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Mesenchymal Stem cells from human lung squamous cell carcinoma modulate NK phenotype and function

Thèse de doctorat

MD-PhD

Présentée à la

Faculté de Biologie et de Médecine
de l'Université de Lausanne

par

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Mesenchymal Stem cells from human lung squamous cell carcinoma modulate NK phenotype and function

Lausanne, le 5 octobre 2017

pour Le Doyen
de la Faculté de Biologie et de Médecine

Prof. Pedro Romero

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This thesis is dedicated to Christine Clarke

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"One word frees us of all the weight and pain of life: That word is love."

Sophocles, Oedipus at Colonus

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 of paired intra-tumor (T-) and adjacent non-tumor tissue (N-)-derived MSCs from patients with squamous cell lung carcinoma (SCC) on circulating natural killer (NK) cells from healthy donors. 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 $CD56^{dim}$ phenotype and differentially modulated $CD56^{bright/dim}$ subset functions. Whereas MSCs affected both degranulation and expression of activating receptors in the $CD56^{dim}$ subset, they primarily inhibited interferon-γ production in the $CD56^{bright}$ subset. Pharmacological inhibition of prostaglandin E2 (PGE2) synthesis, and in some MSCs, 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.

RÉSUMÉ

Les cellules mésenchymateuses souches (CMS) sont des cellules du stroma (tissu de soutien), avec des fonctions multiples, dont des propriétés immunosuppressives impliquées dans la progression tumorale et nécessitant la sécrétion de facteurs solubles. Dans ce travail, nous avons comparé les effets immunomodulatoires de CMS humaines isolées de pièces de résection de cancer pulmonaire épidermoïde (T-CMS), avec ceux de CMS issues du tissu pulmonaire non tumoral adjacent (N-CMS), prélevé chez les mêmes patients. Nous nous sommes intéressés à l'effet des CMS sur l'immunité innée, en particulier sur les cellules tueuses naturelles (NK). Nous avons observé que les T-CMS sont plus immunosuppressives que les N-CMS et affectent aussi bien la fonction que le phénotype des NK, y compris leur expression du CD56. Les T-CMS induisent préférentiellement des NK $CD56^{dim}$, et ont des effets différents sur les fonctions des sous-populations $CD56^{dim}$ et $CD56^{bright}$. Les CMS affectent à la fois la degranulation et l'expression des récepteurs activateurs des $CD56^{dim}$, alors qu'elles inhibent principalement la sécrétion d'IFN-γ dans la sous-population $CD56^{bright}$. L'inhibition pharmacologique de la synthèse de la prostaglandine E2 (PGE2), et pour certaines CMS, de l'activité de l'IL-6, est capable de restaurer la fonction des NK, alors que la stimulation par la PGE2 seule reproduit l'effet suppresseur des T-CMS. Nos observations permettent une meilleure compréhension de la réponse stromale et de son caractère immunosuppresseur sur les NK dans le cancer pulmonaire chez l'homme.

ABBREVIATIONS

ADAP: Adhesion and degranulation-promoting adapter protein
ADCC: Antibody-dependent cell-mediated cytotoxicity
Ang: Angiopoietin
APC: Antigen-Presenting Cells
a-SMA: alpha-smooth muscle actin
BAG: Bcl2-associated athanogene
BM: Bone-marrow
CAF: Cancer-Associated Fibroblasts
CBM: CBM signalosome (which consists of the adaptors Carma1, Bcl-10 and MALT1)
CD105/ENG: Endoglin
CD107a: Lysosomal-associated membrane protein-1
CD112/Nectin-2/PVRL2: Poliovirus receptor-related 2
CD11b/ITGAM: Integrin alpha M
CD155/PVR: Poliovirus receptor
CD16: Fc Fragment of IgG Receptor
CD25/IL-2Ra: Interleukin-2 receptor alpha chain
CD45/LCA: Leukocyte common antigen
CD56/NCAM: Neural cell adhesion molecule
CD73/5'-NT: 5'-nucleotidase
CD90/Thy-1: Thy-1 cell surface antigen
COX-2: Cyclo-oxygenase-2
CTLs: Cytotoxic T Lymphocytes
DC: Dendritic Cells
DNAM-1/ CD226: DNAX Accessory Molecule-1
ECM: Extracellular matrix
EGF: Epidermal growth factor
EMT: Epithelial–Mesenchymal Transition
EV: Extracellular vesicles
FasL: Fas ligand
FGF: Fibroblast growth factor

FoxP3: Forkhead box P3

GM-CSF: Granulocyte-macrophage colony-stimulating factor

GvL: Graft-versus-Leukemia

HGF: Hepatocyte Growth Factor

HLA: Human Leukocyte Antigen

HLA-DR: Human Leukocyte Antigen - antigen D Related

HLA-G: Human leukocyte antigen G

HO-1/HMOX1: Heme oxygenase

HSC: Hematopoietic stem cells

HSCT: Hematopoietic Stem Cell Transplantation

ICAM-1: Intercellular Adhesion Molecule 1

IDO: Indoleamine 2,3-dioxygenase

IFN-γ: Interferon-gamma

IGF-1: Insulin-like growth factor-1

IHC: Immunohistochemical

IL-10: Interleukin 10

IL-1β: Interleukin-1b

IL-6: Interleukin 6

ISCT: International Society for Cellular Therapy

ITAM: Immunoreceptor Tyrosine-based Activation Motif

ITIM: Immunoreceptor Tyrosine-based Inhibition Motif

K562: Human immortalised myelogenous leukemia line

KGF: Keratinocyte growth factor

KIR: Killer- cell Immunoglubulin-like Receptors

LIF: Leukemia inhibitory factor

MFI: Mean fluorescence intensity

MHC: Major Histocompatibility Complex

MICA/B: MHC class I chain-related proteins A and B

MIP: Macrophage Inflammatory Proteins

MSC: Mesenchymal Stem Cells

NCR: Natural Cytotoxicity Receptors

NK: Natural Killer Cells

NKG2A/ KLRC1: Killer cell lectin like receptor C1

NKG2D/ KLRK1: NKG2-D-Activating NK Receptor/Killer Cell Lectin Like Receptor K1

NKp30/NCR3: Natural Cytotoxicity Triggering Receptor 3

NKp44/NCR2: Natural Cytotoxicity Triggering Receptor 2

NKp46/NCR1: Natural Cytotoxicity Triggering Receptor 1

NKp80/ KLRF1: Killer Cell Lectin Like Receptor F1

NS-398: COX-2 inhibitor

NSCLC: Non Small-Cell Lung Carcinoma

NSG mouse: NOD scid gamma mouse

PBMC: Peripheral blood mononuclear cells

PCC: Primary cancer cells

PDGF: Platelet-derived growth factor

PD-1/CD279: Programmed cell death protein 1/cluster of differentiation 279

PD-L1/CD274/ B7-H1: Programmed death-ligand 1/cluster of differentiation 274/B7 homolog 1

PGE2: Prostaglandin E2

SCC: Squamous Cell Carcinoma

SDF-1: Stromal cell-derived factor 1 (CXCL12)

TAF: Tumor-associated Fibroblasts

TGFβ: Transforming growth factor beta

Th1: T helper 1 cells

TLR: Toll-like receptor

TME: Tumor microenvironment

TNFα: Tumor necrosis factor alpha

TRAIL: Tumor-necrosis-factor related apoptosis inducing ligand

Treg: Regulatory T cells

TSG-6/TNFAIP6: TNF-stimulated gene 6 protein / Tumor necrosis factor-inducible gene 6 protein

TW: Transwell

ULBP: UL16-binding protein

VCAM-1: Vascular cell adhesion protein 1

VEGF: Vascular endothelial growth factor

1. INTRODUCTION

1.1 General introduction

Cancer progression requires interactions between tumor cells and the host microenvironment, which includes, among others, a variety of stromal and immune cells. The immune system's natural capacity to detect and destroy abnormal cells may prevent the development of many cancers. However, cancer cells are sometimes able to avoid detection and destruction by the immune system. These dual effects of the immune system on developing tumors are referred to as "cancer immunoediting", a process comprised of three phases that are collectively denoted as the three "Es" of cancer immunoediting: Elimination (1), Equilibrium (2), and Escape (3). (1) Native immune effector cells, such as natural killer (NK) cells, can eliminate nascent transformed cells in an initial innate immune response. (2) During tumor growth, tumor cell variants weakly immunogenic and resistant to immune attack are generated. These cells proceed to the equilibrium phase even though the elimination phase continues through immune selection pressure. (3) Tumor progression leads to the release of tumor-derived soluble factors that underlie several mechanisms of immune evasion in the escape phase (Kim et al., 2007).

The tumor microenvironment is composed of a network of diverse cellular and structural constituents whose functions are interdependent and recapitulate a wound healing response: inflammatory and immune cells, vascular cells, and a variety of stromal cells, among which are MSCs and numerous components of the extracellular matrix (ECM) along with their biologically active degradation products. The relative abundance of the different constituents may vary widely among different tumor types and among specimens of the same tumor type in different individuals. Such variation may be important in determining the anti-tumor immune response. However, the resemblance between tumor-associated tissue remodeling and physiologic tissue

repair suggests that the wound healing response that they generate may be a shared feature among divergent solid tumors. Despite variable intensity among individual tumors, it may provide an array of common therapeutic targets. MSCs may provide a particularly relevant therapeutic target within the tumor stroma based on their pleiotropic effects on tumor growth and the immune response. MSCs may both promote and constrain tumor growth, although their net effect appears to be predominantly protumorigenic (Berger et al., 2016; Djouad et al., 2007; Karnoub et al., 2007; Khakoo et al., 2006; Lazennec and Jorgensen, 2008). In addition, they have opposite effects on immune cell modulation, promoting inflammation on the one hand and exhibiting an immunosuppressive profile, which favors tumor progression, on the other. Thus, harnessing MSC plasticity toward the expression of anti-tumorigenic, anti-inflammatory and pro-immunogenic properties may be an attractive therapeutic option. However, it requires in-depth understanding of the functional relationship between tumor cells, MSCs and immune cells and particularly how tumor cells subvert MSCs to function in their favor.

1.2 Lung cancers

Lung cancer is the second most common malignancy and the leading cancer in terms of lethality worldwide (Siegel et al., 2015). The two major forms of lung cancer are non–small-cell lung cancer (NSCLC) and small-cell lung cancer. More than 85% of cases fall into the NSCLC class, which is associated with a predicted 5-year survival of 17.8% (Chen et al., 2014). Non–small-cell lung cancer can be divided into three major histological subtypes: squamous-cell carcinoma (SCC, ~40%), adenocarcinoma (50%), and large-cell lung cancer. Smoking causes all types of lung cancer but is most strongly linked with small-cell lung cancer and squamous-cell carcinoma, whereas adenocarcinoma is the most common type in patients who have never smoked (Herbst et al., 2008). Despite advances in early detection, NSCLC is often diagnosed at

an advanced stage and has a poor prognosis. Treatment depends on the stage of the tumor and includes surgery, radiation therapy, chemotherapy, a variety of targeted therapies (e.g. receptor tyrosine kinase enzyme inhibitors) and immunotherapy — alone or in combination (Peters et al., 2017). However, the 5-year survival for advanced non-surgical stages is poor (about 5-10%). NSCLC that has spread to other parts of the body is often refractory to any currently available form of treatment. Metastatic, or stage IV NSCLC, has a 5-year survival of about 1%. Whereas other cancers such as breast cancer and melanoma have benefited from advances in targeted therapies, such as checkpoint inhibitors, most types of lung cancer have not shown similarly promising results. Nevertheless, other forms of immunotherapy need to be explored as an adjunct to standard therapy, in an effort to reverse immunologic tolerance in the tumor microenvironment (Bremnes et al., 2011).

The tumor microenvironment provides a wide range of resources that support NSCLC progression (Chen et al., 2014; Wood et al., 2014) among which are diverse stromal cells, including activated MSCs (Bussard et al., 2016; Raffaghello and Dazzi, 2015; Shi et al., 2017).

In this work, we used MSCs isolated from human lung squamous cell carcinoma samples and from non-tumor adjacent lung tissue to explore and compare their immunomodulatory potential.

1.3 Mesenchymal Stem Cells

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). They are a heterogeneous stromal cell population and characterization of their sub-populations has been hampered by the lack of specific markers. Their defining properties include: the capacity to differentiate into mesodermal- and non-

mesodermal-derived tissues (multilineage differentiation into osteocytes, adipocytes and chondrocytes) (Dominici et al., 2006), but also into endodermal and neuroectodermal lineages (Williams and Hare, 2011); recurrent surface marker expression patterns, expressing CD105, CD73 and CD90 and lacking CD45, CD34, CD14, CD11b, CD79a, CD19 and HLA-DR (Ma et al., 2014a); low expression levels of MHC class I molecules, and minimal or no MHC class II molecules nor co-stimulatory molecules, such as CD40, CD80, CD86, precluding antigen presenting activity (Fibbe and Noort, 2003); and the capacity to adhere to cell culture plates (Dominici et al., 2006). MSCs can take the morphological appearance of fibroblasts and they are in fact considered to be fibroblast progenitors.

MSCs are believed to play a critical role in tissue repair. Mediators of inflammation and immune cells, together with endothelial cells and fibroblasts, orchestrate changes in the microenvironment that result in the mobilization and differentiation of MSCs into diverse stromal components that replace damaged tissue cells. These MSCs can be tissue-resident or recruited from the bone marrow. However, the mechanisms by which MSCs are mobilized and recruited to damaged sites are not fully understood. In addition, how they survive and differentiate into distinct cell types *in vivo* is still not clear (Shi et al., 2012a). Once MSCs have entered the microenvironment of injured tissues, many factors, including cytokines such as tumor necrosis factor-α (TNF-α), interleukin-1 (IL-1), interferon-γ (IFN-γ), toxins of infectious agents and hypoxia can stimulate the release of an array of growth factors by MSCs, including epidermal growth factor (EGF), fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), transforming growth factor β (TGFβ), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), insulin-like growth factor-1 (IGF-1), angiopoietin-1 (Ang-1), keratinocyte growth factor (KGF) and stromal cell-derived factor-1 (SDF-1). These growth

factors, in turn, promote the activation of fibroblasts, endothelial cells and tissue progenitor cells, which ensure tissue regeneration and repair (Lee et al., 2011; Ma et al., 2014b; Shi et al., 2012a).

In addition to tissue regeneration, MSCs have been reported to exhibit various functions, such as enhancement of angiogenesis, prevention of apoptosis and fibrosis through the secretion of paracrine mediators, and many others summarized in Figure 1.

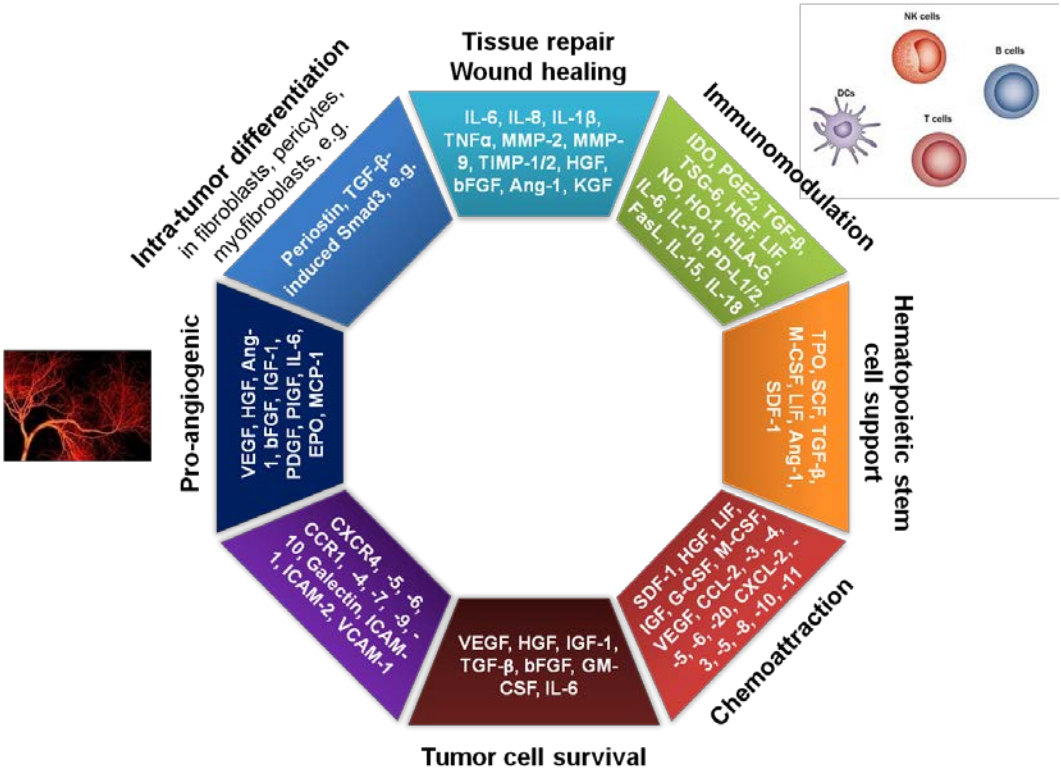


Figure 1: Summary of MSC functions (Bergfeld and DeClerck, 2010; Maumus et al., 2011; Pittenger et al., 1999; Shi et al., 2012b).

Among this plethora of effector functions, MSCs have been reported to exert different immunomodulatory activities after priming by cytokines from a pro-inflammatory microenvironment (inflammation, cancer or infection, e.g.), particularly IFN-γ, TNFα and IL-1β

or through toll-like receptor (TLR) stimulation (Bernardo and Fibbe, 2013; Dumitru et al., 2014; Krampera, 2011).

MSCs express TLR1-6 at mRNA levels, and 2-4 and 7 and 9 also at the protein level. MSC activation through TLR3 and 4 leads to the secretion of IL-1, IL-6, IL-8, TRAIL and CCL5 (Romieu-Mourez et al., 2009). Experimentally, the duration of TLR stimulation on MSCs seems to play a role in the subsequent activation profile developed by MSCs. Short term stimulation with ligands for TLR can induce MSC polarization to MSC1 or MSC2 types. TLR4 stimulation promotes IL-6, IL-8 and TGFβ1 secretion, whereas TLR3 stimulation increases IL-4, IL-1RA, indoleamine 2,3-dioxygenase 1 (IDO), and prostaglandin E2 (PGE2) (Giuliani et al., 2014; Krampera, 2011; Waterman et al., 2010). Thus, TLR4-primed MSCs are polarized into pro-inflammatory MSC1 phenotype (tumor-growth inhibition), whereas TLR3-primed MSCs are polarized into the classical immunosuppressive MSC2 phenotype (Waterman et al., 2010, 2012). The level of inflammation is also critical in the polarization of MSCs. MSCs become immunosuppressive only when exposed to sufficiently high levels of pro-inflammatory cytokines (TNFα, IFN-γ) (Li et al., 2012; Ren et al., 2010) and this immune-suppressive phenotype of MSCs (MSC2) is influenced by TLR3 stimulation and late stages of inflammation (Li et al., 2012). In the presence of low levels of TNFα and IFN-γ (early phase of inflammation), MSCs may adopt a pro-inflammatory phenotype (MSC1) and enhance immune responses, in part through TLR4 expressed on their surface (Waterman et al., 2010). IFN-γ, TNFα and/or TLR3 are able to increase MSC production of PGE2 and IDO. Some studies showed that both TLR3 and 4 enhance immunosuppression through IDO (Opitz et al., 2009); and others showed increase of proinflammatory cytokines in both (Romieu-Mourez et al., 2009).

Thus, MSC licensing to become activated cells depends on activation by proinflammatory cytokines, on priming stimuli such as TLR, and on timing of MSC engagement in the activation process of immune effector cells (Krampera, 2011).

The immunosuppressive effects of MSCs require proximity to their target cells, which include T and B lymphocytes as well as NK cells, and are mediated by secretion of soluble factors and cell-to-cell contact (Figure 2). Activated/primed MSCs upregulate MHC class I molecules, ICAM-1 and VCAM-1 adhesion molecules, and the immunosuppressive molecule PD-L1. These latter three molecules recognize ligands on immune cells, promote cell-to-cell adhesion and then expose immune cells to secreted immunosuppressive molecules (Ren et al., 2010).

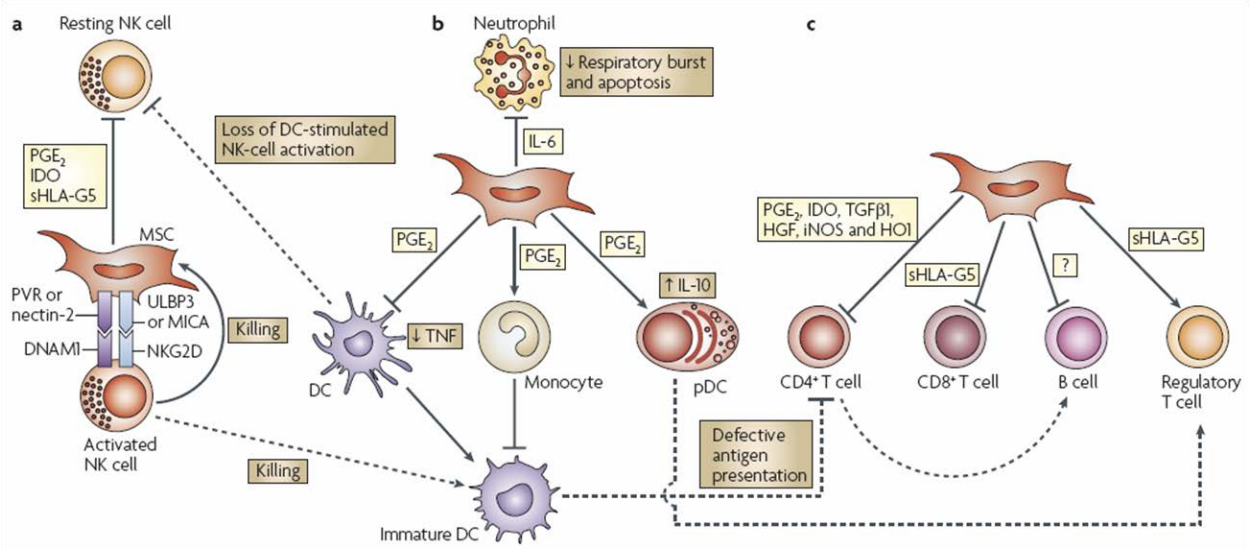


Figure 2: Possible interaction mechanisms between MSCs and cells of the innate and adaptive immune systems (Uccelli et al., 2008).

Following activation, the spectrum of MSC immunosuppressive activity in humans includes secretion of human leukocyte antigen (HLA-G), TGFβ, PGE2, tumor necrosis factor-inducible protein 6 (TSG-6), heme oxygenase 1 (HO-1), IL-10, IL-6, IDO1, HGF and leukemia inhibitory factor (LIF) as well as programmed death ligand (PD-L1/2) and Fas Ligand (FasL) signaling

(Kansy et al., 2014; Krampera et al., 2013; Ma et al., 2014; Poggi and Giuliani, 2016; Poggi et al., 2014; Spaggiari and Moretta, 2013; Stagg and Galipeau, 2013; Turley et al., 2015; Uccelli et al., 2006; Wang et al., 2014; Le Blanc and Davies, 2015).

Moreover, thanks to their immunomodulatory properties and their role in tissue repair, MSCs have held promise, from animal models to clinical trials, in the treatment of numerous connective tissue and immune disorders (Figure 3). Indeed, from a therapeutic perspective, and facilitated by the ease of preparation and immunologic privilege (HLA-I and II negative), MSCs are emerging as a promising therapeutic agent for tissue regeneration (Williams and Hare, 2011).

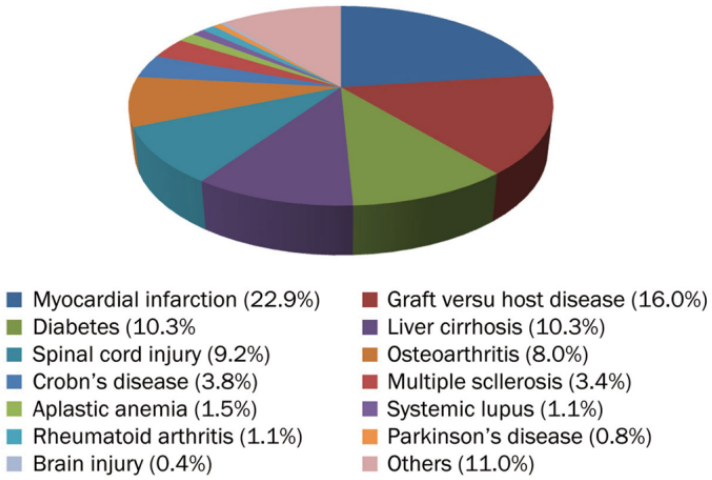


Figure 3: Percentages of the common diseases now treated with mesenchymal stem cells (Wei et al., 2013).

1.4 Mesenchymal Stem Cells and Cancer

Accumulating evidence suggests that MSCs have the ability to migrate and engraft into tumor sites (Kidd et al., 2009) and MSC mobilization has been observed with almost all tested types of solid cancer, including lung cancer (Loebinger et al., 2009).

MSC modulation of tumor growth and immune cells: In the tumor, MSCs can exert stimulatory (Beckermann et al., 2008) or inhibitory (Nomoto-Kojima et al., 2011) effects on cancer cell

growth, invasion, and metastasis through direct or indirect interactions with tumor cells (Karnoub et al., 2007; Liu et al., 2011). Thus, although MSCs can have opposing effects on tumor growth, their net effect seems to be protumorigenic and reflects the imbalance between pro- and antitumorigenic activities that can vary depending on tumor type (and regionally within the tumors), the ecology of the host milieu and the stage in the evolution of a particular tumor. Moreover, MSCs could contribute to tumor development through the modulation of immune surveillance.

MSCs deposit ECM, which constitutes a physical barrier to immune cell infiltration but also provides an essential substratum to immune cell migration (Puré and Lo, 2016). MSCs also secrete IL-1β and IL-6 that regulate the desmoplastic response in tumors, which may limit infiltration and regulate the function of infiltrating leukocytes, and affect T cell proliferation (Hong et al., 2014). Recently, Ridge et al. proposed a comprehensive review of the role of MSCs in tumor progression (Ridge et al., 2017). As mentioned above, MSCs modulate the immune response through secreted immunomodulatory molecules but also by cell-to-cell contact. Moreover, several studies highlight the role of exosomes and other extracellular vesicles (EVs) on immune modulation induced by MSCs as they could be potential carriers of PGE2, TGFβ, and IDO (Burrello et al., 2016; Di Trapani et al., 2016). Exosomes are EVs of 30–100 nm of diameter secreted by different types of living cells, among which MSCs and tumor cells. These EVs can carry a plethora of molecules, including a variety of receptors, adhesion molecules, cytokines, chemokines, as well as cell-specific antigens. Moreover, they are also enriched in a range of nucleic acids (Robbins and Morelli, 2014; Yáñez-Mó et al., 2015). The immunomodulatory effects of MSCs can therefore be speculated to be achieved either through the release of the above mentioned factors and proteins directly into the extracellular milieu as

soluble molecules, or they may be packaged into EVs together with nucleic acids and other post-transcriptional modulators which could influence the inflammatory response when released (Robbins and Morelli, 2014).

Despite these pro-tumorigenic effects, MSCs have also been proposed as an attractive candidate for delivery of anti-tumor agents, based on their ability to home to tumor sites and to secrete cytokines (Seo et al., 2011).

Effect of the tumor microenvironment on MSC phenotype: The tumor microenvironment exhibits the molecular characteristics of a “wound that never heals” that continuously releases various cytokines and other mediators, which establish a persistent stalemate between inflammation and repair in the tissue (Dvorak, 1986). Tumor-derived signals have the capacity to modulate the phenotype of tissue resident and tumor-attracted MSCs (T-MSCs) that become constituents of the tumor niche and harbor distinct features from those of healthy tissue MSCs (N-MSCs) or bone marrow MSCs (BM-MSCs) (Sun et al., 2014) (Figure 4). Differences between non-tumor associated MSCs and T-MSCs are probably mainly caused by the cytokines present in the inflammatory tumor microenvironment. For example, MSCs treated with IFN-γ and TNFα have been shown to upregulate TGFβ and VEGF expression (Jing et al., 2012; Liu et al., 2011). TGFβ could then promote epithelial–mesenchymal transition (EMT) and metastasis (Jing et al., 2012). In addition, IFN-γ or TNFα can also enhance the immunosuppressive effects of MSCs (Ren et al., 2012).

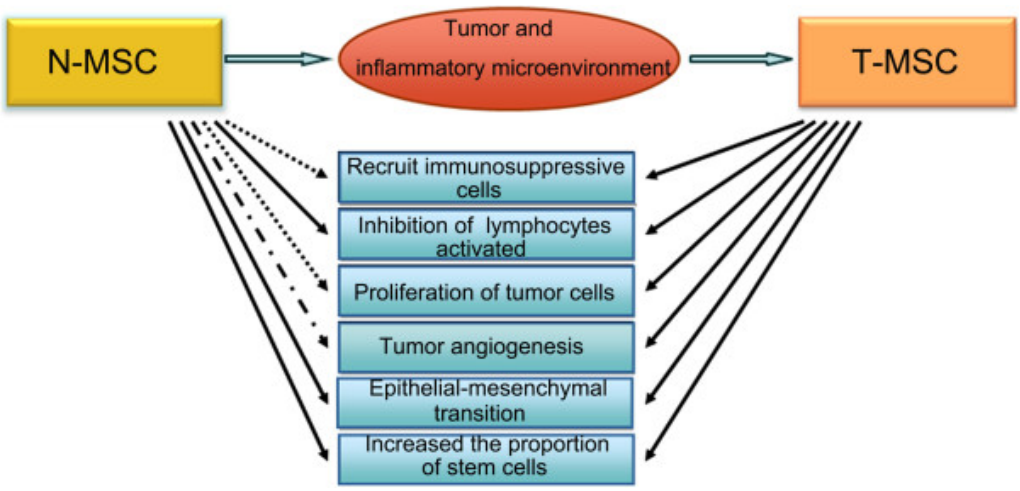


Figure 4: Different effects of non-tumor associated MSCs (N-MSCs) and Tumor-derived MSCs (T-MSCs) on tumors. N-MSCs are "educated" by the tumor inflammatory microenvironment after homing to tumor tissues, and their transformation to T-MSCs exerts different effects on tumor development. ⟶ Promote ⋯▶ Inhibit - - ▶ Controversial. Taken from Sun et al., 2014.

Another component of the tumor microenvironment that could participate in the modulation of MSC phenotype are exosomes. Exosomes derived from breast and ovarian cancer cells can cause adipose-derived MSCs to adopt Tumor-associated Fibroblasts (TAF) phenotypes, with upregulated α-SMA expression. Additionally, they can promote MSC expression of the tumor-associated factors SDF-1, VEGF, CCL5, and TGFβ (Cho et al., 2012; Dostert et al., 2017)

The characteristics of T-MSCs: MSCs in tumor tissues are significantly affected by both tumor cells and the chronically inflammatory tumor microenvironment. MSCs are non-neoplastic and chromosomally normal in solid tumors (Brune et al., 2011; McLean et al., 2011). Moreover, MSCs are more prevalent in tumor tissues than in adjacent normal tissues (Hernanda et al., 2013) and exhibit a significantly greater proliferative capacity (Ding et al., 2012; Gottschling et al., 2013; Johann et al., 2010; McLean et al., 2011; Xu et al., 2011). In addition, T-MSCs exhibit stronger migratory capabilities than non-tumor MSCs (Xu et al., 2011) and exhibit strong drug

resistance. Gottschling et al. performed a comparative molecular and functional analysis of MSCs derived from non-small cell lung cancer and corresponding normal lung tissue (Gottschling et al., 2013). NSCLC-MSCs showed accelerated growth kinetics and reduced sensitivity to cisplatin, as well as an altered gene expression profile (Gottschling et al., 2013). T-MSCs also exhibit stronger immnosuppressive activity compared to BM-MSCs (Johann et al., 2010; Razmkhah et al., 2011; Ren et al., 2012). Finally, T-MSCs have been shown to promote tumor cell proliferation (McLean et al., 2011) and have the ability to increase the proportion of cancer stem cells (Luo et al., 2014; McLean et al., 2011) (Figure 4).

In this work, we focused on the immunomodulatory functions of lung tumor-associated MSCs in comparison with paired non-tumor associated MSCs from the same patients, by studying their modulation of NK cell function. In particular, we focused on the role of PGE2 and IL-6 secreted by lung MSCs in MSC-NK interactions.

1.5 Natural Killer Cells

NK cells are involved in both the adaptive and innate immune responses. They have the intrinsic ability to kill their cellular targets without prior sensitization and without MHC (Major histocompatibility complex) restriction, and to recognize virus-infected cells. They are effector lymphocytes of the innate immune system that control several types of tumors and microbial infections by limiting their spread and subsequent tissue damage. They were first described in mice in the beginning of the 1970s as a new lymphocyte population: large granular lymphocytes of innate immunity. Recent evidence highlights a role for NK cells in “memory” adaptive immunity (Sun and Lanier, 2009). NK cells represent 5 to 20% of circulating lymphocytes and are widely present in tissues, particularly in secondary lymphoid tissues (lymph node, spleen,

tonsils) and in inflamed tissues (Grégoire et al., 2007). Natural killer cells are characterized by the expression of CD56 and the absence of CD3. According to the level of CD56 expression, they are subdivided into two functional subpopulations: $CD56^{bright}$ and $CD56^{dim}$. $CD56^{bright}$ and $CD56^{dim}$ expressions are associated with elevated cytokine production and high degranulation potential, respectively (Caligiuri, 2008; Cooper et al., 2001b, 2001a; Lanier et al., 1986). The $CD56^{bright}$ population produces immunomodulatory cytokines, including IFN-γ (Cooper et al., 2001b). They are considered to be a less mature $CD56^{dim}$ precursor. $CD56^{dim}$ is the terminally differentiated NK subset and is primarily responsible for exerting cytolytic functions (Chan et al., 2007). However, $CD56^{dim}$ NK cells can also produce cytokines, specifically IFN-γ, after triggering *via* NKp46 and NKp30 activating receptors or after stimulation with combinations of IL-2, IL-12 and IL-15 (De Maria and Moretta, 2011). CD56 is also expressed by a T cell subtype and some cancers cells (Lanier et al., 1989). Its role on NK cell function is still unknown. NK cells, in particular the $CD56^{dim}$ subset, also express the low-affinity receptor for the Fc portion of IgG CD16 (FcγRIIIA), whose expression is not restricted to these cells, and which is involved in antibody dependent cell cytotoxicity (ADCC).

