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RESEARCH PAPER

α-4/β-1 and α-L/β-2 integrins mediate cytokine induced lung leukocyte-epithelial adhesion and injury

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Background and purpose: Injury to the alveolar epithelium is a critical feature of acute lung injury (ALI). Using a cytokine model of ALI we demonstrated previously that newly recruited mononuclear phagocytes (MNP) contributed to lung inflammation and injury. We hypothesized that cytokines delivered into the alveolar airspace would have multiple effects on the lung that may contribute to lung injury.
Experimental approach: Intratracheal cytokine insufflation and leukocyte adoptive transfer *in vivo* were combined with *in vitro* analyses of lung epithelial cell-MNP adhesion and injury. Lung inflammatory injury was assessed by histology, leukocyte infiltration, and release of LDH and RAGE.
Key results: Cytokine insufflation was associated with apparent MNP-epithelial adhesion, up-regulation of alveolar ICAM-1 and VCAM-1, and the release of LDH and RAGE into the bronchoalveolar lavage. Insufflation of small molecule integrin antagonists suppressed adhesion of MNP and modulated release of LDH and RAGE. Adoptive transfer of MNP purified from cytokine insufflated lungs into leukopenic rats demonstrated the requirement of MNP for release of LDH that was not induced by cytokine alone. Corroboration that disrupting the ICAM/LFA1 interaction or the VCAM/VLA4 interaction blocked MNP-epithelial cell interaction and injury was obtained *in vitro* using both blocking monoclonal antibodies and the small molecule integrin antagonists, BIO5192 and XVA143.
Conclusions and implications: MNP recruited following cytokine insufflation contributed to lung injury. Further, integrin antagonists reduced alveolar epithelial cell injury induced during lung inflammation. Intratracheal delivery of small molecule antagonsists of leukocyte-epithelial adhesion that prevent lung injury may have significant clinical utility.
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Keywords: acute lung injury; mononuclear phagocyte; alveolar epithelium; α-4/β-1; α-L/β-2; integrin antagonist

Abbreviations: ALI, acute lung injury; AM, alveolar macrophage; A-MNP, alveolar MNP; IFN-γ, interferon-γ; IL-1, interleukin-1; MNP, mononuclear phagocyte; PMN, polymorphonuclear phagocyte or neutrophil; RAGE, receptor for advanced glycation end products

Introduction

Inflammation contributes to many clinical lung disorders including ventilator-induced lung injury, chronic obstructive pulmonary disease, acute lung injury (ALI) and acute respiratory distress syndrome (de Boer *et al*., 2000; Belperio *et al*., 2002; Matthay *et al*., 2003; Prince *et al*., 2003; Rose *et al*., 2003). While the immunological mechanisms mediating lung inflammation may involve cells of both innate and adaptive immunity, mononuclear phagocytes (MNPs) of innate immunity appear to play decisive roles in many inflammatory states. For example, MNPs and the MNP chemokine, monocyte chemotactic protein-1 (MCP-1), contribute significantly to the inflammation associated with chronic obstructive pulmonary disease (de Boer *et al*., 2000), and MNPs are critically involved in the pathogenesis of ventilator-induced lung injury, ALI and acute respiratory distress syndrome (Belperio *et al*., 2002; Matthay *et al*., 2003; Prince *et al*., 2003; Rose *et al*., 2003; Frank *et al*., 2006).

The role played by MNPs in inflammatory disorders is not well understood and is rapidly evolving. In the rat, the resting lung contains a resident population of mature alveolar macrophages (AMs) that are renewed by constitutive

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recruitment (Srivastava *et al*., 2005). Following the induction of inflammation, monocytes are recruited to the lung primarily, but not exclusively, in response to the chemokines, MCP-1 and MCP-2, or leukotrienes (Gunn *et al*., 1997; Maus *et al*., 2001b, 2002a). Once established in the lung, monocytes rapidly differentiate into macrophages by a complex process initiated following transendothelial migration out of the blood stream (Valledor *et al*., 1998; Rosseau *et al*., 2000a; Maus *et al*., 2001a). This effectively defines two subpopulations of MNPs in the inflamed lung: the resident AMs and the newly recruited and differentiating inflammatory alveolar mononuclear phagocytes (A-MNPs) (Maus *et al*., 2006). Whether distinct biological roles can be attributed to these different MNP subpopulations is unknown. However, in humans and mice, the AM population and the newly recruited MNPs induced by inflammatory stimuli can be distinguished by distinct surface markers, mechanism of recruitment (Sunderkotter *et al*., 2004; Garn *et al*., 2006; Tacke and Randolph, 2006) and gene expression profiles (Srivastava *et al*., 2005) indicative of distinct MNP subpopulations. In the present study, and until the language used in reference to both populations has become codified, we have adopted the use of the term A-MNPs to refer to the entire population of MNPs, including the resident AMs and the inflammatory MNPs, harvested from the lung lavage. AMs refer strictly to the resident MNP population harvested from lungs of untreated and control rats.

MNPs in general are potent sources of pro-inflammatory mediators such as tumor necrosis factor-α, interleukin (IL)-1, IL-8, or reactive oxygen species that promote inflammation (Matthay *et al*., 2003; Prince *et al*., 2003; Rose *et al*., 2003; Desouza *et al*., 2005; Smith *et al*., 2006). MNPs respond to many activating signals including the bacterial lipopolysaccharide and cytokines such as IL-1 or interferon-γ (IFN-γ) (Kovarik *et al*., 1998; Ehrt *et al*., 2001; Dobrovolskaia and Vogel, 2002; Beutler and Rietschel, 2003). Upon activation, MNPs secrete cytokines involved in the orchestration of the inflammatory response, activate the respiratory burst nicotinamide adenine dinucleotide phosphate oxidase and release reactive oxygen species. For example, MNPs express and secrete proinflammatory chemokines including regulated upon activation, normal T-cell expressed and secreted, macrophage inflammatory protein-1α, MCP-1, MCP-2, tumor necrosis factor-α and IL-8 (Lucas *et al*., 1998; Foey *et al*., 2000; Gavrilin *et al*., 2000; Wetzel *et al*., 2000; Ragno *et al*., 2001; Carvalho-Pinto *et al*., 2002). Recently, A-MNPs were found to contribute to neutrophil (polymorphonuclear phagocyte or neutrophil (PMN)) immigration, recruitment and infiltration following intratracheal instillation of bacterial lipopolysaccharide or tumor necrosis factor-α in mice (Maus *et al*., 2002b, 2003; Woo *et al*., 2005), or cytokine in rats (Wright *et al*., 2004). As sources of both peptide (IL-8, Cinc) and lipid (LTB_4) chemotactic factors, A-MNPs may contribute to expansion of the inflammatory cell population. The extent to which A-MNPs may also contribute to lung injury *per se* is unknown. However, the resident AMs specifically appear to exert little injury to the lung in the absence of an inflammatory inducer (Wright *et al*., 2004). Furthermore, lung injury during inflammation may result from contributions by AMs (Frank *et al*., 2006), the inflammatory A-MNP population, PMNs (Patton *et al*., 1995) or reflect the combined effects of cytotoxic agents released into the inflammatory environment.

Instillation of acute-phase cytokines into the airway of rodents has been used to study the specific effects of cytokines found in the lavage fluid of clinical ALI/acute respiratory distress syndrome patients (Tsan *et al*., 1992; Patton *et al*., 1995; Guidot *et al*., 1996; Kang *et al*., 1996; Maus *et al*., 2001a; Hybertson *et al*., 2003; Matthay *et al*., 2003; Wright *et al*., 2004). We previously demonstrated the vigorous inflammatory response induced by intratracheal insufflation of IL-1 (Leff *et al*., 1992, 1994a, 1995) or IL-1 in combination with IFN-γ (Wright *et al*., 2004). Both PMNs and MNPs were increased rapidly in the alveoli following cytokine insufflation; and lung leak, oxidative stress and alveolar epithelial cell apoptosis were concomitantly increased following cytokine-induced lung inflammation. Direct evidence that A-MNPs participated in leukocyte recruitment and inflammation was obtained in adoptive cell transfer experiments using A-MNPs obtained from the bronchoalveolar lavage (BAL) of rats previously insufflated with cytokine. We observed high-level expression of the reactive oxygen species generator, xanthine oxidoreductase, in the newly recruited A-MNPs, but none in the resident AMs. Furthermore, inhibition of xanthine oxidoreductase in the newly recruited A-MNPs prevented recruitment of PMNs during adoptive transfer demonstrating a critical role for xanthine oxidoreductase in A-MNP-mediated neutrophil recruitment (Wright *et al*., 2004). These data, therefore, identify another potential distinction between the resident AMs and the newly recruited A-MNPs.

