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Review

The 'kiss of death' by dendritic cells to cancer cells

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Dendritic cells (DCs) are professional antigen-presenting cells (APCs) specialized in the stimulation of naïve T lymphocytes, which are key components of antiviral and antitumor immunity. DCs are 'sentinels' of the immune system endowed with the mission to (1) sense invading pathogens as well as any form of tissue distress and (2) alert the effectors of the immune response. They represent a very heterogeneous population including subsets characterized by their anatomical locations and specific missions. Beyond their unique APC features, DCs exhibit a large array of effector functions that play critical roles in the induction and regulation of the cell-mediated as well as humoral immune responses. In the course of the antitumor immune response, DCs are unique in engulfing tumor cells killed by natural killer (NK) cells and cross-presenting tumor-associated antigens to cytotoxic T lymphocytes (CTLs). However, while DCs mediate antitumor immune responses by stimulating tumor-specific CTLs and NK cells, direct tumoricidal mechanisms have been recently evoked. This review addresses the other face of DCs to directly deliver apoptotic signals to stressed cells, their role in tumor cell death, and its implication in the design of DC-based cancer immunotherapies.
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Tumor immunosurveillance, a mechanism to protect immunocompetent hosts against the emergence of spontaneous tumors, mainly relies on the initial recognition of stressed cells by natural killer (NK) cells and the subsequent triggering of tumor-specific cytotoxic T lymphocyte (CTL) responses.[1] While both NK cells and CTLs are responsible for killing tumor cells via direct contacts, unlike CTLs, NK cells exhibit killing without prior sensitization.[2] CTLs are $CD8^+$ T lymphocytes that recognize tumor-associated antigens (TAAs) as processed peptides bound to major histocompatibility complex (MHC) class I (MHC-I) molecules. Their priming requires concomitant triggering of $CD4^+$ T helper (Th) lymphocytes.[3] Although in certain circumstances tumor cells can directly present TAAs to CTLs,[4] it is commonly assumed that this direct presentation is mostly tolerogenic.[5] Indeed, professional antigen-presenting cells (APCs), particularly dendritic cells (DCs), remain the principal initiators of $CD4^+$ and $CD8^+$ T cell-mediated immune responses.[6] DCs are broadly distributed in peripheral tissues, and function as scavenger cells that continuously sample antigens to subsequently vehicle them to the lymphoid organs, the exclusive site of naïve lymphocyte priming.[6] The cardinal role of DCs in the development of the antitumor immune response resides in their unique abilities to engulf dying tumor cells, a principal source of TAAs, and cross-present TAAs to $CD8^+$ T cells in the lymph nodes (LNs).[7,8] Indeed, the presence of DCs in human carcinomas has been largely documented[9] and certain studies associated the quality and/or quantity of tumor-infiltrating DCs to a favorable prognosis for the patient.[10–13] It is traditionally believed that apoptotic bodies or necrotic debris are generated as a result of direct killing of tumor cells by NK cells and CTLs.[1] However, there is growing evidence that conventional DCs or certain DC subsets are endowed with a direct tumoricidal property by delivering cell death signals to pre-malignant/malignant cells, and also by providing signals required for their own maturation into immunostimulatory APCs. The vision that DCs can detect tumor cells, directly kill them to sample antigens and alert the immune system, would challenge the textbook picture of the traditional NK/DC/T-cell cooperation in tumor immunosurveillance. Here, we review the cell death pathways mediated by NK cells and CTLs on tumor cells, the recent findings on

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Abbreviations: Apaf-1, apoptotic protease-activating factor 1; APC, antigen-presenting cell; Bak, Bcl2-antagonist/killer; Bax, Bcl2-associated X protein; Bid, BH3-interacting domain death agonist; CRT, calreticulin; CTL, cytotoxic T cell; DC, dendritic cell; DcR, decoy receptor; DD, death domain; DED, death effector domain; Diablo, direct IAP-binding family protein; DISC, death-inducing signaling complex; FADD, Fas-associated death domain; Gas6, growth arrest specific gene 6; GZM, granzyme; IKDCs, interferon-producing killer DCs; IM, Imatinib mesylate; MFG-E8, milk fat globule EGF 8; MHC, major histocompatibility complex; NK, natural killer; pDCs, plasmacytoid precursor DCs; PFR, perforin; ROS, reactive oxygen species; Smac, second mitochondria-derived activator of caspase; TAA, tumor-associated antigen; TGF, tumor growth factor; Th, T helper; TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand; T_{reg}, regulatory T cells

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killer properties of DCs, and their implications in tumor immunosurveillance and future development in cancer immunotherapies.

Cytotoxic Leukocyte-Mediated Tumor Cell Death as a Source of TAA

There are numerous mechanisms for cell death and each pathway differentially influences immunogenicity (for review see Zitvogel *et al*.[14]). Classically, cell death was subdivided into programmed cell death and necrosis or accidental cell death.[15] Programmed cell death is a finely regulated physiologic process triggered by diverse forms of cellular stress and leading to nucleus fragmentation, whereas necrotic cell death results from a rapid loss of integrity of the plasma membrane with no energy consumption.[15]

NK cells and CTLs are the principal cytotoxic effectors of the antitumor immune response. They kill tumor cells by two major apoptotic mechanisms that require direct cellular contact. The first pathway is known as granule exocytosis, which delivers perforin (PFR), a membrane-disrupting molecule, and granzymes (GZMs), pro-apoptotic serine proteases. The second pathway is mediated by pro-apoptotic members of the tumor necrosis factor (TNF) family, including FasL and TNF-related apoptosis-inducing ligand (TRAIL).

PFR/GZM-mediated tumor killing. Granule-mediated cytotoxicity is a finely regulated, receptor-triggered secretory pathway, which involves recognition of a target cell, polarization of cytotoxic granules to the immunologic synapse, and release of their contents into the synapse to induce target cell apoptosis[16] (Figure 1).

PFR, which delivers GZMs into the cytosol of target cells, is a key molecule in the cytotoxic activity of NK cells and CTLs. $Pfr^{-/-}$ mice are profoundly immunocompromised and demonstrated an increased susceptibility to spontaneous tumor development.[17] In the granule, PFR and GZMs are bound to the proteoglycan serglycin, which maintains PFR in a monomeric inactive form.[18] The neutral pH in the synapse allows PFR to dissociate from serglycin and multimerize on the target cell membrane in a Ca^{2+}-dependent fashion. The mechanism used by PFR to deliver GZMs remains unclear. Lieberman's group[18] proposed a model in which PFR allows Ca^{2+} influx through the plasma membrane, thus triggering a membrane repair response and the rapid formation of large endosomes that simultaneously engulf PFR and GZM.

