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Reactive Oxygen Species in TNF α -Induced Signaling and Cell Death

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Abstract

TNF α is a pleiotropic cytokine that initiates many downstream signaling pathways, including NF- κ B activation, MAP kinase activation and the induction of both apoptosis and necrosis. TNF α has shown to lead to reactive oxygen species generation through activation of NADPH oxidase, through mitochondrial pathways, or other enzymes. As discussed, ROS play a role in potentiation or inhibition of many of these signaling pathways. We particularly discuss the role of sustained JNK activation potentiated by ROS, which generally is supportive of apoptosis and “necrotic cell death” through various mechanisms, while ROS could have inhibitory or stimulatory roles in NF- κ B signaling.

Keywords

TNF α ; ROS; JNK; NF-kappaB; necrotic cell death

ROS and TNF

Reactive oxygen species, or ROS, are produced in the cell primarily by three sources. The mitochondria are by far the greatest source of ROS. The reactions that occur during oxidative phosphorylation processes by which ATP is generated in the mitochondria are not completely efficient and it has been estimated at between 2 to 5% of electrons are lost as they are transferred between electron transport chain complexes (Boveris and Chance, 1973; Boveris and Cadenas, 1975). Secondly there are NADPH oxidases, which uses NADPH to reduce molecular oxygen resulting in superoxide (Brown and Griendling, 2009; Lambeth, 2004), which are used as a defense against infectious pathogens (Quinn et al., 2006). It has recently become apparent that many cytokines and growth factors that have previously been reported to generate ROS as second messengers in their signaling pathways do so through the activation of locally recruited NADPH oxidases (Ushio-Fukai, 2009). Lastly, there are other enzymes that cause the generation ROS in different ways, although usually less robustly than the NADPH oxidases, including, but not limited to, lipoxygenases, cyclooxygenases, myeloperoxidases, xanthine oxidase, heme oxygenase, monoamine oxidases, and aldehyde oxidase, as well as cytochrome P450-based enzymes.

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Although certain increases of ROS occur during signal transduction events, the low overall cellular levels of ROS are usually maintained by systems of antioxidant enzymes and their substrates, such as the glutathione and thioredoxin systems, superoxide dismutases, catalase, and peroxiredoxins, as well as other non-enzymatic antioxidants (Holmgren, 2000; Rhee et al., 2005; Sies, 1997) Nevertheless, ROS play a substantial role in many signal transduction pathways, and this is especially true of the TNF α signaling pathway.

In order to discuss the role of ROS in TNF α signaling, we must first briefly touch on the various pathways that are initiated by this prolific cytokine. The primary receptor for TNF α , TNFR1, is the prototypical member of the death receptor subfamily (see (Guicciardi and Gores, 2009; Wajant, 2003) for review) of the TNF Receptor superfamily, which mediate downstream signaling events through an intracellular “death domain”. The death domain of the activated receptor interacts the death domains of the adaptor proteins TRADD, and RIP1, which then recruit TRAF2 and other various cellular signaling machinery that initiate the downstream signals. TRADD is the primary adapter molecule and is required for virtually all of the downstream signaling pathways, including NF- κ B activation, MAP kinase activation and both apoptosis and necrosis, though some weak signaling remains in its absence in some cell types with high RIP1 expression.

NF- κ B

Perhaps the most important signaling event that occurs during TNF α signaling is the activation of the transcription factor NF- κ B, which has a substantial role in innate immunity and inflammation (Beinke and Ley, 2004; Bonizzi and Karin, 2004; Hayden and Ghosh, 2008; Vallabhapurapu and Karin, 2009). RIP1 and TRAF2 play important roles in its activation, and though NF- κ B activation occurs to some extent in the absence of one or the other (Yeh et al., 1997; Wong et al., 2010), likely due to some redundancies in IKK recruitment (Devin et al., 2000; 2001; Tada et al., 2001), a majority of the data in the literature support the notion that they are central molecules in the process (Devin et al., 2000; 2001; Ea et al., 2006; Kelliher et al., 1998; Poyet et al., 2000; Tada et al., 2001; Wu et al., 2006). The p105/*nfkb1* gene product is constitutively processed by the proteasome into an active p50 product, but is prevented from interacting with DNA through its interaction with I κ B α , which has strong nuclear export signal and keeps it in the cytoplasm as a heterodimer with p65 (Beinke and Ley, 2004; Bonizzi and Karin, 2004; Hayden and Ghosh, 2008; Vallabhapurapu and Karin, 2009). NF- κ B is activated by TNF α through recruitment of an IKK signaling complex consisting of two kinase subunits, IKK α and IKK β , and a regulatory subunit, IKK γ . While TRAF2 is capable of interacting with IKK α and IKK β directly (Devin et al., 2000; 2001), it also recruits cIAP1 and cIAP2, which polyubiquitinate RIP1 (Bertrand et al., 2008; Mahoney et al., 2008; Varfolomeev et al., 2008). Polyubiquitinated RIP1 recruits the IKK γ subunit and stabilizes complex formation (Ea et al., 2006; Poyet et al., 2000; Wu et al., 2006). Likely recruited by RIP1 (Blonska et al., 2005; Lee et al., 2004), the TAK1 kinase appears to be required at this point for the activation of the IKK complex (Blonska et al., 2005; Liu et al., 2006; Sato et al., 2005; Shim et al., 2005; Takaesu et al., 2003), and MEKK3 may also be required as well (Blonska et al., 2005; Yang et al., 2001), but their exact roles are not yet completely clear. The activated complex leads to phosphorylation of I κ B α , which is primarily mediated by IKK β , and leads to the

subsequent ubiquitination and degradation by the proteasome, allowing the p50/RelA heterodimer to translocate to the nucleus and activate transcription (Beinke and Ley, 2004; Bonizzi and Karin, 2004; Hayden and Ghosh, 2008; Vallabhapurapu and Karin, 2009).

