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# Severity of Elastase-Induced Emphysema Is Decreased in Tumor Necrosis Factor-$\alpha$ and Interleukin-1$\beta$ Receptor-Deficient Mice

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**SUMMARY:** A single intratracheal dose of porcine pancreatic elastase, which is cleared from the lung by 24 hours, was administered to wild-type, IL-1$\beta$ type 1 receptor-deficient, double TNF-$\alpha$ (type 1 and type 2) receptor-deficient, and combined TNF-$\alpha$ (type 1 receptor) plus IL-1$\beta$ receptor-deficient mice. The mean linear intercept (Lm) of saline-treated mice was 32(3) $\mu$m [mean(SE)]. For wild-type elastase-treated mice, Lm was 81(6) $\mu$m at 21 days versus 52(5) $\mu$m at 5 days after treatment, indicating that alveolar wall remodeling occurs long after the elastase injury. At 21 days, Lm values were 67(10), 62(3), and 39(5) $\mu$m in elastase-treated mice deficient in the IL-1$\beta$ receptor, double TNF-$\alpha$ receptors, and combined receptors, respectively. The level of apoptosis assessed by a terminal deoxynucleotidyl transferase-catalyzed in situ nick end-labeling assay was increased at 5 days after elastase treatment and was markedly and similarly attenuated in the IL-1$\beta$, the double TNF-$\alpha$, and the combined receptor-deficient mice. Our results indicate that inflammatory mediators exacerbate elastase-induced emphysema. We estimate that in the combined TNF-$\alpha$ + IL-1$\beta$ receptor-deficient mice, inflammation accounts for about 80% of the emphysema that develops after elastase treatment; decreased apoptosis of lung cells likely contributes to decreased severity of emphysema. (*Lab Invest 2002, 82:79–85*).

Pulmonary emphysema is defined as abnormal enlargement of respiratory spaces with destruction of the alveolar walls. Experimental evidence supports the concept that alveolar units are damaged when activated macrophages and neutrophils elaborate proteases that degrade elastin and other structural proteins.

The proinflammatory cytokines IL-1$\beta$ and TNF-$\alpha$ are released during inflammatory reactions induced by infection or injury. These effecter substances have overlapping biologic functions, suggesting that they share some common signal transduction pathways (Dinarello, 1997; Kusano et al, 1998; Ledgerwood et al, 1999; Muegge et al, 1989; O' Neill and Greene, 1998; Stewart and Marsden, 1995).

In experimental animals, IL-1$\beta$ stimulates several components of the acute-phase response. The cytokine stimulates the expression of metalloproteinase and other enzymes involved in the degradation of connective tissue proteins (Kusano et al, 1998), and it also stimulates apoptosis (Dinarello, 1998). IL-1$\beta$ utilizes a single signaling receptor to activate two IL-1 receptor-associated kinases (IRAK-1 and IRAK-2), to recruit TNF receptor-associated factor 6 (TRAF6) and activate nuclear factor-$\kappa$B (NF-$\kappa$B) (Cao et al, 1996). Activation of NF-$\kappa$B results in nuclear translocation and alterations in the rate of transcription of certain target genes (Baldwin, 1996; Gilmore, 1999). NF-$\kappa$B also either increases or decreases apoptosis depending on the cell type (Barkett and Gilmore, 1999). IL-1$\beta$ affects gene transcription via several other transacting factors including transcription factor AP-1 proteins and CCAAT-enhancer binding proteins (C/EBP) (Muegge et al, 1989; Osborn et al, 1989).

TNF-$\alpha$ uses two receptors for signal transduction that are referred to as the p55 (type 1) receptor and the p75 (type 2) receptor (Baker and Reddy, 1996; De Cesaris et al, 1999; Jain et al, 1999; Ledgerwood et al, 1999; Pfeffer et al, 1993; Read et al, 1997; Tartaglia et al, 1993; Zhao et al, 1996). Both receptors are expressed on most cell types and function by recruitment of adaptor proteins. The type 1 receptor activates apoptosis and expression of NF-$\kappa$B and affects the steady-state levels of various genes (Baker and Reddy, 1996; Jain et al, 1999; Ledgerwood et al, 1999). Type 1 receptor knock-out mice are resistant to the lethal effects of administered endotoxin (Pfeffer et al, 1993). The type 2 receptor helps deliver the cytokine to the type 1 receptor and can stimulate NF-$\kappa$B activation in lymphocytes (Baker and Reddy, 1996). It

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is uncertain whether the type 2 receptor functions independently of the type 1 receptor.

