells, and both myeloid and lymphoid progenitors likely give rise to DCs (2–7). Detailed analyses of DCs isolated from various sites such as skin, liver, thymus, spleen, and bone marrow have led to the recognition that multiple subsets of DCs exist, some of which are potent inducers of immunity, and some of which are able to induce tolerance (8–13).

Analysis of the antigen-presenting ability of DCs has revealed two distinct stages of functional development. DCs in the tissues are characterized by high endocytic function and low major histocompatibility complex (MHC) class II/costimulatory molecule expression. Once these cells take up antigen, they express high levels of MHC class II and costimulation/activation molecules, lose their endocytic ability, and migrate to lymph nodes (8,14–16). These cells have been called "immature" and "mature" DCs, respectively, on the basis of their ability to present antigen to T-cells (8,14,17). Various in vitro studies have demonstrated that the culture of bone marrow cells (myeloid progenitors) with GM-CSF (granulocyte/macrophage colony-stimulating factor) results in the growth and differentiation of macrophages, neutrophils, and DCs. Furthermore, manipulation of the culture system with the addition of interleukin (IL)-4 allows the generation of large numbers of more mature DCs that are potent APCs (12,18). Final maturation of DCs can be achieved by further stimulation with inflammatory stimuli or after CD40 ligation (19,20).

The NOD mouse spontaneously develops autoimmune diabetes characterized by the destruction of β-cells in the islets of Langerhans. Antibody and T-cell responses to various islet autoantigens, including GAD65, heat shock protein 60 (hsp60), and insulin, have been detected in NOD mice (21–23), and Th1 responses to these autoantigens have been implicated in disease development (24–26). The development of diabetes could be inhibited if the islet-specific response were shifted from a Th1 to a Th2 response (26–28). Several studies have suggested that the T-cell autoreactivity in NOD mice is the result of defects in APC function and differentiation that lead to a failure to generate sufficient regulatory T-cells (29–31). Recent studies have implicated DCs in the induction of diabetes (32), and administration of IL-12 in NOD mice was shown to induce diabetes with an accumulation of DCs in the pancreatic islets (25). Most recently, early infiltration of DCs and macrophages has been shown to be responsible for tumor necrosis factor-α production in the islets that is independent of T-cells (33), thus implicating DCs in the early pathogenesis of insulitis. On the other hand, Clare-Salzler et al. (34) demonstrated that the injection of DCs isolated from pancreatic lymph nodes could delay the onset of diabetes in prediabetic syngeneic NOD recipients, whereas DCs isolated from other lymph nodes or spleen had

From the Departments of Medicine (M.F.-H., P.A.M.), Pathology (X.D., R.D.S.), Cell Biology and Physiology (S.M.A., S.C.W.), and Molecular Genetics and Biochemistry (P.A.M.), University of Pittsburgh School of Medicine, University of Pittsburgh Cancer Institute (M.F.-H., X.D., S.M.A., S.C.W., R.D.S., P.A.M.), Pittsburgh, Pennsylvania.

Address correspondence and reprint requests to Penelope A. Morel, MD, University of Pittsburgh Cancer Institute, Biomedical Science Tower W1057, 200 Lothrop St., Pittsburgh, PA 15213. E-mail: morel+@pitt.edu.

Received for publication 23 February 1999 and accepted in revised form 26 August 1999.

APC, antigen-presenting cell; DC, dendritic cell; DCmed, dendritic cells pulsed in medium; DCpep, dendritic cells pulsed in a mixture of three peptides; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; FITC, fluorescein isothiocyanate; GM-CSF, granulocyte/macrophage colony-stimulating factor; hsp60, heat shock protein 60; IFN-γ, γ-interferon; IL, interleukin; mAb, monoclonal antibody; mBSA, mannosylated bovine serum albumin; MHC, major histocompatibility complex; MLR, mixed lymphocyte reaction; PBS, phosphate-buffered saline; PE, phycoerythrin.

no effect. These results suggest that pancreatic lymph node DCs induced the development of regulatory T-cells (34).

In this study, we examined the ability of two distinct DC populations to modulate diabetes development in NOD mice. The two DC populations differed in the expression of selected cell surface markers and in their ability to take up antigen and to stimulate naive T-cell in vitro. Both DC populations prevented disease development, but the more mature DC population was more effective than the immature DC population. Furthermore, mice treated with more mature DCs produced GAD65 peptide–specific IgG1 antibodies. These results suggest that stimulatory DCs can activate regulatory Th2 cells and thus prevent spontaneous diabetes.

RESEARCH DESIGN AND METHODS

Mice. This study involved female NOD/Lt mice 5–10 weeks of age and SWR ($H2^q$) mice 8–12 weeks of age (Jackson Laboratory, Bar Harbor, ME).

Peptides. Three diabetogenic (21,35,36) synthetic peptides representing residues 437–460 of the human hsp60 and 509–528 and 524–543 of murine GAD65 were prepared and purified by the Peptide Facility of the University of Pittsburgh Cancer Institute as previously described (37).

Generation and purification of DCs. Bone marrow cells were prepared as described previously (18) by depletion of $I\text{-}A^+$ cells, B-cells, and T-cells by using the monoclonal antibodies (mAbs) 10.3.6 (anti-$I\text{-}A^k$), RA3.13A (anti-B220), and J1J.10 (anti-Thy 1.2) (ATCC, Rockville, MD) and complement treatment. The remaining cells were cultured in RPMI-1640 (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS), 2 mmol/l L-glutamine, 50 IU/ml penicillin, 50 μg/ml streptomycin, 5×10^{-5} mol/l 2-mercaptoethanol, 1 mmol/l sodium pyruvate, 0.1 mmol/l nonessential amino acids, and 1 ng/ml recombinant mouse GM-CSF (R&D Systems, Minneapolis, MN) with or without 1 ng/ml recombinant mouse IL-4 (PeproTech, Rocky Hill, NJ). DCs were purified after 4 days of culture on metrizamide as previously described (18).

Flow cytometry of purified DC. Purified DCs (2×10^5 in 50 μl RPMI) were incubated with fluorescein isothiocyanate (FITC)-conjugated mAbs specific for CD80, CD86, CD54, CD11a (LFA1.α), and class I ($H\text{-}2K^d$) (PharMingen, San Diego, CA); phycoerythrin (PE)-conjugated mAb specific for CD40 (Caltag Laboratories, San Francisco, CA); and appropriate isotype controls for 30 min on ice. The indirect staining of DCs was performed by using mAbs specific for $I\text{-}A^k$ (10.3.6), CD11c (N418), and DEC-205 (NLDC145) molecules and appropriate antibodies as isotype controls. The cells were stained with 10 μg/ml of FITC-conjugated goat $F(ab')_2$ anti-mouse IgG, anti-hamster IgG, or anti-rat IgG antibody (Cappel, West Chester, PA) as second antibodies, respectively. After staining, the cells were washed twice in phosphate-buffered saline (PBS) containing 2% FBS and 0.02% NaN_3 and fixed in 2% paraformaldehyde.

