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: Adenosine blunts pneumococcal epithelial binding

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Summary

Extracellular adenosine production is crucial for host resistance against *Streptococcus pneumoniae* (pneumococcus) and is thought to 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 signaling via A1 adenosine receptor significantly reduced the ability of pneumococci to bind human pulmonary epithelial cells. A1 receptor signaling blocked 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 required for control of pneumococcal pneumonia as inhibiting it resulted in increased host susceptibility. 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 host resistance of old mice to *S. pneumoniae* pulmonary infection. This study demonstrates a novel mechanism by which extracellular adenosine modulates resistance to lung infection by targeting bacterial-host interactions.

Introduction

Streptococcus pneumoniae (pneumococcus) typically reside asymptomatically in the nasopharynx of healthy individuals but can cause life-threatening invasive diseases resulting in more than 1 million deaths per year worldwide (Kadioglu, Weiser, Paton, & Andrew, 2008; van der Poll & Opal, 2009). The majority of invasive pneumococcal infections occur in elderly individuals ≥65 years of age (Chong & Street, 2008). As the number of elderly individuals is projected to double in the coming decades, reaching 2 billion by 2050, invasive pneumococcal infections pose a serious health concern and an economic burden which calls for novel interventions to fight this disease (Boe, Boule, & Kovacs, 2017).

Lung infection is the most common form of invasive pneumococcal disease (Centers for Disease Control and Prevention. 2016. Active Bacterial Core Surveillance Report, 2016) and the ability of *S. pneumoniae* to bind to and invade the airway epithelium is a crucial step for invasive infection (Kadioglu et al., 2008; van der Poll & Opal, 2009). *S. pneumoniae* express several factors that mediate binding to mammalian host cells and in fact many bacterial virulence elements with diverse functions also double as adherence factors (Kadioglu et al., 2008; van der Poll & Opal, 2009). This includes phosphorylcholine displayed on the bacterial cell wall that mimics moieties found on host platelet-activating factor (PAF) and allows the bacteria to bind to the host PAF receptor (Briles & Tomasz, 1973; Cundell, Gerard, Gerard, Idanpaan-Heikkila, & Tuomanen, 1995). Platelet-activating factor receptor (PAFR) is expressed on airway epithelial cells

68 (Duitman et al., 2012; Ishizuka et al., 2001; Shivshankar, Boyd, Le Saux, Yeh, &
69 Orihuela) and is upregulated during pneumococcal pneumonia (Duitman et al.,
70 2012). PAFR mediates pneumococcal adhesion to airway epithelial cells
71 (Cundell, Gerard, et al., 1995) and pharmacologically blocking this receptor
72 decreased bacterial binding *in vitro* (Cundell, Gerard, et al., 1995; Shukla et al.,
73 2016). Importantly blocking PAFR also attenuated *S. pneumoniae* infection in
74 animal models and PAFR$^{-/-}$ mice were more resistant to pneumococcal lung
75 infection (Cundell, Gerard, et al., 1995; Rijneveld et al., 2004).
76 The extracellular adenosine pathway is an important regulator of host
77 defense against *S. pneumoniae* infection (Bajgar & Dolezal, 2018; Bou Ghanem,
78 Clark, Roggensack, et al., 2015). Upon cellular injury including that caused by
79 infection, ATP is released from cells and converted to adenosine by the
80 sequential action of two extracellular enzymes, CD39, which metabolizes ATP to
81 AMP, and CD73 that de-phosphorylates AMP to adenosine (Hasko, Linden,
82 Cronstein, & Pacher, 2008). Extracellular adenosine can then act as a signaling
83 molecule by binding to and activating one of four G-protein coupled adenosine
84 receptors, A1, A2A, A2B and A3 (Hasko et al., 2008). The enzymes and
85 receptors of the extracellular adenosine pathway are expressed by lung epithelial
86 cells (Burnstock, Brouns, Adriaensen, & Timmermans, 2012) and were shown to
87 control acute lung injury (Hoegl et al., 2015), ciliary motility (Allen-Gipson et al.,
88 2011), wound healing (Allen-Gipson, Wong, Spurzem, Sisson, & Wyatt, 2006),
89 activation of ion channels (Factor et al., 2007), surfactant secretion (Gobran &
90 Rooney, 1990), fibronectin release (Roman, Rivera, Roser-Page, Sitaraman, &

Ritzenthaler, 2006), mucin expression (McNamara et al., 2004) and production of cytokines (Zhong, Wu, Belardinelli, & Zeng, 2006).

Extracellular adenosine is thought to regulate host resistance to lung infections by regulating recruitment and function of innate immune cells (Aeffner, Woods, & Davis, 2014, 2015; Barletta, Cagnina, Burdick, Linden, & Mehrad, 2012; Barletta, Ley, & Mehrad, 2012; Bou Ghanem, Clark, Roggensack, et al., 2015; Theatre et al., 2012). For example, we previously found that CD73 deficient mice had a thousand-fold more *S. pneumoniae* in their lungs as compared to wild type controls following lung challenge (Bou Ghanem, Clark, Roggensack, et al., 2015). However, those studies did not assess whether decreased adenosine functioned to enhance bacterial binding to lung epithelial cells. Here, we report that adenosine A1 receptor signaling inhibited binding of *S. pneumoniae* to human lung epithelial cells *in vitro* by reducing the expression of PAFR on infected host cells. Signaling via the A1 receptor was further required for control of bacterial numbers *in vivo* upon lung infection of mice. Importantly triggering A1 receptor signaling reversed the susceptibility of old mice to *S. pneumoniae* infection. This study identifies a novel mechanism by which adenosine regulates host resistance to bacterial pneumonia and demonstrates for the first time the feasibility of targeting this pathway to boost resistance of vulnerable hosts against pneumococcal pneumonia.

Results

113 *Adenosine and AMP, but not ATP reduces binding of S. pneumoniae to H292*
114 *pulmonary epithelial cells*
115 We previously found that extracellular adenosine was required for host
116 resistance against *S. pneumoniae* lung infection (Bou Ghanem, Clark,
117 Roggensack, et al., 2015). Mice lacking CD73, an enzyme required for
118 extracellular adenosine production, had significantly higher bacterial burdens in
119 their lungs as compared to wild type mice (Bou Ghanem, Clark, Roggensack, et
120 al., 2015). Since a crucial step for establishing lung infection is the ability of *S.*
121 *pneumoniae* to bind to the pulmonary epithelium (Kadioglu et al., 2008; van der
122 Poll & Opal, 2009), we tested whether extracellular adenosine directly modulated
123 pneumococcal binding to respiratory epithelial cells. To test that, we used H292
124 cells, a human lung epithelial cell line extensively used in modeling pulmonary
125 bacterial host interaction (Bhowmick et al., 2013; Choi, Lee, Lee, Park, & Lee,
126 2008; R. T. Clark, Hope, Lopez-Fraga, Schiller, & Lo, 2009; Marks,
127 Parameswaran, & Hakansson, 2012; Wagner et al., 2007; Yonker et al., 2017).
128 H292 cells were treated with extracellular adenosine or vehicle control, infected
129 with *S. pneumoniae* and the amount of bacteria that adhered to the host cells
130 measured. We found that incubation with 100μM of extracellular adenosine,
131 which is close to the alveolar concentration of adenosine in human subjects
132 (Driver, Kukoly, Ali, & Mustafa, 1993), significantly decreased the amount of
133 adherent bacteria that were attached to the lung epithelial cells by 3-fold (Fig 1A).
134 This was not due to direct toxicity to the bacteria, as adenosine at the
135 concentration we tested had no effect on bacterial growth as previously

