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Review

TNF and ROS Crosstalk in Inflammation

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Tumor necrosis factor (TNF) is tremendously important for mammalian immunity and cellular homeostasis. The role of TNF as a master regulator in balancing cell survival, apoptosis and necroptosis has been extensively studied in various cell types and tissues. Although these findings have revealed much about the direct impact of TNF on the regulation of NF-κB and JNK, there is now rising interest in understanding the emerging function of TNF as a regulator of the generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS). In this review we summarize work aimed at defining the role of TNF in the control of ROS/RNS signaling that influences innate immune cells under both physiological and inflammatory conditions.

Trends

TNF is a proinflammatory cytokine with important functions in mammalian immunity and cellular homeostasis. Deregulation of TNFR signaling is associated with inflammatory diseases.

ROS at low concentrations have important functions in regulating pathways such as TNFR1 signaling, but high ROS concentrations ultimately lead to DNA damage and cell death.

Signaling pathways culminating in NF-κB activation are influenced by ROS and lead to upregulation of antioxidant proteins, demonstrating that TNF and ROS influence each other in a positive feedback loop.

Regulation of the redox state and signaling is further complicated by TNF-induced production $NO^{\bullet}$ and the formation of RNS.

A better understanding of the interplay between TNF and ROS/RNS could reveal new therapeutic targets for many inflammatory diseases.

TNF and ROS Are Interconnected

Tumor necrosis factor (TNF) (see Glossary) plays crucial roles in both normal and malignant cells, and thus is an intensely studied cytokine. After its discovery in the 1970s it became clear that TNF is a central player in many processes including cell survival, apoptosis, and necroptosis as well as intercellular communication. Dysregulation of these processes is a hallmark of inflammatory diseases and cancers. In these contexts, TNF regulates a complex signaling network that can trigger either cell survival or cell death [1] (Box 1).

More than a decade ago TNF-dependent but caspase-independent necrotic cell death (necroptosis) was shown to involve **reactive oxygen species** (ROS) that could be derived from either **mitochondrial** or non-mitochondrial sources [2,3]. More recently, RIPK1/3-mediated phosphorylation of MLKL during TNF-induced necroptosis was demonstrated to generate ROS and activate JNK [4]. Accordingly, TNF-induced mitochondrial ROS production was abrogated in RIPK1/3- or MLKL-deficient cells, which failed to undergo necroptosis [4–7]. This link between TNF and ROS adds another layer of complexity to the TNF signaling network because ROS act on many proteins needed to regulate cellular homeostasis, including those mediating cell proliferation, survival, death, differentiation, DNA repair, and metabolism. This review examines the molecular connections between ROS and TNF signaling under physiological and pathophysiological conditions.

ROS and TNF Signaling

TNF signaling is multi-faceted – TNF may be soluble (sTNF) or membrane-bound (mTNF), and two TNF receptors, TNFR1 and TNFR2, exist. TNFR1 is ubiquitously expressed on almost all cell types and can be activated by both sTNF and mTNF, whereas TNFR2 is restricted to immune and endothelial cells and is dependent on the presence of mTNF [8]. The binding of sTNF to TNFR1 can lead to activation of **nuclear factor κB** (NF-κB), the key transcription factor driving cell survival signaling, as well as to cell death. By contrast, TNFR2 has been mainly associated with NF-κB and implicated in tissue regeneration and immune modulation [9,10] (Box 2). It is now acknowledged that ROS are important regulators of TNF–TNFR signaling leading to cell

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Box 1. TNFR Complexes

TNF binding to TNFR1 or TNFR2 causes different molecular complexes to form that transduce signals with differing biological effects. TNF–TNFR1 engagement induces a conformational change in the receptor cytoplasmic domain that recruits the adaptor protein TRADD. Also recruited to TNF-TNFR1 is RIPK1 (TRADD-dependent and -independent recruitment are possible) [116–118]. TNFR1, TRADD, and RIPK1 initiate the assembly of membrane-bound TNFR complex I: binding of TRAF2 (or TRAF5) to the TRADD N-terminal TRAF-binding domain, followed by the binding of TRAF2 to cellular inhibitor of apoptosis protein-1 (cIAP1) and -2. At this point RIPK1 acts as a molecular switch between cell survival and cell death, depending on its state of ubiquitination. cIAP1 and cIAP2 attach K63-linked polyubiquitin chains to RIPK1, which facilitate to recruit the linear ubiquitin chain assembly complex (LUBAC) to complex I [1,116,119]. LUBAC stabilizes complex I by attaching linear M1-linked linear polyubiquitin chains to RIPK1 and prevents inflammation [120]. Polyubiquitination of RIPK1 in complex I is essential for the recruitment of TAB2/3 and TAK1, as well as for NF-κB activation that prevents cell death [1,121]. However, when RIPK1 in complex I is deubiquitinated by cylindromatosis (CYLD), RIPK1 dissociates from the membrane-bound TNFR1 signaling core. Deubiquitinated RIPK1 then assembles in the cytosol with TRADD, FADD, procaspase 8, and $cFLIP_L$ to form complex IIa. An alternative cytoplasmic complex IIb is assembled under conditions in which cIAP1/cIAP2 are depleted and cannot ubiquitinate RIPK1 in membrane-bound complex I. Once again, non-ubiquitinated RIPK1 dissociates from complex I, but assembles with FADD (not TRADD), procaspase 8, c-$FLIP_L$, and RIPK3 to form complex IIb [1].

