Activating A1 adenosine receptor signaling boosts early pulmonary neutrophil recruitment in aged mice in response to *Streptococcus pneumoniae* infection

Shaunna R. Simmons, Sydney E. Herring, Essi Y.I Tchalla, Alexsandra P. Lenhard, Manmeet Bhalla, and Elsa N. Bou Ghanem*

Department of Microbiology and Immunology, School of Medicine, University at Buffalo, Buffalo, NY, USA

Running title: A1 receptor signaling recruits PMNs to the lungs

Key words: extracellular adenosine, pneumococcus, PMNs, A1R, pneumonia, migration, inflammation, immunosenescence, lung

* Corresponding author

E-mail: Elsaboug@buffalo.edu

ABSTRACT

Background: *Streptococcus pneumoniae* (pneumococcus) is a leading cause of pneumonia in older adults. Successful control of pneumococci requires robust pulmonary neutrophil influx early in infection. However, aging is associated with aberrant neutrophil recruitment and the mechanisms behind that are not understood. Here we explored how neutrophil recruitment following pneumococcal infection changes with age and the host pathways regulating this.

Results: Following pneumococcal infection there was a significant delay in early neutrophil recruitment to the lungs of aged mice. Neutrophils from aged mice showed defects in trans-endothelial migration *in vitro* compared to young controls. To understand the pathways involved, we examined immune modulatory extracellular adenosine (EAD) signaling, that is activated upon cellular damage. Signaling through the lower affinity A2A and A2B adenosine receptors had no effect on neutrophil recruitment to infected lungs. In contrast, inhibition of the high affinity A1 receptor in young mice blunted neutrophil recruitment to the lungs following infection. A1 receptor inhibition decreased expression of CXCR2 on circulating neutrophils, which is required for trans-endothelial migration. Indeed, A1 receptor signaling on neutrophils was required for their ability to migrate across endothelial cells in response to infection. Aging was not associated with defects in EAD production or receptor expression on neutrophils. However, agonism of A1 receptor in aged mice rescued the early defect in neutrophil migration to the lungs and improved control of bacterial burden.

Conclusions: This study suggests age-driven defects in EAD damage signaling can be targeted to rescue the delay in pulmonary neutrophil migration in response to bacterial pneumonia.

47 BACKGROUND

48 *Streptococcus pneumoniae* (pneumococcus) are encapsulated, Gram positive bacteria that
49 are responsible for an estimated 1.6 million deaths each year (1). *S. pneumoniae* remain the leading
50 cause of community acquired bacterial pneumonia in individuals over the age of 65 (2-4). Disease
51 and mortality caused by these bacteria persist despite available vaccines and antibiotics (3, 5, 6).
52 Older adults are at an increased risk of infection and serious disease from pneumococcal
53 pneumonia due to progressive deleterious molecular, cellular and systemic changes termed the
54 "Hallmarks of Aging" (7, 8), that can affect the ability of the host to mount an efficient and
55 balanced immune response. These hallmarks include aberrant genetic changes, altered metabolic
56 responses, cellular senescence and chronic inflammation(7, 8). Cellular senescence affects several
57 cell types including immune cells, causing them to be less efficient at pathogen clearance (7, 8).
58 Cellular senescence is also associated with increased production of pro-inflammatory cytokines
59 leading to an increase in basal inflammation known as inflammaging (7, 8). Increased
60 inflammation with age contributes to a reduction in the ability of immune cells to respond to acute
61 stimuli, further dysregulating the immune response (7-9). A better understanding of how immune
62 cell responses change with host aging upon pneumococcal infection, and the signaling pathways
63 that control these responses, is necessary to promote better protection against disease.

64 An important mediator of host protection following pneumococcal pneumonia is the
65 polymorphonuclear leukocyte (PMNs) response. PMNs are the first cell to be recruited to the lungs
66 following pneumococcal infection (10, 11) and this response is necessary for host protection.
67 Individuals with neutropenia are more susceptible to pneumonia and experimental depletion of
68 PMNs early in infection result in reduced ability to control bacterial numbers and accelerated death
69 in murine models of infection (12, 13). Upon pulmonary infection, PMNs are recruited from the

70 circulation into the lung tissue where they kill bacteria via several antimicrobial effector functions,
71 such as release of preformed antimicrobial granules, reactive oxygen species, phagocytosis, and
72 neutrophil extracellular traps (14). However, an effective PMN response in the pulmonary
73 environment must be balanced, where PMNs need to be recruited in quickly to clear bacteria and
74 then resolve. An exacerbated PMN response without resolution is damaging to host tissue due to
75 the antimicrobial products produced by PMNs, and without proper resolution PMNs contribute to
76 lung damage which worsens disease outcomes (12, 15). There is evidence that PMN influx to the
77 lungs later in infection is dysregulated with age as biopsy samples from older adults show an
78 increase in the percentage of PMNs in the lungs compared to younger patients following
79 pneumococcal pneumonia, and this can be recapitulated in mouse models (16, 17). However, how
80 neutrophil recruitment changes with age early in infection, when PMNs are beneficial to the host
81 in clearing the pathogen, and the pathways controlling PMN influx remain unexplored.

82 We previously found that the production of extracellular adenosine (EAD) is crucial for
83 regulating PMN influx to the lungs in young mice (10). Adenosine accumulates in the extracellular
84 space as a result of cellular damage. Upon tissue injury by a variety of insults, including infection,
85 ATP released from cells is metabolized to EAD by the sequential action of the extracellular
86 ectonucleotidases CD39 and CD73 (18). At baseline, EAD levels are very low/ undetectable in
87 extracellular spaces but can increase more than 10-fold upon infection (19). EAD can signal via 4
88 G-protein coupled receptors, A1, A2A, A2B and A3 that respond to EAD in a dose-dependent
89 manner (18, 20). A1 is high affinity and is activated by lower levels of adenosine while A2A and
90 A2B are lower affinity and require higher levels of adenosine to become activated (18). The
91 different adenosine receptors are also coupled to different G proteins that can inhibit (Gi) or
92 stimulate (Gs) adenylyl cyclase or activate phospholipase C (Gq), and therefore can have opposite

93 effects on downstream signaling and cell function. These receptors are ubiquitously expressed on
94 many cell types including PMNs (21).

95 Changes in EAD production and signaling occur with aging (22-27). Although the role of
96 this pathway in immunosenescence remains largely unexplored, we previously found that aging is
97 accompanied by changes in expression of EAD enzymes on murine PMNs at baseline and that
98 EAD signaling controlled PMN antibacterial function (21, 28, 29). However, how the levels of
99 extracellular adenosine and the receptors signaled through change after pneumococcal infection
100 and across host age, and their role in PMN migration, is unknown. In this study, we asked how
101 early PMN recruitment changes with host aging and if the extracellular adenosine pathway
102 regulates that during bacterial pneumonia. We tested the hypothesis that signaling through the high
103 affinity A1 adenosine receptor would be important early in pneumococcal infection when
104 adenosine levels are lower to help draw the PMNs to the lung, while signaling through the lower
105 affinity receptors may be required late in infection to help resolve PMN influx and that these
106 dynamics change during host aging.

107

108 METHODS

109 **Mice**

110 Young (8-12 weeks) and old (18-22 months) C57BL/6 (B6) male mice were obtained from the
111 National Institute on Aging colony or from Jackson Laboratories (Bar Harbor, ME). A2BR$^{-/-}$ mice
112 on a B6 background (B6.129P2-Adora2btm1Till/J) and A2AR$^{-/-}$ mice on a Balb/c background
113 (C;129S-Adora2atm1Jfc/J) were purchased from Jackson Laboratory and bred at our facility. Wild
114 type C57BL/6J and Balb/cJ mice were used as controls. Since males are more susceptible to
115 pneumococcal infection compared to females (30) and due to the limited availability of aged

116 cohorts, experiments were performed in male mice. All mice were housed at the University at
117 Buffalo in specific pathogen free housing for at least 2 weeks before use in experiments. All
118 experiments were conducted in accordance with the Institutional Animal Care and Use Committee
119 guidelines.

120 **Bacteria**

121 *Streptococcus pneumoniae* TIGR4 strain (serotype 4) was a kind gift from Andrew Camilli.
122 Briefly, bacteria were grown to mid-exponential phase at 37ºC at 5% CO_2 in Todd Hewitt broth
123 supplemented with 0.5% yeast extract and oxyrase as previously described (20).

124 **PMN Isolation**

125 Bone marrow cells were collected from the femurs and tibias of uninfected mice by flushing with
126 RPMI supplemented with 10% FBS and 2mM EDTA. Red blood cells were then lysed, and
127 remaining cells were washed, and resuspended in PBS. PMNs were separated via density
128 centrifugation using histopaque 1119 and 1077 (Sigma) as previously described (20, 21, 31, 32).
129 PMNs were then resuspended at the desired concentration in Hank's Balanced Salt Solution/0.1%
130 gelatin with no Ca^{2+} or Mg^{2+} and kept on ice until use. The purity of PMNs was confirmed using
131 flow cytometry and 85-90% of enriched cells were positive for Ly6G and CD11b.

132 **Mouse Infections and Treatments**

133 Mice were infected intratracheally (i.t.) with 50ul of *S. pneumoniae* TIGR4 at the indicated
134 concentrations. Mice were first anesthetized using isoflurane and the bacterial inoculum pipetted
135 into the trachea with the tongue gently pulled out to ensure direct delivery into the lungs. Following
136 infection, mice were euthanized at the indicated time points, blood was collected by portal vein
137 snip to determine bacteremia. Mice were then perfused with PBS through the right ventricle and
138 the lungs harvested. Following harvest, the lungs were minced into small pieces, mixed well and

139 half of each lung sample was homogenized in sterile PBS to plate for CFU, and the remaining half
140 was digested and used for flow cytometry analysis. Lung and blood samples were diluted in sterile
141 PBS and plated on blood agar to enumerate CFU. For experiments where the effect of A1 receptor
142 signaling was measured, the A1 receptor inhibitor, 8-Cyclopentyl-1,3-dipropylxanthine
143 (DPCPX)(28, 33, 34) or A1 receptor agonist 2-Chloro-N6-cyclopentyladenosine (35) were
144 purchased from Sigma Aldrich, dissolved in DMSO and filter sterilized by passing through a
145 0.22µm filter prior to use. Mice were given intraperitoneal (i.p.) injections of 1mg/kg of the A1
146 inhibitor or 0.1mg/kg of the A1 agonist or vehicle control at -1 and 0 (immediately before
147 challenge) days relative to infection. For experiments where the effect of A2A receptor signaling
148 was measured, the A2A receptor inhibitor, 3,7-Dimethyl-1-propargylxanthine (D134) (36), or A2A
149 receptor agonist CGS21680 (37), were purchased from Sigma, dissolved in DMSO and filter
150 sterilized by passing through a 0.22 µm filter prior to use. Mice were given i.p injections of 5mg/kg
151 of the A2A antagonist or 2mg/kg of the A2A agonist or vehicle control at -18 and 0 hours,
152 immediately before challenge for pre-treated (designated as PRE) and 18 hours post challenge for
153 post-treated groups (designated as POST). Control animals were treated with vehicle control.

