The role of Receptor Interacting Protein Kinases in Necroptosis and Inflammation

Grainne Delaney
Senior Sophister
Immunology

Inflammation is a defensive response against pathogens. During the initial inflammatory response, white blood cells are recruited to the site of infection to aid elimination of the pathogen. However, any dysregulation in the immune response pathways can lead to severe acute, or chronic inflammatory diseases. Intracellular pathogens, those that reproduce within the host cell, can induced cell death by apoptosis, which destroys their replication niche. Some microbes exhibit evasive strategies to avoid this form of cell death. When this occurs the host cell can employ alternative countermeasures of programmed inflammatory cell death. Programmed necrosis, or necroptosis is one such mechanism. The important roles of the RIP kinase family and the defensive strategy of necroptosis in relation to the inflammatory response they induce relies on interconnected molecular pathways, which can be disrupted in pathology.

Introduction

Necrosis is a form of cell death defined by characteristic changes to cell morphology, including disruption of the plasma membrane and cytoplasmic swelling. Historically, necrosis is associated with cellular damage due to physical stress or other extracellular conditions, such as osmotic change, strong acidity, severe temperature and depletion of nutrients and oxygen (Vaupel *et al.* 1989, Boujrad *et al.* 2007). All of these observations resulted in the assumption that necrosis is an unregulated and passive form of cell death. However, in recent years necrosis has been conditionally shown to be elicited by specific signalling pathways (Chan 2012). This regulated type of necrosis is called programmed necrosis or 'necroptosis'.

Cellular stresses that can induce cell death and inflammation include cytokine stimulation, inflammation or DNA damage (Labbe *et al.* 2008). Pattern recognition receptors (PRRs) are protein receptors expressed on the surface of cells which identify molecular structures found on invading pathogens known as pathogen-associated molecular patterns (PAMPs). PAMPs induce the production of pro-inflammatory cytokines and can trigger apoptosis. PRRs also recognise danger-associated molecular patterns (DAMPs) which are produced by damaged cells in response to pathogens or by self-reactive cells (Mills *et al.* 2011). Recognition initiates a number of cascades resulting in the activation of transcription factors such as nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) and activator protein 1 (AP-1), the transcription of suitable pro-inflammatory genes and members of the interferon regulatory factor (IRF) gene family. (O'Neill *et al.* 2013, Akira *et al.* 2006). Types of PRRs include toll like receptors (TLRs) which are transmembrane protein receptors, RIG-I-like receptors (RIGs), Nod like receptors (NLRs) and also DNA sensors (O'Neill *et al.* 2013, Kawai *et al.* 2008, Creagh *et al.* 2006, Khanna *et al.* 2001). The successful activation of the transcription factor NFκB leads to the secretion of numerous inflammatory cytokines, including tumour necrosis factor alpha (TNFα) and interleukin-1β (IL-1β), that recruit immune cells to inflammatory site. Receptors of these cytokines can further activate NFκB, to produce more pro-inflammatory cytokines, which in turn recruit leukocytes (neutrophils, monocytes and macrophages etc.) to the site of infection and subsequent elimination of the pathogen.

Cell death is also an important cell defence which integrates closely with inflammation within a multi protein oligo structure called the inflammasome. Initiation of inflammatory cascades by the inflammasome act as a driving force behind some forms of inflammatory defence (Vandenabeele *et al.* 2010). The nature of cell death can have varying effects on the inflammatory response (Bergsbaken *et al.* 2009). Programmed cell death by apoptosis is mainly regarded as a less disruptive, safer form of cell death. It is a highly regulated process where the cell membrane remains intact and the apoptotic bodies are cleared by phagocytosis, resulting in lower amounts of extracellular inflammation. By contrast, necrosis is highly disruptive, resulting in the cell membrane rupturing and release of cell contents (adenosine triphosphate (ATP), nuclear fragments, mitochondrial proteins, lysosomal digestive enzymes, high mobility group box 1 protein (HMGB1) etc.). These endogenous compounds are danger signals, which enhance inflammation. The receptor-interacting protein (RIP) kinase family have emerged as important sensors of both internal and external cellular stress signals and therefore play an essential role in amplifying inflammation, immune responses and also in death-inducing processes (Declercq *et al.* 2009). RIP1 is of particular importance as it's kinase activity is crucial in triggering apoptosis while both RIP1 and RIP3 are required in conjunction with each other to elicit cell death through necroptosis.

