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β_2 integrins rather than β_1 integrins mediate *Alternaria*-induced ILC2 trafficking to the lung

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Abstract

Background—Group 2 innate lymphoid cells (ILC2s) expand in the lungs of mice during type 2 inflammation induced by the fungal allergen *Alternaria alternata*. The increase in ILC2 numbers in the lung has been largely attributed to local proliferation and whether ILC2s migrate from the circulation to the lung following *Alternaria* exposure is unknown.

Objective—We examined whether human (lung, lymph node, blood) and mouse lung ILC2s express β_1 and β_2 integrin adhesion molecules, and whether these integrins are required for trafficking of ILC2 into the lungs of mice

Methods—Human and mouse ILC2s were assessed for surface expression of β_1 and β_2 integrins adhesion molecules by flow cytometry. The role of β_1 and β_2 integrins in ILC2 trafficking to the lungs was assessed by *in vivo* blocking of these integrins prior to airway exposure to *Alternaria* in mice.

Results—Both human and mouse lung ILC2s express high levels of β_1 and β_2 integrin adhesion receptors. Intranasal administration of *Alternaria* challenge reduced ILC2s in the bone marrow and concurrently increased blood and lung ILC2 levels. *In vivo* blocking of β_2 integrins (CD18) significantly reduced ILC2 levels in the lungs, but did not alter ILC2 proliferation, apoptosis, and function. In contrast, *in vivo* blocking of β_1 integrins or α_4 integrins did not affect lung ILC2 levels.

Conclusion—ILC2s increase in number in the mouse lung not only through local proliferation, but also through trafficking from the circulation into the lung using β_2 rather than β_1 or α_4 integrins.

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Keywords

ILC2; Integrin; Adhesion; *Alternaria alternata*

INTRODUCTION

As ILC2s express high levels of Th2 cytokines (IL-4, IL-5, and IL-13), studies have focused on understanding their role in the pathogenesis of human diseases including asthma, allergic rhinitis, and atopic dermatitis.[1–5] While ILC2s and Th2 lymphocytes are both prominent sources of Th2 cytokines, ILC2s do not express antigen receptors and are instead activated by cytokines (*i.e.* IL-33, TSLP, IL-25) and lipid mediators (*i.e.* cysteinyl leukotrienes and prostaglandin D_2).[6, 7] Prostaglandin D_2 induces ILC2 Th2 cytokine production and is also a chemotactic factor for ILC2s through the binding to CRTH2, which is highly expressed by ILC2s.[8] Mediators that activate ILC2s are known to be elevated in different Th2 disease states, including asthma and allergic rhinitis. The number of ILC2s is increased in the peripheral blood, BAL, and sputum of allergic and/or asthmatic individuals, and several studies demonstrate increased ILC2s in the lung of mice during Th2 inflammation.[9–13]

While it is well accepted that T lymphocytes utilize adhesion molecules to traffic from the circulation to the lung following inhalation of allergen[14], studies have not yet examined whether ILC2s migrate from the circulation to the lung following allergen challenge, and if so which adhesion receptors expressed by ILC2s may mediate this function. Several reports have demonstrated that local proliferation of ILC2s occurs in the tissues, including the lung.[15–17] However, ILC2s are bone marrow-derived cells and mechanisms that regulate trafficking from blood to tissues could be important during inflammatory responses.[18] Mice deficient in bone marrow CRTH2-expressing ILC2s had diminished ILC2 levels in the lungs following *N. brasiliensis* infection[19], suggesting that a trafficking defect may be involved, but whether ILC2-expressed adhesion receptors may account for this reduction in ILC2 levels in the lung was not examined. We hypothesized that bone marrow derived ILC2s migrating through the blood express adhesion molecules that firmly bind to counter-receptors expressed by lung endothelium during allergic inflammation. This firm adhesion of ILC2s to pulmonary endothelium then allows for their subsequent migration into the tissues through chemotactic mediators, such as prostaglandin D_2.

The most common method by which leukocytes (other than ILC2s) are known to migrate from the blood into tissues and specifically the airways is mediated by leukocyte-expressed selectins and integrins.[20] Leukocyte-expressed selectins, such as L-selectin (CD62L), mediate the first step of leukocyte tethering to endothelium *in vivo*, whereas leukocyte-expressed β_1 and β_2 integrins mediate leukocyte firm adhesion to endothelium. Integrins are heterodimers comprised of α and β subunits. β_1 (CD29) integrins, such as VLA-4 (α_4/β_1 or CD49d/CD29), are highly expressed on eosinophils, T lymphocytes, and basophils, but not neutrophils.[21, 22] VLA-4 expressed by leukocytes binds to vascular cell adhesion molecule (VCAM-1 or CD106) expressed by endothelium. In addition to β_1 integrins, β_2 and β_7 integrins expressed by leukocytes also mediate firm adhesion. For example, lymphocytes utilize β_2 (CD18) integrins, such as LFA-1 (α_L/β_2 or CD11a/CD18), to firmly adhere to

intercellular adhesion molecule 1 (ICAM-1 or CD54) expressed by endothelium.[23] Although lymphocytes also utilize β_7 integrins to firmly adhere to endothelium, this interaction of lymphocyte expressed β_7 integrins (*i.e.* α_4/β_7) with endothelium is more prominent in migration of lymphocytes to the gut[24], as the counter-receptor for α_4/β_7 (*i.e.* MAdCAM-1) is most highly expressed in the gastro-intestinal tract rather than the lung, which is the focus of our ILC2 studies.

Overall, studies of β integrins and leukocyte trafficking to sites of allergic inflammation have demonstrated an important role for both β_1 and β_2 integrins in eosinophil adhesion and trafficking to tissues.[25] In contrast, for neutrophils β_2 integrins, rather than β_1 integrins, predominantly mediate neutrophil trafficking to tissue sites of inflammation.[26] We therefore profiled expression of integrin adhesion molecules on human and mouse ILC2s to determine which adhesion receptors were highly expressed by ILC2s, and used neutralizing antibodies to demonstrate that ILC2s traffic from the circulation using β_2, but not β_1, integrins during *Alternaria* induced lung inflammation.

METHODS

Human ILC2

Human ILC2s were identified by flow cytometry as $CD45^+Lin^-CRTH2^+$ lymphoid cells[8, 27] in single cell suspensions of either post mortem human lungs and lymph nodes, or human peripheral blood, obtained as described in the online supplement.

Mouse ILC2: Airway administration of *Alternaria* and *in vivo* blocking of integrins

Mice were challenged intranasally with *Alternaria Alternata* three times (days 0, 3, and 6) and were sacrificed one day after the final challenge (day 7) as previously described[10] and detailed in the online supplement. In experiments involving *in vivo* blocking of integrins, mice were administered intraperitoneally an integrin specific blocking or control antibody (β_1, β_2, α_4, or α_L antibody) (see Supplement Table EII) prior to each *Alternaria* challenge (days 0, 3, and 6) as detailed in the online supplement.

