

## Review

# Interactions of tumor cells with dendritic cells: balancing immunity and tolerance

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**Dendritic cells (DCs) are antigen-presenting cells specialized to initiate and maintain immunity and tolerance. DCs initiate immune responses in a manner that depends on signals they receive from pathogens, surrounding cells and their products. Most tumors are infiltrated by DCs. Thus, interactions between DCs and dying tumor cells may determine the balance between immunity and tolerance to tumor cells. In addition, DCs also display non-immunologic effects on tumors and the tumor microenvironment. Therefore, improved understanding of the cross talk between tumor cells and DCs may suggest new approaches to improve cancer therapy.**

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During homeostatic turnover, each day billions of cells undergo death to be replaced by newly differentiated progeny. This balance is critical to the maintenance of organ size and tissue architecture. Malignancy is characterized by altered growth of a malignant clone resulting in disrupted tissue architecture<sup>1</sup> Malignant cells are also genetically abnormal, with aberrant production of proteins, lipids and sugars that can in principle, be recognized by the immune system.

Tumor cells dying either naturally or as a result of anti-tumor therapies are rapidly cleared by phagocytes. Several types of phagocytes, including macrophages, dendritic cells (DCs) and neutrophils are thought to play a role in the removal of the dying corpse. This process potentially exposes the immune system to self (non-mutated) as well as mutated unique antigens.<sup>2</sup> Of the various phagocytes involved in the removal of dying cells, DCs are the professional antigen-presenting cells (APCs) that play a central role in the initiation and regulation of immune responses. DCs regulate both the innate (e.g. macrophages, granulocytes and natural killer (NK) cells) and the adaptive (e.g. T and B cells) immunity.<sup>3,4</sup> DCs can present captured antigens to T cells in an immunogenic or tolerogenic fashion. The nature of T-cell response induced by DCs largely depends on signals the DCs receive from surrounding cells.

DCs are found to infiltrate several types of tumors in both human and mice.<sup>3,5–8</sup> Therefore, understanding how tumor

cells interact with DCs may have major implications for the development of immunity or tolerance against tumor cells. In this review, we will discuss recent insights into interactions between DCs and tumor cells in the context of DC biology. We will also discuss how these newly unraveled pathways might be harnessed for improved immunotherapy of cancer.

## Dendritic Cells as Critical Antigen-Presenting Cells in Tumor Immunity

DCs are APCs that initiate and regulate immune responses (Figure 1).<sup>3</sup> Their central role in regulating immunity and tolerance is emphasized by their ability to control both innate and adaptive lymphocytes. Peripheral tissue antigen-loaded DCs migrate through the afferent lymphatics into the draining lymph nodes where they present processed protein and lipid antigens to T cells via both classical (major histocompatibility complex (MHC) class I and class II) and non-classical (CD1 family) antigen-presenting molecules. Non-activated (immature) DCs present antigens to T cells, which in the absence of appropriate costimulation leads to tolerance.<sup>9,10</sup> Upon encounter with pathogens or other 'danger'-associated stimuli, such as cytokines, products of damaged tissues or innate lymphocytes, DCs undergo a process of activation (maturation), wherein they acquire the capacity to activate immunity.<sup>11–13</sup> This process of activation has profound effects on DC biology and function. It includes translocation of MHC to

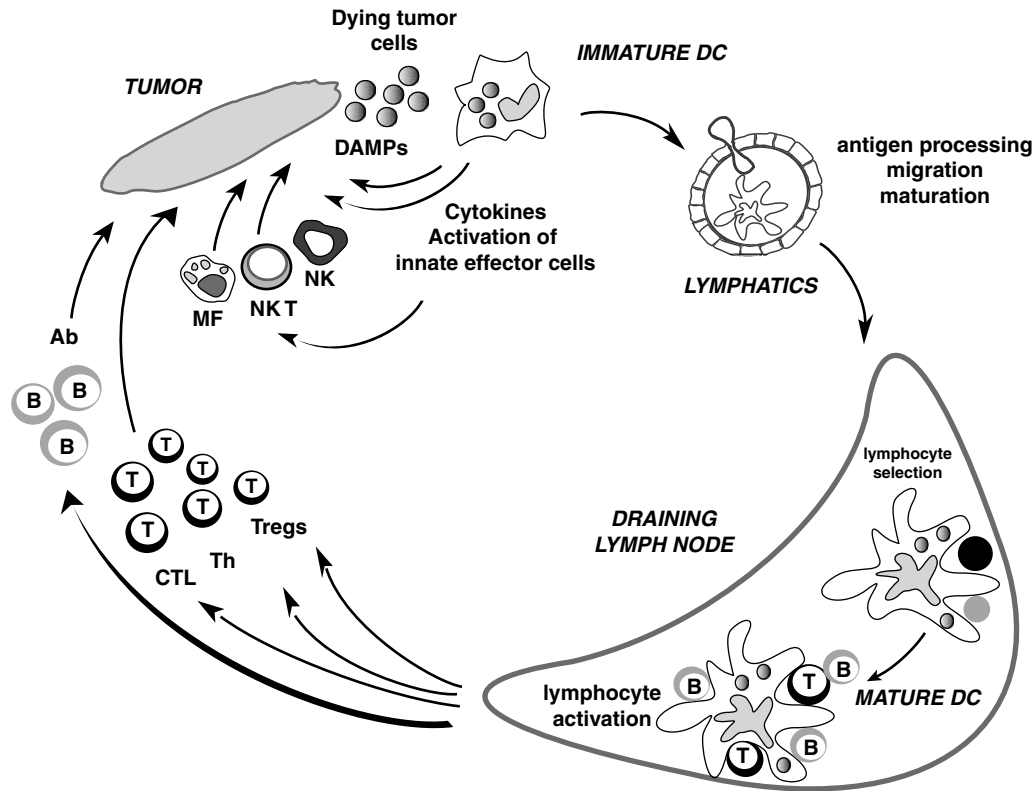
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**Abbreviations:** AC, apoptotic cells; APC, antigen presenting cells; CLR, C-type lectin receptors; CR3, complement receptor 3; CRD, carbohydrate recognition domains; CTL, cytolytic T cells; DAMP, damage associated molecular patterns; DC, dendritic cells; HMGB1, high-mobility box 1; IFN, Interferon; ITAM, immune tyrosine activation motif; ITIM, immune tyrosine inhibitory motif; LC, langerhans cells; MF, macrophage; MHC, major histocompatibility complex; NK, natural killer cells; NLR, nucleotide oligomerization domain-like receptors; PS, phosphatidylserine; RAGE, receptor for advanced glycation end products; TGF- $\beta$ , transforming growth factor beta; TIRAP, Toll IL1 receptor adaptor protein; TLR, Toll-like receptors; TNF, tumor necrosis factor; TRAM, TRIF-related adaptor molecule; TRIF, TIR domain-containing adaptor protein inducing interferon; Tregs, regulatory T cells; TSLP, thymic stromal lymphopoietin