136 described (Bou Ghanem, Clark, Roggensack, et al., 2015) or bacterial viability
137 within the timeframe of this assay (Fig S1).
138 Adenosine in the extracellular milieu is produced when ATP that has
139 leaked from damaged cells is metabolized by two surface enzymes, CD39 that
140 converts ATP to AMP, and CD73, an ecto-5'-nucleotidase that de-phosphorylates
141 AMP to adenosine (Hasko et al., 2008). When we measured the expression of
142 these enzymes in H292 cells, we found that mRNA levels of CD73 were readily
143 detectable. However, CD39 mRNA levels were very low (Fig 1B). Consistent with
144 that, CD73 protein was abundantly expressed on H292 cells, while CD39 was not
145 detectable (Fig 1C). To confirm that the antibody we used for detection of CD39
146 was functional, we stained PMNs isolated from human donors as a positive
147 control and were able to easily detect CD39 on those cells (Fig 1C).
148 Since H292 cells expressed CD73, but not CD39, we hypothesized that
149 AMP will also be able to decrease binding of *S. pneumoniae* to these cells, since
150 CD73 can convert it to adenosine, while ATP will have no effect since CD39 is
151 not present to initiate its dephosphorylation into adenosine. When we performed
152 the binding assays, we found that treatment of H292 cells with adenosine or AMP
153 reduced the percentage of bacteria that bound when compared to vehicle control
154 treatment (Fig 1D). However, we did not observe any significant effect on
155 bacterial binding to host cells upon treatment with ATP. Taken together, these
156 data suggest that extracellular adenosine production inhibits the ability of *S.*
157 *pneumoniae* to bind to lung epithelial cells *in vitro*.
158

159 *Extracellular adenosine receptor signaling is required for reduction of S.*
160 *pneumoniae binding to pulmonary epithelial cells*

161 Extracellular adenosine can directly alter bacterial virulence (Crane &
162 Shulgina, 2009; Kao et al., 2017) and also signal in the mammalian host via four
163 known receptors (Hasko et al., 2008). Therefore we tested whether adenosine
164 was altering bacterial binding by acting on the bacteria, the host or both. Capsule
165 expression regulates the ability of *S. pneumoniae* to bind host cells (Cundell,
166 Weiser, Shen, Young, & Tuomanen, 1995; Hammerschmidt et al., 2005;
167 Sanchez, Hinojosa, et al., 2011; Sanchez, Kumar, et al., 2011; Trzcinski et al.,
168 2015). However, treatment of *S. pneumoniae* with adenosine had no effect on
169 expression of the capsular polysaccharide (Fig S2A). As expected capsular
170 deficient (Δ*cps)* bacteria bound significantly better than wild type (WT) bacteria
171 but adenosine treatment of H292 cells still reduced binding of Δ*cps S.*
172 *pneumoniae* by 3-fold, comparable to its effect on WT bacteria (Fig S2B).
173 Further, when we pre-treated *S. pneumoniae* only with adenosine, we saw no
174 consistent decrease in bacterial binding (Fig 2). This suggests that adenosine
175 was not mediating its effect through acting directly on the bacteria.

176 To test if adenosine was mediating its effect by acting on host cells, we
177 blocked adenosine receptor signaling using a pan-adenosine receptor inhibitor
178 CGS 15943. We found that the ability of adenosine to blunt bacterial binding to
179 H292 cells was abrogated when adenosine receptors signaling was blocked (Fig
180 2). Further, treatment of H292 cells with NECA, a pan adenosine receptors
181 agonist, mimicked the effects of adenosine, where bacterial binding was reduced

182 to 39% compared to the vehicle treated controls (Fig 2). The drugs had no direct
183 effect on bacterial viability as previously shown (Bou Ghanem, Clark,
184 Roggensack, et al., 2015). These findings suggest that extracellular adenosine
185 signaling in mammalian hosts inhibits the ability of *S. pneumoniae* to bind to lung
186 epithelial cells.
187
188 *H292 cells express extracellular adenosine receptors*
189 There are currently four known adenosine receptors A1, A2A, A2B and
190 A3. First, we wanted to determine which adenosine receptors are expressed by
191 H292 cells and whether their expression is affected by bacterial infection. To do
192 so, we measured expression of adenosine receptors mRNA as is commonly
193 done in most studies (Factor et al., 2007; Giacomelli et al., 2018; Roman et al.,
194 2006; Zhong et al., 2006). mRNA for A1, A2A and A2B receptors was readily
195 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 reduces 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 (Hasko et al., 2008). To
217 probe which receptor(s) is involved, we did a dose response, testing the effect of
218 100, 10, 1 and 0.1μM of adenosine on bacterial binding to H292 cells. We found
219 that even lower concentrations of adenosine at 10 and 1μM were as equally
220 efficient at decreasing adherence of *S. pneumoniae* as 100μM, where bacterial
221 binding was reduced to 35% relative to vehicle treated controls (Fig 4). These
222 results suggested that the higher affinity adenosine receptors A1, A2A or A3 but
223 not the low affinity receptor A2B were mediating the effect on bacterial binding
224 observed.
225 To pinpoint the exact receptor involved, we treated H292 cells with
226 specific agonists for each of the different receptors. Since mRNA levels of A3
227 (Fig 3A) were barely detectable, we decided to proceed with testing the role of

228 the other three receptors. Consistent with the results of adenosine doses,
229 agonism of A2B receptor on H292 cells did not result in inhibition of *S.*
230 *pneumoniae* binding to these pulmonary epithelial cells and the percentage of
231 bacteria bound to treated cells were not statistically different than those bound to
232 control treated cells (Fig 4). Similarly, treatment with the A2A receptor agonist did
233 not alter bacterial binding to H292 cells (Fig 4). In contrast treatment with the A1
234 receptor agonist significantly reduced bacterial binding to H292 cells in a dose-
235 dependent manner (Fig 4). We observed a 3-fold and 2-fold reduction in the
236 amount of bound bacteria in comparison to control treated cells when cells were
237 treated with 10 or 1 nM (corresponding to respectively 10x and 1x the drug's
238 reported Ki (Fig 4). None of the adenosine receptor agonists had a direct effect
239 on bacterial viability (Fig S1). These findings suggest that specific signaling via
240 the A1 receptor reduces binding of *S. pneumoniae* to pulmonary epithelial cells.
241
242 *A1 receptor signaling reduces expression of PAFR on infected pulmonary*
243 *epithelial cells*
244 Next we wanted to identify the mechanisms by which adenosine regulated
245 bacterial binding to lung epithelial cells. *S. pneumoniae* is known to co-opt
246 several well-characterized host proteins to adhere to mammalian cells including
247 the Polymeric Immunoglobulin Receptor (PIGR) (Zhang et al., 2000) and PAFR
248 (Cundell, Gerard, et al., 1995). Therefore, we examined the effect of adenosine
249 on the expression of these host proteins by flow cytometry at baseline and during
250 infection. As a control, we also examined the expression of ICAM-1 a host

251 protein whose expression is upregulated during infection but is not thought to be
252 a receptor for *S. pneumoniae* (Murdoch, Read, Zhang, & Finn, 2002). We found
253 that consistent with previous studies, infection of H292 lung epithelial cells by *S.*
254 *pneumoniae* increased the expression of all three proteins we examined (Fig 5A-
255 C). Treatment of H292 cells with adenosine had no significant effect on the
256 expression of ICAM-1 or PIGR (Fig 5A-B). In contrast treatment of the epithelial
257 cells with adenosine or A1 receptor agonist prevented upregulation of PAFR in
258 response to infection and *S. pneumoniae* infected H292 cells treated with these
259 compounds had significantly lower expression of PAFR as compared to vehicle
260 treated controls (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 (Cundell, Gerard, et al., 1995; Shukla et al.,
264 2016), inhibition of PAFR signaling significantly reduced the ability of *S.*
265 *pneumoniae* to bind to H292 cells (Fig 6). Addition of the A1 receptor agonists or
266 adenosine did not further decreased bacterial binding in the presence of the
267 PAFR inhibitor (Fig 6). These findings suggest that A1 adenosine receptor
268 signaling reduces bacterial binding to lung epithelial cells by targeting PAFR
269 receptor.
270
271 *A1 receptor signaling is required for host resistance to S. pneumoniae lung*
272 *infection*

