Title

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Journal

Current Protocols in Immunology, 110(1)

ISSN

1934-3671

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Publication Date

2015-08-01

DOI

10.1002/0471142735.im1437s110

Peer reviewed

HHS Public Access

Author manuscript

Curr Protoc Immunol. Author manuscript; available in PMC 2016 March 01.

Published in final edited form as:

Curr Protoc Immunol. ; 110: 14.37.1–14.37.14. doi:10.1002/0471142735.im1437s110.

Models to study NK cell biology and possible clinical application

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Abstract

Natural killer (NK) cells are large granular lymphocytes of the innate immune system, responsible for direct targeting and killing of both virally infected and transformed cells. NK cells rapidly recognize and respond to abnormal cells in the absence of prior sensitization due to their wide array of germline-encoded inhibitory and activating receptors, which differs from the receptor diversity found in B and T lymphocytes resulting from the use of recombination-activation gene (RAG) enzymes. Although NK cells have traditionally been described as natural killers that provide a first line of defense prior to the induction of adaptive immunity, a more complex view of NK cells is beginning to emerge indicating they may also function in various immunoregulatory roles and have the capacity to shape adaptive immune responses. With the growing appreciation for the diverse functions of NK cells and recent technological advancements that allow for a more in-depth understanding of NK cell biology, we can now begin to explore new ways to manipulate NK cells to increase their clinical utility. In this overview unit, we introduce the reader to various aspects of NK cell biology by reviewing topics ranging from NK cell diversity and function, mouse models and the roles of NK cells in health and disease, to potential clinical applications.

Keywords

NK cell biology; mouse models; clinical application; natural killer cells

INTRODUCTION

Historical Perspective

In the early 1970s, Cudkowicz and Bennett observed a phenomenon that could not be explained by the current thinking in the field of transplant biology. In their studies, they described a process where parental or allogeneic bone marrow cell (BMC) allografts were spontaneously rejected by lethally irradiated F1 hybrid mice - a phenomenon eventually ascribed to NK cells and termed “hybrid resistance” (Cudkowicz and Bennett, 1971). This form of rejection defied the laws of transplantation, which stated that F1 hybrid offspring expressing all of the major histocompatibility complex (MHC) molecules from both parental

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strains should accept parental BMC as has been observed in solid tissue allografts. Although these earlier pioneering studies played a contributing role in the eventual identification of NK cells, it wasn't until the mid 1970s that NK cells were independently discovered by Kiessling and Herberman and defined as "natural killers" owing to their ability to spontaneously kill tumor cells in a non-MHC restricted manner without prior immunization (Herberman et al., 1975; Kiessling et al., 1975). Although additional studies during this time supported the notion that NK cells were discrete from B and T lymphocytes, the mechanism by which NK cells recognized and eliminated tumor cells remained elusive. A decade later based in part on mouse studies using mice deficient in MHC class I expression, Kärre proposed the "missing self" hypothesis, which stated that NK cells preferentially kill tumor cells with reduced or aberrant expression of MHC class I antigens (Karre et al., 1986). Under steady-state conditions MHC class I is ubiquitously expressed, thus the "missing self hypothesis" simultaneously helped shape predictions for the existence of inhibitory receptors that could modulate NK cell responses and also provided the foundation for later explanations describing the potential mechanisms of NK cell self-tolerance. The hypothesis set forth by Kärre provided a unifying explanation for the earlier observations of hybrid resistance and the findings that NK cells could spontaneously kill tumor cells. As the characterization of the NK receptor systems become more understood, it was clear that this hypothesis needed to be modified as recognition-based activation was occurring which was then modified by suppressive signaling.

Phenotype

NK cells predominantly circulate in the blood, however, they also reside in several lymphoid and non-lymphoid organs such as the spleen, liver, lung, intestine, uterus, and to a lesser extent lymph nodes (Sun and Lanier, 2011). This absence in lymph nodes is particularly striking in the mouse whereas in humans, a significant population of $CD56^{hi}$ NK cells is present. Mature NK cells are morphologically referred to as large granular lymphocytes due to the presence of azurophilic granules and similar appearance to other lymphocytes (T and B cells). Additionally, NK cells are frequently defined by both the presence and absence of phenotypic markers to distinguish them from other immune cell-types (particularly T cells) such as CD3−CD56+CD16+CD122+ (in humans) and CD3−CD122+NK1.1+CD16+ (in C57BL/6 mice) and CD3−CD122+DX5+CD16+ (in mouse strains that are NK1.1-) (Cichocki et al., 2014; Di Santo, 2006).

NK CELL DEVELOPMENT

NK cells primarily develop in the bone marrow, although immature NK cells can also be found in several extramedullary sites, from hematopoietic stem cells that undergo sequential differentiation steps where they gradually become more lineage restricted. Mouse NK cell development involves distinct stages marked by differences in cell surface marker expression and comprised of functionally distinct NK cell subsets (discussed further below). The surface expression of CD122 (IL-2/IL-15Rβ chain) and lack of lineage-specific markers (i.e., CD3, CD19, CD4, CD8, Gr-1, etc.) on precursor NK cells (pNK) marks one of the earliest steps in NK cell commitment. As pNKs gradually mature, they acquire additional receptors seen on mature NK cells. The next stage of development begins with immature

NK (iNK) cell acquisition of NK1.1 (in C57BL/6 mice) and CD94-NKG2 receptors. Expression of Ly49 receptors also begins during this stage, with the eventual expression of DX5 (CD49b) and acquisition of cytolytic and cytokine production characterizing mature NK (mNK) cells (Hesslein and Lanier, 2011).

