NEMO/IKK γ regulates an early NF- κ B-independent cell-death checkpoint during TNF signaling

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TNF receptor 1 (TNFR1) ligation can result in cell survival or cell death. What determines which of the two opposing responses is triggered is not fully understood. The current model suggests that it is the activation of the NF- κ B pathway and its induction of prosurvival genes, or the lack thereof, which determines the outcome. NF- κ B essential modifier (NEMO)/IKB kinase- γ (IKK γ)-deficient cells are highly sensitive to apoptosis, and as NEMO is essential for NF- κ B activation, it has been assumed that this is due to the lack of NF- κ B. This study demonstrates that this assumption was incorrect and that NEMO has another antiapoptotic function that is independent of its role in the NF- κ B pathway. NEMO prevents receptor interacting protein-1 (RIP1) from engaging CASPASE-8 before NF- κ B-mediated induction of antiapoptotic genes. Without NEMO, RIP1 associates with CASPASE-8 resulting in rapid tumor necrosis factor (TNF)-induced apoptosis. These results suggest that there are two cell-death checkpoints following TNF stimulation: an early transcription-independent checkpoint whereby NEMO restrains RIP1 from activating the caspase cascade, followed by a later checkpoint dependent on NF- κ B-mediated transcription of prosurvival genes. *Cell Death and Differentiation* (2009) 16, 1279–1288; doi:10.1038/cdd.2009.41; published online 17 April 2009

A major quest in TNF signaling has been to understand the molecular mechanism that determines whether the survival or the death pathway is triggered after receptor ligation.^{1,2} Studies over the past decade have led to the model that it is the activation of the NF- κ B signaling pathway on TNF receptor 1 (TNFR1)³ ligation that acts as a cell-death determinant because NF- κ B transcription factors induce the expression of antiapoptotic genes. It is also known that the components of the TNFR1 death pathway are preexisting and do not require *de novo* protein synthesis. In fact historically, apoptosis is induced in most cell types by treating the cells with a combination of TNF and cycloheximide, a protein synthesis inhibitor. Thus the apoptotic pathway does not require new protein synthesis whereas the NF- κ B-dependent survival pathway requires transcription and translation of new proteins, and yet most cells are largely resistant to apoptosis when treated with TNF alone. This paradoxical situation suggests that there must be additional mechanisms that keep the apoptotic pathway in check before NF- κ B-mediated induction of antiapoptotic genes.

A central regulatory step in NF- κ B signaling is the activation of the I κ B kinase (IKK) complex. The IKK complex, which consists of the catalytic subunits IKK α and IKK β and the regulatory subunit NEMO, phosphorylates the inhibitory molecule I κ B resulting in its degradation and the translocation of NF- κ B transcription factors to the nucleus. In the absence of NEMO, the IKK complex cannot be activated and the canonical NF- κ B signaling pathway is blocked.³ We and others have previously shown that NEMO-deficient cells are

highly sensitive to TNF-induced cell death.^{4–6} Because of the essential role of NEMO in IKK activity, it has been assumed that the sensitivity to TNF-induced apoptosis in NEMO-deficient cells was due to the lack of NF- κ B-mediated gene transcription. However, whether this is the case or whether NEMO possesses additional mechanisms to inhibit apoptosis has not been directly examined. We, therefore, examined more closely the apoptosis induced by TNF in the NEMO-deficient Jurkat T-cell mutant 8321 that we previously isolated.^{5,7} This study now demonstrates that NEMO has another antiapoptotic function, which is independent of its role in NF- κ B signaling. Early during TNF signaling, NEMO inhibits the activation of the apoptosis pathway by restraining RIP1 from interacting with CASPASE-8.

Results

NEMO-deficient cells are more sensitive to TNF-induced cell-death independent of NF- κ B. We have previously reported that NEMO-deficient Jurkat T cells (clone 8321) are highly sensitive to TNF-induced cell death when compared to wild-type cells and this phenotype in the mutant clone can be reverted on complementation with a NEMO cDNA.⁵ In this study we compared the cell-death sensitivity of the NEMO-deficient cells to that of wild-type parental cells rendered sensitive to apoptosis by stable transfection of the I κ B α super-repressor (I κ B α SR), a potent inhibitor of NF- κ B nuclear translocation. As expected, increased cell death was observed in the 3T8 cells transfected with I κ B α SR

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Abbreviations: I κ B α SR, I κ B α super repressor; IKK γ , I κ B kinase- γ ; NEMO, NF- κ B essential modifier; RIP1, receptor interacting protein 1; TNFR1, TNF receptor 1; shRNA, short-hairpin RNA

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(3T8/I κ B α SR) compared to the 3T8 cells transfected with bacterial GST as an negative control (3T8/Con) at 4 and 8 h after TNF stimulation, consistent with published observations that NF- κ B blockade renders cells sensitive to TNF-mediated

apoptosis. What was surprising was the observation that NEMO-deficient 8321 cells displayed greater sensitivity to cell death when compared to the 3T8/I κ B α SR cells at both time points (Figure 1a). As the 8321 and the

