Tumor-Derived Mesenchymal Stem Cells Use Distinct Mechanisms to Block the Activity of Natural Killer Cell Subsets

**Graphical Abstract**

**Highlights**

- Lung-tumor-derived MSCs (T-MSCs) reduce NK cell function and modulate NK phenotype
- T-MSCs are more immunosuppressive than their non-tumor-associated counterparts
- CD56 dim/bright and functional NK cell subsets are differentially modulated by MSCs
- Modulation of NK cell function and phenotype by MSCs occurs mainly through PGE2

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**In Brief**

Galland et al. compare natural killer (NK) cell immunosuppression by mesenchymal stem cells (MSCs) from primary human squamous cell carcinomas and adjacent normal lung tissue. Tumor-associated MSCs exert stronger immunosuppression than normal-tissue-derived MSCs and modulate different NK functions by distinct mechanisms.

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Tumor-Derived Mesenchymal Stem Cells Use Distinct Mechanisms to Block the Activity of Natural Killer Cell Subsets

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SUMMARY

Mesenchymal stem cells (MSCs) display pleiotropic functions, which include secretion of soluble factors with immunosuppressive activity implicated in cancer progression. We compared the immunomodulatory effects on natural killer (NK) cells of paired intra-tumor (T)- and adjacent non-tumor tissue (N)-derived MSCs from patients with squamous cell lung carcinoma (SCC). We observed that T-MSCs were more strongly immunosuppressive than N-MSCs and affected both NK function and phenotype, as defined by CD56 expression. T-MSCs shifted NK cells toward the CD56dim phenotype and differentially modulated CD56bright/dim subset functions. Whereas MSCs affected both degranulation and activating receptor expression in the CD56dim subset, they primarily inhibited interferon-γ production in the CD56bright subset. Pharmacological inhibition of prostaglandin E2 (PGE2) synthesis and, in some MSCs, interleukin-6 (IL-6) activity restored NK function, whereas NK cell stimulation by PGE2 alone mimicked T-MSC-mediated immunosuppression. Our observations provide insight into how stromal responses to cancer dampen NK cell activity in human lung SCC.

INTRODUCTION

Lung cancer is the second most common malignancy and the leading cancer in terms of lethality worldwide. More than 85% of cases fall into the non-small-cell lung cancer (NSCLC) class, which is associated with a predicted 5-year survival of 17.8% and whose predominant histological subtypes are adenocarcinoma (~50%) and squamous cell carcinoma (SCC) (~40%; Chen et al., 2014). The tumor microenvironment provides a wide range of resources that support NSCLC progression (Wood et al., 2014), among which are diverse stromal cells, including activated mesenchymal stem cells (MSCs) (Bussard et al., 2016; Raffaghello and Dazzi, 2015).

Although they were initially described in the bone marrow (BM), MSCs display a broad tissue distribution and are found in adipose, synovial, and lung tissue as well as in umbilical cord and peripheral blood (Williams and Hare, 2011). MSCs are a heterogeneous stromal cell population defined based on three functional and phenotypic criteria: adherence to plastic; expression of selected and lack of lineage-specific cell surface markers; and the capacity to differentiate toward a variety of mesenchymal lineages (Dominici et al., 2006). Among a plethora of effector functions, MSCs have been reported to exert immunosuppressive activity after priming by cytokines from a pro-inflammatory microenvironment, particularly interferon-γ (IFN-γ), tumor necrosis factor α (TNF-α), and interleukin-1β (IL-1β) or through toll-like receptor (TLR) stimulation (Bernardo and Fibbe, 2013; Dumitruc et al., 2014; Krampera, 2011). Following activation, the spectrum of MSC immunosuppressive activity in humans includes secretion of human leukocyte antigen (HLA-G), transforming growth factor β (TGFβ), prostaglandin E2 (PGE2), tumor necrosis factor alpha-inducible protein 6 (TNFAIP6/TSG-6), heme oxygenase 1 (HO-1/HMox1), IL-10, IL-6, indoleamine 2,3-dioxygenase 1 (IDO1), hepatocyte growth factor (HGF), and leukemia inhibitory factor (LIF) as well as programmed death ligand (PD-L1/2) and Fas ligand (FasL) signaling (Poggi and Giuliani, 2016; Poggi et al., 2014; Spaggiari and Morletta, 2013; Stagg and Galipeau, 2013; Turley et al., 2015; Uccelli et al., 2006; Le Blanc and Davies, 2015). The immunosuppressive effects of MSCs require proximity to their target cells, which include T and B lymphocytes as well as natural killer (NK) cells (Aggarwal and Pittenger, 2005; Uccelli et al., 2008).

As a first line of defense against tumors and pathogens, NK cells patrol tissues and can exert antitumor immunosurveillance by secreting cytokines, including IFN-γ, TNF-α, and IL-10, and releasing cytotoxic granules whose contents kill tumor cells. Not surprisingly, NK cell infiltration of tumor tissue correlates with better prognosis in diverse cancer types, including lung carcinomas (Platonova et al., 2011; Villegas et al., 2002), and low NK cell activity is associated with increased cancer risk in adults (Imai et al., 2000). NK cell detection of and response to target cells are regulated by cell surface activating and inhibitory receptors. Activation of NK cells therefore requires integration of signals from their target cells that may be subject to modulation...
by diverse cell types, including MSCs (Moretta et al., 2001; Spaggiari et al., 2008; Vivier et al., 2008).

