A1 Adenosine Receptor Signaling Reduces *Streptococcus pneumoniae* Adherence to Pulmonary Epithelial Cells by Targeting Expression of Platelet-Activating Factor Receptor

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Short Title: Extracellular adenosine inhibits pneumococcal epithelial binding

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Abstract

Extracellular adenosine production is crucial for host resistance against *Streptococcus pneumoniae* (pneumococcus) and is thought to primarily affect antibacterial immune responses by neutrophils. However, whether extracellular adenosine alters direct host-pathogen interaction remains unexplored. An important determinant for lung infection by *S. pneumoniae* is its ability to adhere to the pulmonary epithelium. Here we explored whether extracellular adenosine can directly impact bacterial adherence to lung epithelial cells. We found that treatment of cultured human pulmonary epithelial cells with adenosine significantly reduced the ability of pneumococci to bind to these cells. Extracellular adenosine reduced *S. pneumoniae* adherence by acting on the host. Inhibition of adenosine receptor signaling using a pan antagonist blocked adenosine's ability to inhibit bacterial binding while treatment with a pan adenosine receptor agonist mimicked the effects of adenosine. Using specific receptor agonists, we found that signaling through the A1 adenosine receptor mediated the reduction in bacterial binding to pulmonary epithelial cells. A1 receptor signaling inhibited bacterial binding by reducing the expression of platelet-activating factor receptor, a host protein used by *S. pneumoniae* to adhere to host cells. *In vivo*, A1 was expressed in the lungs and was required for control of pneumococcal pneumonia as inhibiting it resulted in an increase in bacterial numbers and reduced host survival in mice. As *S. pneumoniae* remain a leading cause of community-acquired pneumonia in the elderly, we explored the role of A1 in the age-driven susceptibility to infection. We found no difference in

A1 pulmonary expression in young versus old mice. Strikingly, triggering A1 signaling boosted the ability of old mice to control *S. pneumoniae* pulmonary infection. A1 agonist-treated old mice displayed lower bacterial loads, reduced clinical signs of disease and prolonged survival. This study demonstrates a novel mechanism by which extracellular adenosine modulates resistance to lung infection by targeting bacterial-host interactions.

Author Summary

Streptococcus pneumoniae (pneumococcus) can cause life-threatening invasive infections, such as pneumonia, bacteremia and meningitis worldwide, particularly in the elderly. Understanding the pathways governing bacterial-host interactions can help us design therapies to better fight infection. Extracellular adenosine, a signaling molecule produced by the host, is crucial for resistance against pneumococcal pneumonia. Extracellular adenosine is thought to control the host immune responses, however, whether it affects host-pathogen interaction remains unexplored. Here we found that extracellular adenosine signaling directly affects pneumococcal-epithelial cell interactions. The ability of *S. pneumoniae* to adhere to epithelial cells is important for establishing lung infection. Extracellular adenosine signaling through A1, one of its receptors expressed in the lungs, inhibited the ability of *S. pneumoniae* to adhere to lung epithelial cells. Extracellular adenosine acted by reducing the expression of a host protein used by *S. pneumoniae* to adhere to epithelial cells. Importantly, A1 signaling was crucial for controlling lung infection by *S. pneumoniae* in young

68 mice and could be targeted to boost the ability of old mice to fight lung infection.
69 This study may have future implications for using clinically available adenosine-
70 based therapies to boost the resistance of vulnerable hosts like the elderly to
71 pneumococcal pneumonia.
72
73 **Introduction**
74 *Streptococcus pneumoniae* (pneumococcus) typically reside
75 asymptomatically in the nasopharynx of healthy individuals but can cause life-
76 threatening invasive diseases resulting in more than 1 million deaths per year
77 worldwide (1, 2). The majority of invasive pneumococcal infections occur in
78 elderly individuals ≥65 years of age (3). As the number of elderly individuals is
79 projected to double in the coming decades, reaching 2 billion by 2050, invasive
80 pneumococcal infections pose a serious health concern and an economic burden
81 which calls for novel interventions to fight this disease (4).
82 Lung infection is the most common form of invasive pneumococcal
83 disease (5) and the ability of *S. pneumoniae* to bind to and invade the airway
84 epithelium is a crucial step for invasive infection (1, 2). *S. pneumoniae* express
85 several factors that mediate binding to mammalian host cells and in fact many
86 bacterial virulence elements with diverse functions also double as adherence
87 factors (1, 2). This includes phosphorylcholine displayed on the bacterial cell wall
88 that mimics moieties found on host platelet-activating factor (PAF) and allows the
89 bacteria to bind to the host PAF receptor (6, 7). Platelet-activating factor receptor
90 (PAFR) is expressed on airway epithelial cells (8-10) and is upregulated during

91 pneumococcal pneumonia (8). PAFR mediates pneumococcal adhesion to
92 airway epithelial cells (7) and pharmacologically blocking this receptor decreased
93 bacterial binding *in vitro* (7, 11). Importantly blocking PAFR also attenuated *S.*
94 *pneumoniae* infection in animal models and PAFR$^{-/-}$ mice were more resistant to
95 pneumococcal lung infection (7, 12).

96 The extracellular adenosine pathway is an important regulator of host
97 defense against *S. pneumoniae* infection (13, 14). Upon cellular injury including
98 that caused by infection, ATP is released from cells and converted to adenosine
99 by the sequential action of two extracellular enzymes, CD39, which metabolizes
100 ATP to AMP, and CD73 that de-phosphorylates AMP to adenosine (15).
101 Extracellular adenosine can then act as a signaling molecule by binding to and
102 activating one of four G-protein coupled adenosine receptors, A1, A2A, A2B and
103 A3 (15). The enzymes and receptors of the extracellular adenosine pathway are
104 expressed by lung epithelial cells (16) and were shown to control acute lung
105 injury (17), ciliary motility (18), wound healing (19), activation of ion channels
106 (20), surfactant secretion (21), fibronectin release (22), mucin expression (23)
107 and production of cytokines (24).

108 Extracellular adenosine is thought to regulate host resistance to lung
109 infections by regulating recruitment and function of innate immune cells (13, 25-
110 29). For example, we previously found that CD73 deficient mice had a thousand-
111 fold more *S. pneumoniae* in their lungs as compared to wild type controls
112 following lung challenge (13). However, those studies did not assess whether
113 decreased adenosine functioned to enhance bacterial binding to lung epithelial

114 cells. Here, we report that adenosine A1 receptor signaling inhibited binding of
115 *S. pneumoniae* to human lung epithelial cells *in vitro* by reducing the expression
116 of PAFR on infected host cells. Signaling via the A1 receptor was further required
117 for control of bacterial numbers *in vivo* upon lung infection of mice. Importantly
118 triggering A1 receptor signaling reversed the susceptibility of old mice to *S.*
119 *pneumoniae* infection. This study identifies a novel mechanism by which
120 adenosine regulates host resistance to bacterial pneumonia and demonstrates
121 for the first time the feasibility of targeting this pathway to boost resistance of
122 vulnerable hosts against pneumococcal pneumonia.
123

124 **Results**

125 *Adenosine and AMP, but not ATP inhibits binding of S. pneumoniae to H292*
126 *pulmonary epithelial cells*

127 We previously found that extracellular adenosine was required for host
128 resistance against *S. pneumoniae* lung infection (13). Mice lacking CD73, an
129 enzyme required for extracellular adenosine production, had significantly higher
130 bacterial burdens in their lungs as compared to wild type mice (13). Since a
131 crucial step for establishing lung infection is the ability of *S. pneumoniae* to bind
132 to the pulmonary epithelium (1, 2), we tested whether extracellular adenosine
133 directly modulated pneumococcal binding to respiratory epithelial cells. To test
134 that, human H292 lung epithelial cells were treated with extracellular adenosine
135 or vehicle control, infected with *S. pneumoniae* and the amount of bacteria that
136 adhered to the host cells measured. We found that incubation with 100μM of

137 extracellular adenosine, which is close to the alveolar concentration of adenosine
138 in human subjects (30), significantly decreased the amount of adherent bacteria
139 that were attached to the lung epithelial cells by 3-fold (Fig 1A). This was not due
140 to direct toxicity to the bacteria, as adenosine at the concentration we tested had
141 no effect on bacterial growth as previously described (13) or bacterial viability
142 within the timeframe of this assay (Fig S1).

143 Adenosine in the extracellular milieu is produced when ATP that has
144 leaked from damaged cells is metabolized by two surface enzymes, CD39 that
145 converts ATP to AMP, and CD73, an ecto-5'-nucleotidase that de-phosphorylates
146 AMP to adenosine (15). When we measured the expression of these enzymes in
147 H292 cells, we found that mRNA levels of CD73 were readily detectable.
148 However, CD39 mRNA levels were very low (Fig 1B). Consistent with that, CD73
149 protein was abundantly expressed on H292 cells, while CD39 was not detectable
150 (Fig 1C). To confirm that the antibody we used for detection of CD39 was
151 functional, we stained PMNs isolated from human donors as a positive control
152 and were able to easily detect CD39 on those cells (Fig 1C).

