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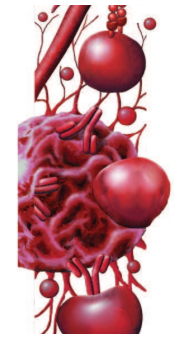
Immunotherapy



Bidirectional interactions of NK cells and dendritic cells in immunotherapy: current and future perspective

NK cells and dendritic cells (DC) are innate cellular components that regulate adaptive immune responses in the immune surveillance of cancer and infections. Interactions of NK and DC are bidirectional. In this mini review, we summarized how NK cells regulate immature DC editing and maturation, how DC regulate NK-cell functions reciprocally in the NK–DC crosstalk, and the importance of NK–DC crosstalk in antitumor immunity. Enhancing NK–DC crosstalk by cellular factor(s), antibodies or creating a microenvironment that promote NK activations, DC maturation and NK–DC crosstalk will provide new insights into future development of DC-based immunotherapy.

Keywords: cell migration • dendritic cells • immune regulation • immunotherapy • natural killer cells

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NK cells

NK cells are large granular lymphocytes constituting a heterogeneous population of the innate immune system. Coordinated actions of soluble factors (such as cytokines) and transcription factors turn hematopoietic stem cells into committed NK progenitors and their subsequent maturation in a stromal cell-, IL-15-dependent [1]. Conventional NK cells are recently classified as members of the innate lymphoid cells which, unlike T and B lymphocytes, do not express T-cell receptor and immunoglobulin genes, respectively [2]. Our earlier work and others have demonstrated that individual NK cell expressed a repertoire of surface receptors (activating and inhibitory) that signaled to the cell upon a target cell interaction. The balance of these inhibitory and activating signals, regulated by the SHP-1 and SHP-2 phosphatase activities, determines the outcome of the NK cell activities [3]. NK cells can also be activated by either cytokine (such as IL-2, IL-12, IL-15, IL-18) [4] or dendritic cells (DC) in specific microenvironments created by infections and tumors [5]. Through their cell-mediated cytotoxicity and/or production of cytokines, activated NK cells are involved in the immune surveillance of cancer and infections, in pregnancy and stress-induced responses [6–9].

Dendritic cells

DC are both professional antigen presenting cells and master regulators of the innate and adaptive immunity [10]. There exists heterogeneous DC subtypes in the body. Classifications of these DC subtypes are based on the phenotypes, locations where they reside and their unique functions [11]. Conventional DCs (cDC) predominantly reside in the lymphoid tissues such as spleen, thymus and secondary lymph nodes (LNs). These classical DC expresses higher levels of MHC-II and CD11c [12], and can be further divided into $CD8\alpha^+CD4^-CD11b^-CD205^+$ and $CD8\alpha^-CD4^+CD11b^+CD205^-$ subsets in mice [13]. When compared with the $CD8\alpha^-$ cDC population which resides in the marginal zones and induces Th-2 cytokine responses, the $CD8\alpha^+$ DC are predominantly found in the T-cell zone and promotes Th-1 responses. In a steady state, immature DC from most lymphoid organs are capable of taking up and presenting self-Ags to autoreactive T cells, thus tolerizing rather than activating these self-reactive cells [14]. Migratory DC, such

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as Langerhans cells, reside in the epidermis layer of the skin in steady state. Plasmacytoid DC, activated through Toll-like receptor (TLR)-7 and TLR9 stimulations, produce profound amounts of IFN-I, IL-12, TNF and chemokines (such as CCL3, CCL5, CXCL10) that promote NK-cell cytotoxicity, survival of T cells and antibody production by B-cells [15]. Recently, other DC subsets such as inflammatory DCs and the Th2-polarizing DC are reported [16], demonstrating the complexity and diverse immune functions these specialized DC subsets regulate *in vivo*.

A large number of clinical and preclinical studies demonstrated the beneficial role of tumor-specific T-cell responses in anticancer activities [17]. Because of the capability of DC to present tumor antigens in inducing tumor-specific T-cell responses, DC have been a promising candidate for antitumor vaccine [18]. However, such promise is complicated by the diverse regulatory functions DC can exert. Under different stimulations, DC can induce either immunological tolerogenic or differentiation of specific T helper (Th1, Th2, Th17) subsets [10]. It is therefore critical to understand how such diverse DC function is regulated, in order to induce a specific function of DC that is beneficial in augmenting antitumor immunity in the DC-cell based immunotherapy. In a recent study that evaluated clinical efficacy of an IL-12p70 based DC vaccine, six of seven stage 4 melanoma patients showed $CD8^+$ T-cell responses when they were treated with CD40L/IFN-γ-matured, IL-12p70-producing DC [19]. The design of this IL-12p70 based DC vaccine capitalized on our current understanding of factor(s) that promote strong T-cell activations, which includes maturation status of DC required for proper stimulations of T cells; capacity to secrete IL-12, a cytokine critical for induction of type I immunity; and capacity to migrate toward LN through acquisition of CCR7 chemokine receptor on the surface.

