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Immunomodulation of endothelial differentiated mesenchymal stromal cells: impact on T and NK cells

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Wharton's jelly mesenchymal stromal cells (WJ-MSCs) are promising candidates for tissue engineering, as their immunomodulatory activity allows them to escape immune recognition and to suppress several immune cell functions. To date, however, few studies have investigated the effect of differentiation of the MSCs on this immunomodulation. To address this question, we sought to determine the impact of differentiation toward endothelial cells on immunoregulation by WJ-MSCs. Following differentiation, the endothelial-like cells (ELCs) were positive for CD31, vascular endothelial cadherin and vascular endothelial growth factor receptor 2, and able to take up acetylated low-density lipoproteins. The expression of HLA-DR and CD86, which contribute to MSCs immunoprivilege, was still weak after differentiation. We then co-cultured un- and differentiated MSCs with immune cells, under conditions of both direct and indirect contact. The proliferation and phenotype of the immune cells were analyzed and the mediators secreted by both ELCs and WJ-MSCs quantified. Interleukin (IL)-6, IL-1β, prostaglandin E2 and in particular indoleamine-2,3-dioxygenase expression were upregulated in ELCs on stimulation by T and NK cells, suggesting the possible involvement of these factors in allosuppression. ELCs co-cultured with T cells were able to generate $CD25^+$ T cells, which were shown to be of the $CD4^+CD25^+FoxP3^+$ regulatory subset. Direct contact between NK cells and ELCs or WJ-MSCs decreased the level of NK-activating receptor natural-killer group 2, member D. Moreover, direct co-culturing with ELCs stimulates CD73 acquisition on NK cells, a mechanism which may induce adenosine secretion by the cells and lead to an immunosuppressive function. Taken together, our results show that ELCs obtained following differentiation of WJ-MSCs remain largely immunosuppressive.

Vascular occlusion remains the leading cause of death in Western countries, despite the availability of several treatments. Among the most widespread is vascular surgery incorporating coronary artery bypass, which requires biologically-responsive vascular substitutes. The shortage of autologous grafts has spurred significant advances in vascular tissue engineering over the last decade. In this context, autologous cells are considered the engineering gold standard, as they are not associated with the immunological and ethical issues of allo- or xenogenic cells. However, autologous cells such as mature endothelial cells (ECs) and smooth muscle cells are inherently heterogeneous and dedifferentiate following prolonged culturing *in vitro*. An attractive alternative is therefore to use allogeneic stem cells, as these boast a high proliferation potential and the ability to differentiate into cells useful for vascular tissue engineering.[1] Mesenchymal stromal cells (MSCs) are multipotent precursors that can be isolated from several tissue sources such as the bone marrow (BM) or adipose tissue and differentiated into multiple lineages under appropriate induction conditions.[2] Among the sources of MSCs, Wharton's jelly (WJ), a neonatal connective tissue of the umbilical cord, is among the most promising.[3] Notably, WJ-MSCs are more primitive than other MSCs and their use does not trigger ethical controversy. Their significant proliferative potential, differentiation capacity and immunomodulatory properties also make them good candidates for deployment in an allogeneic context. Indeed, MSCs are considered to be 'immunoprivileged cells,' because expression on the cell surface of class II human leukocyte antigen (HLA-II) and co-stimulatory molecules is weak.[4] MSCs modulate the immune system through several mechanisms.

They act as immunosuppressants through release of multiple anti-inflammatory and immune-suppressive factors, inhibiting several functions of diverse immune cells such as T, B, dendritic and natural killer (NK) cells[5] including proliferation, production of soluble factors and cellular toxicity. They can also modulate the immune response through the generation of regulatory T cells (Tregs).[6] Immune suppression by MSCs appears to involve both direct cell-to-cell interactions and contact-independent mechanisms such as secretion of soluble factors.[7] Direct contact immunosuppressive effects appear to be mediated by molecular interactions, which lead to the inhibition of activation signals. Among the immunomodulatory molecules expressed on the surface of MSCs, molecules from the B7 family,[8] class 1b major histocompatibility complex molecules, CD200 and its receptor CD200R[9,10] all seem to be involved. Soluble factors including transforming growth factor-β (TGF-β), indoleamine-2,3- dioxygenase (IDO), interleukins (ILs)-6 and -10, prostaglandin E2 (PGE2), matrix metalloproteinases (MMPs) or adenosine are also under investigation.[4,11] Specifically, PGE2 and IDO have been shown to inhibit proliferation of peripheral blood mononuclear cells (PBMCs) or T cells and appear to be involved in the inhibition of NK cells by downregulating their functional receptors.[12,13] Notwithstanding these insights, many aspects of MSC immuno- suppression remain to be elucidated.

The bulk of studies *in vitro* and *in vivo* on MSC hypoimmuno- genicity have been carried out on undifferentiated cells. It is thus of critical interest in regenerative medicine, to determine whether these cells will retain their immunoprivileged and immunomodulatory properties on differentiation into the local cell types at the trans- plantation site, because allogeneic engineered tissue must be tolerated by the host immune system to remain functional and avoid rejection. Although numerous studies have investigated the immunological features of BM-MSCs after differentiation into adipose, cartilage or bone cells,[14,15] neurons[16] or ECs,[17,18] few have addressed the done immunological properties of WJ-MSCs following differentiation.[19] In the present study, we investigated the immunosuppressive and immunoprivileged character of WJ-MSCs after differentiation towards an endothelial phenotype, in order to permit their use in vascular tissue engineering. After 2 weeks of differentiation, co-cultures were then carried out between the resulting endothelial-like cells (ELCs) and PBMCs, T cells or NK cells. Taken together, our data show that WJ-MSCs and their derived ELCs are non-immunogenic and immunosuppressive. Both cell types can impair T cell, can induce the production of Tregs and impair NK-cell functions.

RESULTS

WJ-MSCs acquire endothelial phenotype and function on differentiation

Morphological changes. To confirm their mesenchymal nature before inducing differentiation, WJ-MSCs were immunophenotyped by flow cytometry and found as expected to be positive for expression of CD73, -90 and -105, and negative for CD34 and CD45 (data not shown). Endothelial growth medium-2 (EGM-2) has been shown to induce endothelial differentiation in MSCs.[20,21] Previsous studies in our lab have also demonstrated that poly(allylamine hydrochloride) (PAH) and poly(styrene sulfonate) (PSS) polyelectrolyte multilayer films improve culturing of WJ-MSCs and ECs.[22,23] We therefore used these two stimuli alone and in combination, to induce endothelial differentiation of WJ-MSCs. The obtained data showed that the differentiation into an endothelial phenotype was induced by cultivating WJ-MSCs at the fourth passage, seeded on $(PAH/PSS)_4$ film-coated glass slides, in the presence of EGM-2. After 2 weeks of culture, the cells had clearly undergone a distinct morphological change, transforming from a spindle to a caudate or oval shape with eccentric nuclei (Figure 1A (a)). Cells seeded on $(PAH/PSS)_4$ films or on glass slides in endothelial basal medium-2 (EBM-2), as well as on glass slides in EGM-2 maintained WJ-MSCs morphology (Figure 1A (b, c and d)).

Phenotype, expression of HLA-DR and CD86. To determine whether expression of WJ-MSCs markers was modified during the differentia- tion process, expression levels of CD73, -90 and 105 were monitored by flow cytometry at 5, 10 and 15 days of culture. Regarding the mean fluorescence intensity (MFI), an increase of the mesenchymal markers CD90 and CD73 was observed between day 5 and day 15, but those of CD105 showed a drop. The absence of HLA-DR and CD86 (a co-stimulatory molecule that combines with CD28 on T cells during their differentiation) is mainly responsible of MSCs immunoprivilege; thus, we decided to examine the expression of these proteins. Expression of both HLA-DR and CD86 increased to a minor extent in ELCs (HLA-DR MFI: 1.0 ± 0.7 at day 5 to 2.2 ± 1.3 at day 15; CD86 MFI: 0.7 ± 0.6 at day 5 to 1.3 ± 0.4 at day 15; Figure 1B).

Acquisition of endothelial markers and functionality. Expression of endothelial markers CD31, vascular endothelial (VE) cadherin and VE growth factor receptor 2 was evaluated by western blotting. ELCs seeded on films and treated with EGM-2 (Figure 1C, lane c) expressed all of these markers, as did human umbilical vein endothelial cells (HUVECs; Figure 1C, lane b), whereas naive WJ-MSCs were negative (Figure 1C, lane a). No endothelial protein expression was detected when cells were cultured on $(PAH/PSS)_4$ in EBM-2 (Figure 1C, lane e) or on glass slides either with EGM-2 or EBM-2 (Figure 1C, lane d and f).

We next evaluated the ability to take up acetylated low-density lipoproteins (Ac-LDLs). We observed that differentiated cells also had the ability to incorporate Ac-LDL, a typical function of ECs (Figure 1D (c)) relative to HUVECs. We also investigated the ability of the ELCs to undergo angiogenesis. Indeed, differentiated cells cultured with growth factors on polyelectrolyte multilayer films formed a capillary network (Figure 1E (a and b)) similar to that produced by HUVECs (Figure 1E (c and d)). On the contrary, undifferentiated WJ-MSCs failed to form capillary-like structures (Figure 1E (e and f)).

ELCs do not stimulate proliferation of allogeneic PBMCs and inhibit the proliferation of mitogen-stimulated PBMCs

Several studies have reported that MSC-mediated immunosuppression involves both cell contact-dependent and -independent mechanisms, with the latter likely mediated via release of soluble factors.[24] In order to probe the role of cell contact in ELCs immunomodulation, experiments were performed with and without a Transwell (TW) that prevented direct contact between the two cell populations.

To address whether ELCs could act as antigen-presenting cells and stimulate the proliferation of PBMCs, the cells were incubated with an equal number of non-activated PBMCs for 3 days either with or without cell contact, in a one-way mixed lymphocyte reaction assay.

As shown in Figure 2a upper graph, ELCs and WJ-MSCs significantly suppressed the proliferation of PBMCs (respectively 0.13 ± 0.006 and 0.16 ± 0.03 versus $0.4 \pm 0.04\ \mu g\ ml^{-1}$ for PBMCs alone; Po0.05) and this effect was mainly observed when cells were cultured in direct contact.

