Review

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Natural killer cell-based immunotherapy in cancer: current insights and future prospects

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As our understanding of the molecular mechanisms governing natural killer (NK) cell activity increases, their potential in cancer immunotherapy is growing increasingly prominent. This review analyses the currently available preclinical and clinical data regarding NK cell-based immunotherapeutic approaches in cancer starting from a historical background and an overview of molecular mechanisms taking part in NK cell responses. The status of NK cells in cancer patients, currently investigated clinical applications such as *in vivo* modulation of NK cell activity, *ex vivo* purification/expansion and adoptive transfer as well as future possibilities such as genetic modifications are discussed in detail.

Keywords: cancer immunotherapy, clinical trials, cytokines, *ex vivo* expansion, gene therapy, natural killer cells.

Abbreviations: ADCC, antibody-dependent cellular cytotoxicity; ALL, acute lymphoblastic leukaemia; AML, acute myeloid leukaemia; BM, bone marrow; BMT, bone marrow transplantation; CML, chronic myelogenous leukaemia; CR, complete remission; CRC, colorectal carcinoma; DC, dendritic cell; DLI, donor lymphocyte infusion; G-CSF, granulocyte colony stimulating factor; GM-CSF, granulocyte-macrophage colony stimulating factor; GMP, good manufacturing practice; GvHD, graft-versus-host disease; HCC, hepatocellular carcinoma; HLA, human leukocyte antigen; HSCT, haematopoietic stem cell transplantation; IFN, interferon; IL, interleukin; KIR, killer-cell immunoglobulin-like receptor; LAK cells, lymphokine-activated killer cells; LGL, large granular lymphocyte; MHC, major histocompatibility complex; MM, multiple myeloma; NB, neuroblastoma; NCR, natural cytotoxicity receptor; PBMC, peripheral blood mononuclear cell; PBSC, peripheral blood stem cell; PHA, phytohaemagglutinin; PR, partial remission; RCC, renal cell carcinoma; ROS, reactive oxygen species; SCID, severe combined immunodeficiency; SCT, stem cell transplantation; TCR, T-cell receptor; TNF, tumour necrosis factor; TRAIL, TNF-related apoptosis inducing ligand; T_{reg}, regulatory T cell; WBC, white blood cell.

Natural killer cells: a historical background

Initially regarded as an 'experimental artifact' in T-cell cytotoxicity assays, natural killer (NK) cells were first discovered in mice more than 30 years ago by Kiessling *et al.*, who also named them natural killer cells [1, 2] and in parallel by Herberman *et al.* [3, 4]. Human NK cells were initially described as nonadherent, nonphagocytic, $Fc\gamma R^+$, large granular lymphocytes (LGL) [5]. Later it was, however, appreciated that NK cells not only shared the LGL phenotype and some NK cells also displayed normal small lymphocyte morphology, depending on their activation status [6]. This made it difficult to detect the NK cell population just by the size and morphology. The identification of the NKR-Pl [7] and NK1.1 [8] made

it possible to define the murine NK cells roughly as NK1.1$^+$ TCR$^-$ sIg$^-$ CD16$^+$. Today, human NK cells are defined as CD3$^-$CD56$^+$ lymphocytes. They comprise ~10–15% of all circulating lymphocytes and are also found in peripheral tissues, including the liver, peritoneal cavity and placenta. Resting NK cells circulate in the blood, but following activation by cytokines, they are capable of extravasation and infiltration into most tissues that contain pathogen-infected or malignant cells [9–11].

The discovery of NK cells suggested a possible effector mechanism behind the phenomenon of 'hybrid resistance'. Skin and organ transplantations had shown that allogeneic grafts were rejected whilst syngeneic grafts were tolerated, i.e. rejection only took place when the grafts had MHC molecules differing from the host. This rejection was mediated by T cells, which could induce either a graft-versus-host or a host-versus-graft reaction. Irradiated (AxB)F_1 mice rejected BM transplants from either parent, despite the fact that the transplant did not express any foreign MHC molecules. This was not in accordance with the reigning dogmas of T-cell-mediated rejection. The BM rejection could still be observed in severe combined immunodeficient (SCID) mice, which have no T and B cells but have functional NK cells [12].

