Endocrine Research

# Human Mesenchymal Stem Cells Modulate Cellular Immune Response to Islet Antigen Glutamic Acid Decarboxylase in Type 1 Diabetes

Maria M. Zanone, Enrica Favaro, Ilaria Miceli, Giorgio Grassi, Elisa Camussi, Cristiana Caorsi, Antonio Amoroso, Mirella Giovarelli, Paolo Cavallo Perin, and Giovanni Camussi

Department of Internal Medicine (M.M.Z., E.F., I.M., G.G., E.C., P.C.P., G.C.), Medicine and Experimental Oncology (C.C., M.G.), and Genetics, Biology, and Biochemistry (A.A.), University of Turin, 10126 Turin, Italy

**Context:** Mesenchymal stem cells (MSCs) exert an immunosuppressive effect on the immune system. However, studies on the immunomodulatory potential of MSCs in type 1 diabetes are lacking.

**Objective:** We aimed to evaluate whether human MSCs may inhibit *in vitro* pancreatic islet antigen-specific T cell activation in type 1 diabetes.

**Design:** Human MSCs were isolated and characterized. Peripheral blood mononuclear cells (PBMCs) were obtained from nine type 1 diabetic patients at disease onset and 13 healthy control subjects. IFN-$\gamma$, IL-10, and IL-4 enzyme-linked immunospot responses of lymphocytes incubated with glutamic acid decarboxylase 65 (GAD65) were investigated in PBMC cultures and PBMC/MSC cocultures. Levels of prostaglandin $E_2$ (PGE$_2$), IFN-$\gamma$, IL-4, and IL-10 in supernatants were measured by ELISA. PGE$_2$ inhibition experiments with NS-398 and indomethacin were also performed.

**Results:** Five diabetic patients were identified with a positive PBMC IFN-$\gamma$ response to GAD65 and negative IL-10 and IL-4 response. PBMC/MSC cocultures resulted in a significant decrease in the number of spots and in detection of IL-4-secreting cells. PGE$_2$ inhibitors abrogated the immune-suppressive effect, indicating an involvement of PGE$_2$ production, and the constitutive production of PGE$_2$ by MSCs was enhanced in PBMC/MSC coculture. Moreover, in GAD-responder patients, GAD-stimulated PBMC/MSC cocultures significantly decreased secretion of IFN-$\gamma$ and IL-10 and increased secretion of IL-4.

**Conclusions:** These results provide evidence that human MSCs abrogate *in vitro* a proinflammatory T helper type 1 response to an islet antigenic stimulus in type 1 diabetes. MSCs induce IL-4-producing cells, suggesting a possible switch to an antiinflammatory T helper type 2 signaling of T cells. (*J Clin Endocrinol Metab* 95: 3788–3797, 2010)

It is widely assumed that T lymphocytes play the major role in the autoimmune destruction of islet $\beta$-cells in type 1 diabetes, and several abnormalities of immunophenotype and function of T cells have been highlighted (1–4). Strategies to inhibit T cell activation may allow the designing of new targeted intervention to preserve insulin-producing $\beta$-cells.

Recent studies have generated great research interest on the antiinflammatory and immunomodulatory potential of mesenchymal stem cells (MSCs) (5) beyond their use in regenerative medicine. MSCs can exert an immunoregulatory activity, modulating several T cell functions and exerting a profound immunosuppressive effect on virtually any component of the immune system (6–11). MSCs

Abbreviations: DC2, Dendritic cells type 2; ELISPOT, enzyme-linked immunospot; EPC, endothelial progenitor cell; GAD, glutamic acid decarboxylase; HLA, human leukocyte antigen; IA-2, insulinoma-associated antigen-2; IFN-$\gamma$, interferon-$\gamma$; MSC, mesenchymal stem cell; PBMC, peripheral blood mononuclear cell; PGE$_2$, prostaglandin E$_2$; PMA, phorbol myristate acetate; SI, stimulation index; Th1, T helper type 1.

in fact lack major histocompatibility complex class II molecules and do not express key costimulatory molecules B7-1, B7-2, CD40, and CD40L. Neither apoptosis nor induced T cell anergy is responsible for the MSC-mediated immunosuppressive action. MSCs have been shown to reduce the expression of lymphocyte activation markers, and the analysis of the cytokine profile of dendritic cells, naive and activated T cells, and NK cells indicates the induction of an antiinflammatory phenotype and an increase of the regulatory T cell population (12, 13). The MSC-induced suppression has been ascribed to several soluble factors, including hepatocyte growth factor, TGF-$\beta$1, and prostaglandin $E_2$ ($PGE_2$) (6, 10, 12).

Notably, bone marrow MSCs have been shown not only to inhibit T cell proliferation to polyclonal stimuli but also to inhibit the response of naive and memory antigen-specific T cells to their cognate peptide in mice (14). In this model, MSCs inhibited the antigen-specific proliferation, the IFN-$\gamma$ production, and the cytotoxic activity, suggesting that MSCs prevent T cell activation.

The susceptibility of naive and memory T cells to immunoregulatory stimuli could have profound implications when considering the potential clinical applications of MSCs. *In vivo*, there is evidence that administration of human MSCs improves the outcome of allogeneic transplantation and hampers graft-*vs.*-host disease (15–18). MSCs have also been proposed as a treatment for several autoimmune diseases. Indeed, MSCs have been exploited in a variety of clinical trials aimed at reducing the burden of immune-mediated disease and in experimental animal models of rheumatoid arthritis, systemic lupus erythematosus, and encephalomyelitis (9, 19–23).

Studies on the immunomodulatory potential of MSCs in human type 1 diabetes, a T helper type 1 (Th1)-mediated autoimmune disease, are still lacking. At present, there are reports on the regenerative potential of MSCs in diabetic NOD/SCID mice, in which injection of human MSCs was shown to result in an increased number of pancreatic islets and $\beta$-cells (24). Genetically modified MSCs by recombinant Pdx-1 adenovirus or by nonviral gene transduction were able to express insulin sufficient to reduce blood glucose in the streptozotocin mouse model of diabetes (25, 26). More recently, allogeneic MSCs obtained from diabetes-prone as well as -resistant mice and injected into NOD mice have been shown to delay the onset of diabetes or to reverse hyperglycemia (27).

