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Abstract

Aims/hypothesis

Mesenchymal stem cells (MSCs) have been shown to abrogate in vitro the proinflammatory response in type 1 diabetes. The mechanism involves paracrine factors, which may include microvesicles (MVs). We evaluated whether MVs derived from heterologous bone-marrow MSCs exert an immunomodulatory effect on T cell responses against GAD (glutamic acid decarboxylase) antigen in type 1 diabetes.

Methods

MVs were purified from heterologous human MSCs by differential centrifugation. Peripheral blood mononuclear cells (PBMCs) were obtained from patients with type 1 diabetes at disease onset, and responses to GAD65 stimulation were assessed by IFN-γ enzyme-linked immunosorbent spot analysis. Levels of cytokines and prostaglandin E_2 (PGE_2) were measured in the supernatant fraction, and T helper 17 (Th17) and regulatory T cell analysis was performed.

Results

MVs were internalised by PBMCs, as assessed by confocal microscopy and flow cytometry analyses. MVs significantly decreased IFN-γ spots and levels in GAD65-stimulated PBMCs, and significantly increased transforming growth factor-β (TGF-β), IL-10, IL-6 and PGE_2 levels. Furthermore, MVs decreased the number of Th17 cells and the levels of IL-17, and increased $FoxP3^+$ regulatory T cells in GAD65-stimulated PBMCs.

Conclusions/interpretation

These results provide evidence that MSC-derived MVs can inhibit in vitro a proinflammatory response to an islet antigenic stimulus in type 1 diabetes. The action of MVs involves PGE_2 and TGF-β signalling pathways and IL-10 secretion, suggesting a switch to an anti-inflammatory response of T cells.

Abbreviations

BrdU	5-Bromo-2′-deoxyuridine
ELISPOT	Enzyme-linked immunosorbent spot
GAD	Glutamic acid decarboxylase
HLA-G5	Human leucocyte antigen G5
IA2	Insulinoma-associated protein 2
ICA	Islet-cell antibody
IDO	Indoleamine 2,3-dioxygenase
miRNA	MicroRNA
MSC	Mesenchymal stem cell
MV	Microvesicle
PBMC	Peripheral blood mononuclear cell
PD1	Programmed cell death protein 1
PD-L1	Programmed cell death 1 ligand 1
PGE_2	Prostaglandin E_2

PMA	Phorbol myristate acetate
PV	Pentavac
SI	Stimulation index
TGF-β1	Transforming growth factor-β1
Th2	T helper 2
Th17	T helper 17
Treg	Regulatory T cell

Introduction

The pathogenesis of type 1 diabetes is associated with several abnormalities of T cell immunophenotype and function [1]. These abnormalities are potential targets of immune interventions to preserve insulin-producing beta cells. However, as immunosuppression represents an unacceptable risk in these patients an immunomodulatory approach to restore tolerance to beta cells is crucial. This is potentially achievable by combining synergic immunotherapies [2]. Recent studies, in vitro and in animal models of autoimmune diseases such as rheumatoid arthritis, systemic lupus erythematosus and encephalomyelitis, indicate that mesenchymal stem cells (MSCs) are promising immune regulators [3, 4]. In particular, analysis of the phenotype and cytokine profile of dendritic cells, naive and activated T cells and natural killer cells indicates that MSCs induce an anti-inflammatory phenotype and increase the regulatory T and dendritic cell populations [5]. The immunomodulatory potential of MSCs could be of relevance in the clinical setting of autoimmune diabetes [6–8]. At present there are at least three ongoing Phase I/II clinical trials based on autologous or allogeneic MSC administration in new-onset type 1 diabetes [9].

The immunomodulatory effects of MSCs require both soluble factors, including hepatocyte growth factor, transforming growth factor-β1 (TGF-β1), human leucocyte antigen G5 (HLA-G5) and prostaglandin E_2 (PGE_2) and cell contact [5, 7, 10, 11]. Recent studies suggest that microvescicles (MVs) released from MSCs may mimic their regenerative action [12–16]. MVs, now recognised as an integral component of the cell-to-cell communication network, may mediate the paracrine/endocrine action of stem cells. MVs express surface receptors and carry biologically active proteins, lipids, mRNAs, long non-coding RNAs and microRNAs (miRNAs) [17]. In the context of immune response, and depending on the cellular source, MVs display different functions resulting in immune cell activation or inhibition [18].

The aim of the present study was to investigate whether MVs derived from bone-marrow MSCs inhibit in vitro islet antigen T cell activation at type 1 diabetes onset. The number of IFN-γ-producing T cells, the release of IFN-γ and the induction of a switch to an anti-inflammatory T helper 2 (Th2) signalling in the presence of MVs were studied.

