IL-6 Protects against Hyperoxia-Induced Mitochondrial Damage via Bcl-2-Induced Bak Interactions with Mitofusions

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Overexpression of IL-6 markedly diminishes hyperoxic lung injury, hyperoxia-induced cell death, and DNA fragmentation, and enhances Bcl-2 expression. We hypothesized that changes in the interactions between Bcl-2 family members play an important role in the IL-6-mediated protective response to oxidative stress. Consistent with this hypothesis, we found that IL-6 induced Bcl-2 expression, both *in vivo* and *in vitro*, disrupted interactions between proapoptotic and antiapoptotic factors, and suppressed H₂O₂-induced loss of mitochondrial membrane potential *in vitro*. In addition, IL-6 overexpression in mice protects against hyperoxia-induced lung mitochondrial damage. The overexpression of Bcl-2 *in vivo* prolonged the survival of mice exposed to hyperoxia and inhibited alveolar capillary protein leakage. In addition, apoptosis-associated DNA fragmentation was substantially reduced in these animals. This IL-6-mediated protection was lost when Bcl-2 was silenced, demonstrating that Bcl-2 is an essential mediator of IL-6 cytoprotection. Finally, Bcl-2 blocked the dissociation of Bak from mitofusion protein (Mfn) 2, and inhibited the interaction between Bak and Mfn1. Taken together, our results suggest that IL-6 induces Bcl-2 expression to perform cytoprotective functions in response to oxygen toxicity, and that this effect is mediated by alterations in the interactions between Bak and Mfns.

Keywords: lung injury; mitochondria; apoptosis; cytochrome c; Bax

Supplemental oxygen is commonly given to patients to enhance tissue oxygen delivery. However, prolonged administration of fractional inspired concentrations of oxygen greater than 50 to 60% can lead to lung tissue damage. The underlying mechanisms of pulmonary oxygen toxicity have been well characterized in animal models (1–5). Toxic concentrations of oxygen generate reactive oxygen species (ROS) that damage lung epithelial and endothelial cells, causing leakage of a protein-rich edema fluid that fills the alveolar space. In addition, several studies have demonstrated that hyperoxic lung injury is associated with cell death, which has features of both necrosis and apoptosis (6, 7).

We have previously shown that IL-6 protects against hyperoxic lung injury *in vivo* and oxidant-mediated cell death *in vitro* (6, 8, 9). IL-6 is a pleiotropic cytokine produced at sites of tissue inflammation, such as hyperoxic lung injury. IL-6 belongs to a family of cytokines that are characterized by their overlapping effector profiles and their shared use of gp130 as the α subunit in

CLINICAL RELEVANCE

Our study shows that IL-6 regulates mitochondrial fusion interactions with the proapoptotic protein Bak. Our data suggest that IL-6-induced increases in Bcl-2 regulate Bak interactions with mitofusions and Bcl-2 inhibits Bak dissociation from mitofusion protein (Mfn) 2. In addition, Bcl-2 inhibits Bak interaction with Mfn1. In this way, IL-6-induced Bcl-2 inhibits hyperoxia-induced cell death and imparts improved survival in IL-6-transgenic animals.

their multimeric receptor complexes (6, 9). The IL-6-mediated protective effects in response to oxidative toxicity are associated with markedly diminished alveolar capillary protein leakage, decreased endothelial and epithelial membrane injury, and reduced peroxidation of lung lipids. When IL-6 is specifically overexpressed in lung tissue, there is a potent increase in pulmonary Bcl-2 protein levels (9). This suggests that IL-6-induced Bcl-2 production and accumulation in the lung may play an important role in IL-6-mediated protection.

The Bcl-2 family proteins are key regulators of cell death and survival that can either inhibit or promote apoptosis. Family members include antiapoptotic Bcl-2 and Bcl-xl and proapoptotic Bax, Bad, truncated Bid (tBid), and Bim. Interactions between these antiapoptotic and proapoptotic Bcl-2 proteins exist in a delicate balance at the mitochondrial membrane that determines cell fate. Heterodimerization of antiapoptotic members, such as Bcl-2 or Bcl-xl, with proapoptotic members, such as Bax, Bad, tBid, or Bim, can inhibit or activate apoptosis depending on the relative levels of each protein. Recent reports suggest that mitochondrial fragmentation is a central mechanism of apoptosis. Mitochondrial outer membrane permeabilization causes mitochondrial fragmentation and leads to the release of apoptotic factors. Bax and Bak, two proapoptotic multidomain Bcl-2 proteins, are critical mediators of mitochondrial outer membrane permeabilization (10). Recent studies suggest that, during apoptosis, Bak regulates mitochondrial morphology by interacting with the mitofusion proteins (Mfns), Mfn1 and Mfn2. Upon induction of apoptosis, Bak dissociates from Mfn2 and associates with Mfn1 (10). The functions of Bcl-2 family members in IL-6-mediated protection of lung tissue from oxidative injury remain to be determined.

The studies described in this report define the roles of Bcl-2 proteins in IL-6-mediated protection. Our results confirm that Bcl-2 is induced by IL-6 and confers protection against hyperoxic damage *in vivo* and oxidant (H₂O₂) injury *in vitro*. Furthermore, we show that the ratio of proapoptotic to antiapoptotic factors is increased with hyperoxia, but that increased levels of IL-6 alter this ratio in favor of cell survival, both in IL-6-treated cells and IL-6-expressing transgenic mice. In addition, overexpression of Bcl-2 in the lung tissue of these mice inhibits hyperoxic acute lung injury (HALI). Notably, this protection against apoptosis is abrogated when cells are treated with Bcl-2 short hairpin RNA

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(shRNA) *in vitro*. Lastly, IL-6-induced Bcl-2 inhibits Bak dissociation from Mfn2 and inhibits Bak interaction with Mfn1.

