

Review

Uterine Natural Killer Cells: Their Choices, Their Missions

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Uterine natural killer (uNK) cells, sharing many characters with peripheral blood natural killer (pNK) cells, are a major uterine lymphocyte population at early gestational stages during normal pregnancy in placental mammals. The functions of uNK cells include cytokine production and cytotoxicity that are regulated by signals through activating and inhibitory receptors. uNK cells differ from pNK cells however and contribute to the structural changes that accompany the differentiation of the maternal-fetal interface. Immunological mechanisms must provide a balanced environment for uNK cell proliferation, differentiation and activation through intricate signaling pathways. An improved knowledge of mechanisms regulating uNK cells development and the cytokine network at the maternal-fetal interface of mice and humans might be useful to harness the power of these cells for maintenance of pregnancy. *Cellular & Molecular Immunology*. 2005;2(2):123-129.

Key Words: uNK cell, pregnancy, lymphocyte differentiation, lymphocyte activation, cytokine

Introduction

For years, scientists regarded natural killer (NK) cells as a blunt instrument in the body's immune defense system (1). Born to kill, these cells were thought to travel straight from the bone marrow, where they are manufactured, to the blood, circulating there and infiltrating the sites of early tumors or infectious agents to provide non specific, early self-defense. NK cells constitute around 5%-15% of circulating lymphocytes, and they have been identified in the spleen, lungs, gastrointestinal tract, liver and uterine decidua (2). Interestingly, NK cells are abundant in the pregnant uterus where, in primates, they express high levels of CD56 and little or no CD16. These large granulated lymphocytes are thought to contribute to success of pregnancy and are termed uterine natural killer (uNK) cells (3, 4). Koopman and her colleagues consider that uNK cells represent a distinct lineage of NK cells, possibly arising from a special hematopoietic precursor,

or that their differences from pNK reflect subsequent maturation of peripheral (p) NK cells (most likely the CD56^{bright} subset) in the decidual microenvironment (5, 6). Most uNK cells appear to be activated, cytokine-producing NK cells during the uterine decidualization in women, non-human primates and rodents in early gestation (7). Mature uNK cells are not frequent at the end of normal mammalian pregnancy. These cells have recently become a hot topic in reproductive immunology.

NK cells act as a first line of defense before the development of adaptive immunity by B and T lymphocytes, and have multiple pathways (choices) for activation. Cytokines, immune complexes, adhesion molecules, classical and nonclassical major histocompatibility complex (MHC) molecules, pathogen-associated ligands and stress-induced ligands initiate multiple and possibly redundant pathways that culminate in activation of cytotoxicity and/or production of cytokines. The signaling pathways that regulate B and T lymphocytes are conserved between human and mice, but differ for NK cells. The different pathways may converge on shared signaling modules (8). Placental mammals have been subjected to two opposing selective pressures during evolution, as survival of the species depends on the ability to eliminate microbial pathogens while at the same time protecting fetuses from immune rejection. During pregnancy, it is necessary for uNK cells to reach a decision choosing between the final goals of reproduction and maintenance of maternal and fetal health. This is a tough choice and we do not yet understand the rules that govern decision making by these cells.

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Abbreviations: uNK cell, uterine natural killer cell; DB, decidual basalis; MLAp, mesometrial lymphoid aggregate of pregnancy; KIR, killer inhibitory receptor; ITIM, immunoreceptor tyrosine-based inhibitory motif; VEGF, vascular endothelial growth factor; IFN, interferon.

Life history of uNK cells

The precise steps in NK cell development are becoming clear. Haematopoietic NK precursors have been identified in many sites during different phases of intra-uterine or adult life, including the yolk sac, aorta–gonad–mesonephros region and liver in the embryo and fetus, and the bone marrow, thymus, spleen, omentum and liver in adults (9).