Both necroptosis and inflammation have closely interconnected signalling pathways and thus exhibit a level of co-ordination between them. Maintaining a tightly controlled balance is vitally important as exaggerated cell death can lead to tissue damage and a great loss in the number of immune cells. These pathways are thus crucial as they dictate the magnitude and duration of inflammation while also being responsible for the fate of the cell (Humphries *et al.* 2014). This is furthermore significant because an increased level of inflammation is the basis for characterisation of inflammatory diseases such as rheumatoid arthritis (Epstein *et al.* 2001). Therefore the kinase family of RIPs are critical to the necroptotic and inflammatory pathways, their significance lying in their determination of the fate of a cell: death via necroptosis or survival.

Inflammation or apoptosis? RIP1s crucial deciding role in Tumour Necrosis Factor (TNF) signalling

Receptor-interacting protein 1(RIP1) kinase activity is instrumental in TNF signalling. RIP1 mediates NFκB activation in response to a number of death receptors such as Fas, TNF-receptor 1 and TNF-related apoptosis-inducing ligand receptor-1 (TRAIL1) (Kreuz *et al.* 2004, Kelliher *et al.* 1998, Ting *et al.*1996, Lin *et al.* 2000). However, because of the important role of TNF in initiating the inflammatory and cell death pathways and ultimately in inflammatory diseases, the downstream signalling pathway of TNF and the TNF receptor 1 have been one of the most studied (Figure 1). TNF binding to the TNF-R1 initiates the extrinsic apoptotic signalling pathway, this interaction recruits TNF receptor-associated death domain protein (TRADD) and RIP1 to the cell membrane where they form a complex. Cellular inhibitor of apoptosis proteins (cIAPs) (1 and 2) and TNF receptor-associated factor (TRAF2) (2 or 5) join the complex and catalyse the polyubiquitination of RIP1. Recognition of the ubiquitinated RIP1 by a complex containing a ubiquitin-binding domain composed of TAK1 and IκB kinase (IKK) enables TAK-1-mediated phosphorylation which then activates the IKKs. IKK phosphorylates the IκB proteins which usually sequester NFκB in an inactive state in the cytoplasm. This results in further ubiquitination and degradation of IκB, which frees NFκB allowing it to relocate to the nucleus. NFκB, acts as a transcription factor by inducing the expression of pro-inflammatory genes such as IL-1 and TNF (Han at al. 2011).

There are many intrinsic regulatory components in this pathway. NFκB can act as an inhibitory regulator of apoptosis by promoting the expression of anti-apoptotic genes including cIAPs. These genes exert a positive feedback control on NFκB via an IκB targeting mechanism and supress TNF activity (Chu *et al.* 1997). NFκB can also induce the expression of a cellular fas-associate death domain (FADD)-like interleukin-1 beta-converting enzyme (FLICE) inhibitory protein (c-FLIP) which is a potent inhibitor of death receptor induced apoptosis, thus promoting a pro-inflammatory phenotype, as opposed to an anti-inflammatory one (Kreuz *et al.* 2004). Alternatively, it was shown that a member of the Pellino E3 ubiquitin ligase family was able to target RIP1 in

such a way that would prevent complex 2 formation hence suppressing cell apoptosis (Yang *et al*. 2013, Tenev *et al*. 2011).

Contrastingly, NFκB acts as part of a negative feedback loop by promoting deubiquitinating enzymes A20 and CLYD to remove the ubiquitin chains from RIP1, preventing further activation of NFκB (Declercq *et al*. 2009,Wilson *et al*. 2009). In this form RIP1 associates with FADD and pro-caspase8 forming a death inducing signalling complex: complex 2 (Figure 2) (Wang *et al*. 2008, Declercq *et al*. 2009). Complex 2 and the ripoptosome induce the processing of pro-caspase-8 to its active form, leading to a caspase cascade that results in apoptosis (Wilson *et al*. 2009). The loss of cIAP proteins would similarly drive the pathway in this direction (Tenev *et al*. 2011). Therefore the RIP1s ubiquitination and kinase activity status decides whether TNF signalling enters the inflammatory or apoptotic pathways. Intriguingly, both branches of the pathway counter-regulate each other.

