Natural Killer Cells: The Secret Weapon in Dendritic Cell Vaccination Strategies

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Abstract
In cancer therapy, dendritic cell (DC) vaccination is still being explored. Clinical responses, however, are diverse and there is a lack of immunologic readout systems that correspond with clinical outcome. Only in the minority of patients, T-cell responses correlate with clinical outcome, indicating that other immune cells also gain anticancer activity. We still have limited knowledge of the effect of DC vaccination on different immune effector cells. However, it has been shown that bidirectional cross-talk between natural killer (NK) cells and DCs is responsible for enhanced activation of both cell types and increases their antitumor activity. In this review, we postulate the possibility that NK cells are the secret weapons in DC vaccination and studying their behavior together with T-cell activation in vaccinated individuals might predict clinical outcome.

Clin Cancer Res; 20(5); 1095–103. ©2014 AACR.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

CME Staff Planners’ Disclosures
The members of the planning committee have no real or apparent conflicts of interest to disclose.

Learning Objectives
Upon completion of this activity, the participant should have a better understanding of the potential positive role of natural killer cells in dendritic cell vaccination in cancer patients and the different tools currently available to assess natural killer cell activity in vaccinated patients.

Acknowledgment of Financial or Other Support
This activity does not receive commercial support.

Introduction
Dendritic cells (DC), the most efficient antigen-presenting cells (APC) of the immune system, have been used in clinical vaccination strategies for cancer. Immune responses induced by DCs inhibit tumor cell growth or even eradicate tumor cells. Although many clinical DC vaccination trials have been performed, the exact immune responses induced by these DCs are not completely elucidated. In most of these trials, specific immune reactions are studied instead of investigating the effect on the whole immune system. Initial vaccination strategies focused on inducing antitumor CTLs (1). However, after understanding the function of MHC class II and the identification of Th cells, the induction of CTLs after vaccination was analyzed in the context of Th-cell polarization (2). This eventually led to development of DC differentiation and maturation cocktails that enhance Th1 responses (3). In clinical DC vaccination strategies, only a minority of patients respond to therapy by tumor regression or stable disease; in some cases, complete remission is reported (4). Clinical response rates may vary between studies, which is mainly due to differences in patient population and different DC maturation and vaccination protocols. As indicated in Table 1, when clinical responses are induced after DC vaccination, this does not always correspond with the induction of tumor-specific CTLs. These poor correlations could indicate that T-cell responses are analyzed at the wrong anatomic site or that other non-T-cell responses are induced.

Besides T-cell responses, B-cell responses were also evaluated by detection of tumor-specific antibodies. Although these tumor-specific antibodies are often produced after vaccination, they correspond only in a minority of cases with clinical responses (5). It took until 2000 for other effector cells to be analyzed in the context of DC vaccination.
### Table 1. T and NK cell activation in clinical DC vaccination trials