MATERIALS AND METHODS

Reagents and Antibodies

The RiboQuant Multi-Probe RNase Protection Assay System and the hAPO-2b Apoptosis Multi-Probe Template Set (BD Biosciences, Franklin Lakes, NJ) were used according to the manufacturer's instructions. The following antibodies were used: rabbit polyclonal anti-Bcl-2 and goat polyclonal anti-tBid (Santa Cruz Biotechnology Inc., Santa Cruz, CA); rabbit polyclonal anti-Bax, anti-Bok, anti-Bik, anti-Puma, and anti-Bad (Cell Signaling Technology Inc., Danvers, MA); anti-Bak (Oncogene Research Products, Nottingham, UK); polyclonal chicken anti-Mfn1 (Novus Biologicals, Littleton, CO); rabbit polyclonal anti-Mfn2 (Sigma-Aldrich, St. Louis, MO). Recombinant human epidermal growth factor (hEGF), hydrocortisone, gentamicin, and amphotericin-B were purchased from Clonetics (San Diego, CA). Secondary antibodies were purchased from Jackson ImmunoResearch (West Grove, PA). Lentiviral-driven shRNA for Bcl-2 and control shRNA were a gift from Dr. María S. Soengas, University of Michigan Comprehensive Cancer Center (Ann Arbor, MI) (11). Recombinant E1-deleted adenovirus carrying the human Bcl-2 gene (AdCMVhBcl-2) without the transmembrane domain was from Dr. David Curiel, Gene Therapy Program, University of Alabama (Birmingham, AL) (12). All other reagents were purchased from Sigma.

Cell Culture

Human umbilical vein endothelial cells (HUVECs) were obtained from Clonetics (San Diego, CA) and grown in endothelial growth medium (EGM; Lonza Inc., Walkersville, MD) supplemented with growth factors and antibiotics according to the manufacturer's instructions. Confluent cultured cells were treated with IL-6 (50 and 100 ng/ml), as described previously (8). Briefly, cells were treated with IL-6 at 37°C, and then the medium was removed and replaced with standard growth medium containing 1 mM H₂O₂. After an additional 1-hour incubation, the cells were evaluated by immunoblot analysis.

JC-1 Staining Assay

Changes in mitochondrial membrane potential ($\Delta\Psi_m$) were detected by the uptake of the cationic carbocyanine dye, JC-1 (Sigma). HUVECs were grown to confluence in six-well, 35-mm plates, incubated in the presence or absence of IL-6 (100 ng/ml) for 1 hour, and treated with or without 1 mM H₂O₂ for 1 hour at 37°C. Cells were stained with JC-1 staining solution (2.5 μ g/ml of JC-1 dye in DMSO), incubated for 15 minutes, and visualized with a Nikon TE2000 Eclipse inverted microscope (Nikon Inc., Melville, NY).

Mice

Homozygous CC10-IL-6 Tg⁺ mice (C57BL/6 strain) were generated as previously described (8). In all cases, transgene-negative littermates served as wild-type (WT) control animals. The Institutional Animal Care and Use Committee at the Massachusetts General Hospital at Harvard Medical School approved all mouse work.

Oxygen Exposure

Mice (4–6 weeks old) were placed in cages in an airtight chamber (50 × 50 × 30 cm) and exposed to 100% oxygen for 72 hours. The oxygen concentration in the chamber was monitored with an oxygen analyzer (Vascular Technology, Inc., Chelmsford, MA), as previously described (6, 9, 13, 14).

Electron Microscopy

Five IL-6 Tg⁺ mice and six littermate control Tg[−] mice were exposed to hyperoxia for 3 days. Mice were anesthetized and median sternotomies performed. The trachea was dissected free and cannulated. The lungs were then perfused *in situ*, inflated to 20 cm of water pressure with 3.5% glutaraldehyde, postfixed for 24 hours in 3.5% glutaraldehyde, and representative areas from the left lung were embedded in epon and

stained with 2% uranyl acetate and osmium oxide. Mitochondrial alterations in endothelial cells of left lobe of the lung were examined using a JEOL 1011 electron microscope (JEOL Ltd., Tokyo, Japan).

Transfection Protocols

Cultured cells were treated with AdCMVhBcl-2, AdCMVGFP, or PBS, as described previously (12). Confluent HUVECs were stably transduced with 10⁹ plaque-forming units of adenoviral stock in the presence of 5 μ g/ml polybrene according to manufacturer's protocol (Millipore, Billerica, MA). This approach yielded greater than 95% stable transductants within 10–14 days. *In vivo* stable transduction of mice was achieved using adenoviral vectors. Mice were anesthetized by intraperitoneal injection of a ketamine/xylazine mixture (0.1 ml of 10 mg/ml ketamine, 1 mg/ml xylazine) in pyrogen-free saline. Before injection, the ventral neck area was cleaned with isopropyl alcohol. The mice were placed on an isothermal pad (Braintree Scientific Inc., Braintree, MA) warmed to 37°C to reduce hypothermia and quicken recovery time. The trachea of each mouse was exposed by a small incision in the neck skin. Next, 50 μ l of PBS containing adenovirus (10⁹ plaque-forming units) was injected into the trachea using a 50- μ l Hamilton syringe fitted with a 26-gauge needle. The incision was closed with wound clips, and the mice were kept warm and monitored until they recovered from anesthesia.

RNA Interference

shRNA-encoding lentivirus for silencing the Bcl-2 gene was generated in human embryonic kidney cells (293T), as previously described (11). A scrambled Bcl-2 oligonucleotide sequence (control-shRNA) was used as a control for these experiments. Supernatant viral particles were collected after 24 hours and used to infect HUVECs in EGM (Lonza) containing 10 μ g/ml sequabrene (Sigma-Aldrich). Four repeated rounds of lentiviral infection lasting 4 hours each attained silencing of Bcl-2 expression. The specificity of the knockdown was confirmed by immunoblot.

Bronchoalveolar Lavage

After anesthesia, a median sternotomy was performed, the trachea was dissected free from the underlying soft tissues, and a 0.6-mm tube was inserted into the trachea through a small incision. Bronchoalveolar lavage (BAL) was performed as previously described (6). The BAL was repeated three times for each mouse. The cell-free BAL fluid was stored at −70°C.

Analysis of BAL Protein and Lung Lipid Peroxidation

BAL protein. BAL protein was assayed and quantified as an index of lung injury and capillary leakage (6, 7, 9).

Lung lipid peroxidation. Lungs were isolated and perfused with ice-cold 0.9% NaCl containing 0.1% glucose, 30 mM HEPES, 6 mM KCl, 0.1 mg/ml streptomycin sulfate, 0.07 mg/ml penicillin G, 0.07 mg/ml EGTA, and 20 mM Tris-HCl (pH 7.4). The homogenates were then centrifuged at 3,000 × *g* at 4°C for 10 minutes, and 200 μ l of the supernatant was removed for the assay. Lipid peroxidation was assessed by quantitating the interaction of a chromogenic reagent with malonaldehyde and 4-hydroxyalkenals (6, 9) using the LPO-586 kit as per manufacturer instructions (Calbiochem Corp., San Diego, CA). Data was expressed as mM of products of lipid peroxidation in 200 μ l of a 20% wt/vol solution of lung homogenate. Reactions were performed at 45°C in acidic conditions, where one molecule of malonaldehyde or 4-hydroxyalkenals condensation with two molecules of chromogenic reagent, forms a stable chromophore with maximal absorbance at 586 nm.

