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The Integrins Mac-1 and $\alpha_4\beta_1$ Perform Crucial Roles in Neutrophil and T Cell Recruitment to Lungs during *Streptococcus pneumoniae* Infection

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Neutrophils and T cells play an important role in host protection against pulmonary infection caused by *Streptococcus pneumoniae*. However, the role of the integrins in recruitment of these cells to infected lungs is not well understood. In this study we used the twin approaches of mAb blockade and gene-deficient mice to investigate the relative impact of specific integrins on cellular recruitment and bacterial loads following pneumococcal infection. We find that both Mac-1 (CD11b/CD18) and $\alpha_4\beta_1$ (CD49d/CD29) integrins, but surprisingly not LFA-1 (CD11a/CD18), contribute to two aspects of the response. In terms of recruitment from the circulation into lungs, neutrophils depend on Mac-1 and $\alpha_4\beta_1$, whereas the T cells are entirely dependent on $\alpha_4\beta_1$. Second, immunohistochemistry results indicate that adhesion also plays a role within infected lung tissue itself. There is widespread expression of ICAM-1 within lung tissue. Use of ICAM-$1^{-/-}$ mice revealed that neutrophils make use of this Mac-1 ligand, not for lung entry or for migration within lung tissue, but for combating the pneumococcal infection. In contrast to ICAM-1, there is restricted and constitutive expression of the $\alpha_4\beta_1$ ligand, VCAM-1, on the bronchioles, allowing direct access of the leukocytes to the airways via this integrin at an early stage of pneumococcal infection. Therefore, integrins Mac-1 and $\alpha_4\beta_1$ have a pivotal role in prevention of pneumococcal outgrowth during disease both in regulating neutrophil and T cell recruitment into infected lungs and by influencing their behavior within the lung tissue itself. ***The Journal of Immunology*****, 2011, 186: 5907–5915.**

Streptococcus pneumoniae (the pneumococcus) is a major human pathogen that is a principal cause of bacterial pneumonia in children and adults worldwide and is also a major cause of otitis media, meningitis, and septicemia (1–3). The pneumococcus is a significant agent of infant mortality in developing countries, particularly in Sub-Saharan Africa and Southeast Asia with an annual toll of >1.3 million infant deaths (4, 5). Approximately 25% of all preventable deaths in children under the age of 5 y are due to pneumonia, septicemia, and meningitis caused by the pneumococcus. Additionally, considerable problems exist with current treatment. Despite appropriate antibiotics, the overall fatality rate for pneumococcal pneumonia remains at ~20% and, for pneumococcal septicemia, the rate has remained unchanged since the 1950s (3, 6). Consequently, faced with these facts and the relatively little we know about the pneumococcus in terms of its interactions with different components of the host immune system, investigations of these interactions are of important basic scientific and clinical interest.

It is well established that the host response to pneumococcal lung infection is characterized by an intense inflammatory reaction involving heavy infiltration of neutrophils into alveolar air spaces (7–10). In addition to recruitment of neutrophils, we have also shown that there is a significant role for CD4 T cells in host immunity to pulmonary pneumococcal infection. The evidence comes from their early rapid infiltration into regions of heavy pneumococcal invasion and the increased host susceptibility of $CD4^{-/-}$ mice to infection with significantly elevated bacterial loads in both lungs and blood (8, 10, 11). In mice where the IL-2–dependent activation pathway is blocked in vivo, and subsequent proliferation of CD4 T cells is prevented, *S. pneumoniae* infection causes significantly exacerbated levels of disease, with 40- to 50-fold higher pneumococcal numbers both in blood and lungs and increased mortality rates compared with mice with normal CD4 T cell responses (12).

More recently, protection studies in mice have confirmed the role of CD4 T cells as part of an Ab-independent immune response to pneumococcal infection (13). Intranasal immunization with live pneumococci or with killed, nonencapsulated whole cell vaccine protected Ab-deficient mice, but not CD4 T cell-deficient mice, against subsequent pneumococcal intranasal challenge. CD4 T cell-deficient mice also failed to efficiently clear nasopharyngeal pneumococcal colonization, in contrast to their wild-type parents (14). There is also evidence from the clinical setting of the importance of CD4 T cells in pneumococcal disease. For example, patients with AIDS frequently develop severe pneumococcal infections (15). Indeed, HIV-infected patients with lower CD4 T cell counts are significantly more likely to be persistent pneumococcal carriers than non–HIV-infected patients (16), further supporting the evidence that CD4 T cells are necessary for control and clearance of infection.

Therefore, a better understanding of the mechanisms underlying the recruitment of neutrophils and CD4 T cells to lungs during pneumococcal infection is central to developing therapies to

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augment host defense and in suppression of detrimental lung inflammation. However, there are conflicting reports about the specific adhesion molecules that neutrophils and CD4 T cells use to emigrate from blood into lungs during infection. Adherence of leukocytes to the vasculature and their recruitment into infected tissues usually involves the adhesion molecules termed integrins, of which the CD18 integrins LFA-1 (CD11a/CD18, $\alpha_L\beta_2$) and Mac-1 (CD11b/CD18, $\alpha_M\beta_2$), as well as the CD29 integrin $\alpha_4\beta_1$ (CD49d/CD29), have major roles. Interestingly, the nature of the inflammatory stimulus may dictate the adhesion pathway used in lung infections. Whereas *Escherichia coli* and *Pseudomonas aeruginosa* have been shown to elicit a β_2 integrin-dependent neutrophil infiltration into the lungs, infection with high doses of *S. pneumoniae* appears to be β_2 integrin-independent (17–21). Furthermore, $\alpha_4\beta_1$ is expressed by neutrophils (22), yet its role during pneumococcal infection has not been investigated (23–25).

In this study we have used the two approaches of Ab blockade and mice deficient in LFA-$1^{-/-}$ (CD11a), Mac-$1^{-/-}$ (CD11b), and their ligand ICAM-1 (CD54) to investigate the relative impact of individual integrins on intranasal pneumococcal infection. We find that pneumococcal growth in the lungs and blood is dependent on Mac-1, $\alpha_4\beta_1$, and ICAM-1, but surprisingly not on LFA-1. Furthermore, neutrophil accumulation in the lungs is dependent on Mac-1 and $\alpha_4\beta_1$, whereas that of T cells is entirely dependent on $\alpha_4\beta_1$.

