### Title

Platelets attach to lung type 2 innate lymphoid cells (ILC2s) expressing P-selectin glycoprotein ligand 1 and influence ILC2 function




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### Authors

Karta, Maya R
Cavagnero, Kellen
Miller, Marina
et al.



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

**Maya R. Karta, PhD**[a,b], **Kellen Cavagnero, BS**[a,b], **Marina Miller, MD PhD**[a], **Jana Badrani, MS**[a], **Luay Naji, BS**[a], **Taylor A. Doherty, MD**[a], **David H. Broide, MB ChB**[a]

[a]Department of Medicine, University of California San Diego, La Jolla, California

## 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)

To the Editor:

ILC2 are an important source of Th2 cytokines relevant to allergic inflammation, nasal polyps, asthma and atopic dermatitis[1]. 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 lung[5]. 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 β1 integrins, β2 integrins, and L-selectin[3], and that inhibiting β2 integrins reduced ILC2 trafficking from the bone marrow via the blood stream to the lung following *Alternaria* challenge in wild type (WT) mice[3]. 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)

Corresponding author: David Broide, MB ChB., University of California San Diego, 9500 Gilman Drive, MC 0635, La Jolla, CA 92093-0635, Fax: (858) 534-2110 Tel: (858) 534-2374, dbroide@ucsd.edu.

[b]These authors contributed equally to the research and are both listed as first authors.

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 αIIb, 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-33[8], 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 production[9], 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.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

Conflict of Interest: This study was supported by NIH grants AI 070535, AI 107779, AI 242236, and AI 038425 (DHB). T.A.D is supported by NIH AI 114585 and AI 070535. M.R.K was supported by T32 AI 007469. The authors have no other conflicts of interest to disclose related to this research.

## Abbreviations:

| | |
|---|---|
| **ILC2** | Innate lymphoid cell type 2 |
| **PSGL-1** | P-selectin glycoprotein ligand-1 |

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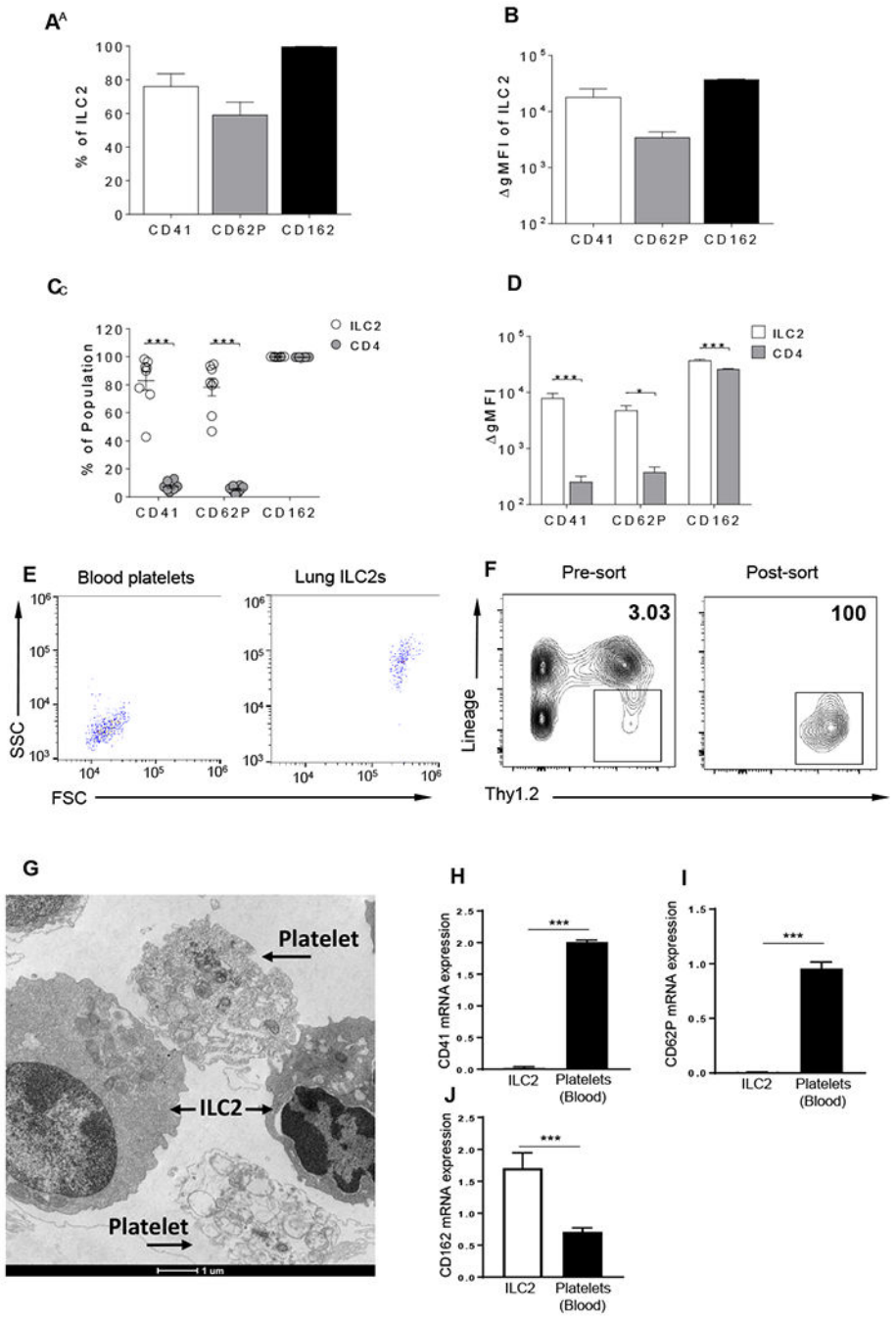