Light Microscopy

Mice were anesthetized and median sternotomies were performed. The trachea was dissected free and cannulated, as described above. The lungs were then perfused *in situ*, inflated to 20 cm of water pressure with 10% formalin in PBS (pH 7.4), removed, and postfixed in 10% formalin in PBS for 24 hours. Tissues from all lung lobes were dehydrated, infiltrated, embedded in paraffin, and stained with hematoxylin and eosin, as previously described (6, 9).

Measurement of DNA Fragmentation

DNA fragmentation was quantified using the transferase-mediated dUTP nick end labeling (TUNEL) method according to the manufacturer's instructions (Calbiochem). For TUNEL analyses, mice were exposed to hyperoxia for 3 days. Mice were then anesthetized, median sternotomies performed, and trachea dissected free and cannulated as described above. The lungs were perfused *in situ*, inflated to 20 cm of water pressure with 10% formalin in PBS (pH 7.4), removed, and postfixed in 10% formalin in PBS for 24 hours. Tissues from all lung lobes were dehydrated, infiltrated, embedded in paraffin, and TUNEL analyses were performed (6, 9). A total of three mice were used from each group (AdCMVhBcl-2, AdCMVGFP, or PBS).

Immunoblotting

Cells were harvested and washed twice with ice-cold PBS containing 1 mM sodium orthovanadate and 1 mM sodium fluoride, and lysed with ice-cold radioimmuno precipitation assay lysis buffer (PBS, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM PMSF, 10 µg/ml leupeptin, 1 mM sodium orthovanadate). Cell lysates were clarified by centrifugation at 10,000 × *g* for 15 minutes, and the supernatant protein concentrations were determined using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA). Lysates were prepared for SDS-PAGE by adding an equal volume of 2× SDS-PAGE sample buffer (100 mM Tris-Cl [pH 6.8], 200 mM dithiothreitol, 4% SDS, 0.2% bromphenol blue, 20% glycerol), and heating the mixture at 100°C for 3 minutes; 20 µg of protein was separated on an SDS-PAGE gel and transferred to a polyvinylidene difluoride membrane by electrophoresis (Millipore, Bedford, MA). After blocking with Tris-buffered saline plus Tween (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 0.05% Tween 20) containing 5% nonfat dry milk for 1 hour at room temperature, the membranes were incubated with blocking solution containing primary antibody (diluted 1:1,000–1:2,000) overnight at 4°C. Membranes were washed

Figure 1. Induction of Bcl-2 by IL-6 with and without H₂O₂. Human umbilical vein endothelial cells (HUVECs) were cultured in the presence or absence of IL-6, with or without H₂O₂, and analyzed for Bcl-2 family proteins by immunoblot alternately with the indicated antibodies. Each panel is representative of a minimum of three similar evaluations. IB, immunoblot; p-Bad, phosphorylated Bad.

and incubated with horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch). Horseradish peroxidase activity was detected using an enhanced chemiluminescence kit according to the manufacturer's instructions (Pierce Biotechnology, Inc., Rockford, IL). Exposed films were scanned using a laser densitometer (Fast Scan, Series 300; Molecular Dynamics, Sunnyvale, CA).

Coimmunoprecipitation

Coimmunoprecipitation analysis was preformed as previously described (10) using the Seize Classic Mammalian Immunoprecipitation kit according to manufacturer's protocol (Pierce Biotechnology Inc.). Briefly, HUVEC lysates or lung homogenates were collected with a CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) buffer (2% CHAPS, 500 mM NaCl, 0.1% SDS, 1% NP-40); 500 µg of protein lysates were immunoprecipitated with 1 µg of anti-Bax or anti-Bcl2 antibody at 4°C overnight with agitation. The protein-antibody complex was further incubated with protein (25 µl) A/G-agarose beads (Pierce Biotechnology Inc.) for 2 hours. The resulting immunoprecipitates were dissolved in 2% SDS buffer for electrophoresis, and immunoblot analysis was used to detect the presence of Bax and Bcl-2.

Isolation of Mitochondria

Mitochondria were isolated from HUVECs or total lung homogenates by using the Mitochondria Isolation Kit according to the manufac-

Figure 2. Effect of IL-6 overexpression on the expression of Bcl-2 family proteins in hyperoxia. Whole-lung homogenate lysates from wild-type (WT) and IL-6 Tg⁺ mice before (RA, room air) and after exposure (3O₂) to 100% oxygen were analyzed by immunoblot, as indicated. Each panel is representative of a minimum of three similar evaluations.

turer's instructions (Pierce Biotechnology Inc.) and as previously described (15). The mitochondrial fraction was identified based on high expression of cytochrome C.

Isolation and Detection of RNA

Expression of mRNA was quantified by Northern blot analysis. Briefly, mice were anesthetized as described above, the right heart perfused with ice-cold PBS (pH 7.4), and the lungs removed and homogenized in TRIzol Reagent (Life Technologies, Grand Island, NY). Total RNA was obtained by processing the tissues according to the manufacturer's specifications. The mRNA was isolated using the Oligotex mRNA kit (Qiagen, Chatsworth, CA). The RNase protection assays were performed with RiboQuant kits (BD Biosciences-Pharmingen, Franklin Lakes, NJ) according to the manufacturer's instructions. Radiolabeled mRNA was visualized by autoradiography and quantified by densitometry (7).

Statistical Analysis

In all experiments, with the exception of survival analysis, *n* is equal to six in each group. Survival studies are based on 12 mice per group. Where appropriate, data are expressed as means ($\pm$ SEM). Data sets were examined by one- and two-way ANOVA, and individual group means were then compared with the Student's unpaired *t* test. Survival analysis was performed by Kaplan-Meier analysis. Differences in survival between treatment groups were assessed using χ^2 analysis.

