CONNECTING THE DOTS BETWEEN INFLAMMATION AND THE INNER WORKINGS OF PROGRAMMED CELL DEATH

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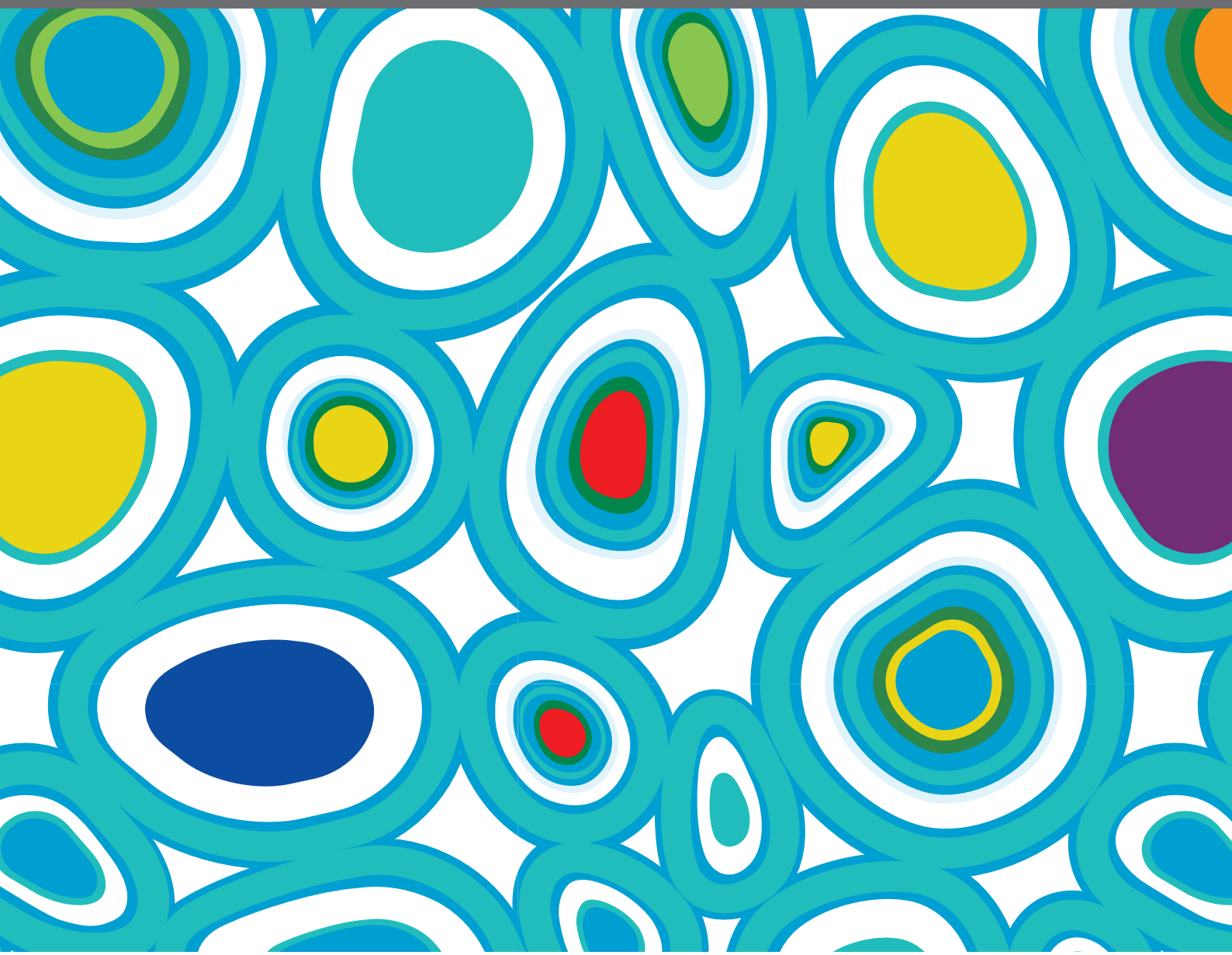

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CONNECTING THE DOTS BETWEEN INFLAMMATION AND THE INNER WORKINGS OF PROGRAMMED CELL DEATH

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Programmed cell death (PCD) is central in maintaining the life of multicellular organisms, during development as well as in healthy adulthood or in the context of disease. The best understood form of PCD is apoptosis, a caspase mediated, immunologically silent cell death that can be initiated in probably all cell types upon aging, lack of growth support, critical damage or infection. One of the key pathways of apoptosis involves mitochondrial outer membrane permeabilization (MOMP), a process tightly regulated by members of the BCL-2 family. Whereas PCD and apoptosis were used synonymously in the past, other forms of PCD have been discovered more recently, including RIPK1/3- and MLKL-dependent necroptosis, resulting in a necrotic phenotype, and pyroptosis. Interestingly, key components of the necroptotic pathway are actively suppressed by apoptotic caspases, and this interconnection allows a switch in cell death modalities with greatly impact on the host's immune response. Recent findings link mitochondria and/or MOMP to non-apoptotic forms of PCD, including ferroptosis and necroptosis, putting this organelle even more in the center of cellular death. This article collection highlights the exciting potential and as yet undiscovered regulation of programmed cell death that can impact the immune system and its response.

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BRIEF RESEARCH REPORT
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Combined Knockout of RIPK3 and MLKL Reveals Unexpected Outcome in Tissue Injury and Inflammation

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Necroptosis, initially identified as a backup cell death program when apoptosis is hindered, is a prominent feature in the etiology and progression of many human diseases, such as ischemic injury and sepsis. Receptor-interacting protein kinase 3 (RIPK3) is the cardinal regulator of this cell death modality, recruiting and phosphorylating the executioner mixed lineage kinase domain-like protein (MLKL) to signal necroptosis, which is terminated by a cellular plasma membrane rupture and the leakage of intracellular contents from dying cells. Experimental data to date indicate that RIPK3 and MLKL is the core machinery essential for all necroptotic cell death responses. By using CRISPR/Cas9 (clustered regularly interspaced short palindromic repeat/CRISPR-associated protein 9) technology, we showed that *Ripk3* and *Mlkl* knockout and *Ripk3/Mlkl* double-knockout in necroptosis-sensitive cell lines extensively block susceptibility to necroptosis, in each case to an indistinguishable degree. *In vivo* studies using *Ripk3*- or *Mlkl*-deficient mice validated kidney ischemia reperfusion injury and high-dose tumor necrosis factor (TNF) availability, as druggable targets in necroptotic-mediated pathologies. Here, we demonstrated that *Ripk3* or *Mlkl*-deficient mice are protected to a similar extent from kidney ischemia reperfusion injury and TNF-induced toxicity. Remarkably, in contrast to each single knockout, *Ripk3/Mlkl* double-deficient mice did not have appreciable protection from either of the above necroptotic-mediated pathologies. Paradoxically, the double-knockout mice resembled, in each case, the vulnerable wild-type mice, revealing novel complexities in the mechanisms of inflammation-driven diseases, due to aberrant cell death.

Keywords: regulated cell death (RCD), necroptosis, *Ripk3/Mlkl*, ischemia- reperfusion injury, TNF-induced shock

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INTRODUCTION

Necroptosis is a caspase-independent programmed cell death mediated by receptor-interacting protein kinase 3 (RIPK3) activation (Cho et al., 2009; He et al., 2009; Zhang et al., 2009) and the pursuant RIPK3-mediated phosphorylation of its pseudokinase substrate mixed lineage kinase domain-like protein (MLKL) (Sun et al., 2012). This initial stimulus prompts a conformational change that results in MLKL oligomerization, plasma membrane translocation, and lethal permeation of the lipid bilayer, leading to the release of cellular content, which triggers an inflammatory response (Rickard et al., 2014b). However, the exact mechanism by which activated MLKL kills cells remains unclear (Petrie et al., 2017). The availability of pharmacological inhibitors,

especially mice harboring deletions that are indispensable for necroptotic pathway signaling, facilitates research investigating the mechanisms of necroptosis and its relevance to diseases such as ischemic injury and sepsis (Krautwald et al., 2016). However, the role of natural necroptosis in human diseases remains controversial, and the potential off-target effects of the applied inhibitors besides kinase activity and existing scaffold functions of the involved proteins often complicate the interpretation of findings. In this context, our previously published data verify that pharmacologically blocking necroptosis may worsen diseases such as acute pancreatitis or vascular leakage syndrome, which is triggered by a high-dose tumor necrosis factor (TNF). The latter is often considered a model for systemic inflammatory response syndrome (SIRS) (Duprez et al., 2011). We recently discovered that necroptosis and ferroptosis, a caspase-independent regulated cell death modality characterized by the accumulation of lethal lipid reactive oxygen species (ROS) which is produced through iron-dependent lipid peroxidation, are alternative cell death pathways that operate in acute kidney failure, where each death modality can compensate for another when one is compromised (Müller et al., 2017). In contrast, we, along with others have reported that *Ripk3* deficiency and catalytically inactive RIPK1 are beneficial in renal ischemia-reperfusion injury (IRI), Gaucher's disease, myocardial infarction, and the high-dose TNF shock model (Linkermann et al., 2013; Polykratis et al., 2014; Vitner et al., 2014; Zhang et al., 2016). Deleting either *Ripk3* or *Mlkl* can suppress skin inflammation in *RIPK1*-deficient mice (Dannappel et al., 2014), and the fact that *Ripk3* or *Mlkl* deficiency ameliorates liver inflammation and splenomegaly in *Sharpin*-deficient mice (Rickard et al., 2014a), suggesting that MLKL follows RIPK3 directly in necroptotic signaling, therefore confers a similar degree of protection against the abovementioned necroptotic-mediated injuries. Nevertheless, there are also studies indicating that *Mlkl* deficiency confers less protection in the kidney IRI model compared to *Ripk3* deficiency, and in contrast to *Ripk3*-deficient mice, *Mlkl*-deficient mice resemble wild-type mice in their sensitivity to hypothermia induced by low-dose TNF (Newton et al., 2016a). However, our present findings reveal that when mice experience severe renal IRI, or when treated intravenously with a high-dose TNF, the differences between *Ripk3*-deficient and *Mlkl*-deficient mice are less apparent, substantiating the premise that RIPK3 cannot exacerbate these injuries independently of MLKL. Interestingly, our findings describe for the first time that combined knockout of the necrosome members *Ripk3* and *Mlkl* in an entire organism antagonizes the beneficial effect of the respective single knockouts in necroptotic cell death processes of severe IRI and TNF-induced shock.

MATERIALS AND METHODS

Cell Culture

NIH3T3 cells (American Type Culture Collection) were cultured in Dulbecco's modified Eagle's medium (Gibco/Thermo Fisher Scientific, Darmstadt, Germany) supplemented with 10% (vol/vol) fetal calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere containing 5% CO_2. Generation of the CRISPR/Cas9 NIH3T3 knockout cells has been described previously (Müller et al., 2017). To exclude feasible off-target effects or clonal variations within the cell population, we generated and analyzed three guide RNAs per target gene and observed congruent outcomes in each case. Each gene knockout was validated via a western blot analysis of the protein expression, as described previously (Müller et al., 2017).

Reagents and Antibodies

Recombinant purified TNFα, annexin V-fluorescein isothiocyanate (FITC) antibody, and 7-amino-actinomycin D (7-AAD) antibody was obtained from BioLegend (London, United Kingdom). The zVAD-fmk (herein referred to as zVAD) was obtained from Bachem (Weil, Germany); erastin and 1S,3R-RSL3 (herein referred to as RSL3) obtained from Tocris, Bio-Techne (Wiesbaden, Germany).

Cell Death Detection *in vitro*

Phosphatidylserine exposure to the outer cell membrane of apoptotic cells, or at the inner plasma membrane of necrotic cells and 7-AAD incorporation into necrotic cells, was quantified by fluorescence-activated cell sorting (FACS). Staining was performed according to the manufacturer's instructions (BioLegend). Fluorescence was analyzed using an FC 500 flow cytometer (Beckman Coulter, Krefeld, Germany).

Mice

All mice (8 weeks old) used were on C57BL/6 background and age-, sex-, and weight-matched. The mice were received and independently bred as wild-type, *Ripk3* knockout, *Mlkl* knockout, and *Ripk3/Mlkl* double-knockout colonies, and mice of different genotypes were not housed in the same cages. The *Ripk3* and *Mlkl* single knockout mice as well as the *Ripk3/Mlkl* double-knockout (dko) mice have been described previously (Newton et al., 2004; Murphy et al., 2013; Tanzer et al., 2015). All mice were kept on a standard diet and a 12-h day/night rhythm. All *in vivo* experiments were performed according to the Protection of Animals Act, after receiving approval from the German local authorities (MELUND, Kiel, Germany, application nos. V311-72241.121-4 and V242-30421/2016).

TNFα-Induced Shock Model

Recombinant carrier-free murine TNFα was obtained from R&D Systems (Bio-Techne, Wiesbaden, Germany). Each female mouse received a single bolus of 1 mg murine TNFα/kg body weight in a total volume of 200 μl phosphate-buffered saline, via the tail vein. The animals were placed under permanent observation and survival was checked every 15 min.

Ischemia-Reperfusion Injury (IRI)

Kidney IRI was induced via a midline abdominal incision and 40-min bilateral renal pedicle clamping using microaneurysm clamps (Aesculap, Inc.) as described previously (Moerke et al., 2018). Male mice were sacrificed 48 h after reperfusion, and serum urea and creatinine values were measured.

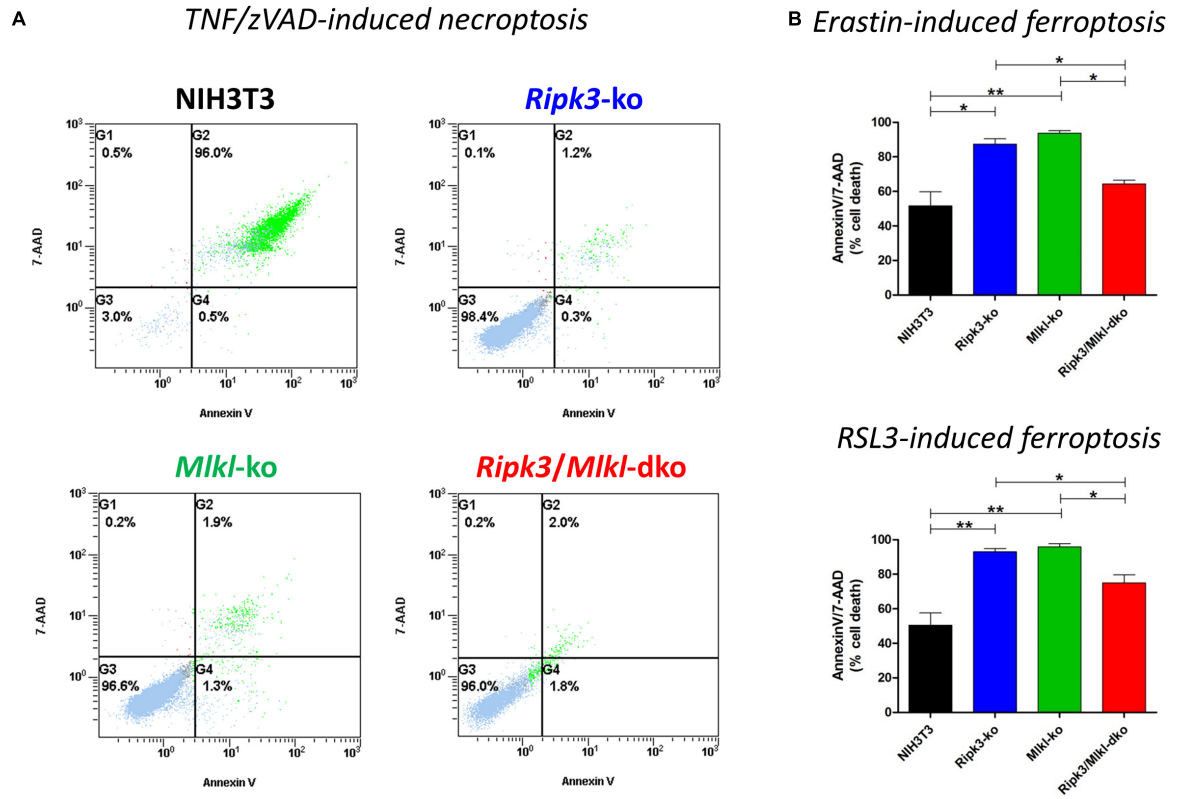

FIGURE 1 | In contrast to the parental NIH3T3 cells, *Ripk*3 knockout (ko) NIH3T3 cells, *Mlkl* knockout (ko) and *Ripk3/Mlkl* double-knockout (dko) NIH3T3 cells are protected from necroptosis. **(A)** Cell death was induced by the addition of 100 ng/ml TNFα + 25 μM zVAD for 24 h at 37°C. Necroptotic cell death was quantified by FACS using 7-AAD and phosphatidylserine accessibility (annexin V staining) as markers. A representative FACS dot plot of three independent experiments is shown. **(B)** Detection of ferroptosis in NIH3T3 clones. All cells were treated for 24 h at 37°C with 2 μM erastin or 2.5 μM RSL3. Ferroptotic cell death was quantified by FACS using 7-ADD and annexin V. Results are the mean ± SD of three independent experiments. *$p < 0.05$ and **$p < 0.02$.

Statistical Methods and Analyses

For all experiments, dataset differences were considered statistically significant when p-values were lower than 0.05, unless otherwise specified. Statistical comparisons were performed using the Mann–Whitney U-test with exception of the survival curves which were analyzed using the Gehan-Breslow-Wilcoxon test and the log rank test (Mantel-Cox). Asterisks in the figures/legends specify statistical significance (*$p < 0.05$, **$p < 0.02$, and ***$p < 0.001$). Statistics are indicated as SD, unless otherwise specified.

RESULTS

Recently, we have shown that two forms of regulated cell death, necroptosis and ferroptosis, are alternative, in that resistance to one pathway sensitizes cells to death via the other pathway, suggesting a mechanism by which one regulated pathway compensates for the other, when one is compromised (Müller et al., 2017). Regarding increased susceptibility to ferroptosis, we obtained these novel insights by specific deletion of *Mlkl*. In contrast to RIPK3, MLKL is so far known to merely play a role in necroptosis (Alvarez-Diaz et al., 2016). Here, however, we confirmed *in vitro* the aforementioned hypersensitization to ferroptosis and consequently coordinated the regulation of these two pathways in a similar manner by deleting *Ripk3* instead of *Mlkl*. However, as RIPK3 exerts its functions independently of necroptosis (Moriwaki and Chan, 2017), we were interested in examining whether the combined loss of *Ripk3* and *Mlkl* genes was equivalent to single gene knockouts. As illustrated in **Figure 1A**, the *Ripk3/Mlkl* dko protected the cells from TNF/zVAD-induced necroptosis just as effectively as each single knockout. As expected, our previously described time- and concentration-dependent hypersensitivity to ferroptosis in *Mlkl* knockout cells, was also present in the *Ripk3* knockout cells. To prove this, we depicted a representative experiment in which cells were treated for 24 h at 37°C with 2 μM erastin and with 2.5 μM RSL3 (**Figure 1B**), small molecules that trigger this unique iron-dependent form of regulated cell death (Dixon et al., 2012). However, even more astonishing were the findings regarding the *Ripk3/Mlkl* dko cells in this setting. Paradoxically, hypersensitivity of the dko cells, to erastin- and RSL3-induced ferroptosis, which was detectable across a range of erastin and RSL3 concentrations (1–10 μM, data not shown) was, in contrast to each single knockout, almost completely abrogated (**Figure 1B**). Although the dko cells were still protected

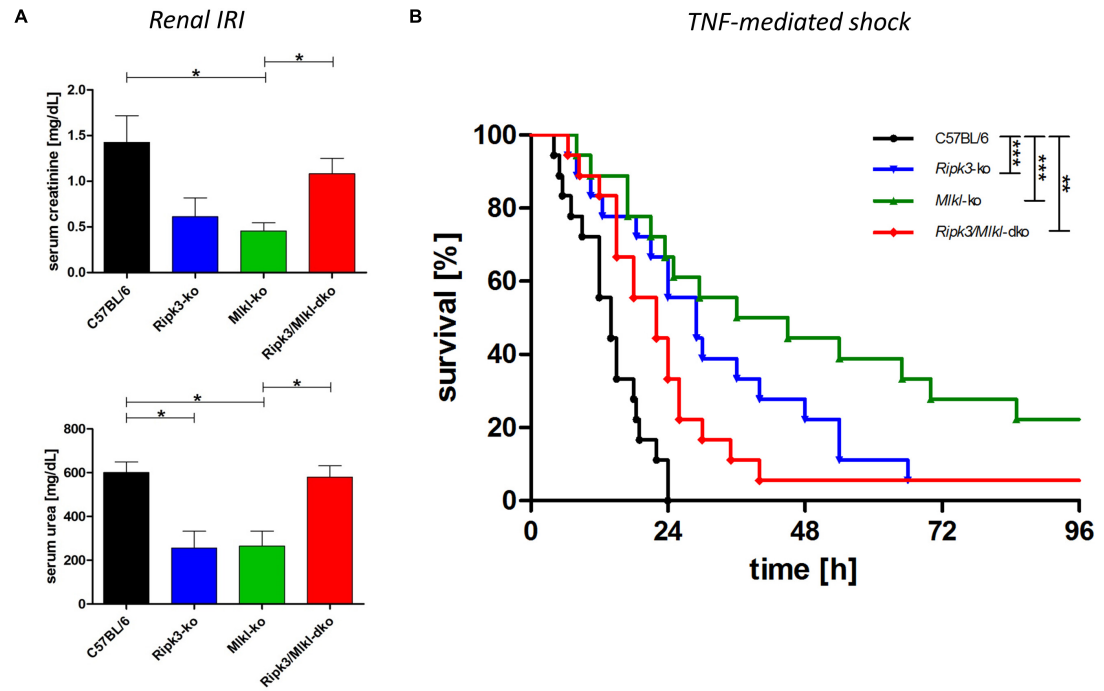

FIGURE 2 | Combined knockout of *Ripk3* and *Mlkl* in an entire organism antagonizes the beneficial effect of each single knockout in ongoing necroptotic cell death processes. **(A)** For establishing renal IRI, mice (n = 16 per group) underwent 40-min bilateral renal pedicle clamping followed by 48-h reperfusion. The wild-type mice had notably higher plasma levels of serum creatinine and urea than the *Ripk3* and *Mlkl* knockout animals. The remarkable protection of each single knockout in the model could not be detected in the *Ripk3/Mlkl* dko animals. **(B)** *Ripk3* and *Mlkl* deficiency protects against TNF-mediated shock, induced by a high-dose TNF (1 mg murine TNFα/kg body weight). Mice with combined knockout of both genes resembled the vulnerable wild-type mice and the superior effect and survival benefit of each single knockout was abolished nearly completely in the dko model (n = 18 per group). Survival is presented as a Kaplan–Meier plot. $^{*}p < 0.05$, $^{**}p < 0.02$, and $^{***}p < 0.001$.

completely from TNF/zVAD-induced necroptosis (**Figure 1A**), in contrast to each single knockout, the dko conferred no increased sensitization to ferroptosis and the cells behaved more like the parental unedited NIH3T3 cells. Initially, we thought that this was probably a cell-specific phenomenon, especially as the observed effect of the absent hypersensitivity of the dko cells to ferroptosis could also not be reproduced using CRISPR/Cas9-edited *Ripk1/Mlkl* double-deficient NIH3T3 cells (data not shown). However, the strong contrast of the dko of the immediately adjacent necrosome members RIPK3 and MLKL perplexed us.

Mouse models have been the key biological tools for defining regulated cell death in development, physiology, and homeostasis. So far, the major phenotypes observed in mice deficient in cell death pathway genes, by the intercrossing of different null alleles, have been described (reviewed in Belizario et al., 2015). Crossing transgenic knockout mouse models deficient in two and more genes has helped elucidate cell death pathways and the essential role of the downstream regulator genes involved in several inflammatory pathologies. However, in contrast to *Ripk1/Mlkl* dko animals, *Ripk3/Mlkl* dko animals are anatomically normal, viable, and fertile (Tanzer et al., 2015). For this reason, we tested *Ripk3/Mlkl* dko animals in direct comparison with both single knockouts in an acute kidney IRI model and in TNF-mediated shock. To track the benefit of genetic deficiency in core necroptotic signaling pathway components, we induced a severe renal IRI in which mice underwent 40-min of bilateral renal pedicle clamping followed by 48-h reperfusion. As shown in **Figure 2A**, the wild-type mice exhibited elevated serum creatinine and urea levels, indicating compromised kidney function. As described previously, *Ripk3* (Linkermann et al., 2013) and *Mlkl* knockout (Müller et al., 2017) conferred distinct protection in this model. As mentioned before, the extent of kidney damage and protection in this model depends greatly on the duration of ischemia and following reperfusion. Nevertheless, the *Ripk3/Mlkl* dko animals were, astonishingly, in contrast to the single knockout animals, barely protected in this cell death modality, but were rather comparable to their genetically unedited wild-type counterparts. Interestingly, the TNFα-mediated inflammatory *in vivo* model presented a similar scenario. *Ripk3* and *Mlkl* single deficiency each protected against TNF-mediated shock convincingly (**Figure 2B**), whereas dko did not. However, as we have reported previously, *Ripk3* knockout mice exhibit prolonged survival following high-dose TNFα injection (Linkermann et al., 2013). In contrast to the missing protection in this pathophysiological model of TNF-induced shock, when *Mlkl*-deficient mice received intravenous low-dose TNF (Newton et al., 2016a), their substantially prolonged survival in this setting (high-dose TNFα) resembled that of the *Ripk3*-deficient mice. However, the combined *Ripk3/Mlkl*

dko nearly completely abolished the superior effect and survival benefit of each single knockout (**Figure 2B**), indicating that the complex regulation and interconnectivity among a single regulated necrosis pathway is still not fully understood.

DISCUSSION

Necroptotic cell lysis and the resultant release of proinflammatory mediators are thought to cause inflammation in necroptotic disease models. Preferentially, investigators have utilized mice lacking *Ripk3* as a necroptosis deficiency model, even where caspase inhibitors were absent under the conditions investigated. However, it is now emerging that RIPK3 almost certainly has pro-inflammatory effects clearly separable from its role in necroptosis (Kearney and Martin, 2017). Therefore, pseudokinase MLKL is currently viewed as the sole and main effector of necroptosis (Hildebrand et al., 2014). Nevertheless, it was shown recently that *Staphylococcus aureus* infection in *Ripk3*- versus *Mlkl*-deficient animals has opposing outcomes: *Mlkl* deficiency led to a delayed clearance of the bacterium, increased inflammation, and a worse outcome, whereas *Ripk3* deficiency led to an improved bacterial clearance and reduced inflammation (Kitur et al., 2016), showing that the loss of *Ripk3* is not equivalent to the loss of *Mlkl* and/or necroptosis and verifying that signaling is usually far more complex. Interestingly, to date, virtually no *in vivo* data have been published using *Ripk3*/*Mlkl* double-deficient animals.

Our investigation of *Ripk3* and *Mlkl* single knockout mice, in established preclinical models of severe AKI and high-dose TNF-induced shock, respectively, did not yield contrasting data. In our experience, knocking out *Ripk3* or *Mlkl* leads to remarkable and quantitatively indistinguishable protection from injury in both *in vivo* models. However, reported differences of these single-null animals are often evident in rather mild conditions triggering the abovementioned clinical disorders (Newton et al., 2016a). Nevertheless, our results regarding the *Ripk3*/*Mlkl* dko mice remain a conundrum. We predicted that the dko animals would receive the same protective effect in the AKI and TNF-induced shock models, as each single knockout, or rather that in their limited ability to stimulate inflammasomes and the inability to activate necroptosis, the dko mice would have increased protection against these pathologies. However, none of this occurred. The dko mice in each case unexpectedly resembled the vulnerable wild-type mice, and the former protective effect in both *in vivo* models, which was mediated by *Ripk3* or *Mlkl* loss, respectively, was completely abrogated. As all mice were backcrossed to an identical C57BL/6 background and as all animals were obtained from our facility, we postulate that differences in colony microflora, or similar, were not responsible for this discovery. Mechanistically, we eschew the hypothesis that immunoreactivity may explain our controversial findings, particularly when in a published animal model of TNF hypersensitivity, the skin principally underwent apoptosis while the spleen and liver in these identical mice were sensitized to necroptosis (Rickard et al., 2014a). So far, the physiological stimulus or insult that dictates the regulated death modality induced *in vivo* remains unmanageable, but a feasible possibility is that merely effector abundance might dictate which death signaling occurs. Nevertheless, we assessed pooled data from several independent experiments to increase the statistical power. The AKI approach was repeated twice with eight mice per group; the TNF-induced shock model was replicated three times with six animals per group. As we obtained identical results in each case, we are convinced that it is a reliable consequence of the simultaneous *Ripk3* and *Mlkl* knockout. Our data unambiguously demonstrates that necroptosis is not the only cell death modality implicated in both abovementioned pathologies and raises the question about the existing functions of RIPK3, and above all, of MLKL beyond necroptosis, and if so, how are they differentially regulated? The concept that combined deficiency in pro-inflammatory and necroptotic signaling in these models switches the etiopathology at onset completely toward ferroptosis, which we have proved, although only in the AKI model with necroptotic-resistant *Mlkl* knockout mice (Müller et al., 2017), can be verified only after developing durable, less serum-labile ferroptosis inhibitors. Furthermore, there is justifiable doubt that necroptosis is the sole initiator of inflammation. Interestingly, *Ripk3* knockout mice in a dextran sulfate sodium (DSS)-induced colitis model had enhanced sensitivity, suggesting that RIPK3 may also have tissue regenerative functions (Moriwaki et al., 2014). Presumably, processes such as mitochondrial ROS generation can contribute to necroptosis, but will be bypassed, activating the necroptotic pathway downstream at the RIPK3 or MLKL level. However, our own discovery of MLKL-independent necroptosis (Günther et al., 2016) and the recently published report of RIPK3-independent necroptosis (Zhang et al., 2016), and the unexpected function of ZBP1 (Z-DNA binding protein 1) in the skin (Lin et al., 2016) and thymus (Newton et al., 2016b), suggests that regulated necrosis pathways still have secrets and further related work would be both essential and highly informative.

AUTHOR CONTRIBUTIONS

SK and UK designed the research. CM and FB performed the experiments. CM, FB, UK, and SK analyzed the data. CM prepared the figures. SK wrote the paper.

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Exploiting Necroptosis for Therapy of Acute Lymphoblastic Leukemia

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Escape from chemotherapy-induced apoptosis is a hallmark of drug resistance in cancer. The recent identification of alternative programmed cell death pathways opens up for possibilities to circumvent the apoptotic blockade in drug resistant cancer and eliminate malignant cells. Indeed, we have recently shown that programmed necrosis, termed necroptosis, could be triggered to induce cell death in a subgroup of primary acute lymphoblastic leukemia (ALL) including highly refractory relapsed cases. In this review we focus on molecular mechanisms that drive drug resistance in ALL of childhood and discuss the potential of necroptosis activation to eradicate resistant disease.

Keywords: necroptosis, leukemia, drug resistance, necroptotic compounds, apoptosis dysregulation

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INTRODUCTION

Acute leukemia is a hematological malignancy that perturbs the normal function of the hematopoietic system with fatal outcome if left untreated. Substantial improvement in the treatment of patients with childhood acute lymphoblastic leukemia (ALL) has been achieved over the last decades (Pui et al., 2015). Despite this success that is based on intensive chemotherapy protocols established in international collaborative studies (Schrappe et al., 2013), relapsed leukemia still ranks among the most common diagnoses of childhood malignancies, and survival rates of relapsed ALL remain low (Bhojwani and Pui, 2013). Thus, new treatment approaches have to be developed, in particular for relapsed ALL patients. In addition to immunotherapy, most current treatment approaches focus on targeting oncogenic lesions to induce cell death (Muschen, 2018). Enormous efforts over the recent years have identified and characterized the genomic lesions that occur in ALL (Mullighan et al., 2007; Fischer et al., 2015; Richter-Pechanska et al., 2018). Chromosomal translocations frequently affecting transcription factors combine with deletions in genes that regulate B- and T-cell development and mutations in genes that drive proliferation (e.g., CRLF2, RAS, ILR7, STAT5, Notch) (Mullighan et al., 2007; Fischer et al., 2015; Richter-Pechanska et al., 2018). The latter frequently occur at subclonal level. This heterogeneity and diversity of molecular lesions in ALL (Mullighan et al., 2007; Liu et al., 2017) has rendered the development of targeted therapies very challenging. In particular, chimeric translocations remain largely undruggable, and direct targeting of deletions is obviously not possible. Many of these alterations lead to reprogramming of hematopoietic differentiation and deregulation of molecular mechanisms that balance cell death and survival, providing the basis for poor response to chemotherapy and failure to undergo apoptosis. At the same time, this deregulation of signaling pathways also identifies nodes that could be targeted using small molecules and novel approaches. Among these, exploiting cell death mechanisms independent on classical apoptosis and caspases activation represents a particularly attractive alternative, with the potential to activate cell death responses under circumstances that prevent caspase-dependent cell death. Indeed, activation of necroptosis using the small molecule SMAC mimetic birinapant eliminated refractory leukemia

cells in samples from highly resistant ALL patients (McComb et al., 2016). Corroborating these results, several compounds such as other SMAC mimetics or natural products are able to trigger the necroptotic pathway in leukemia but also in different carcinomas (Han et al., 2007; Fu et al., 2013; McCabe et al., 2014; Brumatti et al., 2016; Hannes et al., 2016; He et al., 2017; Safferthal et al., 2017). The possibility to develop and use drugs to induce necroptosis render this cell death mechanism very attractive for therapeutic approaches to eradicate malignant cells.

ALTERATION OF CELL DEATH AND SURVIVAL SIGNALING AS MECHANISMS OF DRUG RESISTANCE IN ALL

Comparison of ALL samples at diagnosis and relapse identified genomic and cytogenetic changes (Raimondi et al., 1993; Mullighan et al., 2008; Muschen, 2018; Richter-Pechanska et al., 2018) that are disease-driving and contribute to occurrence of relapse. Indeed, refractory ALL samples frequently present with secondary genetic alterations that arise from a minor subclone at diagnosis, which becomes predominant at relapse conferring drug resistance. Many of these alterations induce deregulation of pro- and anti-survival signaling pathways. Aberrant activation of the PI3K/AKT/mTOR axis is associated with poor clinical outcome in ALL, and its dysregulation can induce cell survival and resistance to cytotoxic drugs (Batista et al., 2011; Gomes et al., 2014; Khanna et al., 2018). Inhibition of this key pro-survival pathway, for instance using arsenic trioxide treatment, can resensitize steroid poor responder patients to glucocorticoids, key components of first-line ALL therapy. Arsenic trioxide increases protein levels of the BH3-only protein BAD, a pro-apoptotic member of BCL2 family and decreases the levels of the caspase inhibitor XIAP (Bornhauser et al., 2007). As shown in a case report of a refractory T-ALL patient, treatment with arsenic trioxide could induce complete remission without minimal residual disease (Wu et al., 2016). More direct inhibitors of this pathway, such as PI3K inhibitors or dual PI3K/mTOR inhibitors have shown promising activity in preclinical ALL models (Fruman et al., 2017). ALL refractory to glucocorticoids presented with high expression levels of the anti-apoptotic BCL2 family protein MCL1, due to a hyper activation of the PI3K/AKT/mTOR network (Wei et al., 2006), and specific MCL1 inhibitors are currently under evaluation for anti-leukemia activity (Ramsey et al., 2018). In refractory ALL, other possible dysregulation may more directly involve the apoptotic pathway and mitochondrial activity, which is controlled by the BCL2 family members. Indeed, correlation of drug resistance and alterations of BCL2 family proteins has been extensively described in leukemia (Letai et al., 2004; Campbell et al., 2010). Next to association of BCL2 family protein expression and drug resistance, these anti-apoptotic proteins also contribute to leukemogenesis. A transgenic mouse model showed a synergistic effect between BCL2 and c-MYC in malignant transformation of B-cells (Strasser et al., 1990). Moreover, an adaptation of the same mouse model demonstrated that presence of BCL-XL (anti-apoptotic BCL2 member) accelerates the development of MYC-driven leukemia (Swanson et al., 2004). Increased leukemia development was observed also in Eμ-Myc transgenic mice upon genetic disruption of one BIM (BCL2 pro-apoptotic protein) allele (Egle et al., 2004). Thus, dysregulation of pro- or anti-apoptotic BCL2 proteins can support malignant cell maintenance and survival also once the tumor is established. Recently developed diagnostic procedures with functional analysis of BCL2 family protein dependence using BH3 profiling (Ryan and Letai, 2013; Ryan et al., 2016; Touzeau et al., 2016) can be used to predict chemotherapeutic sensitivity in several cancer types (Ni Chonghaile et al., 2011). It has become clear from these approaches that a subset of ALL cases heavily depend on specific BCL2 family members. In order to target the interaction between pro- and anti-apoptotic BCL2 proteins in cancer, a new class of compounds, the BH3-mimetics, has been developed. In particular the BCL2-specific BH3-mimetic venetoclax (ABT-199) has shown high activity *ex vivo* and *in vivo* in a subset of B-cell precursor ALL (Fischer et al., 2015) and in some T-cell leukemia samples (Chonghaile et al., 2014; Peirs et al., 2014; Frismantas et al., 2017). Moreover, venetoclax has shown promising results also in clinical trials for other hematologic malignancies (Konopleva et al., 2016). However, high expression of MCL1 (Choudhary et al., 2015) or low expression ratio of BCL2 vs. BCL-XL may underlie a potential resistance to venetoclax. To overcome this, it is possible to combine MCL1 inhibitors with BCL2 inhibitors, which was shown to have a synergistic effect in preclinical studies (Leverson et al., 2015). While representing an important factor for drug resistance, dysregulation of BCL2 proteins is not the only cause for apoptotic rescue in malignant cells. Alterations in genes that drive metabolism have also been described to underlie drug resistance in ALL. Mutations in the nucleotidase NT5C that are recurrent in T-ALL (Tzoneva et al., 2013, 2018) may confer resistance to mercaptopurine, a key element in ALL therapy, representing a typical example of gain-of-function mutations that are difficult to target, which is in addition also associated with occurrence of relapse. Recent discoveries have highlighted the occurrence of the deletion of the B-cell transcription factor IKZF1 together with CDKN2A, CDKN2B, PAX5, or PAR1 to identify a subgroup of B-cell precursor ALL patients with exceedingly bad outcome (Stanulla et al., 2018). We are only at the beginning of understanding the consequences of such deletions on drug resistance. In addition to drive B-cell development, IKZF1 controls a metabolic program that includes regulation of responses to steroids (Marke et al., 2016; Chan et al., 2017), and its loss may be directly linked to steroid resistance. Next to metabolic alterations, a second group of pro-survival proteins, the inhibitor of apoptosis proteins (IAPs), are frequently highly expressed in leukemia (Tamm et al., 2004; Hundsdoerfer et al., 2010) and constitute relevant targets for intervention. The pro-survival activity of cIAP1/2 is linked with their ubiquitination activity and the ability to interact with and promote the survival activity of receptor-interacting protein kinase 1 (RIPK1) (Peltzer et al., 2016; Lalaoui and Vaux, 2018). Ubiquitination of RIPK1 enables its Nuclear Factor kappa B (NF-kB) activating potential, supporting survival also in cancer cells (Bertrand et al., 2008; Varfolomeev et al., 2008). Small molecules SMAC mimetics can target and inhibit

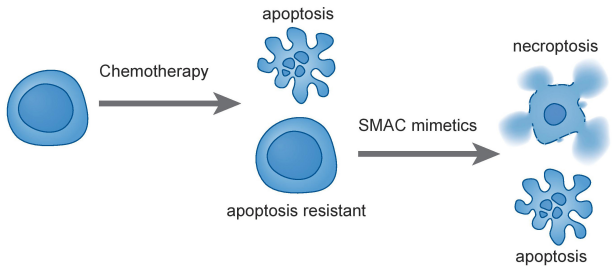

FIGURE 1 | Treatment of leukemia cells with traditional chemotherapies can select for apoptotic-resistance. SMAC mimetics such as birinapant induce concurrent apoptosis and necroptosis in ALL and are thus active against apoptosis-resistant cells. This may contribute to eradication of resistant and refractory leukemia.

the cIAPs, which induces deubiquitination of RIPK1 in the TNF receptor 1 (TNFR1) complex and subsequent activation of RIPK1-dependent death. These agents have shown anti-cancer activity in different solid tumor cell line models (Fulda, 2015). Moreover, primary ALL and acute myeloid leukemia (AML) samples undergo RIPK1-dependent death upon SMAC mimetics treatment (Brumatti et al., 2016; Lalaoui et al., 2016; McComb et al., 2016; Richmond et al., 2016). The tumor suppressor role of RIPK3 for AML development in mice (Hockendorf et al., 2016) further underscores the importance of this pathway in hematological malignancies. Interestingly, treatment with SMAC mimetics induced RIPK1-dependent concurrent apoptosis and necroptosis in primary ALL samples, both *in vitro* and *in vivo* in the xenograft model (McComb et al., 2016). The high anti-leukemic activity of SMAC mimetics is thus based on their potential to trigger necroptosis, to eradicate also refractory ALL cells that are unable to mount an apoptotic response (**Figure 1**). To further characterize and understand the potential of necroptosis activation for anti-leukemia therapy, it will be important to develop biomarkers that brand a response and to determine strategies to identify those patients who may benefit from such an approach.

ACTIVATION OF NECROPTOSIS AS ANTI-LEUKEMIA THERAPY

Despite its relatively recent description (Degterev et al., 2005), necroptosis ranks among the best described non-apoptotic and caspase-independent forms of cell death. It is a caspase-independent cell death mechanism, which presents necrotic features that are highly regulated (Wang et al., 2018). The signal transduction steps that govern necroptosis induce initiation and execution of this cell death pathway controlled by the RIP Kinases, ending with cell swelling and rupture of the cellular membrane, leading to the release of cellular content into the extracellular space (Kaczmarek et al., 2013). The main regulating players of this programmed cell death are RIPK1, RIPK3, and the mixed lineage kinase domain-like protein (MLKL) (Vanden Berghe et al., 2014). Experimentally, necroptosis is frequently triggered by exogenous tumor necrosis factor alpha (TNFα) in combination with pharmacological caspase inhibition using, e.g., zVAD, QVD, or emricasan. Other death receptors that can activate necroptosis in presence of their respective ligands include FAS (also known as CD95 or APO-1), DR3, TRAILR1, TRAILR2, and DR6 (Wilson et al., 2009). Mechanistically, TNFα binding induces oligomerization of TNFR1 and the formation of complex-I at the plasma membrane. Complex-I is a multiprotein complex that includes TNFRI, TNFR-associated death domain protein (TRADD), TNFR-associated factor-2 and 5 (TRAF2/TRAF5), the cIAPs1/2 and RIPK1 (Vanden Berghe et al., 2014). At this level, cell fate decisions are taken, with RIPK1 having multiple functions. Depending on its post-translational modifications, in particular ubiquitination status, RIPK1 controls cell survival or can activate cell death trough apoptosis and necroptosis. Poly-ubiquitination of RIPK1 driven by cIAPs1/2 and LUBAC triggers survival through NF-kB signaling, which leads to mitogen-activated protein kinase (MAPK) activation (Pasparakis and Vandenabeele, 2015). Simultaneously, ubiquitination of RIPK1 prevents necroptosis and RIPK1-dependent apoptosis activation. Deubiquitination of RIPK1 can induce the formation of the cytosolic complex-IIb, which comes in two different flavors. Under caspase-8 proficient conditions, deubiquitination of RIPK1 leads to formation of the ripoptosome leading to apoptosis through caspase-8 dependent mechanisms, while the necrosome is formed if caspase-8 is non-active (Wegner et al., 2017) (**Figure 2**). In the necrosome, RIPK1 associates with and phosphorylates RIPK3 leading to the oligomerization and translocation of MLKL to the plasma membrane (Zhao et al., 2012; Huang et al., 2017). It is worth noting that in particular the ripoptosome is fairly short lived and can usually only be detected under experimental caspase blockade using zVAD. The deubiquitination of RIPK1 may occur through activity of the deubiquitinases CYLD and A20 (Wright et al., 2007; Bonapace et al., 2010; Wegner et al., 2017) or through depletion of cIAP1/2 by SMAC mimetics treatment. To guide decisions between RIPK1-dependent apoptosis or necroptosis, autophagy genes were shown to play an important scaffolding role (Goodall et al., 2016). MLKL can be considered the executor of necroptosis as it induces formation of pores on the plasma membrane, which becomes permeable releasing damage-associated molecular patterns (DAMPs), thus ending into necroptosis (Dondelinger et al., 2014; Wang et al., 2014; Xia et al., 2016). The identification of RIPK1-dependent necroptosis to underlie the extraordinary sensitivity to SMAC mimetics in a subgroup of pediatric ALL represents an example in which experimental inhibition of caspase-8 is not required. Rather, we hypothesize that this may be due to the existence of specific but varying RIPK1-associated protein complexes within the cells. We could not identify any association of protein expression of either caspase-8, RIPK3, MLKL, cIAP1/2, or RIPK1 with sensitivity to SMAC mimetics in ALL (McComb et al., 2016), suggesting that the regulation and sensitivity will be more complicated than mere expression levels. Interestingly, our own data (McComb et al., 2016) demonstrated a TNFα-independent effect of SMAC mimetics, suggesting that auto- or para-crine regulation of TNFα by RIPK1 does not seem to play a major

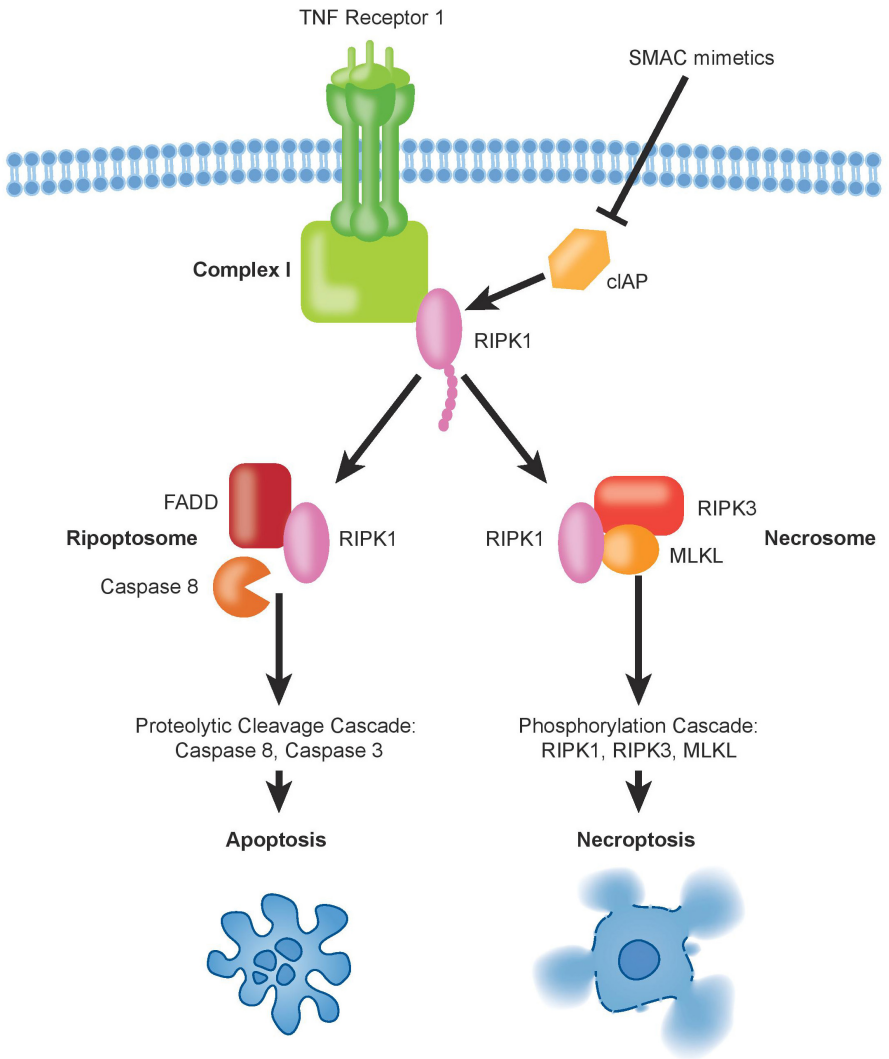

FIGURE 2 | The binding of TNFα to TNFR1 induces the formation of complex-I, which contains also ubiquitinated RIPK1. Deubiquitination of RIPK1, upon inhibition of cIAPs by SMAC mimetics, can trigger formation of pro-death signaling complexes, either via apoptosis and/or necroptosis.

role for sensitivity. Comparative gene expression analyses suggest association of the Philadelphia-like ALL subgroup with sensitivity to SMAC mimetics, with TNFR1 expression correlating with response, while cFLIP did not appear amongst the most highly regulated genes (Richmond et al., 2016). Mutations in caspase-8 or epigenetic silencing has not been described in ALL so far (Mullighan et al., 2007; Liu et al., 2017), indicating that the underlying molecular mechanisms that determine sensitivity will be more complex than anticipated.

POTENTIAL OF NECROPTOSIS-INDUCING COMPOUNDS IN ALL

Triggering necroptotic cell death should be considered as a new therapeutic approach in cancer treatment in order to eradicate malignant cells that are refractory to apoptotic drugs. Several agents, including natural and targeted compounds, have been shown to induce necroptosis in ALL, frequently also in combinatorial approaches. In particular combination of SMAC mimetics with the steroid dexamethasone (Rohde et al., 2017) and with demethylating agents (DAC) (Gerges et al., 2016), as well as inhibition of NF-kB (Meng et al., 2010) activate necroptosis in ALL cells, while hypertonicity enhanced activity of SMAC mimetics by combination of apoptosis and necroptosis (Bittner et al., 2017). The best well-known drugs that can induce necroptotic cell death are indeed the SMAC mimetics combined with caspase-8 inhibition (McCabe et al., 2014; Brumatti et al., 2016; Hannes et al., 2016). This type of treatment can push the cells to necroptosis due to inhibition of cIAPs, thereby inhibiting the pro-survival function of RIPK1, and on the other hand caspase inhibition confers a block in apoptosis. Interestingly, we have observed that refractory ALL samples could undergo

necroptosis cell death also in absence of caspases inhibition upon the SMAC mimetic compound birinapant as single agent (McComb et al., 2016). Several SMAC mimetic compounds are already in phases I or II of clinical trials to treat leukemia and solid tumors (NCT02098161, NCT01188499, NCT01486784). It will be interesting to see if necroptosis contributes to a potential anti-tumor effect in these trials. Still, the most promising anti-tumor activity of SMAC mimetics may be achieved if combined with other anti-cancer agents. For instance, the SMAC mimetic compound BV6 synergizes with DAC, cytarabine, or HDAC inhibitors in acute myeloid leukemia (AML) (Steinhart et al., 2013; Chromik et al., 2014; Steinwascher et al., 2015). This activity required necroptosis for full efficacy. Antagonism of cIAPs may boost both innate and adaptive immune responses and increase tumor cell killing (Beug et al., 2017; Dougan and Dougan, 2018; Michie et al., 2019). In addition to SMAC mimetics, other agents are able to trigger a necroptosis response. Activation of necroptosis using drugs as 5-fluorouracil or staurosporine (Dunai et al., 2012; Grassilli et al., 2013; Oliver Metzig et al., 2016), again if caspases are inhibited, showed high anti-cancer potential. Moreover, necroptosis could be activated by shikonin, a natural compound derived from a plant extract, in leukemia and in multiple myeloma (Han et al., 2007; Wada et al., 2015). This compound and other analogs may overcome drug resistance due to expression of MRP1, BCRP1, P-glycoprotein, BCL2 and BCL-XL through necroptotic cell death (Han et al., 2007; Xuan and Hu, 2009). Furthermore, necroptosis has been described in some cases to depend on autophagy. In fact, the pan-BCL2 inhibitor obatoclax triggered autophagy-dependent necroptosis, thus restoring the response to the glucocorticoid dexamethasone in steroid-resistant ALL (Bonapace et al., 2010). Moreover, bypassing chemoresistance through autophagy-mediated necroptosis is possible upon chalcone treatment or using the tyrosine kinase inhibitor sorafenib (He et al., 2014; Kharaziha et al., 2015). One important aspect to be taken into account when considering necroptosis activation in cancer therapy is its potential immunogenicity. Disruption of the cellular membrane and release of DAMPs may activate immune responses that potentially can also act on the malignant cells. Indeed, vaccination with necroptotic cancer cells induces an adaptive immune response through cytotoxic CD8a+ T cells *in vivo*, which mediates efficient anti-tumor immunity (Aaes et al., 2016). Sometimes though, the release of DAMPs may not be sufficient for CD8+ T cell cross-priming, and RIPK1 signaling and activation of NF-κB within dying cells is in addition required to boost the response (Yatim et al., 2015). The question to what extent activation of necroptosis in ALL in particular, but also in other hematological malignancies such as AML (Brumatti et al., 2016) is immunogenic remains open. Some data from solid tumors suggest that necroptosis does not necessarily always have to be pro-inflammatory and immunogenic (Brouckaert et al., 2004; Lohmann et al., 2009). Still, while TNFα-induced necroptosis may even shut down inflammatory responses (Kearney et al., 2015), data with respect to cytokine release and inflammatory responses on necroptosis induced by SMAC mimetics are lacking, in particular also in the context of refractory ALL. Clearly, susceptibility to necroptosis-mediated cell death does represent a specific vulnerability of lymphoid cells, even without necessity to experimentally inhibit caspases. In the future, potential immunogenicity and inflammatory responses of necroptosis induction will have to be investigated carefully, in order to evaluate the therapeutic anti-leukemia potential of necroptosis induction.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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Immunogenic Cell Death and Immunotherapy of Multiple Myeloma

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Over the past decades, immunotherapy has demonstrated a prominent clinical efficacy in a wide variety of human tumors. For many years, apoptosis has been considered a non-immunogenic or tolerogenic process whereas necrosis or necroptosis has long been acknowledged to play a key role in inflammation and immune-related processes. However, the new concept of "immunogenic cell death" (ICD) has challenged this traditional view and has granted apoptosis with immunogenic abilities. This paradigm shift offers clear implications in designing novel anti-cancer therapeutic approaches. To date, several screening studies have been carried out to discover *bona fide* ICD inducers and reveal the inherent capacity of a wide variety of drugs to induce cell death-associated exposure of danger signals and to bring about *in vivo* anti-cancer immune responses. Recent shreds of evidence place ER stress at the core of all the scenarios where ICD occur. Furthermore, ER stress and the unfolded protein response (UPR) have emerged as important targets in different human cancers. Notably, in multiple myeloma (MM), a lethal plasma cell disorder, the elevated production of immunoglobulins leaves these cells heavily reliant on the survival arm of the UPR. For that reason, drugs that disrupt ER homeostasis and engage ER stress-associated cell death, such as proteasome inhibitors, which are currently used for the treatment of MM, as well as novel ER stressors are intended to be promising therapeutic agents in MM. This not only holds true for their capacity to induce cell death, but also to their potential ability to activate the immunogenic arm of the ER stress response, with the ensuing exposure of danger signals. We provide here an overview of the up-to-date knowledge regarding the cell death mechanisms involved in situations of ER stress with a special focus on the connections with the drug-induced ER stress pathways that evoke ICD. We will also discuss how this could assist in optimizing and developing better immunotherapeutic approaches, especially in MM treatment.

Keywords: immunogenic cell death, multiple myeloma, ER stress, danger-associated molecular pattern, immunotherapy

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INTRODUCTION

Every day in the human body, billions of cells pass away and are kindly replaced by newborn members leaving no trace behind, allowing in this way conservation of whole-body homeostasis. In order to occur without catastrophic consequences, this process must remain almost completely unnoticed to the immune system. During this physiological, programmed cell death, mainly in the

form of apoptosis, intracellular content is confined within membranous bodies that are rapidly cleared by phagocytes in an immunological "silent" manner. Hence, apoptosis has long been considered a non-immunogenic or even tolerogenic process, whereas necrosis and necroptosis have been shown to play a key role in inflammation and immune related processes (Poon et al., 2014; Yatim et al., 2015). However, the new concept of "immunogenic cell death" (ICD) has challenged this traditional view and has granted apoptosis with immunogenic abilities. This immunostimulatory kind of apoptosis is characterized by the ability of dying cells to elicit robust adaptive immune responses against altered self-antigens/cancer-derived neo-epitopes, in the case of tumor cells, or against pathogen-derived antigens (Ags) during the course of an infection (Galluzzi et al., 2017). Besides antigenicity, another vital factor needed to unleash a genuine immune response is adjuvanticity, which is conferred by microorganism- and/or danger-associated molecular patterns (MAMPs and DAMPs, respectively). These are molecules that are exposed or released by dying cells and let the immune system know the existence of a menace to the organism (Fuchs and Steller, 2015). This "danger" state is sensed in the human body by pattern recognition receptors (PRRs) displayed by innate immune cells such as monocytes, macrophages and dendritic cells (DCs), hence promoting activation and maturation of these cells to engage the adaptive arm of the immune system (Matzinger, 2002).

Screening studies have been carried out to unveil the immunogenic potential of myriads of anti-cancer agents (Sukkurwala et al., 2014). To date, only a small yet diverse collection of anti-cancer therapies, whether chemotherapeutic drugs (e.g., anthracyclines, oxaliplatin, bortezomib) (Obeid et al., 2007; Garg et al., 2017) or physical modalities [e.g., radiotherapy, hypericin-based photodynamic therapy (Hyp-PDT), and high hydrostatic pressure (HHP)] (Golden et al., 2012; Adkins et al., 2014) have been shown to induce bona-fide ICD. However, a common denominator can be extracted from the action mechanisms of all these approaches: ER stress and ROS generation. Thus, activation of the ER stress pathways also known as the unfolded protein response (UPR), and specially, the PERK-mediated arm of the UPR is vital for the vast majority, if not all, the scenarios where ICD occurs (Rufo et al., 2017). Moreover, during tumor development, cancer cells have to cope with harsh conditions that trigger ER stress. Thus, UPR activation constitutes an important hallmark of several human cancers that endow cancer cells with the ability to acquire essential characteristics required for tumor progression (Corazzari et al., 2017). Of note, although UPR activation is initially intended to restore cell homeostasis, it can also shift the cellular fate toward cell death. All the aforementioned has clear implications for cancer therapy. The UPR-dependency of tumor cells together with the connection of ER-stress and the emission of danger signals (or ER stress-ICD connection), can be harnessed to design novel therapeutic tools. These therapeutic approaches not only would reduce tumor burden, but also improve the immunogenic capacity of dying cancer cells to elicit long-term adaptive immune responses. In particular, in multiple myeloma (MM), a lethal plasma cell disorder, the elevated production of immunoglobulins leaves these cells heavily reliant on the survival arm of the UPR. Nevertheless, although myeloma cells rely on the UPR to thrive, they are extremely sensitive to ER-stress associated cell death. This feature explains why proteasome inhibitors show a prominent clinical efficacy in the treatment of MM (Merin and Kelly, 2014; Scalzulli et al., 2018). Sadly, resistance to therapy is recurrent, and in most of the cases accounts for the lethality of the disease (Robak et al., 2018). MM is also a genuine neoplasia where the immune system is compromised. Nonetheless, immunotherapeutic interventions in this disease have potential to be successful, as graft-vs-myeloma effect has been evidenced in patients subjected to allogenic stem-cell transplantation or under donor lymphocyte infusions (Ladetto et al., 2016). In fact, current immunotherapeutic approaches are giving promising results in relapsed and refractory patients. Among the novel and more promising immune-based therapies that are under investigation, we can include: (1) Antibody-based therapies with daratumumab and elotuzumab as the flagships of this kind of approach, (2) Boost the immune effector line of defense with adoptive cell therapy (ACT), either with expanded tumor-infiltrating lymphocytes (TILs), NK cells or CAR-T cells, (3) Releasing the brakes of immune response with immune-checkpoint blockade, (4) Enhancing general anti-tumor immunity through vaccination strategies, and finally (5) Combinatorial strategies of the immunotherapies themselves or combined with immunogenic chemo- or radiotherapies. Noteworthy, all of these approaches can theoretically be benefited by ICD. Hence, the immunostimulatory potential of chemotherapeutics or other ICD-related modalities could be exploited to enhance general immunity or at least create an immune-friendly tumor microenvironment. This way, some of the drawbacks occurring in the clinical setting could be circumvented to achieve an effective immune response in cancer patients (Montico et al., 2018).