NK cell function correlates with CD56 expression, where high (CD56 bright) and low (CD56 dim) expression are associated with elevated cytokine production and high degranulation potential, respectively (Caligiuri, 2008; Cooper et al., 2001; Lanier et al., 1986). NK cells are found in the stroma of human lung tumors (Jin et al., 2014), where they primarily display a CD56 dim phenotype, low expression of multiple activating receptors (Platonova et al., 2011; Vitale et al., 2014), and reduced function (Cremer et al., 2012; Gillard-Bocquet et al., 2013; Hodge et al., 2014; Pross and Lotzova, 1993).

Bone-marrow-derived MSCs (BM-MSCs) can inhibit NK cell proliferation, cytotoxicity, and cytokine production by secreting IDO1, TGFβ, HLA-G, and PGE2 (Casado et al., 2013; Krampera et al., 2006; Rasmusson et al., 2003; Spaggiari et al., 2008). However, they can also be lysed by activated NK cells, depending on their expression of activating NK receptor ligands, including MHC class I polypeptide-related sequence (MICA, B), UL16 binding proteins (ULBPs), CD112, and CD155 (Poggi et al., 2005; Spaggiari et al., 2006, 2008). Most of our understanding of the functional MSC-NK cell relationship stems from experiments using normal BM-MSCs, peripheral blood NK cells, and tumor cell lines. In tumors, however, MSCs may become constituents of the tumor niche and display distinct features from those of MSCs derived from healthy tissues or the BM (DelaRosa et al., 2012; Di Trapani et al., 2013; Gottschling et al., 2013; Johann et al., 2010; Liu et al., 2014).

Here, we provide insight into the effect of tumor-associated MSCs on NK cell activity by comparing the immunomodulatory activity toward freshly isolated NK cells from healthy donors of paired samples of MSCs isolated from tumor tissue (T-MSCs) and normal adjacent lung tissue (N-MSCs) of patients with squamous cell lung carcinoma. We observed marked differences between T- and N-MSCs, from their phenotype to their immunosuppressive function. Despite the ability of MSCs to secrete a variety of mediators with immunosuppressive effects, T-MSCs could mediate inhibition of NK cell function primarily through PGE2. Our observations provide insight into how stromal responses to cancer growth blunt NK cell activity in human lung SCC.

**RESULTS**

**Characterization of MSCs Isolated from Patients with Lung SCC**

Stromal cells were isolated from dissociated primary lung SCC (Figure 1A) as well as from adjacent non-tumor tissue from five patients and verified for functional and phenotypic MSC features as defined by the International Society for Cellular Therapy (ISCT) (Dominici et al., 2006). Accordingly, both tumor and

![Figure 1. Clinical Data and MSC Characterization](image-url)

(A) Clinical data related to the five patients. See also Table S1. (B–D) Characterization of N- and T-MSCs from one representative patient. (B) Expression of CD90, CD105, CD73, and lineage (Lin: anti-CD14, -CD20, -CD34, -CD45 antibody cocktail) markers by flow cytometry is shown. Cell passage (p) is shown in brackets. Cells stained with isotype-matched antibodies provided the negative control. (C) Adipogenic (Oil Red O), osteogenic (Von Kossa), and chondrogenic (Alcian blue) MSC differentiation potentials are shown. Cells cultured in standard medium provided the control condition. The scale bar represents 100 μm. (D) H&E, α-SMA, and vimentin IHC stainings are shown. The scale bar represents 50 μm.
A

Day 0

DIRECT

INDIRECT

NK
MSC

Day 4

addition of K562 or PCC

B

DIRECT

C

INDIRECT

with N-MSC
with T-MSC

NK+K562

NK+PCC

NK+K562

NK+PCC

0 50 100 150

% of IFNγ+ NK

D

with N-MSC
with T-MSC

NK+K562

NK+PCC

NK+K562

NK+PCC

0 50 100 150

% of CD107a+ NK

E

IFNγ secretion (pg/ml)

NK
NK+K562
NK+N-MSC
NK+N-MSC+K562
NK+T-MSC
NK+T-MSC+K562

D10
MCC1
MCC2
MCC3
MCC4

F

with N-MSC
with T-MSC

NK+K562

% of IFNγ+CD107a NK

G

DIRECT

D5 NK+K562

+N-MSC3

+T-MSC3

IFNγ (pg/ml)

CD107a

(legend on next page)
tumor-free lung-tissue-derived stromal cells, which we selected for our study, were adherent to plastic under standard culture conditions; expressed comparable levels of CD105, CD73, and CD90 and lacked expression of lineage markers (Figure 1B); and underwent differentiation to osteocytes, adipocytes, and chondrocytes in vitro in response to appropriate stimulation (Figure 1C). Because of the uncertainty as to the distinction between tumor-associated MSCs and myofibroblasts, often termed cancer-associated fibroblasts (CAFs) (Kalluri, 2016), we assessed the expression of the intermediate filament vimentin, a marker of mesenchymal cells, and α-smooth muscle actin (α-SMA), a hallmark of myofibroblasts (Desmoulière et al., 2004) in our stromal cells. Immunohistochemical (IHC) analysis using specific anti-vimentin and anti-α-SMA antibodies revealed that, as expected, the majority of MSCs expressed vimentin but displayed undetectable levels of α-SMA (Figure 1D).

