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Platelets attach to lung ILC2 expressing PSGL-1 and influence ILC2 function

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Capsule Summary:

Electron microscopy demonstrates that mouse lung ILC2 expressing PSGL-1 have platelets attached to their surface and that platelet depletion reduces lung ILC2 proliferation and Th2 cytokines suggesting ILC2 function is influenced by attachment to platelets.

Keywords:

CD41

ILC2

Platelets

P-selectin (CD62P)

P-selectin glycoprotein ligand-1 (PSGL-1 also known as CD162)

Abbreviations:

ILC2 Innate lymphoid cell type 2

PSGL-1 P-selectin glycoprotein ligand-1

To the Editor:

ILC2 are an important source of Th2 cytokines relevant to allergic inflammation, nasal polyps, asthma and atopic dermatitis¹. ILC2 are tissue resident cells and can rapidly release Th2 cytokines in response to innate stimuli^{1,2}. In addition to their tissue residency, ILC2 can also be recruited from the bone marrow through the circulation to the lung^{3,4}, as well as from the gastrointestinal tract to the lung⁵. In this study we provide evidence for a novel pathway whereby platelets attached to mouse lung ILC2 influence the ability of ILC2 to proliferate and express Th2 cytokines.

In prior studies we have demonstrated that ILC2 express adhesion molecules including β 1 integrins, β 2 integrins, and L-selectin³, and that inhibiting β 2 integrins reduced ILC2 trafficking from the bone marrow via the blood stream to the lung following *Alternaria* challenge in wild type (WT) mice³. In this study we extended these observations of adhesion molecule expression by ILC2 to demonstrate by flow cytometry (all methods described in the **Supplementary Online Repository Methods**) the novel observation that naïve mouse lung ILC2 highly express the adhesion molecule P-selectin glycoprotein ligand-1 (PSGL-1 or CD162) (**Fig 1A-B, Supplement Fig E1**). PSGL-1 binds to its ligand P-selectin (CD62P) which is highly expressed by platelets^{E1}. Thus, our observation that ILC2 express PSGL-1 suggests a potential ILC2 interaction with platelets expressing its ligand P-selectin. Interestingly, in addition to PSGL-1, P-selectin (or CD62P)^{E2} and the platelet specific marker CD41 (also known as integrin α IIb, a heterodimeric integral membrane protein whose expression is restricted to cells of the megakaryocyte lineage)⁶ were detected on ILC2s by FACS analysis (**Fig 1A-B**). As platelets have been reported to bind to leukocytes and alter leukocyte function^{E1-E7}, we initially examined

whether levels of platelet markers we detected on lung ILC2 were similar or different compared to other lymphoid cells present in the lung pertinent to Th2 responses such as CD4⁺ T cells. These studies demonstrated that naïve lung CD4⁺ T cells (**Fig 1 C-D**) expressed very low levels of platelet markers (CD41 and CD62P) compared to lung ILC2 (P <0.001). Although CD4⁺ T cells expressed significantly lower levels of platelet markers compared to ILC2, they expressed similar levels of the leukocyte adhesion molecule PSGL-1 (CD162)(**Fig 1C-D**).

To determine whether detection on ILC2 of platelet specific markers such as CD41 was due to attachment of platelets (expressing CD41) to ILC2 (which were not intrinsically expressing CD41), we performed electron microscopy studies of purified populations of sorted naïve mouse lung ILC2 (**Fig 1 E-F; Fig E1**). These studies demonstrated the novel observation that intact platelets were attached to mouse lung ILC2 (**Fig 1G**). To obtain sufficient mRNA from lung ILC2 for qPCR studies, we expanded the number of lung ILC2 by challenging mice with *Alternaria* for 10 days (**Fig E3**) and sorted these lung ILC for qPCR studies. We readily detected high levels of CD41 (**Fig 1H**) and CD62P (**Fig 1I**) mRNA by qPCR in platelets from peripheral blood. In contrast, qPCR of sorted mouse lung ILC2s expanded by *Alternaria* challenge revealed minimal expression of CD41 (**Fig 1H**) or CD62P (**Fig 1I**) mRNA. As platelets highly express CD41 and CD62P (**Fig 1H and 1I**) we believe that most of the CD41 and CD62P we detect on mouse lung ILC2 (**Fig 1A and 1B**) is derived from attached platelets and not from the ILC2. ILC2 expressed much higher levels of CD162 mRNA compared to platelets (**Fig 1J**). The low level of expression of CD41 and CD62P mRNA expression by the sorted ILC2 compared to the blood platelets may be due to the much lower number of platelets in the sorted ILC2 compared to

platelets in blood, as well as the detachment of platelets from lung ILC2 in the *Alternaria* challenge protocol used to expand lung ILC2 for these qPCR studies.

