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Necroptosis and its role in inflammation

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Regulated cell death has essential functions in development and in adult tissue homeostasis. Necroptosis is a newly discovered pathway of regulated necrosis that requires the proteins RIPK3 and MLKL and is induced by death receptors, interferons, toll-like receptors, intracellular RNA and DNA sensors, and probably other mediators. RIPK1 has important kinase-dependent and scaffolding functions that inhibit or trigger necroptosis and apoptosis. Mouse-model studies have revealed important functions for necroptosis in inflammation and suggested that it could be implicated in the pathogenesis of many human inflammatory diseases. We discuss the mechanisms regulating necroptosis and its potential role in inflammation and disease.

Cell death is intricately connected with life in multicellular organisms. The balance between cell death, proliferation and differentiation is crucial for the maintenance of tissue homeostasis throughout life. Programmed cell death (PCD) is essential for many physiological processes, including the shaping of developing organs, epithelial cell renewal and lymphocyte selection. However, cell death that is not developmentally programmed is a sign of stress, injury or infection and is linked to tissue damage and disease pathogenesis. Inflammation is a reaction of the immune system induced in response to infection or tissue injury that is essential for efficient host defence and tissue repair. However, uncontrolled excessive and/or prolonged inflammatory responses cause tissue damage and contribute to the pathogenesis of acute and chronic inflammatory diseases. Although recognized more than 150 years ago as a central component of inflamed tissues, the potential role of cell death as an active component that contributes to tissue homeostasis, inflammation and disease pathogenesis has only recently gained attention[1]. Many of the receptors involved in inflammation can also induce cell death in addition to their potent capacity to drive inflammatory cytokine expression. Recent findings suggest that in addition to the transcriptional regulation of inflammatory genes, the cell-death-inducing properties of these receptors also crucially contribute to inflammation.

For many years apoptosis was considered to be the only form of regulated cell death (RCD), whereas necrosis was seen as an unregulated accidental cell death (ACD) process. Genetic, biochemical and functional evidence, and the discovery of specific chemical inhibitors of necrosis have redefined this process as a molecularly controlled regulated form of cell death[2]. Regulated necrosis includes several cell-death modalities such as necroptosis, parthanatos, ferroptosis or oxytosis, mitochondrial permeability transition (MPT)-dependent necrosis, pyroptosis and pyronecrosis, and cell death associated with the release of (neutrophil) extracellular traps, which is described as NETosis or ETosis (Box 1). Pyroptosis and necroptosis are the best characterized forms of regulated necrosis. Pyroptosis is an inflammatory form of cell death induced by inflammasome activation and has important functions in host defence and inflammation[3]. Necroptosis, mediated by receptor interacting protein kinase-3 (RIPK3) and its substrate mixed lineage kinase like (MLKL), is the best-characterized form of regulated necrosis. Recent studies have provided new and exciting insights into the mechanisms controlling necroptosis and its *in vivo* relevance and suggested that necroptosis could have important functions in the pathogenesis of several human diseases. This Review discusses the mechanisms controlling regulated necrosis and its physiological relevance for tissue homeostasis and inflammation, focusing particularly on necroptosis.

Mechanisms and regulation of necroptosis

Much of our knowledge of necroptosis comes from studies of tumour necrosis factor (TNF) signalling. TNF is a pleiotropic cytokine that has a key role in inflammation induced by infection or tissue injury. TNF signalling, primarily through TNF receptor 1 (TNFR1), induces the expression of many genes that regulate inflammation, but under some conditions TNF is also a potent inducer of cell death[4]. Despite its name and early evidence that TNF also induces caspase-independent cell death[5] by a mechanism involving RIPK1 (ref. 6), for many years most studies of TNF-induced cell death focused on apoptosis. The identification of necrostatins as necrosis inhibitors targeting RIPK1 provided evidence that TNF-induced necrosis is a kinase-regulated process, and it was dubbed necroptosis[7,8]. A key step in unravelling the necroptosis pathway was the discovery of RIPK3 as an essential regulator of TNF-induced necrosis[9–11]. The RIP homotypic interaction motif (RHIM) on RIPK3 and RIPK1 allows their interaction and is required for necroptosis induction[9,10]. The necrosome was defined as the complex containing RIPK1 and RIPK3 that was involved in the initiation of necroptosis[12]. RIPK1 and RIPK3 were later shown to form large amyloid-like structures[13], although it is unclear whether these represent a real signalling platform or a post-event accumulation of these two interacting kinases. The important physiological role of necroptosis was highlighted by a number of genetic studies showing that caspase-8 or Fas-associated protein with death domain (FADD) deficiency cause embryonic lethality and trigger inflammation *in vivo* by sensitizing cells to RIPK3-mediated necroptosis[14–18].

Execution of necroptosis

The identification of MLKL pseudokinase as a substrate of RIPK3 required for necroptosis sheds light on the mechanisms involved in executing necrotic cell death downstream of RIPK3 (refs 19, 20). Although initially MLKL was proposed to be associated with the regulation of mitochondrial fission through the proteins phosphoglycerate mutase family member 5 (PGAM5) and dynamin-related protein 1 (DRP1)[21], the causality of mitochondrial fission in necroptosis has been challenged[22,23].

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BOX 1

Regulated necrosis pathways

Several types of regulated necrosis exist in addition to necroptosis (reviewed in ref. 2). Parthanatos involves hyperactivation of poly(ADP-ribose) (PAR) polymerase 1 (PARP1), an enzyme originally characterized by its role in DNA-repair mechanisms following a DNA-damage response. The massive PARylation of target proteins leads to cellular depletion of NAD^+ (and consequently of ATP), resulting in a bioenergetic crisis and a form of regulated necrosis. The term ferroptosis was recently coined to describe a type of regulated necrosis that is characterized by iron-dependent production of reactive oxygen species (ROS), which can be blocked by the iron chelator desferrioxamine (DFO). It is elicited by pharmacological inhibition of the antiporter system x_c^-, which exchanges extracellular cystine for intracellular glutamate. Glutamate toxicity on neurons works by blocking the same antiporter system and has been termed oxytosis. Mitochondrial permeability transition (MPT)-mediated regulated necrosis is another cell-death modality. Cyclophilin D (CYPD) is the sole genetically confirmed component of the permeability transition pore complex (PTPC), which is implicated in MPT-mediated regulated necrosis. Pyroptosis and pyronecrosis are highly inflammatory cell-death modalities characterized by cellular swelling and plasma membrane permeabilization. Pyroptosis occurs after canonical and non-canonical inflammasome stimulation, leading to caspase-1 and caspase-11 activation, respectively. Some reports distinguish pyronecrosis as a cell-death modality occurring during infection that does not depend on caspase-1 or caspase-11, but requires cathepsin B release following lysosomal membrane permeabilization. NETosis/ETosis (NET is neutrophil extracellular trap; ET is extracelluar trap) is also implicated in protection against microbial and viral infection. This modality occurs in neutrophils, eosinophils, mast cells and macrophages. It is associated with chromatin decondensation and release of NETs, which are composed of DNA, chromatin and histones, and allow immune cells to immobilize and kill infectious agents. Besides apoptosis and the various forms of regulated necrosis, a third modality is often put forward: autophagic cell death. The name is slightly misleading because autophagy is in the first instance a crucial mechanism in cellular homeostasis and adaptive responses, however, it can also become a cell-death mechanism. Autophagic cell death is biochemically characterized by markers of autophagy such as lipidation of LC3 (microtubule-associated protein 1 light chain 3) and the degradation of p62, a ubiquitin-binding scaffold protein, and blocked by genetically or pharmacologically targeting the members of the autophagy pathway. Inhibitors that interfere with cell death modalities are shown in Table 1.