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**Figure 1** The life cycle of dendritic cells in the context of tumor immunity. Circulating precursor DCs enter tissues as immature DC where they can encounter dying tumor cells and their products. This can lead to the recognition of dying tumor cells via DAMPs, capture of dying tumor cells and migration towards draining lymph nodes as well as secretion of cytokines. Cytokines secreted by DCs in turn activate effector cells of innate immunity such as eosinophils, macrophages and NK cells. DCs migration towards secondary lymphoid organs might be associated with their simultaneous activation (maturation) by the products of dying cells such as uric acid or HMGB1 or by cytokines in autocrine or paracrine fashion. These activated migratory DCs that enter lymphoid organs display pMHC complexes, which allow selection of rare circulating antigen-specific T lymphocytes. Activated T cells help DCs for their terminal maturation, which allows lymphocyte expansion and differentiation leading to generation of effector cells and antibodies. However, tumor microenvironment and/or dying tumor cells might inhibit DC maturation leading to the generation of Tregs rather than effector cells. It remains to be determined whether tumor antigens can also reach draining lymph nodes without involvement of peripheral tissue DCs and be captured by lymph node-resident DCs.<sup>164</sup> Abbreviations: DAMP, damage-associated molecular patterns; NKT, natural killer T; MF, macrophage; Ab, antibody; Th, T helper cells; CTL, cytolytic T cells

the cell surface, increased expression of costimulatory molecules, changes in motility and acquisition of dendrites, which among others create an enormous surface to present antigen and interact with lymphocytes.<sup>14,11</sup> Several stimuli, such as signals from inflammatory cytokines, innate lymphocytes, CD40 ligand and pattern recognition receptors (or combinations thereof), have been shown to mediate DC activation, and in some instances, license them to induce immunity.<sup>15–17</sup> However, the nature of specific T-cell responses generated may differ between different stimuli for DC activation. Some activation stimuli that mediate phenotypic changes associated with DC activation may not suffice to elicit protective T-cell immunity.<sup>18,19</sup>

Tumors by themselves are poor APCs. Studies in murine models have suggested that the generation of protective anti-tumor immunity depends on the presentation of tumor antigens by bone marrow-derived APCs, most likely by DCs.<sup>20</sup> Indeed, DCs can efficiently present captured tumor antigens not only on MHCII, as is the case for other APCs, but also MHCI, a process termed as ‘cross presentation’.<sup>21,22</sup> This ability of DCs to cross present antigen from dying cells may be critical for the induction of anti-tumor immunity *in vivo*.<sup>22</sup> Under steady state, this pathway may also contribute to the induction

of tumor and tissue-specific tolerance.<sup>23</sup> Accordingly, defects in uptake of apoptotic cells (ACs) have been implicated in the development of autoimmunity.<sup>24</sup> Induction of T-cell immunity in response to dying tumor cells *in vivo* in mice requires a DC activation stimulus, which can be delivered via innate lymphocytes, CD40 or Toll receptor ligands.<sup>12</sup>

Importantly, DCs are not only efficient at stimulating helper and cytolytic T cells (CTLs), but can also generate regulatory T cells (Tregs) both *in vitro* and *in vivo*.<sup>9</sup> Initial studies demonstrated that immature DCs can lead to the activation of IL10-producing Tregs.<sup>25,26</sup> Recent studies have shown that activated DCs are also efficient at activating both naturally occurring and antigen-specific FoxP3 + CD4 + CD25 + Tregs.<sup>27–29</sup> Generation of Tregs by activated DCs might actually permit resolution of anti-microbial immune responses.<sup>30</sup> The capacity of DCs to induce Tregs is significantly enhanced by cytokines such as transforming growth factor beta (TGF- $\beta$ ) or IL10.<sup>31,32</sup> Uptake of tumor cells by DCs, in the context of immune suppressive cytokine milieu, may therefore promote the induction of tumor-specific Tregs. A growing body of evidence points to an important role for Tregs as an obstacle to tumor immunity.<sup>33,34</sup> Uptake of dying tumor cells by DCs may play a critical role in the induction and

maintenance of tumor-specific Tregs *in vivo* and deserves further study.

An additional layer of complexity is imparted by the different DC subsets that reside in different tissues (or are recruited to them) that may carry different degrees of specialization with regard to activation of different components of the immune system. Such diversity might allow the adaptive immune system to mount functionally distinct types of responses. This is important because the type of immune response itself can be a matter of life or death. For example, in leprosy, the tuberculoid form of the disease is characterized by a 'type 1' response and low morbidity, but the lepromatous form which is characterized by a 'type 2' response, often kills the host.<sup>35</sup> Thus, DCs and their subsets may serve as a link between innate and adaptive immunity by integrating the information from the environment to direct the quality of induced adaptive immunity.<sup>15</sup>

In addition to adaptive immunity, DCs are also efficient in their ability to interact with and activate innate lymphocytes such as NK cells.<sup>36</sup> Due to their ability to regulate diverse aspects of the immune response, DCs are an attractive candidates for vaccination against cancer, either by injection of DCs generated *ex vivo* from bone marrow progenitors or blood precursors and loaded with antigens; or by their specific targeting *in vivo* with anti-DC antibodies fused to antigen.<sup>37,38</sup> Understanding how tumor cells may impact the biology and function of DCs in the tumor microenvironment, therefore, has direct implications for exploiting DCs for cancer immunotherapy.

