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Review

License to kill, regulate and remember: The many roles of natural killer cells

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ABSTRACT

Natural killer (NK) cells are one of the first lines of defense against a wide range of pathogens and cancers. They express a variety of both inhibitory and activating receptors and antigens that can be used to classify cells as a particular subset population with differing regulatory and effector functions. New studies reveal previously uncharacterized complexities of NK development, functions, roles, and responses to stimuli within these subsets of NK cells. These new characteristics include populations of NK cells that are involved in regulation, memory, hematopoiesis, positive and negative selection, and interactions between adaptive and innate cells. NK cells thus serve as an important innate, anti-pathogen and anti-tumor cell with possible adaptive immunity features.

KEYWORDS: natural killer cells, licensing, education, arming, adaptive, innate, cell interactions, immunotherapy, development, regulation

INTRODUCTION

Natural killer (NK) cells are large, granular lymphocytes that are a part of the innate immune system and primarily play a role in anti-tumor and anti-viral responses [1-3]. Though initially assumed to be fairly simplistic cytotoxic innate cells, recent findings have highlighted their complexities and nuances that were not considered previously. Increasing work in both humans and mice has shown the existence of various subpopulations of NK cells with potentially different capabilities and roles in the immune system [4-16]. Numerous characteristics and attributes of adaptive immune cells have started to be applied to NK cells [17-26]. The idea of NK cell "education" or "licensing" is akin to positive selection in T cells whereby NK cells are only fully functional upon capability of binding to host MHC I molecules and showing they can be inhibited [27-29]. The discovery of specialization of NK cells in terms of certain populations primarily producing only cytokines, others that are immunosuppressive, cytotoxic, or promote hematopoiesis further exemplify the multifaceted roles NK cells have [4, 16, 30, 31]. Additionally, selective expansion of certain subsets and potential memory NK cells suggest there might be much more about these cells that we do not understand or consider currently [21, 32-36]. This review will highlight the history and general background of NK cells in both humans and mice, and delve into the new discoveries and characteristics of NK cells that make them such a unique cell. Finally, the clinical significance and potential of NK cells will be highlighted due to the role they have in numerous disease processes and medical conditions.

Natural killer cell history and background

Natural killer (NK) cells were first discovered by Kiessling and Herberman in 1975 [37, 38]. These studies looked at the unique capabilities of these cells to "naturally" eliminate leukemia cells without

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any immunization beforehand [39]. However, the first studies that identified the presence of NK cell activity involved bone marrow rejection. In these studies F1 hybrid mice were able to reject bone marrow cells injected from parental or allogeneic bone marrow allografts. These findings were confusing since F1 host T cells should not be able to reject parental bone marrow cells due to the laws of transplantation that state MHC-encoded transplantation antigens are expressed co-dominantly [40, 41]. Thus there appeared to be a unique lymphocyte population capable of mediating this hybrid rejection, which initiated the study of NK cells.

It was originally proposed that NK cells targeted cells based on "missing self" [42-45]. This hypothesis stated that if an NK cell was not inhibited by the presence of major histocompatibility complex (MHC) class I molecules on a potential target, then it would exhibit cytotoxicity towards that cell. This fit with the potential targets for NK cells that included virally infected and transformed cells that often times down-regulate MHC I expression levels and would make them a prime target due to lack of inhibition of the NK cells [42, 43]. However there are normal cells, including red blood cells, that have low MHC I expression and NK cells have not been shown to attack them normally. Thus the missing-self hypothesis was modified to also include the need for activating signals from the potential targets to signify the cell is a threat to the host [46-48]. NK cells recognize their targets through germline-encoded pattern recognition receptors that are not MHC I restricted [3, 49-53].

The best-characterized targets of NK cells are viruses and tumor cells. Initial studies looking at the activity of NK cells showed high levels of cytotoxicity towards blood-borne cancer cells like various types of leukemia in both mice [37, 54-56] and humans [57-59]. Since NK cells have a prominent role in anti-tumor protection in the blood, their role in protection against metastatic tumors cells has also be discovered. NK cells are capable of targeting and eliminating the metastatic cells from solid tumors that enter the blood stream and help reduce spread of the cancer to other organs and tissues [60-64]. In addition to the anti-tumoral protection provided by natural killer cells, the anti-viral capabilities of NK cells are a significant aspect of the immune system's role in handling viral infections. They have been shown to either cause direct lysis of virally infected cells or respond to the infection by producing IFNγ and other pro-inflammatory cytokines to help eliminate the virus from the host [65-68]. NK cells have been shown to have important roles against cytomegalovirus [69-72], herpes simplex virus [73], various hepatitis viruses [74-76], influenza [77, 78], human immunodeficiency virus [79-81], as well as various other viruses [82-85]. NK cells aid in both the anti-viral and tumor responses through direct ligand recognition and cytotoxicity, antibody-dependent cell-mediated cytotoxicity (ADCC), immunostimulatory and inflammatory cytokines release like TNF-α or IFN-γ, and other mechanisms that will be addressed in more detail later on.

