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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.

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.

KEY WORDS

- ILC2
- Integrin
- Adhesion
- *Alternaria alternata*

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

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 D₂).^{6, 7} Prostaglandin D₂ induces ILC2 Th2 cytokine production and is also a chemotactic factor for ILC2s through the binding to CCR2, 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 allergen¹⁴, 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 CCR2-expressing ILC2s had diminished ILC2 levels in the lungs following *N. brasiliensis* infection¹⁹, 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 D₂.

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 gut²⁴, 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,

120 predominantly mediate neutrophil trafficking to tissue sites of inflammation.²⁶ We therefore
121 profiled expression of integrin adhesion molecules on human and mouse ILC2s to determine
122 which adhesion receptors were highly expressed by ILC2s, and used neutralizing antibodies to
123 demonstrate that ILC2s traffic from the circulation using β_2 , but not β_1 , integrins during
124 *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 described¹⁰ 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 β_1 and β_2 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 Δ gMFI between the ILC2s and CD4⁺Thy1.2⁺ populations (**Figure 2C**). ILC2s showed higher Δ gMFI for the β_2 integrins CD18 ($\Delta\Delta$ gMFI = 744) and CD11a ($\Delta\Delta$ gMFI = 29,590), as well as the β_1 integrins CD29 ($\Delta\Delta$ gMFI = 5,658) and CD49d ($\Delta\Delta$ gMFI = 1,399), and ICAM-1 ($\Delta\Delta$ gMFI = 4,896). However, ILC2s had decreased Δ gMFI of CD62L compared to the CD4⁺Thy1.2⁺ population ($\Delta\Delta$ gMFI = 6,615; **Figure 2C**). As expected, ILC2 and CD4⁺Thy1.2⁺ populations had minimal expression or Δ gMFI 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 Δ gMFI of mouse lung ILC2 β_2 integrin subunits CD18 ($\Delta\Delta$ gMFI = 4,954) and CD11a ($\Delta\Delta$ gMFI = 19,114), as well as in the β_1 integrin subunit CD49d ($\Delta\Delta$ gMFI = 2,723) after *Alternaria* challenge (**Figure 2E**). In addition, after *Alternaria* challenge, levels of lung ILC2 Δ gMFI 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 Δ gMFI 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 α CD18 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 α CD18 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 α CD18 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 α CD18 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 α CD18 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 α CD18 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 α CD18 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 α CD18 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 (α CD11a) antibody³⁰ 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 (α CD29) antibody³¹ had no reduction in total lung ILC2 (**Figure 5A**). Administration of the α CD29 antibody to *Alternaria* challenged mice did not inhibit lung ILC2 proliferation (**Figure 5B**). Similarly, administration of the α CD29 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 (α CD49d) antibody³² also had no reduction in total lung ILC2 (**Figure 5F**). Administration of the α CD49d antibody to *Alternaria* challenged mice did not inhibit lung ILC2 proliferation (**Figure 5G**). Similarly, administration of the α CD49d 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 alone¹⁷ 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 α CD18 blocking antibody was specific to trafficking, as α CD18 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 α CD11a 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 α CD18 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 α CD18 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 marrow¹⁸, 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 state³⁷ 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 ILC2s¹⁷, 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

409 recruitment of ILC2s to the lung, as demonstrated in *in vivo* studies blocking β_2 integrins prior to
410 *Alternaria* allergen challenge. In contrast, *in vivo* blocking of β_1 integrins, or α_4 integrins, did not
411 affect ILC2 levels in the airways. *In vivo* blocking of β_2 integrins did not inhibit ILC2
412 proliferation, induce ILC2 apoptosis, or inhibit Th2 cytokine responses. Thus, ILC2 numbers
413 increase in the lung following *Alternaria* allergen challenge through mechanisms that include β_2
414 integrin, but not β_1 integrin, mediated trafficking of ILC2s from the circulation to the lung, as
415 well as due to local proliferation of ILC2s in response to local inflammatory mediators.