Distribution of $CD56^{dim}$ and $CD56^{bright}$ subsets is different according to the organ. In the blood, the $CD56^{dim}$ subpopulation is dominant and represents 90% of NK cells. NK cells do not undergo sustained proliferation *in vitro*, with differences between CD56 subtypes. $CD56^{bright}$ cells express the high affinity IL-2 receptor (IL2Rαβγ) and expand 10-fold more than $CD56^{dim}$ subset upon low-doses of IL-2. $CD56^{dim}$ cells express the intermediate affinity IL-2 receptor (IL2Rβγ) and do not respond to IL-2 stimulation by increasing their proliferation but rather display enhanced cytotoxicity (Caligiuri et al., 1993; Henney et al., 1981).

NK cells are able to lyse target cells through two main cell-to-cell contact dependent mechanisms: ADCC and the Natural Cytotoxicity. NK cells can also indirectly participate in target cell death by secreting pro-inflammatory cytokines (IFN-γ and TNFα).

NK cells are an important source of cytokines and chemokines, with immunomodulatory functions in the immune response and involvement in the control of adaptive immune responses. Secretion of cytokines and chemokines is highly regulated in NK cells and is dependent on the origin of the stimulation: monokine-dependent (IL-12, IL-15 and IL-18 e.g.) or target-dependent.

IFN-γ: IFN-γ is the most abundant cytokine produced by NK cells. It plays multiple roles in innate and adaptive immune responses against viruses and intracellular bacteria and in tumor control (stimulation of immune response toward cancer cells and enhancement of tumor cell immunogenicity). In this latter process NK cells provide early and important sources of IFN-γ production as they might be the first to recognize developing tumors. IFN-γ secreted at the tumor site augments MHC expression on tumor cells increasing their immunogenicity which in turn can induce tumor cell elimination. NK cells not only operate in early stages of tumor development but also act as helpers in the priming of $CD8^+$ and T helper (Th1) cells by producing IFN-γ (Alshaker and Matalka, 2011; Schoenborn and Wilson, 2007). On the other hand, the pro-tumor functions of IFN-γ involve proliferative and anti-apoptotic signals, as well as escape of the tumor cells from recognition and cytolysis by Cytotoxic T lymphocytes (CTLs) and NK cells. Which face is ultimately displayed may depend on the contexts of tumor specificity, micro environmental factors, and signaling (Zaidi and Merlino, 2011). Other molecules secreted by NK cells include TNFα, TNFβ, GM-CSF, IL-5, IL-8, IL-10, MIP-1α, MIP-1β, and IL-13 (Cooper et al., 2001a).

Trotta et al. have shown that production of IFN-γ is regulated *in vitro* and *in vivo* by the SHIP1 phosphatase whose expression is higher in $CD56^{bright}$ (Trotta et al., 2005). This subset usually requires two signals to produce IFN-γ, and one of them is almost always IL-12. The second signal consists of IL-1, IL-2, IL-15 or IL-18, or engagement of NK activating receptors (CD16 or NKG2D e.g.) (Bryceson et al., 2006). Chemokine production by $CD56^{bright}$ cells is highly dependent on the precise monokine combination used for stimulation. For IFN-γ, optimal stimuli are IL-12 and IL-18 together. Thus, the monokine environment determines the NK response profile. NK cells are also able to secrete cytokines following target cell recognition. Surprisingly, $CD56^{dim}$ NK subtype was reported to be the main producer of IFN-γ following stimulation by K562 cells (Fauriat et al., 2010).

In the present work, we used IL-2 stimulated NK cells and it has been shown that after IL-2 *in vitro* stimulation, $CD56^{bright}$ cells show a higher proliferative potential and cytotoxic activity compared to $CD56^{dim}$ cells (Nagler et al., 1989).

NK cell activation: NK transition from a quiescent state to activation requires the integration of different signals from activating and inhibitory NK receptors which determine whether or not the NK cell will become cytotoxic. Thus the balance between positive and negative signals derived from membrane receptors determines NK activation (Figure 5). The lack of inhibition is not sufficient to induce NK activation and a positive signal is always required. Cytotoxicity is primarily based on granule exocytosis upon formation of an immunological synapse. Granules contain preformed perforin and granzymes. The granule membrane fuses with the plasma membrane, externalizes, and releases the cytotoxic contents. Perforin penetrates the target cell membrane and granzymes (a family of serine proteases) trigger cell apoptosis (Lieberman, 2003). Lining the membrane of the lytic granules is the lysosomal-associated-membrane protein-

1 (LAMP-1, or CD107a). Up-regulation of this molecule on the NK cell membrane is a marker of NK cell degranulation and strongly correlates with both cytokine secretion and NK cell-mediated lysis of target cells (Alter et al., 2004).

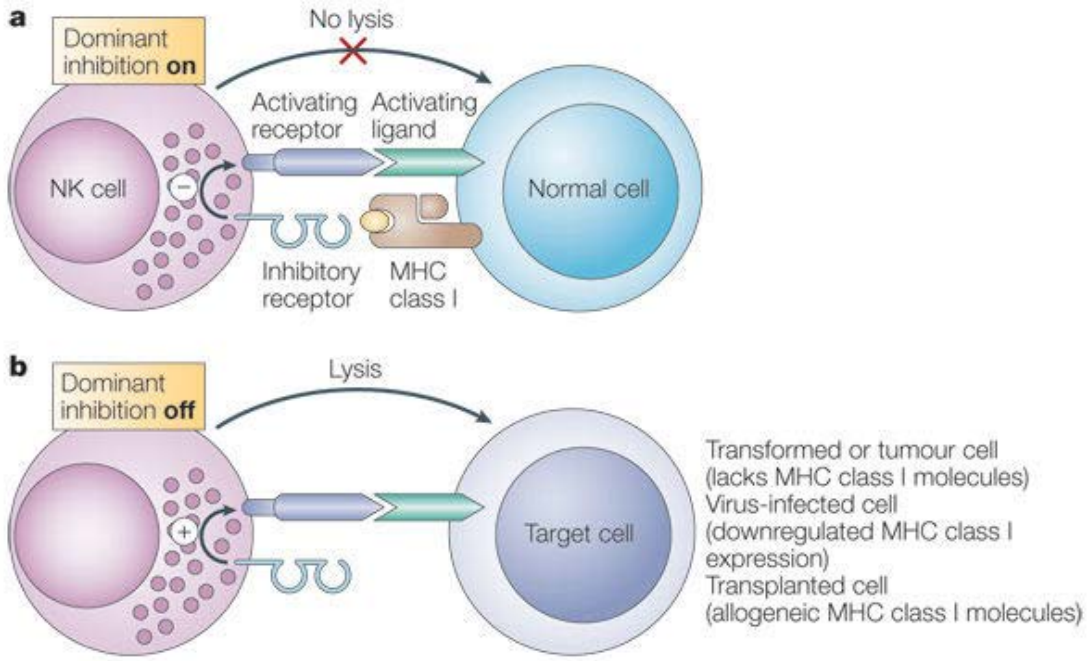


Figure 5: Natural-killer-cell recognition of target cells (Fauci et al., 2005).

Immunological synapse: The immunological synapse was originally defined in the late 1990s (Grakoui et al., 1999; Monks et al., 1998) as the crucial junction between a T cell and an antigen-presenting cell (APC) at which T-cell receptors interact with MHC molecules. Subsequent studies extended these observations and identified relevant immunological synapses between different types of immune cells, as well as between immune cells and non-immune cells. Thus, an immunological synapse can be defined as the intentional arrangement of molecules in an immune cell at the interface with another cell (Orange, 2008). The NK-cell lytic synapse (Figure 6) is formed in a series of stages that are required for cytolytic function. These include recognition/initiation, effector and termination stages that together enable the precise delivery of

lytic-granule contents onto a susceptible target cell (Orange, 2008). Numerous additional functions have been proposed for the immunological synapse: ligand recognition, signal amplification and integration, costimulation, precise targeting of cytotoxicity (McCann et al., 2003), directed secretion (arrangement of molecules at the synapse can create a conduit through which cellular components can be secreted) (Davis et al., 1999; Vyas et al., 2001), protein transfer, inhibition/prevention of activation and signal termination.

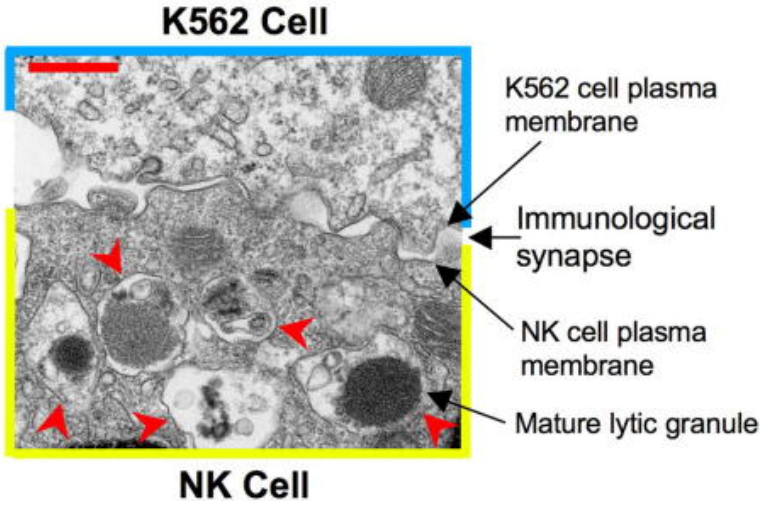


Figure 6: NK cell lytic granules. NK cells contain various related organelles with distinct morphology, including mature lytic granules, lytic granule precursors, multivesicular bodies and late endosomes (see figure – red arrowheads denote lytic granules and their precursory forms). These can polarize to the immunological synapse where their contents can be secreted into a cleft formed between NK and target cell (Orange, 2008).

NK cell activating receptors: Activating receptors induce NK cell activation through combinatorial synergy (Long et al., 2013). Only when the resulting signal from activation of multiple engaged receptors surpasses the activation threshold, do NK cells trigger cytokine secretion or direct cellular cytotoxicity. Activating receptors include NKG2D and the Natural Cytotoxicity Receptors (NCR) NKp30, NKp44 and NKp46 (Figure 7, in green). The latter is sometimes considered as a co-stimulatory receptor (Figure 7, in yellow). Other molecules,

formerly considered as co-receptors (Figure 7, in yellow), participate in the activation of NK cell cytotoxicity: the DNAX accessory molecule (DNAM-1, CD226), signaling lymphocytic activation molecule (SLAM) family, molecule 2B4 (CD244) and NKp80. CD16 is also an activating receptor and exerts a direct cytotoxic function through the binding of appropriate ligands on target cells (Mandelboim et al., 1999).

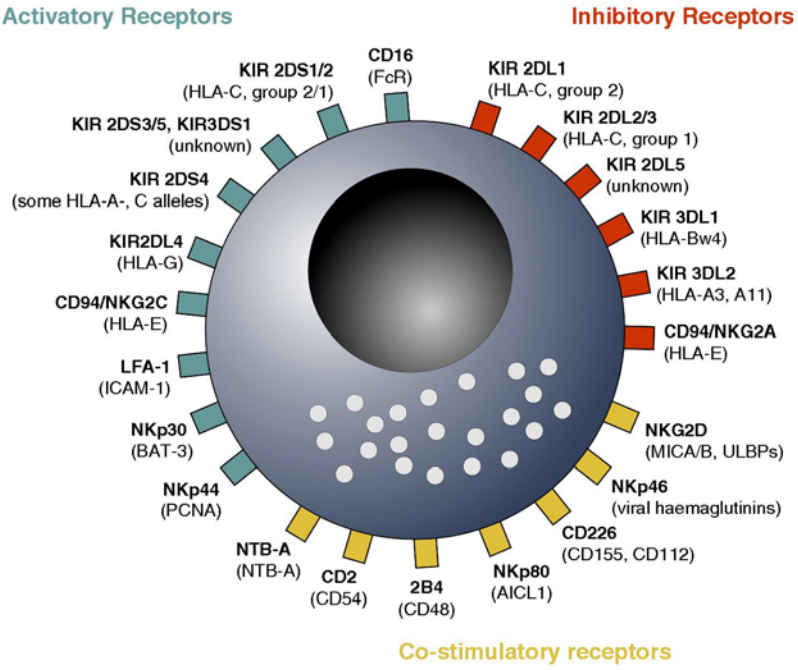


Figure 7: NK cell surface receptors and their ligands. The known ligands are reported in brackets. Other families of receptors are not shown, including cytokine receptors, chemotactic, adhesion receptors, and inhibitory co-receptors (Handgretinger et al., 2016).

NKG2D is one of the best characterized activating NK cell receptors that works both as activating and co-stimulatory receptor. Ligands of the human NKG2D receptor are the MHC I-related molecules MICA/MICB, and the UL16-binding proteins (ULBP-1 to ULBP-6). These ligands are rarely expressed (except for MICA) in healthy tissues but induced by various cellular stresses, such as tumor cell transformation. Although primary tumors frequently express NKG2D

ligands, they also develop mechanisms for NK cell evasion, for example systemic release of NKG2D ligands by tumors, which causes downregulation of NK cell-expressed NKG2D (Groh et al., 2002). Constitutive expression of ligands can also induce the downregulation of NKG2D receptor leading to reduced immune surveillance (Oppenheim et al., 2005). NKG2D expression can be positively or negatively regulated by cytokines: it can be induced by IL-2, IL-12, IL-15, IL-18, TNFα, and IFNα, and diminished by TGFβ (Castriconi et al., 2003).

NKp30 and NKp46 are constitutively expressed on NK cells, whereas NKp44 is only expressed on activated NK cells. NKp46 (NCR1) is involved in tumor eradication but also in the process of tumor immunoediting (Elboim et al., 2010). Potential ligands of NKp46 on tumor cells are yet unknown, except for the hemagglutinin antigens (HA) expressed on virus-infected cells. NKp44 (NCR2) binds to viral hemagglutinins for the recognition of infected cells and to tumor cells in a heparan sulfate proteoglycan (HSPG)-dependent manner (Hershkovitz et al., 2007). In addition, NKp44 receptor recognizes Proliferating Cell Nuclear Antigen (PCNA), which is overexpressed in cancer cells. Their interaction paradoxically inhibits NK cell function and PCNA can promote cancer survival by immune evasion through inhibition of NKp44-mediated NK cell attack (Rosental et al., 2011). NKp30 (NCR3) is involved in NK-mediated recognition and lysis of multiple tumor cell lines and primary tumor cells. NKp30 ligands are poorly defined, except for B7-H6 restricted to malignant tissues (Brandt et al., 2009). Others ligands are BAT3 (BAG6) and pp65. The B7-H6 cell surface molecule can be upregulated upon TLR stimulation of myeloid cells in inflammatory conditions (Pesce et al., 2015). This molecule can be secreted as a soluble form and binds to the NKp30 receptor. Some tumor cells, like K562 or ovarian carcinoma cells, express high levels of surface B7-H6 (Brandt et al., 2009; Pesce et al., 2015). Patients expressing low levels of NKp30 display impaired NK-mediated antitumor cytolytic activity and low IFN-γ

production in response to B7-H6^{+} target cells. Interestingly, the soluble and exosomal form of the NKp30 ligand BAG6 has been described in leukemias. Thus, the chronic receptor-ligand interactions that in the tumor environment induce loss of NKp30 may be due to continuous perturbation by soluble or surface-bound B7-H6 molecules.

DNAM-1 (CD226) is a leukocyte adhesion molecule involved in the induction phase of NK cell activation. Its expression is dependent on the surface expression of lymphocyte function associated-antigen (LFA-1) (Shibuya et al., 1999). The αLβ2 integrin, LFA-1 (CD11a/CD18), is expressed on most leukocytes and mediates cell-cell adhesion upon binding to its ligands, the intercellular adhesion molecules ICAM-1 (CD54), ICAM-2 (CD102), or ICAM-3 (CD50) (Fawcett et al., 1992; Staunton et al., 1989). Upon target cell recognition, the conformational state of LFA-1 changes in educated NK cells and rapid colocalization of active LFA-1 and DNAM-1 occurs at the immunological synapse (Enqvist et al., 2015). CD112 (Nectin-2) and CD155 (PVR: poliovirus receptor), two nectin family proteins regulated by cellular stress, have been identified as the ligands for DNAM-1. They are strongly expressed by tumor cell lines (Bottino et al., 2003). In association with NCR or NKG2D receptors, DNAM-1 is involved in the recognition and lysis of different types of cancer cells. Its role in anti-tumor immunity is more important when ligands for other activating receptors are poorly expressed (Gilfillan et al., 2008). Decreased DNAM-1 expression on NK cells was described in lung carcinoma patients (Platonova et al., 2011a). DNAM-1 is part of an important family of adhesion molecules that bind nectins and nectin-like family proteins (Martinet and Smyth, 2015). These molecules include DNAM-1, T cell immunoreceptor with immunoglobulin and ITIM domains (TIGIT), CD96, and cytotoxic and regulatory T cell molecule (CRTAM). DNAM-1 has been studied as an adhesion molecule that controls NK cell-mediated cytotoxicity. Interestingly, the two other

molecules that contain inhibitory motifs — CD96 and TIGIT — also bind to DNAM-1 ligands and were recently found to counterbalance the DNAM-1-mediated activation of NK cells (Bottino et al., 2003; Chan et al., 2014; Stanietsky et al., 2009).

NK cell inhibitory receptors: As NK cell activation is regulated by classical MHC class I molecules, KIRs (Killer Cell Immunoglobulin-Like Receptors) are the main receptors involved in the recognition of specific HLA-I alleles. KIRs bind to the self-MHC class I ligands (HLA-A, -B, -C) and upon ligation transmit signals that abrogate the effects of activating receptors (Vilches and Parham, 2002) (Figure 5). The KIR superfamily comprises activating (Figure 7, in green) and inhibitory receptors (Figure 7, in red). Inhibitory KIRs are characterized by a long cytoplasmic tail carrying two ITIMs (Immunoreceptor Tyrosine-based Inhibitory Motifs), whereas activating receptors lack ITIMs and have short cytoplasmic tails associated with adaptor molecules containing ITAMs (Immunoreceptor Tyrosine-based Activation Motifs). Each individual has his own repartition of KIR genes, which are highly polymorph. Most NK cells express one or two KIRs with at least one inhibitory receptor specific for self-HLA class I molecule.

Another inhibitory receptor is NKG2A (Figure 7, in red). NKG2A contains two ITIM motifs and forms a heterodimer with CD94 molecule. A natural ligand of CD94/NKG2A heterodimer is HLA-E, a non-classical HLA class I molecule expressed on the cell surface of most leukocytes and on transformed cells. Ligation of CD94/NKG2A by HLA-E transmits inhibitory signaling that suppresses NK cell effector functions, resulting in decreased cytotoxicity and cytokine secretion. In patients with non-small cell lung cancer, intratumoral NK cells display higher gene expression levels of NKG2A relative to non-tumor NK cells (Gillard-Bocquet et al., 2013). Non-classical HLA-E and HLA-G molecules are often overexpressed by tumor cells, participating in

tumor escape through interaction with inhibitory receptors and subsequent inhibition of NK and CTLs. Soluble HLA (sHLA) production has been described as another mechanism of tumor escape.

Interestingly, cytokine secretion and cytotoxicity can be uncoupled when NK cells contact their target cells. Eric Vivier's laboratory has studied NK cell activation and response and the transformation of quantitative differences (strength of stimulation) into qualitative differences (cytotoxicity only *versus* cytotoxicity and cytokine production) (Vivier et al., 2013). His group identified distinct signaling pathways, triggering either cytotoxicity or cytokine and chemokine production. Cytotoxicity triggered by the engagement of cell-surface receptors involves the sequential activation of PI(3)K, Rac1, PAK, MEK1 and MEK2 and finally MAPKs Erk1 and Erk2. The coupling of NKG2D and CD137, or the coupling of several KIR to an intracellular cascade (ADAP and the CARD11-BCL10-MALT1 (CBM) signalosome), exclusively triggers cytokine and chemokine production by NK cells but not cytotoxicity. There are also ADAP-CBM–independent mechanisms that lead to cytokine secretion but not cytotoxicity (as the stimulation mediated by IL-12 and IL-18). The fraction of NK cells that become cytolytic after contact with target cells (cell degranulation) is higher than the fraction of NK cells that produce IFN-γ in the same conditions. ADAP might be activated only above a certain threshold of NK cell activation, whereas triggering of the cytotoxic module might occur at lower strength of stimulation (Vivier et al., 2013).

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).

In this thesis, we looked at the phenotype (receptors expressed) and the function (IFN-γ production and degranulation) of freshly isolated NK cells from healthy donors subjected to IL-2 activation and MSC modulation *in vitro.*

1.6 Natural Killer Cells and Cancer

Generally, tumor cells downregulate MHC class I molecule expression and upregulate ligands for NK cell activating receptors, and thereby overcome the threshold for NK cell activation and become potential targets for NK cell lysis (Figure 8).

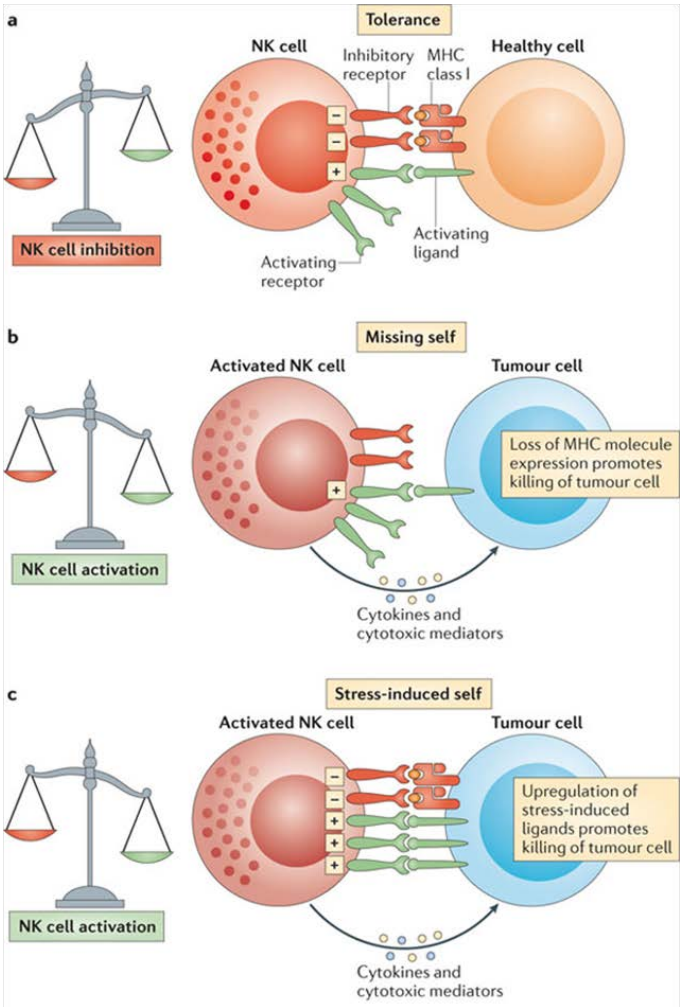


Figure 8: Recognition of tumor cells by NK cells (Vivier et al., 2012).

The major receptors involved in NK-tumor cells recognition are recapitulated in Figure 9. Thus, 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, TNFα, and IL-10, and releasing cytotoxic granules whose contents kill tumor cells. However, tumor cells use direct and indirect mechanisms to escape NK surveillance. Direct mechanisms consist of shedding soluble ligands for NK cell-activating receptors (shed ligands can block tumor cell recognition by NK cells by preventing receptor-ligand interaction (Groh et al., 2002)), upregulation of HLA molecules, and release of inhibitory cytokines. Indirect mechanisms are activation of Treg, DC killing, and phagocyte-derived inhibitory cytokines.

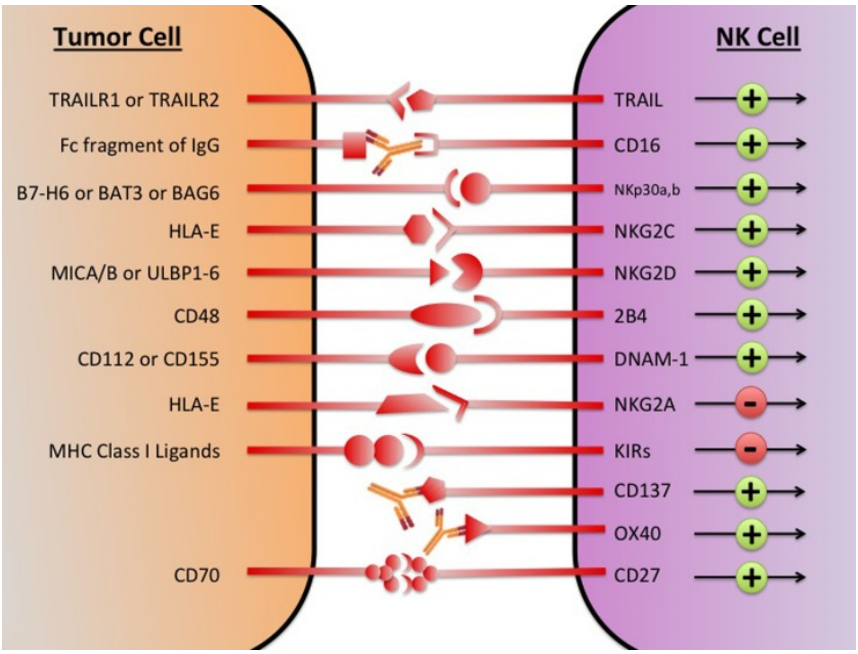


Figure 9: NK receptors and their cellular ligands expressed by tumor cells (Chester et al., 2015).

An 11-year follow-up epidemiologic survey has shown that a low NK cell activity is associated with increased cancer risk in adults (Imai et al., 2000). In lung cancer patients, a low NK cytotoxicity in peripheral blood is associated with a poor prognosis (Cremer et al., 2012;

Platonova et al., 2011a). In selected human malignancies such as acute myeloid leukemia, allogeneic hematopoietic cell transplantations (HSCT) have shown that the development of donor NK cells in recipient tumor patients lacking donor KIR ligands (HLA) can lead to improved engraftment and post-transplant survival (Ruggeri et al., 2006). NK cells are the first lymphocyte population to recover after HSCT and participate in graft-versus-leukemia (GvL) effect (donor NK cells mismatched to the host leukemia HLA trigger cytotoxicity due to a lack of the self-inhibitory signal) (Ruggeri et al., 2006; Velardi, 2008; Velardi et al., 2009).

Other evidence for the role of NK cells in tumor control relies on the observation that NK cells infiltrate diverse solid tumors, amongst others clear-cell renal cell carcinoma, melanoma, non-small cell lung cancer, gastrointestinal stromal tumors (GIST) and colorectal carcinoma (CRC) (Carrega et al., 2008; Delahaye et al., 2011; Kornstein et al., 1987; Platonova et al., 2011a; Schleypen et al., 2003; Villegas et al., 2002; Wittnebel et al., 2007).

NK cells in lung cancer: NK cells are found in the stroma of human lung tumors, especially in squamous cell carcinoma (Jin et al., 2014a) where they primarily display a $CD56^{dim}$ phenotype, low expression of multiple activating receptors (Platonova et al., 2011b; Vitale et al., 2014), and reduced function (Cremer et al., 2012; Gillard-Bocquet et al., 2013; Hodge et al., 2014; Pross and Lotzová, 1993). Platonova et al. showed that intratumoral NK cells displayed a profound alteration of their phenotype, with a drastic reduction of receptor expression level (NKp30, NKp80, CD16, NKG2D, and DNAM-1, among others) (Platonova et al., 2011b). Another group (Esendagli et al., 2008) observed a low expression of CD56, NKG2D, and NKp46 in lung cancer. Low NK cell activity in cancer-diagnosed patients is associated with poor prognosis, and those with advanced stage cancer often possess minimally cytotoxic NK cells (Pross and Lotzová, 1993). In early NSCLC Vitale et al. observed (2014) that the majority of NK cells are

$CD56^{dim}$, with a decrease of NKp30, NKp80, CD16, DNAM-1 and of cytokine production and degranulation (Vitale et al., 2014). Several groups also observed an increase of the NK inhibitory receptors (Jin et al., 2014b).

In lung cancer, the number of NK cell has been shown to be associated with the histology (more NK cell infiltration in SCC than in ADC), the size of primary tumor, the smoking status and, in some cases, with the prognosis (Jin et al., 2014b). Intratumor NK cells are mainly localized at the invasive margin. In NSCLC, NK cells represent 1.7-34.4% of tumor-infiltrating lymphocytes (TIL) and are mainly $CD56^{dim}$ and $NKp46^{+}$, with a decrease in NKp30, NKp80, CD16, DNAM-1, and an increase in CD69 and NKp44. In SCC, 7-10% of TIL are NK cells and 98% are dim. Peripheral blood NK cells are associated with prognosis but not intratumor NK cells, and clinical outcome is more associated with NK phenotype and function than with NK density (Cremer et al., 2012; Platonova et al., 2011b). Others studies have shown an increase in $CD56^{bright}CD16^{-}$ $NKp44^{+}CD69^{+}$ NK subset (CD69 is a marker of residency) (Carrega et al., 2014; Marquardt et al., 2017a), with decrease in cytotoxicity against cancer cells *in vitro*, whereas they release abundant cytokines (Carrega et al., 2008). IFN-γ, TNFα, IL-4 or IL-10 secreted by NK cells can contribute to tumor rejection by modulating tumor stroma and inhibiting tumor-induced angiogenesis (Blankenstein, 2005). Thus, a lower cytolytic activity in NSCLC-NK cells does not necessarily mean an ineffective anticancer activity. NK cells found in the immune infiltrate of NSCLC seem to be different from those associated with the non-tumor lungs. The percentage of bright NK cells in lung tumors remains less than half the total NK population. Generally, the HLA class I allele specific KIR receptors are expressed on subsets of $CD56^{dim}CD16^{+}$ cytolytic NK cells, whereas the immunomodulatory $CD56^{bright}CD16^{-}$ NK subset uniformly expresses CD94/NKG2A and lacks KIRs. Indeed and surprisingly, in NSCLC NK, an evident expression

of KIR was detectable in $CD56^{bright}CD16^{-}$ tumor NK cells, possibly as a consequence of tumor microenvironment-derived signals.

1.7 Natural Killer Cells and Mesenchymal Stem Cells

Recent studies have indicated that activated NK cells, but not freshly isolated resting NK cells, were capable of effectively lysing MSCs *via* their activating receptors (NKp30, NKG2D, and DNAM-1) (Casado et al., 2013; Sotiropoulou et al., 2006a; Spaggiari et al., 2006a, 2008) (Figure 10). Indeed, human BM-MSCs express several ligands for NK cell receptors, such as MICA/B, ULBPs, CD112, CD155 (Poggi et al., 2005; Spaggiari et al., 2006b, 2008), and ICAM-1 (CD54). Lysis of target cells by NK cells requires a number of steps: target cell binding, calcium entry, and release of cytolytic enzymes. Stromal cells are killed by IL-2-activated NK cells upon LFA1-ICAM-1 interactions, and NKG2D-MICA/B and ULBP3 are involved in the delivery of the lethal hit. Among the surface structures involved in effector-target cell interaction, LFA1 plays a key role in cell-to-cell adhesion as well as in delivering an activating signal (Poggi et al., 2005). NK-mediated lysis of MSCs could be inhibited when MSCs are exposed to IFN-γ as a consequence of the up-regulation of HLA class I molecules at the surface of MSC target cells (Spaggiari et al., 2006a). Licensed MSCs exposed to IFN-γ are protected from NK lysis also through the downregulation of ULBP3 ligands and the increase of PGE2 and IDO1 secretion. IFN-γ also increases ICAM-1 expression, which increases physical contacts between NK and their target cells (Chang et al., 2002).

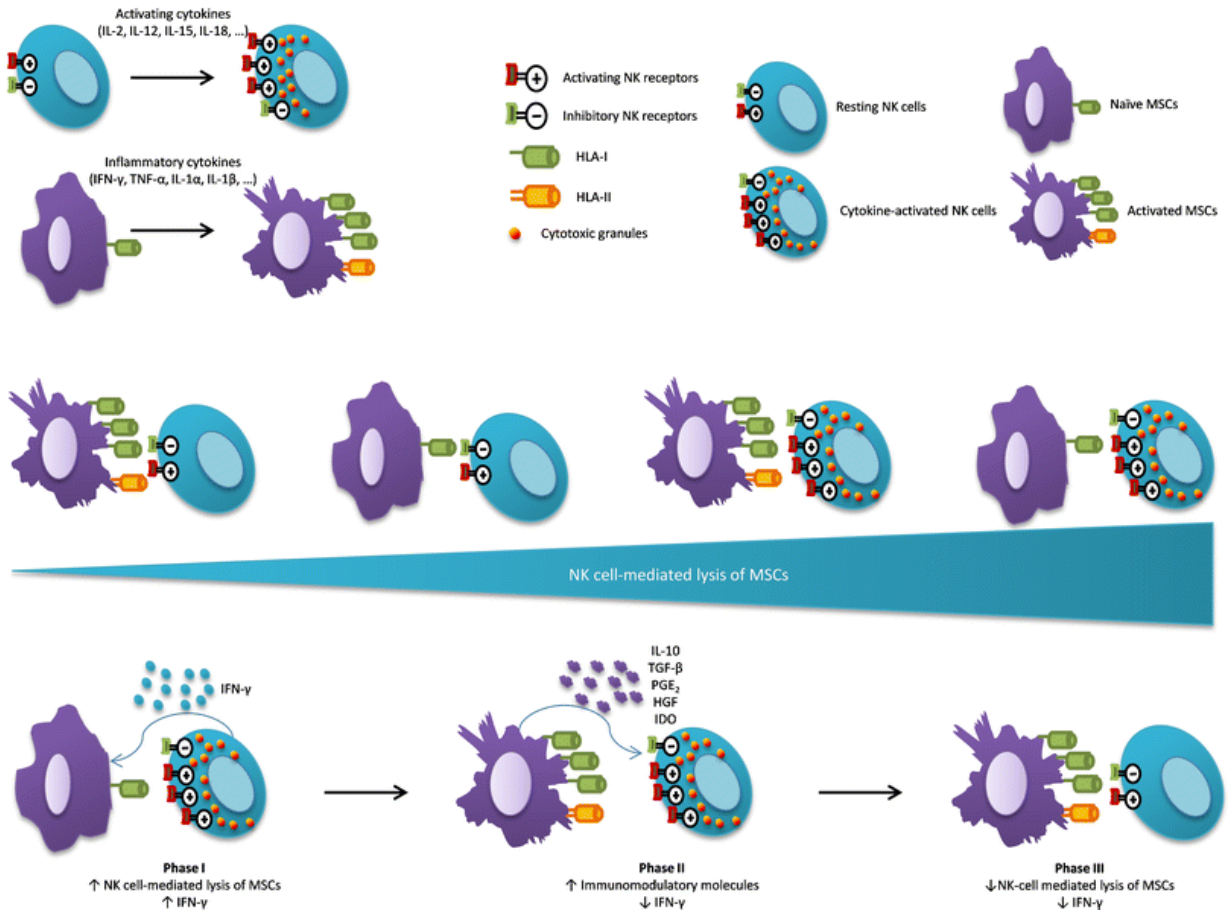


Figure 10: Interplay of NK cells and MSCs in NK cell-mediated lysis and immunomodulatory role on NK cell activity (Casado et al., 2013).