Materials and Methods

Source of mice

Female outbred MF1 mice were obtained from Harlan Olac (Bicester, U.K.) and were maintained under barrier conditions. LFA-$1^{-/-}$ (CD11a) (26), Mac-$1^{-/-}$(CD11b) (27), ICAM-$1^{-/-}$ (CD54) (28), and littermate control C57BL/6J mice were bred at Cancer Research UK London Research Institute (London, U.K.). All mice were at least 8 wk old at use, were sex-matched, and did not have detectable levels of anti-pneumococcal type 2 Abs. All experimental procedures were carried out at the University of Leicester and were approved by the U.K. Home Office and by the University of Leicester Ethical Committee.

Bacteria

S. pneumoniae serotype 2, strain D39, was obtained from the National Collection of Type Cultures (London, U.K.; NCTC 7466). Bacteria were identified as pneumococci prior to experiments by Gram stain, catalase test, α-hemolysis on blood agar plates, and by optochin sensitivity. Before use in infection studies, bacteria were passaged through mice as described previously (8) and were stored at −80°C. When required, suspensions were thawed at room temperature and bacteria harvested by centrifugation before resuspension in sterile PBS (8).

Preparation of mAbs for blockade experiments

The following purified rat anti-mouse mAbs were used to block integrin function: H68 (anti–LFA-1, anti-CD11a; IgG2a) (29), PS2/3 (anti-α_4 integrin, anti-CD49d; IgG2b) (30), and 5C6 (anti–Mac-1, anti-CD11b; IgG2b) (31). These mAbs plus control mAbs, PyLT-1 (IgG2b) and Y13-238 (IgG2a), were prepared in endotoxin-free form (<0.03 EU/ml) by the Cancer Research UK Monoclonal Antibody Service.

Intranasal challenge of mice with S. pneumoniae

As described previously (8, 9, 11), mice under anesthesia were infected intranasally with 1×10^6 CFU *S. pneumoniae*. Where appropriate, in experiments involving mAb blockade, 100 μg purified mAb was administered to each mouse via the dorsal tail vein, 1 h before intranasal challenge and 12 h postchallenge, as determined previously to achieve maximum blockade conditions (32). At 24 h following infection, mice were deeply anesthetized with 5% halothane (Astra-Zeneca, Macclesfield, U.K.) and blood was collected by cardiac puncture. Mice were killed by cervical dislocation, and the lungs were removed separately into 10 ml sterile PBS, weighed, and then homogenized with an Ultra-Turrax T8 homogenizer (IKA, Staufen, Germany). Viable bacterial counts in total lung homogenates and blood were determined by serial dilution in sterile PBS and plating onto blood agar plates as previously described (8).

Enumeration and differential analysis of lung leukocyte count

At 24 h following infections, lungs were removed from preselected groups of mice and leukocytes prepared using a modification of a previous method (9). Briefly, the lungs were placed into 10 ml HBSS, cut into small pieces, homogenized in 5 ml digestion buffer (5% FBS in RPMI 1640 with 0.5 mg/ml [207 collagen digestion units/ml] collagenase [Sigma-Aldrich, Dorset, U.K.] and 30 μg/ml [87 U] DNAase I [Sigma-Aldrich]) and passed through a nylon mesh three times. After homogenization, lung samples were incubated at 37°C for 30 min and passed through a column containing ~1 cm nonabsorbent cotton wool in a glass Pasteur pipette to remove large pieces of debris. Cells were collected in Falcon 2052 tubes (BD Biosciences, Oxford, U.K.) and centrifuged at $322 \times g$ and 4°C for 5 min. The supernatant was removed and the cells were resuspended in 1 ml 1× RBC lysing solution (BD Pharmingen, San Diego, CA). After 5 min at room temperature to lyse the RBC, the remaining cells were brought to isotonicity by adding an excess volume of ice-cold PBS. Following centrifugation at $322 \times g$ for a further 5 min, cells were washed with 1 ml PBS before final resuspension in 1 ml 5% FBS in RPMI 1640. The cell suspension was diluted with 5% FBS in RPMI 1640 to give between 1.4×10^6 and 2×10^6 cells/ml.

For differential leukocyte analysis, a 50 μl cell suspension was centrifuged onto cytospin slides (Thermo Shandon, Runcorn, U.K.) using a cytocentrifuge (Cytospin 2; Thermo Shandon) at $108 \times g$ for 3 min. Following centrifugation, slides were air-dried briefly, fixed in 100% methanol for 10 min, and differentially stained using Giemsa stain (BDH, Lutterworth, U.K.). Slides were analyzed at ×400 magnification with a graticule-equipped eyepiece, independently by two observers and in a blinded manner. At least 200 cells were counted on each slide.

Immunohistochemistry and mAbs

Mouse lung tissue was harvested in one piece and fixed for 1 h at room temperature in 10% neutral buffered formalin. The tissues were then processed, paraffin embedded, and sectioned at 4 μm. For all lung samples, sections were cut at three levels at 100-μm intervals and analyzed for consistency of observations. Following de-waxing, endogenous peroxidase was blocked using 1.6% hydrogen peroxide in PBS for 10 min, followed by incubation with 10% normal rabbit serum for 30 min to block nonspecific staining. The fixed tissue sections were then stained with anti-CD3 mAb (T cells) or with mAb 2B10 (S100A9) to detect neutrophils (33), for 1 h at room temperature. After washes in PBS, biotinylated rabbit anti-rat Ab (Vector Laboratories) was applied for 45 min followed by incubation with ABC complex solution (Vector Laboratories) for 30 min. Finally, the staining was developed using diaminobenzidine chromogen solution (BioGenex).

For immunofluorescence, fresh-frozen lung sections were treated with anti–ICAM-1 (CD54) mAb YN1.1.7 and anti–VCAM-1 (CD106) mAbs M/K-2.7 or 429 (MVCAM.A; BD Biosciences), respectively, for 1 h at room temperature followed by goat anti-rat polyclonal Ab (Molecular Probes) for 1 h. Finally, slides were immersed in Sudan black (0.1% in 70% industrial methylated spirits) to reduce the autofluorescence of paraffin sections. The slides were mounted in hard-set VectaMount, which included DAPI to highlight nuclei (Vector Laboratories). Images were acquired on an i90 Nikon microscope using NIS-Elements software.