154 **Flow Cytometry**

155 The lungs were digested into a single cell suspension in RPMI 1640 containing 10% FBS, 2mg/mL
156 type 2 collagenase and 50 U/mL DNase (Worthington) at 37°C/ 5% CO_2 as previously described
157 (20). Blood was collected by portal vein snips in EDTA as an anticoagulant. Red blood cells were
158 removed with a hypotonic lysis buffer (Lonza). Cells were resuspended in FACS buffer (HBSS/
159 1% FBS), treated with Fc block (anti-mouse clone 2.4G2) and stained with the following
160 antibodies purchased from BD Biosciences or Invitrogen: Anti-Ly6G (IA8), anti-CD11b (M1/70),
161 Anti-CD45 (30-F11). For adenosine receptor staining, the BD Cytofix/Cytoperm kit was used to

162 permeabilize the cells and the cells stained with the following unconjugated rabbit anti-adenosine
163 receptor antibodies from Abcam as previously described (21): A2a (ab3461), A2b (ab222901), A3
164 (ab203298) and A1 (ab82477). Rabbit polyclonal IgG (ab37415) was used as an isotype control
165 and secondary PE-conjugated anti-Rabbit IgG was used (12473981; Invitrogen). Fluorescent
166 intensities were measured on a BD Fortessa and at least 20,000 events were analyzed using FloJo.

167 **Trans Endothelial Migration Assay**

168 To determine trans-endothelial migration of PMNs *in vitro*, C57BL/6 murine primary lung
169 endothelial cells were grown according to manufacturer's protocol (Cell Biologics) and 5µm
170 transwells (Corning) were seeded at 10^6 cells/transwell and grown for 2 days at 37°C/ 5% CO_2
171 until confluent. The bottom of the transwell was infected with *S. pneumoniae* TIGR4 at an MOI
172 of 40. PMNs were isolated from the bone marrow of indicated mouse strains and $5x10^5$ PMNs
173 placed in the top of the transwell and incubated at 37°C at 5% CO2 for 1.5 hours. The number of
174 PMNs that migrated through the endothelial layer into the bottom of the transwell was determined
175 by collecting the cells from the bottom well and lysing them with 10% Triton X-100 to release
176 MPO. MPO was measured in each well by using 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic
177 acid) diammonium salt (ABST) in the presence of hydrogen peroxide (10) and serial dilutions of
178 a known numbers of PMNs were used to establish a standard curve, which was then used to
179 quantify the number of migrated neutrophils as previously described (10).

180 **Measurement of Adenosine**

181 Mice were infected i.t with $2.5x10^4$ CFU *S. pneumoniae* TIGR4. Following infection, mice were
182 euthanized at indicated time points and blood was collected and placed into microtainer tubes (BD)
183 and centrifuged at 9000 rpm to collect sera. Circulating levels of adenosine were measured using

184 the Adenosine Assay Kit (Fluorometric) from BioVision per manufacturer's instructions. Results
185 were read using a Biotek plate reader.

186 **Statistics**

187 Statistics were analyzed using Prism 9 (GraphPad). Bar graph and scatter plot data are presented
188 as Mean +/- SD, and line graph data are presented as Mean +/- SEM. All data were tested for
189 normality using Shapiro-Wilk test prior to statistical analysis. Significant difference between
190 groups was determined by Student's t-test, Mann-Whitney, Kruskal Wallis or one-way ANOVA as
191 indicated. Difference in survival was determined by the log-rank (Mantel-Cox) test. p values <0.05
192 were considered significant. * indicates $p<0.05$, ** indicates $p<0.001$, *** indicates $p<0.0001$.

193

194 RESULTS

195 **Rapid PMN influx to the lungs following *S. pneumoniae* infection is impaired with aging**

196 To determine how PMN influx to the lungs changes with age throughout the course of
197 pneumococcal infection, young and aged mice were infected i.t. with 2.5×10^4 colony forming units
198 (CFU) of *S. pneumoniae* TIGR4 and PMN recruitment to the lungs was measured by flow
199 cytometry over time following infection. Young mice had significant PMN influx to the lungs
200 within 6 hours of infection, and further increase in pulmonary PMN numbers by 18 and 48 hours
201 post infection (hpi) (**Fig 1A**). With age there was a delay in PMN influx early in infection with no
202 increase from baseline by 6 hours, but a significant increase by 18 and 48 hpi (**Fig 1A**). When
203 compared to young controls, aged mice had approximately 4-fold lower PMNs in the lungs at 6
204 hpi (**Fig 1A**). By 18 and 48 hpi, PMNs were present in the lungs of aged mice at similar levels to
205 young mice. This delay in PMN influx to the lungs with age was associated with higher
206 pneumococcal CFU in the lungs throughout the course of infection (**Fig 1B**) and significantly

207 higher bacteremia at 18 hpi (**Fig 1C**). When normalized to bacterial burden, aged mice displayed
208 significantly reduced PMN numbers in the lungs compared to young controls at 6 hours and
209 significantly higher numbers of PMNs at 48 hpi (**Fig S1A)**. These data indicate that with age there
210 is an initial delay in neutrophil influx to the lungs following pneumococcal infection that is
211 associated with impaired bacterial control.

212 **PMN trans-endothelial migration in response to *S. pneumoniae* is impaired with aging**

213 For PMNs to enter the lungs to control pneumococcal infection, these cells must be
214 recruited from the circulation and migrate through the endothelium to the infected pulmonary
215 tissue. To better understand the age-related impairment in PMN influx, we measured neutrophil
216 trans-endothelial migration. Using an *in vitro* transwell model, we measured neutrophil migration
217 across primary mouse lung endothelial cells (isolated from young mice) in response to *S.*
218 *pneumoniae* as a stimulus. At baseline there was no difference in neutrophil migration, however
219 upon infection, there was a significant decline in the number of PMNs that migrated through the
220 endothelial cell layer when PMNs were isolated from aged mice compared to young controls (**Fig**
221 **2**). These data suggest that there is an intrinsic defect in trans-endothelial migration by PMNs from
222 aged mice.

223 ***S. pneumoniae* infection alters expression of extracellular adenosine pathway components on**
224 **PMNs**

225 To test if the extracellular adenosine (EAD) pathway regulates the age-driven changes in
226 PMN pulmonary influx following *S. pneumoniae* infection, we first needed to understand its role
227 in young hosts. To do so, we tracked the expression of the different pathway components over time
228 following infection (gating strategy Fig S2). As this is a pulmonary infection and the damage
229 response is initiated in the lungs, we first tracked expression of the EAD producing and degrading

230 enzymes in the lungs of young mice. We measured geometric mean florescence intensity (MFI) on
231 total lung cells as an indicator of expression levels and found that the EAD producing enzymes
232 CD39 and CD73 as well the EAD degrading enzyme ADA were expressed in the lung tissues and
233 that their levels were maintained constant over 18 hours of infection (**Fig S3)**. We had challenges
234 measuring EAD levels in the bronchioalveolar lavage fluid (not shown), so instead we assessed
235 circulating levels and found overall levels of adenosine in the circulation did not significantly
236 change over the course of infection in young mice (**Fig 3**). We then tracked the expression of the
237 four adenosine receptors on the surface of PMNs in the lungs using flow cytometry. We found that
238 at baseline, the majority of PMNs in the lungs of young mice express A1, A2A, and A2B while
239 only about half of PMNs express A3 on the cell surface (**Fig 4A**). To determine if the level of
240 receptor expression on PMNs changes over time, we stained for adenosine receptor expression
241 following infection. We found that all four adenosine receptors showed a significant decrease in
242 expression within the first 18 hours of infection, followed by a recovery of levels comparable to
243 baseline by 48 hpi in young mice (**Fig 4B**). When we measured fold change from baseline, we
244 observed around 2-fold decrease by 6 hours for A2A and A2B and by 18 hours for A1 and A3
245 followed by recovery for all four receptors by 48 hpi in young mice (**Fig 4C**).

246 As PMNs are recruited from the blood, we also measured receptor expression in the
247 circulation. In circulating PMNs of uninfected young mice, A1, A2A, and A2B receptors were
248 highly expressed, however, very few PMNs expressed A3 (**Fig 5A**). When we measured how
249 receptor expression changes over time in circulating PMNs, we found that overall receptor
250 expression remains constant in young mice (**Fig 5B**). Unlike in the lungs, circulating PMNs in
251 young mice expressed A2A, A2B, and A3 at similar level over the course of infection (**Fig 5C**).
252 The expression of A1 in the circulation showed a slight, but significant 1.5-fold decrease at 6 hpi

253 but returned to baseline levels by 18 hpi) (**Fig 5C**). These data show that PMNs in the lungs and
254 circulation of young mice highly express adenosine receptors A1, A2A, and A2B on their surface,
255 and that there are dynamic changes in adenosine receptor expression on PMNs in response to
256 pneumococcal infection.

