Extracellular adenosine enhances the ability of PMNs to kill *Streptococcus pneumoniae* by inhibiting IL-10 production

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Summary Sentence: Extracellular adenosine produced by CD73 promotes the ability of PMNs to kill *Streptococcus pneumoniae* by blunting IL-10 production

Running Title: PMN anti-bacterial phenotype requires CD73

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24 **Abbreviations**

25 CFU: Colony Forming Units

26 CT: Cycle thresh-hold

27 EAD: Extracellular adenosine

28 I.P: Intra peritoneal

29 I.T: Intra tracheal

30 MFI (Mean Fluorescent Intensity)

31 MOI: Multiplicity of infection

32 MPO: Myeloperoxidase

33 NE: Neutrophil Elastase

34 OPH: Opsonophagocytic

35 PLY: Pneumolysin

36 PMA: Phorbol 12-myristate 13-acetate

37 PMNs: Polymorphonuclear leukocytes

38 Pneumococcus: *Streptococcus pneumoniae*

39 ROS: Reactive oxygen species

40 WT: Wild type

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Abstract

PMNs are crucial for initial control of *Streptococcus pneumoniae* (pneumococcus) lung infection; however, as the infection progresses their persistence in the lungs is detrimental. We found that the progressive inability of PMNs to control infection correlated with phenotypic differences characterized by a decrease in CD73 expression, an enzyme required for production of extracellular adenosine (EAD). EAD production by CD73 was crucial for PMN function as PMNs from CD73$^{-/-}$ mice failed to kill pneumococci and supplementation with EAD reversed this defect. Further, adoptive transfer of PMNs from wild type mice prior to lung challenge was sufficient to boost the resistance of CD73$^{-/-}$ mice to infection. Importantly, CD73 activity was important for the antimicrobial function of PMNs from human donors. We found that CD73-mediated resistance is due to its inhibitory effects on IL-10. PMNs from CD73$^{-/-}$, but not wild type mice, up-regulated IL-10 production upon pneumococcal infection in the lungs and *in vitro*. IL-10 inhibited PMN function, as addition of recombinant IL-10 impaired the ability of PMNs from wild type mice to kill pneumococci *ex vivo*, and treatment with anti-IL-10 boosted the bactericidal activity of CD73$^{-/-}$ PMNs. Blocking IL-10 also boosted the resistance of *CD73$^{-/-}$* mice to pneumococcal pneumonia. CD73/IL-10 did not affect apoptosis, bacterial uptake and intracellular killing or production of anti-microbial Neutrophil Elastase and Myeloperoxidase. Rather, inhibition of IL-10 production by CD73 was important for production of extracellular ROS by PMNs upon *S. pneumoniae*

69 infection. This study demonstrates that CD73 regulates PMN anti-microbial
70 phenotype during pneumococcal pneumonia.
71
72 **1. Introduction**
73 Neutrophils, also known as polymorphonuclear leukocytes or PMNs, play
74 a major role in host defense against *S. pneumoniae* lung infection (1, 2). PMNs
75 are required to control bacterial burden early in the infectious process. In mouse
76 models, early PMN recruitment into the lungs within the first 12 hours following
77 pneumococcal pulmonary infection coincided with a decrease in bacterial
78 numbers and immunodepletion of PMNs prior to infection resulted in host lethality
79 (3). *Ex vivo*, PMNs are thought to kill *S. pneumoniae* by engulfing the bacteria
80 and producing serine proteases including cathepsin G (CG) and neutrophil
81 elastase (NE) (4), which were also required for controlling bacterial numbers in
82 murine models of pneumonia (5). Interestingly, later during infection, PMN
83 persistence in the lungs promotes disease (3, 6, 7). In fact, we previously found
84 that depletion of PMNs 18 hours after pneumococcal lung infection resulted in
85 reduced bacterial numbers and enhanced mouse survival (3). These findings
86 demonstrated that while PMNs are required at the start of infection, their
87 persistence is detrimental for host survival. This also suggested that the
88 antibacterial function of these innate immune cells and their ability to clear
89 pneumococci is altered during the course of infection (3). However the
90 mechanisms of this alteration in PMN phenotype remain unexplored.
91 A major regulator of host resistance to pneumococcal infection is

extracellular adenosine (EAD) (3). Upon damage due to a variety of insults including infection, ATP is thought to leak from damaged cells into the extracellular space and is converted into EAD by the sequential action of two exonucleosidases, CD39 and CD73 (8). EAD can then signal via four known receptors, i.e. A1, A2A, A2B and A3 (9). Several drugs targeting this pathway are in clinical studies or in use to treat a variety of inflammatory diseases (9), making it an attractive avenue for modulating immune responses during infection. We previously found that blocking EAD production by CD73 dramatically increased bacterial numbers in organs and resulted in host lethality upon *S. pneumoniae* lung infection in mice (3). EAD production by CD73 regulated PMN recruitment to the lungs and potentially boosted PMN function (3).

IL-10 is an anti-inflammatory cytokine associated with impaired control of pneumococcal pneumonia (10, 11). In mouse models, IL-10 levels increase in the lungs within 12 hours following pneumococcal challenge (10). Intranasal administration of IL-10 at the time of bacterial challenge results in decreased host survival and increased bacterial numbers in the lungs and blood, while blocking IL-10 prior to infection enhances bacterial clearance and boosts host survival (10). Interestingly, while IL-10$^{-/-}$ mice have reduced lung and systemic bacterial loads, these mice suffer increased mortality due to excessive pulmonary inflammation (12). IL-10 can be produced by many types of immune cells and it is now appreciated that murine PMNs can also produce this anti-inflammatory cytokine during infections (13-16). PMNs are a significant source of IL-10 in sepsis models (17) and stimulation with several bacterial pathogens, including

115 *Escherichia coli*, *Shigella flexneri* and mycobacterial BCG, triggers IL-10
116 production by PMNs both *in vitro* and *in vivo* within 8 hours of infection (16).
117 Parasitic infections such as *Leishmania major* (14) and *Trypanosoma cruzi* (15)
118 also induce IL-10-producing PMNs during mouse infection. IL-10 production by
119 PMNs may reduce their microbicidal capacity, as *in vitro* treatment of PMNs with
120 IL-10 impairs their ability to phagocytose *Staphylococcus aureus*, *Candida*
121 *albicans* and *E. coli* and blunts superoxide production (18, 19). Further, in a *S.*
122 *aureus* burn infection model, a subset of IL-10 producing PMNs is associated
123 with impaired host resistance to infection (13). It is not currently known if *S.*
124 *pneumoniae* infection triggers IL-10 production by PMNs.
125 In this study, we explored the involvement of CD73 in the diminished
126 efficacy of PMNs during the course of pneumococcal pneumonia. We found that
127 during the course of lung infection, CD73 expression on PMNs decreased.
128 Notably, this change in CD73 expression coincided with an increase in IL-10-
129 producing PMNs, and we showed that CD73 suppressed IL-10 production by
130 PMNs during infection and was required for the ability of these cells to kill *S.*
131 *pneumoniae ex vivo.* This study identifies a role for CD73 in maintaining the
132 antimicrobial phenotype of PMNs and elucidates the mechanisms by which CD73
133 regulates PMN anti-microbial function and host resistance to pneumococcal
134 pneumonia.
135
136 **2. Materials and Methods**
137 *2.1 Mice*

138 All experiments were conducted in accordance with Institutional Animal Care and
139 Use Committee (IACUC) guidelines. Wild type (WT) C57BL/6 mice were
140 purchased from Jackson Laboratories (Bar Harbor, ME). CD73$^{-/-}$ mice on
141 C57BL/6 background were purchased from Jackson Laboratories and bred at a
142 specific-pathogen free facility at Tufts University and The University at Buffalo.
143 Female 8-12 week old mice were used in all experiments.
144
145 *2.2 Bacteria*
146 Wild type *S. pneumoniae* TIGR4 strain (serotype 4) and pneumolysin-deletion
147 mutant (ΔPLY) *S. pneumoniae* (20) were kind gifts from Andrew Camilli. Bacteria
148 were grown at 37°C in 5% CO_2 in Todd-Hewitt broth supplemented with 0.5%
149 yeast extract and oxyrase until culture reaches mid-exponential phase. Bacterial
150 aliquots were frozen at -80˚C in the growth media with 20% (v/v) glycerol. Prior to
151 use, aliquots were thawed on ice, washed and suspended in PBS to obtain the
152 appropriate concentration. The inoculums were then confirmed by serial dilution
153 and dribble plating on Tryptic Soy Agar plates supplemented with 5% sheep
154 blood agar.
155
156 *2.3 Isolation of PMNs*
157 Bone marrow cells were harvested from femurs and tibias of uninfected mice,
158 flushed with RPMI 1640 supplemented with 10% FBS and 2 mM EDTA, and
159 resuspended in PBS. Neutrophils were then separated from the rest of the bone
160 marrow cells through density gradient centrifugation, using Histopaque 1119 and

161 Histopaque 1077 as previously described (21). The isolated neutrophils
162 were resuspended in Hanks' Balanced Salt Solution (HBSS)/0.1% gelatin
163 without Ca^{2+} and Mg^{2+}, and used in subsequent assays. The purity of neutrophils
164 was also measured by flow cytometry using APC-conjugated anti-Ly6G and 85-
165 90% of enriched cells were Ly6G+.
166
167 *2.4 Opsonophagocytic killing assay*
168 The ability of PMNs to kill pneumococci was assessed *ex vivo* through an
169 Opsonophagocytic (OPH) killing assay as previously described (3, 22). Briefly,
170 100µl reactions in HBSS/0.1% gelatin consisted of $1x10^5$ PMNs incubated with
171 $1x10^3$ bacteria grown to mid log phase and pre-opsonized with 3% mouse sera.
172 The reactions were incubated rotating for 45 minutes at 37°C. Where indicated,
173 PMNs were incubated as indicated with adenosine (100µM) or rIL-10 (50ng/mL)
174 or anti-IL10 (1ug/mL JES5-2A5) or isotype control (1ug/mL) or 1x protease
175 inhibitor cocktail for 30 minutes prior to adding pre-opsonized bacteria. HBSS
176 with 3% sera was added to PMNs without treatment as the control. Percent killing
177 was determined by dribble plating on blood agar plates and calculated in
178 comparison to no PMN control under the exact same conditions (+/- treatments).
179
180 *2.5 IL-10 Enzyme-Linked Immunosorbent Assay (ELISA)*
181 $1x10^6$ Bone marrow PMNs were incubated with HBSS/0.1% gelatin or adenosine
182 for 30 minutes, followed by 45 minutes infection at 37° C with pre-opsonized *S.*
183 *pneumoniae* TIGR4 at an MOI of 2 or mock treated with HBSS and sera only

