

## The role of natural killer cells in pulmonary immunosurveillance

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### 1. ABSTRACT

Natural killer (NK) cells were originally identified as lymphocytes capable of killing cancer cells without prior sensitization (1). Further characterization of these cells in both humans and rodent models has expanded their role towards a broad-based immunosurveillance of diseased and healthy peripheral tissues. Among peripheral organs, the lung contains the largest percentage of NK cells. Accordingly, NK cells are implicated in many immunological responses within the lung, including innate effector functions as well as initiation of the adaptive immune response. In this article, we review the characteristics of NK cells, current models of NK maturation and cell activation, migration of NKs to the lung, and effector functions of NKs in cancer and infection in the airways. Specific emphasis is placed on the functional significance of NKs in cancer immunosurveillance. Therapeutic modulation of NK cells appears to be a challenging but promising approach to limit cancer, inflammation, and infection in the lung.

### 2. INTRODUCTION: FUNCTIONAL AND MOLECULAR CHARACTERIZATION OF NK CELLS

Natural killer cells were originally described in 1975 as lymphocytes capable of spontaneously and specifically killing tumor targets (1-3). Kiessling and colleagues showed that in the mouse there exists a population of cells that is able to spontaneously kill Moloney leukemia cells. Using the methods of characterization available in 1975, the investigators further defined them as lymphocytes, based upon morphology, but could purify and separate them from T and B lymphocyte populations. Furthermore their development did not require the thymus, which is a critical site of T lymphocyte development (1). Subsequently, a similar population of cells was isolated from human peripheral blood (3). Today, it is clear that the range of target cells extends to some infected and/or stressed cells, and NK cells are more generously defined as large granular lymphocytes with an intrinsic capacity to kill a wide variety of target cells

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without prior sensitization (4). A critical element to their proper function is the ability of NK cells to distinguish pathologic from healthy cells. Target cell recognition involves a complex interplay of multiple receptor-ligand interactions with activating, inhibiting, co-stimulatory, and adhesion functions. NK function and activation is determined by the sum of activating and inhibitory receptors engaged during an encounter with a target cell. For each encounter, the NK cell can mediate several effector functions, including exocytosis of cytotoxic granules containing perforin and granzyme B and synthesis of cytokines such as interferon gamma (IFN  $\gamma$ ), tumor necrosis factor alpha (TNF  $\alpha$ ), and granulocyte-macrophage colony stimulating factor (GM-CSF) (4-6).

Despite major scientific advances in the 40 years since the initial identification of this cell population, a complete molecular definition of NK cells remains elusive, and the NK cell is still identified predominately by its natural killing function (4). This intrinsic killing function is independent of prior exposure to the target cell, thus defining the NK cell population as a separate entity from cytolytic T lymphocytes. NK cells share some developmental requirements with other lymphocytes, such as a requirement for transcription programs mediated by Ikaros, PU.1, Ets-1, and Runx (4, 7), but previous research using genetically deficient mouse models indicates that it is possible to reduce T and B lymphocyte function without affecting NK cell activity and vice versa. For example, athymic *nu/nu* (NUDE) mice have stunted T lymphocyte development, and transgenic *Rag1*<sup>-/-</sup> and *Rag2*<sup>-/-</sup> mice have stunted T, B, and NKT lymphocyte development, but these mutations have no detected effect on NK cell development (8). In contrast, mice deficient in the common gamma chain ( $\gamma$ ) subunit of many cytokine receptors, including IL-2 and IL-15, lack NK cells but produce small numbers of T lymphocytes and normal numbers of B lymphocytes (9, 10). Several transcription factors that drive NK cell development from common lymphocyte precursors have been identified. For example, E4bp4 is expressed in developing and mature NK cells, but not B or T cells. E4bp4-deficient mice have normal numbers of B and T cells but development of mature NK cells from bone marrow precursors is arrested at an NK precursor stage (7). Similarly, mice deficient in the transcription factors MEF, Id2, or IRF-2 have a reduced number of NK cells but normal numbers of B and T cells. The importance of Id2 has been confirmed in developing human NK cells (7). Using another transgenic mouse model, deficiency of T-bet was shown to prevent NK cell development by blocking the migration of NK cells from the bone marrow to peripheral tissues. Migratory defects are also apparent in mice with reduced expression of Eomes, CBF  $\beta$ , and Gata-3 (7). These mouse models demonstrate that although NK cells share many developmental requirements with other lymphocyte populations, they can be identified as a unique population of cells with distinct transcriptional programs. On a cellular level, NK cells are distinguished from T lymphocytes based upon the absence of the T-cell receptor (surface CD3 expression) in combination with the presence of surface receptors NK1.1 (in C57BL/6 strain

only), NKp46, or DX5 in mice, or CD16 (Fc RIII) and CD56 in humans. More recently, NK cells have been classified into a family of innate lymphoid cells (ILCs). ILCs are defined as lymphocytes which lack rearranged antigen receptors. NK cells are distinguished from other subsets of ILCs, namely Ror t<sup>+</sup> and type 2 ILCs, by both their stimulating cytokines, including IL-18, IL-2, IL-12, and IL-15, and their production of IFN  $\gamma$  (11, 12).

