New genetic evidence reignites a cold case: the role of RIP kinases in death and disease

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Abstract

Inflammation is a healthy response to infection or danger and should be rapid, specific and terminated once the danger has passed. Inflammatory diseases, where this regulation fails, account for a large toll of human suffering. Treatments have been directed against Tumor Necrosis Factor (TNF); the "master regulator" of inflammation because production of this cytokine is an outcome of inflammatory signalling pathways and it in turn directly generates many mediators of inflammation. TNF may also directly induce cell death and this cell death can itself provoke inflammation. This potential viscious cycle makes it difficult to know whether TNF induced cell death plays a role in inflammatory diseases, yet if it does then inhibiting it could alleviate inflammatory disease. This review examines the role of cell death, and necroptosis in particular, in inflammation, in the context of recent work that provides significant insights into the roles of the key necroptosis effector molecules; RIPK1, RIPK3 and MLKL.

Getting away with murder – reviewing recent advances in necroptosis research

It has become almost axiomatic that apoptosis, the regulated autonomous destruction of a cell, is not inflammatory, whereas cellular necrosis that allows the release of the cellular contents is. Genetically programmed cellular necrosis, now called necroptosis, requires the activity of RIPK3 and MLKL and is expected to be similarly inflammatory. Befitting an emerging field it is however still contentious whether necroptosis is a physiological inducer of inflammation in either a regulated or pathological response. One of the reasons for the lack of clarity on these questions is that molecules that initiate and carry out necroptosis, such as TNF, RIPK1 and RIPK3, also directly initiate or regulate inflammatory signalling pathways. Another significant limitation is that there is a dearth of markers that can be used to unambiguously identify necroptotic cells in vivo. Therefore genetic experiments have led the way in tackling these questions, but because proteins like RIPK1 have pleiotropic functions in regulating inflammation and cell death it is still not possible to make definitive conclusions about whether necroptosis causes inflammation in vivo. This review presents recent insights into the necroptotic cell death pathway and its inflammatory role with newly developed genetic tools. Some of these insights were gained by trying to understand why *Ripk1*$^{-/-}$ mice die soon after birth, a mystery for the last 15 years {Kelliher 1998} (Figure 1).

Inflammation

It has been recognised for centuries that inflammation is characterised by redness, heat, swelling and pain in the affected tissue (superbly reviewed in {Wallach 2014}). These symptoms are primarily driven by cells within the tissue that respond to damage or danger by producing and releasing cytokines and chemokines that permeabilise blood vessels and recruit cells of the innate and adaptive immune system to allow these professional cells to deal with the "agent provocateur".

While inflammation is a protective response it can also be damaging if unregulated; exemplified by the fact that several tissues/organs can repress potentially damaging inflammatory responses that normally occur in other tissues {Matzinger 1998}. The inflammation-inciting agent may be a foreign body or molecule, frequently pathogen associated, or it may be an endogenous but inappropriate body or molecule. Agents that provoke inflammation have therefore been categorised as Pathogen Associated Molecular Patterns (PAMPs) or Damage Associated Molecular Patterns (DAMPs). Consistent with omnipresent and ubiquitous threats, DAMPs and PAMPs are detected by a host of, usually constitutively expressed, intracellular and extracellular receptors, including proteins of the NOD like Receptor (NLR, intra), Toll Like Receptor (TLR, extra), MAVS/RIGI (intra) and AIM2 (intra) families of proteins. These signalling receptors use similar transcriptional cassettes, such as NF-κB, Fos/Jun and IRFs to activate the production of inflammatory cytokines and chemokines.

PAMPs?

Pathogen Associated Molecular Patterns (PAMPs) are almost self-explanatory; evolution has selected for the ability of multicellular organisms to immediately respond to bacterial, viral and parasitic pathogens by recognising pathogen associated molecules with the receptors described above. Less intuitively, the detection/response system is stunningly context sensitive. Non pathogenic organisms often express the same inflammation inducing molecules as pathogenic ones, yet mammals, for example, co-exist harmoniously with billions of such commensal organisms. Without this context sensitivity, these "harmless" commensals can trigger a fierce inflammatory response and provoke debilitating inflammation such as inflammatory bowel disease. There are many evolutionary solutions to the problem of context and the subject is beyond the scope of this review. But if, for example, we consider cytosolic PAMPs these are extremely unlikely to be benign. Thus PAMP location can provide some context and accordingly there are many intracellular PAMP receptors to respond to this dangerous contingency.

DAMPs?

It was Polly Matzinger's insight that context sensitivity could be achieved if the immune system recognised "danger", rather than a pathogen *per se* {Matzinger 1998}. But how does a cell recognise danger, other than the example raised above? One obvious alarm to a cell that its tissue residence is in danger is dying or damaged neighbours. Thus the concept of Damage Associated Molecular Patterns (DAMP) was introduced. But this raises subsidiary questions; what might a cell death associated DAMP (^{cd}DAMP) be? In theory any intracellular molecule, including DNA, that becomes released from a dying cell could serve. But such molecules are likely to have an essential "day job" within living cells making it difficult to distinguish a "secondary" role. Secondly, what differentiates the billions of homeostatic cell deaths occurring in our bodies everyday from dangerous cell death? The second question has typically been answered by stating that normal homeostatic and developmental cell death occurs via apoptosis, which restricts leaking contents, degrades DNA, and generates "find-me" and "eat-me" signals to stimulate a rapid clean-up by living cells {Martin 2012}. However this explanation does not sit well with another established dogma that apoptosis is a cell suicide mechanism evolved to limit viral replication and survival {Vaux 1994}. Clearly a system whereby cells die of a viral infection but fail to alert the immune system seems less than ideal. One way out of this contradiction could be that a viral infection generates additional DAMPs that override the anti-inflammatory nature of an apoptotic death {Gallucci 1999}. Alternatively excessive viral induced apoptosis overwhelms the local clean-up crew allowing leakage of DAMPs. In support of this idea, failure to clear DNA from apoptotic cells can result in auto-immune disease {Kawane 2001}. These explanations are however simply another way of restating that the context of cell death is potentially as important as the type of cell death in determining whether it is recognised as dangerous or not.

Finally, during trauma-induced cell death the clean-up crew will not be alerted and recruited by apoptotic signals and the contents of the dying cells will be released into the tissue. This type of cell death has been referred to as cellular necrosis {Linkermann 2014}. Recently it has become apparent that cellular necrosis may also be genetically programmed, and this has been dubbed with the portmanteau, necroptosis.

TNF and IL-1; DAMP amplifiers, DAMP generators or DAMPs themselves?