We next examined whether depleting platelets in vivo in WT mice with an anti-CD41 antibody (CD41 expression is restricted to cells of the megakaryocyte lineage)⁶ would influence naïve lung ILC2 numbers, proliferation, or apoptosis. These studies demonstrated that the anti-CD41 antibody significantly depleted platelets by >90% at 24 hours post administration of anti-CD41, and the effect persisted for 48 hours as previously described (**Fig 2A**)^{7,E8}. The depletion of platelets with the anti-CD41 antibody was associated with a significant reduction in the levels of platelet markers detected on lung ILC2 (CD41 as well as CD62P)(**Fig 2B**). In contrast, as anticipated depletion of platelets was not associated with any change in levels of PSGL-1 expressed by ILC2 (**Fig 2B**), as PSGL-1 is expressed on leukocytes and is not a platelet specific marker. Thus, these studies demonstrate that depletion of platelets in vivo is associated with reduced number of lung ILC2 attached to platelets. To determine whether the reduction in platelet markers detected on lung ILC2 was associated with any effect on naïve ILC2 numbers we quantitated levels of lung ILC2 in platelet depleted mice. These studies demonstrated that platelet depleted mice had a significant reduction in the number of total lung ILC2 during the time period that platelets were depleted (**Fig 2C**). The effect of platelet depletion on lung ILC2 numbers was rapidly reversible, for as soon as platelet levels returned to normal, total lung ILC2 numbers also returned to normal (**Fig 2C**). Administration of an anti-CD62P antibody to WT mice in vivo did not deplete platelets and did not reduce total lung ILC2 numbers (**Fig 2 D-E**). Similarly, administration of an anti-PSGL-1 antibody did not deplete platelets and did not reduce total lung ILC2 numbers (**Fig 2F-G**). Administration of the anti-CD41 antibody significantly

reduced lung ILC proliferation as assessed by Ki-67 FACS staining (**Fig 2H-I; Fig E2**), but did not significantly affect the % of apoptotic lung ILC2 as assessed by the % of caspase 3 positive lung ILC2 quantitated by FACS (**Fig 2J**). These studies suggest that depletion of platelets in naïve mouse lung is associated with reduced lung ILC2 proliferation (with no effect on lung ILC2 apoptosis) which results in reduced lung ILC2 numbers. Thus, platelets interacting with lung ILC2 may normally maintain the proliferative capacity of naïve lung ILC2.

We also performed experiments examining the ILC2 platelet interaction following *Alternaria* challenge^{E9} (**Fig E4**). These studies demonstrated that *Alternaria* challenge of WT mice resulted in a significant reduction of the platelet markers (CD41 and CD62P) as quantitated by gMFI for ILC2 CD41 (**Figure 2K**), and gMFI for ILC2 CD62P (**Figure 2L**) compared to PBS challenged mice suggesting that many platelets attached to lung ILC2 had detached. Interestingly, the gMFI for ILC2 PSGL-1 (**Figure 2M**) increased following *Alternaria* challenge. As PSGL-1 is the ligand for P-selectin, when platelets expressing P-selectin detach from PSGL-1 expressed by ILC2 this can expose additional PSGL-1 on ILC2 and account for the increased gMFI for ILC2 PSGL-1 following *Alternaria* challenge. This platelet ILC2 complex concealment of their receptors may also potentially explain why treatment with anti-CD62P and anti-CD162 antibodies could not deplete both platelets and ILC2s as the platelet attached ILC2 may prevent both anti-CD62P and anti-CD162 antibodies from accessing receptors in the ILC2-platelet complexes. Further study is needed to directly demonstrate that ILC2 attach to platelets through a PSGL-1 interaction with P-selectin and/or another mechanism.

To determine whether the attachment of platelets to ILC2 had any functional consequences we compared the total number of lung ILC2 expressing IL-5 and IL-13^{E10,E11} in WT mice to WT mice depleted of platelets (**Fig E5, Fig E6**). These results demonstrated that WT mice challenged with *Alternaria* (in the absence of platelet depletion) had a significant increase in total IL-5+ ILC2 (**Figure 2N**), and total IL-13+ ILC2 (**Figure 2O**). In contrast, in the presence of platelet depletion, WT mice challenged with *Alternaria* had a significantly smaller increase in total IL-5+ ILC2 (**Figure 2N**), and total IL-13+ ILC2 (**Figure 2O**). Taken together, these results suggest that platelet attachment to lung ILC2 promotes IL-5 and IL-13 production by ILC2 following *Alternaria* challenge, while detachment of platelets is associated with reduced IL-5 and IL-13 production by ILC2 following *Alternaria* challenge. As platelets produce IL-33⁸, cysteinyl leukotrienes, and other mediators their attachment to ILC2 could provide an additional signal to enhance ILC2 IL-5 and IL-13 responses.

In addition to examining ILC2 Th2 responses we examined the effect of anti-CD41 on *Alternaria* induced ILC2 proliferation and BAL eosinophils. *Alternaria* challenge in WT mice significantly increased the total number of lung ILC2, while anti-CD41 reduced the total number of lung ILC2 following *Alternaria* challenge (**Figure 2P**). In addition, anti-CD41 reduced the total number of lung Ki67+ ILC2 following *Alternaria* challenge (**Figure 2Q**). Thus, platelet attachment to ILC2 in the lung likely supports their proliferation following *Alternaria* challenge. Anti-CD41 reduced the % of BAL eosinophils following *Alternaria* challenge but this reduction only approximated significance ($p=0.09$)(**Figure 2R**). One of the limitations of depleting platelets in a mouse model is that it will likely deplete free platelets as well as the platelets present in the platelet ILC2 conjugates.