### Interaction of DCs with Dying Tumor Cells

In view of the important role of DCs in immune regulation, several studies have attempted to examine the interaction of dying cells with DCs.<sup>39</sup> This interaction involves several steps beginning with the recognition and uptake of dying cells, processing of the cargo, activation of DCs and eventually, the activation of innate and adaptive lymphocytes. An emerging theme from these studies is that the interaction between these cells and its functional consequences involves several players and pathways highly conserved through evolution. Importantly, many of the steps are regulated by a balance between positive and negative signals (Table 1). Activation of each step often requires not just the provision of a positive stimulus, but also the removal of a negative one. The existence of multiple pathways creates the potential for synergy and pattern recognition through combinatorial diversity. Some molecules impact more than one aspect of these interactions and, to some degree, there may be a division of labor. Below we discuss and exemplify these emerging themes.

**Recognition and uptake of dying cells by DCs.** Dying cells have been postulated to engage several mechanisms to signal the innate immune system for disposal of the corpse (Figure 2). Three types of signals are operative: (i) 'find me', (ii) 'eat me' and (iii) 'stay away.'

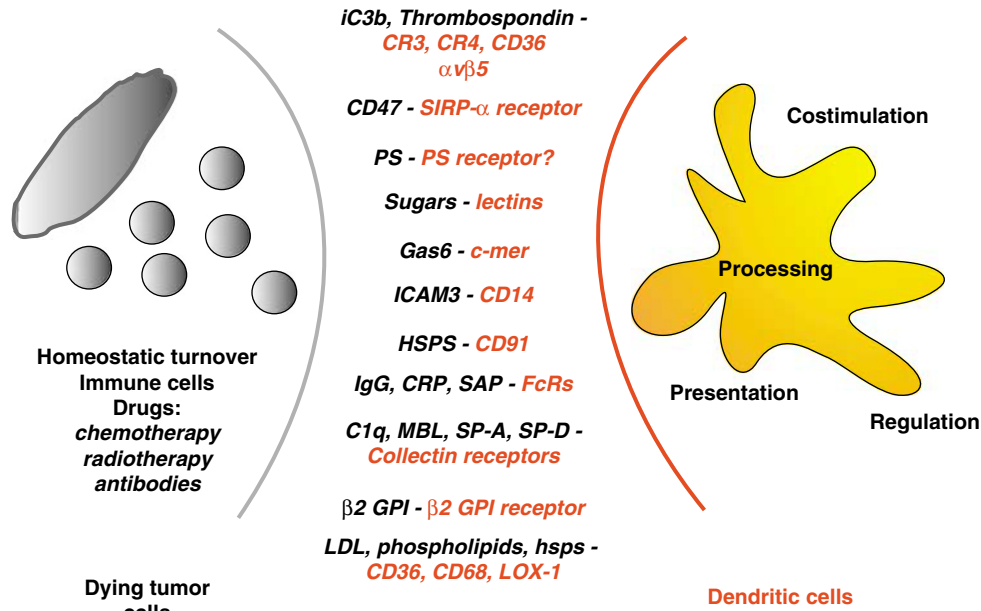
(i) 'Find me' signals are soluble factors, such as lysophosphatidylcholine, and might induce migration of phagocytes to ACs.<sup>40</sup>

**Table 1** Some examples of putative opposing signals that regulate immunity to dying cells

Process	Promotes	Inhibits	References
Recognition	PS and PS bridging signals Heat-shock proteins CD36, CR3, c-mer, integrins Lyso-PC	CD47 CD31	40–44
Uptake	Integrins, activating Fc $\gamma$ R CD36	CD47	42,45–47
DC activation	Activating Fc $\gamma$ Rs TLRs	CR3 c-mer Inhibitory FcRs ?CLRs	42,48–50
Phagosome maturation	? TLR	?	51–54
Antigen processing/presentation	TLR, Activating Fc $\gamma$ Rs, CLRs	Inhibitory FcRs	55–58

- (ii) 'Eat me' signals are usually membrane bound and serve as markers for phagocytes to recognize and ingest ACs. These include alterations in cell surface phospholipid composition with exposure of phosphatidylserine (PS), alterations in cell surface charge or expression of specific molecules.<sup>41</sup> A number of receptors expressed on immature DCs such as  $\alpha$ v $\beta$ 5 integrin, complement receptors and CD36 are thought to be involved in AC uptake.<sup>42,43</sup> The exposure of PS is among the best-characterized events in the interaction of ACs and DCs. It is possibly mediated by increased phospholipid scrambling and the loss of aminophospholipid translocase activity.<sup>41</sup> Several bridge molecules that bind to PS and link to DC receptors have also been identified. These include milk fat globulin E8, which binds  $\alpha$ v integrins;<sup>59</sup> Gas-6 and protein S, which interact with Mer tyrosine kinase.<sup>60</sup> Another pathway may involve collectin family of pattern recognition molecules (such as C1q, mannose binding lectin and surfactant protein-D), which may bind to calreticulin complexed with CD91 or low-density LRP1.<sup>61–63</sup> Furthermore, 'eat me' signals might be indirect, that is, mediated by serum or phagocyte-derived proteins, which can opsonize ACs, and thereby promoting their capture.<sup>64–67</sup> Complement receptors, scavenger receptors as well as Fc receptors can be involved here as well and each will be discussed further hereunder.
- (iii) 'Stay away' (or 'do not eat me') signals may be critical to prevent the uptake of activated but live cells, which might express positive signals such as calreticulin.<sup>45</sup> These serve as negative regulators and include CD47 or CD31.<sup>46</sup>

Capture of ACs leads to activation of several signaling pathways. The downstream convergence of several pathways



**Figure 2** Some phagocyte surface receptors and their putative ligands implicated in the recognition of dying cells. Dying tumor cells, generated either as a result of tissue turnover or the anti-tumor activity of immune cells or therapy, express several ligands that can be recognized by DCs. Ligands expressed by dying cells can function as 'eat me' (e.g. PS) or 'stay away' (do not eat me) signals (for example CD47). In turn, DCs express an armamentarium of receptors that are involved in the recognition and capture of dying tumor cells. This will ultimately lead to tumor antigen processing and presentation to T cells. Additional factors such as expression of costimulatory molecules by DCs and/or cytokines secreted by DCs may regulate the final outcome of antigen presentation and generation of immunity or tolerance

may involve activation of the low-molecular weight GTPase Rac1, which is required for uptake and cytoskeletal rearrangement.<sup>68</sup> For example, Rac1 can be activated via the formation of CrkII–Dock180 complex by both by PS engagement, as well as by integrins.<sup>59</sup> This pathway appears to be evolutionarily conserved and for example, bears considerable homology to activation of ced-10 in *Caenorhabditis elegans*. CD47 can serve as an example of the control elements in the cell death recognition/uptake pathway. Interactions of SIRP $\alpha$  on phagocytes with CD47 leads to activation of Src-homology-containing tyrosine phosphatase-1 (SHP-1) and therefore inhibit Src family kinases.<sup>45</sup> Uptake of dying cells therefore may require not just one (or more) positive signal(s), but also the loss of a negative signal.