Bidirectional NK–DC crosstalk

Human NK cells express high level of CCR7 and L-selectin to reside in the LN at steady state. Mouse NK cells, however, will upregulate their homing receptors to migrate to LNs during infections in a CXCR3-dependent manner [20]. Immature DC predominantly reside in the skin and intestinal mucosa. Upon sensing of invading pathogens, they undergo a maturation process that upregulates MHC-II and co-stimulatory molecules (CD40, CD80, CD86), produces specific cytokines and chemokines, and migrates to LNs where DC and NK meet [21]. In addition to the trafficking to the LN, NK cells can acquire expression of other chemokine receptors (such as ChemR93, CX3CR1, CXCR1) in the trafficking to non-lymphoid tissues at the inflammatory, tumor or infection sites [22,23]. At the sites where NK and DC meet, bidirectional interactions of the NK and DC will modify functional activities of NK and/or DC that in turn regulate initiation and shaping of subsequent cellular and humoral immune responses.

Molecular mechanism(s) underlying NK–DC crosstalks

In a process termed DC editing, activated NK cells are capable of eliminating uninfected, autologous immature DC. In contrast, mature DC are relatively resistant to NK-mediated killing because of their substantial higher level of MHC expression. In several viral infection models, NK cells killed infected or 'deregulated' DC that expressed a reduced level of surface MHC-I molecules and/or the respective ligands for specific NK activation receptors (NKG2D, NKp46 and DNAM-1) [21]. The physiological relevance of NK-cell-mediated DC editing in the regulation of adaptive immunity remains to be fully delineated *in vivo*. It might be an important checkpoint to ensure the quality/quantity of the subsequent adaptive immune responses [24,25], and regulation of DC homeostasis that maintains a balance between immunity and tolerance. Studies have shown that the frequency as well as the activation of DC in the LN is drastically compromised following NK depletion [26], suggesting that NK cells can accelerate also DC maturation and activation. Indeed, NK cells have been shown to induce DC to upregulate HLA molecule expression and to secrete IL-18, IL-12p70 via upregulation of CD86 molecules on DC, and activation of TREM2 and NKp30 signaling in NK cells [27,28].

DC, classically described as the sentinels of the immune system, can stimulate NK-cells proliferation and to mount an effective *in vivo* antiviral and antitumor immune responses [29,30]. DC-derived IL-12 is critical in the generation of IFN-γ producing NK cells. In combination with IFN-I, IL-12 further promotes perforin-mediated cell cytotoxicity in NK cells. Other DC-derived soluble factors such as IL-1 and IL-18 have also been implicated in the acquisition of IL-12-receptor on NK cells [31]. DC-bound IL-15 and TNF promote NK-cell survival, proliferation, cytotoxicity as well as production of pro-inflammatory cytokines [30]. DC produce prostaglandin, a lipid mediator that regulates DC- and NK-cell functions. Prostaglandin-pulsed DC were unable to stimulate resting NK cells to produce IFN-γ and exhibit cell cytotoxicity [32,33].

Ratios of NK:DC cell number and specific engagement of different NK cell receptors that recognize their cognate ligands on the cell surfaces of uninfected immature DC (or virus-infected DC) are factors that will determine NK-cell responses to immature DC [21].

Recently, CD30–CD30L interaction has been reported to be another critical factor that regulates NK cells to secrete IFN-γ, TNF-α and DC maturation [34]. However, the molecular characterizations of other putative receptor/ligand(s) and/or soluble factors in directing NK–DC crosstalk (NK-mediated DC maturation vs DC-editing, and DC-induced NK activation) remain to be fully delineated.

