Immunomodulatory effect of decoy receptor 3 on the differentiation and function of bone marrow-derived dendritic cells in nonobese diabetic mice: from regulatory mechanism to clinical implication

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Abstract: To investigate the regulatory effects of decoy receptor 3 (DcR3) on the differentiation and function of dendritic cells (DCs), bone marrow-derived DCs (BM-DCs) from nonobese diabetic (NOD) mice were cultured with recombinant DcR3.Fc protein. Their differentiating phenotypes and T cell-stimulating functions were then evaluated. Expression of CD11c, CD40, CD54, and major histocompatibility complex I-A^{g7} was reduced in cells cultured with additional DcR3.Fc, compared with DCs incubated with granulocyte macrophage-colony stimulating factor and interleukin (IL)-4, indicating that DcR3 interferes with the differentiation and maturation of BM-DCs. One of the most striking effects of DcR3.Fc on the differentiation of DCs was the up-regulation of CD86 and down-regulation of CD80, suggesting a modulatory potential to skew the T cell response toward the T helper cell type 2 (Th2) phenotype. Consistent with this, the proliferation of $CD4^+$ T cells cocultured with DcR3.Fc-treated DCs was significantly reduced compared with that of T cells stimulated by normal DCs. Moreover, the secretion of interferon-γ from T cells cocultured with DcR3.Fc-treated DCs was profoundly suppressed, indicating that DcR3 exerts a Th1-suppressing effect on differentiating DCs. Furthermore, adoptive transfer experiments revealed that NOD/severe combined immunodeficiency mice received DcR3.Fc-treated DCs, and subsequently, autoreactive T cells showed delayed onset of diabetes and a decrease in diabetic severity compared with mice that received normal DCs and T cells, suggesting a future therapeutic potential in autoimmune diabetes. Data from two-dimensional gel electrophoresis and matrix-assisted laser desorption/ionization–time-of-flight analysis show an up-regulation of some proteins—such as mitogen-activated protein kinase p38 β, cyclin-dependent kinase 6, and signal-induced proliferation-associated gene 1—and a down-regulation of the IL-17 precursor; tumor necrosis factor-related apoptosis-inducing ligand family member-associated nuclear factor-κB activator-binding kinase 1; and Golgi *S*-nitroso-*N*-acetylpenicillamine in cells treated with DcR3, further demonstrating its effect on DC differentiation and function. *J. Leukoc. Biol.* 75: 293–306; 2004.

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INTRODUCTION

Hematopoiesis is a multiple developmental process in which pluripotential stem cells give rise to committed progeny cells, which undergo proliferation, differentiation, or apoptosis, resulting in the continuous production of appropriate numbers of mature and functional lymphoid and myeloid cells throughout the lifetime of a vertebrate organism [1–3]. Among these cells, dendritic cells (DCs) are unique as professional antigen (Ag)-presenting cells (APCs), which occur throughout the peripheral tissues and act as sentinels against invading pathogens [4–6]. DCs are the most efficient APCs and play a crucial role in controlling immune responses [4, 7–10]. Immature DCs, triggered by Ag or pathogens, undergo phenotypic and functional changes from Ag-capturing cells to mature APCs. DC maturation is a complex and continuous process initiated at the periphery after Ag stimulation and is completed during the DC/T cell interaction [4, 11]. Many factors influence DC mat-

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uration, including pathogenic molecules such as lipopolysaccharide (LPS), bacterial DNA, and double-stranded RNA [4]; local microenvironmental factors, including proinflammatory and anti-inflammatory cytokines [4]; and T cell-derived signals such as CD40L [12]. To understand the impact of environmental factors on DCs, several studies have investigated the mechanisms regulating the differentiation, maturation, and function of DCs by examining the effects of and interactions among various cytokines [13–17]. Despite these, the potential regulatory effects of some newly identified cytokines, such as decoy receptor 3 (DcR3), on the differentiation and function of DCs remain to be elucidated.

DcR3, also known as TR6 or M68, belongs to the tumor necrosis factor (TNF) receptor superfamily and is a decoy receptor for Fas ligand (FasL) [18], a receptor homologous to lymphotoxins (LTs), exhibits inducible expression and competes with Herpes simplex virus glycoprotein D for the *Herpesvirus* entry mediator (HVEM) and is expressed by T lymphocytes (LIGHT) [19], and TNF-like molecule 1A (TL1A) [20]. Like osteoprotegerin [21], DcR3 lacks a transmembrane domain and is regarded as a secreted rather than a membrane-bound protein, with a molecular weight (Mw) of 35 kDa. DcR3 binds to FasL and LIGHT, thereby neutralizing the proapoptotic actions induced by the Fas–FasL interaction or inhibiting LIGHT-mediated biological effects by blocking the interaction between LIGHT and the LT-β receptor (LTßR) or HVEM. As *DcR3* is genetically amplified in colon and lung carcinomas [18] and is frequently overexpressed by malignant tumors arising from the pulmonary or gastrointestinal tracts [22], it has been postulated that DcR3 promotes tumor growth by escaping FasL- and LIGHT-mediated immunosurveillance. As in cancer patients, the *DcR3* gene is also overexpressed in patients with silicosis or systemic lupus erythematosus [23]. As LIGHT is also expressed in DCs and acts as a costimulatory factor in priming T cell responses [13, 24, 25], we examined whether DcR3 modulates T cell-mediated immunity by interfering with the differentiation and function of DCs.

We recently demonstrated that soluble DcR3.Fc binds to $CD14^+$ monocytes and modulates their differentiation and maturation into DCs [14]. The expression of human leukocyte antigen-DR, CD1a, CD40, CD54, and CD80/B7.1 was reduced in DcR3.Fc-treated DCs. However, the expression of CD86/B7.2 was up-regulated under the same conditions. It is interesting that DcR3.Fc-treated DCs caused T cell differentiation to be biased toward the T helper 2 (Th2) phenotype in allogenic mixed lymphocyte reactions (MLR) [14]. DCs are not only critical for the induction of primary immune responses but are also important for the induction of immunological tolerance, as well as for the regulation of some types of T cell-mediated autoimmune diseases. Therefore, in this study, we further tested whether DcR3 modulates the differentiation and function of bone marrow-derived DCs (BM-DCs) from nonobese diabetic (NOD) mice, which have been well characterized as a model of Th1-mediated autoimmune disease that resembles human insulin-dependent diabetes mellitus (IDDM).

IDDM is caused by progressive autoimmune destruction of the insulin-producing β cells in the pancreatic islets of Langerhans. Genetic predisposition and environmental factors contribute to its pathogenesis. Extensive studies of the immunodiabetogenic mechanisms of this disease indicate that Th1 lymphocytes play an important role in the initiation and propagation of the diabetogenic process in NOD mice. Therefore, we characterized the modulatory effects of DcR3 on NOD BM-DCs, seeking to take advantage of the biased Th2 phenotype induced by DcR3.Fc-treated DCs as a potential therapy in this disease model.

Our results reveal that the expression of CD11c, CD40, CD54, and major histocompatibility complex (MHC) I-A^{g7} was reduced in cells cultured with additional DcR3.Fc, compared with DCs incubated with granulocyte macrophage-colony stimulating factor (GM-CSF) and interleukin (IL)-4, indicating that DcR3 interferes with the differentiation and maturation of BM-DCs. It is interesting that CD86 was up-regulated, and CD80 was down-regulated in DcR3.Fc-treated DCs, suggesting a modulatory potential to skew the T cell response toward the Th2 phenotype. Moreover, the proliferation of $CD4^+$ T cells cocultured with DcR3.Fc-treated DCs was significantly reduced compared with that of T cells stimulated by normal DCs. Furthermore, the secretion of interferon (IFN)-γ from T cells cocultured with DcR3.Fc-treated DCs was profoundly suppressed, indicating that DcR3 has a Th1-suppressing effect on differentiating DCs. To further investigate quantitative changes in proteins differentially expressed in normal and DcR3.Fc-treated BM-DCs, we performed two-dimensional (2D) gel electrophoresis and matrix-assisted laser desorption ionization–time-of-flight (MALDI–TOF) analysis. Our data show an up-regulation of some proteins, such as mitogen-activated protein kinase (MAPK) p38 β, cyclin-dependent kinase 6, and signal-induced proliferation-associated gene 1, and a down-regulation of the IL-17 precursor, TNF-related apoptosis-inducing ligand family member-associated nuclear factor-κB (NF-κB) activator (TANK)-binding kinase 1, and Golgi *S*-nitroso-*N*-acetylpenicillamine (SNAP) in cells incubated with DcR3.Fc. This confirms the modulatory effects of DcR3 on DC differentiation and function. In summary, our results indicate that DcR3 may act not only as a decoy receptor for its known ligands but also as an effector molecule that modulates the differentiation and function of DCs, thus down-regulating the Th1 immune response. This modulatory effect of DcR3 in restoring or enhancing the Th2 immune response in NOD mice may provide the basis for a future therapeutic application in autoimmune diabetes.

MATERIALS AND METHODS

Animals

Female NOD/Sytwu (K^d, D^b, L^d, I-A^{g7}) mice were initially purchased from the Jackson Laboratory (Bar Harbor, ME) and were subsequently bred and raised at the Animal Center of the National Defense Medical Center (Taipei, Taiwan) under specific, pathogen-free conditions. The spontaneous incidence of diabetes in the colony is currently 80–90% in females and 20–30% in males by 25 weeks of age. The mice that used to provide BM cells were aged from 6 to 8 weeks, and those providing splenocytes for proliferation and cytokine assays were aged from 6 to 10 weeks. No autoimmune-prone animal had developed autoimmune diabetes when BM or splenocytes were isolated. NOD/severe combined immunodeficiency (SCID) mice were also purchased from the Jackson Laboratory and raised at the Animal Center of the National Defense Medical Center under specific, pathogen-free conditions. Female BALB/c

($H\text{-}2^d$) mice were obtained from the Animal Center of the National Defense Medical Center under specific, pathogen-free conditions.

