ORIGINAL RESEARCH

Activated β₂ Integrins Restrict Neutrophil Recruitment during Murine Acute Pseudomonal Pneumonia

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Abstract

Rapid neutrophil recruitment is critical for the efficient clearance of bacterial pathogens from the lungs. Although β₂ integrins and their activation are required for neutrophil recruitment from postcapillary venules of the systemic circulation into inflamed tissues, the involvement of integrins in neutrophil recruitment in response to respiratory infection varies among bacterial pathogens. For stimuli eliciting β₂ integrin–dependent neutrophil influx, including *Pseudomonas aeruginosa*, it remains unclear whether the activation of β₂ integrins is an essential step in this process. In the current study, we analyze neutrophil trafficking within the lungs of mice infected with *Pseudomonas aeruginosa* and evaluate the role of β₂ integrin activation through genetic deletion of talin-1 or Kindlin-3 or by pharmacological inhibition of high-affinity β₂ integrins using a small molecule allosteric antagonist. We observe that attenuation of high-affinity β₂ integrins leads to an enhancement of neutrophil emigration into lung interstitium and airspaces. Neutrophil effector functions, including the production of reactive oxygen species and the phagocytosis of bacteria, are only partially dependent on high-affinity β₂ integrins. These results reveal a mechanism by which activated β₂ integrins limit neutrophil entry into the lung tissue and airspaces during acute pseudomonal pneumonia and suggest potential strategies for modulating neutrophil-mediated host defense.

Keywords: neutrophils; pseudomonal pneumonia; *Pseudomonas aeruginosa*; adhesion; integrin activation

Clinical Relevance

Neutrophils are essential for host defense against respiratory infection but can also induce tissue damage associated with disease. Our work provides mechanistic insight into the recruitment of neutrophils into the lung tissue and airspaces in response to *Pseudomonas aeruginosa*, thus informing potential strategies to modulate this process during disease.

Acute respiratory infections remain a significant public health burden, one that is expected to grow with the continued emergence of drug-resistant pathogens. Neutrophil recruitment is a critical early event in innate host defense against bacteria. Bacterial and fungal infections are more frequent and more severe under conditions in which neutrophil recruitment is reduced, such as neutropenia associated with cancer treatments (1). In this context, promoting an influx of neutrophils into infected lung airspaces represents a potential immunomodulatory therapy to complement antibiotic approaches.

In the canonical neutrophil recruitment cascade that underlies trafficking from postcapillary venules of the systemic circulation, selectin ligands and integrins expressed on the neutrophil surface mediate rolling and adhesive interactions with inflamed endothelia that precede migration into tissue (2). In the context of respiratory infection, neutrophil recruitment into the lung airspaces occurs primarily from the extensive alveolar capillary network (3–5). The diameter of the neutrophil exceeds that of most alveolar capillary segments, necessitating neutrophil deformation for passage (6). Under homeostatic conditions, neutrophils and other leukocyte populations are highly enriched in the pulmonary circulation because of the geometric constraints that prolong leukocyte transit time compared with that of erythrocytes (7). Sequestration and further concentration of neutrophils within alveolar capillaries in response to inflammatory stimuli occurs as a result of reduced deformability of activated neutrophils in a manner that does not depend on integrin-mediated adhesion (8, 9). However, β_2 integrins do play a role in the retention of sequestered neutrophils (10) and in their recruitment into the bronchoalveolar airspaces in response to certain stimuli, including *Pseudomonas aeruginosa* (11, 12). Thus, although the activation of β_2 integrins is obligatory for neutrophil recruitment from the systemic circulation (13, 14), it remains unclear whether this mechanism applies to neutrophil recruitment from the pulmonary vasculature.

Integrin affinity for ligand is regulated in an "inside-out" manner by intracellular signal transduction that triggers the binding of talin and Kindlin family proteins to the short cytosolic tail of integrin β subunits. talin-1 and Kindlin-3 associate with distinct binding motifs in the β_2 integrin tail and induce structural changes that are transmitted through the transmembrane domains to the extracellular legs and headpiece, ultimately altering the ligand-binding affinity of the headpiece (15). We found previously that talin-1 is responsible for the conversion of the extracellular domain of the β_2 integrin family member lymphocyte function–associated antigen 1 (LFA-1) from a bent to an extended form, whereas Kindlin-3 is needed only for the transition from an intermediate-affinity extended (closed headpiece) state to the high-affinity state with an open headpiece (14). In the absence of Kindlin-3, neutrophils retain certain adhesive functions mediated by partially active β_2 integrins (14). In a similar manner, α/β I-like allosteric antagonists, including the small molecule XVA143, prevent the β_2 integrin headpiece from opening to reach the high-affinity state, but permit the partially active conformer (16–18).

In this study, we evaluated the requirement for β_2 integrin activation in neutrophil recruitment during acute respiratory infection of mice with *P. aeruginosa*. We demonstrate that blocking the activation of β_2 integrins, either genetically or pharmacologically, promotes neutrophil emigration into the lung interstitium and airspaces.