Table 1 | Inhibitors that interfere with cell-death modalities

Apoptosis		Regulated necrosis					
Morphology							
Cytoplasmic shrinkage Chromatin condensation (pyknosis) Nuclear fragmentation (karyorrhexis) Blebbing of the plasma membrane Shedding of apoptotic bodies		Increasingly translucent cytoplasm Swelling of organelles; lysosomal membrane permeabilization Increased cell volume (oncosis) Permeabilization of the plasma membrane Mild chromatin condensation; nuclei remain intact					Loss of nuclear integrity Massive chromatin decondensation
Death modality							
Intrinsic apoptosis	Extrinsic apoptosis	Necroptosis	Ferroptosis	MPT-mediated regulated necrosis	Parthanatos	Pyroptosis	NETosis/ETosis
Death regulatory factors							
BID, BAX/BAK Cytochrome *c* APAF1 CASP9	RIPK1* RIPK3† FADD CASP8	RIPK1† RIPK1* RIPK3*	GPX4	CYPD	PARP1	Inflammasome Canonical NLR–ASC–CASP1 Non-canonical (sensor/adaptor?)-CASP11	NOX
Death execution factors							
CASP3 CASP7		MLKL ion channels	GSH decrease Fe^{2+}	Ca^{2+} increase	NAD^+ increase ATP increase	ROS increase	
		Lipid peroxidation, energetic catastrophe, and lysosomal and plasma-membrane permeabilization					
Synthetic inhibitor (factor they inhibit)							
zVAD-fmk (CASP) q-VD-Oph (CASP)		NEC1 and NEC1s GSK Cpd27 (RIPK1) GSK843 (RIPK3) GSK872 (RIPK3) NSA (hMLKL) GW906742X (MLKL)	Fer-1 DFO	SfA (CYPD) CsA (CYPD)	3-AB (PARP1) PJ-34 (PARP1)	VX-740 (CASP1) VX-765 (CASP1)	DPI (NOX) GKT137831 (NOX1 and NOX4)
Physiology							
Controlling cell numbers during embryogenesis and homeostasis Immune regulation Pathogen defence		Embryogenesis? Homeostasis? Inflammation IR-injury Thrombosis Neurodegen.	Glu toxicity IR-injury Neurodeg. Transplant.	IR-injury Thrombosis Transplant.	DNA damage Neurodegen.	Inflammation Pathogen defence	Inflammation Extracellular trap formation Pathogen defence

3-AB, 3-aminobenzamide; APAF1, apoptotic protease-activating factor 1; BAK, apoptosis regulator BAK; BAX, apoptosis regulator BAX; BID, BH3-interacting domain death agonist; CASP, caspase; Cpd27, compound 27 or fluro[2,3-d]pyrimidine 27; CsA: cyclosporine A; DPI, diphenylene iodonium; FADD, Fas-associated death domain; Fer-1, ferrostatin-1; Glu, glutamate; GSH, reduced glutathione; GPX4, glutathione peroxidase 4; MLKL, mixed lineage kinase like; NSA, necrosulfonamide MLKL inhibitor; NEC1, necrostatin-1; NEC1s, necrostatin-1s; Neurodegen., neurodegeneration; NLR, NOD-like receptor; NOX, NADPH oxidase; RIPK, receptor-interacting serine/threonine-protein kinase; qVD-Oph, quinolyl-Val-Asp-Oph; SfA, sanglifehrin a; Transplant., transplantation; TRPM7, transient receptor potential cation channel subfamily M member 7; zVAD-fmk, carbobenzoxy-valyl-alanyl-aspartyl-[*O*-methyl]-fluoromethylketone. *Kinase active form, †kinase inactive form.

Moreover, depletion of mitochondria by parkin RBR E3 ubiquitin protein ligase (PARK2) and/or carbonyl cyanide *m*-chlorophenylhydrazone (CCCP)-induced mitophagy activation did not prevent TNF-induced necroptosis, arguing against mitochondrial reactive oxygen species (ROS) as a final executioner mechanism[24]. Evidence suggests that RHIM-dependent oligomerization and intramolecular autophosphorylation of RIPK3 results in the recruitment and phosphorylation of MLKL[25,26], which leads to a conformational change in the pseudokinase domain leading to the exposure of the 4-helical bundle domain[22]. Two non-exclusive models are proposed for the executioner mechanism of MLKL: one as a platform at the plasma membrane for the recruitment of Ca^{2+} or Na^{+} ion channels[27,28], and one as a direct pore-forming complex that is recruited through binding of the amino-terminus of the 4-helical bundle domain of MLKL to negatively charged phosphatidylinositolphosphates[29–31].

Mechanisms regulating TNFR1–induced necroptosis

In most cells, TNFR1 stimulation is not cytotoxic and induces direct pro-inflammatory signalling through the formation of a membrane-associated protein complex (complex I) (Fig. 1a)[4]. In sensitized cells, TNFR1 induces apoptosis through cytosolic complexes IIa and IIb, and, in particular conditions or cells, necroptosis by the necrosome can be executed. TNF-induced signalling towards necroptosis is prevented by several brakes on RIPK1, as the presence or absence of RIPK1 and its post-translational modifications such as ubiquitylation and phosphorylation are imperative for the biological outcome. RIPK1 comes in several flavours in the four different complexes (I, IIa, IIb and IIc/necrosome), and these are induced in a dynamic way after TNF binding to TNFR1. Ligation of TNFR1 induces the formation of complex I, comprising TNFR1-associated death domain protein (TRADD), RIPK1 and the E3 ubiquitin ligases TNF-receptor-associated factor 2 (TRAF2), the cellular inhibitors of apoptosis (cIAP1 or cIAP2) and the linear ubiquitin chain assembly complex (LUBAC, consisting of haem-oxidized IRP2 ubiquitin ligase-1, HOIL-1L; HOIL-1-interacting protein, HOIP; and SHANK-associated RH domain-interacting protein, SHARPIN). TRADD is required for the recruitment of TRAF2, cIAP1/2 and LUBAC, and for the ubiquitylation of RIPK1 with K63 and linear chains within complex I. This complex favours proinflammatory signalling and prevents cell death through the recruitment of the TGF-activated kinase 1 (TAK1)–TAK1-binding protein (TAB) complex and of the IκB kinase (IKK) complex consisting of IKK1, IKK2 and NF-κB essential modulator (NEMO), leading to NF-κB and mitogen-activated protein kinase (MAPK) activation (reviewed in ref. 32).