In the present study, we evaluated whether human bone marrow-derived MSCs may inhibit *in vitro* islet antigen-specific T cell activation in type 1 diabetes. To elucidate the MSCs' immunoregulatory activity, coculture experiments of MSCs with glutamic acid decarboxylase (GAD)-challenged peripheral blood mononuclear cells (PBMCs)

from type 1 diabetic patients at disease onset were performed, and the number of interferon-$\gamma$ (IFN-$\gamma$)-producing T cells and the release of IFN-$\gamma$ were evaluated. To investigate the underlining mechanisms, changes in T cell cytokine profile and the role of $PGE_2$ were explored.

## Subjects and Methods

### Characterization of human MSCs

Human bone marrow cells were obtained by Lonza (Basel, Switzerland). Bone marrow cells were layered on a Ficoll gradient (Sigma-Aldrich, St. Louis, MO) and centrifuged at 1500 rpm for 30 min. The mononucleated cells were cultured in the presence of the MSC basal medium (MSCBM; Lonza). After 5 d culture, the medium was changed. To expand the isolated cells, the adherent monolayer was detached by trypsin after 15 d for the first passage and every 7 d for successive passages. Cells were seeded at a density of 10,000 cells/cm² and used within the passage 6. All experiments were performed using a single batch of MSCs (donor 1). In the presence of a positive IFN-$\gamma$ response to GAD65, experiments were repeated using a MSC batch from a different bone marrow donor (donor 2).

At each passage, cells were counted and analyzed for immunophenotype by cytofluorimetric analysis, performed as described (28), and indirect immunofluorescence, performed using mouse monoclonal antivimentin (Sigma) and rabbit polyclonal anti-von Willebrand factor (Dakocytomation, Copenhagen, Denmark) antibodies. Alexa Fluor 488 antirabbit and antimouse Texas Red (Molecular Probes, Leiden, The Netherlands) were used as secondary antibodies.

MSC preparations did not express hematopoietic markers like CD45, CD14, and CD34; the costimulatory molecules (CD80, CD86, and CD40); and the endothelial markers (CD31, von Willebrand Factor, and kinase domain receptor). All cell preparations at different passages of culture expressed the typical MSC markers: CD105, CD73, CD44, CD90, CD166 and CD146. They also expressed human leukocyte antigen (HLA) class I but not class II, both at baseline and after stimulation with 100 IU/ml IFN-$\gamma$ for 24, 48, or 72 h (Fig. 1A).

MSCs were shown to undergo adipogenic, osteogenic, and chondrogenic differentiation as previously described (29).

### Subjects and cytokine enzyme-linked immunospot (ELISPOT) analyses

Fresh heparinized blood samples were obtained from nine Caucasian new-onset adult (mean age 25.5 ± 7 yr) type 1 diabetic patients with acute onset of symptoms requiring permanent insulin treatment from the time of diagnosis (30), recruited from the Diabetes Registry of the province of Turin, Italy. Blood was drawn within 3 months from diagnosis. All patients had metabolically controlled disease, were free of recent (<2 wk) infectious or inflammatory conditions. Patients were HLA-DRB1 typed by PCR-Sequence Specific Oligonucleotides methods (LabType; One Lambda, Canoga Park, CA), screened for islet cell antibodies by indirect immunofluorescence and GAD and insulinoma-associated antigen-2 (IA-2) autoantibodies by RIA (Medipan Gmbh, Dahlewitz/Berlin, Germany and Euroimmun, Lübeck, Germany, respectively) (Table 1).

