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THE NK CELLS PHENOTYPE AND FUNCTION IN RESECTED NON SMALL CELL LUNG CANCER: DIFFERENCES BETWEEN SQUAMOUS AND ADENOCARCINOMA AND RELATION TO TUMOR ANGIOGENESIS

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SUMMARY

The tumor microenvironment has come to light as a key player in carcinogenesis and progression. Tumors affect many host cell types, in particular immune cells. The immune system appears to select particularly fit tumor cells in the process of immuno-editing, while the tumor cells influence the polarization of immune cells towards phenotypes that favor tumor growth and vascularization. Here we investigated the phenotype of tumor infiltrating natural killer (NK) cells, focusing on angiogenesis associated cytokines and activities in patient-derived material from non-small cell lung cancer (NSCLC). Samples from the tumor and adjacent normal tissues, as well as peripheral blood and lung samples as well as from non-oncologic patients with bullous emphysema were collected and rapidly processed to obtain single cell suspensions. Flow cytometry (FC) analyses were performed to evaluate specific markers (CD3, CD56, CD16) to identify NK cell subsets. We observed that in the NSCLC samples, the $CD56^{+}CD16^{-}$ NK phenotype, associated with cytokine production, predominated in the tumor samples while the $CD56^{dim}CD16^{+}$ cytotoxic phenotype dominated in the adjacent normal tissues and in lung tissue derived from non-oncologic patients. This was independent of tumor histotype and smoking status. We examined the angiogenic potential of tumor infiltrating NK cells by intracellular staining for production of VEGF, PlGF, IL-8 (CXCL8), IFNγ and other markers. The $CD56^{+}CD16^{-}$ subset was clearly associated with production of angiogenic cytokines in all samples. However, patients with squamous carcinoma histotypes showed remarkably and significantly higher production of angiogenic factors in tumor infiltrating, adjacent tissue and especially in peripheral blood $CD56^{+}CD16^{-}$ NK cells than patients with adenocarcinomas. Following surgical intervention, these levels were reduced in disease-free patients. Moreover, supernatants derived from the tumor infiltrating $CD56^{+}CD16^{-}$ NK cells were able to induce endothelial cell chemotaxis and formation of capillary-like structures in vitro; this was particularly evident for NK cells isolated from squamous cell carcinomas. Our data suggest that squamous NSCLC tumors have a significant systemic effect on NK cells, enhancing angiogenic cytokine production in a manner dependent on the presence of disease. NK cells appear to participate in tumor neovascularization and could represent a peripheral marker for disease progression, angiogenesis and response to therapies in some tumor subsets.

1. INTRODUCTION

1. 1 Angiogenesis and tumor angiogenesis

Angiogenesis, the formation of new blood vessels, is a crucial process in a number of physiological events, including reproduction, development, tissue repair and wound healing. The angiogenesis process depends on a balance between angiogenic promotors and inhibitors. An excess of angiogenic factors over angiogenesis inhibitors, determines a break of this equilibrium and the onset of "angiogenic diseases", including cancer.
Angiogenesis is characterized by phases involving diverse factors and cell types as well as their interactions. These phases include:

- Production of angiogenic factors – often due to hypoxia
- Release of factors and their interaction with their specific receptors on the surface of endothelial cells
- Endothelial cell activation and proliferation
- Directional migration of endothelial cells
- Degradation of the extracellular matrix (ECM) (by enzymes) and ECM remodelling
- Morphogenic reorganization into a network, the formation of lumens making capillary tubes
- Recruitment of supporting cells: pericites, smooth muscle cells, and elaboration of the basement membrane
- Vascular stabilization

Experimental studies have shown that tumors without a vasculature are limited to only a few mm^3 in dimension; most tumors require the capacity of inducing the formation of a new blood supply to overcome the physical limitations on the diffusion of nutrients and oxygen within the tumor (Ferrara N. and Kerbel RS, 2005; Folkman J, 2006; Hanahan D and Folkman J, 1996; Hanahan D and Weinberg RA, 2000; Kerbel R and Folkman J, 2002), a critical step in progression known as the "angiogenic switch" (Hanahan D et al.,1996).
Tumor blood vessels are characterized by profound differences in structure and organization respect to normal vessels. Tumor vessels are tortuous, leaky and poorly functional, they display altered expression of surface antigens, have few pericites. Tumor cells are able to produce numerous angiogenic factors including cytokines, chemokines, growth factors, cell adhesion molecules, extracellular matrix components, proteolytic enzymes. Angiogenesis-associated factors

include the vascular endothelial growth factors (VEGFs), the fibroblast growth factors (FGFs), hepatocyte growth factor (HGF), epidermal growth factor (EGFs), interleukin-8 (IL-8), placental growth factor (PlGF), platelet derived growth factor (PDGF), insulin like growth factor (IGF-I), transforming growth factor (TGF-α, TGF-β), tumor necrosis factor (TNF-α), macrophage colony stimulating factor (M-CSF), Angiopoietins, Angiogenin.

Recent studies on the tumor microenvironment suggest that it is an integral and essential part of cancer (Noonan DM et al., 2008; Albini A et al., 2007; de Visser KE et al., 2006).

The tumour microenvironment is a complex society of many cell types, including endothelial cells (ECs) and their precursors, pericytes, smooth-muscle cells, fibroblasts of various phenotype, myofibroblasts, neutrophils, eosinophils, basophils, mast cells, lymphocytes (T, B, NK cells) and antigen-presenting cells (APCs) such as macrophages and dendritic cells. All these cells have a crucial role in tumor progression, stimulating the formation of the blood vessels (Albini A, Sporn MB, 2007). All these factors and cells producing them are potential targets of new therapeutic strategies against the so called "angiogenic diseases"

1. 2 Role of innate immunity in tumor angiogenesis

1. 2. 1 Monocytes/macrophages and Tumor Associated Macrophages (TAM)

Macrophage heterogeneity reflects the plasticity of these cells in response to microenvironmental signals, such as cytokines and microbial products, which in turn produces diverse functional programs and influences polarization of the immune system. Macrophages have a key role in promoting the orientation of the adaptive immune response typically towards either a Th1 or Th2 response, as well as by expressing specialized and polarized effector functions themselves (Goerdt S and Orfanos CE, 1999; Gordon S, 2003; Mantovani A et al., 2002; Sica A and Bronte V, 2007). Numerous data indicate that macrophages can be phenotypically polarized by the microenvironment to mount specific functional programs: either the classical (or M1) activation of effector cells able to kill tumor cells, or the alternative activation (M2) closely related to the tumor-associated macrophage (TAM) profile. M1 macrophages are generally characterized by an IL–12^{high}, IL-23^{high}, IL-10^{low} phenotype, with obvious consequences in driving a Th1 response and can also produce copious amounts of reactive oxygen and nitrogen intermediates (Goerdt S and Orfanos CE, 1999; Gordon S, 2003; Mantovani A et al., 2002; Sica A and Bronte V, 2007). The M1 macrophages, a terminology mirroring the Th1 nomenclature, are part of the afferent and efferent limbs of Th1 responses and help mediating resistance against intracellular parasites and tumors. On the other

extreme we have alternatively activated (M2) macrophages induced by cytokines such as IL-4 and IL-13, which share an IL-12^{low}, IL-23^{low}, IL-10^{high} phenotype. These M2-polarized leukocytes are generally oriented to tissue remodeling and repair, immunosuppression and angiogenesis (Mantovani A et al., 2008) and substantial data show that an M2 polarized inflammation is a key feature of the tumor microenvironment (Mantovani A et al., 2002; Sica A and Bronte V, 2007) influencing angiogenesis, invasion and metastasis, as well as subversion of adaptive immunity.

The diverse polarized macrophages differ in terms of receptor expression, cytokine production profiles, chemokine repertoires and effector function. Induction of an M2 phenotype appears to be due to STAT3 activation and/or inhibition of STAT1 phosphorylation (Hu X et al., 2003; Ito S et al., 1999; Mantovani A et al., 2002; Riley JK et al., 1999), down-regulation of p65 containing NF-κB and generation of p50 NF-κB homodimers (Porta C et al., 2009). NF-κB is a key axis in tumor angiogenesis, induction of the angiogenic program in ECs involves NF-κB activation (Karin M, 2006), and numerous angiogenesis inhibitors repress NF-κB (Albini A, Noonan DM and Ferrari N, 2007). In addition, chemotherapy and radiotherapy widely used in treatment of cancer patients also induces NF-κB both in normal and cancer cells (Nakanishi C and Toi M, 2005), resulting in induction of inflammation, angiogenesis and tumor reconstruction. Tumor inflammation was historically considered to be part of an abortive rejection process, now the role of tumor associated macrophages (TAMs), has undergone extensive re-evaluation, expressing properties that facilitate tumor growth and progression by stimulating tumor-cell proliferation, angiogenesis and favoring invasion and metastasis (Balkwill F and Mantovani A, 2001; Lewis C and Murdoch C, 2005; Mantovani A et al., 2002; Pollard JW, 2004). TAMs derived from circulating monocytic precursors are directed into the tumor by chemokines, largely through hypoxia-dependent mechanisms involving VEGF and the CXCL12/CXCR4 axis (Mantovani A et al., 2002; Saccani A et al., 2006; Schioppa T et al., 2003). Analyses of TAMs indicate that these express an M2-associated phenotype, with a similar transcriptome profile oriented toward tissue remodeling and repair, immuno-suppression and angiogenesis (Biswas SK et al., 2006; Mantovani A et al., 2002; Murdoch C et al., 2008).

