DOI:10.1093/jnci/dju200
First published online September 1, 2014

REVIEW

A Think Tank of TINK/TANKs: Tumor-Infiltrating/Tumor-Associated Natural Killer Cells in Tumor Progression and Angiogenesis

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Manuscript received October 31, 2013; revised May 8, 2014; accepted May 31, 2014.

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Tumor-infiltrating leukocytes are often induced by the cancer microenvironment to display a protumor, proangiogenic phenotype. This "polarization" has been described for several myeloid cells, in particular macrophages. Natural killer (NK) cells represent another population of innate immune cells able to infiltrate tumors. The role of NK in tumor progression and angiogenesis has not yet been fully investigated. Several studies have shown that tumor-infiltrating NK (here referred to as "TINKs") and tumor-associated NK (altered peripheral NK cells, which here we call "TANKs") are compromised in their ability to lysew tumor cells. Recent data have suggested that they are potentially protumorigenic and can also acquire a proangiogenic phenotype. Here we review the properties of TINKs and TANKs and compare their activities to that of NK cells endowed with a physiological proangiogenic phenotype, in particular decidual NK cells. We speculate on the potential origins of TINKs and TANKs and on the immune signals involved in their differentiation and polarization. The TINK and TANK phenotype has broad implications in the immune response to tumors, ranging from a deficient control of cancer and cancer stem cells to an altered crosstalk with other relevant players of the immune response, such as dendritic cells, to induction of cancer angiogenesis. With this recently acquired knowledge that has not yet been put into perspective, we point out new potential avenues for therapeutic intervention involving NK cells as a target or an ally in oncology.

JNCI J Natl Cancer Inst (2014) 106(8): dju200 doi:10.1093/jnci/dju200

Natural killer (NK) cells, the first innate lymphoid cells discovered, are the most widely distributed and were originally described as large granular lymphocytes able to lyse tumor cells without requiring prior activation (1). NK cell biology is quite complex and has been reviewed in detail elsewhere (2–4); here we discuss the role of NK cells in angiogenesis, tumor tolerance, and progression. Two major subsets of peripheral blood NK cells have been identified in humans, on the basis of surface density expression of CD56, an isoform of the human neural cell adhesion molecule, and of CD16, the low-affinity Fc receptor. The $CD56^{dim}CD16^{+}$ NK cell subset constitutes about 90–95% of peripheral blood NKs that show higher amounts of cytolytic granules, such as perforin and granzyme, and are cytotoxic when encountering nonself (see below) or mediating antibody-dependent cell cytotoxicity (ADCC) (5). Although poor long-term cytokine producers, these cells have recently been shown to rapidly (2 to 4 hours) release substantial amounts of cytokines (6,7). The other relevant peripheral blood NK cell subset is $CD56^{bright}CD16^{-/low}$ cells (about 5–10% of peripheral blood NKs). While weakly cytotoxic, they can produce large amounts of some cytokines, including IFNγ, TNFα, and GM-CSF. The $CD56^{bright}CD16^{-/low}$ cells are considered critical for development of type 1 T-cell responses, since they provide an important innate source of interferon γ (IFNγ), conditioning the microenvironment during antigen presentation in secondary lymphoid organs (8) as well as for other immune reactions. The cytokine-producing $CD56^{bright}CD16^{-/low}$ NK cells are recognized as NK cells that have not yet reached a terminal differentiation into cytotoxic NK cells. These cells can undergo further maturation upon exposure to specific cytokines (interleukin [IL]-2, IL-12, and/or IL-15) into $CD56^{dim}CD16^{+}$ cells, displaying higher levels of perforin and more effective cytolytic capability (9,10).

The acquisition of NK cell cytotoxicity during evolution has been associated with development of highly sophisticated and robust mechanisms controlling NK cytolysis in order to avoid tissue damage. Cytolysis is activated through a variety of cell surface receptors that modulate NK cell functions (11–14). Current data are compatible with the concept that the ligands for activating NK receptors are expressed primarily by "stressed" cells (including tumor- or virus-infected cells). NKp46, NKp30, and NKp44 are activating receptors that have been collectively named "natural cytotoxicity receptors" (NCRs). They were the first human activating receptors mediating NK cytotoxicity to be identified and molecularly characterized (14). Although some viral glycoproteins have been found to bind to NCRs (15), the tumor ligands for NK cells are not fully defined. B7-H6 and, very recently, a novel isoform of the mixed-lineage leukemia (MLL5) protein have been identified that bind to NKp30 and

NKp44, respectively, and are expressed on a large panel of tumors (16–19).

A direct association has been established between the surface density of NCR on NK cells and the intensity of NK-mediated anti-tumor cytolytic activity (20). NKG2D is a different type of NK-activating receptor that is expressed also by cytotoxic T lymphocytes. NKG2D recognizes the stress-inducible MHC class I chain-related A and B genes (MICA/B) (21) and UL16-binding protein (ULBP) proteins (22). Lastly, it has been demonstrated that DNAM-1 (DNAX accessory molecule-1), a triggering receptor expressed by virtually all NK cells (but also by T-lymphocyte subsets and monocytes), is able to specifically bind CD155 and Nectin-2 (CD112), two members of the nectin family present on most tumor cell lines (23). NK cells also produce activating coreceptors recognizing molecules that are mostly restricted to hematopoietic cells: in particular, 2B4 interacts with CD48 (24), and NTB-A displays homophilic recognition (25).

Human NK cells express a wide array of inhibitory receptors, all specific for distinct human leukocyte antigen (HLA) class I molecules (13,26,27). Relevant inhibitory receptors are represented by the killer Ig-like receptors (KIRs) which detect allelic determinants on HLA class I molecules. NK cells lyse target cells that have lost (or express low amounts of) MHC class I molecules, as frequently occurs in tumor cells or cells infected by some viruses, such as certain Herpes viruses or Adenoviruses (13,26,28,29). Given these general concepts, several scenarios of NK interactions with other cells can be envisioned (Supplementary Figure 1, available online): 1) Normal cells expressing even low levels of MHC I, yet lacking stress-related activating ligands, are not lysed; 2) stressed normal cells expressing activating and inhibitory (MHC I) ligands are spared; 3) cells expressing activating ligands and low levels of inhibitory ligands are lysed; 4) cells expressing high levels of activating ligands, even in the presence of inhibitory ligands, can be lysed.

When NK cells are generated in the bone marrow, they express a limited array of inhibitory receptors; only those harboring an inhibitory receptor recognizing self ligands are "licensed to kill," while those expressing inhibitory receptors that do not recognize self (ie, MHC I polymorphisms not present in the individual) are rendered anergic. Current concepts are that NK cells undergo a series of maturation phases, with the $CD56^{bright}CD16^{-}$ cells representing an immature stage that does not express killer cell immunoglobulin-like receptors (KIRs), while the $CD56^{dim}CD16^{+}$ cells are fully differentiated, KIR-expressing cells.