In both complexes IIa and IIb, procaspase 8 forms homodimers and heterodimers with c-$FLIP_L$. RIPK1/RIPK3 can be inactivated by cleavage either by fully activated caspase 8 (leading to apoptosis) or the caspase 8–$FLIP_L$ heterodimer (leading to survival). Full inactivation of RIPK1 and RIPK3 is crucial to prevent necroptosis [1].

If RIPK1/RIPK3 are not inactivated, for example under conditions where caspases are inhibited and deubiquitinated RIPK1 is present, necroptosis will be initiated. A crucial downstream mediator of necroptosis is the pseudokinase mixed-lineage kinase domain-like (MLKL), which is phosphorylated by RIPK3 (see [1,121] for a comprehensive overview of TNFR1 signaling in ubiquitination and cell death).

survival or death (Table 1) [11]. Based on the available data, this review focuses mainly on TNF–ROS signaling crosstalk mediated by TNFR1.

ROS and NF-κB Signaling

Intracellular ROS are generated either from extracellular sources of oxygen species that arise as a result of the action of NADPH oxidase (NOX) or from the mitochondrial respiratory chain. Although it is clear that ROS are crucial for NF-κB signaling downstream of TNF [12], debate is ongoing over whether mitochondrial ROS are involved in NF-κB activation or inactivation. The generally accepted hypothesis holds that TNF-induced ROS suppress NF-κB activation [13], decreasing NF-κB-mediated survival signaling and accounting for the cell death associated with high ROS levels. Conversely, significant data exist indicating that mitochondrial ROS can promote, rather than inhibit, TNF-mediated NF-κB activation [14]. For example, specific inhibition of mitochondrial ROS in human monocytes and T cells using the mitochondria-specific antioxidant MitoVit E has confirmed that mitochondrial ROS are important for NF-κB activation [15]. Under normal physiological conditions, TNF simultaneously induces the pro-apoptotic cascade triggered by procaspase 8 cleavage and the anti-apoptotic pathway mediated by NF-κB activation. Interestingly, in cells where mitochondrial ROS generation is blocked by MitoVit E, TNF-induced procaspase 8 activation proceeds normally, but activation of caspase 3, cleavage of the pro-apoptotic Bcl-2 family member BID, and release of cytochrome *c* from mitochondria are significantly increased. Thus, inhibition of TNF-mediated mitochondrial ROS production apparently diminishes NF-κB activation, suggesting that mitochondrial ROS can positively control NF-κB signaling [15]. It is not yet understood how mitochondrial ROS activate NF-κB, but it is assumed that ROS inactivate the phosphatases that regulate the activity of the kinases controlling NF-κB signaling. Such ROS-mediated phosphatase inhibition would lead to enhanced phosphorylation of IκB, triggering its degradation and permitting NF-κB activation [14,16].

ROS and JNK Signaling

TNF-induced ROS production is also important for crosstalk between the NF-κB-induced cell survival pathway and the JNK-induced cell death pathway [17–19]. Current understanding of the

Box 2. TNFR2 as an Anti-Inflammatory Mediator

Whereas TNF–TNFR1 signaling promotes inflammatory disease, TNF–TNFR2 signaling appears to have protective anti-inflammatory effects [9]. Rat cardiac myocytes can utilize TNF–TNFR2 signaling to counteract TNF–TNFR1-induced ROS production and prevent cell death [122]. TNF can have both neurodegenerative and neuroregenerative effects. Although signaling through TNFR1 is mostly associated with damaging effects resulting from inflammation, oxidative stress, and apoptosis, TNFR2 activity has neuroprotective effects by stimulating NF-κB and AKT-dependent signaling pathways in neurons [10]. TNFR2 signaling results in the release of anti-inflammatory and neurotrophic factors from microglia and astrocytes. In mice, mTNF-TNFR2 signaling activates myeloid-derived suppressor cells (MDSC), leading to the upregulation of NOS2 and arginase-1 (ARG1), the activation of p38 and NF-κB, and the secretion of ROS/NO/RNS, TGF-β, and IL-10. These MDSCs show enhanced suppressive activities that ultimately inhibit T cell proliferation resulting in increased tumor progression [123]. Another possible mediator of TNFR2 signaling effects are regulatory T cells (Treg). It has been described that TNFR2 is expressed at high levels in a population of Treg cells with high suppressive capacity ($CD4^+$ $CD25^+$ $FOXP3^+$) [124]. Activation of TNFR2 is important for the proliferation and function of these Tregs, indicating an important role of TNFR2 in the regulation and suppression of the immune response. Importantly, inhibition of Treg function leads to an increased risk of autoimmune disease. These results and others further support an immunosuppressive function for TNFR2 that contrasts with the proinflammatory function of TNFR1 [125].

relationship between JNK and ROS is that there is a positive feedback loop between ROS-dependent JNK activation and the generation of JNK/SAB-dependent mitochondrial ROS [20,21]. The outer mitochondrial membrane protein SAB (SH3 homology associated BTK binding protein) binds to and recruits activated JNK, increasing mitochondrial ROS generation and sustaining JNK activation in a self-amplifying loop. Notably, TNF-stimulated ROS production occurs in wild-type mouse embryonic fibroblasts (MEFs) but not in $Jnk^{-/-}$ MEFs [22]. This observation indicates that JNK contributes to TNF-induced ROS generation, which in turn stimulates persistent JNK activation. The interplay among TNF, JNK, and ROS in promoting cell death is illustrated in Figure 1.

