Chapter 2

NK Cells and Cancer

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Abstract

NK cells play an important role in host immunity against cancer by exerting cytotoxicity and secreting a wide variety of cytokines to inhibit tumour progression. Their effector functions are regulated by the integration of opposing signals from activating and inhibitory receptors, which determine NK cell activity against tumour targets. NK cell cytotoxicity requires successful progression through discrete activation events that begin with NK cell adhesion to a tumour target cell and culminate in the polarized release of cytotoxic granules into the immunological synapse. Tumour cells can evade NK cell attack through numerous mechanisms such as shedding of activating ligands, upregulation of inhibitory ligands, or stimulation of inhibitory regulatory T lymphocytes. A better understanding of specific NK cell responses to tumour targets can generate better NK cell-based immunotherapeutic strategies for cancer. This chapter discusses NK cell immunosurveillance of cancer, NK cell tumour recognition strategies, cancer immune evasion from NK cells, and different approaches to NK cell modulation for cancer therapy.

Keywords: natural killer, cancer, cytokines, target recognition

1. Introduction: cancer immunosurveillance by NK cells

Natural killer (NK) cells were discovered more than four decades ago and were the focus of some of the earliest trials of cancer immunotherapy. With our more sophisticated understanding of their functional requirements, NK cells are once again attracting attention for their potential in cancer therapy [1]. Initially thought to be an artefact in cytotoxicity assays, NK cells are now known to play an important role in host immunity against tumourigenesis. The theory of cancer immunosurveillance was proposed by Burnet and Thomas in 1957, postulating that immune cells continuously monitor the body such that any threat to the immune system is detected and eliminated [2]. In 1975, NK cells were discovered in mice as a subpopulation of lymphocytes capable of killing tumour cells without prior sensitization [3–5]. This led

to considerable enthusiasm over the possibility that they function as one of the main effector cells of immunosurveillance. Several studies in the 1980s reported a higher cancer incidence in individuals with genetic disorders such as Chediak-Higashi syndrome and X-linked lymphoproliferative syndrome, which lead to defective NK cell function [6, 7]. Subsequent mouse studies showed increased tumour growth in mice with impaired NK cell activity or mice treated with an NK cell-depleting agent [8, 9]. A long-term epidemiological study following cancer patients reported that subjects with lower NK cell activity had a higher incidence of several types of cancer [10]. Collectively, data from both mouse and human studies support the theory of cancer immunosurveillance and the concept that NK cells play a critical role in tumour control and eradication [11, 12]. The two main effector functions observed by NK cells against tumour targets are target cell elimination and cytokine secretion [13]. Until recently, these two effector functions were thought to follow similar mechanisms of activation, but now it is recognized that cytokine secretion by NK cells is distinct from cytotoxicity [14].

1.1. Target cell elimination

NK cells kill tumour cells through granule exocytosis or death receptor ligation. Following NK cell activation, NK cells release the contents of their granules for target cell elimination. The membrane disrupting protein perforin, and a family of serine proteases termed granzymes, are the critical effector molecules contained in their granules [15]. Perforin results in the disruption of endosomal trafficking and binds in a calcium-dependent manner to phospholipid components of the lipid bilayer to facilitate entry of granzymes into the target cell cytosol [16]. Once granzymes enter the target cell, they induce apoptosis. In addition to granule exocytosis, NK cells can directly eliminate target cells through the engagement of cell surface death receptors. NK cells express Fas ligand (FasL) and TNF-related apoptosis-inducing ligand (TRAIL), which are both members of the TNF family and are shown to induce target cell apoptosis once bound to their respective receptors on target cells [16].

