TNF-Induced Activation of the Nox1 NADPH Oxidase and Its Role in the Induction of Necrotic Cell Death

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SUMMARY

Tumor necrosis factor (TNF) is an important cytokine in immunity and inflammation and induces many cellular responses, including apoptosis and necrosis. TNF signaling enables the generation of superoxide in phagocytic and vascular cells through the activation of the NADPH oxidase Nox2/gp91. Here we show that TNF also activates the Nox1 NADPH oxidase in mouse fibroblasts when cells undergo necrosis. TNF treatment induces the formation of a signaling complex containing TRADD, RIP1, Nox1, and the small GTPase Rac1. TNF-treated RIP1-deficient fibroblasts fail to form such a complex, indicating that RIP1 is essential for Nox1 recruitment. Moreover, the prevention of TNF-induced superoxide generation with dominant-negative mutants of TRADD or Rac1, as well as knockdown of Nox1 using siRNA, inhibits necrosis. Thus our study suggests that activation of Nox1 through forming a complex with TNF signaling components plays a key role in TNF-induced necrotic cell death.

INTRODUCTION

Tumor necrosis factor (TNF) is a pleiotropic inflammatory cytokine and plays a critical role in diverse cellular events, including cell proliferation, differentiation, apoptosis, and necrosis (Chen and Goeddel, 2002; Wajant et al., 2003). Through binding to its two receptors, TNF-R1 (p55) and TNF-R2 (p75), TNF is a major mediator of both inflammation and immunity and has thus been implicated in a variety of pathological inflammatory conditions and autoimmune diseases. Since the discovery of its tumoricidal activity, the TNF pathway has become one of the most studied signaling pathways, resulting in the characterization of a vast superfamily of receptors, the TNF receptor superfamily, and their ligands. In response to TNF treatment, the transcription factor NF-κB and MAP kinases, such as c-Jun N-terminal kinase (JNK), are activated in most types of cells and, in some cases, apoptosis or necrosis can also be induced (Fiers et al., 1999; Karin and Lin, 2002).

Much is known about the molecular mechanisms of TNF signaling. Engagement of TNF-R1 by the TNF homotrimer initiates the binding of the adaptor protein, TNF-R1-associated death domain protein (TRADD), which then recruits other effector proteins, such as receptor interacting protein 1 (RIP1) and TNFR-associated factor 2 (TRAF2) to form a TNF-R1 signaling complex leading to the activation of several pathways, including NF-κB and MAP kinases (Chen and Goeddel, 2002; Wajant et al., 2003). Both TRAF2 and RIP1 play essential roles in the activation of NF-κB and MAP kinase pathways through recruitment and activation of IKK (IkB kinase) and MAP3Ks (Chen and Goeddel, 2002; Wajant et al., 2003). Under certain conditions, it is thought that the TRADD, RIP1, and TRAF2 proteins dissociate from the receptor and form a secondary protein complex containing other proteins (Micheau and Tschopp, 2003). Apoptosis is primarily initiated through the recruitment of Fas-associated death domain protein (FADD) to this secondary complex. FADD then mediates the activation of the initiator cysteine proteases caspase-8 and caspase-10, which drives apoptosis. Although caspase cleavage is the primary means of initiating apoptotic cell death by TNF, cell death still occurs (or is even enhanced) in some types of cells in the absence of caspase activity (Fiers et al., 1999).

While the mechanism of TNF-induced apoptotic cell death is well elucidated, the signaling events that lead to TNF-initiated caspase-independent death are largely unknown. Caspase-independent necrotic cell death has been proposed to involve reactive oxygen species (ROS), which some studies suggest are derived from the mitochondria (Fiers et al., 1999). ROS can inactivate MPK phosphatases, leading to sustained JNK activation and resulting in eventual cell death (Kamata et al., 2005). RIP1 is necessary for the generation of ROS by TNF and is required for the initiation of necrotic cell death (Lin et al., 2004). During apoptotic cell death, RIP1 is cleaved by caspase-8 (Lin et al., 1999), limiting its ability to activate the pathway(s) of ROS generation.