Immunophenotype of Tumor-Infiltrating Cells and Primary Cancer Cell Characterization

Untreated, surgically removed primary lung tumors from five patients with a smoking history were subjected to histological analysis and diagnosed as moderately to poorly differentiated, invasive SCC (TNM pT2-pT3; Figure 1A; Table S1). Immune cell infiltrates were assessed by immunostaining of tissue sections from four patients for CD3, CD4, CD8, CD20, CD68 KP1, Granzyme B, CD56, PD-L1, PD1, and FoxP3 (Table S1) and by flow cytometry of cells from three of the tumors (patients 1, 2, and 5). NK cells constituted less than 1% of the total CD45+ cell infiltrate in all three tumors (0.2% of CD45+ cells for patient 1, 0.59% for patient 2, and 0.55% for patient 5). The relative abundance of T cells (CD4+, CD8+, and FoxP3+ CD25+ T reg cells), B cells, and myeloid cells was variable among the samples, reflecting IHC staining (Figure S1; Table S1).

Primary cancer cells (PCC) from three of the patients (patients 2, 3, and 4) were obtained by culturing single cells from the tumor bulk in ultra-low attachment flasks and in knockout (KO) medium supplemented with growth factors. Cells were selected for their ability to form spheres in culture and tested for tumorigenicity following injection beneath the kidney capsule of NOD-SCID common γ-chain knock out (NSG) mice. We then addressed the potential of the PCC to become NK target cells by assessing their expression of NK activating receptor ligands (Figures S2A and S2B) and HLA class I molecules (Figure S2C) in comparison to that of the K562 leukemia cell line, a classical NK target.

Primary cells from lung SCC displayed lower expression of several NK cell ligands than the K562 cell line, including CD112 (NECTIN2), CD155 (PVR), and ULBP1. In contrast, expression of the NKG2D ligands MICA and MICB was higher than that of other ligands in PCC (Figure S2A) and comparable to their expression in K562 cells (Figure S2B). MICA and MICB are involved in NK cell activation by interacting with NKG2D (Jamieson et al., 2002), on the one hand, and in NK exhaustion upon extended stimulation on the other (Groh et al., 2002; Chretien et al., 2014; Oppenheim et al., 2005). HLA class I expression tended to increase over time in PCC co-cultured with NK cells (Figures S2Ci and S2Cii), which may provide a mechanism of resistance to NK cell killing. Although PCC appeared to display a phenotype that is less prone to induce NK activation than that of K562 cells, they were nevertheless able to stimulate NK cell degranulation at different effectortarget ratios (Figure S2D). Thus, primary lung SCC cells are sensitive to NK-mediated immunosurveillance.

T-MSCs Suppress the NK Cell Response to K562 and Primary SCC Cells More Strongly Than N-MSCs

The effect of T- and N-MSCs on NK cell function was investigated in direct and indirect (Transwell) co-culture conditions (Figure 2A). Freshly isolated NK cells were cultured with T- and N-MSCs for 4 days, following which their activation by tumor cells was assessed in a 4-hr assay. NK cells from different donors displayed variable activation in response to K562 cells and PCC (Figure S3A). Nevertheless, in direct co-culture,
T-MSCs were consistently and significantly more immunosuppressive than N-MSCs toward NK cells exposed to K562 cells, as assessed by IFN-γ production and CD107a (lysosomal-associated membrane protein-1) expression, commonly used as a marker of degranulation (Alter et al., 2004; Figures 2B and S3Bi). Following activation by PCC, production of IFN-γ as well as degranulation were slightly, but not significantly, more reduced in the presence of T-MSCs than in that of N-MSCs (Figures 2B and S3Bi). T-MSCs also exerted stronger suppression of IFN-γ production than of degranulation (Figure 2B).

In indirect co-culture, T-MSCs were less immunosuppressive than in direct co-culture and their inhibition of NK cell IFN-γ production and degranulation was comparable to that of N-MSCs (Figures 2C and S3Bi). In contrast to direct co-culture, NK cell production of IFN-γ in response to PCC was more strongly inhibited by N- than by T-MSCs. Thus, the robust immunosuppressive activity of T-MSCs toward NK cells appeared to be contact dependent and effective in the presence of both K562 cells and PCC. Because the immunosuppressive potential of MSHs has thus far been studied mainly using BM-MSCs, we included one sample isolated from an adult healthy donor as a reference for MSC-mediated NK inhibition. Bone marrow MSCs displayed strong inhibitory activity toward both IFN-γ production and degranulation by NK cells (Figure 2D) that was more closely reminiscent of T- than of N-MSCs.

Inhibition by MSCs (T-MSC > N-MSC) of intracellular IFN-γ production was reflected at the secretory level (Figure 2E, dark bars). Moreover, the inhibition was observed even before NK cell activation by K562 cells and PCC (Figure 2E, light bars). Similar to IFN-γ secretion, TNFα secretion by NK cells after activation by K562 cells was inhibited in the presence of MSCs and more strongly so in that of T- than of N-MSCs. Although the inhibition was less marked when K562 cells were substituted by PCC, the tendency was similar (Figure S3C).

We next interrogated the response to T- and N-MSCs of the different functional NK cell subpopulations, including IFN-γ+CD107a+ double-positive, IFN-γ+CD107a− single-positive, and IFN-γ−CD107a− single-positive cells (Figures 2F, 2G, and S3D). The strongest and most selective inhibition by T-MSCs was observed in the NK double-positive subpopulation. The IFN-γ−CD107a− single-positive subpopulation was equally inhibited by N- and T-MSCs, whereas the IFN-γ+CD107a− single-positive NK subpopulation was virtually unaffected (Figure 2F). Thus, the NK subpopulations that are the most strongly inhibited by MSCs, particularly in direct co-culture, are those capable of secreting IFN-γ (Figures 2F and 2G).

