Title: Natural Killer Cells in Placentation and Cancer: Implications for Hypertension during Pregnancy.

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Abstract

Hypertension during pregnancy is the most common medical condition encountered during gestation. Despite this, knowledge of the mechanisms that underlie the disease and the development of new therapies are limited. Hypertension during pregnancy and some forms of cancer confer an increased risk to the development of cardiovascular disease later in life; one mechanism which may link these conditions is the involvement of natural killer (NK) cells. Whilst immunology and immunotherapy are well-developed areas in oncology; the complex mechanisms of the immune system in health and disease at the maternal-fetal interface are less well-defined. Natural killer (NK) cells have emerged as key immune cells involved in physiology and pathology of pregnancy. These small lymphocytes are present in the decidua (the uterine-specific uNK cells) and are distinct from peripheral NK cells. The uNK cell population plays a vital role in mediating trophoblast invasion and affecting decidual vascular remodelling whereas the role of the peripheral NK cell population during pregnancy is less well-defined. This review will give an overview of NK cell biology followed by a discussion of the current evidence for the role of uterine and peripheral NK cells at the maternal-fetal interface in health and disease. Furthermore, examples of NK cell research from cancer biology will be employed to inform future directions of research. By combining this knowledge from oncology where the field of immunotherapy has now matured into clinical trials; it is hopeful that new mechanisms can be elucidated to generate targets for similar therapeutic strategies for women with hypertensive pregnancies where interventions are needed.

Keywords: placenta, cancer, immunology, natural killer cells

Introduction

Hypertensive complications, including preeclampsia, pregnancy-induced hypertension (PIH) and maternal chronic hypertension, are the most common conditions encountered during pregnancy (1). Hypertension during pregnancy has a profound impact on the immediate and future health of both the mother and child. In this review, the hypertensive complications of pregnancy are considered together and not as discrete entities. The presence of maternal chronic hypertension predisposes the development of preeclampsia and PIH (2); concurrently, the development of PE and PIH lead to an increased incidence of cardiovascular disease later in life (3). The development of hypertension during pregnancy confers increased risk of cardiovascular disease later in life (4); as do some forms of cancer (5); one mechanism that may link these conditions is the involvement of natural killer (NK) cells. While several aspects of the immune system could be involved, pathological changes in NK cells which are capable of recognizing and responding to ligands presented by the feto-placental unit during a normal pregnancy may facilitate communication between placental physiology and the maternal cardiovascular system. This review will focus on one particular member at the interface of the innate and adaptive immune system: the natural killer (NK) cells. The aim of this review is to summarise the role of NK cells during healthy and hypertensive pregnancy and how the future direction of this field of research could benefit from what is known about these cells in oncology.

Natural Killer Cell Biology

NK cells are lymphocytes that exhibit traits of both the innate and adaptive immune system that differentiate and mature in the bone marrow, lymph nodes, spleen, tonsils, and thymus, where they then enter into the circulation (6). NK cells were once thought to be a unique example of an innate lymphoid cell (ILC); however recent work has identified that the NK cell belongs to a much larger family of ILCs and is representative of a heterogeneous immune cell population (7). NK cells have been divided into several groups depending on their cytokine profiles and functions which include cytotoxic and regulatory cells. Moreover, it is apparent that tissue resident NK cells may differ from classical peripheral blood NK cells. This distinction is particularly important at the maternal-fetal interface where uterine-specific uNK cells in the decidua have immunophenotypic and functional properties distinct from the peripheral blood NK cells (8).

Cytotoxic NK cells, which form the majority (95%) of peripheral NK cells, are large granular lymphocytes defined in human peripheral blood as $CD56^{Dim}$ with potent cytotoxic ability (as their granules contain perforin and granzyme) and the ability to produce interferon γ (IFNγ) (9). In contrast, regulatory NK cells are agranular, smaller lymphocytes which are $CD56^{Bright}$ with limited cytotoxic ability but principally produce cytokines (such as IL-5, IL-13, TNFα, LT) upon activation (10). Other regulatory subsets of NK cells exist but are less well defined, they are principally characterised by the release of anti-inflammatory and regulatory cytokines such as transforming growth factor β1 (TGFβ1) and interleukin 10 (IL-10) (11). A similar attempt has been made to delineate cytotoxic and regulatory subtypes using $CD27^{+/-}$ as a marker in mice and Ly49s3 or NKR-P1B receptor in rats, however in both cases the distinction between the NK subsets is not as marked as in humans

(12). The remaining subsets of NK cells have not yet been subject to extensive pre-clinical studies. This review will principally focus on the cytotoxic subset of NK cells.