TNF and the IL-1 family of cytokines are potent inducers of a host of chemokines, cytokines and small molecules that together co-ordinate the inflammatory response and drive permeabilisation of blood vessels and recruitment of innate and adaptive immune cells that in turn secrete more inflammatory cytokines, etc. TNF & IL-1 can therefore aggravate or amplify the inflammatory response and blocking TNF signalling has proven spectacularly effective in treating some, but not all, inflammatory diseases {Feldmann 2010}. Likewise blocking IL-1 signalling has proved efficacious in treating rheumatoid arthritis {Dinarello 2012}. Not surprisingly therefore levels of these potent cytokines are tightly regulated and they are usually only present at very low levels until induced in response to PAMPs and DAMPs. IL-1ß is fascinating because it is produced in a pro-form in response to danger signals but requires a second independent danger signal to cause its processing and release from cells, in a caspase-1 dependent manner {Moltke 2013; Croker 2014}. In these cells caspase-1 also causes cell death, dubbed pyroptosis because it is associated with the release of the pyrogen IL-1ß. Although IL-1ß and IL-1α are released from cells undergoing a pyroptotic death and could therefore also be considered to be ^{cd}DAMPs, kinetic analyses suggest that both are processed and released prior to cell death {Gross 2012}. Without getting distracted by semantics it is therefore unclear whether either can be considered as true ^{cd}DAMPs in all cases.

TNF induces the production of inflammatory cytokines but to fully appreciate its role in inflammation we must be aware that TNF can, in certain circumstances, cause cell death and therefore potentially generate ^{cd}DAMPs (Figure 2). This is also true for other TNFSF members such as Fas and TRAIL but, for simplicity's sake, we will restrict our discussion to TNF and its ubiquitously expressed receptor, TNFR1. TNF binding to TNFR1 stimulates the formation of an intracellular complex consisting of recruitment and effector proteins {Silke 2011}, which are not the same for all cell types but we will consider an idealised cell that recruits the adaptors TRADD and RIPK1 to TNFR1 via their respective death domains. TRADD recruits TRAFs and thereby cIAPs. Thereupon, cIAPs decorate proteins within this complex with ubiquitin chains that recruit a secondary E3 ligase complex called LUBAC, which in turn generates linear ubiquitin chains. Together these ubiquitin chains serve as a platform upon which the IKK1/IKK2/NEMO and TAB2/TAB3/TAK complexes are activated which leads to activation of NF-κB and AP-1 transcription factors that drive the production of inflammatory cytokines and also the caspase-8 inhibitor, cFLIP (Figure 2A). A similar ubiquitin platform generating cassette has also been described for TLR receptors that detect PAMPs {Wertz 2010; Silke 2010}.

TNF as a potential inducer of ^{cd}DAMPs

Because pathogens have evolved to limit inflammation by targeting TNF/TNFR1 and TLR induced cytokine production, it is believed that these signalling pathways have evolved to counter such manipulation. If the cIAP/LUBAC dependent NF-κB response is disrupted, cytokine production is limited but so is production of cFLIP. Furthermore inhibition or loss of cIAP or LUBAC activity favours the formation of a secondary signalling complex containing caspase-8 that in the absence of cFLIP can promote caspase-8 dependent apoptosis (Figure 2A1&2) {Peltzer 2014; Silke 2014}. In response, pathogens have evolved to also target the cytotoxic activity of TNF by encoding inhibitors of caspase-8 {Silke 2013; Benedict 2003}. However a further cellular countermeasure

appears to have evolved because caspase-8 activity is also required to inhibit a necroptotic cell death pathway that proceeds in a RIPK1 and RIPK3 dependent manner (Figure 2). Formation of this necroptotic signalling complex is also favored by lack of ubiquitin chains on RIPK1 because inhibition or loss of cIAP or LUBAC activity or the activity of the deubiquitylating enzyme CYLD promote TNF induced necroptosis {Wang 2008; Hitomi 2008; Bonnet 2011; Gerlach 2011}. Thus, if pathogens inhibit the TNFR1 transcriptional response and caspase-8 activation their anti-inflammatory strategies may still be thwarted by the cell undergoing a necroptotic cell death and inducing inflammation via ^{cd}DAMP release (Figure 2).

Proposed mechanism for TNF-TNFR1, RIPK1, RIPK3 and MLKL induced necroptosis

The prevailing model for the role of RIP kinases in necroptosis is that RIPK1 activates RIPK3 (Figure 2). There is no data showing direct phosphorylation of RIPK3 by RIPK1 therefore it is likely that RIPK3 oligomerisation driven by the RHIM domain of RIPK1 and RIPK3 leads to RIPK3 auto-activation {Li 2012} (Figure 2B). Consistent with this mode of activation, other RHIM containing proteins such as TRIF and DAI can promote RIPK3 activation {Mocarski 2012; Murphy 2014} (Figure 2C, D & E). Activation of RIPK3 leads to MLKL phosphorylation, membrane translocation and plasma membrane disruption (Figure 2). Thus MLKL appears to be a key necroptotic effector protein but exactly how it disrupts membranes is unclear because recent reports have provided different and in some cases conflicting mechanisms {Chen 2014; Wang 2014; Cai 2014; Dondelinger 2014; Hildebrand 2014}.

Genetic evidence for the proposed mechanism

Evidence for these models comes from knock-out mice (Summarised in Figure 1). *Casp8*$^{-/-}$ mice, or mice expressing only the DED domains, *Casp8*$^{DED/DED}$, die between embryonic day (E) 10.5-12.5 with heart defects {Varfolomeev 1998; Sakamaki 2002}. Because culturing the *Casp8*$^{DED/DED}$ embryos *ex vivo* rescued the heart defects this suggested that the defects were a consequence, not a cause, of the underlying problem {Sakamaki 2002}. Indeed, mice deficient for *Casp8* in the endothelium display the same heart abnormalities and lethality as the complete *Casp8* knock-outs implicating a defective vasculature as the cause of the lethality {Sakamaki 2002; Kang 2004}. The same problems are observed in *Fadd*$^{-/-}$, *cFlip(cFlar)*$^{-/-}$ mice and *Casp8* catalytically inactive knock-in mice {Yeh 1998; Yeh 2000}. Because FADD is required to activate caspase-8 while cFLIP inhibits it, the similar phenotype of these knock-out mice is completely unexpected. However because *Casp8*$^{-/-}$ and *Fadd*$^{-/-}$ lethality can be rescued by loss of RIPK3 and RIPK1 respectively it has been proposed that caspase-8 activity is required to inhibit a necroptotic cell death at this embryonic stage and that this cell death results in the observed lethality {Kaiser 2011; Oberst 2011; Zhang 2011}. Indeed it may be a caspase-8/cFLIP$_L$ heterodimer (that retains a restricted catalytic activity compared to the caspase-8 homodimer {Pop 2011}) that is ultimately responsible for inhibiting RIPK3 activation {Oberst 2011}, neatly explaining the similar *in vivo* phenotypes. This is also consistent with data showing that cFLIP$_L$ prevented, while cFLIP$_S$, that is unable to create an active heterodimer with caspase-8, sensitised cells to Fas and TLR induced necroptosis {Geserick 2009; Feoktistova 2011}.