TAMs recruited into tumors are primarily attracted and/or immobilized into avascular (Leek RD et al., 1996) and necrotic (Leek RD et al., 1999) areas of hypoxia, (O'Sullivan C and Lewis CE, 1994; Harmey JH et al., 1998). Recent in vitro data show that TAMs are stimulated by hypoxia in such sites to co-operate with tumor cells to promote revascularization (Murdoch C et al., 2008; Schioppa T et al., 2003) in fact the level of hypoxia present in these areas stimulates the release of VEGF from human macrophages (Lewis JS et al., 1999) through up-regulation of transcriptional factors including hypoxia inducible factors (HIFs 1 and 2) that activate genes leading to the release of

glycolytic, proangiogenic and proinvasive molecules. (Leek RD et al., 2000), such as TNFa (Pusztai L et al., 1994), IL-8 (Fujimoto J et al., 2000), and CXCL12 (Schioppa T et al., 2003), matrix metalloproteinases (MMPs 1, 2, 3, 9, and 12) (Deryugina EI and Quigley JP, 2009; Egeblad M and Werb Z, 2002), plasmin, urokinase plasminogen activator and its receptor (Pyke C et al., 1993). The proteolytic activity exerted contributes to the migration and proliferation of ECs and also to the migration and extravasation of tumor cells during the metastatic process (Deryugina EI and Quigley JP, 2009; Egeblad M and Werb Z, 2002). A series of studies have found that hypoxia itself promotes the invasive program that favors metastatic dissemination, and that VEGF based angiogenesis inhibition can result in a greater incidence in metastasis (Ebos JM et al,2009; Paez-Ribes M et al., 2009). A TAM subset that appears to have a particularly intriguing role in angiogenesis is the one of Tie2 expressing macrophages or TEMs (De Palma M et al., 2007; De Palma M and Naldini L, 2009), closely associated with the forming vasculature but that do not appear to incorporate into it as other precursors do. Specific deletion of this TAM subset results in inhibition of angiogenesis and repression of tumor growth, indicating that TEMs are key targets for anti-angiogenic therapy (De Palma M et al., 2007; De Palma M and Naldini L, 2009; Pulaski HL et al., 2009). Further, these cells can also be used as "Trojan horses" to vehicle anti-angiogenic and anti-tumor therapies into the tumor (De Palma M and Naldini L, 2009).

1. 2. 2 Myeloid derived Suppressor Cells (MDSC)

Myeloid derived suppressor cells (MDSCs) represent a range of poorly differentiated myeloid precursor that shares markers for both monocyte and granulocytes (Serafini P, Borrello I and Bronte V, 2006). These cells, originally identified in murine models are characterized by potent immuno-suppressive activity expressed through a variety of mechanisms, including production of TGFb, high level of inducible nitric oxide synthase (NOS2) and arginase (Arg1), which deplete arginine within the tumor microenvironment, as well as mechanims that deplete cystine and cysteine (Srivastava MK et al., 2010), all of which contribute to T cell suppression and apoptosis (Bronte V and Zanovello P, 2005; Kusmartsev S and Gabrilovich DI, 2006; Bronte V, 2009; De Santo C et al., 2005; Dolcetti L et al., 2010). MDSCs are also powerful promoters of angiogenesis by MMP (Yang L et al., 2004) and VEGF production (Sica A and Bronte V, 2007) subsequent to Stat3 activation (Kujawski M et al., 2008). MDSC tumor infiltrates have been reported to be responsible for the resistance of tumors to anti-angiogenic therapy (Shojaei F et al., 2007) (Shojaei F et al., 2009), largely via production of diverse angiogenic factors such as Bv8 (Shojaei F et al., 2007, Shojaei F and Ferrara N, 2008).

Myeloid precursors have also been found to be elevated in human cancers (Almand B et al., 2001; Diaz-Montero CM et al., 2009; Ostrand-Rosenberg S and Sinha P, 2009; Rodriguez PC et al., 2009) and to correlate with disease status (Diaz-Montero CM et al., 2009) where a variety of myeloid cells, blocked at different stages of maturation along the monocytic or granulocytic pathways, are present (Ostrand-Rosenberg S and Sinha P, 2009). The role of human MDSCs in tumor angiogenesis is not fully understood and the main question is how to target these cells: approaches were largely directed towards immune-suppressive activity including use of nitric oxide donors, such as Nitroaspirin (De Santo C et al., 2005; Ugel S et al., 2009), that interfer with recruitment and subsequent angiogenesis by blocking the CSF-1 receptor (Priceman SJ et al., 2009)

1. 2. 3 Neutrophils

Neutrophils are the most abundant circulating leukocyte in humans and the primary granulocytes involved in angiogenesis. These cells play a primary role in responses to diverse infective agents, based mostly on pattern recognition mechanisms. They are quickly and massively recruited into areas producing danger signals and store in granules several soluble mediators that can be rapidly secreted in both physiological and pathological conditions as well as respiratory burst production of reactive oxygen species (O_2^-, H_2O_2, HOCl). These factors can produce tissue destruction and lysis, resulting in significant anti-tumor activity (Di Carlo E et al., 2001). Neutrophils are also sources for several cytokines (including TNFα, IL-1β, IL-1Rα, IL-12 and VEGF), and chemokines (including CXCL1, CXCL-8, CXCL9, CXCL10, CCL3, and CCL4) (Scapini P et al., 2000). These products can influence the immune response and polarization as well as promote tissue reconstruction and angiogenesis. Neutrophils are able to produce a broad range of angiogenic factors (Schruefer R et al., 2005; Schruefer R et al., 2006), we noted that neutrophils appeared to lead the invasion of vessel sprouts into a matrigel sponge containing tat (Benelli R et al., 2000). The use of corneal pocket assays indicated that angiogenesis induced by IL-8 (CXCL8), a chemokine highly active on neutrophils and a known angiogenic factor, acted through CXCR2 receptors expressed on ECs (Addison CL et al., 2000). However, not all endothelial cells express CXCR2 and subsequent studies demonstrated that neutrophils were required for the angiogenic response to IL-8 stimulation (Benelli R et al., 2002). The link between IL-8 production and subsequent tumor-associated neutrophil (TAN) recruitment has been confirmed in tumor models, where this cascade was shown to be critical in linking ras oncogene expression to tumor growth and angiogenesis (Sparmann A and Bar-Sagi D, 2004). TANs have also been shown to be a key source of Metallo-Matrix-Proteinase 9 (MMP9), a protease required for the angiogenic switch and tumor growth in skin

carcinogenesis and in Rip-Tag pancreatic cancer models (Coussens LM et al., 2000; Nozawa H, Chiu C and Hanahan D, 2006). The inflammation-associated angiogenic response during wound healing was significantly delayed in animals harboring genetic defects that compromise neutrophil recruitment, in particular deletion of CXCR2 (Devalaraja RM et al., 2000) or CD18 (Schruefer R et al., 2006). Similarly, a dual src kinase knock-out that compromised neutrophil function also blocked the ability to induce angiogenesis in response to CXCL1 (Scapini P et al., 2004). In this case the neutrophils were recruited into the site, but were unable to release VEGF, a factor critical for induction of the angiogenic response. Intriguingly, in a major site of physiological angiogenesis in adults, the endometrium, neutrophils closely associate with the growing vessels and appear to be the primary source of VEGF for these vessels (Gargett CE et al., 2001; Heryanto B, Girling JE and Rogers PA, 2004; Lin YJ et al., 2006; Na YJ et al., 2006), thus intimately nurture angiogenesis in this organ.

The divergent roles for neutrophils, on one hand able to produce anti-tumor tissue destruction, on the other to supply angiogenic factors and tissue regeneration signals, and the reports of multiple neutrophil subsets(Tsuda Y et al., 2004) lead to the speculation that several subsets of neutrophils may exist, in particular pro- and anti-angiogenic subsets (Noonan DM et al., 2008). This hypothesis is supported also by the role that neutrophils recruitment seem to have in PPARa knock-out mouse models (Kaipainen A et al., 2007). In fact tumors injected into PPARa knock-out mice remained in an essentially dormant state, with notable neutrophil recruitment and production of the anti-angiogenic matrix molecule thrombospondin while neutrophil depletion permitted angiogenesis and tumor growth, indicating an anti-tumor phenotype dictated by the loss of PPARa (Kaipainen A et al., 2007). In the other hand instead, in wild-type mice, neutrophil depletion slowed tumor growth consistent with a pro-angiogenic activity (Nozawa H, Chiu C and Hanahan D, 2006; Pekarek LA et al., 1995). Additional support for the existence of multiple neutrophil subsets with either pro- or anti-tumor activity comes from analyses of the effects of TGFb blockades. TGFb is an immunosupressive cytokine that itself has a dichotomous activity in cancer: inhibitory at early stages and enhancer of tumor progression and immunosuppression at later stages when is frequently found in the tumor microenvironment (Bierie B and Moses HL, 2006). Blockage of TGFb in diverse tumor models can lead to enhanced CD8+ T cell activity and to accumulation of tumor associated neutrophils (TAN) within the tumor lead to mass regression in human and mice (Ge R et al., 2006; Nam JS et al., 2008; Suzuki E et al., 2007; Fridlender ZG et al., 2009) while on the contrary depletion of neutrophils under TGFb blockade impaired $CD8^+$ T cell activation and enhanced tumor growth. Even in this case the authors lean towards the idea of the existence of two different neutrophil subsets: pro-tumoral neutrophils, termed “N2” in concordance

with the M2/Th2 paradigm, linked to the presence of TGFb, and anti-tumoral neutrophils, "N1", recruited into tumors when TGFb turn to be inactive. These findings were similar in two different tumor types (NSCLC and mesothelioma) and in three different mice strains, suggesting that the polarization of neutrophils may be a general feature of tumor microenvironment (Fridlender ZG et al., 2009). However the potential role in angiogenesis of these cells was not fully investigated yet

1. 2. 4 Mast cells

Mast cells were initially suggested to be involved in vascularization during rheumatoid arthritis (Maruotti N et al., 2007; Ribatti D, Contino R and Tursi A, 1988), and appear to play a key role in angiogenesis in allergic reactions as well (Crivellato E, Travan L and Ribatti D, 2009). These cells are intimately involved in vascularization of hematologic malignancies, (Ribatti D, Crivellato E and Molica S, 2009), where they appear to integrate into the vessel wall through the process of vascular mimicry (Nico B et al., 2008) while play a significant role in solid tumor angiogenesis (Crivellato E, Nico B and Ribatti D, 2008; Murdoch C et al., 2008; Ribatti D et al., 2001). In fact, mast cells contribute to the angiogenic switch producing numerous angiogenesis-associated cytokines and chemokines (Murdoch C et al., 2008; Ribatti D et al., 2004) and proteases that promote premalignant angiogenesis (Coussens LM et al., 1999; Ranieri G et al., 2009; Soucek L et al., 2007). Therefore mast cells are considered a new target for anti-angiogenic therapies (Galinsky DS and Nechushtan H, 2008; Liu J et al., 2009).