<table>
<thead>
<tr>
<th>Disease</th>
<th>Vaccination</th>
<th>Clinical response</th>
<th>Specific T cells</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>AML</td>
<td>WT1 mRNA–electroporated DCs</td>
<td>5/10 patients (2/10 with long-lasting responses)</td>
<td>2/5 Patients. Best T-cell responses in best clinical responders</td>
<td>(67)</td>
</tr>
<tr>
<td>Melanoma</td>
<td>Wild-type and modifies gp100 peptide-pulsed DCs</td>
<td>9/27 patients (1/27 complete responses)</td>
<td>3/27 Patients. Best T-cell responses in the best clinical responses</td>
<td>(68)</td>
</tr>
<tr>
<td>Breast cancer</td>
<td>MUC1 gene–transfected DCs</td>
<td>1/10 patients</td>
<td>4/10 patients</td>
<td>(69)</td>
</tr>
<tr>
<td>Renal cell cancer</td>
<td>DC vaccination with tumor cell lysate or tumor cell line lysate</td>
<td>10/27 patients (2/27 complete responses)</td>
<td>5/6 Patients. Best T-cell responses in best clinical responders</td>
<td>(70)</td>
</tr>
<tr>
<td>Hepatocellular carcinoma</td>
<td>DC vaccination with tumor cell lysate</td>
<td>7/25 patients</td>
<td>5/10 Patients, no correlation with clinical responses</td>
<td>(71)</td>
</tr>
<tr>
<td>Melanoma</td>
<td>Tumor antigen cocktail–loaded DCs</td>
<td>11/36 patients</td>
<td>6/10 Patients, no correlation with clinical responses</td>
<td>(72)</td>
</tr>
<tr>
<td>Multiple myeloma</td>
<td>Idiotype-pulsed DCs</td>
<td>5/9 patients</td>
<td>4/5 Patients had a clinical response</td>
<td>(73)</td>
</tr>
<tr>
<td>Colorectal cancer</td>
<td>Tumor lysate–loaded DCs</td>
<td>8/24 patients are disease free</td>
<td>15/24 Patients (significantly better survival when specific T-cell responses)</td>
<td>(74)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Disease</th>
<th>Analysis of NK cells responses</th>
<th>NK cell responses and clinical responses</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>AML</td>
<td>&gt;40% of HLA-DR+ NK cells postvaccination</td>
<td>4/5 Clinical responders</td>
<td>(67)</td>
</tr>
<tr>
<td>CEA-positive tumors</td>
<td>Increase in total NK cell numbers</td>
<td>4/5 Clinical responders</td>
<td>(75)</td>
</tr>
<tr>
<td>(Colorectal, lung, urachal)</td>
<td>Increase in cytotoxic activity (K562 and Raji)</td>
<td>0/4 Nonresponders</td>
<td></td>
</tr>
<tr>
<td>Non-Hodgkin lymphoma</td>
<td>Increase in total NK cell numbers</td>
<td>5/6 Clinical responders</td>
<td>(76)</td>
</tr>
<tr>
<td>Advanced cancer (myeloma, anal, renal)</td>
<td>Increase in total NK cell numbers</td>
<td>1/4 Nonresponders</td>
<td>(77)</td>
</tr>
<tr>
<td>Metastatic gastrointestinal cancer</td>
<td>Increase in NK cell cytotoxicity (K562)</td>
<td>2/3 Clinical responders</td>
<td>(78)</td>
</tr>
</tbody>
</table>

Abbreviation: AML, acute myelogenous leukemia.
In mice, DC-induced natural killer (NK) cell activation was proven to be an absolute prerequisite for induction of Th1 polarization (6). Human in vitro data demonstrate that NK cells and DCs cooperate in pathologic conditions (7). This triggered the evaluation of NK cell responses in clinical DC vaccination studies (Table 1). The immunostimulatory effect of NK–DC interaction is based on a bidirectional cross-talk by which both cell types become activated. For DC vaccination techniques, activation of both cell types in vitro has been described, and their effect on other immune and tumor cells has been suggested to be beneficial for antitumor responses (8, 9). We postulate that NK cells are the secret weapons in DC vaccination. Here, we review the different effects of NK–DC interaction and their impact in DC-vaccination strategies.

**NK cell–dependent DC responses**

NK cells hold the capacity to control and enhance DC-mediated antitumor immune responses, by inducing maturation of Th1-polarizing DCs, providing DCs with antigenic material for presentation and killing of inappropriately matured DCs.

**NK cell–induced DC maturation**

NK cells mediate immunoregulatory "helper" functions. These cells were phenotyped as CD83+CCR7+CD56dim NK cells that possess DC-activating capacities (9), which are mediated by both NK cell–derived soluble factors, mainly IFN-γ and TNF-α (10, 11), as well as ligation of surface receptors (Fig. 1A). NK–DC interaction results in the development of stable, type-1 polarized DCs that produce high amounts of proinflammatory cytokines and, thereby, are able to enhance Th1 and CTL-mediated immunity against intracellular pathogens and cancer (Fig. 1A; refs. 10, 12). In the absence of NK cells, Th polarization is biased toward Th2 polarization, without induction of CTLs (13). NK cell–derived IFN-γ induces upregulation of the major Th1 transcription factor T-bet and inhibition of the Th2 transcription factor GATA-3 (6). These "helper" NK cells gained their function after activation by interleukin (IL)-12 and IL-18 (9). Other proinflammatory cytokines (IL-2, IL-15, type-I IFNs: IFN-α/β), NK cell–sensitive tumor cell lines, and opsonizing tumor-specific antibodies can synergize in a

![NK Cell–dependent DC activation.](ccr-reviews.png)
two-signal mechanism (12, 14, 15). Whether these "helper" NK cells also naturally occur in vivo and whether they indeed represent a distinct subpopulation is still unknown. Reports on the IFN-γ-secreting NK cell subpopulation are controversial, and both CD56bright and CD56dim subsets have been shown to produce IFN-γ after NK–DC interaction (8, 9). NK cells display a high plasticity in phenotype and upon stimulation can easily up- and downregulate surface receptor expression. However, after they have acquired the expression of killer immunoglobulin-like receptors (KIR) and perforin, the expression of CD56 and CD16 is stable (16).