Materials and Methods

Additional description of the materials and methods is provided in the online supplement.

Animals
All animal studies were approved by the Lifespan Animal Welfare Committee. Mice were housed in a specific pathogen-free facility at Rhode Island Hospital. Mice harboring floxed *Tln1* alleles (19), floxed *Fermt3* alleles (20), and the Mx1-Cre transgene (21) have been described. To generate mixed chimeras, 8- to 12-week-old C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME) were lethally irradiated (10 Gy, single dose) and then reconstituted by intravenous injection of bone marrow cells from two sources at a 1:1 ratio: a wild-type mouse expressing enhanced green fluorescent protein (EGFP) under the ubiquitin C (UbC) promoter, and either a *Tln1*^flox/flox^Mx1-Cre or a *Fermt3*^flox/flox^Mx1-Cre mouse. Deletion of the gene encoding talin-1 or Kindlin-3 was induced by intraperitoneal injection of 250 μg of polyinosinic–polycytidylic acid (InvivoGen, San Diego, CA), three doses, each 2 days apart, starting 4 weeks after hematopoietic reconstitution, inducing near complete loss of the respective protein in neutrophils (14). Mice were used for experiments 4–8 weeks after polyinosinic-polycytidylic acid administration.

Acute Respiratory Infection
Prior to infection, a sample of peripheral blood was collected by saphenous venipuncture. The cytotoxic *P. aeruginosa* strain PA103 (22) was grown on solid media before culturing to log phase in Luria broth. Bacteria were suspended in sterile 0.9% saline, and the number of colony-forming units (CFUs) was determined by a standard curve according to optical density at 540 nm. Under vaporized isoflurane anesthesia, a 50-μl bolus of 1×10^6 CFU *P. aeruginosa* was administered to mice by intratracheal inhalation. For some experiments, intravenous injection of the blocking anti-CD18 antibody (30 μg, clone GAME-46), anti–intercellular adhesion molecule 1 (ICAM-1) antibody (30 μg, clone YN1), the β_2 integrin allosteric antagonist XVA143 (16) (3 μl of 10 mM XVA143 in 0.1 ml saline), or vehicle (dimethyl sulfoxide) immediately preceded infection. The mice were monitored closely for signs of morbidity and mortality.

Lung Fractionation
Methods to distinguish the intravascular and interstitial cell populations within lung tissue have been described (23). Briefly, at 4 hours after infection, the mice were anesthetized and then given 1 μg phycoerytherin (PE)-conjugated anti-Ly6G (BioLegend) by intravenous injection. Five minutes later, the pulmonary circulation was perfused with 10 ml Hanks Balanced Salt Solution (containing Ca^{2+}/Mg^{2+}) at physiological pressure (25 cm H_2O) into the beating right ventricle, allowing exsanguination through a left-ventricle incision. The bronchoalveolar lavage (BAL) was collected by repeated (at least five times) infusion–withdrawal of 1 ml of phosphate-buffered saline containing 1 mM ethylenediaminetetraacetic acid and 0.5% bovine serum albumin. Lungs were harvested and then digested using an enzyme cocktail (Miltenyi, San Diego, CA). Digested tissue and BAL samples were labeled with allophycocyanin (APC)-conjugated anti-Ly6B2 (Miltenyi) to identify all neutrophils by flow cytometry on a MACSQuant instrument (Miltenyi). In mixed chimeric mice, wild-type and gene-deficient neutrophils were delineated according to expression of EGFP. The number of *P. aeruginosa* CFUs in the BAL was determined by plating serial dilutions on solid media.

Statistical Analysis
For comparison between two groups, two-tailed Student's *t* tests were performed. For comparison among more than two groups,

one-way analysis of variance and Tukey's *post hoc* test was used. For comparison of adjacent compartments (blood, intravascular, interstitial, BAL) within mixed chimeric mice, paired analyses were performed. *P* values <0.05 were considered significant. All analyses were performed using Prism GraphPad software.

Results

Integrin Activation Regulates Neutrophil Recruitment in Response to *P. Aeruginosa*

β_2 integrins play a role in neutrophil influx into bronchoalveolar airspaces in response to acute respiratory infection with *P. aeruginosa* (11, 12). We confirmed this previous finding by observing a significant reduction in recruited neutrophils in the BAL of mice receiving a blocking antibody against β_2 integrins (anti-CD18, clone GAME-46) immediately prior to infection (Figure 1A). There is conflicting prior evidence of a role for ICAM-1, the primary endothelial ligand for β_2 integrins, in pulmonary neutrophil recruitment stimulated by *P. aeruginosa* (24). In our hands, antibody blockade of ICAM-1 did not significantly affect neutrophil recruitment (*see* Figure E1 in the online supplement).