While the role of PMN–epithelial interaction in chronic obstructive pulmonary disease airway inflammation and injury has been clearly identified (Pettersen and Adler, 2002), much less is known about the A-MNP–epithelial interaction in lung inflammation and injury. The potentially complex role played by newly recruited MNPs in lung inflammation was suggested by the increase in alveolar epithelial adhesion by both PMNs and MNPs observed following hypoxia-induced airway inflammation (Beck-Schimmer *et al*., 2001). In the present experiments, we hypothesized that cytokines in the alveolar airspace would have complex effects on the lung including the modulation of surface molecules that promote MNP adhesion where the contribution to lung inflammation and injury may be enhanced. Data shown here indicate that A-MNPs and A-MNP–epithelial adhesion contributes to cytokine-induced inflammatory lung injury and that insufflation of small molecule integrin antagonists into the alveolar air space can reduce cytokine-induced inflammatory lung injury.

Methods

Animal procedures

The use of rats in this study was approved by the University of Colorado Institutional Review Board under the protocol number 4980199(04)1E.

Healthy male Sprague–Dawley rats (300–400 g body weight; Sasco, Omaha, NE, USA) were used in all studies.

Previous experiments had optimized lung inflammation induced by IL-1 at 50 ng (Leff *et al*., 1994a). Insufflation of IFN-γ alone at doses from 0 to 1000 ng promoted apparent dose-dependent leukocyte adhesion that was maximal at 100ng (not shown). Unless otherwise specified, 50 ng of recombinant rat IL-1α and/or 100 ng of IFN-γ (hereafter referred to as cytokine) in 0.5 ml of normal saline were delivered intratracheally into the airway of rats previously anesthetized with ketamine/xylazine (Wright *et al*., 2004) and lung tissues were harvested 24 h later. Control rats were insufflated with normal saline alone. Differential and total inflammatory cell counts were determined on bronchoalveolar lavage fluid (BALF) obtained 24 h following cytokine insufflation as described (Wright *et al*., 2004). Histology and immunofluorescence were performed on lungs harvested 24 h following cytokine insufflation. Lungs were perfused until blood free, removed surgically and divided. One fraction was immediately fixed in paraformaldehyde for histology or immunofluorescence staining. Another fraction was frozen immediately in liquid N_2 for subsequent biochemical analyses. BALF cells were collected from separate rats by pumping 7.0 ml of normal saline into the trachea. Lavage fluid was pumped in and out of the lung three times before being collected.

Leukocyte depletion

To confirm the role of adoptively transferred MNPs in lung injury it was necessary to remove the effect of circulating leukocytes that would be drawn to the lung by cytokine or MNP insufflation. Accordingly, endogenous circulating leukocytes were depleted with intraperitoneal injection of 165 mg kg^{-1} of cyclophosphamide in sterile normal saline as described (Ikezumi *et al*., 2003a, b). A successful depletion of >98% circulating leukocytes was observed after 48 h from the point of cyclophosphamide administration. Leukopenic rats to be used for adoptive transfer of MNPs were treated with cyclophosphamide and 48 h later insufflated with cytokine or saline. Adoptive MNPs transfer was then conducted 24 h later as described in Figure 5. The persistence of cyclophosphamide-induced leukopenia was confirmed throughout the 96 h of the experiment and was consistent with published reports (Ikezumi *et al*., 2003a, b).

Tissue fixation, immunohistochemistry and microscopy

Lung tissue was fixed in 10% neutral-buffered formalin overnight and embedded in paraffin. Two and four micron sections were prepared and hydrated by exposure to xylene for 3- to 5-min periods followed by two sequential 10 min exposures to 100%, 95% ethanol, dH_2O, and finally for 5 min in phosphate-buffered saline (PBS). Hydrated sections were either stained with hematoxylin and eosin or prepared for immunohistochemistry using a modified version of the Cell Signaling protocol for paraffin sections (Cell Signaling, Beverly, MA, USA). Sections prepared for immunohistochemistry were digested in a pepsin digestion solution for 30 min in a 37 °C humidity chamber (1 ml 0.1 N HCl + 9 ml distilled water (dH_2O) + 0.1 ml of 1 g ml^{-1} pepsin). Sections were washed in dH_2O three times for 5 min each, incubated in 1% H_2O_2 for 10 min and washed three times for 5 min each in dH_2O. Sections were finally washed in PBS for 5 min and blocked for 1 h at room temperature in 5% blocking solution (either donkey or goat serum in PBS). The sections were then incubated overnight at 4 °C with the appropriate antibody diluted in blocking solution. Subsequently, the sections were washed in PBS three times for 5 min each. The appropriate secondary antibody diluted in blocking solution was added to each section and incubated for 30 min at room temperature. Once again, the sections were washed in PBS three times for 5 min and the sections were incubated with the 3,3′-diaminobenzidine peroxidase substrate reagent according to the manufacturer's instructions. The sections were rinsed in dH_2O and subsequently stained with hematoxylin. Sections were then dehydrated by incubating twice in 95 and 100% ethanol for 10 s each. Finally, two incubations in xylene for 10 s each were performed. Slides were then mounted with O-phenylene diamine (OPDA) and sealed. Positive cells were visualized with a Nikon Diaphot inverted microscope using visible light.

Purification of mononuclear phagocytes

Resident AMs were purified from the lungs of control rats by lavage as described (Wright *et al*., 2004). A-MNPs comprising both the resident AM population and the newly recruited MNPs were purified by lavage from lungs of rats insufflated with cytokine 24 h before. Cells were washed in RPMI medium and were then plated into flasks with RPMI medium and allowed to adhere for 1 h. Non-adherent cells were removed and the remaining adherent cells were washed four times with PBS and harvested from the plates by scraping into PBS/ethylenediaminetetraacetic acid (Davies and Gordon, 2005). Post-lavage mononuclear phagocytes (PL-MNPs) present in the lung tissue were subsequently purified from the lungs of rats following removal of the A-MNPs. Lungs were lavaged to obtain the A-MNP population, perfused blood free and cells not released by lavage were prepared as described (Steinmuller *et al*., 2000). Briefly, lungs were incubated under moderate agitation for 60 min in RPMI media containing 100 U ml^{-1} or collagenase type 1 and 50 U ml^{-1} DNAse at 37 °C. After incubation, the digested tissue was passed through a sterile sieve to remove tissue fragments, and the cells obtained in the flow through were adhered to flasks as described above. Adherent cells were suspended in saline to a final concentration of 2×10^6 cells ml^{-1}. MNPs comprised >95% of the cells recovered from adhesion as determined by the microscopic examination of Wright's-stained cells (Wright *et al*., 2004) from both the A-MNPs and the PL-MNPs preparations. Fluorescence-activated cell sorter analysis with the MNP-specific markers CD68 and CD163 confirmed that these cells were MNPs. Fluorescence-activated cell sorter analysis was performed on purified MNP populations exactly as described previously (Wright *et al*., 2004), using fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin (Ig)G_1 as a secondary antibody and mouse-derived primary antibodies to rat CD68 (ED1) and CD163 (ED2). Cultivation of both populations in the presence of M-CSF for 7 days resulted in differentiation of 98–100% of the cells into

macrophages, again confirming the absence of PMNs in these preparations.

Cell culture, adherence and injury in vitro

L2 rat lung alveolar type II cells (ATCC, Manassas, VA, USA, number CCL-149) were grown in Ham's F12K medium with 10% heat inactivated fetal bovine serum at pH 7.4 and 37 °C as indicated by the supplier. Cells were seeded in 48-well plates at 1×10^5 cells per well. After 24 h, monolayers were washed and exposed to cytokines as indicated. After 24 h of exposure to cytokine, cells were washed and resupplied with cytokine-free medium at which point 5×10^5 purified MNPs were added either in the presence or absence of blocking monoclonal antibodies, the integrin antagonists, BIO5192 or XVA143, at 1 mM. Non-adherent cells were removed 30 min later by washing twice in PBS. Adherent cells were quantitated exactly as described (Beck-Schimmer *et al*., 2001). Lactate dehydrogenase (LDH) activity was quantitated in the cell-free medium after 24 h of cocultivation.

Lactate dehydrogenase determination

LDH was quantitated in the cell-free BALF using the CytoTox96 kit, a non-radioactive cytotoxicity assay, exactly as indicated by the supplier (Promega Corporation, Madison, WI, USA). To preserve activity of LDH, samples were assayed from fresh, unfrozen BALF within 1 h of lavage. Cell-free BALF was maintained on ice until the assays were performed. LDH was quantitated from the cell-free supernatants of L2 monolayers and from L2/MNP cocultures. Total L2 cell content was determined by lysis of six independent confluent L2 monolayers. Total L2 LDH per well was 112.7 ± 2.48 IU. LDH isozyme content was determined in fresh BALF and in cocultures using the TITAN GEL-LD agarose electrophoresis as described by the supplier (Helena Laboratories, Beaumont, TX, USA). Freshly isolated BALF was concentrated by sequential precipitation in 40 and 70% $(NH_4)_2SO_4$, resuspended in sodium phosphate buffer at pH 7.5, dialysed overnight in sodium phosphate buffer, pH 7.5 and isotype electrophoresis performed. MNPs and neutrophils were isolated from cytokine-insufflated rats on Percoll gradients as described (Eisenhauer *et al*., 1989; Ross *et al*., 1998), lysed and LDH isotype determined. Fresh erythrocytes and serum were prepared from rats at the time of death and LDH isozyme distribution was determined for each.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis and western immunoblot analysis

Cell lysates were prepared as described previously (Wright *et al*., 2004), and the protein concentration determined by using the bicinchoninic acid assay. Aliquots containing 50 μg of protein or a constant volume of cell-free BAL (20 μl) were incubated with equal amounts of loading buffer (5% β-mercaptoethanol, 95% Laemmli loading dye) for 10 min at 37°C, and then boiled for 5 min. Samples were then separated by electrophoresis on 7.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis or 4–20% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels for 40 min at 100 V, transferred to polyvinylidene difluoride membranes (Whatman Inc, Florham Park, NJ, USA). Membranes were blocked overnight at 4°C in 5% non-fat dried milk in Tris-buffered saline (pH 7.6) containing 0.1% Tween. Membranes were then incubated with antibodies as indicated. Antigen-antibody complexes were detected by reaction with an enhanced chemiluminescence western blotting detection kit according to the manufacturer's instruction (Amersham Life Sciences, Piscataway, NJ, USA). Each experiment was run in triplicate, and representative immunoblots are shown.