Destabilization of complex I leads to the formation of a second cytosolic complex IIa, consisting of TRADD, FADD and caspase-8, which signals towards apoptosis[33–35]. In conditions such as TNF stimulation in the presence of IAP inhibitors (Smac mimetics) or knockdown of IAPs[35], TAK1 inhibition or knockdown[36], NEMO knockdown[37] or Pellino knockdown[38], a cytosolic complex IIb forms that is composed of RIPK1, RIPK3, FADD and caspase-8. This composition resembles the cytosolic ripoptosome complex, which forms independently of TNF, Fas ligand (FASL) or TNF-related apoptosis-inducing ligand (TRAIL) following the loss or inhibition of IAPs[39,40]. This complex IIb favours RIPK1-kinase-activity-dependent apoptosis, however when the levels of RIPK3 and MLKL are sufficiently high and caspase-8 activity is reduced, blocked or absent, complex IIb may evolve to form the necrosome. Caspase-8 is reported to inhibit necroptosis by cleaving RIPK1 (ref. 41) and RIPK3 (ref. 42), as well as CYLD[43], a deubiquitinating enzyme that removes ubiquitin chains from RIPK1 and contributes to necroptosis *in vitro* and *in vivo*[17,18,44], but the contribution of the cleavage of each of these proteins to necroptosis inhibition remains unclear. Expression levels of cellular FADD-like interleukin (IL)-1β-converting enzyme (FLICE)-inhibitory protein ($FLIP_L$) are crucial in the control of necroptosis and apoptosis. The presence of high levels of $FLIP_L$ leads to heteromeric caspase-8–$FLIP_L$ in complex II, which is catalytically active but does not lead to full processing of p10–p20 caspase-8 (and consecutive apoptosis) and alters the substrate specificity[45]. Heteromeric caspase-8–$FLIP_L$ prevents complex IIa-dependent apoptosis. The precise mechanism of the paradoxical prosurvival role of caspase-8 in inhibiting necroptosis is not completely clear, but requires the presence of $FLIP_L$[15], catalytically active caspase-8 (ref. 46), but not its proteolytic processing[47] and suppression of RIPK1–RIPK3 activation[15]. Thus, FADD–caspase-8–$FLIP_L$-mediated control of complex IIb is a second important break preventing the induction of necroptosis. The existence of these brakes (IAPs, TAK1, caspase-8 and $FLIP_L$) explains why in most studies of necroptosis they are blocked by a combination of Smac mimetics or TAK1 inhibitor (inhibiting brake 1) and zVAD-fmk (carbobenzoxy-valyl-alanyl-aspartyl-[*O*-methyl]-fluoromethylketone, inhibiting brake 2), facilitating the efficient formation of the necrosome as long as RIPK3 and MLKL are sufficiently expressed. Most cells are not sensitive to TNF-induced cell death. Adding the protein synthesis inhibitor cycloheximide renders cells sensitive for complex IIa-mediated apoptosis[35], and, when caspase-8 is inhibited, to necrosome-mediated necroptosis[9], but the precise protective proteins targeted by cycloheximide remain unclear.

Other stimuli inducing necroptosis

There are six human death receptors (DRs) in the TNF superfamily: TNFR1, FAS (also known as CD95 or APO-1), DR3 (also known as TRAMP or APO-3), TRAILR1 (also known as DR4), TRAILR2 (also known as DR5, TRICK or KILLER), and DR6 (ref. 48). In contrast to TNFR1 signalling, in which the prosurvival signalling complex forms first and the death-inducing complexes form subsequently in sensitized cells (see earlier), binding of FASL to FAS, or of TRAIL to TRAILR1 or TRAILR2 induces the assembly of a membrane-associated death-inducing signalling complex (DISC) through the adaptor protein FADD, leading to recruitment and activation of caspase-8 and consecutive apoptosis (Fig. 1b). Under particular conditions such as the absence of cIAPs, which favours the recruitment of RIPK1 to Fas[49] and the formation of a cytosolic ripoptosome complex IIb[39], these ligands also mediate necroptosis when caspase-8 is blocked.

Cellular stress, damage and infection are sensed by several receptors such as Toll-like receptors (TLRs), nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs), retinoic acid-inducible gene I (RIG-I)-like receptors (RGRs), ripoptosome and protein kinase R (PKR) complexes. Some of these receptors have also been demonstrated to induce necroptosis. Activated TLR3 forms an endosome platform recruiting a cytosolic adaptor, Toll/IL-1 receptor (TIR) domain-containing adaptor protein inducing interferon (IFN)-β (TRIF), which is involved in NF-κB activation and induction of type I IFNs[50]. Besides signalling through the myeloid differentiation primary response 88 (MyD88) adaptor, TLR4 also signals through TRIF[50]. TRIF contains a RHIM-domain, allowing interaction with RIPK1 and RIPK3. Lipopolysaccharide (LPS)–TLR4 or polyinosine–polycytidylic acid (poly(I:C))–TLR3 stimulation in the presence of zVAD-fmk induce TRIF-mediated necroptosis that depends on RIPK3 and MLKL, but may also proceed in the absence of RIPK1 (refs 51, 52). However, inhibition of RIPK1 kinase activity by necrostatin-1 (Nec1)[51,52] or in knock-in macrophages expressing kinase-inactive $RIPK1^{D138N}$ (ref. 53) prevented LPS–poly(I:C)–zVAD-fmk-induced necroptosis. These results suggest that RIPK1 is probably recruited to the TRIF-signalling complex and when its kinase activity is inhibited it acts as a blocker of TLR3- or TLR4-induced RIPK3 activation, although TRIF may also directly recruit and activate RIPK3 in the absence of RIPK1 (Fig. 1c)[51,53]. Another RHIM-domain-containing protein is the cytosolic DNA-dependent activator of IFN regulatory factors (DAI; also known as Z-DNA binding protein 1, ZBP1), which in response to viral double-stranded DNA induces NF-κB activation and type I IFNs, but also RIPK3-mediated necroptosis[54] (Fig. 1d).

In the absence of caspase-8 and FADD, both type I and type II IFNs were recently shown to induce RIPK1–RIPK3 necrosome formation in mouse embryonic fibroblasts (MEFs) that depends on the viral RNA responsive protein kinase R, which interacts with RIPK1 (ref. 55). However, independent experiments in PKR-deficient macrophages do not

support an essential role for this kinase in IFN-α receptor type I-mediated necroptosis. Interestingly, depending on the cell type, autocrine loops may be implicated in the regulation of necroptosis. In macrophages, LPS–TLR4-, TNF–TNFR1- and poly(I:C)–TLR3-mediated necroptosis requires IFN-α receptor type I signalling, suggesting an autocrine loop of type I IFNs[56] (Fig. 1e). Moreover, stimulation of Pam3CysK–TLR2, Flagellin–TLR5, CpG–TLR9 (ref. 52) or etoposide administration[57] leads to an auto- or paracrine TNF-production loop, which in conditions of IAP inhibition or caspase-8 depletion sensitizes cells to TNF-induced death by RIPK1-dependent necroptosis. Therefore, regulation of necroptosis could be a crucial factor in controlling responses to infection, DNA damage and inflammation.

RIPK1 determines cell survival or death

Genetic studies have demonstrated the pivotal role of RIPK1 in cell survival and death. RIPK1 deficiency causes perinatal lethality[34], which is fully prevented by the combined absence of caspase-8 and RIPK3 (refs 58–60). Caspase-8 deficiency did not prolong survival of *Ripk1*$^{-/-}$ mouse neonates, although it prevented apoptosis in many tissues including the intestine, thymus, liver and lung, whereas RIPK3 or MLKL

