Natural Killer Cells: More than Meets the Eye

Molecular Medicine

Karen Slattery

Junior Sophister

Natural killer cells are cytotoxic cells critical to the innate immune response. Their key roles are in detection and killing of virally infected and transformed cells. Since their discovery in the 1970s, they have been shown to have many functions involving a variety of biological processes in the body, other than the killing of their traditional target cells. This includes the regulation of angiogenesis in the reproductive tract during pregnancy and bone remodeling during osteoclastogenesis. More recently, experiments in mice have shown the presence of memory NK cells, an unprecedented discovery that has raised many questions concerning the idea of immunological memory and the innate immune response. Accumulating evidence also supports the existence of organ-specific functions in subsets of NK cells. A key example of this phenomenon can be seen in the liver, where hepatic NK cells have distinct phenotypic and functional characteristics in comparison to their counterparts in the blood. As a result, hepatic NK cells play a significant role in the regulation of liver diseases such as liver fibrosis. As the importance of NK cells grows, so does their potential as a therapeutic target. NK cell-based immunotherapy is becoming increasingly significant and can be applied to a huge number of infections and diseases, particularly cancer. This review will discuss the non-traditional functions of NK cells and how they can be regulated clinically.

Introduction to NK Cells

Natural Killer (NK) cells have classically been described as innate lymphoid cells that function in the protection against virally infected and transformed cells. The innate immune response is the body's first line of defense and provides a non-specific, short-lived response to a pathogen (Tosi 2005). NK cells respond rapidly to their targets by killing them via the induction of apoptosis or cell lysis, and the production of appropriate pro- or anti-inflammatory cytokines such as IFN- γ and TGF- β. Cytokines are small signaling proteins that are secreted to regulate many biological processes including the immune response (Dinarello 2007). In addition to their role in the innate immune response, NK cells have also been shown to function in a range of diverse processes in the body, including those as complex as angiogenesis and immunological memory (Moffett-King 2002).

NK cell recognition in humans is through the CD56 marker, a glycoprotein routinely involved in cell-to-cell adhesion. It defines two subsets of NK cells based on its relative surface expression; $CD56^{bright}$ and $CD56^{dim}$ (Poli *et al.* 2009). In the peripheral blood (PB), $CD56^{dim}$ NK cells constitute 90% of all NK cells and are characterized by their high cytotoxicity. The remaining 10% are $CD56^{bright}$ NK cells which are characterized by their cytokine production, such as tumor necrosis factor- α (TNF- α) production (Cooper *et al.* 2001).

The function of NK cells is primarily in the protection against viral infection and cancer. A wide variety of mechanisms are used by NK cells to kill their targets, including cytolytic granule release of toxins such as perforin and granzymes, antibody-dependent cell-mediated cytotoxicty (ADCC) and expression of cytotoxic molecules such as TNF-related apoptosis-inducing ligand (TRAIL). TRAIL is released by NK cells and binds to specific death receptors on target cells, triggering a caspase-8 dependent signaling pathway that results in programmed cell death (Zamai *et al.* 1998).

NK cells also use cytokines to affect the functions of other cells and shape the adaptive immune response. IFN-γ is one of the key cytokines produced by NK cells and has many immunomodulatory,

anti-viral and anti-tumor properties (Schroder *et al.* 2004). TNF-α has pro-inflammatory effects with functions including the regulation of the inflammatory response via activation of transcription factors, control of cell proliferation and differentiation and activation of apoptotic caspase cascades (Krueger *et al.* 2011). TGF-β and IL-10 are examples of anti-inflammatory cytokines produced by NK cells (Yu *et al.* 2006, Mehrotra *et al.* 1998).