HUMAN AND MOUSE NK RECEPTORS

As opposed to the other lymphocytes such as T and B cells, NK cells possess several enigmatic functional characteristics; namely, they exert effector functions without prior exposure to antigen and rely on germline-encoded receptors for their activation. One of the major paradoxes of early immunology surrounded this inherent competence of NK cells to lyse target cells and at the same time remain self-tolerant. NK cells possess an extensive repertoire of activating and inhibitory receptors that bind both host- and pathogen-encoded ligands, which serve to modulate their activity (Table 1) (Lanier, 2008). Two hypotheses associated with this modulation of NK cell activity are the “missing self” and “induced self” theories, the former corresponding with decreased inhibitory receptor signaling and the later describing the recognition of stress ligands induced during viral infection or malignant transformation and resulting in increased activation receptor signaling.

NK Cell Inhibitory Receptors

In order to maintain self-tolerance toward normal cells, NK cells express a myriad of inhibitory receptors capable of binding the ubiquitously expressed MHC class I molecules with varying affinities and transmitting inhibitory signals. In humans, NK cells predominantly express the killer immunoglobulin-like receptors (KIRs), which are type I integral membrane proteins that form a polymorphic family within the immunoglobulin superfamily. In contrast, mouse NK cells express members of the Ly49 family of C-type lectin type II transmembrane proteins. Although the inhibitory KIRs in humans and Ly49s in mice differ structurally, they have been shown to be functionally analogous and both directly bind classical MHC class I molecules. Both human and mouse NK cells also express conserved CD94/NKG2 heterodimers of C-type lectin type II transmembrane proteins that recognize the nonclassical MHC class I molecules HLA-E (in human) and Qa-1^b (in mouse) (Pegram et al., 2011).

NK Cell Activating Receptors

Cytotoxic function by NK cells is mediated by an array of structurally distinct germ-line encoded activating receptors, which recognize specific ligands on transformed or virally infected cells. Initiation of NK cell effector function is a complex process that proceeds through a series of steps beginning with activation receptor triggering and subsequent adhesion, followed by immune synapse formation, and ending with granule polarization and exocytosis. Perhaps the most potent stimulator of NK cells is the CD16 activating receptor, which is found on both human and mouse NK cells. Upon binding to the Fc portion on antibodies, NK cells are triggered to mediate antibody-dependent cellular cytotoxicity (ADCC). Perhaps the best-characterized activating receptor, also found on human and mouse NK cells, is NKG2D. NKG2D recognizes “induced self” ligands (e.g. MICA/B and ULBP1-6 in humans; RAE-1α–ε and MULT1 in mice), which are molecules that are

expressed at low levels on normal cells, but can be upregulated on unhealthy cells due to malignancy, stress, or infection. Other classes of activating receptors can recognize molecules encoded by viral pathogens, exemplified by Ly49H in mice (binds MCMV m157 viral glycoprotein) and the natural cytotoxicity receptors (NCRs) in humans (NKp44 and NKp46 bind influenza HA protein) (Pegram et al., 2011).

Current Perspectives on NK Cell Tolerance and Licensing

Although the "missing self" hypothesis provided a good framework for understanding the mechanism governing NK cell self-tolerance, subsequent studies characterizing the function of NK cells in MHC class I-deficient mice showed that NK cells from these mice do not display the NK cell auto-reactivity predicted by the "missing self" hypothesis. Subsequent studies have built on the "missing self" hypothesis to account for this discrepancy and have highlighted that NK cell self-tolerance is mediated by the proper pairing of NK cell receptors and their MHC ligands. In order to more fully explain the process of NK cell self-tolerance, Yokoyama and colleagues proposed the NK cell licensing hypothesis, which states that an NK cell must engage self-MHC class I in order to be responsive to subsequent stimuli received by activating receptors, which they termed "licensing." Conversely, those NK cells that failed to engage self-MHC class I were considered "unlicensed" (see Figure 1) (Kim et al., 2005). This process of licensing results in two types of self-tolerant NK cells: (1) the licensed NK cells, which maintain self-tolerance by direct inhibition by binding to self-MHC and (2) the unlicensed NK cells, which cannot engage self-MHC, but are self-tolerant due to their inherent resistance to stimulation received through activating receptors.

Although the licensing hypothesis provides a relatively simple model to distinguish NK cell function based on the host specific Ly49 expression and MHC haplotype, more complex models of NK cell responsiveness have recently been proposed. Due to the stochastic nature of inhibitory receptor expression, many different combinations of inhibitory receptors may be expressed on distinct NK cells. Thus, the rheostat model has proposed a quantitative nature of NK cell responsiveness. This model builds on the licensing model by incorporating evidence that different inhibitory receptors are capable of binding MHC ligands with varying affinities and that the diversity in the inhibitory receptor repertoires, along with the availability of MHC molecules being expressed, will result in varying degrees of inhibition between distinct NK cells allowing for a spectrum of potential NK cell responses (Shifrin et al., 2014).

