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TLR3/TICAM-1 signaling in tumor cell RIP3-dependent necroptosis

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Abbreviations: CTL, cytotoxic T lymphocyte; DAI, DNA-dependent activator of IFN-regulatory factors; DAMP, damage-associated molecular pattern; HMGB1, high-mobility group box 1; HSP, heat shock protein; mDC, myeloid dendritic cell; NK, natural killer; NLR, NOD-like receptor; PAMP, pathogen- associated molecular pattern; PRR, pattern-recognition receptor; RIP, receptor-interacting protein kinase; TICAM-1, Toll-IL-1-homology domain-containing adaptor molecule 1; TLR, Toll-like receptor; TNFα, tumor necrosis factor α; TNFR1, TNFα receptor 1

The engagement of Toll-like receptor 3 (TLR3) leads to the oligomerization of the adaptor TICAM-1 (TRIF), which can induces either of three acute cellular responses, namely, cell survival coupled to Type I interferon production, or cell death, via apoptosis or necrosis. The specific response elicited by TLR3 determines the fate of affected cells, although the switching mechanism between the two cell death pathways in TLR3-stimulated cells remains molecularly unknown. Tumor necrosis factor α (TNFα)-mediated cell death can proceed via apoptosis or via a non-apoptotic pathway, termed necroptosis or programmed necrosis, which have been described in detail. Interestingly, death domain-containing kinases called receptor-interacting protein kinases (RIPs) are involved in the signaling pathways leading to these two cell death pathways. Formation of the RIP1/RIP3 complex (called necrosome) in the absence of caspase 8 activity is crucial for the induction of necroptosis in response to TNFα signaling. On the other hand, RIP1 is known to interact with the C-terminal domain of TICAM-1 and to modulate TLR3 signaling. In macrophages and perhaps tumor cell lines, RIP1/RIP3-mediated necroptotic cell death can ensue the administration of the TLR agonist polyI:C. If this involved the TLR3/TICAM-1 pathway, the innate sensing of viral dsRNA would be linked to cytopathic effects and to persistent inflammation, in turn favoring the release of damage-associated molecular patterns (DAMPs) in the microenvironment. Here, we review accumulating evidence pointing to the involvement of the TLR3/TICAM-1 axis in tumor cell necroptosis and the subsequent release of DAMPs.

Introduction

Cell death is an important process for both development and homeostasis in multicellular organisms. The mode of cell death is closely associated with other biological responses occurring within the host, including inflammation. Cell death has been categorized as apoptotic or necrotic and, until recently, apoptosis had been considered as a synonym of programmed cell death.[1] Caspases are a family of cysteine proteases that mediate apoptotic cell death in response to ligands of death receptors, including tumor necrosis factor α (TNFα), FAS ligand (FASL) and TRAIL, as well as to intracellular damage, upon the induction of pro-apoptotic BH3-only members of the Bcl-2 family. However, it is now clear that apoptosis is not the only cellular mechanism that mediates programmed cell death. Necrotic cell death, which has traditionally been viewed as a form of passive cell death, may also be regulated, and in this case has been called necroptosis or programmed necrosis.[2] Necroptosis may be induced by TNFα receptor 1 (TNFR1) agonists, but also by innate pattern-recognition receptors (PRRs) such as Toll-like receptor (TLR) 3 and TLR4.[1,4] These two TLRs can recruit the adaptor TICAM-1 (also known as TRIF), leading to Type I interferon (IFN) signaling.[3] In line with this notion, the TLR3 ligand polyI:C (a synthetic double-stranded RNA, dsRNA) can activate either apoptosis or necrosis, depending on the cell lines tested. Cell death induced by the TLR3-TICAM-1 axis may therefore be executed through two distinct subroutines.[5] The mechanisms that dictate the cellular decision to undergo apoptosis or necroptosis in response to TLR3 signaling, as well as the mechanisms that mediate the execution of necroptosis, are the subject of intense investigation.