Statistical analyses

Data are expressed as the mean and standard error of the mean and were assessed for significance using the Student's *t*-test or analysis of variance. $P<0.05$ was considered significant.

Reagents

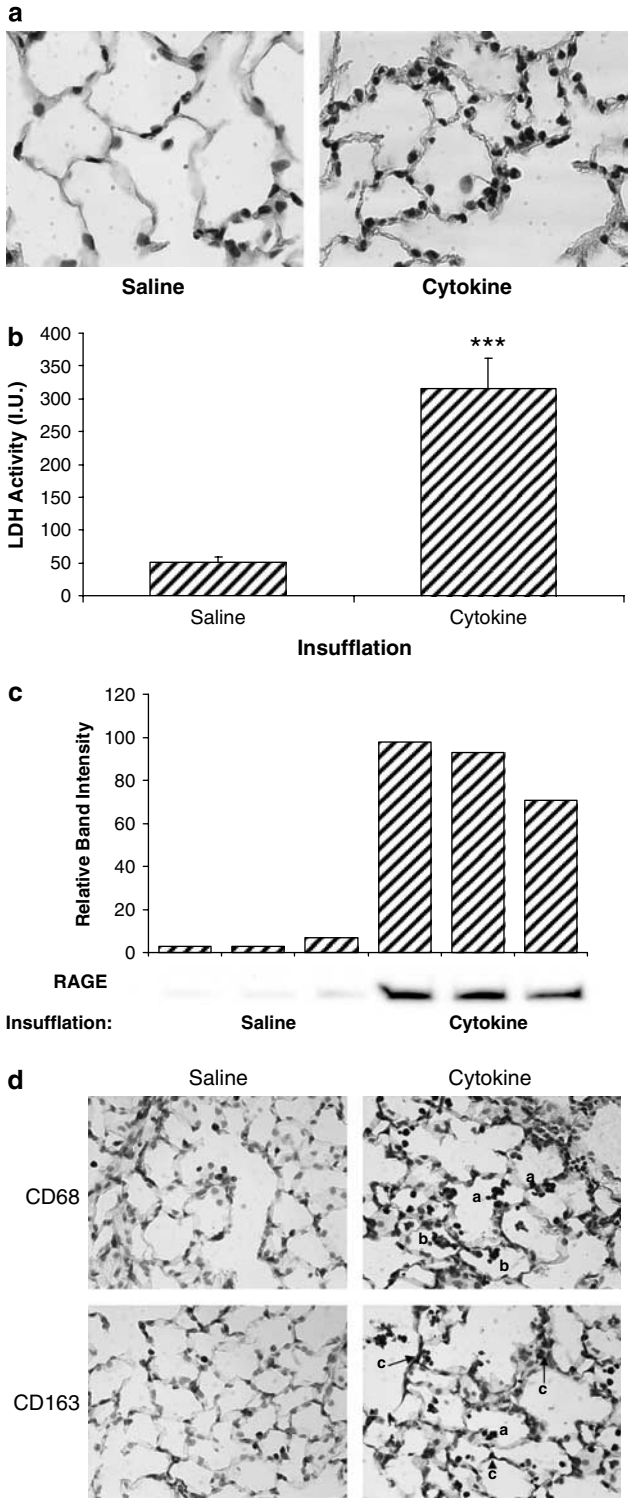
Most reagents, buffers, substrates and inhibitors were purchased from Sigma Chemical Company (St. Louis, MO, USA). Recombinant rat IL-1α (500-RL-005) and IFN-γ (285-IF-100) were purchased from R&D Systems (Minneapolis, MN, USA). Sterile normal saline (0.9% NaCl, pH 6.0) was purchased from Baxter Health Care (Deerfield, IL, USA). Mouse antibodies to the rat-specific MNP surface markers CD68 (MCA341R) and CD163 (MCA342R) were obtained from Serotec (Raleigh, NC, USA). Fluorescein isothiocyanate-conjugated goat anti-mouse IgG_1 (STAR70) was also obtained from Serotec. Goat polyclonal IgG intracellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), donkey anti-goat IgG-HRP and goat anti-mouse IgG-HRP were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-receptor for advanced glycation end products (RAGE) antibody sMAB1179 was purchased from R&D Systems. Normal goat serum was obtained from ICN Biomedicals (Aurora, OH, USA). Donkey serum was purchased from Research Diagnostics (Flanders, NJ, USA). The 3,3′-diaminobenzidine peroxidase substrate kit was obtained from Vector Laboratories (Burlingame, CA, USA). The ICAM/lymphocyte function-associated protein 1 (LFA-1) integrin antagonist, XVA143, was obtained from Hoffmann-La Roche (Nutley, NJ, USA). The VCAM/very late antigen-4 (VLA-4) integrin antagonist, BIO5192, was obtained from Biogen Inc. (Cambridge, MA, USA). Cyclophosphamide was from Sigma.

Results

Cytokine insufflation promoted apparent leukocyte–epithelial adhesion and injury

Our previous experiments demonstrated that cytokine insufflation promoted the rapid influx of MNPs and PMNs into the alveolar airspace that was associated with histological evidence of tissue injury, alveolar wall terminal deoxynucleotide UTP nick-end labeling staining and lung caspase-3 activation, suggesting the development of

significant tissue injury and apoptosis following cytokine insufflation (Wright *et al*., 2004). We observed that apparent adhesion of leukocytes was also promoted by cytokine insufflation, and cytokine-insufflated lungs revealed collapse of the inflamed alveolar walls and increased fragmentation or fragility of the alveoli themselves (Figure 1a).



To further characterize injury associated with cytokine insufflation, we quantified the levels of the intracellular enzyme, LDH, recovered in the cell-free BALF. Consistent with previous observations, lung LDH release was significantly increased 24 h following cytokine insufflation (Figure 1b). The LDH isozyme profile was determined from the cell-free BALF, Percoll gradient purified MNPs and PMNs, erythrocytes and serum from rats following cytokine insufflation. Whole-cell lysates from cultured rat lung epithelial cells, L2 cells, were used for comparison. The LDH isozyme profile from the cell-free BALF was very distinct from that obtained from purified MNPs, PMNs, erythrocytes or from serum (Table 1). Although minor contributions from LDH1, LDH2 and LDH3 were evident in the BALF, the ratio of LDH4 to LDH5 content was closest to that obtained from whole-cell lysates of L2 rat lung alveolar epithelial cells and markedly different from that obtained from lavage leukocytes, erythrocytes or serum (Table 1).

The protein called RAGE is expressed in both human and rat lung alveolar type-I cells (Dahlin *et al*., 2004; Shirasawa *et al*., 2004) and was recently identified as a marker for lung alveolar cell injury and inflammation, including lung injury that arises in human ALI (Morbini *et al*., 2006; Uchida *et al*., 2006). We observed an average 11.2-fold increase in RAGE recovered in the cell-free BALF following cytokine insufflation in rats compared to saline insufflation alone (Figure 1c).

Immunohistochemical staining confirms the presence of MNPs in the apparently adherent leukocytes

Considerable evidence supports the role of PMNs in injury to the lung in both chronic obstructive pulmonary disease airway inflammation and following cytokine insufflation (Leff *et al*., 1994a, b, 1995; Pettersen and Adler, 2002). Since cytokine insufflation could promote adhesion of either PMNs or MNPs to the respiratory epithelium *in vivo*, paraffin-embedded lung tissue specimens were stained for immunoreactivity to the MNP-specific proteins, CD68 and CD163. CD68 and CD163 are surface markers that reflect

Figure 1 Cytokine insufflation induced apparent leukocyte adhesion and lung injury. (**a**) Rats were insufflated with the saline vehicle or cytokine, and 24 h later lungs were perfused free of blood harvested, and prepared for histological examination. Tissue sections were stained by hematoxylin and eosin and representative sections photographed under light microscopy. (**b**) LDH levels were quantitated in the cell-free BALF recovered from rats insufflated as above with saline or cytokine. Six rats were used in each group for quantitation of LDH (***$P<0.01$ by Student's *t*-test). (**c**) Western immunoblot analysis of RAGE from the cell-free BALF of individual rats insufflated with either saline or cytokine. Three rats were randomly selected from each group of six used in panel a. Twenty microliters of cell-free BALF was loaded in each lane, and band intensity obtained from the western blot analysis was quantitated using Kodak imaging software. (**d**) Lung tissue specimens from rats insufflated with either saline or cytokine were stained for the MNP-specific markers CD68 and CD163 and counterstained with hematoxylin. The presence of MNPs in adherent (a), alveolar (b) and interstitial tissue compartments is indicated (c). BALF, bronchoalveolar lavage fluid; LDH, lactate dehydrogenase; MNPs, mononuclear phagocytes; RAGE, receptor for advanced glycation end products.