Antigen uptake experiment. Mannosylated bovine serum albumin (mBSA) (EY Laboratories, San Mateo, CA) was conjugated with Cy3 bisfunctional reactive dye (Amersham Life Science, Pittsburgh, PA) according to the manufacturer's protocols. Purified DCs were washed three times in serum-free RPMI medium. As a negative control, 2×10^5 cells/100 μl of medium were incubated with Cy3-mBSA (20 μg/ml) or in medium for 1 h at 37°C. After incubation, the uptake was stopped by washing the cells three times in cold PBS. Cells were fixed with 2% paraformaldehyde and were analyzed by flow cytometry. In some experiments, DCs were incubated with Cy3-mBSA and were washed once and then stained with mAb specific for DEC-205 molecule or rat mAb as an isotype control. DCs were analyzed for two-color staining of Cy3 and FITC.

Mixed lymphocyte reaction and cytokine enzyme-linked immunosorbent assay. Naive T-cells from SWR mice were enriched by passing spleen cells through a nylon wool column. We placed 2×10^5 T-cells/100 μl of RPMI in 96-well flat-bottom plates with various numbers of irradiated (20 Gy) DCs per 100 μl of medium at 37°C for 4 days. The cells were pulsed with 0.5 μCi/well [^{3}H]thymidine for an additional 18 h, were harvested, and were counted on a β-scintillation counter. T-cells were stimulated by DCs as described above, and culture supernatants were collected from duplicate mixed lymphocyte reaction (MLR) assays and were tested for production of γ-interferon (IFN-γ) and IL-10 by enzyme-linked immunosorbent assay (ELISA) according to PharMingen's protocols. Primary (clone R4-6A2 for IFN-γ, clone JES5-2A5 for IL-10) and biotinylated (clone XMG1.2 for IFN-γ, clone SXC-1 for IL-10) rat anti-mouse IFN-γ and IL-10 antibodies (PharMingen) were used to coat plates and as a second mAb, respectively. Recombinant mouse IFN-γ and IL-10 (Genzyme, Cambridge, MA) were used for standard curves.

Preparation and injection of labeled DCs. DCs were labeled with Cy5-$DilC_{18}(5)$ (Molecular Probes, Eugene, OR) as described previously (38). Briefly, purified DCs were washed in PBS three times, and 10^6 DC/ml were incubated with Cy5-$DilC_{18}(5)$ at 2 μg/ml for 30 min at 37°C. The cells were washed in PBS, and 10^6 DC/mouse were injected intravenously into 7-week-old NOD mice. Mice were killed after 48 h and were perfused through the left ventricle with 2% paraformaldehyde. The spleens and pancreases were removed and fixed in 2% paraformaldehyde on ice for 1 h and were transferred to 30% sucrose overnight. Tissues were then frozen in liquid nitrogen-coated isopentane for 20 s and were then transferred into liquid nitrogen. We cut 6-μm cryosections and stained them immunocytochemically with FITC-conjugated mAb specific for CD80 or PE-conjugated mAb specific for CD40. The sections were mounted under coverslips with Gelvetol (Monsanto, St. Louis, MO) and were imaged with a Leica TCS-NT confocal microscope (Leica Microsystems, Deerfield, IL) by using filters to detect PE, FITC, and Cy5 at 1024×1024 resolution with fixed laser and photomultiplier tube conditions. To generate images of total cell populations and colocalizations of protein and dye throughout the depth of the cells, images were reduced to single-plane projections of the entire image stack by using ImageSpace (Molecular Dynamics, Sunnyvale, CA).

Quantitation of CD80 or CD40 expression on Cy5-labeled DCs in the spleen. To derive quantitative values for the labeling of CD80 or CD40 on the surface of DCs in spleen sections in situ, multicolor confocal immunocytochemical techniques were used. The nature of confocal microscopy ensures that, if fixed laser illumination, pinhole diameter, and photomultiplier settings are used, then comparing the relative staining intensities between sections that have been stained equivalently is possible. To this end, sections were stained with antibodies to detect CD80 or CD40 and were scanned by using confocal microscopy as described above. Images were then ported to Optimas (Bioscan, Seattle, WA), a two-dimensional image analysis package. By using the Cy5 signal (which delineates all injected DCs) as a mask, the CD40 or CD80 images were extracted. The resultant image was ported to ImageSpace, and intensity profiles for CD40 or CD80 staining on $Cy5^+$ cells were extracted. All print images were generated using Photoshop 5.0 (Adobe, Salinas, CA); for the standard two-color images, single monochrome scans were assigned to color channels with no further manipulation or enhancement.

Treatment of mice with DCs. DCs were pulsed for 5 h either in medium (DCmed) or with a mixture of three peptides (DCpep): hsp60 437–460, GAD65 509–528, and GAD65 524–543, each at 60 μg/ml. DCs were washed three times in cold PBS before injection. Three weekly intravenous injections of $4–8 \times 10^5$ DC/mouse in 100 μl PBS were administered to 5-week-old prediabetic NOD mice. The control mice received three injections of PBS. Mice were followed with weekly blood glucose measurements from the tail vein, and a mouse was considered diabetic when the blood glucose level was >300 mg/dl for 2 consecutive weeks.

Antibody detection. Serum samples were collected at 30 weeks of age from the mice treated with DCs and were tested for antibodies to the hsp60 peptide and the two GAD65 peptides by ELISA. Serum samples from diabetic (26–30 weeks of age) and prediabetic mice (5–7 weeks of age) were included as controls. ELISA plates were coated with 50 μl of 10 μg/ml of each peptide overnight. Peptides were fixed by methanol for 5 min at room temperature, the wells were blocked with 1% BSA/PBS, and 50 μl of a 1:100 dilution of serum samples were added to the wells and incubated overnight at 4°C. Plates were washed, and antibodies to the peptides in serum samples were detected by horseradish peroxidase–goat anti-mouse IgG1, IgG2a, and IgG2b isotypes (Caltag, San Francisco, CA). The wells were developed with 2,2′-azino-di[3-ethylbenzthiazoline sulfonate (6)] (ABTS).

Statistical analysis. The statistical significance was determined by Student's *t* test or Kaplan-Meier log-rank test. A P value <0.05 was considered statistically significant.

RESULTS

Phenotype of purified DCs. DCs generated from bone marrow cultured in either GM or GM + IL-4 were 87–92% pure based on the staining for the DC markers (DEC-205 and CD11c), other cell lineage markers (18) (Fig. 1), and by morphology (data not shown). The levels of CD80 and CD40 expression were greatly reduced in the cells cultured in GM alone, and CD86 expression was not detectable in these cells. In addition, the level of MHC class II expression was significantly lower in the GM DCs than in the GM + IL-4 DCs. Furthermore, both populations expressed similar levels of MHC class I, CD54, and CD11a.