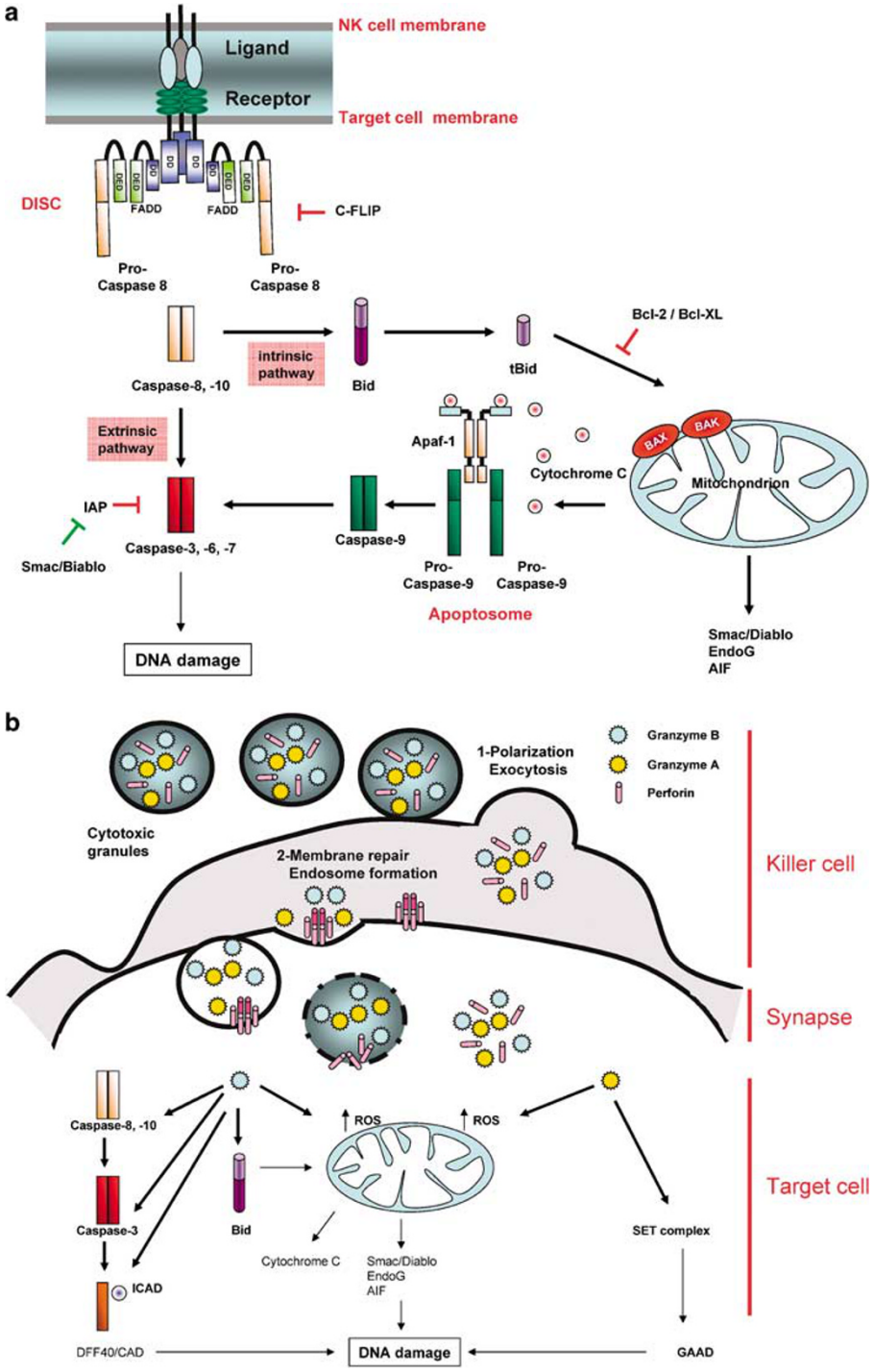
Among GZM family members, GZM A and B are the most abundant in mice and humans.[19] GZM A and K are tryptic proteases that cleave substrates after Arg or Lys residues. Their activity targets the endoplasmic reticulum-associated protein complex (SET complex), resulting in the release of GZM A-activated DNAse and its translocation to the nucleus and single-strand DNA nicks[20] (Figure 1). GZM A and K also trigger a rapid release of reactive oxygen species (ROS) and mitochondrial transmembrane potential loss.[19] GZM B, the most active GZM, is a caspase-like serine protease with a unique specificity for Asp residues to cleave its substrate.[19] GZM B causes a double-stranded DNA fragmentation in both caspase-dependent and -independent pathways (Figure 1). In caspase-dependent cell death, caspase-3 and -8 are cleaved by GZM B, subsequently engaging the cellular apoptotic cascade. In the caspase-independent pathway, GZM B disrupts mitochondrial function through activation of the proapoptotic Bcl2-family member BH3-interacting domain death agonist (Bid), which recruits the proteins Bcl2-associated X protein (Bax) and Bcl2-antagonist/killer (Bak) to change mitochondrial membrane permeability. This causes the release of ROS and cytochrome *c*. In addition, it has been recently shown that GZM B directly cleaves the inhibitor of the caspase-activated deoxynuclease (ICAD), allowing caspase-independent DNA fragmentation.[21] GZM C and M induce cell death when delivered by PFR *in vitro*.[19] Different subsets of cytotoxic cells have been proposed to express different combinations and amounts of PFR and GMZ, which influence the mechanism of targeted cell death.[22] GZMs, even though triggering specific pathways, are somewhat functionally redundant, and can complement the lack of other GZM activity according to the nature of the target and/or the invader. For instance, cytotoxic lymphocytes from GZM A or B-deficient mice maintain their ability to kill target cells, including tumor cells, by granule exocytosis, although this is less efficient when compared to those of wild-type mice.[19]

Death domain receptor-mediated apoptosis: TRAIL and FasL pathways. TRAIL/Apo2L and FasL are homotrimeric type II transmembrane proteins, which belong to the TNF superfamily.[23,24] Their extracellular portion can be cleaved for release as a soluble form. The mechanism by which FasL and TRAIL induce apoptosis are similar, involving recruitment of their receptor in the death-inducing signaling complex (DISC), and activation of caspases and downstream triggering of the same pro-apoptotic cascade of events (Figure 1; and for review see ref Nagata[23] and Schaefer *et al*.[24]).

TRAIL and FasL pro-apoptotic effects rely on a distinct set of receptors. Contrary to FasL, which acts through the ligation of its only receptor Fas,[23] TRAIL interacts with a complex system of receptors that belong to the TNF receptor family.[25] In humans, five binding receptors (TRAIL-Rs) have been identified. Both the TRAIL-R1/death receptor (DR4) and TRAIL-R2/TRICK2 (DR5) contain a functional death domain (DD) and mediate apoptosis. TRAIL-R3/decoy receptor 1 (DcR)1/TRID/LIT and TRAIL-R4/DcR2/TRUNDD are generally referred to as DcRs that inhibit TRAIL-induced death when overexpressed. TRAIL-R4 possesses a truncated cytoplasmic tail lacking a DD, and TRAIL-R3 exists as a GPI-linked protein. TRAIL also binds with a low affinity to osteoprotegerin, an inhibitor of the osteoclasts function. In mice, only one death-inducing receptor, mDR5, has been identified along with two DcRs, mDcTRAIL-1 and mDCTRAIL-2L, distantly functionally similar to the human TRAIL-R3 and -R4, respectively.

