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Review Article

Natural Killer Cells: A Review on Their Role in Cancer Immunotherapy

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ABSTRACT

NK cells are major immunosurveillance cell comprising about 15% of cells in the circulation. Highly controlled regulation between activatory and inhibitory receptors mediates the functions of NK cells. These cells target the tumorous cells that lack MHC (Major histocompatibility Complex) or have altered molecules on their surfaces whereas healthy cells are protected from lysis by NK cells due to inhibitory receptors and MHC I interactions. NK cells, chiefly allogenic variants, are used in the treatment of various cancers such as breast cancer, glioma, neuroblastoma, renal cancer, melanoma etc. NK cells act by release of perforins and granzymes or by activating ADCC (Antibody dependent cellular cytoxicity) pathway.

Though NK cells have evolved as a promising therapeutic regimen for malignancies, their limited concentration in blood has made it difficult to be used widely. Thus researches are being focused on exvivo activation and expansion of NK cells so as to increase the cell number, viability and cytotoxic functions.

Key words: Natural Killer Cells (NK Cells), Major Histocompatibility Complex (MHC), Tumors.

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INTRODUCTION

A consensus in 2012 indicated about millions of new cases, which about 8.2 million deaths related to cancer, which indicates cancer to be one of the major health threats worldwide[1]. The conventional techniques used in the treatment of cancers were surgery or the use of ionising radiations or chemotherapeutics, but the major drawbacks observed in these methods were higher rates of tumor relapse, due to resistance developed by the timorous cells to the drugs and radiations[2]. To overcome these drawbacks, and to make the cancer treatment more reliable, safe and to prevent relapse recently researches are being focused on cancer immunotherapy's, in which several cytokines (IL-2, IL-12 etc.), immune cells (T cells, dendritic cells, natural killer cells and NK cells, cytokine induced killer cells or (IK), monoclonal antibodies are used[3].

In humans, the immune system is actively involved in the prevention of neoplastic development, a mechanism known as immunosurveillance[4]. NK cells being major immune cells in tumor immunosurveillance[5], advanced researches are being conducted on NK cell biology and function which has ked NK cells to emerge as a powerful cancer immunotherapy tools[6].

It was in 1971, natural killer cells were first described when Cudkowizc and Benett showed, and mice undergoing lethod irradiation could reject allogenic parental allograft of bone marrow cells[7]. Later in 1975, cells capable of mediating such action were characterised to be radioresistent[8] having identity similar to lymphoid cells with cytoplasmic granules[6] and ability to spontaneously kill tumours cells in vitro, the mechanism of which was MHC (major histocompatibility complex) restricted. These cells were later recognised as NK cells[9].

However the therapeutic role of NK cells remained hidden till 1990s when target cell recognition phenomenon of NK cells was elucidated and in early 2000s when antileukemic effect of NK cells was demonstrated in HCT (Hematopoietic cell transplantation)[10].

BIOLOGY OF NK CELLS

Keissling and Herberman were the first to name and characterise NK cells[11]. These cells were named considering the fact that, the term natural meant the natural occurs of these cells and killer meant their ability to kill lymphoma and leukemic cells[12]. The primary origin of NK cells that have pivotal role in innate immunity

as well as immunity against tumors and viral infections is lymphoid tissue[13]. Beside, they are also found in spleen, bone marrow and peripheral blood[9]. NK cells make up to 5-15% of circulatory lymphocytes and also present in liver placenta and peritoneal cavity[14].

There are several specific markers for NK cells but some NK cell specific markers are also present on T-cells (NK), DXs in mouse and CD56 and 16 in humans)[9]. The characteristic feature of NK cells is absence of CD3 molecules and presence of CD16 and CD56 as the surface antigen[15]. Therefore, in human NK cells are identifiedas $CD3^{-}CD56^{+}$ lymphocytes which can further be categorised on the basis of level of expression of CD56 as, CD56 dim and CD56 bright[16]. CD 56 dim denotes lower level of expression of CD 56 and comprises about 90% of NK cells in blood whereas CD 56 bright indicates higher expression of CD 56 and comprises only 10% pf NK cells[15]. CD 56 dim type include alloreactive NK cells that can target leukemic cell[17] whereas CD 56 bright type through immature, are more ca[able of appreciable amount of cytokines production and immunoregulatory activities[18].

NK cells, which are mainly produced in bone marrow and hematopoietic stem cells, have five developmental phases:

- **Stage I:** cells express CD34, CD16, CD94 and CD17[19]
- **Stage II:** NK cells are capable of responding to IL-5 which is required for their development[20]
- **Stage III:** cells lose CD4 expression[21]
- **Stage IV:** NK cells differentiate into CD6 bright and produce interferon r (IFNR)[21]
- **Stage V:** NK cells differentiate to CD56dim with the expression of CD16[22]. Cytokines[23] stem cell factor (SCF)[24], stromal cells[25] fetal liver kinase ligand[24] have important roles in differentiation of NK cells.