Figure 1 NEMO inhibits TNF-induced apoptosis in a NF- κ B-independent manner. (a) Parental 3T8 Jurkat T cells transduced with retrovirus encoding either control protein or I κ B α SR, and untransfected NEMO-null 8321 cells were treated with the indicated doses of TNF for 4 and 8 h. Cell death was measured by staining with PE-conjugated annexin V and FACS analysis. (b) Cell lysates from 3T8/Con, 3T8/I κ B α SR, NEMO-null/I κ B α SR, or NEMO-WT/I κ B α SR Jurkat T cells were subject to western blot analysis using anti-NEMO followed by anti-ZAP70 as a loading control. (c) 3T8 cells transfected with control protein or I κ B α SR, and NEMO-null or NEMO-WT Jurkat T cells transfected with I κ B α SR were treated with the indicated doses of TNF for 4, 8, and 24 h. Cell death was analyzed by staining with PE-conjugated annexin V. The experiments shown are representative of three experiments that were conducted. (d) Cell death was analyzed as in (a) after stimulation with 10 ng/ml of TNF for 4, 8, and 24 h. Each bar indicates the mean $\pm$ S.D. of quintuplicate samples treated and analyzed independently. *t*-Test was used to calculate *P* values, and significance was determined at *P* < 0.001. (e) Cells were treated with 5 ng/ml of TNF for the indicated times. Cell death was measured by staining with PE-conjugated annexin V

3T8/ $\text{I}\kappa\text{B}\alpha\text{SR}$ cell lines are both devoid of NF- κ B signaling, the greater sensitivity of the NEMO-deficient 8321 cell line to death suggested that NEMO may provide additional prosurvival signals independent of the NF- κ B signal.

To test the above hypothesis, the 8321 cell line was reconstituted with either a control protein (designated NEMO-null) or wild-type NEMO (designated NEMO-WT). The two cell lines were further transfected with $\text{I}\kappa\text{B}\alpha\text{SR}$ (designated NEMO-null/ $\text{I}\kappa\text{B}\alpha\text{SR}$ and NEMO-WT/ $\text{I}\kappa\text{B}\alpha\text{SR}$) to test the NF- κ B-independent prosurvival function of NEMO. Wild-type Jurkat T cells expressing bacterial GST (3T8/Con) or $\text{I}\kappa\text{B}\alpha\text{SR}$ (3T8/ $\text{I}\kappa\text{B}\alpha\text{SR}$) were included in the analysis as controls. Western blot analysis was performed to confirm that there was equivalent expression of NEMO in the 3T8/Con, 3T8/ $\text{I}\kappa\text{B}\alpha\text{SR}$, and NEMO-WT/ $\text{I}\kappa\text{B}\alpha\text{SR}$ cell lines (Figure 1b). All these cell lines also possess an integrated NF- κ B-Thy1 reporter, which allowed us to verify the ability of $\text{I}\kappa\text{B}\alpha\text{SR}$ to block NF- κ B-mediated gene transcription. On TNF treatment, 3T8/Con expressed Thy1 as a result of NF- κ B activation, whereas the three cell lines transfected with $\text{I}\kappa\text{B}\alpha\text{SR}$ did not express Thy1 (Supplementary Figure 1), thus confirming that $\text{I}\kappa\text{B}\alpha\text{SR}$ does indeed block the NF- κ B pathway. These cell lines were treated with increasing concentrations of TNF for 4, 8, and 24 h, and cell death was examined by annexin V staining. Both the 3T8/ $\text{I}\kappa\text{B}\alpha\text{SR}$ and NEMO-WT/ $\text{I}\kappa\text{B}\alpha\text{SR}$ cell lines exhibited enhanced sensitivity to TNF-induced cell death when compared to 3T8/Con (Figure 1c). The effect of NF- κ B blockade on cell death was minimal at 4 h of TNF treatment and it became more discernable at 8 and 24 h. However, what was striking at the 4 h time point was the noticeably enhanced cell death in NEMO-null/ $\text{I}\kappa\text{B}\alpha\text{SR}$ cells compared to NEMO-WT/ $\text{I}\kappa\text{B}\alpha\text{SR}$ cells (Figure 1c and d). This dose-dependent enhancement in cell death was observed at 4 h, and persisted for up to 24 h (Figure 1c and d). To further confirm these results, the NEMO-null/ $\text{I}\kappa\text{B}\alpha\text{SR}$ and NEMO-WT/ $\text{I}\kappa\text{B}\alpha\text{SR}$ cell lines were treated with TNF for varying lengths of time up to 24 h. NEMO-null/ $\text{I}\kappa\text{B}\alpha\text{SR}$ cells undergo more rapid TNF-induced cell death than NEMO-WT/ $\text{I}\kappa\text{B}\alpha\text{SR}$ cells (Figure 1e). As the two cell lines were both transfected with $\text{I}\kappa\text{B}\alpha\text{SR}$ and thus deficient in NF- κ B-mediated gene transcription, we conclude that NEMO inhibits TNF-induced cell death independently of NF- κ B. Furthermore, the NEMO-deficient cells displayed faster kinetics in their cell death compared to

'NF- κ B-deficient' cells. These results suggest that in addition to its prosurvival function via the induction of NF- κ B-dependent gene transcription, NEMO exerts another inhibitory effect on cell death during TNF signaling before NF- κ B-dependent gene transcription.

NEMO-deficient cells undergo more caspase activation than NEMO-sufficient cells. We next examined whether the effect of NEMO deficiency on cell death was due to enhanced activation of the caspase cascade. NEMO-null/ $\text{I}\kappa\text{B}\alpha\text{SR}$ and NEMO-WT/ $\text{I}\kappa\text{B}\alpha\text{SR}$ cell lines were treated with varying doses of TNF for 2 and 4 h. Western blot analysis of the cleaved forms of CASPASE-8 (p43/p41 and p18 fragments), CASPASE-3 (p17/p19 fragments), and PARP revealed that NEMO-null/ $\text{I}\kappa\text{B}\alpha\text{SR}$ cells exhibited greater activation of the caspase cascade when compared to the NEMO-WT/ $\text{I}\kappa\text{B}\alpha\text{SR}$ cell line (Figure 2a and b). A time-course experiment also revealed a more rapid increase in CASPASE-8, CASPASE-3, and PARP cleavage in NEMO-null/ $\text{I}\kappa\text{B}\alpha\text{SR}$ cells (Figure 2c). These results indicate that an increase in caspase activation is responsible for the enhancement in cell death observed in NEMO-deficient Jurkat T cells.