REFERENCES

1. Doherty TA, Broide DH. Group 2 innate lymphoid cells: new players in human allergic diseases. *J Investig Allergol Clin Immunol* 2015; 25:1-11.
2. Kim BS, Artis D. Group 2 innate lymphoid cells in health and disease. *Cold Spring Harb Perspect Biol* 2015; 7.
3. Bernink JH, Germar K, Spits H. The role of ILC2 in pathology of type 2 inflammatory diseases. *Curr Opin Immunol* 2014; 31:115-20.
4. Nagarkar DR, Poposki JA, Tan BK, Comeau MR, Peters AT, Hulse KE, et al. Thymic stromal lymphopoietin activity is increased in nasal polyps of patients with chronic rhinosinusitis. *J Allergy Clin Immunol* 2013; 132:593-600.
5. Christianson CA, Goplen NP, Zafar I, Irvin C, Good JT, Jr., Rollins DR, et al. Persistence of asthma requires multiple feedback circuits involving type 2 innate lymphoid cells and IL-33. *J Allergy Clin Immunol* 2015; 136:59-68.
6. Karta MR, Broide DH, Doherty TA. Insights into Group 2 Innate Lymphoid Cells in Human Airway Disease. *Curr Allergy Asthma Rep* 2016; 16:8.
7. Barlow JL, McKenzie AN. Type-2 innate lymphoid cells in human allergic disease. *Curr Opin Allergy Clin Immunol* 2014; 14:397-403.

8. Chang JE, Doherty TA, Baum R, Broide D. Prostaglandin D2 regulates human type 2 innate lymphoid cell chemotaxis. *J Allergy Clin Immunol* 2014; 133:899-901.
9. Doherty TA, Scott D, Walford HH, Khorram N, Lund S, Baum R, et al. Allergen challenge in allergic rhinitis rapidly induces increased peripheral blood type 2 innate lymphoid cells that express CD84. *J Allergy Clin Immunol* 2014; 133:1203-5.
10. Bartemes KR, Kephart GM, Fox SJ, Kita H. Enhanced innate type 2 immune response in peripheral blood from patients with asthma. *J Allergy Clin Immunol* 2014; 134:671-8.
11. Smith SG, Chen R, Kjarsgaard M, Huang C, Oliveria JP, O'Byrne PM, et al. Increased numbers of activated group 2 innate lymphoid cells in the airways of patients with severe asthma and persistent airway eosinophilia. *J Allergy Clin Immunol* 2016; 137:75-86.
12. Barlow JL, Bellosi A, Hardman CS, Drynan LF, Wong SH, Cruickshank JP, et al. Innate IL-13-producing nuocytes arise during allergic lung inflammation and contribute to airways hyperreactivity. *J Allergy Clin Immunol* 2012; 129:191-8.
13. Bartemes KR, Iijima K, Kobayashi T, Kephart GM, McKenzie AN, Kita H. IL-33-responsive lineage- CD25⁺ CD44(hi) lymphoid cells mediate innate type 2 immunity and allergic inflammation in the lungs. *J Immunol* 2012; 188:1503-13.
14. Di Rosa F, Pabst R. The bone marrow: a nest for migratory memory T cells. *Trends Immunol* 2005; 26:360-6.

15. Kim HK, Lund S, Baum R, Rosenthal P, Khorram N, Doherty TA. Innate type 2 response to *Alternaria* extract enhances ryegrass-induced lung inflammation. *Int Arch Allergy Immunol* 2014; 163:92-105.
16. Moro K, Kabata H, Tanabe M, Koga S, Takeno N, Mochizuki M, et al. Interferon and IL-27 antagonize the function of group 2 innate lymphoid cells and type 2 innate immune responses. *Nat Immunol* 2016; 17:76-86.
17. Gasteiger G, Fan X, Dikiy S, Lee SY, Rudensky AY. Tissue residency of innate lymphoid cells in lymphoid and nonlymphoid organs. *Science* 2015; 350:981-5.
18. Constantinides MG, McDonald BD, Verhoef PA, Bendelac A. A committed precursor to innate lymphoid cells. *Nature* 2014; 508:397-401.
19. Wojno ED, Monticelli LA, Tran SV, Alenghat T, Osborne LC, Thome JJ, et al. The prostaglandin D(2) receptor CRTH2 regulates accumulation of group 2 innate lymphoid cells in the inflamed lung. *Mucosal Immunol* 2015; 8:1313-23.
20. Vestweber D. How leukocytes cross the vascular endothelium. *Nat Rev Immunol* 2015; 15:692-704.
21. Kinashi T. Intracellular signalling controlling integrin activation in lymphocytes. *Nat Rev Immunol* 2005; 5:546-59.

