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Review Article

Biochemistry Section

Natural Killer Cells - Their Role in Tumour Immunosurveillance

PREETI SHARMA[1], PRADEEP KUMAR[2], RACHNA SHARMA[3]

ABSTRACT

An important component of the innate immune system, the natural killer cells that originate from the lymphoid cell lineage, hold tremendous potential as an effective therapeutic tool to combat a variety of cancers. Their vast capability to kill altered cells such as opsonized cells (antibody coated), tumour cells, genotoxically changed cells without affecting the healthy cells of the body, make them an effective therapeutic agent for various types of cancers. Besides, through interplay and molecular crosstalk via several cytokines, they also augment the adaptive immune response by, promoting the differentiation, activation and recruitment of component cells of the system. With the current advance knowledge of Natural Killer (NK) cells, their receptor-ligand interactions involved in functional regulation, various mechanistic approaches involving the role of cytokines led to desired modulation of NK cell activity in a tailor-made manner, for triggering clinically relevant responces. Several strategies have been adopted by researchers, to augment the efficacy of NK cells. Still many challenges exist for increasing the therapeutic relevance of these cells.

Keywords: Antibody dependent cellular toxicity, Immunotherapy, Mechanism, Natural killer cell receptors, Tumour surveillance

INTRODUCTION

Natural Killer cells (NK) cells are unique in having the quality of recognizing cells that are abnormally stressed, lacking Major Histocompatibility Complex (MHCs) and without antibodies. The term 'natural killer' is relevant in the sense that they do not require prior activation for killing cells lacking MHC class I [1]. This property of NK cells make them efficient in detecting and destroying infected or abnormal cells lacking MHCs and which would otherwise escape detection by immune cells like T lymphocytes [2]. Critical to the innate immune system NK cells are cytotoxic lymphocytes that play a role analogous to cytotoxic T cells in the vertebrate's adaptive immune response [3]. Typically virally infected cells and tumour cells are susceptible to NK cells. These are large granular cells derived from lymphoid progenitor cells generating T and B lymphocytes. Their maturation and differentiation takes place in bone marrow, lymph nodes, spleen, tonsils and thymus from where they directly join the circulation [4-6].

NK cells lack antigen specific cell surface receptors and are capable of immediate reaction without prior antigen exposure [1]. Recently, NK cells have been recognized to play an important role in tumour immunosurveillance i.e., causing direct death of tumour cells without the presence of surface adhesion molecules or antigenic peptides [7-9]. Detection of tumour cells leads to activation of NK cells following cytokines production and release [9].

Natural Killer Cell Receptors, Their Structure And Functions

NK cells are different from NKT cells based on their origin, infact NKT cells promote the activity of NK cells by promoting the secretion of IFNγ [10]. Unlike NKT cells, the NK cells do not express- T-Cell Antigen Receptors (TCRs), CD3 surface marker or surface B cell receptors. They express surface markers CD16 and CD56 on their surfaces in human beings and the NK cells are usually defined as $CD3^-$ $CD56^+$ (5 to 20%) lymphocytes which are primarily divided into two groups namely $CD56^{dim}$ $CD16^+$ and $CD56^{bright}$ CD16 [10,11]. The $CD56^{dim}$ variety has high cytotoxic potential, predominate at the site of inflammation (>90%) and expresses on its surface abundant MHC class I specific inhibitory receptors unlike the $CD56^{bright}$ group of cells which are present predominantly in the lymph nodes, concerned mainly with cytokines secretion and show little toxicity. They are better known as precursors of CD 56^{dim} sub-group [11]. Functional modulation of the NK cells happens through a very well-regulated balance of its activating and inhibitory receptors. Research on NK cells initiated in the mid-1990s, led to cloning and identification of various families of NK cell receptors structural determination of NK cell surface receptors with ligand complexes thereby increasing our understanding of the role of these cells in innate immune response. There are two types of NK cell surface receptors, the inhibitory and activating receptors based on their functions [12]. Also structurally, they can be immunoglobulin like receptor superfamily or the C-type Lectin Like Receptor superfamily (CTLR) [13,14]. The ligands are members of the MHC class-I complex or their homologs [14]. The inhibitory receptors, recognize MHC-I with self-peptide on healthy host cells and provide protection while the activating receptors make facile killing of virally infected and tumour cells through specific ligand recognition system [15].