MAPK pathways

In addition to NF- κ B, TNF α signaling through TNFR1 leads to the activation of all three major Map Kinase signaling cascades, ERK, p38, and JNK. These kinases are activated by upstream MAPK kinase kinases (MAP2Ks), which are in turn activated by MAP3Ks and MAP4Ks. TRAF2 is required for efficient activation of each of these pathways (Devin et al., 2003; Lee et al., 1997; Yeh et al., 1997)

RIP1 is also required for efficient JNK activation, but the kinase activity of RIP1 is not essential (Devin et al., 2003). A significant number of MAP 3/4kinases have been implicated in the activation of JNK downstream of TNF α based on TNF α -induction of their activation and the use of overexpression or dominant negative molecules, including MEKK1 (Baud et al., 1999), ASK1 (Hoeflich et al., 1999; Liu et al., 2000; Nishitoh et al., 1998), TAK1 (Liedtke et al., 2002), MAP3K11 (Sathyanarayana et al., 2002), and the germinal center kinase family members, MAP4K2 (Yuasa et al., 1998), MAP4K5 (Shi and Kehrl, 1997; Shi et al., 1999), TNIK (Fu et al., 1999), NRK (Nakano et al., 2000), MAP4K3 (Diener et al., 1997), and MAP4K4 (Yao et al., 1999). MEKK1, ASK, TNIK, MAP3K11, MAP4K2, and MAP4K5 are all known interact with TRAF2 in a stimulation-dependent manner. TAK1 and MEKK1 and have both been proposed to be essential for TNF α -induction of JNK (Shim et al., 2005; Xia et al., 2000), based on signaling in deficient embryonic fibroblasts. However, there are conflicting reports based on similar studies that show that MEKK1 is not essential for TNF α -induced JNK activation (Devin et al., 2003; Yujiri et al., 2000), so it is possible that there is some redundancy of pathways. Deletion of MAP3K11 (MLK3) reduces, but does not eliminate TNF α -stimulated JNK activation (Brancho et al., 2005). ASK1 is also not essential for transient TNF α -induced JNK activation, but a vital role for ASK1 in the second prolonged phase of TNF α -induced JNK and p38 activation is indicated by experiments in ASK1 deficient mice (Tobiume et al., 2001).

The upstream kinases involved in TNF α -induced p38 and ERK activation are also not well established. Unlike JNK, TAK1 does not seem to play an important role in activation of these kinases, though there is slight reduction in p38 stimulation in TAK $^{-/-}$ cells (Shim et al., 2005). MEKK3 $^{-/-}$ cells have been reported to have a defect in p38 activation in response to TNF α (Lee et al., 2003). However, another report found no difference in p38 activation in MEKK3 or MEKK1 knockout cells (Devin et al., 2003). Interestingly, these two reports are also at odd with respect to the essential role of TRAF2 in p38 activation, with one concluding that TRAF2 is required (Devin et al., 2003) and another concluding that the absence of TRAF2 does not effect p38 activation (Lee et al., 2003). However, it is agreed that RIP is absolutely essential for TNF α -induced p38 activation (Devin et al., 2003; Lee et al., 2003). As with JNK activation, the kinase activity of RIP appears to be dispensable for p38 activation (Devin et al., 2003; Lee et al., 2004), while it does seem to be important for ERK activation (Devin et al., 2003). Likewise, MADD, a splice variant of IG20, binds to TNFR1 directly and appears to be required for ERK activation, but not JNK or p38

activation (Kurada et al., 2009). As loss of MADD expression also results in reduced Grb2 and Sos1/2 recruitment to TNFR1 and decreased Ras and MEKK1/2 activation (Kurada et al., 2009), this may be the pathway by which TNF α activates ERK in some cell types. Alternatively, Syk is an upstream tyrosine kinase can be recruited to RIP1 and TRAF2 that can activate ERK through MAP3K8 (Tpl2/Cot) (Eliopoulos et al., 2006).

While the MAP2 kinases MKK7 and MKK4 are the immediate activators of JNK, only MKK7 is activated by TNF α (Moriguchi et al., 1997). An examination of TNF α -induced JNK activation in MKK4 $^{-/-}$ and MKK7 $^{-/-}$ cells confirmed the requirement for MKK7, while suggesting that a basal level of MKK4 is necessary for the maximal activation of JNK (Tournier et al., 2001). MKK3 appears to be essential for p38 activation in response to TNF α , but MKK3 $^{-/-}$ cells have normal JNK activation (Wysk et al., 1999).

JNK, p38 and ERK activate several different transcription factors by phosphorylation. However, they also have a variety of other cellular targets, and their activation may initiate both pro-death and pro-survival effects, depending on the context of the signal. Of the three map kinases, ERK usually acts in a prosurvival fashion.

Cell death

Cell death is initiated by TNFR1 under specific sets of circumstances, primarily when NF- κ B signaling is decreased or blocked. Depending on the circumstances and the cell type, the characteristics of TNF α -initiated cell death may vary and TNF α may lead to apoptosis or to necrosis.