Facilitation of antigen presentation

NK cells have the natural capacity to kill virally infected or malignantly transformed cells (17). This function is not only of direct use for tumor elimination, but also facilitates uptake and cross-presentation of tumor-related antigenic material by DCs (refs. 18, 19; Fig. 1B). In addition, activated NK cells are induced to express MHC class II and costimulatory molecules (like CD80, CD83, and CD86) and display APC-like properties, with unique pathways for antigen uptake and presentation to T cells (Fig. 1B; refs. 20, 21). These NK cells with DC-like qualities have been described in humans and mice and have been reported as a new immune cell subpopulation referred to as NKDCs as well as iKDCs (IFN-producing killer dendritic cells; refs. 22–24). Developmentally, mouse iKDCs are absent from recombine activating gene-2-null, common γ-chain-null (RAG2−/−/IL2rg−/−) mice, which lack NK cells but not DCs (25, 26). Therefore, although the nomenclature is confusing, it is assumed that these different subsets are most likely to resemble activated NK cells (22).

NK cell–dependent lysis of immature DCs

NK cells also have the capacity to eliminate autologous and alloimmune immature DCs (iDC; ref. 10). Nkp30 has been shown to play a major role in iDC lysis and cooperates with DNAX accessory molecule-1 (DNAM-1; ref. 27; Fig. 1C). Because both mature as well as immature DCs express ligands for Nkp30 and DNAM-1 (27), an additional mechanism must be involved. Analysis of NK cell clones has revealed that killing of iDCs was confined to NK cells that lack expression of inhibitory KIRs specific for self-HLA class I alleles, but do express the HLA-E–specific CD94/NKG2A inhibitory receptor (28). The iDC becomes susceptible to NK cell–mediated cytoxicity because of low expression of HLA-E irrespective of expression of other HLA molecules. In contrast, mature DCs express higher amounts of HLA-E and are, therefore, kept untouched (28, 29). iDCs have been implicated in tolerance and induction of regulatory T cells (30). Thus, it can be hypothesized that by elimination of iDCs, NK cells ensure the activation of adaptive immune responses by preventing inadequately matured DCs from interacting with T cells (31, 32). Although in apparent paradox, NK cells can induce DC maturation as well as lysis of iDCs, depending on the relative numbers of each cell type (10).

DC-induced NK cell responses

Not only are NK cells capable of DC activation, but also reciprocally, DCs stimulate NK cells by soluble as well as contact-dependent activators, thereby enhancing their cytokine production, proliferation, survival, and cytotoxicity.

Induction of cytokine production and proliferation

DCs activate NK cells to produce cytokines (mainly TNF-α and IFN-γ), which are mediated by a DC-derived two-signal mechanism and depend on both soluble and contact-dependent factors (Fig. 2). Depending on their maturation stimuli (33, 34), DCs upregulate the expression of NK cell–activating surface molecules and start producing NK cell-stimulating cytokines.

Among the DC-derived soluble factors, IL-12, IL-18, IL-2, and IL-15 have been shown highly efficient in NK cell activation (8, 35, 36). We and others have shown that NK cell–activating capacity is confined to DCs triggered by pattern recognition receptor signaling (8, 37). IL-15 acts as both a soluble and a contact-dependent factor (38), as DCs and DC exosomes can bind IL-15 to their IL-15Rα, thereby presenting IL-15 in trans. The effect of IL-15 on NK cell proliferation, survival, and cytokine secretion is much stronger when presented in trans than in its soluble form (39).

