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

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ABSTRACT

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. 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 influx 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. 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.

42 INTRODUCTION

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

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

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

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

88 downstream signaling and cell function. These receptors are ubiquitously expressed on many cell
89 types including PMNs [21].

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

102

103 MATERIALS AND METHODS

104 **Mice**

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

111 experiments were performed in male mice. All mice were housed at the University at Buffalo in
112 specific pathogen free housing for at least 2 weeks before use in experiments. All experiments
113 were conducted in accordance with the Institutional Animal Care and Use Committee guidelines.

114 **Bacteria**

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

118 **PMN Isolation**

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

126 **Mouse Infections and Treatments**

127 Mice were infected intratracheally (i.t.) with 50ul of *S. pneumoniae* TIGR4 at the indicated
128 concentrations. Mice were first anesthetized using isoflurane and the bacterial inoculum pipetted
129 into the trachea with the tongue gently pulled out to ensure direct delivery into the lungs. Following
130 infection, mice were euthanized at the indicated time points, blood was collected by portal vein
131 snip to determine bacteremia. Mice were then perfused with PBS through the right ventricle and
132 the lungs harvested. Following harvest, the lungs were minced into small pieces, mixed well and
133 half of each lung sample was homogenized in sterile PBS to plate for CFU, and the remaining half

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

148 **Flow Cytometry**

149 The lungs were digested into a single cell suspension in RPMI 1640 containing 10% FBS, 2mg/mL
150 type 2 collagenase and 50 U/mL DNase (Worthington) at 37°C/ 5% CO_2 as previously described
151 [20]. Blood was collected by portal vein snips in EDTA as an anticoagulant. Red blood cells were
152 removed with a hypotonic lysis buffer (Lonza). Cells were resuspended in FACS buffer (HBSS/
153 1% FBS), treated with Fc block (anti-mouse clone 2.4G2) and stained with the following
154 antibodies purchased from BD Biosciences or Invitrogen: Anti-Ly6G (IA8), anti-CD11b (M1/70),
155 Anti-CD45 (30-F11). For adenosine receptor staining, the BD Cytofix/Cytoperm kit was used to
156 permeabilize the cells and the cells stained with the following unconjugated rabbit anti-adenosine

157 receptor antibodies from Abcam as previously described [21]: A2a (ab3461), A2b (ab222901), A3
158 (ab203298) and A1 (ab82477). Rabbit polyclonal IgG (ab37415) was used as an isotype control
159 and secondary PE-conjugated anti-Rabbit IgG was used (12473981; Invitrogen). Fluorescent
160 intensities were measured on a BD Fortessa and at least 20,000 events were analyzed using FloJo.

161 **Trans Endothelial Migration Assay**

162 To determine trans-endothelial migration of PMNs *in vitro*, C57BL/6 murine primary lung
163 endothelial cells were grown according to manufacturer's protocol (Cell Biologics) and 5µm
164 transwells (Corning) were seeded at 10^6 cells/transwell and grown for 2 days at 37°C/ 5% CO_2
165 until confluent. The bottom of the transwell was infected with *S. pneumoniae* TIGR4 at an MOI
166 of 40. PMNs were isolated from the bone marrow of indicated mouse strains and $5x10^5$ PMNs
167 placed in the top of the transwell and incubated at 37°C at 5% CO2 for 1.5 hours. The number of
168 PMNs that migrated through the endothelial layer into the bottom of the transwell was determined
169 by measuring the amount of MPO in each well compared to a known standard curve by using 2,2′-
170 Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABST) in the presence of
171 hydrogen peroxide as previously described [10].

172 **Measurement of Adenosine**

173 Mice were infected i.t with $2.5x10^4$ CFU *S. pneumoniae* TIGR4. Following infection, mice were
174 euthanized at indicated time points and blood was collected and placed into microtainer tubes (BD)
175 and centrifuged at 9000 rpm to collect sera. Circulating levels of adenosine were measured using
176 the Adenosine Assay Kit (Fluorometric) from BioVision per manufacturer's instructions. Results
177 were read using a Biotek plate reader.

178 **Statistics**

179 Statistics were analyzed using Prism 9 (GraphPad). Bar graph and scatter plot data are presented
180 as Mean +/- SD, and line graph data are presented as Mean +/- SEM. All data were tested for
181 normality using Shapiro-Wilk test prior to statistical analysis. Significant difference between
182 groups was determined by Student's t-test, Mann-Whitney, Kruskal Wallis or one-way ANOVA as
183 indicated. Difference in survival was determined by the log-rank (Mantel-Cox) test. p values <0.05
184 were considered significant. * indicates $p<0.05$, ** indicates $p<0.001$, *** indicates $p<0.0001$.
185

186 RESULTS

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

188 To determine how PMN influx to the lungs changes with age throughout the course of
189 pneumococcal infection, young and aged mice were infected i.t. with 2.5×10^4 colony forming units
190 (CFU) of *S. pneumoniae* TIGR4 and PMN recruitment to the lungs was measured by flow
191 cytometry over time following infection. Young mice had significant PMN influx to the lungs
192 within 6 hours of infection, and further increase in pulmonary PMN numbers by 18 and 48 hours
193 post infection (hpi) (**Fig 1A**). With age there was a delay in PMN influx early in infection with no
194 increase from baseline by 6 hours, but a significant increase by 18 and 48 hpi (**Fig 1A**). When
195 compared to young controls, aged mice had approximately 4-fold lower PMNs in the lungs at 6
196 hpi (**Fig 1A**). By 18 and 48 hpi, PMNs were present in the lungs of aged mice at similar levels to
197 young mice. This delay in PMN influx to the lungs with age was associated with higher
198 pneumococcal CFU in the lungs throughout the course of infection (**Fig 1B**) and significantly
199 higher bacteremia at 18 hpi (**Fig 1C**). When normalized to bacterial burden, aged mice displayed
200 significantly reduced PMN numbers in the lungs compared to young controls at 6- hours and
201 significantly higher numbers at 48 hpi (**Fig S1A)**. These data indicate that with age there is an

202 initial delay in neutrophil influx to the lungs following pneumococcal infection that is associated
203 with impaired bacterial control.