The importance of these functions results in tight regulation of NK cells, which is mediated primarily through the balance of activating and inhibitory receptors. The main groups are the killer immunoglobulin-like receptors (KIR) and the NKG2 receptors. Both are a mixture of activating and inhibitory receptors and interact with a variety of immunoregulatory ligands such as the major histocompatibility complex (MHC) class 1 molecules, HLAs (human leukocyte antigens), and stress induced ligands such as MHC class I polypeptide-related sequence A and B (MICA and MICB) and UL16-bringing proteins (Huntington 2014).

Although the traditional view of NK cells is that they are innate lymphoid cells that protect against cancer and viral infections, research from the past 30 years has revealed that there is a lot more to NK cells than meets the eye. This review will focus on the important functions of NK cells that are often overlooked, with particular emphasis on recent developments and the potential for clinical application of these features.

NK cells and Pregnancy

Uterine NK cells (uNK cells) are critical to angiogenesis of the maternal spiral arteries during pregnancy. Angiogenesis involves the assembly and disassembly of the endothelial lining of the blood vessels. Extravillous trophoblast cells (EVT) grow out from the placenta and penetrate the decidualised uterus (Rossant & Cross 2001). They ensure that there is sufficient blood flow to the intervillous space by infiltrating deep into the uterine wall, encircling the walls of the maternal spiral arteries and destroying them if an increased blood supply is required by the fetus (Pijnenborg 1990). It

is essential that the EVT cells infiltrate correctly to avoid any of the common pregnancy disorders that can arise such as pre-eclampsia or miscarriage.

EVT cells express and secrete soluble HLAs, the ligands of the regulatory KIRs expressed by NK cells (Pijnenborg 1990). Along with the fact that uNK cells are always abundant among EVT cells, this suggests that uNK cells may be involved in controlling the extent of placental invasion by EVT cells. Hiby *et al.* (2010) showed that the presence of maternal genes encoding activating KIRs in combination with HLA-C alleles of the fetus correlated with successful pregnancy, with reduced risk of preeclampsia and recurrent miscarriage. It is believed that typically, upon ligation of the KIRs with the soluble HLA molecules secreted by EVTs, uNK cells are stimulated to secrete various pro-angiogenic cytokines (Moffett-King 2002). These include vascular endothelial growth factor C (VEGFC), which stimulates angiogenesis by binding to the VEGFR2 receptor on the vascular endothelial cell. VEGR2 is a transmembrane tyrosine kinase receptor which initiates a signal cascade involving many secondary messengers such as PIP_2 and DAG (Holmes *et al.* 2007). Placental growth factor (PIGF) binds to the VEGFR1 receptor and functions via a signal cascade similar to that of VEGFC (Luttun & Carmeliet 2002). Angiopoietin 2 (ANG2), another vascular growth factor produced by uNK cells, is an agonist for ANG1 and promotes endothelial cell migration & proliferation in the presence of VEGF (Lim *et al.* 2004). Rajagopalan & Long 2012a) reported that binding of trophoblast-derived HLA-G with its uNK cell receptor induced a senescence-associated secretory phenotype that lead to the sustained secretion of pro-angiogenic factors by uNK cells. More recently, Kieckbusch *et al*. (2014) showed that MHC-dependent inhibition of uNK cells impedes fetal growth and decidual vascular remodeling in mice.

Overall, this suggests that uNK cells are involved in the regulation of angiogenesis of the maternal vasculature by detecting signaling ligands released from EVT cells, and responding via the production of various cytokines involved in angiogenesis regulation. The regulation of angiogenesis during pregnancy is vital in order to achieve appropriate blood flow to the growing fetus. This involvement provides interesting insights into the immunology

of reproduction and also supports the idea of NK cells as multi-functional effectors.