Thirteen age-matched healthy Caucasian nondiabetic subjects without a family history of diabetes (mean age 30 ± 4.9 yr)
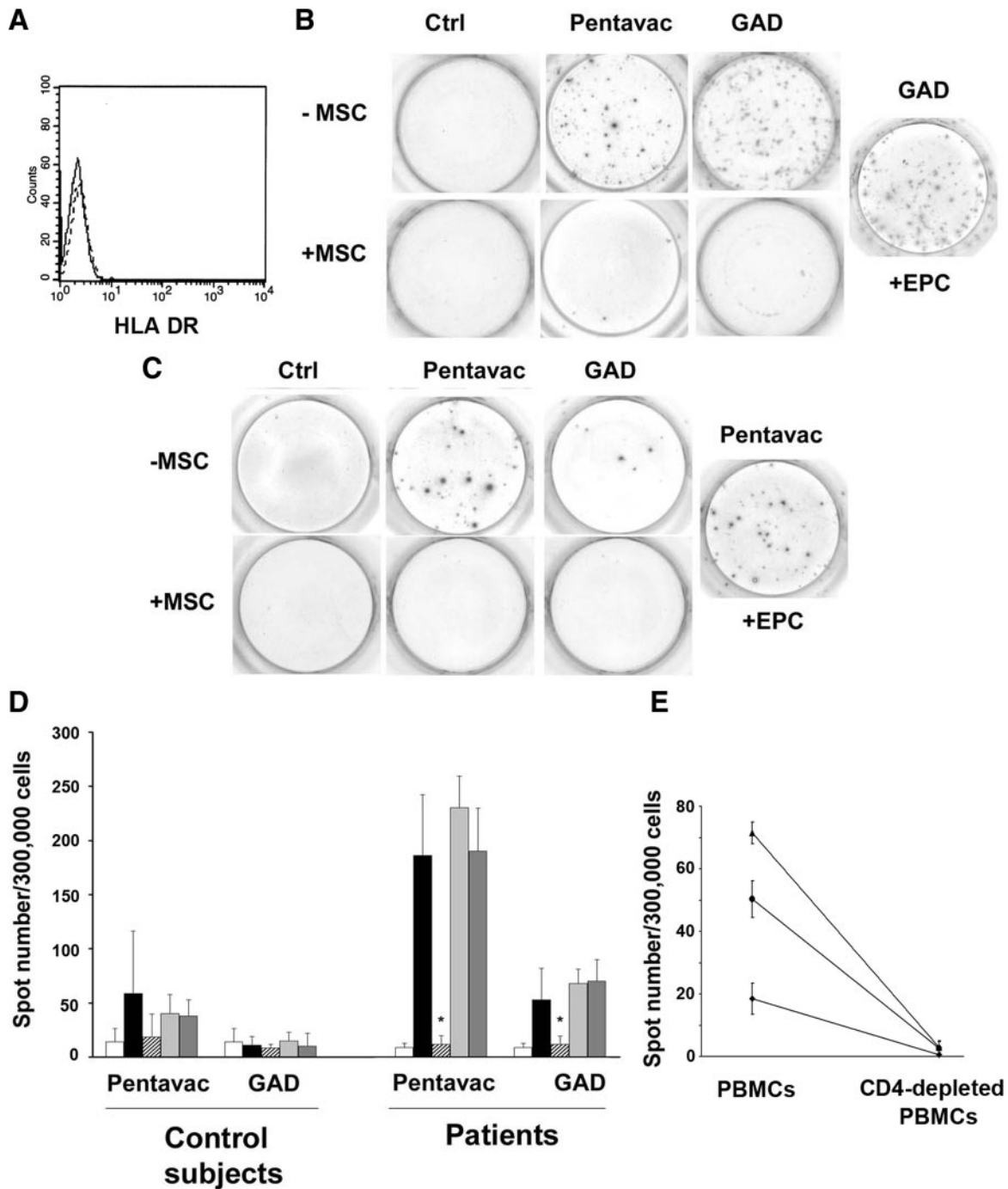
**FIG. 1.** IFN-$\gamma$ ELISPOT response to Pentavac or GAD65 stimulation of PBMCs from nondiabetic control subjects and type 1 diabetic patients. A, Representative flow-cytometric analysis showing lack of class II HLA-DR expression on MSCs after stimulation with IFN-$\gamma$ (100 IU/ml) for 72 h. *Dashed line* histogram represents the corresponding isotype control antibody. B, Representative positive IFN-$\gamma$ ELISPOT response to Pentavac (5 $\mu$g/ml) and GAD65 (10 $\mu$M) of PBMCs ($2 \times 10^{6}$) from a diabetic patient compared with PBMCs challenged with vehicle alone control (ctrl). When stimulated PBMCs were cocultured with MSCs (PBMCs to MSCs, 1:1), both responses became negative. In control coculture experiments using EPCs instead of MSCs, the response persisted positive. C, Representative positive IFN-$\gamma$ ELISPOT response to Pentavac (5 $\mu$g/ml) and negative response to GAD65 (10 $\mu$M) of PBMCs from a nondiabetic control subject. In the presence of MSCs (PBMCs to MCSs, 1:1) the response to Pentavac became negative. In control coculture experiments using EPCs, the response persisted positive. D, Mean value $\pm$ SD of IFN-$\gamma$ spot number per well (300,000 cells) in control subjects and in diabetic patients with a positive response to Pentavac and GAD65 in the following conditions: PBMCs challenged with vehicle alone (*white*), PBMCs stimulated with Pentavac (5 $\mu$g/ml) or GAD65 (10 $\mu$M) (*black*), PBMCs cocultured with MSCs (PBMCs to MSCs, 1:1) and stimulated with Pentavac or GAD65 (*cross-hatched*), Pentavac- or GAD65-stimulated PBMCs/MSCs in Transwell experiments (*gray*), and Pentavac- or GAD65-stimulated PBMCs cultured with supernatants from MSCs (*dark gray*). Data are calculated on three different experiments, performed in triplicate, for each individual patient and control subject. *, $P < 0.05$ *vs.* PBMCs stimulated with Pentavac or GAD65 and *vs.* PBMCs stimulated with Pentavac or GAD65 in Transwell or supernatant conditions. E, Effects of CD4 depletion on IFN-$\gamma$ ELISPOT response to GAD65 in three responsive diabetic patients ($\bullet$, case 1; $\blacklozenge$, case 4; $\blacktriangle$, case 7). Positive responses were reduced to background levels by CD4 depletion. Data are expressed as mean value $\pm$ SD of three experiments for each patient.

**TABLE 1.** Clinical characteristics, HLA genotype, GAD, IA-2 autoantibodies, and islet cell antibodies (ICA) of type 1 diabetic patients

| Case/sex | Age (yr) | HLA-DRB1 genotype | GAD Abs[a] (AU) | IA-2 Abs[a] (AU) | ICA[b] (Juvenile Diabetes Foundation units) |
|---|---|---|---|---|---|
| 1/F | 20 | 03-03 | 2.57 | 0.05 | Negative |
| 2/F | 42 | 01-13 | 25.3 | 51.8 | 320 |
| 3/F | 33 | 03-16 | 24.8 | 55 | Negative |
| 4/F | 26 | 03-04 | 2.21 | 2.03 | 10 |
| 5/M | 25 | 03-13 | 2.22 | 2.23 | Negative |
| 6/M | 27 | 04-07 | 22.14 | 0.05 | 160 |
| 7/F | 21 | 04-16 | 41.65 | 38.3 | 20 |
| 8/M | 21 | 01-03 | 29.7 | 15.7 | 160 |
| 9/F | 19 | 01-03 | 23.8 | 9.8 | 20 |
| Bone marrow | | | | | |
| Donor 1 | | 07-11 | | | |
| Donor 2 | | 01-07 | | | |

Abs, Antibodies; AU, arbitrary units; F, female; M, male.

[a] The 97.5th percentile values 1 and 0.75 AU were used as the cutoff for GAD and IA-2 antibodies, respectively.

[b] Cut-off for ICA was 5 Juvenile Diabetes Foundation units.

served as controls. All subjects gave informed consent, and the study was approved by the local Ethical Review Committee. Fresh PBMCs were isolated by density gradient centrifugation and immediately used.

IFN-γ, IL-4, and IL-10 production, at the single-cell level, was investigated by ELISPOT analysis. ELISPOT was performed as described (4); briefly, fresh PBMCs were dispensed into 48-well plates at a density of at $2 \times 10^6$ in 0.5 ml of AIM V medium (Invitrogen Life Technologies, Grand Island, NY) along with recombinant human IL-2 (0.5 U/ml; R&D Systems, Minneapolis, MN) (31), supplemented with antibiotics and human recombinant GAD65 (10 μM final concentration; Diamyd, Stockholm, Sweden). Control wells contained AIM V medium with peptide diluent alone, a single fixed concentration of polyvalent vaccine Pentavac (Pasteur Mérieux, Lyon, France) (5 μg/ml), or phorbol myristate acetate (PMA) and ionomycin (5 and 745 ng/ml final concentration, respectively). After 48 h at 37 C, nonadherent cells were resuspended, washed and brought to a concentration of $10^6$/300 μl, and 100 μl was dispensed in triplicate into wells of 96-well ELISA plates (Nunc Maxisorp, Poole, UK) preblocked with 1% BSA in PBS and precoated with monoclonal anti-IFN-γ, anti-IL-10, or anti-IL-4 capture antibody (U-Cytech, Utrecht, The Netherlands). After capture at 37 C, 5% $CO_2$ overnight, cells were lysed in ice-cold water, plates were washed in PBS/Tween 20, and spots were developed according to the manufacturer's instructions. Plates were dried and spots of 80–120 μm counted using a computerized digital system (Trantec 1300 ELISpot reader; AMI Bioline, Turin, Italy). Triplicate values were pooled to provide mean spots per 300,000 cells, and mean values in test wells were compared with means of the background wells to derive a stimulation index (SI, ratio of mean spot number in the presence of GAD65 to mean spot number in the presence of diluent alone).

