Autoimmune Diabetes Is Suppressed by Transfer of Proinsulin-Encoding Gr-1$^+$ Myeloid Progenitor Cells That Differentiate In Vivo Into Resting Dendritic Cells

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The nature of the T-cell response to antigen is governed by the activation state of the antigen-presenting dendritic cell (DC). Immature or resting DCs have been shown to induce T-cell responses that may protect against the development of autoimmune disease. Effectively harnessing this "tolerogenic" effect of resting DCs requires that it be disease-specific and that activation of DCs by manipulation ex vivo is avoided. We reasoned that this could be achieved by transferring in vivo partially differentiated myeloid progenitor cells encoding a disease-specific autoantigen. With the aim of preventing autoimmune diabetes, we transferred myeloid progenitor cells encoding proinsulin into NOD mice. Bone marrow (BM) was cultured in granulocyte macrophage colony–stimulating factor (GM-CSF) and transforming growth factor-β1, a cytokine combination that expands myeloid cells but inhibits terminal DC differentiation, to yield Gr-1$^+$/CD11b$^+$/CD11c$^-$ myeloid progenitor cells and a minor population of CD11c$^+$/CD11b$^+$/CD86^{lo} immature DCs. After transfer, Gr-1$^+$ myeloid cells acquired the characteristics of resting DCs (CD11c$^+$/MHC classII^{int}/CD86^{lo}/CD40^{lo}). Gr-1$^+$ myeloid cells generated from transgenic NOD mice that expressed proinsulin controlled by a major histocompatibility complex (MHC) class II promoter, but not from wild-type NOD mice, transferred into 4-week-old female NOD mice significantly suppressed diabetes development. The transfer of DC progenitors encoding a disease-specific autoantigen is, therefore, an effective immunotherapeutic strategy that could be applied to humans. *Diabetes* 54:434–442, 2005

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BM, bone marrow; CFSE, 5(6)-carboxyfluorescein diacetate succinimidyl ester; DC, dendritic cell; FITC, fluorescein isothiocyanate; GM-CSF, granulocyte macrophage colony–stimulating factor; IFN, interferon; IL, interleukin; M-CSF R, macrophage colony–stimulating factor receptor; MHC, major histocompatibility complex; rm, recombinant murine; SA, streptavidin; TGF, transforming growth factor; TNF, tumor necrosis factor.

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Antigen-presenting dendritic cells (DCs) determine the functional properties of the T-cells with which they interact. In the absence of activating signals to DCs, such as those mediated through toll-like receptors (1) or CD40 (2), the outcome of DC/T-cell interaction may be a state of T-cell "tolerance." The stage of differentiation, type, and/or location of DCs can determine the form of tolerance that ensues. For example, DCs in lymphoid tissues exist primarily in a "resting" state (3) and present antigen in a manner that appears to result in tolerance due to deletion of antigen-specific T-cells (2,4,5) or induction of unresponsiveness (6), while DCs in mucosal sites induce cytokine skewing and regulatory T-cells (7). Although DCs are promising immunotherapeutic tools, ideally they would target disease-specific T-cells. In the case of autoimmune diseases, this specificity could be achieved by endowing DCs with the ability to express autoantigen. A potential strategy would be to generate cells ex vivo with the phenotypic and functional properties of resting lymphoid tissue DCs, load them with appropriate antigen, and transfer them to effect T-cell tolerance in vivo. This approach is qualified, however, by the plasticity of DCs and the uncertainty of whether they would localize to microanatomic sites for tolerance induction in lymphoid tissues. Activation of DCs can occur during their preparation and manipulation ex vivo (8) or after transfer (9), which may abrogate their tolerogenic potential. This could be avoided by transfering DC progenitor cells that would then differentiate into inherently tolerogenic DCs in vivo. To this end, we generated partially differentiated myeloid cells that retained DC progenitor properties in vitro and in vivo by culture of bone marrow (BM) in granulocyte macrophage colony–stimulating factor (GM-CSF) and transforming growth factor (TGF)-β1, a cytokine combination that expands myeloid cells but inhibits terminal DC differentiation. We used the NOD mouse model of spontaneous autoimmune type 1 diabetes, in which proinsulin is a key autoantigen (10,11), to test the ability of DC progenitors encoding proinsulin to suppress the development of autoimmune disease in an antigen-specific manner.

RESEARCH DESIGN AND METHODS

Female NODLt/Jax mice were obtained from the Walter and Eliza Hall Institute central breeding facilities. Proinsulin-NOD (PI-NOD) transgenic mice expressing mouse proinsulin II under control of the I-E$_\alpha$^{k} major histocompatibility complex (MHC) class II promoter, described previously (10), were used

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after breeding to homozygosity. Animals were used in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

Reagents, cytokines, and antibodies. Culture medium was RPMI-1640 supplemented with 1 mmol/l sodium pyruvate, 0.1 mmol/l nonessential amino acids (both from Gibco, Rockville, MA), 50 μmol/l 2-mercaptoethanol (Sigma, St. Louis, MO), and 10% vol/vol FCS (R10) (JRH, Lenexa, KS). Recombinant murine (rm) GM-CSF, interleukin (IL)-4, and tumor necrosis factor (TNF)-α were purchased from Peprotech (Rocky Hill, NJ). Recombinant human TGF-β1, rmM-CSF, and rmG-CSF were from R&D Systems (Minneapolis, MN). Interferon (IFN)-γ was kindly provided by Genentech (South San Francisco, CA). Fluorescein isothiocyanate (FITC)-dextran was purchased from Sigma. Antibodies directed against Gr-1 (Ly-6G and RB6-8C5), F4/80 (F4/80), CD11b (M1/70), CD11c (N418), MHC class II (10.2.16 [I-$A^{k,g7,r,f,s}$]), MHC class I (M1/42), macrophage colony–stimulating factor receptor (M-CSF R) (AFS-98), CD40 (FGK-45), B220 (RA3-6B2), CD205 (NLDC-145), CD86 (GL-1), and c-kit (ACK-2) were purified from hybridoma supernatants and used as purified mAb or conjugated in house. Streptavidin (SA)-fluorochrome conjugates (SA-FITC, SA-phycoerythrin, SA-allophycocyanin, and SA–Texas red) were from Caltag (Burlingame, CA). mAb directed to CD40 (3/23), MAC-3 (M3/84), CD13 (R3-242), CD62-l (MEL-14), CD31 (MEC13.3), CD43 (S7), CD11a (2D7), and CD49d (R1-2) were purchased from PharMingen (San Diego, CA). Anti-F4/80 was from Caltag (F4/80). Anti-mouse FIRE (6F12) (12) was provided by Dr. Irene Caminschi, Walter and Eliza Hall Institute. 5(6)-Carboxyfluorescein diacetate succinimidyl ester (CFSE) was purchased from Molecular Probes (Eugene, OR).