FasL and TRAIL induce a potent apoptotic signal through the trimerization of their cell surface receptors, which allow the recruitment of the Fas-associated death domain (FADD) adaptor protein via DD homotypic interactions.[23] This interaction subsequently exposes the death effector domain (DED) of FADD to interact with the DED of procaspase-8 or -10. This ensemble of interactions results in the formation of DISC (Figure 1). The close proximity of caspase-8 molecules

to each other allows dimerization, autocatalytic cleavage, and finally the release of activated caspase-8, which in turn activates caspase-3 and -7, effectors of the cellular apoptotic program. In some target cells (type I cells), the extrinsic TRAIL-R-mediated pathway is sufficient to promote death. However, in some cancer cells (type II cells), where the recruitment of caspase-8 is insufficient, the apoptotic signal has to be amplified by the intrinsic mitochondrial apoptosis pathway, which depends on the activation of Bid.[24] Caspase-8 and -10 cleave Bid, which interacts with Bax and Bak to promote the release of cytochrome *c* from the mitochondrial intermembrane space to the cytosol. Cytochrome *c* associates with the apoptotic protease-activating factor 1 (Apaf-1) in an ATP-dependent manner to form the apoptosome responsible for the activation of the initiator caspase-9, which further activates effector caspase-3, -6, and -7 (Figure 1). In addition,



other proteins including second mitochondria-derived activator of caspase (Smac)/direct IAP-binding family protein (Diablo) are released by stressed mitochondria. Smac/Diablo counteracts the repressing action of inhibitor of apoptosis proteins on pro-caspase-3 cleavage.[25] Interestingly, recent studies demonstrate that recruitment of the TRAIL-R can induce survival signals in certain cell lines. In this case, TRAIL induces the activation of NF-κB and JNK via mobilization of its receptor TNFR1-associated death domain and the receptor-interacting protein in the signaling complex.[26] The outcome of TRAIL signaling is thus the result of a fine balance between the pro-apoptotic and pro-NF-κB pathway.

DCs in Tumor Immunosurveillance

There is evidence to support the fact that our immune system plays a role in the surveillance of spontaneous tumors.[1] One compelling piece of evidence includes the increasing risk of cancer in immunosuppressed patients after organ transplantation, and patients with inherited defects in immune function.[1,27] In addition, animal models have demonstrated that the immune system exerts both host protection and tumor-sculpting/'immunoediting' effects on developing tumors.[1] For instances, 3-methylcholanthrene-induced sarcomas established in immunodeficient SCID or RAG2$^{-/-}$ mice are more immunogenic than sarcomas induced in fully immunocompetent congenic mice (for review see Dunn *et al.*[1]). Repassage of transplantable tumors through immunocompetent hosts selects tumor variants with reduced immunogenicity.[1] These findings suggest that, while immunosurveillance destroys emerging transformed cells to protect the host, 'immunologic pressure' selects tumors to withstand immune defenses. This tumor 'immunoediting' hypothesis is well supported by numerous reports showing that tumors lacking antigen processing and presentation components or elements of the interferon (IFN)-γ signaling are common.[28,29] Thus, it is logical that clinically defined cancer is a result of a long history of interactions between the immune defenses and malignant cells and the selection of tumor adapted to its immunological environment. Efficient antitumor immunotherapies can therefore be envisioned as a way to interfere on the host-tumor equilibrium by boosting immune defense and/or decreasing the immunosuppressive effect of tumors.

The induction of optimal antitumor immunity involves NK cell recognition of tumor cells and priming of both TAA-specific CD4$^+$ Th and CD8$^+$ CTLs.[29] The role of Th cells has been largely attributed to support for the priming of CD8$^+$ CTLs, which serve as the dominant effector cells mediating tumor killing.[3] The activation of NK and T cells (both CD4 and CD8) are highly dependent on the immunostimulatory properties of the APCs, including DCs.[6,30] DCs are heterogeneous and each subset is characterized by specific anatomical locations as well as functional properties.[31] To fully understand the mechanism of tumor-specific CTL priming, it is important to identify the subset that is responsible for TAA cross-presentation. DCs are mainly divided into two major subsets: plasmacytoid precursor DCs (pDCs) and conventional DCs (cDCs). Little is known about the involvement of pDCs in the induction of antitumor immune responses, and their key function relies on production of significant levels of type-I IFNs.[32] While type-I IFN is important for antiviral immunity, it has been reported to activate other DCs, including those involved in cross-priming.[33] The subdivisions of cDCs are rather complex, including Langerhans cells in the epidermis, interstitial cDCs in various tissues, thymic cDCs, and cDCs in lymphoid organs. In mice, cDCs in lymphoid organs are further differentiated by their surface expression of CD4, CD8$\alpha\alpha$ homodimers, CD11b, and the C-type lectins CD205 (DEC205) or DC immunoreceptor (Clec4a2).[34] CD8α^+CD205$^+$ cDCs are mainly found in T cell-rich areas of the peripheral lymphoid organs such as the periarteriolar lymphatic sheaths of the spleen and paracortical regions of the LNs. They appear to be specialized in the uptake of dying cells and cross-presentation of antigens for presentation to T cells by MHC-I molecules.[34] Their ability to cross-present makes them an important player in tolerance induction and in antiviral and antitumor immunity.[6] In contrast, the CD8α^- cDCs localize in splenic marginal zones, subcapsular sinuses, and immediate perifollicular zones of LNs. They appear to be more efficient than CD8α^+CD205$^+$ cDCs at presenting exogenous antigens by MHC class II (MHC-II) molecules.[34] DC heterogeneity has been much more limited in humans. Most of our knowledge on human DCs comes from studies of DCs isolated from blood or *in vitro* cultures.[31]

Figure 1 NK-mediated tumor cell death. NK cells kill their targets via two major pathways: apoptotic TNF family members, TRAIL and FasL, and granule exocytosis involving the synergic activity of PFR and GZM. (**a**) Apoptotic ligand-mediated cell death. Multimerization of TRAIL-R or Fas allows the formation of the DISC, and the activation of 'initiator' caspase-8 and -10, which in turn activate the 'executioner' caspase-3, -6, and -7. Cellular FLICE-inhibitory proteins (C-FLIPs) block the activation of caspase-8 and -10. In certain cells, activation of caspase-8 and -10 is not sufficient to induce DNA fragmentation and cell death requires amplification of the extrinsic death receptor-mediator pathway through the intrinsic mitochondria pathway. The intrinsic pathway is triggered by activation of the pro-apoptotic Bcl-2 family member, Bid. Caspase-8 and -10 cleave Bid into its active form, tBid (truncated Bid), which activates Bax and Bak. Activated Bax and Bak form pores into the outer mitochondrial membrane, causing release from the intermembrane space of the pro-apoptotic molecules cytochrome *c*, apoptosis-inducing factor (AIF), endonuclease G (EndoG), and Smac/Diablo. Cytochrome *c* binds the adaptor Apaf-1 in the cytosol, forming an 'apoptosome' that activates the apoptosis-initiating caspase-9. Caspase-9 activates in turn caspase-3, -6, and -7. Smac/Diablo counteracts the repressing activity of inhibitor of apoptosis proteins (IAP) on the activation of caspase-3, -6, and -7. (**b**) Granule exocytosis pathway. Pro-apoptotic serine proteases, GZMs, are delivered in the cytosol of the target cell via the action of PFR, a membrane-disrupting protein. It was suggested that PFR-induced plasma membrane lesions on the target cells provoke a repair response and the formation of large endosomes containing GZMs and PFR. Then, PFR disrupts the endosome membranes allowing the release of GZMs in the cytosol. GZM B induces cell death via caspase-dependent (activation of caspase-8 and -3) and caspase-independent (activation of Bid) mechanisms. GZM B generates an increased level of ROS by disrupting the mitochondrial transmembrane potential through a Bid-independent mechanism. GZM B also directly cleaves ICAD to free CAD forming the apoptotic nuclease, DNA fragmentation factor (DFF40/CAD) that is primarily responsible for internucleosomal DNA cleavage. GZM A activity is caspase-independent and includes the elevated level of ROS in mitochondria and the degradation of components of the SET complex leading to the release of the Granzyme A-activated DNAse (GAAD)

