Src Phosphorylation of Endothelial Cell Surface Intercellular Adhesion Molecule-1 Mediates Neutrophil Adhesion and Contributes to the Mechanism of Lung Inflammation

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**Objective**—The goal of this study was to determine whether tumor necrosis factor-α (TNF-α)–induced Src activation and intercellular adhesion molecule-1 (ICAM-1) phosphorylation rapidly increase endothelial cell adhesivity and polymorphonuclear leukocyte (PMN) sequestration independently of de novo ICAM-1 synthesis.

**Methods and Results**—TNF-α exposure of mouse lungs for 5 minutes produced a 3-fold increase in 125I-anti-ICAM-1 monoclonal antibody (mAb) binding and 111In oxine-labeled PMN sequestration, as well as Src activation, ICAM-1 Tyr518 phosphorylation, and phospho- (p) Tyr518-ICAM-1 coimmunoprecipitation with actin. The response was absent in Nox2−/− lungs or following Src inhibition. In COS-7 cells transfected with wild-type (WT), phospho-defective (Tyr518Phe), or phospho-mimicking (Tyr518Asp) mouse ICAM-1 cDNA constructs, TNF-α increased the Bmax of YN1/1.7.4 anti-ICAM-1 mAb binding to WT-ICAM-1 but not to Tyr518Phe-ICAM-1, indicating increased binding avidity secondary to ICAM-1 phosphorylation. This effect was mimicked by expression of the Tyr518Asp-ICAM-1 mutant. TNF-α also increased the staining intensity and cell surface clustering of YN1/1.7.4 mAb-labeled WT-ICAM-1 that colocalized with F-actin, which was not observed with Tyr518Phe-ICAM-1 but was recapitulated with Tyr518Asp-ICAM-1. Finally, overexpression of ICAM-1 in mouse lungs significantly increased lipopolysaccharide-induced transvascular albumin leakage and bronchoalveolar lavage PMN counts at 2 and 24 hours after lipopolysaccharide inhalation compared with lungs expressing the Tyr518Phe ICAM-1 mutant.

**Conclusion**—Src-dependent phosphorylation of endothelial cell ICAM-1 Tyr518 induces PMN adhesion by promoting ICAM-1 clustering, which we propose mediates rapid-phase lung vascular accumulation of PMNs during inflammation. (Arterioscler Thromb Vasc Biol. 2011;31:00-00.)

**Key Words:** adhesion molecules ■ endothelium ■ vascular biology ■ inflammation

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The release of the cytokine tumor necrosis factor-α (TNF-α) in inflamed tissues stimulates β2 integrin expression on polymorphonuclear leukocytes (PMNs) and the de novo synthesis of intercellular adhesion molecule-1 (ICAM-1) in endothelial cells.1,2 Newly synthesized ICAM-1 appears on the endothelial cell plasma membrane in significant amounts within 2 to 4 hours of cytokine exposure.3,5 ICAM-1, a member of the immunoglobulin gene superfamily, is the counter-receptor for β2 integrins (CD11/CD18) expressed on the PMN cell surface.6–10 Interactions between endothelial ICAM-1 and different β2 integrins, macrophage-1 antigen (CD11b/CD18) and leukocyte function-associated antigen-1 (LFA-1) (CD11a/CD18),3,4,6–15 are required for firm adhesion of PMNs to the endothelium and PMN transmigration.3,6–8 This binding interaction represents an essential step in PMN extravasation into tissues at sites of infection.1,2,4,16 Endothelial cells constitutively express cell surface ICAM-1, albeit at a low level.3,5,17,18 This basal ICAM-1 expression is thought to support the limited transmigration of PMNs and other leukocytes into tissue, perhaps as an immune surveillance mechanism.3,4,17,18 However, ICAM-1 expression requiring de novo ICAM-protein synthesis increases markedly in response to proinflammatory mediators, such as TNF-α.3,5,17,18 We have shown that TNF-α also induces a rapid phase of endothelial cell surface ICAM-1 adhesion within minutes of TNF-α exposure.11 The purposes of the present study were to address the validity of this phenomenon in the intact circulation and to identify key signaling constituents mediating this posttranslational ICAM-1 modification and its consequences in lung inflammation. We show herein that TNF-α activation of Src and subsequent phosphorylation of ICAM-1 on Tyr518 in mouse endothelial cells induces...
association of ICAM-1 with cortical actin, causes cell surface clustering of ICAM-1, and mediates rapid-phase PMN adhesion to vascular intima. We also demonstrate the importance of the rapid induction of ICAM-1 activation as defined by its phosphorylation in the mechanism of lung inflammation induced by lipopolysaccharide (LPS).

**Methods**

An expanded Methods section is available in the supplemental material, available online at http://atvb.ahajournals.org. Binding kinetics ($K_d$ and $B_{max}$) of cell surface wild-type (WT) and mutant mouse ICAM-1 expressed in COS-7 cells were determined by cell-attached monoclonal antibody (mAb) binding ELISA. Confocal images (1024x1024 pixels) of mouse lung endothelial cells (MLECs) and human umbilical vein endothelial cells stimulated with TNF-α (20 ng/mL) or vehicle for 20 minutes were acquired using a Zeiss LSM510 META confocal imaging system. To measure ICAM-1 mAb-specific binding in the isolated-perfused mouse lung, a dual tracer protocol was implemented. To measure PMN sequestration in mouse lungs, TNF-α and $^{111}$In oxine-labeled PMNs were sequentially infused. $^{111}$Indium oxine tracer washout was determined by monitoring the ratio of recovered tissue counts over infused counts. To assess the role of ICAM-1 phosphorylation in lung PMN infiltration and protein-rich edema formation associated with LPS inhalation, C57BL6 mice injected with liposome/cDNA mixture were used. To determine the binding of labeled PMNs, we used a Zeiss LSM510 META confocal imaging system. To measure ICAM-1 activity of ICAM-1 to anti-ICAM-1 mAb in mouse lung vessels, the fraction of infused counts. TNF-α-induced PMN binding to endothelial cell ICAM-1 in the lungs is supported by the finding that TNF-α-induced increase in PMN binding was not seen in lungs of ICAM-1−/− mice (Figure 1B). As shown in Figure 1C, ICAM-1 expression did not increase for up to 30 minutes after TNF-α treatment of MLECs when binding of the anti-ICAM-1 mAb was maximal; however, predictably, the expression began to increase slightly at 60 minutes but increased substantially after treatment with TNF-α for 2 and 24 hours.