### 3. NK CELL ACTIVATION

As a means of immunosurveillance, NK cells temporarily synapse with other cells which allows for the engagement of surface NK receptors with the ligands present on the surface of the target cell. NK cells simultaneously express multiple receptors that act to inhibit or activate their effector functions. The unique combination of receptor expression and receptor-ligand interaction dictates the overall cellular activity. NK cell activation must be precisely controlled since NK cells have a strong cytotoxic capacity and the potential for deleterious self-reactivity. Thus, the molecular identification of NK cell surface receptors and their function has been extensively studied.

#### 3.1. Human KIRs and LIRs and their functional counterparts in mice

Most NK cell receptors can be grouped into just a few gene families based on structural and functional similarity and location within the genome. Specifically the leukocyte receptor complex (LCR), encoded on chromosome 19 in man encodes multiple receptors belonging to the immunoglobulin superfamily. These receptors are expressed broadly on a wide variety of haematopoietic cells. Another set of genes critical to NK function is encoded in the natural killer gene complex (NKC) located on chromosome 12 in man and 6 in the mouse. These receptors are expressed primarily on NK cells but can also be detected on other subsets of cells with lytic capacity, such as activated CD8<sup>+</sup> cytotoxic T lymphocytes (13). Genes encoded in these regions are members of several gene families including killer immunoglobulin-like receptors (KIRs), leukocyte immunoglobulin-like receptors (LIRs), and C-type lectin-like family of receptors. KIR and LIR family genes encode receptors with extracellular immunoglobulin domains that bind to class I major histocompatibility complex (MHC I) molecules and either short or long cytoplasmic tails which determine if the receptor inhibits or potentiates NK cell activation. Proteins with a long cytoplasmic tail have an immunoreceptor tyrosine-based inhibition motif (ITIM) and are associated with inhibitory signaling. In contrast, a short cytoplasmic tail mediates activation of NK cells and is either dependent or independent of an immunoreceptor tyrosine-based activation motif (ITAM) (14). In mice, through a convergent evolution process, the Ly49 gene family located in the NKC has evolved functions similar to those of human KIRs and LIRs located in the LCR. Ly49 genes encode type II lectin-like molecules as opposed to their functional counterparts in humans that are immunoglobulin-like receptors. However, unlike most lectin or lectin-like receptors which bind carbohydrate

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**Table 1.** NK receptor gene families in human and mouse genomes

Gene Family	Human	Mouse	Function
KIRs	15 genes clustered in LRC on Chr. 19	2 genes on Chr. X	Ig-like receptor family members 9 inhibitory, 6 activating receptors in humans
LIRs	12 genes clustered in LRC on Chr. 19	none known	Ig-like receptor family members 5 inhibitory, 7 activating receptors in humans
Ly49	Pseudogene on Chr. 12	15 genes (C57BL/6 mice) clustered in NKC locus on Chr. 6	Type II lectin-like molecules 9 inhibitory, 2 activating receptors in mice
KLR (eg. CD94) and NKG2 families	4 genes in NKC region of Chr. 12	4 genes clustered in NKC locus on Chr. 6	C-type lectin-like receptors 4 inhibitory receptors in humans and mice

KIRs, killer immunoglobulin-like receptors; LIRs, Leukocyte Ig-like receptors; KLR, killer cell lectin-like receptor; LRC, leukocyte receptor complex; NKC, natural killer gene complex

oieties in a calcium-dependent manner, the Ly49 molecules recognize an epitope of MHC I in a carbohydrate-independent manner (14). The Ly49 gene family is comprised of 15 genes in the C57BL/6 strain of mice but demonstrates extreme quantitative and qualitative polymorphisms in other strains (15). As with KIRs and LIRs in humans, the Ly49 receptor family in mice is comprised of both inhibitory and activating receptors (14). Genes belonging to two other families, killer cell lectin-like receptor (KLR) and NKG2 are also present within the NKC locus in mice and a similar region of chromosome 12 in humans. These family members are also C type lectin-like receptors that bind MHC I and mediate either inhibitory or activating signaling pathways within the NK cell (Table 1) (14).

### 3.2. Detection of MHC I and the licensing hypothesis

Detection of class I major histocompatibility complex (MHC I) molecules by NK cell receptors is a key component of NK cell function. MHC I proteins are critical mediators of the initiation of the adaptive immune response, as they present molecular moieties from intracellular pathogens or transformed cells to cytolytic T cells (CTLs). CTLs kill the infected or transformed target cell. Therefore, infected or malignant cells undergo selection towards the downregulation of MHC I antigens due to the destruction of MHC I<sup>+</sup> cells. Through what was likely to be a co-evolutionary process, host NK cells have developed the capability of detecting expression of the MHC I molecule itself, rather than the antigen presented by the MHC I protein. In a homeostatic state, cells express autologous MHC I molecules on their surface at normal levels that inhibit NK cell-mediated lysis of healthy host cells. Since ligation of MHC I with NK receptors suppresses NK cell function, reduced MHC I expression relieves the inhibitory signaling of NK cells, and infected cells become vulnerable to NK cell mediated killing. Thus, opposing functions by T cells and NK cells provide a coordinated system of immunosurveillance. This two-pronged approach by the immune system to detect antigens presented by MHC I and MHC I molecules themselves limits the viability of infected or malignant cells within the host (16).

