Neutrophils and Neutrophil Products Do Not Mediate Pulmonary Hemodynamic Effects of Endotoxin on Oleic Acid-Induced Lung Injury

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Small-dose endotoxin (Etx) prevents pulmonary perfusion redistribution away from edematous dorsal lung regions after oleic acid (OA)-induced injury in dogs, causing a significant deterioration in oxygenation. We hypothesized that small-dose Etx might mediate this effect via polymorphonuclear neutrophil (PMN) priming with release of inflammatory mediators such as platelet activating factor (PAF) or secretory phospholipase A_2 ($sPLA_2$). To test this hypothesis, we administered specific inhibitors directed against each mediator and used two strategies to generate neutropenia. PAF and $sPLA_2$ inhibitors were administered before OA injury, followed 2 h later by small-dose Etx ($n = 4$ each group). PMN depletion was achieved by hydroxyurea administration for 5 days before the study to achieve absolute neutrophil counts $<1000/mm^3$ ($n = 4$). Inhibition of PMN adherence to lung endothelium was achieved by the administration of an anti-CD18 monoclonal antibody immediately before lung injury ($n = 5$). Positron emission tomography was used to evaluate pulmonary perfusion distribution and lung water content. We observed no effect of these interventions on the perfusion pattern after Etx + OA. Thus, neither neutrophils nor PAF or $sPLA_2$ mediate the effects of Etx on the pattern of perfusion in this model of lung injury.

(Anesth Analg 2004;98:452–7)

The oleic acid (OA) model of experimental acute lung injury (ALI) is characterized by increased pulmonary vascular permeability, proteinaceous alveolar edema, and severe hypoxemia—all cardinal features of the acute respiratory distress syndrome (ARDS) in humans (1). However, another characteristic feature of the OA model is pulmonary perfusion redistribution in which blood flow is directed away from the most edematous portions of lung to more normal regions, thereby helping to preserve gas exchange (2). Interestingly, using the same techniques to measure regional pulmonary blood flow (PBF), significant perfusion redistribution has not been observed in patients with ALI (3).

Small doses of IV endotoxin (Etx), doses that themselves have minimal systemic or pulmonary hemodynamic effects, profoundly alter the biochemical and physiologic expression of OA-induced lung injury (2). Perfusion redistribution is abolished, and oxygenation deteriorates significantly, thereby achieving a pattern similar to that observed in humans (3). In dogs, these changes seem to be mediated by increased prostacyclin production via the cyclooxygenase (COX)-2 pathway (4).

It is likely that the effects of Etx in the OA model are mediated by one or more intermediate proinflammatory molecules, especially those associated with the production of lipid mediators such as prostacyclin. One candidate would be the phospholipase A_2 family of enzymes (5,6), especially secretory phospholipase A_2 ($sPLA_2$), because production of this enzyme is stimulated by Etx (7), increased serum levels have been associated with the development of ARDS in humans (8), and its inhibition experimentally moderates the physiologic consequences of OA-induced lung injury (9,10).

Another possibility is platelet-activating factor (PAF), because this molecule has also been associated with Etx-induced increases in eicosanoids (11) and with the development of ALI in animals and humans (12).

Supported, in part, by the National Institutes of Health grant no. HL32815.

Accepted for publication September 9, 2003.

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DOI: 10.1213/01.ANE.0000097167.39112.A3

0003-2999/04

The recent availability of highly specific inhibitors allowed us to test whether either mediator was involved in generating the effects of small-dose Etx on OA-induced lung injury. Furthermore, because both molecules can be synthesized by neutrophils (5,13), we used two additional strategies to eliminate the potential effects of neutrophils on the Etx-OA lung injury interaction.

Methods

These studies were approved by the Washington University School of Medicine's Animal Studies Committee. Mongrel dogs, weighing 18 to 22 kg, were anesthetized with pentobarbital sodium (40 mg/kg), intubated with a cuffed endotracheal tube, and mechanically ventilated with a Harvard pump respirator (South Natick, MA) using a fraction of inspired oxygen concentration = 1.0 at a tidal volume of 15 mL/kg in the supine position to achieve a peak inspiratory pressure of 18–20 cm H_2O. Respiratory rate was adjusted to achieve a normal $Paco_2$. Positive end-expiratory pressure was not used, consistent with the ventilatory strategy used in our previous studies of OA lung injury (2). An additional barbiturate was administered via continuous infusion to eliminate spontaneous breathing.