22. Ley K, Laudanna C, Cybulsky MI, Nourshargh S. Getting to the site of inflammation: the leukocyte adhesion cascade updated. *Nat Rev Immunol* 2007; 7:678-89.
23. Hogg N, Patzak I, Willenbrock F. The insider's guide to leukocyte integrin signalling and function. *Nat Rev Immunol* 2011; 11:416-26.
24. Sakai Y, Kobayashi M. Lymphocyte 'homing' and chronic inflammation. *Pathol Int* 2015; 65:344-54.
25. Johansson MW, Mosher DF. Integrin activation States and eosinophil recruitment in asthma. *Front Pharmacol* 2013; 4:33.
26. Zarbock A, Ley K. Mechanisms and consequences of neutrophil interaction with the endothelium. *Am J Pathol* 2008; 172:1-7.
27. Doherty TA, Khorram N, Chang JE, Kim HK, Rosenthal P, Croft M, et al. STAT6 regulates natural helper cell proliferation during lung inflammation initiated by *Alternaria*. *Am J Physiol Lung Cell Mol Physiol* 2012; 303:L577-88.
28. Doherty TA, Khorram N, Lund S, Mehta AK, Croft M, Broide DH. Lung type 2 innate lymphoid cells express cysteinyl leukotriene receptor 1, which regulates TH2 cytokine production. *J Allergy Clin Immunol* 2013; 132:205-13.

29. Walford HH, Lund SJ, Baum RE, White AA, Bergeron CM, Husseman J, et al. Increased ILC2s in the eosinophilic nasal polyp endotype are associated with corticosteroid responsiveness. *Clin Immunol* 2014; 155:126-35.
30. Sanchez-Madrid F, Davignon D, Martz E, Springer TA. Antigens involved in mouse cytolytic T-lymphocyte (CTL)-mediated killing: functional screening and topographic relationship. *Cell Immunol* 1982; 73:1-11.
31. Ridger VC, Wagner BE, Wallace WA, Hellewell PG. Differential effects of CD18, CD29, and CD49 integrin subunit inhibition on neutrophil migration in pulmonary inflammation. *J Immunol* 2001; 166:3484-90.
32. Brocke S, Piercy C, Steinman L, Weissman IL, Veromaa T. Antibodies to CD44 and integrin alpha4, but not L-selectin, prevent central nervous system inflammation and experimental encephalomyelitis by blocking secondary leukocyte recruitment. *Proc Natl Acad Sci U S A* 1999; 96:6896-901.
33. Lao-Araya M, Steveling E, Scadding GW, Durham SR, Shamji MH. Seasonal increases in peripheral innate lymphoid type 2 cells are inhibited by subcutaneous grass pollen immunotherapy. *J Allergy Clin Immunol* 2014; 134:1193-5.
34. Maazi H, Patel N, Sankaranarayanan I, Suzuki Y, Rigas D, Soroosh P, et al. ICOS:ICOS-ligand interaction is required for type 2 innate lymphoid cell function, homeostasis, and induction of airway hyperreactivity. *Immunity* 2015; 42:538-51.

35. Ruiter B, Patil SU, Shreffler WG. Vitamins A and D have antagonistic effects on expression of effector cytokines and gut-homing integrin in human innate lymphoid cells. Clin Exp Allergy 2015; 45:1214-25.
36. Demyanets S, Konya V, Kastl SP, Kaun C, Rauscher S, Niessner A, et al. Interleukin-33 induces expression of adhesion molecules and inflammatory activation in human endothelial cells and in human atherosclerotic plaques. Arterioscler Thromb Vasc Biol 2011; 31:2080-9.
37. Sung KL, Li Y, Elices M, Gang J, Sriramaraio P, Broide DH. Granulocyte-macrophage colony-stimulating factor regulates the functional adhesive state of very late antigen-4 expressed by eosinophils. J Immunol 1997; 158:919-27.

FIGURE LEGENDS

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 Δ gMFI 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 $\pm$ 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 Δ gMFI (n=3). Comparison of mouse lung ILC2 (D) percent expression and (E) Δ gMFI 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 $\pm$ 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*; $n \geq 23$), 4-day *Alternaria* challenged (*gray*; $n=8$), and 7-day *Alternaria* challenged mice (*black*; $n \geq 14$). 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 $\pm$ 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 (α CD18) or the isotype control and challenged with *Alternaria* (Alt) (B) Total ILC2s per mouse whole lung ($n \geq 10$). (C) Representative plots of ILC2 Ki-67 and Annexin V expression and summary of the percentage of Ki-67⁺ ILC2s ($n \geq 10$) and live Annexin V⁺ ILC2s ($n \geq 4$) per mouse lung. (D) Summary of total IL-5, IL-13, and IL-5/IL-13 producing ILC2s in the lung ($n \geq 4$; data was log transformed for normalcy). (E) Total number of sorted ILC2s adherent to ICAM-1 coated wells compared to control coated wells $\pm$ preincubation of ILC2s with α CD18 (two independent experiments). (F) Total ILC2s per lung of mice treated $\pm$ Alt and $\pm$ anti-CD11a antibody ($n \geq 6$). Data summarized as bar graphs or individual points; mean represented as a line $\pm$ 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 $\pm$ anti-CD29 antibody (n $\geq$ 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 $\pm$ anti-CD49d antibody (n $\geq$ 5). Mean represented as a line $\pm$ SEM. (NS) not significant, **P* < 0.05, ***P* < 0.01, ****P* < 0.001 by Student's *t* test.