Statistical analysis

Data are shown as mean ± SEM. Data were analyzed using GraphPad Prism software version 4 for Macintosh computers. Unpaired Student *t* test analyses were performed on the pairs of data. Differences were considered significant when $p < 0.05$ ($^*p < 0.05$, $^{**}p < 0.01$). For analysis of more than two data sets, the experimental groups were examined using a Dunnett multiple comparison test. The unpaired Student *t* test was then applied to the samples identified as having significant differences.

Results

Effect of mAb blockade on leukocyte infiltration into lungs infected with S. pneumoniae

We looked first at the total number of leukocytes that infiltrated into the lungs of mice intranasally infected with pneumococci. At 24 h postinfection, there was no difference in mice pretreated either with isotype-matched control mAb or anti–LFA-1 mAb H68 ($p > 0.05$) (Fig. 1*A*). However, there was a significant reduction in total lung leukocyte numbers when mice were pretreated with either anti–Mac-1 mAb 5C6 (52%) or anti-α_4 integrin mAb PS2/3

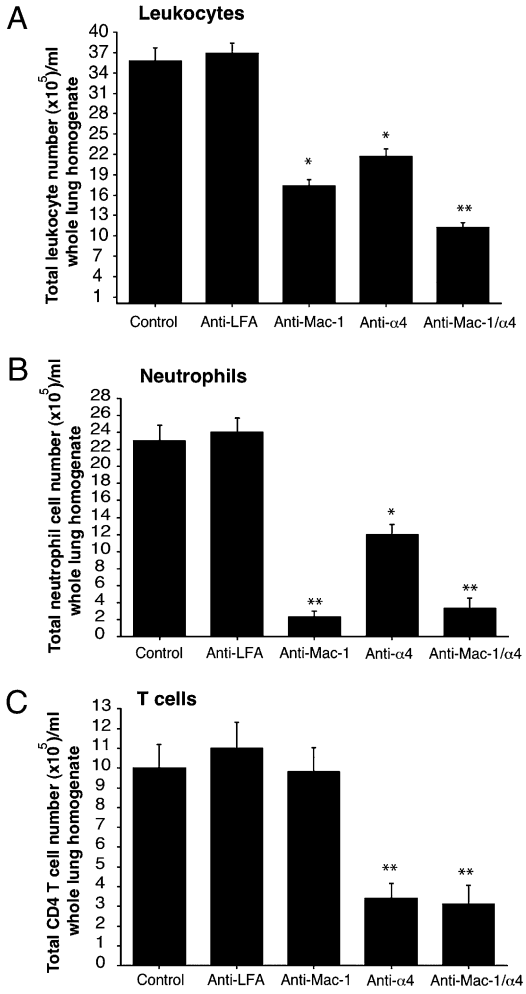

FIGURE 1. *A*, Total leukocyte numbers per milliliter of whole lung homogenate 24 h postinfection with D39 pneumococci (n = 8 mice for each group ± SEM). Mice received either anti–LFA-1 mAb, anti–Mac-1 mAb, anti-α_4 mAb, or a combination of anti–Mac-1 and anti-α_4 mAbs; control mice received appropriate isotype-matched control mAb. *B*, Neutrophil numbers per milliliter of whole lung 24 h postinfection with D39 pneumococci (n = 6 mice for each group ± SEM). Administered Abs as in *A*. *C*, CD4 T lymphocyte numbers per milliliter of whole lung 24 h postinfection with D39 pneumococci (n = 6 mice for each group ± SEM). Administered mAbs as in *A*. *$p < 0.05$, **$p < 0.01$ compared with control.

(39%) mAbs ($p < 0.05$ compared with control). When both anti–Mac-1 and anti-α_4 mAbs were administered together, leukocyte infiltration into lungs was further reduced (69%) compared with control ($p < 0.01$) or either anti–Mac-1 or anti-α_4 integrin alone ($p < 0.05$). The involvement of Mac-1 and α_4 integrin, instead of LFA-1, suggested a distinctive profile of integrin usage for leukocytes emigrating to the lung following *S. pneumoniae* infection.

Effect of Ab blockade on neutrophil and T cell recruitment into lungs infected with S. pneumoniae

When neutrophils were specifically investigated at 24 h postinfection, mAb blockade of Mac-1 reduced recruitment to the lungs by 90% compared with mice given control mAb ($p < 0.01$) (Fig. 1*B*). The mAb blockade of $\alpha_4\beta_1$ integrin had a partial effect on neutrophil infiltration into lungs, indicating that it was less critical than Mac-1 for neutrophil recruitment (49% reduction; $p < 0.05$) (Fig. 1*B*). The combined mAb blockade of both Mac-1 and $\alpha_4\beta_1$ significantly reduced neutrophil infiltration into lungs compared with control ($p < 0.01$), but it was not more effective than blockade of Mac-1 alone ($p > 0.05$). Again, there was no difference in the total number of neutrophils that infiltrated into the lungs of either pneumococci-infected mice pretreated with either anti–LFA-1 or isotype-matched control mAbs ($p > 0.05$).

In contrast to neutrophils, investigation of T cell infiltration into lungs of pneumococcal-infected mice revealed no effect of Mac-1 blockade ($p > 0.05$). It was only $\alpha_4\beta_1$ integrin mAb that significantly reduced the numbers of T cells recruited into infected lungs (67% decrease compared with control mice; $p < 0.01$) (Fig. 1*C*). As expected, the combined mAb blockade of Mac-1 and $\alpha_4\beta_1$ integrins did not further reduce the numbers of T cells. Again, there was no difference in the total number of T cells that infiltrated into the lungs pretreated with either anti–LFA-1 or isotype-matched control mAbs ($p > 0.05$).

It was important to know that the mAbs that exhibited blocking effects in these experiments were acting to prevent T cell and neutrophil recruitment by interfering with integrin activity and not nonspecifically through neutrophil depletion via FcR-mediated uptake. An evaluation of both circulating neutrophil and T cell numbers following an injection of mice with each mAb or PBS showed that the mAbs have no negative effect (Supplemental Fig. 1).