257 **Age-driven changes in EAD-pathway components following *S. pneumoniae* infection**

258 We next wanted to understand how the adenosine pathway components change with host
259 aging. We measured adenosine levels in the circulation of aged mice to see if the dysregulation in
260 PMN influx was due to a lack of extracellular adenosine production. We found that early in
261 infection, there was no significant difference in the amount of adenosine in the circulation when
262 compared to young mice (**Fig 3**). By 48 hpi, aged mice had significantly higher levels of adenosine
263 (**Fig 3**), suggesting exacerbated damage as the infection progressed in aged hosts, which fits with
264 the higher bacterial loads observed in aged hosts (**Fig 1**). When we measured adenosine pathway
265 enzymes in the lungs, we found that at baseline, the lungs of aged mice express significantly higher
266 amounts of the EAD producing enzymes CD73 and CD39, while the levels of the EAD degrading
267 enzyme ADA were higher, but did not reach statistical significance (**Fig S3**). When we tracked
268 enzyme expression through the course of infection, we found that unlike young controls, the
269 expression of all three enzymes had significantly decreased by 18 hpi in lungs of aged mice (**Fig**
270 **S3**).

271 Next, we analyzed receptor expression on PMNs in the lungs and circulation. We found
272 that in the lungs and blood at baseline there was no change in the percentage of PMNs expressing
273 A1, A2A, A2B, or A3 when compared to young mice with the majority of cells expressing A1,
274 A2A, and A2B (**Fig 4A and Fig 5A**). To look at receptor expression changes overtime, we

275 measured MFI and calculated fold change from baseline. In both lung (**Fig 4B**) and blood (**Fig 5B**)
276 there was no significant difference in receptor expression between young and aged mice and the
277 expression of A1 receptor trended to be higher on circulating PMNs at 6 hpi in aged mice compared
278 to young controls (**Fig 5B**). We did find that PMNs in the lungs of aged mice decreased levels of
279 adenosine receptors early in infection respective to their own baseline, however, unlike in young
280 mice where receptor expression returned to baseline levels by 48 hours, with age there was no
281 rebound of adenosine receptor expression at this later time point (**Fig 4C**). A similar pattern of
282 decreased receptor expression over time in aged mice on circulating PMNs was also seen, where
283 circulating PMNs maintained a 2-fold reduction in expression of A1, A2A and A2B following
284 infection (**Fig 5C**). These data show that there is no defect with age in production of extracellular
285 adenosine and or adenosine receptor expression on PMNs. Rather, the decrease in adenosine
286 receptor expression on PMNs during the course of infection is more pronounced and may result in
287 decrease responsiveness to adenosine signaling in the aged hosts.

288 **A2B adenosine receptor plays no role in PMN pulmonary influx following *S. pneumoniae***
289 **infection**

290 To determine if the individual adenosine receptors played a role in PMN pulmonary
291 migration, we focused on the receptors highly expressed on PMNs (A1, A2A and A2B). We first
292 examined the A2B receptor. To examine the role of this receptor in PMN influx to the lungs *in*
293 *vivo,* WT C57BL/6 and A2BR$^{-/-}$ mice were infected intratracheally with 2.5x10^{4} CFU *S.*
294 *pneumoniae* and the total number of PMNs in the lungs was determined by flow cytometry at 6,
295 18, and 48 hpi. We found that there was no difference in the total amount of PMNs in the lungs at
296 any of the time points tested between the mouse strains (**Fig 6**). Bacterial burden in the lungs also
297 did not differ (data not shown and supporting what was previously published (38)) and even when

298 normalized for bacterial numbers, PMN influx into the lungs in response to *S. pneumoniae*
299 infection was comparable between WT and A2BR$^{-/-}$ mice (**Fig S1B**). These data suggest that A2B
300 receptor signaling does not regulate PMN influx to the lung of *S. pneumoniae* infected mice.

301 **A2A adenosine receptor plays no role in PMN pulmonary influx following *S. pneumoniae***
302 **infection**

303 Next, we examined the role of A2A receptor signaling in PMN influx to the lungs following
304 infection. To examine the role of this receptor *in vivo*, A2AR$^{-/-}$ and WT BALB/c controls were
305 infected intratracheally with 2.5x10^4 CFU *S. pneumoniae* TIGR4 and total number of PMNs in the
306 lungs was determined at 6 and 48 hpi by flow cytometry. There was no difference in the number
307 of PMNs in the lungs of A2AR$^{-/-}$ or WT mice at either time point (**Fig 7A**). To confirm that this
308 result was not due to differences in bacterial burden, we analyzed *S. pneumoniae* CFU in both the
309 lung (**Fig 7B**) and blood (**Fig 7C**) and found no difference in bacterial burden across mouse strains.
310 Additionally, there was no difference in survival following infection between A2AR$^{-/-}$ and WT
311 mice (**Fig 7D**). Since these experiments were done using mice on a Balb/c background, we also
312 analyzed the role of A2A in C57BL/6 mice using a specific pharmacological A2A receptor
313 inhibitor, 3,7-Dimethyl-1-propargylxanthine. We first tested the role of A2A early in infection by
314 treating mice with the A2A receptor antagonist or vehicle control 18 hours prior to bacterial
315 challenge (PRE). We found no difference in total number of PMNs in the lungs (**Fig S4A**), total
316 bacterial burden in the lung (**Fig S4B)** or bacteremia (**Fig S4C)** by 48 hpi. Similarly, inhibition of
317 A2A prior to infection had no effect on mouse survival (**Fig S4D).** As A2A is a lower affinity
318 receptor that may play a role later in infection as damage and EAD levels build up, we also tested
319 its role later in infection, by timed inhibition 18 hours post (POST) bacterial challenge. Again, we
320 found no difference in total bacterial burden in the lung (**Fig S4B)** or bacteremia (**Fig S4C)** by 48

321 hpi in POST treated mice. However, there was a decrease in survival in the mice treated with A2A
322 receptor inhibitor post infection (**Fig S4D**) suggesting that A2A receptor may play a protective role
323 later in infection. Overall, these data suggest that A2A receptor signaling does not regulate PMN
324 influx to the lungs of young mice following *S. pneumoniae* challenge.

325 **A1 receptor signaling is required for PMN pulmonary influx in response to *S. pneumoniae***
326 **infection**

327 To determine the role of A1 receptor signaling in PMN migration, young mice were treated
328 i.p. with a specific A1 receptor inhibitor (whose specificity we confirmed in prior publications (28,
329 33)) or vehicle control. Mice were then infected intratracheally with $2.5x10^4$ CFU *S. pneumoniae*
330 TIGR4 one day post drug treatment. Total PMN influx to the lungs of infected mice was measured
331 by flow cytometry at 6, 18, and 48 hours. We found that inhibition of A1 resulted in a significant
332 delay in PMN influx to the lungs at 6 hpi (**Fig 8A-B**). At 6 hpi, there was a significant decrease in
333 total PMN numbers in the lungs (**Fig 8A**) and the amount of MPO (a marker of PMNs) in lung
334 homogenates (**Fig 8B**) of A1 inhibited mice. To understand the mechanism of the decline in PMN
335 recruitment, we examined the expression of CD18 and CXCR2 on the surface of PMNs in the
336 circulation. We found no difference in the numbers of circulating PMNs in A1 receptor inhibited
337 versus vehicle treated mice (**Fig S5)**. We further found that while A1 receptor inhibition had no
338 effect of CD18 expression (**Fig 8C**), it significantly reduced CXCR2 expression at 6 hpi (**Fig 8D**),
339 the time point at which PMN number in the lungs is lower (**Fig 8A**). As CXCR2 was reported to
340 be required for trans-endothelial migration in response to pneumococcal infection (11, 14, 39-41)
341 , we directly assessed the role of A1 on PMN trans-endothelial migration. Young mice were treated
342 i.p. with an A1 receptor inhibitor, and PMNs were then isolated, and trans-endothelial migration
343 was measured using the same *in vitro* transwell assay described above. A1 receptor inhibited PMNs

344 displayed a significant decrease in their ability to migrate trans-endothelially compared to VC
345 treated PMNs, indicating that A1 signaling on PMNs is required for efficient trans-endothelial
346 migration (**Fig 8E**). These data suggest that A1 adenosine receptor signaling is required for
347 recruitment of PMNs to the lungs in response to *S. pneumoniae* infection.

348 **Activation of A1 receptor signaling rescues the ability of PMNs from old mice to be**
349 **recruited to the lungs following *S. pneumoniae* infection**

350 To better understand if adenosine receptor signaling can be targeted to reverse the age
351 associated dysregulation in PMN pulmonary influx following pneumococcal infection, aged mice
352 were treated with specific adenosine receptor agonists to stimulate receptor signaling and then
353 infected intratracheally with $2.5x10^4$ CFU *S. pneumoniae* TIGR4. We first tested A2A receptor as
354 it had a role in controlling survival of young hosts later in infection. We found that activating A2A
355 in aged mice at 18 hpi had no effect on the total number of PMNs in the lungs (**Sup. Fig 6A**), or
356 the number of bacteria in the lungs (**Sup. Fig 6B**), or blood (**Sup. Fig 6C**) by 48 hours post
357 challenge.

358 We then focused on the role of A1 as it was required for early influx of PMNs in young
359 hosts. We tested the effect of A1 agonism in aged mice and determined the total number of PMNs
360 in the lungs at 6 hpi by flow cytometry. We found that agonism of the A1 receptor significantly
361 increased the total number of PMNs in the lungs of aged mice 6 hpi when compared to uninfected
362 mice (**Fig 9A**). Additionally, at 6 hpi, a separate set of A1 agonist treated mice had significantly
363 higher levels of MPO in the lungs compared to vehicle control treated mice (**Fig 9B**). Importantly,
364 A1 receptor agonism decreases bacterial burden in the lungs of aged mice compared to untreated
365 controls (**Fig 9C**). Taken together, these data demonstrate that A1 adenosine receptor signaling can

366 be targeted to reverse the age-related decrease in PMN pulmonary influx and reduce bacterial
367 burden in the lungs of infected aged mice.

