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Immunological modulation following bone marrow-derived mesenchymal stromal cells and Th17 lymphocyte co-cultures

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Abstract

Objective and design The objective of the study is to uncover the influence of human bone marrow-derived mesenchymal stem cells (BM-MSCs) on the generation of Th17 lymphocytes in co-cultures of both BM-MSCs and T cells.

Materials and methods BM-MSCs, characterized according to the international society for cellular therapy (ISCT) criteria, were co-cultured with T cells isolated from peripheral blood. The expression levels of IL-17 receptor, RORγt and IL-23 receptor were evaluated using flow cytometry. The levels of cytokines involved in Th17 immunomodulation were measured using multiplex assay.

Treatment Inflammatory primed and non-primed BM-MSCs were co-cultured with either activated or non-activated T cells either at (1/80) and (1/5) ratio respectively.

Results MSC/T-cell ratio and inflammation significantly influenced the effect of BM-MSCs on the generation of Th17 lymphocytes. Cocultures of either primed or non-primed BM-MSCs with activated T cells significantly induced IL-17A-expressing lymphocytes. Interestingly, the expression of the transcription factor RORγt was significantly increased when compared to levels in activated T cells. Finally, both cell ratio and priming of BM-MSCs with cytokines substantially influenced the cytokine profile of BM-MSCs and T cells.

Conclusion Our findings suggest that BM-MSCs significantly modulate the Th17 lymphocyte pathway in a complex manner.

Keywords Th17 · Cytokines · ROR-γt · MSCs · T cells · IL-23R · Co-culture

Introduction

Mesenchymal stromal cells (MSCs) are multipotent progenitor cells capable of self-renewal and can be induced to differentiate in vitro into multiple cell types [1]. In addition to their plastic adherence properties, they are characterized by specific expression of several markers including

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CD73, CD90, CD105, and lack of the expression CD14, CD19, CD34, CD45, and HLA-DR [2]. By producing several immunoregulatory, cell mobilization and growth factors, MSCs facilitate tissue repair by tissue-resident stem cells [3].

MSCs emerge as an interesting tool to manage inflammation and immunological responses in several diseases as well as to improve the efficiency of hematopoietic stem cell transplantation (HSCT). MSCs were shown to modulate almost all immune cells including monocyte/macrophages, dendritic cells (DC), natural killer (NK) cells, and different subsets of T cells [4]. Importantly, inflammation was reported to be an important regulator of cell biology as it shapes stem cells and stemness during infections and beyond [5]. Thus, MSCs are particularly sensitive to environmental signals such as inflammatory mediators that can alter the immunologic behavior of MSCs, demonstrating the plasticity of their immunomodulatory capacity [3].

T helper 17 (Th17) are a particular subset of T lymphocytes that show pro-inflammatory activities and are highly associated with the development of inflammatory and autoimmune diseases [6]. A multitude of cytokines released during inflammation can influence the shift of naïve CD4+ toward the Th17 lineage pathway [7], particularly, IL-23 signaling via its distinct receptor (IL-23R) which plays a crucial role in the development and maintenance of Th17 cells. In parallel, the retinoic acid receptor-related orphan nuclear receptor (RORγt) was identified as the master transcription factor defining Th17 cells as a distinct lineage [8]. Thus, the main goal of this study is to define the impact of MSCs on the generation of Th17 lymphocytes by analyzing the expression (flow cytometry) of IL-17A, IL-23R as well as RORγt and by evaluating the Th17 cytokine pathway profile (multiplex immunoassay) following the cocultures. Moreover, the influence of the cell ratio used for the co-culture as well as the presence of inflammatory signals for MSCs will be investigated. The MSC/T-cell ratio and the priming of MSCs with inflammatory cytokines are critically important for the immunomodulatory process [9, 10]. MSCs are able to actively adapt their immune-related biology to the environmental context to properly induce the right effect. Our results clearly demonstrated that MSCs substantially influenced the Th17 immune response pathway by modulating the expression and secretion of a network of molecules and cytokines identified as major factors regulating Th17 induction and expansion. The cross-talk between MSCs and Th17 cells and consequent issues seem to be complex and depend on both MSC concentration and priming. These findings should be taken into account when MSCs are considered as immunotherapeutic tools particularly for Th17-associated autoimmune and inflammatory disorders.

Materials and methods

Specimen preparation

Bone marrow was harvested from the sternum of healthy donors ($n=6$) after giving informed written consent. hBM-MSCs were isolated using the classical adhesion method [11]. Briefly, mononuclear cells (MNCs) from the BM aspirate were isolated by density gradient centrifugation (LinfoSep, Biomedics, Madrid, Spain), washed in Hanks' balanced salt solution (HBSS, Lonza Europe, Verviers, Belgium) and seeded at 2×10^4 cells/cm^2 in Dulbecco's modified Eagle's medium with low glucose (DMEM-LG, Lonza) supplemented with 15% fetal bovine serum (FBS, Sigma-Aldrich, Bornem, Belgium), 2 mM L-glutamine and 50 U/mL penicillin (both from Lonza). Cell cultures were incubated at 37 °C in a 5% CO_2 humidified atmosphere. After 48 h, non-adherent cells were removed by washing, and the medium was changed twice a week. When sub-confluence (80–90%) was achieved, adherent cells were harvested using TripLE Select solution (Lonza) and expanded by replating at a lower density (1000 cells/cm2).

Characterization of hBM-MSCs

hBM-MSCs were characterized according to ISCT (International Society for Cellular Therapy) criteria [2]. Their immunophenotype was established by flow cytometry (MACSQuant, Miltenyi Biotec, Netherlands) using the following monoclonal antibodies: anti-CD45-FITC and anti-HLA-DR-PE (Exalpha Biologicals, Maynard, MA), anti-CD34-PE and anti-CD73-PE (BD Biosciences, San Diego, CA, USA), anti-CD14-PE, anti-CD19-PE, anti-CD105-FITC and anti-CD90-PE (R&D systems, Minneapolis, MN, USA). The multilineage potential of hBM-MSCs was confirmed through inducing their differentiation into adipogenic, osteogenic and chondrogenic lineages using the appropriate culture conditions (NH media, Miltenyi Biotec) as previously described [11].