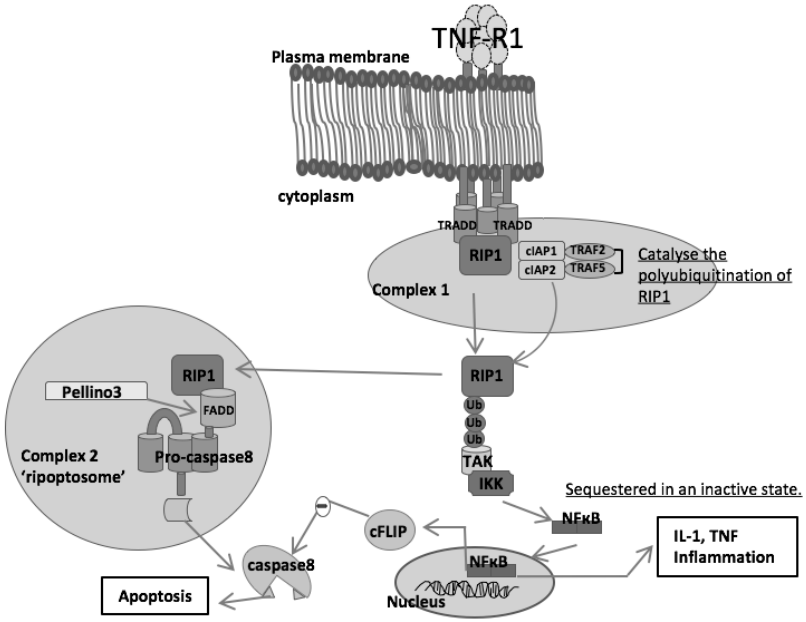

Figure 1. *The functions of RIP1 in TNF in signalling. TRADD and RIP1 are recruited to the receptor and form complex 1. RIP1 is polyubiquitinated in complex 1, which also contains the catalysing agents, cIAP1, cIAP2, TRAF2 and TRAF5. Phosphorylation of RIP1 is mediated by TAK which activates IKK resulting in NFκB activation and expression of pro-inflammatory genes in the nucleus. Inflammatory cytokines such as IL-1β and TNF are secreted along with the anti-apoptotic protein cFLIP. The formation of the complex 2 (known as the ripoptosome in the presence of IAPs) occurs after the deubiquitination of RIP1, along with FADD and pro-caspase 8. Processing of caspase 8 triggers downstream signals which results in apoptosis. Adapted from (Han et al. 2011).*

RIP1-RIP3s Role in Driving Necroptosis

Although apoptosis is a crucial host defence strategy against pathogen replication, other cell death mechanisms are required, as some pathogens have evolved evasive mechanisms to avoid this defence system. One example is cytomegalovirus which encodes a number of viral inhibitors to numerous key regulators in the apoptotic pathways, a specific example being an inhibitor against the caspase8 complex (Zhang *et al.* 2009).

Unlike necrosis driven by a physical trauma, one way to initiate necroptosis is by an underlying pathway composed of the kinases RIP1 and RIP3 (Han *et al.* 2011). RIP1 and RIP3 share a conserved kinase domain in their amino termini. They also contain a unique protein-protein interaction motif called the RIP homotypic interaction motif (RHIM) which is not present in other kinases within the RIP family (Sun *et al.* 2002). Upon stimulation of the TNF receptor, signalling for necroptosis is initiated. TRADD signals to RIP1 which recruits RIP3 (Figure 2). RIP1 is known to bind to RIP3 through their RHIM domain to form a heterodimeric filamentous scaffold, known as the necrosome (Wu *et al.* 2014, Orozco *et al.* 2014). Wu *et al.* 2014 report, using an inducible dimer system, that a signal cascade resulting in necroptosis cannot occur if there is not subsequent recruitment of additional RIP3 proteins to the RIP1-RIP3 heterodimer. This further recruitment of RIP3s is promoted by the initial RIP1-RIP3 interaction. In addition, when necrosis is triggered by death receptors, mediation of RIP1 requires, ripoptosome assembly, caspase 8, as well as inhibition of apoptosis (Feoktistova *et al.* 2011, Chan *et al.* 2012).