Just recently, analysis of this embryonic "check-point" has extended to *Ripk1*$^{-/-}$ mice. As with the *Fadd*$^{-/-}$ perinatal lethality {Zhang 2011}, RIPK1 loss also prevents the *Casp8*$^{-/-}$ lethality at E10.5 {Dillon 2014; Rickard 2014; Kaiser 2014}. Dillon *et al*., also showed that the lethality at E10.5 is due to TNFR1 signaling because the loss of *Tnfr1* also inhibits the *Casp8*$^{-/-}$ lethality {Dillon 2014}. This result is fascinating: firstly the *cIap1*$^{-/-}$*cIap2*$^{-/-}$ mice also die at E10.5 and their death at this embryonic stage is also prevented by loss of either RIPK1, RIPK3 or TNFR1 {Moulin 2012} strongly implying that cIAPs are involved in this same check-point. Secondly this checkpoint appears to show that caspase-8 is activated by TNF. The developmental purpose of such a TNF

signal at E10.5 is unclear because *Tnf* and *Tnfr1* deficient mice live a normal murine life span. Because cIAPs are known to inhibit RIPK1/RIPK3 mediated necroptosis in tissue culture these results again appear to support the idea that there is a necroptotic event in these knock-out animals that causes embryonic lethality.

However an alternative explanation for the lethality is possible. What if TNF signalling amplifies an local inflammatory event that occurs in the *Casp8*$^{-/-}$, *Fadd*$^{-/-}$, *cFlip/Cflar*$^{-/-}$ and *cIap1*$^{-/-}$*cIap2*$^{-/-}$ mice at around E10.5 (Figure 1), but does not initiate the lethal event? For example, genetic defects in the embryonic vasculature could provoke a limited TNF independent cell death that is not by itself lethal to the embryo but which is amplified into a lethal inflammatory event by TNF. Thus, even the apparently straightforward protection of these knock-out animals by loss of TNFR1 encapsulates the "chicken and egg" problems in looking at cell death and inflammation.

Caveats to interpreting the genetics – multiple roles for cIAPs, caspase-8, RIPK1 and RIPK3
The fact that loss of the essential effectors of necroptotic cell death, RIPK1 and RIPK3 (Figure 2), prevents this early lethality (Figure 1) is not as helpful in resolving this problem as it first appears either; because RIPK1 and RIPK3 have roles in TLR, inflammasome and other inflammatory signalling pathways (Figure 3). RIPK1 is a pivotal scaffold element in several inflammatory signalling pathways and RIPK3 is a kinase that is highly likely to have a number of substrates in addition to MLKL. For example, Smac mimetic treatment, that depletes and antagonizes IAPs, induced secretion of TNF and other inflammatory cytokines by BMDM and this was largely prevented by inhibition of RIPK1 or deletion of *Ripk3* {Wong 2014}. Similarly, loss of caspase-8 primes the MAVS/RIG-I complex to respond to viral infection in a RIPK1 dependent manner {Rajput 2011}. Furthermore, treatment of BMDMs or BMDCs with a Smac mimetic resulted in NLRP3 inflammasome activation and IL-1β secretion that was RIPK3 dependent {Vince 2012}. Like the XIAP/cIAP1/cIAP2 deficient cells {Vince 2012}, *Casp8*$^{-/-}$ BMDCs have been shown to have a constitutively active form of the NLRP3 inflammasome whose activity depends upon RIPK3 {Kang 2013} (Figure 3B). These results suggest that IAPs and caspase-8 inhibit RIPK3 dependent activation of the NLRP3 inflammasome.

Unfortunately, as if this picture were not complicated enough, these experiments implicating caspase-8 and RIPK3 in production of inflammatory cytokines are contradicted by others. For example, loss of caspase-8 has also been claimed to result in reduced inflammasome function because *Fadd*$^{-/-}$*Ripk3*$^{-/-}$ and *Casp8*$^{-/-}$*Ripk3*$^{-/-}$ but not *Ripk3*$^{-/-}$ BMDMs are defective both in their response to TLR and NOD ligands and in their ability to activate the inflammasome {Gurung 2014}. Complicating the situation further still, Caspase-8 does not need to activate NLRP inflammasomes and caspase-1 to process IL-1ß into its active inflammatory form, but can do it directly following stimuli such as Smac-mimetics {Vince 2012}, chemotherapeutic drugs {Antonopoulos 2013} and FasL {Bossaller 2012} (Purple dash line, Figure 3). This means that loss of caspase-8 can potentially simultaneously lead to an increase in inflammasome activation and reduction in IL-1ß processing power. Most recently inflammasomes in *Ripk1*$^{-/-}$ BMDMs were shown, like the *Casp8*$^{-/-}$ BMDCs described by Kang *et al*. {Kang 2013} to be in a mildly inflammasome actived state and this was dependent upon the presence of RIPK3 {Rickard 2014}, however, there was little evidence for inflammasome activation or activity in *Ripk1*$^{-/-}$ mice {Rickard 2014}.

Clearly this is an emerging area, with some seemingly contradictory results, but the simplest take home message from these studies is that loss of upstream components of the cell death signaling axis, including RIPK1, RIPK3 and caspase-8 is likely to impact on other inflammatory signalling pathways in ways that we do not yet fully understand and their loss may induce or reduce inflammatory signalling. However, in the absence of strong genetic evidence to the contrary, it is

unlikely that downstream apoptosis or necroptosis effectors such as Caspase Activated Dnase (CAD) or MLKL play multiple roles.

New studies help define role of RIPK1 in cell death pathways

One of the most satisfying recent findings was that two different RIPK1 kinase dead (D138N and K45A) mutant mice are viable and that cells from these mice are completely resistant to necroptotic stimuli {Kaiser 2014; Newton 2014} (Figure 2). However *Ripk1* mutant kinase cells activate NF-κB normally, produce wild type levels of cytokines in response to TNF and are as resistant as wild type cells to apoptotic stimuli {Kaiser 2014; Newton 2014}. Thus the perinatal lethality of *Ripk1*$^{-/-}$ mice is due to the loss of the structural role that RIPK1 plays, not to its kinase activity (Figure 1). This genetic proof provides valuable support to many studies that have shown that most signalling pathways in which RIPK1 is involved, apart from necroptotic cell death, do not require the kinase activity of RIPK1. These RIPK1 kinase dead mutant mice can now be used to validate other pathological scenarios where use of the RIPK1 inhibitor Necrostatin has indicated that RIPK1 kinase activity is required for disease progression (Table 1). Furthermore, and consistent with the fact that both *Ripk3*$^{-/-}$ and *Mlkl*$^{-/-}$ mice are viable {Newton 2004; Wu 2013; Murphy 2013}, RIPK1 kinase activity is not essential for normal development (Figure 1).

These living RIPK1 kinase mutant mice necessarily imply that the *Ripk1*$^{-/-}$ mice die perinatally from aberrant signalling that is not due to necroptosis but apoptosis. However recent work from three independent labs adds yet another unexpected wrinkle to this story. Because, surprisingly, the perinatal lethality of *Ripk1*$^{-/-}$ mice is not prevented by loss of caspase-8 {Dillon 2014; Rickard 2014; Kaiser 2014} although the caspase-8 embryonic lethality was prevented by loss of RIPK1 (Figure 1). Thus, loss of RIPK1 phenocopies loss of RIPK3 in protecting *Casp8*$^{-/-}$ mice from death at E10.5 supporting the idea that the E10.5 checkpoint involves a necroptotic component that requires RIPK1 for its execution. However because the *Ripk3*$^{-/-}$*Casp8*$^{-/-}$ and *Ripk3*$^{-/-}$*Fadd*$^{-/-}$ mice survive past weaning, but the *Ripk1*$^{-/-}$*Casp8*$^{-/-}$ and *Ripk3*$^{-/-}$*Fadd*$^{-/-}$ mice do not, this implies that RIPK1 plays a protective role that RIPK3 does not . Remarkably it turns out that this role is most likely to limit necroptosis because *Ripk1*$^{-/-}$*Ripk3*$^{-/-}$*Casp8*$^{-/-}$ and even *Ripk1*$^{-/-}$*Ripk3*$^{-/+}$*Casp8*$^{-/-}$ mice are viable {Dillon 2014; Rickard 2014; Kaiser 2014}!