NK CELL SUBSETS

In the early 1980s, Lanier and colleagues originally proposed a method of subdividing NK cells into functional subsets. In the late 1990s, this idea was revisited with the discovery that functionally distinct human NK cell subsets could be distinguished based on their level of CD56 expression (Hayakawa et al., 2006). Prior to that, it was clear that mouse NK cell subsets existed based on different Ly49 family member expression. Importantly, there were functional differences attributed to these subsets based on their ability to mediate BMC rejection. Similar subsets exist in human with regard to expression of KIR and other receptors. Since this time, several additional classifications have been used to attempt to

delineate NK cells based on their phenotypic status, differential distribution, and functional status – with the hope of finding parallels between the subsets found in mice and humans.

Phenotypic and functional subsets

In humans, NK cell subsets can be delineated by flow cytometry based on their relative expression of CD56, into $CD56^{bright}$ and $CD56^{dim}$ populations. $CD56^{dim}$ NK cells comprise the majority of the circulating NK pool in the blood and are functionally more cytotoxic, when compared to the $CD56^{bright}$ subset. The $CD56^{bright}$ subset, on the other hand, makes up a small proportion of the circulating NK pool, but are found in greater abundance in the lymph nodes, where they produce greater amounts of cytokines. In mice, Hayakawa and Smyth used CD27 as a marker to delineate mature NK cells ($CD11b^{high}$) into functionally distinct subsets. In their studies, they showed that the $CD27^{low}$ NK subset possessed a higher threshold for stimulation due to increased regulation by inhibitory receptors (Ly49/NKG2A). Conversely, the $CD27^{high}$ NK subset displayed increased effector function and preferential distribution in the bone marrow.(Hayakawa et al., 2006) Although these studies did not find strong parallels between the mouse and human NK cell subsets, subsequent studies by this group and others have suggested that the inclusion of additional markers, most notably the chemokine receptor CXCR3, improves the discrimination of mouse NK cell subsets that more closely mirror those in humans (Marquardt et al., 2010).

FUNCTIONAL CHARACTERISTICS OF NK CELLS

Cytotoxicity

In order for resting NK cells to migrate to the sites of infection and acquire effector function, they must first be primed by either exogenous cytokines or incorporate an activation signal from membrane bound activation receptors. Once a target cell is recognized, an immunological synapse is formed between the two cells. Once activated, NK cells are then able to induce apoptosis in the target cell through a number of mechanisms including exocytosis of cytotoxic granules and death receptor/ligand interactions. The cytotoxic granules contain proteins such as perforin and granzymes, which are able to function by creating pores in the target cells and activating caspases leading to apoptosis. Although perforin-dependent killing of target cells is the major mechanism of NK cell cytotoxicity, NK cells can also kill by alternative mechanisms by utilizing Fas ligand (FasL) or TNF-related apoptosis-inducing ligand (TRAIL). These ligands engage death receptors (Fas and DR4/DR5, respectively) on the surface of target cells, thus facilitating the activation of caspase pathways and initiating target cell apoptosis (Zamai et al., 1998).

Cytokine and Chemokine Production

In addition to their cytotoxicity, NK cells are also known to produce a wide array of cytokines similar to the different types of helper T cells. The two classic cytokines produced by NK cells are IFN-γ and TNF-α. Both play important anti-viral roles during viral infection as well as other pathogenic states. In addition to these, IL-22 has recently been shown to be produced by NK cells during MCMV infection and promote an anti-viral response by neutrophils, whereas IL-22 producing NK cells in the gut mucosa serve to promote mucosal integrity. NK cells have also been shown to produce IL-17 during parasitic infection. GM-

CSF is another cytokine that NK cells have been shown to produce after activation with varying effects including promotion of dendritic cell differentiation and neutrophil activation. Lastly, NK cells can also produce IL-10 and TGF-beta which can serve an immunosuppressive role during infection. The majority of these cytokines are only produced after activation of the NK cell (Sun and Lanier, 2011).

Immunoregulatory NK Cells

As we begin to identify more cytokines produced by NK cells, and different subsets of NK cells, the roles these cells play during different immune responses begins to expand to include more immunoregulatory functions in addition to their cytotoxic potential. NK cells have been shown to contribute to the homeostasis of the immune system and to regulate adaptive immune response via their production of cytokines and chemokines. Most notably, NK cells have begun to be appreciated for their role in modulating the adaptive immune response. Recently, NK cells have also been shown to polarize helper T cells towards a Th1 phenotype via their production of IFNγ. NK cells have also been shown to both promote dendritic cell (DC) maturation and indirectly promote adaptive T cell responses. Conversely, NK cells are also capable of detrimentally affecting adaptive immune responses by directly targeting antigen-specific T cells as has been shown during LCMV infection. Furthermore, NK cells can target virally infected antigen presenting cells during the early stages of MCMV infection, thus limiting the amount of antigen available to initiate a robust T cell response. Many of these inhibitory effects of NK cells can be correlated with lesser pathology resulting from strong inflammatory immune responses(Tian and Zhang, 2010).