The necrosome then phosphorylates Mixed lineage kinase domain like (MLKL) which is a protein classified as a pseudokinase (Murphy *et al.* 2013), MLKL is a vital downstream component of RIP3, for inducing TNF-induced necroptosis (Cai *et al.* 2014). MLKL phosphorylation promotes oligomerization and translocation of the protein towards the plasma membrane. Once MLKL reaches the membrane, it interacts with phospholipids and reduces the integrity of the membrane resulting in cell rupture (Cai *et al.* 2014, Wang *et al.* 2014). MLKL also generates the expression of reactive oxygen species (ROS); the increase in ROS (especially from the mitochondria) is strongly linked with mediating TNF-induced necroptosis (Vandenabeele *et al.* 2010). RIP3 additionally activates a number of enzymes which metabolically contribute to the TNF-induced production of ROS (Zhang *et al.* 2009). These metabolic enzymes include, glutamate dehydrogenase 1 (GLUD 1), glutamate ammonia ligase (GLUL), and glycogen phosphorylase (PYGEL). An alternative mechanism of necroptosis results from stimulation of phosphoglycerate mutase family member 5 (PGAM5), causing PGAM5 to associate with Drp1 (a mitochondrial fission factor) resulting in fragmentation of the membrane (Murphy *et al.* 2013).

It was subsequently shown that RIP3s catalytic ability facilitates the switch between necrosis and TNF-induced apoptosis (Zhang *et al.* 2009). This was demonstrated by RIP3 deficient embryonic fibroblasts, from mice, surviving and reaching maturity, despite TNF exposure, due to TNF-induced necrosis resistance (He *et al.* 2009).

Further regulation is provided by caspase 8, which can block RIP1-RIP3 mediated necrosis by regulating RIP1-RIP3 complex formation with the aid of cFLIP and FADD (Osborn *et al.* 2010, Dillon *et al.* 2012). Caspase 8 also has the ability to deactivate the deubiquitinating enzyme CLYD, that functions by removing the ubiquitin chains from RIP1. When this enzyme is deactivated RIP1 no longer has the ability to activate NFκB, thus repressing necrosis (Wilson *et al.* 2009, O'Donnell *et al.* 2011)