**Regulation of processing and presentation of dying cells.** The pathways for recognition and uptake of dying cells discussed above are not unique to DCs, but also operative in other phagocytes, particularly macrophages. However, DCs and macrophages differ substantially in antigen processing and presentation, and the capacity to prime T cells.<sup>69</sup> Therefore, the immunologic consequences of antigen uptake are expected to be quite distinct for macrophages *versus* DCs. One of the critical differences between DCs and macrophages may be differential lysosomal proteolysis of the phagocytosed antigen.<sup>70–72</sup> While macrophages contain high levels of lysosomal proteases and rapidly degrade proteins, DCs exhibit limited proteolysis, favoring antigen persistence and presentation. DCs are also much more efficient at loading exogenous antigens on MHC1 for cross-presentation of captured antigens to CD8 + killer T cells.<sup>21,73</sup>

Importantly, capture of dying tumor cells is ultimately linked with DC activation, which in turn will determine the type of ensuing T-cell immunity. Several receptors have been shown to be important for antigen uptake and presentation of phagocytosed dying cells by DCs. Of these the major receptors include complement and Fc $\gamma$  receptors, mer family kinases, lectin receptors and toll receptor pathways. The underlying theme appears to be that engagement of certain pathways (for example mer, or complement receptor3, inhibitory FcRs) may mediate tolerance, whereas the engagement of others (for example Toll-like receptors (TLRs), activating FcRs) promote immunity. At least in some instances, such as the uptake of immune complexes or opsonized tumor cells, the same ligand binds both to activating and inhibitory receptors.

**Role of complement and scavenger receptors.** ACs are frequently opsonized by complement component iC3b generated as a result of low level complement activation. Binding of iC3b-opsonized ACs to its receptor, complement receptor 3 (CR3) on APCs has been implicated in tolerance induced by dying cells.<sup>64</sup> Indeed, DCs exposed to ACs opsonized with iC3b are inhibited from activation in response to stimuli such as CD40L and LPS. Recently, two studies with murine bone marrow DCs, as well as human monocyte-derived DCs have shown that engagement of CR3 leads to suppressive effects on DC function, mediated in part via release of TGF- $\beta$  and possibly IL10 in some settings.<sup>48,49</sup> Recent studies also suggest a division of labor between various receptors for dying cells. For example, whereas  $\alpha V\beta 5$  had a significant effect on phagocytosis, CR3 had a more

dominant effect on the suppression of DC activation.<sup>48</sup> Another receptor implicated in immune-suppressive effects of ACs is the scavenger receptor CD36, which binds to oxidized phospholipids and thrombospondin.<sup>74</sup> However, the mechanisms by which these receptors lead to the induction of tolerizing DC states still needs to be clarified. It is also notable that most of the current data regarding the effects of individual ligands/receptors are based on *in vitro* experiments and the relative importance *in vivo* remains to be clarified.

**Role of mer tyrosine kinase.** Recently, the mer/Axl/Tyro3 receptor tyrosine kinase (RTK) family has been implicated in homeostatic regulation of APC activation.<sup>75</sup> Mice lacking all three RTKs develop hyperactivated DCs, and macrophages, which drive lymphoproliferation and systemic autoimmunity.<sup>75</sup> Mice lacking Mer-TK expression also develop a lupus-like syndrome and have defective clearance of ACs.<sup>44,76</sup> A ligand for mer-TK is growth-arrest-specific gene 6 (Gas-6), which binds to PS expressed on ACs.<sup>77</sup> Mer-TK-deficient DCs are resistant to AC-induced inhibition of NF $\kappa$ B pathway, suggesting an important role for this pathway in AC-mediated regulation of DC function.<sup>50</sup> Together these data suggest an important role for this pathway in the regulation of AC–DC interaction and suggest that constitutive engagement of this pathway may be an important control checkpoint for DC activation.

**Role of activating/inhibitory Fc $\gamma$ R balance.** Fc $\gamma$ Rs represent one of the best studied receptors for the uptake and targeting of antigens to DCs, either in the form of immune complexes or opsonized dying cells, such as those generated during antibody therapy of cancer.<sup>78</sup> The Fc $\gamma$ R system consists of a balance of activating and inhibitory FcRs, which carry an immune tyrosine activation (ITAM) or inhibitory (ITIM) motif in their cytoplasmic domain, respectively. Human monocytes and DCs express an array of these receptors, including both activating and inhibitory FcRs.<sup>79,55</sup> Several studies have now shown that antigen uptake in the form of immune complexes or opsonized tumor cells is associated with enhanced antigen presentation by DCs and the generation of antigen-specific T cells.<sup>47,80–82</sup> Selective blockade of inhibitory Fc $\gamma$  receptors is associated with DC activation.<sup>55</sup> DCs from mice deficient in the inhibitory Fc $\gamma$ RII have a more activated phenotype.<sup>56</sup> These data suggest that the balance of activating *versus* inhibitory FcRs may have a major impact on DC activation *in vivo*. The ability of IC to enhance antigen presentation depends on the recruitment of syk to the activating FcRs.<sup>83</sup> Recently, it was shown that selective activation of activating FcRs leads to the induction of a type I interferon response program in human monocytes and monocyte-derived DCs.<sup>84</sup> The balance of FcR signaling may therefore regulate the level of constitutive type I IFN signaling in myeloid cells. Together these data suggest that the engagement of activating Fc $\gamma$ Rs on DCs by opsonized dying cells may lead to major effects on the biology of DCs and generation of adaptive immunity. Harnessing this pathway may allow the recruitment of adaptive immunity and immunologic memory by antibody therapy of cancer.