Methods

Patients

Fresh heparinised blood samples were obtained from nine white patients with new-onset type 1 diabetes [19]. Blood was drawn within 12 months from diagnosis, patients had metabolically controlled disease, and were free of recent infectious or inflammatory conditions. Patients were *HLA-DRB1* typed, screened for islet-cell antibodies (ICAs) by indirect immunofluorescence, glutamic acid decarboxylase (GAD) and insulinoma-associated protein 2 (IA2) autoantibodies by radio-immunoassay (Medipan-Euroimmun, Dahlewitz/Berlin, Germany) (Table 1). Six age-matched healthy white individuals without a family history of diabetes served as controls. The study was approved by the local Ethical Review Committee. Fresh peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation.

Table 1

Clinical characteristics, HLA genotype, GAD, IA2 autoantibodies and ICAs of patients with type 1 diabetes

Case No./Sex	**Age (years)**	***HLA-DRB1* genotype**	**GAD antibodies[a] (arbitrary units)**	**IA2 antibodies[a] (arbitrary units)**	**ICA[b] (JDRF units)**
1/M	29	03-05	119	20.5	120
2/M	21	03-03	0.26	21.7	160
3/F	24	03-03	2.8	0.05	Negative
4/M	27	/-12	22	32	120
5/F	25	04-16	41.7	38.3	20
6/F	28	03-04	2.3	2.03	Negative
7/F	32	04-07	2.06	0.08	Negative
8/F	35	07-11	119.3	7.84	320
9/F	21	04-04	1.6	9.2	20
Bone marrow					
Donor 1		07-11			
Donor 2		01-07			
Donor 3		01-11			

[a]The 97.5th percentile values 1 AU and 0.75 AU were used as cut-off for GAD antibodies and IA2 antibodies, respectively

[b]Cut-off for ICA: 5 JDRF units

Isolation and characterisation of MSC-derived MVs

MSCs were obtained from Lonza (Basel, Switzerland), cultured in Mesenchymal Stem Cells Basal Medium (Lonza) and characterised as described [15]. Human fibroblasts from dermas served as control (Lonza). MVs were obtained from the supernatant fractions of three different heterologous MSCs and of fibroblasts by differential centrifugation, as described [15]. Briefly, after centrifugation at 1,500 *g* and 10,000 *g* for 20 min, cell-free supernatant fractions were centrifuged at 100,000 *g* for 1 h at 4°C, washed in serum-free medium 199/HEPES (Sigma, Milan, Italy) and submitted to further ultracentrifugation. By NanoSight LM10 (NanoSight, Amesbury, UK) equipped with nanoparticle tracking analysis NTA 2.0 analytic software [20], MVs were counted and their size determined (range 60–160 nm, Fig. 1a). Approximately 6400 ± 1200 MVs/cell were obtained.

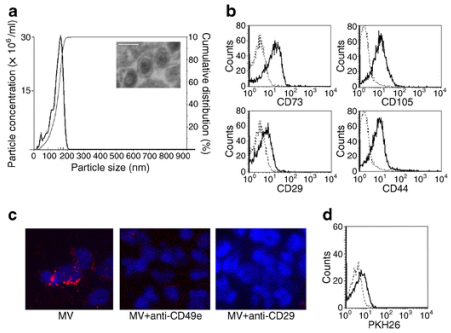

Fig. 1

Characterisation of MSC-derived MVs, and incorporation by PBMCs. (**a**) Nanosizer analysis of purified MSC-derived MVs. Curve 1 describes the relationship between particle distribution (left *y*-axis) and particle size (*x*-axis); curve 2 describes the correlation between cumulative percentage distribution of particles (percentile in right *y*-axis) and particle size (*x*-axis). The inset shows a representative micrograph of transmission electron microscopy of purified MSC-derived MVs showing a spheroid shape (scale bar, 100 nm). (**b**) Representative flow cytometry analysis of surface

molecules (CD73, CD29, CD105 and CD44) expressed by MSC-derived MVs. Dashed line histograms represent the control isotype antibody. (**c**) Representative micrographs of internalisation of PKH26-labelled MSC-derived MVs by PBMCs in the absence or presence of 1 μg/ml blocking antibodies against CD49e and CD29 integrins. (**d**) Representative flow cytometry analysis of internalisation by gated $CD3^+$ cells of PKH26-labelled MSC-derived MVs. Dashed line histogram represents $CD3^+$ cells not incubated with MSC-derived MVs

Transmission electron microscopy was performed on Karnovsky's-fixed, osmium tetraoxide-post-fixed MV pellets embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate and examined with a Jeol JEM 1010 electron microscope (inset in Fig. 1a).