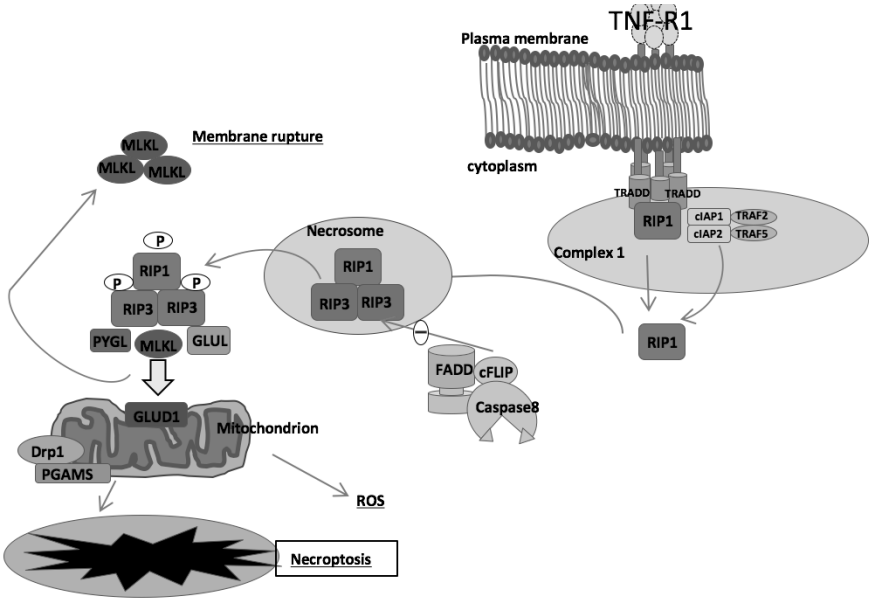

Figure 2. *Upon TNF-R1 stimulation, TRADD, RIP1 and RIP3 form the active necrosome complex. After RIP3 dimerisation, RIP3 intra-molecule auto-phosphorylation occurs. This phosphorylation is required for the recruitment of MLKL which triggers the downstream signalling necessary for necroptosis. It is the signalling events following the RIP1–RIP3 amyloid complex aggregation which are essential in executing necroptosis, RIP3 in the assembled amyloid scaffold recruit the free RIP3 followed by auto-phosphorylation of RIP3 and the subsequent recruitment of MLKL, are all key signalling events. It is proposed the formative events in the interaction between the RIP1-RIP3 heterodimeric amyloid fibril assembly are unlikely to directly instigate necroptosis because the heterodimer itself cannot induce necroptosis. However, they are a key part of the signalling cascade which results ultimately in necroptosis. Adapted from (Han et al. 2011).*

RIP1-RIP3 in a Pathological Context

As a result of necroptosis, the rupturing of the plasma membrane, as well as the lysosomal, and/or the mitochondrial membranes, leads to intracellular danger signals leaking into the extracellular space. These DAMPs include nuclear fragments, ATP and lysosomal enzymes, spilling into the extracellular space which activates PRRs. This promotes further tissue damage and inflammation. Due to the inflammatory nature of necroptosis it is no surprise that RIP1-RIP3 kinases participate in the pathogenesis of many inflammatory diseases and are attractive therapeutic targets. An example of such disease is retinitis pigmentosa (RP).

RP is a progressive genetic condition which results in the degradation of photoreceptor cells and retinal pigment epithelia cells, and eventual loss of vision (Murakami *et al.* 2012). RP can be inherited in an X linked, autosomal dominant, or autosomal recessive manner. It is primarily rod photoreceptors which harbour this deleterious gene, and these cells die by apoptosis, mediated through RIP1 kinase activity in humans (Hartong *et al.* 2006). Furthermore, Murakami *et al.* 2012 showed that in rd10 mice (endogenous mouse model of retinitis pigmentosa), cone cell death can occur through RIP3 mediated necrosis - RIP3 knockout mice thus showed significant preservation of cone cells accordingly. Other visual conditions linked to RIP1-RIP3 kinase activity in humans include, macular degradation and retinal detachment (Hanus *et al.* 2013, Trichonas *et al.* 2010).

Necroptosis plays a major role in the pathogenesis of ischaemic injury, including cerebral and retinal ischemia (Mehta *et al.* 2007, Rosenbaum *et al.* 2010). When the role of necroptosis in neuronal cell death and functional impairment in retinal ischemia was examined in rats, the use of the RIP1 kinase inhibitor nerostatin1 (nec-1) successfully reduced degradation of the inner retina, and induced a functional improvement (Rosenbaum *et al.* 2010). This demonstrates the significance of RIP1 in the impairment of vision in retinal ischemia and suggests exploring this kinase as a possible therapeutic target for treatment. The RIP1/RIP3 mediated necroptotic pathway has been shown to have dominance over apoptosis in kidney injury and has a pivotal role in kidney ischemia (Linkermann *et al.* 2012) and also neurodegeneration in brain ischemia. Mitochondrial dysfunction, excitotoxicity and oxidative stress all contribute towards necroptosis, and are implicated in brain ischemia as well as Alzheimer's and Parkinson's disease (Lin *et al.* 2006). Increased oxidative stress results from the accumulation of copper, iron and zinc as the brain ages (Vandenabeele *et al.* 2010).

Programmed necrotic cell death is thought to be the mechanism by which macrophages present in atherosclerotic lesions die (Figure 3). In a mouse model of early atherosclerosis, aortic lesions in RIP3 deficient mice showed no apparent differences, by comparison to aortic lesions from wild type mice (Lin *et al.* 2013). However, in advanced atherosclerosis, it was shown that significantly lower amounts of RIP3 were present in mice indicating RIP3 is important in preventing progression to advanced plaques (Lin *et al.* 2013).