**T-MSCs Downregulate NK-Cell-Activating Receptors and Induce the CD56dim NK Cell Phenotype**

To understand how MSCs downregulate NK cell function, we interrogated NK receptor modulation in the presence of T- and N-MSCs. MSCs (T-MSC > N-MSC) inhibited cell surface expression of the NK receptors Nkp44, Nkp30, NKG2D, DNAM-I, and NKG2A (Figure 3Aii). Differences between the inhibitory effects of N- and T-MSCs were significant for NKG2D, DNAM-I, and NKG2A receptors. Interestingly, their expression was affected only in direct contact with MSCs (Figure 3Aii), with the exception of Nkp44, whose expression was partially inhibited in indirect co-culture (Figure 3Aii).

Surprisingly, we observed marked changes in the CD56bright/dim ratios of NK subpopulations in response to MSCs. In the absence of MSCs and after 4 days of exposure to IL-2, NK cells were predominantly CD56bright. However, an inversion in the CD56bright/dim NK cell ratio was observed in direct co-culture with MSCs (Figures 3Bii and 3C) that was largely contact dependent (Figures 3B and 3C).

**CD56bright and CD56dim NK Subpopulation Function Is Differentially Modulated by MSCs**

In functional assays where NK cells are activated by tumor cells, the presence of MSCs, particularly T-MSCs, also tipped the balance toward the CD56dim NK phenotype in a contact-dependent manner (Figure 4A). Functional comparison revealed that BM-MSCs were more closely related to T-MSCs than to N-MSCs, as demonstrated by their robust induction of the CD56dim NK subset after activation by K562 cells (Figure 4Bi) and PCC (Figure 4Bii).

Interestingly, inhibition of NK cell function exerted by MSCs differed between the CD56dim/bright subpopulations. In control conditions (NK cells cultured with K562 cells alone), the CD56bright NK cell subset (Figure 4C, first line), associated with elevated cytokine production, was dominant. Upon introduction of MSCs (Figure 4C, lines 2 and 3), the bulk NK cell population decreased its cytokine production and degranulation capacity. However, because the presence of MSCs increased the CD56dim subpopulation, which was associated with degranulation, the change in function of the total NK cell population mostly reflected functional inhibition of the CD56dim subset. Thus, MSC-mediated IFN-γ inhibition occurred predominantly in the CD56bright subpopulation, whereas NK degranulation was inhibited by MSCs in the CD56dim subtype. Inhibition of both functions, particularly cytokine production, was largely contact dependent (Figure 4C). The total NK cell population indirectly co-cultured with MSCs was mainly composed of CD56bright cells, prone to cytokine secretion and comparable to NK cells activated by tumor cells without MSCs.

Modulation of NK receptors also differed between the CD56dim and CD56bright NK subsets. Thus, downregulation of Nkp44, Nkp30, NKG2D, and DNAM-I receptor expression occurred in the CD56dim subpopulation, appeared to be highly contact dependent, and was more pronounced in the presence of T-MSCs than in that of N-MSCs (Figure 4D). In indirect co-culture, only Nkp44 receptor expression was mildly inhibited, whereas NKG2D receptor expression was slightly increased. In the CD56bright subset, only NKG2D expression was slightly decreased in direct co-culture with MSCs. With this exception, receptor expression in CD56bright NK cells remained virtually unaffected by direct or indirect co-cultures with MSCs.

Inhibition of NK cell activating receptors (Nkp44, Nkp30, NKG2D, and DNAM-I) may provide the mechanism that underlies MSC-mediated dysfunction of CD56dim NK cells. In contrast, inhibition of cytokine production in the CD56bright subset was not associated with major changes in receptor expression, except for mild NKG2D downregulation in direct co-culture with MSCs (Figure 4D). We therefore interrogated the mechanisms involved...
Figure 3. The NK Cell Phenotype Is Strongly Modulated by Direct Co-culture with N- and T-MSCs

(A and B) NK phenotype as assessed by flow cytometry after direct (i) and indirect (ii) co-cultures with N- or T-MSCs, normalized to control conditions (NK cells alone, horizontal dashed line). Mean values ± SEM are shown. (A) Expression of NK cell receptors is shown. (i) Data from 4 experiments are shown (four NK cell donors; MSC1 [n = 2]; MSC2 [n = 2]; MSC3 [n = 2]; MSC4 [n = 1]; MSC5 [n = 1]). (ii) Results from 2 experiments are shown (two NK cell donors; MSC1 [n = 2]; MSC2 [n = 1]; MSC3 [n = 2]; MSC4 [n = 1]). Data were analyzed using Wilcoxon matched-pairs signed rank test. (B) Percentages of CD56^dim^ and CD56^bright^ NK subpopulations in control conditions (NK cultured alone, green bar) and in co-culture with N- and T-MSCs are shown. Light tones depict the CD56^dim^ NK subset, dark tones the CD56^bright^ subset. (i) Results from 4 experiments are shown (four NK cell donors; MSC1 [n = 6]; MSC2 [n = 2]; MSC3 [n = 6]; MSC4 [n = 2]). (ii) Results from 2 experiments are shown (two NK cell donors; MSC1 [n = 6]; MSC2 [n = 3]; MSC3 [n = 6]; MSC4 [n = 3]). Data were analyzed using Wilcoxon matched-pairs signed rank test. Data were analyzed using Wilcoxon matched-pairs signed rank test. (C) Representative flow smooth contour plots (with outliers) showing CD16 and CD56 expression by NK cells in direct and indirect co-cultures with and without MSCs (single NK cell donor; MSC3).

