Regulated Cell Death

19

19.1 Introduction

The historical development of the cell death concept has been thoroughly reviewed by Majno and Joris [1]. Interestingly, it was already the German pathologist Rudolf Virchow at the Charité in Berlin who discussed the fact *that cells can perish* in his Lecture XV published in *Cellularpathologie* (Cellular Pathology) under the Chapter "*Passive Vorgänge. Fettige Degeneration, Seite 285*" (passive processes and degenerations) [2]. Topics related to cell death are treated in this lecture held in Berlin on April 10, 1858, at a gross level, under chapters such as "*Die passiven Vorgänge in ihren beiden Hauptrichtungen zur Degeneration: Nekrobiose (Erweichung und Zerfall).*" (The passive processes in their two main directions to degeneration: necrobiosis (softening and decay). Virchow uses the term necrosis to mean an advanced stage of tissue degradation, similar to what is medically now called gangrene. He wrote (p. 287): *Diese letztere Categorie, wo die Elemente unter dem Ablaufe des Prozesses zu Grunde gehen, habe ich vor einigen Jahren vorgeschlagen mit einem Ausdruck zu bezeichnen, welcher von K. H. Schultz für die Krankheit überhaupt gebraucht ist, mit dem der Nekrobiose. Immer nämlich handelt es sich hier um ein Absterben, um ein Zugrundegehen, man möchte fast sagen, um eine Nekrose. Aber der Begriff der Nekrose bietet doch gar keine Analogie mit diesen Vorgängen, insofern wir uns bei der Nekrose den mortificirten Theil als in seiner äusseren Form mehr oder weniger erhalten denken*; *Seite 287*) (loosely translated: This last category where the elements perish during the process, has been proposed by me, a couple of years ago, to denote with a term that was used by K.H. Schultz for the disease specifically, that is, with that of necrobiosis. Namely, it always deals with a mortification, a decay, one would rather like to say necrosis. But the term necrosis does not offer any analogy to these processes, insofar, in necrosis, we conceive the mortified (gangrenous) part to be preserved more or less in its external form). Virchow, a few pages later in his book (p. 305), also refers to cell death in relation to fatty degeneration of cells. He wrote: *Gegenwärtig nennen wir das Ding eine Körnchenkugel, und betrachten es als das erste Stadium der Degeneration, wo nicht mehr die Zelle als Zelle erhalten ist, sondern*

wo bloss noch die rohe Form existirt, mit vollständigem Verlust der eigentlich die Zelle constituirenden Theile, d. h. der Membran und des Kernes (loosely translated: "Currently, we call the thing a granule sphere (obviously meaning a cellular lipid vacuole) and regard it as the first phase of degeneration, where the cell as a cell is not any more preserved, but where only its raw form exists associated with complete loss of those parts that constitute the cell, that is, the membrane and the nucleus").

But back to the present! When—as stressed in the previous chapter—adaptive stress responses such as the UPR and the DDR fail to repair molecular damage and, thus, fail to restore cellular homeostasis, cells generally promote and undergo an RCD as a means to preserve the homeostasis of the whole organism (Fig. 19.1). Doubtlessly, one of the hottest topics in modern biomedicine is the research in the field of RCD. In fact, it is the intersection between cell death as a major source of DAMP emission and execution of innate/adaptive immune responses that is central not only to maintenance and restoration of homeostasis but also, unfortunately, to pathogenesis of many human diseases and—as a consequential necessity—to the development of novel therapeutics. So, let's start here with a brief glance at the background of this topic.

Under stressful and injurious conditions, cells in living tissue have only two options: to survive when the stress responses are successful or to die when they are unsuccessful. The character and the intensity of a cell's death can vary. In principle, cells may die either individually or in groups in an accidental manner, that is, in the form of an ACD or may succumb to an RCD. Accidental cell death is caused by extreme insults such as overwhelming thermal, chemical, or mechanical/physical injuries, a typical example being freezing and thawing procedures. As previously
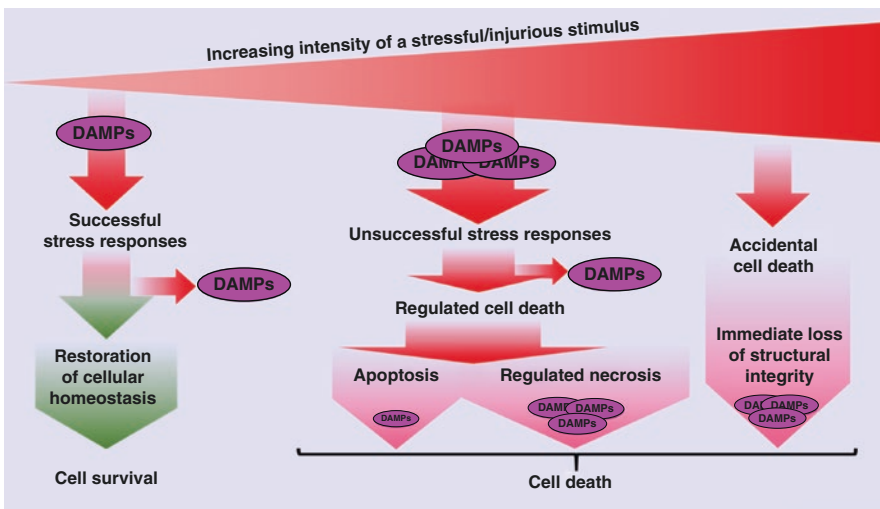

Fig. 19.1 Scenario model of unsuccessful adaptive stress responses that promote regulated cell death. When stress responses fail to repair molecular damage, cells generally promote and undergo a regulated cell death to preserve homeostasis of the whole organism. DAMPs are generated and emitted during stress responses and different forms of regulated cell death (associated with increasing degree of immunogenicity) in relation to the intensity of injurious stimuli. Sources: Refs. [3, 4]
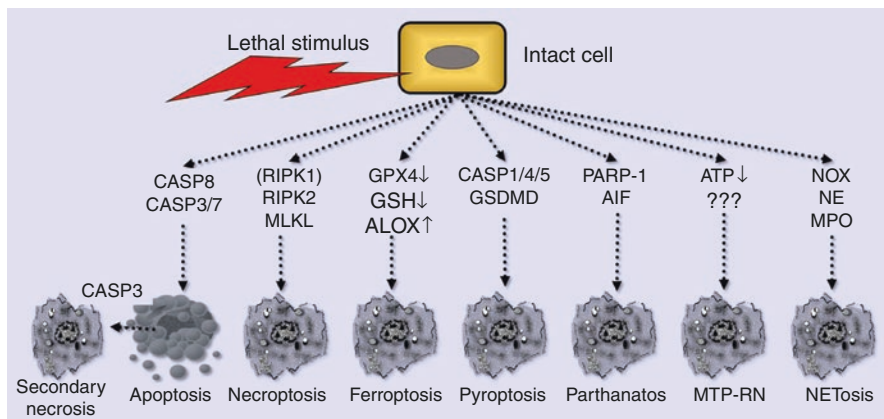
Fig. 19.2 Overview of regulated cell death modalities and the key pathway proteins involved. *AIF* apoptosis-inducing factor, *ALOX* arachidonate lipoxygenase, *ATP* adenosine-5-triphosphate, *CASP* caspase, *GPX4* phospholipid-hydroxyperoxid-glutathione-peroxidase, *GSDMD* gasdermin D, *GSH* (reduced form of) glutathione, *MLKL* mixed lineage kinase domain-like protein, *MPO* myeloperoxidase, *MPT-RN* mitochondrial permeability transition-regulated necrosis, *NE* neutrophil elastase, *NETosis* formation of neutrophil extracellular traps by neutrophils, *NOX* nicotinamide adenine dinucleotide phosphate-dependent oxidase, *PARP-1* poly (ADP-ribose) synthetase-1, *RIPK* receptor-interacting serine/threonine-protein kinase. Sources: Refs. [5–12]

mentioned, this kind of cell death is entirely uncontrolled and virtually immediate. By contrast, RCD is caused by low or moderate insults as can be observed, for example, during various kinds of infections or pathological tissue reperfusion.

Regulated cell death represents an umbrella term that includes several subroutines of cell death which, in principle, can be divided into apoptosis and various forms of RN (Fig. 19.2). By definition, RN is an immunogenic form of cell death because of the inevitable rupture of the plasma membrane and consequent release of large amounts of constitutive DAMPs. Such a process does not happen during apoptosis as a low degree-immunogenic form of cell death because, here, the plasma membrane integrity remains preserved. Expectedly, therefore, the emission of DAMPs by apoptotic cells is weak or even null. Importantly, several different pathways of RN have been recently described, typically associated with variable intensity of emission of inducible DAMPs. Thus, in the perspective of the book, their different mechanistic formations deserve a brief but clear description, before resuming this exciting topic of immunogenicity at the end of the chapter.

19.2 Apoptosis

19.2.1 Introductory Remarks

Apoptosis can be regarded as a form of a cell's suicide and certainly represents one of the cell death types which has been studied for a long time. As a Greek term,

apoptosis translates to the "falling off" of dead leaves from a tree [13]. As also reviewed [1], spontaneous apoptotic cell death as a physiological event was discussed almost as soon as stains became available. And it was the German biologist Walther Flemming, a founder of cytogenetics, who first observed the phenomenon under the microscope and called the process *chromatolysis* [14]. In 1914, the German anatomist Ludwig Gräper interpreted Flemming's observations by claiming that "*chromatolysis* must exist in all organs in which cells must be eliminated" [15]. Many years later in 1951, Glücksmann, a German–British embryologist, in studies on cell death in embryonic tissue, published a finer description of the cell changes now known as apoptosis, such as:

> There is ample support for the morphological identification of dying cells from the following considerations: the degeneration 'granules' are initially Feulgen-positive and have thus originated from nuclear constituents; the stages of cell deaths seen in normal embryos are identical with those produced experimentally and with those observed directly in tissue cultures; degenerating cells react in the same manner to supravital stains in vivo and in vitro. The process of degeneration varies with the degree of specialization of the cell, with its functional state (e.g., mitosis), with the type of animal and under experimental conditions with the causative agents. Cell death may take from less than 1 h to about 7 h, when only a small proportion of a living tissue dies but may be prolonged to days when numerous cells die simultaneously, and their resorption is delayed [16].

Then, in 1972, the finite term *apoptosis* was proposed by the Australian pathologist Kerr who induced liver atrophy in the rat by tying off a large branch of the portal vein. The researcher noticed a discrete drop-off of cells by a sequence of changes that he called at first shrinkage necrosis and a year later apoptosis [17, 18].

Today, it is well known that, in all multicellular organisms, apoptosis is that type of cell death which operates under daily physiological conditions to remove unnecessary, old, unhealthy, and damaged cells with the minimum of disturbance to neighboring cells, aimed at maintaining homeostasis. One has to bring to mind: in healthy human individuals, about 10–100 billion cells die every day and are replaced by new healthy cells to maintain homeostasis of the whole organism [19]. To avoid inflammation, the resulting apoptotic bodies are cleared by phagocytes, that is, they are engulfed before they leak their contents such as DAMPs, a phenomenon called efferocytosis (for efferocytosis, see Part VI, Sect. 22.6.3.3).

