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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Key Words: Neutrophil phenotype, CD73, anti-microbial, pneumonia, reactive oxygen species

Abbreviations

CFU: Colony Forming Units

CT: Cycle thresh-hold

EAD: Extracellular adenosine

I.P: Intra peritoneal

I.T: Intra tracheal

MFI (Mean Fluorescent Intensity)

MOI: Multiplicity of infection

MPO: Myeloperoxidase

NE: Neutrophil Elastase

OPH: Opsonophagocytic

PLY: Pneumolysin

PMA: Phorbol 12-myristate 13-acetate

PMNs: Polymorphonuclear leukocytes

Pneumococcus: *Streptococcus pneumoniae*

ROS: Reactive oxygen species

WT: Wild type

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*

infection. This study demonstrates that CD73 regulates PMN anti-microbial phenotype during pneumococcal pneumonia.

1. Introduction

Neutrophils, also known as polymorphonuclear leukocytes or PMNs, play a major role in host defense against *S. pneumoniae* lung infection (1, 2). PMNs are required to control bacterial burden early in the infectious process. In mouse models, early PMN recruitment into the lungs within the first 12 hours following pneumococcal pulmonary infection coincided with a decrease in bacterial numbers and immunodepletion of PMNs prior to infection resulted in host lethality (3). *Ex vivo*, PMNs are thought to kill *S. pneumoniae* by engulfing the bacteria and producing serine proteases including cathepsin G (CG) and neutrophil elastase (NE) (4), which were also required for controlling bacterial numbers in murine models of pneumonia (5). Interestingly, later during infection, PMN persistence in the lungs promotes disease (3, 6, 7). In fact, we previously found that depletion of PMNs 18 hours after pneumococcal lung infection resulted in reduced bacterial numbers and enhanced mouse survival (3). These findings demonstrated that while PMNs are required at the start of infection, their persistence is detrimental for host survival. This also suggested that the antibacterial function of these innate immune cells and their ability to clear pneumococci is altered during the course of infection (3). However the mechanisms of this alteration in PMN phenotype remain unexplored.

A major regulator of host resistance to pneumococcal infection is

92 extracellular adenosine (EAD) (3). Upon damage due to a variety of insults
93 including infection, ATP is thought to leak from damaged cells into the
94 extracellular space and is converted into EAD by the sequential action of two
95 exonucleosidases, CD39 and CD73 (8). EAD can then signal via four known
96 receptors, i.e. A1, A2A, A2B and A3 (9). Several drugs targeting this pathway are
97 in clinical studies or in use to treat a variety of inflammatory diseases (9), making
98 it an attractive avenue for modulating immune responses during infection. We
99 previously found that blocking EAD production by CD73 dramatically increased
100 bacterial numbers in organs and resulted in host lethality upon *S. pneumoniae*
101 lung infection in mice (3). EAD production by CD73 regulated PMN recruitment to
102 the lungs and potentially boosted PMN function (3).
103 IL-10 is an anti-inflammatory cytokine associated with impaired control of
104 pneumococcal pneumonia (10, 11). In mouse models, IL-10 levels increase in
105 the lungs within 12 hours following pneumococcal challenge (10). Intranasal
106 administration of IL-10 at the time of bacterial challenge results in decreased host
107 survival and increased bacterial numbers in the lungs and blood, while blocking
108 IL-10 prior to infection enhances bacterial clearance and boosts host survival
109 (10). Interestingly, while IL-$10^{-/-}$ mice have reduced lung and systemic bacterial
110 loads, these mice suffer increased mortality due to excessive pulmonary
111 inflammation (12). IL-10 can be produced by many types of immune cells and it is
112 now appreciated that murine PMNs can also produce this anti-inflammatory
113 cytokine during infections (13-16). PMNs are a significant source of IL-10 in
114 sepsis models (17) and stimulation with several bacterial pathogens, including

Escherichia coli, *Shigella flexneri* and mycobacterial BCG, triggers IL-10 production by PMNs both *in vitro* and *in vivo* within 8 hours of infection (16). Parasitic infections such as *Leishmania major* (14) and *Trypanosoma cruzi* (15) also induce IL-10-producing PMNs during mouse infection. IL-10 production by PMNs may reduce their microbicidal capacity, as *in vitro* treatment of PMNs with IL-10 impairs their ability to phagocytose *Staphylococcus aureus*, *Candida albicans* and *E. coli* and blunts superoxide production (18, 19). Further, in a *S. aureus* burn infection model, a subset of IL-10 producing PMNs is associated with impaired host resistance to infection (13). It is not currently known if *S. pneumoniae* infection triggers IL-10 production by PMNs.

In this study, we explored the involvement of CD73 in the diminished efficacy of PMNs during the course of pneumococcal pneumonia. We found that during the course of lung infection, CD73 expression on PMNs decreased. Notably, this change in CD73 expression coincided with an increase in IL-10-producing PMNs, and we showed that CD73 suppressed IL-10 production by PMNs during infection and was required for the ability of these cells to kill *S. pneumoniae ex vivo.* This study identifies a role for CD73 in maintaining the antimicrobial phenotype of PMNs and elucidates the mechanisms by which CD73 regulates PMN anti-microbial function and host resistance to pneumococcal pneumonia.

2. Materials and Methods

2.1 Mice

All experiments were conducted in accordance with Institutional Animal Care and Use Committee (IACUC) guidelines. Wild type (WT) C57BL/6 mice were purchased from Jackson Laboratories (Bar Harbor, ME). $CD73^{-/-}$ mice on C57BL/6 background were purchased from Jackson Laboratories and bred at a specific-pathogen free facility at Tufts University and The University at Buffalo. Female 8-12 week old mice were used in all experiments.

2.2 Bacteria

Wild type *S. pneumoniae* TIGR4 strain (serotype 4) and pneumolysin-deletion mutant (ΔPLY) *S. pneumoniae* (20) were kind gifts from Andrew Camilli. Bacteria were grown at 37°C in 5% CO_2 in Todd-Hewitt broth supplemented with 0.5% yeast extract and oxyrase until culture reaches mid-exponential phase. Bacterial aliquots were frozen at -80˚C in the growth media with 20% (v/v) glycerol. Prior to use, aliquots were thawed on ice, washed and suspended in PBS to obtain the appropriate concentration. The inoculums were then confirmed by serial dilution and dribble plating on Tryptic Soy Agar plates supplemented with 5% sheep blood agar.

2.3 Isolation of PMNs

Bone marrow cells were harvested from femurs and tibias of uninfected mice, flushed with RPMI 1640 supplemented with 10% FBS and 2 mM EDTA, and resuspended in PBS. Neutrophils were then separated from the rest of the bone marrow cells through density gradient centrifugation, using Histopaque 1119 and

Histopaque 1077 as previously described (21). The isolated neutrophils were resuspended in Hanks' Balanced Salt Solution (HBSS)/0.1% gelatin without Ca^{2+} and Mg^{2+}, and used in subsequent assays. The purity of neutrophils was also measured by flow cytometry using APC-conjugated anti-Ly6G and 85-90% of enriched cells were Ly6G+.

2.4 Opsonophagocytic killing assay

The ability of PMNs to kill pneumococci was assessed *ex vivo* through an Opsonophagocytic (OPH) killing assay as previously described (3, 22). Briefly, 100µl reactions in HBSS/0.1% gelatin consisted of $1x10^5$ PMNs incubated with $1x10^3$ bacteria grown to mid log phase and pre-opsonized with 3% mouse sera. The reactions were incubated rotating for 45 minutes at 37°C. Where indicated, PMNs were incubated as indicated with adenosine (100µM) or rIL-10 (50ng/mL) or anti-IL10 (1ug/mL JES5-2A5) or isotype control (1ug/mL) or 1x protease inhibitor cocktail for 30 minutes prior to adding pre-opsonized bacteria. HBSS with 3% sera was added to PMNs without treatment as the control. Percent killing was determined by dribble plating on blood agar plates and calculated in comparison to no PMN control under the exact same conditions (+/- treatments).