There is a growing body of evidence suggesting that some NK cells acquire long-lived "memory" for specific activating ligands that are passed onto progeny (30), although a consensus on this issue is still lacking. Memory NK cells appear to be generated by exposure to pathogen-associated activating ligands, cytokines, and some haptens (30). This memory also seems to be relatively specific to the molecule(s) stimulating this response; it is possible that, as in T cells (31–33), epigenetic changes occur in certain loci of memory NK cells, which could account for transmission to progeny.

As we will see below, tumor-infiltrating NK cells (TINKs) and tumor-associated NK cells (TANKs; altered NK cells in cancer patients, including peritumor and peripheral blood NK cells) can acquire a changed phenotype as compared with NK cells from tissues and peripheral blood of individuals who do not have neoplastic disease. Further, there are numerous functional alterations in both TINKs and TANKs as compared with peripheral blood NK cells of healthy individuals.

Angiogenesis and Immune Cells in Tumor Progression

Angiogenesis is a key early step in cancer development that permits tumor expansion, growth, progression, and dissemination (34–36), as well as being a key target in prevention (37). Several studies have shown that microvascular density is associated with prognosis and vascular endothelial growth factor (VEGF), the major angiogenic factor, has become a target for several cancers (36). However, inhibiting VEGF alone has had limited success in some cancers, suggesting that other approaches are needed (38,39). Immune cells infiltrating tumors often show a skewed phenotype that reflects attenuation of antitumor activity and enhancement of protumor activities, including angiogenesis, in a process defined as tumor-induced polarization (40,41). By this process tumor cells are able to reprogram the host immune system components in favor of neoplastic progression, including angiogenesis, an essential process for tumor survival, and metastasis.

The classic example of tumor-induced polarization occurs in macrophages: M1 macrophages produce Th1 cytokines (such as IL-12 and TNFα) and promote anti-tumor responses, while M2-polarized macrophages produce Th2 cytokines, induce tissue reconstruction, growth promotion, and angiogenesis (42). Several subsets of M2 macrophages are generated by diverse stimuli (IL-4 → M2a; immune complexes/IL-1β → M2b; IL10/TGFβ → M2c). Tumor-associated macrophages (TAMs) show an M2-like profile (42); other tumor-associated M2-like polarizations include myeloid-derived suppressor cells (MDSCs) (43,44) and Tie2 expressing macrophages closely associated with the vasculature (45,46).

Several studies show a protumor and proangiogenic polarization for most immune cells in the tumor microenvironment, including macrophages, dendritic, mast, T and B cells, as well as polymorfonuclear cells (PMN) (40–42,46,47). We had previously hypothesized that this phenotypic polarization might be extended to NK cells (41).

Physiological Proangiogenic Activity of NK Cells

A peculiar behavior of NK cells is found in the developing decidua (Table 1), which converts them from killers to builders (48). Early on in pregnancy, decidual NK cells (dNK) accumulate to become approximately 70% of the local lymphocytes and 30–40% of all decidual cells (49). dNK cells display a $CD56^{superbright}CD16^{-}$ phenotype (49) and, although they express KIRs as well as perforin and granzymes, are poorly cytotoxic (49–52). Further, they have been found to be associated with induction of $CD4^{+}$ T regulatory (Treg) cells (3), and to produce IL-10 (51), thus potentially contributing to the immunosuppressive environment of the placenta. dNK express most of the NK cell hallmarks, including the activating receptors NKp46, NKG2D, and 2B4, as well as KIR and CD94/NKG2A inhibitory receptors, along with markers not found on peripheral blood NK cell subsets, such as CD9 and CD49a (53). Interestingly, the usually activating coreceptor 2B4 acts as an inhibitory receptor in dNK cells (54), suggesting extensive rewiring of the cytoplasmic signals in response to external stimuli. Another notable feature of dNK cells is their close link with vascularization of the decidua and spiral artery formation (51,52) in both humans and mice. dNK

Table 1. List of the key studies concerning the pro-angiogenic and pro-tumor role of Natural Killer cells*

NK Cell system	References	Model(s)	Comments
dNK cells	Li XF et al, J Clin Endocrinol Metab 2001; 86:1823–34	Human endometrium	Intense hybridization for VEGF-C and PlGF mRNAs was found in uterine nature killer cells in secretory phase endometrium and for Ang2 mRNA in the same cells in the late secretory phase. Interleukin-2 (IL-2) and IL-15 up-regulated VEGF-C, but not PlGF or Ang2, mRNA levels in isolated NK cells
	Wang C et al, Microsc Res Tech 2003; 60:420–9.	Murine uterus	NK (termed granulated metrial gland) cells are a major immune cell population in the murine pregnant uterus, and contribute to the maintenance of pregnancy by functioning as uterus-specific natural killer (NK) cells.
	Hanna J et al, Proc Nat Med 2006; 12:1065–74.	Human and murine decidual NK cells	dNK cells are potent secretors of an array of angiogenic factors and induce vascular growth in the decidua. Notably, such functions are regulated by specific interactions between dNK-activating and dNK-inhibitory receptors and their ligands, uniquely expressed at the fetal-maternal interface.
Myocardial NK cells	Ayach et al. (63)	c-kit deficient mice	c-kit deficient mice show dysfunctional NK cells and delayed myocardial repair and angiogenesis.
	Bouchentouf et al. (64)	SCID-NOD/SCID mice	NOD/SCID mice have dysfunctional NK cells and delayed myocardial repair. Mechanisms include engagement of NK $\alpha4\beta7$ integrin KLRG1. No production of hepatocyte growth factor, vascular endothelial growth factor, endothelial growth factor, and CXCL12 by NK cells was found.
Ocular NK cells	Lee et al. (65)	asialo-antibody NK depletion in mice	NK cell depletion leads to reduction of corneal angiogenesis and choroidal neovascularization associated with decreased pro-angiogenic macrophage infiltration into the cornea
Tumor Infiltrating/ Tumor Associated NKs	Carrega et al. (69)	Human NSCLC	$CD56^{bright}CD16^{-}$ NK subset predominates in NSCLC; cells are able to express KIRs but are affected in their cytotoxic activity against K562 cells
	Platonova et al. (70)	Human NSCLC	Tumor infiltrating NKs cells displayed a profound and coordinated alteration of their phenotype, with a drastic reduction of NK cell receptor expression specifically detected in the tumor. TINK cells exhibited profound defects in the ability to activate degranulation and IFN-γ production
	Mamessier E et al. J Clin Invest 2011; 121:3609–22	Human breast cancer	Tumor infiltrating and to a lesser extent tumor associated NKs showed impairment of NK cell function, and cytotoxicity. NKp30, NKG2D, DNAM-1, and CD16 were down-regulated. TGFβ1 and PGE_2 were associated with decreased NK function and tumor progression.
	Rocca YS et al. Innate Immun 2013; 19:76–85.	Human colon cancer	Tumor infiltrating NKs cells displayed a profound alteration of their phenotype with low cytotoxicity, reduced IFNγ production and down-regulation of CD161, CD94, CD158b, NKp30, NKG2D, DNAM-1, and CD16. Contact with colon cancer cells in part reproduced some of these effects.
	Carrega et al. (104)	Human tumors and tissues	NK cells infiltrating the tissues did not substantially change upon malignant transformation, but the relative proportion of NK subsets infiltrating the tissues is different, with a trend toward a tumor-infiltrating NK population enriched in non-cytotoxic cells.
	Bruno et al. (66)	Human NSCLC	NK cells from NSCLC patients produce angiogenic factors and are able to induce endothelial cell recruitment and morphogenesis ex vivo. Exposure to TGFβ1 partially reproduces polarization toward the angiogenic phenotype.