1. 2. 5 Dendritic Cells (DCs)

Dendritic cells (DCs) constitute a heterogeneous population of antigen presenting cells found in virtually every tissue, which through their antigen presentation and cytokine secretion activities contitute the link between innate and adaptive immunity (Lanzavecchia A and Sallusto F, 2001; Steinman RM and Banchereau J, 2007). Although their clinical relevance is a matter of debate DCs recruitment into tumors have been documented in several studies (Balkwill F, 2004; Sozzani S, Allavena P and Mantovani A, 2001; Ueno H et al., 2007; Vicari AP, Treilleux I and Lebecque S, 2004),. Within the tumor microenvironment, DCs generally show an immature phenotype characterized by the low expression of co-stimulatory molecule and IL-12 production (Vermi W et al., 2003; Vicari AP, Treilleux I and Lebecque S, 2004), apparently due to cytokines that block differentiation and maturation (IL-10, IL-6, TGFb, VEGF and M-CSF) (Ratta M et al., 2002; Steinman RM and Banchereau J, 2007). Tumor associated DCs maintain tolerance to tumor

antigens, promote angiogenesis, tumor growth, progression and dissemination (Mantovani A et al., 2002;Sozzani S et al., 2007). DCs express a wide array of pro- and anti-angiogenic mediators that might have a significant role in those pathophysiological settings characterized by DC activation and angiogenesis, including inflammation, wound healing, atherosclerosis and tumor growth (Sozzani S et al., 2007). These mediators, members of distinct families, modulate neovascularization by different mechanisms of action (Mantovani A, 2004; Sozzani S, 2005).
DCs release classical angiogenic growth factors that act on the endothelium by engaging the corresponding signaling receptors on cell surface. Conventional DCs express VEGF-A, FGF2 and ET-1 (Guruli G et al., 2004; Riboldi E et al., 2005; Bourbie-Vaudaine S et al., 2006; Piqueras B et al., 2006). On the other hand these cell subset can release cytokines that repress angiogenesis such as IL-12 (Albini A et al., 2009; Noonan DM et al., 2008; Trinchieri G, 2003), IFNg (Trinchieri G, 2003) and the angiostatic chemokines CXCL9, CXCL10 and CCL21 (Piqueras B et al., 2006). DCs can release cytokines, which although devoid of a direct pro-angiogenic activity, can increase EC responsiveness to classic angiogenic growth factors (such as TNFa (Caux C et al., 1994; de Graaf JH et al., 1996; Verhasselt V et al., 1997), Osteopontin (OPN) (Konno S et al., 2006; Naldini A et al., 2006), IL-6, TGFb, (Verhasselt V et al., 1997), CXCL1/2/3/5/8 and CCL2 (Means TK et al., 2003; Scimone ML et al., 2005; Vermi W et al., 2006;Curiel TJ et al., 2004; Penna G et al., 2002; Piqueras B et al., 2006) or up-regulate the production of angiogenic factors by other cell types. DCs can produce anti-angiogenic ECM components including thrombospondin-1 (TSP-1) (Doyen V et al., 2003; Rusnati M and Presta M, 2006) and long-pentraxin-3 (PTX3) (Doni A et al., 2006; Doni A et al., 2003; Garlanda C et al., 2005) or can transdifferentiate into endothelial cells (Coukos G et al., 2005), similarly to that reported for hematopoietic precursors, circulating endothelial precursors (CEPs) and circulating endothelial cells (CECs) (Dome B et al., 2009).

1. 2. 6 Natural Killer Cells (NKs)

NK cells are classified as the third kind of cells differentiated from the common lymphoid progenitor generating also B and T lymphocytes (Di Santo JP, 2008). They were originally described as large granular lymphocytes active against tumor cells and endowed with both cytotoxic and cytokine-producing functions. NKs serve to contain viral infections while the adaptive immune response is generating antigen-specific cytotoxic T cells that can clear the infection (Vivier E et al., 2004). The acquisition of cytotoxic capabilities during evolution has been associated with the development of highly sophisticated and robust mechanisms controlling the cytolytic processes in order to avoid tissue damage. In fact the NK cell activation system operates through a variety of cell

surface receptors that activate or repress NK cell functions (Chiesa S et al., 2005; Vivier E et al., 2004). Thus, the integration of antagonistic pathways upon interaction with neighboring cells governs the dynamic equilibrium regulating NK cell activation dictates whether or not NK cells are enrolled to kill target cells in response to interferons or macrophage-derived cytokines (Eissman P et al., 2010).

To control their cytotoxic activity, NK cells possess two types of surface receptors: *activating receptors (KARs)* and *inhibitory receptors (KIRs)* (Chiesa S et al., 2005; Vivier E et al., 2004). Most of these receptors are not unique to NK cells and can be present in some T cell subsets as well.

Activating NK cell receptors detect the presence of ligands on cells in 'distress', such as the stress-induced self ligands recognized by NKG2D (ULBP and MIC in human and RAE1, H60 and MULT1 in mouse) or other alert molecules including infectious non-self ligands (for example, the cytomegalovirus-encoded m157 recognized by Ly49H in the mouse) and Toll-like receptor (TLR) ligands. Indeed, NK cells express several TLRs and i*n vitro* exposure of NK cells to TLR ligands induces IFN production and enhances cytotoxicity. However, this process is more efficient when accessory cells are present together with NK cells, suggesting that the role of TLRs in NK cells *in vivo* might be not direct (Gerosa et al, 2005, Hart OM et al, 2005). NK cells also express the low-affinity Fc receptor CD16, that enable them to detect antibody-coated target cells to exert antibody-dependent cell cytotoxicity (ADCC).

NK cells appear to determine whether a cell is infected or not through recognition of an "altered self" state in particular involving the expression levels of Major histocompatibility complex (MHC) class I molecules on the target cell surface, the main mechanism by which cells display viral or tumor antigens to cytotoxic T cells (Chiesa S et al., 2005; Vivier E et al., 2004. A common evolutionary adaption to this, seen in both intracellular microbes and tumours, is a chronic down-regulation of these MHC I molecules, rendering the cell resistant to T cell mediated immunity. It is believed that NK cells, in turn, evolved in response to this adaption, since NKs use their inhibitory receptors to evaluate the absence of MHC class I molecules on susceptible target cells (Yokoyama, W.M et al, 2005, Hart, O.M., Athie-Morales et al, 2005). Inhibition of NK cell activity by MHC class I molecules is crucial to the role played by NK cells. The MHC class I–specific inhibitory receptors include the killer cell immunoglobulin-like receptors (KIRs) in human, the lectin-like Ly49 dimers in mouse and the lectin-like CD94-NKG2A heterodimers in both species (Yokoyama, W.M et al, 2005, Hart, O.M., Athie-Morales et al, 2005) that share conserved intracytoplasmic inhibitory signaling domains called immunoreceptor tyrosine-based inhibition motifs (ITIMs). By interacting with MHC class I molecules, constitutively expressed by healthy cells, inhibitory receptors provide a way for NK cells to ensure tolerance to self while allowing toxicity toward

stressed cells that can down-regolate the expression of the MHC class I molecules. MHC class I is not the only constitutive self signal detected by NK cells as other inhibitory receptors, for example NKR-P1B and 2B4 in mouse and NKR-P1A in human, recognize non-MHC self molecules (Clr-b, CD48 and LLT-1 respectively) and regulate NK cell activation.

Consistent with their function as innate sentinels, NK cells are widespread throughout lymphoid and nonlymphoid tissues where they represent a minor fraction of total lymphocytes (from 2% to 10% in mouse's organs and from 2% to 18% in human peripheral blood (Parham, P. et al, 2005), also distinct NK cell subsets have been defined in mice and humans based on phenotypic, functional and anatomical features (Chiossone L er al., 2007).

In humans, the $CD56^{dim}CD16^{+}$ NK cell subset constitutes about 90-95% of peripheral blood NKs. These cells readily kill target cells upon proper recognition and secrete low cytokine levels (Cooper MA, Fehniger TA and Caligiuri MA, 2001). In contrast, $CD56^{bright}CD16^{-}$ NK cells (about 5-10% of peripheral blood NKs) are poorly cytotoxic but produce large amounts of cytokines, including IFNg, TNFa, and GM-CSF. Only $CD56^{bright}$ NK cells express secondary lymphoid organ homing markers such as CCR7, CD62L and CXCR3, resulting in an enrichment of this subset in lymphoid organs and sites of inflammation (Campbell J et al., 2001; Fehniger TA et al., 2003; Ferlazzo G et al., 2004b).

The question of whether or not the development of the human subsets is interconnected has been under investigation for some time. Recently, a number of studies suggested that $CD56^{bright}CD16^{-}$ NK cells are able to differentiate into $CD56^{dim}CD16^{+}$ NK cells upon prolonged activation (Chan A et al., 2007; Romagnani C et al., 2007), while the reverse may be possible in the presence of TGFβ (Keskin DB et al., 2007).

TGFβ has been reported to be able in polarizing $CD56^{+}CD16^{+}$ subset into $CD56^{dim}CD16^{-}$ during development and differenziation (Allan DS et al., 2010); TGFb is also present in tumor microenvironment (Noonan DM et al., 2008; Serrati S et al., 2008) and its capacity in swincing on $CD56^{+}CD16^{+}$ subset into $CD56^{dim}CD16$ has been already reported (Keskin DB et al., 2007), displaing phenotypic similarityes with $CD56^{bright}CD16^{-}$ decidual NKs (dNKs) that have been shown to produce hight level of pro-angiogenic factors.

NK subset distribution differs between distinct anatomical sites, for example in secondary lymphoid organs, lung, liver, and skin, suggesting specialization (Ferlazzo G and Munz C, 2004; Ferlazzo G et al., 2004; Gregoire C et al., 2007; Trinchieri G, 1989). A particular $CD56^{superbright}CD16^{-}$ NK cell subset (dNKs) is found in the deciduas during implanatation (Hanna J et al., 2006; Hanna J and Mandelboim O, 2007;Kopcow HD et al., 2005). The dNKs have low cytotoxicity and guide decidual angiogenesis by producing high levels of angiogenic factors, in

particular VEGF and PlGF (Hanna J et al., 2006) and promote decidual cellularity (Ashkar AA, Di Santo JP and Croy BA, 2000). The $CD56^{bright}CD16^{-}$ NK cell subset is recruited into tumors (Carrega P et al., 2008) and although NK cells infiltration appears to correlate with a better prognosis in gastric (Ishigami S et al., 2000), colorectal (Coca S et al., 1997), and lung carcinomas (Takeo S et al., 1986; Villegas FR et al., 2002) we have speculated that tumor infiltrating NK cells may be switched to an angiogenic cytokines producing phenotype similar to dNK cells (Noonan DM et al., 2008).

1. 3 Non Small Cell Lung Cancer (NSCLC)

Lung cancer is currently one of the most common malignancies and NSCLC represents about 75-80% af oll cases of lung cancers (Carrega P et al., 2008). The most common types of NSCLC are adenocarcinoma, squamous cell carcinoma (Carega P et al., 2008), and large cell carcinoma, but there are several other types that occur less frequently, and all types can occur in unusual histologic variants. Several risk factors contribute to the development of lung cancer. The risk factors include the following: Tobacco use (cigarette, pipe, or cigar smoking) and exposure to second-hand smoke; chemical or physical agents: radon, arsenic, asbestos, chromates, chloromethyl ethers, nickel, polycyclic aromatic hydrocarbons, radiation (often for therapy) to the breast or chest.