Apoptosis

Apoptosis is usually defined as a type of programmed cell death characterized by the activation of caspases, which are cysteine proteases that cleave cellular substrates and effect specific cellular damage and events. Usually these events involve cellular shrinkage, chromatin condensation and nuclear fragmentation, membrane blebbing, and the formation of membrane-bounded bodies containing the cellular structures and organelles, which are then taken up by surrounding cells or by phagocytic cells of the immune system without inflammation (Fiers et al., 1999; Kroemer et al., 2005).

Caspase activation during TNF α -mediated apoptosis is achieved through the recruitment of Fas-associated death domain (FADD) protein to a secondary complex that is dissociated from the main complex (Micheau and Tschopp, 2003). FADD contains a death-effector domain, which recruits and causes the autocatalytic activation of the initiator caspases -8 and -10, which can directly cleave intracellular substrates or activate other caspases through their proteolytic processing.

In a mitochondrial amplification loop, cell death is amplified by regulated release of cytochrome c from the mitochondria, which binds to the Apaf-1/caspase-9 complex, resulting in the activation of this caspase. Cytochrome c release is positively regulated by Bax and Bak, which are the main proapoptotic members of the Bcl-2 family, and inhibited antiapoptotic members of this family, such as Bcl-2 or Bcl-xL. Cleavage of the Bid protein

by caspase-8 allows it to activate Bax/Bak, providing the main TNF α connection with the mitochondrial cell death pathway.

Necrosis

In addition to apoptosis, TNF α also is capable of activating a programmed necrotic-like cell death that is not dependent on the activity of caspases (Festjens et al., 2006b), and which is characterized by cellular swelling, organelle dysfunction, extensive mitochondrial damage, and plasma membrane rupture (Fiers et al., 1999; Kroemer et al., 2005). Necrotic cell death induced by TNF α requires the production of ROS (Fiers et al., 1999; Festjens et al., 2006b; Goossens et al., 1995; Lin et al., 2004; Sakon et al., 2003; Ventura et al., 2004). The RIP1 protein is required for TNF α -induced ROS production and is also required for TNF α -induced caspase-independent cell death (Festjens et al., 2007; Lin et al., 2004), making RIP1 a central player in the process. Unlike activation of NF- κ B, JNK, and p38, the pronecrotic role of RIP1 requires its kinase activity. A novel drug that prevents programmed necrotic cell death has been recently developed, and appears to be an inhibitor of RIPK1 kinase activity (Degterev et al., 2005; 2008). While caspase inhibitors are known to prevent apoptosis, inhibition of caspases under necrotic cell death conditions potentiates cell death, possibly due, in part, to RIP1 cleavage by caspase-8 during apoptosis (Lin et al., 1999; Vandenberg et al., 2006). In addition to RIP1 there are several other molecules suggested to play a role in necrotic cell death. RIPK3, (Cho et al., 2009; He et al., 2009; Zhang et al., 2009) cyclophilin D, (Li et al., 2004; Nakagawa et al., 2005) and possibly PARPs (Los et al., 2002; Xu et al., 2006) and lysosomal proteases, such as calpains and cathepsins (Luke et al., 2007; Sato et al., 2008), have been identified as being a part of the programmed necrotic execution system.

RIP3 is another member of the RIP kinase family identified by similarity between kinase domains. Using RNA interference screens and differential microarray analysis, RIP3 was independently identified by three separate groups as a downstream component of the TNF-induced necrotic pathway (Cho et al., 2009; He et al., 2009; Zhang et al., 2009). RIP3 lacks a death domain and is therefore recruited to a pronecrotic signaling complex through RIP1, which interacts with RIP3 through its homotypic interaction motif (RHIM). This interaction is associated with RIP1 kinase activity. The downstream effects of RIP3 association are not clear at this point, but one group found RIP3 to be associated with 7 different metabolic enzymes, including glycogen phosphorylase (PYGL), glutamate-ammonia ligase (GLUL), glutamate dehydrogenase 1 (GLUD1), fructose-1,6-bisphosphatase 2 (FBP2), fumarate hydratase (FH), glycosyltransferase 25 domain containing 1 (GLT25D1), and isocitrate dehydrogenase 1 (IDH1) (Zhang et al., 2009). These associations may suggest that RIP3 functions in bioenergetic metabolic pathways that lead to ROS generation.

Cyclophilin D is a component of the mitochondrial permeability transition pore channel, the opening of which results in results in a loss in mitochondrial membrane potential. Despite the fact that Bcl-2 family member-dependent apoptosis is independent of cyclophilin D, fibroblasts and primary hepatocytes isolated from cyclophilin knockout mice are resistant to oxidative stress-mediated necrosis (Li et al., 2004; Nakagawa et al., 2005). This may suggest

that at least in some situations, necrosis may depend on the mitochondrial permeability transition being opened in response to ROS.

PARP-1 also has been reported to being capable of causing mitochondrial dysfunction and JNK activation in a process dependent on RIP1 and TRAF2 (Xu et al., 2006). However, it is a nuclear enzyme, cleaved during apoptosis, that is activated by DNA damage and catalyzes the covalent attachment of poly (ADP-ribose) onto DNA-binding proteins using NAD⁺ as its substrate, making it unclear as to how it is connected with cytoplasmic RIP1 and TRAF2. It is, however, activated in L929 cells when treated with TNF α (Los et al., 2002), and its pharmacological inhibition blocks TNF α -induced necrotic death (Xu et al., 2006). PARP-2 was identified by an siRNA screen for inhibitors of TNF-induced necrotic cell death, and its knockdown causes some reduction of cell death (Hitomi et al., 2008)

Lysosomal calpains and cathepsins are proteases thought to be essential in some necrotic situations. They were identified as necrotic pathway targets in *C. Elegans* (Luke et al., 2007), which were known to require lysosomal function for their necrotic pathways (Artal-Sanz et al., 2006). There is some evidence that this requirement for lysosomal proteases is conserved in mammalian systems (Sato et al., 2008), and lysosomal rupture may occur downstream of ROS damage to their lipids (Boya and Kroemer, 2008), and be important in TNF α -induced necrosis (Boya and Kroemer, 2008).