To evaluate the potential involvement of integrin activation in this process, we took an approach using mixed chimeric mice that allows for the competitive *in vivo* recruitment of wild-type and gene knockout neutrophils in an internally controlled inflammatory environment. We generated two types of mixed chimeric mice that produce both wild-type and either talin-1– or Kindlin-3–deficient leukocytes, distinguished by their expression of EGFP (wild-type leukocytes had a UbC-EGFP transgene that is active in all neutrophils [Figure E2]). In experiments using the mixed chimera format, the frequency of wild-type and knockout neutrophils always sums to 100% within each compartment (e.g., blood, tissue, airspaces). How the frequency of knockout neutrophils changes along the recruitment pathway therefore indicates a competitive advantage or disadvantage relative to wild-type neutrophils and provides insight into the mechanisms of neutrophil trafficking.

Mixed chimeric mice were infected with 1×10^6 CFUs of *P. aeruginosa* strain
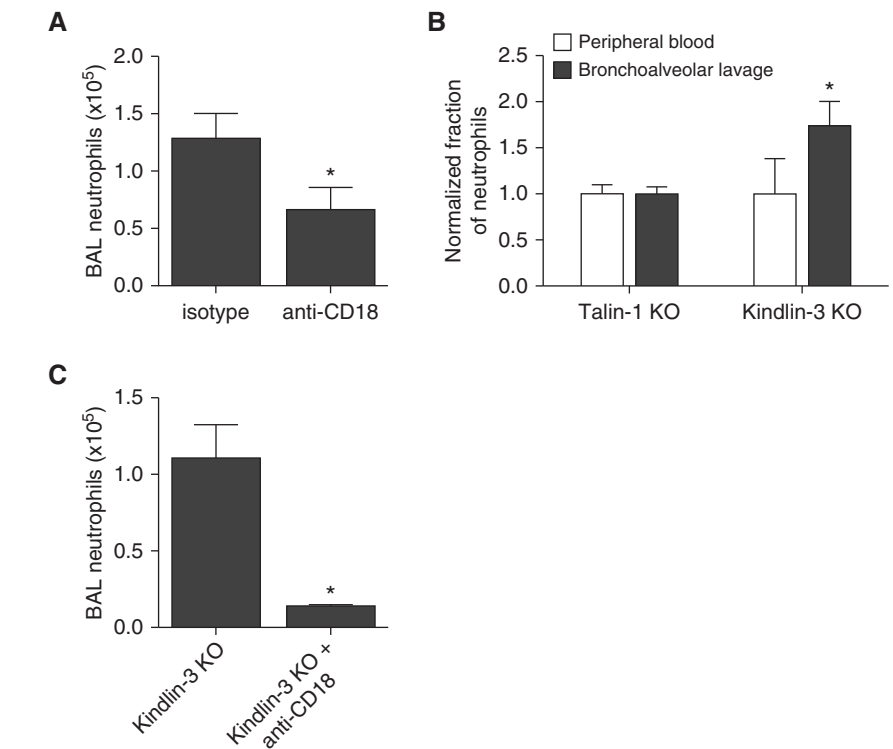
Figure 1. Kindlin-3 deficiency enhances neutrophil recruitment into infected airspaces. (*A*) Neutrophil influx into the airspaces as measured by neutrophil number in the BAL 4 hours after intratracheal instillation of 1×10^6 CFUs of *Pseudomonas aeruginosa*. Mice received either 30 μg isotype or 30 μg anti-CD18 (clone GAME-46) intravenously prior to infection (*n* = 8). *Significantly different from isotype. (*B*) In mixed chimeric mice infected with 1×10^6 CFUs of *P. aeruginosa*, the frequency of talin-1 knockout (KO) (*Tln1*$^{-/-}$) and Kindlin-3 KO (*Fermt3*$^{-/-}$) neutrophils in the peripheral blood and BAL was measured. The data were normalized such that within each experiment, the frequency of KO neutrophils in the peripheral blood was set to 1, and the frequency of KO neutrophils in the BAL was divided by the frequency in the blood (*n* = 4). *Significantly different from peripheral blood frequency. (*C*) In mixed chimeric mice with Kindlin-3 KO neutrophils (49.2 ± 6.9% in control, 46.5 ± 3.3% in anti-CD18 treated), the number of BAL neutrophils was determined 4 hours after infection with 1×10^6 CFUs of *P. aeruginosa* (*n* = 3). *Significantly different from isotype. All data are expressed as mean ± SEM.

