NK Cells: Key to Success of DC-Based Cancer Vaccines?

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LEARNING OBJECTIVES

After completing this course, the reader will be able to:

1. Describe the current in vivo experimental and clinical dendritic cell (DC) vaccination studies encompassing the monitoring of natural killer (NK) cells.

2. Discuss the evaluation of NK cell stimulating potency in the design of DC-based cancer vaccines in the preclinical phase and in clinical trials.

3. Explain the added value of immune monitoring of NK cells in cancer vaccination trials.

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ABSTRACT

The cytotoxic and regulatory antitumor functions of natural killer (NK) cells have become attractive targets for immunotherapy. Manipulation of specific NK cell functions and their reciprocal interactions with dendritic cells (DCs) might hold therapeutic promise. In this review, we focus on the engagement of NK cells in DC-based cancer vaccination strategies, providing a comprehensive overview of current in vivo experimental and clinical DC vaccination studies encompassing the monitoring of NK cells. From these studies, it is clear that NK cells play a key regulatory role in the generation of DC-induced antitumor immunity, favoring the concept that targeting both innate and adaptive immune mechanisms may synergistically promote clinical outcome. However, to date, DC vaccination trials are only infrequently accompanied by NK cell monitoring. Here, we discuss different strategies to improve DC vaccine preparations via exploitation of NK cells and provide a summary of relevant NK cell parameters for immune monitoring. We underscore that the design of DC-based cancer vaccines should include the evaluation of their NK cell stimulating potency both in the preclinical phase and in clinical trials. *The Oncologist* 2012;17:1256–1270

NATURAL KILLER CELLS IN CANCER

In the early 1980s, the role of natural killer (NK) cells in defense against cancer was described in seminal reviews [1, 2]. A myriad of reports rapidly followed, supporting the involvement and therapeutic potential of NK cells in cancer immunity [3, 4]. A range of solid tumors [5–12] and hematological malignancies [13–19] were shown to be associated with significantly impaired NK cell functions. Importantly, NK cell abnormalities have been shown to be, at least in part, responsible for the failure of antitumor immunity. Deficiencies can reside in all NK cell populations, located in peripheral blood, in (lymphoid) organs, and in the tumor itself [16]. Functional impairment can originate from (a) primary NK cell dysfunction (e.g., imbalanced NK cell receptor expression, impaired cytolytic capacity, reduced cytokine secretion potency), (b) insufficient interaction with other immune cells (e.g., impaired killing of dendritic cells [DCs]) [14], (c) active immune suppression (e.g., regulatory T cell [Treg]-mediated suppression) [20, 21], and (d) NK cell resistance mechanisms by tumor cells (e.g., shedding of decoy molecules for activating receptors) [22]. In this regard, multiple cancer studies point toward a prognostic value for NK cells. Table 1 summarizes valuable NK cell parameters used for prognosis of disease progression and patient survival as well as for prediction of therapy efficacy.

In humans, NK cells are characterized by a $CD56^+$ $CD3^-NKp46^+$ phenotype. Based on their CD56 cell-surface density, they can be divided into two subsets with distinct phenotypic properties and key effector functions [23]. The majority ($\sim$90%) of peripheral blood NK cells have a $CD56^{dim}$ $CD16^{bright}$ phenotype and were originally regarded as the more naturally cytotoxic subset, characterized by high cytotoxic granule and perforin expression and lower cytokine-secreting capacity. The smaller $CD56^{bright}CD16^{dim/-}$ NK cell fraction ($\sim$10%) constitutively expresses a higher number of cytokine and chemokine receptors and a lower amount of cytotoxic granules, generally showing a poorer cytotoxic capacity but a superior ability to produce abundant immunoregulatory cytokines following activation, in particular the prototypic cytokine interferon (IFN)-γ. Remarkably, these seemingly subtype-specific effector functions appear to be not as restricted as previously thought. Several research groups recently demonstrated that $CD56^{dim}CD16^{bright}$ NK cells can be more prominent cytokine and chemokine producers than $CD56^{bright}CD16^{dim/-}$ NK cells, depending on the stimuli received [24–26].

Altogether, it is well accepted that NK cells possess potent antitumor functions that could be targeted for immune-based therapy [27–30]. Their direct antitumor effects can be attributed to their cellular cytotoxicity and cytokine-producing capacities. NK cells can also indirectly contribute to tumor control by communicating with other immune cells (e.g., DCs, NKT cells, and T cells), leading to an efficient adaptive antitumor response [31–33]. In this review, we focus on the role of NK cells in DC-based cancer vaccination strategies, providing a comprehensive overview of current in vivo preclinical and clinical DC vaccination studies. We discuss different strategies to improve DC vaccine preparations through engagement of NK cells and we provide a summary of relevant NK cell parameters for immune monitoring.

DC- AND NK CELL–BASED IMMUNOTHERAPY OF CANCER

DCs are widely recognized as key antigen-presenting cells, critical for the induction of cellular immunity and central orchestrators of the immune system, bridging innate and adaptive immunity [34]. In this perspective, it has become clear that NK cells and DCs are in close communication, supporting the development of an efficient antitumor immune response. DC-mediated activation of NK cells contributes to the development of potent innate immunity, whereas, in turn, activated NK cells provide signals for DC activation, maturation, and cytokine production, promoting adaptive immunity (Fig. 1) (extensively reviewed elsewhere [31, 34–37]). Current understanding supports the idea of exploiting NK–DC immune interactions in cancer immunotherapy (Fig. 1). A number of (combined) therapies, for example, cytokine [38], monoclonal antibody [39], and drug [40] therapy, qualify as acting on both immune cell types. Also, tumor cell vaccines that target both the hosts' NK cells and DCs may be successful [41–44]. Overall, improvement in tumor cell recognition by NK cells (cytotoxicity) and DCs (phagocytosis and antigen presentation), including stimulation of NK–DC interaction to advance sustained T cell immunity, should be considered in the design of novel immunotherapeutic protocols.

Several NK cell–based immunotherapeutic strategies have been developed, comprising both direct and indirect targeting of NK cell functions. Two main approaches can be distinguished, that is, adoptive transfer of NK cells [45–47] and in vivo modulation of endogenous NK cells [28, 29, 35, 48, 49], to bring about antitumor effects. Focusing on the latter, the most powerful immunoregulatory approach would be to attract NK cells to the site of interest, to target both their cytotoxic and their immunoregulatory functions, and to break tumor-mediated immune suppression or escape (Fig. 1). Following the expanding knowledge on NK–DC crosstalk, several groups have advocated the need to harness these immune interactions in the design of active specific immunotherapy [48, 50, 51]. In view of this, DC-based vaccination would be an obvious strategy to bring about NK cell activation (Fig. 1).

