Noxa/HSP27 complex delays degradation of ubiquitylated I κ B α in airway epithelial cells to reduce pulmonary inflammation

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IFN- γ is known as a pro-inflammatory cytokine, but can also block inflammation in certain chronic diseases although the underlying mechanisms are poorly understood. We found that IFN- γ rapidly induced Noxa expression and that extent of inflammation by repeated house dust mite exposure was enhanced in *noxa*^{-/-} compared with *noxa*^{+/+} mice. Noxa expression blocked transforming necrosis factor alpha (TNF- α)-induced nuclear translocation of nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) and the production of pro-inflammatory cytokines. Noxa did not affect TNF- α -induced I κ B α phosphorylation but the degradation of 48-chain-ubiquitylated I κ B α . The Cys25 of Noxa was cross-linked with Cys137 of phospho-HSP27 and both proteins were required for blocking the degradation of ubiquitylated I κ B α . Because phospho-HSP27 is present in airway epithelial cells and not in fibroblasts or thymocytes, we generated transgenic mice that inducibly expressed Noxa in airway epithelia. These mice showed protection from allergen-induced inflammation and mucous cell metaplasia by blocking nuclear translocation of NF- κ B. Further, we identified a Noxa-derived peptide that prolonged degradation of 48-chain-ubiquitylated I κ B α , blocked nuclear translocation of NF- κ B, and reduced allergen-induced inflammation in mice. These results suggest that the anti-inflammatory role of the Noxa protein may be restricted to airway epithelial cells and the use of Noxa for therapy of chronic lung diseases may be associated with reduced side effects.

INTRODUCTION

Interferon gamma (IFN- γ) is primarily produced by innate cells, including natural killer and natural killer T cells, non-cytotoxic innate lymphoid cells,¹ and adaptive immune cells, including CD4⁺ T helper (Th) 1 and CD8⁺ cytotoxic T lymphocytes cells.² IFN- γ activates macrophages to produce pro-inflammatory cytokines,³ but can also counteract inflammatory and immunostimulatory pathways.⁴ Transgenic mice engineered to express IFN- γ in airway epithelial cells were found to present with increased inflammation and emphysema⁵ but also with reduced eosinophilia and airway reactivity.⁶ However, because airway epithelial cells do not express IFN- γ ,

the findings in these transgenic mice may not be physiologically relevant.

For clinical applications, IFN- γ has beneficial effects when administered as prophylactic treatment of lupus nephritis⁷ and in cases of rheumatoid arthritis.⁸ IFN- γ signaling inhibits neutrophil accumulation in the lung⁹ or suppresses neutrophil production in the bone marrow.¹⁰ These anti-inflammatory responses are mediated by induction of cytokine decoy receptors, IL-1R α and IL-18BP.² Further, IFN- γ tempers the development of erythrocytes and eosinophilic granulocytes,¹¹ and inhibits Th2 cell function.¹²

Because chronic inflammation promotes chronic lung diseases,¹³ identifying the intracellular factors that mediate

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the anti-inflammatory action of IFN- γ can be of great importance. Our previous studies have shown that IFN- γ causes cell death in airway epithelial cells by activating STAT1,¹⁴ inducing expression of the BH3-only protein, Bik,¹⁵ and suppression of Bmf expression to induce autophagy.¹⁶ Bik and Bmf are Bcl-2 family members that share only the Bcl-2 homology region 3 (BH3) such as Noxa, Puma, Bim, Bid, Bad, Bid, Bnip-3L, and Hrk. This group of proteins is believed to sense cell death signals that emanate from internal stresses, such as DNA damage¹⁷ or CAP-independent translation¹⁸ or from external stimuli, such as IFN- γ ¹⁵ or other cytokines.¹⁹

We found that in airway epithelial cells IFN- γ dramatically induced the BH3-only protein, Noxa, but that Noxa did not induce cell death, yet inhibited inflammation in response to allergen. Noxa required the interaction with phosphorylated HSP27 via a covalent disulfide bond to suppress the nuclear translocation and activation of NF- κ B by inhibiting degradation of ubiquitylated I κ B α . Furthermore, inducible expression of Noxa in airway epithelia of adult mice suppressed allergen-induced inflammation *in vivo*. However, the cross-linking Cys25 was not necessary when the N-terminal peptide was used to block I κ B α degradation and inflammation. Therefore, these studies identify the anti-inflammatory mediator of IFN- γ to be Noxa cross-linked to the non-Bcl-2 protein, pHSP27 that renders N-terminal region of Noxa functional.

RESULTS

IFN- γ rapidly induces Noxa expression to suppress inflammation

Noxa mRNA levels were dramatically induced by 25–50 ng ml⁻¹ IFN- γ treatment over 24–48 h in primary human airway epithelial cells (HAECs) from non-diseased individuals (Supplementary Figure S1A online). Induction occurred within 1 h of IFN- γ treatment both for Noxa mRNA (Figure 1a), and protein levels (Figure 1b), while Hrk, Bad, or Puma showed no change in expression (data not shown). This rapid induction by IFN- γ was consistently observed in HAECs from 14 individuals (Figure 1c). As expected, Noxa protein and mRNA were undetectable in murine airway

epithelial cells (MAECs) from *noxa*^{-/-} mice but Noxa expression was increased by IFN- γ in wild-type MAECs (Supplementary Figure S1B). IFN- γ failed to upregulate Noxa expression in MAECs from *STAT1*^{-/-} mice (Supplementary Figure S1B). However, unlike the DNA damage-induced Noxa expression,²⁰ IFN- γ -induced Noxa expression was independent of p53 (Supplementary Figure S1C).

Noxa deficiency causes increased inflammation

While Noxa mRNA and protein levels were induced robustly by IFN- γ , *noxa*^{+/+} and *noxa*^{-/-} airway cells were both susceptible to IFN- γ -induced cell death (Supplementary Figure S1D). However, we observed that *noxa*^{-/-} compared with *noxa*^{+/+} mice developed increased inflammation following intranasal instillation with house dust mite allergen for 5 consecutive days. The numbers of total cells (Figure 1d) eosinophils and macrophages, but not neutrophils and lymphocytes (Figure 1e), recovered by bronchoalveolar lavage, were significantly higher in *noxa*^{-/-} compared with *noxa*^{+/+} mice. In addition, when sensitized with ovalbumin injection on days 1 and 7 and challenged with ovalbumin aerosols for 5 days, mucous cell numbers were higher (Supplementary Figure S2A) and inflammatory cells were more evident in *noxa*^{-/-} compared with *noxa*^{+/+} mice (Supplementary Figure S2B). These observations suggested that Noxa may play a role in blocking allergen-induced inflammation, but not in the cell death process of hyperplastic airway epithelial cells. Previous studies have shown that Noxa expression sensitizes cells to apoptosis.²¹ To determine whether Noxa sensitizes airway epithelial cells, we first treated HAECs with increasing H₂O₂ concentrations and found that 45 μ M affects cell viability slightly (Supplementary Figure S2C). However, when combined with increasing multiplicity of infection of Ad-Noxa, cell viability was progressively reduced compared to the Ad-GFP controls (Supplementary Figure S2D).