273 We next wanted to confirm the relevance of our findings during lung
274 infection *in vivo* using a mouse model. First we checked for the expression of
275 adenosine receptors in the lungs of mice and found that consistent with previous
276 reports (Factor et al., 2007), A1 and A2A were highly expressed, while less A2B
277 and A3 was detected (Fig 7A-B). As we observed *in vitro*, *S. pneumoniae*
278 infection did not affect the expression of adenosine receptors in mouse lungs (Fig
279 7A-B).
280 We then evaluated the role of A1 receptor signaling in host resistance to
281 infection. We treated mice with i.p. injections of DPCPX, a A1 pharmacological
282 inhibitor that is active *in vivo* prior to infection. The mice were then challenged
283 intra-tracheally with ~$1x10^4$ CFU, a low non-lethal dose of *S. pneumoniae* (Bou
284 Ghanem, Clark, Du, et al., 2015; Bou Ghanem, Clark, Roggensack, et al., 2015),
285 and lung bacterial burdens were determined 6 hours post-infection. Since
286 extracellular adenosine modulates host resistance to infection via regulating the
287 inflammatory immune response following infection (Aeffner et al., 2014, 2015;
288 Barletta, Cagnina, et al., 2012; Barletta, Ley, et al., 2012; Bou Ghanem, Clark,
289 Roggensack, et al., 2015; Theatre et al., 2012), we chose to challenge mice with
290 this low dose of bacteria and examine bacterial numbers in the lungs at this early
291 time point, conditions under which we previously found overt pulmonary
292 inflammation is not yet triggered (Bou Ghanem, Clark, Roggensack, et al., 2015).
293 We further confirmed that the A1 inhibitor had no effect on the baseline numbers
294 of neutrophils, macrophages and T cells in the lungs (data not shown). We found
295 that inhibition of A1 receptor signaling *in vivo* resulted in a significant ~10-fold

296 increase in bacterial numbers in the lungs of young mice following pulmonary
297 challenge (Fig 8A). At 6 hours post infection, the infection was still localized to
298 the lungs and had not spread systemically. However, when we compared
299 bacteremia at 24 hours following infection, we found that while none of the
300 control mice were bacteremic, 25% of A1 inhibited mice had systemic spread of
301 the infection (Fig 8B). Importantly, inhibition of A1 resulted in significantly more
302 host death when mice were challenged at an otherwise non-lethal dose (Fig 8D).
303 These findings demonstrate that specific signaling via the A1 receptor is required
304 for early host resistance to *S. pneumoniae* lung infection.
305
306 *Triggering A1 receptor signaling boosts resistance against S. pneumoniae lung*
307 *infection in aged hosts*
308 As pneumococcal infections are particularly a problem in the elderly, we
309 examined the effect of aging on adenosine receptor expressions in the lung. We
310 compared pulmonary expression levels of the adenosine receptors in young (2
311 months) versus old (18-22 months) mice. We found that there were no significant
312 differences in the expression of any of the adenosine receptors in the lungs of old
313 mice as compared to young controls either at baseline or following lung
314 challenge with *S. pneumoniae* (Fig 7B).
315 We previously found that mice mimic the age-driven susceptibility to *S.*
316 *pneumoniae* lung infection observed in humans (Bou Ghanem, Clark, Du, et al.,
317 2015). To test whether A1 receptor can boost the resistance of old mice to lung
318 infection, mice were treated i.p. with 2-Chloro-N6-cyclopentyladenosine, the A1

319 agonist prior to infection. Mice were then challenged intra-tracheally with *S.*
320 *pneumoniae*, and bacterial burdens in the lung were determined 6 hours post-
321 infection. As expected, vehicle control treated old mice were more susceptible to
322 pneumococcal infection and had ~ 100-fold more bacteria in their lungs at 6
323 hours (Fig 8A) and blood at 24 hours (Fig 8B) as compared to young controls.
324 Strikingly, treatment with the A1 agonist boosted the resistance of old mice to
325 infection. A1 agonist treated old mice had 70-fold less bacteria in their lungs, a
326 bacterial burden that was indistinguishable from that of control treated young
327 mice (Fig 8A). Further, by 24 hours post infection, old mice given the A1 agonist
328 had significantly less bacteria in their systemic circulation when compared to
329 controls (Fig 8B). Importantly, triggering A1 significantly decreased clinical sign of
330 sickness (Fig 8C) and boosted survival of old mice (Fig 8E). We also confirmed
331 that treatment with the A1 agonist boosted survival of young mice that were
332 challenged with a lethal bacterial dose (Fig 8F). These data demonstrate that A1
333 receptor can be targeted to boost the innate resistance of aged hosts to *S.*
334 *pneumoniae* lung infection.
335

336 **Discussion**

337 The role of the extracellular adenosine pathway in modulating host
338 resistance against pulmonary pathogens is becoming better appreciated.
339 Previous studies using murine models of infection showed that deficiency of
340 adenosine A2B or A1 receptors was protective against *Klebsiella pneumoniae*
341 (Barletta, Cagnina, et al., 2012) and influenza (Aeffner et al., 2014) lung infection

342 respectively, whereas overexpression of CD39 on airway epithelial cells
343 promoted *Pseudomonas aeruginosa* clearance from the lungs (Theatre et al.,
344 2012). We previously found that extracellular adenosine production by CD73 was
345 crucial for control of pulmonary bacterial numbers and systemic spread following
346 lung challenge with *S. pneumoniae* (Bou Ghanem, Clark, Roggensack, et al.,
347 2015). In all of the above studies, extracellular adenosine was believed to
348 modulate pathogen clearance by either controlling the recruitment of immune
349 cells, particularly neutrophils and/or regulating their antimicrobial activity (Aeffner
350 et al., 2014, 2015; Barletta, Cagnina, et al., 2012; Barletta, Ley, et al., 2012; Bou
351 Ghanem, Clark, Roggensack, et al., 2015; Theatre et al., 2012). Here we show
352 that apart from its effect on innate immune cells, extracellular adenosine
353 modulates host resistance by acting on pulmonary epithelial cells, targeting
354 expression of mammalian proteins co-opted by *S. pneumoniae* as receptors and
355 reducing the ability of bacteria to bind host cells.
356 Few studies have examined the effect of adenosine on bacterial infection
357 of epithelial cells from different organs (Crane & Shulgina, 2009; Kao et al., 2017;
358 Pettengill, Lam, & Ojcius, 2009; Spooner, DeGuzman, Lee, & Yilmaz, 2014;
359 Walia et al., 2004). Adenosine signaling via the A2B receptor inhibited
360 intracellular persistence of *Chlamydia trachomatis* in cervical epithelial cells
361 (Pettengill et al., 2009) while A2A receptor promoted intracellular replication of
362 *Porphyromonas gingivalis* in gingival epithelial cells (Spooner et al., 2014). In
363 intestinal epithelial cells, adenosine promoted bacterial infections, where addition
364 of exogenous adenosine increased adhesion of *Salmonella* to gut epithelial cells

365 (Walia et al., 2004), and expression of the adenosine producing enzyme CD73
366 on intestinal epithelial cells promoted the intracellular replication as well as
367 translocation and systemic spread of *Salmonella* across the gut epithelium (Kao
368 et al., 2017). Similarly, adenosine altered the adhesion patterns of
369 enteropathogenic *Escherichia coli* to gut epithelial cells and promoted intestinal
370 infection *in vivo* (Crane & Shulgina, 2009). Thus the effect of adenosine on
371 bacterial infection of the epithelium may be organ and adenosine-receptor
372 specific. We demonstrated here that adenosine signaling via the A1 receptor
373 blinted adherence of *S. pneumoniae* to H292 epithelial cells and reduced lung
374 infection *in vivo*, which is to our knowledge, the first report to show an effect of
375 the extracellular adenosine pathway on bacterial infection of pulmonary epithelial
376 cells. In certain infections adenosine had a direct effect on bacterial viability and
377 expression of virulence genes (Crane & Shulgina, 2009; Kao et al., 2017).
378 However, that was not the case here as adenosine neither affected *S.*
379 *pneumoniae* viability or expression of capsular polysaccharide, a major
380 determinate of bacterial adhesion, nor did treatment of the bacteria alone alter
381 their ability to adhere to lung epithelial cells.
382 Purinergic signaling plays a crucial role in regulating responses in the
383 airways (Burnstock et al., 2012). We found here that adenosine was exerting its
384 effect on bacterial binding through signaling via adenosine receptor A1 on host
385 epithelial cells. The pulmonary epithelial cells used *in vitro* in this study were
386 H292 human lung epithelial cells that are a mix of type I and type II pneumocytes
387 (Bhowmick et al., 2013). We found that H292 cells expressed mRNA for A2B, A1