Initially, it was not clear how NK cells distinguished the target cells they should kill from those that they should spare. When Kärre summarized his and other people's work for his doctoral thesis, he found a common denominator not about what was commonly expressed on target cells but about what was commonly missing. This lead him to formulate the *missing-self* hypothesis, where he suggested that NK cells kill target cells lacking expression of self MHC class-I molecules although the mechanism was unclear [13, 14] (see Fig. 1). This model was later confirmed by the discovery of inhibitory receptors on NK cells. *Missing-self* could also explain the hybrid resistance phenomenon; the (AxB)F_1 host killed cells from either parent A or B because these cells lacked complete self MHC expression (A+B). To further test the *missing-self* hypothesis, a MHC class I-deficient version of the tumour cell line RMA was established and named RMA-S. C57BL/6 mice inoculated with RMA-S cells rejected the tumours, whilst mice inoculated with RMA developed the tumour. By treating the mice with NK depleting anti-asialo GM1 antibody, the difference in tumour outgrowth disappeared [15]. This confirmed that NK cells-mediated the selective rejection of MHC lacking tumour growth.

Natural killer cells are separated into two subsets based on their CD56 antigen expression. Yet, this separation is not just phenotypic but rather has many functional outcomes. The majority (~90%) of human NK cells have low-density expression of CD56 (CD56^{dim}), whereas ~10% of NK cells are CD56^{bright}. Early functional studies of these subsets revealed that the CD56^{dim} cells are more cytotoxic [16]. However, there are a number of other cell-surface markers that confer unique phenotypic and functional properties to CD56^{bright} and CD56^{dim} NK cell subsets. CD56^{bright} subset is shown to exclusively express IL-2 receptor α chain (IL-2Rα/CD25), whilst they lack or express only at very low levels the FCγRIII (CD16). On the other hand, the CD56^{dim} subset has high expression of CD16 and lacks CD25 expression. These properties set very different roles to the different subsets with regards to antibody dependent cellular cytotoxicity (ADCC) and response to IL-2 stimulation. In addition to distinct expression of adhesion molecules and cytokine receptors, the CD56^{bright} NK cell has the capacity to produce high levels of immunoregulatory cytokines, but has low-level expression of killer-cell immunoglobulin-like receptors (KIRs) and is poorly cytotoxic. By contrast, the CD56^{dim} NK cell appears to produce low levels of cytokines but has high-level expression of KIRs and is a potent cytotoxic effector cell. Such evidence suggests that the CD56^{bright} and CD56^{dim} subsets are distinct lymphocytes with unique roles in the immune system. Thus, studies of the biology of human NK cells are eventually approaching NK cells as separate CD56^{bright} and CD56^{dim} subsets rather than a homogenous population.