BM cultures and cell transfers. Mice were euthanized by CO_2 asphyxiation and femurs and tibiae removed aseptically. BM was flushed with mouse tonicity PBS/2.5% FCS. Cells were collected by centrifugation and erythrocytes removed by distilled water lysis. After washing in RPMI/10% FCS, cells were plated in 6-well plates (NUNC, Roskilde, Denmark) at 2×10^6/ml in 3 ml R-10 per well. Cultures were initially supplemented with GM-CSF (1 ng/ml) and 2 days later nonadherent cells removed. Remaining adherent cells were then cultured in GM-CSF (1 ng/ml) and TGF-β1 (2 ng/ml) for a further 3 days. After a total of 5 days of culture, nonadherent cells were harvested and washed twice in R-10. To generate GM-CSF/IL-4 BMDCs, BM cultures were prepared as described but supplemented with GM-CSF and IL-4 (1 ng/ml of each) for the entire culture period (13). In some experiments, subsets of cells were depleted with either anti–Gr-1 (RB6–8C5) or biotinylated anti-CD11c (N418) and sheep anti-rat Dynabeads or CELLection biotin-binding Dynabeads (Dynal Biotech; Carlton South, Victoria, Australia), respectively, according to the manufacturer's instructions. Alternatively, $CD11c^+$ cells were depleted with CD11c-phycoerythrin and anti-phycoerythrin magnetic beads (MACS; Miltenyi Biotec, Gladbach, Germany) on a magnetic cell sorter (AutoMACS; Miltenyi Biotec). Unless stated otherwise, to prevent engraftment of hematopoietic stem or progenitor cells that may have remained in the cultures, bulk or depleted cell populations were irradiated (2,000 rads, ^{60}Co source), washed twice in R-10, and resuspended in PBS before injection intravenously into 4-week-old female NOD mice. Irradiation of GM-CSF/TGF-β1–generated cells before transfer did not alter their effect on diabetes development. For bulk GM-CSF/TGF-β1–cultured BM, 2×10^6 cells were transferred intravenously. When cell subsets were depleted, the number of cells transferred was equivalent to that in unseparated bulk GM-CSF/TGF-β1–cultured BM, i.e., 1.8 $\times 10^6$ $Gr\text{-}1^+$ (CD11c-depleted) cells or 0.2×10^6 $CD11c^+$ (Gr-1–depleted) cells.

FITC-dextran uptake. Quantitation of endocytosis by FITC-dextran uptake was performed as described (13). Briefly, cells were incubated with FITC dextran (1 mg/ml) for 2 h at 37°C or 4°C. Endocytosis was stopped by washing twice with ice-cold PBS containing 2.5% FCS and 0.02% sodium azide, and samples were maintained at 4°C for immunofluorescence staining and flow cytometry.

Flow cytometric analysis. Immunofluorescence staining of cells was performed as described previously (13) for flow cytometry on a FACScan (Becton Dickinson, San José, CA). Viable cells were gated on the basis of propidium iodide exclusion. For routine analyses, $1\text{–}2 \times 10^4$ live-gated events were collected. For analysis of in vivo DC phenotype, $1\text{–}2 \times 10^6$ live-gated events were collected.

In vitro differentiation assays. BM cultured in GM-CSF/TGF-β1 was harvested and washed twice, and $Gr\text{-}1^+$ or $CD11c^+$ cells were depleted with immunomagnetic beads as described above. After washing, the remaining $CD11c^+$ or $Gr\text{-}1^+$ cells were plated in 24-well tissue culture plates (Falcon; Becton Dickinson, Franklin Lakes, NJ) at 10^6 cells/ml in 1 ml R-10 supplemented with recombinant cytokines (G-CSF, 2 ng/ml; M-CSF, 10 ng/ml; GM-CSF, 5 ng/ml; IL-4, 5 ng/ml; and TNF-α, 10 ng/ml) as described in RESULTS. Cultures were maintained in 5% CO_2 for up to 7 days.

In vivo cell tracking and differentiation. Cells harvested from GM-CSF/TGF-β1–supplemented BM cultures were prepared as described, labeled with 5 μmol/l CFSE (14), and injected intravenously. In some experiments, mice were anesthetized with methoxyflurane. Then, the spleen was everted through a keyhole incision and, after being injected with CFSE-labeled cells (5×10^6 in 50 μl), gently reinserted into the peritoneal cavity. The wound was closed with surgical clips. Control mice received PBS alone. Mice were killed at defined time points after cell transfer and spleens and other tissues removed. For immunohistology, tissues were embedded in Tissue-Tek OCT freezing medium (Miles, Elkhart, IN). For flow cytometric analysis of in vivo DC development, spleen cell suspensions were prepared using collagenase/EDTA as described (13).

Adoptive cotransfer assay for regulatory T-cells. Adoptive cotransfer assays for antidiabetic regulatory cells were performed as described (15). Spleen cells (2×10^7) from recipients of proinsulin-NOD or wild-type NOD GM-CSF/TGF-β1–cultured BM (test cells) and spleen cells (2×10^7) from recently diabetic female NOD mice (diabetogenic cells) were mixed and injected intravenously into NOD.scid mice. Diabetes development was monitored as described below.

Immunohistology. Cryostat sections (5 μm) were cut from frozen OCT-embedded tissues, air dried, and fixed with cold 100% ethanol before immunostaining or mounting. Avidin/biotin binding sites were blocked using avidin/biotin blocking reagents (Vector, Burlingame, CA), and nonspecific protein interactions were blocked with 1% BSA. Biotinylated primary antibodies were applied at predetermined optimal concentrations for 1 h at room temperature. After washing, streptavidin horseradish peroxidase (ABC-Elite; Vector) or streptavidin Texas red was applied for a further hour. Immunoperoxidase slides were washed, and staining was developed with enzyme substrate (VectorRed [Vector] or 3,3′-diaminobenzidine, σ-Fast' [Sigma]) and counterstained with hematoxylin. Immunofluorescence slides were rinsed and mounted in antifade reagent (DAKO, Carpinteria, CA).

Cytospins. Cytospins were prepared in a cytofuge (Shandon, Pittsburgh, PA) and stained with Diff Quik (Lab Aids, Narrabeen, NSW, Australia) or by immunohistochemistry, as described.

Assessment of proinsulin production. $CD11c^+$ and $Gr\text{-}1^+$ cells were purified from day 5 GM-CSF/TGF-β1 BM as described. For GM-CSF/IL-4, whole cultures were analyzed. Cells were cultured for 24 h with cytokine (IFN-γ, 1,000 units/ml, and TNF-α, 10 ng/ml) or anti-CD40 (50 μg/ml) supplementation. Supernatants were harvested and stored at −20°C until assayed. Proinsulin production was measured using an insulin enzyme-linked immunosorbent assay kit (Mercodia, Uppsala, Sweden). Proinsulin production was calculated as nanograms of 10^6 cells per 24 h. The detection limit was <0.1 ng $\cdot 10^6$ cells$^{-1} \cdot 24$ h^{-1}.