RESULTS

Effect of Exogenously Added IL-6 on Bcl-2 Protein Expression

Overexpression of IL-6 in lung tissue results in increased levels of Bcl-2 protein (9). To better understand the role of Bcl-2 in IL-6-mediated protection from oxidant-mediated cell death, HUVECs were treated with IL-6 in the presence or absence of H₂O₂, and the effect on Bcl-2 protein expression was assessed

Figure 3. IL-6 regulates hyperoxia-induced interactions between Bcl-2 family proteins in HUVECs. Cells were treated with or without H₂O₂, in the presence or absence of IL-6, and interactions between Bcl-2 family proteins were analyzed in mitochondrial immunoprecipitates. (A) Bcl-2/Bax interaction was determined by immunoprecipitation with anti-Bcl-2 antibody followed by immunoblot with anti-Bax antibody. (B) Bcl-2/Bad interaction was determined by immunoprecipitation with anti-Bcl-2 antibody followed by immunoblot with anti-Bad antibody. (C) IL-6 treatment inhibits H₂O₂-induced Bid cleavage in HUVECs (lower band); arrow represents cleaved Bid. Each panel is representative of a minimum of three experiments. IP, immunoprecipitation.

by immunoblotting. IL-6 treatment (50 ng/ml) did not alter the protein expression of Bcl-2 (data not shown). However, repeated exposure to IL-6 (50 ng/ml, four times every 15 minutes) significantly enhanced Bcl-2 protein expression (Figure 1). Phospho-signal transducers and activators of transcription-3 protein expression was also enhanced in IL-6-treated cells (data not shown and Ref. 8). In contrast, IL-6 treatment does not alter the expression of other Bcl-2 family members. The expression of proapoptotic Bad is not altered by IL-6, but the inactivated form of Bad (phospho-Bad) is enhanced by IL-6 treatment (Figure 1). However, H₂O₂ treatment on Bcl-2 protein expression was not altered (Figure 1).

Figure 4. IL-6 regulates hyperoxia-induced interactions between Bcl-2 family proteins in mice. (A) Lung protein homogenates were prepared from mice exposed to room air (RA) or 100% oxygen (3O₂). Mitochondria were isolated from the total lung homogenates, and interactions between Bcl-2 family proteins were analyzed by immunoprecipitation and immunoblotting. Bcl-2/Bax interaction was determined by immunoprecipitation with anti-Bcl-2 antibody followed by immunoblot with anti-Bax antibody. (B) Bcl-2/Bad interaction was determined by immunoprecipitation with anti-Bcl-2 antibody followed by immunoblot with anti-Bad antibody. (C) Bax/Bid interaction was determined by immunoprecipitation with anti-Bax antibody followed by immunoblot with anti-Bid antibody; arrows indicate cleaved Bid. Each panel is representative of a minimum of three experiments.

Figure 5. Expression of human Bcl-2 construct in human umbilical vein endothelial cells (HUVECs). (A) RNase protection assays were used to assess the levels of mRNA encoding Bcl-2 family members in HUVECs. (B) HUVECs were transfected with Bcl-2, and cell lysates were extracted and analyzed for Bcl-2 protein expression by immunoblot.

Effect of IL-6 Overexpression on Bcl-2 Family Proteins in Hyperoxic Conditions

To better understand the mechanism(s) of IL-6-mediated protection from hyperoxia-induced cell death, we characterized the expression levels of Bcl-2 family proteins in the lungs of transgenic mice that overexpress IL-6, before and after exposure to 100% oxygen (Figure 2). Consistent with previous results, Bcl-2 expression was enhanced in the IL-6 Tg⁺ mice. Overexpression of IL-6 also augmented Bcl-2 expression in the lungs of mice exposed to 100% oxygen. In contrast, we saw no significant changes in either A1 or Bcl-x. Expression of Bad was slightly increased in IL-6 Tg⁺ mice exposed to room air or

hyperoxia. In addition, phospho-Bad levels were higher in IL-6 Tg⁺ mice, before and after exposure to 100% oxygen, than in littermate control animals. In addition, the expression levels of proapoptotic Bok and Bimf were reduced in IL-6 Tg⁺ mice compared with WT mice, before and after hyperoxia exposure. Bik was induced by hyperoxia in WT mice and, to a lesser extent, in IL-6 Tg⁺ mice (Figure 2).

IL-6 Regulates Hyperoxia-Induced Interactions between Bcl-2 Family Proteins

The relative ratio of Bcl-2 and Bax heterodimers to homodimers is a determinant of the susceptibility of many cell types

Figure 6. Mice treated with Ad Bcl-2 adenovirus. (A) Northern blot for mRNA encoding Bcl-2 at 24, 48, or 72 hours after treatment with virus. (B) Immunoblot for phosphorylation of Bcl-2 after 24, 48, or 96 hours in hyperoxic conditions.

Figure 7. Survival of Ad-Bcl-2 mice exposed to 100% oxygen. Kaplan-Meier plot of survival depicting the percent survival of AdCMVBcl-2-treated (diamonds), AdCMVGFP-treated (squares), and PBS-treated (circles) control mice (in each group, $n = 12$). Significant differences were found between the Ad-Bcl-2-treated mice and the other two groups by log-rank test. GFP, green fluorescent protein.

to apoptosis. The formation of Bcl-2/Bax heterodimers triggers apoptosis (16). We screened for hyperoxia-induced interactions between Bcl-2 and known binding partners by immunoprecipitating Bcl-2 from mitochondrial lysates and analyzing the precipitates by immunoblotting. Treatment of H_2O_2 (Figure 3A) or hyperoxia (Figure 4A) induced coprecipitation of Bax (21 kD) with Bcl-2. These results suggest that the ratio of proapoptotic Bax to antiapoptotic Bcl-2 is increased by hyperoxia or H_2O_2 treatment, but that increased levels of IL-6 alter this ratio in favor of cell survival, both in IL-6-treated cells and IL-6-overexpressing transgenic mice.

Recent studies suggest that the balance between pro- and antiapoptotic Bcl-2 family proteins is critical for apoptosis (16). Therefore, we also examined the interaction between Bcl-2 and Bad before and after exposure to IL-6 or hyperoxia. Extracts were subjected to immunoprecipitation using antibodies specific for Bcl-2. The immunoprecipitates were analyzed for the presence of Bad and Bcl-2 (Figures 3B and 4B). H_2O_2 induced an interaction between Bad and Bcl-2, and this interaction was reduced by IL-6 treatment of HUVECs (Figure 3B). In both IL-6 Tg^+ and WT mice, intact Bad was detected in Bcl-2 immunoprecipitates. Both hyperoxic conditions and oxidative stress enhanced the interaction between Bcl-2 and Bad, yet expression levels of Bad remained unchanged. These results suggest that the ratio of proapoptotic Bad to antiapoptotic Bcl-2 is increased by hyperoxia or in H_2O_2 -treated cells, but that increased levels of IL-6 alter this ratio in favor of cell survival, both in IL-6-treated cells and IL-6-expressing transgenic mice (Figure 4B).