388 and A2A in decreasing levels while A3 levels were extremely low. This is
389 consistent with previous studies with A549 cells, a type II pneumocyte cell line
390 (Roman et al., 2006) as well as primary bronchial epithelial cells (Zhong et al.,
391 2006). Similar to other studies, despite differences at the mRNA levels, we could
392 detect protein expression for all four adenosine receptors (Giacomelli et al.,
393 2018). Lung epithelial cells are also known to express P2 purinergic receptors
394 that respond to ADP and/or ATP (Burnstock et al., 2012) and ATP and/or ADP
395 impaired replication of *Chlamydia trachomatis* in cervical epithelial cells
396 (Pettengill et al., 2012). However, addition of ATP did not alter *S. pneumoniae*
397 binding to the lung epithelial cells *in vitro* while AMP significantly decreased
398 bacterial binding. We believe that AMP but not ATP decreased bacterial binding
399 since H292 cells express CD73 that can dephosphorylate AMP into adenosine
400 but lack CD39 that is required for converting ATP into AMP. *In vivo*, the ability of
401 epithelial cells to produce adenosine themselves may not be relevant as
402 adenosine can be produced by many cells that influx into sites of infection and
403 damage including immune cells (Eltzschig, Macmanus, & Colgan, 2008).
404 Here, A1 receptor signaling was important for control of *S. pneumoniae*
405 lung infection *in vivo*. We found that A1 and A2A receptors were expressed in
406 mouse lungs, while expression of A2B and A3 was lower, similar to what has
407 been described before (Factor et al., 2007). A1 receptor is known to play an
408 important role in acute lung injury by regulating inflammation, particularly
409 pulmonary influx of neutrophils (Aeffner et al., 2014; Fernandez, Sharma, LaPar,
410 Kron, & Laubach, 2013; Ngamsri, Wagner, Vollmer, Stark, & Reutershan, 2010;

411 Sun et al., 2005). While we cannot differentiate whether the effect of adenosine
412 *in vivo* is due to a direct effect on the lung epithelium or innate immune cells, we
413 used a low dose infection and examined bacterial burdens early on after 6 hours
414 of infection since we previously found that at this dose and this time point very
415 little PMNs influx into the airways (Bou Ghanem, Clark, Roggensack, et al.,
416 2015).

417 We found that adenosine and A1 receptor signaling down regulated
418 expression of PAFR on *S. pneumoniae* infected lung epithelial cells. PAFR is a
419 host protein used by *S. pneumoniae* to adhere to airway epithelial cells (Cundell,
420 Gerard, et al., 1995). When PAFR was inhibited, we found that bacterial binding
421 to the epithelium was reduced by half, consistent with previous reports (Cundell,
422 Gerard, et al., 1995; Shukla et al., 2016). Triggering A1 receptor did not further
423 reduce binding when PAFR was inhibited, suggesting that signaling via A1
424 receptor reduces pneumococcal binding via targeting PAFR. PAFR is expressed
425 in the lungs (Duitman et al., 2012) and impairs host resistance against
426 pneumococcal pneumonia (Cundell, Gerard, et al., 1995; Iovino, Brouwer, van de
427 Beek, Molema, & Bijlsma, 2013; Rijneveld et al., 2004). We observed that PAFR
428 expression is up regulated on lung epithelial cells upon infection, consistent with
429 previous reports (Duitman et al., 2012). Therefore the reduced expression of
430 PAFR in infected cells treated with adenosine or the A1 receptor agonist may be
431 an indirect effect of less bacteria bound, however, we believe that this is unlikely
432 to be a simple reflection of bound bacteria since expression of both ICAM-1 and
433 PIGR, two other host proteins up regulated upon infection were not affected by

434 adenosine. It is more likely that adenosine is indirectly regulating PAFR
435 expression by controlling inflammatory responses of epithelial cells to infection.
436 Previous studies found that PAFR was up regulated on epithelial cells activated
437 by cytokines or other factors, which enhances bacterial binding (Cundell, Gerard,
438 et al., 1995; Ishizuka et al., 2001; Mushtaq et al., 2011), and adenosine receptor
439 signaling controlled cytokine responses by epithelial cells (Zhong et al., 2006).
440 This could explain why we only see A1 receptor signaling reducing expression of
441 PAFR in *S. pneumoniae* infected but not resting epithelial cells. In addition to
442 bacterial adherence to the lung epithelium, PAFR also has a role in
443 pneumococcal invasion of epithelial and endothelial cells and systemic spread of
444 the infection from the lungs to the circulation *in vivo* (Cundell, Gerard, et al.,
445 1995; Fillon et al., 2006; Radin et al., 2005). However, we did not detect any
446 bacteremia in either A1 inhibited or control treated mice at the early 6-hour time
447 point following *S. pneumoniae* lung challenge.
448 Changes in extracellular adenosine production, metabolism and signaling
449 occur with aging (Chen & Chern, 2011; Headrick, 1996; Willems, Ashton, &
450 Headrick, 2005). This is reflected by changed levels and activity of the EAD-
451 producing and degrading enzymes (Crosti et al., 1987; Mackiewicz et al., 2006),
452 altered expression of adenosine receptors (Willems et al., 2005), and an overall
453 decline in G-protein coupled receptor signaling (Yeo & Park, 2002). This pathway
454 has been reported to play an important role in the age-related decline of brain
455 (Mackiewicz et al., 2006), metabolic (Rolband, Furth, Staddon, Rogus, &
456 Goldberg, 1990) and cardiac function (Willems et al., 2005), and importantly, can

457 be targeted to reverse this decline in animal models (Headrick et al., 2003;
458 Hesdorffer et al., 2012; Reaven, Chang, & Hoffman, 1989). However, the role of
459 the extracellular adenosine pathway in age-driven susceptibility to infections
460 remains completely unexplored. We found here that there was no difference in
461 the expression levels of adenosine receptors in the lungs of young versus old
462 mice. Strikingly, treatment of old mice with an A1 receptor agonist significantly
463 boosted their resistance to pneumococcal lung infection where they were able to
464 control early pulmonary bacterial burdens, had reduced systemic spread of the
465 infection and extended survival compared to control treated mice. PAFR
466 expression was reported to be upregulated at baseline in the lungs of old mice
467 (Shivshankar et al.). Therefore it is possible that triggering A1 receptors boots the
468 resistance of aged hosts to *S. pneumoniae* by reducing PAFR expression in the
469 lungs. These findings demonstrate that targeting the adenosine pathway may be
470 a feasible strategy to reverse the innate susceptibility of aging to lung infection.
471 *S. pneumoniae* binds to PAFR via phosphorylcholine displayed on the
472 bacterial cell wall that mimics moieties found on host platelet-activating factor.
473 This form of molecular mimicry is shared by other pathogens (S. E. Clark &
474 Weiser, 2013), including *Haemophilus influenzae* (Swords et al., 2000), *Neisseria*
475 (Serino & Virji, 2002; Weiser, Goldberg, Pan, Wilson, & Virji, 1998),
476 *Pseudomonas aeruginosa* (Barbier et al., 2008)*, Acinetobacter baumannii*
477 (Smani et al., 2012) and is used by bacteria to bind to PAFR and adhere to host
478 cells but can also regulate inflammatory responses in the host (S. E. Clark &
479 Weiser, 2013; Hergott et al., 2015). Intriguingly a pilot study found that single

480 nucleotide polymorphisms in *PTAFR* gene coding for PAFR was associated with
481 increased risk of invasive pneumococcal disease in humans, although these
482 results need to be validated as no correction for multiple testing was performed
483 (Lingappa et al., 2011). In conclusion, our findings regarding the effect of
484 adenosine on PAFR expression and bacterial binding on lung epithelial cells may
485 have future implications for using clinically available adenosine-based therapies
486 (Hasko et al., 2008) to boost host resistance to *S. pneumoniae* and other serious
487 lung infections.
488
489 **Experimental Procedures**
490
491 ***Mice***
492 Young (2 months) and Old (18-22 months) C57BL/6 mice were purchased from
493 Jackson Laboratories (Bar Harbor, ME) and the National Institute on Aging
494 colonies and housed in a specific-pathogen free facility at the University at
495 Buffalo. Male mice were used in all experiments. This work was performed in
496 accordance with the recommendations in the Guide for the Care and Use of
497 Laboratory Animals published by the National Institutes of Health. All procedures
498 were reviewed and approved by the University at Buffalo Institutional Animal
499 Care and Use Committee.
500
501 ***Bacteria***