TNF-Induced Antioxidant Signaling

TNF-induced NF-κB signaling leads to the transcription of not only anti-apoptotic genes but also genes involved in decreasing intracellular ROS levels. This production of antioxidants in response to NF-κB activation plays an important role in balancing ROS effects. Two major players in this context are manganese superoxide dismutase-2 (MnSOD2) and catalase, both of which counteract TNF-induced apoptosis by neutralizing mitochondrial ROS [23,24]. Two other key antioxidants are heme oxygenase-1 (HO-1) and H-ferritin (also known as FHC/FTH). HO-1 catalyzes heme degradation, resulting in the formation of CO and biliverdin, which is subsequently reduced to bilirubin, a potent antioxidant [14]. H-ferritin controls ROS generation, which would otherwise drive persistent JNK signaling, through its ferroxidase activity, which sequesters iron atoms that could be used to catalyze ROS generation [13,25,26].

Another important antioxidant in the TNF context is the master transcription factor NRF2. In principle, the NRF2 pathway could support TNF-induced NF-κB-mediated survival signaling by preventing sustained activation of JNK through massive upregulation of antioxidants [27,28]. However, the interaction of NF-κB (p65) with KEAP1 leads to inhibition of the NRF2–ARE pathway [29]. In addition, NRF2 activity is repressed by MAFK, a novel coactivator of NF-κB signaling [30]. In chronically inflamed tissues, the NRF2 pathway attempts to reinstate a redox balance that promotes cellular repair and limits TNF-induced ROS and its associated **inflammation** [31]. Thus, ROS signaling leading to the activation of NRF2-, HO-1-, and/or H-ferritin-mediated pathways can protect against ROS-mediated inflammation induced by TNF.

ROS and TNF-Induced Apoptosis

The decision of whether a particular cell lives or dies is crucial for the survival of an entire organism. TNF and ROS play important roles in this physiologically vital decision-making, which can be modified at various levels. A TNF-initiated death signal can be influenced by mitochondrial ROS, which contribute to apoptosis by inducing **mitochondrial outer-membrane**

Glossary

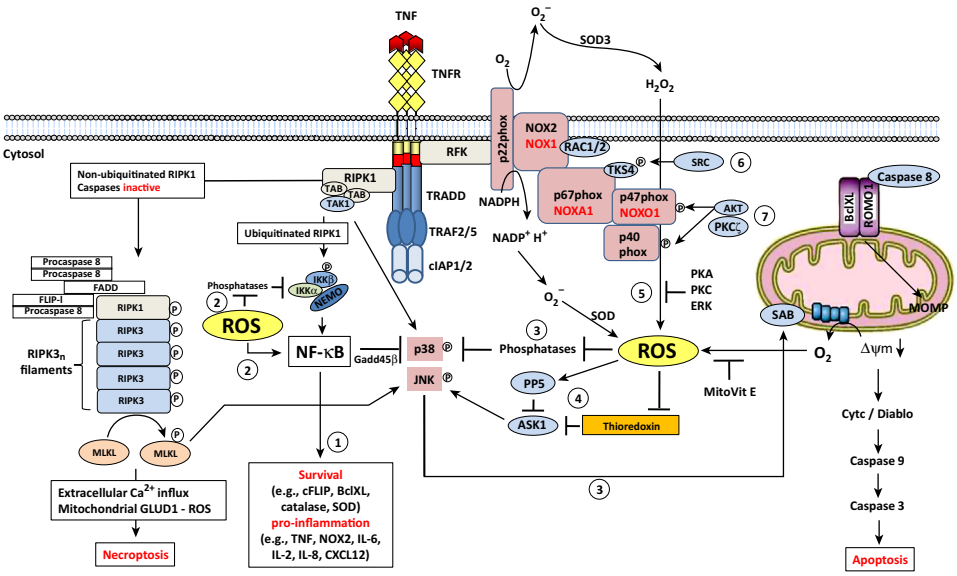
Inflammation: an innate immune response that occurs at a site of tissue damage caused by either physical injury or a chemical or biological agent. Classic signs include heat, redness, pain, swelling, and loss of tissue function. Chronic inflammation can be pathological.
Innate immune system: the collection of leukocytes and their products that provides immediate defense against pathogens. Relies on recognition of common molecular motifs by pattern recognition receptors.
Mitochondrion: a multifunctional organelle that is found in most eukaryotic cells and generates ATP. Considered to be the 'energy powerhouse' of the cell.
Mitochondrial outer-membrane permeabilization (MOMP): process by which specific proteins in a cell disrupt the outer mitochondrial membrane and trigger the release of mitochondrial proteins that promote mitochondria-dependent cell death.
Mitophagy: autophagic removal of mitochondria under conditions of nutrient starvation or mitochondrial stress.
Nuclear factor κB (NF-κB): transcription factor responsible for the expression of key cell survival genes. Following activation of the IKK complex, the inhibitor IκB that holds NF-κB in an inactive state in the cytoplasm is degraded, freeing NF-κB to translocate to the nucleus and drive gene expression.
Reactive oxygen/nitrogen species (ROS/RNS): Chemically reactive oxygen- or nitrogen-derived molecules produced by various cellular mechanisms, including mitochondrial respiration. At low concentrations, ROS/RNS play key roles as messengers during cell signaling and proliferation. However, stress-related increases in ROS/RNS may result in significant damage to cellular components such as DNA and RNA, and trigger cell death.
Tumor necrosis factor (TNF): a cytokine participating in a broad range of cellular processes and responses including survival, differentiation, inflammation, and various forms of cell death.