PA103 by intratracheal inoculation. At 4 hours after infection, we determined the fraction of wild-type and knockout neutrophils that were recruited into the airspaces. Neutrophils lacking talin-1 exhibited a frequency (relative to wild-type neutrophils) in the peripheral blood prior to infection similar to the frequency they exhibited in the BAL of infected mice (Figure 1B), suggesting that talin-1 is not required for neutrophil recruitment during the acute response to *P. aeruginosa*. In contrast, Kindlin-3 knockout neutrophils represented a significantly higher proportion of neutrophils in the BAL than they did in the peripheral blood, indicating that neutrophils lacking Kindlin-3 had a competitive advantage over wild-type

neutrophils in their recruitment into the airspaces (Figure 1B). The recruitment of Kindlin-3 knockout neutrophils into the airspaces was still dependent on β_2 integrins, because anti-CD18 pretreatment reduced their numbers in the BAL (Figure 1C). Together, these results suggest that neutrophils use β_2 integrins to migrate into the airspaces in response to *P. aeruginosa*, but that the talin-1– and Kindlin-3–dependent activation of integrins is not required.

Integrin Activation Restricts Neutrophil Emigration from the Pulmonary Vasculature

To gain further insight into the role of integrin activation in neutrophil

recruitment into the airspaces, we analyzed the distribution of neutrophils within the lung tissue. To do so, we implemented an approach (23) in which neutrophils still within the vasculature are labeled by intravenous delivery of PE–anti-Ly6G mAb just prior to the experimental end point. Cells exposed to this circulating label include those marginated or sequestered in the lung but not those that have emigrated into the parenchyma. The pulmonary circulation is then perfused to remove excess label as well as to wash out those blood cells that are not marginated, and then BAL and lung tissue are collected sequentially. Digested lung tissue is labeled with a second neutrophil marker (APC–anti-Ly6B2) to identify all neutrophils. Thus, by flow cytometry, PE^+APC^+ cells represent intravascular neutrophils, and PE^-APC^+ cells indicate interstitial neutrophils (Figure 2A). Monocytes, which also express Ly6B2, were present in low numbers in the interstitium at this time point, compared with neutrophils, and were excluded from

analyses by their higher Ly6B2 levels and scatter properties (Figure E2).

In the lung tissue of mixed chimeric mice at 4 hours after infection, there was a significant increase in the fraction of talin-1 and Kindlin-3 knockout neutrophils in the interstitium relative to wild-type neutrophils (Figure 2B), indicating a more rapid emigration of neutrophils with defective integrin activation. When viewed in the context of circulating neutrophils, talin-1 knockout neutrophils were less frequent in the lung intravascular compartment than in the peripheral blood prior to infection, whereas Kindlin-3 neutrophils exhibited similar frequencies between these fractions (Figure 2C). Respiratory infection with *P. aeruginosa* significantly reduced the frequency of circulating talin-1 knockout neutrophils relative to wild-type, suggesting that neutrophil release from bone marrow may be defective (Figure 2C). Because the frequency of circulating Kindlin-3 knockout neutrophils was similar to their frequency in lung intravascular fraction, it

suggests that neutrophil sequestration and retention within the pulmonary vasculature may occur independently of Kindlin-3. As seen in the isolated analysis of lung tissue (Figure 2B), side-by-side quantification of all compartments indicates that both talin-1– and Kindlin-3–deficient neutrophils exhibited a competitive advantage over wild-type neutrophils in emigrating into the interstitium (Figure 2C). Together, these results suggest that activated integrins restrict neutrophil emigration from the pulmonary vasculature during acute pseudomonal pneumonia.

Selective Inhibition of High-Affinity β_2 Integrins Modulates Neutrophil Recruitment

XVA143 is in a class of antagonists that prevent the opening of the headpiece that brings β_2 integrins into their high-affinity state (16). Antagonists in this class do not impair the induction of extended, partially active β_2 integrins, the so-called intermediate-affinity state, but do block neutrophil adhesion mediated by high-affinity β_2 integrins (16–18). We have shown previously that Kindlin-3 is not needed for extension of the extracellular domain to activate LFA-1 to its intermediate-affinity state, but is required for achieving the high-affinity state (14). XVA143 thus mimics Kindlin-3 deficiency in its effects on β_2 integrin structure and neutrophil adhesive function (14, 15).