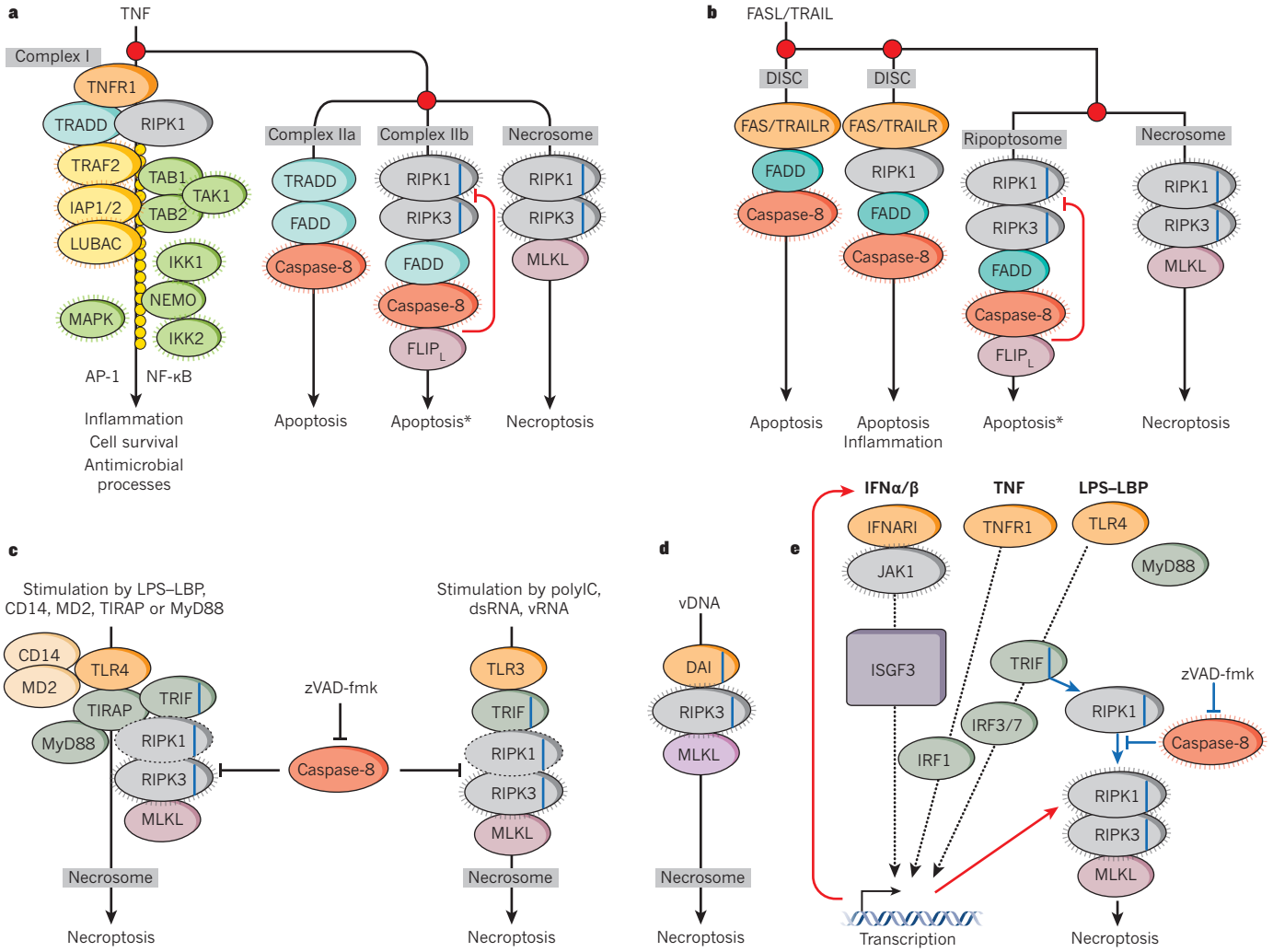

Figure 1 | Pathways leading to necroptosis. **a**, The binding of TNF to its receptor (TNFR1) induces the formation of the receptor-bound complex I (made up of TRADD, RIPK1, TRAF2, IAP1, IAP2 and LUBAC) that activates NF-κB and AP-1 and mitogen-activated protein kinase (MAPK) signalling by the ubiquitin-chain-dependent recruitment of the IKK (made up of NEMO, IKK1 and IKK2) and TAB/TAK-1 complexes. In CHX-treated cells, the cytoplasmic complex IIa (made up of TRADD, FADD and caspase-8) forms, which leads to caspase-8-mediated apoptosis independent of RIPK1. In cells treated with IAP antagonists, TAK1 inhibition or knockdown, or NEMO knockdown the cytoplasmic complex IIb (ripoptosome-like) forms (made up of RIPK1, RIPK3, FADD and caspase-8), resulting in caspase-8-dependent apoptosis, which depends on RIPK1 kinase activity and an RIPK3 platform (apoptosis*). FLIP$_L$ keeps caspase-8 in a heteromeric complex that controls RIPK1 and RIPK3 levels by proteolysis. When caspase-8 is inhibited complex IIc or the necrosome (made up of RIPK1, RIPK3 and MLKL) is formed, inducing RIPK1 kinase activity and RIPK3-kinase-activity-dependent necroptosis. **b**, Stimulation of Fas or TRAILR induces the formation of the receptor-bound death-inducing signalling complex (DISC) that triggers caspase-8-mediated apoptosis independent of RIPK1. In the presence of IAP antagonists, Fas and TRAILR stimulation results in the recruitment of RIPK1 generating a complex similar to complex I in (a), which can progress either in the formation of the cytosolic ripoptosome that induces caspase-8-mediated apoptosis that depends on RIPK1 kinase activity or, when caspase-8 activity is inhibited, RIPK1-kinase-activity-dependent necroptosis. **c**, TLR4 or TLR3 stimulation triggers formation of the necrosome through the RHIM-containing adapter TRIF, resulting in RIPK3-dependent necroptosis in which the role of RIPK1 depends on the cellular context. **d**, DNA-dependent activator of IFN regulatory factors (DAI) recognizes viral double-stranded DNA and through its RHIM domain recruits RIPK3 and induces the formation of the necrosome without RIPK1 and triggers RIPK1-independent RIPK3-kinase-activity-dependent necroptosis. **e**, In bone-marrow-derived macrophages type I interferons (IFNα and β) induce necroptosis through their cognate receptor, IFNARI, leading to activation of JAK1. These cause the formation of the ISGF3 complex (STAT1–STAT2–IRF9), which in a transcription-dependent way causes induction and activation of the necrosome complex. TNF (IRF1) and lipopolysaccharide (LPS; IRF3/7) signalling can induce an autocrine loop for IFNβ. In macrophages, IFNARI is required for TNF- and LPS-induced necroptosis. In the presence of zVAD-fmk, sustained activation of the necrosome eventually results in necroptosis. The 'glowing' symbols represent enzymatic activity. Homotypic interaction motif (RHIM domain) is indicated by the blue line.

deficiency ameliorated systemic inflammation, prevented epidermal hyperplasia and marginally prolonged the survival of *Ripk1*$^{-/-}$ pups, revealing distinct functions for apoptosis and necroptosis in the multiorgan pathology and perinatal death of *Ripk1*$^{-/-}$ mice[58,60]. In addition, intestinal epithelial cell (IEC)-specific knockout of *Ripk1* caused severe lethal intestinal pathology due to FADD–caspase-8-mediated apoptosis of IECs induced primarily, but not exclusively, by TNF[61,62]. Importantly, raising IEC-specific *Ripk1*-knockout mice or *Ripk1*$^{-/-}$*Ripk3*$^{-/-}$ mice under germ-free conditions did not prevent apoptosis of IECs and intestinal pathology, demonstrating that the microbiota is not essential to trigger the death of RIPK1-deficient epithelial cells[60,61], although antibiotic studies suggested that bacteria may aggravate the phenotype[62]. Epidermal-keratinocyte-specific *Ripk1* knockout, however, triggered severe skin inflammation by sensitizing keratinocytes to RIPK3–MLKL-dependent necroptosis, revealing a novel role for RIPK1 as an inhibitor of necroptosis in keratinocytes[61]. Furthermore, RIPK1 deficiency in haematopoietic cells caused bone marrow failure owing to haematopoietic cell apoptosis and necroptosis[60,63]. Collectively, these studies demonstrated the essential role of RIPK1 in preventing apoptosis and necroptosis *in vivo*. Knock-in mice expressing kinase inactive *Ripk1* alleles did not show postnatal lethality or tissue pathology[53,59,61,62,64,65], demonstrating that RIPK1 mediates cell survival by kinase-independent scaffolding functions. The pro-survival functions of RIPK1 that are important for preventing IEC apoptosis seem to be independent of NF-κB activation[61,62]. Although the exact mechanisms by which RIPK1 prevents cell death remain to be elucidated, RIPK1 deficiency but not lack of its kinase activity resulted in degradation of cIAP1, $FLIP_L$ and TRAF2 *in vivo* and in response to TNF stimulation *in vitro*, suggesting that kinase-independent RIPK1 scaffolding properties are important to sustain pro-survival signalling platforms[61,62,66].