Table 1. TNF Signaling Pathways and their Outcomes

Receptor	Ligand[a]	Induced Signaling (Outcome)	Reactive Species	Reactive Species-Induced Signaling
TNFR1	sTNF	TRADD/RIP–MLKL–JNK [4] (mitochondria involvement through SAB)	ROS ↑	Activate ASK1–JNK–TNF (pro-necroptosis) Inhibit NF-κB (cell death) [13] Activate NF-κB–BclXL–Bcl2 (pro-survival) [12] Activate Bim/Puma (pro-apoptosis)
		TRADD/RIP and/or RAC/RFK/NOX1	ROS ↑	Activate JNK (pro-necroptosis) [58–62]
		NF-κB → increased transcription: cFLIP, BclXL, catalase, SOD (pro-survival)	ROS ↓	Opposes cell death via reduced cellular ROS
		NF-κB → increased transcription of TNF, NOX2, IL-6, IL-2, IL-8, CXCL12 (proinflammation)	ROS ↑	Activate JNK (pro-necroptosis)
		Caspase 8–ROMO–BclXL–MOPS (mitochondria involvement) [39–41] Caspase 8–caspase 3 (pro-apoptosis)	ROS ↑	Activate ASK1–JNK (cell death)
		NF-κB–NOS2 [14] NF-κB–KEAP1–NRF2–HO-1–H-ferritin (pro-survival)	$NO^{\bullet}$ ↑	Activate NRF2–HO-1–H-ferritin (pro-survival) $NO^{\bullet}$–ROS interaction → RNS (cell death)
	mTNF	RIP1-independent, CAPK[b]-dependent (mitochondria involvement) [126]	ROS ↑	(pro-necroptosis)
TNFR2	mTNF	RIP1-independent, CAPK-dependent, TNFR1-independent (mitochondria involvement) [126] TNF production	ROS ↑	TNFR2 activation can support TNFR1-triggered oxidative burst (pro-necroptosis)
		cPLA2, ERK, MSK1, PKCζ, CaMKII, PLB (pro-survival) [122]	ROS ↓	Inhibit cell death owing to reduced cellular ROS
		NF-κB, Bcl2, SOD2 (pro-survival) [10,125] AKT caspase 9 inactivation (pro-survival) [10]	ROS ↓	Inhibit cell death owing to reduced cellular ROS
		TRAF1/2, p38, NF-κB, NOS2, ARG1 [123]	Secreted $NO^{\bullet}$ ↑ ROS ↑	Activate secretion of $NO^{\bullet}$ and ROS to inhibit T cell proliferation (immunosuppression)

[a]sTNF, soluble/secreted TNF; mTNF, membrane-bound TNF.
[b]CAPK, ceramide-activated protein kinase.

permeabilization (MOMP) and JNK activation [21]. Mitochondrial contributions to apoptosis are largely controlled by proteins of the Bcl2 family. For example, ROS induce expression of the pro-apoptotic Bcl2 proteins Puma and Bim [32,33]. However, the pro-apoptotic activities of these molecules can be neutralized by the anti-apoptotic proteins Bcl-XL and Bcl2, whose expression is controlled by NF-κB-induced survival signaling [34]. When the pro-apoptotic signals in a particular cell outweigh the anti-apoptotic signals, MOMP is induced. MOMP results



Trends in Cell Biology

Figure 1. TNF-Induced ROS Signaling through NOX1/2 Complexes. Engagement of TNFR1 by TNF activates the NOX1 or NOX2 complex, depending on cell type (NOX1 complex subunits are labeled in red, NOX2 complex subunits are labeled in black, p22phox and p40phox are shared). The first step of NOX1/2 activation is the interaction of the cytosolic domain of TNFR1 with RFK and p22phox. The activated NOX complex converts extracellular O_2 into O_2^-, which extracellular SOD3 then converts into extracellular H_2O_2. This H_2O_2 passes freely through the plasma membrane and acts as a major source of intracellular ROS. Second, TNF-induced formation of complex II leads to interactions between activated caspase 8, ROMO1, and Bcl-XL in the outer mitochondrial membrane. These interactions reduce mitochondrial membrane potential, triggering MOMP and the production of mitochondrial ROS. Also illustrated are several feedback loops that regulate TNF-induced ROS signaling. (1) TNF-mediated NF-κB activation upregulates catalase and SOD expression, leading to an antioxidant response, but also induces TNF and NOX2 expression that feed back into ROS generation. (2) ROS activate NF-κB directly or indirectly by inhibiting IKK phosphatases. (3) ROS activate the JNK pathway by inhibiting MAPK phosphatases, while JNK stimulates mitochondrial ROS production through SAB. (4) Although ROS block the interaction of thioredoxin with ASK1, which frees ASK1 to activate JNK, ROS can also activate PP5, which negatively regulates ASK1. (5) PKA, PKC, and ERK block NOX1-induced ROS production by inhibiting NOXA1 function. (6) SRC-mediated activation of TKS4 positively regulates NOX-induced ROS production. (7) AKT and PKCζ promote NOX complex activation. Please note that the negative regulation of ROS–JNK signaling by the NRF2/HO-1/H-ferritin pathway is shown in the context of RNS in Figure 2.

in the cytosolic release of mitochondria-derived pro-apoptotic factors such as cytochrome *c* and Smac/Diablo [35]. As a result, caspase 9 and caspase 3 are activated and execute classical apoptosis. More detailed descriptions of ROS in apoptosis and the mitochondrial death cascade appear elsewhere [36,37].