Monitoring of diabetes development. Mice were tested weekly for urine glucose (Diastix; Bayer, Pymble, NSW, Australia), and if they were glycosuric, they were then tested for blood glucose (Accu-Chek; Roche, Castle Hill, NSW, Australia). Mice were scored diabetic when two consecutive blood glucose readings were >12.0 mmol/l, and then they were killed. Diabetes incidence was plotted as Kaplan-Meier survival curves (GraphPad Prism; GraphPad Software, San Diego, CA).

Statistical analysis. Student's t test was used for comparison of means (Microsoft Excel). One-way ANOVA with Neuman-Keul's post test was used for comparisons of multiple groups (GraphPad Prism). Statistical differences in diabetes incidence were analyzed by log-rank test (GraphPad Prism).

RESULTS

BM cultured in GM-CSF/TGF-β1 contains predominantly partially differentiated $Gr\text{-}1^+$ myeloid cells. Addition of TGF-β1 to GM-CSF–supplemented BM cultures allows myeloid cell expansion but inhibits terminal differentiation of DCs (9,16). Therefore, we surmised that GM-CSF/TGF-β1 could be used to generate partially differentiated myeloid progenitors that retain DC development potential. GM-CSF/TGF-β1 BM cultures contained a mixture of cell types but were dominated by small round cells with annular or segmented nuclei (Fig. 1*A*), expressing the myeloid differentiation antigen Gr-1 (Ly-6G; Fig. 1*B*). These features are characteristic of partially differentiated myeloid cells (17). Florescence-activated cell sorter analysis confirmed the preponderance of $Gr\text{-}1^+$ cells and their expression of the myeloid marker CD11b but not the DC-specific marker CD11c (Fig. 2). $Gr\text{-}1^+$ cells expressed negligible levels of MHC class I, MHC class II, F4/80, FIRE, MAC 3, CD13 M-CSF R, and c-kit (CD117) (Fig. 2). The

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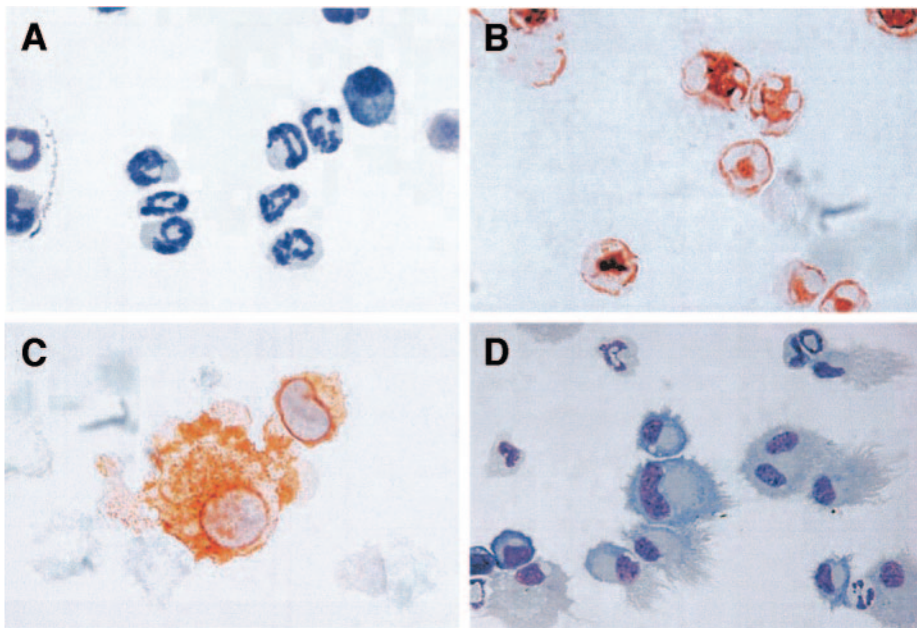

FIG. 1. BM cultured in GM-CSF/TGF-β1 containing predominantly partially differentiated Gr-1$^+$ myeloid cells. Cytospins were prepared from BM cultured in GM-CSF/TGF-β1 (*A*, *B*, and *C*) or GM-CSF/IL-4 (*D*) and stained with Diff Quik (*A* and *D*), antibodies to Gr-1 (*B*), or MHC class II (*C*).

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adhesion molecules CD49d and CD11a (18,19) used by mature neutrophils to home to sites of inflammation were either not expressed or were expressed at a relatively low level, respectively, by Gr-1$^+$ cells, signifying that these cells were not mature neutrophils. Expression of the myeloid differentiation markers CD43 and CD31 (17,20,21) on Gr-1$^+$ cells was heterogenous, indicating that these cells were present in various differentiation states.

A small proportion (8.5 ± 2.1% [mean ± SD], $n = 20$) of GM-CSF/TGF-β1 BM cells had a monocyte-like or immature DC-like appearance (Fig. 1*C*) and expressed low levels of MHC class II restricted primarily to intracellular granules (Fig. 1*C*). Florescence-activated cell sorter analysis showed that this Gr-1$^-$ fraction comprised almost exclusively cells that expressed low or intermediate levels of CD11c. In addition to MHC class I and moderate levels of MHC class II, CD11c$^+$ cells expressed F4/80, FIRE, MAC 3, CD13, and c-kit (CD117) (Fig. 2*A*), molecules expressed by immature BMDCs or DCs in vivo. CD11c$^+$ cells also expressed CD62L (L-selectin) used by DC/monocyte precursors to home from blood to lymph nodes (22). The majority of the CD11c$^+$ cells expressed M-CSF R and high levels of CD11b, as described previously for immature BMDCs from NOD mice (13).

To further define CD11c$^+$ cells from GM-CSF/TGF-β1 BM, we compared BM cultured in GM-CSF/TGF-β1 and GM-CSF/IL-4, as the latter contains a mix of phenotypically mature and immature DCs (Fig. 1*D* and online appendix available at http://diabetes.diabetesjournals.org). CD11c$^+$ cells from GM-CSF/TGF-β1 BM were phenotypically similar to the immature subset of DCs generated in GM-CSF/IL-4 (online appendix). Endocytic activity, a hallmark of functionally immature (CD11c$^+$/CD86^{lo}) DCs (online appendix), measured in GM-CSF/TGF-β1 BM by FITC-dextran uptake, was restricted to CD11c$^+$ cells (online appendix). In contrast to Gr-1$^+$ myeloid cells, CD11c$^+$ cells harvested from GM-CSF/TGF-β BM were more differentiated, as evidenced by their rapid development (within 2 days) into DCs in GM-CSF, IL-4, and TNF-α or into macrophages in M-CSF or GM-CSF/M-CSF. CD11c$^+$ cells rapidly died in cultures supplemented only with G-CSF (not shown). Collectively, these findings indicate that Gr-1$^+$ cells in GM-CSF/TGF-β1 BM are partially differentiated myeloid cells, whereas the minor population of CD11c$^+$ cells are phenotypically and functionally immature DCs.