502 Wild type *S. pneumoniae* TIGR4 strain (serotype 4), were grown at 37°C with 5%
503 CO_2 in Todd-Hewitt broth (BD Biosciences) supplemented with 0.5% yeast
504 extract and Oxyrase (Oxyrase) untill mid-exponential phase. Aliquots were then
505 frozen at -80°C in the growth media with 25% (v/v) glycerol. Before use,
506 bacterial aliquots were thawed on ice, washed once and diluted in PBS to the
507 required concentrations. Bacterial titers were confirmed by plating on Tryptic Soy
508 Agar plates supplemented with 5% sheep blood agar (blood agar plates-
509 Northeast Laboratory Services). The Δ*cps* deletion mutant was a kind gift from
510 Andrew Camilli (Shainheit, Mule, & Camilli, 2014).
511
512 ***PMN isolation***
513 PMNs were isolated from the peripheral blood of healthy donors as previously
514 described (Bou Ghanem et al., 2017). Volunteers were recruited in accordance
515 with the University at Buffalo Human Investigation Review Board (IRB) and
516 signed informed consent forms.
517
518 ***Bacterial binding assay with H292 cells***
519 Human pulmonary mucopidermoid carcinoma-derived NCI-H292 (H292) cells
520 were purchased from ATCC and grown at 37°C/ CO_2 in "H292" media consisting
521 of RPMI 1640 medium (ATCC) with 2 mM L-glutamine, 10% FBS, and 100 U
522 penicillin/streptomycin following a previously described (Bhowmick et al., 2013).
523 The day before the binding assay 2.5×10^5 H292 cells were seeded in tissue
524 culture-treated flat bottom 96-well plates (Corning) and allowed to adhere

525 overnight. The following day, cells were washed in PBS and antibiotic-free H292
526 media was added. The cells were then pretreated for 30 minutes with the
527 indicated drugs or PBS as a vehicle control and then infected with *S.*
528 *pneumoniae* at an MOI of 10-20. The plates were spun down and incubated for 1
529 hour at 37°C/ CO_2. To determine the amount of bacteria that adhered, the cells
530 were washed with PBS, lifted with 0.05% Trypsin/EDTA (Invitrogen) and
531 vigorously vortexed to produce a homogeneous solution. Serial dilutions were
532 then plated on blood agar plates for determination of bacterial colony forming
533 units (CFU). The percent of bacteria bound were determined with respect to a no
534 cell control where bacteria were added to the wells and incubated for an hour
535 under the same experimental conditions (+/-drugs) and then viable bacteria
536 enumerated by plating on blood agar plates. The amount of bacteria bound to
537 control treated cells was set as 100 % and relative changes in bacterial binding in
538 treated cells was then calculated and is presented as % bound relative to vehicle
539 treated controls on the graphs. The effect of adenosine treatment on bacterial
540 binding was also confirmed by performing binding assays on H292 cells seeded
541 on glass slides and lysed with water (not shown).
542
543 ***Adenosine pathway and PAFR drugs***
544 The effect of the extracellular adenosine pathway was tested using the following:
545 Adenosine (Sigma) diluted in PBS and used at the concentrations indicated on
546 the graphs. The pan adenosine receptor agonist 5′-(N-Ethylcarboxamido)
547 adenosine (NECA) used at 1μM. This agonist targets all four receptor with

548 reported Ki values of 6.2, 14, and 20 nM for human A3, A1 and A2A receptors
549 respectively and EC50 of 2.4 μM for human A2B. The pan adenosine receptor
550 inhibitor (ADRI) CGS-15943 used at 1μM. This inhibitor targets all four adenosine
551 receptors, with Ki values of 3.5, 4.2, 16 and 51 nM for human A1, A2A, A2B and
552 A3 receptors respectively. The A1 agonist 2-Chloro-N6-cyclopentyladenosine
553 used at 10, 1, 0.1 nM, A2A agonist CGS21680 used at 270, 27, 2.7nM and A2B
554 agonist BAY 60-6583 used at 200, 20, 2nM. These concentrations correspond to
555 the approximate 10xKi, Ki or 0.1Ki for each receptor. Adenosine receptor drugs
556 were purchased from Tocris or Sigma Aldrich, dissolved in DMSO and dilutions
557 performed in PBS. The potent PAFR antagonist Apafant was also purchased
558 from Tocris and used at 40nM corresponding to 2xKi.
559
560 ***Mouse infections***
561 The effect A1 receptor on lung infection was determined using the competitive
562 inhibitor of A1 receptor, 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX) or A1
563 agonist 2-Chloro-N6-cyclopentyladenosine. All chemicals were purchased from
564 Sigma Aldrich, dissolved in DMSO and filter sterilized by passing through a
565 0.22μm filter. The mice were then given intraperitoneal (i.p.) injections of 1mg/kg
566 of the A1 inhibitor or 0.1mg/Kg of the A1 agonist or vehicle control at days -1 and
567 0 (immediately before challenge) relative to infection. Control mice were mock-
568 treated with the vehicle control. Mice were challenged intra-tracheally with 1-
569 $2x10^4$ *S. pneumoniae* TIGR4 as previously described (Bou Ghanem, Clark,
570 Roggensack, et al., 2015). Six hours post-infection, mice were euthanized and

571 lung and blood samples were harvested and plated on blood agar plates for
572 enumeration of bacterial loads. Following the infection, mice were monitored
573 daily and scored blindly for signs of sickness including weight loss, activity,
574 posture and breathing. Based on these criteria, the mice were given a clinical
575 score of healthy [0] to severely sick [21] as previously described (Mook-
576 Kanamori, Geldhoff, Troost, van der Poll, & van de Beek, 2012).
577
578 ***Flow cytometry***
579 For examining adenosine receptor expression in the lungs, mice were perfused
580 with 10ml PBS, the lungs harvested and minced into small pieces. The lungs
581 were then digested for 1 hour with RPMI 1640 supplemented with 10% FBS, 1
582 mg/ml Type II collagenase (Worthington), and 50 U/ml Deoxyribonuclease I
583 (Worthington) at 37°C/ 5% CO_2. Single-cell suspensions were obtained by
584 mashing the digested lungs, and the red blood cells were removed by treatment
585 with a hypotonic lysis buffer (Lonza). For examining expression of host proteins
586 on H292 cells, the cells were infected with *S. pneumoniae* at an MOI of 2 as
587 described in the binding assay. The cells were resuspended in FACS buffer
588 (HBSS/ 1% FBS) then treated with Fc block (anti-mouse clone 2.4G2 and anti-
589 human clone 3G8) purchased from BD and stained with specific antibodies. The
590 following conjugated anti-human antibodies were used: PAFR (040582;
591 Cederlane), ICAM (clone HA58; Biolegend), CD73 (clone TY/11.8; eBioscience),
592 CD39 (clone24DMS-1; eBioscience) and PIGR (PA535340; Invitrogen). For
593 staining for adenosine receptor antibodies, the cells were permeabilized using

594 the BD Cytofix/Cytoperm kit. The following unconjugated primary rabbit
595 polyclonal anti-adenosine receptor antibodies were purchased from abcam and
596 used: anti-mouse and human A2a (ab3461), A2b (ab222901) and A3 (ab203298)
597 as well as anti-mouse A1 (ab82477). Rabbit polyclonal IgG (ab37415) purchased
598 from abcam and used as an isotype control. For anti-human A1 a primary rabbit
599 monoclonal antibody was used (clone ERP6 179, ab124780). Secondary PE-
600 conjugated anti-Rabbit IgG was used (12473981; Invitrogen). Fluorescence
601 intensities were measured on a BD FACS Fortessa and data were analyzed
602 using FlowJo.
603
604 **RNA extraction and qPCR**
605 RNA was extracted from 2×10^5 H292 using the RNeasy Mini Kit (Qiagen) as per
606 manufacturer's protocol. TURBO DNA-free kit (Invitrogen) was used to digest
607 DNA from the RNA samples prior conversion into cDNA. For each sample, 500ng
608 of RNA was converted into cDNA using SuperScript VILO™ cDNA synthesis kit
609 (Life Technologies) according to the manufacturer's protocol. RT-PCR was
610 performed using CFX96 Touch™ Real-Time PCR Detection System from Bio-
611 Rad and CT (cycle thresh-hold) values were determined using the following
612 TaqMan probes from Life Technologies (Thermo Fischer Scientific): GAPDH
613 (Hs99999905_m1), A1 (Hs00181231_m1), A2A (Hs00169123_m1), A3
614 (Hs00252933_m1), and A2B (Hs00386497_m1). All samples were run in
615 duplicates. Data were analyzed by the comparative threshold cycle ($2^{-\Delta CT}$)
616 method, normalizing the CT values obtained for target gene expression to those