We demonstrated previously that Kindlin-3–deficient neutrophils fail to convert from a rolling to an arrested phenotype in postcapillary venules in response to the chemokine (C-X-C) motif ligand 1 (CXCL1) (14). To confirm the *in vivo* efficacy of XVA143 in blocking neutrophil arrest in postcapillary venules of the systemic circulation, we performed intravital imaging of the mouse cremaster muscle. Surgical exteriorization of the cremaster induces a local inflammatory response and P-selectin–dependent neutrophil rolling in postcapillary venules, with few arrested neutrophils (25). Using this model, we have shown previously that rapid neutrophil arrest in response to intravenous injection of the chemokine CXCL1 is dependent on both talin-1 and Kindlin-3 (14). In the studies performed here, CXCL1-induced neutrophil arrest in postcapillary venules of the cremaster muscle was almost entirely blocked by pretreatment of mice with XVA143, similar
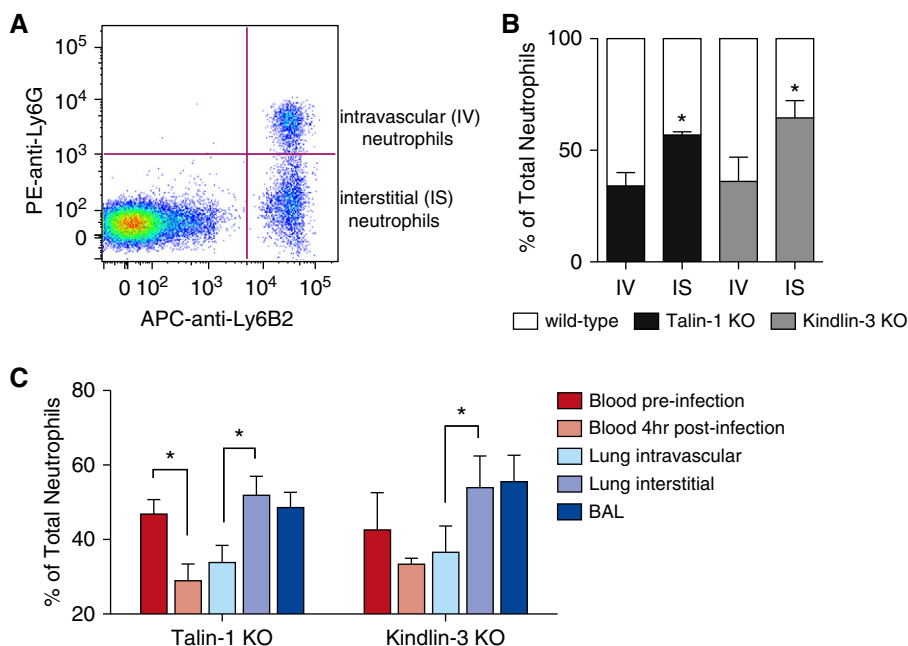
Figure 2. Activated integrins restrict neutrophil emigration. (*A*) Flow cytometry analysis of intravascular (IV; phycoerytherin [PE]$^+$allophycocyanin [APC]$^+$) and interstitial (IS; PE$^-$APC$^+$) neutrophil populations within digested lung tissue. Wild-type, talin-1 KO, and Kindlin-3 KO neutrophil frequency in either (*B*) the lung tissue fraction or (*C*) the peripheral blood, lung tissue, and BAL fractions, of mixed chimeric mice at 4 hours after infection with 1×10^6 CFUs of *P. aeruginosa*. In *B* and *C*, data are expressed as the absolute frequency of the KO neutrophil population within each fraction (the remaining are wild type). Data are expressed as mean ± SEM (*n* = 4 each). Statistical comparisons were performed between adjacent compartments. In *B*, *significant difference between IS and IV fraction of KO neutrophils. In *C*, *significant difference between indicated adjacent fractions.

to the effect of antibody blockade of LFA-1 (Figure 3).

We then assessed the effect of XVA143 on neutrophil recruitment in response to respiratory *P. aeruginosa* infection of C57BL/6 mice. Pretreatment of mice with XVA143 enhanced neutrophil influx into the bronchoalveolar airspaces and promoted emigration from the vasculature into the interstitium (Figure 4A). Thus, XVA143 modulates neutrophil recruitment in the lungs during acute pseudomonal pneumonia in a manner similar to genetic deficiency of Kindlin-3. Because neutrophils can induce bystander tissue injury, we measured the wet-to-dry ratio of the lungs to assess pulmonary edema. At 4 hours after infection, there was a significant increase in the wet-to-dry ratio, but the extent of edema was no different between mice receiving vehicle and those treated with XVA143 (Figure 4B). Histological analyses of lung tissue sections supported these findings (Figure E3). Finally, we measured the clearance of bacteria from lung airspaces by measuring the number of CFUs in the BAL 4 hours after intratracheal administration of 1×10^6 CFU *P. aeruginosa*. In mice pretreated with either XVA143 or vehicle control, the burden of *P. aeruginosa* was reduced by 30- to 40-fold; XVA143 did not significantly affect the clearance of *P. aeruginosa* from the airspaces (Figure 4C).
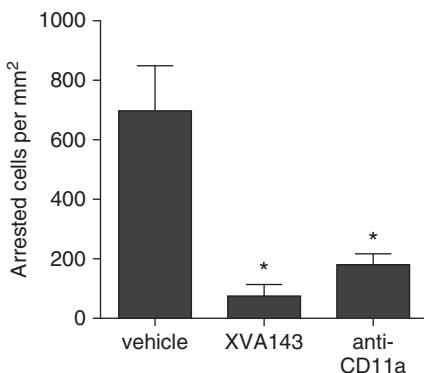
Figure 3. XVA143 blocks neutrophil adhesion in postcapillary venules. C57BL/6 mice were pretreated with either vehicle (DMSO), XVA143, or anti-CD11a (clone M17/4) by intravenous injection. During intravital imaging of the cremaster muscle microvasculature, the number of arrested neutrophils per vessel wall area in a postcapillary venule was determined 30 seconds after intravenous injection of 600 ng chemokine (C-X-C) motif ligand 1 (*n* = 3 per condition). *Significantly different from vehicle.