Proapoptotic Bid undergoes proteolytic cleavage to form truncated active tBid (16). H_2O_2 induced Bid cleavage and IL-6 inhibited H_2O_2 -induced Bid cleavage in HUVECs (Figure 3C). Intact Bid was detected in both IL-6 Tg^+ and WT mice in room air (Figure 4C). However, significant hyperoxia-induced Bid cleavage occurs in WT but not IL-6 Tg^+ mice. tBid plays a crucial role in Bax insertion into mitochondrial membranes

Figure 8. Effect of Ad-Bcl-2 on alveolar capillary protein leakage. (A) Graph depicting alveolar capillary protein leakage in mice treated with PBS (control), AdCMVGFP, or AdCMVBcl-2. After 72 hours of exposure to 100% oxygen, the protein concentration in the bronchoalveolar lavage (BAL) fluid from each group was determined. Administration of AdCMVBcl-2 significantly decreased alveolar capillary protein leakage ($*P < 0.01$; $n = 6$). (B) Lung lipid peroxidation in mice treated with PBS (control), AdCMVGFP, or AdCMVBcl-2. After 72 hours of exposure to 100% oxygen, the malonaldehyde (MDA) concentration in the lung homogenates from each group was determined. Administration of AdCMVBcl-2 significantly decreased MDA and 4-HNE levels ($*P < 0.01$; $n = 6$).

and apoptosis (16). To examine whether hyperoxia affects the association of tBid with Bax, we analyzed the interactions between tBid and Bax before and after exposure to hypoxia. Lysates were immunoprecipitated with antibodies against Bax, and the precipitated proteins were subsequently analyzed for the presence of tBid and Bax. Hyperoxia induced an interaction between tBid and Bax, but the expression levels of Bid were not altered by oxygen exposure level or mice genotype. These results suggest that the ratio of proapoptotic tBid to antiapoptotic Bcl-2 is increased by hyperoxia or H_2O_2 treatment, but that increased levels of IL-6 alter this ratio in favor of cell survival, both in IL-6-treated cells and IL-6-expressing transgenic mice (Figure 4C).

Expression of Bcl-2 in HUVECs

In cell culture systems, the protein encoded by the Bcl-2 gene enhances cell survival by blocking cell death (17, 18). To study the *in vivo* function of Bcl-2 in mice, we made use of replication-defective adenovirus containing a constitutively active form of Bcl-2 (AdhCMVBcl-2). The functional activity of AdhCMVBcl-2 was first assessed by infecting HUVECs with either AdhCMVBcl-2 or control replication-defective adenovirus, Ad-CMVGFP. Changes in Bcl-2 mRNA were evaluated using RNase protection assays, and Bcl-2 expression was monitored with immunoblots. Levels of Bcl-2 mRNA were significantly higher in HUVECs treated with AdCMVBcl-2, even in the absence of H_2O_2 , than in HUVECs that had been treated with

control virus in either the presence or absence of H_2O_2 (Figure 5A). Immunoblots detected higher Bcl-2 expression in HUVECs treated with AdCMVBcl-2 than in cells treated with control virus (AdCMVGFP) (Figure 5B). These data show that transfection of HUVECs with Ad-Bcl-2 results in enhanced expression of Bcl-2 protein *in vitro*.

Time Course of Alterations of Bcl-2 mRNA and Protein in Mouse Lung Tissue

To assess changes in Bcl-2 mRNA levels, mice were treated with AdCMVBcl-2 via tracheal injection. Bcl-2 mRNA increased 24 hours after treatment (Figure 6A), after which the mRNA returned to baseline levels for the duration of the time course. Bcl-2 mRNA levels did not change in the lungs of mice treated with control virus or with PBS. To confirm the expression and activation of Bcl-2 in the lungs of infected mice, lung extracts were analyzed by immunoblotting with antibodies to both the phosphorylated (activated) and nonphosphorylated Bcl-2 protein (Figure 6B). Adenovirus-derived transgenic expression of Bcl-2 is evident within 24 hours of infection with AdCMVBcl-2, but not in mice infected with the control virus. To detect what cells the adenoviral vectors were being taken up by, we also treated mice with AdCMVGFP and then examined where the fluorescent protein was located (*see* Figure E1 in the online supplement).

Overexpression of Bcl-2 Prolongs Survival of Hyperoxic Mice and Inhibits Oxidative Lung Damage

Having confirmed that AdCMVBcl-2 increases Bcl-2 protein both *in vitro* and *in vivo*, we investigated whether overexpression of Bcl-2 could prevent oxidative lung damage and death of hyperoxic mice. In these experiments, control adenovirus, PBS, or the adenovirus expressing AdCMVBcl-2 were introduced intratracheally into mice. After 24 hours, mice were exposed to 100% oxygen. Control mice began to die within 72 hours, and all of the control mice were dead by 96 hours of continuous exposure to 100% oxygen. In contrast, mice infected with AdCMVBcl-2 lived significantly longer when compared with control mice treated with PBS ($P < 0.002$) or AdCMVGFP ($P < 0.005$) (Figure 7).

Effect of AdCMVBcl-2 on Alveolar Capillary Protein Leakage

Hyperoxic lung damage is characterized by leakage of a protein-rich edema fluid that fills the alveolar space. To further characterize the protective effects of AdCMVBcl-2, we compared the levels of protein in the BAL fluids from AdCMVBcl-2-treated mice to those from control animals. Before oxygen exposure (baseline), similar levels of BAL protein were detected in AdCMVBcl-2-treated and control mice. Exposure to 100% oxygen caused a significant increase in alveolar capillary leakage and BAL fluid protein levels. This response was significantly greater in control mice compared with the AdCMVBcl-2-treated mice. Within 72 hours of exposure to 100% oxygen, the BAL fluid from control animals contained $2.03 (\pm 0.14) \mu\text{g}/\mu\text{l}$ protein, whereas AdCMVBcl-2-treated mouse BAL fluid contained $1.38 (\pm 0.45) \mu\text{g}/\mu\text{l}$ of protein ($P < 0.05$) (Figure 8A). These studies suggest that increased Bcl-2 decreases hyperoxia-induced alveolar capillary protein leakage.

