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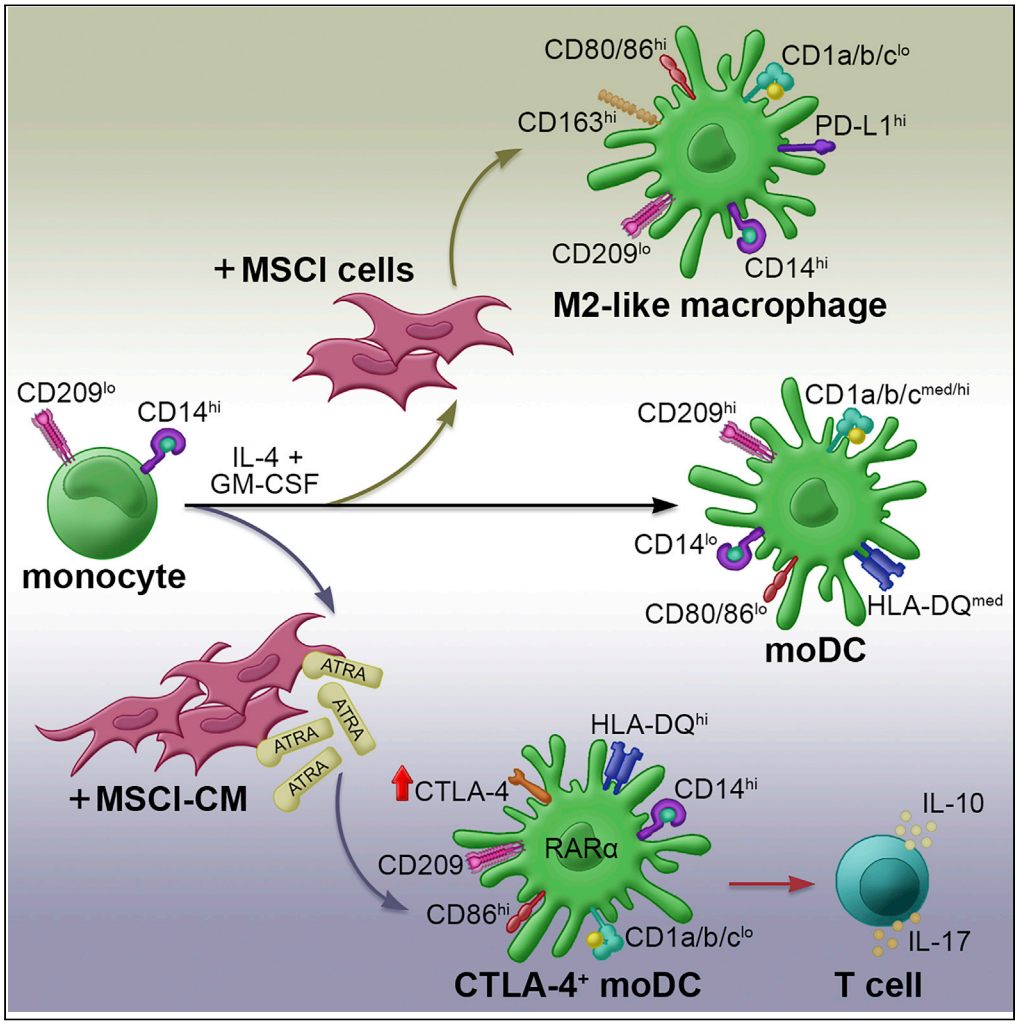

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Highlights

Mesenchymal stromal cell-like cells alter moDC differentiation via RARα activation

Mesenchymal stromal cell-like cells express genes known to play role in ATRA synthesis

MoDCs, differentiated in the presence of MSCI-derived factors, express CTLA-4

CTLA-4^{+} moDCs are able to induce polarization of IL-10- and IL-17-producing helper T cells

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MSC-like cells increase ability of monocyte-derived dendritic cells to polarize IL-17-/IL-10-producing T cells via CTLA-4

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SUMMARY

Mesenchymal stromal cell-like (MSCl) cells generated from human embryonic stem cells are considered to be an eligible cell line to model the immunomodulatory behavior of mesenchymal stromal cells (MSCs) *in vitro*. Dendritic cells (DCs) are essential players in the maintenance and restoration of the sensitive balance between tolerance and immunity. Here, the effects of MSCl cells on the *in vitro* differentiation of human monocytes into DCs were investigated. MSCl cells promote the differentiation of CTLA-4 expressing DCs via the production of all-trans retinoic acid (ATRA) functioning as a ligand of RARα, a key nuclear receptor in DC development. These semi-matured DCs exhibit an ability to activate allogeneic, naive T cells and polarize them into IL-10 + IL-17 + double-positive T helper cells in a CTLA-4-dependent manner. Mapping the molecular mechanisms of MSC-mediated indirect modulation of DC differentiation may help to expand MSCs' clinical application in cell-free therapies.

INTRODUCTION

Due to their multipotent differentiation ability and strong immunomodulatory potential, mesenchymal stromal cells (MSCs) are promising candidates for cell-based therapy of several inflammatory, immune-mediated, and degenerative diseases (Saeedi et al., 2019). There are a number of ongoing clinical trials related to the immunomodulatory effects of MSCs or linked to graft enhancement utilizing their immunosuppressive functions (Wang et al., 2018). However, the exact cellular and molecular mechanisms underlying the MSC-mediated immunomodulation have yet to be revealed. Despite a large number of studies focusing on changes in cells and tissues following MSCs administration, several pieces of the puzzle are still missing and results are often inconsistent. Possible reasons include that MSCs from different sources and under different culture conditions may have diverse phenotype and epigenetic background, secrete distinct patterns of soluble factors and possess different capacities to proliferate (Weiss and Dahlke, 2019). One of the strategies to overcome these limitations is the use of MSC-like (MSCl) cell lines derived from human pluripotent stem cells, generating unlimited numbers of early passage MSCs with consistent quality and immune suppressive properties (Kimbrel et al., 2014).

We have earlier demonstrated in a collaborative study that based on their phenotypic and functional properties such as differentiation potential and immunomodulatory activity, human MSCl cells generated from pluripotent HUES9 embryonic stem cells are eligible to model the behavior of bone marrow-derived MSCs *in vitro* (Varga et al., 2011). In a recent study, we have provided a body of evidence that these MSCl cells are able to modulate the responses of monocyte-derived dendritic cells (moDCs) to retinoic acid-inducible gene I receptor-mediated activation (Bacskai et al., 2015). Human moDCs stimulated in the presence of MSCl cells exhibited reduced expression of phenotypic indicators of DC activation, lower production of TNF-α, CXCL10, IL-12 and IFNγ, as well as a decreased migration and T cell polarization as compared to moDCs activated in the absence of MSCl cells (Bacskai et al., 2015). These prior observations indicate that activation of mature moDCs can be efficiently suppressed by MSCl cell-derived signals and mechanisms. In this study, we investigated whether and how the *in vitro* differentiation of human monocytes into DCs is altered by MSCl cells.

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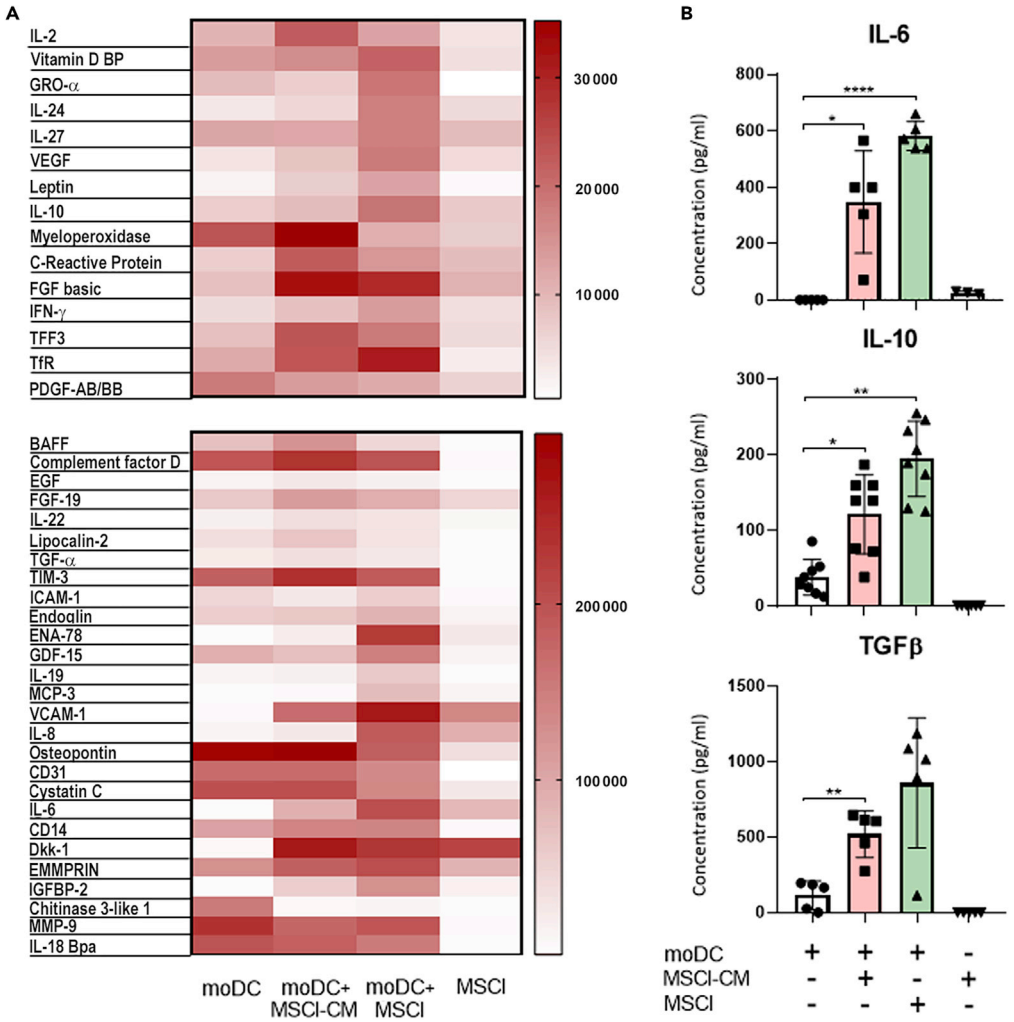

Figure 1. The cytokine and chemokine production of moDCs is modulated by MSCI cells

To examine the regulatory effects of MSCI cells or the MSCI-CM on the cytokine and chemokine production of moDCs, on day 4 of moDC differentiation the integrated density of soluble mediators was measured by Human XL Cytokine Array Kit (A) and the concentration of secreted cytokine IL-6, IL-10, TGFβ was detected by ELISA (B).

In case of co-cultures, the diagrams represent the secretion of cytokines released by moDCs and MSCI cells. Mean values of relative cytokine levels and concentrations were calculated from 4 or more independent experiments. Data are represented as individual data points with the mean ± standard deviation. In the statistical analysis, one-way ANOVA followed by Bonferroni's multiple comparison test was used with significance defined as *$p < 0.05$, **$p < 0.01$ and ****$p < 0.0001$.