NK cells are defined phenotypically by the presence and absence of particular antigens. No one specific marker exists to identify them. Most of the typical NK markers are also located on T cells, mast cells, or macrophages and include the C-type lectin NK-cell receptor protein 1 A (NK1.1 in mice or CD161 in humans) [86], integrin-α2 (CD49b) in mice [87], the neuronal cell adhesion molecule CD56 in humans [88], CD11b and asialoganglio-N-tetraosyceramide (asilo-GM1) [89]. Therefore it is usually a combination of expression of these antigens along with a lack of typical T cell markers like CD3 or the T cell receptor that identifies a NK cell.

NK cell development and maturation occurs primarily in the bone marrow (though the fetal liver is the primary hematopoietic organ before birth [90]) deriving from the common lymphoid progenitor (CLP) [91, 92]. The stages of development can be divided into four primary stages. The first stage is defined by the expression of IL-2 receptor β (IL-2Rβ/CD122). NKG2D, a NK activating receptor is also expressed early on during development, though the precursor NK cells do not appear to be capable of cytotoxic capability at this point. NK cells then develop into the immature stage with the acquisition of NK1.1 in mice or CD161 in humans with the expression of DX5 in mice or CD56 in humans along with NKG2A/CD94 complex expression following shortly after.

The C type lectins (Ly49s) in mice and the killer immunoglobulin-like receptors (KIRs) in humans are the last markers to be expressed before becoming mature NK cells and going out into the periphery [91, 93-96]. The maturation of NK cells can be defined by the varying expression patterns of CD11b and CD27. The four stages according to the expression of these receptors are: $CD11b^{low}CD27^{low}$, $CD11b^{low}CD27^{high}$, $CD11b^{high}CD27^{high}$, $CD11b^{high}CD27^{low}$ [97]. This final stage is the predominant NK population in the liver, spleen, blood, and lungs, while $CD11b^{low}CD27^{high}$ is found more in the lymph nodes and bone marrow with smaller populations also in the spleen and liver [12, 14, 98]. Additionally, the relative expression levels of CD94 could also be indicative of the maturation status and functional capabilities of the NK cells. NK populations can be divided into $CD94^{high}$ or $CD94^{low}$ populations, with the $CD94^{high}$ population being capable of greater levels of proliferation, cytotoxicity, and IFNγ production. The $CD94^{low}$ population was shown to be capable of increasing expression of CD94 and becoming the $CD94^{high}$ population. However the $CD94^{high}$ population was not capable of becoming the $CD94^{low}$ population suggesting the $CD94^{high}$ population to be a more mature NK cell [99].

Human and mouse NK cell differences

Though there are numerous similarities between NK cells found in mice and humans, NK cells are more divergent between species than many adaptive immune cells including T cells. A major difference between human and mouse NK cells involves the differential expression of various receptors and antigens. One such marker is CD56, which is only found in humans and help distinguish between two different populations of NK cells that are either more cytotoxic or produce more cytokines based on having either low or high expression of CD56 respectively [4, 30, 31, 100]. The CD56 bright population is also found primarily in the lymph nodes (LN) in normal conditions, whereas NK cells in mice typically are only found in the lymph node after they have been activated or stimulated and still in relatively low amounts [101]. Correlations of these populations have been made with $CD11b^{low}CD27^{high}$ and $CD11b^{high}CD27^{low}$NK cells in mice with CD56 bright and CD56 low populations respectively [11]. These populations of cells will be discussed more later.

Another difference alluded to before involves the receptor families responsible for binding to self-MHC class I. In mice this family is the C type lectins, Ly49s, and in humans the killer immunoglobulin-like receptors (KIRs). There are structural differences between the two receptor families, but functionally they are very similar. They both have receptors that bind to self-MHC class I, signal through SHP-1/2 or SHIP-1, and inhibit the NK cells [49, 71, 72]. Both receptor families also contain activating receptors that bind to MHC class I-like molecules [49, 102, 103].

There are also significant differences in terms of *in vitro* culture and activity of freshly isolated NK cells from humans and mice. NK cells newly isolated from humans show strong cytotoxic capability and are able to be maintained in culture for long periods of time in the presence of IL-2 and/or IL-15. They maintain expression of normal receptors, including KIR expression, for months in culture and can be greatly expanded [104-106]. Murine NK cells on the other hand exhibit poor cytotoxicity and cytokine production when they are isolated from mice. They need stimulation through cytokines or other activating signals to be able to exhibit these normal functions. Additionally, *in vitro* culture with IL-2 is much more limited, with the majority of NK cells dying or exhibiting reduced cytotoxicity and cytokine production after 2 weeks in culture [107, 108].

There are also differences in the basal activity and expression of granzyme B and perforin between human and murine NK cells. In murine resting NK cells, granzyme B and perforin mRNA is constitutively present, but only minimal levels of protein is detected. Upon stimulation or activation of the NK cells, the protein is rapidly translated and the NK cells can respond and kill the target cell. In contrast, human NK cells constitutively express high levels of both granzyme B and perforin protein. This grants the NK cells a basal level of cytotoxicity and allows for rapid response of the NK cells to targets. IL-2 or IL-15 stimulation can further increase this level of granzyme B and perforin that is present in NK cells and further enhance the inherent cytotoxicity capabilities [109-111].

