Adenosine A_{2B} Receptor Deficiency Promotes Host Defenses against Gram-Negative Bacterial Pneumonia

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***Rationale*: Activation of the adenosine A_{2B} receptor ($A_{2B}R$) promotes antiinflammatory effects in diverse biological settings, but the role of this receptor in antimicrobial host defense in the lung has not been established. Gram-negative bacillary pneumonia is a common and serious illness associated with high morbidity and mortality, the treatment of which is complicated by increasing rates of antibiotic resistance.**

***Objectives*: To test the hypothesis that absence of adenosine A_{2B} receptor signaling promotes host defense against bacterial pneumonia.**

***Methods*: We used a model of *Klebsiella pneumoniae* pneumonia in wild-type mice and mice with targeted deletion of the $A_{2B}R$. Host responses were compared *in vivo* and leukocyte responses to the bacteria were examined *in vitro*.**

***Measurements and Main Results*: $A_{2B}R^{-/-}$ mice demonstrated enhanced bacterial clearance from the lung and improved survival after infection with *K. pneumoniae* compared with wild-type controls, an effect that was mediated by bone marrow–derived cells. Leukocyte recruitment to the lungs and expression of inflammatory cytokines did not differ between $A_{2B}R^{-/-}$ and wild-type mice, but $A_{2B}R^{-/-}$ neutrophils exhibited sixfold greater bactericidal activity and enhanced production of neutrophil extracellular traps compared with wild-type neutrophils when incubated with *K. pneumoniae*. Consistent with this finding, bronchoalveolar lavage fluid from $A_{2B}R^{-/-}$ mice with *Klebsiella* pneumonia contained more extracellular DNA compared with wild-type mice with pneumonia.**

***Conclusions*: These data suggest that the absence of $A_{2B}R$ signaling enhances antimicrobial activity in gram-negative bacterial pneumonia.**

Keywords: neutrophil; extracellular traps; pneumonia; adenosine

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

New therapies are needed to combat lethal lung infections by multidrug-resistant strains of gram-negative bacteria. Activation of the adenosine A_{2B} receptor has been shown to promote antiinflammatory effects during lung injury. The role of adenosine A_{2B} receptor signaling in the setting of pneumonia is not known.

What This Study Adds to the Field

We report that adenosine A_{2B} receptor deficiency improves survival and enhances bacterial clearance from the lung in a mouse model of *Klebsiella* pneumonia. This effect was in part attributable to enhanced neutrophil extracellular trap (NET) production and extracellular killing of bacteria by A_{2B} receptor–deficient neutrophils. Modulation of the A_{2B} receptor may provide a novel therapeutic target for drug-resistant bacterial pneumonia.

Pneumonia is a leading cause of hospitalization in the United States and is the most common infectious cause of death (1, 2). Aerobic gram-negative bacilli are the most common cause of health care–associated pneumonia; mortality rates from gram-negative pneumonia range from 30 to 60% with antimicrobial therapy (3, 4). The emergence of multiresistant strains of gram-negative bacteria, combined with limited development of new antimicrobial therapies, has exacerbated the need for new approaches to combating these pathogens (5–7). Therapy aimed at augmenting the host response has the potential to enhance bacterial clearance and improve outcomes, and could provide a new avenue for therapeutic advances in combating gram-negative pneumonia, including infections that are caused by antibiotic-resistant pathogens.

Adenosine, a breakdown product of ATP, is a potent signaling molecule released from a variety of cells to dampen inflammation, limit tissue destruction, and promote repair (8, 9). In response to cellular stress or tissue injury, the extracellular concentration of adenosine can increase 100-fold. Adenosine acts on four widely expressed G protein–coupled receptors: A_1, A_{2A}, A_{2B}, and A_3, with variable expression among different cells. The adenosine A_{2B} receptor ($A_{2B}R$) has been shown to enhance the production of IL-6 by endothelial cells, epithelial cells, and macrophages (10, 11). On the other hand, $A_{2B}R$ activation attenuates inflammation by effects on endothelial adhesion molecule expression and reduced macrophage tumor necrosis factor-α production. Activation of the $A_{2B}R$ on endothelial cells also enhances barrier function, which limits leukocyte recruitment, rolling, and transmigration and decreases vascular leak and edema formation (12–14). Activation of $A_{2B}R$s on certain macrophages inhibits their proliferation, MHC class II expression, and inducible nitric oxide synthase production (15, 16). In addition, dendritic cell immunogenicity and neutrophil oxidative burst are inhibited by $A_{2B}R$ agonist binding (17, 18). Although the ability of adenosine to limit tissue inflammatory damage is beneficial, it also inhibits the host response to infection and may indirectly impair bacterial clearance and recovery from infection.

Because the preponderance of evidence suggests that $A_{2B}R$ activation acutely inhibits inflammation, we posited that $A_{2B}R$ antagonism could enhance the innate immune response to invading pathogens and may represent a novel mode of antimicrobial

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therapy. Thus, we tested the hypothesis that the deletion of $A_{2B}R$ signaling promotes host defenses against bacterial pneumonia.