THE UNFOLDED PROTEIN RESPONSE

Tumor cells are constantly coping with aggressive insults and subjected to different types of cellular stress. Some of these extrinsic (hypoxia, nutrient deprivation, acidosis) and intrinsic (oncogenic activation, genetic alterations, exacerbated secretory capacity) factors are common instigators of ER stress (Dufey et al., 2016). To cope with ER stress, cells activate an adaptive and well-conserved mechanism called UPR. The UPR is a fine-tuned process controlled by three membrane-bound ER stress sensors: Protein Kinase RNA-activated (PKR)-like ER Kinase (PERK), Inositol-Requiring transmembrane kinase/Endonuclease (IRE1) and Activating Transcription Factor 6 (ATF6). These sensors remain inactive in basal conditions due to the interaction with Binding Immunoglobulin Protein (BIP, also known as GRP78) through their ER luminal domains. Under ER stress conditions, BIP dissociates from the ER stress sensors to help in protein folding (Almanza et al., 2018). This event allows ER stress sensors to self-activate by homodimerization/oligomerization and trans-auto-phosphorylation in the case of PERK and IRE1, and translocation to the Golgi in the case of ATF6. First, the UPR tries

to restore cell homeostasis, by attenuating protein translation, enhancing degradation of misfolded proteins and increasing levels of ER chaperones and redox enzymes to increase folding capacity (Almanza et al., 2018). However, if ER stress persists, the UPR can trigger proapoptotic programs controlled mainly by the IRE1 and PERK arms. IRE1 is a Ser/Thr kinase that also has an endoribonuclease domain. When activated, IRE1 drives XBP1 mRNA splicing, leading to a more stable XBP1s protein that acts as a transcription factor upregulating genes controlling ER homeostasis maintenance (Sano and Reed, 2013). Moreover, during the chronic phase of ER stress, IRE1 is also able to degrade many ER-targeted mRNAs through regulated IRE1-dependent mRNA decay (RIDD) process. Activation of PERK signaling leads to phosphorylation of eIF2α, which results in inhibition of global protein translation in order to reduce protein load. Nonetheless, some transcripts like ATF4 are translated more efficiently during ER stress. ATF4 increases the expression of genes involved in aminoacid and redox metabolism, ubiquitin ligases and the transcription factor CAAT/enhancer-binding protein (C/EBP) homologous protein (CHOP/GADD153). ATF4 and CHOP are also key determinants of ER stress-induced cell death. Finally, the cytosolic domain of ATF6 also acts as a transcription factor that mainly regulates the expression of genes involved in the ER-associated degradation (ERAD) pathway (Dufey et al., 2016).

IMMUNOGENIC CELL DEATH

During the last decade, our conception of the characteristics of different types of cell death has significantly changed. Necrosis was first conceived as an accidental, pathological and pro-inflammatory form of cell death, whereas apoptosis was recognized to be a non-immunogenic, physiological and regulated way of cell demise (Poon et al., 2014). However, these features are no longer so clear-cut since programmed necrosis (necroptosis) has been shown to be triggered by a genetically encoded, well-regulated molecular program (Golstein and Kroemer, 2007; Vanden Berghe et al., 2009; Dhuriya and Sharma, 2018). On the other hand, apoptosis is no longer considered to be an immunologically "silent" process, since some apoptotic cells are able to induce antigen-specific immune responses (Obeid et al., 2007). In cancer research, the role of the immune system has been overlooked for many years due, in part, to the way chemotherapy and other anticancer therapies were usually tested. Particularly, the frequent use of immunodeficient mice to assess the efficacy of these treatments has precluded from gaining insight on the precise role of the immune system in cancer therapy (Krysko et al., 2012). Nonetheless, the re-evaluation of concepts like cancer antigenicity and ICD, as well as the interpretations from Danger Theory, has redirected the focus in oncological research toward novel or improved immunotherapeutic protocols (Garg et al., 2015a).

The ICD concept has been defined as an unique class of regulated cell death capable of eliciting complete antigen-specific adaptive immune responses through the emission of a spatiotemporally defined set of danger signals or DAMPs (Casares et al., 2005; Kroemer et al., 2013). These signals are endogenous molecules that perform conventional intracellular functions but when extracellularly exposed, gain immunogenic competences. The release or membrane exposure of these molecules, allow their interaction with their cognate PRRs displayed by innate immune cells such as monocytes, macrophages and DCs. This leads to activation and maturation of these cells that migrate to draining lymph nodes loaded with cancer-derived antigen-specific cargoes. Cancer antigens are then presented to T cells ($CD4^+$ and $CD8^+$ T lymphocytes) which enable a potent anticancer adaptive immune response (Chen and Mellman, 2013). To date, four modes of ICD have been described, each related to a particular type of inducing stimulus and to the emission of a specific set of danger signals (Galluzzi et al., 2017) (see **Figure 1**): (1) Pathogen-driven ICD, as one of the defense mechanisms against invading pathogens; (2) ICD exhibited by physical cues, such as Hyp-PDT, irradiation and HHP; (3) Necroptosis, but not accidental necrosis, since this regulated form of cell death was able to vaccinate syngenic mice against a rechallenge with cells of the same type (Aaes et al., 2016). According to this, RIPK3 or MLKL deficiency abrogated the ability of these cells to secrete the required immunogenic signals that lead to an anticancer immune response in mice (Yang et al., 2016); and (4) ICD evoked by some chemotherapeutics targeting different types of essential cell components or processes that induce cell death pathways. It has been demonstrated that a diverse panel of drugs can elicit protective immune responses in mice (Apetoh et al., 2007; Obeid et al., 2007; Michaud et al., 2011). Of note, despite some screening studies using large drug libraries have been performed, only a small group of candidates have emerged to be valid ICD inducers (Obeid et al., 2007; Martins et al., 2011; Menger et al., 2012; Sukkurwala et al., 2014). The chemical nature of these agents, is considerably diverse: oxazophorines like cyclophosphamide (Schiavoni et al., 2011); Pt-based compounds as oxaliplatin (Tesniere et al., 2009); anthracyclines (Minotti et al., 2004) such as idarubicin and doxorubicin; anthracenediones such as mitoxantrone and dipeptides such as bortezomib (Merin and Kelly, 2014). Similar to bortezomib, carfilzomib another proteasome inhibitor used in the treatment of MM, has also shown to expose CRT in different MM cell lines (Jarauta et al., 2016). Although it may appear attractive, no simple structure-function relationship has been found that could predict the suitability of drugs to trigger ICD. This is clearly exemplified by the oxaliplatin-cisplatin or the melphalan-cyclophosphamide paradigms (Tesniere et al., 2009; Martins et al., 2011; Dudek-Perić et al., 2015). Several factors, such as the type of cell death, the ICD stimuli and the interconnection between various cellular stress responses, influence the type of danger signals emitted during the course of cell death (Agostinis, 2017). On the other hand, combinatorial strategies can be exploited to compensate for DAMPs generation scarcity displayed by some agents, restore immunogenicity and hence transform tolerogenic cell death into immunogenic modalities (Martins et al., 2011; Bezu et al., 2015). Furthermore, not all the DAMPs exposed during cell death are immunostimulatory. In fact, there are some molecules (Prostaglandin E2, adenosine, etc.) (Agostinis, 2017; Galluzzi et al., 2017) that exhibit immunosuppressive properties and play

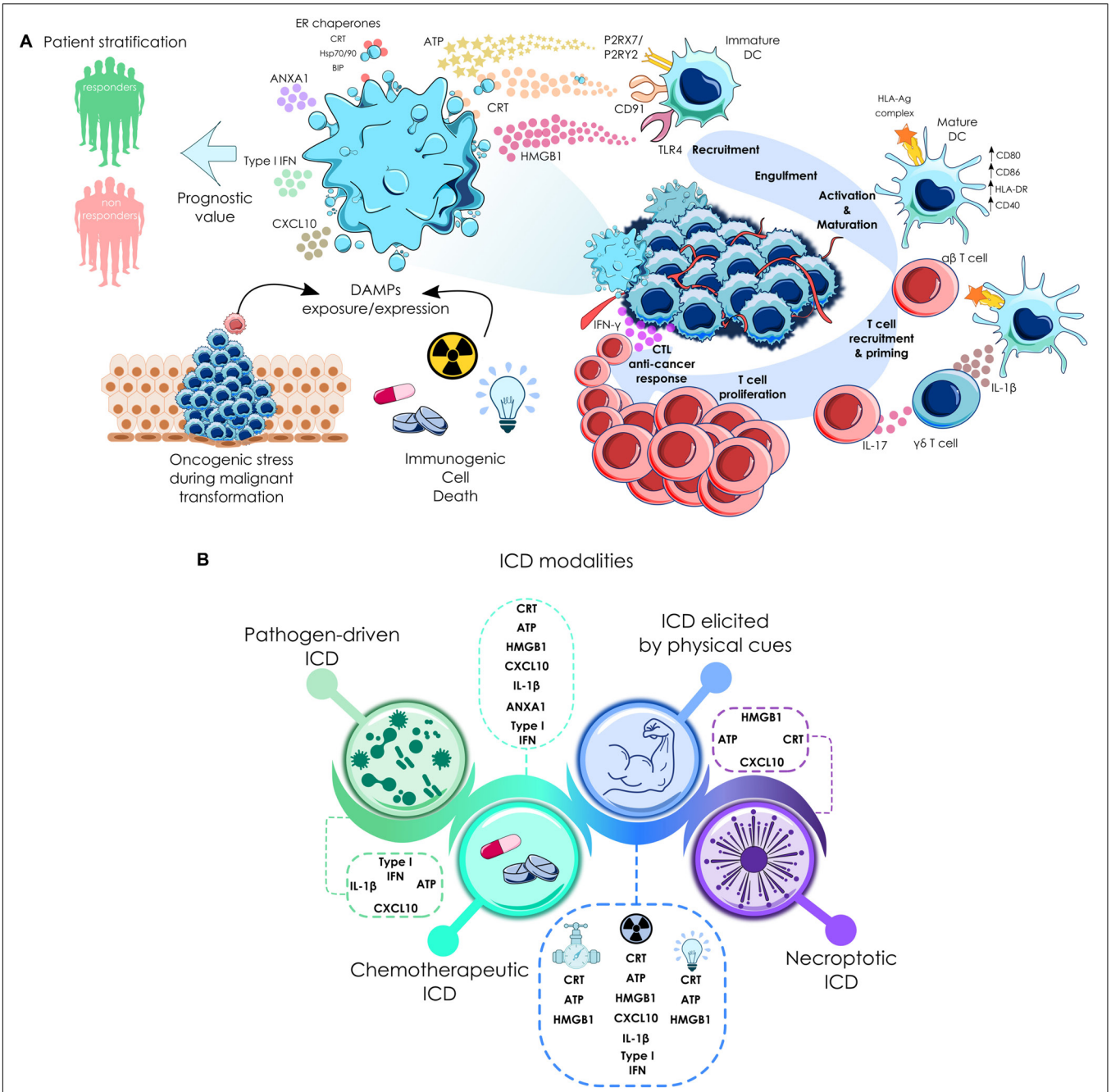

FIGURE 1 | Immunogenic cell death cycle and forms of ICD. **(A)** Cancer cells subjected to some type of chemotherapeutics and other anti-cancer therapies expose calreticulin (CRT) and other endoplasmic reticulum chaperones, such as Hsp70, Hsp90 or Bip on their surface, secrete ATP, initiate type I interferon (IFN) response that is able to trigger the production of CXC-chemokineligand 10 (CXCL10), and release high-mobility group box 1 (HMGB1) and annexin A1 (ANXA1). When secreted or exposed extracellularly, they bind to their cognate receptors on the surface of myeloid or lymphoid cells, which enables the engulfment of cell corpses by antigen-presenting cells, including DCs. This process in the context of proper immunostimulatory signals, eventually leads to the priming of an adaptive immune response involving both αβ and γδ T cells. This culminates in the establishment of a CTL-mediated anti-cancer immune response with potential to kill therapy-resistant cancer cells via an IFNγ-dependent mechanism. In the clinical setting, cancer cells with higher expression of some DAMPs have been found. Depending on the cancer type, this could be correlated with good or bad prognosis, as well as to markers of an active anti-cancer immune response. **(B)** Forms of ICD. Different variants of ICD could be evoked by distinct types of stimuli that are associated with a differential set of danger signals. Even in the form of immunogenic chemotherapy, each drug could instigate differential danger signaling pathways (Galluzzi et al., 2017); P2RX7, purinergic receptor P2X7; P2RY2, purinergic receptor P2Y2; TLR4, Toll-like receptor 4.

important roles in tolerance to dead cells. Among all members of DAMP family, the best studied, and those who have been shown to be pivotal for ICD are described in the next section.

Calreticulin and ER Chaperones

Calreticulin (CRT) is a, highly conserved, soluble, ER-associated chaperone with numerous functions inside and outside the ER (calcium homeostasis, assembly of MHC-I, etc.) (Johnson et al., 2001; Michalak et al., 2009). In stressed or dying cells, CRT is exposed in the outer leaflet of the plasma membrane (ecto-CRT) where it functions as a potent "eat-me" signal. CRT binds to LRP1 (also known as CD91), and possibly other scavenger receptors, displayed by phagocytic cells. This role in phagocytic clearance of dead cells was first described by Gardai et al. (2005). Nonetheless, Obeid et al. (2007) went a step further and demonstrated that CRT exposure was a key determinant in ICD-driven anticancer immunity. Actually, cancer cells undergoing cell death triggered by certain chemotherapeutics, expose CRT on their surface. This event leads to the engulfment of cancer material by DCs and, more importantly, to tumor antigen presentation and anticancer cytotoxic T lymphocyte (CTL) specific responses (Kroemer et al., 2013). Furthermore, ecto-CRT has been shown to prompt IL-6 and TNF expression on DCs, priming pro-inflammatory T-helper type 17 (Th17) polarization (Pawaria and Binder, 2011). Likewise, other ER-resident chaperones such as heat-shock protein 70 (Hsp70) and Hsp90, play also an important role in the immunogenicity of dying cancer cells. Thus, ecto-Hsp90 has been reported to enhance DC uptake of bortezomib-treated MM cells, including primary cells isolated from MM patients and induction of anticancer immunity (Spisek et al., 2007). On the contrary, Dudek-Perić et al. (2015), using blocking antibodies against Hsp90 in a DC maturation assay, reported that this chaperone was not (or at least partially) involved in the immunogenicity of melanoma cells treated with melphalan. The specific role of Hsp70 in the immunogenicity of cancer cells has not been studied so extensively. However, it has been reported that in shikonin- or gemcitabine-treated cells, Hsp70 was involved in DC-mediated activation of $CD4^+$ and $CD8^+$ T cells (Pei et al., 2014; Lin et al., 2015). In the case of Hyp-PDT treatment, Hsp70 promotes nitric oxide (NO) generation in innate immune cells (Song et al., 2013). In a different context, Hsp70 has shown to efficiently vaccinate mice against murine MM cells using a DNA-based vaccination strategy (Liu et al., 2018). BIP, a fundamental regulator of ER function and the UPR, has been described to be secreted and participate in the cross-presentation of tumor-derived Ags in DCs, inducing Ag-specific CTL immune responses (Tamura et al., 2011). Indeed, chaperones as efficient protein folding mediators, are often present bound to antigenic peptides. When released, these chaperone-peptides complexes enter APCs by endocytosis via CD91 receptors and are cross-presented on MHC-I and MHC-II molecules to $CD8^+$ and $CD4^+$ T cells (Feng et al., 2001, 2003). Thereby, these molecules not only potentiate immunogenicity of dying cancer cells by acting merely as potent danger signals, but also contribute to boost cancer antigenicity assisting in the cross-presentation process.

With regards to the kinetics and the cellular pathways involved in the exposure of CRT (depicted in **Figure 2**), it has been documented that they may differ depending on both the apoptotic phase under evaluation and the inducing stimulus (Krysko et al., 2012). For example, there are some instances where ecto-CRT exposure precedes phosphatidylserine externalization (Panaretakis et al., 2009; Osman et al., 2017), is systemically accompanied by ERp57 to the plasma membrane and requires PERK-mediated phosphorylation of eIF2α. This is followed by caspase-8 activation and specific cleavage of BAP31, leading to the subsequent activation of BAX and BAK. CRT relocation also requires anterograde ER-Golgi trafficking and the exocytic pathway in a SNAP23-dependent manner (Panaretakis et al., 2009). On the contrary, Hyp-PDT mediated CRT exposure requires PERK, BAX, BAK and the secretory pathway but not eIF2α phosphorylation and caspase-8 activation (Garg et al., 2012c). However, there are other ways by which CRT can be relocated to the cell surface and that are independent from the aforementioned mechanisms. Other studies claimed that CRT can bind with high-affinity to phosphatydilserine (Païdassi et al., 2011; Wijeyesakere et al., 2016) in a Ca^{2+}-dependent manner, and thus during cell death these two molecules are co-translocated at the same time in a caspase-independent fashion (Tarr et al., 2010).

Many studies investigating the role of CRT in ICD, carried out either *in vitro* or using *in vivo* animal models, assume the fact that CRT exposure is a consequence of the therapy itself. However, these studies have not considered basal surface expression of CRT on cancer cells and its potential implication on immunogenicity. Clinical studies supporting tumor cell-dependent immunity associated to basal CRT exposure are scarce and direct immunogenic effects of cells killed by chemotherapy in cancer patients have been rarely observed. It has been proposed that this is probably due to the fact that the chemotherapeutic dose needed to efficiently induce ICD is not reached in the clinical practice (Montico et al., 2018). Most of the available data indicate that tumor tissues express higher levels of CRT than healthy tissues, and that CRT expression may correlate with cancer progression and aggressiveness (Fucikova et al., 2018). Moreover, increasing clinical evidence is supporting the notion that CRT exposure, as well as other DAMPs may serve as important prognostic biomarkers in cancer patients (Fucikova et al., 2018). Different studies have shown that, depending on the cancer cell type, CRT expression could stand as a positive or negative prognostic factor for cancer patients. For example, in acute myeloid leukemia (AML), indolent B-cell lymphoma, non-small cell lung cancer (NSCLC), ovarian cancer, glioblastoma, endometrial cancer or colon cancer, the increased expression of CRT correlates with a favorable clinical outcome, as well as (in some cases) with increased levels of biological markers related to an active anti-cancer immune response (Peng et al., 2010; Zappasodi et al., 2010; Garg et al., 2015b; Stoll et al., 2016; Fucikova et al., 2016a,b, 2018; Xu et al., 2018). Meanwhile, in other cancer types like gastric cancer, pancreatic cancer, neuroblastoma, bladder carcinoma and mantle cell lymphoma, higher CRT levels were related to a poor clinical outcome (Chen et al., 2009; Chao et al., 2010; Sheng et al., 2014). In some cases like in esophageal squamous carcinoma, no differences in overall survival between CRT-high and low expression groups

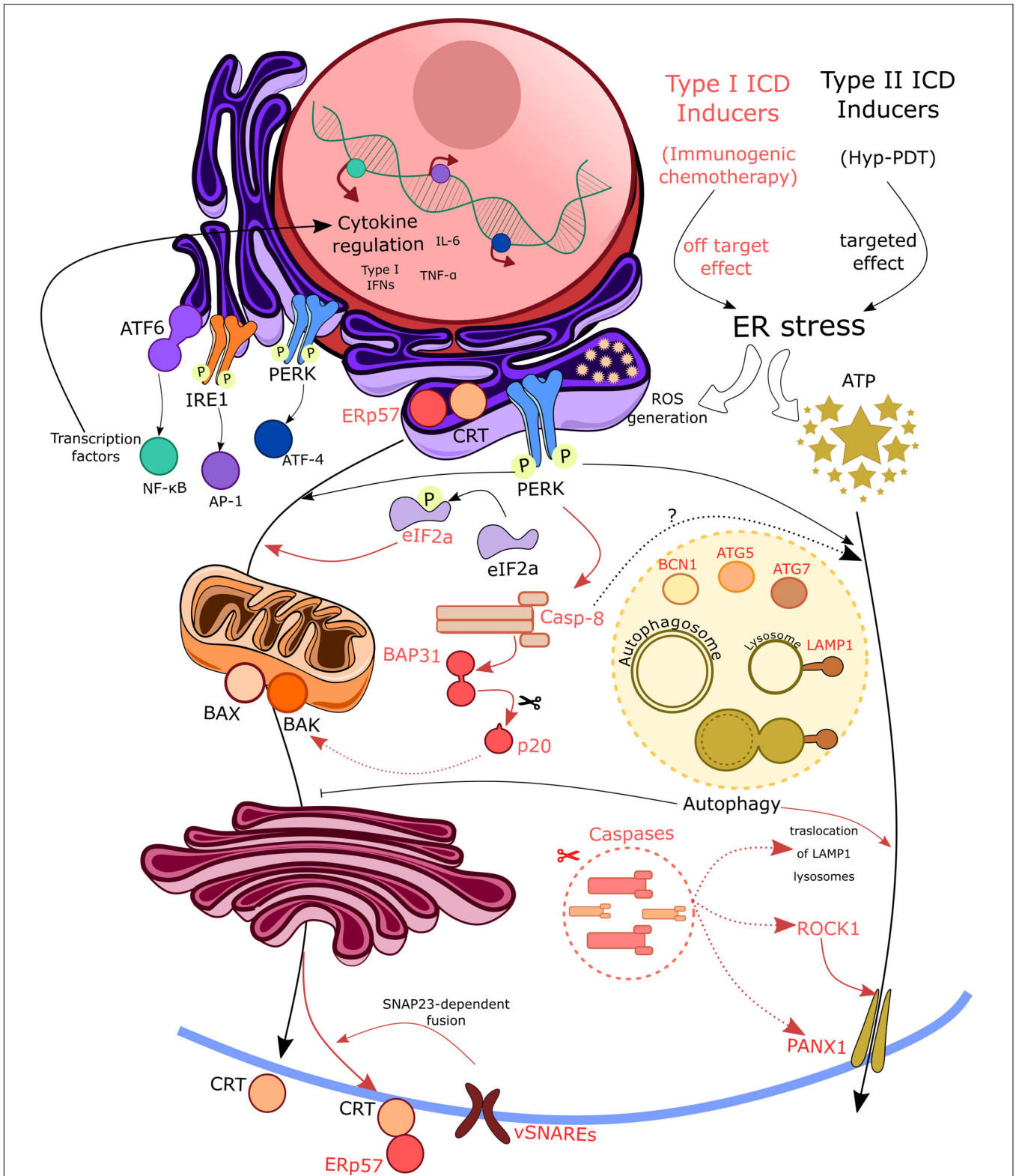

FIGURE 2 | Mechanisms of DAMPs exposure. Differential mobilization pathways can be observed between Type I and Type II ICD inducers, defined by their off-targeted or targeted effect on the ER, respectively. Exposure of CRT in the plasma membrane upon treatment with Type I ICD inducers requires an intrincate pathway with activation of the ER stress–ROS signaling mediated by the activation of the PERK, and the ensuing phosphorylation of eiF2α. This is followed by the required cleavage of B-cell receptor-associated protein 31 (BAP31) by preapoptotic caspase-8. Bax/Bak activation is also mandatory in this process. Finally CRT *(Continued)*

FIGURE 2 | Continued
relocation also requires anterograde ER-Golgi trafficking and the exocytic pathway in a SNAP23-dependent manner (Panaretakis et al., 2009). Along all the way from the ER to the plasma membrane, CRT is accompanied by ERp57. Upon treatment with Type II ICD inducers fewer requirements are needed, since this pathway only relies on PERK, Bax, Bak, and the secretory pathway. Regarding ATP secretion upon type II ICD inducers treatment, it follows a pathway quite similar to that of CRT, except for Bax/Bak and involving partially caspase 8. Type I ICD inducers require an independent pathway mediated by autophagy as ATG5, ATG7 and BCN1 are required in ATP release. Moreover, other molecules involved in different cellular processes like lysosomal exocytosis (LAMP1), membrane blebbing (ROCK1), apoptotic machinery (caspases) and membrane permeabilization (PANX1) have been shown to be essential in type I ICD-induced ATP externalization (Martins et al., 2014). CRT, calreticulin; eIF2a, eukaryotic initiation factor 2; ER, endoplasmic reticulum; ICD, immunogenic cancer cell death; PANX1, Pannexin 1; PERK, protein kinase R-like ER kinase; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; ROCK1, rho-associated, coiled-coil-containing protein kinase 1; ROS, reactive oxygen species; SNARE, SNAP (soluble N-ethylmaleimide-sensitive factor attachment protein) receptor.

were found (Suzuki et al., 2012; Fucikova et al., 2018). In some of these studies, other markers involved in ICD or ER stress response such as phosphorylation of eIF2α, Hsp70, Hsp90 and BiP (GRP78/HSPA5), correlated with CRT expression and patient prognosis (Uramoto et al., 2005; He et al., 2011; Fucikova et al., 2016a,b). As mentioned above, only in a few studies a correlation between increased CRT expression and the chemotherapy regimen and good prognosis was found. For example, ovarian tumors from patients that displayed high levels of CRT showed a good clinical response to radiotherapy or treatment with paclitaxel (which are well-known ICD inducers) (Garg et al., 2015b). Similarly, in endometrial cancer patients, low CRT expression was associated with poor survival rates and resistance to doxorubicin (another reported ICD inducer) (Xu et al., 2018). However, in other cases such as in patients with NSCLC or AML, cancer cells exposed heterogeneous levels of CRT, regardless of the treatment received. Cancer cells can also experiment stress prior to chemotherapy, perhaps due to the oncogenic malignant transformation itself (Fucikova et al., 2018). This alternative source of stress also activates ER stress responses culminating in CRT translocation and danger signaling (Fucikova et al., 2018). This process facilitates anti-cancer immunosurveillance, represented by the higher amount of infiltrating mature DCs and effector T cells in the case of NSCLC patients (Stoll et al., 2016) and increased numbers of circulating NK cells and IFN-γ producing $CD4^+$ and $CD8^+$ T cells in AML patients (Fucikova et al., 2016b). Moreover, cancer cells that express low levels of CRT have shown to correlate, in some cases, with therapy resistance, such as in endometrial cancer patients (Xu et al., 2018). It is possible that this reduced CRT expression may arise from the ability of cancer cells to resist ER stress conditions (whether oncogenic- or chemotherapy-driven). Therefore, this situation might be overcome by using ER stressors that directly target ER stress response, possibly sensitizing to conventional chemotherapy and restoring danger signaling and the ensuing anti-cancer immunosurveillance.

ATP

During the course of ICD, dying cells expel ATP (Ghiringhelli et al., 2009; Michaud et al., 2011) to the extracellular milieu where it functions as a powerful short-range "find me" signal (Elliott et al., 2009). Once secreted, ATP binds to ionotropic (P2X7) and metabotropic (P2Y2) purinergic receptors on APCs (Elliott et al., 2009; Ghiringhelli et al., 2009), stimulating their phenotypic maturation and chemotactic attraction, respectively (Galluzzi et al., 2015). In particular, extracellular ATP can activate the caspase-1 dependent NLRP3 complex (the so called inflammasome) triggering IL-1β secretion (Ghiringhelli et al., 2009), which in turn promotes $CD8^+$ T cell (Ghiringhelli et al., 2009), as well as, IL-17 producing-γδ T cell (Ma et al., 2011) anti-tumor responses. According to this, mice lacking any of these components ($Nlrp3^{-/-}$, $P2rx7^{-/-}$ or $Casp1^{-/-}$) seem to be incapable of promoting adaptive immune responses during drug-induced ICD (Ghiringhelli et al., 2009; Ma et al., 2011). The molecular mechanisms of ATP secretion during ICD are also dependent on ICD-inducing stimulus. In mitoxantrone- or oxaliplatin-driven early apoptotic ATP secretion, autophagy has been demonstrated to be mandatory, since depletion of important autophagy proteins (ATG5, ATG7 and BCN1) prevented ATP release (Martins et al., 2014). Moreover, other molecules involved in other cellular processes such as lysosomal exocytosis (LAMP1, VAMP1), membrane blebbing (ROCK1, myosin II), apoptotic machinery (caspases) and membrane permeabilization (pannexin 1, PANX1) have been shown to be essential for ICD-induced ATP release (Martins et al., 2014). Interestingly, PANX1 activation and surface exposure, as well as, LAMP1 translocation are strongly dependent on caspases rather than on the autophagic machinery (Martins et al., 2014). In fact, it is possible that remodeling of autophagic effectors and lysosomal effectors or PANX1 hemichannels by caspases rather than the mere presence of these components *per se*, are the real originators of ATP secretion (Garg et al., 2014; Martins et al., 2014). However, as it occurs in Hyp-PDT induced CRT relocation, ATP secretion mechanisms may differ from those described for chemotherapy-induced ICD. In particular, Hyp-PDT mediated ATP is autophagy independent (Garg et al., 2013) and rather requires the PERK-mediated proximal secretory pathway and PI3K-regulated exocytosis (Garg et al., 2012c).

HMGB1

High mobility group Box 1 is a non-histone chromatin-binding protein localized in the nucleus, where it interacts with DNA and regulates transcription (Garg et al., 2010). In particular, it regulates the activity of NF-κB and p53 and other transcription factors and favors VD(J) recombination (Müller et al., 2004; Krysko et al., 2012). Extracellularly, HMGB1 can perform cytokine-based (distinct from DAMP-based) functions in monocytes and macrophages under the influence of pro-inflammatory molecules (TNF, LPS, IL-1β) (Scaffidi et al., 2002; Müller et al., 2004; Krysko et al., 2012). When released from dying cells, HMBG1 exerts potent immunostimulatory effects by interacting with distinct PRRs (TLR2, TLR4 and RAGE) (Sims et al., 2010). During chemotherapy- or radiotherapy-induced cell death, HMGB1 is released from dying cells and

signals through TLR4-MyD88 axis on DCs, facilitating antigen processing and presentation (Apetoh et al., 2007; Saenz et al., 2014). The molecular pathways that participate in release of this DAMP remains to be elucidated. It has been documented that necrotic cells passively release huge amounts of HMGB1, acting as a potent mediator of inflammation (Scaffidi et al., 2002). Similarly, HMGB1 is also released by secondary necrotic cells and the use of Z-VAD-fmk (a broad caspase inhibitor that delays secondary necrosis) impede HMGB1 discharge in cells undergoing ICD (Bell et al., 2006; Apetoh et al., 2007). The immune related features of HMBG1 are strongly influenced by its redox status (Venereau et al., 2012; Yang et al., 2012), and this may account for the observed contradictory results (Palumbo et al., 2004; Jube et al., 2012). This redox modulation as well as the different behaviors observed in different studies have precluded from drawing definitive conclusions (Garg et al., 2014).

ICD – ER STRESS CONNECTION

As stated before, numerous studies have been carried out to decipher ICD mechanisms and large screening studies (Martins et al., 2011; Menger et al., 2012; Sukkurwala et al., 2014) have been performed to unveil the immunogenic potential of myriads of anti-cancer agents. All this work has converged toward a common denominator in ICD molecular pathways: ER stress and ROS generation (Tesniere et al., 2008; Rufo et al., 2017). Then, activation of the ER stress control pathways, also known as the UPR, and specially the PERK-mediated arm, is vital for the vast majority if not all the scenarios where ICD occurs (Panaretakis et al., 2009; Rufo et al., 2017). As mentioned in previous sections, CRT exposure induced by chemotherapeutics requires ER stress with a decisive participation of PERK-mediated phosphorylation of eIF2α (Panaretakis et al., 2009). Meanwhile, in hypericin-PDT induced ICD, the ER stress module is similarly required being PERK fundamental, but not eIF2α phosphorylation. Here, PERK may modulate proper secretory pathway functioning, in both ecto-CRT induction and ATP secretion (Garg et al., 2012c; van Vliet et al., 2015). Regardless of these dissimilarities, PERK abrogation through genetic maneuvers, significantly diminished (but not completely abolished) the immunogenicity of stressed cancer cells *in vivo* (Panaretakis et al., 2009; Garg et al., 2012c). Altogether, PERK have shown to be a major player in ICD-derived emission of danger signal(s). Depending on the trigger stimuli it could be involved only in CRT emission or both in ATP and CRT emission (Kepp et al., 2013; van Vliet et al., 2015; Rufo et al., 2017). Nevertheless, this context dependency determines whether PERK contribution arise from its UPR-related function (Panaretakis et al., 2009) or through its ability to modulate the proximal secretory pathway (Garg et al., 2012c). Moreover, other novel PERK cellular functions related to actin cytoskeleton dynamics and formation of ER-plasma membrane contact sites, may sustain DAMP trafficking in ICD (van Vliet et al., 2015, 2017; van Vliet and Agostinis, 2016; Rufo et al., 2017). Interestingly, although the three branches of the UPR (PERK, IRE1α and ATF6) were triggered under cardiac glycoside treatment (Menger et al., 2012), abrogation of IRE1α and ATF6 pathways through genetic interventions did not alter CRT exposure in dying cells under the influence of different types of therapies (mitoxantrone, oxaliplatin, UVC irradiation) (Panaretakis et al., 2009). Furthermore, tunicamycin and thapsigargin, two potent chemical ER stressors, both of which induce strong UPR responses (Obeng et al., 2006; Almanza et al., 2018; Shen et al., 2018), have been shown to efficiently restore CRT relocation and/or *in vivo* immunogenicity of cis-platinum or mytomicin C (Martins et al., 2011), two reported non-ICD inducers. Of note, it seems that ER stress alone is not sufficient to trigger CRT translocation or *in vivo* immune responses (Kepp et al., 2013). In line with this, tunicamycin and thapsigargin have been shown to be ineffective (or at least less effective as other *bona fide* ICD inducers) in eliciting ICD (Martins et al., 2011; Kepp et al., 2013). In contrast, thapsigargin has reflected the opposite in some scenarios (Peters and Raghavan, 2011). The relative importance of ER stress (the process itself and also its kinetics and intensity) is underscored by the classification of ICD inducers. There are two main groups of ICD inducers, type I and type II (Krysko et al., 2012; Rufo et al., 2017), depending on cell death is either a consequence of a primary effect of ER stress or death is triggered through a different path and ER stress is merely a secondary effect of the therapeutic agent under consideration (Krysko et al., 2012). For example, some oncolytic viruses (Newcastle disease virus) (van Vloten et al., 2018; Ye et al., 2018), Pt(II) N-heterocyclic carbene complex (Wong et al., 2015) and hypericin-PDT (Garg et al., 2012c) fall within type II ICD inducer category as they selectively target the ER provoking intense ROS-based ER stress (Krysko et al., 2012; Rufo et al., 2017). Conversely, anthracyclines (type I ICD inducers) exert its cytotoxic effects primarily on the nucleus, where they are mainly localized (Minotti et al., 2004) and leave the ER stress as a secondary side-effect. Bortezomib is also considered a type I ICD inducer. Although bortezomib affects ER homeostasis generating a potent ER stress response (Obeng et al., 2006; Verfaillie et al., 2013; Gandolfi et al., 2017; Manasanch and Orlowski, 2017) and elevation cellular ROS levels (Lipchick et al., 2016), its direct cellular target is the inhibition of 26S proteasome (Gandolfi et al., 2017). Thus, as the cellular targets of these two types of ICD inducers are different, it is conceivable that the cellular responses triggered (particularly in the ER stress context) are different both in their kinetics and potency. Consequently, this has clear implications in the quality and amount of danger signals emitted. In fact, it has been shown that hypericin-PDT (a type II ICD inducer) has a superior capacity of emitting faster, a higher number and a broader spectrum of DAMPs, compared to type I ICD inducers (Garg et al., 2012a,b,c; Krysko et al., 2012; Rufo et al., 2017).

It's important to mention that, in some regulated variants of cell demise, ROS-mediated ER stress may be dispensable for triggering ICD and the ensuing *in vivo* immune responses (Aaes et al., 2016; Rufo et al., 2017). Specially, different to hypericin-PDT based and anthracycline-induced ICD, the necroptotic variant occurred in absence of apparent/perceptible ER stress or PERK activation (Aaes et al., 2016). This reveals that there may be alternative mechanisms that may take part in the induction

of danger signaling and further reinforce the idea that ICD induction may be stimulus and context-dependent.

ER stress could also instigate immunosuppressive effects in the tumor microenvironment. In particular, transmissible ER stress has been observed in myeloid cells incubated with tumor supernatants obtained under ER stress conditions (Mahadevan et al., 2011; Colegio et al., 2014; Parker et al., 2015; De Sanctis et al., 2016). Moreover, tumor cells can activate in a paracrine fashion the UPR in tumor-infiltrated myeloid cells (DCs, MDSCs) that adopt an immunosuppressive phenotype, showing an impaired antigen presenting capacity, secretion of pro-inflammatory cytokines (IL-6, TNFα, IL-23, ...) as well as other immune-restraining factors (Mahadevan et al., 2011, 2012). Supporting this notion, mice tumors exposed to thapsigargin displayed exacerbated tumor growth which correlated with the increased numbers and aggressive phenotype of MDSCs (Lee et al., 2014). To our knowledge, although transmissible ER stress has not been directly demonstrated in MM, this system share common players with MM pathogenesis (IL-6, MDSCs, alteration of DCs). Therefore, as MM cell suffer from ER stress, it is not rare to think that transmissible ER stress might contribute to the characteristic immunosuppressive BM microenvironment in MM patients. Collectively, all these data seem to point to the fact that low to moderate ER stress may contribute to create an immunosuppressive environment, whereas high-level ER stress, such as the one occurred in ICD, could bring about immunostimulatory responses (Cubillos-Ruiz et al., 2017).

Besides the contributions to ICD stated before, ER stress may further boost DAMP signaling abilities of stressed cancer cells through the induction of autophagy (Martins et al., 2014; van Vliet et al., 2015). It is known that upon UPR activation, autophagy is activated as a defense mechanism to promote cell survival (Høyer-Hansen and Jäättelä, 2007; Velasco et al., 2010; Michallet et al., 2011; Corazzari et al., 2017). Moreover, as mentioned in previous sections, autophagy plays a crucial role in ATP secretion during ICD driven by chemotherapeutics (Martins et al., 2014). For these reasons, it may seem feasible that ER stress-induced autophagy triggered by ICD inducers further contributes to the immunogenicity of dying cancer cells. However, whether autophagy is directly induced by these drugs or is just a consequence of ROS-based ER stress in the context of ICD, needs to be thoroughly explored. Nonetheless, there are at least three facts that question the involvement of ER stress-induced autophagy in ICD: (1) The extensive characterization of molecular pathways involved in autophagy-mediated ATP secretion comprise molecular mechanisms (caspases, LAMP1-dependent trafficking, PANX1 channels lysosomal exocytosis) that seem to be independent of ER stress/UPR pathways (Martins et al., 2014). (2) In chemotherapy-induced ICD, autophagy do not regulate the emission of DAMPs which are dependent on ER stress pathways (Panaretakis et al., 2009; Michaud et al., 2011; Martins et al., 2014). (3) Finally, ATP secretion and CRT exposure appear to follow a different time-course, since CRT mobilization has been shown to occur prior phosphatidylserine externalization, whereas ATP is expelled during the blebbing phase of apoptosis. Altogether, these considerations may point to ER stress and autophagy as two independent constituents of ICD, at least in chemotherapeutic-driven ICD. On the other side, under Hyp-PDT treatment, autophagy has also been shown to be activated and to confer resistance against ROS-mediated cytotoxicity of stressed cancer cells (Dewaele et al., 2011; Rubio et al., 2012). One might argue that as hypericin is a direct ER sensitizer (Garg et al., 2012a) (type II ICD inducer), autophagy is triggered as a consequence of ER stress induction. Meanwhile in type I ICD inducers, as ER stress is not the primary target, autophagy could be induced upon interaction with other cellular targets. Furthermore, the ICD pathways involved in danger signaling are not identical when triggered by type I or type II ICD inducers. Thus, contrary to chemotherapy-induced ATP secretion, in the Hyp-PDT scenario ATP secretion is not dependent on autophagy machinery (Garg et al., 2013). Outstandingly, autophagy was found to attenuate CRT translocation and DCs maturation as well as suppress DC-mediated proliferation of CD4 and CD8 T cells (Garg et al., 2013). This has been rationalized as the autophagy machinery is able to clear oxidized proteins and organelles (Rubio et al., 2012; Garg et al., 2013), which in turn would alleviate the ER retention system that becomes overwhelmed under ER stress conditions (Johnson et al., 2001; Wiersma et al., 2015). Hence, during Hyp-PDT treatment, ER stress and ROS production allow oxidized proteins to accumulate leaving the ER retention system saturated (Dewaele et al., 2011; Rubio et al., 2012). Under these conditions, autophagy inhibition would increase the amount of oxidized proteins (possibly by augmenting ROS-based ER stress) and would favor that ER resident chaperones such as CRT could escape from ER confinement (Johnson et al., 2001; Peters and Raghavan, 2011; Garg et al., 2013). Similarly, in a model of melanoma, in wild-type as well as in BRAF-resistant cells, concurrent silencing of ATG5 and treatment with a MEK-inhibitor (U0126), amplified the levels of ecto-CRT and ecto-HSP90 compared to those cells in which autophagy was intact (Martin et al., 2015). Additionally, emerging mechanisms underpinning the crosstalk between the autophagic flux and the endosomal pathway could contribute to unravel the interplay of autophagy in modulation of ER-stress driven DAMP trafficking (Kim et al., 2012; Hyttinen et al., 2013; McKnight et al., 2014; van Vliet et al., 2015). ER stress could also have an impact over intracellular ATP levels through stimulation of mitochondrial respiration and bioenergetics (Bravo et al., 2012). This way the cell fill their bioenergetic stores to restore cell homeostasis. Given the chemotactic power of ATP, by increasing its cellular levels, the cell may also be preparing to alert the immune system that something is wrong. Finally, ER stress and the UPR could also impact on cytokine production in multiple levels (PRRs, transcription factors involved in cytokine production, etc.) (Smith et al., 2018). The mechanisms involved in this process are out of the scope of this manuscript and have been recently reviewed in Reverendo et al. (2019) and Smith et al. (2018).

ER STRESS-ASSOCIATED CELL DEATH

With all these players around the table, it seems tempting to target PERK and/or ER stress in cancer. In fact, ER stress

as a target, is increasingly getting more adepts in the cancer crusade. During tumor development cancer cells have to cope with harsh conditions that are widely known to trigger ER stress (e.g., nutrient deprivation, hypoxia, acidic pH) (Sano and Reed, 2013). Thus, UPR activation constitutes an important hallmark of numerous human cancers (Riha et al., 2017). This process endows cancer cells with the ability to acquire essential characteristics (dormancy, resistance to therapy, tumor-driven angiogenesis, etc.) required for tumor progression (Sano and Reed, 2013; Corazzari et al., 2017; Mohamed et al., 2017). As stated before, ER stress could also negatively influence immunity at different levels, favoring this way tumor development (Mahadevan et al., 2011; De Sanctis et al., 2016; Cubillos-Ruiz et al., 2017). In the particular case of MM, their exacerbated secretory phenotype leave these cells heavily reliant on the survival arm of the UPR. Therefore, as plasma cell development and survival strongly relies on an intact UPR (Reimold et al., 2001; Iwakoshi et al., 2003), it does not seem unusual that UPR activity increases with MM progression (Nakamura et al., 2006). Furthermore, whole genome sequencing studies have revealed that MM patients frequently harbor mutations in genes related to the UPR (Chapman et al., 2011). Among the UPR mediators, XBP1 has been found to be overexpressed in MM and has also been identified to be mutated in a small subpopulation of patients (Carrasco et al., 2007; Bagratuni et al., 2010; Chapman et al., 2011; Nikesitch et al., 2018). Nevertheless, although myeloma cells count on the UPR to thrive, they are extremely sensitive to ER stress-associated cell death (Obeng et al., 2006; Ling et al., 2012; Gandolfi et al., 2017). This feature explains why proteasome inhibitors, have shown a prominent clinical efficacy in the treatment of MM (Leleu et al., 2018; Scalzulli et al., 2018), although resistance to therapy is recurrent and in most of the cases accounts for the lethality of the disease (Nikesitch et al., 2018; Robak et al., 2018). For these reasons, novel ER stress/UPR-targeting therapies have emerged. Given its important role in myeloma pathogenesis, novel drugs targeting the RNAse domain of IRE1 (4μ8C, MKC-3946, STF083010) have been developed. These drugs showed significant tumor growth inhibition in mouse myeloma models (Papandreou et al., 2011; Mimura et al., 2012), as well as in primary myeloma plasma cells (Papandreou et al., 2011). In addition, new potent and selective first-in-class inhibitors have been developed against PERK (GSK2606414 and the derived form GSK2656157) (Atkins et al., 2013; Hoi et al., 2013). These drugs have shown promising pre-clinical results in a model of pancreatic cancer (Atkins et al., 2013; van Vliet et al., 2015). Nonetheless, given the dual role of ER stress and UPR related pathways in cancer, a word of caution about needs to be taken when targeting these cellular pathways. On one side we may be inhibiting the pro-tumorigenic role of UPR mediators but in the other, we may reduce the immunogenicity of cancer cells dampening danger signaling (or vice versa). Therefore, future investigations assessing the repercussion on overall immunity, as well as cell-autonomous responses on cancer cells, on immunocompetent mice models are needed in order to truly evaluate the therapeutic relevance of these approaches in cancer.

Although UPR activation is initially conceived to restore cell homeostasis, it is also able to shift the cellular demise toward cell death. When ER stress persists, the UPR is able to trigger proapoptotic programs controlled mainly by IRE1 and PERK arms. Activated IRE1 can act as a docking platform to recruit other proteins such as the adaptor protein TRAF2, that subsequently tethers ASK1 which causes activation of JNK/p38 MAPK pathway. These downstream stress kinases, are reported to promote apoptosis in several ways. For example, JNK phosphorylation has been shown to inhibit the anti-apoptotic members Bcl-2, Bcl-xL and Mcl-1, while activating pro-apoptotic members BID and BIM (Deng et al., 2001; Lei and Davis, 2003; Almanza et al., 2018). As regards to p38 MAPK, it phosphorylates and activates transcription factor CHOP which contributes to apoptosis controlling several Bcl-2 family members (Yamaguchi and Wang, 2004; Puthalakath et al., 2007). As in the case of PERK signaling, it increases the expression of ATF4 and CHOP, two key determinants of ER stress-induced cell death. CHOP can increase the transcription of BH3-only proteins BIM (Puthalakath et al., 2007) and PUMA (Cazanave et al., 2010). Moreover, Noxa has been reported to be upregulated by ATF4 (Armstrong et al., 2010). ATF4/CHOP pathway also downregulates the expression of Bcl-2 and Mcl-1 anti-apoptotic proteins, contributing in this way to cell death (Puthalakath et al., 2007; Gomez-Bougie et al., 2016). Moreover, PUMA, BID and BIM deficient cells, as well as BAX and BAK double-knock-out cells, are protected from cell death by external ER insults (Ren et al., 2010; Almanza et al., 2018). The extrinsic apoptotic pathway could also be upregulated under ER stress conditions. Thus, CHOP and ATF4 have been shown to increase the expression of DR4 and DR5 receptors (Hiramatsu et al., 2015; Iurlaro et al., 2017). In fact, bortezomib have been shown to cooperate and potentiate cell death induced by Apo2L/TRAIL in MM cell lines (Balsas et al., 2009).

Bcl-2 family are better known for their roles in controlling mitochondrial permeability and cell death mechanisms. However, they also play important roles in regulating calcium ER homeostasis and ER stress-induced cell death. Interestingly, an intense crosstalk between mitochondria and ER organelles exists, which even increases during ER stress conditions (Bhat et al., 2017). For example, BAX and BAK are capable of modulating IRE1 activity during ER stress by interacting with IRE1 (Hetz, 2006). In similar way to mitochondria, BAX and BAK can also oligomerize at the ER membrane under ER stress conditions. This results in an increase of ER-membrane permeability and the release of ER resident proteins such as calreticulin, BIP, PDI and GRP94, which could aggravate ER stress and ROS production (Rodriguez et al., 2011; Pihán et al., 2017). This process is triggered by BH3-only members and counteracted by Bcl-2 and Bcl-x_L (Wang et al., 2011; Kanekura et al., 2015). The mechanism by which ER permeabilization leads to cell death is still unknown. However, it has been speculated that ER permeabilization could bring about release of ER-Ca^{2+} stores and increase Ca^{2+} flux to the mitochondria through mitochondria ER-associated membranes (MAMs). This would instigate cell death by mitochondrial permeabilization transition pore (mPTP) (Pihán et al., 2017). Taken together, these studies

delineate the ER as an important stress sensor and integrator where also cell fate decisions may take place, with Bcl-2 family as the critical circuitry that connect and modulate the mechanisms involved in cell fate (UPR, apoptosis and also autophagy).

IMMUNOTHERAPY IN MM

Multiple myeloma is a hematological malignancy that arises due to uncontrolled proliferation of abnormal plasma cells. It accounts for 10–20% of all hematological neoplasms and 0.9% of all newly diagnosed cancer cases worldwide (Bray et al., 2018). Over the past two decades, treatment regimens and survival rates of myeloma patients have witnessed a radical improvement, with ASCT, IMiDs, proteasome inhibitors and monoclonal antibodies as the contributors to this advance. Among them, proteasome inhibitors, stand out as the cornerstone of this scientific and medical achievement (Scalzulli et al., 2018). However, although overall survival and patient outcomes have considerably improved, drug resistance is still a major concern and accounts for the fatality of the disease (Nikesitch et al., 2018; Robak et al., 2018). That is why novel and more efficient (immuno)therapeutic approaches may take the relief. It is important to point out that MM is a genuine example where the immune system is compromised. Deficits in antibody production/immunoglobulin levels due to a reduction of bone marrow (BM) B-cell progenitors are common in MM (Rawstron et al., 1998). General disruption of T-cell immune profile has also been observed, characterized by increased numbers of regulatory T cells (Tregs), aberrant CD4/CD8 ratios and altered CD4+ T cell numbers among others (Braga et al., 2014; Joshua et al., 2016; Chen et al., 2017). MM is also characterized by augmented expression of programmed cell death ligand 1 (PD-L1), one of the immune checkpoint inhibitory ligands that counterbalance T cell activity by binding to PD-1 on activated T cells (Paiva et al., 2015; Jung et al., 2017). MDSCs are also a major issue in MM, as expansion of this population usually correlates with disease progression and a negative clinical outcome (Malek et al., 2016). In addition, MM also finds good allies in BM stromal cells (BMSCs), which are important players sculpting a permissive BM microenvironment (Mahindra et al., 2010). Through cell-to-cell (Mondello et al., 2017) o exosome-mediated contacts (Wang et al., 2014) with MM cells, they secrete cytokines that favor the recruitment of immunosupressive populations such as Tregs and MDSCs (Giallongo et al., 2016; Malek et al., 2016). Finally, several studies have documented an impaired DC function and although contradictory results have been reported, alterations in DCs frequencies and phenotypes have been found in in MM patients (Pasiarski et al., 2013; Leone et al., 2015; Brown et al., 2018). Despite all these stones in the immunotherapeutic path, immune-interventions have potential to be successful in this disease. Graft-vs-myeloma effect was firstly evidenced in patients subjected to ASCT or under donor lymphocyte infusions, suggesting an active immune response against myelomatous cells (Ladetto et al., 2016). Current immunotherapeutic approaches that are giving positive results in relapsed and refractory patients are going to be described below (see also **Figure 3**).

Antibody-Based Therapy

Although monoclonal antibodies (moAbs) have been in the anticancer therapeutic armamentarium for some years, effectively treating some solid and hematological cancers, it was only a few years ago that Daratumumab was approved for the treatment of MM. Daratumumab is a moAb that selectively targets CD38, an antigen highly expressed in aberrant plasma cells and at relatively low levels on normal lymphoid and myeloid cells, including normal PCs. Similarly, other anti-CD38 moAbs are currently under investigation such as isatuximab and MOR22. As single agent, Daratumumab showed a promising efficacy, observing objective response rates (ORRs) of approximately 30%, progression free survival (PFS) of 4 months and overall survival (OS) of 20 months, in relapsed and refractory MM (RRMM) patients heavily treated with at least two prior lines of therapy (Lokhorst et al., 2015; Lonial et al., 2016; Rodríguez-Otero et al., 2017). Daratumumab has been shown to kill MM cells through a plethora of mechanisms ranging from antibody-dependent cell mediated cytotoxicity (ADCC) mediated by NK cells, complement-medicated cytotoxicity (CDC), antibody-dependent cell phagocytosis (ADCP) mediated by macrophages and even apoptosis via direct cross-linking (van de Donk and Usmani, 2018). NK cell-mediated cytotoxicity seems to be one of the main mechanisms, and since patient NK cell status may vary, this could explain differences in response between patients (van der Veer et al., 2011). Nowadays, another moAb, elotuzumab, has been approved in MM therapy targeting the SLAMF7 molecule expressed among normal and myeloma PCs, NK, and T cells. The mechanism of action of Elotuzumab is thought to differ from that of daratumumab. This thought is based on the fact that elotuzumab alone has not reached objective responses in MM patients but when combined with lenalidomide and dexamethasone, in a phase II trial and afterward in the Eloquent-2 phase III trial, significantly improved ORRs and OS in RRMM patients (Lonial et al., 2015; Rodríguez-Otero et al., 2017).

Combination of chemotherapy with this kind of approach could render synergistic effects and improve patient's outcomes. Interestingly, IMiDs have shown to prime MM cell lines to Daratumumab-induced NK cell-mediated cell death (Fedele et al., 2018). In fact, several clinical trials combining IMiDs and Daratumumab have been performed obtaining good results (Gavriatopoulou et al., 2018). Similarly, the efficacy of Daratumumab alone was even improved with combination regimens of daratumumab plus lenalidomide and dexamethasone or daratumumab with bortezomib plus dexamethasone, significantly extending PFS period with strong and durable responses (Blair, 2017; Rodríguez-Otero et al., 2017). As Carfilzomib has shown better survival curves compared to bortezomib, combinations of Daratumumab plus carfilzomib and dexamethasone are currently under phase I investigation (clinical trial NCT03158688).

Other novel and promising designs of these kind of therapy are the conjugated antibodies and the bi-specific T

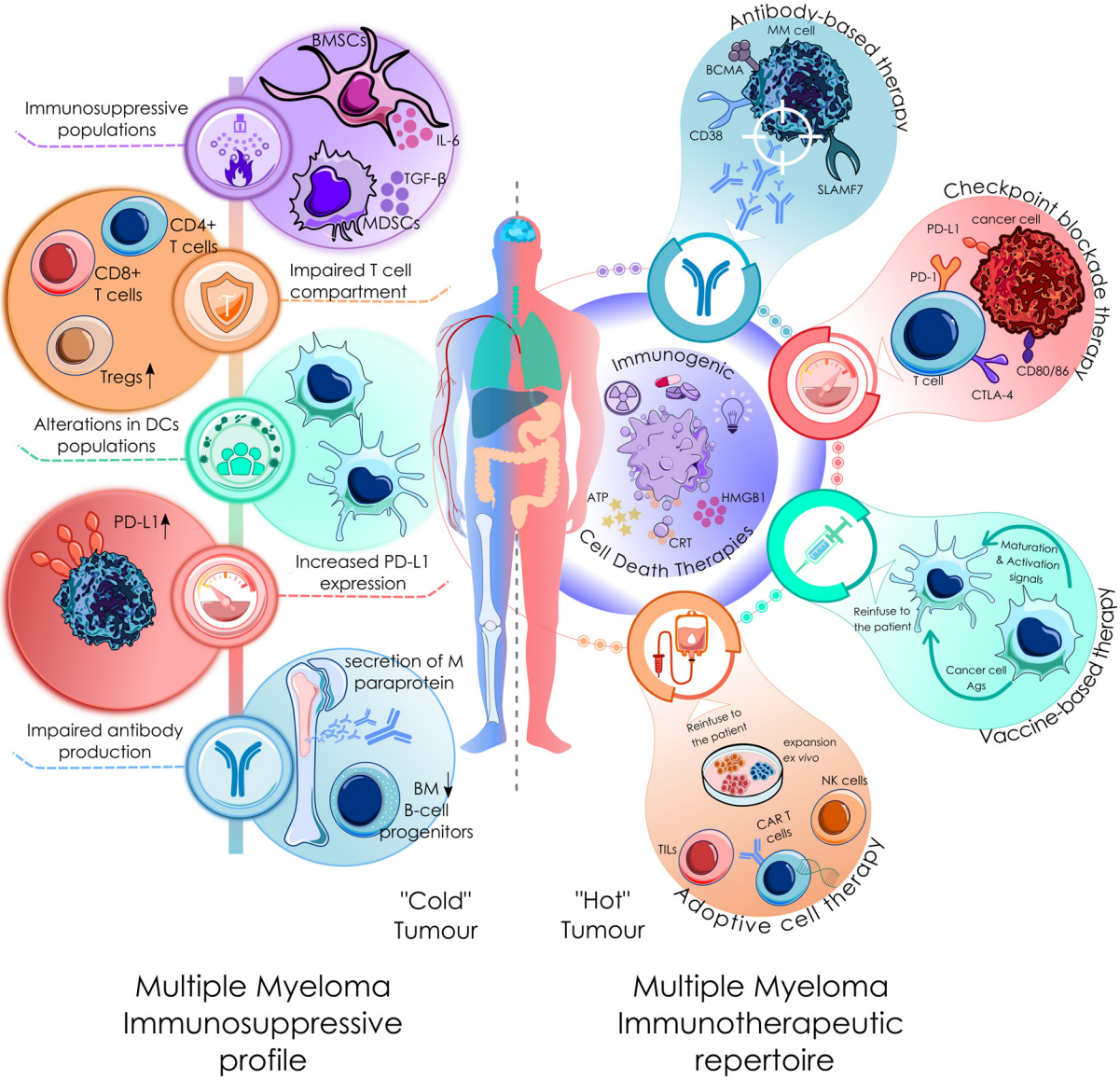

FIGURE 3 | Current clinical immunological scenario in MM. MM is a genuine example where the immune system is compromised. It is characterized by: (1) Deficits in antibody production due to a reduction of bone marrow (BM) B-cell progenitors (Rawstron et al., 1998). (2) General disruption of T-cell immune profile, characterized by increased numbers of regulatory T cells (Tregs), aberrant CD4/CD8 ratios and altered CD4+ T cell numbers among others (Braga et al., 2014; Joshua et al., 2016; Chen et al., 2017). (3) Increased expression of programmed cell death ligand 1 (PD-L1), one of the immune checkpoint inhibitory ligands that counterbalance T cell activity (Paiva et al., 2015; Jung et al., 2017). (4) MDSCs and BMSCs are also a major issue in MM. They are important players sculpting a permissive BM microenvironment, through cell-to-cell (Mondello et al., 2017) o exosome-mediated contacts (Wang et al., 2014) with MM cells, they secrete cytokines that favor the recruitment of immunosupressive populations such as Tregs. (5) An impaired DC function and alterations in DCs frequencies and phenotypes have been found in MM patients (Pasiarski et al., 2013; Leone et al., 2015; Brown et al., 2018). At the right is depicted the current immunotherapeutic repertoire in MM therapy. All these immunotherapeutic approaches could be virtually benefited from the immunostiumulatory effect of ICD-related therapies.

cell engagers (BiTEs). Conjugated antibodies carry in their structure cytotoxic molecules that are guided by the specificity of the antibody part and delivered directly into the target. In particular, an anti-BCMA specific antibody linked to a new class of antimitotic agent, monomethyl auristatin F, has been developed (GSK2857916). This formulation has demonstrated in a phase I trial a 60% response rate and PFS of 7.9 months in RRMM patients with at least three prior lines of therapy (Trudel et al., 2018). Regarding the BiTEs, these are bispecific antibodies that hold on one side specificity for the target cancer cell epitope and on the other recognizes (generally) CD3 molecules on T cells facilitating the contact between them. This way, contact between effector cells and cancer cells is facilitated. There are several BiTEs targeting the BCMA antigen that are currently under development (BI 836909, EM801 and JNJ-64007957) and showed positive results in preclinical

models (Cho et al., 2018). Some of these have now entered clinical trials (NCT02514239, NCT03145181, NCT03269136 and NCT03269136), we will have to wait to new updates of these and other studies to check the efficacy of these new formulations.

