Title

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Journal

Critical Reviews™ in Oncogenesis, 19(1-2)

ISSN

0893-9675

Authors

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Publication Date

2014

DOI

10.1615/critrevoncog.2014010805

Peer reviewed

Critical Reviews™ *in Oncogenesis*, 19(1-2):57–66 (2014)

Positive and Negative Regulation by NK Cells in Cancer

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ABSTRACT: Our understanding of NK biology has expanded immensely since the initial discovery of natural killer cells in 1975. New studies have uncovered various levels of immune regulation both on and by unique subsets of NK cells, which go well beyond simple receptor–ligand interactions between NK cells and target cancer cells. Distinct suppressor and effector populations of NK cells have been delineated in both viral and tumor models. Interactions between NK cells and dendritic cells, T cells, and B cells also dramatically alter the overall immune response to cancer. To exploit the diverse functional abilities of NK cell subsets for cancer immunotherapies, it is important to understand NK cell biology and NK regulator mechanisms.

KEY WORDS: Cancer immunology, natural killer cells, cancer immunoevasion

ABBREVIATIONS: ADCC: antibody-dependent cell-mediated cytotoxicity; **CSC:** cancer stem cell; **DC:** dendritic cell; **GM-CSF:** granulocyte macrophage colony-stimulating factor; **IFNγ:** interferon gamma; **IL**: interleukin; **KIR:** killer immunoglobulin-like receptor; **MHC:** major histocompatibility complex; **mRNA:** messenger ribonucleic acid; **NK:** natural killer; **NKG:** natural killer cell group; TGFβ: tumor growth factor beta; **TNFα:** tumor necrosis factor alpha; **Treg:** T regulatory cell.

I. INTRODUCTION

A. History

Natural killer (NK) cells are a vital component of the innate immune system; their roles in nearly all aspects of immunological responses have been increasingly revealed. Initial observations of previously unidentified effector cells (later recognized as NK cells) involved their ability to spontaneously reject bone marrow allografts, even parental donor bone marrow cells, in F1 mice.[1,2] This phenomenon was termed "hybrid resistance" and appeared to oppose the laws of transplantation because antigens involved in bone marrow rejection were thought to be co-dominantly expressed.[3,4] This rejection was negated upon depletion of bone marrow-derived cells through the utilization of strontium-89, suggesting the presence of a bone marrow-derived cell mediating this resistance.[5] These cells were identified in 1975 through the independent studies of Drs. Herberman and Kiessling, who each uncovered "natural" cytotoxicity in killing assays that utilized both syngeneic and allogeneic tumors, even in the absence of T cells.[6–9] The name "natural killer cells" was given to this cell population since they required no prior sensitization or immunization and their cytotoxic capabilities were non-MHC restricted.

Recently, the field of NK cell biology has expanded well beyond simply describing the cytotoxic functions of these cells, with new roles attributed to the vast array of cytokines NK cells are capable of producing and the potential targets NK cells can recognize and bind. NK cells are now known to play a role in not only viral[10] and tumor[11] resistance but also bacterial[12] and fungal[13,14] immune responses. NKs have also been shown to play a role in both bone marrow rejection[15] and bone marrow cell engraftment.[16] Additional immunoregulatory[17] and tissue regenerative properties [18] have been discovered in viral resistance models. Therefore, to better understand the role NK cells play in immune responses, particularly

11040-8401/14/$35.00

in cancer responses, the multifaceted functions these cells exhibit, the pathways of regulation, and the interactions with other cell types, including cancer cells themselves, need to be considered for potential therapeutic utilization and for targeting these cells.

B. Human versus Mouse NK Cells

The study of NK cells and the roles they play in cancer immunology have been complicated by the differences between human and mouse NK cells, which require careful interpretation when extrapolating findings from murine models. However, by acknowledging these differences and creating models and studies that take these differences into consideration, vast amounts of knowledge are obtained about NK biology through murine studies. Differences in receptor and antigen expression exist between human and mouse NK cells. Human NK cells express CD56 (which mice do not), that differentiate the cells into two separate subsets with preferential locations and functions. The $CD56^{dim}$ NK subset population is found in circulation and is predominately cytotoxic. The $CD56^{bright}$ population is found in the lymph nodes and produces high levels of cytokines but exhibits weak cytotoxic effects.[19,20] Importantly, mouse NK cells are not found in the lymph nodes until immunological stimulation,[21] in contrast with human NK cells, which are normally found in the lymph nodes. There are also differences in the receptors that bind MHC class I and related molecules. In mice, these receptors are the C-type lectin family, Ly49s, while in humans they are the functionally identical, but structurally different, killer immunoglobulin-like receptors (KIRs).[22,23]