2.5 IL-10 Enzyme-Linked Immunosorbent Assay (ELISA)

$1x10^6$ Bone marrow PMNs were incubated with HBSS/0.1% gelatin or adenosine for 30 minutes, followed by 45 minutes infection at 37^o C with pre-opsonized *S. pneumoniae* TIGR4 at an MOI of 2 or mock treated with HBSS and sera only

(uninfected). The reactions were spun down and the supernatants were obtained to measure IL-10 production using Mouse IL-10 ELISA kit (e-bioscience) according to manufacturer's instructions.

2.6 ROS Assay

Following isolation from the bone marrow, PMNs were re-suspended in HBSS (Ca^{2+} and Mg^{2+} free) and acclimated at room temperature for one hour. The cells were then spun down and re-suspended in KRP buffer (Phosphate buffered saline with 5mM glucose, 1mM $CaCl_2$ and 1mM $MgSO_4$) and equilibrated at room temperature for 30 minutes. The cells were then seeded in 96-well white LUMITRAC™ plates (Greiner Bio-One) at $5x10^5$ PMNs per well, treated with anti-IL-10 or rIL-10 for 30 minutes at 37° C. For detection of extracellular ROS, 50μM Isoluminol (Sigma) plus 10U/ml HRP (Sigma) were added, while for detection of intracellular ROS, 50μM Luminol (Sigma) was added to the wells as previously described (23-26). The cells were infected with pre-opsonized *S. pneumoniae* TIGR4 at an MOI of 2 or mock treated with buffer containing 3% mouse sera (uninfected). PMNs treated with 100 nM Phorbol 12-myristate 13-acetate (PMA) (Sigma) were used as a positive control. Luminescence was immediately read (following infection) over a period of one hour at 37° C in a pre-warmed Biotek Plate reader. Wells containing buffer and Isoluminol plus HRP or Luminol alone were used as blanks.

2.7 Animal infections and scoring

Mice were lightly anesthetized with isoflurane and challenged intra-tracheally (i.t.) with $5x10^5$ colony-forming units (CFU) of WT *S. pneumoniae* TIGR4 strain by instilling 50μl of bacteria directly into the trachea with the tongue pulled out to facilitate delivery of bacteria directly into the lungs (3). Following the infection, mice were monitored daily for weight loss and blindly scored for signs of sickness including weight loss, activity level, posture and breathing, scored as healthy [0] to severely sick [21] as previously described (27). The lung and brain were harvested and homogenized in sterile PBS. Blood was collected to follow bacteremia. Each sample was diluted in sterile PBS and dribble plated on blood agar plates to enumerate bacterial numbers.

2.8 Isolation of cells from the lungs and flow cytometry

Mice were perfused with 10ml PBS, the lungs removed, washed in PBS, and minced into small pieces. The lungs were then digested for 1 hour with RPMI 1640 supplemented with 10% FBS, 1 mg/ml Type II collagenase (Worthington), and 50 U/ml Deoxyribonuclease I (Worthington) at 37° C/ 5% CO_2. Single-cell suspensions were obtained by mashing the digested lungs, and the red blood cells were removed by treatment with a hypotonic lysis buffer (Lonza). Cells were analyzed using flow cytometry. Intracellular cytokine staining (ICS) was performed using the Cytofix/Cytoperm kit (BD Biosciences). GolgiPlug was added to the digestion media and following red blood cell lysis, the cells were incubated with RPMI 1640 supplemented with 10% FBS and Golgi Plug for 3 more hours at 37° C/ 5% CO_2. Cells were surface stained with anti-mouse CD45

(clone 30-F11, e-Bioscience), Ly6G (clone 1A8, BD Biosciences) and CD73 (Clone TY/11.8, e-Bioscience). For intracellular staining, cells were permeabilized and stained with IL-10 (clone JES5-16E3, e-Bioscience) or isotype control (Rat IgG2b K Isotype Control, Biolegend). Fluorescence intensities were measured on a FACSCalibur and at least 25,000 events for lung tissue were analyzed using FlowJo.

2.9 Adoptive transfer of PMNs

Bone marrow PMNs were isolated from uninfected mice using density gradient centrifugation as described above and resuspended in PBS. Mice either received $2.5x10^6$ cells via intraperitoneal (i.p.) injection or mock-treated (PBS). One hour post transfer, mice were challenged i.t. with $5x10^5$ CFU of *S. pneumoniae.* Mice were euthanized at 24 hours post infection, and the lungs, brain, and blood were collected and plated on blood agar plates for CFU.

2.10 Blocking IL-10 in vivo

Mice were treated with 0.1mg/mouse of IL-10 blocking antibody (JES52A5, Biolegend) or the isotype control (Rat IgG1 K Isotype Control, Biolegend) through i.p. injection. After 2 hours, mice were then challenged i.t. with $5x10^5$ CFU of *S. pneumoniae.* The lungs and blood were collected two days post-infection to determine bacterial burden.

2.11 Assays with human PMNs

Young (23-38 years old), male and female healthy human volunteers were recruited in accordance with The University at Buffalo Human Investigation Review Board (IRB) and signed informed consent forms. Individuals taking medication, reporting symptoms of infection within the last 2 weeks or that were pregnant were excluded from the study. Whole blood was obtained using acid citrate/dextrose as an anti-coagulant. PMNs were isolated using a 2% gelatin sedimentation technique as previously described (28) which allows for isolation of active PMNs with ~90% purity. 5 x 10^5 PMNs were treated with the selective and competitive inhibitor of CD73, α,β methylene ADP (1 or 10μM) or DMSO vehicle control for 30 minutes at 37^o C. The PMNs were then infected with 10^3 CFU *S. pneumoniae* pre-opsonized in 10% (v/v) baby rabbit serum (Pel-Freeze) for 45 minutes at 37°C with rotation. Samples were then placed on ice to stop the process of opsonophagocytosis followed by serial dilution and plating on blood agar plates to enumerate viable CFU. The percentage of bacterial killing was calculated relative to no PMN controls incubated under the same treatment conditions.

2.12 mRNA measurement

RNA was extracted from 2×10^6 human PMNs using the RNeasy Mini Kit (Qiagen) as per manufacturer's protocol. TURBO DNA-free kit (Invitrogen) was used to digest DNA from the RNA samples prior conversion into cDNA. RNA concentration and 260/280 ratio were determined using Nano-drop (Thermo Fischer Scientific). For each sample, 500ng of RNA was converted into cDNA

using SuperScript VILO™ cDNA synthesis kit (Life Technologies) according to the manufacturer's protocol. RT-PCR was performed using CFX96 Touch™ Real-Time PCR Detection System from Bio-Rad and CT (cycle thresh-hold) values were determined using the following TaqMan probes from Life Technologies (Thermo Fischer Scientific): GAPDH (human Hs99999905_m1) CD73 (human Hs00159686_m1) and IL-10 (human Hs00961622_m1). Each sample was run in duplicates. Data were analyzed by the comparative threshold cycle ($2^{-\Delta CT}$) method, normalizing the CT values obtained for CD73 and IL-10 expression to those for GAPDH of the same sample. For comparison of IL-10 levels upon infection, relative quantity of transcripts (RQ) values were calculated by the ΔΔCT method by using the formula RQ =2^– (ΔΔCT) (29). The ΔΔCT values were obtained by subtracting ΔCT value of the infected (test) from that of the uninfected control.

2.13 Statistics

All statistical analysis was performed using Prism7 (Graph Pad). CFU data were log-transformed to normalize distribution. For all graphs, the mean values +/- SD are shown. Significant differences were determined by 2-way ANOVA followed by Sidak's multiple comparisons test or Student's t-test where *p* values less than 0.05 were considered significant (as indicated by asterisks). Pearson test was used to determine correlation.