TNF-induced apoptosis in NEMO-deficient cells is dependent on RIP1. We next determined the NF- κ B-independent mechanism that could account for the enhanced cell death in the NEMO-deficient cells. NEMO can bind directly to RIP1 through at least two different domains. First, NEMO can bind constitutively to RIP1 as evidenced by their interactions in yeast two-hybrid analysis^{8,9} and this occurs via a pseudo- α -helix present in residues 194–260 of NEMO.¹⁰ Second, NEMO was recently shown to further bind to RIP1 that has undergone K63-linked polyubiquitination after TNF stimulation^{11,12} via an ubiquitin-binding domain present on residues 292–322 of NEMO. We also found that RIP1 serves as a potent death signaling molecule in TNFR1 signaling if it is not ubiquitinated because it is able to bind and rapidly activate CASPASE-8.¹³ Based on these observations, we hypothesized that one function of NEMO may be to 'restrain' RIP1 from engaging CASPASE-8 and, therefore, in NEMO-deficient cells RIP1 is not restrained and it is able to rapidly activate the caspase cascade. This

Figure 2 NEMO-deficient cells undergo more caspase activation than NEMO-sufficient cells. (a, b) NEMO-null/ $\text{I}\kappa\text{B}\alpha\text{SR}$ or NEMO-WT/ $\text{I}\kappa\text{B}\alpha\text{SR}$ cells were treated with indicated doses of TNF for 2 and 4 h (a and b, respectively). SDS-soluble cell extracts were subject to western blot analysis using antibodies specific to CASPASE-8, CASPASE-3, PARP, and ZAP70 (loading control). The experiments shown are representative of three experiments that were conducted. (c) Cell lines were treated with 2 ng/ml of TNF for the indicated times. SDS-soluble cell extracts were subject to western blot analysis using antibodies specific to CASPASE-8, CASPASE-3, PARP, and ZAP70. The experiments shown are representative of three experiments that were conducted

Figure 3 TNF-induced cell death in NEMO-deficient cells requires RIP1. **(a)** 3T8/lkB α SR and NEMO-null/lkB α SR cells were infected with retroviruses generated with pSUPERretro-GFP vector encoding shRNA directed against nonsilencing target (si-NS) or RIP1 (si-RIP1). Transduced cells were selected by cell sorting for high GFP expression. Knockdown of RIP1 expression was determined by western blot analysis for RIP1. Anti-ZAP70 blotting was performed as a loading control. **(b)** The four cell lines used in **(a)** were treated with the indicated doses of TNF for 4, 8, and 24 h. Cell death was measured by staining with PE-conjugated annexin V and FACS analysis. The experiments shown are representative of three experiments that were conducted. **(c)** Cell death was analyzed as in **(b)** after stimulation with 10 ng/ml of TNF for 4, 8, and 24 h. Each bar represents the mean $\pm$ S.D. of quintuplicate samples treated and analyzed independently. *P* values < 0.001 are indicated

hypothesis predicts that TNF-mediated apoptosis in the NEMO-deficient cells is RIP1-dependent whereas in NEMO-sufficient cells, RIP1 is not involved in apoptosis. To test this, we knocked down RIP1 expression in NEMO-null/lkB α SR cell lines using a vector encoding a nonsilencing short hairpin RNA (si-NS) or one targeting RIP1 (si-RIP1). The efficiency of RIP1 knockdown was confirmed by western blot analysis (Figure 3a). Consistent with our hypothesis, there is significantly less apoptosis in NEMO-null/lkB α SR cells on RIP1 knockdown (Figure 3b, open circles *versus* closed circles; Figure 3c, dark gray bars *versus* black bars). These results demonstrate that TNF-induced apoptosis in NEMO-deficient cells requires RIP1. We also tested the effect of RIP1 knockdown on TNF-induced apoptosis in the 3T8/lkB α SR cells. Despite a similar reduction in RIP1 expression (Figure 3a), RIP1 knockdown had no significant effect on apoptosis in the 3T8/lkB α SR cells (Figure 3b, open squares *versus* closed squares; 3C, white bars *versus* light gray bars). Thus as predicted by our hypothesis, cells with wild-type expression of NEMO that are rendered sensitive to TNF-induced apoptosis by NF- κ B blockade (i.e., 'NF- κ B-deficient' cells) do not require RIP1 to undergo apoptosis. In contrast, NEMO-deficient cells require RIP1 to undergo TNF-induced apoptosis. This demonstrates that the TNF cell-death pathway is mechanistically different in cells that are 'NF- κ B-deficient' *versus* cells that are NEMO-deficient.

