Ablation of the complement C3a anaphylatoxin receptor causes enhanced killing of *Pseudomonas aeruginosa* in a mouse model of pneumonia

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Mueller-Ortiz, Stacey L., Travis J. Hollmann, David L. Haviland, and Rick A. Wetsel. Ablation of the complement C3a anaphylatoxin receptor causes enhanced killing of *Pseudomonas aeruginosa* in a mouse model of pneumonia. *Am J Physiol Lung Cell Mol Physiol* 291: L157–L165, 2006. First published February 3, 2006; doi:10.1152/ajplung.00358.2005.—The C3a anaphylatoxin is a 77-amino acid peptide that is generated by enzymatic cleavage of C3 during activation of the complement system. C3a mediates numerous biological functions on binding its receptor (C3aR), which is present on both myeloid and nonmyeloid cells. To investigate the biological impact of C3a-mediated effects during acute pneumonia caused by *Pseudomonas aeruginosa*, we subjected C3aR-deficient mice and matched wild-type (WT) mice to *P. aeruginosa* pulmonary infection. C3aR-deficient mice exhibited increased killing of *P. aeruginosa* in the lungs, less dissemination of bacteria into the bloodstream, and a decreased inflammatory response to *P. aeruginosa* pulmonary infection compared with WT mice. To examine whether the absence of C3aR would impact the humoral immune response to *P. aeruginosa*, we immunized WT and C3aR-deficient mice via intraperitoneal injection with live *P. aeruginosa*. Both groups of mice developed similar levels of antibody specific to *P. aeruginosa*. Immunized C3aR-deficient and WT mice were subjected to *P. aeruginosa* pulmonary infection, and C3aR-deficient mice again displayed increased killing of *P. aeruginosa* in the lungs, less dissemination of bacteria into the bloodstream, and a decreased inflammatory response in the lungs. Collectively, these data demonstrate that independently of antibody production, absence of C3aR causes enhanced killing of *P. aeruginosa* despite a diminished inflammatory response in a mouse model of pneumonia.

THE COMPLEMENT SYSTEM is activated by three pathways that are termed classical, alternative, and lectin. All three pathways result in C3 activation (33). A potent C3 activation fragment, C5a, is a 77-amino acid peptide, known for mediating numerous inflammatory activities on binding to its receptor (C5aR). C5aR is a member of the rhodopsin superfamily of seven-transmembrane G protein-coupled receptors (1, 6, 49) and is expressed on both bone marrow–derived myeloid and lymphoid cells, including monocytes/macrophages, dendritic cells, neutrophils, basophils, eosinophils, mast cells, platelets, T lymphocytes, and B lymphocytes (19, 22, 35, 61–63). In addition, C5aR is found on parenchymal cells of the central nervous system (8, 23), lungs (11), and kidney (5).

C3a exerts multiple inflammatory effects on binding to C3aR, including contraction of smooth muscle, vasodilation, increased vascular permeability, and histamine release from mast cells and basophils (reviewed in Ref. 58). In addition, C3a induces respiratory burst in macrophages (44), neutrophils (16, 18), and eosinophils (17) and is also chemotactic for eosinophils (7) and mast cells (27, 45). C3a causes cytokine release from multiple cell types in vitro, including IL-1β (25, 55), TNF-α (55), and IL-6 (20, 56) from mononuclear cells, IL-8 from epithelial cells (39), and IL-8 and IL-1β from endothelial cells (40). In addition to its well-established role as a proinflammatory molecule, C3a also has been shown to have anti-inflammatory properties. For example, C3a suppresses cytokine production in LPS-stimulated nonadherent peripheral blood mononuclear cells in vitro (55, 56), and C3aR-deficient mice are more susceptible to the effects of LPS (34), indicating an anti-inflammatory role for C3a and C3aR in a model of endotoxemia.

*Pseudomonas aeruginosa* is a gram-negative bacillus and is a leading cause of hospital-acquired pneumonia. Pulmonary infection with *P. aeruginosa* is characterized by a strong recruitment of neutrophils. The elaboration of neutrophil enzymes and oxidants, combined with numerous other inflammatory mediators, leads to extensive damage to the lung tissue (38). During the past several years, studies by our laboratory as well as those by others have demonstrated the importance of C3aR in causing acute inflammation in experimental lung disease (2, 12, 32). Despite the flurry of activity devoted to delineating the biological role of C3aR in pulmonary allergy, little attention has been paid to the impact of this receptor or its ligand in infectious lung disease. Accordingly, to investigate the overall significance and biological function of C3aR in the host immune response to *P. aeruginosa* pneumonia, C3aR-deficient mice were subjected to *P. aeruginosa* pulmonary infection.