Memory NK Cells

While memory has traditionally been attributed to cells of the adaptive immune system, NK cells have also been shown to display memory-like phenotypes. Memory NK cells were first identified using a model of hapten-induced contact hypersensitivity in immunodeficient mice lacking T and B cells. Contact hypersensitivity responses were thought to only be mediated by T cells, yet these immunodeficient mice retained the ability to mount these types of responses, which was abrogated with NK cell depletion. The researchers then found that after initial priming with a specific hapten to induce contact hypersensitivity, hepatic NK cells could then be adoptively transferred to a naïve host and upon challenge with the same hapten, the transferred NK cells would be sufficient to initiate a contact hypersensitivity response in the naïve host implying NK cell memory. Memory NK cells have also been identified after MCMV infection. C57BL/6 mice provide a relevant model to study memory NK cells due to their expression of the activating receptor Ly49H which is specific for the viral glycoprotein m157 expressed on virally infected cells. These antigen specific cells have been shown to clonally expand following MCMV infection, and a small memory population persists after NK cell contraction. Upon isolation and adoptive transfer to naïve neonate mice which lack effective MCMV defense, these memory NK cells are better able to protect and prevent MCMV mediated death than NK cells isolated from naïve hosts. Further research has gone on to show that a memory phenotype could be induced in NK cells *ex vivo* through stimulation with IL-12 and IL-18. Once adoptively transferred back into mice, these NK cells displayed enhanced IFN-γ secretion for several weeks, even after reverting back to a more quiescent phenotype. This heightened responsiveness could

also be detected in the progeny of the transferred NK cells indicating homeostatic proliferation as a potential mechanism of memory maintenance (Rolle et al., 2013).

MOUSE MODELS OF NK CELL BIOLOGY

The recent technological advancements, routine development, and expanding utilization of immunodeficient, knockout, transgenic, and humanized mouse models, have resulted in an expansion of our understanding and a greater appreciation for the complexities regarding the biology and clinical applications of NK cells. Prior studies have extensively relied on antibody depletion of total NK cell populations or of subsets to ascertain function consequences. The principal problem with antibody depletion is the lack of a truly NK cell specific marker resulting in depletion of other cell-types (i.e. in mouse the two primary antibodies used are to NK1.1, which is present on NK/T cells and anti-asialo GM1, which is also present on activated T cells and macrophages). The evaluation of NK cell development and function in vivo is seen as increasingly important due to the inter-relatedness of various cell types and concerns regarding whether isolated cells maintain and exhibit normal physiological functions when cultured in vitro. Interestingly, it has been extremely difficult to use the xenograft model to observe human NK cells in immunodeficient mice possibly due to lack of a critical cytokines. In this section, we will begin by briefly describing the characteristics of a selected group of mutant mice with known NK cell functional and developmental alterations and then we will end with a summary table highlighting additional models and references.

Beige Mice

The beige mouse model was one of the earliest examples of a selective NK cell deficient mouse characterized by its lack of NK cell cytolytic function in both natural cytotoxicity and antibody-dependent cytotoxicity (ADCC), which was the result of defective degranulation. In 1979, *beige* mice were shown to exhibit profound deficiencies in NK cell function resulting from a spontaneous point mutation, called beige, in C57BL/6 mice and leading to their increased susceptibility to infection (Roder, 1979). Additional studies seeking to further characterize these mice noted that beige mice shared a similar phenotype to that of the human Chediak-Higashi syndrome (CHS) – a rare and often fatal disease in humans characterized by neutropenia, diluted pigmentation, increased susceptibility to infection, and lack of NK cell function (Brandt et al., 1975). These studies played a pivotal role in determining the functional and protective characteristics of NK cells and helped establish suitable experimental models for CHS.

β2-microglobulin deficient mice

The β2-microglobulin ($\beta2m^{-/-}$) deficient mouse model has been used to decipher several aspects of NK cell self-tolerance and function, most notably, this model has been used to determine the role of MHC class I molecules on NK cell education (outline above). $\beta2m^{-/-}$ deficient mice were generated by the inactivation of the β2m gene via homologous recombination in embryonic stem (ES) cells. Due to the critical nature of β2m for proper stability of the peptide in the binding groove and surface expression of MHC class I molecules, cells from $\beta2m^{-/-}$ mice contain extremely low levels of MHC class I surface

expression. As described by the "missing-self" hypothesis, $\beta 2m^{-/-}$ blast T cells or fetal liver cells (which express low levels of MHC class I molecules and thus do not bind inhibitory receptors on NK cells) are susceptible to killing by WT NK cells in both in vivo and in vitro killing. Paradoxically, NK cells from $\beta 2m^{-/-}$ mice have been shown to be present in normal numbers, but NK cells from these mice are functionally deficient. Thus, $\beta 2m^{-/-}$ deficient mice exhibit a striking deficiency in NK cell activity in recognizing "missing self," suggesting the absence of MHC class I molecules during development renders these cells tolerant (additional characteristics of these mice provided in Table 2).

Interleukin (IL)-2 and IL-2 Receptor (IL-2R) Mutant Mice

It is well established that IL-2 is an important cytokine for the activation of NK cells, however, several early studies delineating the role of IL-2 versus components of the IL-2R (i.e., the α, β, and γ_c chains) resulted in seminal findings regarding the relative importance of each of these components in NK cell development. Surprisingly, these studies showed that NK cells from IL-2-deficient mice remained functional and maintained relatively normal numbers. Conversely, mice deficient in the IL-2Rβ chain (CD122, which is also a component of the IL-15 receptor) exhibited NK cells with significantly reduced cytotoxic activity and numbers. Additionally, IL-2Rγ_c chain (CD132, which is also a component of the IL-4, IL-7, IL-9, and IL-15 receptors) knockout mice completely lacked mature NK cells in peripheral organs (DiSanto et al., 1995; Kundig et al., 1993; Suzuki et al., 1997).