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

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

215

216 ***S. pneumoniae* infection alters expression of extracellular adenosine pathway components on**
217 **PMNs**

218 To test if the extracellular adenosine (EAD) pathway regulates the age-driven changes in
219 PMN pulmonary influx following *S. pneumoniae* infection, we first needed to understand its role
220 in young hosts. To do so, we tracked the expression of the different pathway components over time
221 following infection. As this is a pulmonary infection and the damage response is initiated in the
222 lungs, we first tracked expression of the EAD producing and degrading enzymes in the lungs of
223 young mice. We measured geometric mean florescence intensity (MFI) on total lung cells as an
224 indicator of expression levels and found that the EAD producing enzymes CD39 and CD73 as well

225 the EAD degrading enzyme ADA were expressed in the lung tissues and that their levels were
226 maintained constant over 18 hours of infection (**Fig S2)**. We had challenges measuring EAD levels
227 in the bronchioalveolar lavage fluid (not shown), so instead we assessed circulating levels and
228 found overall levels of adenosine in the circulation did not significantly change over the course of
229 infection in young mice (**Fig 3**). We then tracked the expression of the four adenosine receptors on
230 the surface of PMNs in the lungs using flow cytometry. We found that at baseline, the majority of
231 PMNs in the lungs of young mice express A1, A2A, and A2B while only about half of PMNs
232 express A3 on the cell surface (**Fig 4A**). To determine if the level of receptor expression on PMNs
233 changes over time, we stained for adenosine receptor expression following infection. We found
234 that all four adenosine receptors showed a significant decrease in expression within the first 18
235 hours of infection, followed by a recovery of levels comparable to baseline by 48 hpi in young
236 mice (**Fig 4B**). When we measured fold change from baseline, we observed around 2-fold decrease
237 by 6 hours for A2A and A2B and by 18 hours for A1 and A3 followed by recovery for all four
238 receptors by 48 hpi in young mice (**Fig 4C**).

239 As PMNs are recruited from the blood, we also measured receptor expression in the
240 circulation. In circulating PMNs of uninfected young mice, A1, A2A, and A2B receptors were
241 highly expressed, however, very few PMNs expressed A3 (**Fig 5A**). When we measured how
242 receptor expression changes over time in circulating PMNs, we found that overall receptor
243 expression remains constant in young mice (**Fig 5B**). Unlike in the lungs, circulating PMNs in
244 young mice expressed A2A, A2B, and A3 at similar level over the course of infection (**Fig 5C**).
245 The expression of A1 in the circulation showed a slight, but significant 1.5-fold decrease at 6 hpi
246 but returned to baseline levels by 18 hpi) (**Fig 5C**). These data show that PMNs in the lungs and
247 circulation of young mice highly express adenosine receptors A1, A2A, and A2B on their surface,

248 and that there are dynamic changes in adenosine receptor expression on PMNs in response to
249 pneumococcal infection.

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

252 To determine if the individual adenosine receptors played a role in PMN pulmonary
253 migration, we focused on the receptors highly expressed on PMNs (A1, A2A and A2B). We first
254 examined the A2B receptor. To examine the role of this receptor on PMN influx to the lungs *in*
255 *vivo,* WT C57BL/6 and A2BR$^{-/-}$ mice were infected intratracheally with 2.5x10^4 CFU *S.*
256 *pneumoniae* and the total number of PMNs in the lungs was determined by flow cytometry at 6,
257 18, and 48 hpi. We found that there was no difference in the total amount of PMNs in the lungs at
258 any of the time points tested between the mouse strains (**Fig 6**). Bacterial burden in the lungs also
259 did not differ (data not shown and supporting what was previously published [33]) and even when
260 normalized for bacterial numbers, PMN influx into the lungs in response to *S. pneumoniae*
261 infection was comparable between WT and A2BR$^{-/-}$ mice (**Fig S1B**). These data suggest that A2B
262 receptor signaling does not regulate PMN influx to the lung of *S. pneumoniae* infected mice.

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

265 Next, we examined the role of A2A receptor signaling on PMN influx to the lungs
266 following infection. To examine the role of this receptor *in vivo*, A2AR$^{-/-}$ and WT BALB/c controls
267 were infected intratracheally with 2.5x10^4 CFU *S. pneumoniae* TIGR4 and total number of PMNs
268 in the lungs was determined at 6 and 48 hpi by flow cytometry. There was no difference in the
269 number of PMNs in the lungs of A2AR$^{-/-}$ or WT mice at either time point (**Fig 7A**). To confirm

270 that this result was not due to differences in bacterial burden, we analyzed *S. pneumoniae* CFU in
271 both the lung (**Fig 7B**) and blood (**Fig 7C**) and found no difference in bacterial burden across
272 mouse strains. Additionally, there was no difference in survival following infection between
273 A2AR$^{-/-}$ and WT mice (**Fig 7D**). Since these experiments were done using mice on a Balb/c
274 background, we also analyzed the role of A2A in C57BL/6 mice using specific pharmacological
275 receptor inhibitor. We first tested the role of A2A early in infection by treating mice with the A2A
276 receptor antagonist or vehicle control 18 hours prior to bacterial challenge (PRE). We found no
277 difference in total number of PMNs in the lungs (**Fig S3A**), total bacterial burden in the lung (**Fig**
278 **S3B)** or bacteremia (**Fig S3C)** by 48 hpi. Similarly, inhibition of A2A prior to infection had no
279 effect on mouse survival (**Fig S3D).** As A2A is a lower affinity receptor that may play a role later
280 in infection as damage and EAD levels build up, we also tested its role later in infection, by timed
281 inhibition 18 hours post (POST) bacterial challenge. Again, we found no difference in total
282 bacterial burden in the lung (**Fig S3B)** or bacteremia (**Fig S3C)** by 48 hpi in POST treated mice.
283 However, there was a decrease in survival in the mice treated with A2A receptor inhibitor post
284 infection (**Fig S3D**) suggesting that A2A receptor may play a protective role later in infection.
285 Overall, these data suggest that A2A receptor signaling does not regulate PMN influx to the lungs
286 of young mice following *S. pneumoniae* challenge.