MATERIALS AND METHODS

Mice. The C3aR-deficient (C3aR−/−) mice used in these studies were generated in our laboratory and have been described previously (34). The C3aR−/− mice were backcrossed 10 generations onto the C57BL/6 background. C57BL/6 mice served as wild-type (WT) controls and were purchased from Jackson Labs (Bal Harbor, ME). Both male and female mice that were age (8–12 wk old) and sex matched were used for these studies. All mouse protocols followed institutional guidelines for animal care and welfare.

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Intranasal infection. P. aeruginosa strain PA103 (37), kindly provided by Barbara Iglewski, University of Rochester Medical Center, was used to induce pneumonia in mice via intranasal inoculation. Bacteria were cultured in tryptic soy broth at 37°C to midlogarithmic phase, harvested by centrifugation, washed twice in sterile, nonpyrogenic 0.9% NaCl, and resuspended in 0.9% NaCl. Mice that had been anesthetized with an intraperitoneal injection of 2.5% tribromoethanol (0.016 ml/g body wt; Sigma-Aldrich, St. Louis, MO) (30) were given 1 x 10⁸ PA103 in a volume of 20 μl intranasally. Control mice received 20 μl of sterile, nonpyrogenic 0.9% NaCl intranasally. The number of bacteria present in the inoculum was verified by culturing serial dilutions of the inoculum on tryptic soy agar (TSA) plates.

Bacterial counts in lungs and blood. C3aR−/− and WT mice that had been infected intranasally with P. aeruginosa were killed either 10 min or 24 h after infection. To determine how much of the initial inoculum was received into the lungs of the mice, three mice per group were infected and then euthanized 10 min later. Either 10 min or 24 h after infection, lungs were collected and placed in sterile 0.9% NaCl on ice. The lungs were homogenized in 2 ml of sterile 0.9% NaCl by pressing the lungs through 100-μm cell strainers (BD Biosciences, San Diego, CA). Serial 10-fold dilutions of the lung homogenates were plated on TSA plates to determine the number of colony-forming units (CFU). Before the lungs were harvested at 24 h postinfection, the mice were terminally bled through syringes containing 50 units of heparin sulfate (Sigma-Aldrich), and 25 and 50 μl of blood from each mouse was plated on TSA plates. Data are expressed as CFU per total lungs (±SE) or CFU per ml of blood (±SE).

Lung histology. Lungs were perfused with 0.3 ml of 10% buffered formalin 24 h after infection, and the trachea was tied off with suture material. The lungs were removed from the mice and placed in 10% buffered formalin overnight at 4°C. The lungs were dehydrated with increasing concentrations of ethanol, embedded in paraffin, cut into 5-μm sections, and stained with hematoxylin and eosin. The stained sections were examined under light microscopy to assess the level of inflammation.

Quantitation of inflammatory cells in the lungs. The types and numbers of inflammatory cells present in the lungs 24 h after infection were assessed by bronchoalveolar lavage (BAL). The lungs were lavaged three times with 0.5 ml of PBS, and the lavage fluid was placed on ice. The total number of cells present in the lavage fluid was determined using a hemacytometer. BAL fluid was centrifuged onto slides with a cytospin centrifuge (Shandon Lipshaw, Pittsburgh, PA), and the slides were stained with Diff-Quik stain (Fisher Scientific, Pittsburgh, PA). Two hundred cells per slide were classified by cell type, neutrophil (PMN), macrophage, or lymphocyte, under light microscopy with the aid of characteristic morphology. Absolute numbers of specific cell types were calculated from the recovered BAL volume, total cell count, and percent abundance of specific cells.