Can RIPK1 inhibit necroptosis?

The fact that loss of RIPK3 protected *Ripk1*$^{-/-}$*Casp8*$^{-/-}$ mice raises the awkward question of whether it is necroptosis that is killing the *Ripk1*$^{-/-}$ mice ({Dillon 2014; Rickard 2014; Kaiser 2014}, Figure 1). Awkward because it has been widely assumed that RIPK1 is required for necroptosis, exemplified by the fact that the RIPK1 kinase inhibitor Nec-1 is protective against TNF and TLR induced necroptosis (Figure 2). Surprisingly, loss of *Ripk3* does indeed provide some protection to *Ripk1*$^{-/-}$ mice {Dillon 2014; Rickard 2014; Kaiser 2014} (Figure 1). Perhaps the protection afforded by loss of RIPK3 is due its role in limiting inflammation? Speaking against this interpretation is the fact that the *Ripk1*$^{-/-}$*Ripk3*$^{-/-}$ and *Ripk1*$^{-/-}$*Mlkl*$^{-/-}$ mice have an equivalent phenotype ({Rickard 2014}, Figure 1). Both *Ripk1*$^{-/-}$*Ripk3*$^{-/-}$ and *Ripk1*$^{-/-}$*Mlkl*$^{-/-}$ mice survive longer than the *Ripk1*$^{-/-}$ mice by a few days but eventually succumb to an intestinal defect associated with excessive apoptosis in this tissue {Dillon 2014; Rickard 2014}. Because the pseudokinase MLKL is the terminal effector of the necroptotic pathway and so far does not appear to have a significant role in regulating production of inflammatory cytokines our interpretation of these experiments is that the *Ripk1*$^{-/-}$ mice die perinatally due to excessive necroptosis.

Combined loss of TNF signalling partially suppressed the excessive cell death phenotype in the intestine of the *Ripk1*$^{-/-}$*Ripk3*$^{-/-}$ mice, and caspase-8 deficiency completely suppressed it, with the increase in survival corresponding to the extent of the suppression (Figure 1, {Dillon 2014; Rickard

2014; Kaiser 2014}). Because the *Ripk1*$^{-/-}$*Mlkl*$^{-/-}$*Casp8*$^{-/-}$ cross was not generated we cannot definitively conclude that combined necroptosis and apoptosis in *Ripk1*$^{-/-}$ mice results in their premature death, however that is the parsimonious conclusion.

What necroptosis does RIPK1 inhibit?

But what is driving this necroptosis in the *Ripk1*$^{-/-}$ mice? Interferon signalling might provide a partial answer because the survival of *Ripk1*$^{-/-}$*Tnfr1*$^{-/-}$*Ifnar*$^{-/-}$ mice was extended beyond that of the *Ripk1*$^{-/-}$*Tnfr1*$^{-/-}$ mice. Furthermore, *Ripk1*$^{-/-}$ MEFs were hypersensitive to cell death induced by either IFNγ or IFNβ {Dillon 2014; Kaiser 2014} and *Ripk1*$^{-/-}$*Casp8*$^{-/-}$ cells were hypersensitive to IFNβ induced death. Furthermore, this death could be inhibited by a RIPK3 kinase inhibitor and or RIPK3, or MLKL, knock-down {Kaiser 2014}. That said, aberrant type I IFN signalling is unlikely to be the only source of necroptosis because *Ripk1*$^{-/-}$*Tnfr1*$^{-/-}$*Ifnar*$^{-/-}$ mice were not protected to the same extent as *Ripk1*$^{-/-}$*Ripk3*$^{-/-}$*Casp8*$^{-/-}$ mice (Figure 1, {Dillon 2014}). TLRs signal through TRIF which can activate RIPK3 via it own RHIM domain, however TLR signalling cannot account for all the increase in necroptosis in *Ripk1*$^{-/-}$ mice because the survival of *Ripk1*$^{-/-}$*Tnfr1*$^{-/-}$*Trif*$^{/-}$ mice was similar *Ripk1*$^{-/-}$*Tnfr1*$^{-/-}$*Ifnar*$^{-/-}$ mice (Figure 1, {Dillon 2014}). It is possible that loss of TLR and IFN signalling together will provide the same degree of protection, but the quadruple knock-out cross may be an experimental bridge too far. A further intriguing possibility is that RIPK1 directly inhibits autonomous RIPK3 activation. In support of this idea, Orozco *et al* recently demonstrated that RIPK1 inhibits auto-activation of an artificially dimerisable form of RIPK3 {Orozco 2014}. Consistent with the knock-out data and the idea that RIPK1 inhibits RIPK3 activation Kearney and colleagues have shown that cells with RIPK1 knock-down are actually more sensitive to necroptosis in response to both TNF and TLR induced necroptosis {Kearney 2014}. Based on these results, these authors put forward the interesting proposition that Necrostatin, rather than blocking the ability of RIPK1 to activate necroptosis, might block necroptosis by enhancing RIPK1's inhibitory properties {Kearney 2014}.

Evidence for Necroptotic DAMPs

So why do the *Ripk1*$^{-/-}$ mice die around birth? The most striking feature of the *Ripk1*$^{-/-}$ mice is systemic and multi-organ inflammation, which is reduced in the *Ripk1*$^{-/-}$*Ripk3*$^{-/-}$ and *Ripk1*$^{-/-}$*Mlkl*$^{-/-}$ mice (Figure 1 & Table 1, {Rickard 2014}). Furthermore deletion of *Myd88*, a key adaptor in DAMP signalling through TLRs, as well as RAGE, IL-1R1 and ST2L/IL-1 Receptor Accessory Protein (Figure 3,{Kaczmarek 2013}) also reduced the *Ripk1*$^{-/-}$ systemic and multi-organ inflammation (Figure 1 & Table1, {Rickard 2014}). These results together suggest that necroptosis induced DAMPs are driving the inflammation and lethality in *Ripk1*$^{-/-}$ mice. There are several proposed DAMPs that signal through TLRs such as HMGB1, mitochondrial DNA and HSPs however HMGB1 levels were not increased in the plasma of *Ripk1*$^{-/-}$ mice. *Myd88* deficiency can also inhibit IL-1ß signalling but there was likewise little evidence for inflammasome/caspase-1 processed IL-1ß in *Ripk1*$^{-/-}$ mice {Rickard 2014}. However levels of Il-1α and IL-33 that also signal through the IL-1 receptor family were readily detected in the plasma of *Ripk1*$^{-/-}$, but not *Ripk1*$^{-/-}$*Ripk3*$^{-/-}$ or *Ripk1*$^{-/-}$*Mlkl*$^{-/-}$ mice, suggesting that necroptosis induced release of IL-1α and IL-33 may be an important driver of inflammatory disease *in vivo* (Table 1, {Rickard 2014}. With reference to the earlier discussion about ^{cd}DAMPs, IL-33 is a particularly interesting molecule. It is a chromatin-associated protein in healthy cells, mostly constitutively expressed in epithelial and endothelial cells {Palmer 2011}. It does not appear to be conventionally secreted but is rather released from dying cells. It is highly inflammatory and levels are increased in inflammatory diseases {Palmer 2011}. Furthermore it is inactivated by apoptotic effector caspases 3 & 7 {Lüthi 2009; Cayrol 2009}. Thus it fulfills many of the requirements of an ideal ^{cd}DAMP; that is activated following a dangerous death but inactivated by a scheduled apoptotic death. Interestingly, although it can be inactivated by apoptotic caspases IL-33 can also be processed by proteases present in neutrophils and cleavage by