Gene knockout studies have shown the Fas signaling adapter Fadd to be critical in apoptosis in mouse fibroblasts triggered by a combination of TNF and cycloheximide.¹⁴ To examine whether FADD plays a role in the TNF-induced cell death observed in NEMO-deficient cells, we knocked down FADD expression in NEMO-null/lkB α SR cells using a lentiviral vector encoding a short hairpin RNA targeting FADD

(si-FADD) and as a negative control, a nonsilencing hairpin (si-NS). The efficiency of FADD silencing was confirmed by western blot analysis (Supplementary Figure 2A). Silencing of FADD had no effect on TNF-induced apoptosis in the NEMO-null/lkB α SR cells. As a positive control, we examined FAS-mediated apoptosis in the same cells. As expected, FADD silencing resulted in decreased sensitivity to FAS-induced cell death demonstrating the effectiveness of the FADD silencing (Supplementary Figure 2B and C). These results suggest that FADD has no role in TNFR1-mediated apoptosis in the NEMO-deficient cells. Recent studies have also reported that FADD knockdown had no effect on cell death caused by TNF plus cycloheximide¹⁵ or TNF plus SMAC mimetics.¹⁶

NEMO requires its ubiquitin-binding domain for its NF- κ B-independent antiapoptotic function. Recent studies have demonstrated that NEMO contains an ubiquitin-binding domain that is able to bind to K63-linked polyubiquitin chains on RIP1.^{11,12} Another prediction of the model that NEMO restrains RIP1 is that mutations in NEMO that compromise its binding to ubiquitinated RIP1 will also lead to enhanced sensitivity to apoptosis in an NF- κ B-independent manner. To test this prediction, we generated three FLAG-tagged NEMO constructs that contain three substitution mutations (NEMO-Y308S, NEMO-F312A, NEMO-L329P) within the ubiquitin-binding domain that have been shown to abrogate the ability of NEMO to bind to K63-linked polyubiquitin chains.^{11,12} NEMO-deficient Jurkat T cells that stably express lkB α SR were then reconstituted with a control protein, FLAG NEMO-WT, FLAG NEMO-Y308S, FLAG NEMO-F312A, and FLAG NEMO-L329P. Expression of wild-type NEMO and its mutations were confirmed by western blot analysis (Figure 4a).

Figure 4 NEMO requires its ubiquitin-binding domain for its NF- κ B-independent antiapoptotic function. (a) Cell lysates from NEMO-null/lκBαSR, NEMO-WT/lκBαSR, NEMO-Y308S/lκBαSR, NEMO-F312A/lκBαSR, NEMO-L329P/lκBαSR cells were subject to western blot analysis using anti-NEMO followed by anti-ZAP70 as a loading control. (b) Cell lines from (a) were treated with 5 or 10 ng/ml of TNF for 4, 8, and 24 h. Cell death was measured by staining with PE-conjugated annexin V and FACS analysis. Each bar represents the mean $\pm$ S.D. of triplicate samples treated and analyzed independently. *t*-Test was used to calculate *P* values and asterisks indicate *P* < 0.01 when compared to values from the NEMO-WT/lκBαSR cell line. (c) Cell lines from (a) were treated with 100 ng/ml of TNF for 15 min. FLAG-tagged wild-type NEMO or NEMO mutants were immunoprecipitated from total cell lysates using anti-FLAG M2 affinity beads. The immunoprecipitates were sequentially blotted with anti-RIP1 (first and second panels) and anti-FLAG (third panel) antibodies. Blotting with the two antibodies on 1% of the total cell extracts are shown in the bottom two panels. The experiment shown is representative of three experiments that were conducted

The absence of NF- κ B-mediated gene transcription in all cell lines was confirmed by detection of an NF- κ B-Thy1 reporter (Supplementary Figure 3). We treated the NEMO-null/lκBαSR, NEMO-WT/lκBαSR, NEMO-Y308S/lκBαSR, NEMO-F312A/lκBαSR, and NEMO-L329P/lκBαSR cell lines with 5 and 10 ng/ml of TNF for 4, 8, and 24 h. As observed previously, reconstitution with NEMO-WT protected cells from TNF-induced apoptosis, independent of NF- κ B (Figure 4b). NEMO-F312A/lκBαSR and NEMO-L329P/lκBαSR exhibited slightly more cell death at 4 h of TNF treatment, as compared to NEMO-WT/lκBαSR (Figure 4b). At 8 and 24 h of TNF treatment, cell lines expressing the three NEMO substitution mutations exhibited significantly more death than cells expressing NEMO-WT (Figure 4b). Thus, as predicted, substitution mutations in the ubiquitin-binding region of NEMO enhanced sensitivity to TNF-induced apoptosis in cells devoid of NF- κ B-mediated transcription. We then confirmed that the NEMO point mutants were indeed unable to associate with ubiquitinated RIP1 on TNFR1 engagement in the context of lκBαSR expression. Consistent with published data,^{11,12} only NEMO-WT was able to associate with ubiquitinated RIP1 in TNF-stimulated cells (Figure 4c, lane 4). Therefore, the inability to associate with ubiquitinated RIP1 in the NEMO substitution mutants correlated with enhanced apoptosis. Although all three point mutations within the ubiquitin-binding domain abrogated NEMO's interaction with ubiquitinated RIP1 to a similar degree (Figure 4c), there appears to be some small differences in their ability to block cell death (Figure 4b). It is

unclear why we did not observe a complete correlation between binding to ubiquitinated RIP1 and blockade of cell death. One possibility could be that the cell-death assay is more sensitive and quantitative than the 'RIP1-laddering' analysis in the NEMO-RIP1 co-immunoprecipitations. Therefore, subtle differences in these mutants may not be discernable by the 'RIP1-laddering' assay in Figure 4c. Alternatively, the three mutations may have different affects on binding to additional partner proteins involved in the regulation of this response that have yet to be characterized. NEMO mutants defective in ubiquitin binding were still able to associate with unmodified RIP1 (Figures 4c, lanes 3–10), consistent with reports that NEMO and RIP1 can also associate independently of ubiquitination as evidenced by their ability to associate in yeast two-hybrid analysis.^{8,9} This ubiquitin-independent association could account for the faster kinetics of cell death in the NEMO-null cells compared to cells reconstituted with the NEMO ubiquitin-binding mutants (Figure 4b). Taken together, these results support the model that NEMO sequesters and restrains RIP1 from activating caspases.