Purification of recombinant DcR3.Fc (rDcR3.FC) protein

DcR3.Fc protein was produced as described previously [14]. Briefly, the open-reading frame of the human *DcR3* gene was isolated by reverse transcriptase-polymerase chain reaction using the forward primer, 5′-GGAATTCAAGGACCATGAGGGCGCTG-3′, and the reverse primer, 5′-GGAATTCGTGCACAGGGAGGAAGCGC-3′. The amplified product was ligated in-frame into the *Eco*RI-cut pUC19-immunoglobulin G (IgG)1-Fc vector containing the cDNA for human (h)IgG1 Fc. The fusion gene was then subcloned into the pBacPAK9 vector (Clontech Laboratories, Palo Alto, CA) and was cotransfected with linearized BacPAK6 DNA (Clontech Laboratories) into Sf21 cells. The supernatant from recombinant virus-infected Sf21 cells was filtered and purified on protein A-Sepharose beads (Amersham Pharmacia Biotech, Uppsala, Sweden). Bound DcR3.Fc protein was then eluted with 0.1 M citric acid buffer (pH 3.0; Sigma Chemical Co., St. Louis, MO) followed by dialysis against phosphate-buffered saline (PBS). Purified, recombinant protein was desalted on NAP™ 10 columns (Amersham Pharmacia Biotech) before use.

Western blot analysis

Purified DcR3.Fc protein (10 μg/lane) was separated on 12% polyacrylamide gels and blotted onto nitrocellulose membranes by standard procedures. The membranes were washed, incubated with anti-DcR3 (Anawrahta Biotech, Taipei, Taiwan) or anti-hIgG1 (Chemicon, El Segundo, CA) primary antibodies (Ab), washed, and then incubated with horseradish peroxidase-conjugated goat anti-mouse Ig secondary Ab (Chemicon). Enhanced chemiluminescence reagents (Amersham Pharmacia Biotech) were used to detect positive signals.

Assessment of DcR3-mediated inhibition of activation-induced cell death

Jurkat cells were seeded in 96-well plates at a density of 10^5/well, stimulated with phorbol 12-myristate-13-acetate (PMA; 30 ng/ml) plus ionomycin (2 μM) overnight at 37°C, and then incubated with DcR3.Fc (10 μg/ml), Fas.Fc (10 μg/ml), or hIgG1 (10 μg/ml) for 20 h. $CD4^+$ T cells were purified from spleen cells by magnetic cell sorting. Briefly, single-cell suspensions of NOD splenocytes were incubated with magnetic cell sorter (MACS) CD8α and B220 microbeads (Miltenyi Biotec, Germany) for 15 min at 6°C. Non-Ab-bound cells were sorted by negative selection using autoMACS (Miltenyi Biotec) according to the manufacturer's instructions. The sorted $CD4^+$ T cells were checked by flow cytometric analysis, and the purity was 90–95%. These freshly prepared $CD4^+$ T cells were then added to anti-CD3 (0.3 μg/ml) Ab-coated plates and were supplied with 5 μg/ml IL-2 for 4 days. $CD4^+$ T cells were incubated for 24 h without anti-CD3 stimulation and at a density of 5×10^5/well, were restimulated with anti-CD3 Ab (1 μg/ml). They were then incubated with DcR3.Fc (10 μg/ml), Fas.Fc (10 μg/ml), or hIgG1 (10 μg/ml) in 96-well plates for 20 h. To quantitate FasL-induced cell apoptosis and DcR3-mediated inhibition, cells were stained with propidium iodide (PI) or annexin V (PharMingen, San Diego, CA) for 15 min at room temperature. Apoptotic cells were then measured by flow cytometry using a Becton Dickinson (San Jose, CA) FACSCalibur cytometer and CellQuest software.

BM cell isolation and culture

DCs were obtained in vitro by growing BM stem cells from tibias and femurs in minimum essential medium (Gibco-BRL, UK), supplemented with 5% fetal calf serum (Gibco-BRL), 5×10^{-5} M 2-mercaptoethanol (Gibco-BRL), 5 mg/ml L-glutamine (Sigma, UK), 100 U/ml penicillin, 100 μg/ml streptomycin (Gibco-BRL), and $NaHCO_3$ (Sigma, UK), and containing 1000 U/ml GM-CSF and 1000 U/ml IL-4. Myeloid progenitor cells were cultured in 24-well plates at 10^6 cells/well. Cultures were fed every 2 days by aspirating off 75% of the medium and adding fresh medium containing cytokines. On day 4 of culture, nonadherent and loosely adherent clusters were transferred to new plates for subsequent culture, and IL-4 was added to a concentration of 3000 U/ml from this time point. In addition to this normal culture of BM-DCs from NOD mice, some BM cells were originally incubated with additional rDcR3.Fc protein at a concentration of 5 μg/ml. GM-CSF and IL-4 used in these experiments were kindly provided by Dr. Tao Mi-Hua of the Institute of Biomedical Science, Academic Sinica (Taipei, Taiwan).

Flow cytometric analysis of cell-surface markers

After 4, 6, or 8 days of culture under different conditions, NOD or BALB/c BM-DCs were harvested, washed, and resuspended at 1×10^6 cells/ml. DCs (2×10^5) were incubated in 100 μl buffer with fluorescein-5-isothiocyanate (FITC)-conjugated monoclonal Ab (mAb) specific for B220, DEC205, CD40, CD80, or CD86 (PharMingen), phycoerythrin-conjugated mAb specific for CD11c or CD54, or allophycocyanin-conjugated mAb for CD86 and the appropriate isotype-matched controls for 30 min on ice. The NOD-specific MHC class II Ab used in the study was anti-I-A^{g7} clone 10-2.16 (purchased from American Type Culture Collection, Manassas, VA), and subsequent staining was performed using FITC-conjugated goat anti-mouse IgG Ab (PharMingen).

T cell proliferation assay

$CD4^+$ T cells were purified as described above. Enriched $CD4^+$ T cells (1×10^5) were seeded in triplicate in a 96-well flat-bottom plate (Falcon, Becton Dickinson) together with titrated numbers of normal or DcR3.Fc-treated BM-DCs. In a glutamate decarboxylase 65 (GAD65)-specific T cell proliferation assay, the synthetic GAD peptides (p247–266) were added in culture wells at the concentration of 15 μg/ml. After 3 days, cell cultures were pulsed with 1 μCi/well ^{3}H-methyl thymidine (Amersham Pharmacia Biotech) overnight for 16 h. The plates were then harvested onto glass fiber, and the incorporated ^{3}H-methyl thymidine was detected with TopCount (Packard, PerkinElmer, Boston, MA).

Cytokine assay

The concentrations of cytokines IL-4, IL-10, IL-13, and IFN-γ secreted by $CD4^+$ T cells cocultured with normal BM-DCs or DcR3.Fc-treated BM-DCs were determined by enzyme-linked immunosorbent assay (ELISA). Briefly, the cultured supernatants were harvested after 48 h, and cytokine ELISAs were performed using purified mAb-coated plates. All procedures followed the standard protocols provided by R&D Systems (Minneapolis, MN). Cytokine concentrations were read with an MRX microplate reader (Dynex Technologies, Chantilly, VA) at 450 nm.

Flow cytometric detection of DcR3.Fc-binding on BM-DC

To detect DcR3.Fc-binding on NOD BM-DC, cells cultured for 6 days after IL-4/GM-CSF treatment were harvested and washed twice in PBS (pH 7.4) containing 1% bovine serum albumin and 0.01% sodium azide. The samples were then incubated with different biotin-conjugated proteins including DcR3.Fc–biotin, LTßR.Fc–biotin, control hIgG1–biotin, or anti-FasL Ab (PharMingen) for 30 min. After two additional washes with PBS buffer, the samples were incubated with streptavidin conjugated with allophycocyanin (PharMingen) for 25 min at 4°C. After a final wash, the cells were analyzed by flow cytometer (Becton Dickinson FACSCalibur with CellQuest software).

Adoptive transfer of DCs and T cells

To investigate the therapeutic potential of DcR3.Fc-treated DCs in autoimmune diabetes, three groups—regular, hIgG-treated, or DcR3.Fc-treated BM-DCs (2×10^5 cells/group)—on day 8 of culture were injected intravenously (i.v.) into 8-week-old NOD/SCID. One week after DC transfer, mice subsequently received 2×10^7 splenocytes isolated from 12-week-old NOD mice. After T cell transfer, mice were then checked for blood sugar levels and glycosuria every week using Optium detection kit (Abbott Laboratories, MediSense Products, Abbott Park, IL) and Chemstrip UG (Boehringer Mannheim, Indianapolis, IN), respectively. When animals were positive (≥250 mg/dl) in two consecutive blood tests, they were scored as diabetic.