Leukocyte pulmonary infiltration in LFA-1$^{-/-}$ and Mac-1$^{-/-}$ mice infected with S. pneumoniae

The results obtained with mAb blockade of the β_2 integrins were unexpected given the predominance of LFA-1 in neutrophil recruitment in other tissues and infections. We therefore sought confirmation of these results through the use of LFA-1$^{-/-}$ and Mac-1$^{-/-}$ mice. When neutrophil numbers in infected lungs of LFA-1$^{-/-}$ mice were analyzed, there was no significant difference at 24 h postinfection between the number of neutrophils that infiltrated into their lungs compared with wild-type littermates ($p > 0.05$) (Fig. 2). However anti–Mac-1 mAb blockade in these mice reduced neutrophil infiltration by 87% ($p < 0.01$) compared with controls (Fig. 2). Similarly, Mac-1$^{-/-}$ mice had significantly reduced neutrophil numbers in their lungs (reduced by 76%) compared with wild-type controls ($p < 0.01$) postinfection, confirming the mAb blockade results (Fig. 1*B*). Inhibition of α_4 integrin in LFA-1$^{-/-}$ mice reduced neutrophil infiltration by 36% ($p < 0.05$). Again, these results are consistent those obtained with anti-α_4 integrin mAb blockade of wild-type mice (see Fig. 1*B*).

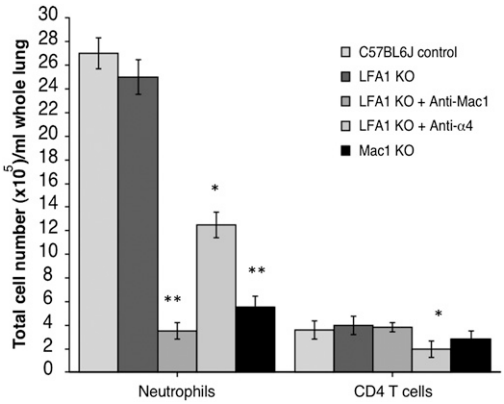

FIGURE 2. Numbers per milliliter of neutrophils and CD4 T cells in whole lung homogenate 24 h postinfection with D39 pneumococci in LFA-1$^{-/-}$ and Mac-1$^{-/-}$ mice and their isogenic parent strain C57BL/6J mice. mAbs were administered as described in the Fig. 1*A* legend (n = 6 mice for each strain ± SEM). *$p < 0.05$, **$p < 0.01$ compared with control.

When T cell infiltration was similarly analyzed, there was no significant difference ($p > 0.05$) between the total numbers of T cells that infiltrated the lungs of LFA-$1^{-/-}$ mice at 24 h postinfection compared with wild-type controls (Fig. 2). There was also no difference in T cell numbers when Mac-1 was blocked using mAb in LFA-$1^{-/-}$ mice or when Mac-$1^{-/-}$ mice were used ($p > 0.05$). There was, however, a reduction of 48% in T cell numbers when α_4 integrin was blocked using mAb in LFA-$1^{-/-}$ mice ($p < 0.05$) (Fig. 2). Again, these results mirrored those obtained with α_4 integrin mAb blockade of T cells in wild-type mice (see Fig. 1*C*).

We next investigated the numbers of circulating T cells and neutrophils in LFA-$1^{-/-}$ and Mac-$1^{-/-}$ mice compared with wild-type mice to see whether any imbalance in numbers might influence their recruitment. T cells numbers were equivalent between the three strains of mice, highlighting the major use of α_4 integrin for tissue recruitment of these leukocytes (Supplemental Fig. 2). However, circulating neutrophil numbers of both LFA-$1^{-/-}$ and Mac-$1^{-/-}$ mice were increased compared with control mice. An increase in circulating neutrophils has been reported previously for LFA-$1^{-/-}$ mice, but not for Mac-$1^{-/-}$ mice (22, 27). As circulating neutrophil numbers from both LFA-$1^{-/-}$ and Mac-$1^{-/-}$ strains appear both to be increased compared with control mice, such a difference does not explain the altered usage of Mac-1 over LFA-1 in the pulmonary cellular infiltration response to pneumococcal infection.

We also checked the expression levels of Mac-1 on LFA-$1^{-/-}$ neutrophils compared with wild-type mice with the thought that there might be a compensatory increase in expression. However, the level of Mac-1 remained unaltered (data not shown). T cells were Mac-1 nonexpressors whether from wild-type mice or from the LFA-$1^{-/-}$ mice (data not shown).

Effect of integrin deficiency or blockade on S. pneumoniae *growth in lungs and blood*

The LFA-$1^{-/-}$ and Mac-$1^{-/-}$ mice were then investigated to determine the impact that the lack of these integrins had on pneumococcal growth in tissues following infection. There was no difference in pneumococcal CFU in lungs or blood of LFA-$1^{-/-}$ mice compared with wild-type controls ($p > 0.05$) (Fig. 3). However, mAb blockade of Mac-1 and α_4 integrin in LFA-$1^{-/-}$ mice significantly increased pneumococcal CFU in both the lungs and blood compared with infected isogenic parent controls ($p < 0.01$ and $p < 0.05$, respectively; Fig. 3). Specifically, pneumococcal numbers increased in both lungs (1.7 log/ml) and blood (1.5 log/ml) of LFA$^{-/-}$ mice at 24 h postinfection when Mac-1 was blocked ($p < 0.01$) and a 1 log/ml increase in lungs and blood when α_4 integrin was blocked ($p < 0.05$) in LFA-$1^{-/-}$, both compared with control (Fig. 3). When Mac-$1^{-/-}$ mice were infected with pneumococci, there was a 2 log/ml increase in pneumococcal loads in both lungs and blood compared with isogenic parent controls ($p < 0.01$) (Fig. 3).

These findings were replicated in wild-type mice when the integrins were blocked exclusively using mAbs. Thus, blockade with anti–LFA-1 mAb had no effect on numbers of bacteria in the lungs and blood, whereas blockade with anti–Mac-1 and/or anti-α_4 integrin resulted in significantly more pneumococci in the lungs and blood (Supplemental Fig. 3).

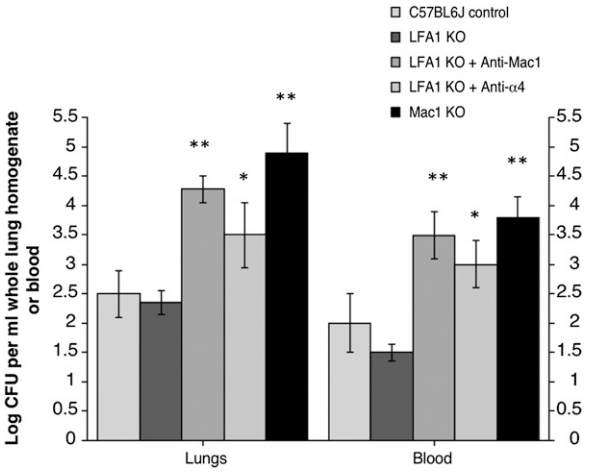

FIGURE 3. Log CFU D39 pneumococci 24 h postinfection in lungs and blood of LFA-$1^{-/-}$ and Mac-$1^{-/-}$ mice and their isogenic parent strain C57BL/6J mice. mAbs were administered as described in the Fig. 1*A* legend ($n = 6$ mice for each strain ± SEM) *$p < 0.05$, **$p < 0.01$ compared with control.