DC-based therapy originates from the unique capacity of DCs to present captured antigens to T cells, which is essential for generating specific T cell immunity [51–54]. Loading of DCs with tumor-associated antigens (TAAs) has therefore become an attractive therapeutic tool in order to generate tumor-specific T cells. The immunological outcome of DC vaccination strategies is predominantly evaluated based on induction of specific T cell responses. Indeed, most (pre)clinical trials provide evidence of tumor-specific $CD8^+$ and/or $CD4^+$ T cell responses. However, a discrepancy between these immunological responses and clinical outcome remains [55]. Studies that could demonstrate clinical tumor regression without detecting specific T cell generation prompted the investigation of other mechanisms that could be attributed to the observed effect [56, 57]. Given the importance of NK–DC

Table 1. Prognostic and predictive NK cell parameters

NK cell parameter	Association	Malignancy
Extensive NK cell tumor infiltration, high frequency of circulating NK cells	Favorable outcome	Lung cancer [154, 155], colorectal cancer [143, 156–158], gastric cancer [159], renal cancer [142, 160], AML [138]
NK cell cytolytic activity (typically against K562 target cells)	Inversely correlated with disease progression and relapse rate	Head and neck cancer [161, 162], hepatic cancer [163], AML [144, 164–168], melanoma [81, 169], various malignancies [170]
NK cell IFN-γ secretion potency	Favorable outcome	Leukemia [167, 171], GIST [146, 172]
Dull (altered) NK cell–activating receptor phenotype (e.g., NCR, NKG2D)	Active disease and poor survival outcome	AML [13], MDS [173], MM [174]
Predominant expression of NKp30c isoform (over the NKp30a and NKp30b isoforms)	Shorter survival time	GIST [175]
Impaired DC-stimulating capacity of NK cells	Disease progression	AML [14]
Low membrane or high soluble NKG2D ligand expression (i.e., MIC-A or MIC-B or ULBP-1 to ULBP-4)	Lower susceptibility to NK cell–mediated killing	Hematological malignancies [176, 177], MM [178], prostate cancer [179], colorectal cancer [158], various malignancies [180, 181]
Missing one or more KIR ligands versus all ligands	Protection against relapse	Leukemia, MDS [182]

Abbreviations: AML, acute myeloid leukemia; GIST, gastrointestinal stromal tumor; IFN, interferon; KIR, killer cell immunoglobulin-like receptor; MDS, myelodysplastic syndrome; MIC, MHC class I chain-related; MM, multiple myeloma; NCR, natural cytotoxicity receptor; NK, natural killer; NKG2D, NK cell receptor D; ULBP, UL-16-binding protein.

crosstalk in the development of an immune response, specific monitoring of NK cells and their responses is emerging as a valuable avenue in DC vaccine protocols. Vaccine DCs can stimulate the cytotoxic activity, cytokine secretion (IFN-γ, tumor necrosis factor [TNF]), and migration of NK cells by soluble (e.g., interleukin [IL]-12, IL-15, IL-18, type I IFN, CXC chemokine receptor ligand 9 [CXCL9], CXCL10, CXCL11, C-C chemokine ligand 5 [CCL5]) and contact-dependent (e.g., NKp30, NKG2D) signals, contributing to the development of potent innate immunity (Fig. 1). In turn, activated NK cells can further stimulate vaccine and host DCs, contributing to recruitment, activation or maturation, and lysis of DCs to advance sustained antitumor T cell immunity and limit inappropriate T cell tolerization (Fig. 1A). Additionally, enhanced killing of antigen-expressing target cells by NK cells can directly reduce tumor burden and provide (extra) tumor cell material for uptake by DCs and antigen presentation to T cells (Fig. 1B). Furthermore, both NK cell regulatory and lytic activities can facilitate robust T cell activation in direct (e.g., IFN-γ, killing) and indirect (e.g., T helper 1 [Th1] DC polarization, lysis of immature DCs [iDCs] to prevent inappropriate T cell tolerization) ways, whereas production of Th1 cytokines by T cells, in turn, can further promote NK cell activation (Fig. 1C).

NK CELLS IN PRECLINICAL DC-BASED VACCINATION
Fernandez and colleagues were the first to address NK–DC crosstalk in cancer, uncovering that DCs support the antitumor activity of NK cells [58]. They demonstrated, in mice, significant NK cell–mediated tumor growth delay upon adoptive transfer of DCs and confirmed the NK–DC interaction in vitro by showing DC-induced improvement in NK cell–mediated cytotoxic and IFN-γ–producing capacity. Ensuing from these data, they emphasized that DC-based immunotherapy may not

only promote T cell–dependent antitumor immunity, but it may also directly trigger NK cells in vivo. At the same time, Cayeux et al. [59] demonstrated that DCs, either peptide pulsed, antigen loaded, or gene modified, generated specific cytotoxic T lymphocytes (CTLs) and rejected in vivo tumor challenge, together with an increase in splenic NK cells and lysis of DCs by IL-2–activated NK cells, inferring that antigens released from lysed DC vaccines could in turn be taken up by host antigen-presenting cells to further enhance immunity [59].

A plethora of research publications on this novel topic of NK–DC interaction rapidly followed, although up until now the effectiveness of DC-based vaccines in clinical settings has rarely been accompanied by NK cell data. Some groups have put forward the potential of DC vaccination to stimulate NK cell functions and supported the idea that DC vaccine efficacy is likely to be dependent on NK cell stimulatory capacity [31, 60–62]. Although, in general, DC-based vaccines are aimed to elicit antitumor T cell responses, in fact, a range of mouse in vivo studies demonstrated that immunization with activated DCs leads to significant antitumor immunity in an NK cell–dependent manner, regulated via direct NK cell effects and/or a critical interplay among NK cells, T cells, and DCs. Table 2 provides a comprehensive overview of preclinical DC vaccination studies exploring the involvement of NK cells. Routinely, bone marrow–derived DCs were loaded with tumor antigens (peptide, protein, lysate) via pulsing or viral transduction and administered through various routes. In some cases, a surrogate tumor antigen, such as ovalbumin (Ova) or β-galactosidase (β-gal), was used to verify antigen specificity. The involvement of NK cells was principally evidenced by in vivo antibody-mediated NK cell depletion or the use of beige NK cell–deficient mice (Table 2).
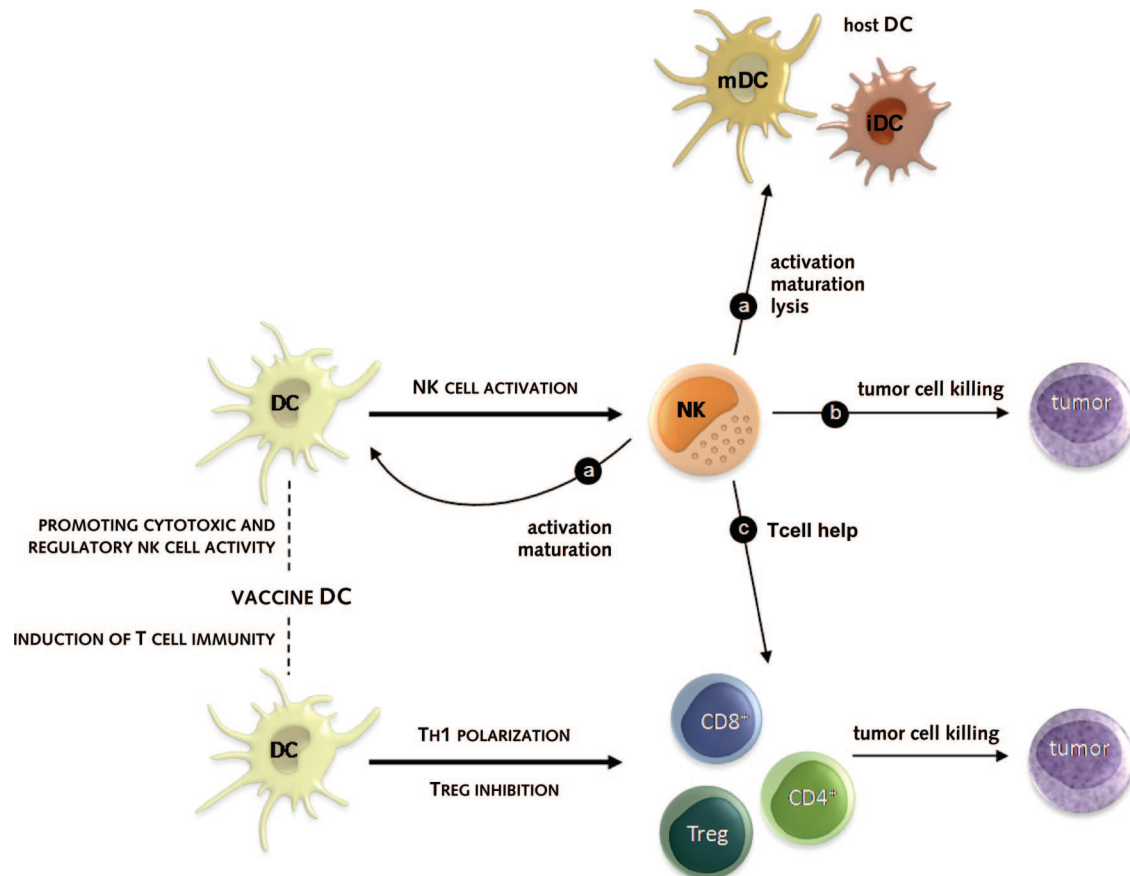
Figure 1. How NK cells can contribute to the antitumor efficacy of DC-based vaccination. Vaccine DCs can activate NK cells to **(A)** further stimulate vaccine and host DCs to advance sustained antitumor T-cell immunity, to **(B)** directly kill tumor cells, reducing the tumor burden and providing tumor cell material for further processing, and to **(C)** facilitate robust T-cell activation.