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 ΔPLY *S. pneumoniae* (MOI=2) for
309 10 minutes at 37^{o}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^{o}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^{o} 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^{o}
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^{o} 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^{o} 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^{o} 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^{o} 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

3. Results

3.1 CD73 expression on PMNs decreases over the course of infection

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

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

drop started to occur after 12 hours post-infection, a time point after which PMN presence in the lungs no longer correlated with control of bacterial numbers (Fig 1A) (3). The decrease of CD73 expression also occurred on circulating PMNs and bone marrow PMNs (Fig. 1C), suggesting that this was a systemic decrease and not only localized in the lungs. A recent study identified a population of PMNs in the spleen with intermediate expression of Ly6G on their surface that exhibited a lowered ability to engulf *S. pneumoniae* during i.v. infection (30). When we compared Ly6G expression, we found that CD73 positive pulmonary PMNs had significantly higher expression of Ly6G (MFI) on their surface at all the time points tested (Fig. 1D). These data show that there are changes in PMNs over the course of pneumococcal pneumonia and that low CD73 expression on PMNs may be indicative of a PMN subset that is associated with lower anti-microbial activity.

3.2 CD73 and extracellular adenosine are required for the ability of PMNs to kill *S. pneumoniae*

Prior studies demonstrated that EAD is required for protection against *S. pneumoniae* lung infection (3). To test if EAD production has a role in anti-pneumococcal function of PMNs, we compared *ex vivo* opsonophagocytic killing of pneumococci by PMNs isolated from the bone marrow of *CD73*$^{-/-}$ or wild type C57BL/6 (WT) mice. Strikingly, *CD73*$^{-/-}$ PMNs completely failed to kill *S. pneumoniae* and in fact the presence of these PMNs promoted an increase in bacterial numbers as indicated by negative bacterial killing on the graph (Fig. 2).

To test if the inability of *CD73*$^{-/-}$ PMNs to kill bacteria was due to a defect in EAD production, we added back adenosine to the opsonophagocytic reactions. Upon infection, adenosine levels in the sera of WT mice varied considerably, but on average significantly increased five-fold to 52.6 +/- 29.0 μM as measured by a fluorometric kit (Fig S 1A). We found that supplementing *CD73*$^{-/-}$ PMNs with EAD at 100 μM (closer to the higher levels found in infected mice) restored the ability of these cells to kill bacteria back to levels comparable with WT PMNs. The difference in bacterial killing observed was not due to difference in bacterial survival in WT versus *CD73*$^{-/-}$ sera or direct toxic effects of EAD on bacterial viability (Fig. S1B and C). Also, addition of EAD had no significant effect on bacterial killing by WT PMNs (Fig 2).

To test the clinical relevance of our findings, we tested the effect of CD73 inhibition on the anti-bacterial activity of PMNs from human donors. PMNs were isolated from the blood of young healthy donors and treated with the selective and competitive inhibitor of CD73, α,β methylene ADP or vehicle control *in vitro* and their ability to kill pneumococci was assessed. We found that in over half (i.e. 4 out of 7) of the donors tested, CD73 inhibition completely abrogated the ability of PMNs to kill pneumococci and in fact promoted bacterial growth in the presence of PMNs instead (Fig. 3A). The inhibitor had no direct effect on the viability of *S. pneumoniae* (data not shown) as previously described (3). CD73 mRNA was detected in all five donors we tested (Fig. 3B) and relative expression did not correlate with the responsiveness of donor PMNs to CD73 inhibition.

These findings demonstrate that production of EAD by CD73 is required for the ability of PMNs to kill pneumococci.

3.3 Adoptive transfer of PMNs from wild type mice boosts resistance of CD73$^{-/-}$ mice to *S. pneumoniae*

To test the role of EAD production by PMNs *in vivo*, we adoptively transferred 2.5x10^{6} PMNs isolated from WT or CD73$^{-/-}$ mice into CD73$^{-/-}$ mice, one hour later infected the mice with 5x10^{5} CFU of *S. pneumoniae* i.t. and then compared bacterial burdens in the lung, blood and brain at 24 hours post infection. We found that although transfer of WT PMNs had no impact on bacterial numbers in the lungs (Fig. 4A), it significantly reduced the systemic spread of pneumococci and resulted in a 50 and 5-fold reduction in blood and brain bacterial loads respectively (Fig. 4B and C) when compared to no transfer controls. Surprisingly, we found that adoptive transfer of CD73$^{-/-}$ PMNs worsened susceptibility to infection and resulted in a significant ~10-fold increase in bacterial numbers in all sites (lung, blood, brain) tested (Fig. 4A-C) when compared to no transfer controls. This was consistent with our *in vitro* findings where bacterial numbers increased in the presence of CD73$^{-/-}$ PMNs. These findings suggest that EAD production by PMNs is required and sufficient to promote resistance to *S. pneumoniae* infection.

3.4 Bacterial uptake and intracellular killing by PMNs are not altered by 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

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

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

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

Although the role of IL-10 during *S. pneumoniae* infection in mice is well 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

apoptosis and necrosis are known to play a role in the anti-bacterial function of PMNs (39), we compared the percentage of apoptotic and necrotic cells using Annexin V and propidium iodide staining and flow cytometry. We found that as expected (40), pneumococcal infection induced pore formation and necrosis of PMNs (PI and Annexin V double positive cells) (Fig S4A). However, there was no difference in viability of PMNs at baseline or upon infection between WT and CD73$^{-/-}$ PMNs and addition or inhibition of IL-10 did not further alter the percentage of necrotic PMNs (Fig S4A). We also tested the effect of IL-10 on the association of PMNs with GFP-labeled pneumococci and found that neither addition nor blocking of IL-10 altered association (data not shown). When we examined the effect of IL-10 on NE activity, we found that neither addition of recombinant IL-10 nor blocking IL-10 significantly altered the activity of the enzyme by PMNs (Fig S3A).

3.10 CD73 and IL-10 regulate extracellular ROS production by PMNs upon *S. pneumoniae* infection

IL-10 was reported to down regulate reactive oxygen species (ROS) production by PMNs (18). To determine whether the CD73/IL-10 axis regulated ROS production upon pneumococcal infection, we first compared production of both intracellular and extracellular ROS by WT and CD73$^{-/-}$ PMNs. When we measured intracellular ROS production over time, we found that the infection up regulated ROS production rapidly within minutes to more than 50-fold increase 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

peak of the response at 5 minutes post infection (Fig. 7D). Taken together, these data demonstrate that inhibition of IL-10 production by CD73 is important for an optimal extracellular ROS response by PMNs during *S. pneumoniae* infection.

The enzyme myeloperoxidase (MPO) was reported to play a crucial role in clearance of *S. pneumoniae* and that it exerted its function partially by facilitating ROS production (41). Therefore we compared the effect of IL-10 and CD73 on MPO production by PMNs. We found that pneumococcal infection significantly up regulated MPO levels (Fig S4C) as previously reported (41). However, there was no difference in the levels of the enzyme between WT and $CD73^{-/-}$ PMNs, nor was its levels altered by blocking or adding IL-10 (Fig S4C). This suggests that the effect of IL-10 and EAD on extracellular ROS production by PMNs upon pneumococcal infection is not mediated via MPO.