Adoptive Cell Therapy

Another way to confront the tumor is by directly using and improving patient's own defenses (immune effector cells) to kill cancer cells with ACT. By expanding, activating and even engineering NK or T cells outside the immunosuppressive tumor microenvironment, some of the immune barriers may be successfully, or at least partially overcome. As mentioned earlier, graft versus myeloma effect has been observed in patients subjected to autologous stem cell transplantation (ASCT). This effect is thought to be mainly mediated by T cells. Therefore, this population and more specifically, tumor infiltrating lymphocytes (TILs), MILs in the case of myeloma, represents one of the major immune effector cells that could be used to fight MM. Although clinical data in this issue is still scarce, encouraging results has been reported. Noonan et al. (2015) reported that a 90% reduction of tumor burden was achieved with a PFS of 25.1 months, hence demonstrating the feasibility and efficacy of this approach. Genetically engineered T cells stand as a novel and a leading therapeutic opportunity in cancer in general and also in MM. There are two categories: (1) Transgenic TCRs, with specificity toward a tumor antigen in the context of MHC molecule and (2) chimeric antigen receptor (CAR) T cells, which are fusion proteins composed of a single-chain variable fragment (scFv) that directs the specificity toward the cancer cell antigen, coupled to intracellular signaling modules (CD3ζ) or costimulatory molecules (CD28 or CD137/4-1BB). TCR engineered T cells have the advantage to recognize both intracellular and surface antigens, therefore virtually any tumor antigen could be targeted. However, they are restricted to the HLA-I type limiting the patient eligibility criteria (Rodríguez-Otero et al., 2017). Moreover, potential recombination with TCR α and β chains could lead to off-target toxicities due to generation of unexpected MHC-TCR-peptide complex (Cohen, 2018). Fatal and sudden toxicities have been observed in two patients receiving transgenic TCR T cells with specificity to MAGE-A3 class I peptide, due to unwanted specificity of transgenic TCR toward the myocardial protein titin (Linette et al., 2013). Therefore, caution in selecting the proper Ag must be taken. In myeloma, transgenic TCR T cells for NY-ESO1 peptide and its homolog LAGE are currently under clinical testing (Rapoport et al., 2015). Regarding the use of CAR T cells, one of its limitations is that only surface antigens can be targeted, so the number of available targets is lower with this approach. Therefore, the success of this therapy relies on selecting the appropriate target, to selectively kill the cancer cell limiting off-target and targeted-toxicities on healthy tissue. To date CD19 CAR T cells has shown remarkable results on acute lymphoblastic leukemia, chronic lymphocytic leukemia and non-Hodgkin lymphoma (Porter et al., 2015; Maude et al., 2018). Nowadays, there are several antigens in the anti-myeloma CAR T cell repertoire including CD19, CD138, CD38 and SLAMF7. To date BCMA CAR T cell formulation is the one that has been developed in further extent (Cohen, 2018). Several clinical trials have tested or are currently testing BCMA CAR T cells in heavily treated RRMM patients reporting encouraging results. In these studies, overall response rates were close to 80% or even higher and CRs were achieved in an important proportion of patients (Castella et al., 2018; Cohen, 2018).

Similarly, NK cells also pose as a committed ally in cancer therapy. They do not rely on MHC restriction or antigen recognition, but rather they are dependent on the balance between activating and inhibitory receptors. In MM, NK cell numbers and functionality are usually altered, therefore it is feasible to think that restoration of NK cell compartment with ACT could represent a suitable opportunity to face this disease. There are many therapeutic options that are currently under clinical evaluation. They mainly differ in their source (umbilical cord vs. peripheral blood), in their allo-reactivity (autologous vs. allogeneic), and the expansion and stimulation protocols used to prepare and improve these cells (Fionda et al., 2018). One conclusion may be drawn out from all these studies and that is the superior capacity of allo-reactive NK cells to bring myeloma down. Regarding the use of CAR NK cells in MM, they are still under preclinical studies and have not move yet to clinical investigation.

Here chemotherapy could also improve the effectiveness of these approaches. In particular, Lenalidomide has shown to improve the function and persistence of anti-myeloma CS1 CAR T cells *in vivo* (Wang et al., 2018). Carfilzomib has also shown activating and sensitizing activities over NK cells and MM cells, respectively (Chang et al., 2018). In addition, the combination of expanded and activated allogeneic NK cells (eNK) with therapeutic mAbs directed against tumor antigens (e.g., daratumumab in the case of MM), could give excellent results through ADCC mediated by eNK cells (Sanchez-Martinez et al., 2018).

Releasing the Brakes With Checkpoint Blockade

T cell activation is a complex and well-regulated process. When the menace have been removed, returning to the homeostatic state and preventing damage of tissues requires negative feedback signals that terminate with the immune response. To that end, checkpoint inhibitors are the major class of receptors that provide these attenuation signals to limit the T cell response. Multiple inhibitory checkpoints have been discovered so far: CTLA-4, PD-1, LAG-3, TIM-3, etc. Although, currently both stimulatory and inhibitory checkpoints are under investigation, the checkpoint drugs on which clinical therapies have been developed are CTLA-4, PD-1 and PD-L1. CTLA-4 is an inhibitory receptor expressed on activated T cells and binds to B7 costimulatory molecules on APCs with higher affinity than CD28. Therefore, CTLA-4 blocks and displaces costimulatory interactions eventually leading to abrogation of T cell activation. Ipilimumab, a blocking antibody against CTLA-4, was the first of these type of drugs clinically tested, showing important improvements in metastatic

melanoma patients (Robert et al., 2011; Sharma and Allison, 2015). Like CTLA-4, PD-1 is also a checkpoint inhibitory receptor expressed on activated T cells and has two known ligands, PD-L1 and PD-L2. PD-1/PD-L1 (PD-L2) signaling axis interferes with TCR signaling and contributes to T cell exhaustion. PD-L1 / PD-L2 are widely expressed among different cell types and their expression is known to increase under IFN-γ exposure (Sharma and Allison, 2015). Hence, it is thought that this pathway is a late mechanism of protection from T cell activation and represents a physiological way to regulate termination of inflammatory reactions (Sharma and Allison, 2015; Cogdill et al., 2017). PD-L1 is upregulated in tumor cells acting as a disguise mechanism that allow them to escape from T cell-mediated tumor surveillance. Moreover, PD-L1 expression has been linked with poor prognosis in a variety of human cancers (Ghebeh et al., 2006; Mu et al., 2011). On the other hand, probably due to the immunosuppressive character of the tumor microenvironment, TILs show higher expression of PD-1 (Fourcade et al., 2010; Zhang et al., 2010). In MM, PD-L1 expression is upregulated on myeloma cells but not in normal plasma cells from healthy donors (Liu et al., 2007; Tamura et al., 2012; Paiva et al., 2015; Yousef et al., 2015). In fact, higher PD-L1 expression in MM cells was associated with disease progression as shown in the differences of PD-L1 expression between MGUS, MM and relapsed/refractory MM (RRMM) patients (Paiva et al., 2015). Blocking PD-1 alone with nivolumab has not reached good clinical objective responses with half of the patients experiencing disease stabilization in a phase I study (Lesokhin et al., 2016; Rodríguez-Otero et al., 2017). Similarly, on KEYNOTE-013 study, Ribrag and colleagues assessed the clinical efficacy of the anti-PD-1 mAb pembrolizumab as single agent in patients with RRMM. No patient of the 30 enrolled in the study experienced any response and the best outcome observed was again disease stabilization (Paul et al., 2018).

Although checkpoint blockade therapy alone has shown promising results in some cancer patients, this response is not universal and strongly relies on the tumor microenvironment. Thus, checkpoint blockade efficacy may also be refined by induction of more propitious immunogenic conditions in the tumor tissue through ICD. Recent preclinical studies have shown that immunogenic chemotherapy may sensitize cancer cells to checkpoint blockade leading to synergistic responses. In a lung mouse cancer model, an approved clinical chemotherapy regimen (Oxaliplatin plus cyclophosphamide) were able to foster $CD8^+$ T cell infiltration and increase $TLR4^+$ DCs in tumor tissue, which leads to sensitization of tumors to immune checkpoint therapy (Pfirschke et al., 2016). Another study also showed that the CDK inhibitor dinaciclib was able to increase immune infiltration and activation within tumors and combination with anti-PD1 therapy resulted in enhanced anticancer activity in three different syngeneic mouse cancer models (Varpe et al., 2012). In the clinical practice, NSCLC patients treated with combined regimens of chemotherapy (platinum-based) with different anti-PD1 agents have demonstrated considerable higher response rates and improved clinical outcome compared to that seen on single-agent modalities (Mathew et al., 2018). In patients with metastatic renal cell carcinoma, combination of anti-PD1 (nivolumab) plus pazopanib or sunitib also showed promising clinical responses (Amin et al., 2014).

In MM, preclinical data shows that lenalidomide, one of the so-called immunomodulatory drugs (IMiDs), reduce the expression of PD-1 and PD-L1 in MM cells and BM accessory cells isolated from RRMM patients. Moreover, a synergistic effect between lenalidomide and anti-PD-1 or anti-PD-L1 was observed (Görgün et al., 2015). These results encouraged the rationale of using PD-1/PD-L1 blockade in combination with IMiDs in the treatment of MM. Hence, phase I and phase II clinical trials on RRMM patients who underwent at least three prior lines of therapy have been conducted (Wilson et al., 2016; Badros et al., 2017). These studies showed ORRs of 60% with even some cases of complete response. Therefore, development of phase III clinical trials were the following step to test these combination modalities (Malavasi et al., 2018). Pembrolizumab plus Len and Dex (KEYNOTE-185, NTC02579863), Pembrolizumab plus Pom and Dex (KEYNOTE-183, NTC02576977) and another phase III study testing three different combination regimens (Poma and Dex vs. nivolumab, Pom and Dex vs. nivolumab, elotuzumab, Pom and Dex; CheckMate 602, NCT02726581) were developed. However, these studies were discontinued due to an increase of unprecedented deaths in the pembrolizumab group as well as that no objective responses were observed in the tested groups.

DC-Based Vaccines and Its Enhancement/Upgrade With ICD

Due to its particular nature, DCs are at the fine-tuned crossroads between innate and adaptive immunity, playing a pivotal role in anti-cancer host immune responses. Therefore, DC-based vaccines seem to be a good option to re-educate the host immune system against myeloma, leading not only to the expansion of anti-tumor specific T cells, but also to long-term memory generation. Since its first documented clinical use on melanoma patients in 1995 (Mukherji et al., 1995), DC-based vaccines have gained momentum in anti-cancer therapy. In fact, this approach has showed positive survival benefits in a diverse set of human cancers (Kantoff et al., 2010; Nakai et al., 2010; Anguille et al., 2014; Cao et al., 2014). In the particular case of MM, DC-MM fusion vaccines achieved anti-cancer immune responses and disease stabilization in the vast majority of patients (Rosenblatt et al., 2011; Rosenblatt et al., 2013). In hematological cancers, following ASCT a complete "resetting" of the hematological system occurs, leaving a huge opening to vaccination strategies to succeed (Rodríguez-Otero et al., 2017). However, although considerable objective clinical responses have been observed, the overall clinical outcome still has not reached the expected standards (Anguille et al., 2014; Vandenberk et al., 2015). As mentioned earlier, due to the hostile microenvironment surrounding MM cells, DC populations are dysfunctional in MM, showing impaired T-cell stimulation capacity (Guillerey et al., 2016; Chung, 2017). Moreover, it is said that the antigens

displayed by myeloma cells are presented to DCs in absence of the appropriate costimulatory signals. Therefore, these interactions lead to inadequate immune responses and even create tolerance against cancer Ags (Chung, 2017). For these reasons, there is a consensus that DC vaccines may need to be optimized and standardized in order to enhance their clinical efficacy. There are several factors that have a direct impact on DC biology and the quality and potency of the ensuing T cell responses: route of administration and frequency of injection, delivery system, use and type of adjuvants, nature of DC vaccine formulations, and nature of tumor cell lysates/antigen cargo (Vandenberk et al., 2015; Rodríguez-Otero et al., 2017). Among them, the immunogenicity of dying cancer cells used to load DCs could be easily and notably improved by using ICD-inducers. Numerous studies have proven the potential of ICD-inducers to have a huge impact on DC biology and improve the ability of DCs to stimulate effector cells and enhance anti-cancer T cell responses *in vivo*. For example, γ-irradiation, has been shown to effectively induce DCs maturation and stimulate *in vivo* CTL responses (Goldszmid et al., 2003). Moreover, γ-irradiated cells efficiently immunized mice against a subsequent rechallenge with live syngeneic cancer cells in various preclinical models (Strome et al., 2002). Different ICD-related modalities such as UV light (Brusa et al., 2008), oncolytic viruses (Donnelly et al., 2011), HHP (Mikyšková et al., 2016), heat shock (Adkins et al., 2017) among others have shown to upregulate maturation markers in DCs as well as prime antigen specific T-cell responses both *in vitro* and *in vivo*. Hyp-PDT is also equally effective in inducing complete tumor regression *in vivo* both in curative and prophylactic vaccination settings (Sanovic et al., 2011). DCs charged with Hyp-PDT treated cells significantly enhanced CTL responses, IFN-γ producing $CD8^+$ T cells and Th1-driven immunity in ectopic murine mammary tumors (Jung et al., 2012) as well as orthotopic glioma mice models (Garg et al., 2016).

In the clinical practice, melanoma and high-grade glioma patients have successfully been treated with DC vaccines loaded with γ-irradiated tumor cells (Cho et al., 2012). In the case of glioblastoma multiforme, patients who underwent conventional treatment plus DC-based therapy showed an increased short-term (1–3 years) survival rates compared to control group receiving conventional therapy (Cho et al., 2012). Relapsed Non-Hodgkin's B-cell lymphoma (NHL) patients have also benefited from DC vaccines pulsed with γ-irradiated, heat shock or UV light-treated tumor cells (Zappasodi et al., 2010). Accordingly, CRT and HSP90 expression levels on NHL cells positively correlated with the observed clinical and immune responses (Zappasodi et al., 2010).

In MM, data regarding the use of ICD-dying cells to provide an enhanced immunogenic feed to DCs and the expected *in vivo* anti-cancer immune responses are still lacking. In particular lenalidomide has shown to impact DCs biology and enhance $CD8^+$ T cell cross-priming by primed DCs (Henry et al., 2013). Another study evaluated ICD induced by bortezomib in MM cell lines and MM primary cells, as well as the capacity of bortezomib-treated cells to increase maturation markers in DCs and to induce proliferation and polarization toward IFN-γ producing T cells *in vitro* (Spisek et al., 2007). There is currently an ongoing phase II clinical trial testing DC/MM fusion vaccines in combination with lenalidomide and GM-CSF (NCT02728102). We will need to wait for further studies to see the clinical advantages of combining this type of approaches.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Over the past years, ICD and ER stress are gaining momentum in anti-cancer therapy. The ability of chemotherapeutics and other anti-cancer therapies, not only to mount an active immune response against the tumor, but also to modulate the cancer immune environment, has transformed the therapeutic scenario in oncoimmunology. Moreover, understanding of the molecular pathways involved in all these processes, is uncovering a whole new set of potential prognostic biomarkers with which cancer patients could be better monitored and stratified to determine their optimal therapeutic regimen. However, given that certain danger signaling markers have been found both in treated and untreated patients, further investigations are needed to unravel the real repercussion of therapy driven-ICD, as well as oncogenic-driven DAMP exposure in the clinical setting. Furthermore, special caution is needed when targeting ER stress and UPR pathways, as it could pose both beneficial and detrimental consequences on patient's outcome. On one sid, we may be enhancing cell death pathways or boosting immunogenicity of cell death, but on the other we could also be fostering the cytoprotective function of the UPR as well as some ER stress-related immunosuppressive effects. Nonetheless, given the adaptability and complexity of cancer, it is becoming increasingly clear that future anti-cancer therapeutic approaches will take advantage from combination of immunogenic (chemo)therapeutic modalities with current and novel immunotherapeutic regimens. In particular, in MM, this type of combinatorial approaches have a great opportunity to success, since encouraging results have been already obtained. Nonetheless further investigations awaits to circumvent and manage some of the basic problems and clinical adverse events that arise with these novel kind of approaches.

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MINI REVIEW
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Death Receptor Interactions With the Mitochondrial Cell Death Pathway During Immune Cell-, Drug- and Toxin-Induced Liver Damage

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Due to its extensive vascularization and physiological function as a filter and storage organ, the liver is constantly exposed to infectious and tumorigenic threat, as well as damaging actions of xenobiotics. Detoxification reactions are essential for the excretion of harmful substances, but harbor also the risk of "side effects" leading to dangerous metabolites of otherwise harmless substances, a well known effect during paracetamol overdose. These drugs can have detrimental effects, which often involves the induction of sterile inflammation and activation of the immune system. Therefore, the role of certain immune cells and their effector molecules in the regulation of drug-induced liver damage are of special interest. Hepatocytes are type II cells, and death receptor (DR)-induced cell death (CD) requires amplification via the mitochondrial pathway. However, this important role of the mitochondria and associated CD-regulating signaling complexes appears to be not restricted to DR signaling, but to extend to drug-induced activation of mitochondrial CD pathways. We here discuss the role of members of the TNF family, with a focus on TRAIL, and their interactions with the Bcl-2 family in the crosstalk between the extrinsic and intrinsic CD pathway during xenobiotic-induced liver damage.

Keywords: death receptor, TRAIL, DILI, Bcl2 family, Bim, JNK, APAP

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LIVER DAMAGE BY XENOBIOTICS

One major task of the liver is to maintain metabolic homeostasis. It processes and stores nutrients absorbed in the gut and delivered by the portal vein. In addition, as part of the enterohepatic circulation, the liver is the first organ to receive absorbed xenobiotics and toxins. Therefore, it provides a plethora of biochemical tools to metabolize, activate or inactivate drugs and poisons. Mainly involved are enzymes of the cytochrome P450 (CYP) family, which introduce functional groups. The subsequent conjugation and detoxification reactions enable the secretion of harmful chemicals via bile and kidney. Additionally, CYP enzymes are also involved in the chemical activation of inactive pro-drugs (e.g., cortisone or prednisone). These pharmacologically important reactions, called first-pass effect, are important for the regulation of activity and dosage of many

Abbreviations: APAP, acetaminophen, paracetamol; CD, cell death; cytC, cytochrome C; DAMP, danger associated molecular pattern; DILI, drug induced liver injury; DR, death receptor; JNK, c-Jun N-terminal kinase; KC, Kupffer cells; MOMP, mitochondrial outer membrane permeabilization; PAMPs, pathogen-associated molecular pattern; TNF, Tumor Necrosis Factor; TRAIL, Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand.

drugs. Next to beneficial activation and detoxification processes, these enzymatic reactions may also result in a detrimental outcome, e.g., via the chemical activation of otherwise harmless compounds, thereby gaining substantial toxic potential. Examples are N-acetyl-p-benzochinonimin (NAPQI), a reactive metabolite of acetaminophen, or aflatoxin B1 (AFB1). These reactive metabolites rather affect centrilobular hepatocytes, which have high CYP enzyme activity. Besides such indirect "side effects" of detoxification reactions, other chemicals may also directly induce liver toxicity. Such drugs or poisons can affect parenchymal and non-parenchymal cells of the liver, promoting a typically early onset of disease within a few days. Especially, hepatocytes in periportal regions of the liver lobules as well as endothelial cells are exposed to high levels of such xenobiotics. Altogether, these different forms of drug-induced liver injury (DILI) account for 50% of all cases with acute liver failure. Examples of medically relevant substances are acetaminophen (APAP, paracetamol), environmental toxins (AFB1), alcohol, carbon tetrachloride (CCl_4), as well as antineoplastic agents. However, the bulk of DILI cases is attributed to APAP overdose. Generally, liver damage by xenobiotics can be induced by protein adducts and dysfunction, lipid peroxidation, DNA damage and glutathione depletion due to increased reactive oxygen species (ROS) levels, thereby inducing mitochondrial damage and impaired energy supply. Subsequent lytic necrosis and/or apoptosis can promote the release of cellular content, and associated induction of immune cell activation and sterile inflammation (**Figure 1** upper part).

THE ROLE OF THE IMMUNE SYSTEM IN LIVER DAMAGE

Liver-resident immune cells are important in the protection from enteric and liver-specific infections, as well as immune surveillance of liver metastases. Deregulated host immune reactions, however, are also a frequent cause of severe hepatitis. Similarly, metabolic disorders can induce detrimental liver damage and thereby activate effector mechanisms of the host immune system, e.g., as seen during metabolic syndrome, non-alcoholic fatty liver disease (NAFLD), non-alcoholic steatohepatitis (NASH), and alcoholic liver disease (ALD).

In this context also DILI is not necessarily only established by direct action of the respective compound on liver cells, but may involve a secondary response of the immune system. Important mediators during initiation and deregulation of such sterile inflammatory processes are so-called danger-associated molecular patterns (DAMPs). These normally intracellular molecules are typically released during necrotic CD due to loss of membrane integrity, but the process is likewise also relevant for apoptotic CD, though to a lesser extent. Immune cells are thus capable to distinguish "self-safe" and "self-dangerous" by evolutionary conserved pattern recognition receptors. Multiple transmembrane and intracellular receptors can sense DAMPs. They activate preferentially myeloid cells, resulting in the secretion of pro-inflammatory cytokines, like TNF and IFNγ, the recruitment of innate immune cells and further damage of the affected tissue. The vicious cycle of massive hepatocyte damage and extensive DAMP release is a well-known feed-forward loop interconnecting inflammation and CD during liver damage.

Kupffer cells (KC) are liver-resident tissue macrophages and the most abundant innate immune cells of the liver. Therefore, depletion of these phagocytic cells can have protective effects (Zhao et al., 2008; Kiso et al., 2012) but also exacerbate drug-induced liver damage due to their additional anti-inflammatory and tissue-protecting functions (Bourdi et al., 2002). Importantly, studies in human patients also suggest a role for infiltrating mononuclear cells in tissue repair processes, rather than promoting tissue damage (Antoniades et al., 2012). Natural killer cells (NK) and NKT cells are likewise part of the liver's innate immune defense. Though liver-resident cells, they accumulate as infiltrates after initial liver damage. For APAP-induced DILI it was reported that NK and NKT cells play a disease-promoting role since increased NKT cell infiltrates and effector molecules, such as IFNγ, were observed. Additionally, NK and NKT depletion had a protective effect in APAP-induced DILI in mice (Liu et al., 2004). Though, this claim was challenged by others, and reported to be caused by side-effects of the solvent DMSO (Masson et al., 2008).

Independent of direct liver damage are immune reactions of the adaptive immune system, which are implicated in so-called unpredictable reactions, called idiosyncratic DILI (IDILI). These destructive mechanisms are not well understood, but are generally believed to be based on immune-mediated hypersensitivity (Schnyder et al., 1997; Chen et al., 2018).

IMMUNE CELL-DERIVED DEATH LIGANDS AFFECTING DRUG-INDUCED HEPATITIS

Drug- and toxin-induced hepatic insults, either by direct toxicity or caused by the adaptive immune system, are associated with deregulated inflammatory responses mediated by members of the TNF superfamily. This family comprises a multitude of membranous and soluble molecules. A subset of this family, including TNF, Fas ligand (FasL, CD95L) and TRAIL can activate so-called DRs and thereby the extrinsic CD pathway. Generally, sensitivity of CD induction by members of the TNF superfamily is highly regulated.

The most detrimental effect on hepatocytes is mediated by FasL, which is involved in various forms of immune cell-mediated acute liver damage. Activation of the Fas receptor on e.g., virus-infected or transformed hepatocytes leads to their rapid death. Released cellular content can modulate cells in close proximity to increase their FasL susceptibility, thereby further increasing bystander killing. Due to the abundant expression of Fas throughout the liver, systemic administration of FasL or agonistic anti-Fas antibodies results in acute and mostly fatal hepatitis (Ogasawara et al., 1993). Consequently, FasL expression and Fas-induced hepatocyte death need to be tightly controlled, e.g., by transcriptional control, post-translational regulation like intracellular storage and activation-dependent mobilization, and shedding by metalloproteases (Brunner et al., 2003).

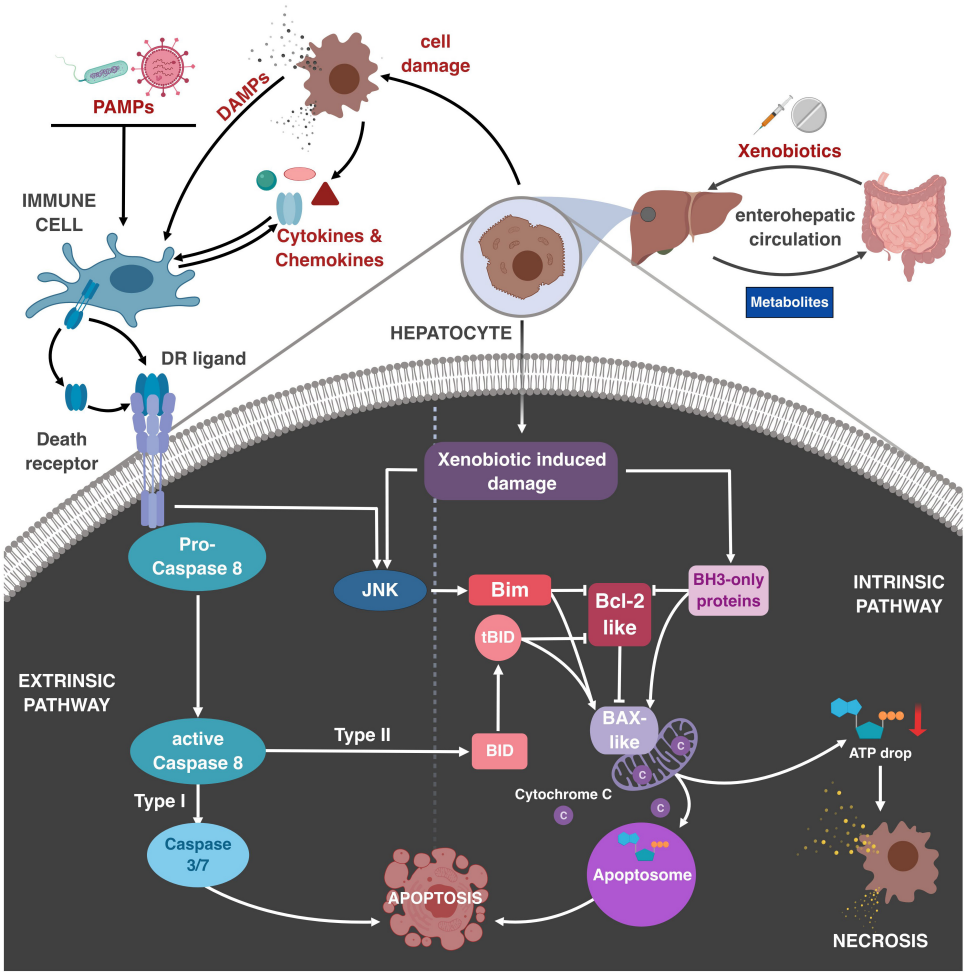

FIGURE 1 | Role of immune cells and TNF family members in regulating drug-induced liver damage. Xenobiotics are absorbed in the gut and transported to the liver, where they become metabolized. During the metabolization, toxic intermediates may be generated, inducing damage to liver parenchymal cells, most importantly hepatocytes, via activation of the Bcl-2-regulated mitochondrial apoptosis pathway. This can lead to apoptosis via permeabilization of the outer mitochondrial membrane, cytC release and subsequent apoptosome formation, or necrotic CD if ATP levels are too low. Resulting DAMP (danger–associated molecular pattern) release by necrosis or late apoptosis can initiate sterile inflammation by activation of liver-resident immune cells, e.g., KC. DAMPs and PAMPs can stimulate immune cells, resulting in the release of effector molecules, e.g., cytokines and chemokines, which recruit and activate other immune cells. Additionally, DR ligands induce the extrinsic CD pathway. In hepatocytes already affected by xenobiotics DR activation promotes synergistic CD at otherwise sublethal concentrations. This crosstalk between DR and xenobiotics involves caspase-8-mediated cleavage of Bid, DR-induced activation of JNK and Bim, and xenobiotic-induced induction and activation of other BH3-only proteins. Resulting neutralization of pro-survival Bcl-2-like and activation of Bax-like molecules results in mitochondrial apoptosis or necrosis.

Functionally, FasL-induced apoptosis serves as a key effector mechanism in T- and NK cell-mediated cytotoxicity against Fas-expressing target cells.

Tumor Necrosis Factor plays a prominent role in the context of KC activation and associated liver pathology. It causes the induction of pro-inflammatory cytokines and chemokines, which promote recruitment of other inflammatory cells, induction of hepatocyte death or proliferation for wound healing responses. These pleiotropic effects on different cell types highlight it's complex signaling output. In the healthy liver, for example, TNF produces non-apoptotic signals via activation of the MAP kinases JNK or p38, or NFκB, thereby triggering pro-survival and pro-inflammatory responses. Bifurcation of the signaling pathway is eventually regulated in a context-dependent manner. Thus, hepatocytes are rather insensitive to TNF-induced CD when administered alone. However, in combination with the liver-specific transcriptional inhibitor D-(+)-galactosamine (GalN) it causes massive CD. In this situation, the transcription-dependent pro-survival signaling of TNF cannot restrict the secondary activation of CD pathways, i.e., apoptosis or necroptosis.

Immediately after its discovery, the TNF homolog TRAIL gained extensive clinical interest due to its rather tumor-selective CD-inducing activity, even though this was also stated for untransformed cells. In the liver, TRAIL was reported to be involved in immune surveillance of tumors and metastases, but also in the control of viral infections. TRAIL is predominantly expressed by liver NK cells, where it contributes to their cytotoxic effector mechanisms together with perforin and FasL

(Kayagaki et al., 1999; Takeda et al., 2001). TRAIL expression is regulated by IFNγ, which triggers TRAIL expression not only in NK cells, but also in monocytes and dendritic cells (DCs). Autocrine IFNγ production has been reported to promote constitutive TRAIL expression by liver-resident NK cells (Takeda et al., 2001). Receptor expression is, however, not sufficient to induce apoptosis by TRAIL, as the ratio of activating and decoy receptors, as well as anti-apoptotic proteins define sensitivity (Sarhan et al., 2014). Sensitivity might also be regulated by the crosstalk with the intrinsic apoptosis pathway. Many drugs that induce ER-stress, DNA damage or ROS sensitize otherwise TRAIL-resistant cells, leading to synergistic CD induction (Ganten et al., 2005; Koschny et al., 2007; Schneider-Jakob et al., 2010; Badmann et al., 2011).

Synergy Between Death Ligands and Xenobiotics in Liver Toxicity

In contrast to FasL, TNF, and TRAIL may not only directly trigger the extrinsic CD pathway, but can also stimulate signaling pathways, which modify apoptosis initiated by other triggers. The cellular context seems to be especially relevant in this crosstalk between DR signaling and the intrinsic apoptosis pathway. A well-described player is the Bcl-2 family member Bid. In so-called type I cells, caspase activation after DR activation is sufficient to directly cause apoptosis. Type II cells, though, rely on the amplification of the extrinsic signal via the mitochondrial pathway (Yin et al., 1999). The best-characterized type II cells are hepatocytes. Low level of caspase-8 activation upon DR activation promotes cleavage of Bid, and its truncated form (tBid) mediates MOMP by direct activation of the pore-forming Bcl-2 family member Bax and neutralization of anti-apoptotic Bcl-2 homologs (Li et al., 1998). Besides this Bid-mediated crosstalk, other connections between different DRs and stress signaling pathways have added further levels of complexity. Initially, a crosstalk between TRAIL and the intrinsic pathway has been described in thymocytes. Thus, it was observed that TRAIL-deficiency results in reduced activation-induced thymocyte apoptosis upon T cell receptor (TCR) crosslinking, which is Bim-dependent (Corazza et al., 2004; Kassahn et al., 2008). Subsequently it was found that TRAIL enhances not only TCR-mediated apoptosis, but extends to other apoptosis triggers, like UV- and γ-irradiation, and glucocorticoids, but not Fas crosslinking. Given that thymocytes are type I cells, these findings suggested that TRAIL may specifically enhance the mitochondrial CD pathway. As in type II cells the Fas pathway is also amplified via mitochondria, it was tempting to speculate that TRAIL would also enhance Fas-induced hepatocyte apoptosis and associated liver damage. Indeed, it was found that Fas-induced hepatocyte apoptosis could be synergistically enhanced by TRAIL receptor activation (Corazza et al., 2006). Interestingly, this CD amplifying pathway initiated by TRAIL did not seem to depend on direct caspase activation, but rather on TRAIL receptor-initiated JNK activation and associated Bim phosphorylation. Consequently, TRAIL- and Bim-deficient mice, as well as mice treated with JNK inhibitors, were protected from anti-Fas-induced acute liver damage. Interestingly, similar observations have also been made for TNF. Kaufmann et al., 2009 described that both, Bid and Bim contribute to TNF-dependent LPS/GalN-induced liver damage (Kaufmann et al., 2009), whereas other publications observed a similar enhancing effect of TNF on Fas-induced hepatocyte apoptosis and associated liver damage, which was dependent on JNK and Bim (Schmich et al., 2011; Faletti et al., 2018). Thus, while lacking a direct hepatotoxic activity both TRAIL and TNF can activate the JNK-Bim axis and thereby enhance Fas-induced apoptosis in type II cells (**Figure 1** lower left part).

Tumor Necrosis Factor-Related Apoptosis-Inducing Ligand, which induces CD in a p53-independent manner, has been regarded as an exciting alternative to conventional p53-dependent chemotherapy (Hellwig and Rehm, 2012). Various studies in different types of tumors revealed additive or even synergistic CD induction when TRAIL was combined with chemotherapy. Given the CD-enhancing effect of TRAIL in hepatocytes via activating the JNK-Bim axis, an obvious idea was that TRAIL would similarly regulate chemotherapy-induced apoptosis in tumor cells. Indeed, in hepatocellular carcinoma cells TRAIL stimulation results in JNK activation and Bim hyperphosphorylation (Schneider-Jakob et al., 2010). Furthermore, while tumor cells were relatively insensitive to TRAIL and chemotherapeutic drugs, a profound synergistic CD induction was seen when used in combination. This synergistic CD induction was strongly attenuated upon either pharmacological inhibition of JNK, or knockdown of Bim and Bid, confirming that TRAIL receptor-mediated activation of the JNK-Bim axis, likely together with the activation or transcriptional induction of other BH3-only proteins, represents the molecular basis for this synergy. Likely synchronized TRAIL- and chemotherapy-induced changes in the Bcl-2 interactome, ultimately resulting in efficient activation of Bax and Bak, and MOMP, are key events (Hantusch et al., 2018). TRAIL-induced and JNK-mediated phosphorylation of Bim appears to be an important switch in this process. It has been previously shown that Bim_L and Bim_{EL} interact with dynein light chain 1 (DLC1). While it was previously thought that DCL1 sequesters Bim at the cytoskeleton (Puthalakath et al., 1999), thereby inhibiting its apoptosis-inducing activity, more recent data shows that DLC1 is important for oligomerization of Bim and clustering it in high-molecular-weight complexes together with Mcl-1 at the mitochondrial outer membrane (Singh et al., 2017). Interestingly, the DLC1-binding domain of Bim overlaps with the phosphorylation site of JNK (T112). Thus, JNK-mediated phosphorylation appears to release Bim from this complex and thereby unleash its apoptosis-inducing activity by initiating Bax activation and neutralization of anti-apoptotic Bcl-2 homologs, such as Bcl-x_L. Thereby it limits its Bax retrotranslocating activity, which seems to be critical for controlling Bax oligomerization and MOMP (Todt et al., 2015; Hantusch et al., 2018). Further adding to the level of complexity is the fact that some of the JNK-mediated phopsphorylation sites in Bim are overlapping with those of ERK1/2 (Lei and Davis, 2003; Ley et al., 2003). Yet, while JNK appears to have in general an activating activity on Bim, ERK1/2-mediated phosphorylation rather leads to Bim degradation and survival. Synergistic induction of CD in tumor cells by TRAIL and chemotherapy may

represent promising strategies to overcome therapy resistance in tumor patient. However, it is worth mentioning that the mechanism *per se* seems to be far more general, as also primary human hepatocytes are sensitized by TRAIL to chemotherapeutic drug-induced apoptosis (Schneider-Jakob et al., 2010).

Role of the TRAIL-JNK-Bim Axis in Enhancing Drug-Induced Liver Necrosis

As discussed above, APAP overdose is responsible for the vast majority of DILI cases. Interestingly, APAP overdose leads to necrotic lesions, rather than apoptotic liver CD. In addition, RIP1 and probably also RIP3 deficiency rescues from APAP toxicity, implicating a necrotic or necroptotic form of CD (Ramachandran et al., 2013; Dara et al., 2015). However, since MLKL inhibition has no beneficial effect on APAP pathology, necrosis remains to be the most relevant pathway. At the same time, APAP toxicity includes upstream apoptotic signaling events, like induction of pro-apoptotic Bcl-2 homologs, Bax activation and MOMP with release of cytC and Smac/DIABLO. Surprisingly, at least *in vivo* no caspase activation is seen, and caspase inhibitors do not prevent APAP-induced liver damage (Jaeschke et al., 2006). The question remains why caspases are not activated despite the extensive activation of the mitochondrial apoptosis pathway. It is well known that APAP treatment causes mitochondrial impairment and associated drop in ATP levels (Jaeschke, 1990). Furthermore, low ATP levels prevent apoptosome formation and caspase activation, shifting the CD execution toward necrosis (Nicotera et al., 1998). Therefore, it was suggested that APAP-induced decrease in ATP levels is responsible for shifting apoptotic processes toward a necrotic outcome. Indeed, preventing APAP-induced mitochondrial permeability transition by cyclosporine A, or increasing intracellular ATP by providing the glycolytic substrate fructose increases APAP-induced caspase activation in hepatocytes (Kon et al., 2004). Thus, current knowledge indicates that APAP-induced liver damage represents an interplay of several distinct CD mechanisms, including the activation of Bcl-2-family members and induction of MOMP, yet resulting in a necrotic outcome. Despite the lack of evidence for apoptosis induction, a role of certain Bcl-2 family members in the regulation of APAP-induced liver toxicity is well documented. Most importantly, the TRAIL-JNK-Bim axis seems to play also an important role in APAP-induced liver necrosis. Astonishingly, TRAIL or Bim deletion not only resulted in reduced APAP-induced hepatocyte death (Badmann et al., 2011), but also reduced death of liver sinusoidal endothelial cells (LSEC) (Badmann et al., 2012). Similarly, a profound role of JNK in the transcriptional upregulation of Bim, and the subsequent phosphorylation of Bim was observed (Badmann et al., 2011). These results clearly demonstrate that CD amplification via the TRAIL-JNK-Bim axis goes far beyond Bcl-2 family-regulated apoptosis induction via the mitochondrial pathway, but extends to necrotic form of liver CD and is likely not limited to APAP-induced liver damage. The question, however, remains how TRAIL can enhance hepatocyte necrosis. Does TRAIL indeed amplify APAP-induced necrosis and if so how? Or does it shift the cellular response from necrosis to apoptosis, which would likely involve a stabilization of intracellular ATP levels and apoptosome formation? And finally, how are these processes regulated by the Bcl-2 family members and their interactions? Especially in LSECs it could be shown that APAP and TRAIL synergistically induce apoptotic events followed by substantial caspase activation, which could indeed be rescued by the pan-caspase inhibitor zVAD (Badmann et al., 2012; **Figure 1** lower right part). Mechanistically, the role of TRAIL and APAP in transcriptional and post-translational activation of Bim and other BH3-family members certainly has to be further addressed. In addition, the role of TRAIL in switching the APAP-induced CD pathway toward necrosis or apoptosis likewise remains an unsolved open question. Similarly, it remains to be investigated whether also other forms of DILI are regulated by TRAIL and the Bcl-2 family.

CONCLUSION

Initial hepatocyte CD and subsequent liver damage induced by deregulated immune reactions are the common denominator of many severe forms of acute and chronic liver pathologies. However, the exact sequence of certain events, like e.g., the initial hepatotoxic insult, involvement of infiltrating and resident immune cells, cytokines, and secondary immune reactions, still need further clarification. Especially in the case of most immune cell-derived factors it needs to be carefully addressed whether they are cause or consequence of on-going CD events. In this regard, the role of death ligands in disease progression is of special interest, since they may add an additional complexity to the picture. Particularly the role of TRAIL, but also other modulating factors of intrinsic apoptosis pathways in liver parenchymal and endothelial cells, have not been taken into account by most *in vitro* studies. They need to be carefully interpreted since they do not reflect the *in vivo* situation where e.g., TRAIL is readily present by infiltrating, and most importantly resident, immune cells to modify xenobiotic- and drug-induced hepatotoxicity. The identification of the relevant underlying mechanisms of DILI, either mediated by apoptosis or necrosis, may help to develop effective emergency treatments to prevent drug-induced liver failure.

AUTHOR CONTRIBUTIONS

All authors drafted the manuscript. JD generated the illustration. VS and TB wrote the manuscript.

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REVIEW
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Supramolecular Complexes in Cell Death and Inflammation and Their Regulation by Autophagy

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Signaling activation is a tightly regulated process involving myriad posttranslational modifications such as phosphorylation/dephosphorylation, ubiquitylation/deubiquitylation, proteolytical cleavage events as well as translocation of proteins to new compartments within the cell. In addition to each of these events potentially regulating individual proteins, the assembly of very large supramolecular complexes has emerged as a common theme in signal transduction and is now known to regulate many signaling events. This is particularly evident in pathways regulating both inflammation and cell death/survival. Regulation of the assembly and silencing of these complexes plays important roles in immune signaling and inflammation and the fate of cells to either die or survive. Here we will give a summary of some of the better studied supramolecular complexes involved in inflammation and cell death, particularly with a focus on diseases caused by their autoactivation and the role autophagy either plays or may be playing in their regulation.

Keywords: inflammation, cell death, death domain, RHIM, supramolecular complexes, innate immunity, autophagy, cargo receptors

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STRUCTURAL ELEMENTS FOR SUPRAMOLECULAR SIGNALING COMPLEXES

In order to assemble supramolecular signaling complexes in a tightly regulated fashion, certain protein–protein interaction domains and motifs have evolved. By using shared interaction mechanisms, these structures can assemble many subunits of differing function in a rapid and modular fashion to facilitate signal transduction. There are likely many other examples of proteins and domains that fall into this category, but in this review we have chosen to focus on the following structural elements due to their significant representation in the pathways regulating cell death and inflammation.

Death Domain Family

The death domain containing protein family is involved in numerous aspects of cell signaling and fate and contains several subfamilies including Caspase Activation and Recruitment Domain (CARD), Death Effector Domain (DED), Pyrin Domain (PYD), and the Death domain (DD) itself (Nanson et al., 2018). These domains share structural and sequence similarity but they show specificity with their interactions and tend to interact within each subfamily specifically, CARD–CARD or DD–DD interactions for example. Each member of the family mediates protein–protein interactions and seem to typically form Helical assemblies, some of which are fibrillar in nature such as ASC in inflammasomes (Lu et al., 2014; Li et al., 2018). Members of the Death Domain family interact through three distinct interaction types known as

Types I–III (**Figure 1A**). Depending on the arrangement and combination of the different interaction interfaces between DD family proteins, different structural arrangements can be generated (**Figure 1**). Clustering of death domain family proteins leads to recruitment of effector proteins including caspases but also ubiquitin ligases and deubiquitinases (DUBs), kinases and other regulatory proteins involved in signal transduction. Through the ordered clustering provided by death domain structures, proteins requiring oligomerization such as caspases are locally concentrated to stimulate interaction and subsequent activation. Interruption of these Death Domain family interactions blocks function suggesting that their assembly is required for activity (Park et al., 2007).

Receptor Homotypic Interaction Motif (RHIM)

Another structural element contained in supramolecular complexes discussed in this review and which has been shown to play a crucial role in inflammatory and cell death signaling is the Receptor Homotypic Interaction Motif (RHIM) (Sun et al., 2002). RHIMs are relatively short motifs characterized by a core sequence that has the property of folding into a highly stable amyloid structure (Pham et al., 2019) (**Figure 1B**). Proteins that include RHIM motifs interact with each other via these RHIM–RHIM interaction and include RIPK1, RIPK3, TRIF, DAI/ZBP1. Each of these may potentially interact with the other, however, it is not clear that all combinations are seen under normal situations in the cell (Pham et al., 2018). Mutation of RHIM motifs in RIPK3 for example is enough to abolish its activity suggesting that its role in polymerizing partners together is not separable from other functions it may have (Li et al., 2012). Large structures have been demonstrated for both RIPK1 and 3 as well as TRIF, which are dependent on RHIM interactions (Li et al., 2012; Gentle et al., 2017; Samie et al., 2018). In the case of TRIF, we have shown that these structures are fibrillar complexes that contain at least RIPK1 as well and probably also RIPK3 and can activate caspase-8 and other signaling outcomes of TRIF mediated signaling (Gentle et al., 2017). RHIM–RHIM interaction also provide important scaffolds for recognition of viral infection and subsequent cell death, such as is observed in influenza A virus infections triggering a DAI/ZBP1 and RIPK3 dependent cell death (Thapa et al., 2016). RHIM containing proteins are often recruited to complexes formed through Death Domain family interactions and RIPK1 indeed, has both a RHIM and Death Domain to promote this. How the architecture of such supramolecular complexes formed through RHIM and Death Domain scaffolds looks is still an unknown question, but what is clear is that loss of either of them can drastically alter signaling outcomes.

Ubiquitin

A common component and key player in the regulation of supramolecular signaling complexes is ubiquitin. In all complexes described in this review, polyubiquitin chains are attached to one or more of the subunits. A common signal activating function of ubiquitin chains in these complexes is to recruit kinase complexes IKK and TAB/TAK to activate NF-κB. However, the same K63 chains are also involved in the eventual silencing of the signaling complexes (see later). Other linkage specific chains are also present including K48 and Met1. Met1 which is added by the Linear UBiquitin chain Assembly Complex (LUBAC) is also important for NF-κB activation through recruitment of IKK complexes in a similar fashion to and in cooperation with K63 chains (Hrdinka and Gyrd-Hansen, 2017). K48 chains are typically associated with proteasomal turnover and are also important for regulation of signals such as from TNFR1 through proteasomal degradation of RIPK1 (Grice and Nathan, 2016; Annibaldi et al., 2018). Depending on the substrate and site of attachment, these ubiquitin chains may also regulate interactions with partner proteins and thus activity, for example recruitment of the NF-kB inducing kinases. The ubiquitin network within a signaling complex is a dynamic one, with the competing actions of ligases and deubiquitinases (DUBs) modifying the overall outcome of the signaling event, regardless of the receptor complex in question. This is a very complicated system and beyond the scope of this review to cover in any depth and has been reviewed thoroughly (Swatek and Komander, 2016). We will focus instead mostly on the role ubiquitin plays regulating recruitment to the autophagy machinery and activation of NF-κB.

SWITCHING OFF LARGE SIGNALLNG PLATFORMS

Assembly for supramolecular complexes such as those discussed throughout this review, presents a potential problem in terms of switching the signal off. Given the damaging outcome of overactivation of inflammatory or cell death promoting complexes, these structures need to be silenced before they can lead to cellular or tissue damage and disease. While this may in part be regulated by post translational modifications such as (de)ubiquitination, and (de)phosphorylation the likely stability and energetic requirement to break apart complexes such as RHIM or CARD mediated fibrils make this an unlikely mechanism to completely explain disassembly and inactivation of the fully assembled complexes. Degradation of subunits of these complexes by the proteasome may also play some role, however, the size of the complexes discussed in this review make it unlikely that proteasomal degradation of the assembled complexes occurs. Access to the proteasome typically requires unfolding of the substrate to allow it to fit within the catalytic barrel (Collins and Goldberg, 2017). Individually unfolding proteins assembled into supramolecular complexes, while perhaps possible, is likely an inefficient way to silence the activity of these complexes, however, is certainly a relevant regulatory mechanism for preventing their assembly through degrading members of the complexes before they can be recruited. Autophagy represents an existing mechanism capable of dealing with very large protein aggregates.

Autophagy is at its heart a cellular recycling mechanism to provide energy in times of nutrient stress, but has been adapted in multicellular organisms to also regulate multiple aspects of cellular biology. The canonical pathway also

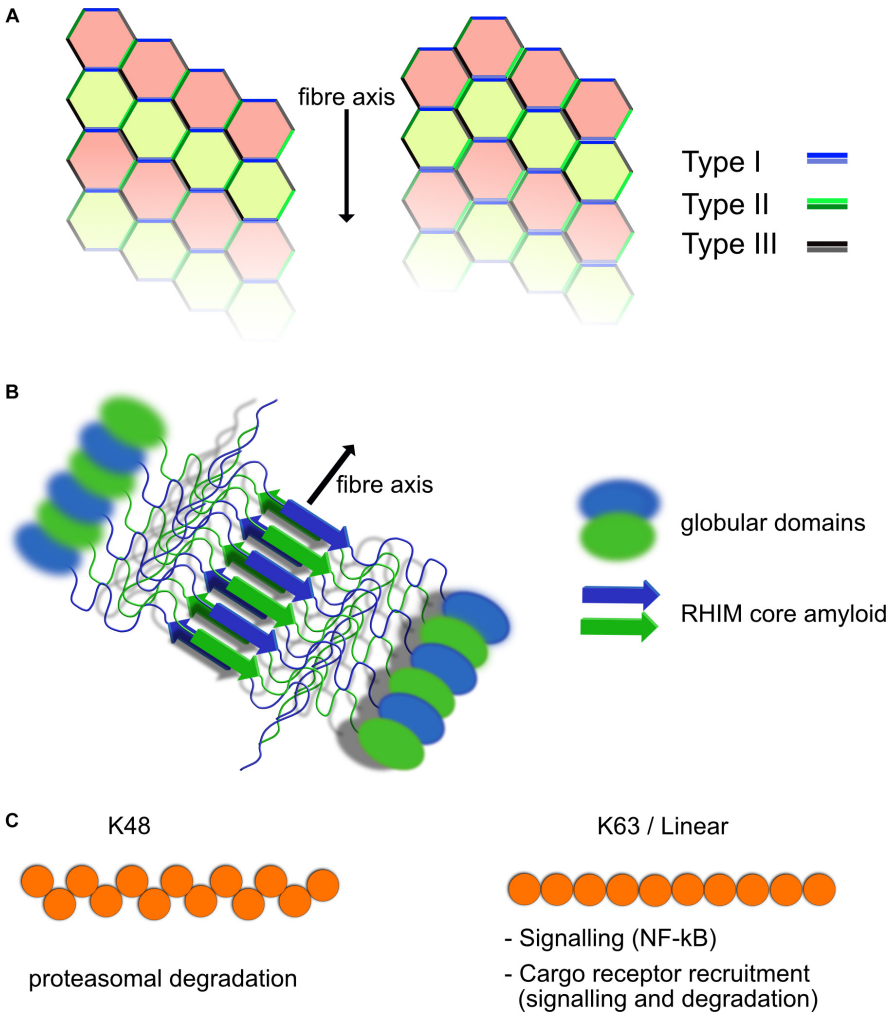

FIGURE 1 | Structural elements of death domain family, RHIM and ubiquitin scaffolds. **(A)** Death domain family interaction can form fibrillar structures. Death domain family members interact with themselves through three different interaction modes known as Types I–III, depending on the surfaces of the domain that interact. Shown are two possible helical fibrillar assemblies using differing combinations of interfaces. Multiple forms of this type of interaction have been identified, allowing for modular assembly of different complexes. **(B)** RHIM–RHIM scaffolds form amyloid fibrils. Shown is a fibril of two proteins containing a RHIM and globular domains. The fibril is formed by two parallel beta amyloid sheets coming together. This brings the globular domains into close proximity for interaction and potential activation such as kinase domains of RIPK1/3. RHIM fibrils can be mixed or homogeneous (RIPK1–RIPK3 or RIPK3–RIPK3 fibers form example). **(C)** Polyubiquitin chains have different functional roles. Shown are K48 and K63/linear ubiquitin chains. The structural layout of the individual chains is different resulting in recruitment of different ubiquitin binding proteins. K63 and linear ubiquitin chains are similar in their layout, although still functionally distinct. K48 chains are predominantly used for proteasomal degradation, whereas K63 and linear ubiquitin chains are used for recruitment of NF-κB activating complexes such as TAB/TAK and IKK as well as linking to autophagic cargo receptors among other functions.

known as Macroautophagy creates so-called autophagosomes, membranous vesicles, which engulf random parts of the cytosol and organelles (Zhao and Zhang, 2018) (**Figure 2A**). These autophagosomes fuse with lysosomes and degrade the contents which are then recycled for use in energy catabolism or for synthesis of new molecules. More specific forms of autophagy have evolved to degrade particular targets including mitochondria (mitophagy), intracellular bacteria and viruses (Xenophagy) and also aggregated proteins (aggrephagy) among others. In each of these targeted autophagic pathways, the target is ubiquitylated, typically by K63 ubiquitin chains (Sharma et al., 2018). These ubiquitylated targets are detected by so-called cargo receptors through binding to ubiquitin chains. This process is now known to be regulated by phosphorylation by TBK1, which will be discussed later in the review more specifically (**Figure 2A**). The receptors then recruit LC3, a component of the forming autophagophores causing engulfment of the cargo receptor bound target in autophagosomes (**Figure 2B**). The cargo containing autophagosomes can then fuse with lysosomes and degrade the contents (**Figure 2B**). Thus, autophagy is ideal for switching off signals from supramolecular signaling complexes. It is of note that many of the signaling pathways using supramolecular complexes such as toll like receptors (TLR) and TNF Receptor

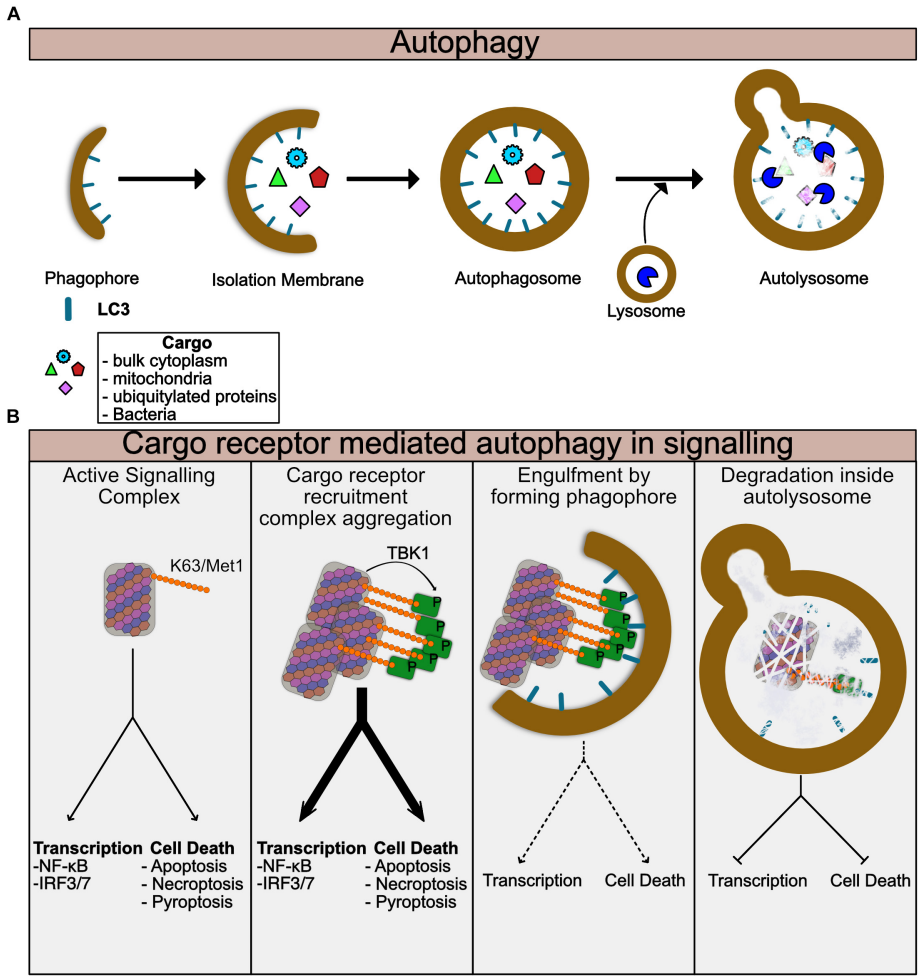

FIGURE 2 | Autophagy regulates turnover of Supramolecular complexes. **(A)** Summary of general autophagic process. The autophagic machinery including LC3 is recruited to the donour membrane and forms the phagophore. This then extends to form the isolation membrane which begins to engulf cytoplasmic or ubiquitylated contents. Eventually the enclosed autophagosome is formed which then can fuse with lysosomes to form the autolysosome. Lysosomal enzymes then degrade the contents of the autolysosome. **(B)** Summary of specific autophagy mediated by cargo receptors. Assembled supramolecular signaling complexes begin their signaling response. Cargo receptors such as p62 are recruited via K63 ubiquitin chains on the complex. TBK1 which has been activated and recruited to the complex phosphorylates the cargo receptor to enhance its recruitment. At this stage the signal from the complex may be amplified due to clustering of multiple complexes together or perhaps further enhancement of the localized proximity of kinases, caspases, and ubiquitin ligases for example. As the cargo:cargo-receptor complexes are engulfed by the forming autophagosome, signaling will be reduced. Finally, degradation of the complex within the autolysosome completes the cycle and the complex is destroyed.

family signals, also induce autophagy, thus stimulating the pathways involved in their silencing (Lee et al., 2018; Liu Y. et al., 2018). Examples of specific autophagy of signaling molecules are given throughout this review.

SUPRAMOLECULAR SIGNALING COMPLEXES IN CELL DEATH AND INFLAMMATION

TNF Family Receptors

Much of the best studied signaling complexes are in the TNF Super Family (TNFSFR), including TNFR1, FAS, TRAIL among others. Each of these receptors ultimately leads to activation of caspase-8 as well as activating transcriptional programs, particularly NF-κB. TNFSFR use death domain family interactions to recruit both scaffolding proteins such as TRADD and FADD, as well as effector proteins such as Caspase-8/10, and RIPK1, although many of the effector proteins also exhibit some scaffolding function, independent of their catalytic activities. Using TNFR1 as a well-studied example, TNFR1 recruits the adapter TRADD, which in turn recruits both RIPK1 and/or FADD. Recruitment of TRAF2/5 along with cIAP1/2 triggers k63 linked ubiquitylation of RIPK1 as well as other components. These ubiquitin chains recruit IKK as well as the TAB/TAK complexes and the linear ubiquitin chain assembly complex (LUBAC) which further adds linear ubiquitin

chains. The IKK and TAB/TAK complexes then both activate NF-κB as well as phosphorylate RIPK1 to prevent its activation (**Figure 3**). This results in upregulation of inflammatory cytokines and cell survival. Loss of RIPK1 ubiquitylation, and thus recruitment of the IKK and TAB/TAK complexes, results in cell death via apoptosis when the so-called complex-II containing RIPK1-FADD-caspase-8 separates from the receptor and activates cytosolic caspase-8 leading to apoptosis (**Figure 4**) (Vince et al., 2007; Dondelinger et al., 2013, 2015, 2017; Jaco et al., 2017; Menon et al., 2017; Annibaldi et al., 2018). This cytosolic amplification of complex-II formation is likely also mediated by supramolecular assembly of the complex-II through DD and RHIM interactions. Structural models have been developed for the assembly of FADD and Caspase-8 into fibrillar complexes in response to TRAIL ligand (Dickens et al., 2012) or FasL (Fu et al., 2016). While these models differ in their assembly, the principle remains that large elongated networks of Caspase-8 are assembled to trigger its activation through proximity (**Figure 4**). It seems likely that RIPK1 dependent Caspase-8 activation in the cytosol via Complex-II follows a similar mechanism. To date no auto-activating mutants of FADD or caspase-8 have been identified in disease, this is likely due to their propensity to trigger apoptosis, however, somatic loss of function mutants or repression of expression of FADD and caspase-8 are associated with numerous cancers, and seem to drive NF-κB (Teitz et al., 2000; Shin et al., 2002; Kim et al., 2003; Tourneur et al., 2004; Soung et al., 2005; Ando et al., 2013). This speaks to the role that supramolecular complex assembly has in regulating the complicated network of signaling molecules that are recruited and that loss of function of one, can lead to hyperactivation of another or vice versa.