These differences highlight a potential evolutionary timeframe for NK cells. Though T cells and adaptive immune cells have traditionally been classified as having evolved more recently due to the adaptive immune system being seemingly more complex and nuanced, the differences between human and mouse T and B cells are very limited [112, 113]. The significant differences between human and mouse NK cells could suggest a more recent evolutionary development as compared to T and B cells, highlighting the novelty and significance of NK cells [114, 115].

Though there are a number of differences between human and mouse NK cells, the importance and knowledge to be obtained from doing mouse work is vast. The fundamental principles of target recognition, cytotoxic activity, cytokine production, inhibition, and regulation are similar between human and mouse NK cells [115]. Correlations and extrapolations of findings discovered in mice to humans are numerous. The mouse models also allow for a setting of high amounts of potential manipulations and experimentation that cannot be done in humans. Additionally with the availability of reagents, techniques, varying strains of mice with different knock-outs and knock-ins, and xenogenic mouse models, the mouse models allow for extensive and detailed experimentation to be performed.

Cytotoxicity

Natural killer cells can exhibit a wide range of functions beyond simple killing of target cells. However, the best-characterized aspect of NK cells is their cytotoxicity. NK cells are able to determine target cells with a combination of a lack of inhibition (missing-self hypothesis as previously described [42, 43]) and activating signals from various danger signals that are released by stressed cells [46, 116, 117]. NK cells are able to primarily recognize cells that are either virally infected or transformed, but NK cells have also been shown to have a role in anti-bacterial [118-120] and fungal [121-123] responses. NK cells recognize their targets through various activating receptors. Some are very specific for a certain target like the Ly49H receptor found in C57BL/6 background mice that is able to bind with the murine cytomegalovirus (MCMV) glycoprotein m157 and lead to activation of the NK cell and killing of the target cell [32, 33, 48, 124]. There are also more general activating receptors that bind to more universal targets like NKG2D, NK1.1, DNAX accessory molecule-1 (DNAM-1), CD16, 2B4, CD94 that dimerizes with NKG2C, and the natural cytotoxicity receptors (NCRs) that include NKp30, NKp44, and NKp46 [3, 49, 52, 125-129].

The NKG2D receptor is found in NK, T, NKT, and γδ T cells. It is a type II transmembrane anchored C-type lectin-like glycoprotein that binds MHC class I-related and similar proteins. This receptor signals through the adaptor proteins Dap10 (in humans and mice) or Dap12 (mouse only), which signal through the PI3K and AKT signaling pathways and lead to phosphorylation of Janus kinase 2 (JAK2), STAT5, ERK1/2, and MEK1/2. This signaling leads to the activation of the NK cells and cytotoxicity towards the target cell the receptor bound to. This activation also leads to secretion of IFNγ and granzyme degranulation. The ligands for this receptor in humans include MHC class I related proteins A and B (MICA/B) and UL16-binding protein (ULBPs). In mice they include retinoic acid early inducible-1 (Rae-1), minor histocompatibility antigen H60, and murine UL16-binding protein like transcript-1 (MULT-1). These ligands can be normally expressed on cells, but are upregulated when the cells are stressed or undergoing high rates of proliferation, which would include most cancer cells [50, 130-134]. NKG2D is thus considered one of the major receptors for NK cells identifying target cancer and virally infected cells to eliminate.

Natural cytotoxicity receptors (NCRs) have also been shown to play a role in NK cell tumor recognition and killing with blockade of these receptors resulting in reduced killing of tumor cells [98, 99]. NKp30 and NKp44 are expressed only on human NK cells, but NKp46 can be found on both human and mouse cells. NKp46 and 30 are constitutively expressed on NK cells, but NKp44 expression needs to be induced through stimulation like IL-2 binding [135, 136]. The ligands for these receptors are still being researched, but the human leukocyte antigen B associated transcript-3 (BAT-3) and B7-H6 have been identified as ligands for NKp30 [135-140].

Additionally, unique isoforms of NKp30 have been discovered with different signaling pathways that lead to subsets of NK cells with varying roles. In a study looking at gastrointestinal stromal tumors, three isoforms of NKp30 were isolated and expressed by different populations of NK cells. Two of the isoforms had the expected outcome of mediating NK cell activation and leading to cytotoxicity of the tumor cells, but one was found to be inhibitory and lead to NK cells producing an immunosuppressive cytokine, IL-10 [16]. This lead to the identification of differing roles of the NKp30 receptor based on the expressed isoform and to the presence of different subsets of NK cells, cytotoxic NK cells and suppressive NK cells, that could greatly impact the immunological response to a tumor challenge.

It is thought that influenza hemagglutinin is a potential ligand for NKp46, but more ligands are suspected [141-143]. There have been conflicting data looking at the importance of NKp46 with some studies showing NKp46 deficient mice unable to control tumor growth while others show the opposite outcome of NKp46 knockout mice resulting in improved tumor control [144]. These studies suggest a potential inhibitory role of this receptor that has not be clarified. There could be different isoforms of NKp46 similar to NKp30 that result in either cytotoxic or suppressive NK cells based on the isoform expression. Thus, our current understanding of the various NCRs is limited and greatly needs to be expanded.