Gr-1$^+$ cells are multipotent myeloid progenitors. To investigate their differentiation potential, Gr-1$^+$ cells isolated from GM-CSF/TGF-β1 BM were exposed to cytokines that drive different myeloid differentiation pathways. In G-CSF, Gr-1$^+$ cells retained their small rounded profile but rapidly acquired highly segmented nuclei and a higher level of Gr-1 expression characteristic of mature granulocytes (Fig. 3). Cells with DC- or macrophage-like characteristics were not detected after 2 days in G-CSF. Consistent with their short lifespan, the numbers of mature granulocytes diminished after 2 days in G-CSF (not shown). In the DC-inducing combination of GM-CSF, IL-4, and TNF-α, small numbers of DC-like cells were seen as early as 2 days, and by 7 days large numbers of cells displayed classic DC morphology (Fig. 3). Flow cytometry revealed that exposure to GM-CSF, IL-4, and TNF-α resulted in loss of Gr-1 and acquisition of CD11c expression by the majority of cells (Fig. 3). CD11c$^+$ cells were approximately equally distributed between phenotypically mature (MHC class II^{hi} or CD86^{hi}) and immature (MHC class II^{lo} or CD86^{lo}) DC subsets (Fig. 3). In M-CSF, Gr-1 expression was lost and adherent macrophage-like cells that retained CD11b expression and acquired low-level expression of the macrophage marker F4/80 appeared.

Similar phenotype of GM-CSF/TGF-β1 BM cells from PI-NOD and wild-type NOD mice. No differences were detected between cells generated from PI-NOD and NOD mice in the presence of either GM-CSF/TGF-β1 (Fig. 4) or GM-CSF/IL-4 (not shown).

CD11c$^+$ DCs but not Gr-1$^+$ myeloid cells from PI-NOD transgenic mouse BM produce proinsulin. CD11c$^+$ DCs generated from PI-NOD transgenic mouse BM in GM-CSF/IL-4 produced threefold more proinsulin than CD11c$^+$ DCs from GM-CSF/TGF-β1 BM (4.2 ± 2.3 vs.

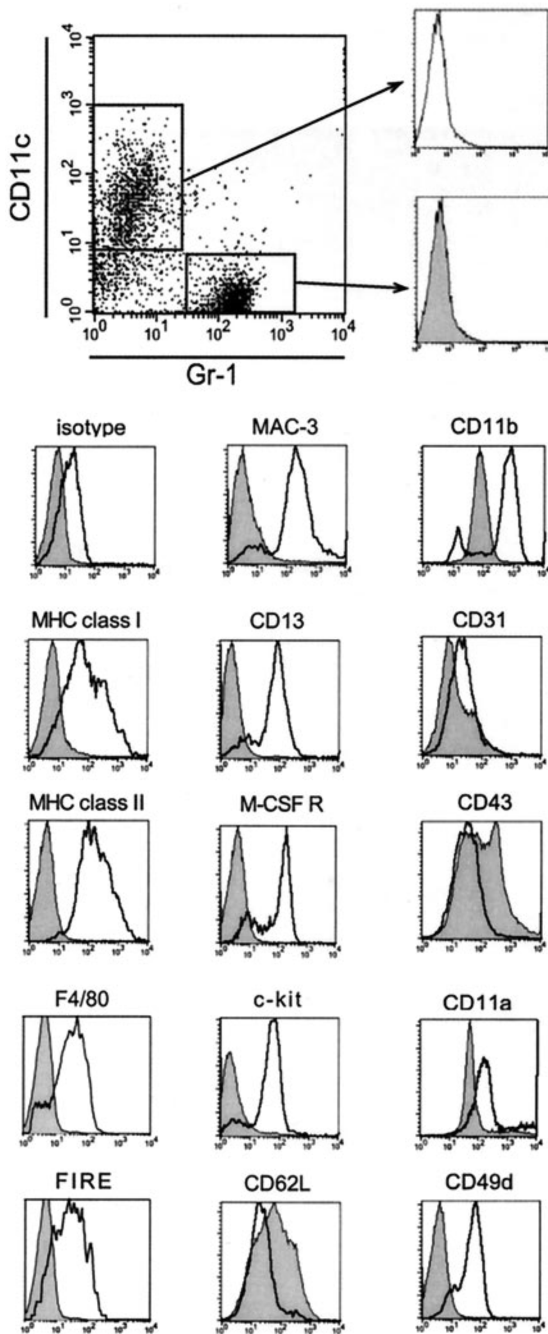

FIG. 2. Undifferentiated myeloid cells are abundant in GM-CSF/TGF-β1–cultured BM. BM was cultured in GM-CSF/TGF-β1 and cells harvested at day 5. Cell surface markers expressed on Gr-1$^+$ and CD11c$^+$ cells were analyzed using four-color flow cytometry. Upper left dot plot shows gating used for analysis of Gr-1$^+$ and CD11c$^+$ cells. Histogram overlays show Gr-1–gated (shaded) and CD11c-gated (open) cells.

1.3 ± 1.1 ng · 10^6 cells^{-1} · 24 h^{-1}). Cytokines that upregulate MHC class II expression (IFN-γ/TNF-α) enhanced proinsulin production by CD11c$^+$ DCs from GM-CSF/TGF-β1 cultures (to 3.4 ± 1.7 ng · 10^6 cells^{-1} · 24 h^{-1}, $P < 0.05$), whereas agonistic anti-CD40 mAb enhanced proinsulin production by DCs from GM-CSF/IL-4 cultures (to 8.0 ± 2.9 ng · 10^6 cells^{-1} · 24 h^{-1}, $P < 0.05$) but not GM-CSF/TGF-β1 cultures. This is consistent with the expression of CD40 by DCs from GM-CSF/IL-4 BM but not GM-CSF/TGF-β1 BM. Purified Gr-1$^+$ cells did not produce detectable proinsulin, even after stimulation with GM-CSF/IFN-γ/TNF-α.

Diabetes development is suppressed by transfer of proinsulin-encoding Gr-1$^+$ undifferentiated myeloid cells. GM-CSF/TGF-β1 BM from PI-NOD mice transferred to 4-week-old female NOD mice suppressed diabetes development (Fig. 5*A*). To identify the cell responsible for this protective effect, CD11c$^+$ or Gr-1$^+$ cells were depleted from GM-CSF/TGF-β1 BM before transfer. Depletion of Gr-1$^+$, but not CD11c$^+$, cells abolished the protective effect (Fig. 5*B*). This was then demonstrated directly and shown to be proinsulin dependent by transfer of purified Gr-1$^+$ cells from GM-CSF/TGF-β1 BM of PI-NOD mice (Fig. 5*C*). Thus, diabetes development was suppressed by transfer of Gr-1$^+$ partially differentiated myeloid cells and not by the CD11c$^+$/CD86^{lo} immature DCs present in GM-CSF/TGF-β1 NOD-PI BM.