368

369 DISCUSSION

370 Cellular damage resulting from infection is proposed to shape the outcome of host-
371 pathogen interactions (42-44) and damage-associated molecular patters (DAMPS) can act as
372 signals that initiate the immune response (45). One such molecule is extracellular adenosine
373 (EAD). At baseline homeostatic levels, adenosine levels in extracellular microenvironments are
374 very low, however, upon tissue damage resulting from insults such as infection, EAD levels can
375 rise more than 10 fold (18). We previously found that EAD production and signaling are crucial
376 for host resistance against pneumococcal infection (10, 20, 21, 38, 46). We show here that EAD
377 signaling through the high affinity A1 receptor is required for initiating the recruitment of PMNs
378 into the lungs following bacterial challenge. This initiation of early PMN recruitment was defective
379 in aged hosts but could be reversed by activation of A1 receptor signaling, suggesting that with
380 age there are changes in extracellular adenosine signaling that impair the ability of PMNs to rapidly
381 respond to pneumococcal infection. One of the hallmarks of aging is altered intercellular
382 communication, including endocrine and neuronal signaling (7) and the findings here suggest that
383 damage signaling is also altered with aging and contributes to the age-driven changes in immune
384 response to infection.

385 PMNs have a dynamic role during pneumococcal pneumonia with their early presence in
386 the lungs being protective vs later persistence being detrimental (47). In murine models of
387 infection, PMN influx to the lungs within the first 12 hpi correlated with control of bacterial burden
388 and PMN depletion prior to infection resulted in increased bacterial burden in the lung and host

389 lethality (48, 49). In humans, patients with neutropenia are at an increased risk for pneumonia (13).
390 These findings indicate that early influx of PMNs to the lungs is required for host protection against
391 pneumococcal pneumonia. In contrast, PMN persistence in the pulmonary environment promotes
392 disease (10, 15, 17). Depletion of PMNs 18 hours after pneumococcal lung infection resulted in
393 reduced bacterial numbers and reduced lethality (10). These findings demonstrate that the ability
394 of PMNs to kill pneumococci is altered during infection (10). Here we found that with aging there
395 is a delay in PMN influx early within the first 6 hours of infection accompanied by persistent
396 presence in higher levels later in infection compared to young mice infected with the same dose of
397 *S. pneumoniae*. This delay left aged mice more susceptible to disease as it was accompanied by
398 increased bacterial burden in the lungs throughout the course of infection and higher incidence of
399 bacteremia compared to young mice. Therefore, with age there is a delay in the protective early
400 PMN recruitment to the site of infection. The majority of studies examining PMN influx in
401 response to infection in aged hosts have focused on later timepoints (9, 10, 17, 50, 51). In humans,
402 elderly pneumococcal pneumonia patients had higher percentages of neutrophils in terminal lung
403 biopsies compared to younger patients (16) and in several animal models of bacterial and viral
404 pneumonia, PMN numbers in the lungs of aged mice were higher than in young controls at later
405 timepoints (9, 10, 17, 50, 51), similar to what we found here. The dynamics of earlier influx and
406 how that is altered with aging have not been as thoroughly addressed in the literature and there are
407 no data from human pneumococcal pneumonia patients. Our findings suggest that the kinetics of
408 PMN influx to the lungs in response to pneumococcal pneumonia is altered with age.
409 Following lung challenge, *S. pneumoniae* localizes within the airways and binds to lung
410 tissues as well (10, 17). For PMNs to reach the site of infection in the lungs, they have to move
411 from the circulation across the endothelium, enter the interstitial space, then migrate across the

412 lung epithelium into the airways (11, 52). We found here that PMN migration across pulmonary
413 endothelial cells in impaired with aging. Prior work found that PMNs from elderly human donors
414 did not show defects in transepithelial migration in response to pneumococcal infection (53) but
415 rather that chemotaxis (or directional movement) of PMNs is diminished with aging (51, 54-58).
416 PMNs isolated from both healthy controls and pneumonia patients above 60 years of age
417 displayed diminished chemotaxis compared with younger individuals (54, 55) which was linked
418 to over activation of phosphoinositide-3-kinase (PI3K) signaling (54). PI3K is controlled
419 downstream of GPCRs including adenosine receptors (54, 59). Here we found that A1 receptor
420 signaling on PMNs is required for their migration across pulmonary endothelial cells in response
421 to infection and that activating A1 receptor reverses the defect in early PMN pulmonary influx
422 observed in aged hosts. These findings are supported by prior work showing that A1 receptor
423 signaling on PMNs is required for their adherence to the endothelium in response to N-
424 Formylmethionyl-leucyl-phenylalanine (fMLP) or Phorbol 12-myristate 13-acetate (PMA)
425 stimulation (60, 61). In this study we found that A1 receptor signaling was needed for the ability
426 of PMNs to upregulate the expression of the chemokine receptor CXCR2 early on in response to
427 infection. During *S. pneumoniae* infection, CXCR2 is required for PMN influx to the lungs (39)
428 and in aged mice, expression of CXCR2 on PMNs was reported to be lower than young hosts
429 (56). Therefore, it is possible that activating A1 receptor signaling in aged hosts boosts PMN
430 migration by upregulating expression of CXCR2.

431 The role of A1 receptor signaling in PMN recruitment to the lungs seem to play opposing
432 roles during pathogen driven vs sterile injury. In LPS or ischemia reperfusion lung injury, A1
433 receptor signaling attenuated PMN trafficking to the lungs (62-65). In contrast and similar to what
434 we observed here, A1 receptor signaling was required for PMN pulmonary influx during infection

435 with H1N1 influenza virus (66). This highlights that pathogens can alter the dynamics of the
436 immune response and that the role of the adenosine signaling may differ in sterile vs. infection
437 driven injury.

438 Extracellular adenosine is known to regulate PMN function (18) and play an important role
439 in host resistance against pulmonary infections (67). Aging is associated with aberrant PMN
440 responses and overall increased susceptibility to pulmonary infections (47) . However,
441 surprisingly, the role of EAD in immunosenescence remains largely unexplored. In prior work we
442 found that there are age driven changes in the expression of the enzymes that make and break EAD
443 on PMNs and that EAD signaling on PMNs could be targeted to reverse the decline in their
444 antibacterial activity (33). In this study, to examine the role extracellular adenosine signaling in
445 the age-related delay in PMN influx we tracked how this pathway changes with host aging. We
446 found that circulating levels of adenosine were no different in young and aged mice, in fact aged
447 mice had significantly more adenosine in circulation at 48 hpi. While this increase in adenosine
448 later on in infection may reflect the increased damage that is occurring in these mice (due to higher
449 bacterial burden), these data also indicate that it is not that adenosine is not being produced with
450 age, but that EAD signaling in PMNs of aged mice may be dysregulated. This is in line with prior
451 work showing that aging is associated with defects in overall GPCR signaling and that alterations
452 in EAD receptor signaling contribute to the age-associated decline of brain and heart organ
453 functions (25) (68) (23). When looking at adenosine receptor expression on the surface of PMNs
454 from aged mice following pneumococcal infection, there was a decrease in receptor expression
455 from baseline in both you and aged mice. However, in PMNs isolated from young mice these
456 receptor levels rebounded by 48hpi, while receptor expression on PMNs from aged mice did not
457 recover. Following activation by an agonist, GPCR signaling is desensitized by internalization of

458 the receptor into the cell, this is a result of interaction of the G protein with β-arrestins to stop
459 signaling and additional scaffold proteins which guide trafficking the internalized GPCR (69-71).
460 The decrease in adenosine receptor expression following pneumococcal infection may be due to
461 this process of GPCR desensitization. Following desensitization, GPCRs may be degraded but
462 may also recycle back to the cell membrane surface for resensitization, where the receptor can be
463 activated again (70). In PMNs isolated from aged mice adenosine receptor expression does not
464 rebound following the initial drop post infection. This would indicate that EAD receptors are not
465 being recycled back to the cell surface, and throughout infection, PMNs of aged mice may be less
466 responsive to activation by adenosine. This could be in part be driven by the consistently elevated
467 levels of EAD later in infection observed in old but not young hosts. Additionally, the differences
468 in receptor levels between young and aged mice later in infection could indicate that there are
469 changes in GPCR desensitization and re-sensitization in PMNs with host age.

470 A balanced PMN response where PMNs are recruited to the lungs and resolve at the proper
471 time is essential for effective clearance of pneumococcus and efficient host protection (12, 13, 15,
472 16). In prior work, we found that in young mice, EAD production by CD73 was needed for
473 regulation of PMN influx later on during infection, where by day 2 following pulmonary challenge,
474 $CD73^{-/-}$ mice had significantly more PMNs in the lungs compared to wildtype controls (10). We
475 hypothesized that extracellular adenosine signaling via the low affinity receptors A2A and A2B
476 receptors regulate PMN resolution from the lungs later in infection when adenosine levels are
477 higher due to increased inflammation and host damage. It has been shown previously that under
478 hypoxia conditions adenosine produced by the endothelium reduced PMN accumulation by
479 activating A2A and A2B receptors on the PMN surface (72). Surprisingly, we found here that
480 young mice lacking A2A or A2B receptors showed no changes in PMN presence in the lungs

481 thorough the course of pneumococcal infection. As both A2A or A2B receptors are expressed on
482 PMNs and are coupled to Gs (18), it is possible that they play a redundant in PMN movement and
483 that removal of both would be needed to observe a significant effect. An alternative explanation is
484 that the levels of adenosine receptors do decline on PMNs through infection even in young hosts,
485 suggesting that PMNs may become less responsive to adenosine signaling with time.

486 In summary, we have shown that A1 receptor signaling is required for early PMN influx to
487 the lungs following pneumococcal infection. Importantly, activation of the A1 receptor using A1
488 receptor agonist, 2-Chloro-N6-cyclopentyladenosine increased PMN influx to the lung in aged
489 mice, which resulted in lower bacterial burden in the lung. This enhanced ability to control
490 bacterial infection could be a combination of increase PMN influx as shown here and improved
491 function as previously reported (33). These findings suggest that extracellular adenosine signaling
492 may be dysregulated with host aging and that pharmacologically targeting this pathway can
493 enhance the immune response in aged mice leading to more effective clearance of pneumococcus.
494 Identifying a potential target to enhance the immune response to pneumococcal pneumonia in aged
495 hosts is important as this disease persists in older adults despite the availability of antibiotics and
496 vaccines (3, 5, 6). In addition to being a potential pharmacological target, the extracellular
497 adenosine pathway is an important pathway to understand in the context of host-pathogen
498 interaction as it could be a marker of host damage. This study adds dysregulation of the EAD
499 pathway with age under the aging hallmark of altered intercellular signaling, which in turn controls
500 immunosenescence.