Table 1 LDH isozyme profile

	LDH1	*LDH2*	*LDH3*	*LDH4*	*LDH5*	*LDH4/LDH5*
BALF	1.26±0.096	1.30±0.88	1.44±0.097	11.9±0.66	84.1±3.2	0.142
MNP	0	0	0.99±0.011	4.0±0.033	95.1±5.5	0.042
PMN	0	0	0	0	100±2.0	ND
RBC	0	0	0	0	100±2.0	ND
L2	0	0	0	12.2±0.94	87.8±4.4	0.139
Serum	17.2±1.45	25.3±1.85	15.6±1.13	6.6±0.025	35.3±1.9	0.187

Abbreviations: BALF, bronchoalveolar lavage fluid; LDH, lactate dehydrogenase; MNP, mononuclear phagocyte; RBC, red blood cell.
LDH isozymes were separated and identified as described in Materials and methods using cell-free BALF or whole-cell lysates of MNP, PMN, erythrocytes (RBC) and serum from cytokine insufflated rats 24 h following cytokine treatment. Lysates of L2 cells were prepared from confluent monolayers. Isozyme bands were quantitated by scanning dosimetry following electrophoresis (see Materials and methods). Individual isozyme bands are expressed as percent contribution to the total isozyme content that was set at 100%. Data show the mean±s.e.m. of six independent determinations.

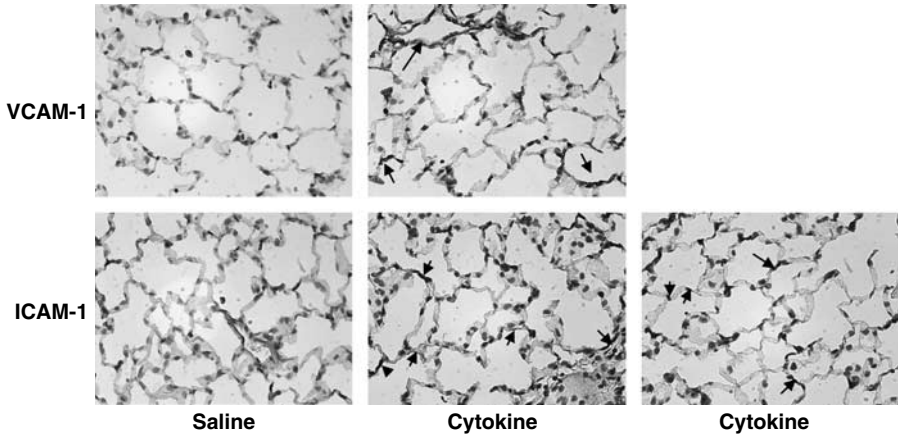


Figure 2 Immunohistochemical staining of alveolar ICAM-1 and VCAM-1. Lungs were insufflated with either saline or cytokine and 24 h later lungs were perfused free of blood, the BALF removed and the tissues stained for immunoreactive VCAM-1 or ICAM-1. Staining of alveolar ICAM-1 took on an apparently focal character (arrows) following cytokine insufflation (shown in two randomly selected fields). BALF, bronchoalveolar lavage fluid; ICAM-1, intracellular adhesion molecule 1; VCAM-1, vascular cell adhesion molecule-1.

differentiation and localization of MNPs in rats. While CD163 exhibits preferred binding to MNPs in the lung tissue and CD68 is expressed largely but not exclusively on A-MNPs, neither marker is expressed on PMNs (Covin *et al*., 1998). Fluorescence-activated cell sorter analysis had previously identified high-level CD68 expression on A-MNPs recovered from the lung lavage of rats insufflated with cytokines 24 h before (Wright *et al*., 2004). CD68 and CD163 immunoreactivity were detected in the A-MNPs and in MNPs found in the perivascular tissue space, the interstitial alveolar compartment and in the MNPs localized at the alveolar epithelium 24 h following cytokine insufflation (Figure 1c). These data confirm that many of the apparently adherent cells were MNPs.

Intratracheal cytokine insufflation stimulated alveolar ICAM-1/VCAM-1 expression

Adhesion and migration of MNPs into the alveolar compartment is thought to depend in part upon epithelial ICAM-1/MNP LFA-1 interaction and epithelial VCAM-1/MNP VLA-4 interaction, and alveolar epithelial cells are known to express ICAM-1 and VCAM-1 (Beck-Schimmer *et al*., 2001; Williams, 2003), while MNPs express LFA-1 and VLA-4 (Rabb *et al*., 1994; Li *et al*., 1998). Immunohistochemical analysis of cytokine-insufflated lungs showed enhanced staining of alveolar ICAM-1 and markedly increased staining of alveolar VCAM-1 24 h following cytokine treatment compared to saline-insufflated sham controls (Figure 2). Staining of alveolar epithelial ICAM-1 acquired an intense focal character 24 h following cytokine insufflation in contrast to VCAM staining that appeared more uniform (Figure 2).

Small molecule integrin antagonists suppressed cytokine-induced lung injury

The small molecule antagonist of α-L/β-2 integrin interaction, XVA143, blocks ICAM-LFA-1 interaction and leukocyte adhesion by tight binding to the α-chain I-domain, restricting ICAM to an inactive conformation that can inhibit inflammation *in vivo* (Lin *et al*., 1999; Welzenbach *et al*., 2002; Shimaoka *et al*., 2003; Li *et al*., 2004). We insufflated rats concurrently with cytokines and XVA143 and 24 h later lungs were harvested, leukocytes counted, and the cell-free BALF assayed for LDH and RAGE content. We observed that XVA143 coinsufflation did not prevent leukocyte infiltration (Figure 3a). In contrast, LDH release was significantly reduced by XVA143 coinsufflation at both high- and

