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Mesenchymal Stromal Cells Prevent Allostimulation *in vivo* and Control CheckPoints of Th1 Priming: Migration of Human DC to Lymph Nodes and NK Cell Activation

C. CONSENTIUS[1,2]; L. AKYÜZ[1,3]; J. A. SCHMIDT-LUCKE[4,x]; C. TSCHÖPE[1,5]; L. PINZUR[6]; R. OFIR[6], P. REINKE[1,7]; H.-D. VOLK[1,3]; K. JUELKE[1,*;#]

Key words. mesenchymal stromal cells • myeloid dendritic cells • natural killer cells • IL-10 • Th1 priming • immunomodulation

ABSTRACT

Although the immunomodulatory potency of mesenchymal stromal cells (MSC) is well established, the mechanisms behind are still not clear. The crosstalk between myeloid dendritic cells (mDC) and natural killer (NK) cells and especially NK cell-derived interferon-gamma (IFN-g) play a pivotal role in the development of Type 1 helper (Th1) cell immune responses. While many studies explored the isolated impact of MSC on either *in vitro* generated DC, NK or T cells, there are only few data available on the complex interplay between these cells. Here we investigated the impact of MSC on the functionality of human mDC and the consequences for NK cell and Th1 priming *in vitro* and *in vivo*. In critical limb ischemia (CLI) patients, who have been treated with allogeneic placenta-derived mesenchymal-like stromal cells (PLX-PAD), no *in vivo* priming of Th1 responses towards the major histocompatibility complex (MHC) mismatches could be detected. Further *in vitro* studies revealed that mDC reprogramming could play a central role for these effects. Following crosstalk with MSC, activated mDC acquired a tolerogenic phenotype characterized by reduced migration towards CCR7 ligand and impaired ability to stimulate NK cell-derived IFN-g production. These effects, which were strongly related to an altered interleukin (IL)-12/IL-10 production by mDC, were accompanied by an effective prevention of Th1 priming *in vivo.* Our findings provide novel evidence for the regulation of Th1 priming by MSC via modulation of mDC and NK cell crosstalk and show that off-the-shelf produced MHC-mismatched PLX-PAD can be used in patients without any sign of immunogenicity. STEM CELLS *2015; 00:000–000*

SIGNIFICANCE STATEMENT

While MHC-mismatched mesenchymal stromal cells (MSC) are already used in clinical trials for tissue regeneration and treatment of immune-related disorders, these studies did not sufficiently address alloreactivity. In the present study, we provide to our knowledge for the first time immunological data from a clinical trial demonstrating that patients treated with MHC-unmatched MSC did not develop a memory T cell response specific to the mismatch. Moreover, we demonstrate that MSC control important checkpoints of Th1 priming by inhibiting migration and cytokine production of myeloid dendritic cells resulting in a diminished ability to prime natural killer cells and Th1 cells.

[1] Berlin-Brandenburg Center for Regenerative Therapies (BCRT), Charité University Medicine, Berlin, Germany; [2] Berlin-Brandenburg School for Regenerative Therapies (BSRT), Charité University Medicine, Berlin, Germany; [3] Institute for Medical Immunology, Charité University Medicine, Berlin, Germany; [4] Franziskus Hospital, Berlin, Germany; [5] Dept. for Cardiology, CVK, Charité University Medicine, Berlin, Germany; [6] Pluristem Therapeutics Inc., Haifa, Israel; [7] Dept. for Nephrology and Intensive Care, CVK, Charité University Medicine, Berlin, Germany

[#]Correspondent author: Dr. Kerstin Juelke, PhD, BCRT c/o Deutsches Rheuma Forschungszentrum (DRFZ), Charitéplatz 1, 10117 Berlin, Germany, mail: kerstin.juelke@drfz.de, phone: +49 (0)30 284 60 682, fax: +49 (0)30 284 60 603; [x] present address: Medical Center Kurfürstendamm, Berlin, Germany; * present address: German Rheumatism Research Center (DRFZ), a Leibniz Institute, Berlin, Germany; Funding: This work was kindly supported in part by the Federal Ministry of Education and Research (BCRT), DFG (BSRT), a grant from Sanofi and Pluristem Therapeutics Inc.

Received March 16, 2015; accepted for publication May 27, 2015; available online without subscription through the open access option.

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1066-5099/2015/$30.00/0

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/stem.2104

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INTRODUCTION

MSC represent multipotent progenitor cells capable of differentiating into adipocytes, chondroblasts and osteoblasts [1–3]. In addition to their potential for tissue repair, MSC display immunomodulatory properties and regulate both adaptive and innate immune cells [3–5]. In particular, MSC have been used in preclinical and clinical studies for the treatment of immune-related disorders including autoimmune diseases, transplant rejection and graft-versus-host disease [6–10]. Since autologous MSC often display disease- and age-related impairments [11, 12] and as their preparation is logistically challenging, cost and time consuming, the use of off-the-shelf MHC-unmatched MSC would be of great advantage, provided they do not elicitate an immune response. Indeed, transplantation of allogeneic MSC has been reported without clear evidences on alloreactivity supporting their immunomodulatory potency [8, 13, 14]. Nevertheless, in many models the efficacy of MSC is less clear and phase III clinical trials in patients failed by lacking efficacy so far. Moreover, evidences suggest triggering of alloimmunity in both preclinical models and patients [15–20]. Accordingly, a better understanding of the mechanisms behind the immunomodulatory effects of MSC is essential to improve the selection of the correct indication and patients and thereby the therapeutical outcome.

Multiple checkpoints control the development of a T cell response. These include maturation of DC, migration of DC to draining lymph nodes (LN), recruitment of T, B and NK cells by releasing chemokines, activation of NK cells to produce IFN-g, being essential for Th1 priming [21–23], and finally antigen presentation and co-stimulation. This results in clonal expansion and differentiation of naive towards memory/effector T cells [24–27]. Several authors investigated the isolated impact of MSC on either DC, NK or T cells *in vitro* [28–30]. However, these studies did not address the complex interplay of DC with NK and T cells [31–33], especially considering their interaction in inflamed peripheral tissues or secondary lymphoid organs (SLO) as well as the potential of MCS to interfere with this communication network.

In the early phases of an immune response, DC encounter LN-NK cells, i.e. before antigen-driven activation of naïve T cells [23, 34]. NK cells comprise various subsets that differ in function, phenotype and tissue localization. Virtually all NK cells in the LN belong to the CD56brightCD16- compartment [35], which respond very efficiently to DC-derived cytokines by proliferation and IFN-g production [33, 36–41]. In turn, IFN-g promotes DC maturation and sustains Th1 immune responses by providing a conditioning signal that increases the capacity of DC to produce IL-12 [21–23, 42]. In contrast, the CD56dimCD16+ NK cell subset enriched in peripheral blood expresses homing receptors for inflamed tissues and rapidly mediates cytotoxicity, which can be enhanced by DC-derived IL-12 [38, 41]. By inhibiting NK cell or DC effector functions, MSC would interfere with the ability of both cell types to induce Th1 immune responses, which could be part of their beneficial effect in Th1-dependent immune disorders. To fine-tune an immune response, numerous phenotypic and functional subsets of DC exist. Stimulatory DC are involved in the initiation of an immune response, whereas tolerogenic DC (TolDC) are required for the development and maintenance of immunological tolerance [43]. There is good evidence *in vitro* that MSC promote the generation of TolDC by inhibiting the differentiation of human DC derived from monocytes or CD34+ precursors [29, 44–47]. It is less clear whether MSC interfere also with DC maturation. So far, contradictory results were obtained on this topic showing both, inhibition or enhancement of maturation by MSC [45, 46]. Yet, MSC might not exclusively meet DC precursors *in vivo* but will most likely also interact with already differentiated immature DC. Therefore, it would be of great importance to answer this question for further clinical applications. Another limitation of most experimental studies addressing the effects of MSC on DC is the DC source. Due to the low accessibility of DC in the body, most studies used *in vitro* generated DC derived from either murine bone marrow or human monocytes. *In vivo*, monocytes give rise to a subset of macrophages and inflammatory DC that share many phenotypic and functional features with DC. However, it is now widely accepted that monocytes do not give rise to conventional mDC [48, 49]. Conventional CD1c+ mDC can be obtained from blood, the only readily available source in humans. Only little is known about the effect of MSC on the maturation of *ex vivo* isolated immature mDC [50] and nothing on the consequences for their ability to activate NK or T cells. Given that MSC are already used in clinical trials and taking into account the important role of mDC and NK cell cross talk for the regulation of Th1 immune responses in such settings, our study aimed to gain better understanding of how MSC interfere with the interaction between *in vivo* generated mDC and NK cells as well as the consequences for Th1 priming *in vitro* and *in vivo*. We demonstrated that distinct checkpoints of Th1 priming were regulated by MSC. mDC activated in the presence of MSC displayed reduced migratory capacity towards CCR7 ligand, considerably limited ability to activate NK cells especially in terms of IFN-g production and lower capacity to induce Th1 differentiation of naïve T cells. This was mainly a consequence of a cytokine shift from pro-inflammatory IL-12 towards anti-inflammatory IL-10 in mDC that have been matured in the presence of MSC ((MSC)mDC). These *in vitro* data could be strengthened by analysis of clinical study samples derived from CLI patients treated with MHC-unmatched PLX-PAD cells, which have been shown before to support revascularization in a hind limb ischemia mouse model [51]. We did not observe any significant induction of Th1 alloreactivity supporting

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our *in vitro* finding that priming of T cells is prevented by mesenchymal cells. Our data provide novel evidence for the regulation of several checkpoints of Th1 priming by MSC via reprogramming of human mDC and NK cell crosstalk and functionality.