The animals were percutaneously instrumented with a 20-gauge femoral artery catheter (Arrow, Reading, PA) and an 8.5F femoral venous sheath (Baxter, Irvine, CA). A 7.5F balloon-tipped pulmonary artery catheter (Baxter) was placed in the pulmonary artery and advanced to wedge position using pressure waveform guidance. An internal jugular venous catheter (Cook, Bloomington, IN) was placed for drug and radionuclide infusion.

Cardiac output was measured using the thermodilution technique with an Edwards Laboratories cardiac output computer (Santa Ana, CA). Blood gases were analyzed using an Instrumentation Laboratories model 1306 blood gas analyzer (Milan, Italy).

Regional PBF and regional lung water concentration (LWC) were measured with positron emission tomography (PET) imaging. These measurements were obtained with a "Super PETT" 3000 system built in-house. The design features, methods for calibration, corrections for activity decay, and corrections for photon attenuation of the system have been described previously (14).

The animals were placed in the scanner in the supine position with the most caudal tomographic slice about 1 to 2 cm below the dome of the diaphragm. Data were recorded simultaneously from 7 slices with a center-to-center separation of 1.05 cm and an in-plane, full-width, half-maximum spatial resolution of 0.85 cm. The image reconstruction was set at 12 mm.

The methods used to measure PBF and LWC, including supporting validation studies, have been previously described (14). In general, PET is used to measure the tissue concentration and distribution of a positron-emitting radionuclide, which in the present study was $H_2{}^{15}O$. The activity data measured with PET, when combined with blood activity (used as a reference) and analyzed with an appropriate compartmental mathematical model, yield tomographic images representative of PBF and LWC.

We studied 32 dogs, divided into seven groups. One group of three dogs, in which no interventions were made, served as a control. In a second group (OA only; $n = 6$), the only intervention was the induction of ALI with OA. In a third group (OA+Etx only; $n = 6$), Etx was administered in addition to OA.

In two additional groups, we used interventions to nonspecifically decrease neutrophil effects in the OA+Etx model. Animals in the anti-CD18 group ($n = 5$) received an anti-CD18 monoclonal antibody, designed to eliminate adherence to activated pulmonary endothelium and thus decrease penetrance into the lungs (R15.7, a gift from Boehringer-Ingelheim Pharmaceuticals, Ridgefield, CT). Animals received 1 mg/kg administered into the central venous circulation via 30-min infusion immediately before lung injury (see below).

In the other group, or polymorphonuclear neutrophil (PMN) depl ($n = 4$), neutrophil depletion was achieved by daily oral administration of hydroxyurea 200 mg/kg for 5 days before the study to achieve circulating absolute neutrophil counts of $<1000/mm^3$.

In the final two groups, we used specific inhibitors of PAF and $sPLA_2$. In the Group PAF ($n = 4$), PAF inhibition was achieved by IV administration of a recombinant form of the enzyme responsible for PAF degradation *in vivo* (recombinant PAF-acetylhydrolase [rPAF-AH], a gift from ICOS Corporation, Bothell, WA). The dosing regimen was based on previous work by Hofbauer et al. (15), demonstrating reduction in PAF to background levels and prevention of ALI in a rat model of acute pancreatitis, and pharmacokinetic studies provided by the manufacturer. Blood samples sent to the manufacturer from dogs studied in these experiments documented that drug levels were consistently more than the target level shown to inhibit PAF in multiple species (>50 $\mu g/mL$). Animals received 5 mg/kg via slow injection over 3 min into the central venous circulation, after baseline measurements, and 30 min before lung injury.

In the Group $sPLA_2$ ($n = 4$), a potent, $sPLA_2$ inhibitor (LY311727, a gift from Eli Lilly and Co, Indianapolis, IN) was administered IV before lung injury. Based on reported $sPLA_2$ levels from patients with severe sepsis (16,17) and the known pharmacokinetics of the drug as provided by the manufacturer (personal communication), we administered an initial dose of

0.3 mg/kg over 15 min, followed by a continuous infusion of 0.75 $mg \cdot kg^{-1} \cdot h^{-1}$, a dosing regimen expected to provide complete inhibition of $sPLA_2$.

Lung injury was produced in all groups (but the control group) by administration of 0.08 mL/kg of OA through the proximal port of the pulmonary artery catheter.