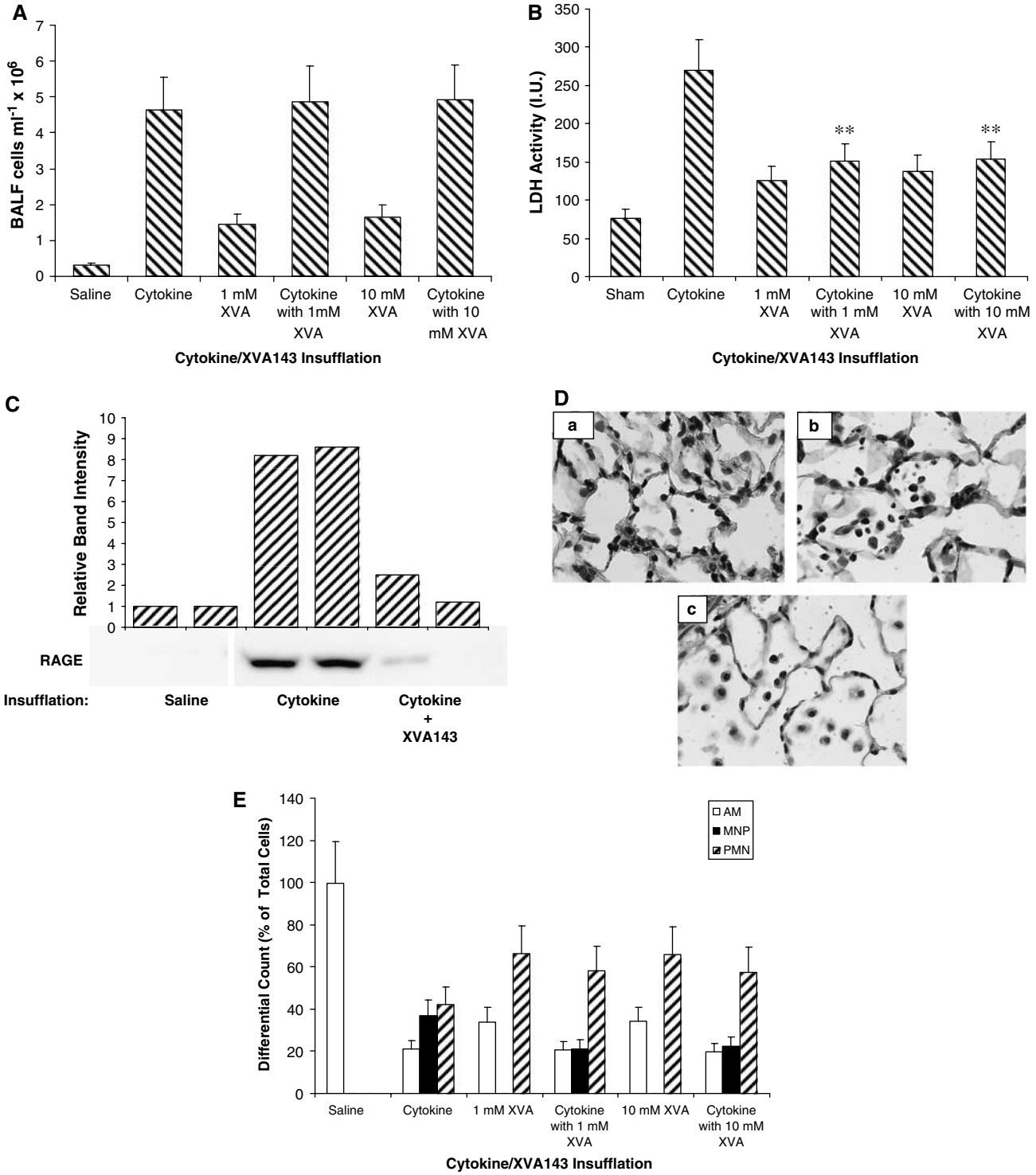


Figure 3 A small molecule antagonist of the α-L/β-2 integrin blocks cytokine-induced lung injury. Rats were insufflated with either saline, cytokine, XVA143, or coinsufflated with cytokine and XVA143. After 24 h, BALF was harvested, inflammatory leukocytes counted, and LDH and RAGE levels quantitated. (**A**) The effect of 1 and 10 mM XVA143 on the recovery of inflammatory cells from the BALF was determined by hemocytometer counting after Wright's staining. (**B**) The effect of high- and low-dose XVA143 on LDH activity found in the cell-free BALF was determined as described above. Statistical differences were calculated for comparison between rats receiving cytokine and no XVA143 or cytokine and 1 or 10 mM XVA143 (**$P<0.02$ by Student's *t*-test). (**C**) Western immunoblot analysis of RAGE obtained in the cell-free BALF. Twenty microliters of BALF was run per lane as described in Figure 1. Two rats from each group were randomly selected for illustration and similar results were obtained for all six rats in each group. Band intensity was normalized to the saline control. (**D**) The effect of XVA143 on the histological architecture. Both low-dose (box b) and high-dose (box c) XVA143 suppressed evidence of inflammation and leukocyte adherence compared to cytokine insufflation without XVA143 (box a). (**E**) Differential cell counts obtained from the BALF were determined following Wright's staining. Data shown are the mean and standard error for six rats in each group and represent the percentile contribution of each group to the total cell population, which was set at 100%. Note: an essentially pure AM population is routinely obtained from sham insufflation with saline alone. BALF, bronchoalveolar lavage fluid; LDH, lactate dehydrogenase; RAGE, receptor for advanced glycation end products.

low-dose treatment (Figure 3b), and RAGE levels recovered in the cell-free BALF were also dramatically reduced by XVA143 coinsufflation (Figure 3c). Histological examination of lung tissues from cytokine-insufflated rats with either high- or low-dose XVA143 coinsufflation revealed preservation of lung tissue architecture and diminished alveolar inflammation (Figure 3d). Thus, the α-L/β-2 antagonist, XVA143, decreased evidence of lung injury in cytokine-insufflated rats. We observed that XVA143 given alone promoted a small but consistent PMNs infiltration (Figures 3a and e) without significantly increasing LDH release or reducing the AM levels. XVA143 treatment alone did not increase the level of newly recruited A-MNPs (Figure 3e).

Small molecule antagonists of the α-4/β-1 integrin interaction, BIO1211 and BIO5192, have been used in rats and sheep to block experimentally induced airway inflammation and autoimmune encephalomyelitis (Lin *et al.*, 1999; Abraham *et al.*, 2000; Leone *et al.*, 2003; Theien *et al.*, 2003). Both intraperitoneal and intratracheal administration of BIO1211 reduced airway inflammation and LDH release in animal models of inflammation (Lin *et al.*, 1999; Abraham *et al.*, 2000). BIO5192 is a highly selective, and potent inhibitor of α-4/β-1 (VLA-4) interaction with VCAM-1 and possesses a K_D of <10 pM *in vitro* (Leone *et al.*, 2003; Theien *et al.*, 2003). We examined the capacity of BIO5192 to block cytokine-induced injury in rats when insufflated directly into the airway. BIO5192 had no significant affect on cytokine-induced recruitment of leukocytes when coinsufflated with cytokine (Figure 4a). In contrast, BIO5192 dose-dependently suppressed LDH release when coadministered with cytokines (Figure 4b). At doses of 10 μg and below, BIO5192 began to lose effectiveness 24 h following cytokine insufflation. However, LDH release was reduced to sham-treated levels at a dose of 10 μg. Higher doses of BIO5192 promoted LDH release. Recovery of RAGE in the cell-free BALF was reduced over threefold by coinsufflation of BIO5192 (Figure 4c).

While XVA143 did not produce the finely graded dose response observed here for BIO5192, both high- and low-dose XVA143 generated statistically significant reduction in LDH release and recovery of RAGE in the cell-free BALF. Thus, antagonists of both α-L/β-2 and α-4/β-1 blocked LDH and RAGE release when coadministered with cytokine.

LDH release requires both cytokine priming and the presence of MNPs

As reported for the insufflation of endotoxin in mice (Maus *et al.*, 2006), cytokine insufflation in rats produces rapid influx into the lung of newly recruited MNPs and PMNs that effectively dilute the resident AM population. We observed

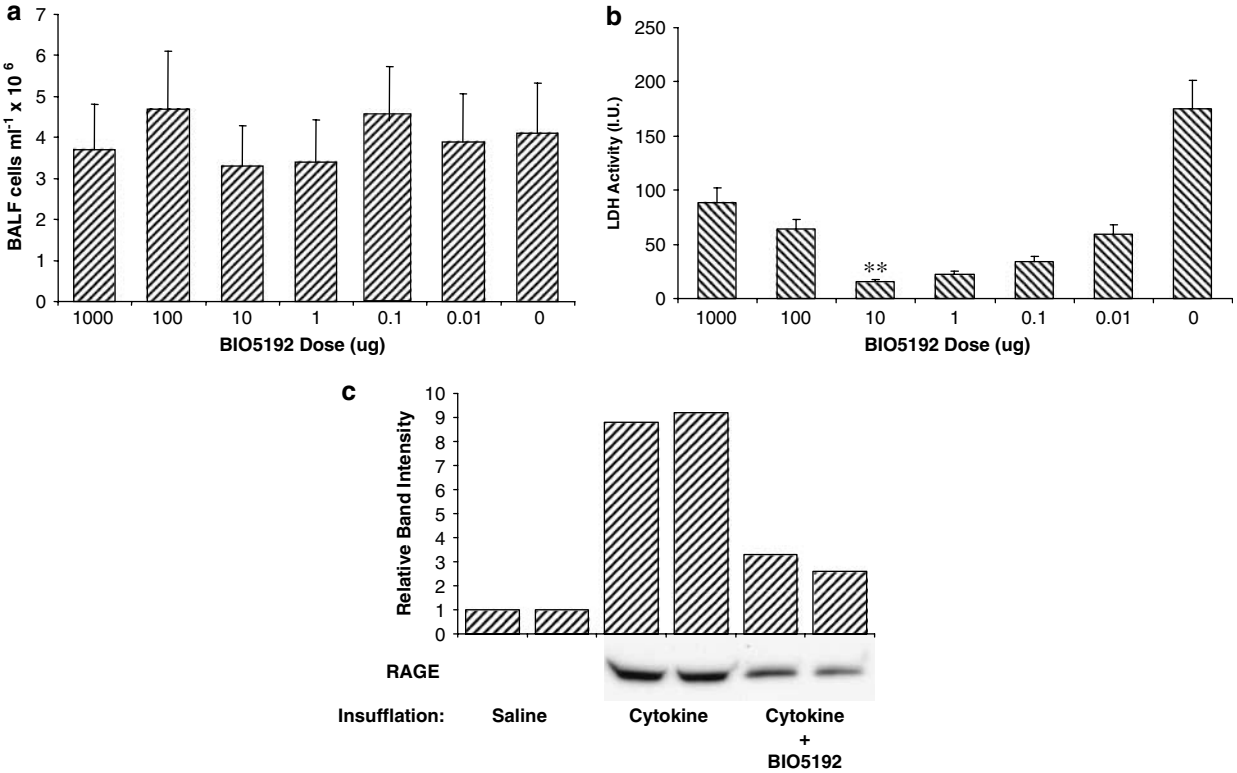


Figure 4 A small molecule antagonist of the α-4/β-1 integrin blocks cytokine-induced lung injury. Rats were insufflated with cytokine in the presence or absence of BIO5192 over a broad concentration range; and after 24 h, BALF was harvested, inflammatory leukocytes counted and levels of LDH and RAGE quantitated in the cell-free BALF. (**a**) The effect of a range of doses of BIO5192 on cytokine-induced BALF cell counts was determined as described in Figure 3. (**b**) The effect of BIO5192 dose on cell-free BALF LDH levels. Six rats were used in each group of both (**a**) and (**b**), except for the highest dose of BIO5192 where two rats were used. Statistical differences were calculated for comparison between rats receiving cytokine and no BIO5192 or cytokine and BIO5192 (***$P<0.01$ determined by Student's *t*-test). (**c**) Representative western immunoblot analysis of two rats from each group. BIO5192 was used at 10 μg for rats receiving both cytokine and BIO5192. BALF, bronchoalveolar lavage fluid; LDH, lactate dehydrogenase; RAGE, receptor for advanced glycation end products.