Abbreviations: iDC, immature dendritic cell; mDC, mature dendritic cell; NK, natural killer cell; Th1, T helper 1; Treg, regulatory T cell.

TAA-loaded DC vaccines were most frequently studied in the B16 murine melanoma model (Table 2). Immunization of mice with melanoma antigen–transduced DCs [63–66] or tumor lysate–pulsed DCs [67] resulted in an NK cell– and T cell–mediated complete or partial protection against tumor challenge. The outcome was typically measured by the absence or significant control of pulmonary metastases. TAA-loaded DC immunization has also led to efficient NK cell– and T cell–dependent antitumor immunity in other tumor models, resulting in protection against tumor challenge and prevention or retardation of established tumor growth [68, 69]. Additionally, it was demonstrated that, in combination with preventive anti-CD25 depletion to block Treg cells, the DC-mediated antitumor effect was more beneficial, allowing rejection of a much higher tumor burden and the development of long-lasting tumor protective immunity [68]. This is in agreement with the work of Ghiringhelli and colleagues, demonstrating that Treg cells inhibit NK cell functions [20]. The approach of DC–tumor cell fusions exploiting the whole tumor cell antigenic profile has also shown potent NK cell stimulatory capacities, resulting in NK cell–dependent protective immunity against tumor challenge [70–72] and long-term systemic immunity

[72] (Table 2). Interestingly, several groups documented the ability of nonantigen-loaded DCs (lacking TAAs), alone or with an adjuvant, to exert protection against tumor challenge, to prevent the development of tumor metastases, to establish long-term survival, and to clear established tumor in an NK cell–dependent manner [73–84] (Table 2). Also, in control experiments of DC vaccination studies, the nonantigen-loaded DC control was able to induce NK cell–mediated immunity [64, 66, 68, 78, 85, 86].

In an alternative (nonantigen-loaded) strategy, DCs are modified to express high amounts of defined proinflammatory cytokines (e.g., IL-12, IL-15, IFN-γ, IFN-α, IL-18, IL-2) [53]. Cytokines play a pivotal role in the polarization and skewing of adaptive immunity and hence in DC-mediated T cell activation. Importantly, these cytokines could also play a key role in the activation of NK cells. Monitoring of NK cells following cytokine-secreting DC vaccination was performed in only four mouse studies, all evaluating DCs modified to secrete IL-12 (Th1-skewing cytokine and important NK cell activation signal) [86–88], including one additionally testing IL-15–secreting DCs (T cell– and NK cell–activating signal) [89]. Vaccination with IL-12– and IL-15–secreting DCs resulted in

Table 2. Preclinical DC-based cancer vaccination models evaluating involvement of NK cells

DC product		Tumor model	Outcome	NK cell monitoring	Comments	Reference
Antigen-loaded DCs						
Human melanoma Ag gp100 AdV-DCs	s.c.	B16 melanoma	CD4$^+$ T- and NK cell–dependent complete protection against tumor challenge	No functional NK cell-directed assays	Anti-NK1.1 Ab, beige mice	[63]
MART-1 or mock AdV-DCs	s.c.	B16 melanoma	↑ NK cell–mediated Ag nonspecific protection against tumor challenge in CD8αKO mice (70%), mediated by interaction of lytic NK cells and IFN-γ producing CD4$^+$ T cells	↑ NK cell cytolytic activity against B16 cells	Anti-NK1.1 Ab in CD8αKO mice	[64]
MART-1 AdV-DCs	s.c.	B16 melanoma	Complete or partial protection against tumor challenge, dependent on CD8$^+$ T, CD4$^+$ T, and NK cell collaboration	↑ IFN-γ–secreting splenic (partly) NK cells upon MART-1 peptide stimulation; ↑ splenic NK cell lytic activity against B16 cells	Anti-NK1.1 Ab, beige mice	[65]
Necrotic tumor lysate pulsed DCs	s.c.	B16F10 melanoma	NK cell–dependent protection against lung metastases upon tumor challenge	↑ LAK cell lysis of YAC-1 cells; ↑ IFN-γ mRNA; ↑ in vitro NK cell IFN-γ	Anti-NK1.1 Ab, beige mice	[67]
SIINFEKL-encoding delta51-VSV-DCs	s.c.f.p.	B16F10 melanoma	NK and CD8$^+$ T cell–dependent specific and nonantigen-specific protection against lung metastases upon tumor challenge	↑ CD69$^+$ and IFN-γ$^+$ splenic NK cells	Anti-NK1.1 Ab	[66]
β-gal antigen pulsed, protein pulsed, or gene modified DCs	i.v.	β-gal–expressing tumors	Moderate rejection of tumor challenge	↑ NK1.1$^+$/Ly49C$^-$ cells; no ↑ LAK cell lysis of YAC-1 cells; ↑ LAK cell lysis of DCs	No in vivo NK cell depletion or reconstitution	[59]
Ova or mock AdV-DCs	i.p.	Ova$^+$ EG-7 lymphoma	Specific T- and NK cell–dependent protective immunity against tumor challenge (78%)	↑ splenic NK cell lytic activity against YAC-1 cells	Anti-NK1.1 Ab	[85]
Tumor-derived gp96-pulsed DCs	s.c.	LLC	Retardation of established tumor growth and protection against tumor challenge	↑ splenocyte lytic activity against LLC, MBL-2 and YAC-1 cells	Anti-NK1.1 Ab	[69]
C1498-extracted peptide or non-Ag-loaded LPS-mDCs	i.p.	C1498 AML	Natural NK cell- and partial NK and CD4$^+$ T cell–mediated prevention of leukemia outgrowth; survival advantage if CD25$^+$ T cells are depleted	↑ NK cell lytic activity against C1498 and YAC-1 cells	Anti-NK1.1 Ab	[68]
Peptide-loaded iDCs or mDCs	i.v., i.p., s.c.	OVA$_{257-264}$, E7$_{49-57}$	mDC- but no iDC-mediated protection against tumor challenge by in vivo NK cell- and TRAIL-mediated elimination of iDCs	↑ CTL activity by neutralizing NK cells or TRAIL	Anti-NK1.1 Ab, anti-TRAIL Ab and deficient mice	[91]
DC or tumor cell fusions						
Irradiated DC–tumor cell cocultures	s.c.	CT26 colon carcinoma	Protective immunity against tumor challenge	↑ splenocyte lytic activity against YAC-1 cells	No in vivo NK cell depletion or reconstitution	[71]
Irradiated DC–tumor cell cocultures	s.c.	CT26 colon carcinoma	NK cell–dependent protective immunity against tumor challenge (100%)	↑ CTL and NK cell cytolytic activity; ↑ IFN-γ mRNA	Anti-asialo-GM1	[70]
Syngeneic DC–allogeneic fibroblast fusions transfected with tumor-derived genomic DNA	s.c.	B16 melanoma	NK cell–dependent protective immunity against tumor challenge and long-term systemic immunity against parental tumor	↑ splenocyte lytic activity against YAC-1 and B16 cells	Anti-asialo-GM1	[72]
Non-Ag-loaded DCs						
Non-Ag-loaded DCs or D1	i.t.	AK7 mesothelioma	Significant NK cell–dependent tumor growth delay	↑ cytolytic activity; ↑ IFN-γ production	Anti-NK1.1 Ab, beige mice	[58]
Non-Ag-loaded mDCs	i.v., i.p., s.c.	CT26 colon carcinoma; LL/2 lung carcinoma	Protection against tumor lung metastases upon tumor challenge: correlated with NK cell activity; CD4$^+$ T cell requirement for induction of NK cell antitumor response; DC-derived IL-12 and IL-15 independent	↑ LAK cell lysis of YAC-1 and CT26 cells; ↑ NK cell lung infiltration upon tumor challenge	Anti-asialo-GM1, beige mice	[80]
Non-Ag-loaded LPS-mDCs	s.c.	A20 B cell lymphoma	NK cell–dependent protection against lethal dose A20 cells	↑ NK cell lytic activity of YAC-1 and A20 cells; NKG2D mediated	Anti-asialo-GM1 or TMβ1 Ab	[73]
Non-Ag-loaded iDCs	s.c., i.c.	B16 melanoma	CD4$^+$ T and NK cell–dependent (CNS) antitumor immunity against tumor challenge	↑ splenic NK cell lytic activity against B16 cells; ↑ DLN NK cell expansion; ↑ i.t. CNS NK cell infiltration	Anti-NK1.1 Ab in CD8αKO mice	[77]
Non-Ag-loaded CpG-mDCs	f.p.	MO5 melanoma	NK cell- and CTL-dependent protection against tumor challenge	↑ NK cell expansion; ↑ DLN NK cells; ↑ CD69$^+$ popliteal NK cells; ↑ IFN-γ$^+$ popliteal and splenic NK cells; ↑ splenic NK cell lytic activity against MO5 cells	Anti-NK1.1 Ab	[74]
α-GalCer-pulsed or unloaded LPS-mDCs	i.v.	B16 melanoma	Protection against tumor challenge; long-term CD4$^+$ T cell–dependent activation of NK cells (pulsed > unpulsed DCs)	↑ CD69$^+$ and IFN-γ$^+$ splenic NK cells dependent on recipient T cells and DCs; continuous NK cell generation	Anti-asialo-GM1 and NK1.1 Ab, IFN-γ$^{-/-}$ mice	[78]
α-GalCer-pulsed DCs	i.t.	CMS4 liver sarcoma	NK cell–dependent tumor rejection and established long-term survival	↑ serum IFN-γ; ↑ hepatic NK cell lytic activity against YAC-1 cells	Anti-asialo-GM1	[79]