In another set of experiments, we investigated whether ELCs can inhibit the proliferation of mitogen-stimulated PBMCs (Figure 2a, lower graph). For this, ELCs were incubated with equal number of PBMCs in the presence of phytohemagglutinin (PHA) for 3 days, with the proliferation of PBMCs stimulated with PHA serving as a positive control. The obtained data show, first, that the ELCs significantly inhibited the proliferation of the PBMCs in response to PHA and, second, that this inhibition only occurred in co-cultures in which cells were in direct contact (0.32 ± 0.12

versus 0.75 ± 0.06 μg ml^{-1} for PHA-PBMC; Po0.01). The immunosuppressive effect of WJ-MSCs was also confirmed, as proliferation of PBMCs stimulated by PHA was significantly inhibited by direct cellular contact with WJ-MSCs (0.43 ± 0.06 versus 0.75 ± 0.06 μg ml^{-1} for PHA-PBMCs alone; Po0.05). Mitogen-stimulated PBMC proliferation was almost nearly restored in TW co-cultures (0.76 ± 0.09 μg ml^{-1} for both co-cultures versus 0.75 ± 0.06 μg ml^{-1} for PHA-PBMCs alone), showing a major role for cellular contact in the immunosuppressive effect mediated by ELCs and WJ-MSCs.

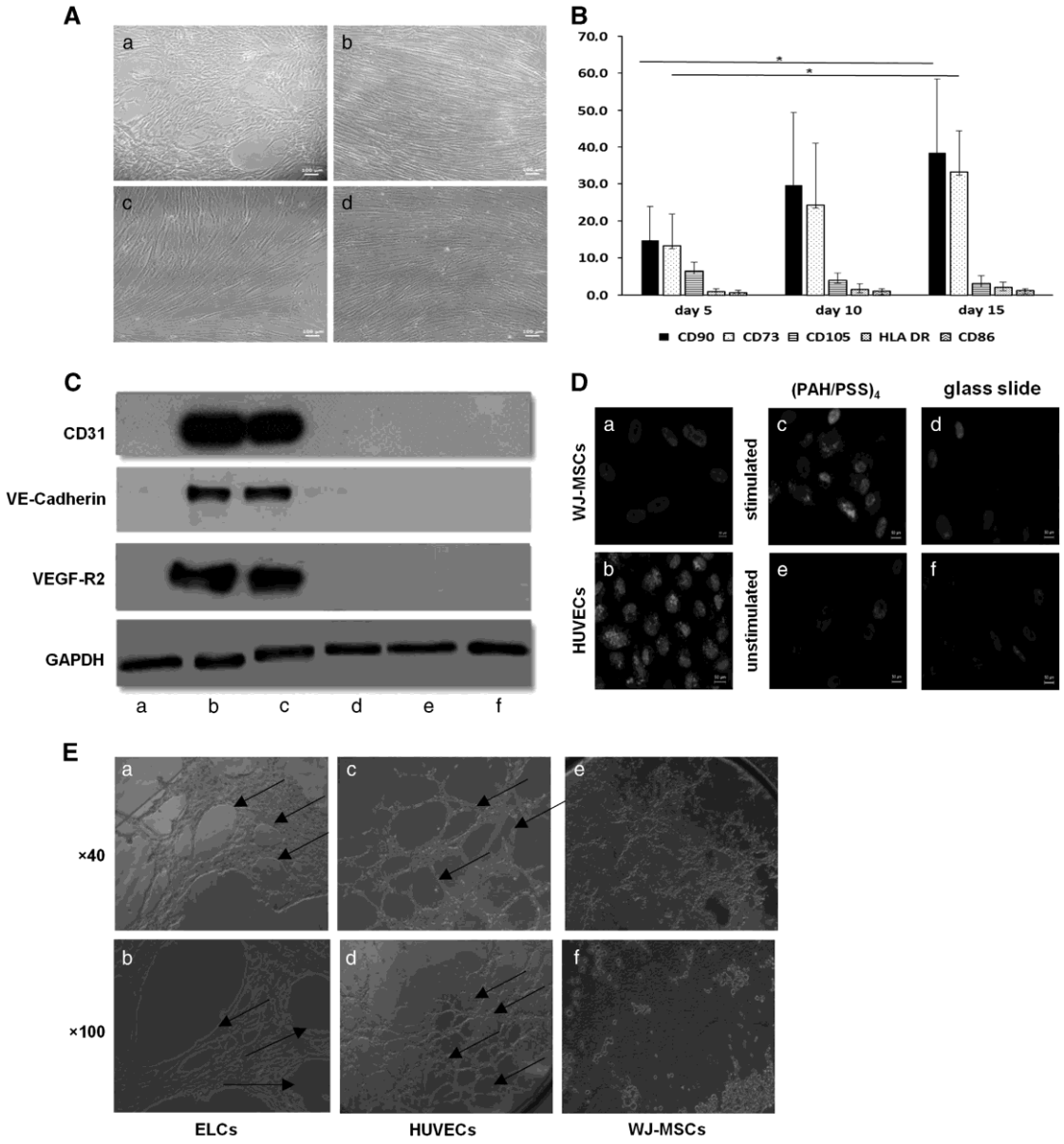


Figure 1 WJ-MSCs change morphology and phenotype on endothelial differentiation, take up Ac-LDL and form tubular structures. (A) EGM-2 stimulated (a and b) and unstimulated (c and d) WJ-MSCs morphology on (PAH-PSS)$_4$-coated glass slides (a and c) and on uncoated glass slides (b and d) was observed by inverted optical microscopy (Nikon Diaphot, UFX-DX, Japan; ×10 magnification; scale bar = 100 μm). Only EGM-2 stimulated WJ-MSCs (a) showed a slight morphological evolution from fibroblastic to polygonal shape. (B) Expression of three mesenchymal markers (CD73, CD90 and CD105) and two immune markers (HLA-DR and CD86) was evaluated after 5, 10 and 15 days of differentiation by flow cytometry. Marker expression was determined for WJ-MSCs seeded on (PAH-PSS)$_4$ films and differentiated in EGM-2. Data represent the MFI of expression of each markers from three independent experiments. Error bars indicate s.e.m. (C) The expression of CD31, VE-cadherin and VE growth factor receptor 2 (VEGF-R2) by cells cultured under the four conditions (d–e) was determined by western blotting: (a) WJ-MSCs (negative control); (b) endothelial cells extracted from human vein umbilical cord

(HUVECs) (positive control); (c) EGM-2/$(PAH\text{-}PSS)_4$; (d) EGM-2/glass slide; (e) EBM-2/$(PAH\text{-}PSS)_4$; (f) EBM-2/glass slide. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. (D) Ac-LDL (the first column shows the staining of isotype controls Ac-LDL) uptake by unstimulated (e and f) and EGM-2 stimulated (c and d) WJ-MSCs on $(PAH\text{-}PSS)_4$. HUVECs were used as positive control (b) and WJ-MSCs as negative control (a). Nuclei counter-staining with DAPI appears in blue, whereas Ac-LDL uptake appears in red (×40 oil objective; scale bar = 50 μm). (E) *In vitro* angiogenesis assay. Lumen-like structures were spontaneously formed by differentiated cells derived from WJ-MSCs (a and b) and by HUVECs (c and d) but not by WJ-MSCs (e and f).

To identify whether the suppressive effect was influenced by cytokines secreted by ELCs and WJ-MSCs, IL-1β, IL-6, IL-8, IL-10, IL-12p70 and tumor necrosis factor-α were quantified in cell culture supernatants. The concentration of cytokines detected in the medium of PHA-activated PBMCs cultured in the absence of WJ-MSCs or ELCs, was used as a positive control.

WJ-MSCs and ELCs secreted less IL-12p70 and IL-10 compared with the positive control and a significant reduction in the concentrations of tumor necrosis factor-α (6.78 ± 2 and 50 ± 28 pg ml^{-1} for WJ-MSCs/PBMCs and ELCs/PBMCs, respectively, versus 652.9 ± 221 pg ml^{-1} for PHA-PBMCs alone; *P*o0.05). The concentration of IL-8 was also significantly decreased in WJ-MSCs/ PBMCs co-culture (20 495 ± 718 versus 36470.7 ± 7192 pg ml^{-1} for PHA-PBMCs alone; *P*o0.05). We further noted an increase in concentration of IL-6 in both co-cultures, with higher levels observed in the ELCs/PBMCs co-culture (12092.3 ± 5.9 versus 21 865 ± 84 pg ml^{-1} for PHA-PBMCs alone; *P*o0.005) in comparison with WJ-MSCs/PBMCs co-culture (19 697 ± 824.8 versus 12092.3 ± 5.9 pg- ml^{-1} for ELCs/PBMCs co-culture; *P*o0.005). Finally, the concentra- tion of IL-1β was higher in both co-cultures with greater amounts observed in WJ-MSCs/PBMCs co-culture (669.4 ± 179.8 and 214.1 ± 82 pg ml^{-1} for WJ-MSCs/PBMCs and ELCs/PBMCs, respec- tively, versus 64 ± 28 pg ml^{-1} for PHA-PBMCs alone; *P*o0.05).

The immunomodulatory properties of WJ-MSCs are moderately altered after endothelial differentiation: impact on T and NK cells To reduce interference from other cellular components such as B cells, monocytes and dendritic cells, we decided to investigate the effects of WJ-MSCs and ELCs on T and NK cells, by allowing or preventing direct cell–cell contact. Proliferation of activated T or NK cells was considered to be a 100% proliferative response (positive control).