Inhibitory Receptors

Belonging to multigene family, Ig-like extracellular domain receptors, are killer cell receptors structurally resembling immunoglobulins. They are present in nonhuman primates, are the main receptor for both classical MHC class I and nonclassical Mamu-G (HLA-G). Inhibitory and dominant in nature, few Killer Inhibitory Receptors (KIRs) are specific to some HLA subtypes. Regular cell killing is inhibited by NK cells because they express MHC class I and these are recognized by KIRs. Leucocyte Inhibitory Receptors (LIR) are the members of the Ig receptor family discovered recently. Ly49 are other homodimeric receptors which are both inhibitory and activating in nature [14-16].

Activating Receptors

Natural Cytotoxic Receptors (NCR) on being stimulated cause NK cell mediated killing and release of INFγ. Ly49 (homodimers) and CD94:- NKG2 (heterodimers) both belong to CTLR. Ly49 are the

receptors for classical polymorphic MHC I molecules while CD94:-NKG2 identifies nonclassical and nonpolymorphic MHC I molecules such as HLA-E at the cell surface and it also requires the presence of nonamer peptide epitope derived from the signal sequence of classical MHC I molecules. CD16 or Fcy III are other types of receptors which are involved in Antibody Dependent Cell Mediated Cytotoxicity (ADCC) [17-19].

NK cells contain small granules called granzymes like perforin and proteases. During the killing process the NK cells come closer to the altered cell, perforins form small pores in the target cell to create an aqueous channel through which all contents of NK cells including granzymes are transferred to the cell and induce apoptosis [20]. Cell death can also be caused by lysis due to osmotic effects [20,21]. Apoptosis leads to the destruction of viruses inside the cell only while lysis could release virions. Alpha defensin is an antimicrobial substance, secreted by NK cells and can directly kill bacteria. It disrupts and destroys the bacterial cell wall in a similar manner to neutrophils. Opsonized infected cells (with antibodies) can be recognised by FcRIII or CD16 receptors expressed on NK cells leading to their activation. They in turn release cytolytic granules and cause cell death. The ADCC mediated killing of tumour cells can be estimated by transfecting NK-92 with high affinity FcR and comparison with wild type NK-92 which do not express FcRs [22].

Cytokines that are released from virally infected cells also play a prominent role in NK cell activation by signalling the NK cells about the infection. IL-12, IL-15, IL-18, IL-2 and CCL5 are the cytokines involved in NK cell activation [23]. Moreover IFN-γ derived from macrophages also activate NK cells. NK cells control viral infection by secreting INF-γ and TNF-α, role of which are to activate macrophages for phagocytosis and lysis and to cause direct killing by NK cells respectively [24].

Mechanism Of Action Of NK Cells

Scientists have been baffled for years regarding the mechanism by which these cells discriminate between altered and normal cell, as these cells do not possess antigen-specific receptors. NK cells and cytotoxic T-cells work in a complementary manner to produce an immune response directed against viruses and tumour cells. Specific to antigen presented over MHC I, the cytotoxic T-cells recognise antigenic peptides derived from viruses and tumours. In most instances, MHC class I expression is down-regulated by tumours and virus infected cells. The cells are then not recognizable by cytotoxic T-cells i.e., because of which the adaptive immune response fail. However, killing of these cells is then mediated by NK cells. These cells recognize MHC downregulated altered cells which escape Cytotoxic T-Lymphocyte (CTL) killing. The MHC class I molecules are identified by inhibitory receptors of the NK cells while this ligation inhibits the activation of NK cells. The opposite to this is that in cells lacking the MHC I for ligating inhibitory receptors, leads to the activation of NK cells [25,26]. Conversely, it can be said that due to lack of engagement of inhibitory receptors, in addition to other signals results in the activation of cytotoxicity of the NK cells. During viral infection and some forms of malignancy, these altered cells start inducing the expression of MHC class I chain related molecules like MICA, MICB and UL-16 binding proteins. These molecules are recognized by NKG2D receptors and ligand binding can lead to signalling to kill the target cell [27-29].