The primary biochemical and morphological "hallmarks" of apoptosis include cell shrinkage, chromatin condensation, chromosomal/internucleosomal DNA cleavage and fragmentation, nuclear fragmentation, plasma membrane blebbing, apoptotic body formation, and, most important, maintenance of intact cellular membranes to prevent massive protein release. It is only the latter that distinguishes apoptosis from RN pathways described below. Of note, this form of cell death is an active procedure controlled by a mechanism programmed by the cell itself. The core apoptotic enzymatic machinery consists of caspases—a subset of the cysteine-dependent aspartate-specific protease family—which in an orchestrated way ultimately cause the morphologic features of this type of cell death (for reviews, see [20–24]).

19.2.2 Activation of Apoptosis

19.2.2.1   General Remarks

Typically, apoptosis is activated by default when a cell is deprived of essential pro-survival factors. Alternatively, apoptosis may be activated deliberately by a variety of injurious stimuli, including certain types of severe cell stress and insults or in response to extracellular signals triggered by specific death-inducing ligands, for example, TNF. The caspases operating in apoptosis are divided into two broad categories: initiator caspases (e.g., caspase-8 and caspase-9) and executioner or effector caspases (e.g., caspase-3 and caspase-7). The initiators trigger a cascade-like proteolytic stimulation of effector caspase zymogens. In turn, the latter products drive the execution phase of the apoptotic death program by cleaving hundreds or even thousands of structurally and functionally critical proteins within the cell (reviewed in [24]).

In mammals, apoptosis can be instigated by two major cellular signalling pathways: an intrinsic pathway that is initiated inside cells by mitochondrial release of pro-apoptotic factors or an extrinsic pathway that is initiated at the cell surface by death receptors that are members of the TNF receptor gene family (Fig. 19.3). These two pathways often act directly and/or indirectly to reinforce one another [3, 21, 24, 33, 34].

19.2.2.2   The Intrinsic Apoptotic Pathway

The intrinsic pathway, often induced by unsuccessful stress responses such as the oxidative stress response, UPR, or the DDR (but also by nutrient deprivation) [35], is regulated by *B cell lymphoma-2* (BCL-2) family proteins, which control the release of specific caspase-activating factors from mitochondria and have either anti-apoptotic or pro-apoptotic functions. As discussed [24, 36, 37], the BCL-2 gene family encodes two major subclasses of apoptosis-regulating factors, which contain different numbers of *BCL-2 homology* (BH) motifs: (1) single BH motif (BH3-only) proteins (e.g., BID, BAD, BIK, BMF, PUMA, and NOXA) which typically act pro-apoptotic, that is, as death agonists, and (2) multi-BH motif proteins which possess three or four BH regions and act, respectively, as agonists (e.g., BAX, BAK, BOK) or anti-apoptotic as antagonists (e.g., BCL-2, BCL-XL, BCL-w, A1, MCL-1) of apoptotic stimulation (Fig. 19.3).

The interplay between members of these subgroups in each individual cell determines whether apoptosis is turned on or off. For example, the anti-apoptotic BCL-2 proteins inhibit apoptosis by inhibiting pro-apoptotic BCL-2 family proteins BAX and BAK. On the other hand, the anti-apoptotic BCL-2 proteins are themselves inhibited by the BH3-only proteins in response to an intrinsic signal. This results in BAX and BAK homo-oligomerization that creates mitochondrial membrane permeabilisation (MMP) associated with formation of membrane pores, releasing mitochondrial cytochrome C into the cytoplasm. Following, cytochrome C triggers a conformational change in *apoptotic protease-activating factor-1* (Apaf-1), resulting in ATP exchange and oligomerization to form a complex, called the "apoptosome" that converts pro-caspase-9 to active caspase-9. Caspase-9 then activates the
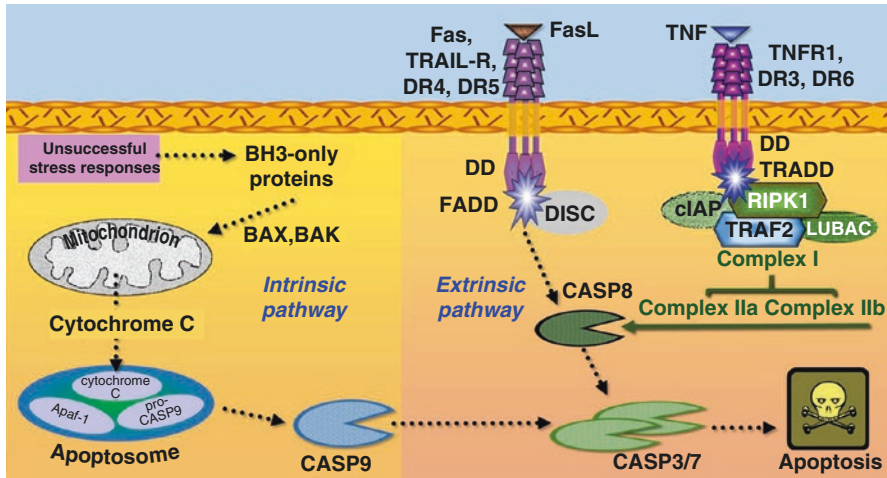
Fig. 19.3 Simplified scenario model illustrating the intrinsic and extrinsic apoptotic pathways. Apoptosis can be instigated by two major cellular signalling pathways: an intrinsic pathway that is initiated inside cells by mitochondrial release of pro-apoptotic factors or an extrinsic pathway that is initiated at the cell surface by death receptors that are members of the TNF receptor gene family. The intrinsic pathway is controlled by members of the Bcl-2 protein family such as the BH3-only proteins which, when activated, stimulate pro-apoptotic BAX and BAK that form pores in the mitochondrial outer membrane to provide release of cytochrome C. Following, cytochrome C associates with Apaf1 to form an apoptosome that drives activation of caspase-9, which then stimulates caspase-3 and caspase-7. The extrinsic apoptotic pathway can be divided into two paths according to two groups of death receptors. The first group (Fas, TRAILR, DR4, DR5) recruits DISC followed a series of events, that is, caspase-8-mediated activation of caspase-3 and caspase-7. The second group (TNFR1, DR3, DR6) recruits the adaptor protein TRADD that links death receptor to TRAF2, RIPK1, cIAPs, and LUBAC, forming the signalling complex I. Further forms complexes IIa and IIb lead to activation of the caspase cascade that results in apoptosis. *Apaf-1* apoptotic protease-activating factor-1, *BH* BCL-2 homology, *CASP* caspase, *cIAPs* cellular inhibitor of apoptosis protein, *DD* death domain, *DISC* death-inducing signalling complex, *FADD* Fas-associated protein with death domain, *LUBAC* linear ubiquitin chain assembly complex, *RIPK1* receptor-interacting serine/threonine-protein kinase 1, *TNF* tumor necrosis factor, *TNFR1* tumor necrosis factor receptor 1, *TRADD* tumor necrosis factor receptor type 1-associated death domain, *TRAILR* tumor necrosis factor-related apoptosis-inducing ligand receptor, *TRAF2* tumor necrosis factor-receptor-associated factor 2. Sources: Refs. [25–32]

executioner caspase-3 and caspase-7, leading to cell death. Caspase-3, caspase-7, and caspase-9 are kept in check by *X-linked inhibitor of apoptosis protein* (XIAP); however, BAX and BAK also release from mitochondria a protein called *second mitochondria-derived activator of caspase* (SMAC, also known as DIABLO) and cytochrome C. The protein SMAC/DIABLO sequesters XIAP, thereby facilitating effector caspase activation (for the original articles, see [25–27]).

19.2.2.3   The Extrinsic Apoptotic Pathway
The extrinsic apoptotic pathway is governed by specialized TNF family death receptors such as TNFR1 and TNFR2, *TNF-related apoptosis-inducing ligand*

(TRAIL) receptor (TRAILR), and Fas (Fig. 19.3). As reviewed [38], death receptors contain a conserved cytosolic death domain (DD) (for death domain, see Box 19.1). The eight kinds of death receptors have different amino acid sequences that determine ligand specificity, and they can be divided into two groups according to the cytosolic adaptor protein that makes a distinct complex. These death receptors transmit signals from extracellular death ligands across the plasma membrane to engage the intracellular caspase machinery. Such death ligands include Fas ligand (FasL), TNF, and TRAIL (reviewed in [28–32]).

Box 19.1 What Is a Death Domain?

The death domain has initially been characterized as an intracellular homology domain of FAS (Apo-1) and the TNF receptor, which is important for apoptotic signalling in both receptors. Subsequently, several other proteins have been found to interact with the cytoplasmic part of either FAS or the TNF receptor in the region of the death domain. Overexpression of these proteins usually leads to cell death. By profile analysis, it was shown that several other proteins contain regions with significant similarity to the death domain. Interestingly, several of these proteins also work in the context of cell death signalling. The list of death domain-containing proteins comprises the FAS, TNF receptor, NGF receptor, several receptor-interacting proteins (MORT1/FADD, TRADD, RIP) all isoforms of ankyrin, the human "death-associated protein kinase" (DAP-kinase), and the adaptor molecule MyD88.

Further reading: Park HH, Lo YC, Lin SC, Wang L, Yang JK, Wu H. The death domain superfamily in intracellular signaling of apoptosis and inflammation. Annu Rev Immunol 2007;25:561–86.

When these ligands bind to their respective receptors, they recruit an adaptor protein such as *Fas-associated protein with death domain* (FADD) (reviewed in [38]). The protein FADD is composed of two domains, the DD and *death effector domain* (DED). The DD of FADD binds to the DD of the death receptor, and FADD recruits pro-caspase-8 through the DED–DED interaction, forming a *death-inducing signalling complex* (DISC), where pro-caspase-8 is activated by self-cleavage. Active caspase-8 and caspase-10 lead to proteolytic stimulation of downstream effector caspase-3, caspase-6, and caspase-7 to induce apoptotic cell death (Fig. 19.3).

The two groups of death receptors deserve some more words. The first group includes CD95/Fas, DR4/TRAIL-R1, and DR5/TRAIL-R2, all of which recruit DISC. This series of events, namely, caspase-8 directly mediated activation of caspase-3 and caspase-7, is sufficient to induce apoptotic cell death in a minority of cell types, also called type I cells such as lymphocytes and thymocytes. However, these consecutive events are insufficient in type II cells such as hepatocytes and pancreatic β cells because of the relatively low levels of DISC in spite of comparable levels of the DISC components. Thus, more often, caspase-8 drives apoptotic execution less directly by cleaving and thereby stimulating the

"BH3-only protein" BID. Truncated BID then engages the above-described cell-intrinsic pathway via BAX and BAK via activation of effector caspases through mitochondria (reviewed in [24]).

The second group of death receptors, including TNFR1, DR3, and DR6, recruit *the tumor necrosis factor receptor type 1-associated death domain* (TRADD) protein for an adaptor protein that links death receptors to TRAF2, RIPK1, *cellular inhibitor of apoptosis protein* (cIAPs), and *linear ubiquitin chain assembly complex* (LUBAC), forming a signalling complex called "complex I" (reviewed in [29, 30, 38]). Upon the ligation of death receptors with their specific ligands, complex I is assembled close to the plasma membrane to stimulate MAPK/JNK involved in cell survival, proliferation, or apoptosis. The RIP1-deubiquitinating enzymes remove K63-linked polyubiquitination, leading to internalization of this receptor complex. (Besides MAPK-signalling cascades, complex I also stimulates the NF-κB pathway, thereby promoting inflammation, host defense, and cell proliferation and survival.)