184 (uninfected). The reactions were spun down and the supernatants were obtained
185 to measure IL-10 production using Mouse IL-10 ELISA kit (e-bioscience)
186 according to manufacturer's instructions.
187
188 *2.6 ROS Assay*
189 Following isolation from the bone marrow, PMNs were re-suspended in HBSS
190 (Ca^{2+} and Mg^{2+} free) and acclimated at room temperature for one hour. The cells
191 were then spun down and re-suspended in KRP buffer (Phosphate buffered
192 saline with 5mM glucose, 1mM $CaCl_2$ and 1mM $MgSO_4$) and equilibrated at room
193 temperature for 30 minutes. The cells were then seeded in 96-well white
194 LUMITRAC™ plates (Greiner Bio-One) at $5x10^5$ PMNs per well, treated with anti-
195 IL-10 or rIL-10 for 30 minutes at 37° C. For detection of extracellular ROS, 50μM
196 Isoluminol (Sigma) plus 10U/ml HRP (Sigma) were added, while for detection of
197 intracellular ROS, 50μM Luminol (Sigma) was added to the wells as previously
198 described (23-26). The cells were infected with pre-opsonized *S. pneumoniae*
199 TIGR4 at an MOI of 2 or mock treated with buffer containing 3% mouse sera
200 (uninfected). PMNs treated with 100 nM Phorbol 12-myristate 13-acetate (PMA)
201 (Sigma) were used as a positive control. Luminescence was immediately read
202 (following infection) over a period of one hour at 37° C in a pre-warmed Biotek
203 Plate reader. Wells containing buffer and Isoluminol plus HRP or Luminol alone
204 were used as blanks.
205
206 *2.7 Animal infections and scoring*

207 Mice were lightly anesthetized with isoflurane and challenged intra-tracheally (i.t.)
208 with $5x10^5$ colony-forming units (CFU) of WT *S. pneumoniae* TIGR4 strain by
209 instilling 50μl of bacteria directly into the trachea with the tongue pulled out to
210 facilitate delivery of bacteria directly into the lungs (3). Following the infection,
211 mice were monitored daily for weight loss and blindly scored for signs of sickness
212 including weight loss, activity level, posture and breathing, scored as healthy [0]
213 to severely sick [21] as previously described (27). The lung and brain were
214 harvested and homogenized in sterile PBS. Blood was collected to follow
215 bacteremia. Each sample was diluted in sterile PBS and dribble plated on blood
216 agar plates to enumerate bacterial numbers.
217
218 *2.8 Isolation of cells from the lungs and flow cytometry*
219 Mice were perfused with 10ml PBS, the lungs removed, washed in PBS, and
220 minced into small pieces. The lungs were then digested for 1 hour with RPMI
221 1640 supplemented with 10% FBS, 1 mg/ml Type II collagenase (Worthington),
222 and 50 U/ml Deoxyribonuclease I (Worthington) at 37° C/ 5% CO_2. Single-cell
223 suspensions were obtained by mashing the digested lungs, and the red blood
224 cells were removed by treatment with a hypotonic lysis buffer (Lonza). Cells were
225 analyzed using flow cytometry. Intracellular cytokine staining (ICS) was
226 performed using the Cytofix/Cytoperm kit (BD Biosciences). GolgiPlug was
227 added to the digestion media and following red blood cell lysis, the cells were
228 incubated with RPMI 1640 supplemented with 10% FBS and Golgi Plug for 3
229 more hours at 37° C/ 5% CO_2. Cells were surface stained with anti-mouse CD45

230 (clone 30-F11, e-Bioscience), Ly6G (clone 1A8, BD Biosciences) and CD73
231 (Clone TY/11.8, e-Bioscience). For intracellular staining, cells were
232 permeabilized and stained with IL-10 (clone JES5-16E3, e-Bioscience) or isotype
233 control (Rat IgG2b K Isotype Control, Biolegend). Fluorescence intensities were
234 measured on a FACSCalibur and at least 25,000 events for lung tissue were
235 analyzed using FlowJo.
236
237 *2.9 Adoptive transfer of PMNs*
238 Bone marrow PMNs were isolated from uninfected mice using density gradient
239 centrifugation as described above and resuspended in PBS. Mice either received
240 $2.5x10^6$ cells via intraperitoneal (i.p.) injection or mock-treated (PBS). One hour
241 post transfer, mice were challenged i.t. with $5x10^5$ CFU of *S. pneumoniae.* Mice
242 were euthanized at 24 hours post infection, and the lungs, brain, and blood were
243 collected and plated on blood agar plates for CFU.
244
245 *2.10 Blocking IL-10 in vivo*
246 Mice were treated with 0.1mg/mouse of IL-10 blocking antibody (JES52A5,
247 Biolegend) or the isotype control (Rat IgG1 K Isotype Control, Biolegend) through
248 i.p. injection. After 2 hours, mice were then challenged i.t. with $5x10^5$ CFU of *S.*
249 *pneumoniae.* The lungs and blood were collected two days post-infection to
250 determine bacterial burden.
251
252 *2.11 Assays with human PMNs*

253 Young (23-38 years old), male and female healthy human volunteers were
254 recruited in accordance with The University at Buffalo Human Investigation
255 Review Board (IRB) and signed informed consent forms. Individuals taking
256 medication, reporting symptoms of infection within the last 2 weeks or that were
257 pregnant were excluded from the study. Whole blood was obtained using acid
258 citrate/dextrose as an anti-coagulant. PMNs were isolated using a 2% gelatin
259 sedimentation technique as previously described (28) which allows for isolation
260 of active PMNs with ~90% purity. 5 x 10^5 PMNs were treated with the selective
261 and competitive inhibitor of CD73, α,β methylene ADP (1 or 10μM) or DMSO
262 vehicle control for 30 minutes at 37° C. The PMNs were then infected with 10^3
263 CFU *S. pneumoniae* pre-opsonized in 10% (v/v) baby rabbit serum (Pel-Freeze)
264 for 45 minutes at 37°C with rotation. Samples were then placed on ice to stop the
265 process of opsonophagocytosis followed by serial dilution and plating on blood
266 agar plates to enumerate viable CFU. The percentage of bacterial killing was
267 calculated relative to no PMN controls incubated under the same treatment
268 conditions.
269
270 *2.12 mRNA measurement*
271 RNA was extracted from 2×10^6 human PMNs using the RNeasy Mini Kit (Qiagen)
272 as per manufacturer's protocol. TURBO DNA-free kit (Invitrogen) was used to
273 digest DNA from the RNA samples prior conversion into cDNA. RNA
274 concentration and 260/280 ratio were determined using Nano-drop (Thermo
275 Fischer Scientific). For each sample, 500ng of RNA was converted into cDNA

276 using SuperScript VILO™ cDNA synthesis kit (Life Technologies) according to
277 the manufacturer's protocol. RT-PCR was performed using CFX96 Touch™
278 Real-Time PCR Detection System from Bio-Rad and CT (cycle thresh-hold)
279 values were determined using the following TaqMan probes from Life
280 Technologies (Thermo Fischer Scientific): GAPDH (human Hs99999905_m1)
281 CD73 (human Hs00159686_m1) and IL-10 (human Hs00961622_m1). Each
282 sample was run in duplicates. Data were analyzed by the comparative threshold
283 cycle ($2^{-\Delta CT}$) method, normalizing the CT values obtained for CD73 and IL-10
284 expression to those for GAPDH of the same sample. For comparison of IL-10
285 levels upon infection, relative quantity of transcripts (RQ) values were calculated
286 by the ΔΔCT method by using the formula RQ =2^– (ΔΔCT) (29). The ΔΔCT
287 values were obtained by subtracting ΔCT value of the infected (test) from that of
288 the uninfected control.
289
290 *2.13 Statistics*
291 All statistical analysis was performed using Prism7 (Graph Pad). CFU data were
292 log-transformed to normalize distribution. For all graphs, the mean values +/- SD
293 are shown. Significant differences were determined by 2-way ANOVA followed
294 by Sidak's multiple comparisons test or Student's t-test where *p* values less than
295 0.05 were considered significant (as indicated by asterisks). Pearson test was
296 used to determine correlation.
297
298 **Online Supplemental Material**

299 *Adenosine measurement*

300 Blood was collected from mice by venipuncture in microtainer tubes. The tubes
301 were centrifuged at 9000rpm for 2 minutes and the sera obtained. Adenosine
302 level in the sera was measured using the Adenosine Assay Fluorometric Kit
303 (BioVision) as per manufacturer's instructions.

304

305 *Bacterial uptake and intracellular killing assay*

306 To determine uptake/phagocytosis and intracellular killing by PMNs, we
307 performed Gentamicin-protection assay. Isolated PMNs from uninfected mice
308 were incubated with pre-opsonized wild type or ∆PLY *S. pneumoniae* (MOI=2) for
309 10 minutes at 37°C. Gentamycin (100ug/ml) was then added for 40 minutes to kill
310 extracellular bacteria. The reactions were washed three times with HBSS and
311 resuspended in 100ul HBSS/0.1% gelatin. To measure initial bacterial uptake,
312 the reactions were diluted and plated on blood agar plates and the percentage of
313 the input inoculum that was engulfed was calculated. To determine intracellular
314 killing, the reactions were returned to 37°C and incubated for 30 minutes and 90
315 minutes. The reactions were diluted and plated on blood agar plates and the
316 percentage of the engulfed inoculum (at 10 minutes) that was killed was then
317 calculated.

318

319 *Neutrophil Elastase and Cathepsin G activity assay*

320 1x10^{6} Bone marrow PMNs were treated with adenosine, anti-IL-10 or rIL-10 or
321 mock treated with HBSS/0.1% gelatin for 30 minutes at 37° C. The PMNs were

322 then infected with pre-opsonized *S. pneumoniae* TIGR4 at an MOI of 2 or mock
323 treated with buffer containing 3% mouse sera (uninfected) for 45 minutes at 37°
324 C. The reactions were spun down and the supernatants were used to measure
325 neutrophil elastase and cathepsin G activities with the Neutrophil Elastase
326 Activity Assay Kit Fluorometric (Abcam) and the Cathepsin G Activity Assay Kit
327 (Abcam) respectively (28).
328
329 *Apoptosis Assay*
330 PMNs were pre-treated with buffer or incubated with anti-IL-10 or rIL-10 for 30
331 minutes at 37° C then infected with pre-opsonized *S. pneumoniae* TIGR4 at an
332 MOI of 2 or mock treated with buffer containing 3% mouse sera (uninfected) for
333 45 minutes at 37° C. The percentage of apoptotic cells were then determined by
334 flow cytometry using the FITC Annexin V apoptosis detection kit with PI
335 (BioLegend) following manufacturer's instructions.
336
337 *Myeloperoxidase (MPO) levels*
338 1x10^{6} Bone marrow PMNs were treated with anti-IL-10 or rIL-10 or mock treated
339 with HBSS/0.1% gelatin for 30 minutes at 37° C. The PMNs were then infected
340 with pre-opsonized *S. pneumoniae* TIGR4 at an MOI of 2 or mock treated with
341 buffer containing 3% mouse sera (uninfected) for 30 minutes at 37° C. The cells
342 were lysed with lysis buffer containing 0.1% Triton-X and MPO levels measured
343 by ELISA (Invitrogen) according to manufacturer's instructions.
344

345 **3. Results**

346 **3.1 CD73 expression on PMNs decreases over the course of infection**

347 We previously found that PMNs are required for protection at the
348 beginning of *S. pneumoniae* lung infection (3), but are detrimental at later times.
349 To test if there was a correlation between PMN numbers in the lungs and
350 bacterial burden, we infected young C57BL/6 mice intra-tracheally (i.t.) with
351 $5x10^5$ colony-forming units (CFU) of *S. pneumoniae* TIGR4 strain and monitored
352 bacterial burden and pulmonary influx of PMNs over the first 72 hours. We then
353 performed correlation analysis between PMN number and lung CFU for 0-12
354 hours vs. 18-72 hours following infection. We found that PMN influx into the lungs
355 strongly correlated with a decrease in bacterial burden for the first 12 hours
356 following infection (Fig 1A left panel, R-squared =0.71, *p=0.0001*). However,
357 there was no correlation between PMN presence in the lungs and bacterial
358 numbers for the remainder of the infection (Fig 1A right panel) suggesting that
359 PMNs are no longer able to control the infection.