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

289 To determine the role of A1 receptor signaling in PMN migration, young mice were treated
290 i.p. with a specific A1 receptor inhibitor (whose specificity we confirmed in prior publications [28,
291 34]) or vehicle control. Mice were then infected intratracheally with 2.5x10^4 CFU *S. pneumoniae*
292 TIGR4 one day post drug treatment. Total PMN influx to the lungs of infected mice was measured

293 by flow cytometry at 6, 18, and 48 hours. We found that inhibition of A1 resulted in a significant
294 delay in PMN influx to the lungs at 6 hpi (**Fig 8A-C**). At 6 hpi, there was a significant decrease in
295 total PMN numbers in the lungs (**Fig 8A**) and the amount of MPO (a marker of PMNs) in lung
296 homogenates (**Fig 8B**) of A1 inhibited mice. To understand the mechanism of the decline in PMN
297 recruitment, we examined the expression of CD18 and CXCR2 on the surface of PMNs in the
298 circulation. We found no difference in the numbers of circulating PMNs in A1 receptor inhibited
299 versus vehicle treated mice (**Fig S4)**. We further found that while A1 receptor inhibition had no
300 effect of CD18 expression (**Fig 8C**), it significantly lowered CXCR2 expression at 6 hpi (**Fig 8D**),
301 the time point at which PMN influx to the lungs is lowered (**Fig 8A**). As CXCR2 was reported to
302 be required for trans-endothelial migration in response to pneumococcal infection [11, 14, 35-37] , we
303 directly assessed the role of A1 on PMN trans-endothelial migration. Young mice were treated i.p.
304 with the specific A1 receptor inhibitor, and PMNs were then isolated, and trans-endothelial
305 migration was measured using the same *in vitro* transwell assay described above. A1 receptor
306 inhibited PMNs displayed a significant 2.3-fold decrease in their ability to migrate trans-
307 endothelially compared to VC treated PMNs, indicating that A1 signaling on PMNs is required for
308 efficient trans-endothelial migration (**Fig 8E**). These data suggest that A1 adenosine receptor
309 signaling is required for influx of PMNs to the lungs in response to *S. pneumoniae* infection.

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

311 We next wanted to understand how the adenosine pathway components change with host
312 aging. We measured adenosine levels in the circulation of aged mice to see if the dysregulation in
313 PMN influx was due to a lack of extracellular adenosine production. We found that early in
314 infection, there was no significant difference in the amount of adenosine in the circulation when
315 compared to young mice (**Fig 3**). By 48 hpi, aged mice had significantly higher levels of adenosine

316 (**Fig 3**), suggesting exacerbated damage as the infection progressed in aged hosts. When we
317 measured adenosine pathway enzymes in the lungs, we found that at baseline, the lungs of aged
318 mice express significantly higher amounts of the EAD producing enzymes CD73 and CD39, while
319 the levels of the EAD degrading enzyme ADA were higher, but did not reach statistical significance
320 (**Fig S2**). When we tracked enzyme expression through the course of infection, we found that
321 unlike young controls, the expression of all three enzymes had significantly decreased by 18 hpi
322 in lungs of aged mice (**Fig S2**).

323 Next, we analyzed receptor expression on PMNs in the lungs and circulation. We found
324 that in the lungs and blood at baseline there was no change in the percentage of PMNs expressing
325 A1, A2A, A2B, or A3 when compared to young mice with the majority of cells expressing A1,
326 A2A, and A2B (**Fig 4A and Fig 5A**). To look at receptor expression changes overtime, we
327 measured MFI and calculated fold change from baseline. In both lung (**Fig 4B**) and blood (**Fig 5B**)
328 there was no significant difference in receptor expression between young and aged mice and the
329 expression of A1 receptor trended to be higher on circulating PMNs at 6 hpi in aged mice compared
330 to young controls (**Fig 5B**). We did find that PMNs in the lungs of aged mice decreased levels of
331 adenosine receptors early in infection respective to their own baseline, however, unlike in young
332 mice where receptor expression returned to baseline levels by 48 hours, with age there was no
333 rebound of adenosine receptor expression at this later time point (**Fig 4C**). A similar pattern of
334 decreased receptor expression over time in aged mice on circulating PMNs was also seen, where
335 circulating PMNs maintained a 2-fold reduction in expression of A1, A2A and A2B following
336 infection (**Fig 5C**). These data show that there is no defect with age in production of extracellular
337 adenosine and or adenosine receptor expression on PMNs. Rather, the decrease in adenosine

338 receptor expression on PMNs during the course of infection is more pronounced and may result in
339 decrease responsiveness to adenosine signaling in the aged hosts.

340 **Activation of A1 receptor signaling rescues the ability of PMNs from old mice to influx to**
341 **the lungs following *S. pneumoniae* infection**

342 To better understand if adenosine receptor signaling can be targeted to reverse the age
343 associated dysregulation in PMN pulmonary influx following pneumococcal infection, aged mice
344 were treated with specific adenosine receptor agonists to stimulate receptor signaling and then
345 infected intratracheally with $2.5x10^4$ CFU *S. pneumoniae* TIGR4. We first tested A2A receptor as
346 it had a role in controlling survival of young hosts later in infection. We found that activating A2A
347 in aged mice at 18 hpi had no effect on the total number of PMNs in the lungs (**Sup. Fig 5A**), or
348 the number of bacteria in the lungs (**Sup. Fig 5B**), or blood (**Sup. Fig 5C**) by 48 hours post
349 challenge.

350 We then focused on the role of A1 as it was required for early influx of PMNs in young
351 hosts. We tested the effect of A1 agonism in aged mice and determined the total number of PMNs
352 in the lungs at 6 hpi by flow cytometry. We found that agonism of the A1 receptor significantly
353 increased the total number of PMNs in the lungs of aged mice 6 hpi when compared to uninfected
354 mice (**Fig 9A**). Additionally, at 6 hpi, a separate set of A1 agonist treated mice had significantly
355 higher levels of MPO in the lungs compared to vehicle control treated mice (**Fig 9B**). Importantly,
356 A1 receptor agonism decreases bacterial burden in the lungs of aged mice compared to untreated
357 controls (**Fig 9C**). Taken together, these data demonstrate that A1 adenosine receptor signaling can
358 be targeted to reverse the age-related decrease in PMN pulmonary influx and reduce bacterial
359 burden in the lungs of infected aged mice.