Moreover, the second group of death receptors can form further complexes, that is, complexes IIa, IIb, and IIc, which are assembled in the cytoplasm and have distinct signalling and functional outcomes (reviewed in [29, 30, 38]). Thus, the protein TRADD recruits FADD and caspase-8, forming complex IIa, whereas complex IIb is composed of RIPK1 and FADD/caspase-8 and is negatively regulated by cIAPs. Both the TRADD-dependent complex IIa and the RIPK1-dependent complex IIb lead to activation of a caspase cascade that results in TNF-induced cell death via apoptosis under specific circumstances. Of note, complex IIc also activates the necroptosis effector MLKL by a RIPK3-dependent mechanism—as will be outlined in more detail below in Sect. 19.3.2.

Notably, several regulatory types of machineries are involved in the death receptor-mediated extrinsic apoptosis pathway. For example, cellular *FLICE-like inhibitory proteins* (cFLIPs) are crucial regulators of death receptor signalling. In fact, cFLIP lacks protease function and is an essential regulator of apoptotic as well as non-apoptotic functions of caspase-8 (for review, see [39]).

19.2.3 Apoptosis and the Emission of DAMPs ("Immunogenic" Apoptosis)

Apoptosis has also been observed to emit DAMPs though by far less compared to subroutines of RN. The reason is clear: there is no rupture of the plasma membrane allowing DAMPs to leave the cell. Nevertheless, apoptotic cells have been shown to be able to secrete DAMPs actively, partially in terms of inducible DAMPs. For example, as documented in studies on a model of dying cancer cells [40], ATP can be released into the extracellular milieu from apoptotic cells in a controlled way and under involvement of pannexin-transmembrane protein channels that connect the intracellular with the extracellular space. In the extracellular compartment, eATP predominantly functions as a signalling molecule through the activation of purinergic P2 receptors (see also Part II, Sect. 5.3.4.3). Via this pathway, eATP acts as an inducible (Subclass IIIA-1) DAMP, thereby conferring a certain a degree of

immunogenicity to an apoptotic cell ("immunogenic apoptosis") (compare Part IV, Sect. 14.2.2.3).

Of note, there is another way of how apoptosis can gain immunogenic properties. If apoptotic cells are not removed by phagocytosis (efferocytosis), they progress to a lytic and inflammatory phase called secondary necrosis/pyroptosis. In this case, proteolytic cleavage of *deafness-associated tumor suppressor* (DFNA5) by caspase-3 triggers secondary necrosis. The protein DFNA5 belongs to the same gasdermin superfamily as GSDMD and has been implicated in the induction of cell death and as a putative tumor suppressor [5] (for GSDMD, see below, Sect. 19.3.4). Recent data demonstrate that this form of secondary necrosis is not an accidental epiphenomenon of apoptosis, but a finely regulated process with characteristic pathophysiological and therapeutic implications. The molecular machinery that controls secondary necrosis protrudes as a promising target for the development of innovative drugs that may increase the immunogenicity of cancer cells succumbing to treatment [41].

19.2.4 Résumé

Strikingly, humans—like all metazoans—have evolved a specialized ability to deliberately eliminate unwanted, old, unhealthy, or damaged cells through a form of regulated cell death that avoids induction of inflammation but maintains homeostasis: the apoptosis. The evolutionarily developed trick of this type of cell death is impressive and unique: engulfment of apoptotic cells by phagocytes before the plasma membrane gets disrupted, namely, the process of efferocytosis, prevents release of large amounts of DAMPs and, thus, avoids immunogenic properties of the dying cell (see Part VI, Sect. 22.6.3.3). Only in certain circumstances, via active secretion of DAMPs or development to secondary necrosis, apoptosis can be associated with minimal/moderate immunogenicity.

Of high interest is also the existence of two pathways: the intrinsic and extrinsic apoptosis signalling paths which converge at the level of the executioner caspases. Studies in invertebrates (*C. elegans*) revealed that the intrinsic pathway is relatively ancient and plays a crucial role in directing cell elimination during development and in tissue homeostasis. The fact that this path is often observed as the result of unsuccessful cell-intrinsic stress responses supports this assumption. By contrast, the extrinsic pathway appears to be a more recent evolutionary adaptation and is involved—when associated with cellular immunogenicity—in injury-induced immune function.

19.3   Regulated Necrosis

19.3.1 Introductory Remarks

Formerly, the definition of necrosis was restricted to an irreversible cell death in terms of an ACD, characterized by irreversible changes in the nucleus (karyolysis, pyknosis, and karyorrhexis) and in the cytoplasm (condensation and intense

eosinophilia, loss of structure, and fragmentation) [1]. Modern definition emphasizes the additional incidence of plasma membrane rupture.

However, the biological phenomenon of an ACD is not always and only the result of a severe excessive injury [42]. Indeed, more often, it does not occur in an uncontrolled, random, or arbitrary manner, at least not in non-traumatic infectious diseases. It is regulated by genetically determined signalling pathways, and cell death researchers who have explored the question of uncontrolled cell death for years have now unmasked the molecular pathways of RN. Excitingly, many RN pathways have meanwhile been described, and it has been and still is a major task to unravel the potential overlap and indistinguishable features of these paths. Indeed, this issue required some guidance from an active highly specialized expert in this field, which has been summarized in the *Guidelines of the Nomenclature Committee on Cell Death*, the most current version of which being in line with this subchapter [3].

As already often mentioned, necrosis, defined by plasma membrane rupture (PM-rupture)/membrane permeabilization, inevitably results in death of this particular cell. At this point the cell is dead but still existing and not gone. Simply by PM-rupture, the intracellular content is released into the interstitial environment, spreading out to other cells, ECM components, etc., and, importantly, gaining access to phagocytic cells such as leukocytes, macrophages, and APCs, thereby inducing innate and adaptive immune responses. For documentation, intravital microscopy has visualized the process of necrosis in vivo [43], and the factors that are released are described in Part IV, Sect. 12.2, as (Class IA) DAMPs, though, in some cases, they are also referred to as cell death-associated molecular patterns (CDAMPs) [44]. In the following, some subroutines of RN are outlined with a particular reference to molecular mechanisms of induction and signalling pathways.

19.3.2 Necroptosis

19.3.2.1 General Remarks

Among the pathways of RN, necroptosis can be regarded as the by far best studied RN subroutine, and translational medicine on necroptosis prevention has already reached phase II clinical trials. The biochemical processes in necroptosis are distinct from those found in apoptosis; in particular, there is no caspase activation. As such, necroptosis is a kinase-mediated cell death that relies on RIPK3-mediated phosphorylation of the pseudokinase MLKL (Fig. 19.4). Instead of reviewing all details of our current understanding of the necroptosis pathways, some aspects are sketched here that are biased to what in the perspective of the book is considered the most important facts to understand necroptosis as the origin of "necroinflammation" and "necroimmunity"; the quintessence of this topic in one sentence articulated is "dying cells actively regulate inflammatory and adaptive immune responses" [54, 55]. For comprehensive and competent reviews, the reader is directed to articles listed under Refs. [6, 7, 45–53].
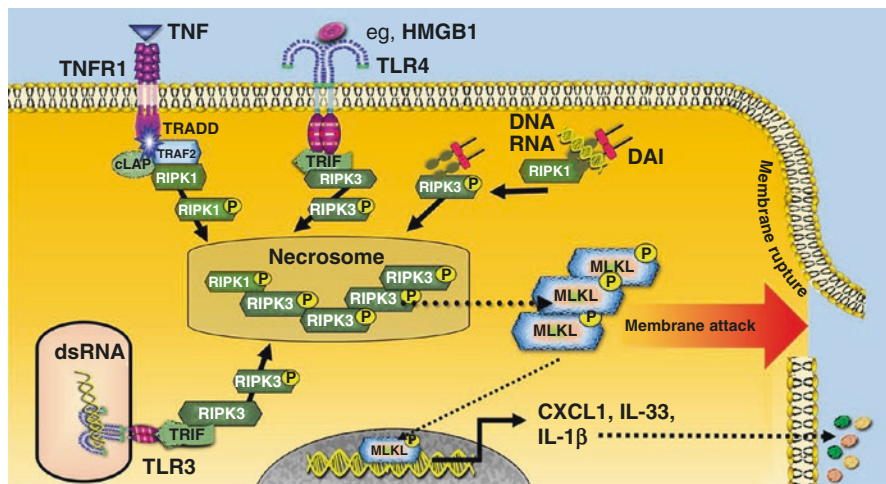
Fig. 19.4 Simplified scenario model of the necroptotic pathway. Necroptosis can be triggered by engagement of TNF to TNF receptor superfamily (e.g., TNFR1), Toll-like receptors (e.g., TLR3 and TLR4), or the DNA receptor DAI. In TNF-induced necroptosis, TNF binds to the TNF receptors and engages a complex formation, which includes TRAF2, cIAP, and RIPK1. RIPK1 recruits and activates RIPK3 that oligomerizes resulting in the formation of the necrosome. RIPK3 phosphorylates MLKL which functions as a necroptosis inducer by causing plasma membrane rupture. The concise mechanisms of plasma membrane rupture have yet to be defined. In parallel, phosphorylated MLKL translocates to the nucleus and results in transcription of CXCL1, IL-33, and IL-1β which are actively secreted. Engagement of the necrosome can also be a consequence of TLR signalling through TRIF/RIPK3 or DAI signalling through DAI/RIPK3. *cIAPs* cellular inhibitor of apoptosis protein, *DAI* DNA-dependent activator of IFN regulatory factors, *dsRNA* double-stranded RNA, *HMGB1* high mobility group box 1, *IL* interleukin, *MLKL* mixed lineage kinase domain-like protein, *RIPK1/3* receptor-interacting serine/threonine-protein kinase 1/3, *TLR* Toll-like receptor, *TNF* tumor necrosis factor, *TNFR1* tumor necrosis factor receptor 1, *TRADD* tumor necrosis factor receptor type 1-associated death domain, *TRAF2* tumor necrosis factor-receptor-associated factor 2, *TRIF* Toll/IL-1(TIR) domain-containing adaptor-inducing interferon-β. Sources: Refs. [6, 7, 45–53]

19.3.2.2   Induction of Necroptosis and Its Three Main Mediator Proteins

Necroptosis can be instigated by a variety of triggers, including TNF, Fas, TRAIL, LPS, DNA, dsRNA, IFNs, ER stress, viral infection, and anticancer drugs [6, 7]. Together—and notably—most of these triggers may be summarized as MAMPs and constitutive and inducible DAMPs. Receptors involved in the recognition of these triggers include death receptors, the members of the TNF receptor superfamily, IFN receptor, TLRs, NLRs, intracellular RNA and DNA sensors (e.g., ZBP1/DAI, see Part II, Sect. 5.2.6.3), and probably other mediators (Fig. 19.4) (for further details of those receptors, see Part II, Chap. 5, and for IFN/IFN receptors and TNF/TNF receptors, see Part VI, Sects. 22.5.5 and 22.5.7).

The enzyme RIPK1 was the first protein shown to be essential for TNF-induced necroptosis and possesses several domains that can activate different cellular

pathways: an N-terminal kinase domain, an intermediary domain, a C-terminal *RIP homotypic interaction motif* (RHIM), and a death domain. The enzyme RIPK3 is a homologous kinase of RIPK1 with a similar N-terminal kinase domain and an intermediate domain that can contribute to TNF-induced activation of NF-κB, if overexpressed. RIPK3 also contains an RHIM domain at its C-terminus but lacks the C-terminal death domain compared to RIPK1. RIPK3 kinase activity appears to be essential for necroptosis. The pseudokinase MLKL is another critical mediator of necroptosis and is regarded as the sole and central effector of necroptosis. The protein possesses an N-terminal four-helical bundle domain, which directly or indirectly results in membrane pore formation.