We postulated that IL-1$\beta$ and TNF-$\alpha$ might be involved in the inflammatory process related to emphysema and we undertook to test this hypothesis by determining whether elastase-induced emphysema would be modulated in mice whose receptors for either or both of these two mediators had been knocked out. We found that, compared with wild-type mice, 21 days after elastase treatment, emphysema was significantly less severe in TNF-$\alpha$ (types 1 and 2) receptor-deficient knock-out mice and in mice deficient in both TNF-$\alpha$(1) and IL-1$\beta$ receptors.

## Results

We examined the time course of development of emphysema after porcine pancreatic elastase (PPE) treatment in wild-type mice (Fig. 1). Mice treated with PPE showed increases in Lm at 1 and 3 days that are statistically insignificant compared with saline controls. The mean values of Lm at 5, 10, and 21 days were significantly greater than the saline control value. There was no statistically significant difference between the 10- and 21-day Lm values; both were more than double the saline control Lm values. The 10- and 21-day values were significantly different from the 1-, 3-, and 5-day values.

Microscopic examination showed the saline controls of all three groups of knock-out mice to be indistinguishable from that of the saline-treated wild-type mice. The appearance of the 5- and 21-day saline groups was similar; accordingly the saline groups were pooled for statistical analysis. In wild-type PPE-treated mice, there was marked enlargement of alveoli, with breaks in alveolar walls compatible with destruction (Fig. 2). Emphysema was least severe in the TNF receptor(1)–IL-1 receptor [TNFR(1)–IL-1R] knock-out group (Fig. 2).

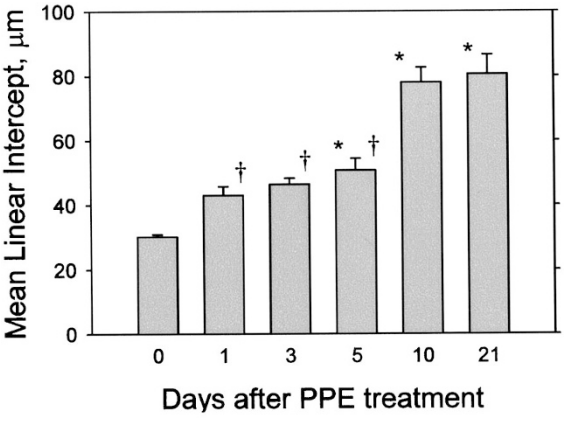


**Figure 1.**
Time course of lung airspace size among wild-type mice, measured by mean linear intercept. Mice were given 0.1 ml of saline or 30 $\mu$g of porcine pancreatic elastase (PPE) in 0.1 ml of saline. Data are shown as means (SEM). Values for saline-treated mice are given at 0 days. Sample sizes for Day 0, 1, 3, 5, 10, and 21 values are 17, 4, 4, 10, 4 and 9 mice, respectively. With the Kruskal-Wallis test, the values of the Day 5, 10, and 21 groups are significantly greater than the saline-treated group (indicated by *asterisks*); the Day 1, 3, and 5 values are significantly less than the Day 10 and 21 values (indicated by *daggers*).