360 DISCUSSION

361 Cellular damage resulting from infection is proposed to shape the outcome of host-
362 pathogen interactions [38-40] and damage-associated molecular patters (DAMPS) can act as signals
363 that initiate the immune response [41]. One such molecule is extracellular adenosine (EAD). At
364 baseline homeostatic levels, adenosine levels in extracellular microenvironments are very low,
365 however, upon tissue damage resulting from insults such as infection, EAD levels can rise more
366 than 10 fold [18]. We previously found that EAD production and signaling are crucial for host
367 resistance against pneumococcal infection [10, 20, 21, 33, 42]. We show here that EAD signaling through
368 the high affinity A1 receptor is required for initiating the recruitment of PMNs into the lungs
369 following bacterial challenge. This initiation of early PMN recruitment was defective in aged hosts
370 but could be reversed by activation of A1 receptor signaling, suggesting that with age there are
371 changes in extracellular adenosine signaling that impair the ability of PMNs to rapidly respond to
372 pneumococcal infection. One of the hallmarks of aging is altered intercellular communication,
373 including endocrine and neuronal signaling [7] and the findings here suggest that damage signaling
374 is also altered with aging and contributes to the age-driven changes in immune response to
375 infection.

376 PMNs have a dynamic role during pneumococcal pneumonia with their early presence in
377 the lungs being protective vs later persistence being detrimental [43]. In murine models of infection,
378 PMN influx to the lungs within the first 12 hpi correlated with control of bacterial burden and
379 PMN depletion prior to infection resulted in increased bacterial burden in the lung and host
380 lethality [44, 45]. In humans, patients with neutropenia are at an increased risk for pneumonia [13]. These
381 findings indicate that early influx of PMNs to the lungs is required for host protection against
382 pneumococcal pneumonia. In contrast, PMN persistence in the pulmonary environment promotes

383 disease [10, 15, 17]. Depletion of PMNs 18 hours after pneumococcal lung infection resulted in reduced
384 bacterial numbers and reduced lethality [10]. These findings demonstrate that the ability of PMNs to
385 kill pneumococci is altered during infection [10]. Here we found that with aging there is a delay in
386 PMN influx early within the first 6 hours of infection accompanied by persistent presence in higher
387 levels later in infection compared to young mice infected with the same dose of *S. pneumoniae*.
388 This delay left aged mice more susceptible to disease as it was accompanied by increased bacterial
389 burden in the lungs throughout the course of infection and higher incidence of bacteremia
390 compared to young mice. Therefore, with age there is a delay in the protective early PMN
391 recruitment to the site of infection. The majority of studies examining PMN influx in response to
392 infection in aged hosts have focused on later timepoints [9, 10, 17, 46, 47]. In humans, elderly
393 pneumococcal pneumonia patients had higher percentages of neutrophils in terminal lung biopsies
394 compared to younger patients [16] and in several animal models of bacterial and viral pneumonia,
395 PMN numbers in the lungs of aged mice were higher than in young controls at later timepoints [9,
396 10, 17, 46, 47], similar to what we found here. The dynamics of earlier influx and how that is altered
397 with aging have not been as thoroughly addressed in the literature and there are no data from
398 human pneumococcal pneumonia patients. Our findings suggest that the kinetics of PMN influx
399 to the lungs in response to pneumococcal pneumonia is altered with age.

400 Following lung challenge, *S. pneumoniae* localizes within the airways and binds to lung
401 tissues as well [10, 17]. For PMNs to reach the site of infection in the lungs, they have to move from
402 the circulation across the endothelium, enter the interstitial space, then migrate across the lung
403 epithelium into the airways [11, 48]. We found here that PMN migration across pulmonary
404 endothelial cells in impaired with aging. Prior work found that PMNs from elderly human donors
405 did not show defects in transepithelial migration in response to pneumococcal infection [49] but

406 rather that chemotaxis (or directional movement) of PMNs is diminished with aging [47, 50-54].
407 PMNs isolated from both healthy controls and pneumonia patients above 60 years of age
408 displayed diminished chemotaxis compared with younger individuals [50, 51] which was linked to
409 over activation of phosphoinositide-3-kinase (PI3K) signaling [50]. PI3K is controlled downstream
410 of GPCRs including adenosine receptors [50, 55]. Here we found that A1 receptor signaling on
411 PMNs is required for their migration across pulmonary endothelial cells in response to infection
412 and that activating A1 receptor reverses the defect in early PMN pulmonary influx observed in
413 aged hosts. These findings are supported by prior work showing that A1 receptor signaling on
414 PMNs is required for their adherence to the endothelium in response to N-Formylmethionyl-
415 leucyl-phenylalanine (fMLP) or Phorbol 12-myristate 13-acetate (PMA) stimulation [56, 57]. In this
416 study we found that A1 receptor signaling was needed for the ability of PMNs to upregulate the
417 expression of the chemokine receptor CXCR2 early on in response to infection. During *S.*
418 *pneumoniae* infection, CXCR2 is required for PMN influx to the lungs [35] and in aged mice,
419 expression of CXCR2 on PMNs was reported to be lower than young hosts [52]. Therefore, it is
420 possible that activating A1 receptor signaling in aged hosts boosts PMN migration by
421 upregulating expression of CXCR2.
422 The role of A1 receptor signaling in PMN recruitment to the lungs seem to play opposing
423 roles during pathogen driven vs sterile injury. In LPS or ischemia reperfusion lung injury, A1
424 receptor signaling attenuated PMN trafficking to the lungs [58-61]. In contrast and similar to what we
425 observed here, A1 receptor signaling was required for PMN pulmonary influx during infection
426 with H1N1 influenza virus [62]. This highlights that pathogens can alter the dynamics of the immune
427 response and that the role of the adenosine signaling may differ in sterile vs. infection driven injury.