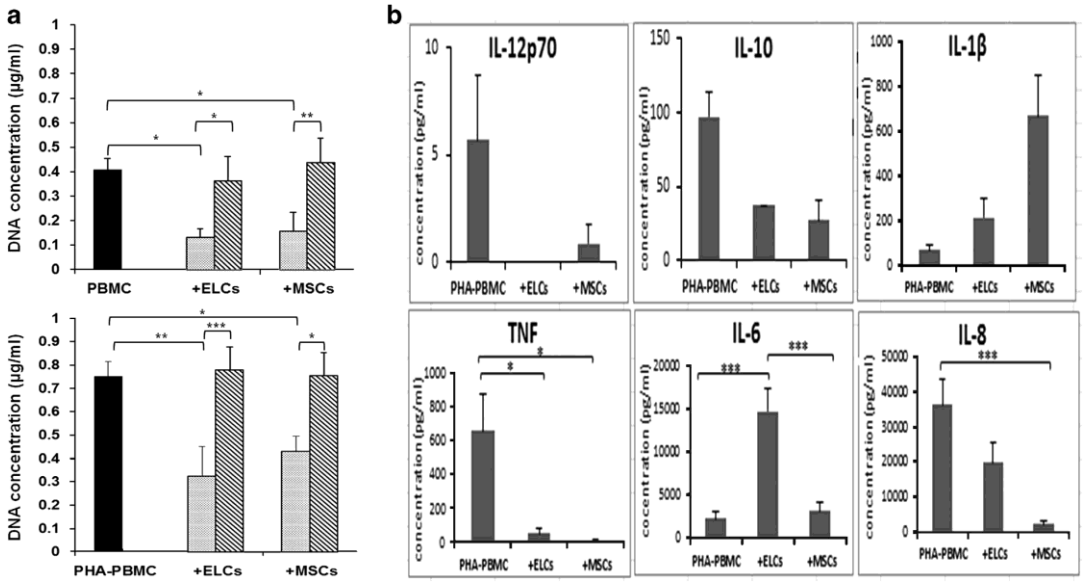
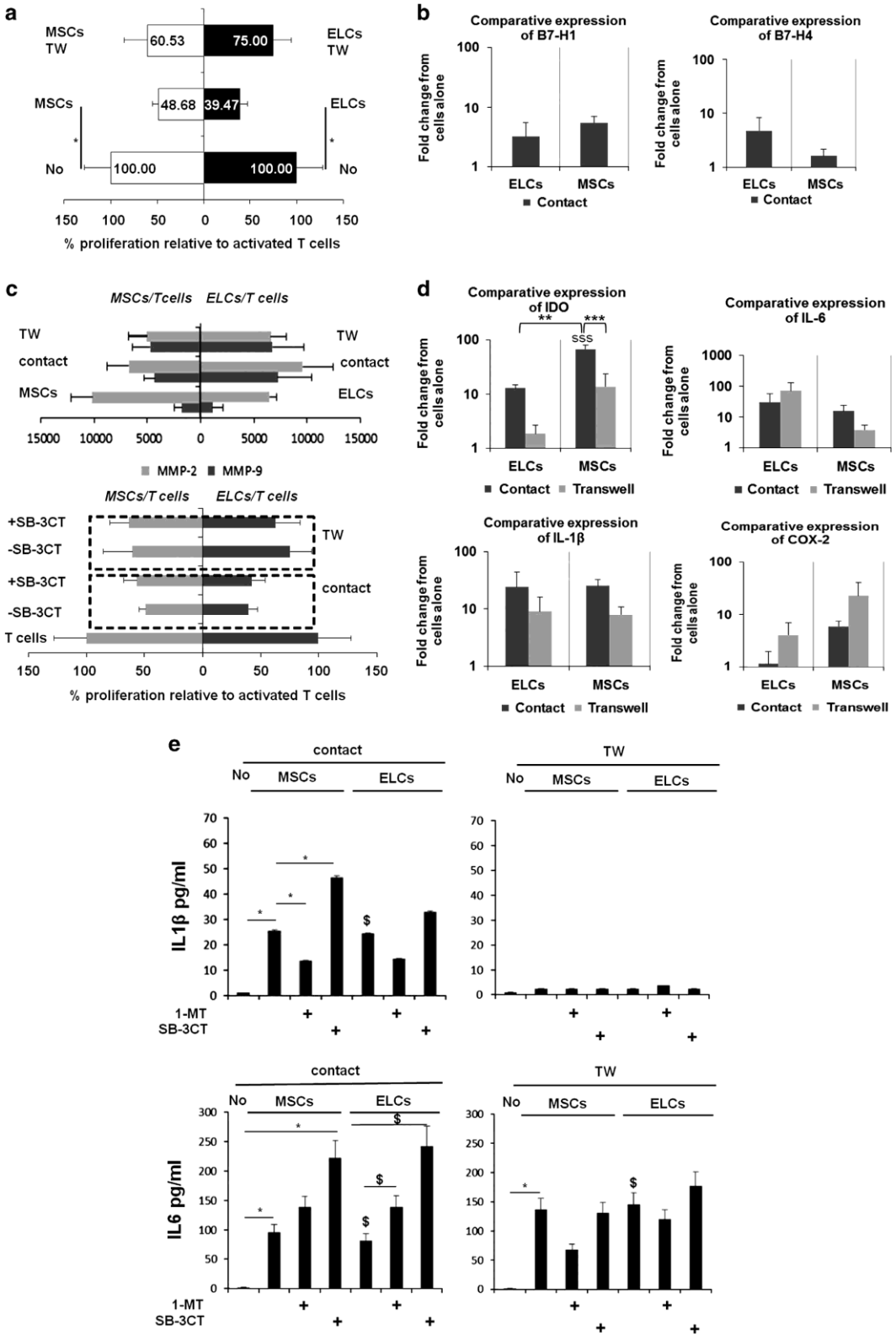


Figure 2 Immune characterization of ELCs: interactions with PBMCs. (a) Upper graph: WJ-MSCs and ELCs inhibit proliferation of PBMCs. Lower graph: non-activated and PHA-activated PBMCs were co-cultured in the presence of WJ-MSCs or ELCs at a 1:1 ratio in the presence or absence of cell–cell contact; the latter were performed using TW chambers. Data are shown as mean ± s.e.m. (*n* = 3). (b) PHA-activated PBMCs were co-cultured in the presence or absence of WJ-MSCs and ELCs at 1:1 ratio and IL12p70, TNF-α, IL-6, IL-8, IL-10 and IL-1β concentrations were determined in cell-free supernatants after 3 days of culture using the cytometric bead array assay. The concentration of cytokines detected in conditioned medium of activated PBMCs alone was used as a control. Data are shown as the mean ± s.e.m. of the cytokine concentration in pg ml^{-1} (*n* = 3). *, ** and ***Statistically significant difference with *P*o0.05, *P*o0.01 and *P*o0.005, respectively.


a
MSCs TW
60.53
75.00
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MSCs
48.68
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ELCs
No
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150 100 50 0 50 100 150
% proliferation relative to activated T cells
b
Comparative expression of B7-H1
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Fold change from cells alone
100
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ELCs MSCs
Contact
c
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contact
MSCs
ELCs
15000 10000 5000 0 5000 10000 15000
MMP-2 MMP-9
MSCs/T cells ELCs/T cells
+SB-3CT
-SB-3CT
+SB-3CT
-SB-3CT
T cells
TW
contact
150 100 50 0 50 100 150
% proliferation relative to activated T cells
d
Comparative expression of IDO
Comparative expression of IL-6
Comparative expression of IL-1β
Comparative expression of COX-2
Fold change from cells alone
ELCs MSCs
Contact Transwell
e
contact
TW
No MSCs ELCs
IL1β pg/ml
IL6 pg/ml
1-MT
SB-3CT

Figure 3 Comparison of the immunomodulatory effects of ELCs and WJ-MSCs on T cells. (a) WJ-MSCs and ELCs inhibit T-cell proliferation. Activated T cells were co-cultured in the presence of WJ-MSCs or ELCs at a 1:1 ratio either with or without cell–cell contact. Proliferation of activated T cells alone served as the positive control (100% of proliferation). (b) ELCs and WJ-MSCs express the contact molecules B7-H1 and B7-H4 mRNA after direct cell–cell contact with T cells. WJ-MSCs and ELCs cultured alone were used as controls. Expression of the contact molecules was determined by quantitative reverse-transcriptase PCR (qRT-PCR) and normalized using the housekeeping gene *RP29* and expressed as fold change from WJ-MSCs or ELCs. (c) Upper graph: levels of MMP-2 (63 kDa) and MMP-9 (82 kDa) in the conditioned medium of ELCs (right) or WJ-MSCs (left) after co-culture with T cells in the absence and presence of cell–cell contact were measured as band intensity by gelatin zymography. ELCs and WJ-MSCs were employed as controls. Lower graph: impact of the inhibition of MMPs by SB-3CT on T-cell proliferation during co-culture with ELCs (right) or WJ-MSCs (left). Proliferation of activated T cells alone was the positive control (100% of proliferation). (d) IDO, IL-6, IL-1β and COX-2 mRNA expression in WJ-MSCs and ELCs after direct and TW co-culturing with activated T cells, as determined by qRT-PCR. WJ-MSCs and ELCs cultured alone were used as controls. The data were normalized using the housekeeping gene *RP29* and compared with controls. (e) IL-6 and IL-1β concentrations determined in cell-free supernatants after 3 days of co-culturing between WJ-MSCs and T cells or ELCs and T cells using the cytometric bead array (CBA) assay. IL-6 was detected under both TW and contact conditions, whereas IL-1β was detected mainly under contact conditions. *Statistically significant difference between the represented conditions with *P*o0.05, *P*o0.01 and *P*o0.005, respectively (*, ** and ***, respectively, indicate the same statistically significant difference between a condition and the native non-stimulated cells). All data are mean ± s.e.m. ($n = 3$).

Immunosuppressive effects of ELCs on T cells

Our data show that WJ-MSCs significantly reduced T-cell proliferation (487 ± 65.9%), which was partially restored by preventing cell–cell contact (60.5 ± 25%). Only 395 ± 8% proliferation was observed when T cells were in direct contact with ELCs. This inhibition was partially reversed in a TW with 75 ± 19% (Figure 3a). We next explored the expression of two contact molecules B7-H1 and B7-H4, which are possibly involved in the contact inhibition of T-cell proliferation. Our data revealed weak expression of B7-H1 mRNA in both ELCs and WJ- MSCs (Figure 3b, left panel). B7-H4, on the other hand, was mainly expressed in ELCs (Figure 3b, right panel), with only weak expression detected in WJ-MSCs. However, our flow cytometry analysis could not confirm the expression of B7-H4 and B7-H1 at the surface of ELCs or WJ-MSCs (data not shown).

MSCs and mature ECs are able to secrete MMP-2 and -9, two factors which were recently shown to have immunomodulatory effects.[25,26] We therefore aimed to determine whether WJ-MSCs and ELCs express MMP-2-soluble, active forms of MMP-2 and -9, using gelatin zymography. As shown in Figure 3c upper graph, both enzymes were readily detected in supernatants of WJ-MSCs and ELCs, with expression of MMP-2 approximately sixfold higher than that of MMP-9. No increase in MMP-2 concentration was detected after co-culture with WJ-MSCs, whether they were in direct contact or not, whereas a small increase was noticed after co-culture with ELCs under direct contact conditions. The largest increases in MMP-9 expression were observed following direct contact with ELCs. To assess the suppressive effect of MMPs on T-cell proliferation, we next examined the ability of an MMP inhibitor called SB-3CT,[27] to restore T-cell proliferation. Addition of SB-3CT restored the proliferation of T cells in direct contact co-cultures to a minor degree (2.6 ± 0.1% and 3.9 ± 0.1% of restoration for, respectively, co-cultures with ELCs and WJ-MSCs; Figure 3c, lower graph), indicating that MMP-2 and -9 are only weakly involved in T-cell immunosuppression mediated by ELCs and WJ-MSCs. In contrast, no effect of the inhibitor was seen in a TW system.