NADPH oxidases are enzymes specifically dedicated to the production of ROS (Lambeth, 2004). In macrophages and neutrophils, TNF is known to stimulate the activity of...
one such oxidase, Nox2/gp91phox, resulting in the generation of superoxide ($\mathrm{O}_2^-$) that is important in the ability of these cells to kill invasive microorganisms. Nox2, which is a membrane glycoprotein that exists as a heterodimer with a 22 kDa subunit (p22phox), is activated by the p47phox and p67phox proteins. Mutations in any of the four oxidase subunits can result in chronic granulomatous disease (CGD), characterized by a susceptibility to severe and recurrent bacterial and fungal infections derived from the inability of phagocytic cells to destroy pathogens (Heyworth et al., 2003). When phosphorylated, the p47phox subunit binds to membrane phospholipids, interacts with p22phox, and recruits the p67phox subunit to the complex. The p67phox activator binds and stabilizes an interaction of the complex with the small GTPase Rac, and the fully formed complex is able to generate $\mathrm{O}_2^-$ in the presence of NADPH. TNF is a potent activator of Nox2 through a little-understood mechanism that may involve phosphorylation of p47phox (Frey et al., 2002; Dewas et al., 2003; Dang et al., 2006).

NADPH oxidases have been characterized in nonphagocytic cell types, along with other regulatory adaptor proteins (Lambeth, 2004). The regulatory p41NOXO1 and p51NOXO1 subunits may function in some of these oxidase complexes similarly to p47phox and p67phox, respectively. Unlike p47phox, NOXO1 lacks an autoinhibitory region, binds to different lipids, and does not require phosphorylation for membrane translocation (Lambeth, 2004).

Nox1 and Nox4 appear to be more ubiquitous than other NADPH oxidases in nonphagocytic cells. However, Nox4 is constitutively active when complexed with p22phox, does not appear to require additional regulatory subunits (Martyn et al., 2006), and may be regulated solely by its expression level. Thus, Nox1 may be the most likely source of immediate NADPH oxidase activity in response to TNF in nonphagocytic cell types where Nox2 is not expressed.

In this study, we show that the Nox1 NADPH oxidase is activated during TNF-induced necrotic cell death by forming a complex with TRADD, RIP1, and Rac1. Interactions of NOXO1 with TRADD and RIP1 are critical for the activation of Nox1 by TNF, and RIP1 is essential for recruiting Nox1 to the complex in MEF cells when necrosis is triggered. Importantly, Nox1-mediated production of $\mathrm{O}_2^-$ is critical for TNF-induced necrotic cell death because such death is inhibited by ROS scavengers, downregulation of the Nox1 protein by siRNA, or expression of dominant-negative versions of TRADD or Rac1. Our data also suggest that Nox1 may mediate the induction of necrosis through regulating sustained JNK activation.

RESULTS

TNF-Induced $\mathrm{O}_2^-$ Generation and Necrotic Cell Death Are Inhibited by BHA

Many experiments have implicated ROS in TNF-induced necrotic cell death, some of which have suggested mitochondrial involvement (Fiers et al., 1999). To address whether the ROS generated by NADPH oxidase also plays a role, we first examined whether $\mathrm{O}_2^-$ was generated under such conditions. Lucigenin, which reacts specifically with $\mathrm{O}_2^-$ and produces chemiluminescence (Teixeira et al., 1999), was used to measure $\mathrm{O}_2^-$ production in murine fibrosarcoma L929 cells, which undergo necrotic cell death in response to TNF treatment. The amount of chemiluminescence increased dramatically 30–40 min after addition of TNF to the cells (Figure 1A), indicating that the production of $\mathrm{O}_2^-$ followed exponential kinetics at this time period, while steady levels of luminescence were achieved 75 min after TNF treatment (see Figure 1B). Cells treated with another inflammatory cytokine, IL-1, failed to produce a detectable amount of $\mathrm{O}_2^-$, indicating that the $\mathrm{O}_2^-$ generation in this case is specific to TNF (see Figure S1A in the Supplemental Data available with this article online). Notably, treatment with a pan-caspase inhibitor, z-VAD-fmk, which has been shown to enhance TNF-induced cell death in L929 cells, significantly increased TNF-stimulated $\mathrm{O}_2^-$ generation (Figure S1B), suggesting that the $\mathrm{O}_2^-$ production was correlated with cell death. To test whether $\mathrm{O}_2^-$ is produced in other cell types under necrotic conditions, we used MEF cells deficient in the RelA subunit of NF-kB (p65$^{-/-}$). These cells preferentially undergo apoptotic cell death in response to treatment with TNF alone but undergo necrosis when caspase inhibitor is present (Sakon et al., 2003). As shown in Figure 1B, TNF treatment induced the formation of $\mathrm{O}_2^-$ only under necrotic cell death conditions. This suggests that the generation of $\mathrm{O}_2^-$ is correlated with caspase-independent necrotic cell death in response to TNF.