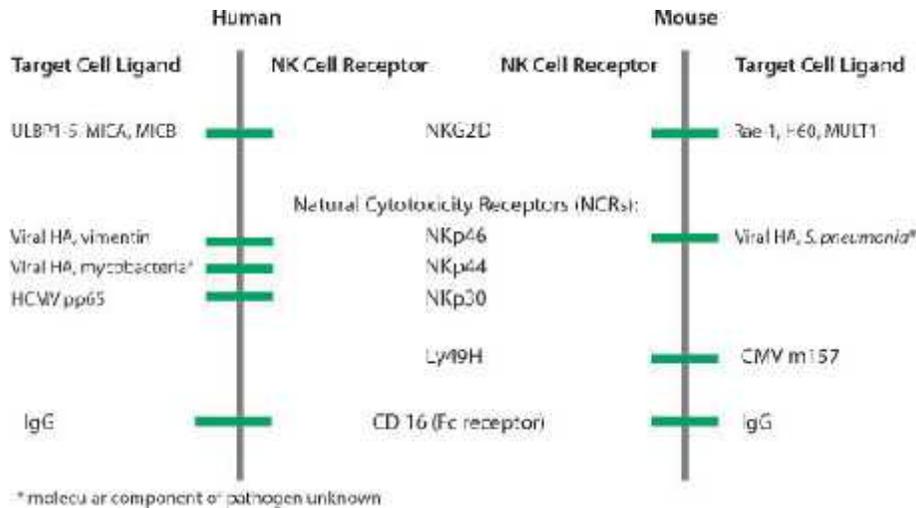
The original concept of NK cell activation proposed that a lack of engagement of MHC I receptors was necessary and sufficient for target cell lysis. The importance of self-MHC expression in the regulation of NK cells was demonstrated by Karre, *et al.* in 1986. Murine

lymphoma cells with a loss of H-2 expression were less malignant than WT cells, suggesting that an immunological defense system was aimed at detecting and eliminating tumor cells with deleted or reduced expression of self-MHC molecules (17). In another seminal study, a 2-microglobulin knock-in transgene was used to restore H-2 expression in an H-2 deficient lymphoma cell line, A.H.2<sup>7</sup> YAC-1. Concomitant with induced expression of MHC I molecules, the transgenic cancer cells were protected from NK cell-mediated lysis (18). The interaction of MHC I and inhibitory receptors fueled a concept referred to as the “missing-self hypothesis.” However, NK cell function cannot be fully described by this simplistic model. Mice with a targeted deletion of H-2K and H-2D MHC class I heavy chains or the 2-microglobulin subunit lack surface MHC I expression and MHC I-mediated inhibitory receptor engagement, but, surprisingly, NK cells from these mice are not hyperactive and instead are hyporesponsive to target cell killing (19). This phenomenon fueled the “licensing hypothesis,” which states that some MHC I expression is required for NK cell activation. Inhibitory receptor engagement with self-specific MHC I molecules expressed by autologous cells will permit, or license, the NK cell to respond during an encounter with a target cell (20). Recent studies suggest that the level of MHC I expression on the target cell influences the responsiveness of NK cells to activating signals on the target cell. Detection of high levels of MHC I, or paradoxically, no detection of self-specific MHC I molecules turns down the reactivity of NK cells, while a normal or low level of MHC I promotes activation of cytolytic function, even in response to weaker activation signals (21). In summary, both the downregulation of MHC class I inhibitory signals and the presence of activating signals influence the function of NK cells.

### 3.3. Activating receptors bind stress ligands

In contrast to inhibitory receptors that suppress NK cell function, activating receptors stimulate effector responses. Activating receptors engage ligands on target cells that are either derived from an intracellular pathogen or endogenous ligands that are overexpressed in malignant or stressed cells. NKG2D and NKp46 in both humans and mice, and NKp44 and NKp30 in humans, have defined ligands of endogenous or viral origin (Figure 1). Engagement of these receptors activates cytotoxic responses directed at the cell expressing the ligand, i.e. the target cell. Members of another gene family in mouse, NKPR, encode lectin-like molecules and appear to function

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**Figure 1.** Pathogen and stress-induced ligands bind to activating NK cell receptors. Activating ligands, described to date, are glycoproteins that are derived from either the pathogen itself or from the host cell upon infection, malignant transformation, or cellular stress. UL16 binding protein 1-5 (ULBP1-5) and MHC class I-like polypeptide-related sequences MICA and MICB in humans and RAE-1 family members, H60, and MULT1 in mice are endogenous stress-induced ligands that bind to NKG2D. Pathogen-derived components including influenza hemagglutinin (HA), *Mycobacterium tuberculosis* vimentin, and human cytomegalovirus (HCMV) pp65 in humans and HA in mice are recognized by natural cytotoxicity receptors (NCRs). Cytomegalovirus (CMV) m157 binds to the Ly49H expressed on mouse NK cells.

as activating receptors. The human genome contains at least one known homology, NKR-P1A. Their ligands are not well-defined, but these receptors are believed to recognize self-molecules (22). NK cells are also capable of detecting cells against which a humoral antibody response has been elicited. CD16 (Fc receptor) can bind the Fc portion of antibodies and lyse cells in a phenomenon termed antibody-dependent cellular cytotoxicity (ADCC). Receptors that modulate NK cell function by acting as co-stimulatory receptors or adhesion receptors have been described, including CD2, CD28, and Nectin (23, 24). Continued research to identify more ligands expressed by target cells will undoubtedly further our understanding of NK cell activation.