See also Figure S1.

RESULTS

The cytokine and chemokine production of moDCs is modulated by MSCI cells

To analyze the direct and indirect immunomodulatory effects of MSCI cells, moDCs were differentiated in the presence of MSCI-CM or MSCI cells and relative cytokine, chemokine and other soluble protein levels in the cell culture supernatants were investigated using a protein array (Figure S1). It was observed that soluble factors released by MSCI cells remarkably altered the protein secretion profile of moDCs. Elevated levels of BAFF, complement factor D, EGF, IL-2, IL-22, lipocalin-2, TGFα, TIM3, myeloperoxidase, C-reactive protein, and Dkk-1, while decreased productions of ICAM-1 and EMMPRIN were detected in the supernatant of moDCs differentiated in the presence of MSCI-CM (Figure 1A). However, increased levels of Vitamin D BP, Endoglin, ENA78, GDF-15, GRO-α, IL-24, MCP-3, VEGF, IL-8, IL-10, and IFNγ, but reduced amounts of FGF-19, Osteopontin, CD31, and IL-18 Bpa were found in the supernatant of moDC-MSCI cell

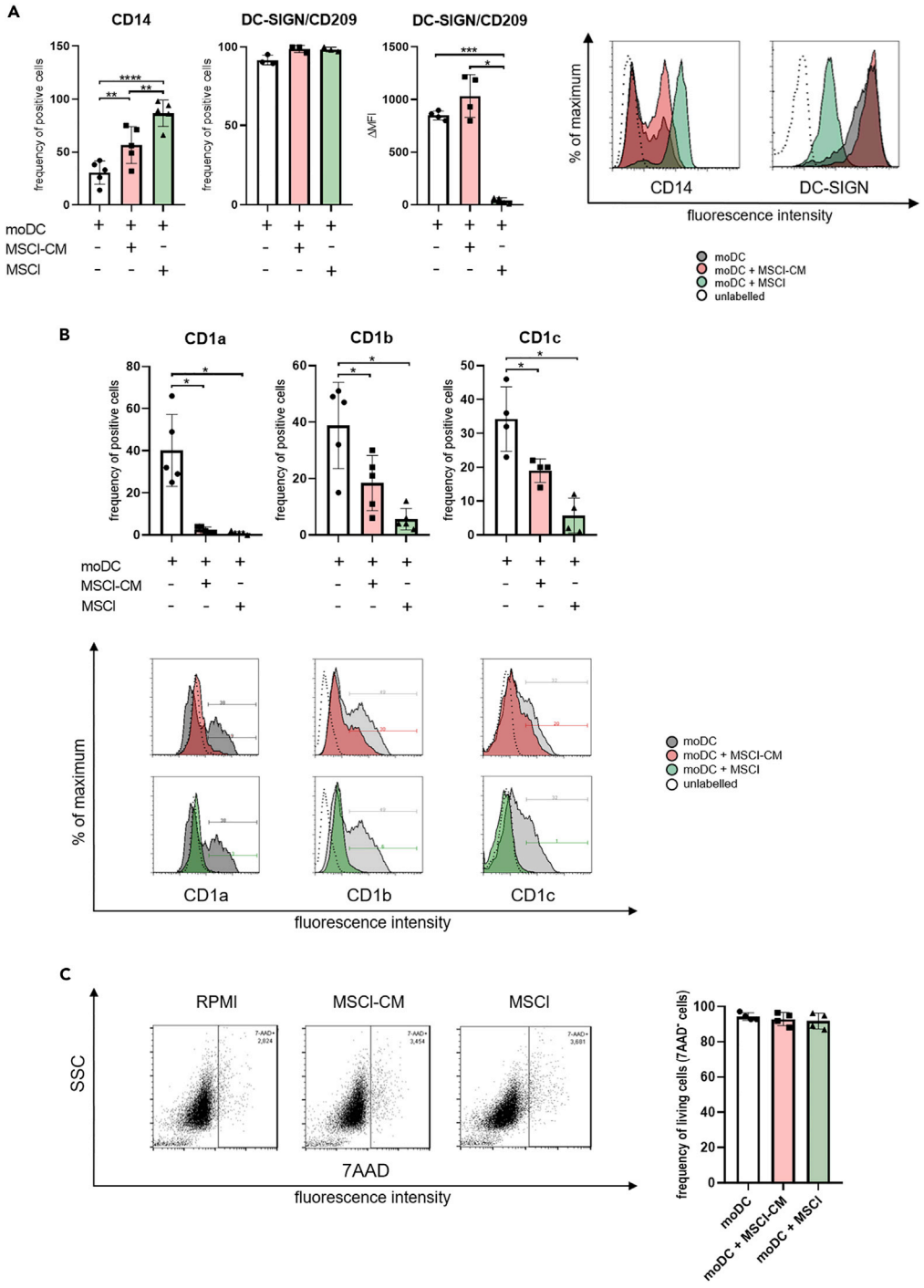

Figure 2. MSCI cells and MSCI-CM modify the cell surface expression of CD14, DC-SIGN/CD209 and group1 CD1 family members

CD14^{+} monocytes were cultured with recombinant IL-4 and GM-CSF ± MSCI cells or MSCI-CM for 4 days. On day 4, the cell surface expression of CD14 and DC-SIGN (A), and that of CD1a, CD1b, and CD1c (B) were analyzed by flow cytometry on monocyte-derived cells.

To exclude the possibility of unspecific staining because of the presence of dead cells, the viability of cells was measured by 7-aminoactinomycin D (7-AAD) staining using flow cytometry (C). The MFI (median fluorescence intensity) and the mean values of the ratio of cells positive for the measured surface molecules were calculated from at least three independent experiments. Data are represented as individual data points with the mean ± standard deviation.

Figure 2. *Continued*
Histograms show one of at least four independent experiments. In the statistical analysis, one-way ANOVA followed by Bonferroni's multiple comparison test was used with significance defined as *p < 0.05, **p < 0.01, ***p < 0.001 and ****p < 0.0001.
See also Figure S2.

co-cultures (Figure 1A). Both exposure to MSCl-CM and direct moDC-MSCl cell contact raised the secretion of IL-19, VCAM-1, leptin, IL-6, CD14, FGF basic, IGFBP2, TFF3 and TfR, while the production of IL-27, Cystatin C, chitinase 3-like 1, MMP-9, and PDGF-AB/BB was diminished in the presence of either MSCl-CM or MSCl cells (Figure 1A). To validate the protein array results, concentrations of some inflammatory (IL-6) and anti-inflammatory (IL-10 and TGFβ) mediators were determined by ELISA (Figure 1B). In the supernatant of moDC-MSCl cell co-cultures significantly enhanced IL-6 and IL-10 productions were detected, while in the presence of MSCl-CM moDCs secreted significantly more IL-6, IL-10 and TGFβ than control cells (Figure 1B). These results demonstrate that the baseline levels of both inflammatory and anti-inflammatory mediators in moDCs cultures can be modulated by MSCl cells through the action of soluble or membrane-bound molecules.

MSCl cells modify the phenotype of monocyte-derived cells

To get insight into how the presence of MSCl-CM or MSCl cells affects the phenotypic characteristics of moDC differentiated *in vitro*, we monitored the expression of cell surface molecules by flow cytometry. During the differentiation of moDCs in the presence of GM-CSF and IL-4, the expression of CD14 is downregulated (Sallusto and Lanzavecchia, 1994), while that of DC-SIGN (Geijtenbeek et al., 2000) and group 1 CD1 family members (Sallusto and Lanzavecchia, 1994) is upregulated. In our experiments, the percentage of $CD14^+$ cells was higher in the presence of MSCl-CM or MSCl cells than in control cell cultures (Figure 2A). While the ratio of DC-$SIGN^+$ cells was similar in treated and control cell cultures, the cell surface expression of DC-SIGN molecule was significantly downregulated on MSCl-exposed cells (Figure 2A). We found that under our experimental conditions, approx. 40% of untreated cells expressed group 1 CD1 family members including CD1a, CD1b, and CD1c (Figure 2B). Exposure to either MSCl-CM or MSCl cells significantly reduced the cell surface expression of these glycolipid receptors on monocyte-derived cells, while these treatments did not modify their viability (Figure 2C). This observation suggests that the presentation of lipid and glycolipid antigens by monocyte-derived cells through group 1 CD1 proteins may be negatively modulated by MSCl cells either in a direct or an indirect manner. For further characterization of phenotypic changes of MSCl-CM- and MSCl cell-treated monocyte-derived cells, expression of CD163, a macrophage scavenger receptor (Skytthe et al., 2020), was investigated. Interestingly, treatment with MSCl-CM induced only a slight increase in the frequency of $CD163^+$ cells, whereas exposure to MSCl cells triggered a significant rise in the ratio of the macrophage marker-expressing cells (Figure S2A).

We also found that MSCl-CM significantly increased the cell surface levels of the peptide antigen-presenting HLA-DQ and the co-stimulatory molecule CD86, whereas contact with MSCl cells significantly enhanced the expression of both CD80 and CD86 on monocyte-derived cells (Figure 3A). Augmented expression of PD-L1, a co-inhibitory molecule, could also be detected on monocyte-derived cells in the presence of MSCl cells (Figure 3B). Importantly, an increased expression of another co-inhibitory molecule CTLA-4 on monocyte-derived cells was induced exclusively by MSCl-CM (Figure 3B). Elevated expression of CTLA-4 was induced by MSCl-CM in a time-dependent manner and a statistically significant difference in CTLA-4 levels on treated and control cells was found on day 4 of treatment (Figure 3C, left panel). A four-fold increase in the expression of CTLA-4 at mRNA level was also detected in four-day-old monocyte-derived cells differentiated in the presence of MSCl-CM compared to control moDCs (Figure 3C, right panel). When four-day-old immature moDCs were co-cultured with MSCl cells for 48 hr a remarkable increase in the expression of CD80, CD86, and PD-L1 on moDCs was observed (Figure S3). However, when immature moDCs were treated with MSCl-CM for a same period of time no or minor changes in the levels of co-stimulatory and co-inhibitory molecules on moDCs could be detected, suggesting that MSCl-derived soluble factors are able to modify the immune regulatory phenotype of moDCs only at the early stage of their development (Figure S3). These observations together with microscopic morphology of the cells (Figure S2B) indicate that MSCl-CM has a potential to regulate the differentiation of monocytes toward $CD1a^-$DC-$SIGN^+CD163^{low}$ semi-matured moDCs, whereas MSCl cells promote the differentiation of $CD1a^-$DC-$SIGN^{low}CD163^{high}$ M2 macrophage-like cells.