**Results**

**TNF-α Rapidly Increases Anti-ICAM-1 mAb Binding Activity and PMN Uptake in Mouse Lungs**

We first determined the effects of TNF-α on the binding activity of ICAM-1 to anti-ICAM-1 mAb in mouse lungs using the dual tracer method, in which we used a mouse-specific anti-ICAM-1 mAb and another control mAb (see supplemental Methods section). TNF-α infusion in WT lungs within 5 minutes resulted in a 2-fold increase in the binding of $^{125}$I-labeled anti-ICAM-1-YN1/1.7.4 mAb (Figure 1A). To address whether the binding was specific, we added a 10-fold excess unlabeled anti-ICAM-1 mAb to the perfusate just before addition of radiolabeled mAb. The excess mAb abolished TNF-α-stimulated binding of tracer mAb (see Figure 1A). Exposure of lung vessels to TNF-α for up to 30 minutes showed that the increase in $^{125}$I-labeled mAb binding was maximal within the first 10 minutes (Figure 1A). We also observed an additional increase in anti-125I-ICAM-1 mAb binding after a prolonged (4-hour) period of TNF-α exposure (Figure 1A), consistent with the increase in ICAM-1 protein expression in response to TNF-α in mouse lungs seen at this later time.¹⁹

We next addressed whether TNF-α produces a similar rapid increase in PMN binding in situ in lung vessels. The vasculature of isolated-perfused mouse lungs was treated with saline or TNF-α (2000 U/mL) for 5 minutes, lungs were cleared of the TNF-α for 1 minute by adding fresh perfusate, and $^{10^5}$ $^{111}$In oxine-labeled mouse PMNs were added to the perfusate over 5 minutes. After a washout period to remove the unbound PMNs, we determined tissue counts as the fraction of infused counts. TNF-α increased the fraction of infused PMNs retained in the lung 3-fold (from 0.15±0.03 to 0.47±0.11; P<0.05). The ICAM-1 dependence of TNF-α-induced PMN sequestration 3-fold above basal level in lungs of WT but not ICAM-1−/− mice. Plotted values are mean±SD (n=3 per group); *P<0.01 compared with basal PMN uptake. C, Western blot showing the absence of increase in lung ICAM-1 expression within the first 30 minutes after TNF-α but a progressive increase thereafter. Results are representative of 3 similar experiments.

**Rapid Phase of TNF-α-Induced Increase in ICAM-1 Binding Activity and PMN Uptake in Lung Vessels Requires Nox2**

TNF-α activates NADPH oxidase in endothelial cells by inducing the phosphorylation of the cytosolic regulatory
binding to ICAM-1. TNF-α had no effect on 125I-ICAM-1 mAb binding to lungs from p47phox−/− mice. Mean values ± SD (n = 3 to 4) are plotted; *P < 0.05 vs basal value. B, Isolated-perfused mouse lungs were exposed to TNF-α for 5 minutes and then perfused with WT PMNs labeled with 111In oxine. TNF-α increased PMN uptake 3-fold above basal level in WT mice, whereas deletion of gp91phox or p47phox abrogated the effect of TNF-α. In other experiments, Nox2-deficient lungs (gp91phox−/−) received superoxide, generated in the perfusate for 10 minutes using the xanthine/xanthine oxidase (Xan/Xan Ox) system (see Methods), which reconstituted the 3-fold increase in PMN accumulation in lungs. Control values for WT lungs were replotted from data in Figure 1B. Values shown are mean ± SD (n = 3 to 5); *P < 0.01 vs basal value.

We also addressed the role of Nox2-derived oxidants in the rapid induction of PMN binding in lungs of p47phox−/− or gp91phox−/− mice (the latter carrying a deletion of the catalytic subunit of Nox2). The rapid stimulatory effect on PMN binding seen in WT lungs was absent in lungs of both knockout models (Figure 2B). Nox2-deficient lungs, however, recovered their ability to recruit PMNs after superoxide was generated via perfusion of lungs with a xanthine/xanthine oxidase (X/XO) mixture for 10 minutes (Figure 2B), indicating that PMN uptake could be restored in Nox2-null lungs by chemically generating the oxidants. Superoxide generation by X/XO resulted in a 3-fold increase in the fraction of infused PMNs retained in the lung compared with the unchallenged group (from 0.20 ± 0.02 to 0.67 ± 0.07; P < 0.05). In control experiments, we observed that infusion with X/XO for 10 minutes at the concentrations used above (see Methods) did not injure the vessels of the lung preparation; ie, superoxide generation did not alter 125I-albumin pulmonary transvascular permeability–surface area product (24 ± 6 μL/min per gram of dry lung in control versus 23 ± 3 μL/min per gram of dry lung in X/XO-exposed lungs; n = 3 per group); thus, the oxidant-mediated PMN uptake was not the result of induction of lung vascular injury by oxidants produced by the X/XO reaction.

TNF-α-Induced ICAM-1 Binding Activity and PMN Adhesion Require Phosphoinositide 3-Kinase-γ and Protein Kinase C-ζ Signaling

As activation of protein kinase C (PKC-ζ) has been shown to induce Nox2 activation and ICAM-1 binding activity in endothelial cells,11,21–23 we validated this observation in the present study and observed that TNF-α induced a 7-fold increase in PKC-ζ activity in MLECs within 5 minutes (see Supplemental Figure IA). Furthermore, the TNF-α-induced increase in 111In oxine-PMN uptake in the mouse lung vasculature was blocked by pretreating mice (50 μg/mouse per day for 5 days, IP) with an inhibitory pseudosubstrate peptide specific for PKC-ζ, whereas an inhibitory pseudosubstrate specific for PKCζ had no effect (see Supplemental Figure IB). As we previously showed in endothelial cells that phosphoinositide 3-kinase-γ (PI3-kinase-γ) activates PKC-ζ upstream of Nox2 activation,24 we next addressed the role of PI3-kinase-γ in TNF-α-mediated rapid PMN uptake in lungs.

As shown in Supplemental Figure IB, we did not see any effect of TNF-α in p110γ−/− mice (deleted catalytic subunit of PI3-kinase-γ), indicating the requirement for PI3-kinase p110γ in mediating the rapid TNF-α-induced PMN uptake in lung vessels.

