

A novel signaling mechanism between gas and blood compartments of the lung

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Propagation of inflammatory signals from the airspace to the vascular space is pivotal in lung inflammation, but mechanisms of intercompartmental signaling are not understood. To define signaling mechanisms, we microinfused single alveoli of blood-perfused rat lung with TNF- α , and determined in situ cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) by the fura-2 ratio method, cytosolic phospholipase A_2 (cPLA $_2$) activation and P-selectin expression by indirect immunofluorescence. Alveolar TNF- α increased $[Ca^{2+}]_i$ and activated cPLA $_2$ in alveolar epithelial cells, and increased both endothelial $[Ca^{2+}]_i$ and P-selectin expression in adjoining perialveolar capillaries. All responses were blocked by pretreating alveoli with a mAb against TNF receptor 1 (TNFR1). Crosslinking alveolar TNFR1 also increased endothelial $[Ca^{2+}]_i$. However, the endothelial responses to alveolar TNF- α were blocked by alveolar preinjection of the intracellular Ca^{2+} chelator BAPTA-AM, or the cPLA $_2$ blockers AACOCF $_3$ and MAFP. The gap-junction uncoupler heptanol had no effect. We conclude that TNF- α induces signaling between the alveolar and vascular compartments of the lung. The signaling is attributable to ligation of alveolar TNFR1 followed by receptor-mediated $[Ca^{2+}]_i$ increases and cPLA $_2$ activation in alveolar epithelium. These novel mechanisms may be relevant in the alveolar recruitment of leukocytes.

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Introduction

Airborne pathogens, a major cause of lung inflammatory disease, induce leukocyte influx into alveolar spaces (1). Increasing interest in this pathophysiology has focused on the role of cytokines such as TNF- α that are released by alveolar macrophages and thereby exert the chemotactic effect of recruiting leukocytes from perialveolar capillaries (2, 3). A central unanswered question is how the chemotactic signal crosses the alveolar wall to reach the capillaries.

The alveolar barrier restricts transport of macromolecules of a size similar to that of cytokines (~15–20 kDa). After secretion in alveoli, cytokines such as TNF- α remain in the alveolar space (4, 5) and leak into the circulation only in alveolar barrier injury (6). Hence, with the barrier intact, alveolar cytokines are unlikely to have rapid access to perialveolar capillaries. Despite this poor access, instilled TNF- α in the airspace induces neutrophil influx (7). Although mechanisms are unclear, a receptor-mediated process may be involved, because soluble TNF receptor-IgG chimera, anti-TNF- α Abs, and absence of TNF receptor-1 (TNFR1) attenuate neutrophil influx to airspace pathogens and aerosolized lipopolysaccharide (8–11).

Our main goal was to test the hypothesis that instillation of TNF- α into the alveolar space sends a signal to the adjoining capillary endothelium. Because TNF- α mobilizes Ca^{2+} , we selected cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) as a marker of the signaling (12–15). Frequently, $[Ca^{2+}]_i$ subserves gap junction- or paracrine-based intercellular communication (16, 17) that may be relevant. Endothelial $[Ca^{2+}]_i$ transients induce vascular expression of the leukocyte trafficking receptor P-selectin that may initiate neutrophil influx (18, 19). Using our recently developed methods for quantifying $[Ca^{2+}]_i$ and P-selectin expression in situ (20, 21), we tested these possibilities in intact alveolocapillary units of the rat lung. We report a novel mechanism in which intra-alveolar infusion of TNF- α induced a rapid proinflammatory response in adjacent capillaries.

Methods

Antibodies and other reagents. Goat anti-rat TNFR1 mAb E-20 was purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, California, USA) (22). Rabbit anti-rat cytosolic phospholipase A_2 (cPLA $_2$) Ab was purchased from Genetics Institute Inc. (Cambridge, Massachusetts, USA). Fab fragment of rabbit anti-goat IgG and donkey anti-mouse IgG were purchased from Jackson

ImmunoResearch Laboratories Inc. (West Grove, Pennsylvania, USA). FITC-conjugated donkey anti-goat IgG came from ICN Biomedicals Inc. (Costa Mesa, California, USA). Goat anti-mouse IgG (Fc-specific; Sigma Chemical Co., St. Louis, Missouri, USA) and goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories Inc.) were also purchased. Mouse anti-rat P-selectin mAbs RMP-1 and RP-2 were gifts of A.C. Issekutz (Department of Pediatrics, Microbiology-Immunology and Pathology, Dalhousie University, Halifax, Nova Scotia, Canada) (23).

Other agents used were fura-2 AM and HOECHST 33342 (Molecular Probes Inc., Eugene, Oregon, USA), TNF- α , arachidonic acid, linoleic acid, heptanol, indomethacin (all from Sigma Chemical Co.), saponin (ICN Biomedicals Inc.), the PLA₂ inhibitors AACOCF₃, MAFP, and BEL, the Ca²⁺ chelator 1,2-bis-(*o*-aminophenophenoxyethane)-*N,N,N',N'*-tetraacetic acid tetra-(acetoxymethyl)-ester (BAPTA-AM; Calbiochem-Novabiochem Corp., San Diego, California, USA), MK886 (Biomol Research Laboratories, Plymouth Meeting, Pennsylvania, USA), and WEB 2170BS (gift of Boehringer Ingelheim Inc., Ridgefield, Connecticut, USA). Intracapillary infusions were given in vehicle consisting of 2% dextran (70 kDa; Pharmacia Biotech Inc., Piscataway, New Jersey, USA), 1% FBS (Gemini Bio-Products Inc., Calabasas, California, USA), HEPES solution at pH 7.4 and osmolarity of 295 \pm 5 mOsm, containing (in mmol/L) 150 Na⁺, 5 K⁺, 1 Ca²⁺, and 10 glucose. Intra-alveolar infusions were given in vehicle consisting of 0.1% delipidated BSA (fraction V; Sigma Chemical Co.) and Ringer's solution at pH 7.4 and osmolarity of 295 \pm 5 mOsm, containing (in mmol/L) 137 Na⁺, 2.7 K⁺, 1 Ca²⁺, and 28 lactate.

