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IFNGamma Priming Protects Fetal and Embryonic MSC from NK Cell-Mediated Killing and Improves their Immunosuppressive Properties: Role of Activating and Inhibitory Receptors

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Abstract

The enhancement of Mesenchymal Stem Cells (MSC) immunosuppressing function can favour the engraftment of MSC employed in tissue repair. IFN-γ is a pro-inflammatory cytokine, which induces tolerogenic molecules in bone marrow (BM) MSC. We have investigated the interaction of IFN-γ primed MSC from fetal (FL-MSC) and embryonic (ES-MSC) origin with natural killer (NK) cells. IFN-γ -primed FL-/ES-MSC were less susceptible to NK cell-mediated killing, where a major role was played by the IFN-γ-induced up-regulation of HLA-ABC and HLA-E. Monoclonal antibody-mediated blocking of CD94/NKG2A on NK cells increased killing of IFN-γ-primed MSC, suggesting a role of this NK cell inhibitory receptor in MSC protection. NKG2D ligands (such as MICA), LFA-1 and ICAM1 expressed on MSC were also involved in NK cell-mediated killing of un-primed, but not of IFN-γ-primed, FL-/ES-MSC. Importantly, NK cells from IFN-γ-primed FL-/ES-MSC-NK cell co-cultures displayed a reduced intracellular free calcium increase, pERK activation, degranulation, cytolysis and IFN-γ production upon interaction with the NK sensitive target cell K562 compared to NK cells from un-primed FL/ES-MSC-NK cell co-cultures. Finally, PGE-2, increased during NK/MSC co-cultures, appeared to be a key soluble factor responsible for FL-/ES-MSC-mediated immunosuppressive effect. These results suggest that surface molecules such as MICA, HLA-E and ICAM1 can play a role in recognizing un-primed FL/ES-MSC but not IFN-γ-primed MSC where HLA-I is a key molecule for NK cell-mediated recognition. Further, the strong immunosuppressive effect of IFN-γ-primed FL-/ES-MSC on NK cells could be exploited in cellular therapy protocols.

Keywords: MSC; ES; Fetal cells; NK cells; Priming; Immunotolerance; Inflammation; GvDH

Abbreviations:

ES: Embryonic Stem Cells; FL: Fetal Liver; MIC: Major Histocompatibility Complex class I-related chain; MSC: Mesenchymal Stem Cells; NCR: Natural Cytotoxicity Receptors; NK: Natural Killer; NKG2D: Natural Killer Group 2D; PGE-2: Prostaglandin-2; IDO: Indolamine 2,3-dyoxigenase

Introduction

Natural killer (NK) cells are characterized for their ability to kill transformed or malignant cells without prior activation [1,2]. They are not only involved in immune responses against viral pathogens, but they play a critical role in organ transplantation and GvHD treatment [3,4]. Adhesion molecules expressed on NK cells such as Leukocyte Function Antigen (LFA)-1 play a key role in recognition of target cells expressing Intercellular Adhesion Molecule-1 (ICAM1, or CD54) [5]. NK cell activity is regulated by a balance of inhibitory receptors [such as NKG2A and KIRs (Killer Inhibitory Receptor)] that override activating signals represented by the family of natural cytotoxicity receptors (NCR), including NKp46, NKp30, NKp44 and NKG2D [6-8]. While several NCR ligands are still unknown, NKG2D recognizes the UL16-Binding Proteins (ULBP) family and MHC class I–related molecules A and B (MICA/B), expressed not only in tumor cells [9], but also in Bone Marrow Mesenchymal Stem Cells (BM-MSC) [10] which are strong negative regulators of immune responses and are of great interest in cellular therapy [11,12]. In particular BM-MSC inhibits NK cells proliferation and cytotoxic functions [10,13,14]. However BM-MSC immunomodulatory functions are time-limited in vitro and other sources of MSC than BM such as cord blood [15-17], placenta [18-20], Wharton Jelly [21-26], adipose tissues [21,22,27,28],Embryonic cells [29-33] and Fetal liver [34-39] have to be considered. Recently, several groups have begun to optimize MSC regulatory functions and to better understand their behaviour in inflammatory conditions, which could be important in MSC-mediated therapies. IFN gamma (IFN-γ) is a classical Th1 pro-inflammatory cytokine largely secreted during infection or inflammatory response, and it is known to induce tolerogenic molecules (such as IDO, B7-H1 and HLA-G)in licensed (primed)human MSC [16,17,19,23,40-45].By consequence, IFN-γ primed MSC better inhibit T cell proliferation and increase their HLA class I expression that plays a critical role in protecting adult from NK killing [10,14].Nevertheless, a beneficial effect of IFN-γ on MSC activity is found not only in vitro but also in

vivo, by preventing GvHD mortality in patients receiving allogeneic bone marrow transplantation (BMT) [46]. Recently, Gotherstrom et al. [35] showed that fetal MSC are susceptible to NK cell-mediated killing. They compared FL-MSC with the adult counterpart, by showing that HLA-I molecules and several activating ligands expressed on FL-MSC are modulated after IFN-γ stimulation.