IL-15 and IL-15 Receptor Mutant Mice

Similar studies as those carried out in IL-2 and IL-2R deficient mice were also performed with IL-15 and IL-15R-deficient mice, partially due to the similarities seen between these two cytokines with regard to T cell stimulatory activity and the use of IL2 to expand and activate NK cells in vitro. However, the need for trans presentation of IL15 complicated the role of this cytokine with regard to direct and indirect means of signaling. In stark contrast to the findings made in IL-2 deficient mice, IL-15 or IL-15Rα deficient mice not only lacked NK cells but the IL15 deficient mice also did not support survival of mature NK cells transferred into them, suggesting that IL-15 played a critical role in both regulating the development and survival of NK cells in vivo (Cooper et al., 2002; Kennedy et al., 2000).

NK CELLS IN HEALTH AND DISEASE

NK Cells in Viral Immunity

Although NK cells were initially identified by their ability to spontaneously kill tumor cells, the relevance of NK cells in mediating antiviral effector functions is evident by the array of evasion mechanisms evolved by viruses to evade NK cell-mediated responses. NK cells are critical components of innate immunity functioning to help contain viral replication during the development of the adaptive immune response. NK cells have been shown to play a key role in protecting both humans and mice from influenza virus, poxvirus, human immunodeficiency virus (HIV), and several herpesviruses, including varicella zoster virus (VSV), Epstein-Barr virus (EBV), herpes simplex virus (HSV), and most notably cytomegalorvirus (CMV).

Pioneering studies utilizing mouse models suggested that NK cells play a key role in innate immunity to herpesviruses, as mice deficient or depleted of NK cells showed higher viral titers. Additional studies supporting the protective role of NK cells against viruses came from studies utilizing mouse cytomegalovirus (MCMV) models, where adoptively transferred NK cells played a direct antiviral role. Shortly thereafter, studies by Biron et al. described a case of a young girl with a rare NK cell deficiency where her NK cells were nonfunctional resulting in a series of viral infections during childhood, including infections with multiple herpesviruses (Biron et al., 1999). In the mouse, it was observed that an activating Ly49 family member, Ly49H, specifically recognized MCMV determinants and played a critical role in viral clearance. Furthermore, analogous expansion of human NK cells expressing NKG2C were observed expanded after CMV infection following HSCT indicating yet another example of NK cell subsets specific for a particular virus. Importantly, it is in these two models that evidence that NK cell “memory” was observed resulting in expansion and heightened responses.

NK Cells in Cancer Immunity

A role for NK cells in cancer immunity was first identified by the ability of so-called large granular lymphocytes to lyse tumor cells without prior exposure to antigen. These cells were found to be uniquely capable of lysing tumor cells that had lost or down regulated expression of major histocompatibility complex class I proteins on their cell surface. Because of these initial discoveries, harnessing the anti-cancer activity of NK cells was thought to represent a promising immunotherapeutic strategy for cancer. Further advances in the field on NK cell biology have led to our current understanding of the role that NK cells play in cancer immunity, and have provided insights into how to best utilize this unique immune cell pool to target and kill malignant cells.

Based on their expression of activating and inhibitory receptors NK cells are ideally suited to survey the blood and parenchymal tissues for the presence of stressed or malignant cells and are thus thought to play a critical role in immunosurveillance and cancer. Upon activation NK cells can mediate their effects on cancer cells through several mechanisms including; perforin mediated cytolysis, the binding of apoptosis inducing ligands expressed by malignant cells, or via the secretion of cytokines such as IFN-γ and TNF-α. Cancer cells originating from various cell types are known to express several NK activation ligands, making immunotherapies targeting NK cell activation a promising strategy (Dambrauskas et al., 2014).

The role of NK cells in cancer immunity has been extensively examined in the context of hematopoietic stem cell transplantation (HSCT). NK cells have been identified as the first lymphoid cell type to repopulate the recipient’s hematopoietic system following HSCT. In animal models of allogeneic HSCT NK cells have proven to be key mediators of graft versus tumor effects, while limiting the onset of graft versus host disease via the direct regulation of T cells (Small et al., 1999). In another study, it was observed that recipient patients who lacked the ligand for the KIRs expressed by allogeneic donor cells displayed reduced GVHD, lower rates of AML relapse, and a greater probability of event free survival after 5 years, presumably through an enhanced graft versus tumor mechanism (Ruggeri et al.,

2002). Similar observations, with regard to KIR/KIR ligand mismatch, have also been observed in patients undergoing autologous HSCT. In this setting, high risk neuroblastoma patients who lacked ligands for at least one of their own inhibitory KIRs displayed improved outcomes, including greater progression free survival (Venstrom et al., 2009).

Clinical Utilization of NK Cells

As our knowledge of NK cells and the roles they play in cancer has increased, so too has the desire to use them therapeutically to treat these malignancies. Clinically, the use of natural killer cell therapy has seen the most success in the context of hematological malignancies. This outcome is likely due to several factors, most notably that NK cells are primarily resident in the blood. There are examples, however, of NK cell based therapies being efficacious in solid tumors as well.