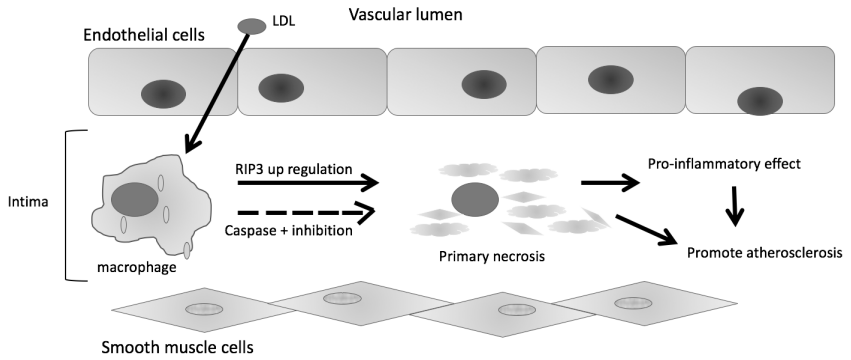

Figure 3. *schematic of macrophage death in atherosclerosis. Increased levels of RIP3 promotes the conversion from apoptosis to programmed necrosis in macrophages. The resulting inflammatory responses promote the development of advanced atherosclerotic lesions in blood vessels. Adapted from (Lin et al. 2013).*

The proteins which inhibit apoptosis are of new interest in cancer treatment. Preclinical drug therapies are trying to induce apoptosis in pre-apoptotic cells, suppressing the overall growth of cancerous cells (Hu *et al.* 2003). IAPs can play a deciding role in regulating whether a cell undergoes TNF-induced apoptosis, or production of pro-inflammatory cytokines. X-linked inhibitor of apoptosis (XIAP) is one of the most potent caspase and cytochrome c inhibitors in mammals, and has been linked with acquired resistance to inflammatory breast cancer (IBC), an extremely aggressive cancer with a very high mortality rate of up to 49% depending on tumour grade and stage of progression (Allensworth *et al.* 2013, http://www.cancer.net/cancer-types/breast-cancer-inflammatory/statistics). Second mitochondrial activator of caspase (Smac) protein is a potent antagonist of IAPs and is the target of Smac mimetic drugs in inflammatory conditions (Allensworth *et al.* 2013). Smac mimetic Birinapant is a drug currently in phase 2 in clinical trials that reduces formation of refractory solid tumors, inflammatory breast cancer cells and lymphomas by priming the cancer cells for death in an IAP dependant mechanism (Amaravadi *et al.* 2015).

Conclusion

Historically our understanding of inflammation and cell death overlap, but there is a necessary divergence between the two pathways that helps improves our molecular understanding of the underlying signalling pathways which control them. However, owing to the inherent complexity of the interconnected pathways

between the pro-inflammatory response and the cell death response to a pathogen, it is necessary to acknowledge both pathways within the same context. Progress has been made in understanding the biological roles of RIP kinases, specifically RIP1 in relation to its inflammatory role in intracellular death receptor signalling. RIP1 is a key regulator of necroptosis, it dissects a delicate balance between its role in complex 1 RIP1 kinase activity, its scaffolding forming function in the necrosome, and TNF-dependent inflammation which is crucial for maintaining cell homeostasis, and is regulated with the help of inhibitors.

Recent discoveries have further elucidated RIP3s functionality and exactly how it induces cell death through pro-inflammatory signalling, but like RIP1 it must also be tightly controlled, with failure to do so leading to hyper-inflammation (Yabal *et al*. 2014).

Most of the studies thus far have examined the role of receptor interacting proteins (RIPs) in cell death, and it is evident that they have many pathophysiological functions. Our current understanding of the these kinases and their role in biological functions implicates them as a potential therapeutic target for treatment of neurodegenerative, inflammatory, and ischemic diseases. Future research efforts should strive to increase our knowledge of RIP kinase biology into targeting and developing RIP derived therapeutic treatments for inflammatory disease.

Acknowledgements

The author would like to thank Ms. A. Worrall, Mr. D. O'Driscoll, Ms. M. Lundahl, Mr. P. Fox and Prof. R. McLoughlin for their edits and advice. This work was adapted from an essay entitled 'The role of RIP kinases and necroptosis in inflammation' for the course BI3020.

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