501

502 DECLARATIONS

503 **Ethics approval and consent to participate**

All animal experiments were conducted in accordance with the Institutional Animal Care and Use Committee guidelines under the approved protocol number MIC33018Y

Consent for publication

Not applicable

Availability of data and materials

All data generated or analyzed during this study are included in this published article [and its supplementary information files].

Competing interests

The authors declare no conflict of interest.

Funding

This work was supported by National Institute of Health grants R00AG051784 and R01 AG068568-01A1 to ENBG and F31 AI169889-01A1 to SRS.

Authors' contributions

SRS designed research, conducted research, analyzed data, and wrote paper. SES designed research, conducted research, and analyzed data. EYIT, APL and MB conducted research and analyzed data. ENBG designed research, wrote the paper, and had responsibility for final content. All authors read and approved the final manuscript.

Acknowledgements

We would like to thank Dr. Michael Battaglia for critical feedback on the manuscript.

525 REFERENCES:

526 1. WHO. Pneumococcal disease. World Health Organization. 2014.

527 2. Grudzinska FS, Brodlie M, Scholefield BR, Jackson T, Scott A, Thickett DR, et al.
528 Neutrophils in community-acquired pneumonia: parallels in dysfunction at the extremes of age.
529 Thorax. 2020;75(2):164-71.

530 3. Simell B, Vuorela A, Ekstrom N, Palmu A, Reunanen A, Meri S, et al. Aging reduces the
531 functionality of anti-pneumococcal antibodies and the killing of Streptococcus pneumoniae by
532 neutrophil phagocytosis. Vaccine. 2011;29(10):1929-34.

533 4. Gonçalves MT, Mitchell TJ, Lord JM. Immune ageing and susceptibility to Streptococcus
534 pneumoniae. Biogerontology. 2016;17(3):449-65.

535 5. CDC. Pneumococcal Disease Global Pneumococcal Disease and Vaccine CDC: Centers
536 for Disease Control and Prevention; 2018 [updated 2018, November 15. Available from:
537 https://www.cdc.gov/pneumococcal/global.html.

538 6. Matanock A LG, Gierke R, Kobayashi M, Leidner A, Pilishvili T. Use of 13-Valent
539 Pneumococcal Conjugate Vaccine and 23-Valent Pneumococcal Polysaccharide Vaccine Among
540 Adults Aged ≥65 Years: Updated Recommendations of the Advisory Committee on Immunization
541 Practices. MMWR Morb Mortal Wkly Rep 2019;68:1069–1075. . CDC; 2019.

542 7. Lopez-Otin C, Blasco MA, Partridge L, Serrano M, Kroemer G. The hallmarks of aging.
543 Cell. 2013;153(6):1194-217.

544 8. López-Otín C, Blasco MA, Partridge L, Serrano M, Kroemer G. Hallmarks of aging: An
545 expanding universe. Cell. 2023;186(2):243-78.

546 9. Boyd AR, Shivshankar P, Jiang S, Berton MT, Orihuela CJ. Age-related defects in TLR2
547 signaling diminish the cytokine response by alveolar macrophages during murine pneumococcal
548 pneumonia. Exp Gerontol. 2012;47(7):507-18.
549 10. Bou Ghanem EN, Clark S, Roggensack SE, McIver SR, Alcaide P, Haydon PG, et al.
550 Extracellular Adenosine Protects against Streptococcus pneumoniae Lung Infection by Regulating
551 Pulmonary Neutrophil Recruitment. PLoS Pathog. 2015;11(8):e1005126.
552 11. Palmer CS, Kimmey JM. Neutrophil Recruitment in Pneumococcal Pneumonia. Frontiers
553 in Cellular and Infection Microbiology. 2022;12.
554 12. Pechous RD. With Friends Like These: The Complex Role of Neutrophils in the
555 Progression of Severe Pneumonia. Front Cell Infect Microbiol. 2017;7:160.
556 13. Rolston KV. The spectrum of pulmonary infections in cancer patients. Curr Opin Oncol.
557 2001;13(4):218-23.
558 14. Kolaczkowska E, Kubes P. Neutrophil recruitment and function in health and
559 inflammation. Nat Rev Immunol. 2013;13(3):159-75.
560 15. Bhowmick R, Tin Maung NH, Hurley BP, Ghanem EB, Gronert K, McCormick BA, et al.
561 Systemic disease during Streptococcus pneumoniae acute lung infection requires 12-lipoxygenase-
562 dependent inflammation. J Immunol. 2013;191(10):5115-23.
563 16. Menter T, Giefing-Kroell C, Grubeck-Loebenstein B, Tzankov A. Characterization of the
564 inflammatory infiltrate in Streptococcus pneumoniae pneumonia in young and elderly patients.
565 Pathobiology. 2014;81(3):160-7.
566 17. Bou Ghanem EN, Clark S, Du X, Wu D, Camilli A, Leong JM, et al. The alpha-tocopherol
567 form of vitamin E reverses age-associated susceptibility to streptococcus pneumoniae lung
568 infection by modulating pulmonary neutrophil recruitment. J Immunol. 2015;194(3):1090-9.

569 18. Barletta KE, Ley K, Mehrad B. Regulation of neutrophil function by adenosine.
570 Arterioscler Thromb Vasc Biol. 2012;32(4):856-64.
571 19. Hasko G, Linden J, Cronstein B, Pacher P. Adenosine receptors: therapeutic aspects for
572 inflammatory and immune diseases. Nat Rev Drug Discov. 2008;7(9):759-70.
573 20. Siwapornchai N, Lee JN, Tchalla EYI, Bhalla M, Yeoh JH, Roggensack SE, et al.
574 Extracellular adenosine enhances the ability of PMNs to kill Streptococcus pneumoniae by
575 inhibiting IL-10 production. J Leukoc Biol. 2020.
576 21. Bhalla M, Simmons SR, Abamonte A, Herring SE, Roggensack SE, Bou Ghanem EN.
577 Extracellular adenosine signaling reverses the age-driven decline in the ability of neutrophils to
578 kill Streptococcus pneumoniae. Aging Cell. 2020:e13218.
579 22. Chen JF, Chern Y. Impacts of methylxanthines and adenosine receptors on
580 neurodegeneration: human and experimental studies. Handb Exp Pharmacol. 2011(200):267-310.
581 23. Willems L, Ashton KJ, Headrick JP. Adenosine-mediated cardioprotection in the aging
582 myocardium. Cardiovasc Res. 2005;66(2):245-55.
583 24. Headrick JP. Impact of aging on adenosine levels, A1/A2 responses, arrhythmogenesis, and
584 energy metabolism in rat heart. Am J Physiol. 1996;270(3 Pt 2):H897-906.
585 25. Mackiewicz M, Nikonova EV, Zimmermann JE, Romer MA, Cater J, Galante RJ, et al.
586 Age-related changes in adenosine metabolic enzymes in sleep/wake regulatory areas of the brain.
587 Neurobiol Aging. 2006;27(2):351-60.
588 26. Crosti F, Ciboddo GF, Barbieri MC, Inversi F, Pavoni D, Quarenghi S, et al. Evidence for
589 adenosine adenosinedeaminase lymphocyte system impairment in ageing. Boll Ist Sieroter Milan.
590 1987;66(4):282-8.

591 27. Yeo EJ, Park SC. Age-dependent agonist-specific dysregulation of membrane-mediated
592 signal transduction: emergence of the gate theory of aging. Mech Ageing Dev. 2002;123(12):1563-
593 78.
594 28. Bhalla M, Hui Yeoh J, Lamneck C, Herring SE, Tchalla EYI, Heinzinger LR, et al. A1
595 adenosine receptor signaling reduces Streptococcus pneumoniae adherence to pulmonary
596 epithelial cells by targeting expression of platelet-activating factor receptor. Cell Microbiol.
597 2019:e13141.
598 29. Bhalla M, Heinzinger LR, Morenikeji OB, Marzullo B, Thomas BN, Bou Ghanem EN.
599 Transcriptome profiling reveals CD73 and age-driven changes in neutrophil responses against
600 Streptococcus pneumoniae. bioRxiv. 2021:2021.04.14.439887.
601 30. Gutierrez F, Masia M, Mirete C, Soldan B, Rodriguez JC, Padilla S, et al. The influence of
602 age and gender on the population-based incidence of community-acquired pneumonia caused by
603 different microbial pathogens. J Infect. 2006;53(3):166-74.
604 31. Swamydas M, Lionakis MS. Isolation, purification and labeling of mouse bone marrow
605 neutrophils for functional studies and adoptive transfer experiments. J Vis Exp. 2013(77):e50586.
606 32. Tchalla EYI, Bhalla M, Wohlfert EA, Bou Ghanem EN. Neutrophils are required during
607 immunization with the pneumococcal conjugate vaccine for protective antibody responses and
608 host defense against infection. J Infect Dis. 2020.
609 33. Bhalla M, Simmons SR, Abamonte A, Herring SE, Roggensack SE, Bou Ghanem EN.
610 Extracellular adenosine signaling reverses the age-driven decline in the ability of neutrophils to
611 kill Streptococcus pneumoniae. Aging Cell. 2020;19(10):e13218.

