

## Polarization of Tumor Infiltrating Leukocytes from Innate Immunity and their role in the Pro-angiogenic Phenotype in NSCLC

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### Abstract

Non-small cell lung cancer (NSCLC), the most frequent lung cancer (80%), can be phenotypically classified into two main subtypes: squamous cell carcinoma (SCC) and adenocarcinoma (ADC). While SCC has relatively rapid doubling times from the onset, ADC has longer doubling times initially that become reduced during tumor progression, suggesting a key role for the microenvironment. During lung tumor progression, a complex and dynamic interplay occurs between proliferating tumor cells and stromal, endothelial and immune tumor-conditioned host cells within the tumor microenvironment (TUMIC). Several factors within the TUMIC, such as hypoxia, cytokines and soluble factors, appear to blunt the anti-tumor immune response and polarize immune cells towards a pro-tumor phenotype. Phenotypically and functionally altered immune cells found in cancer patients include macrophages, neutrophils, myeloid, dendritic, and even NK cells. We studied tumor infiltrating (TINK) and tumor associated (TANK) NK cells in NSCLC. NSCLC TINKs and TANKs show similarities to decidual NK cells, being polarized toward tissue builders, rather than killers, and producing pro-angiogenic cytokines. The functionally polarized immune cells in NSCLC provide the stromal support and neovascularization required for NSCLC tumor expansion and progression in a feed-forward mechanism, leading to tumor progression. Further, systemic alterations of immune cells are also present in NSCLC patients. The precise knowledge of these immune cell alterations within the TUMIC has become crucial for diagnosis, targeted therapeutic intervention, as well as prevention, of NSCLC cancer.

**Keywords:** Innate immune cells; Polarization; Non-small cell lung cancer; Inflammation; tumor microenvironment; Angiogenesis

### Introduction

Lung cancer, a predominant cause of cancer-related mortality in the developed world, is a heterogeneous disease with several histological subtypes. Approximately 80% of all lung cancers are non-small cell lung cancer (NSCLC). NSCLC can be phenotypically further divided into two main subtypes: squamous cell carcinoma (SCC) and adenocarcinoma (ADC) [1]. Standard chemotherapeutic treatments for this devastating disease are only partially effective, and show considerable toxicity, with only fewer than 20% of patients surviving after 5 years from diagnosis; the median survival is less than one year for metastatic disease [1,2]. Surgery represents a valid treatment for early disease, yet still most lung cancers are diagnosed when they become symptomatic and therefore at very late stages. Hence, alternative and innovative therapeutic strategies, including immunotherapies, are urgently needed.

Although in the past lung cancer was not considered an immune-sensitive malignancy, currently there is increasing evidence that both principal NSCLC subtypes can evoke specific B and T cell immune responses. For this reason, in addition to standard intervention approaches, numerous research groups focus their efforts on studying the active specific stimulation of the host's own immune system,

termed "therapeutic vaccination", such as genetically modified autologous tumor cells secreting immune-modulating cytokines, allogenic tumor cells, tumor antigen-pulsed dendritic cells (DC), or therapeutic immune-modulating agents reversing check-point blockades, in particular anti-CTLA-4 or anti-PD-1.

When isolated from host components, tumors cannot expand in mass, they remain a small, clinically indolent disease [3]. For tumor expansion and progression, tumor cells must interact with several host-derived cells consisting of stromal, inflammatory, immune and endothelial cells (ECs) that delineate a complex modified tissue compartment, termed the tumor microenvironment (TUMIC) [4]. Accumulating evidence shows the importance of TUMIC in shaping the tumor mass fate, regulating growth, progression, invasiveness, dissemination and clinical outcome. Among the host-dependent biological features of the tumor hallmarks defined by Hanahan and Weinberg [4] there are "evading immune destruction" and "tumor-promoting inflammation", which together with the immune orchestration of angiogenesis, points out the key role of the immune system in neoplastic disease [5,6].

Tumor progression leads to tumor immune escape through an array of known and as yet unknown mechanisms. Thorough knowledge of these mechanisms and the resulting clinical situation will be fundamental to plan adequate active anti-tumor intervention in combination with conventional treatment modalities such as surgery and chemotherapy.

It is now clear that the TUMIC polarizes immune cells, in particular the innate compartment, (macrophages, myeloid-derived suppressor cells (MDSCs), regulatory dendritic cells (regDCs)), yet the TUMIC also has an influence on mast cells, cancer-associated fibroblasts (CAFs) and adaptive immunity. Recent data have shown that NK cells are also skewed to a pro-tumor pro-angiogenic phenotype in NSCLC [7-9]. The inflammatory response originating within the TUMIC is a crucial step of the disease, tightly linked to the tumor angiogenesis along with repression of adaptive immune system [6,10]. Tumorigenesis and progression are promoted by different molecules produced within the TUMIC, including pro-angiogenic factors and extracellular matrix-modifying enzymes that facilitate angiogenesis, invasion, and metastasis [11-14]. Moreover, inflammatory cells, by releasing reactive oxygen and nitrogen species, can accelerate genetic mutation events, thereby inducing a faster evolution toward malignancy [12,15].

### Major NSCLC subtypes: adenocarcinoma and squamous cell carcinoma

In the past the two major clinical subtypes of NSCLC, adenocarcinoma and squamous cell carcinoma, were treated more or

less the same. However, they are quite distinct biologically and clinically. More than two decades ago the studies of Arai et al. [16] showed that surgically resectable SCC features a volume doubling time about 25% shorter compared to resectable ADC. In the same case series, the faster doubling time was a negative prognostic factor, as the SCCs were, on average, more aggressive clinically [16]. At the Center for Thoracic Surgery of the University of Insubria we studied a series of 116 resectable lung cancers, and confirmed that the median tumor volume doubling time of SCC is significantly shorter than that of ADC, both in screen-detected and in incidentally detected NSCLC patients (Table 1). In addition, among the NSCLC cases diagnosed by CT imaging, a technology that identifies tumors at a very early stage, the ADCs with a minimally invasive phenotype (formerly known as bronchioloalveolar carcinomas-BAC) show very long doubling times [17,18]. However, ADCs at later stages appear to have more rapid doubling times, suggesting that while SCC starts out with a rapid doubling time, ADC shows a more complex evolution over time. The biological diversity of the two main NSCLC subtypes is further underlined by their different response to currently available chemotherapy regimens and targeted therapies.