Freshly isolated human NK cells also exhibit strong cytotoxic capabilities due to the constitutively high expression of granzyme B and perforin proteins.[24] Human NK cells can also be cultured for weeks with feeder cells or IL-2 and IL-15 while maintaining normal expression of various receptors including KIRs.[25] Mouse NK cells, on the other hand, exhibit weak cytotoxic functionality when freshly isolated due to minimal amounts of granzyme B and perforin protein present but high levels of their respective mRNA transcripts, which require additional activation for translation. *In vitro* culture of murine NK cells is also relatively limited due to the occurrence of rapid cell death and decreased function after 2 weeks in culture.[26,27]

These differences between mouse and human NK cells highlight important aspects of NK cells that need to be considered during experimentation. The divergences suggest a more recent evolutionary development of NK cells when compared to the more conserved T and B cells that are analogous between humans and mice.[28,29] This highlights the novelty of NK cells, which possess functional differences that are continuing to evolve, but it also underscores the nuances that can be encountered when applying findings from mouse models to humans.

II. NK REGULATION DURING CANCER

A. Tumor Microenvironment

Cancer cells are able to evade immune responses by NK cells through a number of mechanisms. Cancer cells can increase the expression of MHC class I molecules to inhibit NK cell cytotoxic functions [30,31] and decrease the expression of NKG2D ligands to impair NK cell recognition. Inhibitory cytokines such as IL-10 and TGF-β are also elevated in the tumor due to secretion by the tumor itself, T regulatory cells, or myeloid derived suppressor cells, which makes the tumor environment highly suppressive and limits the efficacy of NK anti-tumor functions.[32–34]

B. Subsets

Recent studies have discerned unique functional and receptor repertoires on subpopulations of NK cells, suggesting that NK cells are not a uniform population of cells but might be more analogous to T cells, with unique subsets. As mentioned earlier, human NK cells exhibit differential expression of CD56, with cells that have low expression of CD56 having greater cytotoxic function and cells that have high expression of CD56 producing greater levels

of cytokines, but exhibiting reduced cytotoxicity. CD11b and CD27 expression correlates to CD56 subset differentiation with the $CD11b^{low}CD27^{high}$ being analogous to the $CD56^{bright}$ population and the $CD11b^{high}CD27^{low}$ being analogous to the $CD56^{dim}$ population functionally.[35]

NK cells also show unique patterns of inhibitory receptor expression that differentiates the functional responses of NK subsets. NK cells that express inhibitory receptors that have high binding affinity to self-MHC class I molecules are considered "licensed" or "educated" NK cells with high IFNγ production and cytotoxicity. Cells that do not express inhibitory receptors that can bind to self are considered unlicensed or uneducated NK cells and are considered to be hyporesponsive.[36–38] Studies looking at the differential functionality of these NK subsets *in vivo* found that after hematopoietic stem cell transplantation and lethal irradiation, licensed NK cells showed greater protection against MCMV than the unlicensed population with significantly greater expansion and IFNγ production.[39,40] Recent studies in our lab have expanded on these findings and showed differential immunoregulatory and functional roles of the subsets throughout the course of infection. The licensed population was shown to function as the effector population with an anti-viral role early during infection and a suppressive role during late stages of infection, owing to their ability to eliminate T cells. The unlicensed population was shown to function as a helper population early during viral responses by producing GM-CSF, which aided in DC expansion and in turn T-cell expansion. Late during immune responses, the unlicensed population was found to traffic to sites of tissue damage and produced IL-22 to aid in tissue repair. Thus, based on licensing or education, NK cells can be classified into subsets with distinct functional responses.

Subsets of NK cells with unique functions have also been differentiated based on the isoform of receptors expressed. Delahaye et al. described unique isoforms of the activating receptor NKp30 that had contrasting responses to gastrointestinal stromal tumors. NK cells that express isoforms NKp30a or NKp30b exhibited characteristic cytotoxicity and cytokine production to tumor targets. However, NK cells that express the NKp30c isoform were actually suppressive and produced high levels of IL-10 and correlated with worse outcomes and increased morbidity and mortality in patients that had a high percentage of NK cells that expressed this isoform.[41]

As increasingly more studies are uncovering NK cells to be a heterogenous population[42] with unique functional subsets, the NK response to various tumors becomes complicated. Changes in the distribution of these populations could dramatically alter the progression of disease and mortality rates. Additionally, therapeutic targeting or adoptive transfer of specific, highly efficacious NK cells could result in improved immunotherapy approaches.