Differential ability of DCs to take up mBSA. In these experiments, purified DC subsets were examined with flow cytometry for their ability to take up Cy3-mBSA. GM DCs

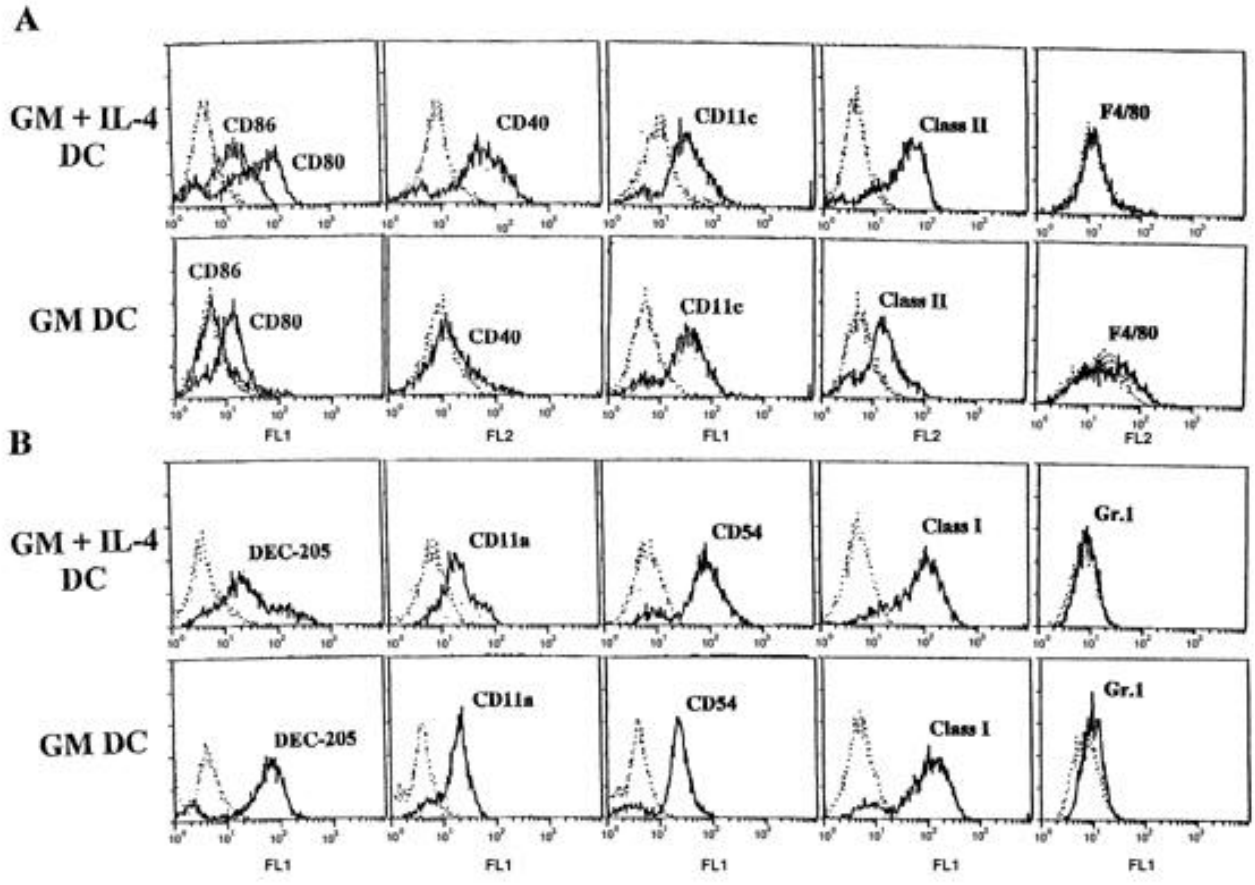

FIG. 1. ***A*** **and** ***B*****: Comparison of the phenotype of bone marrow–derived GM + IL-4 DCs and GM DCs. DCs were generated from bone marrow cells cultured for 4 days with GM + IL-4 or GM alone and were purified on metrizamide gradients. DCs (2×10^5) were stained with indicated mAbs (—) and appropriate isotype controls (···). The histograms are representative of two to five different experiments.**