The precise molecular mechanism by which TNF stimulation leads to increased mitochondrial ROS within a cell is not clear. In response to TNF, complex II containing procaspase 8 can be formed [38]. Activated caspase 8 can bind to ROS modulator-1 (ROMO1), which is located in the mitochondrial outer membrane. ROMO1 then sequesters Bcl-XL, which reduces mitochondrial membrane potential. As a result, ROS are produced that trigger JNK activation and apoptosis [39–41]. A central element in this pathway is apoptosis signal-regulating kinase-1 (ASK1). ASK1 is a mitogen-activated protein kinase kinase kinase (MAPKKK) that activates the JNK and p38 pathways and is required for TNF-induced apoptosis [42]. ASK1 is inactive as long as it is bound by reduced thioredoxin. When thioredoxin is oxidized by ROS, ASK1 is released and activates downstream targets such as JNK and p38 in a TRAF2/TRAF6-dependent manner [43–45]. Alternatively, ASK1 can be inactivated by protein phosphatase

5 (PP5), which is regulated by ROS [46]. This observation suggests the existence of a ROS-dependent feedback loop that controls ASK1 activity and regulates ASK1-induced cell death based on temporal and spatial variations in ROS levels. ROS can further support apoptosis by inactivating JNK-inactivating phosphatases [47]. This regulation leads to sustained JNK activation, which is required for cytochrome *c* release and caspase 3 activation during apoptosis.

ROS and TNF-Induced Necroptosis

ROS have a significant effect on necroptosis, particularly the mitochondrial ROS generated in response to TNF/TNFR1 engagement [48–54]. However, experiments in which mitochondria were depleted by **mitophagy** have indicated that mitochondrial ROS are not essential for necroptosis and can be bypassed if caspases are inhibited [55]. Non-mitochondrial derived ROS have also been implicated in TNF-induced necroptosis [50]. Thus, there are probably complementary pathways that lead to necroptosis, and non-mitochondrial ROS can drive this form of cell death in some cell types.

A significant source of non-mitochondrial ROS participating in TNF-induced necroptosis is the plasma membrane-associated NOX1 complex, which is expressed in non-phagocytic cells [56,57]. When these cells respond to TNF, recruitment of the NOX1 complex to TNFR1 is facilitated by TRADD/RIPK and/or RAC1/riboflavin kinase (RFK) [58–60]. This juxtaposition with TNFR1 leads to NOX1 activation, and the NOX1 complex then transiently produces ROS in a mitochondria-independent manner. These non-mitochondrial ROS contribute to persistent RIPK1-dependent JNK activation that precipitates necroptosis [50,61,62]. The role of RAC1 in this process has been confirmed by a dominant-negative RAC1 mutant that exhibits reduced TNF-induced NOX1 activation, O_2^- generation, and necroptosis [50]. ROS from different sources, either from the mitochondria (RIPK1/3, MLKL-dependent) or the NOX1 complex (RIPK1-dependent), seem to be important for necroptosis [4,50]. Considering these different findings, however, there must be cell-specific regulation of these pathways. More molecular work is needed to clarify whether, under which conditions, and in which cells these pathways can compensate for each other.

Once activated by TNF signaling, the NOX1 complex is controlled at several levels. The NOX1 complex contains five subunits: NOX1, NOXA1, NOXO1, p22phox, and p40phox. NOX1 and p22phox are constitutively localized in the plasma membrane. Upon activation of p22phox/NOX1, the cytosolic subunits NOXO1, NOXA1, and p40phox colocalize with p22phox and NOX1 to form an inactive NOX1 complex at the membrane. A fully-active NOX1 complex is formed when the small GTPase RAC is recruited to the complex and activated [63,64]. ROS are produced by the activated NOX1 complex when the TKS4 protein that interacts with NOXA1 is phosphorylated and activated by SRC kinase [65]. Conversely, ROS production by the NOX1 complex is inhibited when NOXA1 is phosphorylated by protein kinase A (PKA) or protein kinase C (PKC). This phosphorylation allows 14-3-3 protein binding, which induces NOXA1 to dissociate from the NOX1 complex and shuts down ROS generation. Similarly, phosphorylation of NOXA1 by extracellular signal-regulated kinase (ERK) negatively regulates NOX1 complex activity [66]. These findings illustrate the important functional link between TNF/TNFR1 signaling and the NOX1 complex in necroptosis. The actual mechanisms by which the NOX1 complex generates ROS are shared by the NOX2 complex which is crucial for phagocytosis in **innate immune** cells.

TNF Signaling and ROS/RNS Production in the Immune System

ROS are essential components of the innate immune response against microbial pathogens (Box 3). This crucial function of ROS first came to light in studies of phagocytosis. Details of the mechanics of phagocytosis can be found elsewhere [67].

Box 3. The Oxidative Burst

Innate immune cells such as neutrophils and macrophages act as a first line of defense against infection by microbial pathogens. Both of these cell types mediate effective innate immune responses by means of the 'oxidative burst', which is characterized by the rapid production of large amounts of intracellular ROS and the activation of proteases that degrade phagocytosed microbes [127,128]. ROS production during the oxidative burst is non-mitochondrial and results from the tightly regulated activation of NOX proteins. In contrast to the NOX1 complex that generates ROS in non-phagocytic cells, phagocytes such as granulocytes, neutrophils, monocytes, and macrophages produce ROS by the use of an analogous NADPH oxidase complex termed NOX2 [70,129]. NOX2 complex activation is triggered when microbes bearing common molecular patterns are recognized by specific pattern recognition receptors, or when complement components or growth factors bind to the appropriate surface receptors [130]. Exposure of a phagocyte to proinflammatory cytokines such as TNF, IFN-γ, and/or IL-1β induces NOX2 complex formation and thus significantly increases the ROS levels achieved within the cell.