Toll-like receptors and other PRRs harbor the ability to specifically recognize microbal molecules, known as pathogen-associated molecular patterns (PAMPs).[6] PAMPs trigger the maturation of myeloid dendritic cells (mDCs) through the activation of TLR and/or other pathways, eventually eliciting cellular immunity.[7] In mDCs, nucleic acid-recognizing TLRs (i.e., TLR3, TLR7, TLR8 and TLR9) reside in endosomes and sense their ligands only when they are internalized.[8] The uptake of DNA or RNA of microbial origin therefore allows cross-presentation to T cells and the exposure of natural killer (NK) cell-activating ligands. Besides this extrinsic maturation route, it is known that the formation of autophagosomes may deliver cytoplasmic nucleic acids of viral origin to the endosome via autophagy.[9] In either route, TLR signaling links immunological events to pathological cell death.

Recently accumulated evidence suggests that TLRs serve as receptors not only for foreign PAMPs but also for cellular

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Table 1. Host response to nucleic acids and other DAMPs

PAMP/DAMP	Receptors
Microbial nucleic acids(PAMP)	
cytosolic long dsRNA	MDA5
cytosolic 5'-PPP-RNA	RIG-I
endosomal >140 bp dsRNA	TLR3
nonmethylated CpG DNA	TLR9
cytosolic dsDNA	DNA sensors*
Self molecular patterns(DAMP)	
HMGB1	RAGE, TLR2/4
Uric acid	CD14, TLR2/4
HSPs	CD14, TLR2/4,**
S100 proteins	RAGE
Self nucleic acids (DAMP)	
Self DNA	DNA sensors*
Self mRNA	TLR3

*See **Table 2**; ** D40, CD91, Scavenger receptors etc.

Table 2. RNA·DNA recognition molecules in vertebrates

Receptors	Adaptors	Ligands	Induction of Type I IFN
TLR family			
TLR3	TICAM-1	dsRNA, stem RNA	+
TLR7/8	MyD88	ssRNA	+
TLR22	TICAM-1	dsRNA	+
PKR	?	dsRNA	-
RLR family			
RIG-I	MAVS	5'-PPP RNA, dsRNA	+
MDA5	MAVS	dsRNA (long)	+
NLR family			
NALP3	ASC	dsRNA	+
NOD2	MAVS	ssRNA	+
DDX family			
DDX1	TICAM-1	dsRNA	+
DDX21	TICAM-1	dsRNA	+
DHX36	TICAM-1	dsRNA	+
DNA sensors			
TLR9	MyD88	CpG DNA	+
DAI	TBK1	dsDNA	+
Pol3/RIG-I	MAVS	dsDNA	+
IFI16	TBK1	dsDNA	+
DDX41	STING	dsDNA	+
DHX9	MyD88	dsDNA	+
DDX36	MyD88	dsDNA	+
ZAPS	?	dsDNA	+

constituents that are liberated from damaged or necrotic cells.[10] Thus, innate pattern-recognition is not only a mechanism for discriminating pathogens from the host, but also a means for inspecting cellular homeostasis. Molecules that, upon release from damaged/necrotic cells, activate the immune system are called damage-associated molecular patterns (DAMPs).[11] The most popular TLR adaptor MYD88 is known to contain death domains, and some reports have suggested that TLR signaling may be involved in cell death secondary to PAMP/DAMP-stimulation. Necroptotic or damaged cells may thus represent a result of TLR death signaling, and generate a functional complex consisting of sources of DAMPs as well as of the phagocytic response.[11,12]

DAMPs refer to intracellular molecules that acquire inflammation-inducing capacities when released from cells. DAMPs do not belong to the cytokine family but rather resemble PAMP in their functional properties, in particular with regard to mDC and macrophages. The functions of DAMPs may be associated with responses including regeneration and tumorigenesis. During the past 5 years, necroptotic cell death has been closely connected with innate immune responses involving pattern-sensing.[12,13] DAMPs include a large number of cytosolic or nuclear molecules (**Table 1**), as well as, surprisingly, self nucleic acids.[14] This implies that, like viral DNA and RNA, autologous nucleic acids can evoke inflammation. Here, we discuss the importance of the immune modulation induced by nucleic acids and necroptotic host cells.