Another way NK cells can become activated and result in cytotoxicity of targets cells is through antibody-dependent cell-mediated cytotoxicity (ADCC). NK cells can express an Fc receptor (CD16) that binds to the Fc portion of an antibody. This can lead to activation of the NK cell and triggering of the cell to release various cytokines or perforin and granzyme to lyze the potential target cell bound by the antibody [81, 145]. This is a great potential therapeutic tool in both tumor or viral situations since using antibodies directed against ligands found specifically on targets could lead to ADCC of these targets by NK cells. This has been tried in a number of scenarios, including various cancers and viruses [79, 81, 145-149]. Utilization of specific monoclonal antibodies and ADCC remains a source of hope for therapies for many difficult viruses and cancers to eliminate.

NK cells can mediate lysis of target cells through a variety of ways including both apoptotic and necrotic pathways. The primary method involves the release of perforin and granzyme. Perforin functions by essentially creating pores on the surface of the target cell which leads to escape of the cellular components of the target cell and necrosis to occur. Additionally, the pores allow for the entry of granzyme, which can lead to the activation of the caspase signaling pathway and cause apoptosis of the target [150, 151]. NK cells can also kill through various death receptors including TRAIL and FAS to initiate apoptosis, but these pathways take a longer amount of time than the rapid killing induced by perforin/granzyme [152-154].

Cytokine production

Cytokine production is an essential component of NK activity through the release of a wide range of cytokines both inhibitory and stimulatory. NK cells store their cytokines and perforin/granzyme in granules in the cytosol to be released upon activation or stimulation [155]. This allows for the NK cells to respond rapidly to immunological challenge without the need to wait for production of the various cytokines to be released. In humans there is a subset of NK cells that are considered to be primarily involved in cytokine production. This is the $CD56^{bright}$ population that is found in the lymph nodes. The $CD56^{dim}$ population is found in the periphery and is the typical killer NK cells that do not produce high levels of cytokines [4, 30, 31]. It is currently under debate if the $CD56^{bright}$ population is actually a maturation stage of NK cells and is a less mature population than the $CD56^{dim}$ population. It is thought that CD56 expression is reduced as the NK cell matures and becomes the typical killer cell and moves out into the periphery to have its effector functions [100, 156-158]. CD56 is only found on human NK cells, but potential mouse markers that reflect these similar patterns of NK activity have been identified. The $CD11b^{low}CD27^{high}$ and $CD11b^{high}$ $CD27^{low}$ correlate with $CD56^{bright}$ and $CD56^{dim}$ respectively. The $CD11b^{low}CD27^{high}$ population was found to primarily produce cytokines and

exhibited low levels of cytotoxicity in killing assays, while the $CD11b^{high}CD27^{low}$ population had high levels of cytotoxicity and low levels of cytokine production [12, 159]. Thus different functional subsets of NK cells exist in both humans and mice that are not simple killer cells as predicted for NK cells.

The primary cytokine produced by NK cells is IFNγ. It is a dimerized soluble cytokine that is a member of the type II class of interferons. This cytokine is involved in anti-viral and anti-intracellular bacteria capabilities and results in an inflammatory response. IFNγ has been shown to be essential in the response against numerous viruses including CMV by inhibiting viral replication directly by interfering with viral transcription [160]. It is also immunostimulatory by producing a Th1 response in T cells by upregulating the transcription factor T-bet, increasing antigen presentation and lysosome activity in macrophages, and promoting adhesion for leukocyte migration [161]. NK cells not capable of producing IFNγ have been shown to have dramatically reduced anti-MCMV capabilities [162].

Other immunostimulatory cytokines include TNF-α [163] and IL-6 [164]. These cytokines are both proinflammatory and can be released due to a variety of immunological responses including viral and tumoral challenge.

NK cells can also produce numerous immunosuppressive and regulatory cytokines. These include IL-10 [165] and TGF-β [166]. These cytokines can suppress the activity of a variety of immune cells including T cells, macrophages, DCs, and other NK cells as well. They can also facilitate the activation and skewing of other immunosuppressive cells like T regulatory cells [165, 166]. Some studies have suggested the possibility of certain subsets of NK cells being primarily suppressive and regulating the function and activity of the NK response. This was seen with the NKp30c isoform in GIST tumors that produced IL-10 and were not cytotoxic [16]. The possibility of other suppressive NK populations also exists.

A number of cytokines are also involved in hematopoiesis, suggesting a largely overlooked aspect of NK cells involved in cell development and regulation. NK cells are able to produce both granulocyte colony stimulating factor (G-CSF) and granulocyte macrophage colony stimulating factor (GM-CSF), which results in granulocyte and macrophage production in the bone marrow as the names suggest. The production of these cytokines can be important during immunological challenge to facilitate production of cells that can aid in the response [167, 168]. It is also important after hematopoietic stem cell transplantation and irradiation when many of the cells in the bone marrow have been eliminated. NK cells are more tolerant to high levels of irradiation than other lymphocytes and can survive for a number of days after exposure to irradiation allowing them to help facilitate repopulation of the bone marrow [169]. Thus certain populations could potentially by NK "helper" cells whereby they facilitate repopulation and expansion of certain immune cell populations to aid in immune responses.