1.2. Cytokine secretion

Resting NK cells secrete a plethora of cytokines that help eliminate target cells and amplify activation signals for a more efficient immune response. NK stimulation results in enhanced secretion of cytokines, which in turn influence the activity of other immune cells. Pro-inflammatory cytokines secreted by NK cells, which include interleukin (IL)-1, IL-6, IL-12, and the chemokine CXCL8 (also known as IL-8), can enhance the activation and proliferation of T cells, dendritic cells (DCs) and macrophages [17]. By contrast, anti-inflammatory cytokines such as IL-4 and IL-10 suppress T cell and macrophage function, but activate humoral responses. Chemokines, which are chemotactic cytokines, play an important role in directing various immune cells to target sites, such that more potent responses are achieved. Chemokines released by NK cells include, in addition to CXCL8, the macrophage inflammatory protein (MIP)-1α and MIP-1β; chemokine (C-C motif) ligand 5 (CCL5), also known as "regulated on activation normal T cell expressed and secreted" (RANTES); monocytes chemoattractant protein (MCP)-1; and eotaxin [18, 19]. The signalling pathways and mechanisms required for cytokine secretion also appear to be distinct from secretion of cytotoxic granules [14]. The localization and trafficking of IFN-γ and TNF-α were shown to take place in compartments and vesicles that do not overlap with

perforin or other late endosome granule markers. Recycling endosomes (REs) are not needed for release of perforin, but are required for cytokine secretion in NK cells. Although perforin granules are released in a polarized fashion at lytic synapses, distinct carriers transport both IFN-γ and TNF-α to points all over the cell surface, including within the synapse, for non-polarized release.

2. NK tumour recognition

Prior to the discovery of NK cell receptors, it was unclear how NK cells could identify tumour targets for lysis. The 'missing-self' recognition model was initially proposed based on the observation that NK cells kill targets with reduced or absent expression of major histocompatibility complex class I (MHC I) molecules [20, 21]. This model explains why tumour or virally-infected cells with deficient MHC class I expression are targeted by NK cells, whereas healthy autologous cells remain protected. It also explains the hybrid resistance phenomenon, in which F_1 hybrid mice reject parental bone marrow cells donated by either parent, despite the fact that the transplant does not express foreign MHC molecules [22]. Early experiments supported the 'missing-self' model by demonstrating selective rejection of an MHC class I-deficient version of the tumour cell line RMA in mouse models, in which the results were reversed after treating mice with an NK-depleting agent [20]. The characterization of NK cell inhibitory receptors further supported this recognition model by explaining the molecular mechanisms by which NK cells sensed the downregulation of MHC class I expression [23–29]. NK cell-mediated killing of MHC class I-deficient cells also provides a safeguard mechanism for MHC class I-restricted elimination by cytotoxic T lymphocytes. However, the 'missing-self' hypothesis alone failed to explain why NK cells spare autologous cells with absent MHC class I expression or kill tumour cells with sufficient MHC class I expression [30, 31]. The discovery of a wide array of activating NK cell receptors that detect stress-induced ligands on damaged or stressed cells led to the proposition of the 'induced-self' model, by which NK cells kill targets with upregulated expression of activating ligands. It is now understood that NK cell functions are tightly regulated by the integration of opposing signals from activating and inhibitory receptors [32]. Together these models suggest that NK cells detect changes in self-ligands on the surface of autologous cells. NK cells can also be activated through antibody-dependent cellular cytotoxicity (ADCC) whereby the NK cells are triggered directly through ligation of CD16 to kill tumour target cells to which the antibody has bound. The anti-CD20 antibody, Rituximab mediated lysis of CD20+ve lymphoma cells through this mechanism. **Figure 1** summarizes tumour recognition strategies by NK cells.