(continued)

Table 2. (Continued)

DC product		Tumor model	Outcome	NK cell monitoring	Comments	Reference
Non-Ag-loaded CpG activated pDCs	i.t.	B16 melanoma; MCA205 sarcoma	Inhibition and regression of tumor growth via sequential activation of NK cells, conventional DCs and CD8$^+$ T cells; IFN-γ- and perforin-dependent NK cell–mediated T cell crosspriming	↑ CCR5-dependent i.t. NK cell infiltration; ↑ CD69$^+$ i.t. NK cells; ↑ type I IFN-dependent NK cell lytic activity against YAC-1 and B16 target cells; ↑ contact-, IFN-α-, and IL-12—dependent in vitro NK cell IFN-γ release	Anti-NK1.1 Ab or anti-asialo-GM1, ccr5$^{-/-}$ mice, OX40L$^{-/-}$ mice, IFNg$^{-/-}$ and perforin$^{-/-}$ mice	[75]
Unpulsed Dex from iDCs	i.d.	B16F10 melanoma	NK cell–dependent protection against tumor challenge	↑ IL-15Rα–dependent DLN NK cell proliferation; ↑ CD69$^+$ NKG2D-mediated DLN NK cells; ↑ splenocyte lytic activity against YAC-1 cells	Anti-NK1.1 Ab	[81]
Non-Ag-loaded iDCs + 5-FU	s.c.	MC38 colorectal cancer	No in vivo results	↑ splenic NK cell TNF-α expression; ↑ TNF-α–mediated splenic NK cell lytic activity against YAC-1 and MC38 cells	Magnetic in vitro NK cell depletion, no in vivo NK cell depletion or reconstitution	[76]
Delta51-VSV-DCs	s.c.f.p.	B16F10-OVA	Prophylactic and therapeutic type I IFN- and IL-15–dependent NK cell–mediated tumor protection	In vitro and in vivo ↑ IFN-γ^+ splenic NK cells	IFNAR$^{-/-}$, IL-15$^{-/-}$, IL-15R$\alpha^{-/-}$ mice; no in vivo NK cell depletion or reconstitution	[84]
Non-Ag-loaded LPS-mDCs	i.v.	Sarcoma	Protection against tumor challenge; long-term endogenous DC/CD4$^+$ T cell–dependent NK cell antitumor reactivity	↑ CD69$^+$ and IFN-γ^+ splenic NK cells, dependent on IL-2 secreted by CD4$^+$ T cells; ↑ CXCL10-dependent NK cell recruitment to T cell-rich splenic areas	Anti-asialo-GM1, anti-NK1.1 Ab, NOG mice	[83]
Non-Ag-loaded virally (rSeV)-activated DCs	Bolus and i.v.	RM-9 prostate cancer	CD4$^+$ T and NK cell–dependent prevention against lung metastasis	↑ splenocyte lytic activity against YAC-1 cells	Anti-asialo-GM1	[82]
Cytokine-transduced DCs						
IL-12-AdV-DCs	i.t.	CT26 colon carcinoma	IL-12–dependent CD4$^+$ T and NK cell–mediated tumor eradication; IFN-γ–dependent therapeutic effect	↑ cytolytic activity against YAC-1 cells	Anti-asialo-GM1, anti-IFN-γ Ab	[88]
IL-12-AdV-DCs	i.p.	B16 melanoma	NK cell–mediated protection against hepatic metastases	In vitro and in vivo ↑ splenic NK cell IFN-γ and ↑ lytic activity against YAC-1 cells; ↑ serum IFN-γ; no in vivo NK cell memory and NK cell expansion	Anti-asialo-GM1	[87]
IL-12 or mock AdV-DCs	i.t.	CMS4 liver sarcoma	NK, CD4$^+$ T and CD8$^+$ T cell-mediated tumor rejection and established long-term survival (IL-12DC > mockDC)	↑ hepatic NK cell lytic activity against YAC-1 and CMS4 cells; ↑ serum IFN-γ	Anti-asialo-GM1	[86]
IL-12 or IL-15 SV40-DCs	i.t.	CT26 colon carcinoma	Tumor remission upon IL-12 (40%) and IL-15 (73%) transduced DC immunization; CD8$^+$ T and NK cell–dependent IL-15–mediated antitumor immunity	No functional NK cell–directed assays	Anti-asialo-GM1	[89]

Abbreviations: 5-FU, 5-fluorouracil; α-GalCer, α-galactosylceramide; Ab, antibody; AdV, adenovirus-transduced; Ag, antigen; AML, acute myeloid leukemia; β-gal, β-galactosidase; beige mice, lack NK cell function; CNS, central nervous system; CpG, DNA sequence TLR9 ligand; CTL, cytotoxic T lymphocyte; D1, splenic immature dendritic cell line; DC, bone marrow-derived dendritic cell; Dex, DC-derived exosomes; DLN, draining lymph nodes; f.p., footpad; gp, glycoprotein; i.c., intracranial; i.d., intradermal; iDC, immature DC; IL, interleukin; i.p., intraperitoneal; i.t., intratumoral; KO, knockout; LAK, lymphokine-activated killer; LLC, Lewis lung cancer; LPS, lipopolysaccharide TLR4 ligand; MART-1, melanoma antigen recognized by T cells; MBL-2, murine lymphoma cell line; mDC, mature DC; MO5, B16F10 melanoma derivative; NK cell, natural killer cell; NOG mice, nonobese diabetic severe combined immunodeficient IL2Rγ^{null} mice lacking lymphocytes; pDC, plasmacytoid DC; rSeV, recombinant Sendai virus; SV40, simian virus 40; TAA, tumor-associated antigen; TLR, Toll-like receptor; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; VSV, vesicular stomatitis virus-transduced; YAC-1, NK-sensitive mouse T cell lymphoma.

combinatorial NK cell– and T cell–dependent tumor eradication in all studies [86–89] and induced long-term protection against rechallenge with tumor cells [86] (Table 2). Rodriguez-Calvillo et al. [88] showed that tumor rejection was completely reliant on IFN-γ and IL-12. In an equivalent in vitro study with human DCs, the cytolytic activity and cytokine-secreting capacity of autologous NK cells was improved only if DCs were transfected to express human IL-12. Later on, that study was followed by a phase I clinical study in patients with metastatic gastrointestinal carcinomas, resulting in activated NK cells in five of 17 patients [90] (Table 3).