### 3.4. Priming of NK cells

While the effector mechanisms of NK cells are ultimately dependent upon surface receptor engagement with pathogen and/or stress-induced ligands, other components of the immune system, such as cytokines, also affect NK cell activity. Cytokines including interleukins 2, 12, 15, and 18, and type I interferons modulate the activity of NK cells (6, 25, 26). *In vitro* exposure of human or mouse NK cells to IL-15 or IL-2 “primes” NK cells. Priming can be depicted as NK cells having a lower threshold of activation. Activated NK cells display increased sensitivity to target cells and kill a broader range of target cells (5, 27). Furthermore, recombinant IL-15 and IL-2, which both bind to the IL-2 receptor of NK cells, can induce proliferation of both human and mouse NK cells *in vitro* and *in vivo* (5, 26). Longer, overnight exposure (13 – 15h) to IL-12 and low-dose IL-15 has also been shown to induce a memory NK cell phenotype, even in daughter cells, following transfer of memory cells into syngeneic mouse recipients. These memory cells show increased reactivity to tumor cell

targets, cytokine exposure, and stimulation by antibodies directed against NK cell receptors (28). Prolonged exposure to IL-2 for 5 days leads to a phenotypic change of NK cells into a new cell type referred to as lymphocyte activated killer cells (LAKs) (29). NK cells also express Toll-like receptors, including TLRs 2, 3, 4, 7, and 8 (30), and accordingly, polyI:C is commonly used in experimental systems to prime NK cells (31). However, other studies indicate that the effect of TLR agonists to prime NK cells may be an indirect effect mediated through Type I IFN released by accessory cells such as dendritic cells and macrophages (32, 33). More experimental work will help to dissect the importance of accessory cells to priming of NK cells upon exposure to TLR agonists.

## 4. MECHANISMS OF EFFECTOR FUNCTIONS

Activation of NK cells leads to several effector mechanisms, including: 1) release of cytotoxic granules that lyse target cells, 2) upregulation of death receptor ligand expression and the engagement of cognate death receptors on target cells, which can lead to apoptosis of target cells, 3) release of chemokines and cytokines that promote recruitment and activation of NKs and other immune cells, and 4) release of other soluble mediators, such as PGE<sub>2</sub>, which shape responses of the immune system. Effector functions will be described in further detail later in this review in the context of immunosurveillance in the lung.

## 5. GENETIC INFLUENCES ON NK CELL FUNCTION IN MICE AND MAN

Unlike the T and B cell receptors of the adaptive immune system, which undergo somatic cell gene

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rearrangement, NK receptor diversity is dictated solely by inheritance through the germ line. A link between genetic inheritance and NK cell function has been demonstrated in family studies. In one study, for example, two male siblings almost completely lacked natural killer activity against human melanoma target cells. The functional defect is likely a result of a common genetic mutation(s), because stimulation with IL-2 or IFN failed to rescue NK cell mediated killing in both siblings (34). Furthermore, analysis of the human genome was used to identify two groups of KIR haplotypes within the human population. In haplotype A, several inhibitory but only one activating KIR gene is present. In haplotype B groups, several activating and inhibitory KIRs are encoded in the germ-line. Thus, an individual homozygous for the A haplotype is likely to have hyporesponsive NK cells in comparison to an individual homozygous for the B haplotype (35). The clinical implications of such polymorphisms are unknown. The chromosomal regions encoding the NK cell receptor families contain polymorphisms reflected in allelic variation and gene copy number in humans as well as mouse models. Polymorphisms in the NKC locus in mice are being used to elucidate the complex interplay between proteins encoded within the gene-rich NKC locus. Among different inbred mouse strains, different haplotypes of the NCR emerge, and to a large extent, these correspond to functional differences in NK cells (15). An additional level of regulation occurs during gene processing. An alternatively-splice isoform of NKp30, NKp30c, which has immunosuppressive function, is more prevalent in patients with gastrointestinal sarcoma compared to healthy controls. Among patients, its expression level relative to NKp30a and NKp30b is associated with clinical outcome (36). Future studies to evaluate how genetic polymorphisms influence NK cell receptor expression on an individual cell basis will advance our understanding of how NK cell receptors work synergistically to promote and tailor NK cell responses to be appropriate to the pathological stimuli.