360 To determine if this was associated with a difference in the phenotype of
361 pulmonary PMNs over time, we monitored the expression of the EAD-producing
362 enzyme CD73 on PMNs in the lungs for 72 hours following lung challenge. We
363 found that in the first 18 hours of infection, the majority (~75-80%) of PMNs in the
364 lungs expressed CD73 (Fig. 1B). However, both the percentage of PMNs
365 expressing CD73 and the amount (measured by the mean fluorescent intensity
366 or MFI) of CD73 expressed on PMNs in the lungs significantly dropped over the
367 course of infection. CD73 expression had dropped to half by 72 hours and the

368 drop started to occur after 12 hours post-infection, a time point after which PMN
369 presence in the lungs no longer correlated with control of bacterial numbers (Fig
370 1A) (3). The decrease of CD73 expression also occurred on circulating PMNs
371 and bone marrow PMNs (Fig. 1C), suggesting that this was a systemic decrease
372 and not only localized in the lungs. A recent study identified a population of
373 PMNs in the spleen with intermediate expression of Ly6G on their surface that
374 exhibited a lowered ability to engulf *S. pneumoniae* during i.v. infection (30).
375 When we compared Ly6G expression, we found that CD73 positive pulmonary
376 PMNs had significantly higher expression of Ly6G (MFI) on their surface at all the
377 time points tested (Fig. 1D). These data show that there are changes in PMNs
378 over the course of pneumococcal pneumonia and that low CD73 expression on
379 PMNs may be indicative of a PMN subset that is associated with lower anti-
380 microbial activity.
381
382 **3.2 CD73 and extracellular adenosine are required for the ability of PMNs to**
383 **kill *S. pneumoniae***
384 Prior studies demonstrated that EAD is required for protection against *S.*
385 *pneumoniae* lung infection (3). To test if EAD production has a role in anti-
386 pneumococcal function of PMNs, we compared *ex vivo* opsonophagocytic killing
387 of pneumococci by PMNs isolated from the bone marrow of *CD73*$^{-/-}$ or wild type
388 C57BL/6 (WT) mice. Strikingly, *CD73*$^{-/-}$ PMNs completely failed to kill *S.*
389 *pneumoniae* and in fact the presence of these PMNs promoted an increase in
390 bacterial numbers as indicated by negative bacterial killing on the graph (Fig. 2).

391 To test if the inability of *CD73*$^{-/-}$ PMNs to kill bacteria was due to a defect in EAD
392 production, we added back adenosine to the opsonophagocytic reactions. Upon
393 infection, adenosine levels in the sera of WT mice varied considerably, but on
394 average significantly increased five-fold to 52.6 +/- 29.0 μM as measured by a
395 fluorometric kit (Fig S 1A). We found that supplementing *CD73*$^{-/-}$ PMNs with EAD
396 at 100 μM (closer to the higher levels found in infected mice) restored the ability
397 of these cells to kill bacteria back to levels comparable with WT PMNs. The
398 difference in bacterial killing observed was not due to difference in bacterial
399 survival in WT versus *CD73*$^{-/-}$ sera or direct toxic effects of EAD on bacterial
400 viability (Fig. S1B and C). Also, addition of EAD had no significant effect on
401 bacterial killing by WT PMNs (Fig 2).
402 To test the clinical relevance of our findings, we tested the effect of CD73
403 inhibition on the anti-bacterial activity of PMNs from human donors. PMNs were
404 isolated from the blood of young healthy donors and treated with the selective
405 and competitive inhibitor of CD73, α,β methylene ADP or vehicle control *in vitro*
406 and their ability to kill pneumococci was assessed. We found that in over half (i.e.
407 4 out of 7) of the donors tested, CD73 inhibition completely abrogated the ability
408 of PMNs to kill pneumococci and in fact promoted bacterial growth in the
409 presence of PMNs instead (Fig. 3A). The inhibitor had no direct effect on the
410 viability of *S. pneumoniae* (data not shown) as previously described (3). CD73
411 mRNA was detected in all five donors we tested (Fig. 3B) and relative expression
412 did not correlate with the responsiveness of donor PMNs to CD73 inhibition.

413 These findings demonstrate that production of EAD by CD73 is required for the
414 ability of PMNs to kill pneumococci.
415
416 **3.3 Adoptive transfer of PMNs from wild type mice boosts resistance of**
417 **CD73$^{-/-}$ mice to *S. pneumoniae***
418 To test the role of EAD production by PMNs *in vivo*, we adoptively
419 transferred 2.5x10^{6} PMNs isolated from WT or CD73$^{-/-}$ mice into CD73$^{-/-}$ mice,
420 one hour later infected the mice with 5x10^{5} CFU of *S. pneumoniae* i.t. and then
421 compared bacterial burdens in the lung, blood and brain at 24 hours post
422 infection. We found that although transfer of WT PMNs had no impact on
423 bacterial numbers in the lungs (Fig. 4A), it significantly reduced the systemic
424 spread of pneumococci and resulted in a 50 and 5-fold reduction in blood and
425 brain bacterial loads respectively (Fig. 4B and C) when compared to no transfer
426 controls. Surprisingly, we found that adoptive transfer of CD73$^{-/-}$ PMNs worsened
427 susceptibility to infection and resulted in a significant ~10-fold increase in
428 bacterial numbers in all sites (lung, blood, brain) tested (Fig. 4A-C) when
429 compared to no transfer controls. This was consistent with our *in vitro* findings
430 where bacterial numbers increased in the presence of CD73$^{-/-}$ PMNs. These
431 findings suggest that EAD production by PMNs is required and sufficient to
432 promote resistance to *S. pneumoniae* infection.
433
434 **3.4 Bacterial uptake and intracellular killing by PMNs are not altered by**
435 **CD73 and EAD**

436 We next wanted to identify how CD73 and adenosine were regulating the
437 ability of PMNs to kill *S. pneumoniae*. Pneumococcal killing by PMNs was
438 previously shown to depend on uptake and serine proteases (4, 5). To test if
439 EAD production was affecting bacterial phagocytosis, we used a gentamicin-
440 protection assay where gentamicin was added following PMN incubation with
441 pre-opsonized pneumococci to kill any extracellular bacteria. A potentially
442 confounding factor is that wild type *S. pneumoniae* produce pneumolysin (PLY),
443 which creates pores in the mammalian cell membranes and may allow
444 gentamicin to enter the host cell and kill intracellular bacteria. In fact, a PLY-
445 deletion mutant demonstrated 2-fold greater gentamicin protection than wild type
446 *S. pneumoniae* (Fig. S2A). This was not due to difference in sensitivity to
447 gentamicin (Fig. S2B) or susceptibility to overall opsonophagocytic killing by
448 PMNs (Fig. S2C) which is consistent with previous reports that PLY has no
449 significant role in bacterial killing by PMNs (31). These data suggest that using
450 gentamicin protection assays to determine intracellular entry/growth of bacteria
451 such as *S. pneumoniae* that produce pore forming toxins may underestimate the
452 true numbers of intracellular bacteria.
453 We proceeded with using *S. pneumoniae* ΔPLY to compare phagocytosis
454 between WT and CD73$^{-/-}$ PMNs. To differentiate uptake from intracellular killing,
455 we looked at an early time point 10 minutes post infection, and found no
456 significant difference in the percentage of engulfed bacteria between the different
457 mouse strains (Fig. S2D). We also found that the engulfed bacteria were very
458 efficiently killed by both WT and CD73$^{-/-}$ PMNs (Fig. S2E) with 50% and 100% of

459 the engulfed inoculum being killed at 30 and 90 minutes post-uptake
460 respectively. These findings suggest that CD73/EAD does not affect bacterial
461 uptake or intracellular killing by PMNs.
462
463 **3.5 Neutrohil Elastase levels are not altered by CD73 and EAD**
464 Serine protease such as cathepsin G (CG) and neutrophil elastase (NE)
465 were shown to be essential for the ability of PMNs to kill *S. pneumoniae* (4, 5).
466 We therefore measured if CD73 and EAD altered the activities of serine
467 proteases Cathepsin G and NE. We were unable to detect Cathepsin G
468 enzymatic activity either at baseline or upon pneumococcal infection in any of the
469 samples we collected. NE activity was easily detected, however, we found no
470 significant difference in NE activity between WT and CD73$^{-/-}$ PMNs at baseline or
471 upon infection. Further, addition of adenosine did not alter this response (Fig
472 S3A). To test if neutrophil proteases were important for the ability of adenosine to
473 rescue CD73$^{-/-}$ PMN function, we used a protease inhibitor cocktail to inhibit
474 protease activity. Consistent with previous reports, inhibition of neutrophil
475 proteases completely abrogated the ability of WT PMNs to kill bacteria (Fig. S3B)
476 and also prevented the ability of EAD to boost CD73$^{-/-}$ PMN function. These
477 findings show that functional protease activity is important for the ability of EAD
478 to boost PMN anti-pneumococcal function, however, CD73 and EAD had no
479 effect on the activity of NE, a major granular component and serine protease
480 important for killing pneumococci (4, 5, 32).
481

482 **3.6 Extracellular adenosine dampens IL-10 production from PMNs following**
483 **infection**

484 EAD was previously shown to play a role in regulating production of
485 cytokines by immune cells (33) including IL-10 by macrophages (34-36), an anti-
486 inflammatory cytokine whose production early on during pneumococcal infection
487 was reported to be detrimental for host resistance (10). To test whether EAD was
488 targeting IL-10, we compared IL-10 production by WT and CD73$^{-/-}$ PMNs at
489 baseline and upon *ex vivo* infection with *S. pneumoniae.* We found that at
490 baseline, there was no significant difference in IL-10, however, upon infection,
491 CD73$^{-/-}$ PMNs produced significantly more IL-10 as compared to WT PMNs (Fig.
492 5A). Further, while WT PMNs did not upregulate IL-10 production upon
493 pneumococcal infection, CD73$^{-/-}$ PMNs displayed a ~ 2.5-fold increase in IL-10
494 above resting baseline levels (Fig 5A and B) and this upregulation was
495 significantly blunted (Fig. 5B) upon exposure to EAD. When we examined PMNs
496 isolated from three human donors, we could not detect any upregulation in IL-10
497 mRNA in response to *S. pneumoniae* infection (data not shown). These findings
498 show that, in contrast to wild type PMNs, CD73-deficient PMNs produce IL-10 in
499 response to *S. pneumoniae*, and that the induction of IL-10 production upon
500 infection can be blocked by EAD.
501

502 **3.8 IL-10 impairs the ability of PMNs to kill *S. pneumoniae***

503 Although the role of IL-10 during *S. pneumoniae* infection in mice is well
504 established (10-12, 37), its effect on PMN anti-pneumococcal function has not

505 been elucidated. To test that, WT PMNs were treated with either recombinant IL-
506 10 or IL-10 blocking antibody and their ability to kill pneumococci measured in
507 our opsonophagocytic assay. We found that addition of IL-10 abrogated the
508 ability of WT PMNs to kill pneumococci while blocking this cytokine boosted the
509 anti-bacterial efficiency of PMNs (Fig 6).