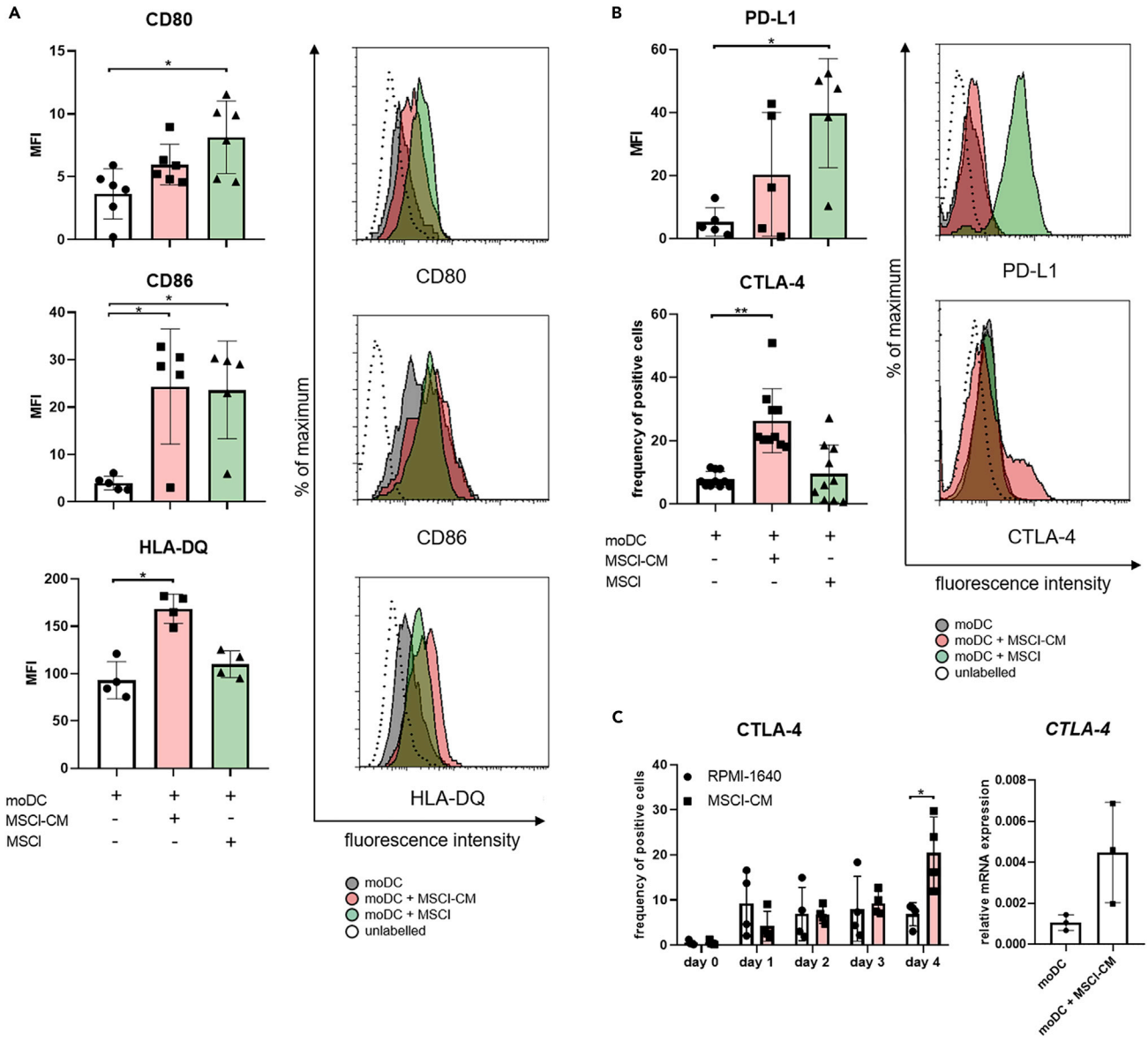

Figure 3. MSCI cells and MSCI-CM alter the cell surface expression of molecules involved in T cell activation

$CD14^+$ monocytes were cultured with recombinant IL-4 and GM-CSF ± MSCI cells or MSCI-CM for 4 days. On day 4, monocyte-derived cells were analyzed for the cell surface expression of the T cell stimulatory CD80, CD86, HLA-DQ (A) and the regulatory CTLA-4 and PD-L1 (B) molecules by flow cytometry. The frequency of CTLA-4-expressing cells was monitored on each day during the differentiation process (C).

The gene expression level of CTLA-4 was measured by qPCR on day 4 (C). The MFI and the mean values of the ratio of cells positive for the measured cell surface molecules were calculated from at least four independent experiments. Mean values of relative mRNA levels were calculated from three independent experiments. Data are represented as individual data points with the mean ± standard deviation. Histograms show one of at least four independent experiments. In the statistical analysis, one-way ANOVA followed by Bonferroni's multiple comparison test (A and B), as well as Student's t-test and Mann-Whitney rank-sum test (C) were used with significance defined as *$p < 0.05$, **$p < 0.01$.

See also Figure S3.

MSCI cells change the phenotypic characteristics of moDCs at least partially via ATRA production

It has recently been reported that RARα is able to regulate the differentiation of specialized DCs from human blood monocytes (Hashimoto-Hill et al., 2018). Therefore, to investigate the molecular mechanisms guiding the phenotypic changes of moDCs in the presence of MSCI-CM, we analyzed the role of RARα in this process using all-trans retinoic acid (ATRA) as an agonist and BMS614 as a selective antagonist of this nuclear receptor.

The expression level of CD1a on moDCs was significantly lowered by soluble factors in MSCl-CM (Figures 2C and 4A) and when cells were treated with ATRA (Figure 4A). As expected, BMS614 prevented the effect of ATRA on CD1a expression. The addition of BMS614 to MSCl-CM induced some but statistically insignificant increase in the expression of CD1a (Figure 4A). Inhibition of RARα blocked the effect of ATRA on HLA-DQ expression and also decreased the ability of MSCl-CM to intensify peptide antigen presentation; however, the observed differences were statistically non-significant (Figure 4A). The enhancement in the expression of T cell co-stimulatory and co-inhibitory molecules on moDCs by ATRA or also by MSCl-CM was largely dependent on functional RARα receptor (Figure 4A). Indeed, the upregulation of CD86 expression on moDCs by ATRA or MSCl-CM was significantly reduced when the function of RARα was inhibited. Furthermore, enhanced ratio of CTLA-4^+ moDCs induced by ATRA or MSCl-CM was also significantly diminished by the selective blockade of RARα (Figure 4A).

Next, it was further analyzed whether MSCl cells modify the immune regulatory potential of moDCs via ATRA production. First, expression of genes known to play a role in ATRA synthesis was investigated in MSCl cells and found that these cells express retinol dehydrogenase 10 (*RDH10*), and aldehyde dehydrogenase 1 family members *ALDH1A1* and *ALDH1A3*, but not *ALDH1A2* (Figure 4B). In next series of experiments, the activity of aldehyde dehydrogenase isoenzymes was inhibited using a highly selective inhibitor DEAB (Morgan et al., 2015) in MSCl cells and the differentiation of moDCs in the presence of ATRA deficient MSCl-CM was monitored. MSCl-CM collected from cultures of MSCl cells with inhibited ATRA synthesis was still able to significantly enhance the cell surface expression of HLA-DQ molecule on moDCs (Figure 4C). In contrast to HLA-DQ, changes in the expression of CD1a, CD86 and CTLA-4 on moDCs exposed to MSCl-CM was dependent on ATRA production by MSCl cell (Figure 4C).

Taken together, these results suggest that MSCl cells bring about the phenotypic changes of moDCs at least partially via ATRA production.

CTLA-4 expression on moDCs differentiated in the presence of MSCl-CM is essential to drive the development of IL-17- and IL-10-producing T cells

To further dissect the immune regulatory capacity of moDCs exposed to MSCl-CM, ELISPOT assays appropriate to monitor the moDC-mediated allogeneic T-lymphocyte activation were used. It was found that moDCs treated with MSCl-CM have a potential to significantly increase the polarization of T cells producing IL-17 (Figure 5A) and IL-10 (Figure 5B). Moreover, flow cytometric analysis revealed that moDCs cultured in the presence of MSCl-CM triggered the polarization of IL-10^+IL-17^+ double-positive T helper cells (Figure 5C).

To analyze the role of CTLA-4 in moDC-mediated T cell polarization, the CTLA-4-mediated signaling pathway was inhibited using specific antibodies. Unexpectedly, a blockade of CTLA-4 signaling in MSCl-CM-treated moDCs significantly reduced their ability to trigger the development of IL-17- and IL-10-producing T cells (Figures 5A–5C). Importantly, isotype control antibodies did not affect the IL-10 and IL-17 production of T cells primed by MSCl-CM-treated moDCs (Figure S4). In parallel experiments, a CTLA-4 blockade did not modify significantly the Th1- and Th2-polarizing capacity of MSCl-CM-exposed moDCs (Figure S5). When moDCs were treated with MSCl-CM in the presence of the specific RARα antagonist (BMS614), which partially prevented the induction of CTLA-4 expression on moDCs by MSCl-CM (Figure 4A), they also displayed a reduced capability to bring about the polarization of IL-17- and IL-10-producing T cells (Figure S6). Although the observed decreases in the number of polarized T cells in the presence of the RARα inhibitor were not statistically significant (Figure S6).

These results clearly demonstrate that the T cell-polarizing capacity of moDCs can be modulated by soluble factors derived from the MSCl cells. Furthermore, the ability of MSCl-CM-treated moDCs to induce polarization of IL-17- and IL-10-producing T cells is dependent on the level of functional CTLA-4 molecules on their surface.