Peroxidation of lipids in the lungs is another indicator of oxygen toxicity and lung damage. Therefore, we examined lipid peroxidation in lung homogenates from AdCMVBcl-2-treated and control mice, both before and after exposure to hyperoxia. At baseline, similar levels of lipid peroxidation byproducts existed in the lungs of AdCMVBcl-2-treated and control mice (Figure 8B). After 72 hours of exposure to 100% oxygen, both the control mice and the AdCMVBcl-2-treated mice had increased levels of lung lipid peroxidation. No statistically significant differences were detected between any of the control mice and those treated with AdCMVBcl2. These data suggest that, despite the observed survival benefit, overexpression of Bcl-2 in the lungs of mice has no significant impact on hyperoxia-induced membrane lipid peroxidation.

Effect of AdCMVBcl-2 on Nuclear DNA Fragmentation

An increase in cell death often accompanies hyperoxic lung damage. This cell death results from a combination of necrosis and apoptosis, both of which cause DNA fragmentation. Therefore, TUNEL assays were used to assess the levels of DNA fragmentation and cell death in AdCMVBcl-2-treated and control mice, both before and after exposure to 100% oxygen. In these experiments, lung sections from mice infected

Figure 9. Transferase-mediated dUTP nick end labeling (TUNEL) assay depicting the effect of AdCMVBcl-2 on cellular DNA fragmentation. (A) DNA fragmentation in mice treated with AdCMVGFP and exposed to 100% oxygen for 72 hours. (B) DNA fragmentation in mice treated with AdCMVBcl-2 and exposed to 100% oxygen. Stars represent positive TUNEL staining.

Figure 10. IL-6 regulates Bak interactions with mitofusions. (A) Immunoblot and immunoprecipitation of lung homogenates from WT and IL-6 Tg⁺ mice exposed to room air (RA) or 100% oxygen (3O₂). Whole-lung lysates were collected with 2% CHAPS buffer and subjected to immunoprecipitation using an anti-Bak antibody. The immunoprecipitates were analyzed for Mfn1, Mfn2, and Bak by immunoblotting. (B) Immunoblot of HUVECs treated with or without IL-6 in the presence or absence of H₂O₂. Whole-cell lysates were immunoprecipitated using an anti-Bak antibody and probed for Mfn1 and Mfn2. (C) Immunoblot of HUVECs transfected with Bcl-2. Cells were mock treated or exposed to H₂O₂, and whole-cell lysates were collected for immunoprecipitation using an anti-Bak antibody. The resultant immunoprecipitates were analyzed for Bak, Mfn1, and Mfn2 by immunoblotting.

with adenovirus expressing constitutively active Bcl-2 or control adenovirus were assayed for nuclear DNA fragmentation (Figure 9). Mice infected with control virus and subjected to hyperoxia displayed extensive TUNEL-positive nuclei in the airway epithelium after 72 hours of exposure. In contrast, DNA fragmentation was almost completely blocked in mice infected with AdhCMVBcl-2. After 72 hours of hyperoxia exposure, 54.2 (±5.0)% of the cells in the lungs from the control virus-treated mice (AdCMVGFP) had detectable DNA fragmentation versus

13.5 (±5.0)% of the cells in the lungs from the AdCMVBcl-2-treated mice ($P < 0.01$). These studies confirm that hyperoxia induces DNA fragmentation in lung tissue, and suggest that increased Bcl-2 expression exerts a protective effect in part by inhibiting DNA fragmentation.

IL-6 Regulates Hyperoxia-Induced Bak Interactions with Mfns

Mitochondrial fragmentation is an important indicator of apoptosis-induced cell death. The overexpression of Bcl-2 inhibits mitochondrial fragmentation during apoptosis (10). Interactions between Bak and the mitochondrial fusion proteins, Mfn1 and Mfn2, are also critical regulators of mitochondrial fragmentation (10). Upon induction of apoptosis, Bak dissociates from Mfn2 and associates with Mfn1. To determine whether IL-6-induced Bcl-2 expression regulates mitofusion interactions with Bak, thus protecting against cell death, we analyzed endogenous protein interactions by coimmunoprecipitation. Immunoprecipitation was performed with anti-Bak antibodies, and the precipitates were immunoblotted for Mfn1 and Mfn2 (Figure 10). Upon apoptotic induction by H₂O₂ or hyperoxia in WT mice, Bak dissociates from Mfn2 and has increased interaction with Mfn1. Bak interacted with Mfn2 in the lung tissue of IL-6 Tg⁺ mice before and after exposure to hyperoxia (Figure 10A). Interestingly, IL-6 treatment blocked the dissociation of Bak from Mfn2, and inhibited the interaction between Bak and Mfn1 (Figure 10B).

To further examine the effect of Bcl-2 overexpression on Bak interactions with Mfn1 and Mfn2, we transfected HUVECs with a Bcl-2 expression plasmid and analyzed Bak/Mfn interactions by immunoblotting (Figure 10C). Bcl-2 overexpression inhibited the interaction between Mfn1 and Bak. This suggests that either IL-6 overexpression in mice or Bcl-2 overexpression in HUVECs regulates Bak interactions with mitofusions in response to oxidative stress.

IL-6 Overexpression Protects against Hyperoxia-Induced Lung Mitochondrial Damage

In the course of causing cell damage and death, hyperoxia leads to alterations in mitochondrial morphology (1). To assess the role of IL-6 overexpression in limiting hyperoxic damage, we compared the ultrastructural features of mitochondria in the left lobe of the lungs of WT and IL-6 Tg⁺ mice after exposure to 100% oxygen for 72 hours (Figure 11A). WT mice exposed to hyperoxia had shrunken lung mitochondria with irregular outer membranes and concentric lamination of the cristae. In addition, these mitochondria showed lytic changes, with loss of outer membrane integrity and marked swelling of the matrix. In contrast, the lung mitochondria of IL-6 Tg⁺ mice exposed to hyperoxia had well preserved outer membranes, mild swelling, and minimal alteration of the cristae (Figure 11A).