**Figure 2.** TNF-α-induced anti-ICAM-1 mAb binding and PMN sequestration are abolished in Nox2-null mouse lungs. A, Isolated-perfused lungs from WT or p47phox−/− mice were challenged with TNF-α (2000 U/mL) for 10 minutes, and specific anti-125I-ICAM-1 mAb binding was determined during the last 5 minutes of the treatment period. Unlabeled ICAM-1 mAb nearly abolished anti-125I-ICAM-1 mAb binding, indicating the specificity of subunit p47phox, mediating its subsequent translocation to the plasma membrane and association with other subunits to form an active NADPH oxidase (Nox2) complex.20 To address whether Nox2 is required for the observed rapid increase in binding of anti-125I-ICAM-1 mAb, we perfused isolated lungs from WT and p47phox−/− mice with TNF-α for 10 minutes and measured mAb binding. TNF-α treatment doubled the binding of labeled anti-ICAM-1 mAb in lungs of WT mice, whereas no significant change was observed in lungs of p47phox−/− mice (Figure 2A).

TNF-α Induction of Nox2-Dependent Src Activation Mediates ICAM-1 Phosphorylation

In Vivo

Studies have demonstrated that oxidants activate Src25,26 and that Src is capable of inducing the phosphorylation of ICAM-1 Tyr518 (27–32). To address whether TNF-α-induced oxidant production and subsequent Src activation mediates phosphorylation of ICAM-1 Tyr518 and thus induces the rapid increase in PMN binding in lungs, we administered TNF-α (2000 U/mL) to the isolated lung perfusate for 10 minutes, snap-froze the tissue in liquid nitrogen, and analyzed homogenates for phosho-ICAM-1 by immunoblotting using an antibody that recognizes the phosphorylated form of mouse ICAM-1 Tyr518. This brief TNF-α exposure resulted in 2-fold increase in phosho-ICAM-1 (Figure 3A and 3B). To test the requirement for Nox2-dependent oxidant generation in the response, we repeated the procedure in lungs from gp91phox−/− mice. In these mice, the level of phosho-ICAM-1 in unstimulated conditions was the same as in WT mice. However, TNF-α perfusion did not increase pICAM-1 Tyr518 phosphorylation (Figure 3A and 3B). In other exper-
ments, we immunoprecipitated ICAM-1 from homogenates obtained from TNF-α-perfused lungs and immunoblotted these with anti-phosphoserine and anti-phosphothreonine Abs. These studies showed that phosphorylation of ICAM-1 on either serine or threonine residues did not increase on exposure to TNF-α (data not shown).

Because the tyrosine kinase c-Src may have a role in ICAM-1 activation,27–32 we assessed the function of c-Src in mediating TNF-α-induced ICAM-1 phosphorylation and increase in PMN sequestration in mouse lungs. Lungs were perfused with or without TNF-α (10 minutes), snap-frozen, homogenized, and immunoblotted for active Src using anti-pTyr418-Src pAb as well as for pTyr518-ICAM-1 using anti-pTyr518-ICAM-1 pAb. We observed that TNF-α rapidly induced both c-Src and ICAM-1 phosphorylation on Tyr518 (Figure 3A and 3B). To address the requirement for oxidant generation in signaling Src activation and thereby in mediating ICAM-1 phosphorylation, we performed the same experiment in lungs from gp91 phox null mice. Basal Src phosphorylation was not significantly different in gp91phox−/− mice. Basal Src phosphorylation was not significantly different in gp91phox−/− mouse lungs versus WT lungs, and importantly, TNF-α perfusion did not induce Src activation or ICAM-1 phosphorylation (Figure 3A and 3C), indicating that Nox2 is required for TNF-α-induced Src Tyr418 phosphorylation (activation) and the resultant ICAM-1 phosphorylation.

**Src-Mediated Phosphorylation of Endothelial Cell ICAM-1 Induces Rapid Lung PMN Sequestration Through an ICAM-1/Actin-Cytoskeleton–Dependent Interaction**

We next determined whether Src-dependent phosphorylation of ICAM-1 Tyr518 in its intracellular C-terminal tail induces association of ICAM-1 with the endothelial cell cytoskeleton, which may induce ICAM-1 clustering and thereby increase availability of ICAM-1 binding epitopes, i.e., increased avidity or “functional affinity”14,19,28 of ICAM-1 for PMN CD11/CD18 β2 integrins. To assess this concept, we treated mouse lungs with TNF-α in the presence and absence of Src inhibitor PP1. As shown in Figure 4A, TNF-α treatment increased ICAM-1 Tyr518 phosphorylation, and this response was blocked by PP1 pretreatment, indicating TNF-α-induced Src activation is responsible for ICAM-1 Tyr518 phosphorylation. To show whether Src phosphorylation of ICAM-1 Tyr518 in vivo plays a role in rapid PMN sequestration induced by TNF-α, isolated-perfused mouse lungs were pretreated with Src inhibitor PP1 for 20 minutes before 5 minutes of TNF-α exposure, followed by perfusion of 111In oxine-labeled PMNs for 10 minutes. As shown in Figure 4B, PP1 blocked the rapid TNF-α-induced PMN sequestration in mouse lungs.

To assess mechanisms of phospho-ICAM-1-mediated PMN sequestration, MLECs were grown to confluence; serum deprived for 2 hours; treated for 20 minutes with vehicle (cell culture medium) or the Src inhibitor PP2; and stimulated for 0.10, 20, or 120 minutes with TNF-α. As shown in Figure 4C, we observed that TNF-α induced an increase in Src activation (autophosphorylation of Tyr418) and ICAM-1 Tyr518 phosphorylation, both of which were blocked by PP2. Furthermore, immunoprecipitation of ICAM-1 revealed increased association of ICAM-1 with β2 integrin, which peaked at 20 minutes and returned to the basal level at 2 hours (Figure 4C). Pretreatment of cells with Src inhibitor PP2 prevented the increased association between ICAM-1 and activin (Figure 4C), indicating that this interaction was the result of Src-dependent ICAM-1 phosphorylation.