153 Since H292 cells expressed CD73, but not CD39, we hypothesized that
154 AMP will also be able to decrease binding of *S. pneumoniae* to these cells, since
155 CD73 can convert it to adenosine, while ATP will have no effect since CD39 is
156 not present to initiate its dephosphorylation into adenosine. When we performed
157 the binding assays, we found that treatment of H292 cells with adenosine or AMP
158 significantly reduced the percentage of bacteria that bound when compared to
159 vehicle control treatment (Fig 1D). However, we did not observe any significant

160 effect on bacterial binding to host cells upon treatment with ATP. Taken together,
161 these data suggest that extracellular adenosine production inhibits the ability of
162 *S. pneumoniae* to bind to lung epithelial cells *in vitro*.
163
164 *Extracellular adenosine receptor signaling is required for reduction of S.*
165 *pneumoniae binding to pulmonary epithelial cells*
166 Extracellular adenosine can directly alter bacterial virulence (31, 32) and
167 also signal in the mammalian host via four known receptors (15). Therefore we
168 tested whether adenosine was altering bacterial binding by acting on the
169 bacteria, the host or both. Capsule expression regulates the ability of *S.*
170 *pneumoniae* to bind host cells (33-37). However, treatment of *S. pneumoniae*
171 with adenosine had no effect on expression of the capsular polysaccharide (Fig
172 S2A). As expected capsular deficient (Δ*cps)* bacteria bound significantly better
173 than wild type (WT) bacteria but adenosine treatment of H292 cells still reduced
174 binding of Δ*cps S. pneumoniae* by 3-fold, comparable to its effect on WT bacteria
175 (Fig S2B). Further, when we pre-treated *S. pneumoniae* only with adenosine, we
176 saw no consistent decrease in bacterial binding (Fig 2). This suggests that
177 adenosine was not mediating its effect through acting on the bacteria.
178 To test if adenosine was mediating its effect by acting on host cells, we
179 blocked adenosine receptor signaling using a pan-adenosine receptor inhibitor
180 CGS 15943. We found that the ability of adenosine to blunt bacterial binding to
181 H292 cells was abrogated when adenosine receptors signaling was blocked (Fig
182 2). Further, treatment of H292 cells with NECA, a pan adenosine receptors

183 agonist, mimicked the effects of adenosine, where bacterial binding was reduced
184 to 39% compared to the vehicle treated controls (Fig 2). The drugs had no direct
185 effect on bacterial viability as previously shown (13). These findings suggest that
186 extracellular adenosine signaling in mammalian hosts inhibits the ability of *S.*
187 *pneumoniae* to bind to lung epithelial cells.
188
189 *H292 cells express extracellular adenosine receptors*
190 There are currently four known adenosine receptors A1, A2A, A2B and
191 A3. First, we wanted to determine which adenosine receptors are expressed by
192 H292 cells and whether their expression is affected by bacterial infection. To do
193 so, we measured expression of adenosine receptors mRNA as is commonly
194 done in most studies (20, 22, 24, 38). mRNA for A1, A2A and A2B receptors was
195 readily detectable, while A3 mRNA was very low (Fig 3A). Upon infection with *S.*
196 *pneumoniae*, we saw a slight but not significant increase in mRNA levels for A1,
197 A2A and A2B with no change in mRNA levels of A3 as compared to uninfected
198 controls (Fig 3B). We then determined protein levels by flow cytometry and found
199 that H292 cells expressed all four adenosine receptors and that A2A was more
200 highly expressed on these cells compared to the other three receptors (Fig 3C).
201 Adenosine receptor protein levels were not altered upon infection of H292 cells
202 with *S. pneumoniae* (Fig 3C). We further confirmed our results with western blots
203 and found that similar to flow cytometry, all 4 adenosine receptors were detected
204 and *S. pneumoniae* infection slightly (but not significantly) increased levels of A1
205 by 1.4-fold but did not alter the expression of the other receptors (Fig 3D-E).

206 These results demonstrate that receptors for extracellular adenosine are
207 expressed in H292 pulmonary epithelial cells and that infection does not
208 significantly alter their levels.
209
210 *A1 receptor signaling inhibits binding of S. pneumoniae to pulmonary epithelial*
211 *cells*
212 Next, we wanted to identify which adenosine receptor(s) was required for
213 inhibiting bacterial binding to lung epithelial cells. These receptors bind to
214 adenosine with different affinities where the higher affinity receptors A1 and A3
215 have an EC_{50} <0.5μM whereas the intermediate affinity A2A has an EC_{50} >0.6μM
216 and the low affinity A2B an EC_{50} between 16-64μM (15). To probe which
217 receptor(s) is involved, we did a dose response, testing the effect of 100, 10, 1
218 and 0.1μM of adenosine on bacterial binding to H292 cells. We found that even
219 lower concentrations of adenosine at 10 and 1μM were as equally efficient at
220 decreasing adherence of *S. pneumoniae* as 100μM, where bacterial binding was
221 reduced to 35% relative to vehicle treated controls (Fig 4). These results
222 suggested that the higher affinity adenosine receptors A1, A2A or A3 but not the
223 low affinity receptor A2B were mediating the effect on bacterial binding observed.
224 To pinpoint the exact receptor involved, we treated H292 cells with
225 specific agonists for each of the different receptors. Since mRNA levels of A3
226 (Fig 3A) were barely detectable, we decided to proceed with testing the role of
227 the other three receptors. Consistent with the results of adenosine doses,
228 agonism of A2B receptor on H292 cells did not result in inhibition of *S.*

229 *pneumoniae* binding to these pulmonary epithelial cells and the percentage of
230 bacteria bound to treated cells were not statistically different than those bound to
231 control treated cells (Fig 4). Similarly, treatment with the A2A receptor agonist did
232 not alter bacterial binding to H292 cells (Fig 4). In contrast treatment with the A1
233 receptor agonist significantly reduced bacterial binding to H292 cells in a dose-
234 dependent manner (Fig 4). We observed a 3-fold and 2-fold reduction in the
235 amount of bound bacteria in comparison to control treated cells when cells were
236 treated with 10 or 1 nM (corresponding to respectively 10x and 1x the drug's
237 reported Ki (Fig 4). None of the adenosine receptor agonists had a direct effect
238 on bacterial viability (Fig S1). These findings suggest that specific signaling via
239 the A1 receptor reduces binding of *S. pneumoniae* to pulmonary epithelial cells.
240
241 *A1 receptor signaling reduces expression of PAFR on infected pulmonary*
242 *epithelial cells*
243 Next we wanted to identify the mechanisms by which adenosine regulated
244 bacterial binding to lung epithelial cells. *S. pneumoniae* is known to co-opt
245 several well-characterized host proteins to adhere to mammalian cells including
246 the Polymeric Immunoglobulin Receptor (PIGR) (39) and PAFR (7). Therefore,
247 we examined the effect of adenosine on the expression of these host proteins by
248 flow cytometry at baseline and during infection. As a control, we also examined
249 the expression of ICAM-1 a host protein whose expression is upregulated during
250 infection but is not thought to be a receptor for *S. pneumoniae* (40). We found
251 that consistent with previous studies, infection of H292 lung epithelial cells by *S.*

252 *pneumoniae* increased the expression of all three proteins we examined (Fig 5A-
253 C). Treatment of H292 cells with adenosine had no significant effect on the
254 expression of ICAM-1 or PIGR (Fig 5A-B). In contrast treatment of the epithelial
255 cells with adenosine moderately, but significantly reduced expression of PAFR
256 on resting uninfected epithelial cells (Fig 5C). Further treatment with adenosine
257 or A1 receptor agonist prevented upregulation of PAFR in response to infection
258 and *S. pneumoniae* infected H292 cells treated with these compounds had
259 significantly lower expression of PAFR as compared to vehicle treated controls
260 (Fig 5C).
261 To test if adenosine was mediating its effect through decreasing
262 expression of PAFR, we blocked PAFR signaling using the specific inhibitor
263 Apafant. We found that as expected (7, 11), inhibition of PAFR signaling
264 significantly reduced the ability of *S. pneumoniae* to bind to H292 cells (Fig 6).
265 Addition of the A1 receptor agonists or adenosine did not further decreased
266 bacterial binding in the presence of the PAFR inhibitor (Fig 6). These findings
267 suggest that A1 adenosine receptor signaling reduces bacterial binding to lung
268 epithelial cells by targeting PAFR receptor.
269
270 *A1 receptor signaling is required for host resistance to S. pneumoniae lung*
271 *infection*
272 We next wanted to confirm the relevance of our findings during lung
273 infection *in vivo* using a mouse model. First we checked for the expression of
274 adenosine receptors in the lungs of mice and found that consistent with previous

275 reports (20), A1 and A2A were highly expressed, while less A2B and A3 was
276 detected (Fig 7A-B). As we observed *in vitro*, *S. pneumoniae* infection did not
277 affect the expression of adenosine receptors in mouse lungs (Fig 7A-B).
278 We then evaluated the role of A1 receptor signaling in host resistance to
279 infection. We treated mice with i.p. injections of DPCPX, a A1 pharmacological
280 inhibitor that is active *in vivo* prior to infection. The mice were then challenged
281 intra-tracheally with ~$1x10^4$ CFU, a low non-lethal dose of *S. pneumoniae* (13,
282 41), and lung bacterial burdens were determined 6 hours post-infection. Since
283 extracellular adenosine modulates host resistance to infection via regulating the
284 inflammatory immune response following infection (13, 25-29), we chose to
285 challenge mice with this low dose of bacteria and examine bacterial numbers in
286 the lungs at this early time point, conditions under which we previously found
287 overt pulmonary inflammation is not yet triggered (13). We further confirmed that
288 the A1 inhibitor had no effect on the baseline numbers of neutrophils,
289 macrophages and T cells in the lungs (data not shown). We found that inhibition
290 of A1 receptor signaling *in vivo* resulted in a significant ~10-fold increase in
291 bacterial numbers in the lungs following pulmonary challenge (Fig 8A). At 6 hours
292 post infection, the infection was still localized to the lungs and had not spread
293 systemically. However, when we compared bacteremia at 24 hours following
294 infection, we found that while none of the control mice were bacteremic, 25% of
295 A1 inhibited mice had systemic spread of the infection (Fig 8B). Importantly,
296 inhibition of A1 significantly resulted in increased signs of clinical disease (Fig
297 8C) and significantly more host death when mice were challenged at an