Expression of Immunosuppressive Mediators in T- and N-MSCs

First, we addressed the expression of genes implicated in immune regulation in T-, N-, and BM-MSCs, including IDO1, IL10, TGFβ1, TNFAIP6 (TSG6), HMOX1, HLAG, IL6, and PTGS2 (COX-2; Figures 5A and S4A). Whereas IDO1, IL10, and HLAG transcripts were almost undetectable at day 0 in culture (data not shown), IL6 and PTGS2 were expressed at variable levels in T- and N-MSCs from different patients (Figures 5A and S4A). Expression of some of these genes differed between BM-MSCs and lung tissue MSCs (Figure S4A). Thus, IL6 expression in BM-MSCs was lower than in most T-MSCs and TNFAIP6 was almost undetectable (Figure S4A). Conversely, TGFβ1 and PTGS2 were more highly expressed in BM-MSCs than in T-MSCs, with the exception of T-MSC1 (Figure S4A). T-MSCs from patients 2 and 5 expressed higher levels of IL6 than their non-tumor-tissue-derived counterparts, whereas PTGS2 expression was comparable in the two MSC subsets (Figure 5A). However, T- and N-MSCs displayed distinct secretion levels of IL-6 and PGE2 after 4 days in culture (even though the statistical values for the latter were not significant; Figure 5B), which could explain, at least in part, the differential impairment by the MSC subsets of NK cell function. Expression of IL6 and PTGS2 by T-MSCs increased over time in culture and was strongly induced by the presence of NK cells, whereas expression of TGFβ1 remained constant (Figure 5C). In co-culture with tumor-activated NK cells, MSCs secreted high levels of IL-6, TGFβ1, and HGF, but of the three cytokines, IL-6 was the most differentially secreted between T- and N-MSCs and more so in the presence of PCC-activated NK cells (Figure S4B). Comparison between BM- and lung tissue MSC secretion of IL-6 and PGE2 (Figure S4C) in direct co-culture with NK cells and following activation by tumor cells revealed lower secretion of IL-6 by BM-MSCs than by T-MSCs but comparable secretion of PGE2 by the two MSC populations. In addition, BM-MSCs bore similarities to lung tissue MSCs for MICA and TLR3 expression but also a distinct profile with higher TLR4 and lower CD274, NECTIN2,
and PVR expression (Figure S4D). MSCs expressed low levels of NK-activating receptor ligands, except for the DNAM-1 ligand PVR (CD155; Figures S4D and S4E).

**IL-6 and PGE2 Are Implicated in T-MSC-Mediated Inhibition of NK Cell Function**

Neutralization of IL-6 with a specific antibody (MAB206) and inhibition of COX-2 using a specific inhibitor (NS-398) were each partially effective in restoring NK function that had been suppressed by MSCs. However, the efficacy of the inhibitors appeared to be highly individual patient MSC dependent. COX-2 inhibition was the most effective in restoring NK cell function, whereas IL-6 had more heterogeneous effects. Analysis of co-culture supernatants demonstrated the level of inhibition reached using the inhibitors (Figure S5A). Interestingly, IL-6 inhibition increased PGE2 secretion (Figure S5Aii).

Three experiments highlighted the heterogeneity of immunomodulatory mechanisms used by primary cells. In experiments using patient 2 MSCs, neutralization of IL-6 with antibody partially restored NK cell function, as measured by IFN-γ expression in response to K562 cells (Figure 6A). Similarly, inhibition of PGE2 synthesis using the COX-2 inhibitor NS-398 resulted in partial reversion of NK loss of IFN-γ expression induced by MSCs (Figure 6A). In contrast, neither inhibitor had a marked effect on NK cell degranulation (Figure S5Bi). Interestingly, rescue of NK cell cytokine expression resulting from inhibiting IL-6 and COX-2 function correlated with restoration of the CD56bright NK cell phenotype (Figures 6Aii and 6Aiii).

Surprisingly, in experiments with patient 5 MSCs (n = 2), addition of anti-IL-6 antibody did not restore NK cell function, whereas COX-2 inhibition partially reversed MSC-mediated blockade of IFN-γ expression and degranulation (Figures 6Bi and S5Ci). Nevertheless, COX-2 and IL-6 inhibition appeared to have a synergistic effect. Partial restoration of both NK cell cytokine production and degranulation by inhibition of MSC-derived immunosuppressive molecules was significantly correlated with an increase in the CD56bright NK cell phenotype (Figures 6Bii, 6Biii, and S5Ci).

We next investigated the mechanisms involved in the restoration of NK function and CD69 and CD56 expression and assessed NK receptor expression. Addition of IL-6 inhibitor partially restored NKG2D expression in NK cells cultured with T-MSCs from patient 2 (Figure 6C). However, anti-IL-6 enhanced the inhibition of NKp30 and DNA-M-I expression induced by T-MSCs. COX-2 inhibition increased NKp30 and NKp44 expression slightly and NKG2D expression more strongly. Neutralization of IL-6 from patient 5 MSCs had the same effect on NKG2D and NKp30 as its neutralization in patient 2 MSCs, increasing and decreasing their expression, respectively (Figure 6Ci). COX-2 inhibition partially restored NKp44, NKG2D, and NKp30 expression that had been downregulated by T-MSCs (Figure 6Ci). Combination of anti-IL-6 and the COX-2 inhibitor was comparable to the effect of the COX-2 inhibitor alone. Hence, COX-2 inhibitor administration to MSCs from both patients was effective in at least partially restoring IFN-γ production and NK receptor (NKG2D, NKp30, and NKp44) expression, whereas anti-IL-6 effects were patient MSC dependent.