19.3.2.3   Signalling Pathways of Necroptosis and Formation of Necrosomes

The necroptosis signalling pathway is controlled predominantly by kinases and E3 ligases, and the three most important proteins dominating this pathway are the already mentioned kinases RIPK1 and RIPK3 and the pseudokinase MLKL. These proteins lead to the formation of multiprotein complexes, the so-called canonical necrosome representing the RIPK1-RIPK3 model and the non-canonical necrosome which does not involve RIPK1 kinase activity but is dependent on the cytosolic DNA sensor ZBP1/DAI. In fact, the signalling mechanisms involved in the necroptotic pathway are complex and complicated. Guided by recent reviews [6, 7], they are sketched here in an oversimplified fashion only, by focusing on TNF-induced necroptosis as the best characterized necroptotic pathway. Notably, the TNF-induced signal transduction complexes can end up with either cell survival or cell death.

Canonical Necrosome Formation

Upon TNF stimulation, RIPK1 and the intracellular adapter molecule TRADD are independently recruited to the TNFR1 by their DD domains to form the TNF receptor complex (denoted as complex I) which essentially prevents necrotic cell death [56–61] (for complex I, compare its role in apoptosis above in Sect. 19.2.2.3). At this complex I, RIPK1 is ubiquitylated in its intermediate domain, allowing the recruitment of the IκB kinase complex (*NF-kappa-B essential modulator* (NEMO), IκB kinase alpha (IKKα), and IKKβ; see also Part VI, Sect. 22.3.3.2). Subsequent activation of NF-κB and MAP kinases leads to transcription of pro-survival genes [62–64]. Thus, IKKα- and IKKβ-dependent phosphorylation of RIPK1 prevents its dissociation from the receptor and hence prevents the formation of a cytosolic pro-cell death complex, denoted as complex II.

Importantly, upon deubiquitination of RIPK1, pro-caspase-8 may be recruited to this complex via a DD that binds the DD of FADD. This results in the forced proximity of pro-caspase-8 and activation by proteolytic cleavage of the caspases to form a functional caspase-8 homodimer capable of cleaving effector caspases, such as caspase-3, caspase-6, and caspase-7, to mediate and execute apoptosis [64]. On the other hand, de-ubiquitylation and particular dephosphorylation may lead to dissociation of RIPK1, allowing it to form the cytosolic pro-cell death

complex II. Two types of complex II can be distinguished (IIa and IIb) depending on the composition of complex II and the activity of the proteins therein. Importantly, while complex IIa and IIb induce apoptosis, they can also induce necroptosis if caspase-8 is inactive or absent. In case caspase-8 is inactive or absent, RIPK1 in complex II recruits and activates RIPK3 resulting in the formation of the necrosome. The necrosome formed from complex IIa consists of TRADD, RIPK1, RIPK3, and FADD, while the necrosome resulting from complex IIb lacks TRADD (Fig. 19.4) (reviewed in [65–73]). The enzyme RIPK3 in the necrosome is present in a phosphorylated manner, and it is not entirely clear to which extent RIPK1 or RIPK3 itself mediates this phosphorylation. However, the necrosome is stabilized by HSP90 and CDC-37, two chaperones that are required for the fully active necrosome activity [74, 75]. As a constitutive binding partner of RIPK3, MLKL is incorporated in the necrosome. Taken together, and as competently argued by Grootjans et al. [7], "*a working model on the interaction between RIPK1 and RIPK3 emerges where inactive RIPK1 initially exists in a closed conformation at the TNFR1, where its DD and RHIM are unavailable for the formation of pro-cell death complexes. De-ubiquitylation and particular dephosphorylation may open up RIPK1, allowing it to form a cytosolic complex II. Activation of RIPK1 by a still to be discovered mechanism then results in its autophosphorylation and activation. Depending on the availability of caspase-8, activation of RIPK1 then allows either activation of caspase-8 or RIPK3 within complex II, which results in, respectively, apoptosis or necroptosis*."

Non-canonical Necrosome Formation

By contrast to canonical RIPK1 → RIPK3 activation model, non-canonical necrosome formation has been proposed for the scenarios of dsRNA-induced necroptosis in fibroblasts and ECs and CMV-induced necroptosis [7]. Thus, CMV viral infection of primary target cells results in RIPK3-dependent necroptosis, which does not involve RIPK1 kinase activity but is reliant on the sensor DAI [76–78]. Of note, as recently reported [79], interrogation of murine CMV-encoded cell death suppressors revealed that necroptosis functions as a trap door to eliminate virally infected cells, thereby stressing necroptosis as a robust innate immune tool of antiviral defense.

Similarly, in other lines of studies, upon infection with IAV, the receptor DAI was demonstrated to recognize IAV genomic RNA, associated with RIPK3 and to be required for recruitment of MLKL and RIPK1 to RIPK3. These results identify DAI as a link between IAV replication and RIPK3 activation and implicate DAI as a sensor of RNA viruses [80]. In other words: these recent findings show that viruses such as murine CMV and IAV infections drive DAI association with RIPK3, leading to its activation and necroptosis [81] (Fig. 19.5). The process of necroptosis then is associated with passive release of large amounts of DAMPs. Intriguingly, these scenarios are in strong support of the concept that DAMPs—here emitted in the course of virus-induced necroptosis—mediate an efficient and robust antiviral defense program. In fact—as will be outlined in detail in Part VI and Part VIII—DAMPs activate cells of the innate immune system, for example, macrophages to mount an
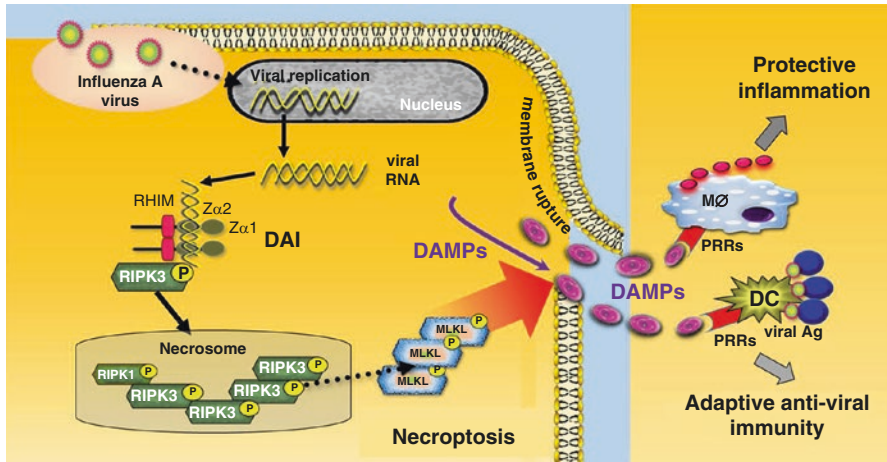
Fig. 19.5 Simplified scenario model of necroptosis caused by infectious agents. Upon infection with Influenza A virus, the receptor DAI senses genomic viral RNA and associates with RIPK3 to recruit RIPK1, RIPK3, and MLKL. This scenario identifies DAI as a link between viral replication and RIPK3 activation. Subsequent necroptosis is associated with passive release of DAMPs which activate innate immune cells such as macrophages and dendritic cells to elicit a protective inflammatory response and antigen-specific adaptive antiviral immune response. *Ag* antigen, *DAI* DNA-dependent activator of IFN regulatory factors, *DC* dendritic cell, *MØ* macrophage, *MLKL* mixed lineage kinase domain-like protein, *PRRs* pattern recognition receptors, *RHIM* RIP homotypic interaction motif, *RIPK1/3* receptor-interacting serine/threonine-protein kinase 1/3, *Zα1/2* Z-form nucleic acid-binding domain ½. Source: Ref. [80]

inflammatory response, and promote the capacity of DCs to present and cross-present viral antigens to T cells, thereby eliciting adaptive antiviral immunity.

19.3.2.4   Direct and Indirect Necrosome Formation by Different Stimuli

Finally, it is worthwhile to stress again that many stimuli can elicit necroptosis in a direct and indirect way. The direct necrosome formation involves the canonical pathway that requires RIPK1 kinase activity and the non-canonical pathway that is dependent on the TRIF adaptor or the cytosolic DNA sensor DAI. In the indirect way, stimuli elicit the production of TNF which on its turn activates the canonical pathway. These stimuli include the activation of death receptors, stimulation of TLRs such as TLR4 or TLR3, and the RNA receptor RIG-I (as highlighted in [7]). For example, in fibroblasts and ECs, TLR3 stimulation was found to result in RIPK3- and MLKL-dependent necroptosis which can proceed independently of RIPK1 kinase activity but requires the adapter protein TRIF [82] (for TRIF, see also Part VI, Sect. 22.3.3.4). In other words, the emission of DAMPs during a first round of cell injury may lead to necroptosis which is associated with another round of DAMP emission serving as stimuli for PRMs. It should be allowed here to discuss whether this scenario may contribute to clinical manifestations such as chronic inflammation and autoimmunity.

19.3.2.5   Execution of Necroptosis: MLKL Activation

In humans, the execution of necroptosis begins with active RIPK3-mediated phosphorylation of the activation loop of the pseudokinase MLKL (pMLKL), a process that requires an active necrosome structure (Fig. 19.4). The enzyme exposes a four-helical bundle domain upon (1) phosphorylation by RIPK3 in its activation loop and (2) the dephosphorylation of a persistent phosphate residue in the hinge region between the helical bundle and the default protein [83–85]. Once pMLKL is fully active, it reportedly oligomerizes and binds to *phosphatidylinositol-4,5-bisphosphate* (PIP2) located in the plasma membrane. This molecule is a multifunctional lipid that regulates several essential subcellular processes in eukaryotic cells [86, 87]. It has been proposed that pMLKL forms pores in the plasma membrane to mediate plasma membrane rupture [87], but this remains to be demonstrated in cells or in vivo.

Of note, more recent studies provided evidence indicating that MLKL can activate the innate immune receptor NLRP3 in a cell-intrinsic manner [88]. Indeed, these new data emphasize that MLKL-mediated NLRP3 and caspase-1 activation and the secretion of IL-1β—operating as an inducible DAMP—are a significant determinant of necroptotic-derived inflammatory signals. Notably, however, GSDMD, the pore-forming caspase-1 substrate required for efficient NLRP3-triggered pyroptosis and IL-1β release, was not essential for MLKL-dependent death or IL-1β secretion (for activation of NLRP3, see Part VI, Sect. 22.4.2, and for pyroptosis, see below, Sect. 19.3.4).

Interestingly, other lines of studies showed that, during necroptosis, MLKL-dependent calcium influx and PS exposure on the outer leaflet of the plasma membrane preceded loss of plasma membrane integrity. Activation of MLKL results in the generation of broken, membrane "bubbles" with exposed PS that are released from the surface of the otherwise intact cell. A distinct machinery, the so-called *endosomal sorting complexes required for transport*-III (ESCRT-III) machinery, is needed for the formation of these bubbles and acts to sustain survival of the cell when MLKL activation is limited or reversed. Under conditions of necroptotic cell death, ESCRT-III controls the duration of plasma membrane integrity [89]. In other words, the ESCRT-III-mediated plasma membrane repair provides an extension of the time to death during necroptosis. As a consequence of this action of ESCRT-III, cells undergoing necroptosis can express regulatory immunomodulatory molecules such as CXCL1, IL-33, and IL-1β and promote antigenic cross-priming of CD8+ T cells [89–92] (for IL-33, see Part VI, Sect. 22.5.6.4; for cross-priming of CD8+ T cells, see Part VIII, Sects. 31.3.5 and 32.5.4).