617 for GAPDH of the same sample. For comparison of adenosine receptor levels
618 upon infection, relative quantity of transcripts (RQ) values were calculated by the
619 ΔΔCT method by using the formula RQ =2^– (ΔΔCT) (Livak & Schmittgen, 2001).
620 The ΔΔCT values were obtained by subtracting ΔCT value of the infected from
621 that of the uninfected control.
622
623 ***Western blotting***
624 H292 cells were solubilized in radioimmunoprecipitation assay (RIPA) buffer (1%
625 Triton X-100, 0.25% sodium deoxycholate, 0.05% SDS, 50 mM Tris-HCl [pH 7.5],
626 2 mM EDTA, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium
627 orthovanadate, 10 mg/liter each of aprotinin and leupeptin). Lysates were
628 centrifuged and protein concentrations of the supernatants were quantified using
629 bicinchoninic acid kit (Pierce). Equal protein quantities of each sample were
630 migrated on Mini-PROTEAN TGX Stain-Free Precast Gels (BioRad) and
631 transferred to polyvinylidene difluoride (PVDF) membranes. Incubations with
632 primary antibodies at 1:1000 dilutions were done overnight at 4°C. Incubation
633 with secondary antibodies (1: 5000 dilutions) coupled to horseradish peroxidase
634 was done for 1 hour at room temperature. ChemiDoc XRS+ system (BioRad)
635 was used for detection of all Western blots using Clarity Western ECL Substrate
636 (BioRad). Blots were stripped in stripping buffer containing glycine, Triton-X 100
637 and SDS, and probed for GAPDH, which served as a loading control. Primary
638 antibodies against A1 (ab124780), A2A (ab3461), A2B (ab222901) and A3
639 (ab203298) were all obtained from Abcam. Primary antibodies against GAPDH

640 (MA5-15738) and horseradish peroxidase-conjugated secondary antibodies
641 (31460 and 31430) were purchased from Invitrogen. Images were quantified
642 using ImageJ (version 1.51e) software. Integrated pixel densities in bands
643 corresponding to proteins of interest were measured, and the background was
644 subtracted. Integrated pixel densities for GAPDH as loading controls were also
645 determined and corrected for the background. Background-corrected integrated
646 pixel densities of the protein of interest were normalized to those of the loading
647 control.
648
649 ***Statistics***
650 All statistical analysis was performed using Prism7 (Graph Pad). CFU data were
651 log-transformed to normalize distribution. For all graphs, the mean values +/- SD
652 are presented. Significant differences were determined by 1-sample t-test,
653 Student's t-test or one-way ANOVA followed by the Tukey's multiple
654 comparisons test as indicated. Survival analysis was performed using the log-
655 rank (Mantel-Cox) test. *p* values less than 0.05 were considered significant (as
656 indicated by asterisks).
657
658
659
660
661
662

663 **Figure Legends**

664 **Fig 1. Extracellular adenosine reduces binding of *S. pneumoniae* to**
665 **pulmonary epithelial cells.** (A) H292 cells were treated with adenosine (ADE,
666 100μM) or vehicle control (VC) for 30 minutes and then infected with *S.*
667 *pneumoniae* TIGR4 for 1 hour. The cells were then washed and the amount of
668 bound bacteria were enumerated by plating on blood agar plates. (B) RNA was
669 isolated from uninfected H292 cells and the expression of the indicated enzyme
670 mRNA was measured by RT-qPCR and normalized to GAPDH. (C) Expression
671 of CD73 (left panel) on H292 cells and CD39 on H292 cells and PMNs (right
672 panel) was assessed by flow cytometry. Histograms shown are representative
673 data from one of three separate experiments. (D) H292 cells were treated with
674 either ADE, ATP, AMP (100μM) or vehicle control (VC) for 30 minutes and then
675 infected with *S. pneumoniae* TIGR4 for 1 hour. The number of bound bacteria
676 was enumerated by plating on blood agar plates. The percent of bacterial
677 binding was then calculated relative to vehicle control treatment that was set to a
678 100. (A and D) Data shown are pooled from four separate experiments (n=4
679 biological replicates) where each condition was tested in triplicate (n=3 technical
680 replicates) per experiment. Asterisks indicate significance calculated by (A)
681 Student's t-test and (B) one-way ANOVA followed by Tukey's test.

682

683 **Fig 2. Signaling via extracellular adenosine receptors is required for**
684 **reduction of *S. pneumoniae* binding to pulmonary epithelial cells.** H292
685 cells were treated with either 100μM ADE, 100μM ADE in the presence of 1μM

686 the pan adenosine receptors inhibitor (ADRI) CGS 15943, 1μM the pan
687 adenosine receptors agonist 5'-*N*-Ethylcarboxamidoadenosine (NECA) or vehicle
688 control (VC) for 30 minutes and then infected with *S. pneumoniae* TIGR4 for 1
689 hour. The number of bound bacteria was enumerated by plating on blood agar
690 plates and the percent bacterial binding calculated relative to vehicle control.
691 Data shown are pooled from three separate experiments where each condition
692 was tested in triplicate. Asterisks indicate significance calculated by one-way
693 ANOVA followed by Tukey's test.
694
695 **Fig 3. Expression of adenosine receptors on H292 cells.** (A) RNA was
696 isolated from uninfected H292 cells and the expression of the indicated
697 adenosine receptor mRNA was measured by RT-qPCR and normalized to
698 GAPDH. (B) RNA was isolated from H292 cells mock treated (uninfected) or
699 challenged with *S. pneumoniae* TIGR4 MOI of 2 for 1 hour and the relative
700 change in expression of the indicated adenosine receptor mRNA upon infection
701 was calculated by the ΔΔCT method. Data shown are pooled from three separate
702 experiments. H292 cells were infected with *S. pneumoniae* TIGR4 MOI of 2 for 1
703 hour or mock treated with PBS (uninfected). (C) Expression of adenosine
704 receptors was assessed by flow cytometry. The percentage of cells expressing
705 the receptors was then measured. Data shown are pooled from three separate
706 experiments. (D) Expression of adenosine receptors was assessed by Western
707 Blotting. One representative blot for each receptor is shown. (E) Western Blot
708 band intensities were analyzed using ImageJ and expression of infected sample

709 relative to uninfected controls is shown. Data presented are pooled from two
710 experiments. (B and E) Adenosine receptor mRNA or protein levels upon
711 infection were not significantly different from 1 by one-sample t-test.
712
713 **Fig 4. Signaling via A1 receptor is required for reduces binding of *S.***
714 ***pneumoniae in vitro* and *in vivo.*** (A) H292 cells were treated for 30 minutes
715 with either the indicated concentrations of ADE, vehicle control (VC) or at the
716 following concentrations of A1 agonist 2-Chloro-N6-cyclopentyladenosine
717 (10, 1, 0.1 nM), A2A agonist CGS21680 (270, 27, 2.7nM) or A2B agonist BAY
718 60-6583 (200, 20, 2nM) corresponding to the approximate 10xKi, Ki or 0.1Ki for
719 each receptor as indicated on the graph. The cells were then infected with *S.*
720 *pneumoniae* TIGR4 for 1 hour. The number of bound bacteria was determined by
721 plating on blood agar plates and the percent bacterial binding calculated relative
722 to vehicle control treatment. Data shown are pooled from three separate
723 experiments where each condition was tested in triplicate. Asterisks indicate
724 significant differences as compared to VC calculated by one-way ANOVA
725 followed by Tukey's test.
726
727 **Fig 5. Signaling via A1 receptor reduces expression of PAFR on H292 cells.**
728 H292 cells were treated for 30 minutes with vehicle control (VC), ADE (100μM),
729 or A1 agonist 2-Chloro-N6-cyclopentyladenosine (10nM) as indicated. The cells
730 were then infected with *S. pneumoniae* TIGR4 MOI of 2 or mock treated
731 (uninfected) for 1 hour. (A-C) Expression of ICAM-1, PIGR and PAFR was