Expression of integrin ligands in uninfected and S. pneumoniae*-infected lungs*

Integrin usage by leukocytes is dictated by the availability of the ligands that they will recognize. We used immunohistochemistry to explore the distribution of ICAM-1 that serves as a ligand for both LFA-1 and Mac-1 (34) and the $\alpha_4\beta_1$ ligand, VCAM-1. In normal, uninfected lungs, there was constitutive VCAM-1 staining that was restricted to the bronchiolar wall epithelium (Fig. 4*A*). VCAM-1 was particularly strongly expressed on the luminal surface (Fig. 4*B*). This staining was demonstrated with two distinct anti–VCAM-1 mAbs (MK2.7 and 429). Between 3 and 6 h following intranasal infection with pneumococci, there was strong

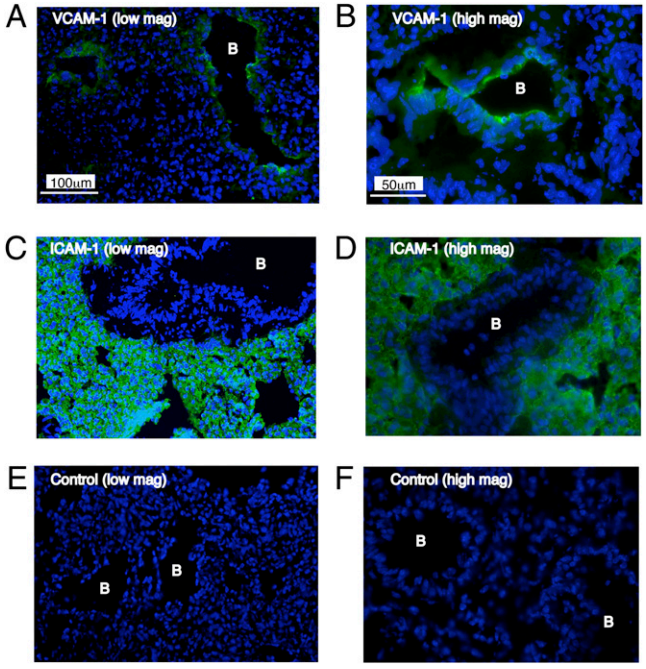

FIGURE 4. Expression of the $\alpha_4\beta_1$ ligand VCAM-1 and the Mac-1/LFA-1 ligand ICAM-1 in uninfected lung tissue. *A*, Immunofluorescence of fresh-frozen lung tissue showing distribution of VCAM-1 expression (green) confined to the bronchioles (*B*); DAPI (nuclei stained blue). *B*, As *A* but higher magnification showing strong VCAM-1 staining on the luminal surface of a bronchiole. *C*, Widespread expression of ICAM-1 (green) on lung cells and lack of ICAM-1 expression on bronchiolar cell membranes (blue, DAPI staining). *D*, As *C* but higher magnification showing more detail of ICAM-1–negative bronchiole. *E* and *F*, Tissue sections stained with control non–type-matched mAb and DAPI staining (blue) (low and higher magnification equivalent to *A–D*).

expression on the bronchioles as before, but also on the walls of the venules. VCAM-1 staining increased in intensity as the infection progressed and was also observed on arterioles at 48 h.

In uninfected lungs, there was also constitutive ICAM-1 staining that was extensive and featured many lung cell types, including endothelial and epithelial cells, which are abundant in lung tissue (Fig. 4*C*). However, in contrast to VCAM-1, there was an absence of direct staining of the bronchioles (Fig. 4*D*). Between 3 and 6 h following pneumococcal intranasal inoculation, the general level of ICAM-1 staining increased but there remained an absence of staining on bronchiole walls. However, by 48 h almost all bronchiole walls are also stained.

*Distribution of T cell and neutrophils in lungs preand post-*S. pneumoniae *infection of wild-type mice*

We next investigated both T cells and neutrophils within lung tissue over time following pneumococcal infection to see how they were distributed following recruitment from the circulation. A low level of T cells, which were already present in the lungs of uninfected mice, were scattered generally within lung tissue and were notably present surrounding the bronchiolar wall tissue (Fig. 5*A*). Following infection at 12 and 24 h, the number of T cells increased substantially, heavy infiltration around selected bronchioles was observed, and T cells were observed within the lumen of the bronchioles (Fig. 5*C*, 5*E*). In contrast, neutrophils were absent from lung tissue of uninfected mice except within venules (Fig. 5*B*), but they rapidly increased in numbers postinfection (Fig. 5*D*, 5*F*). Between 12 and 24 h they were also observed around the bronchioles and infiltrating into the bronchiolar lumen (Fig. 5*D*, 5*F*).