Lung fluorescence microscopy. The microscopy methods have been described (20, 21). Briefly, lungs excised from anesthetized Sprague-Dawley rats were continuously pump-perfused at 14 mL/min with autologous rat blood at 37°C. Lungs were constantly inflated at an airway pressure of 5 cmH₂O. Pulmonary artery and left atrial pressures were held at 10 cmH₂O and 5 cmH₂O, respectively. The lung surface was kept moist with normal saline at 37°C and viewed by epifluorescence microscopy.

Fluorophores were excited by mercury lamp illumination directed through appropriate filters. Fluorescence emission was collected through objective lenses (Wplan FL \times 40 UV; Olympus America Inc., Melville, New York, USA, or Ph3 Neofluar \times 100; Carl Zeiss Jena GmbH, Jena, Germany) and dichroic and emission filters (400DCLPO2 and 510WB40; Omega Optical Inc., Brattleboro, Vermont, USA, or DM500 and O-515; Olympus America Inc.) by image intensifier (KS1381; Video Scope International Ltd., Sterling, Virginia, USA) and video camera (CCD-72; Dage-MTI Inc., Michigan City, Indiana, USA), then subjected to digital image analysis (MCID-M4; Imaging Research Inc., St. Catharines, Ontario, Canada).

Substance delivery. For intra-alveolar infusions, a single alveolus was selected. The microscope objective was adjusted until the maximum diameter of the alveolus came into focus. By micropuncture, the alveolus was infused to fill 3–4 neighboring (nonmicropunctured) alveoli. One of the nonmicropunctured alveoli was selected for imaging. Intracapillary infusions were given either by capillary micropuncture or by a wedged venous microcatheter (PE-10; Baxter Diagnostics Inc., Deerfield, Illinois, USA) as described (20, 21). Agents were infused in the following concentrations: fura-2 AM, 10 μ M; HOECHST 33342, 5 μ g/mL; TNF- α , 10–10,000 U/mL; arachidonic acid, 0.1–10 μ M; linoleic acid, 10 μ M; BAPTA-AM, 40 μ M; heptanol, 3 mM; AACOCF₃, 1 μ M; MAFP, 25 μ M; BEL, 1 μ M; indomethacin, 20 μ M; MK886, 1 μ M; WEB 2170BS, 50 μ M; saponin, 0.01%. These substances were all given by alveolar infusion. TNF- α , fura-2, and heptanol were also given by capillary infusion. BAPTA-AM was given in Ca²⁺-free Ringer's solution. Capillary blood cell-free or Ca²⁺-free conditions were established by infusion of 2% dextran HEPES solution that was either Ca²⁺-rich or Ca²⁺-free (containing 0.5 mmol/L EGTA), 10 minutes before measurements were taken.

[Ca²⁺]_i imaging. Our *in situ* methods have been reported (20). For epithelial and endothelial [Ca²⁺]_i determinations, fura-2 AM (which deesterifies intracellularly to impermeant fura-2), was given for 30 minutes by intra-alveolar or intracapillary infusion, respectively. Infused alveoli were washed with 3% reconstituted bovine surfactant (Survanta; Abbott Laboratories, Columbus, Ohio, USA). Alveoli and capillaries loaded with fura-2 were excited at 340 nm and 380 nm, and [Ca²⁺]_i was determined from the computer-generated 340:380 ratio based on a K_d of 224 nmol/L and appropriate calibration parameters (20, 24). Dominant oscillation frequency and amplitude were determined for [Ca²⁺]_i oscillations of amplitude greater than 15 nM using fast Fourier transformation (Origin V 4.0; Microcal Software Inc., Northampton, Massachusetts, USA).

Indirect immunofluorescence *in situ*. These methods have been described (21). To determine epithelial TNFR1 expression, an alveolus was infused first with E-20 (10 μ g/mL) or control mAb RP-2 (10 μ g/mL) for 10 minutes, then with the secondary FITC-conjugated mAb (75 μ g/mL) for 1 minute. Unbound fluorescence was removed by infusion of Ringer's solution. To determine endothelial P-selectin expression, RMP-1 (35 μ g/mL) was infused into a single capillary for 10 minutes, then secondary FITC-conjugated mAb (1:20) was infused for 1 minute. Unbound fluorescence was removed by blood flow in less than 30 seconds (21). Residual FITC fluorescence indicated expression of the targeted antigen. To assess activation of cPLA₂, we adapted an immunofluorescence assay reported *in vitro* (25) that involves permeabilization of the cell membrane by 0.5% Triton X-100. Membrane permeabilization not only permits cell loading with primary and secondary Abs, but also causes loss of unbound cytoplasmic molecules such as nonactivated cPLA₂ when cells are subsequently