Uterine NK cell development and maturation share some aspects with NK cell development in other tissues, but also display distinctive tissue-specific regulation (10). Murine uNK cells have an absolute requirement for IL-15 in their differentiation (11). Uterine NK cells in rodents and primates also require progesterone-regulated development of decidua which supplies IL-15. Transplants from uNK cell sufficient mice to NK cell deficient have shown that uNK precursors are present in fetal liver and thymus, and in adult thymus, bone marrow, LN and spleen (12). Adult secondary lymphoid organs were the best source of transplantable uNK cell precursors. Precursors of uNK cells have been described as small, non-granulated lymphocytes throughout the uteri of virgin mice (13). The morphology of those cells remains constant until gestation day (gd) 6 (the morning of copulation plug detection which is called gd 0) (14), the earliest time at which they produce interferon (IFN)- γ (15). Then the cells begin to proliferate rapidly, acquire cytoplasmic granules that contain perforin, serine proteases, mucins and other molecules (13). From this stage to late gestational, dynamic changes in murine uNK cells and development of subtypes can be clearly distinguished under light microscopy in sections stained by *Dolichos biflorus* agglutinin (DBA) staining (16). At gd 8-10 (of a 19-20 day gestation period), murine uNK cell numbers are increased and distributed mainly in the decidual basalis (DB) and mesometrial lymphoid aggregate of pregnancy (MLAp, 17). Subsequently, invasive fetal trophoblast cells infiltrate these maternal tissues. A small number of DBA lectin⁺ uNK cells can also be detected in the placenta. Uterine NK cells progressively increased in number with the peak at gd 10-12 in the DB and MLAp. After gd 12, uNK cells undergo a slow form of cell death and disappear from implantation sites. Uterine NK cells enlarge throughout this time, first during the acquisition of more and more cytoplasmic granules and during senescence when they enlarge from a being bloated lymphocytes of 40-45 μm to disintegrating cells $> 80 \mu\text{m}$.

Human and non human primate (18) endometria also host significant numbers of uNK cells, the percentage and phenotype of which change during the menstrual cycle. However, if pregnancy occurs, uNK cells persist in the DB at early gestational stages. The presence of these uNK cells is hard to be identified morphologically without immunohistological or other special stains; the cells are absent from term decidua of normal pregnancy (19).

Function of uNK cells

In secondary lymphoid organs, NK cells await activation

(probably after stimulation by sentinel dendritic cells) (20) before they react in two distinct modes (21). NK cells burst forth from the tonsils, lymph nodes and spleen, and destroy infected and cancerous cells while the immune system's T and B cells are still mobilizing (22, 23). These lymphoid-organ derived cells are mainly CD56^{bright}CD16⁻ and they lack perforin, killer inhibitory receptors (KIRs) and all natural cytotoxicity receptors (NCRs) except low level NKp46 found on natural killer cell precursors. In one activation mode, uNK cells promptly secrete cytokines, chemical messenger proteins, which modulate emerging T and B immune cell responses. In the other, they become potent killers of tumors and virus-infected cells. Without natural killer cells, threatening conditions can get a strong foothold before the adaptive immune response kicks in. Given this heritage, it is an intriguing challenge for immunologists to explain functions of uNK cells in the pregnant uterus. Historically, the mammalian fetus has been regarded as a successful allograft, a tumor or a parasite. The fetus is never in direct contact with uterine/maternal tissues. Instead, the contact is mediated through the placenta, a transient organ rimmed by trophoblast cells preferentially expressing paternal genes. Placental trophoblast cells come in close contact with the maternal tissues forming the so-called maternal-fetal interface. There is no doubt that the maternal immune system is able to recognize and react to fetally-derived antigens. However, the fetus is recognized in such a way that the MHC-specific, acquired arm of the maternal immunity is suppressed (7). Because the interface between trophoblast cells of the fetal placenta and the leukocytes of the maternal decidua is unique, pregnancy cannot be treated simply as a case of acceptance or rejection like a transplant. The immunological mechanism must provide a balanced environment whereby the conceptus is nurtured by the mother, prevented from excessive invasion and protected from pathogens (24).