RIPK1 kinase activity is not required for activation of NF-κB and MAPK signalling but mediates cell death by either apoptosis or necroptosis. On the one hand, lack of RIPK1 kinase activity partly inhibited but did not prevent RIPK3-mediated necroptosis of FADD-deficient IECs and keratinocytes, demonstrating the existence of RIPK1-kinase-activity-dependent and -independent pathways that induce necroptosis *in vivo*[61]. On the other hand, lack of RIPK1 kinase activity as well as RIPK3 deficiency protected mice from TNF-induced systemic inflammatory response syndrome (SIRS)[53,64,65,67], demonstrating that kinase-dependent pro-death functions of RIPK1 rather than direct proinflammatory gene induction are crucial for TNF-induced SIRS, as previously proposed[67].

Mechanisms determining apoptosis or necroptosis

Despite the advances in unravelling the pathways that regulate necroptosis, the precise mechanisms determining the decision whether a cell will die by apoptosis or necroptosis remain poorly understood. On the positive regulatory side, several studies have suggested that expression levels of RIPK3 and MLKL correlate with sensitivity to necroptosis[10,17,31,60,68–70]. However, a potential drawback of these studies is that RIPK3 and MLKL expression was compared between healthy and inflamed tissues; it is, therefore, difficult to conclude that RIPK3 and/or MLKL overexpression has primary causal functions and is not a secondary consequence of ongoing inflammation. Interestingly, some studies suggest that whether RIPK3 is catalytically active or not may determine necroptosis or apoptosis. As a catalytically inactive platform RIPK3 favours RIPK1-dependent apoptosis, whereas catalytically active RIPK3 induces necroptosis[36]. This bifurcation was illustrated *in vivo* by the finding that, although RIPK3-knockout mice have no spontaneous phenotype, $RIPK3^{D161N}$-kinase-inactive knock-in mice die during embryogenesis due to RIPK1–FADD–caspase-8 mediated apoptosis[65], suggesting that kinase-active RIPK3 is required to suppress apoptosis. However, a recent report showed that $RIPK3^{K51A}$ kinase inactive knock-in mice are viable and that RIPK3 inhibitors block TNF-induced necroptosis, but at higher concentrations induce RIPK3-scaffold-dependent apoptosis, suggesting that RIPK3 conformational changes and not inhibition of its kinase activity trigger apoptosis[71].

On the negative regulatory side, caspase-8 is the most crucial factor for preventing necroptosis. Indeed, in most *in vivo* experimental systems thus far, sensitization to necroptosis was achieved by a genetic defect compromising FADD–caspase-8 signalling, and thus inhibiting apoptosis[14–18]. cIAP loss sensitizes cells to apoptosis[72–74] and — when caspase-8 is inhibited — to necroptosis[10], suggesting that cIAP inhibition confers susceptibility to cell death but that the decision whether a cell dies by apoptosis or necroptosis depends on caspase-8 activity. As already discussed, RIPK1 deficiency sensitizes cells to both apoptosis and necroptosis[58–63], although the factors determining whether a cell that lacks RIPK1 will die by apoptosis or necroptosis remain elusive. Therefore, more *in vivo* studies are required to understand the mechanisms that determine the susceptibility of different cell types to necroptosis or apoptosis.

Regulated cell death in inflammation

Although initially cell death was considered to be the result of inflammation, more recently the concept that cell death may precede, trigger or amplify the inflammatory response has gained increasing attention[1]. Establishing cell death as an initiator of inflammation *in vivo* is challenging, because it is very difficult to temporally resolve the two responses to demonstrate causality. In the next section, we discuss the evidence implicating necroptosis as an active mediator of inflammation, focusing particularly on the implications of these findings for the pathogenesis of inflammatory diseases — other aspects related to the role of regulated cell death in pathogen defence have been covered in other recent reviews[75,76].

Necroptosis as a trigger of inflammation

A major obstacle in studying the role of necroptosis *in vivo* has been the lack of a definite molecular marker for the *in situ* identification of necroptotic cells. Antibodies that detect human phosphorylated MLKL, which were recently reported to detect necrotic hepatocytes in the liver of people with drug-induced liver injury[31], promise to provide such a tool, but additional validation will be required to demonstrate their applicability for necroptosis detection in other human tissues. The development of mouse-specific phospho-MLKL antibodies will be invaluable for the preclinical validation of phosphorylated MLKL as a marker of necroptotic cells in mouse models of human disease. So far, in most published studies necroptotic cells are described by necrotic morphology and concomitant absence of caspase-3 activation, which cannot distinguish between different types of necrotic cell death (Box 1). In the absence of a specific molecular marker, the only definite criterion for necroptosis has been dependence on RIPK3 and MLKL. Although NEC1 has been used extensively as an inhibitor of necroptosis, in light of the most recent findings that show the existence of RIPK1-kinase-activity-dependent and -independent necroptosis *in vivo*[61], as well as the complex functions of RIPK1 kinase activity in regulating both necroptosis and apoptosis[77] and the inhibitory effects of NEC1 on indoleamine 2,3-oxygenase[78], studies based on NEC1 cannot be considered as definite evidence for necroptosis and need to be reproduced in a RIPK3- and/or MLKL-deficient background. Therefore, in this Review, we will only discuss *in vivo* studies that support a proinflammatory role for necroptosis by demonstrating that cell death and inflammation are prevented by RIPK3 and/or MLKL deficiency.

In vivo evidence to support a proinflammatory function for necroptosis was initially provided by studies in mice that lack FADD in IECs, which had increased numbers of necrotic IECs and developed spontaneous colitis and ileitis with loss of Paneth cells[17]. RIPK3 deficiency prevented epithelial cell death and inflammation in both the colon and the small intestine of these mice, providing *in vivo* experimental evidence that RIPK3-mediated necroptosis of epithelial cells causes intestinal inflammation[17]. Germ-free conditions and MyD88 or TNF deficiency prevented colitis, but not Paneth-cell loss and ileitis in mice lacking FADD in IECs, suggesting that different mechanisms trigger RIPK3-mediated epithelial cell necroptosis and inflammation in the colon and the small intestine[17]. IEC-specific knockout of caspase-8 caused Paneth cell loss and ileitis[79], which was subsequently shown to depend on RIPK3, but not on TNFR1 as initially suggested[80]. Interestingly, in contrast to IEC-specific FADD knockouts, IEC-specific caspase-8 knockout mice did not develop colitis, suggesting that FADD and caspase-8 have different functions in the colonic epithelium.