In addition to these soluble factors, NK–DC cross-talk is enhanced when both cell types are in contact with each other, suggesting that contact-dependent factors are involved (8). These factors include surface molecules expressed on DCs that ligate with activating NK cell receptors. One of these NK cell receptors is NKG2D, which is a G-type lectin coactivation receptor that binds to stress-inducible members of the polymorphic MHC class I–related chain A/B (MICA/MICB) family (40, 41). The NK cell–activating receptors Nkp30 and Nkp46 are also involved in NK–DC cross-talk (42–44). Moreover, 2B4, which ligates with CD48, enhances NK–DC cross-talk (45). Its function, however, is still promiscuous as the 2B4 pathway can be both activating as well as inhibitory (46, 47). Which pathway is induced depends on the NK cell maturation status and localization (48).

All the previously mentioned surface molecules are stress-dependent factors that can be upregulated during DC maturation, thereby mediating danger signals to the surrounding tissue.

Induction of NK cell–mediated cytotoxicity

The cytotoxic activity of NK cells is based on a delicate balance of inhibitory and activating signals and depends on the expression of MHC class I and activating ligands on the target cell and the expression of the respective receptors by NK cells (49, 50). NK cell cytotoxicity can be enhanced by DC-dependent mechanisms (Fig. 2; refs. 51, 52), including DC-derived IL-12 (8), IL-2, and IL-15 (53) as well as contact-dependent factors like NKG2D, Nkp46, and Nkp30 (43). The NK cell–activating mechanism used by DCs has been shown to relate to the stimuli used in DC maturation (54).
Common playground for NK cells and DCs?

For reciprocal NK–DC activation, it is obligatory for the two cell types to meet. It has been proposed that NK–DC interaction takes place at the site of inflammation as well as in the lymph nodes (7). During an inflammatory response, a burst of immunoactive molecules is secreted, including chemokines, resulting in immune cell recruitment. For NK–DC cross-talk specifically, it is necessary that one cell type produces chemokines, important for recruitment of the other cell type. We have shown that upon maturation, Toll-like receptor (TLR)–triggered DCs produce large quantities of many different chemokines (55, 56). Among these, there are NK cell–recruiting chemokines produced, like CCL5, CXCL10, and CCL19. Different chemokines and chemokine receptors have been shown to be involved in DC-mediated NK cell recruitment (6, 8). Therefore, it seems that there are multiple, nonredundant mechanisms to recruit NK cells to the site of inflammation. Which of these mechanisms is used depends on the DC maturation triggers.

Because maturing DCs migrate into the draining lymph nodes, chemokine production by mature DCs is possibly also responsible for the recruitment of NK cells into the lymph node. It has been shown in CCR3−/− mice that Th1 polarization was inhibited, due to impaired recruitment of NK cells into the lymph nodes (6). In humans, however, additional NK cell–recruiting mechanisms have been proposed. NK cells are able to upregulate their CCR7 expression and subsequently their responsiveness to the lymph node–homing chemokine CCL19 upon activation with IL-18 and after NK–DC cross-talk (8, 9). This would represent an additional mechanism by which DCs induce NK cell migration into the draining lymph nodes (Fig. 3).

Role of NK cells in DC vaccination

The reciprocal effects of NK–DC interaction provide a strong rationale for the combined use of NK cells and DCs in immunotherapy. The use of allogeneic NK cells has shown promising results in cancer immunotherapy (57), and these cells have been proven to be the main effector cells in haploidentical stem cell transplantation in acute myelogenous leukemia (AML; ref. 58). However, in these therapies, NK cells were always used as lonely killers. With our recent understanding of the NK–DC interaction, one could argue that DCs used in vaccination strategies should be in vitro matured in the presence of NK cells (14). From another point of view, there is also a rationale for the in vivo use of DCs that are able to optimally activate and recruit NK cells (8, 37). We and others have shown that for optimal NK cell activation and recruitment, DC maturation protocols should contain TLR agonists, whereas PGE2, another DC-activating molecule produced during inflammation, has a negative effect on NK–DC cross-talk (37, 59). Despite the
reports on the negative effects of PGE2-mediated DC maturation, most clinical trials use PGE2-matured DCs. The rationale behind this is the in vitro capacity of these DCs to migrate (60). However, in in vivo studies, these DCs migrate just as poorly as non-PGE2–matured DCs (61). The lack of migratory capacity of TLR-matured DCs’ combination with their NK cell–recruiting capability opens the possibility for the combination of NK–DC therapy. In these therapies, NK cells and DCs can either be simultaneously injected or only DCs are injected to recruit NK cells.