DISCUSSION

Human DCs represent a very heterogeneous cell population, including distinct subsets associated with both inflammatory and regulatory functions, and with remarkable phenotypic and functional plasticity (Qian and Cao, 2018). The interplays between DCs and other cell types depend on the developmental status and the functional activities of DCs determined by current humoral and cellular components of the

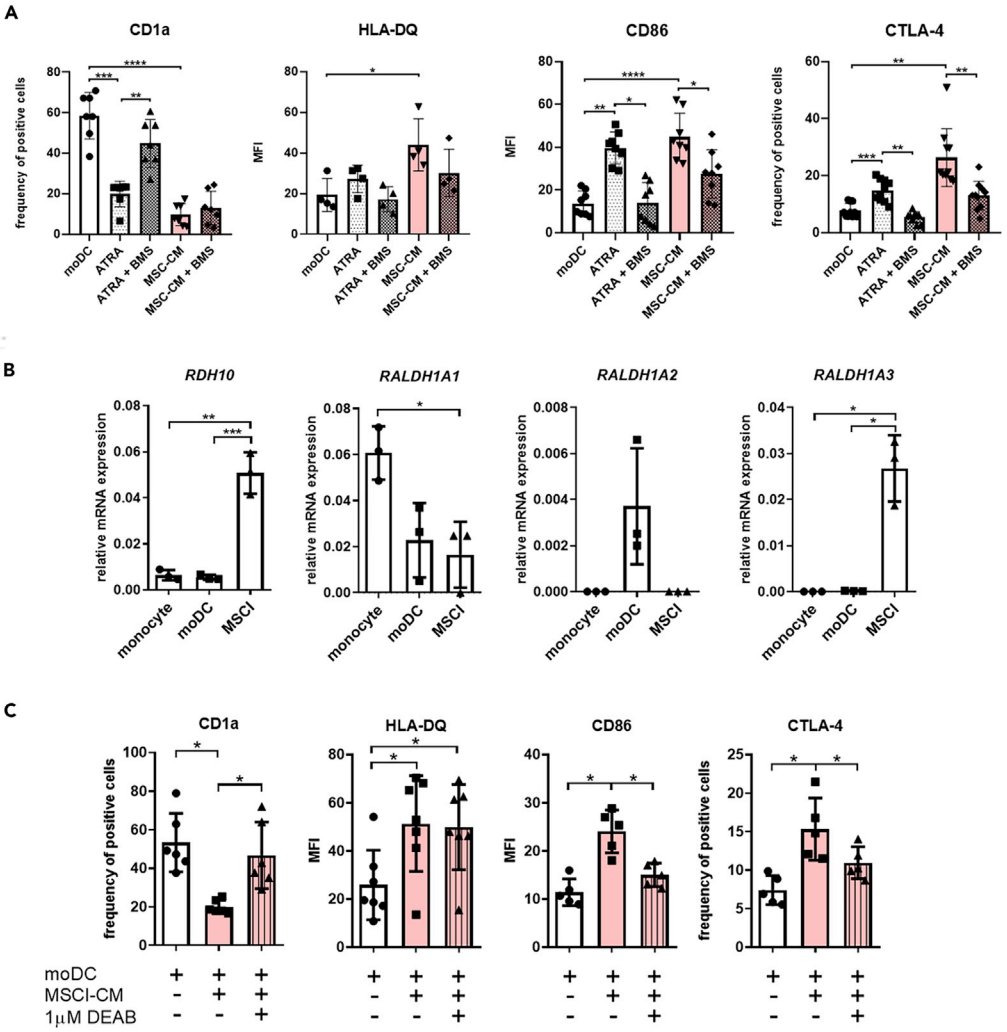

Figure 4. MSCI cells change the immune regulatory potential of moDCs at least partially via nuclear factor RARα and ATRA

To investigate how MSCI-CM affects the differentiation of moDC, monocytes were differentiated in the presence or absence of 1 nM RARα activator, ATRA followed by a 75 min incubation period with or without 1 μM BMS614 (BMS) specific RARα-antagonist prior to exchange the cell culture medium to RPMI-1640 or MSCI-CM. Monocytes were differentiated into moDC in the presence or absence of MSCI-CM for 4 days. On day 4, moDCs were analyzed for the cell surface expression of CD1a, the T cell stimulatory HLA-DQ, CD86 and the T cell co-inhibitory CTLA-4 proteins by flow cytometry (A).

To test and compare the capability of freshly isolated monocytes, moDCs and MSCI to produce ATRA, mRNA was extracted from the different cell types. The relative mRNA expression level of target genes *RDH10, ALDH1A1, ALDH1A2,* and *ALDH1A3* was measured by qRT-PCR (B).

To prove the effect of ATRA to the differentiation process of monocytes into moDCs, the RALDH enzymes essential for the synthesis of ATRA were inhibited specifically by 1μM DEAB in the MSCI cultures for 24 hr before the collection of MSCI-CM. $CD14^+$ monocytes were cultured with recombinant IL-4 and GM-CSF and MSCI-CM with or without ATRA for 4 days. On day 4, moDCs were analyzed for the cell surface expression level of CD1a, HLA-DQ, CD86 and the regulatory CTLA-4 molecules by flow cytometry (C).

Mean values of MFI and moDCs positive for the measured cell surface antigen were calculated from at least four independent experiments. Mean values of relative mRNA levels were calculated from three independent experiments. Data are represented as individual data points with the mean ± standard deviation. In the statistical analysis, one-way ANOVA followed by Bonferroni's multiple comparison test was used with significance defined as *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$, ****$p < 0.0001$.

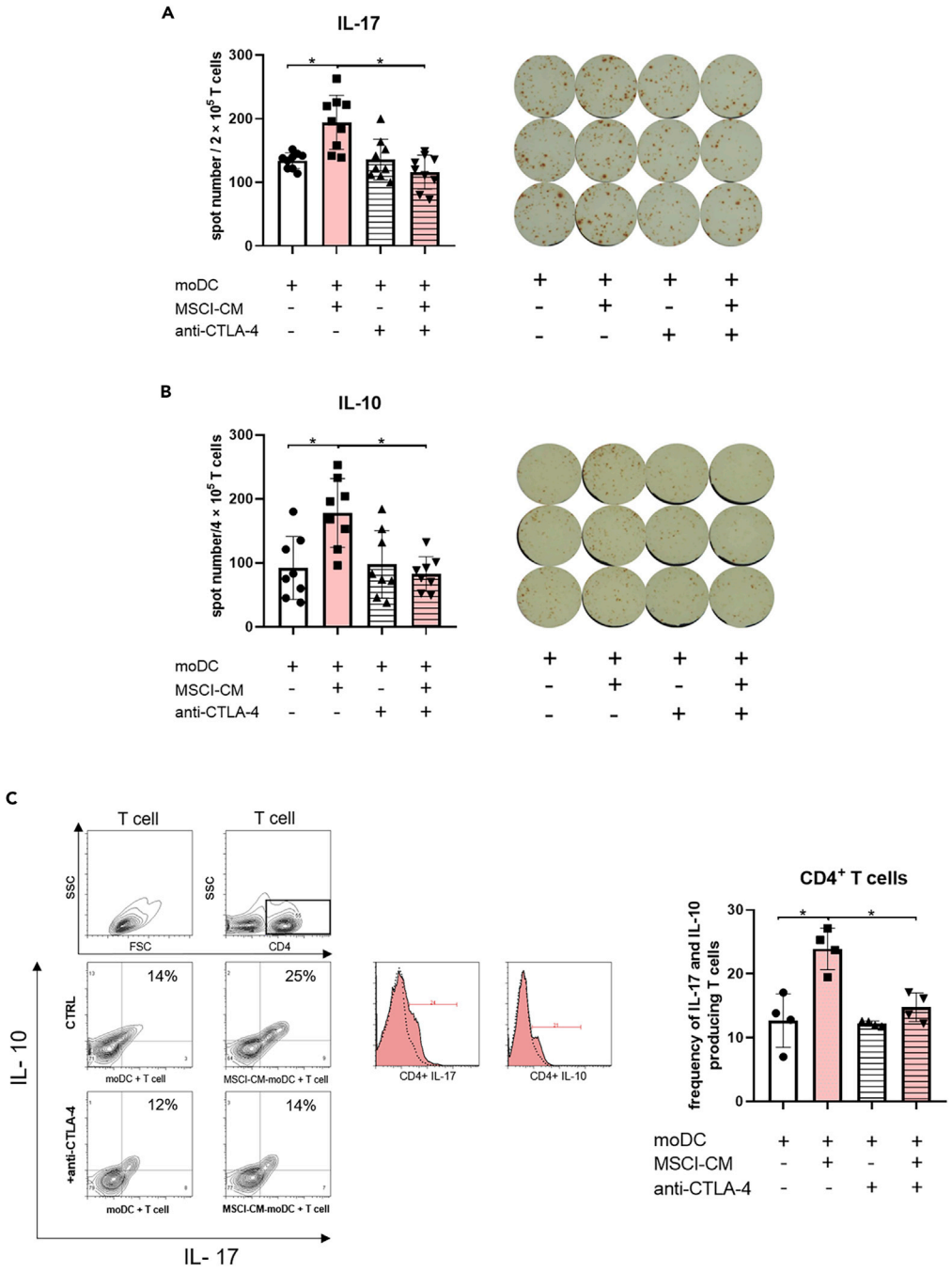

Figure 5. CTLA-4 expression on moDCs differentiated in the presence of MSCI-CM is essential to drive the development of IL-10- and IL-17-producing T cells

$CD14^+$ monocytes were differentiated into moDC in the presence or absence of MSCI-CM for 4 days. On day 4, the moDCs were treated with anti-CTLA-4 blocking antibody and were co-cultured with allogeneic T lymphocytes for further 3, 5 or 9 days. ELISPOT assays were used to determine the number of IL-17- (A) and IL-10- (B) producing T cells. The average values of spot numbers indicating T-lymphocyte responses were counted from 3 micro-wells. Mean values of spot numbers were calculated from 3 independent experiments. IL-10- and IL-17-producing $CD4^+$ T cells were detected by flow cytometry (C).

Mean values of the ratio of T cells positive for the measured cell intracellular cytokines were calculated from four independent experiments. Data are represented as individual data points with the mean ± standard deviation. Contour

Figure 5. *Continued*
plots show one of the three independent experiments. In the statistical analysis, one-way ANOVA followed by Bonferroni's multiple comparison test was used with significance defined as *p < 0.05.
See also Figures S4–S6.

given tissue milieu (Dong et al., 2015). MSCs have the potential to modulate the differentiation and the functions of T and B lymphocytes as well as innate immune cells including granulocytes (Park et al., 2012), natural killer (NK) cells (Abumaree et al., 2018), monocytes (Chen et al., 2014), macrophages (Vasandan et al., 2016), and DCs (Jiang et al., 2005). However, the capacity of MSCl cells and MSCl-CM to alter the initial differentiation of human DCs from monocytes has not yet been investigated.

During both homeostasis and inflammation, circulating monocytes leave the blood circulation and migrate into tissues where they are able to differentiate into DCs (Shi and Pamer, 2011). Several previous studies have examined the effects of MSCs on the differentiation of human moDCs. When irradiated (30 Gy) bone marrow-derived MSCs were co-cultured with freshly isolated monocytes (1:5) together with GM-CSF and IL-4, MSCs greatly inhibited monocyte differentiation into immature DCs (Zhang et al., 2004). Most of cultured cells in the presence of MSCs were negative for CD1a, indicating that the cells had not differentiated into immature DCs; however, MSCs did not affect the expression of CD14. Co-culture with MSCs substantially prevented the upregulation of the co-stimulatory molecules CD40, CD86, and CD80, as well as down-regulated HLA-DR expression. On the contrary, MSCs' supernatants had no effects on DC differentiation (Zhang et al., 2004). In another study, the transwell chamber system was used to separate monocytes from irradiated (15 Gy) bone marrow-derived MSCs during differentiation (Jiang et al., 2005). The MSC/monocyte ratio of 1:10 could completely prevent monocyte differentiation to DCs. Monocytes in the presence of MSC-released factors retained CD14-positive cells without acquisition of CD1a and displayed no up-regulation of CD83 and CD80 (Jiang et al., 2005). In a latter study, in the presence of GM-CSF, IL-4 and non-irradiated bone marrow-derived MSCs, monocytes also did not acquire the surface phenotype typical of immature ($CD14^-$, $CD1a^+$) DCs (Spaggiari et al., 2009). In a recent publication, it has been reported that after co-culture with MSCs from human induced pluripotent stem cells, monocytes displayed a low expression of CD1a, but a high expression of CD14, while exhibiting low expression of CD40, CD80, CD83, and HLA-DR (Gao et al., 2017).