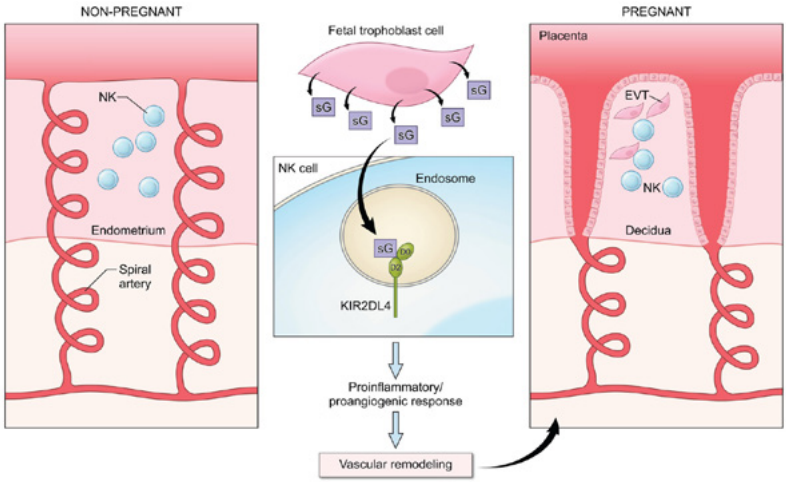

Figure 1: The role of NK cells in vascular remodeling during pregnancy. *Fetal trophoblast cells secrete soluble HLA-G (sG) during pregnancy that is endocytosed by KIR2DL4 receptor. Endosomal signaling results in a sustained proangiogenic secretory response resulting in vascular remodeling and increased blood flow to the fetus (Rajagopalan & Long 2012b).*

NK Cells and Bone Remodeling

In addition to their role in vascular remodeling during pregnancy, NK cells are believed to play an important role in bone remodeling during osteoclastogenesis, which is associated with rheumatoid arthritis (RA) (Söderström *et al.* 2010). Osteoclasts are monocyte-derived cells which break down bone in the presence of the receptor activator of NF-kappaB ligand (RANKL) and macrophage colony-stimulating factor (M-CSF) (Nakagawa *et al.* 1998). Söderström *et al.* (2010) showed that NK cells and monocytes are frequently found in excess at the site of the inflamed joint and that these NK cells express RANKL and M-CSF. Coculturing of NK cells extracted from the synovial fluid of mice with monocytes resulted in differentiation of the monocytes into osteoclasts, and depletion of NK cells resulted in a decrease in the severity of arthritis and prevention of bone destruction (Söderström *et al.* 2010). Overall, this suggests that

NK cells are capable of initiating bone remodeling during RA by triggering the development of bone destroying osteoclasts and activating them via production of RANKL and M-CSF.

Anti-TNF drugs are often used in treatment of RA. For example, infliximab is a monoclonal IgG antibody that binds to TNF-α, one of the key pro-inflammatory cytokines produced by NK cells. Administration of infliximab during randomised, double blinded clincial trials produced significant improvements in all measures of rheumatic disease activity when compared with the placebo resulting in its FDA approval in 2000 (Elliott *et al.* 1994). Perhaps inhibition of NK cells at the site of inflammation would decrease production of these receptor activators and pro-inflammatory cytokines, reducing the severity of the disease and the need for costly drugs such as infliximab. Many experiments have shown that alteration of NK cell activity is a feasible therapeutic strategy. For example, Binyamin *et al.* (2008) reported that antibody-mediated KIR blockade significantly increased NK cell ADCC in human cell lines, while Romagné *et al.* (2009) augmented NK cell killing of tumor cells using the novel monoclonal antibody 1-7F9, which also binds specific KIRs (Binyamin *et al.* 2008, Romagne *et al.* 2009). By adjusting these novel antibodies to block NK cell activating receptors, inappropriate NK cell activity could be stopped and autoimmune disorders such as RA could be treated effectively.