**Role of C-type lectin receptors.** C-type lectin receptors (CLRs) represent a family of calcium-dependent lectins that share primary structural homology in their carbohydrate recognition domains (CRDs).<sup>85</sup> CLRs bind to self and non-self sugars, including those expressed on tumor cells. Several members of CLR family are expressed on myeloid cells including DCs. Some of the best studied members on myeloid APCs include the mannose receptor, DEC-205, DC-SIGN, and Dectin-1, which have endocytic activity.<sup>85</sup> Signaling pathways engaged by these receptors have not yet been fully defined, however, the functional effects of targeting antigens to these receptors are being elucidated. Much of the work relates to recognition of pathogen-derived ligands, however, it is likely that these receptors are engaged by normal tissues as well as by transformed cells.<sup>57</sup> For example, targeting antigen to DEC-205 in the steady state *in vivo* leads to immune tolerance.<sup>10,58</sup> Targeting DC-SIGN leads to inhibition of TLR-mediated IL12 production and DC maturation.<sup>57,86</sup> A recent study showed that the engagement of Dectin1, which contains a hemi-ITAM motif, leads to induction of Th17 cells via syk- and CARD9-dependent mechanism.<sup>87</sup> Although the diversity and signaling mediated by these receptors remains to be clarified, engagement of these receptors by tumor cells may have major effects on antigen presentation. Some examples of potential CLR ligands expressed by tumor cells include muc1, which binds to the mannose receptor and galactose-type c-lectin<sup>88</sup> and the carcinoembryonic antigen (CEA), which binds to DC-SIGN.<sup>89</sup>

**Role of TOLL-like receptors.** TLRs are pattern-recognition receptors that mediate the recognition of pathogen-associated molecular patterns.<sup>90</sup> TLRs initiate signaling via myeloid differentiation primary response gene (MyD88), Toll-IL1 receptor adaptor protein (TIRAP), TIR domain-containing adaptor protein inducing interferon (TRIF) and TRIF-related adaptor molecule. TLR signaling can lead to the activation of several signaling pathways including mitogen-activated protein kinases (MAPK) such as p38, ERK and JNK, NF $\kappa$ B and interferon response genes pivotal to immune response. Recruitment of TLRs to newly formed phagosomes suggested that this may form a mechanism to monitor the nature of phagocytic cargo.<sup>91</sup> Current data suggest that TLRs do not serve directly as phagocytic receptors, and abrogation of TLR signaling does not prevent the uptake of TLR containing particles.<sup>92</sup> TLR signaling may, however, impact the fusion of phagosomes with lysosomes and endosomes, a process collectively termed as 'phagosome maturation'.<sup>51</sup> One mechanism by which this might occur is p38-MAPK-mediated recruitment of Rab family of small GTPases, which are considered as critical regulators of endosomal traffic. However, the impact of TLRs on phagosome maturation is still somewhat controversial and has not been observed in other experimental settings.<sup>52</sup>

Interestingly, an elegant study by Blander and Medzhitov<sup>53,54</sup> has shown that the engagement of TLR signaling by phagocytic cargo can dramatically impact the MHCII presentation of antigens from that cargo by targeting li degradation. Phagosomes that do not engage TLRs fail to process li and do not accumulate mature MHCII dimers. It is notable that

this TLR-mediated control of presentation appears to be phagosome autonomous. In other words, antigen processing within the phagosomes may be uncoupled from the activation status of DCs themselves. One possible mechanism is via compartmentalized assembly of the TLR signaling complex. One mechanism by which TLRs might influence proteolysis and antigen processing might be by relieving negative regulation (e.g. by cystatin c),<sup>93</sup> or inducing optimal phagosomal pH, perhaps by regulating the activities of vacuolar ATPase and the NADPH oxidase NOX2.<sup>69,94,95</sup>

The concept that phagosomes containing TLR ligands might be processed differently provides a subcellular level of self/non-self discrimination, depending on the content of the phagosomal cargo. This process may also be operative in other pathways. For example, after uptake of a mixture of mAb-opsonized and non-opsonized dying myeloma cells, only the antigens from the former subset were efficiently presented by human DCs.<sup>80</sup> Schulz *et al.*<sup>96</sup> have shown that murine CD8 $\alpha$  + DCs can be activated by double-stranded RNA present in virally infected dying cells via a TLR3-dependent mechanism. However, this has not yet been demonstrated with human cells, and the human counterpart of CD8 + DCs remains to be clarified. TLR-mediated enhancement of antigen presentation also has major implications for vaccine design. For example, conjugation of TLR ligands with antigen was shown to enhance generation of T-cell responses.<sup>97</sup>

**Role of cytokines and other soluble factors.** In addition to signals generated from cellular receptors, uptake of dying cells is also associated with altered release of cytokines that can impact the generation of immunity. For example, ACs can specifically inhibit the expression of several cytokines, particularly IL12.<sup>98</sup> Uptake of ACs can also lead to the production of immune suppressive cytokines such as TGF- $\beta$ .<sup>99</sup> Phagocytosis of microbially infected cells, in contrast, is designed to eliminate pathogens and trigger the inflammatory response, including the production of tumor necrosis factor (TNF), IL1 and IL6. The microenvironment of tumors is already enriched for immune suppressive cytokines such as VEGF, TGF- $\beta$ , IL10 and IL13.<sup>33</sup> Some of these cytokines are produced directly by tumors while others are produced by immune cells recruited to the tumor bed. The pattern of cytokines in the tumor bed may depend on the specific tumor and involved tissue. These cytokines can have a major impact on the nature of T cell response elicited by DCs. For example, TGF- $\beta$  and IL10 may promote the induction of regulatory cells by DCs.<sup>31,32</sup> The immunologic outcome of uptake of tumor cells by DCs will therefore likely depend on the specific tumor type and its unique microenvironment.