Interaction between MSCs and NK cells are complex and evolve over time. NK cytotoxicity rises when the degree of NK cell activation increases and is inversely correlated with activation of MSCs (Figure 10). Soluble mediators secreted by the two cell types explain, a least in part, this bidirectional exchange. The IFN-γ secreted by activated NK cells may induce the release of immunomodulatory factors from MSCs (e.g. IL-10, TGFβ, PGE2 or HGF) which in turn, inhibit NK cell-mediated cytotoxicity against MSCs (Casado et al., 2013; Tseng et al., 2014).

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).

Freshly versus previously activated NK cells: BM-MSCs alter the phenotype of circulating NK cells and suppress proliferation and cytokine secretion of resting and activated NK cells (Aggarwal and Pittenger, 2005; Sotiropoulou et al., 2006a; Spaggiari et al., 2006a, 2008). BM-MSCs suppress IL-2 and IL-15 induced proliferation and IFN-γ production, but not the cytotoxicity of freshly isolated NK cells (Spaggiari et al., 2008). In contrast, when confronted with previously activated NK cells, MSCs can interfere with NK mediated cytotoxicity, cytokine production, the expression of activating receptors (NKp30, NKp44, NKG2D) and granzyme B release. Their inhibitory effects are primarily mediated by cell-cell contact and secretion of IDO, PGE2, TGFβ1, HLA-G5 and Activin (Chatterjee et al., 2014; Le Blanc and Mougiakakos, 2012; Spaggiari et al., 2008). MSCs cannot be lysed by freshly isolated NK cells, but only by pre activated NK cells (MSCs allo and autogenic to NK cells) (Rasmusson et al., 2003).

Proliferation: Inhibition of NK-cell proliferation is mostly mediated by soluble factors (Sotiropoulou et al., 2006a; Spaggiari and Moretta, 2013) such as IDO and PGE2. Krampera et al. have shown that the suppressive activity of MSCs required the presence of IFN-γ produced by activated T cells and NK cells, and was related to the ability of IFN-γ to stimulate the production of IDO by MSCs, which in turn inhibited the proliferation of activated T or NK cells (Krampera et al., 2006). In their model, they used BM-MSCs and did not observe any effect of TGFβ1. Similarly, Spaggiari et al. studied the interaction between BM-MSCs and IL-2/IL-15 stimulated NK cells. While they did not observe any effect of TGFβ1 or PGE2 alone, they identified a

synergistic effect of PGE2 and IDO and showed that IDO alone was sufficient for inhibiting NK cell proliferation (Spaggiari et al., 2008). In contrast, using BM-MSCs and IL-15 stimulated NK cells, Sotiropoulou et al. observed an inhibition of NK proliferation in direct and indirect co-cultures mediated by PGE2 and TGFβ1, with an additive effect of both soluble factors (Sotiropoulou et al., 2006a). Pradier et al. supported the hypothesis of a combination of inhibitory molecules involved in MSC-mediated immunosuppression (Pradier et al., 2011).

Cytokines: MSCs are able to inhibit NK cell cytokine (IFN-γ and IL-10) production (Pradier et al., 2011; Sotiropoulou et al., 2006a; Spaggiari et al., 2008), particularly in the $CD56^{bright}$ NK cell subset, but the mechanisms are not fully understood. PGE2 and IDO might be involved but with divergent results *in vitro* (Sotiropoulou et al., 2006b; Spaggiari et al., 2008). Finally, Chatterjee has shown the role of Activin-A, a member of the TGFβ family, in cytokine inhibition (Chatterjee et al., 2014).

NK receptors and cytotoxicity: It has been shown that BM-MSCs could induce a down-regulation of NKp30, NKp44, NKG2D receptors without any changes in CD56, KIR, NKp46, CD69, CD16 and NKG2A expression, mediated by PGE2 and IDO in a contact-dependent manner and associated with decreased NK cytotoxicity (Pradier et al., 2011; Spaggiari et al., 2008). In addition, Sotiropoulou et al. observed a downregulation of 2B4, CD132 and DNAM-1 expression (Sotiropoulou et al., 2006b). Although inhibition of cytotoxicity by MSCs appears to rely mostly on cell-to-cell contact (Spaggiari and Moretta, 2013) (through receptors), it also involves secreted mediators. NK cells cultured shortly with IL-2 in the presence of MSCs showed reduced cytotoxic potential against tumor cells (Krampera et al., 2006), which was more pronounced against HLA class I-positive than HLA class I-negative cells (Sotiropoulou et al., 2006b). The role of PGE2 and IDO is frequently observed in different studies, whereas TGFβ is

more rarely involved (Sotiropoulou et al., 2006a; Spaggiari et al., 2008). To exclude that the reduced cytotoxicity of co-cultured NK cells was due to their exhaustion after killing of MSCs, freshly isolated NK cells were used in the majority of the studies.

CD56 subsets: The interaction between MSCs and CD56 NK subtypes has been previously studied by Sotiropoulou et al. who identified a selective inhibition of the $CD56^{bright}$ subtype rather than a selective expansion of the dim subtype (Sotiropoulou et al., 2006b). CD56 expression was also reduced after co-culture with the tumor stroma (Johann et al., 2010).

TLRs: As mentioned previously, MSCs from different tissues express several TLR that promote their viability, proliferation, and cytokine secretion. TLR ligand stimulation modulates NKG2D ligands expressed on MSCs. After TLR priming, the surface expression of MICA protects them from NK cells (decrease at the surface and increase of shedding). In addition, others ligands expressed by MSCs participate in MSC protection. Inhibition by specific mAbs against DNAM-1 and ULPB3 significantly modifies NK-induced MSC lysis. Moreover, CD112 and ULPB3 (but not CD155) are slightly increased by TLR3 treatment. TLR3-primed adult BM and embryonic MSCs are more resistant than unprimed MSCs to IL-2-activated NK-induced killing. After TLR priming, Giuliani et al. observed an increased production by MSCs of proinflammatory and angiogenic factors, and PGE2. MICA, with DNAM-1 and ULBP3 ligands, is part of a network, which contributes to insure immunoprotection of MSCs against NK cytolytic activity in a context of proinflammatory microenvironment mediated by TLR ligands (Giuliani et al., 2014).

It has been shown that MSCs derived from lung cancer tissues have higher expressions of TLR4 mRNA compared to normal bone marrow-derived MSCs. $TLR4^{+}$ MSCs have a stronger

inhibitory effect on NK cell proliferation and cytotoxicity (Lu et al., 2015a). NKG2D receptor expression on NK cells is also inhibited by sorted $TLR4^{+}$MSCs (Lu et al., 2015b).

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). These features remain to be fully elucidated.

In this work, we provided insights into the effects of lung tumor-associated MSCs on freshly isolated NK cell activity after IL-2 treatment. Indeed, we compared the immunosuppressive effects of T-MSCs with those of N-MSCs isolated from the same patient, and with those of BM-MSCs isolated from healthy donors. We observed marked differences between those three MSC samples, concerning their phenotype and immunosuppressive function.

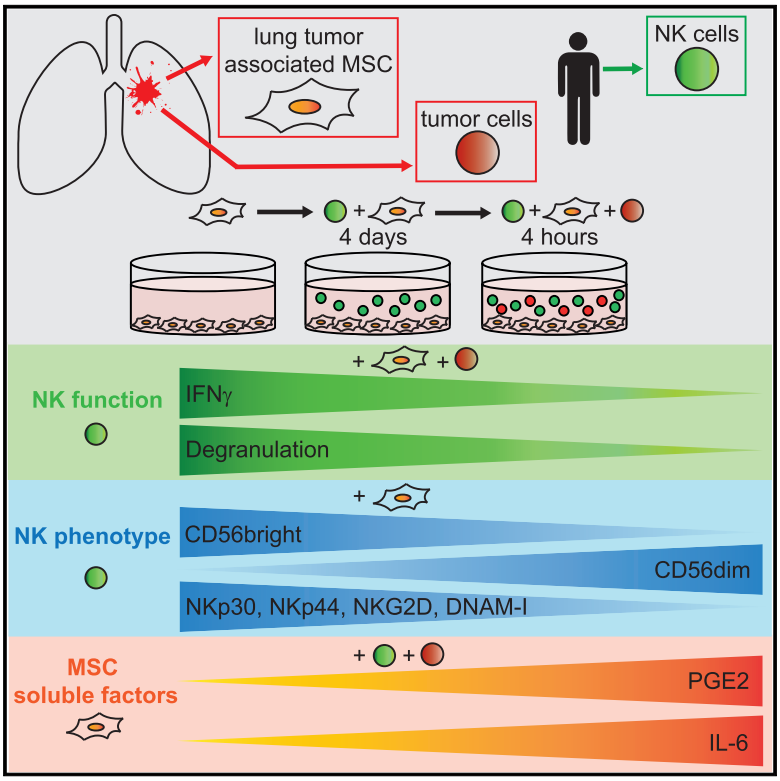
2. PART I: MANUSCRIPT PUBLISHED IN CELL REPORTS

Article

Cell Reports

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 intratumor (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 $CD56^{dim}$ phenotype and differentially modulated $CD56^{bright/dim}$ subset functions. Whereas MSCs affected both degranulation and activating receptor expression in the $CD56^{dim}$ subset, they primarily inhibited interferon-γ production in the $CD56^{bright}$ 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 (TNFα), and interleukin-1β (IL-1β) or through toll-like receptor (TLR) stimulation (Bernardo and Fibbe, 2013; Dumitru 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 Moretta, 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, TNFα, 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

A

	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
Tumor type	Moderately to poorly differentiated invasive SCC	Poorly differentiated SCC	Moderately differentiated keratinizing SCC, partially sarcomatoid	Poorly differentiated SCC, basaloid subtype	Moderately differentiated SCC, with focal keratinization
TNM	pT2b N0 (0/14) Mx	pT2a pN0 (0/20) R0	pT3 pN9 (0/35) Mx R0	pT2a pN0 (0/8)	pT2a pN0 (0/7) pL0 R0
Sex	Female	Female	Male	Male	Male
Age	79	64	70	83	74
Smoking history	Yes	Yes	Yes	Yes	Yes
Neoadjuvant	No	No	No	No	No

SCC: squamous cell carcinoma; p: pathological classification; T: primary tumor; N: node; M: metastasis; X: not assessed histologically; R: residual tumor; L: lymphatic invasion. pTNM classification following the criteria of «The TNM Classification of Malignant Tumours, 8th Edition, UICC», edited by Prof. James Brierle, Wiley Blackwell, 2016.

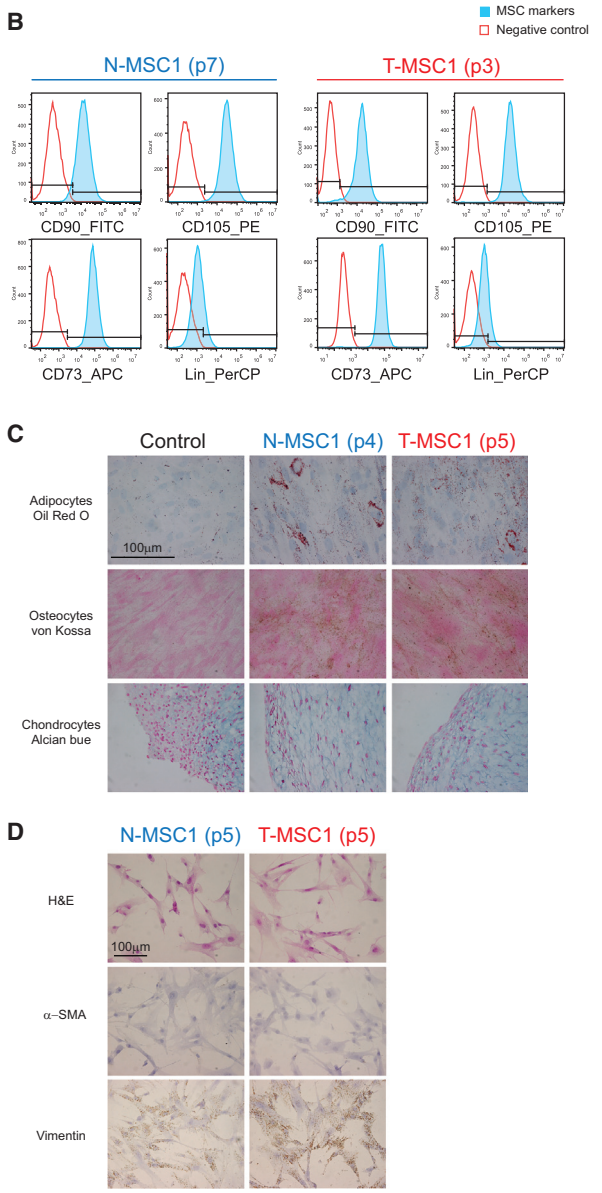


Figure 1. Clinical Data and MSC Characterization

(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, and -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 100 μm.

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 Lotzová, 1993).

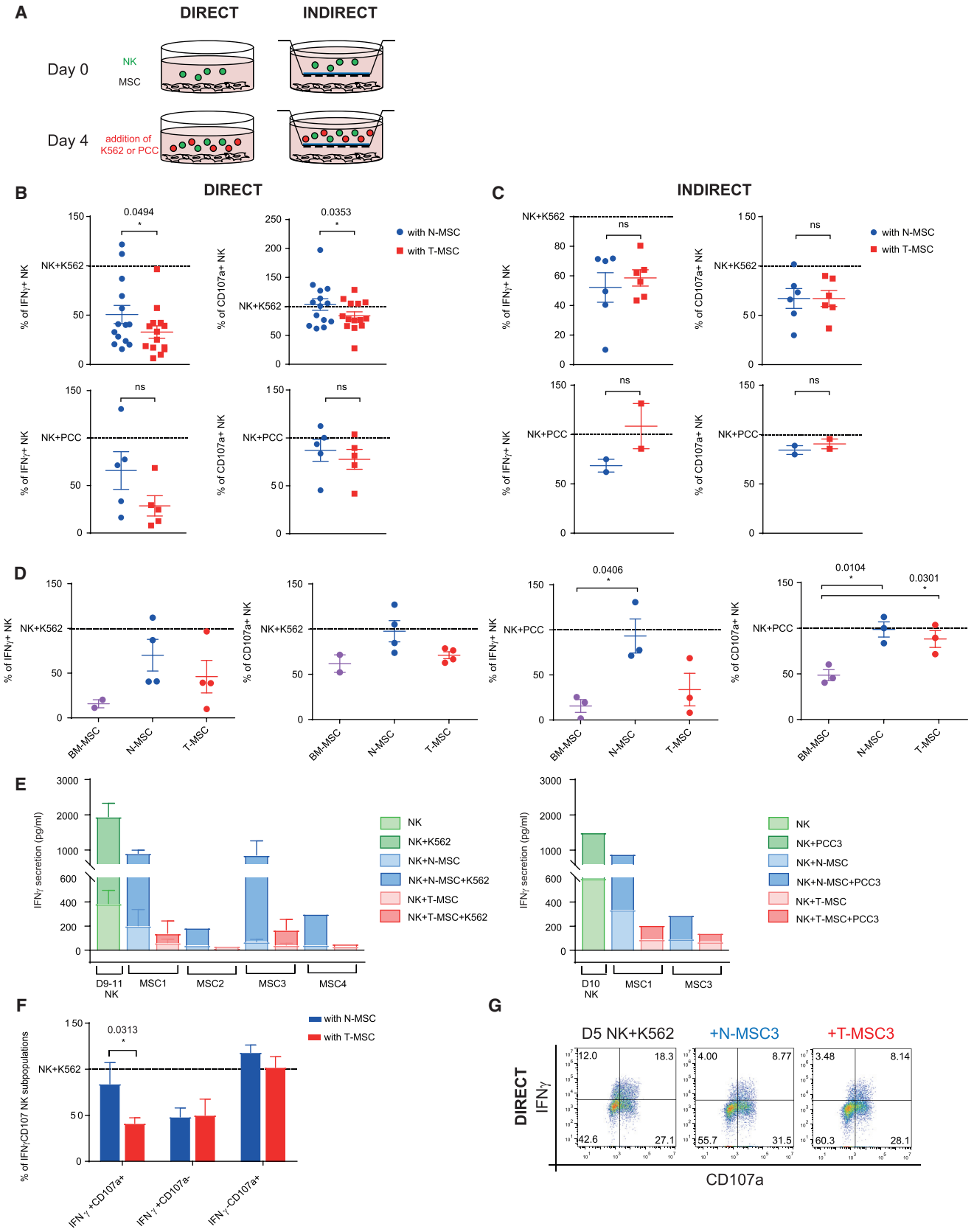
Bone-marrow-derived MSCs (BM-MSCs) can inhibit NK cell proliferation, cytotoxicity, and cytokine production by secreting IDO1, TGFβ, HLA-G, and PGE2 (Casado et al., 2013; Krampera et al., 2006; Rasmusson et al., 2003; Spaggiari et al., 2008). However, they can also be lysed by activated NK cells, depending 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



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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 tumor 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 effector:target 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,

Figure 2. T-MSCs Are More Immunosuppressive Than N-MSCs Toward the NK Response to K562 and PCC

(A) Schematic representation of direct and indirect co-culture experiments.

(B, C, D, and F) NK cell activation (percentages of CD107a and intracellular IFN-γ-positive cells) by K562 or PCC cells following N-, T-, and BM-MSC co-culture, as assessed by flow cytometry. Results were normalized to those of NK cells cultured without MSCs for the same duration and activated by target cells (the control condition is represented by the horizontal dashed line). Results show the mean ± SEM.

(B) Direct co-cultures. Graphs plot individual values of 8 independent experiments with K562 cells (eight different NK cell donors; MSC1 [n = 3 biological replicates]; MSC2 [n = 2]; MSC3 [n = 6]; MSC4 [n = 2]; MSC5 [n = 1]) and 3 experiments with PCC (three NK cell donors; MSC1 [n = 1]; MSC2 [n = 1]; MSC3 [n = 2]; MSC4 [n = 1]).

(C) Indirect co-cultures. Two experiments were done with K562 cells (two NK cell donors; MSC1 [n = 2]; MSC2 [n = 1]; MSC3 [n = 2]; MSC4 [n = 1]) and one with PCC (one NK cell donor; MSC1 [n = 1]; MSC3 [n = 1]).

(D) Comparison of the effects of BM-MSCs to those of lung tissue MSCs. Graphs plot individual values of two experiments in direct MSC-NK co-culture with K562 (two different NK cell donors; MSC2 [n = 1]; MSC3 [n = 2]; MSC4 [n = 1]; BM-MSC [n = 2]) and 2 experiments with PCC (PCC2, 3, and 4; two NK cell donors; MSC2 [n = 1]; MSC3 [n = 1]; MSC4 [n = 1]; BM-MSC [n = 3]).

(E) IFN-γ secretion (pg/mL) by NK cells co-cultured with N- (blue bar) and T-MSCs (red bar) before and after activation by tumor cells (K562, left panel, and PCC, right panel). IFN-γ secretion by NK cells cultured alone is shown in green. Light color bars, basal NK cell secretion (without tumor cells); dark color bars, secretion after activation. Mean values ± SEM for 3 experiments with K562 as target cells (three NK cell donors; MSC1 [n = 2]; MSC2 [n = 1]; MSC3 [n = 3]; MSC4 [n = 1]) and one experiment with PCC as target cells (one single NK cell donor; MSC1 [n = 1] and 3 [n = 1]) are shown.

(F) Percentages of $IFN\text{-}\gamma^{+/-}CD107a^{+/-}$ NK cell subpopulations after 4 days of co-culture with MSCs in 5 independent experiments using NK cells from 5 donors and MSCs from one patient (MSC3; n = 6).

(G) Representative flow density dot plots showing expression of IFN-γ and CD107a by K562-activated NK cells in direct co-culture with and without MSCs (single NK cell donor; MSC3).

Statistical significance was determined by Wilcoxon matched-pairs signed rank test (pairing on MSC patient; B, C, and F) or unpaired t test with Welch's correction (D; * indicates significance at $p < 0.05$; **$p < 0.01$; ns, not significant). See also Figure S3.

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 S3Bii). 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 MSCs 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 CD56^{dim} 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 3Ai). 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 3Ai), with the exception of NKp44, whose expression was partially inhibited in indirect co-culture (Figure 3Aii).

Surprisingly, we observed marked changes in the CD56$^{bright/dim}$ 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 CD56^{bright}. However, an inversion in the CD56$^{bright/dim}$ NK cell ratio was observed in direct co-culture with MSCs (Figures 3Bi and 3C) that was largely contact dependent (Figures 3B and 3C).

CD56^{bright} and CD56^{dim} 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 CD56^{dim} 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 CD56^{dim} 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 CD56$^{dim/bright}$ subpopulations. In control conditions (NK cells cultured with K562 cells alone), the CD56^{bright} 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 CD56^{dim} subpopulation, which was associated with degranulation, the change in function of the total NK cell population mostly reflected functional inhibition of the CD56^{dim} subset. Thus, MSC-mediated IFN-γ inhibition occurred predominantly in the CD56^{bright} subpopulation, whereas NK degranulation was inhibited by MSCs in the CD56^{dim} 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 CD56^{bright} cells, prone to cytokine secretion and comparable to NK cells activated by tumor cells without MSCs.

Modulation of NK receptors also differed between the CD56^{dim} and CD56^{bright} NK subsets. Thus, downregulation of NKp44, NKp30, NKG2D, and DNAM-I receptor expression occurred in the CD56^{dim} 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 CD56^{bright} subset, only NKG2D expression was slightly decreased in direct co-culture with MSCs. With this exception, receptor expression in CD56^{bright} 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 CD56^{dim} NK cells. In contrast, inhibition of cytokine production in the CD56^{bright} 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

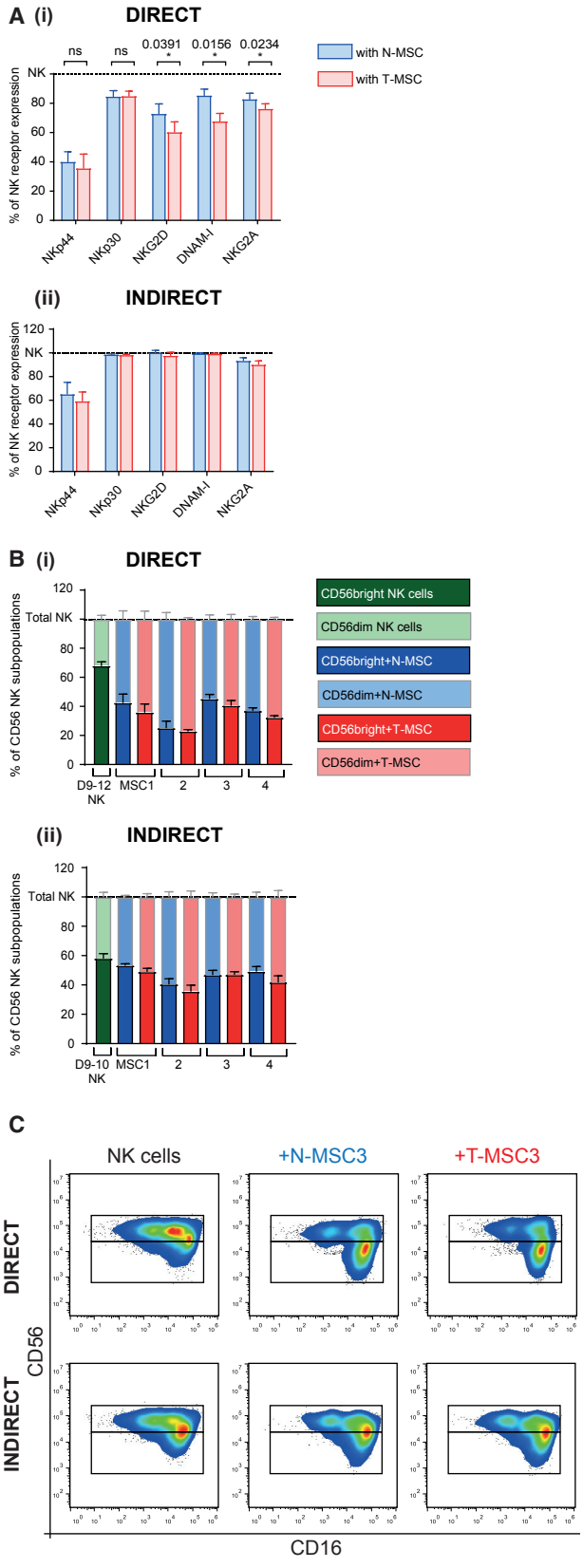


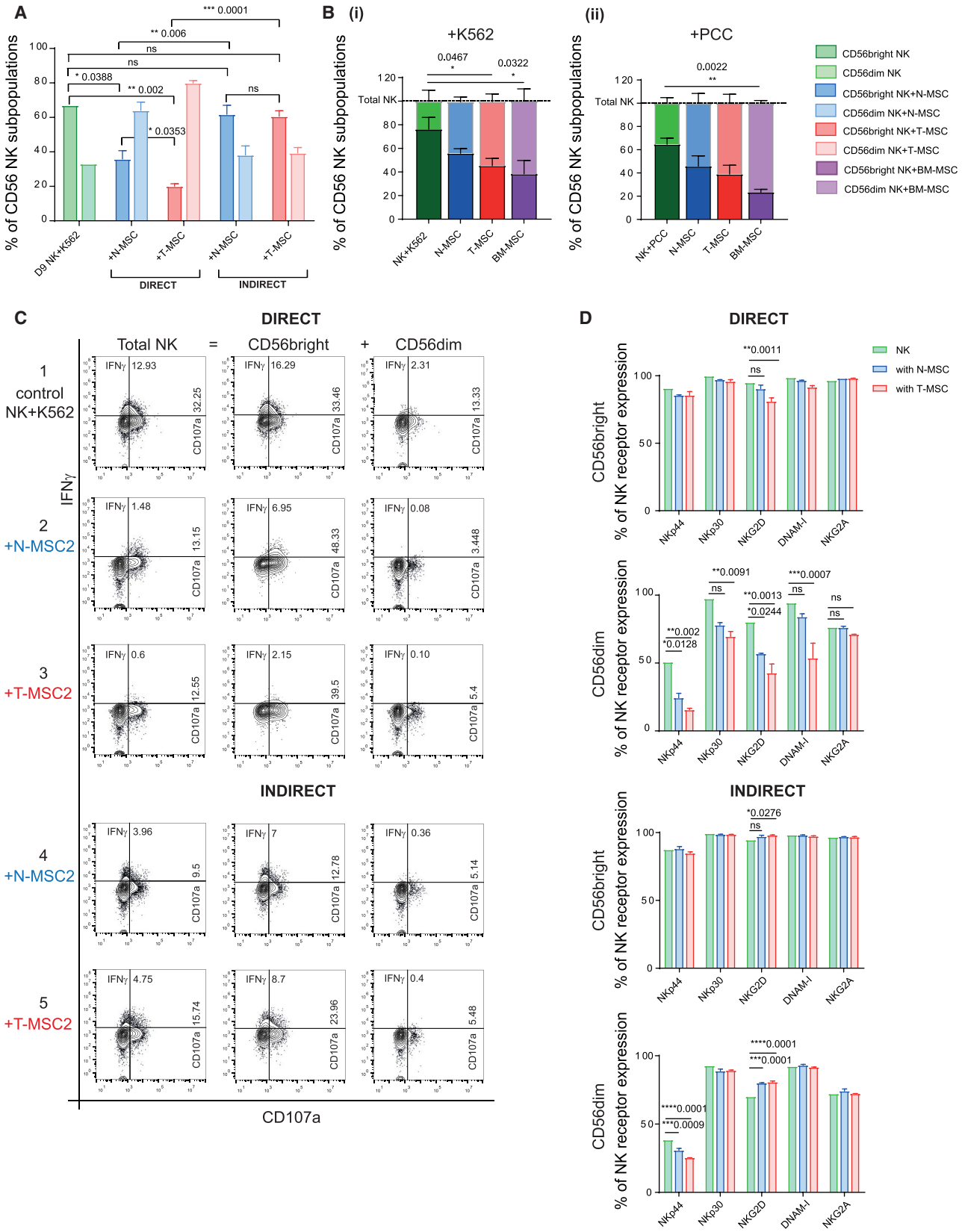
Figure 3. The NK Cell Phenotype Is Strongly Modulated by Direct Co-culture with N- and T-MSCs

(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 $CD56^{bright}$ and $CD56^{dim}$ 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 = 8]; MSC4 [n = 2]). (ii) Data from 2 experiments are shown (two NK cell donors; MSC1 [n = 6]; MSC2 [n = 3]; MSC3 [n = 6]; MSC4 [n = 3]). (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).

in MSC-induced suppression of cytokine production in the $CD56^{bright}$ NK subset.

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*, *TGFB1*, *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, *TGFB1* 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 *TGFB1* 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*,



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and *PVR* expression (Figure S4D). MSCs expressed low levels of NK-activating receptor ligands, except for the DNAM-I 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 S5Aiii).

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 6Ai). 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 6Ai). 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 $CD56^{bright}$ 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 $CD56^{bright}$ NK cell phenotype (Figures 6Bii, 6Biii, and S5Cii).

We next investigated the mechanisms involved in the restoration of NK function 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 6Ci). However, anti-IL-6 enhanced the inhibition of NKp30 and DNAM-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 6Cii). COX-2 inhibition partially restored NKp44, NKG2D, and NKp30 expression that had been downregulated by T-MSCs (Figure 6Cii). 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 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 $CD56^{dim}$ 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

Figure 4. MSC Exert Different Effects on $CD56^{dim}$ and $CD56^{bright}$ NK Subsets

(A and B) Percentages of $CD56^{dim}$ and $CD56^{bright}$ NK cell subpopulations co-cultured with MSCs after 4 hr activation by tumor cells, assessed by flow cytometry. Light tones, $CD56^{dim}$ NK subset; dark tones, $CD56^{bright}$ subset. Tumor-activated NK cells cultured in the absence of MSCs provided a control (green bars). Results show the mean ± SEM. Statistical significance was determined by 2-way ANOVA followed by Tukey's multiple comparisons test. (A) Direct and indirect co-cultures with N- and T-MSCs after 4 hr activation by K562 cells are shown. Data are representative of a single experiment with pooled MSCs from 4 patients (MSC1-4) and a single NK cell donor. (B) Direct co-cultures with N-MSCs, T-MSCs, and BM-MSCs after 4 hr activation by K562 (i) or PCC cells (ii) are shown. Data representative of two experiments with pooled MSCs from 3 patients (MSC2-4), one donor of BM-MSCs, and two NK cell donors are shown. (i) Results from two experiments are shown (two NK cell donors; MSC2 [n = 1]; MSC3 [n = 2]; MSC4 [n = 1]; BM-MSC [n = 2]). (ii) Data from two experiments with PCC2, 3, and 4 are shown (two NK cell donors; MSC2 [n = 1]; MSC3 [n = 1]; MSC4 [n = 1]; BM-MSC [n = 2]).

(C) Representative dot plots showing the percentages of IFN-γ^+ and $CD107a^+$ cells in the total NK cell population and in the $CD56^{bright}$ and $CD56^{dim}$ subsets after direct and indirect co-cultures with MSCs from patient 2 and activation by K562 cells.

(D) Modulation of NK receptor expression in $CD56^{bright}$ and $CD56^{dim}$ subsets in direct and indirect co-cultures with MSCs compared to control conditions (NK cultured alone). Results show the mean ± SEM for a single experiment (single NK cell donor; MSC1 [n = 1] and 3 [n = 1]). Statistical significance was determined by 2-way ANOVA followed by Dunnett's multiple comparisons test with NK cells alone set as the control.

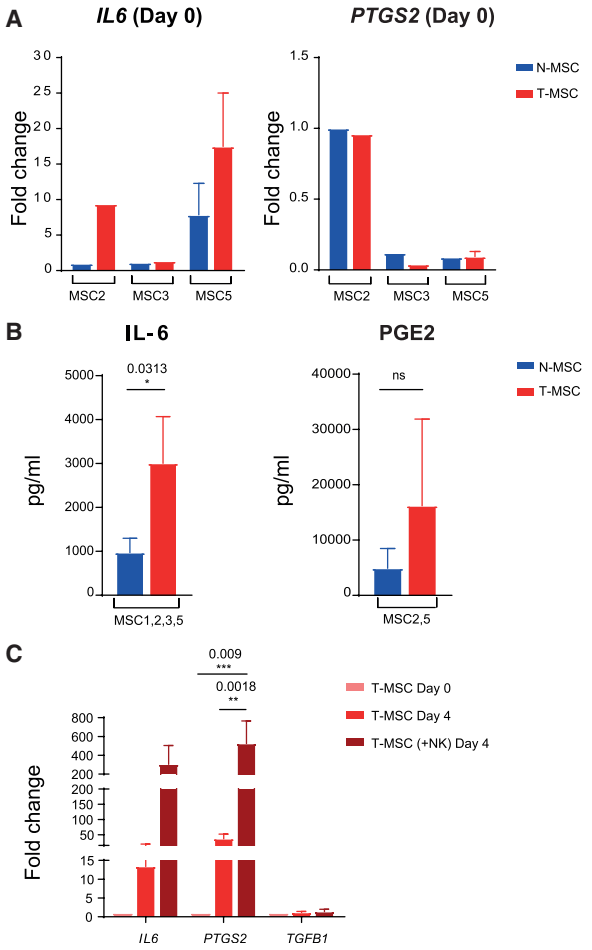

Figure 5. Differential Expression of Immune-Modulating Mediators by T- and N-MSCs

(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 *TGFB1* 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.

See also Figure S4.

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-I, and NKG2A. MSCs also induced an inversion in the $CD56^{bright/dim}$ NK cell ratio in favor of the $CD56^{dim}$ 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-I (Platonova et al., 2011; Esendagli et al., 2008; Levi et al., 2015).

The observed shift toward the $CD56^{dim}$ 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 $CD56^{bright}$ subtype rather than expansion of the $CD56^{dim}$ subpopulation (Sotiropoulou et al., 2006). Following IL-2 stimulation, NK cells acquire a $CD56^{bright}$ phenotype, which correlates with an activated state of the cells in vitro. The $CD56^{dim}$ 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.