Necroptosis: Programmed Necrosis Induced by TNFα

TNFα has been reported to induce two different types of cell death, apoptosis and necrosis, in a cell type-specific manner.[15,16] Through TNFR1, TNFα is implicated in NFκB activation and contributes to cell growth in many cancer cell lines. In parallel TNFα-induced hemorrhagic necrosis has been observed in several cancer cell lines, but the molecular mechanisms underlying these differential responses to TNFα remain poorly understood. Recently, several reports have suggested that the formation of a supracomplex containing the receptor-interacting protein kinase 1 (RIP1) and its homolog RIP3 (which has been named "necrosome") is responsible for the switch from apoptosis to necroptosis.[17,18] RIP1 and RIP3 can assemble only in the absence of functional caspase-8, indicating that this enzyme acts as a key protease for blocking the formation of the necrosome.[5,19] Many viral factors, as well as the genomic instability that frequently characterizes tumor cells, can compromise caspase-8 function, thereby facilitating the induction of necroptosis. Hence, TNFα can promote cell death by signaling through its receptors, including TNRF1 and downstream via RIP1/RIP3, although the output of TNFα signaling is ultimately determined by cell type.

Virus-Mediated Necroptosis

It is notable that a necrotic phenotype has been observed in polyI:C-stimulated bone marrow-derived murine macrophages and other cell lines.[13] TICAM-1 and RIP3 are involved in this process, suggesting the implication of the necrosome pathway in dsRNA-mediated cell death.[12,13] It has been reported that viral