Adherence/phagocytosis and LPS binding. The ability of macrophages from C3aR−/− and WT mice to bind/phagocytose P. aeruginosa was assessed in vitro. Resident peritoneal macrophages were harvested from uninfected WT and C3aR−/− mice by injecting 5 ml of cold HBSS (BioWhittaker, Rockland, ME) into the peritoneal cavity of mice that were anesthetized with 2.5% isoflurane. The abdominal cavity was gently massaged, and the fluid was withdrawn with a syringe and stored on ice. The cells were centrifuged at 500 g for 10 min at 4°C and were resuspended in DMEM (VWR, West Chester, PA). The total number of cells harvested from the mice was determined by counting cells on a hemacytometer. In the presence of 10% serum from either WT or C3aR−/− mice and 250 nM human C3a (Advanced Research Technologies, San Diego, CA), 1 x 10⁴ macrophages were incubated with 1 x 10⁷ P. aeruginosa PA103 that constitutively expressed green fluorescent protein (GFP) for 1 h at 37°C with gentle shaking (100 rpm). The cells were centrifuged at 500 g for 10 min at 4°C, washed one time with PBS, resuspended in PBS, and kept on ice for flow cytometry analysis. Flow cytometry using a FACSCalibur (BD Biosciences) was employed to quantify adherence/phagocytosis of bacteria by analyzing cells positive for GFP. The forward scatter threshold was set to exclude free bacteria from the analysis. Macrophages incubated with non-GFP P. aeruginosa PA103 were used as negative controls, and at least 10,000 cells in all samples were analyzed. This experiment was performed two times in triplicate with similar findings in each experiment. Similarly, LPS binding capacity was determined in C3aR−/− and WT macrophages. Briefly, 1 x 10⁶ macrophages were incubated with 40 μg/ml FITC-LPS (Sigma) for 1 h at 37°C (100 rpm). The cells were washed three times and analyzed using flow cytometry.

Reactive oxygen species production. Production of reactive oxygen species (ROS) by phagocytes from C3aR−/− and WT mice 24 h after P. aeruginosa pulmonary infection was measured using dihydrorhodamine 123 (DHR; Molecular Probes, Eugene, OR). BAL fluid was collected from infected and control mice as described previously and was centrifuged at 200 g for 8 min to pellet the cells. Red blood cells were lysed with ACK lysing buffer (BioWhittaker), and the cells were washed one time with 1% BSA in PBS. The cells were resuspended in 1% BSA in PBS, and 5 x 10⁵ cells from each mouse sample were placed in individual tubes. The total volume in each tube was brought up to 0.4 ml using 1% BSA in PBS. Phorbol myristic acetate (PMA; Sigma-Aldrich) at a final concentration of 10 ng/ml was added to the positive control tubes. A final concentration of 100 μM DHR was added to each tube, except for the negative control tube, which received no DHR. All tubes were incubated at 37°C with gentle shaking for 20 min. The cells were then washed with PBS and resuspended in 0.3 ml of PBS. DHR is freely permeable across most cell membranes, where it becomes oxidized to rhodamine 123 and localizes in the mitochondria. Once DHR is oxidized to rhodamine 123, it emits a bright fluorescent signal upon excitation at 488 nm (59). Fluorescence intensities were measured using a FACSCalibur flow cytometer (BD Biosciences), and at least 10,000 cells in all samples were analyzed. The cells that received no DHR served as the negative controls, and the cells that received PMA served as positive controls.

Cell surface expression of CD11b and CD14. The cell surface expression levels of CD11b and CD14 were quantitated on leukocytes obtained from BAL fluid from mice 24 h postinfection. BAL fluid was collected as described previously and was centrifuged to pellet the cells. The red blood cells were lysed using ACK lysing buffer (BioWhittaker), and then the cells were washed one time with 1% BSA in PBS. The cells were resuspended in 1% BSA in PBS, and total cell counts were determined using a hemacytometer. An equal number of cells (3 x 10⁷) from both the WT and C3aR−/− mice were aliquoted into the appropriate number of tubes for cell surface staining. The cells were incubated with FITC-labeled rat anti-mouse CD11b (CALTAG Laboratories, Burlingame, CA), FITC-labeled rat anti-mouse CD14 (eBioscience, San Diego, CA), PE-labeled rat anti-mouse Gr-1 (Ly-6G; CALTAG Laboratories), or a combination of anti-CD11b and anti-Gr-1 or anti-CD14 and anti-Gr-1. The cells were incubated with a total of 1 μg of each antibody, including the appropriate isotype control antibodies, for 45 min on ice. The cells were then washed one time with PBS and were fixed in 1% paraformaldehyde in PBS. Basal expression levels of CD11b, CD14, and Gr-1 were also measured from untreated WT and C3aR−/− mouse peripheral blood in a similar manner. Fluorescence intensities were measured using a FACSCalibur flow cytometer, and at least 10,000 cells in all samples were analyzed.