The source and nature of the ROS that are generated prior to TNF α -induced necrotic death is still currently a subject of some debate within the field. TNF α -stimulated ROS has been proposed on one hand to come from downstream events involving the mitochondria (Fiers et al., 1999; Goossens et al., 1995). However, that TNF α has been shown to cause the production of superoxide (Meier et al., 1989) through activation of NADPH oxidases (Anilkumar et al., 2008; Kim et al., 2007; Li et al., 2009a; 2010; Woo et al., 2006).

NADPH oxidases

NADPH oxidases are a family of enzymes specifically dedicated to ROS production (Brown and Griendling, 2009; Lambeth, 2004). Activated cells of the innate immune system, such as neutrophils and macrophages, activate the phagocytic form of NADPH oxidase, NOX2 (gp91phox), to produce extensive amounts of superoxide for defense against invading pathogens. Various kinds of nonphagocytic cells including endothelial cells, vascular smooth muscle cells, fibroblasts, and cardiac myocytes, are also known to produce superoxide by NADPH oxidases to regulate intracellular signaling events (Brown and Griendling, 2009; Lambeth, 2004).

Other forms of NADPH oxidase (NOX1, NOX3, and NOX4, NOX5, DUOX1, DUOX 2) have been described in phagocytic and non-phagocytic cell types (Brown and Griendling, 2009; Lambeth, 2004). These other NADPH oxidase family members typically produce small amounts of ROS by for use in intracellular signaling events Most NOX enzymes are heterodimers with, and require the presence of, a 22 kDa subunit (p22phox). In addition to this subunit, many oxidases require other subunits for activity. Nox2 requires a p47phox subunit, which upon phosphorylation, binds to membrane phospholipids, interacts with

p22phox, and recruits the p67phox subunit. The p67phox activator binds the small GTPase, Rac1 or Rac2, and stabilizes its recruitment, which is necessary for an activity. Similar to p47phox and p67phox, respectively, the regulatory p41NOXO1 and p51NOXA1 subunits can function in other oxidase complexes, such as Nox1 (Banfi et al., 2003; Geiszt et al., 2003; Takeya et al., 2003). NOXO1, unlike p47phox, does not require phosphorylation for membrane translocation and activity. Overexpression studies have shown that there is some functionality of the different oxidases when p47phox and/or p67phox are interchanged for NOXO1 and/or NOXA1, though efficiency of superoxide production can be reduced (Banfi et al., 2003; Geiszt et al., 2003; Takeya et al., 2003). This may indicate that there is some flexibility or redundancy in subunit use by Nox enzymes depending on the expression of a given subunit in a specific cell type.

There are multiple ways that NADPH oxidases are regulated by TNF α . Many reports have shown that TNF α signaling initiates increased transcription of various NADPH oxidase components, which contributes to oxidase activity. Among the components that have been shown to be upregulated by TNF are Nox2, Nox3, Nox4, p22phox, p47phox, NOXO1, and p67phox (Condino-Neto and Newburger, 1998; De Keulenaer et al., 1998; Gauss et al., 2007; Kamizato et al., 2009; Li et al., 2010; Moe et al., 2006; Newburger et al., 1991; Yoshida and Tsunawaki, 2008). Other mechanisms for potentiation (or priming) of superoxide production by TNF α have been proposed such as the phosphorylation of p47phox by downstream kinases, including PKC ζ (Frey et al., 2002), tyrosine kinases (Akimaru et al., 1992; Deras et al., 2003; Utsumi et al., 1992), or p38 MAPK (Dang et al., 2006), perhaps through mobilization of subcellular granules (Onnheim et al., 2008). TNF α has also been suggested to regulate NADPH oxidase activity through regulation of the associated hydrogen ion channels that maintain charge equilibrium due to the electronic charge translocated during Nox activation (Chenevier-Gobeaux et al., 2007).

We and others have also recently shown that TNF α is a direct activator NADPH oxidases in various cell types (Kim et al., 2007). In L929 cells, Nox1, NOXO1, and Rac1 form a complex with TNF receptor signaling components in a TNF-dependent manner and lead to cell death (Kim et al., 2007). In this case the tyrosine kinase inhibitor genestein, the general PKC inhibitor, bisindolymaleimide, or a specific PKC ζ peptide inhibitor had no effect on the superoxide production or cell death in response to TNF α . One mechanism of activation of Nox1 by TNF α is suggested by protein interactions between the TNF-R1 adapter proteins RIP1 and TRADD, and NOXO1. RIP1 interacts strongly with NOXO1 protein through an as yet undefined domain, while the polyproline-rich region of TRADD interacts with the N-terminal SH3 domain of NOXO1. Consistent with the known requirement for RIP1 in TNF α -dependent ROS generation and necrotic cell death, RIP1 is absolutely required for the Nox1 and Rac1 recruitment to the signaling complex. Expression of a dominant negative TRADD protein with a mutation in its polyproline region eliminates binding to NOXO1 and diminishes superoxide formation and cell death in response to TNF α . Based on our estimation of the NOXO1 affinities for RIP1 and TRADD, we have proposed that RIP1 recruits NOXO1 and the other signaling components (Nox1/NOXA1/Rac1) to the complex, where the NOXO1-TRADD interaction promotes oxidase activation (Kim et al., 2007). TNF α -induced superoxide mediated by Nox1 contributes considerably to necrotic cell death since the siRNA knockdown of Nox1 prevents both superoxide generation and cell death in

response to TNF α . Therefore, Nox1 appears to be a primary source of initial ROS involved in cell death in L929 cells by this stimulus.