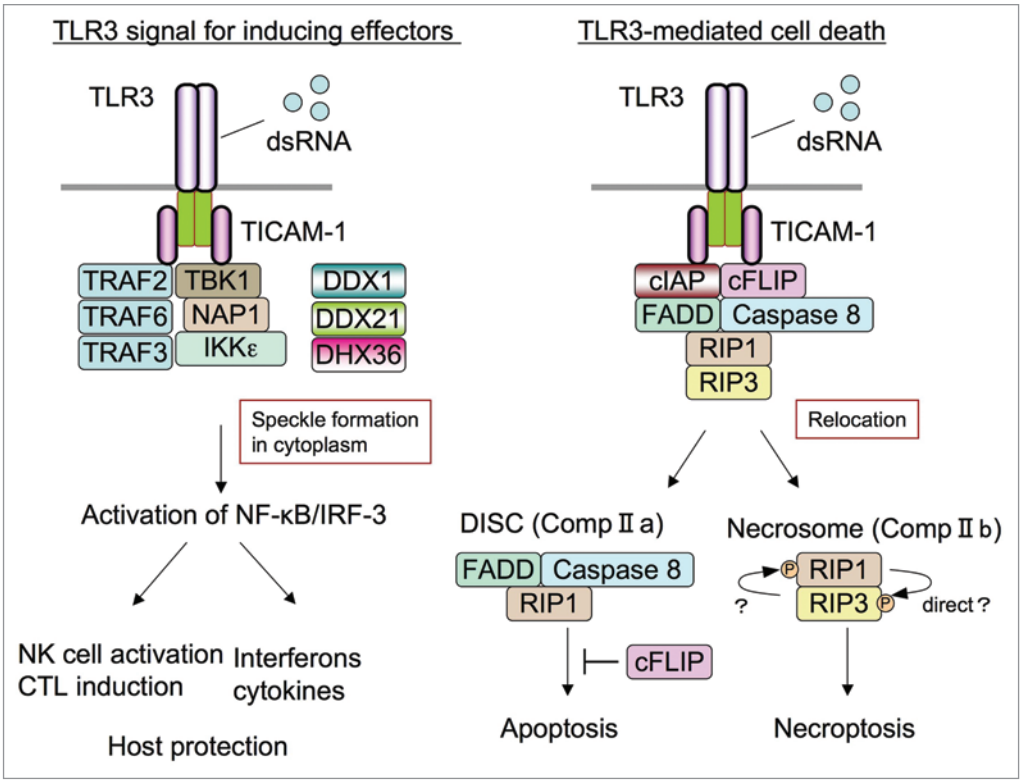

Figure 1. TLR3 signals inducing cell death or effector functions in myeloid cells. Cell survival (left panel) and cell death (right panel) signals are schematically depicted. TICAM-1 assembles in a supramolecular complex around oligomerized Toll-like receptor 3 (TLR3) in the endosome. The complex (named Speckle) then dissociates from TLR3, tranlocating to the cytoplasm. IRF-3 and NFκB are activated by Speckle, leading to their nuclear translocation and induction of Type I interferon (IFN) and inflammatory cytokines, respectively. In dendritic cells (DCs), natural killer (NK) cell-activating ligands and factors for cross-presentation are induced downstream of IRF-3/7 (left panel). In contrast, cell death signaling culminates in apoptosis and/or necrosis depending on downstream signal transducers (right panel). TLR3-dependent apoptosis has been reported in several cancer cell lines,[√] while TLR3-dependent necroptosis has been observed in mouse bone marrow-derived macrophages.[13] These events rely on RIP1/RIP3 activation, similar to those elicited upon ligation of the tumor necrosis factor α receptor 1 (TNFR1). Whether or not the translocation of the TICAM-1 complex is required for the cell death signaling, as well as the mechanisms determining either cytokine secretion or cell death, remain unknown.

dsRNA frequently induces apoptosis in infected cells, a process that in general is known as cytopathic effect.[20] TICAM-1 and RIPs, mainly RIP1, may also be involved in virus-derived necrotic cell death.[5,13] This is relatively rare compared with apoptosis since it occurs only when the viral genome encodes caspase-8 inhibitors.[19] Furthermore, this process requires viral dsRNA to be delivered from the cytosol to the endosomes (where TLR3 is situated) of infected cells. This may happen if the dsRNA is engulfed by autophagosomes, which ensure its transfer to endosomes. The possible involvement of another PRR that sense viral RNA, RIG-I/MDA5, in cell death as induced by viral infection cannot be always ruled out. TNFα can be produced downstream of the TLR3- and RIG-I-mediated RNA-sensing pathways and may induce necrotic cell death. Many RNA viruses trigger cell death,[20] but the factors determining the induction of necroptosis in virus-infected cells remain to be clarified.

DNA viruses can induce necroptosis via another mechanism, which involves the DNA-dependent activator of IFN-regulatory factors (DAI, also known as DLM-1/ZBP1).[21] DAI is a DNA sensor[22] and directly activates RIP3 in the absence of Type I IFN induction.[21] This said, the sensing of DNA in the cytoplasm of virus-infected cells is complex, and it may be that DAI is not the only molecule linked to such a necroptotic response. It is unknown whether RIP3-mediated necroptosis can be induced even if caspase-8 is blocked upon the recognition of viral DNA by DAI or via other mechanisms.[20] In fact, this type of virus-derived necrosis has been reported with DNA viruses that encode caspase inhibitors including vaccinia virus (VV), which encodes B13R/Spi2, poxvirus, encoding CrmA, the Kaposi sarcoma-associated herpesvirus (KSHV), encoding K13 and the molluscum contagiosum virus (MCV), which encodes MC159.[20,23] Generally speaking, the mode of cell death secondary to virus infection differ as a function of viral species. The physiological role of TLR3- and DAI-mediated necroptosis should therefore be analyzed in a virus-specific fashion.

Necroptosis in Inflammation

Apoptosis plays a major role in physiological contexts, while necrosis is very common under pathological conditions.[1] Necroptosis differs from accidental necrosis in its programmed nature, and differs from apoptosis in that necroptosis often stimulates inflammation. When virus-infected cells undergo apoptosis, they are removed by phagocytosis. Viral genomes, be they either