NK CELL RECEPTORS

Highly sophisticated receptors network control the function of NK cells, aiding them to differentiate to abnormal cells from normal[26]. There is a well regulated balance between the inhibiting the activating signals derived from various receptors[27]. When inhibiting signal is dominated by activating signal, lysis of target cells occurs[14].

NK cell receptors are of two types namely activating receptors and inhibitory receptors[28]. Major inhibitory receptors include killer Ig ike receptors (KIR) and C-type leptinreceptors (NKG2A)[29], whereas activating receptors are natural cytotoxic receptors (NCRs), NKp30, Nkp44, NKG2D etc[30].

The first NK cell receptor named Ly-49 (MHC-1 specific) was discovered in mouse. Ly-49 receptors are integral membrane proteins type II and homodimers that can recognize MHC-1 molecules such as H-2d and H-2k[31]. Several receptors about more than 20, belonging to Ly-49 have been characterized, out of which 13 are inhibitory and 8 are stimulatory to the NK cells[32]. The other category of receptors, that have been discovered and which are chiefly expressed in man, are the transmembrane proteins related to the Ig (immunoglobulin) superfamily and can recognize the groups such as HLA-C (Human leukocyte and HLA-A alleles[33]. Of these, HLA-C is mainly involved in the regulation of NK cell functions[34].

The receptors (both KIRs and Ly-49), that are inhibitory in nature, conduct their action via ITIMs (Immunoreceptor tyrosine-based inhibitory motifs)[35]. On binding of specific MHC molecules, the tyrosine residues are phosphorylated causing inhibition of stimulatory signals. On the other hand, stimulatory receptors lack ITIMs and mediate their action via association with DAP 12, which is a ITAM (Immunoreceptor tyrosine based activation motif) bearing adaptor molecule[36]. Receptors associated with NKG2 family have also been identified in human and mice. Such receptors contain CD94 molecule (a C type lectin) which can exert both excitatory and inhibitory signals[37]. Inhibitory signals occur when CD94 binds with Qa-1b (in case of mouse) and HLA-E (in case of human in target cells[9].

Another receptor recently identified is KLRE1 (leptin like receptor) which is an inhibitory transmembrane protein expressed in rats and mice[38]. NK cells also possess ILT (Ig like transcript), that binds with HLA-G[38], expressed on fetus, protecting fetus and placenta from rejection[39]. NCRs are another group of diversed NK cell receptors that comprise NKp44, NKp46, NKp30 and NKG2D[38]. These receptors recognise MHC-1 negative target cells[40] NK cells also express FcγRIII receptors, which can bind with Fc fragment of associated antibody causing antibody dependent cellular cytotoxicity (ADCC)[41].

Recognition of tumorous cells by NK cells via inhibitory and excitatory receptors is a complex process. Three recognition models so far have been identified viz: Missing self-model, Self recognition model and Stress induced recognition model[42]. When cells are at steady state, the inhibitory receptors bind to MHC-I molecules and thereby prevent NK cell activation as well as function. However, under the conditions of stress, there is down regulation of MHC-I molecules leading to the loss of inhibitory signals and thus promoting the activation of NK cells via the mechanism known as missing self recognition[43]. In contrast to the inhibitory receptors, the activating receptors of NK cells have the ability to recognize either the pathogen encoded molecules, not expressed in host (via a mechanism known as non self-recognition)[6] or the proteins that are self-expressed and upregulated by the tumor cells or viral infected cell (via the mechanism known as stress induced recognition)[42].

MECHANISM OF ACTION

Studies have shown that CD56 dim type NK cells are the major circulating fraction and most potent against tumor cells[44]. Similarly, a 11 year follow up study has demonstrate that, reduced NK cell cytotoxicity is associated with the increased risk of cancer[45]. An increased infiltration of NK cells in cancer cells have proven to be a positive prognostic marker in carcinomas of colorectum, stomach and lungs[46].

Direct clearance of tumor cells

The cells deficit of MCH-I Or the cells with stress induced protein molecules are targeted by NK cell[47]. Moreover, certain cells with expressed MHC-I are also targeted via activating receptor induced recognition of stress induced ligand[48]. Several mechanisms have been developed to explain the direct killing of tumor cells by NK cells. These include:

- Release of cytoplasmic granules with perforin and granzymes causing caspase dependent or independent apoptosis of tumor cells[49].
- FasL (Fas ligand), TRAIL (Tumor necrosis factor related apoptosis inducing ligand) dependent mechanism[49].