ILC2 proliferation, apoptosis, and Th2 cytokines

ILC2 proliferation (Ki-67 staining), apoptosis (annexin V staining) and Th2 cytokines (IL-5, IL-13 staining) were quantitated by flow cytometry as described in the online supplement.

Statistical analysis

Statistical analysis was performed using Prism software (Graphpad, La Jolla, CA) as described in the online supplement. $P < 0.05$ was considered statistically significant.

RESULTS

Human ILC2s express β_1 and β_2 integrins

To determine whether human ILC2s express adhesion molecules commonly associated with migration of other leukocytes, human ILC2s derived from peripheral blood, lung tissue, and intrathoracic lymph nodes were analyzed for expression of adhesion molecules by flow

cytometry. Human ILC2s were gated as $CD45^+Lin^-CRTH2^+$ lymphoid cells (Figure 1A) and were detected at a mean frequency of 0.15% of $CD45^+$ cells in the blood, 0.04% in the lungs and 0.06% in lung lymph nodes (Figure 1B). Human ILC2s highly express both the integrin chains for β_2 integrins (CD18 and CD11a) and moderately express both the integrin chains for β_1 integrins (CD29 and CD49d) (Figures 1C–E and Supplement Figures E1–E2). Compared with human CD4 cells, human ILC2s express similar levels of β1 and β2 integrin adhesion molecules (Figure 1F–H).

Mouse ILC2s express β_1 and β_2 integrins

To study the functional importance of ILC2 expression of adhesion molecules to recruitment of ILC2 to the lung, we used a well validated model of *Alternaria* allergen challenge in non-sensitized wildtype mice which induces a rapid innate mediated increase in the number of ILC2s.[13] To validate that mouse ILC2s expressed a similar profile of adhesion molecules as human ILC2s, the expression of the same adhesion molecules were assessed by flow cytometry on ILC2s from mouse lungs. Mouse ILC2s were identified as $Lin^-Thy1.2^+$ from the $CD45^+$ lymphoid cell population (Figure 2A). We have previously used this gating strategy to show that the $Lin^-Thy1.2^+$ are ILC2s in naïve and *Alternaria*-challenged mice.[28] To determine relative levels of adhesion molecule expression, ILC2s and $CD4^+Thy1.2^+$ cells from the same naïve mice lungs were analyzed (Figure 2B–C and Supplement Figure E3).

ILC2s showed slightly higher levels of expression compared to CD4 cells of the β_1 integrin CD29 (mean 99.1% compared to 79.8%) and CD49d (mean 90.5% compared to 79.4%), as well as the β_2 integrin CD18 (mean 88.0% compared to 49.0%). In addition, ICAM-1 was more highly expressed on ILC2 compared to CD4 cells (mean 98.5% compared to 65.2%), while both populations showed equally high percentages of CD11a expression (mean > 99%; Figure 2B). Interestingly, the percent of lung CD4 cells expressing L-selectin (CD62L; mean = 80.1%) was markedly higher compared to lung ILC2 cells expressing L-selectin (mean = 4.9%; $p < 0.0001$) (Figure 2B).

These differences were further demonstrated by the comparison of ΔgMFI between the ILC2s and $CD4^+Thy1.2^+$ populations (Figure 2C). ILC2s showed higher ΔgMFI for the β_2 integrins CD18 (ΔΔgMFI = 744) and CD11a (ΔΔgMFI = 29,590), as well as the β_1 integrins CD29 (ΔΔgMFI = 5,658) and CD49d (ΔΔgMFI = 1,399), and ICAM-1 (ΔΔgMFI = 4,896). However, ILC2s had decreased ΔgMFI of CD62L compared to the $CD4^+Thy1.2^+$ population (ΔΔgMFI = 6,615; Figure 2C). As expected, ILC2 and $CD4^+Thy1.2^+$ populations had minimal expression or ΔgMFI for the endothelial adhesion molecule VCAM-1 (CD106). Thus, compared with human ILC2s, mouse lung ILC2s express high levels of both β_2 (CD18/CD11a) and β_1 (CD29/CD49d) integrins, which are at slightly higher levels compared to CD4 cells.

Effect of *Alternaria* challenge on levels of ILC2 adhesion molecule expression

Non-sensitized naïve mice challenged with the fungal allergen *Alternaria alternata* over three days develop innate type 2 inflammation and ILC2 activation.[27] Therefore, we assessed lung ILC2 adhesion molecule expression after activation *in vivo* by *Alternaria* challenge (Figure 2D–E). Similar to human lung ILC2s, the majority of naïve non-*Alternaria* challenged

mouse lung ILC2s expressed high levels of the β_2 integrins CD18 (mean = 97.7%) and CD11a (mean = 98.6%) as well as the β_1 integrins CD29 (mean = 98.0%) and CD49d (mean = 86.9%). Levels of ICAM-1 were highly expressed in mouse lung ILC2 (mean = 98.0%). In contrast, CD62L (mean = 2.5%) and VCAM-1 (mean = 8.2%) were expressed at low levels (Figure 2B). After *Alternaria* challenge, levels of lung ILC2 expression of the β_2 integrin CD18 and the β_1 integrin CD29, as well as CD62L, ICAM-1, and VCAM-1 did not change. However, there was a minimal but statistically significant increase in the percentage of ILC2s expressing the β_2 integrin CD11a and the β_1 integrin CD49d (Figure 2D). There was a statistically significant increase in the ΔgMFI of mouse lung ILC2 β_2 integrin subunits CD18 (ΔΔgMFI = 4,954) and CD11a (ΔΔgMFI = 19,114), as well as in the β_1 integrin subunit CD49d (ΔΔgMFI = 2,723) after *Alternaria* challenge (Figure 2E). In addition, after *Alternaria* challenge, levels of lung ILC2 ΔgMFI of CD29, CD62L, ICAM-1, and VCAM-1 did not change (Figure 2E). Thus, *Alternaria* challenge in mice led to modest increases in β_1 and β_2 integrin ΔgMFI adhesion molecule expression, but did not alter the overall expression profile.

Alternaria challenge reduced ILC2s in the bone marrow and increased ILC2s in the blood and lungs

To determine whether ILC2s circulate from the bone marrow to the lungs following *Alternaria* challenge, we assessed ILC2 levels in the bone marrow, blood, and lungs of mice challenged every three days with *Alternaria* allergen. Mice were sacrificed 24 hours after either the second challenge on day 4, or the third challenge on day 7 (Figure 3). Mouse bone marrow and blood ILC2s were characterized as $Lin^{-}Thy1.2^{+}CD127^{+}ST2^{+}$ from the $CD45^{+}$ lymphoid cell population (Supplement Figure E4). Intranasal administration of *Alternaria* significantly diminished bone marrow ILC2 levels on day 7 compared to both naïve and day 4 mice (Figure 3A). There were little to no detectable blood ILC2s in the naïve and day 4 mice. However, *Alternaria* challenge significantly increased blood ILC2 levels on day 7 compared to naïve and day 4 mice (Figure 3B). In addition, ILC2 levels in the lung lymph nodes and spleen were found to be increased on day 7 (Supplemental Figures E4 C–D and E5 A–B). As expected, mice sacrificed on both day 4 and day 7 had increased levels of lung ILC2s compared to naïve mice (Figure 3C). Summary of the changes in overall $CD45^{+}$ cell levels are depicted in Supplement Figure E5 C–G. Together, these studies demonstrate that intranasal administration of *Alternaria* reduced ILC2s in the bone marrow and concurrently increased blood and lung ILC2 levels on day 7.