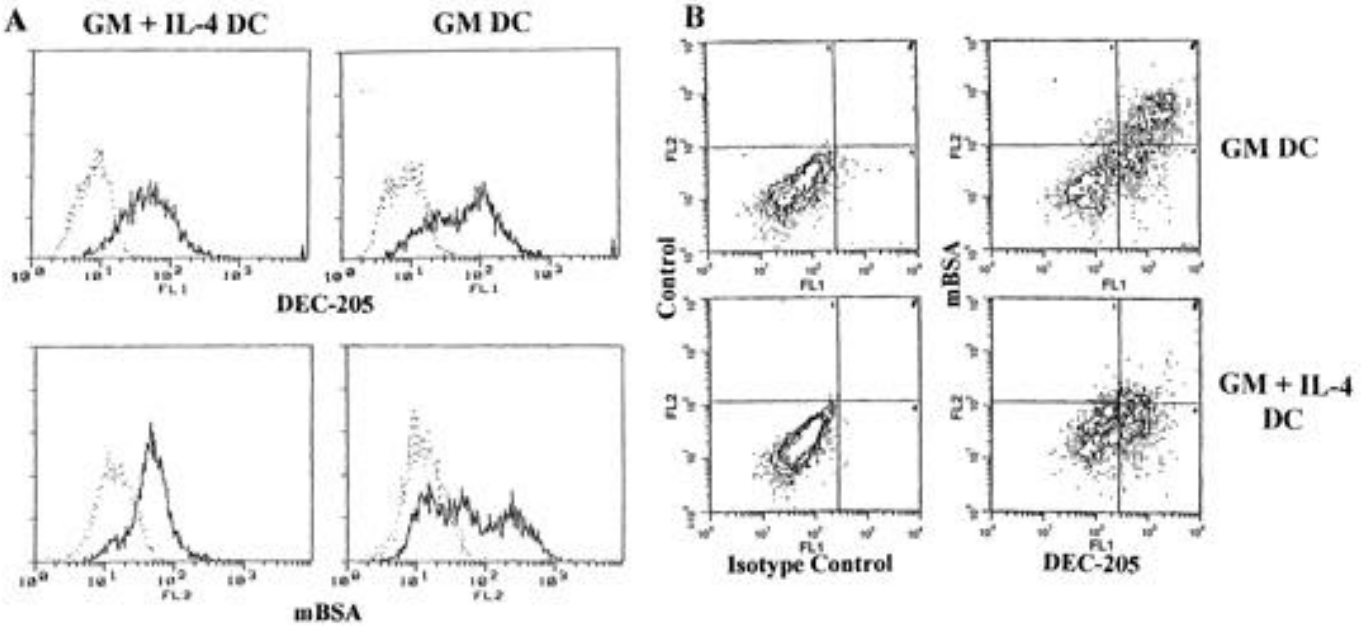

FIG. 2. Flow cytometric analysis of GM + IL-4 DCs and GM DCs for mBSA uptake and DEC-205 expression. ***A*****: DCs (2×10^5) were stained with mAb specific for DEC-205 molecule (—) or rat mAb as an isotype control (···) (*upper panels*), and DCs (2×10^5) were incubated with 20 µg/ml Cy3-mBSA or in medium for 1 h at 37°C (*lower panels*). The uptake was stopped by washing the cells in cold PBS. The cells were fixed and analyzed with flow cytometry.** ***B*****: DCs (2×10^5) were incubated with 20 µg/ml Cy3-mBSA (mBSA) or in medium (control) for 1 h at 37°C. The cells were washed and incubated with mAb specific for DEC-205 molecule or rat mAb as an isotype control. After washing, the cells were stained with FITC-conjugated goat anti-rat IgG and were washed and fixed as described (see RESEARCH DESIGN AND METHODS). Results are representative of five different experiments.**

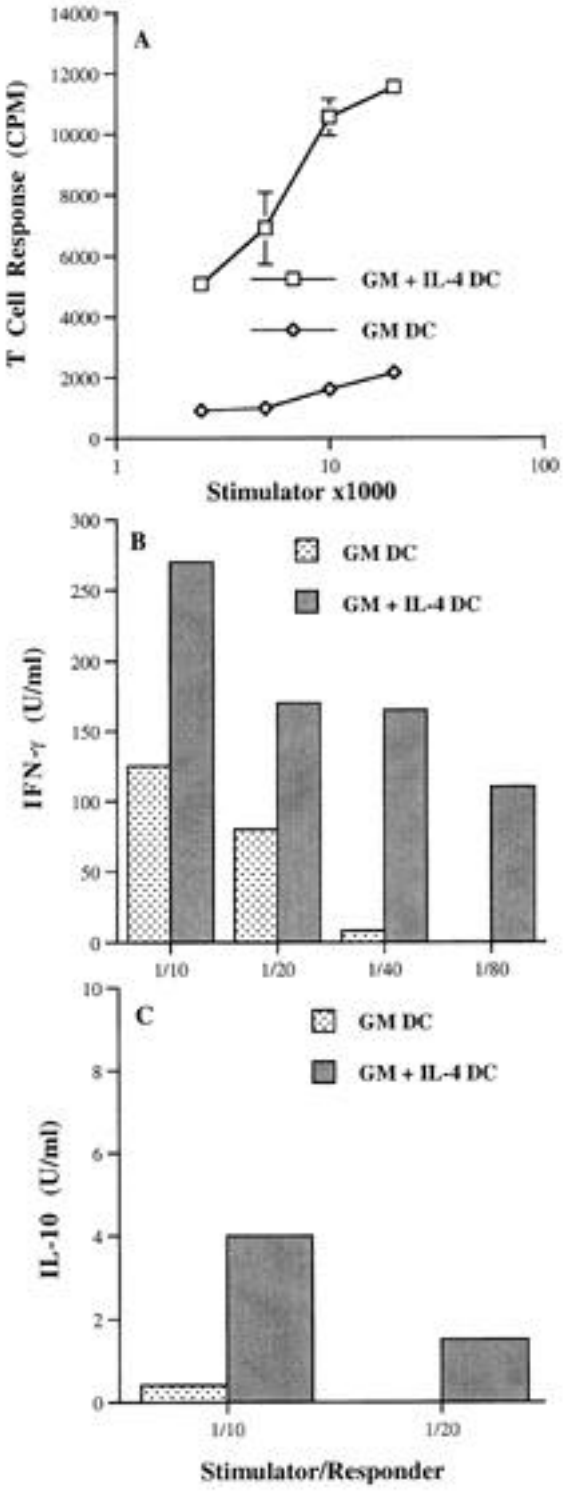

FIG. 3. Comparison of the stimulatory function of GM + IL-4 DCs and GM DCs. *A*: Primary MLR. Nylon wool–enriched allogeneic T-cells (2×10^5/well) from SWR mice were stimulated with the indicated DC numbers as stimulators for 4 days. The cells were pulsed overnight, harvested, and counted on a β-scintillation counter. *B* and *C*: Cytokine production. Nylon wool–enriched allogeneic T-cells (2×10^5/well) from SWR mice as responsers were stimulated with indicated DCs. Culture supernatants were collected after 4 days and were tested for the secretion of IFN-γ (*B*) and IL-10 (*C*) by ELISA. Results are representative of five different experiments.

were more efficient in the uptake of mBSA than GM + IL-4 DCs (Fig. 2*A*, *lower panels*). The increase in the capacity of GM DCs to take up mBSA may have been because of the higher level of DEC-205 expression in GM DCs (Figs. 1*B* and 2*A*, *upper panels*). Experiments were performed in which bone marrow–derived DC subsets were incubated with mBSA first, and then the cells were washed and stained for the expression of DEC-205. As shown in Fig. 2*B*, the DCs that could take up mBSA most efficiently were those stimulated with GM-CSF alone that expressed the highest level of DEC-205. Because DEC-205 is homologous to the mannose receptor (39), this molecule may be partly responsible for the observed uptake.

MLR and cytokine production. The ability of the two DC populations to stimulate T-cells in a primary MLR was studied. As shown in Fig. 3*A*, the purified DCs derived from bone marrow cells cultured with GM + IL-4 were potent stimulators of naive allogeneic T-cells, whereas DCs derived from cultures containing GM alone were poor stimulators. The capacity of the bone marrow–derived DC subsets to stimulate allogeneic T-cells was correlated with the levels of costimulatory CD80, CD86, and MHC class II molecules of these two DC subpopulations.

Because the GM + IL-4 DCs stimulated the allogeneic T-cells more efficiently than the GM DCs, GM + IL-4 DCs may have a capacity to activate T-cells to drive a Th1 response, which was demonstrated recently (40). To assess this possibility, the differential ability of DC subsets to stimulate naive allogeneic T-cells was further studied by measuring IFN-γ and IL-10 levels in MLR culture supernatants. As shown in Fig. 3*B*, high levels of IFN-γ were produced by T-cells cultured for 4 days with GM + IL-4 DCs, whereas IFN-γ production was greatly reduced when GM DCs were used as stimulatory cells in those cultures. IL-10 was produced at low to undetectable levels by both populations (Fig. 3*C*), and no increase was evident in the level of IL-10 produced by GM DCs.