NEMO inhibits apoptosis by restraining RIP1 from associating with CASPASE-8. Another prediction of the model is that in NEMO-sufficient cells, RIP1 will not form a signaling complex with CASPASE-8 whereas in NEMO-deficient cells, RIP1 rapidly associates with CASPASE-8. Lysates from NEMO-null and NEMO-WT cells treated with TNF for 1 and 2 h were subjected to immunoprecipitation

using an antibody to RIP1 followed by blotting with an antibody that recognizes active CASPASE-8. We used the rabbit monoclonal 18C8, which only recognizes active cleaved CASPASE-8 migrating at 43/41 and 18 kD, because PROCASPASE-8 migrated at the same mobility as the immunoglobulin heavy chain from the RIP1 immunoprecipitates. The presence of the heavy chain precluded us from detecting the uncleaved PROCASPASE-8 in western blots. As predicted, we detected RIP1 association with active CASPASE-8 in NEMO-null cells but not in the NEMO-WT cells (Figure 5a). As the experiment shown in Figure 5a was conducted using cells without NF- κ B blockade, we repeated the same experiment in cells that were also transfected with I κ B α SR to confirm that the effect of NEMO on the interaction between RIP1 and CASPASE-8 was indeed NF- κ B-independent. As shown in Figure 5b, the result from cells transfected with I κ B α SR was similar to that observed in cells that were not transfected with I κ B α SR. There was more active CASPASE-8 associated with RIP1 in the NEMO-null/I κ B α SR cells compared to the NEMO-WT/I κ B α SR cells. This demonstrates that NEMO inhibits the association of RIP1 and CASPASE-8 independent of its role in NF- κ B. The RIP1 immunoprecipitates were reprobed with anti-FADD to examine whether NEMO had any effect on the association of RIP1 and FADD. Interestingly, whereas there were no discernable changes in RIP1–FADD interaction in response to TNF stimulation in the NEMO-null/I κ B α SR cells, FADD appeared to disassociate from RIP1 in the NEMO-WT/I κ B α SR cells. This suggests that the absence of NEMO stabilizes the RIP1–FADD interaction and the resultant higher level of FADD in the complex may potentially contribute toward enhancing the interaction between RIP1 and CASPASE-8. Nonetheless, the fact that FADD knockdown had no effect on TNF-induced apoptosis in the NEMO-deficient cells (Supplementary Figure 2) argues against a significant role for FADD in this pathway.

Finally, we tested whether NEMO can function to inhibit the interaction between RIP1 and CASPASE-8 in cotransfection experiments. Equivalent amounts of FLAG-RIP1 and HA-CASPASE-8 were cotransfected into 293EBNA cells in the presence of increasing amounts of NEMO or the negative control GST. RIP1 was immunoprecipitated using anti-FLAG mAb, eluted with the FLAG peptide, and blotted for the presence of HA-CASPASE-8. As shown in Figure 5c, the presence of NEMO reduced the amount of CASPASE-8 associated with RIP1 suggesting that NEMO can disrupt the association of RIP1 with CASPASE-8. This observation provides further evidence to support the model that NEMO acts to restrain RIP1 from associating with CASPASE-8, thus preventing cell death.

Discussion

NEMO-deficient cells are sensitive to TNF-induced apoptosis^{4–6} and as NEMO has been shown to be necessary for NF- κ B activation,^{3,17} it has been assumed that NEMO functioned as an antiapoptotic molecule due to its role in the NF- κ B signaling pathway. However, this study demonstrates an additional, novel role for NEMO in blocking cell death independent of its role in NF- κ B signaling whereby NEMO

restrains RIP1 from engaging CASPASE-8 before the induction of prosurvival genes. In addition to its well-characterized role in TNFR1-to-NF- κ B signaling,^{11,13,18–20} RIP1 is also known to be a potent cell-death inducer.^{21,22} Therefore, it is likely that cells expend considerable efforts to rein in the apoptosis-inducing behavior of RIP1 when death is not the desired outcome. Based on this study and our previous study,¹³ at the minimum, this consists of ubiquitination of RIP1 and sequestration of RIP1 by NEMO away from CASPASE-8. It is likely that E3 ligases such as TRAF2¹³ and CIAPs^{16,23} and deubiquitinases such as CYLD¹⁶ may have a role in regulating this process.