428 Extracellular adenosine is known to regulate PMN function [18] and play an important role
429 in host resistance against pulmonary infections [63]. Aging is associated with aberrant PMN
430 responses and overall increased susceptibility to pulmonary infections [43] . However, surprisingly,
431 the role of EAD in immunosenescence remains largely unexplored. In prior work we found that
432 there are age driven changes in the expression of the enzymes that make and break EAD on PMNs
433 and that EAD signaling on PMNs could be targeted to reverse the decline in their antibacterial
434 activity [34]. In this study, to examine the role extracellular adenosine signaling in the age-related
435 delay in PMN influx we tracked how this pathway changes with host aging. We found that
436 circulating levels of adenosine were no different in young and aged mice, in fact aged mice had
437 significantly more adenosine in circulation at 48 hpi. While this increase in adenosine later on in
438 infection may reflect the increased damage that is occurring in these mice (due to higher bacterial
439 burden), these data also indicate that it is not that adenosine is not being produced with age, but
440 that EAD signaling in PMNs of aged mice may be dysregulated. This is in line with prior work
441 showing that aging is associated with defects in overall GPCR signaling and that alterations in
442 EAD receptor signaling contribute to the age-associated decline of brain and heart organ functions
443 [25 64 23]. When looking at adenosine receptor expression on the surface of PMNs from aged mice
444 following pneumococcal infection, there was a decrease in receptor expression from baseline in
445 both you and aged mice. However, in PMNs isolated from young mice these receptor levels
446 rebounded by 48hpi, while receptor expression on PMNs from aged mice did not recover.
447 Following activation by an agonist, GPCR signaling is desensitized by internalization of the
448 receptor into the cell, this is a result of interaction of the G protein with β-arrestins to stop signaling
449 and additional scaffold proteins which guide trafficking the internalized GPCR [65-67]. The decrease
450 in adenosine receptor expression following pneumococcal infection may be due to this process of

451 GPCR desensitization. Following desensitization, GPCRs may be degraded but may also recycle
452 back to the cell membrane surface for resensitization, where the receptor can be activated again [66].
453 In PMNs isolated from aged mice adenosine receptor expression does not rebound following the
454 initial drop post infection. This would indicate that EAD receptors are not being recycled back to
455 the cell surface, and throughout infection, PMNs of aged mice may be less responsive to activation
456 by adenosine. This could be in part be driven by the consistently elevated levels of EAD later in
457 infection observed in old but not young hosts. Additionally, the differences in receptor levels
458 between young and aged mice later in infection could indicate that there are changes in GPCR
459 desensitization and re-sensitization in PMNs with host age.

460 A balanced PMN response where PMNs influx to the lungs and resolve at the proper time
461 is essential for effective clearance of pneumococcus and efficient host protection [12, 13, 15, 16]. In prior
462 work, we found that in young mice, EAD production by CD73 was needed for regulation of PMN
463 influx later on during infection, where by day 2 following pulmonary challenge, CD73$^{-/-}$ mice had
464 significantly more PMNs in the lungs compared to wildtype controls [10]. We hypothesized that
465 extracellular adenosine signaling via the low affinity receptors A2A and A2B receptors regulate
466 PMN resolution from the lungs later in infection when adenosine levels are higher due to increased
467 inflammation and host damage. It has been shown previously that under hypoxia conditions
468 adenosine produced by the endothelium reduced PMN accumulation by activating A2A and A2B
469 receptors on the PMN surface [68]. Surprisingly, we found here that young mice lacking A2A or A2B
470 receptors showed no changes in PMN presence in the lungs thorough the course of pneumococcal
471 infection. As both A2A or A2B receptors are expressed on PMNs and are coupled to Gs [18], it is
472 possible that they play a redundant in PMN movement and that removal of both would be needed
473 to observe a significant effect. An alternative explanation is that the levels of adenosine receptors

474 do decline on PMNs through infection even in young hosts, suggesting that PMNs may become
475 less responsive to adenosine signaling with time.

476 In summary, we have shown that A1 receptor signaling is required for early PMN influx to
477 the lungs following pneumococcal infection. Importantly, activation of the A1 receptor using a
478 specific A1 receptor agonist increased PMN influx to the lung in aged mice, which resulted in
479 lower bacterial burden in the lung. This enhanced ability to control bacterial infection could be a
480 combination of increase PMN influx as shown here and improved function as previously reported
481 [34]. These findings suggest that extracellular adenosine signaling may be dysregulated with host
482 aging and that pharmacologically targeting this pathway can enhance the immune response in aged
483 mice leading to more effective clearance of pneumococcus. Identifying a potential target to
484 enhance the immune response to pneumococcal pneumonia in aged hosts is important as this
485 disease persists in older adults despite the availability of antibiotics and vaccines [3, 5, 6]. In addition
486 to being a potential pharmacological target, the extracellular adenosine pathway is an important
487 pathway to understand in the context of host-pathogen interaction as it could be a marker of host
488 damage. This study adds dysregulation of the EAD pathway with age under the aging hallmark of
489 altered intercellular signaling, which in turn controls immunosenescence.

490

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493

494 AUTHOR CONTRIBUTIONS

495 SRS designed research, conducted research, analyzed data, and wrote paper. SES designed
496 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.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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FIGURE LEGENDS