Mice with keratinocyte-specific FADD deficiency developed RIPK3-dependent keratinocyte necroptosis and skin inflammation, suggesting that epithelial cell necroptosis also triggers inflammation in the skin[18]. Keratinocyte-specific caspase-8 knockout mice also developed skin inflammation, which was suggested to be caused by the loss of caspase-8-dependent suppression of RIG-I–IRF3 signalling[81,82]. However, mitochondrial antiviral-signalling protein (MAVS) deficiency did not prevent skin inflammation in mice with inducible ablation of caspase-8, arguing against an important role for the RIG-I–MAVS–IRF3 axis[83]. By contrast, RIPK3 deficiency prevented skin inflammation in these mice[83], further supporting the idea that RIPK3-dependent keratinocyte necroptosis triggers inflammation in mice lacking caspase-8 or FADD in the epidermis. In addition, epidermis-specific RIPK1 knockout caused keratinocyte necroptosis and skin inflammation that was prevented by RIPK3 or MLKL deficiency[61], whereas *Ripk1*$^{-/-}$ newborn pups developed RIPK3- and MLKL-dependent skin hyperplasia[61], providing additional evidence that necroptosis triggers inflammation. TNFR1-deficiency ameliorated, but could not fully prevent inflammation in these mice, suggesting that although TNF is a major driver of cell death and inflammation, TNF-independent pathways also contribute in these models.

In addition to intestinal and skin epithelia, necroptosis of several other cell types was shown to trigger inflammation. RIPK3-dependent necrosis of retinal pigment epithelial cells induced inflammation in a mouse model of double-stranded RNA-induced retinal degeneration[84]. Moreover, RIPK3-deficient mice were protected from TNF or TNF–zVAD-fmk induced SIRS, demonstrating that necroptosis has an important role in TNF-induced shock[53,65,67]. Two studies showed that RIPK3-mediated necroptosis contributes to caecal ligation and puncture-induced sepsis[67,85], although another study failed to confirm the function of RIPK3 or MLKL in this model[86] of polymicrobial sepsis. In addition, RIPK3- and MLKL-dependent necroptosis was shown to exacerbate tissue injury and inflammation in a mouse model of acute pancreatitis[10,86], whereas RIPK3 deficiency was protective in a mouse model of Gaucher's disease[87]. Furthermore, RIPK3 deficiency ameliorated ischaemia-reperfusion-induced injury and inflammation and prolonged kidney and heart allograft survival[88–90], suggesting that necroptosis of graft cells could be a crucial factor in triggering inflammation contributing to transplant rejection.

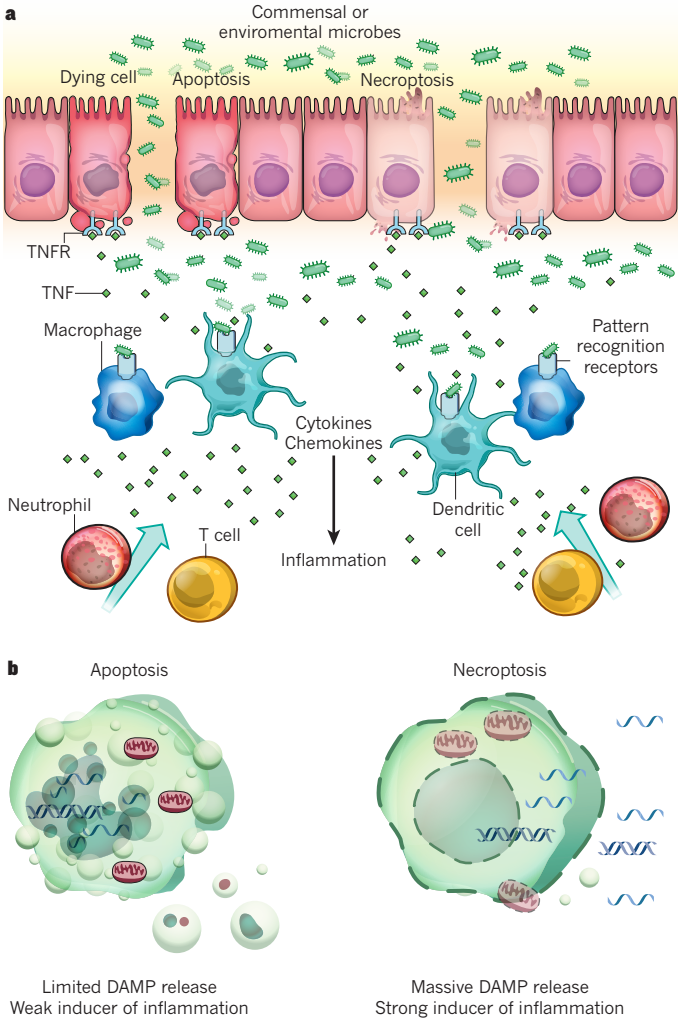

Figure 2 | Regulated cell death triggers inflammation. **a**, Epithelial cell death in barrier tissues can cause barrier disruption, allowing commensal or environmental microbes to invade the tissue. Recognition of microbial pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors on myeloid or stromal cells induces the expression of cytokines and chemokines that attract and activate immune cells resulting in inflammation. Cytokines expressed by immune cells (for example, TNF) could trigger the death of additional epithelial cells, further compromising the barrier in a vicious circle, resulting in chronic non-resolving inflammation. In this setting, DAMP release may have a limited contribution as the presence of microbial PAMPs could fully drive the inflammatory response. It is unclear whether apoptosis and necroptosis have a differential capacity to induce barrier disruption. **b**, In sterile tissues, apoptotic cell death is considered a weak inducer of inflammation, as the orderly disassembly of the dying cell allows no, or limited, release of DAMPs. By contrast, the massive release of DAMPs by disintegrating necroptotic cells is believed to be a strong trigger inducing inflammation (see Box 2).

How does regulated cell death cause inflammation?

Cell death could induce inflammation indirectly, by disrupting epithelial barriers triggering microbe-driven immune responses (Fig. 2a). This is particularly relevant in the intestinal epithelium, which provides a barrier between luminal bacteria and the mucosal immune system. IEC death usually triggers intestinal inflammation in different mouse models and here it is unclear whether apoptosis and necroptosis differ in their capacity to induce inflammation. Because barrier disruption not only depends on cell death but also on the capacity of the remaining cells to proliferate and keep the barrier sealed, it is important to study whether necroptosis and apoptosis differentially regulate key barrier-related properties of neighbouring epithelial cells such as proliferation, migration and adhesion.

In a sterile setting, dying cells could directly trigger inflammation by releasing factors collectively described as damage-associated molecular patterns (DAMPs) (Box 2). Apoptosis is generally considered to be non-immunogenic because the orderly disassembly of apoptotic cells allows no or limited DAMP release (Fig. 2b). However, there is a general consensus that necroptosis directly triggers inflammation by a massive release of DAMPs from the disintegrating cell[91] (Fig. 2b). Although this notion is clearly supported by evidence that DAMPs are released by necrotic cells and that specific DAMPs are important mediators of inflammation *in vivo*[91], at present there are no *in vivo* studies that provide direct experimental proof that necroptosis-induced inflammation depends on specific DAMPs. Such experimental proof might be difficult to obtain using genetic models owing to functional redundancy between DAMPs, and the important intracellular functions of several DAMPs. Indeed, surprisingly, myeloid-, hepatocyte- or pancreas-specific knockout of HMGB1, a prototypic DAMP, did not ameliorate but instead exacerbated LPS- or injury-induced damage and inflammation owing to an important role for HMGB1 in maintaining genome homeostasis and cell survival and preventing histone release[92–94]. Antibody neutralization may therefore be more suitable for addressing the functional role of specific DAMPs such as HMGB1, although this approach can not address the cellular origin of DAMPs to formally demonstrate that DAMP release by specific necrotic cells causes inflammation. IL-1 family cytokines (IL-1α, IL-1β, IL-18, IL-33, IL-36 and IL-37) constitute important DAMPs and their role as mediators of necroptosis-induced inflammation needs to be addressed *in vivo* in relevant genetic models. In the absence of conclusive functional *in vivo* studies, the role of specific DAMPs as mediators of necroptosis-induced inflammation is currently largely based on correlative evidence and nice schemes in reviews, and awaits *in vivo* experimental validation.