Several of the in vivo animal studies analyzed potential mechanisms of DC vaccine–induced antitumor effects in more detail, demonstrating the requirement of either the lytic capacity, cytokine-secreting properties, or both of NK cells and/or T cells (Table 2). For example, Ribas and colleagues demonstrated that effective DC-induced antitumor immunity was mediated by lytic NK cells and IFN-γ–producing CD4$^+$ T cells [64]. Mechanistically, these preclinical studies support the complete or partial CD4$^+$ T cell [64, 68, 77, 78, 82, 83], CD8$^+$ T cell [66, 69, 74, 75, 89], or CD4$^+$CD8$^+$ T cell [65, 85, 86] requirements for DC vaccine–induced NK cell–mediated anti-

Table 3. Clinical DC-based cancer vaccination trials with implementation of NK cell monitoring

DC product	Trial design	Route	Tumor model	NK cell monitoring	Results and conclusions	Reference
Tumor peptide-pulsed DCs	Phase I, $n = 7$	i.d.	Glioma	No NK cell tumor infiltration	Systemic T cell–mediated cytotoxicity (4/7)	[96]
α-GalCer-pulsed CD1d-expressing DCs	Phase I, $n = 12$	i.v. + i.d.	Metastatic malignancy (breast, colon, liver, peritoneal, lung, renal, and prostate carcinomas, melanoma)	↑ mean PB NK cell levels, followed by transient ↓; ↑ in vivo CD69$^+$ NK cells; ↑ in vivo IC IFN-γ^+ NK cells; ↑ serum IFN-γ (10/10); ↑ serum IL-12 (6/9); ↑ K562 killing by PBMC (5/11)	NKT cell–directed therapy inducing a secondary NK cell response	[106, 107]
α-GalCer-pulsed DCs	Phase I, $n = 5$	i.v.	Advanced cancer (myeloma, anal, and renal cancer)	↓ PB NK cell numbers; unchanged PB NK cell CD69, NKp30, NKp44, NKp46, IC IFN-γ expression	NKT cell expansion and activation without PB NK cell–related changes	[105]
CEA-encoding fowlpox vector-loaded DCs	Phase I, $n = 9$	i.d + s.c.	CEA$^+$ tumors (colorectal, lung, and urachal cancer)	NK cell number and frequency: ↑ (5/9), stable (2/9), ↓ (2/9). Lytic activity: ↑ (4/9), stable (2/9), ↓ (3/9); variable NK cell receptor changes	S/NE patients (5/9) with ↑ NK activity correlated with NKp46 and NKG2D expression; PD patients (4/9)	[92]
MAGE-Ag-loaded Dex	Phase I, $n = 4$	s.c. + i.d.	MAGE A3$^+$ or MAGE A4$^+$ nonsmall cell lung carcinoma	Variable NK cell frequency changes; ↑ K562 lytic activity (2/4)	Dex may stimulate innate immunity	[104]
MAGE3 peptide-pulsed Dex	Phase I, $n = 15$	i.d. + s.c.	Melanoma (stage III/IV)	↑ K562 lytic activity; ↑ IFN-γ secretion; ↑ intratumoral NK cell recruitment (1/1); ↑ NK cell number and frequency; ↑ NKG2D$^+$ NK cells related to recovery of K562 lytic activity	Restoration of PB NK cell activity (7/14) upon 4 inoculations with Dex; tumor regression and ongoing therapy ($n = 2$, week 30) exert remaining boosted NK cell effector functions	[56, 81, 102]
IL-12 gene-encoding AdV-DCs	Phase I, $n = 17$	i.t.	Metastatic gastrointestinal carcinomas (pancreatic, colorectal, primary liver malignancies)	↑ K562 lytic activity (5/15); ↑ serum IFN-γ and ↑ lytic activity (4/5)	PR (1/17) and SD (1/17); no correlation between serum IFN-γ and clinical response or toxicity	[90]
MART-1 AdV-DCs	Phase I/II, $n = 10$	i.d.	Metastatic melanoma	↑ IC granzyme B (7/10); ≥1.5-fold ↑ CD25$^+$ (5/10) and ↑ CD69$^+$ (2/10) NK cells	In a subset of immunized patients, DC vaccination may have also led to activation of PB NK cells, next to T cell stimulation	[93]
Autologous heat-shocked UV-C–treated tumor cell-loaded DCs	Pilot study, $n = 18$	s.c.	Non-Hodgkin's lymphoma	↑ CD56^{dim}CD16$^+$ and ↑ CD16 MFI PB NK cells in responders (6/18); ↑ NKp46$^+$ NK cell in responders (4/6)	CR (3/18), PR (3/18), SD (8/18), PD (4/18); correlation of clinical response with ↓ CD4$^+$CD25$^+$FOXP3$^+$ Treg cells and ↑ CD56^{dim}CD16$^+$ NK cells	[98]
Autologous WT1-mRNA electrotransfected DCs	Phase I/II, $n = 10$	i.d.	AML	Unchanged PB NK cell frequencies and numbers; HLA-DR$^+$ NK cells: ≥40% in responders (4/5) and <40% nonresponders (5/5)	Superior levels of activated NK cells in responders	[97]
CEA peptide-loaded OPA-DCs	Phase I/II, $n = 10$	s.c.	Colorectal cancer	↑ NK cell frequency (6/8); ↑ K562 lytic activity (2/8); ↑ CD69$^+$ NK cells (66.7% post highly pure DC injections)	SD (1/8) with ↑ NK cell frequency and ↑ lytic activity; PD (7/8) with normal NK cell proliferation but failure of response	[95]
AFP peptide-pulsed DCs	Phase I/II, $n = 5$	i.d.	Hepatocellular carcinoma	↑ CD69$^+$ NK cells (4/5); ↑ CD25$^+$ NK cells (4/5); CD56^{dim}CD16$^+$ NK cells show greater degree of activation	Increased PB NK cell activation and decreased Treg frequencies	[94]
Autologous tumor lysate–IFN-α/TNF-α/poly(I:C)-matured DCs + cyclophosphamide + GM-CSF + pegIFN-α-2a	Pilot study, $n = 24$	i.n. + i.d.	Metastatic cancer (colorectal, melanoma, hepatocellular, and renal cell, cancer, cholangiocarcinoma, and carcinoid malignancies)	↑ K562 lytic activity (11/17); modest ↑ NK cell frequency (6/17)	Increased PB NK cell activity correlated with serum IL-12 concentrations, but not with clinical response	[100]
Autologous tumor lysate-pulsed DCs + low-dose IL-2	Phase I/II, $n = 10$	s.c.	Renal and breast cancer	↑ K562 lytic activity (6/10); ↑ CD56^{dim}CD16$^+$ NK cells (6/10)	SD (1/10) with ↑ K562 cell lytic activity and ↓ CD4$^+$CD25$^+$ T cells; PD (9/10); no correlations with clinical response	[101]
Autologous tumor lysate-pulsed DCs	Phase I/II, $n = 31$	i.d.	Breast cancer	↑ NK cell frequency	Prolonged 3-year progression-free survival duration	[99]