Mode of lung cancer detection	Adenocarcinoma (n=69)	Squamous cell carcinoma (n=47)	P*
<b>Chest X-ray screening*</b>			
n. of pts	25	16	
Median TVDT, days	109	80	0.0347
(IQR)	(91-185)	(56-108)	
<b>Incidental**</b>			
n. of pts	44	31	
Median TVDT, days	227	95	0.0108
(IQR)	(108-349)	(64-230)	
<b>P° (screening vs. incidental)</b>	0.0099	0.2128	

^TVDT calculated according to Schwartz formula [194] after comparison of two consecutive chest X-rays or CT-scans.  
 \*Mann-Whitney test  
 \*Annual repeat screen during population-based chest X-ray screening programme carried out in the Province of Varese, Italy in 1997-2011 [195].  
 \*\*Asymptomatic subjects with incidental NSCLC detection, for whom previous imaging study was available for tumor size comparisons.  
 TVDT: Tumor Volume Doubling Time; IQR: Interquartile Range (25%-75%).

**Table 1:** Median tumor volume doubling time (TVDT)^ of 116 non-small cell lung cancers (NSCLC) by histology and by mode of detection (case series 1997-2011, Center for Thoracic Surgery, University of Insubria).

Currently, targeted therapies are used almost exclusively in lung ADC, and the distinction between subtypes is imperative not only in advanced but also in early stage NSCLC undergoing surgical resection, to optimize treatment in case of relapsing disease [19]. The epithelial growth factor receptor (EGFR) inhibitors erlotinib, gefitinib and afatinib are selectively indicated for treatment of NSCLCs with EGFR mutations, which predominantly occur in ADCs [20]. Similarly, the EML4-ALK rearrangement occurs predominantly in ADC cells [21], and it is associated with susceptibility to the targeted agent crizotinib, although acquired drug resistance almost inevitably develops [22]. Importantly, the angiogenesis inhibitor bevacizumab and the folate anti-metabolite pemetrexed are excluded from use in patients with SCC, as treatment with these molecules has been associated

respectively with the occurrence of life-threatening hemorrhages or with lack of effectiveness [23].

### Different “actors” and “scenarios” in the tumor microenvironment

#### Macrophages and tumor-associated macrophages:

Macrophages are innate immune cells characterized by high plasticity associated with nearly opposite functional programs induced by different microenvironment signals [24]. Crucial functions of macrophages range from phagocytosis, induction of inflammation, recognition and elimination of invading pathogens, antigen

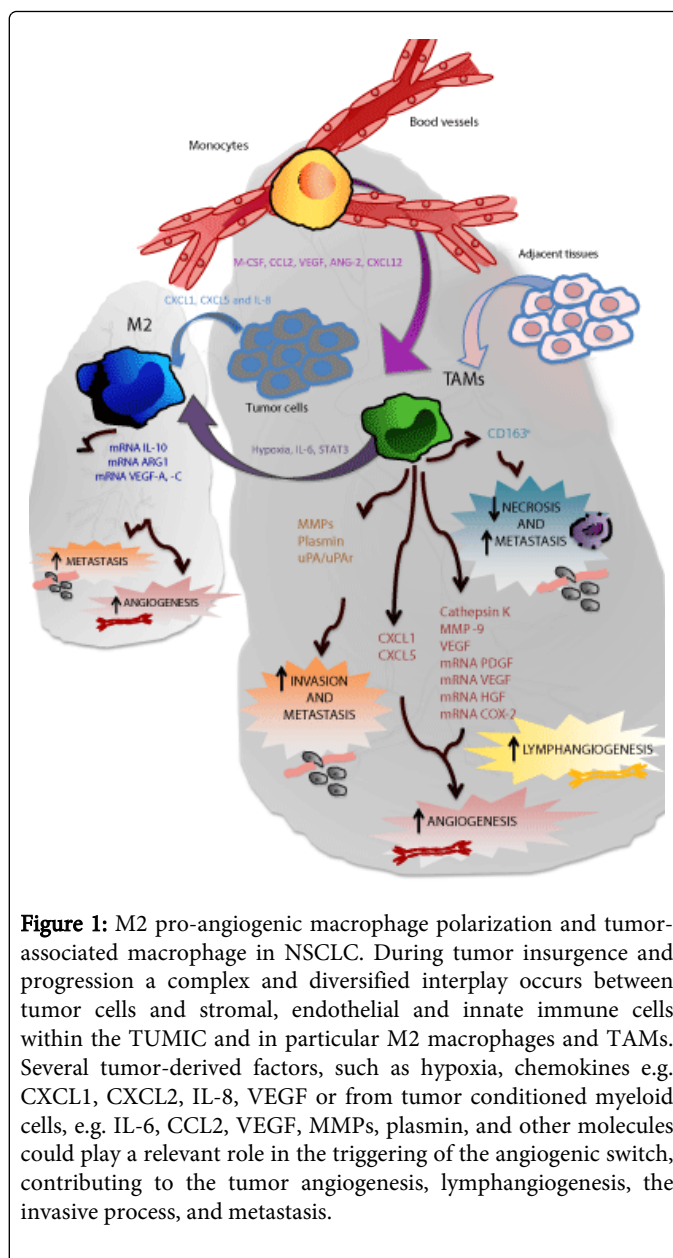
presentation and for resolution of inflammation and tissue repair, remodeling and maintenance of tissue homeostasis [25]. Two main subtypes of macrophages have been characterized: M1 (classically activated) macrophages, that possess high anti-microbial activities, pro-inflammatory function and display anti-tumor activity, mediated for example by production of reactive nitrogen and oxygen intermediates, and by synthesis of pro-inflammatory cytokines, and M2 (alternatively activated) macrophages that, conversely, are involved in tissue remodeling and repair activities, immune-suppressive effects, pro-angiogenic and pro-tumor functions. M1 macrophages are also characterized by a high capacity for IL-12 and IL-23 secretion, related to the induction or development of Th1 response, while M2 macrophages are closely related to the tumor-associated macrophage (TAM) profile and associated with an IL-10 secretory phenotype associated with Th2 and immune-suppression [24,25].

However, the M1/M2 macrophage paradigm represent extremes across a gradient of macrophage polarization, thus macrophages can display both M1 and M2 markers and M1 and M2 macrophages can be present in the same tissue [24]. In this context, it is conceivable that there is an evolving macrophage balance that integrates over time a changing phenotype in response to the microenvironment, depending on soluble tissue-derived signals and on cell-to-cell interactions, including the presence of polarized T lymphocytes, T<sub>reg</sub> and NK cells [26-28]. Experimental results have suggested that tumor-associated macrophages (TAMs) are an heterogeneous cell population with cells showing various degrees of polarization, also comprising Tie2-expressing monocytes/macrophages (TEMs) [29], and other immature myeloid cells, in particular myeloid-derived suppressive cells (MDSCs) [27].