To examine the nature of the responder cells, PBMCs from three responder patients were depleted of CD4 T cells by positive selection (Miltenyi Biotech, Bergisch Gladbach, Germany), and the CD4$^-$ fractions were seeded in parallel with the PBMCs.

### PBMC/MSC cocultures

Human MSCs were seeded into 48-well plates containing $2 \times 10^6$ PBMCs per well and cocultured in AIM V. Preliminary ex-

periments (n = 6) were performed to establish optimal cell ratio, varying the MSC concentration (PBMCs to MSCs 0.2:1, 1:1, 5:1, and 10:1) and by performing IFN-γ ELISPOT analysis of PBMCs stimulated with a single fixed concentration of Pentavac; the ratio inducing a significant reduction of spot number was evaluated. Optimal coculture duration before performing cell stimulation and ELISPOT assay was established as 48 h by assessing the PBMCs viability by trypan blue exclusion. Coculture experiments were performed with PBMCs obtained from all diabetic patients and control subjects. Parallel cultures with PBMCs without MSCs were established for comparison. At 48 h after 10 μM GAD65 stimulation, nonaderent cells were removed and the IFN-γ, IL-4, and IL-10 ELISPOT assays performed as described above.

Human circulating endothelial progenitor cells (EPCs), isolated, cultivated, and characterized as previously described (32), were used in parallel coculture experiments as control non-immunocompetent cells. PBMC survival in parallel cultures and cocultures was assessed by trypan blue exclusion count of nonadherent cells, showing a similar number of viable cells at 24 (99.4 ± 0.9 and 98.7 ± 0.4%), 48 (99.6 ± 0.4 and 98.6 ± 1%), and 72 (96.5 ± 2.6 and 97.4 ± 3.2%) hours of culture.

We assessed in Transwell experiments the need of cell to cell contact. Human MSCs and PBMCs were seeded in 24-well plates on the opposite compartments of a 0.3-μm pore-size membrane (Costar, Life Sciences, Amsterdam, The Netherlands); MSCs were seeded onto the Transwell membrane in the upper chamber, and PBMCs were added 1–2 h thereafter in the lower chamber. PBMCs were then challenged with GAD65 and the IFN-γ ELISPOT assay performed as described.

### Levels of cytokines and $PGE_2$

All culture supernatants were collected immediately before dispensing cells in ELISPOT wells by centrifugation and stored at −20 C before analysis by ELISA for IL-4, IL-10, IFN-γ, and $PGE_2$ (R&D Systems), following the manufacturer's instructions.

For $PGE_2$ synthesis inhibition experiments, MSCs were resuspended in complete medium in the presence or absence of $PGE_2$ inhibitors NS-389 (5 μM; Cayman Chemicals, Ann Arbor, MI) or indomethacin (5 μM; ICN Chemicals, Irvine, CA) (12) for 48 h, and coculture experiments were then carried out as described in the presence of the inhibitors.

## Statistical analysis

A SI (ratio of mean spot number in the presence of GAD65 to mean spot number in the presence of diluent) of 3 or higher was chosen as a positive response in the ELISPOT analysis, established using a receiver-operator characteristic curve (4).

Number of spots and levels of cytokines and PGE$_2$ in different conditions were compared using the Mann-Whitney *U* or the Wilcoxon test for unpaired or paired data, respectively. Data were analyzed using the SPSS statistical package (SPSS, Chicago, IL), and *P* values <0.05 were considered significant.

## Results

### Detection of proinflammatory IFN-γ-secreting T cells in response to GAD65 by ELISPOT

In diabetic patients and in control subjects, spontaneous production of IFN-γ was present at similar very low levels (for all experiments, mean number of spots per 300,000 cells was $11.4 \pm 8$ in diabetic patients and $10.3 \pm 6$ in control subjects). All diabetic and control patients showed a similar, in frequency and magnitude, and significant IFN-γ response to stimulation with the polyclonal T cell stimulus PMA/ionomycin. Similarly, the majority of patients (seven of nine, 78%) and control subjects (eight of 13, 62%) showed positive response to a single fixed concentration of Pentavac polyvalent vaccine (Table 2).

After evaluating the IFN-γ-producing T cells in response to the islet antigen GAD65, five diabetic patients (five of nine, 56%) showing a SI higher than 3 were identified, indicating a positive IFN-γ T cell response to GAD65 (mean $60 \pm 32$ spots), whereas none of the control subjects showed a positive response (mean $11.2 \pm 7.6$ spots) (Fig. 1, B–D, and Table 2). Repeated testing within

1 month in positive response patients using a second blood sample showed that the IFN-γ T cell responses were reproducible over time. Positive responses were entirely abolished when PBMCs were depleted of CD4 T cells, indicating the CD4$^+$ cells were the IFN-γ-producing cells (Fig. 1E).

The polyclonal stimulus PMA/ionomycin induced a significant ELISPOT response for IL-4-producing cells (unstimulated $3.5 \pm 1.5$, stimulated $83.1 \pm 63$ in diabetic patients; unstimulated $6.8 \pm 5.9$, stimulated $55 \pm 34$ in control subjects) and IL-10-producing cells (unstimulated $14.1 \pm 11$, stimulated $152 \pm 56$ in diabetic patients; unstimulated $12.5 \pm 10$, stimulated $57.7 \pm 48$ in control subjects). In contrast, GAD65 did not elicit any positive response in both groups (Fig. 2).

### Effect of MSCs on T cell response to GAD65

MSCs inhibited IFN-γ T cell response to Pentavac both in diabetic patients and control subjects (Table 2 and Fig. 1). The optimal PBMC/MSC (1:1) ratio was defined by evaluating the significant reduction of spot number detected by IFN-γ ELISPOT analysis under Pentavac stimulus (mean spots without MSCs $138.7 \pm 86$, mean spots with MSCs $20.5 \pm 12$; $P < 0.05$).