Flow cytometry analyses for adhesion molecules or typical MSC markers were performed on MVs [20]. A pool of approximately 5×10^9 particles/100 μl was incubated with 5 μl of latex beads (Aldehyde/sulphate latex 4% w/v 4 μmol/l; Molecular Probes, Leiden, the Netherlands) at room temperature for 30 min and then at 4°C for 30 min, then washed and incubated for 30 min with antibodies. After washing, MV-coated beads were analysed by FACScan (Becton Dickinson, Franklin Lakes, NJ, USA). The following phycoerythrin- or fluorescein isothiocyanate-conjugated monoclonal antibodies (1:50) were used: anti-CD105, -CD29 (β1 integrin), -CD44, -CD73, -CD49d (α4 integrin), -CD49e (α5 integrin), -CD49f (α6 integrin) (BD Pharmingen, San Diego, CA, USA), -TGF-β, -HLA-G5 and -PD-L1 (Biolegend, San Diego, CA, USA).

Effect of MVs on PBMCs and cytokine enzyme-linked immunosorbent spot analyses

Internalisation of MVs into PBMCs was evaluated by confocal microscopy (LSM5-PASCAL; Zeiss, Oberkochen, Germany). A pool of approximately 5×10^9 MV particles was labelled with PKH-26 dye (2 μl/ml) for 30 min at 37°C and then washed, ultracentrifuged at 100,000 *g* for 1 h at 4°C and added to PBMCs (1×10^6) for 24 h. In selected experiments, MVs were pre-incubated with blocking monoclonal antibodies (1 μg/ml) against the α/β integrins CD49d, CD49e (Biolegend) and CD29 (BD Pharmingen) on the basis of previous studies [15]. Furthermore, internalisation of MVs into 1×10^5 $CD3^+$ T cells, positively selected with magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany), was assessed by flow cytometry analysis.

Fresh PBMCs, dispensed into 48-well plates at a density of 2×10^6/ml, were stimulated for 48 h with polyvalent vaccine Pentavac (PV; Sanofi Pasteur, Lyon, France) (5 μg/ml) or phorbol myristate acetate (PMA)/ionomycin (5 ng/ml and 745 ng/ml), to evaluate recall and polyclonal aspecific responses, or with human recombinant GAD65 (Diamyd, Stockholm, Sweden) (10 μmol/l). IFN-γ production was investigated by enzyme-linked immunosorbent spot (ELISPOT) analysis [7]. 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). The optimal amount of MVs, selected under PV stimulation, was 2.5×10^8 MV particles/ml per 2×10^6 PBMCs for 24 h (mean spots without MVs 43 ± 16, with MVs 18.4 ± 14, $p < 0.05$).

In selected experiments, 2×10^6 PBMCs were pre-incubated with blocking antibodies (1 μg/ml) against CD49d, CD49e or CD29 or with MV-free supernatant fraction obtained by ultracentrifugation of GAD65 (10 μmol/l) pre-incubated overnight with MVs (2.5×10^8 particles/ml). ELISPOT was then performed.

Proliferation was assessed by detection of incorporated 5-bromo-2'-deoxyuridine (BrdU) using the Cell Proliferation BrdU assay (Roche, Basel, Switzerland), and apoptosis was evaluated by flow cytometry analysis as an expression of annexin V by T cells (BD Pharmingen). Expression of CD69 (using anti-CD69-PE monoclonal antibody; BD Pharmingen), as a marker of T cell activation, was assessed by flow cytometry analysis.

Quantitative real-time PCR, western blot and ELISA analyses

In the different sets of experiments, RNA was collected from MVs and PBMCs using the mirVana RNA kit (Ambion, Austin, TX, USA), according to the manufacturer's protocol, and real-time PCR analyses were performed in duplicate as described [20] (primers are listed in electronic supplementary material [ESM] Table 1). In selected experiments, MVs were treated with 5 U RNAse for 3 h at 37°C; the reaction was stopped by 10 U/ml RNAse inhibitor (Ambion) and MVs were washed by ultracentrifugation.

To detect microRNA-21 (miR-21) by real-time PCR, RNA was collected using mirVana miRNA Isolation Kit (Life Technologies, Grand Island, NY, USA), followed by miScript Reverse Transcription Kit and miScript SYBR Green PCR Kit (Qiagen, Hilden, Germany). RNU48 was used as endogenous control.

Western blot on MVs was performed [12] using anti-IL-10, -IFN-γ, -TGF-β and -HLA-G5 monoclonal antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Supernatant levels of IFN-γ, IL-17, IL-10, IL-6, TGF-β and PGE_2 were measured by ELISA (R&D, Milan, Italy).

To analyse the role of soluble factors, MVs and PBMCs were pre-treated with 5 μmol/l anti-TGF-β monoclonal antibody (Clone 27235; R&D) or with combination of 1 mmol/l 1-methyl-tryptophan (as inhibitor of indoleamine 2,3-dioxygenase [IDO]) and 5 μmol/l NS-398 (as inhibitor of PGE_2) (Sigma).