As a major immune cell population in the mammalian pregnant uterus, uNK cells contribute to the maintenance of pregnancy (2, 5, 6). During the pregnancy, hemiallogeneic fetal cells invade the maternal decidua but remain spared from attack by the maternal immune system, posing a great unsolved paradox of immunology (25, 26). One prominent feature of the pregnant human and murine decidua is the striking abundance of NK cells, which constitute around 70% of resident lymphocytes (7). In contrast, NK cells in peripheral blood usually comprise about 15% of circulating lymphocytes (24). This enrichment suggests NK cells in the uterus have different functions and may respond to novel ligands presented by decidua and/or trophoblast, unique elements of the pregnant uterus. uNK cells are thought to play vital roles in implantation and in pregnancy of human (5, 7), rhesus monkey (18) and mice (2), especially before mid-gestation.

The comprehensive functions of uNK cells still remain unknown, a direct or indirect role in maternal-fetal tolerance has been suggested in early steps of the implantation/pregnancy process. The two possibilities that are currently under investigation (which are not mutually

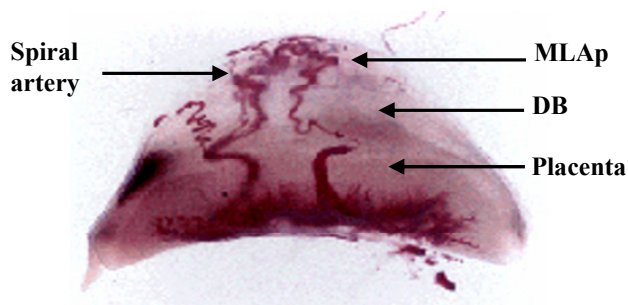


Figure 1. Uterine spiral arteries of an immune competent mouse at gd 10. Uterine NK cells are mainly distributed in decidual basalis (DB) and mesometrial lymphoid aggregate of pregnancy (MLAp), rather than in the placenta. The vascular remodeling increases the bulk flow and the supply of nutrients and oxygen to the fetus. Triggering of decidual spiral artery dilation is largely mediated by IFN- γ derived from uNK cells in normal mice, as well as other cytokines. For human, remodeling of uterine spiral arteries occurs in the first trimester of pregnancy, with failed remodeling a characteristic of preeclampsia (Reproduced with permission from Dr. Y. Kiso and B.A. Croy).

exclusive) are that uNK cells influence maternal mucosal and arterial function (27) and/or placental trophoblast invasion (28). In particular, roles for them in placental development and in vascular remodeling of uterine spiral arteries have been suggested in the mice (29).

During early pregnancy in humans and mice, uNK cells are required for the proper formation of the uterine vasculature and the deciduas (30). Maternal vessels crossing the DB are highly coiled and can be referred to as spiral arteries (SA) (Figure 1). In pregnancy, the architecture of the SA is profoundly modified by angiogenesis and thereby maintains the expanded tissue of the decidua for successful implantation. uNK cells were often located near the blood vessels, and expressed vascular endothelial growth factor (VEGF), a potent growth factor involved in angiogenesis and microvascular hyperpermeability, suggesting a possible role for these highly mobile cells in inducing specifically localized angiogenesis during the development of decidua/MLAp through VEGF production. Other angiogenic factors like FGF, EGF and angiopoietins have been also demonstrated in the decidua and non-pregnant endometrium, especially in uNK cells containing high levels of mRNAs for the angiogenic growth factors, VEGF-C, PlGF, and Ang2 (31). During decidualization and normal pregnancy, IFN- γ production induced by uNK cells has addressed the concentration of IFN- γ needed per implantation site for induction of spiral artery modification (15).