We also assessed NK cell expression of CD25 (IL-2Rα) in co-cultures with MSCs, which was expressed by a small percentage of NK cells. In the presence of T-MSCs, CD25 was strongly downregulated, and its expression was partially and completely restored by anti-IL-6 antibody and COX-2 inhibitor, respectively (Figure 6D).

**NK Stimulation with PGE2 Mimics T-MSC-Mediated IFN-γ Immunosuppression**

To confirm the importance of MSC-mediated PGE2 secretion in NK cell inhibition, we stimulated NK cells with PGE2 in the absence of MSCs (donor 14 NK cells) at doses comparable to those secreted by T-MSCs in the presence of NK cells. PGE2 inhibited cytokine production by NK cells stimulated by K562 cells to a level comparable to that observed in T-MSC co-cultures (Figure 7A). In contrast, IL-6 stimulation did not affect NK function nor did it display synergy with PGE2 (Figure 7A). NK cell degranulation was unchanged after stimulation by PGE2, IL-6, or both, suggesting a role for PGE2 predominantly in the inhibition of cytokine production. Moreover, addition of PGE2 to NK cells increased the CD56dim subpopulation, as did the presence of T-MSCs (Figure 7B). Finally, stimulation with PGE2 alone was sufficient to reproduce the downregulation of NK receptors induced by T-MSCs (Figure 7C).

**DISCUSSION**

Using paired samples of primary MSCs isolated from lung SCC and adjacent tumor-free tissue, we showed that tumor-associated MSCs are more strongly immunosuppressive toward NK cells than their tumor-free tissue-derived counterparts, exerting...
stronger inhibition of NK cell IFN-γ secretion and degranulation in response to K562 and primary cancer cells. Functionally, T-MSCs were more closely related to BM-MSCs than N-MSCs. As two key functions of BM-MSCs are to participate in building a hematopoietic stem cell (HSC) niche and to protect HSCs from injury by mediators of inflammation and inflammatory effector cells, they must display potent immunosuppressive properties (Sotiropoulou et al., 2006; Spaggiari and Moretta, 2013; Spaggiari et al., 2008). In quiescent tissues, in the absence of inflammatory stimuli, MSCs may decrease or even temporarily lose their immunosuppressive features. However, in a tumor microenvironment, which mimics tissue repair and contains a vast array of cytokines derived from inflammatory, tumor, and activated stromal cells, MSCs may regain their full immunosuppressive potential and resemble their BM counterparts. The immunosuppressive mechanisms, as illustrated by the type and quantity of immunosuppressive cytokines produced and the level of NK cell receptor ligands expressed, may differ between BM- and T-MSCs, possibly as a function of the type of stimulatory microenvironment to which the cells are exposed.

MSCs, particularly T-MSCs, displayed markedly different degrees of inhibition of functional NK cell subpopulations, predominantly inhibiting NK cell subsets that produce IFN-γ. Because IFN-γ plays a prominent role in tumor rejection by preventing tumor stroma formation and tumor-induced angiogenesis (Zaidi and Merlino, 2011), as well as by activating the immune system, inhibition of its production by T-MSCs may be highly relevant toward facilitating tumor progression. Consistent with this notion, recent observations suggest that the predominant activity of NK cell subsets recruited to lung cancer is IFN-γ production rather than direct cancer cell killing (Carrega and Ferlazzo, 2017).

In addition to their production of IFN-γ, the activating receptor expression profile of NK cells was significantly affected by MSCs, with downregulation of NKP44, NKP30, NKG2D, DNAM-1, and NKG2A. MSCs also induced an inversion in the CD56bright/dim NK cell ratio in favor of the CD56dim phenotype. These effects were more pronounced in response to T-MSCs than to N-MSCs and, just like inhibition of IFN-γ production, were contact dependent. Consistent with these observations, intratumoral NK cells in human lung cancer display low expression of NKP30, CD56, NKG2D, and DNAM-1 (Platonova et al., 2011; Esendagli et al., 2008; Levi et al., 2015).

The observed shift toward the CD56dim NK cell phenotype after exposure to MSCs is consistent with low expression of CD56 by NK cells in vivo (Esendagli et al., 2008; Platonova et al., 2011; Levi et al., 2015) and selective inhibition of the CD56bright subtype rather than expansion of the CD56dim subpopulation (Sotiropoulou et al., 2006). Following IL-2 stimulation, NK cells acquire a CD56bright phenotype, which correlates with an activated state of the cells in vitro. The CD56dim phenotype observed in the presence of MSCs therefore seems to reflect a decrease in NK cell activation, as suggested by our functional assays and by the reduced NK cell expression of IL-2Rα following T-MSC co-culture.

CD56dim and CD56bright NK cell functions were differentially modulated by MSCs. With the exception of NKG2D, T-MSCs downregulated activating receptor expression exclusively in the CD56dim NK cell subset, where they also inhibited degranulation. In contrast, T-MSCs inhibited IFN-γ production in CD56bright NK cells where no obvious receptor expression changes occurred (except for the mild downregulation of NKG2D). Distinct mechanisms may therefore underlie T-MSC-mediated inhibition of cytokine production by CD56bright and degranulation of CD56dim NK cells, with the possible implication of NKG2D in both functions of the two NK subsets.