732 assessed by flow cytometry. The Mean florescent intensities (MFI) as well as the
733 percentage of cells expressing the indicated ligands were then measured. (A-C)
734 Data shown are pooled from three separate experiments where each condition
735 was tested in triplicate. Asterisks indicate significant differences calculated by
736 one-way ANOVA followed by Tukey's test.
737
738 **Fig 6. A1 receptor signaling does not further decrease bacterial binding**
739 **when PAFR is inhibited.** H292 cells were treated for 30 minutes with the
740 indicated vehicle control (VC), ADE (100μM), A1 agonist 2-Chloro-N6-
741 cyclopentyladenosine (10nM) or PAFR inhibitor (40nM). The cells were then
742 infected with *S. pneumoniae* TIGR4 MOI of 20 for 1 hour. The number of bound
743 bacteria was enumerated by plating on blood agar plates and the percent
744 bacterial binding calculated relative to vehicle control. Data shown are pooled
745 from four separate experiments where each condition was tested in triplicate.
746 Asterisks indicate significant differences vs. vehicle control treated cells
747 calculated by one-way ANOVA followed by Tukey's test.
748
749 **Fig 7. Pulmonary expression of adenosine receptors in mice are not altered**
750 **by aging or *S. pneumoniae* infection.** Young and old C57BL/6J mice were
751 infected with *S. pneumoniae* intra-tracheally. The lungs were harvested six hours
752 post infection and digested into a single cell suspension and the expression of
753 adenosine receptors analyzed by flow cytometry. (A) Histograms shown are
754 representative data from young mice and (B) bar graphs are quantification of the

755 Mean Florescent Intensities (MFI) of the indicated adenosine receptor from
756 young and old mice and are pooled from six separate experiments with n=9-12
757 mice per group.
758
759 **Fig 8. Signaling via A1 receptor is important for the control of pulmonary**
760 **pneumococcal burden *in vivo.*** Young mice mock-treated or treated with
761 DPCPX, an A1 receptor inhibitor and old mice mock-treated or treated with 2-
762 Chloro-N6-cyclopentyladenosine, an A1 receptor agonist were inoculated intra-
763 tracheally with ~1-2x10^4 CFU of *S. pneumoniae* TIGR4. (A) Bacterial burdens in
764 the lungs were determined 6 hours post-infection. Data shown are pooled from
765 n=9 young and n=4 old mice per group. (B-E) To follow the infection over time,
766 mice were challenged with ~1-2x10^4 CFU of *S. pneumoniae* TIGR4. Bacterial
767 burdens in the blood (B) and clinical scores (C) were determined 24 hours post-
768 infection. (A-B) Dashed lines indicate the limit of detection. Asterisks indicate
769 significant differences calculated by one-way ANOVA followed by Tukey's test.
770 Survival of control and A1 inhibited young mice (D) as well as control and A1
771 agonist-treated old mice (E) and was assessed at the indicated time points
772 following challenge. (F) Young mice control treated or administered A1 receptor
773 agonist were infected with ~1-2x10^5 CFU, a lethal dose of *S. pneumoniae* TIGR4
774 and survival was followed over time. Fractions denote survivors at 7 days after
775 challenge over the total number of mice. Asterisks indicate a statistical
776 significance by the log-Rank (Mantel-Cox) test. Data were pooled from two
777 independent experiments.

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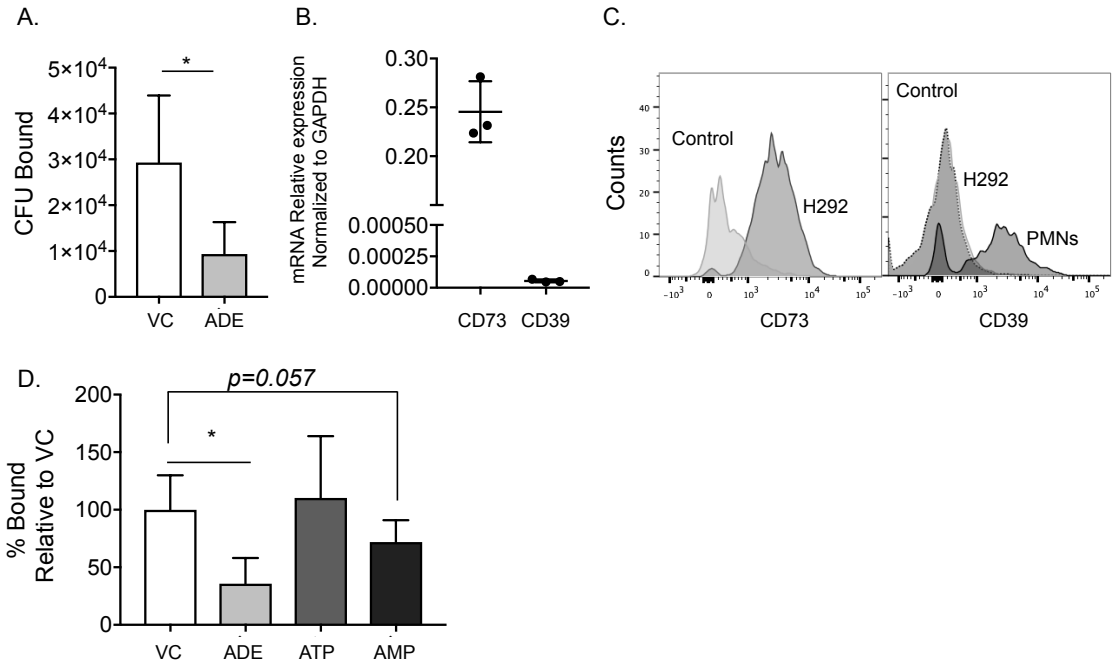
Figures

Figure 1



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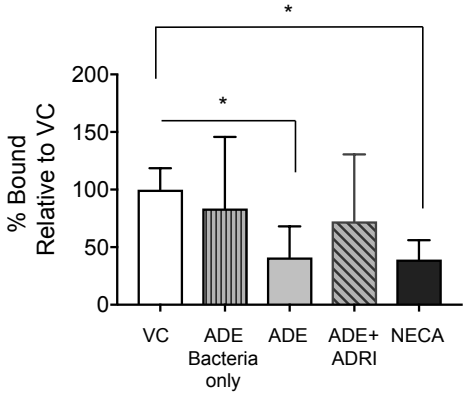

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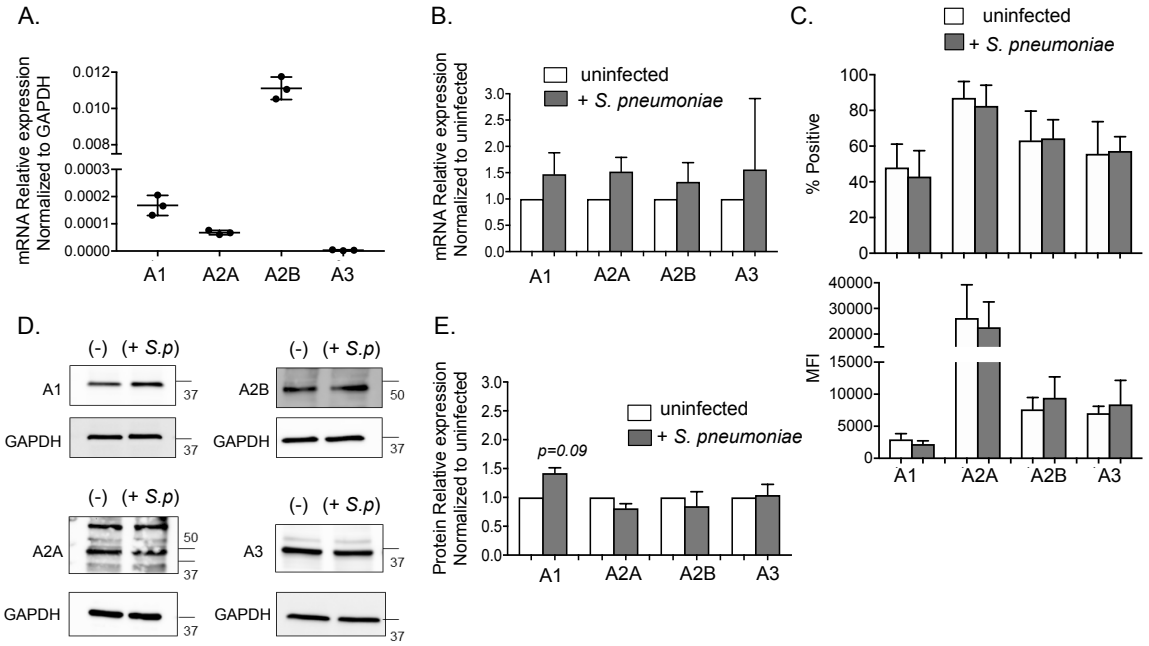
Figure 3