Other groups have also shown that TNF α signaling is coupled with NADPH oxidase activation, albeit by different molecules and mechanism. While others have confirmed TNF α activation of Nox1 (Woo et al., 2006), Nox2 and Nox3 have also more recently been shown to be more directly activated by TNF α (Anilkumar et al., 2008; Li et al., 2008; 2009a). Nox4 has also been reported to be activated downstream of TNF α (St Hilaire et al., 2008), but since it is regulated differently than the other oxidases, this appears to be through a transcriptional mechanism (Moe et al., 2006) and TNF α -dependent ROS generation has been shown to be enhanced in Nox2-overexpressing cells, but is not further enhanced upon Nox4 overexpression (Anilkumar et al., 2008)

One of the most important molecules appears to be that of riboflavin kinase, which has been shown to bind both to the death domains of TNFR1 and TRADD and also to p22phox (Yazdanpanah et al., 2009). Although its platform bridging between the complexes in itself would be a possible potential mechanism of oxidase activation, and is necessary for the recruitment of either Nox1 or Nox2 and Rac1 to TNFR1 in HeLa cells, riboflavin kinase has an enzymatic function as well, which is likely its main mechanism of activation (Yazdanpanah et al., 2009). It converts riboflavin (vitamin B2) to flavin mononucleotide (FMN), which is then converted to Flavin Adenine Dinucleotide (FAD) by the enzyme FAD synthetase, FAD is an essential prosthetic group of NADPH oxidase, and is required for superoxide generation. Localization of the enzyme to the complex likely results in FAD generation in close proximity to the oxidase and allows for FAD loading of the enzyme.

One important concept in TNF α -induced NADPH oxidase activation is that of TNF-initiated endosomal formation. Increasingly, endocytosis of receptor components within endosomes has been identified as having a signaling role far beyond signal termination or the recycling of receptors. TNF receptor components have long been known to associate with specific lipid microdomains (Legler et al., 2003; Muppidi et al., 2004; Shen et al., 2004). This is important because NADPH oxidase components are also often associated with lipid rafts signaling platforms and caveolae (Ushio-Fukai, 2006), and these are often involved in trafficking with endosomes (Pelkmans et al., 2004). One study has shown that TNF α -induced, Nox1-mediated, ROS generation in HeLa cells requires TNFR1 internalization, and that treatment with internalization inhibitor monodansyl cadaverine or expression of a dominant negative dynamin mutant blocks ROS generation in response to TNF α (Woo et al., 2006). This is consistent with another study that reported that in smooth muscle cells where TNF α -stimulated a Nox1-dependent ROS generation in endosomes that was dependent on dynamin (Miller et al., 2010.). A dependence on endocytosis may explain the apparent requirement for gelsolin, an actin regulatory protein, in TNF α -dependent ROS generation (Li et al., 2009b), since actin is involved in endocytosis. Another study reported that Rac1 is specifically recruited to the endosomal compartment after TNF α stimulation and that TNF α -stimulated Nox2-mediated production of endosomal superoxide was dependent on receptor internalization (Li et al., 2009a). These studies suggest that endosomal formation in response to TNF α is important for ROS generation. However, it is important to remember that most of these studies measured the intracellular or endosomal

ROS generation, and did not measure it extracellularly. In our study, which was measured total intracellular and extracellular superoxide, we found that no dependence on endocytosis for Nox1 activation, since neither cytochalasin D or latrunculin A inhibited superoxide formation (Kim et al., 2007). This may suggest either that the process of superoxide generation differs with cell type, or it may suggest that endosomal formation is not necessary for assembly of the oxidase machinery, but only be required for bringing the ROS in to an endosome where it can function within the cell.

Mitochondrial involvement

Cell death stimuli affect mitochondrial function in two ways; the first is the disruption of membrane potential and the initiation of the permeability transition (PT), which generally leads to apoptosis, and the second is mitochondrial generation of ROS, which could potentially contribute to necrosis. However, oxidative stress itself causes the mitochondria to generate further ROS through mitochondrial protein damage, which can act as a positive feedback loop (Ott et al., 2007), which may explain apparently contradictory data on Bcl-2 function in antioxidant pathways through a conditioning effect (Kowaltowski and Fiskum, 2005). However, Bcl-2 is typically thought of in terms of inhibiting pro-oxidant-induced mitochondrial change and subsequent formation of ROS; thus the oxidative stress-induced response is suppressed by Bcl-2 in many cases (Ott et al., 2007). The thiol oxidant diamide, a crosslinker of thiols, mimics disulfide bond formation, and thus induces mitochondrial membrane potential disruption and permeability transition (Costantini et al., 1995), whereas monovalent thiol-reactive compounds inhibit apoptosis (Marchetti et al., 1997).