In all experiments, the presence of MSCs at a 1:1 ratio resulted in a statistically significant decrease in the number of IFN-γ spots in the presence of GAD65 (mean spots without MSCs $52.9 \pm 29$, mean spots with MSCs $12 \pm 7.3$; $P < 0.05$ for paired data), corresponding in all cases to a SI less than 3, indicating an inhibition of the response to GAD65 (Table 2 and Fig. 1). Experiments were repeated within 1 month using a second blood sample and

**TABLE 2.** IFN-γ ELISPOT response to Pentavac and GAD65 in type 1 diabetic patients and nondiabetic control subjects

| | PBMCs | | | | PBMCs + MSCs | | | |
|---|---|---|---|---|---|---|---|---|
| Case | Baseline count | PV | GAD65 | GAD65 SI | Baseline count | PV | GAD65 | GAD65 SI |
| 1 | 5[a] | 94[a] | 48[a] | **9.6** | 5[a] | 9[a] | 13[a] | 2.6 |
| 2 | 10 | 64 | 22 | 2.2 | 9 | 25 | 12 | 1.3 |
| 3 | 4 | 11 | 8 | 2 | 7 | 10 | 14 | 2 |
| 4 | 6 | 77 | 23 | **3.9** | 13 | 23 | 15 | 1.1 |
| 5 | 9 | 235 | 43 | **4.7** | 8 | 9 | 17 | 2.1 |
| 6 | 17 | 82 | 102 | **6** | 9 | 10 | 5 | 0.5 |
| 7 | 6 | 44 | 84 | **14** | 9 | 4 | 2 | 0.2 |
| 8 | 9 | 233 | 11 | 1.2 | 10 | 19 | 5 | 0.5 |
| 9 | 12 | 15 | 16 | 1.3 | 8 | 7 | 10 | 1.2 |
| Control subjects (n = 13) | $14.3 \pm 12.1$ | $58.5 \pm 58.7$ | $11.2 \pm 7.6$ | <3 | $13.8 \pm 9.7$ | $18.5 \pm 21.2$ | $8.2 \pm 3.7$ | <3 |

Baseline count is for PBMC cultures or PBMC/MSC cocultures in vehicle alone; PV is for PBMCs stimulated with Pentavac; and GAD65 is for PBMCs stimulated with GAD65. *Bold* indicates positive response (stimulation index >3). The table is representative of one experiment performed in triplicate for each patient, and each spot number represents the mean of the triplicate wells. Number of spots in control subjects is expressed as mean ± SD for all the subjects. MSCs were derived from donor 1.
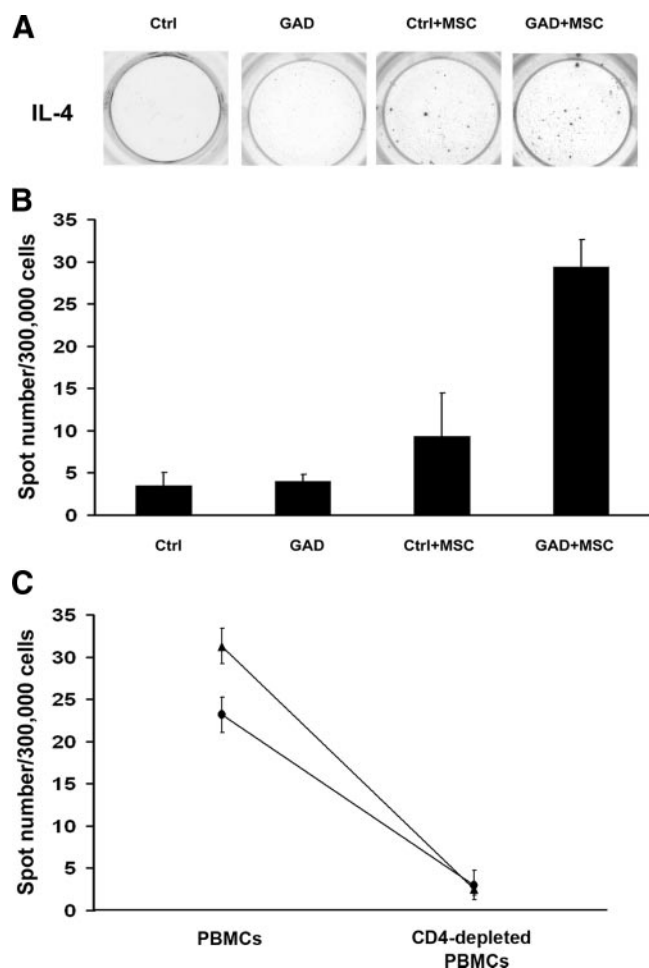
[a] Spot number per 300,000 cells.

**FIG. 2.** IL-4 ELISPOT responses to GAD65 stimulation of PBMCs from type 1 diabetic patients. A, Representative IL-4 ELISPOT response to GAD65 (10 $\mu$M) in PBMCs from one diabetic patient. No spots were detected in wells with PBMCs challenged with vehicle alone control (ctrl) or stimulated with GAD65. The response became positive (SI > 3) when GAD65-stimulated PBMCs were cocultured with MSCs (GAD+MSC). B, Mean value $\pm$ SD of IL-4 spot number per well (300,000 cells) in patients with a positive IFN-$\gamma$ response to GAD65 (10 $\mu$M), in the following conditions: PBMCs challenged with vehicle alone control (ctrl), PBMCs stimulated with GAD65 (GAD), PBMCs cocultured with MSCs (ctrl+MSC), and PBMCs cocultured with MSCs and stimulated with GAD65 (GAD+MSC). Data are calculated on two different experiments in each patient. C, Effects of CD4 depletion on IL-4 ELISPOT response to GAD65 in PBMCs cocultured with MSCs in two diabetic patients (●, case 1; ▲, case 7). Positive responses were reduced to background levels by CD4 depletion. Data are expressed as mean value $\pm$ SD of three experiments for each patient.

confirmed that PBMC/MSC cocultures resulted in a negative IFN-$\gamma$ T cell response (not shown). Experiments repeated with PBMCs from responder patients cocultured with a different MSC batch gave similar immunosuppressive results (data not shown).