510 To test whether the anti-bacterial activity of CD73$^{-/-}$ PMNs can be rescued
511 by targeting IL-10 we treated the PMNs with the IL-10 blocking antibody and
512 performed the opsonophagocytic assay. We found that blocking IL-10 restored
513 the pneumococcal killing ability of CD73$^{-/-}$ PMNs to WT levels (Fig 6). To test if
514 the ability of adenosine to rescue CD73$^{-/-}$ PMN function was dependent on
515 blunting IL-10, we added recombinant IL-10 to CD73$^{-/-}$ PMNs supplemented with
516 adenosine. We found that the addition of recombinant IL-10 had no discernable
517 effect on bacterial killing by CD73$^{-/-}$ PMNs, consistent with the (already high)
518 levels of IL-10 produced by these cells (Fig. 6). In addition, the addition of IL-10
519 prevented the ability of adenosine to boost the anti-microbial function of these
520 cells (Fig 6). These findings demonstrate that EAD's ability to blunt IL-10
521 production is crucial for the anti-pneumococcal function of PMNs.

522

523 **3.9 IL-10 has no effect on PMN viability, bacterial association or NE**
524 **production**

525 We wanted to start exploring potential mechanisms by which IL-10
526 inhibition by CD73 regulates PMN function. It was previously reported that IL-10
527 producing PMNs were apoptotic (38). Since cellular death pathways including

528 apoptosis and necrosis are known to play a role in the anti-bacterial function of
529 PMNs (39), we compared the percentage of apoptotic and necrotic cells using
530 Annexin V and propidium iodide staining and flow cytometry. We found that as
531 expected (40), pneumococcal infection induced pore formation and necrosis of
532 PMNs (PI and Annexin V double positive cells) (Fig S4A). However, there was no
533 difference in viability of PMNs at baseline or upon infection between WT and
534 CD73$^{-/-}$ PMNs and addition or inhibition of IL-10 did not further alter the
535 percentage of necrotic PMNs (Fig S4A). We also tested the effect of IL-10 on the
536 association of PMNs with GFP-labeled pneumococci and found that neither
537 addition nor blocking of IL-10 altered association (data not shown). When we
538 examined the effect of IL-10 on NE activity, we found that neither addition of
539 recombinant IL-10 nor blocking IL-10 significantly altered the activity of the
540 enzyme by PMNs (Fig S3A).
541
542 **3.10 CD73 and IL-10 regulate extracellular ROS production by PMNs upon**
543 ***S. pneumoniae* infection**
544 IL-10 was reported to down regulate reactive oxygen species (ROS)
545 production by PMNs (18). To determine whether the CD73/IL-10 axis regulated
546 ROS production upon pneumococcal infection, we first compared production of
547 both intracellular and extracellular ROS by WT and CD73$^{-/-}$ PMNs. When we
548 measured intracellular ROS production over time, we found that the infection up
549 regulated ROS production rapidly within minutes to more than 50-fold increase
550 around the peak of the response at the 25 minute mark in both strains of mice

551 (Fig. 7A). There was no significant difference in intracellular ROS production by
552 WT vs CD73$^{-/-}$ PMNs in response to pneumococcal infection, consistent with our
553 findings that bacterial uptake and intracellular killing was not affected by CD73.
554 When we examined production of extracellular ROS in uninfected PMNs,
555 no ROS production was observed (Fig. 7B). However, upon exposure to
556 pneumococci, ROS production was rapidly induced by PMNs from both strains of
557 mice. This response was immediate and peaked within the first five minutes of
558 infection and waned over time (Fig. 7B) as previously described (23). Importantly,
559 during the first 10 minutes, PMNs from B6 mice produced significantly higher
560 levels of extracellular ROS than CD73$^{-/-}$ PMNs, producing 3-fold higher levels of
561 ROS at the peak of the response (Fig. 7B at the 5 minute mark). Intriguingly,
562 extracellular ROS production by PMNs in response to the positive control PMA
563 was not different between mouse strains (Fig. S4B), indicating that the blunted
564 response observed in CD73$^{-/-}$ PMNs was not due to a general defect in ROS
565 production, but rather was specific to pneumococcal infection.
566 To determine if IL-10 had a role in production of extracellular ROS by
567 PMNs, we added recombinant IL-10 to B6 PMNs and found that this cytokine
568 significantly blunted the magnitude of the response during pneumococcal
569 infection, which is consistent with its reported anti-inflammatory role (18) (Fig.
570 7C). Next we tested whether the extracellular ROS response of CD73$^{-/-}$ PMNs
571 upon infection can be rescued by treating the PMNs with the IL-10 blocking
572 antibody. We found that blocking IL-10 significantly boosted extracellular ROS
573 production in infected CD73$^{-/-}$ PMNs, resulting in around a 2-fold increase at the

574 peak of the response at 5 minutes post infection (Fig. 7D). Taken together, these
575 data demonstrate that inhibition of IL-10 production by CD73 is important for an
576 optimal extracellular ROS response by PMNs during *S. pneumoniae* infection.
577 The enzyme myeloperoxidase (MPO) was reported to play a crucial role in
578 clearance of *S. pneumoniae* and that it exerted its function partially by facilitating
579 ROS production (41). Therefore we compared the effect of IL-10 and CD73 on
580 MPO production by PMNs. We found that pneumococcal infection significantly up
581 regulated MPO levels (Fig S4C) as previously reported (41). However, there was
582 no difference in the levels of the enzyme between WT and CD73$^{-/-}$ PMNs, nor
583 was its levels altered by blocking or adding IL-10 (Fig S4C). This suggests that
584 the effect of IL-10 and EAD on extracellular ROS production by PMNs upon
585 pneumococcal infection is not mediated via MPO.
586
587 **3.11 Pulmonary PMNs from CD73$^{-/-}$ mice produce IL-10 early on during *S.***
588 ***pneumoniae* infection**
589 Next, we wanted to test the relevance of our findings *in vivo* and assess
590 IL-10 production during lung infection. WT C57BL/6 and CD73$^{-/-}$ mice were
591 infected i.t. with 5x10^5 CFU of *S. pneumoniae* TIGR4 and IL-10 production by
592 pulmonary PMNs was monitored at 6 hours post infection by intracellular
593 cytokine staining. We focused on early time points since that is when PMNs are
594 most relevant for the control of bacterial numbers. When we gated on PMNs
595 (Ly6G+; see Fig. S5 for the gating strategy), we found that a low percentage
596 (~6%) of PMNs in the lungs of WT mice expressed IL-10 and similar to our

597 observations *ex vivo*, there was no increase in IL-10 production upon infection
598 (Fig. 8A and B). However, we observed a significant increase in IL-10 production
599 by pulmonary PMNs in CD73$^{-/-}$ mice where the percentage of IL-10 producing
600 PMNs increased from 5% at baseline to 20% (Fig. 8A) and the amount of IL-10
601 produced (measured by MFI) increased approximately 1.5-fold (Fig. 8B) at 6
602 hours post-infection. When we compared the number of PMNs producing IL-10,
603 we found that in CD73$^{-/-}$ mice, there is a significant increase in IL-10 producing
604 PMNs above baseline by 6 hours post-infection. In contrast, in WT mice, we did
605 not observe an increase in PMNs until 18 hours following infection. Further, as
606 compared to WT, CD73$^{-/-}$ mice had 6-fold and 2-fold more PMNs making IL-10 at
607 6 and 18 hours after lung challenge, respectively. When we measured total IL-10
608 production in the lungs at 6 hours following infection, we found that CD73$^{-/-}$ mice
609 had on average more IL-10 (231.9 +/-20.51 pg/ml) as compared to WT mice (190
610 +/- 86.65 pg/ml), but the difference did not reach statistical significance. These
611 findings show that upon lung infection, in the absence of CD73, IL-10 production
612 by pulmonary PMNs is higher and occurs more rapidly.
613

614 **3.12 Blocking IL-10 pre-infection partially rescues the susceptibility of**
615 ***CD73$^{-/-}$* mice to pneumococcal lung infection**

616 To test whether the early increase in IL-10 production by PMNs in CD73$^{-/-}$
617 mice contribute to their susceptibility to infection, we treated the mice with either
618 isotype control or the IL-10 blocking antibody 2 hours prior to infection. Mice were
619 then challenged i.t. with 5x10^{5} CFU of *S. pneumoniae*, and bacterial burdens in

620 the lung and blood were determined two days post-infection. Blocking IL-10 prior
621 to infection significantly boosted the resistance of CD73$^{-/-}$ mice resulting in a 20
622 and 24-fold reduction in bacterial numbers in the lung and blood respectively as
623 compared to isotype treated controls and rendering bacterial burdens in these
624 mice indistinguishable from those of WT mice (Fig 9). Our data suggest that the
625 increased susceptibility of CD73$^{-/-}$ mice during pneumococcal infection is at least
626 in part mediated by IL-10.
627

628 **4. Discussion**

629 There is mounting evidence that PMNs are plastic and display phenotypic
630 and functional heterogeneity under different disease states (42). These range
631 from traditional pro-inflammatory subsets during infections (13, 42) to B-helper
632 PMNs (30, 43) to suppressive phenotypes in tumor microenvironments (44, 45)
633 and anti-inflammatory in response certain pathogens and microbial products (13-
634 16, 46-48). In the context of pneumococcal pneumonia, we previously found that
635 the efficacy of PMNs during the course of disease changes from clearing bacteria
636 early on to promoting infection at later time points (3). Here we show that this
637 shift away from an anti-microbial phenotype is associated with a decrease in
638 CD73 expression on PMNs. Using *in vitro* bacterial killing assays and adoptive
639 transfer of PMNs into CD73$^{-/-}$ recipients, we found that CD73 expression on
640 PMNs was crucial for the ability of these cells to kill *S. pneumoniae* and mediate
641 protection during lung infection *in vivo.* In fact loss of CD73 expression on PMNs
642 seem to actively promote bacterial growth as *S. pneumoniae* grew in the

643 presence of CD73$^{-/-}$ PMNs *in vitro* and adoptive transfer of CD73$^{-/-}$ PMNs
644 worsened the infection resulting in increased bacterial burdens in the lungs,
645 blood and brain of recipient mice. Importantly, CD73 mRNA was also expressed
646 in PMNs isolated from all the human donors we tested and pharmacological
647 inhibition of this enzyme *in vitro* abrogated the ability of PMNs to kill
648 pneumococci in 60% of donors, highlighting the clinical relevance of this
649 pathway. These findings suggest that CD73 expression on PMNs regulates their
650 antimicrobial phenotype.

651 During systemic infection with *S. pneumoniae,* a subset of immature
652 PMNs with a lower expression of surface Ly6G that exhibited a low ability to bind
653 bacteria was observed immobilized in the spleen (30). When we followed Ly6G
654 expression on PMNs (gated on Ly6G positive cells) over time, we noticed it
655 changed over time where we observed an increase in Ly6G expression on
656 pulmonary PMNs recruited at the 6 hour time point followed by a subsequent
657 decrease after that. However, we observed that CD73 negative PMNs in the
658 lungs had significantly lower amounts of Ly6G on their surface as compared to
659 their CD73^{+} counterparts at all time points suggesting that they could denote a
660 less mature subset. We believe that these CD73-negative pulmonary PMNs
661 migrate from the circulation after originating from the bone marrow, as the
662 reduced expression of CD73 following infection was also observed on circulating
663 PMNs as well as the PMN pool in the bone marrow.