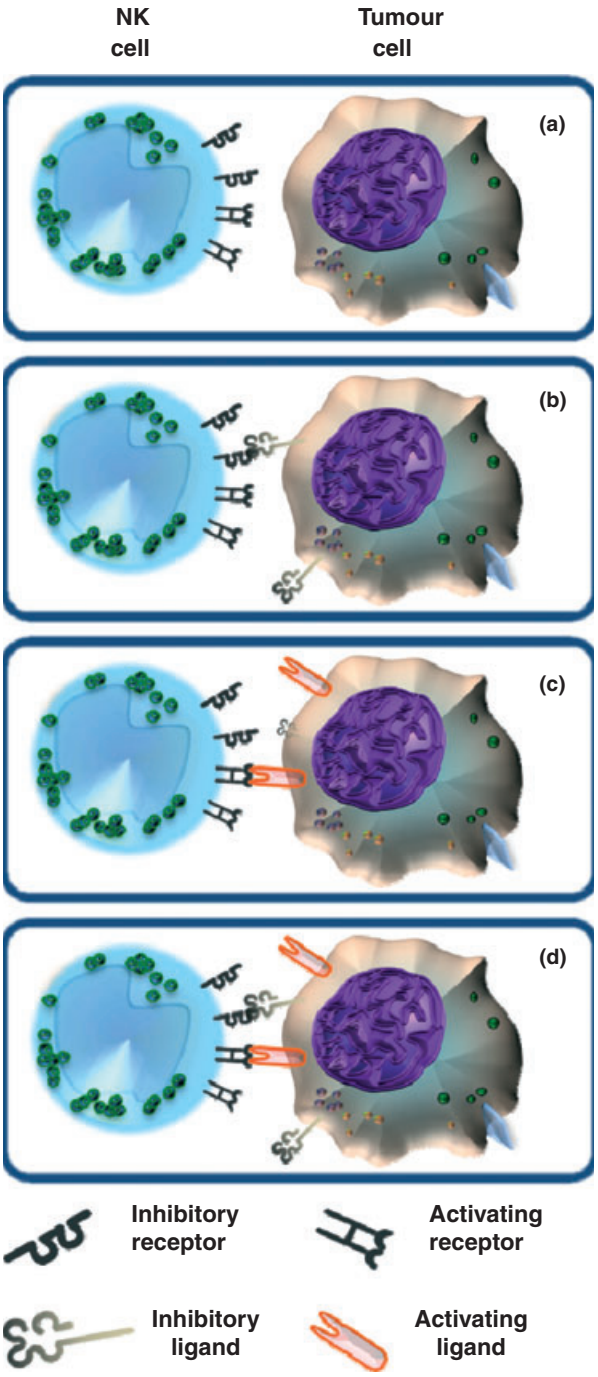

Fig. 1 The recognition of tumour cells by NK cells. The figure presents four hypothetical scenarios for the encounter of an NK cell and a tumour cell. (a) Although the tumour cell does not express any inhibitory ligands, it cannot be killed by the NK cell because it also lacks the expression of any activating ligands. This target is practically invisible to the NK cell and no recognition takes place. (b) The tumour cell expresses ligands for inhibitory receptors, whereas it lacks ligands for activating receptors. The NK cell recognizes the inhibitory ligands and, therefore, no killing takes place. (c) The tumour cell has significantly downregulated or absent expression of inhibitory ligands along with sufficient expression of activating ligands. *Missing-self* recognition takes place and the target is killed. (d) The tumour cell expresses significant levels of both inhibitory and activating ligands. The NK cells recognize both types of ligands and the outcome of this interaction is determined by the balance of inhibitory and activating signals.

As the name implies, NK cells can kill certain cells without prior sensitization, but they are also potent producers of various cytokines, such as IFN-γ, TNF-α, GM-CSF and IL-3 [17]. Therefore, NK cells are also believed to function as regulatory cells in the immune system, influencing other cells and responses and acting as a link between the adaptive and innate immune responses. For example, NK cells seem to participate in the development of the autoimmune disease, myasthenia gravis, by regulating both the autoreactive T and B cells through IFN-γ production [18]. Moreover, it has been observed that depletion of NK cells in C57Bl/6 mice leads to increased engraftment of neuroblastoma (NB) xenografts mainly because of dysregulation of Th1-oriented B-cell responses [19]. These data prove the significant impact of NK cells on adaptive immune responses. Other studies have also shown a close interaction between NK cells and dendritic cells (DC) [20]. In addition to their role as the initiators of antigen specific responses, DCs have been shown to support the activity of NK cells [21], whilst reciprocally, cytokine-preactivated NK cells have been shown to activate DCs and induce their maturation and cytokine production [22–24]. *In vivo* activation of NK cells by a DC vaccine consisting of autologous DCs loaded with a tumour-associated antigen has also been shown [25]. NK cells are also involved in the defence against virus infections and autoimmunity both of which have been elegantly reviewed elsewhere [26, 27].

Today, we know that NK cell cytotoxicity is the result of a complex balance between the inhibitory and activating receptors [28]. Table 1 provides a list of human NK cell activating and inhibitory receptors identified to our knowledge. Upon recognition of the

ligands on the target cell surface by activating NK cell receptors, various intracellular signalling pathways drive NK cells towards cytotoxic action and this results in target cell cytolysis [29].

However, these processes are tightly controlled by a group of inhibitory receptors. These receptors act as negative regulators of NK cytotoxicity and inhibit the action of NK cells against 'self' targets. A main group of this type of receptors is KIRs, which are mainly specific for self MHC Class-I molecules. If the target cell is recognized by inhibitory KIRs, which means, it has sufficient amount of self MHC Class-I molecules on the cell surface, an inhibitory signal from KIRs stops the action of cytotoxic pathways triggered by activating receptors [30, 31]. The KIRs are type I (extracellular amino terminus) membrane proteins that contain either two or three extracellular Ig-like domains [32] and they are designated as KIR2D or KIR3D respectively. The cytoplasmic domains of the KIRs can be either short (S) or long (L), corresponding to their function as either activating or inhibitory receptors respectively. Members of the KIR family recognize HLA-A, HLA-B and HLA-C alleles and KIR2DL4 recognizes HLA-G [33]. The KIR receptors are clonally distributed on NK cells, which provides that even the loss of a single HLA allele (a common event in tumourigenesis and viral infections) can be detected by a pool of NK cells [33, 34].

The activating side of the balance also includes a series of different receptors (see Table 1). The main activating receptor group is called natural cytotoxicity receptors (NCRs) [29] and it is believed that the main control over the NK cell activating pathways is regulated by these receptors. Currently, there are three different NCRs identified: NKp30 [35], NKp44 [36] and NKp46 [37]. NKp30 and NKp46 are expressed both in activated and in nonactivated NK cells, whereas NKp44 expression is restricted to activated NK cells. Most activating receptors do not directly signal through their cytoplasmic tail, but instead associate noncovalently with other molecules containing immunoreceptor tyrosine-based activation motifs (ITAM) that serve as the signal transducing proteins. NKp30 and NKp46 are associated with CD3ζ, whereas NKp44 is associated with DAP12. NK cell activation has been studied extensively in recent years and is discussed elsewhere [38, 39].