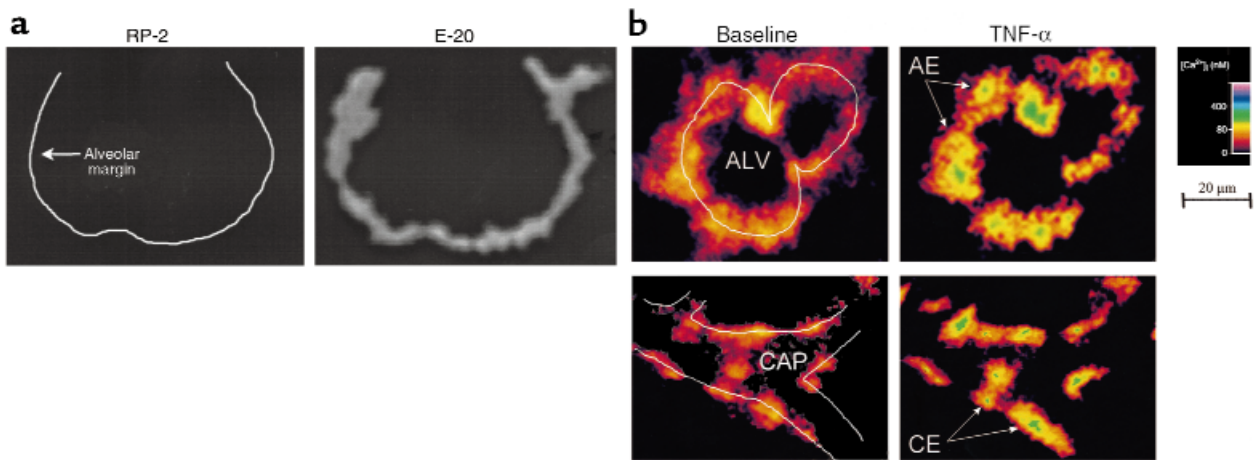


Figure 1

In situ lung imaging. (a) Images of a single alveolus show indirect immunofluorescence for a nonspecific mAb (RP-2) and an anti-TNFR1 mAb (E-20). Line in left image was drawn from parallel bright-field image. Replicated 3 times. (b) Images show the 340:380 ratio color-coded for $[Ca^{2+}]_i$ in an alveolus (ALV) and its adjoining capillary (CAP). Line sketches depict alveolar and capillary margins. Individual alveolar epithelial (AE) and capillary endothelial (CE) cells are indicated. Images were obtained in the same alveolus and capillary at baseline (left) and 5 minutes after alveolar infusion of 1,000 U/mL TNF- α (right). Replicated 8 times.

washed. Hence, no immunofluorescent staining for cytoplasmic cPLA₂ is detectable in unstimulated cells. However, in activated cells, staining occurs on the translocated fraction of cPLA₂ that becomes membrane bound and thus remains intracellular. Therefore, to carry out the assay, we first infused an alveolus for 5 minutes with TNF- α or vehicle, then fixed and permeabilized the alveolus by infusion of 4% paraformaldehyde and 0.5% Triton X-100 for 10 minutes and 4 minutes, respectively. We then infused the alveolus with anti-rat cPLA₂ Ab (1:5) and FITC-conjugated IgG (1:10), followed by alveolar washes with infusions of Ringer's solution. Residual fluorescence indicated the presence

of activated, membrane-bound cPLA₂ (25). Alveolar epithelial nuclei were stained in situ by alveolar infusion of HOECHST 33342 (26) followed by a wash.

Receptor crosslinking in situ. Crosslinking of the alveolar TNFR1 was performed by sequential intra-alveolar infusions of E-20 (10 μ g/mL), secondary anti-goat IgG (60 μ g/mL), and finally Ringer's solution to wash the alveolus. For control, the isotype-matched mAb RP-2 (10 μ g/mL) replaced E-20 in the crosslinking protocol.

Statistics. All data are mean \pm SEM. Values of several groups were compared using the Wilcoxon and Friedman test for dependent groups, and Kruskal-Wallis and Mann-Whitney *U* tests for independent groups. Linear

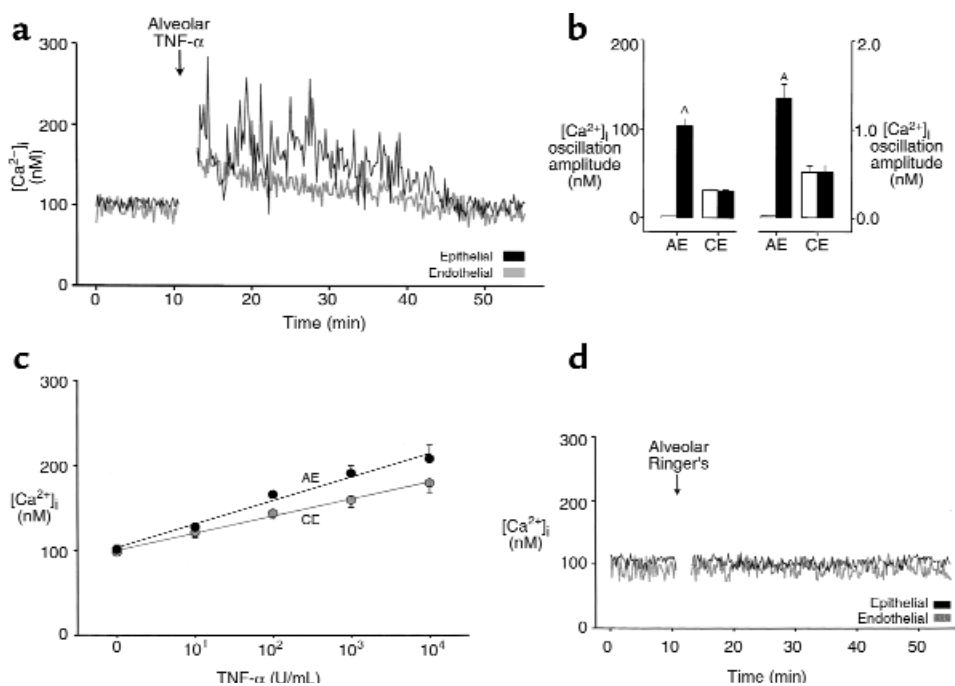


Figure 2

$[Ca^{2+}]_i$ responses to intra-alveolar infusion of TNF- α . (a) Tracings are from an epithelial cell of an alveolus and an endothelial cell of an adjoining capillary as indicated. TNF- α injected at 1,000 U/mL. Replicated 8 times. (b) Data show amplitude (left panel) and frequency (right panel) of $[Ca^{2+}]_i$ oscillations. Open bars, baseline; filled bars, 5 minutes after alveolar TNF- α infusion (1,000 U/mL). ^A*P* < 0.05 vs. baseline; *n* = 8 experiments. AE, alveolar epithelial cells; CE, capillary endothelial cells. (c) Group $[Ca^{2+}]_i$ responses 5 minutes after alveolar TNF- α infusion. Each point is mean \pm SEM of 4 experiments. Lines were drawn by linear regression (*P* < 0.001). (d) Single experiment tracings show $[Ca^{2+}]_i$ responses to intra-alveolar infusion of Ringer's solution. Replicated 4 times.