3.11 Pulmonary PMNs from $CD73^{-/-}$ mice produce IL-10 early on during *S. pneumoniae* infection

Next, we wanted to test the relevance of our findings *in vivo* and assess IL-10 production during lung infection. WT C57BL/6 and $CD73^{-/-}$ mice were infected i.t. with $5x10^5$ CFU of *S. pneumoniae* TIGR4 and IL-10 production by pulmonary PMNs was monitored at 6 hours post infection by intracellular cytokine staining. We focused on early time points since that is when PMNs are most relevant for the control of bacterial numbers. When we gated on PMNs (Ly6G+; see Fig. S5 for the gating strategy), we found that a low percentage (~6%) of PMNs in the lungs of WT mice expressed IL-10 and similar to our

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

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

To test whether the early increase in IL-10 production by PMNs in CD73$^{-/-}$ mice contribute to their susceptibility to infection, we treated the mice with either isotype control or the IL-10 blocking antibody 2 hours prior to infection. Mice were 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

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

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

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

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

Murine PMNs have been described to produce IL-10 in sepsis models and in response to several bacterial infections such as *Escherichia coli*, *Shigella flexneri* (16) and *Staphylococcus aureus* (13) as well as parasitic infections such as *Leishmania major* (14) and *Trypanosoma cruzi* (15). In contrast, IL-10 production by pulmonary PMNs was actively repressed during *Mycobacterium 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.

PMNs were shown to kill *S. pneumoniae* in a manner that requires phagocytosis and serine proteases (4). In exploring how the CD73/IL-10 axis regulated PMN anti-pneumococcal activity we found that apoptosis, bacterial uptake and intracellular killing and production of anti-microbial Neutrophil Elastase and Myeloperoxidase were not affected by this pathway. Rather, inhibition of IL-10 production by CD73 was crucial for optimal production of extracellular ROS by PMNs upon *S. pneumoniae* infection. This is in line with previous findings that adenosine signaling via the A1 receptor enhanced superoxide production by PMNs in response to antibody coated erythrocytes (51) and that IL-10 blunted PMN superoxide production in response to PMA and *C. albicans* hyphae (18, 19).

Previous work showed that *S. pneumoniae* triggered extracellular respiratory burst by PMNs (52) and that PLY released by the bacteria also triggered production of intracellular ROS (23). Similarly, we found here that the bacterial infection triggered both intracellular and extracellular ROS production. NADPH oxidase is known to assemble both at the phagosomal membrane and on the cell surface in response to bacterial infections (53), here we found that CD73 was important for optimal extracellular ROS production but had no effect on intracellular ROS suggesting that this pathway may target cell surface assembly of the NADPH oxidase complex only. The role of ROS production in anti-pneumococcal function of PMNs and host resistance to *S. pneumoniae* is arguable. Studies showed that mice lacking components of the NADPH oxidase 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

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

Acknowledgments

We would like to acknowledge Andrew Camilli for bacterial strains. This work was supported by National Institute of Health grant R00AG051784 to ENBG.

Conflict of Interest Disclosure

None

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Figure Legends

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

asterisks, denotes significant correlation. We gated on PMNs (Ly6G+ cells) and also monitored (B) the percentage of cells expressing CD73 and (B-C) the amounts of CD73 (mean fluorescent intensity or MFI) in the indicated organs, at the indicated time points. (D) We gated on all Ly6G+ cells and then gated on CD73 positive versus negative populations and compared the expression (MFI) of Ly6G. Data are pooled from two separate experiments with 4-5 mice per time point (A) and 4-7 mice per time point (B-D). Asterisks indicate significant differences from uninfected controls calculated by Student's t-test.

Figure 2. CD73 and extracellular adenosine are required for the ability of neutrophils to kill *S. pneumoniae.* (A) PMNs were isolated from the bone marrow of C57BL/6 (WT) or $CD73^{-/-}$ mice and treated with 100μM Adenosine or PBS (vehicle control) for 30 minutes at 37°C. The reactions were then infected with *S. pneumoniae* pre-opsonized with homologous sera for 45 minutes at 37°C. Reactions were then stopped by placing samples on ice and viable CFU were determined after serial dilution and plating. The percentage of bacteria killed upon incubation with PMNs was determined by comparing surviving CFU to a no PMN control. Positive percent killing indicates bacterial death while negative percent indicates bacterial growth. Data shown are pooled from three separate experiments (n=3 biological replicated or mice per strain) where each condition was tested in triplicate (n=3 technical replicates) per experiment. Asterisks indicate significance calculated by Student's t-test.

Figure 3. CD73 is required for the ability of neutrophils isolated from human donors to kill *S. pneumoniae.* PMNs were isolated from the blood of young healthy donors and (A) pre-treated with CD73 inhibitor (α,β methylene ADP) or vehicle control for 30 minutes at 37°C and then incubated for 45 minutes with complement-opsonized *S. pneumoniae* TIGR4. For each donor, the average percent bacterial killing compared to a no PMN control was calculated from triplicate wells per condition. Data from 7 donors are shown. (B) RNA was isolated from resting PMNs of the indicated donors and the expression of CD73 mRNA was measured by RT-qPCR and normalized to GAPDH. Significant differences denoted by asterisk, were determined by Student's t-test.

Figure 4. Adoptive transfer of PMNs from wild type mice boosts resistance of CD73$^{-/-}$ mice to *S. pneumoniae.* CD73$^{-/-}$ mice were mock treated (no transfer) or injected i.p with 2.5x10^{6} PMNs isolated from the bone marrow of C57BL/6 or CD73$^{-/-}$ mice. One hour post transfer, mice were infected i.t with 5x10^{5} CFU of *S. pneumoniae* and bacterial numbers in the lung (A), blood (B) and brain (C) were determined 24 hours post infection. Significant differences, determined by Student's t-test, are indicated by asterisks. Representative data from one of three separate experiments (n=3 mice per group) are shown.

Figure 5. Extracellular adenosine blunts IL-10 production from PMNs following infection. (A) PMNs from the indicated mouse strains were incubated for 45 minutes at 37°C with *S. pneumoniae* pre-opsonized with homologous sera

or mock treated with buffer and homologous sera (uninfected). The supernatants were then collected and assayed for IL-10 production by ELISA. (B) PBS control or adenosine (100μM) were added to the PMNs 20 minutes prior to infection and the fold-change in IL-10 production was calculated by dividing the values of infected reactions by uninfected controls for each condition. Data were pooled from three separate experiments (n=3 mice) with each condition tested in triplicate per experiment. Asterisks indicate significant differences determined by Student's t-test.

Figure 6. IL-10 impairs the ability of PMNs to kill *S. pneumoniae*. PMNs from C57BL/6 or CD73$^{-/-}$ mice were incubated for 20 minutes with the indicated anti-IL-10 (1μg/ml JES5-2A5), isotype control (1μg/ml), rIL-10 (50ng/ml) or adenosine (100μM) and then infected with pre-opsonized *S. pneumoniae* for 45 minutes at 37°C. Reactions were then plated on blood agar plates and the percentage of bacteria killed compared to a no PMN control under the same conditions was calculated. Data shown are pooled from three separate experiments (n=3 mice per strain) with each condition tested in triplicate. Asterisks indicate significant differences determined by Student's t-test.

Figure 7. Inhibition of IL-10 production by CD73 is important for optimal production of extracellular reactive oxygen species by PMNs in response to *S. pneumoniae* infection. PMN were isolated from the bone marrow of the indicated strains of mice and left untreated (A and B) or treated with the indicated

rIL-10 (50ng/ml) (C) or anti-IL-10 (1µg/ml JES5-2A5) or isotype control (1µg/ml) (D). PMNs were then infected with *S. pneumoniae* pre-opsonized with homologous sera (+*Sp*) or treated with 3% matching sera (uninfected) and intracellular ROS production measured by chemiluminescence of Luminol (A) or extracellular ROS production measured by chemiluminescence of Isoluminol in the presence of HRP (B-D). Representative data are shown from one of six (B) and one of four (A, C-D) separate experiments with one mouse per strain per experiment where each condition is tested in quadruplicates. Significant differences ($p<0.05$) were determined by 2-way ANOVA followed by Sidak's multiple comparisons test.