BOX 2

Damage-associated molecular patterns

Damage-associated molecular patterns (DAMPs) collectively describes the molecules and cellular components that are released or exposed by dying, injured or stressed cells and that act as danger signals to alert the immune system. DAMPs include cytokines and alarmins that are released mainly by dying cells such as the interleukin-1 family cytokines IL-1α and IL-33, as well as the S100 proteins S100A8, S100A9 and S100A12. In addition, several cellular components that are normally found inside the cell performing important and predominantly non-immunological functions, are released only by dying or damaged cells to act as DAMPs. These include nucleic acids and ribonucleoproteins, histones and HMGB family members, heat-shock proteins, mitochondrial *N*-formyl peptides and DNA, or even intact mitochondria, F-actin, calreticulin, but also molecules such as monosodium urate and ATP (reviewed in ref. 107). DAMPs are usually detected by pattern recognition receptors that activate immune responses by inducing the expression of cytokines and chemokines. DAMPs are primarily released by cells undergoing necrosis, although apoptotic cells were recently shown to expose or release DAMPs[122]. DAMPs may undergo modifications that inhibit their immunogenicity, for example caspase-dependent proteolysis of IL-33 or oxidation of HMGB1 (refs 123–125). Therefore, the type of cell death may determine the nature, quantity and immunogenicity of the DAMPs released.

Necroptosis-dependent and -independent functions of RIPK3

Genetic rescue experiments using RIPK3 or MLKL knockouts are routinely used to demonstrate that necroptosis triggers inflammation. However, it remains unclear whether in some cases RIPK3 might be capable of triggering inflammation by exerting necroptosis-independent functions. RIPK3 has been implicated in inflammasome activation and IL-1β release in Smac-mimetic-treated macrophages or in caspase-8-deficient dendritic cells[95,96], apparently in a cell-death-independent manner, although multiple independent studies failed to show a role for RIPK3 in canonical or non-canonical inflammasome activation[97–99]. RNA viruses, however, were recently reported to activate the NLRP3 inflammasome in a RIPK1- and RIPK3-dependent but MLKL-independent manner, suggesting that in response to viral RNA detection RIPK1 and RIPK3 induce inflammasome activation in a necroptosis-independent manner that seems to involve mitochondrial fission and ROS production[100]. These studies suggest that regulation of the inflammasome by RIPK3 may contribute to inflammation, although available *in vivo* data do not support an important role for the inflammasome in RIPK3-dependent inflammation, because, for example, double IL-1α and IL-1β deficiency did not prevent inflammation in mice with epidermal keratinocyte-specific knockout of caspase-8 (ref. 81). Furthermore, a recent study reported that RIPK3 regulates LPS-induced NF-κB and caspase-1 activation and inflammatory cytokine production in dendritic cells but not in macrophages, and controls dextran-sodium-sulphate-induced colon inflammation[101].

Several studies have shown that caspase-8 activates the inflammasome and also directly cleaves pro-IL-1β to secrete IL-1β[95,97–99,102–104], suggesting that it could induce inflammation independently of its pro-apoptotic functions. However, most were performed on macrophages lacking both caspase-8 and RIPK3, as caspase-8-knockout macrophages do not survive, raising the question of whether the observed effects might, to some extent, depend on the role of caspase-8 in inhibiting necroptosis. An alternative explanation for these findings could be that inflammasome activation is coupled to cell death in cells that are primed to express inflammasome components and IL-1-family cytokine precursors. Caspase-8 could directly cleave IL-1 precursor proteins and perhaps also caspase-1 during apoptosis, whereas RIPK3–MLKL-dependent necroptosis could trigger inflammasome activation by inducing changes in the redox state, intracellular ion concentrations and the metabolic status of the cell, all of which are well-known inflammasome inducers[3]. This hypothesis is intriguing in light of recent findings showing that IL-1β is released primarily from dying macrophages in response to inflammasome activation[105]. Considering that most stimuli known to act as inflammasome activators in primed macrophages inflict cellular damage, activation of the inflammasome could represent a unifying mechanism endowing primed cells with the capacity to induce inflammation when they are exposed to cell-death-inducing stress. Coupling regulated cell death with inflammasome activation in primed cells could provide a mechanism to ensure that the death of cells exposed to stimuli that activate NF-κB, such as microbial products or inflammatory cytokines, will trigger an immune response to mediate host defence and promote tissue repair. The potent capacity of most stimuli that trigger regulated cell death (for example, TNF, IFNs and LPS) to also induce proinflammatory gene expression ensures that in a population of cells mixed responses occur between cells that survive or die, and that dying cells have also been primed to produce cytokines and chemokines[106]. Therefore, additional studies in relevant *in vivo* models will be needed to assess the contribution of cell-death-dependent and -independent functions of RIPK3 and caspase-8 in inflammation.

Is necroptosis more inflammatory than apoptosis?

Apoptosis is generally considered to be non-immunogenic, based on the concept that developmentally programmed cell death, such as that during thymocyte selection in the thymus, should not trigger inflammation. However, apoptotic cell death that is not developmentally programmed indicates tissue injury and should be detected by the immune system to ensure efficient tissue regeneration and host defence. Indeed, several recent studies supported a proinflammatory role of apoptosis. Apoptosis of cancer cells in response to some chemotherapeutic agents was shown to be highly immunogenic, contributing to therapy responses (reviewed in ref. 107). Mouse-model studies suggested that apoptosis of epithelial cells lacking NEMO or TAK1 triggers chronic inflammation[108–111], although the potential role of necroptosis in these models has not been addressed. Compelling evidence that epithelial cell apoptosis induces inflammation comes from studies showing that inducible $FLIP_L$ knockout caused keratinocyte apoptosis and inflammation[83,112], which could not be prevented by RIPK3 deficiency[83]. In addition, skin inflammation in chronic proliferative dermatitis mice was recently shown to depend primarily on TNFR1–TRADD–RIPK1-dependent death of SHARPIN-deficient keratinocytes mediated primarily by FADD–caspase-8-dependent apoptosis, whereas RIPK3-dependent necroptosis contributes to disease severity, but is not essential for the induction of inflammation[64,113,114].