High-Affinity β₂ Integrins and Neutrophil Effector Function

Despite an augmented neutrophil recruitment into airspaces in response to *P. aeruginosa*, XVA143-treated mice cleared bacteria to a similar extent as control mice at the early 4-hour time point. To address whether high-affinity β₂ integrins are required for neutrophil effector functions that contribute to the eradication of pathogens, we assayed for oxidative burst and phagocytosis of *P. aeruginosa*. Neutrophils used in these assays were derived from conditionally immortalized murine myeloid progenitors that were differentiated in the presence of stem cell factor and granulocyte colony-stimulating factor (26). More than 80% of cells obtained through this method expressed the neutrophil marker Ly6G (Figure E4). Genetic deletion of talin-1, Kindlin-3, or CD18 (integrin β₂ subunit) in myeloid progenitors was achieved through CRISPR-Cas9 targeting (Figure E4). Neutrophils lacking talin-1, Kindlin-3, or β₂ integrins, as well as wild-type neutrophils treated with XVA143, retained the capacity to release extracellular superoxide anion in response to *P. aeruginosa* (Figure 5A). These assays were performed in the presence of an antiadhesive surface to specifically isolate the recognition of *P. aeruginosa* as the stimulus of oxidative activity.

Phagocytosis assays were performed using the pH-sensitive pHrodo Green dye conjugated to heat-killed *P. aeruginosa* (PA103-pHrodo), such that only internalized particles gained fluorescence (27). Neutrophil phagocytosis of *P. aeruginosa* was significantly reduced by either talin-1, Kindlin-3, or β₂ integrin deficiency, or by XVA143 treatment (Figure 5B). As a control, neutrophils exposed to PA103-pHrodo at a low temperature were unable to internalize the particles (Figure 5B). Together, these results indicate that high-affinity β₂ integrins play a role in phagocytosis of *P. aeruginosa* but are not required for the adhesion-independent generation of superoxide in response to *P. aeruginosa*.

Discussion

β₂ integrins represent the dominant neutrophil adhesion receptors, as their loss is the genetic cause of leukocyte adhesion deficiency type I (LAD-I) in humans (28).

LAD-I is characterized by recurrent bacterial and fungal infections. Likewise, leukocyte adhesion deficiency type III resulting from mutation of the *FERMT3* gene encoding Kindlin-3 also manifests in a compromised host defense, at least in part because of neutrophil recruitment defects (13, 14, 29–31). Activation of β₂ integrins by talin-1 and Kindlin-3 is necessary for neutrophil recruitment from postcapillary venules (14). However, the mechanisms of neutrophil trafficking in the lungs are fundamentally different from those that underlie neutrophil recruitment from other vascular beds throughout the body (32). Here, we have found that in the context of acute respiratory infection of mice with *P. aeruginosa*, neutrophil recruitment into the lung interstitium and bronchoalveolar airspaces is augmented when β₂ integrin activation is blocked. By using a technique to distinguish the neutrophil populations in lung tissue, we specifically identified emigration from the pulmonary vasculature as the primary step that is accelerated by antagonizing the activation of β₂ integrins.