Although NSCLCs are associated with cigarette smoke, adenocarcinomas may be found in patients who have never smoked. The single most important risk factor for the development of lung cancer is smoking. For smokers, the risk for lung cancer is on average tenfold higher than in lifetime nonsmokers (defined as a person who has smoked <100 cigarettes in his or her lifetime). The risk increases with the quantity of cigarettes, duration of smoking, and earlier starting age. Smoking cessation results in a decrease in precancerous lesions and a reduction in the risk of developing lung cancer. Former smokers continue to have an elevated risk for lung cancer for years after quitting. Squamous cell carcinomas are linked more strongly with smoking than other forms of NSCLC. The incidence of squamous cell carcinoma of the lung has been decreasing in recent years, such that adenocarcinoma is now the most common histologic subtype in many countries. This is likely due to changes in cigarette manufacture and smoking habits. Currently in most western countries, adenocarcinomas represent about 40% of NSCLC, followed by squamous cell carcinoma (25%), large cell carcinoma (10%), and the remainder several other subtypes with lower frequency.

As a class, NSCLCs are relatively insensitive to chemotherapy and radiation therapy compared with SCLC. Patients with resectable disease may be cured by surgery or surgery followed by chemotherapy. Local control can be achieved with radiation therapy in a large number of

patients with unresectable disease, but complete remission is seen only in a small number of patients. Patients with locally advanced unresectable disease may achieve long-term survival with radiation therapy combined with chemotherapy. Patients with advanced metastatic disease may achieve improved survival and palliation of symptoms with chemotherapy, targeted agents, and other supportive measures.

NSCLC arises from the epithelial cells of the lung of the central bronchi to terminal alveoli. The histological type of NSCLC correlates with site of origin, reflecting the variation in respiratory tract epithelium of the bronchi to alveoli. Squamous cell carcinoma usually starts near a central bronchus. Adenocarcinoma and bronchioloalveolar carcinoma usually originate in peripheral lung tissue. Although NSCLCs represent a heterogeneous aggregate of histologies, approaches to diagnosis, staging, prognosis, and treatment are similar.

In NSCLC, the determination of stage is important in terms of therapeutic and prognostic implications. Careful initial diagnostic evaluation to define the location and to determine the extent of primary and metastatic tumor involvement is critical for the appropriate care of patients. Stage has a critical role in the selection of therapy. The stage of disease is based on a combination of clinical factors and pathological factors (Pfister D.G. et al., 2004). In NSCLC, results of standard treatment are poor except for the most localized cancers. Surgery is the most potentially curative therapeutic option for this disease. Postoperative chemotherapy may provide an additional benefit to patients with resected NSCLC. Radiation therapy combined with chemotherapy can produce a cure in a small number of patients and can provide palliation in most patients.

2. AIMS AND RATIONAL OF THE STUDY

In addition to excessively proliferating neoplastic cells, tumors are tissues that contain host components including stromal cells, a vasculature and a characteristic inflammatory infiltrate associated with the constant remodeling that tumors undergo. It is increasing recognized that these host cells also represent key therapeutic targets, and clinical data have now demonstrated that inhibition of tumor vascularization with specific agents significantly prolongs patient survival. However, the clinical benefit obtained with anti-angiogenic agents is as yet still in terms of months but there is vast room for improvement. Myeloid cell infiltrates have been found to be responsible for the resistance of tumors to anti-angiogenic therapy, largely via production of diverse angiogenic factors such as Bv8, thus it is clear that these cells represent a therapeutic target. Among the mechanisms of immune system tumor promotion, there is rapidly expanding evidence that the innate immune system plays a key role in orchestrating angiogenesis in cancer as well as other pathological and physiological conditions.

IL-12 is a potent Th1 cytokien that is also endowed with significant anti-angiogenic activity. This cytokine plays a key role in angiogenesis inhibiton by at least some endogenous angiogenesis inhibitors such as angiostatin (Morini M et al., 2004). Previous studies using blocking antibodies and bolus protein treatments indicated that the anti-angiogenic activity depended on induction of IFNγ prodiction by T and NK cells. This same IFNγ production is also thought to be responsible for the hepatotoxicity of IL-12. Using a constant, low dose gene-therapy approach to IL-12 delivery, a novel mechanism that plays a key role in the anti-angiogenic properties of IL-12 was discovered (Morini M et al., 2004). Using gene targeted mice lacking IFNγ, IFNγ was found to not be requited for the anti-angiogenic activity (Morini M et al., 2004). Although initial gene targeted and antibody depletion approaches indicated both T and NK cells were not necessary (Morini M et al., 2004), later studies using dual targeted SCID/common gamma chain mice indicated that NK cells are critical for the anti-angiogenic activity of IL-12 (Faggioli F et al., 2008). Our unpublished data support this in other models where both T and NK cells are targeted, as well as those where NK cells alone are depleted.

NK cells are delegated the "missing self" defense mechanism as they are endowed with the capacity to kill host cells that lack expression of MHC class I molecules. Peripheral NK cells are predominantly $CD56^{dim}CD16^{+}$ cytotoxic NK cells (90-95%). There is a minor constituent of peripheral NK cells that are $CD56^{bright}CD16^{-}$ NK (5-10%) and associated with cytokine production. It is now well established that NK cells are a predominant cell type within the developing deciduas

during implantation. These decidual NK cells, or dNK, are also a cytokine producing and highly angiogenic phenotype relatively similar between humans and murine models (Hanna J. et al.,2006). These dNK cells are $CD56^{superbright}CD16^{-}$, produce VEGF, PlGF and IL-8, and have been shown to significantly enhance growth of transplanted tumors by their angiogenic activity. Our hypothesis was that an NK subset similar to the dNK subset, could be present in tumors, potentially favoring tumor angiogenesis. Consistent with this concept, it has recently been shown that the stroma of human NSCLC tumors are infiltrated largely by $CD56^{bright}CD16^{-}$ NK cells (Carega P. et al., 2008) which appear to be selectively recruited into these tissues. Unlike the $CD56^{bright}CD16^{-}$ NK subset found in peripheral blood, the tumor infiltrating cells express KIR receptors and appear to be indolent with reduced killing capacity but intact cytokine production. It is not known if these tumor infiltrating NK cells express VEGF, PlGF or IL-18, nor whether their phenotype can be switched to cytotoxic phenotype by IL-12. Importantly, there is increasing evidence that DC-NK cross-talk is a key regulator of the immune system, and our preliminary data indicate that their interaction also significantly influences the angiogenic phenotype.

The goal of this project is to investigate the role of these cells in tumor angiogenesis in model systems as well as in human tumors. The data on the cellular and molecular mechanisms produced by this project will allow identification of key points that could well lead to development of new pharmaceutical targets for cancer therapy and consequent improvement of both patient survival and quality of life.

This study was designed in order to evaluate:

1. the role of NK cells in tumor angiogenesis by analyzing infiltrating cells in tumors derived from patients with non small cell lung cancer (NSCLC);
2. to confirm the presence of a predominant phenotype in NK cells infiltrating tumors and compare this phenotype with non tumor NK cells;
3. analyse cytokines production profiles of tumor infiltrating NK cells and clarify their role in tumor angiogenesis, according to the phenotype displayed;
4. the possible correlation between tumor infiltrating NKs phenotype/functionality also in relation with tumor histology and patiets smoking habits;
5. identify new targets for anti-angiogenic therapy.

3. MATERIALS AND METHODS

3. 1 Samples and patients selection

Samples (tumor tissue and "normal" adjacent tissues recovered from the margins of the surgically resected material) were obtained from patients with NSCLC during surgical resections after obtaining informed consent. The tissue samples were placed in PBS with 1% Pen/Sstrep at 4°C for no more than 18 hours prior to processing. Peripheral blood samples were drawn on the same patients prior to surgical intervention, stored at 4°C and processed within 18 hours. Patients with diabetes, HIV/HCV/HBV infection, other chronic inflammatory conditions, previously treated with chemotherapy or radiotherapy, or those iatrogenically immunosuppressed or having undergone myeloablative therapies were excluded.

3. 2 Peripheral blood mononuclear cells (PBMCs) isolation

In order to obtain mononuclear cells, a ficoll histopaque gradient was performed on peripheral blood by diluting the blood sample 1:1 with RPMI 1640 (LONZA). This suspension was then carefully stratified on Ficoll (LONZA) and centrifuged at 500 x g for 30 min. at room temperature with no brake. The lymphocyte containing ring at the interface was collected in a new tube and washed twice in PBS by centrifugation.

3. 3 Solid tissue enzymatic digestion

The solid tissues (tumor and adjacent normal tissues) were mechanically minced by scissors to obtain small fragments. The fragments were then digested with an enzymatic cocktail containing DNAse (100 μg/ml, Roche), Collagenase (1 mg/mL, Sigma Aldrich) in RPMI 1640 with 1% penstrep for 1 hour at 37°C. The suspension was then filtered on 50 μm pores cell strainers (BD) to obtain a single cell suspension and washed in PBS by centrifugation to remove residual enzymes.

3. 4 Phenotypic characterization of tumor infiltrating NK cells

Total NK cells obtained as previously described were assessed for subset distribution by Flow Cytometry. $3x10^5$ cells per sample (blood, normal adjacent tissue, tumor tissue) were stained with anti human CD45-FITC, and CD16-FITC, CD3-PerCP and CD56-APC (BD). Briefly, after setting physical parameters (SSC, FSC), lymphocyte populations were individuated by gating on CD45 positive cells, then NK cells population was distinguished by subgating on CD3 negative cells and CD56 positive cells. $CD3^-CD56^+$ population was then evaluated for $CD16^{+/-}$ in order to characterize the distribution of these two NK subsets in blood, adjacent normal tissue and tumor.

3. 5 Intracellular staining for cytokines profile analyses

Total NK cells from blood and tissues were incubated overnight in RPMI 1640 added of 5% FCS, 1% Pen/Strep, IL-2 100 U/ml at 37°C, 5% CO_2. Cells ($5x10^5$/ml) were then stimulated for 6 hours with phorbol 12-myristate 13-acetate (PMA, 10 ng/ml), Ionomycin (Iono, 500 ng/ml) and Brefeldin-A (Golgi Stop, BD). The cells were then treated with Cytofix/Cytoperm fixation and permeabilization solution (BD). The expression of specific cytokines and angiogenic growth factors were then evaluated by flow cytometric analyses after staining with anti human CD45-FITC, and CD16-FITC, CD3-PerCP, CD56-APC combined with different PE-labeled anti-human cytokines antibodies (VEGF, IL-8, Angiopoietin-1, IFNγ).