In vivo migration of DCs. We were interested to determine the in vivo migratory pattern of the two DC populations after intravenous injection. DCs were labeled with Cy5-DilC_{18}(5) as described (see RESEARCH DESIGNANDMETHODS), and 48 h after intravenous injection of NOD mice (n = 3), the labeled DCs could be detected in the T-cell area of the spleen (Figs. 4 and 5), and a small amount was found in the exocrine tissue of the pancreas (Fig. 6). The tissue sections were stained with FITC-conjugated mAbs specific for CD80 and CD40 molecules and were examined for the colocalization of these molecules on the injected labeled DCs. Figure 4 shows that the number of DCs expressing CD80 was higher with GM + IL-4 DCs (*A* and *B*) than with GM DCs (*C* and *D*) in spleen sections. Molecular colocalization is clearly demonstrated in the inset in Fig. 4*A*, enlarged in Fig. 5, which shows a single optical section through the midplane of the projected image stack designated in Fig. 4*A*. The clear yellow color (Fig. 5*C*) is due to colocalization of the Cy5-DilC_{18}(5) labeling dye in red (Fig. 5*B*) and the FITC-conjugated anti-CD80 in green (Fig. 5*A*). The fishnet plots of the confocal images (Fig. 4*B*, *D*, *F*, and *H*) represent the quantitative assessment of CD80 and CD40 expression on the injected cells only. CD40 was expressed at low levels in both populations (Fig. 4*E–H*), which suggests that the high CD40 expression observed in vitro on GM + IL-4 DCs (Fig. 4*E* and *F*) was downregulated in vivo. Quantitation of the level of CD80 expression on the

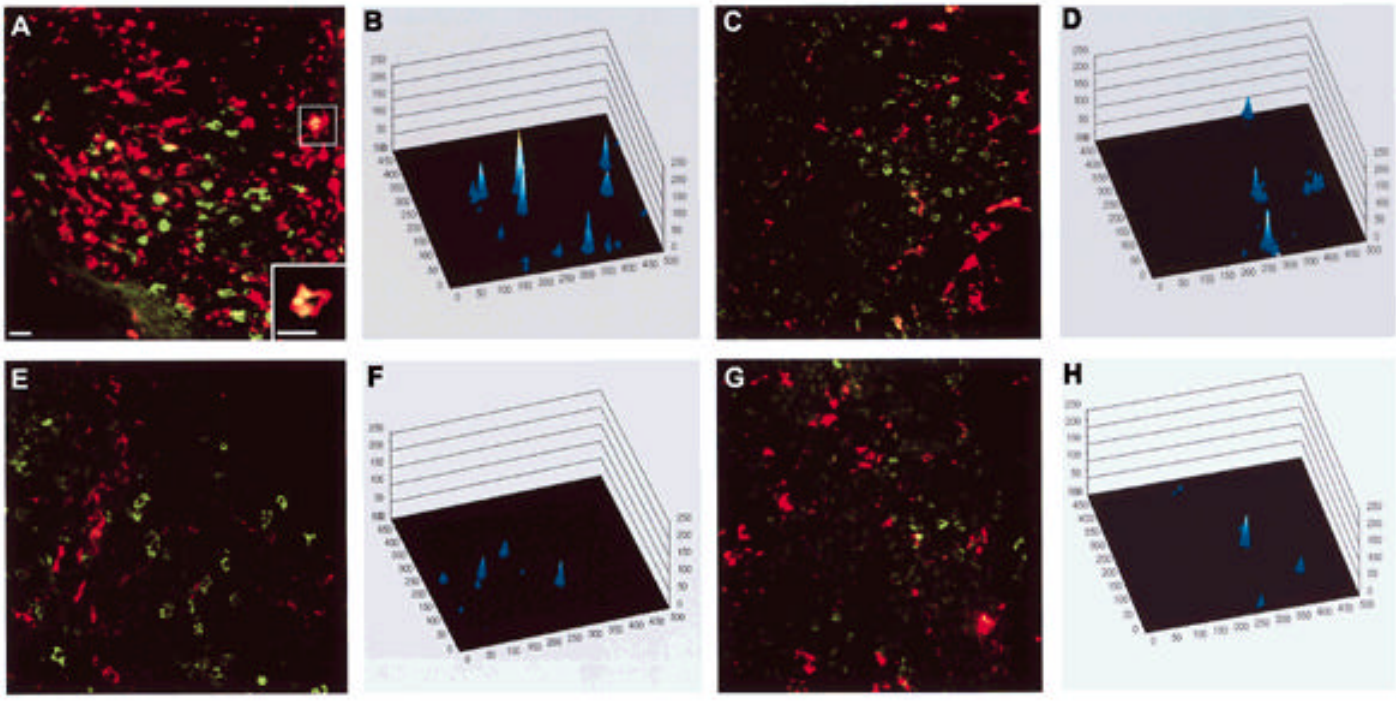

FIG. 4. Both DC populations migrated to the T-cell area of the spleen after intravenous injection. GM + IL-4 DCs or GM DCs were labeled with Cy5-Di1C$_{18}$(5), and 7-week-old mice (n = 3) were given intravenous injections of 10^6 GM DCs or GM + IL4 DCs per mouse. The spleens were harvested after 48 h, and tissue sections were stained with mAbs specific for CD80 (*A–D*) and CD40 (*E–H*) molecules as described (see RESEARCH DESIGN AND METHODS). *A* and *E* are representative spleen sections from the mice injected with GM + IL-4 DCs, and *C* and *G* are representative of spleen sections from the mice injected with GM DCs. The two-color images, which are derived from integration, are shown in *A*, *C*, *E*, and *G*. The Cy5 signal is shown in red, and the CD40 or CD80 stain is shown in green. By using these two color representations, colocalization of the signal will appear as yellow as illustrated more specifically in a single midplane section taken through a single cell (inset in *A*). Signal intensity for either CD80 (*B* and *D*) or CD40 (*F* and *H*) is shown graphically as intensity plots (bar = 50 µm).