Figure 8. Pulmonary PMNs from CD73$^{-/-}$ mice produce IL-10 early on during *S. pneumoniae* infection. Wild-type C57BL/6 or *CD73*$^{-/-}$ mice were mock-infected or i.t challenged with 5 x 10^5 CFU of *S. pneumoniae*. Six (grey bars) and 18 hours (black bars) following challenge, (A) the percentage of IL-10 producing PMNs (Ly6G+), (B) the mean florescent intensities (MFI) of IL-10 in PMNs (Ly6G+) and (C) the number of IL-10 producing PMNs (Ly6G+) recruited into the lungs were determined by intracellular cytokine staining and flow cytometry (see Materials and Methods). Pooled data are from three separate experiments (n=6-9 mice per strain per time point). Significant differences determined by Student's t-test are indicated by asterisks.

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

Figure 1.

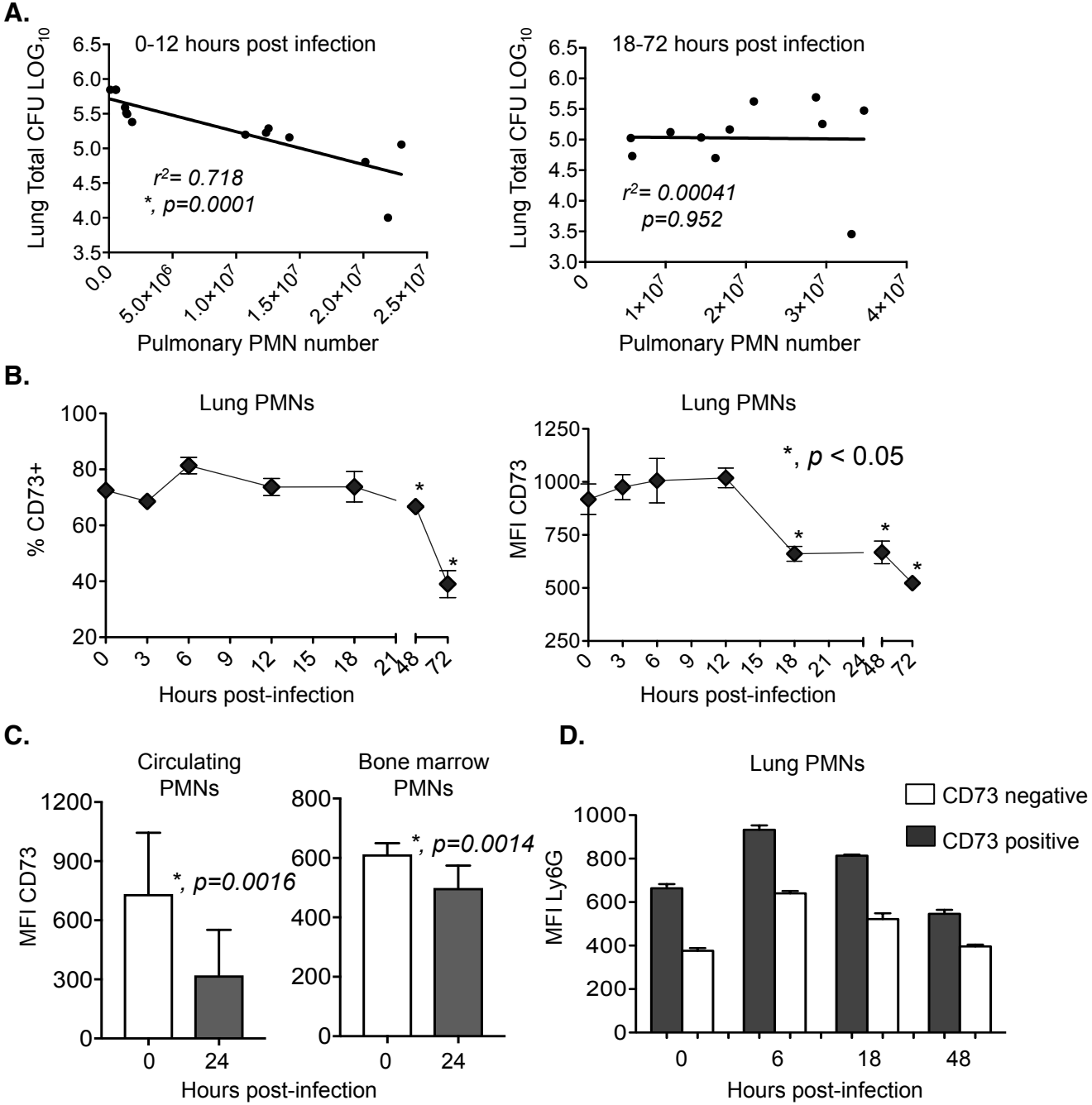

A.
0-12 hours post infection
Lung Total CFU LOG10
r2= 0.718
*, p=0.0001
Pulmonary PMN number
18-72 hours post infection
r2= 0.00041
p=0.952
B.
Lung PMNs
% CD73+
Hours post-infection
MFI CD73
*, p < 0.05
C.
Circulating PMNs
*, p=0.0016
Bone marrow PMNs
*, p=0.0014
D.
Lung PMNs
CD73 negative
CD73 positive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

Figure 2.

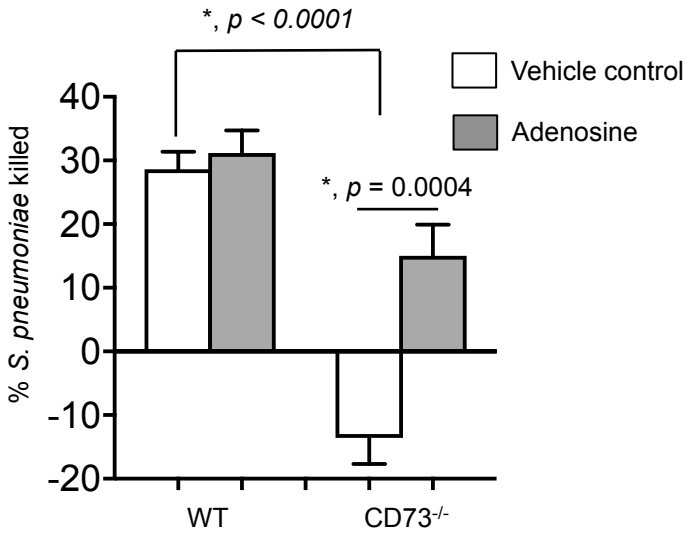
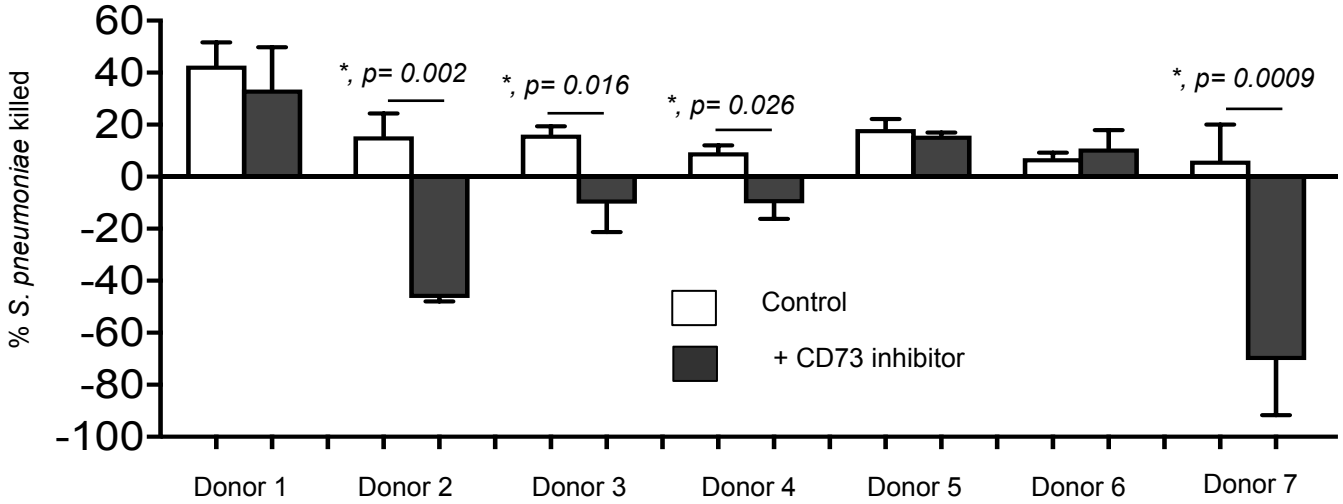

*, p < 0.0001
Vehicle control
Adenosine
*, p = 0.0004
% S. pneumoniae killed
40
30
20
10
0
-10
-20
WT
CD73-/-

Figure 3.