Studies of the immunoregulation of trophoblast invasion are hampered by the difficulty of duplication of the specialized uterine environment *in vitro*. By three-dimensional tissue culture model, composed of rounded fragments of endometrial or decidual tissue and multicellular spheroids of choriocarcinoma cells, it was shown that a few CD56⁺ cells were found directly at the invasion front, as well

as between the choriocarcinoma cells (32). These cells also contained the cytolytic granule protein perforin indicating a migration of NK cells with cytolytic potential toward the potentially invasive cells. Recent data suggest that decidua directly controls trophoblast invasion (33) using members of the $\alpha 2$ macroglobulin gene family of proteases and hormone and cytokine transporters. The role of uNK cells is inducing IFN- γ mediated upregulation of these genes (34).

Roles of uNK cells in the cytokine network

Cells communicate by producing soluble molecules or by expressing cell surface ligands. These signals are recognized by receptors on separate cells and transformed into biochemical information by intracellular adaptors, molecular scaffolds, second messengers and enzymes that regulate cellular responses to the signals.

NK cells are specialized in detecting aberrant cells in the body. Once fully differentiated, human and mouse NK cells also show similarities in cytokine responsiveness. In general, IFN- α , IFN- β , IL-12, IL-15 and IL-18 induce the same biological effects in human and mouse NK cells, in part due to the activation of conserved intracellular signaling pathways. The cellular partners that provide these cytokine signals to NK cells are macrophages and dendritic cells; together these three cells operate in synergy to provide an amplification system involving tumor necrosis factor (TNF) and IFN- γ that primes innate as well as adaptive immunity. This suggests that NK cells are part of an evolutionarily ancient cellular and molecular network, which operated before the advent of the components of adaptive immunity.

The uNK cells can be a significant source of immunoregulatory cytokines that alter local immune responses, such as IFN- γ production (Figure 2). IFN- γ contributes to initiation of uterine vascular modification, decidual integrity and uNK maturation during normal murine pregnancy (35). IFN- γ was also detected in DB of IL-12^{-/-}/IL-18^{-/-} mice by ELISA, this suggests induction (IL-12) and enhancement (IL-18) of IFN- γ production in the implantation site may be partially compensatory (36). Demonstration of IL-23 α (p19) and IL-27 α and β transcription offers potential alternative pathways for uNK cell activation (36-39). The IFN- γ may exert a detrimental effect on vasculature remodeling during pregnancy development. It was observed that some women with recurrent pregnancy loss (RPL) have increased IFN- γ (+874) A/A and T/A genotype (40). Our studies of gene expression in endometrium underlying health and early arrested, normally-conceived littermate pig fetuses have shown messages for IFN- γ and TNF- α are elevated > 10⁵ fold at day 23 of a 150 day pregnancy in association with the incipient pregnancy failure. This suggests enormous changes in cytokines can be highly localized and may participate in fetal loss. Maternal VEGF expression was also lost in implantation sites containing arrested conceptuses, indicating a partnership between implantation site angiogenesis and the immune system (41).