298 otherwise non-lethal dose (Fig 8D). These findings demonstrate that specific
299 signaling via the A1 receptor is required for early host resistance to *S.*
300 *pneumoniae* lung infection.
301
302 *Triggering A1 receptor signaling boosts resistance against S. pneumoniae lung*
303 *infection in aged hosts*
304 As pneumococcal infections are particularly a problem in the elderly, we
305 examined the effect of aging on adenosine receptor expressions in the lung. We
306 compared pulmonary expression levels of the adenosine receptors in young (2
307 months) versus old (18-22 months) mice. We found that there were no significant
308 differences in the expression of any of the adenosine receptors in the lungs of old
309 mice as compared to young controls either at baseline or following lung
310 challenge with *S. pneumoniae* (Fig 7B).
311 We previously found that mice mimic the age-driven susceptibility to *S.*
312 *pneumoniae* lung infection observed in humans (41). To test whether A1 receptor
313 can boost the resistance of old mice to lung infection, mice were treated i.p. with
314 2-Chloro-N6-cyclopentyladenosine, the A1 agonist prior to infection. Mice were
315 then challenged intra-tracheally with *S. pneumoniae*, and bacterial burdens in the
316 lung were determined 6 hours post-infection. As expected, vehicle control treated
317 old mice were more susceptible to pneumococcal infection and had ~ 100-fold
318 more bacteria in their lungs at 6 hours (Fig 8A) and blood at 24 hours (Fig 8B) as
319 compared to young controls. Strikingly, treatment with the A1 agonist boosted the
320 resistance of old mice to infection. A1 agonist treated old mice had 70-fold less

321 bacteria in their lungs, a bacterial burden that was indistinguishable from that of
322 control treated young mice (Fig 8A). Further, by 24 hours post infection, old mice
323 given the A1 agonist had significantly less bacteria in their systemic circulation
324 when compared to controls (Fig 8B). Importantly, triggering A1 significantly
325 decreased clinical sign of sickness (Fig 8C) and slightly delayed host death and
326 boosted survival of old mice (Fig 8E). These data demonstrate that A1 receptor
327 can be targeted to boost the innate resistance of aged hosts to *S. pneumoniae*
328 lung infection.
329

330 **Discussion**

331 The role of the extracellular adenosine pathway in modulating host
332 resistance against pulmonary pathogens is becoming better appreciated.
333 Previous studies using murine models of infection showed that deficiency of
334 adenosine A2B or A1 receptors was protective against *Klebsiella pneumoniae*
335 (28) and influenza (26) lung infection respectively, whereas overexpression of
336 CD39 on airway epithelial cells promoted *Pseudomonas aeruginosa* clearance
337 from the lungs (25). We previously found that extracellular adenosine production
338 by CD73 was crucial for control of pulmonary bacterial numbers and systemic
339 spread following lung challenge with *S. pneumoniae* (13). In all of the above
340 studies, extracellular adenosine was believed to modulate pathogen clearance by
341 either controlling the recruitment of immune cells, particularly neutrophils and/or
342 regulating their antimicrobial activity (13, 25-29). Here we show that apart from its
343 effect on innate immune cells, extracellular adenosine modulates host resistance

344 by acting on pulmonary epithelial cells, targeting expression of mammalian
345 proteins co-opted by *S. pneumoniae* as receptors and reducing the ability of
346 bacteria to bind host cells.

347 Few studies have examined the effect of adenosine on bacterial infection
348 of epithelial cells from different organs (31, 32, 42-44). Adenosine signaling via
349 the A2B receptor inhibited intracellular persistence of *Chlamydia trachomatis* in
350 cervical epithelial cells (44) while A2A receptor promoted intracellular replication
351 of *Porphyromonas gingivalis* in gingival epithelial cells (43). In intestinal epithelial
352 cells, adenosine promoted bacterial infections, where addition of exogenous
353 adenosine increased adhesion of *Salmonella* to gut epithelial cells (42), and
354 expression of the adenosine producing enzyme CD73 on intestinal epithelial cells
355 promoted the intracellular replication as well as translocation and systemic
356 spread of *Salmonella* across the gut epithelium (32). Similarly, adenosine altered
357 the adhesion patterns of enteropathogenic *Escherichia coli* to gut epithelial cells
358 and promoted intestinal infection *in vivo* (31). Thus the effect of adenosine on
359 bacterial infection of the epithelium may be organ and adenosine-receptor
360 specific. We demonstrated here that adenosine signaling via the A1 receptor
361 inhibited adherence of *S. pneumoniae* to H292 epithelial cells and reduced lung
362 infection *in vivo*, which is to our knowledge, the first report to show an effect of
363 the extracellular adenosine pathway on bacterial infection of pulmonary epithelial
364 cells. In certain infections adenosine had a direct effect on bacterial viability and
365 expression of virulence genes (31, 32). However, that was not the case here as
366 adenosine neither affected *S. pneumoniae* viability or expression of capsular

367 polysaccharide, a major determinate of bacterial adhesion, nor did treatment of
368 the bacteria alone alter their ability to adhere to lung epithelial cells.
369 Purinergic signaling plays a crucial role in regulating responses in the
370 airways (16). We found here that adenosine was exerting its effect on bacterial
371 binding through signaling via adenosine receptor A1 on host epithelial cells. The
372 pulmonary epithelial cells used *in vitro* in this study were H292 human lung
373 epithelial cells that are a mix of type I and type II pneumocytes (45). We found
374 that H292 cells expressed mRNA for A2B, A1 and A2A in decreasing levels while
375 A3 levels were extremely low. This is consistent with previous studies with A549
376 cells, a type II pneumocyte cell line (22) as well as primary bronchial epithelial
377 cells (24). Similar to other studies, despite differences at the mRNA levels, we
378 could detect protein expression for all four adenosine receptors (38). Lung
379 epithelial cells are also known to express P2 purinergic receptors that respond to
380 ADP and/or ATP (16) and ATP and/or ADP impaired replication of *Chlamydia*
381 *trachomatis* in cervical epithelial cells (46). However, addition of ATP did not
382 alter *S. pneumoniae* binding to the lung epithelial cells *in vitro* while AMP
383 significantly decreased bacterial binding. We believe that AMP but not ATP
384 decreased bacterial binding since H292 cells express CD73 that can
385 dephosphorylate AMP into adenosine but lack CD39 that is required for
386 converting ATP into AMP. *In vivo*, the ability of epithelial cells to produce
387 adenosine themselves may not be relevant as adenosine can be produced by
388 many cells that influx into sites of infection and damage including immune cells
389 (47).

390 Here, A1 receptor signaling was important for control of *S. pneumoniae*
391 lung infection *in vivo*. We found that A1 and A2A receptors were expressed in
392 mouse lungs, while expression of A2B and A3 was lower, similar to what has
393 been described before (20). A1 receptor is known to play an important role in
394 acute lung injury by regulating inflammation, particularly pulmonary influx of
395 neutrophils (26, 48-50). While we cannot differentiate whether the effect of
396 adenosine *in vivo* is due to a direct effect on the lung epithelium or innate
397 immune cells, we used a low dose infection and examined bacterial burdens
398 early on after 6 hours of infection since we previously found that at this dose and
399 this time point very little PMNs influx into the airways (13).
400 We found that adenosine and A1 receptor signaling down regulated
401 expression of PAFR on *S. pneumoniae* infected lung epithelial cells. PAFR is a
402 host protein used by *S. pneumoniae* to adhere to airway epithelial cells (7). When
403 PAFR was inhibited, we found that bacterial binding to the epithelium was
404 reduced by half, consistent with previous reports (7, 11). Triggering A1 receptor
405 did not further reduce binding when PAFR was inhibited, suggesting that
406 signaling via A1 receptor reduces pneumococcal binding via targeting PAFR.
407 PAFR is expressed in the lungs (8) and impairs host resistance against
408 pneumococcal pneumonia (7, 12, 51). We observed that PAFR expression is up
409 regulated on lung epithelial cells upon infection, consistent with previous reports
410 (8). Therefore the reduced expression of PAFR in infected cells treated with
411 adenosine or the A1 receptor agonist may be an indirect effect of less bacteria
412 bound, however, we believe that this is unlikely to be a simple reflection of bound

413 bacteria since expression of both ICAM-1 and PIGR, two other host proteins up
414 regulated upon infection were not affected by adenosine. It is more likely that
415 adenosine is indirectly regulating PAFR expression by controlling inflammatory
416 responses of epithelial cells to infection. Previous studies found that PAFR was
417 up regulated on epithelial cells activated by cytokines or other factors, which
418 enhances bacterial binding (7, 10, 52), and adenosine receptor signaling
419 controlled cytokine responses by epithelial cells (24). This could explain why we
420 only see A1 receptor signaling reducing expression of PAFR in *S. pneumoniae*
421 infected but not resting epithelial cells. In addition to bacterial adherence to the
422 lung epithelium, PAFR also has a role in pneumococcal invasion of epithelial and
423 endothelial cells and systemic spread of the infection from the lungs to the
424 circulation *in vivo* (7, 53, 54). However, we did not detect any bacteremia in
425 either A1 inhibited or control treated mice at the early 6-hour time point following
426 *S. pneumoniae* lung challenge.
427 Changes in extracellular adenosine production, metabolism and signaling
428 occur with aging (55-57). This is reflected by changed levels and activity of the
429 EAD-producing and degrading enzymes (58, 59), altered expression of
430 adenosine receptors (56), and an overall decline in G-protein coupled receptor
431 signaling (60). This pathway has been reported to play an important role in the
432 age-related decline of brain (58), metabolic (61) and cardiac function (56), and
433 importantly, can be targeted to reverse this decline in animal models (62-64).
434 However, the role of the extracellular adenosine pathway in age-driven
435 susceptibility to infections remains completely unexplored. We found here that

436 there was no difference in the expression levels of adenosine receptors in the
437 lungs of young versus old mice. Strikingly, treatment of old mice with an A1
438 receptor agonist significantly boosted their resistance to pneumococcal lung
439 infection where they were able to control early pulmonary bacterial burdens, had
440 reduced systemic spread of the infection and extended survival compared to
441 control treated mice. PAFR expression was reported to be upregulated at
442 baseline in the lungs of old mice (9). Therefore it is possible that triggering A1
443 receptors boots the resistance of aged hosts to *S. pneumoniae* by reducing
444 PAFR expression in the lungs. These findings demonstrate that targeting the
445 adenosine pathway may be a feasible strategy to reverse the innate susceptibility
446 of aging to lung infection.
447 *S. pneumoniae* binds to PAFR via phosphorylcholine displayed on the
448 bacterial cell wall that mimics moieties found on host platelet-activating factor.
449 This form of molecular mimicry is shared by other pathogens (65), including
450 *Haemophilus influenzae* (66), *Neisseria* (67, 68), *Pseudomonas aeruginosa* (69),
451 *Acinetobacter baumannii* (70) and is used by bacteria to bind to PAFR and
452 adhere to host cells but can also regulate inflammatory responses in the host
453 (65, 71). Intriguingly a pilot study found that single nucleotide polymorphisms in
454 *PTAFR* gene coding for PAFR was associated with increased risk of invasive
455 pneumococcal disease in humans, although these results need to be validated
456 as no correction for multiple testing was performed (72). In conclusion, our
457 findings regarding the effect of adenosine on PAFR expression and bacterial
458 binding on lung epithelial cells may have future implications for using clinically