Abbreviations: α-GalCer, α-galactosylceramide; AdV, adenovirus-transduced; AFP, α-fetoprotein; Ag, antigen; AML, acute myeloid leukemia; CEA carcinoembrionic antigen; CR, complete response; DC, autologous dendritic cell; Dex, DC-derived exosomes; IC, intracellular; i.d., intradermal; IFN, interferon; IL, interleukin; i.n., intranodally; i.t., intratumoral; MAGE, melanoma-associated antigen; MART-1, melanoma antigen recognized by T cells; NK cell, natural killer cell; OPA-DC, monocyte-derived DC matured with OK432 (*Streptococcus pyogenes* preparation), low-dose prostanoid and interferon-α; PB, peripheral blood; PBMC, peripheral blood mononuclear cells; PD patients, patients with progressive disease; pegIFN-α-2a, pegylated interferon-α-2a; PR, partial response; SD stable disease; S/NE patients, patients with stable and no evidence of disease; TNF-α, tumor necrosis factor α; Treg cells, regulatory T cells; WT1, Wilms' tumor protein 1.

tumor effects (Table 2). To a further extent, some immunization protocols could induce long-term activation of NK cells via continuous generation of NK cells, rather than a true memory response [78, 83], whereas transfer of activated splenocytes from DC-immunized mice to naïve mice was shown to exert a similar NK cell–mediated protective antitumor immune response [64]. In the illustrated studies, mostly matured DCs were used, described to be more resistant to NK cell–mediated killing. In this context, Hayakawa et al. [91] demonstrated in vivo that immature peptide-pulsed DCs were eliminated by NK cells via a TNF-related apoptosis-inducing ligand (TRAIL)-dependent pathway. Moreover, NK cell depletion or neutralizing TRAIL led to enhanced CTL activity and a higher tumor rejection rate upon live tumor challenge. Concomitantly, immunization with mature peptide-pulsed DCs showed high protection and, in contrast to iDCs this effect was not improved when NK cells were depleted or when TRAIL was neutralized [91].

NK Cells in Clinical DC-Based Cancer Vaccination

In general, the preclinical studies described above demonstrate that NK cells are necessary but not sufficient to induce adaptive T cell responses following DC vaccination, suggesting critical interplay among NK cells, T lymphocytes, and DCs. These findings provide sound evidence that DC-based strategies designed to engage both NK cell and T cell immunity could markedly improve current DC vaccine efficacy for the treatment of tumors (Fig. 1). In practice, though, only a few groups have implemented evaluation of NK cells in their clinical DC trials. Table 3 provides an exhaustive overview of DC-based tumor vaccination trials implementing NK cell monitoring, either planned or post hoc.

Monitoring of NK cells was most frequently studied in tumor antigen–loaded DC immunization trials (Table 3). Evaluating viral vector–transduced DC vaccination, Osada et al. [92] demonstrated, in patients with a carcinoembryonic antigen–positive tumor, that NKp46 and NKG2D expression correlated with patients' NK cell cytolytic activity and that NK cell cytolytic activity correlated with clinical outcome. Moreover, they postulated that NK cell responses may predict clinical outcome more closely than T cell responses. Butterfield et al. [93] evaluated a melanoma antigen recognized by T cells (MART)-1–transduced DC vaccine that was designed to activate MART-1–specific $CD8^+$ and $CD4^+$ T cells. Based on preclinical work from the same lab [65], they additionally performed NK cell analysis and could demonstrate, 2 weeks postvaccination, increased intracellular granzyme B content in NK cells in seven of 10 patients. These exploratory studies indicated that, in a subset of patients, the DC vaccine may have led to activation of circulating NK cells. Peptide-pulsed DC vaccination was shown to induce NK cell activation in some patients in two studies, demonstrated by increased peripheral blood NK cell frequency, activation status (CD69, CD25), and lytic activity [94, 95]. Bray et al. [94] also demonstrated a parallel decrease in Treg frequencies. A third study did not detect NK cell changes as assessed by NK cell tumor infiltration post-DC

therapy [96]. In a recently published phase I trial by our own group [97], evaluating a therapeutic Wilms' tumor I antigen–targeted DC vaccine in patients with acute myeloid leukemia, we could demonstrate the induction of complete and molecular remission in 50% of patients. Clinical responses were correlated with both innate and adaptive immune responses postvaccination. Four of five patients who responded to the DC vaccine had a highly activated NK cell phenotype, in comparison with none of the nonresponders. Extensive NK cell monitoring in a subsequent phase II trial is ongoing.

All studies with tumor lysate–pulsed DC vaccination, alone [98, 99] or as a part of combination therapy [100, 101], led to NK cell changes (Table 3). Di Nicola and colleagues found a positive correlation between an increased frequency of $CD56^{dim}CD16^+$ NK cells and clinical response post-DC vaccination [98]. Strengthening the clinical observations of others [94, 101], they also found a positive correlation between a decreased frequency of $CD4^+CD25^+FOXP3^+$ Treg cells and clinical response. The other studies also demonstrated favorable NK cell changes (increased NK cell frequency and lytic activity) in some patients, but failed to make NK cell–related correlations with clinical outcome [99–101].

Two groups investigated a cell-free DC-based vaccine preparation consisting of melanoma-associated antigen–pulsed autologous DC-derived exosomes (Dex; exosomes are nanometer-sized membrane vesicles invaginating from multivesicular bodies and secreted from different cell types [102, 103]) (Table 3). Both could demonstrate an increase in NK cell lytic activity in half of the patients receiving Dex [56, 104]. Escudier and coworkers also showed that NK cells were capable of recognizing autologous tumor cells following Dex therapy and, for one patient, recruitment of NK cells inside tumor areas could be documented. Based on these results, they pursued exploring the NK cell–stimulating capacity of Dex vaccination with preclinical and clinical studies, including additional ex vivo experiments [81] of patient samples taken during their phase I study [56]. In line with the murine data, they confirmed in vitro NKG2D-mediated NK cell activation by human Dex. From the phase I study, they additionally showed that Dex boosted recirculation of NK cells and restored NKG2D expression and the cytotoxic capacity of NK cells in half of the immunized patients. In two patients with tumor regression and continuing treatment, NK cell effector functions remained boosted at later time points.

Secondary NK cell responses were monitored in two phase I studies following NKT cell–directed therapy with α-galactosylceramide (α-GalCer)-pulsed DCs in a mixed patient group with metastatic malignancies (Table 3) [105–107]. The glycolipid antigen antitumor compound α-GalCer is a specific NKT cell ligand with the potential to sufficiently activate NKT cells and induce secondary immune effects, including T cell and NK cell activation [107]. Both groups could demonstrate a transient decrease in peripheral blood NK cells. However, Nieda and colleagues demonstrated that α-GalCer–pulsed DCs induced a significant secondary NK cell response [106, 107], whereas Chang et al. [105] could not detect significant differences for any of the NK cell parameters measured.