In the natural history of cancer, TAMs become switched early on to an immature activation state, resembling the M2 macrophage polarization program. This includes low production of reactive oxygen species (ROS), poor release of inflammatory cytokines (IL-1 $\beta$ , IL-6, TNF $\alpha$  and IL-12), enhanced release of anti-inflammatory cytokines (IL-10), decreased efficiency in antigen presentation, an increase in pro-angiogenic features and immune-suppressive functions [30,31].

Several studies performed on different transgenic mouse models evaluating the potential role of TAMs in the tumor progression in vivo demonstrate that up-regulation of TAM number is associated with increased tumor growth [32], while their depletion or inhibition leads to a relevant inhibition of tumor angiogenesis and progression [33]. In multiple murine models of carcinomas, it has been well established that TAMs acquire a predominant role in the regulation of tumor angiogenesis, releasing crucial factors, such as vascular endothelial growth factor A (VEGF-A), matrix metalloproteinase 9 (MMP-9) and placental growth factor (PlGF) [26].

TAMs originate from blood monocytes recruited from the tumor vasculature as well as from adjacent tissues by different types of tumor microenvironment-derived chemokines (Figure 1), in particular M-CSF (CSF-1), CCL2 (MCP-1), VEGF, Angiopoietin-2 (ANG-2), and CXCL12 (SDF-1) [26,27,34-36]. TAMs represent a relevant part of the inflammatory component infiltrating solid cancers, exerting a crucial role in the regulation of cancer-related inflammation and tumor progression [37]. Presence and quantity of TAMs have been correlated with a poor prognosis for patients with diverse types of tumors, including breast, prostate and bladder cancers [38,39].



**Figure 1:** M2 pro-angiogenic macrophage polarization and tumor-associated macrophage in NSCLC. During tumor insurgence and progression a complex and diversified interplay occurs between tumor cells and stromal, endothelial and innate immune cells within the TUMIC and in particular M2 macrophages and TAMs. Several tumor-derived factors, such as hypoxia, chemokines e.g. CXCL1, CXCL2, IL-8, VEGF or from tumor conditioned myeloid cells, e.g. IL-6, CCL2, VEGF, MMPs, plasmin, and other molecules could play a relevant role in the triggering of the angiogenic switch, contributing to the tumor angiogenesis, lymphangiogenesis, the invasive process, and metastasis.

Accumulating evidence has shown that different types of cytokines, chemokines, growth factors and proteolytic enzymes can be produced by TAMs and these molecules have been suggested to act as key effectors in tumor angiogenesis and progression during the “angiogenic switch” [40,41]. However, in the case of NSCLC, current data are not conclusive concerning the prognostic role of the collective TAM tumor infiltrate in term of recurrence-free survival and overall survival. TAM density in NSCLC correlated with tumor IL-8 mRNA levels and high microvessel counts, suggesting a role of TAMs in the tumor angiogenesis [42,43], as well as with accelerated lymphangiogenesis [44] (Figure 1). However, this scenario still requires further investigation since there are still opposite and conflicting results on the role of TAMs in NSCLC [45-49].

In lung cancer, TAMs have been suggested to support tumor progression contributing to stroma formation and angiogenesis induction through the release of platelet-derived growth factor

(PDGF), in association with TGFβ production by the cancer cells [26]. Through production of proteases, including MMP-1,-2,-3,-9, and-12, plasmin, urokinase-type plasminogen activator (uPA) and the uPA receptor, macrophages can also regulate the degradation of the extracellular matrix (ECM), weakening the connective tissue and favoring enhanced infiltration and metastasis.

Interestingly, a gene profile study on primary NSCLC tissues showed that TAMs are associated with higher gene expression of cathepsin K, MMP-9, uPA, VEGF, PDGF, HGF and COX-2 [50] when compared to monocytes from healthy donors. A high expression of cathepsin was observed in NSCLC patients with a poor prognosis [51]. Moreover, higher gene expression of MMP-9 and VEGF-A in TAMs were present in the later stage of disease as compared to early stages (Figures 1 and 2), including in patients with lymph node metastasis [50]. Functional studies using conditioned media derived from TAMs demonstrated that these cells release soluble factors able to improve cell lung cancer invasion and migration in vitro. TAM invasiveness has been associated with their ability to release of MMP-9 and uPA while targeting of these two molecules by mAbs significantly impaired TAM invasion.

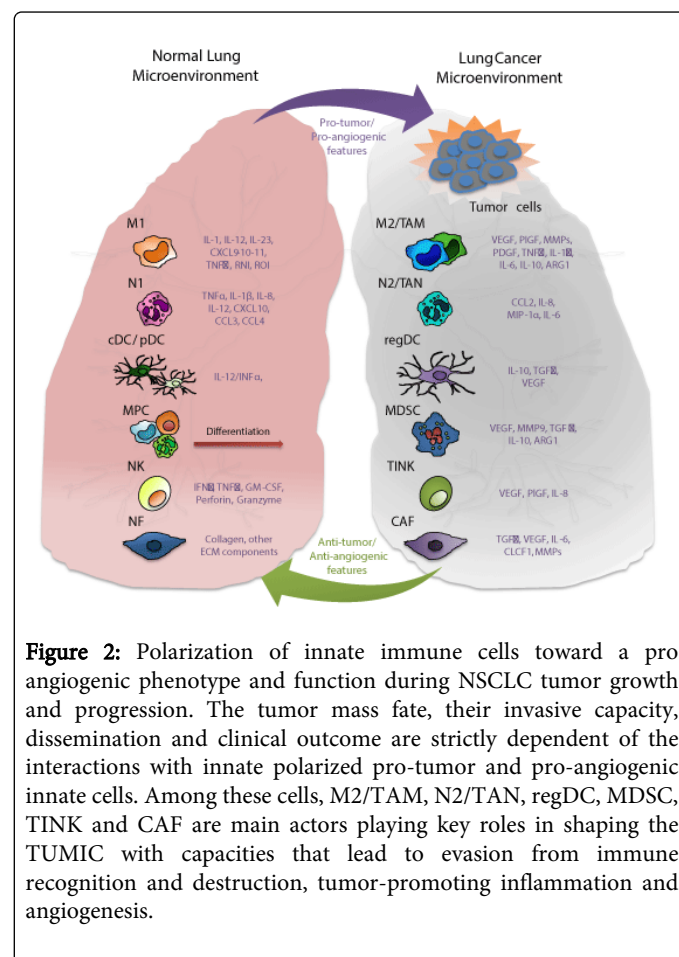
Hypoxia is a crucial condition for the development of several cancer types, inducing the necessary microenvironment for tumor establishment [52,53]. High TAM numbers accumulate in hypoxic areas of tumors and this process can triggers a pro-angiogenic program in these cells [54] as a function of IL-10 release, hypoxia and polarization [31]. Therefore, infiltrating TAMs represent an indirect pathway of promotion of angiogenesis, together with other tumor-derived angiogenic molecules.