In this study we compared the effects of IFN-γ priming on MSC (derived from bone marrow, fetal and embryonic sources) suppressive properties, by focusing our attention on the interaction between MSC and NK cells, and overall the role of activating and inhibitory ligands expressed on NK and MSC, respectively. Our results show that IFN-γ priming renders all MSC sources less susceptible to IL2-activated NK cell-mediated killing. This effect depends on the up-regulation of surface inhibitory HLA-ABC and HLA-E molecules on MSC. Interestingly, LFA-1, NKG2D and NKp30 NK cell receptors played a role in recognition of un-primed, but not of IFN-γ-primed MSC. In addition, NK cells from IFN-γ-primed FL-/ES-MSC-NK cell co-cultures displayed, upon interaction with the NK sensitive target cells, a reduced degranulation and cytolytic ability compared to NK cells from un-primed FL/ES-MSC-NK cell co-cultures. These findings show that IFN-γ-primed FL/ES-MSC can be a really strong immune-regulator of NK cell mediated innate immunity suitable to cellular therapies.

Materials and Methods

Reagents

The antibodies used to assess NK and MSC phenotypes were assessed from Beckman Coulters (CD3, NKp30, NKp44, NKp46, CD16, CD69, NKG2A, CD105, CD73, CD166, HLA-ABC, HLA-DR, CD54, and CD45), Becton Dickinson (CD107a, B7-H1, CD90) and R&D Systems (NKG2D, MICA, ULBP2, ULBP3, IDO). Recombinant human IL-2 and IFN−γ were purchased from Peprotech, whereas Monensin was purchased from Sigma. Monoclonal antibody (mAb) anti-HLA-ABC, anti-B7-H1 and anti-MICA were obtained from eBioscience, whereas mAb anti-NKp30, anti-NKG2D, anti-NKG2A and anti-LFA1 where purchased from BioLegend.

Generation of human mesenchymal stem cells

Human fetal livers (range 7-9 weeks of gestation, n=7) were obtained from eight women after voluntary or therapeutic abortions. Informed written consent was obtained from the patient in accordance with the Declaration of Helsinki, and tissue collection and use were performed according to the guidelines and with the approval of the French Biomedicine Agency. Fetal liver cells were prepared as described previously [34]. Bone marrow samples were obtained from adult bone marrow aspirates (45±15 years, n=6) to isolate MSC. Briefly, bone marrow and fetal mononuclear cells were isolated and plated at 1.6×105cells/cm^2 in α MEM (Life Technologies). This was supplemented with 2mM L glutamine, 1% penicillin/streptomycin solution (all from Gibco), and 10% heat inactivated fetal calf serum (FCS; PAA Laboratories) (complete medium) at 37°C in air with 5% CO_2. After three days, non-adherent cells were discarded and the cells were plated at 4×103cells/cm2 in a α MEM complete medium supplemented with bFGF (1 ng/ml). The medium was changed twice a week. When the cells were 70-80% confluent, the MSC were harvested by treating with trypsin-EDTA (Gibco) (passage 1) and were then cultured in α MEM complete medium. Embryonic-derived MSC differentiation was induced by plating 10.103-25.103 cells/cm^2 on a monolayer of OP9 cells in the presence of 20% heat-inactivated Foetal Bovine Serum (FBS) in α-MEM medium, as described previously [31]. All MSC were characterized according to the International Society for Cellular Therapy (ISCT) criteria [47].

Isolation of NK cells and cell lines

NK cells were isolated by negative selection using the RosetteSep method (StemCellTechnologies), according to the manufacturer's instructions. Freshly purified CD56+/CD3- (93-97%, as checked by flow cytometry) NK cells were stimulated with IL2 (200 U/ml) and were used immediately in functional assays. Cells were plated (10^6 cells/ml) in flat-bottom 96well tissue-culture plates in the presence or absence of MSC at a 4:1 ratio of NK/MSC. After 5 days, NK cells were assessed for their cytolytic activity, cytokine production and surface phenotype. The HLA class I- K562 cell line was used as a target in the NK-cell cytotoxicity analysis.

LDH release cytotoxic assay

The cytolytic activity of activated NK cells against K562 cells and MSC were assessed in a 4h.

Lactate Dehydrogenase (LDH) release assay using CytoTox 96 Non-radioactive Cytotoxicity Assay kit (Promega). Briefly, NK cells were incubated with targets (K562 or MSC) at different effector–target (E:T) ratios for 3h and 15minat 37°C in a humid atmosphere with 5% CO_2, and then 10µl lytic solution was added into target cell maximal release group. After that, the plates were centrifuged and supernatants from each well (50 µl) were transferred into 96 flat bottom microwell plates and 50 µl of lactic acid dehydrogenase substrate mixture was added for 45min. All measurements were done in triplicates. A microtiter plate reader was used for evaluation of the changes in the absorbance at 492 nm. In blocking experiments, targetswere pre-incubated (5µg/ml for 30 min at 37°C) with anti-MICA, anti-B7H1 and anti-HLA class I antibodies, whereas NK cells with anti-NKG2D,anti-NKp30, anti-NKG2A and anti-CD11a antibodies. As control, cells were treated with an isotype IgG1 or IgG2a antibody under the same conditions.