Because expression of many pro-inflammatory genes are regulated by NF- κ B including IL-8,²² we investigated whether Noxa suppresses the synthesis of inflammatory chemokines. The TNF α -induced IL-8 secretion from human airway

Figure 1 IFN- γ induces Noxa expression to inhibit inflammation. (a) AALB cells treated with IFN- γ or vehicle over 48 h and analyzed for Noxa mRNA by q-PCR. Data represented as the mean $\pm$ s.e.m. ($n = 3$ wells/for each time point and repeated at least three times). (b) Protein extracts from HAECs treated with IFN- γ or vehicle and Noxa protein levels analyzed by western blotting (representative of three experiments). (c) HAECs from 14 individuals treated with 50 ng ml⁻¹ IFN- γ and analyzed for Noxa mRNA levels by q-PCR. (d,e) The *noxa*^{+/+} and *noxa*^{-/-} mice challenged with HDM extracts (50 μ g) intranasally in 50 μ l PBS for 4 days ($n = 5$ mice per group) and the lung inflammatory cells quantified in the bronchoalveolar lavage (BAL) fluid collected 24 h later. BAL cell differentials quantified after staining with Wright Giemsa. The number of total cells (d) and eosinophils, macrophages, neutrophils, and lymphocytes (e). Error bars indicate $\pm$ s.e.m., * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. HAEC, human airway epithelial cell; HDM, house dust mite; q-PCR, quantitative polymerase chain reaction.

Figure 2 Noxa blocks TNF α -induced NF- κ B nuclear translocation and promoter activation by prolonging the half-life of I κ B α . **(a)** AALEB cells were infected with Ad-Noxa or Ad-GFP (80 MOI) and were treated or left untreated with 20 ng ml⁻¹ of TNF α for 24 h. Secreted IL-8 was quantified by Luminex assay ($n = 3$ experiments with three wells in each experiment). **(b)** Luciferase activity in A549-Luc cells when either infected with 100, 200, or 300 MOI Ad-Noxa or 300 MOI Ad-LacZ and stimulated with TNF α for 30 min, or **(c)** when the cells were stably transfected with shNoxa or shCtrl and treated with IFN- γ ; reduced expression of Noxa mRNA and protein by shNoxa was verified by qRT-PCR and western blotting. **(d)** AALEB cells treated with IFN- γ (50 ng ml⁻¹) or vehicle and 24 h later with TNF α for 30 min. Cells immunostained with antibodies to NF- κ B and Noxa and counterstained with Hoechst 33342. **(e)** Immunostaining with antibodies to NF- κ B and Noxa of AALEB cells infected with Ad-Noxa or Ad-LacZ treated with TNF α for 30 min; percentage of cells with nuclear NF- κ B. Error bar indicates $\pm$ s.e.m. ($n = 3$ independent experiments with three wells in each experiment). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. **(f)** Cytosolic and nuclear extracts from *noxa*^{+/+} and *noxa*^{-/-} MAECs treated with IFN- γ and TNF α and analyzed by western blotting. **(g)** AALEB cells infected with Ad-Noxa and Ad-GFP and treated with cyclohexamide. Extracts probed for NF- κ B, I κ B α , Noxa, and GAPDH. **(h,i)** AALEB cells infected with Ad-GFP or Ad-Noxa, treated with 10 μ M MG-132 and 30 min later with vehicle or 10 ng ml⁻¹ TNF α ; cells harvested 10 min later and extracts immunoprecipitated with anti-I κ B α antibodies. **(h)** The levels of p-I κ B α and Noxa levels were determined with the respective controls in input and immunoprecipitates. **(i)** Levels of p-I κ B α and Noxa levels in the input and I κ B α and K48 chain-linked ubiquitin of I κ B α were determined in the immunoprecipitates. Western blots are representative of three different experiments. MOI, multiplicity of infection; q-PCR, quantitative polymerase chain reaction.

epithelial cells was significantly reduced when Noxa was expressed using an adenoviral expression vector (Ad-Noxa) compared to Ad-GFP-infected controls (**Figure 2a**). The possible role of Noxa in affecting NF- κ B activation was tested using the A549-NF- κ B-luc cells that are stably transfected with a luciferase reporter gene driven by a promoter containing several NF- κ B response elements. As expected, TNF α -induced luciferase activity was inhibited when Noxa was expressed in a dose-dependent manner (**Figure 2b**); no cell death was detected by Ad-Noxa even at a multiplicity of infection of 300 (data not shown). Further, suppression of Noxa expression using shRNA enhanced IFN- γ -induced NF- κ B activity in cells treated with TNF α for 30 min (**Figure 2c**). Further, merely expressing Noxa or expressing Noxa in the presence of IFN- γ treatment showed a similar reduction in A549-NF- κ B-luc cells, showing that IFN- γ had no further impact on Noxa-mediated suppression of NF- κ B activity (**Supplementary Figure S2F**). Together, these findings suggest that the anti-inflammatory effect of IFN- γ is mediated by Noxa.

We next analyzed the effects of IFN- γ or Noxa on TNF α -induced change in sub-cellular localization of NF- κ B. While NF- κ B was localized to the nucleus 30 min after TNF α treatment, it remained localized to the cytosol in cells that were pretreated with IFN- γ for 24 h (**Figure 2d**). Similarly, direct expression of Noxa using adenoviral vectors suppressed TNF α -induced nuclear translocation of NF- κ B as shown by immunofluorescence (**Figure 2e**). In addition, IFN- γ was delayed in suppressing nuclear translocation of NF- κ B in *noxa*^{-/-} MAECs (**Figure 2f**), confirming that IFN- γ requires Noxa to delay NF- κ B activation. These findings were further confirmed by immunoblotting of nuclear and cytoplasmic extracts that showed nuclear NF- κ B being significantly reduced in cells pretreated with IFN- γ compared to non-treated controls (**Supplementary Figure S2F**).