these proteases increase activity of IL-33 approximately ten fold {Lefrançais 2012}. Correlating with the high levels of neutrophils and inflammation, this active cleaved form of IL-33 was present in the plasma of *Ripk1*$^{-/-}$ and *Ripk1*$^{-/-}$*Casp8*$^{-/-}$ mice but not *Ripk1*$^{-/-}$*Ripk3*$^{-/-}$ or *Ripk1*$^{-/-}$*Mlkl*$^{-/-}$ mice.

RIP Kinases and MLKL as therapeutic targets in disease

These results indicate that necroptosis can play a role in driving inflammation in development and in sterile inflammation scenarios, but if they are to have any clinical utility we want to know whether inhibiting necroptosis can limit inflammation in disease. Necroptosis, has been implicated as pathogenic in multiple models of tissue damage including heart attack, atherosclerosis, liver injury, retinal injury, pancreatitis and renal injury (see Table I for summary, {Linkermann 2014}). Furthermore, *Ripk1*$^{D138N/\ D138N}$, *Ripk1*$^{K45A/\ K45A}$, *Ripk3*$^{-/-}$ and *Mlkl*$^{-/-}$ mice have no overt phenotype suggesting that necroptosis is dispensable for survival (Figure1, {Newton 2004; Wu 2013; Murphy 2013; Newton 2014; Kaiser 2014}) and may be a safe therapeutic target.

RIPK1 may even be a useful target because kinase dead *Ripk1*$^{D138N/D138N}$ mice were protected from TNF induced hypothermia (Table I, {Newton 2014}) and crossing the *Ripk1*$^{K45A/K45A}$ to the Sharpin mutant *cpdm* mice reduced inflammation in these mice (Table I, {Berger 2014}). These results provide much needed genetic support for observations made with the RIPK1 kinase inhibitor Nec-1, because in addition to its very short half-life *in vivo*, Nec-1 is also an inhibitor of IDO (indoleamine 2,3-dioxygenase) an enzyme also involved in the inflammatory response {Vandenabeele 2013}. Certainly results showing that Necrostatin can increase post injury survival in an ischemia-reperfusion (IR) model that simulates renal damage following kidney transplantation are worth following up (Table I, {Linkermann 2012}. In agreement, *Ripk3*$^{-/-}$ mice were protected from IR injury {Lau 2013} and associated with reduced HMGB1 release (Table I), suggesting that necroptosis inhibition may have therapeutic utility by not only preventing loss of functional kidney tissue but also by preventing DAMP induced cytokine release {Linkermann 2013}.

Whether targeting RIPK3 might have similar utility is however less clear. On one hand, Necrostatin pre-treatment protected mice from TNF-induced systemic inflammatory response syndrome (SIRS) (Table I, {Duprez 2011}) and *Ripk3*$^{-/-}$ mice were protected from both, SIRS and caecal ligation and puncture (CLP) model of sepsis (Table I, {Duprez 2011}). However others were unable to demonstrate that either *Mlkl*$^{-/-}$ mice or *Ripk3*$^{-/-}$ mice were protected in the CLP model (Table I, {Wu 2013}). Even less encouraging, within 2 days of inducibly knocking in the *Ripk3*^{D161N} allele, adult mice died of rapid weight loss and excessive cell death in the intestine {Newton 2014}. Whether this means that RIPK3 should never be targeted is not clear because another RIPK3 kinase dead knock-in mouse is viable (Figure 1, (Kaiser & Mocarski, personal communication)).

One potential insight we can take from the combination of knock-out/in animals described above, and particularly the triple knock-outs, is that targeting necroptosis alone is unlikely to be a panacea for all ills and that a more efficacious strategy might be to block apoptosis, or at least caspase-8, in addition to blocking necroptosis. Ischemia reperfusion injuries with an acknowledged apoptotic and necrotic component seem obvious candidates for this sort of approach.

The roles of Ripk1/3 during infection

Because RIPK1 plays an important structural role in TNF/TLR/NF-κB induced inflammatory cytokine production (Figure 3) it is unsurprising that RIPK1 is functionally implicated in many infections {Christofferson 2014}. In addition to these widely known roles, RIPK1 also plays a role in induced expression of type I Interferons, α and ß, in response to single strand RNA (ssRNA) viruses (Pink circle, Figure 3){Balachandran 2004; Rajput 2011}. In uninfected cells caspase-8 appears to limit activation of this pathway by cleaving RIPK1, because caspase-8 knock-out cells

are "primed" for IRF3 activation {Rajput 2011} a finding that suggests a mechanism for the chronic inflammation observed in *Casp8*$^{-/-}$ mice (Pink circle, Figure 3, {Rajput 2011}).

As discussed, host cells activate the apoptotic pathway to limit viral replication, and this may be through a RIPK1/caspase-8 dependent pathway. One of the first indications that programmed necrosis may play a role in viral defence came from dsDNA *vaccinia virus* (VV), a virus that is capable to inhibit apoptosis (Dobbelstein and Shenk, 1996; Wasilenko et al., 2003) and pyroptosis (Gerlic et al., 2013). Chan *et al* showed that RIPK1 was required for TNF induced necrosis of VV-infected cells {Chan 2003}. TNFR2 signalling potentiated TNFR1 induced necroptosis {Chan 2003}, probably although this was not explored at the time, by depleting TRAF2 and IAPs {Silke 2010}. Subsequently VV-induced necroptosis was shown to require RIPK3 {Cho 2009}. The strongest argument for the importance of necroptosis during viral infection comes from studies of the dsDNA *murine cytomegalovirus* (MCMV). MCMV encodes a protein, vIRA (M45), which interacts via its RHIM domain with both RIPK1 and RIPK3 to inhibit DAI-dependent necroptosis {Upton 2008; Upton 2010; Upton 2012}. Necroptosis may also occur in HIV infected CD4+ T-cells because treatment with Nec-1, or the human MLKL inhibitor NSA restrains HIV-1 cytopathic effect {Pan 2014}.

Bacterial infection can also induce RIPK1/3 dependent cell death. *Salmonella*, which induces pyroptosis in macrophages, was also found to induce a necroptotic autocrine loop via induction of type I IFN to induce RIPK3-dependent necroptosis (Green circle, Figure 2, {Robinson 2012}). Bacteria have also evolved to inhibit cell death and RIPK1 signaling, a recent example provided by the *enteropathogenic Escherichia coli* (EPEC) effector protein, NleB1 that binds to and modifies FADD, TRADD and RIPK1 with its N-acetylglucosamine transferase activity to prevent FasL- or TNF-induced apoptosis {Li 2013; Pearson 2013}. Furthermore, *Porphyromonas gingivalis* was shown to specifically cleave RIPK1 via its lysine-specific (Kgp) protease {Madrigal 2012}.