Flow cytometry analysis of NK ligands expression on MSC surface and activating receptors on NK cells

For surface-marker expression,cells were stained with saturating concentrations of the appropriate mAbs or isotype-matched control Ab, for 30 min at 4°C in the dark. Cells were analysed using a FACScan flow cytometer using CellQuest Analysis software (BD Biosciences).

Cytolytic degranulation by activated NK cells

NK cells were also tested for their cytolytic activity against NK susceptible target cells by quantifying surface expression of CD107a, as previously described [31]. Monensin (10µM) was added for the last 4h to inhibit cytokine secretion.

Cytokine detection by an enzyme-linked immunosorbent assay (ELISA)

Cultured cell supernatants were harvested after 5 days of co-culture and tested in triplicate for production of IFN-γ (eBioscience) and PGE-2 (R&D Systems) by ELISA, according to the manufacturer's instructions. The ELISA plates were read at OD450nm on a Microplate ELISA reader (Titertekmultiskan plus, Puteaux, France).

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ERK signaling

NK cells cultured in presence of MSC for 5 days were subsequently incubated with K562 target cells (2:1) for 15min to evaluate ERK 1/2 (Total) signaling, by using Instant One™ ELISA (eBioscience), according to the manufacturer's instructions.The ELISA plates were read at OD450nm on a Microplate ELISA reader (Titertekmultiskan plus, Puteaux, France).

Intracellular free calcium increase [Ca^{++}]ì assay

Intracellular calcium increase [Ca^{++}] was evaluated as previously described [48]. Briefly NK cells harvested from different co-cultures with mesenchymal stem cells of different origin were labelled with 1µM of acetoxy-methyl ester of Fura-2 at 37°C for 45min. After extensive washing NK cells were added to slide cover slip coated with K562 tumor target cells. [Ca^{++}]I increase induced in NK cells after interaction with K562 cells was monitored under controlled temperature conditions (37°C) with the inverted microscope Olympus IX70 equipped with the analysis image system Cell R (Olympus, Germany). Images were taken with the ORCA camera (Hamamatsu, Japan) every 15sec for at least 1h after the NK-K562 interaction. Data are expressed as nM Ca^{++} and are the mean of at least 20 different NK cells which interacted with K562 target cells.

Data analysis

Statistical analysis was performed using Graph Pad Prism software (Graph-Pad). All experiments were performed in at least three independent assays, which gave highly comparable results. Data are presented as mean +/- SD. The statistical significance (*P* values) of the results was calculated using the two-tailed paired test. A $P<0.05$ was considered statistically significant. Informed written approval was obtained from the INSERM institutional review board for these studies. Informed consent was provided according to the Declaration of Helsinki.

Results

IFNγ-primed FL/ES-MSC are more resistant to NK cell-mediated killing

IFN-γ has been recently proposed as key factor in pro-inflammatory conditions, by playing a critical role in protecting adult and fetal MSC from NK killing [14,35]. After incubation with IFN-γ (20 ng/ml for 48 h), the sensitivity of adult, fetal and embryonic MSC to NK-cell mediated lysis was significantly diminished at every ratio NK/MSC used, compared to the sensitivity of unprimed MSC (Figure 1a and 1b). Similarly, the degranulation ability of NK cells (CD107a assay) was remarkably decreased against IFN-γ-primed MSC as demonstrated by the decreased percentage of CD56+/CD107a+ cells when BM/FL/ES-MSC were primed with IFNγ (Figure 1c and 1d).

IFN-γ modulates the surface HLA molecules expression by MSC

To determine the reason why IFNγ primed FL/ES-MSC were less susceptible to NK cell-mediated lysis, we first evaluated the expression of HLA-I molecules, which role has been shown in MSC protection from NK cells [14] in FL/ES-MSC upon incubation with IFNγ. A remarkable increase of HLA-ABC and HLA-E expression was detected in FL and ES-MSC, besides BM-MSC after IFNγ stimulation (Figure 2). By contrast class II molecules (HLA-DR), not constitutively expressed by both FL- and ES-MSC, were increased mainly in BM- and ES-MSC upon incubation with IFNγ.

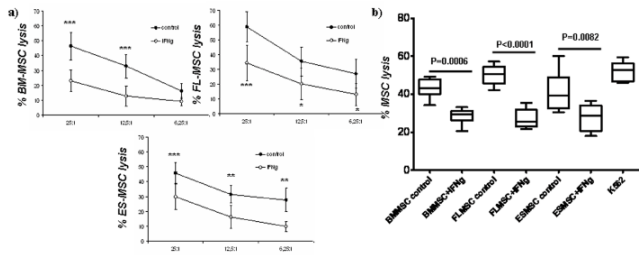

Figure 1(a and b): IFN-γ reduces the sensitivity of MSC to IL2-activated NK killing. A) MSC cultured in the presence or absences of IFN-g (20ng/ml) for 48h were used as target in a non-radioactive cytotoxicity assay (Promega) with NK effector cells at different ratio NK/MSC. B) CD107a degranulation by NK cells against IFN-primed MSC. NK cells were cultured with MSC (ratio NK/MSC, 5:1) for 4h in presence of anti-CD107a antibody and Monensin (2 µM). K562 cell line was used as positive control. ****=p*<0.001, ***=p*<0.05