Next we addressed whether TNF-α stimulated the association of ICAM-1 specifically with polymerized F-actin. MLECs were treated with TNF-α for 20 minutes, lightly fixed with 1% paraformaldehyde on ice to limit permeabilization, and immunostained with ICAM-1 mAb coupled with Alexa 488 goat anti-mouse secondary antibody (green) and Alexa 546 phallolidin (red), a fluorescently tagged small molecule that binds and labels F-actin stress fibers. This technique allowed visualization of membrane-associated ICAM-1 together with actin stress fibers. Figure 4D shows diffuse membrane-associated endogenous ICAM-1 staining and the presence of some actin stress fibers in nonstimulated subconfluent MLECs and little localization of ICAM-1 along stress fibers. However, stimulation of MLECs for 20 minutes with 100 ng/mL TNF-α induced ICAM-1 clustering, which appeared along actin stress fibers.

**Quantification of Anti-ICAM-1 mAb Binding Kinetics and Effects of ICAM-1 Tyr518 Point Mutations on TNF-α-Induced Endothelial Cell Surface ICAM-1 Clustering**

To investigate quantitatively how ICAM-1 Tyr518 phosphorylation mediates a rapid increase in PMN binding, COS-7 cells (Figure 5A and 5B) were transiently transfected with WT, Tyr518Phe-nonphosphorylatable, and Tyr518Asp-phospho-micking mouse ICAM-1 cDNAs. We used COS-7 cells to express WT-, Tyr518Phe-, and Tyr518Asp-ICAM-1.
to determine the role of ICAM-1 phosphorylation induced by TNF-α on anti-ICAM-1 mAb YN1/1.7.4 (YN1) and KAT-1 binding kinetics. To exclude the possibility that differences in transfection efficiency contributed to the differences in YN1 antibody binding, we also measured the binding activity of KAT-1 mAb. YN1 mAb binds to the LFA-1 epitope, whereas eBioKAT-1 mAb binds to a nonoverlapping D1 domain epitope (eBioscience) in the ICAM-1 extracellular domain. Figure 5A shows the effects of TNF-α on YN1 mAb concentration-dependent binding to WT-ICAM-1 (left) and Tyr518Phe-ICAM-1 expressed in COS-7, compared with mAb binding to the phospho-mimicking Tyr518Asp-ICAM-1 mutant in these cells (right). A summary of binding kinetics is shown in Figure 5B. TNF-α induced a 23% increase in B_max of ICAM-1 YN1 mAb binding in COS-7 cells expressing WT-ICAM-1, and this effect was blocked in cells expressing the phospho-defective Tyr518Phe-ICAM-1 mutant. YN1 mAb binding to cells expressing Tyr518Asp-ICAM-1 was 31% greater (P<0.05) than in nonstimulated COS-7 cells expressing WT-ICAM-1. Thus, phosphorylation of ICAM-1 Tyr518 was associated with increased ICAM-1 B_max for YN1 mAb, which was similarly increased with the phospho-mimicking Tyr518Asp ICAM-1 mutant. There was no effect of TNF-α on YN1 mAb binding to phospho-defective Tyr518Phe ICAM-1 mutant. The K_d of mAb YN1 binding did not differ in any of the groups. Interestingly, there was no effect of TNF-α on KAT-1 binding to WT-ICAM-1 and also KAT-1 binding to Tyr518Phe and Tyr518Asp-ICAM-1 mutants was similar (ie, there was no difference in B_max or K_d) (Figure 5B), indicating that WT and mutant ICAM-1 constructs were equally expressed. Thus, ICAM-1 intracellular domain (Tyr518) phosphorylation increases its avidity for LFA-1, as reflected by the specific increase in anti-ICAM-1 YN1 mAb binding.

Cellular localization of WT-ICAM-1, as well as Tyr518Phe and Tyr518Asp-ICAM-1 mutants, is shown in Figure 5C and Supplemental Figure II. Human umbilical vein endothelial cells were transiently transfected with ICAM-1 cDNA constructs. After 48 hours, they were stimulated with 100 ng/mL TNF-α for 20 minutes and immunostained with anti-ICAM-1 mAb and immunoblotted (IB) for ICAM-1 and β-actin. As expected, ICAM-1 constructs were equally expressed. As shown in Figure 5C, in the absence and presence of Src inhibitor PP2, Src was dephosphorylated, as determined by Western blotting with anti-Src phospho-specific antibody (P-Src). Next, we examined the intracellular distribution of WT-ICAM-1 and its mutants. WT-ICAM-1 was uniformly distributed in the plasma membrane, whereas in TNF-α-stimulated cells, ICAM-1 clustering was observed in association with polymerized cortical actin filaments. Thus, membrane-associated ICAM-1 staining in nonstimulated WT-ICAM-1 expressing cells (see also, Supplemental Figure IIA) and TNF-α-stimulated Tyr518Phe-
ICAM-1 expressing cells (see also, Supplemental Figure IIC) was diffuse, whereas in TNF-α-stimulated WT-ICAM-1 (see also, Supplemental Figure IIB) and nonstimulated Tyr518Asp-ICAM-1 (see also, Supplemental Figure IID) expressing cells, ICAM-1 staining intensity increased and was observed in clusters.

Expression of Phospho-Defective ICAM-1 Mutant Prevents LPS-Induced Lung Inflammatory Injury in Mice

Adult male C57BL6 mice (n=3/group) were transduced with pcDNA3.1 (empty vector), WT ICAM-1, or Tyr518Phe ICAM-1 cDNA constructs by retro-orbital injection of liposome-cDNA complexes to assess responses to LPS-induced lung inflammatory injury. After 48 hours, mice were exposed to nebulized LPS (10 mg over 1 hour), and bronchoalveolar lavage fluid was collected at time 0 and at 2 and 24 hours after LPS exposure. LPS induced a significant increase in ICAM-1 Tyr518 phosphorylation at 2 and 24 hours, as well as increased endogenous ICAM-1 expression (Figure 6A). Western blotting of expressed ICAM-1 2 days after cDNA transfection revealed equal levels of green fluorescent protein-tagged WT and green fluorescent protein–tagged Tyr518Phe mutant ICAM-1 expression (Figure 6B, green fluorescent protein blot). PMN counts (Figure 6C) and albumin concentration (Figure 6D) of bronchoalveolar lavage fluid were determined as described in Methods. As shown in Figure 6C, overexpression of WT mouse ICAM-1 in mouse lungs significantly increased PMN transmigration and accumulation in lungs compared with Tyr518Phe phospho-defective ICAM-1 mutant and empty vector transfected lungs at 2 and 24 hours after LPS.