The ability of migrating tumor cells to trigger an effective CTL response depends on the activation of $CD4^+$ Th cells by host APCs. It is commonly accepted that the stimulation of naïve lymphocytes by cells others than professional APCs is rather tolerogenic.[5] Professional APCs, including DCs, have the ability to prime CTLs by sampling TAAs from the tumor and then migrating to LNs to cross-present tumor antigens to $CD8^+$ CTLs in the context of their own MHC-I.[7] While DCs are usually considered as the only APC able to cross-prime naïve T cells, murine macrophages and neutrophils have recently been shown to exhibit the similar property; although their roles in triggering tumor-specific CTLs remains to be determined.[35,36] Cross-presentation of TAAs, even by professional APCs, does not necessarily mean priming of CTLs. Cross-priming of CTLs *in vivo* only occurs in the presence of a certain level of Th response.[3] Thus, DCs must undergo a maturation process to provide all the necessary signals for complete activation of Th and CTLs, since partially activated DCs induce tolerance.[6] Nevertheless, the pro-inflammatory signals classically described for DC maturation are often absent from the bed of poorly immunogenic tumors, and thus tolerance is thought to be the 'default action' of cross-presentation.[37] Cross-presentation is the major mechanism in activating tumor-specific CTLs and the induction of tumor cell death has a critical role in tumor immunosurveillance to provide a source of TAAs for DC presentation to CTLs.[8,14,38]

It was originally proposed that apoptotic cell death was a non-immunogenic type of death in opposition of necrosis, which leads to rapid rupture of the membrane and, supposedly, the delivery of a variety of 'danger signals' to activate DCs.[39,40] Recent findings disprove the concept that the type of cell death, that is apoptosis *versus* necrosis, determines immunogenicity. While Hmgb1, a nuclear protein release from necrotic cells, was reported as a critical inducer of inflammation in response to necrosis,[41] Rock's group[42] demonstrated that IL-1 is more critical for neutrophilic inflammation to sterile cell death, but less important for monocyte-mediated host defense; its direct impact on DC recruitment remains unclear. Meanwhile, Rock and co-workers[43] identified uric acid released by damaged cells as a 'danger signal' to activate DCs. Others have shown that apoptotic cell death, which is a critical physiologic process allowing rapid clearance of unnecessary cells by phagocytes, prevents the release of potentially 'dangerous' material from the dying cells.[14] However, in specific circumstances, apoptotic bodies can trigger the maturation of DC, which in turn prime TAA-specific T cells.[8,44] More recent findings demonstrated that the way tumor cells enter the apoptotic cellular program determines whether the cell death was immunogenic or non-immunogenic.[14] For instance, phagocytes exploit multiple receptors and secreted proteins to affect the clearance of dying cells.[45] Milk fat globule EGF 8 (MFG-E8), Del-1, and growth arrest-specific gene 6 (Gas6) are examples of opsonins secreted by scavenger cells to bind phosphatidylserine exposed at the surface of early apoptotic cells and mediate their rapid engulfment.[45] Jinushi *et al*.[46] demonstrated that GM-CSF-dependent MFG-E8 production by macrophages allowed engulfment of apoptotic cells and expansion of T regulatory (T_{reg}) cells in a tumor growth factor (TGF)-β- and MHC-II-dependent manner. It has been suggested that MFG-E8 production by particular DC subsets could be related to tolerance than protective immunity. A hallmark discovery on the definition of immunogenicity of apoptotic cancer cells has recently been provided by Obeid *et al*.,[47] who showed that exposure of calreticulin (CRT) at the surface membrane of anthracyclin-treated tumor cells provides an 'eat me signal' to DCs to uptake the apoptotic cells. Tumor cells treated with genotoxic agents, which did not expose CRT on the cell surface, succumbed to a non-immunogenic cell death. It thus becomes clear that the nature of the signals exposed at the surface of dying tumor cells determines their fate for DC recognition and phagocytosis, which differentiates immunogenic from non-immunogenic cell death. These findings highlight the notion that certain signals delivered by dying tumors are different from the classical inflammatory signals that might participate in the maturation fate of DCs.

DC-NK Cross Talk is Center Stage of Tumor Immuno-Surveillance

DCs are endowed with the ability to activate the tumoricidal activity of NK cells. *In vitro*, IL-12, IL-18, IL-15, and type-I IFN produced by activated cDCs and pDC activate NK proliferation, cytotoxic activity, and IFN-γ production.[30] Recent clinical investigations showed that the treatment of patients with Imatinib mesylate (IM/Gleevec®/STI571), an inhibitor of c-kit tyrosine kinase, or with Flt3-L, which induces DC expansion and the DC-mediated NK antitumor effect, is associated with prolonged survival.[48,49] The NK/DC cross talk is bidirectional; NK cells reciprocally trigger and/or sustain maturation of DCs, therefore promoting a T cell-mediated antitumor immune response. The NK-mediated maturation of DCs is based on cell-to-cell contact, especially involving NKp30 and proinflammatory cytokines including TNF-α/ IFN-γ (for a complete review see Walzer *et al*.[30]). In addition, NK cells participate in the editing of DCs undergoing maturation by promoting apoptosis of immature DCs. Only fully matured DCs, which have complied with the NK-mediated 'quality inspection', can prime the ensuing protective CTL-mediated antitumor immune responses.[50] It has been shown that the DC-mediated recruitment of NK cells in LNs provides early IFN-γ secretion or induced a strong DC-mediated IL-12 production, thus promoting Th1 immune responses.[51]

Tumors are mostly poorly inflammatory and thus, in theory, do not provide the condition(s) necessary for DC maturation. NK cells are equipped with a complementary set of receptors specialized in 'danger' recognition, which are not found on DCs.[2] NK cells recognize the 'missing self' signal at the surface of tumor cells, which involves mainly the down-regulation of MHC-I.[2] NKG2D, an activating receptor on NK cells, detects the 'induced self', which correspond to stress-induced ligands at the surface of tumor cells and mediates a positive signal for tumoricidal activities.[2] The differential activation of NK cells toward tumor and normal tissue involves a fine balance between inhibitory and activating signals. Thus, NK cells are able to recognize and kill tumor cells without prior sensitization and the activation of NK by tumor cells might be sufficient, in some circumstances, to activate DCs and promote T cell-mediated tumor rejection.[30]