2.1. NK cell inhibitory receptors

Human NK cell inhibitory receptors fall into two groups: the killer immunoglobulin-like receptors (KIRs), and the lectin-like receptor NKG2A, which forms a heterodimeric complex with CD94. KIRs bind to human leukocyte antigen (HLA)-A, -B, or -C, whereas the NKG2/CD94 complexes ligate HLA-E. Human KIRs contain either two (KIR2D) or three (KIR3D) immunoglobulin (Ig)-like domains in their extracellular domain. KIR2D receptors recognize

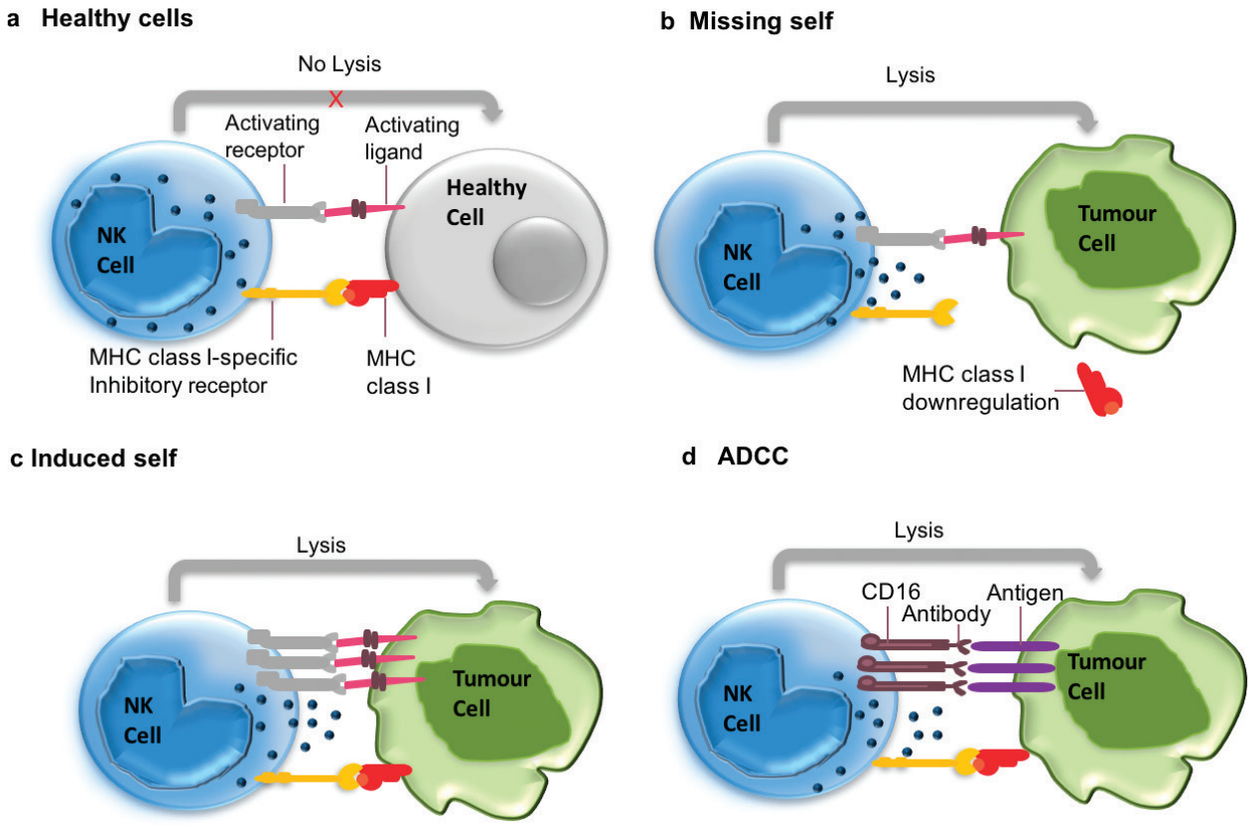

Figure 1. Tumour recognition strategies by NK cells. A) Balanced signals delivered by activating and inhibitory NK cells receptors are recognized as healthy and spared from NK cell-mediated lysis. B) Tumour cells downregulate MHC class I molecules, and are recognized by NK cells through 'missing self' for lysis. C) The upregulation of stress- or damage-related ligands is recognized by activating NK cell receptors and can overcome inhibitory signals to result in tumour lysis through the 'induced-self'. D) Antigen-specific antibodies can bind CD16 on NK cells to result in ADCC. ADCC: antibody-dependent cell-mediated cytotoxicity; MHC: major histocompatibility complex; NK: natural killer cell.