MATERIALS AND METHODS

Immune cell preparation

Peripheral blood leukocyte concentrates were used to isolate immune cells. The relevant institutional review boards approved the study and all patients gave their written informed consent according to the Declaration of Helsinki. To perform phenotypic and functional analysis, immune cell subpopulation were separated by magnetic cell sorting (MACS) and subsequently sorted by FACSAria II (BD). Further details about cell isolation procedures are included in Supporting information.

BM-MSC and PLX-PAD

Third party human BM-MSC were purchased at ScienCell (Cat.No 7500). PLX-PAD were obtained from Pluristem Therapeutics Ltd., Haifa, Israel. Details for maintenance and expansion of BM-MSC and PLX-PAD are provided in Supporting information. For co-culture experiments, BM-MSC or PLX-PAD were thawed and seeded one day in advance in complete RPMI-1640.

Cell culture

Cells were cultured in complete RPMI-1640 medium supplemented with 10% human AB serum (Lonza), 100U/ml penicillin, 0.1mg/ml streptomycin, 2mM L-Glutamine and 20mM Hepes (all Biochrom). When indicated, HTS Transwell plates (Corning, 0,4µm pore size polycarbonate membranes) were used. In this case, MSC were seeded in the lower chamber and mDC in the upper chamber.

Maturation of mDC

$1x10^5$ mDC were matured in the presence or absence of $2x10^4$ BM-MSC or PLX-PAD in 200µl medium in 96well flat bottom plates by 100pg/ml lipopolysaccharide (LPS) alone (Sigma-Aldrich) or 100ng/ml LPS plus 10µg/ml resiquimod (R848, Alexis) for 18hrs.

Details for generation of moDC, migration assay of mDC and multiplex analysis of cytokine production are provided in Supporting information.

NK cell activation by mDC

mDC matured in the presence or absence of MSC were used in a second step to activate NK cells.

Analysis for IFN-g production

$5x10^4$ NK cells were co-cultured with $2{,}5x10^4$ mature mDC in 200µl medium in 96well round bottom plates for 24hrs. Brefeldin A (Sigma Aldrich, 10µg/ml) was added for the last 8hrs.

Analysis of cytotoxic potential

After pre-activation of CD56dim NK cells with mature mDC (NK:mDC ratio 2:1) for 18hrs, NK cells were co-cultured with the MHC-class I negative target cell line *K562 (ATTC) in an effector to target ratio of 5:1* at 37°C for 6hrs. A CD107a Mobilization Assay or a flow cytometric assay for NK cell killing was performed [52, 53]. Further details are included in Supporting information.

Analysis of proliferation

$1x10^5$ NK cells labeled with 1µM carboxyfluorescein succinimidyl ester (CFSE) were co-cultured with $5x10^4$ mature mDC in 200µl medium in 96well round bottom plates for 5 days. Proliferation was measured by dilution of CFSE after gating on living CD56+ NK cells.

Priming of allogeneic naïve T cells by mDC

mDC matured in the presence or absence of MSC were used to activate allogeneic naïve T cells. $1x10^5$ T cells were co-cultured with $2x10^4$ mature mDC in 200µl medium in 96well round bottom plates for 5 days. Cytokine levels were analyzed in cell culture supernatants by cytometric bead assay (CBA, BD, Human Th1/Th2/Th17 Cytokine Kit).

Flow cytometry

A list of mAbs used in the study is included in Supporting information. Staining for CCR7 was performed at 37°C for 15min. All other surface molecules were stained at 4°C for 10min. Intracellular staining for IFN-g was assessed after surface marker staining, fixation and permeabilization (BD) at room temperature for 20min. Data were acquired on a LSRII™ flow cytometer and analyzed using FlowJo software (Tree Star, Inc). When MSC were present in the culture, they were excluded from analysis by gating on CD45+ cells.

Clinical study design

Allospecific T cell response to PLX-PAD was measured in PBMC samples from an open-label phase I dose-escalation study in 15 CLI patients (EudraCT number 2008-003711-13). The patients received a single intramuscular (i.m.) treatment with PLX-PAD. Immunological analyses were performed before, 3 days, 1 week and 4 weeks after treatment. To detect memory/effector T cells specific for PLX-PAD, an IFN-g Elispot (AID, Germany) was used [54]. Further details are provided in Supporting information.

Statistical analysis

Statistical analysis was performed in GraphPad Prism using Wilcoxon matched pairs test. All values are shown as mean ± standard deviation (SD). *=P ≤ 0.05; **=P ≤ 0.01; ***=P ≤ 0.001.

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RESULTS

BM-MSC impair CCR7-dependent migration of mDC

Upon antigen encounter DC acquire the chemokine receptor CCR7 and migrate from sites of inflammation to the closest draining LN in order to activate LN-NK and naïve T cells [55]. Accordingly, we analyzed the influence of BM-MSC on CCR7 expression as well as on migration of mDC towards its ligand CCL21. After maturation by low dose LPS, approximately 60% of mDC upregulated CCR7 (Fig. 1A and 1B). The percentage of CCR7+ cells and the level of CCR7 expression per cell, measured by median fluorescence intensity (MFI) of the CCR7+ cells, were significantly reduced in the presence of BM-MSC (Fig. 1A). Consequently, considerably less mDC migrated towards CCL21 (Fig. 1C). The inhibitory effect on CCR7 expression was mediated by soluble factors produced by BM-MSC and not due to competition for LPS since we observed the same effect when using BM-MSC-conditioned medium supplemented by LPS (Fig. S1). Nevertheless, this inhibition could be overcame when mDC were matured using a combination of high dose LPS and R848, which is required for efficient *in vitro* cytokine production by mDC [56]. Under these conditions CCR7 acquisition and migration towards CCL21 were only marginally reduced by BM-MSC (Fig. 1A-C).

BM-MSC show only minor effects on maturation of in vivo generated DC

In order to activate naïve T cells in the LN, DC need to upregulate co-stimulatory molecules. While several studies demonstrated that MSC inhibit the *in vitro* differentiation of human DC generated from monocytes or CD34+ precursors [29, 45], it is not entirely clear whether they also interfere with DC maturation. Therefore, we analyzed the effect of BM-MSC on the maturation of blood-derived *in vivo* generated mDC and compared it to their impact on differentiation and maturation of *in vitro* generated moDC. In line with data from previous studies [29, 57], we could confirm that BM-MSC strongly inhibited the generation of moDC when they were present during *in vitro* differentiation, while there was no effect on the maturation process (Fig. S2). When *ex vivo* isolated immature mDC were matured in the presence of BM-MSC ([BM-MSC]mDC), we observed only a minor reduction of CD83 and CD40 acquisition, while the co-stimulatory molecules CD80 and CD86 were even marginally upregulated and human leukocyte antigen (HLA)-DR was not altered (Fig. 1D and 1E).

BM-MSC induce an anti-inflammatory cytokine profile in mDC

mDC-derived cytokines and chemokines play an important role for the recruitment and stimulation of NK and T cells. By performing multiplex analysis, we measured 27 cytokines, chemokines and growth factors in cell culture supernatants of LPS/R848-activated mDC/BM-MSC co-cultures (Fig. 2A and Tab. S1). We found that in the presence of BM-MSC, mDC secreted less of the pro-inflammatory cytokines IL-12p70 and IL-1b, while they produced more anti-inflammatory cytokines IL-10 and IL-1 Receptor antagonist (IL-1Ra, Fig. 2A). Moreover, IL-6 was increased in the co-culture of mDC and BM-MSC. By calculating the ratios between IL-12p70 and IL-10 as well as IL-1b and IL-1Ra, we could show that BM-MSC shift the cytokine production of mDC towards an anti-inflammatory profile (Fig. 2B).