Priming of hBM-MSCs with inflammatory cytokines

hBM-MSCs were primed overnight using a cocktail of proinflammatory cytokines, specifically 25 ng/mL IL-1β, 103 U/mL IFN-γ, 50 ng/mL TNF-α and 3 × 103 U/m IFN-α (all from Peprotech, Rocky Hill, NJ, USA).

Co-culture assays

Co-cultures of hBM-MSCs (250,000) vs and T cells (3000) were performed as previously described [12]. Mononuclear

cells were obtained by Ficoll-Hypaque gradient centrifugation of peripheral blood from healthy donors that provided an informed consent ($n=6$). Purification of T cells (> 95% purity) was performed using positive selection (MACS system; Miltenyi Biotec). Activation of T lymphocytes was accomplished using mitogenic stimuli [phytohemagglutinin (PHA, 5 µg/mL; Remel) and interleukin-2 (IL-2, 20 U/mL; Biotest AG)]. Activated T cells and hBM-MSCs (either primed with inflammatory cytokines or not) were co-cultured for 5 days in 1/80 and 1/5 cell ratio.

Th17 cell phenotyping

The percentage of Th17 T cells during the co-culture with MSCs was determined by flow cytometry analysis using the Human Th17 Phenotyping Kit (BD Pharmingen™) and according to the manufacturer's protocol.

ROR-γt expression

The expression level of ROR-γt in Th17 cells following their co-cultures with hBM-MSCs was analyzed by flow cytometry using the human/mouse RORγt/RORC2/NR1F3 APC-conjugated antibody according to the manufacturer's protocol (R&D Biosystems).

IL-23 receptor expression

The expression level of IL-23 receptor (IL-23R) in Th17 cells following their co-cultures with hBM-MSCs was analyzed by flow cytometry using the Human IL-23R PE-conjugated antibody according to the manufacturer's protocol (R&D Biosystems).

Cytokine multiplex immunoassay

The supernatants from the different culture conditions were collected and frozen at -20 °C for measuring the cytokines involved in the Th17 cell immune response pathway. The following cytokines (IL-1β, IL-4, IL-6, IL-10, IL-17A, IL-17F, IL-21, IL-22, IL-23, IL-25, IL-31, IL-33, IFNγ, soluble CD40 ligand (sCD40L), and TNFα) were measured using the Bio-Plex Pro™ Human Th17 Cytokine Assays® (Bio-Rad Laboratories, Inc.). This multiplex format enables robust and reproducible cytokine measurement. Briefly, samples were diluted (1:10) with the appropriate solution and then centrifuged at 10,000×*g* for 5 min. 50 µL of the supernatant was used for the cytokine assay according to the manufacturer's instructions.

Statistics

Results are expressed as the mean ± standard error of the mean (SEM) of at least five independent experiments, each performed in duplicate. Statistical analysis was performed by analysis of variance (ANOVA). Bonferroni post hoc test was used for multiple comparisons between groups. Results are considered significant when $P<0.05$ and statistical significance was set at *0.05, **0.01, ***0.001. All analyses used GraphPad Prism software version 5 (USA).

Results

Characterization of hBM-MSCs

hBM-MSCs were characterized according to the criteria of the ISCT [2]. MSCs showed fibroblastic shape with plastic adherence capacities during culture. Regarding their multilineage potential, they were able to differentiate into osteoblasts, chondrocytes and adipocytes. Furthermore, MSCs presented a specific immunophenotype by expressing the mesenchymal markers CD73, CD90 and CD105, and lacking expression of hematopoietic or immune markers including CD45, CD14, CD19, CD34 and HLA-DR (data not shown).

IL-17A expression is differentially modulated by MSCs

The influence of MSCs cultured at two cell ratios (1/80 vs 1/5) and primed or not with inflammatory cytokines on the generation of Th17 cells was investigated. Flow cytometry analysis of IL-17A expression was used as an indication of Th17 cell generation from PHA/IL-2-activated T cells. The expression levels of IL-17A were significantly increased in PHA/IL-2-activated T cells (0.82666667 ± 0.10405) when compared to un-activated T cells (0.201666 ± 0.03763). Modulation of IL-17A expression and, therefore, Th17 cell generation are likely depending on cell ratio of the co-culture and significantly increase when MSCs are primed with inflammatory cytokines. Constitutively, MSCs at (1/80) cell ratio allowed a significant reduction of IL-17A expression (0.263333 ± 0.0625) by PHA/IL-2-activated T cells, whilst at (1/5) cell ratio MSCs substantially increased the IL-17A expression levels (1.46166 ± 0.1123). In the primed groups, only MSCs at (1/5) cell ratio were able to further enhance IL-17A expression ($2.1483 \pm 0.0.744$). Optimal and efficient increase in IL-17A expression as induced by primed MSCs at high cell ratio may, therefore, indicate a possible expansion of IL-17 cells (Fig. 1a).