As previously reported, the antioxidant BHA significantly blocked TNF-induced cell death in both L929 and p65$^{-/-}$ cells (Figures 1C and 1D and Figures S1C and S1D). While BHA did have some protection in TNF-treated p65$^{-/-}$ MEFs, it was far less efficient than in L929 cells (compare Figure 1C and Figure S1C with Figure 1D and Figure S1D). However, BHA prevented TNF-induced cell death more efficiently in the presence of zVAD (Figure 1D and Figure S1D), indicating that BHA more efficiently protects these cells from necrosis than from apoptosis. Pretreatment with the ROS scavengers NAC and TEMPOL gave similar results (Figure S2A). We then tested whether BHA had an effect on $\mathrm{O}_2^-$ generation. As shown in Figure 1E, BHA pretreatment abolished TNF-induced $\mathrm{O}_2^-$ formation in L929 cells. Similarly, the addition of superoxide dismutase (SOD), NAC, or TEMPOL reduced $\mathrm{O}_2^-$ to background levels (Figures S2B–S2D). Pretreatment for 30 min with cycloheximide did not block $\mathrm{O}_2^-$ generation in L929 or p65$^{-/-}$ cells (data not shown), indicating that protein synthesis was not required. Taken together, these results indicated that antioxidants block both TNF-induced necrotic cell death and $\mathrm{O}_2^-$ accumulation.

Nox1 NADPH Oxidase Is Responsible for TNF-Induced $\mathrm{O}_2^-$ Generation

The production of $\mathrm{O}_2^-$ in response to TNF suggests the activation of an NADPH oxidase. Of the known NADPH oxidases, only Nox2/gp91 has been previously shown to
be activated in response to TNF (Frey et al., 2002). While Nox2 is found primarily in phagocytic cells, it is also expressed in several other cell types, including endothelial cells and vascular fibroblasts (Gorlach et al., 2000; Chameseddine and Miller, 2003). We therefore sought to determine whether Nox2 is expressed in L929 and p65−/− MEF cells. As shown in Figure 2A, Nox2 was not detected by western blot in either of these two types of cells, though it was readily detected in the J774 and RAW264.7 macrophage cell lines. We then tested whether Nox1 is expressed in L929 and p65−/− MEF cells because it has been reported to be expressed in several types of nonphagocytic cells (Suh et al., 1999; Takeya et al., 2003; Geiszt et al., 2003). We probed the same lysates with a Nox1 antibody and detected a 46 kDa band in several cell types, including L929 and p65−/− MEFs (Figure 2A). This band ran below the expected Nox1 size, which is predicted to be 65 kDa. However, Nox2, before it is glycosylated, also runs lower than its actual molecular weight (Yu et al., 1999; Nguyen and Tidball, 2003), as is not uncommon for an integrated membrane protein. Nevertheless, we sought to more clearly establish the identity of this band as Nox1. Treatment of HL-60 cells with PMA or DMSO induced expression of this band (Figure 2A) in a manner similar to several other oxidase components, including p47phox and p67phox (Figure S3). In order to further verify the identity of the band, we performed in vitro translation of an untagged Nox1 cDNA with S35...
methionine and detected a band at about 49 kDa (Figure 2B, top), which includes the 3 kDa signal peptide cleaved from the endogenous protein. We also expressed the full-length Nox1 and several potential Nox1 variants with an N-terminal Xpress tag (Xp), (3.5 kDa) for comparison (Figure 2B, bottom). In vitro translated Xp-Nox1 proteins gave a single band at about 51 kDa, while an Xpress-tagged naturally occurring C-terminal splice variant of Nox1 (Nox1-lv) had a smaller size than this (49.5 kDa). Cloning from the next in-frame start codon (Nox1-trunc) gave an even smaller in vitro translated product of just over 47 kDa. These results indicated that the higher mobility of the endogenous band detected by the Nox1 antibody was not likely due to alternative translation or splice variation, as adjustment for the signal peptide and tag removal makes the full-length Nox1 in cells likely to
be observed at 46 kDa, which is the size we observed. Transfection of a C-terminal Myc-tagged Nox1 construct in several cell types resulted in little expression as detected by anti-myc antibody for unknown reasons (data not shown). However, we were able to immunoprecipitate the ectopically expressed Myc-tagged Nox1 protein from a large number of cells with an anti-Myc antibody and detected the precipitated protein with the anti-Nox1 antibody at just under 50 kDa (Figure 2C). Immunoprecipitation of in vitro translated Nox1-Myc with an anti-Myc antibody also gave a protein recognized by the Nox1 antibody at the expected size (data not shown). These data suggest that the Nox1 antibody properly recognizes the Nox1 protein at an observed weight of ∼46 kDa.