In agreement with these previous findings both MSCl cells and MSCl-CM have the potential to regulate the differentiation of monocytes toward $CD14^+CD1a^-$ cells. In our experimental setup MSCl-CM had only a minor effect on DC-SIGN levels, whereas exposure to MSCl cells significantly reduced its expression. Furthermore, MSCl cells, but not MSCl-CM, triggered a significant increase in the frequency of cells expressing CD163, an M2 macrophage marker (Hu et al., 2017). These findings indicate that MSCl-CM regulates the differentiation of monocytes toward a DC-like phenotype, while MSCl cells switch monocyte differentiation to M2 macrophage rather than DC direction. In line with our observations, it was reported in a prior study that bone marrow-derived MSCs drive the differentiation of monocytes to macrophages (Jiang et al., 2005). We have found that MSCl cells produce several inflammatory and anti-inflammatory factors, among them IL-6 and IL-10. It comes as no surprise because several soluble factors have been reported to contribute to the immunomodulatory effects of MSCs, and the roles of IL-6 and IL-10 along with PGE2 and TSG-6 have been especially well established (Weiss and Dahlke, 2019). It is important to mention that anti-inflammatory mediators such as IL-6 and IL-10 induce the expression of CD163 (Etzerodt and Moestrup, 2013); therefore, these cytokines may have a pivotal role in the induction of the differentiation of M2 macrophage-like cells in our experiments. On the other hand, cytokine-independent pathways may also be involved in the MSC-induced polarization of monocytes/macrophages. In a mouse model of asthma, the phagocytosis of MSCs caused lung macrophages to turn into a type 2 immunosuppressive phenotype (Braza et al., 2016). In a recent study, it has been found that the phagocytosis of MSCs caused $CD14^{++}CD16^-$ classical monocytes to polarize toward a $CD14^{++}CD16^+CD206^+$ immune regulatory intermediate subtype with anti-inflammatory properties, increased expression of PD-L1 and production of IL-10 (De Witte et al., 2018). After phagocytosis of MSCs, these primed monocytes were able to induce $CD4^+CD25^{hi}$ Treg formation *in vitro* to a significantly higher extent than un-primed monocytes (De Witte et al., 2018). Furthermore, different Notch ligands could modulate monocyte/macrophage function and determine the M1 versus M2 macrophage polarization (Wang et al., 2010). Since Notch-mediated signals play a role in the protection against inflammation by MSCs (Li et al., 2008; Zhang et al., 2009; Lu et al., 2020), it is possible that the Notch pathway becomes activated during the direct contact between MSCl cells and monocytes. Further studies might reveal the exact mechanisms contributing to the different effects of MSCl cells and MSCl-CM on the monocyte differentiation.

Semi-mature moDCs differentiated in the presence of MSCl-CM express a low level of group 1 CD1 proteins responsible for the presentation of lipid and glycolipid antigens, while expressing increased levels of class II MHC molecules and co-stimulatory B7 family members that are essential for the activation of naive $CD4^+$ T cells. Elevated expression of CTLA-4 on semi-mature moDCs was also induced by MSCl-CM. This co-inhibitory molecule can be expressed or produced by cells with either myeloid or lymphoid origin and competes with CD28 on T lymphocytes for binding to co-stimulatory B7 family members (Halpert et al., 2016; Wang et al., 2002). CTLA-4-expressing DCs were detected in patients suffering from certain carcinomas, highlighting the importance of this molecule during tumor progression (Han et al., 2014). Furthermore, genetically modified DCs expressing CTLA-4-Ig fusion protein prevented alloimmune activity in inflammatory conditions and ensured the survival of allografts through the induction of IL-10 production by Th17 cells, indicating a tolerogenic role of CTLA-4-expressing DCs in chronic inflammation (Watanabe et al., 2016). In addition to DCs, monocytes are also able to express CTLA-4, which can be downmodulated during the differentiation process into moDCs (Laurent et al., 2010). However, our results demonstrate that the ratio of CTLA-4-expressing cells was very low in the freshly isolated monocyte population and it was enhanced from the first day of moDC differentiation and further increased in the presence of MSCl cell-derived factors on day 4. There are no available data in the literature about CTLA-4 expression on M2 macrophages that further confirms the observed distinct effects of MSCl cells and MSC-CM on the differentiation of monocyte-derived cells.

According to our observations, MSCl-CM changed the expression levels of CD1a, CD86, and CTLA-4 on semi-mature moDCs at least partially via ATRA production. We have demonstrated that MSCl cells express genes essential for ATRA synthesis. The treatment of moDCs with ATRA mimicked the effects of MSCl-CM on the expression of CD1a, CD86, and CTLA-4 molecules. ATRA is derived from retinol and acts as a ligand of nuclear hormone receptors to drive the differentiation program of moDCs (Henning et al., 2015; Nagy et al., 2012; Bene et al., 2017). The lipid-ligation of nuclear hormone receptors such as PPARγ and RARα results in altered transcriptional regulation of moDC development, metabolism, and T cell polarizing capacity (Szatmari et al., 2004). Indeed, the ability of MSCl-CM to trigger phenotypic alterations was significantly diminished by either selective blockade of RARα in moDCs or by that of ATRA synthesis in MSCl cells. The fact that in the *lamina propria* of gut, DC subpopulations and MSCs constitutively express the enzymatic machinery of ATRA production even in the absence of dietary vitamin A (Vicente-Suarez et al., 2015) further supports our *in vitro* observations.

We have found that moDCs treated with MSCl-CM have the potential to trigger the polarization of $IL\text{-}10^+IL\text{-}17^+$ double-positive T helper cells in a CTLA-4-dependent manner. Blocking of CTLA-4 signaling by specific antibodies significantly reduced the ability of MSCl-CM-treated moDCs to trigger the development of IL-17- and IL-10-producing T cells, whereas inhibition of CTLA-4 expression in MSCl-CM-primed moDCs by applying BMS614, the RARα antagonist, induced only a slight, statistically insignificant decrease in the number of polarized T cells. This phenomenon can be explained by the fact that BMS614 only partially prevented the induction of CTLA-4 expression on moDCs by MSCl-CM (Figure 4A).

An increased secretion of IL-6 and TGFβ by moDCs differentiated in the presence of MSCl-CM was detected, and these cytokines are known to be crucial regulators of IL-10 production by Th17 cells (Mcgeachy et al., 2007). We presume that CTLA-4 expressed by semi-matured DCs decreases the levels of available co-stimulatory molecules for CD28 receptors on the surface of interacting T cells. Our hypothesis is supported by a recent study showing that myeloid DCs constitutively secrete CTLA-4 in microvesicular structures (Halpert et al., 2016). $CTLA\text{-}4^+$ microvesicles can competitively bind B7 co-stimulatory molecules on bystander DC, resulting in the downregulation of B7 surface expression with significant functional consequences for T cell responses (Halpert et al., 2016). On the other hand, CTLA-4-mediated signals can also modulate cytokine secretion, as CTLA-4 triggering on mDCs increases IL-10 and reduces IL-8 and IL-12p70 production (Laurent et al., 2010). IL-17/IL-10 double-producing Th cells are indicative of a nonpathogenic Th17 cell population that has been shown to have a critical role in restraining Th17 cell-mediated inflammatory and autoimmune diseases (Mcgeachy et al., 2007; Gagliani et al., 2015). Furthermore, a significantly higher frequency of IL-17/IL-10 double-producing Th cells was found in acute myeloid leukemia patients than in healthy controls (Musuraca et al., 2015). The substantial imbalance between IL-17-/IL-10-producing cells and IL-17/IFN-γ-producing cells, together with a reduced frequency in Th1 and Th2 cells, may act as an immunosuppressive factor in these patients, altering the physiological role of Th17, contributing to the infections and probably promoting leukemia escape (Musuraca et al., 2015).

In summary, in this work we identified a previously undescribed mechanism for the immunomodulatory effects of MSCl cells on human DCs. In particular, we showed that monocyte-derived cells differentiated in the presence of MSCl cells or MSCl-CM could be characterized by a unique phenotype and functional profile. We demonstrated that despite the increased levels of class II MHC and co-stimulatory molecules on MSCl-CM-treated moDCs, these cells have the ability to induce immunosuppression via promoting the development of IL-17/IL-10 double-producing Th cells in a CTLA-4-dependent manner.

Limitations of the study

Human MSCs with different origins and under diverse culture conditions may exhibit different capacities to proliferate and may have diverse phenotypic and functional properties. To overcome these limitations, human MSC-like cells generated from pluripotent HUES9 embryonic stem cells were used in this study. These cells proved to be eligible to model the behavior of bone marrow-derived MSCs *in vitro* (Varga et al., 2011). In our experimental conditions, MSCl cells promote the differentiation of CTLA-4 expressing DCs by production of ATRA, functioning as a ligand of nuclear receptor RARα. These semi-matured DCs activate allogeneic, naive T cells and polarize them into IL-10- and IL-17-producing T helper cells in a CTLA-4-dependent manner. Further work is required to confirm these findings in primary human MSCs from different sources and also to reveal the *in vivo* relevance of this regulatory mechanism.

Resource availability

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Attila Bácsi (etele@med.unideb.hu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

Data and codes are available from the corresponding author upon request.

METHODS

All methods can be found in the accompanying Transparent Methods supplemental file.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2021.102312.

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AUTHOR CONTRIBUTIONS

Conceptualization, A.M., Z.V., K.B., and É.R.; formal analysis, A.M., K.B., and A.B.; funding acquisition, É.R., T.B., and A.B.; investigation, A.M., K.B., R.K., M.T., M.N., I.B., and K.P.; methodology, A.M., M.T., and I.B.;

resources, Z.V. and Á.A.; supervision, É.R., T.B., and A.B.; validation, A.M., K.B., M.T., and I.B.; visualization, K.Sz.; writing – original draft preparation, A.M., K.B., and A.B.; writing – review & editing, A.M., K.B., and A.B.

DECLARATION OF INTERESTS

The authors declare no conflicts of interest related to this research.