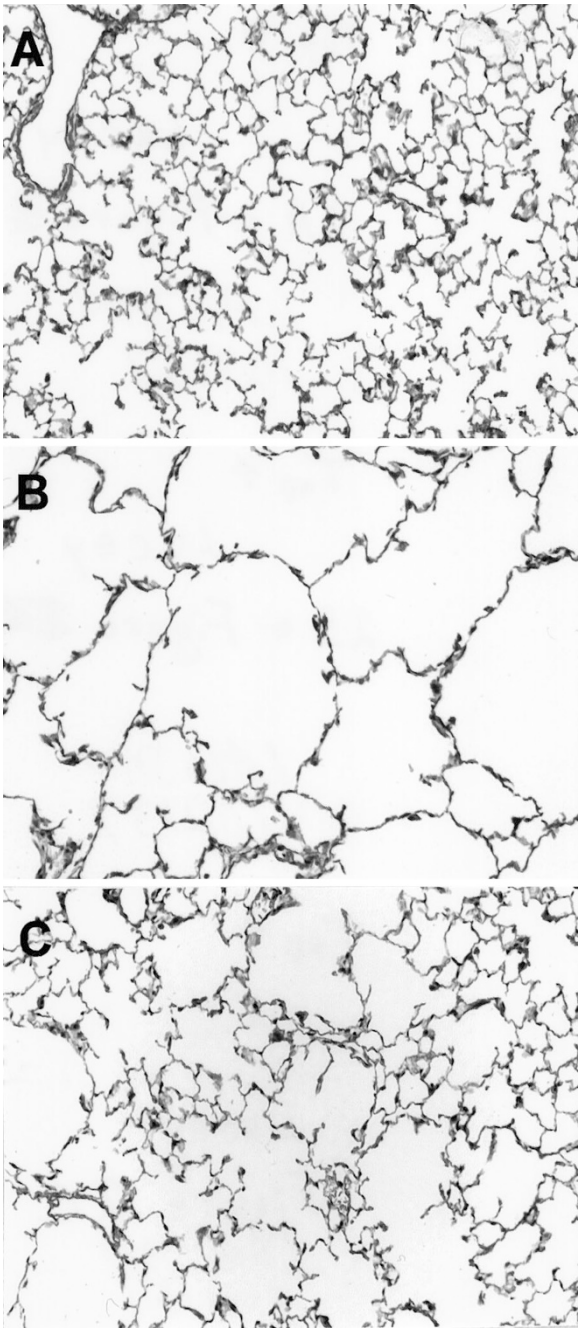


**Figure 2.**
Photomicrographs of lung parenchyma after treatment with 30 $\mu$g of PPE or vehicle (original magnification $\times$200, Verhoeff's van Gieson stain). A, Wild-type mice 21 days after saline. B, PPE-treated wild-type mice 21 days after treatment. C, PPE-treated TNFR(1)–IL-1R mice 21 days after PPE treatment. Alveolar size and structure are normal in panel A. There is alveolar wall destruction and marked enlargement of airspaces in panel B. The severity of emphysema is considerably less in panel C than in panel B.

Figure 3 summarizes the Lm values at 5 and 21 days after treatment in the wild-type and knock-out mice and in their saline controls. The 5-day post-PPE mean Lm values of the wild-type group and the TNFR(1 and 2) knock-out group were significantly lower ($p < 0.05$) than their respective 21-day values; the other geno-

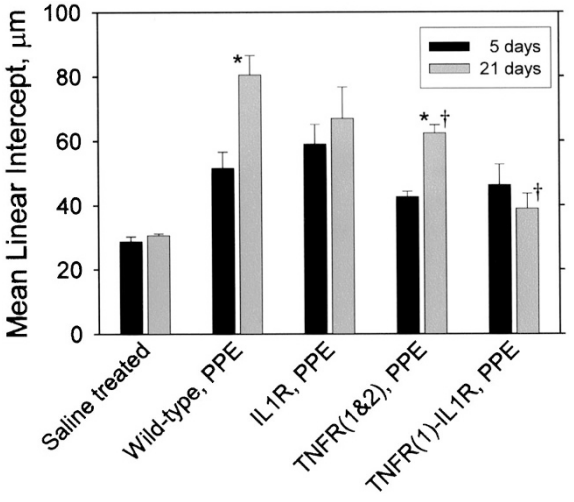


**Figure 3.**

Effect of PPE treatment (30 $\mu$g in 0.1 ml of saline) on mice deficient in either IL-1$\beta$ receptor [IL1R], TNF-$\alpha$ receptors 1 and 2 [TNFR(1&2)], or a combination of TNF-$\alpha$ type 1 receptor and the IL-1$\beta$ receptor [TNFR(1)-IL1R]. Controls received 0.1 ml of saline. All saline-treated groups, regardless of genotype, had similar mean linear intercept values and their values were combined. Values are shown as means ( SEM). Sample sizes for the 10 bars, from left to right, are as follows: 12, 22, 6, 9, 3, 5, 6, 8, 6, and 6 mice. The 21-day post-PPE mean Lm values of wild-type and TNFR(1)–IL-1R-deficient groups were significantly higher ($p < 0.05$, Kruskal-Wallis) than the 5-day values (indicated by *asterisks*). At 21 days both the TNFR(1 and 2) group and the TNFR(1)–IL-1R group showed significantly lower values than the 21-day wild-type group (indicated by *daggers*). The statistical comparisons among the eight PPE-treated groups were done with a two-way ANOVA test, with day of death considered as one treatment and genotype as the other.

types were similar at 5 and 21 days. At 21 days both the TNFR(1 and 2) group and the TNFR(1)–IL-R knock-out group showed significantly lower Lm values than the 21-day wild-type group. The mean Lm value was lowest in the TNFR(1)–IL-R knock-out group.