664 In exploring mechanisms of how CD73 controls the PMN antimicrobial
665 phenotype, we found that it inhibited IL-10 production in response to

666 pneumococcal infection. In wild type mice, we did not see any up regulation in IL-
667 10 production by PMNs early in infection and IL-10 producing PMNs only started
668 to accumulate at the 18h time point that coincided with a decrease in CD73 on
669 pulmonary PMNs and their inability to control bacterial numbers (3). In contrast,
670 PMNs from CD73$^{-/-}$ mice up regulated IL-10 production early on within 6 hours of
671 infection, and our IL-10 blocking experiments both *in vitro* and *in vivo* suggest
672 that this impaired the ability of these mice to control the infection. Our data align
673 with previous reports that administration of IL-10 early in infection impairs the
674 ability of the host to control pneumococcal numbers (10). However, studies have
675 also found that mice that lack IL-10 had exacerbated PMN influx in their lungs
676 (12, 37) and succumbed to the infection despite having lower bacterial burden in
677 their organs (12). These findings suggest that early production of IL-10 is
678 deleterious for the host's ability to control the infection, however, it may be
679 required later on for resolution of inflammation and return to homeostasis.
680 Therefore, the eventual accumulation of IL-10 producing PMNs in wild type mice
681 we observed here may be beneficial in resolution of pulmonary inflammation
682 observed in healthy hosts (3).

683 Murine PMNs have been described to produce IL-10 in sepsis models and
684 in response to several bacterial infections such as *Escherichia coli*, *Shigella*
685 *flexneri* (16) and *Staphylococcus aureus* (13) as well as parasitic infections such
686 as *Leishmania major* (14) and *Trypanosoma cruzi* (15). In contrast, IL-10
687 production by pulmonary PMNs was actively repressed during *Mycobacterium*
688 *tuberculosis* (49). In this study, we did not detect an up regulation in IL-10

689 production by PMNs in response to *S. pneumoniae* infection in wild type mice
690 either *in vivo* during lung challenge or *in vitro* in response to direct bacterial
691 infection. Further, we were unable to detect an increase in IL-10 mRNA in PMNs
692 isolated from human donors in response to stimulation with *S. pneumoniae*. This
693 fits with previous studies demonstrating that resting human PMNs have histone
694 modifications at the IL-10 locus rendering it transcriptionally silent (50) and that
695 induction of IL-10 production by PMNs requires further stimulation such as direct
696 contact with LPS-treated Tregs or IL-10 itself that promote histone
697 posttranslational modifications that can activate IL-10 transcription (38).
698 Our data suggests that IL-10 production by PMNs in response to *S.*
699 *pneumoniae* is actively down regulated by EAD production by CD73 as addition
700 of exogenous adenosine to CD73$^{-/-}$ PMNs prevented the increase in IL-10
701 production following infection. This is in contrast to what has been described for
702 macrophages where adenosine induced IL-10 production by macrophages in
703 response to LPS stimulation (35, 36) or *E. coli* infection (34) by acting via the
704 A2A or A2B receptor to facilitate binding of transcription factors to the IL-10 locus
705 and enhancing transcription (34) or via posttranscriptional modifications of the 3'-
706 UTR that facilitated translation (35) respectively. These differences observed
707 could be accounted for by the cell type i.e. macrophages vs. PMNs or the
708 stimulation where the macrophage studies have been performed with Gram-
709 negative bacteria and their products (LPS) while we are examining responses to
710 a Gram positive organism.

711 PMNs were shown to kill *S. pneumoniae* in a manner that requires
712 phagocytosis and serine proteases (4). In exploring how the CD73/IL-10 axis
713 regulated PMN anti-pneumococcal activity we found that apoptosis, bacterial
714 uptake and intracellular killing and production of anti-microbial Neutrophil
715 Elastase and Myeloperoxidase were not affected by this pathway. Rather,
716 inhibition of IL-10 production by CD73 was crucial for optimal production of
717 extracellular ROS by PMNs upon *S. pneumoniae* infection. This is in line with
718 previous findings that adenosine signaling via the A1 receptor enhanced
719 superoxide production by PMNs in response to antibody coated erythrocytes (51)
720 and that IL-10 blunted PMN superoxide production in response to PMA and *C.*
721 *albicans* hyphae (18, 19).
722 Previous work showed that *S. pneumoniae* triggered extracellular
723 respiratory burst by PMNs (52) and that PLY released by the bacteria also
724 triggered production of intracellular ROS (23). Similarly, we found here that the
725 bacterial infection triggered both intracellular and extracellular ROS production.
726 NADPH oxidase is known to assemble both at the phagosomal membrane and
727 on the cell surface in response to bacterial infections (53), here we found that
728 CD73 was important for optimal extracellular ROS production but had no effect
729 on intracellular ROS suggesting that this pathway may target cell surface
730 assembly of the NADPH oxidase complex only. The role of ROS production in
731 anti-pneumococcal function of PMNs and host resistance to *S. pneumoniae* is
732 arguable. Studies showed that mice lacking components of the NADPH oxidase
733 are not more susceptible to pneumococcal lung infection (30, 54) and that

734 inhibition of respiratory burst has no effect on the ability of PMNs to kill *S.*
735 *pneumoniae* (4). In contrast, ROS production by PMN-derived MPO was
736 important for bacterial clearance during pneumococcal otitis media (41) and
737 bacterial mutants lacking components that detoxify ROS die more readily in
738 response to oxidative stress (55-57).
739 Adenosine is a well-known regulator of PMN function and is known to
740 regulate PMN recruitment, release of inflammatory cytokines, phagocytic
741 capacity and oxidative burst (33). However, the majority of those studies have
742 been conducted in the context of sterile inflammation or in response to *in vitro*
743 stimulation by inflammatory mediators such as fMLP, LPS, TNF or inert particles
744 (33). The role of the EAD pathway in PMN response to infections is now better
745 appreciated and was shown to play a role in host resistance during pulmonary
746 infections with influenza A virus (58, 59), *Klebsiella pneumoniae* (60) and *S.*
747 *pneumoniae* (3). Here, we identified the mechanisms by which EAD production
748 by CD73 regulated PMN anti-pneumococcal function and further found it was
749 relevant for the function of PMNs from human donors. This may be relevant for
750 incorporating clinically available adenosine-based drugs to combat
751 pneumococcal pneumonia and other serious lung infections in the future.
752
753 **Authorship**
754 NS and JNL conducted research, analyzed data and wrote the paper. EYIT, MB
755 and JYH conducted research, JML provided essential expertise and reviewed the

756 paper. ENBG designed research, conducted research, analyzed data, wrote the
757 paper and had primary responsibility for final content.
758

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762

763 **Conflict of Interest Disclosure**

764 None
765

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1041

1042 **Figure Legends**

1043 **Figure 1. Expression of CD73 on pulmonary PMNs changes over the course**
1044 **of infection.** C57BL/6 mice were inoculated i.t with $5x10^5$ CFU of *S. pneumoniae*
1045 TIGR4. The blood, bone marrow and lungs were harvested, plated for bacterial
1046 enumeration and analyzed by flow cytometry. (A) Mice were euthanized at 0, 6,
1047 12, 18, 24 and 48 hours following infection. We then compared bacterial numbers
1048 in the lungs to PMN numbers (Ly6G+ cells) for the first 0-12 and subsequent 18-
1049 72 hours post infection and performed Pearson correlation analysis where

1050 asterisks, denotes significant correlation. We gated on PMNs (Ly6G+ cells) and
1051 also monitored (B) the percentage of cells expressing CD73 and (B-C) the
1052 amounts of CD73 (mean fluorescent intensity or MFI) in the indicated organs, at
1053 the indicated time points. (D) We gated on all Ly6G+ cells and then gated on
1054 CD73 positive versus negative populations and compared the expression (MFI)
1055 of Ly6G. Data are pooled from two separate experiments with 4-5 mice per time
1056 point (A) and 4-7 mice per time point (B-D). Asterisks indicate significant
1057 differences from uninfected controls calculated by Student's t-test.
1058
1059 **Figure 2. CD73 and extracellular adenosine are required for the ability of**
1060 **neutrophils to kill *S. pneumoniae.*** (A) PMNs were isolated from the bone
1061 marrow of C57BL/6 (WT) or CD73$^{-/-}$ mice and treated with 100μM Adenosine or
1062 PBS (vehicle control) for 30 minutes at 37°C. The reactions were then infected
1063 with *S. pneumoniae* pre-opsonized with homologous sera for 45 minutes at 37°C.
1064 Reactions were then stopped by placing samples on ice and viable CFU were
1065 determined after serial dilution and plating. The percentage of bacteria killed
1066 upon incubation with PMNs was determined by comparing surviving CFU to a no
1067 PMN control. Positive percent killing indicates bacterial death while negative
1068 percent indicates bacterial growth. Data shown are pooled from three separate
1069 experiments (n=3 biological replicated or mice per strain) where each condition
1070 was tested in triplicate (n=3 technical replicates) per experiment. Asterisks
1071 indicate significance calculated by Student's t-test.
1072

1073 **Figure 3. CD73 is required for the ability of neutrophils isolated from**
1074 **human donors to kill *S. pneumoniae.*** PMNs were isolated from the blood of
1075 young healthy donors and (A) pre-treated with CD73 inhibitor (α,β methylene
1076 ADP) or vehicle control for 30 minutes at 37°C and then incubated for 45 minutes
1077 with complement-opsonized *S. pneumoniae* TIGR4. For each donor, the average
1078 percent bacterial killing compared to a no PMN control was calculated from
1079 triplicate wells per condition. Data from 7 donors are shown. (B) RNA was
1080 isolated from resting PMNs of the indicated donors and the expression of CD73
1081 mRNA was measured by RT-qPCR and normalized to GAPDH. Significant
1082 differences denoted by asterisk, were determined by Student's t-test.
1083
1084 **Figure 4. Adoptive transfer of PMNs from wild type mice boosts resistance**
1085 **of CD73$^{-/-}$ mice to *S. pneumoniae.*** CD73$^{-/-}$ mice were mock treated (no transfer)
1086 or injected i.p with 2.5x10^{6} PMNs isolated from the bone marrow of C57BL/6 or
1087 CD73$^{-/-}$ mice. One hour post transfer, mice were infected i.t with 5x10^{5} CFU of *S.*
1088 *pneumoniae* and bacterial numbers in the lung (A), blood (B) and brain (C) were
1089 determined 24 hours post infection. Significant differences, determined by
1090 Student's t-test, are indicated by asterisks. Representative data from one of three
1091 separate experiments (n=3 mice per group) are shown.
1092
1093 **Figure 5. Extracellular adenosine blunts IL-10 production from PMNs**
1094 **following infection.** (A) PMNs from the indicated mouse strains were incubated
1095 for 45 minutes at 37°C with *S. pneumoniae* pre-opsonized with homologous sera