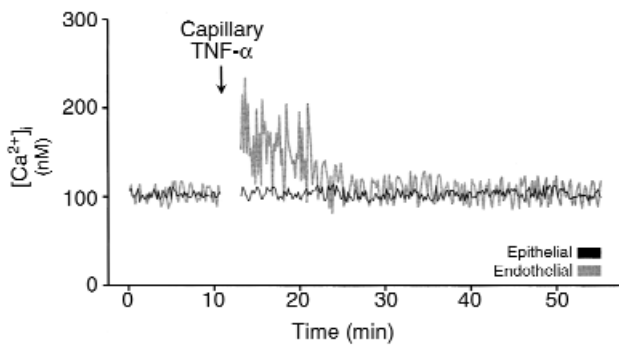


Figure 3
Single experiment tracings show $[Ca^{2+}]_i$ responses to intracapillary infusion of TNF- α (1,000 U/mL). Replicated 5 times.

regression analysis was performed using SigmaPlot (Jandel Scientific Software, San Rafael, California, USA). Statistical significance was accepted at $P < 0.05$.

Results

Using indirect immunofluorescence in situ, we confirmed the uniform presence of TNFR1 in alveoli (Figure 1a). No alveolar fluorescence was detected with the isotype-matched control mAb RP-2, which recognizes rat tissue but not alveolar epithelial antigen. Epithelial $[Ca^{2+}]_i$ was determined in several cells of the imaged alveolus. Endothelial responses were similar in lung venular and alveolar septal capillaries, therefore we have not distinguished between these locations in reporting the data.

Because our procedures involved alveolar micropuncture, we confirmed that no capillary fluorescence was detectable 30 minutes after an intra-alveolar infusion of FITC-conjugated IgG, which ruled out macromolecular leakage across the alveolar membrane ($n = 3$; not shown).

Intra-alveolar TNF- α . We gave intra-alveolar TNF- α infusion for 10 seconds. As exemplified by the alveolo-capillary unit shown in Figure 1b, $[Ca^{2+}]_i$ increased in all epithelial and endothelial cells in the imaged area ($P < 0.05$). The $[Ca^{2+}]_i$ increases, consisting of marked $[Ca^{2+}]_i$ oscillations in epithelial cells (Figure 2, a and b), occurred in less than 2 minutes and persisted for more than 20 minutes. By contrast, oscillations were not enhanced in endothelial cells. Increases in mean $[Ca^{2+}]_i$ were concentration dependent for both cell types (Figure 2c). Alveolar infusion of Ringer's solution had no effect on $[Ca^{2+}]_i$ (Figure 2d).

Intracapillary TNF- α . The single experiment tracings in Figure 3 depict $[Ca^{2+}]_i$ responses to intracapillary infusion of TNF- α . Not only did mean $[Ca^{2+}]_i$ increase ($P < 0.05$), but there was a marked increase in the amplitude of $[Ca^{2+}]_i$ oscillations ($P < 0.05$). Moreover, the $[Ca^{2+}]_i$ increases were sustained for shorter durations than were those for alveolar infusions ($P < 0.05$). Epithelial $[Ca^{2+}]_i$ did not increase.

E-20 ligation. Figure 4a shows that alveolar infusion of mAb E-20 alone had no effect on $[Ca^{2+}]_i$, although a

subsequent alveolar infusion of the crosslinking secondary Ab induced $[Ca^{2+}]_i$ increases in epithelial and endothelial cells ($P < 0.05$). These responses were not different from those for alveolar infusion of TNF- α . The left panel in Figure 4b shows that alveolar infusion of E-20 followed by infusion of TNF- α did not increase epithelial or endothelial $[Ca^{2+}]_i$. Therefore, E-20 blocked $[Ca^{2+}]_i$ responses to alveolar TNF- α ($P < 0.05$). The right panel are data from the same alveolo-capillary unit, showing that with E-20 in the alveolus, capillary TNF- α still induced endothelial $[Ca^{2+}]_i$ increases ($P < 0.05$). Therefore, alveolar infusion of E-20 did not block endothelial $[Ca^{2+}]_i$ responses to TNF- α given by capillary infusion.

In experiments that are not shown here, we confirmed that the nonspecific mAb RP-2 caused no $[Ca^{2+}]_i$ responses, either as the primary mAb in the crosslinking protocol ($n = 3$) or as a control for the inhibitory effect of E-20 ($n = 3$). Hence, the crosslinking and blocking effects of E-20 were specific to receptor ligation. We also confirmed that when E-20 and TNF- α were both given sequentially by capillary infusion, endothelial $[Ca^{2+}]_i$ increases were blocked ($P < 0.05$). However, capillary infusion of E-20 did not block $[Ca^{2+}]_i$ increases to alveolar infusion of TNF- α ($n = 4$; not shown).

Ca^{2+} chelation. A 15-minute alveolar infusion of the membrane-permeable intracellular Ca^{2+} chelator BAPTA-AM blocked both epithelial and endothelial

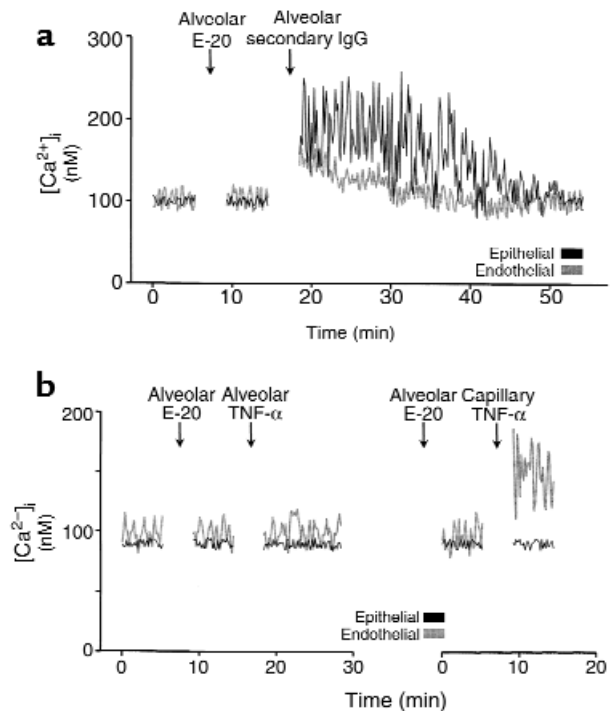


Figure 4
E-20 ligation in a single alveolocapillary unit. Intra-alveolar infusion of Ab E-20 was followed by alveolar infusion of (a) secondary IgG or (b) TNF- α (1,000 U/mL), given either by alveolar (left panel) or capillary (right panel) infusion. Replicated 4 times.