Flow cytometry analysis of regulatory T cells and T helper 17 cells

PBMCs from diabetic patients were stimulated for 48 h with GAD65 and cultured with or without MSC-derived MVs to analyse regulatory T cells (Tregs) and T helper 17 (Th17) cells. PBMCs were stained with anti-CD4-PerCP, -CD25-PE, -CD127-APC monoclonal antibodies (Biolegend), fixed and permeabilised and then incubated with anti-FoxP3-FITC monoclonal antibody (eBiosciences, San Diego, CA, USA). For analysis of T helper 17 (Th17) cells, PBMCs were stimulated overnight with PMA (50 ng/ml) and ionomycin (2 μg/ml) in the presence of brefeldin A (Sigma), labelled with anti-CD4-PerCP monoclonal antibody (Biolegend), fixed, permeabilised and incubated with anti-IL-17-APC monoclonal antibody (Biolegend).

Statistical analysis

A SI ≥ 3 was chosen as a positive response in the ELISPOT analysis [7]. Spots and levels of cytokines and PGE_2 were compared using the Mann–Whitney *U* test or the Wilcoxon test, for unpaired or paired data, respectively. Data were analysed using the SPSS statistical package (SPSS, Chicago, USA), and $p < 0.05$ was considered significant.

Results

Characterisation of MVs and internalisation by PBMCs

Cytofluorimetric analysis showed the presence of several adhesion molecules known to be expressed on MSCs (Fig. 1b). Fibroblast-derived MVs showed a different molecule pattern, in which CD49d and CD49e were expressed but not CD44, CD29 or CD73 (data not shown). Confocal microscopy indicated that MSC-derived MVs were internalised by PBMCs. Anti-CD49e and anti-CD29 (Fig. 1c), but not anti-CD49d, blocking antibodies reduced internalisation. Figure 1d shows internalisation of MVs by $CD3^+$ T cells.

Effect of MSC-derived MVs on T cell response

In diabetic and control individuals, spontaneous production of IFN-γ was present at similar very low levels (Fig. 2), and all individuals showed a similar, significant IFN-γ response to polyclonal T cell stimulus PMA/ionomycin. All patients and three control individuals showed a positive response to PV. Six diabetic patients showed a positive response to GAD65 (Fig. 2a–c).

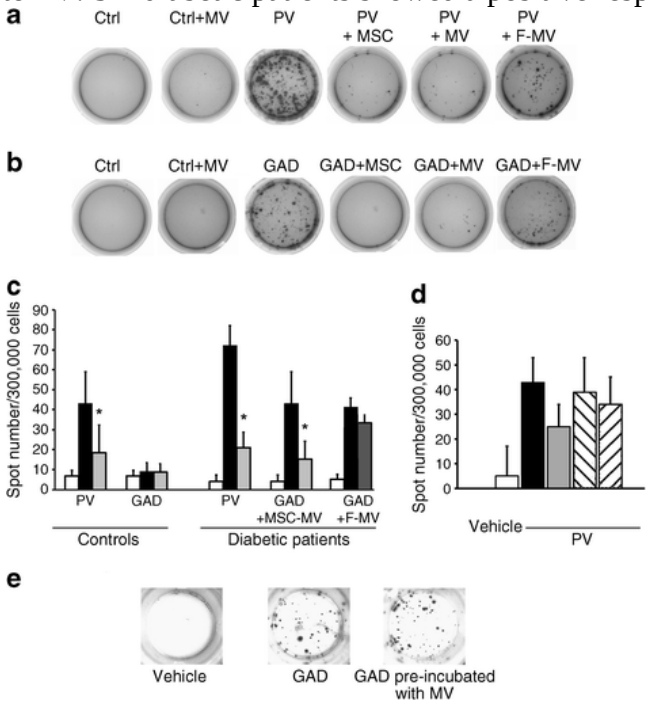

Fig. 2

IFN-γ ELISPOT response of PBMCs to PV or GAD65. (**a**) Representative positive IFN-γ ELISPOT response to PV of PBMCs from a control individual, compared with the PBMCs challenged with vehicle alone (Ctrl). When PMBCs were co-cultured with MSCs (1:1) or in the presence of MSC-derived MVs (2.5×10^8 particles/ml), the response became negative. In control experiments using fibroblast-derived MVs (F-MV), the response remained positive. (**b**) Representative positive IFN-γ ELISPOT response to GAD65 (GAD) of PBMCs from a diabetic patient. When co-cultured with MSCs (1:1) or in presence of MSC-derived MVs (2.5×10^8 particle/ml) response became negative. In control experiments using F-MVs, the response remained positive. (**c**) Mean value ± SD of IFN-γ spot number per well (300,000 cells) in control individuals and diabetic patients with a positive response to PV and GAD65, in the following conditions: PBMCs challenged with vehicle alone (white bars); PBMCs stimulated with PV or GAD65 (black bars); PBMCs stimulated with PV or GAD65 and cultured with MSC-derived MVs (grey bars); PBMCs stimulated with GAD65 and cultured with F-MVs (dark grey bars). *$p < 0.05$ vs PBMCs stimulated with PV or GAD65 without MVs. (**d**) Mean value ± SD of IFN-γ spot number per well in individuals with a positive response to PV in the following conditions: PBMCs challenged with vehicle alone (white bars); PBMCs challenged with PV (black bars); PBMCs challenged with PV and cultured with MSC-derived MVs (grey bars); PBMCs challenged with PV and cultured with MSC-derived MVs in the presence of anti-TGF-β monoclonal antibodies (downwards-hatched bars) or 1-methyl-tryptophan and NS-398 (upwards-hatched bars). Data are calculated from two different experiments, performed in triplicate, for each individual patient and control individual. (**e**) PBMC response to GAD65 following pre-incubation with MVs. Representative IFN-γ ELISPOT response of PBMCs from a diabetic patient, challenged with vehicle alone, GAD65 and MV-free supernatant fraction obtained by ultracentrifugation of GAD65 pre-incubated with MVs (2.5×10^8)