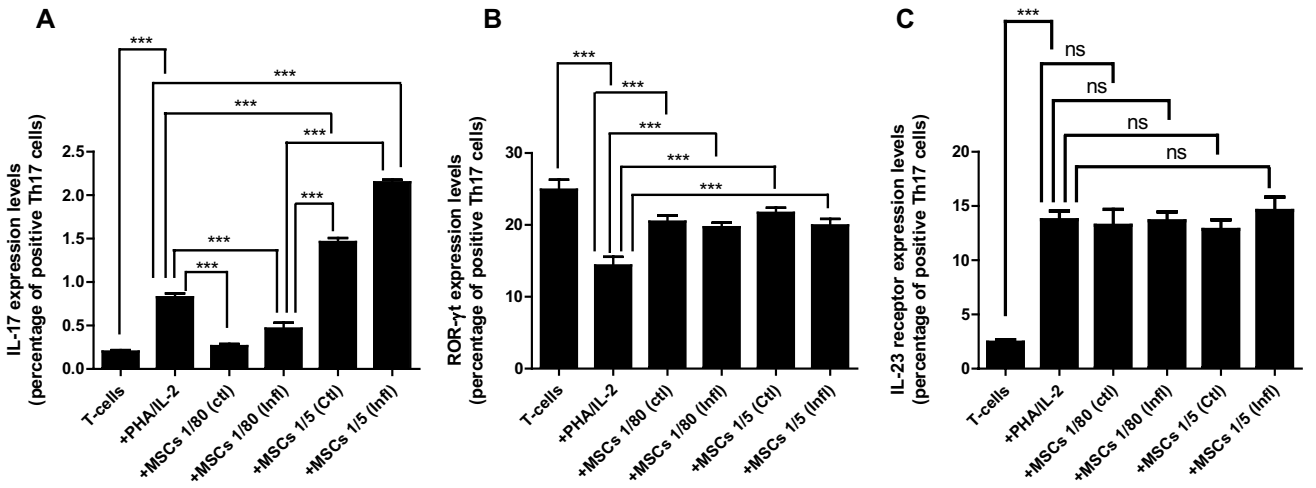

Fig. 1 MSCs modulated the levels of Th17 generation as well as IL-23 receptor and RORγt expression in MSC:T cell co-cultures. Un-activated or inflammatory-primed MSCs with cytokines are co-cultured with either un-activated or PHA/IL-2 activated T cells at low (1/5) or high (1/80) ratio as indicated. Then levels of IL-17A (a), RORγt (**b**), and IL-23 (**c**) receptor expression, which represent the levels of percentage of positive Th17 cells, were analyzed by flow cytometry (mean ± SEM from six independent experiments). $*P<0.05$, $**P<0.01$, $***P<0.01$ vs the corresponding control

ROR-γt expression is consistently promoted by MSCs

We then evaluated the impact of MSCs cultured at two cell ratios (1/80 vs 1/5) and primed or not with inflammatory cytokines on the expression of ROR-γt known as the master regulator of Th17 cell development. Flow cytometry analysis demonstrated that in PHA/IL-2-activated T cells, RORγt expression level (14.355 ± 2.9470) is significantly lower than that of un-activated T cells (24.9216 ± 3.3606) suggesting that T-cell activation seems to inhibit the expression of ROR-γt. Independently of the cell ratio used in the co-culture as well as their priming with inflammatory cytokines, MSCs constantly induced a clear increase of RORγt expression in PHA/IL-2-activated T cells. By promoting the expression of their master transcription factor, MSCs are likely favoring the shift of T cells toward a specific Th17 subset lineage (Fig. 1b).

IL-23 receptor expression is not modulated by MSCs

We further determined the effect of MSCs cultured at two cell ratios (1/80 vs /5) and primed or not with inflammatory cytokines on the expression of IL-23 receptor (IL-23R) which mediate the signaling of IL-23 cytokine important for the maintenance of Th17 cells. Flow cytometry analysis showed that T-cell activation greatly induced the expression IL-23R as shown by the high expression in PHA/IL-2-activated T cells (13.74 ± 1.983) compared to un-activated T cells (2.443 ± 0.5857). Interestingly, IL-23R expression in PHA/IL-2-activated T cells was not modulated by MSCs regardless of the cell ratio used in the co-culture as well as their inflammatory priming. Such observation infers that PHA/IL-2-activated T cells are already engaged toward the Th17 cell pathway and MSCs favor the maintenance of Th17 cells (Fig. 1c).

The Th17 cell cytokine pathway profiling

Finally, we used immunoassay strategy to analyze the expression profile of T cells and MSCs for cytokines mainly involved in the Th17 immune response pathway. The importance of the cell ratio (1/80 vs high 1/5), inflammatory priming of MSCs and co-cultures of both population on such profile were also investigated.

T cells

Except IL-25 and IL-33, activated T cells highly produced most of cytokines associated with Th17 immune response pathway. PHA/IL-2-activated T cells when compared to un-activated T cells, respectively, produced a highly significant amount of IL-1β (17.67 ± 1.55; 0.37 ± 0.09), IL-4 (547.28 ± 38.38; 0 ± 0), IL-6 (648.785 ± 68.02; 8.98 ± 1.57), IL-10 (287.83 ± 43.16; 0 ± 0), IL-17A (280.91 ± 36.18; 4.05 ± 1.25), IL-17F (312.92 ± 23.77; 7.43 ± 1.23), IL-21 (241.41 ± 33.42; 36.43 ± 5.30), IL-22 (169.61 ± 22.07; 7.60 ± 1.65), IL-23 (103.89 ± 3.77 ; 53.53 ± 5.34), IL-31 (678.715 ± 39.1366; 5.1975 ± 0.890), IFN-γ (4708 ± 539.96; 24.04 ± 3.76), sCD40 (817.7475 ± 86.8919; 18.10 ± 4.17) and TNF-α (1437.915 ± 196.617; 45.0975 ± 7.8442). In contrast, the levels of IL-25 (1.76 ± 0.4) and IL-33