Cell death may also release some proinflammatory molecules. Damage-associated molecular pattern (DAMPs) are largely intracellular molecules that can act extracellularly to initiate inflammation.<sup>100</sup> Several receptors, including TLRs and receptor for advanced glycation products, probably play a role in recognition of DAMPs. Several molecules such as high-mobility box1 (HMGB1),<sup>101</sup> heat-shock proteins,<sup>102</sup> purine metabolites such as uric acid,<sup>103</sup> matrix components such as hyaluronan<sup>104</sup> and heparan sulfate, may play a role in the recognition of damage-associated cell death. Nucleotide

oligomerization domain-like receptors (NLRs) are a family of germline-encoded pattern recognition receptors that allow the host to respond rapidly to several pathogenic microorganisms.<sup>105</sup> A breakthrough in the field was the identification of inflammasomes, which are NLR containing large multiprotein complexes that recruit inflammatory caspases and trigger their activation.<sup>106</sup> At least four pathogen-sensing inflammasomes have been identified and at least one of them (cryopyrin/NALP3), also mediates the recognition of endogenous danger signals such as uric acid.<sup>107</sup> In a recent study, activation of IL1R-MyD88 pathway was shown to be critical for neutrophil but not monocyte response to inflammation induced by necrotic cells.<sup>108</sup> Therefore, both the nature of the soluble factor(s), as well as the responding cell type may determine the outcome of response to dying cells.

An important recent insight is the crosstalk between the release of danger-associated soluble mediators such as HMGB1 and TLR-mediated activation of APCs in the immune response to dying cells and immune complexes. For example, Apetoh *et al.*<sup>109</sup> recently demonstrated that both HMGB1 and TLR4-MyD88 signaling pathways were required for generating immune responses against dying tumor cells. Tian *et al.*<sup>110</sup> demonstrated that HMGB1 was an essential component of DNA-containing immune complexes that mediated activation of plasmacytoid DCs (pDCs), through a TLR9/MyD88 pathway involving receptor for advanced glycation end products (RAGE). Although the specific receptors for HMGB1 remain somewhat controversial and require further study, these studies do point to the emerging importance of the crosstalk between different 'sensing' mechanisms, such as TLRs, Fc $\gamma$  receptors, RAGE and possibly other receptors for determining immunity to dying cells and immune complexes.

**Integration of signals.** As discussed above, DCs must integrate (often opposing) signals from several pathways and receptors, including the uptake- and pattern-recognition receptors and inflammatory/anti-inflammatory cytokines. Although the current knowledge of signaling pathways engaged by dying cells is evolving, data suggest a role for several pathways, including the Jak-STAT pathway, NF $\kappa$ B pathway and TLR-dependent signaling in regulating immune activation.<sup>50,111,112</sup> A critical pathway in this regard may be the Jak-STAT pathway, as activation of STAT3 in tumor infiltrating DCs can promote tolerance in several models.<sup>113</sup> Alternately, activation of type I IFN signaling and STAT1 may promote induction of immunity. Engagement of some pathways such as Mer-TK may promote the inhibition of NF $\kappa$ B activation.<sup>50</sup> A greater degree of complexity emerges from the recognition of crosstalk between these pathways, such as between the FcR pathway and type I IFN signaling.<sup>84</sup> Furthermore, the responsiveness of DCs (or a certain subset thereof) to engagement of a certain pathway may depend on several factors including the strength of the signal, as well as the basal state of signaling in that cell.<sup>111</sup>

### Role of DC Subsets in the Interaction with Dying Tumor Cells

Just as lymphocytes, DCs are composed of distinct subsets with common and unique functions. Different subsets may

mediate distinct functions in response to dying cells.<sup>114</sup> Indeed, the requirement for immunogenic or tolerogenic presentation of tissue-derived antigens may be different for different subsets of DCs. For example, uptake of ACs by a subset of B220-PDCA1- lymphoid DCs in the mouse can result in the production of type I IFN by a TRIF- and MyD88-independent pathway, as well as the ability to prime CD4<sup>+</sup> and CD8<sup>+</sup> T-cell responses to antigens derived from dying cells.<sup>115</sup> In contrast, GM-CSF-derived myeloid murine DCs can phagocytose ACs, but generally do not induce immunity in the absence of type I IFN signaling, such as that initiated by Toll receptors. Different subsets of DCs may also be specialized to activate different arms of the immune system.<sup>116</sup> For example, mouse splenic CD8<sup>+</sup> DCs, but not CD8<sup>-</sup> subset were shown to be able to cross-present antigens *in vitro* and *in vivo*.<sup>117,118</sup> CD8<sup>+</sup> DCs express relatively higher level of proteins involved in MHC I presentation, and may be specialized for presenting antigens to CD8<sup>+</sup> T cells, whereas the CD8<sup>-</sup> DC subset may be more specialized to present antigens on MHC II to CD4 T cells.<sup>116</sup> Pathways that regulate the recognition and processing of antigens from dying cells in these subsets may differ, and therefore the nature of immune response generated may depend not only on the nature of signals from the dying cells, but also the nature of the processing APC. Unfortunately, at present, it remains difficult to translate the findings from mouse DC subsets to humans as the human counterparts of some of the murine DC subsets (e.g. CD8<sup>+</sup> DCs) remain elusive.

Nonetheless, different subsets of DCs exist in humans as well. Much alike in the mouse, in the human two major DC pathways are found, that is, plasmacytoid DCs and myeloid DCs.<sup>119</sup> Two types of myeloid DCs are found in the human skin: Langerhans cells (LCs) in the epidermis, and interstitial DCs (intDCs) in the dermis.<sup>120</sup> Cells with corresponding phenotypic and functional profiles can also be generated by culturing human CD34<sup>+</sup> HPCs with GM-CSF and TNF.<sup>121</sup> These subsets display different phenotypes and biological functions. For example, intDCs, but not LCs, express nonspecific esterases.<sup>122</sup> This is important because different enzymatic activity might yield different peptides that will be presented to T cells as distinct pMHC complexes eventually generating different repertoires. IntDCs, but not LCs, express IL10 and induce the differentiation of naïve B cells into IgM-secreting plasma cells.<sup>122,123</sup> Accordingly, intDCs activate CD4<sup>+</sup> follicular helper T cells. In contrast, LCs are particularly efficient at inducing high-avidity cytotoxic CD8<sup>+</sup> T cells. These recent findings led us to propose that intDCs (dermal DCs) preferentially induce humoral immunity, whereas LCs preferentially induce cellular immunity (Klechevsky *et al*, submitted).