Cytokine and chemokine analysis. The levels of the cytokines IL-1β, IL-6, and TNF-α and the chemokines monocyte chemotactic protein-1 (MCP-1)/JE and KC were examined in mice 24 h postinfection. BAL fluid was collected as described previously and was centrifuged to remove cells. The BAL fluid supernatants were then assayed for the presence of these cytokines and chemokines by performing ELISA (R&D Systems, Minneapolis, MN) per the manufacturer’s instructions. IL-6 and TNF-α levels were determined.
similarly from supernatants of cultured peritoneal macrophages isolated from C3aR−/− and WT animals. Macrophages were stimulated for 24 h with LPS (10 ng/ml), LPS (10 ng/ml) with C3a (1.000 nM; Advanced Research Technologies), or LPS (10 ng/ml) with C3adesArg (1.000 nM; Advanced Research Technologies).

Measurement of vascular permeability. Vascular permeability changes indicated using Evans blue dye were measured in WT and C3aR−/− mice 24 h postinfection. Twenty hours postinfection, 200 µl of 1% Evans blue dye (made in PBS and sterile-filtered; Sigma-Aldrich) was given to each mouse via an intraperitoneal injection (26).

Four hours after injection of the dye, the mice were terminally bled using EDTA as an anticoagulant, and BAL was performed. The blood was centrifuged at 510 g for 15 min to obtain plasma, and the BAL fluid was centrifuged at 510 g for 8 min to pellet cells. The optical density at 600 nm (OD600) of the supernatant fraction of the BAL fluid was measured. The plasma samples were diluted as follows: 300 µl of plasma plus 700 µl of PBS. The OD600 values of the diluted plasma samples were measured. The permeability index is defined as the ratio of the OD600 of the BAL fluid to the OD600 of the plasma (31).

Immunization and challenge of mice. Using a protocol similar to that described in (48), we immunized C3aR−/− and WT mice via an intraperitoneal injection once a week for 3 wk with 2 × 107 live P. aeruginosa PA103 to elicit the production of antibodies specific to P. aeruginosa. Two weeks after the final immunization, C3aR−/− and WT mice were challenged intranasally with 1 × 105 P. aeruginosa PA103 organisms as described previously.

Measurement of antibodies specific to P. aeruginosa. Ten milliliters of an overnight culture of P. aeruginosa PA103 were centrifuged, washed once with PBS, and resuspended in 10.5 ml of PBS. All 96 wells of a 96-well plate were coated with 100 µl of P. aeruginosa, and the plate was incubated overnight at 4°C. The next day, the wells were washed three times with 0.05% Tween 20 in PBS and were blocked for 1 h at room temperature with 1% BSA in PBS. Serum samples that had been diluted 1:500, 1:1,000, and 1:5,000 in 1% BSA in PBS were added to the wells and were incubated for 2 h at room temperature. Control wells received only 1% BSA in PBS. The wells were washed as above and incubated for 1 h in either alkaline phosphatase-conjugated goat anti-mouse IgG diluted 1:1,000 (Sigma-Aldrich), alkaline phosphatase-conjugated goat anti-mouse IgM diluted 1:5,000 (Sigma-Aldrich), or alkaline phosphatase-conjugated goat anti-mouse IgG2a diluted 1:100 (Southern Biotech). The wells were washed as above. Detection was performed using a p-nitrophenyl phosphate liquid substrate system (Sigma-Aldrich), and development was stopped using 2 M NaOH. The absorbance was read at 405 nm on a plate reader.

Statistical analyses. Comparisons between C3aR−/− and WT mice were assessed with GraphPad Prism (San Diego, CA) software, using the unpaired two-tailed Student’s t-test, with P values <0.05 considered significant. Data are expressed as means ± SE.

RESULTS

C3aR−/− mice have fewer bacteria in lungs and blood. To determine whether absence of the C3aR hinders the ability of C3aR−/− mice to control a pulmonary infection with P. aeruginosa, WT and C3aR−/− mice were infected intranasally with P. aeruginosa. Twenty-four hours after infection, lungs were removed from the mice, homogenized, and plated on TSA plates. Blood was also drawn from the mice 24 h postinfection and plated on TSA plates. Both groups of mice received an equal initial dose of bacteria into the lungs, as assessed from lungs removed from the mice 10 min after infection (data not shown). Lungs from the infected WT mice contained 2.8 times more P. aeruginosa than lungs from infected C3aR−/− mice 24 h after infection (P < 0.05) (Fig. 1A). The WT mice also had 4 times more P. aeruginosa present in the bloodstream 24 h after infection than the C3aR−/− mice (P < 0.05) (Fig. 1B).