Neutralization of IL-10 reconstitutes the balance between pro- and anti-inflammatory cytokines

In order to understand which factors mediate the switch towards an anti-inflammatory cytokine profile of (BM-MSC)mDC, we blocked different soluble factors in the mDC/BM-MSC co-culture and measured the cytokine levels by multiplex analysis. The blockade of IL-10/IL-10 Receptor (IL-10R), IL-1Ra or IL-6 completely reconstituted the level of IL-12p70, whereas the production of IL-1b could only be restored in part (Fig. 3A). The increase of IL-1Ra could exclusively be compensated by addition of anti-IL-10/anti-IL-10R suggesting that IL-1Ra expression is regulated by IL-10. The level of biologically active IL-10 could only be reduced by addition of anti-IL-10/anti-IL-10R but not by neutralizing IL-1Ra or IL-6. While the neutralizing antibodies for IL-10/IL-10R or IL-6 strongly diminished the detectable level of their respective target cytokine, IL-1Ra could still be measured in the presence of anti-IL-1Ra (Fig. S3). This was most likely due to distinct epitopes recognized by neutralizing and detection antibody. Therefore, we could not specifically quantify biologically active IL-1Ra under neutralizing conditions. Importantly, the ratio of biologically active IL-12p70/IL-10 could only be restored in the presence of anti-IL-10/anti-IL-10R, whereas it remained reduced in the presence of anti-IL-1Ra or anti-IL-6 (Fig. 3B). Additionally, anti-IL-10/anti-IL-10R, but not anti-IL-6, increased the IL-1b/IL-1Ra ratio. Taken together, these data demonstrate that predominantly IL-10 regulated the balance between pro- and anti-inflammatory cytokines produced by mDC.

BM-MSC inhibit the ability of mDC to activate NK cells

DC-derived cytokines induce proliferation and IFN-g production in NK cells and increase their cytotoxic potential. The observed changes in the cytokine profile of (BM-MSC)mDC raised the question whether they lead to a decreased ability of mDC to activate NK cells. For this purpose, we stimulated NK cells with mDC, which have been matured in the presence or absence of BM-MSC beforehand and measured their cytotoxic ability, proliferation and IFN-g production. Compared to resting NK cells, short term pre-activation by mDC increased the cytotoxic potential of CD56dim NK cells towards

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MHC class I negative tumor target cells K562. In contrast, pre-activation by (BM-MSC)mDC was slightly less efficient in enhancing NK cell cytotoxic potential (Fig. 4A). Furthermore, CD56bright as well as CD56dim NK cells proliferated less upon stimulation with (BM-MSC)mDC compared to mDC (Fig. 4B). Most strikingly, (BM-MSC)mDC induced significantly lower IFN-g production in both CD56bright and CD56dim NK cells (Fig. 4C and 4D). The percentage of IFN-g+ cells as well as the amount of IFN-g produced per cell, measured by MFI of the IFN-g+ cells, was strongly reduced. The inhibitory effects on NK cell activation were not mediated by contaminating BM-MSC during NK/mDC co-culture, as it could also be seen when mDC and BM-MSC were co-cultured in transwell plates (Fig. 5C). These findings clearly show that BM-MSC indirectly inhibit the effector functions of NK cells by modulating the stimulatory capacity of mDC.

Decreased IL-12 production by (BM-MSC)mDC accounts for reduced NK cell activation

The production of IFN-g by NK cells can be induced by DC-derived cytokines or by ligation of activating receptors [41, 58]. To understand the dominant activating mechanism in our setting, mDC and CD56bright NK cells were co-cultured in transwell plates, either separated by the membrane or in contact in the lower chamber. We observed comparable frequencies of IFN-g producing NK cells under both conditions, demonstrating that IFN-g production in NK cells was mainly induced by mDC-derived cytokines (Fig. 5A). Furthermore, IFN-g production was still decreased when NK cells and (BM-MSC)mDC were separated by the transwell membrane suggesting that (BM-MSC)mDC either secreted less of an activating or an additional inhibitory factor. Since we observed a decreased IL-12p70 secretion by (BM-MSC)mDC, we analyzed the role of this cytokine for the observed effect. We could show that IL-12 blocking in mDC/NK cell co-cultures completely prevented IFN-g production by NK cells, demonstrating its essential role (Fig. 5B). Furthermore, the addition of recombinant IL-12 fully restored the IFN-g production by NK cells, which have been activated by (BM-MSC)mDC (Fig. 5B and Fig. S4A). This finding demonstrates that the decreased production of IL-12 by (BM-MSC)mDC is responsible for the reduction of NK cell-derived IFN-g.

Increased production of IL-10 is responsible for reduced functionality of (BM-MSC)mDC

Next, we wanted to understand how BM-MSC inhibit the capacity of mDC to activate NK cells. By performing transwell experiments, we could show that BM-MSC did not need to be in contact with mDC to inhibit their ability to stimulate NK cells. The level of inhibition by BM-MSC was still the same indicating that a soluble factor was responsible for this effect. The level of IL-12p70 in mDC/BM-MSC co-cultures was regulated by IL-10, IL-6 or IL-1Ra (Fig. 3). As shown in Fig. 5D, neutralization of IL-10/IL-10R in mDC/BM-MSC co-cultures restored the ability of (BM-MSC)mDC to induce IFN-g production in NK cells. In contrast, blocking of IL-1Ra or IL-6 in mDC/BM-MSC co-cultures (Fig. S4B) or blocking of IL-10, IL-1Ra or IL-6 in mDC/NK cell co-cultures (Fig. 5B and Fig. S4A) had no effect on the percentage of IFN-g producing NK cells. In conclusion, we could show that by increasing the IL-10 production of mDC, BM-MSC shifted their cytokine profile towards an anti-inflammatory status characterized among others by low IL-12 production, which is the reason for a diminished ability of (BM-MSC)mDC to activate NK cells.

BM-MSC and PLX-PAD inhibit Th1 priming in vitro and in vivo

Since IFN-g produced by NK cells promotes Th1 priming [21], inhibition of DC/NK cell cross talk combined with the diminished migration of DC to SLO could be an explanation for the described inhibition of Th1-driven immune-pathological processes *in vivo*. To test our hypothesis, we investigated clinical study samples derived from CLI patients that had received HLA-unmatched PLX-PAD cells into their ischemic limb. PLX-PAD share many but not all properties with BM-MSC [59, 60]. Since most of our *in vitro* data were obtained using BM-MSC, we compared the immunomodulatory properties of PLX-PAD and BM-MSC *in vitro* with regard to the regulation of mDC effector function. As shown in Fig. 6A, BM-MSC as well as PLX-PAD exhibited a comparable capacity to inhibit the ability of mDC to activate NK cells. In addition, mDC matured in the presence of BM-MSC or PLX-PAD were less efficient in inducing Th1 priming of allogeneic naïve T cells *in vitro* as shown by reduced production of IFN-g after 5 days of co-culture (Fig. 6B). In contrast to some reports on other MSC [61], we also did not observe any Th1 priming specific to allogeneic BM-MSC or PLX-PAD cells *in vitro* (Fig. 6B).

Next, we investigated whether HLA-unmatched PLX-PAD cells induced *in vivo* priming of Th1 cells specific for the respective mismatches. Therefore, PBMC, isolated from the patients at different time points after PLX-PAD injection, were restimulated *ex vivo* with the corresponding PLX-PAD cells or unrelated third party donor PBMC. The *in vivo* induced memory Th1 response was measured by using the sensitive IFN-g Elispot test (Fig. 6C). We observed no or only very marginal Th1 priming specific for the MHC-mismatch even after application of high-dose allogeneic PLX-PAD cells (Fig. 6C). Only three patients developed minimal reactivity towards the mismatch (indicated with open symbols). One of these patients (open triangle) already had few pre-existing donor cell-specific memory Th1 cells before cell transplantation and low immune reactivity towards unrelated third party donor cells, suggesting an unspecific “bystander” response, which can also occur after vaccination. Two other patients (open circle and open square) showed a minimal response at week +1 or week +4 post-treatment respectively, which could not be seen to third party cells. Yet, considering only responses as pos-

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itive when they exceed the background + 2-fold SD (up to 23 IFN-g spots/300,000 PBMC) none of the patients reached a response of more than 25 IFN-g spots/ 300,000 PBMC, which has been defined as clinically relevant [62]. Taken together, our data indicate that MSC inhibit the priming of Th1-driven immune responses via modulating mDC and NK cell function (Fig. 7).

DISCUSSION

In the present study, we provide novel evidence that mesenchymal cells, like PLX-PAD and BM-MSC, regulate important checkpoints for Th1 priming such as DC migration from inflamed tissue to SLO, activation of NK cells to produce IFN-g and Th1 priming of naive T cells (Fig. 7). These *in vitro* data are in line with our *in vivo* observations demonstrating that allogeneic PLX-PAD cells did not trigger a specific alloresponse in patients treated with the HLA-unmatched off-the-shelf product.