NK licensing/arming/education

NK cell can possess a wide range of inhibitory receptors that are acquired randomly during the development of the NK cell. These receptors can be NKG2A or the Ly49 (mice) or KIR (human) receptor families [126]. The Ly49s and KIRs bind to specific MHC class I haplotypes with each receptor having greater or weaker affinity to a certain haplotype [52, 170]. Regardless of the MHC background of the host, an NK cell can possess any of the possible LY49s or KIRs [49]. This suggested that there were potential NK cells that possessed inhibitory receptors that could not bind to the host MHC I and thus would not be inhibited normally. To help explain why there is not a high level of self-reactivity from this population of NK cells, the notion of licensing/education was developed [10, 27, 28, 171]. This hypothesis states that as a NK cell develops, it goes through essentially a process analogous to positive selection of thymocytes in the thymus [172]. If an NK cell is able to bind to the host MHC I molecule, it is considered licensed/educated and is then capable of a wide range of effector functions including cytotoxicity and cytokine production and can be inhibited normally. The other population of NK cells that possess inhibitory receptors with low affinity to host

MHC I are considered unlicensed/uneducated and are hyporesponsive [10, 27]. This idea would explain the lack of high levels of self-reactivity from the NK cells that lack inhibitory receptors with high binding capability to self. Additionally, the amount of interaction amongst the various inhibitory receptors and MHC-class I molecules seems to alter the functional capabilities of the specific NK cell as well. It was shown that the greater number of inhibitory receptors a NK cell possessed that could bind to self-MHC, the greater the level of functional activity of the cell including cytokine production and cytotoxicity [173, 174], expanding upon the idea of licensing.

Much of the work concerning licensing has been done *in vitro* with isolated subsets of NK cells based on inhibitory receptor expression patterns. These studies have shown increased cytotoxicity of the licensed NK cells towards targets as well as increased degranulation and IFNγ production as compared to unlicensed [27]. However, exogenous stimulation of the unlicensed population through various cytokines or stimulatory molecules, including IL-2, IL-15, polyinosinic:polycytidylic acid (poly I:C), or activating receptor stimulation has shown this typically hyporesponsive population to exhibit levels of cytotoxicity, degranulation, and cytokine production typical of the licensed population [10, 27]. *In vivo* support for licensing has typically been shown in areas concerning bone marrow rejection and engraftment after hematopoietic stem cell transplantation (HSCT) [10]. In these studies, the licensed population of NK cells resulted in greater rejection of bone marrow grafts than the unlicensed. These results also occurred in bone marrow cells injected from MHC class I knockout mice suggesting it is not due to potential differences in inhibition of the licensed versus the unlicensed NK cells, but rather intrinsic differences in there capabilities to reject the donor cells [7, 175]. Human studies have also shown evidence for licensing in terms if specific KIR-HLA combinations. After HSCT, licensed NK cells with KIRs specific for host were able to expand and respond to CMV reactivation greater than unlicensed cells [69]. They were also shown to exhibit greater cytotoxicity and cytokine production in patients with acute myleogenous leukemia [171] and in *ex vivo* culture with target cells [173].

The actual process NK cells go through to become licensed/educated/armed is currently not well understood in terms of what cells are involved, where it occurs, or when it occurs during the development of the NK cell. One study showed that the responsiveness and licensed status of the NK cell can be altered depending on the presence or absence of MHC I. When mature, functional NK cells were transferred to MHC I-deficient hosts, they became hyporesponsive and essentially unlicensed. When hyporesponsive NK cells from MHC I-deficient mice were transferred into wild type hosts, then they became responsive and fully functional. This suggested that this licensing phenomenon is not necessarily a singular event that occurs only during the initial stages of NK development, but is instead more of a dynamic process that can be altered based on the environment that the NK cells are in. This could also lead to potential alterations in NK activity in the cases of various diseases or cancers that have reduced or altered MHC I expression [176].

Contrary to the *in vitro* findings and the limited comparisons between licensed and unlicensed NK cells seen *in vivo*, a study looking at MCMV resistances and licensing showed the opposite predicted effect of licensing. In this study, either licensed or unlicensed NK cells were depleted based on antibodies against the various Ly49 receptors prior to MCMV infection. There were significantly greater viral titers in mice that were depleted of the unlicensed population than depletion of the licensed cells or undepleted mice, suggesting a greater role of the unlicensed cells in MCMV response. It was thought that any potentially beneficial effects the licensing phenomenon had on NK cell activity was offset by the fact that these cells possessed inhibitory receptors that can bind to self and thus be inhibited by potential host cells infected with MCMV. The unlicensed cells, on the other hand, do not have such inhibitory receptors and would not be as inhibited as the licensed cells and could thus have greater anti-MCMV effects [177]. However, this study did not factor in the kinetics of the NK cells, in terms of the licensed NK cells being the initial and faster responders prior to the unlicensed population that requires a greater amount of activating signals and cytokines to be equally active. It also did not address the potential

of other cells, including T regulatory cells, affecting the licensed and unlicensed populations in potentially different ways and degrees. In a setting of immunodepletion, such as what occurs after irradiation and bone marrow transplantation, the activity levels of the different NK populations could be drastically different and that initial, fast responding licensed population may have the greatest clinical effect in responding to viruses or tumors.