Table 1 DC-mediated cytotoxicity

Specie	Phenotype	Activation	Target	Mechanism	Reference
Human	Blood, $CD11c^+$ M-$DC8^+$ DC	IFN-II	Tumor cell lines	TNF-α	71
Human	Blood, $CD11c^+$ DC	TLR-7/8	K562	Perforin	54
Human	Blood, $CD11c^+$ DC	IFNs I and II	Tumor cell lines	TRAIL	52
Human	Blood, $CD11c^+$ DC		Fresh tumors	TRAIL	75,76
	In vitro monocyte-derived DC	Immature	Tumor cell lines	FasL	
			Endothelium	LT-$\alpha1\beta2$	
Human	*In vitro* monocyte-derived DC	Immature	Tumor cell lines	Unknown cell-to-cell contacts	70
			LPS, IFN-γ		
Human	*In vitro* monocyte-derived DC	Immature or activated	Tumor cell lines	Caspase 8-dependent, TNF-α, TRAIL and FasL independent	57
Human	CD34 stem cell-derived DC	DsRNA, CD40L	Tumor cell lines	TRAIL (IFN-β),	73
	In vitro monocyte-derived DC			TRAIL independent (TNF-α)	
Human	Blood $CD11c^-$ PDC	TLR-7/8	Jurkat	TRAIL	54
Human	Blood $CD11c^-$ PDC	TLR-9	K562	TRAIL	KA Palucka, personal communication
Rat	$CD103^+/CD4^-$	Immature	Tumor cell lines	Caspase and Ca^{2+}-independent	59,60
Rat	BMDC	NKG2D cross-linking	Tumor	Nitric oxide	77
Mouse	$CD8\alpha^+$ $CD11c^+$	Immature	$CD4^+$ T cells	FasL	69
Mouse	NKDC (C57BL/6),	*Ex vivo* TLR-9/IL-18	YAC-1	Perforin/granzyme (?)	53,55,58
	$CD11c^+NK1.1^+CD3^-$	*In vitro* CpG/IL-4			
Mouse	IKDC (C57BL/6)	*Ex vivo* IL-2/ Gleevec	Tumor cell lines	TRAIL	78
Mouse	IKDC (Balb/C)	*Ex vivo* Listeria	YAC-1	Perforin/granzyme	56
		In vitro TLR-9			

Killer DCs

In addition to the ability of DCs to activate $CD4^+$ and $CD8^+$ T cells and cross talk with NK cells, a novel, direct tumoricidal activity of DCs has recently been described by various groups (reviewed in Table 1). Activated human DCs suppress the growth of a variety of tumor cell lines *in vitro*, addressing the possibility that besides their role in orchestrating antitumor immune responses, DCs may exhibit direct antitumor effector functions.[69,70] Human blood cDCs acquire the ability to kill tumor cells via TRAIL expression or TNF-α secretion, upon IFN-α or IFN-γ stimulation,[52,71] and monocyte-derived DCs upon IFN-β stimulation or measles virus infection.[72,73] Immature DCs have also been shown to kill freshly isolated tumors.[74–76] Although *in vitro*-derived immature DCs kill target cells very efficiently at low effector/target ratios via an apoptotic mechanism involving DNA fragmentation, mitochondrial dysfunction, and late membrane disruption, the level of cytotoxicity found in the freshly isolated, immature DCs remains low and questionable.[75] To note, while Chen's group[70] demonstrated that the tumor killing by immature DCs is mediated through direct cell-to-cell contact, Vujanovic's group[75] showed, using the same type of immature DCs, that the killing mechanism was mediated by both cell-to-cell contact and soluble mediators. This discrepancy pointed out that the killing activity of DCs was rather eclectic and was highly dependent on the nature of targets. Furthermore, the cytotoxicity may be a function of DC maturation since LPS or IFN-γ enhances the cytotoxicity of DCs,[70] whereas CD40L or TNF-α does not enhance or even abrogate the killing activity.[76] Furthermore, Vidalain *et al*.[73] showed that poly(I:C) stimulation of the monocyte-derived human DCs leads to an increased cytotoxic activity, via type-I IFN-mediated TRAIL upregulation on DCs. Surprisingly, CD40L-activated DCs with no upregulation of TRAIL expression remained cytotoxic to certain types of tumor cells. It is believed that CD40L-induced robust TNF-α secretion exerts a differential effect on target cell death according to the balance of pro-apoptotic signals/pro-NF-κB signals. Huang *et al*.[74] recently reported that bone marrow-derived DCs mediate cytotoxic activity toward B-cell lymphoma. Interestingly, they showed that by disrupting the balance of pro-apoptotic *versus* anti-apoptotic signals, target cells can be sensitized to TNF family ligand-mediated apoptosis. In addition to the TRAIL-mediated killing activity of DCs, FasL-mediated killing of ovarian carcinoma cells by immature human DCs has been shown.[64] In mice, Shortman's group[69] described the expression of FasL on a subset of $CD8^+$ DCs that allow them to kill Fas^+ activated T cells. A FADD-independent mechanism used by human monocyte-derived DCs has also been reported.[57] DCs kill certain tumor cells via a caspase-8-dependent apoptotic signal, which does not involve FasL, TRAIL, or TNF-α. The finding remains unclear since it contradicts previous findings.[76] Surprisingly, TLR-7/8-activated human blood-derived DCs demonstrated a Ca^{2+}-dependent cytotoxicity toward tumor cells, evoking a granule exocytosis-dependent mechanism.[54] The authors observed that the basal skin carcinoma-infiltrating $CD11c^+$ DCs co-stained for PRF and GZM and DCs isolated from peripheral blood of those cancer patients exhibit cytotoxicity. However, there was no direct evidence that the cytotoxic DCs isolated from peripheral blood were indeed the same as the PRF/GRZ^+ $CD11c^+$ DC infiltrating the tumors.

Interestingly, although others have reported that pDCs are poorly cytotoxic,[52,71] Stary *et al*.[54] demonstrated that blood-derived pDCs kill tumor cells upon TLR-7/8 activation via a TRAIL-dependent mechanism. Palucka's group also recently described a $CD2^+$ pDC subset found in human blood that kills tumors *in vitro* in a TRAIL-dependent fashion (KA Palucka,

personal communication). These findings encourage a closer look at the role of PDCs in antitumor immune responses.

In rats, Josien's group[59,60] identified a subset of $CD4^-$/$CD103^+$ DCs that mediate tumor cell killing independently of granule exocytosis or apoptotic TNF family ligands. Srivastava *et al.*[77] showed that the rat cytotoxic DCs express NKR-P2, an ortholog of mouse and human NKG2D. They reported that the crosslinking of NKR-P2 induced maturation of DCs and triggers apoptosis of tumor cells via the release of high amounts of nitric oxide.