HLA-C alleles, whereas KIR3D receptors recognize HLA-A or HLA-B alleles. The common pathway generated by ligation of inhibitory receptors is characterized by tyrosine phosphorylation of immune tyrosine-based inhibitory motifs (ITIM) that recruit tyrosine phosphatases such as the Src homology 2 domain-containing phosphatase (SHP)-1 and SHP-2, which are responsible for the inhibition of various NK cell effector functions [33].

2.2. NK cell education

NK cell education refers to the mechanisms through which inhibitory input by MHC class I during development translates into functional responsiveness in mature NK cells [34]. Unlike the educational processes in T- or B-cell development, NK cell education remains a topic of intense debate, with several models proposed to explain how NK cell responsiveness relates to inhibitory signalling. NK cells that lack ITIM-bearing inhibitory receptors for self-MHC-I and NK cells from hosts that lack MHC-I ligands for ITIM-bearing inhibitory receptors have a reduced responsiveness to activation signals, such as stimulation by sensitive target cells or cross-linking of NK cell activating receptors [34–37]. These results have led to the two main models in NK cell education. The first 'disarming' model

proposes that in the absence of inhibition, continuous stimulation of NK cells leads to a state of hyporesponsiveness [38]. The second model proposes that inhibitory receptors provide an ITIM-dependent signal to the NK cells that renders them responsive [39]. This model is referred to as 'arming' or 'licensing', although the latter term is now understood to include any process by which NK cells that receive signals through inhibitory receptors for self-MHC-I gain responsiveness [40]. Studies reporting that NK cell responsiveness is calibrated according to the strength of inhibitory signals received [36, 41, 42], have led to a third 'rheostat' model that aimed to reconcile the two opposing models, and account for the quantitative tuning of NK cell responsiveness [42–44]. The rheostat model postulates that NK cell responsiveness is dynamically calibrated based on the strength of inhibitory signals received. More recent data demonstrating that NK cell 'tuning' or 'licensing' may be set by transient signals and can be reversible have led to an updated model known as the 'revocable license' [45]. The revocable license model argues that NK cells can keep their license as long as they are tightly regulated by inhibitory signals, but once this inhibitory input is lost, their license is revoked. Many questions regarding the molecular basis of licensing and the effect of subsequent activation signals on licensed vs. unlicensed cells remain unanswered. In many cases, the original concept of 'missing-self' and the self-tolerance of NK cells in an MHC-I-devoid environment cannot be explained without the involvement of NK cell activating receptors.

2.3. NK cell activation receptors

NK cell activation receptors can be grouped into three categories: those that associate with immunoreceptor tyrosine-based activation motif (ITAM)-containing subunits, the DAP10-associated NK group 2 member D (NKG2D) receptor and a number of other receptors including DNAX accessory molecule-1 (DNAM-1), CD2 and 2B4. Receptors that associate with the ITAM-containing adapter proteins transmit signals through the recruitment of tyrosine kinases Syk or ZAP70, and include CD16, which mediates antibody-dependent cellular cytotoxicity, and the natural cytotoxicity receptors (NCRs) NKp30, NKp44, NKp46 and NKp80, which are known to play an important role in NK-mediated cytotoxicity against tumour cells [46]. NKp30 and NKp46 are constitutively expressed on all peripheral blood NK cells, whereas NKp44 is expressed only on activated NK cells. NKp30 binds the nuclear factor HLA-B-associated transcript (BAT)-3, NKp46 binds to influenza haemagglutinin and the cellular ligand for NKp80 is the activation-induced C-type lectin (AICL) [47]. NKG2D associates with the DAP10 adaptor protein and signals through a phosphoinositide 3-kinase (PI3K)-binding motif. It binds several ligands associated with stress, infection or transformation including MHC I chain-related protein A and B (MICA/B) and the UL16-binding proteins 1–4 (UBLP1-4) [48].