459 available adenosine-based therapies (15) to boost host resistance to *S.*
460 *pneumoniae* and other serious lung infections.
461
462 **Materials and Methods**
463 **Ethics statement**
464 This work was performed in accordance with the recommendations in the Guide
465 for the Care and Use of Laboratory Animals published by the National Institutes
466 of Health. All procedures were reviewed and approved by the University at
467 Buffalo Institutional Animal Care and Use Committee (IACUC) and are under
468 protocol # MIC33018Y.
469
470 ***Mice***
471 Young (2 months) and Old (18-22 months) C57BL/6 mice were purchased from
472 Jackson Laboratories (Bar Harbor, ME) and the National Institute on Aging
473 colonies and housed in a specific-pathogen free facility at the University at
474 Buffalo. Male mice were used in all experiments.
475
476 ***Bacteria***
477 Wild type *S. pneumoniae* TIGR4 strain (serotype 4), were grown at 37°C with 5%
478 CO_2 in Todd-Hewitt broth (BD Biosciences) supplemented with 0.5% yeast
479 extract and Oxyrase (Oxyrase) untill mid-exponential phase. Aliquots were then
480 frozen at -80°C in the growth media with 25% (v/v) glycerol. Before use,
481 bacterial aliquots were thawed on ice, washed once and diluted in PBS to the

482 required concentrations. Bacterial titers were confirmed by plating on Tryptic Soy
483 Agar plates supplemented with 5% sheep blood agar (blood agar plates-
484 Northeast Laboratory Services). The Δ*cps* deletion mutant was a kind gift from
485 Andrew Camilli (73).
486
487 ***PMN isolation***
488 PMNs were isolated from the peripheral blood of healthy donors as previously
489 described (74). Volunteers were recruited in accordance with the University at
490 Buffalo Human Investigation Review Board (IRB) and signed informed consent
491 forms.
492
493 ***Bacterial binding assay with H292 cells***
494 Human pulmonary mucopidermoid carcinoma-derived NCI-H292 (H292) cells
495 were purchased from ATCC and grown at 37°C/ CO_2 in "H292" media consisting
496 of RPMI 1640 medium (ATCC) with 2 mM L-glutamine, 10% FBS, and 100 U
497 penicillin/streptomycin following a previously described (45). The day before the
498 binding assay 2.5 x 10^5 H292 cells were seeded in tissue culture-treated flat
499 bottom 96-well plates (Corning) and allowed to adhere overnight. The following
500 day, cells were washed in PBS and antibiotic-free H292 media was added. The
501 cells were then pretreated for 30 minutes with the indicated drugs or PBS as a
502 vehicle control and then infected with *S. pneumoniae* at an MOI of 10-20. The
503 plates were spun down and incubated for 1 hour at 37°C/ CO_2. To determine the
504 amount of bacteria that adhered, the cells were washed with PBS, lifted with

505 0.05% Trypsin/EDTA (Invitrogen). Serial dilutions were then plated on blood agar
506 plates for determination of bacterial colony forming units (CFU). The percent of
507 bacteria bound were determined with respect to a no cell control where bacteria
508 were added to the wells and incubated for an hour under the same experimental
509 conditions (+/-drugs) and then viable bacteria enumerated by plating on blood
510 agar plates. The amount of bacteria bound to control treated cells was set as 100
511 % and relative changes in bacterial binding in treated cells was then calculated
512 and is presented as % bound relative to vehicle treated controls on the graphs.
513
514 ***Adenosine pathway and PAFR drugs***
515 The effect of the extracellular adenosine pathway was tested using the following:
516 Adenosine (Sigma) diluted in PBS and used at the concentrations indicated on
517 the graphs. The pan adenosine receptor agonist 5′-(N-Ethylcarboxamido)
518 adenosine (NECA) used at 1μM. This agonist targets all four receptor with
519 reported Ki values of 6.2, 14, and 20 nM for human A3, A1 and A2A receptors
520 respectively and EC50 of 2.4 μM for human A2B. The pan adenosine receptor
521 inhibitor (ADRI) CGS-15943 used at 1μM. This inhibitor targets all four adenosine
522 receptors, with Ki values of 3.5, 4.2, 16 and 51 nM for human A1, A2A, A2B and
523 A3 receptors respectively. The A1 agonist 2-Chloro-N6-cyclopentyladenosine
524 used at 10, 1, 0.1 nM, A2A agonist CGS21680 used at 270, 27, 2.7nM and A2B
525 agonist BAY 60-6583 used at 200, 20, 2nM. These concentrations correspond to
526 the approximate 10xKi, Ki or 0.1Ki for each receptor. Adenosine receptor drugs
527 were purchased from Tocris or Sigma Aldrich, dissolved in DMSO and dilutions

528 performed in PBS. The potent PAFR antagonist Apafant was also purchased
529 from Tocris and used at 40nM corresponding to 2xKi.
530
531 ***Mouse infections***
532 The effect A1 receptor on lung infection was determined using the competitive
533 inhibitor of A1 receptor, 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX) or A1
534 agonist 2-Chloro-N6-cyclopentyladenosine. All chemicals were purchased from
535 Sigma Aldrich, dissolved in DMSO and filter sterilized by passing through a
536 0.22μm filter. The mice were then given intraperitoneal (i.p.) injections of 1mg/kg
537 of the A1 inhibitor or 0.1mg/Kg of the A1 agonist or vehicle control at days -1 and
538 0 (immediately before challenge) relative to infection. Control mice were mock-
539 treated with the vehicle control. Mice were challenged intra-tracheally with 1-
540 $2x10^4$ *S. pneumoniae* TIGR4 as previously described (13). Six hours post-
541 infection, mice were euthanized and lung and blood samples were harvested and
542 plated on blood agar plates for enumeration of bacterial loads. Following the
543 infection, mice were monitored daily and scored blindly for signs of sickness
544 including weight loss, activity, posture and breathing. Based on these criteria, the
545 mice were given a clinical score of healthy [0] to severely sick [21] as previously
546 described (75).
547
548 ***Flow cytometry***
549 For examining adenosine receptor expression in the lungs, mice were perfused
550 with 10ml PBS, the lungs harvested and minced into small pieces. The lungs

551 were then digested for 1 hour with RPMI 1640 supplemented with 10% FBS, 1
552 mg/ml Type II collagenase (Worthington), and 50 U/ml Deoxyribonuclease I
553 (Worthington) at 37°C/ 5% CO_2. Single-cell suspensions were obtained by
554 mashing the digested lungs, and the red blood cells were removed by treatment
555 with a hypotonic lysis buffer (Lonza). For examining expression of host proteins
556 on H292 cells, the cells were infected with *S. pneumoniae* at an MOI of 2 as
557 described in the binding assay. The cells were resuspended in FACS buffer
558 (HBSS/ 1% FBS) then treated with Fc block (anti-mouse clone 2.4G2 and anti-
559 human clone 3G8) purchased from BD and stained with specific antibodies. The
560 following conjugated anti-human antibodies were used: PAFR (040582;
561 Cederlane), ICAM (clone HA58; Biolegend), CD73 (clone TY/11.8; eBioscience),
562 CD39 (clone24DMS-1; eBioscience) and PIGR (PA535340; Invitrogen). For
563 staining for adenosine receptor antibodies, the cells were permeabilized using
564 the BD Cytofix/Cytoperm kit. The following unconjugated primary rabbit
565 polyclonal anti-adenosine receptor antibodies were purchased from abcam and
566 used: anti-mouse and human A2a (ab3461), A2b (ab222901) and A3 (ab203298)
567 as well as anti-mouse A1 (ab82477). Rabbit polyclonal IgG (ab37415) purchased
568 from abcam and used as an isotype control. For anti-human A1 a primary rabbit
569 monoclonal antibody was used (clone ERP6 179, ab124780). Secondary PE-
570 conjugated anti-Rabbit IgG was used (12473981; Invitrogen). Fluorescence
571 intensities were measured on a BD FACS Fortessa and data were analyzed
572 using FlowJo.
573

574 **RNA extraction and qPCR**

575 RNA was extracted from 2×10^5 H292 using the RNeasy Mini Kit (Qiagen) as per
576 manufacturer's protocol. TURBO DNA-free kit (Invitrogen) was used to digest
577 DNA from the RNA samples prior conversion into cDNA. For each sample, 500ng
578 of RNA was converted into cDNA using SuperScript VILO™ cDNA synthesis kit
579 (Life Technologies) according to the manufacturer's protocol. RT-PCR was
580 performed using CFX96 Touch™ Real-Time PCR Detection System from Bio-
581 Rad and CT (cycle thresh-hold) values were determined using the following
582 TaqMan probes from Life Technologies (Thermo Fischer Scientific): GAPDH
583 (Hs99999905_m1), A1 (Hs00181231_m1), A2A (Hs00169123_m1), A3
584 (Hs00252933_m1), and A2B (Hs00386497_m1). All samples were run in
585 duplicates. Data were analyzed by the comparative threshold cycle ($2^{-\Delta CT}$)
586 method, normalizing the CT values obtained for target gene expression to those
587 for GAPDH of the same sample. For comparison of adenosine receptor levels
588 upon infection, relative quantity of transcripts (RQ) values were calculated by the
589 ΔΔCT method by using the formula RQ =2^– (ΔΔCT) (76). The ΔΔCT values
590 were obtained by subtracting ΔCT value of the infected from that of the
591 uninfected control.