Noxa blocks the degradation of ubiquitylated I κ B α

In non-stimulated cells, the inhibitory I κ B α protein sequesters NF- κ B to the cytoplasm and inflammatory stimuli activate

IKK α and IKK β to phosphorylate I κ B α , which leads to its ubiquitylation and proteasomal degradation, releasing NF- κ B to translocate to the nucleus.^{23,24} Over 0, 15, and 30 min of TNF α treatment, I κ B α levels were reduced in the cytosolic extracts of *noxa*^{-/-} but not *noxa*^{+/+} MAECs (Supplementary Figure S2G), suggesting that Noxa stabilizes I κ B α . TNF α -induced nuclear translocation of NF- κ B was also significantly reduced following overexpression of Noxa using Ad-Noxa compared to cells infected with Ad-GFP (Figure 2g), suggesting that Noxa expression is sufficient to stabilize I κ B α . Interestingly, after 10 min

of TNF- α treatment the levels of phosphorylated I κ B α were similar in HAECs infected with Ad-Noxa and Ad-GFP as control (Figure 2h). Lysine-48 (K48)-linked polyubiquitin chains are well established as the canonical signal for proteasomal degradation.²⁵ Interestingly, the levels of I κ B α with homotypic K48-linked ubiquitin chains were increased in HAECs infected with Ad-Noxa and expressed Noxa compared with Ad-GFP-infected controls (Figure 2i). This finding suggests that Noxa blocks the proteasomal degradation of I κ B α that is already destined for degradation.

Figure 3. The cross-linking of Noxa and p-HSP27 is essential for blocking NF- κ B activation. (a) Noxa (6 kDa) and a 32 kDa cross-reactive protein detected by Noxa antibodies in IFN- γ -treated AALEB cells. (b) Immunoprecipitates using anti-Noxa antibody from IFN- γ -treated cells analyzed by immunoblotting. (c) Immunoprecipitates using IgG as control or p-S82HSP27 antibodies from IFN- γ -treated AALEB cells analyzed by immunoblotting with antibodies to p-S82HSP27 and Noxa. (d) GST or GST-Noxa proteins bound to glutathione-sepharose beads used to pull down proteins from AALEB cell lysates and analyzed by western blotting. (e) Protein extracts from AALEB cells transfected with HSP27 expression vector and treated with the p38MAPK inhibitor, SB203580, were immunoprecipitated using anti-Noxa antibodies. (f) HSP27 was co-transfected with Noxa, NoxaC25S, NoxaC51S, Noxa2CS, or Noxa3KR and immunoprecipitated using anti-p-S82HSP27. (g) Immunoprecipitates using anti-p-S82HSP27. (h) Immunofluorescence of shCtrl and shHSP27 cells treated with IFN- γ and/or TNF α or vehicle using antibodies to NF- κ B and p-S82HSP27 and counterstained with Hoechst 33342. (i) Immunoprecipitates using anti-NF- κ B from shCtrl and shHSP27 cells treated with IFN- γ and TNF α or vehicle and analyzed by immunoblotting. (j) Lysates from shCtrl and shHSP27 cells pre-treated with CHX and 10 ng ml⁻¹ TNF α analyzed by western blotting. All western blots are representative of at least three different experiments.

Noxa is cross-linked to phosphorylated HSP27

Because Noxa is known to inactivate Mcl-1 and thereby sensitize cells to apoptotic death,^{26,27} we tested the hypothesis that Noxa may interact with other protein(s) to gain anti-inflammatory properties. Noxa antibodies consistently detected a cross-reacting protein of approximately 32 kDa in size only in IFN- γ -treated HAECs (Figure 3a). Peptide mass fingerprint analysis of the cross-reacting the 32 kDa band excised following immunoprecipitation repeatedly revealed a high score for HSP27 (Supplementary Table S1). This observation was confirmed by immunoblotting of anti-Noxa immunoprecipitated proteins with an antibody to HSP27 phosphorylated at Ser₈₂ (Figure 3b). Interestingly, Mcl-1 and Bcl-x_L, proteins known to interact with Noxa,²⁷ were not detected in the immunoprecipitates (Figure 3b). Immunoprecipitation with p-S₈₂HSP27 antibodies confirmed that Noxa forms a complex with phosphorylated HSP27 (pHSP27) (Figure 3c). The interaction between Noxa and pHSP27 was further verified by pull-down assays from extracts prepared from AAELB cells using recombinant, purified GST-Noxa protein and detecting pHSP27 protein by western blot analysis (Figure 3d). Blocking the phosphorylation of expressed HSP27 using the p38MAPK inhibitor, SB203580, reduced the levels of immunoprecipitated pHSP27-Noxa complex (Figure 3e), further supporting that pHSP27 rather than total HSP27 needs to be present to form the Noxa/pHSP27 interaction. Interestingly, merely overexpressing Noxa by infecting cells with Ad-Noxa in the absence of IFN- γ also resulted in the appearance of the 32 kDa Noxa-pHSP27 complex (Supplementary Figure S3A), suggesting that IFN- γ was not inducing a cross-linking enzyme to facilitate this complex formation. The idea that Noxa and p-HSP27 are linked by disulfide bond(s) was confirmed by the absence of the p-HSP27-GST-Noxa complex in pull-down products from cell extracts treated with the reducing reagent, DTT that cleaves disulfide bonds (Supplementary Figure S3B).

To identify the amino acids within Noxa that are responsible for the Noxa-HSP27 complex, we generated various mutant GST-Noxa proteins that were tested in pull-down assays. Deletions of 10 amino acids from the N-terminus (Noxa- Δ 5') or 14 amino acids from the C-terminus (Noxa- Δ 3') abrogated the interactions of Noxa and pHSP27 (Supplementary Figure S3C). Because 293T cells express no detectable levels of endogenous HSP27, these cells were co-transfected with an HSP27-expression vector along with wild-type Noxa, Noxa-2CS, and Noxa-3KR. Mutations of cysteines 25 and 51 to serines (Noxa-2CS) also disrupted the interactions while mutation of lysines 35, 38, and 41 to arginines (Noxa-3KR) did not (Figure 3f). Further mutagenesis experiments demonstrated that only Cys₂₅ residue was essential for the formation of Noxa-phospho-HSP27 complex (Figure 3f) confirming the hypothesis that Noxa and HSP27 are linked by an intermolecular disulfide bond.