Although it is clear that RIPK1 and RIPK3 play an important role during infection, distinguishing between the direct inflammatory effect (cytokine) and the cell death (necroptosis) is still one of the most challenging and unsolved questions in this field, similar to caspase-1's role in cytokine production versus pyroptotic cell death {Croker 2014}. For example the *Yersinia pestis* outer protein P (YopP) was shown to inhibit NF-κB leading to cFLIP degradation, caspase-8 activation, apoptosis, RIPK1 cleavage via a "Ripoptosome"; although this was not the terminology used {Gröbner 2007}. More recently a RIPK1, caspase-8 pathway was shown to be required for *Y. pestis* infection-induced production of IL-1β, IL-18, TNF, and IL-6 and processing of IL-1β and IL-18 by caspase-1 {Weng 2014; Philip 2014}.

The dog that didn't bark: what RIPK1 doesn't do

As we've recorded above, RIPK1 has been implicated in contributing to signalling from almost every known innate immune signalling complex: TNFR1, TLRs, NLRP inflammasomes etc. This ubiquitous presence would lead one to expect that *Ripk1*$^{-/-}$*Ripk3*$^{-/-}$*Casp8*$^{-/-}$ mice are devastatingly immunodeficient; but on the contrary they live an approximately normal life span, have a remarkably normal distribution of lymphocytes, succumb to the *Casp8*$^{-/-}$*Ripk3*$^{-/-}$ ALPS syndrome only later in life {Dillon 2014; Rickard 2014; Kaiser 2014} and generate a robust antigen-specific T-cell response to MCMV viral infection {Kaiser 2014}.

Not proven

The fundamental questions in the field of necroptosis research are, how, where and when does it occur. The lack of markers for necroptotic cells has hampered our ability to address the where and when questions and a phospho-MLKL antibody may go some way to address the deficiency in

markers for necroptosis, at least in humans {Wang 2014}. Such studies may help to answer the "why should I care" question because until these are answered we are unable to definitively address whether necroptosis is important in human disease. In the meantime the results from the extensive genetic crosses described in this review (Figure 1) provide strong support for the idea that inflammatory diseases can have a necroptotic component and that it may be suited to targeting with small molecules in the clinic. Moreover they provide an impetus to target the pathway and suggest other biomarkers for necroptotic inflammation that can be looked at in other disease scenarios.

The how question turns out to have some intriguing answers and the studies discussed in this review have almost laid to rest the long-standing mystery of why the *Ripk1*$^{-/-}$ mice die and in the process brought to light unexpected roles for RIPK1 in inhibiting necroptosis while confirming other known roles. They suggest that RIPK3 may also have roles in inhibiting apoptosis and they have provided some, albeit contradictory, data to suggest how MLKL kills necroptotic cells. They certainly provide more than enough justification for going back over old cases {Kelliher 1998} to look for new insights {Dillon 2014; Rickard 2014; Kaiser 2014}.

Tissue	Model / investigation	Nec-1	Nec-1s	*Ripk3* Deletion	*Mlkl* Deletion	*Myd88* Deletion	DAMP involvem
Heart	Myocardial infarction / cardiac hypoxia	Protects					
Skin	*Fadd* epidermal deletion *Casp8* epidermal deletion *Ripk1*$^{-/-}$ mice: epidermal hyperplasia *Sharpin*$^{cpdm/cpdm}$ dermatitis			Prevents Prevents Protects		Slight delay No effect Protects	Extracellu HMGB1 ↑IL-33 a S10(↑IL-33
Pancreas	Cerulein induced pancreatitis	Worsens		Protects	Protects		
Liver	Ethanol induced liver injury Fas-induced hepatitis Liver parenchymal cell TAK1 deletion induced inflammatory hepatocarcinogenesis	No effect		Protects No effect Worsens			
Eye	dsRNA induced retinal degeneration Retinitis pigmentosa; cone cell death (*Rd10*$^{-/-}$ mice) Retinitis pigmentosa (*Irbp*$^{-/-}$ mice) Retinal detachment injury	Protects Protects No effect	Protects	Protects Protects			HMGB1
Kidney	Renal ischaemia reperfusion injury Renal transplantation	↑survival		↑survival Protects			HMGB1
Brain	Ischaemic brain injury Trauma (controlled cortical impact) Huntington's disease model	Protects Protects Protects					
Intestine	Intestinal epithelial cell *Fadd* deletion Tamoxifen inducible *Casp8* deletion (CreER) *Ripk1*$^{-/-}$ mice: intestinal phenotype Crohn's disease patients (CD) CD and ulcerative colitis patients			Prevents Prevents No effect		Prevents colitis$^{\Psi}$ No effect	

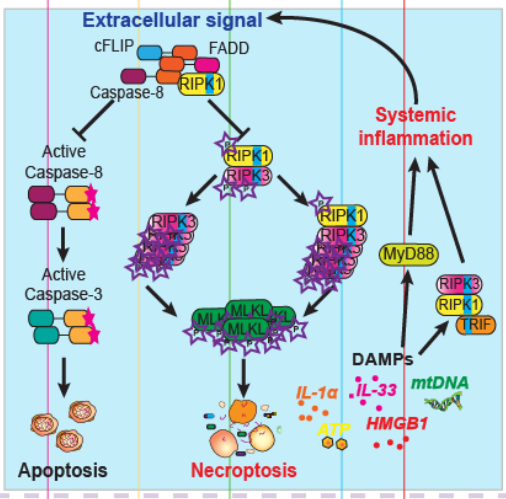
Vascular	Atherosclerosis; *Ldlr*$^{-/-}$ and *Apoe*$^{-/-}$ mice			Protects			
Systemic	TNF injected mice (SIRS) Caecal ligation puncture SIRS/sepsis LPS injected mice LPS/Z-VAD injected mice LPS/GalN injected mice TNF/Z-VAD injected mice (hyper acute shock) *Ripk1*$^{-/-}$ mice: inflammatory phenotype	Protects/ Worsens * Worsens No effect on cytokines No effect Protects/ Worsens	Protects	Protects Protects/ No effect No effect on cytokines Protects Protects Protects	Protects No effect Protects	Protects	Plasma mtDNA Plasma Il and Il-33

Table 1. Evidence for RIPK1 and RIPK3 regulation of models of inflammation and tissue damage. *Both Nec-1i (inactive) and Nec-1 equally effective, but at low doses paradoxically both drugs worsened; attributed to off-target effects of Nec-1[34]. Φ*Sharpin*$^{cpdm/cpdm}$*Ripk1*$^{K45A/K45A}$ kinase dead (KD) mice also have no multi-organ pathology seen in *Sharpin*$^{cpdm/cpdm}$ mice. Ψ*Myd88*$^{-/-}$ prevents colitis but not Paneth cell loss and small intestine phenotype.