- Secretion of effector molecules like IFNs, IL-2, IL-12 etc[50] which not only stop tumor angiogenesis and stimulate adoptive immunity but also induce production of nitric oxide causing the destruction of tumor cells via NO signaling mechanisms[51].
- ADCC

Type IIIA Fc receptors on NK cells can recognise target cells coated by antibodies, enhancing the NK cell mediated ADCC with rapid activation and degranulation of NK cells[52]. Immunocytokines (also known as antibodies with Fc end linked to cytokines), promote the synapsis between monoclonal antibody coated cancer cell and NK cell, thereby facilitating the Fc and cytokine receptor binding[53]. As for example, use of rituximab linked to antibody which blocks immunomodulatory agents like linalidomide, upregulates the activation of of NK cells facilitating the lysis of target cells[54], in non-Hodgkin Lymphoma[55].

Romain et al, who introduced triple mutation in Fc fragment of monoclonal antibody HuM195 that targeted acute myeloid leukemia (AML) antigen, stated that HuM195 increased both number and the cytotoxic ability of NK cells, mediated by CD16 signaling[56]. However, cytotoxic ability of NK cells during the use of non-modified monoclonal antibody is governed by KIR dependent inhibition. In this case, anti-KIR antibodies must be used to block iKIRs interaction with MHC-I ligands, so as to potentiate the actions of NK cells[54].

Indirect clearance of tumor cells

In this case, NK cells are first activated, which then induces dendritic cells that enhance the antigen specific cytolytic responses, as they can cross the tumor specific antigens obtained from NK cell directed cell lysis, to T cell[6].

NK CELLS IN IMMUNOTHERAPY

NK cells used for cancer therapy may be obtained from both autologous and allogenic sources[1].

Autologous NK cell

Previously, NK cell immunotherapy involved the endogenous activation and proliferation of NK cells to induce antitumor activity, via administration of cytokines like IL-2[57] or IL-12 and IL-15[58]. Such stimulated NK cell exhibited greater cytotoxicity in the form of LAK (Lymphokine activated killer cell)[59] and were used in the treatment for various cancers like breast cancer, glioma, adenocarcinoma and renal cancer[60]. However, the clinical outcomes were poor[59], therefore, as alternative technique was developed in which allogenic NK cells were used in place of autologous NK cells[61].

Allogenic NK cells

Allogenic NK cells are more successfully used in cancer treatment as they are not susceptible to the inhibition that arises due to self MHC recognition by NK cells[62]. Several cancers like leukemia, solid tumors, metastatic melanoma, renal cancer, Hodgkin's disease, AML are treated[63]. Miller et al, showed that allogenic NK cells are safe and donor induce graft versus host disease (GVHD)[63]. Another pilot study, demonstrated that transfusion of NK cells from KIR-HLA mismatch donor reduced the relapse risk of AML in children[64]. However, the risk of GVHD, if present can be reduced by the use of immunosuppression techniques, use of CD3 depleted NK cells and selection of donor with HLA matched to host[65]. To date allogenic NK cells developed from peripheral blood and CD34+ cells have presented the promising outcomes in cancer immunotherapy[66].

NK cell lines

About seven NK cell lines have been developed, namely NK92, YT, HANK-1, KHYG-1, NKL, NKYS and NKG[67] which are used in the production of "Off the shelf" anticancer products[68]. Of these cell lines NK92, NKL, KHYG-1 and NKG are well known for the antitumor function[69]. NK92, clinically the most studied cell line[68], is FDA approved safe and beneficial therapy for a wide range of tumors[70]. In the study of Tonn et al, Nk92 was used to treat both solid tumors and hematological malignancies. Use of NK92 cells showed partial efficacy in some end stage cancer like renal cancer and melanoma[71].

STEPS OF NK CELL BASE IMMUNOTHERAPY

NK cell based immunotherapy involves three major steps as follows:

KIR typing

Of several NK cell receptors identified, KIR family is the major determinant in NK cell response[26]. KIR, since is stongly polymorphic, its typing is very critical. The advantage of KIR typing is that it facilitates the selection of allogenic donor and prognostication of autologous NK therapy[72]. KIR typing involves genotyping, categorisation of A/B haplotypes, phenotyping and alleotyping[9]. Gene expression can be assessed by real time PCR or flow cytometry[73]. Allele polymorphism is seen in all KIR genes that are inhibitory in nature[74]. Therefore single nucleotide polymormism assays are being used for rapid clinical typing[75]. Patients receiving grafts with stronger KIR allele demonstrated lower relapse rate, better survival and lower mortality rates[76].