METHODS

Animals, *In Vivo* Procedures, and Tissue Harvest

C57BL/6 mice and homozygous mice with β-galactosidase (β-Gal) knockin at the $A_{2B}R$ locus on a C57BL/6 background (12) were maintained under specific pathogen-free conditions and in compliance with institutional animal care regulations. Experimental *Klebsiella pneumoniae* pneumonia and generation of bone marrow–chimeric mice were performed as described (19). *In vivo* neutrophil depletion was induced by intraperitoneal administration of 400 μg of monoclonal antibody (mAb) against Ly6G (clone 1A8; Bio X Cell, West Lebanon, NH) or isotype control mAb (clone 2A3; Bio X Cell) as described (20). Methods for tissue harvest (21), bronchoalveolar lavage, and isolation of bone marrow neutrophils (22) and histology (19, 21) have been described previously.

Bacterial Content, Cytokine Assays, Quantitative RT-PCR, and Flow Cytometry

Sample bacterial content was determined by serial dilution and culture as previously described (19). Samples were processed for cytokine analysis as described (19, 21, 23) and cytokine levels were measured with commercial ELISA kits (DuoSet ELISA development; R&D Systems, Minneapolis, MN) or multiplex bead array kits (MILLIPLEX map; Millipore, Billerica, MA) according to the manufacturer's instructions. For quantitative RT-PCR of the $A_{2B}R$ gene, RNA was isolated from sorted cells, cDNA was generated, and quantitative real-time reverse transcriptase PCR was performed as previously described (24). Cell suspensions for flow cytometric analysis and sorting were prepared as described (19, 21) according to a published gating strategy (25), as detailed in the online supplement.

Ex Vivo Assays

To opsonize bacteria, 2×10^9 bacteria were incubated in 5 ml of freshly collected mouse serum for 30 minutes at 37°C, washed with sterile water, and resuspended in Hanks' balanced salt solution (HBSS). For all coculture experiments, 4×10^5 leukocytes and an equal number of bacteria in 200 μl of HBSS were placed in round-bottom 96-well plates (Falcon), centrifuged to achieve cell contact, and incubated at 37°C. Neutrophil and macrophage bactericidal assays were performed according to a modification of a previously described protocol (19); cells were incubated for 3 hours. In some experiments, gentamicin was added to the wells after 1 hour (1 μg/ml for 10 min at 37°C) to kill extracellular bacteria, and cells were then washed twice with HBSS and incubated for an additional 2 hours to allow intracellular killing of bacteria. In other experiments, bactericidal assays were performed in the presence of DNase (final concentration, 10 U/ml; Sigma-Aldrich, St. Louis, MO). After the incubation steps, cells were centrifuged and resuspended in 200 μl of sterile H_2O to lyse neutrophils, and surviving bacteria were quantified by serial dilution and culture. Phagocytic capacity, measured as the proportion of leukocytes that contain fluorescently labeled bacteria after quenching of extracellular fluorescence, was determined as described previously (23). Oxidative burst of bone marrow neutrophils was determined by preincubation with 1 μM dihydrorhodamine 123 (Invitrogen, Grand Island, NY) in HBSS as described (26). Indicated cell stimulants were applied and resultant reactive oxygen species (ROS) production was detected with a fluorescence plate reader. Neutrophil extracellular traps were quantified as described (27) after 3 hours of coculture of neutrophils and bacteria or in bronchoalveolar lavage fluid after cells were pelleted.

RESULTS

The $A_{2B}R$ Is Highly Expressed in the Setting of Gram-Negative Pneumonia

Previous studies have demonstrated that, in the uninfected lung, the $A_{2B}R$ is highly expressed by type II alveolar epithelial cells with relatively less expression by alveolar macrophages (24). Because the A_{2B} receptor is also expressed on several leukocyte subsets (28), we sought to determine impact of bacterial pneumonia on the cellular distribution $A_{2B}R$ mRNA in the lungs. In cells sorted from lungs of mice with *Klebsiella* pneumonia, we found that neutrophils and recruited macrophages demonstrated nine- and threefold greater expression of $A_{2B}R$ mRNA as compared with nonleukocyte lung cells, indicating that neutrophils are the predominant population of cells expressing $A_{2B}R$ transcript in the infected lung (Figure 1). Interestingly, expression of $A_{2B}R$ mRNA was more than 30-fold greater in neutrophils isolated from infected lungs as compared with resting bone marrow neutrophils, suggesting that neutrophil expression of the $A_{2B}R$ is enhanced during infection.

Deletion of the $A_{2B}R$ on Bone Marrow–derived Cells Results in Improved Outcome of Gram-Negative Bacterial Pneumonia

Given the high expression of the $A_{2B}R$ in infected lungs and its previously described antiinflammatory effects, we next compared the outcome of *Klebsiella* pneumonia in mice lacking the $A_{2B}R$ with wild-type controls. We found that $A_{2B}R^{-/-}$ mice with bacterial pneumonia have a survival advantage over wild-type controls (Figure 2A). Because most deaths from the infection occurred beyond Day 4, we limited subsequent studies to the first 3 days of infection to avoid survival bias. Quantification of bacterial burden on Day 3 of infection revealed lungs of wild-type animals to contain 29-fold more viable bacteria as compared with $A_{2B}R^{-/-}$ animals; the degree of bacteremia in wild-type animals similarly exceeded that of $A_{2B}R$-deficient animals (Figure 2B). Lung histology revealed similar degrees of consolidation with intraalveolar neutrophils in wild-type and $A_{2B}R$-deficient animals; bacteria were visible in wild-type but not $A_{2B}R$-deficient lungs (Figure 2C). Because the $A_{2B}R$ signaling has been linked to reduced extent of lung injury in several animal models, including in response to LPS (14, 29), we also assessed the extent of lung injury