ILC2s in the lungs following *Alternaria* challenge originate in bone marrow in mice

In order to determine whether ILC2s migrate from the bone marrow to the airways, we generated bone marrow transplanted (BMT) mice in which either CD45.1 or CD45.2 donor bone marrow was transplanted into irradiated recipient CD45.2 mice, and challenged them with *Alternaria* (as described in the Methods in the Online Supplement). In both the CD45.1 and the CD45.2 BMT mice, over 95% of all the hematopoietic cells in the blood were of donor origin (Figure 3D–E). *Alternaria* challenge significantly increased CD45.1 ILC2 levels in the blood and lungs of the CD45.1 donor BMT mice, but did not increase CD45.2 ILC2 levels (Figure 3F–G and Supplemental figure E6 A–C). In addition, there was a significant increase in the total number of proliferating (Ki-67 expressing) CD45.1 ILC2s in

the *Alternaria* challenged CD45.1 BMT mice compared to the naïve CD45.1 BMT mice (Figure 3H). There was little Ki-67 expressing CD45.2 ILC2s in both the naïve and *Alternaria* challenged CD45.1 BMT lungs (Supplemental Figure E6 D). These results suggest that ILC2s migrate from the bone marrow to the lungs.

In vivo blocking of β_2 integrins reduced ILC2 recruitment to the lung

Due to the high levels of β_2 integrins we noted to be expressed on both human and mouse ILC2s we examined whether administering neutralizing antibodies to β_2 integrins would inhibit the accumulation of ILC2s in the lungs of *Alternaria* challenged mice (Figure 4A). *Alternaria* challenged mice treated with control antibody had significantly higher total numbers of ILC2s in the lungs compared to naïve mice (Figure 4B). In contrast, *Alternaria*-challenged mice pretreated with an αCD18 β_2 integrin blocking antibody had significantly reduced absolute ILC2s per lung compared to *Alternaria* challenged mice (61.3% reduction of *Alternaria*-induced ILC2 increase in lung) (Figure 4B).

In vivo blocking of β_2 integrins does not inhibit ILC2 proliferation, or induce ILC2 apoptosis

To confirm that the effect of the αCD18 antibody on the levels of lung ILC2 following *Alternaria* challenge was due to inhibition of ILC2 migration rather than inhibition of ILC2 proliferation or induction of ILC2 apoptosis, ILC2 levels of Ki-67 and Annexin V were determined (Figure 4C). *Alternaria* challenge induced increased lung ILC2 proliferation, as previously described.[29] Administration of the αCD18 antibody to *Alternaria* challenged mice did not inhibit lung ILC2 proliferation (Ki-67$^+$ ILC2s) or ILC2 apoptosis (Annexin V$^+$ ILC2s) (Figure 4C). Thus, β_2 integrins are required for the accumulation of lung ILC2s from the blood without affecting proliferation or apoptosis.

Effect of *in vivo* blocking of β_2 integrins on ILC2 Th2 cytokine expression

To determine whether pretreatment with the αCD18 antibody had an effect on the activation of ILC2s, we assessed IL-5 and IL-13 production. *Alternaria* allergen challenge induced increased numbers of IL-5$^+$ ILC2, IL-13$^+$ ILC2, as well as ILC2s that were double positive for IL-5 and IL-13 (Figure 4D). The ILC2s in the lung following *Alternaria* allergen challenge include ILC2 expressing Th2 cytokines IL-5 and IL-13 (approximately 23% and 17% of total lung ILC2 respectively), as well as a population of ILC2 not expressing Th2 cytokines. Administration of the αCD18 antibody did not reduce cytokine-producing ILC2 numbers (Figure 4D), despite reducing the total number of ILC2 (Figure 4B) suggesting a reduction in the number of ILC2s that do not express Th2 cytokines after administration of the αCD18 antibody (Supplement Figure E7).

In vitro adhesion of ILC2s to ICAM-1 is dependent on β_2 integrins

In order to determine direct interaction between ILC2s and ICAM-1, sorted mouse lung ILC2s were plated on ICAM-1 coated plates and allowed to adhere for 90 minutes (as described in the Methods in the Online Supplement). There was a significant increase in the number of adherent ILC2s in the ICAM-1 coated wells compared to control coated wells (Figure 4E). To determine the role of β_2 in the adherence of ILC2s to ICAM-1, sorted ILC2s

were preincubated in the presence or absence of the αCD18 antibody prior to plating on ICAM-1 coated plates. There was a significant reduction in the number of ICAM-1 adherent ILC2s in wells containing ILC2s preincubated with αCD18 compared to control ILC2s (Figure 4E). This demonstrates the direct interaction between ILC2s and ICAM-1 *in vitro*, which is dependent on ILC2 expression of CD18.

In vivo blocking of the β_2 binding integrin, α_L, inhibits ILC2 recruitment to the airways

Both human and mouse ILC2s had high expression of α_L, which is one of the α integrins that interacts with β_2. Therefore, we tested whether blocking the integrin $\alpha_L\beta_2$ (LFA-1), would also inhibit ILC2 recruitment to the airways. *Alternaria* challenged mice pretreated with a blocking anti-α_L integrin (αCD11a) antibody[30] had a significant reduction in lung ILC2 levels compared to the lung ILC2 levels of *Alternaria* challenged mice that were pretreated with the isotype control (Figure 4F).

In vivo blocking of either β_1 or α_4 integrins does not inhibit ILC2 recruitment to the airways

As ILC2s also express high levels of β_1 integrins, we investigated whether blocking this pathway would inhibit ILC2 numbers in the lung following *Alternaria* challenge. *Alternaria* challenged mice pretreated with a blocking anti-β_1 integrin (αCD29) antibody[31] had no reduction in total lung ILC2 (Figure 5A). Administration of the αCD29 antibody to *Alternaria* challenged mice did not inhibit lung ILC2 proliferation (Figure 5B). Similarly, administration of the αCD29 antibody to *Alternaria* challenged mice did not induce lung ILC2 apoptosis (Figure 5C).