* The table lists selected references concerning the role of Natural Killer cells (NK) in tumor progression and angiogenesis and summarizes the principal findings grouped as: Decidual NK (dNK) cells, which represent the most investigated subset in relation to angiogenesis. Since there are numerous articles, only a few are cited. NK cells during myocardial repair; the studies were all performed on murine models. NK cells during angiogenesis and repair in murine ocular models, where the pro-angiogenic function exerted by NK cells is associated with macrophages. Tumor infiltrating/associated NK cells, most of the references regard the impairment of NK cytotoxic activities with only one reference demonstrating a direct association between Tumor infiltrating/associated NK cells and angiogenesis. SCID-severe combined immunodeficiency; NOD-non obese diabetic.

cells produce substantial quantities of angiogenic factors (Table 1), including VEGF, PlGF, as well as IL-8 (CXCL8), and show potent angiogenic activity in vitro and in vivo (51). This activity has been linked to tissue construction (49) that is critical for implantation and fetal development. Low levels of dNK cells are associated with miscarriage (49). In a different microenvironment, such as that of tumors, this proangiogenic activity could have detrimental actions. It has been shown that addition of angiogenic dNK cells to tumor cell xenografts markedly enhanced growth of tumors (51). The origin of dNK cells is controversial; expansion in loco from NK

cell precursors (55) and conversion of peripheral blood NK cells to dNK upon exposure to decidual endothelial and stromal cells (56,57) have been proposed. Decidual stromal cells produce high levels of TGFβ that has been reported to convert isolated peripheral blood $CD56^{dim}CD16^{+}$ NK cells into $CD56^{bright}CD16^{dim}$ cells displaying poor cytotoxicity and expressing other markers, such as CD9, in common with dNKs (58,59). A combination of TGFβ, hypoxia, and a demethylating agent has been found to convert sorted peripheral blood $CD56^{dim}CD16^{+}$NK cells into a dNK-like phenotype (59). These cells show low cytotoxicity (59), high levels of VEGF expression (induced by hypoxia), expression of the dNK marker CD9 (induced by TGFβ), and of KIRs (maintained by the demethylating agent). dNK cells tend to preferentially express CXCR3 and CXCR4 (60–62), in part in response to hormones (57,60), and the respective ligands of these receptors are able to preferentially induce chemotaxis of $CD56^{bright}CD16^{dim}$ NK cells (57,60).

NK cells have also been linked with angiogenesis during tissue repair (Table 1). Mice deficient in c-kit showed defective myocardial repair, and gene expression profiling and immunohistochemistry indicated a role for NK cells (63). Further analyses showed that NK cells were required for angiogenesis in the damaged murine myocardium (64) and that engagement of KLRG1 (killer cell lectin-like receptor 1) by cadherins and α4β7 integrin by VCAMs (vascular cell adhesion proteins) were important for NK activation. In models of bFGF (basic-fibroblast growth factor)-induced corneal and laser-induced choroidal angiogenesis, NK cell depletion led to a substantial reduction of neovascularization (65). In NK-depleted animals, reduced macrophage infiltration into the cornea and lower levels of VEGF-A, VEGF-C, and IFNγ mRNAs were observed. In vitro, a coculture of NK, macrophages, and endothelial cells showed an IFNγ-dependent increase in VEGF (65).

Similarities and Diversities Between Angiogenic NKs and TINKs/TANKs

Given the angiogenic tissue construction activity of dNK and several similarities between the decidual, tissue repair, and tumor microenvironments, a working hypothesis (41) is that TINKs or TANKs could have a proangiogenic activity, resulting in tumor promotion rather than tumor inhibition (Table 1). Our recent data in the context of non-small cell lung cancer (NSCLC) supports this hypothesis (66). Lung tissues are relatively rich in NK cells (66–68), where the predominant subset in normal parenchyma is $CD56^{dim}CD16^{+}$ (66, 69). In contrast, the major NK subset in NSCLC tissues appears to be cytokine-producing $CD56^{bright}CD16^{-}$ NK cells (66,69). The NSCLC TINKs are localized in the tumor stroma (69) and at the invasive margin (70) of the tumor. These TINKs express activating receptors and some of the $CD56^{bright}Perforin^{low}$ NK cells display a discrete expression of KIRs (69,70). In patients with pleural effusions, including some lung cancer patients, the $CD56^{bright}CD16^{dim}$ NK subset predominated; these cells expressed high levels of IFNγ upon activation with IL-2 and became efficient in killing suitable target cells (71).

When examined for angiogenic cytokine production, NK cells of NSCLC patients were found to produce relatively high levels of VEGF, placental-derived growth factor (PlGF), and IL-8 (66). Interestingly, this was not limited to the TINK cells, as pro-angiogenic factor production was also observed in adjacent tissues and even peripheral blood (TANKs). Further, NK cells in NSCLC patients with squamous cell carcinoma produced substantially higher levels of VEGF and PlGF as compared with those with adenocarcinomas (66). The levels of VEGF and PlGF in TANKs from adenocarcinoma patients were comparable with those from healthy controls, while that of TANKs from squamous cell carcinoma patients was markedly higher. Further, supernatants of NSCLC TINKs showed angiogenesis-associated activities in vitro, in particular those of squamous cell carcinomas (66). Most NSCLC TINKs show low levels of the NK maturation markers CD11b and CD27 (72), while the increased numbers of fully mature NK cells indicated by the CD57 marker were a positive prognostic indicator in NSCLC squamous cell carcinomas (73). These data imply that like dNK cells, TINKs and TANKs have some markers of mature NK cells, including granzyme and perforin, yet display low cytotoxicity, while producing angiogenesis-promoting factors.

In the case of squamous NSCLC, a potent effect of the tumor on the systemic NK phenotype was observed, since NK polarization was found even in peripheral blood. Transcriptome profiling studies indicate that NSCLC has a distinct effect on peripheral blood mononuclear cell gene expression (74,75). This was true in particular for squamous cell NSCLC, and many genes making up a prognostic signature using these profiles were NK associated (74,75). The data show that even a squamous cell carcinoma of small (operable) size had a substantial systemic effect on the immune system, in particular NK cells. A similar systemic alteration of TANKs has been observed for colorectal (76) and breast (77) cancers, where these cells showed repression of NK functions. In breast cancer, the level of functional repression correlated with disease stage (77) and was reverted in long-term survivors. The mechanisms leading to the systemic effect creating TANKs are as yet unknown, and the overall consequences of this remain unclear. NK trafficking through the tumor with local polarization or a systemic release of factors affecting NK cell polarization could result in generation of TANKs. The variable reports of ratios between $CD56^{dim}CD16^{+}$ to $CD56^{bright}CD16^{-}$ within the TINK/TANK in NSCLC surgical samples (66,69,70) may relate to nontumor to tumor tissue ratio of the biopsies.