We then tested whether Nox1 is responsible for TNF-induced \( \text{O}_2^- \) generation. Among the four Nox1 siRNA oligos tested, oligos #1 and #4 reduced the amount of Nox1 protein when transfected into L929 cells (Figure 2D and Figure S4A). Oligos #2 and #3 had little or no effect on Nox1 protein levels (Figure 2D and data not shown). The Lamin A/C control oligo caused no reduction in the Nox1 protein but efficiently reduced both the A and C forms of Lamin in these cells (Figure 2D and Figure S4A). Knockdown of Nox1 by oligos #1 and #4 led to a dramatic decrease in the production of \( \text{O}_2^- \) in these cells (Figure 2E and Figure S4B), but neither of the other siRNA oligos diminished the amount of \( \text{O}_2^- \) generated in response to TNF. In a rescue experiment, we cotransfected an expression plasmid encoding human Nox1 and Nox1 siRNA oligo #4. Because the target sequence of oligo #4 in mouse Nox1 is quite different from the sequence of human Nox1, oligo #4 cannot reduce human Nox1 expression. Cotransfection of the human Nox1 plasmid restored the TNF-induced \( \text{O}_2^- \) activation in siRNA transfected cells, while cotransfection of a catalytically inactive point mutant of Nox1, T341K, which is based on a homologous inactive mutation of Nox2 found in some patients with CGD, failed to restore the TNF-induced activation (Figure 2F). Based on these findings, Nox1 is likely the major NADPH oxidase responsible for TNF-induced \( \text{O}_2^- \) generation in L929 cells.

**Nox1 Forms a Complex with TRADD, RIP1, and Rac1 Following TNF Treatment**

Nox1 is localized to caveolin-enriched lipid rafts on the cell membranes of some cell types (Hilenski et al., 2004). Several TNF receptor machinery components have also been shown to relocalize to similar lipid rafts upon TNF simulation (Legler et al., 2003), suggesting Nox1 activation may be regulated by an interaction with TNF-R1 or its adaptor molecules. We therefore immunoprecipitated the endogenous TRADD protein in the presence or absence of TNF. As has been previously shown, the protein RIP1 communoprecipitated with the TRADD protein upon TNF stimulation, indicating that a signaling complex had been formed upon TNF treatment (Figure 3A). When probed with the Nox1 antibody, a 46 kDa band was detected in the TNF-treated precipitants, but not in the untreated ones, indicating that Nox1 coprecipitated with TRADD. We also sought to determine whether the complex contained Rac1, which is required for an active Nox1 NADPH oxidase complex. Rac1 protein was also found in the TRADD communoprecipitants from the treated cells, but not the untreated cells (Figure 3A, bottom). This suggests that not only is Nox1 found in a complex with TRADD, but also that Nox1 is likely activated in the complex.

We next sought to identify the means by which the Nox1 protein is recruited to the TRADD complex following TNF treatment since we did not observe a direct Nox1 interaction with TRADD or RIP1 (data not shown). Although little is known about Nox1 activation, it is thought to involve the NOXO1 and NOXA1 subunits. We therefore tested whether TRADD and/or RIP1 interacts with these two proteins upon overexpression in HEK293 cells. As shown in Figure 3B, left panel, RIP1 shows a strong interaction with NOXO1, but little interaction with NOXA1. Similarly, a specific interaction was also found between TRADD and NOXO1 (Figure 3B, right). None of the other known NADPH oxidase subunits including p22phox, p47phox, or p67phox interacted significantly with RIP1 or TRADD (data not shown). Therefore, the interaction between NOXO1 and RIP1 or TRADD provides a potential mechanism for recruiting Nox1 to the TNF-induced signaling complex.