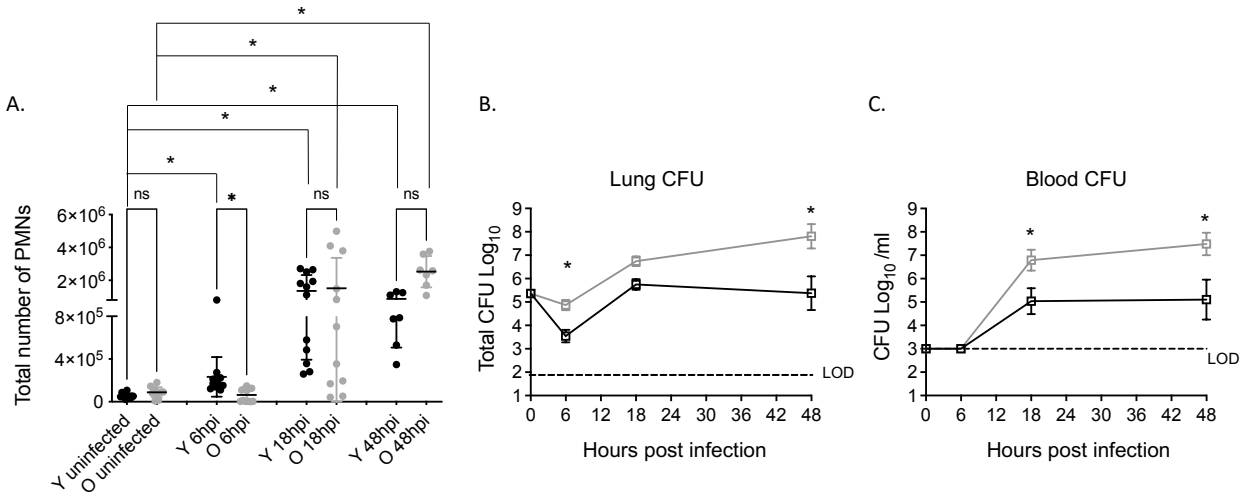
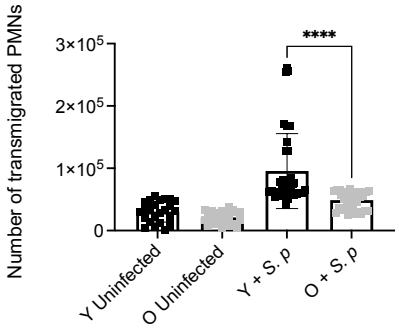


Figure 1: Old mice have delayed early pulmonary PMN influx. Young and old male C57BL/6 mice were infected i.t. with $5x10^4$ CFU *S. pneumoniae* TIGR4. (A) Lungs were harvested, digested, and stained for PMNs and analyzed by flow cytometry at the indicated timepoints following infection. (B) Lung and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



760

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

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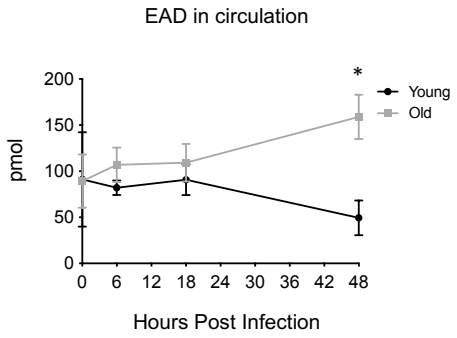

775

776 **Figure 3: Adenosine levels in circulation change with age over time** Young and old male
777 C57BL/6 mice were infected i.t. with $5x10^4$ CFU *S. pneumoniae* TIGR4 and sera was collected at
778 6, 18, and 48 hpi. Adenosine levels in the circulation were measured from two separate
779 experiments with n=3 mice at each individual time point. Asterix indicates significantly different
780 from young at the indicated timepoint as calculated by Kruskal Wallis followed by Dunn's multiple
781 comparison test.

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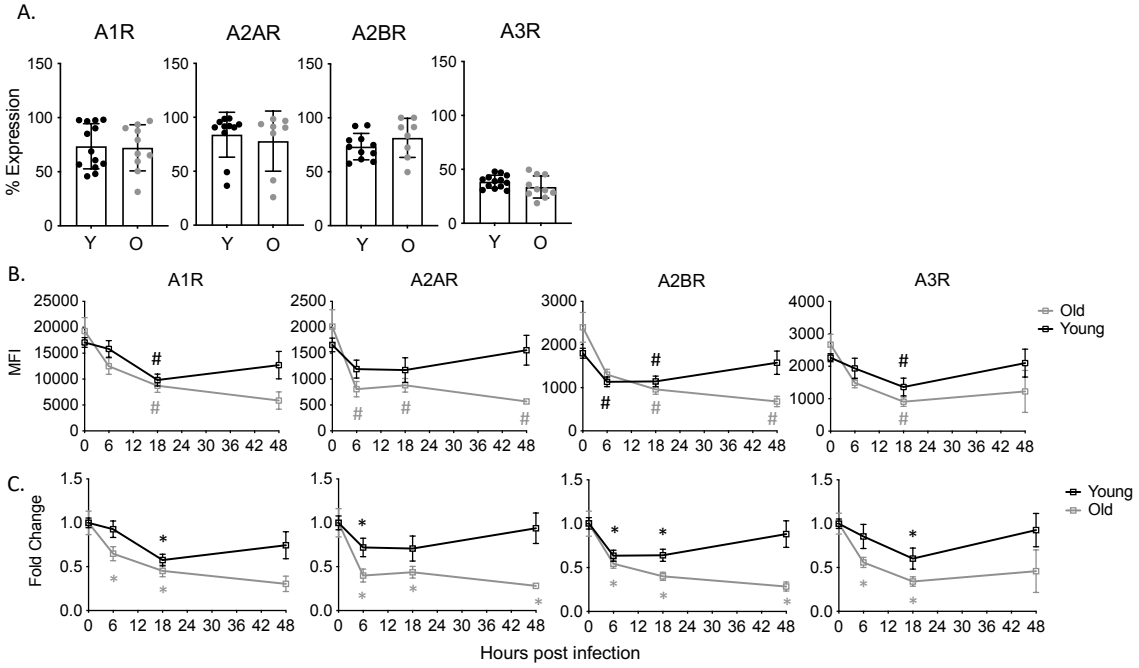
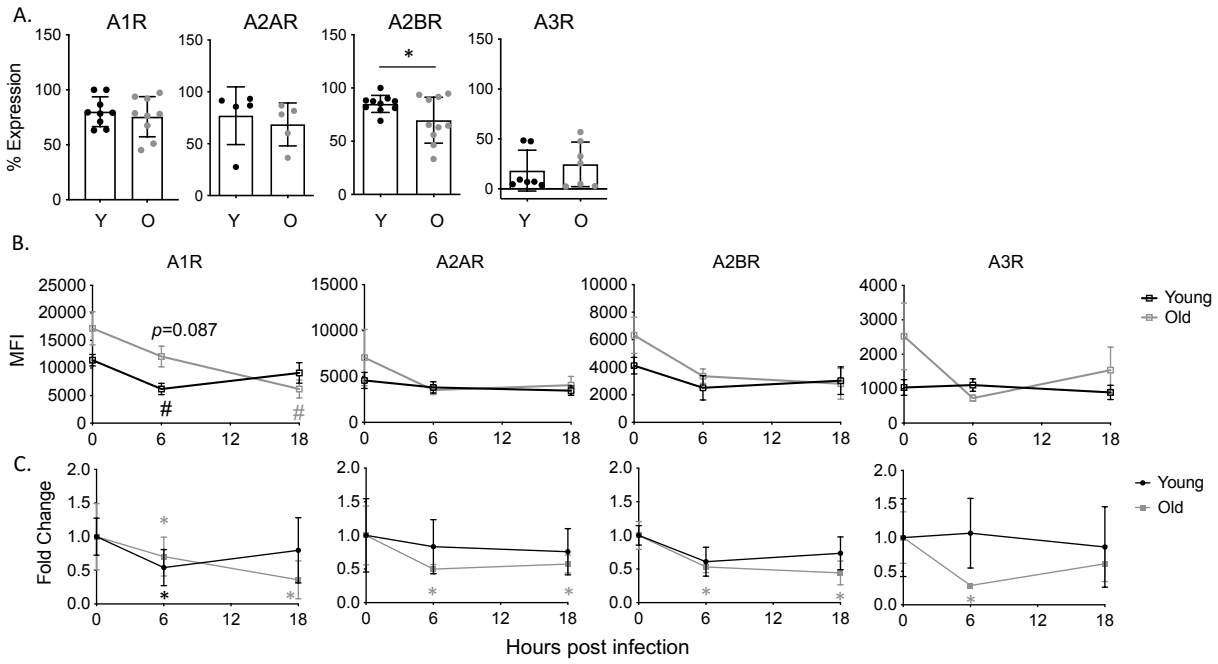