From the clinical trials discussed above, we can conclude that DC-based cancer vaccination can have a significant impact on primary and secondary changes in the NK cell compartment. The results strongly indicate that increased DC vaccine–induced NK cell activity, in terms of phenotype, lytic, and/or regulatory functions, points to a more beneficial outcome [56, 81, 95, 97] and show that some DC-mediated NK cell changes are correlated with clinical response [92, 98] (Table 3). However, the limited number of (uniform) trials (e.g., mixed patient groups) and the diversity of DC vaccines (preparation, routes of administration, vaccine doses, and intervals) and of NK cell immunomonitoring assays do not allow the drawing of solid conclusions. For example, some studies demonstrated an increase [81, 95, 98–100] in peripheral blood NK cell frequency after DC vaccination, whereas others saw no [97] or variable [92, 104] changes or a decrease [105–107]. In this context, scrutiny of changes in NK cell frequency and function in blood, lymphoid, and tumor tissues is needed to fully judge the impact of DC vaccines on NK cells in vivo in humans. In accordance with the preclinical data of Martin-Fontecha and colleagues [108], DC-mediated transient NK cell recruitment to lymphoid tissue, where NK–DC interactions are likely to take place, could account for the peripheral decrease in NK cells observed in a number of clinical trials [105–107]. Further exploration of NK cell engagement and consistent monitoring in DC-based trials is therefore of utmost importance to learn more about the relevance of NK cells in diverse DC-based cancer vaccination approaches.

IMPROVEMENT OF DC VACCINES VIA NK CELL ENGAGEMENT

Given the limited clinical benefit so far, increasing attention is being paid to the amelioration of the therapeutic efficacy of DC vaccines. The list of approaches is growing and several have been identified as highly innovative [53, 54, 62, 109]. As highlighted in the present review, a promising strategy to improve DC-based vaccination is through ex vivo instruction of DCs to preferentially activate NK cells. Based on the current knowledge on NK–DC crosstalk, DC preparations could be optimized for a variety of parameters to harness DCs for NK cell activation during immunotherapy for cancer. Activated NK cells can, in turn, lead to further stimulation of vaccine and host DCs (Fig. 1).

According to the DC subset and differentiation or maturation protocol used, DCs can have different effects on NK cells [110, 111]. It has been demonstrated that DC generation with inclusion of IFN-α [112–116] or IL-15 [117–119] in differentiation cocktails and the use of Toll-like receptor (TLR) agonists in maturation cocktails [115, 117, 120–124] hold promise in enhancing the NK cell–activating capacity of DC vaccines and their reciprocal crosstalk and T cell activation. As illustrated in a human in vitro study [116], IFN-α–treated monocyte-derived DCs (IFN-DCs) induced specific CD8$^+$ T cell responses against melanoma peptides only in the presence of NK cells. IFN-DCs were also shown to recruit NK cells [113] and to directly activate NK cells, as measured by CD69 expression and IFN-γ production [112]. Depending on their matura-

tion status, DCs are sensitive to NK cell–mediated killing [59, 91, 125], which can affect the survival and immunogenicity of the DC vaccine.

The mode of transfection or transduction and the antigen preparation are critical for the DC activation status and for communication with different immune cells. Numerous antigen preparations (peptide, protein, cell lysate, apoptotic tumor cells, nucleic acids) and loading methods (pulsing, viral transduction, electroporation, lipofection, gene transduction) have been investigated to optimize T cell–stimulating capacity, but some were also examined for their NK cell–activating competence (Table 2). For example, the use of apoptotic tumor cell–loaded DCs was demonstrated to induce an NK cell–dependent T cell response [126, 127]. Considering the mode of delivery, Vujanovic and colleagues [124] demonstrated, in a recent human trial, that adenoviral transduction, like TLR4 ligand/IFN-γ maturation, increased the expression of transmembrane TNF and IL-15 on DCs, resulting in enhanced NK cell activation, IFN-γ secretion, proliferation, and antitumor activities [93, 124]. They showed, in vitro, that adenovirus-transduced DCs, as compared with protein- or peptide-loaded DCs, are the most successful at inducing NK cell activation [94]. It was confirmed, in mouse models, that adenovirus-transduced DCs induced significant NK cell antitumor activity in vivo [85, 124]. Analogous results were found for prophylactic and therapeutic vesicular stomatitis virus–transduced DC vaccination in mice [66, 84] (Table 2).

Recruiting immune cells to the sites of interest (tumor and immune cell–enriched sites) and bringing them in proximity of each other is believed to be fruitful for immune activation [48, 128]. In this regard, the route of vaccine administration is an important determinant (Tables 2 and 3) [52]. A number of preclinical and clinical studies demonstrated that defined DC preparations were able to elicit the recruitment of NK cells through specific mechanisms. A first observation by Martin-Fontecha et al. [108] showed, in mice, that s.c. injection of mature DCs led to rapid recruitment of NK cells into the lymph nodes, providing IFN-γ for Th1 priming. In line with these findings, others have demonstrated the induction of NK cell infiltration in lymph nodes [83, 129] as well as in tumor [75] following immunization of mice with TLR ligand–activated DCs, resulting in NK cell and T cell activation as well as tumor regression. The NK cell recruiting capacities of diverse human DC vaccines have also been examined in multiple in vitro studies. Mature IFN-DCs [112, 113], DCs loaded with reovirus-infected melanoma cells [130], and DCs matured with bacterial fragments [123] were shown to secrete distinct NK cell–attracting chemokines (i.e., the CXCR3 ligands CXCL9, CXCL10, CXCL11 and the CCR5 ligand CCL5), mediating in vitro migration and stimulating NK cell activation, NK cell–mediated cytotoxicity, and IFN-γ production. In parallel, the chemokine receptor profile of NK cells (CCR5, CCR7, CXCR1, CXCR3, CX3CR1) can be differentially regulated by DC vaccines to promote NK cell trafficking [131].

A more direct approach is the transfection or transduction of DCs with NK cell stimulatory molecules, such as genes encoding IL-12, IL-15, or other NK cell–stimulating factors (so-

called "designer" DCs). This strategy has already been demonstrated to be effective in activating NK cells and T cells, for example, by vaccinating metastatic gastrointestinal cancer patients with nonantigen-loaded IL-12–secreting DCs [90] (Table 3). Also, cotransfection (by electroporation) of TAAs with IL-12– encoding genes into mature human DCs induced an increase in activated NK cells and TAA-specific CTLs in vitro [131].

Finally, NK cells themselves can be used to improve the therapeutic efficacy of the DC vaccine [31, 50]. The generation of DC preparations in the presence of NK cells or NK cell–related factors was carried out by several groups in the preclinical setting [126, 132–135]. Using this strategy, ex vivo–generated DCs are brought into contact with NK cell–related signals with or without adjuvant, already bringing DCs to an activated status and priming the DCs for Th1 polarization.

In conclusion, these data underscore why future research efforts should also focus on optimizing the NK cell–activating properties of DC vaccines, in addition to improving their T cell stimulatory and Treg inhibitory capacities (Fig. 1). The NK cell–activating character of DC vaccine preparations can be imprinted at multiple levels: (a) DC differentiation and maturation, (b) source of antigen and delivery mode to DCs, (c) NK cell–trafficking potential, (d) direct DC modulation, and (e) with support of other immune cells such as NK cells themselves. DC vaccination trials should carefully evaluate how NK cell–mediated surveillance against tumor cells can be incorporated into DC-based therapy.