	IL-1β (pg/ml)		IL-4 (pg/ml)		IL-6 (pg/ml)		IL-10 (pg/ml)		IL-17A (pg/ml)		IL-17F (pg/ml)		IL-21 (pg/ml)	
BM-MSCs	Mean	STDEVPA	Mean	STDEVPA	Mean	STDEVPA	Mean	STDEVPA	Mean	STDEVPA	Mean	STDEVPA	Mean	STDEVPA
UST	0.37	0.09	0	0	8.98	1.57	0	0	4.05	1.25	7.43	1.23	36.43	5.30
ST	17.67	1.55	547.28	38.38	648.785	68.02	287.83	43.16	280.91	36.18	312.92	23.77	241.41	33.42
MSCs (1/80)	0.00	0.00	0	0	678.89	37.17	0	0	0	0	11.19	4.88	13.12	3.82
MSC^{i} (1/80)	73.99	4.50	7.27	0.53	23905.71	671.23	10.63	1.88	7.74	0.90	62.10	4.45	238.56	11.95
ST + MSCs (1/80)	34.98	2.58	268.68	51.68	24048.69	636.77	367.59	56.77	392.00	31.84	945.03	42.83	403.80	95.98
ST + MSCs^{i} (1/80)	160.64	7.11	57.50	8.16	24232.74	641.38	464.50	50.07	343.64	16.14	909.12	91.24	417.41	62.10
MSCs (1/5)	1.35	0.19	0	0	3851.02	508.18	0	0	2.77	0.65	47.33	6.84	107.10	22.62
MSCs^{i} (1/5)	111.01	5.25	7.21	0.26	23693.91	819.97	7.72	0.43	7.00	0.86	66.49	1.46	191.39	8.44
ST + MSCs (1/5)	154.46	5.51	70.08	11.53	24444.34	134.13	866.95	73.54	403.65	91.35	920.05	105.56	318.96	49.72
ST + MSCs^{i} (1/5)	197.21	6.89	80.55	14.11	24181.53	571.37	411.09	65.29	447.65	62.82	813.53	89.06	371.62	37.76

IL-22 (pg/ml)		IL-23 (pg/ml)		IL-25 (pg/ml)		IL-31 (pg/ml)		IL-33 (pg/ml)		sCD40 (pg/ml)		TNF-α (pg/ml)		IFN-γ (pg/ml)	
Mean	STDEVPA	Mean	STDEVPA	Mean	STDEVPA	Mean	STDEVPA	Mean	STDEVPA	Mean	STDEVPA	Mean	STDEVPA	Mean	STDEVPA
7.60	1.65	53.53	5.34	0	0	5.197	0.89	0	0	18.10	4.17	45.0975	7.84	24.04	3.76
169.61	22.07	103.89	3.77	1.76	0.40	678.71	39.136	11.25	2.39	817.74	86.89	1437.91	196.61	4708.20	539.96
0	0	14.78	4.37	0	0	0	0	0	0	10.99	4.57	0	0	3.73	2.08
0	0	108.52	13.69	2.67	0.37	239.03	47.19	0	0	109.72	1.84	261.76	75.96	1634.77	70.95
65.51	6.12	145.32	2.66	3.06	0.54	606.92	86.18	0	0	677.19	140.12	668.21	94.31	3639.93	454.37
53.50	3.63	151.00	1.30	3.30	0.74	709.09	92.88	0	0	686.25	156.19	1281.07	186.33	5967.38	800.06
0	0	12.31	2.39	1.69	0.37	9.79	4.47	0	0	64.48	7.68	0	0	18.50	8.07
0	0	106.48	7.47	2.54	0.005	176.72	28.51	0	0	89.64	6.46	198.89	42.42	1766.21	591.15
45.92	8.33	132.02	15.39	3.41	0.35	313.05	28.88	0	0	487.12	47.81	221.96	26.63	2894.36	587.28
48.06	2.59	144.39	10.96	3.48	0.44	550.36	72.81	0	0	520.15	36.61	419.62	92.96	4850.98	705.96

Fig. 2 Cytokine profile released in (1/5) and (1/80) MSC:T-cell ratio co-cultures and known to modulate Th17 activity. Un-activated or inflammatory-primed MSCs with cytokines are co-cultured with either un-activated or PHA/IL-2 activated T cells at low (1/5) or high (1/80) ratio as indicated. Then levels of the cytokine profile released by co-cultures were analyzed as indicated in "Materials and methods" (mean ± STDEVPA from at least five independent experiments)

(11.25 ± 2.39) were considerably low in PHA/IL-2-activated T cells (Figs. 2, 3).

MSCs

MSCs in contrast to activated T cells do not constitutively produce a wide range of Th17-associated cytokines. In both un-primed MSCs (1/80) and (1/5) cultures, the levels of IL-1β, IL-4, IL-10, IL-17A, IL-22, IL-25, IL-31, IL-33, IFN-γ, and TNF-α were similarly negligible. In contrast, un-primed MSC (1/5) cultures when compared to un-primed MSC (1/80) cultures showed highly significant amount of IL-6 (3851.02 ± 508.18; 678.89 ± 37.17), IL-17F (47.33 ± 6.84; 11.19 ± 4.88), IL-21 (107.10 ± 22.62; 13.12 ± 3.82), and sCD40 (64.48 ± 7.68; 10.99 ± 4.57), respectively. However, MSCs (1/5) and (1/80) produced similar detectable amount of IL-23 (12.31 ± 2.39; 14.78 ± 4.37), respectively (Figs. 2, 3).

Inflammation priming of MSCs

Inflammation clearly induced the secretion of a selective panel of cytokines related to Th17 cell pathway. Both inflammatory-primed MSCs (1/80) and (1/5) when compared to un-primed (1/80) and (1/5), respectively, displayed highly significant levels of IL-1β (73.99 ± 4.50; 111.01 ± 5.25), IL-6 (23905.71 ± 671.23; 23693.91 ± 819.97), IL-17F (62.10 ± 4.45; 66.49 ± 1.46), IL-21 (238.56 ± 11.95; 191.39 ± 8.44), IL-23 (108.52 ± 13.69; 106.48 ± 7.47), IL-31 (239.03 ± 47.19; 176.72 ± 28.51), IFN-γ (1634.77 ± 70.95 ; 1766.21 ± 591.15), sCD40 (109.72 ± 1.84; 89.64 ± 6.46) and TNF-α (261.76 ± 75.96; 198.89 ± 42.42). On the other hand, the cytokines levels of IL-4 (7.27 ± 0.53; 7.21 ± 0.26), IL-10 (10.63 ± 1.88; 7.72 ± 0.43), IL-17A (7.74 ± 0.90; 7.00 ± 0.86), IL-22 (0), IL-25 (2.67 ± 0.37; 2.54 ± 0.005) and IL-33 (0.00; 0.00) were extremely low and comparable to those of un-primed MSCs (Figs. 2, 3).