C3aR−/− mice display a decrease in pulmonary inflammatory infiltrates after infection. To determine whether the decreased number of bacteria in the lungs of the C3aR−/− mice was due to an elevated pulmonary cellular response, lungs were removed from both groups of mice for histology 24 h after challenge with either saline or P. aeruginosa. Lung sections from WT and C3aR−/− mice challenged with only saline revealed normal lung architecture when stained with hematoxylin and eosin (Fig. 2, top). In contrast, lung sections from WT and C3aR−/− mice that had been infected with P. aeruginosa from the lungs and dissemination into the bloodstream after intranasal infection. Wild-type (WT) and C3a receptor-deficient (C3aR−/−) mice were infected intranasally with P. aeruginosa, and 24 h after infection, the lungs were removed and homogenized, and serial dilutions of the homogenates were plated on agar plates (A). Blood was drawn from the mice using heparin as an anticoagulant 24 h after infection and was plated on agar plates (B). Data in A are expressed as mean colony-forming units (CFU) per total lungs (±SE), and 12–14 mice per group were used. Data in B are expressed as mean CFU per ml of blood (±SE), and 7–10 mice per group were used. Significant differences between WT and C3aR−/− mice are indicated *(P < 0.05).

Fig. 1. Clearance of Pseudomonas aeruginosa from the lungs and dissemination into the bloodstream after intranasal infection. Wild-type (WT) and C3a receptor-deficient (C3aR−/−) mice were infected intranasally with P. aeruginosa, and 24 h after infection, the lungs were removed and homogenized, and serial dilutions of the homogenates were plated on agar plates (A). Blood was drawn from the mice using heparin as an anticoagulant 24 h after infection and was plated on agar plates (B). Data in A are expressed as mean colony-forming units (CFU) per total lungs (±SE), and 12–14 mice per group were used. Data in B are expressed as mean CFU per ml of blood (±SE), and 7–10 mice per group were used. Significant differences between WT and C3aR−/− mice are indicated *(P < 0.05).
**aeruginosa** revealed the presence of a large number of inflammatory infiltrates, with an apparent increase in inflammatory cells in the lung tissue of the WT mice compared with the C3aR−/− mice (Fig. 2, bottom). Inflammatory cells were quantified in BAL fluid taken from the mice 24 h postinfection. C3aR−/− and WT mice treated with saline had similar numbers of total cells in the BAL fluid that were predominantly macrophages (Table 1). After infection with **P. aeruginosa**, C3aR−/− mice had 48% fewer total leukocytes and polymorphonuclear (PMN) cells than WT mice (P < 0.05) (Table 1), which is in agreement with the histological data.

**Phagocytes from C3aR−/− and WT mice exhibit similar adherence/phagocytosis of P. aeruginosa and generate similar levels of ROS.** In vitro uptake studies were conducted using a strain of **P. aeruginosa** expressing GFP. Peritoneal macrophages (1 × 10⁶) from uninfected WT or C3aR−/− mice were incubated with 1 × 10⁷ **P. aeruginosa** organisms in the presence of 250 nM C3a and 10% serum from WT or C3aR−/− mice for 1 h at 37°C. As determined by flow cytometry, macrophages isolated from both groups of mice incubated with GFP-expressing **P. aeruginosa** had similar numbers of bacteria associated with them (data not shown), indicating that C3aR−/− and WT macrophages do not differ in adherence/phagocytosis of **P. aeruginosa**. In support of these data, macrophages from C3aR−/− and WT mice showed no difference in LPS binding capacity (data not shown). To examine whether macrophages and neutrophils from C3aR−/− mice are more activated and therefore able to kill **P. aeruginosa** more efficiently compared with phagocytes from WT mice, macrophages and neutrophils that were isolated from the BAL fluid of infected WT and C3aR−/− mice 24 h after infection with **P. aeruginosa** were examined for production of ROS. DHR has been demonstrated to be a good indicator for production of the respiratory burst in phagocytes by flow cytometry analysis (59). Equal numbers of phagocytes from the BAL fluid of infected WT and C3aR−/− mice were incubated with DHR and were shown to produce similar amounts of ROS 24 h after infection (Fig. 3, A and B). In addition, phagocytes from the BAL fluid of saline-treated C3aR−/− and WT mice were activated in vitro with PMA and produced comparable amounts of ROS in response to PMA stimulation (data not shown), indicating that phagocytes from C3aR−/− and WT mice do not differ in ROS production upon activation. These data demonstrate that phagocytes from WT and C3aR−/− mice were equally activated in response to **P. aeruginosa** pulmonary infection.