MSC have previously been shown to inhibit the differentiation of human DC from both monocytes and CD34+ precursors *in vitro* resulting in a reduced ability of these DC to stimulate T cells [29, 45–47]. Yet, most of the studies have been performed with DC generated from monocytes, which do not give rise to conventional mDC *in vivo* [48, 49]. Moreover, while there is clear evidence that MSC inhibit DC differentiation *in vitro*, there are contradictory results on their capacity to interfere with DC maturation [45, 46]. Interpretation of the results provided on this topic is further complicated by differences with respect to the DC source or the time of MSC administration. Here, we demonstrate that MSC present during maturation of human immature mDC, which were freshly isolated from blood, did not inhibit the upregulation of molecules important for antigen-presentation and co-stimulation of naïve T cells. The levels of HLA-DR, CD80 and CD86 on mDC were even slightly enhanced, which is comparable to data obtained by Spaggiari et al. for the maturation of *in vitro* generated moDC [45]. In contrast, the expression of CD83 and CD40 on (BM-MSC)mDC was marginally reduced compared to mDC matured in the absence of BM-MSC. However, apart from CD83, of which the function is not known yet, the effects observed are very low and the biological significance of these changes remains questionable. In order to initiate an immune response, DC must also acquire CCR7 expression and migrate to the next draining LN. So far, there are no data available on the capacity of MSC to interfere with the migration of human DC. We prove for the first time that BM-MSC impair CCR7 acquisition in human mDC resulting in a lower ability to migrate towards CCR7 ligand CCL21. While most studies use high concentrations of TLR ligands in order to achieve efficient cytokine production in mDC *in vitro* [56], CCR7 (and co-stimulatory molecules, data not shown) could be upregulated already at low dose LPS, which was significantly inhibited by BM-MSC. This finding suggests that BM-MSC might effectively suppress the ability of DC to stimulate e.g. NK and naïve T cells *in vivo* due to their incapability to home to draining LN. Our data are in line with prior *in vitro* and *in vivo* studies in mice that have documented an inhibitory effect of MSC on LPS-or TNF-a-induced CCR7 acquisition followed by diminished migration towards its ligands [63, 64] and hindered local priming of antigen-specific CD4+ T cells *in vivo* [63]. In mice, experiments with supernatants of MSC cultures and the fact that intravenously administered MSC are largely sequestered in the lung and might not be able to reach the subcutaneously implanted DC within the limited time required suggest a major role for soluble factors [63]. Accordingly, we show that maturation of human mDC in the presence of BM-MSC-derived supernatants led to a comparable inhibition of CCR7 expression. In inflamed peripheral tissues and LN, DC encounter NK cells, which respond very efficiently to DC-derived cytokines [34–36, 41]. IL-12 is known to be a potent inducer of IFN-g production especially in CD56bright NK cells and to rapidly enhance cytotoxicity in CD56dim NK cells [38]. In turn, IFN-g primes macrophages and DC to secrete pro-inflammatory cytokines thus increasing their potential for Th1 priming [42]. While it has been demonstrated that MSC inhibit the generation of IL-12 producing moDC *in vitro* by interfering with their differentiation [29, 46], it is controversially discussed whether they inhibit IL-12 production when present only during the maturation process [45, 46, 65]. By performing an extensive analysis of secreted cytokines, chemokines and growth factors, we could show that (BM-MSC)mDC produced significantly lower levels of IL-12p70 and IL-1b and increased levels of IL-10 and IL-1Ra, representing a cytokine profile typical for TolDC. As a consequence of reduced IL-12 production, (BM-MSC)mDC were much less effective in inducing IFN-g production in NK cells. This finding was not only true with respect to the percentage of IFN-g producing cells but also in terms of IFN-g quantity per cell suggesting that BM-MSC very efficiently inhibited the amount of early NK cell-derived IFN-g. Since NK cell-derived IFN-g promotes and sustains Th1 immune responses, inhibition of DC/NK cell cross talk in addition to the diminished migration of DC to SLO could be one explanation for inhibition of Th1-driven immune-pathological processes by MSC *in vivo*. Indeed, in CLI patients treated with allogeneic placenta-derived MSC-like PLX-PAD cells no clinically relevant Th1 priming specific for the MHC-mismatch could be detected. Remarkably, while BM-MSC control the ability of mDC to migrate towards SLO and to stimulate NK cells, they do not affect the expression of HLA-DR and co-stimulatory molecules by mDC. This suggests that antigen-presentation is preserved. Nevertheless, (MSC)mDC displayed a lower ability to induce differentiation of allo-reactive naïve T cells into IFN-g producing Th1 memory cells most likely due to their diminished expression of IL-12.

The known modes of action used by MSC to generate TolDC are diverse and influenced by the experimental system, the species and the context in which

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MSC meet DC or their precursors. Among others, IL-6, Prostaglandin E2 (PGE2) and IL-10 have been reported to participate in the generation of human TolDC by MSC *in vitro*, but only when present during early DC differentiation [29, 46, 47]. On the contrary, two other studies did not confirm the role for IL-6 but supported a role for PGE2 and Notch signalling, respectively [45, 66]. IL-10 production by DC is crucial in the bidirectional communication with responding T cells, as it directs the polarization of naïve T cells into IL-10-secreting regulatory T cells [67]. In addition to its effect on T cells, IL-10 serves as a positive feedback loop, potentiating the tolerogenic circuit in an auto/paracrine manner by influencing DC and other immune cells in the microenvironment, such as Th1 cells, NK cells, macrophages and others [68, 69]. Earlier work showed that IL-10 inhibits the production of many pro-inflammatory cytokines including IL-12 and IL-1b and at the same time enhances the expression of their natural antagonists, for example IL-1Ra [68, 70]. IL-1Ra blocks signalling of IL-1b [71], which in turn has been shown to be important for IL-12-induced IFN-g production by NK cells [72]. The origin of IL-10 or IL-1Ra in our mDC/BM-MSC co-cultures is not entirely clear. In spite of reports claiming that MSC are able to express IL-10 themselves [73–75], we could only detect IL-10 in co-cultures with mDC. This confirms results of others who neither detected IL-10 when BM-MSC were cultured alone [47, 50]. IL-1Ra was shown earlier to be expressed by murine MSC and to mediate the anti-inflammatory effects in a mouse model of lung injury [76]. In our hands, BM-MSC alone did not secrete IL-1Ra but strongly enhanced its generation by activated mDC, while at the same time reducing the production of IL-1b. By performing blocking experiments, we could show that neutralization of IL-10, IL-6 or IL-1Ra completely restored IL-12p70 production, while the level of IL-1b only partially depended on these factors. Importantly, only neutralization of IL-10, but not of IL-6 or IL-1Ra, decreased the amount of biologically active IL-1Ra as well as IL-10 and at the same time increased IL-12 and IL-1b production therefore restoring a pro-inflammatory cytokine profile of stimulatory mDC. Furthermore, only IL-10 neutralization in mDC/BM-MSC but not in mDC/NK cell co-cultures re-established IFN-g production in NK cells. This suggests that IL-10 preferentially acts on mDC by suppressing their expression of pro-inflammatory cytokines and not on NK cells directly.

In summary, we propose that MSC present during DC maturation induce in an IL-10-dependent manner the generation of TolDC that are incompetent in priming an appropriate Type 1 immune response.

CONCLUSION

Our data demonstrate for the first time that off-the-shelf produced HLA-unmatched PLX-PAD cells do not induce T-cell alloreactivity in CLI patients. We could show that the interaction of MSC with mDC during maturation leads to the generation of TolmDC (Fig. 7). These mDC are incapable to migrate towards LN indicating a diminished ability to prime LN-NK and naïve T cells *in vivo*. Moreover, MSC shift the cytokine profile of mDC in an IL-10-dependent manner towards an anti-inflammatory state characterized by low IL-12 and IL-1b as well as high IL-10 and IL-1Ra production. As a consequence, (MSC)mDC display a strongly reduced ability to activate NK cells, which is important for Th1 priming *in vivo*. In addition, (MSC)mDC directly induce less Th1 priming due to their lower IL-12 production and reduced availability in LN. In conclusion, while the complete mechanism of immunomodulation by MSC requires further investigation and will depend on the clinical setting for their application, this study adds further evidence on how MSC inhibit Th1 immune responses and offers a test opportunity to pre-check immunogenicity of allogeneic MSC-like cell products.

ACKNOWLEDGMENTS

We thank Dr. Arne Sattler for discussions and critical reading of the manuscript. We would also like to acknowledge the assistance of Dr. Desiree Kunkel, BCRT Core Facility Flow Cytometry for cell sorting and Dr. Reinke, BCRT Core Facility Cell Harvesting and Dr. Ringe/Prof. Sittinger, BCRT and Cellserve GmbH for help in MSC generation.

COMPETING INTERESTS

L.P. and R.O. are employed by Pluristem Therapeutics. H.-D.V. is consultant at Pluristem Therapeutics. All other authors declare that they have no competing interests. Pluristem Therapeutics provided partial research funding for data analysis of clinical study samples.