Co-cultures of MSCs and activated T cells

The profile of the cytokine associated with the Th17 cell pathway was substantially modulated following the co-cultures of MSCs and activated T cells. Thus, both increase and decrease of distinct cytokines were observed. Moreover, the changes in the cytokine levels during these co-cultures were also greatly influenced by the cell ratio used and the inflammatory priming of MSCs.

(1/80) cell ratio co-cultures of either un-primed or inflammatory-primed MSCs with PHA/IL-2-activated T cells, respectively, demonstrated differentially altered cytokine

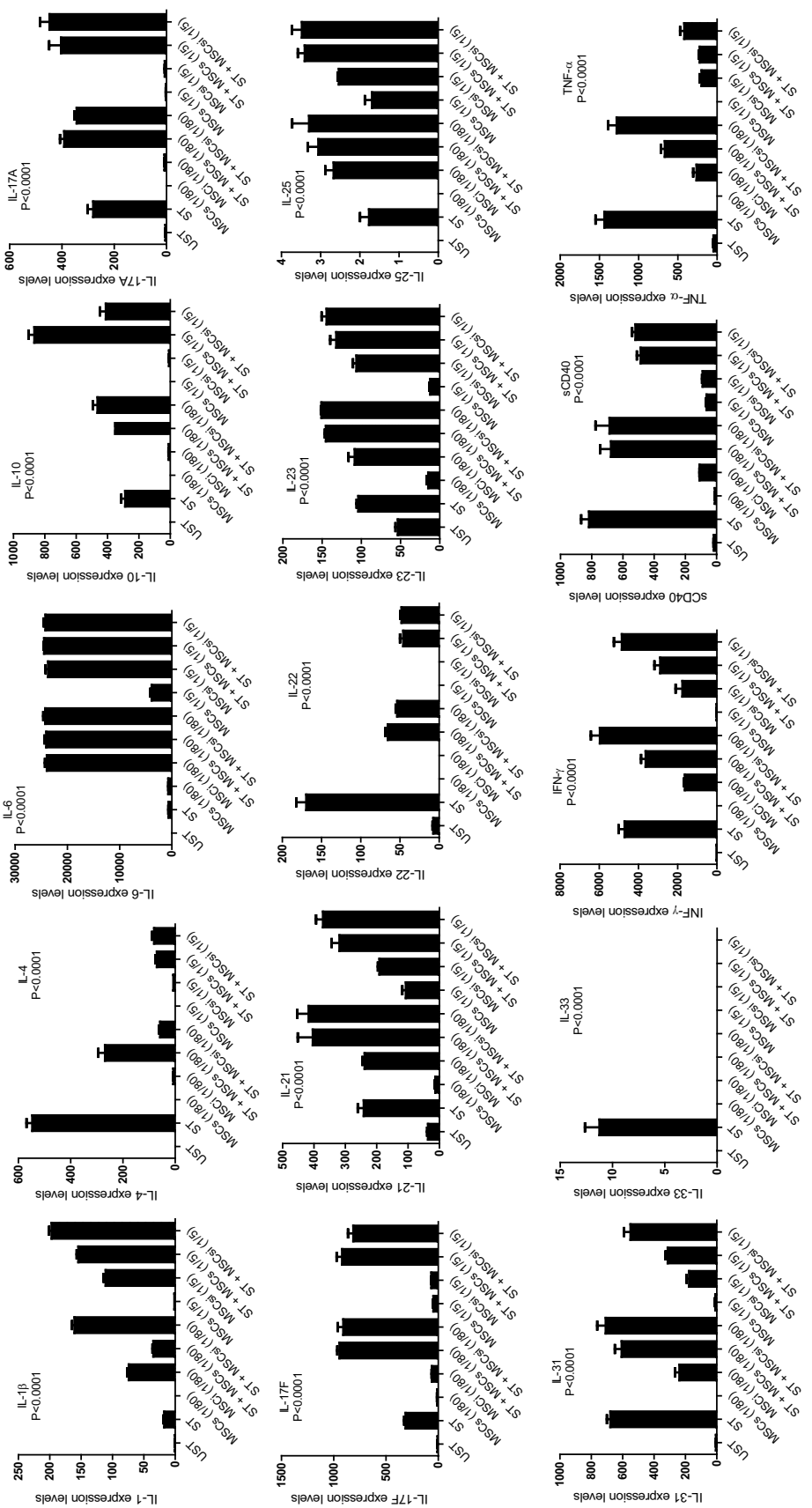

Fig. 3 Cytokine profile released in (1/5) and (1/80) MSC:T-cell ratio co-cultures and known to modulate Th17 activity. Un-activated or inflammatory-primed MSCs with cytokines are co-cultured with either un-activated or PHA/IL-2 activated T cells at low (1/5) or high (1/80) ratio as indicated. Then levels of the cytokine profile released by co-cultures were analyzed as indicated in "Materials and Methods". $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.01$ vs the corresponding control