2D gel electrophoresis and image analysis

BM-DC cell pellets (5×10^6 cells) were solubilized in lysis buffer containing 7 M urea (Boehringer Mannheim, Germany), 2 M thiourea (Aldrich, Milwaukee,

WI), and 4% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonate (CHAPS; J. T. Baker, Phillipsburg, NJ). After sonication, 1 mg total protein was loaded onto immobilized pH gradient (IPG) gel strips (pH 3–10, 18 cm long; Amersham Pharmacia Biotech) that had been rehydrated overnight before use in a solution of 7 M urea, 2 M thiourea, 4% CHAPS, 4 mM Tris base, 2% IPG ampholyte, 65 mM dithioerythritol (DTE), and 0.0002% bromophenol blue. For separation in the first dimension, isoelectric focusing was performed using the IPGphor system (Amersham Pharmacia Biotech) at 20°C and 8000 V for a total of 75 kVh. After isoelectric focusing, the IPG strips were equilibrated for 15 min in equilibration solution [50 mM Tris-HCl, pH 8.8, 6 M urea, 2% sodium dodecyl sulfate (SDS), 30% glycerol, 2% DTE] and were then attached with 0.5% agarose to the top of a vertical 10–15% linear gradient SDS-polyacrylamide gel. Second-dimensional electrophoresis was performed with a Protean II multicell (Bio-Rad, Hercules, CA) at 45 mA per gel for 5 h until the bromophenol blue reached the bottom of the gel. The gels were fixed in 10% methanol and 7% acetic acid for 30 min, stained in 350 ml Sypro Ruby Protein gel-stain solution overnight, and then soaked in deionized water for 20 min to wash residual dye from the polyacrylamide matrix. The developed gels were digitally scanned as 2D images using a Typhoon 9200 fluorescence image scanner (Amersham Pharmacia Biotech) and were then analyzed using ImageMaster software (Amersham Pharmacia Biotech) to detect and quantify protein spots automatically. Intensity levels were normalized between gels as a proportion of the total protein intensity detected for the entire gel. Normalized protein intensity data for the matched spots with a twofold difference were exported to Gel Report (Amersham Pharmacia Biotech).

In-gel digestion and MALDI–TOF–mass spectrometry (MS)

From the 2D gel analysis of control and induced samples, MS selected differentially expressed proteins for further identification. These spots were cut from the 2D gels, sliced into 1–2 mm^2 pieces, and then destained three times with 23 mM ammonium bicarbonate buffer (pH 8.0) in 50% acetonitrile for 15 min. The gel pieces were dehydrated in 100% acetonitrile for 5 min and then dried for 20 min in a vacuum centrifuge. Enzymatic digestion was achieved by adding 15 μl trypsin in 25 mM ammonium bicarbonate to a final concentration of 0.0225 μg per sample at 37°C for 16 h. Peptide fragments were extracted twice with 50 μl 50% acetonitrile–0.1% trifluoroacetic acid. Acetonitrile was removed by centrifugation under vacuum, and the peptides were concentrated using C18 Zip-Tip, eluted with 2 μl 100% acetonitrile, and directly spotted onto the sample plate of a MALDI–TOF–MS. Finally, 0.5 μl α-cyano-4-hydroxycinnamic acid (10 mg/ml) was applied to each spot, and the spots were air-dried at room temperature before mass spectra were acquired (M@LDI™, Micromass, Manchester, UK). The resultant peptide masses were matched with the theoretical peptide masses of all proteins from all species in the SWISS-PROT database using Masslynx 3.4 software. Peptide mass mapping is particularly successful for the identification of proteins, as described in the literature [26–28], so we chose this method to identify proteins. Our protein selection criteria were a match of at least four fragments from a single 2D gel spot against a single protein sequence entry in the database; a high coverage value; and a sequence of mouse origin. This protein was then considered as a candidate [29].

RESULTS

Characterization of DcR3.Fc protein

To investigate the potential effects of DcR3 on NOD BM-DCs, we produced and purified rDcR3.Fc protein from a baculoviral expression system and further characterized its biochemical and biological properties. Results from Western blot analysis probed with anti-DcR3 Ab (**Fig. 1A**, lane 2) or anti-hIgG1 Ab (Fig. 1A, lane 3) indicate that the specific signal of this fusion protein is approximately 62 kDa. As soluble DcR3 binds specifically to FasL and LIGHT and potentially inhibits the cytotoxicity induced by their receptor/ligand interactions, we tested whether purified DcR3.Fc functionally blocks the activation-induced death of Jurkat cells and $CD4^+$ cells. Our results demonstrate that the DcR3.Fc fusion protein inhibits PMA/ionomycin-induced Jurkat cell apoptosis (Fig. 1B, $P<0.05$) and anti-CD3-induced $CD4^+$ T cell apoptosis (Fig. 1C, $P<0.05$) to a degree similar to that induced by soluble Fas.Fc. To confirm further the binding specificity of DcR3.Fc to FasL and LIGHT, we also performed immunoprecipitation experiments in which DcR3.Fc precipitated FasL and LIGHT (data not shown).

Microscopic examination of BM-DCs cultured with DcR3.Fc

To check whether DcR3 affects the differentiation and maturation of BM-DCs from NOD mice, we examined the growth and morphology of BM cells incubated with DcR3.Fc. The myeloid progenitors from NOD BM cells were cultured with GM-CSF and IL-4 to induce differentiation into DCs. As microscopically observed on day 4, loosely adherent aggregates of growing DCs were apparent in normal and DcR3.Fc-treated, cultured cells (data not shown). On day 8, most of the nonadherent cells in both groups showed veil- or sheet-like processes (**Fig. 2**). In the presence or absence of DcR3.Fc, the differentiated BM-DCs revealed the fine dendrites that are morphologically typical of DCs. Pictures in Figure 2 clearly show the typical membranous or spine-like projections in regular cultured (Fig. 2C) or DcR3-incubated DCs (Fig. 2D). As DcR3 caused an up-regulation of several B cell-related genes (data presented in **Table 1**) and altered the differentiation and maturation in NOD BM cultures, we characterized further the cells generated in the presence of DcR3 with additional cell-specific markers. More specifically, flow cytometric analysis with B cell-specific markers revealed that a low percentage of regular (less than 3%) or DcR3-treated cells (less than 4%) are B220- or CD19-positive, indicating that DcR3 does not drive BM cell differentiation to B cells. In addition, flow cytometric characterization of cultured cells with macrophage/monocyte- or granulocyte-restricted markers, e.g., CD23, CD32, revealed that a low percentage of cells (less than 6%) expressed those markers. To further confirm the characteristics of DCs, we stained cultured cells with DC-specific marker DEC205 on day 8. The result revealed that the expression of DEC205 in two groups of cells is quite similar (Fig. 2, E and F), indicating that in the presence or absence of DcR3, GM-CSF- and IL-4-driven NOD BM cells mainly differentiated into DCs, consistent with their culture characteristics, morphological analysis, and cell-surface marker expression. However, DcR3.Fc-treated BM-DCs grew slowly, with a significant reduction in cell number and cluster size relative to cells to which DcR3.Fc was not added (Fig. 2, B and D). To address this point, we stained the cultured cells with Annexin V and PI at different time courses. The percentage of apoptotic cells in both groups at different time points is quite similar (data not shown), indicating that DcR3 does not cause excessive cell death during the culture course. This result also suggests that DcR3 may interfere with the GM-CSF/IL-4-mediated expansion of cultured BM-DCs. This result indicates that DcR3 interferes with the growth of differentiating BM-DCs from NOD mice and is consistent with our previous finding that DcR3 affects the growth and maturation of human $CD14^+$ monocyte-derived DCs [14].

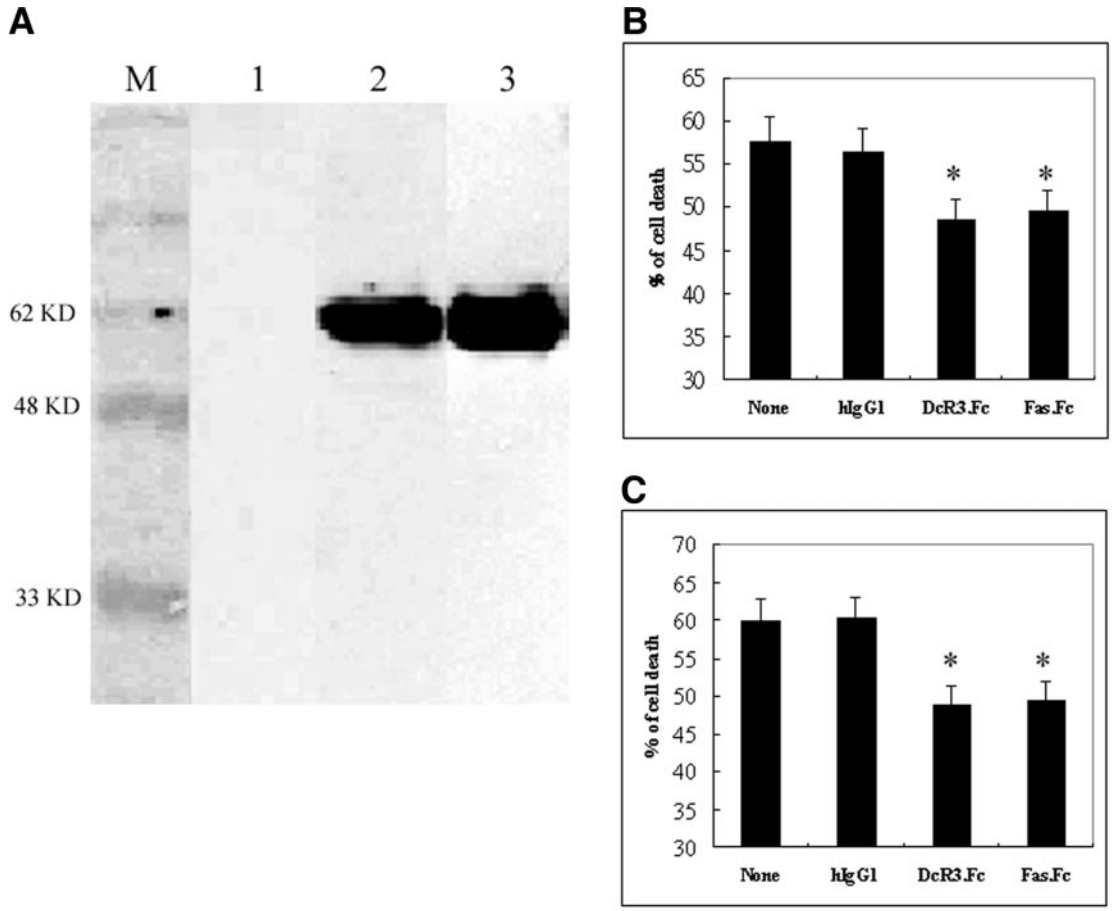

Fig. 1. Characterization of DcR3.Fc protein. (A) Western blot analysis of DcR3.Fc protein. rDcR3.Fc fusion protein was produced from a baculoviral expression system and analyzed by a Western blot probed with anti-DcR3 Ab (lane 2) or anti-hIgG Ab (lane 3). Lane 1 is a negative control probed with anti-LTßR Ab. (B and C) Inhibition of activation-induced apoptosis in Jurkat cells and $CD4^+$ T cells by DcR3.Fc. Jurkat cells (10^5/well) were stimulated with PMA (30 ng/ml) and ionomycin (2 μM) for 20 h (B), and $CD4^+$ T cells (5×10^5/well) were activated with immobilized anti-CD3 (0.3 μg/ml) for 5 days (C) in 96-well microtiter plates, followed by restimulation for 20 h in the presence of Fas.Fc (10 μg/ml), DcR3.Fc (10 μg/ml), or hIgG1 (10 μg/ml). PI staining and flow cytometric analysis determined percentage cell death.

