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Title: Erythropoietin inhibits respiratory epithelial cell apoptosis in a model of ALI.

Running title: EPO: cytoprotective in lung epithelium.

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Abstract

Background: Fas-mediated apoptosis of the alveolar epithelium is important in the pathogenesis of ARDS. Erythropoietin has cytoprotective properties in other organ systems, and is relatively deficient in critical illness. This study investigates a potential role for erythropoietin in reducing apoptosis in a model of acute lung injury.

Methods: Apoptosis was induced in A549 or NHBE cells by Fas activation with CH-11 Fas-cross-linking antibody or by co-culture with peripheral blood neutrophils in a transwell system. The effect of rhEPO on apoptosis was measured by PARP-cleavage and Cell Death Detection Assay. Specific EPO-EPOR mediated effect was determined using EPO-blocking antibody or EPOR siRNA.

Results: Expression of EPOR was demonstrated in A549, NHBE and normal human alveolar epithelium. Fas- and neutrophil-mediated apoptosis of A549 and NHBE cells was inhibited by rhEPO by specific EPO-EPOR mediated mechanism. This anti-apoptotic effect was associated with induction of a pro-apoptotic Bcl-xL/Bax ratio.

Conclusion: Erythropoietin has cytoprotective properties in respiratory epithelium in an *in vitro* model which may indicate a potential therapeutic role in acute lung injury.

Introduction

Acute lung injury (ALI)/ Acute Respiratory Distress Syndrome (ARDS) is a complex devastating disease complicating acute inflammatory conditions including sepsis, trauma and pneumonia. Despite advances in supportive care, mortality remains high at 30-50% (1).

ARDS is characterised by neutrophilic inflammation and injury to the endothelium and epithelium. Activation of the vascular endothelium occurs rapidly in response to local or systemic inflammation, resulting in increased permeability, extravasation of protein rich fluid and interstitial oedema along with adherence and margination of neutrophils. Alveolar flooding results if microvascular and interstitial pressures are high. Intact alveolar epithelium allows active clearance of alveolar oedema by active sodium and chloride transport. Damage to the alveolar epithelium, however, results in disruption of normal epithelial fluid transport (2), along with reduced production and turnover of surfactant (3), and loss of the tight epithelial barrier allowing flooding of the alveoli with high molecular weight proteins and fluid. The degree of epithelial damage in ARDS is predictive of outcome (4).

Studies of humans and animals point to apoptosis as an important mechanism of epithelial cell death in ARDS. Histopathological changes of apoptosis have been demonstrated in both early and fatal ARDS (5, 6) in humans and in animal models of LPS-induced acute lung injury (7, 8). Apoptosis occurs in response to activation of specific cell membrane receptors, termed "death receptors", of which the Fas receptor (CD95) is the best characterized. Several lines of evidence point to the importance of Fas/FasL signalling in ALI. Soluble FasL (sFasL) is found in the BAL of patients with ARDS, where levels correlate with the severity of disease (9), while Fas and FasL expression and co-localisation in alveolar epihlteial cells has been shown in post mortem specimens from patients who died of ARDS (10). In animal models, administration of inhaled agonist anti-Fas antibody (Jo2) induces alveolar cell apoptosis and other pathological changes of ALI (11, 12). LPS-induced lung injury in mice is associated with increased epithelial cell expression of Fas, and infiltration of FasL expressing inflammatory cells (8) while mice with mutations of Fas (*lpr*) or FasL (gld), or those treated with antagonist anti-Fas antibody, are protected against LPS-induced ALI (8, 13).

Erythropoietin (EPO) is a 30.4 kDa glycoprotien, which by activation of the EPO receptor (EPOR) is the critical modulator of erythroid production in response to anaemia (14). The stimulus for EPO production and EPOR expression is not red cell mass however, but rather tissue oxygen supply via upregulation of the hypoxia-inducible factor (HIF)-1α. The kidney is the major site of production and secretion of EPO; however EPO secretion has also been demonstrated in diverse tissues including nervous, vascular, liver, intestinal epithelium and reproductive organs (14). This wide distribution of EPO and EPOR expression has stimulated great interest in the other biological roles of EPO, particularly with regard to cytoprotection.

EPO confers cytoprotection by a number of pathways involving Janus-kinase 2 (Jak2) signalling including upregulation of anti-apoptotic genes such as Bcl-xL and Bcl-2 (15) and inactivation of pro-apoptotic caspase-9 and Bad (16). The cytoprotective properties of EPO have been demonstrated in numerous models of ischemic and inflammatory injury, largely in neuronal, vascular and cardiac tissues (17). Little is known regarding expression or function of EPO/EPOR in pulmonary tissues or the potential role or erythropoietin in pathological processes in the lung.

In this study we demonstrate for the first time expression of EPO and its receptor in respiratory epithelial cells. Using a cell-culture model of ALI, we show anti-apoptotic activity of rhEPO in Fas- and neutrophil- mediated epithelial cell apoptosis, and that this effect is mediated by specific EPO-EPOR interactions. We propose induction of an anti-apoptotic Bcl-xL/Bax phenotype as a mechanism of the observed cytoprotective effect. Administration of rhEPO may thus represent a potential therapy for ARDS if additional studies confirm its protective role in animal models of ALI.

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Materials and methods:

Reagents: Recombinant human Interferon-gamma (IFN-γ) (# 285-IF) and anti-EPO neutralising anti-body (MAB287) were purchased from R&D Systems Inc (Minneapolis, USA). Anti-human Bcl-xL (#sc-1413), anti-human EPO (#SC-7956) and anti-human EPOR (#sc-697) were purchased from Santa Cruz Biotechnology Inc., Santa Cruz, CA. 95060. Anti-human Bax (# 554104) was purchased from BD Pharmingen (Mississauga, ON). β-actin monoclonal antibody (# AC-74) and E. Coli LPS (L4391) were purchased from Sigma Aldrich (Oakville, ON). PARP mouse monoclonal antibody (clone C-2-10) was purchased from Biomol Research labs (Plymouth Meeting, PA) and anti-Fas mAB (Clone CH-11) from MBL International, (Woburn, MA 01801). EPOR siRNA (Hs_EPOR_5 HP Validated siRNA (NM_000121) was purchased from Qiagen (Mississauga, Ontario L5N 8L2).