Figure 4: EAD pathway expression on pulmonary PMNs. Young and old male C57BL/6 mice were infected i.t. with $5x10^4$ CFU *S. pneumoniae* TIGR4. The lungs were harvested, and lung digests were stained for PMNs and each adenosine receptor. Percent expression at baseline (A) and MFI over time (B) of the 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.



802

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

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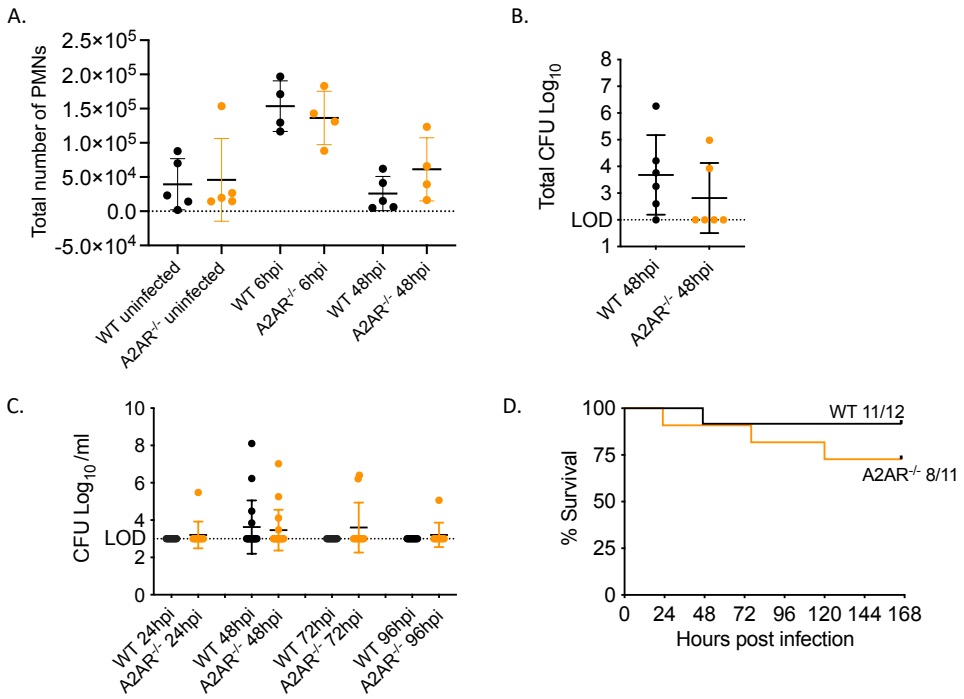

Figure 6: A2B receptor does not affect PMN pulmonary numbers *in vivo.* WT and A2BR$^{-/-}$ mice were infected i.t with 5x10^4 CFU *S. pneumoniae* TIGR4. At the indicated timepoints following bacterial challenge, lungs were harvested, digested, and stained for PMNs and analyzed by flow cytometry. Data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