Modulatory effects of DcR3 on surface-marker expression in cultured BM-DCs

We previously demonstrated that DcR3.Fc but not LTßR.Fc, Fas.Fc, or hIgG1 affects the differentiation and maturation of human $CD14^+$ monocyte-derived DCs by modulating surface-marker expression and subsequent T cell stimulation [14]. In this study, we further investigated whether DcR3 similarly modulates the differentiation and maturation of BM-DCs from NOD mice. Results of flow cytometric analysis indicate that the expression of cell-surface markers changed in NOD BM-DCs when incubated with DcR3.Fc. The expression of CD11c, CD40, CD54, and MHC I-A^{g7} (**Fig. 3, A, E, F,** and **D**, respectively) was reduced in cells cultured with additional DcR3.Fc compared with DCs incubated with normal GM-CSF and IL-4, indicating that DcR3 interferes with the differentiation and maturation of BM-DCs from NOD mice. However, the down-regulation of class II MHC or CD40 is not very significant, especially compared with that of monocyte-derived DCs treated with DcR3.Fc in our previous publication [14]. It is likely that the discrepancy between these two results is a result of the different nature of BM-DCs and monocyte-derived DCs and/or intrinsic property of DCs in NOD strain. However, to further back-up the down- or up-regulation of these markers, we analyzed the mean fluorescence intensity (MFI) of those markers on cultured cells (**Table 2**). One of the most striking effects of DcR3.Fc on the differentiation of DCs was the up-regulation of CD86 and the down-regulation of CD80 (Fig. 3, C and B, respectively; Table 2), suggesting a modulatory potential to skew the T cell response toward the Th2 phenotype. This effect induced by DcR3.Fc on NOD BM-DCs is very similar to that induced in human monocyte-derived DCs, suggesting that DcR3 has a broad modulatory effect on the differentiation of different kinds of DCs.

Binding of DcR3.Fc on BM-DCs

In this study, we found that DcR3.Fc but not LTßR.Fc or hIgG1 modulates the expression of several surface molecules on BM-DCs. We previously demonstrated that DcR3.Fc but not LTßR.Fc binds freshly isolated $CD14^+$ monocytes and that anti-LIGHT, anti-LTα, and anti-FasL Ab do not bind these cells. This suggests that DcR3.Fc interacts with a surface molecule on $CD14^+$ monocytes that is distinct from LIGHT, the membrane form of LT, and FasL.

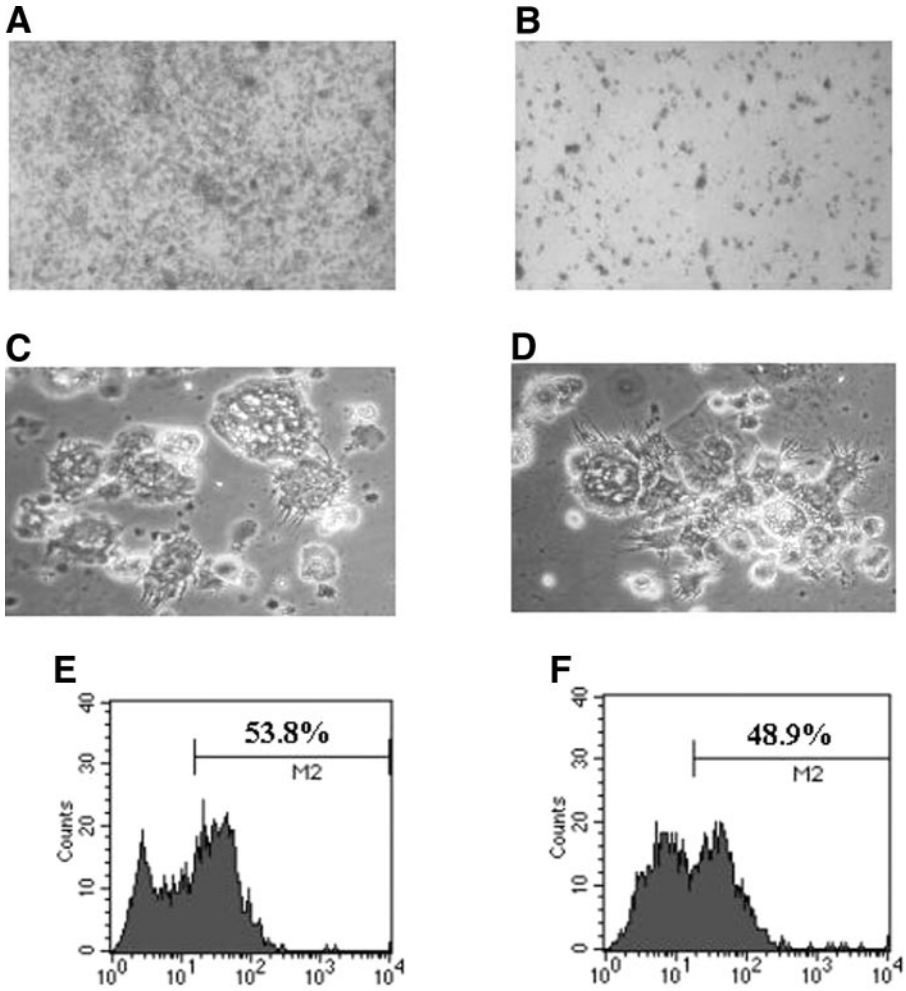

Fig. 2. Microscopic examination of BM-DCs from NOD mice. BM cells from 8-week-old NOD mice were cultured with IL-4 and GM-CSF (A and C) or incubated with additional DcR3.Fc (B and D). On day 8, cells were observed microscopically (original magnification, 40×, A and B; original magnification, 200×, C and D). Expression of DEC205 on cultured cells was further characterized by flow cytometic analysis (E, cells cultured with IL-4/GM-CSF; F, cells cultured with additional DcR3.Fc).

To address this finding further, we used biotin-conjugated DcR3.Fc, LTßR.Fc, or Fas.Fc fusion proteins as well as anti-FasL and anti-LIGHT Ab to stain BM-DCs from NOD mice. As shown in **Figure 4A**, strong fluorescence was detected in cultured BM-DCs stained with DcR3.Fc–biotin and subsequently with streptavidin–allophycocyanin, whereas LTßR.Fc–biotin only detected a weak signal on those cells. No signal was detected on cells stained with Fas.Fc–biotin (data not shown) or anti-FasL (Fig. 4B), suggesting that FasL plays no role in the DcR3-driven differentiation and maturation of BM-DCs. In previous studies, we demonstrated that LIGHT is expressed on the surfaces of immature human DCs but is lost from mature DCs and is barely detectable by LTßR.Fc [14]. Whereas the strong signal did not change in cells stained with DcR3.Fc–biotin when those cells were treated with LPS, the weak signal detected in cultured cells stained with LTßR.Fc–biotin disappeared (data not shown). From these results, we conclude that the molecule detected by DcR3.Fc on mature DCs is distinct from LIGHT, indicating that DcR3 binds to LIGHT and an unidentified novel ligand in immature DCs.

Functional analysis of DcR3.Fc-treated BM-DCs

DcR3 is up-regulated in certain tumors [18, 22], and tumor-associated DCs usually have lower T cell-stimulatory potentials [30]. Moreover, DcR3.Fc modulates the expression of cell-surface molecules, which are important for Ag processing and presentation. We therefore investigated the potential of DcR3.Fc-treated BM-DCs to modulate T cell proliferation and function. As accumulating evidence indicated that DCs are capable of inducing T cell activation and proliferation through an Ag-independent interaction with T cells [31], we thus investigated this idea in our NOD BM-DCs. As shown in **Figure 5A**, irradiated BM-DCs, in the absence of exogenous Ag, had a much stronger stimulatory effect on autologous T cell proliferation (Fig. 5A, lane 3) than did irradiated splenocytes (Fig. 5A, lane 2). This result supports the idea that DCs, with their high density of surface MHC class II and costimulatory-adhesive molecules, represent the most potent APCs signaling T cells in the absence of exogenous Ag. However, BM-DCs incubated with DcR3 down-regulated this autologous T cell proliferation (Fig. 5A, lane 4) compared with that of normal BM-DCs. This result is also consistent with our previous finding that the proliferation of $CD4^+$ T cells cocultured with allogenic DCs treated with DcR3 was significantly reduced [14]. We also performed the GAD65-specific T cell proliferation assay in this system. The immune response against GAD65, a key target auto-Ag of IDDM, is mediated by $CD4^+$ T cells and is highly correlated with the diabetogenic process in NOD mice. As we expected, the proliferative response of T cells stimulated with GAD65-incubated splenocytes increased (Fig. 5B, lane