In a recent study on NSCLC adenocarcinomas [55], tumor infiltration by M2 macrophages was directly correlated with metastasis (Figure 1). These data highlight the role exerted by hypoxia in the development of polarized TAMs through the activation of ERK pathway. Further studies will be necessary to clarify which specific transcription factors are involved in the macrophage polarization process. Studies performed in the murine Lewis lung carcinoma (LLC) model also demonstrated that TAMs were associated with metastasis and angiogenesis both in vitro and in vivo. Tumor hypoxia selectively promoted the in vitro M2 polarization of murine macrophages in conjunction with stimulation of IL-6 [56]. IL-6 has been shown to be involved as a crucial activator for the oncogenic transcription factor STAT3 [56] that has been suggested to be involved in the M2 macrophage polarization [27] (Figure 1). Noteworthy, when hypoxia and IL-6-induced murine M2 macrophages were injected with LLC cells in vivo, the metastasis rate increased dramatically as well as the proportion of CD31-positive cells as compared to controls, suggesting a clear correlation between M2 macrophages and tumor angiogenesis [56].

Lactic acid released from hypoxic murine tumor (LLC or B16) cells supported the role of hypoxia in skewing functional M2 macrophage polarization [57]. A by-product of anaerobic glycolysis, lactic acid might play a crucial role in inducing expression of VEGF and arginase 1 (ARG1) in TAMs, a process that was mediated by hypoxia-inducible factor 1 alpha (HIF-1α). These studies directly suggest that the possibility to interfere with the M2-type polarization during tumor progression could represent an innovative therapeutic intervention strategy [58,59]. In vitro endothelial cell chemotaxis assays performed on human peripheral mononuclear cells exposed to conditioned media of the Calu 6 and A549 NSCLC cell lines showed a marked increased in monocyte angiogenic activity. This was correlated with enhanced

levels of CXC chemokines: IL-8 (CXCL8), CXCL5, and CXCL1. Interestingly, macrophage angiogenic activity was enhanced by tumor-derived macrophage inhibitory factor (MIF).

A xenograft model using the highly metastatic NCI-H460-LNM35 (LNM35) human lung carcinoma cell line, in comparison to the low metastatic cell line NCI-H460-N15 (N15), found that the highly metastatic cancer cells were associated with increased macrophage and neutrophil infiltration and lymph node metastasis in vivo. This phenomenon was correlated with human and murine expression of IL-1, and VEGF-A,-C and-D molecules [60]. Interestingly, the enhanced F4/80<sup>+</sup> infiltrating macrophages, detected by immunohistochemistry, expressed specific markers for the M2-type, i.e. IL-10 and ARG1 mRNAs, as well as for both VEGF-A and VEGF-C mRNAs (Figures 1 and 2). The high angiogenic and lymphangiogenic potential of LNM35 tumors appeared to be mediated by induction of CXC chemokines: IL-8 (CXCL8), CXCL5 and CXCL1 by cancer cells (Figure 1), in conjunction with a IL-1-driven inflammatory signaling that lead to the recruitment of M2-type macrophages.



**Figure 2:** Polarization of innate immune cells toward a pro-angiogenic phenotype and function during NSCLC tumor growth and progression. The tumor mass fate, their invasive capacity, dissemination and clinical outcome are strictly dependent of the interactions with innate polarized pro-tumor and pro-angiogenic innate cells. Among these cells, M2/TAM, N2/TAN, regDC, MDSC, TINK and CAF are main actors playing key roles in shaping the TUMIC with capacities that lead to evasion from immune recognition and destruction, tumor-promoting inflammation and angiogenesis.

Concerning the role of the TEM subset in tumor vascularization and progression, it has been reported that selective depletion, or inhibition of their migration capacities, results in substantial inhibition of both tumor angiogenesis and progression in various mouse tumor models [61,62]. TEMs represent a subset of already pre-programmed highly pro-angiogenic circulating human monocytes that can be detected in several tumor infiltrates, including lung carcinomas,

near to the tumor blood vasculature as well as in the stroma [63]. In contrast to Tie2-monocytes, TEMs showed higher expression of MMP-9, VEGF-A, COX-2, and WNT5A [64]. Furthermore, exposure to ANG-2 induced enhanced tumor-promoting functions of TEMs, in particular increasing expression of two important pro-angiogenic enzymes: thymidine phosphorylase (TP) and cathepsin B (CTSB) [64]. Purified peripheral TEMs from healthy human donors, induced angiogenesis in xenotransplanted human tumors while Tie2-monocytes did not, suggesting a key role for TEMs in human cancer growth [63]. ANG-2 has also been implicated in lung metastasis and formation of the pre-metastatic niche by TEMs [65].

## Neutrophils

Neutrophils are the most abundant circulating leukocytes and represent the first-line host defense against infectious microorganisms. In response to diverse inflammatory stimuli, neutrophils migrate from blood to infected tissues, where they efficiently contain pathogens through several strategies: phagocytosis which involves the microorganism engulfment and subsequent elimination in phagolysosomes and degranulation which results in potent antimicrobial lytic molecule release (including activation of myeloperoxidase (MPO) and the respiratory burst) resulting in copious production of reactive oxygen species (ROS) ( $O_2^-$ ,  $H_2O_2$ , HOCl) [66,67] and formation of neutrophil extracellular traps. In addition, neutrophils are also able to produce several cytokines (TNF $\alpha$ , IL-1 $\beta$ , IL-12) and chemokines (CXCL8, CXCL10, CCL3, and CCL4) (Figure 2) directly involved in the complex immune cell cross-talk in order to regulate the balance between humoral and cell-mediated immunity [68,69].

Neutrophils represent a significant inflammatory cell infiltrate component observed in a wide array of murine and human tumors, including head and neck cancer [70], renal cell carcinoma [71], melanoma [72], hepatocellular cancer [73], colon cancer [74], gastric cancer [75] and lung cancer [46,76], suggesting a potential involvement in tumor development.

Although the role of tumor-associated neutrophils (TANs) in cancer progression remains unclear and is currently debated, increased systemic neutrophil levels [77] and high intra-tumor neutrophil density were found to be a negative prognostic factor [76]. The first evidence of neutrophil infiltrated BAC was reviewed in 1998 when Bellocoq et al. reported that an enhanced TAN number was significantly associated with a poor outcome in BAC patients [78]. Increased blood neutrophil numbers was associated with a negative prognosis and a poor overall survival (OS) in patients with advanced NSCLC [79] and with small cell lung carcinoma (SCLC) [80]. Intra-tumor CD66b<sup>+</sup> neutrophils in resectable NSCLC were correlated with a significantly increased relapse incidence [76].