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Supplemental information

MSC-like cells increase ability of monocyte-derived dendritic cells to polarize IL-17-/IL-10-producing T cells via CTLA-4

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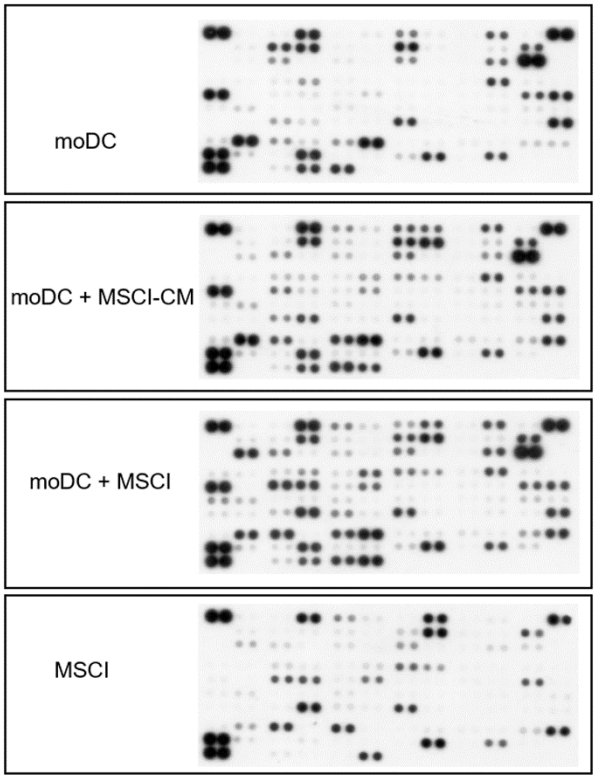
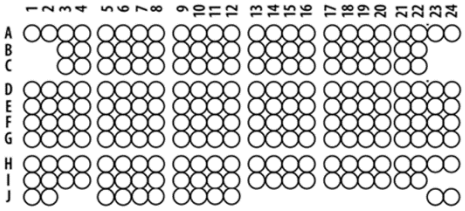
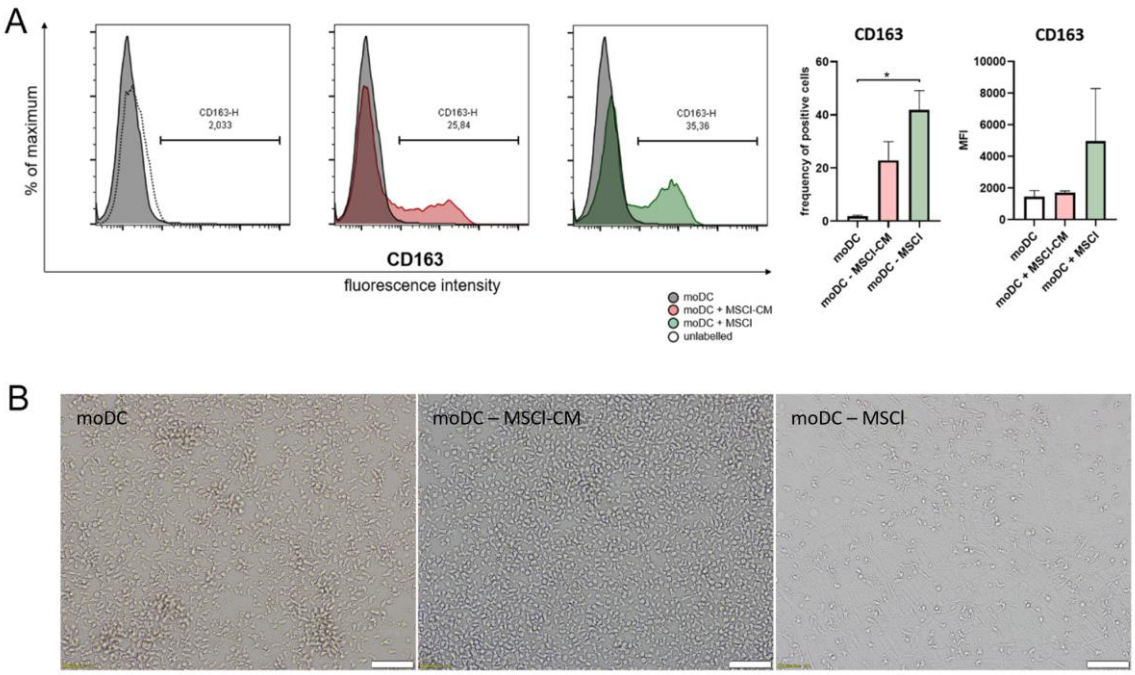
Supplemental Figures and Legends



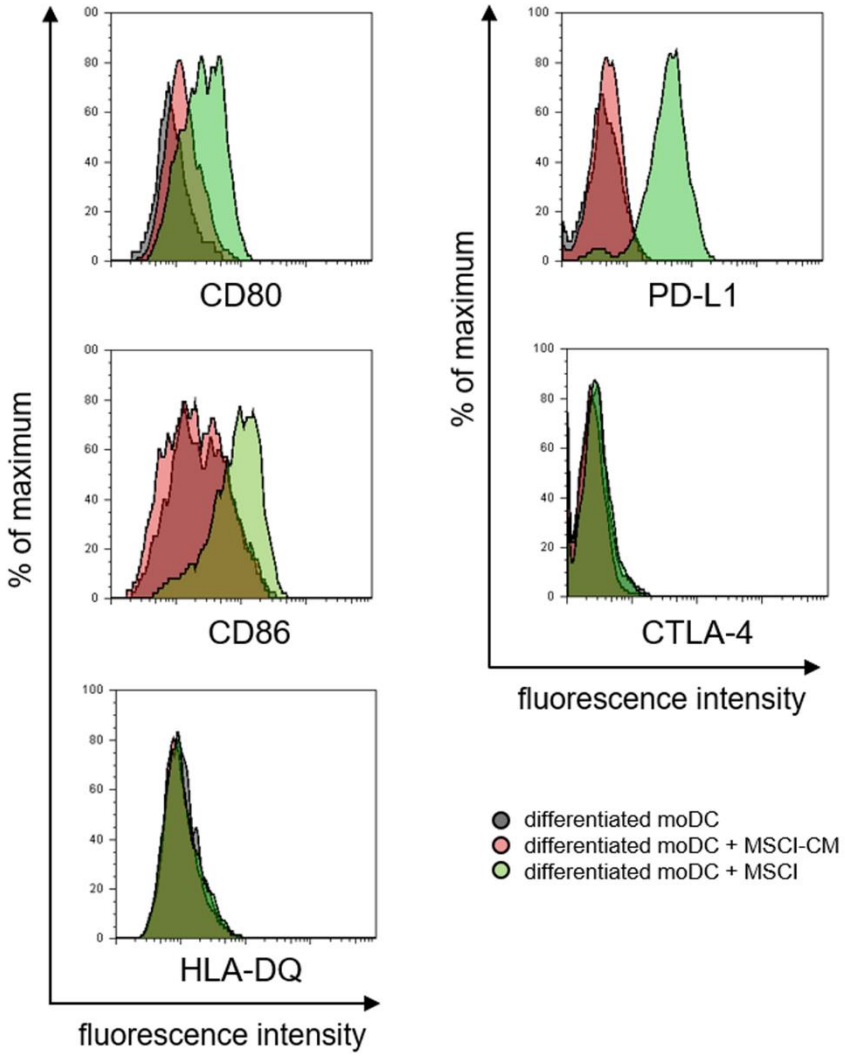


A1	A2	Reference Spots	C15	C16	Flt-3 Ligand	E23	E24	IL-18 Bpa	H5	H6	PDGF-AA
A3	A4	Adiponectin	C17	C18	G-CSF	F1	F2	IL-19	H7	H8	PDGF-AB/BB
A5	A6	Apolipoprotein A-1	C19	C20	GDF-15	F3	F4	IL-22	H9	H10	Pentraxin-3
A7	A8	Angiogenin	C21	C22	GM-CSF	F5	F6	IL-23	H11	H12	PF4
A9	A10	Angiopoietin-1	D1	D2	GRO-α	F7	F8	IL-24	H13	H14	RAGE
A11	A12	Angiopoietin-2	D3	D4	Growth Hormone	F9	F10	IL-27	H15	H16	RANTES
A13	A14	BAFF	D5	D6	HGF	F11	F12	IL-31	H17	H18	RBP4
A15	A16	BDNF	D7	D8	ICAM-1	F13	F14	IL-32α/β/γ	H19	H20	Relaxin-2
A17	A18	Complement Component C5/C5a	D9	D10	IFN-γ	F15	F16	IL-33	H21	H22	Resistin
A19	A20	CD14	D11	D12	IGFBP-2	F17	F18	IL-34	H23	H24	SDF-1α
A21	A22	CD30	D13	D14	IGFBP-3	F19	F20	IP-10	I1	I2	Serpin E1
A23	A24	Reference Spots	D15	D16	IL-1α	F21	F22	I-TAC	I3	I4	SHBG
B3	B4	CD40 ligand	D17	D18	IL-1β	F23	F24	Kallikrein 3	I5	I6	ST2
B5	B6	Chitinase 3-like 1	D19	D20	IL-1ra	G1	G2	Leptin	I7	I8	TARC
B7	B8	Complement Factor D	D21	D22	IL-2	G3	G4	LIF	I9	I10	TFF3
B9	B10	C-Reactive Protein	D23	D24	IL-3	G5	G6	Lipocalin-2	I11	I12	TfR
B11	B12	Cripto-1	E1	E2	IL-4	G7	G8	MCP-1	I13	I14	TGF-α
B13	B14	Cystatin C	E3	E4	IL-5	G9	G10	MCP-3	I15	I16	Thrombospondin-1
B15	B16	Dkk-1	E5	E6	IL-6	G11	G12	M-CSF	I17	I18	TNF-alpha
B17	B18	DPPIV	E7	E8	IL-8	G13	G14	MIF	I19	I20	uPAR
B19	B20	EGF	E9	E10	IL-10	G15	G16	MIG	I21	I22	VEGF
B21	B22	EMMPRIN	E11	E12	IL-11	G17	G18	MIP-1α/MIP-1β	J1	J2	Reference Spots
C3	C4	ENA-78	E13	E14	IL-12 p70	G19	G20	MIP-3α	J5	J6	Vitamin D BP
C5	C6	Endoglin	E15	E16	IL-13	G21	G22	MIP-3β	J7	J8	CD31
C7	C8	Fas Ligand	E17	E18	IL-15	G23	G24	MMP-9	J9	J10	TIM-3
C9	C10	FGF basic	E19	E20	IL-16	H1	H2	Myeloperoxidase	J11	J12	VCAM-1
C11	C12	FGF-7	E21	E22	IL-17A	H3	H4	Osteopontin	J23	J24	Negative Controls
C13	C14	FGF-19									