592

593 ***Western blotting***

594 H292 cells were solubilized in radioimmunoprecipitation assay (RIPA) buffer (1%
595 Triton X-100, 0.25% sodium deoxycholate, 0.05% SDS, 50 mM Tris-HCl [pH 7.5],
596 2 mM EDTA, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium

597 orthovanadate, 10 mg/liter each of aprotinin and leupeptin). Lysates were
598 centrifuged and protein concentrations of the supernatants were quantified using
599 bicinchoninic acid kit (Pierce). Equal protein quantities of each sample were
600 migrated on Mini-PROTEAN TGX Stain-Free Precast Gels (BioRad) and
601 transferred to polyvinylidene difluoride (PVDF) membranes. Incubations with
602 primary antibodies at 1:1000 dilutions were done overnight at 4°C. Incubation
603 with secondary antibodies (1: 5000 dilutions) coupled to horseradish peroxidase
604 was done for 1 hour at room temperature. ChemiDoc XRS+ system (BioRad)
605 was used for detection of all Western blots using Clarity Western ECL Substrate
606 (BioRad). Blots were stripped in stripping buffer containing glycine, Triton-X 100
607 and SDS, and probed for GAPDH, which served as a loading control. Primary
608 antibodies against A1 (ab124780), A2A (ab3461), A2B (ab222901) and A3
609 (ab203298) were all obtained from Abcam. Primary antibodies against GAPDH
610 (MA5-15738) and horseradish peroxidase-conjugated secondary antibodies
611 (31460 and 31430) were purchased from Invitrogen. Images were quantified
612 using ImageJ (version 1.51e) software. Integrated pixel densities in bands
613 corresponding to proteins of interest were measured, and the background was
614 subtracted. Integrated pixel densities for GAPDH as loading controls were also
615 determined and corrected for the background. Background-corrected integrated
616 pixel densities of the protein of interest were normalized to those of the loading
617 control.
618
619 ***Statistics***

620 All statistical analysis was performed using Prism7 (Graph Pad). CFU data were
621 log-transformed to normalize distribution. For all graphs, the mean values +/- SD
622 are presented. Significant differences were determined by 1-sample t-test or
623 Student's t-test as indicated. Survival analysis was performed using the log-rank
624 (Mantel-Cox) test. *p* values less than 0.05 were considered significant (as
625 indicated by asterisks).

626

627 **Acknowledgments**

628 We would like to acknowledge Andrew Camilli for bacterial strains as well as
629 Anthony Campagnari and Ira Blader for their feedback on the manuscript.

630

631 **Legends**

632 **Fig 1. Extracellular adenosine reduces binding of *S. pneumoniae* to**
633 **pulmonary epithelial cells.** (A) H292 cells were treated with adenosine (ADE,
634 100μM) or vehicle control (VC) for 30 minutes and then infected with *S.*
635 *pneumoniae* TIGR4 for 1 hour. The cells were then washed and the amount of
636 bound bacteria were enumerated by plating on blood agar plates. (B) RNA was
637 isolated from uninfected H292 cells and the expression of the indicated enzyme
638 mRNA was measured by RT-qPCR and normalized to GAPDH. (C) Expression
639 of CD73 (left panel) on H292 cells and CD39 on H292 cells and PMNs (right
640 panel) was assessed by flow cytometry. Histograms shown are representative
641 data from one of three separate experiments. (D) H292 cells were treated with
642 either ADE, ATP, AMP (100μM) or vehicle control (VC) for 30 minutes and then

643 infected with *S. pneumoniae* TIGR4 for 1 hour. The number of bound bacteria
644 was enumerated by plating on blood agar plates. The percent of bacterial
645 binding was then calculated relative to vehicle control treatment that was set to a
646 100. (A and D) Data shown are pooled from four separate experiments (n=4
647 biological replicates) where each condition was tested in triplicate (n=3 technical
648 replicates) per experiment. Asterisks indicate significance calculated by Student's
649 t-test.
650
651 **Fig 2. Signaling via extracellular adenosine receptors is required for**
652 **reduction of *S. pneumoniae* binding to pulmonary epithelial cells.** H292
653 cells were treated with either 100μM ADE, 100μM ADE in the presence of 1μM
654 the pan adenosine receptors inhibitor (ADRI) CGS 15943, 1μM the pan
655 adenosine receptors agonist 5'-*N*-Ethylcarboxamidoadenosine (NECA) or vehicle
656 control (VC) for 30 minutes and then infected with *S. pneumoniae* TIGR4 for 1
657 hour. The number of bound bacteria was enumerated by plating on blood agar
658 plates and the percent bacterial binding calculated relative to vehicle control.
659 Data shown are pooled from three separate experiments where each condition
660 was tested in triplicate. Asterisks indicate significance calculated by Student's t-
661 test.
662
663 **Fig 3. Expression of adenosine receptors on H292 cells.** (A) RNA was
664 isolated from uninfected H292 cells and the expression of the indicated
665 adenosine receptor mRNA was measured by RT-qPCR and normalized to

666 GAPDH. (B) RNA was isolated from H292 cells mock treated (uninfected) or
667 challenged with *S. pneumoniae* TIGR4 MOI of 2 for 1 hour and the relative
668 change in expression of the indicated adenosine receptor mRNA upon infection
669 was calculated by the ΔΔCT method. Data shown are pooled from three separate
670 experiments. H292 cells were infected with *S. pneumoniae* TIGR4 MOI of 2 for 1
671 hour or mock treated with PBS (uninfected). (C) Expression of adenosine
672 receptors was assessed by flow cytometry. The percentage of cells expressing
673 the receptors was then measured. Data shown are pooled from three separate
674 experiments. (D) Expression of adenosine receptors was assessed by Western
675 Blotting. One representative blot for each receptor is shown. (E) Western Blot
676 band intensities were analyzed using ImageJ and expression of infected sample
677 relative to uninfected controls is shown. Data presented are pooled from two
678 experiments. (B and E) Adenosine receptor mRNA or protein levels upon
679 infection were not significantly different from 1 by one-sample t-test.
680
681 **Fig 4. Signaling via A1 receptor is required for inhibiting binding of *S.***
682 ***pneumoniae in vitro* and *in vivo.*** (A) H292 cells were treated for 30 minutes
683 with either the indicated concentrations of ADE, vehicle control (VC) or at the
684 following concentrations of A1 agonist 2-Chloro-N6-cyclopentyladenosine
685 (10, 1, 0.1 nM), A2A agonist CGS21680 (270, 27, 2.7nM) or A2B agonist BAY
686 60-6583 (200, 20, 2nM) corresponding to the approximate 10xKi, Ki or 0.1Ki for
687 each receptor as indicated on the graph. The cells were then infected with *S.*
688 *pneumoniae* TIGR4 for 1 hour. The number of bound bacteria was determined by

689 plating on blood agar plates and the percent bacterial binding calculated relative
690 to vehicle control treatment. Data shown are pooled from three separate
691 experiments where each condition was tested in triplicate. Asterisks indicate
692 significant differences as compared to VC calculated by Student's t-test.
693
694 **Fig 5. Signaling via A1 receptor reduces expression of PAFR on H292 cells.**
695 H292 cells were treated for 30 minutes with vehicle control (VC), ADE (100μM),
696 or A1 agonist 2-Chloro-N6-cyclopentyladenosine (10nM) as indicated. The cells
697 were then infected with *S. pneumoniae* TIGR4 MOI of 2 or mock treated
698 (uninfected) for 1 hour. (A-C) Expression of ICAM-1, PIGR and PAFR was
699 assessed by flow cytometry. The Mean florescent intensities (MFI) as well as the
700 percentage of cells expressing the indicated ligands were then measured. (A-C)
701 Data shown are pooled from three separate experiments where each condition
702 was tested in triplicate. Asterisks indicate significant differences calculated by
703 Student's t-test.
704
705 **Fig 6. A1 receptor signaling does not further decrease bacterial binding**
706 **when PAFR is inhibited.** H292 cells were treated for 30 minutes with the
707 indicated vehicle control (VC), ADE (100μM), A1 agonist 2-Chloro-N6-
708 cyclopentyladenosine (10nM) or PAFR inhibitor (40nM). The cells were then
709 infected with *S. pneumoniae* TIGR4 MOI of 20 for 1 hour. The number of bound
710 bacteria was enumerated by plating on blood agar plates and the percent
711 bacterial binding calculated relative to vehicle control. Data shown are pooled

712 from four separate experiments where each condition was tested in triplicate.
713 Asterisks indicate significant differences vs. vehicle control treated cells
714 calculated by Student's t-test.
715
716 **Fig 7. Pulmonary expression of adenosine receptors in mice are not altered**
717 **by aging or *S. pneumoniae* infection.** Young and old C57BL/6J mice were
718 infected with *S. pneumoniae* intra-tracheally. The lungs were harvested six hours
719 post infection and digested into a single cell suspension and the expression of
720 adenosine receptors analyzed by flow cytometry. (A) Histograms shown are
721 representative data from young mice and (B) bar graphs are quantification of the
722 Mean Florescent Intensities (MFI) of the indicated adenosine receptor from
723 young and old mice and are pooled from six separate experiments with n=9-12
724 mice per group.
725
726 **Fig 8. Signaling via A1 receptor is important for the control of pulmonary**
727 **pneumococcal burden *in vivo.*** Young mice mock-treated or treated with
728 DPCPX, an A1 receptor inhibitor and old mice mock-treated or treated with 2-
729 Chloro-N6-cyclopentyladenosine, an A1 receptor agonist were inoculated intra-
730 tracheally with ~1-2x10^4 CFU of *S. pneumoniae* TIGR4. (A) Bacterial burdens in
731 the lungs were determined 6 hours post-infection. Data shown are pooled from
732 n=9 young and n=4 old mice per group. (B-E) To follow the infection over time,
733 mice were challenged with ~1-2x10^4 CFU of *S. pneumoniae* TIGR4. Bacterial
734 burdens in the blood (B) and clinical scores (C) were determined 24 hours post-

735 infection. Asterisks indicate significant differences calculated by Student's t-test.
736 Survival of control and A1 inhibited young mice (D) as well as control and A1
737 agonist-treated old mice (E) and was assessed at the indicated time points
738 following challenge. Fractions denote survivors at 7 days after challenge over the
739 total number of mice. Asterisks indicate a statistical significance by the log-Rank
740 (Mantel-Cox) test. Data were pooled from two independent experiments.
741