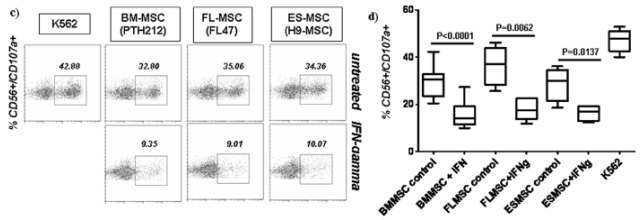

Figure 1(c and d): Degranulation ability of NK cells (CD107a assay) was remarkably decreased against IFN-γ-primed MSC as demonstrated by the decreased percentage of CD56+/CD107a+ cells when BM/FL/ES-MSC were primed with IFN-γ

We next evaluated whether IFNγ primed FL- and ES-MSC display a different expression of ligands which engagement can deliver a positive or negative signal in NK cells. We found a strong increase of expression of CD54 in FL-and ES-MSC, besides BM-MSC, associated with a statistically significant up-regulation of Programmed Death Ligand-1 (PDL-1, also named B7-H1) expression. Interestingly, compared to BM-MSC, FL-MSC and ES-MSC expressed higher basal levels of CD54. By contrast MICA, a ligand of the activating NK cell receptor NKG2D was not significantly modulated in all MSC sources. This may be mainly due to the heterogeneity of MICA expression among the different donors (overall in adult and fetal MSC).

Activating and inhibitory ligands as possible escape mechanism used by MSC

Given that IFNγ increased the expression of HLA-I, HLA-E and B7-H1 on MSC, we explored if these molecules were involved in MSC protection to NK lysis (Figure 3a).

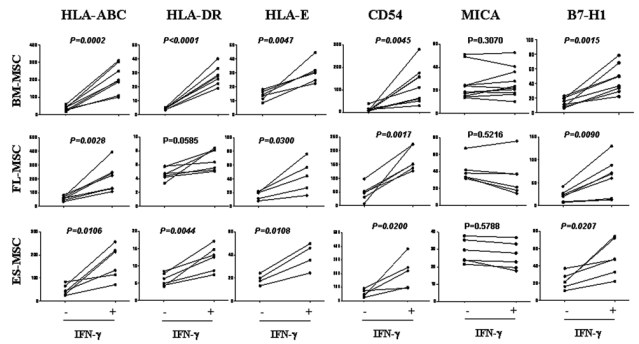

Figure 2: Modulation of activating and inhibitory ligands expression after IFN-γ treatment. MSC were incubated for 48 hr in the absence or presence of IFN-γ (20ng/ml). Surface expression of class I, class II, HLA-E, MICA, CD54 (ICAM1) and B7-H1 molecules was determined by flow cytometry. Histograms show marker expression on IFN-γ-treated (bold black lines) and untreated (thin black lines) MSC. The surface expression of class I, class II, HLA-E, MICA, CD54 (ICAM1) and B7-H1 molecules is indicated as mean fluorescence intensities (MFI) (indicated as MFI ±SD; gray profile = MSC control, black profile = IFN-primed MSC) of five independent experiments. In Italic is represented the significant modulation induced by IFN−γ

For this purpose, HLA class I and B7-H1 molecules on MSC were blocked with specific antagonistic antibodies (mAb) (W6/32 and B7-H1, respectively) before the cytolytic assay, while the interaction with HLA-E was impaired using anti-CD94/NKG2A mAb to block the HLA-E receptors on NK cells. Our results showed that the addition of antagonist blocking mAbs had no effects on the lysis of unprimed FL/ES-MSC. By contrast, in IFNγ primed MSC the incubation with anti-HLA-I or anti-CD94/NKG2A mAb increased the NK cell-mediated killing. These results show that the IFN-mediated class I and HLA-E increased expression played an important role in NK/MSC interaction. By contrast, even if increased after IFN-γ priming, B7-H1 does not play a significant role in MSC protection from activated NK cells.

Furthermore, since a role for NKG2D and NKp30 in the interaction between NK cells and BM-MSC has been shown [10], we investigated whether these molecules were also involved in NK cell-FL- and ES-MSC killing. Blocking NKG2D-mediated recognition of MICA expressed on FL- and ES-MSC with either anti-NKG2D or anti-MICA mAb, we observed a remarkable reduction of unprimed FL- and ES-MSC lysis (Figure 3b). Similar results were obtained blocking the activating receptor NKp30 and the adhesion molecules LFA-1 on NK cells. However, neither NKG2D nor NKp30 appeared to be relevant molecules in recognition of IFN-γ-primed FL- and ES-MSC. Furthermore, blocking LFA1-ICAM1 molecules interaction did not appear to reduce the IFN-primed MSC killing. Altogether, these results show that surface receptor-ligand pairs involved in the recognition and lysis of IFNγ-primed FL- and ES-MSC are different from those relevant in unprimed MSC killing.