C. Immunoregulation

1. T Cells

The interaction between NK cells and T cells plays a crucial role in shaping the overall immune response. One significant aspect of this interaction is the role T regulatory (Tregs) cells play in the NK immune response. A set of recent publications demonstrated that Tregs play a role in NK cell homeostasis and sensitivity to target cells by limiting the availability of IL-2 to NK cells. Thus, competition for IL-2 between T cells and NK cells serves as a significant regulatory mechanism for both populations.[43,44] A recent publication from our lab also demonstrated that Tregs significantly limit the functionality and expansion of the licensed or educated NK cells that are able to recognize self during murine cytomegalovirus infection.[40] By regulating this active, fast-responding effector population, Tregs help reduce potential immunopathology and minimize the inflammatory conditions promoted by this NK population. However, this regulation of NK cells by Tregs can also be detrimental if Tregs are recruited to tumor sites as a mechanism of reducing the immune response against the tumor. The highly effective licensed population may thus be severely limited in its functionality due to this increased presence of Tregs.

Reciprocally, NK cells have recently been found to regulate antigen-specific T cells during various viral

responses, with the depletion of NK cells resulting in significantly greater numbers of antigen-specific T cells. Depending on the viral inoculation given to mice, this regulation could be beneficial or harmful to the mice. At low doses of virus, the reduced T-cell numbers were a detriment to the host due to reduced capability of eliminating the virus. At higher doses of virus, the T cells mounted too strong of a response, resulting in severe immunopathology. Therefore, in high viral dose settings, NK cells reduce the immunopathology that occurs from the over-reactive T cells, which is beneficial to the host. These regulatory effects of NK cells on T cells could have dramatic effects on the anti-tumor role of T cells. Therefore, NK cells may actually hamper the immune response to tumors by directly reducing the number of T cells.[17]

2. B Cells

NK cells and B cells also interact through both indirect and direct pathways. NK cells lead to B-cell activation and induce class switching of immunoglobulins produced through CD40-CD40 ligand interaction and through the production of various cytokines, including IFNγ that can lead to class switching.[45,46] Additionally, the antibodies secreted by B cells can lead to antibody-dependent cell-mediated cytotoxicity (ADCC) by NK cells through CD16, Fc receptor expression on NK cells that serves as a strong activator of NK cells. Thus B cells can aid in NK responses to various pathogens or cancers by antibody release and mediating ADCC.[47]

3. Dendritic Cells

The bidirectional regulation and interaction between dendritic cells (DCs) and NK cells is multifaceted and consists of both direct and indirect pathways. DCs are capable of directly stimulating NK cells through trans-presentation of IL-15, which leads to NK activation. DCs also produce a number of cytokines that facilitate activation and expansion of NK cells, including production of type I interferons, IL-18, and IL-12. Direct binding has also been demonstrated to lead to bidirectional activation of both DCs and NK cells.[48,49] CD30L on DCs is capable of binding to CD30 on NK, leading to the maturation of DCs and release of pro-inflammatory cytokines. Additionally, this engagement results in NK cell activation and the release of IFNγ and TNFα.[50]

In addition to directly affecting DCs, NK cells can also indirectly regulate adaptive immune cells through DC regulation. NK cells are capable of lysing DCs that have phagocytized pathogens or antigens, which reduces the number of antigen presenting cells to activate T cells.[51] This DC lysis also reduces the various cytokines produced by DCs, which can hamper the overall immune response as well. NK cells can also promote DCs through IFNγ production,[52] CD40-CD40L interaction,[53] or GM-CSF production. Thus, a number of pathways and mechanisms exist for NK–DC interactions and reciprocal regulation of both populations during immune challenge.

D. Exhaustion

An important phenomenon that is often overlooked in NK responses to various tumors is NK cell exhaustion. Similar to what occurs with T cells, continuous exposure to certain target antigens results in the exhaustion of NK cells. Studies have suggested that the reason the clinical effects of adoptive transfer of NK cells have been limited may be due to rapid exhaustion of NK cells to tumor antigens and targets. NK cells exhibited strong anti-tumor functions and cytokine production early after adoptive transfer; however, starting at day 5 post-transfer, NK cells exhibited weak IFNγ production and cytotoxicity, despite being present at the tumor sites.[54] Exhaustion thus appears to be a significant concern in clinical utilization and targeting of NK cells during cancer, as rapid exhaustion of NK cells impairs their anti-tumor functions. Repeated adoptive transfers or additional immunotherapy to increase the functionality of NK cells may be needed to maintain the strong anti-tumor role of NK cells.