While much recent data have established that the non-mitochondrial NADPH oxidases are involved in TNF α -initiated necrotic cell death, the source of the ROS is by no means exclusive. In fact, previous data point to the involvement in mitochondrial derived ROS (Fiers et al., 1999; Goossens et al., 1995; 1999; Schulze-Osthoff et al., 1992; 1993). In other experiments in less sensitive cell lines, our own group has detected what appears to be ROS derived from the mitochondrial generated several hours after the addition of TNF α , and at a much later time point than we detected NADPH oxidase activity (unpublished data). While this could indicate the appearance of non-specific ROS due to the onset of cell death, it may also suggest that Nox1-produced superoxide and mitochondrial-produced ROS are linked together in programmed necrosis. It is possible that the superoxide produced by NADPH oxidases may lead to mitochondrial-produced ROS, or vice versa. One group has suggested that in necrotic cell death induced by serum withdrawal that Nox1 activation is actually downstream of the production of mitochondrial ROS (Lee et al., 2006). Indeed, another group, (which also agrees that Nox1 is critical for TNF α -induced necrosis) has suggested that Nox1 is present in both the plasma membrane and the mitochondria (Byun et al., 2008). However, the kinetics of complex formation suggests that the Nox1-produced superoxide generation is a fairly early event after TNF α treatment. Additionally, although it is clear that TNF α -induced ROS causes mitochondrial damage (Mariappan et al., 2009). ROS damage to the mitochondria does not seem to be the sole determinant of cell death as several groups have demonstrated that mitochondrial targeted antioxidants do not substantially protect from TNF α -induced ROS or necrosis (Goossens et al., 1999; Jarvis et al., 2007).

Additional evidence for a mitochondrial role in TNF α -induced ROS and its associated necrosis comes from the more recent suggestion that two BH3-only subunit of Bcl2-family proteins are important for this necrotic cell death. Over-expression of BNIP3 causes the mitochondrial permeability transition (Kim et al., 2002) leading to necrosis-like form of cell death independent of caspases (Vande Velde et al., 2000). A dominant negative mutant of BNIP3 (Bcl2/E1B 19kD interacting protein) prevented loss mitochondrial membrane potential, counteracted ROS-dependent lysosomal damage, and protected against TNF α -induced necrosis in L929 cells (Ghavami et al., 2009), while knockdown of Bmf using siRNA prevents TNF α -induced necrosis (Hitomi et al., 2008).

ROS and cell death

Reactive oxygen species play a role in both apoptotic and necrotic cell death. In general, moderate oxidative stress induces apoptosis, whereas necrotic cell death is triggered when cells have a higher exposure to ROS (Saito et al., 2006; Takeda et al., 1999; Teramoto et al., 1999). How then does ROS initiated by TNF α play a part in cell death? Some molecular targets for TNF α -induced ROS have been found, but much is yet to be discovered. ROS have been shown to induce apoptosis through the production of ceramide, the p53 activation, and the induction of the regulatory protein of the PI3-kinase, p85 (Andrieu-Abadie et al., 2001; Liu et al., 2008a; Yin et al., 1998). However, it is unclear as to whether these pathways have a significant impact on the TNF-ROS cell death pathways.

A problem with defining ROS targets is that ROS have a mixture of roles in cell death since ROS may directly oxidize cellular proteins, lipids, or nucleic acids and therefore cause general cellular damage, or ROS can initiate cell death through acting as initiators or second messengers in various signaling pathways (Morgan et al., 2007). Another problem with defining the role of ROS is when they may function at different points within a given pathway. For instance, ROS may act upstream or downstream of p53 in causing DNA damage and also affect the downstream p53-mediated pathway (Liu et al., 2008a). Similarly, ROS may act upstream or downstream of JNK, the mitochondria, and caspase activation to lead to cell death (Festjens et al., 2006a; Kamata et al., 2005; Nakajima et al., 2008; Omori et al., 2008; Sidoti-de Fraise et al., 1998; Ventura et al., 2004; Wicovsky et al., 2007).

One of the major ways that ROS affects signaling is through ROS reaction with cysteine (Paulsen and Carroll, 2010), especially at the catalytic sites of enzymes. It has long been established that a classical protein tyrosine phosphatases can be inactivated by ROS oxidation of their catalytic cysteine (Groen et al., 2005; Nakashima et al., 2002; 2005). However ROS can also inactivate the dual specificity phosphatases (Kamata et al., 2005), which are capable of dephosphorylating tyrosine and serine/threonine residues, as well as phospholipids. Oxidation of catalytic cysteines leads to reversible or irreversible inactivation of the phosphatases by ROS depending on the oxidation state of the cysteine (den Hertog et al., 2005; Groen et al., 2005). A prolonged phosphorylation status affects the activity of kinases within the cell, including the stress-activated MAP kinases, p38 and JNK (Kamata et al., 2005).

The TNF α activation of JNK has two phases that differ in how they affect cell death and also in their relationship to the generation of ROS. The first phase protects from cell death and is independent of ROS (Lamb et al., 2003; Ventura et al., 2006), while the second phase, sustained phase, is either dependent on caspase cleavage of MEKK1 (Cardone et al., 1997; Nakajima et al., 2008; Wicovsky et al., 2007) or the activation of ASK1 (see below), as well as ROS. This phase is the phase that usually contributes to both apoptosis and necrosis (Chang et al., 2006; Kamata et al., 2005; Sakon et al., 2003; Tobiume et al., 2001; Ventura et al., 2004; 2006)

Various Nox isoforms have been shown to mediate the TNF α -induced activation of JNK. We have shown that Nox1 is required for sustained activation of JNK by TNF α (Kim et al., 2007). JNK is augmented in Nox2 overexpressing 293 cells when further treated with TNF α (Anilkumar et al., 2008), while endogenous NOX3 has recently been shown to mediate the TNF α activation of JNK in HepG2 hepatocytes (Li et al., 2010).