An additional role for the unlicensed population of NK cells was also discovered in bone marrow cell rejection/engraftment after hematopoietic stem cell transplantation. Typically, depletion of the host NK cells prior to lethal irradiation and bone marrow cell injection in mice results in greater engraftment of the donor cells, abrogating the rejection facilitated by NK cells. Depletion of the unlicensed population results in enhanced rejection of bone marrow cells by the licensed population, suggesting that the unlicensed cells were either inhibiting or reducing the functional capabilities of the licensed population [7]. In addition, depletion of the licensed population of NK cells results in improved engraftment, even greater than total NK depletion. Since the only population remaining is the unlicensed NK cells, these results suggest the unlicensed are actually promoting engraftment of the donor cells. This is a unique and previously undescribed capability of NK cells to serve as essentially "helper" cells. It also gives a potential new role for unlicensed NK cells to serve as Helper NK cells and promote donor cell engraftment after transplantation. This engraftment could be facilitated by certain sets of cytokines produced by the unlicensed population of NK cells including G-CSF and GM-CSF (manuscript in preparation).

Overall there are still numerous unanswered questions about licensing. We still do not know about the actual clinical significance of licensing or what role the unlicensed cells play since they are able to get out into the periphery. Fundamental differences between unlicensed and licensed cells are also not known including differences in cytokine production, transcription factor expression differences, trafficking, activation, and more. Additional work is required to improve and expand upon this concept to better understand natural killer cell subset differences.

NK cell negative selection

Since licensing is analogous to T cell positive selection in the thymus, the potential for negative selection of NK cells in the bone marrow was also addressed in a pair of articles. MCMV glycoprotein m157 expression was induced in either bone marrow stromal cells [25] or systemically [26] to see if the NK activating receptor Ly49H would become desensitized and hyporesponsive to the actual virus. In both cases, NK cells that developed in the presence of m157 were hyporesponive to MCMV and had significantly reduced anti-MCMV capabilities as compared to wild type mice. Even with transfer of these NK cells into wild type hosts, there was still dramatically reduced anti-MCMV function [25, 26]. This suggested that NK cells go through processes similar to both negative and positive selection during their development in the bone marrow that is analogous to T cell development. Unlike what occurs with T cells if they fail to pass these two selective processes, NK cells are still able to get out into the periphery and are not capable of any sort of receptor rearrangement.

NK cell memory

Expanding on the T cell and NK cell similarities, recent studies in both mice and humans have shown the potential of memory-like NK cells with specific expansion of certain subsets. After exposure to CMV or MCMV, certain populations of NK cells (NKG2C+ in humans [69] and Ly49H+ [17] in mice) had longer survival times, expansion, and stronger secondary responses against viral rechallenge than other NK cell populations [17, 22, 35, 36, 178]. This phenomenon was also induced by hapten-specific contact hypersentivity and cytokine stimulation [21, 34]. However it is difficult to discern if the memory like longevity and increased response to rechallenge is truly due to a memory phenotype, or due to continuous low-level exposure to the pathogen or hapten. If the Ly49H+ NK cells are continuously being exposed to the low levels of latent CMV that is potentially in the host or in the transferred cells, the NK cells could remain active and continue to expand greater than other cell populations resulting in the memory-like pattern observed. Both the specific expansion of certain NK populations and the potential memory-like

characteristics of NK cells are representative of the sophistication of NK cells beyond what is typically characterized of innate cells. These traits are more characteristic of adaptive immune cells and hint at the limited understanding we have of NK cells currently.

NK and dendritic cell crosstalk

NK cells and dendritic cells (DCs) can interact in a number of ways to alter both the adaptive and innate immune response. DCs are capable of stimulating and activating NK cells through the trans-presentation of IL-15. IL-15 is best capable of signaling when it is trans-presented on a receptor to the target cell and DCs facilitate this presentation leading to NK cell activation [179, 180]. IL-15 was shown to be essential for NK survival and maturation since mice that were deficient in IL-15 production had severe defects in NK populations impairment in maturation [181]. DCs also produce type I IFNs, IL-18, and IL-12 which activate and expand NK cells [182, 183, 184]. These cytokines, as well as signaling through activating receptors such as Ly49H, have been shown to be able to overcome the need for IL-15 [124]. DCs can thus act to stimulate and activate NK cells and help expand the NK response to pathogens or transformed cells.

The interface of the interaction between DCs and NK cells is still not completely known. A recent paper showed an interaction between CD30 on NK cells and CD30L on DCs that lead to a pro-inflammatory immune response. Signaling from this interaction lead to differentiation of DCs to a more mature status and also release pro-inflammatory cytokines through the mitogen-activated protein kinase pathway. This engagement also resulted in NK cells releasing IFNγ and TNFα resulting in a strong pro-inflammatory immune response [185].