MVs inhibited IFN-γ T cell response to PV both in diabetic patients and in control individuals (Fig. 2a–c). In GAD65-responder patients, MVs significantly decreased the number of IFN-γ spots (mean number of spots: without MVs 42.8 ± 12.7, with MVs 15.2 ± 9, $p < 0.05$ for paired data), resulting in an SI < 3 (Fig. 2a–c and Table 2). Experiments repeated within 1 month, using a second blood sample from the same patient and a different MV batch, gave similar results.

Table 2

IFN-γ ELISPOT response to PV and GAD65 in patients with type 1 diabetes and non-diabetic control individuals

Case No./Sex	PBMCs				PBMCs + MSC-derived MVs[a]			
	Baseline count[b]	PV[b]	GAD_{65} [b]	GAD_{65} SI[c]	Baseline count[b]	PV[b]	GAD_{65} [b]	GAD_{65} SI[c]
1/M	3	86	26	8.6	8	12	18	2.2
2/M	2	65	30	15	4	26	9	2.2
3/F	3	68	28	9.3	3	26	3	1
4/M	2	76	35	17.5	9	21	17	1.8
5/F	10	40	64	6.4	12	5	21	1.7
6/F	5	38	10	2	5	21	6	1.2
7/F	4	41	13	3.2	3	30	7	2.3
8/F	3	70	6	2	4	21	6	1.5
9/F	5	60	6	1.2	6	25	9	1.5
Control individuals[d]	6.8 (2.9)	43 (16.7)	9 (4.5)	< 3	13.3 (8.9)	18.4 (14)	8.7 (4.2)	< 3

The table is representative of one experiment, performed in triplicate for each patient. Each spot number represents the mean of the triplicate wells

[a]MSC-derived MVs were from donor 1 or donor 2

[b]Spot number/300.000 cells. Baseline count, PBMC or MV-stimulated PBMC cultures in vehicle alone; PV, PBMCs stimulated with PV; GAD65, PBMCs stimulated with GAD65

[c]SI > 3 indicates a positive response

[d]In control individuals, spot numbers are expressed as mean (SD) for all the individuals ($n = 6$)

Fibroblast-derived MVs did not statistically significantly reduce the SI after PV or GAD65 stimulation (Fig. 2a–c).

Diabetic patients showed a similar IFN-γ ELISPOT response to stimulation with GAD65 (10 μmol/l) in the absence (mean spots 51 ± 3.5) or presence of anti-CD29, -CD49d, -CD49e blocking antibodies (mean spots 48 ± 8.0) or of MV-free supernatant fraction obtained by ultracentrifugation of GAD65 pre-incubated with MVs (mean spots 47 ± 7.1) (Fig. 2e). Therefore, MVs do not act by antigen sequestering.

Incubation with MVs attenuated, but did not abrogate, PBMC proliferation in response to GAD antigen (Fig. 3a), and MV treatment did not significantly increase cell apoptosis assessed by annexin V expression (Fig. 3b). The early T cell activation marker CD69 was significantly reduced by MV treatment (Fig. 3c, d).

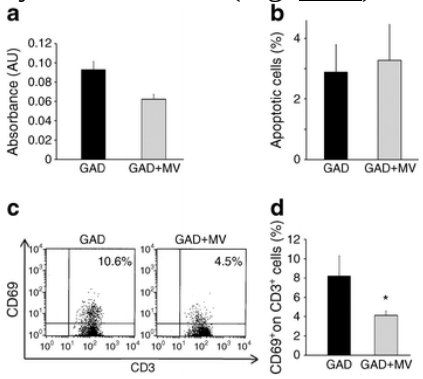

Fig. 3

Proliferation, apoptosis and T cell activation. (**a**) Mean ± SD proliferation, assessed as BrdU incorporation (arbitrary units [AU]), of diabetic PBMCs stimulated with GAD65 (GAD) and cultured without (black bar) or with MSC-derived MVs (grey bar). (**b**) Mean ± SD percentage of annexin V-positive apoptotic T cells from diabetic PBMCs stimulated with GAD65 and cultured without (black bar) or with MSC-derived MVs (grey bar). (**c**) Representative flow cytometry analysis of activated $CD69^+$ T cells of the same diabetic patient's PBMCs stimulated with GAD65 and cultured without or with MSC-derived MVs. (**d**) Mean ± SD percentages of $CD69^+$ of the $CD3^+$ T cell population from diabetic PBMCs stimulated with GAD65 and cultured without (black bar) or with MSC-derived MVs (grey bar). *$p < 0.05$ for unpaired and paired data. Data represent the mean of two experiments for each responder patient analysed