Discussion

In the present study, we addressed the molecular mechanism and functional significance of rapid-phase TNF-α-induced phosphorylation of Tyr518 in the C-terminal intracellular tail
of ICAM-1 in mouse lungs and cultured MLECs. Our results demonstrate that TNF-α increases anti-ICAM-1 mAb binding to ICAM-1 and recruitment of PMNs in lungs that is dependent on Nox2 and its upstream activators, PI3-kinase- and PKC-ζ, and on Src activation. PMNs were not recruited into lungs of ICAM-1−/− mice after TNF-α challenge, indicating the requirement of Src-dependent phosphorylation of ICAM-1 in mediating the response. To assess changes in ICAM-1 adhesivity in vessels, we measured TNF-α-induced changes in binding activity of ICAM-1 using a specific 125I-labeled anti-ICAM-1 mAb (YN1/1.7.4), which we corrected for nonspecific binding by coadministering a nonbinding 131I-labeled control mAb as described.12,13 The rapid increase in anti-ICAM-1 mAb binding occurred within 5 minutes of TNF-α treatment and was thus independent of de novo ICAM-1 synthesis which requires at least 2 hours. We detected a further increase in mAb binding in mouse lungs that resulted from de novo ICAM-1 synthesis only after 2 hours of TNF-α exposure, consistent with previous observations.1,5,18,19,31 Thus, TNF-α mediates rapid recruitment and sequestration of PMNs through the phosphorylation-dependent modification of endothelial cell surface ICAM-1.

As TNF-α has been shown to stimulate the generation of reactive oxygen species (ROS) in endothelial cells,17,21,22,24,25,26 we investigated whether NADPH oxidase, specifically Nox2, plays a role in signaling the rapid increase in ICAM-1 binding and recruitment of PMNs. We found that p47phox−/− and gp91phox−/− subunits, essential protein constituents of Nox2 NADPH oxidase, were required for the rapid-phase TNF-α-induced increase in ICAM-1 binding activity and lung PMN sequestration. To address the requirement for ROS, we demonstrated that brief exposure of lung vessels to subtoxic levels of superoxide, generated by addition of xanthine and xanthine oxidase, stimulated PMN recruitment in gp91phox−/−/− mouse lung preparations.

Because PI3-kinase-γ and PKC-ζ are sequential upstream activators of Nox2 in endothelial cells,24 we predicted that TNF-α should activate both kinases. Using histone H1 as a substrate to measure PKC-ζ activity,11 we observed that TNF-α induced PKC-ζ activation in MLECs within 5 minutes. We also established the physiological relevance of increased PKC-ζ activity using a specific PKC-ζ blocking peptide, which was shown to prevent TNF-α-induced PMN sequestration in murine lungs. Our results fit with the role of PKC-ζ activation of NADPH oxidase assembly through serine-phosphorylation of cytoplasmic p47phox and its subsequent translocation to the membrane to induce NADPH oxidase complex formation.17,21,22,24

Because PKC-ζ-dependent activation of NADPH oxidase was required for the TNF-α-induced increase in PMN binding to endothelial cells, we surmised that ROS generated downstream of PKC-ζ might activate tyrosine kinases, such as c-Src.25,26 We observed that TNF-α caused a rapid phosphorylation of ICAM-1 on Tyr518. We did not detect an increase in the phosphorylation of ICAM-1 on serine or threonine residues, making it unlikely that PKC-ζ directly phosphorylated ICAM-1 in MLECs stimulated with TNF-α; thus, PKC-ζ most likely acts upstream of a tyrosine kinase such as Src. Studies have shown that Src is important in the ICAM-1-dependent PMN transmigration response.19,27–32 Data presented here support such a mechanism because TNF-α rapidly activated c-Src kinase in lungs of WT but not Nox2-deficient mice. On the basis of our findings, we propose that TNF-α increases PI3-kinase-γ activity, which stimulates PKC-ζ-dependent phosphorylation of p47phox, thereby promoting Nox2 assembly and ROS production. Thus, in this scheme TNF-α induces the activation of c-Src by oxidants, which in turn leads to Src-mediated phosphorylation of ICAM-1 Tyr518.

Cell surface β2 integrins on PMNs bind endothelial expressed ICAM-1.6–10 The effect of TNF-α in inducing Tyr518 phosphorylation-dependent ICAM-1 binding may be due to increased ICAM-1 affinity or ICAM-1 avidity.14,15 Both alterations in ICAM-1 can mediate increased PMN binding and sequestration of PMNs in lungs. To address whether...
phospho- (p) Tyr518-ICAM-1 could account for the enhanced adhesivity of ICAM-1, we first investigated whether TNF-α induces ICAM-1 clustering through association with the actin cytoskeleton. Src inhibition in endothelial cells blocked TNF-α-induced ICAM-1 tyrosine phosphorylation and ICAM-1 association with actin. Furthermore, inhibition of Src blocked PMN sequestration in mouse lungs induced by a 10-minute exposure to TNF-α, indicating that ICAM-1 phosphorylation by Src mediates the rapid recruitment of PMNs to lungs.

How ICAM-1 phosphorylation modification increases binding to CD11/CD18 integrins on PMNs is not clear. To identify whether ICAM-1 binding affinity or avidity were modified by the phosphorylation of ICAM-1 at Tyr518, we assessed ICAM-1 binding kinetics and membrane localization. For this we generated a nonphosphorylatable (Tyr518Phe) ICAM-1 mutant mouse cDNA as well as a phospho-mimicking (Tyr518Asp) mutant and studied binding kinetics of nonoverlapping rat anti-mouse ICAM-1 extracellular D1 domain mAbs YN1/1.7.4 and eBioKAT-1 (eBio-science), as well as ICAM-1 localization in the plasma membrane. The data presented show that ICAM-1 Tyr518 phosphorylation leads to ICAM-1 clustering, increased binding of YN1/1.7.4 mAb, and a 3-fold increase in PMN adhesion. Because YN1/1.7.4 recognizes the neutrophil LFA-1 integrin binding site on ICAM-1, we surmise that TNF-α-induced ICAM-1 phosphorylation at Tyr518 by Src makes the extracellular ICAM-1 LFA-1-interacting epitope more available for binding to PMNs via β2 integrin. Furthermore, we did not observe an increase in KAT-1 mAb binding (Bmax or Kd) to WT-ICAM-1 following treatment of cells with TNF-α or to phospho-mimicking Tyr518Asp-ICAM-1 mutant relative to phospho-defective ICAM-1 mutant Tyr518Phe. Thus, consistent with the hypothesis that ICAM-1 phosphorylation increases its avidity, only YN1/1.7.4 mAb binding increased. Therefore, we conclude that TNF-α-induced rapid PMN sequestration in lung microvessels is due to increased LFA-1 integrin/ICAM-1 interaction rather than increased expression level of ICAM-1.