Memory NK Cells

Although it was originally believed that immunological memory was a feature unique to the adaptive immune response, recent evidence suggests that certain subsets of innate immune cells may also have memory. Immunological memory refers to the ability of the immune system to respond more effectively and rapidly to a pathogen upon secondary exposure, due to prior development of antigen specific lymphocytes. O'Leary *et al.* (2006) studied the immune response of T and B cell-deficient mice exposed to haptens. Haptens are small molecules that become immunogenic when bound to a carrier molecule (Lemus & Karol 2008). They found that adoptive transfer of hepatic NK cells from a sensitized donor resulted in a hypersensitive

response upon exposure to specific antigens at least four weeks later (O'Leary *et al.* 2006). Subsequent work found that hepatic NK cells, but not splenic or naïve NK cells, developed memory to vaccines containing antigens from influenza, vesicular stomatitis virus (VSV) and HIV-1, and that it was dependent on the chemokine receptor CXCR6 (Paust *et al.* 2010a). The exact role of CXCR6 in NK cells has not yet been investigated, however it appears to regulate the effector functions and survival of hepatic memory NK cells. Interestingly, NK cells do not express recombination-activating gene (RAG) proteins which are required by T and B cells for their ability to produce the huge numbers of antigen-specific receptors they do (Paust *et al.* 2010b).

This revelation raises many questions concerning the traditional view of NK cells and the innate immune response. The most important mysteries lie behind the molecular mechanisms NK cells use to mediate this antigen-specific response independent of RAG proteins, yet a similar phenomenon has been reported in sea lampreys and hagfish, where recombinatorial assembly of gene segments is used to produce variable lymphocyte receptors (Cooper & Alder 2006). As immunological memory is the primary aim of any successful vaccine, memory NK cells may be used as a novel approach in future vaccine developments, particularly in patients suffering from T or B cell deficiencies, such as in HIV. This comes at a time where vaccine development is one of the top priorities in biomedical research, with increasing antimicrobial resistance and decreasing antibiotic reliability (Davies & Davies 2010).

Tissue-Specific Functions of NK Cells

The distribution of certain subsets of NK cells varies dramatically throughout the body (Carrega & Ferlazzo 2012). One of the key examples of this phenomenon is in the liver. The liver represents a unique immunological environment and has a cellular repertoire enriched with lymphoid cells such as NK cells, NKT cells and γδ T cells (Crispe 2009). Hepatic NK cells are located in the hepatic sinusoids, often adhering to the endothelial cells where they are under constant exposure to gut-derived antigens. While NK cells

normally constitute 5-15% of the total number of lymphocytes in the PB, they can reach up to 25-40% of the total number of lymphocytes in the non-diseased liver (Tian *et al.* 2013).

Hepatic NK cells contain a unique composition of markers on their surface. In contrast to NK cells in the PB where 90% are $CD56^{dim}$ NK cells, $CD56^{dim}$ NK cells & $CD56^{bright}$ NK cells are expressed in equal amounts in the liver (Krueger *et al.* 2011). While the PB $CD56^{bright}$ NK cells are characterized by potent cytokine production, the hepatic $CD56^{bright}$ NK cells have been shown to display increased cytotoxicity & degranulation in response to target cells. This was shown in functional studies carried out by Moroso *et al.* (2010) on NK cell subsets during human liver transplantation. They also showed increased expression of the toxic molecules perforin & granzyme in $CD56^{bright}$ cells via intracellular staining. This, along with upregulated TRAIL expression, results in hepatic NK cells having increased cytotoxicity towards target cells in comparison to those in the PB.

The importance of this increased cytotoxicity can be seen in the role of hepatic NK cell in liver fibrosis. Liver fibrosis is an innate tissue repairing mechanism that involves the formation of excess fibrous connective tissue. When a tissue is damaged, the immune system reacts by producing a variety of immune cells, such as macrophages, that secrete pro-inflammatory and pro-fibrogenic cytokines, leading to the activation and proliferation of hepatic stellate cells (HSCs) (Pellicoro *et al.* 2014). These are induced to transdifferentiate into myofibroblast cells, which are characterized by secretion of extracellular matrix (ECM) proteins such as collagens and glycoproteins. Upon persistent tissue damage, such as in HCV infection or alcohol abuse, fibrosis can progress to a pathological state involving scar formation and impaired organ function. Hepatic NK cells protect against fibrosis by killing early-activated HSCs via mechanisms including cytolytic granule mediated apoptosis and release of TRAIL. They also produce the anti-fibrotic cytokine IFN- γ, which works by antagonizing the effects of the pro-fibrotic cytokine TGF- β (Weng *et al.* 2007, Dooley & ten Dijke 2012). It does this by inducing the expression of inhibitory proteins, known as inhibitory Smads, involved in the TGF- β signaling pathway, and