Spontaneous cytotoxic ability of NK cells is mediated through the perforin/granzyme B pathway leading to apoptosis and/or lysis of virus-infected or cancerous cells. They also produce a number of cytokines which recruit and regulate cells of the adaptive immune system (13). NK cells are described as immune sentinels and thus have a widespread distribution throughout the body. They are unique in that they are able to identify stressed or infected cells without antibody-based recognition therefore providing a rapid immune reaction. NK cells normally represent a small percentage of the lymphocyte population, for example, 5-10% in the spleen and 2-18% of the peripheral blood mononuclear cell (PBMC) lymphocyte population in humans (14). The potent cytotoxic ability of NK cells is regulated on three levels. Firstly, NK cells undergo an education process whereby only those that recognise “self” are promoted to having cytotoxic ability (15). Secondly, these cytotoxic cells are tightly regulated by a sophisticated system involving a complex of interactions between the target cell and either activating or inhibitory receptors on the NK cell surface. NK cell surface receptors classically recognise MHC class I ligands as “self” or when these are up-regulated due to stress, “dangerous self”, or missing, “missing self”, such as during infection or in cancerous cells. Only NK cells that recognize “self” become tolerant, hence why they act when there is "dangerous self" or "missing self". NK cells can also recognise cell adhesion or virally-derived molecules. Finally, resting NK cells have a relatively low cytotoxic ability compared to “primed” NK cells. Priming involves a translational switch of the mRNA of cytotoxic molecules abundant in resting NK cells resulting in activation of the cytotoxicity (16). Priming is regulated by the cytokine microenvironment in which the NK cell is

present; such as type-1 interferons, IL-12, IL-18 and IL-15 to varying degrees in humans and rodents (17). It can also be regulated by close proximity of other immune cell types such as T cells, monocytes and dendritic cells (18-20).

Receptors on NK cells belong to the family of killer cell Ig-like receptors (KIRs) and can be predicted to be either activating or inhibitory based upon a characteristic immune-receptor tyrosine-based activation or inhibitory motif; ITAM or ITIM, respectively. These specialised domains are phosphorylated by Src kinases resulting in the recruitment of scaffolding proteins for further signal transduction in the case of ITAMs; or recruitment of protein phosphatases to turn off signalling in ITIMs (21) . Whilst the general regulatory mechanisms of NK cell surface receptors are conserved between humans and rodents; the identity of these receptors are not directly comparable (Tables 1 & 2). For example, CD56, the main extracellular pan-marker of NK cell populations in humans, is not expressed in rodents. In addition, receptors which are shared between species bind different ligands (12). Therefore, direct comparison of NK cell populations between rodents and humans requires care. Inter-species variation in cell surface receptors also exists between rats and mice. Additionally, further complexity exists as intra-species variation is observed in different strains of laboratory mice (22).

Natural Killer Cells at the Maternal-Fetal Interface

The study of NK cells in pregnancy has principally focused on the uterine specific population of these cells; known as uNK cells. These cells are considered to be a specialised, tissue-specific population whereby they exhibit a characteristic granular appearance but display significantly less cytotoxicity towards, for example, cancer cell lines than peripheral NK cells (23), thus they do not normally kill the

invading trophoblast. This aspect of biology of uNK cells is particularly interesting as they still contain large granules with granzyme B and perforin, pointing to possible alternate functions in these unique NK cells. In contrast to peripheral blood NK cells, where CD16 allows for discrimination of cell cytotoxic and regulatory subsets, uNK cells in humans are almost exclusively $CD16^{-}CD56^{Bright}$ (24); however, the uNK cell population does not fit exactly with the NK2 phenotype despite the $CD56^{Bright}$ phenotype but rather a variety of NK cell subtypes are represented. Both large granular and small agranular lymphocytes are identifiable microscopically, these uNK express a unique repertoire of receptors, and they have more potent cytokine producing ability (24). The major population of uNK cells in the decidua are of the TGFβ producing NK3 type; accounting for 20% of the uNK population (11). A similar characterisation of uNK cell markers has been conducted in mice which showed a unique $CD3^{-}CD122^{+}NK1.1^{-}DX5^{-}$ population[1] which was considered to represent murine uNK cells (25). In contrast to human uNK cells, murine uNK express high amounts of CD16 (25). A similar repertoire of receptors expressed by the rat uNK cell population has not yet been reported in the literature partly due to the limited commercial availability of rat-specific NK cell receptor antibodies, however ANK61 (26) or perforin (27) has been used as a semi-quantitative measure for uNK cells using immunohistochemistry.