### 6. MATURATION, MIGRATION, PERIPHERAL SEEDING, AND THE INFLUENCE OF THE ENVIRONMENTAL MILIEU ON NK CELLS

The development, maturation, and activation potential of subsets of NK cells continues to be unraveled. NK cells emerge from hematopoietic stem cells that differentiate to common lymphoid progenitor cells, then to bipotential T/NK progenitor cells, and finally to lineage-committed NK precursor cells. Subsequent maturation of NK precursor cells occurs in the bone marrow and can be distinguished by ordered acquisition of NK cell receptors involved in the activation or inhibition of NK cell effector functions (4). In both mice and humans, the bone marrow microenvironment is critical for the generation of at least the majority NK cells *in vivo*, as bone marrow ablation or congenital bone marrow defects lead to an abnormal or absent NK cell population (27, 37, 38). However, an infrequent subset of NK cells with a distinct transcriptional program and NK receptor profile was shown to develop in the mouse thymus (39). Analogously, in humans, production of a distinct subset of NKs by lymph nodes during a stress response has been described (40). Recently,

another investigator used a flow cytometry-based approach to identify seven maturation stages of NK cells in humans, and cells in the first two stages were detected in only the bone marrow (41). Thus, while traditional NK cell development almost always depends on the bone marrow environment, alternative sites of NK cell development do occur under certain inflammatory states. The production of NK cells is an ongoing process, as turnover of NK cells occurs in about two weeks in both humans and mice (42, 43).

NK cells are present in peripheral organs, even in the absence of inflammation, compatible with their established role in surveillance for infected, malignant, stressed, or damaged cells. The frequency of NK cells is much higher in peripheral organs such as the lung and liver compared to blood or the central immune organs, including the spleen, bone marrow, lymph nodes, and thymus of untreated mice (44). NK cells comprise about 10% of the lymphocytes in the lungs of mice (6), which is consistent with their important role in pulmonary immune responses. More recent studies in mice and humans have identified NK cells in the gut, skin, uterus, pancreas, joints, and brain (45).

As evidence emerges that there are NK cell subsets with different surface receptor expression and functional capacities, it is becoming more apparent that the function of NK cells is influenced by their maturity and environmental milieu. The immune profile varies between different organs and influences the local immune response. Soluble mediators released from DCs, macrophages, and T cells, and cell-cell contact between these cells and NKs influences the effector function(s) of NK cells (44). Subsets of NK cells differ in their natural killing potential and cytokine production. For example, the surface antigen CD56 is an isoform of neural-cell adhesion molecule (NCAM) and has unknown function in NK cells. However, due to initial hypotheses that it mediated cell-cell interactions with target cells, CD56 has been traditionally used and can successfully differentiate subsets of human NK cells. Specifically CD56<sup>dim</sup> NK cells release cytotoxic granules, whereas CD56<sup>bright</sup> NK cells mediate an effector function predominantly characterized by cytokine production (5). It is unclear if these subsets reflect different states of maturation or if there is bidirectional transition between these subsets which is controlled by the environmental milieu. Such experimental questions are inherently difficult to answer in man. In mice, however, the maturation state of NK cells can be defined by the co-expression of surface CD27 and CD11b. CD27 is a member of the TNF receptor superfamily, and engagement with its ligand, CD70, induces proliferation and production of IFN- by NK cells (46). CD11b (Mac-1/CD18) is a 2 integrin that has been implicated in development, differentiation, and survival of NK cells (47). Hayakawa and Smyth found that CD11b<sup>high</sup> NK cells can be separated into two subsets with categorical tissue distribution and activation thresholds, based upon their expression of CD27 (48). Chiossone, *et al.* proposed a model of murine NK cell maturation with four sequential stages: CD11b<sup>low</sup>CD27<sup>low</sup>, CD11b<sup>low</sup>CD27<sup>high</sup>, CD11b<sup>high</sup>CD27<sup>high</sup>, and CD11b<sup>high</sup>CD27<sup>low</sup>. The stages of developmental were associated with progressive acquisition of effector

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functions. CD27+CD11b+ NK cells seem to parallel CD56<sup>bright</sup> human NK cells which produce high levels of IFN  $\gamma$ , and CD27+CD11b- are more similar to CD56<sup>dim</sup> NK cells which have high cytolytic function (49). Further studies using mouse models will help to clarify how the local environment influences the development and differentiation of NK cell subsets with unique effector functions.

### 7. THE ROLE OF NK CELLS IN PULMONARY IMMUNOSURVEILLANCE

The lungs represent a unique immunological environment, as their constant exposure to a vast array of exogenous particles requires precise mechanisms to distinguish the harmful from innocuous stimuli. Tight control over the immune response is necessary to both eliminate harmful materials and prevent unnecessary inflammation and damage to normal pulmonary tissue, which may compromise the normal function of the lung (50, 51). NK cells account for 10% of the resident lymphocytes in the lung, suggesting that they may play a role in immunosurveillance for pathogens or transformed cells. Additional NK cells are recruited just hours after the induction of inflammation. In the following sections, we discuss the importance of NK cells in the immunosurveillance of cancer and the response to infection in the lungs. Many parallels are seen in the mechanisms used by NK cells to increase the organism's resistance to tumors and infections (14), and an understanding of their role across multiple immune challenges will be useful for developing therapeutics aimed at modulating NK cell functions.