We next explored other candidates as mediators of the suppression driven by ELCs. IDO, IL-1β, IL-6, PGE2, hepatocyte growth factor and TGF-β have been reported to trigger T-cell suppression by WJ-MSCs;[28] thus, we examined the expression of these genes before and after endothelial differentiation on co-culture with T cells. WJ-MSCs or ELCs incubated in the absence of T cells were used as controls. In the presence of a direct interaction between T cells and WJ-MSCs, IDO expression was high; separation of the cells reduced the expression fivefold. After co-culture with ELCs, IDO expression was 12.8-fold higher than in the control and sevenfold higher in the contact condition than in TW. However, expression of IDO in ELCs was 5.2- and 7.4-fold lower, respectively, relative to WJ-MSCs in contact or in TW conditions. After co-culture with T cells, expression of IL-6 was 30- and 15-fold higher, respectively, than the controls for ELCs and WJ-MSCs, whereas its expression was reduced for WJ-MSCs and increased for ELCs in TW culture. IL-1β expression after co-culture was 24- and 25-fold higher for ELCs and WJ-MSCs, respectively, relative to controls. Furthermore, we detected a higher expression of IL-1β in ELCs than in WJ-MSCs, with a marked increase in the TW condition for ELCs. The expression level of cyclooxygenase (COX)-2, the enzyme producing PGE2, was essentially identical under all the conditions (Figure 3d). No changes in the expression of hepatocyte growth factor, COX-1 and TGF-β were detected under any of these conditions (data not shown).

As expression of IL-1β and IL-6 was particularly upregulated in ELCs, we investigated whether the IDO inhibitor 1-methyl tryptophan (1MT) and

the MMP inhibitor SB-3CT could influence their secretion (Figure 3e). The highest levels of IL-1β were observed after culturing T cells in direct contact with ELCs or WJ-MSCs, concentrations after co-culture was 24.3 ± 0.2 and 25.5 ± 0.3 pg ml^{-1} for ELCs and WJ-MSCs, respectively, which were higher than for T cells alone (1.01 ± 0.01 pg ml^{-1}). IL-1β was significantly inhibited in the presence of 1MT and induced by SB-3CT for MSCs but not ELCs ($P \leq 0.05$). A weak expression of IL1β was detected in the TW conditions (Figure 3e: first and second graphs).

IL-6 was readily detected in the surpernatant of T cells co-cultured with ELCs (81.6 ± 11.3 pg ml^{-1}) or WJ-MSCs (95.6 ± 13.2 pg ml^{-1}), with the highest levels seen under direct contact conditions. IL-6 secretion was significantly enhanced after incubating the cells co-cultured with WJ-MSCs either with 1MT or SB-3CT (138.4 ± 19.1 and 222.0 ± 30.7 pg ml^{-1}, respectively; P p 0.05). The same results were observed after co-culture with ELCs (139.1 ± 19.2 and (222.0 ± 30.7 pg ml^{-1}) in presence of 1MT and SB-3CT (Figure 3e: third and fourth graphs).

ELCs induce the generation of T-cell subsets displaying a regulatory phenotype. Previous work has shown that MMPs secreted by MSCs are involved in the inhibition of T-cell proliferation via shedding of the IL-2 receptor CD25.[25] We explored whether MMPs in the cell supernatant of ELCs and WJ-MSCs co-cultured with T cells could affect CD25 expression. Flow cytometry analysis showed a clear increase in the percentage of $CD25^+$ T cells after co-culture with ELCs or WJ-MSCs under direct contact conditions (62.1 ± 4% and 59.4 ± 19%, respectively) compared with T cells alone (37.8 ± 8%; Figure 4b). The percentage of cells expressing CD25 was also slightly increased in the presence of a TW (39.9 ± 10% and 40 ± 14%, respectively, for ELCs and WJ-MSCs TW co-cultures). If MMPs were involved in the shedding of CD25, addition of MMPs inhibitor should increase the percentage of cells expressing CD25. However, addition of SB-3CT resulted in a slight decrease of the $CD25^+$ population after co-culture with WJ-MSCs whether in direct contact or not, but not following co-culture with ELCs under equivalent conditions (Figure 4b).

Among candidates investigated as mediators of the immuno- suppression, IDO expression was notably increased after co-culture with WJ-MSCs or ELCs. Thus, we next explored the effect of its inhibitor, 1MT, on CD25. The percentage of $CD25^+$ T cells was lower after culture with WJ-MSCs separated by a TW in the presence of 1MT (26.8 ± 6.4%versus 37.8 ± 8% for T cells alone; Figure 4b). No significant effect of the IDO inhibitor was seen on CD25 expression by T cells co-cultured with ELCs.

As IDO has been shown to promote Treg generation,[29] we investigated whether the $CD25^+$ population expressed forkhead box P3 (FoxP3), which is a main marker of Tregs. Interestingly, after performing a gating on the $CD4^+$ population, we detected an increase in the $CD25^+FoxP3^+$ population in co-cultures relative to activated T cells alone, which was higher in co-cultures with ELCs (75 ± 2.5% versus 23 ± 1.5% for T cells alone) than with WJ-MSCs (62 ± 3.8% versus 23 ± 1.5% for T cells alone; Figure 4c). Thus, we confirmed that WJ-MSCs generate Tregs, as demonstrated previously.[30–32] Moreover, we showed that ELCs were able to induce a larger population of Tregs than WJ-MSCs. We next explored the effect of an IDO inhibitor (1MT) on Tregs generation. After co-culture of T cells with WJ-MSCs, we observed a moderate inhibition of 3 ± 0.8% under direct contact conditions, whereas the percentage reached 21 ± 2.3% when the cells were separated. No effect of the IDO inhibitor was detectable on Tregs generated by ELCs co-culturing (Figure 4c).

We also investigated the effect of MMPs on Tregs. Surprisingly, a decrease of the Tregs percentage was observed in every condition with the MMP inhibitor SB-3CT either in the presence of cell–cell contact or in a TW. Maximum inhibition of Tregs generation was seen when T cells were cultured in direct contact with ELCs where the percentage of Tregs dropped from 75 ± 3% to 64 ± 4% (Figure 4c).

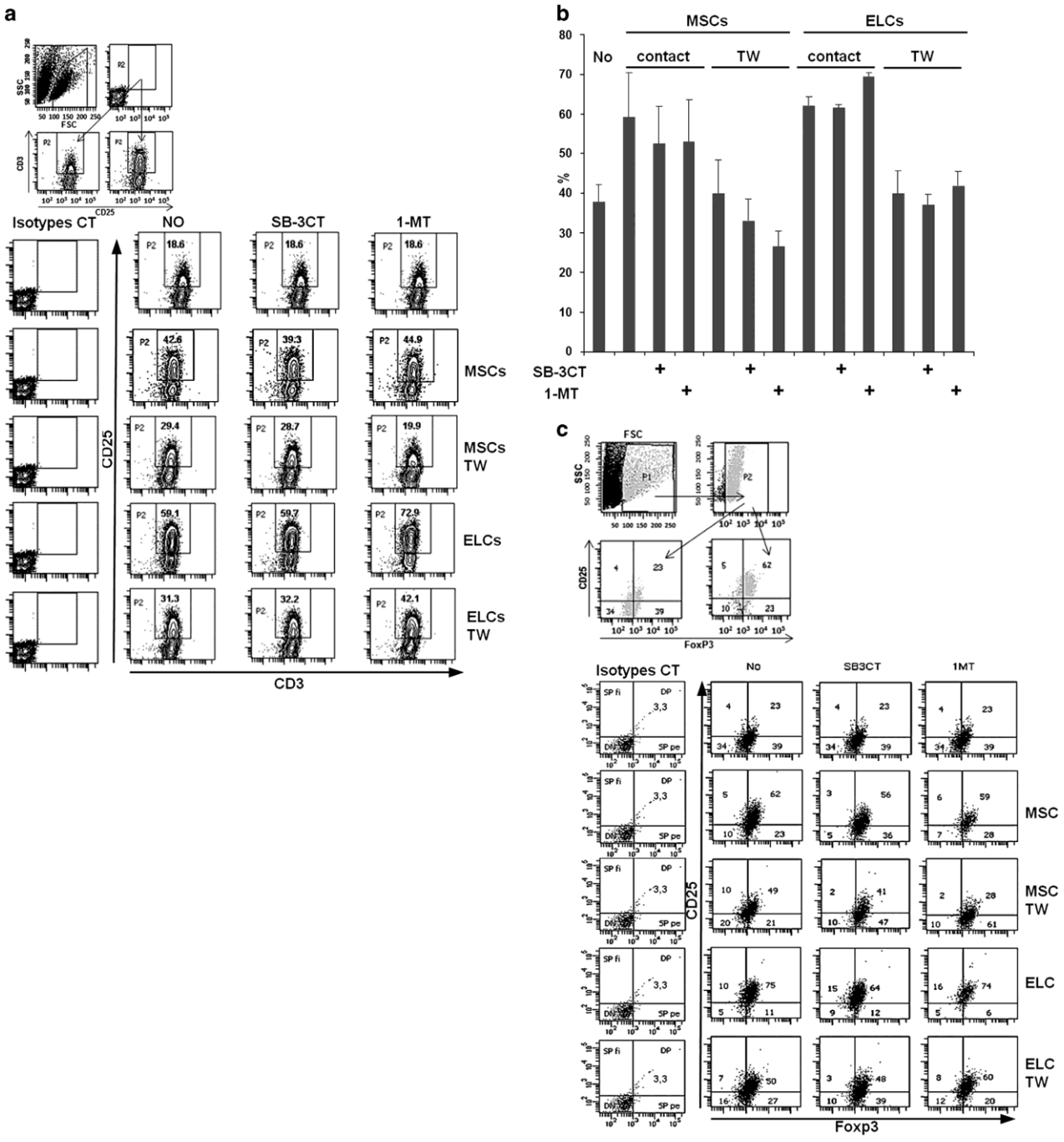


Figure 4 WJ-MSCs and ELCs induce the generation of T-cell population subsets displaying a regulatory phenotype. (a) On the top of the figure is included the gating strategy. Cells are gated by taking into account SSC (side scatter) and FSC (forward scatter) to select the lymphocyte population (P1 gate). The P2 gate was next set regarding the staining of control isotypes for anti-CD25 and anti-CD3 antibodies. The cells gated into the P2 gate were considered positive for CD25 and CD3. Representative dot plot of the percentage of $CD3^{+}CD25^{+}$ population after co-culturing of activated T cells in the absence or presence of WJ-MSCs and ELCs with or without SB-3CT (MMPs inhibitor) or 1MT (IDO inhibitor). These data show that CD25 is induced on T cells mainly following contact with WJ-MSCs or ELCs. The first column shows the staining of isotype control. (b) Mean percentage of $CD3^{+}CD25^{+}$ populations determined from three independent experiments ($n = 3$). These data show a significant effect of 1MT on CD25 expression on T cells co-cultured with ELCs. (c) On the top of the figure is included the gating strategy. Cells are gated by taking into account SSC (side scatter) and FSC (forward scatter), to select the lymphocyte population (P1 gate). The P2 gate was next set on the CD4 positive population. On the cells gated into the P2 gate ($CD4^{+}$ cells), the staining of anti-CD25 (*Y* axis and anti-FoxP3 antibody (*X* axis) was determined after placing the quadrants taking into account the isotype control staining. Representative dot plot

showing the percentage of $CD3^+CD25^+FoxP3^+$ cells determined on $CD4^+$ T cells after co-culture with WJ-MSCs and ELCs under direct contact conditions. T cells cultured alone were employed as controls. The first column shows the staining of the isotype control and the four quadrants were defined as SP FI or PE (simple positive FITC or PE), DN (double negative) or DP (double positive). *, ** and ***Statistically significant difference between the represented conditions and the control with *P*o0.05, *P*o0.01 and *P*o0.005, respectively.