In the case of TNF family receptors, one important aspect of their activity is their endocytosis. Endocytosis is required for apoptosis from TNFR1 for example (Micheau and Tschopp, 2003; Schütze et al., 2008). Endocytosed receptors are thought to be able to either traffic back to the plasma membrane or are

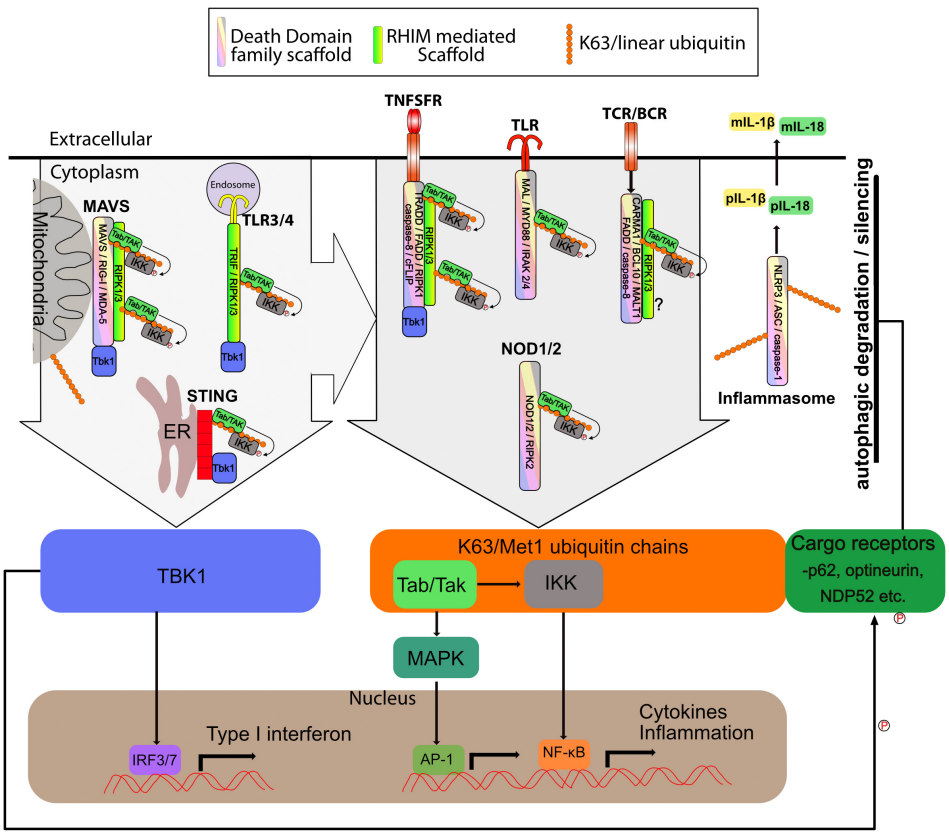

FIGURE 3 | Supramolecular signaling complexes in inflammation share common scaffolds and signaling pathways. The supramolecular signaling complexes formed by TLR, TNFSR, NOD1/2, STING, MAVS, Carma, BCL-10, MALT1 (CBM) and inflammasome complexes are indicated. Shown are the core scaffolds formed through death domain family interactions as well as RHIM interactions. STING contains no death domain family member or RHIM. Specific proteins known to interact through death domain interactions and RHIM interaction are indicated within each complex. Recruitment of TBK1 and/or K63/linear ubiquitin is indicated. TBK1 recruitment activates IRF3 to induce interferon responses. K63/linear ubiquitin recruits the TAB/TAK and IKK complexes which result in activation of NF-kB and Map kinase transcriptional responses. Also shown is linear/K63 ubiquitination of ASC of inflammasomes which can promote their assembly and activation or degradation. Complexes and organelles such as mitochondria that are k63 ubiquitylated recruit autophagy cargo receptors such as p62. This leads ultimately to degradation and silencing of the complexes. This is enhanced by TBK1 mediated phosphorylation of the cargo receptors. Other scaffolds such as those mediated via TRAF proteins are also present, but for simplicity have been omitted from the figure. DD family and RHIM scaffold are not meant to be to scale or reflect the actual organization of the scaffold, but simply indicate that each of these scaffolds are present.

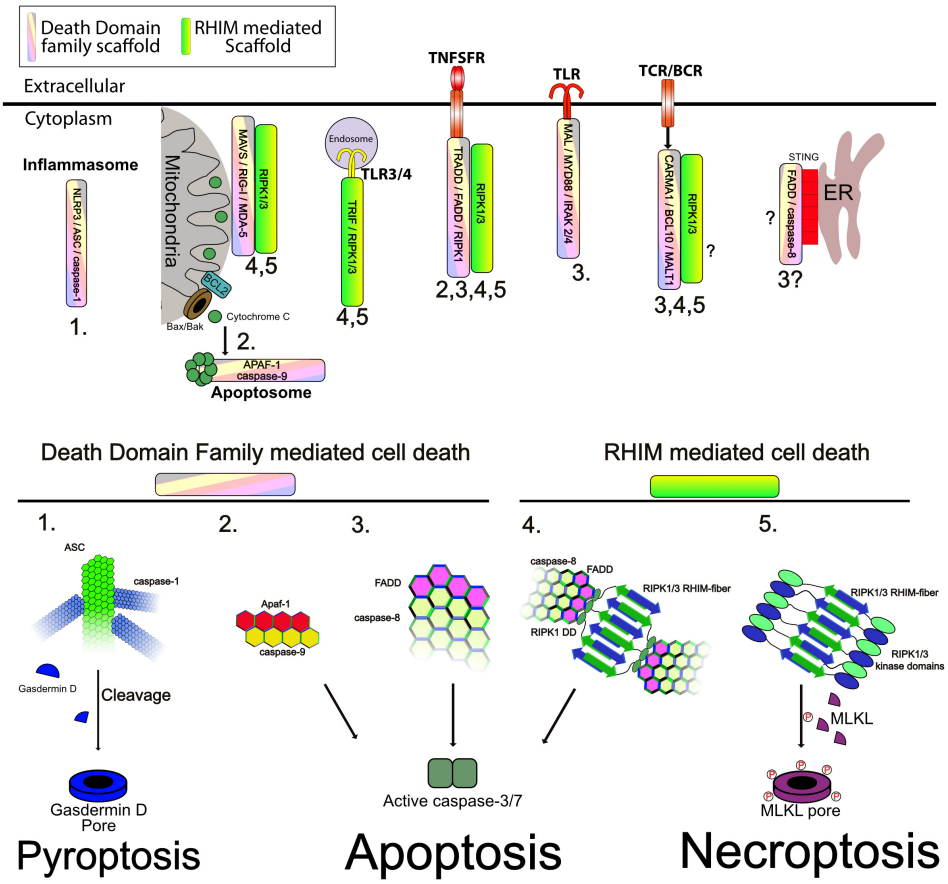

FIGURE 4 | Supramolecular signaling complexes can activate cell death. **(Upper)** The indicated complexes and the respective scaffolds recruited are indicated. Numbers correspond to the modes of cell death shown in the lower panel ad indicate the modes shown to be triggered by the respective complexes. **(Lower)** Different modes of cell death and the role of the respective scaffold in activating caspases, or RIP kinases is shown. (1) Inflammasomes assemble a core filament of ASC which in turn recruits filaments of caspase-1, to cluster it and trigger its activation. Active caspase-1 then cleaves Gasdermin D to allow the cleaved form to assemble into multimeric pores in the plasma membrane, causing pyroptosis. (2) Release of cytochrome C through Bax/Bak pores on the mitochondrial outer membrane triggers formation of the apoptosome. The apoptosome consists of a core of APAF1 and caspase-9 interacting through CARD–CARD interactions as indicated. This allows activation of caspase-9. Caspase-9 then cleaves and activates caspase-3/7 to trigger apoptosis. (3) Caspase-8 clustering onto FADD causes its autoactivation. Clustering is mediated through DED–DED interactions between FADD and caspase-8 and caspase-8 itself (not shown is cFLIP which can commingle with caspase-8 to regulate its activation). Active caspase-8 can cleave caspase-3/7 and trigger apoptosis. This scaffold can be activated by numerous receptors as indicated. (4) Recruitment and activation of RIPK1/3 through RHIM–RHIM mediated amyloid fibers can trigger FADD recruitment and clustering through the DD of RIPK1. Clustered FADD recruits caspase-8, probably causing similar aggregation as shown in mode 3. Active caspase-8 cleaves caspase-3/7 to trigger apoptosis. (5) The kinase domains of RIPK1 and RIPK3 are also brought into close proximity by RHIM fibers. Normally, the capase-8 indicated in mode 4 will inactivate these through cleavage of their kinase domains, however, when no caspase-8 activity is present, as is shown, RIPK3 is activated and phosphorylates MLKL. Phosphorylated MLKL assembles into pore complexes in the plasma membrane, causing necroptosis.

packaged into multivesicular bodies (MVB) that ultimately fuse with lysosomes and are degraded. In this regard, endocytosis and trafficking via vesicles acts as an in-built silencing mechanism for TNFSFR proteins. However, targeted autophagy was recently identified to regulate the levels of fn14 suggesting that autophagic degradation may also occur in other TNFSFR too (Winer et al., 2018). Supporting this, optineurin (a homolog of NEMO and a functioning autophagic cargo receptor) blocks TNF induced NF-κB by binding ubiquitylated RIPK1 (Zhu et al., 2007). While not shown in this study, this likely also results in degradation of RIPK1/3 containing complexes. More recently, optineurin, mutations of which are associated with amyotrophic lateral sclerosis (ALS), was shown to trigger degradation of RIPK1 and its loss lead to axonal degeneration (Ito et al., 2016). Optineurin loss also sensitized L929 cells to necroptosis induction supporting its role in targeting RIPK1 and RIPK3 for autophagic degradation to prevent their accumulation and activation (Ito et al., 2016). Optineurin loss has also been shown to sensitize to TNF induced caspase-8 activation (Nakazawa et al., 2016), supporting it as having a negative regulation of TNFR1 signaling.

Toll Like Receptors (TLRs)

Toll Like receptors, like TNFSFR, also signal through large complexes which may promote both inflammation and cell death. TLR's, use specific adapter proteins to recruit signaling complexes to the receptor. More specifically, TLRs that signal

through the adaptor TRIF/TICAM1 are able to recruit RIPK1 and through RIPK1 activate caspase-8 and or RIPK3 to trigger apoptosis or necroptosis (Kaiser and Offermann, 2005; Weber et al., 2010; Kaiser et al., 2013) (**Figures 3**, **4**) TRIF is recruited to both TLR3, where it is the sole adapter, and TLR4, which also uses MYD88 like all other TLR's (Hoebe et al., 2003; Oshiumi et al., 2003). Normally, TRIF mediated signaling results in assembly of K63 and linear ubiquitin chains that recruit the TAB/TAK and IKK complexes to trigger NF-κB (**Figure 3**) (Shim et al., 2005; Zinngrebe et al., 2016). TRIF also activates IRF3/7 mediated interferon responses via TBK1 activation (Fitzgerald et al., 2003; Hemmi et al., 2004) (**Figure 3**). In either case TRIF signaling complexes are restricted to endosomes (**Figures 3**, **4**). Myd88 dependent signaling occurs through formation of the myddosome (Motshwene et al., 2009). The myddosome is a helical complex consisting of Myd88, IRAK4, and IRAK1/2 stacked together through Death Domain interactions in a manner similar to that shown in **Figure 1A** (Lin et al., 2010). This platform recruits TRAF6, leading to IKK activation and NF-κB (Häcker et al., 2006) (**Figure 3**). While some evidence exists for apoptosis induced through MYD88 from TLR2 through recruitment of FADD and caspase-8, this seems to be the exception rather than the rule (**Figure 4**) (Aliprantis et al., 2000).

In either case, both MYDD88 and TRIF adapters have been shown to form supramolecular complexes which are required for several aspects of their signaling activity (Funami et al., 2007; Tatematsu et al., 2010; Guven-Maiorov et al., 2015; Gentle et al., 2017; Samie et al., 2018). Indeed Mutations in Myd88 that are associated with Lymphoma trigger auto-assembly of the TIR domain and the myddosome without receptor ligation (Avbelj et al., 2014). To date no auto-activating mutations of TRIF have been identified, possibly due to its capacity to trigger cell death when overactive. Mutations in FADD that are associated with cancers have also been shown to interfere with the association of FADD with MYD88, which may in turn promote myddosome assembly (Guven-Maiorov et al., 2015).

A number of studies have now associated regulation of TLR signaling via autophagy. Specifically Myd88 was reported to be recruited to p62 and Histone deacetylase 6 (HDAC6) positive structures upon TLR4 stimulation (Into et al., 2010; Fujita et al., 2011). p62 acts as a cargo receptor for selective autophagy and HDAC6 is thought to help traffic the ubiquitylated complexes to bring them together and promote fusion of the autophagosomes with lysosomes (Kawaguchi et al., 2003; Lee et al., 2010). Loss of p62 or HDAC6 resulted in enhanced JNK, p38, and ERK signaling in response to TLR ligands, but appeared to have little effect on NF-κB. While it was not specifically demonstrated in these studies, the enhanced signaling, albeit through specific pathways, is suggestive of the myddosome being targeted by autophagy, loss of which prevents the signals being dampened.

TRIF can form large fibrillar complexes that have been variably reported to interact with the autophagy cargo receptors p62, Tax1BP1, and NDP52 to target them for degradation (Inomata et al., 2012; Gentle et al., 2017; Yang et al., 2017; Samie et al., 2018). Our own study showed that if autophagy is inhibited, not only did the transcriptional activity of TRIF signaling become enhanced resulting in increased cytokine production, but also the cell death inducing activity, with enhanced caspase-8 activation being detected upon TLR3 stimulation of melanoma cells (Gentle et al., 2017). Similar results were shown in the other studies indicating enhanced IFN production in response to polyI:C or LPS when autophagy was inhibited (Samie et al., 2018). TRIF can also interact with the ubiquitin like protein ubiquitilin1 driving association with autophagosomes and degradation of TRIF (Biswas et al., 2011). Loss of ubiquitilin1 leads to excessive type I interferon responses from TLR4 and TLR3 (Biswas et al., 2011). Additionally TRIF can also induce autophagy as is the case with most of the complexes discussed here (Gentle et al., 2017).

As with TNFRSF receptors that become endocytosed, TLRs, in part at least, are associated with endosomes, an aspect which is essential for their activity (Petes et al., 2017). In addition to the autophagic regulation discussed above, a similar fate probably awaits activated TLRs that are present on endosomes such as TLR3 and TLR4 containing TRIF complexes (Wang et al., 2007). What ultimately happens to plasma membrane bound TLRs after assembly of myddosomes is unclear. They may also be trafficked via general endocytosis or they may, as is the case with TNFR1 complex II dissociate from the receptors themselves and then become targeted by autophagy separately.

Inflammasomes

Inflammasomes are another example of a death domain based supramolecular complex that has potent inflammatory signaling activity but can also trigger death of cells. There are a number of inflammasomes, however, the majority share a core structure, with a receptor subunit such as NLRP3 activating and recruiting Apoptosis-associated speck-like protein containing a CARD (ASC), which is able to then polymerize into a helical fibrillar structure that recruits Caspase-1 (Lu et al., 2014; Li et al., 2018). This brings caspase-1 into close proximity with other caspase-1 molecules, triggering auto-processing and activation of the zymogen into its functional form. Caspase-1 then cleaves the cytokines IL-1β and IL-18 to trigger further inflammation, but can also cleave substrates including Gasdermin D which then forms pores in the plasma membrane leading to a type of cell death called Pyroptosis (Martinon et al., 2002; He et al., 2015; Shi et al., 2015; Liu et al., 2016) (**Figures 3**, **4**). Pyroptotic death is additionally thought to release danger associated molecular patterns (DAMPS) that trigger inflammation in and of themselves, thus promoting an inflammatory environment (Frank and Vince, 2018). Auto-activating mutations in inflammasome components are known and cause diseases such as Familial Mediterranean Fever (FMF) and cryopyrin-associated periodic syndrome (CAPS) (Cordero et al., 2018). These mutations typically lead to auto-assembly of the inflammasome and lead to severe inflammatory pathologies due to the increased secretion of IL-1β and II-18 and pyroptosis (Cordero et al., 2018). Efficiently regulating the signal strength and length of signaling through inflammasomes is therefore essential. Autophagy plays a number of roles in regulating inflammasomes. Both the AIM2 and NLRP3 inflammasomes can be recruited to p62 and engulfed by autophagosomes and later associate with lysosomes for degradation (Shi et al., 2012). Blocking autophagy enhances

inflammasome activity and stimulating autophagy reduces it (Shi et al., 2012). Loss of autophagy is also associated with enhanced NLRP3 activation and IL-1β secretion (Saitoh et al., 2008), although the mechanism behind this activation is not yet clear. A number of studies have suggested that mitochondrial defects, possibly through insufficient mitophagy, may promote inflammasome activation through excessive ROS production or possibly release of ligands such as mtDNA (Ip et al., 2017). Stimuli that induce inflammasome activation also induce autophagy (Shi et al., 2012), supporting its role as a negative feedback system for these complexes.

STING

STING is activated by cyclic dinucleotides (cGAMP) produced by cGAS upon detection of cytoplasmic DNA (Ablasser et al., 2013). cGAMP binding causes STING to assemble into a large ER associated complex that leads to activation of NF-κB through IKK activation upon recruitment to K63 and linear polyubiquitin chains, but also to IRF3 through recruitment and activation of TBK1 (Abe and Barber, 2014) (**Figure 3**).

STING activation can also induce apoptosis in T cells and B-cell lymphomas although the mechanism is still unclear (Tang et al., 2016; Gulen et al., 2017; Larkin et al., 2017). Auto activating mutations of STING cause STING-associated vasculopathy with onset in infancy (SAVI), which among other problems results in T-cell cytopenia. This is independent of IRF-3 and could be due to cell death of developing or mature T-cells based on mouse knock-in models (Warner et al., 2017; Wu and Yan, 2018). Autoactivating mutations of STING trigger dimerization in the absence of ligand, allowing the signaling complex to form (Konno et al., 2018). FADD deficiency can block IFN activation in STING activated cells, suggesting that STING requires FADD and providing a possible mechanism for how STING may induce cell death through apoptosis (Ishikawa and Barber, 2008) (**Figure 4**). Additionally, STING can also cause necroptotic cell death indirectly through induction of TNF and interferon in macrophages and dendritic cells (Brault et al., 2018).

STING has also recently been identified as an autophagic substrate. Upon activation it binds to p62 and is targeted for degradation through autophagy (Liu D. et al., 2018; Prabakaran et al., 2018). STING itself can also act as an autophagy cargo receptor through an LIR motif to directly recruit LC3 and form autophagosomes around it after activation (Liu D. et al., 2018), although why it should need its own LIR when p62 is also recruited remains to be seen. Loss of p62 causes strongly enhanced IFN stimulated gene expression in response to cytosolic DNA indicating that STING activity is much higher. STING also induces autophagy in order to stimulate its own degradation (Liu D. et al., 2018) and the autophagy inducing kinase ULK1 also phosphorylates activated STING to negatively regulate its function (Konno et al., 2013). STING directly promotes the lipidation of LC3 at the ER to promote autophagosome production (Gui et al., 2019). This function of STING appears to have evolved before interferon activation as demonstrated by a lack of TBK1 and IRF activation by both Xenopus and sea anemone STING homologs, and is important for clearance of HSV-1 (Gui et al., 2019). Together, these studies support the picture of autophagy regulating signaling platforms and vice versa.

Mitochondrial Antiviral-Signaling Protein (MAVS)

Mitochondrial antiviral-signaling protein (MAVS) is a signaling scaffold for detection of viral RNA products. The receptors RIG-I and MDA5 bind to viral dsRNA molecules and then oligomerize (Berke et al., 2012; Jiang et al., 2012). Exposure of their CARD domains allows them to bind to the card domains of MAVS on the mitochondrial outer membrane whereby MAVS polymerizes into supramolecular fibrillar complexes to act as a scaffold for recruitment of IKK and TBK1 complexes for the activation of NF-κB and IRF3 (**Figure 3**) (Seth et al., 2005; Hou et al., 2011; Xu et al., 2014). Additionally FADD, RIPK1, RIPK3, and caspase-8 can be recruited to these complexes (Kawai et al., 2005; Downey et al., 2017). Infection with Semliki Forest virus induces apoptotic cell death through caspase-8 activation via the MAVS pathway highlighting that there can be a direct death inducing signal triggered by MAVS (**Figure 4**) (El Maadidi et al., 2014). Again, the MAVS complexes assemble through CARD–CARD interactions, and while no auto-activating mutations are known in humans for MAVS itself, gain of function mutations are found in both mda5 and RIG-I and are associated with type I interferonopathies such as Aicardi-Goutières syndrome (AGS) and Atypical Singleton-Merten syndrome (SMS) (Rice et al., 2014; Jang et al., 2015; Rutsch et al., 2015). These mutations can lead to enhanced fibril formation of the MAVS complex through an unknown mechanism that may be due to enhanced activation of the receptors themselves leading to excessive MAVS polymerization (Funabiki et al., 2014; Rice et al., 2014).

While MAVS appears to be an autophagy substrate it is perhaps a somewhat special case in this regard due to its location on the mitochondria. MAVS is reported to contain an LIR motif for direct recruitment of LC3 and MAVS activation and assembly into its large fibrillar state can stimulate mitophagy (Sun et al., 2016), thus removing MAVS along with the mitochondrial it is associated with. Additionally, MAVS can be degraded by autophagy through an NDP52 dependent mechanism regulated by the interferon response gene tetherin and loss of NDP52 gives an enhanced interferon response, albeit minor (Jin et al., 2017). The relatively minor effect that loss of NDP52 has on MAVS signaling, may be due to MAVS acting as its own cargo receptor and inducing autophagy/mitophagy. Clearly, however, MAVS adheres to the pattern shown for the other complexes so far discussed in using autophagy as a silencing mechanism.

NOD2

NOD2 is another CARD containing PRR that recognizes bacterial muramyl dipeptide (MDP) (Girardin et al., 2003). Through its CARD domain NOD2 recruits RIPK2, which is then ubiquitylated by XIAP resulting in the recruitment of the TAB/TAK and IKK complexes and NF-κB activation through addition of linear ubiquitin chains via LUBAC (**Figure 3**) (Damgaard et al., 2012, 2013). Recently, RIPK2 was shown to also form filaments through CARD–CARD interactions

and filament formation and scaffolding function is required for NOD2 activity (Hrdinka et al., 2018; Pellegrini et al., 2018). Interestingly, crosstalk between the NOD2 and MAVS pathway has been demonstrated during viral infection. ssRNA is recognized by NOD2 and it then interacts with MAVS triggering IRF3 activation through TBK1, although surprisingly, not through CARD–CARD interactions (Sabbah et al., 2009). Mutations in NOD2 are associated predominantly with Crohn's Disease and result in a failure to induce NF-κB in response to ligand (Hampe et al., 2001; Hugot et al., 2001; Ogura et al., 2001; Bonen et al., 2003). However, there is some question as to whether this is the causative defect in Crohn's Disease (Eckmann and Karin, 2005). Another disease thought to be caused by autoactivating mutations of NOD2 is Blau syndrome. Patients with Blau syndrome exhibit granulomatous dermatitis, arthritis, and uveitis (Kanazawa et al., 2005; Parkhouse et al., 2014) Although, again the autoactivation of NOD2 driving disease has also been questioned (Dugan et al., 2015). Given the fibril forming nature of NOD2-RIPK2 complexes, it seems likely that hyperactivation could result from aberrant assembly of RIPK2 fibrils, however, this has not been demonstrated yet.

NOD2 has a complex interaction with the autophagy machinery. As is the case with most of the complexes discussed here, NOD2 can induce autophagy. It is thought that this occurs through a RIPK2 kinase dependent mechanism (Homer et al., 2012). Additionally, NOD2 is also involved in the recruitment of autophagic machinery to sites of invading bacteria at the plasma membrane through interaction with Atg16. This targets the bacteria for degradation via xenophagy (Cooney et al., 2010; Homer et al., 2010, 2012; Travassos et al., 2010; Anand et al., 2011; Negroni et al., 2016). To date no degradation of NOD2 or its complexes have been shown via autophagy. NOD2 is thought to be a proteasomal substrate under conditions where HSP90 is blocked or its access to NOD2 is restricted (Normand et al., 2018). RIPK2, however, has been identified as interacting with p62, although autophagic degradation was not shown in this study (Park et al., 2013). The NOD2-RIPK2 fibril may therefore also be a target of autophagic degradation given this association and may play a role in dampening NOD driven inflammation.

CBM Signalosome

In a similar manner to inflammasomes, the CARMA-BCL10-MALT1 (CBM) signaling complex consists of a core of BCL10 that recruits the paracaspase MALT-1. Different CARD containing adapters can recruit BCL10 to trigger its assembly including CARD9, -10, -11, and CARD14 (Gehring et al., 2018; Juilland and Thome, 2018). CARD11/CARMA1 is one of the best studied to date and is activated by TCR and BCR signaling. CARMA1 recruits BCL10 through CARD–CARD interactions, whereupon BCL10 forms filamentous helical structures in a manner similar to what has been described here for other CARD mediated structures such as inflammasomes (Qiao et al., 2013; David et al., 2018; Schlauderer et al., 2018). These filaments assemble in a star like formation radiating out from CARMA1 nucleation points (David et al., 2018). MALT1 is a paracaspase which requires recruitment to BCL10 for activation. TRAF6 is also recruited via interaction with MALT1 and NF-κB is subsequently activated through recruitment of IKK complexes to ubiquitin chains (**Figure 3**) (Sun et al., 2004). Recently it was shown that LUBAC is also required for NF-κB activation, although this may be due to a scaffolding role as catalytically inactive HOIP could also restore NF-κB signals in HOIP deficient cells (Dubois et al., 2014). FADD and caspase-8 are also recruited to the CBM complex and caspase-8 catalytic activity is required for effective activation of NF-κB by the CBM (Su et al., 2005). As loss of caspase-8 activity is a known trigger for necroptosis in stimulated T cells, these data also suggest that recruitment of FADD and caspase-8 are accompanied by RIPK1 and that this could be the route for activation of necroptosis in the absence of caspase-8 in stimulated lymphocytes (**Figure 4**).

Interestingly, mutations in CARD11 (Carma1) are causative of B cell expansion with NF-κB and T cell anergy (BENTA), an immunodeficiency caused by overactivation of the CBM complex. These mutations cause CARD11 to aggregate and recruit BCL10 and MALT1 into large complexes (Snow et al., 2012; Brohl et al., 2015). They are also often associated with diffuse large B cell lymphoma and other lymphomas (Lenz et al., 2008; Chan et al., 2013). CARMA3 has also been identified as a negative regulator of MAVS oligomerization and IRF3 activation (Jiang et al., 2016). This highlights again, that supramolecular signaling complexes can be prone to autoactivating mutations due to their ability to rapidly assemble into ordered signaling hubs, and the complexity with which they share common interactions and regulation.

As with the other supramolecular complexes discussed so far, a link with autophagy has been shown with CBM signalosomes too. Degradation of BCL10 in response to TCR stimulation occurs through a proteasome independent lysosomal dependent mechanism (Scharschmidt et al., 2004), suggesting autophagy as a likely route. As discussed later, the CBM complex also associates with p62 during TCR signaling in order to regulate the intensity of the signal (Paul et al., 2012).

MITOCHONDRIA

Mitochondria act not only as the metabolic engine of the cell, but also as a key signaling hub filtering signals for cellular growth and energetics, innate immune and inflammatory signals, and also decisions to survive or die. The classical intrinsic mitochondrial apoptotic machinery comprised of Bax and Bak mediated pores regulated by BCL2 family pro and anti-apoptotic proteins is well characterized, if not completely understood (Edlich, 2018). As with the death domain family, the BCL2 family of proteins share common domains that they use for their interaction, namely the BCL2 Homology (BH) domain. Mechanistically, Bax and Bak form pores in the outer membrane of mitochondria upon an apoptotic stimulus. Contents of the mitochondrial intermembrane space then leak out, including cytochrome C and SMAC/DIABLO. Cytochrome C then binds APAF1 triggering the formation of the apoptosome thus activating caspase-9. Caspase-9 in turn activates effector caspases such as caspase-3 which carry out the end points of apoptosis (**Figure 4**). The BCL2 family proteins also form

large complexes which are quite dynamic in their composition and function depending on the state of the cell, and may be antiapoptotic or proapoptotic [reviewed in Westphal et al. (2014), Cosentino and García-Sáez (2017), and Kale et al. (2018)]. As such they can be considered a kind of dynamic supramolecular complexes themselves that also use common domains (BH) to interact. In another link between mitochondria and inflammatory signaling, release of mitochondrial DNA (mtDNA) after Bak/Bak mediated permeabilization can also activate STING to trigger inflammation (McArthur et al., 2018; Riley et al., 2018).

The central role mitochondria play in regulating so many aspects of cellular homeostasis and immunity mean that they can in a sense be considered as a supramolecular signaling hub themselves. Given their importance it is not surprising then, that they are also heavily regulated and prone to quality control. Mitophagy is a specialized form of autophagy that causes the degradation of damaged mitochondria (Narendra et al., 2014). Mitophagy is analogous to aggrephagy in that targets on the mitochondria are ubiquitylated and cargo receptors such as p62 are recruited to the mitochondria followed by engulfment in autophagosomes and subsequent degradation in lysosomes (**Figures 2**, **3**) (Geisler et al., 2010). Recent work by a number of labs has shown that mitophagy plays a role in regulating aspects of apoptosis and also associated inflammation. A recent study has shown the activation of Bax and Bak on mitochondria is associated with induction of autophagy, and that subsequently, apoptotic mitochondria are engulfed and degraded through autophagy (Lindqvist et al., 2017). Blockade of autophagy lead to enhanced production of interferon β (Lindqvist et al., 2017). Release of other mitochondrial contents in to the cytosol after outer membrane permeabilization can also be a stimulatory event, inducing cytokine production and DNA damage (Ichim et al., 2015; McArthur et al., 2018; Riley et al., 2018). BCL2 family of proteins integration into the outer membranes of mitochondria likely makes them a difficult target for direct action by specific degradation through autophagy, however, evidence exists for a role of Parkin (E3 ubiquitin ligase responsible for ubiquitylating damaged mitochondria during mitophagy) in regulating BCL2 complexes as well as mitophagy. Parkin can ubiquitylate MCL-1 leading to its degradation and cell death in response to mitochondrial damaging agents such as valinomycin (Zhang et al., 2014). Less damaging treatments such as cccp are reported to induce mitophagy instead (Zhang et al., 2014). Loss of mitophagy may contribute to inflammation in a number of ways including accumulation of ROS, release of mtDNA from damaged mitochondria as well as persistence of signaling platforms such as MAVS that are localized on the mitochondrial membrane.

TBK1 as a Central Regulator

An interesting connection between the activation of these receptor signaling complexes and their degradation by autophagy is the activation of the Tank Binding Kinase 1 (TBK1). TBK1 is required for IRF activation from many of the receptors complexes described in this review including TLR3/4 via TRIF, STING and MAVS. TBK1 is also a well characterized activator of autophagic cargo receptors including p62, optineurin and NDP52 (Pilli et al., 2012; Korac et al., 2013; Matsumoto et al., 2015; Yang et al., 2016; Oakes et al., 2017; Cho et al., 2018). TBK1 phosphorylates these receptors, enhancing their recruitment of the autophagic machinery and therefore promoting degradation of the target complexes (**Figure 2**). This was shown directly for STING, but also for mitochondria and bacteria. Recently TBK1 was also identified as being recruited to TNFR1 where it does not activate IRF3, but instead played an essential role in regulating survival (**Figure 3**). Loss of TBK1 expression leads to RIPK1 dependent apoptosis and reduced levels can replicate ALS in mice (Lafont et al., 2018; Xu et al., 2018). This was shown to be dependent on TBK1 mediated phosphorylation of RIPK1. Given the recent observations about TNFR1 and TBK1 mediated survival, it is possible that TBK1 activation may also promote the degradation of RIPK1 complexes in reducing the amount of activated complex-II in response to TNF, however, this remains to be shown. Of note is that TBK1 is also a critical component of enhanced autophagy and NF-κB activation in K-Ras-dependent non-small cell lung carcinoma (NSCLC) (Newman et al., 2012). The role of TBK1 in activating autophagy receptors and its recruitment to and activation by supramolecular complexes described here is indicative of the importance of this pathway in dampening inflammatory and cell death signals.

AUTOPHAGY RECEPTORS ACTING AS SIGNALING SCAFFOLDS TOO

In another twist to the role of autophagy in regulating supramolecular signaling complexes, some cargo receptors such as p62 can promote signaling and assembly of the complexes prior to their degradation (**Figure 2B**). This has been reported for caspase-8 activation upon TRAIL stimulation (Jin et al., 2009), whereby p62 promoted the aggregation of caspase-8 in a cullin-3 dependent fashion, thus suggesting that aggregation by p62 enhanced the signal triggering apoptosis, but at the same time lead to the ultimate degradation of the complex and its silencing.

Recently a role for HDAC6 has been shown in clearance of *Listeria monocytogenes* (Moreno-Gonzalo et al., 2017). In this context loss of HDAC6 in DCs resulted in enhanced bacterial load, due to defects in autophagy, and also strongly reduced activation of NF-κB and MAPK pathways. It was proposed that by interacting with MYD88 that HDAC6 promoted its aggregation and activation of downstream signaling pathways. While no specific mechanism for this is given, HDAC6 may promote association of MYD88 with autophagy receptors such as p62 to enhance its activity before being degraded. Additionally loss of p62 was shown to reduce cytokine production, NF-κB and ERK activation in response to TLR2 and TLR6 activation in keratinocytes (Lee et al., 2011). In a similar fashion to the above examples, p62 promoted NF-κB activation prior to degradation of BCL10 in TCR signaling (Paul et al., 2012, 2014), however, BCl10 degradation ultimately silences NF-κB activation (Scharschmidt et al., 2004). NOD2 also shows reduced NF-κB activation in response to ligand in the absence of p62 (Park et al., 2013). Of note is that is required for TRAF6 dependent ubiquitylation of NEMO/IKKγ, and loss of p62 blocks IL-1β induced NF-κB

substantially (Zotti et al., 2014). Additionally p62 is required for RAS induced NF-κB in cancer through TRAF6 ubiquitylation and IKK activation (Durán et al., 2008). Together these data support the idea that these large signaling complexes that become ubiquitylated also use this aggregation phase to enhance signaling prior to silencing. While p62 is by far the most studied of the autophagy cargo receptors, it is likely that there is some redundancy and that the other cargo receptors also exhibit signal amplifying activities prior to their degradation. Thinking of these adapters as cargo receptors may actually be too simplistic for their role in signal regulation, and instead perhaps they should be thought of more as generalized modulators or scaffolds for tuning signal strength and duration.

Loss of Autophagy in Various Diseases Associated With Inflammation and Cell Death

A number of diseases are associated with deficiencies in autophagy, many of which are inflammatory in nature and in a number of cases show direct links to proteins from supramolecular signaling complexes involved in cell death and inflammatory signaling. Gaucher's Disease is a lipid storage disease caused by mutations in glucocerebrosidase that results in accumulation of the sphingolipid glucocerebroside in lysosomes, effectively blocking their function. Thus, as a byproduct, the autophagy pathway is also backed-up and blocked by a failure to degrade targets in the lysosome (Settembre et al., 2008). Gaucher's disease is associated with a strong hyperinflammation and splenomegaly and interestingly, in mouse models, it was shown that it could be largely blocked by loss of RIPK3, suggesting a potential role for RIPK3 mediated cell death as a possible driver of the disease (Vitner et al., 2014). While it has yet to be shown, it is intriguing to speculate that active RIPK3, assembled into fibrillar complexes through the RHIM domain do not get degraded, and then promote either cell death or inflammation directly. Indeed increased RIPK3 levels are seen in Gaucher's patients (Vitner et al., 2014).

Niemann Pick disease is another lysosomal disease that is associated with inflammatory pathology, particularly Crohn's disease like symptoms. Niemann Pick diseases are caused by failure to metabolize Sphingomyelin for various reasons, leading to lysosomal disfunction (Guo et al., 2016). While it has not directly been shown that Niemann Pick is regulated by RIPK3 in a similar fashion to Gaucher's disease, the possibility remains. As mentioned, a particular pathology associated with Niemann-Pick is the development of Crohn's Disease like pathology. This was reported to be associated with decreased xenophagy in a manner similar to loss of function of two other well-known Crohn's Disease associated proteins, Nod2 and XIAP (Schwerd et al., 2017), both of which also positively regulate autophagy (Homer et al., 2010; Gradzka et al., 2018). Mutations in NOD2 lead to loss of NF-κB activation, as do many of the mutations in XIAP that are associated with disease, suggesting that failure to activate this pathway is the initial cause of the pathology, however, recent studies have shown that NOD2 and XIAP both also have roles in targeting invasive bacteria for xenophagy (Homer et al., 2010; Gradzka et al., 2018). How this may be coordinated is not clear, but the association of other autophagy related mutations such as the Atg16L1, NDP52, Optineurin, and others CD suggest that specific defects in the autophagy process may be a key trigger as well. At least part of the inflammation seen may be associated with a concomitant failure to silence assembled signaling complexes within the usual time frame. Additionally, there are a number of neuronal diseases associated with defects in autophagy that may also have an inflammatory element. These include Alzheimer's disease and Parkinson's disease to give just two prominent examples. Again, a role for degradation of signaling complexes may also play a significant role in the inflammation and cell death seen in these diseases.

CONCLUSION

Formation of large supramolecular complexes as signaling hubs is emerging as a unifying theme in signaling and it is likely that many more receptor complexes will demonstrate this capacity. The fact that so many receptors all share common structural domains and signaling targets supports this and highlights the crosstalk that many of these receptors have in regulating the strength, and severity of signals that are produced. The propensity of these complexes to assemble is also highlighted by the numerous, although rare, genetic immune diseases associated with auto-activation of components of the complexes discussed here. The common theme of induction of autophagy, recruitment to cargo receptors to amplify signals prior to their degradation provides a tightly regulatable circuit for negative regulation of these important signaling complexes and suggest that autophagy induction may provide a useful target in helping to prevent some of these diseases at their source.

AUTHOR CONTRIBUTIONS

IG conceived of and wrote the manuscript.

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REVIEW
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TNFR1 and TNFR2 in the Control of the Life and Death Balance of Macrophages

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Macrophages stand in the first line of defense against a variety of pathogens but are also involved in the maintenance of tissue homeostasis. To fulfill their functions macrophages sense a broad range of pathogen- and damage-associated molecular patterns (PAMPs/DAMPs) by plasma membrane and intracellular pattern recognition receptors (PRRs). Intriguingly, the overwhelming majority of PPRs trigger the production of the pleiotropic cytokine tumor necrosis factor-alpha (TNF). TNF affects almost any type of cell including macrophages themselves. TNF promotes the inflammatory activity of macrophages but also controls macrophage survival and death. TNF exerts its activities by stimulation of two different types of receptors, TNF receptor-1 (TNFR1) and TNFR2, which are both expressed by macrophages. The two TNF receptor types trigger distinct and common signaling pathways that can work in an interconnected manner. Based on a brief general description of major TNF receptor-associated signaling pathways, we focus in this review on research of recent years that revealed insights into the molecular mechanisms how the TNFR1-TNFR2 signaling network controls the life and death balance of macrophages. In particular, we discuss how the TNFR1-TNFR2 signaling network is integrated into PRR signaling.

Keywords: apoptosis, necroptosis, TNF, TNFR1, TNFR2, ripk1, ripk3, caspase-8

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INTRODUCTION

Tumor necrosis factor-alpha (TNF) is a highly pleiotropic cytokine that affects practically any type of cell. It triggers cellular responses reaching from the induction of inflammatory gene expression programs, over the stimulation of cellular proliferation and differentiation to the activation of cellular suicide programs such as apoptosis and necroptosis (Wajant et al., 2003; Wajant and Scheurich, 2011; Brenner et al., 2015).

Tumor necrosis factor-alpha is the name giving and prototypic ligand of the TNF superfamily (TNFSF). It is expressed (i) as a type II single spanning transmembrane protein and (ii) as a soluble variant which is released from the transmembrane form by proteolytic processing in the stalk region which separates the characteristic TNF homology domain (THD) from the transmembrane and the intracellular domain (Locksley et al., 2001; Bodmer et al., 2002; **Figure 1**). Since the THD mediates self-assembly into trimeric molecules and receptor binding, both the transmembrane and soluble form of TNF interact with the two know receptors of TNF, TNF receptor 1 (TNFR1), and TNFR2 (**Figure 1**). Both receptors of TNF are typical representatives of the TNF receptor

superfamily (TNFRSF). As such, TNFR1 and TNFR2 are single-spanning type I transmembrane proteins characterized by having several cysteine-rich domains (CRDs) in their extracellular domain (Locksley et al., 2001; Bodmer et al., 2002). Soluble forms of TNFR1 and TNFR2 have also been described and result from alternative splicing or shedding (Philippe et al., 1993; Taylor, 1994; Galve-de Rochemonteix et al., 1996; Lainez et al., 2004; Gregory et al., 2012). The soluble TNF receptor variants inhibit TNF by competing with the cellular receptor species for TNF binding but possibly also by acting as dominant-negative molecules. Indeed, the N-terminal CRDs of TNFR1 and TNFR2 are not directly involved in ligand binding but mediate inactive self-association in the absence of ligand (Chan et al., 2000). This part of the TNF receptors has therefore been named pre-ligand binding assembly domain (PLAD) and seems to be a prerequisite for ligand binding and subsequent formation of active receptor complexes (Chan et al., 2000). Thus, soluble TNF receptor molecules might also act as TNF inhibitors by formation of inactive complexes with cellular TNF receptors by PLAD-PLAD interaction, but this issue has not been clarified yet.

TNFR1 and TNFR2 belong to different subgroups of the TNFRSF. TNFR1 is a death receptor (DR) and harbors a death domain (DD) in its cytoplasmic part (Tartaglia et al., 1993). The DD is a conserved type of protein-protein interaction domain which enables DRs to interact homotypically with cytoplasmic proteins also harboring a DD (Park et al., 2007). DD-containing signaling proteins link TNFR1 to cytotoxic signaling pathways triggering apoptosis or necroptosis but also allow engagement of signaling pathways that activate transcription factors of the nuclear factor of kappa B (NFκB) family or kinases of the MAP kinase family (Wajant et al., 2003; Wajant and Scheurich, 2011; Brenner et al., 2015). There exist several mechanisms, described below in more detail, that suppress cytotoxic signaling by TNFR1 so that proinflammtory, gene inductive signaling can be considered as the default mode of TNFR1 activity. TNFR2 has no DD and is a prototypic TNF receptor associated factor (TRAF)-interacting TNFRSF receptor (Xie, 2013). Thus, there is a short amino acid motif near the C-terminus of TNFR2 which enables recruitment of the adapter protein TRAF2 and TRAF2-associated proteins such as TRAF1 and cellular inhibitor of apoptosis protein 1 (cIAP1) and cIAP2 (Xie, 2013). TNFR2 has therefore no intrinsic cell death inducing activity but stimulates NFκB signaling and activation of various kinases (Wajant et al., 2003). The transmembrane and soluble form of TNF bind with high affinity to the two TNF receptor types and crystallographic data revealed a similar structural mode of ligand binding by TNFR1 and TNFR2 (Banner et al., 1993; Mukai et al., 2010). Nevertheless, there is a striking difference in the TNF receptor-stimulating activity of the two TNF forms. While transmembrane TNF activates TNFR1 and TNFR2 signaling with high efficacy, binding of soluble TNF results only in the case of TNFR1 in strong and general receptor activation (Wajant et al., 2003; **Figure 1**). TNFR1 is expressed by almost any cell type. TNFR2 expression, however, is rather restricted to certain cell types, including myeloid cells, regulatory T-cells, glial cells and some endothelial cell types, but can also be induced in epithelial cells, fibroblasts and certain T- and B-cell subsets (Medler and Wajant, 2019). TNFR2 is furthermore frequently expressed on hematopoietic malignancies and some solid tumors. TNF is not constitutively expressed and is instead readily induced in activated immune cells but it is also expressed by fibroblasts and endothelial and epithelia cells in response to proinflammatory triggers and cytokines including TNF itself (Pauli, 1994; Medler and Wajant, 2019).

TNFR1-RELATED SIGNALING PATHWAYS

After binding of soluble or membrane-bound TNF, the DD-containing cytoplasmic proteins TNFR1-associated death domain (TRADD) and receptor interacting protein kinase-1 (RIPK1) recruit to TNFR1 due to DD–DD interactions (**Figure 2**). Deficiency or knock-down of RIPK1 enhance recruitment of TRADD and TRAF2 to TNFR1 (Devin et al., 2001; Jin and El-Deiry, 2006; Zheng et al., 2006; Fullsack et al., 2019). Deficiency or knock-down of TRADD, in contrast, consistently reduced TRAF2 recruitment in various studies and showed varying effects on RIPK1 recruitment (Jin and El-Deiry, 2006; Zheng et al., 2006; Ermolaeva et al., 2008; Pobezinskaya et al., 2008; Fullsack et al., 2019). Since TRAF2 interacts furthermore with high affinity with TRADD outside its DD (Park et al., 2000), these findings suggest that TNFR1-bound TRADD, and to a lesser extent TNFR1-bound RIPK1, recruit TRAF2 homotrimers (or TRAF1-TRAF2 heterotrimers) into the TNFR1 signaling complex. With TRAF2 the E3 ligases cIAP1 and cIAP2, which already form complexes with TRAF2 homotrimers (or TRAF1-TRAF2 heterotrimers) in the cytoplasm, become co-recruited to the TNFR1 signaling complex (Wajant and Scheurich, 2011). The cIAPs modify various components of the TNFR1 signaling complex, in particular RIPK1, with K63-linked ubiquitin chains and create so binding sites for the E3 ligase linear ubiquitin chain assembly complex (LUBAC). The LUBAC then further modifies RIPK1 with linearly linked ubiquitin chains which allow the recruitment of the MAP3K transforming growth factor-β (TGFβ)–activated kinase-1 (TAK1) via the adapter protein TAK1-binding protein-2 (TAB2) and of the inhibitor of kappa B kinases (IKK) complex (Wajant and Scheurich, 2011; Brenner et al., 2015). TAK1 can now phosphorylate and activate the IKK2 subunit of the IKK complex and triggers this way the events of the classical NFκB pathway including phosphorylation and degradation of inhibitor of kappa B-alpha (IκBα), release and nuclear translocation of previously IkBα-inhibited NFκB dimers and transcription of various NFκB-regulated targets. The latter include IκBα itself but also other factors that in feedback loops can modulate TNFR1 signaling, e.g., the FADD-like ICE-inhibitory proteins (FLIPs), cIAP2, A20 and TRAF1 (Wajant et al., 2003). The described chain of events emerge from the plasma membrane located TNFR1 signaling complex (also named complex I) within seconds to very few minutes and allows production of functional relevant amounts of NFκB-regulated proteins in less than 1 h (Wajant and Scheurich, 2011; Brenner et al., 2015).

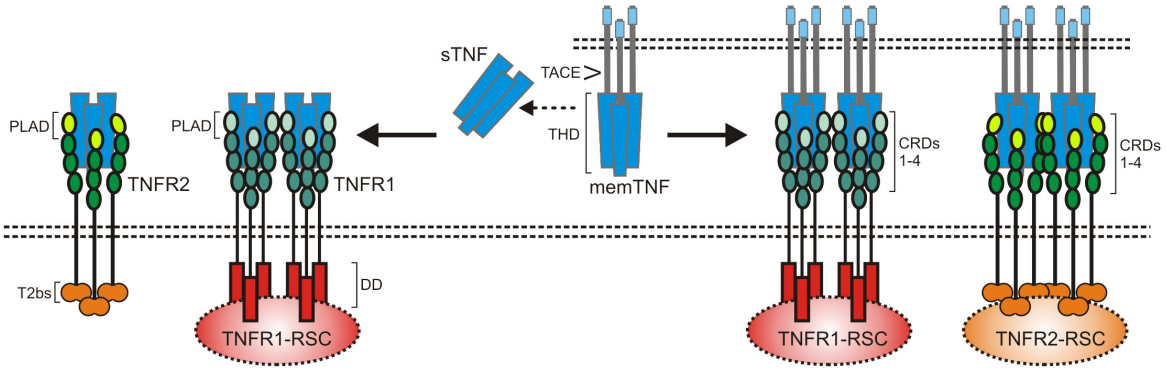

FIGURE 1 | The TNF-TNFR1-TNFR2 system. As other receptors of the TNFRSF, TNFR1 and TNFR2 are characterized by cysteine-rich domains (CRD) in their extracellular part. TNFR1 harbors furthermore a death domain (DD) and TNFR2 a TRAF2 binding site (T2bs). TNF occurs in two forms, as a membrane-bound trimeric ligand (memTNF) and as a soluble likewise trimeric molecule (sTNF). TACE processes memTNF to sTNF. Please note, memTNF stimulates both TNF receptors while sTNF largely fails to stimulate TNFR2 despite high-affinity binding. PLAD, pre-ligand binding assembly domain.

With time the TNFR1 signaling complex starts to internalize and this comes along with the release of the TNFR1-bound signaling molecules (Fritsch et al., 2017). In the cytoplasm the latter can form receptor-free cytoplasmic complexes (also named complex IIa and IIb) with FADD, caspase-8 and RIPK3 which context-dependent enable induction of apoptosis or necroptosis (Brenner et al., 2015). Since TRADD, RIPK1, TRAF2 and the cIAPs are also part of the initially formed TNFR1-associated signaling complex, it is tempting to speculate that these molecules as a whole can dissociate from TNFR1 to recruit then FADD/caspase-8 and to act as a "condensation nucleus" to recruit RIPK3 and additional RIPK1 and RIPK3 molecules (Li et al., 2012). Noteworthy, there is evidence that TNFR1-induced activation of p38 and its downstream target MAPK kinase-activated kinase-2 (MK2) results in MK2-mediated phosphorylation of RIPK1 on serine 320 (human)/321(mouse) of TNFR1-associated RIPK1 but also of "free" cytosolic RIPK1 which hinders RIPK1 from acting as "condensation nucleus" of cytosolic complexes containing kinase active RIPK1 (Li et al., 2012; Jaco et al., 2017). Complex IIa-associated maturation of procaspase-8 dimers results in the release of mature heterotetrameric caspase-8 molecules composed of the two p18 and p10 subunits of a procaspase-8 dimer. Subsequent apoptosis induction is typically suppressed by constitutive and NFκB-induced expression of FLIP proteins and K63-ubiquitination of RIPK1 (Brenner et al., 2015). Thus, cells are normally resistant against TNF-induced apoptosis as long as FLIP expression/induction is not inhibited (e.g., by CHX or IKK inhibitors) and/or RIPK1 K63 ubiquitination is not prevented (e.g., by SMAC mimetics or TRAF2 depletion) (Brenner et al., 2015; Annibaldi and Meier, 2018). Noteworthy, complex IIa-associated active caspase-8 and caspase-8/FLIP$_L$ heterodimers, which have a limited enzymatic activity, cleave RIPK1 and RIPK3 and prevent so complex IIb triggered formation of necroptosis-inducing oligomeric RIPK1/RIPK3 aggregates (Brenner et al., 2015). Moreover, K63- and linear ubiquitination of RIPK1 and activation of TAK1 and IKK not only stimulate the anti-apoptotic classical NFκB pathway but also inhibits cytotoxic RIPK1 activation by phosphorylation in its intermediate domain (S320 of human RIPK1, S321 of murine RIPK1) directly (IKK and TAK1) and indirectly (TAK1) via activation of the p38-MK2 dyad (Dondelinger et al., 2015, 2017; Geng et al., 2017; Jaco et al., 2017; Menon et al., 2017). TNF-induced necroptosis therefore only occurs when caspase-8 activation fails in cells with a compromised TNFR1-TRAF2-cIAP1/2-LUBAC-TAK1-IKK sequence (**Figure 2**). Apoptotic cells release membrane-enclosed apoptotic vesicles containing the cellular content of the dying cell which are cleared by macrophages without triggering inflammation. In contrast, necroptosis is a lytic form of cell death releasing intracellular DAMPs and proinflammatory cytokines and thus promotes inflammation (Kearney and Martin, 2017; Frank and Vince, 2019). Since ongoing TNFR1 signaling is *per se* highly inflammatory, TNF-induced necroptosis might nevertheless dampen inflammatory TNF effects under certain circumstances (Kearney and Martin, 2017). Thus, the inflammatory net effect of TNFR1 *in vivo* is determined by the complex interplay of TNFR1-induced classical NFκB signaling, apoptosis and necroptosis.

In context of TNFR1 signaling, TRADD, RIPK1, TRAF2 and TAK1 are not only of central relevance for the activation of the classical NFκB pathway and suppression of the cell death inducing capacity of TNFR1 but are also responsible for triggering the MAP kinase cascades leading to the activation of JNK and p38 (Wajant et al., 2003). Since the relevance of TNFR1-induced activation of JNK and p38 signaling have been poorly addressed so far in macrophages, it will not been addressed further in this review. The same applies for TNFR1-induced DD-independent activation of the neutral sphingomyelinase and ERK signaling pathways.

TNFR2-RELATED SIGNALING PATHWAYS

Initially, oligomerized TNFR2 recruit TRAF2 along with its tightly associated binding partners TRAF1, cIAP1 and

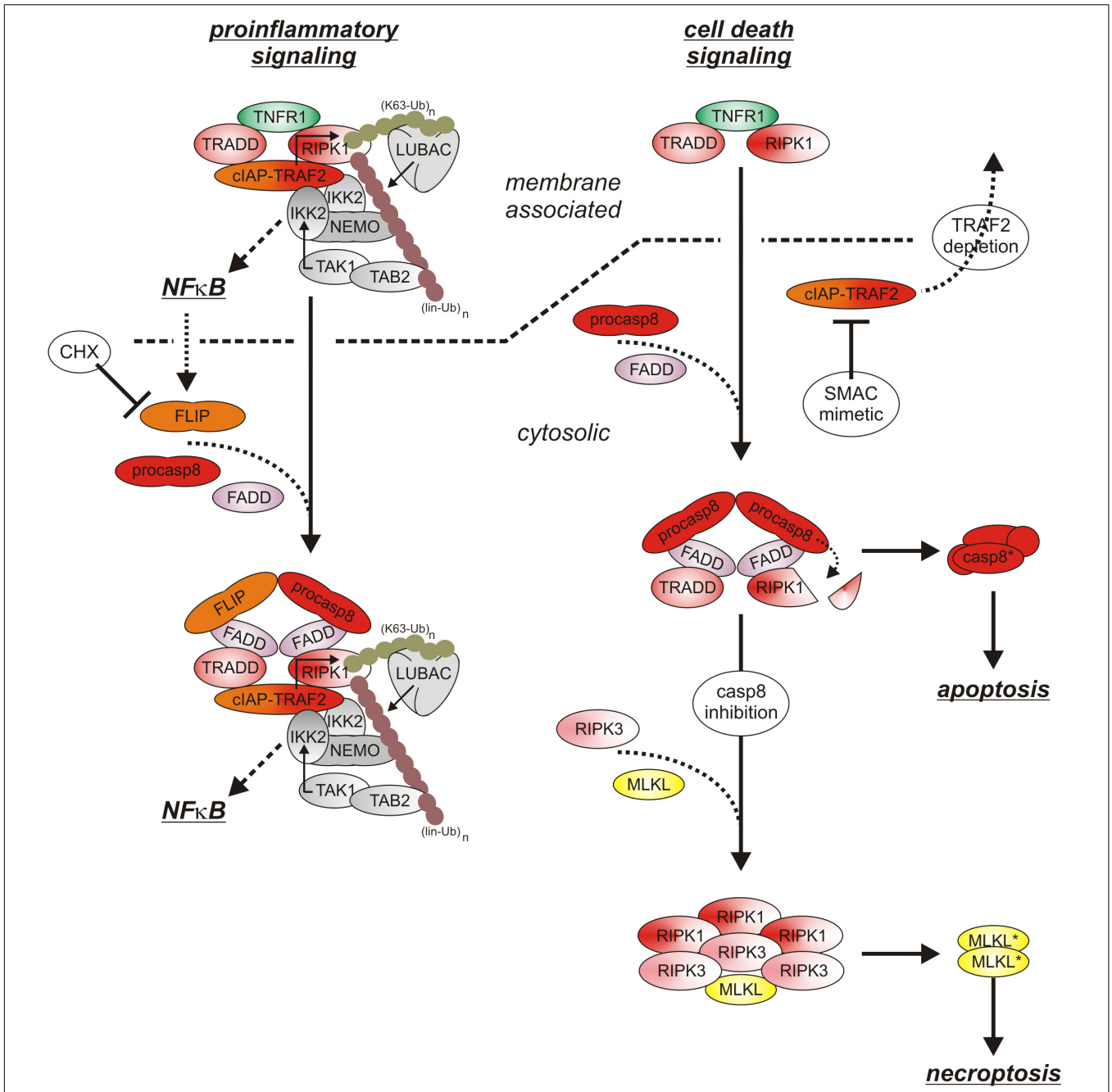

FIGURE 2 | TNFR1 signaling. The default state of TNFR1 signaling results in activation of proinflammatory pathways such as the classical NFκB pathway. If FLIP proteins and the TRAF2-cIAP1/2 complexes are limited, e.g., due to CHX treatment and/or TRAF2-cIAP1/2 depletion), the quality of TNFR1 signaling shifts to apoptosis. Not before caspase-8 activity is limited under apoptotic conditions, there is finally necroptosis induction. For more details see text.

cIAP2 what resembles the indirect, TRADD/RIPK1-mediated recruitment of these proteins in context of TNFR1 signaling. Although there is no evidence for a role of TRADD and/or RIPK1 in TNR2 signaling, the LUBAC as well as the IKK complex are also recruited to the TNFR2 signaling complex (**Figure 3**), but less efficient as in the case of TNFR1 (Wicovsky et al., 2009; Borghi et al., 2018). TNFR2 stimulation results therefore in activation of the classical NFκB pathway, too.

Cell surface expression levels of TNFR2 reach often > 10000 molecules per cell (e.g., Gehr et al., 1992; Medvedev et al., 1996) and are regularly much higher than those of TNFR1 which are typically in the range of a few hundred to 1-3 thousand molecules per cell (e.g., Thoma et al., 1990; Gehr et al., 1992). Recruitment of TRAF2-cIAP1/2 and TRAF1-TRAF2-cIAP1/2 complexes to TNFR2 can therefore lead to a significant depletion of these complexes in the cytoplasm and may thus affect other

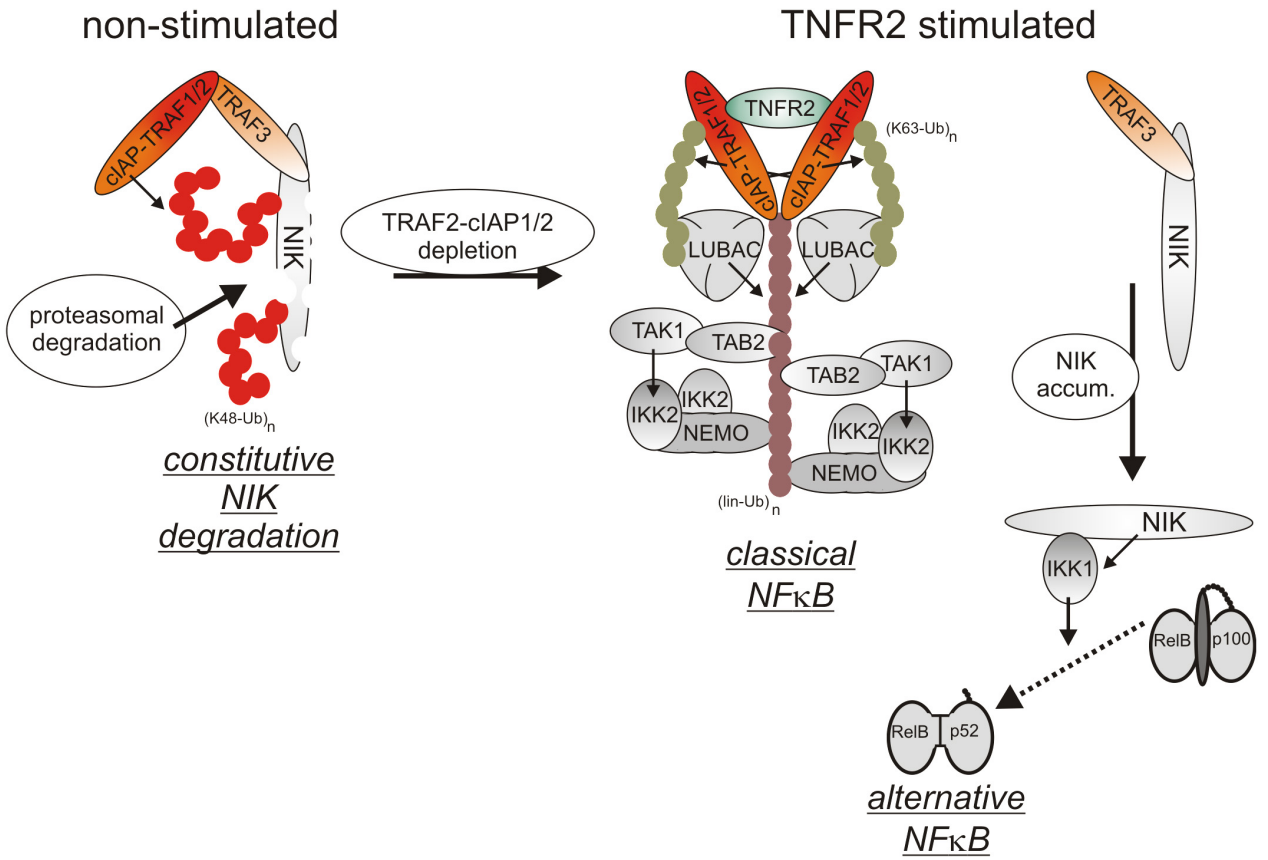

FIGURE 3 | TNFR2 signaling. TNFR2 efficiently recruits TRAF2-cIAP1/2 and TRAF1-TRAF2-cIAP1/2 complexes which allow activation of the classical NFκB pathway. TNFR2 recruitment of TRAF2-cIAP1/2 and TRAF1-TRAF2-cIAP1/2 complexes concomitantly depletes the cytosolic pool of these proteins and hinder them so to trigger degradation of the alternative NFκB pathway stimulating kinase NIK. Thus, TNFR2 also activates the alternative NFκB pathway.

activities of these molecules (Duckett and Thompson, 1997; Fotin-Mleczek et al., 2002; Li X. et al., 2002). Indeed, TRAF2 and the cIAPs are constitutively engaged in the cytoplasm in the inhibition of the alternative/non-canonical NFκB pathway which is of special relevance for the control of the activity of p52-RelB NFκB dimers (Sun, 2017). By virtue of its ability to reduce the cytosolic pool of TRAF2-containing complexes, TNFR2 is thus able to activate the alternative NFκB pathway (Rauert et al., 2010). In detail, TRAF2 recruits its binding partners cIAP1/2 to TRAF3 and the TRAF3 interacting MAP3-kinase NIK. The cIAPs ubiquitinate NIK with K48-linked ubiquitin chains and thereby promote the proteasomal degradation of this constitutively active kinase. TNFR2-induced depletion of cytosolic TRAF2-cIAP1/2 complexes results therefore in the accumulation of active NIK and NIK-mediated phosphorylation of the NFκB precursor protein p100. Phosphorylated p100 becomes K48-ubiquitinated and is then processed by the proteasome to the NFκB transcription factor subunit p52, thus resulting in the activation of p100-containing NFκB dimers (Sun, 2017). Although it has been found that depletion of TRAF2-cIAP1/2 and TRAF1-TRAF2-cIAP1/2 complexes is fully sufficient to interfere with the cytoplasmic activities of these complexes (Fotin-Mleczek et al., 2002), the depletion effect might be enhanced and sustained further by TNFR2-triggered proteasomal degradation of TRAF2 and cIAP2 (Duckett and Thompson, 1997; Li X. et al., 2002). TNFR2-induced depletion of the cytosolic pool of TRAF2-cIAP1/2 and TRAF1-TRAF2-cIAP1/2 complexes can also limit the availability of these proteins for other receptors. Indeed, it has been observed that prestimulation of TNFR2 (or similarly acting TNFRSF receptors such as Fn14) affects recruitment of TRAF2 and cIAPs to TNFR1 and thereby attenuates the ability of TNFR1 to stimulate classical NFκB signaling (Fotin-Mleczek et al., 2002). Due to the relevance of TRAF2 and cIAPs for preventing apoptosis and necroptosis in context of TNFR1 signaling, TNFR2-mediated depletion/degradation of these molecules can result in enhanced TNFR1-induced cell death in macrophages as is discussed below in detail.