#### 7.1. Primary lung cancer

Pulmonary malignancies are the leading cause of cancer-related deaths, and the second most frequent type of newly diagnosed cancer in both men and women (52). Unlike other malignancies, such as colorectal cancer, the number of newly diagnosed cases of lung cancer has nearly paralleled the number of lung cancer-related deaths since 1975 (52), which reflects the inadequacy of current treatment options. Thus, new therapeutic strategies to complement surgical resection, irradiation, and chemotherapy are necessary. Modulation of the immune system may be a promising future therapeutic option for lung cancer.

Immunotherapy is already a promising treatment option for certain types of malignancies, such as melanoma, renal cell carcinoma, and ovarian cancer (53). For example, ipilimumab, which is an antibody that block human cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), increased antitumor T-cell response, and in phase 3 clinical trials, improved the overall survival of patients previously treated for metastatic melanoma (54). For patients with metastatic renal cell carcinoma a successful therapy for those with widely metastatic disease involves systemic administration of high-dose IL-2. Although its side effects can be severe a complete and durable pathologic response can be evident in a fraction of patients (55). In a pilot study, the poxviral-based vaccine

PANVAC was beneficial to some patients with metastatic breast or ovarian cancer. PANVAC contains transgenes that encode 2 tumor-associated antigens, MUC-1 and CEA, and 3 T-cell costimulatory molecules (56).

The immune system can eliminate transformed cells that express a number of unique surface antigens. These include tumor-associated antigens, which represent mutated or ectopically-expressed proteins that are recognized by T lymphocytes, or stress-associated antigens recognized by cells of the innate immune system (56, 57). Supporting this idea, referred to as "tumor immunosurveillance," previous studies demonstrate that mice with complete or partial deficiencies in certain immune effector pathways have an earlier onset and increased penetrance of cancer (57, 58). Immunosurveillance is a combined effort from both the innate and adaptive immune systems and NK cells, NKT cells, DCs, and macrophages have all been implicated in the detection and elimination of tumor cells during the initial immune response to developing tumors (57, 59). Tumor-antigen specific T cells mediate a second and adaptive immune response that leads to further elimination of tumor cells. The majority of studies to define the contribution of individual immune components to tumor resistance have used models of carcinogen-induced fibrosarcoma (8), so the mechanisms of immunosurveillance for other malignancies, including lung cancer, are less clear.

Immunosurveillance of the human lung is evidenced by an increased risk of lung cancer in immunocompromised patients. Serraino, P., *et al.* found that the number of observed cases of lung cancer was greater than the number expected, when patients were immunocompromised due to either human immunodeficiency virus (HIV) or therapeutic immunosuppression following an organ transplantation (60). Surprisingly, previous studies using a primary lung carcinogenesis murine model seem to indicate that the adaptive immune system does not significantly contribute to immunosurveillance for lung cancer. For example, in a urethane-induced model of lung cancer, the latency period, penetrance, and total number of tumors was similar between athymic nude (nu/nu) mice, which lack a thymus and thus thymically-derived T cells (61, 62), and euthymic (nu/+) littermate controls (63). Since the thymus is involved in the development of the majority of T cells (61, 62), these studies suggest that T cells do not significantly contribute to tumor immunosurveillance in the lung. Furthermore, nu/nu and nu/+ mice responded similarly to ionizing radiation treatment of urethane-induced lung cancer (64).

NK cells are implicated in immunosurveillance for tumor cells within the lung. A clear correlation between NK cell activity in the lung and tumor cell clearance from the lung has been reported in both mouse and human studies (35, 59, 65). NK cells activated *ex vivo* with IL-2 and adoptively transferred by systemic *i.v.* injection to allogeneic mice were able to localize to the lungs of recipient mice, infiltrate tumors, and significantly

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reduce tumor size (65). Similarly, localization of NK cells to the lung tumor microenvironment has been visualized in human patients with non-small cell lung cancer (NSCLC). Using an antibody to Nkp46, a common marker used to identify NK cell populations, NK cells were detected at the invasive margin of NSCLC tumor samples (59). Interestingly, in another study, the NK cell populations that were found infiltrating NSCLC tumors were CD56<sup>bright</sup>, which is the NK cell subset associated with cytokine production rather than cytotoxic function (35). At the molecular level, the expression of activation markers on NK cells is significantly different in patients with non-small cell lung cancer (NSCLC) or small cell lung cancer (SCLC) compared to healthy donors (35, 59). In one study, the percentage of peripheral blood NK cells expressing the activating receptor Nkp46 was significantly reduced in patients with NSCLC or SCLC compared to healthy donors (35). Additionally, the percentage of Nkp46+ cells was lower in patients with stage IV disease than in patients with stage III disease, and mean expression levels of Nkp46 were reduced in correlation with advancing stage (35). This recent study extended beyond NK cell receptor expression to look at NK cell function as well. PBMCs harvested from lung cancer patients and healthy controls were tested *ex vivo* for their ability to kill tumor target cells and produce IFN  $\gamma$ . PBMCs of NSCLC and SCLC patients had a reduced ability to kill K562 myelogenous leukemia cells in a standard <sup>51</sup>Cr release assay that is used to detect cancer cell death. Additionally, the percentage of NK cells stimulated to produce IFN  $\gamma$ , by *ex vivo* treatment with IL-12 or IL-18, was reduced in PBMC samples from lung cancer patients compared to healthy donors. Similarly, the expression of several NK receptors was altered for tumor-infiltrating NK cells compared to peripheral blood NK cells from the same patient. The overall pattern of receptor expression suggests that the function of tumor-infiltrating NK cells is reduced compared to peripheral blood NK cells. Indeed, CD107a and IFN  $\gamma$  assays showed reduced function as expected (59). Together, these data suggest that NK cell effector functions, including cell-specific lysis and cytokine production, can at least partially dictate the detection of tumors, the rate of growth, and the metastatic potential of lung cancer in humans. Cancer progression, therefore, may depend on local impairment of NK cell activity.