$3x10^5$cells per facs tube were stained for 30 min. with anti-human CD3-PerCP (Biolegend), CD56-APC (BD), CD16-FITC (BD), washed in PBS, then permeabilized with saponin (BD-cytofix/citoperm kit) and stained with VEGF-PE (R&D), IL-8 (R&D), PlGF–PE (ABCAM), Ang-1-PE (ABCAM). Excess of Abs was washed with PBS 1x at 500 x g, 4°C for 8 minutes. After 2 washes, cells were resuspended in 500 μL of PBS + 0.5% BSA for citofluorimetric analyses (BD-FACS Canto I).

3. 6 Immunohistochemistry of tumor samples

A portion of each tumor sample was retained fixed in formalin and embedded in paraffin for routine histopathology. Additional serial sections were stained with CD57 (indicative of activated NK cells), CD56 and CD3. Sections showing $CD3^-CD56^+$ cells were considered to be NK cells. The percentage of NK cells observed in the tissue samples was low and consistent the frequencies observed in flow cytometry

3. 7 NK cells enrichment for chemotaxis and morphogenesis assay

Total NKs, derived from blood and tissues samples were enriched by immunosorting with the MagCellect NK cell negative selection kit (R&D) from the cell suspensions obtained from blood, tumor and adjacent normal tissues. Briefly, cells were resuspendend into R&D MagCellect Buffer, then negative selection was performed by incubation for 15 min. with MagCellect Human NK Cell Biotinylated antibody Cocktail and 15 min. with MagCellect Streptoavidin Ferrofluidin reagent. Purity was greater than 80 % as determined by flow cytometry.

3. 8 Chemotaxis and morphogenesis assay of lung tumor infiltrating NKs on Endothelial cells (HUVECs)

NK cells purified from blood and tissues were incubated overnight as above. Cells ($50x10^3$ cells/ml) were then incubated in RPMI 1640 with 1% Pen/Strep and were either stimulated for 6 hours with phorbol 12-myristate 13-acetate (PMA, 10 ng/ml) and Ionomycin (Iono, 500 ng/ml) or untreated in the same culture medium. The cells were then removed by centrifugation and the supernatants recovered, filtered twice by 50 µm pore size filters to remove residual cells and debris, then concentrated with Concentricons (Millipore) devices with 5 kDa pore membranes, followed by reconstitution in PBS and re-concentration twice in order to remove residual PMA and Iono. The samples were then concentrated 10 fold and frozen at -20°C until use, and the samples were diluted to 1x when used.

In order to evaluate the capacity of tumor infiltrating NKs to induce angiogenesis associated activities, we performed endothelial cell chemotaxis and morphogenesis assays *in vitro*. Since angiogenic factors induce endothelial cell migration and invasion in vitro, we evaluated the ability of NK cell products to induce chemotaxis of human umbilical vein endothelial cells (HUVECs, Promocell) in the Boyden chamber migration assay. HUVECs were seeded ($5x10^4$ cells/well) in the upper compartment of a modified Boyden chamber; the lower compartment was filled with Cell culture supernatants derived from purified mitogen-stimulated or unstimulated NKs cells as described above. As a positive control M199 endothelial growth medium (Sigma, St Louis, MO, USA), supplemented with 10% heat-inactivated FBS, 1% glutamine, fibroblast growth factors (1 µg acid-fibroblast growth factor plus 1 µg basic-fibroblast growth factor /100 ml), epidermal growth factor (1 µg/100 ml), heparin (10 mg/100 ml) and hydrocortisone (0.1 mg/100 ml) was used. After incubation (6 hours, 37°C, 5% CO_2) filters were collected and cells were fixes and stained with DAPI, filter attached migrated cells were then counted.

Angiogenic stimuli also induce the formation of capillary-like networks by endothelial cells seeded on matrigel *in vitro*. For these morphogenesis assays, a 24-well plate, pre-chilled at -20°C, was carefully filled with 300 µl/well of liquid Matrigel (10 mg/ml) avoiding bubbles with a pre-chilled pipette, and allowed to polymerize for 1 h at 37°C. HUVECs ($5x10^4$ cells/well) were plated after resuspension in 1 ml of tumor infiltrating stimulated (PMA/Iono) or unstimulated NK cell supernatants as described above. The effects on the growth and morphogenesis of endothelial cells were recorded after 6 and 24 h with an inverted microscope (Leitz DM-IRB) equipped with CCD optics and a digital analysis system.

4. RESULTS

4. 1 Patient Characteristics

NK cells were isolated from 31 lung cancer patients undergoing tumor resection (median age was 71, range: 44-79), as well as 10 patients undergoing resection for pneumothorax, (median age 27, range 16-69), whose characteristics are shown in Tables 1 and 2. Consistent with the population at risk, the majority of the cancer patients were males (90%) and either former or current smokers (90%). The most frequent histologic type was adenocarcinoma (55%), followed by squamous cell carcinoma (29%), and tumors of other histologic subtypes. The controls (Table 1) were notably younger in age, predominantly male (90%) and current or former smokers (70%). As previously observed (Carega P. et al., 2008), NK cells represent approximately 5% of the total $CD45^+$ cells within tumors, adjacent lung tissues and in the peripheral blood (Table 2).

4. 2 The $CD56^{bright}CD16^-$ NK cells subset predominates in NSCLC tumor infiltrating NK cells as compared to control lung tissue compartments

NKs can infiltrate solid tumors, displaying different phenotypes that correlate with functional activity in terms of cytokine production and cytotoxicity, and potentially inhibiting or promoting tumor angiogenesis. Previous studies have shown that tumor samples from patients with NSCLC are enriched in the $CD56^+CD16^-$ NK subset (Carrega P. et al., 2008). These $CD56^+CD16^-$ tumor infiltrating cells have a limited capacity to kill tumor cells through an IFNγ and TNFα mediated mechanism. Here we confirm that the $CD56^+CD16^-$ NK subset is predominant in lung tumor samples (Fig. 1A) with respect to the related peripheral blood and normal adjacent lung tissue. The average level of these cells is significantly higher ($P<0.001$) than that in the adjacent lung tissue and peripheral blood samples. Although the average age of the control population was significantly different from that of the cancer patient population, the lung tissues from non-oncologic patients showed a predominant $CD56^{dim}CD16^+$ profile similar to that of the resected normal adjacent tissues lung tissues of oncologic patients (Fig. 1B). We did not observe any significant differences regarding the predominance of the $CD56^+CD16^-$ NK subset between histological subtypes: the $CD56^+CD16^-$ NK subset predominated in adenocarcinomas and squamous cell carcinomas (Fig. 1C), as well as occasional mixed adeno-squamous or large cell carcinomas (data not shown). Further, we did not observe any difference in distribution of NK cell

phenotype on the basis of smoking status, as samples from non-smokers, former smokers and current smokers showed essentially the same distribution in tumor and adjacent lung tissues (Fig. 1D). Smoking status also did not affect the distribution of the $CD56^{+}CD16^{-}$ NK subset in control patients as well (data not shown).

4. 3 The $CD56^{bright}CD16^{-}$ NK subset is associated with production of angiogenic cytokines

The $CD56^{+}CD16^{-}$ subset has been described as a potent producer of several cytokines (Cooper M. A. et al., 2001). Since dNKs have been reported to produce angiogenic factors (Hanna J. et al., 2006), we evaluated the capacity of the $CD56^{+}CD16^{-}$ NK subset, predominant in NSCLC tumors, to produce several angiogenic factors, in particular VEGF, PlGF, IL-8, and Angiopoietin-1 (Ang-1). Further, production of IFNγ, a key immunomodulatory cytokine also endowed with anti-angiogenic potential (Indraccolo S. et al., 2007) was investigated. The tumor $CD56^{+}CD16^{-}$ NK cell subset was clearly associated with significantly higher production of pro-angiogenic factors (Fig. 2), in particular VEGF, PlGF and IL-8. Interestingly, only peripheral blood $CD56^{+}CD16^{-}$ NK cells significantly produced the anti-angiogenic cytokine IFNγ, which instead was produced at very low levels by the tissue and tumor infiltrating NK cells. A similar profile was observed with production of Ang-1. These data suggest a possible role of the $CD56^{+}CD16^{-}$ NK cell subset in tumor angiogenesis in NSCLC.

4. 4 Squamous cell carcinomas show NK cells producing very high levels of angiogenic factors

We then examined the distribution of cytokine and angiogenic factor production as a function of histotype and clinical parameters. The adenocarcinomas all showed a similar lower level of production of angiogenic growth factors with the $CD56^{+}CD16^{-}$ subset producing VEGF and PlGF (Fig. 3). However, we noted that VEGF production by NK cells in patients with squamous cell carcinomas was significantly higher than in those with adenocarcinomas in tumor and adjacent lung tissue as well as in peripheral blood NK cells (Fig. 3). NK cells from patients with squamous cell carcinomas also produced significantly higher levels of PlGF than in those with adenocarcinomas in the adjacent lung tissue and peripheral blood compartments (Fig. 3). We then examined the correlation of the patients with high angiogenic cytokine production with that of histological and clinical parameters within the squamous carcinoma samples. Expression of $CD56^{+}$ was limited to few cells showing characteristics of NK morphology in most of the NSCLC samples, with occasional staining of the tumor epithelial compartment. Most of the $CD56^{+}$ cells with an NK

phenotype were $CD3^-$, and the numbers of these cells correlated well with those identified by flow cytometry (Table 2). The samples all showed highly vascularized tumors as determined by CD31 staining (Fig. 4), although this was characteristic of most of the NSCLC samples. We did not find other clinical parameters within the squamous carcinoma patient group that overtly correlated with the high level of production of VEGF and PlGF by NK cells in the tumor, adjacent tissue or peripheral blood.

4. 5 Tumor infiltrating NK $CD56^+CD16^-$ functionally promotes angiogenesis by recruiting endothelial cells and inducing formation of capillary like networks

Angiogenesis is a necessary process for tumor growth, survival and metastasis. Immune cells can infiltrate solid tumors displaying different phenotypes that correlate with tumor angiogenesis. Here we confirmed that NSCLC tumor infiltrating NK cells are enriched in the $CD56^+CD16^-$ subset, and demonstrate that this phenotype is associated with production of the angiogenic factors VEGF, PLGF, IL-8, and Angiopoietin-1. In order to evaluate the functional capacity of these cells to promote tumor angiogenesis, we examined the effects of NK cell products on *in vitro* biological correlates of angiogenesis, in particular endothelial cell recruitment and morphogenesis.