The activation of JNK is upregulated by ROS in several ways. Firstly, JNK activity is maintained by the ROS-mediated inactivation of JNK phosphatases (Kamata et al., 2005). The glutathione S-transferase Pi (GST π) monomers have been reported to bind directly to the JNK C-terminus and inhibit its activity (Wang et al., 2001). Since ROS causes the oligomerization of GST π , this prevents monomeric GST π from JNK binding, resulting in JNK activity (Adler et al., 1999). As mentioned, ASK1 is a MAP3K that is important for sustained JNK phosphorylation under conditions when ROS is present (Tobiume et al., 2001). The reduced form of thioredoxin has been reported to bind to ASK1 and prevents the activation of its kinase activity. This occurs in a large complex with TRAFs and is designated as the ASK1 signalosome (Liu et al., 2000; Noguchi et al., 2005; Saitoh et al., 1998). When oxidized, thioredoxin is released and ASK1 is capable of binding to signaling proteins that activate it (Noguchi et al., 2005). Other reports suggest that thioredoxin inhibits ASK1 activation through an inducement of its ubiquitination and subsequent degradation (Liu and Min, 2002). Additionally, the SUMO-specific protease, SENP1 is also complexed with thioredoxin in resting endothelial cells. Upon ROS dependent oxidation of the complex, SENP1 translocates to the nucleus where it desumoylates HIPK1, which then translocates to the cytoplasm and potentiates ASK1 activation (Li et al., 2008). Since ASK1 $^{-/-}$ MEFs are substantially resistant to the sustained JNK activity and apoptosis initiated by ROS such as H₂O₂ (Tobiume et al., 2001), the ROS-thioredoxin-ASK1 axis is believed to be an important molecular switch that may mediate second messenger ROS signals to JNK resulting in its activation.

JNK and p38 are the two main MAP kinases that are readily activated in response to stress and these two kinases play significant roles in the processes determining cellular fate during cellular stress. The activation of JNK has been shown to play central roles in many ROS-dependent apoptotic processes (Liu and Lin, 2005; Nakano et al., 2006; Shen and Liu, 2006). For instance, the suppression of JNK activity using genetic or pharmacological approaches substantially protects cells from ROS-induced apoptosis (Shen and Liu, 2006). JNK mediates phosphorylation and activation of the Itch E3 ubiquitin ligase, which ubiquitinates c-FLIP and causes its proteasomal degradation, thus preventing its inhibition of caspase-8 (Chang et al., 2006). The main mechanistic site of action for JNK in apoptosis is

likely the mitochondria. The most conclusive evidence of this is from a study using JNK1-/-JNK2-/- primary MEFs which were significantly resistant to UV radiation due to protection from cytochrome c release or mitochondrial depolarization (Tournier et al., 2000). Further evidence comes from the observation that most members of the Bcl-2 family, including Bcl-2 itself, are phosphorylation targets of JNK. This suggests that the proapoptotic activity of JNK can be executed via phosphorylative regulation of Bcl-2 family members (Bogoyevitch and Kobe, 2006; Nakano et al., 2006). For instance, the phosphorylation of Bax by JNK and p38 leads to Bax activation and its translocation to mitochondria (Kim et al., 2006). This could indeed be the main molecular links between JNK and the mitochondria apoptotic machinery, as Bax and Bak are required for JNK-dependent apoptosis (Lei et al., 2002). In some reports, ROS have been shown to be downstream of JNK (Ventura et al., 2004), or dependent on caspase activation of JNK (Nakajima et al., 2008), and this may be likely to JNK-dependent induction of mitochondrial dysfunction. ROS have also been shown to lead to the p38/JNK dependent upregulation of TNF α in macrophages (Nakao et al., 2008), which, if true as in other cell types where p38 or JNK upregulate TNF (Lee et al., 1994; 2005; Yao et al., 1997) would act as an amplification loop of the TNF signal through further production of the more potent membrane form of membrane TNF α (Weingartner et al., 2002).

In addition to its role in apoptosis, significant evidence supports the role of ROS-mediated JNK activation in necrotic cell death (Shen and Liu, 2006). Exogenous ROS induce necrotic cell death via JNK activation (Shen et al., 2004) therefore JNK is now suggested to be one essential mediator of necrotic cell death pathways (Shen and Pervaiz, 2006). ROS are crucial coactivators in TNFR1-mediated sustained JNK activation, and sustained JNK activation represents an important event in both apoptotic and necrotic TNF α -induced cell death (Chang et al., 2006; Kamata et al., 2005; Ventura et al., 2004; 2006). In L929 cells, which undergo default necrotic cell death in response to TNF α , the sustained JNK directly correlates with superoxide generation and necrotic cell death, and inhibition of JNK completely blocks TNF α -induced necrotic cell death of these cells (Kim et al., 2007). Other reports have shown similar results from the inhibition of sustained JNK activation using genetic or pharmacological manipulation (Kamata et al., 2005; Ventura et al., 2004; 2006), though ROS may also have JNK independent effects (Wicovsky et al., 2007).