**Figure 1. Lung ILC2s attach to platelets and express leukocyte adhesion molecule PSGL-1**

**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 ΔgMFI (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) ΔgMFI (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 ± SEM. * $p < 0.05$, ** $p < 0.01$, and ** * $p < 0.0001$.

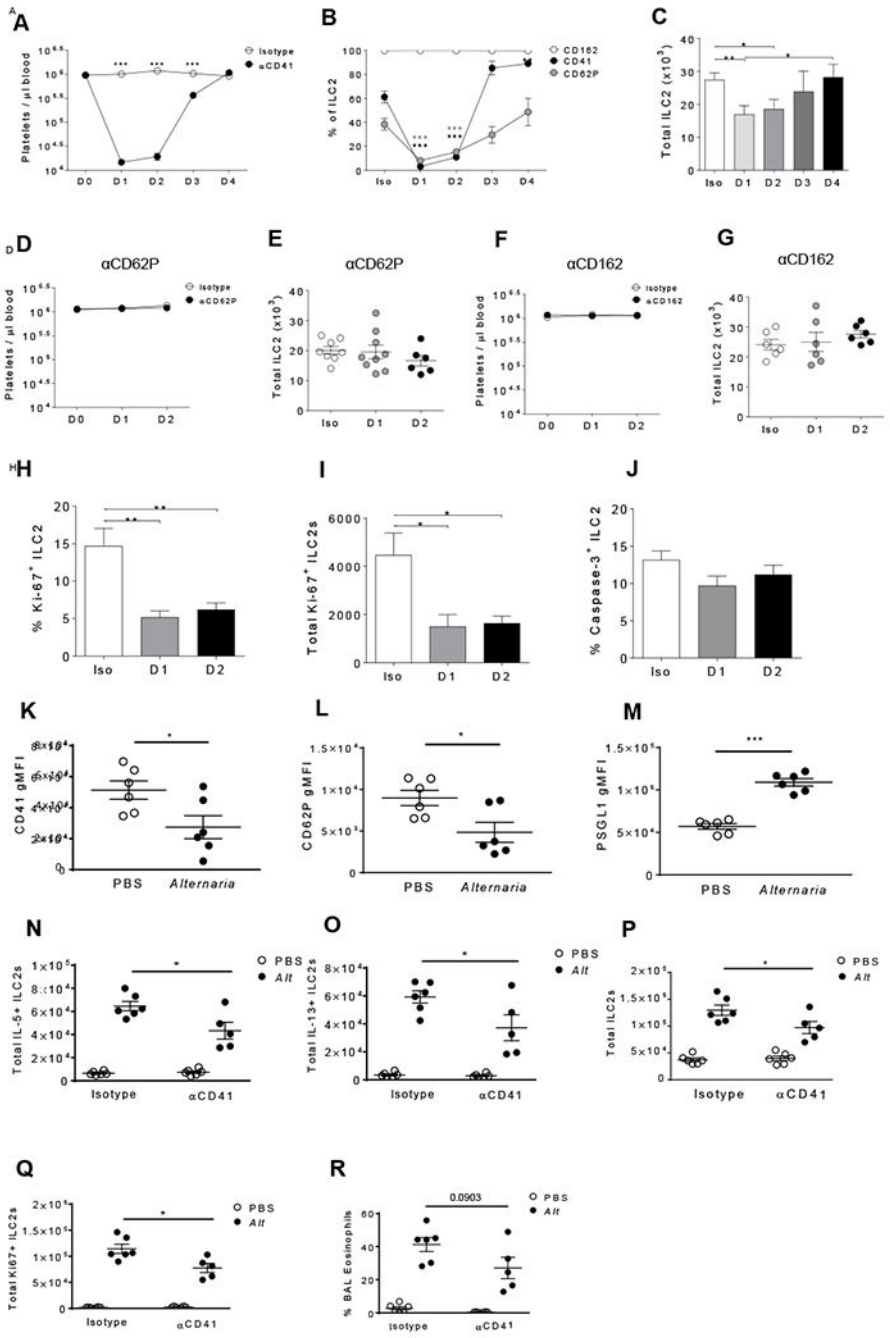


**Figure 2. Platelet depletion in vivo reduces lung ILC2 proliferation, numbers of lung ILC2, and ILC2 Th2 cytokine expression.**

**(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 ± SEM. * $p < 0.05$, ** $p < 0.01$, ** * $p < 0.0001$.