Supernatants from stimulated NSCLC tumor infiltrating NK cells were able to induce HUVEC chemotaxis (Fig. 5), while that of unstimulated cells was significantly less, suggesting this effect was due to NK cells and not eventual contaminating tumor cells. The chemotaxis effect of supernatants from adenocarcinoma cells was more apparent than that from squamous carcinomas (Fig. 5).

We then examined the ability of NK cell supernatants to promote capillary-like remodeling of HUVE cells seeded onto matrigel 3D support. Supernatants from NSCLC adenocarcinoma infiltrating NK cells stimulated by PMA and Iono induced endothelial cell morphogenesis in vitro following stimulation (Fig. 6). Interestingly, in this assay the unstimulated NK cells derived from squamous cell carcinomas showed a baseline angiogenic activity that was greatly enhanced following stimulation (Fig. 6). Network formation in the presence of supernatants of NK cells isolated from control non-oncologic tissue and peripheral blood NK cells was very limited (fig 6). Taken together, these data suggest that NK cells infiltrating into NSCLC tumors have an enhanced angiogenic potential as compared to non-tumor NK cells.

5. DISCUSSION

Squamous cell carcinomas show some distinguishing characteristics as compared to adenocarcinomas, including their response to anti-angiogenic therapy. Previous studies have shown that surgically resectable samples of squamous cell carcinomas have significantly more rapid doubling times (25% less) that those of surgically resectable adenocarcinomas (Arai T. et al., 1994). In the same case series, the faster doubling time was clearly a prognostic factor (Arai T. et al., 1994), thus in this series the squamous cell carcinomas were on the average more aggressive clinically.

Our data shows that the $CD56^{+}CD16^{-}$ NK cell subset expresses proangiogenic cytokines that could contribute to tumor angiogenesis in NSCLC. The levels of these cytokines were particularly high in $CD56^{+}$ $CD16^{-}$ NK cells of patients with squamous cell carcinoma, both tumor infiltrating but to an even greater extent in tissue and peripheral blood NK cells. These observations indicate that squamous NSCLCs have a potent influence on NK cells, exerting alterations in the phenotype of these cells both locally and systemically. The presence of a squamous cell carcinoma, even of modest dimensions, appears to have a potent effect on the phenotype of the $CD56^{+}CD16^{-}$ NK cells at a systemic level. In patients with squamous cell carcinomas, significantly higher levels of production of VEGF, a principal ligand of VEGFR1 and VEGFR2, by $CD56^{+}CD16^{-}$ NK cells is found in both tumor tissue, adjacent lung tissue and in circulating peripheral blood. Further, in patients with squamous cell carcinomas we observed significantly higher levels of PlGF production, a specific VEGFR1 agonist, by $CD56^{+}CD16^{-}$ NK cells only in the adjacent lung tissue and in circulating peripheral blood. The levels of cytokine production in peripheral blood $CD56^{+}CD16^{-}$ NK cells dropped to baseline levels following surgical intervention in subsequently disease-free patients. These data indicate that these cells could have a diagnostic, prognostic or monitoring significance in squamous NSCLC.

We note that several studies have found lower vessel densities in normal tissues as compared to tumor tissues, in particular lung (Eberhard A. et al., 2000; Regina S. et al., 2008). Two studies reported lower microvessel density (MVD) in squamous cell carcinomas as compared to adenocarcinoms (Ozbudak I. et al., 2009), while another article observed a similar trend in surgically resected NSCLC that, however, was not statistically significant (Imoto H. et al, 1998). Interestingly, VEGF staining was found to be an independent prognostic indicator of survival for resectable lung cancer patients, particularly for those of the squamous histotype (Imoto H. et al, 1998). A subsequent study, however, found no difference in the production of VEGF165 between tumor and adjacent lung tissues, while VEGF189 was found to be lower in the tumor tissues

(Regina S. et al, 2008). We sought to correlate the production of pro-angiogenic cytokines by NK cells with the MVD in the squamous cell carcinomas studied here, however no clear correlations were found. The high systemic production of these cytokines could produce a reduction in local vessel density, as circulating VEGF could "compete" for local VEGF, reducing the gradient effect and effectively lowering angiogenesis. Further, the high levels of systemic VEGF and PlGF are likely to enhance vessel permeability.

Bevacizumab has now entered into clinical use for NSCLC adenocarcinomas as an anti-angiogenic agent in combination chemotherapy. In contrast, it is not used for squamous cell carcinomas as these patients showed a tendency for life-threatening hemorrhages (Reck et al., 2009) Thus, identification of groups that may respond well to anti-angiogenic agents or those likely or not to have hemorrhages would permit entry of these drugs into the squamous NSCLC subset as potential therapy options.

6.TABLES

	Lung cancer patients (n=31)	*Control subjects (n=10)*
Age at diagnosis, mean	63	31
Age, median (range)	71 (52-78)	27 (16-69)
Male/female	28/3	9/1
Risk factors		
Smoker (%)	12 (38%)	6 (60%)
Former smoker (%)	16 (52%)	1 (10%)
Never smoker (%)	3 (10%)	3 (30%)
Histology of LC cases		
Adenocarcinoma (%)	17 (55%)	
Squamous cell carcinoma (%)	9 (29%)	
Large cell carcinoma (%)	2 (6%)	
Other NSCLC (%)	3 (10%)	
Stage of LC cases		
I A	9 (29%)	
I B	9 (29%)	
II A	4 (12%)	
II B	3 (9,6%)	
III A	6 (19,4%)	
III B	0 (0%)	
IV	0 (0%)	

Table1: patients classification by age, sex, histology and stage in tumor samples and related controls.

Patient #	Gender	Age (yrs)	Histology	T	N	M	Stage*	Smoking status	Tumor infiltrating NK		Lung tissue infiltrating NK		Peripheral Blood NK	
									Total°	CD3-CD56+CD16-	Total°	CD3-CD56+CD16-	Total°	CD3-CD56+CD16-
1	M	77	ADK	4	0	0	IIIA	Former	1.4	77.7	0.7	1	9.9	2.8
2	M	70	ADK	1a	0	0	IA	Former	0.4	83	0.1	40.5	15	2.3
3	M	77	ADK	2a	0	0	IB	Former	1.6	69.3	0.8	13.2	35.9	3.9
4	M	54	ADK	2a	0	0	IB	Former	1.2	74.8	0.7	16.1	20.9	2
5	M	60	NLC	1b	2	0	IIIA	Current	0.2	59.1	2	18.4	12.9	10.3
6	M	75	ADK	3	1	0	IIIA	Former	23.3	83.3	0.2	32.1	21.3	3.2
7	M	67	ADK	2b	0	0	IIA	Former	1.7	64.9	7.5	13.9	0.5	28.4
8	M	71	St	3	1	0	IIIA	Former	7.5	72.4	0.7	21	36.8	1.1
9	M	59	SQK	1a	0	0	IA	Current	0.2	52.1	0.9	19.8	3.3	10.2
10	M	73	ADK	2a	0	0	IB	Current	1.9	87.3	0.3	21.4	0.6	13.5
11	M	56	ADK	2a	0	0	IB	Current	0.3	55.9	0.2	38.5	30.7	1.6
12	F	72	LCC	3	0	0	IIB	Current	0.8	75	6.9	27.4	0.2	43.3
13	M	78	SQK	1a	0	0	IA	Former	0.6	64	8.8	18	1.4	22.6
14	M	70	ADK	1a	0	0	IA	Current	5.5	55.5	0.8	19.7	0.6	9.7
15	M	67	ADK	2a	0	0	IB	Current	2.4	56.9	0.2	47.3	2	33.3
16	M	73	ADK	2a	0	0	IB	Former	0.2	88.5	1.1	14.3	5.2	33.3
17	M	56	LCC	2a	1	0	IIA	Current	0.1	87.5	1.7	14.4	0.1	22.7
18	M	67	SQK	2b	0	0	IIA	Current	0.9	98.3	3.1	21.1	7.9	7.6
19	M	79	ADK	2a	0	0	IB	Former	0.6	89.9	0.7	0.3	0.1	43.3
20	M	52	ADK	1b	1	0	IIA	Never	3.3	96.1	0.9	4.1	0.7	1.4
21	M	73	SQK	2a	0	0	IB	Former	1.6	72.7	0.1	25	0.8	25
22	M	66	SQK	1b	0	0	IA	Former	0.1	92.4	0.6	6.3	2	17.4
23	M	73	SQK	1b	2	0	IIIA	Former	2.9	73	2	8.3	0.3	23.7
24	M	66	SQK	1b	0	0	IA	Former	1.3	89.9	0.8	20.8	0.8	19
25	M	72	SQK	2	2	0	IIIA	Current	ND	ND	ND	ND	12.3	ND
26	M	74	SQK	3	0	0	IIB	Former	ND	ND	ND	ND	0.1	ND
27	M	71	ADK	2	1	0	IIB	Current	ND	ND	ND	ND	0.1	ND
28	M	63	ADK	1b	1	0	IA	Current	ND	ND	0.4	ND	1.4	ND
29	F	74	ADK	1b	0	0	IA	Never	1	ND	0.3	ND	0.1	ND
30	M	73	ADK	2a	0	0	IB	Former	0.2	ND	2.3	ND	0.3	ND
31	F	72	SQK	1	0	0	IA	Never	0.4	ND	0.3	ND	13	ND

Table 2: percentage of $CD3^{-}CD56^{+}CD16^{-}$ tumor infiltrating NKs over total NKs ($CD45^{+}CD3^{-}CD56^{+}$ cells) in comparison with blood and adjacent normal tssues from patients with NSCLC. $CD3^{-}CD56^{+}CD16^{-}$ NKs subset represents the predominalt part of tumor infiltrating cells both in tumor histotypes (ADK and SQK) and indipendently to stage, smoking habits and TNM classification.