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**Figure 5. Differential Expression of Immune-Modulating Mediators by T- and N-MSCs**

(A) Relative gene expression of IL6 and PTGS2 by N- and T-MSCs in the basal state (day 0 of co-culture) as assessed by qPCR and shown as a fold change in expression (N-MSC2 expression set as the control condition). Data are representative of four experiments (four NK cell donors; MSC2, 3, and 5), and results show the mean ± SEM. (B) IL-6 and PGE2 secretion (pg/mL) by MSCs after 4 days in culture alone. Results show the mean ± SEM: MSC1, 2, 3, and 5 for IL-6 and MSC2 and 5 for PGE2. Statistical significance was determined by Wilcoxon matched-pairs signed rank test ("*" indicates significance at p < 0.05; "**" p < 0.01). (C) Relative gene expression of IL6, PTGS2, and TGFBI by T-MSCs collected at the indicated time points with and without direct co-culture with NK cells (four NK cell donors; MSC2, 3, and 5). Results show fold change in gene expression compared to T-MSCs at day 0. Statistical significance was determined by 2-way ANOVA with Tukey’s multiple comparisons test.
Figure 6. Targeting T-MSC-Derived Immunosuppressive Mediators Rescues NK Cell Activity
Data were analyzed by flow cytometry, and results show mean values ± SEM.
(A) Results are from one rescue experiment with a single NK cell donor and MSCs from patient 2.

(legend continued on next page)
Marked differences in expression and secretion of IL-6 and PGE2 by N- and T-MSCs suggested that both mediators may be implicated in T-MSC-mediated immunosuppression. Interleukin-6 and COX-2 inhibitors revealed that T-MSCs rely on PGE2 to mediate much of their immunosuppressive effect. Accordingly, addition of COX-2 inhibitor to MSC-NK cell co-cultures partially restored NK cell IFN-γ expression, the proportion of CD56bright cells, and Nkp30, Nkp44, NKGD2D, and IL-2Rα expression. COX-2 inhibition in different experiments and using MSCs from different patients consistently restored NK function, suggesting that PGE2 secretion provides an immunosuppressive mechanism common to MSCs, irrespective of their origin.

**PGE2 Is a Key Mediator of T-MSC-Dependent Immunosuppression**

Bone marrow MSCs have been shown to exert a profound inhibitory effect on NK-cell function, outside of the tumor context, mediated by PGE2, IDO1, and/or TGFβ (Sotiroupolou et al., 2006; Spaggiari and Moretta, 2013; Spaggiari et al., 2008). Our observations suggest that T-MSCs suppress NK cell function primarily by PGE2, as neither TGFβ nor IDO1 were upregulated in T-MSCs. Interestingly, plasma PGE2 levels in NSCLC patients have been found to be elevated compared to healthy subjects (Hidalgo et al., 2002), and high expression of COX-2 in tumor sections of NSCLC has been associated with poor prognosis (Bhooshan et al., 2016; Brown and DuBois, 2004; Khuri et al., 2001). Stimulation of NK cells with PGE2 alone reproduced all of the principal effects of T-MSCs, including inhibition of IFN-γ production, the shift toward the CD56dim phenotype, and down-regulation of NK-cell-activating receptors.

Prostaglandin E2 secretion can occur through an IL-6-dependent mechanism, which has been suggested to provide an anti-inflammatory mediator in arthritis (Bouffi et al., 2010). Abrogation of IL-6 increased PGE2 secretion by MSCs, which may constitute a candidate mechanism to explain why the combined inhibition of both was able to better rescue NK cell function. However, the implication of IL-6 in MSC-mediated NK cell immunomodulation was variable. Whereas in one patient, IL-6 inhibition partially restored NK cell function, the same did not hold true for MSC-inhibited NK cells of another patient. Thus, whereas PGE2 secretion appeared to be a common mediator of immunosuppression by MSCs from different patients, IL-6 seemed to provide a more patient-specific immunosuppressive function. Nevertheless, we identified a novel (possibly context-dependent) role for IL-6 in permitting tumor-subverted MSCs to help establish an immunosuppressive microenvironment that impairs NK cell function.

Taken together, our observations show that, despite the variability of MSCs (including the patient origin of the cells, the stage of the tumor from which they were extracted, and the variable expression of ligands for NK cells) and of donor NK cells (from 14 donors, with variations in the panel of receptors expressed and response to tumor cells), which may influence NK cell activation and the degree of inhibition mediated by MSCs, T-MSCs consistently exert stronger immunosuppression of NK cells than N-MSCs. They also provide a mechanistic explanation for differences between the immunomodulatory potential of T- and N-MSCs and highlight the notion that T-MSCs may largely rely on PGE2 and to a lesser extent on IL-6 to exert their immunosuppressive effects. Selection of immunosuppressive mediators by T-MSCs may be determined by signals derived from the tumor cells or from the microenvironment they condition, which may vary from patient to patient. Efforts to discover drugs that could affect MSC behavior by blocking their inhibitory effects may provide promising options to improve anti-tumor NK cell function.