At present it is not known what signal(s) from the platelet to the lung ILC2 might regulate its proliferation and function. Platelets are known to generate a variety of mediators including cytokines, chemokines, and lipid mediators which could influence lung ILC2 proliferation and function^{E7}. Further study is needed to determine which platelet derived mediator influences lung ILC2 proliferation and function. Interestingly, recent studies have demonstrated the lungs as a primary site of terminal platelet production⁹, and thus the lung provides a ready source of platelets to interact with lung ILC2.

In summary, in this study we have made the novel observation using electron microscopy that mouse lung ILC2 expressing PSGL-1 have platelets attached to their surface and that platelet depletion reduces lung ILC2 proliferation and Th2 cytokine expression. Thus, platelet attachment to lung ILC2 may play an important role in ILC2 function.

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FIGURE LEGENDS

Figure 1. Lung ILC2s attach to platelets and express leukocyte adhesion molecule PSGL-1

A-B. FACS analysis demonstrates that mouse lung ILC2 express platelet markers CD41 and CD62P (P-selectin) as well as the leukocyte adhesion molecule CD162 (PSGL-1). Results expressed as (A) percentage of ILC2s expressing marker, and (B) as ILC2 Δ gMFI (n=11, 3 independent experiments). **C-D.** Comparison of lung ILC2s and lung CD4⁺Thy1.2⁺ lymphocyte cell surface expression of CD41, CD62P, and CD162. Results expressed as (C) percentage and (D) Δ gMFI (n=8; 2-way ANOVA). **E.** FACS plots demonstrate that based on FSC and SSC, blood platelets (smaller size) and lung ILC2 (larger size) localize in different regions. **F.** Lineage negative and Thy1.2 positive ILC2 enriched from 3.03% pre-sort to 100% post-sort. **G.** Electron microscopy image^{E12} of sorted population of lung ILC2 demonstrating platelet (lack nucleus and contain characteristic cytoplasmic granules) attached to ILC2. **H-J.** qPCR of peripheral blood platelets and sorted lung ILC2 to detect CD41 (**H**), CD62P (**I**), and CD162 (**J**) mRNA. Data represented as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.0001$.

Figure 2. Platelet depletion in vivo reduces lung ILC2 proliferation, numbers of lung ILC2, and ILC2 Th2 cytokine expression.

(A-C). Platelets were depleted in naïve C57BL/6 mice with an anti-CD41 antibody. From Day 0 to 4 (**A**) platelets, (**B**) CD41, CD62P, and CD162 on lung ILC2s, (**C**) the number of total lung ILC2s were quantitated. **(D-E)** The effect of *in vivo* administration to C57BL/6 mice of an anti-CD62P antibody, or **(F-G)** an anti-CD162 antibody was assessed (Day 0 to 2) on (**D**, **F**) platelets,

(E, G) total lung ILC2s. (H-J). Platelets were depleted in naïve C57BL/6 mice with an anti-CD41 antibody. On Day 0 and 2 (H) the percentage ILC2s expressing Ki-67, (I) the total number of lung ILC2s expressing Ki-67, (J) the percentage of lung ILC2s expressing active caspase 3 were quantitated. (K-O) FACS studies quantitated gMFI for ILC2 CD41 (K), CD62P (L), and PSGL-1 (M), lung IL-5+ ILC2 (N), lung IL-13+ ILC2 (O) in *Alternaria* challenged mice. (P-R). The total number of lung ILC2 (P), as well as the total number of lung Ki67+ ILC2 (Q) and % BAL eosinophils (R) were quantitated post-*Alternaria* challenge in mice depleted of platelets (anti-CD41) or not depleted of platelets (isotype). Data represented as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$.