Natural killer cells have been described as 'large granular lymphocytes' and their granularity is their means for target cell killing (see Fig. 2). These granules contain perforin and granzyme B [40] and it is postulated that granzymes and perforin both bind to the target surface as part of a single macromolecular complex [41]. When an NK cell encounters a target cell, perforin and granzyme B are released; granzyme enters the target cell and mediates apoptosis, whilst perforin disrupts endosomal trafficking [42, 43]. NK cells can also express FasL and TNF-related apoptosis-inducing ligand (TRAIL), which are both members of the TNF family and are shown to induce target cell apoptosis when they bind their receptors on target cells [44, 45]. TNF-α has also been suggested to mediate activation-induced cell death by NK cells [46].

NK cells in cancer

The development of any malignancy is under close surveillance by NK cells as well as other members of the immune system. Nevertheless, malignant cells obtain means to escape from the immune system and proliferate. General mechanisms include saturation of the immune system by the rapid growth of the tumour, inaccessibility of the tumour owing to defective vascularization, its large dimension or its localization in immune-privileged sites and resistance to the Fas- or perforin-mediated apoptosis. The expression of FasL by tumour cells as a counterattack strategy against immune effector such as T cells and NK cells is also common [47–49]. Additionally, the defective expression activation receptors and various intracellular signalling molecules by T cells and NK cells in cancer patients was observed and reported to correlate with disease progression [50]. It has also been shown that malignant cells secrete immunosuppressive factors that inhibit T and NK cell proliferation [51]. As a result of all these events, defective immunity secondary to tumour development has been a well-established phenomenon [52]. Table 2 presents a selection of previously defined NK cell abnormalities in cancer patients.

Table 1 Activating and inhibitory receptors on human NK cells

CD	Alternative name	Type of signal	Ligand	Distribution on NK cells
CD2	LFA-2	Activation	CD58 (LFA-3)	All
CD7	LEU-9	Activation	SECTM1, Galectin	All
CD11a	LFA-1	Activation	ICAM-1,-2,-3,-4,-5	All
CD11b	Mac-1	Activation	ICAM-1, Fibrinogen	All
CD16	FcγRIII	Activation	IgG	Mainly $CD56^{dim}$ Negative/dim on $CD56^{bright}$
CD27	TNFRSF7	?	CD70	Mainly on $CD56^{bright}$ Negative/dim on $CD56^{dim}$
CD44	Hyalunorate receptor	Activation	Hyalouronan	All
CD59	Protectin	Activation	C8, C9	All
CD69	CLEC2C	Activation	Unkown	Activated
CD85j	ILT-2	Inhibition	HLA-A, -B, -G	Subset
CD94/CD159a	CD94/NKG2A	Inhibition	HLA-E	Most
CD94/CD159c	CD94/NKG2C	Activation	HLA-E	Most
CD96	TACTILE	Activation	CD155	Activated low expression on resting
CD160	BY55	Activation	HLA-C	All
CD161	NKR-P1	Activation/ Inhibition	LLT1	Subset
CD223	Lag3	Activation	HLA Class II	Activated
CD226	DNAM-1	Activation	CD112, CD155	All
CD244	2B4	Activation/ Inhibition	CD48	All
CD314	NKG2D	Activation	MICA, MICB, ULB-1,-2,-3,-4	All
CD319	CRACC	Activation	CRACC	Mature NK cells
CD328	Siglec-7	Inhibition	Sialic acid	Subset
CD329	Siglec-9	Inhibition	Sialic acid	Subset
CD335	NKp46	Activation	Viral haemagglutinin (?)	All
CD336	NKp44	Activation	Viral haemagglutinin (?)	Activated
CD337	NKp30	Activation	Viral haemagglutinin (?)	All
Various	KIR2DS, KIR3DS	Activation	HLA Class I	Subsets
Various	KIR2DL, KIR3DL	Inhibition	HLA Class I	Subsets
–	NTB-A	Activation	NTB-A	All
–	KLRG1	Inhibition	E-,N-,P-cadherin	All

Potential of NK cells in cancer immunotherapy

Modulation of NK cell activity

IL-2 alone. The cDNA encoding for the human IL-2 gene was cloned in 1983 [53] after a long search starting in 1965 for the soluble factors in lymphocyte conditioned media that could sustain the proliferation of T cells in culture [54, 55]. It is now well known that IL-2 effects many types of cells in the immune system including cytotoxic T cells, helper T cells, regulatory T cells, B cells and NK cells. Currently, there are three distinct chains of the IL-2 receptor identified; the α

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