As the α_4 integrin (CD49d), may associate with either a β_1 integrin ($\alpha_4\beta_1$) or a β_7 integrin ($\alpha_4\beta_7$), we also examined whether they expressed β_7 integrins. Human ILC2s from PBMCs, lymph nodes, and lungs were assessed for the surface expression of integrin β_7 alone and for dual expression of CD49d and β_7 (Figure 5D). Human ILC2s, regardless of tissue source, showed low percentages of ILC2s expressing β_7 and even lower levels of ILC2s expressing both CD49d and β_7 (Figure 5D). In contrast to human ILC2s, mouse ILC2s expressed high levels of β_7 integrin in both naïve mice (mean = 67.8%) and *Alternaria* challenged mice (mean = 74.0%; Figure 5E). Due to the high levels of $\alpha_4\beta_1$ and $\alpha_4\beta_7$ expression on mouse lung ILC2s, the role of blocking α_4 integrins in *Alternaria*-induced ILC2 recruitment was assessed.

Alternaria challenged mice pretreated with a blocking anti-α_4 integrin (αCD49d) antibody[32] also had no reduction in total lung ILC2 (Figure 5F). Administration of the αCD49d antibody to *Alternaria* challenged mice did not inhibit lung ILC2 proliferation (Figure 5G). Similarly, administration of the αCD49d antibody to *Alternaria* challenged mice did not induce lung ILC2 apoptosis (Figure 5H). These studies suggest that neither $\alpha_4\beta_1$ nor $\alpha_4\beta_7$ integrins play a significant role in ILC2 recruitment to the lung.

DISCUSSION

ILC2s expand in the lungs of mice during type 2 inflammation induced by *Alternaria* challenge.[13, 27] The increase in ILC2 numbers in the lung has previously been attributed to local proliferation alone[17] as studies have not demonstrated that ILC2 migrate from the

circulation to the lung following allergen challenge. We report that both human and mouse ILC2s express high levels of β_1 and β_2 integrin adhesion receptors, known to be used by other circulating leukocytes to adhere to endothelium and traffic to the lung. In addition, we show that after the 7 day *Alternaria* challenge ILC2 levels in the bone marrow decreased, while they increased in the circulation and lungs of mice, suggesting that ILC2s are released from the bone marrow to traffic to the lung following *Alternaria* challenge. In addition, BMT studies demonstrated that ILC2s derived from the bone marrow traffic to the lung following *Alternaria* challenge. *In vivo* blocking of β_2 integrins (CD18) prior to *Alternaria* allergen challenge in mice significantly diminished ILC2 levels in the lungs. The effect of the αCD18 blocking antibody was specific to trafficking, as αCD18 treatment did not affect *Alternaria*-induced ILC2 proliferation, apoptosis, or Th2 cytokine expression. Moreover, *in vitro* we demonstrated that ILC2s adhere to ICAM-1 and that the adherence was inhibited by blocking antibodies to the β_2 integrin. This was further confirmed by *in vivo* blocking of the α_L integrin, a subunit of integrin $\alpha_L\beta_2$ (LFA-1), which showed a reduction in lung ILC2 levels when αCD11a was administered. Importantly, we also demonstrate that *in vivo* blocking of β_1 or α_4 integrins did not affect *Alternaria*-induced ILC2 levels in the airways. Therefore, we demonstrate an additional novel mechanism by which *Alternaria* allergen challenge increases airway ILC2 levels by recruiting ILC2s from the bone marrow to the circulation through integrin mediated trafficking that is dependent on β_2 integrins, but not β_1 integrins.

We have previously demonstrated that human subjects with allergic rhinitis due to cat allergen exposure, when challenged with cat allergen in the laboratory develop a significant increase in numbers of peripheral blood ILC2s, which is not noted on a separate diluent nasal challenge visit.[9] In addition, ILC2s levels in grass pollen allergic individuals increase in the blood during the grass pollen season.[33] These studies demonstrate that exposure to inhaled allergens in allergic subjects can significantly increase peripheral blood ILC2 levels. At present it is not known in humans whether the increase in peripheral blood ILC2s following exposure to inhaled allergens is due to a signal to the bone marrow to release ILC2 from the bone marrow which then traffic to tissue sites such as the upper or lower airway, where they can express Th2 cytokines and contribute to allergic inflammation. In contrast, studies in mice have provided evidence that ILC2s have tissue residency in the airways and do not recirculate.[16, 17] However, these studies have not examined whether the expansion of ILC2 numbers in the lung following *Alternaria* challenge may be mediated by trafficking of ILC2 from the bone marrow to the lung. Our study provides evidence that ILC2 numbers increase in the mouse lung following *Alternaria* allergen challenge through mechanisms that include β_2 integrin, but not β_1 integrin, mediated trafficking from the circulation to the lung, as well as due to local proliferation of ILC2s in response to local inflammatory mediators. These results are in agreement with a prior study by our group, showing *Alternaria* challenge increases the percentage of Ki-67 proliferating ILC2s in the lungs.[15] When considered together, it is likely that one subset of ILC2s that are increased in the lung following *Alternaria* challenge are a result of migration from the blood, while a second subset of ILC2s are the pre-existing lung ILC2s that proliferate and increase ILC2 lung numbers independent of migration from the circulation.

As ILC2 express high levels of the β_2 integrin which is known to mediate firm adhesion of other leukocytes to ICAM-1 expressed by endothelium, the reduction in lung ILC2 we have noted in *Alternaria* challenged mice treated with an αCD18 antibody can best be explained by reduced ILC2 β_2 integrin adhesion to ICAM-1 expressed by endothelium in the lung. Our *in vitro* studies demonstrated that ILC2s adhere to ICAM-1 in a β_2 integrin dependent manner. As ILC2s and endothelium both express ICAM-1, administration of an anti-ICAM-1 antibody to *Alternaria* challenged mice would unfortunately not only block ICAM-1 function on endothelium, but also ICAM-1 function on ILC2 making interpretation of this *in vivo* experiment problematic. One of the weaknesses of this study is the lack of the blocking of CD18 specifically on ILC2s, which would demonstrate direct CD18 expression on ILC2 involvement in ILC2 migration. In addition, our study demonstrates that the αCD18 antibody does not inhibit the numbers of ILC2 in the subset of ILC2 expressing Th2 cytokines. This lack of inhibition of ILC2 Th2 cytokine expression would be anticipated as there is currently no evidence that inhibition of β_2 integrins inhibits Th2 cytokine responses.

Although we demonstrated that both human and mouse ILC2s expressed significant levels of β_1 integrins ($\alpha_4\beta_1$ or VLA-4), blocking β_1 integrin did not reduce the number of ILC2 in the lungs of *Alternaria* challenged mice. Thus, β_1 integrins, are not as central as β_2 integrins in mediating the increase in number of lung ILC2 following *Alternaria* challenge. In addition to β_1 and β_2 integrins, β_7 integrins (in particular $\alpha_4\beta_7$) can mediate adhesion to MAdCAM-1 expressed by endothelium. In this study we found that β_7 integrin is expressed at low levels on human ILC2s isolated from PBMCs, lung tissue, and lung lymph nodes. Prior studies have also not detected significant expression of β_7 integrin on mouse lung ILC2s and human peripheral blood ILC2s.[34, 35] Thus, β_7 integrins are unlikely to play a major role in human ILC2 adhesion. In contrast to human lung ILC2s, we did note high levels of expression of β_7 integrin on mouse lung ILC2s. Prior studies have reported the expression of CD49d/β_7 integrin on the surface of mouse ILC precursors in the bone marrow[18], but minimal expression on mouse lung ILC2s.[34, 35] Despite the varying levels of mouse ILC2 CD49d/β_7 integrin expression reported in different studies, we demonstrated that the inhibition of CD49d did not alter ILC2 levels in the airways and therefore CD49d/β_7 is unlikely to play a significant role in ILC2 migration to the lungs following *Alternaria* challenge.