$CD56^{dim}$ and $CD56^{bright}$ NK cell functions were differentially modulated by MSCs. With the exception of NKG2D, T-MSCs downregulated activating receptor expression exclusively in the $CD56^{dim}$ NK cell subset, where they also inhibited degranulation. In contrast, T-MSCs inhibited IFN-γ production in $CD56^{bright}$ 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 $CD56^{bright}$ and degranulation of $CD56^{dim}$ NK cells, with the possible implication of NKG2D in both functions of the two NK subsets.

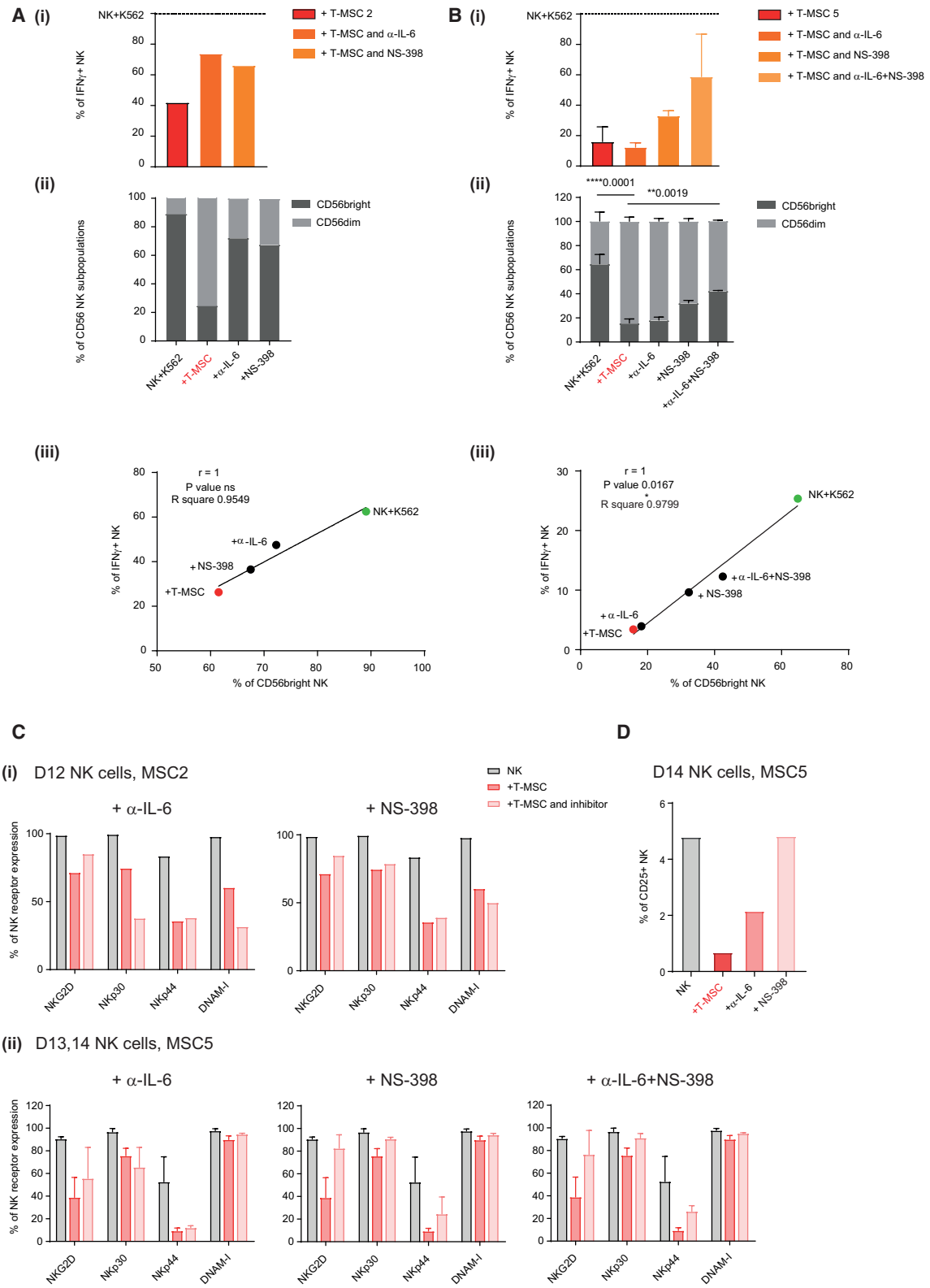

Figure 6. Targeting T-MSC-Derived Immunosuppressive Mediators Rescues NK Cell Activity

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.

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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 $CD56^{bright}$ cells, and NKp30, NKp44, NKG2D, 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 (Sotiropoulou 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 $CD56^{dim}$ 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), 1% non-essential amino acids (NEAA) (Gibco), and 10 ng/mL platelet-derived growth factor (PDGF) (Prospec; 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 (Prospec). PCCs were assessed by flow

(B) Summary of two rescue experiments with MSCs from patient 5 (two NK cell donors).

(A and B) (i) Percentage of IFN-γ^+ NK cells after activation by K562 cells in the presence of T-MSCs with or without anti-IL-6-neutralizing antibody and/or NS-398. Results are normalized to NK cell activation by K562 cells in the absence of MSCs, considered to be 100% (horizontal dashed line). (ii) Percentages of K562-activated $CD56^{bright}$ and $CD56^{dim}$ NK subsets cultured alone or with T-MSCs in the presence or absence of inhibitors are shown. Statistical significance was determined by 2-way ANOVA followed by Dunnett's multiple comparisons test, with K562-activated NK cells co-cultured with T-MSCs set as the control (red). (iii) Correlation between the percentage of $CD56^{bright}$ NK cells and intracellular IFN-γ production in different culture conditions is shown. The statistical test used was the Spearman correlation coefficient r, p values are reported, and linear regression line with R square for goodness of fit.

(C) Percentage of NK receptor expression after 4 days in culture alone (gray bar) or with T-MSCs in the absence (dark red bar) or presence of the indicated inhibitors (light red bar). (i) MSC2 is shown; one NK cell donor. (ii) MSC5 is shown; two NK cell donors.

(D) Percentage of IL-2Rα (CD25) expression on NK cells cultured alone (gray bar) or in the presence of T-MSCs (red bars) with or without the indicated inhibitors. Data are representative of a single experiment with a single NK cell donor and MSC5.

See also Figure S5.

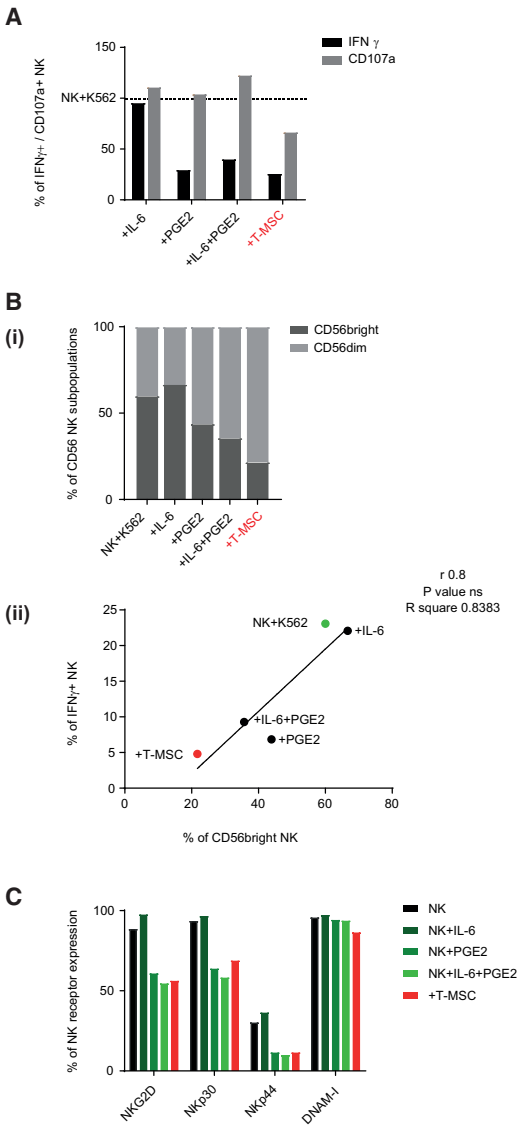

Figure 7. NK Stimulation with PGE2 Mimics MSC-Mediated IFN-γ Immunosuppression

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$^+$ NK cells (gray bar) after activation by K562 cells in different culture conditions: with IL-6, PGE2, a combination of 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 CD56^{bright} and CD56^{dim} NK subsets after activation by K562 cells under conditions described in (A). (ii) Correlation between the percentages of CD56^{bright} and IFN-γ^+ NK cells is shown. 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.

cytometry for HLA-ABC expression (see Supplemental Experimental Procedures). The K562 human leukemia cell line (ATCC no. CCL-243) was cultured in RPMI1640 supplemented with 10% FCS and 1% PS.

Tumor-Infiltrating Immune Cell Characterization

Immune cell infiltration in the tumor bulk was assessed by IHC on whole-tissue sections from SCCs of four patients and by flow cytometry of tumor bulk from three patients after dissociation (see Supplemental Experimental Procedures).

Microscopy

Images were taken with a Nikon Eclipse E800, digital camera DXM1200, at 20× or 40× magnification and at a resolution of 1,280 × 1,024, and analyzed with the ACT-1 (v.2) software.

NK Cell Isolation and Culture

NK cells were isolated from peripheral blood mononuclear cells derived from buffy coats obtained from 14 healthy volunteer donors with donor consent and approval from the Ethics Committee of the Canton de Vaud (project authorization no. P-108). NK cells were cultured in RPMI1640 (Gibco) supplemented with 10% heat-inactivated human serum (Sigma and Life Technologies), 1% PS, and IL-2 (10 ng/mL; R&D Systems; NK medium; see Supplemental Experimental Procedures).

Co-culture Experiments

T-MSCs and N-MSCs were plated in 96-well plates 24 hr before co-culture (0.5×10^5 cells/well). MSCs were treated with mitomycin C 50 μg/mL (Sigma), and freshly isolated NK cells (10^5 cells) were added at 1:2 MSC:NK ratio. Media were supplemented with IL-2 10 ng/mL (R&D). After 4 days of NK-MSC co-culture in NK medium, 10^5 K562 target cells or PCC were added and maintained in co-culture for 4 hr.

Flow Cytometry

See Supplemental Experimental Procedures for more detailed information.

MSC Characterization

Cells were stained with the « human MSC phenotyping kit » (no. 130-095-198; Miltenyi Biotec) according to the manufacturer's protocol.

NK Phenotype

Before and after 4 days of co-culture with MSCs, the NK cell phenotype was assessed using anti-NKp30 (phycoerythrin [PE]; no. 558407; BD Biosciences; 1:50), -NKG2D (antigen-presenting cell [APC]; no. 562064; BD Biosciences; 1:10), -NKG2A (fluorescein isothiocyanate [FITC]; no. 130-098-817; Miltenyi; 1:20), -NKp44 (PE; no. 558563, BD Biosciences; 1:15), -DNAM-I (PE; no. 130-100-461; Miltenyi; 1:20), and -CD25 (APC; no. 555434; BD Biosciences; 1:10) antibodies among CD45$^+$CD3$^-$CD56$^+$CD16$^{+/-}$ cells (anti-CD45 Alexa Fluor 700, no. 560566, BD Biosciences, 1:20; anti-CD3 PC7, no. 41116015, Beckman Coulter, 1:20; anti-CD56 ECD, no. 41116015, Beckman Coulter, 1:40; anti-CD16 PerCP-Cy5.5, no. 560717, BD Biosciences, 1:20).

CD107a Degranulation and Intracellular IFN-γ

After 4 hr activation by target cells, NK cells were stained with anti-CD107a (FITC; no. 555800; BD Biosciences; 1:10), -CD3, -CD45, -CD56, and -CD16 antibodies. Intracellular IFN-γ expression was assessed with APC-conjugated anti-IFN-γ antibody (no. 130-097-944; Miltenyi; 1:25) after cell fixation (PFA) and permeabilization (PBS + BSA 0.2% + saponine 0.1% buffer, for 30 min at room temperature). NK degranulation and IFN-γ expression were quantified within the CD45$^+$CD3$^-$CD56$^+$CD16$^{+/-}$ cell population. NK cells without target tumor cells were used as a control.

Inhibitors/Stimulatory Molecules

Inhibitors

Human anti-IL-6 antibody (MAB206; clone no. 6708; R&D) was added at the final concentration of 2 μg/mL at the start of co-culture. Inhibition of PGE2 was achieved using NS-398 (no. 70590; Cayman Chemical) at the final concentration of 5 μM. Medium + DMSO or + PBS were used as negative controls for NS-398 and IL-6 treatment, respectively.

NK Stimulation

Human recombinant IL-6 (SRP3096; Sigma) was used at a final concentration of 25 ng/mL. PGE2 was obtained from Sigma (P0409) and used at 1 μM

(350 ng/mL). 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.

IFN-γ, TNFα, HGF, IL-6, and TGFβ1 were assessed in the co-culture supernatants by Luminex assay (Bio-Plex Pro Human Cytokine/TGFβ; Bio-Rad).

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 $2^{-\Delta\Delta Ct}$ 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 BM-MSCs and lung tissue MSCs was determined by an unpaired t test with Welch's correction. For qRT-PCR data, multiple t tests corrected for multiple comparisons using the Holm-Sidak method were used to compare mean expression levels 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 R^2 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

The accession numbers for the data reported in this paper are Flow Repository: FR-FCM-ZYA4; FR-FCM-ZYAE; FR-FCM-ZYAY; FR-FCM-ZYAD; FR-FCM-ZYAC; FR-FCM-ZYAB; FR-FCM-ZYAA; FR-FCM-ZYA9; FR-FCM-ZYA8; FR-FCM-ZYA7; FR-FCM-ZYA6; FR-FCM-ZYA5; FR-FCM-ZYA3; FR-FCM-ZYA2; FR-FCM-ZYAZ; and FR-FCM-ZY9V.

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.celrep.2017.08.089.

AUTHOR CONTRIBUTIONS

Conceptualization, S.G., G.F., and I.S.; Methodology, S.G. and G.F.; Investigation, S.G., G.F., P.M., and J.V.; Writing – Original Draft, S.G.; Writing – Review & Editing, S.G., G.F., A.C., and I.S.; Funding Acquisition, I.S. and S.G.; Resources, I.S. and I.L.; Supervision, G.F. and I.S.

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REFERENCES

Aggarwal, S., and Pittenger, M.F. (2005). Human mesenchymal stem cells modulate allogeneic immune cell responses. Blood *105*, 1815–1822.

Alter, G., Malenfant, J.M., and Altfeld, M. (2004). CD107a as a functional marker for the identification of natural killer cell activity. J. Immunol. Methods *294*, 15–22.

Bernardo, M.E., and Fibbe, W.E. (2013). Mesenchymal stromal cells: sensors and switchers of inflammation. Cell Stem Cell *13*, 392–402.

Bhooshan, N., Staats, P.N., Fulton, A.M., Feliciano, J.L., and Edelman, M.J. (2016). Prostaglandin E receptor EP4 expression, survival and pattern of recurrence in locally advanced NSCLC. Lung Cancer *101*, 88–91.

Bouffi, C., Bony, C., Courties, G., Jorgensen, C., and Noël, D. (2010). IL-6-dependent PGE2 secretion by mesenchymal stem cells inhibits local inflammation in experimental arthritis. PLoS ONE *5*, e14247.

Brown, J.R., and DuBois, R.N. (2004). Cyclooxygenase as a target in lung cancer. Clin. Cancer Res. *10*, 4266s–4269s.

Bussard, K.M., Mutkus, L., Stumpf, K., Gomez-Manzano, C., and Marini, F.C. (2016). Tumor-associated stromal cells as key contributors to the tumor microenvironment. Breast Cancer Res. *18*, 84.

Caligiuri, M.A. (2008). Human natural killer cells. Blood *112*, 461–469.

Carrega, P., and Ferlazzo, G. (2017). Natural killers are made not born: how to exploit NK cells in lung malignancies. Front. Immunol. *8*, 277.

Casado, J.G., Tarazona, R., and Sanchez-Margallo, F.M. (2013). NK and MSCs crosstalk: the sense of immunomodulation and their sensitivity. Stem Cell Rev. *9*, 184–189.

Chen, Z., Fillmore, C.M., Hammerman, P.S., Kim, C.F., and Wong, K.-K. (2014). Non-small-cell lung cancers: a heterogeneous set of diseases. Nat. Rev. Cancer *14*, 535–546.

Chretien, A.-S., Le Roy, A., Vey, N., Prebet, T., Blaise, D., Fauriat, C., and Olive, D. (2014). Cancer-induced alterations of NK-mediated target recognition: current and investigational pharmacological strategies aiming at restoring NK-mediated anti-tumor activity. Front. Immunol. *5*, 122.

Cooper, M.A., Fehniger, T.A., and Caligiuri, M.A. (2001). The biology of human natural killer-cell subsets. Trends Immunol. *22*, 633–640.

Cremer, I., Fridman, W.H., and Sautès-Fridman, C. (2012). Tumor microenvironment in NSCLC suppresses NK cells function. OncoImmunology *1*, 244–246.

DelaRosa, O., Sánchez-Correa, B., Morgado, S., Ramírez, C., del Río, B., Menta, R., Lombardo, E., Tarazona, R., and Casado, J.G. (2012). Human adipose-derived stem cells impair natural killer cell function and exhibit low susceptibility to natural killer-mediated lysis. Stem Cells Dev. *21*, 1333–1343.

Desmoulière, A., Guyot, C., and Gabbiani, G. (2004). The stroma reaction myofibroblast: a key player in the control of tumor cell behavior. Int. J. Dev. Biol. *48*, 509–517.

Di Trapani, M., Bassi, G., Ricciardi, M., Fontana, E., Bifari, F., Pacelli, L., Giacomello, L., Pozzobon, M., Féron, F., De Coppi, P., et al. (2013). Comparative study of immune regulatory properties of stem cells derived from different tissues. Stem Cells Dev. *22*, 2990–3002.

Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F., Krause, D., Deans, R., Keating, A., Prockop, Dj., and Horwitz, E. (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy *8*, 315–317.

Dumitru, C.A., Hemeda, H., Jakob, M., Lang, S., and Brandau, S. (2014). Stimulation of mesenchymal stromal cells (MSCs) via TLR3 reveals a novel mechanism of autocrine priming. FASEB J. *28*, 3856–3866.

Esendagli, G., Bruderek, K., Goldmann, T., Busche, A., Branscheid, D., Vollmer, E., and Brandau, S. (2008). Malignant and non-malignant lung tissue areas are differentially populated by natural killer cells and regulatory T cells in non-small cell lung cancer. Lung Cancer *59*, 32–40.

Gillard-Bocquet, M., Caer, C., Cagnard, N., Crozet, L., Perez, M., Fridman, W.H., Sautès-Fridman, C., and Cremer, I. (2013). Lung tumor microenvironment induces specific gene expression signature in intratumoral NK cells. Front. Immunol. *4*, 19.

Gottschling, S., Granzow, M., Kuner, R., Jauch, A., Herpel, E., Xu, E.C., Muley, T., Schnabel, P.A., Herth, F.J.F., and Meister, M. (2013). Mesenchymal stem cells in non-small cell lung cancer–different from others? Insights from comparative molecular and functional analyses. Lung Cancer *80*, 19–29.

Groh, V., Wu, J., Yee, C., and Spies, T. (2002). Tumour-derived soluble MIC ligands impair expression of NKG2D and T-cell activation. Nature *419*, 734–738.

Hidalgo, G.E., Zhong, L., Doherty, D.E., and Hirschowitz, E.A. (2002). Plasma PGE-2 levels and altered cytokine profiles in adherent peripheral blood mononuclear cells in non-small cell lung cancer (NSCLC). Mol. Cancer *1*, 5.

Hodge, G., Barnawi, J., Jurisevic, C., Moffat, D., Holmes, M., Reynolds, P.N., Jersmann, H., and Hodge, S. (2014). Lung cancer is associated with decreased expression of perforin, granzyme B and interferon (IFN)-γ by infiltrating lung tissue T cells, natural killer (NK) T-like and NK cells. Clin. Exp. Immunol. *178*, 79–85.

Imai, K., Matsuyama, S., Miyake, S., Suga, K., and Nakachi, K. (2000). Natural cytotoxic activity of peripheral-blood lymphocytes and cancer incidence: an 11-year follow-up study of a general population. Lancet *356*, 1795–1799.

Jamieson, A.M., Diefenbach, A., McMahon, C.W., Xiong, N., Carlyle, J.R., and Raulet, D.H. (2002). The role of the NKG2D immunoreceptor in immune cell activation and natural killing. Immunity *17*, 19–29.

Jin, S., Deng, Y., Hao, J.-W., Li, Y., Liu, B., Yu, Y., Shi, F.-D., and Zhou, Q.-H. (2014). NK cell phenotypic modulation in lung cancer environment. PLoS ONE *9*, e109976.

Johann, P.-D., Vaegler, M., Gieseke, F., Mang, P., Armeanu-Ebinger, S., Kluba, T., Handgretinger, R., and Müller, I. (2010). Tumour stromal cells derived from paediatric malignancies display MSC-like properties and impair NK cell cytotoxicity. BMC Cancer *10*, 501.

Kalluri, R. (2016). The biology and function of fibroblasts in cancer. Nat. Rev. Cancer *16*, 582–598.

Khuri, F.R., Wu, H., Lee, J.J., Kemp, B.L., Lotan, R., Lippman, S.M., Feng, L., Hong, W.K., and Xu, X.-C. (2001). Cyclooxygenase-2 overexpression is a marker of poor prognosis in stage I non-small cell lung cancer. Clin. Cancer Res. *7*, 861–867.

Krampera, M. (2011). Mesenchymal stromal cell 'licensing': a multistep process. Leukemia *25*, 1408–1414.

Krampera, M., Cosmi, L., Angeli, R., Pasini, A., Liotta, F., Andreini, A., Santarlasci, V., Mazzinghi, B., Pizzolo, G., Vinante, F., et al. (2006). Role for interferon-gamma in the immunomodulatory activity of human bone marrow mesenchymal stem cells. Stem Cells *24*, 386–398.

Lanier, L.L., Le, A.M., Civin, C.I., Loken, M.R., and Phillips, J.H. (1986). The relationship of CD16 (Leu-11) and Leu-19 (NKH-1) antigen expression on human peripheral blood NK cells and cytotoxic T lymphocytes. J. Immunol. *136*, 4480–4486.

Le Blanc, K., and Davies, L.C. (2015). Mesenchymal stromal cells and the innate immune response. Immunol. Lett. *168*, 140–146.

Levi, I., Amsalem, H., Nissan, A., Darash-Yahana, M., Peretz, T., Mandelboim, O., and Rachmilewitz, J. (2015). Characterization of tumor infiltrating natural killer cell subset. Oncotarget *6*, 13835–13843.

Liu, R., Wei, S., Chen, J., and Xu, S. (2014). Mesenchymal stem cells in lung cancer tumor microenvironment: their biological properties, influence on tumor growth and therapeutic implications. Cancer Lett. *353*, 145–152.

Moretta, L., Biassoni, R., Bottino, C., Mingari, M.C., and Moretta, A. (2001). Immunobiology of human NK cells. Transplant. Proc. *33*, 60–61.

Oppenheim, D.E., Roberts, S.J., Clarke, S.L., Filler, R., Lewis, J.M., Tigelaar, R.E., Girardi, M., and Hayday, A.C. (2005). Sustained localized expression of ligand for the activating NKG2D receptor impairs natural cytotoxicity in vivo and reduces tumor immunosurveillance. Nat. Immunol. *6*, 928–937.

Platonova, S., Cherfils-Vicini, J., Damotte, D., Crozet, L., Vieillard, V., Validire, P., André, P., Dieu-Nosjean, M.-C., Alifano, M., Régnard, J.-F., et al. (2011). Profound coordinated alterations of intratumoral NK cell phenotype and function in lung carcinoma. Cancer Res. *71*, 5412–5422.

Poggi, A., and Giuliani, M. (2016). Mesenchymal stromal cells can regulate the immune response in the tumor microenvironment. Vaccines (Basel) *4*, E41.

Poggi, A., Prevosto, C., Massaro, A.-M., Negrini, S., Urbani, S., Pierri, I., Saccardi, R., Gobbi, M., and Zocchi, M.R. (2005). Interaction between human NK cells and bone marrow stromal cells induces NK cell triggering: role of NKp30 and NKG2D receptors. J. Immunol. *175*, 6352–6360.

Poggi, A., Musso, A., Dapino, I., and Zocchi, M.R. (2014). Mechanisms of tumor escape from immune system: role of mesenchymal stromal cells. Immunol. Lett. *159*, 55–72.

Pross, H.F., and Lotzová, E. (1993). Role of natural killer cells in cancer. Nat. Immun. *12*, 279–292.

Raffaghello, L., and Dazzi, F. (2015). Classification and biology of tumour associated stromal cells. Immunol. Lett. *168*, 175–182.

Rasmusson, I., Ringdén, O., Sundberg, B., and Le Blanc, K. (2003). Mesenchymal stem cells inhibit the formation of cytotoxic T lymphocytes, but not activated cytotoxic T lymphocytes or natural killer cells. Transplantation *76*, 1208–1213.

Sotiropoulou, P.A., Perez, S.A., Gritzapis, A.D., Baxevanis, C.N., and Papamichail, M. (2006). Interactions between human mesenchymal stem cells and natural killer cells. Stem Cells *24*, 74–85.

Spaggiari, G.M., and Moretta, L. (2013). Cellular and molecular interactions of mesenchymal stem cells in innate immunity. Immunol. Cell Biol. *91*, 27–31.

Spaggiari, G.M., Capobianco, A., Becchetti, S., Mingari, M.C., and Moretta, L. (2006). Mesenchymal stem cell-natural killer cell interactions: evidence that activated NK cells are capable of killing MSCs, whereas MSCs can inhibit IL-2-induced NK-cell proliferation. Blood *107*, 1484–1490.

Spaggiari, G.M., Capobianco, A., Abdelrazik, H., Becchetti, F., Mingari, M.C., and Moretta, L. (2008). Mesenchymal stem cells inhibit natural killer-cell proliferation, cytotoxicity, and cytokine production: role of indoleamine 2,3-dioxygenase and prostaglandin E2. Blood *111*, 1327–1333.

Stagg, J., and Galipeau, J. (2013). Mechanisms of immune modulation by mesenchymal stromal cells and clinical translation. Curr. Mol. Med. *13*, 856–867.

Turley, S.J., Cremasco, V., and Astarita, J.L. (2015). Immunological hallmarks of stromal cells in the tumour microenvironment. Nat. Rev. Immunol. *15*, 669–682.

Uccelli, A., Moretta, L., and Pistoia, V. (2006). Immunoregulatory function of mesenchymal stem cells. Eur. J. Immunol. *36*, 2566–2573.

Uccelli, A., Moretta, L., and Pistoia, V. (2008). Mesenchymal stem cells in health and disease. Nat. Rev. Immunol. *8*, 726–736.

Villegas, F.R., Coca, S., Villarrubia, V.G., Jiménez, R., Chillón, M.J., Jareño, J., Zuil, M., and Callol, L. (2002). Prognostic significance of tumor infiltrating natural killer cells subset CD57 in patients with squamous cell lung cancer. Lung Cancer *35*, 23–28.

Vitale, M., Cantoni, C., Pietra, G., Mingari, M.C., and Moretta, L. (2014). Effect of tumor cells and tumor microenvironment on NK-cell function. Eur. J. Immunol. *44*, 1582–1592.

Vivier, E., Tomasello, E., Baratin, M., Walzer, T., and Ugolini, S. (2008). Functions of natural killer cells. Nat. Immunol. *9*, 503–510.

Williams, A.R., and Hare, J.M. (2011). Mesenchymal stem cells: biology, pathophysiology, translational findings, and therapeutic implications for cardiac disease. Circ. Res. *109*, 923–940.

Wood, S.L., Pernemalm, M., Crosbie, P.A., and Whetton, A.D. (2014). The role of the tumor-microenvironment in lung cancer-metastasis and its relationship to potential therapeutic targets. Cancer Treat. Rev. *40*, 558–566.

Zaidi, M.R., and Merlino, G. (2011). The two faces of interferon-γ in cancer. Clin. Cancer Res. *17*, 6118–6124.

Cell Reports, Volume *20*

Supplemental Information

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

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Table S1

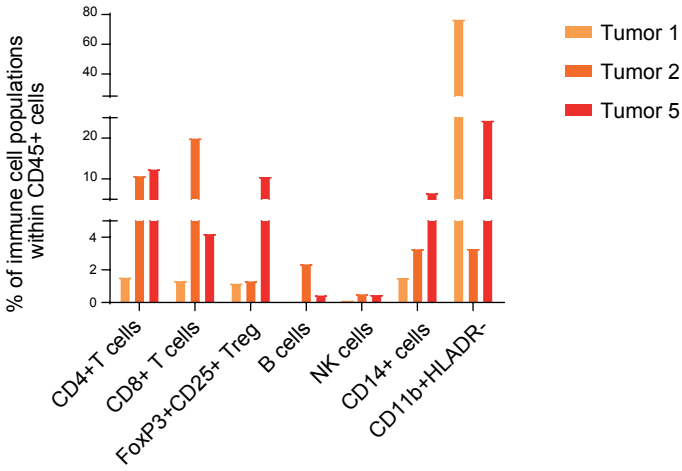
	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5
Histology	Abundant stroma, necrosis and desmoplasia; Smaller intratumoral immune infiltrate compared to others tumors	Tumor with very scant stroma; Large lymphocytic infiltrate, organized in clusters	Highly invasive tumor; necrotic areas, desmoplastic stroma (similar to patient 1); Less immune infiltrate compared to all others tumors	Tumor with scant stroma (comparable to patient 2); Abundant lymphocytic infiltrate	not available
Others			Partially bullous emphysema in non tumoral lung tissue	Diffuse emphysema in lung parenchyma	
IHC	CD3 > CD20 CD4 > CD8 (5:1) very few CD56, GrzB	CD3+++ (tumor and stroma totally infiltrated) >CD20 CD8 > CD4 CD20 in the periphery Lymphoid follicules no CD56, GrzB+++(CD8) FoxP3: small amount, in the periphery	CD20 > CD3 CD4 > CD8 (intratumor+periphery) CD20 in follicules (GC-like), CD4 also very few CD56 (everywhere) FoxP3: ++, in the stroma and in the tumor	CD3 > CD20 CD4 >CD8, diffuse (B cells more organized) lot of T cells, diffuse, septa+invasive margin lot of B cells, more organized very few CD56, not in contact with tumor cells lot of FoxP3 at the interface tumor-stroma	not available
PDL1 status	tumor PDL1-	tumor PDL1+++	tumor PDL1+ and PDL1- (two areas)	tumor PDL1+	
FACS	92.9% of CD45+ cells in total living cells CD11b+HLA-DR- 77.49% 4.42% CD3+ within CD45+ cells 42.11% CD8+ within CD3+ cells 49.08% CD4+ within CD3+ cells 23.11% of Treg within CD3+ cells 0.2% of NK cells within CD45+ cells	95.7% of CD45+ cells in total living cells CD11b+HLA-DR- 3.38% 44.74% CD3+ within CD45+ cells 62.05% CD8+ within CD3+ cells 32.29% CD4+ within CD3+ cells 2.96% Treg within CD3+ cells 0.59% of NK cells within CD45+ cells	not available	not available	80.2% of CD45+ cells in total living cells CD11b+HLA-DR- 24.61% 17.26% CD3+ within CD45+ cells 24.87% CD8+ within CD3+ cells 73.46% CD4+ within CD3+ cells 25.54% of Treg within CD3+ cells 0.55% of NK cells within CD45+ cells

+, ++, +++: relative abundance of immune infiltrate; GC-like: germinal center-like

Supplemental Table Legends

Table S1. Related to Figure 1. Tumor histology. Description of tumor histology for patients 1 to 4. Immune cell infiltrate in the tumor bulk was assessed by immunohistochemistry for patients 1, 2, 3 and 4 and by flow cytometry for patients 1, 2 and 5. PDL1 expression in tumor cells is also indicated.

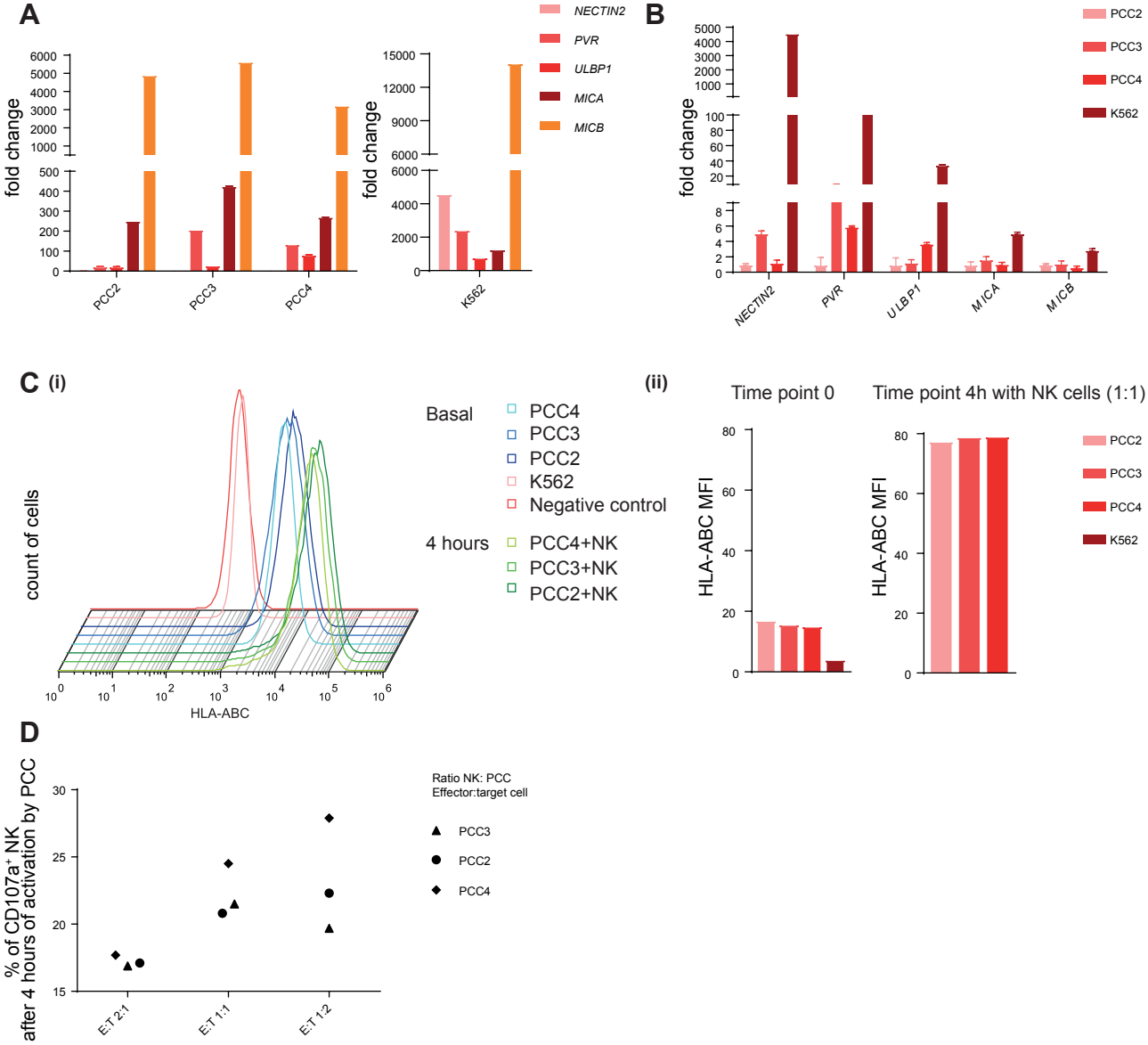
Figure S1


% of immune cell populations within CD45+ cells
80
60
40
20
10
4
2
0
Tumor 1
Tumor 2
Tumor 5
CD4+T cells
CD8+ T cells
FoxP3+CD25+ Treg
B cells
NK cells
CD14+ cells
CD11b+HLADR-

Supplemental Figure Legends

Figure S1. Related to Figure 1. Tumor-infiltrating immune cells. Characterization of tumor-infiltrating immune cells from patients 1, 2 and 5 by flow cytometry. Percentages of different immune cell populations were calculated in the tumor bulk within $CD45^+$ cells: $CD4^+$ T cells, $CD8^+$ T cells, $FoxP3^+CD25^+$ Treg, $CD19^+$ B cells, $CD3^-CD56^+$ NK cells, $CD14^+$ cells, $CD11b^+HLA\text{-}DR^-$ myeloid cells.