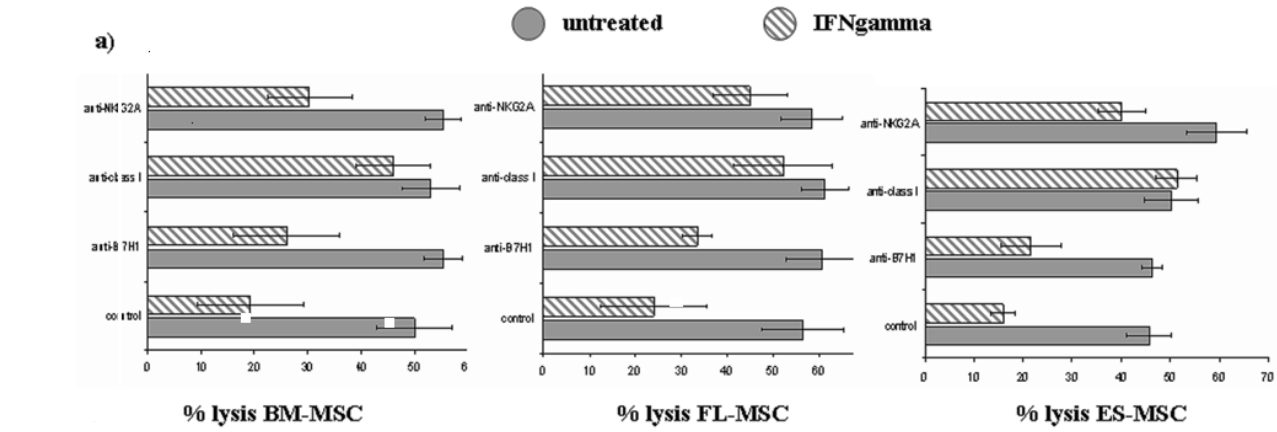

Figure 3a: Involvement of activating and inhibitory receptors in MSC/NK interaction. To determine the impact of class I, HLA-E and B7-H1 molecules, as well as the impact of MICA, CD54, NKp30, NKG2D and LFA1 on MSC killing

IFNγ primed FL-/ES-MSC inhibit NK cytolytic and degranulation abilities

The IL2-induced up-regulation of activating receptor expression in NK cells, the cytolytic granules exocytosis, as well as the NK cell-mediated killing of target cells is usually inhibited after co-culture with MSC [10,14,31]. Purified NK cells cultured with IL-2 in the presence of FL- and ES-MSC for 5 days displayed an increased reduction of cytolytic (Figure 4a) and CD107a expression when incubated with the sensitive target cell K562 (Figure 4b). In addition, secretion of IFN-γ by NK cells (Figure 4c), the ERK signalling, which is critical for cytotoxic granules exocytosis [5] (Figure 4d) as well as the intracellular calcium increase upon NK-K562 interaction (Figure 1, supplementary data) were also inhibited. IFN-γ primed BM-MSC and ES-MSC better inhibited NK cell functions compared to unprimed MSC, whereas IFN-γ primed FL-MSC did not always share enhanced suppressive properties compared to unprimed cells.

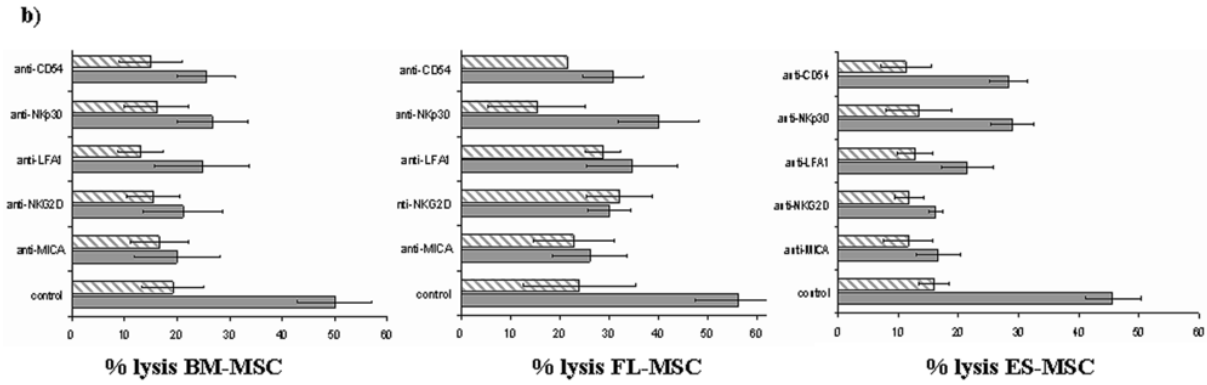

Figure 3b: Involvement of activating and inhibitory receptors in MSC/NK interaction.IL2-activated NK cells were incubated with specific blocking mAb or with an isotype matched control mAb, 30min prior to co-incubation with MSC, in a non-radioactive cytotoxicity assay. One representative out of 6 independent experiments is presented

Intriguingly, while IFNγ-primed FL-MSC better down-regulate activating receptors expression on NK cells, they do not seem to further inhibit **intracellular** calcium release compared to unprimed cells. As shown in the supplementary data, Figure 2, we also found that FL- and ES-MSC strongly impaired on NK cells the expression of activating receptors as NKp30, NKp44 and NKG2D as well as of the adhesion molecule LFA-1. A slight decrease of CD16 was also observed. By contrast, IFN-γ priming only ameliorates the down-regulation of NKp30, since NKG2D was further decreased only in FL-MSC, NKp44 was further decreases only in BM-MSC and ES-MSC, but not FL-MSC, and LFA1 was further decreased in ES-MSC and FL-MSC, but not in BM-MSC. Interestingly NKG2A and CD158a,b expression was not significantly modulated neither by unprimed, nor IFNγ-primed FL- and ES-MSC.