Here, the ESCRT-III phenomenon raises a word of caution in general regarding the functional consequences of necroptosis. In fact, as also discussed by Garg and Agostinis [93] and Kearney and Martin [94], despite its primarily dominant pro-inflammatory/immunogenic phenotype, additional release/secretion of SAMPs and/or anti-inflammatory/immunosuppressive cytokines may immunomodulate the final outcome of this type of cell death. For example, consequent processes such as mitigated acute inflammation → chronic inflammation or decreased immunogenicity → tolerogenicity may context-dependently develop. Indeed, this kind of discussion is

emerging regarding the issue of different immunogenicity associated with the various subunits of RCD, a point that will be resumed below in Sect. 19.3.7.

Howsoever the precise execution mechanism of necroptosis may be arranged, necroptosis can be understood as a signalling pathway to defend against microbes, especially viruses that express caspase inhibitors [45, 95]. Interestingly, as insinuated elsewhere [96], this pathway can be regarded as a form of cell suicide, where specific pro-death proteins such as RIPK3 and MLKL are newly expressed and up-regulated by various stimuli to promote death (in contrast to the ferroptotic pathway that is regarded as sabotage—see next section).

19.3.2.6   Concluding Remarks

Necroptosis has attracted tremendous attention because it impressively reflects a paradigm for the new concept of injury-/cell death-induced immunity in general, probably the most widespread unmet clinical need in modern medicine. In fact, necrosis occurs during many pathologies, such as trauma and infections, stroke, myocardial infarction, and transplantation, just to mention a few of them. Of note, since necroptosis has been shown to be also induced by viruses, it is increasingly discussed as a robust DAMP-based cell death-induced antiviral defense program.

And, again, we meet the golden thread of this book here: When controlled, necroptosis leads to rapid homeostatic recovery; however, when uncontrolled, for example, when associated with excessive or chronic-repetitive emission of large amounts of DAMPs, this cell death may be promoting acute hyperinflammation-based disorders such as ARD and SIRS or chronic autoinflammatory diseases.

Hence, not surprisingly, therapeutic strategies have been envisioned to prevent necroptosis, for example, by use of RIPK1 kinase inhibitors. Accordingly, the first compound developed under this notion was necrostatin-1 (Nec-1) [97], a small molecule and a hydantoin [98–100]. Several other agents followed, but currently, a certain disillusion regarding their effectivity so far observed can be noticed. Although necroptosis inhibitors tested so far showed inhibitory effects against several inflammation-mediated disorders, only a few have passed to the stage of clinical testing and need extensive research for therapeutic practice. As stated elsewhere [101], "*revisiting the existing drugs and developing novel necroptosis inhibiting agents as well as understanding their mechanism are essential. A detailed study of necroptosis function in animal models of inflammation may provide us an alternative strategy for the development of drug-like necroptosis inhibitors*."

19.3.3   Ferroptosis

19.3.3.1   General Remarks

Ferroptosis is an iron-dependent, oxidative form of RN that does not share morphological, biochemical, or genetic similarities with other forms of non-apoptotic cell death. Instead, morphological features of ferroptosis include normal-sized nucleus free of chromatin condensation and dense miniature mitochondria with vestigial cristae. Biochemically, this subroutine of RCD is characterized by the accumulation

of ROS from iron metabolism, NADPH oxidase activity, and lipid peroxidation products, predominantly derived from peroxidation of PUFAs.

Lipid peroxidation is generally considered as a process under which oxidants such as ROS attack lipids containing carbon–carbon double bond(s), especially PUFAs that involve hydrogen abstraction from a carbon, with oxygen insertion resulting in lipid peroxyl radicals and hydroperoxides. Oxidation of lipids can occur via three distinct mechanisms: (1) enzymatic oxidation by enzymes such as lipoxygenases, cyclooxygenases and cytochrome P450, (2) non-enzymatic, ROS-mediated oxidation, and (3) non-enzymatic, non-free radical oxidation. Each oxidation mechanism leads to generation of specific products with high stereospecificity (for reviews, see [102–106]). Major products of lipoxygenase oxidation of PUFAs depend on their substrate and refer to various oxidized molecules including leukotrienes, lipoxins, and hydroperoxyeicosatetraenoic acids (HpETEs). Oxygenation by cyclooxygenases results in the generation of prostaglandins, prostacyclins, and thromboxanes, whereas cytochrome P450 generates epoxyeicosatrienoic acids (EETs), thromboxanes, and prostacyclins (reviewed in [107, 108]). Non-enzymatic mechanisms are mediated by ROS that are generated by ROS-producing enzyme systems such as NADPH oxidases and nitric oxide synthases in the presence of transition metal ions (Fe^{2+} and Cu^{2+}) and result in a mixture of non-specific stereoisomers (reviewed in [106, 108]). Moreover, the enzyme myeloperoxidase has also been shown to instigate non-enzymatic lipid peroxidation through the generation of ROS (discussed in [106, 109]). Notably, by contrast to products of enzymatic lipid oxidation, the products of directly ROS-mediated non-enzymatic processes are regarded to be more toxic and harmful to the host [110].

As recently comprehensively reviewed by Bochkov et al. [106], OxPLs can be divided into two structural groups: non-fragmented (full-chain) OxPLs or oxidatively fragmented OxPLs. Both full-chain and fragmented OxPLs were found to contain one or more oxygen-containing functional groups such as hydroperoxy, hydroxy, keto, epoxy, and prostane groups as well as aldehydic or carboxylic ω-terminal groups. Notably, particularly oxidatively fragmented OxPLs can be chemically reactive, as, for example, α,β-unsaturated aldehydes that are highly electrophilic and rapidly interact with nucleophilic groups which often leads to the damage of biomolecules. In fact, these highly reactive terminal degradation products refer to reactive aldehydes 4-HNE, MDA, and CEP [111, 112], that is, OSEs described in Part IV, Sect. 13.3.2.3, as Subclass IIB-1 DAMPs.

Under homeostatic conditions, such lipid peroxidation products that are generated by both enzymatic and ROS-mediated mechanisms modulate and orchestrate a multitude of physiological processes; however, when present in too high concentration, they can cause cell death in the form of ferroptosis. Guided by recent review articles ([8, 96, 113–116]), some aspects of this emerging form of RN are briefly sketched here.

19.3.3.2   The Signalling Pathways of Ferroptosis

The ferroptosis pathway has been recently reviewed in detail [8, 117] and may be best explained by firstly pointing toward homeostatic conditions concerned. Thus,

under normal circumstances, free highly reactive intracellular iron is dangerous and therefore bound to ferritin [118, 119], thereby controlling the oxidative capacity of free iron and the amount of oxygen radicals. For example, heavy chain ferritin was demonstrated to be protective in models of IRI [120], and the role of iron in AKI has been well established [121]. When the term ferroptosis was first ascribed to the process of iron-catalyzed RN, iron chelation (a type of bonding of ions and molecules to iron ions) was demonstrated to prevent this deadly signal [122]. Also, under homeostatic conditions, the enzyme phospholipid-hydroxyperoxid-glutathione-peroxidase (GPX4 or PHGPx), a selenoprotein, plays an essential role in avoiding iron-dependent lipid peroxidation which is mediated by a unique enzyme, the arachidonate lipoxygenase (ALOX). In fact, constitutive ALOX activation is antagonized by GPX4 which employs glutathione (GSH) to reduce H_2O_2 to GSSG (oxidized glutathione) and H_2O. Of note, besides recycling by the glutathione reductase, intracellular concentrations of GSH are maintained by the activity of the GSH synthase which requires the substrates glutamine, cysteine, and glycine to function. A glutamate/cystine "antiporter" (i.e., a co-transporter involved in secondary active transport of molecules or ions across a membrane) in the plasma membrane referred to as "system XC-minus" provides the cells with cystine (Cys–Cys disulfide, Cys_2). This amino acid is then intracellularly metabolized to cysteine, the rate-limiting amino acid for GSH synthesis [123]. Together, these observations show that GPX4—indirectly—inhibits lipid peroxidation and, thus, ferroptosis. On the other hand, the loss or inducible depletion of GPX4 was found to result in lethality, for example, in early embryonic lethality, or in death of neurons or renal tubular cells later during life [100, 124–128]. It is worth noting here that, under normal conditions, production of ROS as generated in the course of cellular metabolism is kept in check by antioxidative mechanisms operating in the course of the oxidative stress response (see above, Sect. 18.3.3).

From this scenario, it becomes clear that disruption of this intracellular thiol antioxidant network, caused by (1) loss/inactivity of intracellular GSH, (2) pharmacological inhibition of GPX4, and/or (3) loss of the GPX4 protein, results in spontaneously occurring ferroptosis. Hence, iron-dependent ALOX-mediated peroxidation of lipids (predominantly PUFAs but also other membrane lipids such as phosphatidylethanolamine and phosphatidylinositol-4,5-bisphosphate) leads to the production of toxic "lipid ROS" (i.e., lipid hydroperoxides and other products) which lead to subsequent loss of plasma membrane integrity, that is, ferroptosis [100, 115, 129] (Fig. 19.6). Intriguingly, this ferroptotic pathway—in comparison to the necroptotic path that is regarded as a form of cell suicide—can be seen as a form of "sabotage" [96], where cell death results from disruption of the operation of an essential process leading to death without the involvement of dedicated cell death machinery. No death-promoting molecules are newly up-regulated, no caspases, no kinases whatsoever!

Another possibility that may lead to lipid peroxidation and subsequent ferroptosis is the production of ROS in excess as, for example, observed in pathological situations of IRI [130, 131]. In this case, the oxidative stress responses are overcharged to scavenge the free radicals. Involvement of such a mechanism is supported by
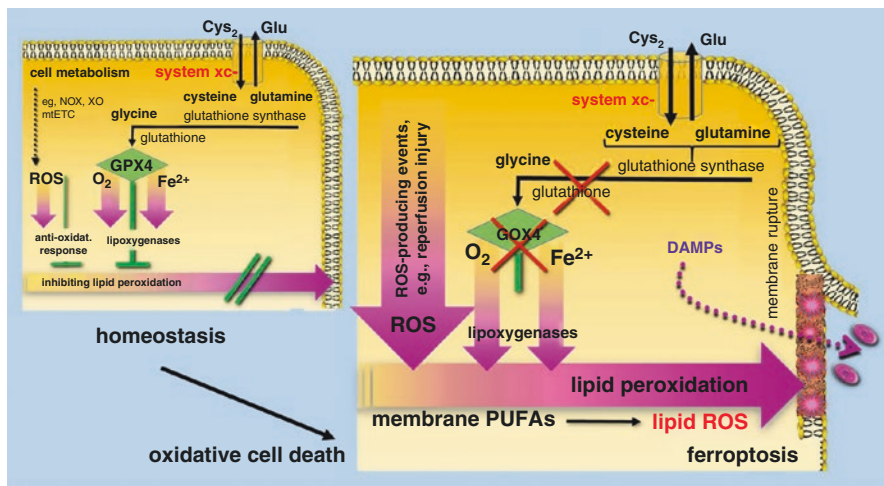
Fig. 19.6 Simplified scenario model of the ferroptotic pathway. This figure shows the homeostatic scenario (left) and the oxidative form of a non-apoptotic cell death, that is, ferroptosis (right). Ferroptosis is due to disruption of the intracellular thiol antioxidant network, caused by loss/inactivity of intracellular GSH, pharmacological inhibition of GPX4, and/or loss of the GPX4 protein. Iron-dependent lipoxygenase-mediated peroxidation of lipids (predominantly PUFAs) leads to the production of toxic "lipid ROS" which lead to subsequent loss of plasma membrane integrity associated with passive release of DAMPs. In addition, production of ROS in excess may lead to lipid peroxidation. *anti-oxid. response* antioxidative stress response, *Cys₂* cystine, *Glu* glutamate, *GPX4* phospholipid-hydroxyperoxid-glutathione-peroxidase, *mtECT* mitochondrial electron transport chain, *NOX* nicotinamide adenine dinucleotide phosphate-dependent oxidase, *PUFAs* polyunsaturated fatty acids, *ROS* reactive oxygen species, *XO* xanthine oxidase. Sources: Refs. [100, 115, 129]

recent studies providing first evidence that the Keap1 ↔ Nrf2 pathway as described above in Sect. 18.3.3.3 diminishes the ferroptotic cell death [132].