A.


60
40
20
0
-20
-40
-60
-80
-100
% S. pneumoniae killed
*, 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.

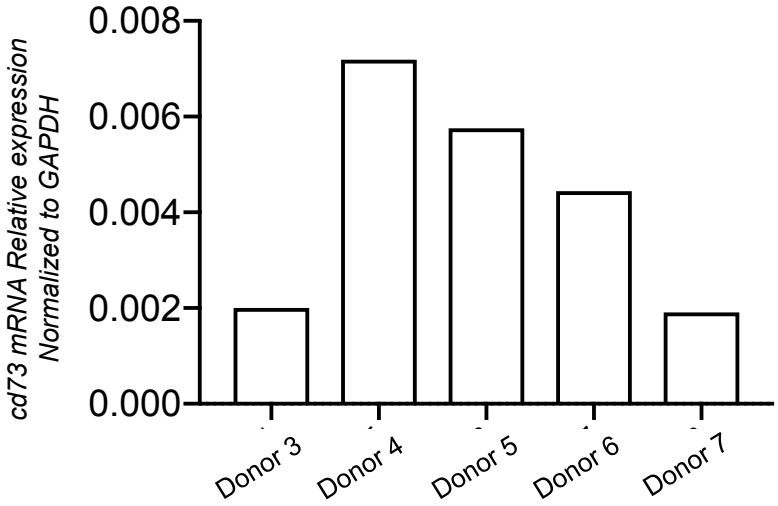

0.008
0.006
0.004
0.002
0.000
cd73 mRNA Relative expression
Normalized to GAPDH
Donor 3
Donor 4
Donor 5
Donor 6
Donor 7

Figure 4.

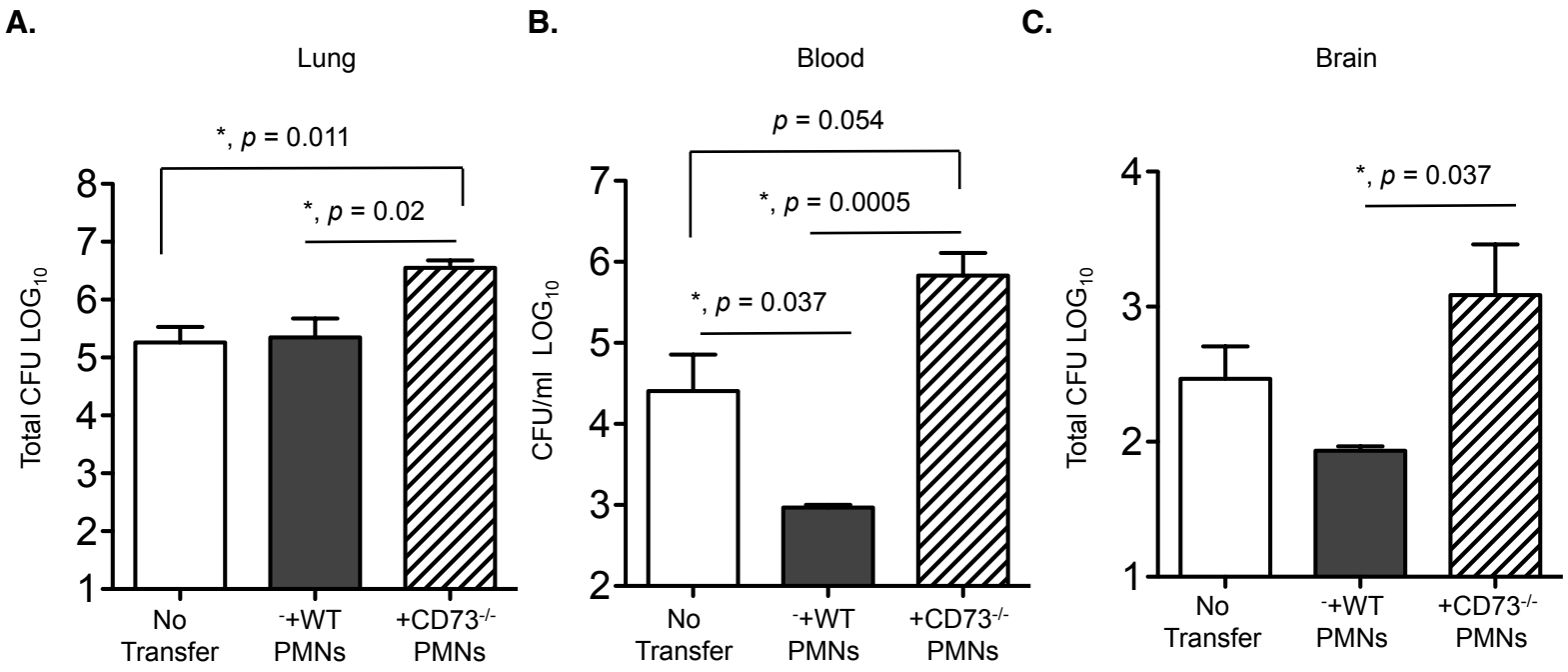

A.
Lung
*, p = 0.011
*, p = 0.02
Total CFU LOG10
8
7
6
5
4
3
2
1
No Transfer
-+WT PMNs
+CD73-/- PMNs
B.
Blood
p = 0.054
*, p = 0.0005
*, p = 0.037
CFU/ml LOG10
7
6
5
4
3
2
No Transfer
-+WT PMNs
+CD73-/- PMNs
C.
Brain
*, p = 0.037
Total CFU LOG10
4
3
2
1
No Transfer
-+WT PMNs
+CD73-/- PMNs

Figure 5.