To assess the role of apoptosis in elastase-induced emphysema, we performed a terminal deoxynucleotidyl transferase (TdT)-mediated dNTP nick end-labeling (TUNEL) assay on the lung tissue. Elastase treatment resulted in increases in TUNEL-positive cells in the lungs of wild-type, PPE-treated mice (Fig. 4). Brown staining was detected in cells in the alveolar wall but was predominantly found in the cytoplasm of macrophages. Quantification of TUNEL-positive material by computer image analysis showed a comparable degree of attenuation of apoptosis in all three groups of receptor-deficient mice (Fig. 5).

## Discussion

Studies in humans with severe $\alpha$1-antitrypsin deficiency and in animal models suggest that smoking-induced emphysema results from proteolytic attack on structural proteins in the alveolar wall, including elastin, by enzymes released from inflammatory cells (Jones et al, 1985; Laurell and Ericksson, 1963). Controversy remains regarding the relative contribution of macrophage-derived versus neutrophil-derived enzymes in initiating the injury. Recent information strongly supports the role of macrophage-derived metalloproteinases in smoke-induced emphysema. Mice deficient in macrophage elastase and rats depleted of macrophages did not develop emphysema after long-term smoke exposure (Hautamaki et al, 1997; Ofulue and Ko, 1999).

Administration of a single endotracheal dose of pancreatic elastase to rodents induces a rapid increase in airspace size and an inflammatory reaction. Kinetic studies indicate that the exogenous elastase is removed from the lung within 24 hours (Stone et al, 1988). Our experimental results on the time-course of development of emphysema in wild-type mice after PPE treatment reveal that airspace enlargement continues for 10 or more days after the administered elastase is cleared from the lung (Fig. 1). This phenomenon is well known (Snider and Sherter, 1977) and has raised the possibility that progression of emphysema may relate to the inflammatory process. Hayes and colleagues (Hayes et al, 1975) described a mild infiltration of neutrophils 4 hours after PPE instillation to hamsters, which peaked by 1 day. However, neutrophilic infiltration was focal and not dramatic. Macrophages were increased by 8 hours and reached a maximum at 24 hours. The interstitial infiltrate had largely cleared by Day 4, but intraalveolar collections of macrophages, many hemosiderin laden, persisted up to Day 16.

Microscopic examination of the lungs of our mice showed cellular infiltration in the lungs of 5 day animals, which appeared to be less in the receptor-deficient mice than in the wild-type mice. To obtain a preliminary measurement of the severity of lung inflammation, two of us (ECL and GLS) graded inflammation on a 5 point scale (0 to 4) on one coded slide, from 6 animals each, from the 5 and 21 day wild-type and TNFR(1)-IL1R groups. Scores for each slide in a group were summed to give a group score, which was expressed as a percentage of the maximum possible score of 24. The inflammatory score for the 5 day wild type group was 50% and that for the TNFR(1)-IL1R group was 29% ($p < 0.05$, Mann-Whitney test). The 21 day values were 25% and 29%, respectively (NS).

All mice received the same dose of PPE that presumably induced a similar amount of emphysema during the initial 24-hour period. Our 5-day Lm results show variable data but generally support that assumption. The difference in Lm values between PPE and saline-treated mice (delta Lm) represents emphysema as a result of a combination of proteolysis caused by PPE and by the inflammatory response. The difference between the delta Lm of the wild-type group and the delta Lm of a receptor-deficient group represents the amount of inflammation-induced emphysema related to the mediator whose receptor is deficient.