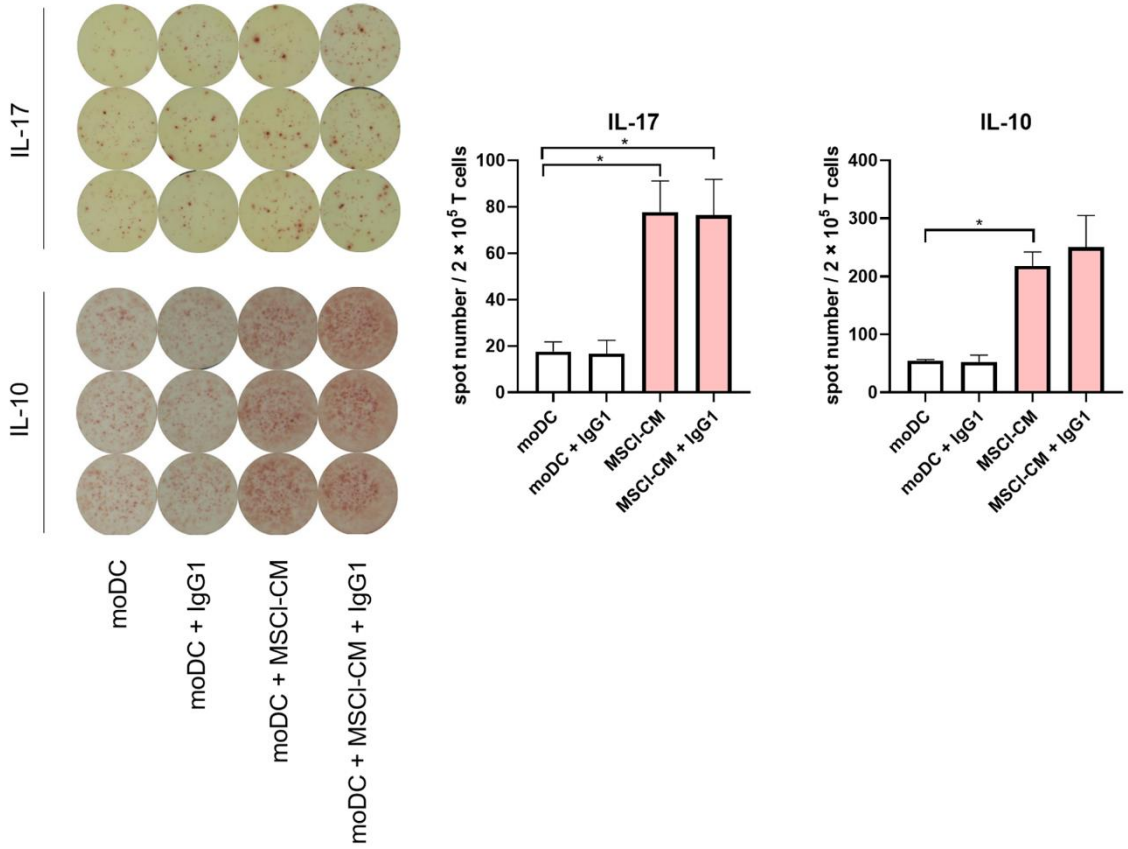
SFigure 1. The cytokine and chemokine production of moDCs is modulated by MSCl cells (Related to Figure 1A). To examine the regulatory effects of MSCl cells or the MSCl-CM on protein production of moDCs, on day 4 of moDC differentiation the integrated density of produced mediators was measured by Human XL Cytokine Array Kit.



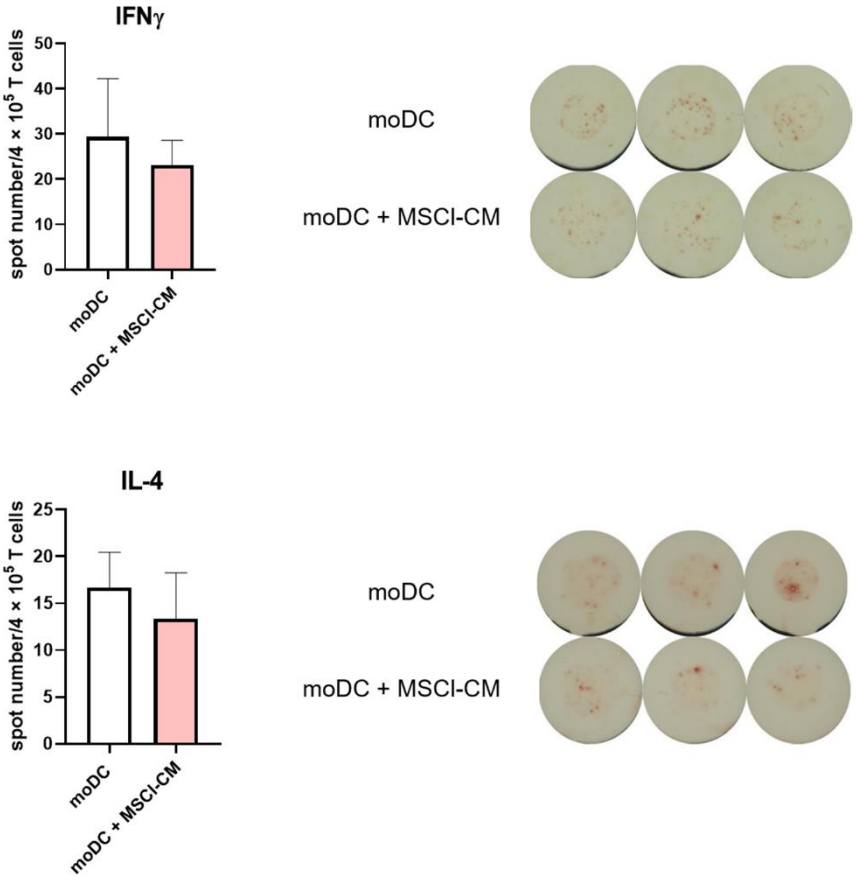
SFigure 2. Cell surface expression of CD163 molecule and morphology of monocyte-derived cells differentiated in the presence of MSCl cells or MSCl-CM (Related to Figure 2 and Figure 3). $CD14^+$ monocytes were cultured with recombinant IL-4 and GM-CSF ± MSCl cells or MSCl-CM for 4 days. On day 4, the cell surface expression of CD163, a macrophage scavenger receptor, was analyzed by flow cytometry (**A**) and the morphology of monocyte-derived cells was investigated using an OLYMPUS IX-81 microscope. Images were taken at 10x magnification (scale bars, 100 µm) (**B**). The mean values of the ratio of cells positive for the measured cell surface molecules were calculated from three independent experiments. Data are represented as mean ± standard deviation. Histograms show one of three independent experiments. In the statistical analysis, one-way ANOVA followed by Bonferroni's multiple comparison test was used with significance defined as *$P < 0.05$.



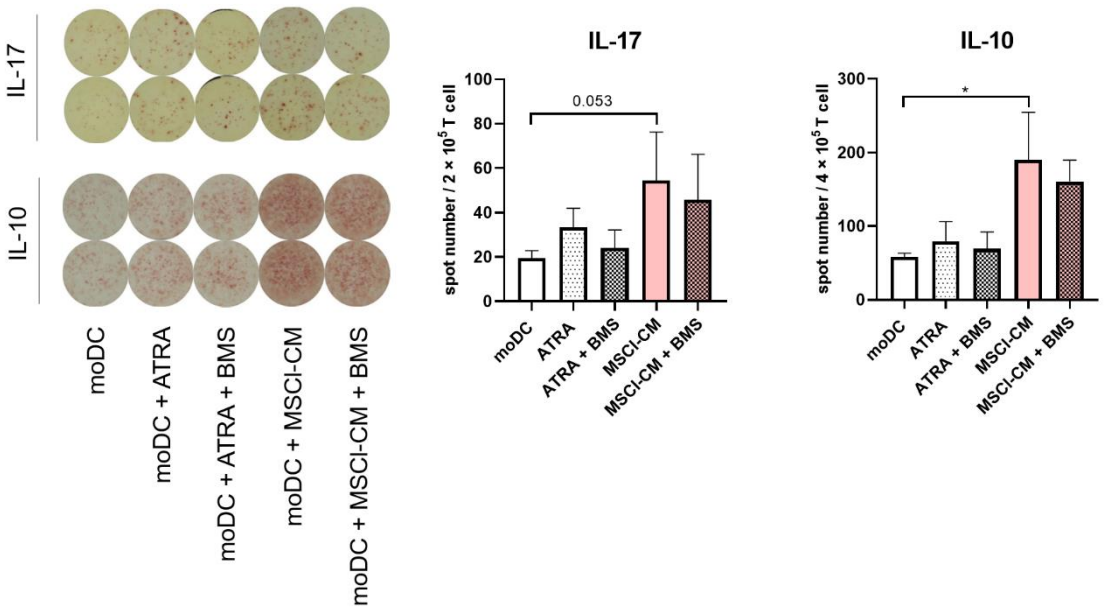
SFigure 3. The effects of MSCl-CM on the phenotype of immature moDCs (Related to Figure 3). Freshly isolated $CD14^+$ monocytes were differentiated in the presence of recombinant IL-4 and GM-CSF for 4 days. On day 4, differentiated moDCs were treated with MSCl-CM or were co-cultured with MSCl cells. After 48 hours moDCs were washed from MSCl-CM or separated from MSCl cells by using DC-SIGN specific antibodies conjugated with paramagnetic beads. The cell surface expression of CD80, CD86, HLA-DQ, PD-L1 and CTLA-4 was measured by flow cytometry.



SFigure 4. Effect of isotype control antibody on the T cell polarizing capacity of moDCs differentiated in the presence of MSCl-CM (Related to Figure 5). $CD14^+$ monocytes were cultured with recombinant IL-4, GM-CSF and MSCl-CM for 4 days. On day 4, the moDCs were incubated with IgG1 isotype control antibodies and co-cultured with T cells for nine days at a moDC : T-cell ratio of 1 : 10. ELISPOT assays were used to determine the number of IL-17 and IL-10 producing T cells. The average values of spot numbers indicating T-lymphocyte responses were counted from 3 micro-wells. Mean values of spot numbers were calculated from 3 independent experiments. Data are represented as mean ± standard deviation. In the statistical analysis, one-way ANOVA followed by Bonferroni's multiple comparison test was used with significance defined as *$P < 0.05$.



SFigure 5. Th1- and Th2-polarizing capacity of moDCs differentiated in the presence of MSCI-CM (Related to Figure 5). $CD14^+$ monocytes were cultured with recombinant IL-4, GM-CSF and MSCI-CM for 4 days. On day 4, the moDCs were co-cultured with allogeneic T-lymphocytes for further 3 or 5 days. ELISPOT assays were used to determine the number of IFNγ (Th1) and IL-4 (Th2) producing T cells. The average values of spot numbers indicating T-lymphocyte responses were counted from 3 micro-wells. Mean values of spot numbers were calculated from 3 independent experiments. Data are represented as mean ± standard deviation. In the statistical analysis, unpaired two-tailed Student's t-test was used.



SFigure 6. Effect of RARα inhibition on moDCs differentiated in the presence of MSCl-CM to drive the development of IL-10 and IL-17 producing T cells (Related to Figure 4A and Figure 5). To determine the role of nuclear receptors, moDCs were differentiated in the presence or absence of 1 nM RARα activator, ATRA followed by a 75-min incubation period with or without 1 µM BMS614 (BMS) specific RARα antagonist for 75 min at 37°C atmosphere containing 5% CO_2 prior to exchange the cell culture medium with or without MSCl-derived conditioned medium (MSCl-CM). $CD14^+$ monocytes were cultured with recombinant IL-4, GM-CSF and MSCl-CM for 4 days. On day 4, the moDCs were co-cultured with allogeneic T-lymphocytes for further five or 9 days. ELISPOT assays were used to measure the number of IL-17- and IL-10-producing T cells. The average values of spot numbers indicating T-lymphocyte responses were counted from 2 micro-wells. Mean values of spot numbers were calculated from 3 independent experiments. Data are represented as mean ± standard deviation. In the statistical analysis, one-way ANOVA followed by Bonferroni's multiple comparison test was used with significance defined as *P < 0.05.