also by the inhibition of TGF- β dependent collagen expression through competition with the transcriptional coactivators p300 and CBP (Ghosh *et al.* 2001). IFN- γ also induces HSC cell cycle arrest and amplifies NK cell activity via the upregulation of the activating NKG2D receptor and TRAIL expression.

This feature of NK cells can be seen throughout the body, in organs such as the lungs, the spleen and the kidneys, and suggests that the microenvironment of NK cells has a strong influence in the role they play in the immune response. Little is known about the exact mechanism behind this, yet it is likely that there are a multitude of factors involved. For example, macrophages have been shown to influence the phenotypes and functions of lung and splenic NK cells, while gut NK cells may be regulated by signals from dendritic cells, epithelial cells and the luminal contents (Reynders *et al.* 2011, Michel *et al.* 2012). Further research is required to clarify the origin and actions of these tissue-specific NK cells.

NK Cells and Immunotherapy

As the mechanisms that control NK cell cytotoxcity towards infection and disease have been revealed, a wide range of NK-cell based therapies have become possible (Farag *et al.* 2003). Initial trials attempted to use *in vitro* generated NK cells and *in vivo* cytokine therapy, such as IL-2 therapy, to treat cancer, yet the results were not substantial and so researchers are now looking in different directions.

Monoclonal antibodies that bind to cell surface antigens on tumor cells have been developed which aim to attract NK cells to the site of tumorigenesis and induce ADCC. For example, FDA approved rituximab and ofatumumab binds to CD20 on tumor cells, while trastuzumab binds to Her2 (Adams & Weiner 2005). Another potential approach is to block the interaction between NK cell inhibitory receptors and their ligands. Koh *et al.* (2001) reported that blocking of Ly49C and I inhibitory receptors in mice using monoclonal antibodies increased NK cell cytotoxicity towards tumors and decreased tumor growth, suggesting that NK cell

receptor blockade is feasible and would be effective in the treatment of cancer.

Finally, the ability to modulate the balance of activating and inhibitory NK cell receptors and ligands would be a powerful tool in the treatment of disease. Santourlidis *et al.* (2002) showed that KIR expression is controlled epigenetically via DNA methylation. They then used the gene-demethylating agent decitabine to induce the rapid and stable transcription of all KIR receptors in NK cell lines, NK cell clones and freshly isolated NK cells. These advances come at an important time, as the development of resistant tumors and increased relapse has diminished the effectiveness of common cancer treatments such as chemotherapy and ionizing radiation. Each of these approaches also has the potential to be adapted to treat numerous pathological diseases, including fibrosis and viral infections.

Conclusions

Its is clear that defining NK cells simply as innate lymphoid cells may not be an accurate description of these multifunctional cells. The diverse roles of NK cells range from the killing of tumor and virally infected cells, to producing long lasting antigen specific receptors independent of RAG proteins, and even stretch as far as regulating tissue remodeling and repair, as seen in angiogenesis, osteoclastogenesis and fibrosis. The more we discover about the role of NK cells, the less clear the line between the innate and adaptive immune response becomes.

With this increase in the functions of NK cells comes an increase in their potential as a therapeutic target. The possibilities of NK cell based therapies are growing and are relevant to a huge number of infections and diseases, including the one with the most sought after treatment, cancer. Future research must concentrate on determining the underlying mechanisms used by NK cells to carry out these functions, so that diseases can be diagnosed effectively, understood at a molecular basis, and treated using novel immunotherapies.

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