**EXPERIMENTAL PROCEDURES**

**Primary MSC and Tumor Cell Isolation**

**MSCs**

Fresh primary human tumor samples and adjacent macroscopically normal lung tissue were obtained at surgery from 5 SCC patients at the Centre Hospitalier Universitaire Vaudois (CHUV) (Lausanne) with the approval and according to the guidelines of the Ethics Committee of the Canton de Vaud (project authorization no. 131/12). Patients 1–5 were aged 79, 64, 70, 83, and 74, respectively, at the time of surgery. Patients 1 and 2 were female, and patients 3–5 were male. Pathologic tumor staging varied among patients from T2a to T3 and was performed at the CHUV. T-MSCs and N-MSCs were isolated after tissue disruption (see Supplemental Experimental Procedures) and cultured in Iscove’s Modified Dulbecco’s Medium (IMDM) supplemented with 10% fetal calf serum (FCS) (PAN-Biotech), 1% penicillin/streptomycin (PS) (Gibco), and 10 ng/mL platelet-derived growth factor (PDGF) (Prospect; MSC medium). Cells were used at early passages for all experiments below p 7. MSCs were assessed for differentiation potential (to osteocytic, chondrocytic, and adipocytic lineages) and membrane expression of selected markers (see Supplemental Experimental Procedures). Fresh human BM-MSCs were obtained from a healthy donor (male donor; 78 years old; project authorization no. 131/12) and harvested as described above.

**Tumor Cells**

From 3 out of 5 patient samples, PCCs were isolated and cultured as spheres in low-attachment conditions in IMDM supplemented with 20% knockout serum (Gibco), 1% PS, and 20 ng/mL epidermal growth factor (EGF), fibroblast growth factor (FGF), and LIF (Prospect). PCCs were assessed by flow
Percentages of CD56bright and IFN-γ+ NK cells under conditions described in (A). (ii) Correlation between the percentages of CD56bright and IFN-γ+ NK cells. Results are normalized to 100% as the level of IFN-γ in NK+K562 condition, red). Results are normalized to 100% as the level of IFN-γ production and CD107a expression by NK cells activated by K562 cells in different culture conditions: with IL-6, PGE2, or both or co-cultured with T-MSCs.

Figure 7. NK Stimulation with PGE2 Mimics MSC-Mediated IFN-γ Immunosuppression

Data were assessed by flow cytometry on NK cells from a single donor. T-MSCs used for co-culture were from patient 5. (A) Percentages of IFN-γ+ (black bar) and CD107a+ (gray bar) NK cells after activation by K562 cells in different culture conditions: with IL-6, PGE2, or both or co-cultured with T-MSCs for 4 days (set as the control condition, red). Results are normalized to 100% as the level of IFN-γ production and CD107a expression by NK cells activated by K562 cells (horizontal dashed line). (B) (i) Percentages of CD56bright and CD56dim NK subsets after activation by K562 cells under conditions described in (A). (ii) Correlation between the percentages of CD56bright and IFN-γ+ NK cells. The statistical test used was the Spearman correlation coefficient r, significance determined at p < 0.05, and linear regression line and significance determined with R square value.

(C) Percentage of indicated receptor expression on NK cells alone, stimulated with IL-6, PGE2, or both or co-cultured with T-MSCs.
Medium + EtOH was used as a negative control. NK cells were treated for 4 days.

ELISA

IFN-γ ELISA (Human IFN-γ DuoSet ELISA; no. DY285-05; R&D) was performed on MSC/NK/K562 and MSC/NK/PCC co-culture supernatants, according to the manufacturer’s instructions. Controls for baseline IFN-γ secretion were supernatants from NK cells alone and NK cells cultured with K562 cells. Samples were diluted 2-fold and analyzed in duplicates. Quantification of IL-6 secretion by MScs was performed by ELISA (Human IL-6 DuoSet ELISA; no. DY206-05; R&D) in culture supernatants, according to the manufacturer’s instructions. Samples were diluted 4- to 8-fold. PGE2 was quantified using the Prostaglandin E2 Parameter Assay Kit (no. KGE004B; R&D) according to the manufacturer’s protocol. Samples were diluted 3- to 7-fold.

RNA Extraction, cDNA Synthesis, and qRT-PCR

Total RNA was extracted from MSCs using the RNeasy mini Kit (Qiagen), following standard procedures. cDNA was synthesized by reverse transcription using M-MLV Reverse Transcriptase (Promega) according to the manufacturer’s instructions. qRT-PCR amplification was performed using TaqMan Universal PCR mastermix or SYBR Green mix (Applied Biosystems). Samples were analyzed in triplicates (37.5 ng of cDNA/reaction). Data were analyzed by the 2^(-ΔΔCT) method, normalizing threshold cycles first to housekeeping gene expression (PPIA [protein phosphatase 1; TaqMan probe; Hs99999904_m1], GAPDH [SYBR Green], or TBP [SYBR Green]) and then to controls. Primers were purchased from Microsynth AG (see Supplemental Experimental Procedures).

Statistics

Wilcoxon matched-pairs signed rank test was used for nonparametric data and for comparing two matched groups (N- and T-MSCs). Multiple group analysis was performed by 2-way ANOVA test followed by Tukey’s multiple comparisons test or Dunnett’s multiple comparisons test for nonparametric data. Statistical significance (adjusted p value) of the comparison between N- and T-MSCs for each patient. For correlation analysis, the Spearman test was used with the correlation coefficient r. Figures also showed linear regression line with R² for the goodness of fit. Calculations were performed in Prism 7 (GraphPad Software), p values < 0.05 were considered statistically significant and are denoted by asterisks: "p < 0.05; "*p < 0.01; "**p < 0.001; ns, not significant. Error bars represent the SEM, unless stated otherwise.

ACCESSION NUMBERS


SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, five figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.cellrep.2017.08.089.

AUTHOR CONTRIBUTIONS


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