C3aR−/− mice produce lower levels of cytokines and chemokines after infection. To further evaluate the inflammatory response in the C3aR−/− and WT mice, levels of the proinflammatory cytokines were measured by ELISA in BAL fluid 24 h after infection with **P. aeruginosa**. Levels of IL-1β, IL-6, and TNF-α were significantly lower in C3aR−/− mice compared with WT mice (data not shown). In addition, C3aR−/− mice had decreased levels of reactive oxygen species (ROS) and NO compared with WT mice (data not shown). These data suggest that C3aR−/− mice have a more attenuated inflammatory response to **P. aeruginosa** infection.

**Table 1. Leukocyte counts and differentials in BAL fluid 24 h after pulmonary infection with Pseudomonas aeruginosa**

<table>
<thead>
<tr>
<th>Mouse Genotype</th>
<th>Treatment</th>
<th>Total Leukocyte Count, × 10⁶ cells</th>
<th>PMNs, × 10⁶ cells</th>
<th>Macrophages, × 10⁶ cells</th>
<th>Lymphocytes, × 10⁶ cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>Saline</td>
<td>0.18 ± 0.04</td>
<td>0</td>
<td>0.18 ± 0.04</td>
<td>0</td>
</tr>
<tr>
<td>C3aR−/−</td>
<td>Saline</td>
<td>0.15 ± 0.04</td>
<td>0</td>
<td>0.15 ± 0.04</td>
<td>0</td>
</tr>
<tr>
<td>WT</td>
<td><strong>P. aeruginosa</strong></td>
<td>25.70 ± 3.64</td>
<td>24.33 ± 3.52</td>
<td>1.23 ± 0.16</td>
<td>0.14 ± 0.03</td>
</tr>
<tr>
<td>C3aR−/−</td>
<td><strong>P. aeruginosa</strong></td>
<td>13.81 ± 2.83*</td>
<td>12.80 ± 2.74*</td>
<td>0.95 ± 0.12</td>
<td>0.07 ± 0.02</td>
</tr>
</tbody>
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Bronchoalveolar lavage (BAL) was performed on the mice 24 h after intranasal challenge with either saline or **P. aeruginosa**. Absolute numbers of specific cell types were calculated from the recovered BAL volume, total cell count, and percent abundance of specific cells. Results are expressed as means ± SE. Three mice per group were used for saline treatment. Twelve mice per group were used for **Pseudomonas** infection. Significant differences between WT and C3aR−/− mice infected with **P. aeruginosa** are indicated (*P < 0.05).
Inflammatory cytokines IL-1β, IL-6, and TNF-α were measured from BAL fluid taken from the mice 24 h postinfection. Compared with WT mice, C3aR<sup>-/-</sup> mice had 53% lower production of IL-1β (<i>P</i> < 0.05), 63% lower production of TNF-α (<i>P</i> < 0.05), and 64% lower production of IL-6 (<i>P</i> < 0.05) (Fig. 4, A and B). In vitro stimulation of C3aR<sup>-/-</sup> peritoneal macrophages with LPS alone or LPS with C3a yielded near identical levels of TNF-α and IL-6 compared with stimulated cells from WT animals (data not shown). This finding suggests that the reduced cytokine levels in the BAL of C3aR<sup>-/-</sup> mice did not result from impaired or altered LPS-mediated cytokine production by C3aR<sup>-/-</sup> macrophages. To determine whether the WT mice had greater production of chemokines compared with the C3aR<sup>-/-</sup> mice after infection, levels of MCP-1/JE and KC were measured in BAL fluid taken 24 h after infection with <i>P. aeruginosa</i>. The mouse chemokine MCP-1/JE is chemotactic for monocytes, macrophages, and lymphocytes (3, 24), and the mouse chemokine KC is chemotactic for neutrophils (10, 57). Compared with WT mice, C3aR<sup>-/-</sup> mice had 60% lower production of MCP-1/JE (<i>P</i> < 0.05) and 69% lower production of KC (<i>P</i> < 0.0001) (Fig. 4, A and B). These data support the results shown earlier in Fig. 2 and Table 1 and provide a possible mechanism for the decreased cellular pulmonary infiltration in the C3aR<sup>-/-</sup> mice after <i>P. aeruginosa</i> pulmonary infection.