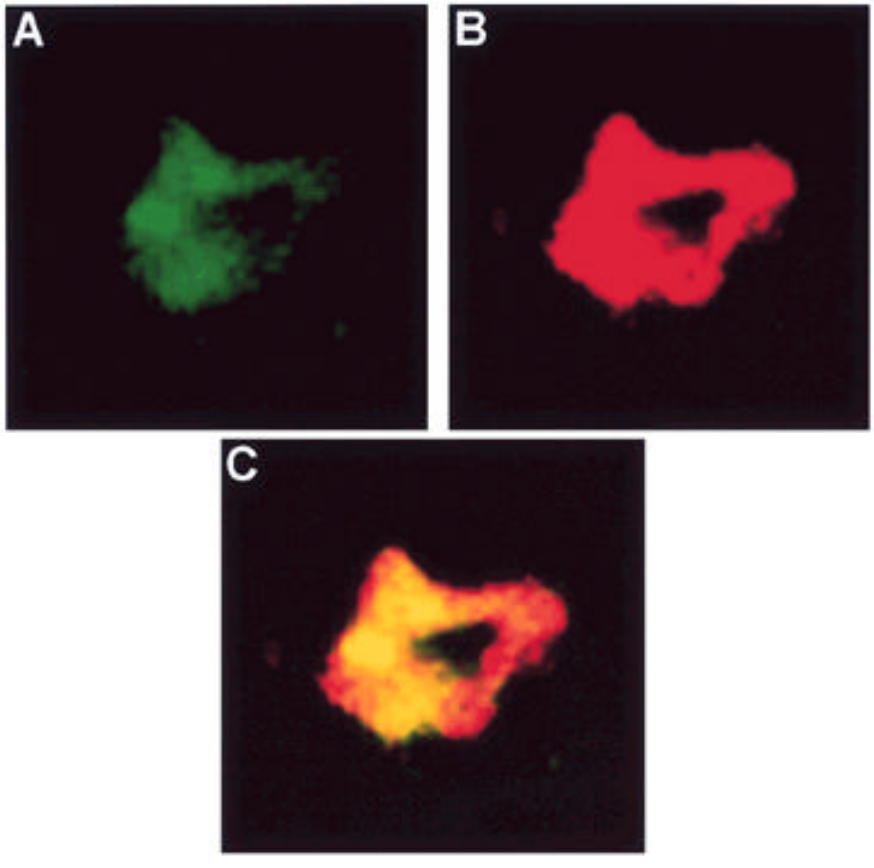

FIG. 5. Colocalization of CD80 on a Cy5-D1C$_{18}$(5)-labeled DC in the spleen. The image is of the cell identified by the inset in Fig. 4*A*. The labeled DC is shown in red (*B*), and the staining with FITC-conjugated anti-CD80 mAb is shown in green (*A*). When the two images are superimposed, the total cellular labeling with Cy5 is colocalized with the CD80, which appears as a yellow color (*C*).

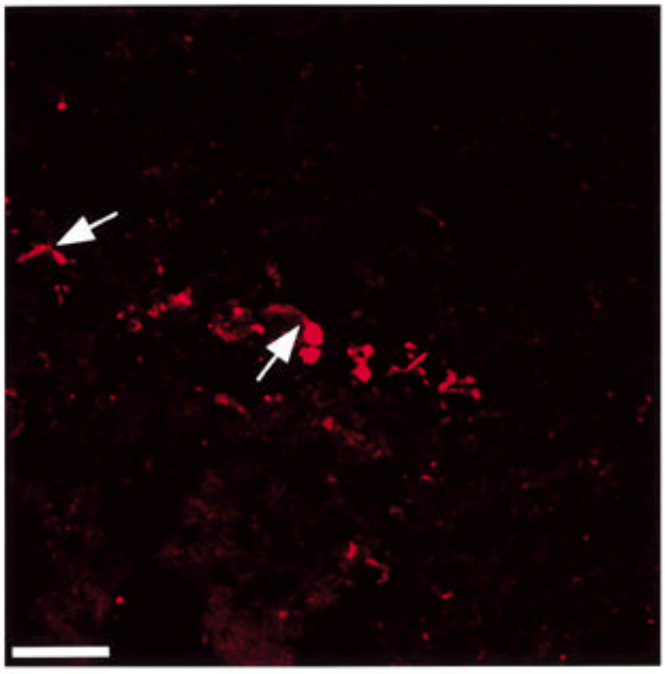

FIG. 6. DCs migrated to the exocrine tissue of the pancreas after intravenous injection. GM DCs or GM + IL4 DCs were labeled with Cy5-$Di1C_{18}$(5), and 7-week-old mice (n = 3) were intravenously injected with 10^6 GM DCs or GM + IL4 DCs per mouse as described in Fig. 4. The pancreases were harvested after 48 h, and a representative section from the mice injected with labeled GM + IL-4 DCs is shown. DCs are shown in red (bar = 50 μm).

injected DCs (Fig. 4*B* and *D*) suggested that both bright and dim $CD80^+$ cells could be detected in the GM + IL-4–derived population. In contrast, only a few bright $CD80^+$ DCs and no dim $CD80^+$ DCs were detected in the GM-derived population. These results suggest that in vitro phenotypic differences have been maintained in vivo, at least in the case of CD80 expression.

DCs inhibit spontaneous development of diabetes. We have previously shown that a single intravenous injection of GM DCs pulsed either in medium or in a mixture of three peptides reduced the incidence of diabetes in NOD mice (18). We performed preliminary experiments to determine the ability of the more stimulatory GM + IL-4 DC population to modulate diabetes in NOD mice and found that a single injection of these DCs could delay the onset of disease (data not shown). Because the results obtained with a single injection failed to reach statistical significance (18) (data not shown), our subsequent experiments involved the use of multiple DC injections.

To further examine the in vivo effect of the GM + IL-4 DC population, we performed experiments in which 5-week-old mice were treated with three weekly intravenous injections of 4–8 × 10^5 DC/mouse. Before injection, GM + IL-4 DCs were pulsed either in medium or in a mixture of three peptides for 5 h. Control mice received three weekly injections of PBS, and mice were followed for the development of diabetes as previously described. The incidence of diabetes was reduced to 40% (4/10) in mice treated with GM + IL-4 DCpep (P = 0.003) and 20% (2/10) in mice treated with GM + IL-4 DCmed (P = 0.002) compared with 90% in mice treated with PBS (9/10); however, no significant difference in the incidence of the disease between the two DC-treated groups was observed (P > 0.05) (Fig. 7*A*). Similar experiments were performed by using multiple injections of GM DCs, and in these experiments, the incidence of diabetes was 57% (8/14) in mice treated with GM DCpep (P = 0.045) and 70% (7/10) in mice treated with GM DCmed (P = 0.4) compared with 100% (9/9) in mice treated with PBS (Fig. 7*B*). These results demonstrate that multiple injections of the more stimulatory GM + IL-4 DC population were more effective in reducing the incidence of diabetes.