no detectable LDH or RAGE release in the BALF of saline-insufflated rats, expressing an essentially pure AM population. On the other hand, LDH and RAGE were released during cytokine insufflation, which may reflect injury induced by PMNs, AMs, or newly recruited A-MNPs. Since cytokine insufflation necessarily mixes resident AMs with newly recruited MNPs, MNPs were purified from cytokine-insufflated rats, comprising A-MNPs, and from the post-lavage lung tissue, comprising PL-MNPs. The A-MNP population contains both the newly recruited MNPs and the resident AMs present before cytokine insufflation, whereas MNPs purified from the post-lavage lung tissue (PL-MNPs) contain few or no AMs. Fluorescence-activated cell sorter analyses of A-MNPs and post-lavage PL-MNPs using antibodies to CD68 and CD163 confirmed the absence of PMNs or AMs in the purified PL-MNPs (not shown). A-MNP and PL-MNP populations were purified from the lungs of rats insufflated with cytokine 24 h before, and 1×10^6 cells were then insufflated into naïve rats without cytokine priming. Cell-free BALF was prepared from these rats with adoptively transferred MNPs 24 h later and LDH levels were quantitated in the cell-free BALF. Although a minor trend in injury produced was apparent, no statistically significant difference between PL-MNPs, A-MNPs or control treatments was observed (data not shown). Thus, in rats not previously primed with cytokine, transfer of neither population of insufflated MNPs was able to induce significant LDH release. Since the A-MNPs comprise both AMs and newly recruited MNPs, we infer that neither population induced lung injury in the absence of cytokine insufflation.

Cyclophosphamide pretreatment was used to deplete circulating leukocytes using the regimen indicated (Figure 5a). As described previously (Ikezumi *et al.*, 2003a, b), cyclophosphamide treatment produced a persistent and profound leukopenia throughout the subsequent 96 h following treatment. At no point in this period did the circulating leukocyte population exceed 5% of the native level (Figure 5b). We treated 15 rats with cyclophosphamide or with no cyclophosphamide, and 48 h later 12 rats were insufflated with cytokine. Twenty-four hours following cytokine insufflation, three rats were insufflated with A-MNPs and three rats were insufflated with PL-MNPs. LDH was measured from rats receiving either cyclophosphamide or cyclophosphamide and cytokine at 72 h (i.e. 24 h exposure to cytokine). LDH was measured from rats receiving either cyclophosphamide or cyclophosphamide at 48 h, followed by cytokine and 24 h later insufflated with 1×10^6 A-MNPs or PL-MNPs. Thus, these rats were exposed to cyclophosphamide for 96 h, cytokine and no cells for 24 h, and subsequently MNP cells for an additional 24 h (total of 96 h). Rats receiving cytokine but no cyclophosphamide were harvested

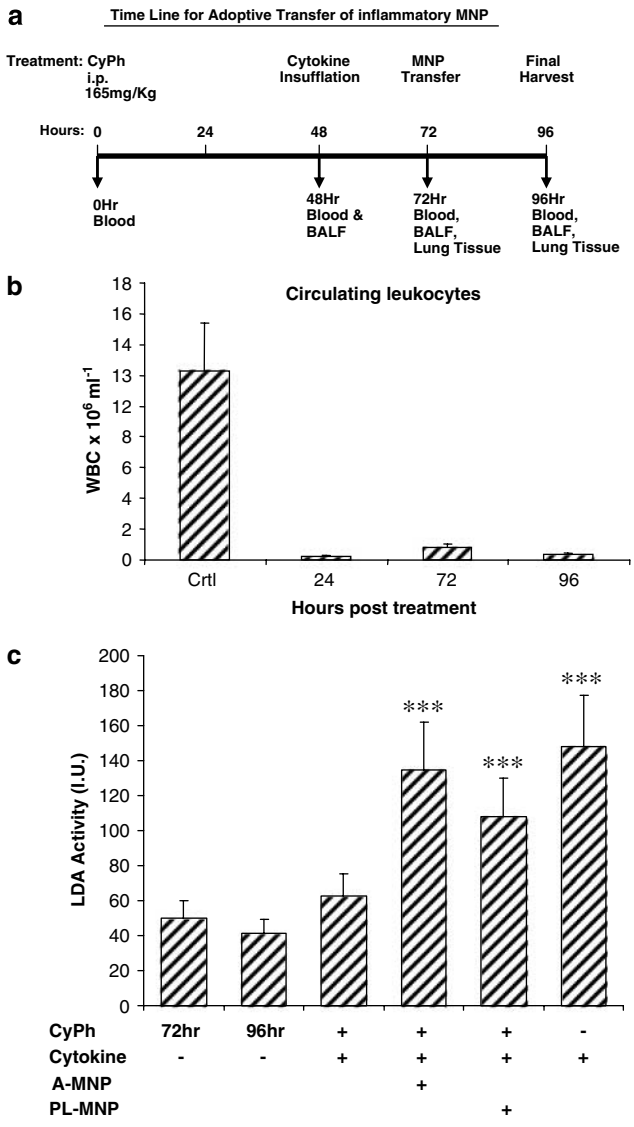
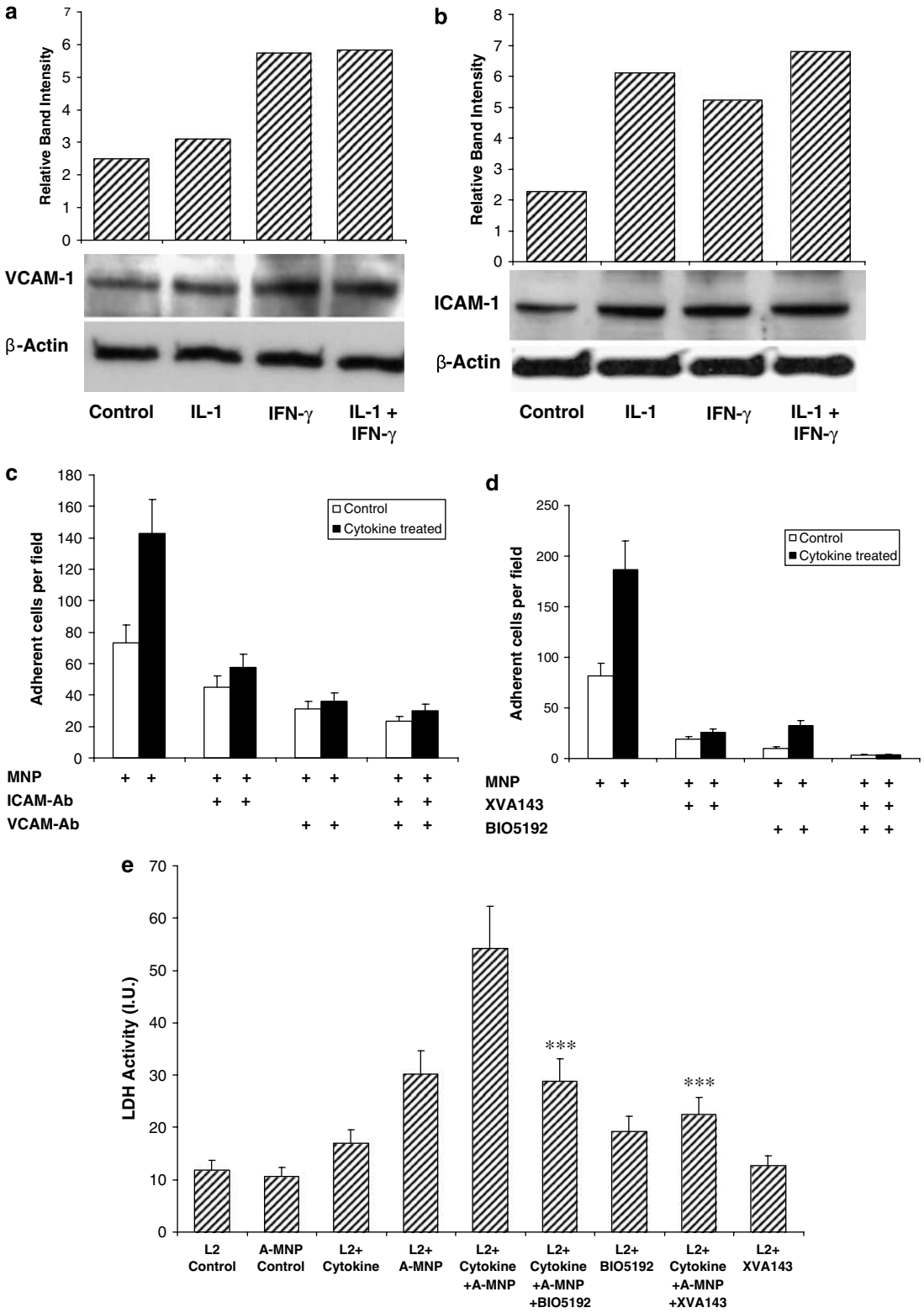