Of note, the molecular events that occur downstream of lipid ROS to cause irreversible cell death are unclear. Fragmentation of PUFA and membrane lipid damage may be sufficient to permeabilize the plasma membrane irreversibly. Alternatively or in parallel, reactive lipid intermediates generated following PUFA oxidation could promote cell death by covalently modifying and inactivating essential intracellular proteins (discussed in [116].

19.3.3.3   Induction of Ferroptosis

There are several compounds identified as ferroptosis inducers (FINs). Ferroptosis can be induced by experimental compounds such as erastin, *Ras-selective lethal small molecule 3* (RSL3), and buthionine sulfoximine or clinical drugs including sulfasalazine, sorafenib, and artesunate in cancer cells and certain normal cells (e.g., kidney tubule cells, neurons, fibroblasts, and T cells) [96, 113]. The compound erastin compromises the activity of system XC-minus by shifting the balance to less GSH production, less redox capacity of GPX4, and more lipid peroxidation and

therefore is one of the FINs. Alternatively, ferroptosis can be experimentally induced by the direct GPX4 inhibitor RSL3 and several other ferroptosis-inducing agents [129, 133]. Importantly, as a characteristic sign of RCD pathways, ferroptosis was shown to be extensively metabolically regulated [134], and several proteins involved in ferroptosis have been detected in a human haploid-cell screen [135], though their exact role in the pathway is still elusive. More clearly, in a genetic approach, the deletion of the GPX4 gene locus was found to result in lethality [124, 125, 128], and the conditional deletion of GPX4 from renal tubular cells reportedly resulted in tubular cell ferroptosis within 48 h following the inducible gene knockout.

19.3.3.4   Ferrostatins

Discovering compounds and mechanisms for inhibiting ferroptosis has been of considerable interest in recent years. This has led to the development of ferrostatins that were found to be the most potent small molecules for the prevention of IRI by a single compound [131, 136]. No other compounds yielded a comparable level of protection from isolated renal tubules or in vivo in kidney and liver models. Originally found in a screen for inhibitors of erastin-induced ferroptosis in HT1080 cells (a fibrosarcoma cell line), ferrostatin-1 (Fer-1) was identified [122] that basically acts as a lipid antioxidant. Fer-1 is the most commonly used compound to study ferroptosis in cells and thought to be as efficient as Nec-1, which also functions as an effective inhibitor of ferroptosis [100]. Moreover, the plasma half-life and the absorption in liver liposomes along with the plasma stability are far from optimal, and the statistically significant effects seen in in vivo models [131, 137] most likely underestimate the therapeutic preclinical potential of ferrostatins. Another inhibitor, the compound 11–92, was much more efficient than Fer-1 in preventing tubular necrosis [138], and another inhibitor, the compound 16–86, was shown to convey strong effects in preclinical models of AKI. Further compounds are currently under development such as liproxstatin-1 that was developed and investigated in a model of liver IRI with substantial beneficial effects [100]. Future studies will undoubtedly lead to the development of safer and more efficient agents.

19.3.3.5   Concluding Remarks

Ferroptosis has been recently identified as a new subroutine of immunogenic RN that, like necroptosis, confirms the concept of cell death-induced immunity. Ferroptosis is activated explicitly by failing or dysfunctioning GPX4 activity but probably also by GPX4-independent mechanisms such as failure of the oxidative stress response in case of excessive ROS production. For the future, it will be interesting to know whether ferroptosis involves the release of large amounts of specific DAMPs (what can be expected) and, if so, whether these are unique to ferroptosis or common across different forms of RN. Notably, induction of ferroptosis is increasingly recognized as a critical form of cell death that may contribute to acute and chronic human diseases, such as IRI following myocardial infarction or cerebral stroke, major neurodegeneration, and neuroinflammation, that is, disorders associated with significant cell death. Plausibly, this new knowledge has motivated researchers and the pharmaceutical industry to search for safe and effective compounds able to inhibit this form of RN. Doubtlessly, new developments in this field of modern therapeutic modalities will be soon published.

19.3.4 Pyroptosis

19.3.4.1 General Remarks

Different from necroptosis and ferroptosis but like apoptosis, pyroptosis is executed by caspases, here denoted as inflammatory caspases. This subroutine of RCD can be induced via caspase-1, human caspase-4 and caspase-5, or mouse caspase-11. The pyroptotic cell death has mainly been observed to occur in phagocytes of the myeloid lineage, such as macrophages and DCs, but is also documented in enterocytes, epithelial cells, ECs, and keratinocytes. This may be due to the fact that these cell types may express higher levels of the inflammatory caspases that drive pyroptosis. The activation process of these caspases is of utmost importance and, thus, concisely outlined here.

19.3.4.2 Activation of Pyroptosis-Driving Caspases Within Inflammasomes

The pyroptosis-driving caspases are activated within inflammasomes, which are macromolecular protein complexes composed of inflammasome-initiating receptors/sensors and inflammatory caspases, in the presence or absence of the inflammasome adapter ASC. Previously, in Part II, Sects. 5.2.3.3 and 5.2.5.2, the inflammasome-forming NOD receptors/sensors and AIM2 receptor have already been briefly introduced. These inflammasome-forming PRMs are known to sense MAMPs derived from a variety of bacteria (e.g., via NLRP3) and viruses (via AIM2) as well as DAMPs emitted during various sterile stressful and injurious conditions. Activation of inflammasome-associated inflammatory caspases promotes cleavage of pro-IL-1β and pro-IL-18 to form mature cytokines IL-1β and IL-18. In parallel, these caspases drive cleavage of the pro-pyroptotic factor GSDMD by generating an N-terminal fragment that oligomerizes to form pores on the host cell membrane and cause the lytic demise of the cell. The pyroptotic cell death leads to the release of the IL-1β/IL-18 inflammatory hallmarks associated with inflammasome activation. According to new notions, the key cytokine IL-1β can be regarded as an inducible DAMP (see Part IV, Sect. 14.3.2.3).

The diverse inflammasomes and their mechanisms of activation are discussed in the next Part VI, Sect. 22.4; here, the focus will be directed to the inflammatory caspase-driven, GSDMD-induced lytic cell death. Remarkably, a wealth of reports on pyroptosis and inflammasomes has recently been published which can be found under Refs. [9, 121–124, 139–150]).

19.3.4.3 Gasdermin D: The Key Executer of Pyroptosis via Pore Formation

In the initial phase of the pyroptotic pathway, the caspases are activated within the inflammasomes whose activation is the consequence of the interaction of their sensors/receptors with MAMPs and/or DAMPs (Fig. 19.7). Although the molecular mechanisms of pyroptosis have not been fully elucidated, it was recently demonstrated that cleavage of GSDMD by these inflammatory caspases is critical for pyroptosis to occur, that is, caspase-1 and caspase-11 in murine macrophages and their human orthologues caspase-1, caspase-4, and caspase-5. Gasdermin D is a
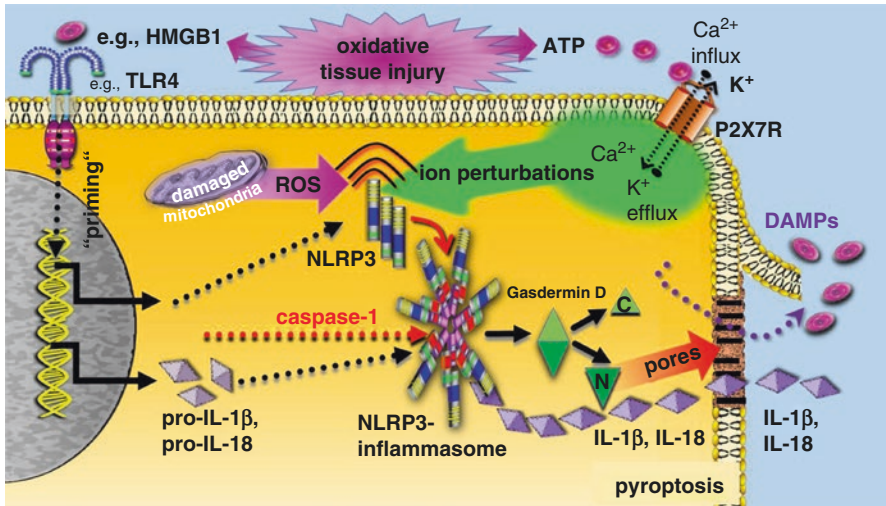
Fig. 19.7 Simplified scenario model of the pyroptotic pathway. Pyroptosis is induced by caspases which are activated within inflammasomes, here exemplified by the NLRP3 inflammasome as activated by DAMPs (HMGB1 and ATP) emitted in the course of oxidative tissue injury. In this example of canonical inflammasome activation, HMGB1 triggers TLR4-mediated transcription and translation of NLRP3 and pro-IL-1β/pro-IL18 (priming step), whereas ATP leads indirectly via induction of ion perturbation (K+ efflux) to final activation (activation step). Additionally, mitochondria-derived ROS serve as co-activating signal (for further details of activation, see Part VI, Sect. 22.4.2.2). Upon activation, the human inflammatory caspase-1 cleaves Gasdermin D, releasing the N-terminal domain from the autoinhibitory Gasdermin D–C domain. Activated Gasdermin D-N then binds to lipids in the plasma membrane and forms large pores, leading to release of cellular contents such as mature processed IL-1β and IL-18 as well as cell death. *ATP* adenosine-5-triphosphate, *HMGB1* high mobility group box 1, *K+* potassium, *NLRP3* nucleotide-binding oligomerization domain-like receptor family pyrin domain-containing 3, *P2X7R* P2X purinoceptor 7, *ROS* reactive oxygen species. Sources: Refs. [9, 139–145]

member of the GSDM family which is emerging as mediators of RCD in a variety of processes that regulate cellular differentiation and proliferation. The GSDM family of proteins is conserved in vertebrates and comprises, in humans, four paralogous termed GSDMA, GSDMB, GSDMC, and GSDMD [142].