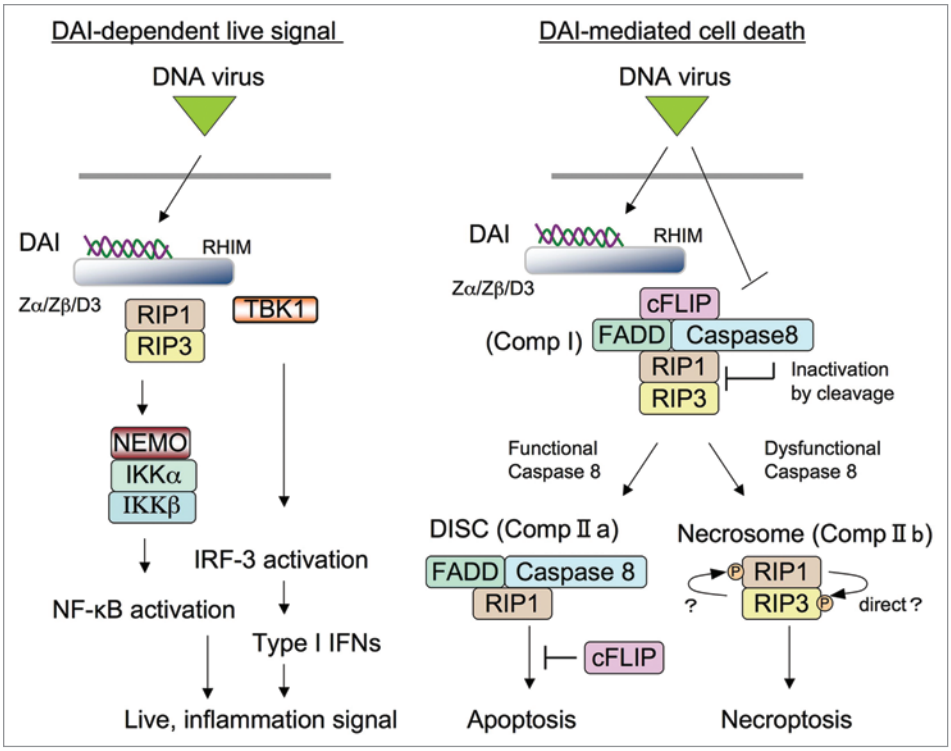

Figure 2. Necroptosis induced by the DAI pathway. Cell survival (left panel) and cell death (right panel) signals transmitted by the DNA-dependent activator of IFN-regulatory factors (DAI) are schematically depicted. Pro-survival signaling involves the activation of IRF-3 and NFκB to support antiviral responses (left panel). Type I IFNs and inflammatory cytokines are the main effectors induced by IRF-3/NFκB activation. In contrast, DAI activates RIP3 to induce necroptosis during viral infection, provided that caspases are inhibited. When viruses express caspase inhibitors, the RIP1/RIP3 necrosome plays a dominant role in the activation of cell death via necroptosis (right panel). If caspase-8 is active, RIP3 should get inactivated and apoptosis should be the dominant phenotype, though this scheme has not yet been experimentally confirmed. The mechanisms determining the choice between these two signaling pathways are unknown.

DNA- or RNA-based, are degraded in infected cells, thus being able neither to stimulate phagocytes including macrophages and DCs, nor to favor the liberation of DAMPs. In contrast, non-apoptotic cell death is accompanied by the release of DAMPs and viral products, resulting in the activation of macrophages,[13] as it occurs during chronic infection, in which viruses produce caspase inhibitors or render infected cells resistant to apoptosis.[24] A typical model of necroptosis evokes two effectors, namely, viral nucleic acids and DAMPs, to modulate immune and bystander cells of the host. In the context of necroptosis, these effectors allow for the amplification of inflammatory responses by myeloid phagocytes (mDCs and macrophages). These cells accumulate in inflammation as induced by persistent viral infection, and mediate the secondary release of cytokines and other biologically active molecules. In addition, viral factors can result in incipient inflammation, as observed in chronic infections by the hepatitis B or C virus.[24] This, in conjunction with viral nucleic acids and DAMPs, may modify the features of the infectious milieu. Further studies are needed to clarify the importance of viral nucleic acids and DAMPs in the context of virus-dependent chronic inflammation, as it may facilitate tumor progression.

Necroptosis and Oncogenesis

Accumulating evidence indicates that pro-inflammatory signals, including those following the activation of NFκB, are crucial for oncogenesis. Moreover, DAMPs have been associated with tumorigenesis as well as with antitumor immune responses.[25,26] Tumor progression is not always accompanied by viral infections, and it remains unclear whether DAMPs released from non-infected tumor cells are sufficient to support tumor growth. It has been reported that self mRNA acts as a TLR3 ligand[14] and that self DNA can stimulate host cell sensors.[22,27] Due to the uncomplete identification and functional characterization of DNA sensors and their signaling pathways, however, it is unknown whether host nucleic acids are potent inducers of inflammation as compared with viral RNA or unmethylated CpG DNA of bacterial origin. Moreover, the role of RNA sensors in the tumor microenvironment has not yet been clarified (**Table 2**).