To further confirm the involvement of NOXO1 in the formation of the Nox1/TRADD/RIP1 complex, we immunoprecipitated NOXO1 in L929 cells in the presence or absence of TNF. We detected a constitutive interaction between the NOXO1 and Nox1 in untreated cells (Figure 3C), consistent with previous data (Cheng and Lambeth, 2004). In contrast, RIP1 and Rac1 were not coprecipitated with NOXO1 in untreated cells. RIP1 and Rac1 were observed in the complex 30 min after TNF treatment, suggesting that TNF induced the formation of a Nox1/TRADD/RIP1 signaling complex that also contained NOXO1 (Figure 3C).

To determine whether endocytosis of the receptor was responsible for bringing the TNF signaling components into contact with the Nox1 machinery, we treated cells with Cytochalasin D or Latrunculin A, which inhibit actin polymerization by different mechanisms. Neither drug had any effect on TNF-induced JNK activation or I\( \kappa_B \) degradation, nor prevented recruitment of Nox1 to TRADD (Figures S5A and S5B). The levels of \( \text{O}_2^- \) were increased upon drug treatment (Figure S5C), suggesting that inhibition of endocytosis may increase the amount of detectable extracellular \( \text{O}_2^- \) to some extent by keeping the complex in the cell membrane. Thus endocytosis is not responsible for the Nox1 recruitment.

**RIP1 Is Required for the Recruitment of Endogenous Nox1 in TNF-Treated Cells**

Because RIP1 showed a strong interaction with NOXO1, we then tested whether RIP1 was required for the formation of the endogenous Nox1/TRADD/RIP1 complex. Detection of the Nox1/TRADD/RIP1 complex in wild-type MEFs was seen under necrotic conditions (with TNF,
CHX, and zVAD), but not under apoptotic conditions (TNF and CHX) or in cells treated with TNF alone (data not shown). Therefore, coimmunoprecipitation experiments were performed in wild-type, RIP1−/−/−, and TRAF2−/−/− MEF cells under necrotic conditions. While the anti-TRADD antibody brought down Nox1 in the wild-type and TRAF2−/−/− cells, this interaction was not seen in RIP1−/−/− MEFs, though Nox1 was present in the cell lysates (Figure 3D). This indicates that RIP1 was required for recruitment of endogenous Nox1 to the complex in response to TNF and implies that the activation of Nox1 requires the RIP1 protein.

TRADD Association with NOXO1 Requires a Polyproline Region that Is a Consensus SH3 Ligand Motif

While the N terminus of TRADD contains a TRAF binding region and the C terminus contains a death domain that interacts with TNF-R1, RIP1, and FADD, the central portion of the molecule has no ascribed function, except that it contains a nuclear export sequence. This uncharacterized region of TRADD contains a consensus SH3 ligand motif with seven prolines flanked by an N-terminal lysine. This region could be responsible for the interaction with NOXO1, since NOXO1 contains two SH3 domains. Therefore, we hypothesized that the interface between TRADD and NOXO1 might involve this polyproline region. We created a TRADD protein with alanine substitutions in the proline region (P-A). These mutations caused a small shift in the mobility of the protein, possibly due to the increased flexibility (Figure 4). When coexpressed with NOXO1, this mutant protein failed to interact with NOXO1 as the wild-type protein did (Figure 4A). Because the mobility of the mutant TRADD protein had changed, we checked whether the overall structure of the mutant was altered by examining its interaction with RIP1, FADD, and TRAF2. As shown in Figure 4B, the P-A mutant retained the interaction with these proteins, and coimmunoprecipitated with them as efficiently as the nonmutant TRADD protein did. These results suggest that the TRADD P-A mutant protein loses its interaction with NOXO1 specifically. To test whether the SH3 domains of NOXO1 were involved in binding to TRADD, we mutated the conserved tryptophan in the binding pockets of NOXO1. The resulting W196K and W268K mutations would not be expected to bind to proline-rich ligand sequences any longer but should retain the structure of the SH3 domains. As shown in Figure 4C, mutation of the first SH3 domain (W196K), but not the second domain (W268K), prevented the binding of NOXO1 to TRADD. The mutations on both SH3...
domains also abolished the interaction between TRADD and NOXO1. These data suggest that the first SH3 domain is critical for binding to TRADD.