Although the focus in recent years has been on NF- κ B as the determinant of survival *versus* death following TNF stimulation, our molecular characterization of an early cell-death checkpoint that is NF- κ B-independent suggests that the current model of TNF signaling should be revised to incorporate this new understanding. We, therefore, propose that there are two cell-death checkpoints during TNF signaling. An early checkpoint occurs when RIP1 undergoes ubiquitination mediated by TRAF2 or c-IAPs^{16,23,24} and this enhances its association with NEMO and restrains RIP1 from engaging CASPASE-8 (Figure 5d). The main function of this first checkpoint is to ensure that RIP1 does not trigger the caspase cascade. At the same time, formation of the RIP1–NEMO complex also leads to IKK activation and this leads to a later cell-death checkpoint in which NF- κ B now induces prosurvival gene expression, which now provides for a long lasting protection from cell death. At the first cell-death checkpoint, if ubiquitination of RIP1 is prevented or if a regulatory component such as NEMO is absent, RIP1 is now free to engage CASPASE-8 and death rapidly ensues (Figure 5e). This model, which shows that there are two cell-death checkpoints during TNF signaling provides an explanation for the paradoxical observations that the TNF death signaling machinery is preexisting in cells whereas the survival response is dependent on transcription, yet cells are largely resistant to TNF-induced cell death. Soon after TNFR1 is ligated, RIP1 is rapidly ubiquitinated and sequestered from preexisting components of the death pathway such as CASPASE-8. This transcription-independent event then allows sufficient time for the NF- κ B transcription machinery to induce the array of prosurvival genes necessary to permanently disable the cell-death pathway. Conversely, if the desired biological outcome is death, disabling the first cell-death checkpoint will render cells susceptible to rapid TNF-mediated cell death. Physiologically, this may be achieved by altering the levels of ubiquitin-modifying enzymes of RIP1 including E3 ligases and deubiquitinases. These ubiquitin-modifying enzymes, as well as nonenzymatic molecules such as NEMO that regulate the interaction of RIP1 with the caspase pathway, are attractive targets for pharmacological modulation. In this regard, two recent studies reported that SMAC mimetics, which cause the autodegradation of CIAP1 and CIAP2, render tumor cell lines sensitive to TNF-mediated apoptosis through a RIP1-dependent manner.^{16,23} In the absence of both CIAP1 and CIAP2, RIP1 does not undergo ubiquitination and converts to a death signaling molecule, an effect analogous to the loss of the ubiquitin acceptor site lysine 377 on RIP1 we previously described.¹³ Therefore, it seems

Figure 5 NEMO inhibits RIP1 from associating with CASPASE-8. (a) NEMO-null and NEMO-WT Jurkat T cells were treated with 25 ng/ml TNF for 1 and 2 h. RIP1 was immunoprecipitated from the lysates using anti-RIP1 antibody. The protein complexes were eluted and subject to sequential western blot analysis using anticlaved CASPASE-8 and anti-RIP1 antibodies. (b) NEMO-null/I κ B α SR and NEMO-WT/I κ B α SR cells were treated as in panel (a). Blotting was performed sequentially with anticlaved CASPASE-8, anti-FADD, and anti-RIP1 antibodies. (c) 293EBNA cells were transfected with FLAG-RIP1, and HA-CASPASE-8 in the presence of increasing amounts of myc-NEMO or the negative control GST. Anti-FLAG immunoprecipitations were eluted with the FLAG peptide and sequentially blotted with anti-HA, anti-myc, and anti-FLAG. The intensities of the CASPASE-8 bands were determined by densitometry and normalized to the corresponding RIP1 band. The ratios of the intensity in the different samples are presented relative to the ratio in the sample with no GST or NEMO. (d) Model of TNFR1 signaling showing two cell-death checkpoints. On ligation of TNFR1, ubiquitination of RIP1 occurs rapidly leading to its association with NEMO and other components of the IKK signalosome. This sequesters RIP1 away from CASPASE-8 and serves as the early transcription-independent cell-death checkpoint. The formation of the IKK signalosome subsequently leads to the induction of NF- κ B-dependent antiapoptotic genes and this serves as a second cell-death checkpoint, which provides for a long-lasting genetically programmed protection from death. (e) If the early cell-death checkpoint fails, such as when ubiquitination of RIP1 is prevented or if a regulatory component such as NEMO is absent, RIP1 can engage CASPASE-8 and death rapidly ensues. This early NF- κ B-independent cell-death checkpoint serves to determine whether the survival or death pathway is triggered following TNFR1 ligation

likely that the loss of K377 on RIP1,¹³ inhibition of TRAF2,¹³ loss of CIAP1 and CIAP2 caused by SMAC mimetics,^{16,23} and the loss of NEMO described in this study all function to disrupt the early NF- κ B-independent cell-death checkpoint in TNF signaling.

It is very likely that disruption of the first cell-death checkpoint generates a qualitatively and quantitatively different response to TNF-induced cell death when compared to disruption of the second cell-death checkpoint. Death sensitivity conferred by the loss of the first checkpoint is dependent on RIP1 and is a direct effect of the formation of the signaling complex between RIP1 and CASPASE-8 (Figure 3b, open circles *versus* closed circles). In contrast, death sensitivity conferred by loss of the second checkpoint does not require RIP1 and is an indirect effect caused by the loss of caspase inhibitors such as c-FLIP secondary to the loss of NF- κ B (Figure 3b, open squares *versus* closed squares). The faster kinetics and greater magnitude of death in the NEMO-deficient cells, which has a disruption in both the first and second checkpoint, as compared to the I κ B α SR-transfected cells, which has a disruption in the second checkpoint, indicated that disruption of the first checkpoint generates a more potent death signal.

The observation that nonubiquitinated RIP1 forms a complex with CASPASE-8 to trigger apoptosis^{13,16,23} raises a number of questions. For instance, a vast proportion of RIP1 in wild-type cells does not undergo ubiquitination after TNF stimulation and yet they do not associate with CASPASE-8 to trigger apoptosis. Therefore, it is likely that preventative mechanisms must be in place in quiescent cells to limit these potentially deadly associations from occurring. These preventative mechanisms are poorly understood and may involve (1) keeping the localization of these molecules apart, (2) stimulus-dependent posttranslational modifications needed for the associations to occur, and (3) inhibitors bound to RIP1 or CASPASE-8 that prevent them from interacting. It is potentially interesting that NEMO and RIP1 can interact with each other in yeast^{8,9} and in nonstimulated cells (see Figure 4c) leading to the speculation that under basal condition, binding of NEMO to RIP1 may serve to sequester RIP1 from CASPASE-8. The observation in Figure 5c that transfection of NEMO displaces CASPASE-8 from RIP1 is also consistent with this speculation. Future experiments will be directed at testing which of these preventative mechanisms may be involved in the TNF pathway. Exactly how NEMO binding to ubiquitinated RIP1 prevents RIP1 from associating with CASPASE-8 remains to be discerned. Binding of ubiquitinated RIP1 to NEMO may result in RIP1's localization to a cellular compartment that is not accessible to CASPASE-8. In addition, NEMO binding to ubiquitinated RIP1 may also serve to stabilize this posttranslational modification by preventing deubiquitinases such as A20 and CYLD from gaining access to the polyubiquitin chains on RIP1. Whether the attachment or removal of polyubiquitin chains on RIP1 is altered in the NEMO-deficient cells is unclear at the moment. It is possible that in the NEMO-deficient cells, ubiquitination of RIP1 is normal but due to the lack of binding to NEMO, the ubiquitinated RIP1 can now form a complex with CASPASE-8. These possibilities will be tested in the future.