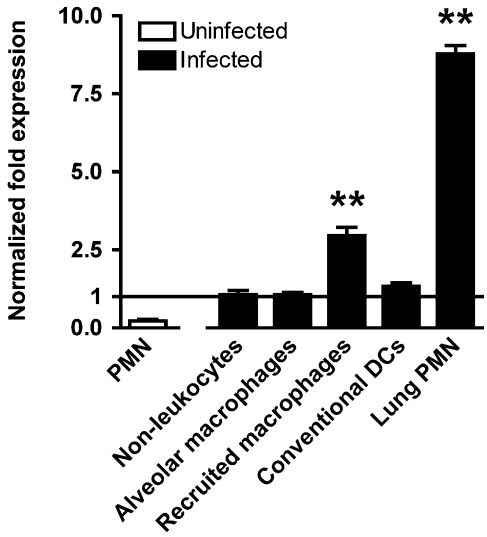

Figure 1. Adenosine A_{2B} receptor is highly expressed in the lungs in bacterial pneumonia. Shown is relative adenosine A_{2B} receptor mRNA expression in cells sorted from lungs on Day 3 of pneumonia. Expression is normalized to that of nonleukocyte cells in the lung. Data shown represent means ± SEM of triplicates representative of two experiments; $^{**}P < 0.001$ for recruited macrophages and lung polymorphonuclear neutrophils (PMN) compared with other groups (one-way analysis of variance). DCs = dendritic cells.

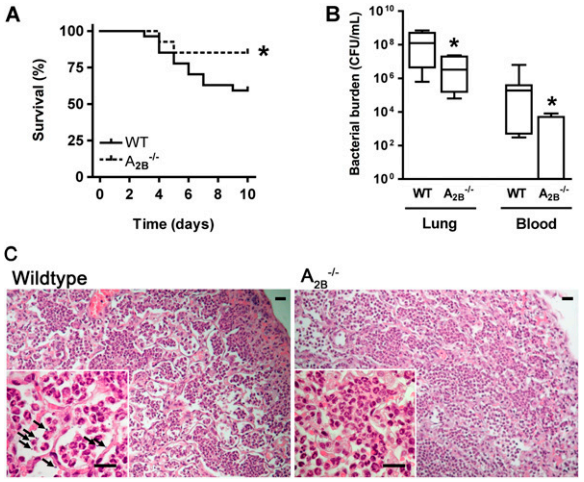

Figure 2. Absence of the adenosine A_{2B} receptor is protective in *Klebsiella* pneumonia. (*A*) Survival of wild-type and $A_{2B}^{-/-}$ mice after pulmonary infection with *K. pneumoniae* (n = 29 or 30 mice per group in two independent experiments), $^*P < 0.05$ compared with wild-type mice (log-rank test). (*B*) Number of bacteria recovered from lungs and blood of *Klebsiella*-infected wild-type and $A_{2B}^{-/-}$ mice on Day 3 postinfection (n = 6 or 7 mice per group); $^*P < 0.05$ compared with wild-type mice (Mann-Whitney). (*C*) Representative lung hematoxylin and eosin stains 3 days after onset of infection show consolidation of lung parenchyma; high-power micrographs (*insets*) show intraalveolar cells to be polymorphonuclear cells (n = 3 mice per group). *Arrows* point to visible bacteria. All *scale bars* represent a length of 20 μm. Original magnification: (*A*–C) ×200; *insets,* ×1,000. WT = wild type.

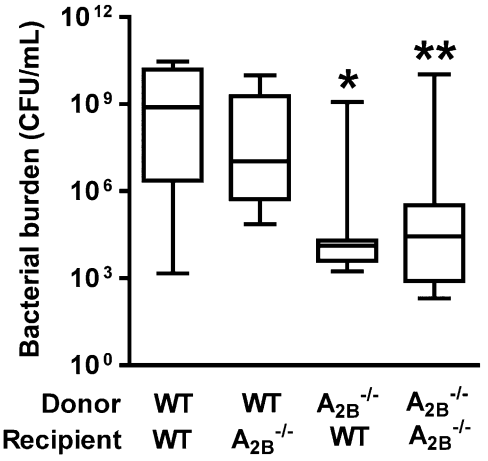

Figure 3. Absence of adenosine A_{2B} receptor on bone marrow–derived cells mediates protection against *Klebsiella* pneumonia. Shown is the number of bacteria recovered from the lungs of bone marrow–transplanted mice on Day 3 postinfection (n = 9–12 mice per group). Lethally irradiated recipient mice (wild-type or $A_{2B}^{-/-}$) received either wild-type or $A_{2B}^{-/-}$ donor marrow for reconstitution, resulting in the four indicated groups; $^*P < 0.05$ for $A_{2B}^{-/-}$ donor/wild-type recipient versus wild-type donor/wild-type recipient; $^{**}P < 0.01$ for $A_{2B}^{-/-}$ donor/$A_{2B}^{-/-}$ recipient versus wild-type donor/wild-type recipient (one-way analysis of variance).

in wild-type and $A_{2B}R^{-/-}$ mice with bacterial pneumonia; we found $A_{2B}R$-deficient mice with pneumonia to have reduced lung injury as compared with wild-type animals, a finding that we attribute to improved bacterial clearance (*see* Figure E2 in the online supplement).