DATA AND MATERIALS AVAILABILITY

EudraCT number 2008-003711-13. Inquiries should be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

C.C.: collection of data, data analysis and interpretation, manuscript writing; L.A.: data analysis and interpretation; J.A.S.-L. Co-PI clinical phase I study with PLX-PAD cells; C.T. PI clinical phase I study with PLX-PAD cells; P.R. Co-PI clinical phase I study with PLX-PAD cells; L.P. and R.O.: PLX-PAD cell characterization and data analysis of PLX-PAD clinical study; H.-D.V. contribution to the concept, final approval of the manuscript; K.J. concept and design, data analysis and interpretation, manuscript writing and final approval of the manuscript.

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Figure. 1. BM-MSC inhibit CCR7-dependent migration but only marginally maturation of mDC. (A-E) mDC were matured in the presence (grey) or absence (black) of BM-MSC by high dose (100ng/ml LPS+10µg/ml R848) or low dose (100pg/ml LPS alone) TLR ligands for 18hrs. **(A-C)** CCR7 Expression was examined by flow cytometry. Transwell migration assay was performed towards CCL21. **(A)** The level of CCR7 on CCR7high mDC and percentage of CCR7high mDC are depicted as mean +/- SEM (n=13-15). **(B)** Histograms of one representative experiment are shown. (**C**) The number of migrated mDC is shown as mean +/- SEM (n=6-8). **(D-E)** Expression of CD86, CD80, CD83, CD40 and HLA-DR of mDC was analyzed by flow cytometry after maturation by LPS/R848 for 18hrs (n=7). **(D)** Mean MFI values for the indicated cell surface markers of (BM-MSC)mDC relative to mDC alone +/- SEM are shown. **(E)** Representative histograms for (BM-MSC)mDC and mDC are depicted for one donor.

Abbreviations: BM-MSC, bone marrow-derived mesenchymal stromal cells; (BM-MSC)mDC, mDC matured in the presence of BM-MSC; CD, cluster of differentiation; MFI, mean fluorescence intensity; LPS, lipopolysaccharide; mDC, myeloid dendritic cells; R848, resiquimod; SEM, standard error mean; TLR, Toll Like Receptor

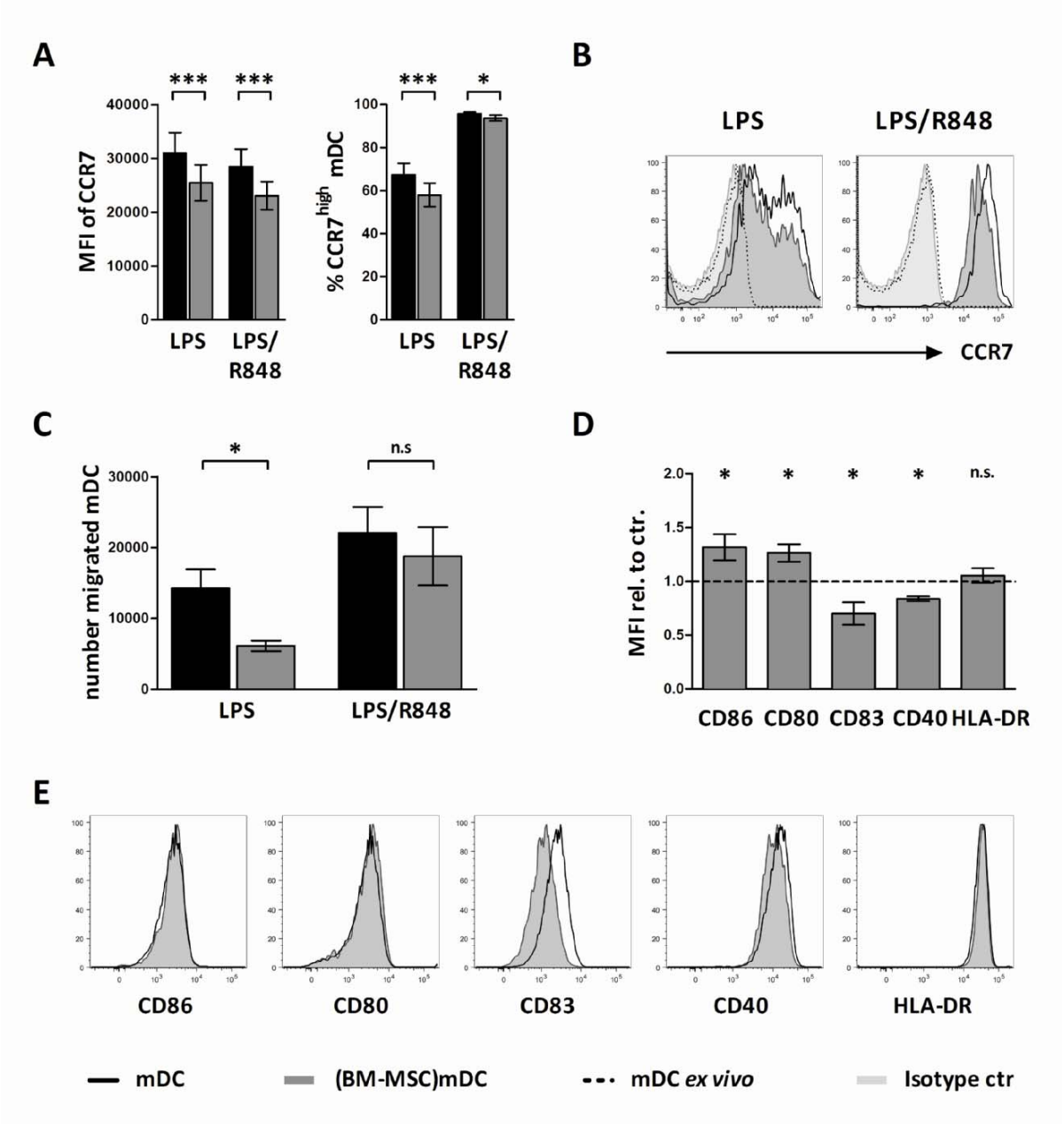

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Figure. 2. BM-MSC induce an anti-inflammatory cytokine profile in mDC. (A, B) Cytokine levels in mDC/BM-MSC co-culture supernatants were determined by multiplex analysis (n=21). mDC alone (black), (BM-MSC)mDC (grey) or BM-MSC alone (white) were cultured in medium with or without LPS/R848 for 18hrs. **(A)** Concentrations of the respective cytokines are presented as mean +/- SEM. **(B)** Ratios between IL-1b and IL-1Ra as well as IL-12p70 and IL-10 are shown as mean +/- SEM.

Abbreviations: BM-MSC, bone marrow-derived mesenchymal stromal cells; (BM-MSC)mDC, mDC matured in the presence of BM-MSC; IL, interleukin; IL-1Ra, interleukin-1 Receptor antagonist; LPS, lipopolysaccharide; mDC, myeloid dendritic cells; R848, resiquimod; SEM, standard error mean

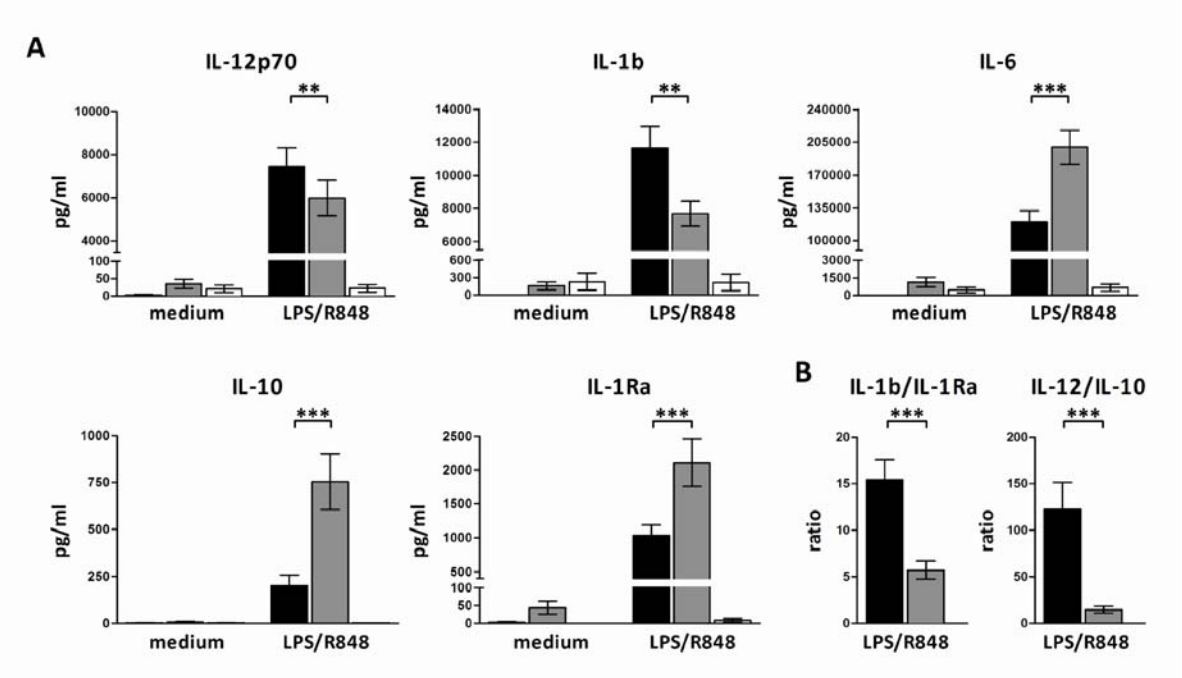

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Figure. 3. IL-10 regulates the balance between pro- and anti-inflammatory cytokines. (A, B) Cytokine levels of mDC/BM-MSC co-culture supernatants were determined by multiplex analysis (n=3-5). mDC alone (black) or (BM-MSC)mDC (grey) were cultured in medium with LPS/R848 and anti-IL-10/anti-IL-10R, anti-IL-1Ra or anti-IL-6 as indicated for 18hrs. **(A)** Concentrations of the specified cytokines in mDC/BM-MSC co-cultures in relation to mDC alone are depicted as mean +/- SEM. **(B)** Ratios between IL-1b and IL-1Ra as well as IL-12p70 and IL-10 are shown as mean +/- SEM.