Figure S2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fold change
NECTIN2
PVR
ULBP1
MICA
MICB
PCC2
PCC3
PCC4
K562
B
fold change
PCC2
PCC3
PCC4
K562
NECTIN2
PVR
ULBP1
MICA
MICB
C (i)
count of cells
HLA-ABC
Basal
PCC4
PCC3
PCC2
K562
Negative control
4 hours
PCC4+NK
PCC3+NK
PCC2+NK
(ii)
Time point 0
Time point 4h with NK cells (1:1)
HLA-ABC MFI
PCC2
PCC3
PCC4
K562
D
% of CD107a+ NK after 4 hours of activation by PCC
Ratio NK: PCC
Effector:target cell
PCC3
PCC2
PCC4
E:T 2:1
E:T 1:1
E:T 1:2

Figure S2. Primary cancer cell profile. (A) (B) Relative gene expression of *NECTIN2*, *PVR*, *ULBP1*, *MICA* and *MICB* NK receptor ligands by PCC from patients 2, 3, and 4 and K562 as assessed by qPCR and analyzed as a fold change in expression. Results show the mean ± SD of triplicates, normalized to *GAPDH* expression. (A) For each tumor cell type, *NECTIN2* expression was set as the control condition. (B) For each NK cell receptor ligand, PCC2 expression set as the control condition. (C) HLA class I expression by PCC2, 3 and 4 and K562 cells in the basal condition and after 4 hours of co-culture with NK cells, analyzed by flow cytometry (i) Representative histograms (count of cells); (ii) HLA class I expression level expressed as mean fluorescence intensity (MFI). (D) Percentage of NK cell degranulation (CD107a expression) after 4 hours of activation by PCC from patients 2, 3 and 4 at different effector:target (E:T) ratios (2:1; 1:1; 1:2) (NK from one donor). Results were assessed by flow cytometry and analyzed with FlowJo software.

Figure S3

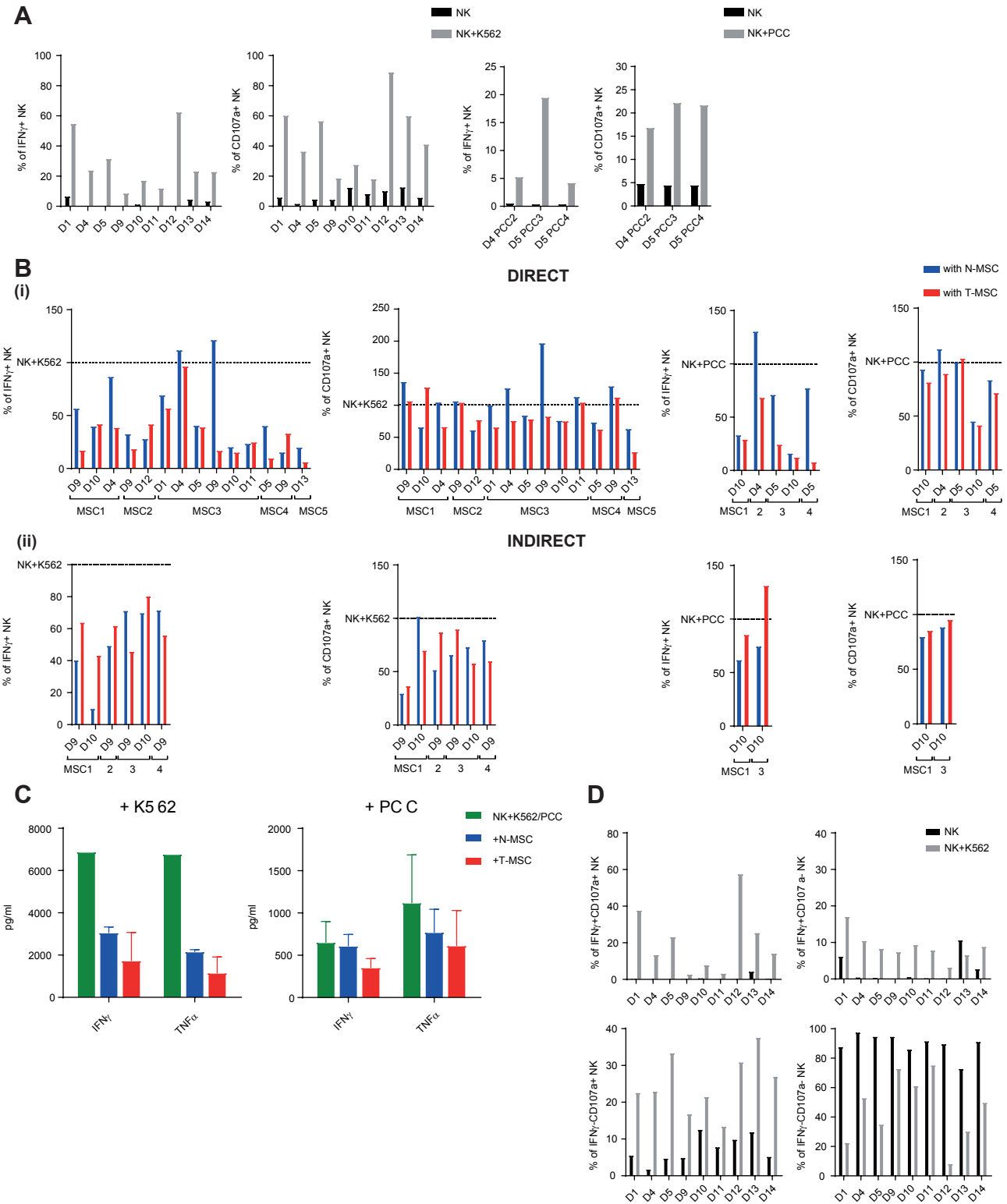
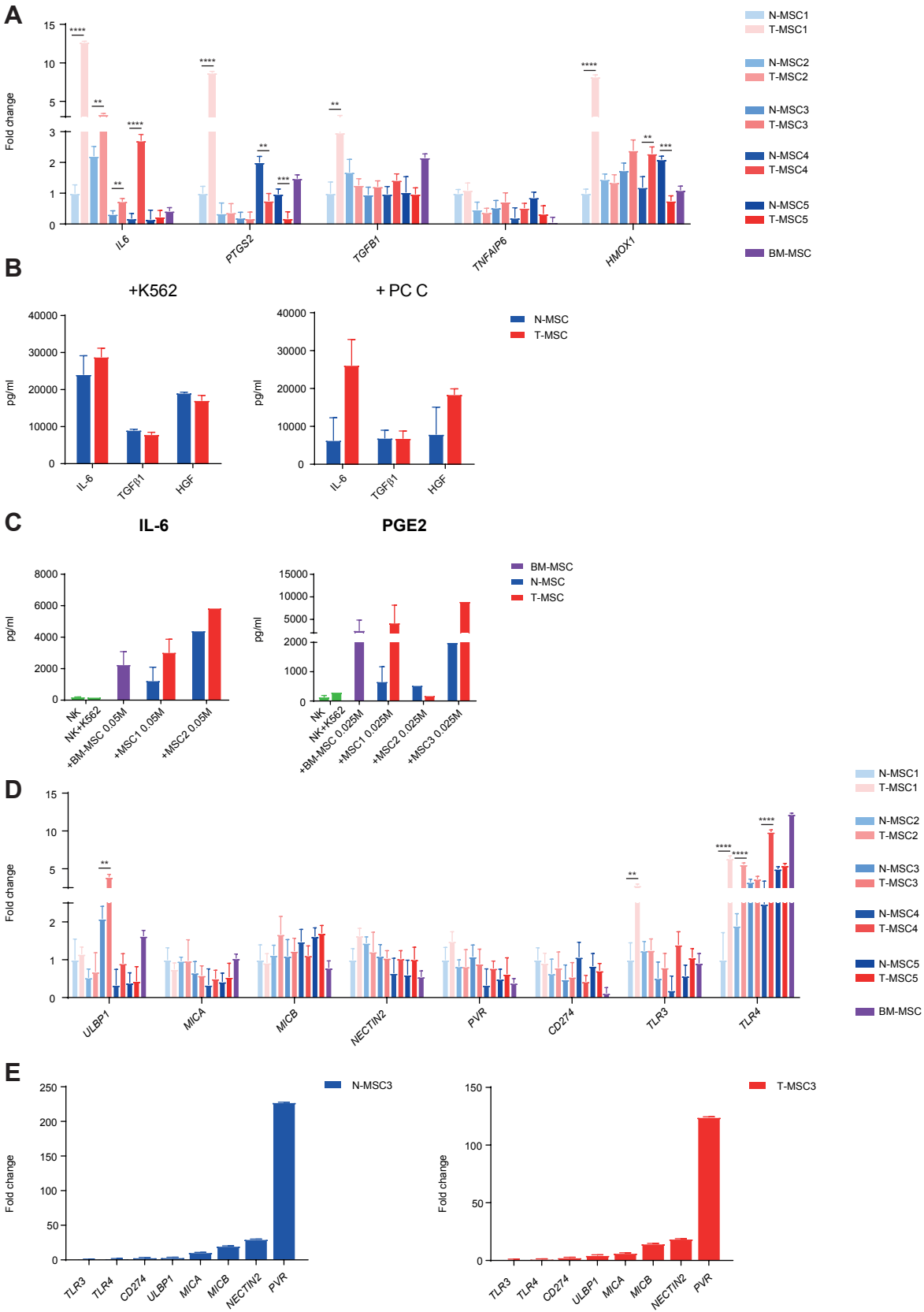

A
NK
NK+K562
NK+PCC
% of IFNγ+ NK
% of CD107a+ NK
D1 D4 D5 D9 D10 D11 D12 D13 D14
D4 PCC2 D5 PCC3 D5 PCC4
B
DIRECT
(i)
with N-MSC
with T-MSC
NK+K562
NK+PCC
MSC1 MSC2 MSC3 MSC4 MSC5
MSC1 2 3 4
(ii)
INDIRECT
C
+ K5 62
+ PC C
NK+K562/PCC
+N-MSC
+T-MSC
pg/ml
IFNγ
TNFα
D
NK
NK+K562
% of IFNγ+CD107a+ NK
% of IFNγ+CD107 a- NK
% of IFNγ-CD107a+ NK
% of IFNγ-CD107a- NK

Figure S3. Related to Figure 2. Heterogeneity of different donor NK cells. Comparison of NK cell activation levels between the different donors. (A) Percentages of IFN-γ^+ and CD107a$^+$ NK cells assessed by flow cytometry. NK cells were stimulated for 4 days with IL-2 (black bars) and activated by K562 cells (left panels) or PCC (right panels) for 4 hours (gray bars). (B) and (C): Percentages of IFN-γ^+ and CD107a$^+$ NK cells co-cultured with N-MSCs (blue) or T-MSCs (red) and activated by K562 cells (left panels) or PCC (right panels). Data were obtained by flow cytometry and normalized to those of NK cells cultured alone for the same duration and activated by K562 cells or PCC considered as 100 percent (horizontal dashed lines). (B) Graphs show the mean ± SEM of (i) eight experiments in direct MSC-NK co-culture with K562 (eight different NK cell donors, MSC1 (n=3 biological replicates); MSC2 (n=2); MSC3 (n=6); MSC4 (n=2); MSC5 (n=1)); and 3 experiments with PCC (three NK cell donors, MSC1 (n=1); MSC2 (n=1); MSC3 (n=2); MSC4) (ii) two experiments in indirect MSC-NK co-culture with K562 (two NK cell donors, MSC1 (n=2); MSC2 (n=1); MSC3 (n=2); MSC4 (n=1); one experiment with PCC (one NK cell donor, MSC1 (n=1); MSC3 (n=1)). (C) IFN-γ and TNFα secretion (pg/ml) by IL-2 stimulated NK cells cultured alone (green bars) or co-cultured with N- (blue bars) or T-MSCs (red bars), after activation by K562 or PCC target cells (one NK cell donor, MSC3 and MSC4). (D) Percentages of functional NK subpopulations from each donor: IFN-γ^+CD107a$^+$, IFN-γ^+CD107a$^-$, IFN-γ^-CD107a$^+$ and IFN-γ^-CD107a$^-$ NK cells, stimulated for 4 days with IL-2 and activated by K562 cells. Data were assessed by flow cytometry.

Figure S4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Fold change
IL6
PTGS2
TGFB1
TNFAIP6
HMOX1
N-MSC1
T-MSC1
N-MSC2
T-MSC2
N-MSC3
T-MSC3
N-MSC4
T-MSC4
N-MSC5
T-MSC5
BM-MSC
B
+K562
+ PC C
pg/ml
IL-6
TGFβ1
HGF
N-MSC
T-MSC
C
IL-6
PGE2
pg/ml
NK
NK+K562
+BM-MSC 0.05M
+MSC1 0.05M
+MSC2 0.05M
+BM-MSC 0.025M
+MSC1 0.025M
+MSC2 0.025M
+MSC3 0.025M
BM-MSC
N-MSC
T-MSC
D
Fold change
ULBP1
MICA
MICB
NECTIN2
PVR
CD274
TLR3
TLR4
E
Fold change
N-MSC3
T-MSC3
TLR3
TLR4
CD274
ULBP1
MICA
MICB
NECTIN2
PVR

Figure S4. Related to Figure 5. MSC profile. (A), (D) and (E) Relative gene expression by N- (blue bars), T-MSCs (red bars) and BM-MSCs (purple bars) assessed by qPCR and analyzed as a fold change in expression. Graphs show the mean of triplicates ± SD. *TBP* (A and D) or *GAPDH* (E) were used as housekeeping genes. Statistical significance was determined by a multiple T-test, corrected for multiple comparisons using the Holm-Sidak method and the p-value for significance (* indicates significance at $P < 0.05$; **, $P < 0.01$; ns, not significant). Paired N- and T-MSCs were compared for each patient. (A) Relative expression of *IL6*, *PTGS2*, *TGFB1*, *TNFAIP6* and *HMOX1* in MSCs from patients 1 to 5 and from the bone-marrow of one donor. N-MSC1 expression was set as the control condition. (B) Graph shows the concentration of IL-6, TGFβ1, and HGF secreted by MSCs (1, 3, and 4) in direct co-culture with one NK cell donor for 4 days and activated for 4 hours by K562 cells or PCC. (C) IL-6 and PGE2 secretion (pg/ml) by MSCs (N-MSCs (blue bar), T-MSCs (red bar) and BM-MSCs (purple bar)) co-cultured with NK cells at a 1:2 MSC:NK ratio (IL-6) and 1:4 ratio (PGE2), after activation by K562 cells. Secretion by NK cells cultured alone and activated by tumor cells is shown in green. Data are displayed as the mean ± SEM of 2 experiments (two NK cell donors, MSC1 (n=2); MSC2 (n=1); MSC3 (n=1) and BM-MSCs (n=2)) (D) Relative gene expression of NK receptor ligands, *ULBP1*, *MICA*, *MICB*, *NECTIN2*, *PVR*, *CD274*, *TLR3* and *TLR4* by MSCs from patients 1 to 5, and BM-MSCs. N-MSC1 expression was set as the control condition. (E) Relative gene expression of the same genes as in (D) by MSCs from patient 3. *TLR3* expression level was set as the control condition.

Figure S5

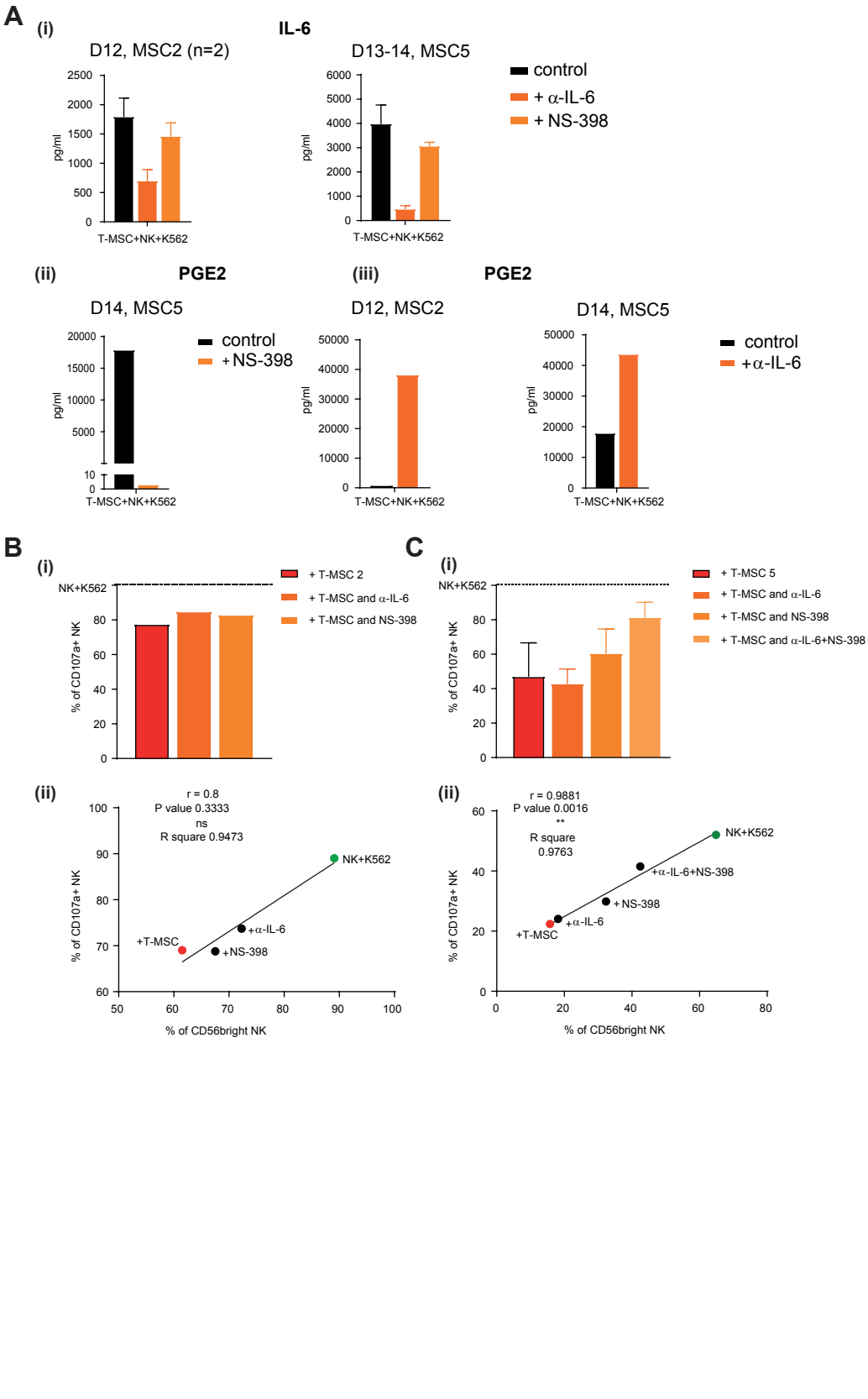

A
(i)
IL-6
D12, MSC2 (n=2)
D13-14, MSC5
control
+ α-IL-6
+ NS-398
pg/ml
T-MSC+NK+K562
(ii)
PGE2
D14, MSC5
control
+NS-398
(iii)
PGE2
D12, MSC2
D14, MSC5
control
+α-IL-6
B
(i)
NK+K562
+ T-MSC 2
+ T-MSC and α-IL-6
+ T-MSC and NS-398
% of CD107a+ NK
(ii)
r = 0.8
P value 0.3333
ns
R square 0.9473
NK+K562
+α-IL-6
+T-MSC
+NS-398
% of CD56bright NK
C
(i)
NK+K562
+ T-MSC 5
+ T-MSC and α-IL-6
+ T-MSC and NS-398
+ T-MSC and α-IL-6+NS-398
% of CD107a+ NK
(ii)
r = 0.9881
P value 0.0016
**
R square
0.9763
NK+K562
+α-IL-6+NS-398
+ NS-398
+α-IL-6
+T-MSC
% of CD56bright NK

Figure S5. Related to Figure 6. Targeting T-MSC immunosuppressive activity. (A) Graphs show the concentrations of IL-6 and PGE2 (pg/ml) secreted by T-MSCs alone or T-MSCs with the indicated inhibitors (anti-IL-6 neutralizing antibody and NS-398) in the supernatant of a 4 days MSC-NK co-culture and after NK activation by K562 target cells. (i) Level of IL-6 secretion inhibition obtained with the indicated inhibitors and MSC2 in one experiment (one NK donor, in duplicate), and MSC5 in two experiments (two NK donors). (ii) Level of PGE2 secretion obtained with NS-398 and (iii) anti-IL-6 antibody. Results are representative of one experiment with MSC5 and one with MSC2. (B) and (C) (i) Percentage of $CD107a^{+}$ NK cells co-cultured with T-MSCs alone or T-MSCs with the indicated inhibitors and activated by K562 cells. Data were analyzed by flow cytometry and normalized to the control condition (NK cells alone activated by K562 cells, horizontal dashed line). (ii) Correlation between the percentage of $CD56^{bright}$ NK cells and of $CD107a^{+}$ NK cells in different culture conditions. The statistical test used was the Spearman correlation coefficient r; significance determined at $P < 0.05$ *; $P < 0.01$ **; ns, not significant, p-values are reported; linear regression line with R square for goodness of fit. (B) Results are shown for one rescue experiment (single NK cell donor, MSC2). (C) Results obtained from two experiments (two NK cell donors, MSC5 (n=2)).

Supplemental Experimental Procedures

Primary MSC isolation
T-MSCs and N-MSCs were isolated after mechanical and enzymatic disruption of the fresh primary human tumor samples and adjacent macroscopically normal lung tissue (project authorization n°131/12), in IMDM (GIBCO) supplemented with collagenase II and IV (0.5 mg/ml, GIBCO) and DNAse I (0.1 mg/ml, Roche) for 2h at 37°C.

Multilineage differentiation potential of MSCs
MSC differentiation potential along osteocytic, chondrocytic and adipocytic lineages was assessed in culture using three different conditioned media. For induction of osteocytic and adipocytic differentiation, MSCs were seeded at 0.1 M cells/well in 12-well plates, in 1 ml medium/well. When MSCs reached 80% confluence, cells for osteocytic induction were treated with DMEM (GIBCO), supplemented with PS 1%, FCS 10%, NEAA 1%, APP (Ascorbic Acid P; 50 µg/ml, Sigma), Dexamethasone (10^{-7} M, Sigma) and βGP (betaGlycerophospate; 5 mM, Sigma). For adipocytic differentiation, MSCs were treated with IMDM, supplemented with 1% PS, 10% FCS and 1% NEAA, ITSS (Insulin Transferin Sodium Selenite; 10 µg/ml, Roche), Dexamethasone (10^{-6} M), Indometacine (100 µM, Fluka) and IBMX (3 Isobutyl 1 Methylxanthine, 100 µM, Sigma). Control conditions for both inductions are MSCs cultured in MSC medium. For chondrocytic induction, MSCs were cultured as a pellet at 0.5 M cells/pellet, in 1ml medium. Induction medium was composed of DMEM (high glucose), 1% PS, ITSS (10 µg/ml) and linoleic acid (1 mg/ml, Sigma), with AAP (50 µg/ml), Dexamethasone (10^{-7} M), and TGFβ (10 ng/ml, Miltenyi Biotec). Control medium consisted of DMEM, 1% PS, ITSS (10 µg/ml) and linoleic acid (1 mg/ml). Following the treatments, cells were fixed and stained with oil red O (adipocytes), silver nitrate for von Kossa staining (osteocytes), and Alcian blue (chondrocytes).

Immunohistochemistry (IHC)
T- and N-MSCs grown in 24-well plates (40,000 cells/well) for 24 hours were washed twice in PBS, then were fixed at room temperature in 4% PFA, and were stained with anti-vimentin (#M0725, Dako) and anti-α-SMA (#ab5694, Abcam) antibodies.

Characterization of tumor-infiltrating immune cells by IHC
Whole-tissue sections from the SCC were formalin-fixed and paraffin-embedded (FFPE) and stained with antibodies to assess immune cell infiltration. Haematoxylin/eosin staining (J.T. Baker; Merk) and staining with anti-CD3 (2GV6, #790-4341, Roche), -CD20 (#CD20-L26-L-CE, Novocastra), -CD68 KP1 (#GA60961-2, DAKO), -PD-L1 (#SP263, Roche Ventana), -Granzyme B (GrB-7, #MON7029-1, MONOSAN), -CD56 (#NCL-L-CD56-1B6, Novocastra), -CD8 (C8/144B, #MA5-13473, DAKO), -PD1 (#AF1086, R&D), -CD4 (SP35, #790-4423, Ventana), -FoxP3 (236A/E7, #ab20034, Abcam) antibodies were performed.

Flow Cytometry
For all experiments, cells were first incubated 15 minutes with FcR Blocking Reagent (Miltenyi), and then stained for 30 min with conjugated antibodies at 4°C. Before acquisition, cells were washed in PBS and fixed in 1% PFA. All samples were acquired with a Gallios Cytometer (Beckman Coulter) and data analyzed using FlowJo version10 software. The initial gating strategy included cell size selection, cell doublet and dead cell exclusion (with fixable viability dye (LIVE/DEAD®, #L-34963, Life Technology) or DAPI). Beads were used to set compensation for multicolor flow cytometry experiments (VersaComp Antibody Capture Bead kit, #22804, Beckman Coulter). Figures and graphs were made with GraphPad Prism7 software.

Characterization of tumor-infiltrating immune cells. Immune infiltrates in the tumor bulk of Patient 1, 2 and 5 were assessed using anti-CD45 (Alexa700, #560566, BD, 1:20), -CD4 (PE, #555347, BD, 1:40), -CD8 (Pacific Blue, #558207, BD, 1:20), -CD3 (PC7, #41116015, BC, 1:20), -CD19 (FITC, #11-0199-41, eBioscience, 1:40), -CD56 (ECD, #41116015, BC, 1:40), -HLA-DR (APC, #559866, BD, 1:20), -CD14 (PE, #557154, BD, 1:10), -CD16 (PerCP-Cy5.5, #560717, BD, 1:20), -CD11b (APC-eFluor780, #47-0118-41, eBioscience, 1:20) antibodies. Regulatory T cells (Tregs) were stained with anti-human FoxP3 Staining Set FITC from eBioscience (#71-5776-40), according to the manufacturer's protocol. $CD4^+$ T cells were defined as $CD45^+CD3^+CD4^+$, $CD8^+$ T cells as $CD45^+CD3^+CD8^+$, regulatory T cells as $CD45^+CD3^+CD4^+CD25^+FoxP3^+$, B cells as $CD45^+CD3^-CD19^+CD14^-CD16^-CD56^-$, NK cells as $CD45^+CD3^-HLA\text{-}DR^-CD56^+CD16^{+/-}$, myeloid cells as $CD45^+CD3^-CD11b^+HLA\text{-}DR^{+/-}$, and $CD14^+$ cells as $CD45^+CD3^-CD16^{+/-}CD56^-HLA\text{-}DR^+CD14^+$cells.

Characterization of tumor cells. K562 cells and primary lung carcinoma cells (PCC) were thawed, blocked with FcR Blocking Reagent and stained with anti-HLA-ABC (VioGreen, #130-101-449, Miltenyi) antibodies. DAPI was used to mark living cells. Signal-to-noise ratio was calculated as the MFI (mean fluorescence intensity) of the positive cells divided by the MFI of the negative cells.

NK cell isolation and culture

Buffy coats (project authorization n° P-108) were diluted 1:4 in PBS and NK cells isolated from PBMC (Lymphoprep, StemCell Technologies) using a negative sorting kit (NK Isolation Kit, #130-092-657, Miltenyi), with 95% purity.

Primers used in this work

Genes	Primers	Amplicon size (bp)
PTGS2	FW 5' – CTG GCG CTC AGC CAT ACA G – 3'	94
	RV 5' – CGC ACT TAT ACT GGT CAA ATC CC – 3'	
TGFB1	FW 5' – CAG ATC CTG TCC AAG CTG – 3'	270
	RV 5' – TCG GAG CTC TGA TGT GTT – 3'	
IL6	FW 5' – CAG TTG CCT TCT CCC TGG G – 3'	111
	RV 5' – TGA GTG GCT GTC TGT GTG GG – 3'	
IL2RA	FW 5' – CGC AGA ATA AAA AGC GGG TCA – 3'	116
	RV 5' – ACT TGT TTC GTT GTG TTC CGA – 3'	
MICA	FW 5' – CTT CAG AGT CAT TGG CAG ACA T – 3'	172
	RV 5' – TGT GGT CAC TCG TCC CAA CT – 3'	
MICB	FW 5' – TCT TCG TTA CAA CCT CAT GGT G – 3'	168
	RV 5' – TCC CAG GTC TTA GCT CCC AG – 3'	
NECTIN2(CD112)	FW 5'- GGA TGT GCG AGT TCA AGT GCT – 3'	179
	RV 5' – TGG GAC CCA TCT TAG GGT GG – 3'	
PVR (CD155)	FW 5' – TGG AGG TGA CGC ATG TGT C – 3'	105
	RV 5' – GTT TGG ACT CCG AAT AGC TGG – 3'	
ULBP1	FW 5' – TAA GTC CAG ACC TGA ACC ACA – 3'	174
	RV 5' – TCC ACC ACG TCT CTT AGT GTT – 3'	
TLR3	FW 5' – TTA AAG AGT TTT CTC CAG GGT GTT TT – 3'	125
	RV 5' – AAT GCT TGT GTT TGC TAA TTC CAA – 3'	
TLR4	FW 5' – CCC CTT CTC AAC CAA GAA CCT – 3'	123
	RV 5' – ATT GTC TGG ATT TCA CAC CTG GAT – 3'	
CD274 (PDL1)	FW 5' - TGGCATTTGCTGAACGCATTT – 3'	120
	RV 5' – TGCAGCCAGGTCTAATTGTTTT – 3'	
HMOX1	FW 5' – AAGACTGCGTTCCTGCTCAAC – 3'	247
	RV 5' – AAAGCCCTACAGCAACTGTCG – 3'	
TNFAIP6	FW 5' – TCTGGCAAATACAAGCTCACC – 3'	143
	RV 5' – CTGCCCTTAGCCATCCATCC – 3'	
GAPDH	FW 5' – GGC TCT CCA GAA CAT CAT CC – 3'	
	RV 5' – CCT GCT TCA CCA CCT TCT TG – 3'	
PPIA	FW 5' – ACC GTG TTC TTC GAC ATT GC – 3'	
	RV 5' – TTA TGC CGT GTG AAG TCA CC – 3'	
TBP	FW 5' – CGG CTG TTT AAC TTC GCT TC – 3'	
	RV 5' – CAC ACG CCA AGA AAC AGT GA – 3'	

3. PART II: COMPLEMENTARY UNPUBLISHED DATA

3.1 Results

In view of the large amount of data collected during this thesis, some of them were not included in the peer-reviewed manuscript but are presented here as complements to specific sections of the article, or as preliminary data. We start by giving more detailed descriptions and discussions of some figures of the paper in sections 3.1.1 to 3.1.3. Then, in sections 3.1.4 to 3.1.6, we present supplemental data which directly complement the manuscript and reveal additional details of the MSC-NK interaction. Finally, section 3.1.7 presents preliminary data on the role of the tumor cells in T-MSC specific profile induction.