Talins and Kindlins bind directly to the short cytosolic tail of integrin β chains and induce changes in integrin structure that regulate affinity for ligand (33). We demonstrated previously that talin-1 and Kindlin-3 play distinct roles in the process of activating the β₂ integrin LFA-1 (14). Those studies on murine neutrophils found that talin-1 stabilizes the extended form of LFA-1, even in the absence of Kindlin-3, but both proteins are required for neutrophil arrest and recruitment to the inflamed peritoneal cavity (14). In the current study of acute respiratory infection with *P. aeruginosa*, talin-1– and Kindlin-3–deficient neutrophils also exhibit phenotypic differences and similarities. When compared with wild-type neutrophils in mixed chimeric mice, Kindlin-3 knockout neutrophil influx into the airspaces was augmented, whereas talin-1 knockout neutrophils showed no overall difference with their wild-type counterpart. This latter outcome may be the additive result of reduced neutrophil release from the bone marrow and/or reduced retention within pulmonary capillaries, both of which counteract the enhanced emigration of talin-1 knockout neutrophils into the interstitium. By contrast, our data indicate that Kindlin-3 deficiency does not significantly affect circulating neutrophils or their sequestration/retention in alveolar
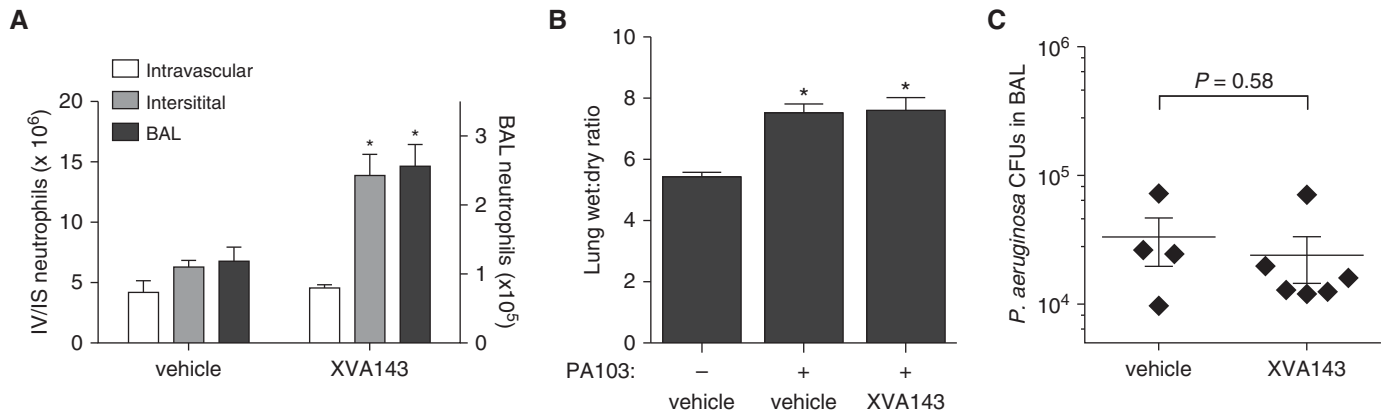
Figure 4. XVA143 promotes neutrophil recruitment into interstitium and airspaces. C57BL/6 mice were pretreated with either vehicle (DMSO) or XVA143 by intravenous injection immediately prior to intratracheal instillation of 1×10^6 CFUs of *P. aeruginosa*. (*A*) The number of neutrophils in the lung tissue intravascular and interstitial fractions, and BAL, measured at 4 hours after infection. A representative experiment is shown (*n* = 3). *Significantly different from the same fraction from vehicle control. (*B*) At 4 hours after infection (control mice received saline), the lungs were harvested and weighed. After drying for 72 hours, the lungs were again weighed, and the wet-to-dry weight ratio was calculated. *Significantly different from uninfected vehicle control. (*C*) Bacterial load in the BAL at 4 hours after infection.

capillaries, but Kindlin-3–deficient neutrophils exhibit a more robust emigration than do wild-type neutrophils. These results suggest that high-affinity β₂ integrins restrict neutrophil transmigration out of the pulmonary vasculature and into the interstitial spaces, where neutrophils can then cross the epithelium and enter the airspaces in an integrin activation–independent manner. The precise mechanisms of this restriction on neutrophil emigration from the

pulmonary circulation remain unclear. Inactivation of β₂ integrins is important for the cyclic adhesion and de-adhesion that is needed for efficient leukocyte motility (34, 35). The integrin inactivation process may be a rate-limiting step in neutrophil recruitment in the context of acute pseudomonal pneumonia. Another possibility is that engaged high-affinity β₂ integrins may transduce outside-in signals that attenuate the molecular processes involved in transendothelial migration in the lungs.

Our studies do not directly address the integrin subtype(s) whose activation is involved in regulating neutrophil trafficking during acute pseudomonal pneumonia. Previous work has shown that both of the β₂ integrin subtypes that are highly expressed by neutrophils, LFA-1 and macrophage antigen-1, play a nonredundant role in neutrophil recruitment during the acute response to *P. aeruginosa* (24). Talins and Kindlins are common activators of all integrin subtypes; thus, β1 integrin
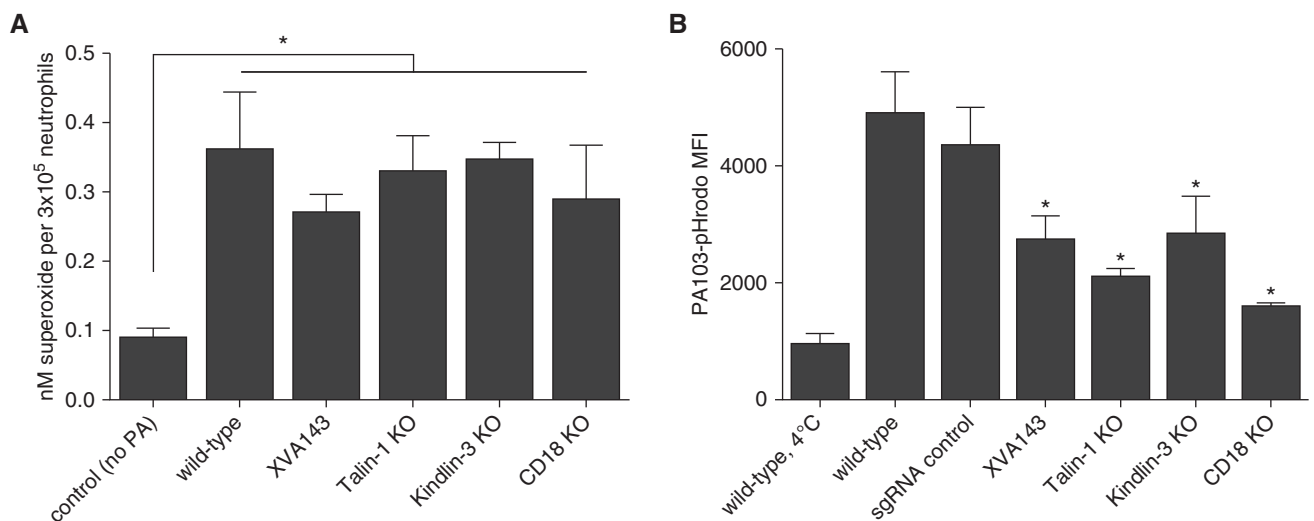
Figure 5. Impaired β₂ integrin activation attenuates neutrophil effector function. (*A*) Murine neutrophil release of superoxide anion was quantified by measuring the reduction of ferricytochrome C over the course of 2 hours. Oxidative burst was stimulated by exposing neutrophils to *P. aeruginosa* (PA) (*n* = 3). *Significantly different from control wild-type neutrophils not exposed to PA. (*B*) Murine neutrophil phagocytosis of heat-killed *P. aeruginosa* conjugated to pHrodo Green dye was measured after 2 hours of exposure at 37°C by flow cytometry of Ly6G-expressing cells. Data are expressed as pHrodo Green mean fluorescence intensity (MFI) ± SEM (*n* = 3). *Significantly different from wild-type. sgRNA, single guide RNA.