TABLE 1. Identification by MS of the Protein Spots During DcR3.Fc-Treated DCs

Spot #	Identification	NCBI accession no.	Match	Coverage (%)	pI	Mw (kDa)
Up-regulated						
1	Antihuman ovarian carcinoma antibody heavy chain variable region	2828739	4/8	50	13.2	8.0
2	Chitinase-related membrane complement regulatory protein	1336166	4/8	31	28.8	5.1
3	Telomerase-binding protein, p23	12846222	5/9	30	27.6	8.9
4	MAPK p38 β	6273387	5/9	42	35.8	6.5
5	Reduced nicotinamide adenine dinucleotide-ubiquinone oxidoreductase	20178012	5/9	33	27.3	7.0
6	mAb heavy chain	2906094	4/7	46	15.1	9.2
7	Hypothetical protein MGC7474	13529659	5/9	43	25.8	9.3
8	Ig heavy chain	293448	4/7	63	10.3	9.2
9	Homolog to retinal dehydrogenase type I	12841680	4/8	37	18.2	9.8
10	Dehydrogenase anti-DNA Ig light chain IgG	1870294	4/8	45	10.6	8.6
11	Ig heavy chain variable region	15407470	4/8	54	13.0	9.1
12	Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	6679937	8/16	33	35.8	8.4
13	Aldehyde reductase	12847479	4/8	26	36.6	6.9
14	Similar to protein disulfide isomerase-related protein	13905146	7/13	31	48.0	5.0
15	Similar to neurofibromatosis 2	13529410	5/10	28	41.5	8.6
16	Ras-GTPase-activating protein Src Homology (SH3) domain-binding protein	7305075	5/10	25	51.8	5.4
17	Similar to protein tyrosine phosphatase, receptor-type, F-interacting protein, binding protein 2	13278277	8/16	29	48.7	9.2
18	Cyclin-dependent kinase 6	5453257	5/10	30	32.8	6.2
19	Vimentin	2078001	17/34	49	51.6	5.0
20	Protein disulfide isomerase precursor	129729	13/26	32	57.1	4.8
21	Vimentin	2078001	18/36	55	51.6	5.0
22	Adenosine 5′-triphosphate (ATP)-dependent cecal ligation and puncture (CLP) protease ATP-binding subunit ClpX-like, mitochondrial precursor	14916542	8/16	21	69.3	8.1
23	ATP-dependent DNA helicase II, 80 kDa subunit	125732	8/16	18	83.3	5.1
24	Signal-induced proliferation-associated gene 1	6755520	6/12	15	75.1	5.6
25	Betaine-homocysteine methyltransferase	7709990	9/18	29	45.0	8.0
Down-regulated						
26	IL-17 precursor	2498483	4/8	44	16.9	8.9
27	Fatty acid-binding protein 5	4557581	5/10	45	11.0	6.1
28	T cell receptor-α (TCR-α) chain c region	135512	4/8	56	15.5	4.5
29	TANK-binding kinase 1	9790253	4/8	29	15.7	5.6
30	Retinol-binding protein 4	20888903	4/8	40	23.2	5.7
31	Adenylate kinase isoenzyme 1	13959400	4/8	31	21.5	5.7
32	Glutathione *S*-transferase μ3	121720	4/8	29	25.8	7.6
33	Golgi SNAP	14250239	5/10	26	28.5	9.3
34	α-Enolase	12963491	13/24	38	47.1	6.4
35	Phosphatidylinositol-4-phosphate 5-kinase type II α	6760471	5/10	25	46.2	6.5

pI, isoelectric point; NCBI, National Center for Biotechnology Information.

2) compared with that of splenocytes without the incubation of GAD65 (Fig. 5A, lane 2). However, irradiated BM-DCs, in the presence of exogenous Ag, GAD65, still showed a much stronger stimulatory effect on $CD4^+$ T cell proliferation (Fig. 5B, lane 3) than did irradiated splenocytes incubated with the same Ag. It is interesting that in the presence of GAD65, irradiated DCs showed quite a similar stimulatory effect on T cell proliferation compared with DCs without GAD65. We assumed that the experimented DCs on day 8 were relatively mature, even in the presence of GAD65, as they gradually lost the ability of Ag-up-taking and processing. Similarly, in the presence of Ag, DCs incubated with DcR3 still down-regulated this auto-Ag-specific T cell proliferation (Fig. 5B, lane 4) compared with that of normal BM-DCs (Fig. 5B, lane 3). In addition to its suppressive effect on T cell proliferation, DcR3.Fc also modulated the secretion of IFN-γ from naïve $CD4^+$ T cells cocultured with DcR3.Fc-treated BM-DCs. The secretion of IFN-γ from T cells stimulated with BM-DCs that had been pretreated with DcR3.Fc is much lower than that from T cells incubated with normally cultured DCs (**Fig. 6A**). This is also consistent with our previous finding that $CD14^+$ monocyte-derived DCs treated with DcR3.Fc down-regulated the secretion of IFN-γ from $CD4^+$ T cells in allogenic MLR. Despite the presence of IFN-γ, the secretion of IL-10 (Fig. 6B), IL-13 (Fig. 6C), and IL-4 (Fig. 6D) from T cells incubated with the two types of DCs did not differ. Unexpectedly, the secretion of IL-4 from T cells stimulated with DcR3.Fc-treated DCs did not increase, suggesting an intrinsic defect in IL-4 production in NOD mice. To investigate this point further, we performed the similar experiment in a non-NOD mouse strain. Results shown in **Figure 7D** indicated that the

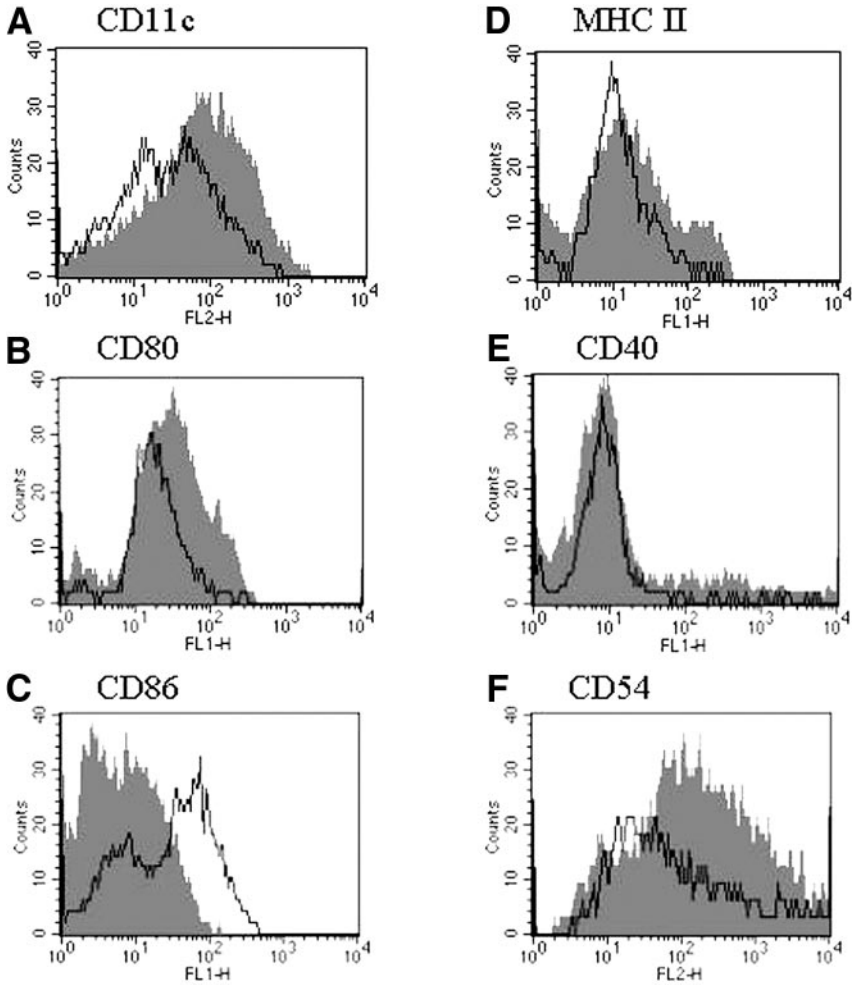

Fig. 3. Flow cytometric analysis of BM-DCs generated in the presence or absence of DcR3.Fc protein. Flow cytometric analysis was used to characterize the expression of CD11c (A), CD80 (B), CD86 (C), MHC class II I-A^{g7} (D), CD40 (E), and CD54 (F) by DCs treated with (open histograms) or without (solid histograms) DcR3.Fc at day 8 of culture. Composite data are from two of four representative experiments. Compared with DCs incubated only with GM-CSF and IL-4, the expression of CD11c (A), CD40 (E), CD54 (F), CD80 (B), and MHC I-A^{g7} (D) was reduced in cells cultured with additional DcR3.Fc, but the expression of CD86 (C) in these cells was up-regulated.

secretion of IL-4 from $CD4^+$ T cells incubated with DcR3.Fc-treated DCs in a BALB/c background is significantly increased ($P<0.01$), demonstrating that the failure of IL-4 up-regulation in NOD mice is an intrinsic feature of this strain.

Therapeutic potential of DcR3.Fc-treated DCs

To test the idea that the strong effects of DcR3.Fc-treated DCs on the down-regulation of IFN-γ secretion by NOD T cells may provide a potential basis for clinical application, we performed DC and T cell transfer experiments. NOD/SCID mice that received DcR3.Fc-treated DCs and subsequently, autoreactive T cells showed delayed onset of diabetes and a decrease in diabetic frequency compared with NOD/SCID mice that received normal DCs and T cells (**Fig. 8**, $P<0.05$). Forty-one days after T cell transfer, 60% of mice that originally received regular DCs developed diabetes. However, none of the mice that originally received DcR3.Fc-treated DCs and subsequently, T cells developed diabetes at this time. All mice that received regular DCs developed diabetes 61 days after T cell transfer, and less than 40% of mice that received DcR3.Fc-treated DCs developed diabetes. This result suggests a future therapeutic potential of DcR3-modulated DCs in autoimmune diabetes.