Gr-1$^+$ myeloid cells differentiate into DCs in vivo. Our hypothesis was that PI-encoding Gr-1$^+$ myeloid cells would protect against diabetes because they differentiated into "tolerogenic" resting DC in vivo. To examine their fate, Gr-1$^+$ myeloid cells purified from PI-NOD GM-CSF/TGF-β1 BM were labeled with CFSE to enable visualization after transfer. Two days after intravenous transfer into NOD mice, CFSE-labeled cells (~1–2 per 10× field) could be detected in cryostat sections of spleen, lung, and liver but not in peripheral blood or other tissues (thymus, pancreas, small intestine, kidney, inguinal lymph node, and pancreatic lymph node). CFSE-labeled cells in liver and lung diminished rapidly in number after 2 days but were still detected in spleen (≥1 per 10× field) at 4 and 6 days posttransfer. Because they were preferentially retained in spleen, we injected CFSE-labeled Gr-1$^+$ cells directly into spleen to examine their differentiation. One day after injection, abundant CFSE-labeled cells with a rounded profile that stained for CD11b and Gr-1, but rarely for MHC class II, were clearly visible. Three days after injection, CFSE-labeled cells were present predominantly in the T-cell areas and had developed a larger, more stellate appearance. Many continued to express CD11b but had lost Gr-1 expression, and 30–50% had acquired substantial expression of MHC class II and CD11c (Fig. 6). To determine the phenotype of the CD11c$^+$ DCs that developed in vivo, spleens were collected after 3 days for flow cytometry. CD11c$^+$ cells comprised a large proportion of the CFSE-labeled cells in the spleen (Fig. 7*A*). Three-color flow-cytometry revealed that CFSE$^+$/CD11c$^+$ DCs were almost equally distributed between the CD11c$^+$/CD8$^+$ and CD11c$^+$/CD11b$^+$ subtypes. This contrasted with the dominance of CD11c$^+$/CD11b$^+$ or CD11c$^+$/CD8$^-$ DC subtype (Fig. 7*B*) normally found in NOD mice (13). Analysis of DEC-205, normally expressed on CD8$^+$ splenic DCs, confirmed the distribution of CFSE$^+$ DCs between CD8$^+$ and CD8$^-$ subtypes (Fig. 7*B*). CFSE$^+$ DCs derived from Gr-1$^+$ progenitors exhibited a "resting" phenotype, expressing intermediate levels of MHC class II and low levels of the costimulatory/signaling molecules CD86 and CD40, identical to those of unlabeled recipient DCs (Fig. 7*C*).

Absence of regulatory, antidiabetogenic cells after transfer of GM-CSF/TGF-β1–cultured BM. A potential mechanism for the diabetes protective effect of Gr-1$^+$ myeloid cells is induction of regulatory, antidiabetic T-

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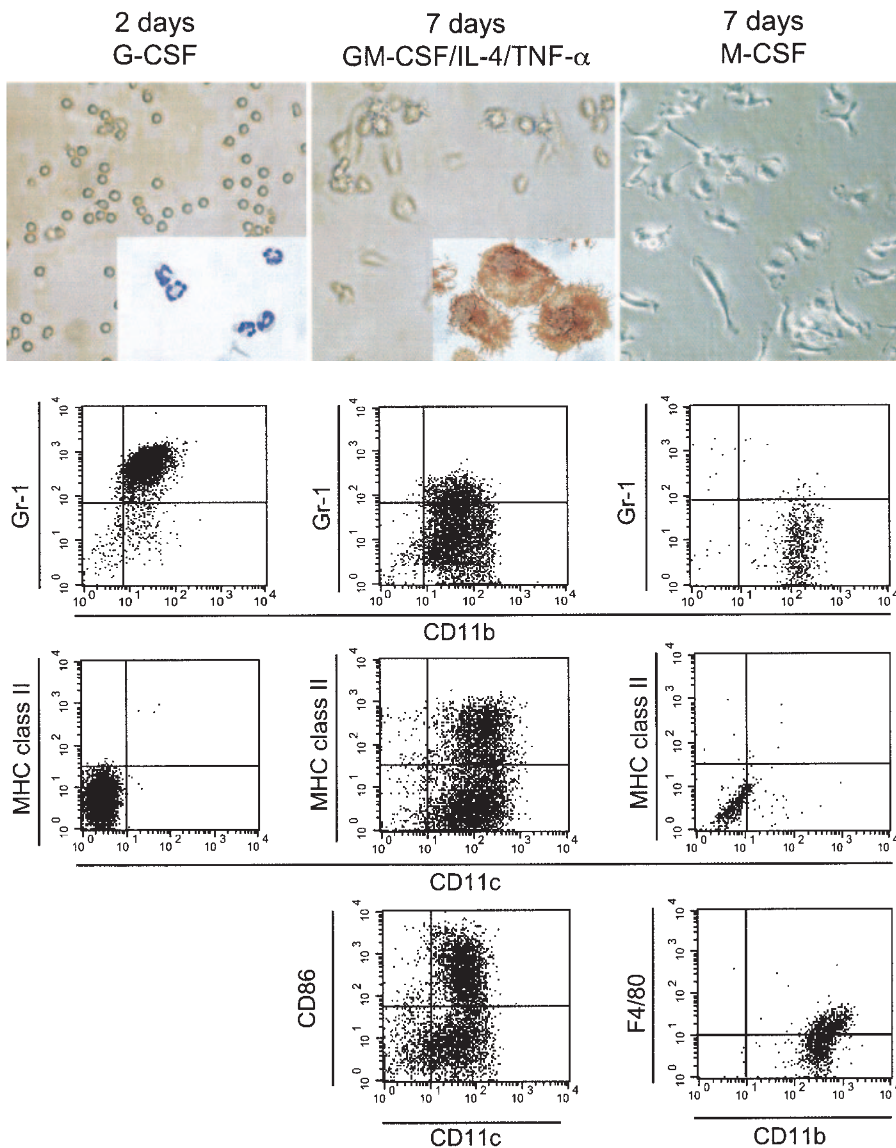

FIG. 3. Gr-1$^+$ cells are multipotent myeloid progenitors. Gr-1$^+$ cells were purified from GM-CSF/TGF-β1–cultured BM and cultured in the presence of the cytokines shown. Photomicrographs show appearance in culture (phase contrast microscopy) or in cytospin preparations stained with Diff Quik (G-CSF) or anti-MHC class II (GM-CSF/IL-4/TNF-α). Lower panels show flow cytometric analysis of surface markers.

cells. To test for regulatory T-cells, spleen cells from NOD mice that had received NOD or NOD-PI GM-CSF/TGF-β1 BM 4 weeks previously were cotransferred with diabetogenic spleen cells from recently diabetic female NOD mice into immunodeficient NOD.scid recipients. The proportion of mice that developed diabetes by 12 weeks after transfer was similar in recipients of spleen cells from mice that received GM-CSF/TGF-β1 BM from NOD-PI (78 ± 21%) or wild-type NOD (69 ± 32%) mice.