Cell processing

Nk cell purification

After selection of suitable donor via KIR typing, the NK cells are purified and processed exvivo. The quality of NK cells are assessed in terms of cell count, function, purity, viability and phenotype[77]. Immunogenetic separation technique yields purified form of NK cell mixture that consists of more that 90% of NK cells with very little amount of T and B lymphocytes[78].

NK cell processing or activation exvivo

Purified Nk cells can be activated in several ways. One of the common method is by using cytokines such as IL 2, IL 12, IL 15 or IFN type I[79]. An alternative to this method is to activate NK cells by artificially presenting the cells with membrane bound ligands like IL 15 and IL 21[80].

Use of chimeric antigen receptor (CAR) also aids to potentiate NK cells. Such receptor insertion may be retroviral, lentiviral or transposon system mediated[81]. Similarly culture along with unmodified CD56 and stimulating cytokine scan also result in activation of NK cells[82]. Such activated cells show higher affinity to neuroblastoma both invitro or invivo via the involvement of NCR, DNAM-1 and granzymes[83]. NK cell adhesion to tumor cells can also be enhanced by immunocytokines. One common example is the antibody against ganglioside GD2 that is conjugated to IL 2[84]. These antibodies on one part binds with GD2 on neuroblastoma and on the other part to IL 2 receptors on NK cells[85]. Though these techniques are safe, lack of donors for NK cell[86] and requirement of sophisticated infrastructure and instruments have limited the effective use of these approaches[87].

Augmentation of NK cells

It can be mediated via

Combination of NK cells with therapeutic antibody/ Chimeric protein

One of the important NK cell dependent therapy for cancer is ADCC[88]. Use of monoclonal antibody against CD137 or blocking antibodies for KIR or NKG2A increases NK cell induced cell lysis[89].

Another method of augmentation is incubation of NK cells with specific killer cell engager. This not only stimulates cytolytic activity of NK cells but also increases production of INF ɣ, TNFα and IL8[90].

Combination with medication

NK cells that are adoptively transferred not only target cancerous cells, but also interact with immune system, therefore combination therapy should be applied to improve efficacy[91]. Most of the combined regimen includes injection of IL 2 in low dose after NK cell infusion but recent studies are being focused on newer agents. As for example, non-Hodgkin lymphoma is being treated using granzyme B and FasL along with lenalidomide as medical supplement which enhances ADCC[9]. Drugs such as spironolactone upregulates NKG2D[92] whereas doxorubicin upregulates TRAIL R2 causing enhancement of antitumor functions of NK cells. NKG2D can also be upregulated by histone deacetylase inhibitor[93].

Modulation of tumor cells

Tumor cells can be epigenetically modulated with histone deacetylase inhibitor, demethylating agents and proteosome imhibitors[9]. Such modulation upregulates NKG2D and prevents its loss from NK cells[94]. In a study of William et al, lentiviral shRNA was used to target almost 10000 human genes so as to silence JAK 1 and JAK 2. This increased susceptibility of tumor cells to lysis by NK cells[9].

Genetic modifications of NK cells

Recent advances in the field of NK cell immunotherapy include genetic manipulation of NK genes via siRNA, that involves suppression of inhibitory receptors and over expression of activating receptors[95]. Further NK cells can also be engineered to express chimeric antigen receptor[96] and cytokines (via transfer of cytokine genes)[97], which not only activate but also improve proliferation, survival and antitumor activity of NK cells[98].

CONCLUSION

NK cells have been recently evolved as the potential therapeutic agent in cancer, which are resistant to the contemporary therapies used. NK cells, major cells in tumor immunosurviellance, can destroy tumor cells that lack or have altered MHC-I proteins, via release of cytotoxic granules. Normal cells are protected from lysis due to interactions between KIRs and MHC-I protein on the cell surface.

However, NK cells, though evolved as a novel immunotherapeutic strategy, have some limitations which hinder their widespread use. NK cell therapy, both autologous and allogenic, requires about 10^{11} lymphocytes. Since the peripheral blood from donor comprises only 15% of NK cells, it possesses a challenge in acquiring NK cells in sufficient amount as well as difficulties in expanding the cell exvivo. Besides, in spite of presence of efficient immunosurveillance system, many tumors can still develop in body creating the disease. Therefore, it is necessary to understand the mechanism by which the tumor cells escape immunosurveillance and researches are to be focused on development of more promising approaches for expansion and activation of immune cells involved in cancer therapy so as to attain higher cytotoxicity ability, survival rates and desirable clinical outcomes.

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