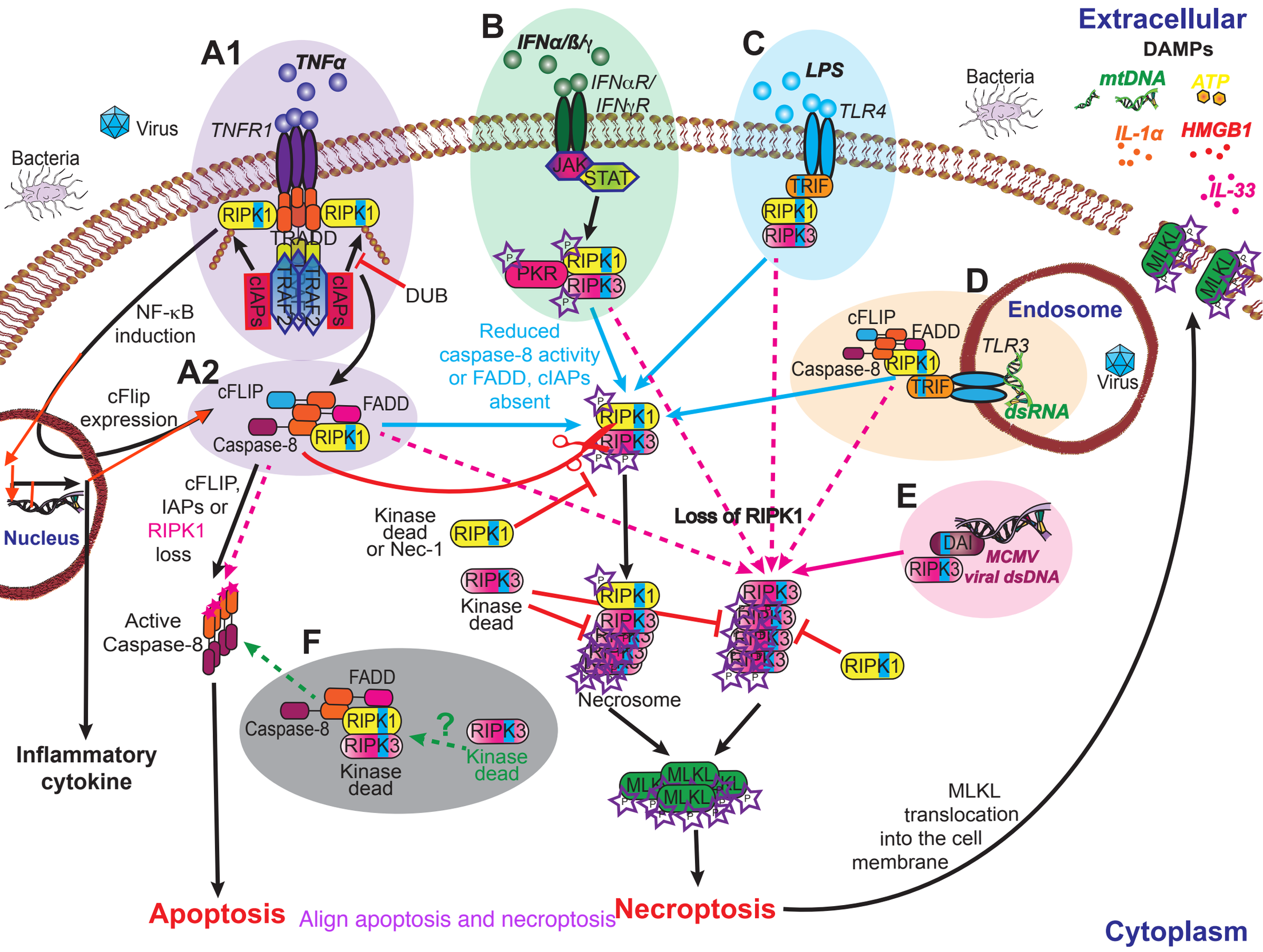
References

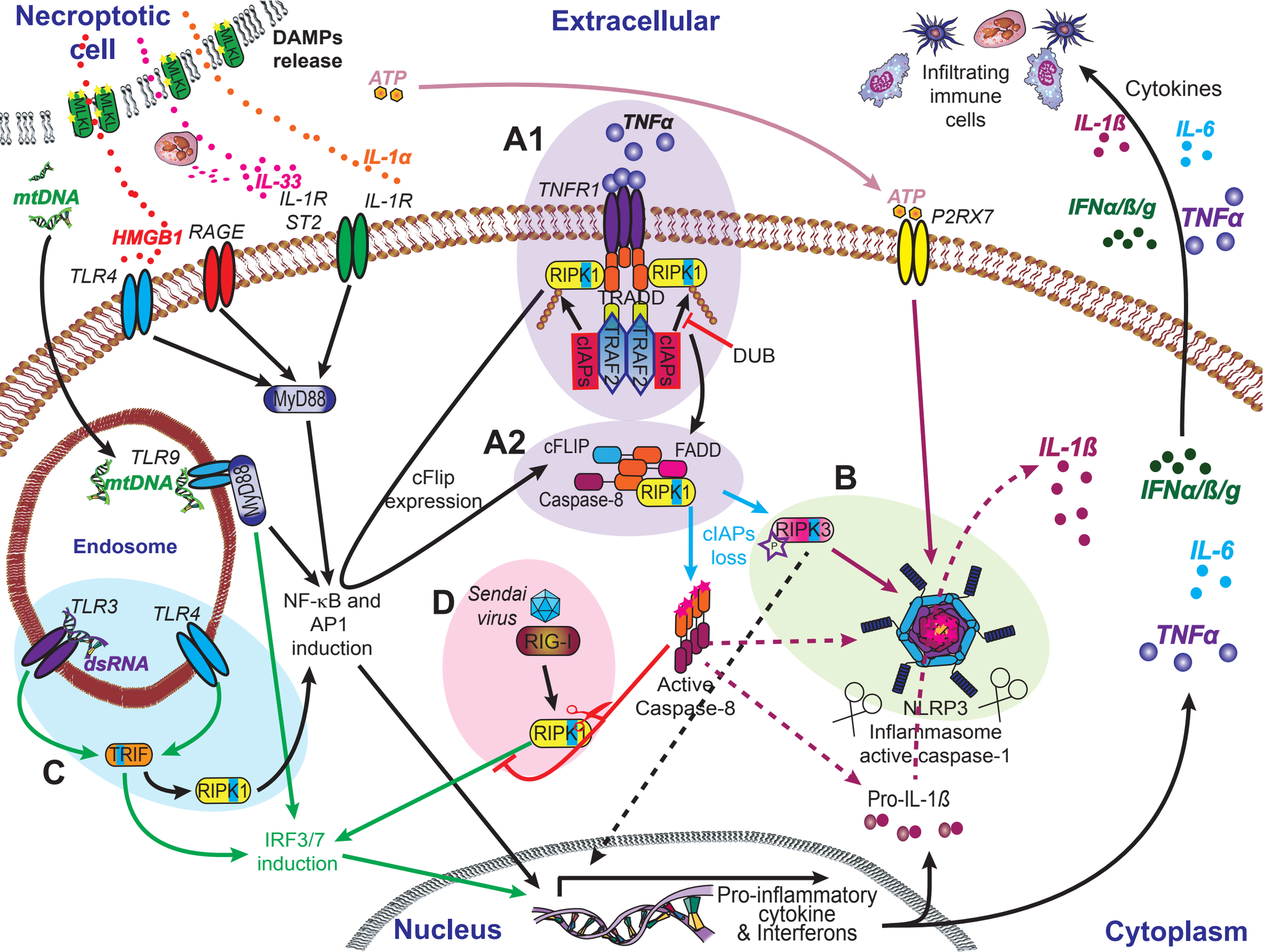
{bibliography}

Background — E10.5 — E12.5 — E14.5 — E19/P0 — P3/P4 — Weaned — Adult — Reference



mixed C57BL/6 129/Sv Ripk1$^{-/-}$	**Kelliher 1998**
+*Trif$^{-/-}$ or +Ifnar$^{-/-}$or +Nik1$^{-/-}$*	**Dillon 2014**
+*Casp8$^{-/-}$*	**Dillon 2014; Kaiser 2014**
+*Ripk3$^{-/-}$*	**Dillon 2014; Kaiser 2014**
+*Tnf$^{-/-}$*	**Kaiser 2014**
+*Tnfr1$^{-/-}$*	**Cusson 2002**
+*Ripk3$^{-/-}$Tnf$^{-/-}$*	**Kaiser 2014**
+*Tnfr1$^{-/-}$Ifnar$^{-/-}$*	**Dillon 2014**
+*Ripk3$^{-/-}$Tnfr1$^{-/-}$*	**Dillon 2014**
+*Ripk3$^{+/-}$Casp8$^{-/-}$*	**Kaiser 2014**
+*Ripk3$^{-/-}$Fadd$^{-/-}$*	**Dillon 2014**
+*Ripk3$^{-/-}$Casp8$^{-/-}$*	**Dillon 2014; Kaiser 2014**
C57BL/6 Ripk1$^{-/-}$	**Rickard 2014**
+*Casp8$^{-/-}$*	**Rickard 2014**
+*Tnf$^{-/-}$Fasl$^{gld/gld}$*	**Rickard 2014**
+*Ripk3$^{-/-}$ or +Mlkl$^{-/-}$*	**Rickard 2014**
+*Myd88$^{-/-}$*	**Rickard 2014**
+*Ripk3$^{-/-}$Casp8$^{-/-}$*	**Rickard 2014**
Ripk1$^{D138N/D138N}$	**Newton 2014**
Ripk1$^{K45A/K45A}$	**Kaiser 2014**
Ripk3$^{-/-}$	**Newton 2004**
Ripk3$^{D161N/D161N}$	**Newton 2014**
+*Mlkl$^{-/-}$or +Trif$^{-/-}$or +Dai$^{-/-}$or +Cyld$^{-/-}$or +Tnfr1$^{-/-}$or +Tnfr1$^{-/-}$Dr3$^{-/-}$or +cFlip$^{-/-}$or +Ripk1$^{D138N/D138N}$*	**Newton 2014**
+*Ripk1$^{-/-}$*	**Newton 2014**
+*Casp8$^{-/-}$*	**Newton 2014**
Ripk3$^{K51A/K51A}$	**Kaiser and Mocarski Personal communication**
Mlkl$^{-/-}$	**Wu 2013; Murphy 2013**
Casp8$^{-/-}$	**Varfolomeev 1998**
+*Tnfr1$^{-/-}$*	**Dillon 2014**
+*Ripk1$^{-/-}$*	**Rickard 2014**
+*Ripk3$^{-/-}$*	**Oberst 2011; Kaiser 2011**
+*Mlkl$^{-/-}$*	**Alvarez-Diaz, Strasser and Alexander Personal communication**
cFLIP$^{-/-}$	**Yeh 2000**
+*Ripk3$^{-/-}$*	**Dillon 2012**