Identification of killer DC subsets: NKDCs *versus* IKDCs. In mice, two subsets of cells with bitypic NK and DC phenotypic and functional characteristics have been recently discovered and termed NKDCs and interferon-producing killer DCs (IKDCs).[55,56,78] It remains unclear how closely related these two subsets are or whether they are indeed identical. The phenotypic and functional characteristics of both subsets are summarized in Table 2. Dematteo's group[55] first described NKDCs as a subset of DCs capable to kill tumors and present antigens. NKDCs are mainly phenotypically defined by their coexpression of CD11c and NK1.1. They are characterized by the production of large amounts of IFN-γ secretion in response to a combination of CpG + IL-4 or IL-12 + IL-18.[55,58] Hence, the authors demonstrated that contamination of DC preparations with NKDCs accounted for the controversial production of IFN-γ by DCs. More interestingly, the authors showed that, subsequent to OVA-loaded YAC-1 cell killing, NKDCs up-took and presented OVA antigen to specific class-I restricted T cells, thus claiming the benefit of these cells to kill their target and directly cross-present resultant antigen to specific T cells.[53] However, several major pitfalls resided in the recognition of NKDCs as a cell type *per se*, with a dual functional identity directly linking innate and adaptive immunity. First, the isolation of this subset is based on CD11c and NK1.1 expression, which does not allow a clear distinction from classical NK cells (for extensive comments see Spits and Lanier[61]). Second, the phenotypic characterization of NKDCs showed a significant phenotypic heterogeneity with, for example, $NK1.1^+CD49b^-$ cells.[55] As a consequence, the ability of NKDCs to cross-present antigens provided by their apoptotic target cells remained questionable by the possible contamination with APCs and/or NK cells during isolation.

Meanwhile, the related IKDCs have also raised a lot of interest and controversy on their own.[61,62] IKDCs, in naïve mice, are characterized by the coexpression of CD11c, B220, and a variety of NK markers including CD49b, NK1.1, and IL-2Rβ.[56,78] Although the phenotypic definition of IKDC suffers the same 'glitch' regarding the use of CD11c and B220 to distinguish them from NK cells,[61] we and others have

Table 2 Phenotypic and functional comparison of IKDCs and NKDCs

	IKDC	NKDC
Mice	Balb/c, C57Bl/6[56,78]	C57BL/6[55]
Tissues	Spleen, LN, thymus, liver, lung, gut[56]	Spleen, LN, thymus, liver, lung[53]
Phenotype		
Definition	$CD11c^{int}/B220^+/CD49b^+$[56] $CD11c^+/B220^+/NK1.1^+$[78]	$NK1.1^+/CD11c^+/CD3^-$[55]
MHC-II	Low in spleen; higher in LN; upregulated upon stimulation[56]	Low; upregulated upon stimulation[55]
Costimulatory	$CD80^+$, $CD86^+$, $CD40^+$ (LN); upregulated upon stimulation[56] $OX40L^-$, 4-1BB[a] $PDL\text{-}1^+$ upon stimulation[a] $PDL\text{-}2^-$[a]	$CD80^{low}$, $CD86^{low}$; upregulated upon stimulation[55] $CD40^-$[55]
Lineage markers	$Ly6G^-$, $PDCA1^-$, $CD3\varepsilon^-$, $CD19^-$, $CD4^-$, and $CD8^-$[56] $CD11b^{+/-}$[56,78]	$Ly6G^-$, $CD19^-$, $CD3^-$, $CD4^-$, and $CD8^-$[55] $CD11b^{+/-}$[55]
NK markers	$Ly49^+$, NKG2A/C/E$^+$, $NKG2D^+$[56] $2B4^+$[a], KLRG1$^+$ (MAFA), $CD122^+$ (IL-2R β)[a]	$Ly49^+$, $NKG2D^+$, $2B4^+$[55] CD122 (IL-2R β)[58]
TLR	TLR-1, TLR-2, TLR-9[a] No TLR-3, TLR-4[56b]	TLR-4, TLR-7, TLR-9[53 b] No TLR-3 and TLR-5[53, b]
Function		
Cytotoxicity	Yes; perforin[67]/TRAIL[78]	Yes;?[55]
Cytokine secretion	IFN-γ, IL-12, IFN-α/β, TNF-α[56]	IFN-γ IL-12, TNF-α, IL-6, IL-10[55]
Antitumor	Inhibit B16 growth *in vivo*[78]	Inhibit B16 growth *in vivo*[53]
APC	Yes; MHC-II restricted[56]	Yes; MHC-I and II-restricted[55,53]
Expansion	IL-15/stroma cell help[63]	CpG/IL-18; FLT-3[53]

[a]F Housseau, unpublished observation. [b]Demonstrated by functional *in vitro* assay.

clearly showed that the hallmark of IKDCs is the coexpression of MHC-II and NK markers upon activation.[56,78] IKDCs are distinguishable from NK cells by their ability, as naïve cells, to secrete large amount of IFN-γ when cocultured with a broad array of tumor cells or MCMV-infected fibroblasts but not with normal or uninfected cells (Bonmort *et al.*[63]; F Housseau, unpublished observations). The uniqueness of this subset stands mainly in their functional duality, clearly illustrated by two observations. First, upon activation with virus or TLR-9 ligands, IKDCs are characterized by their ability to 'switch' from a NK-type of cell to a DC-related APC.[56] IKDCs transiently acquired natural killing functions toward typical NK targets. This is illustrated by the upregulation of NK markers including NKG2D, the development of granule exocytosis-mediated killing, and the upregulation of TRAIL.[56,78] Over time, IKDCs downregulate NKG2D and lose their cytotoxic potential associated with an upregulation of MHC-II and costimulatory molecules, the hallmarks of APCs.[56] Second, upon activation *in vitro* and *in vivo*, IKDCs secrete rapidly but temporary low amounts of IFN-α as well as IL-12 (Chan *et al.*[56] and unpublished data). It appears that this autocrine production of IFN-α and IL-12 are essential for IKDC functions. Indeed, a profound defect in maturation of IKDCs was observed in type-I IFN-R knockout mice (F Housseau, unpublished observation). In particular, our group found that the recruitment of MHC-II^{high}CD40^{+} IKDCs following MCMV infection was significantly impaired. The early production of type-I IFN is likely essential for the maturation of IKDC into a MHC-II^{high} APC. By analogy with the work of Pillarisetty *et al.*[55] on NKDC, we postulate that IL-12 plays a crucial role in regulating IFN-γ production by IKDCs. The roles of IL-12 and type-I IFN in IKDC functions are currently under investigation.