Cell lines and culture: Human alveolar epithelial cells (A549, European Collection of Cell Cultures, Porton Down, UK) were cultured at 37°C in 5% CO_2 in Ham's F12 (Gibco-BRL), 10% FCS, 1% penicillin/streptomycin. Normal Human Bronchial Epithelial (NHBE) cells were isolated and cultured as described by Gray et al (18). Bronchi were obtained from patients undergoing lung resection due to lung cancer or benign lung tumors. The Ethics Committee of Providence Healthcare approved the study and all patients were provided informed consent to participate in the investigation. Prior to agonist treatment, cells were washed with serum-free F-12 and placed under serum-free conditions or in serum containing 1% FCS for LPS stimulations.

Real Time PCR: mRNA was quantified using commercially available SYBR Green assays. The results are expressed as the ratio of the mean of triplicate target gene cDNA measurements to the triplicate housekeeping gene (β-actin) measurement. Primers used were: Bcl-xL F 5' GAT GCA CAT AGC GTT CCC CT 3', R 5' CCC TAG CAG ATA AAG TGA CGG G 3'; Bax F 5' GGT TTC ATC CAG GAT CGA GCA GG 3'

R 5'ACA AAG ATG GTC ACG GTC TGC C 3'; Actin F 5' TGG AGA AGA GCT ATG AGC TGC CTG 3', R 5' GTG CCA GAC AGC ACT GTG TTG 3'. EPO F 5' GCC AGA GGA ACT GTC GAG AG, R' ATG GTA GGT GCC AAA ACA GG 3'and EPOR F' GAG CAT GCC CAG GAT ACC TA, R 5' TAC TCA AAG CTG GCA GCA GA 3'.

Flow cytometry: Flow cytometric analyses were performed as before (19) by standard techniques using murine immunoglobulin G (IgG) isotype controls (PharMingen, San Diego, CA) and specific anti-Fas (clones ZB4; PanVera, Madison, WI) as primary antibodies.

Cell Death Detection ELISA: Apoptosis was demonstrated in treated A549 or NHBE cells utilizing the ELISA kit from Roche Applied Science, [#1774425] (Mannheim, Germany). This technique uses mouse monoclonal antibodies directed against DNA and histones. This allows the specific determination of mono- and oligonucleosomes in the cytoplasmic fractions of cell lysates (20, 21).

Western Blot: Protein was collected from total cell lysates and western blots were performed as previously described (22). Nitrocellulose membranes were stripped and reprobed for β-actin to normalize differences in protein loading. Densitometry was performed on autoradiographs to quantitate expression and normalized to β-actin protein expression.

Immunohistochemistry: Ethics approval for the use of human tissues was granted by the St Paul's Hospital Ethics Review Board. Paraffin-embedded sections of normal lung parenchyma from patients undergoing lung resection were obtained from the Tissue Registry. Slides were processed with standard techniques of Citra buffer heat-induced antigen retrieval procedure (Dako, Mississauga, Ontario), Following blocking, slides were incubated in primary antibody (rabbit anti-EPOR 1:50) or rabbit IgG isotype control 1:200) overnight at 4ºC and immunostained as previously described (23).

Transfection and siRNA: Cells were transfected with Hs EPOR_5 HP Validated siRNA (NM_000121) or scramble RNA (Qiagen, Mississauga, ON) using the HiPerfect transfection reagent (Qiagen, Mississauga, ON) in 24 well plates according to manufacturers instructions and using standard optimisation procedures. After incubation in transfection medium for 24 hours, medium containing the transfection reagent was discarded, and the cells were maintained in fresh medium for another 24hours prior to stimulation with IFN-γ, CH-11 and rhEPO as indicated.

Isolation of peripheral blood neutrophils (PMNs): Neutrophils were isolated from blood drawn from healthy control subjects using standard methodology of Hypaque-Ficoll density-gradient centrifugation, dextran sedimentation, and hypotonic lysis of erythrocytes (24). Neutrophils were maintained in Iscove's modified Dulbecco's medium. Purity and viability were confirmed by May-Grunwald-Giemsa (Sigma) and Trypan Blue (Sigma) staining respectively.

Statistical analysis: Data were analyzed with GraphPad Prism 3.0 software package (GraphPad Software, San Diego, CA). Values are presented as means ± SEM. The significance of differences between means was assessed by Student t–test. Multiple groups were compared by one-way analysis of variance (ANOVA) and by Bonferoni's t test post hoc. A p value < 05 was considered significant.

Results

<u>Respiratory epithelial cells express EPO and EPOR</u>

Gene and protein expression of EPO was demonstrated in A549 cells and was downregulated by LPS at 24 hours (Figure 1A). EPO expression was not found in NHBE cells (data not shown). EPOR mRNA and protein were expressed in A549 (Figure 1B) and primary NHBE cells (Figure 1C), and expression was not regulated by LPS. EPOR expression is also demonstrated in normal human alveolar epithelial cells by immunohistochemistry (Figure 1D), however EPO expression was not seen (data not shown).

<u>Fas-mediated apoptosis of A549 cells is inhibited by rhEPO.</u>

A549 cells have previously been shown to be resistant to Fas-mediated apoptosis due to failure to express Fas receptor on the cell membrane. Susceptibility to Fas-mediated apoptosis by CH-11 anti-Fas agonist antibody is incurred following upregulation of surface Fas expression by IFN-γ (10). We reproduce this result here. There is no membrane expression of Fas above isotype control in unstimulated A549 cells as demonstrated by flow cytometric analysis, and surface expression is induced following incubation with IFN-γ (Figure 2A). Using cleavage of PARP and detection of the p85 cleavage product as a marker of apoptosis (Figure 2B), we see that apoptosis is induced in a dose dependent fashion by incubation with IFN-γ and CH-11 in combination (lanes 4 and 5) but not by either stimulus used alone (lanes 2 and 3). Co-incubation with rhEPO completely abrogated this effect (lane 6). Semi-quantitative analysis of the effect by densitometry demonstrates significant inhibition of PARP cleavage by EPO (Figure 2C).

We went on to quantify this effect by measurement of free nucleosome release into the supernatant (Figure 2D). Free nucleosome release was significantly increased by IFN-γ and CH-11, and there was a dose-dependent inhibition of this effect by co-incubation with rhEPO.