Because HSP27 contains only one cysteine residue at position 137, the role of this residue was investigated by comparing wild-type and C137A mutant HSP27 constructs expressed in 293T cells that were treated with IFN- γ to induce Noxa. Although expression levels of pHSP27 were equal in cells transfected with either construct, immunoprecipitation with

Noxa antibodies showed that the formation of Noxa-pHSP27 was impaired in cells transfected with the C137A mutant and was accompanied by the loss of interactions with I κ B α /NF- κ B complex (Figure 3g). These studies demonstrated that an intermolecular disulfide bond between Cys₂₅ in Noxa and Cys₁₃₇ in HSP27 cross-links Noxa to pHSP27 to allow the formation of a complex with I κ B α and NF- κ B.

HSP27 is essential for Noxa-I κ B α -NF- κ B interaction

Suppression of HSP27 in AAELB cells by stably expressing shHSP27 did not affect IFN- γ -induced Noxa expression (Supplementary Figure S3D). These cells were used to investigate the contribution of pHSP27 in blocking nuclear translocation of NF- κ B. As shown by immunofluorescence staining IFN- γ blocked nuclear translocation of NF- κ B in shCtrl but not in shHSP27 cells (Figure 3h). In addition, when Noxa or HSP27 expression in AAELB cells was reduced using shNoxa or shHSP27, IFN- γ no longer suppressed secretion of IL-8 (Supplementary Figure S3E) or IL-6 (Supplementary Figure S3F). Therefore, we proceeded to determine whether the interaction of Noxa with I κ B α /NF- κ B would be disrupted by reducing HSP27 levels. Although NF- κ B and I κ B α were equally expressed in shCtrl and shHSP27 cells, the interactions between Noxa and NF- κ B or I κ B α were abolished in shHSP27 cells (Figure 3i). Immunoprecipitation with NF- κ B (p65) antibodies from shHSP27 cell extracts showed that the interaction of NF- κ B with I κ B α was essentially completely disrupted when cells were treated with TNF α , suggesting that I κ B α degradation occurred faster when HSP27 was suppressed (Figure 3i). Further, the NF- κ B-Noxa interaction was disrupted in shHSP27 cells but not in shCtrl cells (Figure 3i) and TNF α -induced degradation of I κ B α was enhanced in shHSP27 compared with shCtrl cells (Figure 3j), suggesting that pHSP27 also is crucial in stabilizing I κ B α . Therefore, Noxa requires the presence of pHSP27 to interact with the I κ B α /NF- κ B complex and the cross-linking of pHSP27 and Noxa appears to play a critical role in stabilizing I κ B α .

Inducible expression of Noxa in AECs protects from allergic inflammation and mucous cell hyperplasia

Constitutive expression of HSP27 has been detected in the lungs of mice²⁸ but the presence of phosphorylated HSP27 in various tissues and cell types have not been studied. Because the cross-linking of Noxa to pHSP27 may be specific to airway epithelia, we compared pHSP27 levels in various tissues and cell types. Phospho-HSP27 was detected in MAECs and in lung tissues but not in murine embryo fibroblasts, thymus, and liver tissues (Figure 4a). To investigate the anti-inflammatory and possibly the therapeutic role of Noxa expression in airway epithelial cells *in vivo*, we generated a conditional inducible transgenic (Noxa^{Ind}) mouse using the tetO-CCSP promoter system. Immunofluorescence analyses showed induced levels of Noxa in the bronchial epithelial cells of three founder Noxa^{Ind} mouse lines compared with wild-type littermates (Figure 4b). These mice expressed Noxa mRNA and protein in the lung tissues 24 h post doxycycline (dox)-diet (Figure 4c). These transgenic mice and littermate controls were instilled with house dust mite (HDM) extract for 5 consecutive days,

Figure 4 Inducible transgenic expression of Noxa in airway epithelial cells protects mice from inflammation. **(a)** pHSP27 levels in HAECs, MAECs, MEFs and in murine lung, thymus, and liver tissues tested by western blotting. **(b)** Immunostaining with Noxa antibodies of tissue sections from the left lungs of Noxa^{Ind} and Noxa^{WT} littermates. **(c)** Noxa protein and mRNA levels in the right lungs analyzed by western blotting and q-PCR, respectively. **(d)** Noxa^{Ind} and Noxa^{WT} mice were challenged with HDM extracts intranasally for 5 days, fed dox diet for 2 days, and lungs lavaged 24 h later ($n = 8$ mice per group). The number of total cells, eosinophils, and lymphocytes were quantified. **(e)** Mucous cell numbers per mm basal lamina (BL) were quantified using VisioMorph system on Alcian blue/hematoxylin and eosin (AB/H&E)-stained sections ($n = 8$ per group). Representative images of AB/H&E-stained airway epithelium from Noxa^{Ind} and Noxa^{WT} mice. **(f)** Co-immunostaining with Noxa (red) and NF- κ B (green) antibodies of airway epithelium from Noxa^{Ind} and Noxa^{WT} littermates following HDM challenge and dox-diet. Quantification of percentage airway epithelial cells with nuclear NF- κ B in Noxa^{Ind} and Noxa^{WT} mice exposed to HDM ($n = 5$ per group). Error bars indicate $\pm$ s.e.m. * $P < 0.05$. HAECs, human airway epithelial cell; MAECs, murine airway epithelial cell; MEF, mouse embryo fibroblast; q-PCR, quantitative polymerase chain reaction.

given the dox-diet on days 6 and 7, and killed on day 8. The number of total inflammatory cells in the bronchoalveolar lavage fluid was reduced in Noxa^{Ind} mice compared to littermate controls and these reduced numbers were due to reduced bronchoalveolar lavage eosinophils and lymphocytes (**Figure 4d**). The no dox, no HDM, dox-fed but not HDM mice controls showed only macrophages and no inflammation (data not shown). Transgenic expression of Noxa also resulted in a threefold reduced number of mucous cells per mm basal lamina compared with littermate controls (**Figure 4e**). Immunofluorescence analysis showed that expressed Noxa was co-localized with NF- κ B in the cytosol resulting in the lower percentage of airway epithelial cells with nuclear NF- κ B in Noxa^{Ind} mice (**Figure 4f**). Further, pHSP27 was co-localized with induced Noxa (**Supplementary Figure S4A**) and NF- κ B (**Supplementary Figure S4B**) in airway epithelial cells of Noxa^{Ind} mice.