We also assessed the pathophysiological significance of ICAM-1 phosphorylation in LPS-induced lung PMN infiltration and vascular hyperpermeability. For these studies, control mice were injected intravenously with liposome-ICAM-1 cDNA complexes and after 48 hours (when the proteins are expressed), the mice were challenged with LPS. Inhalation of nebulized LPS induced a significant increase in ICAM-1 Tyr518 phosphorylation, lung lavage PMN counts, and albumin concentration by 2 hours, which increased further at 24 hours. LPS-induced increase in expression of ICAM-1 at 24 hours significantly increased PMN infiltrates in lungs, and this was associated with increased albumin levels in lavage fluid, indicative of inflammation and vascular hyperpermeability, the hallmarks of acute lung injury. Interestingly, in mice transduced with the phospho-defective Tyr518Phe ICAM-1 mutant, LPS-induced increase in lavage PMN counts and albumin accumulation in lungs did not increase compared with values observed in mice transduced with the empty vector. Thus, ICAM-1 phosphorylation at Tyr518 plays a pivotal role in vascular inflammation and lung vascular hyperpermeability induced by endotoxin.

In summary, we showed that TNF-α rapidly increased both ICAM-1 binding avidity and PMN sequestration in lung microvessels and cultured endothelial cells. These events were independent of de novo ICAM-1 synthesis but required generation of ROS through Nox2, Src activation, and Src-dependent ICAM-1 phosphorylation at Tyr518. The phosphorylation of ICAM-1 induced ICAM-1 clustering in endothelial cells and its increased avidity for PMNs, resulting in PMN adhesion and recruitment in tissue. We posit that this rapid posttranslational modification-dependent mechanism of ICAM-1 binding to PMNs may play an important role in inflammatory diseases such as acute lung injury.

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**Disclosures**

None.

**References**


Src Phosphorylation of Endothelial Cell Surface Intercellular Adhesion Molecule-1 Mediates Neutrophil Adhesion and Contributes to the Mechanism of Lung Inflammation
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Supplement Material

Methods

Mice

Wild-type (WT) C57BL/6 and genetic knockout strains (ICAM-1\(^{-/-}\), p47\(^{phox-/-}\), gp91\(^{phox-/-}\), and PI-3 kinase p110\(^{\gamma -/-}\)) were from Jackson Laboratories (Bar Harbor, ME). Male mice weighing 25-30 g were used for all isolated perfused lung experiments and for peripheral blood PMN isolation. C57 mouse pups, 3-5 days old, were euthanitized and used for isolation and culture of lung microvascular endothelial cells as described\(^1\). All animal procedures were approved by the University of Illinois Animal Care and Use Committee, in accordance with AAALAC guidelines.

Antibodies and Other Reagents

The following primary antibodies from Santa Cruz Biotechnology (Santa Cruz, CA) were used: mouse anti-ICAM-1 monoclonal antibody (mAb), rabbit anti-PKC\(\zeta\) polyclonal Ab (pAb), mouse anti-\(\beta\)-actin mAb, rabbit anti-c\(Src\) pAb, and rabbit anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) pAb. Other antibodies were purchased as identified: rabbit anti-ICAM-1 pAb and rabbit anti-phospho-(Y418)-Src pAb, Cell Signaling (Beverly, MA); rabbit anti-phospho-(Y518)-ICAM-1 polyclonal Ab (equivalent to human Y512 phospho Ab), Abcam (Cambridge, MA) and GenWay Biotech (San Diego, CA); eBioKAT-1 (KAT-1), a rat anti-mouse ICAM-1 mAb that recognizes a different epitope than the YN1/1.7.4 mAb, eBioscience (San Diego, CA); YN1/1.7.4, a rat anti-mouse ICAM-1 mAb (adhesion blocking Ab), and P-23, a control non-binding mouse anti-human P-selectin mAb, were generous gifts of Dr. Neil Granger.\(^2\) Goat anti-mouse and anti-rabbit horseradish peroxidase (HRP)-conjugated secondary
antibodies (IgG) were from Santa Cruz. Mouse TNFα was purchased from Roche Applied Science (Indianapolis, IN). PP1 (4-amino-5-(4-methylphenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine) and PP2 (4-amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo[3,4-d]pyrimidine), and all other chemicals were from Sigma-Aldrich (St. Louis, MO).

**Mouse Lung Perfusion Protocol**

The isolated-perfused mouse lung was instrumented with arterial and left atrial cannulas and perfused at 2 ml/min to equilibrate (20 minutes) with a 3% albumin-Krebs solution (37°C) from a reservoir with aid of a peristaltic pump as described. All preparations were mechanically ventilated and arterial and venous pressures were continuously recorded. Perfusion pressure of lung preparations varied from 7 to 12 cm H2O, and remained within ~1 cm H2O of the initial value over the course of an experiment. The perfusion rate was reduced from 2 ml/min to 0.5 ml/min for the dual tracer protocol and for the 111Indium oxine-labeled PMN experiments to 0.2 ml/min (see below).

**Dual Tracer Protocol for ICAM-1 mAb Uptake in Perfused Lung Preparations**

The 125I-labeled anti-ICAM-1 mAb (YN1/1.7.4) and 131I-labeled control mAb (P-23) were prepared with isotopes purchased from Perkin-Elmer using the chloramine-T method. After a 20 minute steady-state perfusion, TNFα was infused for a total of 10 minutes via a side-port of the pulmonary artery cannula at 0.05 ml/min at a final concentration, after-10 fold dilution into perfusing liquid, of 2,000 U/ml. After 5 minutes of TNFα perfusion, we implemented a dual tracer protocol to measure ICAM-1 mAb-specific binding activity. The 125I-labeled anti-ICAM-1 mAb (YN1/1.7.4) and 131I-labeled control mAb (P23), each at 60,000 cpm/ml/100 µg mAb, were simultaneously administered for 5 minutes and then rinsed out with 3% albumin-Krebs solution for 5 minutes to remove unbound tracer. We collected 10 µl
samples of each undiluted tracer and ~ 1 ml samples of the venous effluent at 30 sec intervals over the 5 min of tracer infusion. $^{125}$I and $^{131}$I radioactivities in lung tissue and effluent were counted using a calibrated gamma counter corrected for the minor spectral overlap. Specific uptake of the $^{125}$I-ICAM-1 mAb was calculated and reported as cpm/gram dry lung using the following formula:

$$\left[ \frac{^{125}\text{I cpm/g lung}}{^{125}\text{I cpm injected}} - \frac{^{131}\text{I cpm/g lung}}{^{131}\text{I cpm injected}} \right] \times ^{125}\text{I cpm lung},$$

where cpm/g lung is tissue counts normalized by lung dry weight, cpm injected is total infused counts, and cpm lung is absolute tissue counts.