Abbreviations: BM-MSC, bone marrow-derived mesenchymal stromal cells; (BM-MSC)mDC, mDC matured in the presence of BM-MSC; IL, interleukin; IL-1Ra, interleukin-1 Receptor antagonist; IL-10R, interleukin-10 Receptor; LPS, lipopolysaccharide; mDC, myeloid dendritic cells; R848, resiquimod; rel. to ctr., relative to control; SEM, standard error mean

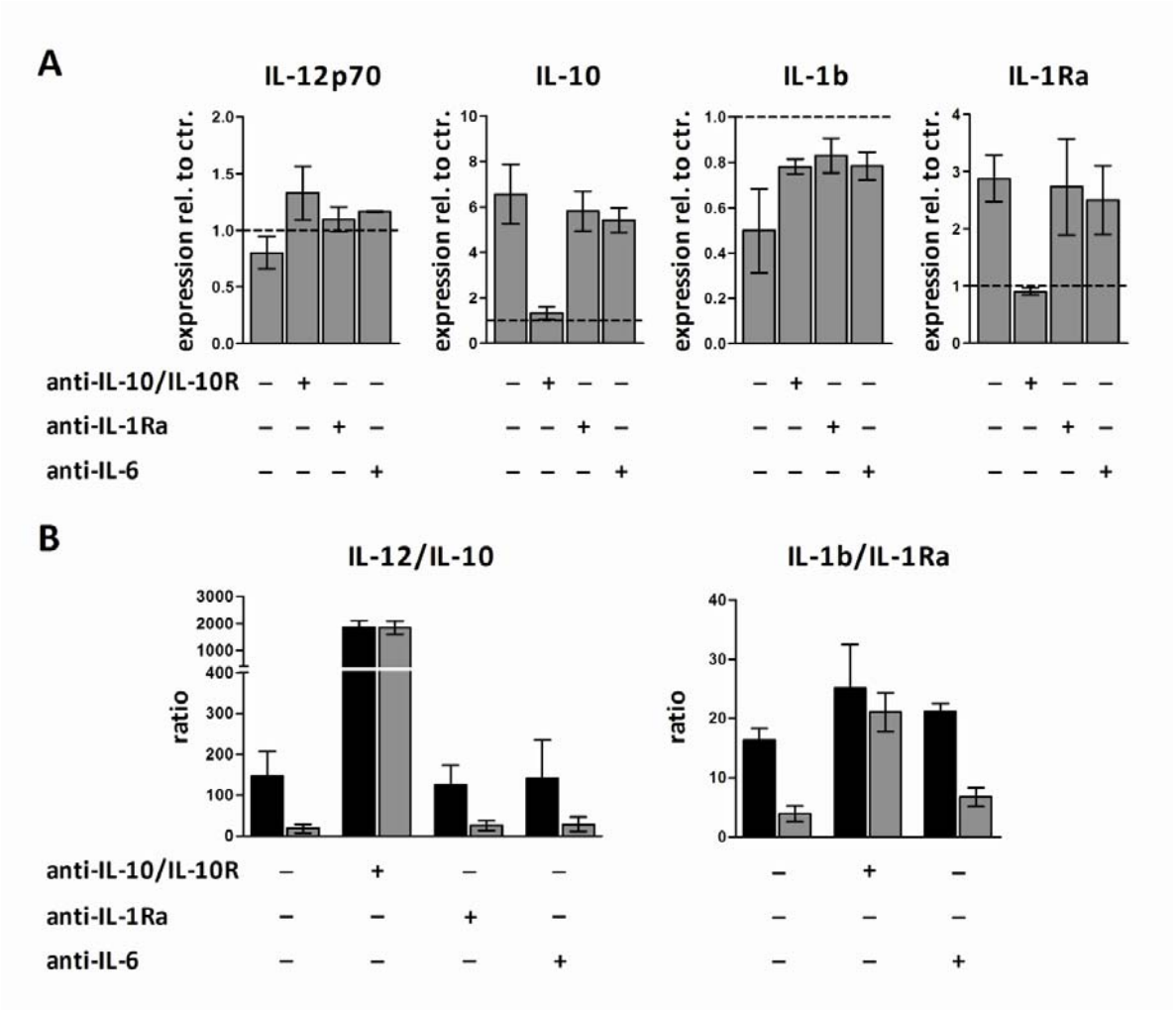

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Figure. 4. BM-MSC inhibit the ability of mDC to activate NK cells. (A-D) Mature mDC (grey) or (BM-MSC)mDC (black) were used to activate sorted CD56bright or CD56dim NK cells. Resting NK cells were used as control (white). **(A)** CD56dim NK cells were pre-activated by the indicated mDC for 18hrs before stimulation with K562 cells for 6hrs. CD107a expression on NK cells and killing of K562 are shown as mean +/- SEM (n=5). **(B)** Proliferation of NK cell subsets was measured by dilution of CFSE after 5 days of co-culture with mDC. Data for one representative donor out of 4 are shown. **(C-D)** Intracellular staining of IFN-g was analyzed by flow cytometry after stimulation of NK cell subsets with mDC for 24hrs. Mean percentage and mean MFI values of IFN-g+ cells +/- SEM **(C**, n=14) as well as representative dot plots for one donor **(D)** are shown.

Abbreviations: BM-MSC, bone marrow-derived mesenchymal stromal cells; (BM-MSC)mDC, mDC matured in the presence of BM-MSC; CD, cluster of differentiation; CFSE, carboxyfluorescein succinimidyl ester; ctr., control; IFN-g, interferon-gamma; LPS, lipopolysaccharide; MFI, mean fluorescence intensity; NK cells, natural killer cells; mDC, myeloid dendritic cells; R848, resiquimod; SEM, standard error mean