Immunosuppressive effects of ELCs observed on NK cells

We next evaluated the effects of WJ-MSCs and ELCs on the proliferation of activated NK cells. We used the proliferation of IL-15-stimulated NK cells as a control, scaling it to 100%. ELCs were able to reduce the proliferation of NK cells by 44.2 ± 16.2% in contact cultures versus 48.7 ± 7.8% in a TW. We also observed, in agreement with the literature,[33] that WJ-MSCs inhibit NK cell proliferation, either by direct contact or in a TW context, with 55.2 ± 14% and 59.1 ± 16% proliferation, respectively, relative to the control (Figure 5a).

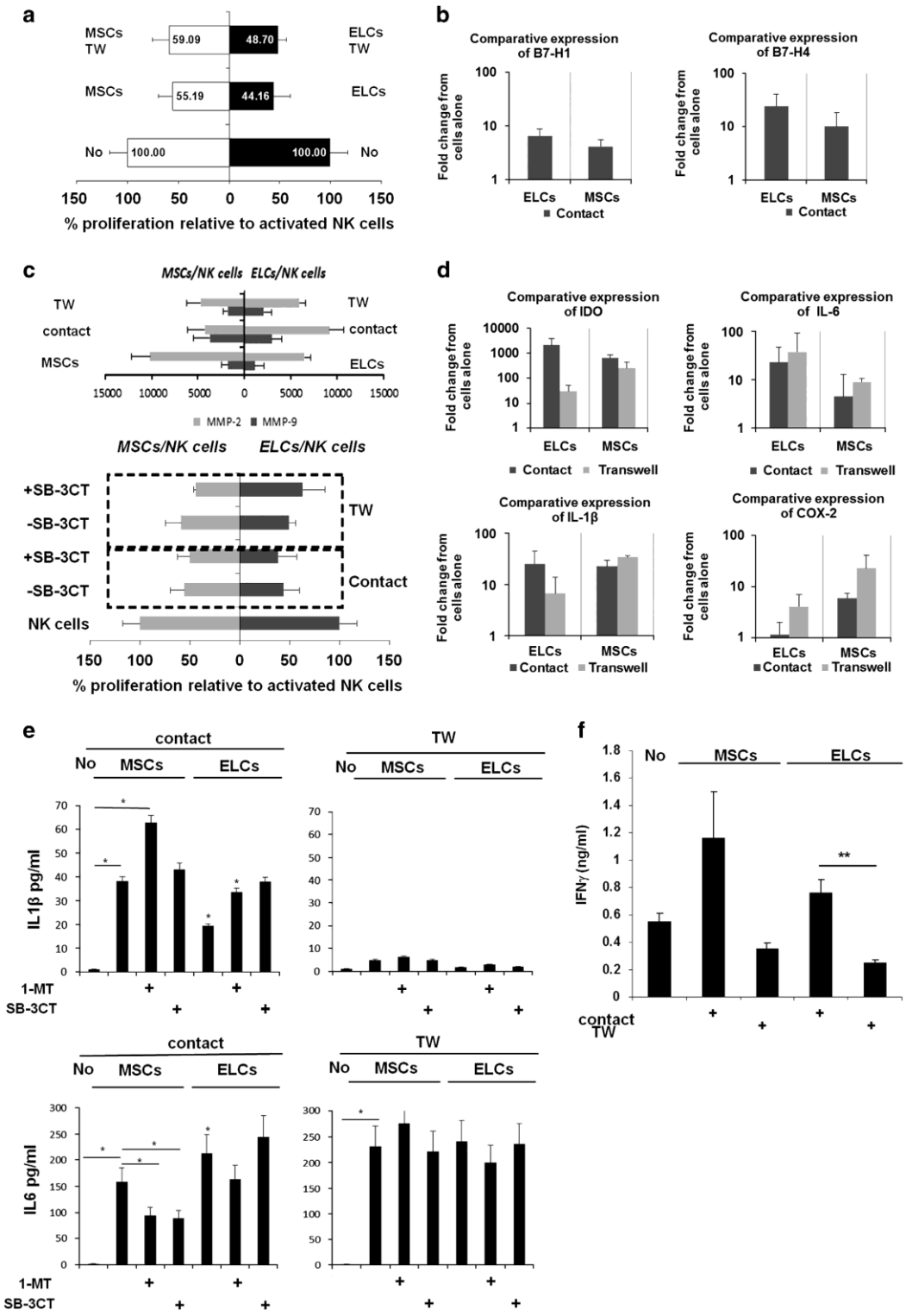
We next examined the levels of B7H1 and B7H4 mRNA in ELCs and WJ-MSCs after contact with NK cells (Figure 5b, left and right panels). The levels of both mRNAs were enhanced in ELCs and WJ-MSCs after co-culture. However, flow cytometry analysis failed to reveal the encoded proteins on the surfaces of ELCs and WJ-MSCs (data not shown).

As MMPs have been shown to have immunomodulatory effects on NK cells,[34] the same procedure described above was applied to detect the activity of MMP-2 and MMP-9 in NK co-cultures. We observed higher levels of MMP-2 in direct co-cultures of ELCs with NK cells than in a TW and versus ELCs alone (Figure 5c, upper graph). In contrast, MMP-2 activity was not enhanced in a co-culture of WJ- MSCs with NK cells, either under contact or separated conditions. MMP-9 was slightly enhanced in the supernatant of both ELCs and WJ-MSCs cultured in contact with NK cells. However, the addition of SB-3CT to the co-cultures was not able to restore proliferation of NK cells (Figure 5c, lower graph). Taken together, these results suggest that inhibition of MMPs alone is not sufficient to restore NK-cell proliferation, and therefore that other mediators may be involved in this phenomenon. In follow-up experiments, we carried out co-culture with activated NK cells for 3 days and then evaluated the expression by ELCs and WJ-MSCs of the same genes that were tested above (Figure 5d). The expression profile changed from that observed after co-culture with T cells. In fact, IDO, IL-6 and IL-1β expression was higher in ELCs than in WJ-MSCs grown in direct co-cultures (3.2-, 5.1- and 1.1-fold higher, respectively), but the expression of COX-2 was lower in ELCs than in WJ-MSCs. Hepatocyte growth factor, COX-1 and TGF-β showed no change of expression under all conditions tested (data not shown). Quantification of IL-1 and IL-6 in NK cells supernatants further showed that IL-1 was largely secreted when NK cells were in contact with ELCs or WJ-MSCs (19.3 ± 0.8 and 38.3 ± 1.8 pg ml^{-1}, respectively, versus 1.1 ± 0.1 pg ml^{-1} for NK cells alone; $P<0.05$; Figure 5e, left panel), a phenomenon that could be enhanced by addition of 1MT ($P<0.05$) and to a lesser extent SB-3CT (ns). No significant secretion of IL-1 was seen in TW conditions (Figure 5e, right panel). IL-6 was detected in supernatant of NK cells after co-culture with ELCs (212 ± 35 pg ml^{-1}) or WJ-MSCs (158 ± 26 pg ml^{-1}) under both contact and TW conditions (240 ± 40 and 230 ± 40 pg ml^{-1} for ELCs and WJ-MSCs, respectively). Inhibition of IL-6 secretion was observed in the presence of 1MT in both WJ-MSCs and ELCs (41% and 23% of inhibition, respectively) cultured in direct contact but reach significance only for WJ-MCSs, although the same tendency was observed with ELCs. A significant effect of SB-3CT on IL-6 secretion was observed only for WJ-MCSs. No significant effect of SB-3CT of 1MT supplementation was observed in the TW condition.

As IDO was strongly induced after co-culture with ELCs and WJ-MSCs, we next evaluated whether interferon-γ, a major cytokine secreted by NK cells, could be involved in the activation of IDO. The obtained data clearly show that direct contact between ELCs and WJ-MSCs with NK cells strongly induces interferon-γ secretion (0.76 ± 0.1 and 1.1 ± 0.3 ng ml^{-1} for ELCs and WJ-MSCs, respectively, versus 0.56 ± 0.07 ng ml^{-1} for NK cells alone).

Impact of co-culture on expression of NK cell markers. To investigate whether WJ-MSCs and ELCs could have an effect on NK cells, expression of the NK cell receptors CD16, CD56 and natural-killer group 2, member D (NKG2D) was analyzed and compared with that detected after co-culturing with WJ-MSCs or ELCs in the absence or presence of cell–cell contact (Figures 6a and b). After 15 days of expansion in the presence of IL-15, NK-cell phenotyping revealed two populations: $CD16^{+}CD56^{+}$ and $CD16^{-}CD56^{+}$. The intensity of expression of CD56 was similar for the two subsets. After co-culture with WJ-MSCs, however, the partitioning between the two populations altered. As shown by the black arrow on the left, the cell population expressing CD16 and $CD56^{bright}$ was no longer detected, while a new population appeared, which was $CD16^{+}$ but which exhibited a lower level of CD56 (Figure 6a). Furthermore, we also observed an increase in the CD16 $CD56^{bright}$ population, which reached 75.7%. In contrast, the percentage of the population corresponding to $CD16^{+}CD56^{bright}$ was increased in co-cultures of WJ-MSCs and NK cells under TW conditions, relative to isolated NK cells or those in direct contact with WJ-MSCs. The percentage of the $CD16^{-}CD56^{bright}$ population decreased from 75.1 to 38% when cells were not in direct contact. No change in the partitioning between the two NK populations was observed in direct or TW-separated ELCs/NK cell co-cultures. As MMPs have been shown to modify CD16 expression on NK cells,[35,36] we next examined whether the