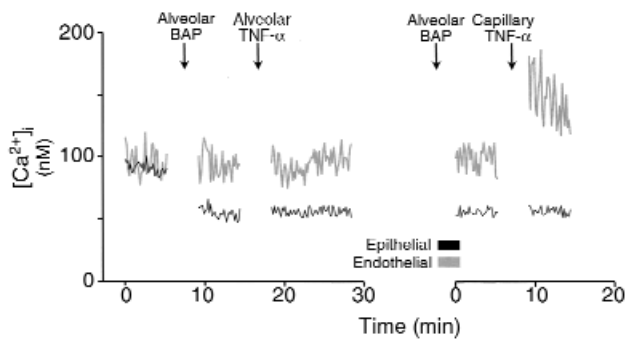


Figure 5 Ca^{2+} chelation in a single alveolocapillary unit. BAP (BAPTA-AM), 40 μM . $\text{TNF-}\alpha$, 1,000 U/mL. Alveolar or capillary routes of infusion are indicated. Replicated 4 times.

$[\text{Ca}^{2+}]_i$ responses to alveolar $\text{TNF-}\alpha$ ($P < 0.05$; Figure 5, left). However, capillary infusion of $\text{TNF-}\alpha$ still induced an endothelial $[\text{Ca}^{2+}]_i$ increase ($P < 0.05$; Figure 5, right). Therefore, alveolar infusion of BAPTA-AM blocked the endothelial response because it blocked the epithelial response. Capillary perfusion with Ca^{2+} -free HEPES also blocked endothelial $[\text{Ca}^{2+}]_i$ increases induced by alveolar $\text{TNF-}\alpha$ ($P < 0.05$), indicating that endothelial Ca^{2+} entry was required for the response ($n = 4$; not shown).

Not shown are experiments in which we determined that heptanol (a cell uncoupler; ref. 27), given by either the alveolar or the capillary route, had no inhibitory effects on either epithelial or endothelial responses to alveolar infusion of $\text{TNF-}\alpha$ ($n = 4$ each). Capillary perfusion with blood cell-free, Ca^{2+} -rich HEPES solution did not abolish the endothelial $[\text{Ca}^{2+}]_i$ response to alveolar $\text{TNF-}\alpha$ ($n = 4$, not shown). Hence, the $[\text{Ca}^{2+}]_i$ signal in endothelial cells did not result from adherence and emigration of intravascular neutrophils.

Activation of cPLA₂. $\text{TNF-}\alpha$ causes Ca^{2+} -dependent activation of cPLA₂, leading to arachidonate release (28). Activated cPLA₂ translocates to cellular membranes and the nuclear envelope (25). Immunofluorescence of membrane-bound cPLA₂ was detectable in several epithelial cells in single alveoli infused with $\text{TNF-}\alpha$ (Figure 6a). Overlaying images for cPLA₂ immunofluorescence (Figure 6b) and nuclear fluorescence (Figure 6c) revealed close apposition of cPLA₂ to the nucleus (Figure 6d), indicating that cPLA₂ was translocated to the perinuclear region. No cPLA₂ staining was detected when alveoli were infused with Ringer's solution before immunofluorescence labeling (Figure 6e), although nuclear staining was positive (Figure 6f).

Alveolar infusions of the cPLA₂ inhibitor AACOCF₃, given 10 minutes before alveolar $\text{TNF-}\alpha$ infusion, blocked the endothelial $[\text{Ca}^{2+}]_i$ response ($P < 0.05$) but not the epithelial response (Figure 7a, left). Endothelial $[\text{Ca}^{2+}]_i$ increases were rescued in the presence of intra-alveolar AACOCF₃ by capillary infusion of $\text{TNF-}\alpha$ ($P < 0.05$; Figure 7a, right). We interpret this to mean that

AACOCF₃ given by the alveolar route did not block direct endothelial responses to $\text{TNF-}\alpha$.

AACOCF₃ given by alveolar infusion 5 minutes after alveolar infusion of $\text{TNF-}\alpha$ terminated the endothelial $[\text{Ca}^{2+}]_i$ response in less than 2 minutes ($P < 0.05$; Figure 7b), indicating that even after the onset of the endothelial response it was quickly abrogated by the cPLA₂ inhibitor. Similarly to AACOCF₃, alveolar infusion of MAFP, an inhibitor of both Ca^{2+} -dependent PLA₂ (cPLA₂) and Ca^{2+} -independent PLA₂ (iPLA₂), blocked endothelial responses to alveolar $\text{TNF-}\alpha$ ($P < 0.05$). However, BEL, which inhibits iPLA₂, had no inhibitory effect on the endothelial $[\text{Ca}^{2+}]_i$ response ($n = 4$ each; not shown).

Endothelial responses to alveolar infusion of $\text{TNF-}\alpha$ were not blocked by preinfusing alveoli with indomethacin (a cyclooxygenase blocker), MK886 (a 5-lipoxygenase-activating protein inhibitor), or WEB 2170BS (the platelet-activating factor receptor antagonist) (Figure 7c), suggesting that these mechanisms were not essential to the observed responses. However,