Environmental and genetic components may influence the incidence and outcome of lung cancer. Human population studies indicate that smoking is an independent risk factor for cancer (66). Interestingly, smoking can reduce the natural killing activity of human peripheral blood leukocytes. NK cells of patients who smoked had a reduced ability to kill MM200 melanoma target cells (67). Melanoma cancer cells are typically used in these studies, so further studies are necessary to determine if the effect of smoking on NK cells also inhibits their ability to kill lung cancer cells. Previous research has identified eleven genes associated with increased risk for lung cancer, and the majority of patients have mutations in epidermal growth factor receptor (EGFR) or v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (KRAS) (68). However, to date, none of these genes appear to be especially important in NK cell function or even immune

system function at all. Further studies are necessary to clarify whether or not genetic risk factors are consistent between different types of lung cancer and how they are influenced by environmental risk factors. It will also be enlightening to identify how various cell types, including NK cells, are affected by these environmental and genetic risk factors.

### 7.2. Metastatic cancer to the lung

The number of studies using a primary model of carcinogenesis to evaluate immune pathways involved in the control of lung cancer is limited. However, some information can be gleaned from studies using tumors metastatic to the lung. In mice, for example, *in vivo* exposure to cigarette smoke increases the number of metastatic melanoma tumors in the lung (69). The contributions of the adaptive immune system and of NK cells in cancer immunosurveillance were tested using *Rag2*<sup>-/-</sup> and *Rag2*<sup>-/-</sup> *c*<sup>-/-</sup> mice in this model. *Rag2*<sup>-/-</sup> mice had low tumor burdens in mice that were not exposed to cigarette smoke, and smoking exacerbated the tumor burden, indicating that even in the absence of T and B lymphocytes, smoking can further impair lung immunosurveillance mechanisms. *Rag2*<sup>-/-</sup> *c*<sup>-/-</sup> mice displayed high tumor burdens, even without exposure to cigarette smoke, suggesting that NK cells limit the number of metastatic melanoma lung tumors. Adoptive transfer experiments were used to further investigate the effect of cigarette smoke on NK cell function. Donors were exposed to cigarette smoke or sham, and isolated NK cells were transferred to recipients challenged concurrently with melanoma. Tumor burden was measured in the recipients and was higher in recipients receiving NK cells from cigarette smoke-exposed donors compared to sham-exposed donors (69). Whether or not the effect of smoking on immunosurveillance mechanisms is similar in primary lung cancer is unclear and warrants further investigation. Previous studies indicate that the cellular components involved in tumor immunosurveillance are partially dependent upon the origin of the tumor, the local tumor environment, the cause of malignant transformation, and the aggressiveness of the tumor (57). The efficacy of immunomodulation in the lung is likely to be complicated by the strong tolerogenic environment of the lung (70). Further studies utilizing mouse models of primary lung cancer and analysis of tumor samples collected from lung cancer patients will be a crucial step in understanding how immunosurveillance mechanisms differ between pulmonary malignancies and those, such as fibrosarcoma, that arise in other systemic sites.

### 7.3. The role of NK cells in pulmonary infection

While much of our knowledge regarding the physiology of NK cells stems from tumor biology, further work has identified the role of this cell population in a multitude of pulmonary inflammatory responses. In the next sections, the contribution of NK cells to immune function in the lung following certain viral and bacterial infections, including tuberculosis (TB), will be discussed. Some elements of NK cell responses are conserved following exposure to multiple types of infection. Other

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activities which differ may be the result of a tailored response to the pathogen, or alternatively, may be the consequence of functional impairment induced by the pathogen.