This analytic approach is shown schematically in Figure 6. The delta Lm of the wild-type, 21-day, PPE-treated mice is set at 100%. The difference between the delta Lm of each receptor-deficient group and the wild-type group, expressed as a percentage of the wild-type delta Lm, expresses the amount of emphysema related to that receptor. Approximately 27% of total emphysema is related to

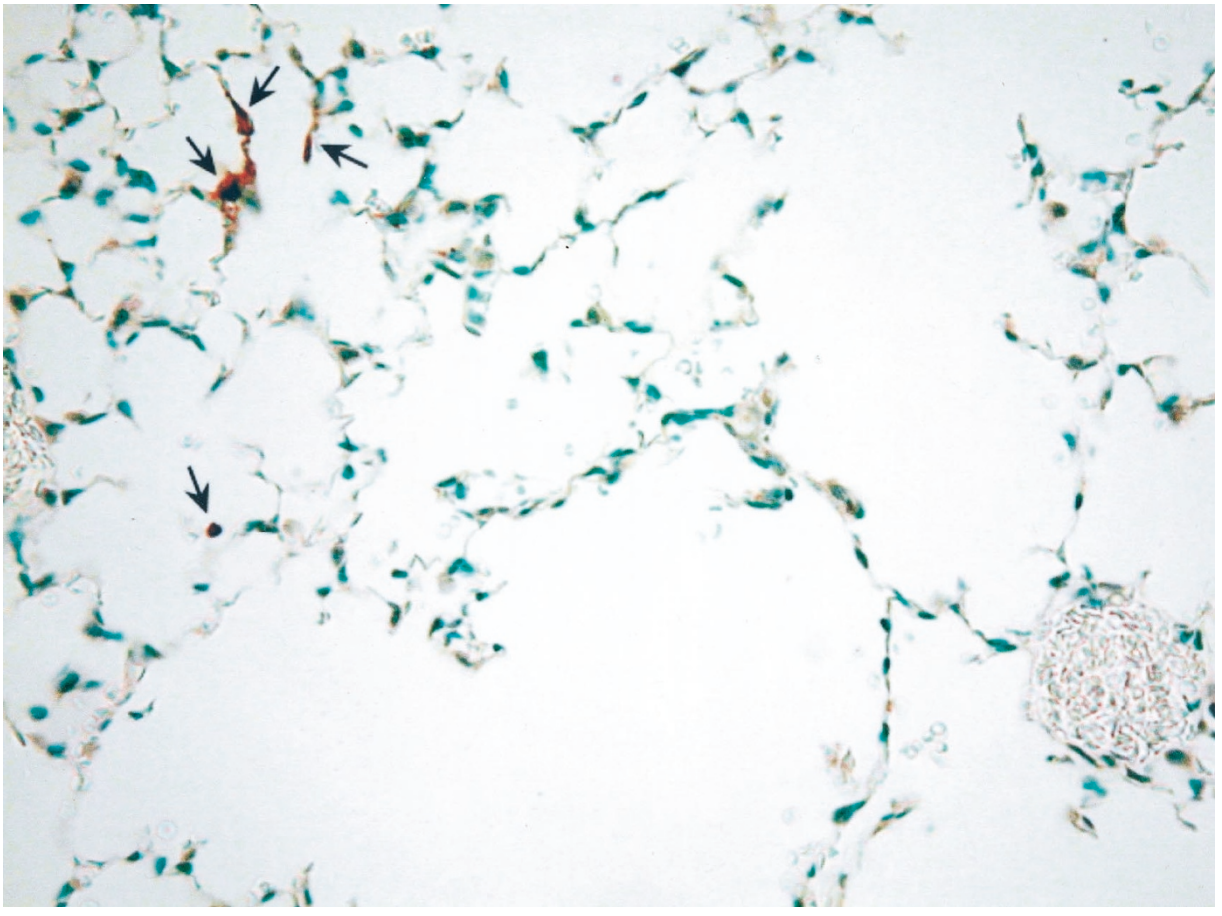

**Figure 4.**
Photomicrographs of lung parenchyma showing terminal deoxynucleotidyl transferase (TdT)-mediated dNTP nick end-labeling (TUNEL)-positive (brown-stained) cells 1 day after PPE treatment of a wild-type mouse (original magnification ×200, methyl green counterstain). The *arrows* indicate some of the TUNEL-positive areas.

IL-1$\beta$ type 1 receptor and 36% to TNF-$\alpha$ type 1 and 2 receptors. Knocking out both the TNF-$\alpha$ type 1 and the IL-1$\beta$ type1 receptors in mice results in an 81% decrease in the amount of emphysema or about 18% more than an additive response. This analysis suggests that only about 20% of the emphysema observed at 21 days is related to the direct effect of PPE, whereas about 80% is related to the effects of inflammation.