It was demonstrated that uNK cells require IL-15 for their

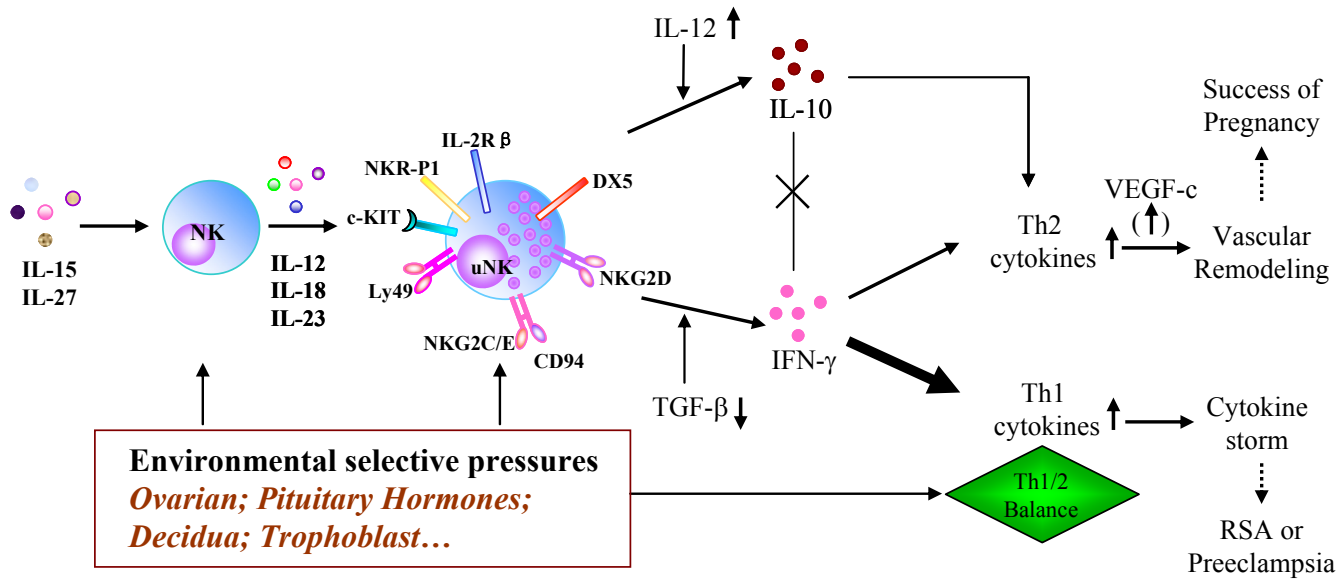


Figure 2. Roles of uNK cells during the pregnancy. Uterine natural killer cells are small and agranular in the earlier proliferation phase, then they proliferate, differentiation, enlarge and become increasingly granulated lymphocytes mediated by the IL-15, IL-27 and other cytokines including IL-12, IL-18 and IL-23. Different receptors or ligands were known to regulate the activities of NK cells, and their unusual phenotypic and functional properties were balanced in the immune response of pregnancy under the multiple environmental pressures. There are still questions remained to elucidate the Th1/Th2 paradigm on the outcome of the materno-fetal relationship.

differentiation during pregnancy. Implantation sites of IL-15^{-/-} mice had no uNK cells, no spiral-artery modification. Ashkar suggested that, in decidua, the expected IFN-regulatory factor (IRF)-1 regulation of IL-15 does not occur (11). Locally produced TGF-β1 (42) and mHLA-G (43) are likely mechanisms that regulate NK cell proliferation and cytokine production in human endometrium. Human uNK cells express genes for the suppressive cytokine IL-10 and its receptor (IL-10R). IL-10 was produced by uNK cells upon stimulation with IL-2 and IL-12 in culture, but have no direct effect was observed on IFN-γ production (44) (Figure 2).

Surface markers of uNK cells

Cell-surface molecules are sequentially expressed by maturing NK cells and can be used as markers of developmental intermediates in mice and humans. Although some markers differ between the two species, many are shared and can be used to delineate common stages of NK-cell differentiation. A current view is that the dynamic balance between activatory and inhibitory receptors is the means by NK cells can be tolerant to self while being armed and ready to fight infection (45). NK cell activation is multifactorial and does not rely on a single signaling adapter.

Inhibitory receptors

The first inhibitory MHC-specific receptors to be discovered were the Ly49 receptors in rodents (46, 47), which bind

directly to classical MHC class I molecules. More than 10 different Ly49 receptors have been identified in C57BL/6J. The second family discovered was the killer cell immunoglobulin-like receptors (KIR) family (48), which appears to be functional in primates but not in rodents. The third family was functional in both primates and rodents, and it consists of CD94/NKG2 heterodimers (49). All three of the families of inhibitory receptors signal through motifs in their cytoplasmic domains, called immunoreceptor tyrosine-based inhibitory motifs (ITIM). Multiple receptors are usually expressed on each cell, and a complex combinatorial repertoire of NK specificities is generated. Our laboratory has shown that the virgin B6 uterus contains transcripts for all of the Ly49 activation receptors and 2 inhibitory receptors. Mating brings on expression of all of the inhibitory receptors by gd 6. The pregnancy pattern of Ly49 expression is stable to at least gd 12, the latest timepoint we studied.