DISCUSSION

Proinsulin-encoding myeloid DC progenitors generated in BM cultured in GM-CSF/TGF-β1 differentiate into resting DCs in vivo and suppress the development of autoimmune diabetes in NOD mice. Achieving expression of antigen in resting DCs by transfering antigen-encoding DC progenitors is a novel strategy for preventing autoimmune disease. Previously, the addition of TGF-β1 to GM-CSF–supplemented BM cultures was shown to inhibit the final maturation steps of DC development, leading to the generation of phenotypically and functionally immature DCs (9,16). We found that this combination of cytokines also leads to the accumulation of partially differentiated myeloid cells. While Gr-1 is routinely used as a neutrophil marker, it is expressed by a range of myeloid progenitors and, at least transiently, by monocytic cells such as those elicited to the peritoneal cavity (19), as well as by a recently described population of murine blood monocytes that exhibits DC differentiation capacity (23). Our finding that Gr-1$^+$ cells can give rise to DCs and macrophages in vitro is consistent with the observations of others (17,24). In addition, we now show that these cells also differentiate into both major subtypes (CD8$^+$ and CD8$^-$) of lymphoid tissue DCs in vivo, complementing other evidence (25,26) that myeloid-committed cells are capable of giving rise to both CD8$^+$ and CD8$^-$ DCs in vivo. Undifferentiated myeloid cells (CD11b$^+$/Gr-1$^+$/CD31$^+$) that have been termed natural suppressor cells inhibit CD8$^+$ T-cell activation by antigen-nonspecific nitric oxide–dependent mechanisms (27). A role for these myeloid suppressor cells in our experiments is excluded by their absence of MHC class II expression (27). The crucial requirement for MHC class II$^+$ progeny of Gr-1$^+$ cells for diabetes suppression was demonstrated by the lack of effect of wild-type Gr-1$^+$ cells

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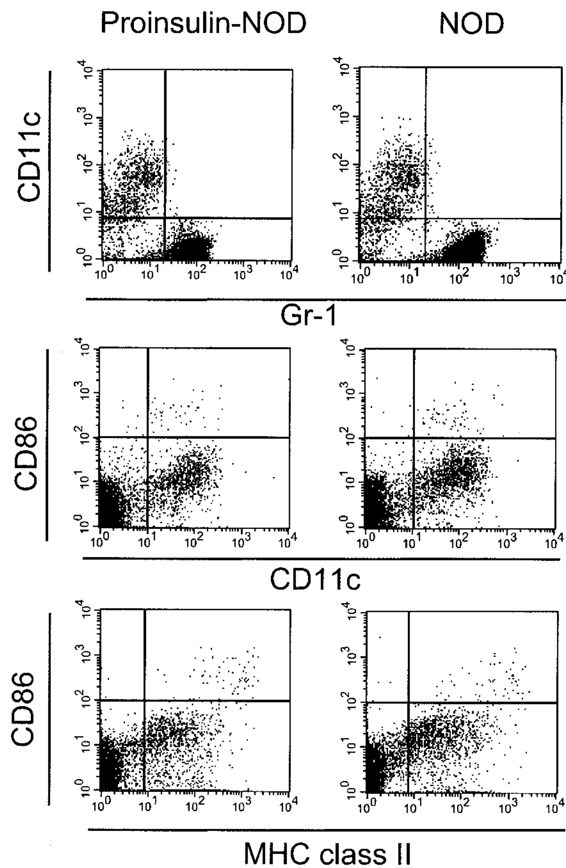

FIG. 4. Similar phenotype of GM-CSF/TGF-β1 BM cells from PI-NOD and wild-type NOD mice. BM from PI-NOD or wild-type NOD mice was cultured in GM-CSF/TGF-β1 for 5 days, cells were harvested, and surface marker expression was analyzed by flow cytometry.

without the MHC class II–driven proinsulin transgene. Additionally, the low expression of CD31 on Gr-1$^+$ cells generated in GM-CSF/TGF-β1 indicates that they are more differentiated than CD31$^+$ myeloid suppressor cells (21,28).

In contrast to proinsulin-encoding Gr-1$^+$ myeloid cells, proinsulin-encoding immature DCs were not protective. This may be because they were activated in vitro or because exposure to TGF-β1 decreases the expression of CCR7 (29), which coordinates DC migration and interaction with T-cells in secondary lymphoid tissues (30). Our findings suggest that Gr-1$^+$ myeloid progenitors may have been overlooked in previous studies examining the tolerance-inducing capacity of GM-CSF/TGF-β1–generated immature DCs (9). Because Gr-1$^+$ myeloid cells represent a large proportion of cells in BM (21), they are a potentially important source of DC progenitors that could be harnessed for tolerance induction.

The mechanism by which proinsulin-encoding myeloid progenitor cells suppress diabetes development remains unclear. Because transferred CFSE-labeled Gr-1$^+$ cells migrated primarily to spleen, liver, and lung, and not thymus, protection from diabetes is likely to be due to peripheral rather than central tolerance. While there is debate about whether specific subsets of DCs, e.g., CD8$^+$ or CD8$^-$, are "specialized" for peripheral tolerance induction, it is nevertheless clear that DCs must be in a nonactivated or resting state for tolerance to ensue