3.1.1 Tumor-Infiltrating Immune Cells. Related to Table S1 and Figure S1.

Surgically-removed primary tumors from the five studied patients were subjected to a histological analysis and immunostaining was performed on tissue sections for CD3, CD4, CD8, CD20, CD68 KP1, Granzyme B, CD56, PD-L1, PD1, and FoxP3 (Table S1). The immune cell infiltrate of tumors from three patients (patients 1, 2 and 5) was assessed by flow cytometry for the expression of lineage-specific markers, using specific gating strategies (Fig. S6 A-D). Myeloid cells, defined as $CD45^{+}CD11b^{+}HLA\text{-}DR^{-}$, constituted the major (77.49%) infiltrating sub-population in the tumor bulk of patient 1, with only 4.42% of cells corresponding to T lymphocytes, defined as $CD45^{+}CD3^{+}$ cells. In contrast, T lymphocytes were the dominant sub-population in the infiltrate of the tumor bulk of patient 2 (44.74%), with only 3.38% of cells displaying a myeloid phenotype. The infiltrate of the tumor bulk from patient 5 fell between those of the other two patients, with 24.61% of myeloid cells and 17.26% of T lymphocytes. Among the $CD3^{+}$ cells in the tumor infiltrate of patient 2, 62.05% were $CD8^{+}$ and 32.29% were $CD4^{+}$. The $CD45^{+}CD3^{+}$ infiltrate in the tumor from patient 1 was composed of 49.08% $CD4^{+}$

Figure S6

A

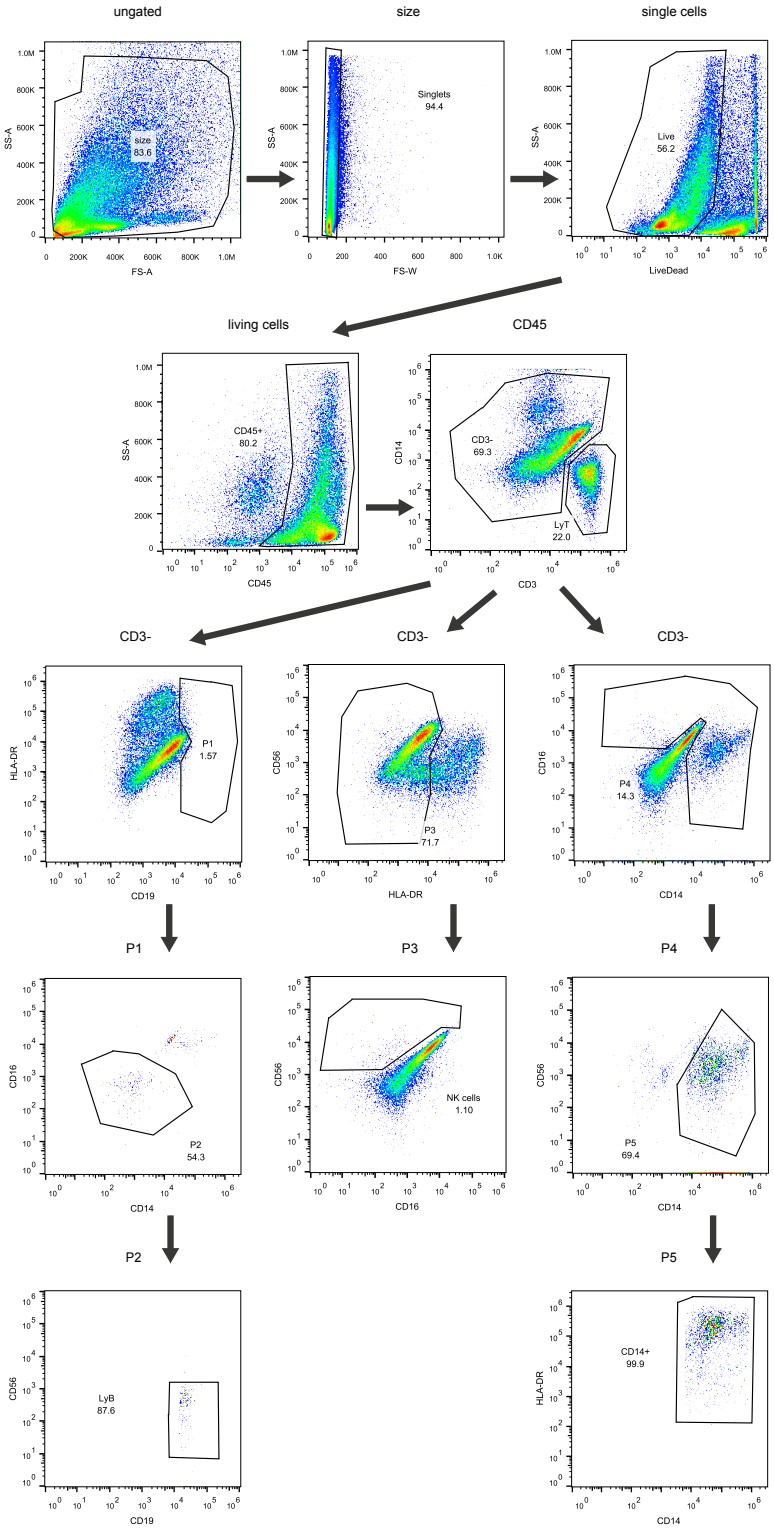
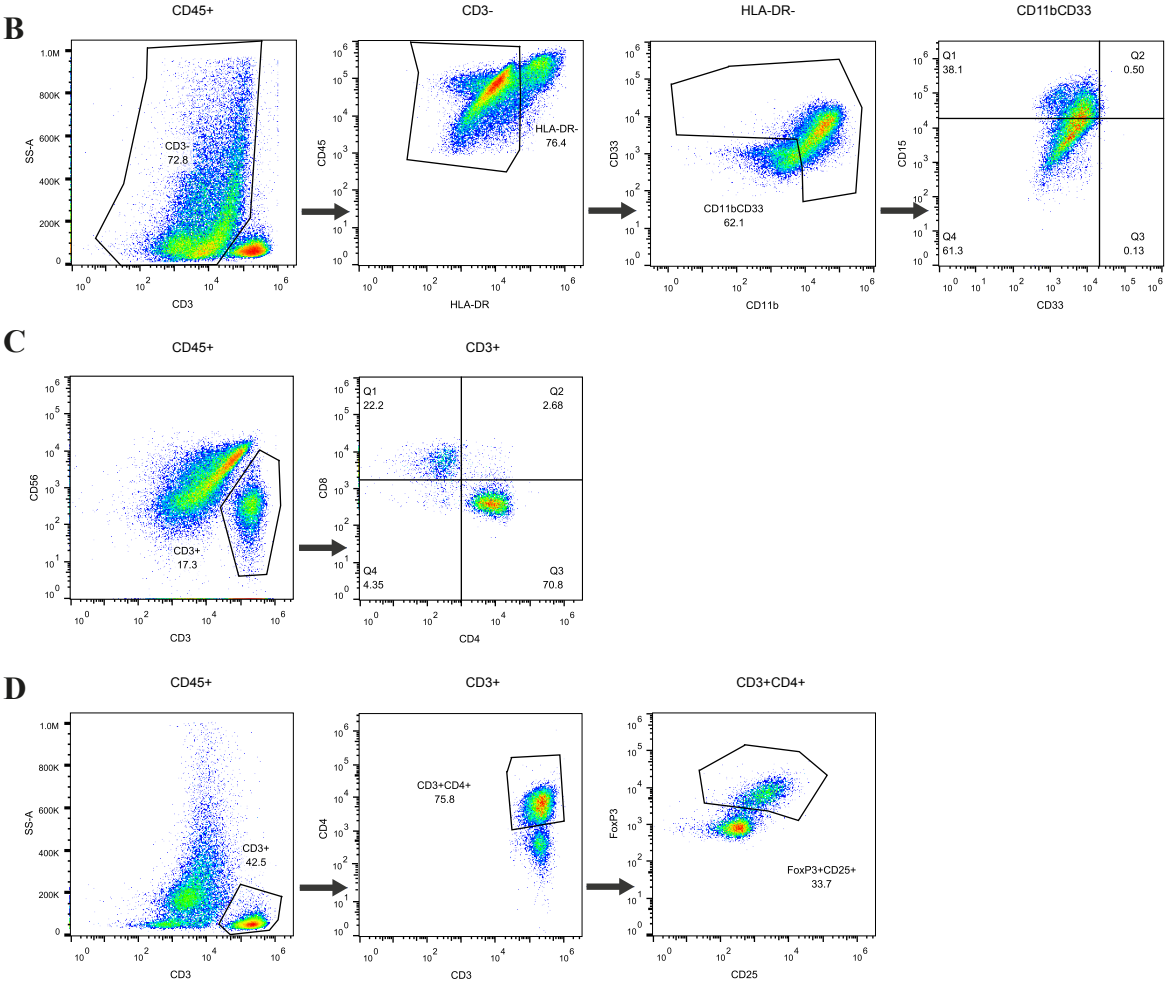


Figure S6. Gating procedure for characterization of tumor-infiltrating immune cells. Gating strategy used for flow cytometry data on tumor bulk sample from one representative patient (patient 5) to determine the frequencies of (A) NK, T, B and $CD14^+$ cells; (B) Myeloid cells; (C) $CD8^+$ T cells; (D) $FoxP3^+CD25^+$ Treg starting from $CD45^+$ gated cells.

Figure S6 bis



and 42.11% $CD8^+$ cells. Patient 5 tumor infiltrating $CD3^+$ cells were composed of 73.46% $CD4^+$ and 24.87% $CD8^+$ cells. Interestingly, regulatory ($FoxP3^+$) T cells represented a substantial fraction of $CD3^+$ cells (23.11%), in patient 1 and 5 (25.54%) but only 2.96% in patient 2. NK cells constituted less than 1% of the total $CD45^+$ tumor infiltrate in all three patients (0.2% of $CD45^+$ cells for patient 1, 0.59% for patient 2 and 0.55% for patient 5). These data were consistent with IHC staining (Fig. S1).

The percentage of T-MSCs in the tumor bulks was also assessed by flow cytometry and was higher than the percentage of N-MSCs in normal tissues, but represented less than 1% of the total bulk population for all patients (data not shown). An analysis from other human cancers also showed that MSCs represented about 1% of total cells present in the tumor (Brennen et al., 2012).

3.1.2 Ligands for NK receptors expressed by tumor cells. Related to figures S1, S2 and Table S1

K562 cell line is known to be a NK preferential target due to the lack of HLA class I expression and the expression of different ligands for NK activating receptors. We confirmed that the K562 cell line transcriptionally expressed high levels of *CD112*, *CD155*, *ULBP1*, *MICA*, and *MICB*. In contrast, PCC from lung carcinoma did not express *CD112* and showed only low levels of *CD155* and *ULBP1*, whereas they expressed the NKG2D ligands *MICA* and *MICB* (Fig. S2 A and B). Expression also differed between patients. PCC isolated from patient 2 were the less immunogenic according to their lower levels of expression of all tested ligands (Fig. S2 B). It is interesting to note that, for this patient, expression of NK ligands by tumor cells and immune infiltrate of the tumor bulk differed from others patients. Flow cytometry and IHC revealed a

higher content of $CD8^+$ T cells in the tumor, compared to $CD4^+$ cells (Fig. S1 and Table S1). $CD8^+$ T cells, in contrast to NK cells, required HLA-I expression to be activated and to kill tumor cells. PCC from all patients expressed HLA-I, and this surface expression was induced by the presence of NK cells in co-culture (Fig. S2 C). Compared to other patients, Treg infiltration was very low in patient 2 tumor bulk. Patient 2 tumor was also highly positive for PD-L1 and more immune-infiltrated compared to all other tumors (Table S1).

3.1.3 Ligands for NK receptors expressed by MSCs. Related to figure S4

Lung MSCs expressed several ligands for NK activating receptors (Fig. S4 D) that have been studied in NK-mediated MSC killing (Spaggiari et al., 2006b). However, these ligands also contribute to NK cell exhaustion and subsequent down-regulation of NK receptors, similarly to prolonged contact with tumor cells inducing NK cell anergy. In this work, we showed that lung MSCs highly expressed Nectin-2 and PVR even more, which are ligands for DNAM-1. Besides, compared to DNAM-1 ligands, MICA/B (ligands for NKG2D) were expressed at a lower level.

MSCs can bear ligands for surface molecules involved either in the activation or inhibition of lymphocyte functions (Poggi and Zocchi, 2015; Turley et al., 2015). Indeed, DNAM-1 can react with MSC-expressing PVR and Nectin-2 (Bottino et al., 2003). This recognition generally leads to the killing of the DNAM-1L-bearing cell. However, it is also possible that DNAM-1L can bind TIGIT or CD96 inhibitory molecules on lymphocytes, leading to the impairment of an immune response (Blake et al., 2016). Moreover, NKG2DL and DNAM-1L are widely expressed in several carcinomas and leukemic disorders, suggesting that MSCs, rather than tumor cells, can be a suitable alternative target in TME.

Other groups have shown that BM-MSCs can secrete Matrix Metalloproteinases (MMPs) and other proteases able to shed ligands from their surface (Giuliani et al., 2014; Nausch and Cerwenka, 2008) and to induce NK cell exhaustion. NKG2D ligand (NKG2DL) expressed on MSCs can be shed in the extracellular milieu (sMICA e.g.), thus interfering with NKG2D-mediated recognition of tumor target cells (Poggi et al., 2014). Indeed, ADAMs can function as sheddases of NKG2DL (Zocchi et al., 2015), contributing to the immune cell escape of neoplastic cells. Recently, a soluble form of PVR (sCD155) was detected in patients with various tumors (Iguchi-Manaka et al., 2016). Soluble CD155 levels were significantly higher in the sera of patients with lung, gastrointestinal, breast, and gynecologic cancers than in sera from healthy donors (Iguchi-Manaka et al., 2016).

Future investigations of the mechanisms controlling the regulation of NKG2D and DNAM-1 ligands should offer a better understanding of the MSC-NK interaction and a better assessment of NK ligands as potential targets for therapy. The idea would be to avoid overexposure of NK cells to a continuous positive signal leading to their exhaustion and their inability to respond to later stimulations by tumor cells. Interestingly, MSCs (T-MSCs more than N-MSCs) also expressed PD-L1 (Fig. S4 D), that can directly inhibit NK cells and we would like to test this interaction in future co-culture experiments.

3.1.4 MSC: NK ratios in co-culture. Related to the set-up of co-culture experiments

Before choosing the 1:2 ratio for subsequent functional co-culture experiments, different MSC:NK ratios were tested (from 1:1 to 1:16) (Fig. S7). At 1:2 MSC:NK ratio, a recurrent immunosuppressive effect of MSCs was observed, with large differences between N- and T-MSCs (Fig. S7). Comparable results were observed with 1:4 MSC:NK ratio.

Figure S7

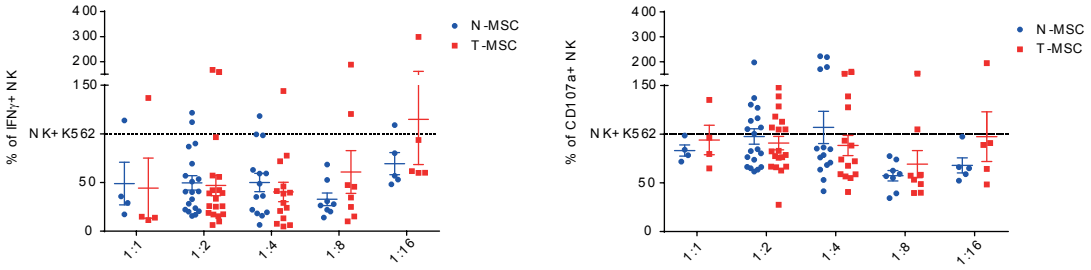


Figure S7. NK:MSC ratios. NK cell activation (percentages of CD107a and intracellular IFN-γ positive cells) by K562 following N- and T-MSC co-culture at different NK: MSC ratio (1:1 to 1:16), as assessed by flow cytometry. Results were normalized to those of NK cells cultured without MSCs for the same duration and activated by target cells (the control condition is represented by the horizontal dashed line). Results show the mean ± SEM. For 1:1 MSC:NK ratio: two experiments with MSC1 (n=1), 2 (n=1), 3 (n=1) and 4 (n=1)); 1:2 ratio (nine experiments, MSC1 (n=4), 2 (n=3), 3 (n=8), 4 (n=2), 5 (n=2)); 1:4 ratio (five experiments, MSC1 (n=4), 2 (n=2), 3 (n=6), 4 (n=2)); 1:8 ratio (3 experiments, MSC1 (n=2), 2 (n=1), 3 (n=4), 4 (n=1)); 1:16 ratio (2 experiments, MSC1 (n=1), 2 (n=1), 3 (n=3)).

3.1.5 Inhibition of NK cell proliferation by MSCs. Related to MSC effect on NK function

BM-MSCs have been largely studied for their ability to decrease T and NK cell proliferation (Krampera et al., 2006; Spaggiari et al., 2008), through PGE2 and IDO secreted factors. Lung MSCs reduced NK proliferation when co-cultured at 1:4 MSC:NK ratio (Fig. S8 A). Interestingly, at 1:2 MSC:NK ratio, at which a decreased NK function and a reduced expression of NK activating receptors were observed, NK proliferation was less affected and even preserved for some MSC patients (Fig. S8 A). Whereas the modulation of lung MSCs on NK cells was dependent on MSC:NK ratios, BM-MSCs exerted their inhibition in all tested conditions. Furthermore, MSC modulation of NK proliferation occurred similarly in both CD56 NK subsets (Fig. S8 B), and did not explain the enrichment in the $CD56^{dim}$ subset through a selective inhibition of the $CD56^{bright}$ population.

3.1.6 ICAM-1 expressed by T-MSCs modulates NK degranulation but not cytokine secretion. Related to the investigation of molecules potentially involved in NK function modulation.

MSCs express high levels of several molecules, namely IL-6, COX-2/PGE2 and ICAM-1. IL-6 and COX-2 have been deeply investigated in this work and presented in the article, but ICAM-1 also seemed to play a role in NK function modulation by MSCs. At the basal level, before co-culture, T-MSCs from Patients 2 and 5 expressed higher levels of *ICAM1* than their non-tumor tissue-derived counterparts (Fig. S9 A), and *ICAM1* was highly induced over time in co-culture with NK cells (Fig. S9 A). The antibody blockade of ICAM-1 (MAB720) in MSCs from Patient 5 (Fig. S9 B) failed to restore IFN-γ production by NK cells in response to K562. In contrast, NK degranulation was restored at a similar level to the one reached by COX-2 inhibition. Thus,

Figure S8

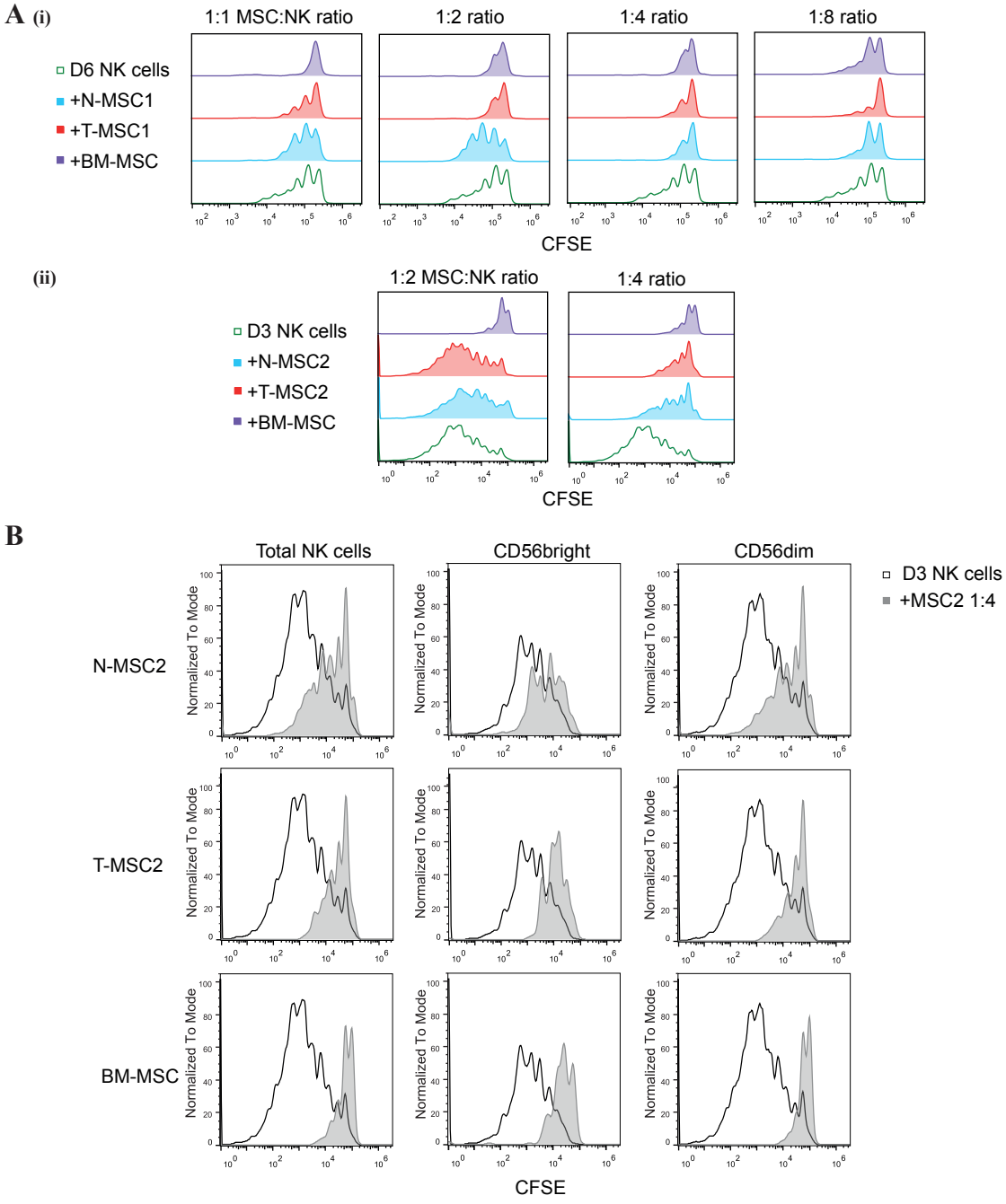
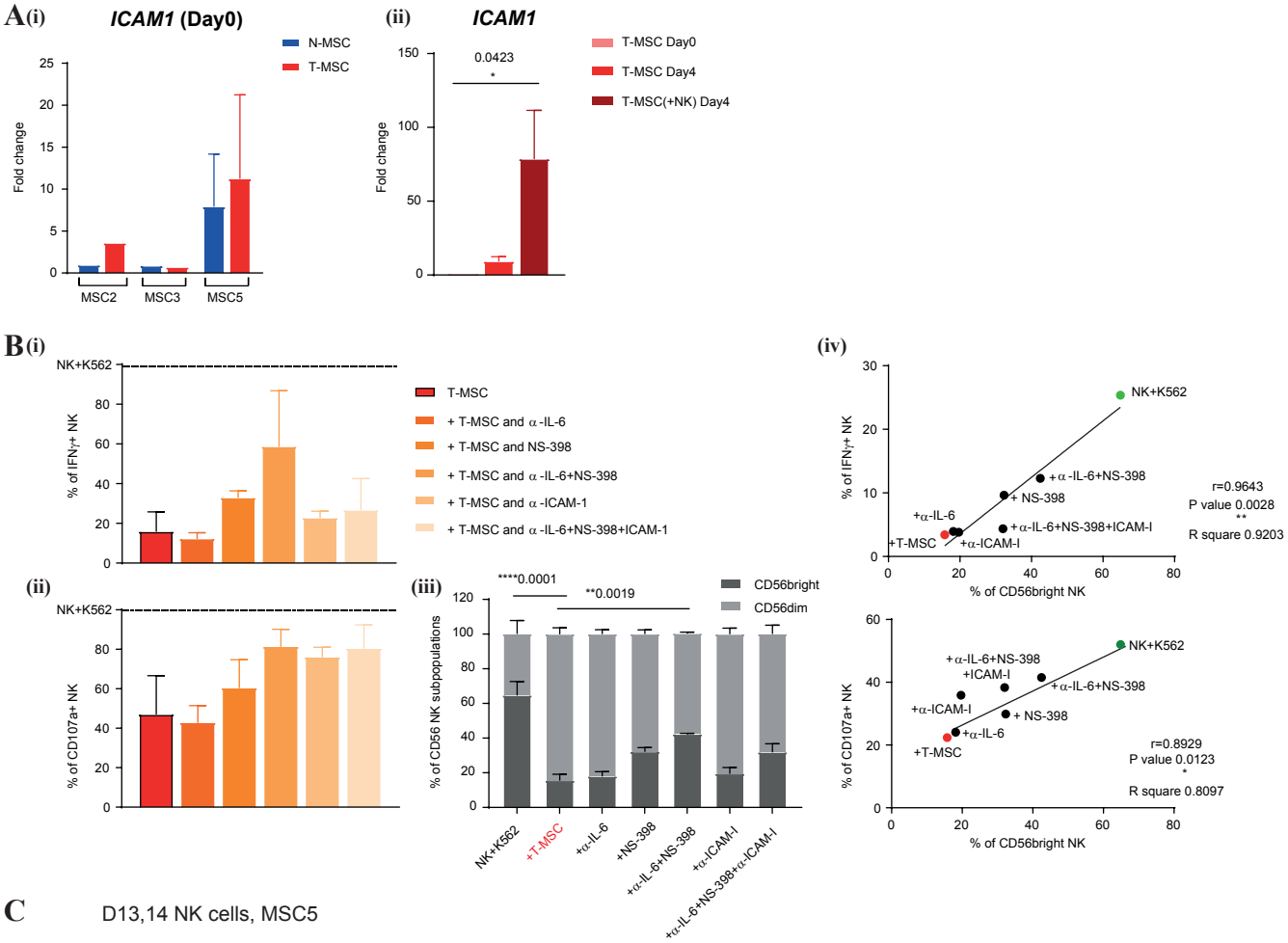


Figure S8. NK proliferation. (A) and (B): Histograms showing the distinct generations of proliferating NK cells, stained with CFSE beforehand and cultured alone or in the presence of MSCs (N-, T- or BM-MSCs) for 4 days. NK cells are defined as $CD45^{+}CD3^{-}CD56^{+}CD16^{+/-}$ cells and histograms are normalized to mode. (A) (i) one experiment with a single NK cell donor and MSCs from patient 1 and BM-MSCs, at different MSC: NK ratios (1:1 to 1:8); (ii) one experiment with a single NK cell donor and MSCs from patient 2 and BM-MSCs, at 1:2 and 1:4 MSC:NK ratios. (B) Histograms showing the proliferative peaks of the total NK cell population and of the $CD56^{bright}$ and $CD56^{dim}$ subsets, after co-culture with MSCs from patient 2, at 1:4 MSC:NK ratio.

Figure S9


A(i)
ICAM1 (Day0)
N-MSC
T-MSC
Fold change
MSC2
MSC3
MSC5
(ii)
ICAM1
T-MSC Day0
T-MSC Day4
T-MSC(+NK) Day4
0.0423
*
B(i)
NK+K562
% of IFNγ+ NK
T-MSC
+ T-MSC and α -IL-6
+ T-MSC and NS-398
+ T-MSC and α -IL-6+NS-398
+ T-MSC and α -ICAM-1
+ T-MSC and α -IL-6+NS-398+ICAM-1
(ii)
% of CD107a+ NK
(iii)
****0.0001
**0.0019
% of CD56 NK subpopulations
CD56bright
CD56dim
NK+K562
+T-MSC
+α-IL-6
+NS-398
+α-IL-6+NS-398
+α-ICAM-I
+α-IL-6+NS-398+α-ICAM-I
(iv)
% of IFNγ+ NK
% of CD56bright NK
r=0.9643
P value 0.0028
**
R square 0.9203
+α-IL-6+NS-398
+ NS-398
+α-IL-6
+α-IL-6+NS-398+ICAM-I
+T-MSC
+α-ICAM-I
% of CD107a+ NK
+α-IL-6+NS-398
+ICAM-I
+α-ICAM-I
r=0.8929
P value 0.0123
*
R square 0.8097

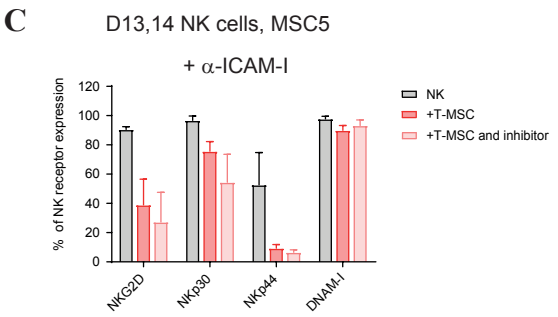

C
D13,14 NK cells, MSC5
+ α-ICAM-I
% of NK receptor expression
NK
+T-MSC
+T-MSC and inhibitor
NKG2D
NKp30
NKp44
DNAM-I

Figure S9: ICAM-1 is highly expressed by T-MSCs. (A) Relative gene expression of *ICAM1* by N- and T-MSCs 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, 5 (n=2)) and results show the mean ± SEM. (i) in the basal state (day 0 of co-culture); (ii) relative gene expression of *ICAM1* by T-MSCs collected at the indicated time points with and without direct co-culture with NK cells (four NK cell donors, MSC2, 3, 5(n=2)). Results show fold change in gene expression compared to T-MSCs at day 0. Statistical significance was determined by one-way ANOVA with Tukey's multiple comparisons test (* indicates significance at $P < 0.05$; **, $P < 0.01$; ns, not significant). (B) and (C) Data were analyzed by flow cytometry and results show mean values ± SEM. (B) Results are from two rescue experiments with two NK cell donor and MSCs from patients 3 and 5. Percentage of IFN-γ^+ (i) and CD107a$^+$ (ii) NK cells after activation by K562 cells in the presence of T-MSCs with or without anti-IL-6 neutralizing antibody and/or NS-398 and/or anti-ICAM-1 antibody. Results are normalized to NK cell activation by K562 cells in the absence of MSCs, considered to be 100 percent (horizontal dashed line). (iii) Percentage of K562-activated CD56^{bright} and CD56^{dim} NK subsets cultured alone or with T-MSCs in the presence or absence of inhibitors. Statistical significance was determined by 2-way ANOVA followed by Dunnett's multiple comparisons test, with K562-activated NK cells co-cultured with T-MSCs set as the control (red). (iv) Correlation between the percentage of CD56^{bright} NK cells and intracellular IFN-γ production/CD107a surface expression in different culture conditions. The statistical test used was the Spearman correlation coefficient r; p-values are reported; linear regression line with R square for goodness of fit. (C) Percentage of NK receptor expression after 4 days in culture alone (gray bar) or with T-MSCs in the absence (dark red bar) or presence of anti-ICAM-1 neutralizing antibody (light red bar). MSC5 (n=2), two NK cell donors.

ICAM-1 expressed by T-MSCs appeared to be more closely correlated with the regulation of NK cytotoxicity than with the cytokine production. In addition, NK cell brightness was almost not affected (Fig. S9 B (iii) and (iv)), in line with the inability of ICAM-1 blockade to restore NK cytokine production. Surprisingly, ICAM-1 inhibition slightly exacerbated NK receptor downregulation induced by Patient 5 T-MSCs, with the exception of DNAM-1 (Fig. S9 C).

Exploration of the role of ICAM-1 in MSC-mediated immunosuppression underscores the complexity of MSC-NK crosstalk, also highlighted by others (Casado et al., 2013) and the plasticity of MSCs in terms of their immunomodulatory functions. It is known that IFN-γ induces MSC expression of the enzymes IDO and COX-2 (Le Blanc and Davies, 2015), which generate immunosuppressive mediators, and of the adhesion receptor ICAM-1 (Ren et al., 2010). Ren et al. found that ICAM-1 and VCAM-1 were critical for MSC-mediated immunosuppression (Ren et al., 2010). Furthermore, ICAM-1 and VCAM-1 were found to be inducible by the concomitant presence of IFN-γ and inflammatory cytokines (TNFα or IL-1). ICAM-1 and VCAM-1 were required for lymphocyte–MSC adhesion and, when the function of the adhesion molecules was inhibited by blocking Abs or gene knockout, MSC-mediated immunosuppression was significantly reversed *in vitro* and *in vivo*. However, we did not confirm their observations in our human model, where only the cytotoxic function of NK cells was restored by adhesion blockade. In addition, NK activating receptors, that are commonly associated with NK cytotoxicity, were even more down-regulated in the concomitant presence of MSCs and anti-ICAM-1, than in the presence of T-MSCs alone.

As previously described, we observed a major role of PGE2 in MSC-mediated NK cytokine inhibition. We hypothesize that PGE2 was still secreted, even in the presence of anti-ICAM-1 antibody, due to the experimental setup of the co-culture that allowed close proximity between

cells. Blocking MSC-NK adhesion disturbed the balance that favored MSC immunosuppression, but the mechanisms are not fully understood and require more investigations.

3.1.7 MSC-tumor cell co-cultures. Preliminary results on the modulation of MSC phenotype by tumor cells

We have shown that N- and T-MSCs differed in their gene expression profile and their immunosuppressive potential toward NK cells, but how does the tumor – or the tumor microenvironment – contribute to the T-MSC specific characteristics? MSCs in tumors have been described as originating from various sources: either from recruited BM-MSCs, or from adipose tissue-derived stem cells, or from perivascular MSCs, or even from resident N-MSCs that, in contact with tumor cells and other components of the tumor microenvironment, could switch to a T-MSC phenotype (Karnoub et al., 2007; Kidd et al., 2012; Kucerova et al., 2010; Prantl et al., 2010; de Souza et al., 2016; Suzuki et al., 2011). Therefore, we co-cultured N-MSCs from patients 2, 3 and 4 for seven days with growing amounts of paired PCC or K562 at 1:2 and 1:5 MSC:tumor cell ratios and assessed their gene expression (Fig. S10 A) and secretory profiles (Fig. S10 B). *IL6* and *PTGS2* were significantly induced in N-MSCs in indirect co-culture and this phenomenon followed the increasing MSC:PCC ratios (Fig. S10 A). For patient 2 and 4, the presence of K562 also increased *IL6* gene expression (Fig. S10 A) and secretion (Fig. S10 B) by N-MSCs. Interestingly, PCC from patient 3 expressed *IL6* (data not shown), unlike other PCCs, suggesting that the tumor of patient 3 was enriched in IL-6 cytokine coming from at least these two sources: MSCs and PCC. *PTGS2* expression was induced in the presence of K562 cells in MSCs from all patients (Fig. S10 A). In contrast, *TGFB1* expression remained unchanged, except in patient 3 MSCs co-cultured with K562 at the highest MSC:tumor cell ratio, where its expression increased (Fig. S10 A).