GM + IL-4 DC mice produced GAD65 peptide–specific IgG1 antibodies. To determine whether any humoral responses were generated in mice treated with GM + IL-4 DC, ELISA was performed to detect peptide-specific antibodies. In these experiments, the serum samples were tested for the presence of IgG1 and IgG2b antibody isotypes against hsp60 (437–460), GAD65 (509–528), and GAD65 (524–543) peptides. The serum samples were collected from DC-treated NOD mice, and serum samples from diabetic and prediabetic mice were included as controls. Significant differences were found for GAD65 peptide–specific IgG1 antibodies (Fig. 8). Mice treated with DCmed produced higher levels of IgG1 specific for GAD65 (509–528) compared with the diabetic control

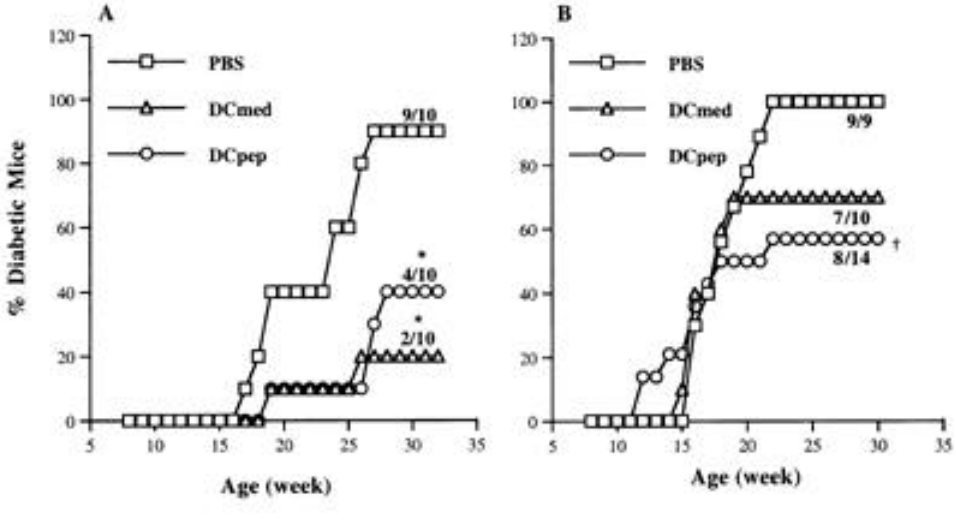

FIG. 7. Comparison of the in vivo effects of GM + IL-4 DC (*A*) and GM DC (*B*) populations. Five-week-old NOD mice were given three weekly intravenous injections of 4–8 × 10^5 DC/mouse. Before injection, DCs were pulsed for 5 h either in medium (DCmed) or in a mixture of three peptides (DCpep). Control mice received three weekly injections of PBS, and the mice were followed as described previously. *P = 0.002, GM + IL-4 DCmed vs. PBS; *P = 0.003, GM + IL-4 DCpep vs. PBS; and †P = 0.045, GM DCpep vs. PBS.

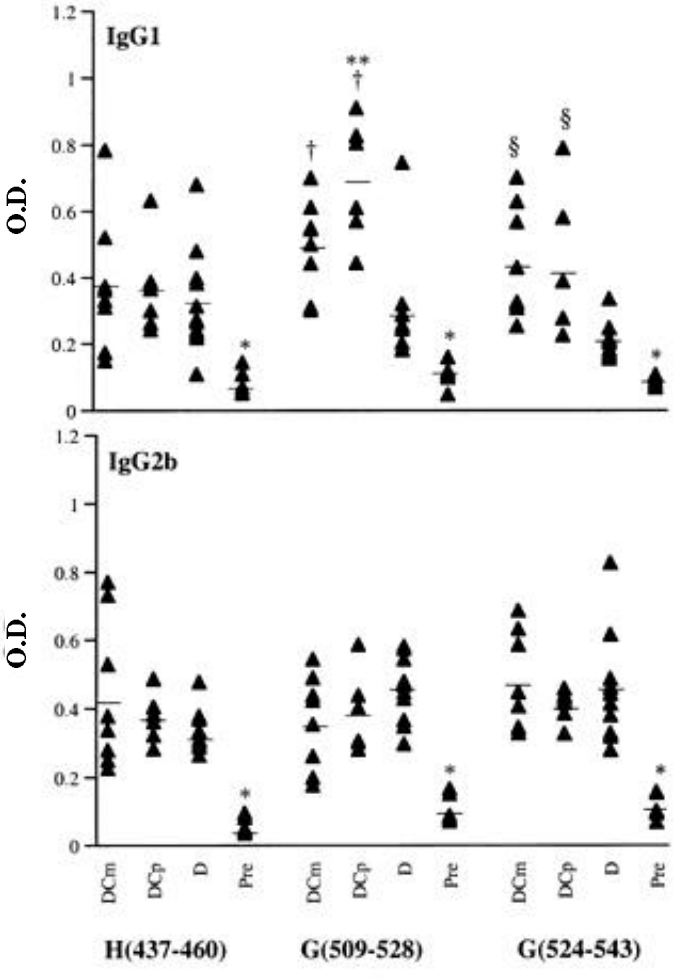

FIG. 8. GM + IL-4 DC-treated mice produced GAD65 peptide–specific IgG1 antibodies. Serum samples were collected from 30-week-old DCmed-treated (DCm) (n = 8) or DCpep-treated (DCp) (n = 6) NOD mice. Sera from 26- to 30-week-old diabetic mice (D) (n = 11) and 5- to 7-week-old prediabetic mice (Pre) (n = 5) were included as controls. The serum samples were tested for the presence of IgG1 and IgG2b antibodies to hsp60, H(437–460); GAD65, G(509–528); and GAD65, G(524–543) peptides by ELISA as described (see RESEARCHDESIGNAND METHODS). P values for the G(509–528)-specific IgG1 are †P = 0.009, DCmed vs. diabetic mice; †P = 0.0002, DCpep vs. diabetic mice; and **P = 0.04, DCpep vs. DCmed mice. P values for the G(524–543)-specific IgG1 are §P = 0.0004, DCmed vs. diabetic mice; and §P = 0.006, DCpep vs. diabetic mice. P values for the IgG1 and IgG2b levels are *P < 0.05 for diabetic, DCmed, and DCpep vs. prediabetic mice. O.D., optical density.

mice (P = 0.009). Interestingly, GAD65 (509–528)-specific IgG1 antibody was induced at higher levels in mice treated with DCpep compared with both the diabetic control mice (P = 0.0002) and mice treated with DCmed (P = 0.04). The GAD65 (524–543)-specific IgG1 antibody levels in mice treated with either DCmed or DCpep were higher than in the diabetic mice (P = 0.0004 and P = 0.006, respectively); however, no significant difference in the level of GAD65 (524–534)-specific IgG1 antibodies was found between DCmed- and DCpep-treated mice (P = 0.86). We observed no increase in the production of IgG2b in the sera collected from the DCmed- and DCpep-treated mice compared with IgG2b levels in the sera of diabetic control mice (P > 0.05). However, IgG2b antibodies in the serum samples from the prediabetic mice were detected at significantly lower levels compared with the sera from DC-treated groups and the diabetic group (P < 0.05), which is consistent with previous studies (41). We also tested the serum samples for production of the peptide-specific IgG2a isotype, but we were unable to detect this antibody. This is not surprising because a study recently showed that commercial anti-IgG2a antibodies do not detect the IgG2a isotype from NOD because NOD mice have the IgG2c isotype (42). Thus, the increase in GAD65 peptide–specific IgG1 antibodies in mice treated with the DCpep and DCmed suggests that Th2 responses were generated in the protected NOD mice.