Enhancing NK–DC crosstalk in cancer immunotherapy

A seminal study by Fernandez *et al.* demonstrated that DC were able to induce NK-cell mediated cytotoxicity and IFN-γ production *in vitro*, and that adoptively transferred DC induced NK-cell dependent, NK-cell mediated antitumor functions *in vivo* [35]. Cellular factors that foster NK–DC crosstalk has emerged to be critical in the regulation of antitumor responses. Gene silencing or gene deficiency of WAS protein in DC impaired NK–DC conjugate formation, impaired NK activation, enhanced tumor growth and enhanced metastasis of melanoma cells [36,37]. Enhanced tumor incidence and onsets of B16 melanoma and methylcholanthrene-induced fibrosarcoma were observed in the conditional knockout mice in which IL-27 was specifically deleted in DC [38]. Reconstituting IL-27 in these animal models was able to restore NK activation and inhibition of tumor growth, thus further supporting a role of the DC-derived IL-27 in NK–DC crosstalk in antitumor immunity [38]. Shimizu and Fujii demonstrated that bone-marrow-derived DC immunization generated long-term NK-dependent antitumor immunity in a mouse model of melanoma, and that the long-lasting antitumor NK response required endogenous DC [39]. Bouwer *et al.* reported also the important contribution of NK cells in B16 melanoma challenge and their helper role in enhancing antitumor immunity [40]. Morandi *et al.* recently demonstrated the *in vivo* relevance of DC editing by NK cells in expanding cancer-specific cytotoxic T lymphocytes (CTLs) in a TS/A mouse model of mammary adenocarcinoma. In this study, inoculation of NK-sensitive YAC-1 in mice induced NK activation *in vivo*, and perforin-dependent reduction of total CD11c$^+$ DC in the draining LNs. Of interest, such a decrease in the total CD11c$^+$ DC cells was accompanied by an improved capability to induce tumor-specific CTL *in vitro* and *in vivo*. Although the underlying mechanism is unclear, it suggested that NK cells could selectively destroy non-immunogenic DC to 'select' the most immunogenic DCs to expand CTL for the enhanced survival of mice upon a lethal challenge of tumor cells [24].

The importance of NK–DC crosstalk in orchestrating antiviral and antitumor immunity *in vivo* therefore provided a rationale for the rational designs of antitumor immunotherapy that aims at enhancing NK–DC crosstalk *in vivo* [24]. Current approaches include the use of monoclonal antibodies (mAb) to activate NK cells; specific augmentation of DC maturation to produce specific cytokines and/or chemokines that promote NK-cell, DC-cell migrations in the NK–DC crosstalk.

Cetuximab is a chimeric mAb that targets epidermal growth factor receptor on head and neck or breast cancers. Srivastava *et al.* showed that NK cells, when activated by Cetuximab-coated PCI-15B head and neck cancer cells, produced a significant higher amount of IFN-γ that promoted better DC cross-presentation in co-cultures [41]. In another recent study, Deauvieau *et al.* demonstrated that therapeutic mAb herceptin-coated HER2$^+$ BT474 breast tumor cells activated human NK cells to promote cross-presentation of tumor antigens in the monocytes-derived DC in an IFN-γ- and TNF-dependent manner [42].

Direct genetic modification of DC to manipulate factors that foster NK–DC crosstalk can be beneficial in augmenting immunological outcomes in antitumor therapy. Enhancing CD40L expression on DC cells, for example, enhanced NK–DC interactions and the maturation of DC, which in turn enhanced the production of appropriate cytokines that stimulated NK and enhanced the ability of these NK cells' cytotoxic ability toward certain tumor [43]. DC can also be activated by the innate recognitions of certain recombinant viruses or viral vectors (such as vesicular stomatitis virus, canarypox virus and adenoviral virus) to promote NK activation and prolonged antitumor immunity [44,45]. Boudreau *et al.* demonstrated that recombinant vesicular stomatitis virus transduction of DC supported greater IFN-I production and IL-15-mediated activation of NK cells [46]. Naveh *et al.* demonstrated that DC transduced by human adenoviral vectors induced NK-cell activation to promote the differentiation of CTL responses to the melanoma antigens [45]. Brandstadter *et al.* demonstrated that vaccinia virus infection induced NK activation in an IL-18- and DC-dependent manner [47]. Intracellular delivery of poly-inosinic-cytidylic (I:C) into epithelial ovarian cancer cells activated MDA-5 to induce production of IFN-α and other pro-inflammatory cytokines/chemokines, as well as cell death in the cancer cells. Engulfment of such apoptotic cells by monocytes and monocyte-derived DC promoted DC maturation (upregulation of HLA molecules, co-stimulatory molecules and CXCL10), which collectively creates an inflammatory environment to recruit NK cells trafficking to the site and induce NK-cell activation via soluble factors and NK cell–DC cell contacts [48].