Like macrophages, neutrophils have also been reported to be able to display at least two different phenotypes associated with diverse biological behavior. Fridlender et al. demonstrated that in mouse models of lung cancer, TGF $\beta$  represents a crucial factor that induces a shift from an anti-tumor (N1) to a pro-tumor (N2) neutrophil phenotype (Figure 2). When TGF $\beta$  activity was blocked, N1 neutrophils were observed to be associated with direct tumor cell killing and CD8<sup>+</sup> T cell activation, whereas N2 neutrophils were predominant in the control animals bearing tumors [81].

Early-stage NSCLC TANs displayed a CD62L<sup>low</sup>CD54<sup>high</sup> phenotype with a characteristic pattern of chemokine receptors

including CCR5, CCR7, CXCR3, and CXCR4 [82]. These TANs were able to release pro-inflammatory cytokines (CCL2, IL-8, CCL3, and IL-6) as well as the anti-inflammatory IL-1R antagonist (Figure 2). Both TANs and neutrophils isolated from distant non-malignant lung tissues were able to induce T cell proliferation and IFN $\gamma$  production. TAN-T cell cross-talk induced an increase of CD54, CD86, OX40L, and 4-1BBL co-stimulatory molecules on the neutrophil surface, which fostered T cell proliferation in a positive-feedback loop [82].

Several recent studies have highlighted the importance of the neutrophil-to-lymphocyte ratio, a biomarker of the host systemic inflammatory response whose increase has been reported to be associated with a poor outcome in patients with SCLC [83] and NSCLC patients receiving standard chemotherapy [84]. In an in-vitro study, Hattar et al. demonstrated that the co-incubation of A549 with neutrophils induced proliferation of A549 cells by an elastase-dependent mechanism. Moreover, a specific COX-2 inhibitor was able to decrease A549 proliferation in the presence of neutrophils [85]. Inhibition of COX-2 has been associated with reduction of lung cancer incidence [3]. Together these data suggested the potential relevance of the interactions between neutrophils and tumor cells that could lead to the release of inflammatory mediators that potentially are able to enhance tumor cell growth and inhibition of this process may help delay tumor onset.

## Dendritic cells

Dendritic cells (DCs) are the most important antigen presenting cells (APCs) primarily involved in inducing the adaptive immune response, whereas immature DCs are able to prevent the activation of auto-reactive T cells and the onset of autoimmunity [86,87].

Following maturation, DCs give rise to a heterogeneous population of cells in which the two main actors are conventional DCs (cDCs) (also called myeloid (mDCs)) and plasmacytoid (pDCs) [88]. cDCs are able to secrete IL-12 and can be reproduced in vitro using CD34<sup>+</sup> precursors or blood monocytes using appropriate stimuli; when exposed to TGF $\beta$  they are able to differentiate into Langerhans cells (LCs). On the other hand, pDCs can release interferon-alpha (IFN $\alpha$ ) and are derived from lymphoid precursors, express the surface marker CD123 (interleukin IL-3 receptor, IL-3R) and their growth is dependent on the presence of IL-3 [89-91].

During cancer promotion and progression, the maturation of DCs is strongly inhibited by different signals from the microenvironment and DCs remain in an immature state, thereby acquiring a tolerogenic and immunosuppressive properties [92,93].

Patients with NSCLC are characterized by reduced CD11c<sup>+</sup> cDCs, in particular in lymph nodes of patients with metastasis or in lymph nodes located adjacent to the primary tumor in subjects without metastasis. Moreover, DCs within the tumor show low expression of HLA-DR antigens, suggesting that their ability for antigen presentation is severely impaired [94]. Among CD11c<sup>+</sup> cDCs present in lung cancer, three major subpopulations have been identified in NSCLC: CD1a<sup>+</sup> (a marker that specifically identifies immature DCs [95]) Langerin<sup>+</sup> cells, CD1a<sup>+</sup>Langerin<sup>-</sup> cells, and CD1a<sup>-</sup>DC<sup>+</sup>SIGN<sup>+</sup>CD68<sup>+</sup> cells [96,97]. CD123<sup>+</sup> pDCs have also been observed in lower numbers in tumor tissues as compared to healthy tissue [98].

CD83<sup>+</sup> mature DCs are found in a very low numbers in tumor tissues, higher numbers are found in adjacent tissues [100]. NSCLC tumor tissues also contain three subsets of cDCs based on CD11c expression: high (CD11c<sup>high</sup> cDC), intermediate (CD11c<sup>int</sup> cDC) and

low (CD11c<sup>pDC</sup>). Tumor-infiltrating CD11c<sup>high</sup> cDCs show a lower state of maturation as compared with DCs from peripheral blood, and the stimulation of tumor-infiltrating cDCs by TLR4 or TLR8 ligands are only able to induce secretion of limited amounts of cytokines. CD11c<sup>int</sup> DCs represent a quarter of total DCs found in tumor and adjacent tissues, they express low levels of co-stimulatory molecules and high levels of immune-inhibitory molecules, such as B7-H1. The decreased numbers of CD11c<sup>pDC</sup> found in tumors represent immature cells that are able to secrete low amount of IFN $\alpha$  upon TLR9 stimulation [100].

High VEGF expression and DC infiltration have been reported to be inversely correlated in tumor specimens of NSCLC and are associated with a poor prognosis [101] suggesting that VEGF might inhibit or regulate DC recruitment and/or activation. Conversely, a high density of mature DCs can be considered a good predictor for clinical outcome in NSCLC, as it is correlated with prolonged survival and it may identify patients with early-stage tumors [102].

DCs with immunosuppressive functions (Figure 2) are termed regulatory DCs (regDCs) [103]. It was recently demonstrated that, in mice, development of LLC was associated with intra-tumor accumulation of regDCs [104].

NSCLC cells that are positive for programmed-death receptor ligand 1 (PD-L1) are associated with histological subtypes and overall survival. Patients with ADC or prognosis after surgery less than 3 years show higher expression rate of PD-L1, thus this molecule might be regarded as a poor prognostic factor. PD-L1 was detected in CD1a<sup>+</sup> immature DCs in NSCLC, potentially maintaining DC in an immature state and contributing to the immune escape and disease progression [105]. The B7-H3 co-inhibitory molecule (a member of the PD-L family) is up-regulated in NSCLC-residing DC, this phenomenon correlates with lymph node metastasis [106]. High levels of circulating B7-H3<sup>+</sup> DCs are associated with tumor stage and metastasis diffusion. NSCLC-derived DCs show immunosuppressive activities and are able to release large amounts of IL-10 (Figure 2), contributing to an immature phenotype and tolerogenic state [106]. DCs, like other immune cells, can be conditioned and polarized by tumor microenvironment into tolerogenic cells able to contribute to tumor progression, immunosuppression and angiogenesis by releasing TGF $\beta$ , polarizing T lymphocytes into pro-tumor Th2 cells (Figure 2) and activating T<sub>reg</sub> cells [104].