Like the dNK cells of the decidua, the origin of the angiogenic cytokine-producing TINKs is not clear (Figure 1). Cytotoxic NK cells have a stable and apparently terminal phenotype (10), although $CD56^{dim}$ NK cells can become $CD56^{bright}$ upon in vitro activation and, at the same time, CD16 may be downregulated (10). The low expression of perforin and granzyme on a large part of TINKs, similar to that of $CD16^{-}$ peripheral blood NK cells, has suggested that TINKs are derived from $CD56^{bright}CD16^{-}$ NK cells, and that factors within the tumor microenvironment might induce expression of some KIRs on these cells (69). Microarray studies (78) have suggested that dNK cells express a very different molecular profile as compared with $CD56^{bright}CD16^{low/neg}$ and $CD56^{dim}CD16^{+}$ NK cells. A microarray analysis on seven adenocarcinoma and five squamous NSCLC-derived NKs (sorted as $CD3^{-}CD56^{+}$ cells) indicated distinct profiles between patient and control samples (79). Interestingly, as compared with other NK cells, both dNK and NSCLC TINKs showed upregulation of several activation markers and in particular Granzyme A (78–79). dNK cells also showed

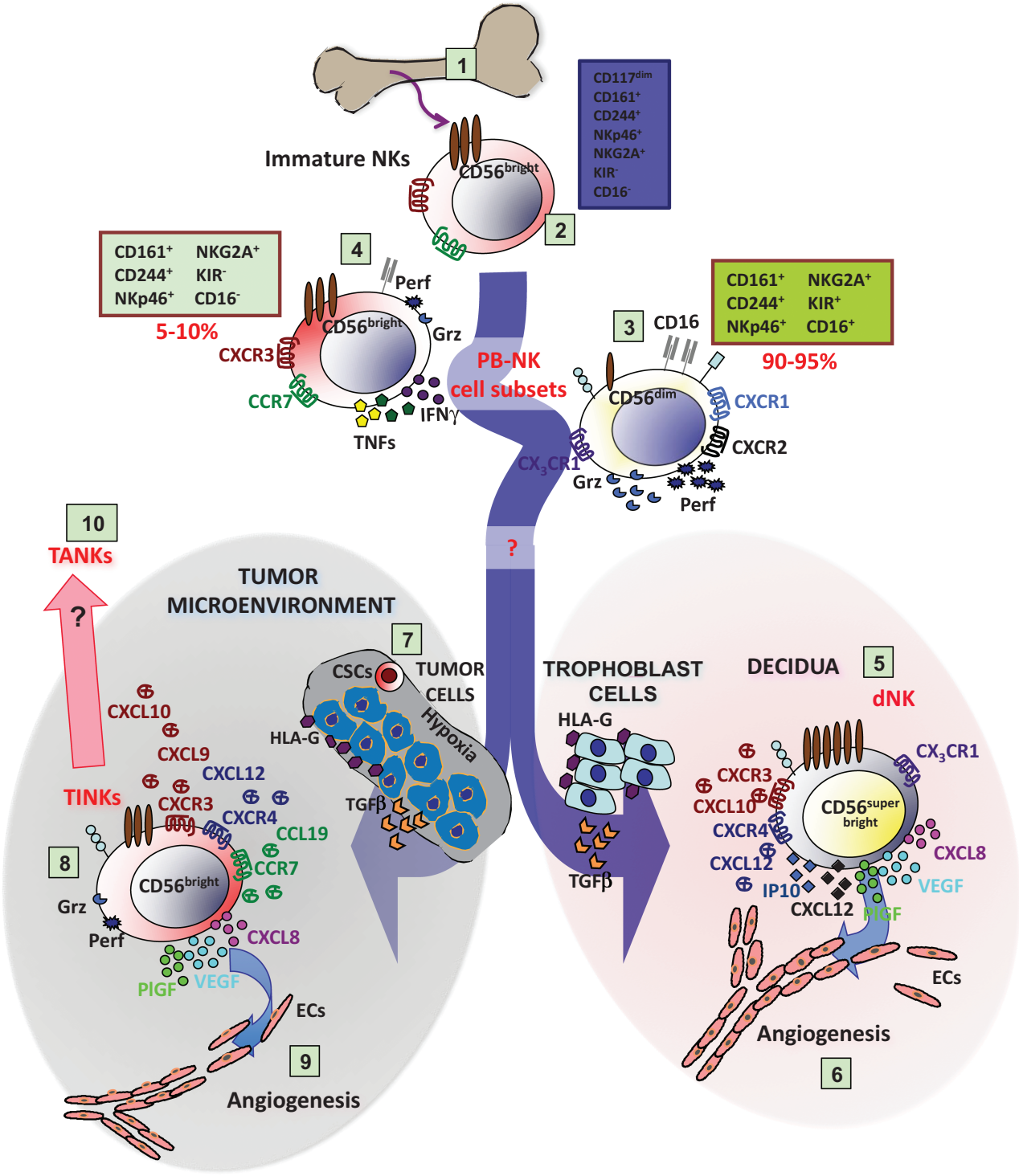

Figure 1: Hypothesis of natural killer (NK) cell subset differentiation and functions in decidual and tumoral tissues. NK precursors are generated in the bone marrow [1] and undergo several steps toward maturation into the major peripheral blood (PB) NK cell subsets [2]. The main PB subsets are the $CD56^{dim}CD16^{+}$ [3] (90%–95% of circulating NK cells) and $CD56^{bright}CD16^{dim/-}$ NK cells [4](5–10% of circulating NK cells) associated with lower cytotoxic activity and production of IFNγ and other cytokines, expressing the CXCR3 and CCR7 chemokine receptors. These cells [2] also appear to be a precursor to the cytotoxic $CD56^{dim/+}CD16^{+/bright}$ NK [3], showing high levels of perforin and granzyme and expression of CX3CR1, CXCR1 and CXCR2. The developing decidua contain high levels of dNK [5] cells that are recruited into this tissue. Within the decidua (shown as a pink area) trophoblast cells release transforming growth factor (TGFβ) and express HLA-G molecules associated with reduction of NK cytotoxicity and promotion of pro-angiogenic activity [6]. The dNK [5] cells express CX3CR1, CXCR3 and CXCR4, and secrete angiogenic factors including VEGF, PIGF, CXCL8, CXCL12. Within many tumor microenvironments (shown as a grey area), tumor cells can express HLA-G and produce TGFβ along with hypoxia [7]. These elements have been associated with conversion of PB NK [3] cells into poorly cytolytic dNK-like tumor infiltrating natural killer cells (TINKs) cells able to release vascular endothelial (VEGF), placental (PlGF) growth factors and IL8 (CXCL-8) [8], thus sustaining angiogenesis [9]. It is not clear if tumor associated natural killer cells (TANKs) [10] are the result of emigration of TINKs [8] from the tumor microenvironment or due to systemic effects on NK cells of cancer-related products.

increased expression levels of Granzyme B, while TINKs showed high production of Granzyme K and Fas.