In a cell type-specific manner TNFR2 can also activate the tyrosine kinase bone marrow kinase on chromosome X (BMX) and the phosphatidylinositol 3-kinase (PI3K)/protein kinase B(Akt) pathway (Pan et al., 2002; So and Croft, 2013). The latter presumably occurs TRAF2-dependent because TRAF2 has been implicated in Akt activation by various receptors including TNFR1 and the TNFR2-related TNFRSF receptor CD40 (Davies et al., 2005; Zhu et al., 2016). In contrast, TNFR2-induced BMX activation occurs independently from TRAF2 (Pan et al., 2002).

The molecular mechanisms used by TNFR2 to activate BMX and PI3K/Akt are, however, poorly investigated and their relevance in macrophages has not been addressed so far.

In sum, TNFR1 and TNFR2 are differently activated by soluble and membrane TNF, induce production of their own ligand in some cells, engage receptor-specific but also common pathways and the various TNFR1/2-associated signaling pathways are, last but not least, interconnected by regulatory circuits. TNF and its receptors therefore constitute a complex signaling network what is reflected by the fact that additive, synergistic or even antagonistic effects have been reported for the two TNF receptor types.

THE TNF-INDUCED CYTOTOXIC SIGNALING NETWORK IN MACROPHAGES

The complexity of the TNF-TNFR1-TNFR2 signaling system is especially relevant in macrophages because this cell type not only co-expresses TNFR1 and TNFR2 but also produces high amounts of TNF upon stimulation of a variety of receptors including all types of PRRs and various members of the TNFRSF. Of central relevance for the upregulation of TNF is the activation of the classical NFκB pathway. Macrophage-produced TNF not only mediates the proinflammatory and cytotoxic activities of this cell type but also regulates in an autocrine fashion the viability and activation status of macrophages. Indeed, there are a variety of examples of pathogen-induced macrophage cell death that crucially involves TNF (**Table 1**). An intensively studied example is killing of human alveolar and monocyte-derived macrophages by *in vitro* infection with mycobacteria (Keane et al., 1997; Balcewicz-Sablinska et al., 1998, 1999; Bermudez et al., 1999). Interestingly, mycobacteria infection also results in macrophage production of IL-10 which in turn triggers TNFR2 shedding resulting in TNF neutralization by soluble TNFR2 and reduced apoptosis (Balcewicz-Sablinska et al., 1998, 1999). TNF-induced cell death in mycobacteria-infected murine macrophages and macrophage cell lines has been attributed to TNFR1-induced caspase-8 activation and concomitant TNFR1-induced reactive oxygen species (ROS)-mediated activation of apoptosis signaling kinase-1 (ASK1) which promotes c-Cbl-mediated ubiquitination and degradation of the short FLIP isoform ($FLIP_S$) (Bhattacharyya et al., 2003; Kundu et al., 2009). Worth mentioning *Mycobacterium tuberculosis* can also trigger RIPK3/MLKL-mediated cell death by tuberculosis necrotizing toxin-mediated NAD^+ depletion independently from TNF and RIPK1 signaling (Pajuelo et al., 2018).

TABLE 1 | Pathogen-induced TNF-mediated macrophage killing.

Pathogen	TNF-inducing factor/ mechanisms	Evidence	References
Mycobacterium avium	–	Anti-TNF	Balcewicz-Sablinska et al., 1999; Bermudez et al., 1999
Mycobacterium tuberculosis	–	Anti-TNF	Balcewicz-Sablinska et al., 1998; Rojas et al., 1999
Mycobacterium tuberculosis	Mce4	Anti-TNF	Saini et al., 2016
Mycobacterium tuberculosis	PGRS33 > TLR2	Anti-TNF	Basu et al., 2007
Helicobacter pylori	JHP0290	Anti-TNF	Pathak et al., 2013
Salmonella enterica	OMP96-induced cell stress	Anti-TNF	Chanana et al., 2006, 2007
Ureaplasma urealyticum	–	Anti-TNF	Li Y.H. et al., 2002
–	LPS	Anti-TNF	Xaus et al., 2000
Bacillus Calmette-Guérin	–	TNFR1 KO	Rodrigues et al., 2013
Yersinia pseudotuberculosis	–	TNFR1 KO	Peterson et al., 2016

TNFR1-DEPENDENT NECROPTOSIS IN MACROPHAGES

Studies with SMAC mimetics (IAP antagonists) depleting cIAP1/2 and the cIAP1/2-related XIAP molecule as well as evaluation of XIAP and cIAP1/2 knockout cells revealed that these molecules are crucial for the survival of primary murine bone marrow-derived macrophages (McComb et al., 2012; Wong et al., 2014). The survival function of XIAP, cIAP1 and cIAP2 in this scenario could be traced back to the suppression of TNF-induced necroptosis (McComb et al., 2012; Wong et al., 2014). Studies with murine macrophages genetically deficient for TNFR1, TNFR2 and TNF together with the use of TNFR1- and TNFR2-specific agonists and antagonists revealed furthermore that both TNF receptors cooperate in triggering necroptotic cell death (Legarda et al., 2016; Siegmund et al., 2016; Lawlor et al., 2017). While exogenous TNF and/or autocrine TNF produced in response to TNFR1 and TNFR2 activation deliver a potential trigger for TNFR1-induced necroptosis, TNFR2 signaling enables realization of the necroptotic potential of activated TNFR1 by depletion of the cytosolic pool of TRAF2-cIAP1/2 complexes (Ruspi et al., 2014; Siegmund et al., 2016). As already mentioned above, some pathogens, e.g., mycobacteria, trigger IL-10-mediated shedding of TNFR2 to dampen/escape autocrine TNF killing (Balcewicz-Sablinska et al., 1998, 1999). The protective effect of TNFR2 shedding has so far mainly be attributed to the neutralization of TNF by the soluble TNFR2 ectodomain (Balcewicz-Sablinska et al., 1998, 1999). In light of the pro-necroptotic activity of TNFR2 in macrophages identified in recent years, however, it appears plausible that the protective effect of TNFR2 shedding is also due to the inhibition of the pro-cell death activities of TNFR2. RIPK1 and RIPK3 trigger execution of necroptotic cell death mainly by activation of mixed lineage kinase domain-like (MLKL) protein which forms cell-lytic plasma membrane pores and stimulation of mitochondrial production of reactive oxygen species (ROS) (Roca and Ramakrishnan, 2013; Fulda, 2016). Since ROS can promote TNF mRNA expression in human and murine macrophages (Gossart et al., 1996; Chandel et al., 2000;

Kono et al., 2000; Brown et al., 2004), this arm of the necroptotic response might close a feed forward loop in TNF-induced necroptosis.

In accordance with the established anti-necroptotic activity of caspase-8, robust/maximal necroptosis induction by TNF in macrophages requires caspase-8 inhibition (McComb et al., 2012; Siegmund et al., 2016). In most studies, the latter has been achieved artificially by the use of caspase-8 inhibitors or genetic deletion of the caspase-8 gene. This raises the question for the physiological/pathophysiological conditions under which necroptosis occurs. A first pathophysiological scenario where caspase-8 is limited are tumor cells that evade from apoptotic surveillance mechanisms by down-regulation of caspase-8 expression, for example by DNA methylation or caspase-8 gene deletion (e.g., references Grotzer et al., 2000; Teitz et al., 2000; Fulda et al., 2001; Shivapurkar et al., 2002; Hopkins-Donaldson et al., 2003). Necroptosis induction in caspase-8 low tumor types, however, has been poorly investigated so far. A second scenario where caspase-8 activity is limited and necroptosis gain relevance is in cells infected by viruses encoding caspase-8 inhibitory proteins. Several viral inhibitors of caspase-8 have been identified in recent years and include CrmA from cowpox virus and the baculovirus-encoded p35 protein (Ray et al., 1992; Zhou et al., 1997; Xu et al., 2001) but also proteins of clinically important human viruses. The human cytomegalovirus (CMV) gene UL36 encodes the viral inhibitor of caspase-8-induced apoptosis (vICA) protein which is conserved in primates and rodents and the ribonucleotide reductase R1 subunits of herpes simplex virus types 1 and 2 also act as caspase-8 inhibitors (McCormick, 2008; Dufour et al., 2011). Please be aware that some of these viral proteins not only inhibit caspase-8 but also other caspases including caspase-1 which is of crucial relevance for signaling by inflammasomes. The relevance of caspase-8 inhibition and sensitization for necroptosis for the *in vivo* effects of these pathogenic factors can therefore be difficult to define.

TNF-INDUCED NECROPTOSIS AND ITS INTEGRATION IN PRR SIGNALING

In accordance with the fact that PRRs strongly stimulate TNF production by macrophages, it has been described that induction of TNF and TNFR2-mediated TRAF2 depletion contribute to macrophage necroptosis induced by certain TLRs, including TLR4 and TLR3 (Kaiser et al., 2013; Legarda et al., 2016; Siegmund et al., 2016; Lawlor et al., 2017). TNF-independent necroptosis induction by TLR4 and by TLR3, however, has also been described (He et al., 2011; McComb et al., 2014). The varying relevance of autocrine TNF production for TLR4-induced necroptosis presumably reflects the use of different doses of the TLR4 agonist lipopolysaccharide (LPS) in the cited studies. Indeed, it has been described that high doses of LPS directly activate necroptotic signaling so that the effect/contribution of autocrine TNF-induced necroptosis is masked (Legarda et al., 2016). Studies with XIAP-deficient bone marrow progenitor cell-derived dendritic cells revealed an unexpected inhibitory role of XIAP on TNF signaling (Yabal et al., 2014) which was later on confirmed in murine bone marrow-derived macrophages (Lawlor et al., 2015, 2017). It turned out that LPS-stimulated XIAP-deficient macrophages, despite unchanged TNF production, elicit an enhanced cell death response, inflammasome activation and IL-1β secretion in a TNF-dependent manner (Yabal et al., 2014). Worth mentioning XIAP-deficiency showed no effect on TNF-induced activation of the classical NFκB pathway, p38 MAPK activation and TNFR1 signaling complex formation (Yabal et al., 2014). Thus, XIAP might preferentially affect TNF-related activities in macrophages that require RIPK3. Indeed, TNF-induced IL-1β secretion, caspase-8 activation and cell death induction were blocked in dendritic cells derived of XIAP/RIPK3 DKO mice (Yabal et al., 2014).

Lipopolysaccharide-induced TLR4 signaling has not only the potential to trigger necroptosis via induction of endogenous TNF but also adjusts the necroptotic sensitivity of macrophages for TNF in a complex manner (**Figure 4**). On the one side, TLR4 induces type I interferons which have various pro-necroptotic effects as is discussed below in detail. One the other side, TLR4 activates caspase-8 in a TNF-independent manner and promotes so the "inactivating" cleavage of the deubiquitinase Cyld (Legarda et al., 2016; **Figure 4**). Cyld removes K63-linked polyubiquitin chains from RIPK1 and interferes so in context of TNFR1 signaling with the recruitment and survival functions of the TAB2-TAK1 and IKK complexes. The LPS-induced caspase-8-mediated degradation of Cyld thus desensitizes macrophages for necroptosis. TLR4-induced caspase-8 activation requires the TLR4-RIPK1 linking adapter protein Toll/interleukin-1 receptor domain-containing adaptor protein inducing interferon (TRIF) and is dependent on RIPK1 and FADD (Weng et al., 2014; Legarda et al., 2016; Peterson et al., 2016). Caspase-8 activation by TLR4 thereby closely resembles the mechanisms of TNFR1-induced TRADD-RIPK1-mediated stimulation of the FADD-caspase-8 dyad.

Studies with human-induced pluripotent stem cells (hiPSCs) showed that differentiated RIPK1-deficient macrophages progressively undergo autocrine TNF-dependent cell death (Buchrieser et al., 2018). Thus, RIPK1 can also elicit anti-necroptotic activity in context of TNF signaling in macrophages. However, the cues defining the net quality of RIPK1 effects in macrophages are elusive. Noteworthy, addition of exogenous soluble TNF not further enhanced endogenous-TNF dependent cell death of RIPK1 KO hiPSCs while LPS- and poly(I:C)-induced TNF-independent necroptosis was enhanced (Buchrieser et al., 2018). Since soluble TNF poorly stimulate TNFR2, it is tempting to speculate that TNFR2-mediated cell death sensitization is the limiting step in TNF-induced cell death in this model.

COOPERATION OF TNF AND TYPE I INTERFERONS IN NECROPTOTIC SIGNALING IN MACROPHAGES

Besides the classical NFκB pathway, the type I interferon pathway is a second major signaling pathway which is regularly engaged by PRRs (**Figure 5**). The central elements of the type I interferon

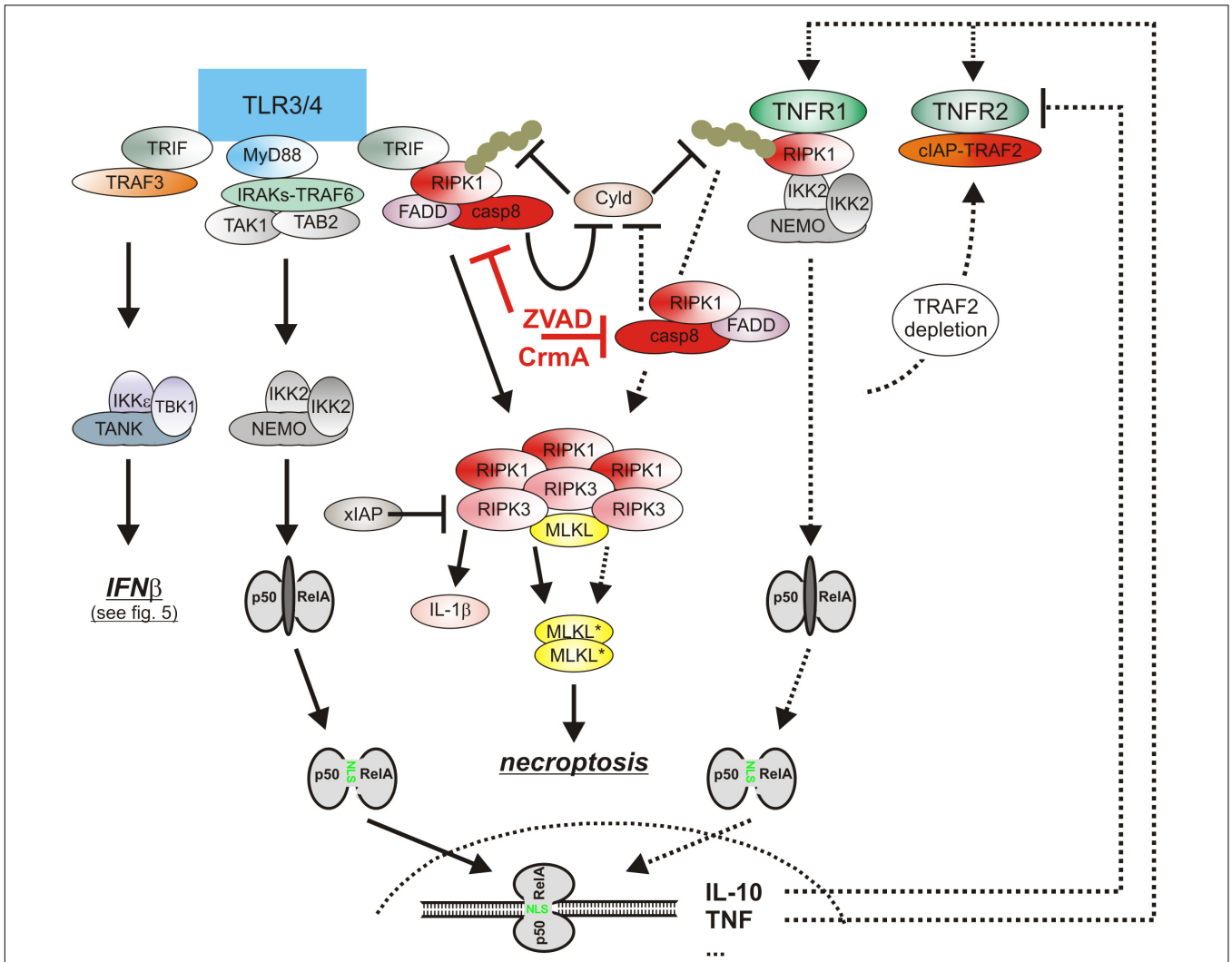

FIGURE 4 | The cytotoxic TLR-TNF signaling network. TNF expression is strongly upregulated by PRRs such as TLR3 and TLR4 via the classical NFκB pathway. TNF via the two TNF receptors can then cooperate with TLR3/4-TRIF-RIPK1 axis to trigger apoptosis or necroptosis. For details see text. Please note, necroptosis only takes place when caspase-8 activity is limited, e.g., by viral caspase-8 inhibitors.

pathway are the kinases TANK-binding kinase 1 (TBK1) and inhibitor of kappaB kinase ε (IKKε) which phosphorylate and activate the interferon-β gene inducing transcription factor interferon regulatory factor 3 (IRF3). In context of TLR3 and TLR4 signaling the adapter proteins TRIF and TRAF3 mediate activation of TBK1/IKKε and IRF3. Together with the in parallel activated classical NFκB pathway, the type I interferon pathway stimulates the production of the type I interferons (Ikushima et al., 2013). Thus, TNF and type I interferons are co-produced by pathogen challenged macrophages. Similarly to TNF, type I interferons not only act as effector molecules of activated macrophages but also retroact on the macrophages. Moreover, there is growing evidence that TNF and type I interferons cooperated in the control of macrophage viability (**Figure 4**). So it has been found that TNF/ZVAD- and LPS/ZVAD-induced necroptosis are blocked in macrophages derived of Interferon-α/β receptor alpha chain (IFNAR1) knockout mice (McComb et al., 2014; Legarda et al., 2016). The compromised necroptosis sensitivity correlated with reduced expression of the "pro-necroptotic" proteins TNFR2 and MLKL (Legarda et al., 2016). Reconstitution experiments revealed, however, that reexpression of TNFR2 and MLKL alone is not sufficient to restore necroptotic sensitivity for LPS pointing to additional type I interferon targets which are of relevance in necroptotic signaling. Indeed, there is evidence that TRIF-dependent induced type I interferons also promote the expression of Z-DNA binding protein-1 (Zbp1) and gasdermin D (GSDMD), which after cleavage can also cause lytic cell death (see below), via KAT2B- and p300-mediated histone 3 acetylation at lysine 27 (Li et al., 2018). Moreover, it has been shown that interferon-β activates protein kinase R (PKR) and promotes so its interaction with RIPK1 to trigger RIPK1/RIPK3-mediated necroptotic cell death (Thapa et al., 2013). The relevance of this mechanism for TNF-induced IFNAR1-dependent necroptosis in murine macrophages

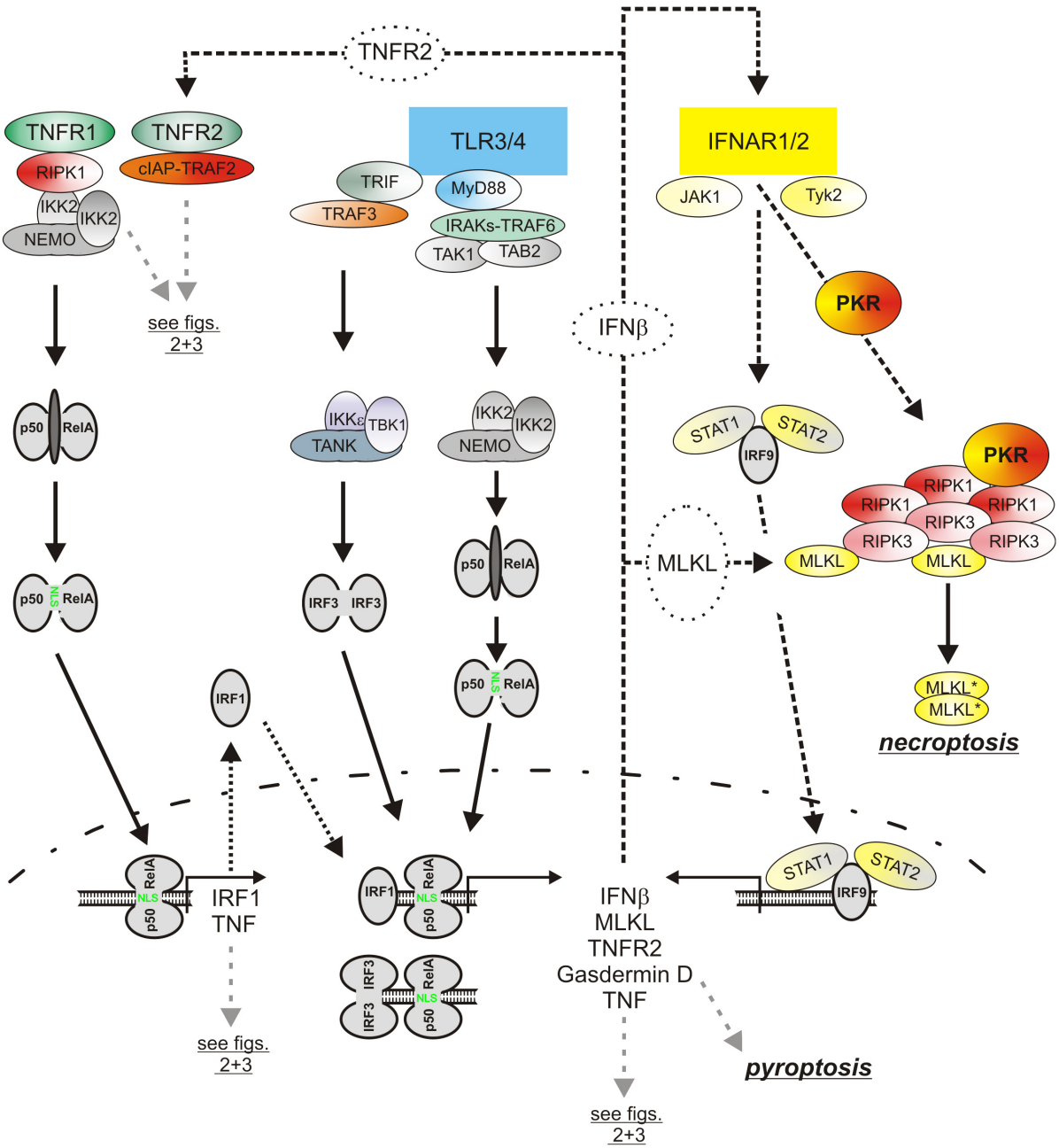

FIGURE 5 | The role of type I interferons in the cytotoxic TLR-TNF signaling network. TNF via the classical NFκB pathway induces IRF1 which in turn stimulates in cooperation with the classical NFκB pathway the expression of low amounts of IFNβ. The latter boosts its own expression by autocrine signaling but also the expression of pro-necroptotic proteins, such as TNFR2 and MLKL. PRRs, e.g., TLR4, often co-induce TNF and IFNβ expression. Thus, cytotoxic signaling by PRRs, IFNβ, and TNF are interconnected in various ways in macrophages by feed forward loops. Please be aware, for simplicity functional relevant phosphorylation and ubiquitination events are not included. For more details see text.

is, however, unclear. Last but not least, it has been found in caspase-8-inhibited macrophages that LPS initially induces TRIF-mediated type I interferon production which then trigger via the interferon-stimulated gene factor-3 (ISGF3) complex sustained RIPK3 phosphorylation and necroptosis by a yet poorly understood mechanism independent from PKR (McComb et al., 2014; Saleh et al., 2017).

Noteworthy, there is not only evidence that type I interferons contribute to TNF-induced necroptosis but vice versa also that TNF- and/or necroptosis-associated signaling contribute to the induction of type I interferons. TNF induces in macrophages not only NFκB-regulated genes but also, with delay, typical signal transducer and activator of transcription-1 (STAT1)- and interferon response proteins such as MX1, IRF7 and

STAT1 itself (Yarilina et al., 2008). The stimulation of the transcription of the latter proteins is due to an indirect mechanism involving NFκB-mediated upregulation of IRF1 via both TNF receptors and subsequent induction of low concentrations of IFNβ by the joint action of IRF1 and NFκB transcriptions factors (Yarilina et al., 2008). TNF and TNF-induced IFNβ cooperate then in the sustained and strong expression of NFκB/STAT co-regulated inflammatory factors such as CCL5, CXCL10 and CXCL11 and also maintain IFNβ expression (Yarilina et al., 2008). Whether TNF-induced IFNβ production is of relevance for TLR4-triggered necroptosis appears, however, unlikely as the TLR4-TRIF-IRF3 axis is already sufficient to mount a strong type I interferon response. There is, however, evidence from studies with LPS/ZVAD-treated macrophages that TBK1, IKKε, RIPK1 and RIPK3 form a high molecular weight complex which via RIPK3 promote TBK1/IKKε signaling (Saleh et al., 2017). Thus, TNF-induced RIPK1/RIPK3 activation in course of necroptotic signaling might have the potential to boost IFNβ production but this has not been evaluated yet.

TNF AND CASPASE-8-MEDIATED INFLAMMASOME ACTIVATION AND PYROPTOSIS

Besides apoptosis and necroptosis, pyroptosis is a third form of programmed cell death which is of particular relevance in macrophages (Man et al., 2017). Pyroptosis is a strongly proinflammatory form of lytic cell death which is triggered downstream of inflammasome complexes by caspase-1 mediated cleavage of gasdermin D (GSDMD). The N-terminal p30 cleavage product of GSDMD forms then large pores in the plasma membrane and executes so cell lysis (Man et al., 2017). Inflammasome-triggered pyroptosis of macrophages and intestinal epithelial cells is of special relevance for combating infection by intracellular bacteria (Frank and Vince, 2019). Recent studies showed that Yersinia bacteria by help of their TAK1 inhibitory acetyltransferease YopJ triggers RIPK1-mediated activation of caspase-8 (Orning et al., 2018; Sarhan et al., 2018). Interestingly, this not only results in effector caspase activation and apoptosis but also in caspase-8-mediated, thus non-canonical GSDMD cleavage, GSDMD-mediated NLRP3 inflammasome activation and pyroptosis (Orning et al., 2018; Sarhan et al., 2018). In one of these reports, it has been furthermore shown that cell death induction and IL-1β production by a mixture of pharmacological TAK1 inhibitors and TNF are reduced in GSDMD-deficient murine macrophages (Orning et al., 2018). Moreover, Yersinia-induced cell death was reduced in murine macrophages deficient for TLR4, TRIF or TNFR1 (Orning et al., 2018). Thus, under appropriate conditions TNF may also trigger GSDMD-dependent non-canonical inflammasome activation and pyroptosis, too (**Figure 5**). GSDMD is presumably directly cleaved by caspase-8 in these scenarios because TAK1 inhibition/LPS-induced generation of the pore forming p30 GSDMD fragment occurred in caspase-3/-7 double-deficient macrophages and GSDMD coimmunoprecipitated furthermore with caspase-8 (Orning et al., 2018; Sarhan et al., 2018). The mechanisms described are presumably of broader relevance as various other pathogenic bacteria and viruses, e.g., enteroviruses, pseudomonas and vibrio also utilize TAK1 inhibitory proteins (Zhou et al., 2013; Lei et al., 2014; He et al., 2017; Rui et al., 2017). RIPK1-mediated caspase-8 activation is inhibited by cIAPs (see above). Since Yersinia, LPS and TNF trigger cIAP depletion via the TLR4-TRIF pathway and the TNFR2-TRAF2 axis, it appears possible that cIAP depletion contributes to the pyroptotic RIPK1-caspase-8-GSDMD signaling branch but this issue has not been experimentally addressed yet.

Most pathogens activate in macrophages several types of inflammasome complexes. *Yersinia pestis* for example not only activates the NLRP3 inflammasome but also the pyrin inflammasome (Philip and Brodsky, 2012; Jamilloux et al., 2018). The sensor protein pyrin detects Rho GTPases molecule species which are inhibited by bacterial toxins and forms then an inflammasome with ASC and procaspase-1 (Jamilloux et al., 2018). TNF and various other PPR-induced cytokines, including type I interferons, stimulate the expression of pyrin in macrophages (Centola et al., 2000). Thus, the TNF triggered pyroptotic RIPK1-caspase-8-GSDMD signaling axis might further cooperate with TNF/interferon-induced pyrin expression and enhanced pyrin inflammasome activity to promote macrophage pyroptosis (**Figure 5**). Indeed, a contribution of TNF-induced pyrin expression to pyrin inflammasome activation, IL-1β production and pyroptosis induction has been recently demonstrated for clostridium difficile toxin B (Sharma et al., 2019).

Just recently two studies demonstrated that intrinsic, thus mitochondria-dependent apoptosis in bone marrow-derived macrophages is accompanied by activation of the NLPR3 inflammasome and IL-1β activation (Chauhan et al., 2018; Vince et al., 2018). Noteworthy, the latter was not only due to activation of the NLRP3 inflammasome but has also been traced back to inflammasome-independent IL-1β processing by caspase-8. In context of intrinsic apoptosis caspase-8 is directly activated by processing by effector caspases and indirectly by cIAP1/2 depletion and subsequent RIPK1 kinase activation. Caspase-8-activation and cIAP1/2 depletion can also be triggered by TNFR1 (or other death receptors) and TNFR2. It is thus well conceivable that TNF triggers this unusual proinflammatory mode of apoptosis, especially under circumstances where TNFR1-induced caspase-8 is insufficiently blocked. Future studies have to show whether the proinflammatory activities of caspase-8 (GSDMD cleavage, IL-1β processing) gain relevance for the biology and pathophysiology of TNF *in vivo*. Noteworthy, the pyroptotic GSDMD p30 fragment is able to trigger mitochondrial ROS production (Platnich et al., 2018) and the ROS in turn are established inducers of the NLRP3 inflammasome (Tschopp and Schroder, 2010) and, as mentioned before, of TNF expression (Gossart et al., 1996; Chandel et al., 2000; Kono et al., 2000; Brown et al., 2004). Thus, the GSDMD p30-ROS axis might auto-amplify p30 production by two feed forward loops, first by NLRP3 inflammasome activation and second by TNF-induced caspase-8 activation. TNF itself

is furthermore able to trigger mitochondrial production of ROS in macrophages and thus might further enhance these feed forward loops.

CONCLUSION AND PERSPECTIVE

There are a considerable number of high quality publications addressing the role of TNF in the life death balance of macrophages. In sum, these studies show that the effect of TNF on macrophage viability not only depends on the integrated and complex activity of the TNFR1-TNFR2 signaling network but also from its crosstalk with other, equally complex signaling systems engaged by PRR-, inflammasomes and interferons. It is thus not really surprising that the precise net-effects of TNF on macrophages in infection diseases and cancer are still poorly predictable. Indeed, it is not even clear whether and if yes to which extend, the reported effects of TNF on macrophages are generalizable to all types of macrophages. Likewise, it is unclear under which *in vivo* conditions which of the various individual TNF-related signaling mechanisms gain dominance. Thus, future studies must show whether there are key factors that determine the quality of TNF signaling on macrophage viability. It appears particularly important to learn more about the crosstalk of concomitantly occurring signaling paths engaged by TNF and other inducers of macrophage cell death. Last but not least, it will be important for the understanding of the role of TNF for macrophage biology to learn more about the systemic immunological net-effects triggered by timely limited "immunogenic" death versus persistent inflammatory activation of macrophages.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Sepsis: Inflammation Is a Necessary Evil

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Sepsis is one of the leading causes of deaths world-wide and yet there are no therapies available other than ICU treatment. The patient outcome is determined by a complex interplay between the pro and anti-inflammatory responses of the body i.e., a homeostatic balance between these two competing events to be achieved for the patient's recovery. The initial attempts on drug development mainly focused on controlling inflammation, however, without any tangible outcome. This was despite most deaths occurring during the immune paralysis stage of this biphasic disease. Recently, the focus has been shifting to understand immune paralysis (caused by apoptosis and by anti-inflammatory cytokines) to develop therapeutic drugs. In this review we put forth an argument for a proper understanding of the molecular basis of inflammation as well as apoptosis for developing an effective therapy.

Keywords: inflammation, sepsis, apoptosis, programmed cell death, immune suppression

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INTRODUCTION

Early medical records have documented infectious diseases in humans as far back as 1000 BC, and yet, pathogenic infection remains as the leading cause of morbidity and mortality (Ruffer and Ferguson, 1911; Cossart, 2014). Infection leading to sepsis continues to be one of the biggest health problems world-wide. Although difficult to discern the absolute global burden of the disease, it is estimated that thirty million people are affected each year (Reinhart et al., 2013). The disease predominantly affects low- to middle-income countries and is responsible for an estimated six million deaths (Fleischmann et al., 2016). In addition, every year one million deaths of newborns are due to maternal/neonatal sepsis (Vogel, 2017). In the United States alone, costs associated with this disease can exceed $16 billion dollars, as most patients admitted to ICU require mechanical ventilation to stay alive (Angus et al., 2001).

Despite the heavy cost of sepsis, the etiology of the disease continues to be enigmatic. In the past, it was believed that the primary source of infection originated solely from the gut microbiota (Friedman et al., 1998). However, subsequent studies showed that *Pseudomonas* sp. that colonizes and causes infection of the upper respiratory tracts was the most commonly associated infection in sepsis (Rangel-Frausto, 1999; Mayr et al., 2014). Now we know that sepsis is a highly heterogenous disease both in terms of its cause and its progression. Before the 90s, the majority of septic patients who presented at the clinic showed gram-negative organisms in their blood (Polat et al., 2017). This lead some scientists to establish diagnostic criteria for the sepsis syndrome – claiming specific medical symptoms and known cause of infection are central for diagnosis (Bone et al., 1989). Within the following decade it became evident that although gram-negative bacteria are still prevalent in septic patients, gram-positive microbiota became more apparent within patient sera (Friedman et al., 1998). In fact, almost the same number of gram-negative and gram-positive bacteria are

today associated with the disease (Vincent and Abraham, 2006). However, the causative agent is not always bacteria as parasites and fungus can also cause sepsis (Hubner et al., 2013; Florescu and Kalil, 2014; Liang, 2016). Furthermore, in about a third of patients an infectious pathogen is not detectable (Bone et al., 1989; Liang, 2016). This includes trauma patients whom frequently displayed clinical signs of sepsis but lacked bacteria in the blood (Goris et al., 1985). These discrepancies forced physicians to modify the diagnostic criteria for sepsis in 1992 at a Consensus Conference in Chicago (Bone et al., 1992). These new criteria suggested that infection did not have to be limited to bacteria and systemic inflammatory response syndrome – SIRS – became the new age term to describe the disease (Bone et al., 1992).

Although diagnostic criteria were being updated regularly – one aspect of sepsis drew the attention of researchers and remained constant - the presence of inflammation during disease. The inflammatory nature of sepsis was investigated as far back as 1960 – where the first clinical trial commenced to attenuate the inflammatory response (Bennett et al., 1962). These studies led to the use of corticosteroids; however, no therapeutic benefit was noted (Bennett et al., 1962). Drug trials which target the inflammatory phase of sepsis would continue well into the 2000s without any tangible benefits in patient survival (Polat et al., 2017). A recent shift in the paradigm would lead researchers to believe that inflammation is in fact necessary to fight infection associated with disease (Ding et al., 2018). Nevertheless, these revelations are relatively new and therapies to treat the disease are still under investigation.

ROLE OF INFLAMMATION IN SEPSIS PATHOLOGY: A DOUBLE-EDGED SWORD

Sepsis is fundamentally an inflammatory disease mediated by the host immune response. The innate immune response is facilitated by the activation of pattern recognition receptors (PRR) during early sepsis. The receptor-response is highly dynamic and can be elicited by both pathogen-associated molecular patterns (PAMPs) and/or damage-associated molecular patterns (DAMPs) such as mitochondria released from injured tissues (Mogensen, 2009; Hauser and Otterbein, 2018). At an organism level, complement, surface-receptors of epithelial, endothelial and disseminated immune surveillance cells incite such responses (Takeuchi and Akira, 2010). Intracellular signaling process is highly complex – with complementary and/or redundant roles for numerous signaling pathways, ultimately leading to expression of genes involved in adaptive immunity and inflammation. However, the deregulated hyperinflammation can lead to the many symptoms seen in the early phase of sepsis including disseminated intravascular coagulation (DIC) and subsequent multi-organ dysfunction syndrome (MODS), inflammation-coagulation due to aberrant platelet activation, peripheral vasodilation leading to low blood pressure ensuing hypoperfusion of the kidney and kidney failure (Dhooria et al., 2016; Wang et al., 2018). Thus, sepsis is a multifaceted disease manifested in many ways including endocrine disorder, coagulopathy, polyneuropathy, complement activation and polyneuropathy, all emanating from dysregulated inflammation (**Figure 1**).

Inflammation is an essential step in alerting the immune system to the presence of infection so that the hosts white blood cells can quickly locate and combat the pathogen (Weighardt et al., 2000). This response is typically tightly controlled, with inflammation waning after infection is resolved – returning to basal levels with the host's white blood cells following suit. When homeostasis is maintained, excessive inflammation and immune cell activity is avoided, and the immune system can prime itself for effective response to future infection (Newton and Dixit, 2012). During sepsis, the stimulus that is recognized by the immune system, ranging from PAMP's like endotoxins and viruses to DAMP's during serious trauma, is far greater than in regular infections (Hotchkiss et al., 2016). The immediate result is a cytokine storm brought on due to the overstimulation of the numerous white blood cells that recognize those factors. This dysregulation in response causes a myriad of symptoms that make sepsis distinctly different to regular infections, regardless of severity (Martin, 2012). When functioning normally, the immune system can combat most infections, with an imperceptible amount of inflammation occurring before the pathogens are cleared from the host. Resolving most infections so rapidly, with little damage to the host, depends on the strict regulation of cytokines. Cytokines are essential in the process of initiating and escalating the innate immune response as well as the adaptive immune response (Banyer et al., 2000). However, high levels of inflammatory cytokines can co-exist with a significant innate immune suppression, which can lead to nosocomial infections (Hall et al., 2013).

MOLECULAR MECHANISMS OF INFLAMMATION

Many different antigenic constituents from bacteria, viruses and fungi, as well as tissue trauma are known causative agents of sepsis. Common pathogens recurrently isolated from septic patients include gram-positive *Staphylococcus aureus* and *Streptococcus pneumoniae* and gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa* (Martin et al., 2003). PAMPs such as LPS are recognized by toll-like receptors (TLRs) expressed on antigen presenting cells (APCs), such as macrophages and dendritic cells (Poltorak et al., 1998). APCs express a variety of these TLRs containing leucine-rich repeats, which act to sense and elicit responses against these antigens (Kawai and Akira, 2010). Upon receptor contact with their cognate ligands, pro-inflammatory intermediates are recruited, some of which include mitogen activated protein kinases (MAPKs) – which are activated upon phosphorylation – signal transducers and activators of transcription (STAT), Janus kinases (JAK) and nuclear factor κ (kappa)-light-chain-enhancer of activated B cells (NF-κB) – which translocates to the nucleus. As a result, gene expression is initiated to promote inflammatory cytokine and chemokine production (Johnston et al., 1995). This fine-tuned process is dependent on the repertoire of

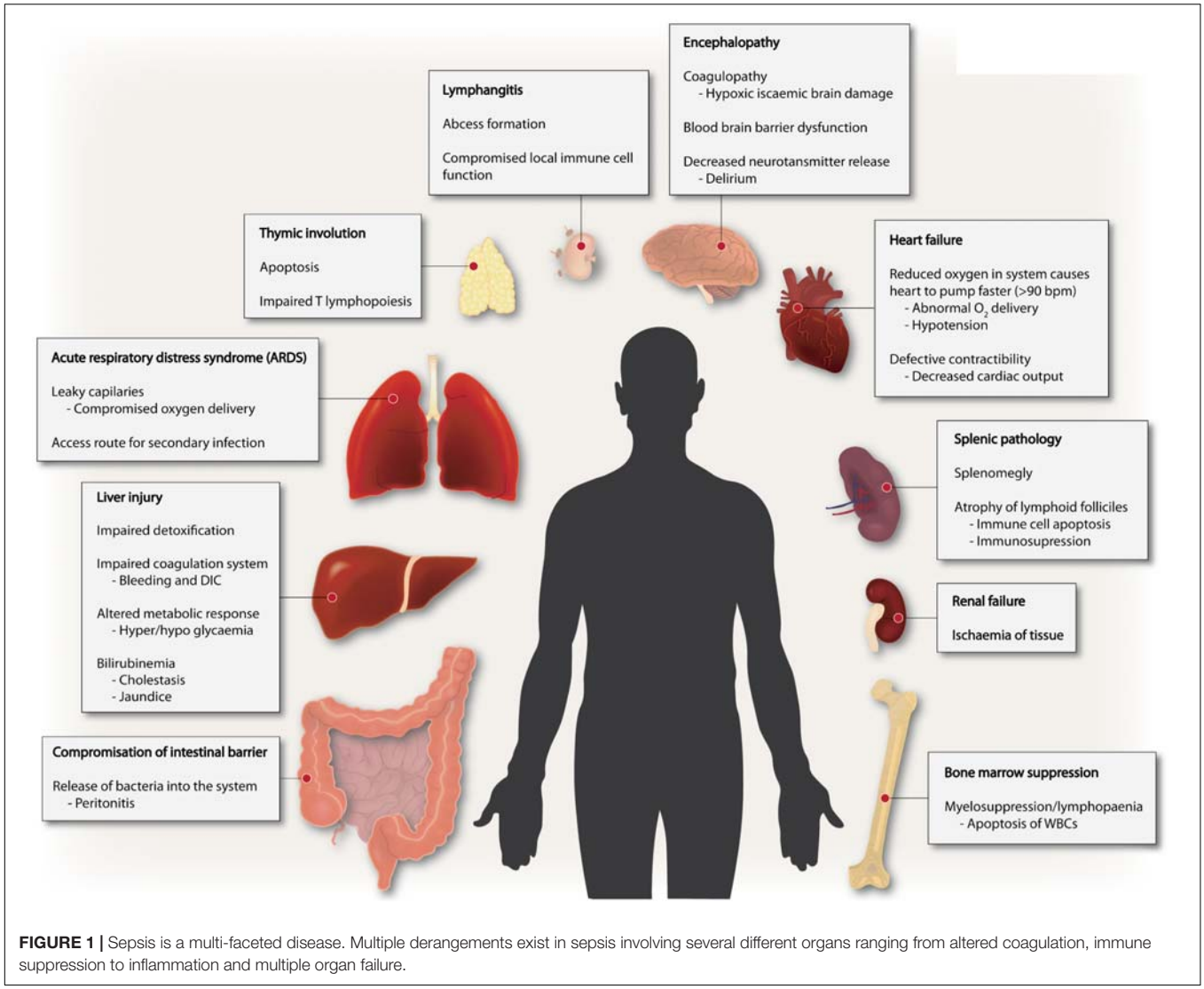

FIGURE 1 | Sepsis is a multi-faceted disease. Multiple derangements exist in sepsis involving several different organs ranging from altered coagulation, immune suppression to inflammation and multiple organ failure.

PAMPs, DAMPs and signaling pathways stimulated, to determine intensity and route of response, in an effort to re-establish host homeostasis. In the septic response, excessive inflammation due to deregulated intrinsic mechanisms is associated with pathology (Surbatovic et al., 2013).

IMMUNE ACTIVATION GENES

The transcription complex, NF-κB, is triggered in response to numerous extracellular inflammatory stimuli (Sen and Baltimore, 1986; Pahl, 1999). Activation of NF-κB by post-translational mechanisms induces expression of early activation genes including IL-1/12/18 and type-1 IFNs – to name a few (Naumann and Scheidereit, 1994). These inflammatory cytokines initiate synthesis of other cytokines and chemokines, such as IL-6/8, IFN-γ and CXC-chemokine ligands – exacerbating the inflammatory response. Stimulation of PRRs leading to the inflammatory cascade causes adaptive immune constituents to either become reactive or suppressive (Hayden et al., 2006). Such canonical pathways have shown to instigate the hyperinflammation observed in sepsis. Hence, studies have aimed to block NF-κB – as well as other intermediates – to attenuate hyper-responsiveness, however, results are conflictive (Sha et al., 1995; Gjertsson et al., 2001). Studies investigating differentially expressed genes in sepsis demonstrate genetic aberrations associated with disease, which could potentially be used as diagnostic markers (Prabhakar et al., 2005; Zhang et al., 2017). Interleukin-1 receptor-associated kinase 3 (IRAK3) is one such marker, which is specifically elevated in blood monocytes of septic patients and can possibly possess diagnostic value (Escoll et al., 2003).

ENDOCRINOPATHY

Sepsis is a highly inflammatory disorder with the presence of organ dysfunction in severe cases and mostly caused by bacterial

infection (Bone et al., 1989). These obvious characteristics of the disease prompted galvanize the belief that inflammation solely was responsible for sepsis related mortality. This claim was supported by endotoxemia models, which were deemed appropriate as they recapitulate obvious pathogenic features of the disease (Heppner and Weiss, 1965; Hardaway et al., 1996; Deitch, 2005). Hence, therapies were designed to attenuate host inflammatory responses evident in sepsis. One of the first anti-inflammatory treatments was the use of corticosteroids (Bennett et al., 1962). Evidence of adrenal gland insufficiency in patients with sepsis initially encouraged the use of steroids (Melby and Spink, 1958). Indeed, endotoxemia animal models of disease supported these findings (Mckechnie et al., 1985) and led to the use of steroids in a human study. The trial consisted of septic patients administered with high doses of methylprednisolone, leading to significant reduction in mortality with (Schumer, 1976). In a subsequent study, high dose steroid administration was found to have adverse effects (Warrington and Bostwick, 2006). Also, with adequate vasopressor therapy and fluid resuscitation (Colling et al., 2018), the use of steroids for treating sepsis became obsolete. Recently, a meta-analysis re-visited the applicability of steroids in sepsis – suggesting that low doses could be advantageous (Rochwerg et al., 2018). Another larger randomized study – comprised of 3800 subjects – measured survival in septic shock patients infused with hydrocortisone. They concluded that hydrocortisone did not reduce 90-day mortality when compared to placebo (Venkatesh et al., 2018). As it stands, the benefits of steroid use for treating sepsis remain vague and lack promise.

COAGULATION CASCADE

Coagulopathy associated with sepsis has long been identified as a clinical feature of disease (Martinez et al., 1966). Of those who present to the clinic, 35% meet criteria for DIC, which is a robust predictor of mortality (Wheeler and Bernard, 1999; Bakhtiari et al., 2004). During early DIC, activation of thrombin leads to the formation of fibrin complexes followed by thrombocytopenia. Late progressive DIC is characterized by the deposition of fibrin in the small blood vessels of the body, causing dissemination of micro-thrombi, which is associated with organ failure (Taylor et al., 2001; Gando et al., 2016). In order to prevent mortality in septic patients with microthrombus development, studies have used high-dose anti-thrombin therapy, however, no benefit was noted with the treatment (Warren et al., 2001). Other studies investigating the antithrombotic activity of heparin – unfractionated or low-dose – have also showed lack of effectiveness in preventing sepsis-related mortality (Zhang and Ma, 2006; Jaimes et al., 2009). The biggest drawback of such studies seems to be their single facet approach, where in fact a multi-facet approach is required for treating heterogeneous disease such as sepsis. Also to be taken into consideration is the close relationship between the innate immune system and the coagulation cascade (Esmon, 2003). Ample evidence suggest that hemostatic changes in sepsis can be regulated by pro-inflammatory mediators such as TNF-α during the "cytokine storm" (Zimmerman et al., 2002; Levi and Van Der Poll, 2010). Hence, the pathological "cross-talk" between coagulation and inflammation during septic shock warranted further investigation. In this context, the coagulation mediator, activated protein C, readily became of interest as a treatment option as this protein has important roles in coagulation and in attenuating immune responses (Opal, 2004). Identification of this protein as a putative therapeutic target for sepsis led to the controversial Recombinant Human Activated Protein C Worldwide Evaluation in Severe Sepsis (PROWESS) study (Bernard et al., 2001). Recombinant human activated protein C, marketed by Eli Lilly as Drotrecogin alpha activated (DrotAA) or Xigris, was used in this study. However, the study showed a modest 6.1% decrease in 28-day mortality in severe septic shock patients treated with DrotAA (Bernard et al., 2001). Similar studies using DrotAA in Early Stage Severe Sepsis (ADDRESS) and Extended Evaluation of Recombinant Human Activated Protein C United States Trial (ENHANCE), showed lack of drug efficacy with increased side effects such as hemorrhage (Abraham et al., 2005; Vincent et al., 2005).

Translation from "bench to bedside" appears to be the biggest hurdle for researchers in the development of successful treatments for sepsis. The disconnect has indeed been highlighted in the failure of past drug trials, especially those impeding inflammatory pathways associated with disease. However, in recent years researchers have shifted their focus to immune activation in sepsis – as inflammation is critical for clearing infection. Hence, immune stimulating strategies reveal an innovative focal point for treating sepsis pathogenesis.

CYTOKINE AND COMPLEMENT ACTIVATION

Cytokines TNF-α and IL-1 are the most extensively studied pro-inflammatory mediators in sepsis. These cytokines are capable of activating target immune cells to produce additional inflammatory mediators and as a consequence, a heightening immune responses. This prompted an increased focus on these cytokines to develop a therapeutic strategy to treat sepsis (Schulte et al., 2013). Other cytokines with anti-inflammatory property such as IL-6, IL-8, IL-12, IFN-γ, and IL-10 could dampen the inflammatory response (Van Der Poll and Opal, 2008). The cytokine predominantly produced by Th17 T-cells, IL-17, possesses the capacity to provoke a pro-inflammatory immune response by eliciting the production of TNF-α, which in turn provides a route for cross-talk between lymphocytes and phagocytes (Weaver et al., 2007). Murine studies have shown that blockage of IL-17 is associated with marginal survival advantage (Flierl et al., 2008). The sepsis inflammatory response has also been shown to be regulated by macrophage migration inhibitory factor (MIF). MIF has been shown to be vital for the regulation of host immune responses via modulation of TLR4. Mice lacking MIF have been shown to have a defective response to intravenous LPS introduction, due to reduced TLR4 expression (Calandra et al., 2000).

Apart from the production of inflammatory cytokines, complement activation is also associated with sepsis onset. This in turn has profound effects on coagulation, compromising the endothelial barrier integrity transitioning into a pro-coagulant state. Similar to cytokines, complement activation is also initiated by PAMPs and DAMPs. During sepsis, complement peptide C5a is converted to a potent chemo-attractant state, causing derangement in neutrophil function, that results in tissue damage (Guo and Ward, 2005). Additionally, this potent peptide amplifies inflammatory responses by stimulating production of pro-inflammatory cytokines, which is thought to contribute to organ failure in acute sepsis (Ward, 2010). Furthermore, complement factors have also been detected in clinical settings of disease suggesting a role in sepsis pathogenesis. However, treatment methods to prevent complement activation are ineffectual in reducing mortality associated with sepsis (Markiewski et al., 2008).

IMMUNE CELL DEATH

Sepsis pathogenesis was believed to consist of two distinct phases in response to systemic infection, which included the initial pronounced inflammation phase – or cytokine storm – that transitioned into a phase of prolonged immune suppression (Hotchkiss et al., 2013). Patients who survive the initial phase can enter a protracted hypo-inflammatory phase know as PICS – persistent inflammation/immunosuppression and catabolism syndrome (**Figure 2**). PICS is characterized by organ failure, persistent inflammation, protein catabolism/cachexia, ineffectual wound healing and increased susceptibility to infection due to immune suppression (Gentile et al., 2012). The propensity for patients with sepsis to readily develop persistent, recurrent and nosocomial infections suggested the existence of immune suppression in sepsis (Grimaldi et al., 2011). In further support of this idea, patients with sepsis have a higher rate of latent virus reactivation and blood cultures positive for opportunistic organisms (Otto et al., 2011; Walton et al., 2014). Irrespective of the disease classification, the immune suppression and dysregulation associated with disease is undeniably the major cause of sepsis related fatalities (Daviaud et al., 2015).

During sepsis, cells from both the innate and adaptive immune system are affected. The immune cells displaying marked depletion during sepsis include B cells, $CD4^+$, and $CD8^+$ T cells and dendritic cells in lymphoid organs such as the thymus, spleen and lymph nodes (Hotchkiss et al., 2005). Apoptosis, both extrinsic and intrinsic, has proved to be the driver of this depletion, with dying cells positive for active caspases and enhanced expression of pro-apoptotic BH3-only proteins. Multiple studies have shown, using various models such as transgenic mice and caspase-inhibitors, that blockade of lymphocyte apoptosis improves survival in sepsis (Hotchkiss et al., 1999a; Peck-Palmer et al., 2009). Indeed, autopsy studies of patients with sepsis revealed that immune cell apoptosis was the underlying cause of mortality (Torgersen et al., 2009). Furthermore, survival had a strong negative correlation with immune cell apoptosis during sepsis in mice (Hotchkiss et al., 1999b). The apparent linear relationship between disease severity and apoptosis is due to less circulating immune cells, hence, decreased surveillance and detection of infectious pathogens. In turn, compromising the hosts ability to successfully clear what should be a "mild" secondary infection. The negative effects of cell death during sepsis also impacts apoptotic cell uptake and clearance by surviving immune cells. Loss of follicular dendritic cells causes considerable impairment of T and B cell function, with $CD4^+$ T cell deficit impeding macrophage activation (Tinsley et al., 2003). Consequently, impaired macrophages are unable to mount the suitable inflammatory response toward the invading agent.

IMMUNE CELL TOLERANCE AND DYSFUNCTION

Lymphocytes undergoing apoptosis – during sepsis – can also serve to further suppress immune functions via interactions with macrophages, monocytes or dendritic cells. Phagocytotic cells are triggered to release anti-inflammatory cytokines – such as IL-10 and TGFβ – upon engulfment of apoptotic cells rendering them anergic. Additionally, this process can cause aberrations at the transcriptional level – preventing pro-inflammatory cytokine production – thus further contributing to immune paralysis (Coopersmith et al., 2003). Immune tolerance caused by excessive exposure to endotoxin can similarly have major consequences on macrophage functionality. In addition to excess release of immunosuppressive mediators, endotoxin tolerant macrophages possess relatively low levels of HLA-DR on their surface, resulting in a lack of antigen presentation (Saenz et al., 2001). Malfunction of sentinel first line defense immune cells combined with pronounced apoptosis is associated with a poor outcome in sepsis (Huang et al., 2009). Innate defenses are further compromised during disease pathogenesis due to impaired function of neutrophils and natural killer (NK) cells. During sepsis, circulating neutrophils exhibit an immature phenotype affecting transmigration, adhesion and the formation of neutrophil extracellular traps (NETs) (Kovach and Standiford, 2012). Indeed, neutrophils isolated from patients with sepsis showed to lack maturity, evidently having chemotaxis defects and reduced oxidative capacity. The depressed effector functions alter neutrophil antimicrobial defenses and is reported to be associated with the development of secondary infections in *in vivo* and in clinical settings (Demaret et al., 2015). This is also the case with NK cells, which are heavily depleted during sepsis. Animal and human studies have shown that as well as being reduced in number, remaining NK cells have defective cytotoxic function (Forel et al., 2012). Amongst the vast magnitude of cellular aberrations occurring during sepsis, exhaustion of T cells is characteristic of prolonged septic insult. Onset of T cell exhaustion is caused by a high load of antigen and amplified levels of anti- and pro-inflammatory cytokines, characteristic of the host septic environment. A recent study using cecal-ligation and puncture (CLP) showed that exhaustion of $CD8^+$ T cells can extend beyond initial septic insult and can inflict long-lasting changes in T cells leading to compromised reactivity toward

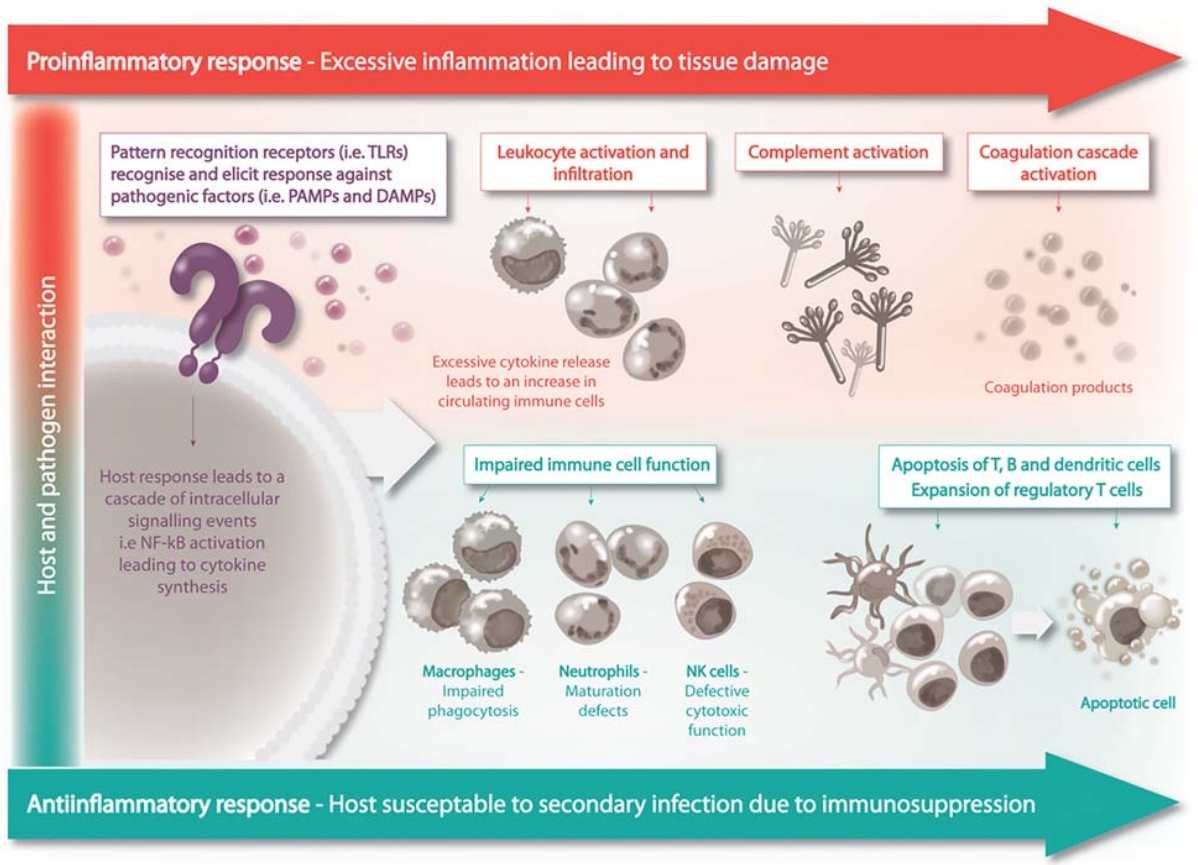

FIGURE 2 | Sepsis is a biphasic disease, the initial phase is characterized by overwhelming inflammation followed by immuno-suppression. A homeostatic balance between these two competing events is to be achieved for the patient's recovery.

future infections (Condotta et al., 2015). Hence, detrimental effects of immune cell death and dysregulation result in a long-term immunological "scar" causing substantial mortality of patients many years later when initial disease has been resolved.