1096 or mock treated with buffer and homologous sera (uninfected). The supernatants
1097 were then collected and assayed for IL-10 production by ELISA. (B) PBS control
1098 or adenosine (100μM) were added to the PMNs 20 minutes prior to infection and
1099 the fold-change in IL-10 production was calculated by dividing the values of
1100 infected reactions by uninfected controls for each condition. Data were pooled
1101 from three separate experiments (n=3 mice) with each condition tested in
1102 triplicate per experiment. Asterisks indicate significant differences determined by
1103 Student's t-test.
1104
1105 **Figure 6. IL-10 impairs the ability of PMNs to kill *S. pneumoniae*.** PMNs from
1106 C57BL/6 or CD73$^{-/-}$ mice were incubated for 20 minutes with the indicated anti-IL-
1107 10 (1μg/ml JES5-2A5), isotype control (1μg/ml), rIL-10 (50ng/ml) or adenosine
1108 (100μM) and then infected with pre-opsonized *S. pneumoniae* for 45 minutes at
1109 37°C. Reactions were then plated on blood agar plates and the percentage of
1110 bacteria killed compared to a no PMN control under the same conditions was
1111 calculated. Data shown are pooled from three separate experiments (n=3 mice
1112 per strain) with each condition tested in triplicate. Asterisks indicate significant
1113 differences determined by Student's t-test.
1114
1115 **Figure 7. Inhibition of IL-10 production by CD73 is important for optimal**
1116 **production of extracellular reactive oxygen species by PMNs in response**
1117 **to *S. pneumoniae* infection.** PMN were isolated from the bone marrow of the
1118 indicated strains of mice and left untreated (A and B) or treated with the indicated

1119 rIL-10 (50ng/ml) (C) or anti-IL-10 (1μg/ml JES5-2A5) or isotype control (1μg/ml)
1120 (D). PMNs were then infected with *S. pneumoniae* pre-opsonized with
1121 homologous sera (+*Sp*) or treated with 3% matching sera (uninfected) and
1122 intracellular ROS production measured by chemiluminescence of Luminol (A) or
1123 extracellular ROS production measured by chemiluminescence of Isoluminol in
1124 the presence of HRP (B-D). Representative data are shown from one of six (B)
1125 and one of four (A, C-D) separate experiments with one mouse per strain per
1126 experiment where each condition is tested in quadruplicates. Significant
1127 differences ($p<0.05$) were determined by 2-way ANOVA followed by Sidak's
1128 multiple comparisons test.
1129
1130 **Figure 8. Pulmonary PMNs from CD73$^{-/-}$ mice produce IL-10 early on during**
1131 ***S. pneumoniae* infection.** Wild-type C57BL/6 or *CD73$^{-/-}$* mice were mock-
1132 infected or i.t challenged with 5 x 10^5 CFU of *S. pneumoniae*. Six (grey bars) and
1133 18 hours (black bars) following challenge, (A) the percentage of IL-10 producing
1134 PMNs (Ly6G+), (B) the mean florescent intensities (MFI) of IL-10 in PMNs
1135 (Ly6G+) and (C) the number of IL-10 producing PMNs (Ly6G+) recruited into the
1136 lungs were determined by intracellular cytokine staining and flow cytometry (see
1137 Materials and Methods). Pooled data are from three separate experiments (n=6-9
1138 mice per strain per time point). Significant differences determined by Student's t-
1139 test are indicated by asterisks.
1140

1141 **Figure 9. Blocking IL-10 pre-infection partially reverses the susceptibility of**
1142 ***CD73*$^{-/-}$ mice to pneumococcal challenge.** CD73$^{-/-}$ mice were treated i.p with IL-
1143 10 blocking antibodies (anti-IL-10) or isotype control two hours prior to pulmonary
1144 challenge with 5x10^{5} CFU of *S. pneumoniae*. Pneumococcal burdens in the lungs
1145 (A) and blood (B) were determined 48 hours post-infection. Pooled data from two
1146 separate experiments with 8 mice per group are shown. Values significantly
1147 different by Student's t-test are indicated by asterisk.
1148

Figure 1.

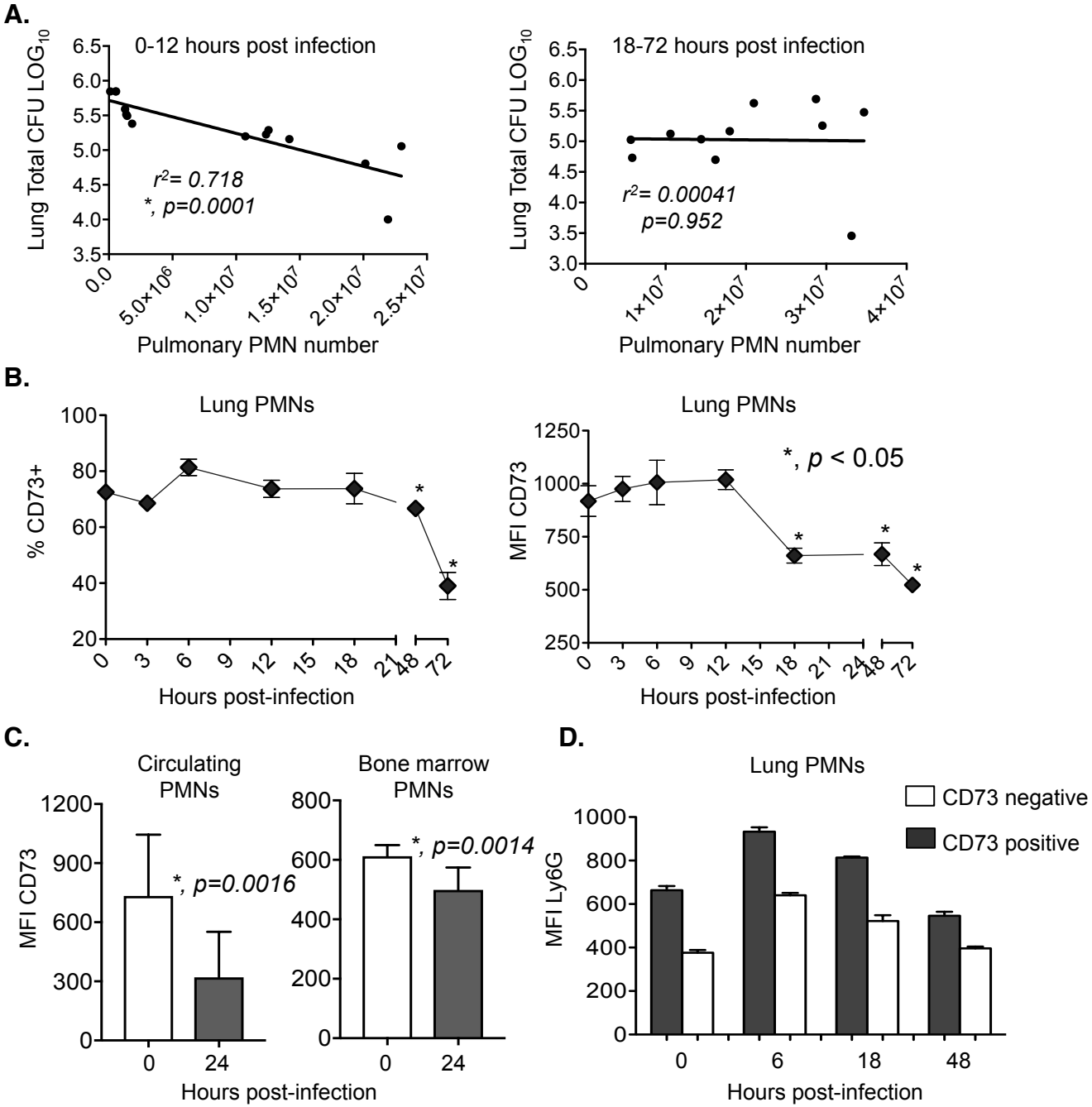

A.
0-12 hours post infection
Lung Total CFU LOG10
r2= 0.718
*, p=0.0001
Pulmonary PMN number
18-72 hours post infection
r2= 0.00041
p=0.952
B.
Lung PMNs
% CD73+
Hours post-infection
MFI CD73
*, p < 0.05
C.
Circulating PMNs
Bone marrow PMNs
*, p=0.0016
*, p=0.0014
D.
Lung PMNs
CD73 negative
CD73 positive
MFI Ly6G

Figure 2.

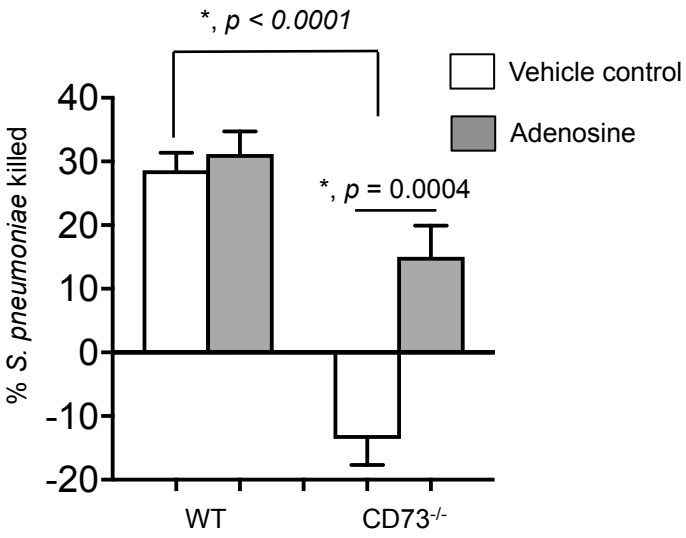
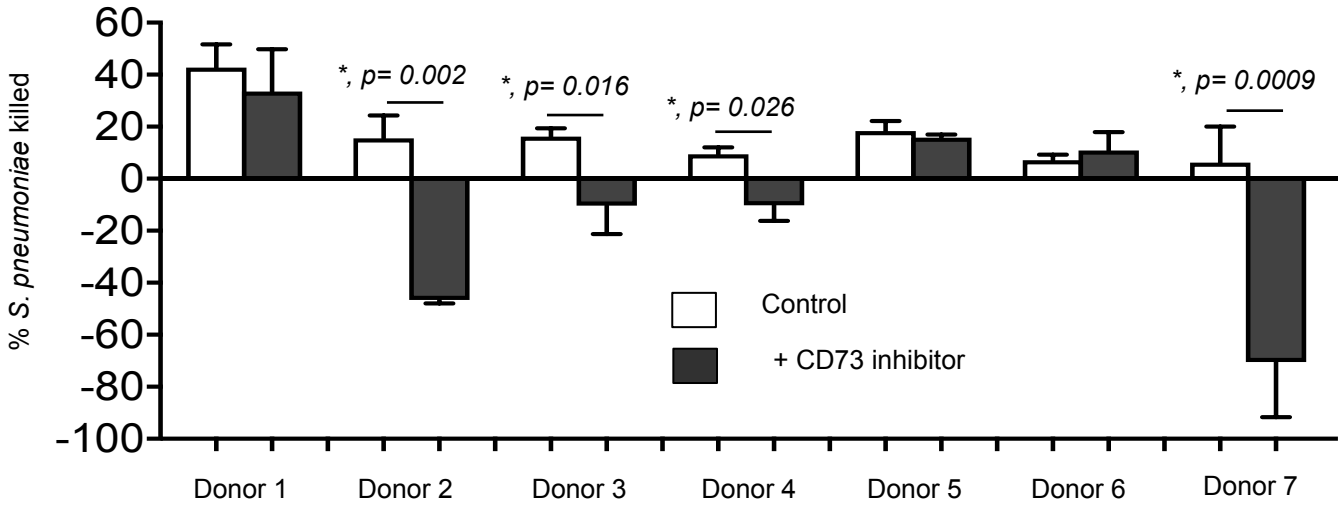

*, p < 0.0001
Vehicle control
Adenosine
*, p = 0.0004
% S. pneumoniae killed
40
30
20
10
0
-10
-20
WT
CD73-/-

Figure 3.

A.


% S. pneumoniae killed
60
40
20
0
-20
-40
-60
-80
-100
*, p= 0.002
*, p= 0.016
*, p= 0.026
*, p= 0.0009
Control
+ CD73 inhibitor
Donor 1
Donor 2
Donor 3
Donor 4
Donor 5
Donor 6
Donor 7

B.