Transparent Methods

Human moDC cultures

Heparinized leukocyte-enriched buffy coats were obtained from healthy blood donors drawn at the Regional Blood Center of the Hungarian National Blood Transfusion Service (Debrecen, Hungary) in accordance with the written approval of the Director of the National Blood Transfusion Service and the Regional and Institutional Research Ethical Committee of the University of Debrecen, Faculty of Medicine (Debrecen, Hungary). Written, informed consent was obtained from the blood donors prior blood donation, their data were processed and stored according to the directives of the European Union.

Peripheral blood mononuclear cells (PBMCs) were separated from buffy coats by Ficoll-Paque Plus (Amersham Biosciences) gradient centrifugation. Monocytes were purified from PBMCs by positive selection using immunomagnetic cell separation and anti-CD14-conjugated microbeads (Miltenyi Biotec), according to the manufacturer's instruction. After separation on a VarioMACS magnet, 96–99% of the cells were shown to be $CD14^+$ monocytes, as measured by flow cytometry. Isolated monocytes were plated at $1.5x10^6$ cell/ml concentration in RPMI (Sigma-Aldrich) supplemented by 10% FCS (Gibco) and 1% antibiotic/antimycotic solution (Hyclone) in the presence of 100 ng/ml IL-4 (PeproTech EC) and 80 ng/ml GM-CSF (Gentaur Molecular Products) added on day 0. Monocytes were cultured for four days in 12-well tissue culture plates at a density of 1.5×10^6 cells/ml in conditioned MSCl medium or in RPMI-1640 medium or were cultured for 4 days in T75 culture flasks at a density of 1.5×10^6 cells/ml in RPMI-1640 medium (Sigma-Aldrich).

To determine the role of nuclear receptors, moDCs were differentiated in the presence or absence of 1 nM RARα activator, ATRA followed by a 75-min incubation period with or without 1 μM BMS-195614 (BMS614) specific RARα antagonist (Sigma-Aldrich) for 75 min at 37°C atmosphere containing 5% CO_2 prior to exchange the cell culture medium with or without MSCl-derived conditioned medium (MSCl-CM).

Generation of MSCl cells

Mesenchymal stromal cell-like cells derived from the human embryonic stem cell line HUES9 was kindly provided by Douglas Melton, HHMI (Howard Hughes Medical Institute). MSCl cells were used according to the ethical permission 6681/2012/EHR. The cells were cultured on mitotically inactivated mouse embryonic fibroblast (MEF) to form embryonic bodies (EB) followed by trypsinization to obtain single cell cultures, which were further cultured on gelatin covered 10 cm plates in DMEM (Gibco) containing 10% FBS. Cells of the confluent cultures exhibited fibroblast like morphology and were further characterized in a collaborative study with the laboratory of Balázs Sarkadi, Membrane Research Group of the Hungarian Academy of Sciences, Semmelweis University and National Blood Service, Budapest, Hungary (Varga et al., 2011). MSCl cell passages in opto-mechanical-treated polystyrene flasks (TPP) provided coherent cell layers in the presence of L-glutamine, 10% FCS (Gibco) and 1% anti-mycoticum/antibioticum solution (Hyclone) in low glucose DMEM (Gibco). The cultured MSCl cells were used after 10 passages. Confluent layer of MSCl cells was cultured in 12 ml RPMI (Sigma-Aldrich) supplemented with 10% FCS (Gibco) and 1% antibiotic/antimycotic solution

(Hyclone) for 48 hours to get the MSCl-CM. Synthesis of ATRA by MSCl cells was inhibited specifically by 1 µM N,N-diethylaminobenzaldehyde (DEAB) (Sigma-Aldrich) in the MSCl cultures for 24 h. After this incubation time MSCl cells were washed and fresh RPMI was added to the cells for 48 h.

Co-cultures of moDC and MSCl cells

MSCl cells were collected by using 0.05 –0.02% Trypsin/EDTA solution in Dulbecco's PBS (DPBS), washed and cultured at a cell density of $3.6x10^6$ cells in opto-mechanical treated T75 flask for optimal cell growth (TPP). To achieve confluent cell layers the MSCl cells were cultured in 12 ml RPMI (Sigma-Aldrich) supplemented with 10% FCS (Gibco) containing 1% antimycotic/antibiotic solution (Hyclone) for minimum 12 hours. $1.8x10^7$ freshly isolated monocytes were placed directly on the top of the adherent MSCl cells and the co-cultured moDCs were differentiated for four days in the presence of 80 ng/ml GM-CSF (Gentaur Molecular Products) and 100 ng/ml IL-4 (PeproTech). After the differentiation process moDCs were separated from MSCl cells by positive selection using immunomagnetic cell separation and anti-CD209/DC-SIGN microbeads, according to the manufacturer's instruction (Miltenyi Biotec).

Flow cytometry

Phenotyping of resting, conditioned moDCs in the presence of MSCl-CM or with MSCl cells was performed by flow cytometry using anti-human CD14-fluorescein isothiocyanate (FITC), CD209/DC-SIGN-phycoerythrin (PE), CD1a-FITC, CD80-FITC, CD86-PE, PD-L1-PE, CD163-PE (BioLegend), CTLA-4-PE, CD1b-FITC, CD1c-allophycocyanin (APC) (Sony Biotechnology Inc.), HLA-DR-FITC (BD Biosciences). Cell viability was assessed by 7-aminoactinomycin-D (7-AAD; 10 µg/ml; Sigma–Aldrich) staining for 15 minutes immediately before flow cytometric analysis. Fluorescence intensities were measured by FACS Calibur cytometer (BD Biosciences) and data were analyzed by the FlowJo software (Tree Star).

Measurement of the cytokine concentrations

Culture supernatants from moDCs, MSCl-CM exposed moDCs (CM-moDCs) or from co-cultures containing moDCs and MSCl cells were harvested 4 days after monocyte separation and the relative levels of selected human cytokines and chemokines were determined by Human XL Cytokine Array Kit following the manufacturer's instructions (R&D Systems). Culture supernatants from moDCs, CM-moDCs or from co-cultures containing moDCs and MSCl cells were harvested 4 days after monocyte separation, and the concentration of IL-6, IL-10 and TGFβ cytokines was measured and validated by using OptEIA kits (BD Biosciences) following the manufacturer's instructions.

Treatment of moDCs to measure T-lymphocyte polarization

Control, ATRA- or BMS614-pretreated moDCs and CM-moDCs were counted, washed and co-cultured with allogenic peripheral blood lymphocytes (PBL) for three, five or nine days in RPMI-1640 medium (Sigma-Aldrich) at a moDC : T-cell ratio of 1 : 10 at 37°C. Parallel cultures of control moDCs and CM-moDCs were incubated with anti-CTLA-4 neutralizing antibody or with its isotype control IgG1 antibody (LifeSpan BioSciences) on ice for 1.5 h and then the cells

were washed and co-cultured with T cells for three, five or nine days in RPMI-1640 medium (Sigma) at a moDC : T-cell ratio of 1 : 10 at 37°C. The T cells were analyzed for IFNγ, IL-4, IL-17 and IL-10 secretion by the avidin-horseradish peroxidase-based enzyme-linked ImmunoSpot (ELISPOT) system (NatuTec GmbH). Cultures including T cells or moDCs alone served as negative controls. To detect IFNγ, IL-4, IL-17 or IL-10 secretion the plates were coated with 0.5 µg/ml mouse anti-hCD3 antibody (BD Biosciences). The plates were analyzed by using the ImmunoScan plate reader (Cell Technology Limited).

To determine which T-lymphocyte populations are responsible for the cytokine production, after three, five or nine days the T cells were stimulated with 1µg/ml ionomycin and 20 ng/ml phorbol-myristic acetate (PMA) for 4 hours and the vesicular transport was inhibited by BD GolgiStop™ protein transport inhibitor (BD Biosciences) four hours before the cell staining. The cells were labelled with anti-human CD4-Peridinin Chlorophyll Protein Complex (PerCP) conjugated antibodies (BioLegend). Following this, they were fixed and permeabilized by using BD Cytofix/Cytoperm™ Plus Fixation/Permeabilization Kit (BD Biosciences) and labelled with anti-human IFNγ-APC (BD Biosciences), anti-human IL-4-PE (R&D Systems), anti-human IL-10-Alexa Fluor 488 and anti-human IL-17-PE antibodies (BioLegend). Fluorescence intensities were measured by FACS Calibur cytometer (BD Biosciences) and data were analyzed by the FlowJo software (Tree Star).

Naïve CD4+ T-cell isolation

Naive T-cells were separated from human blood mononuclear cells using the naive CD4+ T-cell isolation kit based on negative selection according to the manufacturer's instruction (Miltenyi Biotec). Using the CD4+ T Cell Isolation Kit, human CD4+ T helper cells are isolated by negative selection. Non-target cells are labeled with a cocktail of biotin-conjugated monoclonal antibodies and the CD4+ T Cell MicroBead Cocktail. The magnetically labeled non-target T cells are depleted by retaining them on a MACS® Column in the magnetic field of a MACS Separator, while the unlabeled T helper cells pass through the column.

RNA isolation, cDNA synthesis and real time quantitative PCR (RT-qPCR)

Briefly, total RNA was isolated by TriReagent (Molecular Research Center). Total RNA was reverse-transcribed using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Gene-specific TaqMan pre-made assays (Applied Biosystems) were used to perform qPCR in a final volume of 12.5 µl in duplicates using DreamTaq DNA polymerase and ABI StepOnePlus real-time PCR instrument. Amplification of h36B4 was used as normalizing controls using specific primers and probe (Integrated DNA Technologies). Cycle threshold values were determined using the StepOne Software, version 2.1 (Applied Biosystems). The sequences of the primers and probes are available upon request.

Statistical analysis

Comparisons between two groups were performed using unpaired two-tailed Student's t-test for normally distributed variables with equal variance and Mann–Whitney rank sum test for data that did not pass normality or equal variance test. Normality was assessed both visually (Q-Q plots) and through Shapiro-Wilk test. One-way ANOVA followed by Bonferroni's post hoc test was used for comparisons for more than 2 groups. The results were expressed as mean ±

standard deviation. Analyses were performed by using Excel (Microsoft Corporation) and GraphPad Prism Version 6.0 (GraphPad Software Inc.) software. Differences were considered to be statistically significant at $P < 0.05$. Significance was indicated as *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.

Supplemental References

VARGA, N., VEREB, Z., RAJNAVOLGYI, E., NEMET, K., UHER, F., SARKADI, B. & APATI, A. 2011. Mesenchymal stem cell like (MSCl) cells generated from human embryonic stem cells support pluripotent cell growth. *Biochem Biophys Res Commun,* 414**,** 474-80.