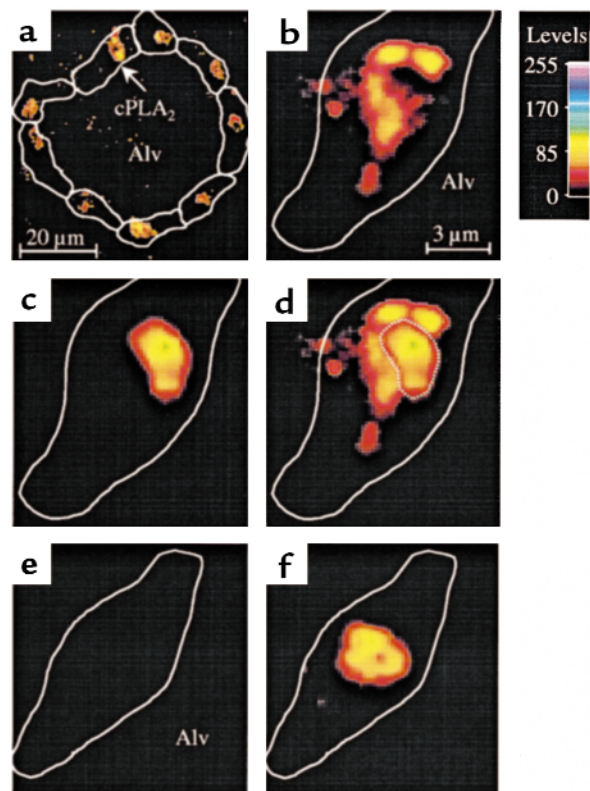


Figure 6 Translocation of cPLA₂ in single alveoli. An alveolus was infused with 1,000 U/mL $\text{TNF-}\alpha$ (a-d) or with Ringer's solution (e and f). Then the alveolus was stained for both the immunofluorescence of cPLA₂ (a, b, e) and the nuclear fluorescence of HOECHST 33324 (c and f). The alveolus was washed and imaged at low (a) and high (b-f) magnifications as indicated by the scale bars. Color code shows fluorescence intensities. Cell margins identified by bright-field microscopy are depicted by line sketches. Alv, alveolar lumen. Images for cPLA₂ and nuclear fluorescence were combined by image overlay (d, f). Nuclear outline is indicated by dotted line (d). Replicated 4 times.

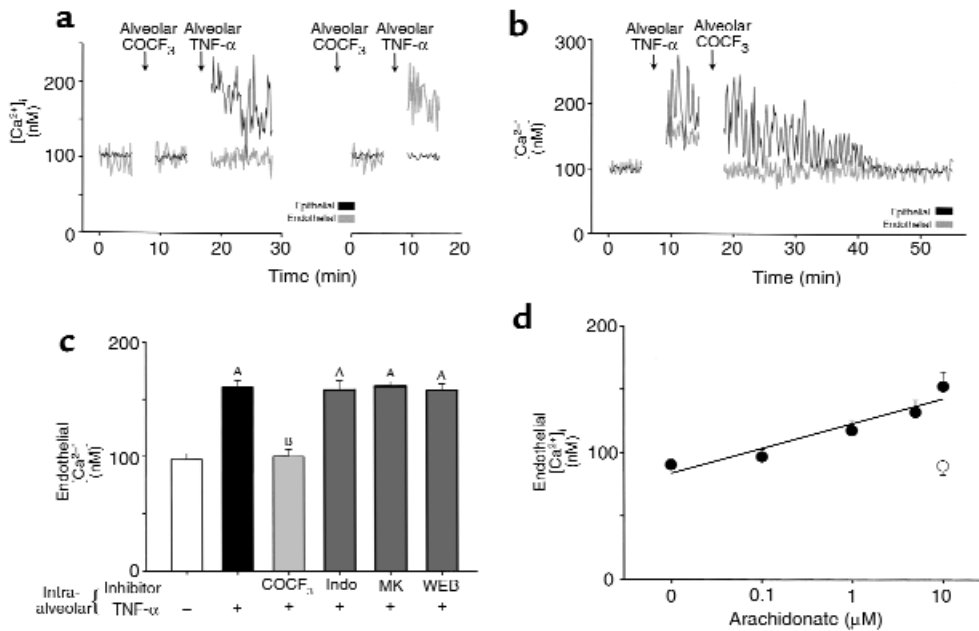


Figure 7

Effects of cPLA₂ and its products. (a and b) Tracings from single experiments. Alveolar or capillary routes of TNF-α infusion are indicated. Replicated 4 times each. (c) Group data for indicated inhibitors given in 10-minute alveolar infusions before alveolar infusion of TNF-α; *n* = 4 for each. ^A*P* < 0.05 vs. baseline (open bar). ^B*P* < 0.05 vs. TNF-α (solid bar). COCF₃, AACOCF₃; indo, indomethacin; MK, MK886; WEB, WEB 2170BS. (d) Alveoli were permeabilized by 10-minute saponin infusions. Then arachidonate was infused into permeabilized (closed circles) and control (open circle) alveoli. Each point is the mean ± SEM of 4 experiments. Line was drawn by linear regression (*P* < 0.001).

in permeabilized alveoli, arachidonate infusion induced concentration-dependent increases of endothelial [Ca²⁺]_i (*P* < 0.05; Figure 7d). Hence, arachidonate induced endothelial responses from the alveolar aspect of the capillary. No arachidonate effects occurred in nonpermeabilized alveoli. These findings were replicated by intra-alveolar linoleate (10 μM), which also caused endothelial [Ca²⁺]_i increases in capillaries adjacent to permeabilized, but not nonpermeabilized, alveoli (*P* < 0.05, *n* = 4; not shown).

P-selectin. Endothelial [Ca²⁺]_i increase may induce expression of the leukocyte-endothelial adhesion molecule P-selectin (18, 21). We quantified P-selectin expression in intact venular capillaries using our previously described methods (21). As shown in Figure 8, within 5 minutes of alveolar infusion of TNF-α, P-selectin expression increased approximately 10-fold above baseline. This increase was blocked by anti-TNFR1 mAb, BAPTA-AM, and AACOCF₃. Neither infusion with Ringer's solution nor the isotype-matched control mAb RP-2 had any observable effects.

Discussion

We show here the existence of novel epithelial-endothelial signaling mechanisms in the lung. To summarize our findings: (a) Alveolar infusion of TNF-α increased endothelial [Ca²⁺]_i. (b) Crosslinking alveolar TNFR1 replicated the response. (c) Immune inhibition of alveolar TNFR1 blocked the response. (d) The response was also blocked by alveolar injections of the intracellular [Ca²⁺]_i chelator BAPTA-AM and

cPLA₂ blockers. (e) Alveolar infusion of TNF-α increased [Ca²⁺]_i and activated cPLA₂ in alveolar epithelial cells. (f) Alveolar infusion of TNF-α enhanced P-selectin expression in endothelial cells.