We reasoned that because the $A_{2B}R$ is expressed by both leukocyte and nonleukocyte populations in the infected lungs, the lack of expression on one or both of these populations may explain the protection noted in $A_{2B}R$-deficient animals. To address this issue, we examined the outcome of infection in reciprocal bone marrow–chimeric animals. Consistent with prior findings, $A_{2B}R$-deficient recipients of $A_{2B}R$-deficient bone marrow exhibited significantly lower lung bacterial burden as compared with wild-type recipients of wild-type bone marrow. Furthermore, wild-type recipients of $A_{2B}R$-deficient bone marrow demonstrated a comparable degree of protection as globally $A_{2B}R$-deficient animals; conversely, both groups of recipients of wild-type bone marrow exhibited less effective bacterial clearance (Figure 3). These data suggest that deletion of the $A_{2B}R$ on bone marrow–derived cells mediates improved antimicrobial defense against *Klebsiella* pneumonia.

Inflammatory Cell Recruitment and Cytokine Levels Do Not Differ between Wild-Type and $A_{2B}R^{-/-}$ Mice

Given that bone marrow–derived cells are important for decreasing bacterial burden in infected $A_{2B}R^{-/-}$ mouse lungs, we reasoned that $A_{2B}R^{-/-}$ mice might exhibit enhanced cellular recruitment to the lung after infection. However, we found no significant difference between wild-type and $A_{2B}R^{-/-}$ mice in the absolute number of neutrophils, alveolar macrophages, recruited macrophages, conventional $CD11b^+$ dendritic cells, activated $CD4^+$ T cells, or activated $CD8^+$ T cells (Figures 4A–4F) recruited to the lung after infection. In addition, there was no significant difference in recruitment of invariant NKT cells or NK cells (*see* Figures E3A and E3B) to the lung between the two groups over time. Given data that $A_{2B}R$ signaling can inhibit leukocyte activation (17, 30), we examined the activation phenotype of lung neutrophils, macrophages, and dendritic cells and found no significant difference between wild-type and $A_{2B}R^{-/-}$ mice with *Klebsiella* pneumonia (Figures E3C–E3H). Similarly, although $A_{2B}R$ signaling has been linked to enhanced production of certain inflammatory cytokines (31, 32), we found no difference between levels of these mediators in lungs of wild-type and $A_{2B}R$-deficient mice with *Klebsiella* pneumonia (Figure E4). These data suggest that leukocyte recruitment to the lung during early infection is not significantly different between wild-type and $A_{2B}R^{-/-}$ mice.

$A_{2B}R$-Deficient Neutrophils Demonstrate Enhanced Extracellular Bactericidal Activity

We next assessed whether functional differences between wild-type and $A_{2B}R^{-/-}$ leukocytes recruited to the lung might explain the difference in outcome in response to infection. We focused on neutrophils because of their known role in the clearance of bacteria during pneumonia and because of the high expression level of $A_{2B}R$ mRNA on neutrophils in the setting of pneumonia (Figure 1). Because $A_{2B}R$ gene expression appeared to be much higher in lung neutrophils during pneumonia as compared with resting bone marrow neutrophils (Figure 1), we began by evaluating the expression of $A_{2B}R$ in resting neutrophils after exposure to bacteria *in vitro*. As compared with freshly isolated cells and neutrophils incubated *ex vivo* in the absence of microorganisms, neutrophils incubated with bacteria had approximately 500-fold induction of $A_{2B}R$ mRNA (Figure 5A). We next assessed the contribution of $A_{2B}R$ to neutrophil bactericidal activity. Mature neutrophils obtained from the bone marrow of uninfected $A_{2B}R$-deficient donors were found to have greatly enhanced ability to kill bacteria when compared with wild-type neutrophils, with more than 80-fold greater bactericidal activity against nonopsonized *K. pneumoniae* and more