The tumor microenvironment likely affects the polarization of $CD56^{bright}CD16^{low/neg}$ NK cells toward a proangiogenic phenotype, inducing the production of cytokines such as VEGF, PlGF, and IL-8. High levels of TGFβ and hypoxia are also features typical of the tumor microenvironment (Figure 1) that could impact the nature and function of $CD56^{bright}CD16^{low/neg}$ TINKs. In cancer, TGFβ1 acts as a "Janus-like" cytokine: In the initial phases of cancer formation, it behaves as a tumor suppressor, inhibiting tumor cell replication and favoring apoptosis (80,81). In contrast, in later stages of tumor progression, TGFβ1 exerts a protumorigenic role, promoting survival and epithelial-mesenchymal transition and invasion, as well as acting within the tumor microenvironment as an immune-suppressive and angiogenic agent (80,81). TGFβ appears to be a strong immune-cell–polarizing agent, active on most immune cells (81).

As mentioned above, several studies from the Strominger/Kopcow groups have indicated that TGFβ, eventually together with other factors, can induce a dNK-like differentiation from peripheral blood $CD56^{dim}CD16^{+}$ NK cells (58,59,82). TGFβ has been associated with a functional impairment of NK cells (77). We found that TGFβ produces polarization of peripheral blood NK cells from healthy subjects toward VEGF-, PlGF-, and IL-8-producing NK cells (66), again indicating a parallel with the dNK-like phenotype. These data suggest that, within the decidua and the tumor microenvironment, TGFβ, along with other cues such as hypoxia, might result in the polarization of NK cell precursors or even mature NK cells toward angiogenic dNK-like cells. This would be in keeping with the immunomodulatory role of TGFβ in several tumors and tumor models (80,83). Tumor-derived TGFβ has been shown to upregulate CXCR3 and CXCR4 in NK cells (84), similar to what occurs for dNK cells (60–62), and to exert an immunosuppressive activity (85). Blocking of TGFβ has been shown to overcome immune suppression (85) and enhance the effects of NK cell therapy (86). Plasma TGFβ levels have been shown to be substantially upregulated in lung and colon cancer patient immunosuppression linked to downregulation of NKG2D (87–90). It is also possible that tumor-microenvironment–associated factors induce a further modulation of $CD56^{dim}CD16^{+}$ cells into the TINK phenotype.

HLA-G, a unique immunoregulatory class I MHC molecule expressed by decidual trophoblasts (91) and in diverse tumor tissues (91,92) might play a role in TINK and, through release of soluble forms, TANK polarization (Figure 1). HLA-G is expressed by trophoblasts in the decidua (91) and is upregulated in several tumor tissues (70,91,92). Tumor and serum HLA-G expression levels have been found to be an independent marker of poor prognosis in NSCLC, ovarian, breast, colorectal, esophageal, and gastric cancers, as well as hepatocellular and endometrial carcinoma (91–94). HLA-G expression can be quite heterogeneous within the tumor; however, a mechanism involving exosome release and trogocytosis by neighboring tumor and immune cells, including NK cells, has been evoked to explain the apparent bystander effect of HLA-G–mediated immune suppression (91,92). HLA-G seems to exert its immunosuppressive effects through interactions with three key inhibitory receptors: immunoglobulin-like transcripts ILT-2 (found on macrophages, dendritic, decidual and peripheral NK cells, as well as B and T lymphocytes), ILT-4 (expressed by macrophages and dendritic cells), and KIR2DL4 (in decidual and peripheral blood NK cells) (91,92). Through these receptors, HLA-G inhibits $CD8^{+}$ T cells and NK cytotoxicity, alters dendritic cell (DC) maturation, trafficking, antigen presentation, and cross talk with T and NK cells. HLA-G interaction with the nonclassical KIR2DL4 (CD158d) has been shown to stimulate resting NK cells to secrete proinflammatory and proangiogenic factors (95) through induction of a senescence-associated secretory phenotype (96). Frequently strongly expressed in ovarian cancer, HLA-G in a metastasis model was associated with increased metastases and reduced NK cell cytotoxicity (97). Among other effects, soluble HLA-G differentially modulated chemokine receptor expression between $CD56^{bright}$ and $CD56^{dim}$ populations, downmodulating CCR2 in the $CD56^{bright}$ cells and CXCR3 in both populations, while having little effect on CXCR4 (98), suggesting modulation of NK cell recruitment by microenvironments expressing HLA-G (Figure 1). Other NK suppressive factors in common between the decidua and tumor microenvironment are indoleamine 2,3-dioxygenase (IDO) and prostaglandin E2 (99).

In terms of angiogenic cytokine production within the tumor microenvironment, the relative contribution of NK cells remains to be determined, which likely depends on the tissue of origin. Unlike other innate cells, within the tumor microenvironment there is a strong presence of NK-activating ligands that could enhance the cytokine-producing activity of TINKs, as has been found in the decidua (100). NK cells also interact with other immune cells, modulating both their presence and activation state. As examples, in the cornea and choroid, NK depletion was associated with reduced macrophage recruitment and loss of angiogenic potential (65); in the premetastatic niche, NK cells are associated with MDSC accumulation (101). Interestingly, NK cells have also been reported to convert into MDSCs within the tumor microenvironment (102).

TINKs Have a Reduced Cytotoxic Potential Toward Cancer and Cancer Stem Cells

Tumor cells and the tumor microenvironment substantially suppress the antitumor function of TINKs through a variety of mechanisms (103). In NSCLC, TINKs have been shown to be highly enriched in the $CD56^{bright}Perforin^{low}$ NK cell subset (66,69), a phenotype quite similar to that of TINKs from colorectal (76) and breast (77) cancers. Consistent with the lower cytotoxicity of the $CD56^{bright}Perforin^{low}$ NK cell subset, TINKs uniformly show poor cytotoxicity (66,69,76,77,103).

In terms of total lymphocytes, NK cell frequency varies depending on the tissue, ranging from relatively high (approximately 25% in kidney; 15% in liver and subcutaneous adipose tissues) to quite low (1–4% in colon, stomach, adrenal gland) (104). We observed that in cancers, the frequency of $CD56^{bright}Perforin^{low}$ NK cells among the total NK cell compartment may be substantially higher than in matched normal tissues, as in the case of lung and breast cancers (54% vs 9.2% and 30% vs 4.1%, respectively) (104). The comparative analyses of the chemokine expression patterns of TINKs with those derived from matched healthy tissues showed that the tumor microenvironment might account for this specific accumulation of NK with the TINK phenotype. Neoplastic lung

tissues showed a marked downregulation of CXCL2, a chemokine able to specifically attract $CD56^{dim}$ NK cells, since its cognate receptor, CXCR2, is expressed by $CD56^{dim}$ cytotoxic NK cells but not on the $CD56^{bright}$ counterpart. At the same time, the upregulation of CCL19, CXCL9, and CXCL10, chemokines more specific for $CD56^{bright}$ NK cells, occur in lung tissues following neoplastic transformation. A similar trend is observed in breast cancers, where the $CD56^{dim}$-associated chemokines, CXCL2, CX3CL1, CXCL1, and CXCL8, are downregulated in the tumor tissue, and the upregulation of CCL5 and CCL19 is simultaneously observed (104).