2.4. NK cell activation

NK cells require the co-engagement of multiple activating receptors in order to exhibit natural cytotoxicity against tumour target cells [49]. Upon encounter with potential target cells, an immunological synapse forms at the point of contact between the NK cell and the target cell,

where NK cell receptors can interact with their respective ligands. Given sufficient activation signals, NK cell cytoskeletal rearrangements are initiated, which result in the polarization of NK cell lytic granules toward the immunological synapse, where they eventually fuse and release their cytotoxic contents on to the target cell [50]. In contrast to CTLs, NK cells have their cytotoxic granules preformed before target cell recognition, and so their release is initially constrained until sufficient signalling is achieved. NK cells have also been shown to establish cytoskeletal polarity more slowly than CTLs, and to have a unique sensitivity to minor interference with cytoskeletal dynamics [51]. This stepwise progression in activation events with specific requirements for synergistic signalling may provide a mechanistic explanation of how the spontaneous cytotoxic capacity of NK cells is regulated [52]. **Figure 2** outlines NK cell activation events at the immunological synapse with a tumour target cell.

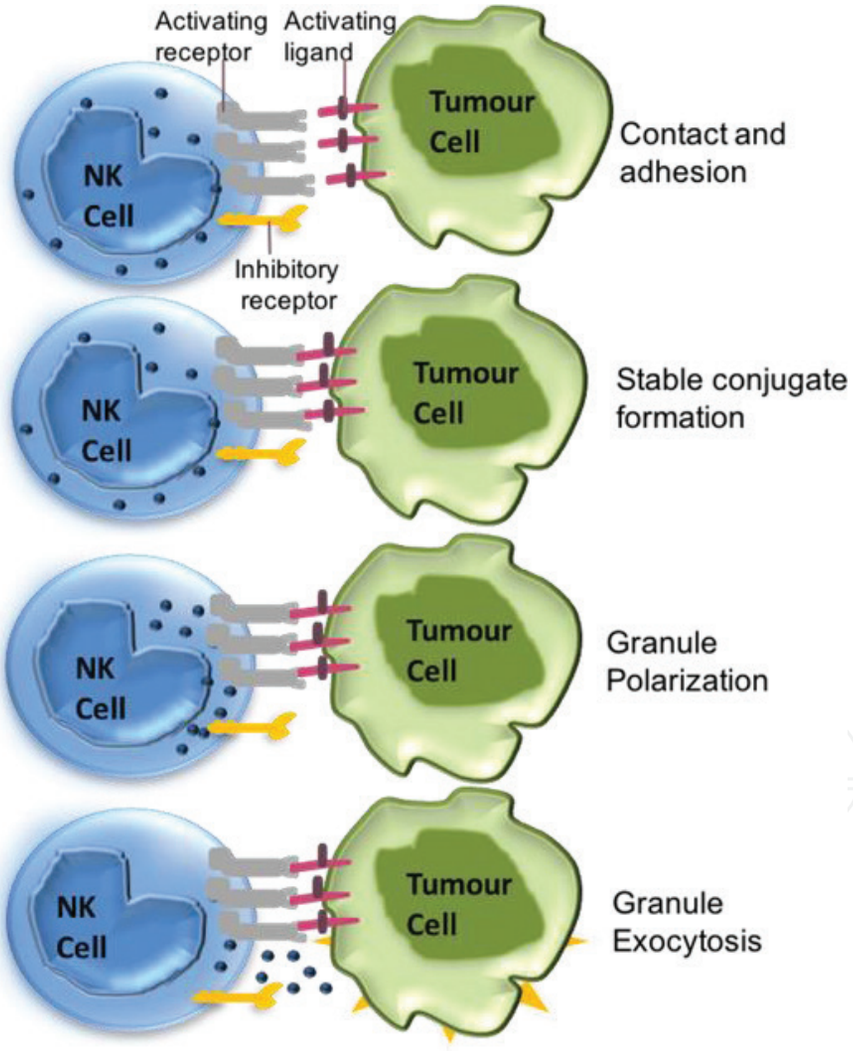

Figure 2. Activating immunological synapse between NK cell and tumour target. NK cell encounter with a tumour cell target generates an immunological synapse at the point of contact. If the ligand combination on the tumour target engages NK cell activating receptors sufficiently, cytoskeletal rearrangements take place resulting in granule polarization and the eventual release of cytotoxic granules on to the target cell. NK: natural killer cell.

3. Cancer immune evasion from NK cells

Although the development of any malignancy is under surveillance by immune cells, tumour cells can still obtain means to escape from the immune system and proliferate. The recent addition of immune evasion as an emerging 'hallmark' of cancer, sheds lights on growing evidence in support of cancer evasion of immune cells [53]. Malignant cells acquire a set of biological capabilities during their development, allowing them to overcome recognition and elimination by the immune system. These capabilities are acquired with the assistance of inflammatory cells and soluble factors in the tumour microenvironment, which play an active role in the tumour development process. Kiessling et al. proposed that cancer evasion from NK cells involves an early stage of tumour formation and growth, which is associated with antigen-specific tolerance, and a later stage, which induces a state of immunodeficiency [54]. Cancer immunoediting, as proposed by Dunn et al. argues that less immunogenic variants are positively selected during tumour formation as they have a better chance of survival in a normal immunological environment [55]. This led to the formulation of the three Es of cancer immunoediting; elimination, equilibrium and escape [56]. The elimination phase involves tumour eradication by immune cells. Any tumour cells that survive the elimination phase enter the equilibrium phase. During this phase, immune cells and tumour cells are in a dynamic equilibrium, with selective pressure exerted on tumour cells, such that only the less immunogenic variants survive. In the escape phase, tumour cell variants which are positively selected in the equilibrium phase continue to grow.

Tumours can evade NK cell attack directly by insufficient expression of ligands for NK cell activation receptors, such that the activation threshold for NK cell granule exocytosis is not met. Once successful evasion from NK cell attack is achieved, the tumour cells create the microenvironment necessary for continued growth. There are several strategies for direct evasion from NK cells by tumour targets. For example, tumours have been shown to reduce expression or shed ligands for important NK cell receptors. The NKG2D ligands UBLP2, MICA and MICB are commonly shed by tumour cells to evade NK cell attack through NKG2D recognition. Alternatively, tumour cells can increase MHC class I, soluble MIC and FasL expression in order to increase NK cell inhibitory signalling [30, 57, 58]. The secretion of soluble factors such as IL-10, TGF-β and indoleamine 2, 3-dioxygense (IDO) by tumour targets suppresses the adaptive immune response to exhibit significantly less anti-tumour capacity [59–63].

Tumour cells employ numerous cell types from the immune system for indirect NK cell evasion mechanisms. Tumour cells have been reported to recruit, myeloid-derived suppressor cells (MDSCs), regulatory T cells, which release immunosuppressive Th2 cytokines and phagocytes, which release reactive oxygen species (ROS) to inhibit NK cell function [64]. Macrophages found in the tumour microenvironment can be classified as M1 or M2. The M1 subtype is associated with tumour control, through pro-inflammatory cytokine and ROS secretion, whereas the M2 subtype promotes tumour growth and invasion through the production of anti-inflammatory cytokines, upregulation of scavenging receptors and tissue remodelling [65]. Excess ROS in the tumour microenvironment can lead to tumour cell lysis. The Warburg

effect, by which tumour cells rely mostly on glycolysis for energy production, enables cancer cells to resist ROS-related death and gain survival advantage for metastasis [66].