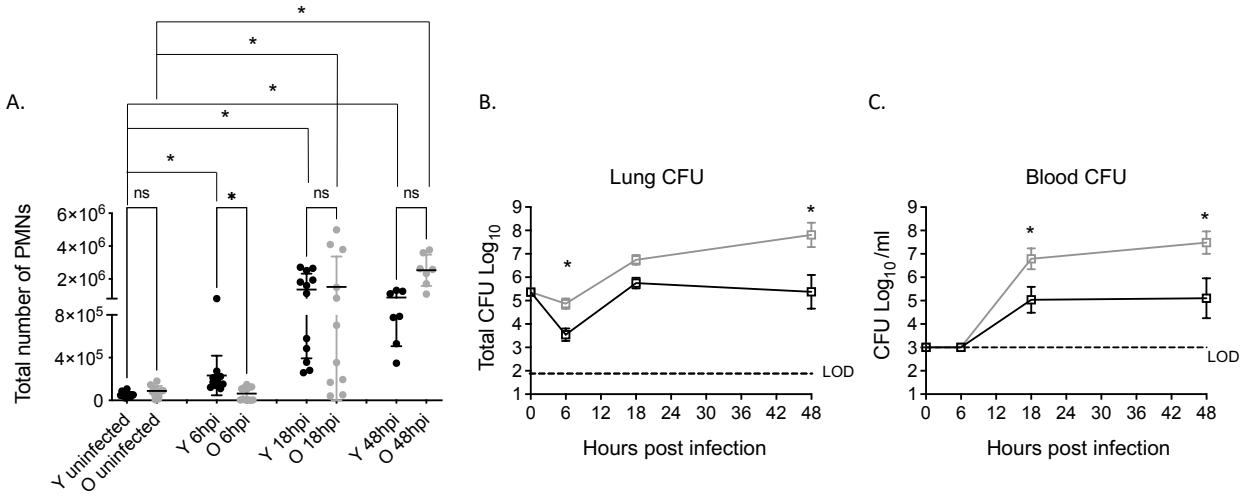
612 34. Zhou Y, Tong L, Chu X, Deng F, Tang J, Tang Y, et al. The Adenosine A1 Receptor
613 Antagonist DPCPX Inhibits Tumor Progression via the ERK/JNK Pathway in Renal Cell
614 Carcinoma. Cell Physiol Biochem. 2017;43(2):733-42.
615 35. Zhang X, Xia J, Qian D, Wang Y, Lin Y, Huang X, et al. An adenosine A1 agonist 2-chloro-
616 N6 cyclopentyladenosine inhibits the angiotensin II-induced cardiomyocyte hypertrophy through
617 the calcineurin pathway. Cardiology. 2014;129(3):153-62.
618 36. Yao L, McFarland K, Fan P, Jiang Z, Ueda T, Diamond I. Adenosine A2a blockade prevents
619 synergy between mu-opiate and cannabinoid CB1 receptors and eliminates heroin-seeking
620 behavior in addicted rats. Proc Natl Acad Sci U S A. 2006;103(20):7877-82.
621 37. Phillis JW. The selective adenosine A2 receptor agonist, CGS 21680, is a potent depressant
622 of cerebral cortical neuronal activity. Brain Res. 1990;509(2):328-30.
623 38. Herring SE, Mao S, Bhalla M, Tchalla EYI, Kramer JM, Bou Ghanem EN. Mitochondrial
624 ROS production by neutrophils is required for host antimicrobial function against Streptococcus
625 pneumoniae and is controlled by A2B adenosine receptor signaling. PLoS Pathog.
626 2022;18(11):e1010700.
627 39. Herbold W, Maus R, Hahn I, Ding N, Srivastava M, Christman JW, et al. Importance of
628 CXC chemokine receptor 2 in alveolar neutrophil and exudate macrophage recruitment in response
629 to pneumococcal lung infection. Infect Immun. 2010;78(6):2620-30.
630 40. Reutershan J, Morris MA, Burcin TL, Smith DF, Chang D, Saprito MS, et al. Critical role
631 of endothelial CXCR2 in LPS-induced neutrophil migration into the lung. J Clin Invest.
632 2006;116(3):695-702.
633 41. Williams MR, Azcutia V, Newton G, Alcaide P, Luscinskas FW. Emerging mechanisms of
634 neutrophil recruitment across endothelium. Trends Immunol. 2011;32(10):461-9.

635 42. Casadevall A, Pirofski LA. The damage-response framework of microbial pathogenesis.
636 Nat Rev Microbiol. 2003;1(1):17-24.
637 43. Pirofski LA, Casadevall A. The damage-response framework of microbial pathogenesis
638 and infectious diseases. Adv Exp Med Biol. 2008;635:135-46.
639 44. Pirofski LA, Casadevall A. Pathogenesis of COVID-19 from the Perspective of the
640 Damage-Response Framework. mBio. 2020;11(4).
641 45. Burgoyne RA, Fisher AJ, Borthwick LA. The Role of Epithelial Damage in the Pulmonary
642 Immune Response. Cells. 2021;10(10).
643 46. Bhalla M, Hui Yeoh J, Lamneck C, Herring SE, Tchalla EYI, Heinzinger LR, et al. A1
644 adenosine receptor signaling reduces Streptococcus pneumoniae adherence to pulmonary
645 epithelial cells by targeting expression of platelet-activating factor receptor. Cell Microbiol.
646 2020;22(2):e13141.
647 47. Simmons SR, Bhalla M, Herring SE, Tchalla EYI, Bou Ghanem EN. Older but Not Wiser:
648 the Age-Driven Changes in Neutrophil Responses during Pulmonary Infections. Infect Immun.
649 2021;89(4).
650 48. Garvy BA, Harmsen AG. The importance of neutrophils in resistance to pneumococcal
651 pneumonia in adult and neonatal mice. Inflammation. 1996;20(5):499-512.
652 49. Hahn I, Klaus A, Janze AK, Steinwede K, Ding N, Bohling J, et al. Cathepsin G and
653 neutrophil elastase play critical and nonredundant roles in lung-protective immunity against
654 Streptococcus pneumoniae in mice. Infect Immun. 2011;79(12):4893-901.
655 50. Kulkarni U, Zemans RL, Smith CA, Wood SC, Deng JC, Goldstein DR. Excessive
656 neutrophil levels in the lung underlie the age-associated increase in influenza mortality. Mucosal
657 Immunol. 2019;12(2):545-54.

658 51. Toapanta FR, Ross TM. Impaired immune responses in the lungs of aged mice following
659 influenza infection. Respir Res. 2009;10:112.
660 52. Craig A, Mai J, Cai S, Jeyaseelan S. Neutrophil recruitment to the lungs during bacterial
661 pneumonia. Infect Immun. 2009;77(2):568-75.
662 53. Bou Ghanem EN, Lee JN, Joma BH, Meydani SN, Leong JM, Panda A. The Alpha-
663 Tocopherol Form of Vitamin E Boosts Elastase Activity of Human PMNs and Their Ability to Kill
664 Streptococcus pneumoniae. Front Cell Infect Microbiol. 2017;7:161.
665 54. Sapey E, Greenwood H, Walton G, Mann E, Love A, Aaronson N, et al. Phosphoinositide
666 3-kinase inhibition restores neutrophil accuracy in the elderly: toward targeted treatments for
667 immunosenescence. Blood. 2014;123(2):239-48.
668 55. Sapey E, Patel JM, Greenwood HL, Walton GM, Hazeldine J, Sadhra C, et al. Pulmonary
669 Infections in the Elderly Lead to Impaired Neutrophil Targeting, Which Is Improved by
670 Simvastatin. Am J Respir Crit Care Med. 2017;196(10):1325-36.
671 56. Nomellini V, Brubaker AL, Mahbub S, Palmer JL, Gomez CR, Kovacs EJ. Dysregulation
672 of neutrophil CXCR2 and pulmonary endothelial icam-1 promotes age-related pulmonary
673 inflammation. Aging Dis. 2012;3(3):234-47.
674 57. Ren Z, Gay R, Thomas A, Pae M, Wu D, Logsdon L, et al. Effect of age on susceptibility
675 to Salmonella Typhimurium infection in C57BL/6 mice. J Med Microbiol. 2009;58(Pt 12):1559-
676 67.
677 58. Van Avondt K, Strecker J-K, Tulotta C, Minnerup J, Schulz C, Soehnlein O. Neutrophils
678 in aging and aging-related pathologies. Immunological Reviews. 2023;314(1):357-75.

679 59. Shi L, Wu Z, Miao J, Du S, Ai S, Xu E, et al. Adenosine interaction with adenosine receptor
680 A2a promotes gastric cancer metastasis by enhancing PI3K-AKT-mTOR signaling. Mol Biol Cell.
681 2019;30(19):2527-34.
682 60. Cronstein BN, Levin RI, Philips M, Hirschhorn R, Abramson SB, Weissmann G.
683 Neutrophil adherence to endothelium is enhanced via adenosine A1 receptors and inhibited via
684 adenosine A2 receptors. J Immunol. 1992;148(7):2201-6.
685 61. Felsch A, Stocker K, Borchard U. Phorbol ester-stimulated adherence of neutrophils to
686 endothelial cells is reduced by adenosine A2 receptor agonists. J Immunol. 1995;155(1):333-8.
687 62. Sun CX, Young HW, Molina JG, Volmer JB, Schnermann J, Blackburn MR. A protective
688 role for the A1 adenosine receptor in adenosine-dependent pulmonary injury. J Clin Invest.
689 2005;115(1):35-43.
690 63. Fernandez LG, Sharma AK, LaPar DJ, Kron IL, Laubach VE. Adenosine A1 receptor
691 activation attenuates lung ischemia-reperfusion injury. J Thorac Cardiovasc Surg.
692 2013;145(6):1654-9.
693 64. Ngamsri KC, Wagner R, Vollmer I, Stark S, Reutershan J. Adenosine receptor A1 regulates
694 polymorphonuclear cell trafficking and microvascular permeability in lipopolysaccharide-induced
695 lung injury. J Immunol. 2010;185(7):4374-84.
696 65. Gazoni LM, Walters DM, Unger EB, Linden J, Kron IL, Laubach VE. Activation of A1,
697 A2A, or A3 adenosine receptors attenuates lung ischemia-reperfusion injury. J Thorac Cardiovasc
698 Surg. 2010;140(2):440-6.
699 66. Aeffner F, Woods PS, Davis IC. Activation of A1-adenosine receptors promotes leukocyte
700 recruitment to the lung and attenuates acute lung injury in mice infected with influenza A/WSN/33
701 (H1N1) virus. J Virol. 2014;88(17):10214-27.