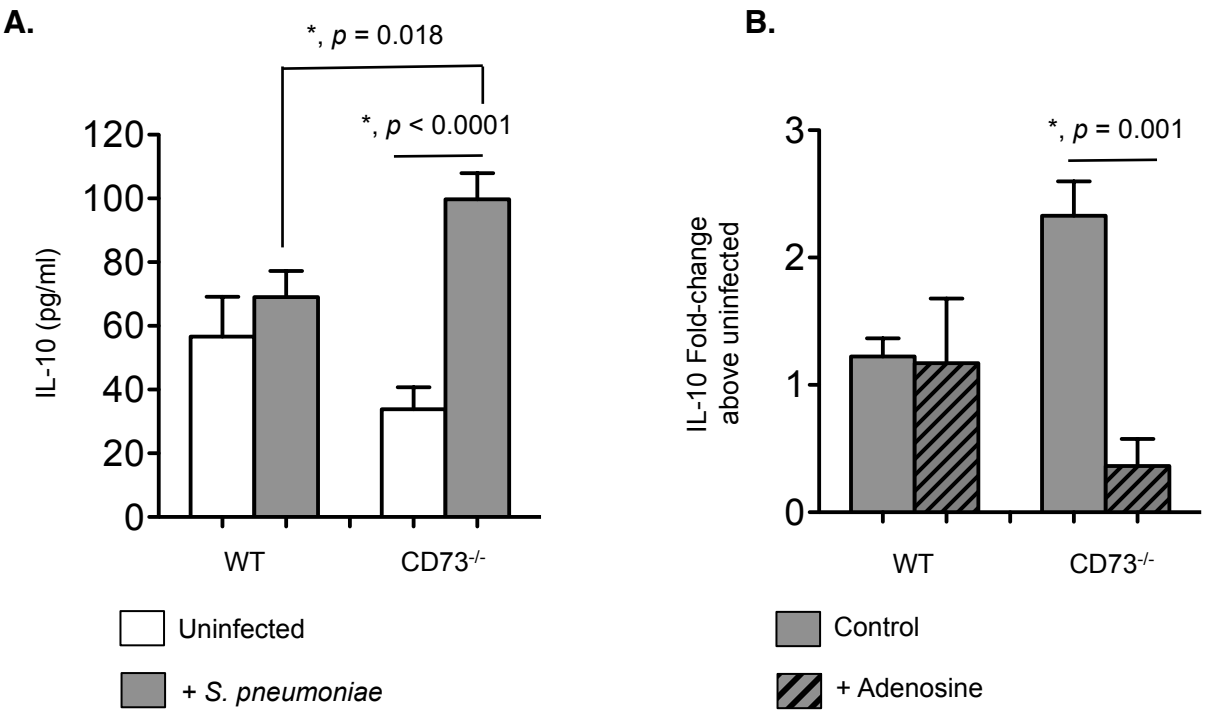

A.
*, p = 0.018
*, p < 0.0001
IL-10 (pg/ml)
120
100
80
60
40
20
0
WT
CD73-/-
Uninfected
+ S. pneumoniae
B.
*, p = 0.001
IL-10 Fold-change above uninfected
3
2
1
0
WT
CD73-/-
Control
+ Adenosine

Figure 6.

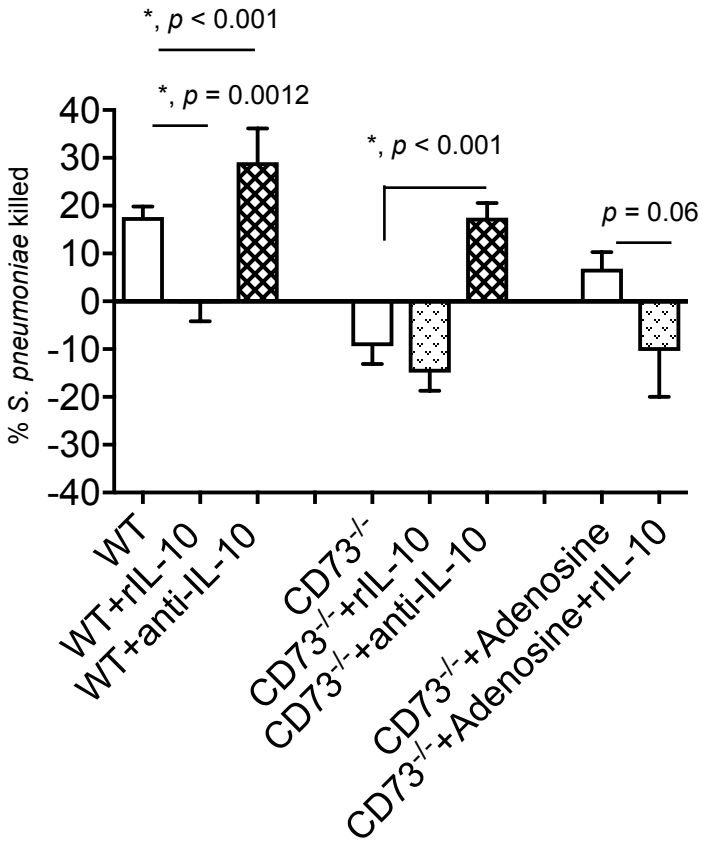

*, p < 0.001
*, p = 0.0012
*, p < 0.001
p = 0.06
% S. pneumoniae killed
40
30
20
10
0
-10
-20
-30
-40
WT
WT+rIL-10
WT+anti-IL-10
CD73-/-
CD73-/-+rIL-10
CD73-/-+anti-IL-10
CD73-/-+Adenosine
CD73-/-+Adenosine+rIL-10

Figure 7.

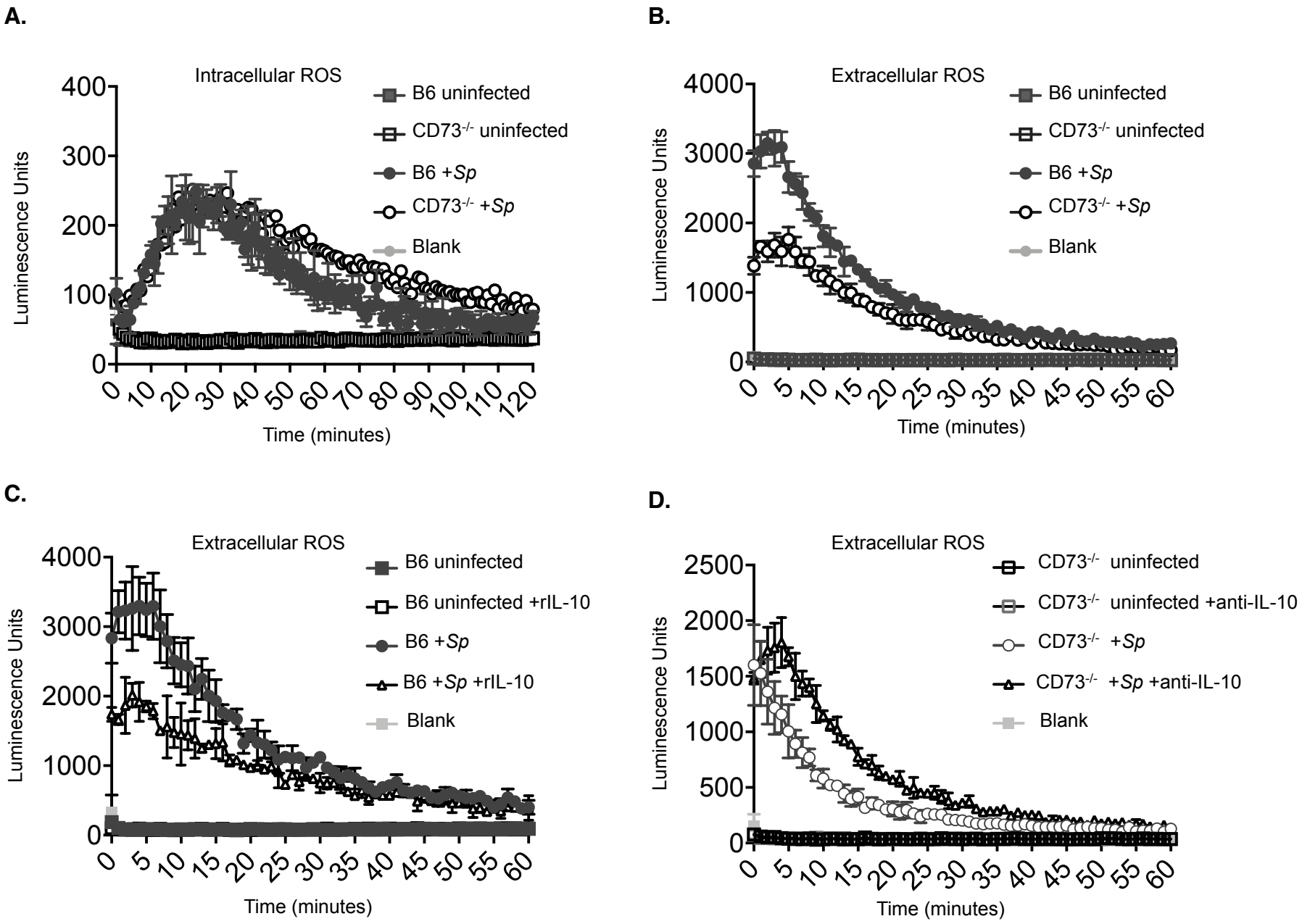

A.
Intracellular ROS
Luminescence Units
Time (minutes)
B6 uninfected
CD73-/- uninfected
B6 +Sp
CD73-/- +Sp
Blank
B.
Extracellular ROS
Luminescence Units
Time (minutes)
B6 uninfected
CD73-/- uninfected
B6 +Sp
CD73-/- +Sp
Blank
C.
Extracellular ROS
Luminescence Units
Time (minutes)
B6 uninfected
B6 uninfected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