The TRADD P-A Mutant Blocks TNF-Induced Nox1 Activation and Necrotic Cell Death

To test the role of TRADD in Nox1 activation, several L929 cell lines were created in which the Flag vector, Flag-TRADD, and Flag-TRADD P-A mutant were stably expressed (Figure 5A). We used these cell lines to examine the effect of the TRADD P-A mutant on different TNF signaling pathways. As shown in Figure 5B, no deficiencies in immediate JNK activation and IkB degradation were found in these cells, but less Nox1 coimmunoprecipitated with TRADD upon TNF treatment (Figure 5C), suggesting a specific dominant-negative effect of this TRADD mutant on Nox1 recruitment. We then measured the TNF-induced O$_2^-$ production in these cell lines. The O$_2^-$ production was dramatically impaired in the cells with ectopic expression of TRADD P-A mutant protein, but not in the other stable cell lines (Figure 5D). To further confirm these results, we produced a second set of stable cell lines and obtained similar data (Figure S6A). These results indicated that the TRADD P-A mutant selectively blocks Nox1 activation.

The above data suggest that the TRADD P-A mutant acts as a dominant-negative molecule with respect to the activation of NADPH oxidase by TNF but has no effect on TNF-induced activation of NF-$\kappa$B and JNK. We then tested whether the specific reduction in O$_2^-$ generation by Nox1 affected TNF-induced necrotic cell death. We treated stable cell lines for 10 hr with TNF and observed the resulting cell death. A significant increase of cell survival was seen in the cells expressing the TRADD P-A mutant protein compared to cells having the empty vector or the nonmutant TRADD, as measured by a tetrazolium dye colorimetric test (MTT) (Figure 5E, left). Phase contrast microscopy verified these results (Figure 5E, right), and a decrease in TNF-induced cytotoxicity was also seen in the other independently derived stable cell lines when compared to controls (Figure S6B). In the presence of zVAD, where NADPH oxidase activity was much higher and cell death occurred quickly, a difference in cell viability between the Flag-TRADD P-A mutant and the other cell lines could be easily detected as early as 2 hr (Figure S6C). These data suggest...
that a specific reduction in Nox1 activation by TNF results in an increase in cell viability and implicates the essential role of \( \text{O}_2^- \) in TNF-induced necrotic cell death.

**The Dominant-Negative Rac1 Mutant, N17Rac1, Reduces TNF-Induced Nox1 Activity and Necrotic Cell Death**

To further verify that a reduction in \( \text{O}_2^- \) production in response to TNF leads to a decrease in cell death, we sought to reduce the Nox1 activation in other ways.

Rac1 has been recently shown by several groups to be essential for Nox1 activity (Cheng et al., 2006; Miyano et al., 2006; Ueyama et al., 2006) and was detected in the Nox1/TRADD/RIP1 complex (Figure 3A). Therefore, we stably expressed a Rac1 mutant (T17N) in L929 cells (Figure 6A). This mutant, which acts as a dominant-negative molecule by preventing GDP-GTP exchange, had no detectable effect on early JNK activation and activation of NF-\( \kappa \)B by TNF (Figure 6B). Expression of N17Rac1 significantly reduced the amount of \( \text{O}_2^- \) produced in response to TNF.
when compared with the empty vector transfected cells (Figure 6C). We next examined the effect of N17Rac1 on TNF-induced necrotic cell death. As shown in Figure 6D, a significant reduction in the amount of cell death was seen in cells stably expressing N17Rac1 compared to control cells. Therefore, like the TRADD P-A mutant,
N17Rac1 also specifically inhibits TNF-induced Nox1 activation and necrotic cell death. Knockdown of Nox1 Reduces TNF-Induced Necrotic Cell Death

To eliminate the possibility that unexpected nonspecific effects of the TRADD and Rac1 mutants contribute to the decrease in TNF-induced necrotic cell death, we knocked down the expression of Nox1 protein with the Nox1 siRNA oligo #4 as described in Figure 2. Knocking down Nox1 protein level significantly protects the cells against TNF-induced necrotic cell death in L929 cells (Figure 6E). A similar reduction in death was seen using a second siRNA oligo (#1) to Nox1 (Figure S7), but not the Lamin A/C oligo, or the Nox1 oligo (#2) that fails to reduce Nox1 protein levels (Figures 6E and 2D). Cell death due to TNF could be rescued by cotransfection of the human Nox1 plasmid, but not its catalytically inactive point mutant T341K or empty vector (Figure 6F).