$^{111}$Indium oxine-Labeled PMN Sequestration in Mouse Lungs

PMNs were isolated from WT C57BL/6 mice using a Polymorph-prep density gradient (Axis Shield, Oslo, Norway) per manufacturer’s instructions. Mice were anesthetized with ketamin/xylazine (100/5 mg/kg) and when non-responsive to touch, peripheral blood was collected via venous canula using a hetastarch exchange technique as described7. Red blood cells were lysed by hypotonic shock, and the isolated PMNs were incubated with $^{111}$Indium oxine (Amersham, GE Healthcare: Life Sciences, Piscataway, NJ) under serum-free conditions for 15 minutes at room temperature. Under these conditions, 75 to 85% of tracer activity was incorporated into leukocytes8,9. PMNs were washed twice with phosphate buffered saline/pH = 7.4 (PBS) and re-suspended at 1 x 10$^6$ cells/ml. A 10 µl sample of undiluted PMNs was counted to determine total gamma radioactivity infused/lung preparation. TNFα and PMNs were sequentially infused via a side-arm of the arterial cannula. Excess TNFα in the vascular compartment was removed by a 1 minute rinse with 3% albumin-Kreb’s solution. $^{111}$Indium oxine tracer washout was monitored at 5 minute intervals; after 30 minutes of washout, effluent
Radioactivity was negligible. PMN binding was determined by calculating the ratio of recovered tissue counts over infused counts.

In additional experiments, we replaced TNFα with a superoxide generating system by co-perfusing lungs with xanthine (400 mM) and xanthine oxidase (X/XO; 0.040 U/ml) for 10 minutes. We verified the presence of superoxide in the perfusate by electron paramagnetic resonance spectroscopy (data not shown). After 10 minutes, the lungs were rinsed for 1 minute as before and 111Indium oxine-labeled PMNs were perfused as described above.

**Cell Culture**

Mouse lung endothelial cells (MLEC) were isolated and cultured as described in endothelial cell growth medium 2 (EGM-2) supplemented with the BulletKit® (Biowhittaker, Walkersville, MD) and 10% fetal bovine serum (FBS). Confluent monolayers were incubated for 4 hours in EGM-2 + 1% FBS for 2 hour prior to experiments.

**Mouse ICAM-1 cDNA Mutagenesis and Cell Expression**

WT and mutant mouse ICAM-1 cDNAs were subcloned into the pcDNA3.1 expression vector. The mutants were made using a site-directed mutagenesis kit (Stratagene, La Jolla, CA) with primers: The primers used were as follows:

**mICAM1-518F-F:**

5’- GATCAGGATATTCAAGTTACAG- 3’

**mICAM1-518F-R:**

5’- CTGTAACTTGATAATATCCTGATC- 3’

**mICAM1-518D-F:**

5’- GAAAGATCAGGATAAGACAAGTTACAGAAGGCTCAGGAG- 3’

**mICAM1-518D-R:**
5’- CTTCTGTAACCTTTGTATCCTGATCTTTCTTGCGGTT- 3’

COS-7 cells were cultured in DMEM with 10 % FBS and transfected with lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to manufacturer’s protocol. HUVEC (Lonza, Walkersville, MD) at passage 4-7 were cultured with Clonetics EBM-2 media containing growth factors and 10 % FBS and transiently transfected by Amoza nucleofection (Lonza Nucleofector kit, Köln, Germany) according to the manufacturer’s instructions.

**Anti-ICAM-1 mAb Binding to Endothelial and COS-7 Cell Monolayers**

Binding kinetics ($K_d$ and $B_{max}$) of cell surface WT and mutant mouse ICAM-1 48 hr after transient transfection of HUVEC and COS-7 cell monolayers were determined from concentration-dependent mAb binding data measured by ELISA. Cells were grown to confluence in 96-well plates and challenged with TNF$\alpha$ (200 U/ml, 10 minutes) or mock-treated with saline. Cells were incubated with rat anti-mouse ICAM-1 mAbs (YN1/1.7.4 and eBioKAT-1) (for 1 hr at 4°C) and were then fixed with 2% paraformaldehyde for 15 minutes at 4°C followed by secondary Ab detection using anti-rat alkaline phosphatase to generate saturation binding isotherms. Binding kinetics were analyzed by non-linear curve fitting using GraphPad Prism software (San Diego, CA). Anti-ICAM-1 mAb concentration (0.1 – 30 $\mu$g/ml) plotted against alkaline phosphatase OD450 nm values, which indicated cell surface ICAM-1 binding, was used to calculate the $K_d$ (equilibrium dissociation constant) and $B_{max}$ (maximum density of binding sites) observed with ICAM-1 mAbs.

**Western Blotting and ICAM-1 Immunoprecipitation Analysis**

Murine lung tissue and cultured cells were lysed at 4°C and protein samples of the clarified supernatant [10 $\mu$g of protein (Bio-Rad) unless otherwise indicated] were separated on 8% SDS-polyacrylamide gels and transferred to nitrocellulose (Millipore, Billerica, MA).
ICAM-1 and c-Src, in their native and phosphorylated forms, i.e., p-(Y518)-ICAM-1 and p-(Y418)-Src, were detected with specific antibodies. We probed for GAPDH as a loading control. For co-immunoprecipitation studies, MLEC lysates, treated for 0-120 minutes with TNFα in the absence or presence of Src inhibitor PP2, were incubated with anti-ICAM-1 mAb and Protein A/G beads, and then proteins were collected on the beads separated by SDS-PAGE and blotted as above for ICAM-1 and β-actin. Membranes were developed using an enhanced chemiluminescence kit (Amersham, GE Healthcare: Life Sciences, Piscataway, NJ). Band intensity was quantified using NIH ImageJ software and values expressed relative to GAPDH or total ICAM-1 intensity.