DRUG TRIALS FOR SEPSIS: A CATALOG OF FAILURES

Severe immune dysregulation is the prominent hallmark of sepsis – rendering the disease biologically complex and consequently a challenge to treat (Sprung et al., 2006). More than 100 clinical trials have investigated putative treatments for sepsis, yet a cure still remains elusive (**Table 1**). Past failures of clinical trials can largely be attributed to disinclination of researchers to abandon ineffectual sepsis models. For instance, multiple past trials were based on studies which used experimental mice dosed with abnormal amounts of pathogen to mimic sepsis (Fink, 2014). Models such as this have since been largely discredited as they cause inflammation at a supra-physiological level (Lewis et al., 2016). The use of such imprecise models to study sepsis – in-turn – led to the development of non-targeted drugs which were unable to resolve disease in the clinic.

In the late 1960s, many researchers began to trial immunomodulatory agents for the treatment of sepsis (Davis et al., 1969). One of the most potent immune activators that gained prominence was bacterial cell wall component – LPS (Okeke and Uzonna, 2016). Studies demonstrated that lethality, associated with high doses of endotoxin in mice, was reverted with IgG infusion (Davis et al., 1969; Rubenstein and Worcester, 1969). Subsequently, the first anti-endotoxin trial commenced, which investigated the level of antiserum in patients infected with gram-negative bacteria. The study showed a reduction in mortality upon bacterial vaccination (Ziegler et al., 1982). Promising pre-clinical results led to the development of the monoclonal antibody – HA-1A – directed against the toxic lipid A component of LPS. Initial human trials showed that patients with sepsis tolerated the antibody well and were thought to benefit from treatment (Ziegler et al., 1991). However, lack of data reproducibility raised doubts against the study leading to a second trial. Further investigation revealed that the HA-1A treated groups had an increase in mortality – leading to drug withdrawal from the market (Costongs et al., 1993; Mccloskey et al., 1994). Homologs of LPS have also been designed to antagonize the activity of endotoxin at the receptor level. One such study used potent LPS antagonist E5531, which blocked

endotoxin response in healthy volunteers infused with small amounts of LPS (Bunnell et al., 2000). However, LPS homologs lost credibility when applied in a clinically relevant setting of sepsis. A study using eritoran – a synthetic TLR4 antagonist – demonstrated the drug's inability to reduce 28-day mortality in septic patients (Opal et al., 2013). The lack of effectiveness seen with anti-endotoxin treatment is perhaps not surprising since only about half of septic patients present with gram-negative infections. This suggested that pre-clinical models of disease do not appropriately reflect the heterogeneity of the human condition (Hotchkiss and Karl, 2003; Buras et al., 2005). Additionally, studies have used adoptive transfer of bone marrow cells between LPS-sensitive and LPS-resistant mice, which found that transferred bone marrow cells rendered mice susceptible to endotoxin lethality (Michalek et al., 1980). However, this study added credence to the fact that endotoxin wasn't killing the mice directly, rather their response to that exposure was.

For the last two decades, anti-cytokine strategies were thought to have boundless therapeutic potential. However, despite this optimism, they've shown to have little use in the treatment of sepsis. This was the case for extensively studied adjunctive therapies targeting tumor necrosis factor or TNF-α. Neutralization of this target receptors entailed the use of monoclonal antibodies as well as soluble TNF-α receptors as decoy receptors (Tracey et al., 1987; Vacheron et al., 1992; Borrelli et al., 1996). Many of these studies showed promise in rodents, however, could not demonstrate the same effect in human clinical trials. A notable study performed by Fisher et al., 1996, revealed that targeting inflammatory mediators could even be harmful. In this randomized, double-blinded study, septic patients were administered with recombinant soluble TNF-α receptor. Recombinant protein did not reduce mortality in septic patients and high doses were even associated with increased mortality (Fisher et al., 1996. In fact, clinical use of anti-TNF-α therapy has been linked to increased risk of infections (Ali et al., 2013). On the contrary, studies have shed light on the benefit of cytokine activation during sepsis (Echtenacher et al., 2001). Other studies have similarly used IL-1 receptor antagonists to test prognostic value in clinical sepsis, yet, fail to demonstrate significant reduction in mortality (Fisher et al., 1994). Failed trials that target inflammation in sepsis highlight the disconnect between laboratory experiments and clinical outcome and this warrants an urgent recalibration in research approach.

TABLE 1 | Sepsis therapy, a catalog of failures.

Target	Strategy	References
Lps/Endotoxin	HA-1A	Ziegler et al., 1991
	E5531	Bunnell et al., 2000
	Anti-CD14	Reinhart et al., 2004
	Eritoran	Opal et al., 2013
	Polymyxin B conjugate	Payen et al., 2015
Endocrinopathy	Methylprednisolone	Bone et al., 1989
	Vasopressin	Ohsugi et al., 2016
Hypercoagulability /Disseminated Intravascular Coagulation (DIC)	Activated Protein C	Bernard et al., 2001
	Anti-thrombin	Warren et al., 2001
	Heparin	Zhang and Ma, 2006
	Thrombomodulin	Hagiwara et al., 2016
Cytokines	Anti-TNF-α	Tracey et al., 1987
	IL-1 receptor Antagonists	Fisher et al., 1994
	Soluble TNF-α receptor	Borrelli et al., 1996
	Diacerhein	Calisto et al., 2012
Eicosanoids	Ibuprofen	Bernard et al., 1997
Nitric Oxide	L-NMMA	Petros et al., 1994
Oxidat1ve Stress	Statins	Patel et al., 2012
	Selenium	Sakr et al., 2014
Nf-Kb Transcription	Curcumin	Zhong et al., 2016
Apoptosis	Caspase inhibitors	Weber et al., 2009

Most, if not all, were targeting inflammation including caspase inhibitors. Caspases do have a central role in inflammation (Mandal et al., 2018).

CURRENT TRIALS

Many recent therapeutics have targeted endotoxins and cytokines circulating through patients with sepsis at dangerously high levels, in an attempt to reduce the inflammation and the associated pathology (Polat et al., 2017). However, all these therapies, while showed promise in animal models, showed no change in patient outcome or overall survival, and in some cases increased mortality. These therapeutics often had the desired effect of reducing the levels of its target endotoxin or cytokine, however, they consistently failed to improve short term clinical outcomes observed during SIRS i.e., organ failure, elevated heart rates and blood pressure or longer-term outcomes such as: time spent in ICU, rate of opportunistic infection and mortality. The failure to improve patient outcome by any of these measures' highlights both the complexity of the disorder as well as the flaw of targeting the initial acute phase of sepsis. Lack of correlation between cytokine levels and mortality was highlighted in a retrospective study published 20 years ago (Antonelli, 1999). Recently, focus has shifted to the chronic immunosuppressive phase as the cause of most sepsis-related deaths.

The subsequent immunosuppressive phase of sepsis is characterized by a drop in pro-inflammatory cytokine levels and leukopenia, frequently culminating in infection by opportunistic pathogens and death (Polat et al., 2017). During sepsis both myeloid cells and lymphocytes undergo high levels of apoptosis, with many of the remaining cells entering a state of anergy, rendering both the innate and adaptive arms of the immune system ineffective (Hotchkiss et al., 2013). The magnitude of the drop of total and functioning leukocytes is directly correlated with patient survival. Coupled with consistently falling mortality rates during the acute phase of sepsis (Levy et al., 2010), increasing the number of healthy leukocytes presents a promising therapeutic target that is now beginning to be explored

(Meisel et al., 2009; Chousterman and Arnaud, 2018). Immunostimulatory adjuvant therapies intend to counter the immune-paralysis that occurs in the chronic phase of sepsis (Meisel et al., 2009; Chousterman and Arnaud, 2018; Francois et al., 2018). These therapies aim to reduce apoptosis of leukocytes allowing their numbers to increase and revert them to a functional phenotype. Such therapies currently being investigated include the growth factors granulocyte-macrophage colony stimulating factor (GM-CSF) and IL-7 and the receptor, programmed cell death 1 (PD-1).

GM-CSF is a potent cytokine that stimulates the generation and maturation of monocytes and neutrophils, allowing them to effectively respond to pathogens (Mathias et al., 2015; Shindo et al., 2015). This effect has been demonstrated both *ex vivo* and *in vitro*. Addition of recombinant human GM-CSF to whole blood of septic patients recapitulates their phenotype closer to that of a healthy person. Treatment re-sensitizes both neutrophils and macrophages to LPS, with treated cells releasing significantly higher levels of pro inflammatory cytokines including TNF-α, IL-6, and IL-8, all of which are released at significantly lower levels in many patients during the later stages of sepsis (Mathias et al., 2015). Another marker of immune cell dysfunction is prolonged downregulation of membrane-associated human leukocyte antigen receptors (mHLA-DR) (Landelle et al., 2010). Lower expression levels of mHLA-DR have been associated with poorer outcomes and lower patient survival (Landelle et al., 2010), with GM-CSF demonstrating the capacity to restore mHLA-DR expression. A clinical trial involving patients with severe sepsis or septic shock conducted by Meisel et al. (2009) corroborated these findings. Patients were initially treated with 4 μg/kg daily for the first five days, then depending on the response, given 4 or 8 μg/kg daily for the next 3 days. *Ex vivo* analysis of monocytic function demonstrated that the monocytes from patients treated with GM-CSF – that were then stimulated with LPS – secreted higher levels of TNF-α, IL-6, and IL-8. Additionally, mHLA-DR expression was significantly upregulated, compared to that of normal levels, and less of the anti-inflammatory cytokine IL-10 was expressed (Meisel et al., 2009). Analysis of patient serum revealed that absolute neutrophil and monocyte count increased by a factor of four, with all patients approaching a normal white blood cell count after treatment. Additionally, TNF-α levels were increased, however, all other cytokines remained unchanged relative to the placebo group. Other clinical outcomes included treated patients spending less time on mechanical ventilation and reduced APACHE-II scores. Despite these favorable short- and long-term changes to many clinical outcomes, 28-day mortality was not reduced (Meisel et al., 2009).

IL-7 is a growth factor that stimulates the proliferation and maturation of many cell types, in particular T lymphocytes. IL-7 also causes many desirable changes in T lymphocytes (that may prove beneficial) in the context of sepsis disease progression, including: upregulation of Bcl-2 proteins and resistance to apoptosis, proliferation and enhanced function (Francois et al., 2018). During sepsis both $CD4^+$ and $CD8^+$ T cell populations drop considerably, and, like myeloid cells, the magnitude of the drop is correlated closely with patient survival (Drewry et al., 2014). In a phase II trial investigating CYT107, a recombinant form of human IL-7 in treating patients with septic shock and severe lymphopaenia (Francois et al., 2018). The most significant finding of this study was that, despite the complexity of the inflammation and immunosuppression seen during sepsis, there was a four-fold increase in absolute T lymphocyte count, which persisted well beyond the completion of therapy (Francois et al., 2018). However, much like the trial of GM-CSF, there was no significant difference in 28- or 90-day survival.

One of the main contributing factors of lethality in sepsis is immune tolerance, the mechanisms of which are only beginning to be understood. One pathway in which this occurs is the upregulation of PD-1 on T lymphocytes and PD-L1 specifically on APC's (Watanabe et al., 2018). During the immunosuppressive phase of sepsis, APCs upregulate PD-L1 further impairing remaining T-lymphocytes and compounding the effects of the suppressive cytokine profile of patients (Shindo et al., 2015; Liu et al., 2017). When T cells expressing PD-1 interact with cells expressing high levels of PD-L1, any response the T cells would have otherwise mounted is suppressed. This is compounded by the elevated levels of soluble PD-L1 in the serum of septic patients, leading to further lymphocyte attrition (Liu et al., 2017). PD-1 and PD-L1 antagonists are a new class of therapeutic blocking the interaction between the two molecules (Patera et al., 2016). These monoclonal antibodies are being investigated for use in diseases such as cancer and types of chronic viral infection, where restoring T cell function is of particular importance in fighting the disease (Watanabe et al., 2018). Similar to GM-CSF and IL-7 treatment, the PD-L1 antagonist BMS-936559 is well tolerated, with all therapies having little to no adverse effects when used to treat critically ill patients (Patera et al., 2016). Importantly, all drugs did not elicit an excessive pro inflammatory cytokine response, that would have further harmed patients (Meisel et al., 2009; Francois et al., 2018; Watanabe et al., 2018).

However, these therapies are not without limitations. The absence of improved short-term survival in all immunoadjuvant therapies is the most glaring shortcoming. It is likely due to a complex variety of reasons, though they do offer some clear benefit to sepsis patients. These limitations include the relatively small sample size in all the studies, the severity of sepsis of those included, as well as the use of 28- or 90-day survival as the end point. Severe sepsis and septic shock have the highest mortality rates of all types of sepsis during the acute phase, chronic phase and long after discharge from hospital (Karlsson et al., 2009). Long term mortality in these cases is over 1.5 times higher than in-hospital mortality, with the quality of life of survivors also being lower (Karlsson et al., 2009). It is these deaths that immuno-stimulant adjuvant therapies may offer the greatest benefits i.e., in reducing immune scarring and allowing the survivors of sepsis to reconstitute a functioning immune system. Past trials have not been powered to follow patients for such extended periods of time, but it is possible that is where the greatest benefits will be seen.

CONCLUDING REMARKS

Experimental drug therapies for sepsis are at cross-roads with the withdrawal of the latest drug Xigris (activated protein C, Eli Lilly) from the market following the negative results of the 1,700-person PROWESSSHOCK phase III trial in 2011. Critical-care physicians now have no drugs specifically approved to treat severe sepsis with the failure of *Talactoferrin alfa* (an immunomodulatory lactoferrin, Agennix, Germany) and AstraZeneca's *CytoFab*, an antibody directed against pro-inflammatory tumor necrosis factor-alpha (TNF-α) to name a few. Apart from using incorrect animal models (such as endotoxin-mediated sepsis in the absence of any confirmed infection), these failures could be attributed to the strategy of targeting inflammation, notwithstanding the fact that inflammation contributes to less than 20% of sepsis-related mortality. In the context of sepsis, inflammation is necessary evil as inflammatory cytokines are the activators of both the innate and the adaptive immune systems. Blocking of this pathway proven to be counterproductive in treating sepsis as there is a clear correlation between anti-inflammatory therapies and increased risk of infections (Ali et al., 2013). Use of steroids is yet another controversial topic. Since its inception in 1976 (Schumer, 1976), glucocorticoids are the preferred choice of treatment by great many physicians in spite of the fact that it does not offer any survival advantage (Venkatesh et al., 2018). There is a collective imperative on both the researchers and the physicians to measure the treatment outcome in terms of patient survival rather than reduced economic cost in ICUs. The immune paralysis phase of sepsis accounts for more than 80% of the sepsis-related mortalities and there is an inverse correlation between immune cell apoptosis and patient survival (Hotchkiss et al., 2013). Methods for identifying when patients have entered the immunosuppressive phase of sepsis and for detecting defects in immunity might enable the application of potent new immunotherapies. Therefore, there is a need to identify the factors that lead to immune-suppression by deregulated cytokine production and immune cell apoptosis. To understand the molecular mechanism of immune cell death during sepsis, particular emphasis should be given on the intrinsic or the Bcl-2 family mediated apoptosis.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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MINI REVIEW
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Mitochondria and Inflammation: Cell Death Heats Up

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Mitochondrial outer membrane permeabilization (MOMP) is essential to initiate mitochondrial apoptosis. Due to the disruption of mitochondrial outer membrane integrity, intermembrane space proteins, notably cytochrome *c*, are released into the cytosol whereupon they activate caspase proteases and apoptosis. Beyond its well-established apoptotic role, MOMP has recently been shown to display potent pro-inflammatory effects. These include mitochondrial DNA dependent activation of cGAS-STING signaling leading to a type I interferon response. Secondly, via an IAP-regulated mechanism, MOMP can engage pro-inflammatory NF-κB signaling. During cell death, apoptotic caspase activity inhibits mitochondrial dependent inflammation. Importantly, by engaging an immunogenic form of cell death, inhibiting caspase function can effectively inhibit tumorigenesis. Unexpectedly, these studies reveal mitochondria as inflammatory signaling hubs during cell death and demonstrate its potential for therapeutic exploitation.

Keywords: mitochondria, cell death, inflammation, interferon, NF-κB, apoptosis, caspases, mtDNA

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INTRODUCTION

Mitochondrial outer membrane permeabilization (MOMP), induced by the pro-apoptotic Bcl-2 proteins BAX and BAK, is the essential step in initiating mitochondrial apoptosis. Following MOMP, soluble mitochondrial intermembrane space proteins including cytochrome *c*, SMAC (also called DIABLO) and Omi (also called HtrA2), are released into the cytoplasm. In the cytoplasm, cytochrome *c* binds to APAF-1; this leads to APAF-1 conformational changes and oligomerization into a heptameric wheel-like structure called the apoptosome that recruits and activates the initiator caspase-9 (Bratton and Salvesen, 2010). Active caspase-9 cleaves and activates the executioner caspases-3 and -7, leading to widespread substrate cleavage. Caspase activity is essential for the biochemical and morphological hallmarks of apoptosis, leading to rapid cell death that is considered immunosilent (Arandjelovic and Ravichandran, 2015). Nevertheless, cells usually die irrespective of caspase activation upon MOMP, demarcating it as a point of no return (Tait et al., 2014).

While apoptosis is considered a silent form of cell death, mitochondrial dysfunction (that occurs upon MOMP) is associated with inflammatory effects. For instance, mitochondrial dysfunction can lead to cytosolic exposure of several danger-associated molecular patterns (DAMPs), such as mitochondrial DNA (mtDNA) (Shimada et al., 2012; West et al., 2015) and cardiolipin (Tuominen et al., 2006). Moreover, mitochondrial ROS – increased upon disruption of mitochondrial respiratory chain function – can also promote inflammation (Nakahira et al., 2011; Zhou et al., 2011; Zorov et al., 2014). Once exposed to the cytosol, mitochondrial DAMPs are recognized by various

adaptor molecules or receptors leading to an inflammatory response (Grazoli and Pugin, 2018). When mtDNA is in the cytosol it can be recognized by cyclic GMP-AMP (cGAMP) synthetase (cGAS), toll-like receptor 9 (TLR9), and the NLRP3 inflammasome (West and Shadel, 2017), of which the latter can also be activated by mtROS (Shimada et al., 2012). Upon MOMP, release of intermembrane space proteins (Liu et al., 1998; Adrain et al., 2001; van Loo et al., 2002) and cytosolic exposure of the inner mitochondrial membrane occurs (McArthur et al., 2018; Riley et al., 2018), enabling mtDAMP exposure during apoptosis. Various studies have shown that activation of apoptotic caspases has an immunosilencing effect during cell death. The anti-inflammatory effects of apoptotic caspases are likely to be pleiotropic; for instance, caspases have been shown to directly cleave and inactivate inflammatory pathway components as well as strongly suppress protein translation (Clemens et al., 2000; Ning et al., 2019). At least two parallel inflammatory pathways are activated during caspase-independent cell death (CICD) (Rongvaux et al., 2014; White et al., 2014; Giampazolias et al., 2017; McArthur et al., 2018; Riley et al., 2018). In this minireview, we will discuss how MOMP induces inflammation, focusing primarily on two recently described mechanisms: MOMP-induced cGAS-STING signaling (Rongvaux et al., 2014; White et al., 2014; Giampazolias et al., 2017; McArthur et al., 2018; Riley et al., 2018) and activation of pro-inflammatory NF-κB signaling (Giampazolias et al., 2017).

MITOCHONDRIAL RELEASE OF mtDNA CAUSES A TYPE I INTERFERON RESPONSE

When pathogen-derived, cellular or mitochondrial DNA is present in the cytosol various immunogenic pathways are activated. One of these cytosolic DNA sensors is cGAS, which produces cGAMP, from ATP and GTP, upon DNA binding. cGAMP functions as a secondary messenger and binds to the endoplasmic reticulum (ER) membrane adaptor STING (Cai et al., 2014). Upon binding, STING changes its conformation and becomes activated. Active STING translocates from the ER to an ER-Golgi intermediate apparatus and the Golgi compartment. During this process, the carboxyl terminus of STING recruits and activates TBK1, which in turn phosphorylates the transcription factor IRF3. Phosphorylated IRF3 dimerises and translocates to the nucleus where it initiates a type I interferon response (Chen et al., 2016). The type I interferon response acts in a pleiotropic manner to activate both innate and adaptive immunity (Trinchieri, 2010).

Several years ago, it was found that during mitochondrial apoptosis under caspase-inhibited conditions a type I interferon response is activated (**Figure 1**; Rongvaux et al., 2014; White et al., 2014). Genetically engineered mouse models and corresponding mouse embryonic fibroblasts with deleted caspases-3 and -7, or -9 showed significantly upregulated type I interferon expression and interferon-stimulated gene response following MOMP. Consistent with this, cells were highly resistant to infection by RNA and DNA viruses (Rongvaux et al., 2014). Similar results were obtained in hematopoietic stem cells, as deletion of caspase 9 increased basal levels of type I interferons and cell death in the presence of caspase inhibition stimulated expression of type I interferons (White et al., 2014). Both groups established that this increase in type I interferons during cell death was due to recognition of mtDNA by cGAS and subsequently STING activation (Rongvaux et al., 2014; White et al., 2014).

During mitochondrial apoptosis, only the outer mitochondrial membrane was thought to permeabilise following BAX and BAK activation. This made it challenging to reconcile how matrix localized mtDNA could activate cytosolic cGAS-STING signaling. Toward answering this conundrum, recent studies have shown that subsequent to MOMP, the inner mitochondrial membrane is extruded through expanding, BAX/BAK-dependent, outer membrane pores (McArthur et al., 2018; Riley et al., 2018; Ader et al., 2019). In the cytosol, these mitochondrial herniations can rupture, enabling mtDNA release (McArthur et al., 2018; Riley et al., 2018). Beyond providing a mechanism for mtDNA dependent activation of cGAS-STING signaling these studies demonstrate that BAX and BAK can form huge, expanding pores, termed macropores (McArthur et al., 2018), on the mitochondrial outer membrane. Previous studies have shown that BAX and BAK pores are highly flexible and dynamic in their pore size and shape in order to release proteins into the cytosol (Bleicken et al., 2013; Große et al., 2016; Salvador-Gallego et al., 2016), however, no study had shown before that these pores could be big enough to release mtDNA from the mitochondria. It is unclear beyond the requirement for BAX/BAK activation whether the release of mtDNA is regulated, but it is independent of both mitochondrial dynamics and mitochondrial permeability transition (Riley et al., 2018).

Besides STING activation, other immune sensing pathways can also be activated by mtDNA. Many antigen presenting cells possess TLR9, which is able to recognize mtDNA by virtue of its high content of CpG rich domains. It has been observed that recognition of mtDNA by TLR9 can both lead to NF-κB translocation and a type I interferon response (Oka et al., 2012; Zhang et al., 2014; Saito et al., 2018). An immune response can also be provoked by activation of the NLRP3 inflammasome via mtDNA during cell death (Nakahira et al., 2011; Shimada et al., 2012; Vince et al., 2018). Activation of the NLRP3 inflammasome leads to processing of interleukin (IL) 1β and IL-18, thereby activating monocytes, macrophages, neutrophils, and T-cells (Netea et al., 2010). Nevertheless, the contribution of these two pathways in the immune response following CICD awaits further investigation.

ACTIVATION OF PRO-INFLAMMATORY NF-κB SIGNALING FOLLOWING MOMP

In addition to increased expression of type I interferon genes during CICD, we also observed nuclear translocation of NF-κB, thereby activating transcription of pro-inflammatory genes (Giampazolias et al., 2017). NF-κB has been described as having a key role in inflammation, and can be activated in a canonical and non-canonical manner (Lawrence, 2009).

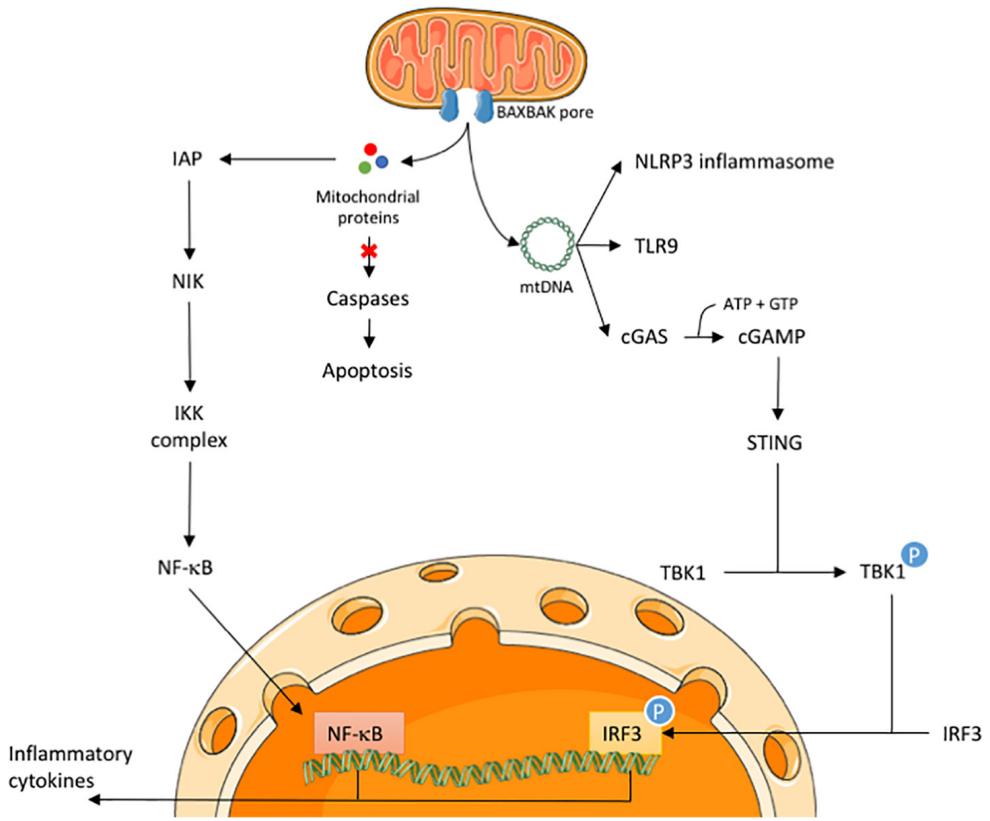

FIGURE 1 | Release of mitochondrial proteins and mtDNA initiates an inflammatory response in CICD. Upon an apoptotic trigger, BAX and BAK form pores in the mitochondrial membrane to allow the release of mitochondrial proteins, such as cytochrome *c* and SMAC/DIABLO, and mtDNA. During CICD, the release of mitochondrial proteins activates the NF-κB pathway via a SMAC-like mechanism. In parallel, release of mtDNA may activate the NLRP3 inflammasome, TLR9 receptor and the cGAS-STING pathway. Activation of the latter will lead to nuclear translocation of IRF3 during CICD. Both NF-κB and IRF3 engage the transcription of various inflammatory cytokines, leading to immune cell activation.

Besides inducing the transcription of various inflammatory genes, NF-κB also regulates the activation, differentiation, and effector function of inflammatory T-cells (Lawrence, 2009; Liu et al., 2017).

One of the genes that is transcribed by NF-κB activation is tumor necrosis factor (TNF). TNF is a pro-inflammatory cytokine that can trigger necroptosis, which is a regulated form of cell death that shares morphological similarities with necrosis. It has been observed that CICD has necroptotic features, as the kinetics of cell death were slowed by genetic alteration or pharmacological inhibition of the necroptotic pathway. Inhibiting TNF signaling with Enbrel showed a decrease in cell death, indicating that TNF is needed to engage necroptosis in CICD (Giampazolias et al., 2017). TNF is a well-known activator of NF-κB (Schütze et al., 1992), however, neither TNF nor the necroptotic pathway is responsible for the activation of NF-κB during CICD. Rather, we found that the increase in TNF expression and NF-κB activation during CICD is wholly dependent on BAX and BAK, indicating that MOMP is essential for initiating the inflammatory response. Because of the observation that MOMP is needed to initiate nuclear translocation of NF-κB in caspase inhibited conditions, we can conclude that necroptosis is not essential for the activation of this pathway but accelerates cell death following TNF expression (Giampazolias et al., 2017).

While NF-κB is robustly activated following MOMP, how this is initiated is unclear. A logical explanation may relate to the release of intermembrane space protein SMAC. When SMAC is present in the cytosol it binds to inhibitor of apoptosis proteins (IAPs) to block their function (Du et al., 2000; Verhagen et al., 2000). Besides the role of IAPs to regulate caspase activity and apoptosis, it is also known that IAPs are able to modulate inflammatory signaling by engaging pro-survival NF-κB activation (Gyrd-Hansen and Meier, 2010). Our lab has shown that upon MOMP, NF-κB becomes activated through a SMAC-like mechanism, whereby IAPs are degraded, leading to the activation of NF-κB-inducing kinase (NIK) (**Figure 1**). Degradation of IAPs occurs in a SMAC-like manner since a non-SMAC binding XIAP mutant is stabilized (Giampazolias et al., 2017). Nevertheless, even in the absence of known intermembrane space IAP-binding proteins, SMAC and Omi, IAPs are degraded following MOMP (Giampazolias et al., 2017). These results demonstrate that neither SMAC or Omi are required for IAP degradation upon

MOMP and that some other factor(s) downregulate IAPs in their absence. Interestingly, recent studies have shown that IAP-depletion following MOMP also contributes to caspase-8 dependent inflammasome activation in macrophages (Chauhan et al., 2018; Vince et al., 2018). MOMP-dependent IAP depletion can therefore promote inflammation by at least two distinct pathways.

The question remains as to how IAP degradation is engaged following MOMP. Are proteins in the mitochondrial intermembrane space responsible for this? Or does it relate to the permeabilization of the mitochondrial inner membrane, leading to the release of proteins from the matrix and the inner mitochondrial membrane into the cytosol? In order to fight microbial pathogens, the NF-κB pathway is commonly used and pathogens often interfere with this pathway to escape the immune response (Rahman and McFadden, 2011). Possibly stemming from their bacterial ancestry, could it be that mitochondria have similar pathogen associated molecular patterns (PAMPs) as invading bacteria, and caspases are capable of silencing the mitochondrial PAMPs that activate the NF-κB pathway?

CELL DEATH ASSOCIATED INFLAMMATION: DISEASE RELEVANCE AND OPEN QUESTIONS

In this mini-review we have described how MOMP can lead to an inflammatory response in two parallel pathways. MOMP allows mtDNA to be released into the cytosol, which will be recognized by cGAS leading to activation of STING. This allows phosphorylation of IRF3 to occur, inducing a type I interferon response. In parallel, MOMP leads to the exposure of factors into the cytosol causing degradation of IAPs in a SMAC-like manner. IAP downregulation activates NIK and subsequently NF-κB activation, leading to the transcription of various cytokines. Several questions remain outstanding: is mtDNA release regulated? What triggers NF-kB activation during CICD? How does caspase activity silence inflammation? Does the immune response only occur in caspase-inhibited conditions during cell death? Most importantly, what are the biological roles of these inflammatory effects during apoptotic cell death?

Although apoptosis is a common cell death mechanism during embryonic development, the expression of many pro-apoptotic proteins is reduced in various adult tissues (Sarosiek et al., 2017). Cardiomyocytes for example do not express APAF-1 (Sanchis et al., 2003), thereby being unable to form the apoptosome following MOMP. As a consequence, caspase activation by cytochrome *c* is strictly regulated by endogenous XIAP (Potts et al., 2005). Although cell death is strongly regulated in cardiomyocytes, apoptosis and necrosis does occur in hearts when reperfused after myocardial infarction (MI) (Zhao et al., 2000). Inhibition of caspases in myocardial ischemia/reperfusion-induced models has shown to limit infarct size and to improve recovery in rabbit and rat hearts (Holly et al., 1999; Mocanu et al., 2000; Kovacs et al., 2001). Even when inhibition of caspases does not fully prevent cardiomyocytes from dying, the observed clinical improvement when reperfusion is performed in combination with caspase inhibition suggests that caspase activity negatively impacts recovery via an unknown mechanism. It is well established that during ischemia and reperfusion injury an inflammatory response is provoked by DAMPs derived from necrotic cells in the core of the infarct site. These DAMPs help to recruit leukocytes to the injured areas for cell debris clearance before heart remodeling can take place (Ong et al., 2018). In contrast to the necrotic core of the infarct, apoptosis is primarily observed in the border zone of the infarct (Cheng et al., 1996; Sarate et al., 1997; Toyoda et al., 1997). It has been speculated that loss of cells in the border zone has negative impact on ventricular remodeling (Balsam et al., 2005). Inhibition of caspases might enhance survival of cardiomyocytes in the border zone when the extrinsic apoptotic pathway is activated, however, when MOMP occurs in these cells the intrinsic apoptotic pathway will still lead to cell death in the presence of caspase inhibitors. Nevertheless, engaging a pro-inflammatory response in the border zone through the release of DAMPs in CICD might have beneficial effects. Especially DAMP-associated TNF upregulation during CICD might play a big role in preventing cell loss in the border zone, as a 50% increase in apoptotic cell death was observed when a TNF inhibitor was administered in a mice suffering from MI (Wang et al., 2018). On the other hand, there is evidence that a type I interferon response, which can potentially be induced by the release of mtDNA during MI (Wang et al., 2015), might be harmful as inhibiting this pathway improves cardiac function and ventricular dysfunction after MI (King et al., 2017). This indicates that a fine balance in pro-inflammatory pathways is needed to have a beneficial effect in MI therapy.

In the context of cancer therapy, it has been described in multiple studies that apoptosis can have a pro-tumorigenic effect instead of being anti-tumorigenic (Ichim and Tait, 2016; Cao and Tait, 2018). One of the implications is that if apoptosis is not executed properly, it can cause DNA damage and genomic instability thereby promoting tumor growth (Lovric and Hawkins, 2010; Ichim et al., 2015; Liu et al., 2015). In contrast, engaging CICD in subcutaneously injected tumors in mice causes 50% of the tumors to completely regress. These tumors showed increased expression of cytokines and T-cell infiltration, which was completely reversed when T-cells were depleted in mice, suggesting that the immune system is of great importance in tumor clearance (Giampazolias et al., 2017). Specific inhibition of pro-apoptotic caspases improves cancer cell death by recruiting immune cells to the tumor site. This shows that there is a potential to significantly improve cancer therapy by changing the way cells die from an immunosilent to immunogenic phenotype.

AUTHOR CONTRIBUTIONS

Both authors contributed to the research, writing, and editing of the manuscript.

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MINI REVIEW
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Keeping Cell Death in Check: Ubiquitylation-Dependent Control of TNFR1 and TLR Signaling

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Pro-inflammatory signaling pathways, induced by pathogens, tissue damage or cytokines, depend on the ubiquitylation of various subunits of receptor signaling complexes, controlled by ubiquitin ligases and deubiquitinases. Ubiquitylation sets the stage for the activation of kinases within these receptor complexes, which ultimately regulate pro-inflammatory gene expression. The receptors, which transduce pro-inflammatory signals, can often induce cell death, which is controlled by ubiquitylation as well. In this review, we discuss the key role of ubiquitylation in pro-inflammatory signaling by TNFR1 and TLRs and its role in setting the threshold for cell death induced by these pro-inflammatory triggers.

Keywords: TLR, TNFR1, TNF, ubiquitin, inflammation, apoptosis, necroptosis

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BUILDING UP SIGNALING COMPLEXES: UBIQUITYLATION IN IMMUNE RECEPTOR SIGNALING

Inflammation is essential for the initial response to a pathogen or to tissue damage. If a pathogen overcomes barrier tissues such as the skin or the intestinal epithelium, an inflammatory response is induced by cells of the innate immune system, such as macrophages and dendritic cells. These cells, but also epithelial cells, endothelial cells as well as fibroblasts express germline-encoded pattern recognition receptors (PRR), which recognize structures or factors typical for pathogenic microbes. Structures associated with pathogens such as bacteria or viruses (but not the host), collectively dubbed as pathogen associated molecular patterns (PAMPS), are recognized by transmembrane TLRs and C-type lectin receptors (CLRs), as well as by cytoplasmic

Abbreviations: BMDM, bone marrow derived macrophages; CYLD, cylindromatosis; DUB, deubiquitinase; FADD, fas -associated protein with death domain; FLIP, flice inhibitory protein; HOIL-1, haem-oxidized IRP2 ubiquitin ligase-1; HOIP, HOIL-1 interacting protein; IAP, inhibitor of apoptosis; IKK, IκB kinase; IL, Interleukin; IRAK, interleukin-1 receptor associated kinase; LPS, lipopolysaccharide; LUBAC, linear ubiquitin chain assembly complex; MAPK, mitogen activated protein kinase; MK2, mitogen activated protein kinase-activated protein kinase 2; MKK, MAPK kinase; MLKL, mixed lineage kinase like; MLKL, mixed lineage kinase like; MyD88, Myeloid differentiation primary response 88; NEMO, NF-κB essential modulator; NF-κB, nuclear factor -κB; OTULIN, OTU deubiquitinase with M1-linkage specificity, SPATA2, spermatogenesis associated protein 2; PIM, PUB interacting motif; PUB, peptide:N-glycanase/UBA- or UBX-containing proteins; RIPK1, receptor interacting kinase 1; SC, signaling complex; SHARPIN, SHANK associated RH domain interactor; TAK1, TGF-beta activated kinase; TBK TANK binding kinase; TBK1, TANK binding kinase; TLR, Toll-like receptor; TNF, tumor necrosis factor; NOD, Nucleotide-binding oligomerization domain-containing protein; TRADD, Tumor necrosis factor receptor type 1-associated death domain protein; UBA, ubiquitin associated; UBD, ubiquitin binding domain.

receptors such as the Retinoic acid-inducible gene (RIG)-I-like receptors (RLRs), DNA sensing receptors (DSRs), and NOD-like receptors (NLRs).

Likewise, molecules indicative of cell damage (danger associated molecular patterns, DAMPS) are recognized by TLRs or IL-1 family receptors (Takeuchi and Akira, 2010; Martin, 2016).

The engagement of PRRs ultimately induces the transcription of genes encoding cytokines, chemokines and interferons. PRR signaling requires ubiquitin ligases, generating ubiquitin chains with different linkage types. The pro-inflammatory cytokines induced by PRR activation, such as TNF or IL-1, again heavily depend on poly-ubiquitylation for the signaling pathways they induce in target cells and tissues. As described in this article, ubiquitylation also has a key role in preventing cell death induced by these triggers.

Ubiquitylation is a posttranslational protein modification by which ubiquitin, a small protein, is reversibly linked to protein substrates. Ubiquitin ligases enzymatically link the C-terminus of ubiquitin, a small, 8 kD protein, to the ε-amino group of a lysine of a given protein, including ubiquitin itself. In addition, ubiquitin can be linked to the free amino group of the methionine of another ubiquitin. This energy-requiring transfer is accomplished in three separate steps. Firstly, upon ATP consumption, a free ubiquitin is transferred to a cysteine of an ubiquitin-activating enzyme, forming a high-energy thioester (E1). Ubiquitin is then transferred from E1 to an E2 enzyme. The human genome encodes only two E1 ligases, but about fourty E2 conjugating enzymes, which exhibit a first level of specificity. The transfer of ubiquitin from E2 to the target protein is mediated by one of several hundred different substrate-specific E3 ligases. E3 ubiquitin ligases can mediate the attachment of either single ubiquitins to target proteins, resulting in protein mono-ubiquitination, or using an attached ubiquitin as an anchor to generate extended polyubiquitin chains (Ebner et al., 2017). The primary structure of ubiquitin contains seven lysines (K6, K11, K27, K29, K33, K48, and K63), the ε-amino group of which can be linked to the C-terminus of an incoming ubiquitin, thereby creating an isopeptide bond. In addition, the free amino-terminus of ubiquitin (M1) can be linked with the C-terminus of another ubiquitin, resulting in a peptide bond, which generating M1-linked ubiquitin chains. Thus, depending on the linkage specificity of the respective E3 ubiquitin ligase, poly-ubiquitin chains with different inter-ubiquitin linkages can be generated (Komander and Rape, 2012; Akutsu et al., 2016).

Ubiquitylation is a reversible posttranslational modification and poly-ubiquitin chains are disassembled by ubiquitin-specific proteases. These deubiquitinases, just as ubiquitin ligases, exhibit substrate specificity with regard to the linkage of the ubiquitin bond they hydrolyze. About one-hundred deubiquitinases exist in humans, which attenuate or erase the signal mediated by ubiquitin ligases (Mevissen and Komander, 2017).

Poly-ubiquitin chains exhibit their function through their recognition by proteins containing ubiquitin binding domains (UBDs), which bind and thereby 'read' those structures (Dikic et al., 2009). UBDs are specific for the structure of ubiquitin chains, depending on the linkage type, or they recognize linker regions directly, and their affinity may depend on the length of the ubiquitin chain (Rahighi and Dikic, 2012). In analogy to chromatin modifiers in epigenetics, ubiquitin ligases can be considered as "writers" and UBDs as "readers." Accordingly, deubiquitinases function as "erasers" (Komander and Rape, 2012).

The recognition of ubiquitylation by UBDs triggers diverse biological processes. The attachment of K48-linked polyubiquitin chains to a protein is a signal for its degradation by the proteasome. K63- and M1-linked polyubiquitin chains have various functions, such as in DNA repair or for the activation of kinases in receptor complexes (Ulrich and Walden, 2010; Jiang and Chen, 2011). The role of K6-, K11-, K27-, K29-, and K33-linked ubiquitylation is comparably less well understood and reviewed elsewhere (Akutsu et al., 2016).

For both TLR and TNF signaling, K63- and M1-linked polyubiquitination is essential for the formation of the signaling complexes, which ultimately mediate NF-κB and MAPK activity and pro-inflammatory gene activation. As a general principle, the stimulation of an innate immune receptor induces the recruitment (via different adaptors) of E3-ligases (such as TRAF6 or cIAP1/2), which conjugate proteins in the receptor complex with K63-linked polyubiquitin chains. Adapter proteins which recognize these chains mediate the recruitment of the kinase TAK1, the activity of which is central to TLR and TNF signaling (Jiang and Chen, 2011). In addition, K63-linked polyubiquitin chains recruit the LUBAC complex, an M1-linkage specific ubiquitin ligase, to the receptor complex. LUBAC decorates proteins in the complex with M1-linked polyubiquitin chains, often by extending K63-linked with M1-linked polyubiquitin chains (Cohen and Strickson, 2017). This promotes the recruitment of the IKK complex, which comprises the kinases IKKα, IKKβ and the adapter NEMO/IKKγ, to the receptor complex, via the interaction of its subunit NEMO with M1-linked polyubiquitin chains. Being in the proximity of TAK1, IKKs are activated by TAK1 through direct phosphorylation. Once activated, the IKKs phosphorylate IκBα, which triggers its K48-linked polyubiquitylation and degradation, thereby liberating NF-κB transcription factors. In addition, TAK1, by phosphorylation of MKKs, activates p38 and JNK signaling, and thereby AP-1 transcription factor activity (Hrdinka and Gyrd-Hansen, 2017). Ubiquitylation in these receptor complexes, is counteracted by deubiquitinases, including CYLD, which exhibits linkage specificity for K63-and M1-linked ubiquitin chains, and OTULIN, which specifically disassembles M1-linked ubiquitin chains (Mevissen and Komander, 2017).

TNFR1 Signaling Controlled by Ubiquitylation

Induction of PRRs result in the induction of various pro-inflammatory cytokines, and a crucial player among those is TNF. TNF is made by macrophages, monocytes, dendritic cells as well as activated lymphocytes, but also by non-professional immune cells such as epithelial and endothelial cells (Takeuchi and Akira, 2010). TNF-induced inflammation is beneficial in containing pathogens, but also has a key role for chronic inflammatory

diseases. In consequence, TNF-inhibitory molecules proved to be successful for the treatment of inflammatory diseases, such as rheumatoid arthritis or psoriasis (Udalova et al., 2016).

There are two receptors for TNF. While TNFR1 is expressed ubiquitously, expression of TNFR2 is restricted to immune cells and endothelia (Wajant et al., 2003). In general, TNF triggers inflammation in tissues by inducing pro-inflammatory gene expression in target cells. It does so by induction of NF-κB and MAPK, which are activated through ubiquitylation-dependent signaling complexes. Upon ligation of the TNFR1, TNFR1 complex I is formed by the interaction of the death domain (DD) of the receptor with the DDs of the kinase RIPK1 and the adaptor TRADD, thereby independently recruiting both proteins to the receptor. TRADD recruits the protein TRAF2 and thereby the E3 ligases cIAP1/2 which decorate RIPK with K63-linked polyubiquitin chains (Bertrand et al., 2008; Varfolomeev et al., 2008).

Both free and attached K63-linked polyubiquitin chains have been reported to activate the kinase TAK1 (Wang et al., 2001; Xia et al., 2009). This is mediated by adaptor proteins (TAB2 and TAB3), which recognize K63-linked poly-ubiquitin chains (but not M1-linked polyubiquitin chains) via their zinc-finger UBD, activating the kinase TAK1 (Wang et al., 2001; Kanayama et al., 2004; Kulathu et al., 2009; Xia et al., 2009). TAK1 activation is a key event for pro-inflammatory gene expression, as TAK1 activates the IKK complex, through IKKβ phosphorylation, and MAPK signaling by the phosphorylation of MKK3, MKK6, and MKK4 (Moriguchi et al., 1996; Shirakabe et al., 1997; Lee et al., 2000; Wang et al., 2001).

However, full activation of the IKK complex requires the activity of another ubiquitin ligase, the LUBAC complex. LUBAC consists of the enzymatic subunit HOIP and the proteins HOIL-1 and SHARPIN and is the only identified E3 ligase capable of generating M1-linked polyubiquitin chains. K63-linked polyubiquitylation is the prerequisite for the association of LUBAC to the receptor complex, as the absence of cIAP1/2 abrogated the recruitment of LUBAC to the TNFR1 signaling complex (TNFR1-SC) (Haas et al., 2009). LUBAC was shown to interact with polyubiquitin via the Npl4 zinc finger (NZF) domains of HOIP and of HOIL-1 (Haas et al., 2009; Peltzer et al., 2018). Once associated with the TNFR1-SC, LUBAC was demonstrated to attach M1-linked polyubiquitin chains to RIPK1, TRADD and TNFR1 itself (Gerlach et al., 2011; Draber et al., 2015). There is convincing evidence that, upon TNF stimulation, LUBAC extends preexisting K63-linked poly-ubiquitin chains in the TNFR1-SC with M1-linked polyubiquitin chains, and those hybrid chains were attached to RIPK1 upon TNFR1 stimulation (Emmerich et al., 2016). In addition, TNFR1 itself is decorated with M1-linked polyubiquitin chains, possibly attached to multi-monoubiquitinated TNFR1.

M1-linked polyubiquitin chains are bound with high affinity by the IKK complex member NEMO, through its UBAN (ubiquitin-binding domain present in ABINs and NEMO), thereby mediating the association of the IKK complex with the TNFR1-SC (Lo et al., 2009; Rahighi et al., 2009). NEMO itself was shown to be subject to M1-linked polyubiquitylation by LUBAC (Tokunaga et al., 2009, 2011; Gerlach et al., 2011), while it was subsequently demonstrated that M1-linked ubiquitylation of NEMO is comparably low, questioning its relevance for signaling (Clark et al., 2013; Emmerich et al., 2013). The interaction of NEMO with K63-/M1-linked hybrid chains might bring the IKK complex, recruited to M1-linked polyubiquitin sections, into proximity of the TAB/TAK1 complex, associated with K63-linked polyubiquitin chain segments, facilitating the activating phosphorylation of the IKKs by TAK1 (Wang et al., 2001; Zhang et al., 2014; Cohen and Strickson, 2017). The activated IKK complex phosphorylates the protein IκBα, which triggers the K48-linked polyubiquitylation and degradation. Freed from IκBα, a NF-κB dimer can enter the nucleus and promote the transcription of genes promoting inflammation. MKKs activated by TAK1 induce the MAP Kinases JNK and p38 and activate the transcription factor AP1, which also induces the transcription of pro-inflammatory cytokines (Moriguchi et al., 1996; Shirakabe et al., 1997; Wang et al., 2001).

In cells stimulated by TNF, this transcriptional program induces a large number of genes, which bring about the changes typical for inflamed tissues. In addition, TNF ubiquitylation-dependently induces genes, which promote cell survival such as c-FLIP, as will be described in detail below.

The disassembly of ubiquitin chains by DUBs has a crucial role in the regulation of TNFR1 signaling, either by mediating destabilization of the receptor complex, attenuating the signal, or by trimming/editing polyubiquitin chains. The K63-and M1-linkage specific DUB CYLD was shown to negatively regulate TNF-induced NF-κB and MAPK activation (Brummelkamp et al., 2003; Kovalenko et al., 2003; Trompouki et al., 2003; Lee et al., 2013). CYLD is recruited to the TNFR1-SC along with the LUBAC complex (Takiuchi et al., 2014; Draber et al., 2015; Hrdinka et al., 2016). This requires the adaptor protein SPATA2, bridging the interaction between CYLD and HOIP (Elliott et al., 2016; Kupka et al., 2016; Schlicher et al., 2016; Wagner et al., 2016; **Figure 1A**). CYLD was shown to counteract M1- and K63-linked ubiquitylation of RIPK1, TRADD and TNFR1 (Draber et al., 2015; Hrdinka et al., 2016). Consistently, the absence of SPATA2 was shown to exhibit increased M1-ubiquitylation in the TNFR1-SC (Schlicher et al., 2016). However, a reduction of RIPK1 ubiquitylation as well as K63- and M1-linked polyubiquitin was observed in cells lacking SPATA2 by other studies (Draber et al., 2015; Schlicher et al., 2016; Wei et al., 2017). While the reasons for those inconsistencies are not clear, different effects of CYLD and SPATA2 on ubiquitylation in the TNFR1-SC may hint at yet unidentified SPATA2 functions, which may not be directly linked to CYLD.

OTULIN specifically degrades M1-linked polyubiquitin chains, generated by LUBAC (Keusekotten et al., 2013; Rivkin et al., 2013). Just like SPATA2, OTULIN interacts with LUBAC through a PUB interacting motif (PIM), which associates with the PUB domain of HOIP, implying a competition of SPATA2 and OTULIN for binding to HOIP (Elliott et al., 2014, 2016; Schaeffer et al., 2014; Takiuchi et al., 2014; Schlicher et al., 2016; Wagner et al., 2016). However, in contrast to SPATA2 and CYLD, OTULIN was not found to be recruited with LUBAC to the TNFR1-SC upon TNFR1 stimulation. Accordingly, the absence of OTULIN did not affect M1-linked polyubiquitylation

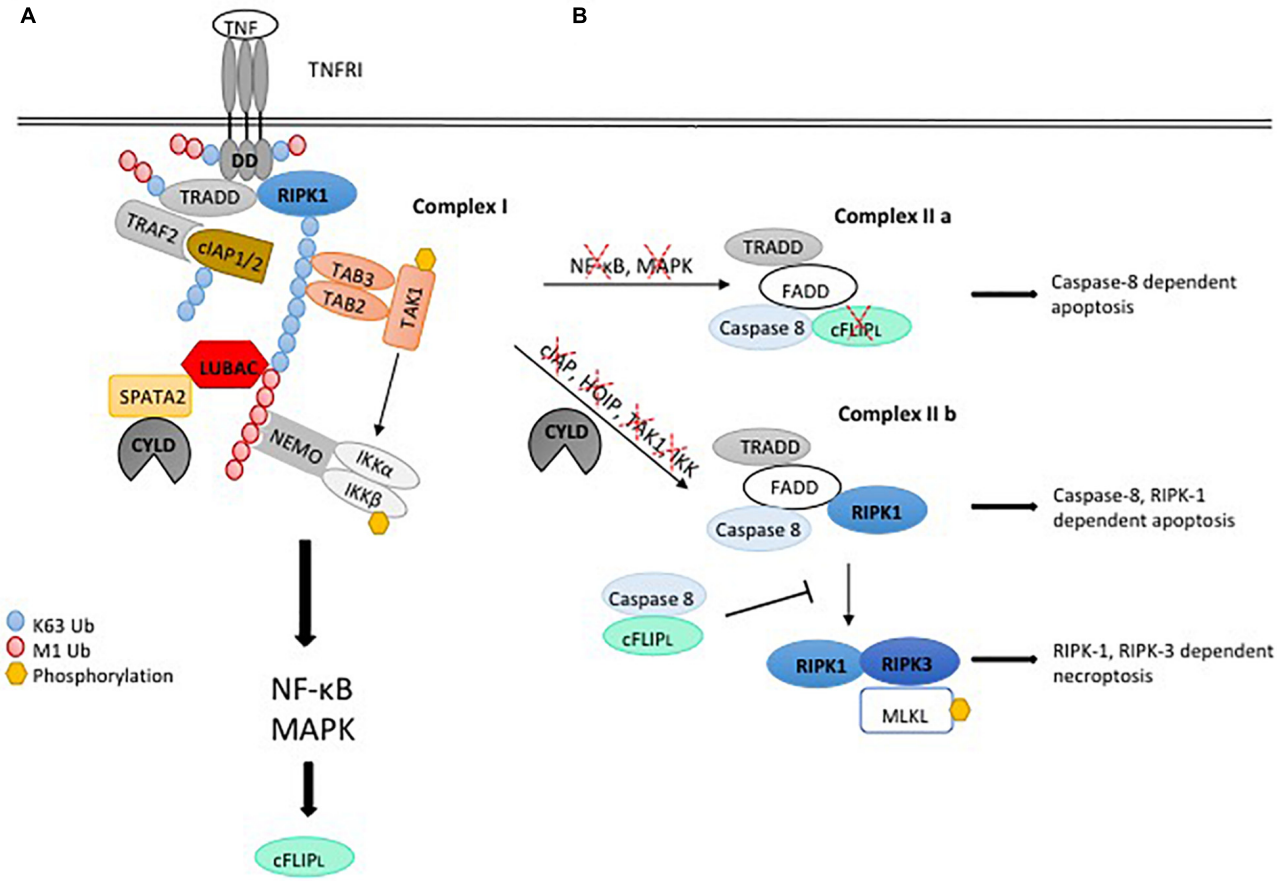

FIGURE 1 | Ubiquitylation regulating pro-inflammatory signaling and cell death induction by the TNFR1-SC. **(A)** Upon binding of TNF to TNFRI, complex I is assembled by binding of the adaptor protein TRADD and the kinase RIPK1 to the receptor. Recruitment of TRAF2 and the E3 ligase cIAP1/2 leads to K63-linked ubiquitylation within the complex, serving as a platform for binding of TAB2/3 and TAK1 to mediate activation of MAPK signaling. In addition, K63-linked poly-ubiquitin chains recruit LUBAC, which mediates M1-linked ubiquitylation of different components within the complex, promoting association of NEMO and activation of IKKα/β and NF-κB. Assembly of the TNFR1-SC is negatively regulated by the DUB CYLD, which interacts with the LUBAC component HOIP via the bridging protein SPATA2 and is recruited to the TNFR1-SC, thereby diminishing M1- and K63-linked ubiquitin chains. **(B)** TNFR1 stimulation induces functional variants of the cell death promoting complex II, consisting of the adaptor TRADD and FADD, RIPK1, caspase-8 and its paralog c-FLIP. Abrogation of NF-κB induced gene expression permits caspase-8 activation in complex II a, leading to cell death by apoptosis. When ubiquitylation of the TNFR1-SC is compromised, caspase-8-activating complex II b is formed, which induces apoptosis depending on RIPK1 kinase activity. Upon loss of caspase activity, RIPK1 associates with RIPK3, which phosphorylates and activates MLKL, leading to necroptotic cell death.

at the TNFR1-SC (Draber et al., 2015). However, different studies indeed detected OTULIN in TNFR1-SC pulldowns (Schaeffer et al., 2014; Wagner et al., 2016), and it was also shown that OTULIN disassembles M1-linked ubiquitin in receptor complexes (Fiil et al., 2013; Keusekotten et al., 2013). This is compatible with a concept that OTULIN functionally counteracts LUBAC, thereby limiting the activation of NF-κB and MAPK signaling. Consistently, mice with acute ablation of OTULIN in bone marrow cells or myeloid cells exhibited massive TNF-dependent systemic or chronic inflammation, respectively, reflecting patients with defective OTULIN, which exhibit multi-organ inflammation (Damgaard et al., 2016).

This view was challenged recently. It had been observed previously that LUBAC ubiquitylates itself, and that OTULIN deubiquitylates LUBAC components (Fiil et al., 2013; Keusekotten et al., 2013; Elliott et al., 2014; Draber et al., 2015; Hrdinka et al., 2016). In a recent study, knock-in MEF expressing catalytic-inactive OTULIN exhibited enhanced LUBAC auto-ubiquitylation with reduced abundance of HOIL-1 HOIP and SHARPIN. The authors reported reduced stability of TNFR1 complex I and enhanced formation of TNFR1 complex II in these cells (Heger et al., 2018). These data suggest that OTULIN promotes LUBAC activity and pro-inflammatory signaling, while preventing cell death, as described further below.

A third DUB implicated in TNFR1 signaling, A20, was recently shown to exhibit its function independently of its enzymatic activity. The recruitment of A20 to the TNFR1-SC required M1-polyubiquitin chains and actually resulted in protection of these chains from degradation. This was shown to depend on the ZnF7 zinc-finger domain of A20 providing the

interaction with polyubiquitin chains and possibly shielding them from binding proteins activating gene expression (Nishimasu et al., 2012; Verhelst et al., 2012; Draber et al., 2015). Consistent with a minor role of A20 enzymatic activity, mice expressing DUB-inactive A20 mutant exhibit normal TNF-induced NF-κB signaling (De et al., 2014).

TLR Signaling Controlled by Ubiquitylation

Pattern recognition receptors of the toll-like-receptor (TLR) family are germline-encoded and expressed by professional innate immune cells such as macrophages, monocytes and dendritic cells, but also epithelial cells, endothelial cells and fibroblasts. They recognize, through their leucine-rich ectodomains, an array of bacterial or viral structures, including lipids, proteins and nucleic acids. TLRs are located either on the plasma membrane or on endosomes and recognize bacterial patterns such as peptidoglycan through the TLR1/2 heterodimer, LPS through TLR4, flagellin through TLR5, and CpG DNA is detected by TLR9. Likewise, viral, double-stranded RNA is recognized by endosomal TLR3 (Takeuchi and Akira, 2010).

Toll-like receptors signal through two different pathways, depending on whether MyD88 or TRIF is recruited as an adapter to the receptor. Signaling of all TLRs except TLR3 requires the adapter protein MyD88, while signaling by TLR3 depends on TRIF. TLR4 is the only TLR, which signals via both MyD88- and TRIF-dependent pathways (Yamamoto et al., 2003a).

The MyD88-dependent pathway is also employed by receptors for cytokines of the IL-1 family (O'Neill, 2008). TLRs and IL-1 receptor share the Toll -and IL-1 (TIR) domain, which interacts with the TIR domain of the adapter MyD88 upon receptor activation. In turn, the DD of MyD88 recruits, through a homotypic interaction, the kinase IRAK4 via its DD, which promotes the additional recruitment of the kinases IRAK1 and IRAK2. The IRAKs now dissociate from the receptor complex and interact with TRAF6, an E3 ubiquitin ligase (Takeuchi and Akira, 2010). By cooperating with the E2 ligases Ubc13 and Uev1A, TRAF6 auto-ubiquitylates, but also generates K63-linked poly-ubiquitin chains which were shown to be attached to IRAK1, IRAK4, and MyD88 (Emmerich et al., 2013). In addition, IRAK1 and IRAK4 can phosphorylate the E3 ligase Pellino1, which also generates K63-linked poly-ubiquitin chains (Ordureau et al., 2008). Those protein-anchored and/or free K63-linked polyubiquitin chains provide the docking sites for the zinc-finger UBD of the adaptors TAB2/3, which bring the kinase TAK1 to the complex and activate it, thereby mediating activation of MAPK signaling (Wang et al., 2001; Kanayama et al., 2004; Kulathu et al., 2009; Xia et al., 2009).

As for TNFR1 signaling, LUBAC is required for full activation of NF-κB by the MyD88-dependent pathway (Cohen and Strickson, 2017). In further similarity to the TNFR1-SC, LUBAC extends preexisting K63-linked poly-ubiquitin chains in the complex with M1-linked polyubiquitin chains (Emmerich et al., 2013). M1-linked polyubiquitin chains, recognized by NEMO, promote the activation of the IKK complex (Lo et al., 2009; Rahighi et al., 2009), by a mechanism which was demonstrated to depend on TAK1-mediated phosphorylation and IKK auto-phosphorylation (Zhang et al., 2014; **Figure 2A**).