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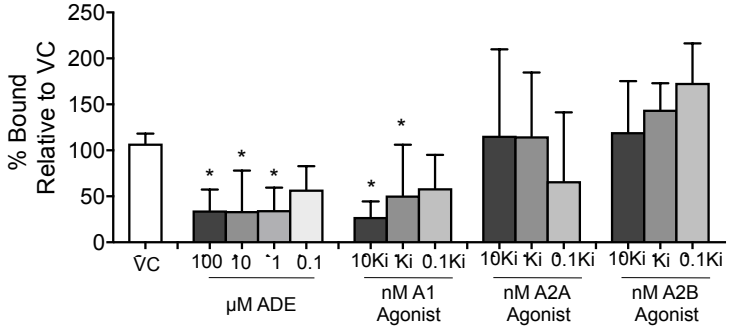

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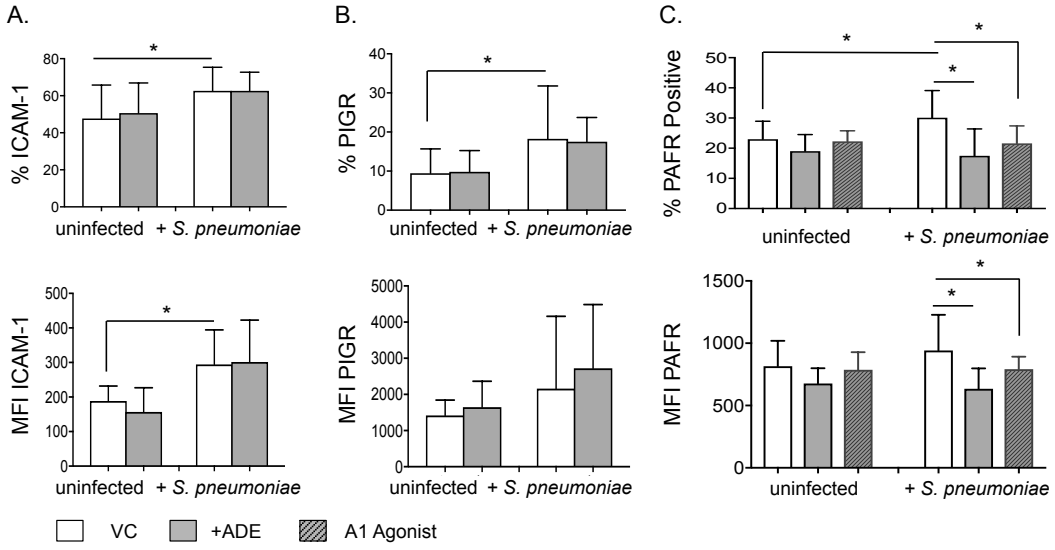

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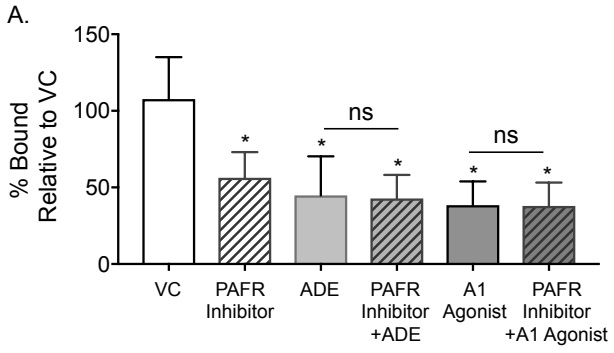

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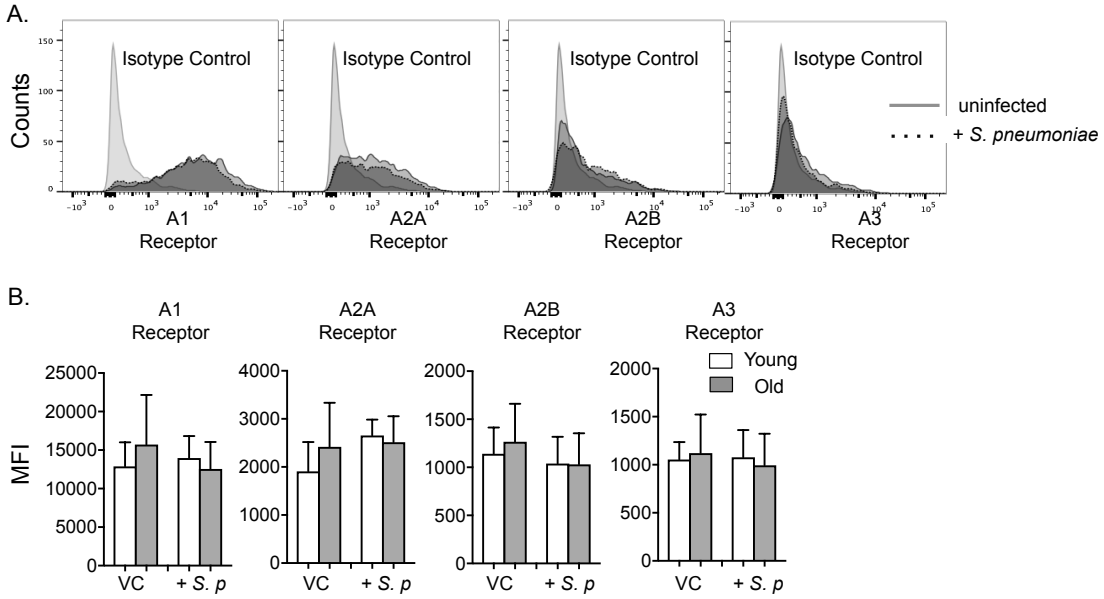

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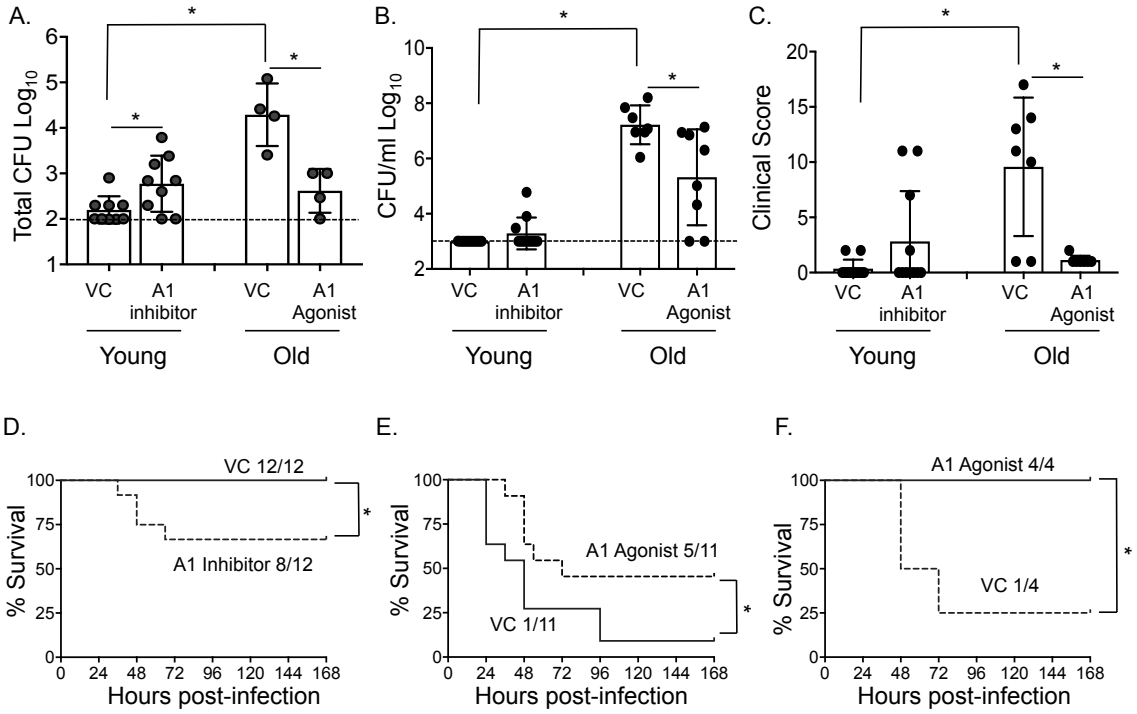
Figure 8



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