Women with alloimmune abortions have a limited inhibiting KIR repertoire and it is postulated that miscarriages may occur because trophoblastic HLA class I molecules are recognized by decidual NK cells lacking the appropriate inhibitory KIRs (50).

Activating receptors

Activating ligands for human NK cells are MICA/B, Rae1, and H60 (which bind to NKG2D), cell bound IgG (which binds to the FcγR), and CD48 (which binds to 2B4 and CD2) (51-53). Candidate receptors for such reactions include

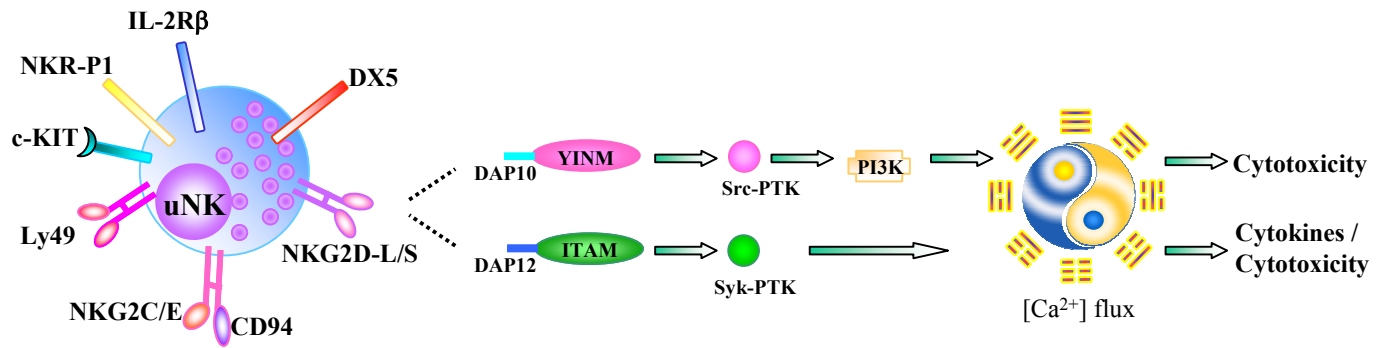


Figure 3. Activating choice of uNK cells by DAP10/12 pathway. NKG2D is a versatile receptor that depending on the availability of adapter partners, mediates costimulation in T cells and/or activation in NK cells. It was indicated that the differential signalling ability of NKG2D depends both on the cell type and the activation state, which is determined by the differential expression of DAP10 versus DAP12, and on the two different NKG2D isoforms. NKG2D was shown to associate either with DAP12, which triggers intracellular calcium concentration ($[Ca^{2+}]$) flux, cytotoxicity and induction of cytokines such as interferon- γ ($IFN-\gamma$), or with DAP10, which mediates only costimulation.

NKp30, NKp44, and NKp46 (54). The KIRs contain the stimulatory KIR2DS and KIR3DS, the NKG2 family contains stimulatory NKG2C and E receptors. The mouse Ly49 family contains stimulatory Ly49D and Ly49H receptors.

NK activating receptors generally associate with small transmembrane adapter proteins that transmit activation signals, including KARAP/DAP12 (55), CD3 ζ , Fc γ R, and DAP10/KAP10 (56). RNA is transcribed in mouse uNK cells for each of these pathways (57). It was thought that each of these adapters is expressed by all NK cells but associates only with a distinct subset of the stimulatory receptors. NK cells from mice or humans with mutations in one of these genes, KARAP/DAP12, exhibit only a subtle phenotype and can still attack most NK target cells. Implantation sites in mice lacking DAP12 also have only subtle changes, with some but not full modification of spiral arteries.