β_1 and β_2 integrins, expressed by ILC2s, bind to known counter-receptors such as VCAM-1 and ICAM-1 expressed by vascular endothelium. *Alternaria* inhalation challenge is known to induce high levels of lung IL-33 within hours of challenge.[13] In addition, IL-33 has been shown to induce VCAM-1 and ICAM-1 expression by endothelial cells.[36] As this study demonstrated that blocking β_2 integrins (*i.e.* the ICAM-1 counter-receptor pathway) reduced numbers of ILC2s in the lung following *Alternaria* challenge, whereas blocking the β_1 integrin pathway (*i.e.* the VCAM-1 counter-receptor pathway) did not reduce lung ILC2 numbers, it suggests that either the VLA-4/VCAM pathway only mediates minor firm adhesion of ILC2s to endothelium pathway that can be bypassed by the β_2 integrin/ICAM-1 pathway, or that VLA-4 expressed on ILC2 is in a low affinity state[37] which does not permit firm adhesion to endothelium. We also demonstrated that mouse lung ILC2s did not express VCAM-1, but did express ICAM-1, which is in agreement with another study.[34]

In summary, this study makes the novel observation that the expansion of ILC2s in the lung following *Alternaria* challenge is not only mediated by the well described local lung proliferation of ILC2s[17], but is also mediated by recruitment of circulating ILC2s from the blood to the lungs. Interestingly, ILC2 expression of β_2 integrins plays an important role in the recruitment of ILC2s to the lung, as demonstrated in *in vivo* studies blocking β_2 integrins prior to *Alternaria* allergen challenge. In contrast, *in vivo* blocking of β_1 integrins, or α_4 integrins, did not affect ILC2 levels in the airways. *In vivo* blocking of β_2 integrins did not inhibit ILC2 proliferation, induce ILC2 apoptosis, or inhibit Th2 cytokine responses. Thus, ILC2 numbers increase in the lung following *Alternaria* allergen challenge through mechanisms that include β_2 integrin, but not β_1 integrin, mediated trafficking of ILC2s from the circulation to the lung, as well as due to local proliferation of ILC2s in response to local inflammatory mediators.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

BM	Bone marrow
BMT	Bone marrow transplanted
CRTH2	Chemoattractant receptor-homologous molecule expressed on Th2 cells
FSC	Forward scatter
gMFI	Geometric mean of fluorescent intensity
ICAM-1	Intercellular adhesion molecule-1
ILC2	Group 2 innate lymphoid cells
LFA-1	Lymphocyte function-associated antigen-1
Lin	Lineage
LN	Lymph nodes
MAdCAM-1	Mucosal vascular addressin cell adhesion molecule-1
PBMCs	Peripheral blood mononuclear cells
SSC	Side scatter
VCAM-1	Vascular cell adhesion molecule-1
VLA-4	Very late antigen-4

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KEY MESSAGES

- Both human and mouse lung ILC2s express high levels of β_1 and β_2 integrin adhesion receptors.
- ILC2s increase in number in the mouse lung not only through local proliferation, but also through trafficking from the circulation into the lung using β_2 rather than β_1 or α_4 integrins.

CAPSULE SUMMARY

ILC2s, which express high levels of Th2 cytokines, are not only resident in the lung, but also traffic from the bone marrow to the lung upon exposure to *Alternaria*, an allergen associated with severe asthma.

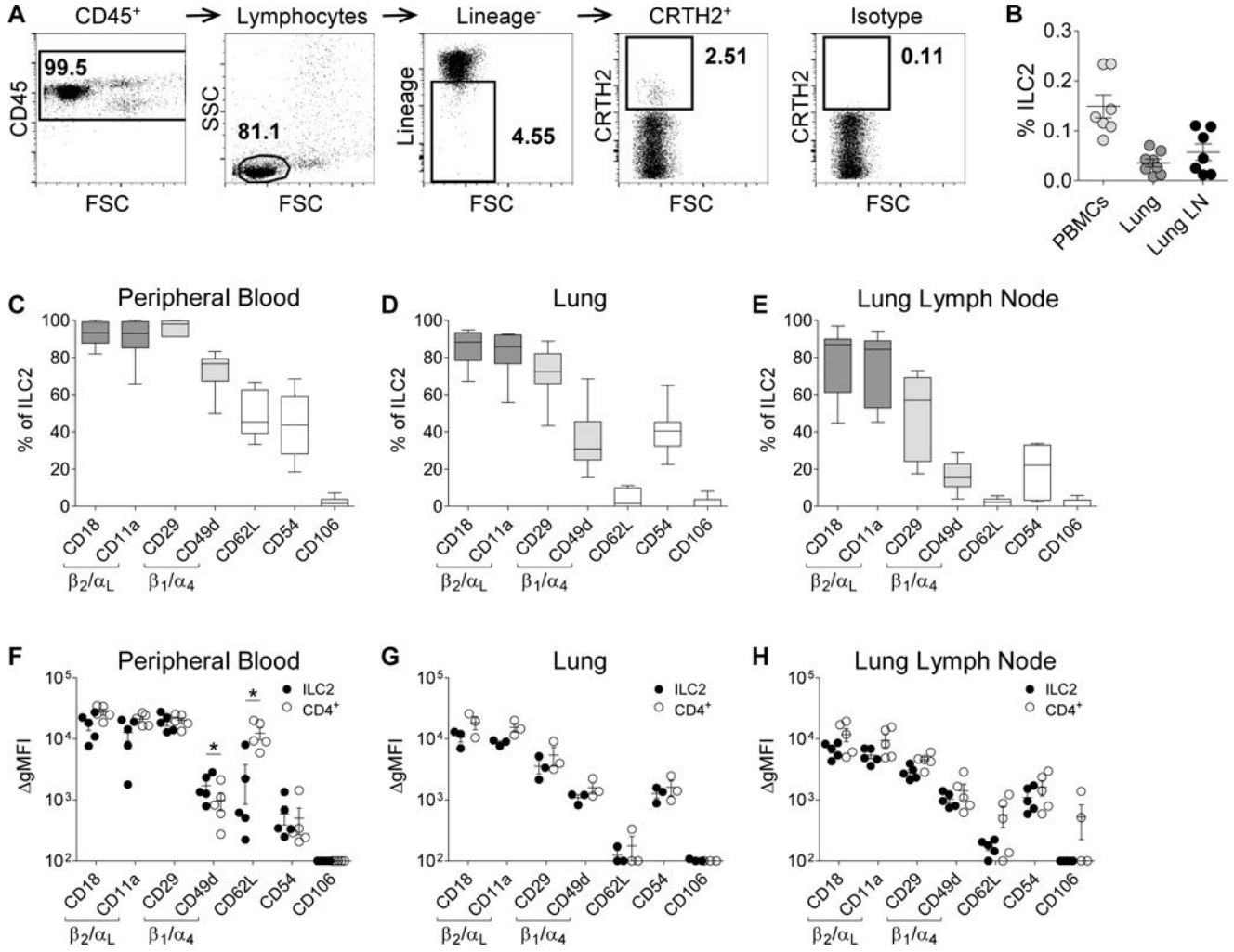

Figure 1. Human ILC2s express CD18/CD11a (β_2/α_L) and CD29/CD49d (β_1/α_4) integrins

