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 necosome) 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.3 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 microbial 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.9 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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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. 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. 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 TNFR1 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. TICAM-1 and RIP3 are involved in this process, suggesting the implication of the necrosome pathway in dsRNA-mediated cell death. It has been reported that viral constituents that are liberated from damaged or necrotic cells. 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). 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.

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. DAMPs include a large number of cytosolic or nuclear molecules (Table 1), as well as, surprisingly, self nucleic acids. 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. 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. 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. 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 TNFR1 and downstream via RIP1/RIP3, although the output of TNFα signaling is ultimately determined by cell type.

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**Table 1. Host response to nucleic acids and other DAMPs**

| PAMP/DAMP       | Receptors          |
|-----------------|--------------------|
| Microbial nucleic acids (PAMP) | MD2, MD2L, TLR2, TLR3 |
| Cytosolic long dsRNA | MDA5, RIG-1       |
| Cytosolic 5’-PPP RNA | RIG-1              |
| Endosomal >140 bp dsRNA | TLR3              |
| Nonmethylated CpG DNA | TLR9, DNA sensors* |
| Cytosolic dsDNA | DNA sensors*       |
| Self molecular patterns (DAMP) | RAGE, TLR2/4     |
| HMGB1 | RAGE, TLR2/4 |
| Uric acid | CD14, TLR2/4 |
| HSPs | CD14, TLR2/4, ** |
| S100 proteins | RAGE              |
| Self nucleic acids (DAMP) | DNA sensors* |
| 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 |
|-----------|----------|---------|------------------------|
| TLR3      | TICAM-1  | dsRNA, stem RNA | +                       |
| TLR7/8    | MyD88    | ssRNA   | +                      |
| TLR22     | TICAM-1  | dsRNA   | +                      |
| PKR       | ?        | dsRNA   | -                      |
| RIG-I     | MAVS     | 5’-PPP RNA, dsRNA | +                     |
| MDA5      | MAVS     | dsRNA (long) | +                      |
| NALP3     | ASC      | dsRNA   | +                      |
| NOD2      | MAVS     | ssRNA   | +                      |
| DDX family | DNA sensors |
| TLR9      | MyD88    | CpG DNA | +                      |
| DAI       | TBK1     | dsDNA   | +                      |
| PoI3/RIg-I | MAVS     | dsDNA   | +                      |
| IFF16     | TBK1     | dsDNA   | +                      |
| DDX41     | STING    | dsDNA   | +                      |
| DHX9      | MyD88    | dsDNA   | +                      |
| DHX36     | MyD88    | dsDNA   | +                      |
| ZAP5      | ?        | dsDNA   | +                      |

*See Table 2; ** D40, CD91, Scavenger receptors etc.
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. 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. 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. 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
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 ligand14 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 level11 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 secretion of IL-1 family proteins. However, not all DAMPs operate as inflammasome activators, even in the broad sense of this term.
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. 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. 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. Complement-mediated opsonization of dead cells strongly alters the functional properties of mDCs and macrophages. Thus, it has been impossible to discriminate apoptotic and necrotic cells based on this. The mechanisms whereby necrotic cells initiate an immune response upon phagocytosis by mDCs and macrophages, compared with apoptotic cells, remain largely uncharacterized.

Elucidating the role of necrotic 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 during inflammation serves as 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. In this context, a region harboring DAMP-mediated tumor-associated macrophages serving a scaffold for invasion and metastasis. The clarification of the mechanisms of adjuvant signaling is being increased to the molecular level. 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 antinecrotic therapeutics.

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