Reactive Oxygen Species from sources other than TNF α initiated ROS potentiates TNF α cytotoxicity in many situations. For instance, sustained JNK activation in human skin fibroblasts that is mediated by substantial ROS derived from integrin-mediated signaling potentiates TNF α /CHX-induced apoptosis without perturbation of NF κ B signaling. Moreover, in HeLa and 293 cells exogenous oxidative stress promotes ligand-independent TNF α signaling and also enhances ligand-mediated TNF signaling (Ozsoy et al., 2008). It is unclear whether endogenous ROS could potentiate in the same way, but overexpression of Nox1, NoxO1, and NoxA1 potentiates TNFR1-dependent activation of JNK but not NF- κ B, and also induces TNFR1- and ASK1- dependent cell death (Pantano et al., 2007).

Cell death, NF- κ B, JNK, and ROS

Typically, treatment with TNF α does not actually result in cell death. This largely due to the activation of NF- κ B, which initiates transcription of pro-survival proteins (Karin and Lin, 2002), such as the cellular inhibitor of apoptosis proteins (cIAPs), the caspase-8 inhibitory protein cFLIP, the ubiquitin-editing enzyme TNFAIP3 (A20), and the antiapoptotic Bcl2 family members A1 and Bcl-xL. These proteins directly or indirectly inhibit apoptosis through their various functions. Crosstalk from NF- κ B to JNK is known to prevent sustained JNK activation and thus prevent cell death (Reuther-Madrid et al., 2002; Tang et al., 2002). Specific NF- κ B controlled gene targets have been shown to affect JNK activation, such as GADD45 β and XIAP, (Papa et al., 2004; Tang et al., 2001). In addition, NF- κ B activation induced by TNF α can directly affect ROS via increased expression of the antioxidant proteins, such as Ferritin Heavy Chain (FHC) and Manganese Superoxide Dismutase (MnSOD) (Jones et al., 1997; Pham et al., 2004). Therefore, NF- κ B regulates genes that suppress ROS and decrease sustained JNK activation, thus preventing both apoptotic and necrotic cell death (Nakano et al., 2006; Papa et al., 2004; Reuther-Madrid et al., 2002; Tang et al., 2002).

ROS and other TNF signals

While the connection between JNK and ROS seem to be very well established, the affect of ROS on the other TNF α -induced pathways has been much more debated. Given that ROS often target the same phosphatases that dephosphorylate JNK, the p38 and ERK pathways are often simultaneously activated with JNK. Initially, reactive oxygen species were proposed to also be involved in NF- κ B activation based on the observed inhibition of TNF α -induced NF- κ B by the antioxidants N-acetyl-L-cysteine (NAC) and pyrrolidine dithiocarbamate (PDTC) (Schreck et al., 1992; Staal et al., 1990). NF- κ B activation was also reduced by overexpression of catalase (Schmidt et al., 1995). It was later shown that the effect of these NAC and PDTC eliminated the binding of the TNF α ligand to its receptor and by inhibiting I κ B α ubiquitin ligase activity, respectively (Hayakawa et al., 2003). It is since been recognized that ROS better serve to modulate NF- κ B rather than activate what NF- κ B has already been activated by other mechanisms (Oliveira-Marques et al., 2009). Nevertheless, reports continue to abound as to both inhibitory and stimulatory effects of ROS on NF- κ B in TNF α signaling.

Just as the effect of ROS vary between the different cell types, so the mechanisms vary widely in the prostimulatory or inhibitory effects on NF- κ B. Some of these reports suggest that TNF α -induced Nox-derived ROS affects NF- κ B signaling one way or another.

The stimulatory effects on NF- κ B activation are less reported and characterized than the inhibitory effects. TNF α treatment was reported downregulate catalase expression in MCF-7, Caco-2 and Hct-116 cancer cells, leading to increased duration of NF- κ B signal (Lupertz et al., 2008). In MCF-7 cells, Nox2 is reported to be required for endosomal superoxide that potentiates the TRAF2 recruitment to endosomal TNFR1, and is thus is required for efficient NF- κ B activation (Li et al., 2009a). Rac1 (Rac1N17) suppressed TNF α -induced ROS generation and subsequent NF- κ B activation in HUVEC cells (Deshpande et

al., 2000). Likewise, DPI, an inhibitor of NADPH oxidases, as well as dominant-negative Rac1 reduce NF- κ B due to TNF α in A549 cells (Kim et al., 2008). TNF α -induced ROS is apparently involved in the pre-transcriptional activation of RelA by phosphorylation at Ser276 through a PKA dependent event in U937 cells (Jamaluddin et al., 2007). Conversely, the phosphorylation of RelA at serine 536, which contributes to its DNA-binding activity, is induced by NAC in HeLa, Hep3B, and A549 cells (Liu et al., 2008b).

The effects of ROS on inhibition of NF- κ B components are more characterized. For instance, NF- κ B has been shown to be glutathionylated causing less transcriptional activity (Klatt and Lamas, 2000). Likewise, I κ B α is glutathionylated at cysteine 189, preventing its phosphorylation and degradation (Kil et al., 2008). Another major target for ROS is the catalytic subunit of IKK, IKK β which is oxidized by ROS at cysteine 179 (Reynaert et al., 2006), thus subduing the NF- κ B signaling by TNF. Thus ROS attenuate the NF κ B signal primarily through oxidation of the many pathway components.

CONCLUSION

In conclusion, ROS affects TNF α signaling in many and diverse ways. ROS plays a role in MAP kinase signaling, NF- κ B activation, apoptosis and necrosis. One of the more characterized roles of ROS is in the sustained activation of JNK, which often acts on different targets to potentiate apoptosis and necrosis. Due to the potential for ROS to act at various points and in multiple pathways during TNF α signaling, the ultimate effects of ROS are quite complex. Though we have learned much about the roles of ROS, doubtless there is still much to learn.

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