When EPCs instead of MSCs were used as control in the coculture experiments, the inhibition of IFN-$\gamma$-secreting T cells was not observed (Fig. 1, B and C).

In paired coculture Transwell experiments, where MSCs were physically separated from PBMCs of patients

with positive response to Pentavac and GAD65, MSCs failed to significantly inhibit the IFN-$\gamma$ ELISPOT response to Pentavac and GAD65 (response to Pentavac: mean spots without MSCs 186.8 $\pm$ 56, mean spots with MSCs in Transwell condition 230.3 $\pm$ 29; response to GAD65: mean spots without MSCs 50.1 $\pm$ 13, mean spots with MSCs in Transwell condition 68.4 $\pm$ 16), indicating that the effect of MSCs required a cell-to-cell physical interaction (Fig. 1D). Furthermore, supernatants from MSCs grown at confluence did not inhibit the positive IFN-$\gamma$ responses (Fig. 1D).

In cocultures, IL-10-secreting cells were not detectable by ELISPOT in response to GAD65, whereas IL-4-secreting cells were detected (unstimulated mean spots 9.3 $\pm$ 5.1, stimulated 29.4 $\pm$ 3.2) in type 1 diabetic patients (Fig. 2). In cocultures, IL-4-secreting cells were also detected under Pentavac stimulus (unstimulated 4.3 $\pm$ 2.7, stimulated 14.7 $\pm$ 6).

Positive IL-4 responses were entirely abolished when PBMCs were depleted of CD4 T cells, indicating the CD4$^+$ cells were the IL-4-producing cells (Fig. 2C).

PGE$_2$ has been described as an MSC-derived immune modulator (12, 23). When MSCs were preincubated with the PGE$_2$ synthesis inhibitors NS-389 and indomethacin followed by cocultures with PBMCs in the presence of PGE$_2$ inhibitors, their immune-suppressive effect on GAD65-induced IFN-$\gamma$ T cell response was abrogated (mean spots with MSCs 13 $\pm$ 5, with NS-389 50.4 $\pm$ 29, and with indomethacin 59.4 $\pm$ 23) (Fig. 3).

## Cytokine and PGE$_2$ production

In patients responding to GAD65, levels of IFN-$\gamma$ in the supernatants were significantly increased with respect to nonresponder patients. In the supernatants obtained by GAD65-stimulated PBMC/MSC cocultures, the level of IFN-$\gamma$ decreased, by approximately 5-fold (mean 407 $\pm$ 190 pg/ml) compared with parallel GAD65-stimulated PBMC cultures (mean 2209 $\pm$ 915 pg/ml, $P < 0.05$; Fig. 4). Concomitantly, in patients responding to GAD65, a decrease of IL-10 levels was detected in GAD65-stimulated PBMC/MSC cocultures (mean 649 $\pm$ 528 pg/ml) compared with parallel GAD65-stimulated PBMC cultures (mean 795 $\pm$ 552 pg/ml) and with IL-10 levels in PBMC/MSC cocultures of nonresponder patients (1913 $\pm$ 710 pg/ml, $P < 0.05$ compared with responder patients; Fig. 4). In contrast, in responder patients, levels of IL-4 significantly increased, by approximately 6-fold, in supernatants of GAD65-stimulated PBMC/MSC cocultures (mean 30 $\pm$ 16 pg/ml) compared with parallel GAD65-stimulated PBMC cultures (mean 4.9 $\pm$ 2.8 pg/ml, $P < 0.05$; Fig. 4).

Levels of PGE$_2$ were higher in all PBMC/MSC coculture supernatants compared with parallel PBMC or MSC cul-
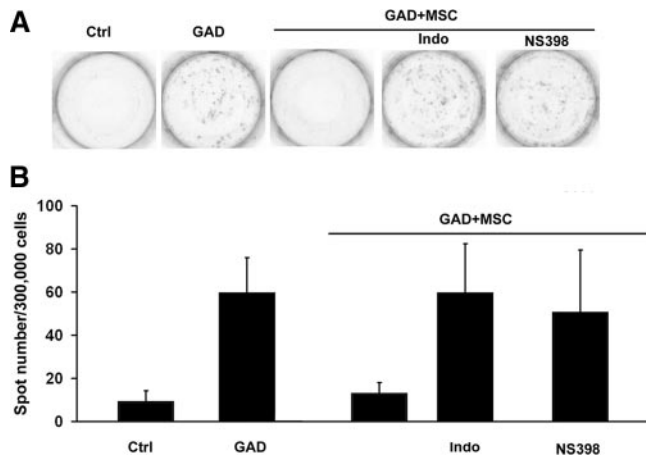
**FIG. 3.** Effects of PGE$_2$ inhibitors on MSC modulation of IFN-γ ELISPOT response. A, Representative positive IFN-γ ELISPOT response to GAD65 (10 μM) in a diabetic patient compared with PBMCs challenged with vehicle alone control (ctrl) and to PBMCs cocultured with MSCs and stimulated with GAD65 (GAD+MSC). Forty-eight hours of incubation of MSCs, before coculture, with PGE$_2$ synthesis inhibitors indomethacin (Indo; 5 μM) and NS398 (5 μM) abrogated the MSC inhibitory effect. B, Mean value ± SD of IFN-γ spot number per well (300,000 cells) in patients with a positive response to GAD65 in the following conditions: PBMCs challenged with vehicle alone control (ctrl), PBMCs stimulated with GAD65 (GAD), and PBMCs cocultured with MSCs and stimulated with GAD65 in the absence (GAD+MSC) or in the presence of indomethacin (Indo) (5 μM) or NS398 (5 μM). Data are calculated on two different experiments in each patient.



**FIG. 4.** Levels of cytokines in supernatants of PBMCs and PBMCs cocultured with MSCs. Mean ± SD release of IFN-γ, IL-10, and IL-4 in the cell-free supernatants was evaluated in the following conditions: PBMCs challenged with vehicle alone (*white*), PBMCs stimulated with GAD65 (*black*), PBMCs cocultured with MSCs and stimulated with GAD65 (*cross-hatched*). Three different study groups are plotted: the nondiabetic control subjects, the GAD65-nonresponder diabetic patients, and the GAD65-responder diabetic patients. Data represent the mean of two different experiments performed in duplicate for each patient. IFN-γ: *, $P < 0.05$ *vs.* all other levels; §, $P < 0.05$ *vs.* parallel PBMCs stimulated with GAD65 in responder patients. IL-10: *, $P < 0.05$ *vs.* PBMCs cocultured with MSCs and stimulated with GAD65 in responder patients. IL-4: §, $P < 0.05$ *vs.* parallel PBMCs stimulated with GAD65 in responder patients.

tures alone in both study groups (Fig. 5A). Levels in supernatants of GAD65-stimulated PBMC/MSC cocultures from responder patients were significantly higher than levels in supernatants of parallel GAD65-stimulated PBMC cultures (Fig. 5B). Preincubation of MSCs with inhibitors NS-398 and indomethacin significantly decreased PGE$_2$ secretion in PBMC/MSC coculture conditions (Fig. 5B).