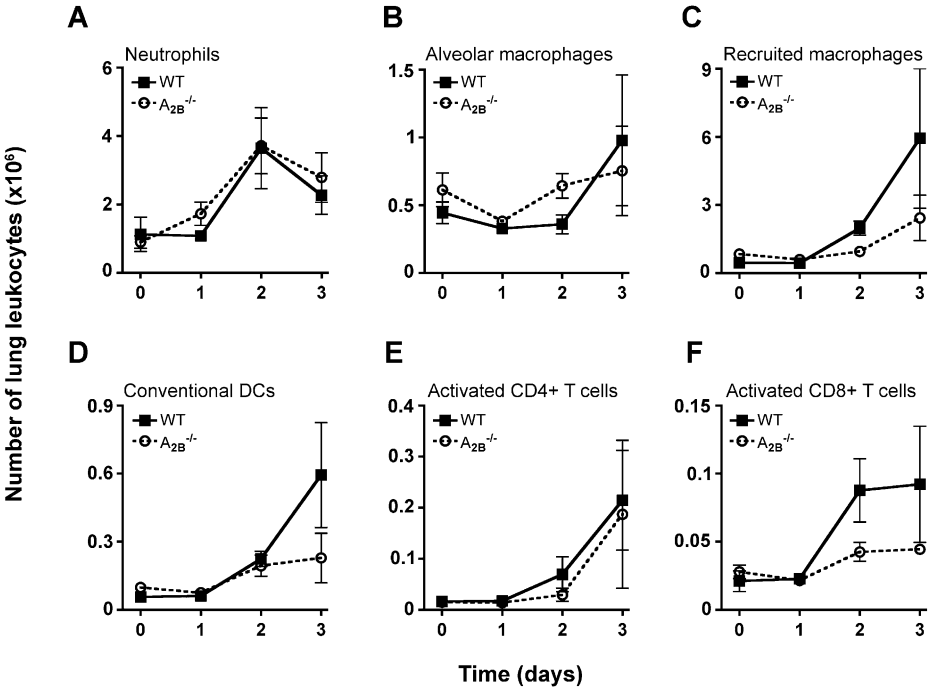

Figure 4. Leukocyte recruitment to infected lungs does not differ between wild-type and $A_{2B}^{-/-}$ mice. The absolute number of lung (*A*) neutrophils, (*B*) alveolar macrophages, (*C*) recruited macrophages, (*D*) conventional dendritic cells, (*E*) activated $CD4^+$ T cells, and (*F*) activated $CD8^+$ T cells recruited to wild-type and $A_{2B}^{-/-}$ lungs on the indicated days postinfection, was quantified by flow cytometry. Data shown represent means ± SEM of triplicates, representative of two experiments (each with n = 4–6 mice per group per time point); no significant difference between groups was found (two-way analysis of variance). DCs = dendritic cells; WT = wild type.

than 600-fold against serum-opsonized bacteria, a condition that likely mimics *in vivo* conditions more closely (Figure 5B).

To determine the mechanism of enhanced bacterial killing by A_{2B}R-deficient neutrophils, we next examined the phagocytic capacity and intracellular bactericidal activity of bone marrow–derived neutrophils from wild-type and $A_{2B}R^{-/-}$ mice. We found no difference in phagocytosis of bacteria between wild-type and A_{2B}R-deficient neutrophils (Figure 5C). In addition, the intracellular killing of bacteria did not differ significantly between the two groups (Figure 5D). Taken together with evidence of greatly enhanced killing in Figure 5A, these data suggest that $A_{2B}R^{-/-}$ neutrophils have greater bactericidal activity against extracellular, but not intracellular, bacteria as compared with wild-type neutrophils.

A major mechanism of neutrophil extracellular antimicrobial activity is the production of chromatin-based extracellular traps (NETs) (33). We quantified NET production by wild-type and $A_{2B}R^{-/-}$ neutrophils *ex vivo*, and found that $A_{2B}R^{-/-}$ neutrophils produce fourfold more NETs at baseline, 2.5-fold more NETs in response to unopsonized *Klebsiella*, and 2.3-fold more NETs in response to opsonized *Klebsiella* than wild-type control neutrophils (Figure 5E). Because oxidative burst production is an essential antimicrobial function of neutrophils and can occur in the extracellular space, we also compared neutrophil oxidative burst between wild-type and $A_{2B}R^{-/-}$ neutrophils, and found no difference in ROS production in response to phorbol myristate acetate or bacteria (Figure 5F). These data suggest the enhanced extracellular bacterial killing observed in A_{2B}R-deficient neutrophils may be the result of enhanced NET production.

Next, we sought to assess the relevance of enhanced NET production to improved host defenses in A_{2B}R-deficient hosts to bacterial pneumonia. We found that, when resting bone marrow neutrophils from wild-type and $A_{2B}R^{-/-}$ mice were incubated with bacteria *ex vivo*, degradation of extracellular DNA resulted in impaired bacterial killing; furthermore, the difference in bactericidal activity between wild-type and A_{2B}R-deficient neutrophils was abrogated (Figure 6A). We then assessed whether the *ex vivo* finding of enhanced NET production in $A_{2B}R^{-/-}$ neutrophils is operational *in vivo*. To achieve this, we quantified extracellular DNA in the alveolar spaces of mice with bacterial pneumonia, reasoning that neutrophils are the predominant cells in this compartment during infection. We found threefold more extracellular DNA in the bronchoalveolar lavage fluid of $A_{2B}R^{-/-}$ mice with pneumonia as compared with wild-type controls (Figure 6B). This difference was not attributable to differences in the total number of neutrophils in the bronchoalveolar lavage fluid of animals with pneumonia, or to DNA release from dead cells, because the number of apoptotic and necrotic neutrophils did not differ between $A_{2B}R^{-/-}$ and wild-type mice (Figure 6C). Finally, we assessed the bronchoalveolar lavage fluid of wild-type and $A_{2B}R^{-/-}$ mice with pneumonia by immunofluorescence microscopy, and found the extracellular DNA to consist of strands of DNA associated with neutrophil elastase (Figure 6D), the typical appearance of NETs (34). These data suggest that the improved bactericidal clearance in A_{2B}R-deficient mice with bacterial pneumonia is, at least in part, attributable to enhanced NET production and extracellular killing of bacteria by A_{2B}R-deficient neutrophils.