profiles. The levels of IL-1β (34.98 ± 2.58; 160.64 ± 7.11), IL-10 (367.59 ± 56.77; 464.50 ± 50.07), IL-21 (403.80 ± 95.98; 417.41 ± 62.10), IL-23 (145.32 ± 2.66; 151.00 ± 1.30), IL-17A (392.00 ± 31.84; 343.64 ± 16.14), IL-17F (945.03 ± 42.83; 909.12 ± 91.24) and IFN-γ (5967.38 ± 800.06) within the co-cultures were consistently increased when compared to PHA/IL-2-activated T cells alone. As noted, the highest increase in cytokine production during the co-culture was generally observed with MSCs primed with inflammatory cytokines. In contrast, the levels of IL-4 (268.68 ± 51.68; 57.50 ± 8.16), IL-22 (65.51 ± 6.12; 53.50 ± 3.63), IFN-γ (3639.93 ± 454.37) and TNF-α (668.21 ± 94.31; 1281.07 ± 186.33) during the co-culture of both populations were significantly reduced when compared to PHA/IL-2-activated T cells. Both un-primed MSC (1/80) cultures and PHA/IL-2-activated T cells produced similar IL-6 (678.89 ± 37.17; 648.785 ± 68.02) levels, while MSCs (1/80) cultures primed with inflammatory cytokines showed a significantly higher amount of IL-6 when compared to PHA/IL-2-activated T cells, respectively [(23905.71 ± 671.33; 648.785 ± 68.02)]. Also, co-cultures of either un-primed or inflammatory-primed MSCs with PHA/IL-2-activated T cells at (1/80) cell ratio showed similar levels of IL-6 when compared to inflammatory-primed MSCs (1/80), respectively [(24048.69 ± 636.77; 23905.71 ± 671.33) (24232 ± 641.38; 23905.71 ± 671.33)]. Of note, levels of IL-31 (606.92 ± 86.18; 709.09 ± 92.88) and sCD40 (677.19 ± 140.12; 686.25 ± 156.19) resulting from the co-cultures remained comparable to levels in PHA/IL-2-activated T cells alone.

(1/5) cell ratio co-cultures of either un-primed or inflammatory-primed MSCs with PHA/IL-2-activated T cells also. respectively, showed differentially altered cytokine profiles. Thus, a significantly higher production levels of IL-1β (154.46 ± 5.51; 197.21 ± 6.89), IL-10 (866.95 ± 73.54; 411.09 ± 65.29), IL-17A (403.65 ± 91.35; 447.65 ± 62.82), IL-17F (920.05 ± 105.56; 813.53 ± 89.06), IL-21 (318.96 ± 49.72; 371.62 ± 37.76) and IL-23 (132.02 ± 15.39; 144.39 ± 10.96) are observed in the co-cultures when compared to PHA/IL-2-activated T cells alone. For IL-4 (70.08 ± 11.53; 80.55 ± 14.11), IL-22 (45.92 ± 8.33; 48.06 ± 2.59), IL-31 (313.05 ± 28.88; 550.36 ± 72.81), IFN-γ (2894.36 ± 587.28), sCD40 (487.12 ± 47.81; 520.15 ± 36.61) and TNF-α (221.96 ± 26.63; 419.62 ± 92.96), the expression levels were significantly reduced when compared to PHA/IL-2-activated alone. Interestingly, the IFN-γ expression level remained unchanged in (1/5) ratio co-cultures of inflammatory-primed MSCs with PHA/IL-2-activated T cells (4850.98 ± 705.96), when compared to levels in PHA/IL-2 activated T cells alone. Un-primed MSC (1/5) cultures produced significantly higher levels of IL-6 when compared to PHA/IL-2-activated T cell levels (3851.0.2 ± 508.18; 648.785 ± 68.02), but were significantly lower than IL-6 levels produced by inflammatory-primed MSCs (648.785 ± 68.02; 23693.91 ± 819.97). Also, co-cultures of either un-primed or inflammatory-primed MSCs with PHA/IL-2-activated T cells at (1/5) cell ratio showed similar levels of IL-6 when compared to inflammatory-primed MSCs (1/5), respectively [(24444.34 ± 636.77; 23693.91 ± 819.97) (24181 ± 571.37; 23693.91 ± 819.97)]. The levels of IL-33 and IL-25 (3.41 ± 0.35; 3.48 ± 0.44) remained very low in both co-cultures without any influence of inflammation priming of MSCs (Figs. 2, 3).

Discussion

Data on MSC modulation of Th17 cells have been reported but are lacking clear evaluation and understanding of the different and multiple factors that could affect such interactions [13, 14]. The mechanism by which MSCs exert their regulatory effects in vivo remains largely unknown thus limiting both the general interest and the clinical impact of these studies [15].

Strong body of evidence consider the immunomodulatory properties of MSCs as adjunctive tools for successful organ/tissue transplantation [16]. However, understanding the signals guiding the interactions with the host immune system is necessary to optimize the functions of MSCs and, therefore, enhance their therapeutic value [17]. Compelling evidences have thus shown that T cell subsets respond differently to MSCs [18]. Th17 cell subset is of particular interest since it plays an important role many autoimmune and inflammatory diseases [19]. Thus, Th17 cell modulation by MSCs might be proposed as a therapeutic strategy.

Th17 cells are IL-17-producing CD4 + T-cell subpopulation characterized by their ability to release IL-17A, IL-17F, IL-21, and IL-22 [6, 14]. Interestingly, our data showed that MSC:T-cell ratio greatly influenced IL-17A expression levels with strong IL-17A induction being obtained with (1/5) MSC:T-cell ratio. Furthermore, additional increased in IL-17A was observed with co-culture containing inflammatory-primed MSCs. The latter increase in IL-17A is only observed in (1/5) MSC:T-cell ratio, while (1/80) MSC:T-cell ratio significantly reduced IL-17A expression regardless of the inflammatory environment. Co-culture with MSCs substantially increased the levels of IL17A and IL-17F secretion regardless of the cell ratio or inflammatory priming. Th17 response were reported to be modulated by adipose tissue (AT)-MSCs in a passage-dependent fashion [19], or when MSCs were pretreated with IL-1β [20]. Moreover, Th17 cells stimulate other cells to release cytokines IL-21 and IL-23 that in turn, can induce differentiation or development of Th17 cells [21].