The origins of uNK cells are of great interest, although remain unclear. They show ability to proliferate and differentiate from early NK progenitor cells or CD34+ cells which are recruited to uterine wall from blood (28). Residual endometrial NK cells may contribute (29) and/or mature NK cells may also be recruited during pregnancy upon release of paracrine factors from the trophoblast (30). These

[1] NK1.1 is also known as CD161b/c and Ly55. DX5 is also known as CD49b.

diverse origins may also be reflected by morphological and functional diversity of the uNK cells.

In humans, decidualisation occurs with each menstrual cycle but in rodents only occurs in response to implantation. Upon decidualisation, endometrial stromal cells become characteristically round in appearance and express factors such as prolactin, growth factors, pro-angiogenic factors and cytokines (IL-11 and IL-15) which stimulate the differentiation and proliferation of the uNK cell (31). Decidualisation is associated with a marked increase in NK cell number; approximately 75% of infiltrating leukocytes within the decidua are NK cells (24). The other 25% are composed of macrophages, few T cells and very few B cells (24). In addition to the invading trophoblast, uNK cells play an important role in maternal spiral artery remodelling. Compelling evidence of this is seen in pregnant mice which are genetically deficient for NK cells where the smooth muscle layer of the uterine spiral arteries remains intact (32). It has been shown in rats that the early increase in uNK cells directs early trophoblast invasion followed by a recession in number during mid-gestation where trophoblasts take over as the main effectors of vascular remodelling (33). However, recent work which employed antibody-based NK cell depletion in a rat model of preeclampsia did not replicate a deficiency in uterine spiral artery remodelling but did identify marked maternal uterine vasculopathy at a later time point in gestation (26). The uNK cells produce several key mediators of vascular remodelling ranging from IFNγ to matrix metalloproteinases (MMPs) and pro-angiogenic factors which can directly promote vascular remodelling (34). They also communicate with the invading trophoblast through the unique human leukocyte antigen (HLA) repertoire found on trophoblasts to indirectly mediate trophoblast-dependent spiral artery remodelling (24). The interrogation of the underlying

mechanisms of uNK cell-mediated vascular remodelling is difficult due to the shallower trophoblast invasion seen in mouse models and the previously discussed strain-specific NK cell adaptations. Methodologically, there are no current knockout models which specifically deplete uNK cells or an antibody-based method of depleting this cell population in particular.

Changes in peripheral NK cells do not directly mirror the status of uNK cells during pregnancy; therefore the two populations should be considered separately (11). Additionally, it has been shown that uNK cells, but not peripheral NK cells, can promote trophoblast invasion and decidual vascular remodelling (35). In humans, circulating NK cell numbers (total $CD56^{+}$) are increased relative to non-pregnant women in the first trimester followed by a decline in late pregnancy. The cytotoxicity of these cells follows this pattern (36). A similar temporal study of peripheral NK cell number over pregnancy has not yet been reported in rodents. Alterations in peripheral NK cells have mostly been associated with recurrent pregnancy loss and infertility; however these findings are based on small, observational studies. A recent meta-analysis of the available literature indicated that an increase in peripheral NK cells, but not uNK cells, is seen in women with recurrent miscarriage. Notably, this analysis did not discriminate between $CD56^{Bright}$ and $CD56^{Dim}$ NK cells (37). The authors correctly identified that there is a pressing need for further research before immunotherapy should be used clinically (37). Other small studies have also explored a pro-inflammatory shift of NK cells in preeclampsia (38). In particular, IFNγ producing peripheral NK cells are increased in women with preeclampsia (39). Recent work has elucidated a causal role for IFNγ produced by NK cells in vascular dysfunction in an angiotensin II dependent mouse model of hypertension (40); such a mechanism has not been explored in hypertensive pregnancy. Whilst peripheral

NK cells may not play a major role in the vascular remodelling at the maternal-fetal interface, peripheral NK cells can produce vascular endothelial growth factor (VEGF) which is important for the maintenance and function of the systemic vasculature; this production has been shown to be impaired in women with preeclampsia (41). In pre-clinical work, studies in a murine model that exhibit a 90% depletion of peripheral NK cells showed that maternal mean arterial pressure (MAP) was increased in mid-gestation indicating that NK cells may play a role in blood pressure regulation (42). As the science which underpins our knowledge of peripheral NK cells and their role in pregnancy is, as yet, in its infancy looking to another field where NK cells are well assessed, such as oncology, may give us clues as to how to proceed in future research.