NOX2 Complex in Phagocytosis

Similarly to the NOX1 complex, the NOX2 complex is composed of five subunits: NOX2 (also known as gp91phox), p67phox (homologous to NOXA1), p47phox (homologous to NOXO1), and the shared p22phox and p40phox subunits (Figure 1). NOX2 and p22phox are localized at the plasma membrane and form the cytochrome *b* (558) complex. Upon pathogen attack, the cytosolic p67phox, p47phox, and p40phox subunits come together and recruit a small GTPase (RAC1 in monocytes and RAC2 in neutrophils). All these elements then colocalize with p22phox and NOX2 at the membrane to form the complete and active NOX2 complex [68]. To generate ROS, the NOX2 complex converts extracellular O_2 into O_2^-, which is further converted into extracellular H_2O_2 by SOD3 (Figure 1). H_2O_2 can penetrate the phagocyte membrane and can act inside the cell to promote pathogen phagocytosis [69,70].

There is growing evidence that intracellular mitochondrial ROS also facilitate phagocytosis [71,72]. However, the exact contributions of mitochondrial and non-mitochondrial ROS to innate immune responses have yet to be defined [73,74]. It should be noted that, as well as being essential for innate immune responses against pathogens, TNF–NOX2 signaling is believed to be responsible for chronic inflammation and its associated tissue damage [14,75].

TNF and ROS Participate in a Positive Feedback Loop

On one hand, ROS generation is induced by cytokines; on the other, ROS can stimulate proinflammatory cytokine production by activating NF-κB [14]. In phagocytic cells, H_2O_2 triggers TNF expression via activation of the p38 and JNK pathways [76]. In addition, H_2O_2 oxidizes the catalytic cysteines of MAPK-inactivating phosphatases, thus activating MAPKs such as p38 [47,77]. This positive feedback loop, in which TNF-induced ROS production subsequently triggers TNF expression via p38, JNK, and NF-κB, emphasizes the importance of proper ROS regulation in executing a successful TNF-mediated innate response.

RFK plays a particularly important role in TNF-triggered ROS generation. The membrane-bound p22phox subunit of the NOX2 complex is coupled to RFK, and RFK can interact with TNFR1. RFK converts riboflavin into flavin mononucleotide and flavin adenine dinucleotide, which are essential cofactors of oxidases such as NOX2 [60]. In line with this observation, phagocytes lacking riboflavin or RFK activity display defective TNF-dependent NOX2 signaling, resulting in reduced ROS production and impaired innate immune responses against pathogens [78]. Although resting phagocytic cells express NOX2 complex components, these proteins are inactive and do not assemble into the NOX2 complex until the cells are stimulated by a pathogen. Indeed, neutrophils simply adhering to the extracellular matrix do not produce high ROS levels. However, upon TNF stimulation, the NOX2 complex is immediately assembled and activated in adherent neutrophils, and ROS are produced via a pathway involving VAV1, RAC2, and proline-rich tyrosine kinase-2 (PYK2) [79]. In addition, TNF signaling leading to NOX2 activation

increases the adherence of macrophages and neutrophils to endothelial cells, enhancing the efficiency of phagocytosis.

RNS as Mediators of TNF Signaling

In the same way as ROS are important for TNF signaling during innate immune responses, **reactive nitrogen species** (RNS) play a prominent role but in a strikingly different way. Paradoxically, the starting point for most RNS generation is the powerful antioxidant nitric oxide ($NO^{\bullet}$). During infection, $NO^{\bullet}$ contributes directly to microbe elimination and inhibits the escape mechanisms these organisms seek to deploy. High levels of $NO^{\bullet}$ also act as a redox balancer to protect a cell from the destructive effects of high intracellular ROS. However, $NO^{\bullet}$ induces the expression of proinflammatory genes such as TNF, at least in human macrophages [80]. If TNF action generates significant ROS in the form of O_2^{-}, this radical reacts with $NO^{\bullet}$ to generate RNS such as nitrite (NO_2), dinitrogen trioxide (N_2O_3), and peroxynitrite (NO_3^{-}) [81], all of which can induce DNA damage and cell death [82]. Thus, $NO^{\bullet}$ serves as a pivot, protecting cells from the effects of high ROS and TNF-mediated cell death, but also generating RNS and promoting inflammation. These findings highlight the functional importance of a proper NO/ROS/RNS balance during TNF signaling. At the organism level, the immune system uses an array of different redox mechanisms to generate and regulate ROS, $NO^{\bullet}$, and RNS to maintain a broad range of immune functionalities [83]. Different pathogens elicit different RNS/ROS combinations, and each of these is aimed at triggering elimination of phagocyte threats.

TNF–NF-κB Signaling as an RNS Inducer

TNF-induced NF-κB signaling drives the transcription of the gene encoding inducible nitric oxide synthase (iNOS, also known as NOS2) [83] (Figure 2). In response to various stimuli, NOS2 produces $NO^{\bullet}$/RNS, which provide the immune system with enormous flexibility when facing diverse challenges. For example, $NO^{\bullet}$ can induce cell death in a BAX/BAK-dependent manner that involves cytochrome *c* and caspase 9 [84]. Alternatively, RNS/ROS-induced NRF2 can trigger HO-1/H-ferritin expression, leading to anti-inflammatory cytokine production that contributes to antioxidant protection and counteracts cell death [85,86]. Indeed, $NO^{\bullet}$/RNS-mediated production of HO-1 and H-ferritin suppresses TNF-induced ROS generation [83,87]. TNF-induced $NO^{\bullet}$ also stimulates the expression of other key molecules involved in the redox response, including hypoxia-inducible factor 1 (HIF-1) and AKT [88,89]. Thus, depending on their molecular species and abundance, RNS can trigger opposite reactions that have profound effects on the redox balance of a cell. Feedback mechanisms are also involved. Although only a short burst of $NO^{\bullet}$ produced in response to TNF is sufficient to activate the powerful NF-κB, JNK, and p38 signaling pathways [90], prolonged $NO^{\bullet}$ exposure serves as a negative feedback trigger and inhibits NF-κB signaling [91–93].