Effect of MSC-derived MVs on cytokine and PGE_2 production

TGFB1 transcript, but not *IL10*, was detected in MVs. TGF-β was also detected by western blot analysis and on the surface of MVs by flow cytometry (Fig. 4a, b). Programmed cell death 1 ligand 1 (PD-L1) was not detected on the MV surface. In GAD65 responder PBMCs, analysis of *IFNG* transcript showed a significant decrease in the presence of MVs, confirming the ELISPOT data (Fig. 4c). Blockade of MV internalisation by anti-CD29 and -CD49e antibodies, as well as pre-treatment of MVs with RNAse, reduced *IL10* and *TGFB1* transcripts in MV-treated PBMCs (Fig. 4d). Furthermore, levels of miR-21 were increased in GAD-stimulated PBMCs in the presence of MVs (Fig. 4e).

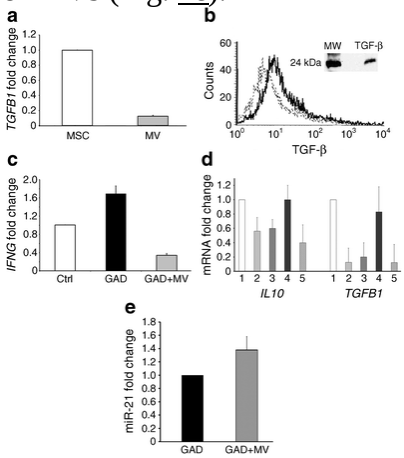

Fig. 4

Real-time PCR, flow cytometric and western blot analyses. (**a**) *TGFB1* mRNA level detected in MVs compared with MSCs. Results were normalised to 18S RNA transcript and expressed as fold change in respect to MSC gene expression ($2^{-\Delta\Delta}$Ct). (**b**) Representative flow cytometry analysis of surface expression of TGF-β in MVs. Dashed line histogram represents the control isotype antibody. Inset shows a representative western blot analysis of TGF-β expression in MSC-derived MVs. (**c**) *IFNG* mRNA level detected in PBMCs with vehicle alone (white bar), with GAD65 (black bar) and with GAD65 and cultured with MSC-derived MVs (grey bar). Results were normalised to 18S RNA and expressed as fold change in respect to PBMC gene expression ($2^{-\Delta\Delta}$Ct). (**d**) *IL10* and *TGFB1* mRNA levels detected in the following conditions: PBMCs stimulated with GAD65 cultured with MSC-derived MVs (1), with blocking antibodies against CD29 (2), with blocking antibodies against CD49e (3), with trypsin-treated MVs (4) or RNAse-treated MVs (5). Results were normalised to 18S RNA and expressed as fold change in respect to gene expression of PBMCs stimulated with GAD65 cultured with MSC-derived MVs ($2^{-\Delta\Delta}$Ct). (**e**) miR-21 level detected in the following conditions: PBMCs stimulated with GAD65 (black bar) and PBMCs stimulated with GAD65 cultured with MSC-derived MVs (grey bar). Results were normalised to RNU48 transcript and expressed as fold change in respect to gene expression of PBMCs stimulated with GAD65 ($2^{-\Delta\Delta}$Ct). All data represent the means ± SD of three independent experiments

MV treatment decreased IFN-γ ($p < 0.05$) and IL-17 supernatant-fraction levels and increased IL-10, TGF-β, PGE_2 ($p < 0.05$) and IL-6 levels in GAD-responder patients (Fig. 5a–f). The addition of anti-TGF-β monoclonal antibody or combination of inhibitor of IDO and of PGE_2 synthesis partially reduced the MV inhibitory effects on IFN-γ spots (Fig. 2d).