<u>EPO mediated anti-apoptotic effect in A549 cells by specific EPO-EPOR interaction.</u>

Two complementary methods were used to confirm that the observed cytoprotective effect is mediated by specific EPO/EPOR signalling. We knocked down expression of EPOR by transfection of A549 cells with EPOR siRNA (Hs_EPOR_5 HP Validated siRNA (NM_000121) . Confirmation of transfection efficiency is demonstrated in Figure 3A, with the highest dose of EPOR siRNA (100 nM) achieving approximately 70% reduction in EPOR expression compared to control transfected cells. rhEPO continues to exert anti-apoptotic effect following transfection of scramble (Scr) RNA or EPOR siRNA 50 nM, with significant reduction in free nucleosome release compared to CH-11 and IFN-γ alone. This anti-apoptotic effect of rhEPO is lost following transfection of the higher dose of EPOR siRNA 100nM, with no significant reduction in free nucleosome release compared to CH-11 + IFN treated cells (Figure 3B). EPOR siRNA 100nM transfected cells undergo significantly more CH-11 induced apoptosis than Scr siRNA transfected cells in the presence of rhEPO (Figure 3B). Experiments were repeated following pre-treatment with the anti-EPO neutralising antibody (MAB287, R&D systems) (25) at 5 μg/ml (Figure 3C). The anti-apoptotic effect of rhEPO was completely abrogated by co-incubation with EPO neutralizing antibody.

EPO upregulates Bcl-xl/Bax expression ratio in the presence of LPS

The balance of anti-apoptotic Bcl proteins such as Bcl-xL to pro-apoptotic proteins such as Bax are important determinants of mitochondrial membrane integrity, preventing cytochrome c release into the cytosol where it forms the apoptosome. We next looked at gene and protein expression of Bcl-xL relative to Bax as a potential mechanism of our observed anti-apoptotic effect (Figure 4). As ALI often occurs in the setting of severe sepsis, we were interested to determine if this effect would be maintained in the setting of endotoxin/LPS, a critical determinant of Gram negative sepsis. Incubation of A549 cells with rhEPO 1unit/ml or LPS 1μg/ml, alone or in combination, for 24 hours results in significant upregulation of Bcl-xL/Bax mRNA relative to control. LPS and EPO treatment alone resulted in a trend towards increased Bcl-xL/Bax protein ($p=0.09$ in each case), while co-incubation with LPS and EPO caused a significant increase in the anti-apoptotic Bcl-xL/Bax protein ratio. When we look at the relative contribution of Bcl-xL and Bax modulation to this effect, there is no significant effect of EPO or LPS alone or in combination on the expression of either molecule, although the effect appears to be driven primarily via upregulation of Bcl-xL, which is increased 1.8 times above control by EPO and LPS in combination ($p=0.07$).

EPO is anti-apoptotic in primary respiratory epithelial cells.

Though widely used in the study of respiratory epithelial cell biology, A549 cells are an immortalized cell line and as such apoptotic responses cannot be predictably assumed to translate into primary tissues. We therefore went on to confirm our findings in primary

NHBE cells. NHBE cells express Fas on their surface (Figure 5A) and therefore did not require IFNγ for induction of apoptosis by CH-11. Incubation of NHBE cells with CH-11 results in significant increase in free nucleosome release, which is inhibited in a dose dependent fashion by co-incubation with rhEPO. Apoptosis is completely abrogated with the higher dose of EPO (Figure 5B). In the presence of LPS, apoptosis is also inhibited but remains significantly increased above control even with high dose EPO (Figure 5C). Apoptosis and cytoprotective effect by EPO is further demonstrated by p85 PARP cleavage (Figure 5D), which is associated with a concordant reduction in Bcl-xL/Bax protein expression in response to CH-11, and restoration to control levels by rhEPO (Figure 5E). We went on to examine the specific effects on Bcl-xL and Bax expression. In contrast to the effects with LPS, the predominant effect of EPO in the setting of Fas activation is seen on Bax expression (Figure 5G), with significant upregulation of Bax in response to CH-11 and abrogation of the effect by co-incubation with rhEPO. Bcl-xL expression (Figure 5F) remained stable under these conditions.

EPO protects epithelial cells from neutrophil-mediated apoptosis.

In order to expand our hypothesis in a more physiologically relevant model pertinent to ARDS, we went on to examine the cytoprotective effect of EPO in a co-culture model of alveolar epithelial cells with peripheral blood neutrophils. Co-incubation of A549 cells with peripheral blood neutrophils in a transwell system to prevent direct cell-cell contact results in apoptosis as demonstrated by PARP cleavage, with a dose-dependent inhibition of apoptosis by rhEPO (Figure 6A). Again apoptosis is associated with a concordant significant downregulation of Bcl-xL/Bax protein expression, which is inhibited by co-incubation with rhEPO (Figure 6B).

Discussion:

ARDS is a severe and frequently fatal condition complicating critical illness of many aetiologies. Apoptotic injury to the type 2 pneumocytes is an important component of the pathophysiology of ARDS and a critical determinant of outcome, while Fas/FasL signaling has been implicated in death signaling in this disease. Current therapy in ARDS consists of supportive care and treatment of the underlying cause, while therapies directed against the inflammatory processes have been unsuccessful in clinical trials (reviewed in (26)). Failure of these therapies likely reflects the complexity and redundancy of the systemic inflammatory response, but also the importance of apoptosis which is not targeted by these agents. In this study, we show for the first time expression of EPO and its receptor in respiratory epithelium. We demonstrate a cytoprotective effect of rhEPO in cultured alveolar and bronchial epithelial cells in models of Fas- and neutrophil- mediated apoptosis, which is mediated by specific EPO-EPOR interactions. This effect is mediated at least in part by induction of an anti-apoptotic Bcl-xL/Bax phenotype.

Expression of EPO and EPOR has been demonstrated in a wide variety of tissues, and recent years has seen an explosion in interest in the anti-apoptotic properties of EPO in a variety of conditions, particularly with respect to ischemia/reperfusion injury to neuronal and cardiac tissue (reviewed in (17)) . While expression of EPO and EPOR has previously been reported in non-small cell lung cancer (27) and foetal lung (28), this is the first time that expression has been demonstrated in normal human lung tissues.