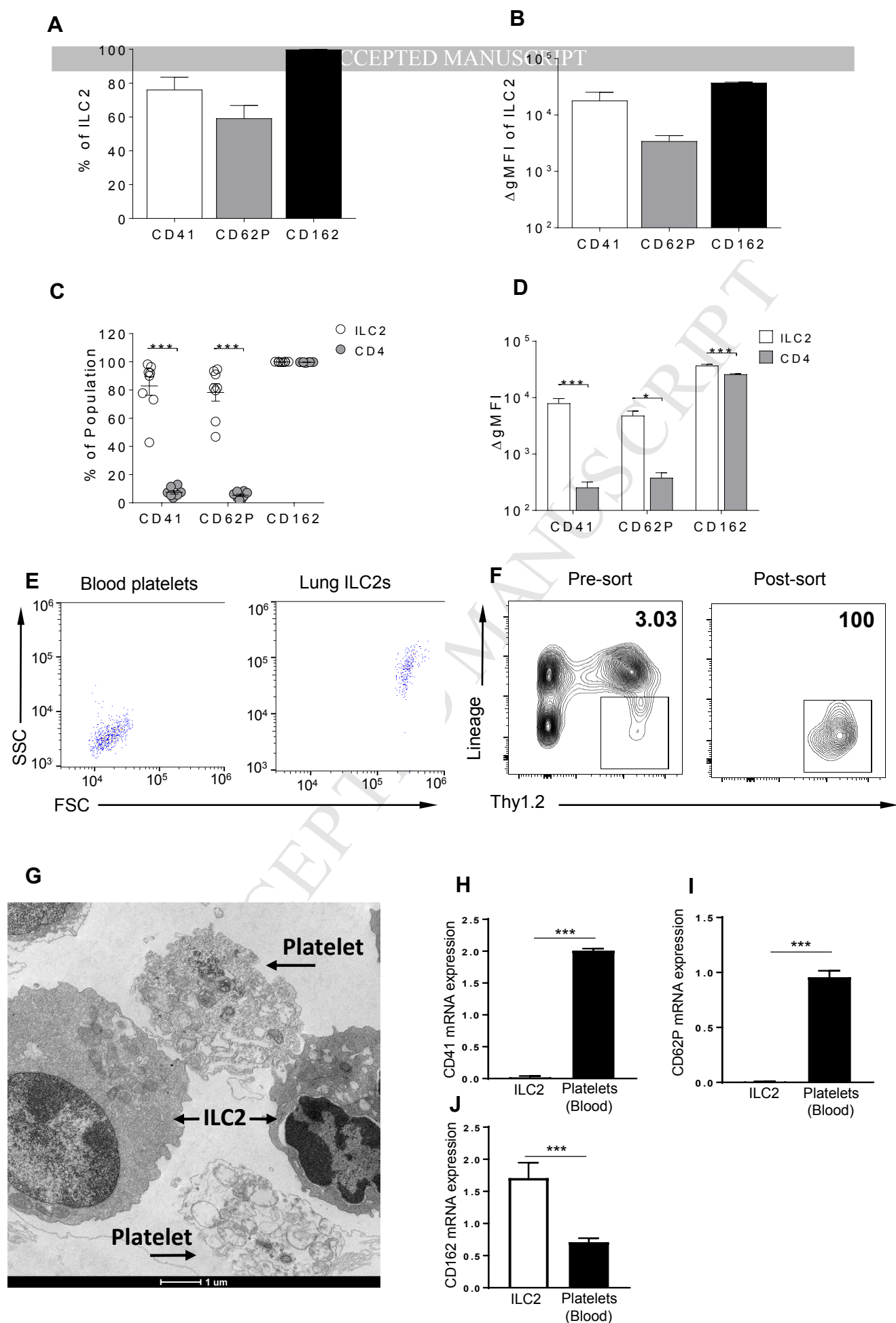


Figure 1

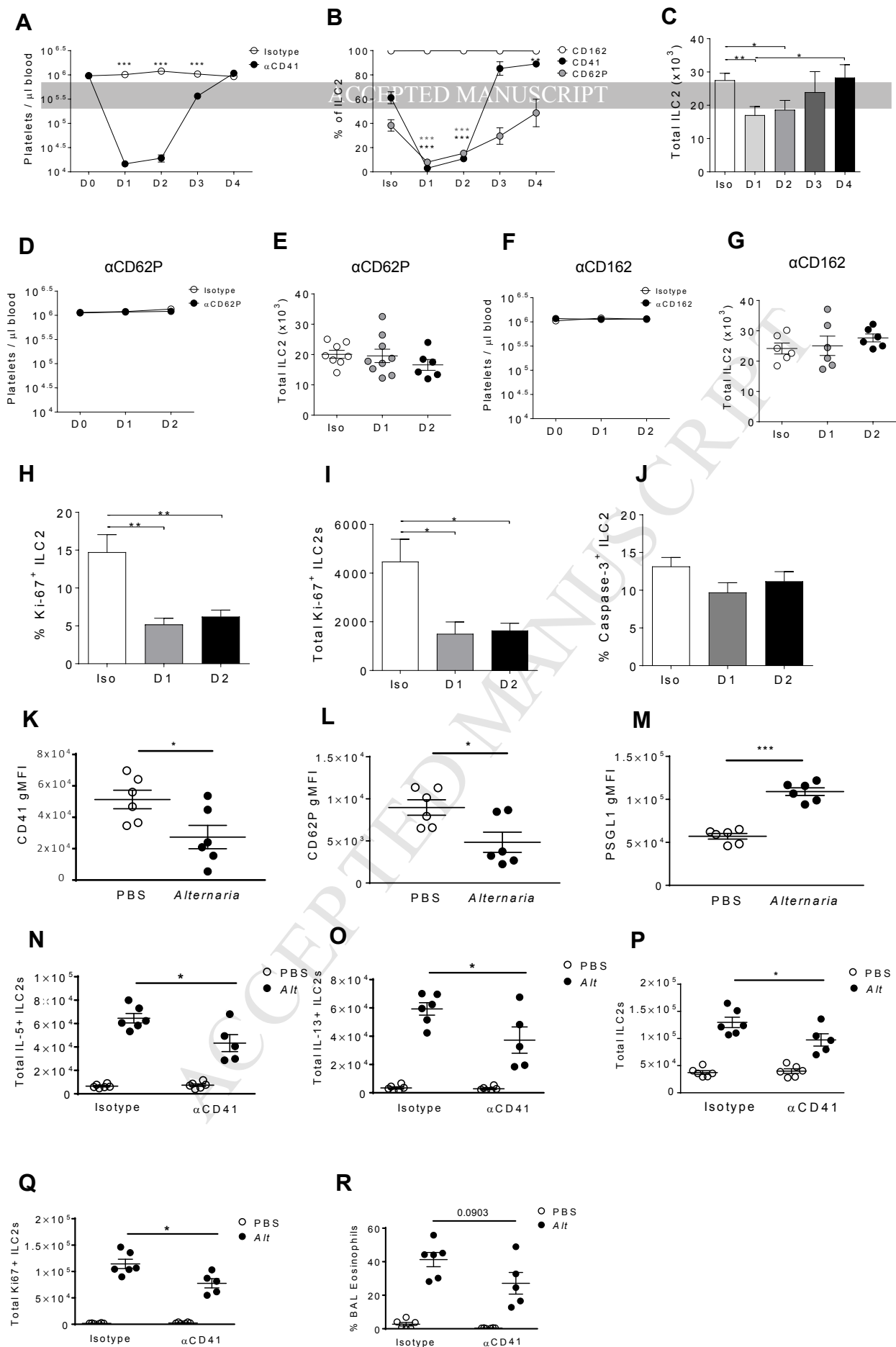


Figure 2

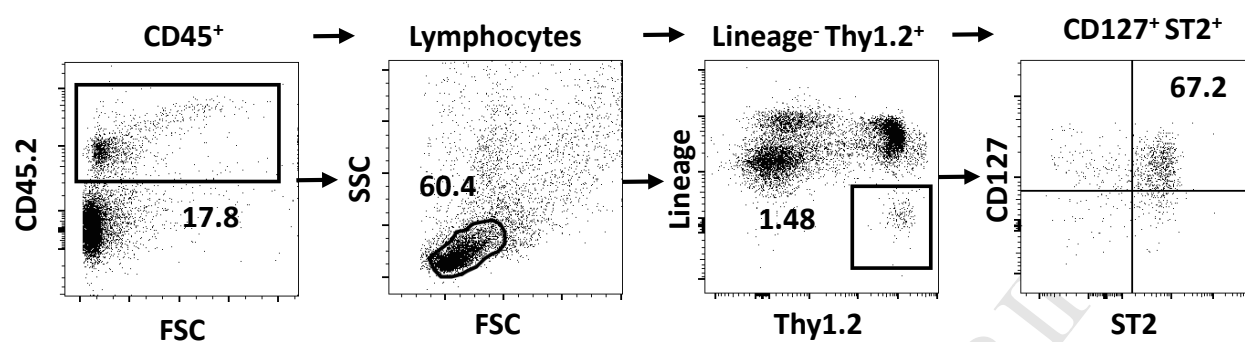


Figure E1

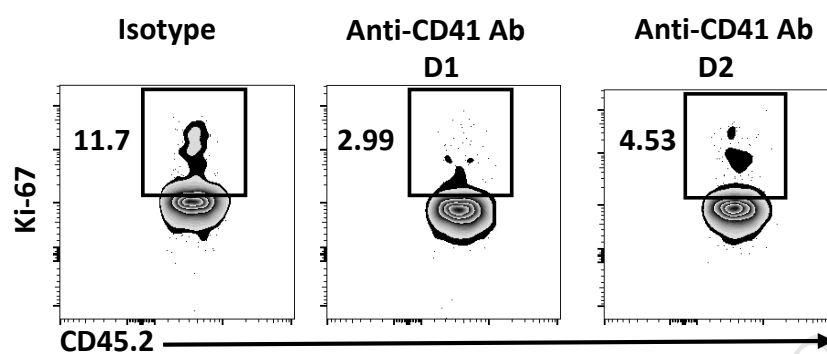


Figure E2

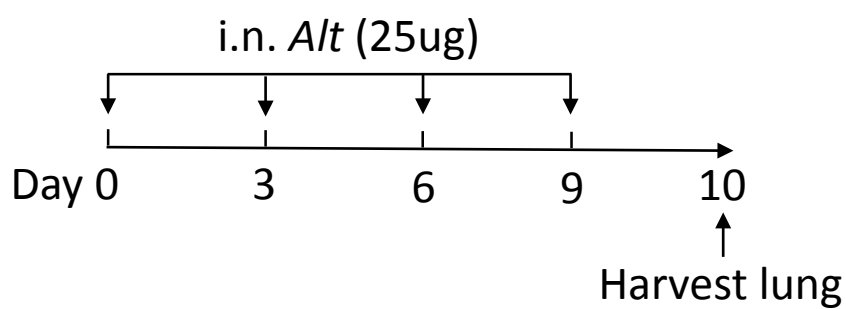


Figure E3

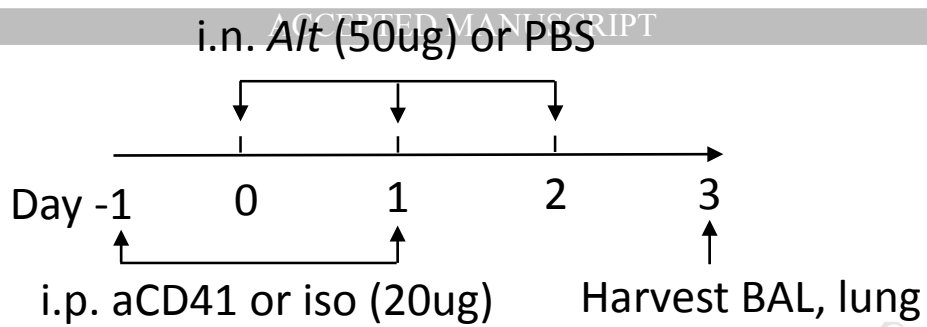


Figure E4

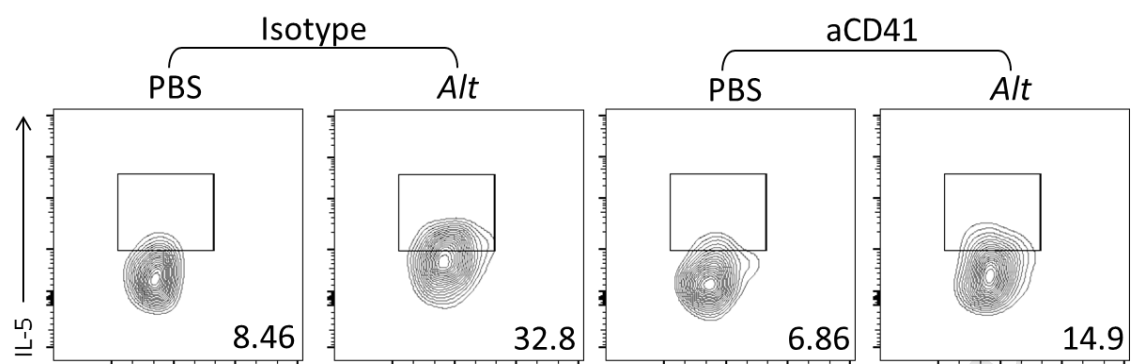


Figure E5

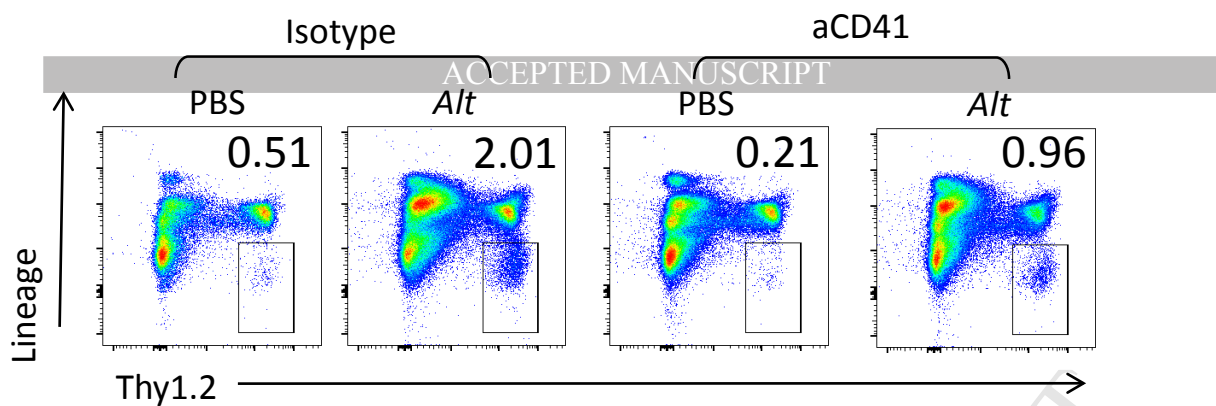


Figure E6

SUPPLEMENTARY ONLINE REPOSITORY**Platelets attach to lung ILC2 expressing PSGL-1 and influence ILC2 function**

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SUPPLEMENTARY METHODS

Mice

C57BL/6 mice ages 6-8 weeks (referred to as WT mice) were obtained from The Jackson Laboratory (Bar Harbor, ME) for studies of ILC2. All animal experiments and protocols were approved by the University of California, San Diego Institutional Animal Care and Use Committee and performed according to the guidelines.

Depletion of platelets in mice in vivo

Mice were depleted of platelets^{E1-E7} by intraperitoneal injection (i.p.) of 1 mg/kg CD41 blocking antibody (BioLegend, San Diego, CA)^{E8}. In other experiments mice were injected intraperitoneally with 100 µg of blocking antibodies against CD162, CD62P or their isotype controls (BD Biosciences, San Jose, CA).