Our main conclusions are as follows. First, from finding (a), we conclude that an alveolar stimulus induced Ca²⁺ signaling in endothelial cells of perialveolar capillaries. Second, from findings [(b) and (c)] we conclude that the TNF-α effect followed ligation of alveolar TNFR1. Third, from findings (d) and (e) we conclude that the endothelial [Ca²⁺]_i response was attributable to the epithelial [Ca²⁺]_i increase and to activation of epithelial cPLA₂. Fourth, from finding (f) we conclude that the endothelial response was proinflammatory. In addition, the gap-junction uncoupler heptanol failed to inhibit endothelial responses to alveolar TNF-α, thereby ruling out a gap-junctional basis for alveolo-capillary signaling. Taken together, these findings are the first evidence that adjacent epithelial and endothelial cells of the alveolocapillary junction communicate Ca²⁺ signals through a receptor-mediated mechanism initiated in the epithelium.

Methodological considerations

Compartmental localization. Alveolar leak of TNF-α given by intra-alveolar infusion could be ruled out because: (a) Intra-alveolar TNF-α-induced endothelial effects were blocked by intra-alveolar infusion of E-20, BAPTA-AM, or cPLA₂ blockers. However, TNF-α given directly in capillaries still caused endothelial [Ca²⁺]_i increases. These results indicate that TNF-α did not

leak across the alveolar wall. (b) Intracapillary infusion of E-20 blocked endothelial $[Ca^{2+}]_i$ increases in response to capillary TNF- α but not to alveolar TNF- α . Hence, again, intra-alveolar TNF- α did not ligate endothelial receptors. (c) FITC-IgG given by intra-alveolar injection was not detectable in the vascular space, showing that macromolecules did not rapidly diffuse across the alveolar epithelial barrier. (d) Capillary infusion of TNF- α did not induce $[Ca^{2+}]_i$ transients in adjacent alveolar epithelial cells, indicating that TNF- α did not freely move between vascular and alveolar spaces.

Antibody ligation. As far as we know, these are the first crosslinking data reported in situ. Crosslinking TNFR1 effectively mimics TNF- α -induced responses (29). We confirmed TNFR1 expression on the alveolar apical surface. Crosslinking resulted in epithelial and endothelial $[Ca^{2+}]_i$ responses comparable to those generated by alveolar infusion of TNF- α . However, no responses were obtained either with the primary mAb alone or by replacing a nonspecific primary mAb in the crosslinking protocol. These findings affirm that TNF- α -induced responses require both ligation and aggregation of TNFR1.

Alveolocapillary $[Ca^{2+}]_i$ oscillations. A consistent feature in these experiments was that the epithelial and endothelial responses to alveolar TNF- α were different. Although mean $[Ca^{2+}]_i$ increased in both cell types, high amplitude $[Ca^{2+}]_i$ oscillations occurred only in epithelial cells, probably because only alveolar TNFR1 was ligated. Thus, enhanced endothelial $[Ca^{2+}]_i$ oscillations could also be induced by directly ligating endothelial TNFR1 by capillary infusion of TNF- α . $[Ca^{2+}]_i$ oscillations are attributed to Ca^{2+} -dependent ligational properties of endosomal inositol triphosphate receptors (1, 4, 5, 30), as well as to oscillatory inositol triphosphate release (31). In most cell types, $[Ca^{2+}]_i$ oscillations are relatively short-lived (30). In contrast, $[Ca^{2+}]_i$ oscillations in our experiments were prolonged well in excess of the duration of TNF- α exposure. Hence, relatively brief periods of alveolar receptor ligation may generate sustained $[Ca^{2+}]_i$ responses in alveolocapillary cells.

Interactions between TNF- α and cPLA₂. Our in situ assay for cPLA₂ activation indicates a novel pulmonary role for cPLA₂, namely its involvement in alveolocapillary signal propagation. Alveolar epithelial cells constitutively express cPLA₂ (32). Both TNF- α and sustained $[Ca^{2+}]_i$ increases activate cPLA₂ (28, 33, 34). By immunofluorescence assay, we determined that intra-alveolar TNF- α induced membrane translocation and probably activation of cPLA₂ in alveolar epithelium (25). Translocation was predominantly localized to the perinuclear region, the preferential site of agonist-stimulated phospholipid hydrolysis by cPLA₂ (35).

Activation of cPLA₂ was supported by the inhibitory effects of the cPLA₂ blockers AACOCF₃ and MAFP, which block both the Ca^{2+} -dependent and the Ca^{2+} -independent cytosolic isoforms (cPLA₂ and iPLA₂, respectively) (36, 37). Although nonspecific effects may occur, we point out that these agents blocked the

endothelial, but not the epithelial $[Ca^{2+}]_i$ responses to alveolar TNF- α . Because the agents did not block Ca^{2+} mobilization per se, we may rule out nonspecific effects in causing the inhibition of the endothelial $[Ca^{2+}]_i$ responses. Interestingly, BEL, which selectively inhibits iPLA₂ (36), had no inhibitory effect. Taken together, these results suggest that the observed endothelial responses resulted from activation of Ca^{2+} -dependent cPLA₂ in alveolar epithelium.

Products of cPLA₂ activation. Activation of cPLA₂ yields arachidonate, prostaglandins, leukotrienes, and platelet-activating factor (38, 39). All of these products could potentially act as paracrine mediators. However, inhibitors of prostaglandin or leukotriene synthesis, or of the platelet-activating factor receptor, had no inhibitory effects on alveolocapillary signal propagation. Hence, to the extent that we can determine, these products did not convey the propagated signal. Although unidentified products may have contributed to the endothelial effect of alveolar TNF- α , cPLA₂ specifically releases arachidonate from the *sn*-2 position of phospholipids (35). Therefore, the alveolocapillary signal may have been conveyed by arachidonate.