The retinoic acid receptor-related orphan nuclear receptor (ROR-γt) and IL-23R play key roles in differentiation of T

cells into Th17 through their ability to control the expression of IL-17A, IL-17F and IL-23 [8, 13] . Furthermore, the differentiation of naïve T cells into Th17 is largely regulated by cytokines that specifically induce RORγt expression by activating STAT3 pathway [22]. Thus, Th17-derived cytokines and RORγt maintain a positive regulatory loop to amplify the Th17 response. Our data demonstrated that MSCs are able to increase ROR-γt expression levels regardless of the MSC:T-cell ratio or the inflammatory environment suggesting that MSCs may constitutively favor and maintain Th17 differentiation. Indeed, an increase in ROR-γt expression may lead to an increase in Th17 pro-inflammatory cytokines and thus sustain the loop that promotes Th17 pathway. Therefore, MSC modulation of RORγt expression known to be associated with Th17 polarization and survival will greatly influence a Th17 shift and may thus promote inflammation.

IL-23 induces the differentiation of naive CD4+ T cells into highly inflammatory helper T cells (Th17/ThIL-17) that produce IL-17, IL-17F, IL-6, and TNF-α, but not IFN-γ and IL-4 [7]. The signaling of IL-23 is driven by IL-23 receptor (IL-23R), which is highly expressed by naive memory T cells [23]. In our model, there was no detectable influence of MSCs either used at different cell ratio or primed by inflammatory cytokines on IL-23 receptor expression. Moreover, we observed that the level of IL-23 secretion was only slightly increased during the co-culture of MSCs and T cells compared to the other cytokines. Similar results with fetal MSCs have reported that the levels of IL-23 remain unchanged during the augmentation of Th17 cells. These data suggest that IL-6 and IL-1, instead of IL-23, may be partly involved in the expansion of Th17 cells [24]. The fact that IL-23 receptor expression was not reduced during the co-cultures could be correlated to the reduced levels of IL-4 but enhanced IFN-γ levels in such conditions. IL-2, IFN-γ, and IL-27 signaling are reported to block Th17 differentiation, but the underlying mechanism remains unclear and possibly related to T-bet repressor activity. Of note IL-4 and IFN-γ negatively regulate IL-23 receptor expression and Th17 cells were efficiently generated in the presence of TGF-β1 and IL-6 that induce IL-23 receptor expression [13]. MSCs by primarily enhancing RORγt expression and maintaining high IL-23R expression unchanged are likely promoting initial polarization of Th17 cells and, therefore, their expansion. Indeed, IL-23 signaling is not required for the initial Th17 polarization but instead stabilizes and expands pathogenic Th17-polarized cells.

MSCs mainly exert their effects through the release of soluble mediators that regulate immune effector cell functions and subsequently control the immune response. For this reason, we have performed Th17 cytokine profiling to characterize the cytokines involved in Th17 pathway response. TGF-β and inflammatory cytokines, such as IL-6, IL-21, IL-1β, and IL-23, play central roles in the generation of Th17 cells [25]. Being secreted by activated T cells, IL-1β, IL-6, IL-21 and IL-23 were substantially induced in inflammatory-primed MSCs. In a dose-dependent manner, co-cultures have also demonstrated increased levels of these cytokines that are sufficient to induce the differentiation of human Th17 cells from naïve T cells. Moreover, elevated IL-1 and IL-6 are important for enhancing the expansion of differentiated and memory Th17 cells. The in vivo milieu must also be taken into account, for increased IL-6 production could theoretically enhance inflammation by promoting effector immune cell differentiation, as in the case of IL-17A-producing T cells [26]. The role of MSC-derived IL-6 is likely to be more complex [12], as up-regulation of IL-6 secretion by MSCs may favor Th17 differentiation aggravating the inflammation response [27]. In contrast, is it required to reduce inflammation by switching the host response from a Th1/Th17 towards a Th2 immune profile in an IL-6-dependent PGE2 secretion-dependent manner [9]. Furthermore, fetal MSCs added to PHA/IL-2 activated T cells at the beginning of cultures and at 1:10 cell ratio, promoted the expansion of human Th17 cells corroborating thus our results. In the presence of pro-inflammatory cytokines, MSCs in parallel to their constitutive TGF-β production secrete significant levels of IL-6, supporting thus RORγt expression and development of Th17 cells [28]. Consistent with the augmentation of Th17 cells, significantly higher levels of IL-6 and IL-1 were observed in co-culture [24]. Within the inflamed joints, MSC was shown to phagocytose apoptotic cells (AC) from rheumatoid arthritis (RA) enhancing thus MSC osteogenic differentiation by increasing IL-17 and RANKL expression on CD4 + T cells [18]. Interestingly, osteoclasts formation and bone resorption were associated with the induction of Th17 cells following increased IL-6 production. The levels of IL-4 and IFN-γ are specifically high in activated T cells compared to their insignificant presence in MSCs even after inflammatory priming. The generation of specific CD4+ T-cell immune responses is reinforced by the ability of both IFN-γ and IL-4 to act in regulatory feedback loops such that they promote further expansion of Th1- and Th2-type cells, respectively. However, in our settings, the levels of IFN-γ and IL-4 were considerably reduced during the co-culture indicating a shift towards another T-cell subset. CD40 is a member of the tumor necrosis factor receptor (TNFR) superfamily, which is constitutively or inducibly expressed on the surface of a variety of immune and non-immune cells. Its ligand, CD40L (also known as CD154), is a type II transmembrane protein belonging to the TNF superfamily and is transiently expressed on the surface of activated CD4 + T cells, but can also be up-regulated on other cell types in the context of autoimmune disease [29]. The CD40/CD154 couple plays a critical role in the activation of both humoral and cellular