Platelet counting

Retroorbital bleeding was performed on mice under isoflurane (Vedco, St. Joseph, MO) anesthesia. Blood was collected in 0.05M EDTA. EDTA-anticoagulated blood was diluted with 1% Ammonium oxalate (Sigma-Aldrich, St. Louis, MO) which lyses all erythrocytes, and incubated on a rotator for 15 minutes. Cells were then placed in hemocytometer and allowed to settle in moist environment prior to counting the number of platelets under a light microscope.

Lung processing to obtain ILC2

Lungs were initially perfused with PBS (to remove any intravascular blood) prior to processing into single cell suspensions using a lung dissociation kit and a tissue dissociator (Miltenyi Biotec, Auburn, CA).

Flow cytometry

Flow cytometry was used to detect lung ILC2, platelet markers and CD4 cells. In addition, flow cytometry was used to detect proliferating and apoptotic ILC2, as well as IL-5⁺ and IL-13⁺ ILC2.

Lung single cell suspensions were pre-incubated with anti-mouse CD16/CD32 for 10-20 minutes on ice. To identify lung ILC2s, lung cells were stained with CD45.2, lineage (Lin) markers [CD3, Gr-1, CD11b, CD45R, Ter-119, CD5 (Biolegend or BD Bioscience), CD11c, FcεR1, NK 1.1, TCRβ, and TCRγδ], Thy1.2 (eBioscience), CD127, and ST2 as previously described^{E9}. Mouse lung ILC2s were identified as CD45.2⁺Lin⁻Thy1.2⁺CD127⁺ST2⁺ lymphoid cells (**Fig E1**).

To identify lung CD4⁺ T cells, lung cells were stained with CD45.2, CD3 (eBioscience), CD4, and Thy1.2. CD4⁺ T cells were identified as CD45.2⁺CD3⁺CD4⁺Thy1.2⁺.

To assess the expression of platelet markers (CD41 and CD62P) as well as the adhesion receptor PSGL-1 (CD162), lung cells were stained with antibodies against CD41(eBioscience), CD62P (eBioscience), and CD162 (BD Biosciences).

To assess cellular proliferation, cells were permeabilized following surface staining using a Foxp3 Staining Buffer Set and stained with anti-mouse Ki-67 (eBioscience) (**Fig E2**) as previously described^{E9}. Apoptotic ILC2s were identified by staining for active Caspase-3 (BD Biosciences) according to manufacturer's protocol.

To determine ILC2 cytokine production^{E10,E11}, lung cells (10⁶/well) were incubated at 37°C for 3 hours with a cell stimulation cocktail consisting of PMA, ionomycin, brefeldin A, and monensin (ThermoFisher) in RPMI 1640 media supplemented with penicillin/streptomycin, 10% FBS, glutamine, 2-Mercaptoethanol (ThermoFisher). Stimulated lung cells were surfaced stained to detect ILC2 as described above, fixed and permeabilized with an intracellular IL-5 or IL-13 cytokine staining kit (BD Biosciences), and stained with PE-conjugated anti-IL-5 or PE-conjugated anti-IL-13 according to the manufacturer's protocol.

In selected experiments to compare platelet size to ILC2 size, peripheral blood platelets were detected by FACS by staining lysed blood with PE-conjugated anti-CD41 (eBioscience) using a no-wash protocol.

Flow cytometry antibodies and the appropriate isotype controls were purchased from BioLegend unless otherwise stated. All sample data was collected using the Novocyte Flow Cytometer (Acea, San Diego, CA) and data was analyzed using FlowJo software version 10.4.1 (Ashland, OR).

FACS sorted lung ILC2

We used FACS to sort a purified population of mouse lung ILC2 (100% Lin⁻ Thy1.2⁺)(**Fig 1F**) from naïve WT mice for electron microscopy to determine whether platelets were attached to ILC2. We used a FACS Aria II (Becton Dickinson) or a FACS Aria Fusion (Becton Dickinson) for ILC2 sorting at the UCSD Human Embryonic Stem Cell Core Facility as previously described^{E11}. Lung single cell suspensions were stained above as described above with Zombie Aqua (BioLegend) to discriminate live and dead cells.

Electron Microscopy

Electron microscopy studies of lung ILC2 were performed by the UCSD Electron microscopy Core as previously described^{E12}. FACS sorted lung ILC2 were immersed in modified Karnovsky's fixative (2.5% glutaraldehyde and 2% paraformaldehyde in 0.15 M sodium cacodylate buffer, pH 7.4) for at least 4 hours, postfixed in 1% osmium tetroxide in 0.15 M cacodylate buffer for 1 hour and stained en bloc in 2% uranyl acetate for 1 hour. ILC2 samples were dehydrated in ethanol, embedded in Durcupan epoxy resin (Sigma-Aldrich), sectioned at 50 to 60 nm on a Leica UCT ultramicrotome, and picked up on Formvar and carbon-coated copper grids. Sections were stained with 2% uranyl acetate for 5 minutes and Sato's lead stain for 1 minute. Grids were viewed using a JEOL 1200EX II (JEOL, Peabody, MA) transmission electron microscope and photographed using a Gatan digital camera (Gatan, Pleasanton, CA). The identification of ILC2 (small lymphocyte with single nucleus) and platelets (lack of nucleus, platelet granules) was determined by the UCSD Electron microscopy Core pathologist.