For the simultaneous injection of NK cells and DCs, intratumoral injection seems an attractive strategy. NK cells lyse tumor cells, thereby making antigenic material available for DCs to take up and present. In addition, NK cells induce maturation of bystander DCs and lyse inappropriately matured iDCs. Reciprocally, mature DCs can activate NK cells to proliferate, secrete cytokines, and augment NK cell cytotoxicity. Another option is the injection of NK cells and DCs in the tumor-draining lymph nodes where the Th1 polarizing capacity of the NK cell can be used to its fullest in this T cell–rich environment. Subcutaneous systemic injection of DCs together with NK cells seems a less attractive strategy. Because of the lack of migration of DCs, both cell types will possibly not home into tissue in the direct tumor vicinity and, thereby, will not stimulate the appropriate effector cells.

In addition to the simultaneous injection of both cell types, we propose a different option for NK–DC interaction in DC vaccination: the development of a tertiary lymphoid structure. In this strategy, only DCs are administered and this option is based on the capacity of TLR-matured DCs to produce chemokines and, thereby, selectively recruit effector cells (55). Because of the lack of in vivo DC migration, DCs could be injected in tumor-draining lymph nodes or into the tumor vicinity, where they can recruit and interact with NK cells. DC-mediated chemokine production is also attractive for recruitment of CTLs and Th1 cells (62–64). We hypothesize that by recruitment of all these effector cells by DCs, the same interactions that take place in lymph nodes can be induced extranodally in a tertiary lymphoid structure. Whether the immunosuppressive effect of the tumor microenvironment has detrimental effects on effector cell induction by DCs remains to be elucidated.

**NK responses in DC vaccination**

The evidence on reciprocal interactions of NK cells and DCs indicates that there is a rationale for monitoring NK activity after immunotherapeutic approaches, including DC vaccination. Notably, there is no consensus on assays to be routinely applied for NK cell monitoring, and as shown in Table 1, the definition of NK cell responsiveness differs between studies. Here, we propose a combination of assays
that should be applied as a means to standardize the definition of NK cell responsiveness.

The different assays include both phenotypic and functional characterizations. Phenotypic characterization is usually performed by flow cytometry. The surface markers CD69 and CD25 allow discrimination between resting, recently activated, and activated NK cells. CD69 expression is transiently present on NK cells after activation and is, therefore, considered a surface marker of recently activated NK cells, whereas CD25 has a more sustained expression profile upon activation (65). Upon activation, NK cells also induce the expression of HLA-DR (22). In addition, these activation markers are often combined markers for the identification of different NK cell subsets, such as CD16 in combination with CD56, KIRs, and natural cytotoxicity receptors (e.g., Nkp30 and Nkp46).

To analyze NK cell functionality, intracellular staining measures their content of granzymes and perforins, which is considered a benchmark for cytotoxic ability. NK cells that have recently degranulated are identified by their surface expression of CD107a (66). The secreted cytokine quantity can be measured by ELISA, and the percentage of cytokine-secreting cells is determined by intracellular staining or ELISPOT. The ultimate readout for assessing NK cell function after immunotherapy is studying whether NK cells increase their cytotoxicity to tumor cells. The K562 tumor cell line is an MHC class I-negative and is, therefore, readily killed by NK cells. The Raji cell line is a more attractive cell line because it is not susceptible to NK-mediated lysis unless the NK cells are preactivated. If patients' tumor cells are accessible, it is possible to assess whether the immunotherapeutic intervention has resulted in increased cytotoxicity to the tumor. An additional advantage of the accessibility of tumor cells is that these cells and their microenvironment can be studied following immunotherapy for production of NK cell-suppressive factors such as prostaglandins or TGF-β.

Conclusions

In this review, we postulate that NK cells might be the secret weapons in DC-vaccination strategies. NK cells can bidirectionally interact with mature DCs and, thereby, enhance both antitumor T-cell responses as well as NK cell responses. This NK–DC interaction might occur at the site of the tumor or in the lymph node. Until now, only limited, albeit promising human in vivo proof has been shown that NK cell activation after DC vaccination correlates with clinical outcome. To learn more about this NK–DC interaction in vivo, it is important for monitoring of NK cell activation to be incorporated into clinical DC vaccination trials. This process may lead to a system by which clinical outcome can be predicted.


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