C3aR<sup>-/-</sup> mice have decreased vascular permeability in lungs after pulmonary infection. WT mice had more neutrophils in the lungs compared with the C3aR<sup>-/-</sup> mice after <i>P. aeruginosa</i>-induced pneumonia. This finding suggests that the WT mice may have increased vascular permeability, which is a feature of acute inflammation (15), in their lungs compared with the C3aR<sup>-/-</sup> mice. To test this possibility, 20 h after intranasal challenge with either saline or <i>P. aeruginosa</i>, C3aR<sup>-/-</sup> and WT mice were injected intraperitoneally with...
Evans blue dye as a marker of vascular permeability. Four hours after the Evans blue dye injection, vascular permeability in the lungs was assessed from BAL fluid obtained from both groups of mice. Saline-treated C3aR−/− and WT mice had a similar low level of vascular permeability (Fig. 5); however, WT mice treated with P. aeruginosa had nearly 50% more vascular permeability than infected C3aR−/− mice (P < 0.05) (Fig. 5).

**Effects of prior immunization on killing of P. aeruginosa and production of an inflammatory response in C3aR−/− and WT mice.** As demonstrated in mice and rats, antibodies to P. aeruginosa antigens protect against P. aeruginosa infection (9, 14, 21, 29, 46–48, 53), and in vitro studies have suggested that C3a significantly suppresses the humoral immune response (19, 41–43). To determine whether the absence of C3aR impairs the antibody response to P. aeruginosa, C3aR−/− and WT mice were immunized intraperitoneally once a week for 3 wk with live P. aeruginosa. Two weeks after the final immunization, sera from C3aR−/− and WT mice contained similar levels of IgM, IgG, IgG1, and IgG2a specific to P. aeruginosa PA103 (Fig. 6). These data indicate that the absence of C3aR does not significantly impact the humoral antibody response in mice when immunized intraperitoneally with P. aeruginosa.

Two weeks after the final immunization with live P. aeruginosa, C3aR−/− and WT mice were challenged intranasally as before with P. aeruginosa. As in the unimmunized mouse studies (see Fig. 1), immunized and challenged C3aR−/− mice had fewer bacteria in the lungs and less dissemination of bacteria into the bloodstream compared with immunized and challenged WT mice 24 h after infection (data not shown). In addition, immunized and challenged C3aR−/− mice had significantly fewer total leukocytes in the BAL fluid (Fig. 7) and significantly lower levels of proinflammatory cytokines and chemokines in the BAL fluid (data not shown) compared with immunized and challenged WT mice. Together, these results indicate that the enhanced killing of P. aeruginosa and the diminished inflammatory response observed in the C3aR−/− mice compared with the WT mice occurs independently of specific antibody production to P. aeruginosa.

**DISCUSSION**

This investigation is the first to document the role of C3aR in the host’s response to P. aeruginosa pulmonary infection and the first to demonstrate the importance of C3aR in mounting a proinflammatory response during a gram-negative bacterial infection. C3aR−/− mice had a decreased inflammatory response [diminished recruitment of neutrophils into the lungs (see Table 1), lower production of proinflammatory cytokines and chemokines (see Fig. 4), and reduced vascular permeability (see Fig. 5)] compared with WT mice after infection with P. aeruginosa. C3a has been shown to increase vascular permeability (58) and to induce activation of NF-κB and activating protein-1 (AP-1), which are important transcription factors for the production of several cytokines and chemokines, including IL-6, IL-1β, TNF-α, and IL-8 (20). Because C3a does not chemotact neutrophils in vitro (16, 18), the diminished recruitment of neutrophils to the lungs in the C3aR−/− mice may be due at least in part to decreased production of the chemokine KC, which is a powerful chemotactic molecule for neutrophils (10, 57). In addition, P. aeruginosa has been shown to be chemotactic for neutrophils in vitro (54, 60). Therefore, fewer P. aeruginosa organisms in the lungs of the C3aR−/− mice also may contribute to the diminished recruitment of neutrophils in these mice compared with WT mice.

Despite having fewer neutrophils present in the lungs, the C3aR−/− mice exhibited increased killing of P. aeruginosa in the lungs and less dissemination of bacteria into the bloodstream (see Fig. 1). Our adherence/phagocytosis and ROS experiments indicated that the enhanced killing of P. aeruginosa observed in the C3aR−/− mice was not due to increased phagocytosis or neutrophil activation by C3aR−/− phagocytes compared with WT phagocytes. CD14 and CD11b are markers for neutrophil activation and also have been shown to mediate uptake of P. aeruginosa by phagocytes (28). Phagocytes taken from the lungs of C3aR−/− and WT mice 24 h after infection with P. aeruginosa had similar cell surface expression of CD14 and CD11b (data not shown), which further supports our phagocytosis and neutrophil activation data.