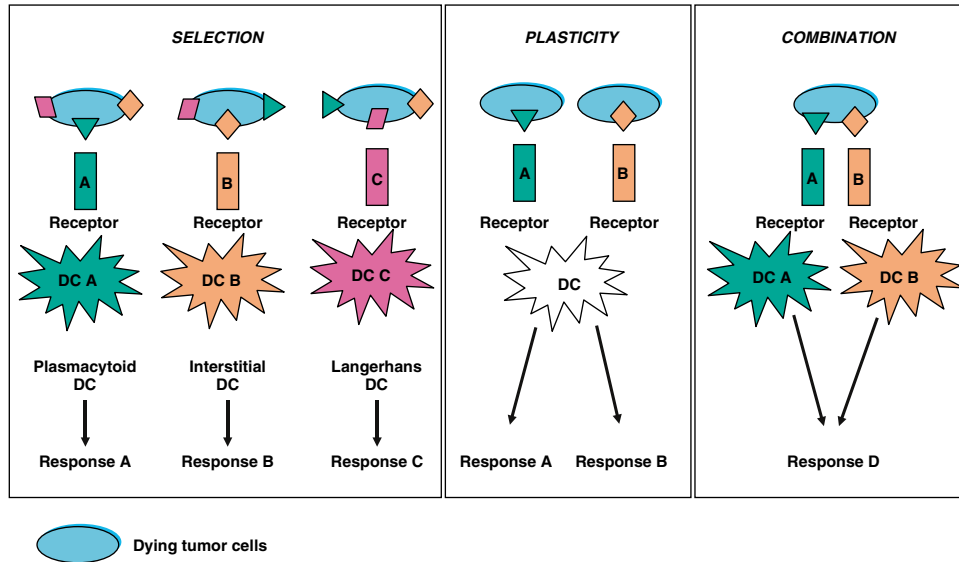
Functional differences apply also to monocyte-derived DCs and different cytokines skew the *in vitro* differentiation of monocytes into DCs with different phenotypes and function. Thus, when activated (for example by GM-CSF) monocytes encounter IL4, they will yield IL4-DCs.<sup>124</sup> By contrast, after encounter with IFN- $\alpha$ , thymic stromal lymphopoietin (TSLP), TNF or IL15, activated monocytes will differentiate into DCs with distinct functional properties, and termed as IFN-DCs,<sup>125</sup> TSLP-DCs,<sup>126,127</sup> TNF-DCs or IL15-DCs,<sup>128</sup> respectively. For

example, myeloid DCs that contain LCs, that is, IFN-DCs or IL15-DCs<sup>129</sup> are more efficient in priming high affinity tumor-specific CTLs.

The existence of DC subsets and their functional plasticity might actually provide a framework for handling the diverse phenotypes of dying tumor cells and generating diverse immune responses (Figure 3). By analogy to how DC handle microbes, the recognition of ACs might be regulated by expression of unique receptor by each DC subset that recognize a unique ligand on ACs. This selective recognition may lead to a specific type of immune response, for example humoral immunity when ACs are taken up by intDCs or cellular immunity when ACs are taken up by LCs. However, DCs and their subsets display a great degree of plasticity in which case a DC may express a combination of receptors that may permit a particular DC to interact with different types of ACs, which will be dictated by the type of ligand expressed on ACs. Thus, DCs may be able to generate different types of responses to different ACs. Finally, a dying tumor cell expressing different ligands might interact with distinct types of DCs thereby eliciting an immune response that will result from an interplay between the two DC subsets. For example, dying tumor cell might be recognized and captured by myeloid DCs, but it may also interact with neighboring plasmacytoid DCs and trigger them to secrete type I interferon. This in turn will activate myeloid DCs and facilitate generation of cellular immunity.<sup>130</sup>

### Interactions of DCs with Living Tumor Cells

Although most of the attention regarding the interaction of tumor cells with DCs to date has focused on dying tumor cells (and its immunologic outcome), it is now increasingly clear that DCs may also have more direct interactions with living cells. DCs can acquire antigen from living cells for cross presentation, by a process termed 'nibbling', which may be a distinct mechanism of antigen uptake.<sup>131,132</sup> Interactions between tumor cells and DCs may render DCs into tolerogenic TGF- $\beta$ -secreting cells that induce CD4<sup>+</sup> CD25<sup>+</sup> Tregs.<sup>133</sup> However, interactions of DCs and tumor cells may also have several non-immunologic consequences. There is already an extensive body of literature about the role of tumor-associated macrophages in promoting tumor progression. However, as discussed above, DCs are functionally and biologically distinct from macrophages. Recent studies from two groups have shown that DCs may have direct effects on survival and clonogenicity of human tumors such as myeloma.<sup>134–136</sup> DCs have also been implicated in promoting angiogenesis in human tumors. DCs may promote angiogenesis both by serving as direct progenitors to tumor-associated neoangiogenesis, as well as by the secretion of proangiogenic cytokines.<sup>137,138</sup> Here again, different subsets of DCs may play distinct roles in modifying the tumor microenvironment. Certain subsets of DCs may mediate a tumor-protective effect. TLR-mediated activation of DCs may enable effector function with tumoricidal properties.<sup>139</sup> Certain subsets of DCs seem to have overlapping phenotype with NK cells.<sup>140</sup> For example, a subset of murine DCs termed interferon producing killer DCs (IKDCs) were shown to play an important role in tumor immune surveillance.<sup>141–143</sup> DCs have also been



**Figure 3** The molecular response of DCs and their subsets to dying tumor cells. Several models can be envisioned: (i) selection, where different apoptotic tumor cells or different ligands expressed on an apoptotic tumor cell target distinct DC subsets through a unique set of receptors, (ii) plasticity, where different apoptotic tumor cells or their ligands act through different receptors on the same DC leading to generation of qualitatively different immune responses, and (iii) a dying tumor cell may target two distinct DC subsets, leading to a mixed response through a combination of signals

shown to be capable of inducing tumor cell death by other mechanisms such as Fas or nitric oxide-mediated interactions.<sup>144</sup> Taken together, these emerging data suggest that different DC subsets may have both tumor-promoting as well as tumor-suppressive properties *in vivo*. This can be exemplified by the demonstration that breast cancer instructs DCs to induce CD4<sup>+</sup> T cells secreting IL13 and promote cancer development by mediating DC polarization.<sup>5</sup> Thus, improved understanding of DCs in the tumor bed, particularly in human tumors is therefore needed to better harness the anti-tumor effects (and avoid the protumor effects) of these cells.