The N-terminal Noxa peptide stabilizes I κ B α and is anti-inflammatory

We further tested whether specific regions of the Noxa protein may act as anti-inflammatory by using Noxa-derived peptides. Based on our findings that Cys25 constitutes the cross-linking amino acid, and because the human and murine Noxa proteins share a high homology, except that the murine Noxa contains the duplicate sequence (**Supplementary Figure S5A**), we selected four regions that are 15 amino acids in length and span the following functional regions of the human Noxa protein. Peptide NoxaA represents the N-terminus, NoxaB contains Cys25, NoxaC comprises the BH3 domain, and NoxaD represents the C-terminal end of the protein (**Supplementary**

Figure S5B). To facilitate uptake into cells, the peptides were synthesized with the HIV-derived TAT sequence on their N-termini. When A549 cells were treated with 10 ng ml^{-1} TNF α , NF- κ B-induced luciferase activity was reduced by NoxaA and NoxaD peptides but not by NoxaB or NoxaC (**Figure 5a**). However, when nuclear localization of NF- κ B was investigated in TNF α -treated MAECs by immunofluorescence, only NoxaA but not NoxaD or the control TAT peptide affected nuclear localization (**Figure 5b**).

To examine the anti-inflammatory effect *in vivo*, noxa^{-/-} mice were intranasally instilled with $50 \mu\text{g}$ house dust mite allergen for 5 consecutive days and then instilled with NoxaA peptide on days 6 and 7. The number of inflammatory cells in the bronchoalveolar lavage fluid mice was significantly reduced by NoxaA (**Figure 5c**) and this reduction was due to reduced numbers of eosinophils (**Figure 5d**). The number of neutrophils, macrophages, and lymphocytes were not affected (data not shown). After 10 min of TNF- α treatment the levels of I κ B α with homotypic K48-linked ubiquitin chains were increased in AALEB cells treated with NoxaA compared to Ctrl peptide (**Figure 5e**).

DISCUSSION

The present studies show that IFN- γ through STAT1 activation rapidly induces Noxa which is cross-linked with a non-Bcl-2 family member, pHSP27, and suppresses the canonical NF- κ B signaling pathway. Therefore, these findings define the mediator by which IFN- γ acts as anti-inflammatory and are the first to show that a Bcl-2 family member could inhibit inflammation by reducing NF- κ B activation.

Members of the extrinsic apoptotic machinery, including the TNFR1 interactors, TRADD, FADD, and RIPK1,²⁹ are known

Figure 5 N-terminal peptide of Noxa is sufficient to prolong I κ B α half-life and block inflammation. (a) Luciferase activity in A549-Luc cells treated with vehicle or peptides A, B, C, and D at 50 nM concentration. (b) Nuclear localization of NF- κ B (p65) following treatment of *noxa*^{-/-} MAECs with 0 or 10 ng ml⁻¹ TNF α in the presence of TAT-Ctrl, and peptides A or D. (c,d) C57Bl/6 mice exposed to 50 μ g house dust mite for 5 consecutive days and instilled with 10 μ M each of TAT-Ctrl or peptide A on days 6 and 7. On day 8, the numbers of total inflammatory cells (c) and eosinophils (d) were assessed in the BAL fluid. Error bar indicates $\pm$ s.e.m. (in two separate experiments with minimum of 4 mice per group each). * P < 0.05. (e) AAIEB cells were treated with TAT-Ctrl or peptide A and 24 h later treated with TNF α for 10 min. Immunoprecipitates with anti-I κ B α antibodies were analyzed for levels of I κ B α and K48 chain-linked ubiquitin of I κ B α and I κ B α and β -actin in the input. Western blots are representative of three different experiments. BAL, bronchoalveolar lavage.

to be required for optimal NF- κ B activation. The present study adds a new paradigm by showing that a member of the intrinsic apoptotic machinery affects the immune response by directly blocking NF- κ B activation. It is well-established that proteins of the Bcl-2 family interact with each other to control the permeabilization of the mitochondrial outer membrane and thereby regulate apoptosis. However, only a limited number of studies have explored their interaction with non-Bcl-2 family members and their roles in cellular processes other than those related to cell death. For example, Bad effects insulin secretion and metabolism³⁰ and BID, contrary to Noxa, by interacting with NOD1, NOD2, and the I κ B kinase (IKK) complex facilitates NF- κ B activation.³¹

Noxa is induced rapidly also by hypoxia via hypoxia-inducible factor (HIF)-1 α ,³² by DNA damage,¹⁷ by ER stress inducing agents.³³ Furthermore, anticancer agents, including the proteasome inhibitor, bortezomib, or ubiquitylation of Mcl-1, induce Noxa expression.³⁴ All these studies used fibroblasts, chronic lymphocytic leukemia, melanoma, neuroblastoma, osteosarcoma, and lung cancer cells and investigated expressed Noxa interacting with the anti-apoptotic Mcl-1 to regulate cell death by affecting Mcl-1 degradation.^{25,35} However, although Mcl-1 was present in airway epithelial cells, the presence

of pHSP27 caused the IFN- γ -induced Noxa to prefer the cross-linking with pHSP27 and not Mcl-1. Ser₁₃ phosphorylation alters Noxa structure and blocks the interaction site with Mcl-1.³⁶ Whether pHSP27 phosphorylates Noxa or just forces the N-terminal region to be exposed for interaction with I κ B α is unclear. While the phosphorylation and ubiquitylation process remained unaffected, Noxa delayed the degradation of I κ B α after it was already tagged by the K48 ubiquitin chain. Small molecules, called ubistatins, block the binding of ubiquitylated substrates to the proteasome by targeting the ubiquitin-ubiquitin interface of K48-linked chains.³⁴ Whether the N-terminus of Noxa interacts with the I κ B α -specific proteins or the ubiquitin-ubiquitin interface or blocks the interaction of ubiquitylated I κ B α to the 19S proteasome before being shuttled for degradation needs further investigation.

The airway epithelium is constantly exposed to the elements of the outer world and therefore has to continuously regulate inflammation. We show that Noxa is rapidly induced within hours and this rapid response allows the fine-tuning or unnecessary enhancement of inflammation. Given that Noxa expression in airway epithelium causes a significant difference in inflammation reveals that this protein contributes to the anti-inflammatory protective role of the airway epithelium. Because many pathways are not necessarily associated with inflammation, including autophagy, regulators of ROS also play a role in regulating inflammation; the induction of Noxa may be one of many mechanisms by which the airway epithelium dampens inflammation. Noxa expression can either sensitize airway cells when cells are damaged or inhibit inflammation. The present studies also suggest that the underlying mechanisms of Noxa-induced cell death in airway epithelial cells need further investigation, because Noxa-induced cell death may not only depend on enhanced degradation of Mcl-1 but also on blocking NF- κ B activation that directly regulates expression of the anti-apoptotic Bcl-2.