MMPs inhibitor SB-3CT could modify the NK-cell phenotype after co-culture. Interestingly, we observed that the $CD16^{+}CD56^{bright}$ population, which disappeared after direct contact with WJ-MSCs in co-culture, was partially restored after addition of SB-3CT (Figure 6a, middle black arrow, and Figure 6b). However, in the case of WJ-MSCs without cell contact or ELCs with or without cell contact, the inhibitor did not significantly modify the partitioning between the cell populations (Figure 6b). As IDO was strongly expressed by ELCs and WJ-MSCs either in cell contact or not, we also examined whether the IDO inhibitor 1MT could modify NK-cell phenotype after co-culture. Surprisingly, when 1MT was added to co-cultures with WJ-MSCs, we observed altered expression of CD56 and CD16 (Figure 6a, right black arrow), which was not seen when the cells were separated. As for co-culturing with ELCs, direct or indirect contact with NK cells did not significantly modify the partitioning between the two populations, nor did addition of SB-3CT. However, supplementation with 1MT decreased the percentage of $CD16^{+}CD56^{+}$ NK cells from 63 to 48% and increased the percentage of $CD16^{-}CD56^{+}$ from 31.7 to 50.2% (Figure 6b).


a
MSCs TW
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Comparative expression of IL-1β
Comparative expression of COX-2
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e
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No MSCs ELCs
IL1β pg/ml
IL6 pg/ml
1-MT
SB-3CT
f
IFNγ (ng/ml)
contact
TW

Figure 5 Comparison of the immunomodulatory effects of ELCs and MSCs on NK cells. (a) ELCs and WJ-MSCs inhibit NK cell proliferation. Activated NK cells were co-cultured in the presence of WJ-MSCs or ELCs at a 1:1 ratio either with or without cell–cell contact during 3 days. Proliferation of activated NK cells alone was used as positive control (100% of proliferation). (b) Expression levels of B7-H1 and B7H4, factors that are potentially involved in direct cell–cell immunosuppression. B7-H1 and B7-H4 mRNA expression in WJ-MSCs and ELCs maintained in contact or not with activated NK cells was determined by quantitative reverse-transcriptase PCR (qRT-PCR), normalized using the housekeeping gene *RP29* and expressed as fold change from WJ-MSCs or ELCs alone. WJ-MSCs and ELCs cultured alone were used as controls. (c) Upper graph: band intensity of MMP-2 (63 kDa) and MMP-9 (82 kDa) as detected by gelatin zymography in the conditioned medium of ELCs (right) or WJ-MSCs (left), following co-culture with NK cells, either allowing for direct cell–cell contact or not. ELCs and WJ-MSCs were employed as controls. Lower graph: impact of the inhibition of MMPs by SB-3CT on NK-cell proliferation during co-culture with ELCs (right) or WJ-MSCs (left). Proliferation of activated NK cells alone was the positive control (100% of proliferation). (d) IDO, IL-6, IL-1β and COX-2 mRNA expression in WJ-MSCs and ELCs after direct and TW co-culturing with activated NK cells, as determined by qRT-PCR. WJ-MSCs or ELCs alone were used as controls and the data were normalized using the housekeeping gene *RP29* and compared with controls. (e) IL-6 and IL-1β concentrations determined in cell-free supernatants after 3 days of culture using the cytometric bead array (CBA) assay. The IL-6 concentration was significantly increased after indirect co-culture, whereas that of IL-1β was increased mainly after cell–cell contact. (f) Interferon (IFN)-γ concentration (ng/ml) as determined by enzyme-linked immunosorbent assay (ELISA) in cell-free supernatants of NK cells after 3 days of co-culturing with WJ-MSCs or ELCs, under contact or TW conditions. The conditioned medium of activated NK cells alone was used as control. All data are shown as the mean ± s.e.m. ($n = 3$).

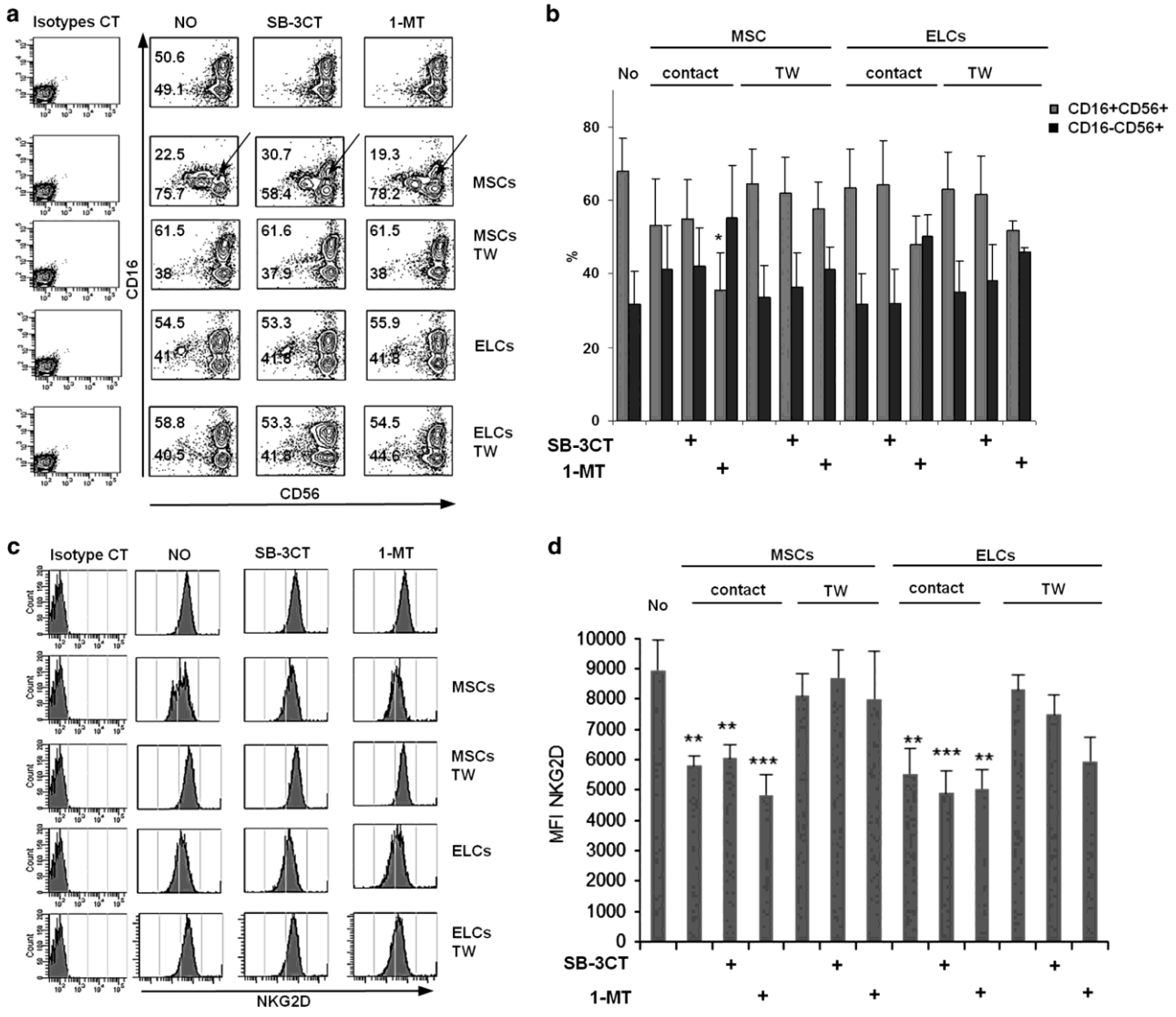

Figure 6 WJ-MSCs and ELCs modify NK-cell phenotype. (a and b) Determination by flow cytometry of the distribution and percentages of $CD16^{+}CD56^{+}$ and $CD16^{-}CD56^{+}$ populations. Activated NK cells were co-cultured in the absence or presence of WJ-MSCs and ELCs, and in the absence or presence of

SB-3CT (MMP-2 and MMP-9 inhibitor) and 1MT (IDO inhibitor). Co-cultures were performed under both direct contact or TW conditions. The first column shows the staining of isotype controls for CD16 and CD56. (c and d) MFI of NKG2D was significantly inhibited after co-culture with WJ-MSCs or ELCs under direct contact conditions. The first colmun shows the staining of isotype control for NKG2D. Data shown in a and c are representative dot plots. Data in b and d are shown as the mean ± s.e.m. ($n = 3$).

We next examined the expression of NKG2D on NK cells after co-culture with ELCs and WJ-MSCs. As shown in Figures 6c and d, NKG2D levels were significantly decreased after co-culturing with WJ-MSCs and ELCs under direct contact conditions, with an MFI of 5804 ± 334 and 5533 ± 840 for NK cells in direct contact, respectively, with WJ-MSCs and ELCs, versus 8952 ± 978 for NK cells alone. Interestingly, expression of NKG2D was partially restored for NK cells cultured without direct contact with WJ-MSCs or ELCs. Inhibition of MMPs and IDO had no noticeable effect on NKG2D expression regardless of the culturing conditions, as their inhibitors did not restore NKG2D expression (Figure 6d).

NK cells acquire CD73 on exposure to WJ-MSCs and ELCs. We observed that CD73 was the only WJ-MSCs marker whose expression was not modified during differentiation. As CD73 produces adenosine, it could have a role in immunosuppression mediated by WJ-MSCs and ELCs (Figure 1B). To test this hypothesis, we examined CD73 expression on NK cells after co-culturing with ELCs and compared it with CD73 expression on NK cells alone. After 3 days of co-culture with ELCs, CD73 expression increased from an MFI of 231 ± 54 to 1961 ± 917, but CD73 uptake exhibited a high variability between cells from different individuals. We also confirmed that the co-culture with WJ-MSCs allowed CD73 transfer on NK cells, with an MFI reaching 989 ± 441, as shown previously[33] (Figure 7). To exclude the possibility that the interaction between the cells can induce the expression of CD73 in NK cells, a real-time PCR for CD73 was performed on purified NK cells and compared with the CD73 expression detected in endothelial cells from the umbilical artery, used as a positive control. The result showed no expression of CD73 mRNA in NK cells (data not shown), confirming that the expression of CD73 on NK cells was due to a transfer.