Lastly, in addition to upregulating NOS2 during inflammation, NF-κB regulates xanthine oxidase/dehydrogenase (XOR), an enzyme that can catalyze both reduction and oxidation reactions [14]. All these observations highlight the major influence of TNF on the intricate balance between inflammatory and non-inflammatory responses that is required for the safe and efficient elimination of pathogens.

TNF and ROS in Inflammatory Diseases

Over the past few decades numerous studies have indicated that TNF, ROS, and NF-κB are inextricably tied together in immunity, inflammation, and cancer [94]. It has long been known that TNF is involved in the clinical symptoms of disorders such as rheumatoid arthritis (RA), inflammatory bowel disease, sepsis, ankylosing spondylitis, systemic lupus erythematosus (SLE), psoriasis, multiple sclerosis (MS), respiratory diseases, vasculitis, type 1 diabetes (T1D), and TNFR1-associated periodic syndrome (TRAPS) [95,96]. More recently, ROS have been implicated in atherosclerosis and pancreatitis [97]. The following subsections outline the roles of TNF and ROS in three common inflammatory disorders.

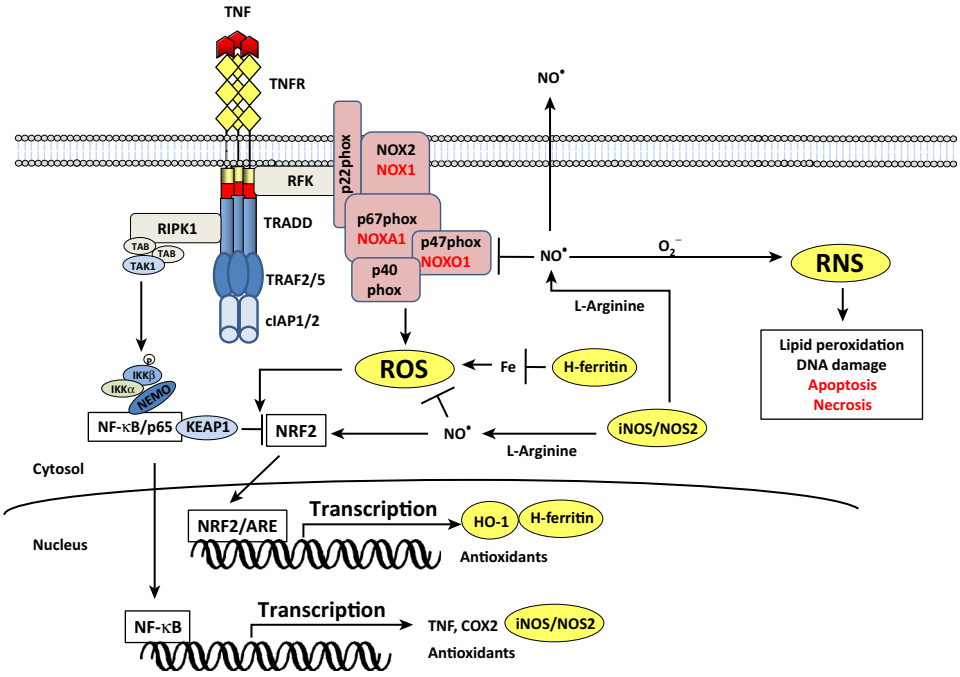


Figure 2. TNF-Induced RNS Production through NOS2. Engagement of TNFR1 by TNF results in NF-κB activation, which drives the transcription of NOS2. NOS2 catalyzes the generation of NO• from L-arginine with concomitant consumption of NADPH and O_2. NO• can diffuse across the plasma membrane to reach the extracellular space and support the phagocytic killing of microorganisms. However, extracellular NO• also contributes to the inflammation associated with RA and septic shock. NO• that remains intracellular interacts with ROS and acts as an antioxidant, but in so doing it creates high levels of RNS that can cause DNA damage or induce apoptosis or necroptosis. Intracellular ROS and NO• also trigger KEAP1 proteolysis and thereby activate NRF2 signaling, which leads to the expression of antioxidant proteins such as HO-1 and H-ferritin. The HO-1/H-ferritin pathway negatively regulates ROS generation by sequestering iron, which is important for NOX- and mitochondria-dependent ROS production. A feedback loop is thus established to generate ROS and NO• while simultaneously controlling the antioxidant response.

TNF and ROS in Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory disease that affects peripheral joints but also the skin, kidneys, heart, and lungs. Macrophages, neutrophils, dendritic cells (DCs), plasma cells, and T and B lymphocytes infiltrate into synovial membranes and secrete proinflammatory cytokines [98]. High levels of TNF, NOX2, and ROS accumulate in inflamed joints [99,100]. Accordingly, therapeutic inhibition of TNF signaling efficiently inhibits ROS production and reduces joint inflammation [101].