NK cells can also limit and regulate both the adaptive and innate immune response by affecting the DC population. This is achieved by directly lysing the DCs that have taken up viral pathogens. By eliminating the DC population, there are reduced antigen presenting cells (APCs) capable of activating the adaptive immune response, which will limit the extent of adaptive immune cell activation and expansion. The innate response is also hampered by the reduced number of cytokines produced by the DCs that could affect pathogens and other immune cells directly [183, 186].

NK and T cell interactions

The NK cells and T cells are capable of modulating and regulating each others' effector functions in both direct and indirect ways. The cytokines produced by NK cells, namely IFN-γ, are able to skew the differentiation of T cells towards a Th1 phenotype, altering the adaptive immune response that occurs [101, 161]. NK cells have also been shown to activate T cells be serving as an antigen presentation cell (APC). Antigen uptake through a variety of activating receptors and increased expression of MHC II and costimulatory ligands for T cell receptors by NK cells in viral infections demonstrated this unique ability of NK cells to stimulate T cells [187].

NK cells have also been shown to be suppressive of the T cell response. IFN-γ has been shown to actually lead to apoptosis of CD8 T cells late in an immune response leading to an immunosuppressive role of NK cells [188-190]. A recent study has also shown NK cells are able to modulate the CD8 adaptive immune response by the direct killing of CD4 T cells that stimulate CD8 T cells. This direct killing of CD4 T cells lead to a reduced CD8 response to various viruses. At low and medium dosages of virus, this was harmful to the host mouse since the adaptive immune response was dampened. However, at high dosages of the virus it was actually beneficial, due to the exacerbated response the T cells normally make to a high viral load that lead to severe immunopathology in a number of organs. NK cells prevented this autoimmune response at high viral doses by the reduction of CD4+ and CD8+ cells [191]. NK cells are also able to indirectly modulate the T cell response by eliminating transformed and virally infected antigen presenting cells (APC) and hamper the activation of T cells [186, 192, 193].

T cells can in turn affect the activity of NK cells. The production of various cytokines can both activate and inhibit NK cells. The production of IL-2 can lead to the activation of all NK cells [30, 194]. Treg cells can also suppress NK function

directly through TGF-β production [195-198]. NK cells can thus serve as a link to the adaptive immune system and alter the T cell response to pathogens. T cells in turn can limit and regulate the NK response and prevent over-reactivity as well creating a balanced dynamic between the two lymphocyte populations.

Clinical use of NK cells

A number of immunotherapies using NK cells have been examined to take advantage of the anti-tumor capabilities of NK cells. One approach was to try and improve the capabilities of the NK cells in the patient. This was done through administration of stimulatory and activating cytokines like IL-2 [199] or IL-15 [180, 200, 201]. IL-2 was shown to be efficacious in mice to improve the anti-tumor response by the NK cells [198] and was approved for clinical use for renal cell carcinoma [202], metastatic melanoma [203], and metastatic breast cancer [199]. IL-2 administration enhanced NK expansion in patients, but had limited long term affects in patients. Tumor relapse and overall survival of patients was not significantly altered [199, 202, 203]. There were also severe toxicities associated with the administration of IL-2. High doses of IL-2 lead to vascular leak syndrome due to increased vascular permeability and extravasation of fluids and proteins into the lung leading to pulmonary edema and cardiovascular failure [204-206]. IL-2 can also lead to expansion and activation of Tregs that can limit the activity of NK cells through the release of TGF-β [207, 208]. IL-15 was a good alternative due to not activating Tregs and not having as severe side effects as IL-2. Numerous approaches are currently being investigated to use IL-15 clinically including giving IL-15/IL-15Rα complexes for the trans-presentation of IL-15 and in combination with other cytokines including IL-6 to enhance NK function and anti-TGF-β to block the inhibition [180, 201, 209, 210].

Another clinical application of NK cells that is actively being pursued is the adoptive transfer of NK cells in conjunction with irradiation and hematopoietic stem cell transplantation in hopes of eliminating any remaining tumor cells that survive the irradiation [197, 211-214]. The NK cells would also help eliminate any viruses that become reactivated in the now immunosuppressed state of the patient. Additionally, NK cells have been observed to potentially be able to target cancer stem cells specifically [215, 216], which are more radioresistant than other cancer populations [217, 218] and are thought to be responsible for the tumor relapse that occurs in patients [219-221]. Adoptive transfer of autologous and allogeneic NK cells have resulted in successful engraftment and expansion of the NK cells with IL-2 administration for maintenance [211], but limited clinical benefit has been seen in a variety of cancers including various leukemias, lymphomas, breast and lung cancers, and metastatic melanoma. Autologous transfer showed minimal clinical benefit, most likely due to the tumor cells being able to inhibit the NK function due to the presence of MHC I molecules [23, 197, 211, 214]. Allogeneic NK cell transfer showed more promise with some studies showing improved survival rates with no side effects, and others showing no significant clinical benefit even with substantial NK expansion after transfer [39, 212, 222-228]. Minor changes in patient survival and metastasis have been observed, despite the clear engraftment, trafficking, and expansion of the transferred NK cells [211, 212].