immune responses. A natural antagonist of CD40/CD154 interaction is the soluble form of CD40 (sCD40) which has been shown to inhibit the binding of CD154–CD40 in vitro [15]. Our results clearly indicated that activation of T cells leads to a significant release of sCD40 compared to the slight effect of inflammation on MSCs. However, co-culture demonstrated a reduced release of sCD40 in a MSC dose-dependent way and regardless of inflammation priming. It has been reported that activated monocytes from both healthy controls and rheumatoid arthritis (RA) patients induce Th17 responses in an IL-1β/TNFα-dependent fashion [30]. Our data showed that activated T cell produced large amount of TNF-α compared to inflammatory-primed MSCs. Importantly, TNF-α levels during MSC and T-cell co-cultures were strongly reduced suggesting an inhibitory effect of MSCs on TNF-α release by activated T cells. Indeed, under inflammatory conditions, skin-MSCs were found to produce high amounts of soluble TNF receptor-1 (sTNFR1), the biologic decoy of TNF-α, which then caused the inhibition of Th17 cell differentiation [31]. It has been also shown that IL-10 can control both Th1 and Th17 immune responses. The level of IL-10 was substantially increased following MSCs and T-cell co-culture suggesting that IL-10-producing Th17 cells prevent overshooting immune responses and the resulting tissue damage in a negative feedback loop. IL-17A-producing CD4+ T cells were found to express high levels of interleukin-10 receptor α (IL-10Rα) and that IL-10 signaling in T cells controlled IL-17A+ IFN-γ− and IL-17A+ IFN-γ+ [32].

IL-21, which is known to control the generation of Th17 cells, in combination with TGF-β, induces IL-17 production from naïve CD4+ T cells as well as the expression of RORγt and IL-23R [13, 33, 34]. On the other hand, IL-6 or IL-21 could induce Th17 cells themselves to produce IL-21. Such endogenous production of IL-21 will function in an autocrine loop to amplify the Th17 response. Furthermore, both IL 21 and IL-6 up-regulated IL-23R, thereby priming Th17 cells to the amplifying and stabilizing effects of IL-23 [14, 35]. By sustaining enhanced levels of Th17-associated cytokines such as IL-6, IL21 and IL-23 and decreasing those of Th-1 and Th-2 response, MSCs create a cytokine milieu with positive loops that favor the differentiation and expansion of activated T cells towards a Th17 population. Conversely, a Th17 pro-inflammatory immune response is, therefore, promoted by MSCs.

In our study, the expression of IL-22, a member of the IL-10 cytokine family, has been specifically restricted to activate T cells as inflammatory-primed MSCs failed to show any induction of it secretion. Surprisingly, co-culture with MSCs significantly reduced the secretion level of IL-22 independently of their cell ratio or inflammatory priming status. IL-22 has protective and regenerative effects and only minor pro-inflammatory effects. Thus, IL-22 seems to be a novel type of immune mediator that increases the innate response of tissue cells, protects tissues from damage, and enhances their regeneration by regulating immunity and inflammation at barrier surfaces [36]. Accordingly, by downregulating the IL-22–IL-22R pathway, MSCs may prevent immune cells from altering the responses and functions tissue-resident stromal cells. Most of IL-17 family members have pro-inflammatory function, while IL-25 has a different responsibility towards the progress of Th2 response. IL-17E (IL-25) is an amplifier of Th2 immune response by promoting the differentiation and effector functions of Th2 cells. IL-25 is capable of regulating immune responses, especially regulation of different types of Th2 cells by controlling the production of several cytokines, chemokines and immunoglobulins [37]. Wang et al. found that MSC-mediated strong Th17 suppression required both IL-25 and CD274 expression [38]. In our setting, the expression of IL-25, mostly known as IL-17E and highly expressed in Th2 cells, was not found in activated T cells or inflammatory-primed MSCs and most importantly unmodulated during their co-culture. Moreover, we have previously demonstrated that the expression of CD274 is only observed when MSCs are primed with inflammatory cytokines [10]. Although being expressed by many cells and tissues, we did not observe any expression and/or induction of IL-33 in our settings. During injury, some of the barrier cells are destroyed and IL-33 is thus released. IL-33, as a guardian of barriers and a local alarmin, is a key initiator of the acute local inflammation and tissue-repair process, and it participates at several points in shaping innate and adaptive immune responses [39]. It is likely that MSCs mediate their natural immunomodulatory and reparative functions independently of IL-33.

In contrast, IL-31 was shown to be highly expressed by activated T cells and also induced in inflammatory-primed MSCs. This cytokine produced mainly by activated CD4+ T cells is a member of the gp130/IL-6 cytokine family that acts on a broad range of immune- and non-immune cells and, therefore, possesses potential pleiotropic physiological functions, including regulating hematopoiesis and immune response [40]. During the co-culture, the secretion level of IL31 was diminished with MSCs at high cell ratio without any significant influence of the inflammation priming. IL-31 was expressed by all of the TH2 clones and not by TH1, TH17, or TH22 in an IL-4-dependent manner. IL-31 was able to induce proinflammatory genes such as CCL2 and granulocyte colony-stimulating factor promoting thus TH2-driven inflammation [41]. Parallel to decreasing the level of IL-4, MSCs are also able to reduce the secretion of IL-31, which collectively may lead to prevent Th-2 response and favor in contrast a shift of T cells toward a Th17 immune response.

In summary, our results show that MSCs may promote the expansion of a pro-inflammatory Th17 phenotype from

activated T cells. It is likely that the activation as well as differentiation state of T cells, the cell ratio, expansion passage and inflammatory priming of MSCs play critical roles in the outcome of these co-cultures. Furthermore, a better understanding of patient heterogeneity, to define patients with an IL-17-driven disease such as during the pathogenesis of inflammatory arthritis may have important issues for the use of MSCs [23]. Thus, long-term side effects associated with potential contribution of MSCs to disease pathogenesis by promoting Th17 response need to be considered when designing future cell therapy strategy as well as the possibility to target some of the key IL-17 pathway components.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This study was conducted in accordance with the Declaration of Helsinki (1964) and the protocol and experiments were approved by the local ethics committee of the "Institut Jules Bordet" (Belgium).

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