Tumour cells can also impair dendritic cell (DC) function to prevent NK cell priming, by changing their expressions of IL-6, IL-10, vascular epithelial growth factor or GM-CSF. Finally, tumour cells have also been shown to lower NK cell count by decreasing the numbers of lymphoid progenitor cells [67]. **Figure 3** describes different tumour immune evasion strategies from NK cells.

4. NK cell modulation for cancer therapy

The ability of NK cells to kill tumour cells has made them very attractive in immunotherapy. NK cell impairments associated with tumour development and progression have been frequently reported in cancer patients, including weakened effector functions and an altered phenotype with downregulation of activating NK cell receptors [68]. Different strategies have been employed to repair, replace or enhance the biological functions of autologous or

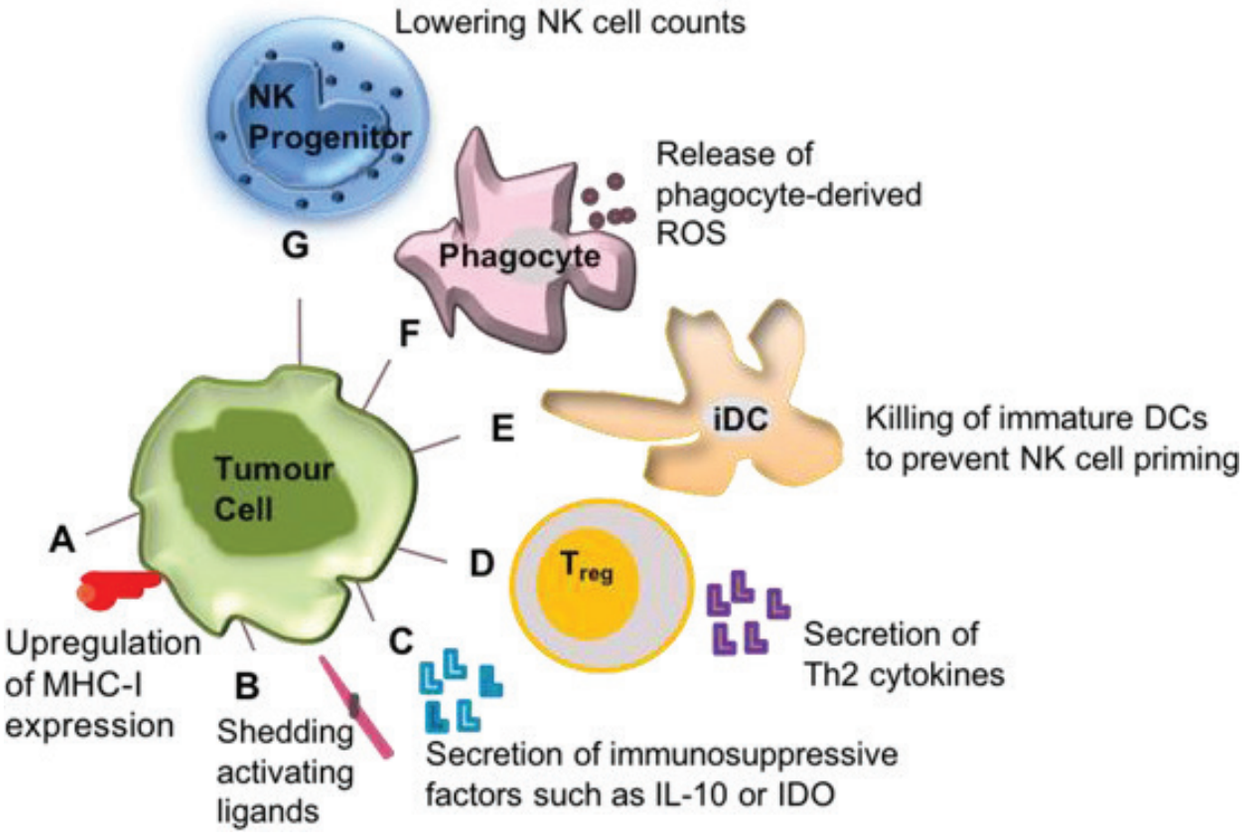

Figure 3. Tumour Evasion from NK cells. Tumour cells use direct and indirect mechanisms to evade NK cell attack. Direct mechanisms include A) upregulation of MHC class I expression B) shedding of soluble ligands for NK cell activation receptors and C) release of inhibitory cytokines. Indirect mechanisms include D) activation of inhibitory regulatory T cells E) killing of immature dendritic cells to prevent NK cell priming F) release of phagocyte-derived inhibitory cytokines and G) reducing the number of NK progenitor cells to lower NK cell counts. NK: natural killer cell; DC: dendritic cell, IL-10: interleukin 10; IDO: indoleamine 2,3-dioxygenase; MHC: major histocompatibility complex; ROS: reactive oxygen species; Th2: T helper cell type 2.

allogeneic NK cells *in vivo* and *ex vivo*. In a clinical setting, the key factors to be considered are the number, purity, proliferative capacity and activation state of NK cells. The most limiting of these factors is obtaining a sufficient number of NK cells, hence the extensive development of *ex vivo* expansion methods for NK cell adoptive immunotherapy applications. The impressive clinical responses seen following administration of chimeric antigen receptor T cells (CAR-T) has led to trials of CAR-NK cells at centres in the US and Europe. Reports of pre-clinical data are encouraging and suggest that the more constrained proliferation of CAR-NK cells *in vivo* and the lower release of inflammatory cytokines may provide improve the safety profile.