Role of IDO and PGE2 in NK killing impairment

MSC immunosuppressive properties are mediated by cell-to-cell contact and/or soluble factor secretion. Indolamine 2,3-dyoxigenase (IDO) and PGE-2 secreted by MSC have been shown to inhibit NK responses [13,23,31]. In our experiments, IDO expression and PGE-2 secretion by FL- and ES-MSC were strongly increased after IFN-γ stimulation and during NK/MSC co-cultures (Figure 3a and 3c supplementary data). Noteworthy, IFNγ **primed FL- and ES-MSC further increased the expression of IDO and secretion of PGE2 in NK-MSC co-cultures (Figure 3b and 3d supplementary data). To determine the impact of IDO and PGE-2 in NK cell killing, we used specific chemical inhibitors (1-Methyl-tryphtophan and Indomethacin, respectively). Our results show that IDO played a significant role in regulating NK cell killing only in IFN-primed MSC. In contrast, the already significant role of PGE-2 in unprimed MSC is further improved in IFN-primed MSC (overall in BM-MSC and FL-MSC), as shown by the restoration of K562 lysis (Figure 5).**

Conclusions

Among their immune-privileged status due to the expression of class I molecules, MSC are also immunosuppressive cells, developing strategies to evade immune surveillance that include cell-contact and the release of immunosuppressive molecules (e.g. HLA-G, Nitric Oxid and PGE-2) [12,49]. In this study, we have found a high susceptibility of fetal and embryonic MSC to activated NK cell-mediated killing, and this susceptibility was strongly decreased when MSC were pre-activated with IFN-γ for 48 h. The ability of granule exocytosis by NK cells against IFNγ-primed FL- and ES-MSC was also markedly reduced. Although IFNγ stimulation did not significantly modulate "classical" MSC surface markers such as CD90, CD105 and CD73, we observed in MSC a strong increased expression in activating and/or inhibitory ligands that interact with the respective counterpart in activated NK cells.

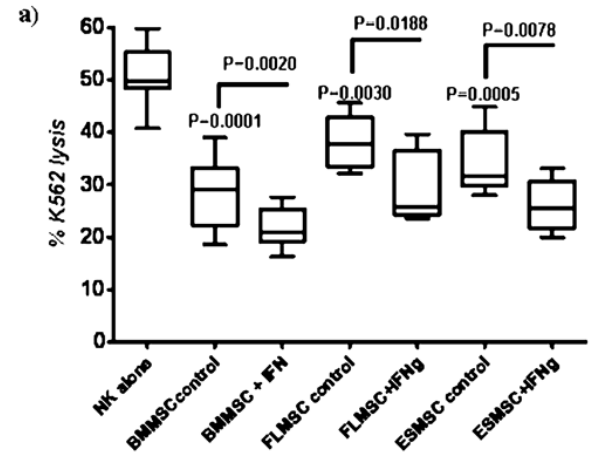

Figure 4a: MSC impair NK cell lytic functions. MSC cultured in the presence or absences of IFN-γ (20ng/ml) for 48h were used as target in a non-radioactive cytotoxicity assay (Promega) with NK effector cells at different ratio NK/MSC. K562 cell line was used as positive control. One representative out of 5 independent experiments is presented. Data are shown as box plots. Each box represents the 25th to 75th percentiles. Lines outside the boxes represent the minimum and maximum values. Lines inside the boxes represent the mean

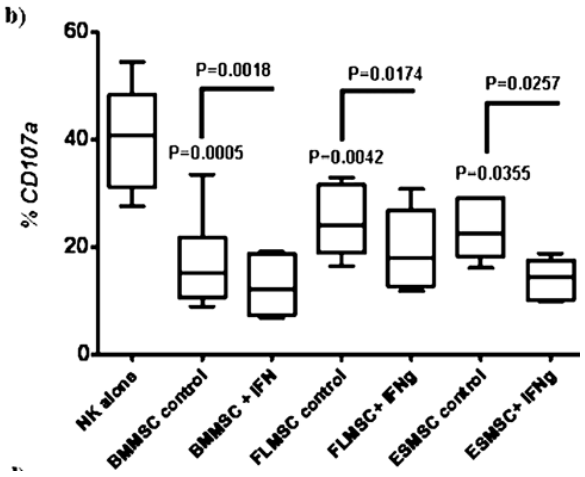

Figure 4b: MSC impair NK cell lytic functions. CD107a degranulation by NK cells against IFN-primed MSC. NK cells were cultured with MSC (ratio NK/MSC, 5:1) for 4h in presence of anti-CD107a antibody and Monensin (2 μM). K562 cell line was used as positive control. One representative out of 5 independent experiments is presented. Data are shown as box plots. Each box represents the 25th to 75th percentiles. Lines outside the boxes represent the minimum and maximum values. Lines inside the boxes represent the mean