Activated macrophages release IL-1$\beta$ and TNF-$\alpha$ that can directly activate signal transduction pathways that affect connective tissue matrix production, proteolytic enzyme synthesis, and apoptosis (Dinarello, 1998; Kollias et al, 1999; Leong and Karsan, 2000). We previously reported that IL-1$\beta$ inhibits elastin production by rat lung interstitial fibroblasts (Berk et al, 1991). Others have reported that TNF-$\alpha$ inhibits both elastin and collagen deposition by lung fibroblasts (Greenwel et al, 2000; Kahari et al, 1992). Both TNF-$\alpha$ and IL-1$\beta$ stimulate apoptosis in many but not all cell types (Segura-Valdez et al, 2000). These results suggest that these effectors could act by inhibiting the resynthesis of connective tissue matrix in the alveolar wall and by increasing the loss of alveolar cells via apoptosis.

Recent evidence implicates alveolar cell loss in the pathogenesis of emphysema (Senior, 2000). Increases in the levels of the proapoptotic factor soluble Fas was reported in patients with chronic obstructive pulmonary disease (Yasuda et al, 1998). We found increases in apoptotic cells in the alveolar wall in elastase-treated mice at 5 days after injury. We also detected an increase in labeling within macrophages, suggesting that these cells ingested fragments from cells that had previously undergone apoptosis. The amount of apoptosis is decreased in TNF-$\alpha$ and IL-1$\beta$ receptor-deficient mice. Taken together our results support the concept that inflammation plays an important role in PPE-induced emphysema in mice. Inflammatory cells responding to the mediators IL-1$\beta$ and TNF-$\alpha$ may exacerbate proteolytic injury. Increased apoptosis of interstitial cells, also controlled in some measure by these mediators, likely also contributes to development of emphysema by impairing the repair process, but it is not the only factor.

## Materials and Methods

Three strains of knock-out mice were obtained from The Jackson Laboratory (Bar Harbor, Maine): (a) mice homozygous for both Tnfrsf1a$^{tm1Imx}$ and Tnfrsf1b$^{tm1Imx}$ targeted mutations (p55 and p75 deficient), (b) mice homozygous for the Il1r1$^{tm1Roml}$ targeted mutation, and

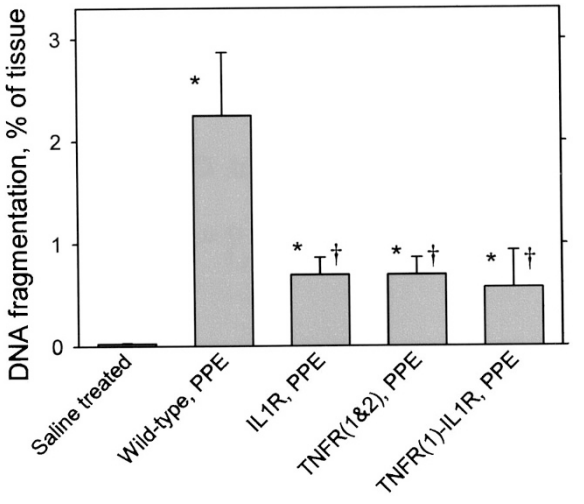


**Figure 5.**

Volume proportion of brown color in lung tissue with the TUNEL assay as an index of DNA fragmentation and apoptosis in mouse lung sections. Values are means (SEM). There is virtually no DNA fragmentation in saline-treated controls. There is a marked increase in DNA fragmentation in wild-type PPE-treated mice. There were smaller but significant increases in DNA fragmentation in IL-1R, TNFR(1 and 2), and TNFR(1)–IL-1R PPE-treated mice (indicated by *asterisks*). Values for the knock-out groups are significantly less, using ANOVA, than in the wild-type PPE-treated group (indicated by *daggers*). Sample sizes are three for all groups.