The delivery of IL-2, IL-12 and IL-15 genes to the human NK cell line NK-92 has also been shown to enhance proliferative and cytotoxic capabilities. These cytokines are known to play important roles in the enhancement of survival and activation of many immune cells including T cells, B cells and NK cells. Strategies to enhance endogenous NK cell function *in vivo* through cytokines were pioneered by Rosenberg et al. who demonstrated great initial potential for IL-2 administration in advanced cancer patients [69]. *In vitro* stimulation of NK cells by activating cytokines such as IL-2 is known as the lymphokine-activated killer (LAK) phenomenon [70]. In early experiments, NK cells were activated *ex vivo* and adoptively transferred to patients with advanced metastatic renal cancer and melanoma along with IL-2 infusions. However, overall data from clinical trials since then have failed to provide a convincing proof of efficacy [68]. The clinical efficacy of LAK therapy was limited by the toxicity of IL-2 and the potential expansion of T regulatory cells. Mouse NK cells stimulated *in vitro* with a combination of IL-12, IL-15 and IL-18 were recently shown to have enhanced effector functions and longer survival after adoptive cell transfer [71]. Target cell stimulation of NK cells is an alternative to *in vitro* cytokine stimulation. Recent studies reported a tumour-priming approach, in which human NK cells are activated by co-incubation with an NK-resistant leukaemia cell line in the absence of IL-2 [72]. The clinical potential of these tumour-primed NK cells has been explored in acute myeloid leukaemia and multiple myeloma with promising results in autologous and allogeneic settings [73].

The last three decades unravelled different molecular mechanisms governing NK cell-mediated anti-tumour functions. This led to the development of a variety of strategies for NK cell-based immunotherapy of cancer. However, many challenges still remain as we better our molecular and functional characterization of NK cells and their receptors, and decipher the different signalling pathways involved in NK cell recognition of targets. NK cell responses can differ according to the type, combination and intensity of signals. Thus, a better understanding of tumour-specific responses at the bench, will lead to novel therapeutic strategies with better efficacy in the clinic.

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