We also examined whether FADD had any role in the cell-death process following disruption of the first cell-death checkpoint caused by the loss of NEMO. Although the loss of NEMO appeared to stabilize the RIP1–FADD interaction (Figure 5b), suggesting FADD may play a role here, knocking down FADD had no effect on the overall cell death triggered by TNF (Supplementary Figure 2) arguing against a role for FADD. Nonetheless, we are unable to rule out that in the absence of FADD, there may be a qualitative change in the cell death that is occurring. Harper *et al.*²⁵ previously reported that FADD deficiency in Jurkat T cells led to increased TNF-mediated necrosis. It is possible that in our NEMO-deficient cells, FADD silencing shifted the death response from apoptosis to necrosis, which we are unable to distinguish by annexin V staining. We are currently exploring whether nonapoptotic cell death may occur in the NEMO-deficient cells and whether FADD and RIP1 have any role in it.

Although this study demonstrates that NEMO plays a prosurvival role in TNF signaling through multiple mechanisms, there is one intriguing observation that suggests perhaps NEMO may have a proapoptotic role as well. In cells that are RIP1 deficient and NF- κ B deficient, the absence of NEMO leads to less cell death (Figure 3b, black squares *versus* black circles) suggesting that under these conditions, NEMO is proapoptotic. The biological relevance of this putative proapoptotic function of NEMO is unclear at the moment because there are currently no reports of RIP1 deficiency in cells other than that achieved by experimental mutagenesis or knockdown. One possibility may be that because RIP1 and NEMO are binding partners, the loss of RIP1 may allow NEMO to now bind to another molecule that is strongly proapoptotic. Future studies will be directed at examining this putative proapoptotic function of NEMO.

TNF is a dual-functioning molecule in that it can promote cell survival or cell death. Cells are able to fine-tune the balance between these two opposing responses to generate the appropriate response. Understanding the mechanism underlying this balance between survival and death may suggest strategies to manipulate this balance for therapeutic purposes in diseases where TNF may have a role such as cancer, inflammatory bowel disease, and rheumatoid arthritis. Our results suggest that disrupting the NEMO–RIP1 interaction, that is, the first cell-death checkpoint in the TNFR1 pathway, may be one such potential strategy.

Materials and Methods

Cell lines. The wild-type and NEMO/IKK γ -deficient Jurkat T cell lines have been described previously.⁵ Cells were maintained in IMDM medium (Sigma-Aldrich) supplemented with 10% bovine calf serum (Hyclone), 50 mM 2-mercaptoethanol (Sigma-Aldrich), 2 mM L-glutamine (Cellgro), and 15 μ g/ml gentamicin. Retroviral transduction of Jurkat T cells was performed as previously described.⁵

Plasmids. I κ B α SR, which contains a serine to alanine substitution mutation at residues 32 and 36, was subcloned into a retroviral expression vector containing an internal ribosome entry site (IRES)-puromycin resistance cassette. Bacterial glutathione S-transferase (GST) cloned into the same retroviral vector was used as a negative control. Bulk populations of puromycin-resistant cells were used in all experiments. Wild-type NEMO/IKK γ containing a FLAG-tag at the N terminus was subcloned into the retroviral expression vector. NEMO substitution mutations tyrosine to serine at residue 308 (NEMO-Y308S), phenylalanine to alanine at

residue 312 (NEMO-F312A), and leucine to proline at residue 329 (NEMO-L329P) were generated using Stratagene Quikchange XL site-directed mutagenesis kit.

RNA interference. ShRNA-mediated gene silencing of RIP1 was performed by using the retroviral expression vector pSUPERretro (Oligoengine) that was modified so that the puromycin-resistance cassette was replaced by green fluorescent protein (GFP). Selection for retrovirus-infected cells expressing high levels of short-hairpin RNA (shRNA) was achieved by sorting for cells with high GFP expression using FACSVantage Cell Sorter (BD Biosciences). The RIP1-specific insert consisted of a 19-nt sequence (GGAGCAAAGTGAATAATGA) separated by a noncomplementary spacer from the reverse complement of the same 19-nt sequence to form the shRNA duplex (si-RIP1). Vector containing a nonsilencing shRNA (TTCTCCGAACGTGTCACGT) was used as a control (si-NS).

ShRNA-mediated gene silencing of FADD was performed using the lentivirus vector pLKO.1 containing scramble shRNA (scramble; Addgene Inc., Cambridge, MA, USA) or shRNA targeting FADD (si-FADD) (Sigma-Aldrich). Lentiviral transduction of Jurkat T cells were performed using VSV-G and psPAX2 (Addgene Inc.) expression plasmids for packaging the lentiviral particles and viral infection carried out as previously described for retrovirus.⁵ Bulk population of drug-resistant cells was used in experiments to avoid clonal variability.