### Myeloid-derived suppressor cells

Myeloid-derived suppressor cells (MDSCs) include a heterogeneous population of immature myeloid and myeloid progenitor cells endowed with immunosuppressive properties, pro-angiogenic potential, and able to support metastatic spread [107,108]. Human MDSCs are described as CD11b<sup>+</sup>, CD33<sup>+</sup>, CD16<sup>low</sup>, HLA-DR<sup>neg/low</sup> and can be classified into two major subsets based on CD14 marker expression: the CD14<sup>+</sup> subtype, termed monocytic MDSCs (M-MDSCs), and the CD14<sup>-</sup>CD15<sup>+</sup> subtype that are polymorphonuclear MDSCs (PMN-MDSCs) [109]. The murine counterpart is characterized by the co-expression of Gr-1 and CD11b [108].

Tumor and inflammatory milieu-derived mediators such as IL-1 $\beta$ , IL-6, IL-10, VEGF and GM-CSF are crucial factors able to induce MDSC recruitment, expansion, and triggering functions [110]. Murine MDSCs exert a direct role in the promotion of tumor angiogenesis through release of soluble factors, such as MMP-9 and VEGF, and by their ability to trans-differentiate into ECs [111,112]. In contrast, the

MMP inhibitor TIMP-2 targets NSCLC MDSCs and impairs their angiogenic and immunosuppressive potential [113]. In addition, it has been proposed that murine MDSCs could act as regDCs, since once isolated from lungs of healthy mice and co-cultured with LCC conditioned medium, they acquire inhibitory functions. [104]. regDCs can also secrete TGF $\beta$  and IL-10 (Figure 2) resulting in the suppression of immune responses, mainly through inhibition of T helper, T cytotoxic lymphocytes and natural killer cells [114] as well as the induction of T<sub>regs</sub> [115].

HLA-DR<sup>-low</sup> M-MDSCs have been found to be increased in the peripheral blood of NSCLC patients as compared to healthy donors [116] and their numbers were associated with extrathoracic metastasis, as well as response to chemotherapy and tumor progression. M-MDSCs appear to exert very low allo-stimulatory activity and show the ability to inhibit both autologous CD4<sup>+</sup> and CD8<sup>+</sup> T cell proliferation and IFN $\gamma$  production in a cell-to-cell contact-dependent manner. M-MDSCs have been shown to express the NADPH oxidase component gp91<sup>phox</sup> and are able to generate high level of reactive oxygen species (ROS) suggesting that their suppressive effect on T cells is mainly mediated by ROS production [116]. In advanced NSCLC patients, it has been shown that PMN-MDSC peripheral count was increased, and this subset, when co-cultured *in vitro* with CD8<sup>+</sup> T cells, was able to reduce the expression of CD3 $\zeta$  chain leading to the suppression of T cell proliferation and induction of apoptosis [117]. In peripheral blood the CD11b<sup>+</sup>CD14<sup>-</sup> MDSC subpopulation is decreased in advanced disease stage patients that are responsive to chemotherapy or in early-stage patients after tumor mass surgical resection [117].

Depletion of L-arginine by the enzyme ARG1 has been reported as an additional mechanism by which MDSCs are able to exert their immunosuppressive role. ARG1 is mainly produced by PMN-MDSC and the ARG1 levels in peripheral blood of NSCLC patients are correlated with PMN-MDSC count [118]. In different types of cancer, including NSCLC, an accumulation in the peripheral blood of immature CD66b<sup>+</sup> PMN-MDSCs in the MDSC fraction with immunosuppressive activities has been found [119]. These cells display altered surface marker expression, longer survival and decreased ability to act as effector cells when compared to neutrophils derived from healthy subjects. Furthermore, PMN-MDSCs lack CXCR1 and CXCR2 receptors, suggesting an extravasation and tumor tissue infiltration defects *in vivo* [119]. In a murine model of lung cancer, treatment with mAb against the Gr1 or Ly6G markers depleted MDSCs, resulting in enhanced anti-tumor immune responses and inhibition of pro-angiogenic activities [120]. When MDSC depletion was induced using LLC-Ovalbumin expressing tumor cells, the treatment resulted in enhanced therapeutic vaccination responses with marked inhibition of tumor growth and strong reduction in tumor burden compared to controls.

Finally, MDSCs have been shown to be crucial in the modulation of immune responses, contributing to angiogenesis and promoting tumor progression and metastases [109]. Accumulating experimental data show an inverse correlation between MDSC number, cancer clinical stage, and prognosis, suggesting that MDSCs could be a potential marker correlating clinical outcome and response to therapy; larger prospective trials are needed to determine the role of MDSC as a biomarker [121].

### Natural killer cells

Natural killer (NK) cells are innate immune cell effectors able to recognize and eliminate tumor and virus-infected cells. NKs constitute

a heterogeneous population of large granular lymphocytes that comprise approximately 10-15% of peripheral blood mononuclear cells (PBMC) in humans. Several human NK cell subsets have been described on the basis of the expression of two main surface antigens, CD56 and CD16. CD56<sup>dim</sup>CD16<sup>+</sup> NK cells constitutes about 90-95% of peripheral blood NK cells and are associated with target cell eradication through the secretion of perforin, granzyme and antibody dependent cellular cytotoxicity (ADCC) [122]. The second NK cell subset, CD56<sup>bright</sup>CD16<sup>-</sup>, represents about 5-10% of peripheral blood NK cells. These NK cells are poorly cytotoxic but able to release large amounts of cytokines, including IFN $\gamma$ , TNF $\alpha$ , and GM-CSF. A peculiar third NK cell subset has been found in the decidua during implantation [123,124]. Decidual NK cells (dNKs) are CD56<sup>superbright</sup>CD16<sup>-</sup>, have low cytotoxicity [125], are tolerogenic, participate in the protection of the developing embryo, and are involved in decidual angiogenesis producing copious quantities of angiogenic factors [125] as well as promoting decidual cellularity [126].

dNKs represent a clear example of the microenvironment involvement in shaping immune cell plasticity and response. A similar scenario has been described in the context of lung cancer. Carrega et al. showed that CD56<sup>bright</sup>CD16<sup>-</sup> subset represent the predominant NK subset infiltrating NSCLC [8]. Tumor infiltrating NK cells have been reported to exert poor cytotoxicity on target tumor cells [8,9,127-129]. We demonstrated that CD56<sup>bright</sup>CD16<sup>-</sup> NK cells infiltrating resectable NSCLC tumors, and even peripheral blood NK cells in patients with NSCLC, express pro-angiogenic cytokines (Figure 2) including VEGF, PlGF and IL-8 [7]. These factors are functionally active *in vitro* on endothelial cells. These data suggest that NK cells contribute to tumor angiogenesis in NSCLC. Like dNK cells and NK cells in some cases of tissue injury, the NSCLC tumor infiltrating NK cells (TINKs) and peripheral blood NK cells from oncology patients (tumor associated NK cells or TANKs) [130] have pro-angiogenic activities [7]. We also observed that the NK-associated pro-angiogenic activity was particularly pronounced in patients with SCC NSCLC, again suggesting that SCC subtype starts aggressively but remains constant over time, while ADC continues to gain increasing malignancy as time passes, in keeping with clinical observations. These data indicate that the NSCLC TUMIC exerts a potent polarizing effect on NK cells, resulting in phenotype and functional alterations of these cells both locally and systemically. Several TUMIC-derived/associated factors have been reported to impair NK "normal function", including acidity, hypoxia, immune suppressive cytokines (in particular TGF $\beta$ ), exosomes.