Figure S10

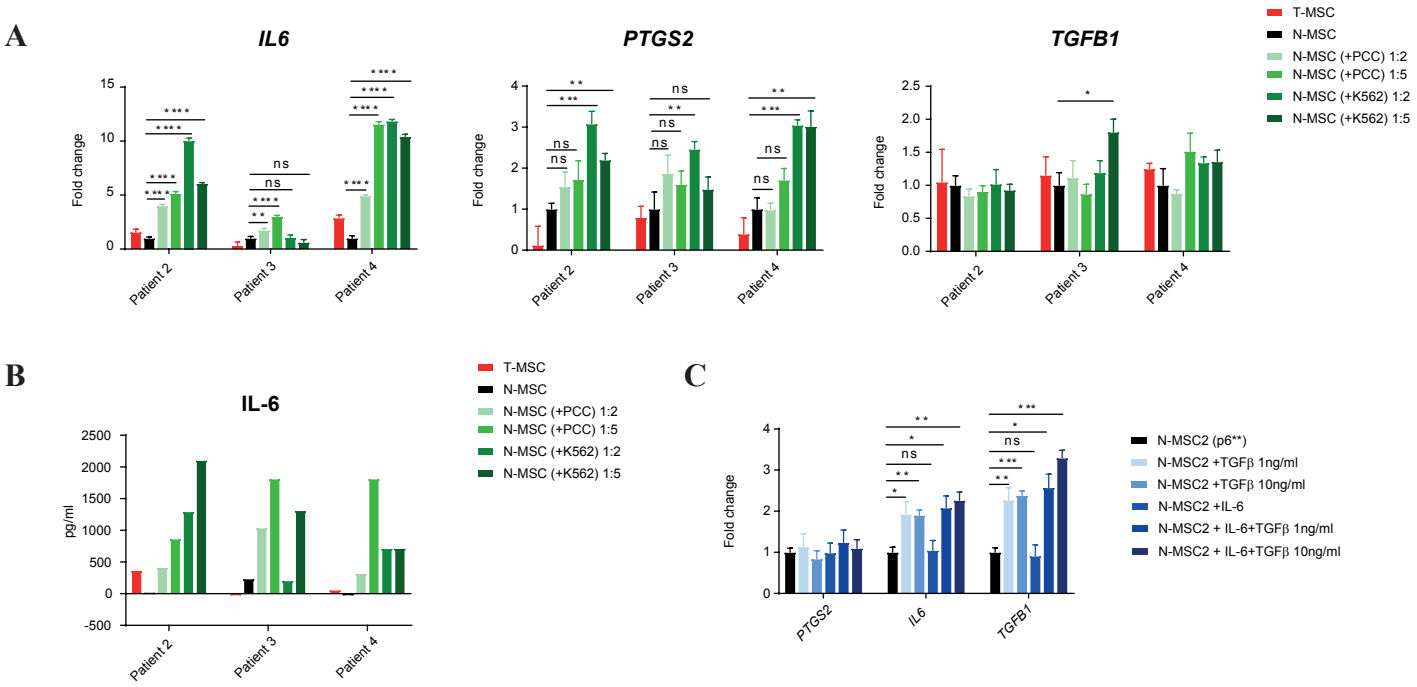


Figure S10: MSC-PCC co-culture. (A) (B) MSCs and K562 or PCC (patient 2, 3 and 4) were co-cultured for 7 days at different MSC:tumor cells ratios (1:2, 1:5) in indirect conditions and analyzed for *IL6*, *PTGS2* and *TGFB1* expression modulation and IL-6 secretion. (A) Relative gene expression of *IL6*, *PTGS2* and *TGFB1* by N-MSCs co-cultured for 7 days with tumor cells, assessed by qPCR and shown as a fold change in expression (N-MSC expression set as the control condition, in black bar). Gene expression by T-MSCs is reported as a control (red bar). Results show the mean ± SD of triplicates, normalized to *GAPDH* expression. Statistical significance was determined by a multiple T-test, corrected for multiple comparisons using the Holm-Sidak method and the adjusted p-value for significance (* indicates significance at $P < 0.05$; **, $P < 0.01$; ns, not significant). (B) IL-6 secretion (pg/ml) by MSCs from patients 2, 3 and 4 after 7 days in culture with or without K562 and PCC. (C) Relative gene expression of *IL6, PTGS2* and *TGFB1* by N-MSCs from patient 2 after 7 days of treatment with TGFβ1 (1 and 10ng/ml) and/or IL-6 (10ng/ml) and in control medium. Results show fold change in gene expression compared to N-MSCs. Statistical significance was determined by a multiple T-test, corrected for multiple comparisons using the Holm-Sidak method and the adjusted p-value for significance (* indicates significance at $P < 0.05$; **, $P < 0.01$; ns, not significant).

On one hand, the tumor is able to prime MSCs, on the other hand, MSCs themselves – as a part of the TME – contribute to create a niche that favors tumor growth (Karnoub et al., 2007; Kucerova et al., 2010; Prantl et al., 2010; Suzuki et al., 2011). T-MSCs expressed and secreted IL-6, TGFβ1 and PGE2 (Fig. S4. A and B, Fig. 5 A). To test the potential role of some of these cytokines likely to be present in the tumor microenvironment, we treated N-MSCs with IL-6 or TGFβ1 alone or in combination for 7 days. IL-6 treatment alone did not bring any significant modulation of *TGFB1*, *PTGS2* or *IL6* gene expression (Fig. S10 D). By contrast, TGFβ1 treatment, both alone and in combination with IL-6, was able to enhance the expression of *TGFB1* (feedback amplification loop) and *IL6* (Fig. S10 D). Although TGFβ1 is produced by MSCs and seemed involved in the production of IL-6, this cytokine failed to fully explain the N-MSC change into an immunosuppressive phenotype, as *PTGS2* expression is not modulated by its presence.

3.2 Complementary Material and Methods

CFSE staining

Freshly isolated NK cells were washed with 10 ml PBS+5%FCS and stained at room temperature with CellTrace™ CSFE (ThermoFisher, C34570) at the final concentration of 5µM (1:1000 dilution from stock solution) for 5 minutes, protected from light. After staining, cells were washed 3 times in PBS+ 5% FCS, and then co-cultured with MSCs in NK medium+IL-2 for 4 days (as described in the section "co-culture experiments"). NK cells treated with Mitomycine C after having been stained with CSFE were used as control sample for non proliferative cells.

After 4 days, cells were harvested for flow cytometry analysis. More precise technical information about antibody staining, sample acquisition and analysis can be found in Supplemental Experimental Procedures.

Inhibitors/ stimulatory molecules

Inhibitors. Human anti-ICAM-1 antibody (MAB720, R&D) was added at the final concentration of 10 µg/ml at the start of co-culture. Medium + PBS was used as negative control.

N-MSC expression analysis after tumor cell co-culture and cytokine treatment

Co-culture. N-MSCs from patients 2, 3 and 4 were co-cultured with K562 cells and with primary cancer cells from the same patient for 7 days in transwell culture conditions at different MSC:tumor cell ratios (1:2 and 1:5). MSCs (20.000 cells/well) were seeded onto six-well plates (Costar, Corning incorporated). PCC or K562 cells were seeded into 1,0 µm-pore insert of PET-membrane (Corning, Falcon) at different amounts according to the two different ratios. Controls

were T-MSCs and N-MSCs cultured alone. Cells were cultured in MSC medium, half of which was refreshed at day 3 and 5 and frozen at -20°C for subsequent analysis of the supernatant. At day 7, cells (tumor cells and MSCs) were harvested, washed twice in ice-cold PBS, snap frozen and stored at -80°C until RNA extraction. Supernatant of the co-culture at day 7 was also frozen for later analysis.

TGFβ1 and IL-6 treatment. N-MSCs from patients 2 were seeded at 20.000 cells per well in six-well plates and treated for 7 days with IL-6 (10ng/ml, ref. SRP3096, Sigma), TGFβ1 (1ng/ml or 10ng/ml, ref. 130-095-066 Miltenyi Biotec) or a combination of both in MSC medium. Controls were N-MSCs cultured in MSC medium. Medium was changed at day 3 and 5. At day 7, N-MSCs were harvested, washed twice in ice-cold PBS, snap frozen and stored at -80°C until RNA extraction.

For both culture experiments, N-MSC expression was assessed by qRT-PCR as described in the previous “RNA extraction, cDNA synthesis and qRT-PCR” section.

4. PERSPECTIVES

In this work, we provided insights into the effects of tumor-associated MSCs on NK cell activity. Indeed, we compared the immunomodulatory activity toward freshly isolated IL-2-activated 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. Interestingly, we observed marked differences between T- and N-MSCs in their phenotype and immunosuppressive function. Despite the ability of MSCs to secrete a variety of mediators with immunosuppressive effects, T-MSCs seemed to 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.

In the future, we would like to follow two major axes in the investigation of MSC-NK interactions. Firstly, we would like to better understand how tumor cells induce stromal cells to produce high levels of IL-6, PGE2 and other mediators, and to become immunosuppressive. Our preliminary studies indicate that TGFβ1 could participate in this process. Additional candidates would be interesting to test, such as IL-1β, which could be involved in MSC conditioning. In a study on the influence of inflammatory microenvironment on human MSCs in the context of polytrauma, it has been shown that COX2, PTGES (which catalyzes the conversion of PGH2 to PGE2) and TSG-6 were upregulated in MSCs upon stimulation with IL-1β (Hengartner et al., 2015). Thus, IL-1β seems to be a promising targetable candidate for future experiments, in order to reverse T-MSC immunosuppressive priming.

Secondly, we would like to address the mechanisms leading to NK receptor downregulation and hyporesponsiveness by looking more precisely at the immunological synapse formation. The role

of PD-L1 and PD1, as well as the role of ICAM-1-LFA1 and other adhesion molecules will be addressed in more detail. We already know, from lung MSC gene expression profile analysis, that PD-L1 (*CD274*) is much more highly expressed by T-MSCs than by N-MSCs. For the study of PD-L1/PD1 interaction, it would be interesting to characterize their expression on both MSCs and NK cells, and test an anti-PD1 receptor-blocking monoclonal antibody (BMS-936558, Nivolumab) (Wang et al., 2014a) in co-culture experiments. ICAM-1 and LFA1 binding could be analyzed using Real-time fluorescent microscopy, ICAM-1 bead binding assay or study of LFA-1–mediated NK spreading, e.g. (Sanborn et al., 2010).

Having examined direct cell-to-cell interactions, we would like to address the potential shedding of NKG2D and DNAM-1 ligands expressed by MSCs, and the role of MSC proteases in this mechanism. For this purpose, we would like to detect the potential presence of sMICA and/or sCD155 in the supernatant of NK:MSC co-cultures. Moreover, data from another project in our laboratory on the secretory and gene expression profile of MSCs indicate that they express/secrete specific proteolytic enzymes, like MMP2. Thus, one interesting experiment would be to target these proteases and analyze the effect on NK receptor expression in co-culture with MSCs.

Interestingly, the secretory profile analysis of lung MSCs from patient 1 (not shown) indicates that T-MSCs have a marked immunosuppressive profile, with significantly higher secretion of TSG-6, PTGD2 (which synthesizes PGH2) and CD81 and CD9 (two exosome-related proteins) compared to N-MSCs. We also found various molecules from the complement system secreted in larger amount by T-MSCs compared to N-MSCs (C4a, CFD, C1r, CFB, C1s). Thus, it would be interesting to explore the role of these molecules in the immunomodulatory potential of T-MSCs.

Regarding the immunomodulatory molecules secreted by MSCs, we largely discussed IL-6 cytokine in previous sections. Its receptor is composed of two subunits: IL-6Rα (CD126) and gp130 (CD130). The latest is ubiquitously expressed, while IL-6Rα is cell specific, but the two subunits are required to allow IL-6 signaling. However, for the cells that do not express the CD126 subunit, it also exists as a soluble form of IL-6Rα, which binds to IL-6, increasing its half-life time and allowing trans-signaling when binding to gp130 on target cells (Hunter and Jones, 2015). Other groups have shown that MSCs secrete IL-6 but also soluble IL-6Rα (Luu et al., 2013). Curiously, we did not detect any IL-6/IL-6 Rα complexes in the supernatant of MSC-NK co-cultures, although this could be due to the detection limit of the kit (78.10 pg/ml; Human IL-6/IL-6 Rα Complex DuoSet ELISA, R&D).

All these data underline how intriguing, complicated and heterogeneous, the profile of T-MSCs and their role in immunosuppression in lung tumors are, and they call for further investigations.

Finally, in parallel to the directions proposed above, it would be of great interest to transpose our observations and investigations to an *in vivo* model. A patient-derived tumor graft model with infused NK cells preconditioned or not with T- or N-MSCs or BM-MSCs would allow us to explore the role of the MSC-NK axis in promoting tumorigenesis.

This work reveals the potential for MSC targeting or reprogramming as an additional anti-tumor therapeutic strategy, considering their immunosuppressive and pro-inflammatory phenotype, particularly through the release of PGE2. To conclude, it is important to add a few general comments regarding the role of TME-targeting therapies, their place in the anti-tumor treatment arsenal, but also their limitations. The TME is a complex ecosystem that supports tumor growth and metastatic dissemination, and that constitutes an integral part of cancer. Accordingly, novel

candidate targets within the TME are constantly being proposed to improve the actions of various anti-cancer therapies, notably immunotherapy, that work by potentiating host anti-tumor immune responses (Pitt et al., 2016). However, the major limitation of TME targeting is the redundancy of its anti-immune and pro-tumor mechanisms, which can occur concurrently within the tumor microenvironment. The complexity of their interplay results in multiple levels of immune suppression, reducing the effectiveness of immunotherapy. As an example, many different cells can be the sources of TGFβ release in the TME, which induces both tumor progression and immunosuppression. To effectively counteract or neutralize tumor-promoting inflammation, simultaneous reprogramming or the repression of multiple immune-response programs activated in cancers will be needed (Coussens et al., 2013). As previously described in this work, the tumor microenvironment, including the MSCs can impede the immune response. Besides, approaches to specifically reduce immune suppression within the tumor microenvironment tend to become part of the immunotherapy strategy.

In the above paragraph, we only briefly discussed therapies targeting the immune responses within the TME, but anti-TME therapies represent a broader spectrum of strategies, including the use of drugs targeting cancer-associated inflammation (e.g. MSCs, TGFβ, COX-2, IL-6 and TNFα), and also targeting the tumor vasculature, the interplay between tumor cells and TME, and hypoxia (Fang and DeClerck, 2013).

We also have to take into consideration the side effects of targeting the TME. First, many agents targeting the TME alter the homeostatic balance in normal organs and tissues and can be toxic. Then, agents targeting the TME often affect cells or pathways that are not necessarily the enemy of cancer cells. In addition, targeting the TME does not prevent resistance and requires optimal biological dosage that may be difficult to determine (Fang and DeClerck, 2013).

Lastly, it is important to remember that there are several questions regarding the activity and place of agents targeting the TME in our therapeutic arsenal against cancer that remain to be answered. Are these agents most effective when used alone or in combination with conventional therapies, such as chemotherapy? When taking into account the dynamic changes in the pro- or anti-tumorigenic functions of the TME during cancer progression (Fang and DeClerck, 2013), which agents are more likely to be effective at early stages of cancer progression and which are the ones more likely to have an impact at later stages (e.g. TGFβ inhibitors)? Most importantly, it is necessary to stratify patients according to the likelihood that they may respond to a specific singular treatment or to combinatorial therapy. This emphasizes the importance of identifying reliable biomarkers that allow determination of the TME properties at different stages of evolution of different tumor types (Fridman et al., 2012; Sun, 2015).

5. REFERENCES

Aggarwal, S., and Pittenger, M.F. (2005). Human mesenchymal stem cells modulate allogeneic immune cell responses. Blood *105*, 1815–1822.

Alshaker, H.A., and Matalka, K.Z. (2011). IFN-γ, IL-17 and TGF-β involvement in shaping the tumor microenvironment: The significance of modulating such cytokines in treating malignant solid tumors. Cancer Cell Int. *11*, 33.

Alter, G., Malenfant, J.M., and Altfeld, M. (2004). CD107a as a functional marker for the identification of natural killer cell activity. J. Immunol. Methods *294*, 15–22.

Beckermann, B.M., Kallifatidis, G., Groth, A., Frommhold, D., Apel, A., Mattern, J., Salnikov, A.V., Moldenhauer, G., Wagner, W., Diehlmann, A., et al. (2008). VEGF expression by mesenchymal stem cells contributes to angiogenesis in pancreatic carcinoma. Br. J. Cancer *99*, 622–631.

Berger, L., Shamai, Y., Skorecki, K.L., and Tzukerman, M. (2016). Tumor Specific Recruitment and Reprogramming of Mesenchymal Stem Cells in Tumorigenesis. Stem Cells Dayt. Ohio *34*, 1011–1026.

Bergfeld, S.A., and DeClerck, Y.A. (2010). Bone marrow-derived mesenchymal stem cells and the tumor microenvironment. Cancer Metastasis Rev. *29*, 249–261.

Bernardo, M.E., and Fibbe, W.E. (2013). Mesenchymal Stromal Cells: Sensors and Switchers of Inflammation. Cell Stem Cell *13*, 392–402.

Bhooshan, N., Staats, P.N., Fulton, A.M., Feliciano, J.L., and Edelman, M.J. (2016). Prostaglandin E Receptor EP4 expression, survival and pattern of recurrence in locally advanced NSCLC. Lung Cancer *101*, 88–91.

Blake, S.J., Dougall, W.C., Miles, J.J., Teng, M.W.L., and Smyth, M.J. (2016). Molecular Pathways: Targeting CD96 and TIGIT for Cancer Immunotherapy. Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res. *22*, 5183–5188.

Blankenstein, T. (2005). The role of tumor stroma in the interaction between tumor and immune system. Curr. Opin. Immunol. *17*, 180–186.

Bottino, C., Castriconi, R., Pende, D., Rivera, P., Nanni, M., Carnemolla, B., Cantoni, C., Grassi, J., Marcenaro, S., Reymond, N., et al. (2003). Identification of PVR (CD155) and Nectin-2 (CD112) as Cell Surface Ligands for the Human DNAM-1 (CD226) Activating Molecule. J. Exp. Med. *198*, 557–567.

Bouffi, C., Bony, C., Courties, G., Jorgensen, C., and Noël, D. (2010). IL-6-Dependent PGE2 Secretion by Mesenchymal Stem Cells Inhibits Local Inflammation in Experimental Arthritis. PLoS ONE *5*.

Brandt, C.S., Baratin, M., Yi, E.C., Kennedy, J., Gao, Z., Fox, B., Haldeman, B., Ostrander, C.D., Kaifu, T., Chabannon, C., et al. (2009). The B7 family member B7-H6 is a tumor cell ligand for the activating natural killer cell receptor NKp30 in humans. J. Exp. Med. *206*, 1495–1503.

Bremnes, R.M., Al-Shibli, K., Donnem, T., Sirera, R., Al-Saad, S., Andersen, S., Stenvold, H., Camps, C., and Busund, L.-T. (2011). The role of tumor-infiltrating immune cells and chronic inflammation at the tumor site on cancer development, progression, and prognosis: emphasis on non-small cell lung cancer. J. Thorac. Oncol. Off. Publ. Int. Assoc. Study Lung Cancer *6*, 824–833.

Brennen, W.N., Chen, S., Denmeade, S.R., and Isaacs, J.T. (2012). Quantification of Mesenchymal Stem Cells (MSCs) at Sites of Human Prostate Cancer. Oncotarget *4*, 106–117.

Brown, J.R., and DuBois, R.N. (2004). Cyclooxygenase as a Target in Lung Cancer. Clin. Cancer Res. *10*, 4266s–4269s.

Brune, J.C., Tormin, A., Johansson, M.C., Rissler, P., Brosjö, O., Löfvenberg, R., von Steyern, F.V., Mertens, F., Rydholm, A., and Scheding, S. (2011). Mesenchymal stromal cells from primary osteosarcoma are non-malignant and strikingly similar to their bone marrow counterparts. Int. J. Cancer *129*, 319–330.

Bryceson, Y.T., March, M.E., Ljunggren, H.-G., and Long, E.O. (2006). Activation, co–activation, and co–stimulation of resting human NK cells. Immunol. Rev. *214*.

Burrello, J., Monticone, S., Gai, C., Gomez, Y., Kholia, S., and Camussi, G. (2016). Stem Cell-Derived Extracellular Vesicles and Immune-Modulation. Front. Cell Dev. Biol. *4*.

Bussard, K.M., Mutkus, L., Stumpf, K., Gomez-Manzano, C., and Marini, F.C. (2016). Tumor-associated stromal cells as key contributors to the tumor microenvironment. Breast Cancer Res. BCR *18*.

Caligiuri, M.A. (2008). Human natural killer cells. Blood *112*, 461–469.

Caligiuri, M.A., Murray, C., Robertson, M.J., Wang, E., Cochran, K., Cameron, C., Schow, P., Ross, M.E., Klumpp, T.R., and Soiffer, R.J. (1993). Selective modulation of human natural killer cells in vivo after prolonged infusion of low dose recombinant interleukin 2. J. Clin. Invest. *91*, 123–132.

Carrega, P., and Ferlazzo, G. (2017). Natural Killers Are Made Not Born: How to Exploit NK Cells in Lung Malignancies. Front. Immunol. *8*.

Carrega, P., Morandi, B., Costa, R., Frumento, G., Forte, G., Altavilla, G., Ratto, G.B., Mingari, M.C., Moretta, L., and Ferlazzo, G. (2008). Natural killer cells infiltrating human nonsmall-cell lung cancer are enriched in CD56brightCD16− cells and display an impaired capability to kill tumor cells. Cancer *112*, 863–875.

Carrega, P., Bonaccorsi, I., Di Carlo, E., Morandi, B., Paul, P., Rizzello, V., Cipollone, G., Navarra, G., Mingari, M.C., Moretta, L., et al. (2014). CD56(bright)perforin(low) noncytotoxic human NK cells are abundant in both healthy and neoplastic solid tissues and recirculate to secondary lymphoid organs via afferent lymph. J. Immunol. Baltim. Md 1950 *192*, 3805–3815.

Casado, J.G., Tarazona, R., and Sanchez-Margallo, F.M. (2013). NK and MSCs crosstalk: the sense of immunomodulation and their sensitivity. Stem Cell Rev. *9*, 184–189.

Castriconi, R., Cantoni, C., Chiesa, M.D., Vitale, M., Marcenaro, E., Conte, R., Biassoni, R., Bottino, C., Moretta, L., and Moretta, A. (2003). Transforming growth factor β1 inhibits expression of NKp30 and NKG2D receptors: Consequences for the NK-mediated killing of dendritic cells. Proc. Natl. Acad. Sci. *100*, 4120–4125.

Chan, A., Hong, D.-L., Atzberger, A., Kollnberger, S., Filer, A.D., Buckley, C.D., McMichael, A., Enver, T., and Bowness, P. (2007). CD56bright Human NK Cells Differentiate into CD56dim Cells: Role of Contact with Peripheral Fibroblasts. J. Immunol. *179*, 89–94.

Chan, C.J., Martinet, L., Gilfillan, S., Souza-Fonseca-Guimaraes, F., Chow, M.T., Town, L., Ritchie, D.S., Colonna, M., Andrews, D.M., and Smyth, M.J. (2014). The receptors CD96 and CD226 oppose each other in the regulation of natural killer cell functions. Nat. Immunol. *15*, 431–438.

Chang, Y.-J., Holtzman, M.J., and Chen, C.-C. (2002). Interferon-gamma-induced epithelial ICAM-1 expression and monocyte adhesion. Involvement of protein kinase C-dependent c-Src tyrosine kinase activation pathway. J. Biol. Chem. *277*, 7118–7126.

Chatterjee, D., Marquardt, N., Tufa, D.M., Beauclair, G., Low, H.Z., Hatlapatka, T., Hass, R., Kasper, C., Kaisenberg, C. von, Schmidt, R.E., et al. (2014). Role of gamma-secretase in human umbilical-cord derived mesenchymal stem cell mediated suppression of NK cell cytotoxicity. Cell Commun. Signal. *12*, 63.

Chen, Z., Fillmore, C.M., Hammerman, P.S., Kim, C.F., and Wong, K.-K. (2014). Non-small-cell lung cancers: a heterogeneous set of diseases. Nat. Rev. Cancer *14*, 535–546.

Chester, C., Fritsch, K., and Kohrt, H.E. (2015). Natural Killer Cell Immunomodulation: Targeting Activating, Inhibitory, and Co-stimulatory Receptor Signaling for Cancer Immunotherapy. NK Cell Biol. 601.

Cho, J.A., Park, H., Lim, E.H., and Lee, K.W. (2012). Exosomes from breast cancer cells can convert adipose tissue-derived mesenchymal stem cells into myofibroblast-like cells. Int. J. Oncol. *40*, 130–138.

Chretien, A.-S., Le Roy, A., Vey, N., Prebet, T., Blaise, D., Fauriat, C., and Olive, D. (2014). Cancer-induced alterations of NK-mediated target recognition: current and investigational pharmacological strategies aiming at restoring NK-mediated anti-tumor activity. NK Cell Biol. *5*, 122.

Cooper, M.A., Fehniger, T.A., Turner, S.C., Chen, K.S., Ghaheri, B.A., Ghayur, T., Carson, W.E., and Caligiuri, M.A. (2001b). Human natural killer cells: a unique innate immunoregulatory role for the CD56bright subset. Blood *97*, 3146–3151.

Cooper, M.A., Fehniger, T.A., and Caligiuri, M.A. (2001a). The biology of human natural killer-cell subsets. Trends Immunol. *22*, 633–640.

Coussens, L.M., Zitvogel, L., and Palucka, A.K. (2013). Neutralizing Tumor-Promoting Chronic Inflammation: A Magic Bullet? Science *339*, 286–291.

Cremer, I., Fridman, W.H., and Sautès-Fridman, C. (2012). Tumor microenvironment in NSCLC suppresses NK cells function. Oncoimmunology *1*, 244–246.

Davis, D.M., Chiu, I., Fassett, M., Cohen, G.B., Mandelboim, O., and Strominger, J.L. (1999). The human natural killer cell immune synapse. Proc. Natl. Acad. Sci. U. S. A. *96*, 15062–15067.

De Maria, A., and Moretta, L. (2011). Revisited function of human NK cell subsets. Cell Cycle Georget. Tex *10*, 1178–1179.

Delahaye, N.F., Rusakiewicz, S., Martins, I., Ménard, C., Roux, S., Lyonnet, L., Paul, P., Sarabi, M., Chaput, N., Semeraro, M., et al. (2011). Alternatively spliced NKp30 isoforms affect the prognosis of gastrointestinal stromal tumors. Nat. Med. *17*, 700–707.

DelaRosa, O., Sánchez-Correa, B., Morgado, S., Ramírez, C., del Río, B., Menta, R., Lombardo, E., Tarazona, R., and Casado, J.G. (2012). Human adipose-derived stem cells impair natural killer cell function and exhibit low susceptibility to natural killer-mediated lysis. Stem Cells Dev. *21*, 1333–1343.

Desmoulière, A., Guyot, C., and Gabbiani, G. (2004). The stroma reaction myofibroblast: a key player in the control of tumor cell behavior. Int. J. Dev. Biol. *48*, 509–517.

Di Trapani, M., Bassi, G., Ricciardi, M., Fontana, E., Bifari, F., Pacelli, L., Giacomello, L., Pozzobon, M., Féron, F., De Coppi, P., et al. (2013). Comparative Study of Immune Regulatory Properties of Stem Cells Derived from Different Tissues. Stem Cells Dev. *22*, 2990–3002.

Di Trapani, M., Bassi, G., Midolo, M., Gatti, A., Kamga, P.T., Cassaro, A., Carusone, R., Adamo, A., and Krampera, M. (2016). Differential and transferable modulatory effects of mesenchymal stromal cell-derived extracellular vesicles on T, B and NK cell functions. Sci. Rep. *6*.

Ding, G., Shao, J., Ding, Q., Fang, Z., Wu, Z., Xu, J., and Gao, P. (2012). Comparison of the characteristics of mesenchymal stem cells obtained from prostate tumors and from bone marrow cultured in conditioned medium. Exp. Ther. Med. *4*, 711–715.

Djouad, F., Charbonnier, L.-M., Bouffi, C., Louis-Plence, P., Bony, C., Apparailly, F., Cantos, C., Jorgensen, C., and Noël, D. (2007). Mesenchymal stem cells inhibit the differentiation of dendritic cells through an interleukin-6-dependent mechanism. Stem Cells Dayt. Ohio *25*, 2025–2032.

Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F., Krause, D., Deans, R., Keating, A., Prockop, D., and Horwitz, E. (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy *8*, 315–317.

Dostert, G., Mesure, B., Menu, P., and Velot, É. (2017). How Do Mesenchymal Stem Cells Influence or Are Influenced by Microenvironment through Extracellular Vesicles Communication? Front. Cell Dev. Biol. *5*.

Dumitru, C.A., Hemeda, H., Jakob, M., Lang, S., and Brandau, S. (2014). Stimulation of mesenchymal stromal cells (MSCs) via TLR3 reveals a novel mechanism of autocrine priming. FASEB J. Off. Publ. Fed. Am. Soc. Exp. Biol. *28*, 3856–3866.

Dvorak, H.F. (1986). Tumors: wounds that do not heal. Similarities between tumor stroma generation and wound healing. N. Engl. J. Med. *315*, 1650–1659.

Elboim, M., Gazit, R., Gur, C., Ghadially, H., Betser-Cohen, G., and Mandelboim, O. (2010). Tumor Immunoediting by NKp46. J. Immunol. *184*, 5637–5644.

Enqvist, M., Ask, E.H., Forslund, E., Carlsten, M., Abrahamsen, G., Béziat, V., Andersson, S., Schaffer, M., Spurkland, A., Bryceson, Y., et al. (2015). Coordinated expression of DNAM-1 and LFA-1 in educated NK cells. J. Immunol. Baltim. Md 1950 *194*, 4518–4527.

Esendagli, G., Bruderek, K., Goldmann, T., Busche, A., Branscheid, D., Vollmer, E., and Brandau, S. (2008). Malignant and non-malignant lung tissue areas are differentially populated by natural killer cells and regulatory T cells in non-small cell lung cancer. Lung Cancer Amst. Neth. *59*, 32–40.

Fang, H., and DeClerck, Y.A. (2013). Targeting the Tumor Microenvironment: From Understanding Pathways to Effective Clinical Trials. Cancer Res. *73*, 4965–4977.

Fauci, A.S., Mavilio, D., and Kottilil, S. (2005). NK cells in HIV infection: paradigm for protection or targets for ambush. Nat. Rev. Immunol. *5*, 835–843.

Fauriat, C., Long, E.O., Ljunggren, H.-G., and Bryceson, Y.T. (2010). Regulation of human NK-cell cytokine and chemokine production by target cell recognition. Blood *115*, 2167–2176.

Fawcett, J., Holness, C.L., Needham, L.A., Turley, H., Gatter, K.C., Mason, D.Y., and Simmons, D.L. (1992). Molecular cloning of ICAM-3, a third ligand for LFA-1, constitutively expressed on resting leukocytes. Nature *360*, 481–484.

Fibbe, W.E., and Noort, W.A. (2003). Mesenchymal Stem Cells and Hematopoietic Stem Cell Transplantation. Ann. N. Y. Acad. Sci. *996*, 235–244.

Fridman, W.H., Pagès, F., Sautès-Fridman, C., and Galon, J. (2012). The immune contexture in human tumours: impact on clinical outcome. Nat. Rev. Cancer *12*, 298–306.

Gilfillan, S., Chan, C.J., Cella, M., Haynes, N.M., Rapaport, A.S., Boles, K.S., Andrews, D.M., Smyth, M.J., and Colonna, M. (2008). DNAM-1 promotes activation of cytotoxic lymphocytes by nonprofessional antigen-presenting cells and tumors. J. Exp. Med. *205*, 2965–2973.

Gillard-Bocquet, M., Caer, C., Cagnard, N., Crozet, L., Perez, M., Fridman, W.H., Sautès-Fridman, C., and Cremer, I. (2013). Lung tumor microenvironment induces specific gene expression signature in intratumoral NK cells. Front. Immunol. *4*, 19.

Giuliani, M., Bennaceur-Griscelli, A., Nanbakhsh, A., Oudrhiri, N., Chouaib, S., Azzarone, B., Durrbach, A., and Lataillade, J.-J. (2014). TLR Ligands Stimulation Protects MSC from NK Killing. STEM CELLS *32*, 290–300.

Glenn, J.D., and Whartenby, K.A. (2014). Mesenchymal stem cells: Emerging mechanisms of immunomodulation and therapy. World J. Stem Cells *6*, 526–539.

Gottschling, S., Granzow, M., Kuner, R., Jauch, A., Herpel, E., Xu, E.C., Muley, T., Schnabel, P.A., Herth, F.J.F., and Meister, M. (2013). Mesenchymal stem cells in non-small cell lung cancer—Different from others? Insights from comparative molecular and functional analyses. Lung Cancer *80*, 19–29.

Grakoui, A., Bromley, S.K., Sumen, C., Davis, M.M., Shaw, A.S., Allen, P.M., and Dustin, M.L. (1999). The immunological synapse: a molecular machine controlling T cell activation. Science *285*, 221–227.

Grégoire, C., Chasson, L., Luci, C., Tomasello, E., Geissmann, F., Vivier, E., and Walzer, T. (2007). The trafficking of natural killer cells. Immunol. Rev. *220*, 169–182.

Groh, V., Wu, J., Yee, C., and Spies, T. (2002). Tumour-derived soluble MIC ligands impair expression of NKG2D and T-cell activation. Nature *419*, 734–738.

Handgretinger, R., Lang, P., and André, M.C. (2016). Exploitation of natural killer cells for the treatment of acute leukemia. Blood *127*, 3341–3349.

Hengartner, N.-E., Fiedler, J., Schrezenmeier, H., Huber-Lang, M., and Brenner, R.E. (2015). Crucial Role of IL1beta and C3a in the In Vitro-Response of Multipotent Mesenchymal Stromal Cells to Inflammatory Mediators of Polytrauma. PLOS ONE *10*, e0116772.

Henney, C.S., Kuribayashi, K., Kern, D.E., and Gillis, S. (1981). Interleukin-2 augments natural killer cell activity. Nature *291*, 335–338.

Herbst, R.S., Heymach, J.V., and Lippman, S.M. (2008). Lung cancer. N. Engl. J. Med. *359*, 1367–1380.

Hernanda, P.Y., Pedroza-Gonzalez, A., van der Laan, L.J.W., Bröker, M.E.E., Hoogduijn, M.J., Ijzermans, J.N.M., Bruno, M.J., Janssen, H.L.A., Peppelenbosch, M.P., and Pan, Q. (2013). Tumor promotion through the mesenchymal stem cell compartment in human hepatocellular carcinoma. Carcinogenesis *34*, 2330–2340.

Hershkovitz, O., Jivov, S., Bloushtain, N., Zilka, A., Landau, G., Bar-Ilan, A., Lichtenstein, R.G., Campbell, K.S., van Kuppevelt, T.H., and Porgador, A. (2007). Characterization of the Recognition of Tumor Cells by the Natural Cytotoxicity Receptor, NKp44. Biochemistry (Mosc.) *46*, 7426–7436.

Hidalgo, G.E., Zhong, L., Doherty, D.E., and Hirschowitz, E.A. (2002). Plasma PGE-2 levels and altered cytokine profiles in adherent peripheral blood mononuclear cells in non-small cell lung cancer (NSCLC). Mol. Cancer *1*, 5.

Hodge, G., Barnawi, J., Jurisevic, C., Moffat, D., Holmes, M., Reynolds, P.N., Jersmann, H., and Hodge, S. (2014). Lung cancer is associated with decreased expression of perforin, granzyme B and interferon (IFN)-γ by infiltrating lung tissue T cells, natural killer (NK) T-like and NK cells. Clin. Exp. Immunol. *178*, 79–85.