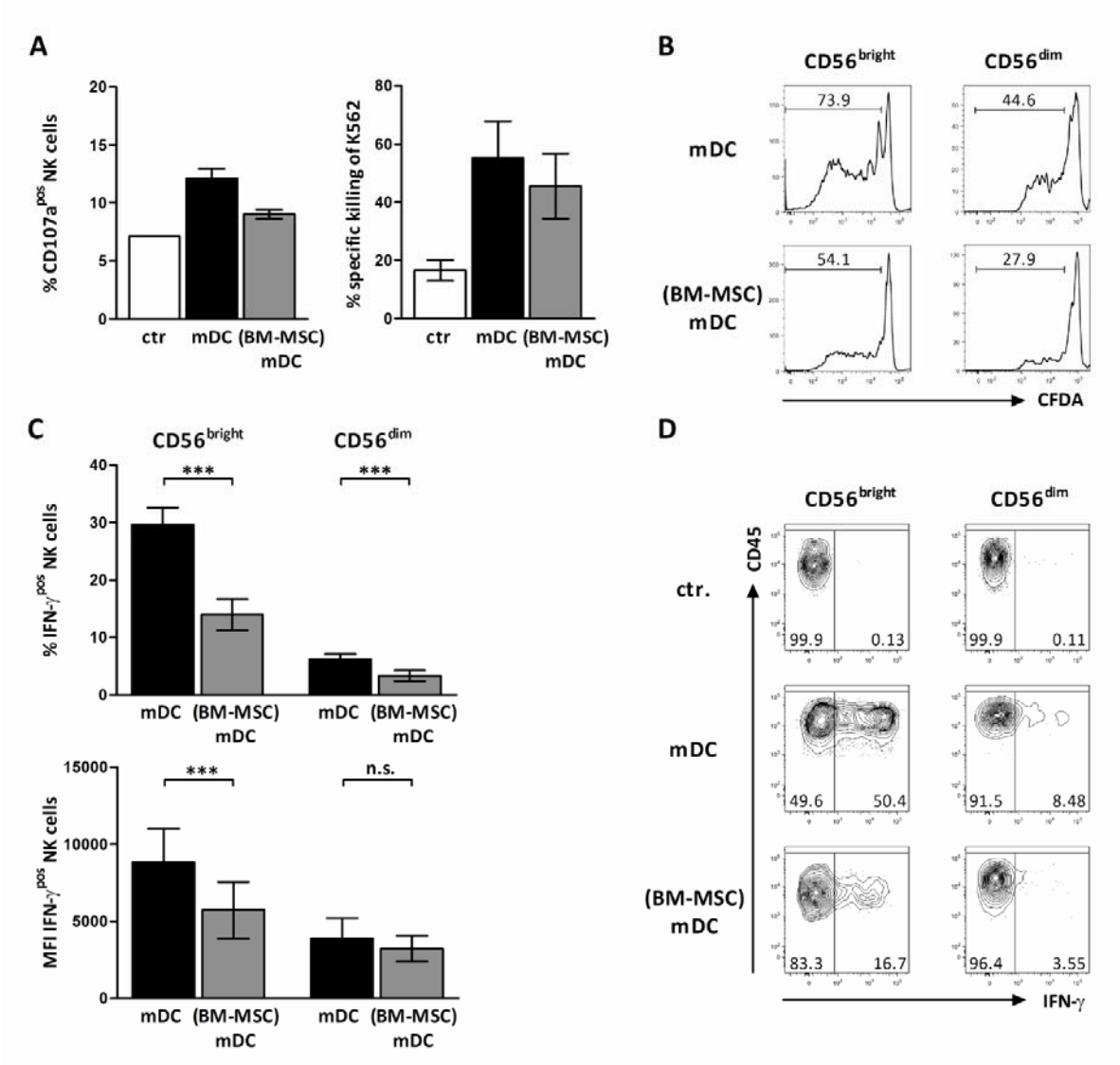

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Figure. 5. Decreased IL-12 production by (BM-MSC)mDC accounts for reduced NK cell activation. (A-D) Mature mDC (grey) or (BM-MSC)mDC (black) were used to activate CD56bright NK cells. IFN-g production was analyzed after 24hrs. **(A)** mDC and NK cells were cultured in transwell plates, either separated by the membrane or in contact in the lower chamber. Histograms are shown for one representative donor out of 3. **(B)** mDC and BM-MSC were cultured in transwell plates before being used for NK cell activation. Blocking antibodies for IL-12 or IL-10/IL-10R or recombinant IL-12 were added during NK cell stimulation. Representative stainings for one donor out of 8 (IL-12/anti-IL-12) or 3 (anti-IL-10/IL-10R) are depicted. **(C)** mDC and BM-MSC were cultured in contact (solid graphs) or separated by a transwell membrane (striped graphs) before being used for NK cell activation (n=11-18). Percentage of IFN-g+ NK cells (upper graph) and percentage of IFN-g+ NK cells relative to control without BM-MSC (lower graph) are shown as mean +/- SEM. **(D)** mDC and BM-MSC were cultured in transwell plates before being used for NK cell activation. Neutralizing antibodies for IL-10/IL-10R were added during mDC stimulation. Representative dot plots are shown for one donor out of 6.

Abbreviations: BM-MSC, bone marrow-derived mesenchymal stromal cells; (BM-MSC)mDC, mDC matured in the presence of BM-MSC; CD, cluster of differentiation; ctr., control; IFN-g, interferon-gamma; IL, interleukin; IL-10R, interleukin-10 receptor; LPS, lipopolysaccharide; MFI, mean fluorescence intensity; NK cells, natural killer cells; mDC, myeloid dendritic cells; R848, resiquimod; rel. to ctr., relative to control; SEM, standard error mean; tw, transwell

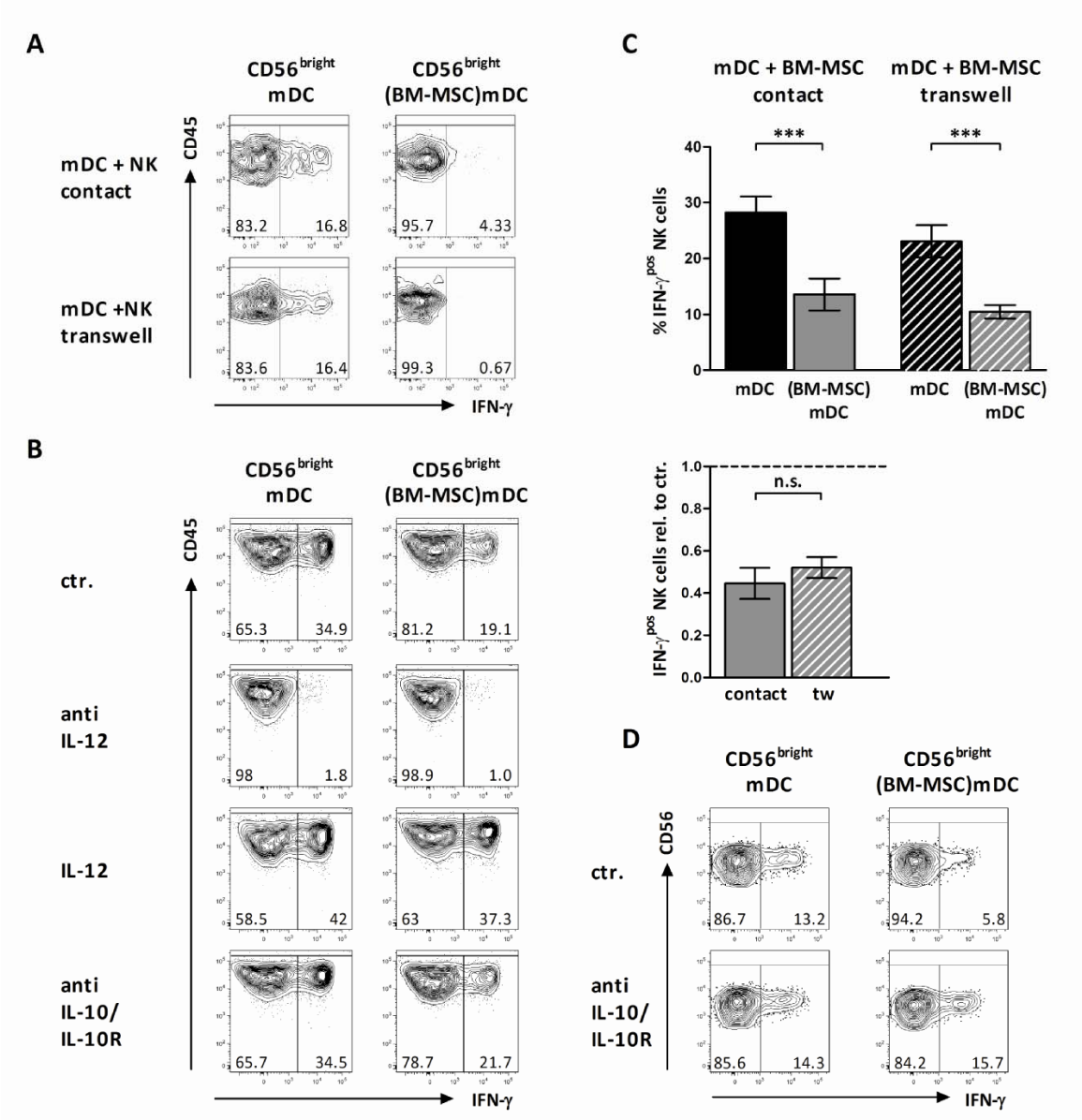

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Figure. 6. BM-MSC and PLX-PAD inhibit Th1 priming. (A-B) Mature mDC (black), (BM-MSC)mDC (dark grey) or (PLX-PAD)mDC (light grey) were used to stimulate CD56bright NK or naïve T cells. IFN-g production was analyzed by flow cytometry **(A)** or CBA **(B)**. **(A)** CD56bright NK cells were stimulated with the indicated mDC for 24hrs. Percentage of IFN-g+ NK cells relative to stimulation with control mDC matured in the absence of MSC is shown as mean +/- SEM. **(B)** Naïve T cells were cultured in the presence of the indicated allogeneic mDC for 5 days. IFN-g levels in the culture supernatants are presented as mean +/- SEM (n=4). **(C)** CLI Patients received one i.m. injection of PLX-PAD cells. Blood was taken before, 3 days, 1 week and 4 weeks after injection. PBMC were stimulated overnight in triplicates at IFN-g Elispot plates by PLX-PAD or third-party allogeneic PBMC. The numbers of spots per 300,000 PBMC corrected for the background detected in unstimulated controls are shown. A response of more than 25 IFN-g spots/ 300,000 PBMC has been set as clinically relevant (dashed line). Open symbols indicate three patients that showed minimal reactivity towards the mismatch.