NK-T Cells at the Maternal-Fetal Interface

NK cell surface markers can also be expressed by a small group of T cells; these are defined as NK-T cells, and have completely distinct origin and effector functions although NK-T cell activity promotes NK cell activity by secreting IFNγ. The majority of NK-T cells are defined as invariant NK-T cells (iNKT) which uniquely express a semi-invariant T cell receptor (TCR) α chain which can be activated by the synthetic glycolipid α-galactosylceramide (43). A smaller, less-characterised population of non-invariant NK-T cells express diverse TCRs (non-iNKT) and have restricted expression and are not activated by α-galactosylceramide (43). NK-T cells are able to produce both T_h1 and T_h2 cytokines.

NK-T cells have also been identified in the human and mouse decidua at a similar frequency of approximately 0.5% (43). Studies have shown that iNK-T cells may play a role in spontaneous pregnancy loss (44) but, thus far, this cell population

234 does not appear to be altered in the peripheral blood of women with hypertensive
235 pregnancy (45). Non-iNK-T cells may play a regulatory role in trophoblast invasion
236 and immune response in the decidua (46).

237 **Natural Killer Cells and Oncogenesis**

238 As sentinels of the immune system, NK cells are intrinsically involved in the
239 identification and clearance of transformed cells. This role is thought to be principally
240 fulfilled by the cytotoxic type population. Indeed, NK cells were first identified as a
241 sub-population of lymphocytes derived from mice which exhibited spontaneous
242 cytotoxicity against a cancer cell line (47). Pre-clinical experiments in NK cell
243 deficient or depleted mice highlighted that these cells have an active role in
244 immunosurveillance (9), directly limiting tumor growth and metastases (48) as well
245 as identifying key cytokines (IL-2, IL-12, IL-15, IL-18 and IL-21) which promote the
246 maturation and recruitment of NK cells leading to greater tumour clearance (49). In
247 support of these findings, epidemiological studies in humans have also identified that
248 both men and women with NK cells of naturally medium or high cytotoxicity have a
249 reduced overall risk of developing any type of cancer relative to those individuals
250 with low NK cell cytotoxic activity (50). The tumour microenvironment subverts the
251 killing potential of these cells by releasing soluble factors such as TGFβ1 (51) as well
252 as co-opting other immune cells such as T regulatory cells (Tregs) (52) or tumour-
253 associated fibroblasts to alter the behaviour of NK cells. With respect to their effector
254 function, NK cells mediate the destruction of cancer cells in two ways: (i) directly
255 through the granzyme B/perforin pathway or through activation of the TNF-related
256 family of receptors (9); (ii) by expression of death receptor ligands such as FasL and
257 TNF-related apoptosis-inducing ligand (TRAIL) and (iii) indirectly through recruitment
258 and activation of other immune cells by production of chemokines and cytokines

such as IFNγ and TNFα among others (53). These mechanisms are responsible for apoptosis and killing of virus infected cells or tumour cells. However tumour microenvironment can significantly affect NK cell function and allows for the failure of immune surveillance (54). Thus in similarity to placentation, NK cells are responsive to their environment which may create significant therapeutic opportunities in both conditions.

Natural Killer Cells in Placentation and Cancer: Future Directions

Research regarding the characterisation and role of peripheral NK cells in the hypertensive conditions of pregnancy lags behind the relatively well-developed cancer literature. Considering the fields of pregnancy and oncology together can be of mutual benefit; however, this review is centred upon pregnancy research. A note of caution for interpreting both of these fields together is that the uNK cell population is a highly-specialised tissue specific group; drawing comparisons between peripheral NK cells only in cancer and pregnancy may be more appropriate. Further studies which span clinical samples and pre-clinical animal models are required to measure the presence of NK cells at the maternal-fetal interface and how this population affects the development and maintenance of the maternal-fetal interface and perhaps further afield in the maternal systemic vasculature. The tumour microenvironment suppresses NK cell behaviour. Do such immunosuppressive mechanisms also exist in a physiological situation such as pregnancy where immune tolerance is vital and NK cells are intimately associated with the maternal-fetal interface? The TGFβ family are expressed in the trophoblast and early placenta (55) whilst a number of studies have proposed a link between mutations in the TGFβ gene and preeclampsia risk (56). TGFβ has also been shown to induce peripheral NK cells to show a uNK cell phenotype (57). The interaction between Tregs and NK

cells is subject to investigation whereby an increase in Tregs during pregnancy is considered to be beneficial to promote fetal tolerance (58).