7. FIGURES

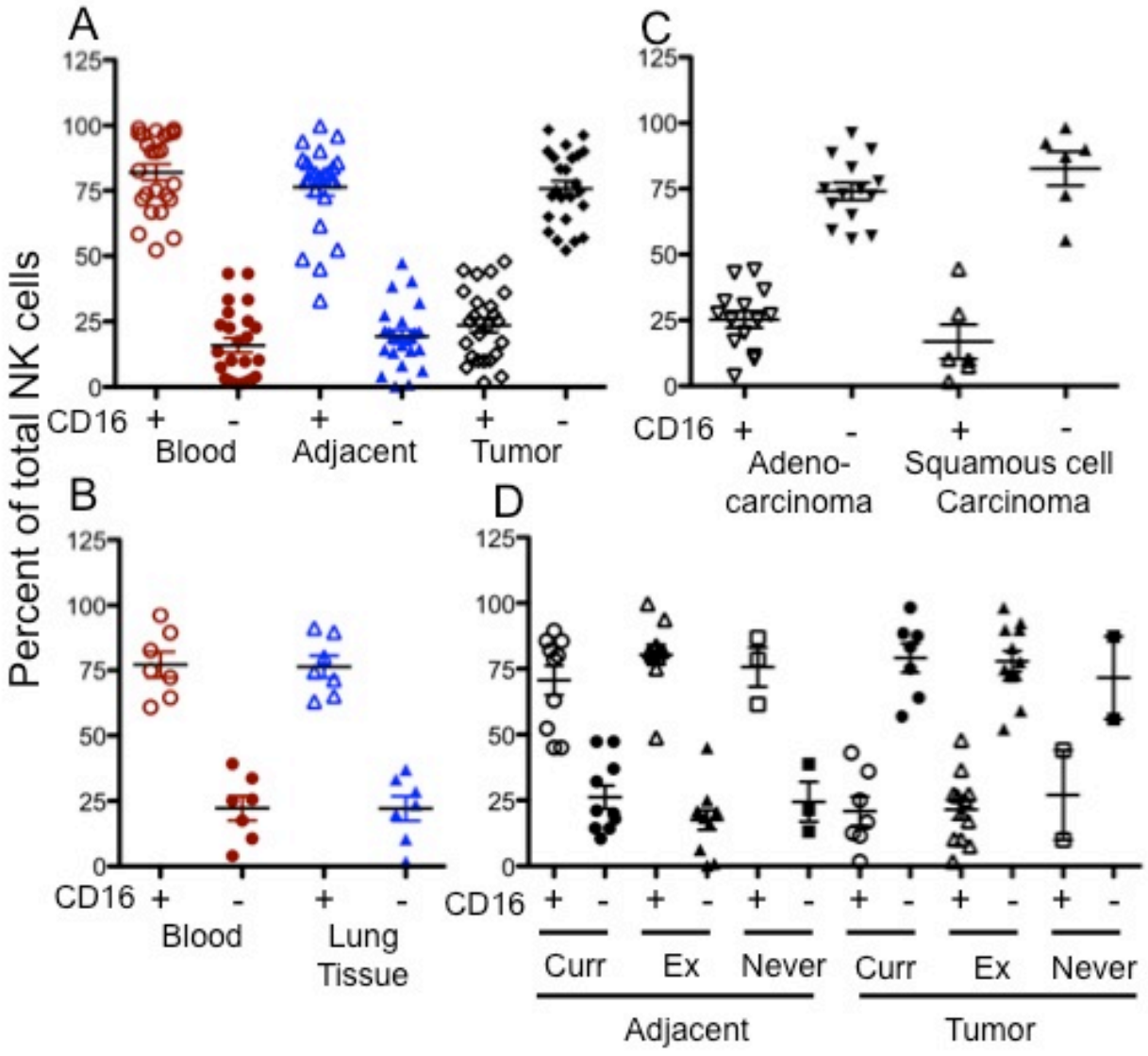

Figure 1. Phenotypic distribution of the $CD3^-/CD56^+/CD16^+$ and $CD3^-/CD56^+/CD16^-$ NK cell subsets as determined by flow cytometry. (A) NK cell distribution in samples derived from peripheral blood, normal adjacent lung tissues and tumor tissues of patients with NSCLC (B) A similar distribution of NK cell subsets is found in peripheral blood and lung tissues from non-oncologic patients. (C) The tumor infiltrating NK cells are primarily of the $CD3^-/CD56^+/CD16^-$ subset in both adenocarcinomas and squamous cell carcinomas. (D) Smoking status does not influence the NK cell subset in tumor or adjacent lung tissues.

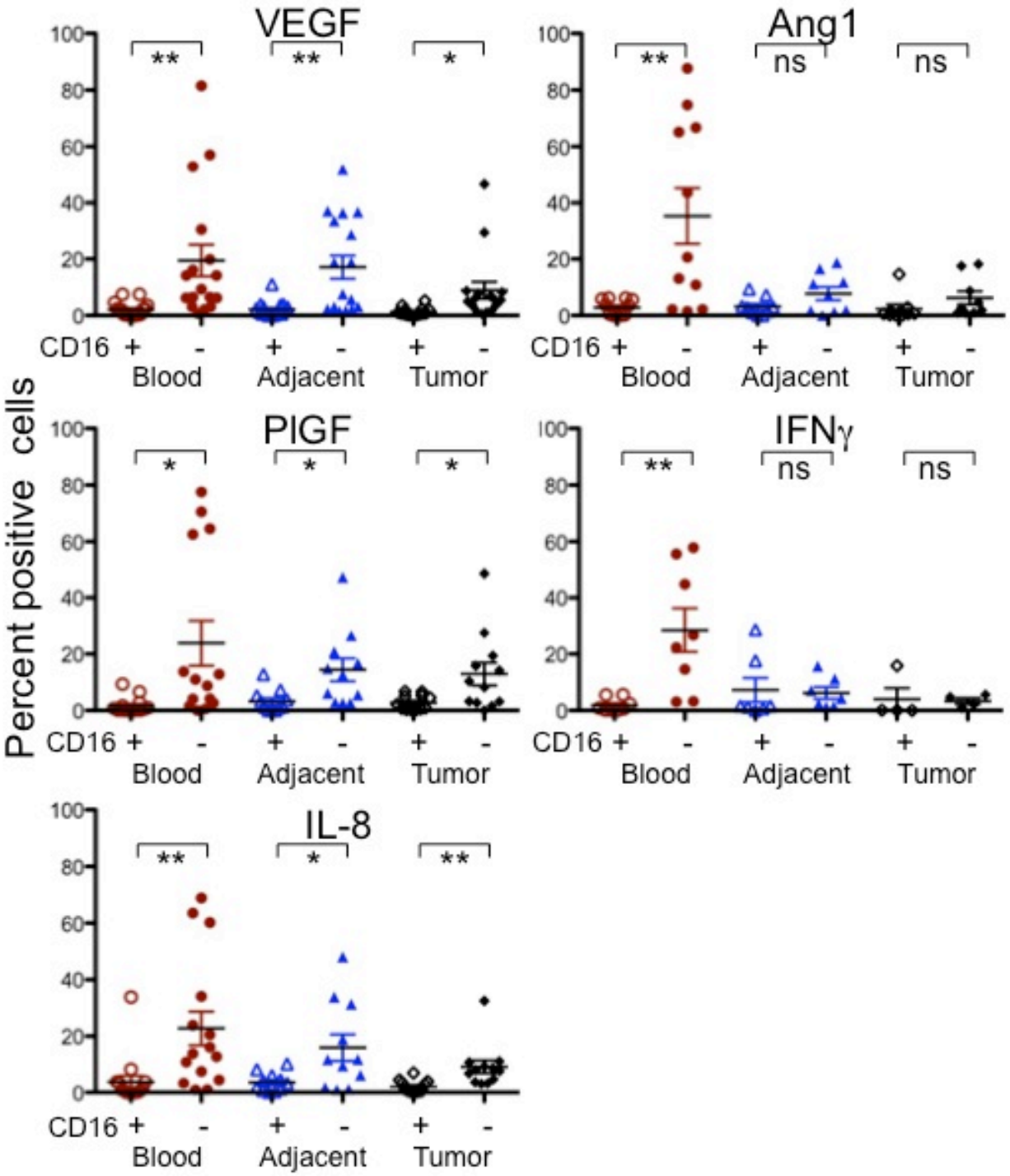

Figure 2. Characterization of tumor infiltrating NK cells by intracellular staining for angiogenic and anti-angiogenic cytokines. The $CD3^{-}CD56^{+}CD16^{-}$ NK cell subset is clearly associated with enhanced cytokine production, including VEGF, PlGF and IL-8 in the tumor and adjacent normal tissues as well as peripheral blood. In the peripheral blood, the $CD3^{-}CD56^{+}CD16^{-}$ NK cell subset was also associated with enhanced production of Angiopoietin 1 and IFNγ.

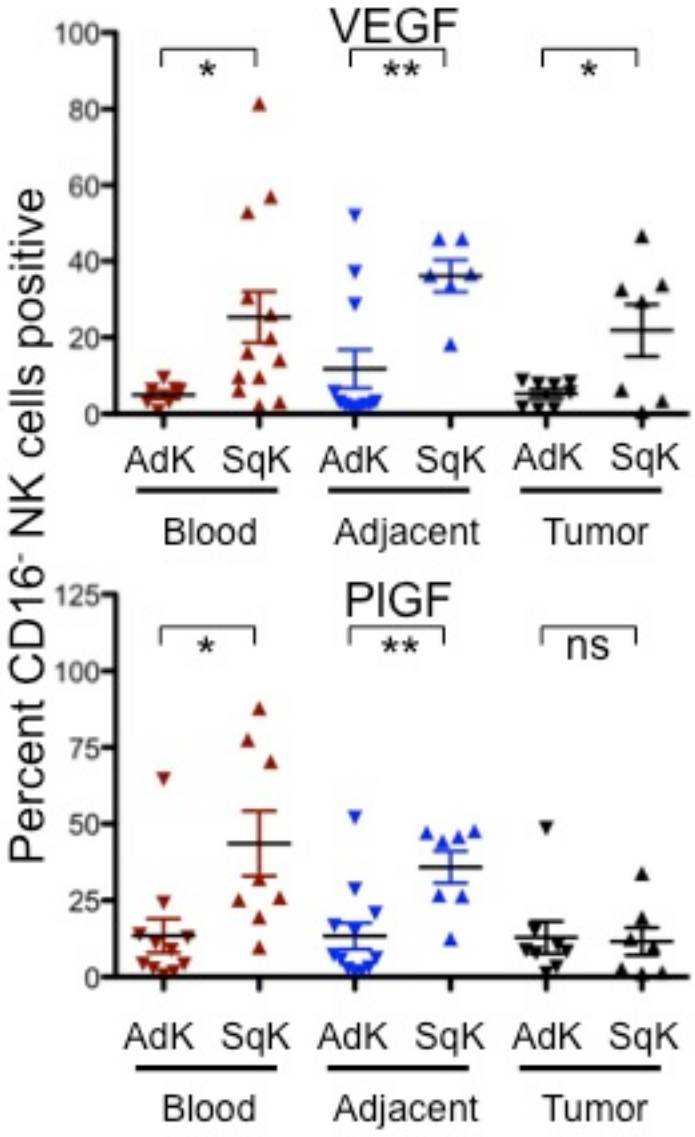

Figure 3: CD3$^-$CD56$^+$CD16$^-$ NK cells from patients with squamous cell carcinomas produce significantly more VEGF than those from patients with adenocarcinomas. This was appaerent both locally in the tumor but also systemically in the adjacent tissue and in the peripheral blood. In addition, CD3$^-$CD56$^+$CD16$^-$ NK cells from peripheral blood and adjacent tissue from patients with squamous cell carcinomas produced significantly more PlGF. Interestingly, the NK cells infiltrating the tumor tissues did not show significant differences in PlGF production.