Anaemia of chronic disease occurs in the setting of persistent acute or chronic immune activation (29), and has multiple causes including inhibition of EPO production and responsiveness. Consistent with reduced EPO production in LPS treated rats (30), we found downregulation of EPO gene and protein expression following LPS treatment in A549 cells. Potential mechanisms include inhibition of EPO gene expression by the transcription factor NF-κB (31), which is upregulated by LPS, or a secondary effect through induction of pro-inflammatory cytokines including IL-1β and TNFα which have also been shown to inhibit EPO gene and protein expression (32). Importantly, EPOR expression was not significantly altered in the epithelium by LPS in our model, permitting robust cytoprotective effect of EPO under conditions of LPS stimulation. We were unable to detect EPO gene or protein expression in primary bronchial epithelial cells or in alveolar epithelial tissue, suggesting that local production of EPO, at least by the epithelium, is less important than circulating levels in critical illness. Whether other resident cells of the lung including the alveolar macrophage are a physiologically relevant source of EPO in ALI has not been determined, although we have demonstrated expression of both EPO and EPOR in alveolar macrophages ex-vivo (data not shown). EPO expression was not demonstrated by immunohistochemistry in the vascular endothelium in our lung sections, however induced expression of EPO has previously been demonstrated in vascular endothelium in a model of compressive spinal injury (33).

Inhibition of EPO-EPOR interaction by gene silencing of EPOR by siRNA or by incubation with EPO neutralising antibody inhibits the cytoprotective effect of rhEPO in our model. Though not significant, there is a trend towards residual anti-apoptotic effect even with EPOR siRNA 100nM. We were unable to completely knock down EPOR expression, and the trend towards cytoprotective effect likely represents signalling through the remaining 20-30% expressed receptor. EPOR-independent EPO signalling has not been described in the literature to date, and while such an effect cannot be discounted based on our data, it is less likely to be the mechanism of the residual effect.

Numerous human studies have implicated Fas-induced apoptosis in the pathogenesis of ARDS (8, 10, 34), while the inflammatory and apoptotic changes of ARDS are induced in animal models by both human sFasL and by the Fas-activating antibody Jo-1 (12, 35). Following induction of membrane Fas expression in A549 cells, apoptosis is induced by cross-linkage and activation of Fas by CH-11 antibody. Lack of Fas expression by A549 cells is likely a characteristic of the tumour origin of these cells as a mechanism of immune resistance rather than a characteristic of alveolar epithelial cells. Fas expression has been demonstrated in alveolar epithelial cells of patients who died of ARDS (10), and in primary distal lung epithelial cells in culture (36). Consistent with previous data (36), NHBE cells express Fas on the membrane and apoptosis is induced by Fas ligation.

LPS induces alveolar epithelial cell apoptosis in whole-animal models of ARDS (7), however in our hands, A549 cells were resistant to apoptosis by LPS, even at high doses up to 10 µg/ml (Data not shown). Apoptosis in cultured A549 (37) and tracheobronchial epithelial cells (38) treated with LPS is reported only with very high doses of LPS (100 µg/ml), which are of questionable clinical significance. LPS-induced apoptosis *in-vivo* likely is the result of more complex convergence of death signals, including the Fas/FasL pathway (8). A549 cells treated with LPS trended towards an anti-apoptotic phenotype. Similar to the observation of LPS-mediated upregulation of Bcl-xL mRNA in murine hepatocytes (39), LPS resulted in upregulation of Bcl-xL/Bax mRNA in A549 cells, although no significant change in protein expression was observed. Co-incubation with rhEPO significantly promotes an anti-apoptotic Bcl-xL/Bax phenotype in A549 cells in the presence of LPS, which may be protective in a more complex model where multiple apoptotic signals are in effect. In NHBE cells, Fas-mediated apoptosis is completely abrogated by rhEPO, and this anti-apoptotic effect is maintained in the presence of LPS. This is important in the clinical context of sepsis as a major pathogenic determinant of ARDS.

Apoptosis is tightly regulated by the balance of a number of anti- and pro-apoptotic molecules including Bcl-xL and Bax, both of which can be modulated by EPO. Fas activation can result in caspase-dependent (Type I) apoptosis or mitochondrial –dependent (type II) apoptosis (40). Type II apoptosis is regulated by members of the the Bcl-2 family. The proapoptotic proteins Bax and Bak induce cell death by oligomerisation and translocation to the mitochondrial membrane resulting in mitochondrial membrane permeabilization , an effect which is inhibited by anti-apoptotic members such as Bcl-xL and Bcl-2 (41). PI3-kinase/Akt mediated upregulation of Bcl-xL has been implicated in EPO-mediated cytoprotection in endothelial cells (42), while downregulation of Bax by rhEPO has been shown in models of ischemia in the kidney and brain (43), (44). Here we demonstrate similar effects in respiratory epithelial cells. In the presence of LPS, the effect of EPO is observed predominantly through upregulation of Bcl-xL. We saw no change in EPOR expression in A549 or NHBE cells with LPS treatment, indicating an effect downstream of the EPO receptor. Bcl-xL gene transcription is regulated by NF-κB, which is a downstream target of both EPOR and the LPS receptor TLR4, and is a potential mediator of the synergistic effect of LPS and EPO. In contrast, our data indicates that Fas-mediated apoptosis of respiratory epithelium is associated with upregulation of Bax. This suggests that activation of the mitochondrial intrinsic apoptotic pathway is important in Fas-mediated apoptosis of the respiratory epithelium, although additional anti-apoptotic mechanisms involving type I apoptosis cannot be discounted. In the context of Fas-mediated apoptosis, downreguation of Bax appears to be the more important mechanism of EPO-mediated cytoprotection, although additional effect via Bcl-xL regulation may be important in the presence of LPS.

Continuing this concept of a more complex model of apoptotic injury, we went on to examine the effect of rhEPO in a model of neutrophil-mediated apoptosis. Animal models show movement of neutrophils through epithelium and endothelial membranes without damage or degranulation (45), while in a rabbit model of streptococcal-induced lung inflammation, the major metabolic activity of neutrophils occurred in the alveolar space rather than the interstitium or microvasculature (46). This data suggests that indirect mediator effects may be more important than direct cell-cell interactions in neutrophil-induced apoptosis in ALI, and sFasL is a candidate mediator (8, 10).