NK cells are professional cytotoxic lymphocytes and, in addition, can potentiate inflammation by the secretion of pro-inflammatory cytokines such as IFNγ and TNFα and through recruitment of other pro-inflammatory cells. This behaviour is common in a number of conditions such as cancer, cardiovascular (59) and autoimmune diseases (60). However, research into the actions of peripheral NK cells is lacking in the area of pregnancy and associated complications. Whilst immunotherapy is a well-developed area in cancer research reaching the stage of clinical trials; it is a relatively new area in treating pregnancy complications. Whilst cancer immunotherapy is focussed on the activation of peripheral NK cells to target and eliminate tumours, in contrast, immunotherapy during pregnancy should be focussed on suppressing the killer potential of these cells. Our own recent work has shown that treatment with etanercept, a TNFα antagonist, improves vascular function and pregnancy outcome in a rat model of chronic hypertension in pregnancy. One source of this detrimental TNFα was found to be peripheral NK cells present in both the circulation and placental tissue (61). In light of the current literature, it is clear that NK cells have a multifaceted role in pregnancy and a delicate balance exists between the regulatory and effector functions of this population of cells. Targets should be identified which are involved in the role of excess activation or number of detrimental NK cells that preserves the vital vascular remodelling and immune regulatory properties of uNK cells.

In summary, NK cells are a diverse population with various regulatory and effector functions that are both context and tissue dependent. Nevertheless, there are properties of NK cells which are common in both the fields of placental biology

and oncology which may inform future research directions. Further, the newly described subsets of NK cells in humans have yet to be identified and characterised in rodents. New insight into these populations of NK cells and investigation into their role in the maintenance of health or pathology of various diseases is warranted. NK cells represent an emerging area of research which has the potential to elucidate new mechanisms and identify potential targets to treat hypertensive conditions of pregnancy where novel therapies are much needed.

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Tables

Table 1: Common Pan-Markers of NK Cells in Humans and Rodents

Species	Common Pan-Marker of NK Cell
Human	$CD3^-$ $CD56^+$
Mouse	$CD3^-$ $CD49b^+$/$CD122^+$
Rat	$CD3^-$ $CD161^+$/ANK61

NK cell markers can also be expressed by a smaller subset of T cells therefore the absence of CD3 is a prerequisite for identifying NK cell populations. CD56, the main pan-marker for human NK cells, is not expressed in rodents. CD122 is a marker of NK cell lineage and is expressed at all stages of mouse NK development and CD49b (also known as DX5) is expressed on the majority of murine NK cells. CD161 (which binds NKR-P1A and NKR-P1B) are expressed on all rat NK cells. CD161 and ANK161 antibodies are raised against the same antigen.

Table 2: Major NK Cell Surface Receptor Families Differ between Humans and Rodents

Humans	Mice	Rats
NKR-P1A	NKR-P1B/-D/-F/-G	NKR-P1A/-B/-F/-G
CD94/NKG2	CD94/NKG2	CD94/NKG2
NKG2D	NKG2D	NKG2D
KIR	Ly49	Ly49
Nkp46	Nkp46	Nkp46
Nkp44	-	-
Nkp30	-	Nkp30

The NKR-P1 family were the first group of receptors to be characterised in NK cells. One inhibitory receptor, NKR-P1A, is present in humans whilst there is an extended family in mice (NKR-P1B/-D/-F/-G) and in rats (NKR-P1A/-B/-F/-G) which act as either activating or inhibitory receptors. CD94/NKG2D expression in all species is dependent upon cytokine environment of the NK cells. NKG2D plays a major role in mediating cytotoxicity against transformed or stressed cells in all species. Human killer cell immunoglobulin-like receptors (KIRs) play a critical role in recognising the invading extravillous trophoblast; however these are not conserved in rodents. The rodent Ly49 receptors are functionally equivalent but structurally dissimilar to the human KIR family. The Nkp family are activating receptors which also play a key role in recognition and clearance of transformed cells. Nkp46 is expressed by all species whereas Nkp44 is human-specific and Nkp30 is in humans and rats only.