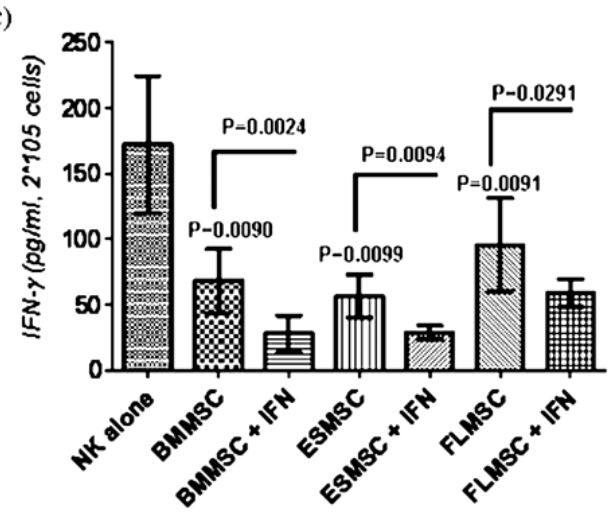

Figure 4c: MSC impair NK cell lytic functions. IFN−γ secretion, K562 cell line was used as positive control. One representative out of 5 independent experiments is presented. Data are shown as box plots. Each box represents the 25th to 75th percentiles. Lines outside the boxes represent the minimum and maximum values. Lines inside the boxes represent the mean

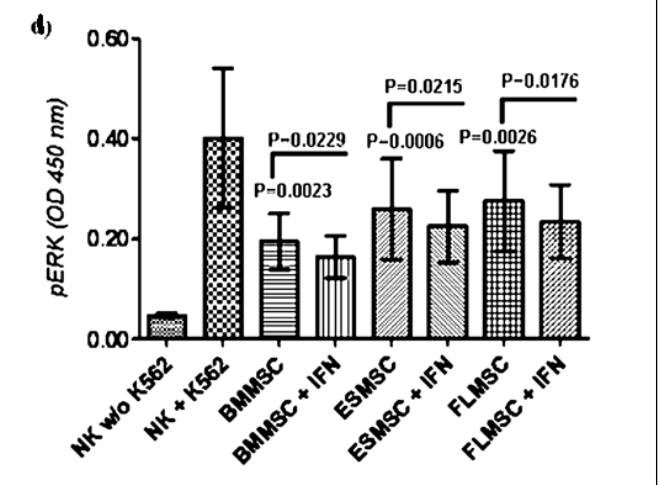

Figure 4d: MSC impair NK cell lytic functions. ERK signalling in NK cells cultured with MSC derived from different tissues. K562 cell line was used as positive control. One representative out of 5 independent experiments is presented. Data are shown as box plots. Each box represents the 25th to 75th percentiles. Lines outside the boxes represent the minimum and maximum values. Lines inside the boxes represent the mean

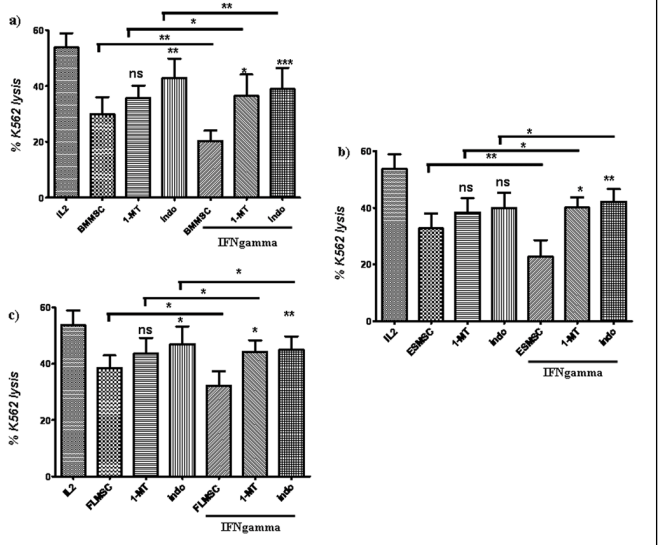

Figure 5: IFN−γ priming enhances immunosuppressive functions of MSC. MSC cultured in the presence of IFN-γ (20 ng/ml) for 48h were used as effectors in co-culture with NK cells (in presence of IL-2) for 5 days. To evaluate the role of IDO and PGE-2, 1-Methyltryptofan and Indomethacin were supplemented in the culture medium. Then NK cells were used as effectors against K562 cells. One representative out of 5 independent experiments is presented