We adopted the model of Serrao and colleagues (47), who demonstrated that apoptosis of A549 cells in transwell co-culture was mediated by sFasL. Consistent with their data, apoptosis was induced in A549 cells by co-culture with peripheral blood neutrophils isolated from healthy volunteers. A recent study reports apoptosis of distal lung epithelial cells induced by neutrophil elastase (NE) (48), however in our hands A549 cells were resistant to apoptosis by NE (data not shown), suggesting that this was not a mechanism here. Abrogation of apoptosis by rhEPO was again associated with a concordant modulation of Bcl-xL/Bax expression. Soluble FasL was measurable in the supernatant by ELISA and levels were not altered by EPO (data not shown) indicating that inhibition of sFasL release was not responsible for the cytoprotective effect in this model.

Taken together, our data suggests that in the context of sepsis-induced acute lung injury, EPO has potential for complementary mechanisms of cytoprotection, the net result of which includes induction of an anti-apoptotic Bcl-xL/Bax phenotype in the epithelium. Our data is however limited to association and does not define regulation of Bcl-xL/Bax as the only mechanism of cytoprotection in our model. While EPO mediated consistent anti-apoptotic effect in different respiratory epithelial cell types under different conditions, with concordant increase in Bcl-xL/Bax ratio, the individual effects on Bcl-xL and Bax expression were inconsistent and at times small. The protective effects of EPO in respiratory epithelium thus almost certainly include additional as yet undefined mechanisms. Candidate mechanisms implicated in other organ systems include regulation of other Bcl-2 family proteins (49), apoptotic protease-activating factor-1 (Apaf-1) and caspase expression and activity (50).

ARDS is characterised by severe tissue and alveolar hypoxemia where microcirculatory dysfunction in sepsis contributes to the local tissue hypoxia. One would expect therefore that EPO and EPOR levels would be high. Critical illness, however, is characterized by decreased EPO production, decreased bone marrow response to EPO and reduced RBC survival (51-53). This, combined with the association of blood transfusion with poorer outcome, led to a number of studies investigating the use of EPO in critically ill patients to reduce transfusion requirements. The recently published multicentre RCT (54) found no reduction in red cell transfusion with use of EPO, possibly reflecting the adoption of more conservative transfusion thresholds. However a reduction in mortality in a pre-specified subgroup analysis of trauma patients hints at clinically relevant cytoprotective effect. The rate of thrombotic vascular events in the EPO group was increased compared to the placebo group, and was increased depending on the number of doses, although this increased thrombotic risk was not seen in patients also receiving heparin prophylaxis.

While many therapies are in development aimed at various components of the apoptotic cascade, including caspase inhibitors, death receptor antagonists and Bcl-2 family modulators, none have yet reached clinical use (55). Recombinant human EPO has a long history of safety and efficacy in treating anaemia in chronic renal disease, while recent reports regarding reduced survival in cancer patients (56) are unsurprising given what is now known regarding EPOR expression in tumour cells (57) and cytoprotective effect of EPO. A number of Phase II trials have demonstrated safety and efficacy of rhEPO and Darbopoietin in acute stroke and myocardial infarction (58, 59), achieving serum concentrations in the range of 5 units/ml with no increase in thrombotic events. Care must be taken in extrapolating this safety data to a critical care population. Our data demonstrating cytoprotective effect in respiratory epithelial cells represents a novel therapeutic opportunity for EPO in critical illness as a cytoprotective agent in ARDS. It is likely that the doses required to achieve such effect will be higher and/or more frequent than those employed in trials aimed at the haematopoietic properties of EPO in critical illness, with potential increased risk of thrombosis. Whether this risk can be offset by the co-administration of anti-coagulants such as heparin, as in the Corwin trial (54) or by the use of newer rhEPO analogues would require vigorous safety evaluation.

Critical illness involves a dysregulated EPO response to global and microvascular hypoxia, and loss of cytoprotective effect may contribute to the apoptotic alveolar epithelial injury of ARDS. This study demonstrates the potential to protect respiratory epithelial cells from apoptosis with exogenously administered EPO, demonstrating an important pathway in the lung epithelium which may be exploited for therapeutic use in the future. Further *in vivo* studies will be required to confirm these effects and to determine optimal timing and duration of therapy.

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Figure Legends

Figure 1. Respiratory epithelial cells express EPOR and EPO.

A549 or NHBE cells were grown to confluence in 6-well plates. Cells were washed, placed in low serum (1% FCS) medium and were left untreated or incubated with LPS 1 ug/ml for 24 hours. Total RNA or protein was extracted for Real Time PCR and Westren Blot analysis.

A & B: Gene and protein expression including representative Western blot images of EPO (A) and EPOR (B) in A549 cells.

C: Gene and protein expression of EPOR in NHBE cells. NHBE cells did not express EPO mRNA or protein.

D: Representative image of immunohistochemical expression of EPOR (i) and isotype control (ii) in normal human alveolar tissue. Positive staining is pink with New Fuschin substrate. Inset shows normal alveolar ultrastructure.

All experiments were performed a minimum of 3 times. Values are expressed as mean +/- S.E. (* $p<0.05$ compared to control).