Remarkably, the frequency of TINKs among the total tumor-infiltrating lymphocytes did not show statistically significant differences when compared to the percent of NK cells as a function of total lymphocytes from normal matched tissues (66,69,104,105). These recent data suggest that neoplastic transformation did not determine an increased migration of NK cells within the tissues. Rather, as a consequence of switches in chemokine expression patterns, cytotoxic NK cells are substantially decreased compared with healthy tissues, at least in some frequently occurring cancers such as breast and lung carcinomas. This new evidence now emerges as a further mechanism of cancer immunoediting with implications for both immunosurveillance and tumor escape from immune attack.

In the context of tumor cell heterogeneity, relapse and metastasis, substantial interest has arisen regarding the concept of cancer-stem or cancer-initiating cells (CSCs/CICs). Current concepts are that these cells, at least for some tumor subtypes, show slow replication and thus are resistant to most chemotherapeutics and radiotherapy, while through asymmetric replication CSCs/CICs give rise to rapidly proliferating cells that make up the bulk of the tumor (106–109). By definition, CSCs/CICs are those cells able to give rise to new tumors, clinically leading to tumor recurrence and metastatic disease. Targeting CSCs/CICs has therefore become of paramount importance, and properly activated NK cells may have the capacity to eliminate this insidious cell population. Glioblastoma stem cells, which express low levels of MHC-class I molecules and high levels of the activating DNAM-1 ligands PVR and Nectin-2, were susceptible to lysis by NK cells following IL-2 or IL-15 activation (110). Unactivated NK cells showed low activity. NK cells have been found to be able to kill oral squamous carcinoma stem cells as well as bona fide embryonic, mesenchymal, and dental pulp stem cells (111). Again, however, microenvironmental effects may modulate and compromise this activity (112). Cell lines bearing the ovarian cancer stem cell marker CD24 are resistant to chemotherapy yet preferentially targeted by NK cells (113). A detailed study on human colorectal carcinoma confirmed that the CICs express lower MHC-class I and higher levels of NK-activating ligands and that CICs were preferentially targeted over bulk tumor by allogenic and autologus NK cells (114).

Increasing experimental (86,115–121) and clinical (86,117,122) evidence indicate that NK cells modulate metastatic dissemination, suggesting that NK cells are able to kill the CSCs/CICs that give rise to metastatic disease. However, most of the experimental studies (86,115–121) used tumor cells injected directly into healthy mice, rather than "conditioning" the host. Attenuation of NK cell activity is associated with generation of the premetastatic niche and metastasis efficiency in murine models (101). Hypoxic tumor-cell–conditioned media increased NK and MDCS recruitment into the premetastatic niche and reduced NK cytolytic activity (101). MDSCs appear to play a role in modulating NK cell function (101), which in part is overcome upon IL-2 cytokine intervention (123). A murine in vivo study showed that TAM family tyrosine kinases are involved in tumor-induced NK attenuation (121). Inhibition of the TAM family tyrosine kinase activity resulted in NK "licensing" to kill tumor and metastatic cells in murine models of both hematogenous and lymphatic routes, resulting in slower tumor growth and substantial reduction in metastases (121). These data indicate that murine NK cells can be "switched" from a poorly antitumor or even protumor phenotype to antitumor activities. It becomes clear that the compromised cytotoxic activity of NK cells is also implicated in the lack of CSC/CIC killing in cancer patients.

Implications of TINK Polarization for Cancer Therapy

The potential tumor-cell–killing capacity of NK cells suggests that these cells could be useful in therapeutic approaches (124). Several chemotherapies and targeted agents can enhance NK function, including metronomic cyclophosphamide (125), imatinib (126,127), gefitinib (128), and sorafenib (129) (although this latter point is controversial [130]). In the case of metronomic cyclophosphamide, a role for VEGF receptors in activating NK cells has been suggested (131). Immunomodulatory drugs such as Lenalidomide are associated with NK activation in vivo and reversion of tumor microenvironment (IL-6, TGFβ) effects on NK cells in vitro through inhibition of STAT3 (132). A key consideration is that NK cells infiltrating cancer tissues display low levels of CD16 on their surface. CD16 (also known as FcγRIII) is an Fc receptor able to recognize IgG that is bound to the surface of target cells (133), and it is expressed at high levels on peripheral blood $CD56^{dim}$ cytotoxic NK cells. Activation of cytotoxic NK cell FcγRIII by IgG causes the release of cytotoxic mediators like perforin and granzymes that enter the target cell and promote cell death by triggering apoptosis. The ADCC process is currently recognized as a major mechanism of action of several targeted therapies for the treatment of neoplastic diseases: these therapies aim to exploit NK cell ADCC by the administration of tumor-specific monoclonal antibodies (mAb) (134).

TINKs of those cancer tissues studied to date express little or no CD16 (66,69,104). In addition, CD16 appears to be downregulated in $CD56^{dim}Perforin^{+}$ NK cells infiltrating NSCLC (69,70). In view of these recent results, the limited expression of FcγRIII on NK cells infiltrating the tumors should be considered when mAb-mediated target therapies are proposed for the treatment of solid tumors, including NSCLC. $CD56^{bright}Perforin^{low}$ NK cells upon cytokine-induced activation (exposure to IL-2, IL-15, or both), can convert into $Perforin^{high}$ NK cells able to efficiently kill cancer cells (9,10). A hypothesis is that sufficient triggering of activating receptors might induce maturation to a $Perforin^{high}$ NK, even in the absence of specific cytokines. Although further studies are warranted to better clarify the functions and the possible differentiation pathways of tumor-infiltrating NK cell subsets, the use of IL-2 or IL-15, currently available as pharmaceutical grade compounds, can be envisaged in association with current tumor-specific, mAb-mediated target therapies (124). This therapeutic association should be advantageous for inducing tumor-cell–killing capability in this $CD56^{bright}CD16^{neg}$ NK cell subset, which abundantly infiltrates