Given the reproducibility of the perfusion response to OA, Etx, or the combination, as documented by numerous studies (2,14,18), and to reduce the costs associated with PET imaging (cyclotron and scanner time), we obtained PET imaging studies only at the end of the experiment (i.e., after the combination of Etx+OA) in Groups OA-only, OA+Etx only, anti-CD18, and PMN depl. Thus, lung injury in these groups was produced first by the administration of 15 μg/kg of *Escherichia coli* Etx (Fisher Scientific, Pittsburgh, PA) into the central venous circulation, followed 30 min later by 0.08 mg/kg of IV OA. A single set of PET images was then obtained 2 h after OA (Fig. 1).

We have also shown in previous studies that the synergistic effect of small-dose Etx and OA lung injury occurs whether the Etx is administered before or after OA (i.e., it is independent of any priming effect) (18). Thus, in Groups anti-CD18 and $sPLA_2$, PET imaging was performed first 2 h after the OA administration to establish the effects, if any, of the inhibitors on the expected perfusion redistribution after OA only and then 30 min after the additional administration of Etx to measure any effects on the expected reversal of perfusion redistribution (Fig. 1).

The following data were obtained at the time of each dataset collection in all groups: (a) a transmission scan used to correct for photon attenuation during emission scans and for placement of regions of interest for later image analysis (see below); (b) a 15-s scan obtained during a continuous infusion of about 60 mCi O-15-labeled water (used for PBF measurement); (c) a 300-s scan obtained after equilibration of the O-15 water (for measurement of LWC and for the apparent blood-tissue partition coefficient for water used in the PBF calculation); and (d) pulmonary artery, pulmonary wedge, and systemic arterial blood pressures, cardiac output, and arterial blood gas analysis.

After final PET data collection, the dogs in the PMN depl and anti-CD18 groups underwent bronchoscopic alveolar lavage (BAL). To obtain BAL fluid, a flexible fiberoptic bronchoscope was wedged into a distal left lower lobe bronchus before lavage with 70 mL of warmed sterile normal saline. Saline was retrieved with low wall suction and divided into 2 aliquots. One sample was spun in a cytocentrifuge and stained with Wright-Giemsa for differential cell counts. The remainder of the fluid was used for total cell count.

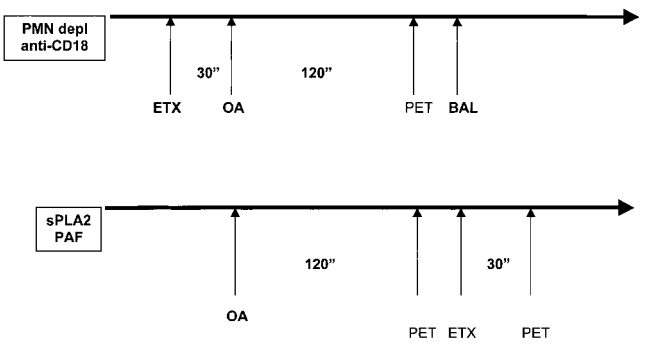

Figure 1. Schematic diagrams showing the protocol timelines for the various experimental groups. OA = oleic acid administration; PET = positron emission tomographic imaging; Etx = endotoxin administration; BAL = bronchoalveolar lavage. For abbreviations related to experimental groups, see text.

At the completion of the study, all animals were euthanized with additional pentobarbital and 15–20 mL of saturated KCl.

From each dog, the four contiguous tomographic slices with the most lung were analyzed from the seven slices reconstructed as part of each PET scan, encompassing most of the caudal lobes. Regions of interest from the right and left lungs were defined on each transmission scan, as previously described (19).

The position of each region was kept in computer memory, and mean values for each region were obtained for all PET measurements made. PBF was measured as milliliter per minute per 100-mL lung. To normalize the regional PBF data for differences in cardiac output, PBF in each picture element (pixel) was expressed as a fraction of the total blood flow to the region.

To evaluate the relationship of PBF to anatomic position within a region, the x and y coordinates for each pixel, along with the respective fractional PBF values for each pixel, were recorded. The pixel data were then sorted, first by their y-coordinate values. Next, within each value for y, the data were sorted again by their x-coordinate values. The result was a listing of the pixels' "y" location, beginning in the most ventromedial portion of the region and ending with the most dorsolateral portion of the region. Each region contained ~400–500 pixels. Arbitrarily, the data were divided into 20 bins stacked vertically in the ventral-dorsal direction so that each bin contained ~20–25 pixels, which could then be averaged. By keeping the number of bins per region and the number of tomographic slices per dog constant, bin values could be averaged across dogs, allowing comparisons between experimental groups.

To quantify perfusion redistribution, we determined the difference in fractional PBF between data sets and summed the difference in those bins in which PBF decreased between the two times (19,20).