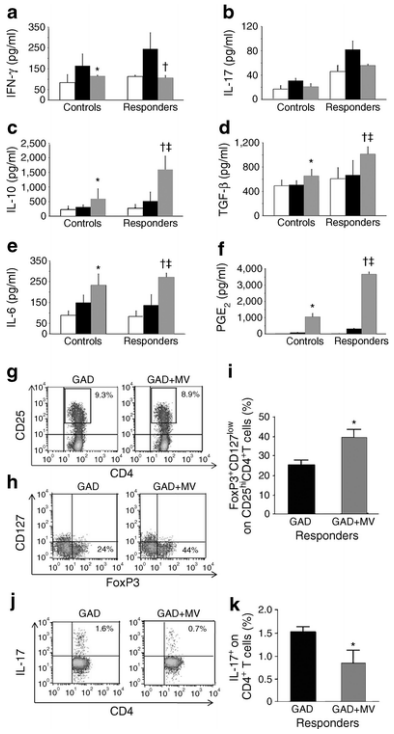

Fig. 5

Levels of cytokines and PGE_2 in the supernatant fraction and flow cytometry analysis of $FoxP3^+$ Tregs and Th17 cells. Mean ± SD release of IFN-γ (**a**), IL-17 (**b**), IL-10 (**c**), TGF-β (**d**), IL-6 (**e**) and PGE_2 (**f**) in the cell-free supernatant fractions evaluated in the following conditions: PBMCs challenged with vehicle alone (white bars), stimulated with PV in control individuals or GAD65 in diabetic patients (black bars), PV or GAD65-stimulated PBMCs cultured with MVs (grey bars). Study groups plotted: PV-responder control individuals, GAD65-responder diabetic patients. Data represent the mean of two different experiments performed in duplicate for each individual. (**a**) $*p < 0.05$ vs PBMCs stimulated with PV in control individuals; † $p < 0.05$ vs paired PBMCs stimulated with GAD65 in responder patients. (**c**) $*p = 0.05$ vs PBMCs stimulated with PV in control individuals; † $p < 0.05$ vs paired PBMCs stimulated with vehicle alone and ‡ $p < 0.05$ vs paired PBMCs stimulated with GAD65 in responder patients. (**d**) $*p < 0.05$ vs paired PBMCs stimulated with PV in control individuals; † $p < 0.05$ vs paired PBMCs stimulated with vehicle alone and ‡ $p < 0.05$ vs paired PBMCs stimulated with GAD65 in responder patients. (**e**) $*p < 0.05$ vs PBMCs stimulated with PV in control individuals; † $p < 0.05$ vs parallel PBMCs stimulated with vehicle alone and ‡ $p = 0.05$ vs PBMCs stimulated with GAD65 in responder patients. (**f**) $*p < 0.05$ vs PBMCs stimulated with PV in control individuals; † $p < 0.05$ vs paired PBMCs stimulated with vehicle alone and ‡ $p < 0.05$ vs paired PBMCs stimulated with GAD65 in responder patients. (**g**) Representative flow cytometry analysis of $CD4^+CD25^{hi}$ T cells (upper right quadrant) of a diabetic patient's PBMCs stimulated with GAD65 and cultured without or with MSC-derived MVs. (**h**) Representative flow cytometry analysis of regulatory $CD127^{low}FoxP3^+$ T cells (lower right quadrant) gated on $CD4^+CD25^{hi}$ population of the same diabetic patient's PBMCs stimulated with GAD65 and cultured without or with MSC-derived MVs. (**i**) Mean ± SEM percentages of $CD127^{low}FoxP3^+$ of the $CD25^{hi}$ $CD4^+$ T cells from diabetic PBMCs stimulated with GAD65 and cultured without (black bar) or with MSC-derived MVs (grey bar). $*p < 0.05$ for unpaired and paired data. Data represent the mean of three experiments for each responder patient. (**j**) Representative flow cytometry analysis of $IL17^+$ of the $CD4^+$ T cell population (upper right quadrant) in a diabetic patient's PBMCs stimulated with GAD65 and cultured without or with MSC-derived MVs. (**k**) Mean ± SEM percentages of $IL17^+$ of the $CD4^+$ T cell population from diabetic PBMCs stimulated with GAD65 and cultured without (black bar) or with MSC-derived MVs (grey bar). $*p < 0.05$ for unpaired and paired data. Data represent the mean of three experiments for each responder patient

Effect of MSC-derived MVs on Tregs and Th17 cells

In three diabetic patients responding to GAD65, flow cytometry analysis showed that MVs significantly increased the proportion of $CD127^{low}$, $FoxP3^+$ T cells (gated on $CD4^+$ $CD25^{high}$) compared with cultures without MVs ($p < 0.05$ for unpaired and paired data) (Fig. 5g–i). MVs significantly lowered the percentage of IL-17-positive T cells within the $CD4^+$ population compared with cultures without MVs ($p < 0.05$ for unpaired and paired data) (Fig. 5j, k). These effects were also observed in PBMCs stimulated with PV (ESM Fig. 1).