DISCUSSION

Fully mature antigen-pulsed DCs have been used in various systems to boost or initiate immune responses, and these cells are currently being used in tumor vaccine protocols (43). Although several DC populations have been described that may induce anergy or kill interacting T-cells, few studies have reported the administration of these cells in autoimmune disease. Recent studies have described the use of $CD86^-$ immature liver-derived DCs to prolong cardiac (44) or islet cell (45) allograft survival, and thymic DCs pulsed with myelin basic protein–derived peptides were shown to prevent the induction of disease in experimental allergic encephalomyelitis (46). In addition, Clare-Salzler et al. (34) described the use of DCs derived from pancreatic lymph nodes to delay diabetes induction in NOD mice. The detailed phenotype of injected cells was not determined, presumably because of the small number of cells recovered; however, the cellular preparations used were not pure and were possibly contaminated with other cells such as T-cells. This protective function of pancreatic lymph node DCs may be due to the presentation of islet cell antigens by these cells. This possibility was supported by studies demonstrating clear DC/T-cell clusters in pancreatic lymph nodes (47), and APCs from these nodes can present antigen to islet-specific T-cell hybridomas (48). In contrast with these studies, we have used highly purified DC subsets from the bone marrow of NOD mice with distinct phenotypic and functional differences to study their function in vivo.

The data presented herein describe differences in the phenotype, the T-cell stimulatory capacity, and the antigen uptake of two DC populations derived from the bone marrow of NOD mice. GM + IL-4 DCs expressed high levels of MHC class II as well as costimulation/activation molecules. These cells were efficient stimulators of naive allogeneic T-cells and induced a strong proliferative response and high levels of IFN-γ production. In contrast, GM DCs expressed low levels of both MHC class II and costimulation/activation molecules and were poorly stimulatory in a primary MLR, both at level of proliferation and in IFN-γ production. Furthermore, the GM DCs were highly efficient in the uptake of mBSA compared with the GM + IL-4 DCs. Our data show that the differential capacity of these two DC populations to take up mBSA correlated with the level of DEC-205 expression because those DCs that were most efficient in mBSA uptake had the highest levels of DEC-205 expression. Studies have shown that the mechanisms of antigen capture in DCs may be via both macropinocytosis and receptor-mediated uptake (49,50). One study showed that human DCs express the mannose receptor, which is involved in antigen uptake (49). In the murine system, one study recently reported that the DEC-205 receptor may have a similar function (39). We are currently

investigating the role of DEC-205 in the uptake of antigens. Thus, based on these in vitro data, the two DC populations used in this study can be classified as immature (GM DCs) and more mature (GM + IL-4 DCs) in agreement with the general concept of DC maturation (17,49).

Previously, we have shown that treatment of prediabetic NOD mice with a single injection of immature DCs reduced the incidence of diabetes (18). In the present study, we examined the ability of a more mature DC population to modulate the development of diabetes. Despite its stimulatory phenotype and function, a single injection of the GM + IL-4 DC population did not accelerate the development of diabetes but in contrast was capable of inducing a delay in disease onset (data not shown). Furthermore, a significant reduction in the incidence of diabetes in mice treated with multiple injections of stimulatory DCs was observed when compared with mice injected with immature DCs. In the case of the GM + IL-4 DCs, the peptide pulsing did not significantly improve the ability of these cells to prevent disease. This suggests that DCs may process and present autoantigens to T-cells in vivo, which leads to the stimulation of a regulatory Th2 response. Another possibility is that the observed effect may be because of a non–islet antigen-specific therapy, such as that seen for Bacille Calmette-Guérin and complete Freund's adjuvant (51,52) because DCs were generated in medium containing FBS. However, this is unlikely in the light of the islet antigen-specific IgG1 responses that are observed in the DC-treated mice. In addition, only peptide-pulsed GM DCs were significantly able to prevent the development of diabetes, whereas medium-pulsed GM DCs did not, despite the presence of FBS in the GM DC cell preparations. Therefore, DC therapy may lead to the development of islet antigen-specific regulatory Th2 cells, which are likely to be critical in controlling disease progression in NOD mice.

Both DC populations migrated to the T-cell area of the spleen and exocrine tissue of the pancreas in the injected mice. The expression of costimulatory CD80 was stable in the injected DC populations in vivo, whereas the CD40 expression seemed to be downregulated in the GM + IL-4 DC population. The maintenance of a higher level of CD80 expression in the GM + IL-4 DC population may correlate with the increased ability of this population to induce a protective response. The initiation of regulatory Th2 responses in the NOD mouse has been shown to be dependent on costimulatory molecules (53), which may be relevant to the in vivo function of these cells. The loss of differential CD40 expression in vivo may also contribute to the development of a Th2 response in the treated mice because CD40 expression is known to be partly responsible for IL-12 production and Th1 development (40).

To determine whether a Th2 response was initiated in the DC-treated mice, we examined the humoral peptide-specific response in these mice. Interestingly, GAD65 peptide–specific IgG1 antibodies were produced by DC-treated mice, and this antibody isotype was produced more significantly in DCpep-treated animals, which suggests the generation of a specific Th2 response. GAD65 has been implicated in the induction of diabetes in NOD mice (21,54), and treatment of NOD mice with GAD65 peptides was shown to protect them by inducing a Th2 response (27). In peptide immunotherapy, the injected peptides likely encounter DCs and stimulate GAD65-specific Th2 cells in vivo. In the experiments presented herein, we have shown that mice administered peptide-pulsed DCs induced a specific Th2 response and were protected from disease, which supports the role of DCs in the initiation of the regulatory Th2 response.

Studies have shown that T-cell autoreactivity in NOD mice is the result of an inability of APCs to activate immunoregulatory T-cells (29,30,55–57), which leads to an imbalance between the effector and regulatory T-cell populations in this model. The existence of regulatory Th2 cell populations has been demonstrated in NOD mice, and their development appears to require expression of costimulatory molecules early in life (53,58). Based on our in vitro data, the GM + IL-4 DC population is a stimulatory APC; therefore, the in vivo results suggest that DC therapy may have stimulated the generation of regulatory Th2 cells and prevented the disease by changing the balance between effector Th1 and regulatory Th2 cell populations in these mice.

In summary, these data suggest that the in vitro–generated DCs from NOD mice do not have the capacity to drive a Th1 response in vivo but in contrast are capable of preventing disease onset. Treatment of NOD mice with DC alone has the potential to be immunotherapy for diabetes in these mice. A more stimulatory DC population is needed for an effective stimulation of the regulatory Th2 population and prevention of the disease; thus, these studies may have important implications for the treatment of human diabetes.

ACKNOWLEDGMENTS

This work was supported by National Institutes of Health Grants AI-25151 (P.M.), CA-73743 (P.M.), and AR-44811 (R.S.).

We thank Dr. Angus Thomson and Dr. Susan McCarthy for critically reading the manuscript and Dewayne Falkner for technical assistance.

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