In accord with our C3aR data, other molecules that promote inflammation during acute P. aeruginosa pneumonia also have been found to impair clearance of P. aeruginosa from the lungs. For example, mice deficient in IL-18 (52), migration inhibitory factor (4), the type 1 IL-1 receptor (51), or the IFN-γ receptor (50) all exhibit enhanced killing of P. aeruginosa in the lungs, despite a reduced inflammatory response, compared with WT control mice. Although these studies as well as ours yield similar supporting findings, to date no mechanism has been delineated in any investigation that would explain why a reduced inflammatory response would lead to increased killing of P. aeruginosa in the lungs.

**Fig. 5.** Vascular permeability changes as measured by Evans blue dye in BAL fluid and plasma 24 h after infection with P. aeruginosa. Twenty hours after intranasal challenge with either saline or P. aeruginosa, WT and C3aR−/− mice were given 200 μl of 1% Evans blue dye intraperitoneally. Four hours later, the mice were bled by using EDTA as an anticoagulant, and BAL was performed. The permeability index is defined as the ratio of the optical density at 600 nm (OD600) of the BAL fluid to the OD600 of the plasma. Three mice per group were used for saline treatment, and 5–6 mice per group were used for P. aeruginosa infection. Data are expressed as means ± SE. Significant difference between WT and C3aR−/− mice is indicated (*P < 0.05).
As stated previously, in vitro experiments have suggested that C3a significantly suppresses the humoral immune response (19, 41–43). Therefore, we wanted to evaluate the role of C3aR in the development of an antibody response specific to P. aeruginosa. C3aR−/− and WT mice that were immunized intraperitoneally with P. aeruginosa once a week for 3 wk had similar levels of P. aeruginosa-specific IgM, IgG, IgG1, and IgG2a in their sera 2 wk after the final immunization (see Fig. 6), indicating that C3aR does not significantly impact the antibody response to P. aeruginosa in this model. Presence of antibody to P. aeruginosa did aid in the clearance of this organism from the lungs of immunized C3aR−/− and WT mice after intranasal challenge with P. aeruginosa, because immunized and challenged C3aR−/− and WT mice had fewer bacteria in their lungs and bloodstream than did unimmunized C3aR−/− and WT mice (data not shown). However, immunized and challenged C3aR−/− mice still had fewer P. aeruginosa present in the lungs after pulmonary infection and less dissemination of bacteria into the bloodstream compared with immunized and challenged WT mice 24 h after infection.

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demonstrating that the enhanced killing of P. aeruginosa in the C3aR/−/− mice was not dependent on antibody.

Several studies using animal models of pulmonary allergy have indicated opposing roles for the C3aR and the C5aR in the allergic response in the lungs. Absence of the C3aR leads to decreased bronchoconstriction and airway hyperresponsiveness in allergen-challenged mice (12, 32) and guinea pigs (2) as well as reduced infiltration of eosinophils and neutrophils into the lungs and attenuated pulmonary Th2 responses (12). However, absence of C5a or the C5aR, through either C5 deficiency (13) or pharmacological blockade of the C5aR (36), leads to enhanced Th2 responses and increased airway hyperresponsiveness (13, 36) as well as increased infiltration of eosinophils into the lungs (13).

The studies we have presented in this article also demonstrate opposite roles for the C3aR and the C5aR in the host pulmonary immune response to P. aeruginosa pneumonia. In stark contrast to our findings in the C3aR/−/− mice, C5aR/−/− mice have increased numbers of bacteria in their lungs, display a heavy infiltration of neutrophils into their lungs, have increased vascular permeability, and have greater mortality than their WT littersmates after P. aeruginosa pulmonary infection (31). In P. aeruginosa pneumonia, the C5aR promotes killing of P. aeruginosa in the lungs and functions to temper the host pulmonary inflammatory response to this organism. In contrast, the C3aR hinders killing of P. aeruginosa in the lungs and mediates a proinflammatory response to P. aeruginosa.

In summary, independently of antibody production, the C3aR impairs killing of P. aeruginosa in the lungs during pulmonary infection, and the C5aR is important in mounting a proinflammatory response to P. aeruginosa in the lungs. These data support a possible therapeutic potential in neutralizing the C3aR during treatment of P. aeruginosa pneumonia, particularly those strains of P. aeruginosa that are resistant to multiple antibiotics, to aid in killing of this pathogen.

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