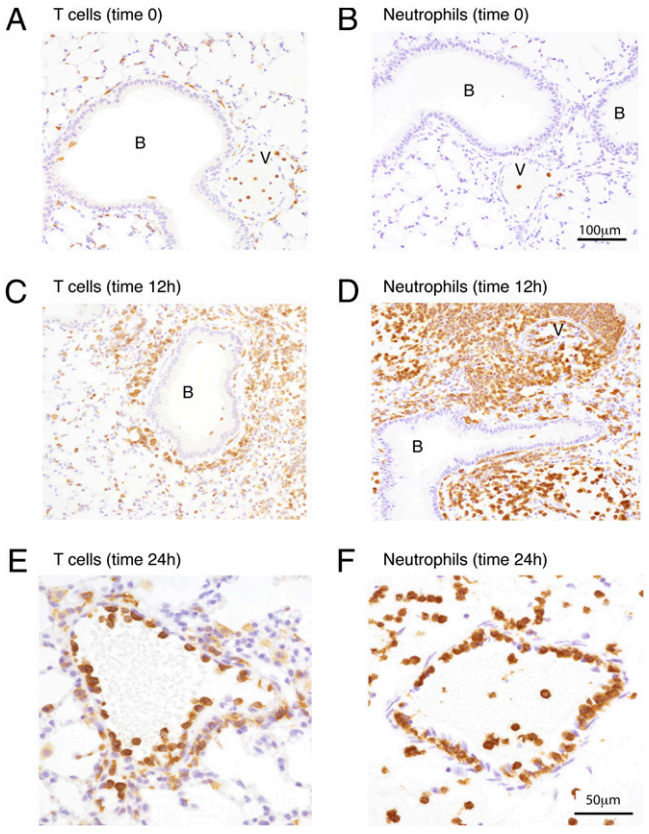

FIGURE 5. Distribution of T cells and neutrophils in lung tissue before and after intranasal infection of C57BL/6 mice with *S. pneumoniae. A*, The distribution of T cells (brown) around a bronchiole and within an adjacent vein in uninfected lung; $n = 3$ mice. *B*, The lack of neutrophils (brown) in uninfected lung except for a few with an intravascular location; $n = 3$ mice. *C*, The distribution of T cells in pneumococci-infected lung at 12 h; $n = 3$ mice. *D*, The distribution of neutrophils in pneumococci-infected lung at 12 h; $n = 3$ mice. *E*, A typical vein in pneumococcal-infected lung at 24 h showing marginated T cells. *F,* A typical artery in pneumococcal-infected lung at 24 h showing marginated neutrophils. The tissue counterstain is hematoxylin; the cellular stain is diaminobenzidine. B, bronchiole; V, vein.

The widespread margination of both T cells and neutrophils was evident within vessels (both venules and arterioles), particularly at 24 h postinfection (Fig. 5*E*, 5*F*). The distribution of concentrated numbers of T cells and neutrophils was not uniform throughout the lung tissue, but it was focused on certain bronchiolar regions, corresponding to regions where the bacteria were present (data not shown).

In terms of integrins, $\alpha_4\beta_1$ was expressed by leukocytes associated with the bronchioles (Supplemental Fig. 4*A*). The lack of use of LFA-1 by the recruited cells raised the issue as to whether their expression of LFA-1 was compromised by conditions within the pneumococci-infected lung tissue. However, staining of infected lung tissue showed that LFA-1 was well expressed on leukocytes associating with the bronchioles (Supplemental Fig. 4*B*).

Distribution of T cells and neutrophils in Mac-1$^{-/-}$ lungs following S. pneumoniae *infection*

Because Mac-1 played such a pivotal role in neutrophil recruitment to the infected lungs, we wanted to investigate the distribution of neutrophils compared with T cells in Mac-1$^{-/-}$ mouse lungs. When consecutive tissue sections of uninfected lungs were viewed, it was evident that there was a complete lack of neutrophils, but a constitutive T cell presence, with distributions of both that resembled the uninfected lungs of the wild-type mice (Fig. 6*A*, 6*B* compared with Fig. 5*A*). Additionally, the distribution of T cells at 24 h postinfection resembled that observed in the wild-type mice with a concentration around the bronchioles (Fig. 6*C* compared with Fig. 5*C*). However, a key difference was that the overall number of neutrophils within the lung tissue was substantially lower compared with infection of a wild-type mouse (compare Fig. 5*D* and Fig. 6*D*) and this was also reflected in a lack of neutrophils associated with the bronchioles. It was also noted at the 24 h time point that there was large-scale disruption of tissue integrity with a heavy presence of erythrocytes throughout the tissue (Fig. 6*C*, *inset*). In terms of the vascular tissue, marginated T cells were observed but no vessels displayed any neutrophils (Fig. 6*E*, 6*F*). These results provide further evidence that the lack of Mac-1 prevented neutrophil recruitment at the level of the vasculature and that this lack of neutrophils in the Mac-1$^{-/-}$ lungs, even in the presence of recruited T cells, severely impaired the response to *S. pneumoniae.*

Role of ICAM-1 in neutrophil recruitment into lungs postinfection with S. pneumoniae

As ICAM-1 is a Mac-1 ligand (34) and, as its expression was widespread in lung tissue, it was of interest to test whether it was used by neutrophil Mac-1 as part of the recruitment into lungs or to migrate more generally within lung tissue. It was also of interest to test the impact of ICAM-1 deficiency on pneumococcal proliferation. Neutrophil numbers were at wild-type levels in uninfected lungs of ICAM-1$^{-/-}$ mice, and they were not significantly different postinfection at 24 h, indicating that there was no problem in neutrophil entry into the pulmonary tissue (Fig. 7*A*). Immunohistochemistry revealed that the recruited neutrophils migrated throughout the lung parenchyma in ICAM-1$^{-/-}$ lungs as in wild-type lungs (data not shown). Thus, in the ICAM-1$^{-/-}$ situation, neutrophils had no difficulty in entering lung tissue; in fact, there was a trend toward increased numbers compared