DAMPs have recently been characterized at the molecular level[11] and representative DAMPs (**Table 1**) include HMGB1,[28] uric acid crystal,[10] S100 proteins,[29] naked actin[30,31] and heat-shock proteins (HSPs).[32] The functional features of DAMPs and the mechanisms whereby they provoke inflammation have been delineated,[11,28,29] and these studies have introduced the concept of "inflammasome" in the field of innate immunity.[33] Caspase-1 is activated upon the administration of NOD-like receptor (NLR) ligands, which include some DAMPs as well as inorganic PAMPs. Active caspase-1, together with the upregulation of the immature variants of IL-1 family proteins that ensues TLR stimulation, accelerates the robust release of IL-1β, IL-18 and IL-33.[34] There are many kinds of NLRs as well as TLRs, and the common pathways (including those centered around the adaptor ASC) can be activated by a variety of cytoplasmic DAMPs and PAMPs.[33,34] The cytoplasmic immature forms of the abovementioned cytokines are activated by limited caspase-1-mediated proteolysis, and then are secreted into the extracellular microenvironment.[34] Hence, IL-1 family proteins require two DAMPs/PAMP signals for their upregulation and activation.[35] Of note, the tumorigenic properties of asbestos and silica are in part attributable to the activation of the inflammasome, leading to the secreton of IL-1 family proteins. However, not all DAMPs operate as inflammasome activators, even in the broad sense of this term.

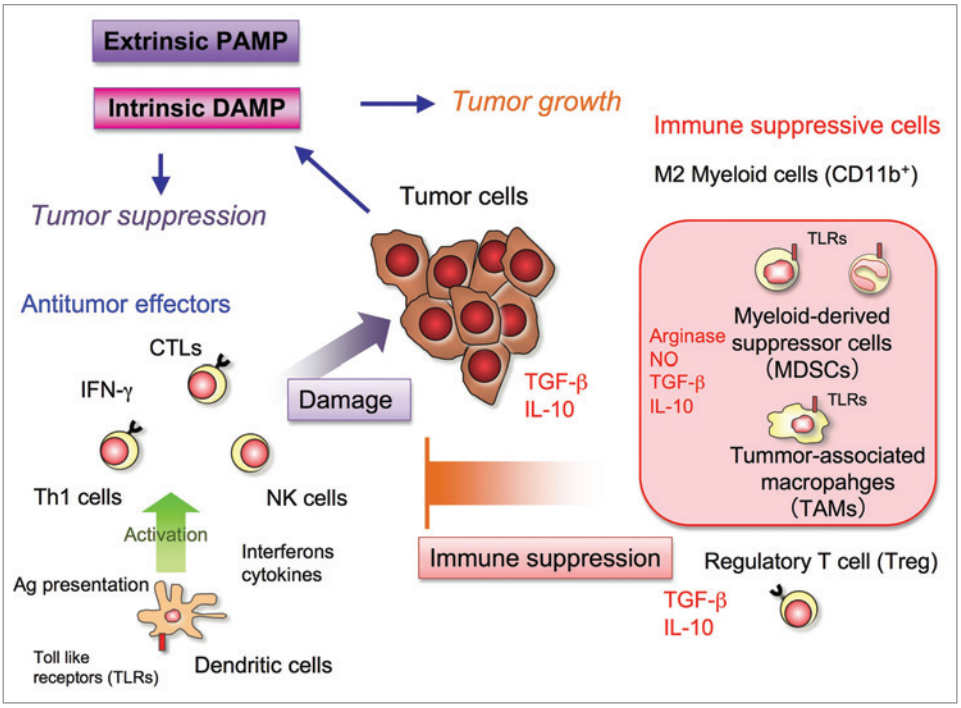

Figure 3. Inflammation provides the microenvironment for infection-related cancer. Immune cells infiltrating the tumor mass may modulate the local microenvironment upon the recognition of pathogen- or damage-associated molecular patterns (PAMP/DAMPs). Cancer cells undergoing necrosis liberate DAMPs and debris containing nucleic acids, which recruit immune cells stimulating an inflammatory response. In some cases, tumors benefit from the inflammatory response, while in other cases they regress following inflammation. The mechanisms determining this switch remain to be clarified.