Specific mutations of genes involved in immune or inflammatory responses or in TNF or ROS biology are also associated with RA development. These mutations affect the genes encoding human leukocyte antigen (HLA); protein tyrosine phosphatase non-receptor, type 22 (PTPN22); TNF receptor-associated factor 1-complement component 5 (TRAF-C5); and p47phox (NCF1) [100,102–104]. The roles of these mutated genes and their links to TNF and ROS during RA development are the subject of ongoing studies.

TNF and ROS in TRAPS

TRAPS is a familial autoinflammatory syndrome characterized by recurrent prolonged episodes of fever, rash, abdominal pain, and systemic amyloidosis. Almost all mutations in the *TNFRSF1A* gene encoding the TNFR1 protein are missense mutations in the receptor extracellular domain

which is responsible for receptor pre-association and TNF binding [105]. Mutant TNFR1 cannot reach the cell surface or interact with the wild-type TNFR1 protein. The majority of mutated TNFR1 proteins are retained in the endoplasmic reticulum as a result of their abnormal protein folding. Cells from TRAPS patients, or from mice with heterozygous *Tnfrsf1a* mutations homologous to those linked to TRAPS, show spontaneously activated JNK and p38 MAPKs [106]. Cells from TRAPS patients also exhibit increased oxygen consumption and respiratory capacity, leading to increased mitochondrial ROS production [96]. It is believed that ROS inactivate MAPK phosphatases, thereby enhancing MAPK activation. Treatment of TRAPS patients with TNF-blocking agents improves their symptoms but does not fully suppress the disease. The mutated TNFR1 subunits in these patients may act as unusual gain-of-function proteins that signal from within the cell to enhance inflammatory responses. However, mutations in TNFR1 are not the sole factor driving this disease because the presence of a functional wild-type TNFR1 protein is still necessary to elicit the clinical signs of TRAPS [107].

TNF and ROS in T1D

In T1D, a proinflammatory response involving TNF, IL-1, and ROS stimulates the destruction of insulin-secreting β cells by activated $CD4^+$ and $CD8^+$ T cells in the pancreatic islets [108,109]. Activated $CD8^+$ T cells can produce TNF that is directly toxic to β cells. Activated $CD4^+$ T cells also secrete TNF that activates natural killer cells, macrophages, and DCs, which enhance β cell destruction [110]. At T1D onset, the pancreas contains high numbers of IFN-γ-producing Th1 cells. Although Th1 cells are deemed to be the major players in T1D, islet-specific Th17 cells can contribute to T1D in the absence of Th1 cells [111]. Thus, TNF produced by either Th1 or Th17 cells can promote T1D onset.

At the molecular level, TNF mediates β cell destruction leading to T1D through its activation of JNK, ROS, and p53 signaling [112]. Recent data suggest that TNF-dependent induction of NOX-derived ROS promotes the differentiation of proinflammatory M1 macrophages that infiltrate pancreatic islets and destroy β cells [113]. However, the available preclinical and clinical data on the effectiveness of blocking TNF activity in T1D patients is conflicting. TNF blockade has been shown to both accelerate and delay T1D development in animal models [114].

Concluding Remarks

TNF is a master regulator of cell survival and cell death. Because TNF affects numerous pathways controlling immune responses and inflammation, its functions must be carefully orchestrated. The major role of TNF is to regulate the immune system through the activation of TNF receptors and downstream pathways involving molecules such as NF-κB, MAPKs, caspases, and ROS/RNS. NF-κB activation protects against cell death because NF-κB governs the transcription of a wide array of genes involved in cell survival, proliferation, and inflammation. However, TNF-induced MAPK activation leads to cell proliferation on one hand but apoptosis on the other. TNF signaling also induces ROS/RNS generation whose crucial role is to control TNF signaling downstream of TNF receptors. This function of ROS has been largely ignored in the past, perhaps because of the major challenge posed by measuring its impact on the several hundred genes involved in signaling downstream of TNF–TNFR engagement. There are multiple sources of ROS both inside and outside the cell, and diverse ROS species that are generated in different places within a cell, at different timepoints, and at different concentrations. This is further complicated by the complex ROS/RNS interplay. All these factors significantly influence the effect of ROS and RNS on a specific pathway or protein. Nevertheless, it is now clear that ROS/RNS are an integral part of TNF signaling because they are intimately involved in the numerous feedback loops that are part of the extensive crosstalk of pathways downstream of TNFR engagement (Table 1). To better understand the roles of ROS and TNF in inflammatory diseases, it will be important to elucidate how ROS regulate TNF-induced pathways, especially NF-κB activation (see Outstanding Questions). In terms of novel therapeutic options for patients with

Outstanding Questions

How do ROS function physiologically, and how do they contribute to the mechanism of inflammation?

How does ROS–TNF crosstalk contribute to life–death decisions of the cell, and how can we modulate this interaction clinically?

Why have clinical trials using antioxidants failed? The intricate relationship between oxidative stress and inflammation needs to be further characterized.

What is the role of TNFR2 signaling and its interactions with TNFR1 and/or ROS?

How and to what extent is TNFR2 involved in RNS generation and signaling?

Does LTα play a role in TNF–ROS crosstalk in inflammatory diseases?

inflammatory disorders, a combination therapy that controls TNF and ROS may represent an entirely new approach to tackling TNF-related immunopathic diseases [115].

Acknowledgments

The authors thank M. Saunders for excellent scientific editing, K. Weger for the contribution of the glossary and research assistance, and M. Brenner for general support. H.B. is funded by a Canadian Institutes of Health Research (CIHR) S2B award (141816), and D.B. is funded by the ATTRACT Programme and a CORE grant (C15/BM/10355103) of the National Research Fund Luxembourg (FNR).

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