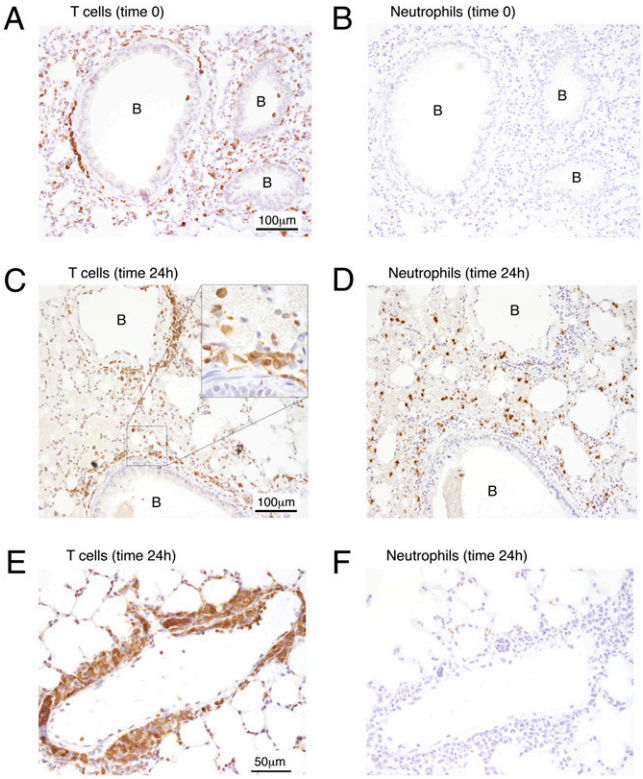

FIGURE 6. Distribution of T cells and neutrophils in lung tissue of Mac-$1^{-/-}$ mice before and after intranasal infection of mice with *S. pneumoniae. A*, The distribution of T cells in uninfected lung; $n = 3$ mice. *B*, The lack of neutrophils in a consecutive section; $n = 3$ mice. *C*, The distribution of T cells in pneumococcal-infected lung at 24 h; *inset*, higher magnification of lung tissue showing T cell concentration around bronchioles and heavy presence of erythrocytes; $n = 4$ mice. *D,* The scattered distribution of neutrophils in a consecutive section; $n = 4$ mice. *E*, A typical vein in pneumococci-infected lung at 24 h showing marginated T cells. *F,* The same vein as in *E* revealing the lack of marginated neutrophils. The tissue counterstain is hematoxylin; the cellular stain is diaminobenzidine. B, bronchiole.

with wild-type mouse lungs. Surprisingly, despite the presence of T cells and neutrophils, the ICAM-$1^{-/-}$ mice were significantly less able than wild-type mice to control the pneumococcal infection (Fig. 7*B*).

Discussion

Neutrophil infiltration into pulmonary tissue space is one of the hallmarks of acute bacterial pneumonia (35). Indeed, one of the essential features of a successful host immune response to bacterial infection in lungs is the early recruitment of neutrophils (36). In addition to this well-documented phagocyte response, the involvement of CD4 T lymphocytes in the early stages of the host pulmonary response has also been recently recognized (8, 10, 11).

In this study we investigated the integrins that are required for the recruitment of neutrophils and T cells into lungs during pneumococcal pneumonia infection and have established that Mac-1 (CD11b/CD18) and $\alpha_4\beta_1$ integrin (CD49d/CD29) have crucial roles. The approach of using Mac-$1^{-/-}$ mice and blockade of the two integrins by mAbs led to substantially reduced recruitment of neutrophils and T cells and significantly increased pneumococcal loads in lungs and blood compared with control mice. A key finding is that LFA-1 has no role in this response. There was no difference postinfection in either leukocyte counts or pneumococcal loads in lungs or blood in either anti–LFA-1 mAb-treated or LFA-$1^{-/-}$ mice compared with the appropriate control mice.

In terms of T cells, we show that $\alpha_4\beta_1$, but not LFA-1 or Mac-1, is necessary for their recruitment into lungs postinfection, and that these T cells supplement those already present. The integrin $\alpha_4\beta_1$ contributes to T cell accumulation during inflammation of the skin (37), joint (38), pancreas (39), and brain (40, 41), but to our knowledge this is the first study to demonstrate both its essential role in the recruitment of T cells into lungs during pneumococcal bronchopneumonia and the effect that blockade of this integrin has on pneumococcal growth in lungs and blood.

Our findings also establish a dominant role for the CD18 integrin, Mac-1, and a partial role for $\alpha_4\beta_1$ in the recruitment of neutrophils into lungs. The lack of neutrophil recruitment when Mac-1 activity is absent indicates its importance in their exiting from the circulation into the lungs. This is in keeping with the evidence in other inflammatory contexts that Mac-1 is the major receptor used by neutrophils in intraluminal migration within the vasculature and also recruitment into tissue sites (42, 43). For reasons that are unclear, our findings lack agreement with another study using Mac-$1^{-/-}$ mice where increased neutrophil infiltration was observed following *S. pneumoniae* infection rather than decreased as in our study, although the increase in bacterial proliferation is similar (44). A role for Mac-1 is consistent with evidence that neutrophils recruited to the lungs of mice during pneumococcal bronchopneumonia exhibit increased expression of Mac-1 and that mice deficient in the urokinase plasminogen activator receptor, which mediates neutrophil migration by an interaction with Mac-1, have decreased neutrophil infiltration into lungs with accompanying increases in pneumococcal numbers (45–47).

Our data are also in agreement with those of Maus et al. (48), who reported that Ab blockade of either β_2 integrins or α_4 integrin led to a >5-fold reduction in infiltrating neutrophils to mouse lungs challenged with the pneumococcal toxin pneumolysin compared with controls. Other studies have also shown that both purified pneumolysin and whole wild-type pneumococci, as used in this study, lead to increased expression of total β_2 integrin on neutrophil membranes and increased neutrophil adhesion (49–51).

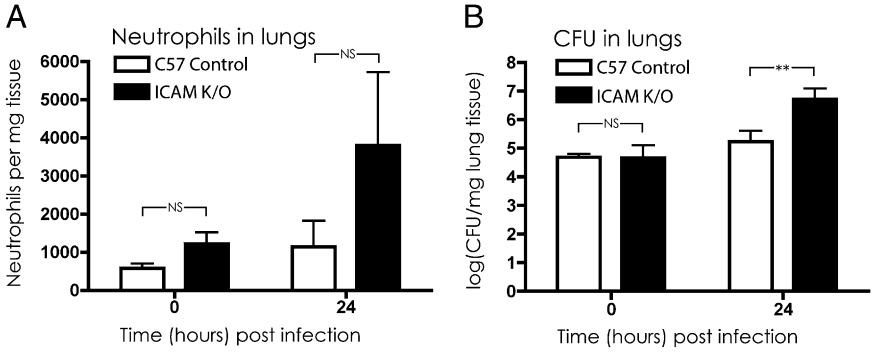

FIGURE 7. Neutrophil recruitment into ICAM-$1^{-/-}$ and wild-type mice following *S. pneumoniae* infection. *A*, Numbers per milliliter of neutrophils in whole lung homogenate at time 0 and 24 h postinfection with D39 pneumococci in ICAM-$1^{-/-}$ mice and wild-type C57BL/6J mice ($n = x$ mice of each strain ± SEM). *B*, Log CFU D39 pneumococci at time 0 and 24 h postinfection in lungs of ICAM-$1^{-/-}$ mice and wild-type C57BL/6J mice ($n = x$ mice of each strain ± SEM). $^{**}p < 0.01$.