TABLE 2. MFI of Surface Markers on Regular and DcR3.Fc-Treated DCs*

Markers	Regular DC	DcR3-DC
CD11c	163 ± 33	85 ± 15
CD80	70 ± 11	50 ± 8
CD86	45 ± 15	70 ± 10
CD40	256 ± 75	171 ± 45
CD54	300 ± 21	200 ± 35
MHC II	95 ± 10	74 ± 8

* NOD BM-DCs were cultured in the presence of GM-CSF and IL-4 (regular DC) or in conjunction with 5 μg/ml DcR3.Fc. After 8 days, the cultured cells were collected and stained with various Ab. The expression of surface markers was analyzed by flow cytometry, and the MFI was calculated by CellQuest software (BD Biosciences, San Jose, CA). The data represent the mean ± SD from three independent experiments.

Proteomic analysis of proteins differentially expressed in normal and DcR3.Fc-treated BM-DCs

DC differentiation from naïve BM precursors is a tightly controlled process. To investigate further the modulatory effects of DcR3 on the expression of potential differentiation factors in BM-DCs, we performed 2D gel electrophoresis and MALDI–TOF–MS analysis to characterize the proteins differentially expressed in normal and DcR3.Fc-treated BM-DCs. Three independent culture experiments were performed, and the dif-

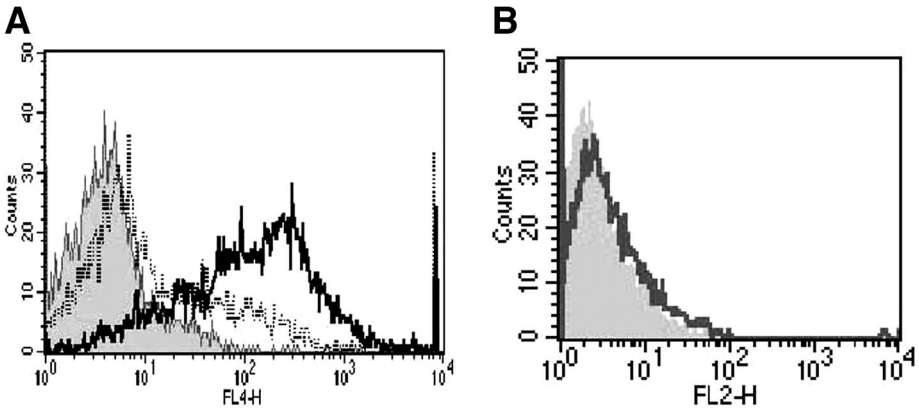

Fig. 4. DcR3.Fc-binding on BM-DCs from NOD mice. Flow cytometric analysis of DcR3.Fc-binding on BM-DCs cultured with GM-CSF and IL-4 for 6 days. (A) The primary staining reagents were DcR3.Fc–biotin (solid line), LTßR.Fc–biotin (dashed line), or hIgG1–biotin (solid histograms), and the secondary reagent was streptavidin conjugated with allophycocyanin. (B) DCs were cultured for 6 days and stained with anti-FasL Ab (open histogram) or Ig-matched control Ab (solid histogram).

ferential gene expression in the two groups of DCs was determined at the protein level on day 8. We identified a subset of genes that differed in their expression levels under DcR3.Fc treatment. More than 500 distinct protein spots were identified in each independent experiment (**Fig. 9**). Thirty-five spots (approximately 7%) were quantitatively different in DcR3.Fc-treated (Fig. 9, left panel) and normal DCs (Fig. 9, right panel). Differentially expressed proteins were further identified by MS and found to represent genes encoding secreted proteins as well as genes involved in cell growth, signaling, and lipid metabolism (Table 1). These data show the up-regulation of 25 proteins, including MAPK p38 β, cyclin-dependent kinase 6, ATP-dependent DNA helicase II, and signal-induced proliferation-associated gene 1, and the down-regulation of 10 proteins, including the IL-17 precursor, TANK-binding kinase 1, and Golgi SNAP, in cells incubated with DcR3.Fc, confirming that DcR3 has a modulatory effect on the differentiation and function of DCs.

DISCUSSION

DcR3, a decoy receptor capable of neutralizing FasL, LIGHT, and TL1A, is overexpressed in tumor cells originating from the gastrointestinal tract or the pulmonary system [18, 22]. Therefore, it has been postulated that DcR3 may play an important role in suppressing host immunity by neutralizing the cytotoxic effects of FasL and LIGHT. Tumor-associated DCs usually have a low T cell-stimulatory capacity [4]. Therefore, we investigated whether DcR3 modulates the T cell-immune response through its initial action on DCs, in addition to directly binding to FasL and LIGHT on activated T cells. A recent report indicated that rDcR3 and DcR3.Fc fusion proteins interact with LIGHT with the same binding affinity and have the same inhibitory effects on the development of cytotoxic T lymphocytes (CTL) in mice, suggesting that the biological effects of DcR3.Fc are equivalent to those of rDcR3 protein [32]. In those studies, the kinetics of binding of LIGHT to the Fc and non-Fc versions of DcR3 was determined by biospecific interaction analysis. The equilibrium constant (Keg) for LIGHT binding to DcR3 or to DcR3.Fc revealed no statistically significant difference, and the experimental data of LIGHT/DcR3 and LIGHT/DcR3.Fc fit well to a 1:1 binding model. Therefore, the immunomodulatory effects of DcR3.Fc observed in this study should reflect the functions of DcR3. The results presented in this study demonstrate that DcR3.Fc modulates NOD BM-DC differentiation and directs its subsequent action on T cells. Apparently, this modulation occurs via an interacting

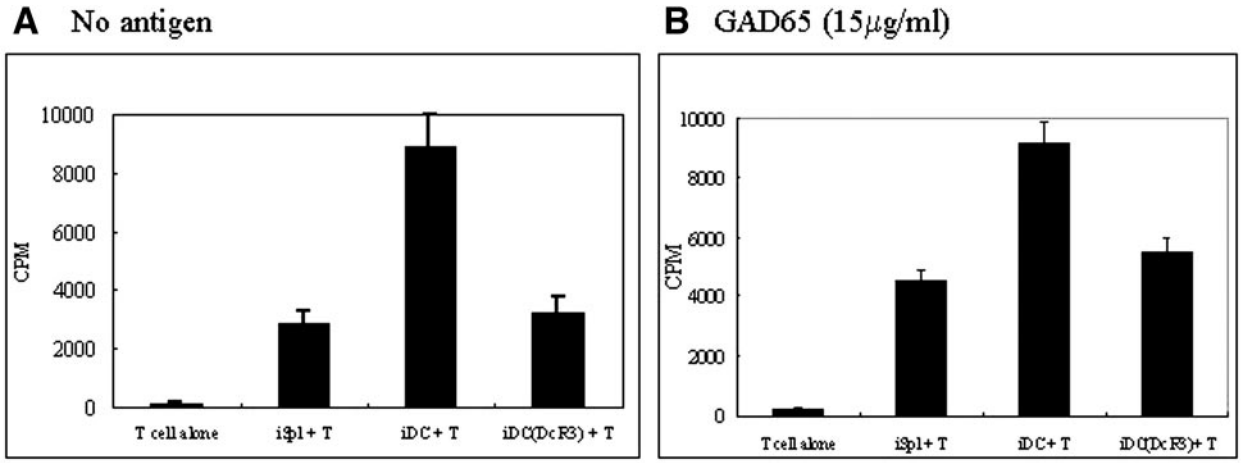

Fig. 5. Modulatory effect of DcR3.Fc-treated DCs on T cell proliferation. Splenic $CD4^+$ T cells were stimulated with irradiated syngenic splenocytes (iSpl + T; A, lane 2), normal cultured DCs (iDC + T; A, lane 3), or DcR3.Fc-treated DCs [iDC(DcR3) + T; A, lane 4] for 3 days. Similar cultures were set up in the presence of GAD65 peptide at the concentration of 15 μg/ml (B). [^{3}H]Thymidine was added to the cultures during the last 16 h before the cells were harvested on day 3. Results are from one of three representative experiments conducted in triplicate (mean±SD).