NK cell adoptive transfer has emerged as an attractive therapeutic strategy for cancer. In this context, NK cells are isolated from either peripheral blood, or differentiated from precursor hematopeitc progenitor cells and expanded in ex vivo culture systems. Several strategies for in vitro cell expansion exist including the use of NK-stimulating cytokine cocktails, or engineered feeder cell line co-cultures. These highly activated cells represent an “off the shelf” treatment modality which can be implanted into tumor bearing hosts in combination with more conventional therapies. Therapies involving infusions of autologous NK cells have been shown to be well tolerated in patients with glioma, renal cell carcinoma, and lymphoma (Dillman et al., 2004). Unfortunately, robust clinical responses have not always been observed. The use of allogeneic NK cells, however, may prove to be a more efficacious strategy, since this scenario takes advantage of the effects of KIR/KIR ligand mismatch noted above. Several studies utilizing this approach have observed significant clinical benefits. Of note, in a recent trial in poor prognosis AML patients, complete remission was observed in 5 of 19 patients receiving allogeneic NK cell infusion (Miller et al., 2005). A number of clinical trials are currently underway assessing the efficacy of both autologous and haploidentical NK cell infusion in combination with other modalities to treat an array of solid and hematological cancers, most notably leukemia and neuroblastoma.

Current research is also focused on utilizing small molecules, or exogenous cytokine administrations in order to improve the NK cell response following HSCT. NK cells are primary mediators of antibody dependent cellular cytotoxicity (ADCC) toward antibody coated tumor cells, via activation through the Fc receptor, CD16. Strategies aimed at improving NK mediated ADCC effects are currently under investigation. Recently it was discovered that combining antibody therapy, targeting tumor associated antigens, along with agonistic antibodies, targeting the NK cell costimulatory ligands, such as CD137, improves the antitumor efficacy of antibody therapy in models of both solid and hematological malignancy (Kohrt et al., 2014).

These therapeutic strategies rely on the broad cytotoxic function of NK cells, and attempts are under way to improve the specificity of NK cell killing towards tumor cells expressing specific antigens. Recently the use of chimeric antigen receptor (CAR) technology has come to the forefront of cancer immunotherapy and while this cutting edge technique has been primarily employed with regard to cytotoxic T cells, the potential expansion of CAR

technology for use in NK cell therapies has also gained traction in the field. For example, NK cells have been engineered to express receptors directed towards CD19 or CD20 and have shown enhanced cytotoxicity against certain lymphomas and leukemias expressing these markers (Boissel et al., 2013).

While the clinical utilization of NK cell immunotherapy has led to some promising results, several challenges remain which must be considered before this strategy can move forward. First among these is the need for exogenous stimulation, such as IL-2 or IL-15, in order to maintain NK cell functions in vivo. These cytokines, when given in large doses, are associated with life threatening toxicities. Furthermore it remains to be seen how and if cancer cells are capable of responding to and resisting NK cell therapy. Recent reports suggest that highly metastatic breast cancers are capable of down regulating expression of activating MHC-Ib (MICA/B) ligands in order to avoid NK cell mediated attack (Wang et al., 2014). Other studies report the ability of cancer cells to shed MHC –Ib ligands into the tumor microenvironment, limiting NK cell function in vivo (Doubrovina et al., 2003). Overcoming these challenges will be crucial for the advancement of NK cell based immunotherapies into the clinic.

CONCLUDING REMARKS

It is without question that our understanding of NK cell biology has grown dramatically since they were initially identified in the early 1970s. Although traditionally considered a homogeneous cohort of killer cells, many researchers now agree that NK cells exhibit a great deal of diversity, especially with regard to their effector functions. Previous and ongoing studies in mice continue to reinforce the idea that this model serves as a powerful tool to better understand the biology and function of NK cells – as recent and ongoing studies continue to shed new light on the regulatory and homeostatic roles of various NK cell subsets. Although substantial gains have been made in our understanding of NK cell biology, there continue to be limitations in translating the information gleaned from mouse NK cells in order to manipulate human NK cells in the clinical setting. Many of these shortcomings are due to the difficulty in identifying phenotypic similarities between these two species that also correspond with functional parallels, however, as more insight is gained into the sophistication of NK cell subsets a clearer picture for how to extrapolate these findings will begin to unfold.

Acknowledgments

Dr. William J. Murphy received funding under NIH grant number R01HL089905. Anthony E. Zamora received funding under NIH grant number 3R01CA102282-09S1. Ethan G. Aguilar received funding under NIH grant number 3R01CA095572-10S1.

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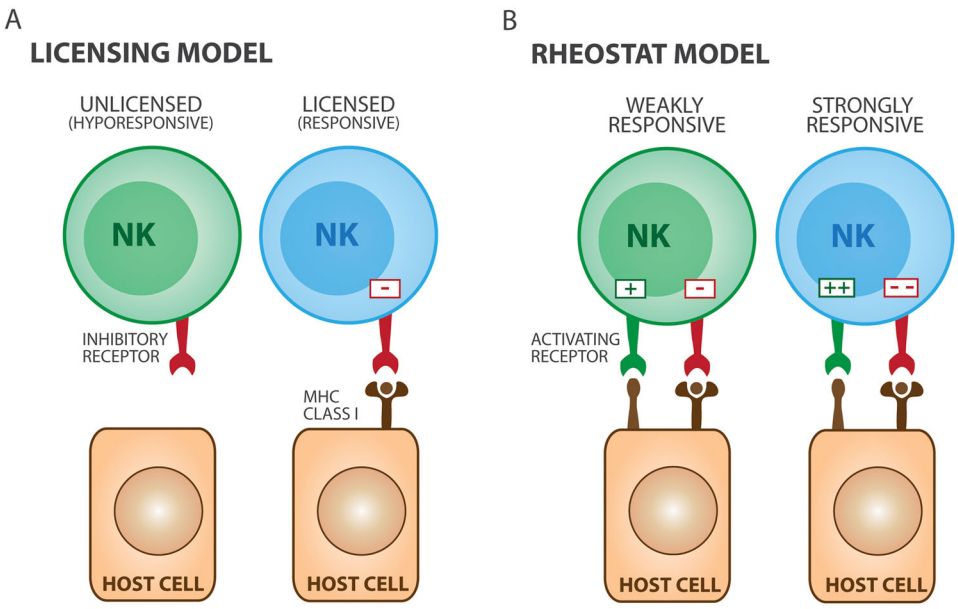

Figure 1.