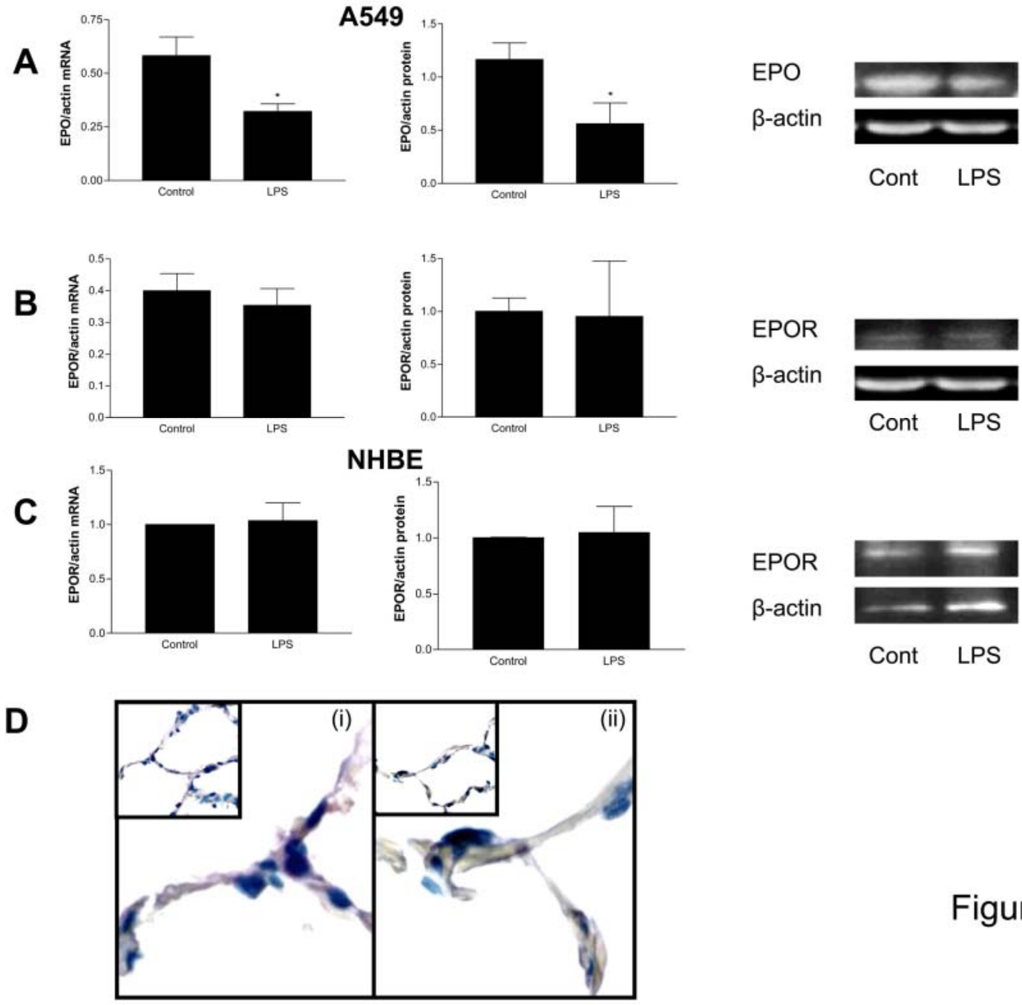

Figure 1

Figure 2. EPO inhibits Fas-mediated apoptosis in A549 cells.

A: A549 cells were incubated with IFNγ 250 units/ml x 24 hours. Cells were then trypsinised, washed and labelled with an isotype control (red dashed) or anti-Fas mAB (black-unstimulated cells; blue-IFNγ-treated cells) antibody and fluorophore-conjugated detection antibodies. Fas expression was quantified by flow cytometry and the graph is representative of three independent experiments.

B & C: A549 cells were incubated with Fas-activating CH-11 0.1 or 1μg/ml and IFNγ 250 units/ml alone and in combination, with or without rhEPO 1unit/ml x 24 hours. Apoptosis was determined by p85 expression in total cell lysates by Western blot. Blot is representative of three independent experiments (B). Densitometric analysis of p85 PARP expression (C).

D: A549 cells were stimulated with IFNγ and CH-11 1μg/ml +/- rhEPO 0.1 or 1 unit/ml and apoptosis was measured by free nucleosome release into the supernatant using Cell Death Detection Elisa (Roche). Data are expressed as mean +/- S.E. and are obtained from three experiments (* $p < 0.05$ vs control; † $p < 0.05$ vs CH-11).

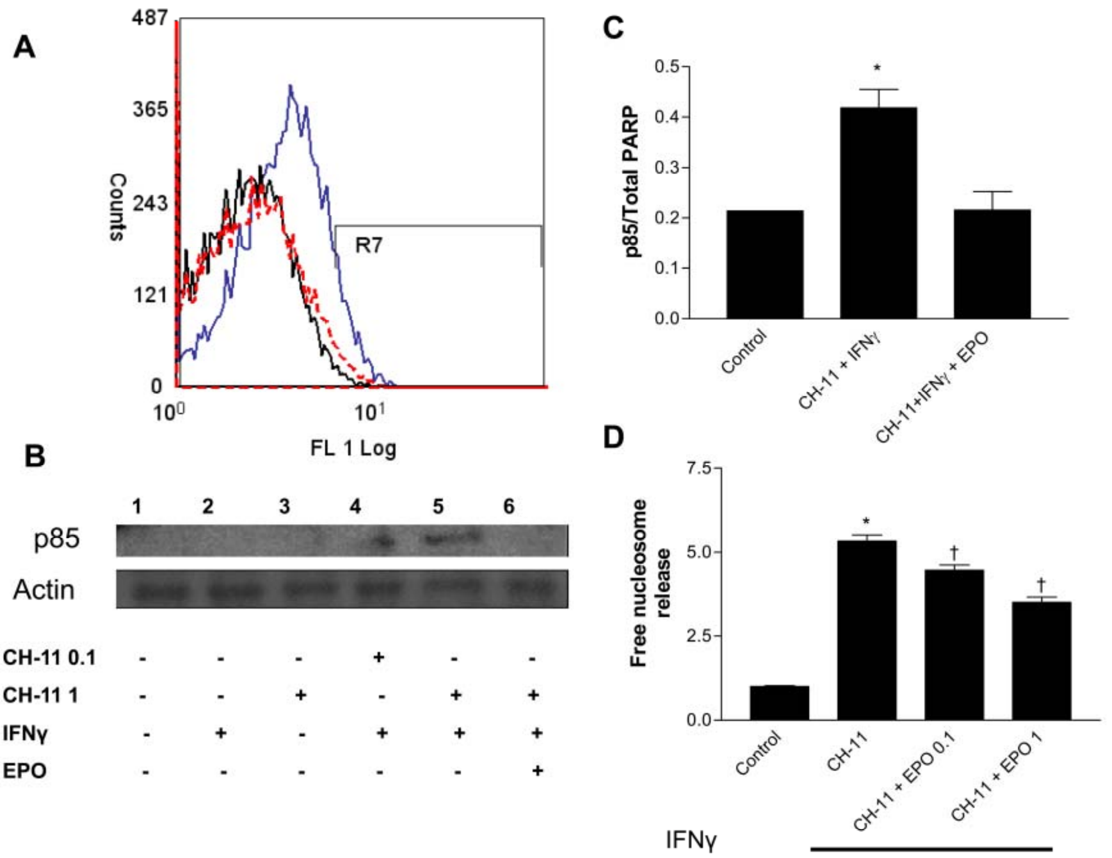

Figure 3. EPO inhibits apoptosis in A549 cells by specific EPO-EPOR interaction.

A: A549 cells were transfected with EPOR siRNA (Hs_EPOR_5 HP Validated siRNA (NM_000121) , using standard techniques for transfection and optimisation. Total protein lysates were collected and densitometric analysis of EPOR/actin expression by Western blot was performed.