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1003

1004 **Supporting Information Legends**

1005 **Figure S1.** Effect of adenosine pathway drugs on bacterial viability. *S.*
1006 *pneumoniae* TIGR4 were treated with PBS control, adenosine (ADE, 100μM), A1
1007 agonist 2-Chloro-N6-cyclopentyladenosine (10, 1, 0.1 nM), A2A agonist
1008 CGS21680 (270, 27, 2.7nM) or A2B agonist BAY 60-6583 (200, 20, 2nM)
1009 corresponding to the approximate 10xKi, Ki or 0.1Ki for each receptor for 1 hour.
1010 The number of viable bacteria was determined by plating on blood agar plates
1011 Data shown are pooled from three separate experiments where each condition
1012 was tested in triplicate.
1013

1014 **Figure S2.** Adenosine does not alter expression of bacterial capsular
1015 polysaccharide. (A) *S. pneumoniae* TIGR4 were treated with adenosine (ADE,
1016 100μM) or vehicle control (VC) for 30 minutes and expression of capsular
1017 polysaccharide measured by flow cytometry. Left panel, representative data from
1018 one of three separate experiments where each condition was tested in triplicate
1019 are shown. Right panel, representative histogram with the gating strategy using
1020 Δcps *S. pneumoniae* TIGR4 as a negative control is shown. (B) H292 cells were
1021 treated with adenosine (ADE, 100μM) or vehicle control (VC) for 30 minutes and
1022 then infected with WT or Δ*cps S. pneumoniae* TIGR4 at an MOI of 20 for 1 hour.

1023 The number of bound bacteria was determined by plating on blood agar plates
1024 and the percent bacterial binding calculated relative to binding of WT bacteria to
1025 vehicle control treated cells. Data shown are pooled from three separate
1026 experiments where each condition was tested in triplicate. Asterisks indicate
1027 significant differences calculated by Student's t-test.
1028

Figure 1

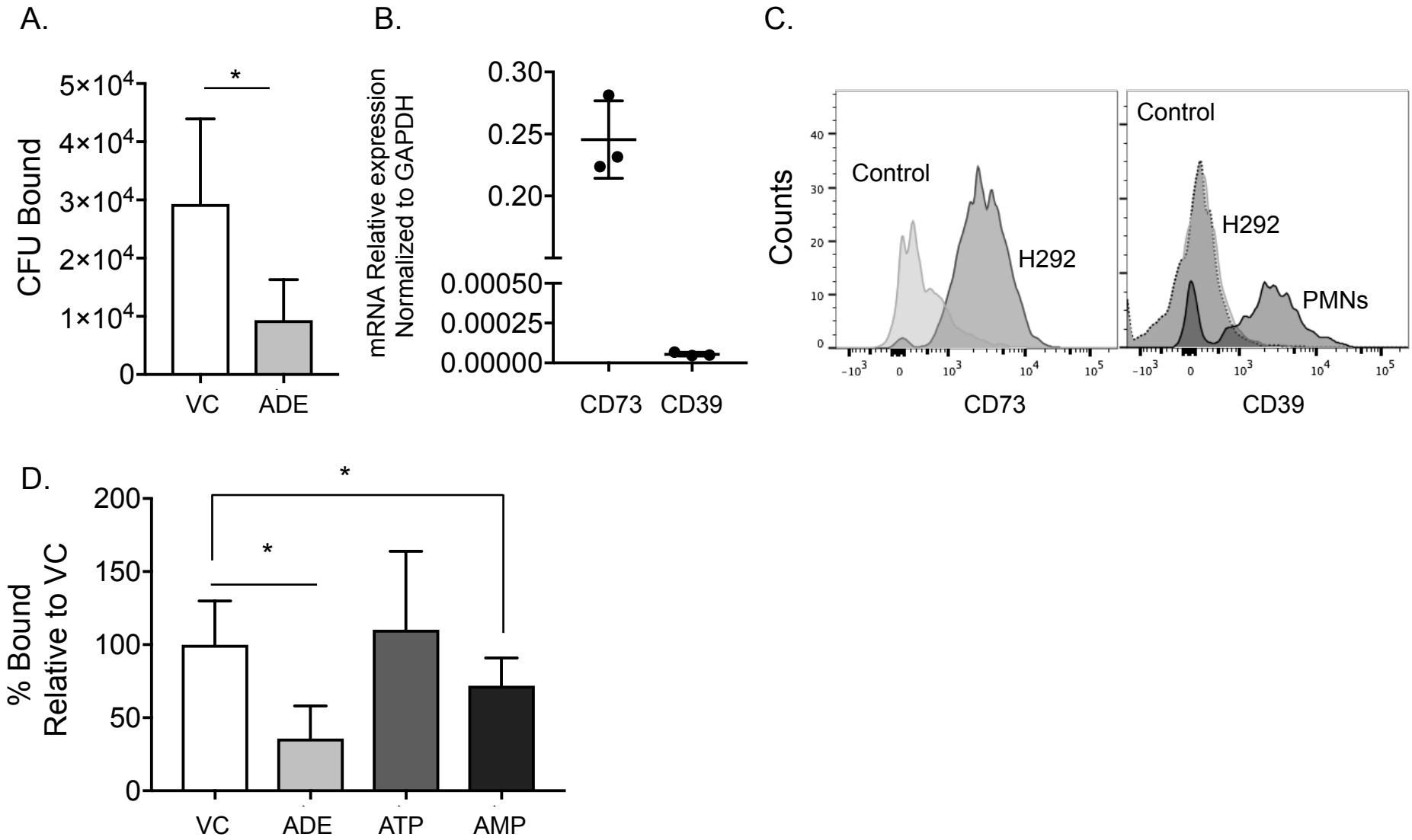

A.
CFU Bound
5×10^4
4×10^4
3×10^4
2×10^4
1×10^4
0
*
VC
ADE
B.
mRNA Relative expression
Normalized to GAPDH
0.30
0.25
0.20
0.00050
0.00025
0.00000
CD73
CD39
C.
Counts
Control
H292
Control
H292
PMNs
CD73
CD39
D.
% Bound
Relative to VC
200
150
100
50
0
*
*
VC
ADE
ATP
AMP

Figure 2

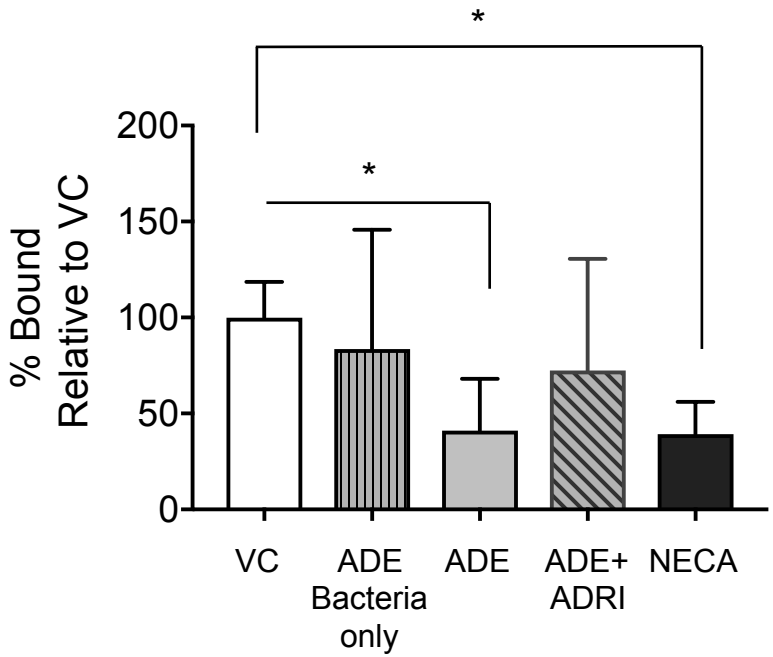

*
*
200
150
100
50
0
% Bound
Relative to VC
VC
ADE
Bacteria
only
ADE
ADE+
ADRI
NECA