IV. THERAPEUTIC UTILIZATION OF NK CELLS DURING CANCER

A. NK Immunotherapy

Preclinical and clinical studies have focused on ways to enhance anti-tumor functionality of NK cells in the host. Administration of stimulatory and activating cytokines such as IL-2 and IL-15 have been utilized with mixed results. IL-2 was shown to be efficacious in mice to improve anti-tumor responses and approved for clinical use in renal cell carcinoma,[55] metastatic melanoma,[56] and metastatic breast cancer.[57] Even though NK expansion was seen after IL-2 injection in patients, tumor relapse rates and overall survival of patients was not significantly altered.

However, high-dose IL-2 administration can result in vascular leak syndrome, pulmonary edema, and eventually cardiovascular failure, making continued utilization of IL-2 problematic.[58–60] IL-15 is an alternative to IL-2 and is potentially superior due to the lack of the side effects associated with IL-2 and no activation of Tregs as seen with IL-2 administration.[61,62] Combined IL-2 and anti-CD25 can also reduce Treg activation and enhance anti-tumor responses.[63] Utilization of IL-15 is being explored through a number of different approaches including giving IL-15/IL-15Rα complexes to mirror the trans-presentation of IL-15 that occurs physiologically [64] and in combination with other immunotherapies, including IL-6 and anti-TGFβ administration.[65]

B. Adoptive Transfer

Beyond enhancing the NK cells present in patients, adoptive transfer of NK cells has been pursued as a novel immunotherapy in a number of cancers. Adoptive transfer of NK cells is usually utilized in conjunction with either irradiation and hematopoietic stem cell transplantation or chemotherapy in hopes of NK cells eliminating surviving cancer cells and reducing cancer relapse rates.[66] Clinical adoptive transfers of either autologous or allogeneic NK cells have resulted in successful engraftment and expansion of NK cells.[66,67] However, only limited clinical benefit was observed when utilized in patients with leukemia, lymphoma, breast and lung cancer, or metastatic melanoma. Allogeneic NK cell transfer has been the most promising therapeutic, with some studies demonstrating improved survival rates and no side effects, but other studies have reported minimal changes in survival and metastasis occurring after NK transfer.[68–72] In all studies, NK trafficking, engraftment, and expansion appeared to be occurring, but potential NK cell exhaustion, the suppressive tumor environment, immunoregulation, or suppressive NK cells subsets could all contribute to the mixed and minimal results obtained in clinical studies.

C. Cancer Stem Cells

The study of cancer biology has greatly expanded with the concept of cancer stem cells (CSCs), or tumor-initiating cells. These cells are a relatively small proportion of cancer cells, but they have the ability to maintain long-term growth potential and are highly resistant to conventional cancer therapies.[73] Avril et al. and Tallerico et al. have suggested that NK cells may preferentially target CSC populations, making NK cells a promising target for immunotherapy.[74,75] Recent work in our lab has confirmed these findings through utilization of cell lines and unmanipulated human primary tumor samples to model NK targeting of CSCs. We have also demonstrated that combined immunotherapy with either a proteasome inhibitor, bortezomib,[76,77] or local radiotherapy and adoptive transfer of NK cells significantly improved survival of mice and reduced tumor burden. This supported the notion of utilizing traditional cancer therapies to debulk and remove the majority of cancer cells and then utilize NK cells to remove the CSC population.

V. CONCLUSION

The nuances of NK cell regulation and function during cancer has become increasingly complicated with new advances in NK cell biology. The discovery of

unique subsets of NK cells with distinct functional capabilities demonstrates the intricacies of NK and cancer cell interactions to be more than just NK cells lysing cancer cells. The multifaceted regulation of NK cells by other immune cells, the cytokine milieu present in the tumor microenvironment, the various levels and diversity of antigens expressed by cancer cells, and NK exhaustion all make the therapeutic targeting and utilization of NK cells in cancer difficult and complex. Only by better understanding the multitude of regulatory pathways and subsets of NK cells can we begin to understand their diverse functions during cancer and better utilize these cells as a cancer therapeutic.

ACKNOWLEDGMENTS

We would like to acknowledge Anthony E. Zamora and Ethan G. Aguilar for their thoughtful review and editing of the manuscript.

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