B: A549 cells were transfected with EPOR siRNA or scramble (Scr) siRNA prior to incubation with IFNγ and CH-11 1μg/ml +/- rhEPO 0.1 or 1 unit/ml for 24 hours. Apoptosis was quantified by Cell Death Detection ELISA Assay.

C: A549 cells were pre-treated with the anti-EPO neutralising anti-body (MAB287, R&D systems) 5ug/ml for thirty minutes prior to incubation with CH-11 1μg/ml +/- rhEPO 0.1 or 1 unit/ml for 24 hours. Apoptosis was quantified by Cell Death Detection ELISA.

Data are expressed as mean +/- S.E. and are obtained from three experiments (* $p < 0.05$ vs. match-transfected CH-11+ IFNγ treated cells; † $p < 0.05$ vs. Scr siRNA transfected cells) .

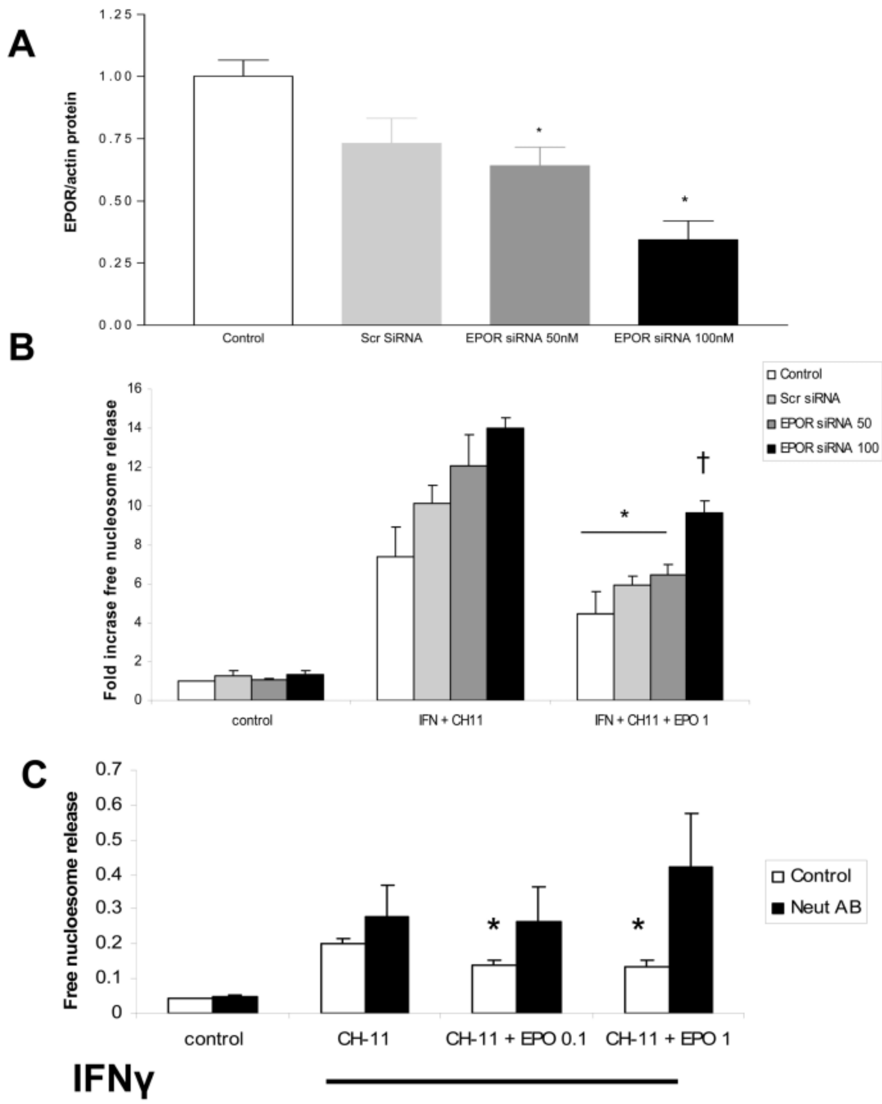

Figure 3

Figure 4: EPO induced an anti-apoptotic Bcl-xL/Bax phenotype in A549 cells.

A549 cells were grown to confluence in 6-well plates and incubated with LPS 1μg/ml and rhEPO 1unit/ml, alone or in combination.

A: Ratio of Bcl-xL/Bax mRNA expression quantified by Real Time PCR.

B-D: Densitometric analysis of Bcl-xL/Bax (B)**,** Bcl-xL/Actin (C) and Bax/Actin (D) protein expression by Western blot of total protein lysates.

E: Representative Western bolt of Bcl-xL, Bax and β-actin protein expression in A549 cells.

Data are expressed as mean +/- S.E. and are obtained from six experiments. (* p< 0.05 compared to control; † p<0.05 vs. LPS/EPO alone).