(**A**) Representative ILC2 gating strategy from human lymph nodes. (**B**) The frequency of ILC2s from CD45$^+$ human PBMCs, lungs, and lung lymph nodes. Summary of ILC2 adhesion molecule expression from (**C**) PBMCs (n=7), (**D**) lung tissue (n=8), and (**E**) lymph nodes (n=7). Comparison of ILC2 and CD4$^+$CRTH2$^+$ cell adhesion molecule ΔgMFI from (**F**) PBMCs (n=5), (**G**) lungs (n=3), and (**H**) lymph nodes (n=5) of the same donor. *Solid circles* represent ILC2 populations and *open circles* represent CD4$^+$CRTH2$^+$ populations. Data depicted as boxplots (whiskers: 10th–90th percentile) or individual points; mean represented as a line ± SEM. *P<0.05 by Student's paired t test.

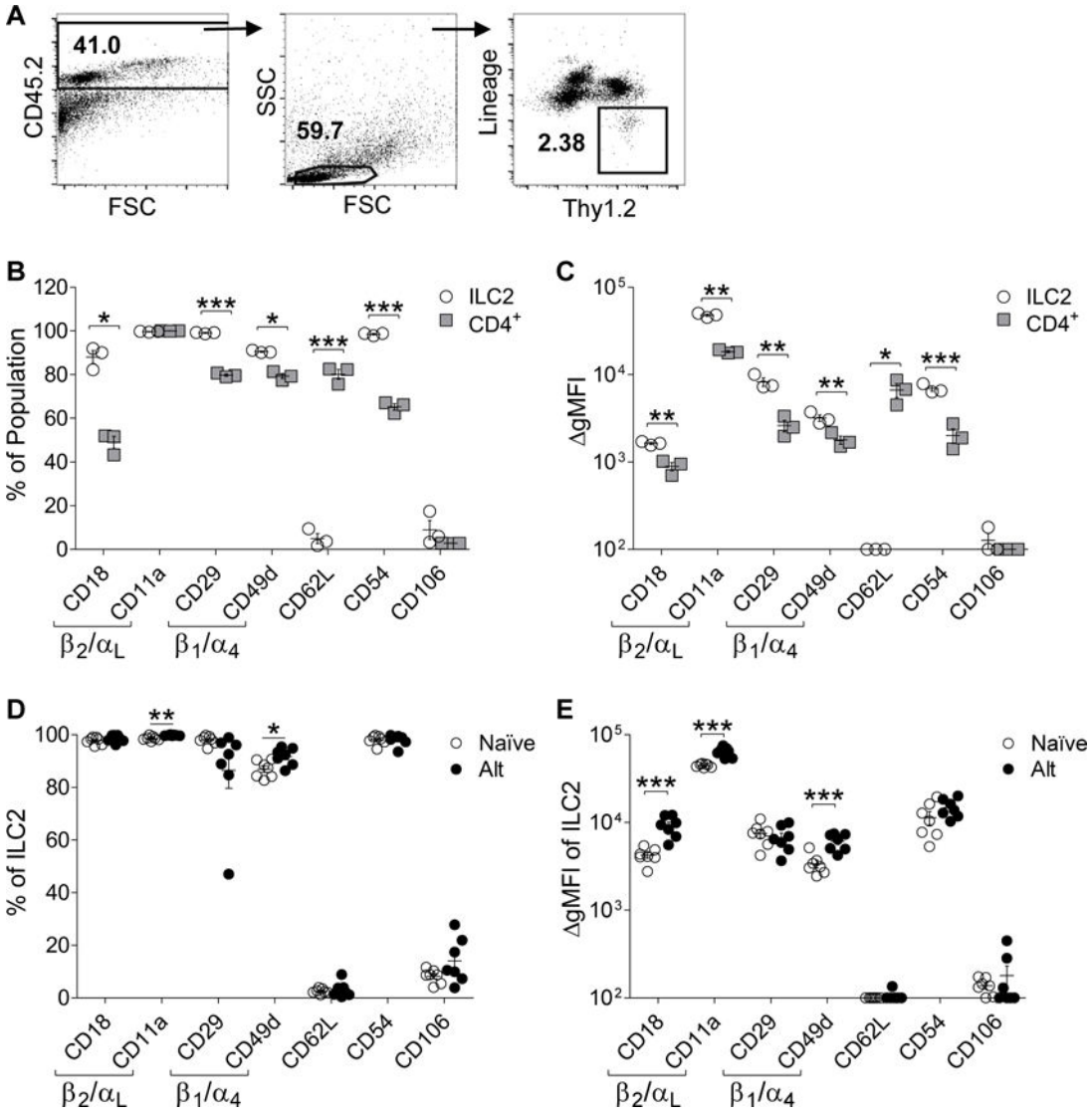

Figure 2. Adhesion molecule expression on mouse lung ILC2s

(**A**) Representative gating strategy of mouse lung ILC2s. Mouse lung ILC2s (*open circles*) compared to lung $CD4^{+}Thy1.2^{+}$ cells (*gray squares*) (**B**) percent adhesion molecule expression and (**C**) adhesion molecule ΔgMFI (n=3).Comparison of mouse lung ILC2 (**D**) percent expression and (**E**) ΔgMFI of adhesion molecules between naïve mice (*open circles*) and *Alternaria* challenged mice (*solid circles*; n=7). Independent experiments depicted as individual points; mean represented as a line ± SEM. *P<0.05, **P<0.01, ***P<0.001 by Student's t test.