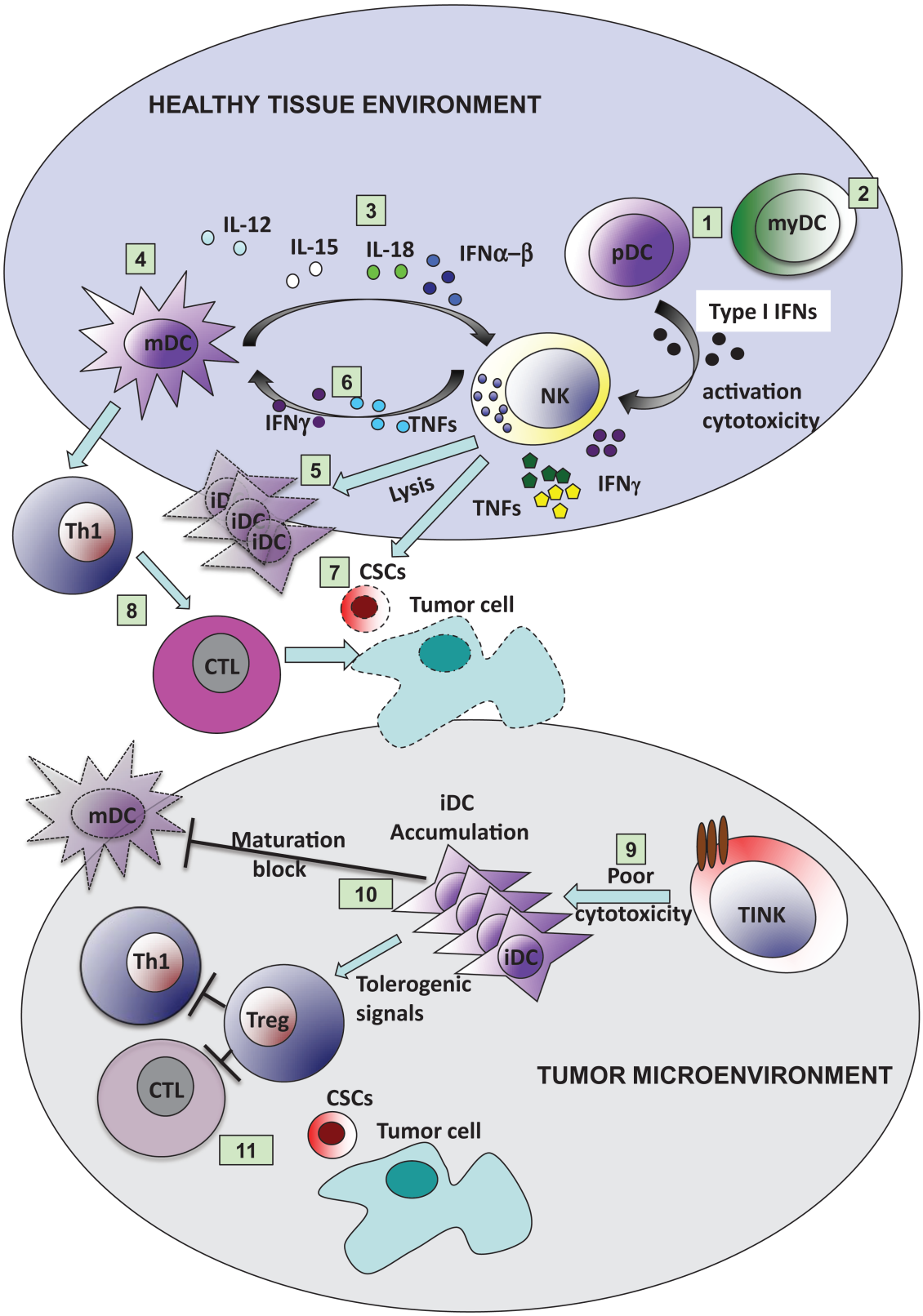

Figure 2: NK-DC cross-talk in both normal and tumor microenvironment contexts. Natural killer (NK) cell editing of dendritic cells (DCs) plays a crucial role in the regulation of both innate and adaptive immunity. In normal tissues (light blue area), plasmacytoid [1] and classical myeloid [2] DCs can activate NK cells via the release of several cytokines [3]. Activated NK cells can edit DCs, either inducing their maturation [4] or eliminating the immature, allegedly tolerogenic [5] DCs (iDCs). NK cells also release IFNγ [6] and can eliminate cancer stem cells (CSCs)[7]. In the normal context/microenvironment, NK cells sustain Th1 polarization, necessary for an effective adaptive immune response against tumors [8]. In the tumor microenvironment (grey area) NK cells are characterized by poor cytotoxicity [9], leading to iDC accumulation [10], block of cytolytic T cell responses [11] and inducing immune tolerance of tumor cells.