Last, we sought to determine whether enhanced neutrophil bactericidal activity is sufficient to explain the improved outcome of A_{2B}R-deficient hosts as compared with wild-type hosts. To address this point, we rendered wild-type and $A_{2B}R^{-/-}$ animals neutropenic before intratracheal challenge with *K. pneumoniae*. As expected, both groups of animals had a 2- to 3-log greater lung bacterial content when rendered neutropenic; in addition, we found that, even in the absence of neutrophils, A_{2B}R deficiency resulted in improved bacterial clearance from the lungs (Figure 7A). Similar to findings in previous studies (35), we found that lung macrophages from A_{2B}R-deficient hosts displayed better bacterial killing as compared with wild-type macrophages (Figure 7B). Taken together, our data suggest that the improved host defense of A_{2B}R-deficient hosts is mediated by bone marrow–derived leukocytes that include, but are not limited to, enhanced neutrophil bactericidal mechanisms.

DISCUSSION

The present work identifies the A_{2B}R as a regulator of host defense in gram-negative bacterial pneumonia. We report that A_{2B}R deficiency improves survival and enhances bacterial clearance in a mouse model of *Klebsiella* pneumonia and that

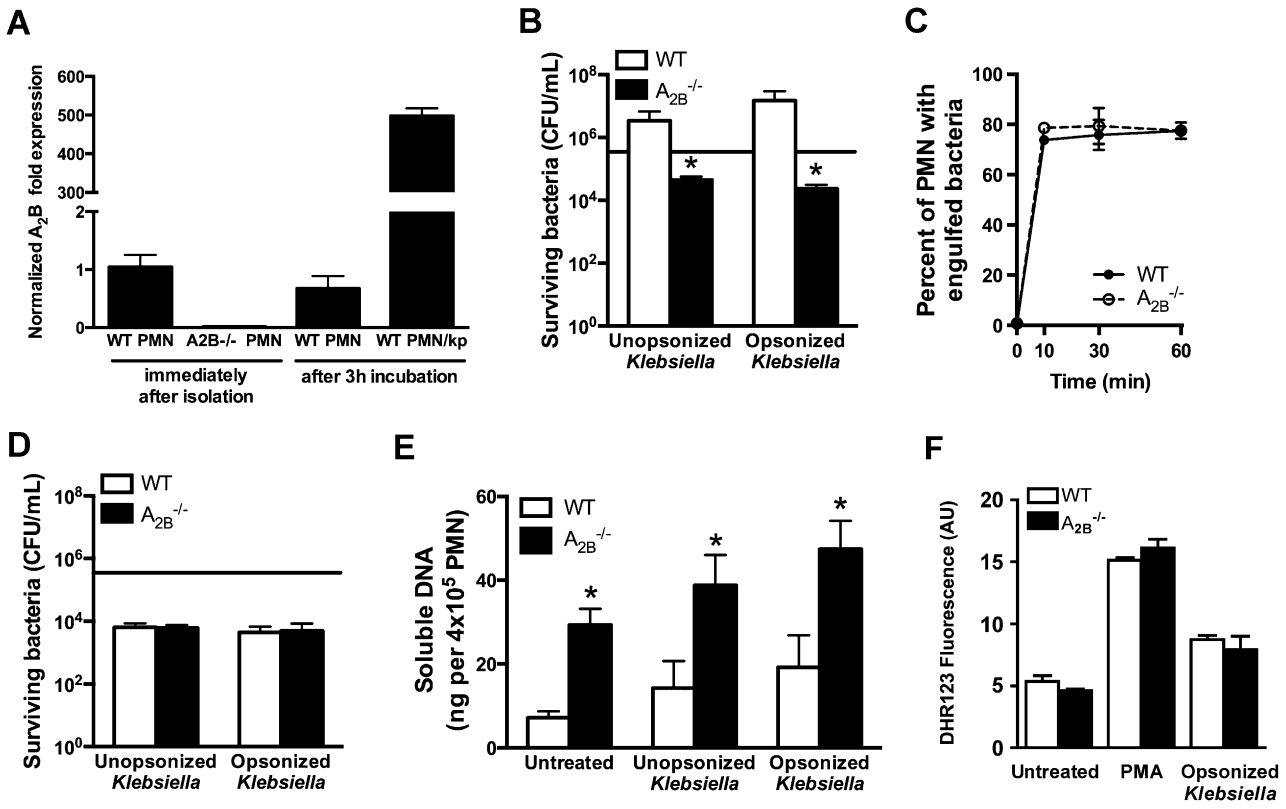

Figure 5. *Ex vivo* antibacterial function of wild-type and $A_{2B}^{-/-}$ neutrophils. *Ex vivo* assays were done to compare wild-type and $A_{2B}^{-/-}$ bone marrow neutrophils. (*A*) Relative expression of $A_{2B}R$ mRNA in resting bone marrow neutrophils after exposure to bacteria; (*B*) combined (extracellular and intracellular) bactericidal activity, $^*P < 0.01$ (Mann-Whitney); (*C*) phagocytic capacity; (*D*) intracellular bactericidal activity; (*E*) neutrophil extracellular trap (NET) production, $^*P < 0.05$ (one-way analysis of variance); (*F*) oxidative burst. Data for all panels represent means ± SEM of triplicates or quadruplicates representative of two to four experiments for each panel. *Horizontal line* in (*B*) and (*D*) represents initial bacterial load per well. PMA = phorbol myristate acetate; PMN = polymorphonuclear neutrophils; Kp = *Klebsiella pneumoniae*; WT = wild type.

this effect is mediated by bone marrow–derived cells. The absence of the $A_{2B}R$ on leukocytes did not influence leukocyte recruitment or expression of inflammatory cytokines, but was associated with altered antibacterial effects of myeloid cells, including enhanced NET production and bactericidal activity.