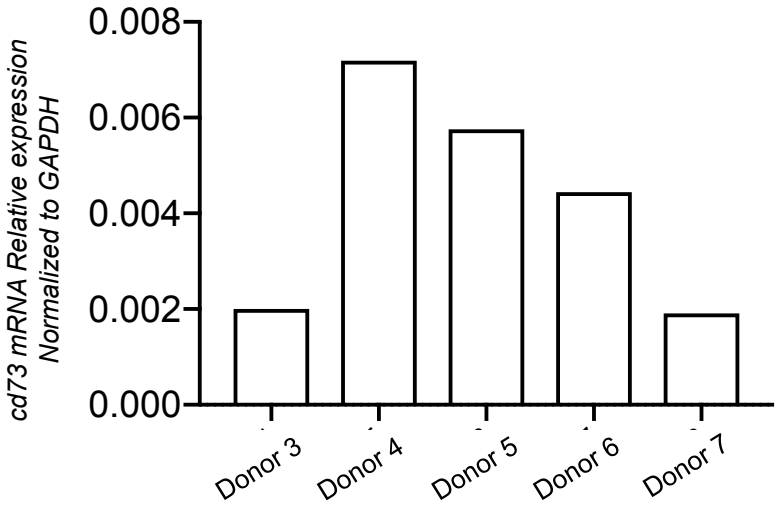

cd73 mRNA Relative expression
Normalized to GAPDH
0.008
0.006
0.004
0.002
0.000
Donor 3
Donor 4
Donor 5
Donor 6
Donor 7

Figure 4.

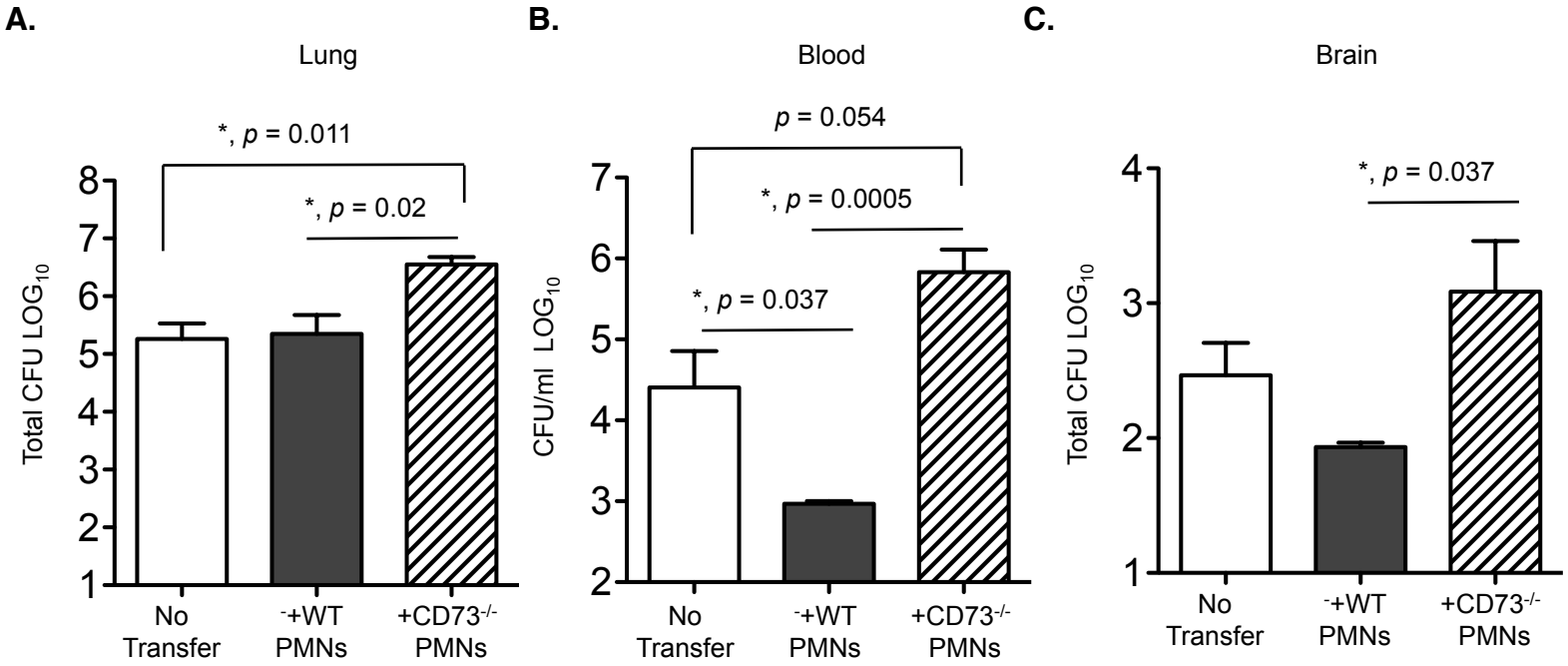

A.
Lung
*, p = 0.011
*, p = 0.02
Total CFU LOG10
8
7
6
5
4
3
2
1
No Transfer
-+WT PMNs
+CD73-/- PMNs
B.
Blood
p = 0.054
*, p = 0.0005
*, p = 0.037
CFU/ml LOG10
7
6
5
4
3
2
No Transfer
-+WT PMNs
+CD73-/- PMNs
C.
Brain
*, p = 0.037
Total CFU LOG10
4
3
2
1
No Transfer
-+WT PMNs
+CD73-/- PMNs

Figure 5.

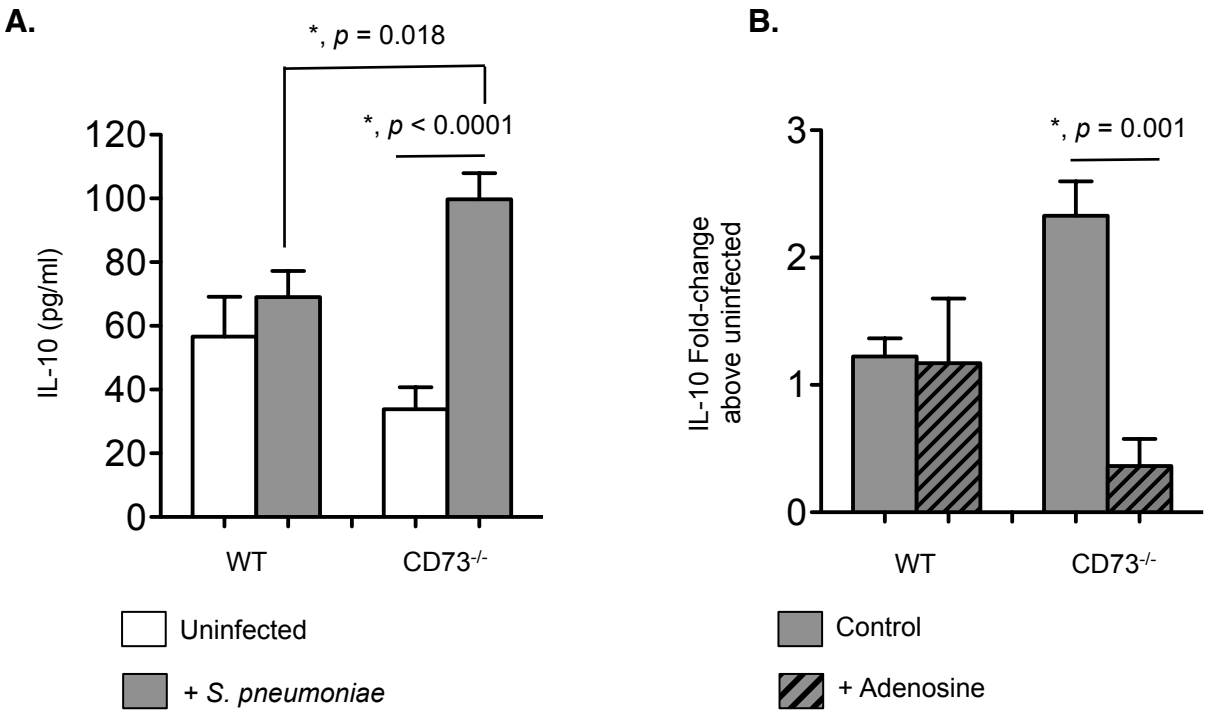

A.
*, p = 0.018
*, p < 0.0001
IL-10 (pg/ml)
120
100
80
60
40
20
0
WT
CD73-/-
Uninfected
+ S. pneumoniae
B.
*, p = 0.001
IL-10 Fold-change above uninfected
3
2
1
0
WT
CD73-/-
Control
+ Adenosine

Figure 6.

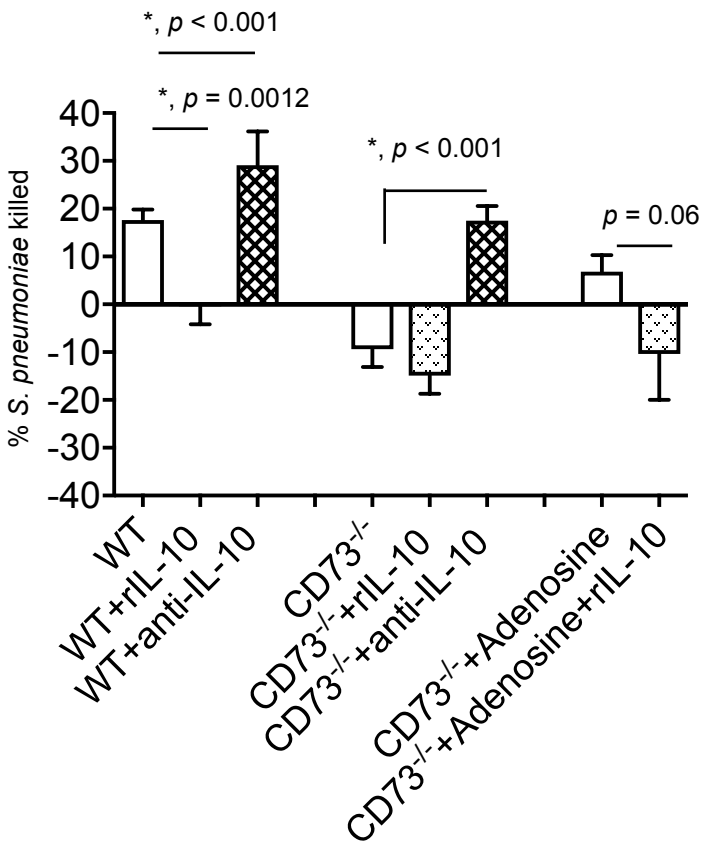

*, p < 0.001
*, p = 0.0012
*, p < 0.001
p = 0.06
% S. pneumoniae killed
40
30
20
10
0
-10
-20
-30
-40
WT
WT+rIL-10
WT+anti-IL-10
CD73-/-
CD73-/-+rIL-10
CD73-/-+anti-IL-10
CD73-/-+Adenosine
CD73-/-+Adenosine+rIL-10

Figure 7.