Interestingly, our group found that the functional and phenotypic 'transformation' of IKDCs is associated *in vivo* with their migration to the LNs, where they encounter T cells.[56]

Whereas splenic IKDCs demonstrated potent natural killing activity, after infection, LN IKDCs do not kill target cells in a classical PRF/GZM-dependent manner. Importantly, the relationship of precursor-product between splenic innate IKDCs and LN mature IKDCs has been clearly demonstrated in the context of an adoptive transfer experiment of MHC-II^{low}CD11c^{int}CD49b^{+} splenic IKDCs. Upon *Listeria monocytogenes* (LM) infection, only MHC-II^{high}CD11c^{+}CD49b^{+}

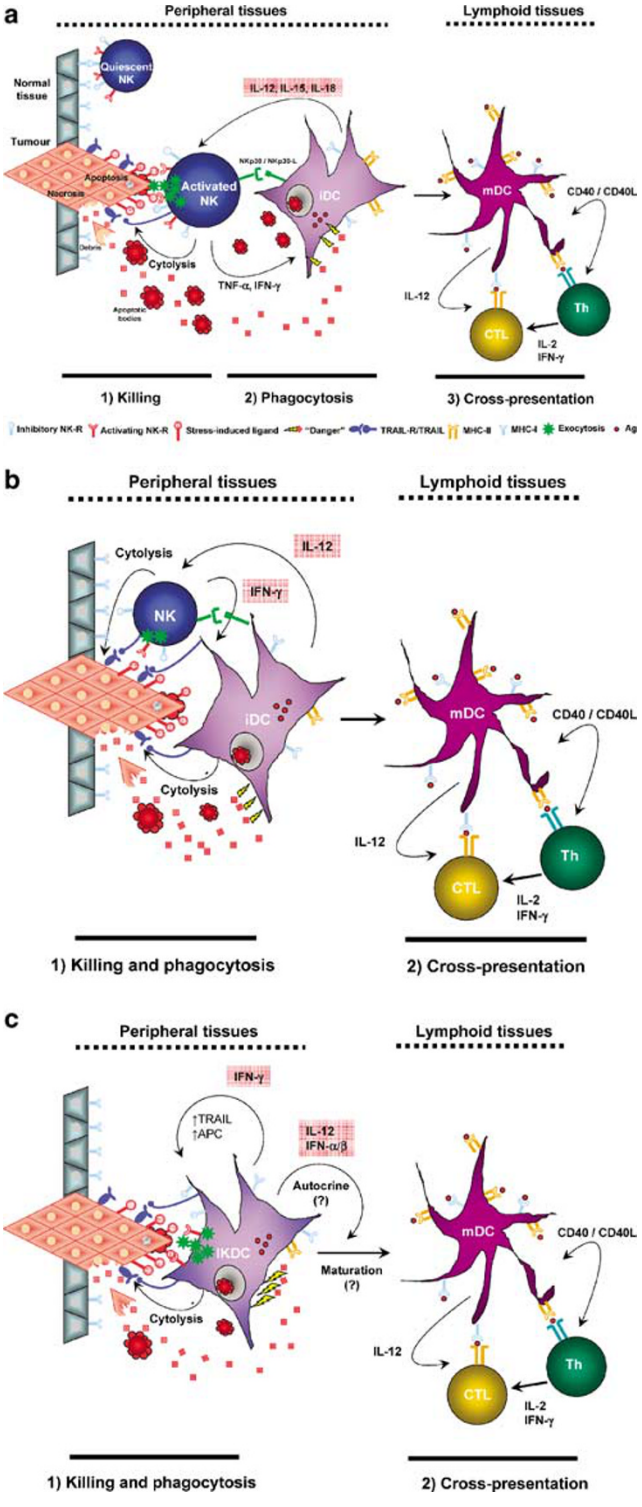


Figure 2 TAA cross-presentation by DCs to CTLs. (**a**) In the classical multi-step model of cross-presentation, immature DCs (iDCs) engulf necrotic debris or apoptotic bodies resulting from the killing activity of activated NK cells toward tumors. The cytotoxicity of NK cells is mediated via granule exocytosis and/or apoptotic ligands like TRAIL. To present TAA to MHC-II-restricted Th lymphocytes and MHC-I-restricted CTLs, iDCs have to migrate to lymphoid organs and differentiate into mature DCs. To do so, DCs sense 'danger signals' delivered by apoptotic bodies, inflammatory mediators released by necrotic cells, or activated NK cells (cell-to-cell contact and soluble mediators). (**b**) In the two-step model of TAA cross-presentation, activated DCs are endowed with tumor killing activities mainly mediated by the upregulation of TRAIL at their membrane surface and the recognition of its receptor(s) at the surface of the tumor cells. Upregulation of TRAIL on DCs is mainly dependent of IFN-γ production by activated NK cells or type I IFN provided by the damaged tissues. DCs are then activated in presence of 'danger signals' at the tumor site and present TAA derived from their killed targets to Th and CTLs in lymphoid organs. The cross-presentation of TAA provided by their killed target remained to be demonstrated. (**c**) IKDCs, which express inhibitory and activating NK receptors, recognize tumor cells without any help from NK cells. Indeed, IKDCs produce high amount of IFN-γ on their own. IFN-γ enhances TRAIL expression and MHC molecules. IKDCs kill their targets via a rapid granule exocytosis-mediated mechanism and/or TRAIL-dependent pathway. In response to the 'danger signals' provided by apoptotic bodies and/or necrosis, IKDCs produce type-I IFN that seems critical for their activation and maturation into a DC-type APC. IL-12 is thought to be essential for the high level of IFN-γ production by IKDCs. The maturation of IKDCs into an APC cross-presenting target tumor cell-derived antigens remains to be established

IKDCs were found in the LNs. Moreover, MHC-II^{high}CD11c^{int} CD49b^{+} LN IKDCs, but not MHC-II^{int}CD11c^{int}CD49b^{+} splenic IKDC, isolated from OVA-expressing LM-infected mice presented OVA *ex vivo* to MHC-II-restricted OVA-specific CD4^{+} T cells.[56]

Classically, the stimulation of tumor-specific T cells is thought to be a result of a multi-step process involving a complex cellular cooperation (Figure 2). Three major events have to take place in a coordinated fashion: (1) killing of the tumor cells by cytotoxic effectors, (2) transfer of TAA from the dying tumor to DCs, and (3) cross-presentation of TAA to T lymphocytes in the LN. The cross-priming of TAA-specific CTLs depends on the activation of DCs induced by dying cells, local inflammatory factors, and/or other innate effectors, including NK cells. The recent identification of killer DCs including IKDCs and NKDCs unveiled an alternative 'two-step process' of immunosurveillance where innate effectors continuously patrolling tissues can, very early on before any sign of inflammation (naïve IKDCs killed spontaneously B16; see Bonmort *et al.*[63] and Taieb *et al.*[78]), detect cells undergoing stress, kill them, and return to lymphoid organs to alert the adaptive components of the immune response (Figure 2). The detection of PFR-producing DCs in human cancers is an intriguing observation that supports this novel tumor immunosurveillance concept.[54] To date, the missing links of IKDCs in tumor immunosurveillance remain in their way to proceed with dying tumor cells and their ability to cross-prime antitumor CTLs.

Therapeutic Implications

Recent attempts have been used to induce effective antitumor immune responses by using DCs or tumor cells as vectors, in association with recombinant cytokines or T-cell costimulation molecules, or with monoclonal antibodies targeting stimulation of specific DCs or T cell subsets, and blocking of T_{reg} cells (reviewed in Schuler *et al.*[65]). DC-based cancer immunotherapies have been traditionally focused on their antigen presentation properties. While traditional DC-based vaccines provide great hope in achieving effective antitumor immune responses, durable clinical responses have rarely been seen.[67] The ability of cytotoxic DCs to directly kill tumor cells clearly makes them a therapeutic target for cancer immunotherapies and should be taken into consideration for future design of effective cancer immunotherapies. For instance, the efficiency of DC-based vaccines might be improved by targeting DCs to upregulate death-triggering receptors. The decision to use mature *versus* immature DCs should also be dictated by the nature and/or the levels of the death-triggering receptors expressed on the DCs. It appears that TNF-α, TRAIL, and FasL are differentially induced on the surface of the killer DCs according to the nature of the stimulating signals.[73] Thus, the type of stimulating reagents used would be tailored to the tumoricidal activity of activated DCs toward a specific tumor.