In particular, HLA-ABC and HLA-E molecules were up-regulated in FL- and ES-MSC, besides BM-MSC, as well as CD54 and B7-H1 (CD274 or PDL-1). In contrast, HLA-DR molecules, not constitutively

expressed in any MSC tested, were increased after IFN-γ stimulation mainly in BM-MSC. Interestingly, the NKG2D ligand MICA, that can play a relevant role in NK/MSC interaction [10], was not significantly modulated by IFNγ in all sources of MSC. Since HLA-I play a key role in NK cell-mediated recognition of target cells; we investigated the role of these molecules in the down-regulation of MSC killing induced by IFN-γ-primed FL- and ES-MSC. Our results show that HLA-ABC played a critical role in protecting IFN-γprimed FL- and ES-MSC. This is in line with what was reported from Spaggiari et al. [14] for BM-MSC and Gotherstrom et al. [35] for FL-MSC, respectively. A similar role was played by HLA-E on IFNγ-primed MSC, as the addition of blocking mAbs directed to the CD94/NKG2A, had no effects on the lysis of adult, fetal and embryonic unprimed MSC, but a slight increased killing was observed in IFN-γ-primed MSC. These results suggest that the increased expression of these molecules after IFNγ treatment has a specific role in MSC protection. A still undefined role can be played by B7-H1. Normally, B7-H1 is mainly expressed in monocytes and dendritic cells [50], but in inflammatory conditions is also found on endothelial cells [51] as well as on adult MSC [45,52,53]. Despite its IFN-γ-induced increased expression, the role of B7-H1 was not evident, neither on MSC protection, nor in MSC-mediated inhibition of NK cells (data not shown), suggesting other possible roles on NK/MSC interaction. These results are in line with results shown by Fazekasova et al. [52], where an anti-B7-H1 antibody did not restore CD3+ lymphocytes proliferation after co-culture with placenta and bone marrow MSC pre-activated with IFN-γ. Importantly, our findings show that activating receptors such as NKp30 and NKG2D, together with the adhesive receptor LFA1, were also involved in NK cell-mediated recognition and killing of FL- and ES-MSC. Interestingly, after IFN-γ priming of FL- and ES-MSC the role of these activating receptors was not as evident as in untreated FL- and ES-MSC. Altogether, these findings suggest that inflammation can reduce the sensitivity of FL- and ES-MSC to NK cell mediated killing interfering with the recognition through NKG2D, NKp30 and LFA1, and is associated with increased negative signals mediated through NK cell receptors for HLA-ABC and HLA-E. Since IFN-γ stimulation renders FL- and ES-MSC less susceptible to NK cell-mediated killing, we also investigated the possible consequences on NK cell triggering exerted by IFN-γ-primed FL- and ES-MSC. To this aim, we tested the ability of NK cells harvested from co-cultures with IFN-γ-primed FL- or ES-MSC to be activated upon recognition of the NK sensitive target cell K562. We found that early (calcium increase and ERK activation) and late (degranulation, lysis and cytokine secretion) activating events markedly impaired the interaction between NK cells and K562. Furthermore, this impairment was more evident when NK cells were harvested after incubation with IFN-γ-primed FL- or ES-MSC than untreated MSC.IDO and PGE2 were strongly enhanced in co-cultures between NK cells and IFN-γ-primed FL- and ES-MSC compared to co-cultures with untreated MSC. In addition, PGE2 appeared to be a more relevant factor in determining the impairment of NK cell functions. A specific down-regulation of NK cell activating receptors such as NKp30, NKp44 and NKG2D, with LFA1, was found on NK cells from IFN-γ-primed FL- and ES-MSC, suggesting that the recognition and killing of target cells expressing the counter-ligands for these receptors can be down-regulated as well.

In this study, MSC incubation with IFN-γmimics an inflammatory environment to which these cells can be exposed upon administration for therapeutic purposes as in tissue repair/regeneration. IFN-γ is a pro-inflammatory cytokine largely secreted by T lymphocytes and NK cells and it can modulate MSC immunoregulatory functions [12,42,54]. Indeed, Polchert et al. [46] demonstrated in a mouse model that MSC pre-activated with IFNγ improved their immunosuppressive effect in a GvHD model, where the dose of IFN-γ was critical. Herein, we point out that IFN-γ-primed MSC can actually down-regulate NK cell functions more than untreated MSC suggesting an employment of IFNγ-primed MSC to further reinforce their immunosuppressive effects. Even if bone marrow MSC remain the main source of MSC for clinical approaches, other MSC sources such as Wharton's jelly-derived MSC display a modulated immune-behaviour upon treatment with pro-inflammatory cytokines [23,43]; in addition, it has been reported an enhanced HLA-G-dependent suppressive effect toward NK cells in the presence of IFNγ with Placenta-derived MSC [55]. Our results, indicate that also FL- and ES-MSC can be a suitable source of MSC able of inhibiting NK cell mediated activities and that this inhibition is enhanced with IFN-γ-primed FL- and ES- MSC. This suggests that FL- and ES-MSC can be a good alternative to MSC from other sources at least for their immunosuppression of NK cell mediated innate immunity. Although other pro-inflammatory cytokines such as TNFα and IL1β are currently used to improve MSC functions [43], on an immunological point of view, we think that IFN-γ can be a suitable priming to protect MSC from NK cell killing. The finding that IFN-γ primimg triggers FL- and ES-MSC to improve their suppressive functions against NK cells, demonstrates the key role of IFN-γ in FL- and ES-MSC immuno-behaviour, besides the reported effects on adult MSC. Therefore, since MSC display a markedly heterogeneous response, the possibility to identify the best MSC source and the good priming might permit to the "bad donor" to restore its suppressive conditions. This study suggests that among other fetal/perinatal sources of MSC, fetal liver and embryonic MSC show attractive characteristics as differentiation potential, fast proliferation and immunosuppressive properties, by proposing themselves as good candidates for cell therapy [34,37,39,56].

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