NK CELL IMMUNOMONITORING

From a variety of cancer immunotherapeutic approaches (e.g., hematopoietic cell transplantation [136–139], cytokine biotherapy [38, 140, 141]), it could be perceived that NK cell activation can contribute to the overall antitumor response. An array of both common and experimental NK cell parameters that have been implemented at the clinical and preclinical level, respectively, are listed in Table 4. Classical NK cell immunomonitoring includes evaluation, prior to and after therapy, of NK cell frequencies and numbers, regularly complemented by multiparametric phenotypic analysis (Table 4). In general, the activity of peripheral blood NK cells is tested, although NK cells residing in lymphoid tissue or infiltrated inside [7, 8, 12, 142] or surrounding [6, 143] the tumor have also been assessed for monitoring. Functional assays are routinely focusing on NK cell–mediated cytotoxic activity against NK-sensitive target cells (K562; standard assay for the majority of studies) and NK-resistant cells (Daudi, Raji) in the presence or absence of the cytotoxicity-promoting cytokine IL-2. NK cell cytolytic activity was demonstrated to be a valuable prognostic marker for several malignancies (Table 1). Importantly, some studies performed killing assays against autologous primary tumor cells to evaluate therapy efficacy [102, 144], which is likely to be of high relevance in the setting of eliminating residual tumor cells to prevent relapse. Commonly relying on the established chromium release killing assay, some clinical studies sought to provide more detailed information on the potential mechanisms of cytotoxicity using single-cell assays (Table

4). Another functional characteristic that has also been assigned as a predictive parameter is NK cell IFN-γ production (Table 1). Higher IFN-γ production is an indicator of immunostimulatory resident NK cells, which is especially interesting in view of DC-based approaches. Other NK cell–related cytokines (e.g., TNF-α, GM-CSF, IL-5, IL-10, IL-13, IL-15) may be considered as interesting for therapy-related effects. For example, GM-CSF is a known DC differentiation factor and IL-10 is an acknowledged immune-inhibitory cytokine, whereas IL-15 favors the proliferation and survival of NK cells and T cells and is involved in NK–DC crosstalk [124, 145]. Of particular interest for DC-based therapy is the examination of the ability of NK cells to activate, maturate, attract, and promote phagocytosis of DCs (Table 4). Only a few studies have evaluated such functionalities in an experimental setting [11, 42, 112, 113, 123, 135, 146]. NK cell–mediated killing of iDCs is also an attractive parameter to follow [14]. Ideally, it could provide useful information on which patient subsets are more likely to respond to DC-based therapy [147]. Finally, indirect NK cell–related parameters are the subject of research, because a range of transformed cancer cells has been shown to downregulate or secrete soluble molecules (e.g., ligands for the activating receptor NKG2D) in order to evade NK cell immunosurveillance [16]. In line with this, evaluation of tumor cell sensitivity to NK cell–mediated killing would be a significant predictive marker for therapy effectiveness.

Since the perception of the clinical significance of NK cell activity, there has been a need for accurate data generation and analysis [148, 149]. Today it is clear that NK cell biology is more complex than initially considered. Consequently, NK cell activity cannot merely be monitored by plain cytotoxicity assays with NK-sensitive target cells or simple phenotypic analyses [48]. As depicted in Table 4, an assortment of supplementary phenotypic and functional NK cell–related parameters have already been applied. Notwithstanding, guiding principles for significant parameters should be developed and standardized, ultimately providing useful information on outcomes and therapies. Just recently, Björkström et al. [150] and Bryceson et al. [151] provided precise, up-to-date protocols for NK cell receptor repertoire analysis and functional analysis of human NK cells with flow cytometry. Importantly, similar to the assessment of T cell responses [152], monitoring NK cell parameters must be done with prudence. Functionally diverse NK cell subsets may act in a distinctive manner per malignancy and per therapy, and the use of various technical protocols may result in significant variable outcomes. For example, we demonstrated variation in the quantification of IFN-γ secreted by human NK cells following tumor cell stimulation using different methods [153].

FUTURE PERSPECTIVES

DC-based vaccines are highly potent NK cell–stimulating candidates. Unlike the growing body of evidence acquired through experimental research (Table 2), there is a relative scarcity of data reporting NK cell function in DC trials (Table 3) [48, 50]. Herein, we provide an overview of mouse and human in vivo

Table 4. Human NK cell parameters for immunomonitoring

NK cell parameter		Comments
Phenotypic		
NK cell frequency, number	CD56$^+$CD3$^-$(NKp46$^+$)	Standard phenotype for NK cell quantification (e.g., in peripheral blood [majority of studies] or NK cell tumor infiltrate [154–159])
NK cell subsets	CD56^{dim}CD16^{bright}, CD56^{bright}CD16$^{dim/-}$	Common subdivision of NK cell types
NK cell activation status	CD69, HLA-DR, CD25, CD107a [7, 94, 95, 97, 144]	Common activation membrane markers
NK cell receptor repertoire	DNAM-1, NKG2A, NKG2D, NKp46, NKp30, NKp44, KIRs, CD158a/b [12, 13, 18, 92, 136, 146, 150, 169, 173, 175, 183]	Common NK cell receptors
NK cell chemokine receptor repertoire	CCR2, CCR5, CCR7, CD62L, ChemR23, CX3CR1, CXCR1, CXCR3, CXCR4, S1P5 [123, 131, 184]	Research level
Functional		
NK cell-mediated cytotoxicity	NK-sensitive K562 target cells; NK-resistant cells (Daudi, Raji) ± IL-2; autologous primary tumor cells [102, 144]	Standard assay for the majority of studies: K562 killing assay (mostly chromium release or flow cytometric cytotoxicity assays); single-cell assays [151]: CD107a [6, 136, 169], NKG2D [81], perforin or granzyme B [93], annexin V expression; antibody-dependent cell cytotoxicity [157]
NK cell cytokine production	IFN-γ, GM-CSF, IL-5, IL-10, IL-13, IL-15, TNF-α	Standard determination of the prototypic NK cell cytokine IFN-γ; intracellular [6, 7, 105–107] or secreted [11, 102, 137, 146, 167, 169, 171, 172, 179] in ex vivo cocultures; monitoring of other cytokines at research level
NK cell migratory capacity	NK cell–attracting chemokines, e.g., CXCL9-CXCL11 (CXCR3L), CCL19, CCL21 (CCR7L), CCL3-CCL5 (CCR5L); NK cell–secreted chemokines, e.g., CCL3 (MIP-1α), CCL4 (MIP-1β), CCL5 (RANTES)	Research level [112, 113, 123, 184]
DC activation or maturation capacity of NK cells	DC expression of MHC and costimulatory molecules, proinflammatory cytokines, Th1-polarizing capacity, phagocytosis	Research level [11, 42, 135, 146]
NK cell–mediated DC killing	Killing of immature DCs	Predictive parameter for acute myeloid leukemia [14]
Indirect NK cell–related		
Cytokine serum concentrations	NK cell–related cytokines (usually IFN-γ); NK cell–activating cytokines (usually IL-12, but also IL-2, IL-18, IL-15, type I IFN)	Informative, not NK cell specific [90, 100, 106, 107, 169]
Tumor cell expression of NK cell–related immune evasion or suppression molecules	Membrane-bound NKG2DL [158, 169, 176, 177]; soluble NKG2DL [11, 169, 173, 176–181, 185]	Predictive parameter for different malignancies

Abbreviations: CCL, C-C chemokine ligand; CCR, C-C chemokine receptor; CXCR, CXC chemokine receptor; DC, dendritic cell; DNAM-1, DNAX accessory molecule 1; FCM, flow cytometry; IFN, interferon; IL, interleukin; KIR, killer cell immunoglobulin-like receptor; MHC, major histocompatibility complex; MIP-1, macrophage inflammatory protein 1; NK cell, natural killer cell; NKG2DL, NK cell receptor D ligand; RANTES, regulated upon activation normal T cell expressed and presumably secreted; Th1, T helper 1; TNF-α, tumor necrosis factor α.

studies that investigated the influence of NK cell participation in antitumor immunity following DC-based vaccination strategies. In summary, these studies underscore a substantial role for both the cytotoxic and the regulatory functions of NK cells in the development of DC-mediated adaptive antitumor immunity, advocating for clear implementation of NK cell monitoring in cancer immunotherapy, in particular for DC-based therapy. Careful consideration of defined NK cell parameters and protocols is needed. Ultimately, this will contribute to a more complete understanding of therapy efficacy, generating valuable information that could be exploited in the development of novel adjuvants to improve anticancer immunotherapies. With the need for surrogate markers that could predict clinical outcome, research on NK cell activation as a prognostic factor warrants further intensification. In conclusion, a clear understanding of NK cell functions and mechanisms is essential to envision therapeutic breakthroughs in DC vaccine design. Hence, the development of DC-based vaccination strategies should implement NK cell–stimulating potency both in the preclinical phase and in clinical trials.

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