To the extent that arachidonate is secreted (37) and directly causes Ca^{2+} entry into cells (40, 41), it may have exerted a paracrine effect. We cannot directly confirm arachidonate secretion into the alveolocapillary space, but there is indirect support of this possibility. First, increases of endothelial $[Ca^{2+}]_i$ induced by alveolar TNF- α were inhibited by external Ca^{2+} depletion, indicating that the response resulted from Ca^{2+} entry, consistent with the action of arachidonate. Second, alveolar arachidonate evoked endothelial $[Ca^{2+}]_i$ increases in permeabilized alveoli. This mimics a possible vascular

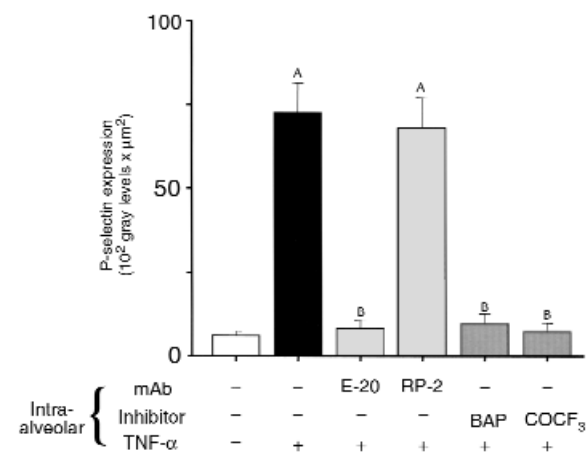


Figure 8 P-selectin expression in lung capillaries. Capillaries were stained for P-selectin expression 5 minutes after alveolar infusion as indicated. AACOCF₃ (COCF₃) or mAbs were given 10 minutes before TNF- α infusion; BAP (BAPTA-AM, 40 μ M) was given 15 minutes before TNF- α infusion (1,000 U/mL). P-selectin expression was quantified as the product of mean fluorescence intensity and fluorescent area 1 minute after washout of unbound FITC-IgG with blood flow; $n = 4$ for each. ^A $P < 0.05$ vs. baseline (open bar). ^B $P < 0.05$ vs. TNF- α alone (black bar).

effect of basolaterally secreted arachidonate from alveolar epithelial cells.

We considered that arachidonate may also be secreted into the alveolar lumen by alveolar epithelial cells, and that the luminal fatty acid may cross the alveoli and act on adjacent capillaries. Although to our knowledge transalveolar fatty acid transport has not been confirmed, fatty acids are capable of crossing cell membranes (42), and when present in the alveolar lumen, they may cross the alveolar barrier by entering cells apically and exiting basolaterally. We tested this possibility for 2 polyunsaturated fatty acids, arachidonate and linoleate, given by intra-alveolar infusion. Both are known to increase $[Ca^{2+}]_i$ in cultured cells (43). However, in intact alveoli, neither was apparently transported across the alveolar barrier at a sufficient level to evoke $[Ca^{2+}]_i$ transients in the adjoining capillary. By contrast, in permeabilized alveoli, arachidonate and linoleate both induced endothelial $[Ca^{2+}]_i$ increases. These findings indicate that an alveolar barrier to FFA transport existed, and that its elimination was necessary to allow sufficient action of alveolar FFAs on adjacent capillaries. The barrier may be attributable to transport limitations across cell membranes as well as to fatty acid metabolism within the cell. We interpret that to the extent that arachidonate released by alveolar epithelial cells caused the observed endothelial responses, its substantive effect resulted from basolateral rather than apical secretion.

P-selectin. The goal of alveolocapillary signaling is to induce enhanced expression of leukocyte adhesion receptors in endothelial cells, in order to activate leukocyte influx. We tested this hypothesis in the context of P-selectin expression. Alveolar TNF- α enhanced endothelial P-selectin expression within 5 minutes. This response resembles the $[Ca^{2+}]_i$ -dependent P-selectin expression we recently reported in pressure-stressed lung capillaries (21). Although platelets also express P-selectin, we previously reported that these capillaries are free of adherent platelets, and that P-selectin expression occurs in the presence of cell-free capillary infusion (21). Because endothelial P-selectin expression is a hallmark of early inflammation (44) and may precede neutrophil adhesion and emigration (19), we interpret that receptor-mediated alveolocapillary signaling evoked a rapid proinflammatory effect in perialveolar capillaries.

Inhibition of the P-selectin response permitted interpretation of the sequence of signaling events. Because alveolar E20 blocked all responses, we consider TNFR1 ligation to be the first step in the sequence. All intra-alveolar interventions such as BAPTA-AM and AACOCF₃ that blocked epithelial responses also blocked endothelial $[Ca^{2+}]_i$ increases and P-selectin expression. Hence, the epithelial responses were causal to endothelial signals that led to P-selectin expression. Although we did not directly determine kinetics along the signaling pathway, we determined that the epithelial $[Ca^{2+}]_i$ increases and the resulting endothelial $[Ca^{2+}]_i$ increases were detectable in 2 minutes, whereas epithelial cPLA₂ activation and endothelial P-selectin

expression were detectable in 5 minutes. These results agree with reported time courses of P-selectin upregulation and cPLA₂ translocation (45, 46). Based on these considerations, we conclude that alveolar TNF- α caused rapid capillary expression of a leukocyte adhesion receptor. This resulted from sequential events in which alveolar TNFR1 ligation caused $[Ca^{2+}]_i$ increase and cPLA₂ activation in alveolar epithelium, which was followed by an increase of endothelial $[Ca^{2+}]_i$ in adjacent capillaries.

Conclusions

Our findings provide the first direct evidence that alveoli communicate inflammatory signals to perialveolar capillaries. This intercompartmental signaling may be essential for effective host defense against airspace pathogens. Previous reports indicate that alveolar neutrophil influx after lung instillation of TNF- α occurs over a period of hours (7). Here we show that intra-alveolar TNF- α causes an endothelial proinflammatory response in minutes. This indicates that an alveolar stimulus is capable of immediately inducing alveolocapillary signaling, although the full scope of the response may develop over a much longer period. To summarize, TNF- α initiates ligation and clustering of the alveolar epithelial TNFR1, mobilization of epithelial $[Ca^{2+}]_i$, and the translocation and perhaps activation of epithelial cPLA₂. This may, through basolateral arachidonate secretion from alveolar epithelial cells, account for endothelial $[Ca^{2+}]_i$ influx and therefore enhanced expression of P-selectin. The extent to which these findings apply to other inflammatory ligands in the alveolus requires further investigation.

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