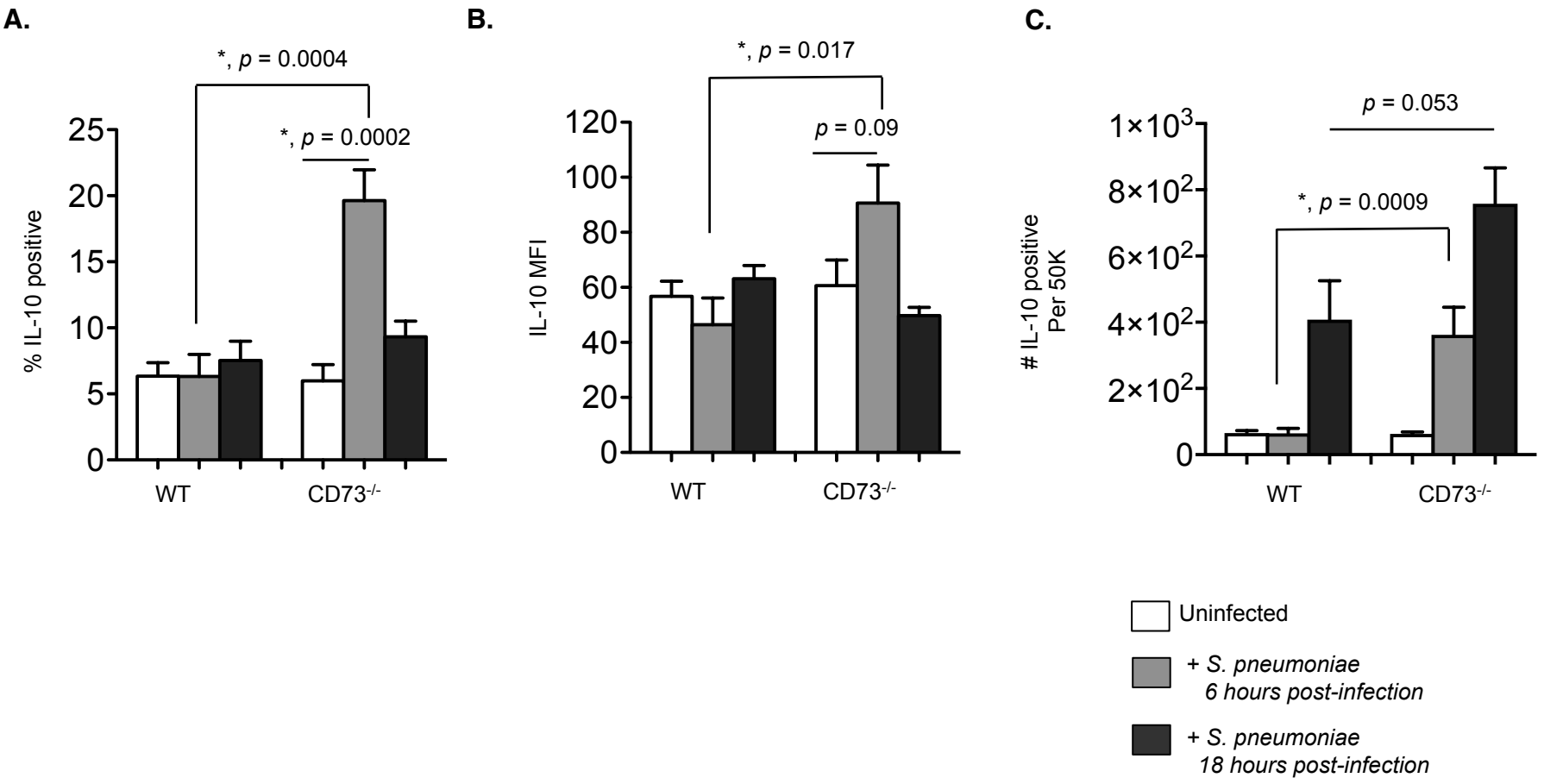
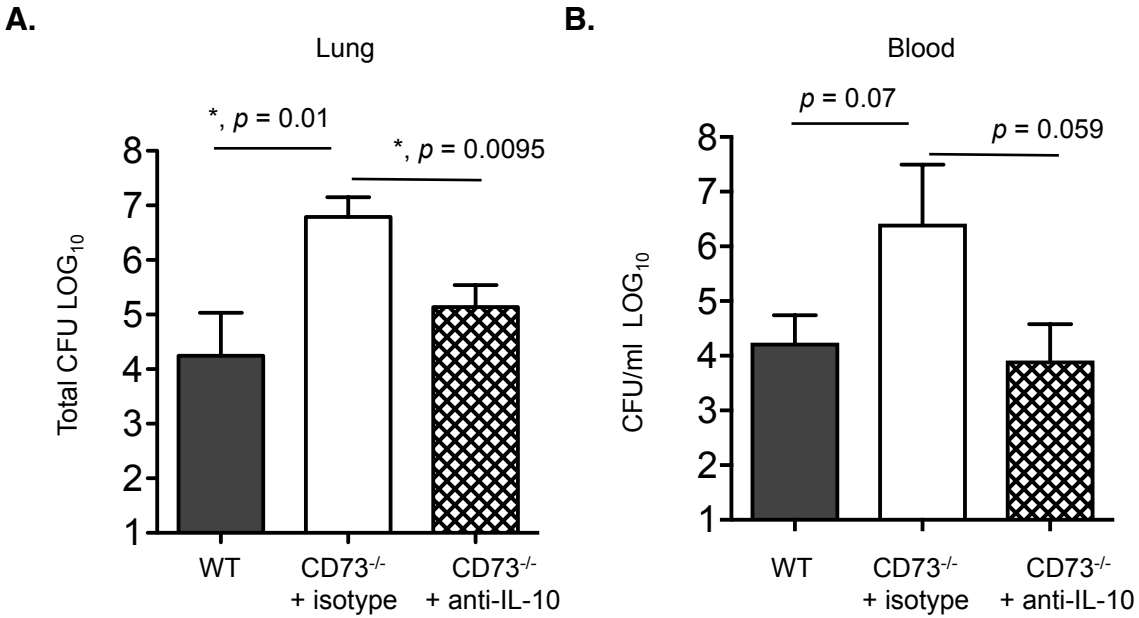

A.
*, p = 0.0004
*, p = 0.0002
% IL-10 positive
25
20
15
10
5
0
WT
CD73-/-
B.
*, p = 0.017
p = 0.09
IL-10 MFI
120
100
80
60
40
20
0
WT
CD73-/-
C.
p = 0.053
*, p = 0.0009
IL-10 positive Per 50K
1×10^3
8×10^2
6×10^2
4×10^2
2×10^2
0
WT
CD73-/-
Uninfected
+ S. pneumoniae 6 hours post-infection
+ S. pneumoniae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