The extracellular concentration of adenosine, normally in the nanomolar range, can increase to the micromolar range during inflammation as a result of accelerated turnover of ATP. Because the affinity of the $A_{2B}R$ for adenosine is 50- to 100-fold lower than the high-affinity states of A_1, A_{2A}, and A_3 receptors, signaling via the A_{2B} receptor may be selectively activated in the setting of inflammation (8, 9). The role of $A_{2B}R$ in disease pathogenesis is highly complex, being determined by rates of local production and clearance of adenosine, the extent of expression of $A_{2B}R$ by resident and recruited cells, and the repertoire of these cells for the other adenosine receptors, which compete with $A_{2B}R$ for adenosine. $A_{2B}R$ signaling has been reported to be broadly antiinflammatory in several models of acute lung injury; in particular, $A_{2B}R$ deficiency is associated with increased lung inflammation after challenge with bleomycin, LPS, ischemia–reperfusion, and ventilator-associated lung injury (29, 36–38). The reported mechanisms of these effects have varied markedly between these models and have included alteration of leukocyte traffic, reduction in vascular permeability mediated by bone marrow–derived cells, and attenuation of the neutrophil oxidative burst (14, 37, 38); on the other hand, work has shown $A_{2B}R$ expression on bone marrow–derived cells to paradoxically enhance neutrophil migration to the lung interstitium in response to LPS (39). We speculate that the reported differences in the mechanisms of $A_{2B}R$ effects in these models reflect differences in dynamics of production and degradation of adenosine, and differences in the number of adenosine-responsive cells as well as their adenosine receptor repertoire in the context of different injuries. Because bacterial pneumonia is an important cause of acute lung injury in the clinical setting, the present study expands current knowledge by providing evidence of a regulatory role for $A_{2B}R$ signaling that impairs the host responses to pneumonia. In particular, our observations and the prior reports in the literature are consistent with the paradigm that some of the purinogenic mechanisms that are beneficial in noninfectious lung injury inhibit host inflammation in response to tissue injury and are harmful in the context of active infection.

Whereas the role of adenosine in dampening inflammation has been an area of active research, the relevance of adenosine signaling to host defense against infection has received less attention. Studies of adenosine receptor signaling in animal models of intraperitoneal sepsis have reported contradictory findings: $A_{2B}R$ deletion was found to increase expression of inflammatory cytokines and worsened survival (40); conversely, $A_{2B}R$ blockade, global deletion, or selective deletion from myeloid cells was associated with accelerated bacterial clearance and improved outcome (35). Because unchecked inflammation and failure to control infection can both lead to death, minor differences in experimental protocols might influence the outcome of such experiments. These differences may relate to the size of the infectious inoculum that determines the relative contribution of bacterial growth on the one hand, and the systemic acute phase