qRT-PCR

To expand the low number of ILC2 present in naïve mouse lung for qRT-PCR studies, mice were challenged with *Alternaria* four times over 10 days (**Fig E3**), and then lung cells sorted by FACS into a pure population of ILC2 as described above. We assessed the levels of expression of CD41, CD62P, and CD162 mRNA in purified lung ILC2 or in peripheral blood by qRT-PCR as previously described in this laboratory^{E10,E11}. In brief, total RNA was extracted with RNA-STAT-60 (Tel-Test) and reverse transcribed with Oligo-dT and SuperScript II kit (Life Technologies). qRT-PCR was performed with TaqMan PCR Master Mix and CD41, CD62P, and CD162 primers (all from Life Technology). The relative amounts of transcripts were normalized

to those of housekeeping gene (GAPDH) mRNA and compared between the different genes by the $\Delta\Delta$ cycle threshold method as previously described in this laboratory^{E10,E11}.

***Alternaria* 3 day challenge of WT mice depleted of platelets with anti-CD41 Ab**

WT mice were intranasally challenged with PBS or 50 μ g of *Alternaria alternata* extract (Greer) daily for 3 days (**Figure E4**) and euthanized 24 hours after the last challenge to quantitate lung ILC2 numbers, lung ILC2 expression of IL-5 and IL-13, as well as lung ILC2 proliferation (Ki67) (**Figure E5, E6**). Mice were administered 1mg/kg of a platelet depleting CD41 antibody (BioLegend) 24 hours prior to the first *Alternaria* intranasal challenge and 24 hours after the first *Alternaria* intranasal challenge.

Starting 24 hours after the first *Alternaria* intranasal challenge, blood was drawn daily via the retro-orbital plexus to quantitate levels of peripheral blood platelets. Red blood cells were subsequently lysed with Red Blood Cell Lysing Buffer (MilliporeSigma).

Statistical analysis

Change in the geometric mean of fluorescent intensity compared to the isotype (Δ gMFI) values were set to a minimum of 1. All comparisons were analyzed using Student's *t* test, unless otherwise stated. Results are reported as the mean and standard error of the mean (SEM). Statistical analysis was performed using Prism software (Graphpad, La Jolla, CA) and a two-sided *P* value of 0.05 was considered statistically significant.

SUPPLEMENTARY DISCUSSION

In addition to their tissue residency, ILC2 can also be recruited from the bone marrow through the circulation to the lung, as well as from the gastro-intestinal tract to the lung^{E13}. Thus, the number of ILC2 in the lung may be regulated both by their local proliferation in the lung, as well as through recruitment from the bone marrow or gastro-intestinal tract. Mediators positively regulating the number of ILC2 include cytokines (IL-7, IL-33, IL-25, TSLP) and lipid mediators (CysLTs, PGD2)^{E13}. Studies have demonstrated that ILCs (including ILC2s) are generated from common lymphoid progenitors, which are then committed to the ILC lineage as early innate lymphoid progenitors, common helper innate lymphoid cell progenitors, innate lymphoid cell progenitors, and ILC2 progenitor populations^{E14}. Stimuli including cytokines (IL-33, TSLP, IL-25) and lipid mediators (PGD2, LTB4, LTC4, LTD4, LTE4) can activate ILC2 to express Th2 cytokines^{E13}.

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SUPPLEMENTARY FIGURE LEGENDS:

Supplementary Figure E1. Lung ILC2 gating strategy

Representative ILC2 gating strategy from mouse lung. *FSC*, Forward scatter; *SSC*, side scatter.

Supplementary Figure E2. Lung ILC2 Ki-67

Representative plots of mouse lung ILC2 Ki-67 expression following in vivo administration of either an isotype control antibody (isotype), or a platelet depleting anti-CD41 antibody for 1 day (D1) or 2 days (D2).

Supplementary Figure E3. Protocol for 10 day *Alternaria* challenge.

Protocol for challenging WT mice with *Alternaria* for 10 days.

Supplementary Figure E4. Protocol for 3 day *Alternaria* challenge.

Protocol for challenging WT mice with *Alternaria* for 3 days.

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241 **Supplementary Figure E5. Lung IL-5+ ILC2 levels following 3 day *Alternaria* challenge.**

242 Representative plots of mouse lung IL-5+ ILC2 levels following in vivo administration of either
243 an isotype control antibody (isotype), or a platelet depleting anti-CD41 antibody prior to
244 *Alternaria* challenge.

245

246 **Supplementary Figure E6. Lung ILC2 levels following 3 day *Alternaria* challenge.**

247 Representative plots of mouse lung ILC2 (CD45.2⁺, Lin⁻, Thy 1.2⁺, CD127⁺, ST2⁺ lymphoid
248 cells) levels following in vivo administration of either an isotype control antibody (isotype), or a
249 platelet depleting anti-CD41 antibody prior to *Alternaria* or PBS challenge.

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