Both the whole Noxa protein when cross-linked with pHSP27 and the peptide comprising the N-terminal region without the cross-linking Cys25 equally delayed the proteasomal degradation of ubiquitylated I κ B α . Therefore, the role of pHSP27 is likely to expose the N-terminal region of Noxa to form a complex with I κ B α and NF- κ B. HSP27 has vastly different functions, including reducing growth or proliferation and increasing differentiation, and protecting against apoptosis.³⁵ This diverse function of HSP27 may depend on its ability as a chaperone protein to interact with other proteins that are present in various cell types or conditions. Further, HSP27 can be phosphorylated at three serine residues, resulting in the redistribution of the large oligomer into smaller tetrameric units, and its dephosphorylation favors the formation of large oligomers, the dimer of HSP27 being the building block for multimeric complexes that can be up to 1000 kDa.³⁷ It is possible that depending on the phosphorylation state, HSP27 interacts with different proteins to ultimately mediate different outcomes. For example, in prostate cancer cells, HSP27-eIF4E interaction defines the chaperoning role of HSP27 to decrease eIF4E ubiquitylation and proteasomal degradation to protect

the protein synthesis initiation process and survival.³⁸ Similarly, hic-5 interacts with HSP27 and blocks the anti-apoptotic role of HSP27.³⁹

Our finding that pHSP27 has an anti-inflammatory role is consistent with the report that HSP27 deficiency in mice augments neutrophil infiltration in wounds.⁴⁰ In other studies, HSP27 can enhance NF- κ B activity by interacting with IKK⁴¹ or by interacting with polyubiquitin chains.⁴² However, these studies focused on analyzing the presence or absence of HSP27 and did not investigate the phosphorylation state of HSP27. Our studies demonstrate a novel observation that Noxa expression being highly induced in airway epithelial cells may define the anti-inflammatory function of pHSP27.

The finding that pHSP27 is primarily detected in lung tissues or airway epithelial cells would suggest that the anti-inflammatory role of Noxa is restricted to AECs. Consistent with this hypothesis, inducible Noxa expression in airway epithelial cells of transgenic mice was effective in suppressing inflammation. Delivering the NoxA peptide directly to the airways may be a useful tool to temper inflammatory responses by prolonging the half-life of I κ B α . Therefore, developing therapeutic vectors that deliver Noxa proteins or peptides to the airway epithelium or molecules that specifically induce Noxa expression in airway cells would represent novel anti-inflammatory tools to control chronic inflammation. Noxa as a treatment may be effective particularly in diseases that are associated with high levels of pHSP27, including COPD,⁴³ asthma,⁴⁴ and cancer.⁴⁵ IFN- γ has been used to treat chronic inflammatory diseases, but as a pleiotropic cytokine, it has many effects. By identifying Noxa as the protein that mediates the anti-inflammatory role of IFN- γ , this study may have uncovered a means to harness the anti-inflammatory benefits of IFN- γ and minimize the other effects of IFN- γ . This would place Noxa in a unique position among anti-inflammatory therapeutic agents, especially when prolonged treatments are expected in chronic inflammatory diseases, such as asthma and COPD.

METHODS

Animals. All mouse studies were performed at the Lovelace Respiratory Research Institute (LRRI), a facility approved by the Association for the Assessment and Accreditation for Laboratory Animal Care International using protocols, pre-approved by the Institutional Animal Care and Use Committee (IACUC), and the Environmental Safety and Health department (ES&H). *Noxa*^{-/-} on C57Bl/6 background mice were provided by Dr A. Strasser (Walter and Eliza Hall Institute, Melbourne, Australia) and *p53*^{+/-} breeders were purchased from The Jackson Laboratory (Bar Harbor, ME) and were bred at LRRI.¹⁷ Mice were housed in isolated cages under specific pathogen-free conditions for the described studies.

The conditional and airway-specific overexpression of Noxa was achieved by mating two lines of transgenic mice, the CCSP-reverse tetracyclineresponsive transactivator (rtTA) mice, bearing the rtTA under the control of the CCSP gene promoter,⁴⁶ and the tetracycline operator (TetO)₇-Noxa mice, containing TetO and minimal cytomegalovirus promoter and the Noxa transgene. (TetO)₇-Noxa mice were generated at the MD Anderson Genetically Engineered Mouse Facility following standard methods and Institutional Animal Care and Use Committee-approved protocols and bred with CCSP-rtTA mice at LRRI. The transgene plasmid was cut with *HindIII* and *NheI*,

and murine Noxa cDNA was separated from the TG-1 vector. Purified murine Noxa cDNA was injected into the pronuclei of zygotes collected from superovulated (C57Bl/6) female mice, and injected zygotes were transferred to pseudopregnant ICR recipient females. Founder animals were identified by polymerase chain reaction amplification of 2.5 μ l of tail DNA segments digested overnight in NaOH at 55 °C. Oligonucleotides were as follows: a, 5'-AGGGATC-CAT GTGTATTGTA TGTGGGCTC TAACCTGGC-3'; b, 5'-TGCAGCTAGG TGAGCGTCCA CAGGACCCTG AGTGGT-3'. To conditionally express murine Noxa in the lung, transgenic mice were fed with doxycycline-containing diet. Founder animals (C57Bl/6J) were bred to C57Bl/6J $\times$ C57Bl/6J animals to evaluate germline transmission. Six lines that transmitted the Noxa sequence were analyzed by quantitative polymerase chain reaction (q-PCR) for expression of Noxa in lung and tracheal tissue. On the basis of these results, three murine lines with expression of Noxa in the airway epithelia were chosen for colony expansion and line 2 was maintained by breeding with wild-type C57Bl/6 mice and using the wild-type littermates as controls for each experiment.

Exposures. At 6–10 weeks of age, mice were entered into the experimental protocols. Mice were instilled with HDM extracts for 5 consecutive days and lung tissues were analyzed 24 or 72 h post the last challenge. HDM (Greer Laboratories, Lenoir, NC) was from *Derma-tophagoides Pteronyssinus* (lot #248041), as 21.5 mg protein per 103.3 mg dry weight in a freeze-dried form was resuspended in sterile phosphate buffered saline for a final concentration of 1 μ g μ l⁻¹ and stored in frozen aliquots at -20 °C. Sensitization and exposure of mice to ovalbumin was as described previously.⁴⁷ Bronchoalveolar lavage and preparation of lung tissues for histopathological examination and staining with Alcian blue/hematoxylin and eosin was as described.⁴⁷