TGF $\beta$  is one of the numerous TUMIC factors involved in the induction of immune cell polarization [131], and is expressed at high levels both in the TUMIC and in the decidua [130]. TGF $\beta$  has been found to polarize the CD56<sup>dim</sup>CD16<sup>-</sup> peripheral NK cells toward a decidual-like phenotype expressing CD56<sup>bright</sup>CD16<sup>-</sup>, KIR<sup>+</sup>CD9<sup>+</sup>CD49a<sup>+</sup> [130]. TGF $\beta$  has been shown to inhibit CD16-mediated human NK cell IFN $\gamma$ -production and ADCC through SMAD3 [132]. TGF $\beta$  appears to contribute to the induction of the angiogenic switch of NK cells from healthy individuals [130].

An hypoxic microenvironment is another common theme between the decidua [133] and the TUMIC [134]. A combination of TGF $\beta$ , hypoxia, and a demethylating agent have been found to convert sorted peripheral blood CD56<sup>dim</sup>CD16<sup>+</sup> NK cells into a dNK-like cell phenotype characterized by low cytotoxicity associated with high expression levels of VEGF, CD9 marker and KIRs [135].

Adenosine is a soluble immunomodulatory molecule acting through adenosine receptors (in particular A1, A2A, A2B, and A3) expressed on multiple immune subsets, including NK cells. Adenosine peaks during decidualization [136] and up to 20-fold increases in the extracellular fluid of solid carcinomas has been reported [137]. Once released in the extracellular environment, adenosine has been reported to impair NK cell normal function by decreasing TNF $\alpha$  secretion (following IL-2 stimulation), inhibiting cytotoxic granule exocytosis, repressing perforin and Fas ligand-mediated cytotoxic activity as well as cytokine production [138].

Finally, exosomes are abundantly released by tumor cells and present in the TUMIC, and represent another mechanism by which tumors regulate NK cell plasticity by impairing NK killing efficiency [139]. This impairment includes the down-regulating perforin/granzyme production and/or NKG2D ligand expression [140]. The production of NKG2D ligand-bearing exosomes has also been proposed as a mechanism for tumor cell escape from immune recognition [141]. Moreover, the granzyme B-inhibitory serpin proteinase inhibitor-9 (PI-9) has also been identified inside exosomes [142] that could also play an important role in the resistance of tumor cells to NK cell lysis.

### Mast Cells

Mast cells (MCs) represent another inflammatory cell type associated with high plasticity that are able to regulate different aspects of inflammatory responses, angiogenesis, allergic reactions, tissue repair, remodeling and tumor. MCs have been reported to accumulate in several types of tumors in response to diverse tumor-derived chemoattractant factors, including RANTES, CCL2, stem cell factor (SCF) [143]. MCs are able to release several pro-angiogenic factors, such as fibroblast growth factor 2 (FGF-2), VEGF-A, -B, -C, -D, IL-8, TNF $\alpha$ , TGF $\beta$  [144]. Moreover they store secretory granules pre-formed active serine proteases, including tryptase and chymase [145]. Tryptase is a strong stimulator of EC proliferation leading to promote vascular tube formation *in vitro*, and also a valuable activator of both MMPs and PA [146].

MC numbers within tumor tissues have been correlated with tumor angiogenesis, cancer progression and poor prognosis in lung adenocarcinomas [147-150]. Interestingly, the accumulation rate of MCs was significantly different between diverse types of NSCLC [151]. Proliferation of SCLC was found to be associated with the expression of H1, H2, H3 and H4 histamine-receptors [151]. H1-receptor inhibition resulted in an improved overall survival rate and decrease tumor proliferation by inhibiting mast cell recruitment and release of VEGF and HIF-1 $\alpha$  [152]. Abundant MC accumulation in solid cancers, including lung carcinomas, has been showed to closely associate with the number of blood vessels surrounding solid tumors apparently sustaining tumor angiogenesis [153]. MC contribution to tumor angiogenesis is exerted by the release of growth factors, including VEGF, IL-8 and MMP-9 secretion, which in turn facilitates tumor invasiveness [144]. Studies using mast cell-deficient Kit<sup>W</sup>/Kit<sup>W<sup>v</sup></sup> or Kit<sup>W-sh</sup>/Kit<sup>W-sh</sup> mice showed that tumor-associated MCs significantly contribute to tumor angiogenesis, enhancing tumor growth and metastasis [154-156]. However, there are some controversial studies in NSCLC concerning the role of MCs as predictors of poor survival [147,148]. Although angiogenesis and MC density were found to be positively associated, only microvascular density, and not the MC count, was correlated with poor survival in SCC NSCLC patients [147]. A positive beneficial role for tumor MC

accumulation together with CD68<sup>+</sup> macrophage infiltration has been reported [47], adding some unresolved and conflicting features for defining the importance of MCs infiltrating NSCLC as a driving force in tumor angiogenesis and progression.

### Cancer-associated fibroblasts

Cancer-associated fibroblasts (CAFs) are a major component of the TUMIC (Figure 2), regulating important tumor cell functions by secreting several cytokines, chemokines and growth factors, such as TGF $\beta$ , VEGF, CXCL12, HGF, FGF, ECM proteins and ECM degrading enzymes (MMPs) [157-159]. CAFs constitute a major portion of the reactive tumor cell stroma and play a crucial role in tumor progression. The main precursors of CAFs are normal fibroblasts, and the transdifferentiation of fibroblasts to CAFs is driven to a great extent by cancer-derived cytokines such as TGF $\beta$  [161]. The tumor-promoting effects of CAFs have been described as a hallmark of cancer, since they are directly associated with evasion of apoptosis, sustained angiogenesis promotion, and tissue invasion and metastasis [162]. CAFs are also able to secrete IL-6 (Figure 2) and cardiotrophin-like cytokine factor 1 (CLCF1), cytokines that play a role in the promotion of NSCLC growth [163]. Compared with normal fibroblasts, CAFs appear to increase the invasiveness of co-cultured NSCLC cells in vitro and also enhanced tumorigenicity of NSCLC cells lines in vivo [164]. Mechanisms involved appear to be enhancement of integrin  $\alpha$ 11, *CTHRC1*, *SULF1*, *MFAP5*, *CLU*, and *THBS2* expression [164]. These genes are regulated by the TGF $\beta$ 1 signaling pathway [165,166], crucial for CAF differentiation and for the induction of epithelial to mesenchymal transition (EMT) [167].