Figure 3

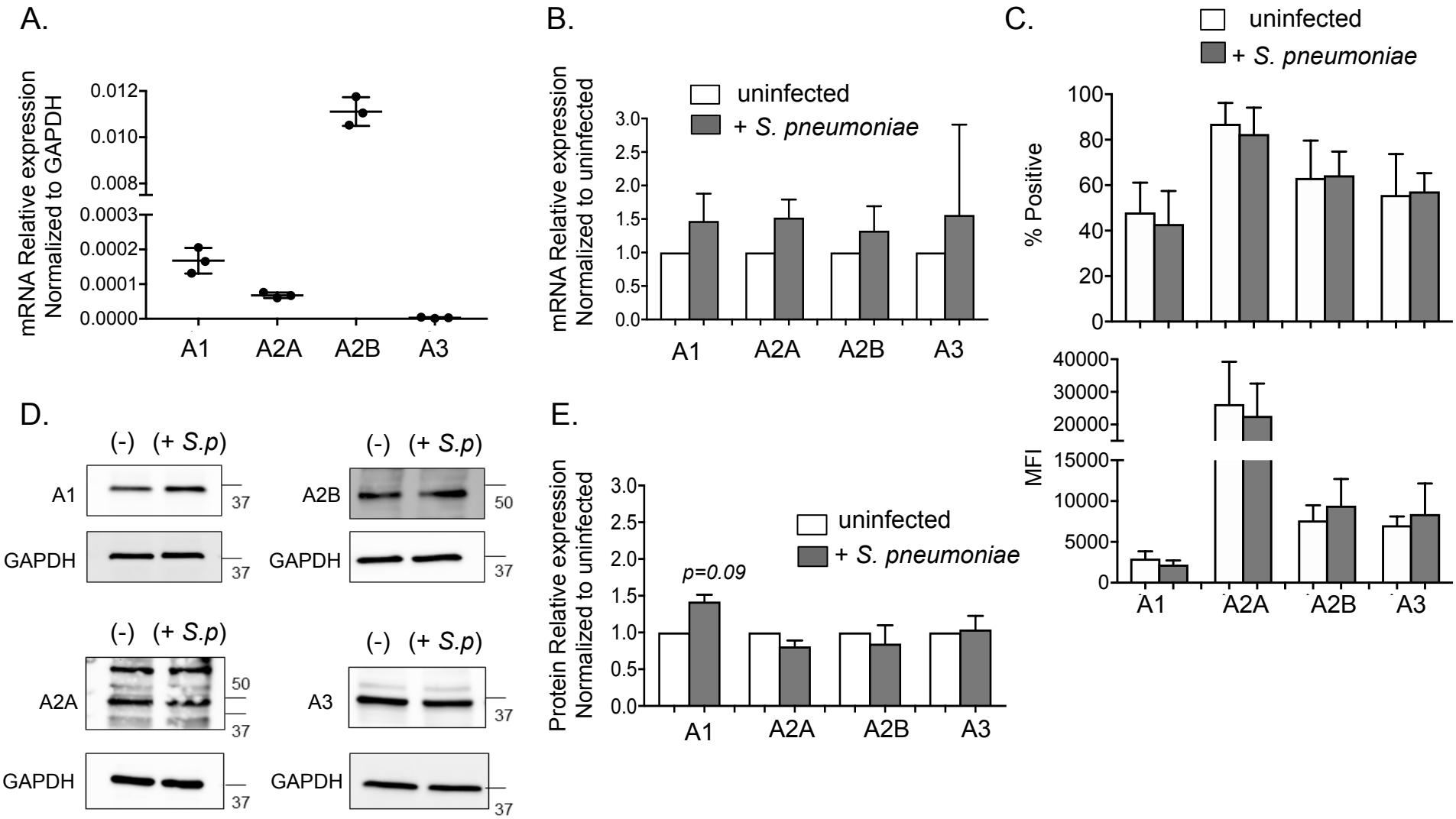

A.
mRNA Relative expression
Normalized to GAPDH
0.012
0.010
0.008
0.0003
0.0002
0.0001
0.0000
A1
A2A
A2B
A3
B.
mRNA Relative expression
Normalized to uninfected
uninfected
+ S. pneumoniae
3.0
2.5
2.0
1.5
1.0
0.5
0.0
A1
A2A
A2B
A3
C.
uninfected
+ S. pneumoniae
% Positive
100
80
60
40
20
0
MFI
40000
30000
20000
15000
10000
5000
0
A1
A2A
A2B
A3
D.
(-) (+ S.p)
A1
37
GAPDH
37
(-) (+ S.p)
A2B
50
GAPDH
37
(-) (+ S.p)
A2A
50
37
GAPDH
37
(-) (+ S.p)
A3
37
GAPDH
37
E.
Protein Relative expression
Normalized to uninfected
uninfected
+ S. pneumoniae
p=0.09
3.0
2.5
2.0
1.5
1.0
0.5
0.0
A1
A2A
A2B
A3

Figure 4

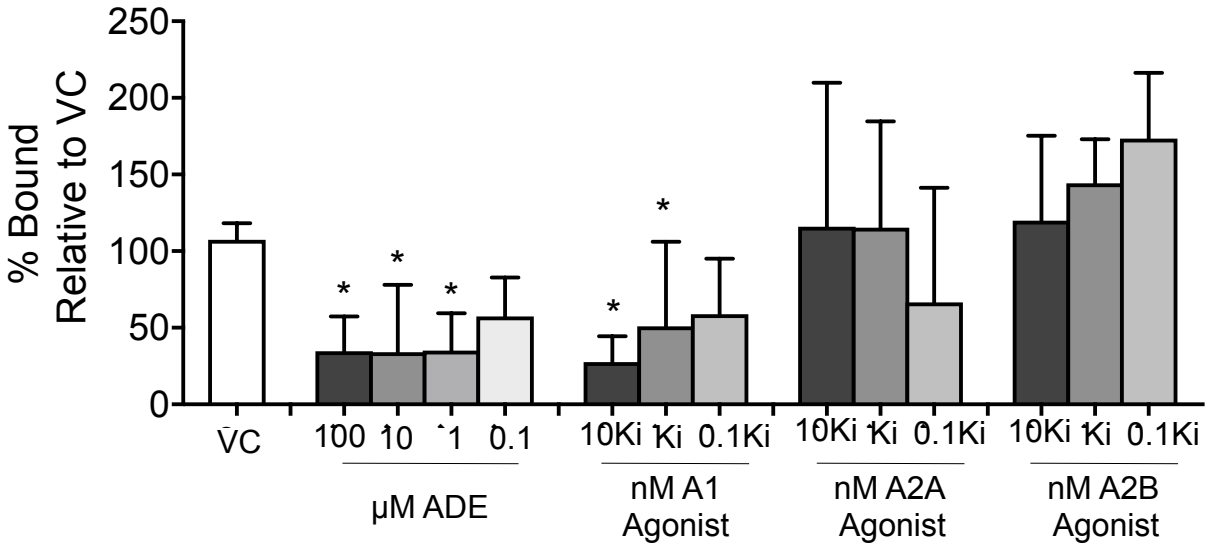

% Bound
Relative to VC
250
200
150
100
50
0
VC
100
10
1
0.1
10Ki
1Ki
0.1Ki
10Ki
1Ki
0.1Ki
10Ki
1Ki
0.1Ki
*
*
*
*
*
μM ADE
nM A1
Agonist
nM A2A
Agonist
nM A2B
Agonist

Figure 5

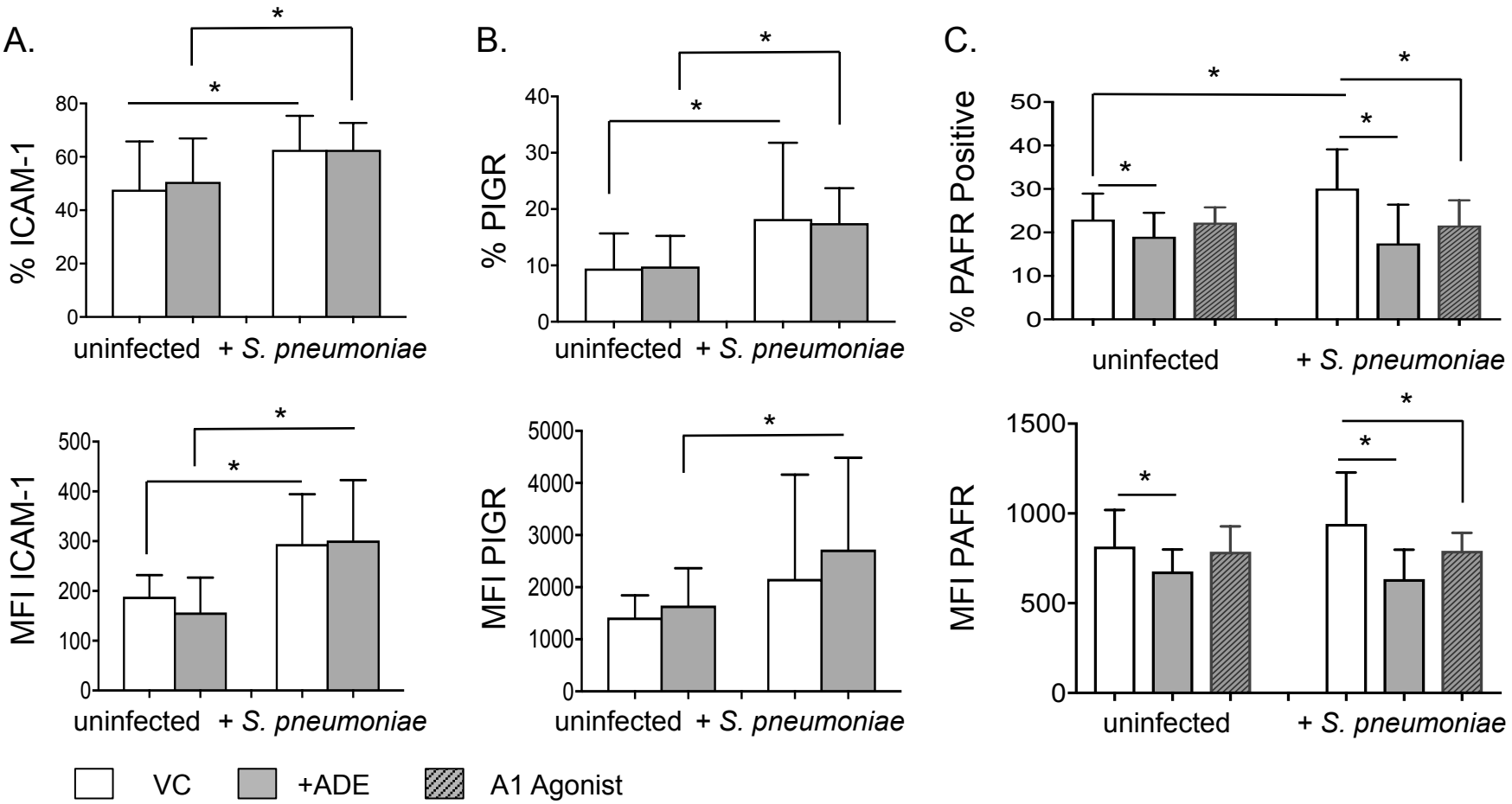

A.
B.
C.
% ICAM-1
% PIGR
% PAFR Positive
MFI ICAM-1
MFI PIGR
MFI PAFR
uninfected
+ S. pneumoniae
VC
+ADE
A1 Agonist

Figure 6

A.