Discussion

MSCs have emerged as promising tools for tissue regeneration after acute or chronic injury and have immunomodulatory potential [5, 21, 22]. In the context of diabetes research, several capabilities of MSCs may therefore be exploited [7, 23–25]. In the present study, we demonstrated that MVs derived from heterologous human bone-marrow MSCs mimic the immunomodulatory

properties of MSCs in type 1 diabetes [7, 26]. This effect was observed not only with GAD but also with a recall antigen, suggesting that MVs interfere with an antigen-elicited immune response. The non-antigen-specific immunoregulatory property does not limit the translation of MSCs to potential therapeutic approaches in several autoimmune diseases and in transplantation [3, 4, 26–28]. In the present study, MVs consistently downregulated in vitro Th1 responses, detected as IFN-γ production, and the number of Th17 cells and levels of proinflammatory IL-17. Th17 effector cells participate in type 1 diabetes pathways, paralleling Th1 cells, and their secreted signature cytokine IL-17 contributes to beta cell death [29]. The immunomodulatory effect of MSC-derived MVs appears to be specific since fibroblast-derived MVs did not significantly inhibit the IFN-γ T cell response.

Previous studies demonstrated that MVs, after receptor-mediated internalisation in target cells and/or membrane fusion, transfer proteins and genetic material, including functional mRNA and miRNA, involved in the control of transcription, proliferation and immunoregulation [12, 15, 18]. In the present study, we found that the immunomodulatory action of MVs depended, at least in part, on their internalisation by PBMCs, as it was reduced by integrin blockade. The involvement of multiple surface molecules is conceivable. The main effects of MVs were on inhibition of antigen-driven inflammatory effector T cell activation and induction of an anti-inflammatory Treg phenotype.

T cells, upon interaction with MVs, produced PGE_2 and TGF-β, known to be involved in the effects of MSCs [28, 30], in an autocrine loop mediating and propagating the immunomodulation. The crucial role of these factors was supported by reduction of the immunomodulatory property of MVs by specific inhibitors, as seen for MSCs [30]. TGF-β was also conveyed as mRNA and as protein within and on the surface of MVs thus acting as a sustained signal upon contact with recipient cells [31]. TGF-β mediates inhibition of lymphocyte proliferation [32–34] and promotes Treg generation [35–37]. Previous studies indicated that MSC-derived MVs express miRNA, including miR-21 known to enhance TGF-β signalling [17, 38]. In the present report, RNA depletion of MVs reduced TGF-β transcripts in PBMCs; this is suggestive of a transfer to the target cells of mRNA or miRNA capable of inducing TGF-β. Thus, after contact with MVs and/or their internalisation, T cells may enhance the TGF-β pathway and TGF-β release in a paracrine/autocrine manner.

Data in experimental autoimmune diabetes strongly suggests a shift towards an anti-inflammatory profile in response to MSC treatment [8, 39–41], partially mediated by the negative co-stimulatory PD1/PD-L1 pathway [42, 43]. In the setting of experimental autoimmune encephalomyelitis, MSC-derived MVs have recently been shown to potentially inhibit autoreactive lymphocyte proliferation and induce tolerogenic signalling, via induction of PD-L1, TGF-β, IL-10 and Tregs [44]. Our report indicates that MSC-derived MVs may restore Th1/Th2 balance and preserve Tregs in type 1 diabetes. MVs increased the production of the regulatory cytokine IL-10 and induced higher frequencies of Foxp3+ phenotype Tregs. A strong relationship exists between Tregs and Th17 cells, with fully differentiated Th17 cells convertible in T cells with immunosuppressive activity in the presence of MSCs and an inflammatory environment [45, 46].

Furthermore, IL-6 production by PBMCs was increased in presence of MVs. IL-6 is a cytokine that has conflicting effects in type 1 diabetes [47]. It has been shown that IL-6 suppresses maturation of inflammatory dendritic cells and mediates beta cell repair induced by MSCs [41, 43].

MSCs represent a potential non-antigen-specific immunoregulatory approach to type 1 diabetes prevention/therapy [9]. It is established that an immunomodulatory approach to restore tolerance to beta cells is achievable with a combination of synergic immunotherapies, which include antigen-specific and non-antigen-specific approaches [48]. The present study indicates that some of the

immunomodulatory actions of MSCs, with induction of a tolerogenic T cell phenotype, can be vicariously exerted by MVs in the context of type 1 diabetes. Besides retaining the reparative and immunomodulatory potentials of the cells of origin, MVs might represent a universal immunomodulator in MHC-mismatched recipients, overcoming the potential immunogenicity of MSCs in an allogenic setting. The intrinsic, long-lasting immunoprivilege of MSCs is in fact still being debated [49].

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Contribution statement
MMZ and EF conceived and designed the study, performed experiments and analysed data. MMZ and GC wrote the manuscript. AC, SL, AF, CC, MCD and SB designed and performed experiments and analysed data. MG and GC contributed to the study design and data analysis and oversaw research. AA, MP, PCP and CT. interpreted data and contributed to the discussion. All the authors critically revised the manuscript and approved the final version of the article to be published. GC and MMZ are the guarantors of this work and, as such, had full access to all the data and take responsibility for the integrity of the data and the accuracy of the data analysis.

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