Group data are presented as the mean ± SD. Group means were compared by standard analysis-of-variance techniques. The Tukey test was used for *post hoc* comparisons.

Results

All groups were similar at baseline with respect to systemic and pulmonary artery pressures and blood gas analysis. There were no significant changes in hemodynamic variables in any of the groups throughout the study period (data not shown).

There was no significant effect on arterial oxygenation after Etx only in the PMN depl and anti-CD18 groups (data not shown). In the OA only group, oxygenation decreased moderately (and significantly compared with baseline) after OA (Fig. 2). Oxygenation also decreased to a similar degree in the $sPLA_2$ and PAF groups after OA but before Etx (data not shown).

All animals that received both OA and Etx showed marked hypoxemia after the combination (Fig. 2). In all cases, the change after OA+Etx was more than after OA alone in the OA-only group (Fig. 2). Overall, these effects on oxygenation were entirely consistent with previously reported effects after OA only, Etx only, or after the combination (14,18).

Blood and BAL fluid were evaluated for total WBC count and differential in the PMN depl and anti-CD18 groups. The animals rendered neutropenic with hydroxyurea demonstrated a mean circulating PMN count of $5.0 \pm 2.2 \times 10^6$ cells/mL before treatment, which decreased to $0.5 \pm 0.1 \times 10^6$ cells/mL by the day of the experiment. After Etx+OA, the circulating PMN count was further reduced to $0.1 \pm 0.1 \times 10^6$ cells/mL. The WBC concentration in the BAL was comparable ($0.2 \pm 0.2 \times 10^6$ cells/mL with 26% neutrophils).

Animals treated with anti-CD18 monoclonal antibody showed a mean circulating PMN count of $3.1 \pm 0.4 \times 10^6$ cells/mL before antibody administration, which was not significantly changed by the end of the experiment ($2.7 \pm 1.0 \times 10^6$ cells/mL). Nevertheless, BAL counts showed that there was virtually no penetration of PMNs into the alveolar compartment ($0.16 \pm 0.36 \times 10^6$ cells/mL with 9% neutrophils).

Although we did not measure LWC at baseline, a range of normal values was established in the control group (Fig. 3). This range is consistent with data from previous studies (14,18). By this standard, animals in all intervention groups developed an increase in LWC after the OA+Etx administration (Fig. 3). The magnitude of this increase in LWC is also comparable to that measured in previous work.

In the PAF and $sPLA_2$ groups, dorsal fractional pulmonary blood flow (fPBF) was 0.25 ± 0.06 and 0.29 ± 0.07, respectively, after OA but before adding Etx.

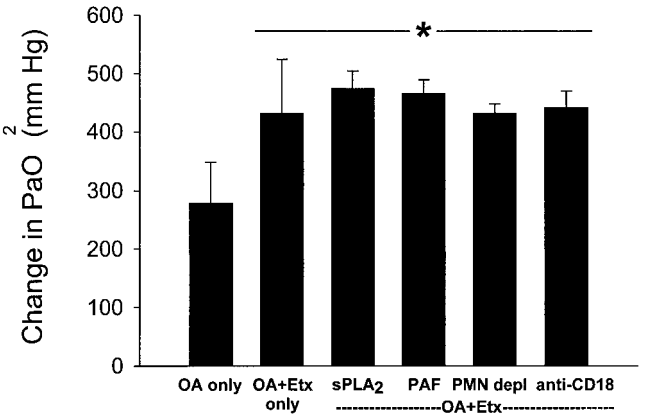

Figure 2. Effect of oleic acid (OA), or OA and endotoxin (OA+Etx), on arterial oxygenation in the various experimental groups. Data are expressed as the change in Pao_2 between baseline and at the time of the final PET imaging study. Pao_2 decreased significantly in all groups compared with baseline; the decrease in Pao_2 was greater in all groups that also received Etx compared with the one group that only received OA (*$P < 0.05$). The changes after OA alone or after OA+Etx are consistent with those observed in previous studies (14,18).