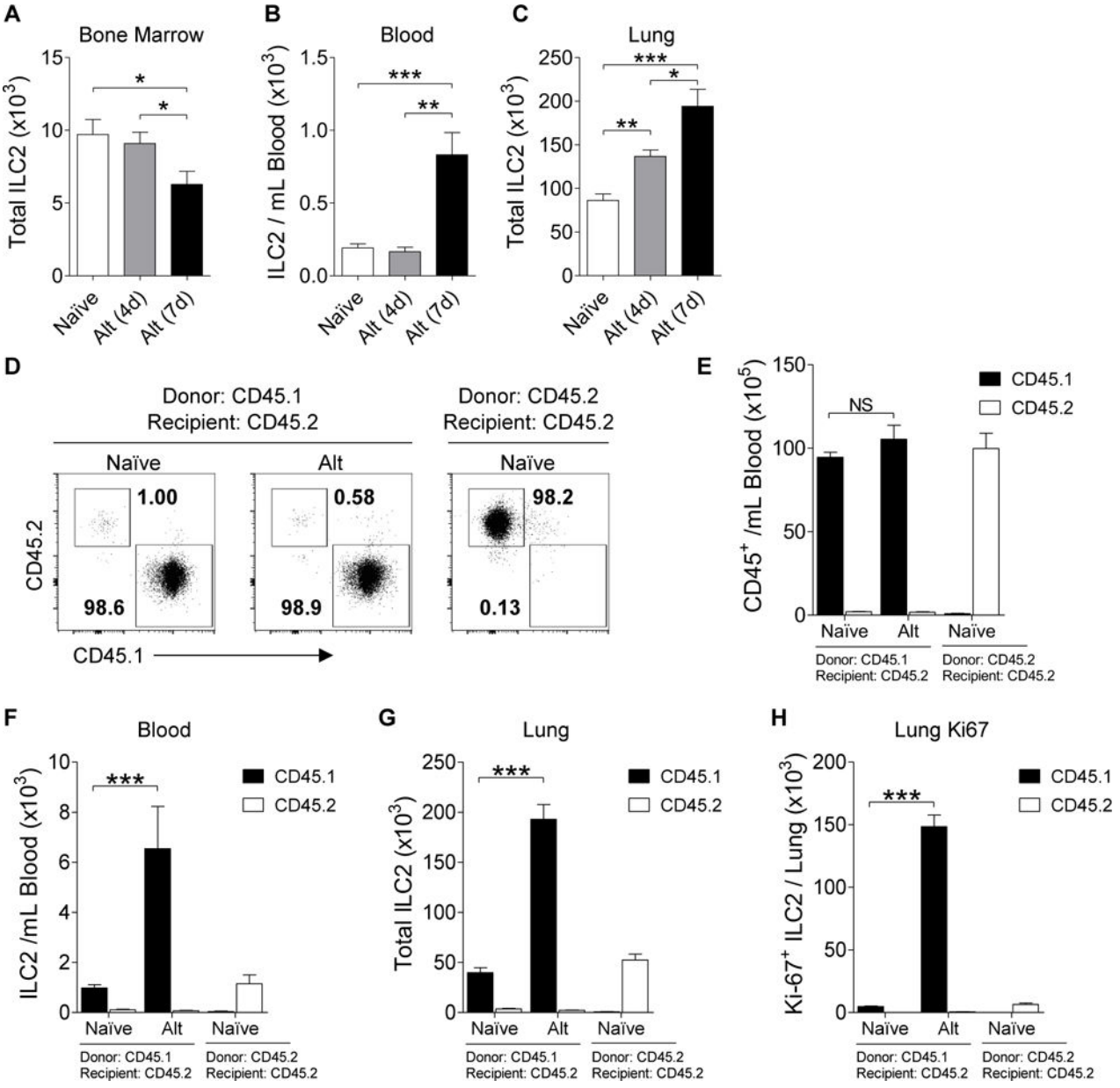

Figure 3. ILC2 levels change in bone marrow, blood, and lungs following *Alternaria* challenge in wt and BMT mice

Total ILC2s from (**A**) bone marrow, (**B**) blood, and (**C**) whole lung from naïve (*white*; n≥23), 4-day *Alternaria* challenged (*gray*; n=8), and 7-day *Alternaria* challenged mice (*black*; n≥14). CD45.1 or CD45.2 donor bone marrow was transplanted into CD45.2 irradiated recipient mice (**D**) Representative plots of BMT mouse blood ILC2 expression of CD45.1 and CD45.2. (**E**) Total CD45.1 (*black*) and CD45.2 (*white*) blood cells from BMT mice. Total ILC2s from (**F**) blood and (**G**) whole lung from naïve (n=8) or *Alternaria* challenged (n=4) BMT mice. (**H**) Total lung Ki-67$^+$ ILC2s from BMT mice. Data summarized as bar graphs representing mean ± SEM. **P*< 0.05, ***P*< 0.01, ****P*< 0.001 by Student's *t* test.