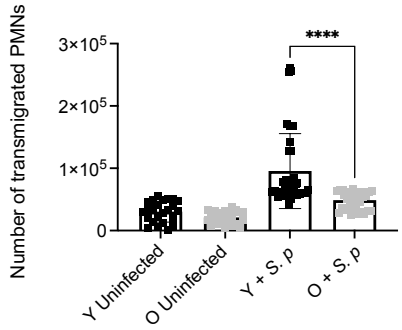
702 67. Lee JS, Yilmaz O. Unfolding Role of a Danger Molecule Adenosine Signaling in
703 Modulation of Microbial Infection and Host Cell Response. Int J Mol Sci. 2018;19(1).
704 68. Rolband GC, Furth ED, Staddon JM, Rogus EM, Goldberg AP. Effects of age and
705 adenosine in the modulation of insulin action on rat adipocyte metabolism. J Gerontol.
706 1990;45(5):B174-8.
707 69. Waldo GL, Northup JK, Perkins JP, Harden TK. Characterization of an altered membrane
708 form of the beta-adrenergic receptor produced during agonist-induced desensitization. J Biol
709 Chem. 1983;258(22):13900-8.
710 70. Yuan L, Barbash S, Kongsamut S, Eishingdrelo A, Sakmar TP, Eishingdrelo H. 14-3-3
711 signal adaptor and scaffold proteins mediate GPCR trafficking. Scientific Reports.
712 2019;9(1):11156.
713 71. Shenoy SK, Lefkowitz RJ. β-Arrestin-mediated receptor trafficking and signal
714 transduction. Trends Pharmacol Sci. 2011;32(9):521-33.
715 72. Eltzschig HK, Thompson LF, Karhausen J, Cotta RJ, Ibla JC, Robson SC, et al.
716 Endogenous adenosine produced during hypoxia attenuates neutrophil accumulation: coordination
717 by extracellular nucleotide metabolism. Blood. 2004;104(13):3986-92.
718
719 FIGURES & LEGENDS



720

721 **Figure 1: Old mice have delayed early pulmonary PMN influx.** Young and old male C57BL/6
722 mice were infected i.t. with $5x10^4$ CFU *S. pneumoniae* TIGR4. (A) Lungs were harvested,
723 digested, and stained for PMNs and analyzed by flow cytometry at the indicated timepoints
724 following infection. (B) Lung and blood (C) were collected, and samples plated on blood agar and
725 CFU was enumerated at the indicated timepoint post infection. Data are pooled from 6 separate
726 experiments with a total of n=7 mice at 48-hour time point and n=12 mice at all other time points.
727 Asterix indicates significant difference between indicated groups and as calculated by (A) Kruskal
728 Wallis followed by Dunn's multiple comparison test and (B and C) one-way ANOVA followed by
729 Sidak's multiple comparison test. ns indicates non-significant.

730



731

732 **Figure 2: PMNs from old mice have a defect in trans-endothelial migration.** PMNs were
733 isolated from the bone marrow of young and old male C57BL/6 mice. Transwells were seeded
734 with murine primary lung endothelial cells and the bottom of the transwell infected with *S.*
735 *pneumoniae* TIGR4 at an MOI of 40. PMNs were placed in the top of the transwell and incubated
736 at 37°C at 5% CO2 for 1.5 hours. PMNs migrated to the bottom of each transwell were enumerated.
737 Technical replicates are pooled from 4 separate experiments (n=4 mice). Asterix indicates
738 significance as determined by unpaired Student's t-test.

739

740

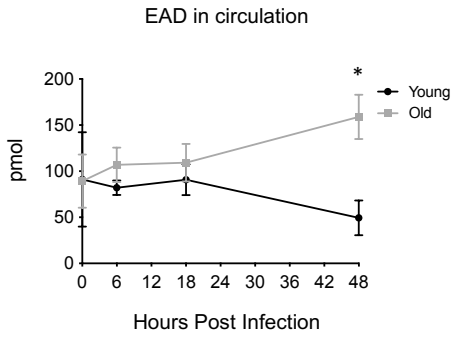
741

742

743

744

745



746

747 **Figure 3: Adenosine levels in circulation change with age over time** Young and old male
748 C57BL/6 mice were infected i.t. with $5x10^4$ CFU *S. pneumoniae* TIGR4 and sera was collected at
749 6, 18, and 48 hpi. Adenosine levels in the circulation were measured from two separate
750 experiments with n=3 mice at each individual time point. Asterix indicates significantly different
751 from young at the indicated timepoint as calculated by Kruskal Wallis followed by Dunn's multiple
752 comparison test.

753

754

755

756

757

758

759

760

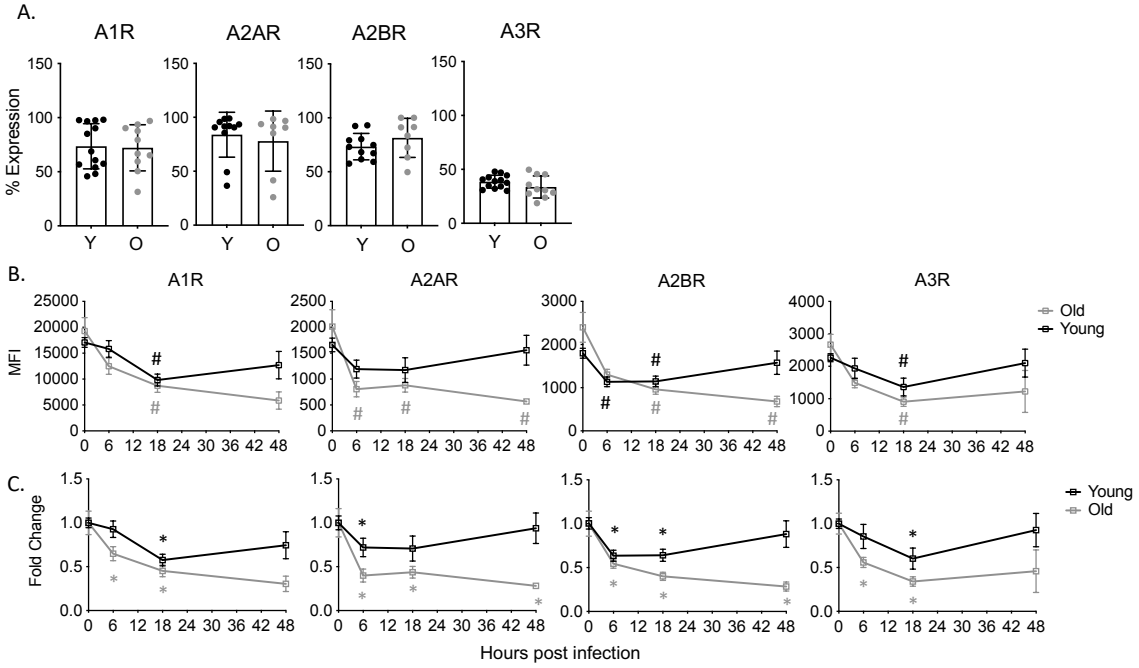
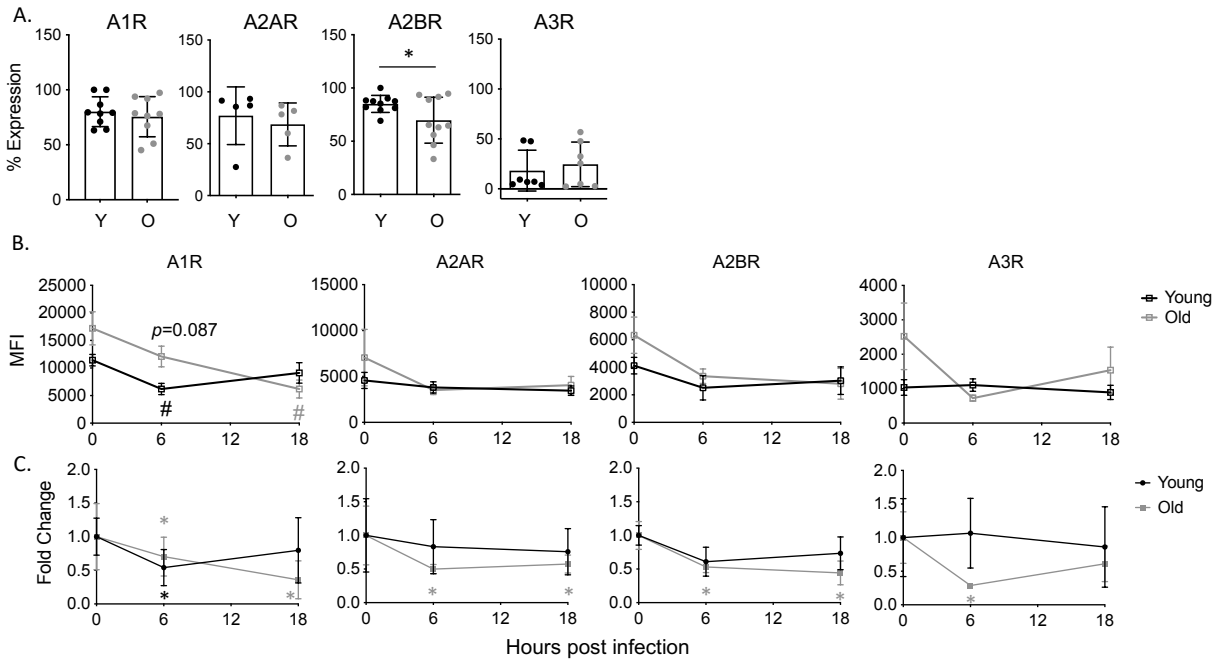


Figure 4: EAD pathway expression on pulmonary PMNs. Young and old male C57BL/6 mice were infected i.t. with $5x10^4$ CFU *S. pneumoniae* TIGR4. The lungs were harvested, and lung digests were stained for PMNs and each adenosine receptor. Percent expression at baseline (A) and MFI over time (B) of the indicated adenosine receptor on PMNs was determined. Replicates are pooled from 6 separate experiments with n=6-12 mice per group. (B) # indicate significance from uninfected baseline as determined by Kruskal Wallis followed by Dunn's Multiple comparison test. (C) Asterix indicate significant difference from one as determined by a 1-sample t-test.



773

774 **Figure 5: EAD pathway expression on circulating PMNs.** Young and old male C57BL/6 mice
775 were infected i.t. with $5x10^4$ CFU *S. pneumoniae* TIGR4. Blood was collected and stained for
776 PMNs and adenosine receptors. Percent expression at baseline (A) and MFI over time (B) was
777 determined using flow cytometry. Replicates are pooled from 4 separate experiments with n=6-9
778 mice per group. Asterix indicate significant difference as determined by unpaired students t-test
779 (A) and significantly different from one as determined by 1-sample t-test (C). (B) # / p-values
780 indicate significance from uninfected baseline as determined by Kruskal Wallis followed by
781 Dunn's Multiple comparison test.