## Discussion

There is compelling evidence that type 1 diabetes is associated with the loss of immunological tolerance to self. Autoreactive T cells that recognize islet autoantigens have been identified, playing a direct role in the disease immunopathogenesis (4). This hypothesis is supported by studies showing that administration of therapeutic agents inhibiting T cell function delays disease progression. In light of the steady increase of type 1 diabetes, the development of safe and effective means affording its prevention or reversal represents a priority challenge (33). Due to their immunomodulatory effects, MSCs are potential candidates, but their effect on T lymphocytes in human type 1 diabetes remains so far unexplored. In the present study, we found that allogeneic human bone marrow-derived MSCs can abrogate *in vitro* a proinflammatory Th1 response to an islet antigenic stimulus in new-onset diabetic
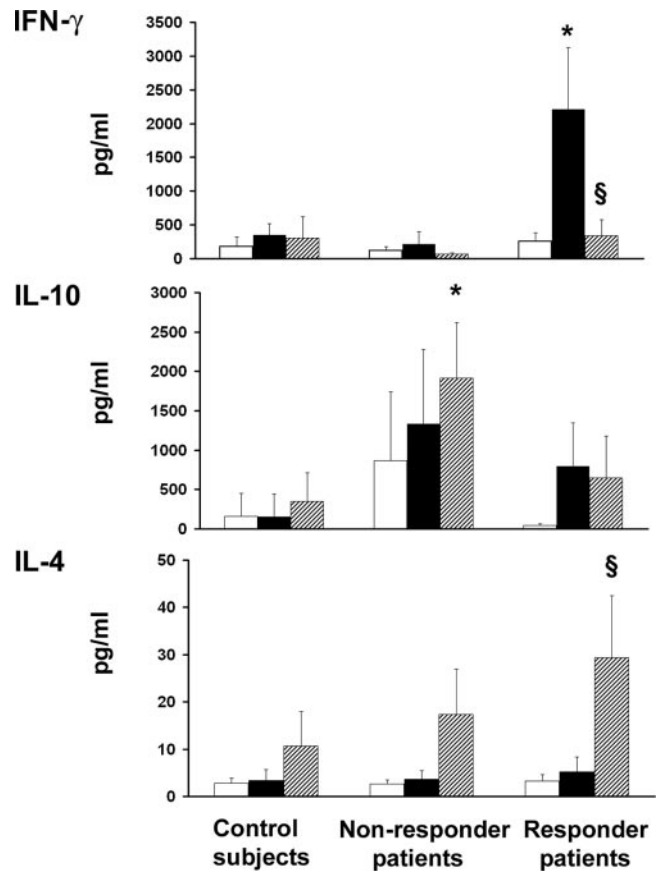
patients. The contact between T cells and MSCs in all patients responding to GAD consistently abrogated the T cell production of IFN-γ, assessed *in vitro* at the single-cell level by ELISPOT and inhibited IFN-γ secretion.

The mechanisms of MSC interaction with the immune system cells are still controversial (5, 23). It has been suggested that MSCs reduce the expression of lymphocyte activation markers; change the cytokine profile of dendritic cells, naive and activated T cells, and NK cells to an antiinflammatory phenotype; and increase the regulatory T cell population (12, 13).

MSCs have been demonstrated to induce mature dendritic cells type 2 (DC2) to increase IL-10 secretion, thus promoting antiinflammatory DC2 signaling (12). In the present study, MSCs did not induce IL-10-secreting cells in
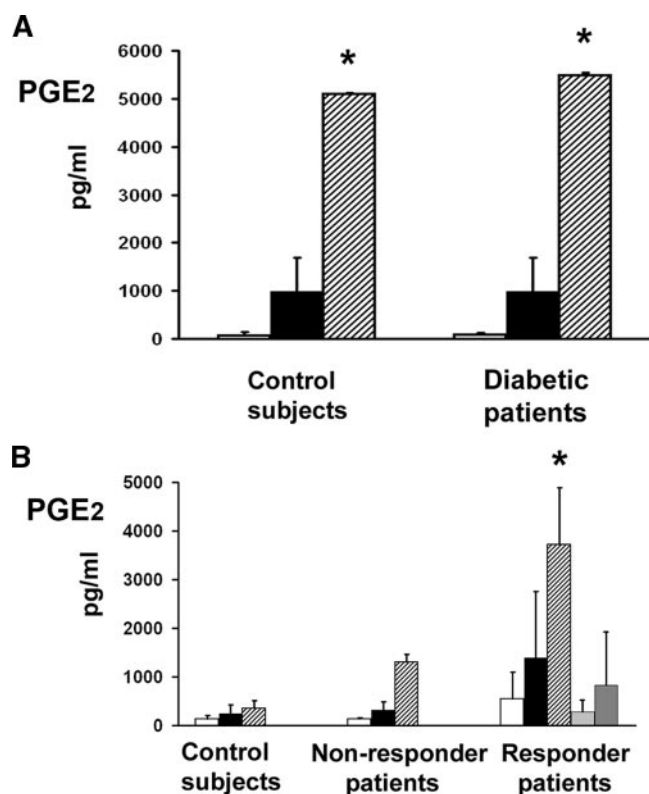
**FIG. 5.** Levels of $PGE_2$ in supernatants of PBMCs, MSCs, and PBMCs cocultured with MSCs in the absence or presence of GAD65. A, Basal production of $PGE_2$ in nondiabetic control subjects and in diabetic patients in three different conditions: PBMCs alone (*white*), MSCs alone (*black*), PBMCs cocultured with MSCs for 48 h (*cross-hatched*). *, $P < 0.05$ *vs.* all other levels. B, Production of $PGE_2$ in nondiabetic control subjects, in GAD65-nonresponder diabetic patients, and in GAD65-responder diabetic patients after stimulation with GAD65 in the following conditions: PBMCs challenged with vehicle alone (*white*), PBMCs stimulated with GAD65 (*black*), PBMCs cocultured with MSCs and stimulated with GAD65 (*cross-hatched*), and GAD65-stimulated PBMCs cocultured with MSCs in the presence of indomethacin (*gray*) or NS-398 (*dark gray*). Data (mean value ± SD) represent the mean of two different experiments performed in duplicate for each patient. *, $P < 0.05$ *vs.* parallel PBMCs stimulated with GAD65 in responder patients and *vs.* PBMCs cocultured with MSCs in the presence of indomethacin and NS-398.