Our current findings, however, also contrast with some earlier studies that demonstrated a β_2-independent neutrophil influx into lungs following pneumococcal infection (17, 20, 21). These differences could be because the previous models were conducted either using a rabbit model of peritonitis or a mouse model induced by intratracheal instillation of a very high-dose bolus of pneumococci that leads to lobar pneumonia. In our model we use a 2.5×10^2-fold smaller inoculum of bacteria introduced by respiratory intranasal inhalation, resulting in bronchopneumonia.

It was unexpected that LFA-1 played no part in either neutrophil or T cell recruitment into lungs because this β_2 integrin has a major impact on leukocyte recruitment to other tissues (52, 53). LFA-1 was highly expressed by the infiltrating leukocytes, indicating that lack of expression was not an issue. It is possible that its activity might be selectively masked during the specific circumstances of pneumococcal infection in lungs, but there is evidence both for the use of LFA-1 in pneumococcal infections at sites other than the lungs and, conversely, use of LFA-1 in lung infections mediated by other pathogens. Specifically, the neutrophil response to pneumococcal infection within the peritoneum is both LFA-1– and Mac-1–dependent (54). Neutrophils also make use of LFA-1 during the course of other pulmonary infections, such as with *Mycobacterium tuberculosis*, but these infections involve areas of the lung that may have other adhesion requirements for leukocyte entry (55) and the long-term nature of the inflammatory and disease process caused by *Mycobacterium* is substantially different from the acute nature of pneumococcal pneumonia.

In many tissues, following selectin-mediated rolling, LFA-1 and Mac-1 cooperate in the adhesion of neutrophils to the vasculature, with LFA-1 supporting initial attaching that sets up more sustained adhesion via Mac-1 (22, 42, 56). The integrin $\alpha_4\beta_1$ is also involved in arrest of leukocytes onto vasculature, and for T cells it precedes the firmer attachment supplied by LFA-1 (57). However, we find that it is α_4 integrin and not LFA-1 that is critical for recruitment of both neutrophils and T cells. As the shear flow rate is lower in the pulmonary capillaries where leukocyte extravasation occurs, rather than the postcapillary venules as in other tissues (18, 58), this may negate the role of the early stage adhesion molecules in attaching leukocytes to the vasculature. Consistent with this view, E- and P-selectin are not involved in neutrophil recruitment into the lungs during pneumococcal infection (21). Moreover, neutrophils must deform to pass through the small diameter of the pulmonary capillaries, and this would also aid the close contact necessary for engaging the integrins (58). Our findings indicate that $\alpha_4\beta_1$ is required, presumably as an attaching receptor and potentially a migrating receptor, for T cells for recruitment into the bronchoalveolar compartment. Neutrophils can also make use of $\alpha_4\beta_1$ to attach (59). However, in an inflammatory setting, neutrophils also use Mac-1 to migrate intraluminally on the vasculature and then into tissues (42, 43). It seems probable that, following attaching, they are making use of Mac-1 in a similar manner to gain entry into the lung parenchyma at an appropriate location following *S. pneumoniae* infection.

It is of interest that ICAM-1, a ligand recognized by Mac-1 (and LFA-1), has widespread constitutive expression by extravascular cells within the lung. Investigating its role with the use of ICAM-$1^{-/-}$ mice, we found that neutrophils were recruited into the infected lungs without hindrance with a trend toward larger numbers than wild-type mice. More investigation is needed to identify the ligands that neutrophil Mac-1 engages to gain entry into lung tissue. In the infected or inflamed liver, both monocytes and neutrophils, respectively, make use of CD44 for recruitment into the sinusoids (60, 61). Another candidate is JAM-C that serves as a ligand for neutrophil Mac-1 (62). In inflammation models, overexpression of JAM-C causes neutrophil influx into lungs (63), although influx is unaffected in JAM-$C^{-/-}$ mice given various inflammatory challenges (64). The fact that neutrophils migrated freely within ICAM-$1^{-/-}$ lung tissue was also of interest. This is also in keeping with evidence that leukocytes do not make use of integrins to migrate within shear-free tissue environments, at least in lymph nodes (65).

It was unexpected that the ICAM-$1^{-/-}$ mice were less able to control the proliferation of the *S. pneumoniae*. The complement component iC3b opsonized to bacteria serves as a ligand for Mac-1 in response to *S. pneumoniae* for phagocytosis and bacterial clearance (66). However, adhesive substrates such as ICAM-1 enhance CD18-dependent neutrophil functions such as production of cytokines and reactive oxygen species that aid bacterial clearance (67, 68). Although not yet directly demonstrated, it is probable that a Mac-1/ICAM-1 adhesive step, although not necessary for migration, plays a similar stimulating role in the neutrophil response to *S. pneumoniae*.

In contrast, the $\alpha_4\beta_1$ ligand VCAM-1, but not ICAM-1, is essentially expressed exclusively by the bronchioles in uninfected lungs. Intranasal inoculation of pneumococci, as in this study, results in infection of the bronchioles, making this lung compartment the first site of bacterial proliferation. Thus, rapid access to these bacteria by leukocytes would be an essential step in successfully combating infection. A population of T cells is already present in the uninfected lung, and T cell numbers increase postinfection. These cells predominantly surround the bronchioles and are therefore in position to bind VCAM-1 and access the bronchiolar cavities with their proliferating bacterial load. VCAM-1 becomes expressed on the lung venules within the first few hours of infection, allowing more $\alpha_4\beta_1$-mediated T cell recruitment. In contrast, there are no neutrophils in the uninfected lung, but they arrive within the first few hours following infection. As with the T cells, they also express $\alpha_4\beta_1$ and have a bronchiolar distribution. A point of interest is whether they are able to use Mac-1, in addition to $\alpha_4\beta_1$, to bind to VCAM-1–expressing bronchiolar membranes when migrating across into the bronchiolar lumen. There is some information that Mac-1, similar to its close structural homolog $\alpha_D\beta_2$, can bind VCAM-1 (69).

In summary, we have used a model of pulmonary *S. pneumoniae* infection and have clearly demonstrated, to our knowledge for the first time, the important role of Mac-1 and $\alpha_4\beta_1$ integrins as well as the Mac-1 ligand ICAM-1 in the host response to this infection. First, by controlling neutrophil and T cell recruitment into infected lungs and, second, dictating interactions within the bronchoalveolar compartment within lung tissue, these adhesion molecules play a pivotal two-stage role in the prevention of pneumococcal outgrowth and dissemination from the lungs during disease.

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Disclosures

The authors have no financial conflicts of interest.

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