Hong, I.-S., Lee, H.-Y., and Kang, K.-S. (2014). Mesenchymal stem cells and cancer: Friends or enemies? Mutat. Res. Mol. Mech. Mutagen. *768*, 98–106.

Hunter, C.A., and Jones, S.A. (2015). IL-6 as a keystone cytokine in health and disease. Nat. Immunol. *16*, 448–457.

Iguchi-Manaka, A., Okumura, G., Kojima, H., Cho, Y., Hirochika, R., Bando, H., Sato, T., Yoshikawa, H., Hara, H., Shibuya, A., et al. (2016). Increased Soluble CD155 in the Serum of Cancer Patients. PLOS ONE *11*, e0152982.

Imai, K., Matsuyama, S., Miyake, S., Suga, K., and Nakachi, K. (2000). Natural cytotoxic activity of peripheral-blood lymphocytes and cancer incidence: an 11-year follow-up study of a general population. The Lancet *356*, 1795–1799.

Jamieson, A.M., Diefenbach, A., McMahon, C.W., Xiong, N., Carlyle, J.R., and Raulet, D.H. (2002). The Role of the NKG2D Immunoreceptor in Immune Cell Activation and Natural Killing. Immunity *17*, 19–29.

Jin, S., Deng, Y., Hao, J.-W., Li, Y., Liu, B., Yu, Y., Shi, F.-D., and Zhou, Q.-H. (2014a). NK Cell Phenotypic Modulation in Lung Cancer Environment. PLoS ONE *9*.

Jin, S., Deng, Y., Hao, J.-W., Li, Y., Liu, B., Yu, Y., Shi, F.-D., and Zhou, Q.-H. (2014b). NK Cell Phenotypic Modulation in Lung Cancer Environment. PLoS ONE *9*.

Jing, Y., Han, Z., Liu, Y., Sun, K., Zhang, S., Jiang, G., Li, R., Gao, L., Zhao, X., Wu, D., et al. (2012). Mesenchymal Stem Cells in Inflammation Microenvironment Accelerates Hepatocellular Carcinoma Metastasis by Inducing Epithelial-Mesenchymal Transition. PLoS ONE *7*.

Johann, P.-D., Vaegler, M., Gieseke, F., Mang, P., Armeanu-Ebinger, S., Kluba, T., Handgretinger, R., and Müller, I. (2010). Tumour stromal cells derived from paediatric malignancies display MSC-like properties and impair NK cell cytotoxicity. BMC Cancer *10*, 501.

Kalluri, R. (2016). The biology and function of fibroblasts in cancer. Nat. Rev. Cancer *16*, 582–598.

Kansy, B.A., Dißmann, P.A., Hemeda, H., Bruderek, K., Westerkamp, A.M., Jagalski, V., Schuler, P., Kansy, K., Lang, S., Dumitru, C.A., et al. (2014). The bidirectional tumor--mesenchymal stromal cell interaction promotes the progression of head and neck cancer. Stem Cell Res. Ther. *5*, 95.

Karnoub, A.E., Dash, A.B., Vo, A.P., Sullivan, A., Brooks, M.W., Bell, G.W., Richardson, A.L., Polyak, K., Tubo, R., and Weinberg, R.A. (2007). Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. Nature *449*, 557–563.

Khakoo, A.Y., Pati, S., Anderson, S.A., Reid, W., Elshal, M.F., Rovira, I.I., Nguyen, A.T., Malide, D., Combs, C.A., Hall, G., et al. (2006). Human mesenchymal stem cells exert potent antitumorigenic effects in a model of Kaposi's sarcoma. J. Exp. Med. *203*, 1235–1247.

Khuri, F.R., Wu, H., Lee, J.J., Kemp, B.L., Lotan, R., Lippman, S.M., Feng, L., Hong, W.K., and Xu, X.-C. (2001). Cyclooxygenase-2 Overexpression Is a Marker of Poor Prognosis in Stage I Non-Small Cell Lung Cancer. Clin. Cancer Res. *7*, 861–867.

Kidd, S., Spaeth, E., Dembinski, J.L., Dietrich, M., Watson, K., Klopp, A., Battula, V.L., Weil, M., Andreeff, M., and Marini, F.C. (2009). Direct evidence of mesenchymal stem cell tropism for tumor and wounding microenvironments using in vivo bioluminescent imaging. Stem Cells Dayt. Ohio *27*, 2614–2623.

Kidd, S., Spaeth, E., Watson, K., Burks, J., Lu, H., Klopp, A., Andreeff, M., and Marini, F.C. (2012). Origins of the tumor microenvironment: quantitative assessment of adipose-derived and bone marrow-derived stroma. PloS One *7*, e30563.

Kim, R., Emi, M., and Tanabe, K. (2007). Cancer immunoediting from immune surveillance to immune escape. Immunology *121*, 1–14.

Kornstein, M.J., Stewart, R., and Elder, D.E. (1987). Natural killer cells in the host response to melanoma. Cancer Res. *47*, 1411–1412.

Krampera, M. (2011). Mesenchymal stromal cell "licensing": a multistep process. Leukemia *25*, 1408–1414.

Krampera, M., Cosmi, L., Angeli, R., Pasini, A., Liotta, F., Andreini, A., Santarlasci, V., Mazzinghi, B., Pizzolo, G., Vinante, F., et al. (2006). Role for interferon-gamma in the immunomodulatory activity of human bone marrow mesenchymal stem cells. Stem Cells Dayt. Ohio *24*, 386–398.

Krampera, M., Galipeau, J., Shi, Y., Tarte, K., and Sensebe, L. (2013). Immunological characterization of multipotent mesenchymal stromal cells—The International Society for Cellular Therapy (ISCT) working proposal. Cytotherapy *15*, 1054–1061.

Kucerova, L., Matuskova, M., Hlubinova, K., Altanerova, V., and Altaner, C. (2010). Tumor cell behaviour modulation by mesenchymal stromal cells. Mol. Cancer *9*, 129.

Lanier, L.L., Le, A.M., Civin, C.I., Loken, M.R., and Phillips, J.H. (1986). The relationship of CD16 (Leu-11) and Leu-19 (NKH-1) antigen expression on human peripheral blood NK cells and cytotoxic T lymphocytes. J. Immunol. Baltim. Md 1950 *136*, 4480–4486.

Lanier, L.L., Testi, R., Bindl, J., and Phillips, J.H. (1989). Identity of Leu-19 (CD56) leukocyte differentiation antigen and neural cell adhesion molecule. J. Exp. Med. *169*, 2233–2238.

Lazennec, G., and Jorgensen, C. (2008). Concise review: adult multipotent stromal cells and cancer: risk or benefit? Stem Cells Dayt. Ohio *26*, 1387–1394.

Le Blanc, K., and Davies, L.C. (2015). Mesenchymal stromal cells and the innate immune response. Immunol. Lett. *168*, 140–146.

Le Blanc, K., and Mougiakakos, D. (2012). Multipotent mesenchymal stromal cells and the innate immune system. Nat. Rev. Immunol. *12*, 383–396.

Lee, J.W., Fang, X., Krasnodembskaya, A., Howard, J.P., and Matthay, M.A. (2011). Concise review: Mesenchymal stem cells for acute lung injury: role of paracrine soluble factors. Stem Cells Dayt. Ohio *29*, 913–919.

Levi, I., Amsalem, H., Nissan, A., Darash-Yahana, M., Peretz, T., Mandelboim, O., and Rachmilewitz, J. (2015). Characterization of tumor infiltrating Natural Killer cell subset. Oncotarget *6*, 13835–13843.

Li, W., Ren, G., Huang, Y., Su, J., Han, Y., Li, J., Chen, X., Cao, K., Chen, Q., Shou, P., et al. (2012). Mesenchymal stem cells: a double-edged sword in regulating immune responses. Cell Death Differ. *19*, 1505–1513.

Li, Y., Qu, Y.H., Wu, Y.F., Liu, L., Lin, X.H., Huang, K., and Wei, J. (2014). Bone marrow mesenchymal stem cells suppressing activation of allogeneic cytokine-induced killer/natural killer cells either by direct or indirect interaction. Cell Biol. Int.

Lieberman, J. (2003). The ABCs of granule-mediated cytotoxicity: new weapons in the arsenal. Nat. Rev. Immunol. *3*, 361–370.

Liu, R., Wei, S., Chen, J., and Xu, S. (2014). Mesenchymal stem cells in lung cancer tumor microenvironment: their biological properties, influence on tumor growth and therapeutic implications. Cancer Lett. *353*, 145–152.

Liu, Y., Han, Z., Zhang, S., Jing, Y., Bu, X., Wang, C., Sun, K., Jiang, G., Zhao, X., Li, R., et al. (2011). Effects of inflammatory factors on mesenchymal stem cells and their role in the promotion of tumor angiogenesis in colon cancer. J. Biol. Chem. *286*, 25007–25015.

Loebinger, M.R., Kyrtatos, P.G., Turmaine, M., Price, A.N., Pankhurst, Q., Lythgoe, M.F., and Janes, S.M. (2009). Magnetic resonance imaging of mesenchymal stem cells homing to pulmonary metastases using biocompatible magnetic nanoparticles. Cancer Res. *69*, 8862–8867.

Long, E.O., Kim, H.S., Liu, D., Peterson, M.E., and Rajagopalan, S. (2013). Controlling Natural Killer Cell Responses: Integration of Signals for Activation and Inhibition. Annu. Rev. Immunol. *31*, 227–258.

Lu, Y., Liu, J., Liu, Y., Qin, Y., Luo, Q., Wang, Q., and Duan, H. (2015a). TLR4 plays a crucial role in MSC-induced inhibition of NK cell function. Biochem. Biophys. Res. Commun. *464*, 541–547.

Lu, Y., Liu, J., Liu, Y., Qin, Y., Luo, Q., Wang, Q., and Duan, H. (2015b). TLR4 plays a crucial role in MSC-induced inhibition of NK cell function. Biochem. Biophys. Res. Commun. *464*, 541–547.

Luo, J., Ok Lee, S., Liang, L., Huang, C.-K., Li, L., Wen, S., and Chang, C. (2014). Infiltrating bone marrow mesenchymal stem cells increase prostate cancer stem cell population and metastatic ability via secreting cytokines to suppress androgen receptor signaling. Oncogene *33*, 2768–2778.

Luu, N.T., McGettrick, H.M., Buckley, C.D., Newsome, P.N., Rainger, G.E., Frampton, J., and Nash, G.B. (2013). Crosstalk between mesenchymal stem cells and endothelial cells leads to downregulation of cytokine-induced leukocyte recruitment. Stem Cells Dayt. Ohio *31*, 2690–2702.

Ma, S., Xie, N., Li, W., Yuan, B., Shi, Y., and Wang, Y. (2014a). Immunobiology of mesenchymal stem cells. Cell Death Differ. *21*, 216–225.

Ma, S., Xie, N., Li, W., Yuan, B., Shi, Y., and Wang, Y. (2014b). Immunobiology of mesenchymal stem cells. Cell Death Differ. *21*, 216–225.

Mandelboim, O., Malik, P., Davis, D.M., Jo, C.H., Boyson, J.E., and Strominger, J.L. (1999). Human CD16 as a lysis receptor mediating direct natural killer cell cytotoxicity. Proc. Natl. Acad. Sci. *96*, 5640–5644.

Marquardt, N., Kekäläinen, E., Chen, P., Kvedaraite, E., Wilson, J.N., Ivarsson, M.A., Mjösberg, J., Berglin, L., Säfholm, J., Manson, M.L., et al. (2017a). Human lung natural killer cells are predominantly comprised of highly differentiated hypofunctional CD69(-)CD56(dim) cells. J. Allergy Clin. Immunol. *139*, 1321–1330.e4.

Marquardt, N., Kekäläinen, E., Chen, P., Kvedaraite, E., Wilson, J.N., Ivarsson, M.A., Mjösberg, J., Berglin, L., Säfholm, J., Manson, M.L., et al. (2017b). Human lung natural killer cells are predominantly comprised of highly differentiated hypofunctional CD69(-)CD56(dim) cells. J. Allergy Clin. Immunol. *139*, 1321–1330.e4.

Martinet, L., and Smyth, M.J. (2015). Balancing natural killer cell activation through paired receptors. Nat. Rev. Immunol. *15*, 243–254.

Maumus, M., Guérit, D., Toupet, K., Jorgensen, C., and Noël, D. (2011). Mesenchymal stem cell-based therapies in regenerative medicine: applications in rheumatology. Stem Cell Res. Ther. *2*, 14.

McCann, F.E., Vanherberghen, B., Eleme, K., Carlin, L.M., Newsam, R.J., Goulding, D., and Davis, D.M. (2003). The size of the synaptic cleft and distinct distributions of filamentous actin, ezrin, CD43, and CD45 at activating and inhibitory human NK cell immune synapses. J. Immunol. Baltim. Md 1950 *170*, 2862–2870.

McLean, K., Gong, Y., Choi, Y., Deng, N., Yang, K., Bai, S., Cabrera, L., Keller, E., McCauley, L., Cho, K.R., et al. (2011). Human ovarian carcinoma–associated mesenchymal stem cells regulate cancer stem cells and tumorigenesis via altered BMP production. J. Clin. Invest. *121*, 3206–3219.

Monks, C.R., Freiberg, B.A., Kupfer, H., Sciaky, N., and Kupfer, A. (1998). Three-dimensional segregation of supramolecular activation clusters in T cells. Nature *395*, 82–86.

Moretta, L., Biassoni, R., Bottino, C., Mingari, M.C., and Moretta, A. (2001). Immunobiology of human NK cells. Transplant. Proc. *33*, 60–61.

Morgado, S., Sanchez-Correa, B., Casado, J.G., Duran, E., Gayoso, I., Labella, F., Solana, R., and Tarazona, R. (2011). NK cell recognition and killing of melanoma cells is controlled by multiple activating receptor-ligand interactions. J. Innate Immun. *3*, 365–373.

Nagler, A., Lanier, L.L., Cwirla, S., and Phillips, J.H. (1989). Comparative studies of human FcRIII-positive and negative natural killer cells. J. Immunol. *143*, 3183–3191.

Nausch, N., and Cerwenka, A. (2008). NKG2D ligands in tumor immunity. Oncogene *27*, 5944–5958.

Nomoto-Kojima, N., Aoki, S., Uchihashi, K., Matsunobu, A., Koike, E., Ootani, A., Yonemitsu, N., Fujimoto, K., and Toda, S. (2011). Interaction between adipose tissue stromal cells and gastric cancer cells in vitro. Cell Tissue Res. *344*, 287–298.

Opitz, C.A., Litzenburger, U.M., Lutz, C., Lanz, T.V., Tritschler, I., Köppel, A., Tolosa, E., Hoberg, M., Anderl, J., Aicher, W.K., et al. (2009). Toll-like receptor engagement enhances the immunosuppressive properties of human bone marrow-derived mesenchymal stem cells by inducing indoleamine-2,3-dioxygenase-1 via interferon-beta and protein kinase R. Stem Cells Dayt. Ohio *27*, 909–919.

Oppenheim, D.E., Roberts, S.J., Clarke, S.L., Filler, R., Lewis, J.M., Tigelaar, R.E., Girardi, M., and Hayday, A.C. (2005). Sustained localized expression of ligand for the activating NKG2D receptor impairs natural cytotoxicity in vivo and reduces tumor immunosurveillance. Nat. Immunol. *6*, 928–937.

Orange, J.S. (2008). Formation and function of the lytic NK-cell immunological synapse. Nat. Rev. Immunol. *8*, 713–725.

Pesce, S., Tabellini, G., Cantoni, C., Patrizi, O., Coltrini, D., Rampinelli, F., Matta, J., Vivier, E., Moretta, A., Parolini, S., et al. (2015). B7-H6-mediated downregulation of NKp30 in NK cells contributes to ovarian carcinoma immune escape. Oncoimmunology *4*, e1001224.

Peters, S., Camidge, D.R., Shaw, A.T., Gadgeel, S., Ahn, J.S., Kim, D.-W., Ou, S.-H.I., Pérol, M., Dziadziuszko, R., Rosell, R., et al. (2017). Alectinib versus Crizotinib in Untreated ALK-Positive Non–Small-Cell Lung Cancer. N. Engl. J. Med. *0*, null.

Pitt, J.M., Marabelle, A., Eggermont, A., Soria, J.-C., Kroemer, G., and Zitvogel, L. (2016). Targeting the tumor microenvironment: removing obstruction to anticancer immune responses and immunotherapy. Ann. Oncol. Off. J. Eur. Soc. Med. Oncol. *27*, 1482–1492.

Pittenger, M.F., Mackay, A.M., Beck, S.C., Jaiswal, R.K., Douglas, R., Mosca, J.D., Moorman, M.A., Simonetti, D.W., Craig, S., and Marshak, D.R. (1999). Multilineage potential of adult human mesenchymal stem cells. Science *284*, 143–147.

Platonova, S., Cherfils-Vicini, J., Damotte, D., Crozet, L., Vieillard, V., Validire, P., André, P., Dieu-Nosjean, M.-C., Alifano, M., Régnard, J.-F., et al. (2011a). Profound Coordinated Alterations of Intratumoral NK Cell Phenotype and Function in Lung Carcinoma. Cancer Res. *71*, 5412–5422.

Platonova, S., Cherfils-Vicini, J., Damotte, D., Crozet, L., Vieillard, V., Validire, P., André, P., Dieu-Nosjean, M.-C., Alifano, M., Régnard, J.-F., et al. (2011b). Profound Coordinated Alterations of Intratumoral NK Cell Phenotype and Function in Lung Carcinoma. Cancer Res. *71*, 5412–5422.

Poggi, A., and Giuliani, M. (2016). Mesenchymal Stromal Cells Can Regulate the Immune Response in the Tumor Microenvironment. Vaccines *4*.

Poggi, A., and Zocchi, M.R. (2015). Mesenchymal stromal cells as regulators of anti-tumour immune response. Indian J. Med. Res. *141*, 139–142.

Poggi, A., Prevosto, C., Massaro, A.-M., Negrini, S., Urbani, S., Pierri, I., Saccardi, R., Gobbi, M., and Zocchi, M.R. (2005). Interaction between human NK cells and bone marrow stromal cells induces NK cell triggering: role of NKp30 and NKG2D receptors. J. Immunol. Baltim. Md 1950 *175*, 6352–6360.

Poggi, A., Musso, A., Dapino, I., and Zocchi, M.R. (2014). Mechanisms of tumor escape from immune system: Role of mesenchymal stromal cells. Immunol. Lett. *159*, 55–72.

Pradier, A., Passweg, J., Villard, J., and Kindler, V. (2011). Human bone marrow stromal cells and skin fibroblasts inhibit natural killer cell proliferation and cytotoxic activity. Cell Transplant. *20*, 681–691.

Prantl, L., Muehlberg, F., Navone, N.M., Song, Y.-H., Vykoukal, J., Logothetis, C.J., and Alt, E.U. (2010). Adipose tissue-derived stem cells promote prostate tumor growth. The Prostate *70*, 1709–1715.

Pross, H.F., and Lotzová, E. (1993). Role of natural killer cells in cancer. Nat. Immun. *12*, 279–292.

Puré, E., and Lo, A. (2016). Can Targeting Stroma Pave the Way to Enhanced Antitumor Immunity and Immunotherapy of Solid Tumors? Cancer Immunol. Res. *4*, 269–278.

Raffaghello, L., and Dazzi, F. (2015). Classification and biology of tumour associated stromal cells. Immunol. Lett. *168*, 175–182.

Rasmusson, I., Ringdén, O., Sundberg, B., and Le Blanc, K. (2003). Mesenchymal stem cells inhibit the formation of cytotoxic T lymphocytes, but not activated cytotoxic T lymphocytes or natural killer cells. Transplantation *76*, 1208–1213.

Razmkhah, M., Jaberipour, M., Erfani, N., Habibagahi, M., Talei, A., and Ghaderi, A. (2011). Adipose derived stem cells (ASCs) isolated from breast cancer tissue express IL-4, IL-10 and TGF-β1 and upregulate expression of regulatory molecules on T cells: do they protect breast cancer cells from the immune response? Cell. Immunol. *266*, 116–122.

Ren, G., Zhao, X., Zhang, L., Zhang, J., L'Huillier, A., Ling, W., Roberts, A.I., Le, A.D., Shi, S., Shao, C., et al. (2010). Inflammatory cytokine-induced intercellular adhesion molecule-1 and vascular cell adhesion molecule-1 in mesenchymal stem cells are critical for immunosuppression. J. Immunol. Baltim. Md 1950 *184*, 2321–2328.

Ren, G., Zhao, X., Wang, Y., Zhang, X., Chen, X., Xu, C., Yuan, Z., Roberts, A.I., Zhang, L., Zheng, B., et al. (2012). CCR2-dependent recruitment of macrophages by tumor-educated mesenchymal stromal cells promotes tumor development and is mimicked by TNFα. Cell Stem Cell *11*, 812–824.

Ridge, S.M., Sullivan, F.J., and Glynn, S.A. (2017). Mesenchymal stem cells: key players in cancer progression. Mol. Cancer *16*, 31.

Robbins, P.D., and Morelli, A.E. (2014). Regulation of immune responses by extracellular vesicles. Nat. Rev. Immunol. *14*, 195–208.

Romieu-Mourez, R., François, M., Boivin, M.-N., Bouchentouf, M., Spaner, D.E., and Galipeau, J. (2009). Cytokine modulation of TLR expression and activation in mesenchymal stromal cells leads to a proinflammatory phenotype. J. Immunol. Baltim. Md 1950 *182*, 7963–7973.

Rosental, B., Brusilovsky, M., Hadad, U., Oz, D., Appel, M.Y., Afergan, F., Yossef, R., Rosenberg, L.A., Aharoni, A., Cerwenka, A., et al. (2011). Proliferating cell nuclear antigen is a novel inhibitory ligand for the natural cytotoxicity receptor NKp44. J. Immunol. Baltim. Md 1950 *187*, 5693–5702.

Ruggeri, L., Mancusi, A., Burchielli, E., Aversa, F., Martelli, M.F., and Velardi, A. (2006). Natural killer cell alloreactivity and haplo-identical hematopoietic transplantation. Cytotherapy *8*, 554–558.

Sanborn, K.B., Rak, G.D., Mentlik, A.N., Banerjee, P.P., and Orange, J.S. (2010). Analysis of the NK cell immunological synapse. Methods Mol. Biol. Clifton NJ *612*, 127–148.

Schleypen, J.S., von Geldern, M., Weiß, E.H., Kotzias, N., Rohrmann, K., Schendel, D.J., Falk, C.S., and Pohla, H. (2003). Renal cell carcinoma-infiltrating natural killer cells express differential repertoires of activating and inhibitory receptors and are inhibited by specific HLA class I allotypes. Int. J. Cancer *106*, 905–912.

Schoenborn, J.R., and Wilson, C.B. (2007). Regulation of Interferon-γ During Innate and Adaptive Immune Responses. B.-A. in Immunology, ed. (Academic Press), pp. 41–101.

Seo, S.H., Kim, K.S., Park, S.H., Suh, Y.S., Kim, S.J., Jeun, S.-S., and Sung, Y.C. (2011). The effects of mesenchymal stem cells injected via different routes on modified IL-12-mediated antitumor activity. Gene Ther. *18*, 488–495.

Shi, Y., Su, J., Roberts, A.I., Shou, P., Rabson, A.B., and Ren, G. (2012a). How mesenchymal stem cells interact with tissue immune responses. Trends Immunol. *33*, 136–143.

Shi, Y., Su, J., Roberts, A.I., Shou, P., Rabson, A.B., and Ren, G. (2012b). How mesenchymal stem cells interact with tissue immune responses. Trends Immunol. *33*, 136–143.

Shi, Y., Du, L., Lin, L., and Wang, Y. (2017). Tumour-associated mesenchymal stem/stromal cells: emerging therapeutic targets. Nat. Rev. Drug Discov. *16*, 35–52.

Shibuya, K., Lanier, L.L., Phillips, J.H., Ochs, H.D., Shimizu, K., Nakayama, E., Nakauchi, H., and Shibuya, A. (1999). Physical and Functional Association of LFA-1 with DNAM-1 Adhesion Molecule. Immunity *11*, 615–623.

Siegel, R.L., Miller, K.D., and Jemal, A. (2015). Cancer statistics, 2015. CA. Cancer J. Clin. *65*, 5–29.

Sotiropoulou, P.A., Perez, S.A., Gritzapis, A.D., Baxevanis, C.N., and Papamichail, M. (2006a). Interactions Between Human Mesenchymal Stem Cells and Natural Killer Cells. STEM CELLS *24*, 74–85.

Sotiropoulou, P.A., Perez, S.A., Gritzapis, A.D., Baxevanis, C.N., and Papamichail, M. (2006b). Interactions between human mesenchymal stem cells and natural killer cells. Stem Cells Dayt. Ohio *24*, 74–85.

de Souza, L.E.B., Malta, T.M., Kashima Haddad, S., and Covas, D.T. (2016). Mesenchymal Stem Cells and Pericytes: To What Extent Are They Related? Stem Cells Dev. *25*, 1843–1852.

Spaggiari, G.M., and Moretta, L. (2013). Cellular and molecular interactions of mesenchymal stem cells in innate immunity. Immunol. Cell Biol. *91*, 27–31.

Spaggiari, G.M., Capobianco, A., Becchetti, S., Mingari, M.C., and Moretta, L. (2006a). Mesenchymal stem cell-natural killer cell interactions: evidence that activated NK cells are

capable of killing MSCs, whereas MSCs can inhibit IL-2-induced NK-cell proliferation. Blood *107*, 1484–1490.

Spaggiari, G.M., Capobianco, A., Becchetti, S., Mingari, M.C., and Moretta, L. (2006b). Mesenchymal stem cell-natural killer cell interactions: evidence that activated NK cells are capable of killing MSCs, whereas MSCs can inhibit IL-2-induced NK-cell proliferation. Blood *107*, 1484–1490.

Spaggiari, G.M., Capobianco, A., Abdelrazik, H., Becchetti, F., Mingari, M.C., and Moretta, L. (2008). Mesenchymal stem cells inhibit natural killer–cell proliferation, cytotoxicity, and cytokine production: role of indoleamine 2,3-dioxygenase and prostaglandin E2. Blood *111*, 1327–1333.

Stagg, J., and Galipeau, J. (2013). Mechanisms of immune modulation by mesenchymal stromal cells and clinical translation. Curr. Mol. Med. *13*, 856–867.

Stanietsky, N., Simic, H., Arapovic, J., Toporik, A., Levy, O., Novik, A., Levine, Z., Beiman, M., Dassa, L., Achdout, H., et al. (2009). The interaction of TIGIT with PVR and PVRL2 inhibits human NK cell cytotoxicity. Proc. Natl. Acad. Sci. U. S. A. *106*, 17858–17863.

Staunton, D.E., Dustin, M.L., and Springer, T.A. (1989). Functional cloning of ICAM-2, a cell adhesion ligand for LFA-1 homologous to ICAM-1. Nature *339*, 61–64.

Sun, Y. (2015). Translational Horizons in the Tumor Microenvironment: Harnessing Breakthroughs and Targeting Cures. Med. Res. Rev. *35*, n/a-n/a.

Sun, J.C., and Lanier, L.L. (2009). Natural killer cells remember: An evolutionary bridge between innate and adaptive immunity? Eur. J. Immunol. *39*, 2059.

Sun, Z., Wang, S., and Zhao, R.C. (2014). The roles of mesenchymal stem cells in tumor inflammatory microenvironment. J. Hematol. Oncol.J Hematol Oncol *7*, 14.

Suzuki, K., Sun, R., Origuchi, M., Kanehira, M., Takahata, T., Itoh, J., Umezawa, A., Kijima, H., Fukuda, S., and Saijo, Y. (2011). Mesenchymal stromal cells promote tumor growth through the enhancement of neovascularization. Mol. Med. Camb. Mass *17*, 579–587.

Trotta, R., Parihar, R., Yu, J., Becknell, B., Allard, J., Wen, J., Ding, W., Mao, H., Tridandapani, S., Carson, W.E., et al. (2005). Differential expression of SHIP1 in CD56bright and CD56dim NK cells provides a molecular basis for distinct functional responses to monokine costimulation. Blood *105*, 3011–3018.

Tseng, H.-C., Bui, V., Man, Y.-G., Cacalano, N., and Jewett, A. (2014). Induction of Split Anergy Conditions Natural Killer Cells to Promote Differentiation of Stem Cells through Cell-Cell Contact and Secreted Factors. Front. Immunol. *5*, 269.

Turley, S.J., Cremasco, V., and Astarita, J.L. (2015). Immunological hallmarks of stromal cells in the tumour microenvironment. Nat. Rev. Immunol. *15*, 669–682.

Uccelli, A., Moretta, L., and Pistoia, V. (2006). Immunoregulatory function of mesenchymal stem cells. Eur. J. Immunol. *36*, 2566–2573.

Uccelli, A., Moretta, L., and Pistoia, V. (2008). Mesenchymal stem cells in health and disease. Nat. Rev. Immunol. *8*, 726–736.

Velardi, A. (2008). Role of KIRs and KIR ligands in hematopoietic transplantation. Curr. Opin. Immunol. *20*, 581–587.

Velardi, A., Ruggeri, L., Mancusi, A., Aversa, F., and Christiansen, F.T. (2009). Natural killer cell allorecognition of missing self in allogeneic hematopoietic transplantation: a tool for immunotherapy of leukemia. Curr. Opin. Immunol. *21*, 525–530.

Vilches, C., and Parham, P. (2002). KIR: Diverse, Rapidly Evolving Receptors of Innate and Adaptive Immunity. Annu. Rev. Immunol. *20*, 217–251.

Villegas, F.R., Coca, S., Villarrubia, V.G., Jiménez, R., Chillón, M.J., Jareño, J., Zuil, M., and Callol, L. (2002). Prognostic significance of tumor infiltrating natural killer cells subset CD57 in patients with squamous cell lung cancer. Lung Cancer *35*, 23–28.

Vitale, M., Cantoni, C., Pietra, G., Mingari, M.C., and Moretta, L. (2014). Effect of tumor cells and tumor microenvironment on NK-cell function. Eur. J. Immunol. *44*, 1582–1592.

Vivier, E., Tomasello, E., Baratin, M., Walzer, T., and Ugolini, S. (2008). Functions of natural killer cells. Nat. Immunol. *9*, 503–510.

Vivier, E., Ugolini, S., Blaise, D., Chabannon, C., and Brossay, L. (2012). Targeting natural killer cells and natural killer T cells in cancer. Nat. Rev. Immunol. *12*, 239–252.

Vivier, E., Ugolini, S., and Nunès, J.A. (2013). ADAPted secretion of cytokines in NK cells. Nat. Immunol. *14*, 1108–1110.

Vyas, Y.M., Mehta, K.M., Morgan, M., Maniar, H., Butros, L., Jung, S., Burkhardt, J.K., and Dupont, B. (2001). Spatial organization of signal transduction molecules in the NK cell immune synapses during MHC class I-regulated noncytolytic and cytolytic interactions. J. Immunol. Baltim. Md 1950 *167*, 4358–4367.

Wang, C., Thudium, K.B., Han, M., Wang, X.-T., Huang, H., Feingersh, D., Garcia, C., Wu, Y., Kuhne, M., Srinivasan, M., et al. (2014a). In vitro characterization of the anti-PD-1 antibody nivolumab, BMS-936558, and in vivo toxicology in non-human primates. Cancer Immunol. Res. *2*, 846–856.

Wang, M., Cao, J.-X., Pan, J.-H., Liu, Y.-S., Xu, B.-L., Li, D., Zhang, X.-Y., Li, J.-L., Liu, J.-L., Wang, H.-B., et al. (2014b). Adoptive Immunotherapy of Cytokine-Induced Killer Cell Therapy in the Treatment of Non-Small Cell Lung Cancer. PloS One *9*, e112662.

Waterman, R.S., Tomchuck, S.L., Henkle, S.L., and Betancourt, A.M. (2010). A New Mesenchymal Stem Cell (MSC) Paradigm: Polarization into a Pro-Inflammatory MSC1 or an Immunosuppressive MSC2 Phenotype. PLoS ONE *5*.

Waterman, R.S., Henkle, S.L., and Betancourt, A.M. (2012). Mesenchymal Stem Cell 1 (MSC1)-Based Therapy Attenuates Tumor Growth Whereas MSC2-Treatment Promotes Tumor Growth and Metastasis. PLoS ONE *7*.

Wei, X., Yang, X., Han, Z., Qu, F., Shao, L., and Shi, Y. (2013). Mesenchymal stem cells: a new trend for cell therapy. Acta Pharmacol. Sin. *34*, 747–754.

Williams, A.R., and Hare, J.M. (2011). Mesenchymal stem cells: Biology, patho-physiology, translational findings, and therapeutic implications for cardiac disease. Circ. Res. *109*, 923–940.

Wittnebel, S., Da Rocha, S., Giron-Michel, J., Jalil, A., Opolon, P., Escudier, B., Validire, P., Khawam, K., Chouaib, S., Azzarone, B., et al. (2007). Membrane-bound interleukin (IL)-15 on renal tumor cells rescues natural killer cells from IL-2 starvation-induced apoptosis. Cancer Res. *67*, 5594–5599.

Wood, S.L., Pernemalm, M., Crosbie, P.A., and Whetton, A.D. (2014). The role of the tumor-microenvironment in lung cancer-metastasis and its relationship to potential therapeutic targets. Cancer Treat. Rev. *40*, 558–566.

Xu, X., Zhang, X., Wang, S., Qian, H., Zhu, W., Cao, H., Wang, M., Chen, Y., and Xu, W. (2011). Isolation and comparison of mesenchymal stem-like cells from human gastric cancer and adjacent non-cancerous tissues. J. Cancer Res. Clin. Oncol. *137*, 495–504.

Yáñez-Mó, M., Siljander, P.R.-M., Andreu, Z., Zavec, A.B., Borràs, F.E., Buzas, E.I., Buzas, K., Casal, E., Cappello, F., Carvalho, J., et al. (2015). Biological properties of extracellular vesicles and their physiological functions. J. Extracell. Vesicles *4*.

Zaidi, M.R., and Merlino, G. (2011). The two faces of interferon-γ in cancer. Clin. Cancer Res. Off. J. Am. Assoc. Cancer Res. *17*, 6118–6124.

Zocchi, M.R., Camodeca, C., Nuti, E., Rossello, A., Venè, R., Tosetti, F., Dapino, I., Costa, D., Musso, A., and Poggi, A. (2015). ADAM10 new selective inhibitors reduce NKG2D ligand release sensitizing Hodgkin lymphoma cells to NKG2D-mediated killing. Oncoimmunology *5*.