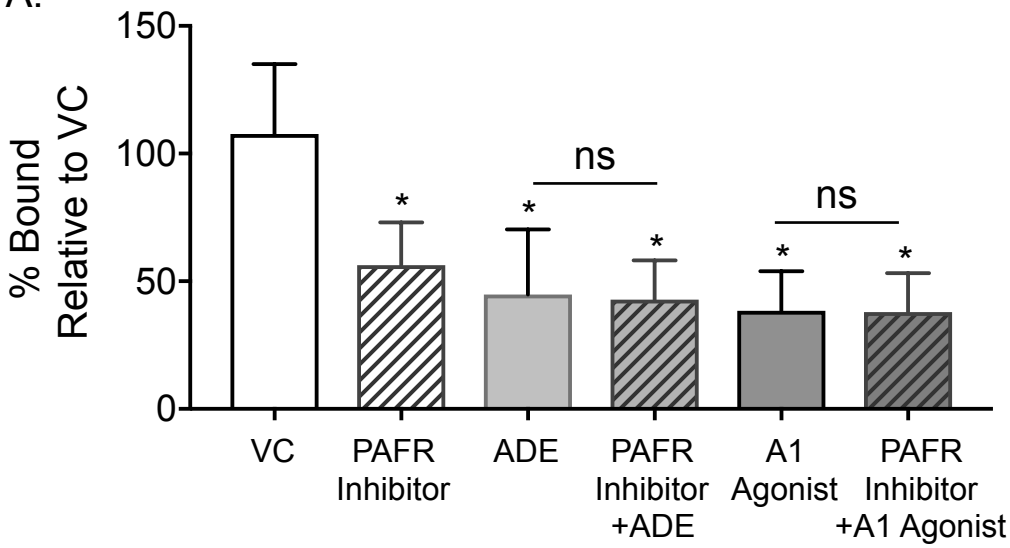
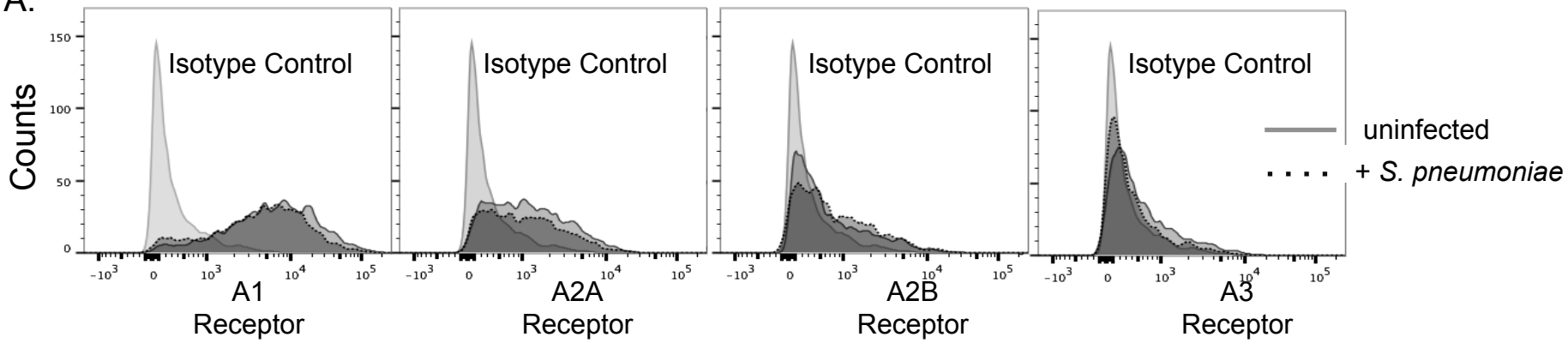

150
100
50
0
% Bound
Relative to VC
ns
ns
*
*
*
*
*
VC
PAFR
Inhibitor
ADE
PAFR
Inhibitor
+ADE
A1
Agonist
PAFR
Inhibitor
+A1 Agonist

Figure 7

A.


Counts
Isotype Control
Isotype Control
Isotype Control
Isotype Control
uninfected
+ S. pneumoniae
A1 Receptor
A2A Receptor
A2B Receptor
A3 Receptor

B.

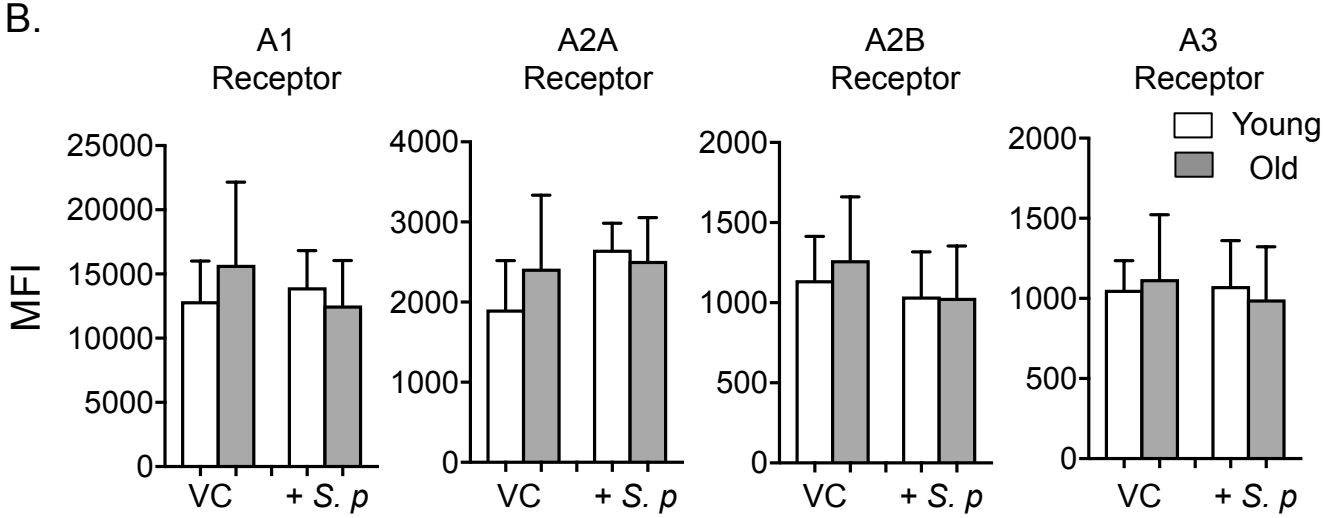
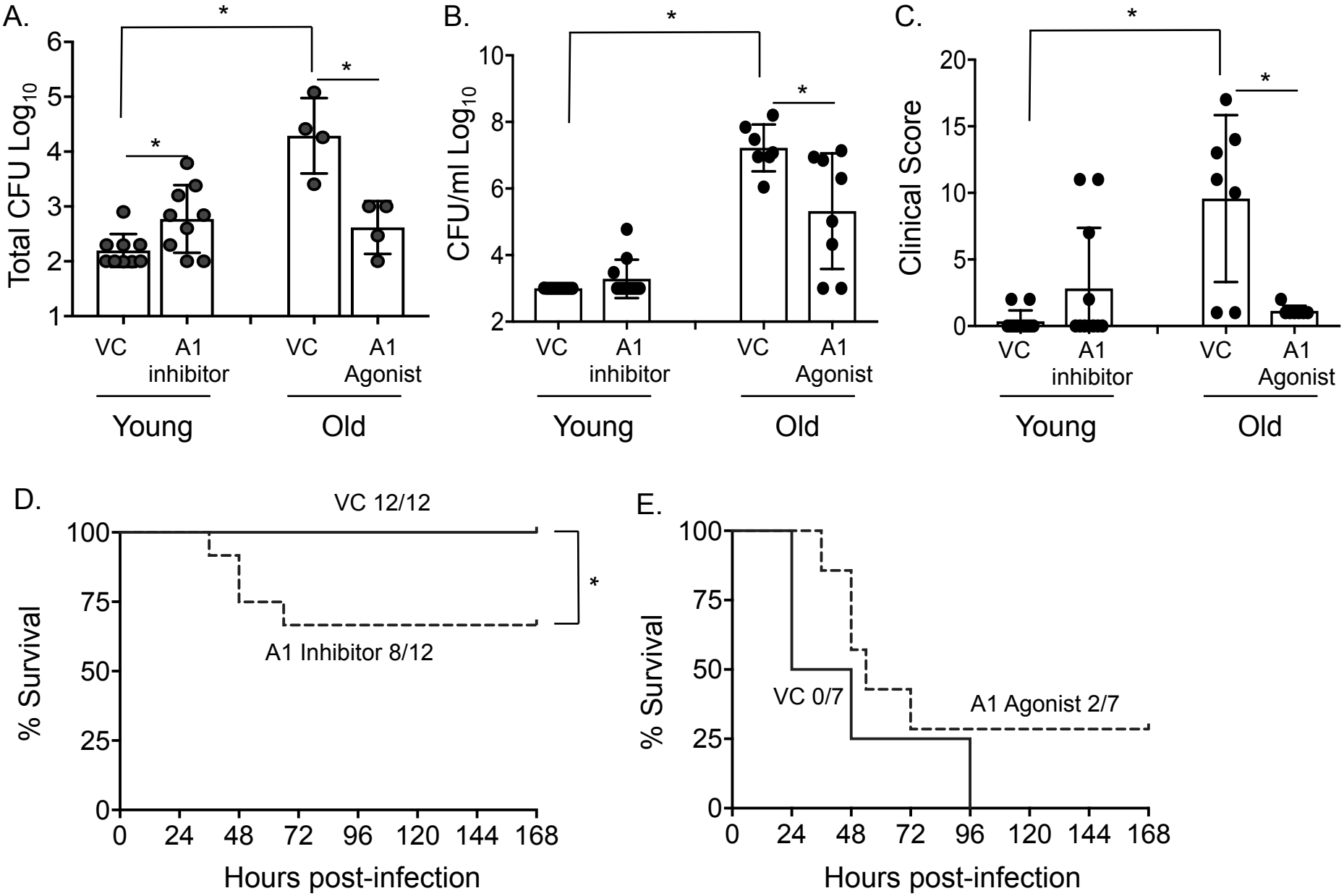

A1 Receptor
A2A Receptor
A2B Receptor
A3 Receptor
Young
Old
MFI
VC
+ S. p

Figure 8


A.
Total CFU Log10
VC
A1 inhibitor
VC
A1 Agonist
Young
Old
B.
CFU/ml Log10
C.
Clinical Score
D.
VC 12/12
A1 Inhibitor 8/12
% Survival
Hours post-infection
E.
VC 0/7
A1 Agonist 2/7