IL-6 Suppresses H₂O₂-Induced Loss of Ψ_m In Vitro

Mitochondrial swelling and damage causes the loss of mitochondrial membrane potential ($\Delta\Psi_m$), which can be measured using the mitochondrial dye JC-1 (19). Under normal circumstances, JC-1 accumulates in the inner mitochondrial membrane where it oligomerizes and fluoresces red. A reduction in $\Delta\Psi_m$ results in diffusion of the dye from the mitochondria, a subsequent reduction in the red fluorescent intensity throughout the entire cell, and a shift from red fluorescence (JC-1 aggregates) to green fluorescence (JC-1 monomers). As expected, control cells showed high levels of red fluorescence after staining with JC-1 (Figure 11B). After treatment with 1 mM H₂O₂ for 1 hour, the red fluorescence changed to green

Figure 11. Effect of IL-6 on mitochondrial morphology and mitochondrial membrane potential (Ψ_m). Transmission electron micrographs of mitochondrial morphology of the lungs of WT and IL-6 Tg⁺ mice after exposure to 100% oxygen for 72 hours. (A) WT mitochondria (I) show relative shrinkage with irregular outer membranes (open arrow) and concentric lamination of the cristae (closed arrows). Transgenic mitochondria (II) show lytic changes with loss of outer membrane integrity (open arrow), and marked swelling (asterisk) of the matrix. N, nucleus. In IL-6 Tg⁺ mice, the mitochondria show well preserved outer membranes (open arrows), and mild swelling and disarray of the cristae (closed arrows). Both panels show representative endothelial cells and are shown at the same original magnification (50,000 $\times$). (B) Control HUVECs with or without IL-6 pretreatment (100 ng/ml, 1 hour) were treated with H₂O₂ (1 mM, 1 hour), stained with JC-1 dye, and visualized with a TE2000 inverted microscope (Nikon, Tokyo, Japan).

fluorescence, indicating that the $\Delta\Psi_m$ had collapsed. HUVECs pretreated with IL-6 (100 ng/ml) showed a reduction in green fluorescence intensity. These results suggest that IL-6 suppress H₂O₂-induced loss of Ψ_m (Figure 11B).

Bcl-2 shRNA Blocks IL-6-Mediated Protection of Cultured HUVECs from H₂O₂-Induced Cell Death

Our findings indicate that Bcl-2 functions as an IL-6-induced cytoprotective molecule in both HUVECs and whole lungs. To further understand the mechanism of this protection against oxidative stress, we used a lentiviral-driven shRNA to block expression of endogenous Bcl-2. HUVECs were transfected with the shRNA vector and examined to assess cell viability (Figure 12A). Disruption of Bcl-2 expression by shRNA blocked IL-6 induced cytoprotection from oxidative stress. Bcl-2 shRNA efficiently reduced Bcl-2 expression (Figure 12B). IL-6 inhibited H₂O₂-induced cell death in control shRNA-treated cells, but IL-6 failed to protect cells transfected with Bcl-2 shRNA from H₂O₂-induced cell death. These results suggest that Bcl-2 is required for IL-6-mediated cytoprotection. The observation that overexpression of either Bcl-2 or IL-6 influences Bak interactions with Mfn1 and Mfn2 led us to ask whether Bcl-2 silencing has any effect on these interactions. We analyzed endogenous protein interactions by immunoblotting Bak immunoprecipitates with Mfn1 and Mfn2 antibodies (Figure 12B). Silencing of Bcl-2 inhibited the effect of IL-6 on the interactions of Bak with Mfns, and also inhibited the association between Bak and Mfn2. Collectively, these results suggest that IL-6 influences the interaction of Bak with Mfns by inducing Bcl-2 expression.

DISCUSSION

The results presented here provide new insight into the roles of IL-6 and Bcl-2 in the protection of lung tissue from hyperoxia. Our studies confirm that Bcl-2 is induced by IL-6 *in vivo* and *in vitro*, and that this induction is a key event in IL-6-mediated cytoprotection from oxygen toxicity. Mice expressing exogenous Bcl-2 in the trachea have significantly enhanced survival in 100% oxygen and reduced HALI. This may be the direct result of the observed inhibition of alveolar capillary protein leakage and DNA fragmentation, both indicators of cell damage or death. Bcl-2 was also shown to mediate cytoprotection from hyperoxia by altering interactions of Bak with Mfns. The ratios between Bcl-2 family proteins were shown to be critical in determining the extent of hyperoxia-mediated lung damage. The novel aspect of the present study is the finding that IL-6 induced Bcl-2 regulates Bak interactions with mitofusions via inhibition of Bak dissociation from Mfn2. In addition Bcl-2 inhibits the interaction of Bak with Mfn1. These two events are considered to be vital signaling determinants in the cell death pathway (10), and thus, when their relative levels are modified by increased IL-6 and Bcl-2, there is inhibition of hyperoxia-induced apoptosis. In our animal model, this translates into improved survival in hyperoxia.

Our previous studies showed that IL-6 is protective against HALI (6, 8, 9). In addition, IL-11 (an IL-6 cytokine family member) is cytoprotective against HALI through Bcl-2-related protein, A1 (7). Overexpression of IL-11 resulted in significant increases in A1 mRNA and protein, and modest increases in BCL-2 and BCL-xL message. Although there were no significant changes in BCL-2 or BCL-xL induced by exposure to room

Figure 12. Bcl-2 silencing restores sensitivity to H₂O₂-induced apoptosis. (A) Survival of HUVECs after Bcl-2 silencing, with or without treatment of IL-6, in the presence or absence of H₂O₂. The asterisk indicates significantly enhanced survival compared to H₂O₂-treated cells (¶) (**P* < 0.05; ***P* < 0.01). (B) Immunoblot showing down-regulation of Bcl-2 by specific short hairpin RNA (shRNA). HUVECs were transfected with Bcl-2 shRNA or control shRNA. After transfection, cells were treated with or without H₂O₂, whole-cell lysates were collected, and lysates analyzed for Bcl-2 expression by immunoblot (top). Whole-cell lysates were used for immunoprecipitation (IP) using an anti-Bak antibody. The resultant immunoprecipitates were analyzed for Bak, Mfn1, and Mfn2 by immunoblotting. The images are representative of a minimum of four experiments.

air, there were modest increases in these proteins after exposure to hyperoxia, which was abrogated in A1-knockout mice (7). This contrasts with our prior (9) and present findings, that IL-6 overexpression leads to significantly increased expression of Bcl-2, but not A1 or BCL-xL, in animals exposed to room air or hyperoxia. IL-6 has also been shown to affect liver regeneration and repair after injury by inducing the expression of downstream antiapoptotic proteins, including Bcl-2 and Bcl-xL through signal transducer and activator of transcription-3-mediated gene regulation (20, 21). Bcl-2 is also protective against diverse cell death stimuli, such as depletion of trophic factors, antitumor drugs, oxygen free radicals, and viral agents in multiple cell types, including epithelial cells, endothelial cells, cardiac myocytes, lymphocytes, and motor neuron hybrid cells (17, 21–23). The ratio between prosurvival and proapoptotic proteins (Bcl-2/Bax) dictates the susceptibility or resistance of cells to apoptotic signals. Thus, we hypothesized that IL-6 may exert a cytoprotective affect against HALI by regulating the expression levels and ratios of Bcl-2 family members. Our results show that the ratios of Bax and Bad to Bcl-2, critical determinants of mitochondria-mediated apoptosis, are higher in hyperoxic WT mice than in IL-6 Tg⁺ mice. This is in contrast to prior work by O'Reilly and colleagues (24), which suggested that hyperoxia-induced lung injury does not depend on the relative expression levels of Bcl-2 members. In the current

study, however, the levels of Bcl-2-related proteins are altered in response to hyperoxia, with overexpression of IL-6 resulting in exaggerated levels of Bcl-2. These results suggest that the relative amounts can be important. It is possible that there is a threshold effect whereby the ratio of these proteins to each other is shifted such that Bcl-2, in this case, overwhelms the effects of the proapoptotic proteins, altering the cell death process.