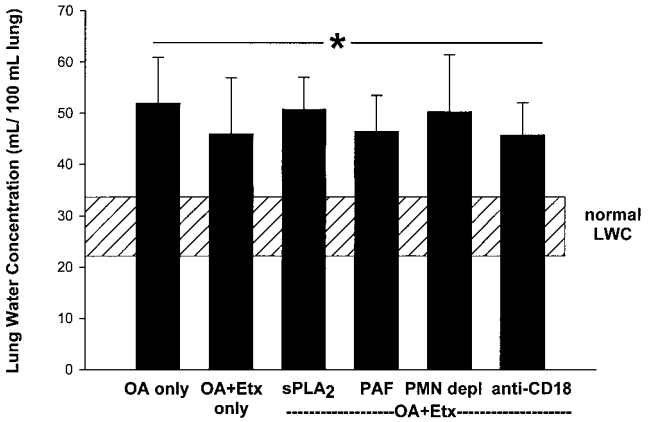

Figure 3. Effect of oleic acid (OA), or OA and endotoxin (OA+Etx), on lung water concentration (LWC) in all experimental groups at the time of the final PET scan. Normal values represent the range of mean ± 1 SD among the three normal control dogs. These normal values are similar to those previously reported (14,18). By the time of the final PET scan, LWC was similar in all experimental groups and was significantly greater than LWC in the normal control animals (*$P < 0.05$).

These values were not significantly different from the dorsal fPBF in the OA-only group (Fig. 4). Again, this reduction in dorsal regional PBF after OA alone is consistent with previous reports with this model (2,18). At this same time, LWC had already increased in these two groups (compared with normal controls; Fig. 3) to 49.7 ± 6.5 and 50.0 ± 6.5 mL H_2O/100-mL lung, respectively. Neither of these values is significantly different from mean LWC after OA+Etx (Fig. 3).

After Etx, this redistribution of blood flow in the lung was abolished (Fig. 4), also consistent with previous reports (18). Similarly, in the PMN depl and

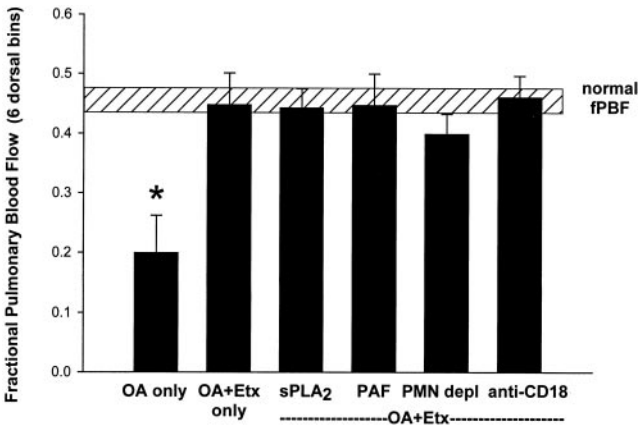

Figure 4. Effect of oleic acid (OA), or OA and endotoxin (OA+Etx), on the pattern of pulmonary blood flow (PBF) to the dorsal six bins on the PET images in all experimental groups at the time of the final PET scan. Normal values represent the range of mean ± 1 SD among the three normal control dogs. These normal values are similar to those previously reported (14,18). In the OA only group, fractional (f)PBF decreased significantly (*$P < 0.05$) compared with normal values, consistent with perfusion redistribution. In contrast, in all other groups (all of which also received Etx), fPBF was not different from normal controls, i.e., Etx prevented (or reversed, depending on the specific experimental group) the perfusion redistribution seen in the OA-only group.

anti-CD18 groups, OA+Etx prevented any significant perfusion distribution (Fig. 4). There was no significant difference in the perfusion pattern in any of the five groups given the combination of OA+Etx (Fig. 4), and in all five groups, the perfusion pattern was different than after OA alone (Fig. 4).

Discussion

The major finding of this study is that neither neutrophils nor the neutrophil products PAF or $sPLA_2$ seem to mediate the effects of small-dose Etx on the pulmonary perfusion pattern seen in the OA-induced model of ALI.

The OA model is a well characterized and frequently used experimental model of ALI (1). After systemic administration of OA, animals develop significant pulmonary edema (14,18). In previous work, we demonstrated that after OA lung injury, the pattern of PBF is altered from normal, with redistribution away from the edematous dorsal lung regions, a process that helps to preserve ventilation-perfusion matching and gas exchange (14,18). This explains the preservation of gas exchange despite lung injury and increases in pulmonary edema in the $sPLA_2$ and PAF groups, before the administration of Etx (Fig. 2A).

Small doses of Etx, which by themselves are largely devoid of significant systemic or pulmonary hemodynamic effects, markedly alter the physiologic expression of OA lung injury. Etx abolishes the expected perfusion redistribution seen after OA, resulting in marked hypoxemia (2,18). This phenomenon was seen in all four groups after the combination of Etx and OA in the present study (Fig. 2). The magnitude of this effect is completely consistent with our previous studies.