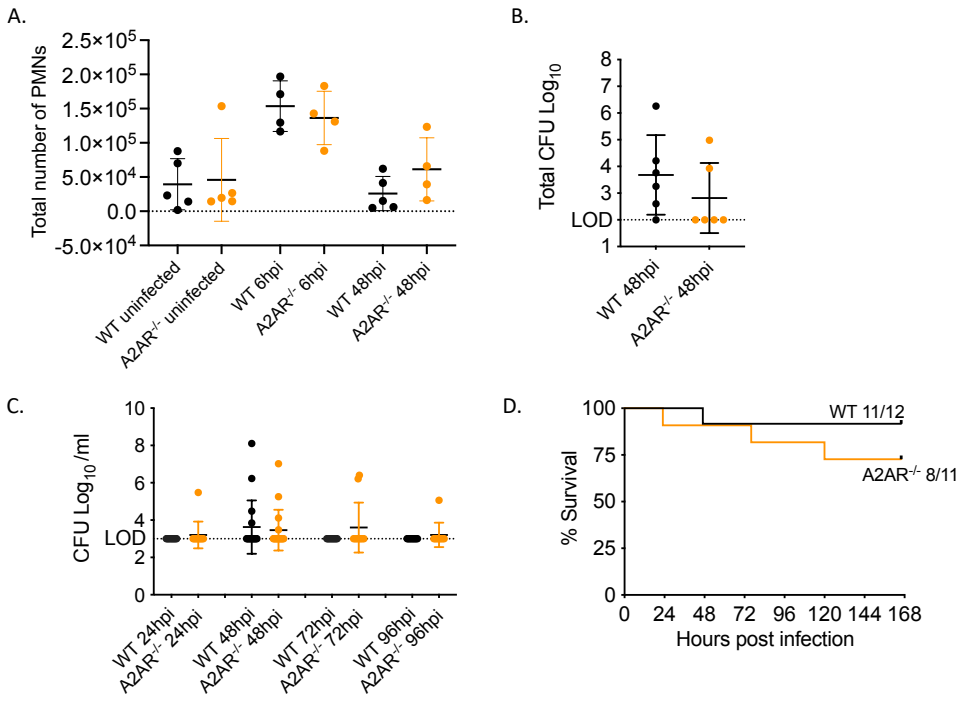

Figure 7: A2A receptor does not affect PMN pulmonary numbers or host resistance to infection. (A) WT and A2AR$^{-/-}$ mice were infected i.t with 5x10^4 CFU *S. pneumoniae* TIGR4. At the indicated timepoints following bacterial challenge, lungs were harvested, digested, and stained for PMNs and analyzed 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.

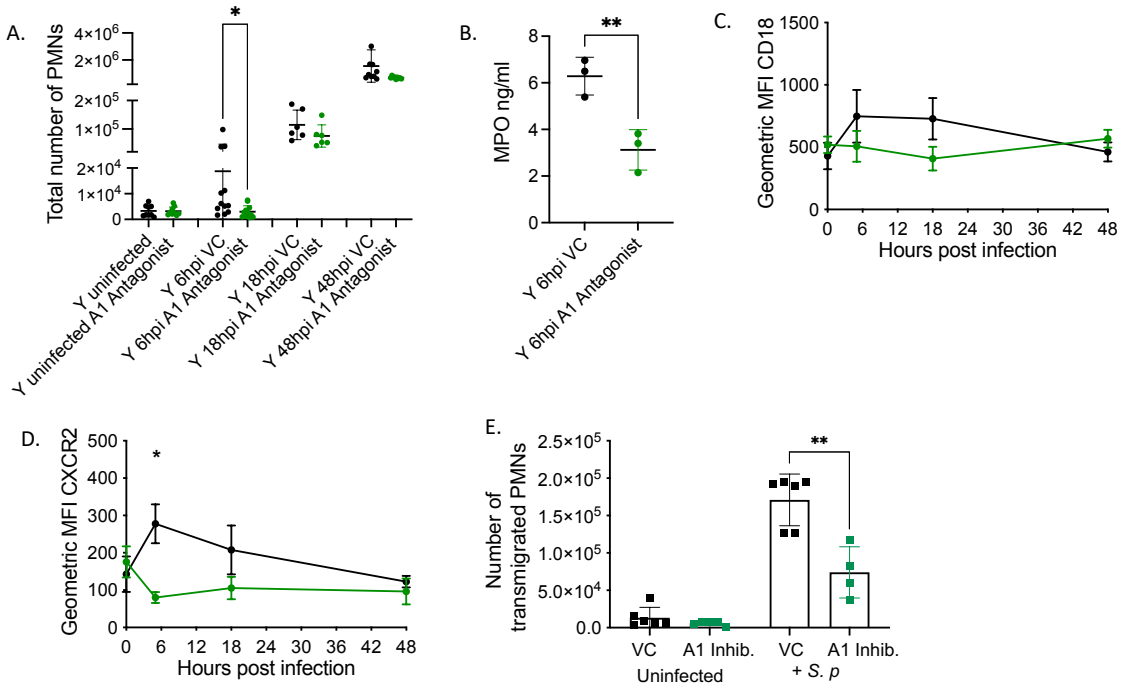

Figure 8: A1 receptor signaling is required for PMN pulmonary influx. (A) Young, male mice were treated i.p with specific A1 inhibitor and infected i.t with $5x10^4$ CFU *S. pneumoniae* TIGR4. At the indicated time points, lungs were harvested, digested, and stained for PMNs (CD45+, Ly6G+ CD11b+) (A) 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 specific A1 inhibitor or vehicle control (VC) and PMNs were isolated from the bone marrow. PMN migration across primary endothelial cells in response to *S. pneumoniae* TIGR4

851 infection was measured. Technical replicates are pooled from 3 separate experiments. Asterix
852 indicates significance as determined by unpaired student t test.

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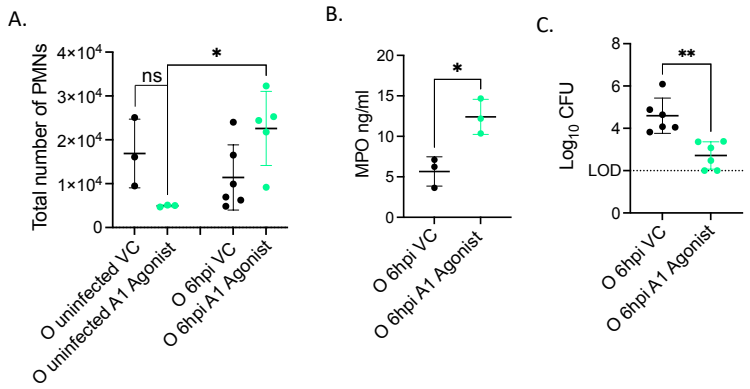

Figure 9: A1 receptor signaling rescues defect in early PMN pulmonary influx in aged mice. (A) Old male mice were treated i.p. with specific A1 agonist or Vehicle Control and infected i.t. with $5x10^4$ CFU *S. pneumoniae* TIGR4 for 6 hours. Lungs were harvested, digested, and stained for PMNs and analyzed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