A different TLR signaling pathway, which is independent from the adaptor MyD88, instead requires the adapter protein TRIF (Yamamoto et al., 2003a). This pathway is employed by TLR3 and TLR4 upon binding of their ligands double-stranded RNA and LPS, respectively. TLR4 additionally requires the adaptor TRAM for the interaction with TRIF (Yamamoto et al., 2003b). TRIF is recruited to TLR3 or TLR4 through its TIR domain, interacting with the TIR domain of the activated receptor. Via its RHIM domain, TRIF recruits the kinase RIPK1 through a homotypic RHIM:RHIM interaction to the receptor, which is required for downstream NF-κB signaling (Meylan et al., 2004; Cusson-Hermance et al., 2005). The further assembly of the receptor complex is mediated by ubiquitylation, by the TRIF-mediated recruitment of the E3-ligases TRAF6 and cIAP1/2. These E3 ligases generate K63-linked polyubiquitin chains, activating the kinase TAK1, which in turn activates IKK and MAPK as described above (Cusson-Hermance et al., 2005; Shim, 2005). In addition, M1-linked ubiquitylation mediated by LUBAC was recently shown to be essential for TLR3 signaling. LUBAC was demonstrated to be recruited to activated TLR3, generating M1-linked polyubiquitin chains, which was essential for the activity of IKKs and MAPKs and the transcriptional induction of TNF or IL-8, as well as secretion of IFN-β. Interestingly, this study showed that the TLR3 receptor SC contains FADD and caspase-8 (Zinngrebe et al., 2016). As in the case of MyD88-mediated signaling, the presence of hybrid K63/M1-linked polyubiquitin chains was observed upon stimulation of TLR3, some of which were attached to RIPK1, while most of these chains were not linked to the kinase (Emmerich et al., 2016; **Figure 2B**). In addition to NF-κB and MAPK/AP-1 activation, TRIF mediates the induction of IFN-α and IFN-β, through phosphorylation of the transcription factors IRF-3 and IRF-7 by the IKK-related kinases TBK-1 and IKKε (Häcker and Karin, 2006). This requires the recruitment to TLR3 of the E3 ligase TRAF3 and its auto-ubiquitylation, resulting in further recruitment of the adaptors TANK, NAP1, and SINTBAD, which in turn recruit TBK-1 and IKKε to the receptor complex (Takeuchi and Akira, 2010).

The role and the regulation of deubiquitylases (DUBs) in MyD88 and TRIF dependent signaling is less well explored. In the MyD88 dependent pathway, CYLD was shown to reduce TLR2-mediated TRAF6 ubiquitylation and NF-κB activation (Kovalenko et al., 2003; Trompouki et al., 2003; Yoshida et al., 2005; Lim et al., 2007; Komander et al., 2009; Ritorto et al., 2014). More recently, CYLD was shown to reduce K63-linked polyubiquitylation of MyD88, thereby limiting MyD88-dependent cytokine induction and inflammation *in vivo* (Lee et al., 2016). This is consistent with data generated with CYLD deficient BMDM, which were shown to exhibit increased JNK activation upon stimulation with LPS (Zhang et al., 2006). In line with the role of SPATA2 being required for CYLD activity in receptor complexes, SPATA2 deficiency resulted in increased JNK signaling and cytokine expression of BMDM treated with LPS (Wei et al., 2017). However, it is noteworthy

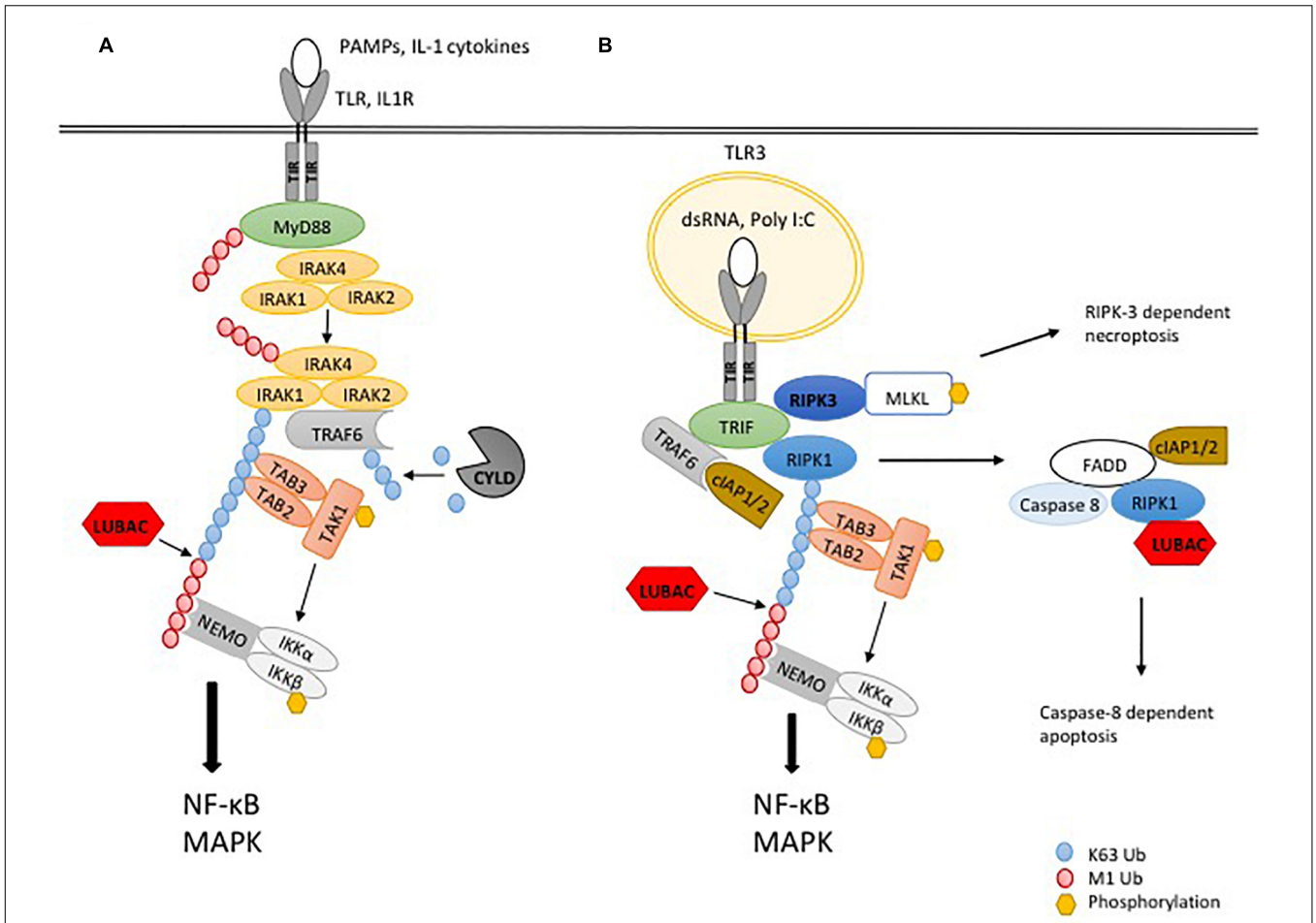

FIGURE 2 | Ubiquitylation in TLR signaling. **(A)** The recognition of PAMPs or IL-1α/β by the respective TLR or IL-1R leads to binding of the adaptor protein MyD88, which interacts with the TIR domain of the receptor, leading to recruitment of the kinases IRAK4, IRAK1 and IRAK2. Dissociation of IRAK1 and IRAK2 and their interaction with the ubiquitin ligase TRAF6 initiates the formation of K63-linked poly-ubiquitin chains, serving as a platform for recruitment of TAB2 and TAB3 to activate the kinase TAK1 and in turn MAPK signaling. In addition, the ubiquitin ligase LUBAC extends K63-linked poly-ubiquitin chains with M1-linked poly-ubiquitin chains, resulting in recruitment and activation of the IKK complex through its adaptor NEMO. Phosphorylation of IKKβ by TAK1 leads to activation of NF-κB and pro-inflammatory signaling. The DUB CYLD counteracts ubiquitylation by TRAF6. **(B)** TLR3 signaling works independently of MyD88 but requires the adaptor TRIF. After binding of dsRNA or Poly I:C to the receptor, the kinase RIPK1 is recruited. The ubiquitin ligases TRAF6 and cIAP1/2 generate K63-linked poly-ubiquitin chains to activate TAK1 and downstream MAPK. To induce the activation of IKKs and NF-κB, LUBAC is recruited to the complex to generate M1-linked poly-ubiquitin chains. If K63- and/or M1-ubiquitylation are blocked, apoptosis is induced by a complex including FADD, caspase-8 and RIPK1.

that another study did not find that the absence of CYLD in BMDM affected LPS-induced NF-κB and MAPK signaling (Reiley et al., 2006).

Together, the generation of ubiquitin chains is key for the induction of pro-inflammatory gene expression by TNFR1, IL-1R, and TLRs. This is reflected by genetic defects in humans, which affect M1-linked ubiquitylation, with severe pathologic consequences. Deficiency in HOIL-1 resulted in invasive pyogenic bacterial infection, likely due to an impaired induction of NF-κB, but also in autoinflammation and glycogen storage disease (Boisson et al., 2012). Similar defects were reported for a patient with a hypomorphic HOIP mutation (Boisson et al., 2015). Of note, the respective defects resulted in a destabilization of all the LUBAC components in these patients. While the observed pathologies can be explained by the inability to raise a pro-inflammatory response, an alternative explanation could be an enhanced susceptibility for cell death, as described below.

UNLEASHING THE CELL DEATH MACHINERY: REGULATION BY (DE-)UBIQUITINATION

Cell Death Induced by TNFR1

The default outcome of the signaling pathways described above is the induction of transcriptional programs, which regulate inflammation. However, upon certain conditions, inflammatory triggers can result in the induction of cell death, and the

regulation of ubiquitylation is central in the decision for or against cell death. The predominant forms of cell death induced by immune/cytokine receptors are apoptosis and necroptosis. Both forms of cell death are triggered by the formation of protein complexes, which provide the platforms to activate the proteolytic activity of caspase-8 or the kinase activity of RIPK3.

TNFR1-induced apoptosis requires the activation of caspase-8 by homodimerization, which results in the cleavage and thereby activation of executioner caspases-3/-7 (Boatright et al., 2003). Caspase activation triggers a controlled form of cell death, leaving the plasma membrane intact and surrounding cells undisturbed. Thus, apoptosis is in general not expected to be pro-inflammatory or immunogenic.

In contrast, necroptosis requires RIPK3 activation by auto-phosphorylation, which is induced by dimerization via its RHIM and kinase domains (Cho et al., 2009; He et al., 2009; Raju et al., 2018). RIPK3 phosphorylates and activates the pseudokinase MLKL, which mediates Ca^{2+} influx and plasma membrane rupture (Sun et al., 2012; Cai, 2013; Khan et al., 2014). Necroptosis is morphologically indistinguishable from uncontrolled necrosis, with spillage of cytoplasmic contents into the environment of a dying cell (Zhang et al., 2016).

Some 20 years ago it had been observed that necrotic cell death induced by TNF occurs in absence of caspase activity (Vercammen et al., 1998). TNF-induced programmed necrosis is indeed repressed by the proteolytic activity of caspase-8, which is functionally separate from the apoptosis-inducing caspase-8 activity, exhibiting a different substrate specificity (Pop et al., 2011). Upon heterodimerization with $cFLIP_L$, caspase-8 does not induce the activation of executioner caspases and apoptosis, but instead cleaves pro-necroptotic proteins such as RIPK1, RIPK3, and CYLD (Feng et al., 2007; O'Donnell et al., 2011; Oberst et al., 2011; Zhang et al., 2019). This pro-survival caspase-8 activity is the reason for the mid-gestation lethality of caspase-8 deficient mice, which was rescued in mice expressing a cleavage-deficient caspase-8 allele (which cannot undergo processing to its pro-apoptotic form) (Varfolomeev et al., 1998; Kang et al., 2008). The rescue of caspase-8 knockout mice upon additional loss of RIPK3 or MLKL provided genetic evidence for the inhibition of necroptosis by caspase-8 (Kaiser et al., 2011; Oberst et al., 2011; Alvarez-Diaz et al., 2016).

TNFR1 stimulation induces cell death via a signaling complex, which is different from the TNFR1-SC described above and therefore dubbed complex II. This complex is not associated with the receptor and comprises RIPK1, the adaptors TRADD and FADD, the initiator caspase-8 as well as the caspase-8 paralog c-FLIP (Micheau and Tschopp, 2003). It constitutes the platform to activate caspase-8 by induced proximity. However, as mentioned above, cell death upon TNFR1 stimulation is not the default outcome, because the TNFR1-SC transcriptionally induces the expression of pro-survival molecules such as c-IAP2 and c-FLIP, the latter coming in two splice forms, $c\text{-}FLIP_s$ and $c\text{-}FLIP_L$ (Chu et al., 1997; Micheau et al., 2001). The $cFLIP_{S/L}$ molecules heterodimerize with caspase-8 and thereby inhibit the pro-apoptotic activity of caspase-8 (Hughes et al., 2016). Thus, TNFR1-SC signaling activates a transcription-dependent anti-apoptotic checkpoint, by the transcriptional induction of pro-survival proteins, preventing pro-apoptotic caspase-8 activation (**Figure 1B**).

Thus, any disturbance of the TNFR1-SC, interfering with M1- or K63-linked polyubiquitination, and/or the activity of the kinases, which require polyubiquitin chains for their activation, will reduce or abrogate the transcription-inducing activity of NF-κB and MAPK. Thereby, expression of pro-survival proteins such as cFLIP is compromised, allowing caspase-8 homodimerization in a complex, which is defined as TNFR1 complex IIa in this context. Many pathogens interfere with and subvert pro-inflammatory signaling [reviewed in Reddick and Alto (2014)], which may accordingly result in cell death. This checkpoint can be experimentally suppressed by inhibitors of transcription or translation such as actinomycin D or cycloheximide, permitting TNF to trigger apoptosis (Kreuz et al., 2001).

A different pathway for TNFR1-induced apoptosis critically depends on RIPK1, which is required for the activity of a death-inducing complex defined as complex IIb. This complex is composed of the proteins FADD, caspase-8 and RIPK1 (Wang et al., 2008). RIPK3 is likely also part of this complex, as it was shown to contribute to RIPK1-dependent apoptosis (Dondelinger et al., 2013). While RIPK1 has a scaffold function in TNFR1 complex I, which is independent of its kinase activity, its kinase activity is required for the activation of caspase-8 in TNFR1 complex IIb (Löder et al., 2012; Dondelinger et al., 2013). Therefore, the regulation of RIPK1 kinase activity, which induces its auto-phosphorylation on S166, is a critical checkpoint for TNF-induced apoptosis. In consequence, apoptosis controlled by this checkpoint can be prevented by RIPK1 inhibitors (Degterev et al., 2008). Moreover, provided that caspase-8 is inhibited, complex IIb can induce necroptosis. This also requires the kinase activity of RIPK1, which mediates the recruitment and activation of RIPK3 through the RHIM domains of both molecules (Cho et al., 2009; Li et al., 2012; Raju et al., 2018).

By default, upon TNFR1 stimulation, RIPK1 auto-activation is prevented by its ubiquitylation and phosphorylation (described in detail below). Thus, complex IIb induces cell death when, upon TNFR1 stimulation (i) the activity of E3 ligases such as the cIAPs and LUBAC is compromised or (ii.) the activity of RIPK1 inhibitory kinases, which are ubiquitylation-dependently recruited to and activated in the TNFR1-SC, is compromised (**Figure 1B**).

Accordingly, the loss of K63-linked polyubiquitylation upon TNFR1 activation results in RIPK1 dependent cell death. Treatment of cells with IAP inhibitors (SMAC mimetics, SM) results in K48-linked autoubiquitylation of cIAP1/2 and their rapid degradation (Petersen et al., 2007; Varfolomeev et al., 2007; Vince et al., 2007; Bertrand et al., 2008). In consequence, upon TNF/SM stimulation, RIPK1 ubiquitylation is reduced and RIPK1-dependent apoptosis ensues (Bertrand et al., 2008; Wang et al., 2008). In a physiological setting, cIAP1 can be degraded upon stimulation of the TNF-superfamily receptor FN14 by its ligand TNF-like weak inducer of apoptosis (TWEAK). Thereby, stimulation of FN14 and TNFR1 can cooperate to induce cell death which is blocked by RIPK1 inhibition (Vince et al., 2008; Wicovsky et al., 2009). Similar effects have been described

with TNFR2, which, upon stimulation with transmembrane TNF, triggers the cytosolic depletion of TRAF2/cIAP1/2 and cooperates with TNFR1 to induce apoptosis (Chan and Lenardo, 2000; Fotin-Mleczek et al., 2002). In similarity to K63-linked ubiquitylation, M1-linked polyubiquitylation by LUBAC is crucial for the prevention of cell death by TNF. Mice deficient for the LUBAC component SHARPIN (*cpdm* mice) develop TNF-dependent dermatitis and multi-organ inflammation, which can be rescued by heterozygosity of caspase-8 or keratinocyte-specific loss of FADD, combined with loss of RIPK3, indicating cell death as the cause of inflammation (HogenEsch et al., 1993; Gerlach et al., 2011; Ikeda et al., 2011; Kumari et al., 2014; Rickard et al., 2014). This was shown to depend on the kinase activity of RIPK1, as $RIPK^{K45R}$ knock-in *cpdm* mice were protected from multi-organ inflammation, and $RIPK^{K45R}$ cells were shown to be protected from necroptosis (Berger et al., 2014). Similarly, MEF lacking HOIP were shown to undergo apoptosis upon stimulation with TNF, which partly depended on RIPK1 activity (Peltzer et al., 2014). Likewise, TNF induced cell death in absence of the LUBAC component HOIL-1, which was in part dependent on RIPK1 (Peltzer et al., 2018).

Consistent with the notion that RIPK1 ubiquitylation prevents TNF-induced RIPK1 activity and cell death, RIPK1 deubiquitylation by CYLD was reported to be required for apoptosis induction by complex IIb (Hitomi et al., 2008; Wang et al., 2008). Underscoring the cell-death promoting role of CYLD, the adaptor for the recruitment of CYLD to the TNFR1-SC, SPATA2, was shown to promote RIPK1-dependent apoptosis (Schlicher et al., 2016; Wei et al., 2017). Similarly, both CYLD and SPATA2 were reported to promote RIPK1 activation and TNF- induced necroptosis (Hitomi et al., 2008; Wang et al., 2008; Kupka et al., 2016; Wei et al., 2017). Counteracting its role in promoting necroptosis, CYLD was shown to be a substrate of non-apoptotic caspase-8 activity (O'Donnell et al., 2011).

The role of OTULIN in the disassembly of M1-linked polyubiquitin chains and the regulation of RIPK1-dependent death appears to be more complex. OTULIN specifically degrades M1-linked polyubiquitin chains, implying that it functionally counteracts LUBAC, thereby promoting TNF-induced cell death. However, a recent study suggests that the M1-linked auto-ubiquitylation of LUBAC inhibits its function, and decreases the abundance of LUBAC components. OTULIN, by deubiquitylating LUBAC, was suggested to promote LUBAC activity and thereby prevent the TNF-induced formation of complex II and cell death (Heger et al., 2018). Accordingly, fibroblasts derived from mice, which homozygously express an inducible, catalytically inactive $OTULIN^{C129A}$ mutant, exhibited a substantial reduction of M1-linked polyubiquitin in the TNFR1-SC and enhanced formation of complex II and cell death upon treatment with TNF. The cell death was partly inhibited by RIPK1 inhibition, indicating that OTULIN activity maintains RIPK1-dependent and -independent pro-survival checkpoints. Consistently, the auto-inflammation in adult mice, which expressed inactive OTULIN, was dependent on cell death, as suggested by the finding that it was largely relieved by the combined loss of caspase-8 and RIPK3. This suggested that OTULIN, in similarity to LUBAC, prevents cell death (Heger et al., 2018). However, another study found that induced OTULIN deficiency in leukocytes did not result in cell death (Damgaard et al., 2016). Moreover, as cells expressing a patient-derived $OTULIN^{G218R}$ mutant conferring pathologic inflammation were not sensitized to TNF-induced cell death, it is not clear how hypomorphic OTULIN mutations found in patients compare to $OTULIN^{C129A}$ (Damgaard et al., 2019).

Together, K63-and M1-linked ubiquitylation in the TNFR1-SC is critical for the prevention of RIPK1-induced cell death. However, the specific requirement of RIPK1 ubiquitylation for the prevention of complex IIb formation was challenged by the finding that RIPK1 was ubiquitylated in complex II as well (Dondelinger et al., 2013; de Almagro et al., 2015). This raised the possibility that the restriction of RIPK1 activity depends on E3-ligases, but not on a direct effect of ubiquitylation on RIPK1.

Ultimately, ubiquitylation in the TNFR1-SC promotes the activity of kinases such as TAK1, IKKκ/β, or p38. Indeed, a number of recent studies showed that the inhibitory phosphorylation of RIPK1 by those ubiquitylation-dependent kinases prevents RIPK1 activity. A first indication came from the finding that TNFR1 ligation in the absence of TAK1 activity results in rapid apoptosis, which was dependent on RIPK1 kinase activity (Dondelinger et al., 2013). Interestingly, TNF-induced cell death upon cIAP inhibition could be reduced by knockdown of CYLD, while cell death by TAK1 inhibition was independent of CYLD, indicating that TAK1 represses RIPK1 activity downstream of ubiquitylation events. Indeed, NEMO/IKKα/β, the activity of which depends on TAK1, prevented RIPK1 activity and cell death independently of the induction of NF-κB (Legarda-Addison et al., 2009; Dondelinger et al., 2015). More recently it was demonstrated that IKKα/β phosphorylates S25 of RIPK1, thereby inhibitising its kinase activity. Accordingly, knock-in of a phospho-mimetic $RIPK1^{S25D}$ mutant prevented RIPK1 auto-phosphorylation and cell death upon TNFR1 stimulation and IKK inhibition (Dondelinger et al., 2019). Underscoring the relevance of this phosphorylation, mice carrying the SHARPIN cpdm mutation, when crossed to $RIPK1^{S25D}$ animals, were completely protected from multi-organ inflammation, in similarity to the protection provided by kinase dead RIPK1S45A (Berger et al., 2014).

In addition to IKK mediated phosphorylation, S321 and S336 of RIPK1 were shown to be phosphorylated in the cytosol by MK2, a downstream kinase of MAPK p38. While inactivation of MK2 by itself had no effect on TNF induced apoptosis, it further sensitized cells lacking IKK activity, or cells treated with SMAC mimetics, to TNF-induced RIPK1 activation, complex II formation and apoptosis (Dondelinger et al., 2017; Jaco et al., 2017; Menon et al., 2017). Consistently, stimulation of cells with TNF and TWEAK, resulting in the loss of TRAF2, reduced the activation of both IKK and MK2, permitting RIPK1 dependent cell death (Dondelinger et al., 2017).

Another study showed that the RIPK1 site shown to be targeted by MK2 by the studies above could be directly phosphorylated by TAK1 *in vitro*, however, the loss of S321 phosphorylation in cells lacking p38/MK2 activity possibly

suggests that the effect of TAK1 on this site is mostly via its downstream kinase p38/MK2 (Dondelinger et al., 2015; Geng et al., 2017; Jaco et al., 2017; Menon et al., 2017).

More recently it was demonstrated that the kinases TBK1 and IKKε are, dependent on LUBAC-mediated M1-linked ubiquitylation and NEMO, recruited to the TNFR1-SC. While these kinases exhibited limited effects on TNF-induced gene expression, they were required to prevent TNF-induced cell death (Lafont et al., 2018; Xu et al., 2018). Direct phosphorylation of RIPK1 by TBK1/IKKε was suggested by RIPK1 gel shifts or the loss of phosphorylation of RIPK1S189 in cells upon TBK1/IKKε inhibition (Lafont et al., 2018; Xu et al., 2018). Furthermore, TNF-induced cell death of TBK1$^{-/-}$ cells required RIPK1 kinase activity (Xu et al., 2018). Here, inhibition or loss of either TBK1 or IKKα/β (targeting different sites in RIPK1) permitted TNF-induced apoptosis, indicating that for prevention of RIPK1 mediated apoptosis, the simultaneous inhibitory phosphorylation of RIPK1 on different sites must be maintained (Lafont et al., 2018). Interestingly, IKKε had previously been reported to phosphorylate and inactivate CYLD, raising the possibility that the negative regulation of CYLD by IKKε also has a role in the prevention of RIPK1 dependent cell death in the context of inflammation as well (Hutti et al., 2009).

A recent study suggested that the prevention of RIPK1 activity by ubiquitylation does not necessarily depend on ubiquitylation-dependent kinases such as TAK1, IKK or p38/MK2. Instead, ubiquitylation, mediated by cIAP1, directly controlled RIPK1 activity. A point mutation in the UBA domain cIAP1, resulted in reduced interaction with TRAF2, but mediated otherwise normal NF-κB and MAPK activation and NIK degradation, indicating regular activation of the upstream kinases. Nevertheless, the reduced cIAP activity rendered cells more sensitive to TNF-induced cell death, due to reduced K48-linked polyubiquitylation of RIPK1, as well as reduced occupancy of lysines of RIPK1 by mono-ubiquitylation, which resulted in accumulation and increased (activating) auto-phosphorylation of RIPK1 (Annibaldi et al., 2018).

Cell Death Induced by TLRs

While TNF-induced cell death has been subject to intense research, cell death induced by TLR signaling is much less investigated. MyD88-dependent TLR signaling does not directly induce cell death, however signaling through TRIF was shown to result in cell death by apoptosis which required FADD, caspase-8 and inhibition of the proteasome, presumably stabilizing IκB (Ruckdeschel et al., 2004). In another study, apoptosis upon TRIF overexpression was shown to require the RHIM domain of TRIF (Kaiser and Offermann, 2005). The involvement of ubiquitylation in the regulation of TRIF-dependent cell death was suggested by a study showing that poly (I:C) induced apoptosis was dependent on TLR3, TRIF, and caspase-8, which was counteracted by cIAP1 (Weber et al., 2010). Importantly, TLR3-induced apoptosis, promoted by the absence of cIAPs, required RIPK1 (Feoktistova et al., 2011; Estornes et al., 2012). One of these studies found active caspase-8 to be associated with the TLR3 (Estornes et al., 2012). In similarity to TNF-induced cell death signaling, upon inhibition of caspases, macrophages were shown to undergo necroptosis upon stimulation of TLR3 and TLR4. This was dependent on the presence of TRIF (He et al., 2011). In this study, RIPK1 knockdown resulted in macrophage death, where as Nec-1 prevented TLR3/4-induced necroptosis, indicating a prosurvival function of RIPK1, but the promotion of TRIF-induced cell death by its kinase activity. Another study on TLR-induced necroptosis confirmed the requirement for TRIF and RIPK1 kinase activity for macrophage necroptosis upon TLR3/4 stimulation and showed that macrophage necroptosis by stimulation of TLR2/5/9 required TNF to induce cell death. However, fibroblasts and endothelial cells did not require RIPK1 to undergo TLR3-induced necroptosis, suggesting a TRIF:RIPK3 complex to activate RIPK3 (Kaiser et al., 2013). This finding is supported by the contribution of TRIF to the perinatal RIPK3-dependent mortality of RIPK1$^{-/-}$ mice (Dillon et al., 2014). Thus, unlike with TNF-dependent necroptosis, there is no absolute requirement for RIPK1 in TLR-induced necroptosis.

More recently, LUBAC was shown to prevent TLR3-induced apoptosis, as loss of HOIP or SHARPIN sensitized cells to cell death induced by poly (I:C) (Zinngrebe et al., 2016). This study identified the formation of a cytosolic death-inducing complex induced by TLR3, containing LUBAC, cIAP1/2, RIPK1, FADD, and caspase-8. Together, these reports demonstrate that K63-and M1-ubiquitylation represent pro-survival checkpoints not only in TNF-, but also in TLR-dependent cell death.

CONCLUSION

The usual outcome of TLR and TNFR1 stimulation is pro-inflammatory gene expression, as different mechanisms or checkpoints prevent cell death upon stimulation of these receptors. In case of TNFR1 signaling, different levels of cell death prevention were defined, and interfering with these checkpoints [as often observed with pathogens (Lamkanfi and Dixit, 2010)] will reduce the threshold for cell death.

Cell death is prevented by TNFR1-induced gene expression, by transcriptional induction of pro-survival proteins. Another level of cell death suppression is the inhibition of the kinase activity of RIPK1, by the activity of E3 ligases such as cIAP1/2 and LUBAC, as well as kinases such as TAK, IKK, p38, and TBK1/IKKε. Interfering with these enzymes will, however, not only promote RIPK1 dependent cell death, but also inactivate the transcription-dependent checkpoint (with the exception of TBK1/IKKε, which have no role in pro-survival NF-κB induction by TNF (Pomerantz and Baltimore, 1999).

The actual and specific roles of cell death, triggered by the mechanisms described, remain to be investigated. It appears that, just as inflammatory gene expression triggered by innate immune and cytokine receptors, cell death induced via these receptors can likewise be beneficial or deleterious. Increasing evidence supports the concept that in addition to unrestricted pro-inflammatory signaling, the cell death-inducing activities of innate immune receptors have a key role for pathological inflammation. Cell death due to dysregulated ubiquitylation, as in LUBAC- or OTULIN defective animals or patients, can be a crucial trigger

of pathologic chronic inflammation, with consequences such as auto-inflammatory disease (HogenEsch et al., 1993; Gerlach et al., 2011; Ikeda et al., 2011; Tokunaga et al., 2011; Berger et al., 2014; Kumari et al., 2014; Rickard et al., 2014; Boisson et al., 2015; Heger et al., 2018; Damgaard et al., 2019).

On the other hand, induction of cell death by apoptosis upon loss of ubiquitylation-dependent kinase activity has been shown to be beneficial. For example, the *Yersinia* protein YopJ, injected into target cells, blocks the kinase activity of TAK1 and thereby pro-inflammatory gene expression (Haase et al., 2005; Mukherjee et al., 2006). However, the resulting caspase-8 activation and cell death is in fact instrumental for defense against the pathogen (Philip et al., 2014; Weng et al., 2014). Accordingly, RIPK1^{K45A} (kinase dead) knock-in mice exhibited reduced macrophage cell death upon Yersinia infection, but succumbed rapidly to the infection (Peterson et al., 2017). Those examples likely reflect only an initial understanding of TLR- and TNFR1- induced cell death for both host defense and disease. Certainly, more work will be required to clarify the role of TLR- and TNFR1-induced cell death for limiting the spread of infection as well as causing human pathology.

AUTHOR CONTRIBUTIONS

LG generated the figures. All authors wrote the review.

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Ripoptocide – A Spark for Inflammation

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The clinical success of biologics that inhibit TNF (Tumor Necrosis Factor) in inflammatory bowel diseases (IBD), psoriasis and rheumatoid arthritis (RA) has clearly established a pathogenic role for this cytokine in these inflammatory disorders. TNF binding to its receptors activates NFκB and MAPK signaling, inducing the expression of downstream pro-inflammatory genes. This is thought to be the primary mechanism by which TNF elicits inflammation. TNF is also a well-known trigger of caspase-dependent apoptosis or caspase-independent necroptosis. Whether cell death has any role in TNF-mediated inflammation has been less clear. Emerging data from animal models now suggest that cellular demise caused by TNF may indeed provoke inflammation. The default response of most cells to TNF stimulation is survival, rather than death, due to the presence of two sequential cell death checkpoints. The early checkpoint is transcription-independent involving the non-degradative ubiquitination of RIPK1 to prevent RIPK1 from becoming a death-signaling molecule. The later checkpoint requires the induction of pro-survival genes by NFκB-mediated transcription. When the early checkpoint is disrupted, RIPK1 initiates cell death and we suggest the term *ripoptocide* to describe this manner of death (encompassing both apoptosis and necroptosis). The sensitivity of a cell to ripoptocide is determined by the balance between regulatory molecules that enforce and those that disassemble the early checkpoint. As there is evidence suggesting that ripoptocide is inflammatory, individuals may develop inflammation due to ripoptocide brought about by genetic, epigenetic or post-translational alteration of these checkpoint regulators. For these individuals, drugs that reinforce the early checkpoint and inhibit ripoptocide could be useful in ameliorating inflammation.

Keywords: TNF, ripoptocide, apoptosis, necroptosis, ubiquitin, E3 ligase, deubiquitinase, RIPK1

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ROLE OF TNF IN INFLAMMATION

Tumor necrosis factor (TNF) was first described in 1975 as a serum factor that could lyse tumor cells present in bacillus Calmette-Guerin (BCG)-infected mice that were challenged with endotoxin (Carswell et al., 1975). It was discovered as part of an effort to uncover factors that could account for the observations of William Coley in the late 1800s, who administered a bacterial cocktail to induce tumor regression in his patients. This bacterial cocktail came to be known as Coley's toxins.

Subsequent efforts to use TNF as an anti-tumor agent in patients largely failed, due in part to the fact that TNF administration induces an intolerable systemic inflammatory response. TNF is now used only in conjunction with melphalan in isolated limb perfusion (TM-ILP) for the localized treatment of soft tissue sarcoma and melanoma of the extremities (Grunhagen et al., 2006). A large body of work over the past few decades has now shown TNF to be highly inflammatory with pleiotropic effects in multiple cells and tissues. This pro-inflammatory function of TNF plays a physiological role in anti-microbial defense (Fiers, 1991). On the other hand, dysregulation of TNF has been linked to the development of inflammatory diseases including rheumatoid arthritis (RA), inflammatory bowel diseases (IBD) and psoriasis. Biologics that block TNF have proven to be highly effective in the treatment of these inflammatory disorders (Taylor and Feldmann, 2009; Blandizzi et al., 2014; Mitoma et al., 2018). This inflammatory role of TNF in both anti-microbial defense and in inflammatory disorders is thought to be due to its induction of NFκB and MAPK signaling, and subsequent transcription of downstream pro-inflammatory genes including other cytokines, chemokines, receptors and adhesion molecules.

Another explanation for why TNF failed as an anti-tumor agent is that TNF is a poor inducer of tumor cell death when used as a single agent, contrary to its initial description as a cytotoxic factor. The initial experiments were carried out using Meth A and L929 mouse tumor lines (Carswell et al., 1975), which are highly sensitive to TNF-induced cell death. In contrast, most transformed cells as well as non-transformed primary cells are largely resistant to TNF-induced cytotoxicity. Indeed, TNF has the opposite effect and induces a pro-survival state in most cells. Nonetheless, extensive studies have demonstrated that TNF has the capability to induce cell death under the right circumstances. Experimentally, this often involved the use of pharmacological agents or genetic manipulation to sensitize cells to death. One manipulation used often to sensitize cells to TNF-induced killing is to treat cells with either actinomycin D or cycloheximide to block new protein synthesis. This indicated that the cell death machinery is pre-existing but since the default response to TNF in most cells is survival, this suggested that there are molecular mechanisms that serve as checkpoints to suppress the cell death machinery. Since the default response is survival rather than death, the physiological and patho-physiological function of TNF-induced cytotoxicity has been difficult to study. While knocking out TNF enables one to ascribe a role for TNF to a particular biological response, one is unable to conclude whether that TNF-mediated response is due to its induction of MAPK/NFκB signaling or cell death. Therefore, a role for cell death in mediating the inflammatory effects of TNF has been unclear. Recent emerging data from mouse genetic models with perturbations that alters the cell death response now support the notion that cell death may play a role in driving inflammatory responses. In this review, we will discuss our current understanding of the molecular mechanisms that determine whether a cell remains resistant or succumb to TNF-induced death and propose that tipping the response to death may be linked to inflammation in some patients.

DUAL SEQUENTIAL CELL DEATH CHECKPOINTS IN THE TNF PATHWAY

The early observation that transcription or translation inhibitors sensitized cells to TNF-induced death pointed toward the presence of a transcription-dependent cell death checkpoint. In the mid-1990s, this checkpoint was attributed to NFκB-dependent transcription of pro-survival genes (Beg and Baltimore, 1996; Van Antwerp et al., 1996; Wang et al., 1996). One critical molecule induced by NFκB is c-FLIP, which binds to unprocessed CASPASE 8 and prevents it from triggering apoptosis (Micheau et al., 2001; Micheau and Tschopp, 2003). c-FLIP is a short-lived protein and if it is not replenished by NFκB-dependent transcription, unprocessed CASPASE 8 undergoes autocatalysis to generate a p18/p10 tetrameric complex that initiates the apoptotic cascade. Other pro-survival molecules induced by NFκB includes members of the BCL2 family and several components of the TNF receptor 1 (TNFR1) signaling complex such as cIAP1/2, TRAF2 and A20 (Wang et al., 1998, 1999; Lee et al., 2000; He and Ting, 2002). Another checkpoint was discovered in 2007 and this was shown to be dependent on the non-degradative ubiquitination of the TNF signaling molecule RIPK1 but did not depend on NFκB-mediated transcription (O'Donnell et al., 2007). We had previously proposed that these two cell death checkpoints function sequentially in the TNFR1 signaling pathway (O'Donnell and Ting, 2010, 2011; Ting and Bertrand, 2016). Ubiquitination of RIPK1 functions as the initial checkpoint and this transcription-independent checkpoint serves to prevent RIPK1 from becoming a survival-signaling molecule (**Figure 1A**). The TRAF2/cIAP1/2 and LUBAC E3 ubiquitin ligases are recruited to TNFR1 to conjugate K63-linked and M1-linked (linear) polyubiquitin chains onto RIPK1, respectively (Hsu et al., 1996; Shu et al., 1996; Bertrand et al., 2008; Wang et al., 2008; Haas et al., 2009; Gerlach et al., 2011). Polyubiquitinated RIPK1 serves as a platform to recruit the TAB2/3-TAK1 and NEMO-IKKα/β kinase complexes allowing TAK1 to phosphorylate and activate IKKα/β (**Figure 1A**). One function of NEMO-IKKα/β is to phosphorylate RIPK1 on residue serine 25 to further suppress its death-signaling capability (Dondelinger et al., 2015, 2019), as well as CYLD to inhibit this deubiquitinase from dismantling K63-linked polyubiquitin chains (Reiley et al., 2005). The death-signaling capability of RIPK1 is additionally suppressed by TAK1, TBK1/IKKε and MK2–mediated phosphorylation (Dondelinger et al., 2017; Geng et al., 2017; Jaco et al., 2017; Menon et al., 2017; Lafont et al., 2018; Xu et al., 2018). These phosphorylation events serve to reinforce the early checkpoint, providing a transient protection against death. Another function of NEMO-IKKα/β is to phosphorylate I-κBα leading eventually to the activation of NFκB and its induction of pro-survival genes (**Figure 1A**). This later checkpoint constitutes a transcription-dependent programing of the cells to provide a more permanent protection

against death. Indeed, gene products of the late NFκB-dependent checkpoint include the E3 ligase for RIPK1 (i.e., cIAP1/2 and TRAF2) thereby functioning in a positive feedback manner to further strengthen the early checkpoint and to suppress RIPK1's death-signaling function. A20/TNFAIP3 is another gene product induced by NFκB that strengthens the early checkpoint by binding to M1-linked ubiquitin chains and preventing their dismantling (Draber et al., 2015).

While disruption of either checkpoint sensitizes cells to TNF-induced death, the manner by which the cells die are different in the two situations. The early checkpoint can be disrupted by inhibiting ubiquitination of RIPK1. This can be done experimentally by treating cells with SMAC mimetics that degrade cIAP1/2 (Bertrand et al., 2008), mutating the ubiquitin acceptor site on RIPK1 (O'Donnell et al., 2007), deleting NEMO (Legarda-Addison et al., 2009) or the E3 ligases that catalyze K63-linked or M1-linked (linear) ubiquitin conjugation (Gerlach et al., 2011; Ikeda et al., 2011; Tokunaga et al., 2011; Moulin et al., 2012). Disrupting ubiquitination 'flips on a death switch' on RIPK1 (O'Donnell et al., 2007), converting it from a survival-signaling molecule to a death-signaling molecule. This enables RIPK1 to associate with the FADD/CASPASE 8 complex leading to the auto-processing of CASPASE 8 to initiate apoptosis (**Figure 1B**). If FADD/CASPASE 8 is absent or defective, RIPK1 forms a complex with RIPK3, leading to the activation of this kinase. RIPK3 phosphorylates MLKL and this initiates an alternative form of cell death known as necroptosis or programed necrosis. In either case, this can be considered 'death by execution' and a hallmark of this death is the requirement for a functional RIPK1 kinase activity. We propose the term *ripoptocide* to describe cell death that is dependent on RIPK1, be it apoptosis or necroptosis. Another route to flip on the death switch on RIPK1 is to activate CYLD, a deubiquitinase that preferentially dismantles K63-linked ubiquitin chains, including from RIPK1. On the other hand, the late checkpoint can be disrupted by pharmacological or genetic inhibition of NFκB-dependent gene expression. This leads to a failure in replenishing c-FLIP, which normally acts as a brake on CASPASE 8. Without c-FLIP present, CASPASE 8 undergoes auto-processing and initiates apoptosis (Micheau and Tschopp, 2003). This can be considered 'death by starvation' and RIPK1 is not involved in turning on this death. While TNF can induce apoptosis if either checkpoint is disrupted, because the mechanisms involved are different, it is likely that the biological effects of 'death by execution' and 'death by starvation' is likely to be different.

APOPTOSIS CONFERRED BY THE LOSS OF THE EARLY CHECKPOINT IS INFLAMMATORY

Apoptotic cell death has been largely assumed to be non-inflammatory and tolerogenic, and there are examples in the immune system that support this idea. For instance, the bulk of thymocytes undergo apoptosis when they failed to be selected (Wiegers et al., 2011; Daley et al., 2017) with no sign of inflammation in the thymus. Similarly, antigen-specific lymphocytes that multiply in response to an infection undergo apoptosis after the infection has been cleared due to cytokine withdrawal without causing inflammation (Snow et al., 2010). The rapid engulfment of apoptotic cellular debris by phagocytic cells to prevent the release of intracellular content, together with presentation of self-antigens on MHC molecules in a non-inflamed environment without costimulatory signals are ways by which apoptosis can induce peripheral tolerance (Ferguson et al., 2011; Martin et al., 2012; Green et al., 2016).

TNF can induce RIPK1/RIPK3-dependent necroptosis, a form of necrotic death marked by the release of endogenous ligands for pattern recognition receptors (known as damage-associated molecular patterns or DAMPs), which can activate innate immune cells to mount an inflammatory response (Wallach et al., 2016). Since existing paradigm considers apoptosis to be tolerogenic, TNF-induced necroptosis rather than apoptosis is thought to induce inflammation. However, emerging animal models are now suggesting that TNF-induced apoptosis can in fact be inflammatory as well. A key model that has shed light on this is the *cpdm* mutant mouse strain. This strain was identified because it spontaneously developed dermatitis and was subsequently shown to possess a loss-of-function mutation in the *Sharpin* gene. In addition to the skin, the *cpdm* strain also exhibits multi-organ inflammation and immunodeficiency (HogenEsch et al., 1999). SHARPIN associates with two RING-containing proteins, HOIP/RNF31 and HOIL1/RBCK1, to form the Linear Ubiquitination Assembly Complex (LUBAC) (Ikeda, 2015). LUBAC is an E3 ligase that catalyzes the addition of linear ubiquitin chains on RIPK1 and NEMO, and this post-translational modification is a critical part of the early checkpoint (**Figure 1B**). SHARPIN deficiency conferred sensitivity to RIPK1-dependent death in cells treated with TNF (Gerlach et al., 2011; Ikeda et al., 2011; Tokunaga et al., 2011). *In vivo*, the dermatitis seen in the *Sharpin*-deficient *cpdm* strain can be reversed by a compound deletion in *Tnf* (Gerlach et al., 2011) indicating that this inflammation is TNF-driven. Furthermore, a K45A knock-in mutation of *Ripk1* that disables its kinase activity (and thus RIPK1-dependent death) also reversed the skin inflammation in the *cpdm* mice (Berger et al., 2014). This observation demonstrated that the SHARPIN deficiency led to the disruption of the early checkpoint and flipped on the death switch on RIPK1. Conditional deletion of the death-signaling molecule *Fadd* in keratinocytes (Kumari et al., 2014) or a heterozygous germline deletion of *Casp8* (Rickard et al., 2014), both in combination with germline *Ripk3* deletion to disable apoptosis and necroptosis, also reversed the inflammation in the *cpdm* strain. However, deletion of just *Ripk3* or *Mlkl* in the *cpdm* strain only partially reversed the skin inflammation (Kumari et al., 2014; Rickard et al., 2014). The $Sharpin^{cpdm/cpdm}Ripk3^{-/-}$ or $Sharpin^{cpdm/cpdm}Mlkl^{-/-}$ mice, which were competent for TNF-induced apoptosis but not necroptosis, still developed dermatitis.

Another insightful model came from the study of mice with a deletion of *Nemo,* a component of the IKK complex, in intestinal epithelial cells (IEC). Prior *in vitro* studies had indicated that NEMO is an essential component of the early

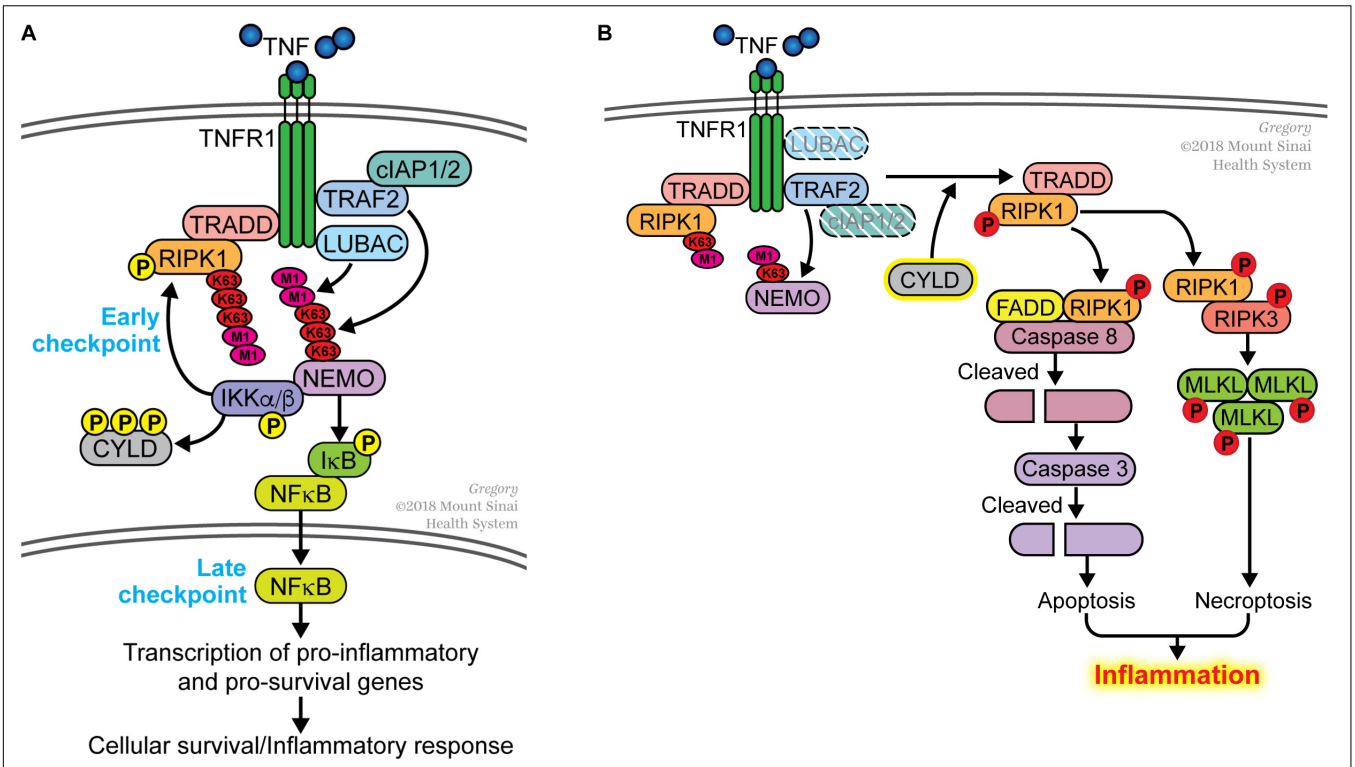

FIGURE 1 | Dual sequential cell death checkpoints in the TNFR1 signaling pathway. **(A)** In most cells, binding of TNF to its receptor TNFR1 does not trigger cell death. This is due to the presence of two sequential cell death checkpoints. The early checkpoint occurs rapidly following TNFR1 ligation when RIPK1 is conjugated with K63- and M1-linked poly-ubiquitin chains catalyzed by the TRAF2/cIAP1/2 and LUBAC E3 ligases, respectively. When this occurs, RIPK1 functions as pro-survival signaling molecule and does not associate with the FADD/CASPASE 8 death-signaling complex. This early checkpoint is reinforced by IKK-mediated phosphorylation of RIPK1 and CYLD to further inhibit their death-signaling capabilities. The late checkpoint occurs when NFκB translocates to the nucleus to upregulate expression of pro-survival genes. The two checkpoints function in different ways to protect cells from death; the early checkpoint does not require *de novo* gene transcription whereas the later checkpoint does. **(B)** The early checkpoint fails when RIPK1 ubiquitination is disrupted. This happens when the ubiquitin E3 ligases (e.g., TRAF2, cIAP1/2, LUBAC) is inhibited or deleted. An increase in the activity of deubiquitinases (e.g., CYLD), which remove ubiquitin from RIPK1, can similarly lead to the disruption of the early checkpoint. Impaired ubiquitination of RIPK1 leads to its association with FADD and CASPASE 8 to initiate apoptosis, or with RIPK3 to induce necroptosis if apoptosis is inhibited. We use the term ripoptocide to denote this RIPK1-dependent death, which can lead to inflammation.

checkpoint (Legarda-Addison et al., 2009; O'Donnell et al., 2012). NEMO can function as a ubiquitin-dependent physical restraint on RIPK1 (Legarda-Addison et al., 2009; O'Donnell et al., 2012) or via IKK-dependent phosphorylation of RIPK1 (Dondelinger et al., 2015) to inhibit the death-signaling function of RIPK1. Since NEMO is also essential for NFκB signaling, loss of NEMO leads to the failure of both checkpoints but cell death in NEMO-deficient cells is dependent on RIPK1 (Legarda-Addison et al., 2009; O'Donnell et al., 2012), indicating that the early checkpoint is central to cell death sensitivity. IEC-specific deletion of *Nemo* led to severe intestinal inflammation that is TNF-dependent and reversed with a loss-of-function in the kinase domain of RIPK1 (Vlantis et al., 2016). In contrast, combined deletion of *Rel* members of the NFκB family in the same tissue (thereby leading to failure of only the late checkpoint with no RIPK1 involvement) did not lead to colitis (Vlantis et al., 2016). Similar to the situation in the *cpdm* mice, deletion of *Ripk3* in the IEC knockout of *Nemo*, still resulted in colitis in a proportion of mice (Vlantis et al., 2016). In both models, blocking RIPK3-dependent necroptosis did not completely reversed the inflammation. Another insightful model is the *Tnfaip3*$^{-/-}$ mice, which succumbed postnatally to multi-organ inflammation (Lee et al., 2000). This postnatal lethality can be partially reversed by inactivating the kinase activity of RIPK1 or by deleting *Ripk3* but not deleting *Mlkl* (Newton et al., 2016). Thus, the inflammation in A20-deficient mice is not due to MLKL-mediated necroptosis but rather, it is caused in part by RIPK1/3-dependent apoptosis or a death-independent function. It is interesting to note that since A20 is also an inhibitor of NFκB signaling, A20-deficient cells also harbor enhanced NFκB activity but despite this, A20-deficient cells are highly sensitive to TNF-induced apoptosis (Lee et al., 2000). This behavior suggests that disruption in the early checkpoint (and therefore ripoptocide) can override a functioning late checkpoint. It should also be noted that the postnatal lethality of the A20-deficient mice could not be fully reversed by the kinase-inactive RIPK1 or RIPK3 removal (Newton et al., 2016), suggesting that the inflammation caused by A20 deficiency may be due to a combination of excessive cell death and NFκB gene transcription. These different mouse knockout models suggest that TNF-driven

apoptosis caused by disruption of the early checkpoint (and therefore dependent on RIPK1) underlies the inflammation. Thus, there may be something uniquely inflammatory about RIPK1-dependent apoptosis. One possibility is that in addition to activating the cell death machinery, RIPK1 can also induce the expression of inflammatory cytokines and chemokines in the dying cells (Yatim et al., 2015; Najjar et al., 2016; Saleh et al., 2017; Zhu et al., 2018). The combined effect of apoptosis with inflammatory cytokines/chemokines may be particularly potent at recruiting and activating inflammatory cells. Another possibility is that TNF-induced cell death is a combination of apoptosis and necroptosis in contexts where RIPK3 is available. RIPK1-dependent apoptosis could also lead to inflammation if this is occurring in cells serving a barrier function. Their inappropriate loss would lead to a breach in barrier and subsequent invasion of the underlying tissue by commensals.

The mouse models described above, which are caused by single gene alteration, provide evidence that disruption of the early checkpoint results in ripoptocide and inflammation. Humans with a genetic defect in the early checkpoint would be similarly expected to develop inflammation. In this regard, humans with a genetic defect in *RNF31* (coding for HOIP) or *RBCK1* (coding for HOIL1) developed autoinflammation and immunodeficiency, which overlap with the phenotype observed in the SHARPIN-deficient mice. Females with a single copy defect in the X-linked *IKBKG* gene (coding for NEMO) develop Incontinentia Pigmenti (IP), which is characterized by skin inflammation during the early stages of life (Fusco et al., 2015). Cells in which X-inactivation occurred on the wild type *IKBKG* allele would be sensitive to TNF-induced ripoptocide. Humans haploinsufficient for *TNFAIP3* (coding for A20) also developed autoinflammation (Zhou et al., 2016), akin to the phenotype of the *Tnfaip3*$^{-/-}$ mice. In addition, polymorphisms in the *TNFAIP3* gene has long been associated with a number of human inflammatory disorders (Vereecke et al., 2011; Ma and Malynn, 2012). While there is no direct evidence currently that the inflammation in these human genetic disorders is RIPK1-dependent, the mouse models strongly suggest that the pathology in these genetic disorders is caused by a failure in the early checkpoint. Therapeutically, these rare patients may benefit from the use of TNF antagonists and RIPK1 kinase inhibitors.

MECHANISMS THAT CONFER SENSITIVITY TO RIPOPTOCIDE AND INFLAMMATION

While the genetic models described above provide insights into the biological consequence of disrupting the early checkpoint, it is less clear how this checkpoint may be disrupted in a normal individual. The TNF cell death pathway likely evolved as an anti-microbial defense mechanism (Old, 1985) and the physiological role of the early checkpoint in this response remains incomplete. Germline deletion of several components of the early checkpoint (e.g., cIAP1/2, HOIP, HOIL1 and TRAF2) resulted in embryonic lethality due to inappropriate cell death (Yeh et al., 1997; Moulin et al., 2012; Peltzer et al., 2014, 2018) demonstrating that these survival molecules are essential for development. However, this checkpoint and more importantly, the capability to actively induce death when it fails, must serve an essential postnatal function evolutionary because there is no selection pressure to have this death response in a developing embryo. The early checkpoint likely evolved to serve a 'trapdoor' function in postnatal life. The molecules that constitute the early checkpoint are often also involved in signaling downstream of pattern recognition and cytokine receptors. Thus, these checkpoint molecules are targeted by microbial-encoded effector molecules to block the pattern recognition and cytokine receptors from signaling (Silke and Hartland, 2013). In so doing, the infected cells become vulnerable to TNF-induced ripoptocide and this could serve to limit infection. The fact that microbes encode molecules that block apoptosis is consistent with the notion that the induced death of host cells serves an anti-microbial function (Silke and Hartland, 2013). In addition, the pro-inflammatory effects of ripoptocide could serve to bypass the inflammatory blockade imposed by the microbial-encoded molecules. There is evidence that an effective response to Yersinia infection requires RIPK1-dependent apoptosis (Peterson et al., 2017; Dondelinger et al., 2019). The Yersinia effector molecules YopJ can target components of the early checkpoint including TAK1 and IKK (Orning et al., 2018; Dondelinger et al., 2019). Recently, it was reported that TBK1 and IKKε phosphorylate RIPK1 to inhibit its death-signaling function (Lafont et al., 2018; Xu et al., 2018) and this constitutes another element of the early checkpoint. Since TBK1 and IKKε play a critical role in the induction of type I interferon, there is speculation that microbial-encoded antagonists of TBK1/IKKε, in attempting to block type I interferon induction, could open the 'trapdoor' leading to TNF-mediated destruction of infected cells.

Since TNF underlies a number of inflammatory disorders, the question arises as to whether the inflammation in these pathologies is caused by excessive TNF-driven NFκB and MAPK signaling, or by a failure in the early checkpoint leading to inappropriate TNF-induced ripoptocide. We propose that this may be dependent on the tissue affected and on the individual. For instance, it is possible that in one inflammatory disorder, it is caused by excessive TNF-driven expression of NFκB-dependent inflammatory genes whereas in a different disorder, it is driven by TNF-mediated death (**Figure 2**). It may be that in a particular tissue, the affected cell type expresses lower level of checkpoint-fortifying molecules rendering this cell more susceptible to ripoptocide. However, in tissues where early checkpoint molecules are highly expressed, inflammation may be due to excessive induction of NFκB and MAPK signaling. It is also possible that within a population of patients with the same disorder, some develop inflammation due to excessive cell death whereas others develop inflammation due to excessive NFκB/MAPK signaling. RIPK1 kinase inhibitors are being developed for inflammatory disorders (Harris et al., 2017) and these may work only in the subset of patients where the cause is a defect in the early checkpoint. The results from these trials will be interesting as

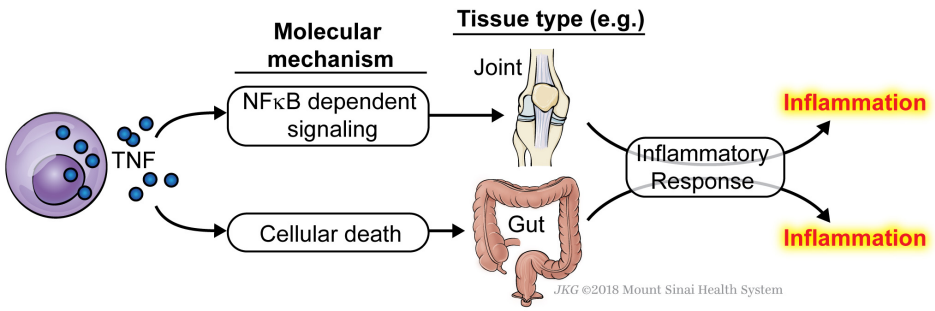

FIGURE 2 | TNF can cause inflammation via induction of either NFκB signaling or ripoptocide. Induction of either arm of the signaling pathways downstream of TNFR1 can lead to inflammation. It may be that in some tissue, the inflammation is caused by excessive TNF-induced NFκB signaling and expression of downstream cytokines, adhesion molecules and other pro-inflammatory genes. In a different tissue, the inflammation may be caused by excessive TNF-induced ripoptocide, which could come about from reduced expression of signaling molecules that fortify the early checkpoint. The affected tissues are drawn for illustrative purposes. Currently, there is no evidence to show tissue-dependent sensitivity to ripoptocide. Alternatively, within a single tissue/disorder, some patients develop inflammation due to excessive NFκB signaling whereas others develop inflammation due to excessive ripoptocide.

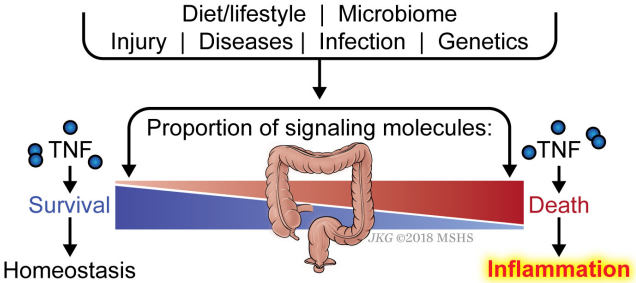

FIGURE 3 | Balance between checkpoint enforcement and disassembly determines sensitivity to ripoptocide-dependent inflammation. Whether a tissue develops sensitivity to TNF-induced ripoptocide and subsequent inflammation may be determined by the level of early checkpoint regulators in the cells within the tissue. For example, if cells from an individual express high level of LUBAC (which enforces checkpoint) but low level of CYLD (which disassembles checkpoint), they would be more resistant to TNF-induced ripoptocide. That individual would be more resistant to ripoptocide-mediated inflammation. Conversely, if cells from an individual express low LUBAC but high level of CYLD expression, that individual would be more prone to ripoptocide and hence inflammation. Individual to individual variation in expression of checkpoint regulators could be due to allelic differences, epigenetic differences and a host of environmental factors extrinsic to the cells. Prior infections or injury could alter expression level via changes in transcription factor activity and chromosomal accessibility. Elements within the diet or byproducts of diet breakdown could directly alter the protein level or functionality of the checkpoint regulators. Encoded gene products or byproducts of metabolism from microbes (commensals or pathogens) may have the same effect. These factors that modulate expression/activity of checkpoint regulators and how they work together to confer sensitivity to ripoptocide are poorly understood.

they would provide evidence for whether ripoptocide underlies inflammatory disorders in humans.