The effects of small-dose Etx on perfusion redistribution in the OA lung injury model are associated with significant increases in prostacyclin levels, which could be prevented by pretreatment with a COX-2 inhibitor (2). Because the effects of Etx on prostacyclin production are only seen with concomitant OA lung injury, we reasoned that the pulmonary endothelium was the likely site of increased prostacyclin production. The rate-limiting step in prostacyclin biosynthesis is the availability of the arachidonic acid substrate, released from membrane phospholipids by the activation of PLA_2.

Etx can activate neutrophils to release oxygen free radicals, which, in turn, can activate COX-2 and PLA_2. In addition, Etx-induced chemokine production can be blocked by PLA_2 inhibitors, suggesting that PLA_2 may play a role in neutrophil sequestration within the lungs (6). Thus, we reasoned that Etx might increase $sPLA_2$ production from neutrophils sequestered in the lungs during lung injury, resulting in increased availability of arachidonic acid in endothelial cells, an effect that might be further amplified by direct membrane damage from OA itself. Finally, PAF, which is also a product of arachidonic acid metabolism and has also been implicated in the effects of Etx on eicosanoid biosynthesis, might mediate these effects (11).

PLA_2 is produced in a variety of tissues, including neutrophils and macrophages, and has been implicated in the inflammatory process associated with sepsis and ARDS (21). PLA_2 may act directly in the lung to produce injury, or it may exert its effects through activation of other proinflammatory molecules (e.g., eicosanoids, PAF, and phospholipids). Both PLA_2 and PAF are associated with the increased eicosanoid production that accompanies ALI in experimental and clinical settings (10,22,23).

Therefore, it seemed reasonable to hypothesize that the synergistic biochemical and physiologic effects of Etx-OA lung injury might require the intermediary action of PAF or PLA_2. However, despite the use of potent inhibitors of both molecules, no redistribution in PBF away from the edematous lung regions was seen in OA+Etx lung injury. In other words, these inhibitors had no effect on the physiologic expression of OA+Etx lung injury, suggesting that PAF and PLA_2 do not mediate the effects of Etx on the pattern of pulmonary perfusion in this model of lung injury.

The major limitation to accepting this conclusion is that we were unable to demonstrate in these studies that the inhibitors used were indeed able to significantly inhibit the mediators in question. However, as noted in the Methods section, based on the known pharmacokinetics of these drugs, the plasma levels achieved in the current studies (in the case of the PAF inhibitor), and the efficacy data in

multiple other species, this limitation is unlikely to explain our results.

Neutrophils are not required for the development of OA-induced lung injury (24,25). However, they are recruited into the airspaces after OA injury (24,26) and could therefore modify the development or resolution of injury in this model. Schoene et al. (27) reported mean BAL neutrophil counts of 158×10^6 cells/mL after OA lung injury in dogs compared with 2.3×10^6 cells/mL in controls. These values after OA injury are almost three orders of magnitude higher than what we observed in the BAL of our animals after PMN depletion, thus providing evidence of effective neutrophil depletion from the airspaces in our study groups.

By either rendering the animals neutropenic before the experiment or inhibiting PMN penetration into the alveolar airspace with a canine-specific murine monoclonal antibody to CD18, we sought to evaluate the role of neutrophils in the expression of OA+Etx lung injury. Administration of hydroxyurea for five days before the study resulted in circulating absolute neutrophil counts of $<1000/mm^3$. Likewise, the administration of the CD18-blocking antibody was effective at preventing alveolar neutrophil recruitment. Although we did not perform BAL in animals injured by OA alone, our results clearly show that we achieved significant degrees of circulating neutropenia and that PMNs did not penetrate into the airspaces. Despite these reductions in the presence of neutrophils in the lungs and therefore presumably their contribution to the development of lung injury in our model, we did not see any change in the robust response to OA+Etx injury that we have seen in previous studies (14,18).

In summary, neither neutrophils nor at least two important neutrophil products often implicated in Etx-stimulated eicosanoid production seem to be responsible for the ability of small-dose Etx to inhibit the perfusion redistribution that is otherwise characteristic of the OA lung injury model. Of course, PMNs may have many other important effects on the evolution of lung injury, both in this model and clinically. Regardless, the mechanism of the Etx-lung injury interaction remains unclear. Other possibilities, such as a direct effect of Etx on endothelial cells or cytokine effects after macrophage-monocyte stimulation by Etx, will have to be addressed in future studies.

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