+*Tnfr1$^{-/-}$ or +Ripk3$^{-/-}$Tnfr1$^{-/-}$*	**Dillon 2014**
+*Ripk3$^{-/-}$Fadd$^{-/-}$*	**Dillon 2012**
cFLIP$^{IEC/IEC}$or cFLIP$^{Hep/Hep}$	**Piao 2012**
+*Tnfr1$^{-/-}$*	**Piao 2012**
Fadd$^{-/-}$	**Yeh 1998**
+*Tnfr1$^{-/-}$*	**Dillon 2014**
+*Ripk1$^{-/-}$*	**Zhang 2011**
+*Ripk3$^{-/-}$*	**Dillon 2012**
clap1$^{-/-}$clap2$^{-/-}$	**Moulin 2012**
+*Ripk1$^{-/-}$*	**Moulin 2012**
+*Ripk3$^{-/-}$*	**Moulin 2012**
+*Tnfr1$^{-/-}$*	**Moulin 2012**


Extracellular
DAMPs
mtDNA
ATP
IL-1α
HMGB1
IL-33
Bacteria
Virus
A1
TNFα
TNFR1
RIPK1
RIPK1
TRADD
TRAF2
TRAF5
cIAPs
cIAPs
DUB
NF-κB induction
B
IFNα/ß/γ
IFNαR/ IFNγR
JAK
STAT
PKR
RIPK1
RIPK3
C
LPS
TLR4
TRIF
RIPK1
RIPK3
Bacteria
D
Endosome
cFLIP
FADD
Caspase-8
RIPK1
TRIF
TLR3
dsRNA
Virus
A2
cFlip expression
cFLIP
FADD
Caspase-8
RIPK1
Reduced caspase-8 activity or FADD, cIAPs absent
RIPK1
RIPK3
Nucleus
cFLIP, IAPs or RIPK1 loss
Kinase dead or Nec-1
RIPK1
Loss of RIPK1
E
DAI
RIPK3
MCMV viral dsDNA
RIPK3
Kinase dead
RIPK1
RIPK3
RIPK3
RIPK1
Active Caspase-8
F
FADD
Caspase-8
RIPK1
RIPK3
Kinase dead
?
RIPK3
Kinase dead
Necrosome
Inflammatory cytokine
MLKL
MLKL
MLKL translocation into the cell membrane
Apoptosis
Align apoptosis and necroptosis
Necroptosis
Cytoplasm


Necroptotic cell
MLKL
DAMPs release
Extracellular
ATP
Infiltrating immune cells
Cytokines
IL-1ß
IL-6
IFNα/ß/g
TNFα
IL-1α
IL-33
mtDNA
HMGB1
RAGE
TLR4
IL-1R ST2
IL-1R
A1
TNFα
TNFR1
RIPK1
TRADD
cIAPs
TRAF2
DUB
P2RX7
MyD88
A2
cFLIP
FADD
Caspase-8
cFlip expression
TLR9
mtDNA
Endosome
B
cIAPs loss
RIPK3
IL-1ß
IFNα/ß/g
IL-6
TNFα
TLR3
TLR4
dsRNA
NF-κB and AP1 induction
D
Sendai virus
RIG-I
Active Caspase-8
NLRP3 Inflammasome active caspase-1
Pro-IL-1ß
C
TRIF
IRF3/7 induction
Pro-inflammatory cytokine & Interferons
Nucleus
Cytoplasm