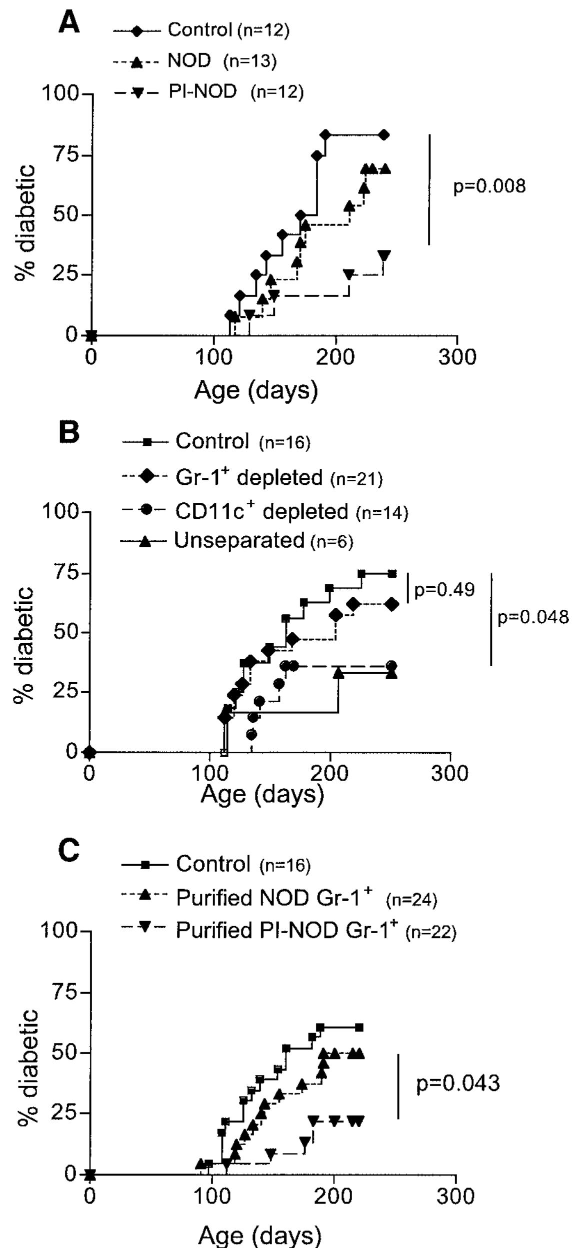

FIG. 5. Diabetes development is suppressed by transfer of proinsulin-encoding Gr-1$^+$ undifferentiated myeloid cells. *A*: GM-CSF/TGF-β1–cultured BM from proinsulin-NOD (t) or NOD (▲) mice was transferred intravenously to 4-week-old female NOD mice and diabetes development determined. Control mice received PBS. Diabetes development was significantly suppressed ($P < 0.01$) by transfer of GM-CSF/TGF-β1–cultured proinsulin-NOD but not wild-type NOD BM. Data were pooled from two separate experiments in which proinsulin-NOD and NOD cells were tested in parallel. *B*: BM from proinsulin-NOD mice was harvested from GM-CSF/TGF-β1–supplemented cultures. Gr-1$^+$ or CD11c$^+$ cells were depleted using immunomagnetic beads, the remaining cells (CD11c$^+$ or Gr-1$^+$, respectively) were transferred intravenously to 4-week-old female NOD mice, and diabetes development was compared with contemporaneous controls. Diabetes development was significantly suppressed ($P < 0.05$) by transfer of CD11c$^+$ cells depleted but not Gr-1$^+$ cell–depleted GM-CSF/TGF-β1–cultured proinsulin-NOD BM. Data were pooled from two separate experiments in which cells were tested in parallel. *C*: BM from proinsulin-NOD or NOD mice was harvested from GM-CSF/TGF-β1–supplemented cultures. CD11c$^+$ cells were depleted using immunomagnetic beads, the remaining Gr-1$^+$ cells were transferred intravenously to 4-week-old female NOD mice, and diabetes development was compared with contemporaneous controls. Diabetes development was significantly suppressed ($P < 0.05$) by transfer of Gr-1$^+$ cells from proinsulin-NOD (t) but not NOD (▲) GM-CSF/TGF-β1–cultured BM. Data were pooled from two separate experiments in which proinsulin-NOD and NOD cells were tested in parallel.

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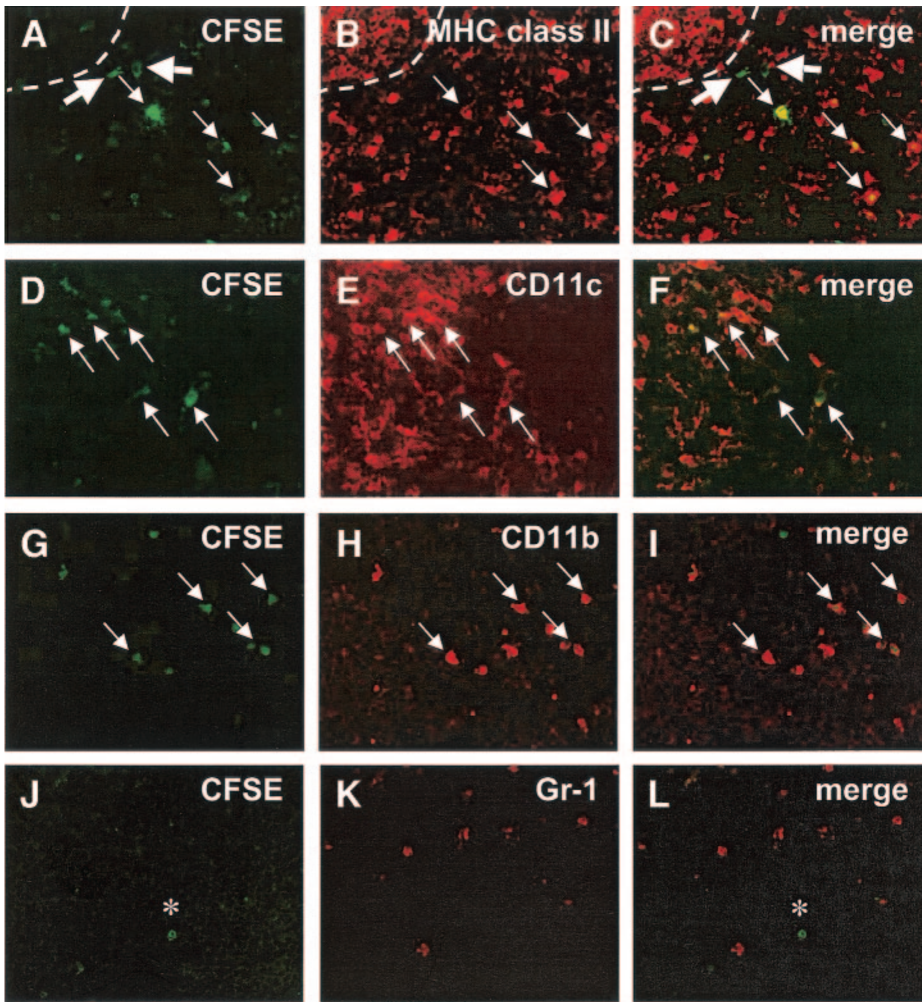

FIG. 6. Gr-1$^+$ myeloid cells differentiate to CD11c$^+$/MHC class II$^+$ DCs in vivo. Gr-1$^+$ cells were purified from GM-CSF/TGF-β1–cultured proinsulin-NOD BM, CFSE labeled, and injected directly into the spleen. After 3 days, frozen sections of spleen were stained for immunofluorescence analysis. Localization of CFSE- and antibody-labeled cells was performed by immunofluorescence microscopy. Panels show CFSE-labeled cells (*A*, *D*, *G*, and *J*) visualized with Texas red–conjugated monoclonal antibody as indicated (*B*, *E*, *H*, and *K*) and the merged images (*C*, *F*, *I*, and *L*). The dashed lines delineate the boundary of the T-cell area (below line) and B-cell area (above line). In *A*, *B*, and *C*, the thin arrows show CFSE$^+$ cells expressing MHC class II and the broad arrows show CFSE$^+$ cells not expressing MHC class II. In *D*, *E*, and *F*, arrows show CFSE$^+$ cells expressing CD11c. In *G*, *H*, and *I*, arrows show CFSE$^+$ cells expressing CD11b. In *J* and *L*, asterisks indicate CFSE$^+$ cells not expressing Gr-1. (Gr-1 cells are normally rare in the spleen.)