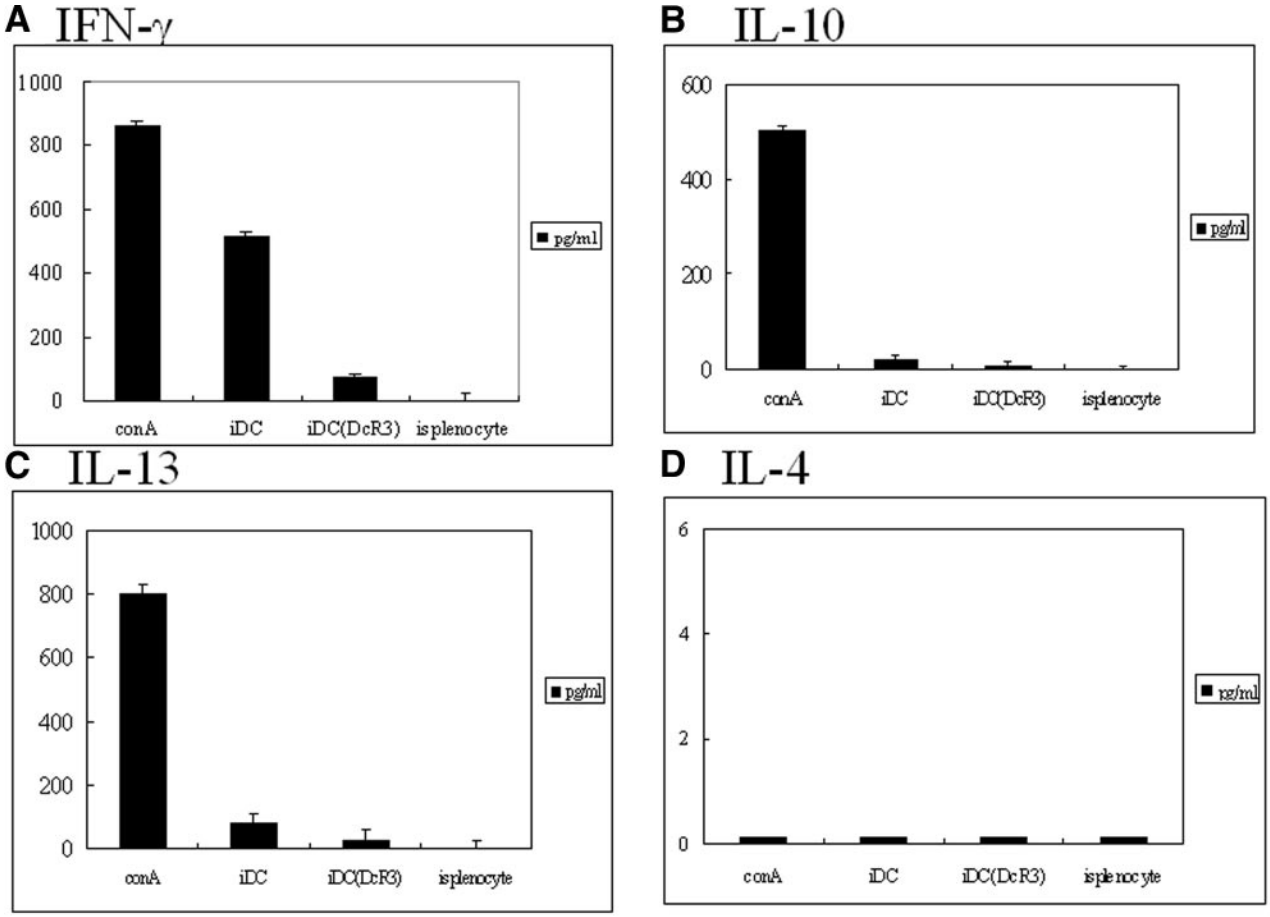

Fig. 6. Modulatory effects of DcR3.Fc-treated DCs on cytokine production in a NOD strain. The secretion of IFN-γ (A), IL-10 (B), IL-13 (C), and IL-4 (D) by NOD $CD4^+$ T cells stimulated with concanavalin A (conA; lane 1) or cocultured with syngenic, irradiated, normal DCs (iDC; lane 2), DcR3-Fc-treated DCs [iDC(DcR3); lane 3], or splenocytes (isplenocyte; lane 4) was detected by ELISA on day 3. Data represent one of three independent experiments conducted in triplicate (mean±SD).

ligand distinct from FasL and LIGHT. These results in BM-DC of the murine (m)NOD system are generally consistent with our previous studies on the human $CD14^+$ monocyte system [14], supporting the hypothesis that a soluble DcR3.Fc fusion protein can trigger "reverse signaling" and that the maturation and function of BM-DCs are thus modulated.

The data presented in Figure 4 suggest that a novel ligand is expressed on DCs other than LIGHT and FasL. Based on the

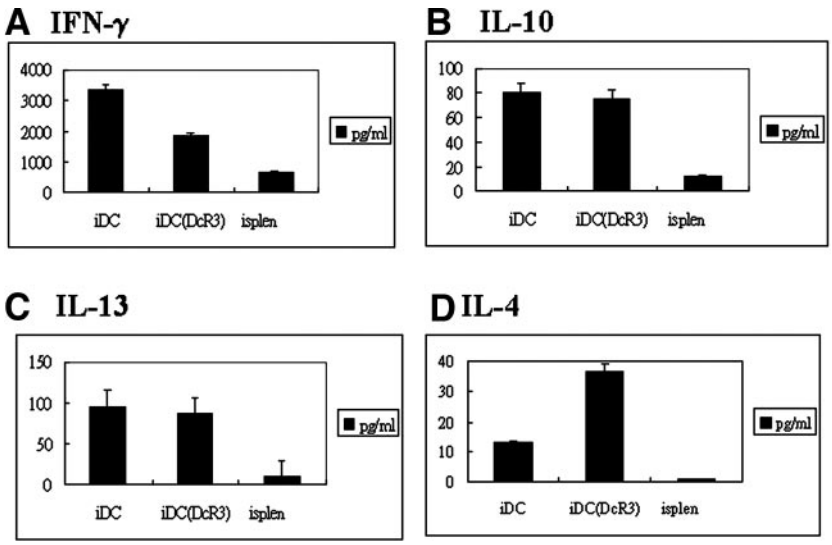

Figure 7. Modulatory effects of DcR3.Fc-treated DCs on cytokine production in BALB/c strain. The secretion of IFN-γ (A), IL-10 (B), IL-13 (C), and IL-4 (D) by BALB/c $CD4^+$ T cells cocultured with syngenic, irradiated, normal DCs (iDC; lane 1), DcR3-Fc-treated DCs [iDC(DcR3); lane 2], or splenocytes (isplen; lane 3) were detected by ELISA on day 3. Data represent one of three independent experiments conducted in triplicate (mean±SD).

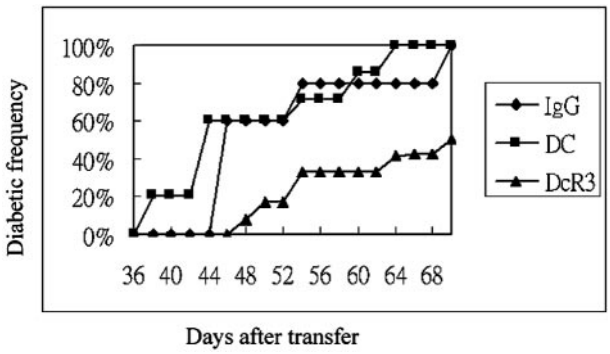

Fig. 8. Adoptive transfer of DCs and T cells. Three groups of BM-DCs—regular (DC), hIgG-treated (IgG), or DcR3.Fc-treated (DcR3; 2×10^5 cells/group)—on day 8 of culture were injected i.v. into 8-week-old NOD/SCID (number of mice for each group: 10, 10, and 12, respectively). One week after DC transfer, mice subsequently received 2×10^7 splenocytes isolated from 12-week-old NOD mice. After T cell transfer, mice were then checked for glycosuria every week. When animals were positive (≥250 mg/dl) in two consecutive urine tests, they were scored as diabetic.

following reasons, we could rule out the possibility that DcR3.Fc–biotin may just be a better reagent than the LTßR.Fc–biotin and further deduce that a novel ligand for DcR3 is expressed on DCs: (1) In this study, we found that DcR3.Fc but not LTßR.Fc or hIgG1 modulates the expression of several surface molecules on BM-DCs. As DcR3.Fc and LTßR.Fc can bind LIGHT, however, only DcR3.Fc modulates DC differentiation, suggesting a biological function-distinct, and LIGHT-independent receptor for DcR3. (2) We previously demonstrated that DcR3.Fc but not LTßR.Fc binds freshly isolated $CD14^+$ monocytes and that anti-LIGHT, anti-LTα, and anti-FasL Ab do not bind these cells. This suggests that DcR3.Fc interacts with a surface molecule on $CD14^+$ monocytes that is distinct from LIGHT, the membrane form of LT, and FasL. (3) We also demonstrated that LIGHT is expressed on the surfaces of immature human DCs but is lost from mature DCs and is barely detectable by LTßR.Fc [14]. Whereas the strong signal did not change in cells stained with DcR3.Fc–biotin when those cells were treated with LPS, the weak signal detected in cultured cells stained with LTßR.Fc–biotin disappeared, indicating that a LIGHT-independent, novel molecule is expressed on culture DCs. (4) It is also unlikely that the DcR3.Fc–biotin may just be a better reagent than the LTßR.Fc–biotin (data presented in Fig. 4), as the data presented in other groups demonstrated that LTßR.Fc and DcR3.Fc bind LIGHT with almost the same fluorescence intensity [19].

The effects of DcR3.Fc on DC differentiation and function in human $CD14^+$ monocytes and NOD BM cells are almost identical in terms of cell-surface marker expression and T cell-stimulating ability, demonstrating the general modulatory potential of this protein on DC differentiation and function. One of the most striking effects of DcR3.Fc on DC differentiation is the up-regulation of CD86/B7.2 and the down-regulation of CD80/B7.1. Cytokines such as IFN-γ up-regulate CD80/B7.1 and CD86/B7.2 expression, whereas IL-10 down-regulates both proteins [33, 34]. Therefore, the unique feature of DcR3-mediated up-regulation of CD86/B7.2 with simultaneous down-regulation of CD80/B7.1 might provide an invaluable tool with which to dissect the mechanisms underlying the differential regulation of CD80/B7.1 and CD86/B7.2 expression. Moreover, it has been demonstrated that although CD80/B7.1 and CD86/B7.2 equivalently costimulate IL-2 and IFN-γ production, CD86/B7.2 preferentially induces greater IL-4 production than CD80/B7.1 [35, 36]. However, in this study, the secretion of IL-4 from T cells incubated with DcR3.Fc-treated DCs in the NOD system was much lower than that from T cells stimulated with DcR3.Fc-treated DCs in the human monocyte system. We previously demonstrated that $CD14^+$ monocyte-derived DCs treated with DcR3.Fc suppressed $CD4^+$ T cell proliferation in allogenic MLR and profoundly up-regulated IL-4 secretion from $CD4^+CD45RA^+$ T cells. NOD mice spontaneously develop autoimmune diabetes, which is characterized by a progressive insulitis followed by selective destruction of β cells in pancreatic islets. Extensive evidence suggests that effector $CD4^+$ T cells, which preferentially secrete IFN-γ and TNF-α (Th1 cells), mediate β cell destruction, at least pre-