Another key question is why an individual develops sensitivity to death and therefore inflammation, whereas another individual does not. The list of signaling molecules that constitute the early checkpoint is quite numerous and is likely to grow as we gain more understanding of this checkpoint. Defective expression in one of these genes or more likely, a combination of several genes, would be expected to render the affected cell sensitive to TNF-induced ripoptocide. It is likely that the balance between signaling molecules that enforce the checkpoint (i.e., pro-survival molecules) versus those that disassemble the checkpoint (i.e., pro-death molecules) determine sensitivity to ripoptocide and subsequent inflammation (**Figure 3**). There is an array of factors that could impact the expression levels of these molecules. Foremost, the genetics of the individual can determine the relative expression of the two opposing classes of molecules in the affected cells. Expression can be further tuned by epigenetic regulation in response to cell extrinsic environmental cues. These cues could come from prior infections, tissue injury, microbiome and diet. In addition to affecting gene expression, microbial-encoded molecules or the products of their metabolism could directly affect the function or availability of these checkpoint molecules via post-translational mechanisms. Cell intrinsic factors and environmental signals likely combine to determine the expression and function of the two opposing classes of molecules. The combinatorial effects from multiple hits may ultimately tip the balance in favor of RIPK1-dependent death and inflammation. Since most human inflammatory disorders are chronic, even a small change in the balance between pro-survival and pro-death molecules in the early checkpoint may lead to disease progression over time without having a significant disruption of homeostasis at any given point.

CONCLUSION

The toggling of RIPK1 between its survival-signaling and death-signaling functions, regulated by an early ubiquitin switch, provides a molecular explanation for the long-known capability of TNF to induce either cell survival or death (O'Donnell et al., 2007). An elaborate machinery exists to regulate the non-degradative ubiquitination of RIPK1 as a checkpoint against death. Failure to hold this checkpoint results in ripoptocide. While a number of molecules are now known to regulate this checkpoint, the list is likely to grow. The quest in future studies will be to understand how the different molecules in the checkpoint themselves are regulated. Currently, we have a limited understanding of the genetic, epigenetic and post-translational mechanisms that determine whether the early checkpoint holds or fails. Further insights into these mechanisms will allow us to fully manipulate this checkpoint for therapeutic purposes. Strategies to reinforce the checkpoint and prevent ripoptocide may be clinically beneficial in inflammatory disorders and transplantation. Conversely, disrupting the checkpoint and inducing ripoptocide may be beneficial in cancer, vaccines and infectious diseases.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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REVIEW
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NOD Signaling and Cell Death

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Innate immune signaling and programmed cell death are intimately linked, and many signaling pathways can regulate and induce both, transcription of inflammatory mediators or autonomous cell death. The best-characterized examples for these dual outcomes are members of the TNF superfamily, the inflammasome receptors, and the toll-like receptors. Signaling via the intracellular peptidoglycan receptors NOD1 and NOD2, however, does not appear to follow this trend, despite involving signaling proteins, or proteins with domains that are linked to programmed cell death, such as RIP kinases, inhibitors of apoptosis (IAP) proteins or the CARD domains on NOD1/2. To better understand the connections between NOD signaling and cell death induction, we here review the latest findings on the molecular regulation of signaling downstream of the NOD receptors and explore the links between this immune signaling pathway and the regulation of cell death.

Keywords: RIPK2, NOD2, ubiquitin, inflammation, cell signaling

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ACTIVATION OF THE NOD PATHWAY

Pattern Recognition Receptors

Sensing of pathogen-associated molecular patterns (PAMPs) is the initiating step in an efficient immune reaction to a bacterial, viral or parasitic threat. The intracellular receptors nucleotide-binding oligomerization domain-containing protein 1 and 2, NOD1 and NOD2, are members of the pattern recognition receptors (PRR) and recognize intracellular bacterial peptidoglycans. The PRR family consists of a range of cytoplasmic or transmembrane stress sensors that recognize PAMPs and damage-associated molecular patterns (DAMPs).

PRRs are divided into two main groups based on their cellular localization: the transmembrane/endosome-associated PRRs, consisting of toll-like receptors (TLRs) and C-type Lectin receptors, and the cytosolic PRRs which are further divided into the RIG-1-like receptors, AIM2-like receptors and the NOD-like receptors (NLRs) (Bertin et al., 1999; Inohara et al., 1999; Ogura et al., 2001b). NLRs are characterized by a central 300–400 amino acid long NACHT domain (also referred to as the NOD or NBD domain) that has predicted nucleoside-triphosphatase activity and facilitates its oligomerization. On the C-terminus, NLRs bear multiple leucine-rich repeats (LRRs) that mediate ligand sensing (**Figure 1**).

The NLRs consists of four subfamilies, based on the nature of their N-terminal effector domain: The NLRC subfamily is characterized by one or multiple N-terminal caspase activation and recruitment domains (CARDs) that allow direct interaction with other CARD-containing proteins. Among the NLRCs, NOD1 and NOD2 represent the two best characterized members and are sensors of intracellular bacterial peptidoglycan (Girardin et al., 2003a,b). The NOD1 and NOD2

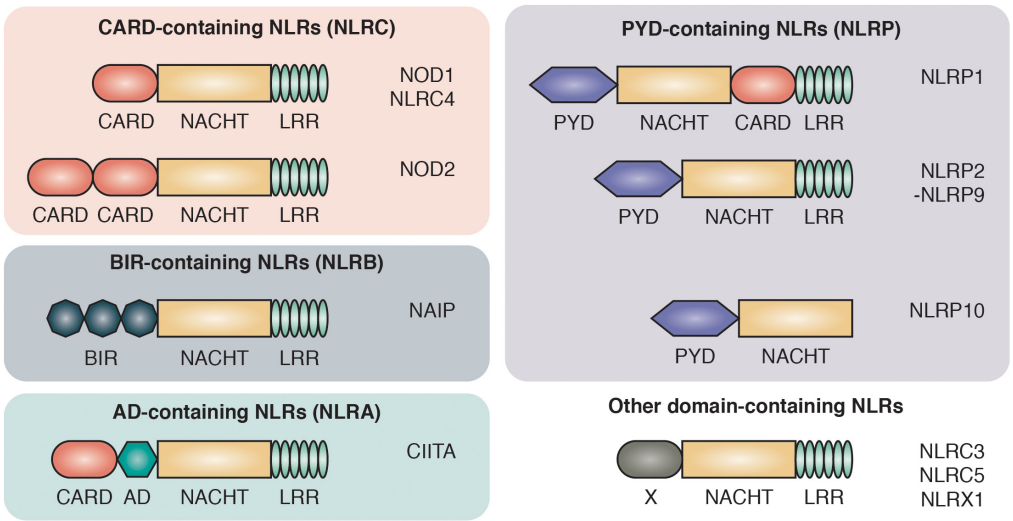

FIGURE 1 | Domain architecture of NOD-like receptors. NLRs are composed of C-terminal leucine-rich repeats (LRR), an intermediate nucleotide-binding-domain (NACHT) and variable N-terminal protein-protein interaction domains that divide NLRs into different protein subfamilies: NLRCs contain one to two caspase activation and recruitment domains (CARD). NLRBs contain multiple baculovirus inhibitor of apoptosis protein repeats (BIR). NLRAs contain at least one acidic transactivating domain (AD) and the NLRP subfamily harbors a pyrin domain (PYD).

pathways have been associated with a range of autoimmune disorders, most prominently with inflammatory bowel disease (IBD). Single nucleotide polymorphisms (SNPs) in the NOD2 gene were the first identified genetic risk factors associated with Crohn's disease (CD) (Hugot et al., 2001; Ogura et al., 2001a).

The second key member of the NLR family are the NLRPs, which are best known for their role in the formation of large oligomeric complexes, the inflammasomes. Inflammasomes mediate the processing, activation and secretion of pro-inflammatory cytokines as well as the induction of pyroptosis through the recruitment and activation of caspase-1 (Martinon et al., 2009). NLRPs contain an N-terminal pyrin domain (PYD) that is also known as a "death fold," which is evolutionary related to the death domain (DD) found in cell death-inducing receptors including Fas, TNFR1 and TRAIL R-1 and R-2 (Fairbrother et al., 2001). The two smaller subfamilies of NLRs are NLRA and NLRB. The NLRA (A for acidic transactivating domain) subfamily only includes one member, class II major histocompatibility complex transactivator (CIITA), that serves as an activator of MHC class II antigen presentation (Nickerson et al., 2001). Members of the NLRBs [B for baculovirus inhibitor of apoptosis protein repeat (BIR)] have one or multiple N-terminal BIR domains. The approximately 70 amino acid zinc-binding BIR domain was first identified through sequence homology among proteins belonging to the Inhibitors of Apoptosis (IAP) family and is mostly recognized for its role in promoting cell survival (Silke and Meier, 2013).

Expression of NOD Receptors

NOD1 and NOD2 both recognize building blocks of bacterial peptidoglycans and share identical downstream signaling pathways. One important difference between these two PRRs is their distinct expression pattern: NOD1 is broadly expressed in a variety of cells including epithelial cells, stromal cells and endothelial cells (Inohara et al., 1999; Park et al., 2007b). In contrast, the expression of NOD2 is more restricted and highest in the hematopoietic compartment, most prominently in cells of myeloid origin such as monocytes (Ogura et al., 2001b), dendritic cells and macrophages (Pashenkov et al., 2010). Furthermore, expression of NOD2 has also been demonstrated in hematopoietic cells of lymphoid origin including B cells (Petterson et al., 2011), $CD4^+$ and $CD8^+$ T cells (Caetano et al., 2011; Lin et al., 2013) and regulatory T cells (Kerns et al., 2009). Notably, NOD2 is also expressed by various epithelial cell types, particularly in Paneth cells located within the ileum of the intestinal tract (Uehara et al., 2007).

Basal expression levels of NOD1 and NOD2 are generally low but can be induced by various immunomodulators. In intestinal epithelial cells, interferon-gamma (IFN-γ) (Rosenstiel et al., 2003), tumor necrosis factor-alpha (TNF-α), lipopolysaccharide (LPS) (Kim Y.G. et al., 2008), 1,25-dihydroxycholecalciferol (Wang et al., 2010), and butyrate (Leung et al., 2009) have been shown to induce the upregulation of NOD2 mRNA. Additionally, we and others observed that IFN-γ increases NOD2 protein levels in bone marrow-derived macrophages and is required for an effective cytokine response after stimulation with the NOD2 ligand muramyl dipeptide (MDP) (Nachbur et al., 2015; Fekete et al., 2017; Stafford et al., 2018).

Once expressed, NOD1 and NOD2 reside in the cytosol and localize to bacterial entry sites at the plasma membrane (Barnich et al., 2005; Kufer et al., 2008). Both receptors constantly interact with the actin cytoskeleton, which facilitates their rapid relocalization upon stimulation (Legrand-Poels et al., 2007). More recent studies indicate that NOD1 and NOD2 are associated with early endosomes that serve as assembly

platforms for NOD signaling complexes (Irving et al., 2014; Nakamura et al., 2014).

Expression levels of NOD1 and NOD2 are held in check through constant degradation by the proteasome. This is counteracted by several chaperones including HSP90, SGT1 (Correia et al., 2007; Mayor et al., 2007; Lee et al., 2012) and HSP70 (Mohanan and Grimes, 2014) which bind and stabilize NOD proteins. Their importance for NOD signaling has been demonstrated using small molecule inhibitors that decrease NOD2 ligand-dependent activation.

A candidate E3 ubiquitin ligase that was shown to ubiquitinate NOD2 and target it for proteasomal degradation is TRIM27 (Zurek et al., 2012). NOD2 was shown to be modified with K48-linked ubiquitin chains after overexpression of TRIM27 in HEK293T cells while siRNA-mediated knockdown of TRIM27 led to the stabilization of NOD2 protein levels. Recently, NLRP12 was shown to indirectly regulate NOD2 protein levels in monocytes. NLRP12 activation leads to the sequestering of HSP90, which in turn promotes K48-linked ubiquitination and degradation of NOD2 in response to MDP. The significance of NLRP12 as a regulator of NOD2 signaling was highlighted by the finding that LPS-primed NLRP12-deficient mice are highly susceptible to secondary challenge by bacterial MDP (Normand et al., 2018).

Canonical Activation of NOD1 and NOD2

Upon its discovery, various groups reported that NOD1 is activated by lipopolysaccharides (LPS) and mediates the activation of NF-κB in a MyD88-independent manner (Girardin et al., 2001; Inohara et al., 2001; Kobayashi et al., 2002). However, through the use of ultra-pure LPS, and synthetic NOD ligands, it has become clear that NOD1 and NOD2 sense distinct monomeric peptidoglycan (PGN) fragments: NOD1 is activated by γ-D-glutamyl-meso-diaminopimelic acid (DAP), a PGN fragment that is present in the cell wall of all Gram-negative and certain Gram-positive bacteria (Chamaillard et al., 2003; Girardin et al., 2003a). NOD2 recognizes muramyl dipeptide (MDP), a PGN fragment found in both Gram-negative and Gram-positive bacteria (Girardin et al., 2003b; Inohara et al., 2003).

Multiple mechanisms of how MDP and DAP are transported into the cytosol to activate NOD1/2 have been reported. In agreement with a role of NODs as sensors of intracellular bacterial infections, NOD1 activation during infection has first been reported with the facultatively intracellular pathogen *Shigella flexneri* (Girardin et al., 2001). Moreover, extracellular DAP can be delivered to the cytosol by type III and IV secretion systems, for instance in *Helicobacter pylori* (Viala et al., 2004), or through bacterial outer membrane vesicles (OMVs). OMVs are small secreted vesicles derived from the outer membrane of Gram-negative bacteria that are able to penetrate the intestinal mucus layer and interact with epithelial cells (Sanchez et al., 2010). Only recently it has been shown that OMVs from probiotic and commensal strains of *Escherichia coli* can be endocytosed by intestinal epithelial cells where they colocalize with NOD1 and trigger its aggregation. OMVs are therefore important contributors to the maintenance of the intestinal homeostasis (Canas et al., 2018). On the other hand, there is substantial findings that OMVs from pathogenic bacteria contribute to their virulence, for instance of *Neisseria gonorrhoeae*, *Pseudomonas aeruginosa* (Kaparakis et al., 2010), *Salmonella enterica* (Yoon et al., 2011), *Brucella abortus* (Pollak et al., 2012), and *Legionella pneumophilia* (Jager et al., 2015; Jung et al., 2016).

Further mechanisms of how NOD ligands translocate to their intracellular receptors include passive transport through oligopeptide transporters such as SLC15A1 (Vavricka et al., 2004; Ismair et al., 2006), or active transport processes such as phagocytosis of live or fragmented bacteria or through epithelial junction transfer (Kasper et al., 2010). In accordance with the localization of NODs to endosomes, endocytosis is another important entry route for NOD ligands (Lee et al., 2009; Marina-Garcia et al., 2009). Certain cell types, in particular dendritic cells express the endosomal peptide transporters SLC15A3 and SLC15A4, that facilitate this process (Nakamura et al., 2014).

Once in the cytoplasm, PGN binds to NOD1/2 and induces a downstream signaling cascade resulting in the induction of a transcriptional response. *In silico* modeling of human and zebrafish NOD2 indicated a hydrophobic pocket on the concave face of the LRR as a putative binding site of MDP to NOD2 (Tanabe et al., 2004; Maharana et al., 2014). This was validated using information gathered from the rabbit NOD2 crystal structure, where mutating amino acids within the hydrophobic core of the LRR reduced, respectively abolished MDP-dependent NF-κB activation (Maekawa et al., 2016). Using surface plasmon resonance (SPR), Grimes et al. provided the first biochemical evidence that MDP bound directly to NOD2 with a relatively high affinity (KD = 51 nM) (Grimes et al., 2012). Interestingly, the affinity of MDP to NOD2 was pH-dependent and highest in the pH range from 5.0 to 6.5. This data suggests that *in vivo* binding could occur in an acidic cellular compartment, for instance in endosomes. Due to the high degree in sequence homology, ligand binding of NOD1 is believed to occur in a similar manner, however a crystal structure that could confirm this theory is still missing. Nevertheless, direct interactions between the NOD1 LRR domain and several agonists, such as TriDAP (L-Ala-D-isoGlu-meso-DAP) have been demonstrated (Laroui et al., 2011). Notably, in their assay, the NOD1 ligand TriDAP bound the NOD1 LRR domain with a KD of 34.5 μM, which raises questions about the physiological relevance of TriDAP.

But how is ligand binding triggering the assembly of the NOD signaling complex? It was difficult to draw conclusions about the mode of action of signaling complex assembly from early models of NOD1 and NOD2, which were based on the crystal structures of homologous receptors such as apoptotic protease-activating factor 1 (Apaf-1) (Riedl et al., 2005; Proell et al., 2008). The recently published crystal structure of NOD2 provides a more detailed view on how structural changes impact on ligand binding and signal transduction. Under steady-state conditions, NOD2 remains in an inactive, closed conformation with tightly packed subdomains by ADP-mediated inter-domain interactions (Maekawa et al., 2016). Ligand binding to the LRRs and the exchange of ADP for ATP triggers the unfolding of the protein and stabilizes it

in an active conformation (Maekawa et al., 2016). NOD2 oligomerizes via its NOD and CARD domains and recruits RIPK2 to form a hetero-CARD complex (Kobayashi et al., 2002; Fridh and Rittinger, 2012). Recent work showed that multiple RIPK2 monomers can then bind via homotypic CARD-CARD interactions to form fibrillar protein assemblies, termed higher-order signalosomes (Gong et al., 2018; Pellegrini et al., 2018). Single amino acid mutations in the CARD domain of RIPK2 that disrupt its oligomerization shut down MDP-dependent NF-κB responses (Pellegrini et al., 2018). Similar structures have been reported in other innate immune signaling pathways such as in the NLRP3, ASC, caspase-1 inflammasome (Lu et al., 2014) and are believed to facilitate and regulate signal transduction (Wu, 2013).

Non-canonical Activation of NOD2

While activation of the NOD pathways through PGN stimulation is well documented, there are more recent reports of activation through PGN-independent pathways. Keestra-Gounder et al. (2016) observed that systemic pro-inflammatory responses triggered by thapsigargin and by infections with the ER-stress-inducing bacterium *Brucella abortus* are blunted in NOD1/2-deficient mice. The underlying mechanisms are still largely unclear, however, experiments conducted with a dominant-negative form of TRAF2 suggested that this process is TRAF2-dependent. The ER stress sensor IRE1 and TRAF2 have been previously shown to interact in overexpression studies and in yeast-two-hybrid screenings (Urano et al., 2000) and this interaction could link NF-κB and MAPK activation with stress pathways (Kaneko et al., 2003). Furthermore, earlier studies suggested that members of the TRAF family interact with the adaptor protein RIPK2, which functions downstream of NOD1 and NOD2 activation (Thome et al., 1998). NOD2 contains a predicted TRAF2-binding motif in its nucleotide-binding oligomerization domain (Schneider et al., 2012) and could therefore function as the link between ER stress and inflammatory signaling. A recent study confirmed that thapsigargin induces NOD-dependent pro-inflammatory signaling, although this was due to the compound's inhibition of the sarcoplasmic or endoplasmic reticulum calcium ATPase family (SERCA), which is responsible for Ca^{2+} movement into the ER and cellular Ca^{2+} regulation (Molinaro et al., 2019). Thapsigargin-induced depletion of Ca^{2+} within the ER led to a rise in intracellular Ca^{2+} levels and enhanced both Ca^{2+} internalization and endocytosis. This endocytosis led to internalization of trace peptidoglycan contaminants in the cell culture grade FCS, which was confirmed using mass spectrometry.

Several pathogenic scenarios also point toward PGN-independent activation of the NOD pathway. Neuropathic pain, mediated by an inflammatory reaction of peripheral macrophages in mice that underwent nerve injury, results in the activation of the NOD2 pathway without the evident involvement of bacterial components (Santa-Cecilia et al., 2019). Furthermore, increased levels of phosphorylated RIPK2, a hallmark of NOD1/2 pathway activation, has been detected in neoplastic tissue of triple-negative breast cancer patients (Mertins et al., 2016). Also in this scenario, it is not directly evident that NOD pathway activation is a direct result of bacterial components and it will be interesting to further investigate the mode of activation in these tissues. It has to be noted though that secreted bacterial components such as OMVs could well be the underlying factor for these apparently non-canonical forms of activation of the NOD pathway.

Signaling Downstream of NOD2 Activation

Binding of PGN to NOD1/2 and subsequent recruitment of RIPK2 results in the ubiquitination of RIPK2 and the recruitment of downstream effector proteins including the IKK complex and TAK1 (Park et al., 2007a; Kim J.Y. et al., 2008; **Figure 2**). The exact role of ubiquitin ligases and the consequence of RIPK2 ubiquitination will be discussed later. RIPK2 ubiquitination ultimately leads to the activation of key transcription factors such as NF-κB and AP-1. Synchronized activation of both transcription factors is required for the transcriptional response, as interference with the timing of activation using a RIPK2 inhibitor resulted in a reduced cytokine response (Nachbur et al., 2015).

Among the strongest induced genes downstream of NOD activation are immunomodulatory cytokines, such as TNF, IL-1β, IL-6, and CC-chemokine ligand 2 (CCL2/MCP-1) (Kobayashi et al., 2005; Gilmore, 2006; Conforti-Andreoni et al., 2010). Transcriptomic profiling of MDP stimulated macrophages revealed a specific gene set downstream of NOD2 compared to general inflammatory stimuli (Tigno-Aranjuez et al., 2014). Members of this set are preferentially involved in immune functions, nucleotide regulation, and cell metabolism. In endothelial cells and Langerhans cells, stimulation with MDP resulted in enhanced IL-6 production and the Th17-differentiation of T cells within the skin (Manni et al., 2011), suggesting that the transcriptional response downstream of NOD stimulation varies considerably between cell types.

While the major outcomes of the NOD1 and NOD2 pathway primarily occur via the activation of NF-κB transcription factors and cytokine production, NOD1 and NOD2 activation can also lead to autophagy induction to clear a bacterial threat. This is in line with NOD2 localization at early endosomes, and the role of NOD1/2 in intestinal homeostasis. Activated NOD has been shown to interact with the autophagy protein ATG16L1 at the site of bacterial entry, although whether RIPK2 is involved in this process is under debate. Two studies show the involvement of RIPK2 in autophagy induction (Cooney et al., 2010; Homer et al., 2010), while a third study observed autophagy induction occurring in the absence of RIPK2 (Travassos et al., 2010). However, the studies agree on the observation that autophagy induction is independent of NF-κB, using both infection models as well as purified ligands. Induction of autophagy downstream of NOD activation can have implications in several pathological conditions, particularly in Crohn's diseases where mutations in

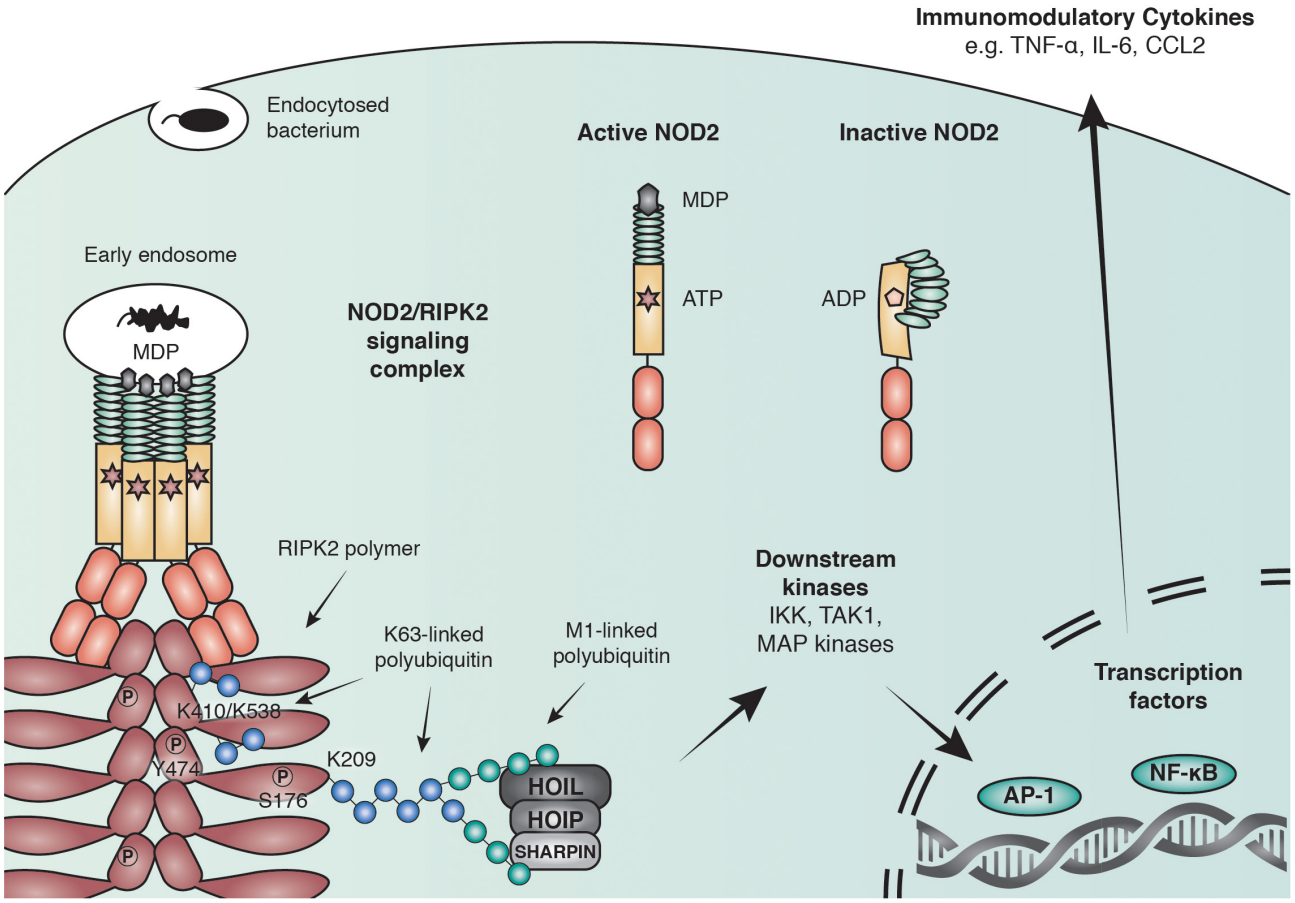

FIGURE 2 | Anti-bacterial signaling mediated by NOD2. Within the family of NLRs, NOD2 represents a particularly well-studied receptor that is activated by binding to the peptidoglycan fragment MDP in the cytosol and at endosomal membranes. NOD2 recruits the adaptor molecule receptor-interacting serine/threonine-protein kinase 2 (RIPK2) through CARD-CARD interactions to form large polymers that facilitate the activation of downstream kinases and lead to the initiation of immune modulatory transcriptional responses through AP-1 and NF-κB transcription factors. RIPK2 is regulated through polyubiquitination by multiple E3 ubiquitin ligases including X-linked inhibitor of apoptosis protein (XIAP) and the linear ubiquitin chain assembly complex (LUBAC) and by phosphorylation of serine and tyrosine residues.

the autophagy protein ATG16L1 are among the highest genetic risk factors to develop the disease.

RIPK2, THE MEDIATOR OF NOD SIGNALING

RIPK2 Is a Member of the RIP Kinase Family

As mentioned above, RIPK2 is the central adaptor kinase in the NOD pathway. RIP kinases represent a class of serine/threonine kinases that play essential roles in the regulation of innate immune signaling. Their functions depend on the highly conserved N-terminal kinase domains and distinct C-terminal interaction motifs. Amongst the RIP kinases, RIPK1 and RIPK3 are the best-characterized members, which are being extensively studied due to their involvement in cell death and their role in chronic diseases and cancer. RIPK1 contains a C-terminal death domain (DD) that mediates direct binding to death receptors of the TNF receptor superfamily members including TNFR1, Fas, and TRAIL, and to adaptor proteins such as FADD or TRADD. Upon binding, oligomeric protein complexes are formed that can regulate survival or cell death. Under specific conditions, RIPK1 associates with RIPK3 through their RIP homotypic interaction motif (RHIM) to activate the pseudokinase mixed lineage kinase domain-like (MLKL) to induce necroptosis, a pro-inflammatory form of programmed cell death (Silke et al., 2015).

Receptor-interacting serine/threonine-protein kinase 2 (RIPK2) represents the next best-characterized member of the RIP kinase family. Compared to RIPK1 and RIPK3, RIPK2 does not have a RHIM or a DD and is therefore unable to interact with these death receptor complexes. RIPK2 is composed of an N-terminal kinase domain, an intermediate domain of unknown function, and a C-terminal CARD that mediates binding to NOD1/2 via homotypic CARD-CARD interactions (Inohara et al., 1998; McCarthy et al., 1998; Thome et al., 1998). Structural data of the kinase domain

(Canning et al., 2015; He et al., 2017; Hrdinka et al., 2018; Suebsuwong et al., 2018) and the CARD of RIPK2 (Lin et al., 2015; Goncharuk et al., 2018) have recently been revealed. The kinase domain shows a typical kinase fold with the catalytic center located between a smaller N- and a larger C-lobe. The C-terminal CARD of RIPK2 displays typical features of a protein from the death domain family, but unlike other CARDs or death domains, the CARD of RIPK2 contains an additional sixth helix. The intermediate domain of RIPK2 is thought to be unstructured and highly flexible, however, posttranslational modifications in this domain upon stimulation could suggest so far unappreciated involvement in RIPK2s signaling function.

RIPK2 is indispensable for NOD-mediated activation of the NF-κB and MAPK pathways and its recruitment to NOD2 occurs via CARD-CARD interaction (Girardin et al., 2001; Park et al., 2007a; Magalhaes et al., 2011). An acidic patch in the NOD1 CARD forms the primary binding interface with basic residues in the RIPK2 CARD. Using mutational analysis and pulldown experiments, Manon et al. (2007) identified three acidic residues (E53, D54, E56) in helix 3 of the NOD1 CARD and three basic residues (R444, R483, R488) in the RIPK2 CARD as the key mediators of the NOD1-RIPK2 interaction. A more recent study proposed that two additional residues in RIPK2 (K443, Y474) are required for NOD1 binding (Mayle et al., 2014).

The NOD2-RIPK2 interface differs from that between NOD1 and RIPK2. Overexpression of both NOD2 CARDs is required for a constitutive NF-κB response (Ogura et al., 2001b). Even though the two CARDs of NOD2 do not act independently, the N-terminal NOD2 CARD (CARDa) is solely responsible for binding to RIPK2 (Fridh and Rittinger, 2012). In contrast to the interaction between NOD1 and RIPK2, the NOD2-RIPK2 interaction motif is made of two basic residues in the NOD2 CARDa (R38, R86) and a set of acidic residues on the RIPK2 CARD (D461, E472, D473, E475 and D492). Intriguingly, direct interaction between NOD2 and RIPK2 has so far only been described using recombinant proteins or in overexpression experiments, which suggests that under physiological conditions the NOD-RIPK2 interaction is either highly transient or unstable.

Structure and Function of RIPK2

RIPK2 was originally identified as a serine-threonine kinase based on sequence homology (Inohara et al., 1998; McCarthy et al., 1998; Thome et al., 1998), but was later reclassified as a dual-specificity kinase that is also able to phosphorylate tyrosine residues (Tigno-Aranjuez et al., 2010). Although the importance of RIPK2 as the central player in NOD signaling has been demonstrated, the function of its kinase activity is still under debate. The active state of the kinase domain is dictated by an invariant lysine within the N-lobe (K47), which contacts and supports ATP. This interaction is supported by the formation of a salt bridge within the middle of the αC-helix (Kornev and Taylor, 2010).

The only substrate of RIPK2 that has been described so far, is RIPK2 itself. Upon activation by dimerization via the CARD domains of NOD1/2, RIPK2 autophosphorylates on S176 in the activation loop of the kinase domain (Dorsch et al., 2006) and on Y474 in its CARD (Tigno-Aranjuez et al., 2010). In overexpression systems, mutation of either of those residues decreased RIPK2's ability to induce downstream signaling.

By comparing the phosphorylation profiles of wild-type RIPK2 vs. catalytically inactive mutants (K47A and D164N), it was observed that besides S176, multiple additional serine residues within the activation loop can be phosphorylated (Pellegrini et al., 2017). More phosphorylated residues have been discovered in large-scale proteomic screenings, however, their functional relevance remains to be evaluated (Daub et al., 2008; Oppermann et al., 2009). *In vitro* auto-phosphorylation assays indicated that catalytically inactive mutants could still be phosphorylated by purified full-length RIPK2, suggesting that autophosphorylation occurs in *trans* (Pellegrini et al., 2017), which requires strong interactions between two or multiple RIPK2 molecules. In line with this theory, biophysical measurements suggested that the active state RIPK2 is a stable dimer whilst the inactive kinase is in a monomer-dimer equilibrium. Supporting this, recently published crystal structures display the phosphorylated form of RIPK2 as a side-by-side dimer, suggesting that dimerization plays a critical role in kinase activation (Tigno-Aranjuez et al., 2010; Tigno-Aranjuez et al., 2014; Canning et al., 2015; Charnley et al., 2015; Nachbur et al., 2015; Haile et al., 2016).

While the ability of phosphorylation by RIPK2 was clearly demonstrated, it is still under debate whether the kinase function is required for NOD signaling. On the one hand side, studies utilizing overexpression of RIPK2 suggested that the kinase activity of RIPK2 is dispensable for NF-κB activation and cytokine production altogether. The catalytically dead mutants of RIPK2 (K47A and D146N) could still activate NF-κB signaling, although this occurred independently of NOD2 engagement with MDP (Inohara et al., 1998; Thome et al., 1998; Eickhoff et al., 2004; Hrdinka et al., 2018). On the other hand side, bone marrow-derived macrophages (BMDMs) from a kinase-dead (K47A) knock-in mouse were defective in signaling (Nembrini et al., 2009). However, kinase-dead RIPK2 was only expressed at very low levels, which could be the reason for deficient NOD signaling in this system and suggests that RIPK2's kinase activity is required for protein stability rather than being an intrinsic requirement for NOD signaling. Recent studies also re-raised questions about the importance of the regulatory autophosphorylation sites S176 and Y474. Overexpression of RIPK2 mutants in HeLa cells showed that wild-type RIPK2 and the S176A mutant resulted in similar amounts of cytokines following infections with *S. flexneri,* while the S176E mutant resulted in reduced cytokine levels (Ellwanger et al., 2019). In contrast, cytokine production was completely abolished in cells expressing the RIPK2 Y474F mutant. The importance of Y474 for signal transduction was also highlighted in two recent studies that utilized cryo-EM to solve RIPK2 structures. Y474 was found to sit at a critical interface in the CARD and to mediate intermolecular interactions during RIPK2 polymerization, which was shown to be essential for NF-κB activation. Thus, it is not surprising and explains that a tyrosine to phenylalanine mutation disrupts RIPK2 activity (Gong et al., 2018; Pellegrini et al., 2018).

RIPK2 Ubiquitination and Scaffolding

While the importance of the kinase activity of RIPK2 remains somewhat dubious, it has become clear that its ubiquitination is a critical determinant of downstream signaling. Ubiquitin is a small, 8.5 kDa protein that can be covalently attached via its C-terminus to lysine residues of target proteins or to the N-terminus of one of the seven lysine residues of a substrate-attached ubiquitin. The diverse biological outcomes of protein ubiquitination are dependent on the complex interplay between the sites of the ubiquitination, chain length, chain type, chain branching as well as posttranslational modifications on ubiquitin itself (Komander and Rape, 2012; Swatek and Komander, 2016).

Within the NOD signaling pathway, RIPK2 is the key substrate for this process. Upon NOD activation, multiple ubiquitination events have been described on RIPK2, which are required to induce the activation of NF-κB and MAPK pathways. Ubiquitination was first observed to regulate the NOD1 and NOD2 pathways in studies that utilized over-expression of RIPK2 and *Mycobacterium tuberculosis* infections in macrophages. RIPK2 was stably ubiquitinated, and this ubiquitination was required for optimal cytokine production (Hasegawa et al., 2008). These results led to several subsequent studies and today it is widely accepted that polyubiquitin chains on RIPK2 serve as binding platforms for downstream signaling proteins that are vital for the activation of NF-κB and MAP kinases. The key events downstream of RIPK2 ubiquitination are the recruitment of the NF-κB-activating IkB kinase (IKK) complex composed of IKKα, IKKβ and NEMO (Inohara et al., 2000; Yang et al., 2007; Hasegawa et al., 2008), TGF-β activated kinase (TAK1), which is recruited via the two ubiquitin-binding scaffold proteins MAP3K7-binding protein 2 and 3 (TAB 2 and 3) (Kanayama et al., 2004) and the linear ubiquitin chain assembly complex (LUBAC), which is composed of a catalytic subunit HOIP and the two regulatory subunits HOIL-1 and SHARPIN (Gerlach et al., 2011).

IAPs: Critical E3 Ligases of RIPK2

So far K48, K63, M1 and more recently K27 ubiquitin linkages have been reported on RIPK2 (Damgaard et al., 2012; Panda and Gekara, 2018). Accordingly, an increasing number of E3 ligases and DUBs have been described to regulate the RIPK2 ubiquitin network (**Figure 3**). Screenings for ubiquitinated lysines within the kinase domain of RIPK2 suggested that ubiquitination of K209 is essential for signaling, and a RIPK2 K209R mutant was unable to activate NF-κB (Hasegawa et al., 2008).

A critical family of E3 ligases regulating NOD signaling are the IAPs. Cellular IAP1 and -2 (cIAP1/cIAP2), as well as X-linked IAP (XIAP), have all been reported to regulate NOD signaling (Bertrand et al., 2009; Krieg et al., 2009). The IAPs represent a group of cell death regulators and were initially described as caspase inhibitors, however, only XIAP is able to inhibit caspases at physiologically relevant concentrations. cIAPs, in turn, regulate cell death indirectly via their E3 ligase activity (Yang and Li, 2000). IAPs are defined by the presence of up to three approximately 70 amino acids long motifs called baculoviral IAP repeats (BIRs) (Birnbaum et al., 1994), which can mediate protein-protein interactions. Moreover, cIAP1, cIAP2, and XIAP contain a ubiquitin-associated domain (UBA) for binding to polyubiquitin chains and a really interesting new gene (RING) domain that provides E3 ligase activity (Silke and Vucic, 2014). The role of cIAP1 and cIAP2 in regulating TNF receptor signaling complexes is well-established: cIAPs directly ubiquitylate RIPK1 to facilitate activation of MAPK and canonical NF-κB pathways (Mahoney et al., 2008; Varfolomeev et al., 2008).

The first evidence that the cIAPs play a role in NOD signaling occurred in 1998. In HEK-293T cells overexpressed cIAPs co-immunoprecipitated with overexpressed RIPK2 (Thome et al., 1998). Bertrand et al. (2009) later showed that mice deficient in cIAP1 and cIAP2 had significantly reduced cytokine production in response to MDP injection compared to wild-type mice. Overexpression and pulldown experiments in HEK293T cells also suggested that cIAP1 and cIAP2 bind to and ubiquitinate RIPK2 independently of their CARD domains. However the role of the cIAPs in NOD signaling is controversial and we and other groups subsequently observed that removal of cIAP1/2 had no significant impact on signaling immediately downstream of NOD2 (Damgaard et al., 2012, 2013; Stafford et al., 2018). The discrepancy between the blunted cytokine response to MDP *in vivo* in cIAP1 and cIAP2-deficient mice but normal signaling in *ex vivo* stimulated BMDMs can be explained by a secondary autocrine loop that drives cIAP-dependent NF-κB and MAPK activation through TNFR1 (Stafford et al., 2018).

While recent studies argue against a critical role of cIAPs in NOD signaling, XIAP has emerged as a critical mediator of RIPK2 ubiquitylation and NOD signaling. The addition of K63-linked ubiquitin chains on RIPK2 is dependent on XIAP (Krieg et al., 2009; Damgaard et al., 2012). Using mouse and human cell lines devoid of XIAP, it was shown that XIAP is an indispensable component of the NOD signaling pathway and is required for the majority of ubiquitination on RIPK2. SPR recently revealed a direct interaction between the RIPK2 kinase domain and the BIR2 of XIAP (Goncharov et al., 2018). Consistently, IAP antagonists specifically targeting XIAP's BIR2 disrupted this interaction, and impair RIPK2 polyubiquitination and downstream activation of MAPK and NF-κB pathways (Goncharov et al., 2018; Hrdinka et al., 2018). Adding to the first discovered ubiquitination site K209, Goncharov et al. also described further XIAP-dependent ubiquitination sites (K410/K538) on RIPK2 that, when mutated, reduce NF-κB activation and cytokine production.

Other E3 Ligases and Deubiquitinases in the NOD Pathway

Ubiquitination of RIPK2 by XIAP is a vital step for subsequent recruitment of LUBAC (Damgaard et al., 2012), which is the only protein complex described so far to have the ability to add linear ubiquitin chains to substrates (Fiil et al., 2013; Tokunaga, 2013). It is not entirely clear whether linear ubiquitin chains are added on a previously non-ubiquitinated lysine residue of RIPK2, or as branching on a pre-existing ubiquitin chain. Cells lacking LUBAC subunits fail to fully activate NF-κB, which highlights the importance of LUBAC for efficient NF-κB and MAPK activation

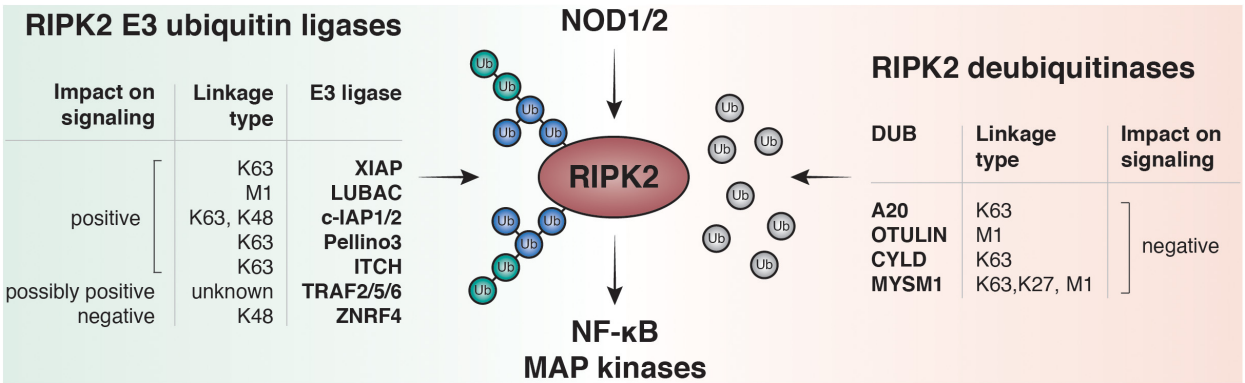

FIGURE 3 | RIPK2 ubiquitination regulates NF-κB and MAPK activation by NOD1 and NOD2. Upon ligand binding to NOD1 or NOD2, RIPK2 is rapidly ubiquitinated with K63- and M1-linked polyubiquitin chains. The K63-specific E3-ligase XIAP and the Linear-Ubiquitin Assembly Complex (LUBAC) have been shown to be essential for downstream responses including activation of NF-κB and MAPK pathways *in vitro* and to induce robust PGN-dependent immune responses *in vivo*. Other E3 ligases are able to bind and ubiquitinate RIPK2 such as cellular inhibitor of apoptosis protein-1 and protein-2 (c-IAP1 and c-IAP2), pellino3, itchy E3 ubiquitin protein ligase (ITCH), TNF receptor associated factor 2, 5, and 6 (TRAF2, TRAF5, and TRAF6) and zinc and ring finger 4 (ZNRF4). Deubiquitinases negatively regulate NOD signaling by removing ubiquitin from RIPK2. Amongst them are A20, OTU deubiquitinase with linear linkage specificity (OTULIN), ubiquitin carboxyl-terminal hydrolase CYLD, and histone H2A deubiquitinase MYSM1.

after NOD2 stimulation, possibly by recruiting and facilitating activation of the IKK complex.

Additional E3 ligases that mediate ubiquitination of RIPK2 to positively regulate NOD signaling have been reported: The TNF Receptor Associated Factors (TRAF) -2, -5, and -6, which are key adaptors in the TNFR1 signaling pathway contain a RING domain with E3 ligase activity. All three of these proteins have been linked to NOD signaling (Xie, 2013). So far there is no evidence for E3 ligase activity of TRAF2 and TRAF5 during NOD signaling, however, TRAF6 has been reported to directly contribute to RIPK2 ubiquitination. The knockdown of TRAF6 by siRNA in HEK293T cells reduced ubiquitination of RIPK2 and the induction of NF-κB following NOD2 stimulation (Yang et al., 2007). In another study, TRAF6 was not required for NOD signaling since TRAF6-deficient mouse embryonic fibroblasts (MEFs) still activated NF-κB and MAP kinases in response to NOD1 agonists (Hasegawa et al., 2008).

The E3 ligase Pellino3 was identified as another positive regulator of the NOD2 pathway, by mediating K63-linked ubiquitination of RIPK2. BMDMs from Pellino3-deficient mice displayed a lower activation of NF-κB and MAPK pathways and produce fewer cytokines after stimulation with MDP (Yang et al., 2013). Of note, the authors found a lower expression of Pellino3 protein in the colons of patients with Crohn's disease, supporting the theory that Pellino3 is an important mediator of NOD2 signaling in the gut.

E3 ubiquitin-protein ligase Itchy homolog (ITCH) was also reported to be a direct E3 ligase for RIPK2, by adding K63-linked ubiquitin chains in *in vitro* ubiquitination assays and pulldown experiments (Tao et al., 2009). BMDMs from ITCH knock-out mice failed to ubiquitinate RIPK2 and had reduced activation of NF-κB and MAPK pathways and consequently reduced expression of NF-κB target genes after MDP-stimulation.

More recently, ZNRF4 was identified as a negative regulator of NOD2-dependent NF-κB activation in a genome-wide RNAi screening in HEK293T cells. ZNRF4 induced K48-linked ubiquitination of RIPK2 and promoted RIPK2 degradation. Moreover, ZNRF4 knockdown macrophages produced higher amounts of pro-inflammatory cytokines in response to MDP and ZNRF4 knockdown mice displayed reduced tolerance to secondary exposure to MDP and *L. monocytogenes* (Bist et al., 2017). To sum up, these data suggest that ZNRF4 could be part of a negative feedback loop to turn off prolonged and aberrant NOD2 signaling after pathway activation.

The removal of ubiquitin by linkage-specific DUBS fine-tunes NOD1 and NOD2 signaling. A20 was the first DUB identified to negatively regulate NOD2 signaling by cleaving non-K48-linked ubiquitin chains (Hitotsumatsu et al., 2008). OTULIN was shown to limit M1-linked ubiquitination of RIPK2 and antagonize LUBAC after NOD2 activation and subsequent NF-κB and MAPK signaling (Fiil et al., 2013). The ubiquitin carboxyl-terminal hydrolase CYLD targets both M1- and K63-linked ubiquitin linkages to limit NOD2 signaling (Hrdinka et al., 2016). Panda et al. showed that RIPK2 is also ubiquitinated with atypical K27-linked chains and Histone H2A deubiquitinase MYSM1 is a DUB that specifically removes K27-, K63- and M1-specific chains to dampen NOD2 signaling. Supporting a role in NOD signaling, MYSM1-deficient mice injected intraperitoneally with MDP exhibited higher recruitment of neutrophils to the peritoneum and peripheral organs (Panda and Gekara, 2018).

SIGNALING OUTCOMES OF NOD ACTIVATION

NOD2 Signaling and Disease

Inflammatory bowel disease, particularly Crohn's disease, is the most commonly associated pathology associated with NOD2 signaling (Caruso et al., 2014; Philpott et al., 2014). However there is compelling evidence that deregulated NOD1/2 signaling

is associated with inflammation-associated diseases such as early-onset sarcoidosis, uveitis, neuropathic pain, rheumatoid arthritis or solid cancers (Caruso et al., 2014; Kim et al., 2016) and more recently with allergic asthma (Miller et al., 2018) and type 2 diabetes mellitus (T2DM) (Amar et al., 2011; Schertzer et al., 2011; Denou et al., 2015; Cavallari et al., 2017). Most of these disease associations have been reviewed extensively elsewhere (Kanneganti et al., 2007; Chen et al., 2009; Philpott et al., 2014; Mukherjee et al., 2019), and we focus here briefly on the most recent understanding of how NOD signaling can contribute to IBD or T2DM.

A clear hot spot for NOD2 related pathologies is the intestinal tract. The two key players in NOD2 signaling, NOD2 and RIPK2 are both highly expressed in intestinal epithelial cells as well as in resident immune cells in the gut. NOD2 seems to have an important role in gut homeostasis as there is evidence that NOD2 directly regulates colonic epithelial cell growth and survival. Nevertheless, NOD2-deficient mice do not have intestinal inflammation and display normal myeloid and lymphoid cellularity in the gut, at least in the absence of stimulation (Kobayashi et al., 2005). However NOD2-deficient mice do have reduced clearance upon oral or intragastric bacterial challenge (Kim et al., 2011). *In vitro*, primary colonic epithelial cells induced cell death in response to treatment with the NOD2 ligand MDP, while cells from NOD2-deficient mice were protected and shRNA-mediated knockdown of NOD2 in human colonic carcinoma cells resulted in increased levels of apoptosis (Cruickshank et al., 2008).

Several studies show an intimate link between NOD signaling and TLR signaling in the gut: NOD2 can significantly inhibit TLR4 signaling in enterocytes of the neonatal small intestine resulting in marked protection from the induction of TLR4-dependent apoptosis (Richardson et al., 2010). Furthermore, NOD2-deficient mice have exacerbated antigen-specific colitis that is dependent on TLR2 function (Watanabe et al., 2006). Subsequently it was shown that NOD2 protects in mouse models of experimental colitis via a cross-tolerance mechanism that dampens TLR responses (Hedl et al., 2007; Watanabe et al., 2008; Hedl and Abraham, 2011b), which relies on the induction of interferon regulatory factor 4 (Watanabe et al., 2014).

In experimental models of type 2 diabetes mellitus (T2DM), alterations in the intestinal barrier lead to increased intestinal permeability and translocation of PAMPs to the bloodstream, a phenomenon named metabolic endotoxemia (Cani et al., 2007). It is a well-established concept, that chronic exposure to low levels of bacterial components in the plasma, such as LPS or MDP, promotes inflammation and contributes to the development of hepatic insulin resistance. Therefore, it is not surprising that NOD1 and NOD2 agonists have been identified as modulators of insulin sensitivity. Intriguingly, the activation of either NOD1 or NOD2 leads to different outcomes in mouse models of T2DM: NOD1/2 double-knockout mice (Schertzer et al., 2011) and NOD1 knockout mice (Amar et al., 2011) were protected from HFD-induced insulin resistance. This effect was due to the role of NOD within immune cells, as bone marrow chimeras using bone marrow from NOD1-deficient mice transplanted into wild-type mice were protected against glucose and insulin tolerance (Chan et al., 2017). Unlike NOD1-knockout mice, animals deficient in NOD2 showed no protection to insulin resistance during HFD and even had increased adipose tissue and liver inflammation as well as exacerbated insulin resistance (Denou et al., 2015). Accordingly, injections of mice with the NOD2 ligands MDP and Mifamurtide reduced insulin resistance in mouse models of HFD-induced obesity and insulin resistance after endotoxic shock, while the NOD1 ligand FK565 worsened glucose tolerance (Cavallari et al., 2017). This divergence between the roles of NOD1 and NOD2 could be explained by the differential tissue and cellular distributions of the receptors.

Pharmacological Inhibition of the NOD2 Pathway

Given the involvement of NOD2 and RIPK2 in a range of diseases, inhibition of RIPK2 could have an application in inflammatory diseases driven by dysregulated NOD signaling pathways. Kinase inhibitors with significant activity toward RIPK2 are already approved for clinical use, such as the multi-tyrosine kinase inhibitor ponatinib and the EGFR inhibitor gefitinib (Canning et al., 2015). Over the last years, significant efforts have been put towards the development of more specific RIPK2 inhibitors and multiple compounds have been successfully tested in mice. Two groups independently developed highly specific RIPK2 inhibitors, that could efficiently block cytokine production *in vivo* after intraperitoneal administration of MDP (Goncharov et al., 2018; Hrdinka et al., 2018). Furthermore, a specific RIPK2 inhibitor WEHI-345, was used to protect against the onset of paralysis in the experimental autoimmune encephalomyelitis (EAE) model for multiple sclerosis (Nachbur et al., 2015). These experiments also showed that even though RIPK2 kinase inhibitors bind into the ATP-binding pocket and block its kinase activity, their real mode of action is by blocking NOD signaling through disruption of the RIPK2-XIAP interaction. Lastly, GlaxoSmithKline has tested their RIPK2 kinase inhibitor GSK-559 in Phase 1 clinical trials for IBD, however, they have recently terminated their RIPK2 program.

An alternative approach to inhibit the NOD pathway is to antagonize the critical E3 ligases IAPs. However, compounds that target cIAPs and XIAP are not tolerated in the clinic as they induce an inflammatory response *in vivo* (Lawlor et al., 2015). Until recently, all reported compounds with activity toward XIAP were pan IAP inhibitors (Condon et al., 2014). Recently new compounds that only target XIAP have been developed and could be promising tools to block NOD signaling without inducing cell death (Goncharov et al., 2018). Similar to RIPK2 inhibitors, these new compounds antagonize NOD signaling by disrupting the RIPK2-XIAP interaction.

IS NOD SIGNALING LINKED TO CELL DEATH?

As discussed in detail above, signaling downstream of NOD1/2 harbors many proteins and protein domains that are closely associated with cell death signaling. A link between NOD

signaling and cell death induction seemed therefore likely ever since NOD signaling was studied.

NOD1, NOD2, and RIPK2 harbor one or multiple highly conserved CARDs, which are known to recruit caspases, the key mediators of apoptosis. It is therefore not surprising that all these proteins have initially been associated with caspase binding and with programmed cell death. Indeed, overexpression studies with NOD2 showed that it could bind to multiple caspases via its CARD, and was able to directly activate caspase-9 and induce apoptosis (Inohara et al., 1999). This was attributed to the analogy to Apaf-1, the well-characterized activator of caspase-9 in the intrinsic apoptosis pathway. Similarly, NOD1 was able to directly activate Caspase-9 in a RIPK2-dependent manner. This was somewhat surprising as also RIPK2 was shown to interact with Caspase-9, but not to activate it. It was therefore suggested that RIPK2 needs to interact with NOD1 for caspase-9 activation (Bertin et al., 1999).

An indirect link between NOD signaling and apoptosis was suggested in early studies on RIPK2, which showed that overexpressed RIPK2 could potentiate CD95-induced apoptosis via caspase-8 and caspase-10 (Inohara et al., 1998). ATP binding to RIPK2 was critical for this function as the mutation of K38 resulted in reduced cell death after CD95L stimulation. Notably, RIPK2 also interacted with various members of the death receptor machinery, including cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein (c-FLIP), cIAP1 and cIAP2 and members of the TNFR-associated factor (TRAF) family (Thome et al., 1998). These findings suggested that RIPK2 may play a role in the regulation of cell death, which was supported by experiments conducted in MCF-7 breast carcinoma cells, where overexpression of RIPK2 induced apoptosis (McCarthy et al., 1998). The cell death-inducing function of RIPK2 was dependent on the CARD and could be blocked with the caspase inhibitor zVAD.

The strongest evidence that suggests direct involvement of NOD signaling pathways in regulating caspase functions stem from observations that NOD1 and NOD2 can induce IL-1β through NF-κB and MAPK pathways in multiple human and mouse cell populations, including myeloid-derived cells (Li et al., 2004; Watanabe et al., 2004; Abraham and Cho, 2009). Moreover, there is evidence that NOD2 directly activates caspase-1 in certain cell lines (Damiano et al., 2004; Ferwerda et al., 2008; Hsu et al., 2008; Marina-Garcia et al., 2008). In human monocyte-derived macrophages (MDMs), activation of NOD2 leads to rapid IL-1β processing and autocrine signaling, a process that was essential for robust cytokine production (Hedl and Abraham, 2011a). The authors measured early MAPK activation, which was dramatically reduced by blocking IL-1β signaling and by inhibiting caspases using zVAD. Since the effects were visible already before transcription, translation, and secretion of IL-1β would be expected to occur, a model where NOD2 stimulation activates caspase-1, leading to the rapid processing of preformed pro-IL-1β, which in turn mediates early MAPK activation was suggested (Hedl and Abraham, 2011a).

A surprising finding was presented later, when it was shown that Bid, a well-characterized pro-apoptotic member of the Bcl2 family, was shown to be required for NOD signaling, as cells and mice deficient in Bid were not able to react to MDP (Yeretssian et al., 2011). However this finding was refuted shortly after (Nachbur et al., 2012) and Bid has since not been linked to NOD signaling, nor has it come up in screens for regulators of the NOD signaling pathway (Warner et al., 2014).

While there seems to be no direct link between NOD signaling and apoptosis, there is a strong link between NOD signaling and autophagy, the disassembly of damaged or unnecessary cellular components, that can result in death. In the context of NOD signaling, autophagy is more likely to be a cellular defense mechanism for bacterial clearance rather than a cell death mechanism.

Taken all together, initial experiments that linked NOD signaling with cell death could not be confirmed when endogenous protein levels and physiological ligands were used in later experiments. While overexpression studies are an important tool to determine molecular mechanisms of cell signaling, it has become clear that one has to be cautious when assessing the effects of overexpressed proteins on cell death. The last decade has seen many advances in establishing the links between innate immune signaling pathways and cell death, using mainly myeloid cells and relevant ligands. It has become clear that the link between NOD signaling and cell death is not as straight forward as initially thought, despite the indisputable involvement of cell death-related proteins and cell death-promoting domains.

CONCLUSION AND PERSPECTIVES

The title of this research topic is "Connecting the dots between inflammatory signaling and the working of cell death." Here we have dissected the molecular mechanisms of signaling downstream of the intracellular PGN receptors NOD1/2. We have found that a critical point of difference between the NOD pathway and other innate immune signaling pathways is its failure to connect these dots. This is somewhat surprising. Not only do most inflammatory signaling pathways directly or indirectly induce cell death, but also have early studies implicated that activation of the NOD signaling pathway results in caspase activation and apoptosis. The development of new reagents and model systems has led to studies using endogenous proteins and specific means to stimulate the NOD pathways, as well as the use of relevant cell types. This is in contrast to earlier studies that were largely based on overexpression of members of the NOD pathway. In these newer work, the initial findings that NOD1/2 activation leads to any form of cell death could not be confirmed.

So what is different between the NOD pathway and other cell death-inducing inflammatory pathways? One reason could be that the NOD pathway is not exclusively pro-inflammatory at all. The best evidence is the strong association of NOD2 mutations with Crohn's disease: These are prominently loss of function mutations within NOD2, suggesting that NOD signaling has an anti-inflammatory role. Conversely, hyperactivation of the NOD pathway is described in other inflammatory diseases and elevated RIPK2 activation, a hallmark of NOD signaling, is observed in many pathologies, intriguingly also in patients with IBD. Therefore, the NOD pathway rather plays an

immunomodulatory role, rather than a pro-inflammatory. A cell that induces inflammation needs to be shut down rapidly to avoid hyperactivation of an inflammatory response, and a potent way to do so is to induce programmed cell death in this cell. If NOD signaling is, however, not as inflammatory at all, there is no need to self-destruct and hence the missing link between NOD signaling and cell death.

Despite the missing link between cell death and NOD signaling, this pathway has emerged as an important contributor to human pathologies. Therefore, significant efforts have been put toward better understanding the molecular mechanisms of NOD signaling. The focus for the development of therapeutics interfering with NOD signaling has been the kinase RIPK2, and several ATP competitive inhibitors have been developed by commercial and academic entities. The most recent data show convincingly, however, that the kinase activity of RIPK2 is dispensable for downstream signaling, and the critical role of RIPK2 is its scaffolding function in the pathway. Therefore, the understanding of protein-protein interactions and the ubiquitin network on RIPK2 and other members of the NOD pathway is pivotal for the development of novel therapeutics in this space.

AUTHOR CONTRIBUTIONS

VH generated the figures. VH, CS, and UN wrote the review.

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