Abbreviations: BM-MSC, bone marrow-derived mesenchymal stromal cells; (BM-MSC)mDC, mDC matured in the presence of BM-MSC; CD, cluster of differentiation; CLI, critical limb ischemia; IFN-g, interferon-gamma; i.m., intramuscular; LPS, lipopolysaccharide; MFI, mean fluorescence intensity; NK cells, natural killer cells; mDC, myeloid dendritic cells; PBMC, peripheral blood mononuclear cells; PLX-PAD, placenta-derived mesenchymal-like adherent stromal cells; (PLX-PAD)mDC, mDC matured in the presence of PLX-PAD; R848, resiquimod; rel. to ctr., relative to control; SEM, standard error mean; Th1, Type 1 helper T cells

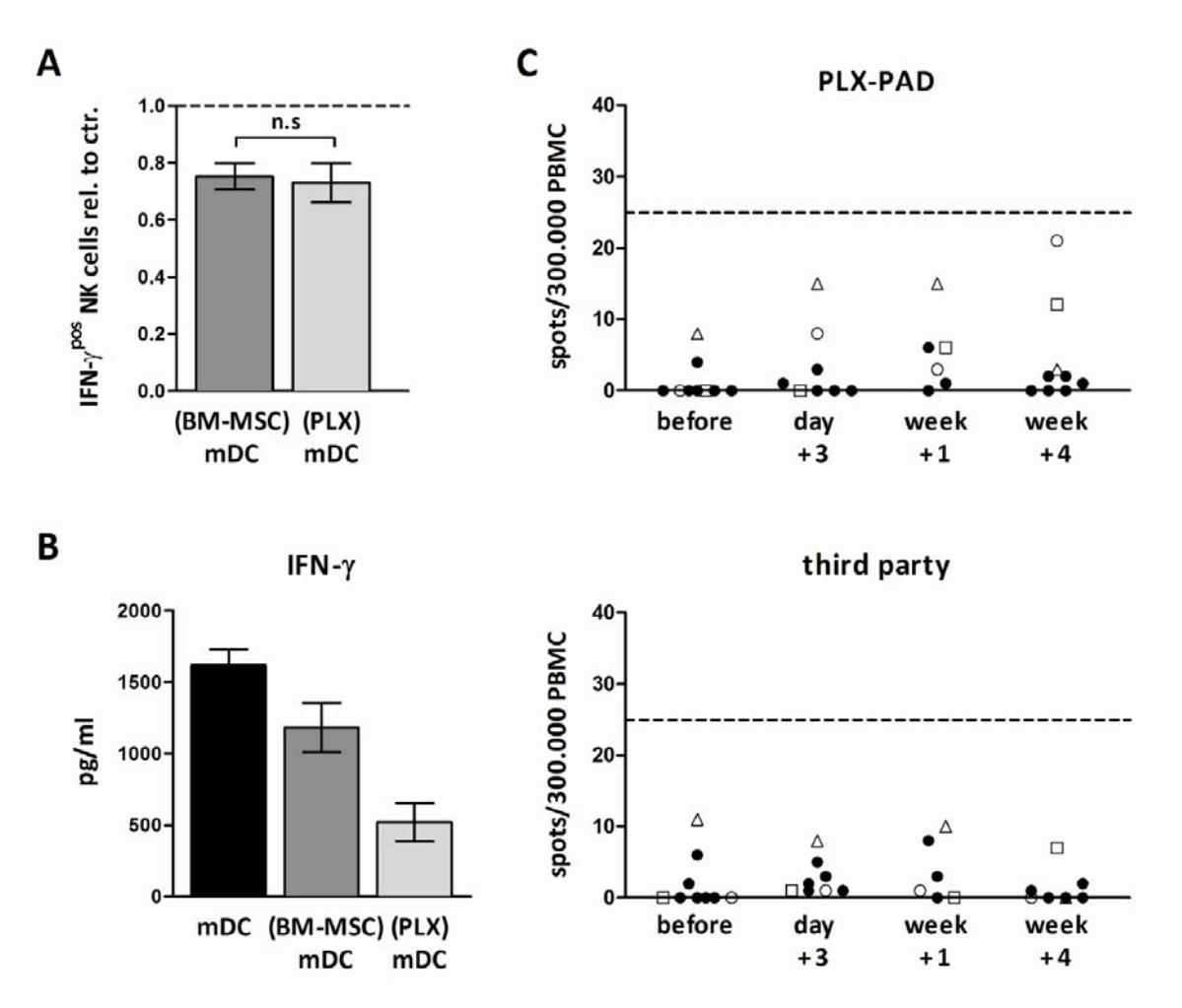

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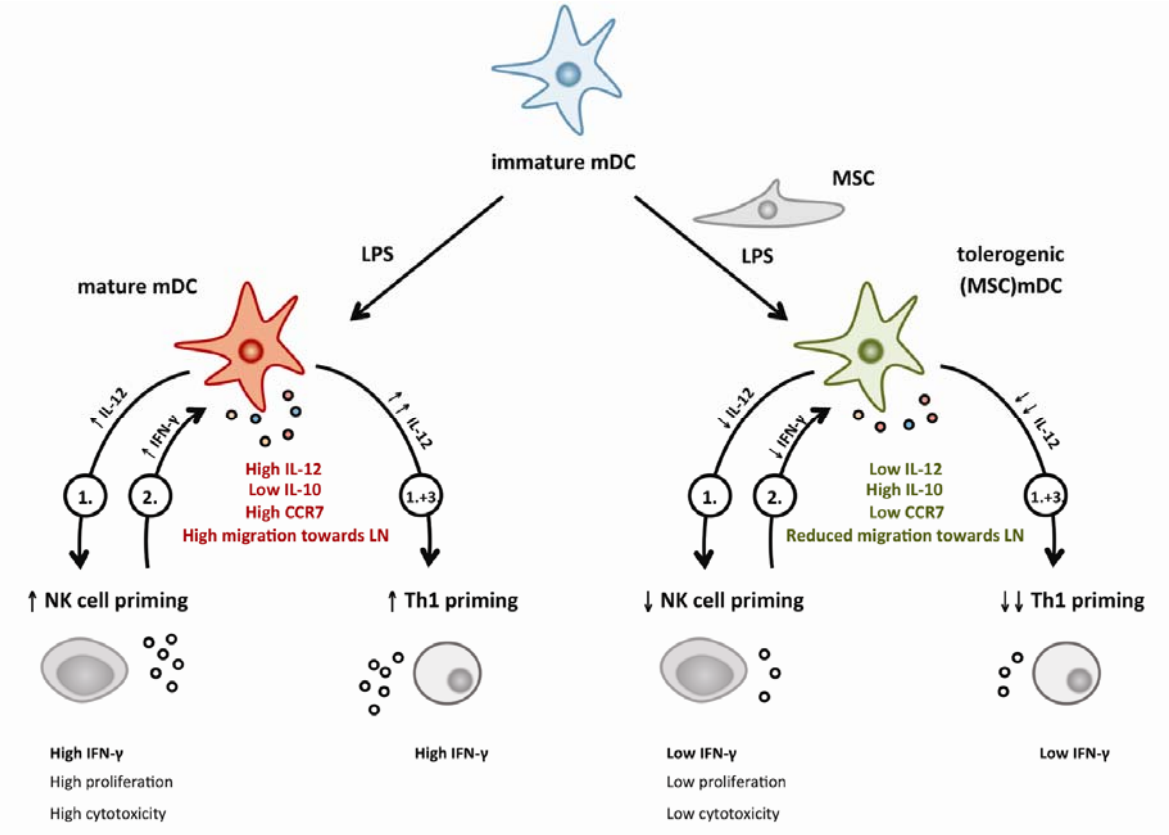
Figure. 7. MSC instruct mDC to be anti-inflammatory and poor triggers of NK cell as well as Th1 activation. After antigen encounter in the periphery, DC start to migrate in a CCR7-dependent manner towards lymph nodes and produce pro-inflammatory cytokines, e.g. IL-12. DC-derived IL-12 induces Th1 priming of naïve T cells and activation of NK cells (①). IFN-g produced by activated NK cells (②) enhances IL-12 secretion by DC, leading to an increased Th1 priming (③). mDC matured in the presence of MSC display a phenotype of TolDC with IL-10 being the key mediator for these changes. (MSC)mDC produce less IL-12, resulting in a lower ability of (MSC)mDC for Th1 priming of allogeneic naïve T cells. Moreover, (MSC)mDC display a decreased potential for the activation of autologous NK cells in terms of proliferation, cytotoxicity and IFN-g production, with the latter being essential for priming of Th1 responses (②+③).

Abbreviations: IL, interleukin; IFN-g, interferon-gamma; LN, lymph node; LPS, lipopolysaccharide; NK cells, natural killer cells; mDC, myeloid dendritic cells; MSC, mesenchymal stromal cells; (MSC)mDC, mDC matured in the presence of MSC; Th1, Type 1 helper T cells.



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