(2,4,5,31). Targeting antigen expression to resting as opposed to activated lymphoid tissue DCs, either by antibody-antigen conjugates or genetically (5), as achieved here for proinsulin, induces deletional tolerance and/or unresponsiveness in antigen-specific T-cells (2,4,6,31). Because both CD8$^+$ and CD8$^-$ DCs that differentiated from transferred Gr-1$^+$ myeloid progenitors exhibited a resting phenotype, we propose that diabetes protection is due to deletion or unresponsiveness of proinsulin-reactive T-cells. This is consistent with our inability to demonstrate the presence of antidiabetogenic Treg. Unfortunately, in common with others (32,33), we are unable to obtain sensitive and reproducible responses to proinsulin by NOD mouse T-cells ex vivo and therefore cannot directly test these possibilities.

Because of their costimulation dependence (34), CD4$^+$/CD25$^+$ Treg require activation by "mature" DCs to elicit regulatory function (35). Similarly, other forms of Treg in NOD mice may be most efficiently induced by mature DCs (36–38). This supports suggestions that the impaired maturation potential of NOD DCs (13,39) could lead to reduced Treg activation in vivo (40,41). In this setting, tolerance can be restored by activating DCs in vivo (42) or in an islet antigen–independent manner by adoptively transferring DCs matured ex vivo (36–38). In contrast, our findings indicate that the ability of resting DCs to induce tolerance is unaffected by the alteration of DC development in NOD mice.

The literature provides little evidence of the ability of DCs to suppress spontaneously as opposed to experimentally induced autoimmune disease. Attempts to generate tolerogenic DCs that suppress diabetes in an autoantigen-dependent manner after transfer to NOD mice have had little success. In instances where diabetes has been suppressed by DCs generated ex vivo, no requirement for presentation of β-cell antigens has been found (36–38,43–45). Our findings show that an autoantigen-dependent protective effect, an important requirement for progressing DC-based immunotherapy to the clinic, can be achieved by transferring genetically modified Gr-1$^+$ myeloid progenitors. DC progenitors encoding autoantigen driven by a differentiation stage-specific promoter have several advantages over other DC-based strategies for autoimmune disease prevention. Activation and the risk of antigen presentation that could elicit a pathogenic immune response is minimized. DC progeny express whole autoantigen protein; therefore, assumptions about epitope determinants that are necessary with peptides are avoided, as is the need for substantial quantities of pure protein autoantigen. This immunotherapeutic strategy could be applied to prevent type 1 diabetes and other autoimmune diseases in humans.

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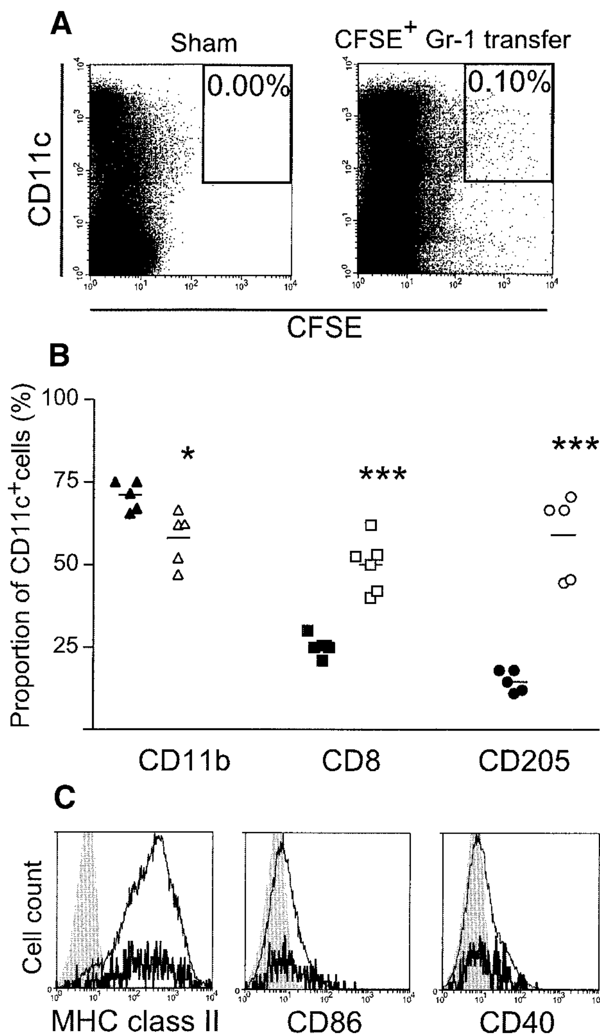

FIG. 7. Gr-1$^+$ myeloid cell–derived DCs have a resting phenotype in vivo. *A–C*: Gr-1$^+$ cells were purified from GM-CSF/TGF-β1–cultured proinsulin-NOD BM, CFSE-labeled, and injected directly into the spleen. Three days later spleens were digested and the phenotype of CFSE-labeled Gr-1$^+$ cell-derived DCs determined by flow cytometry. *A*: Percentage of CFSE-labeled cells that express CD11c in the spleen of mice receiving no cells (left) or CFSE-labeled Gr-1$^+$ cells (right). Similar results were obtained in six mice analyzed in two separate experiments. *B*: Proportion of CFSE$^-$ (closed symbols) or CFSE$^+$ (open symbols) CD11c$^+$ DCs expressing phenotypic markers of splenic DC subtypes. Cells from individual mice were gated on CFSE$^+$ or CFSE$^-$ CD11c$^+$ cells for analysis. Data for each phenotypic marker are pooled from two separate experiments. *Significantly less than CFSE$^-$ DCs ($P < 0.05$); ***significantly greater than CFSE$^-$ DCs ($P < 0.001$). *C*: Histograms depicting expression of MHC class II, CD86, and CD40 on CFSE$^-$ (thin line) or CFSE$^+$ (bold line) DCs in recipients of Gr-1$^+$ myeloid cells. Cells from individual mice were gated on CFSE$^+$/CD11c$^+$ or CFSE$^-$/CD11c$^+$ DC for analysis. Solid gray histograms depict background fluorescence of unstained cells. Similar results were obtained in six mice in two separate experiments.

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