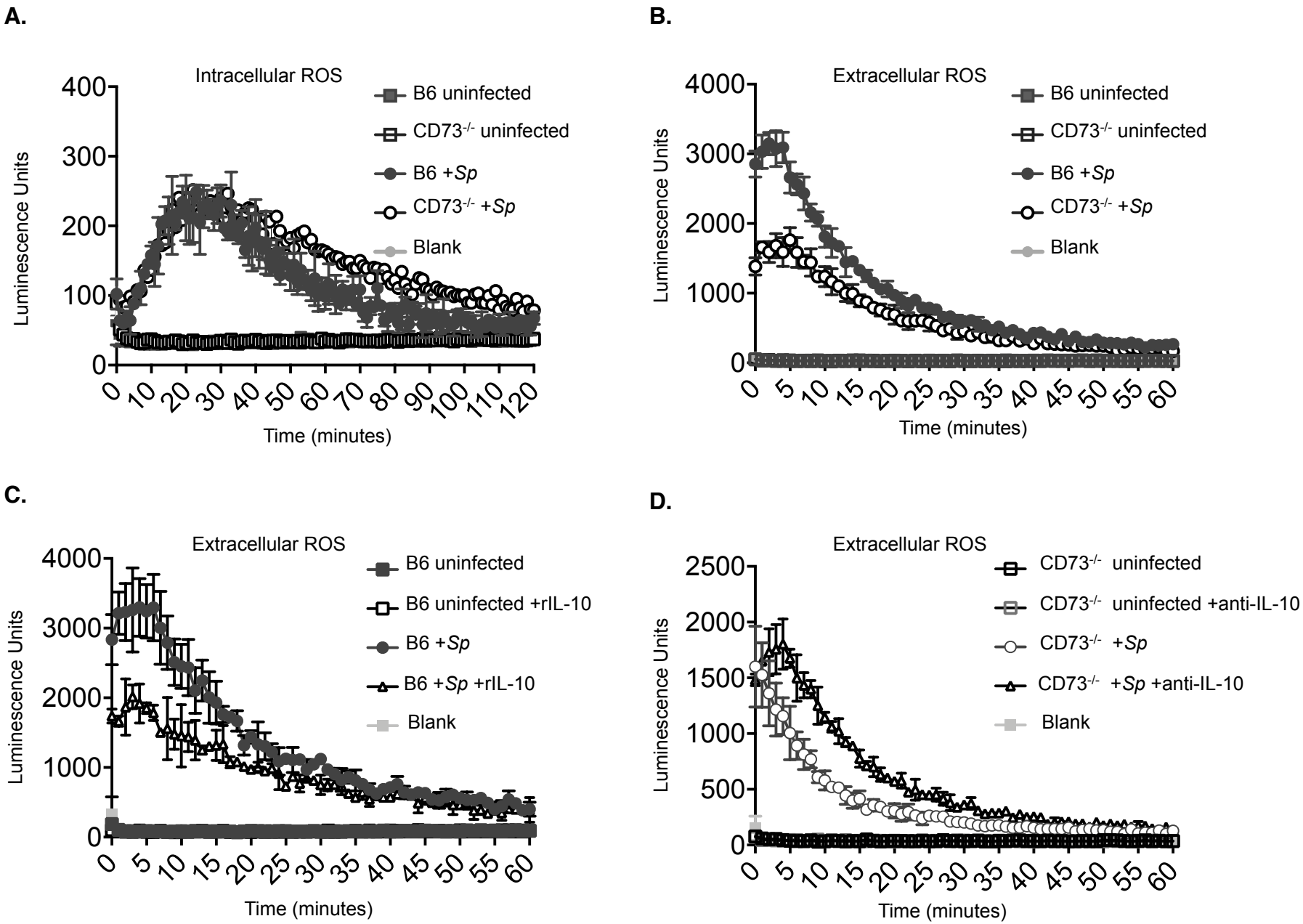

A.
Intracellular ROS
Luminescence Units
Time (minutes)
B6 uninfected
CD73-/- uninfected
B6 +Sp
CD73-/- +Sp
Blank
B.
Extracellular ROS
Luminescence Units
Time (minutes)
B6 uninfected
CD73-/- uninfected
B6 +Sp
CD73-/- +Sp
Blank
C.
Extracellular ROS
Luminescence Units
Time (minutes)
B6 uninfected
B6 uninfected +rIL-10
B6 +Sp
B6 +Sp +rIL-10
Blank
D.
Extracellular ROS
Luminescence Units
Time (minutes)
CD73-/- uninfected
CD73-/- uninfected +anti-IL-10
CD73-/- +Sp
CD73-/- +Sp +anti-IL-10
Blank

Figure 8.

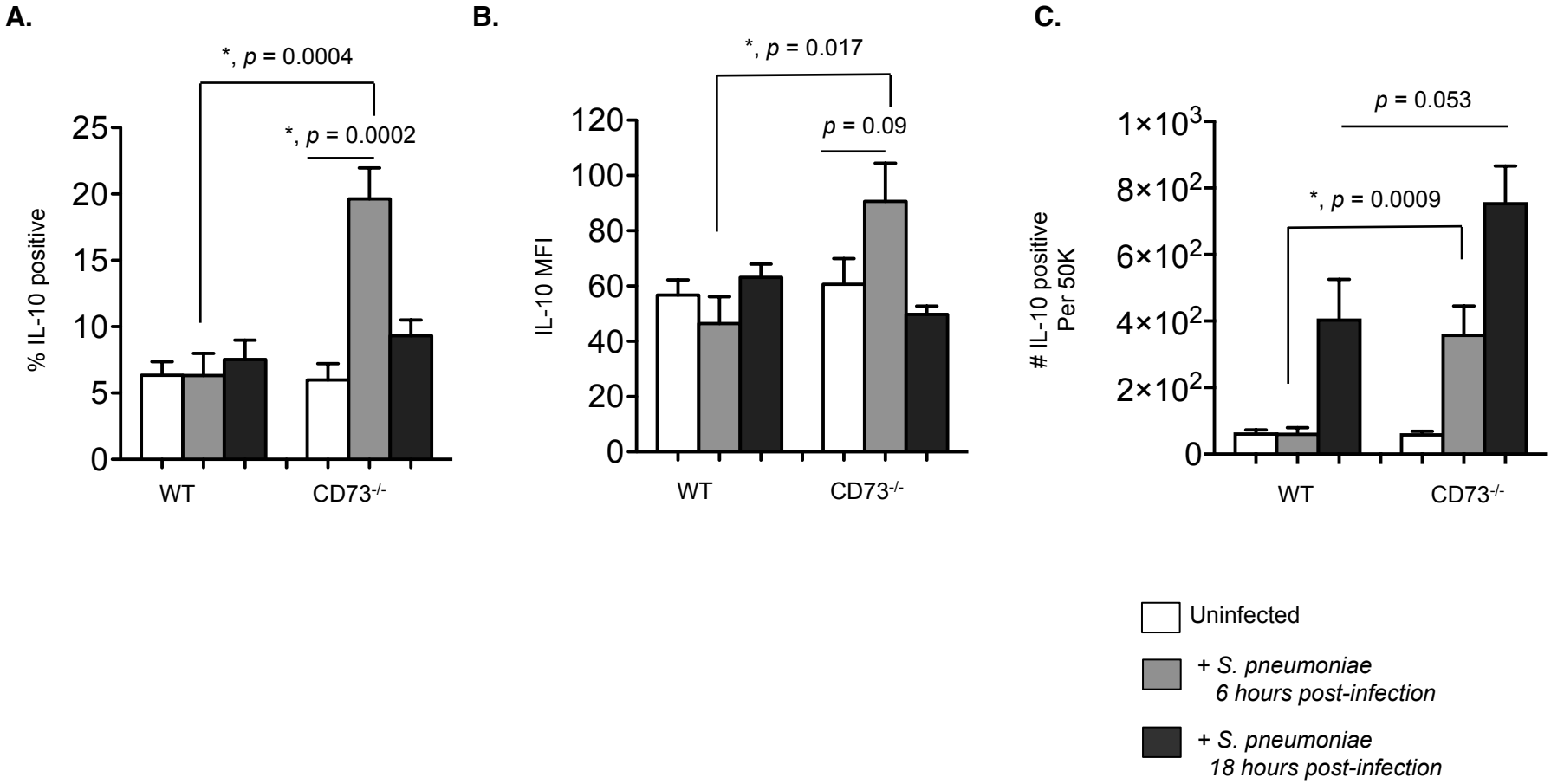
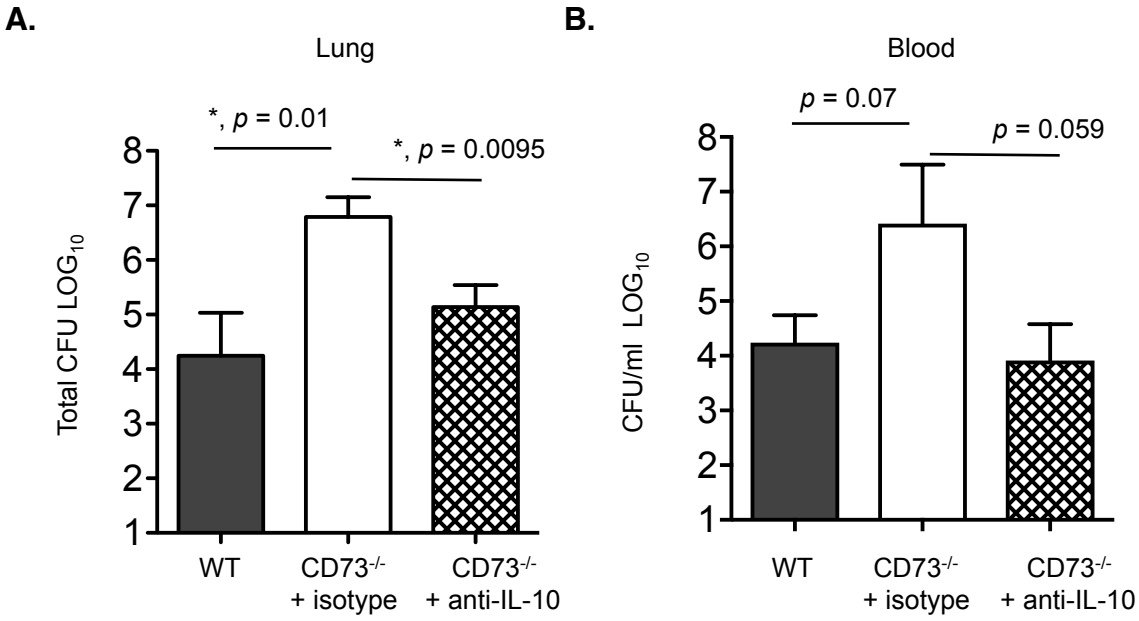

A.
*, p = 0.0004
*, p = 0.0002
% IL-10 positive
25
20
15
10
5
0
WT
CD73-/-
B.
*, p = 0.017
p = 0.09
IL-10 MFI
120
100
80
60
40
20
0
WT
CD73-/-
C.
p = 0.053
*, p = 0.0009
IL-10 positive Per 50K
1×10^3
8×10^2
6×10^2
4×10^2
2×10^2
0
WT
CD73-/-
Uninfected
+ S. pneumoniae 6 hours post-infection
+ S. pneumoniae 18 hours post-infection

Figure 9.


A.
Lung
*, p = 0.01
*, p = 0.0095
Total CFU LOG10
8
7
6
5
4
3
2
1
WT
CD73-/- + isotype
CD73-/- + anti-IL-10
B.
Blood
p = 0.07
p = 0.059
CFU/ml LOG10
8
7
6
5
4
3
2
1
WT
CD73-/- + isotype
CD73-/- + anti-IL-10