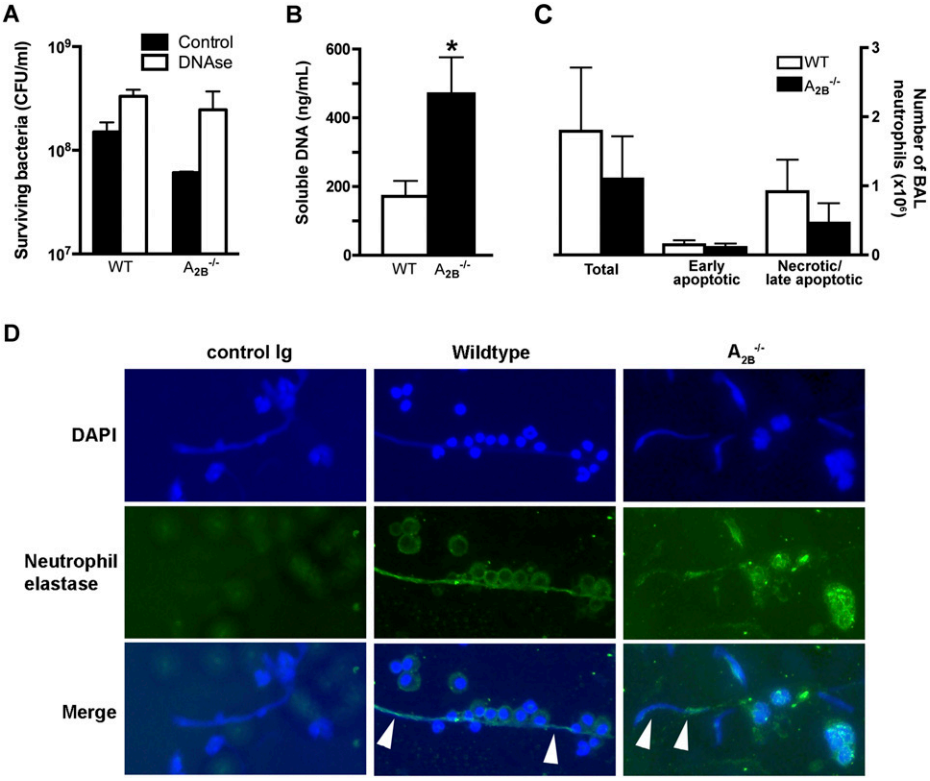

Figure 6. Neutrophil extracellular trap (NET) production in wild-type and $A_{2B}^{-/-}$ mice. (*A*) Role of NETs in bacterial killing *ex vivo*. Resting bone marrow neutrophils from uninfected animals were incubated with bacteria and surviving bacteria were quantified. (*B*) Extracellular DNA content in bronchoalveolar lavage of wild-type and $A_{2B}^{-/-}$ mice on Day 3 postinfection, $^*P < 0.05$ (Mann-Whitney). (*C*) Total live and apoptotic count of harvested bronchoalveolar lavage neutrophils. Data represent means ± SEM (n = 9 mice per group). Data shown in (*A–C*) represent means ± SEM for triplicates or quadruplicates from n = 4–10 mice per group. (*D*) Immunofluorescence microscopy of bronchoalveolar lavage on Day 3 of infection. *Arrowheads* show NETs, identified as strands of extracellular DNA that contain neutrophil elastase. Original magnifications: *left and right panels*, ×200; *middle panels*, ×100. BAL = bronchoalveolar lavage; DAPI = 4′,6-diamidino-2-phenylindole; WT = wild type.

response on the other, to mortality in sepsis models. To our knowledge, ours is the first study of the role of adenosine signaling in pneumonia.

Several lines of evidence support a biologically important effect of adenosine signaling on neutrophil functions, as reviewed (18). One study reported inhibition of neutrophil oxidative burst with an $A_{2B}R$ agonist (41). We did not observe an increase in oxidative burst in $A_{2B}R$-deficient neutrophils; our results may represent cross-talk between neutrophil adenosine receptors, with maintenance of $A_{2A}R$ signaling in $A_{2B}R^{-/-}$ neutrophils, because $A_{2A}R$ activation strongly inhibits oxidative burst (9, 18). Our study also relates to work demonstrating that $A_{2B}R$-deficient macrophages exhibit enhanced phagocytosis and bacterial clearance

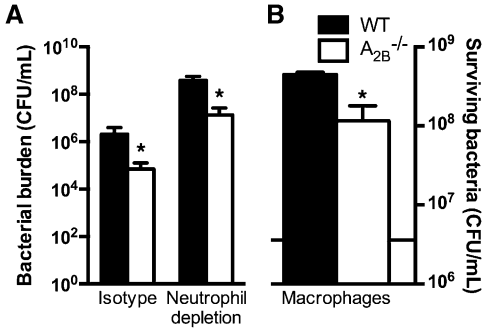

Figure 7. Contribution of nonneutrophil leukocytes to host defense in wild-type and $A_{2B}^{-/-}$ mice. (*A*) Number of bacteria recovered from lungs of *Klebsiella*-infected wild-type and $A_{2B}^{-/-}$ mice after administration of isotype control or neutrophil-depleting monoclonal antibody on Day 3 after infection (n = 8–10 mice per group); $^*P < 0.05$ compared with wild-type mice (Mann-Whitney). (*B*) *Ex vivo* bactericidal activity of alveolar macrophages harvested from wild-type and $A_{2B}^{-/-}$ mice against *K. pneumoniae*; *horizontal line* in (*B*) represents initial bacterial load per well. $^*P < 0.05$ compared with wild-type mice (Mann-Whitney).

in a model of sepsis (35). This study concluded that $A_{2B}R^{-/-}$ neutrophils did not demonstrate enhanced antibacterial activity, but only phagocytic capacity as a measure of bactericidal activity. Our data are in accordance with these findings; we found that $A_{2B}R$-deficient neutrophils exhibit no significant difference in phagocytic capacity, but demonstrate enhanced extracellular killing of bacteria that is not affected by phagocytosis.

NETs are composed of chromatin and antimicrobial cytoplasmic and granular proteins such as elastase and catalase. Exuded NETs form a fibrillar matrix that entrap invading pathogens and bring them into the proximity of antimicrobial proteins, thus facilitating killing (34). Our data suggest a mechanism by which the production of NETs can be modulated by adenosine. The mechanism of NET production is not yet fully defined; it has been suggested that neutrophil populations are heterogeneous in terms of their ability to produce NETs (42). It is possible that $A_{2B}R$ deficiency induces the development of a larger fraction of neutrophils that are able to undergo NETosis; alternatively, adenosine may modulate the amount of NET exuded per neutrophil.

Our work has several implications for future research. First, the signaling mechanisms that lead to NET production are only beginning to be defined; the signaling that links $A_{2B}R$ activation to modulation of NET formation is therefore of interest. Second, a number of gram-negative bacilli including *Klebsiella* possess virulence factors that inhibit neutrophil phagocytosis (43, 44); augmenting extracellular killing by enhancing NETosis via modulation of the $A_{2B}R$ has the potential to overcome these phagocytosis-resistant variants. Finally, a number of $A_{2B}R$ agonists and antagonists are under development as pharmaceutical agents; the present work lays the groundwork for use of these agents as a novel therapeutic option in pneumonia caused by multidrug-resistant gram-negative bacteria.

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