Immunosurvillance Of Tumour Cells

NK cells perform very important role in fighting tumours as the recognition of these tumour cells is not MHC restricted. Hence, there is no compromise between NK cells and decreased expression exhibited by some altered tumour cells. For the last few years there has been a progressive effort made in the field of NK cell research to decipher the functional aspects of these cells. Also, NK cell based immunotherapy is emerging as a novel and promising approach as a therapeutic agent in treating malignancies. The current knowledge about the NK cells characteristics based on the various 'missing self'and 'induced self', model experiments have led to major advancements in our understanding. Other than T and B cells, the NK cells do not recognize foreign peptide or antigens. These are self-centric in nature meaning that, they can detect changes in self molecules which are displayed on the surface of autologous cells [30]. The MHC class I recognition by inhibitory receptors and inactivation of NK cells clearly explains that virally infected and malignant autologous cells having down-regulation of the MHC I molecules, are only susceptible to attack by NK cells while healthy cells remain unaffected from NK cell toxicity [30]. The 'missing self' was not able to explain why human erythrocytes were being spared and why few tumour cells containing abundant quantities of MHC class I molecules, were being killed by NK cells. Ensuing knowledge of various inducible ligands however, have explained that NK cell induction requires the expression of certain inducible ligands for activating NK cell receptors and NK cell response is the sum-total of all the activating and inhibitory signals. However, recognition of the inducible ligand and for validating the activation hypothesis, a thorough investigation and molecular characterization of the ligands is required to better explain NK mediated tumour surveillance phenomenon. Several studies carried out in various animal models have explained the same notion that NK cells are involved in eradication of tumour cells and elimination of these cells leads to more vigorous growth of tumour and its metastasis. Shankaran et al., reported that mice knocked with RAG2 (Recombinase Activating Gene) and STAT1, had a higher rate of development of adenocarcinoma as compared to control and the mice deficient only in RAG2 [31]. Dunn GP et al., and Smyth MJ et al., and Swann JB et al., reported the same finding. Results support that NK cells play a role in tumour surveillance [32-34]. Various cytokines like interleukins i.e., IL-2, IL-12, IL-15, IL-21 and IFN-α/β enhance the activation of NK cells, promote NK cell maturation, surge the cytotoxic activity against tumours and administering these cytokines always heightened the NK cell activity [35,36]. In one of the studies conducted, it was shown that in the initial stages of tumour development, though NK cells arrested and eliminated the tumour but in its dormant stage, these cells were of least importance and adaptive immune system come into play to check the development and chronicity [37].

Several therapeutic interventions including surgery, chemo-therapeutic agents or ionizing radiations, have been adopted as primary strategies to check the tumours. Therapies have been designed to induce potent antitumour response by harnessing the potential of the immune system. But these strategies including cytokines, monoclonal antibodies, vaccines, adoptive cell transfers (T, NK and NKT) and Toll-Like Receptor (TLR) agonists, may not be effective in many of the cases as chances of relapse can occur due to drug resistance or resistance to radiations. The capability of the immune system to eliminate tumour cells till they are fully converted to tumour growth by the phenomenon known as immunosurveillance [38] keeps a dynamic balance in the biological system. The current understanding of NK cell biology, functions etc. has recently led to the development of NK cells as a powerful and potent tool in cancer immunotherapy. NK cell immunotherapy [39] has emerged as a potential alternative through various potential approaches including large-scale production and expansion of the cells for clinical trials and therapeutic purposes, for enhancing the efficacy. Direct killing of the tumour cells is mediated by release of perforins and granzymes leading to cell apoptosis [40], mediated by death receptors and by secreting various effectors like INF-γ [40]. Release of these cytokines is associated with Nitric Oxide (NO) production i.e. the NK cells kill the target cells by NO signalling [41]. Another way of direct killing is through ADCC by expressing CD16 for destroying altered cells. Cytokine like, IL-2, IL-12, IL-18, and IL-15 further stimulate NK cell activity while other induce IFN production. These NK cells

also participate indirectly in the killing process. Acting as regulatory cells, NK cells interact with dendritic cells, macrophages, T-cells and endothelial cells through production of various cytokines such as INF-γ, TNF-α, and IL-10 as well as other chemokines and growth factors. By releasing INF-γ, these cells induce $CD8^+$ cytotoxic cells to differentiate into CTLs. They also help in differentiation of CTLs via induction of $CD4^+$ cells. Moreover, in course of killing of tumour cells by NK cells, antigens produced, are taken up by Antigen Presenting Cells (APCs) for induction of adaptive immune response [42].