Prolonged JNK Activation Is Restrained in Cells in which Nox1 Activation Is Reduced

JNK is activated within 15 min after TNF treatment, but in cell types that are not sensitive to TNF-induced cell death, this activation is transient due to JNK phosphatases and is barely detected after 60 min. It has been previously shown that prolonged JNK activation after this time contributes to TNF-induced cell death and that increased ROS levels play a role in this process (Tang et al., 2001; Sakon et al., 2003). It was proposed that this prolonged JNK activation occurs through activation of the MAP3K ASK1 (Liu et al., 2000; Tobiume et al., 2001) and also through the inactivation of JNK phosphatases by ROS (Kamata et al., 2005). We therefore examined whether prolonged JNK activation was reduced in our stable cell lines expressing the dominant-negative TRADD or Rac1 mutants. Notably, TNF-induced activation of JNK at 15 and 30 min occurs normally in these cells (Figures 5B, 6B, 7A, and 7B). But at extended time points when $O_2^-$ was produced in L929 cells (>45 min), we saw a significant reduction in the amount of JNK phosphorylation in cells expressing the TRADD P-A mutant (Figure 7A) or the N17Rac1 mutant (Figure 7B), in which Nox1 activation is reduced, compared to the control cells. This reduction in sustained JNK activation was especially obvious 3 hr after treatment.

To determine whether prolonged JNK activation was involved in cell death in L929 cells, we treated the cells with a pharmacological inhibitor of JNK, SP600125, and measured TNF-induced cell death. The JNK inhibitor reduced the amount of cell death similarly to treatment with BHA after 8 hr of TNF treatment (Figure 7C), suggesting that JNK activation is an important step in TNF-induced necrotic cell death (Figure 7C). SP600125 also significantly reduced the amount of cell death at early time points (<6 hr) in p65$^{-/-}$ cells treated with TNF and zVAD (data not shown) in a manner similar to BHA. These data imply that sustained JNK activation occurs in response to the production of $O_2^-$ generated downstream of TNF and that prolonged JNK activation contributes to caspase-independent necrotic cell death induced by TNF.

DISCUSSION

The signaling events that mediate TNF-induced caspase-independent necrotic cell death remain largely elusive, though previous studies indicate that the generation of ROS is essential for TNF-induced necrotic cell death (Fiers et al., 1999). ROS may mediate necrotic cell death by inactivating MPK phosphatases, leading to sustained JNK activation (Kamata et al., 2005). In our current study, we have shown that ROS generated through activation of Nox1 plays a critical role in TNF-induced necrotic cell death (Figure 7D).

Our data suggest that Nox1 is the major NADPH oxidase responsible for TNF-induced generation of $O_2^-$ in mouse fibroblasts and is activated through a signaling complex containing Nox1, NOXO1, Rac1, and the TNF effector proteins, TRADD and RIP1. TRADD and RIP1 bind
TNF can induce biphasic activation of JNK; while the prolonged JNK activation contributes to TNF-induced cell death, the transient JNK activation protects cells against death (Karin and Lin, 2002; Ventura et al., 2006). Recent studies suggest that this prolonged JNK activation occurs through activation of the MAP3K ASK1 (Liu et al., 2000; Tobiume et al., 2001) or through the inactivation of JNK phosphatases by ROS reaction with their catalytic cysteine (Kamata et al., 2005). Our current work is consistent with the later observation, particularly, in that the kinetics of $O_2^-$ generation by Nox1 correlates with the profile of prolonged JNK activation.

TNF-R1 appears to be the receptor that activates Nox1, as TRADD is not required to the TNF-R2 complex. This is consistent with our previous results showing that necrotic cell death is induced only through TNF-R1 (Lin et al., 2004). Due to the antibodies involved and the proximity of both bands to the IgG heavy chain, we have not yet determined whether the TNF-R1 protein is physically involved in the Nox1 complex or whether this complex is downstream of the receptor. Nox1 is localized to lipid rafts in some cell types, and TNF-R1 has been reported to relocalize to lipid rafts after TNF stimulation, as has RIP1 and TRAF2 (Legler et al., 2003). Previous data showed the receptor components RIP1 and TRADD bind to TNF-R1 within 5 min upon stimulation and begin to release from the receptor at about 20–30 min. We were able to detect Nox1 recruitment into the complex (with TRADD) within 15 min of TNF treatment, which may suggest TNF-R1 involvement in the complex. The maximal amount of Nox1 coprecipitated with TRADD at about 30 min after TNF treatment. This time point corresponds to the time at which the maximal amount of Rac1 is also recruited to the complex, and we are able to detect a rise in $O_2^-$ levels shortly after this time. RIP1 is heavily modified by K63-linked polyubiquitination at early time points after TNF treatment whereby it interacts with the NEMO subunit of IKK to activate NF-kB. It is possible that this ubiquitination prevents its interaction with NOXO1 until enough nonubiquitinated RIP1 is recruited to the receptor.