NKG2D triggers activity in NK cells in association not only with DAP12, but also with DAP10, via a novel Syk-independent regulatory pathway (Figure 3). In mouse NK cells, NKG2D can associate with either the adapter DAP10, signaling through PI3K in a Syk-PTK-independent fashion, or the ITAM-containing DAP12 adapter, signaling through a Syk-PTK dependent pathway. Both signaling pathways trigger cytotoxicity, but only the DAP12-linked pathway results in induction of cytokine (58, 59). NKG2D, an activating receptor on T and NK cells for the nonclassical MICA and MICB antigens (51), using the DAP10 adaptor protein for signal transduction (56), was expressed on the surface of uNK cells of both virgin and pregnant uteri of C57BL/6J mice at gd 10 by FACS analysis (Yang H, personal communication). A gene expression study of human NK cells has shown that CD9, four integrin subunits, NKG2C and NKG2E, Ly49L, KIRs, galectin-1 and progesterone-associated protein (PP) 14, all of which could have immunomodulatory functions during pregnancy, were overexpressed

in uNK cells (5).

To further characterize uNK cells in relation to pNK cells and to discover genes with relative overexpression that could provide clues to their function, the gene expression profiles of freshly isolated NK cells from human decidual and peripheral blood were compared by J. Strominger's research group. While the samples were not paired for the cell of origin from the same donors and male blood samples were included in peripheral NK cell preparations, the study showed uNK cells are clearly distinct from both CD56^{bright} and CD56^{dim} of pNK cell. Overall, around 70% of the 278 differentially expressed genes were overexpressed in uNK cells (197 genes, including KIR3DL1, KIR3DL2, KIR2DL3, KIR2DL4, granzyme B and perforin) as compared with either CD56^{bright} or CD56^{dim} pNK cells, whereas roughly 30% of genes, such as NKG2C and NKG2E were overexpressed in the three to five fold range in pNK cells versus uNK cells (5). Based on Lorenzo's (60) hypothesis, NK cells would be activated only when needed, that is, when they encounter "stressed" (by cytokine activation, proliferation, high temperature, viral infection or tumor transformation and implantation) cells expressing inducible self ligands that have lost MHC class I molecules.

Future directions

Given the differences in the structures of activating and inhibitory receptors expressed by human and mouse uNK cells, one may ask whether these cells serve divergent functions in the two species. This is a difficult question to answer because the essential physiological roles played *in vivo* by human uNK cells remain to be defined. Human cases of selective NK deficiency are rare and bone marrow transplanted in infancy, providing precursors for the uNK cell lineage. Considering the proven, conserved effector functions of murine and human NK cells (cytotoxicity and cytokine

production), it is plausible that the biological roles of these cells in pregnancy of mice and humans are similar. Because the nature of the precursors and sites of differentiation are not shared by uNK and pNK cells, special attention must be paid to general effects of hormones and decidua in uNK cells biology and to later interactions with trophoblast. These interactions may lead to expression of uNK cell-specific molecules, that have not yet been defined.

Because NK cells are activated by the balance between a variety of activating and inhibiting receptors, it could still be possible that even in NK cells, NKG2D in association with DAP10 acts as a synergistic or costimulating receptor rather than activating on its own (61). More ligand-receptor interactions are being identified in human and mouse uNK cells. New techniques, such as molecular imaging in living cells, gene microarray analysis, RNA interference, conditional gene ablation, proteomics and computer modeling, need be introduced into this field. A good way to get prepared to integrate new and exciting information is to take advantage of the comparisons between mouse and human uNK/pNK cells in order to learn from their differences and similarities and to have a global view on the biology of mammalian uNK cells. In the future years, a better understanding of uNK cell signaling pathways should shed enough light on their biological functions to make them useful in medical treatment as targets of therapeutic strategies in pregnancy related diseases. It has already been documented that neonates born to mothers with pre-eclampsia have significantly higher percentages of NK cells (CD3⁺/CD56⁺CD16⁺) in their cord blood (62). This fetal programming may represent fetal attempts as compensation for a deficient in maternal uNK cell function during pregnancy but it may also indicate long term postnatal skewing of immunity that could have undesirable outcomes in adulthood.

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