Chemokines such as CCL19 and CCL21 play a critical role in the naïve T-cell–DC interaction that in turn leads to T-cell activation. It is possible to enhance T-cell activations via NK–DC crosstalk in antitumor immunity. NK cells activated under different cytokine or culture conditions may activate DC maturation differentially. It has been shown that IL-18 and IFN-α activated NK cells, which in turn led to enhanced priming of DC, enhanced production of chemokines (such as CCL19) in DC, enhanced DC interactions with T cells, and stronger T cells expansion and granzyme B production [49,50]. Nguyen-Pham *et al.* demonstrated that cocultures of immature DC with resting NK cells, in the presence of IL-2, poly-(I:C) and IFN, activated DC to produce higher levels of IL-12p70 cytokine that in turn promote Th1 responses in antitumor immunity [51].

Exosomes are cell-derived vesicles generated by the late endosomal, multivesicular body pathway. They fused with the plasma membrane and are released into the extracellular environment. Surface receptors and soluble factors acquired by the exosomes are able to regulate cellular functions such as gene expression or cell signaling [52]. Exosomes derived from tumor antigen-loaded DC and tumor cells induce antigen-specific CTL and antitumor immunity *in vivo* [53]. Interestingly, activated DC-derived exosomes, but not from the immature DC, showed enhanced NK activation, proliferation and production of cytokines [54].

Conclusion & future perspective

Bidirectional interactions of NK–DC are critical in shaping optimal adaptive immunity. Abilities to activate NK cells to promote their antitumor activities or to activate DC to promote cross-presentation, CTL differentiation and/or selection of potent Ag-presenting DC for CTL expansion have been shown to augment antitumor immunity.

DC maturation can be induced by cytokines, pattern recognition receptors and virus infections [44]. To augment specific antitumor immunity in DC-based immunotherapy, it is possible to create an 'inflammatory' LN environment that enhances NK and DC activations in the NK–DC crosstalk [55]. First, as DCs are activated by the innate recognitions of these recombinant viruses or viral vectors [44], insertion of a transgene (such as IFN-γ, IL-27) that enhances/regulates NK–DC crosstalk could potentiate DC's ability to prime Ag-specific CTL. Second, detailed characterizations of the bidirectional interactions between specific NK and DC preparations (subsets, different cytokine-activated NK or DC activated by different TLR ligands) will identify further soluble factors, surface receptors and exosomes involved in the regulation of NK and DC functions: it will allow one to select the best NK/DC subsets for development of immunotherapy. New factor revealed will allow one to examine specifically its role in the NK–DC crosstalk in tumor environment. Semaphorins, a large family of secreted and membrane-bound proteins, have emerged to be an important regulator of several biological processes (such as vascular, cancer and neurodevelopment), cell morphology and leukocyte trafficking [56,57]. Of interest to us, Semaphorin 3E (its cognate receptor, plexin D1) has been reported to regulate negatively the secretion of IL-12 in DC, a primary cytokine to regulate NK cell activation [58]. The use of Semaphorin 3E-deficient mice will further enrich us the understanding of the role of Semaphorin 3E in the regulation of NK, DC and NK–DC crosstalk in tumor models in animals. One might derive exosomes from a specific DC preparation to activate NK cells *in vitro* to augment the ability of these NK cells in the activation of DC *in vivo* to promote the NK–DC crosstalk in the induction of specific immunological outcomes in future development of immunotherapy. Third, as migrating immune cells at tumor sites are critical in mounting effective antitumor activity, understanding the regulation of NK-cell or DC-cell migrations in NK–DC crosstalk can be critical in future designs of antitumor immunotherapy [59]. Abilities to upregulate specific chemokine receptors for NK-cell trafficking into the tumors will promote infiltration of NK cells at the tumor microenvironment.

Tumor cells can create a microenvironment that favors aberrant or ineffective antitumor immune responses [55]. DC at the tumor sites can become suppressive and impair activation/functions of the infiltrated NK cells. In addition, the tumor microenvironment may prevent immune cells from trafficking into the tumor site. Understanding how NK-cell migration is regulated in a specific tumor microenvironment, analyses of the tumor infiltrating DC and NK cells in NK–DC crosstalk and ability to use specific NK cell preparations (such as cytokine-activated or genetic-modified NK) to 'restore' normal function of the aberrant DC cells at the tumor site will be important for the development of future antitumor therapies.