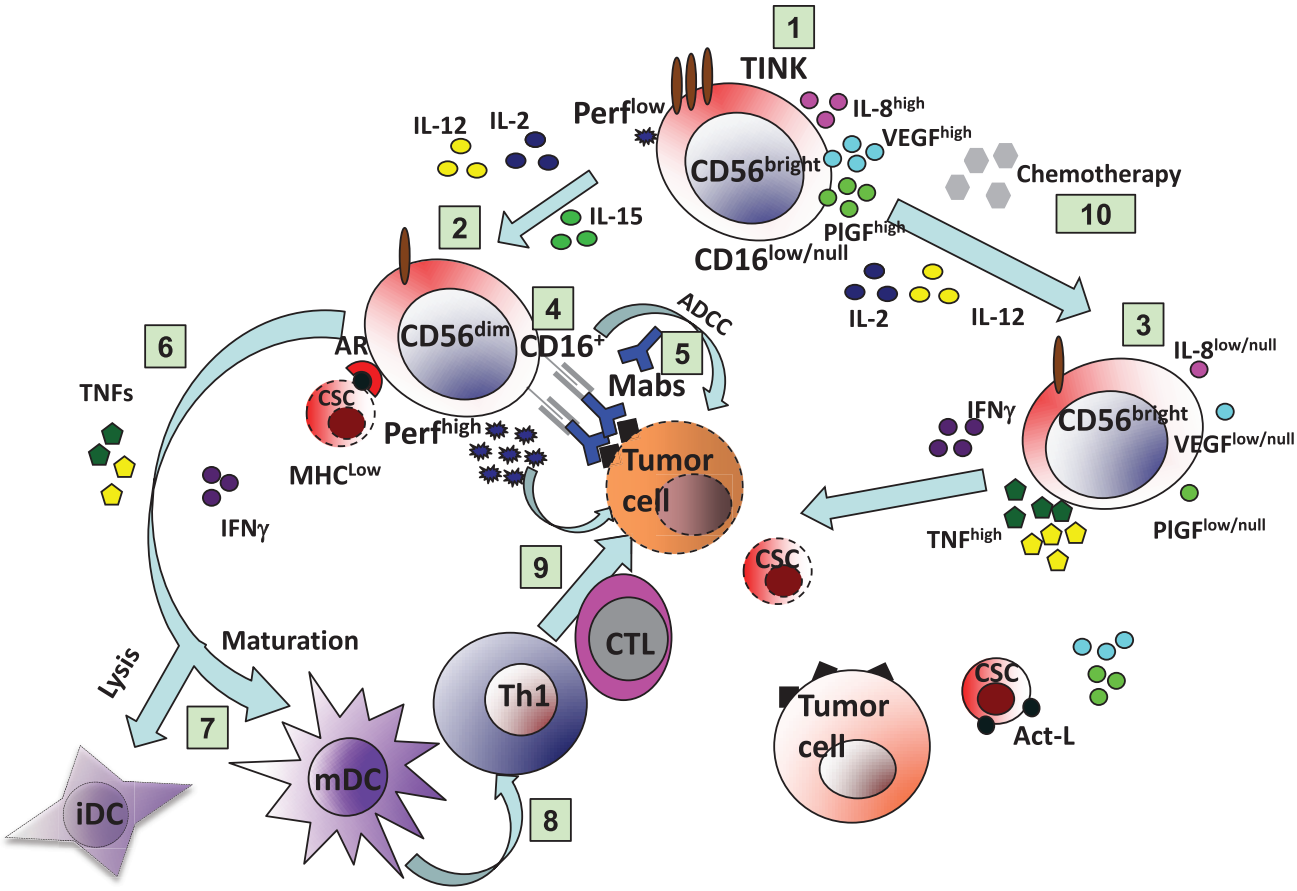

Figure 3: Involvement of TINKs in therapeutic approaches. Tumor infiltrating natural killer cells (TINKs) [1] are characterized by a substantial pro-angiogenic subset able to produce vascular endothelial (VEGF), placental (PlGF) growth factors and IL8 (CXCL-8). Several strategies to revert the "normal" anti-tumor activity are indicated: The cytotoxic activity [2] in response to activating receptors (AR) and tumor necrosis factors (TNFs) and interferon γ (IFNγ) production [3] of perforin-low ($Perf^{low}$) TINKs [1] could be restored by exposure to IL-2, IL-15 and/or IL-12, leading to a $Perf^{high}$ phenotype. Because these cytokines [3] also induce up-regulation of Fc receptor γRIII (CD16) [4], antibody-dependent cell cytotoxicity (ADCC) could be restored [5] by association of IL-2/-15/-12 with monoclonal antibody (Mabs) therapeutic approaches [5]. Cytokine treated $CD56^{dim}$ TINKs [6] could regain the ability of dendritic cell (DC) editing [7], leading to a Th1 polarization [8] and promotion of an adaptive anti-tumor response [9]. In addition, chemotherapy, alone or in association with IL-2 and/or IL-12 [10] could be used to functionally switch TINKs [1] to an anti-tumor phenotype by down-regulating pro-angiogenic factors ($VEGF^{low}$, $PlGF^{low}$, $IL\text{-}8^{low}$) and promoting production of IFNs and TNFs [3].

cancer tissues. Taken together, these data suggest that, with proper activation, NK cells could become effective weapons against tumor cell recurrence following radio- and/or chemotherapy.

The Natural Killer-Dendritic Cell Interface in the Development of Antitumor Immune Responses

NK cells are currently considered in cancer immunotherapy but most studies have focused on the single goal of exploiting their direct antitumor cytotoxic potential. For example, NK cells can rapidly expand in vitro and in vivo, in particular following haploallogenic bone marrow transplantation, where they appear to serve in both host defense as well as inhibition of graft-vs-host reactions (135). However, we must look beyond cytotoxicity to identify other means of intervention. It is now well established that interactions occurring between NK cells and dendritic cells (DCs) play a major role in the priming of antitumor responses (Figure 2). Bidirectional activations occur during the NK/DC cross talk (136–138), which substantially modulates the immune responses of both cells and the adaptive as well as innate arms of immunity. In agreement with the experimental results, a statistically significant association between clinical responses and the presence of activated NK cells postvaccination has been reported in cancer patients who were treated with peptide-loaded, DC-based vaccines (139–141).

Several studies have shown that NK cells can be efficiently activated by DCs to release cytokines, to proliferate, and to enhance their cytotoxicity (136,142,143). Activated NK cells can then kill tumor cells, but, at the same time, play a relevant immunoregulatory role by editing DCs (144) (Figure 2). NK cell-mediated DC editing may occur by eliminating the less immature, allegedly tolerogenic DCs (136,144,145). The relevance of this phenomenon for the generation of an effective tumor-specific adaptive immune response has recently been demonstrated also in vivo (146,147). The decreased cytotoxicity of tumor-infiltrating NK cells could substantially alter DC-NK interactions (Figure 2), where poorly cytotoxic NK cells would not only have reduced capacity to kill tumor cells, but also reduced editing of immature dendritic cells (2). In the tumor microenvironment, NK editing of DC might be compromised, leading to tolerogenic DC (Figure 2), similar to that found in HIV infection, where lack of NK editing leads to increased immature DCs and likely subsequent immune suppression (148).

On the other hand, NK cells can also shape adaptive immune responses by causing DC maturation and influencing the polarization of primary T-cell responses (149–152). This helper role of NK cells for the development of adaptive immunity takes place via the release of soluble factors, such as TNFα and IFNγ, (138,143,153). These cytokines are released following NK recognition of target tumor cells and represent an alternative mode for DC activation apart from direct recognition of pathogen constituents, particularly relevant in the tumor microenvironment, where there are no signals from pathogens and other danger signs, thus impairing proper DC maturation. Taken together, these data show that NK cells are not only activated by DCs but, in turn, can also influence the emerging adaptive immune responses that are initiated by these antigen-presenting cells (Figure 2). This reciprocal activation of NK cells and DCs provides a strong rationale for the combined use of NK cells and DCs in cancer immunotherapy.

Implications for Novel Strategies of Cancer Immunotherapy

In view of the most recent knowledge on NK-DC liaisons, it is possible to envisage use of adoptive immunotherapy with NK cells (Figure 3) for obtaining also an optimal DC maturation (154,155). At the same time, DC-based vaccines should be reconsidered with the aim of activating and recruiting NK cells. In this regard, it is noteworthy that vaccination with unpulsed exogenous DCs (ie, DCs carrying no tumor antigens) has been shown to induce tumor-specific T cells in a tumor-bearing host via the activation of NK cells. In this cellular network, cytokines released by activated NK cells cause the maturation of endogenous DCs (Figure 2) that can eventually present tumor antigens derived by NK cell-mediated tumor cell lysis (142,156). NK cell interactions with antigen-presenting cells need to be considered in future immunotherapies of cancer, aiming to activate NK cells not only for their direct cytotoxic potential but also for enhancing the effectiveness of antitumor T-cell response, including tumor-specific T-cell memory, as required in cancer vaccine approaches (Figure 3).

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Acknowledgments

This work was supported by the AIRC (Associazione Italiana per la Ricerca sul Cancro) (IG10228 to AA and IG11650 to GF); the Italian Ministry of Health Grande Progetto Strategico; by the Ministero dell'Istruzione dell'Università e della Ricerca PRIN (Programmi di Ricerca Scientifica di Rilevante Interesse Nazionale) 2010NECHBX_003; and by the P.O. FESR 2007–2013 - linea d'intervento 4.1.2.A and Fondazione MultiMedica Onlus. AB is a FIRC (Fondazione Italiana per la Ricerca sul Cancro) fellow. We thank Paola Corradino for data management and bibliography and Alessandra Panvini for assistance.

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