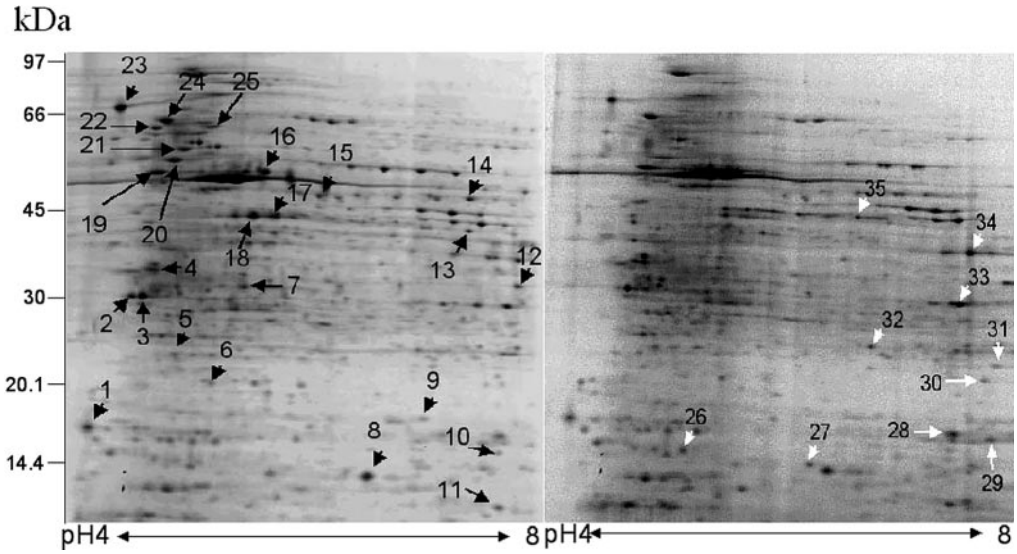

Fig. 9. 2D profiles of NOD BM-DCs. DcR3.Fc-treated (left panel) and normal BM-DCs (right panel) were harvested on day 8 of culture and analyzed by 2D gel electrophoresis. Twenty-five spots, indicated by black arrows (left panel), represent the proteins up-regulated during DcR3.Fc treatment. Ten spots, indicated by white arrows (right panel), represent the proteins down-regulated in DcR3.Fc-treated DCs. These results are representative of three independent experiments.

dominantly [37]. Therefore, the inability of NOD DCs to trigger Th2 effector cells, which secrete IL-4, may contribute to the immunopathogenic process and may have an impact on the breakdown of self-tolerance in autoimmune diabetes. Nevertheless, the strong effects of DcR3.Fc-treated DCs on the down-regulation of IFN-γ secretion by NOD T cells provide a potential basis for clinical application. To test this idea further, we performed DC transfer experiments. NOD/SCID mice that received DcR3.Fc-treated DCs and subsequently, autoreactive T cells showed delayed onset of diabetes and a decrease in diabetic severity compared with NOD/SCID mice that received normal DCs and T cells (Fig. 8). These data are generally consistent with the hypothesis that immature DCs or cytokine (e.g., IL-10)-treated DCs, which express lower levels of MHC proteins or costimulatory molecules and higher levels of the programmed cell death-1 ligand (PD-L1), have a balance of stimulatory versus inhibitory molecules that favors inhibition. They are, therefore, more responsible for the induction of tolerance [38, 39]. The potential role of PD-L1 and its ligands in DcR3-treated, DC-mediated immunomodulation in NOD mice is currently under investigation in our laboratory.

DC differentiation from naïve BM precursor cells is a tightly regulated and highly complicated process influenced by many environmental cytokines. To systematically investigate how DcR3 modulates the differentiation of BM-DCs and their subsequent T cell-stimulatory potential, we took advantage of recently developed proteomic technology to profile changes in gene expression in DcR3.Fc-treated BM-DCs. DCs acquire their function during differentiation, which occurs through the programmed expression of specific proteins. To better identify specific genes involved in the DcR3-mediated modulation of DCs, we performed 2D gel electrophoresis and MALDI–T-OF–MS analysis. This approach, which relies on the quantitative analysis and identification of proteins by proteomic techniques, has additional advantages over the quantitative analysis of mRNA by oligonucleotide microarrays: Proteins represent the most functional compartment of a cell, and the information obtained at the protein level cannot be predicted simply by examining expression at the RNA level; and the proteomics approach can identify post-translational modifications, which may regulate important functions of these proteins. In this study, we identified almost 7% of the proteins shown to be differentially regulated in the two DC groups. These differentially regulated genes were predominantly related to cell-growth control, signaling, the regulation of the immune response, or lipid metabolism. They include some genes previously reported to be modulated during DC differentiation. A large number of additional genes not previously reported in DCs were also expressed during DcR3 modulation.

Immature DCs move from the circulating blood to inflammatory tissues where they take up and process Ag. These cells then migrate to the draining lymphoid organs and become mature with the up-regulation of costimulatory and MHC molecules, which subsequently prime naïve T cells. We previously identified a large number of genes encoding proteins involved in cell adhesion and motility and Ag uptake, processing, and presentation, which are regulated during DC differentiation [40]. For example, the expression of CD11a/lymphocyte function-associated antigen-1a, syndecan 2, CD44E, and presenilin 1, which are involved in cell adhesion and motility, is down-regulated in mature DCs. In contrast, expression of the Fc receptor, MHC class II proteins, and heat shock protein 73, which represent Ag-presentation capacity, is up-regulated in mature DCs, as expected. It is interesting that the expression of most genes involved in cell adhesion and motility or Ag uptake, processing, and presentation, which are regulated during DC differentiation and maturation, did not differ between normal and DcR3.Fc-treated DCs, emphasizing the phenotypic similarity of these two DC groups. However, some genes encoding proteins involved in cell growth, signaling, and metabolism were differentially regulated in DCs after DcR3.Fc treatment. MAPK p38 β is a kinase-phosphorylated protein, which is induced in response to inflammation, stress, and environmental cytokines. Data from kinase assays in our laboratory also demonstrated a significant increase in MAPK p38 activity in DcR3.Fc-incubated DCs treated with LPS (data not shown), suggesting a critical role for this kinase in DC differentiation during DcR3 modulation. As MAPK p38 affects cell-cycle progression and differentiation, and expression of this protein is up-regulated in DCs treated with LPS or other stimuli [41], the up-regulation of this protein in DcR3-modulated DCs may indicate that DcR3 plays a regulatory role in DC differentiation. In addition to MAPK p38, vimentin, GAPDH, cyclin-dependent kinase 6, signal-induced proliferation-associated gene 1, and Ras-GTPase-activating protein SH3 domain-binding protein, which is up-regulated in DcR3.Fc-treated DCs, are all involved in the cell cycle, differentiation, or growth control, indicating a significant effect of DcR3 on DC differentiation and maturation. mIL-17, one of the 10 down-regulated proteins detected in DcR3.Fc-treated DCs, was initially cloned by subtractive hybridization from an activated T cell hybridoma and designated mCTL-associated Ag 8 [42]. IL-17 induces epithelial, endothelial, and fibroblastic cells to activate NF-κB and to produce proinflammatory cytokines such as IL-6, IL-8, GM-CSF, and prostaglandin E2 [43, 44]. It can also induce fibroblasts to support better the growth and differentiation of $CD34^+$ progenitor cells. Therefore, IL-17 is considered to be an inducer of stromal cell proinflammatory and hematopoietic cytokines. Down-regulation of this protein in DcR3.Fc-treated DCs further supports the proposition that DcR3 is negatively modulated in the DC-mediated immune response.

Extensive evidence defines DCs as principal, professional APCs involved in T cell priming. This evidence includes several experiments: On one hand, DCs were compared directly with other APCs, such as B cells and monocytes, in terms of their strong ability to prime alloreactive, naïve TCR transgenic T cells in vitro or to significantly activate and expand Ag-specific, naïve precursors from polyclonal populations [45]. Consistent with these results, our study reveals that NOD BM-DCs have a much stronger stimulatory effect on naïve syngenic $CD4^+$ T cells than on conventional APCs. Conversely, injection of Ag-loaded DCs also induced potent, primary $CD4^+$ and $CD8^+$ T cell responses in vivo [46, 47]. Furthermore, DCs destroy neonatal T cell tolerance [48] and peripheral tolerance against soluble Ag [49], transplantation Ag [50], peripheral tissue Ag [51, 52], and tumor Ag [53, 54]. However, accumulating data also indicate that DCs are involved in central and peripheral tolerance. The presence of

DCs in the thymic medulla suggests that they may participate in the establishment of central tolerance. This point was supported by Brocker et al. [55], who demonstrated that the exclusive expression of MHC class II proteins in DCs was sufficient to delete Vβ-specific T cells through a retrovirally encoded super-Ag. Moreover, several studies reported that thymic [56] and splenic DCs [57–59], in addition to B cells, were involved in Ag presentation after i.v. injections of high doses of tolerogenic Ag. Furthermore, mature DCs from the T cell areas of lymph nodes present self-Ag at the periphery [60, 61], indicating an important role for these cells in the maintenance of tolerance. Finally, DCs also participate in the induction of tolerance against liver allografts [62]. Therefore, the newly identified role of DcR3 in the differentiation and function of BM-DCs in NOD mice further confirms the action of cytokine-modulated DCs in the induction and maintenance of tolerance. This effect of DcR3 in modulating the suppression of Th1 and the restoration or enhancement of the Th2-immune response in NOD mice may provide the basis for future therapeutic applications in autoimmune diabetes.

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