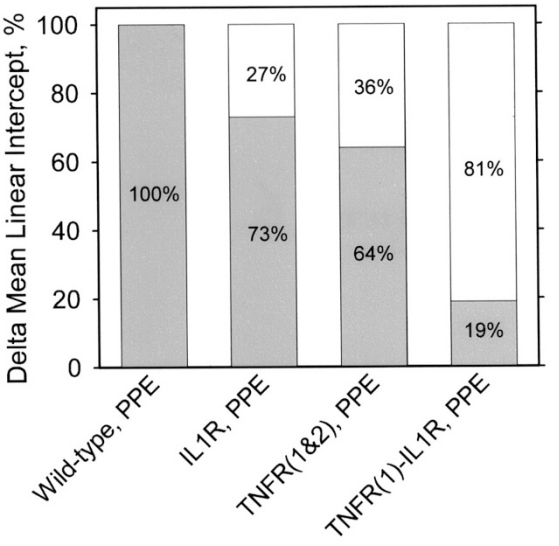


**Figure 6.**

Schema of proportion of emphysema mediated by TNF-$\alpha$- and IL-1$\beta$-related inflammation. The Lm of PPE-treated mice minus the Lm of saline-treated mice is referred to as the delta Lm (*gray portion of bar*). Delta Lm represents emphysema caused by the direct proteolytic effect of PPE plus the effects of inflammatory cells and mediators. For the TNFR(1)–IL-1R PPE-treated group, the delta Lm is 19% of that of the wild-type group. The difference between the delta Lm of the wild-type PPE-treated group and the knock-out PPE-treated group represents inflammation-induced emphysema (*white portion of bars*) related to that mediator or mediators. The sum of the mediator-induced emphysema of the IL-1R and TNFR(1 and 2) groups (27 + 36 = 63%) is 18% less than (81%) for the TNFR(1)–IL-1R group, suggesting that there may be a synergistic effect.

(c) mice homozygous for both the Tnfrsf1a$^{tm1Imx}$ and Il1r1$^{tm1Imx}$ targeted mutation. These mice will be referred to as double TNF-$\alpha$ receptor knock-out mice [TNFR(1 and 2)], IL-1$\beta$ type 1 receptor knock-out mice (IL-1R), and combined TNF-$\alpha$ type 1 and IL-1$\beta$ type 1 receptor knock-out mice [TNFR(1)–IL-1R], respectively. Wild-type mice used as controls were B6129SF2/J.

### *Treatment and Death*

The mice were anesthetized with methoxyflurane and given an intratracheal instillation of 30 $\mu$g of porcine pancreatic elastase (Elastin Products, St. Louis, Missouri) in 0.1 ml of sterile saline solution (0.9% NaCl) or 0.1 ml of saline alone. Groups of mice were killed for study at either 5 or 21 days after treatment. Animals were anesthetized and exsanguinated. The sternum was cut and the diaphragm removed to expose the lungs. The lungs were inflated until visibly taut (maximum volume) with freshly prepared paraformaldehyde through a tracheal cannula, using previously published methods (Lucey et al, 1996, 1998). The maximum volume was maintained for at least 2 minutes before the trachea was tied off to maintain inflation. The lungs, which harden rapidly with paraformaldehyde, were excised and floated in cold fixative. Two transverse slabs of tissue were cut from the left lung and one from the right caudal lobe. The same locations were sampled in all mice. The tissue was embedded in paraffin and 4-$\mu$m serial sections were cut, individually handled and numbered, and transferred to slides.

### *In Situ Localization of Apoptosis*

In situ nick end-labeling (TUNEL) was performed using the TdT-FragEl DNA Fragmentation Detection Kit (Oncogene Research Products, Boston, Massachusetts). Terminal deoxynucleotidyl transferase catalyzes the binding of biotin-labeled dNTP to the free 3'-OH termini of fragmented DNA. The biotinylated nucleotides are detected using a streptavidin-horseradish peroxidase conjugate. Diaminobenzidine reacts with the labeled sample to generate an insoluble brown-yellow product at the site of fragmented DNA.

### *Morphometry*

Quantitative histologic measurements were made with an image analysis system, consisting of a Nikon E600 microscope and a Spot RT Slider digital camera and computer with Image-Pro Plus image analysis software. Six fields from 4-$\mu$m-thick lung tissue sections of each mouse were photographed. From each field five areas of interest, free of airways and muscular blood vessels, were picked for measurement of the number of intersections of virtual lines of known length, with alveolar septa. An increase in the average distance between intercepts (Lm) indicates enlarged airspaces. The areas of interest were also analyzed for tissue area and lung-air area. The brown-yellow TUNEL-positive tissue was identified by a color-cube segmentation standard and expressed as a percentage of the lung tissue.

### *Statistics*

SigmaStat was used for statistical analysis. ANOVA tests were used to compare grouped data, except

were data did not pass tests of normality and equal variance. The Mann-Whitney Rank Sum and Kruskal-Wallis tests were used for nonparametric data. Probability values of $\leq 0.05$ were considered significant.

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