According to current notions, the caspases mentioned above cleave the protein GSDMD releasing its N-terminal domain from inhibition by its C-terminal domain. In fact, evidence suggests that, under homeostatic conditions, the GSDMD C-terminus has an autoinhibitory effect on the intrinsic activity of the pyroptosis-inducing GSDMD N-terminus. This cytotoxic N-terminal fragment is then released and targets/binds to phospholipids (phosphoinositide and cardiolipin) on the host plasma cell membrane [151]. Importantly, before its migration to the membrane, GSDMD N-terminus transits from a monomer to an oligomer molecule that is initiated by caspase-mediated removal of the C-terminal autoinhibitory domain. Oligomerized GSDMD N-terminus leads to its insertion into the membrane resulting in the GSDMD pore formation. The pores mostly have an inner diameter of 12–14 nm with approximately 16 symmetric protomers. Processed GSDMD N-terminus, via its affinity for cardiolipin and phosphatidylserine, may also target and kill intracellular bacteria.

GSDMD N-terminus pore formation in the host cell leads to pyroptosis to produce apoptotic body-like cell protrusions ("blebbing") followed by rapid rupture of the plasma membrane. Notably, the precise mechanism of plasma membrane blebbing and rupture is currently unknown. Importantly, current notion hold that IL-1β and IL-18, formerly believed to be actively secreted, are released from the cell upon this membrane rupture, probably through those pores (reviewed in [143–146, 151, 152]).

Finally, it is also worth mentioning here that the two major functions of the inflammasomes, GSDMD cleavage and maturation of IL-1β and IL-18 (as outlined in more detail in Part VI, Sect. 22.4), appear to happen in a mechanistically distinct fashion, as oligomerization-deficient ASC is required for IL-1β maturation but dispensable for GSDMD cleavage [149].

19.3.4.4 Concluding Remarks

Research in inflammasome biology is a rapidly expanding field, and, during the past 5 years—as reflected by a growing number of publications (see above)—tremendous progress has been made in the understanding of inflammasome activation and function. One hallmark of inflammasomes certainly refers to their role in the execution of pyroptosis. Thus, detecting and responding to microorganisms that invade host cells is critical for clearing life-threatening infections. Again, as with necroptosis, pyroptosis passively releases large amounts of constitutive Class IA DAMPs as well as inducible IIIB-1 DAMPs in the form of processed bioactive IL-1β which all together, via amplification of further innate immune processes and promotion of subsequent adaptive immune responses, mediate robust antibacterial and antiviral defense programs. An example is illustrated in Fig. 19.8.
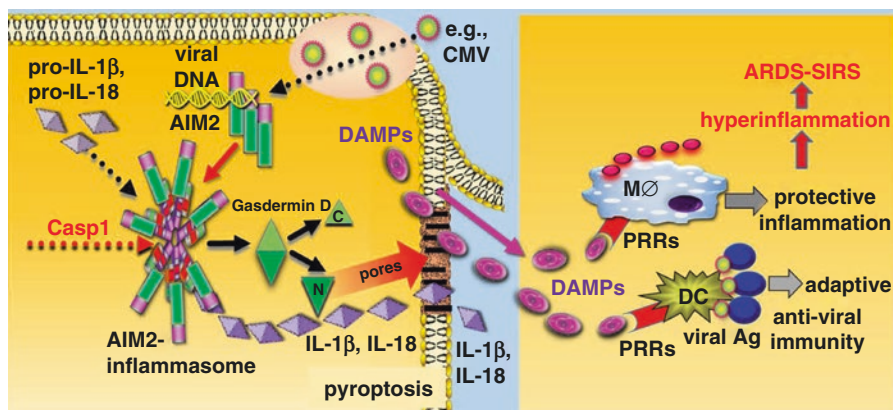

Fig. 19.8 Simplified scenario model of pyroptosis caused by infectious agents (exemplified by virus-activated AIM2 inflammasome). In the course of viral DNA-triggered activation of the AIM2 inflammasome (e.g., by CMV), activated caspase-1 (like NLRP3 inflammasome-activated caspase-1) initiates pyroptosis by cleaving gasdermin D leading to cell death. DAMPs are passively released through Gasdermin D-N-induced pores and activate innate immune cells such as macrophages and dendritic cells to elicit a protective inflammatory response and antigen-specific adaptive antiviral immune response. Of note, excessive/systemic release of DAMPs in this situation can lead to hyperinflammation resulting in ARDS/SIRS. *AIM2* absent in melanoma 2, *ARDS* acute respiratory distress syndrome, *CASP1* caspase-1, *CMV* cytomegalovirus, *DC* dendritic cell, *MØ* macrophage, *SIRS* systemic inflammatory response syndrome. Sources: Refs. [9, 139–145]

However, the "bitter pill is just around the corner": uncontrolled pyroptosis associated with excessive or chronic-repetitive emission of DAMPs (constitutive and inducible DAMPs) can become detrimental in promoting acute hyperinflammation-based disorders such as ARD and SIRS or chronic autoinflammatory diseases. Hence, to prevent such disasters, further research work is necessary to fully understand the exact mechanisms of pyroptosis and its precise roles in vivo. Future success in this field will allow to explore and design effective novel therapeutic strategies aimed at preventing this dark side of DAMP emission.

19.3.5 Mitochondrial Permeability Transition-Driven Necrosis and Parthanatos

19.3.5.1 General Remarks

Two other emerging death pathways which gain increasing attention are the *mitochondrial permeability transition-regulated necrosis* (MTP-RN) and parthanatos. According to Galluzzi et al. [10], the term MPT is commonly employed to indicate an abrupt increase in the permeability of the inner mitochondrial membrane to small solutes, resulting in dissipation of the mitochondrial transmembrane potential (which is required for ATP synthesis and several other mitochondrial functions), massive water intake, and osmotic organelle breakdown. This form of RCD generally manifests with necrotic morphological features. "Parthanatos" is a portmanteau term (parthanatos) derived from *par* (for poly (ADP-ribose [PAR] polymer) and *Thanatos*, the personification of death in Greek mythology [153, 154]. Of particular interest is that parthanatos does not obligatorily require caspases for its execution, a feature that distinguishes this cell death form from caspase-dependent apoptosis. Notably, the identification of the underlying molecular mechanisms of the parthanatic pathway has revealed that the nature of this cell death is unique and separate from those of other forms of cell death such as apoptosis, necroptosis, or ferroptosis.

Extensive details of molecular mechanisms involved in MTP-RN and parthanatos are only beginning to be elucidated, and various aspects continue to be a matter of debate. Because of this, both subroutines of RCD are only briefly touched here.

19.3.5.2 Mitochondrial Permeability Transition-Driven Necrosis

The subroutine of MPT-RN is precipitated by the abrupt loss of permeability to small solutes of the inner mitochondrial membrane (for reviews, see [10, 11, 60, 155, 156]). This death pathway results from relatively severe perturbations of intracellular redox and/or Ca^{2+} homeostasis. According to current notions, the MPT-RN ensures a conformational change in a multiprotein complex assembled at the juxtaposition between the inner and outer mitochondrial membranes, the so-called permeability transition pore complex (PTPC). The precise molecular composition of the PTPC complex is not entirely clear but is discussed to show a degree of context dependency [35, 155]. Apart from this lack of knowledge, it seems to be evident that MPT results in a rapid drop of intracellular ATP availability, driving a peculiar form

of RCD. Together, these remarkable findings and others not mentioned here indicate that mitochondria play a fundamental role in the signalling pathways underlying MPT-driven RN (reviewed in [10]).

19.3.5.3 Parthanatos

Parthanatos is a unique and highly choreographed form of cell death, which occurs through injury-induced overactivation of the nuclear enzyme, *poly (ADP-ribose) synthetase-1* (PARP-1), also known as poly (ADP-ribose) glycohydrolase or poly (ADP-ribose) transferase-1. Such cellular injuries include stimuli which can induce DNA damage directly, such as ROS (e.g., H_2O_2), alkylating agents (e.g., *N*-methyl-*N′*-nitro-*N*-nitrosoguanidine), UV radiation, and ionizing radiation (for reviews, see [153, 154, 157, 158]). The enzyme PARP-1 helps in the regulation of cellular homeostasis and the preservation of genomic integrity and stability under homeostatic conditions. Corresponding to its function in maintaining nuclear homeostasis, PARP-1 constitutes a DNA base-excision repair system by sensing injury-induced DNA strand nicks and breaks and facilitating their repair through the synthesis of PAR polymer. When there is mild or moderate DNA damage, the activity of PARP-1 increases up to 500-fold, and the enzyme makes use of oxidized NAD (NAD^+), produced through the expenditure of ATP, to synthesize PAR polymer [158].

When cellular injury/DNA damage is too severe, PARP-1 becomes rapidly and excessively activated and produces high toxic levels of long-chained, branched polymers of PAR. The PAR polymer is now recognized as an essential signalling molecule in the parthanatos cascade. At first, PAR polymer translocates into the cytosol, where it constitutes a death signal to the mitochondria via dissipation of mitochondrial membrane potential, associated with direct binding to the PAR polymer-binding site on *apoptosis-inducing factor* (AIF). As a result, AIF is released from the mitochondria and translocates to the nucleus. Once in the nucleus, it causes large-scale DNA fragmentation and chromatin condensation through as-of-yet unidentified *parthanatos AIF-associated nuclease* (PAAN). Parthanatic cell death is the eventual outcome. Like necroptosis and ferroptosis, this parthanatic cell death involves loss of cell membrane integrity but unlike them is not accompanied by cell swelling (reviewed in [158]).

19.3.5.4 Concluding Remarks

Both subroutines of RCD, MPT-RN, and parthanatos represent an attractive arena of research. Both cell death forms have been shown to play a role in several human disorders including but not limited to cardiac and cerebral ischemia. For example, myocardial infarction involves cell death forms through pathways that are substantially regulated by permeability changes of mitochondrial membranes [159]. On the other hand, parthanatos is also reportedly implicated in the pathogenesis of several human disorders, in particular, stroke and several neurodegenerative diseases, but also in those who do not directly affect the nervous system [160]. Plausibly, therapeutic opportunities and the design of pharmacological intervention have already been envisaged. Thus, for MPT-RN, pharmacological interventions targeting putative components and upstream regulators of the PTPC complex may be potential

targets for drug design. For parthanatos, the event of AIF translocation from the mitochondria to the nucleus may currently be considered an attractive therapeutic target for drug design. Further progress in the elucidation of the exact molecular mechanisms is expected to promote first approaches to treatment of those diseases that are known to be associated with both MPT-RN and parthanatos.

19.3.6 Neutrophil Extracellular Trap Formation and Release (NETosis)

19.3.6.1   General Remarks

The phenomenon of NETosis has been already briefly touched in Part III, Sect. 8.2.3.2. As mentioned there, NETosis is a form of neutrophil-specific cell death characterized by the release of chromatin-derived weblike structures released into the extracellular space, referred to as NETs. Notably, NETosis is accepted as a specific form of RCD subroutine performed by activated neutrophils. Guided by comprehensive review article (published under Refs. [12, 161–169]), a few main aspects of this unique cell death are outlined here by focusing mainly on molecular mechanisms described for conventional suicidal NETosis.