Necroptosis is believed to be a more potent inducer of inflammation than apoptosis but this concept has not been rigorously tested in relevant *in vivo* experimental models. Clearance of dying cells is crucial in limiting inflammation[115]. Although both apoptotic and necroptotic cells are recognised and cleared by phagocytes, the mechanism may be different owing to profound differences in the morphology, blebbing and apoptotic body formation in apoptosis compared with oncosis and rapid plasma membrane permeabilization in the case of necroptosis. The more acute release of DAMPs could support increased proinflammatory properties of necroptosis; however, as already discussed, the role of specific DAMPs in necroptosis-induced inflammation *in vivo* has not been functionally validated. Particularly considering that co-incubating apoptotic or necrotic cells with macrophages did not result in cytokine induction[116], the *in vivo* context may be very relevant for this type of experiment. To allow a direct comparison of apoptosis and necroptosis, ideally they would need to be induced in a similar number of cells in the same tissue. Recent

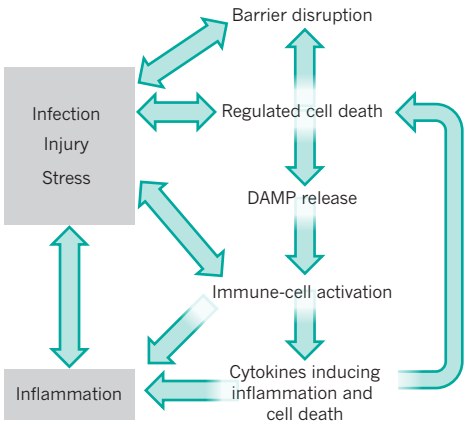

Figure 3 | Regulated cell death fuels the vicious circle in chronic inflammation. Regulated cell death could play a part in the initiation and amplification or chronicity of inflammation. This form of cell death can induce immune activation and cytokine expression indirectly through barrier disruption, allowing microbial entry, or directly by the release of DAMPs. Activated immune cells produce cytokines that induce inflammation directly by activating the expression of proinflammatory genes but also trigger regulated cell death, closing a circle that amplifies the inflammatory response. Infection, injury and stress can initiate the response by inducing regulated cell death directly and/or by triggering immune-cell activation and cytokine production.

results showing that necroptosis, but not apoptosis, of RIPK1-deficient keratinocytes triggers skin inflammation provide initial *in vivo* experimental evidence that, at least in this model, keratinocyte necroptosis is a more potent trigger of skin inflammation compared with apoptosis[61], although, as already discussed, keratinocyte apoptosis has been shown to trigger inflammation in other models. In conclusion, although there are indications that necroptosis is more inflammatory than apoptosis, new and more specific *in vivo* models are needed to directly compare the capacity of these two pathways of regulated cell death to trigger inflammation and address the underlying mechanisms. Such models could take advantage of intracellular mediators that exclusively induce apoptosis (for example, tBid) or necroptosis (for example, constitutively active MLKL mutants) to specifically address the function of each cell-death pathway without interference from extracellular inducers.

Necroptosis in human disease

Studies in mice have provided strong evidence suggesting that necroptosis has a crucial role in disease pathogenesis. In addition to acting as an initiation signal, regulated cell death could contribute to the amplification and chronicity of inflammation since many cytokines produced during the immune response (such as, TNF family members or INFs) are potent cell-death inducers. A vicious circle of cell death, DAMP release, immune-cell activation and release of death-inducing cytokines may fuel prolonged non-resolving inflammatory responses and contribute to the pathogenesis of chronic inflammatory diseases (Fig. 3). It is intriguing to speculate that the pathogenic role of TNF in chronic inflammation could be, at least in part, due to its cell-death inducing properties. However, few studies addressing the role of necroptosis in human inflammatory diseases exist and these rely largely on correlative evidence such as the upregulation of RIPK3, which is only indicative and does not provide proof that necroptosis takes place and is causally associated with disease pathogenesis.

Epithelial cell death has been associated with intestinal inflammation in inflammatory bowel disease (IBD) and although early reports focused on apoptosis it is not clear whether necroptosis also occurs[117], in particular because most studies used TdT-mediated dUTP nick end labelling (TUNEL) assays that cannot distinguish between apoptotic and necroptotic cells. Mouse-model studies showed that epithelial-cell necroptosis induces intestinal inflammation, suggesting that necroptosis could also contribute to the pathogenesis of IBD in humans[17,79,83]. However, evidence that necroptosis is implicated in human IBD remains limited. An initial study identified dying cells with necrotic morphology in ileal crypt sections from patients with Crohn's disease, indicating that Paneth cells undergo necroptosis[79]. A more recent study showed that RIPK3 and MLKL were upregulated, whereas caspase-8 expression was reduced in the inflamed mucosa of children with Crohn's disease, ulcerative colitis or allergic colitis and suggested that necroptosis is a crucial event that amplifies inflammation and contributes to these intestinal pathologies[118]. Although these studies do indicate that necroptosis might be involved in IBD they are based on rather weak and correlative evidence, therefore more research using specific and sensitive molecular markers of necroptosis will be required to establish the potential role of necroptosis in IBD.

Mouse-model experiments identified keratinocyte necroptosis as a potent trigger of skin inflammation[18,61,83], suggesting that keratinocyte necroptosis might also be implicated in the pathogenesis of human inflammatory skin diseases. A recent report suggested that keratinocyte necroptosis contributes to severe cutaneous adverse drug reactions in humans and in a mouse model of the disease[119]. However, the occurrence and potential role of necroptosis in human inflammatory skin diseases such as psoriasis has not been investigated. Recent reports based on mouse models suggested that RIPK3-mediated necroptosis contributes to liver injury and inflammation induced by alcohol but also in non-alcoholic steatohepatitis[120,121]. Upregulation of RIPK3 was detected in hepatocytes in the livers of people with alcohol-induced liver disease and non-alcoholic steatohepatitis, suggesting that RIPK3-dependent necroptosis could contribute to hepatocyte death and inflammation[120,121]. Moreover, immunostaining with antibodies that recognize phosphorylated MLKL, which promise to provide a specific marker of necroptosis, suggested that hepatocyte necroptosis occurs in the livers of people with drug-induced liver injury[31]. Therefore, although mouse-model experiments strongly suggest that necroptosis could be implicated in inflammatory diseases, more studies using definite molecular markers of necroptosis and functional validation using specific inhibitors will be required to establish the involvement of necroptosis in the pathogenesis of human diseases.

Outlook

Necroptosis is now established as an important pathway of regulated cell death, but many questions remain to be addressed. What are the mechanisms controlling the formation and activity of the necrosome? What is the role of specific ubiquitylation events and enzymes? What is the precise mechanism of MLKL-mediated necroptosis? How is the kinase activity of RIPK1 and RIPK3 regulated and what are their substrates? What are the specific kinase-dependent and scaffolding functions of RIPK1 controlling inflammation, cell survival, apoptosis and necroptosis? What is the relative contribution of cell-death-dependent and -independent functions of RIPK3 and RIPK1 in inflammation? How does necroptosis contribute to the initiation, amplification and chronicity of inflammation? Does necroptosis display different properties in regulating inflammation compared with other types of regulated cell death? What is the relationship between RIPKs, necroptosis and inflammasome activation? These questions become particularly important when considering that stimuli triggering cell death such as TNF also potently induce inflammatory gene expression. The clinical efficacy of anti-TNF therapy has established TNF as a key player in chronic inflammatory diseases including IBD, rheumatoid arthritis and psoriasis, but the TNF-dependent pathogenic mechanisms remain unclear. Determining the relative contribution of cell-death-dependent and -independent pathways in TNF-induced chronic inflammation may lead to new and more specific therapeutic targets. New preclinical mouse-model studies will be needed to answer these mechanistic questions. Addressing the role of necroptosis in human disease remains a major challenge and will require the establishment of specific, sensitive and reliable molecular markers of necroptosis. Current data raise the hope that manipulating necroptosis could provide new and urgently needed therapeutic opportunities in acute and chronic inflammatory conditions. It remains to be seen if this promise will be fulfilled. ■

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