**Confocal Microscopy**

Mouse lung endothelial cells (MLECs) and HUVEC were incubated with serum-free EBM-2 medium for 2 hr and stimulated with TNFα (20 ng/ml) or vehicle for 20 min. After washing, the cells were fixed with 2% paraformaldehyde for 15 minutes at 4°C and blocked with 10% serum for 30 min. After incubation with anti-ICAM-1 antibody YN1/1.7.4, the cells were incubated with secondary anti-rat IgG labeled with Alexa 488 (1:500 dilution) and 1:40 dilution of phalloidin labeled with Alexa 546 (Molecular Probes-Invitrogen, Eugene OR). Confocal images (1024 x 1024 pixels) were acquired using a Zeiss LSM510 META imaging system on an Axiovert 200 microscope with a 60X 1.25 NA water immersion objective. Laser excitation (488 nm and 543 nm) and emission filters (BP 505-530 nm and LP560 nm) were used to collect green and red fluorescence in optical sections in 0.44 μm step increments at maximal resolution (pinhole to achieve 1 Airy unit). Roughly 16-20 optical sections were acquired per cell, deconvolved 2-dimensionally, and rendered into 3D orthogonal views and pseudocolored projection images to highlight membrane distribution profiles and peak staining intensities.
Mouse Lung Injury Model

The role of ICAM-1 phosphorylation in lung PMN infiltration and protein-rich edema formation associated with LPS inhalation (the acute lung injury model used) was assessed in 18 male C57BL6 mice aged 8-10 weeks. Mice were anesthetized by i.p. injection of ketamine/xylazine (100/5 mg/kg) and when non-responsive to touch, injected with 150 µl liposome/cDNA mixture (pcDNA 3.1 (empty vector), WT mouse ICAM-1, or Y518F ICAM-1 mutant cDNA reconstituted in liposome emulsion at a ratio of 1 µg cDNA:2 µl liposomes) suspended in sterile PBS into the retro-orbital sinus as described10. Mice (n=3/group) were allowed to recover for 48 hr and then were administered LPS (Sigma, St. Louis MO) by nebulizing 10 mg LPS dissolved in 10 ml water over 1 hr and then mice were euthanatized under anesthesia 2 or 24 hrs later. Bronchoalveolar lavage fluid (3 x 1 ml lung lavages with sterile PBS) was then separated using Cytospin slides into cellular and fractions and analyzed for PMN number and albumin concentration as described7 for determination of neutrophil PMN infiltration in vivo and pulmonary transvascular albumin permeability measurements.

Statistical Analysis

Comparisons to detect significance of differences between group means were made by one way analysis of variance (ANOVA) followed by post hoc comparison of group means using GraphPad Instat software, with P < 0.05 considered to be significant.

Online References


Figure Legends:

**Online Figure I. TNFα induces PKCζ activation in mouse lung endothelial cells (MLECs) and mediates rapid phase of lung PMN uptake.**

A: MLEC monolayers received TNFα as a pretreatment for the indicated times. PKCζ was immunoprecipitated from cell lysates and used to stimulate $^{32}$P incorporation into exogenous histone, a PKCζ substrate. $^{32}$P-labeled protein was resolved by SDS-PAGE, and phosphorylated histone H1 detected by autoradiography was used as a measure of PKCζ activity. Relative phospho-histone band intensity show 7-8 fold increase in PKCζ activity 5 and 30 min after stimulation of cells with TNFα (radiogram shown is representative of data obtained in 3 separate experiments). B: Isolated-perfused mouse lung challenged with TNFα increased lung PMN sequestration 3-fold above basal level. Another
group of mice received 5 daily injections of the inhibitory pseudo-substrate peptide (PS) targeting PKC\(\zeta\) or PKC\(\theta\) (control). PKC\(\zeta\)-PS pretreatment prevented TNF\(\alpha\)-induced PMN uptake. By contrast, control peptide was ineffective. In other studies, deletion of p110\(\gamma\), the catalytic subunit of PI3 kinase-\(\gamma\) known to be upstream of PKC\(\zeta\), abolished the effect of TNF\(\alpha\) on PMN sequestration. Control values for WT lungs plotted from data in Fig. 1B. * = \(P < 0.01\) vs basal level; values shown are mean ± S.D, \(n = 3\) per group.

**Online Figure II. ICAM-1 immunostaining and confocal imaging showing ICAM-1 clustering in endothelial cells challenged with TNF\(\alpha\).** HUVECS were transfected with WT-, Y518F-, and Y518D-ICAM-1 cDNA and 48 hr later the cells were stimulated with TNF\(\alpha\) for 20 min, fixed with 1% paraformaldehyde on ice, and labeled with rat anti-mouse ICAM-1 mAb YN1/1.7.4 followed by Alexa 488 goat-anti mouse secondary Ab to detect ICAM-1 localization and clustering. Confocal images were acquired as described in Methods. We acquired 16-20 optical sections in 0.44 \(\mu\)m step increments and rendered these into 3D orthogonal views (A-D) or projection images which were pseudocolored to highlight sites of ICAM-1 localization and clustering (E-H). We observed WT-ICAM-1 clustering (discrete puncta in yz and xz profiles in orthogonal views and red spots in projection images; B and F), absence of Y518F-ICAM-1 clustering in cells treated with TNF\(\alpha\) (C and G), and abundance of clustered Y518D-ICAM-1 in non-stimulated cells (D and H) compared to non-stimulated WT-ICAM-1 (A and E). Images are representative of 3-5 z-stacks collected from each coverslip and the experiment was repeated twice with equivalent results. Scale bar = 20 \(\mu\)m.
A. PKCζ-mediated Histone Phos.

<table>
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<th>TNFα (min)</th>
<th>0</th>
<th>5</th>
<th>30</th>
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B. ¹¹¹In-PMN Binding (lung cpm/infused cpm)

- Control
- TNFα
- TNFα + PKCζ-PS
- TNFα + PKCθ-PS

WT: P13K-p110γ⁻/⁻
Online Data Supplement: Figure II

Confocal Orthogonal Views of Expressed ICAM-1 Forms

Pseudocolor Map of 3D ICAM-1 Projection