Table 1

NK cell receptors in humans and mice

Receptor class	Species	Receptor	Ligands	Function
C-type lectin	M	Ly49A	H-2D^{d,k,p}	Inhibitory
		Ly49C	H-2D^{b,d,k}, H-2K^{b,d,k}	Inhibitory
		Ly49D	H-2D^{d}	Activating
		Ly49H	m157	Activating
		Ly49I	H-2K/D^{b,d,s,q,v}	Inhibitory
		Ly49P	H-2D^{d}	Inhibitory
KIR	H	KIR2DL1	HLA-C2 (Lys 80)	Inhibitory
		KIR2DL2/3	HLA-C1 (Asn 80)	Inhibitory
		KIR2DL4	HLA-G	Activating
		KIR2DL5	Unknown	Inhibitory
		KIR3DL1	HLA-Bw4	Inhibitory
		KIR3DL2	HLA-A3/A11	Inhibitory
		KIR2DS1	HLA-C2 (Lys 80)	Activating
		KIR2DS2	HLA-C1 (Asn 80)	Activating
		KIR2DS3	Unknown	Activating
		KIR2DS4	HLA-Cw4	Activating
		KIR2DS5	Unknown	Activating
		KIR3DS1	HLA-Bw4	Activating
C-type lectin	H, M	CD94/NKG2A	H: HLA-E, M: Qa-1^{b}	Inhibitory
		CD94/NKG2C	H: HLA-E, M: Qa-1^{b}	Activating
		CD94/NKG2E	H: HLA-E, M: Qa-1^{b}	Activating
		NKG2D	H: MICA/B, ULBPs M: RAE-1, MULT-1, H60	Activating
NCR	M	NCR1	Viral HA	Activating
	H	NKp30	BAT-3, HSPG, B7-H6	Activating
		NKp44	Viral HA, HSPG	Activating
		NKp46	Viral HA, HSPG	Activating
	H, M	2B4	CD48	Activating/Inhibitory
		KLRG1	Cadherins	Activating
		DNAM-1	PVR, CD122	Activating

Abbreviations: M, mouse; H, human; HLA, human leukocyte antigen; KIR, killer immunoglobulin-like receptor; KLRG1, killer cell lectin-like receptor G1; BAT-3, HLA-B-associated transcript 3; HSPG, heparan sulfate proteoglycan; NCR, natural cytotoxicity receptor; MULT-1, mouse UL16-binding-like transcript-1; RAE-1, retinoic acid early transcript-1; H60, histocompatability 60; PVR, polio virus receptor; HA, hemagglutinin