Tumour cells, during progression adopt several mechanisms to escape from NK cell recognition. In cancerous patients, abnormalities of NK cells have been noticed. These cells also cause generation of defective NK cells via down-regulation of adhesion molecules or production of costimulatory ligands, or by upregulating MHC-I molecules, secreting immunosuppressive factors, resisting fas or perforin mediated apoptosis [43-46]. Sialoglycans affects critical steps in tumour immunology. Different sialoglycants are recognized as ligands to sialic acid binding Ig like lectin-7 (siglec-7), which also known as a pan-NK cell marker, and siglec-9 on human NK cells. Siglec-9 was recently expressed in subset of $CD56^{dim}$ NK cell [47,48]. In various tumour types, vulnerability to NK cell cytotoxicity are affected by the advancement of sialylation status of cancer cells. Since, hypersialylation gives an advantage to tumour cells under pressure from NK immunosurveillance. Through the recruitment of the sialic acid binding immunoglobulin like lectin-7 on the cancer cells, activation of natural killer cells inhibited [49]. Siglec-7 also responsible for inhibiting the function of NK cell when combined with other specific antibodies. It has also been concluded from a study that siglec-7(+) NK cell shows more CD107a degranulation and IFN γ production compared siglec-7(-) NK cells [50]. It is apparent from the direct and indirect killing mechanisms of NK cells that they are very efficient and competent immunotherapeutic agents in the clinical context. In early clinical trials conducted in the 1980s, IL-2 activated NK cells were introduced through subcutaneous injections to treat cancerous patients. Results showed 15-30% positive effects in patients with advanced renal cell carcinoma or melanoma. But IL-2 activated NK cells increase sensitivity to apoptosis when in contact with vascular endothelium with slowing of the migratory rate [51]. Furthermore, other inducers of the NK cells like IL-12, IL-15, IL-21 Flt3-L, SCF, and IL-7 have also been used. Flt3-L leads to the expansion of not only NK cells but also of dendritic cells [52,53]. Other than IL-2 and IL-15, IL-12 mainly enhances the production of INF-γ via NK cells [54]. IL-12 and IL-18 manifest their effects through release of INF-γ [55]. Apoptosis of tumour cells is mediated by INF- γ by, induction of TNF-Related Apoptosis-Inducing Ligand (TRAIL) and FasL- mediated cellular susceptibility to apoptosis [56]. NK cell can also be stimulated by direct engagement of surface TLR3 and TLR9 and some synthetic molecules have been found capable of mimicking the immunostimulatory activity of viral and bacterial products via TLRs [57].