The oligomerization and insertion of Bax into the outer mitochondrial membrane is induced by tBid (17). Consistent with this known mechanism, cell death was enhanced when Bax associated with tBid in our studies. This suggests that, when hyperoxia induces tBid expression, tBid interacts with Bax, and Bax oligomerizes and inserts into the outer mitochondrial membrane, leading to the observed cell death in WT mice that was suppressed in IL-6 Tg⁺ mice.

Our data show that the ratio of Bcl-2 to Bax is greater in the lungs IL-6 Tg⁺ mice in comparison to WT mice. This is a possible mechanism through which IL-6 induces protection from hyperoxia. Previous studies suggest that an increased Bcl-2/Bax ratio inhibits the release of cytochrome-c from the mitochondria and reduces the production of ROS during cell death (25). The Bcl-2/Bax ratio in mitochondria also plays a critical role in the regulation of the apoptosis pathway. The antioxidant property of Bcl-2 prevents effector caspase activa-

tion by blocking docking proteins and by decreasing cellular ROS (25, 26). A reduced Bcl-2/Bax ratio and higher Bax/Bcl-2 ratio enhances the mitochondria permeability transition pore permeability (27). In hyperoxic conditions, increased ROS, reduced Bcl-2/Bax ratios, and heightened Bax/Bcl-2 ratios augment mitochondria permeability transition pore opening in mice. Recent reports suggest that a high Bcl-2/Bax ratio, induced by exogenous expression of Bcl-2, inhibits ROS production (27). Thus, protection from hyperoxia in our Bcl-2-overexpressing mice is likely the result of inhibition of ROS production by exogenous Bcl-2 expression. In addition, Bcl-2 has been suggested to inhibit hyperoxia-induced cell death by modulating mitochondria-dependent apoptotic pathways and increasing intracellular antioxidants (28). Recent studies also suggest that glutathione (GSH) binding by Bcl-2 plays a central role in the mitochondrial antioxidant function of Bcl-2 (17, 26).

A critical outcome of this study is that silencing of Bcl-2 in IL-6-treated HUVECs eliminated the cytoprotective effect of IL-6. Although mice infected with AdCMVBcl-2 had significantly enhanced survival in 100% oxygen, the survival benefit was not of the same magnitude as seen in our transgenic mice overexpressing IL-6. This suggests that an increased level of Bcl-2 is not the only mechanism by which IL-6 has protective effects.

These results raise important questions about how Bcl-2 protects the lung from oxidative damage. For example, is the inhibition of the cell death response or the antioxidant activity of Bcl-2 the most important mechanism in the cytoprotection against oxidative damage? Viral gene transfer of AdCMVBcl-2 increases Bcl-2 expression in the lungs of mice, enhances mouse survival in hyperoxic conditions, and decreases hyperoxic lung damage. AdCMVBcl-2-treated mice displayed enhanced phosphorylation of Bcl-2, which has been associated with antiapoptotic activity (29); this may help explain the observed Bcl-2-mediated cytoprotection. Our data demonstrate that Bcl-2 is cytoprotective in a hyperoxic lung injury model, and provide further evidence that IL-6-mediated protection of lung tissue from oxidative damage is mediated in part by Bcl-2 (9). In addition, the reduced capillary leakage observed in AdCMVBcl-2 mice may be due to direct inhibition of apoptosis by Bcl-2.

An important observation, made for the first time in this study, is that IL-6 augments the interaction between Bak and Mfns. Mitochondrial fragmentation is a vital part of mitochondria-mediated apoptosis and is triggered by interactions between Bak and Mfns. The interaction of Bax and Mfn2 occurs in non-apoptotic cells, and has been postulated to inhibit apoptosis (10). Our data show that oxidative stress causes Bak to dissociate from Mfn2 and preferentially associate with Mfn1, tipping the balance of pro- and antiapoptotic factors to favor apoptosis. Consistent with this, IL-6 treatment and Bcl-2 overexpression prevented Bak dissociation from Mfn2 during exposure to oxidative stress, suggesting that this may be an important component of IL-6-mediated cytoprotection from oxidative stress.

Bad is a critical regulator of apoptosis. Phosphorylated Bad binds to 14-3-3, a survival protein that sequesters Bad in the cytoplasm in an inactive form in response to survival signals (30, 31). Once survival signals are abrogated, Bad is dephosphorylated, dissociates from 14-3-3, localizes to the mitochondria, and performs proapoptotic functions. Our data indicate that Bad is phosphorylated in IL-6 Tg⁺ mice and IL-6-treated HUVECs, but not in WT or control cells. Thus, phosphorylated Bad phosphorylation may contribute to the protective effects of IL-6. Free mitochondrial Bad interacts with either Bcl-2 or Bcl-X_L, and neutralizes their antiapoptotic functions (32). The interactions between Bad and Bcl-2 would be expected to be enhanced in WT mice, likely resulting in increased apoptosis in response to oxidative stress. Our studies suggest that the pro-

TECTIVE effects of IL-6 can be explained in part by the regulation of Bcl-2 interaction with Bad.

Results from this study add to our knowledge of the mechanism of this protective response by demonstrating that Bcl-2 is a critical mediator of IL-6-induced cytoprotection. In summary, IL-6-mediated protection against hyperoxia is partly mediated by up-regulation of Bcl-2 expression, regulation of Bcl-2 family member interactions, and the regulation of Bak/Mfn interactions. Further investigation is needed to gain mechanistic insights into the regulation and functionality of the Bak/Mfn interactions. Understanding the mechanisms of IL-6-mediated protection from hyperoxia-mediated lung damage is critical to the development of clinical interventions for the protection of patients requiring supplemental oxygen.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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