Immunoblotting. Cells were treated as indicated, pelleted, and lysed in buffer containing 20 mM Tris pH 7.5, 40 mM sodium chloride, 5 mM EDTA, 50 mM sodium fluoride, 30 mM sodium pyrophosphate, 1% Triton X-100, and Protease Inhibitor Cocktail Set V (EMD Biosciences Inc.) for 10 min on ice. Lysates were clarified by centrifugation at $10\,000 \times g$ at 4°C. Extracts were resolved by SDS-PAGE, transferred onto nitrocellulose membrane, and probed with the indicated antibody and the appropriate secondary antibody conjugated to horseradish peroxidase (Dako Cytomation). Anti-NEMO/IKK γ was obtained from Santa Cruz Biotechnology. Anti-RIP1 antibody was obtained from BD Biosciences. ZAP70 antibody was a generous gift from Dr. Paul Leibson. To analyze the apoptosis pathway, cells were treated with human TNF α (R&D Systems) as indicated. Cells were washed with PBS and lysed in 0.5% SDS by vortexing and boiling for 5 min. Lysates were resolved by SDS-PAGE, transferred onto nitrocellulose membrane, and probed with the indicated antibodies. Caspase 8, cleaved-caspase3, and PARP antibodies were obtained from Cell Signaling Technology.

Flow cytometry. Cells were treated with human TNF α as indicated and stained with either phycoerythrin (PE)-conjugated annexin V (BD Biosciences), or anti-Thy1 (Sera Laboratories) followed by PE-conjugated goat anti-mouse Ig (BD Biosciences). Analysis was performed using a FACScan or FACScalibur flow cytometer and Cell Quest software (Becton Dickinson).

Immunoprecipitation. For FLAG immunoprecipitation, cells were stimulated with TNF as indicated and lysed in buffer containing 20 mM Tris pH 7.5, 150 mM sodium chloride, 1 mM EDTA, 30 mM sodium fluoride, 2 mM sodium pyrophosphate, 5 mM N-ethylmaleimide, 1% Triton X-100, and Protease Inhibitor Cocktail Set V for 10 min on ice.¹² Lysates were clarified by centrifugation at $10\,000 \times g$ at 4°C and precleared with albumin-agarose beads (Sigma-Aldrich) with agitation for 1 h at 4°C. The beads were removed by centrifugation at $8000 \times g$ for 30 s and the lysate was incubated with anti-FLAG M2 agarose affinity beads (Sigma-Aldrich) with agitation for 2 h at 4°C. Beads were collected by centrifugation at $8000 \times g$ for 30 s and washed five times with lysis buffer containing 500 mM NaCl. Protein was eluted from beads by competition with 300 μ g/ml of FLAG peptide (Sigma-Aldrich) in sample buffer. Lysates were resolved by SDS-PAGE and analyzed using anti-RIP1 and anti-FLAG (Sigma-Aldrich) antibodies. RIP1 immunoprecipitation was performed as previously described, with modifications.¹³ Briefly, cells were stimulated with TNF as indicated and lysed in 0.2% NP-40 lysis buffer (20 mM Tris-HCl pH 7.4, 150 mM sodium chloride, 0.2% NP-40, 10% glycerol, Protease Inhibitor Cocktail Set V, 1 ml/ml BSA) for 15 min on ice. Lysates were clarified by centrifugation at $10\,000 \times g$ at 4°C and incubated with albumin-agarose beads (Sigma-Aldrich) with agitation for 1 h at 4°C. The beads were removed by centrifugation at $8000 \times g$ for 30 s, and the lysate was incubated with 2 μ g of anti-RIP1 antibody with agitation for 4 h at 4°C followed by incubation with protein A/G agarose beads (Santa Cruz Biotechnology) for 1 h at 4°C. Beads were washed five times with lysis buffer, and proteins were eluted from the beads at 75°C for 10 min. Samples were resolved by SDS-PAGE and analyzed by immunoblotting using a rabbit mAb clone 18C8 that recognizes only cleaved CASPASE-8 (Cell Signaling Technology), anti-FADD (BD Biosciences), and anti-RIP1 antibodies.

Coexpression and coimmunoprecipitation. 293EBNA cells were transfected with 2 μ g each of plasmids encoding FLAG-RIP1 and HA-CASPASE-8 (kindly provided by Dr. Shahrooz Rabizadeh, Buck Institute for Age Research) in the presence of 0.02, 0.1, and 0.5 μ g of myc-NEMO or the negative control GST. zVAD-FMK (10 μ M) was added to the transfectants to prevent cell death. Twenty-four hours after transfection, cells were lysed in buffer containing 20 mM Tris pH 7.5, 150 mM sodium chloride, 5 mM EDTA, 50 mM sodium fluoride, 30 mM sodium pyrophosphate, 1% Triton X-100, and Protease Inhibitor Cocktail Set V for 10 min on ice. Lysates were clarified by centrifugation at $10\,000 \times g$ at 4°C. FLAG immunoprecipitation was performed as described earlier. Briefly, lysates were precleared with albumin-agarose beads and then incubated with anti-FLAG M2 agarose affinity beads. Protein was eluted from the beads by competition with 300 μ g/ml of FLAG peptide. Lysates were resolved by SDS-PAGE and analyzed by western blot using anti-HA (Roche Diagnostics), anti-c-Myc (Santa Cruz Biotechnology), and anti-FLAG (Sigma-Aldrich) antibodies. The intensities of the CASPASE-8 bands were quantified by densitometry analysis and normalized to the corresponding RIP1 bands using the software Quantity One (BioRad).

Statistical analysis. Data are presented as mean $\pm$ S.E.M. Comparisons between groups were made using Student's *t*-test.

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