Figure 5 Adoptive transfer of MNPs into rats rendered leukopenic by pretreatment with CyPh demonstrated that MNPs but not cytokine alone promote LDH release *in vivo*. (**a**) The time-line used for CyPh treatment and adoptive transfer of MNPs is illustrated. (**b**) Total circulating leukocytes were quantitated at various points following CyPh treatment, and these data confirm the persistence and degree of leukopenia obtained by the CyPh treatment used in panel c. (**c**) Fifteen rats were treated with 165 mg kg^{-1} CyPh or three rats with no CyPh (CyPh −). Forty-eight hours later, 12 rats were insufflated with cytokine (cytokine +). Twenty-four hours following cytokine insufflation, three rats were insufflated with purified A-MNPs and three rats were insufflated with purified PL-MNPs. LDH was measured in the cell-free BALF from rats receiving either CyPh or CyPh and cytokine at 72 h (i.e. 24 h exposure to cytokine). LDH was measured from rats receiving either CyPh or CyPh at 48 h, followed by cytokine and 24 h later insufflated with 1×10^6 A-MNPs or PL-MNPs. Thus, these rats were exposed to CyPh for 96 h, cytokine and no cells for 24 h, and subsequently MNP cells for an additional 24 h (total of 96 h). Rats receiving cytokine but no CyPh were harvested 96 h following cytokine insufflation. LDH data were analysed by analysis of variance and only salient points are indicated (***$P<0.02$ relative to 96 h CyPh with or without cytokine). No statistically significant difference was observed between 72 or 96 h CyPh with or without cytokine insufflation, and no statistically significant difference was observed between rats receiving adoptively transferred A-MNPs, PL-MNPs in the presence of cytokine and CyPh and those insufflated with cytokine in the absence of CyPh treatment. A-MNPs, alveolar mononuclear phagocytes; CyPh, cyclophosphamide; LDH, lactate dehydrogenase; MNPs, mononuclear phagocytes; PL-MNPs, post-lavage mononuclear phagocytes.

24 h following cytokine insufflation, typical of the leukonormic model routinely used. We observed that LDH release was not induced by cytokine insufflation in rats pretreated with cyclophosphamide (Figure 5c). However, significant increase in LDH release was produced when rats were pretreated with cyclophosphamide, insufflated with cytokine 48 h later, and subsequently insufflated with PL-MNPs or A-MNPs (Figure 5c). These data demonstrate that while cytokine alone was unable to induce LDH release, in cytokine-primed lungs adoptively transferred MNPs from



either A-MNP or PL-MNP populations promoted statistically significant release of LDH compared to rats treated in the same way but without adoptive transfer of MNPs. Furthermore, the level of LDH measured in the cell-free BALF of cyclophosphamide-treated rats insufflated with MNPs was not significantly different from that obtained by cytokine insufflation in cyclophosphamide-untreated rats.

Epithelial cell LDH release in vitro *was induced by MNPs and blocked by XVA143 and BIO5192*

To confirm that MNPs recovered from the lungs of cytokine-insufflated rats could promote epithelial injury, A-MNPs were cocultivated on monolayers of L2 cells, a rat type II alveolar epithelial cell line. Both ICAM-1 and VCAM-1 expression were increased in L2 cells by 24 h of cytokine treatment (Figures 6a and b). Adhesion of purified A-MNPs to L2 monolayers was stimulated by cytokine treatment and inhibited by treatment with blocking monoclonal antibodies to ICAM-1 or VCAM-1 (Figure 6c). Similarly, the small molecule integrin antagonists, XVA143 and BIO5192, also blocked A-MNP adhesion to L2 monolayers *in vitro* (Figure 6d). We observed that both antibody to ICAM-1 and VCAM-1 as well as XVA143 and BIO5192 reduced MNP adhesion to control L2 cells, and this most likely reflects the pre-existing level of ICAM-1 and VCAM-1 expressed by cultured L2 cells.

Spontaneous release of LDH from L2 cells (12 IU of activity) over the course of 24 h represented approximately 10% of the total LDH content of L2 cells (112.7 ± 2.48 IU), and this was not significantly elevated by 24 h exposure to cytokine (Figure 6e). Similarly, spontaneous release of LDH from A-MNPs cultivated alone for 24 h (9.8 IU of activity) represented approximately 15% of the total A-MNP LDH (65.33 ± 13.1 IU of activity). Cocultivation of cytokine-treated L2 cells with A-MNPs recovered from the lungs of cytokine-insufflated rats stimulated LDH release to a level of approximately 51% of the total L2 cell LDH after 24 h of cocultivation (Figure 6e). Significant reduction in LDH release was observed in L2/A-MNP cocultures by treatment with BIO5192 or XVA143. Cocultivation of L2 cells and A-MNPs in the presence of both integrin antagonists caused substantial dissociation of the L2 monolayer after 24 h and consequently LDH was not measured in these experiments. We observed that BIO5192 alone, but not XVA143, promoted modest release of LDH from L2 cells. Nonetheless, A-MNPs recovered from the lungs of cytokine-insufflated rats stimulated alveolar epithelial cell LDH release *in vitro* that was inhibited by BIO5192 and XVA143.

Discussion and conclusions

Injury to the alveolar epithelial barrier is a critical component of pathogenesis in many lung inflammatory disorders that may be targeted for therapeutic intervention. However, the relatively poor understanding of injury has limited potential therapeutic opportunities. Our present experiments demonstrate that cytokine-induced lung inflammation promoted alveolar MNP adhesion and lung injury, and that insufflation of α-4/β-1 and α-L/β-2 integrin antagonists disrupted MNP adhesion and lung injury.

Data shown here demonstrate that cytokine-induced inflammation is associated with increased lung injury. Histologic evidence of alveolar inflammation and tissue damage as well as elevation of RAGE and LDH found in the cell-free BALF were induced 24 h following cytokine insufflation. While MNPs and PMNs comprise 99% of the leukocytes present in the alveolar space following cytokine insufflation, the LDH isozyme profile obtained in the cell-free BALF was distinct from that found in either MNPs or PMNs purified from the same rats. Furthermore, while haemorrhage may be expected in cytokine-insufflated lungs, it is apparently accompanied by negligible haemolysis since the LDH isozyme profile was also different from that obtained from erythrocytes. While the ratio of LDH4 to LDH5 found in the cell-free BALF was similar, obtained from L2 rat lung epithelial cells, the clear presence of low levels of

Figure 6 Effects of L2/A-MNP coculture on L2 ICAM/VCAM expression, leukocyte adhesion and cell injury. (**a**) Western immunoblot analysis of VCAM-1 expression in cytokine-treated L2 cells. L2 cells were grown to 80–90% confluency and treated with either 10 ng ml^{-1} rrIL-1α, 25 ng ml^{-1} rrIFN-γ, both cytokines combined or were untreated. After 24 h, whole-cell lysates were prepared and used in western blot analysis for VCAM-1 expression. Band intensity for representative immunoblots was quantitated and normalized to the β-actin control. (**b**) Western immunoblot analysis of ICAM-1 expression in cytokine-treated L2 cells. Whole-cell lysates from (**a**) were used for immunoblot analysis of ICAM-1 expression. (**c**) Adherence assay with purified A-MNPs and blocking monoclonal antibodies to ICAM-1 or VCAM-1. L2 cells were grown in 48-well plates and were treated with IL-1 and IFN-γ or were untreated. After 24 h, cells were washed in PBS and the growth medium replaced. Five micrograms of blocking antibody was then added per well, and 1.5×10^5 purified A-MNPs were added 30 min later. Following 30 min of adherence, media was removed, the non-adherent cells were removed by washing twice with 0.5 ml PBS and the remaining adherent MNPs were counted from two high-power fields per well. Data show the mean and standard error for six wells in each group. (**d**) Adherence assay with purified A-MNPs and XVA143 or BIO5192. L2 cells and A-MNPs were cocultivated exactly as described in panel c except that 1 mM XVA143 or BIO5192 was used instead of the blocking monoclonal antibodies. (**e**) LDH was quantitated in the cell-free medium from cells grown as follows. L2 cells were grown for 72 h alone (L2 control) or grown for 24 h, washed, treated with IL-1 and IFN-γ for 24 h, washed and grown for an additional 24 h (L2 + cytokine). L2 cells cocultivated with MNPs were grown for 24 h, washed, treated with cytokine or vehicle for 24 h, washed, and MNPs or MNPs with 1 mM BIO5192 or 1 mM XVA143 were added as indicated. LDH was quantitated in the cell-free medium 24 h later. Purified A-MNP control cells were cultivated alone in RPMI with 10% heat inactivated FBS for 24 h (A-MNP control). L2 cells were grown alone for 48 h and then exposed to BIO5192 or XVA143 for 24 h after which LDH was quantitated in the cell-free medium. Each point represents the average of six independent experiments and data show the mean LDH activity $\pm$ s.e.m. (***$P < 0.02$ for comparison between L2 + cytokine + A-MNPs in the presence of BIO5192 or XVA143). A-MNPs, alveolar mononuclear phagocytes; FBS, fetal bovine serum; ICAM, intracellular adhesion molecule; IFN-γ, interferon-γ; IL-1, interleukin-1; LDH, lactate dehydrogenase; MNPs, mononuclear phagocytes; PBS, phosphate-buffered saline; VCAM, vascular cell adhesion molecule.