Cells. Primary HAECs (Cambrex Bio Science, Walkersville MD, Inc.), MAECs, AALEB cells, A549 cells stably transfected with multiple copies of the NF- κ B response element driving a luciferase reporter construct (A549-NF- κ B-luc cell line) (Panomics, Inc., Redwood City, CA.), and 293T cells were cultured as described previously.¹⁴ AALEB cells are immortalized HAECs that have been well characterized.⁴⁸ Cells were maintained in bronchial epithelial growth medium BEGM (Lonza, Walkersville, MD) supplemented with growth factors (BEGM Singlequots, Lonza) as described previously.¹⁴ Adenoviral expression vectors for Noxa were provided by Dr G. Shore (McGill University, Montreal, Canada), and cells were infected as previously described.⁴⁹ The A549-NF- κ B-luc cell line was purchased from Panomics, Inc., CA and grown in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen, Waltham, MA) supplemented with 10% fetal bovine serum, 1 mM sodium pyruvate (Invitrogen), penicillin/streptomycin (Invitrogen) and hygromycin. The 293T cells were cultured in DMEM with 10% fetal bovine serum. Transfections were carried out with the TransIT-2020 Transfection Reagent (Mirus Bio, Madison, WI) according to the manufacturer's protocol. Cell viability was determined by trypan blue exclusion.

MAECs were isolated essentially as described previously⁵⁰ by incubation in pronase solution (DMEM, 1.4 mg ml⁻¹ pronase and 0.1 mg ml⁻¹ DNase) overnight at 4 °C to dissociate airway epithelial cells from the basal lamina. Cells were collected by gently rocking trachea in DMEM/Ham H12 media (Invitrogen) followed by centrifugation at 400 g for 10 min at 4 °C. MAECs were grown in media described previously.⁵¹

Generation of expression constructs. The GST-tagged recombinant proteins of the full length Noxa (pGEX-Noxa), 5' or 3' truncated versions (pGEX-Noxa Δ 5' or pGEX-Noxa Δ 3'), C25/51S mutant (pGEX-Noxa-2CS), and C-terminal K35/38/41R mutant (pGEX-Noxa-3KR) were expressed in pGEX-5X-1 Gene Fusion System (GE Health). The constructs were generated by amplifying the desired coding regions with appropriate mutations using PCR and inserting the products into the *BamHI* and *EcoRI* sites of pGEX-5X1 vector. The primers for generating NOXA full-length and truncated constructs

were: NOXA (*Bam*HI)-5'-tcc gga ctc gga tcc cca tgc ct-3'; NOXA (*Eco*RI)-5'-ccg tcg act gca gaa ttc tca gg-3'; NOXA-5' deletion (*Bam*HI)-5'-cgg atc ccc caa cgg agc ccc gcg cgg gct cca gc-3'; NOXA-3' deletion (*Eco*RI)-5'-cag aat tct cac tgc cgg aag ttc agt ttg tct cc-3'. Primers for generating pGEX-Noxa-2CS were: pGEX-NOXA-*Bam*HI-F (5'-aag gtc gtc gga tcc cca tgc ctg gg-3'), NOXA C25S-F (5'-ctg gaa gtc gag tct gct act caa ctc-3'), NOXA C25S-R (5'-gag ttg agt agc aga ctc gac ttc cag-3'), NOXA-C51S-*Eco*RI-R (5'-cga ccc ggg aat tct cag gtt cct gag gag aag agt ttg g-3'). After generating products PCR1 and PCR2 using primers pGEX-NOXA-*Bam*HI-F/NOXA C25S-R, and NOXA C25S-F/NOXA-C51S-*Eco*RI-R, these products were purified and used as the templates to amplify PCR3 product using pGEX-NOXA-*Bam*HI-F and NOXA-C51S-*Eco*RI-R primers. PCR3 was digested with *Bam*HI + *Eco*RI, and cloned into pGEX-5X1. pGEX-Noxa-3KR was constructed using the following primers: pGEX-NOXA-*Bam*HI-F, NOXA-K35R-R (5'-ctg ccg gaa gtt cag gcg gtc tcc aaa tct-3'), NOXA-K41R-R (5'-gat atc aga ttc aga agg cgc tgc cgg aag ttc ag-3'), NOXA-K48R-*Eco*RI-R: (5'-cgg gaa ttc tca ggt tcc tga gca gaa gag gcg gga tat cag att cag-3'). To express Noxa and its mutant proteins in mammalian cells, Noxa-WT, Noxa-2C, and Noxa-3KR were sub-cloned into the *Bam*HI and *Eco*RI sites of pcD β A vector. pcD β A-Noxa-C25S and pcD β A-C51S constructs were generated using pGEX-NOXA-*Bam*HI-F/NOXA-*Eco*RI-R (5'-cga ccc ggg aat tct cag gtt cct gag cag aag agt ttg g-3') to amplify pGEX-Noxa-2C and pGEX-NOXA-*Bam*HI-F/NOXA-C51S-*Eco*RI-R to amplify Noxa-WT. The PCR products were digested with *Bam*HI + *Eco*RI and sub-cloned into the pcD β A vector, where expression of cloned sequences is driven by the cytomegalovirus enhancer. All constructs were verified by sequencing.

Immunofluorescence. Cells were seeded onto 2- or 4-well chamber slides (Labtek, Grand Rapids, MI), fixed with 3.5% paraformaldehyde, permeabilized with 0.1% Triton X-100, and incubated in blocking buffer (3% bovine serum albumin (BSA), 1% normal goat serum in phosphate-buffered saline (PBS)). After incubation with the relevant primary antibody cells were stained with either anti-rabbit Alexa647 or mouse Alexa555 (Invitrogen), mounted with glass coverslips using Fluoromount-G (Southern Biotech, Shijr, Taipei, Taiwan) and immunofluorescence visualized using Axioplan 2 (Carl Zeiss) with a Plan-Aprochromal $\times 63/1.4$ oil objective and a charge-coupled device camera (SensiCam; PCO) using an acquisition and processing software Slidebook 5.0 (Intelligent Imaging Innovation, Denver, CO).

GST pull-down assays. Expression constructs were transformed into ECOS-21 bacterial cells (Yeastern Biotech, Shijr, Taipei, Taiwan) and induced using 1 mM IPTG (Sigma, St. Louis, MO) for 4 h at 37 °C (GE Health, manufacturer's protocol). Cells were lysed by repeated freeze-thawing and sonication. Expression was determined via western blot analysis using an anti-Noxa Mouse mAb (114C307, Calbiochem, Billerica, MA). Fusion proteins were affinity-purified on glutathione-Sepharose beads (Pharmacia, London, England) and quantified. For pull-down assays, lysates of non-treated and IFN- γ -treated cells were incubated for 2 h with GST fusion proteins noncovalently immobilized on glutathione-Sepharose beads. Beads were washed in cell lysis buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.07 trypsin inhibitor units/ml of aprotinin, and 1 μ g ml⁻¹ each of leupeptin, pepstatin, antipain, and chymostatin (Sigma)). Bound proteins were solubilized in sample buffer, resolved by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and subjected to anti-HSP27 immunoblotting.