### Implications for Tumor Immunotherapy

**DC activation as a marker of immunogenic tumor cell death.** The possibility that cell death induced by different chemotherapies might have different consequences for the immune system was first suggested in a landmark study by Casares *et al.*,<sup>39,145</sup> who showed induction of tumor immunity in mice after anthracycline (but not mitomycin) treatment of tumors. A growing body of data now suggests that the immunogenicity of dying cells may be linked to their ability to activate DCs.<sup>146–148</sup> Uptake of myeloma cells killed by bortezomib (a proteasome inhibitor), but not those killed by dexamethasone or  $\gamma$ -irradiation led to activation of DCs.<sup>147</sup> Surprisingly, uptake of bortezomib killed tumor cells was sufficient to induce tumor-specific T cells in culture without the need for an exogenous activation stimulus. The delivery of activating stimulus from bortezomib killed tumor cells to DCs was mediated by the expression of hsp90 on the surface of dying cells. Bortezomib killed murine tumors also seem to deliver a DC activation signal, although the underlying mechanism has not yet been investigated in this system.<sup>149</sup>

Interestingly, Obeid *et al.*<sup>146</sup> have recently demonstrated that induction of protective anti-tumor immunity in mice in response to tumor cell death induced by anthracyclines was linked to the induction of calreticulin (another heat shock protein) on the surface of dying murine tumor cells. Immunostimulatory activity of tumor cells enriched in heat shock proteins after induction of hyperthermia has also been observed in some animal models as well as with human cells in culture.<sup>150–152</sup> This is also in accordance with murine studies showing that cell surface hsp90 represent a distinct immunogenic signal (relative to soluble hsp90) and promotes the development of autoimmunity.<sup>153,154</sup> Together, the common theme from these independent observations is that the exposure of certain heat shock proteins on the surface of dying cells may be a marker for immunogenic forms of cell death and deliver an activating stimulus to DCs.

**Chemotherapy.** The finding that some chemotherapies induce an immunogenic form of cell death in some cell types has major implications for both understanding and improving the results with chemotherapy, as well as combining it with immune-based approaches. Activation of adaptive immunity has the potential to impact the durability of clinical responses due to the recruitment of immunologic memory. For example, the possibility that immune-based mechanisms may underlie the clinical effects of bortezomib may help understand the observed correlation of vasculitic skin rash with clinical response to bortezomib in lymphoma, as well as the observations that the clinical responses may be delayed by several months in many patients with follicular lymphoma.<sup>148,155</sup> One important issue for harnessing the potentially immunogenic property of chemotherapy induced cell death may be the need for tumor-specific targeting. This may be accomplished via coupling to anti-tumor antibodies,



or via advances in nanotechnology. Indeed, targeting 'immunogenic drugs' selectively to tumor cells *in vivo* may serve as a cancer vaccine. Such a strategy might also have the additional advantage that it does not require prior knowledge of antigens expressed by tumor cells, and would be specific for the spectrum of antigens expressed by the patient's own tumor. Improved understanding of the mechanisms of immunogenic cell death might eventually allow us to not only better harness these properties of some agents, but may also allow us to modify cell death induced by other agents. In the end, it may be valuable to view chemotherapy and immunotherapy not as antagonistic, but as synergistic partners for improving outcome in cancer.<sup>156</sup>

It is worth noting that even though certain forms of chemotherapies may be able to mediate an immunogenic form of cell death, this may not suffice for the generation of protective or therapeutic anti-tumor immunity. This may be because the tumor microenvironment presents several challenges for the immune system, including the presence of Tregs (which may be further induced),<sup>33,34</sup> The tumor bed also contains several other immune-suppressive elements such as suppressive cytokines (for example VEGF, IL10 and TGF- $\beta$ ) and cells (such as myeloid suppressor cells).<sup>33,34</sup> Another potential obstacle may be that most chemotherapies fail to kill cancer stem cells, which are inherently resistant to such therapies. This may be important, as recent studies have suggested that immune responses to antigens derived from cancer stem cells are associated with improved outcome in human cancer.<sup>157</sup> Therefore generating clinically meaningful immunity even with immunogenic chemotherapy will require attention to overcoming these obstacles.

**Antibody therapy.** Monoclonal antibodies have already proven to be among the most successful forms of new anti-tumor therapies in the last decade. However, these agents also provide underappreciated opportunities for immunization against cancer.<sup>158,159</sup> Monoclonal antibody therapy-promoted cell death can directly cause tumor destruction, and subsequent antigen uptake, presentation by DCs and related professional APCs, leading to adaptive T-cell-mediated immune responses. Monoclonal antibodies can be engineered to mediate improved antibody-dependent cytotoxicity, which should enhance antigen presentation and T-cell activation.<sup>82</sup> This can be accomplished by increasing antibody affinity for tumor antigen targets, or by manipulating antibody Fc $\gamma$  domains to increase their affinity for Fc $\gamma$  receptor(s).<sup>160</sup> As discussed above, it may prove possible to further refine such antibody engineering to selectively engage activating, as opposed to inhibitory Fc $\gamma$  receptors. Antibody structures can be further modified to contain immunostimulatory motifs that selectively induce and amplify antigen presentation to favor the induction of clinically effective host anti-tumor immunity.<sup>161</sup> In lieu of direct modification of antibody structures, anti-tumor antibodies might also be combined with other agents that promote antigen presentation (e.g., toll receptor agonists), or overcome negative regulation (e.g., anti-CTLA-4 antibody). It is worth noting that several clinically useful antibodies are routinely combined with chemotherapy agents,<sup>162,163</sup> further studies are required to determine if chemotherapy-based

tumor destruction cooperates with monoclonal antibody therapy to promote adaptive, tumor antigen-specific immunity.

## Summary

DCs are critical APCs to initiate and regulate immunity. A growing body of evidence now suggests that these cells also play an important role in shaping the host response to tumors. Interactions between DCs and dying cells are determined by a balance of several (often opposing) molecular interactions that regulate recognition, uptake, processing and ultimately presentation of cellular antigens to the immune system. DCs also interact with living tumor cells and have non-immunologic effects on tumor cells and their microenvironment. The interactions between tumor cells and subsets of DCs are complex and just beginning to be understood. Improved understanding of the interactions between DCs and tumors will provide fundamental insights into the mechanisms of tumor immune surveillance and escape, and yield novel approaches to therapy of cancer.

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