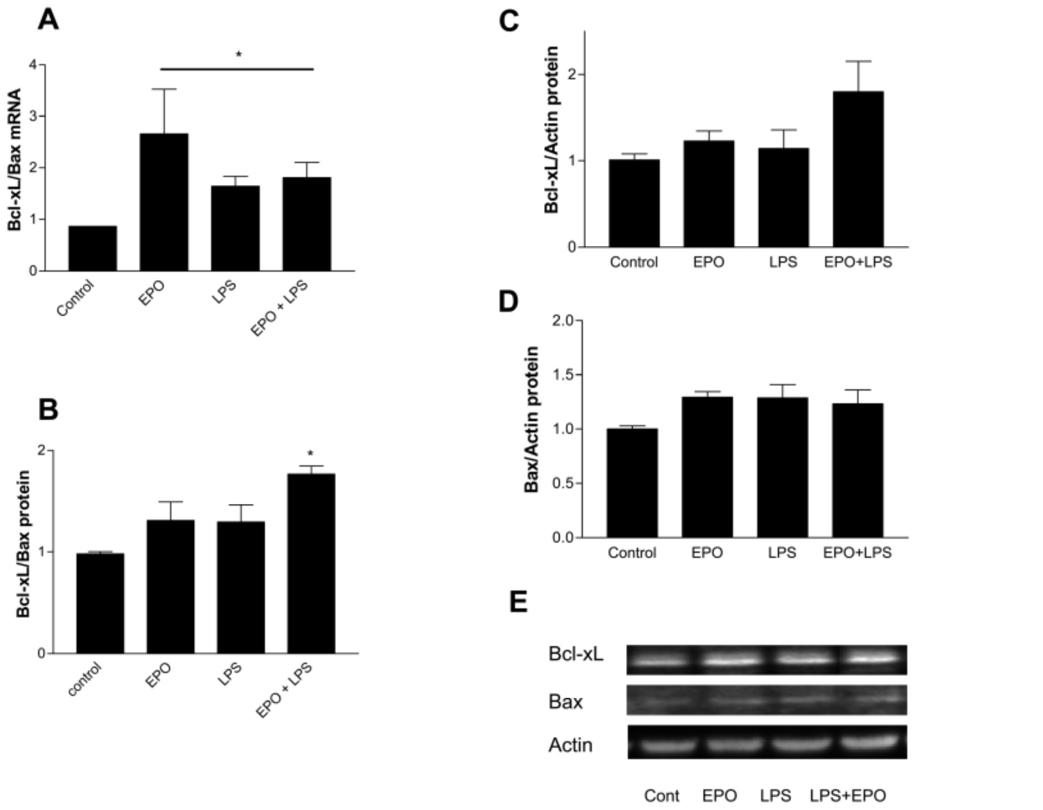

Figure 4

Figure 5. EPO abrogates Fas-mediated apoptosis in NHBE cells.

A: NHBE cells were trypsinised, washed and labelled with an isotype control (solid line) or anti-Fas mAB (broken line) antibody and fluorophore-conjugated detection antibodies. Fas expression was quantified by flow cytometry and the graph is representative of three independent experiments.

B & C: NHBE cells were seeded on 24-well plates and stimulated with CH-11 1μg/ml +/- rhEPO 0.1 or 1 unit/ml for 24 hours and apoptosis quantified by Cell Death Detection ELISA (B). The experiment was repeated in the presence of LPS 1μg/ml (C).

D - G: NHBE cells were grown to confluence in 12-well plates. Cells were washed and placed in low serum (1%FCS) media and incubated with CH-11 1μg/ml +/- rhEPO 0.1 or 1 unit/ml for 24 hours prior. Total protein lysates were collected and Western blot analysis of p85 (D) and Bcl-xL (F) and Bax (G) protein expression relative to β-actin and Bcl-xL/Bax ratio (E) was performed.

H: Representative Western bolts of Bcl-xL, Bax and β-actin protein expression in NHBE cells. CH is CH-11; E is EPO.

Data are expressed as mean +/- S.E. and are obtained a minimum of three experiments. (* $p < 0.05$ compared to control; † $p<0.05$ vs. CH-11 alone.

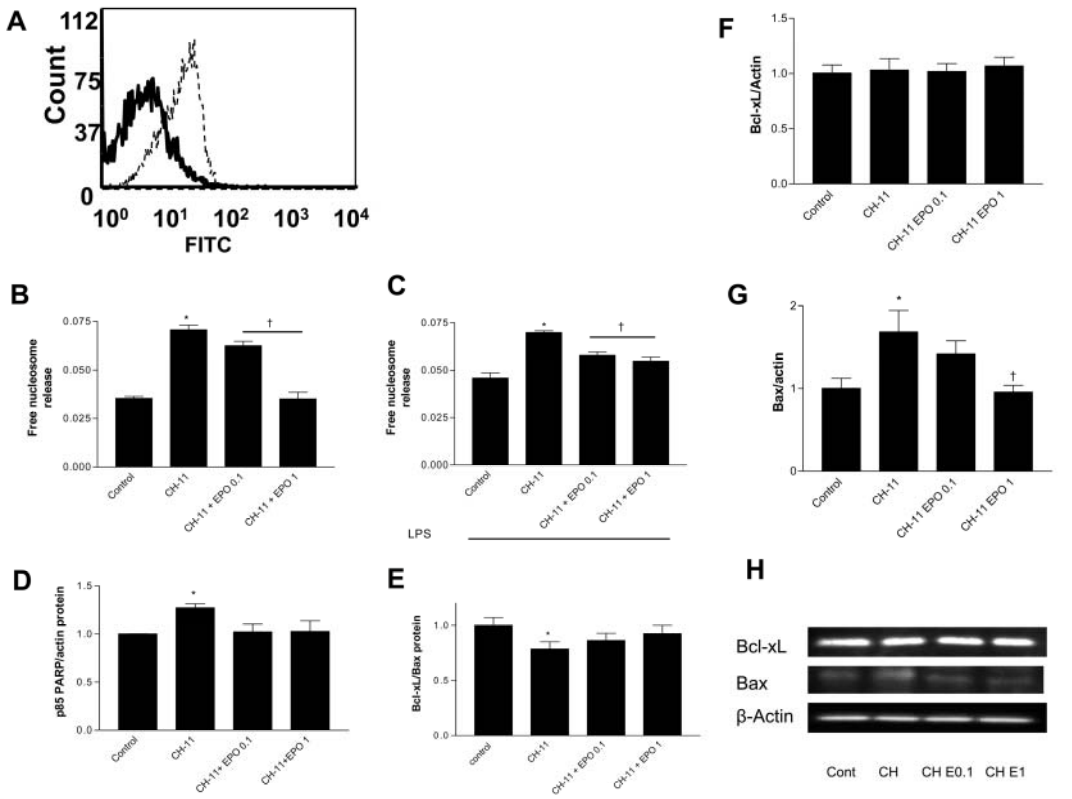

Figure 5

Figure 6: EPO abrogates neutrophil-mediated apoptosis of A549 cells.

PMNs were isolated from healthy volunteers by Hypaque-Ficoll density-gradient separation as described. A549 cells were grown to confluence in 6-well plates, washed and placed in low serum (1% FCS) media +/- rhEPO 0.1 or 1 unit/ml. PMNs (2.5 x 10^5) were placed in the upper chamber of Transwell polycarbonate microporous inserts (0.3 μm membrane) above the epithelial cell monolayer. Total protein lysates were collected from the epithelial layer after 24 hours.

A&B: Densitometric analysis of Western blot determination of p85 (A) and Bcl-xL/Bax (B) protein expression.

Data are expressed as mean +/- S.E. and are obtained a minimum of three experiments. (* $p < 0.05$, ** $P < 0.01$ compared to control; † $p<0.05$, †† $p<0.01$ vs PMN alone).