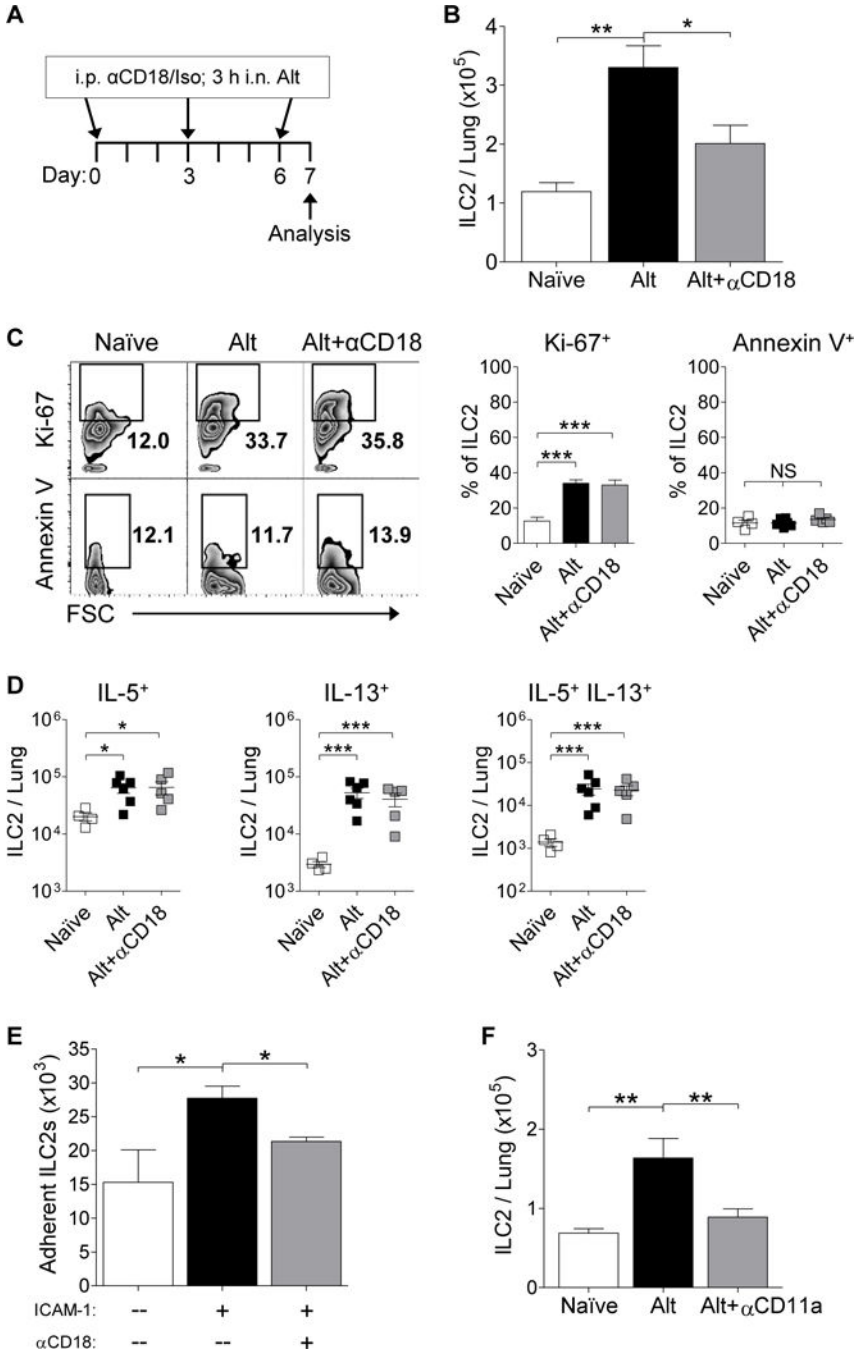

Figure 4. *In vivo* blocking of CD18 diminishes *Alternaria*-induced ILC2 recruitment to the airways

(**A**) Mice treated with anti-CD18 antibody (αCD18) or the isotype control and challenged with *Alternaria* (Alt) (**B**) Total ILC2s per mouse whole lung (n≥10). (**C**) Representative plots of ILC2 Ki-67 and Annexin V expression and summary of the percentage of Ki-67$^+$ ILC2s (n≥10) and live Annexin V$^+$ ILC2s (n≥4) per mouse lung. (**D**) Summary of total IL-5, IL-13, and IL-5/IL-13 producing ILC2s in the lung (n≥4; data was log transformed for normalcy). (**E**) Total number of sorted ILC2s adherent to ICAM-1 coated wells compared to

control coated wells ± preincubation of ILC2s with αCD18 (two independent experiments). (**F**) Total ILC2s per lung of mice treated ± Alt and ± anti-CD11a antibody (n≥6). Data summarized as bar graphs or individual points; mean represented as a line ± SEM. (*NS*) not significant, **P*< 0.05, ***P*< 0.01, ****P*< 0.001 by Student's *t* test.

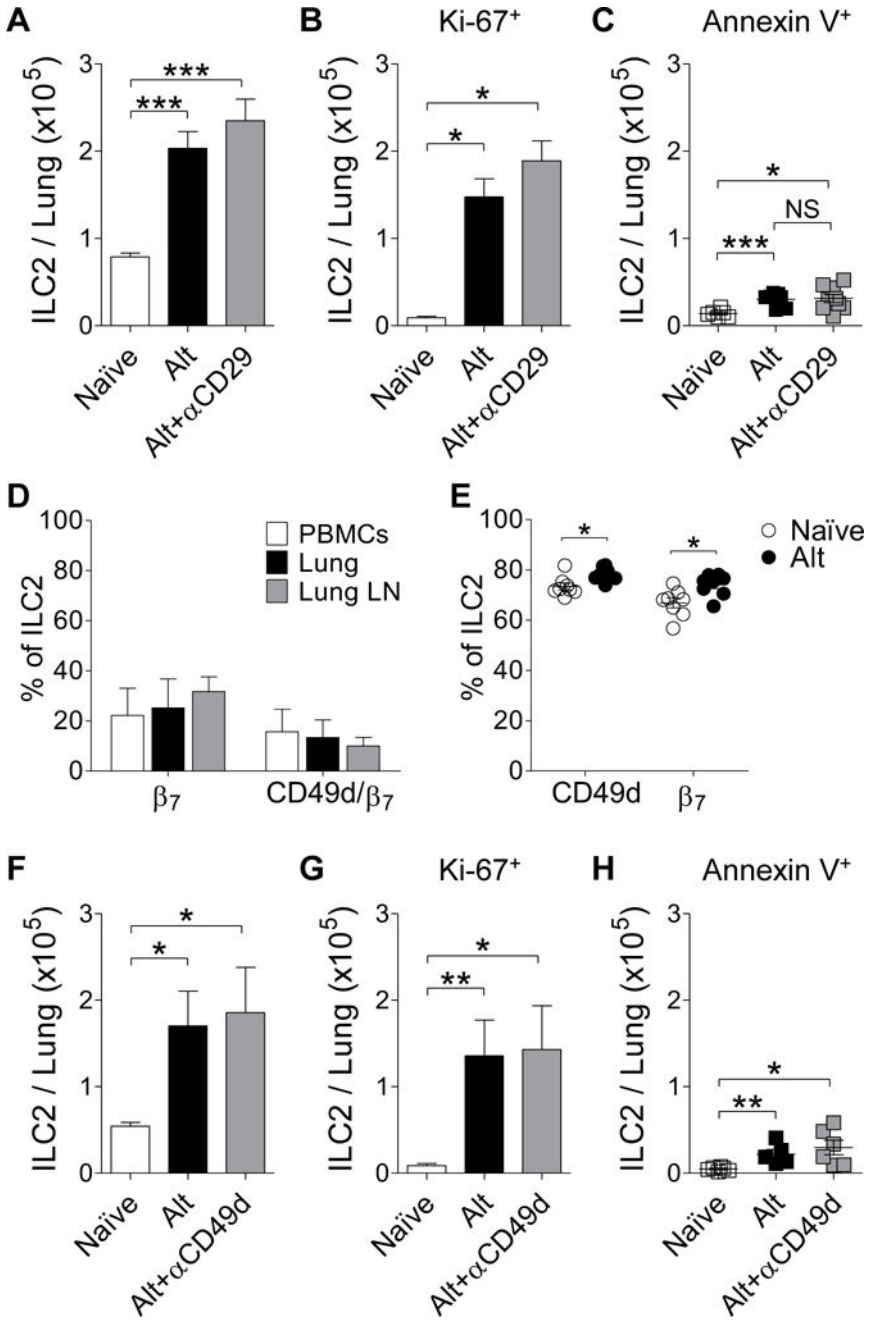

Figure 5. *In vivo* blocking of CD29 and CD49d does not alter *Alternaria*–induced ILC2 recruitment

(**A**) Total number of lung ILC2s, (**B**) total Ki-67$^+$ ILC2s, and (**C**) live Annexin V$^+$ ILC2s per mouse treated ± anti-CD29 antibody (n≥6). (**D**) Percentage of human ILC2s expressing integrin β_7 and both CD49d and β_7 from PBMCs (*white*; n=4), lung tissue (*black*; n=3), and lymph nodes (*gray*; n=4). (**E**) Summary of the percentage of mouse lung ILC2s expressing CD49d and integrin β_7 from both naïve and Alt challenged mice (n=8). (**F**) Total number of lung ILC2s, (**G**) total Ki-67$^+$ ILC2s, and (**H**) live Annexin V$^+$ ILC2s per mouse treated ±

anti-CD49d antibody (n≥5). Mean represented as a line ± SEM. (*NS*) not significant, **P*< 0.05, ***P*< 0.01, ****P*< 0.001 by Student's *t* test.