activation would also be defective in talin-1 and Kindlin-3 knockout neutrophils. XVA143 binds to the β_2 integrin subunit and inhibits the transition of both LFA-1 and macrophage antigen-1 into their high-affinity state (16). The effects of XVA143 on neutrophil recruitment in our study mimic those observed for Kindlin-3 deficiency, which suggests that β_2 integrins, rather than $\beta1$ integrins, are the receptors that restrict neutrophil emigration from the pulmonary vasculature in this context. Furthermore, although the modulation of neutrophil trafficking and phagocytosis by XVA143 is similar to the phenotype of Kindlin-3 knockout neutrophils, because Kindlin-3 also plays a role in downstream signaling (13), there are likely to be some neutrophil functions that are differentially affected by XVA143 versus Kindlin-3 deficiency.

Neutrophils are essential for host defense against *P. aeruginosa* and other bacterial infections of the lungs (32, 36–38). Neutropenic human patients (39, 40) and laboratory mice (36, 41, 42) have enhanced susceptibility to pseudomonal pneumonia. Inefficient rapid, early neutrophil recruitment, even when corrected within 24 hours, resulted in 10-fold more bacteria in the airspaces at 48 hours in a murine model of pneumococcal pneumonia (43). Potentiating the early phase of neutrophil recruitment may represent a therapeutic approach in the age of antibiotic resistance.

For pseudomonal pneumonia, our studies suggest that high-affinity β_2 integrins are a potential target for such a therapeutic strategy. At the same time, the nonspecific nature of the neutrophil antimicrobial response can induce bystander tissue injury (44). Thus, promoting neutrophil recruitment into lung airspaces may come at an overall cost to the host under certain conditions. In our studies, we did not observe additional injury to the lung in mice receiving XVA143. However, our experiments were focused on the acute response to a relatively large bacterial bolus.

P. aeruginosa is a common cause of nosocomial pneumonia, primarily in patients with suppressed immunity (45). Our studies used immunocompetent mice to probe the mechanisms of neutrophil recruitment elicited by *P. aeruginosa*. Despite enhancing neutrophil entry into the airspaces, XVA143 treatment did not significantly affect bacterial clearance. Although it is possible that impaired neutrophil effector function in XVA143-treated mice contributed to unimproved host defense, it seems that both control mice and those receiving XVA143 were able to effectively clear *P. aeruginosa*. Further research is required to determine whether neutrophil recruitment during pseudomonal pneumonia in the presence of underlying innate immune dysfunction

occurs by mechanisms related to those described here, and whether promoting neutrophil recruitment by inhibiting β_2 integrin activity improves host defense in that context.

In summary, we have shown that blockade of β_2 integrin activation, either through genetic deletion of talin-1 or Kindlin-3, or with the allosteric antagonist XVA143, promotes neutrophil emigration from pulmonary vasculature during acute respiratory infection with *P. aeruginosa*. Nonselective blockade of all β_2 integrins inhibits neutrophil recruitment, suggesting that inactive or partially active β_2 integrins are sufficient to mediate the interactions necessary for entry into the tissue and airspaces. Overall, this study furthers our understanding of neutrophil trafficking in the lungs and provides additional evidence that the mechanisms driving neutrophil recruitment during respiratory infection are fundamentally different from those described for neutrophil recruitment from systemic vascular beds. ■

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