Immune Response Elicited by the Phagocytosis of Dead Cells

Phagocytosis of dead cells involves not only cell clearance but also the initiation of an immune response. Dead cell antigens are rapidly presented on MHC Class II molecules after internalization by DCs, driving the recruitment and activation of various $CD4^+$ T cell subsets, including Th1, Th2, Th17 and regulatory T cells (Tregs) (**Fig. 1**). In the presence of a second co-stimulatory signal provided by TLRs, working as an adjuvant, DCs cross-present antigens on MHC Class I molecules to induce the proliferation of $CD8^+$ cytotoxic T lymphocytes (CTLs).[36] The presentation of exogenous antigens by DCs is therefore dependent on the presence of PAMPs/DAMPs.[36] Accordingly, necrotic debris appears to result in CTL cross-priming more efficiently than apoptotic bodies. Cross-presentation is enhanced by molecules such as Type I IFN and CD40, and by immune cells including $CD4^+$ T, NK and NKT cells. Hence, the use of adjuvants to affect many cell types of the immune system other than antigen-presenting cells, and a precise evaluation of the total cross-priming activity appear to be indispensable for the development of efficient adjuvant therapies.

The TLR3/TICAM-1 axis is best known as an inducer of cross-presentation in vivo.[37] The cross-presentation activity of the TLR3 ligands polyI:C and viral dsRNA was first described by Schulz et al. in 2005.[38] While the potency of polyI:C as an adjuvant has been reported by Steinman and colleagues,[37,39] the precise identity of the DAMPs participating in cross-presentation and possessing latent cross-priming (CTL-inducing) capacities has not yet been determined.

It is known that phagocytosis induces functional changes in mDCs and macrophages (**Fig. 2**): phagocytes are skewed toward a regulatory phenotype accompanied by the production of IL-10 and TGFβ during the phagocytosis of apoptotic cell debris, even in the presence of PAMP.[40,41] This suggests that material that cannot be taken up exerts different effects on mDCs than internalizable material during their phagocytic interactions. Phagocytes undergo cytoskeletal rearrangement when they take up cell debris, involving cell adhesion molecules that accelerate the interaction between the phagocyte membrane and cell debris. The opsonization of dead cells further enhances phagocytosis as well as the induction of an immune outcome.[42] Complement-mediated opsonization of dead cells strongly alters the functional properties of mDCs and macrophages.[43] Yet, it has been impossible to discriminate apoptotic and necroptotic cells based on this.[44] Thus, the mechanisms whereby necroptotic cells initiate an immune response upon phagocytosis by mDCs and macrophages, compared with apoptotic cells, remain largely uncharacterized. Elucidating the role of necroptotic cells and DAMPs as adjuvants for NK-cell activation and antigen presentation is highly relevant for antitumor therapy. Since the phagocytosis of dead cells by mDCs usually leads to the generation of tolerogenic mDCs, additional adjuvants appear to be required for mDCs to present tumor antigens in an immunogenic fashion, leading to the induction of an effective immune response against cancer.

Termination of Inflammation

Inflammation often drives tissue repair and regeneration, and the microenvironment formed during inflammation serves a basis for assembling cells that initiate tissue development and reorganization (**Fig. 3**). The pro-inflammatory microenvironment facilitates cell growth as well as genome instability, thus being prone to the accumulation of cells with multiple mutations. Furthermore, incipient inflammation compromises the immune system so that the abnormal proliferation of transformed cells is tolerated. Thus, malignant cells build up a tissue that involves tumor-associated macrophages serving a scaffold for invasion and metastasis.[45] In this context, a region harboring DAMP-mediated persistent inflammation provides a perfect nest for tumor progression (**Fig. 3**). Therapeutics for suppressing inflammation, such as aspirin, may constitute an immune therapy irrespective of the presence of infection.[46] We surmise that two types of inflammation exist, namely tumor-supporting and tumor-suppressing, implying that inflammation is a complex phenomenon consisting of multiple distinct aspects. We have shown that some adjuvants can induce tumor-suppressing inflammation, thereby limiting tumor proliferation by DAMPs.[47] The adjuvant-induced switch of cell death/inflammation signals to an antitumor outcome is an intriguing approach for cancer therapy, particularly in view of the fact that the mechanisms of adjuvant signaling are being increasingly characterized at the molecular level.[48,49] The clarification of the role of adjuvant signaling in compromising tumor progression will lead to the discovery of non-toxic synthetic tumor-regressing molecules with potential as novel anticancer therapeutics.[50]

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