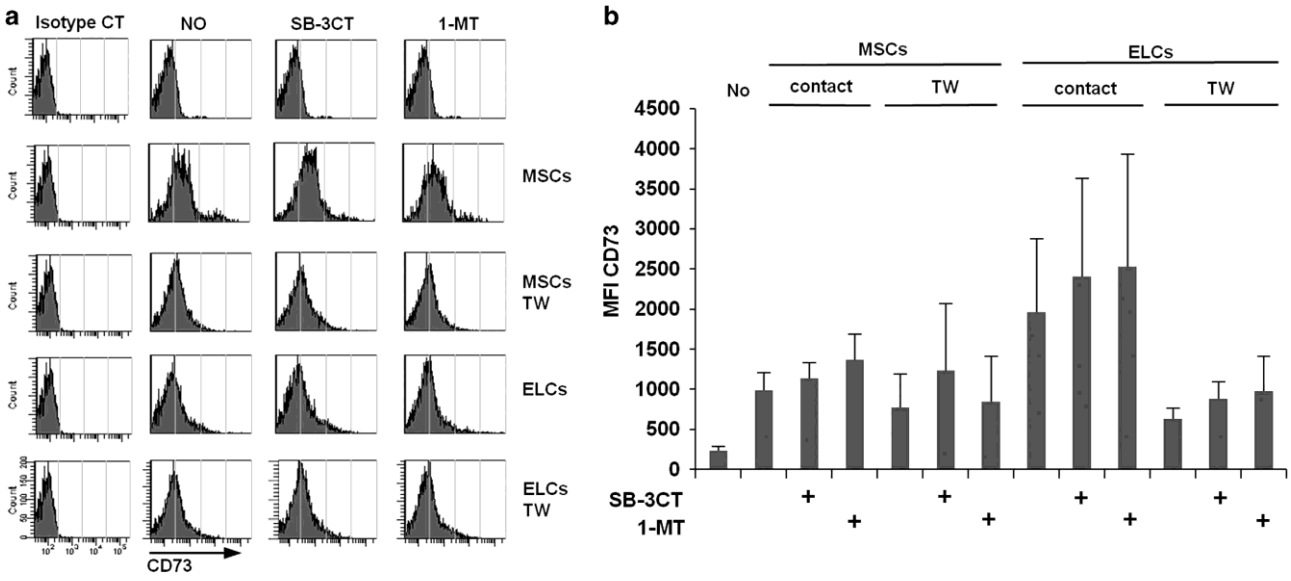

Figure 7 Flow cytometry analysis of CD73 expression on NK cells after co-culture with WJ-MSCs and ELCs under contact or TW conditions.

(a) Representative histogram showing CD73 expression on NK cells after co-culturing with ELCs or WJ-MSCs under direct contact conditions. Activated NK cells were co-cultured in the absence or presence of WJ-MSCs and ELCs with or without SB-3CT (MMP-2 and MMP-9 inhibitor) and 1MT (IDO inhibitor). The first column shows the staining of isotype control for CD73. (b) CD73 MFI from three independent experiments determined under the same culture condition as described in a ($n = 3$).

DISCUSSION

Regeneration of blood vessels for the treatment of vascular-related diseases is one of the most important research areas in tissue engineering based on ECs.[37–39] However, the use of ECs is hampered by a number of factors.[40,41] MSCs have been considered as alternatives, as they can be used in an allogeneic setting without the need for immunosuppressive therapy.[40]

WJ, the main component of the extracellular matrix of umbilical cord, is known to contain a population of fibroblast-like cells that are described as MSCs.[42] The major objective of the present study was to determine whether the immune regulatory properties of MSCs are modified by differentiating them toward an endothelial phenotype. For this, we aimed to obtain ELCs from WJ-MSCs by combining a functionalized cell culture surface and growth factors. Endothelial differentiation was assessed by the expression of CD31, VE-cadherin and VE growth factor receptor 2 on ELCs. These three proteins are endothelial-specific markers known to have an important role in maturation of the endothelium during the angiogenesis process.[43] Our CD31 expression result is at odds with the studies of Oswald *et al.*[44] and Janeczek *et al.*,[21] who reported no change in CD31 expression in differentiated BM-MSCs. Thus, the expression of these markers by WJ-MSCs seems to promote differentiation toward the endothelium. We next demonstrated that our differentiated cells were functional, as we observed both efficient uptake of Ac-LDL and tubular structure formation in an extracellular matrix gel. Although several studies have shown that uptake of Ac-LDL by ECs is modulated by shear stress,[45,46] Ac-LDL was successfully incorporated by ELCs under our static culture conditions.

Previous studies have shown that the hypoimmunogenicity of MSCs derives from the absence of HLA-DR and CD86 expression. In this study, the expression of HLA-DR and CD86 was barely detectable, suggesting that even after differentiation ELCs remained immunopri- vileged. This result contrasts with previous reports, in which *in vitro* differentiation of allogeneic BM-MSCs into myogenic, smooth muscle, endothelial[17] and neuronal[16] lineages significantly increased the expression of major histocompatibility complex-II.

We also observed that our produced ELCs were not immunogenic, as they did not stimulate allogeneic PBMCs in co-culture. Furthermore, they were immunosuppressive, as they modulated PHA-stimulated PBMCs alloproliferation. In fact, the suppressive effect was enhanced by ELCs in comparison with WJ-MSCs and largely exerted under conditions of contact. Similar results were obtained after differentiation of MSCs into adipose, bone and cartilage lineages.[14,15,47] These results led us to investigate the effects of differentiated cells on: (1) a population of cells enriched in $CD3^+$ T cells, the major effector cells of adaptive immunity;[48] and (2) NK cells, as they represent the major effector of the innate immune response exhibiting a critical role in early host defense against infections and cancer.[49]

We noticed that ELCs, and to a lesser extent WJ-MSCs, suppressed the proliferation of activated $CD3^+$ T cells, mainly under cell–cell contact conditions. Inhibition of $CD3^+$ T cells by human BM-MSCs,[50–53] umbilical cord blood MSCs,[50] placenta MSCs[50,54] and umbilical cord MSCs[55,56] had already been described in the literature; however, we are the first to show that endothelial differentiation of WJ-MSCs do not alter this capacity.

Our subsequent goal was to determine the mechanisms by which WJ-MSCs and ELCs exert their immunosuppression. Candidate enzymes investigated in this context were MMPs. In addition to the important role of MMPs in the modification of the extracellular matrix, cell mobilization and cell–extracellular matrix interactions, MMPs act on additional substrates such as cytokines and inflammatory mediators, and thus have an effect on immune response regulation and cell proliferation.[57,58] Ding *et al.*[25] have found that MMP-2 and MMP-9 have a significant role in the immune suppressive activity of murine BM-MSCs, and that blocking these two MMPs completely restores T-cell proliferation. Furthermore, MMPs have been shown to be involved in the shedding of CD25. Here we have shown that downregulation of CD25 expression inhibits T-cell proliferation mediated by IL-2. However, although MMP-2 and MMP-9 were detected in supernatants of ELCs and WJ-MSCs co-cultured with T cells, we observed that (1) addition of SB-3CT did not have a major effect on restoring T-cell proliferation and (2) CD25 expression on T cells increased after co-culture with ELCs or WJ-MSCs.

We hypothesized that this increase was due to proliferation of a Tregs population, as was previously demonstrated by Selmani *et al.*[59] and others.[30,50,60,61] This idea was subsequently confirmed by demon- strating that the population of $CD4^+CD25^+FoxP3^+$ detected in direct co-cultures with ELCs or WJ-MSCs increased significantly relative to appropriate controls. Similarly, addition of SB-3CT decreased Tregs production, consistent with the idea that MMPs have a role in their generation.[62] Among the other mediators identified as actors in MSCs immunomodulation, we observed no TGF-β, hepatocyte growth factor and COX-1 expression by ELCs and WJ-MSCs in all culture condi- tions, a result that contrast with previous studies.[63,64] IL-6 and IL-1β were detected in T cells co-cultured with WJ-MSCs and ELCs. IL-1β has been shown to be involved in the proliferation of Tregs;[65,66] thus, this mediator conjugated with IDO[67–70] could be involved in their expansion. However, our results also

revealed high expression levels of COX-2. This enzyme is responsible for producing PGE2, a prostaglan- din that promotes Tregs differentiation.[71] Thus, we cannot exclude the involvement of PGE2 in the generation of Tregs observed in our study. The immunosuppressive effects of ELCs were also confirmed on NK cells, as their proliferation was partially inhibited. Among the soluble mediators that could be involved in inhibition of NK proliferation, MMP-2 and MMP-9, IDO, IL-6 and IL-1β cannot be excluded, in particular after direct contact between NK cells and ELCs. Recently published data have revealed an autocrine loop between IDO[72] and IL-6.[73] More specifically, IDO-mediated aryl hydrocarbon receptor activation induced IL-6 expression, whereas inhibition or knockdown of the aryl hydrocarbon receptor reduced IL-6 expression. These data explain our observation that the use of an IDO inhibitor resulted in a decrease in IL-6 concentration in NK-cell supernatants. As to the NK-cell phenotype after co-culture culturing with ELCs, no change in the partitioning between cell populations was observed. In contrast, the $CD16^{+}CD56^{+}$ population was no longer observed after co-culturing with WJ-MSCs but partially restored after adding IDO and MMPs inhibitors. This latter result is in agreement with studies showing that MMPs are involved in the shedding of CD16, which occurs after NK-cell activation and places the cells in a refractory phase.[35]

We next examined the impact on NKG2D expression of co-culturing NK cells with ELCs. We focused on this receptor, because several studies have reported that the inhibitory effects on MSCs are closely related to the downregulation of activating receptors, especially NKG2D.[72] The decrease in NKG2D that we observed after contact with ELCs accords with a recent publication by Chauveau *et al.*,[74] in which it was demonstrated that ECs activation and proliferation modulate NKG2D activity. This may have an impact on NK-cell- mediated cytotoxicity. In the case of WJ-MSCs, we observed the same results as Spaggiari *et al.*,[72] which were an inhibition of NK-cell proliferation and modification of phenotype. As Spaggiari *et al.*[72] highlighted the role of IDO and PGE2 in this phenomenon, we tested whether 1MT could restore NKG2D expression. Surprisingly, addition of 1MT did not have the expected effect, suggesting that restoration of NKG2D expression may require the inhibition of more than one mediator.

Finally, we cannot exclude a role of adenosine in the immunosuppressive effects of ELCs that we observed on NK cells, regarding ELCs high expression level of CD73. In addition to the roles of adenosine in the endothelial microenvironment, it has been shown that adenosine is a potent immunosuppressor involved in inhibiting the expression of IL-2, CD25, interferon -γ and tumor necrosis factor-α, and that it decreases the cytolytic activity of cytotoxic T cells.[75,76] Very recently, NK cells were shown to acquire the expression of CD73 after a co- culture with MSCs, and that $CD73^{+}$ NK cells had the ability to convert adenosine monophosphate into adenosine.[33] Our results confirm not only that NK cells are able to acquire CD73 on co-culture with WJ-MSCs, but that the proportion of $CD73^{+}$ NK cells is higher after co-culture with ELCs. Further studies should confirm whether the upregulation of CD73 in NK cells is accompanied by increased enzyme activity.