PBMCs or an increased production of this cytokine. Notably, we detected a decreased secretion of IL-10, concomitant with a decrease of IFN-$\gamma$ in MSC cocultures of responder patients. These data appear in line with the report showing that patients making IL-10 responses to islet IA-2 peptides also make IFN-$\gamma$ responses to the same or other peptides (4). This evidence does not have an immediate explanation, but some CD4 T cells produce both IFN-$\gamma$ and IL-10 (34), and such cells have been described in persistent infections (35), intriguing data in the context of the suspected role of viruses in the pathogenesis of type 1 diabetes. Our results might be limited by the use in the study of PBMCs and not purified DC2 and by the use of a single antigenic stimulus rather then a multi-epitope panel (4, 36).

MSCs induced in PBMCs of responder patients IL-4-producing cells and IL-4 secretion. These data suggest a possible switch to an antiinflammatory Th2 signaling of T cells. Increased IL-4 secretion has been shown in studies of MSCs cocultured with subpopulations of phytohemagglutinin-stimulated immune cells (12) but not in studies of T cells activated by encephalitogenic peptide (19). Lymphocyte activation is extremely complex, and it is likely that several mechanisms are involved in the MSC-mediated immunosuppression and that the specific factors may depend on the lymphocyte population tested, the stimulus used, the timing of analysis, and possibly, the context of the immune disease.

Inhibition of $PGE_2$ production abrogated the MSC-mediated IFN-$\gamma$ suppression, indicating that $PGE_2$ secretion plays an important role in MSC-mediated immune effects. This result is in apparent contrast with the finding that the contact between MSCs and PBMSs was needed for the suppression of IFN-$\gamma$ production and that the MSC supernatants were ineffective. However, we found that the contact between MSCs and PBMCs enhanced the production $PGE_2$, confirming previous reports (12). This observation suggests the requirement of both soluble factors and cell contact in line with interpretation that the immunomodulatory effects of MSCs might require an initial cell-contact phase (14). The requirement of cell contact for MSCs to operate their inhibition is a controversial issue, and the results reported in the literature may depend on the species and the type of stimulus (6, 14).

Studies on the regenerative capabilities of MSCs in diabetic mouse models have suggested that MSCs may be therapeutic themselves (24–26). In particular, the injection of MSCs into immunodeficient diabetic NOD/SCID mice resulted in the selective homing of MSCs to pancreatic islets and in an increased number of pancreatic islets and functioning $\beta$-cells (24). Furthermore, MSCs can be influenced to differentiate into cells with properties of the $\beta$-cell phenotype, becoming more efficient after transplantation in mice (26). A recent study on the immunomodulatory function of MSCs in murine model of autoimmune type 1 diabetes indicates that the beneficial effects observed could also be ascribed to the immunomodulatory capacities of MSCs (27), as for other studies focusing on MSC-induced repair of cell injury (23, 37, 38).

*Ex vivo*-expanded MSCs have been infused in several phase I studies (5, 15, 16, 23). The overall evidence is that administration of MSCs appears to improve the outcome of allogeneic transplantation and of acute graft-*vs.*-host disease (17, 18). These studies paved the way for the clinical use of MSCs also in autoimmune diseases. In experimental autoimmune encephalomyelitis, animal model of multiple sclerosis mediated by autoreactive T cells, in-

jected MSCs home to lymphoid organs where they cluster around T cells and ameliorate the disease onset (20). In this setting, MSCs induce peripheral tolerance, impairing both the cellular and humoral arm of the encephalitogenic immune response, without evidence of transdifferentiation into neural cells (19, 20). Furthermore, in a murine model of rheumatoid arthritis, MSCs have been demonstrated to exert an immunomodulatory effect by educating antigen-specific regulatory T cells (22). MSCs are also able to inhibit autoreactive T and B cells in experimental models of systemic lupus erythematosus (9).

MSCs exhibit ability to suppress alloimmune and autoimmune responses, raising the issue of the target specificity in their potential use as a non-antigen-based immunotherapy. However, studies on murine models of type 1 diabetes (27, 39) and multiple sclerosis (19) as well as nonautoimmune diseases (40) indicate that a key feature of MSCs is their ability to selectively migrate into sites of injury, where they are likely to interact with activated T cells. Diabetogenic T cells are generated in pancreatic lymph nodes where they are introduced to antigens by dendritic cells. The preferential homing of MSCs to pancreatic lymph nodes (27, 39) supports the hypothesis that these cells could directly suppress autoreactive T cells *in vivo* within the pancreatic environment. Furthermore, the desired therapeutic effects could be achieved by modulation of chemokines/receptors to promote the homing of MSCs to specific anatomical sites (41). The first report on transplantation of human allogeneic MSCs, into a patient with autoimmune systemic sclerosis, indicates its safety and, notably, striking efficacy by selective immunosuppression and regeneration of impaired endothelial progenitors (42). Thus, as in clinical settings using MSC infusion for treatment of severe graft-*vs.*-host disease or autoimmune diseases (15–18, 42), infusion of autologous or allogeneic MSCs carries a potential use in patients at risk of type 1 diabetes or at disease onset, to preserve or reduce loss of $\beta$-cells (23, 33).

In conclusion, in the present study, MSCs appeared capable of inhibiting antigen-specific T cell activation by impairing the production of IFN-$\gamma$ and by inducing anti-inflammatory IL-4 production from PBMCs of type 1 diabetic patients. These data stimulate further studies on MSC immunomodulation in diabetes and open a perspective for immuno-intervention strategies.

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Address all correspondence and requests for reprints to: Dr. Maria M. Zanone, Dipartimento di Medicina Interna, Corso Dogliotti 14, 10126 Torino, Italy. E-mail: mmz@libero.it.

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