In addition, the recent identification of IKDCs has fueled a lot of interest and hope. Indeed, Taieb *et al.*[78] showed that

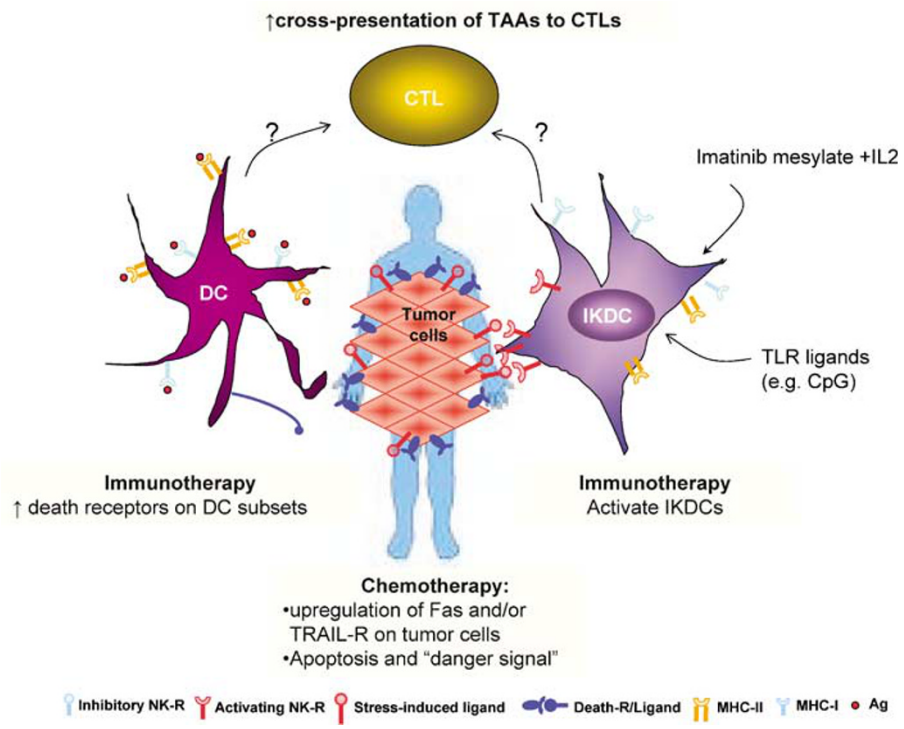


Figure 3 Future development of therapeutic strategies by combining killer DC-based immunotherapies and chemotherapy. Efficiency of DC-based vaccines might be improved by targeting DCs to upregulate various death-triggering receptors based on each tumor cell type. In addition, IKDCs, which mediate direct killing of tumor cells followed by subsequent activation of T cells, clearly represent a promising therapeutic target. The combination of immunotherapy (that activates IKDCs and/or other killer DC subsets, depletes T_{reg}, enhances DC cross-presentation to CTLs) and chemotherapy (that induces expression of Fas or TRAIL-R on tumor cells, apoptosis, and release of 'danger signals') could be an effective means to achieve effective antitumor immune response

IM (Gleevec) + IL-2 increased the survival of B16F10 melanoma-bearing mice. This effect of IM + IL-2 appears to be related to the recruitment of activated IKDCs, which mediates a TRAIL-dependent tumor cell death. IM has already proven its efficiency in the clinic to stimulate the host-dependent antitumor immunity in gastrointestinal stromal tumor patients via the enhancement of DC-mediated NK priming.[48] The adoptive transfer of IKDCs into tumor-bearing immunodeficient mice and TRAIL blocking experiments were the first *in vivo* evidence of KDC involvement in tumor immunosurveillance.[78] It thus becomes critical to identify the human counterpart of IKDCs and evaluate their antitumor activity *in vivo*.

One key obstacle hindering the induction of successful antitumor immune responses is the elevated numbers of circulating and tumor-infiltrating $CD4^+CD25^+$ T_{reg} cells in cancer patients when compared to healthy volunteers.[66,68,79] Not only T_{reg} cells suppress Ag-specific effector T cells, they also express membrane-bound TGF-β, which directly inhibits NK cell effector functions and downregulate NKG2D receptors on the NK cell surface both *in vitro* and *in vivo*.[80] Indeed, depletion of T_{reg} cells using anti-CD25 antibodies or low-dose cyclophosphamide improves T cell-based tumor clearance,[81] augment the response to DC-based therapy,[82] and exacerbate NK cell-mediated tumor suppression and metastases.[80,83] The combination of therapies that deplete/suppress T_{reg} cells, activate IKDCs, enhance the susceptibility of tumor cells to die, promote cross-presentation of apoptotic body-derived antigens, and increase antigenicity could be an effective means in achieving strong effective antitumor immune response (Figure 3). Indeed, certain chemotherapies that induce upregulation of Fas or TRAIL-R[84,85] and/or influence the cell death pathway by a variety of mechanisms promoting the immunologically mediated killing has been employed.[25,86] Although FasL appears to be a potent weapon to fight cancer cells, it suffers from robust side effects when overexpressed and tumor resistance resulting from high frequency of non-functional mutation of its receptor.[23] On the contrary, TRAIL has potent anticancer properties *in vivo* without any toxic effects,[25,87] and targeting certain subsets of DCs or IKDCs to mediate TRAIL-mediated cytotoxicity appears to have great therapeutic potential. However, it has been shown that certain cancer cells can be resistant to TRAIL-mediated apoptosis.[88,89] Some tumor cells have defects in the apoptotic signaling pathway(s) and/or mutations at the level of TRAIL-Rs.[90,91] In addition, many chemotherapy-resistant tumors express high levels of antiapoptosis molecules Bcl-2 and Bcl-xL, which may be protective against TRAIL-induced apoptosis.[92] Thus, targeting different DC subsets with various killing mechanisms according to each tumor cell type appears to be a key step in the design of killer DC-based cancer immunotherapy.

Concluding Remarks

As professional APCs in charge of the orchestration of immune responses, DCs are commonly envisioned as 'sentinels' of the organism, collecting apoptotic cells, cellular debris, or soluble antigens resulting from tumor killing by cytotoxic effectors, or spontaneous cell death. DCs have been extensively proven capable of processing TAA and cross-presenting them to CTLs. However, there is a growing body of literature reporting the direct tumoricidal activity of DCs and demonstrating that DC subsets can directly detect signs of cellular stress via expression of NKG2D and TRAIL. Although the pathways used by DCs to kill tumors remain eclectic, one exciting advance is the identification of DC subsets specialized in the recognition and direct killing of tumor cells that can be therapeutically targeted. Indeed the characterization of IKDCs and their recruitment *in vivo* by chemotherapy treatment based on the combination of IM and IL-2 open an entirely new perspective of immunotherapy. The challenges are now to understand the role of IKDCs *in vivo* for cross-priming efficient antitumor CTLs, and more importantly to identify the human counterpart of murine IKDCs.

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