19.3.6.2   Characteristic Features and Induction of NETosis

Characteristically, NETosis differs from apoptosis, necroptosis, ferroptosis, and pyroptosis. Formation of NETosis is initiated in response to either infectious (e.g., bacterial, viral, fungal) or sterile (e.g., chemical) stimuli which bind to diverse neutrophil receptors including TLRs, Fc receptors, complement receptors, and cytokine receptors. Regarding defense against infectious tissue injury, NETs have been observed to trap microorganisms and exhibit bactericidal activity through the action of NET-associated components (e.g., antimicrobial peptides and neutrophil elastase) [161, 162] (for antimicrobial peptides, see Part VI, Sect. 23.4). This early-observed phenomenon had led to the notion that NETosis is a defense response to infection only. However, increasing evidence now suggests that this process might also occur in non-infectious, sterile inflammation [163]. Since neutrophils via PRR signalling are activated by MAMPs and/or DAMPs, it can be assumed that they are involved in NET formation. In fact, studies in support of this assumption have already been published. For example, in a murine model of IRI, the DAMPs HMGB1 and histones were found to stimulate NET formation through TLR4- and TLR9-MyD88 signalling pathways [170]. In other lines of studies, the DAMP MSU crystals were found to be potent inducers of NETosis [171]. Again different sets of studies provided evidence suggesting that TLRs could be even key regulatory factors in *S. aureus*-stimulated NET formation [172].

Three models for NETosis are being discussed to date: besides conventional suicidal NETosis, which occurs after several hours of stimulation, two types of non-suicidal vital NETosis have recently been reported. Conventional suicidal NETosis is the best-described model that refers to a form of RN associated with final rupture of the plasma membrane. In vital NETosis, neutrophils release NETs without showing disruption of the membrane. Vital NETosis is induced within minutes by *S.*

aureus through both complement receptors (e.g., C3R) and TLR2 ligands or by *E. coli* directly via TLR4 or indirectly via TLR4-activated platelets. Unexpectedly, after the release of the nucleus, these neutrophils are still able to phagocytose pathogens, and their lifespan is not affected by DNA loss [173]. Finally, another type of ROS-dependent vital NETosis has been described in which mtDNA is released instead of nDNA [174]. In these studies, viable neutrophils, following priming with GM-CSF and subsequent short-term TLR4 or complement factor 5a (C5a) receptor stimulation, were shown to generate NETs which contained mtDNA but no nDNA (for C5a and its receptor, see Part VI, Sects. 23.2.5 and 23.2.6).

19.3.6.3 Mechanisms of NETosis

Although the mechanisms regulating formation of NETosis have not been fully elucidated and remain incompletely characterized, some critical cellular events are obviously clear as briefly sketched here for suicidal NETosis (reviewed in more detail in [12, 163, 167, 168]).

Conventional Suicidal NETosis

This conventional type of NET formation is usually initiated upon injury by ligand binding to one of the diverse neutrophil receptors. Upon activation of these receptors, downstream generation of ROS by the NADPH oxidase enzyme complex at the cytoplasmic or phagosomal membrane is accepted as an early dominant step in NET formation. Under the influence of ROS, granules and the nuclear envelope rupture, followed by release of nuclear, granular, and cytoplasmic contents which finally mingle. Then, neutrophil elastase (NE) and MPO are released from a select group of neutrophil granules, called azurophilic granules, into the cytosol and migrate to the nucleus, where they promote histone degradation and chromatin decondensation, respectively. Also, ROS leads to the activation of protein-arginine deiminase 4, an enzyme that converts arginine to citrulline on histones (e.g., histone H3), thereby fostering further chromatin decondensation in the neutrophil nucleus. In fact, citrullination also termed deimination of histones is regarded as another critical step in NET formation that occurs downstream of ROS (for histone modifications, see Part VI, Sect. 24.2.2). As a consequence of these events, the nuclear membrane ruptures, and damaged chromatin is released into the cytosol where it is further decorated with granular and cytosolic proteins to form the NETs, a process that leads to final cell death. Notably, it is not completely understood how these traps are ultimately expelled into the extracellular space; disruption of the plasma membrane, however, is the prevailing view [163, 175].

Vital NETosis (Type I)

The process of vital NETosis has been documented following microbial-specific MAMPs that are recognized by host PRRs. In particular, LPS was shown to induce rapid NET release. The induction of vital NETosis subsequently triggers a typical non-lytic pathway that is characterized by the release of nDNA through three morphological changes: (1) nuclear envelope growth and vesicle release, (2) nuclear decondensation, and (3) nuclear envelope disruption. In fact, electron microscopy revealed that NET release induced by *S. aureus* occurs via blebbing of the nuclear

envelope and vesicular exportation in vitro and in vivo (reviewed in [12, 173, 176]). As a result, this pathway preserved the integrity of the neutrophils' plasma membranes. Hence, NETting neutrophils become "anuclear cytoplasts" capable of detecting, capturing, and trapping pathogens [177].

Vital NETosis (Type II)
Finally, as mentioned above, another type of ROS-dependent vital NETosis has been described in which mtDNA is released instead of nDNA. This process results in NET formation from 80% of neutrophils within 15 min through recognition of the complement fragment C5a or LPS [174]. How vital NETosis may give rise to the release of mtDNA is not entirely clear; recent studies, however, suggest that mtROS are critically implicated in this process [178]. Beyond this suggestion, the type of the ligand and context-dependent factors may also play a role.

19.3.6.4   Concluding Remarks
Though there is still some controversial debate going on about the nature of these three different types of NETosis and their underlying mechanisms, it appears to be evident that this process per se is a powerful instrument of the innate immune system in warding off infectious and sterile injury and by this contributing to homeostasis. Per definition, this RCD is associated with the emission of DAMPs, in particular, nuclear DAMPs, which, when emitted in large amounts in the course of excessive NET formation, aggravates an inflammatory response and, hence, may contribute to diseases characterized by acute hyperinflammation or chronic inflammation. Thus, NETosis has been identified to be implicated in septic infections, ARDS following severe trauma, autoinflammatory, autoimmune, and metabolic diseases. Furthermore, NETs have been observed to act as a scaffold for thrombus formation [179] which is increasingly being recognized as a critical phenomenon linking inflammation with venous thrombosis. Interestingly, recent studies in a mouse model of venous thrombosis provided evidence that platelet-derived disulfide HMGB1 act as a central mediator of the sterile inflammatory process in venous thrombosis [180].

Hence, as with all other efferent actions of the innate immune system, NETosis is a double-edged sword: while it is an effective first-line antimicrobial mechanism, it might also lead to organ failure and death when uncontrolled and unregulated. Accordingly, intense research is on the way to identify agents that can be used to therapeutically modulate the formation of NETs, one example being low-molecular-weight heparins [181]. In Volume 2, this topic will be resumed when appropriate.

19.3.7 Subroutines of Regulated Cell Death Exude Different Immunogenicity

For the cell itself, it does not matter how it dies. However, it does matter for its environment. Most likely, the reason for the genetic conservation of several subroutines of RN is the difference in their immunogenicity as a response to different inciting challenges an organism is persistently exposed to.

Of note, the delay between the decision to undergo a particular type of RN and the final burst of the plasma membrane provides a critical time window of opportunity for these cells to actively produce or mature pro- or anti-inflammatory cytokines which operate as inducible DAMPs. As immunomodulating molecules, they may shape an inflammatory response and, potentially, a subsequent adaptive immune response which is already mediated by passive release of DAMPs. This window is just as narrow as it has to be to prevent viral or bacterial expansion. However, upon massive necrosis, as it happens during severe infection, trauma, and postischemic reperfusion of organs, active secretion of cytokines and chemokines, compared to passive release of constitutive DAMPs in large amounts, can only be of comparably minor importance. According to such a line of consideration, the three major pathways of RN, namely, necroptosis, ferroptosis, and pyroptosis, are briefly explored in the following.

In fact, growing evidence is provided in support of the view that the secretion of certain cytokines or chemokines modulates immunogenicity of a dying cell. Proteases are very active in some necrotic-type cell death subroutines and can process, as in case of pyroptosis, long-lasting cytokines, such as pro-IL-1β and pro-IL-18. When such cells, often macrophages, finally "pyroptise," the immunogenicity is not limited to the standard cytosolic arsenal of constitutive DAMPs which are passively released but contains some "extra flavor" in terms of emission of inducible DAMPs to be "extra immunogenic." In contrast, active transcription of IL-33 during necroptosis may exert an additional anti-inflammatory, immunosuppressive effect; IL-33 has been shown to activate M2 → M2-like macrophages and stabilize Treg cells in the surrounding microenvironment, thereby keeping immunogenicity in check.

Besides pyroptosis and necroptosis, several other RN pathways have not yet been associated with the production of cytokines or chemokines, rendering them just as immunogenic as they are by release of DAMPs. These RN subroutines include ferroptosis, parthanatos, and MPT-RN. Regulated necrosis in neutrophils is explicitly immunogenic because it appears to be often associated with the release of NETs that are released in a highly dynamic, highly immunogenic fashion.

Finally, during apoptosis, there is no plasma membrane rupture and therefore no passive release of DAMPs; however, low/moderate amounts of DAMPs may be actively secreted.

Analysing these factors for each cell death subroutine, a hierarchy for immunogenicity of apoptotic/necrotic cell death pathways is tentatively introduced here. It is primarily because of the immunogenicity of RN that we should understand these pathways and interfere with them therapeutically as indirect, but putatively highly potent immunosuppression.

19.3.8   Résumé

Certainly, the intersection between cell death and the immune system is currently appreciated as one of the hottest topics in biomedical and cell death research. It is critically central to homeostatic healing responses on the one hand and contributes to many human pathologies on the contrary. Most importantly, increasing

knowledge of this phenomenon has paved the way to develop innovative therapeutics for both aims in medical care: to suppress or to enhance cell death-induced disease-causing immune responses.

The "medium" of this scenario can be seen in the emission of various degrees of constitutive and inducible DAMPs which can promote both innate and adaptive immune processes. As ideal diagnostic and therapeutic targets, they are supposed to play a dominant role in future medicine.

19.4   Outlook

First of all, the brief description of different forms of RCD and their consequences contributes to modern notions in immunology holding that immunity is induced by infectious or sterile injury and not primarily by nonself.

However, RCD of infected cells functions as perhaps the most ancient firewall against lethal injury caused by pathogens by limiting replication and dissemination while concomitantly alerting neighboring cells to an impending threat. In fact, of considerable importance are recent reports showing that subroutines of RN such as necroptosis and pyroptosis are induced by viruses. These observations are in strong support of the notion that it is the DAMPs released in large amounts from dying infected cells which elicit and amplify a robust antiviral defense response and not, as formerly proposed, the invading foreign virus per se and alone.

Indeed, much work remains to be done to fully understand how apoptosis, necroptosis, ferroptosis, pyroptosis, and NETosis defend against infectious and sterile injury in vivo. For example, future research issues will address the molecular mechanisms of how the plasma membrane finally gets disrupted, that is, how membrane pores are precisely formed. Further, efforts will have to be devoted to obtaining further insights into the molecular crosstalk between necroptosis and other forms of RN (regulated necrosis) in various pathological settings.

Other more philosophical questions will be raised as well. For example, has evolution "invented" necroptosis, ferroptosis, or pyroptosis to just equip mammals with a robust tool for a successful struggle against pathogen-mediated infections by ensuring the release of appropriate amounts of constitutive and inducible DAMPs? And are those infection-induced subroutines of RCD just used as a blueprint in case of sterile injury-induced cell death?

Finally, a major objective of future efforts will be developing clinically implementable combinatorial strategies for the therapeutic modulation of necroptotic/ferroptotic/pyroptotic cell death and consequent necroptosis-/ferroptosis-/pyroptosis-driven necroinflammation and necroimmunity.

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