### 7.3.1. Viral infections

As previously mentioned, NK cells likely evolved into an independent cell population due to their role in viral clearance. Individuals with NK cells have a selective advantage in the detection of viruses which are capable of reducing MHC I expression to avoid detection by T cells (16). Thus, patients with genetic deficiencies that lead to the loss of NK cell function suffer from recurrent viral infections (71, 72). In 2006, a novel primary human immunodeficiency, predominantly characterized by an NK cell deficit, was identified in four children belonging to a large Irish, nomadic, inbred population. One child developed lymphoproliferative disorder due to lytic activation of the Epstein-Barr virus. Two other children suffered from severe respiratory illnesses likely caused by viruses. A genome-wide scan, guided by the assumption that the emergence of this disorder in an inbred population represents recessive inheritance of the underlying genetic defect, led to the identification of a 12 Mb region on chromosome 8p11.23-q11.21 that was not previously associated with NK cell development or function. Further fine mapping and molecular studies were used to identify the underlying genetic cause to be a hypomorphic variant of minichromosome maintenance-deficient 4 (MCM4) (73, 74). These studies provide substantial support for the hypothesis that genetic components are involved in NK cell-mediated resistance to multiple pathologies, including viral infections (75). While the effect of NK cells to control some viruses, such as lymphocytic choriomeningitis virus (LCMV), appears to be at least partially due to modulation of T cell function by NK cells (76), there is evidence to suggest that influenza viral burden may be limited by a direct effect of NK cells.

Infection by influenza viruses are a current medical burden and also present a serious future pandemic risk. NK cells are recruited within the first few days after influenza virus infection (77) and display increased killing activity and IFN production at the site of infection (78). Using *in vivo* mouse and hamster models, NK cell depletion with anti-asialo GM1 caused increased morbidity and mortality after influenza infection (79). These physiological observations are supported by molecular studies which indicate that haemagglutinins (HAs) present on virus-infected cells are recognized by the activating NK receptors NKp46 and NKp44 in humans and NKp46 in mice (80, 81). Furthermore, expression level of HAs by different influenza viruses is likely to affect the potential for NK cell activation. In addition to direct NCR-mediated detection of infected cells, NK cells also respond to stress signals from influenza-infected monocytes and dendritic cells. The latter effect is through both a NKG2D-mediated contact-dependent mechanism as well as through a response to cytokines such as IFN and IL-12, which are released from infected cells. Several effector functions of NK cells have been shown to limit influenza infection, including target cell killing, IFN production, and ADCC (6).

### 7.3.2. Bacterial infections

Traditionally, NK cells are thought to contribute to tumor and virus resistance, but recent work has identified functional roles during bacterial infections as well. *Streptococcus pneumoniae* can survive in the human respiratory tract as part of the commensal flora, however, it is also responsible of severe infections including pneumoniae, meningitis, and sepsis. It is the leading cause of death of children in the world (82). While the role of NK cells in bacterial infection is less well defined than in viral infections, NK cells are recruited to the lung 6 hours post-infection (hpi), indicating that they may play a role in limiting early bacterial dissemination. Mice with a targeted disruption of NCR1 (NKp46) had higher bacterial loads at 6 hpi. The impairment of viral clearance was likely due to suppressed activation of NK cells and reduced IFN production in the lungs of NCR1-deficient mice. NCR1 was not identified as a direct sensor of *S. pneumoniae*. Instead, macrophages and DCs were shown to mediate the activation of NK cells by *S. pneumoniae* (83). NK cell function is also modulated by pertussis toxin (PT) of *Bordetella pertussis* bacteria, further linking NK cells to bacterial clearance (84).

### 7.3.3. Mycobacterial infection

*Mycobacterium tuberculosis* (TB) is present in one-third of the human population (82). While TB maintains a chronic latent state in most individuals, active disease can become apparent and accounts for nearly 2 million deaths each year (82). Several investigators have demonstrated that TB is associated with altered NK cell function. NKp46 and NKG2D mediate NK cell function by interacting with the stress-related glycoproteins vimentin and ULBP-1 expressed on TB infected monocytes and macrophages (85, 86). Additionally, NKp44 can bind directly to the mycobacterial cell wall through an as of yet undefined ligand. MICA expression is also upregulated in TB-infected cells, suggesting that this ligand may also influence viral pathogenesis through the NKG2D NK cell receptor (6). Interestingly, NK cells from the peripheral blood of patients with active TB have decreased cytotoxic function (87). This perhaps provides evidence that TB influences NK cell responses to cause an active infection. So far, studies using *in vivo* exposure of mice have not corroborated an effect of NK cells on TB burden (6). It is possible that NK cell responses help mediate early innate responses but that redundant mechanisms, likely within the adaptive immune system, compensate for NK cell deficiency during the later stages of TB infection, at least in the mouse.

## 8. CONCLUSION AND PERSPECTIVES

NK cells represent a unique class of immune cells. They are derived from lymphocyte precursors, yet clearly function in an innate capacity. Our current knowledge is based upon studies using human patient samples, predominately NK cells isolated from human peripheral blood, and from studies of wild-type and transgenic mice. While species differences are apparent, the general principles of NK cell biology are conserved

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between mouse and man. Their activation is a rapid process, dictated by a combination of signaling through several germ-line encoded receptors that bind to extracellular ligands. However, it is now apparent that NK cell function is modulated by the local tissue environment. Therefore, we have focused our attention here specifically on NK cell functions within the lung. Modern techniques such as molecular imaging and detection of NK cell receptor expression at the cellular level will undoubtedly further our understanding of the mechanisms regulating NK cell effector functions. Armed with this knowledge, NK cell-based therapeutics may be a promising avenue for the treatment of cancer and inflammation. The short life-span and continuous production of NK cells means that therapeutic strategies could be used to dramatically alter the immune response in a quick and reversible way.

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