The pro-metastatic role of CAFs in NSCLC was confirmed in co-culture studies: CAFs induce an increase in motility of NSCLC cells through the expression of alpha-smooth muscle actin, and a decrease in proliferation through a SMAD3-dependent up-regulation of the growth inhibitory gene p21 (CDKN1A) [167]. CAFs secrete ECM degrading enzymes, in particular MMPs (Figure 2), facilitating tumor invasion and metastasis [159]. Through recruitment of endothelial precursor cells (EPCs) and monocytes [168-170], CAFs contribute to pro-angiogenic and tumor progression. In breast cancer, the pro-angiogenic properties exerted by CAFs is linked to their ability to recruit endothelial precursor cells (EPCs) via SDF-1/CXCL12 secretion [169] and monocytes via CCL2-CCR2A/2B signaling pathway [168]. The role of CAFs in the promotion of angiogenesis in the context NSCLC remains to be elucidated. Tissue fibrosis has been epidemiologically associated with increased risk for tumors, and recent studies have suggested a correlation between matrix rigidity and cancer insurgence [171,172]. However, using a genetic murine model, deletion of  $\alpha$ SMAC<sup>+</sup> fibroblasts actually stimulated progression of pancreatic cancer [173], indicating we still have a great deal to understand in the role of CAFs within the TUMIC.

### T regulatory cells

Regulatory T cells (T<sub>regs</sub>) are immunosuppressive lymphocytes able to impair auto-reactive T cell activity and maintain immunological self-tolerance. In NSCLC, T<sub>regs</sub> (defined as CD4<sup>+</sup>CD25<sup>high</sup>FOXP3<sup>+</sup> [12]) number is associated with advanced tumor growth and poor prognosis [175]. T cell infiltration in malignant and non-malignant lung tissues was found to be similar, and both tissue and tumor-infiltrating T cells show no functional impairment. However, CD4<sup>+</sup>CD25<sup>+</sup>CD127<sup>-</sup>T<sub>reg</sub> cells are present only in malignant tissues [177]. Additional data confirm that NSCLC patients display an

increased percentage of T<sub>reg</sub> cells as compared to controls, and demonstrate that the T<sub>reg</sub> cell count is increased in relation to tumor stage and higher in patients with metastasis as compared to non-metastatic patients [176,177]. Furthermore, T<sub>regs</sub> are found in all lung carcinoma subtypes, but with significant enrichment in adenocarcinoma respect to squamous carcinoma [178].

Increased accumulation of T<sub>regs</sub> in NSCLC tissues is correlated with poor prognosis [179,180], in particular in smoking patients. Smoking patients characterized by a higher number of T<sub>regs</sub> [181] have a significantly higher risk for recurrent disease [179]. Moreover, high T<sub>reg</sub> count in the regional lymph nodes is associated with an unfavorable prognostic factor even in lymph node negative patients [182]. The frequency of peripheral T<sub>regs</sub> was found to be significantly higher in tumor of patients with pleural invasion, vessel invasion, lymphatic vessel invasion and recurrent disease [183].

Forkhead box protein 3 (FOXP3) and toll-like receptor 4 (TLR4) are relevant factors exerting a role in tumor escape and tumor growth. The expression of these two factors in NSCLC is higher than in normal lung tissue. FOXP3 expression correlates with lymph node metastasis and tumor staging and is aged-related, whereas TLR4 expression is related with tumor differentiation [184,185]. Moreover, T cell immunoglobulin-3 (TIM-3) is a negative regulatory molecule that plays a critical role in immune tolerance. Nearly 60% of FOXP3<sup>+</sup> tumor-infiltrating lymphocytes are TIM-3<sup>+</sup>, and TIM-3 expression on CD4<sup>+</sup> T cells correlated with poor clinical-pathological parameters of NSCLC patients, such as nodal metastasis and advanced cancer stages [186].

Induction of CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> T<sub>regs</sub> occurs in vitro when CD4<sup>+</sup> T cells are exposed to antigens or polyclonal activators in the presence of immunosuppressive cytokines, in particular IL-10 or TGF $\beta$  [187]. It is known that TGF $\beta$  is expressed in patients with high risk to develop NSCLC [188]. Both ADC and SSC patients exhibited higher levels of serum IL-10 and TGF $\beta$  than healthy controls [189]. TGF $\beta$ -mediated immunosuppression is in relationship with aberrant inflammation (COX-2/PGE2 pathway) and this is related to the induction of polarization to T<sub>regs</sub>. PGE2 is able to stimulate the development of T<sub>regs</sub>, both in vitro and in vivo, suggesting a crucial role of tumor-derived COX-2 promotion of the T<sub>reg</sub> phenotype. COX-2/PGE2 and TGF $\beta$  are both implicated in tumorigenesis and are capable to generate peripherally induced T<sub>regs</sub> [190,191].

TGF $\beta$  plasma concentration in NSCLC patients directly correlated with the frequency of circulating CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>+</sup> T<sub>regs</sub>. These cells display higher expression levels of FOXP3 if compared to T<sub>regs</sub> of control subjects. It is of note that in resected lung tumor tissue specimens, a co-expression of TGF $\beta$ , COX-2, and FOXP3 is found [192]. Anti-CD25 IgG plasma level was significantly higher in patients with NSCLC than control subjects, in particular in patients at stage III of NSCLC, suggesting that antibody specificity could be used as a biomarker for prognosis of lung cancers in analogy with the enhancement of T<sub>reg</sub> count in peripheral blood [193]. While it is clear that T<sub>regs</sub> play a role in the promotion of tumor tolerance and immunosuppression their specific contribution to tumor angiogenesis in NSCLC still requires further investigation.

### Concluding remarks

The NSCLC TUMIC is able to polarize most innate immune cells toward a pro-tumor phenotype. In some cases this can be extended systemically, with peripheral cells also being affected. Polarization



within the TUMIC is then critical for permitting tumor angiogenesis, suppression and subversion of the adaptive immune system, leading to tumor progression. Thus the TUMIC and the immune cells within the TUMIC can be considered targets for prevention and therapy.

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