Therapies Based On Nk Cells Targeting Cancer

Prior to adoptive infusion of NK cells, they can be activated through short-term exposure in vitro. To obtain clinically significant amounts of these cells, their long-term expansion is required. Also, *in vitro* expansion lead to potential phenotypic changes resulting in selective expansion and reduced cytotoxic killing. These concerns can be addressed by improvement in techniques to achieve clinically relevant NK cells with their in vivo anti-tumour efficacy. Several factors influence the clinical efficacy and relevance of the NK cells. These factors include the source of NK cells, type of cytokines used for stimulation, medium of cell culture and conditions, expansion etc. The Peripheral Blood Mononuclear Cells (PBMC), Umbilical Cord Blood (UCB), cell lines, Human Embryonic Stem Cells (HESC), Induced Pluripotent Stem Cells (iPSCs) have been the source of NK cells [58]. PBMCs are processed via apheresis or Ficoll separation under cGMP conditions for NK cell purification [59]. One unique method was adopted by Sukamoto N et al., to generate a large number of NK cells without prior purification of peripheral blood, that is culturing the PBMCs with autologous plasma, IL-2, OK-432 and γ-irradiated autologous T-cells (FN-CH 296 stimulated). On day 21-22 purity level of NK cells reached upto 90.96% [60]. An immunomagnetic depletion approach is another method of purification and enrichment of NK cells involving depletion of other lymphocytes such as T and B-cells, and myloid cells [61]. Nguyen S et al., have reported the beneficial effects of partial T-cells depletion after Haematopoietic Stem Cell (HSC) transplant, thereby suggesting a positive role of T-cells in in vivo stimulation of NK cells activity [62]. Use of feeder cells and cell lines in in vivo expansion of NK cells has also been reported [63]. Further more, direct enrichment of CD56+ cells via immunomagnetic selection is another useful approach [61]. Use of HSC ($CD34^+$) from bone marrow, peripheral blood or UCB through differentiation and expansion of $CD34^+$, can be another potential source to have clinically relevant antitumour NK cells. Recently, a study has shown that frozen $CBCD34^+$ is most promising HSC source for producing NK cells compared to fresh $CBCD34^+$ and frozen $PBCD34^+$ [64]. NK cells derived from UCB are less active exhibiting reduced killing properties, and can be stimulated by ex vivo treatment with IL-2, IL-12, and IL-15 [61]. One of the important sources of NK cells, HESC and iPSC with reduced risk of immune rejection has been reported by Knorr DA et al., [63]. In this procedure, HESCs and iPSCs underwent two stage culture method to differentiate into $CD34^+$ cells via SPIN-EB system [65]. NK cells derived from human embryonic stem cells has the ability to kill the multiple types of tumours in both in vivo and in vitro. NK cells derived from both HESc and IPSC are able to inhibit the HIV-1 NL4-3 infection from CEM-GFP cells [66]. Additionally, a mouse xenograft model based study also have observed that NK cells derived from PB and iPSC having the ability to mediate killing of ovarian cancer cell [67]. In xeno-free and serum-free conditions, cytotoxic NK cells were generated leading to one step forward towards clinical scale production [63].

For off the shelf anticancer therapy, the cell lines derived from NK cells (NK-92, NKL, KYHG-1, and NKG) are potential source. Moreover, genetically modified NK cell lines expressing intracellular IL-2 and cell surface molecules like CD16, NCRs, or Chimeric Antigen Receptors (CARs) have also been used as possible tools for generating activated NK cells [65]. Many genetically modified NK cells have been choosen for clinical trials but all this is still in a nascent stage and several novel potential strategies are under extensive research. To cope up with tumour microenvironment various immunosuppressive therapies are being developed. Many approaches involve triggering of ADCC through monoclonal antibodies, whose antigen binding fragment (Fab) binds to tumour cells and constant region (Fc) binds to CD16 ligand on the NK cell surface [68]. Anti-CD20 (Rituximab), Anti-Her-2 (Trastuzumab), Anti-CD52 (Alemtuzumab) and Anti-EGFR (Cetuximab) are few examples of monoclonal antibodies used for ADCC triggering phenomenon [69].

CONCLUSION

None of studies are currently done to establish intimate mechanism of NK cells. The multifaceted lytic role of NK cells has been clearly implicated in the tumour cell killing and promising results have been obtained in various experimental models. However, NK cell based immunotherapeutic clinical efficacy in cancer patients is quite moderate and many challenges still need to be overcome.

To increase the activity of NK cells against tumours, various efficacious cytokine combinations with NK cell based immunotherapy have been tried in association of other approaches. For its actual use at the therapeutic level, a good control upon NK cell activity, based on a deep knowledge of their basic physiology at the bench is necessary. Parallel to development of clinically relevant pool of NK cells, there is a need for comparative clinical trials. Various NK

cell based products undergoing multicentric clinical trials, will come into the picture in future to assess its efficacy data. Soon, NK cell therapy will probably be one of the most promising tools for the management of human cancer.

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PARTICULARS OF CONTRIBUTORS:
1. Associate Professor, Department of Biochemistry, Santosh Medical University, Ghaziabad, Uttar Pradesh, India.
2. Professor, Department of Biochemistry, Santosh Medical University, Ghaziabad, Uttar Pradesh, India.
3. Lecturer, Department of Biochemistry, TSM Medical College and Hospital, Lucknow, Uttar Pradesh, India.

NAME, ADDRESS, E-MAIL ID OF THE CORRESPONDING AUTHOR:
Dr. Preeti Sharma,
23 Arya Nagar, Surajkund Road, Meerut-250001, Uttar Pradesh, India.
E-mail: prcdri2003@yahoo.co.in

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