782

783

784

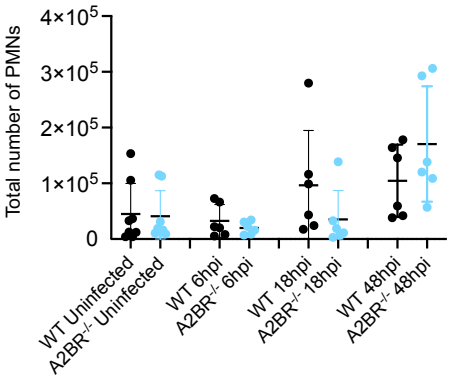


Figure 6: A2B receptor does not affect PMN pulmonary numbers *in vivo.* WT and A2BR$^{-/-}$ mice were infected i.t with 5x10^4 CFU *S. pneumoniae* TIGR4. At the indicated timepoints following bacterial challenge, lungs were harvested, digested, and stained for PMNs and analyzed by flow cytometry. Data are pooled from 4 separate experiments with n=6-8 mice per group.

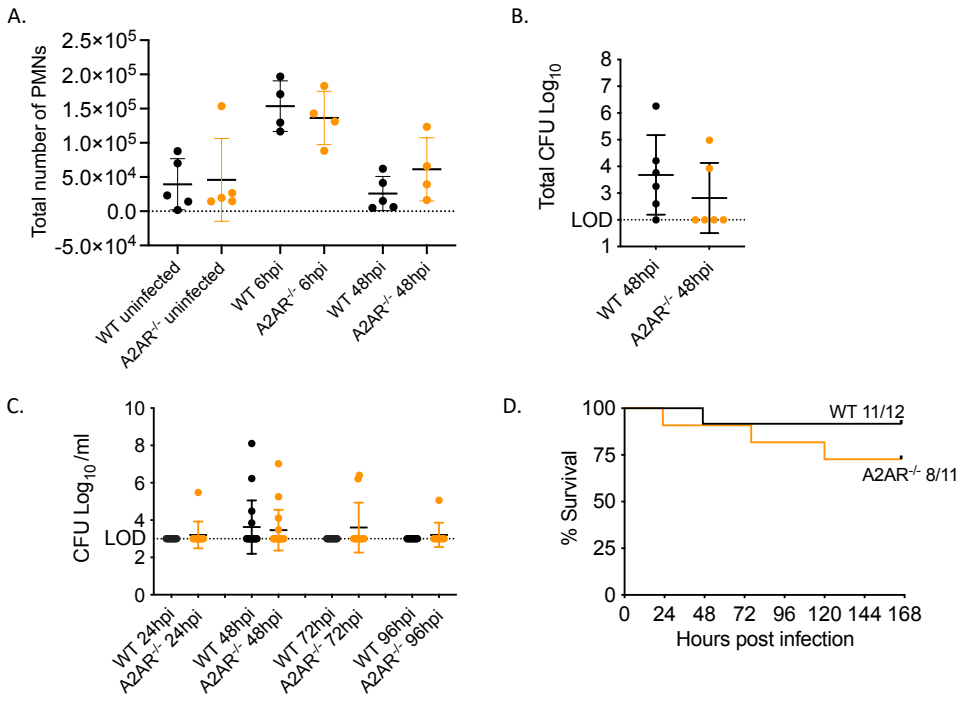


Figure 7: A2A receptor does not affect PMN pulmonary numbers or host resistance to infection. (A) WT and A2AR$^{-/-}$ mice were infected i.t with 5x10^4 CFU *S. pneumoniae* TIGR4. At the indicated timepoints following bacterial challenge, lungs were harvested, digested, and stained for PMNs and analyzed by flow cytometry. Data are pooled from 3 separate experiments with n=4-6 mice per group. Lungs (B) and Blood (C) were harvested at indicated timepoints and plated on blood agar plates and CFU was enumerated. Data were pooled from 2 separate experiments with (C) n=11-12 mice per group and (D) n=6 mice per group. (E) WT and A2AR$^{-/-}$ mice were infected as in (A) and monitored for survival. Data are pooled from 2 experiments with n=11-12 mice per group. No significant differences were found in survival as measured by Log-Rank (Mantle Cox) test.

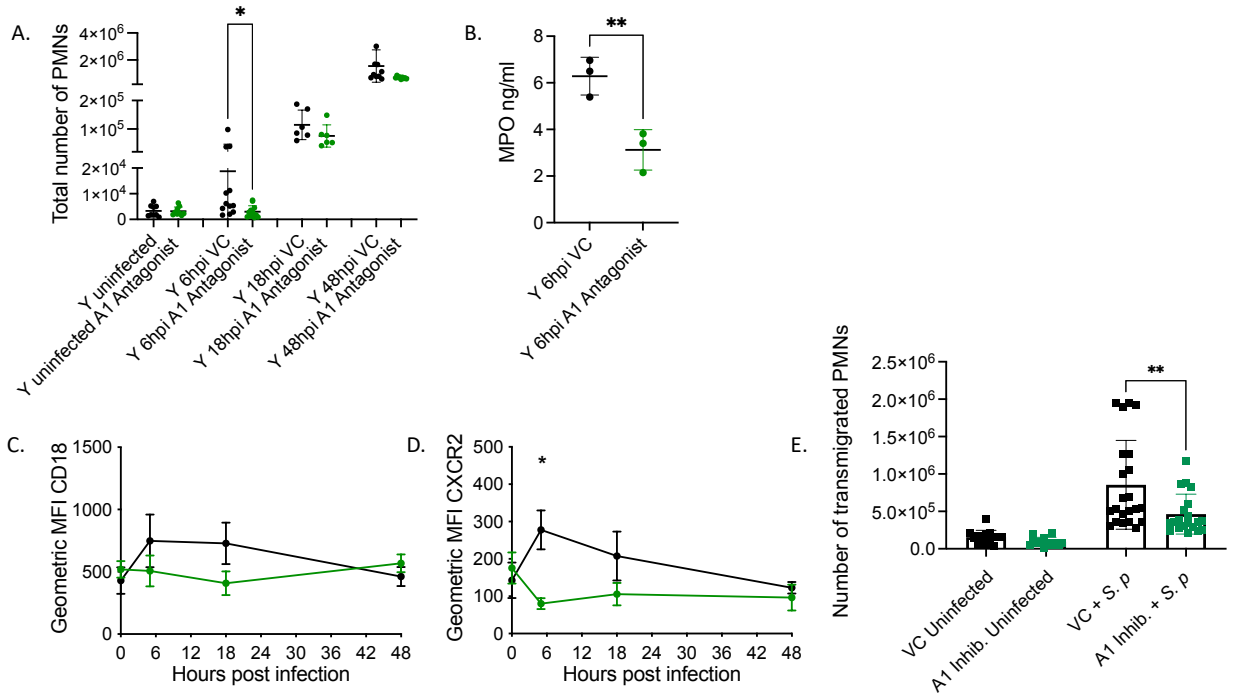


Figure 8: A1 receptor signaling is required for PMN pulmonary influx. (A) Young, male mice were treated i.p with A1 inhibitor DPCPX and infected i.t with $5x10^4$ CFU *S. pneumoniae* TIGR4. At the indicated time points, lungs were harvested, digested, and stained for PMNs (CD45+, Ly6G+ CD11b+) (A) and analyzed by flow cytometry. (B) Lungs of a separate set of treated and infected mice were harvested 6 hpi, homogenized and MPO levels were determined by ELISA. (C-D) Blood was harvested from infected and A1 treated mice (same mice as A) at the indicated time points and stained for PMNs and CD18 (C) or CXCR2 (D). (A, C and D) Data are pooled from 3 separate experiments with n=6-12 mice per group. (A and D) Asterix indicate significance as determined by Kruskal Wallis followed by Dunn's multiple comparison test. (B) Asterix indicate significance as determined by unpaired students t-test. (E) Young, male C57/B6 mice were treated i.p. with A1 inhibitor DPCPX or vehicle control (VC) and PMNs were isolated from the bone marrow. PMN migration across primary endothelial cells in response to *S. pneumoniae* TIGR4

820 infection was measured. Technical replicates are pooled from 4 separate experiments. Asterix
821 indicates significance as determined by Mann-Whitney test.

822

823

824

825

826

827

828

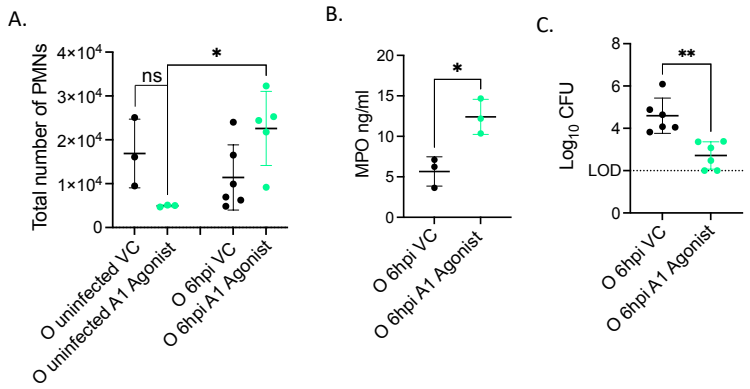
829

830

831

832

833



834

835 **Figure 9: A1 receptor signaling rescues defect in early PMN pulmonary influx in aged mice.**
836 (A) Old male mice were treated i.p. with A1 agonist 2-Chloro-N6-cyclopentyladenosine or Vehicle
837 Control and infected i.t. with $5x10^4$ CFU *S. pneumoniae* TIGR4 for 6 hours. Lungs were harvested,
838 digested, and stained for PMNs and analyzed by flow cytometry. Asterix indicates significance as
839 determined by One- way ANOVA followed by Sidak's multiple comparison test. ns indicates non-
840 significant. (C) Lung homogenates from infected mice were plated on Blood agar and bacterial
841 CFU was enumerated. Data are pooled from two separate experiments and with n=3 uninfected
842 and n=6 infected mice per group. (B) Lungs of a separate set of treated and infected mice were
843 harvested 6 hpi, homogenized and MPO levels were determined by ELISA. Data are pooled from
844 n=3 mice. (B and C) Asterix indicates significance as determined by unpaired Student's t-test.

845