TNF-R1 and Nox1 are expressed ubiquitously, so it is unclear at present under what conditions this necrotic pathway may be induced. In many systems, TNF induces inflammation rather than cell death due to the activation of NF-κB, which not only upregulates proinflammatory genes but also results in the upregulation of antioxidant proteins such as MnSOD and prosurvival proteins such as c-Flip, A20, and cIAPs1/2, which inhibit apoptosis. Many of these proteins may also inhibit necrosis. Nevertheless, there are likely some conditions under which necrotic death is initiated. In model cell culture systems such as ours, the addition of zVAD is sometimes required to initiate necrotic cell death, and, as mentioned before, in many situations the cleavage of RIP1 by caspases during apoptosis (Lin et al., 1999) probably prevents necrotic cell death from occurring. Given that many microbes encode antiapoptotic proteins such as CrmA, E1B19, and p35, many of which directly block caspase activation, the
Nox1-mediated necrotic death pathway may be a default cell death pathway that is activated by TNF following the inflammatory response in order to remove the remaining damaged or infected cells that are not removed by apoptosis. To our knowledge, there are no current reports in the literature where Nox1−/− mice have been challenged by infection (the only described phenotype with regard to these mice is lower than average blood pressure), but such experiments would test this hypothesis. Interestingly, Nox1 did not appear to contribute to necrotic cell death induced by dsRNA and interferon in L929 cells (Figure S8) but did appear to contribute specifically to the TNF-induced death.

Previously, the activation of Nox2 by TNF has been proposed to involve the activation of a tyrosine kinase or PKCζeta. We found that inhibitors of these pathways had no effect on the activation of Nox1 by TNF and did not protect from cell death (Figure S9). It is uncertain as to whether TNF may activate Nox2 by a similar mechanism in phagocytic or cardiovascular cell types, in which the p47phox protein is expressed instead of NOXO1. Though we did not detect an obvious interaction between p47phox and TRADD or RIP1, an interaction with these proteins is still a possibility in a physiological context. Further study is necessary to investigate whether Nox2 is recruited to TNF-R1 complex through RIP1/TRADD similarly as Nox1.

**EXPERIMENTAL PROCEDURES**

**Reagents**

Murine rTNFα was from R&D Systems. Antibodies were from commercial sources: anti-Nox1, anti-TRADD, anti-ixBα, anti-Myc, anti-His, anti-HA, anti-GFP, and anti-Xpress from Santa Cruz; anti-RIP1 from Rockland. SP600125, zVAD, and CHX were from Calbiochem. Lucigenin was from Invitrogen. Anti-actin and anti-Flag antibodies, NADPH, L929, HEK293, and the various 3T3-like immortalized MEF cell lines were cultured in DMEM with 10% fetal bovine serum, 2 mM glutamine, and 100 U/ml penicillin/streptomycin. L929 stable cell lines were established by transfecting cells with the designated vector and then maintaining in media containing 200 μg/mL puromycin. L929 cells were plated in 6-well plates, and cells were transfected with 100 pmol of Nox1- or Lamin A/C-specific RNAi oligo (Dharmacon) using Lipofectamine 2000 reagent (Invitrogen). After 72 hr, knockdown was analyzed by western blotting and the remaining cells were used for the NADPH oxidase and cytotoxicity assays.

**Cytotoxicity Assay**

Cell death was determined using MTT. MTT absorbance was read at 570 nm. Representative images were taken by a phase-contrast microscope.

**In Vitro Translation**

In vitro translation was done using the T7 and SP6 directed TNF-coupled wheat germ and reticulocyte lysate systems (Promega).

**Supplemental Data**

Supplemental Data include nine figures and can be found with this article online at [http://www.molecule.org/cgi/content/full/26/5/675/DC1/](http://www.molecule.org/cgi/content/full/26/5/675/DC1/).

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