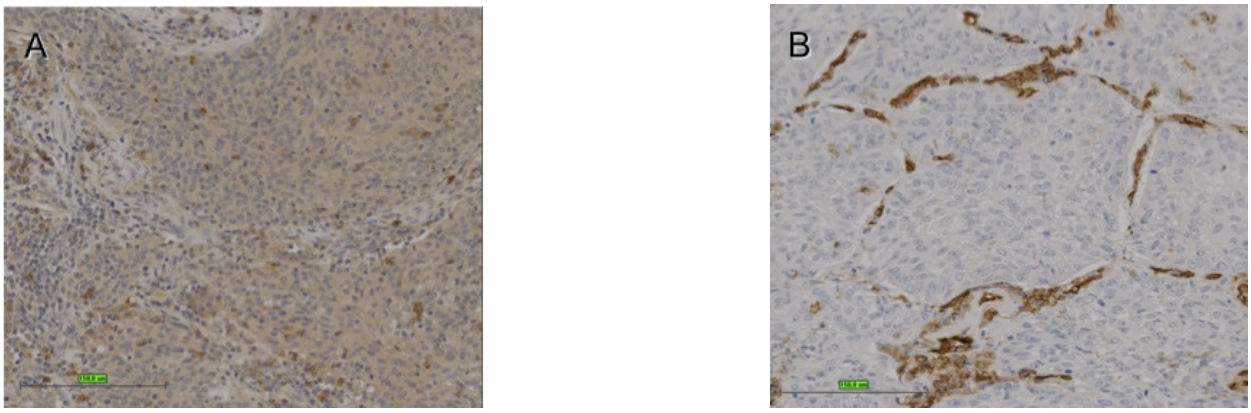

Figure 4. Immuno-histochemistry of squamous cell carcinomas. (A) CD56 staining of squamous cell carcinoms show occasional positive cells scattered throughout the tumor. (B) CD31 staining shows extensive vascularization of the tumors.

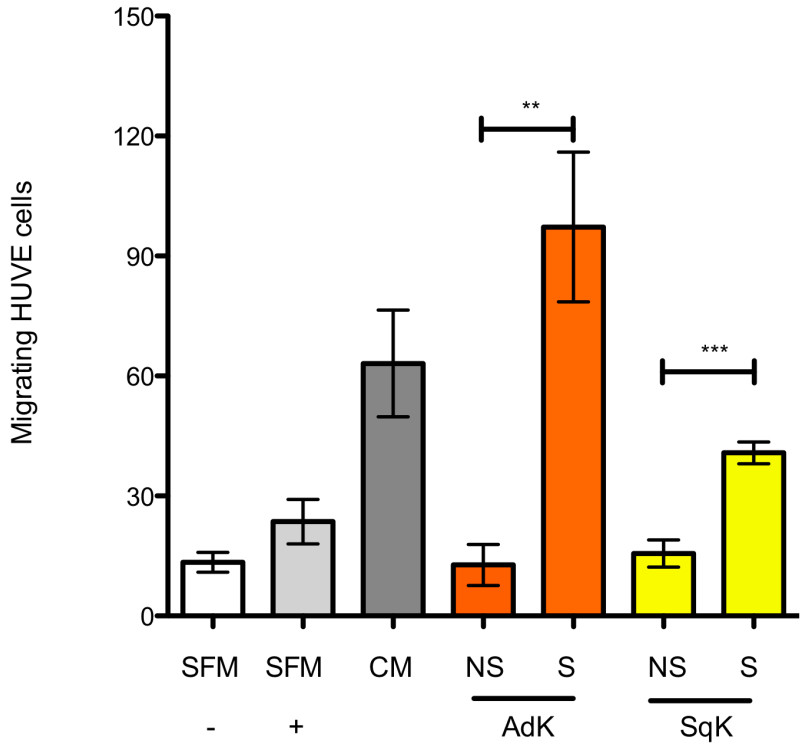

Figure 5. Analysis of the capacity of supernatants from NK cells derived from adenocarcinomas or squamous cell carcinomas to induce endothelial cell chemotaxis. SFM-: serum free medium as a negative control, containing M199 endothelial growth medium alone. SFM+: serum free M199 endothelial growth medium (Sigma, St Louis, MO, USA), 1% -glutamine, fibroblast growth factors (1µg acid-fibroblast growth factor plus 1µg basic-fibroblast growth factor /100ml), epidermal growth factor (1µg/100ml), heparin (10mg/100ml) and hydrocortisone (0.1mg/100ml). CM: complete medium containing SFM+ medium supplemented with 10% heat-inactivated FBS serum as a positive control. Supernatants from NK cells isolated from adenocarcinomas (AdK)

and squamous cell carcinomas (SqK) that were stimulated for 6 hours with PMA and Iono (S) showed enhanced induction of chemotaxis as compared to supernatants from NK cells that were untreated in the same culture medium (NS) for both.

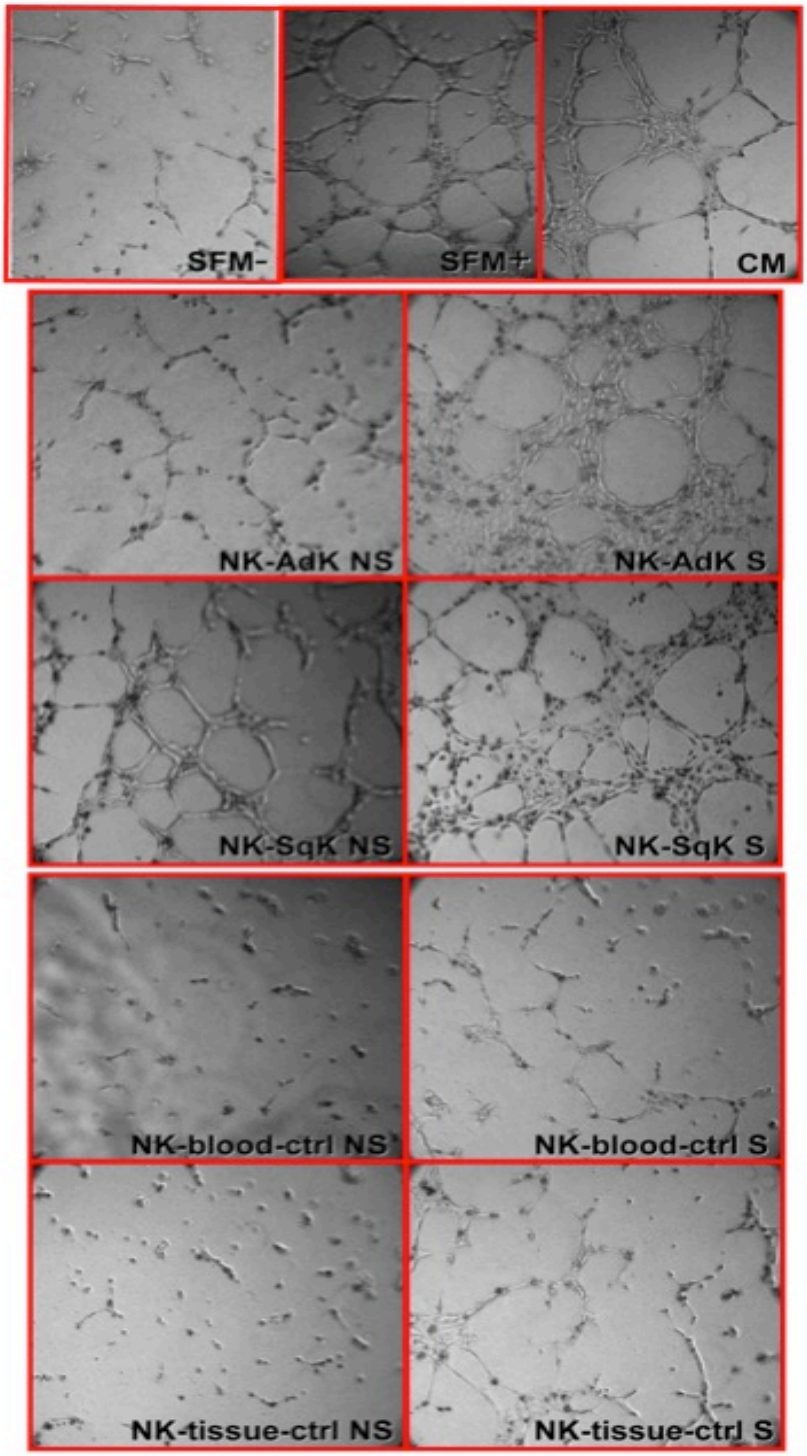

Figure 6. Analysis of the capacity of supernatants from NK cells derived from adenocarcinomas or squamous cell carcinomas to induce endothelial cell morphogenesis. SFM-: serum free medium as a negative control containing M199 endothelial growth medium alone. SFM+: serum free medium containing serum free M199 endothelial growth medium (Sigma, St Louis, MO, USA), 1% -glutamine, fibroblast growth factors (1µg acid-

fibroblast growth factor plus 1µg basic-fibroblast growth factor /100ml), epidermal growth factor (1µg/100ml), heparin (10mg/100ml) and hydrocortisone (0.1mg/100ml). CM: complete medium containing SFM+ medium supplemented with 10% heat-inactivated FBS serum as a positive control. Supernatants from NK cells isolated from adenocarcinomas (AdK) that were stimulated for 6 hours with PMA/Iono (S) showed induction of morphogenesis as compared to supernatants from unstimulated (NS) NK cells. Supernatants from NK cells isolated from squamous cell carcinomas (SqK) showed induction of morphogenesis even when unstimulated (NS). The induction of morphogenesis by these cells was further enhanced upon stmulation (S), indicating that these cells harbor a strong angiogenic activity. NK cells isolated from the peripheral blood (blood) or lung tissues (tissue) of non oncologic patients did not show significant enhancement of morphogenesis in the presence (S) or absence (NS) of stimulation.

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