Table 2

Mouse Models to study NK biology and applications

Model	Description	NK cell status	Applications
C57BL/6	In C57BL/6 mice (H-2^b haplotype), NK cells are found with normal frequency and function. The majority (~85%) of NK cells in this strain express at least one self-MHC-I-specific receptor (Ly49C, Ly49I, and/or NKG2A). Additionally, this strain also expresses the activating Ly49H receptor, which can bind viral (MCMV) m157.	NK cells expressing self-MHC-I-specific receptors show normal responsiveness. NK cells that lack a specific inhibitory receptor for self-MHC are hyporesponsive. MHC-I-deficient target cells are readily rejected. NK cells are able to mediated direct recognition of MCMV-infected cells due to Ly49H expression.	C57BL/6 mice are one of the most commonly used inbred strains of mice. It is a general purpose strain, but is also commonly used in viral studies (MCMV and influenza) due to reagents being readily available. Mouse models of MCMV infection have also been used to gain insight into the immune response and viral pathogenesis with hopes of extrapolating these findings to humans.
Scid (*scid*)	The C.B-17 scid mice are homozygous for the *scid* gene (mutation in *Prkdc*^{scid}: protein kinase, DNA activated, catalytic polypeptide) and display a severe combined immune deficiency (SCID) syndrome. This strain has impaired production of functional B and T cells due to the lack of DNA-PK, which is necessary for joining non-homologous ends of double-stranded DNA.	This strain of mice has been shown to lack T and B cell functions, however, NK cells display normal functional activity.	Often used for transplantation studies of murine or human (xenogeneic) tumors for therapeutic or imaging studies. This strain has low levels of human PBMC and HSC engraftment.
NOD-*scid*-IL2rγ null (NSG, NOD *scid* gamma)	NSG mice do not express the *Prkdc* or the X-linked *IL2rγ* genes, which results in severe impairment in T and B cell development and an absence of NK cells.	Although these mice lack NK cells, they have been used in NK cell adoptive transfer studies.	NSG mice are one of the most highly immunodeficient mice, due to the depletion of the IL-2 receptor γ-chain and mutated *Prkdc* gene. They are capable of engrafting a wide range of human cells and tissues, therefore they are often used for cancer xenograft modeling, to study stem cell biology, or mouse humanization.
Beige	Early studies utilized beige mice as an experimental model for the human Chediak-Higashi syndrome. Beige mice were one of the earliest models used to study the effects of aberrant NK cell function and helped shape our understanding of NK cell function.	NK cells from beige mice lack cytolytic function in both natural cytotoxicity and ADCC, which is the result of defective degranulation. NK cells from these mice are found in normal numbers and still possess the ability to produce characteristic cytokines.	Beige mice are most notable for their utility as an experimental model for Chediak-Higashi syndrome. Although initial studies utilized these mice to study the role of NK cells in mediating protection from pathogenic infections, newer models are now used due to the beige mutation also affecting the granules of many other cell types.
β2m$^{-/-}$	β2m-deficient mice were generated by the inactivation of the β2m gene via homologous recombination in ES cells. Due to the critical nature of β2m for proper stability of the peptide in the binding groove of MHC-I, cells from β2m$^{-/-}$ mice contain extremely low levels of MHC-I surface expression.	The mice have normal numbers of NK cells, however their NK cells are hyporesponsive. NK cells fail to kill ConA blasts and do not reject MHC-I-deficient target cells.	β2m-deficient mice are commonly used to determine several aspects of NK cell self-tolerance and function. This model has also been used to determine the effects of MHC class I molecule binding to various inhibitory Ly49 receptors in order to determine the licensing status of discrete subsets of NK cells.
IL-2$^{-/-}$	IL-2-deficient mice were generated by targeted mutagenesis in order to determine the effects on the immune system. IL-2$^{-/-}$ mice lack many of the common negative regulatory pathways (e.g. negative regulation by Tregs), which leads to a breakdown in self-tolerance and development of autoimmune disease.	NK cells from IL-2-deficient mice remain functional and are present in relatively normal numbers.	IL-2-deficient mice were initially generated to determine the role of this cytokine on the immune system. Subsequent studies have utilized these mice to determine whether IL-2 is necessary for normal NK cell development.
IL-15$^{-/-}$	IL-15-deficient mice were generated by targeted mutagenesis and have been used to help delineate the pleiotropic and distinct functions between IL-2 and IL-15. IL-15$^{-/-}$ mice have been shown to have significant NK, NKT, IEL, and memory CD8 T cell deficiencies.	Although NK cells from IL-2-deficient mice have been shown to develop normally, IL-15-deficient mice completely lack NK cells, suggesting IL-15 plays a critical role in NK cell development.	IL-15$^{-/-}$ mice have historically been used to answer questions regarding the development and survival of different immune cells. Additionally, these mice have been useful for studying the roles of IL-15 in infectious disease, antitumor immunity, and inflammation.

Model	Description	NK cell status	Applications
IL-2/15Rβ$^{-/-}$	IL-2/15Rβ–deficient mice were generated by targeted mutagenesis and have been shown to develop severe autoimmune disorders with age. The importance of IL-2/15Rβ-mediated signaling in the growth and development of IELs and NK cells was shown to be critical as both cell populations have major developmental defects, whereas T cells are spontaneously activated.	IL-2/15Rβ$^{-/-}$ mice have severely reduced (essentially absent) numbers of NK cells. The small population of NK cells that persists do not exhibit cytotoxic activity.	IL-2/15Rβ$^{-/-}$ mice are commonly used to study the development of distinct lymphocyte populations. Additionally, the phenotypic characteristics of these mice have provided important insight into the potential mechanisms leading to severe combined immunodeficiency syndromes in humans.
γc$^{-/-}$ (common gamma chain; IL-2Rγc)	Similar to the other IL-2/15R-deficient mice, γc–deficient mice were also generated by targeted mutagenesis. γc$^{-/-}$ mice show a defect in mature B and T cell development and a substantial reduction in absolute lymphocyte numbers.	γc-deficient mice have severely reduced numbers of NK cells.	γc$^{-/-}$ mice are commonly used to study the development of distinct lymphocyte populations. Additionally, these mice have been used as an experimental model for X-linked severe combined immunodeficiency, as humans with this disease also have a loss of γc function.
Gzm B$^{-/-}$	Gzm B$^{-/-}$ mice were generated in mice with a homozygous null mutation in the granzyme (gzm) B gene. These mice develop normally and have normal lymphopoiesis and hematopoiesis. CTLs and NK cells display slight reductions in their ability to lyse target cells.	NK cells from Gzm B$^{-/-}$ mice develop normally and exhibit virtually normal lytic function.	Gzm B$^{-/-}$ mice have been used to elucidate the capacity of this serine protease to elicit target cell DNA fragmentation and cell death. These mice have also been used to study the mechanisms behind NK cell-mediated lysis of target cells.
Perforin$^{-/-}$	Perforin-deficient mice have been generated by homologous recombination and have been shown to be both viable and fertile. Although these mice have normal numbers of CTLs and NK cells, they are more susceptible to certain viral infections (particularly LCMV).	NK cells from perforin-deficient mice show a greatly reduced ability to kill MHC-I-deficient target cells.	Perforin-deficient mice have primarily been used to glean insight into the mechanisms utilized by CTLs and NK cells to kill target cells.

Abbreviations: MHC, major histocompatability complex; MCMV, mouse cytomegalovirus; NSG, NOD scid gamma; ADCC, antibody-dependent cellular cytotoxicity; Gzm B, granzyme B; LCMV, lymphocytic choriomeningitis virus; ConA, concanavalin A; PBMC, peripheral blood mononuclear cell; IEL, Intraepithelial lymphocyte; DNA, deoxyribonucleic acid; Tregs, regulatory T cells