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Executive summary

NK cells

- Conventional NK cells are recently classified as members of the innate lymphoid cells which, unlike T and B lymphocytes, do not express T-cell receptor and immunoglobulin genes, respectively.
- NK cells are activated by either cytokines (such as IL-2, IL-12, IL-15, IL-18) or dendritic cells (DC) in the microenvironments.
- Through their cell-mediated cytotoxicity and/or production of cytokines, activated NK cells are involved in the immune surveillance of cancer and infections, in pregnancy and stress-induced responses.

DCs

- DC subsets are heterogeneous populations of hematopoietic cells. They are both professional antigen presenting cells and master regulators of the innate and adaptive immunity.
- DC are capable of inducing cytotoxic T lymphocytes (CTL), diverse Th subsets differentiation and immunological tolerance.
- Because of the capability of DC to present tumor antigens in inducing tumor-specific T-cell responses, DC have been a promising candidate for antitumor vaccines.

Bidirectional NK–DC crosstalk

- Through acquisitions of specific chemokine receptors, NK and DC meet in lymphoid tissues (such as lymph nodes) or non-lymphoid tissues (inflammatory, tumor or infection sites).
- At the sites where NK and DC meet, they modify functional activities of NK and/or DC that in turn will regulate initiation and shaping of subsequent adaptive immunity.

Molecular mechanism(s) underlying NK–DC crosstalks

- In a process termed DC editing, activated NK cells are capable of eliminating autologous immature DC.
- NK cells can also accelerate DC maturation and activation. Mature DC are relatively resistant to NK-cell lysis.
- DC provide/secrete a range of signals, which in turn regulate NK cell activation, proliferation and stimulate NK cells to mount an effective *in vivo* antiviral and antitumor immune responses.
- Ratios of NK:DC cell number and specific engagement of different NK cell receptors that recognize their cognate ligands on the cell surfaces of uninfected immature DC (or virus-infected DC) are factors that will determine NK-cell responses to immature DC. However, the molecular characterizations of other putative receptor/ligand(s) and/or soluble factors in directing NK–DC crosstalk (NK-mediated DC maturation vs DC-editing, and DC-induced NK activation) remain to be fully delineated.

Enhancing NK–DC crosstalk in cancer immunotherapy

- Cellular factors (such as WAS protein and IL-27) that foster NK–DC crosstalk have been shown to be critical in the regulation of antitumor immunity.
- NK cells, activated under the antibody-coated tumor cells, produced a significantly higher amount of IFN-γ, and importantly resulted in higher DC cross-presentation.
- Innate recognition of recombinant viruses or viral vectors fosters NK–DC crosstalk in antitumor therapy.
- Co-cultures of immature DC with resting NK cells, in the presence of IL-2, poly-(I:C) and IFN-activated DC, enhanced the production of IL-12p70 cytokine in promoting Th1 response in antitumor immunity.
- Exosomes are cell-derived vesicles generated by the late endosomal, multivesicular body pathway. Exosomes derived from activated DC showed enhanced NK activation, proliferation and production of cytokines.

Conclusion & future perspective

- Abilities to activate NK cells to promote their antitumor activities or to activate DC to promote cross-presentation, CTL differentiation and/or selection of potent Ag-presenting DC for CTL expansion have been shown to augment antitumor immunity. To augment specific antitumor immunity in DC-based immunotherapy, it is possible to create an 'inflammatory' lymph node environment that enhances NK and DC activations in the NK–DC crosstalk. These include:
  - Further development of specific recombinant viruses in the delivery of transgene(s) that will both enhance and regulate NK–DC crosstalk.
  - Detailed characterizations of the bidirectional interactions between specific NK and DC preparations (subsets, different cytokine-activated NK or DC activated by different TLR ligands) to select the best NK/DC subsets for development of immunotherapy, to upregulate specific chemokine receptors for NK-cell trafficking into the tumors will promote infiltration of NK cells at the tumor microenvironment.
  - Analyses of the tumor infiltrating DC and NK cells in NK–DC crosstalk and ability to use specific NK cell preparations (such as cytokine-activated or genetic-modified NK) to 'restore' normal function of the aberrant DC cells at the tumor site will be important for the development of future antitumor therapies.

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