In conclusion, our results show that WJ-MSCs and ELCs share many immunomodulatory properties. Nevertheless, the extent of expression of soluble mediators differed according to the immune cell type with which they were co-cultured and whether direct cell–cell contact was allowed.

Thus, this work shows overall that the immunological features of WJ-MSCs after endothelial differentiation may allow their application in regenerative medicine, as they appear likely to be immunotolerated by immune cells.

METHODS

Reagents were obtained from Sigma-Aldrich (Munich, Germany) and media components by from Lonza (Levallois-Perret, France), unless specified otherwise.

WJ-MSCs isolation and culture

Human umbilical cords were obtained after birth delivery and informed consent using the guidelines approved by the University Hospital Center of Nancy. WJ-MSCs were isolated as described by Petsa *et al.*[77] and cultured in α-minimum eagle medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 $IU\,ml^{-1}$ penicillin, 100 $\mu g\,ml^{-1}$ streptomycin, 2 m_M L-glutamine and 205 mg ml^{-1} Fungizon (Life Technologies, Grand Island, NY, USA).

Induction of endothelial differentiation

After the third passage, WJ-MSCs were collected and then seeded on $(PAH/PSS)_4$ polyelectrolyte multilayer film-coated glass slides, as described elsewhere.[78] Uncoated glass was used as control. After reaching 60% of cell confluence, the medium was replaced by EBM-2 or differentiation medium, which consisted of EGM-2 (EBM-2 supplemented with growth factors) and incubated for 2 weeks. EBM-2 was supplemented with 0.5% heat-inactivated FBS, 100 $IU\,ml^{-1}$ penicillin, 100 $\mu g\,ml^{-1}$ streptomycin, 2 m_M L-glutamine and 205 $mg\,ml^{-1}$ Fungizon. As a control, cells were cultured either on non-coated or coated glass with EBM-2. Cell morphology was monitored daily by optical microscopy (Nikon, UFX-DX, Tochigi, Japan). Following 15 days of culture under the differentiation conditions with

EGM-2, cells were identified to be ELCs. WJ-MSCs and HUVEC cells were respectively used as negative and positive controls.[39,79] HUVEC cells were isolated according to Jaffe *et al*.[80] and cultured in EBM-2 supplemented with 10% heat-inactivated FBS.

Characterization of ELCs

Endothelial differentiation was assessed by western blotting. Cells were collected in RIPA (radioimmunoprecipitation assay buffer) lysis buffer. Twenty micrograms of protein lysates were subjected to SDS–PAGE (polyacrylamide gel electrophoresis) resolution and transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% bovine serum albumin in Tris buffered saline-Tween 20 solution and then probed with the following primary antibodies to endothelial phenotypic markers: CD31 (Dako Agilent Technol- ogies, Santa Clara, CA, USA, M0823), VE-cadherin (Abcam, Cambridge, UK, ab33168), VE growth factor receptor 2 (Cell Signaling Technology, Danvers, MA, USA, 55B11) and to glyceraldehyde 3-phosphate dehydrogenase (Santa Cruz, Dallas, TX, USA, sc-51906) as a loading control. The membranes were then incubated with appropriate horseradish peroxidase-conjugated secondary antibody at room temperature and bands were detected by chemiluminescence.

ECs have the ability to incorporate Ac-LDL. The functionality of the ELCs was thus assessed using an Ac-LDL uptake assay performed as described by Doan *et al*.[81] Analysis of capillary formation was performed with an *in vitro* angiogenesis assay. For this, extracellular matrix gel solution (ScienCell, Carlsbad, CA, USA) maintained at 4 °C was added onto μ-Slide Angiogenesis (Ibidi, Munich, Germany) and incubated for 30 min at 37 °C to allow solidification. Differentiated cells and controls (HUVECs and WJ-MSCs) were suspended in culture medium at a density of 2×10^5 cells per ml and 50 μl of each solution was plated on solidified extracellular matrix gel. After 4 h of incubation (37 °C), capillary formation was analysed by light microscopy.

Isolation and culture of PBMCs, T cells and NK cells

Peripheral blood samples were obtained from healthy volunteers with informed consent for blood collection and biological studies, using the guidelines approved by the French blood collection center Lorraine Champagne. PBMCs, T cells and NK cells were collected and cultured as previously described,[82] except for $CD3^+$ T cells that were cultured in RPMI-1640 medium supplemented with 10% AB serum, 2 m_M glutamine, 1 $\mu g\,ml^{-1}$ streptomycin, 1 IU ml^{-1} penicillin and 5 $\mu g\,ml^{-1}$ PHA (Oxoid, Dardilly, France). After 3 days, 100 $\mu g\,ml^{-1}$ of IL-2 (Biotechne, Lille, France) was added to the culture medium until day 14, when the cells were collected.

In vitro assays for testing the immunomodulatory effects of WJ-MSCs and ELCs on the proliferation of PBMCs

One-way mixed lymphocyte reaction. To determine whether WJ-MSCs and ELCs function as alloantigen presenting cells, one-way mixed lymphocyte reactions were performed. For this, freshly isolated PBMCs were added to WJ-MSCs or ELCs at a 1:1 ratio and allowed to proliferate in co-culture for 3 days. PBMCs seeded alone were used as control. Co-cultures were performed either by putting the two cell populations in direct contact or by separating them using a TW membrane (pore size: 0.4 μm; Millicell Inserts, Dutscher, Brumath, France). After 3 days of culture, non-adherent immune cells, adherent ELCs and WJ-MSCs, and supernatants were respectively collected for DNA quantification, RNA extraction and cytokine dosage.

Mitogen-stimulated proliferation of PBMCs. To determine the effects of WJ-MSCs and ELCs on the PHA-stimulated proliferative response of allogeneic PBMCs, freshly isolated responder PBMCs stimulated with 10 $\mu g\,ml^{-1}$ PHA were added to cultures of WJ-MSCs and ELCs, as described above. Cultures of unstimulated and PHA-stimulated PBMCs seeded alone were used as controls. Co-cultures were performed either by putting the two cell populations in direct contact or by separating them using a TW.

Co-culturing of WJ-MSCs and ELCs with T or NK cells

Immune cells (expanded T or NK cells) were cultured for 3 days with WJ-MSCs and ELCs at a ratio of 1:1 in RPMI-1640 10% FBS containing 100 $\mu g\,ml^{-1}$ IL-2 for co-culturing with T cells and in α-minimum eagle medium 10% FBS IL-15 containing 50 $ng\,ml^{-1}$ for co-culturing with NK cells, either allowing for direct contact or keeping the cells separated by a TW. For inhibition experiments, the cells were cultured in the presence of 60 μ_M SB-3CT, a specific inhibitor of MMP-2 and MMP-9, and 1 m_M of the IDO inhibitor 1MT.

Measure of immune cell proliferation

DNA from immune cells (PBMCs, T cells or NK cells) in co-culture was measured as a marker of cell quantity using Hoechst 33258 (Life Technologies, Grand Island, NY, USA) as shown by Poleni *et al.*[83].

Flow cytometry

Cells were incubated in 0.5% human serum albumin in phosphate buffered saline staining buffer and stained with fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)- or allophycocyanin-conjugated antibodies or correspond- ing isotype controls (CD25 FITC, CD45 FITC, CD90 FITC, B7H1 FITC, FoxP3 PE CD34 PE, CD73 PE, CD105 PE, B7H4 PE, CD3 allophycocyanin from BD Pharmingen, Franklin Lakes, NJ, USA; and CD16 FITC, HLA-DR PE, NKG2D PE, CD86 PE, CD56 allophycocyanin from Miltenyi Biotec, Tubingen, Germany) for 30 min at room temperature followed by two washes in staining buffer. For intracellular staining of FoxP3, cells were fixed with 1% parafor- maldehyde and permeabilized with phosphate buffered saline 0.5% human serum albumin, 0.1% Triton X-100. Labeled cells were acquired by FACS Canto I (BD Biosciences, Francklin Lakes, NJ, USA) and 100 000 events were analyzed using FACS Diva Software (BD Biosciences). For double or triple staining performed by flow cytometry, isotype controls were used to place region and quadrants. Immune cells were immunophenotyped after co-culture with or without WJ-MSCs or ELCs under direct contact or TW conditions, in the presence or absence of IDO and MMPs inhibitors. NK cells were identified as $CD16^+CD56^+$ cells and the expression levels of NKG2D and CD73 were determined by gating this population. For T cells, the CD25 marker was quantified after gating the CD3+ population. Tregs were identified as $CD25^+$ $FoxP3^+$ cells, after gating on the $CD4^+$ population. The WJ-MSCs phenotype was evaluated before and during differentiation into ELCs by verifying the expression of CD73, CD90 and CD105, and the absence of CD34 and CD45. Expression of two immunological markers, HLA-DR and CD86, was also monitored.

Real-time quantitative PCR

After removing the suspension of lymphocytes from co-culture, total RNA from adherent cells was isolated using QIAzol lysis reagent and RNeasy Micro Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. One hundred nanograms of total RNA was reverse transcribed using iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). Real-time PCR was performed using iTaq Universal SYBR Green Supermix (Bio-Rad) and the StepOnePlus system (Life Technologies, Grand Island, NY, USA). The indicated genes (Supplementary Table 1 and Supplementary Material) were analyzed by choosing ribosomal protein 29 (*RP29*) gene as the housekeeping gene for normalization.

Cytokine determination in cell culture supernatants by cytokine bead array

IL-12p70, tumor necrosis factor, IL-10, IL-6, IL-1β and IL-8 concentrations were quantitatively measured in culture supernatants using the BD Cytometric Bead Array Inflammation Kit (BD Biosciences) according to the manufacturer's instructions.

Detection of MMP-2 and MMP-9

Gelatin zymography was performed on culture supernatants to assess secreted MMP-2 and -9 activities, as described previously.[84] Gels were scanned with a calibrated GS-800 imaging densitometer (Bio-Rad) and analyzed using ImageJ (National Institutes of Health, USA).

INF-γ detection by enzyme-linked immunosorbent assay

Cultured cell supernatants were collected after 3 days of co-culture and tested in triplicate for the production of INF-γ (Peprotech, 900-M27; Neuilly sur Seine, France) by enzyme-linked immunosorbent assay according to the manufac- turer's instructions. The enzyme-linked immunosorbent assay plates were read at OD 405 nm with a standard microplate reader (Multiskan EX, Thermo LabSystems, Helsinki, Finland). The results were expressed in ng ml^{-1}.

Statistical analysis

Data are presented as mean ± s.e.m. for each condition. Pairwise comparisons were performed using one-factor analysis of variance with Fisher's correction (Statview IVs, Abacus Concepts Inc., Berkley, CA, USA). Differences were considered significant for *P* (rejection level of the null hypothesis of equal means) values o0.05.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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