LDH1, LDH2 and LDH3 may indicate either a contribution from serum leakage into the lung or from other unidentified cells. However, the LDH isozyme profile obtained in the cell-free BALF was different from that obtained from serum. These observations are consistent with the lung tissue being the primary source of LDH found in the cell-free BALF of cytokine-insufflated rats as previously reported in both human clinical specimens (Cobben *et al*., 1999a) and for rat lung inflammation induced by either chemical means or by pathogen (Schultze *et al*., 1994; Cobben *et al*., 1999b). Elevation of RAGE in the cell-free BALF is also indicative of injury to alveolar epithelial cells, and these data reinforce the observation that lung alveolar apoptosis, oxidative stress and tissue damage were also increased by cytokine-induced inflammation (Wright *et al*., 2004).

The alveolar epithelial adhesion molecules, ICAM-1 and VCAM-1, were stimulated by cytokine insufflation and this was associated with apparent increased alveolar MNP adhesion. Both type I and type II alveolar epithelial cells express ICAM-1 constitutively (Williams, 2003) and alveolar epithelial ICAM-1 is inducible *in vitro* with both IL-1 (Krakauer, 2000) and IFN-γ (Look *et al*., 1992). Importantly, type II cell alveolar epithelial ICAM-1 was induced *in vivo* by IFN-γ in both mice (Kang *et al*., 1996) and rats (present data), and the combined action of IL-1 and IFN-γ further stimulated ICAM-1 staining in rat lungs. Cytokine stimulation of epithelial ICAM-1 is significant in the context of lung injury because ICAM-1 facilitates both adhesion and migration of MNPs over the epithelial surface (Chang *et al*., 2002; Paine *et al*., 2002) and lung inflammation induced by bacterial endotoxin (Beck-Schimmer *et al*., 2002).

The role of alveolar epithelial VCAM-1 is less clearly delineated in cytokine-induced lung inflammation than that of ICAM-1. However, both ICAM-1 and VCAM-1 were required for efficient migration of MNPs through an alveolar epithelial monolayer (Rosseau *et al*., 2000b), and both ICAM and VCAM can be induced on rat alveolar epithelial cells to promote MNP adhesion (Rabb *et al*., 1994; Beck-Schimmer *et al*., 2001). *In vitro*, IL-1 and IFN-γ markedly upregulated VCAM expression in astrocytoma monolayers and also promoted MNP adhesion (Rosenman *et al*., 1995). The present data are consistent with these observations in asmuch as VCAM was induced in rat lung epithelial cells both *in vitro* and *in vivo* by cytokine insufflation. These observations suggest that VCAM may play a significant role in mediating MNP–epithelial interaction in cytokine-induced lung inflammation as it does in mediating endotoxin- or antigen-induced airway inflammation (Rabb *et al*., 1994; Li *et al*., 1998).

Roles for ICAM/LFA-1 and VCAM/VLA-4 interactions in inflammatory disorders are frequently established using monoclonal antibodies directed against ICAM or VLA-4 (Rabb *et al*., 1994; Li *et al*., 1998). However, the observation that statins can block inflammatory processes at the level of integrin function has led to the development of small molecule, statin-like antagonists with high specificity and efficient binding characteristics that avoid some of the disadvantages attributed to the *in vivo* use of monoclonal antibodies. We employed the LFA antagonist, XVA143 (Welzenbach *et al*., 2002; Shimaoka *et al*., 2003), to inhibit ICAM/LFA-1 interaction in the lung, and our data revealed that disruption of ICAM/LFA-1 interaction *in vivo* reduced histologic evidence of inflammation and injury as well as the release of LDH and RAGE in the cell-free BALF.

Immunohistochemical analysis also demonstrated activation of alveolar epithelial VCAM following cytokine insufflation, suggesting that it may also be an important component of MNP/alveolar epithelial interaction. Several small molecule antagonists of $\alpha_4\beta_1$-integrin (VLA-4) interaction with VCAM have been synthesized and used to block airway inflammation *in vivo* (Lin *et al*., 1999; Abraham *et al*., 2000). Importantly, these compounds (especially BIO-1211) protected the airway from injury when delivered either by an intravenous or intratracheal route. We used a derivative of BIO-1211 to inhibit VLA-4/VCAM interaction in our model of cytokine-induced lung inflammation. This compound, BIO5192, is a very selective and potent inhibitor of $\alpha_4\beta_1$-interaction and has been used *in vivo* in rats to block experimentally induced autoimmune encephalomyelitis (Leone *et al*., 2003; Theien *et al*., 2003). Rats insufflated concomitantly with cytokine and BIO5192 showed a dose-dependent decrease in LDH release and reduction of RAGE in the cell-free BALF, consistent with a role for VLA-4/VCAM interaction in cytokine-induced lung injury.

While a statistically significant and dose-dependent reduction in LDH release was produced by XVA143 or BIO5192, it is important to note that, at all doses, both compounds produced a mild neutrophil alveolitis that was independent of cytokine insufflation (data not shown). While the present impression of these compounds is one of monovalent and antagonistic binding (Lin *et al*., 1999; Abraham *et al*., 2000; Leone *et al*., 2003; Theien *et al*., 2003), it is possible that the inhibitors are also capable of producing a chemotactic signal for PMNs. It is unknown how such unexpected signaling would arise. We imagine that in the absence of a specifically activating signal, PMNs concomitantly recruited to the lung during cytokine insufflation do not promote LDH release and this is supported by the absence of LDH in BALF from rats insufflated with XVA143 or BIO5192 alone. Nonetheless, despite the presence of PMNs in the cytokine-insufflated lung, LDH and RAGE release were reduced by each compound in cytokine-insufflated rats. Clearly, further study is required to establish the significance of the low-level neutrophil alveolitis induced by intratracheal use of these compounds.

Apparent adhesion of MNPs to the respiratory epithelium was associated with enhanced lung injury, and disruption of this interaction reduced evidence of lung injury including histologic evidence of inflammation and tissue damage and release of RAGE and LDH into the cell-free BALF. Adoptive transfer of MNPs into the lungs of rats not previously exposed to cytokine failed to promote LDH release, suggesting that injury required both cytokine stimulation and the presence of MNPs. Depletion of circulating leukocytes with cyclophosphamide (Ikezumi *et al*., 2003a, b) showed that LDH release depended on both cytokine priming and the presence of inflammatory MNPs, whereas cytokine alone did not promote LDH release. These observations indicated that the alveolar epithelial cell/MNP interaction mediated lung injury and that inhibitors of ICAM-1/LFA-1 and VCAM-1/

VLA-4 interaction reduced injury. Similarly, intratracheal administration of IFN-γ in rats (Steinmuller *et al*., 2000; Zhang *et al*., 2003; Ikezumi *et al*., 2003a, b) or LPS insufflation in rats and mice (Beck-Schimmer *et al*., 2002; Skerrett *et al*., 2004) also revealed an important role of alveolar epithelial cell/leukocyte interaction in lung inflammation.

The capacity for MNPs derived from the lungs of cytokine-insufflated rats to promote epithelial injury was corroborated *in vitro* using cocultures of MNPs and L2 rat lung epithelial cells. While endogenous levels of ICAM-1 and VCAM-1 promoted MNP adhesion to cultured L2 cells, both MNP adhesion and cell injury were stimulated in cytokine-treated L2 monolayers, and disruption of MNP adhesion with either α-4/β-1, α-L/β-2 antagonists, or monoclonal antibodies to ICAM-1 or VCAM-1 blocked LDH release. L2 cells are considered to be type II epithelial cells and therefore unlikely to express RAGE, and indeed we were unable to detect significant levels of RAGE in these cells.

It is not known how MNPs contribute to injury. MNPs purified from either the lavage of cytokine-insufflated rats or from the post-lavage tissue, where the AMs have been removed, produced statistically indistinguishable injury *in vivo*. However, depletion of AMs in the lungs of rats with clodronate liposomes was also found to reduce alveolar dysfunction in ventilator-induced lung injury (Frank *et al*., 2006), and together these data suggest that the capacity to promote lung injury may be a general property of MNPs induced during inflammation. While the mechanism by which MNPs contribute to lung injury is not well understood, our data demonstrate that injury can be reduced with antagonists of α-4/β-1 and α-L/β-2 interaction.

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Conflict of interest

The authors state no conflict of interest.

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