A recent paper tried to explain why this is the case by stating that NK cells can become exhausted like T cells after continuous exposure to a certain target. The transferred NK cells were shown to be active and capable of anti-tumor functions at day one after transfer, but starting at day five, they became hyporesponive with reduced activating receptors, IFNγ production, cytotoxicity, and transcription factors that regulate NK activity [23]. This suggested that despite the NK cells reaching the target tumor sites, the NK cells become rapidly exhausted and not capable of producing significant anti-tumor effects that would have clinical benefit. Finding ways to reduce this exhaustion and keep NK cells active or giving repeated injections of NK cells might help improve the clinical benefit of performing adoptive transfers of NK cells.

There are many limitations in using NK cells for adoptive transfer currently. One major hurdle is obtaining sufficient, active NK cells to transfer. Short term *in vitro* culture beforehand usually

does not allow for sufficient expansion of the cells. Long term culture is now being used with a number of different cytokine combinations (IL-2, IL-12, IL-15, or IL-21) or co-cultured with feeder cells to provide large numbers of active NK cells for transfer [229-231]. Another issue is how the NK cells are transferred. Since they are usually given after other major therapies, it is difficult to discern if the function and activity of the NK cells are truly due to their innate ability, or if they are actually altered by the treatments the patient has undergone. The determination of the function and activity of the NK cells can also be misleading since the NK cells are obtained from peripheral blood after transfer and then studied through *in vitro* assays which may not be truly reflective of the environment they were in within the patient. More work needs to be done in order to more easily obtain high numbers of functional NK cells and better ways to analyze the activity, function, and phenotype of the transferred cells when they are in the patient and been exposed to the tumor cells.

CONCLUSIONS

The roles and functions of NK cells have greatly expanded from the simple killer cells they were originally thought to be. Through the production of numerous cytokines that can be immunosuppressive, immunostimulatory, or involved in hematopoiesis to helper cells that can aid in donor cell engraftment, NK cells can have many roles. Different subsets and populations of NK cells exist that specialize in these differing roles. The discoveries of specific expansion of

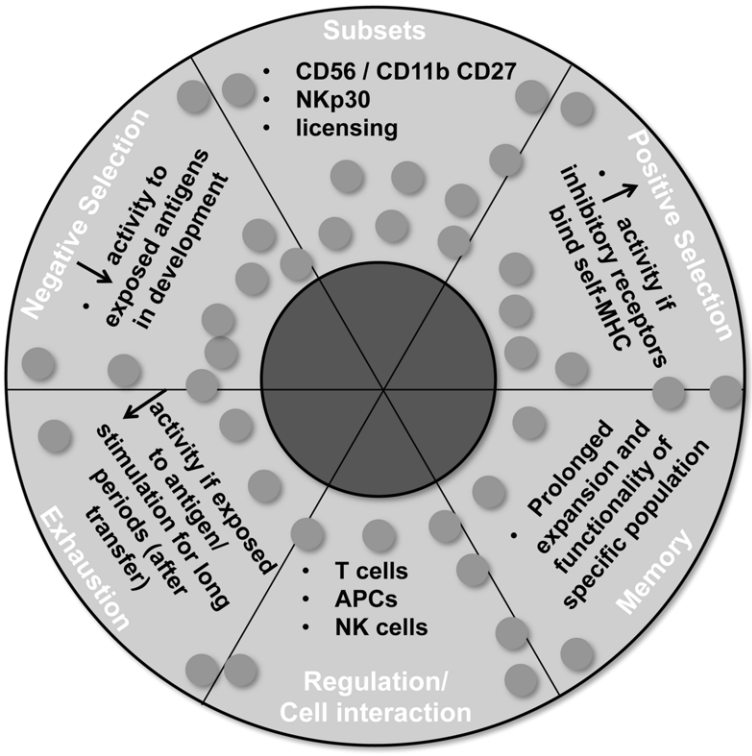

Figure 1. Adaptive features of NK cells. NK cells have many functions similar to those of lymphocytes in the adaptive immune system. Numerous subsets of NK cells are being discovered that specialize in certain functions such as immunoregulation, cytotoxicity, or cytokine production. Both positive and negative selection have been shown to occur during the development of NK cells. Potential memory NK cells have also been discovered that are long lived and respond rapidly to rechallenge. Overexposure to certain antigens or after prolonged stimulation, especially after adoptive transfer into hosts with tumors, have shown signs of NK exhaustion with reduced capabilities. Finally, numerous interactions and regulations of many other immune cells including T cells, dendritic cells, macrophages, and other NK cells have been shown, both positive and negative regulation.

certain NK populations in response to a challenge, NK memory-like properties, positive and negative selection during development, and NK exhaustion blur the line between these innate cells and their adoptive immune system counterparts (Figure 1). Additionally, with the differences seen between human and mouse NK cells, NK cells may represent an evolutionarily new population of lymphocytes that have developed to be a rapid response to various immunological challenges and serve as a link between the innate and adaptive immune system. A greater understanding of the roles, functions, and subsets of NK cells could lead to significant clinical advancements and uses in cancer and viral therapies.

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