Preparation of cytoplasmic and nuclear extracts. Briefly, cells were lysed in 100 μ l lysis buffer (10 mM Tris · HCl, pH 8.0; 60 mM KCl; 1 mM ethylenediaminetetraacetic acid (EDTA); 1 mM dithiothreitol (DTT), protease Inhibitors cocktail, 0.5% NP-40), and nuclei centrifuged at 2000 g. Nuclear extract was obtained by incubating nuclei in nuclear extraction buffer (20 mM Tris · HCl; 420 mM NaCl; 0.2 mM EDTA; 25% glycerol; 1.5 mM MgCl₂; 1 mM DTT and 0.1 mM PMSF) and centrifugation at 10,000 g.¹⁵

Immunoblotting. Western blot analysis was performed as described previously.¹⁵ Briefly, equal amount of proteins were resolved by SDS-PAGE and transferred to PVDF membranes (Immobilon-P, Millipore Corp, Billerica, MA). Membranes were incubated with optimal concentrations of relevant primary antibody and incubated with horseradish peroxidase-conjugated anti-mouse or -rabbit secondary antibody (Sigma), washed 3 $\times$ 5 min in TBS-T and visualized by Western Lightning Plus-ECL according to the manufacturer's protocol (Perkin Elmer, Waltham, MA). The antibodies used were mouse Noxa (114C307) (Calbiochem), rabbit anti-Noxa (FL-54), anti-I κ B α (C-21) and mouse anti-NF- κ B p65 (F-6) (Santa Cruz Biotechnology), rabbit anti-Lamin A/C and mouse HSP27 (Cell Signaling Technology, Danvers, MA), rabbit anti-p-Ser^{15,78,or 82} HSP27 (Stressgen, Ann Arbor, MI), anti-K48-linkage Specific Polyubiquitin mAb (Cell Signaling), mouse anti- β -Actin, and anti- β -tubulin (Sigma).

Immunoprecipitation. The Crosslink IP Kit (Thermo Fisher Scientific, Waltham, MA) was used to cross-link 10 μ g of anti-Noxa, anti-NF- κ B and anti-I κ B α (Santa Cruz) or HSP27 (Stressgen) antibodies to Sepharose protein A/G beads using disuccinimidyl suberate according to the manufacturer's protocol. The associated proteins were immunoprecipitated by gentle mixing equal amounts of protein lysates with the antibody-crosslinked beads at 4 °C overnight. After six washes, bead-bound proteins were eluted and analyzed by immunoblotting.

Retroviral silencing with shRNA. Retroviral silencing vector encoding shHSP27 or shNoxa and the corresponding control vector (shCtrl) were purchased from Origene Technologies, Inc., Rockville, MD. The suppressing effect of the shRNA was first established in AALEB cells and the subsequent amplification, purification, and packaging of the retroviral particles using Phoenix cells (Orbigen, Inc.) were performed as specified by the manufacturer.

Luciferase assay. A549/NF- κ B-luc cell line (Panomics Corp.) was used to monitor the activity of NF- κ B transcription factor in a cell-based assay (R/D Biosystems, Minneapolis, MN). Cells were lysed by rocking for 15 min in passive lysis buffer (Promega Corp., Madison, WI) and luciferase activity was detected with the Luciferase Assay system (Promega) using a Fluoroskan Ascent detector (Labsystems) according to the manufacturer's protocol.

Luminex cytokine assay. The cytokines in cell culture media were quantified by Luminex instrument (Luminex Corp., Austin, TX) using Multiplex Fluorescent Bead-Based Luminex Cytokine Assays (EMD Millipore, Billerica, MA).

MALDI-TOF. To identify interacting proteins, Noxa was immunoprecipitated with antibodies cross-linked to Sepharose protein A beads. The immunoprecipitate was fractionated on an SDS-PAGE and the 32 kDa band along with blank controls excised, eluted, and subjected to tryptic digestion. Samples were dissolved in methanol, mixed at a 1:1 ratio (v/v) with 2,5-dihydrobenzoic acid (20 mg ml⁻¹ in 70% methanol in water) as matrix. Survey scans were acquired from m/z 500 to -4,000. Sequence analysis of the tryptic peptides was performed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (ABI 4700 MALDI/TOF Mass Spectrometer) running 4000 Series Explorer. Precursors for MS/MS were selected on the basis of signal intensity with up to 20 peptides per spot being chosen for MS/MS analysis. Peptide sequences were identified by searching MASCOT[®] database using GPS Explorer Version 3.5 software (Applied Biosystems, Waltham, MA). Both MS and MS/MS spectra were processed by the software. For MS spectra, an S/N threshold of 30 was used, while for MS/MS spectra, a threshold of 20 was used to detect peaks.

Real-time PCR analysis. Total RNA was isolated from HAECs, MAECs and AALEB cells using RNeasy Micro Kit (Qiagen). The mRNA levels of Noxa, IL-1 β , IL-6, Bcl-2, Bcl-x_L were quantified by ABI HT 7900 Real Time PCR system using TaqMan One-step RT-PCR

Gene Expression kit (Life Technologies, Waltham, MA). The mRNA levels of KC from MAECs were quantified using High Capacity RNA-to-cDNA Kit (Life Technologies) and QuantiTect SYBR Green PCR Kit (Qiagen, Germantown, MD).

Statistical analysis. Grouped results from at least three different experiments were expressed as means $\pm$ s.e.m. Results (grouped by time point and treatment) were analyzed using two-way analysis of variance. In the event that significant main effects were detected ($P < 0.05$), Fisher's least significant difference test was used to differentiate between groups. A P -value of 0.05 was considered to indicate statistical significance. Data were analyzed using statistical analysis software (Statistical Analysis Software Institute).

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at <http://www.nature.com/mi>

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AUTHOR CONTRIBUTION

CZ performed most of the experiments and drafted parts of the paper, J TJ conducted the peptide, HDM, and ubiquitylation studies, HSC performed the immunofluorescence studies, MGW provided strategies to generate various mutation constructs, JX propagated the adenoviral constructs, YAM helped with the mouse studies, YT designed the studies and wrote the manuscript for review by all co-authors.

DISCLOSURE

The authors declared no conflict of interest.

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