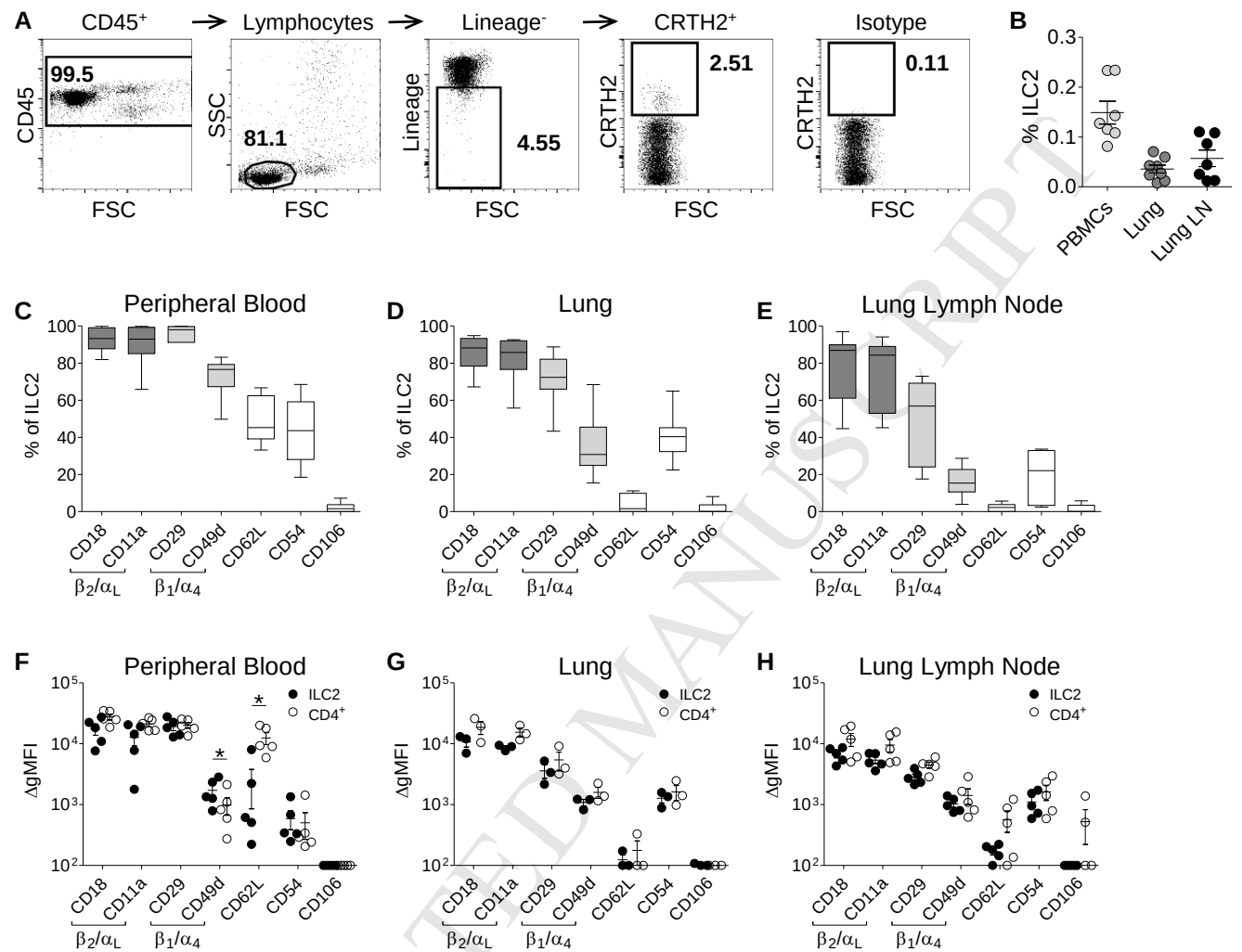
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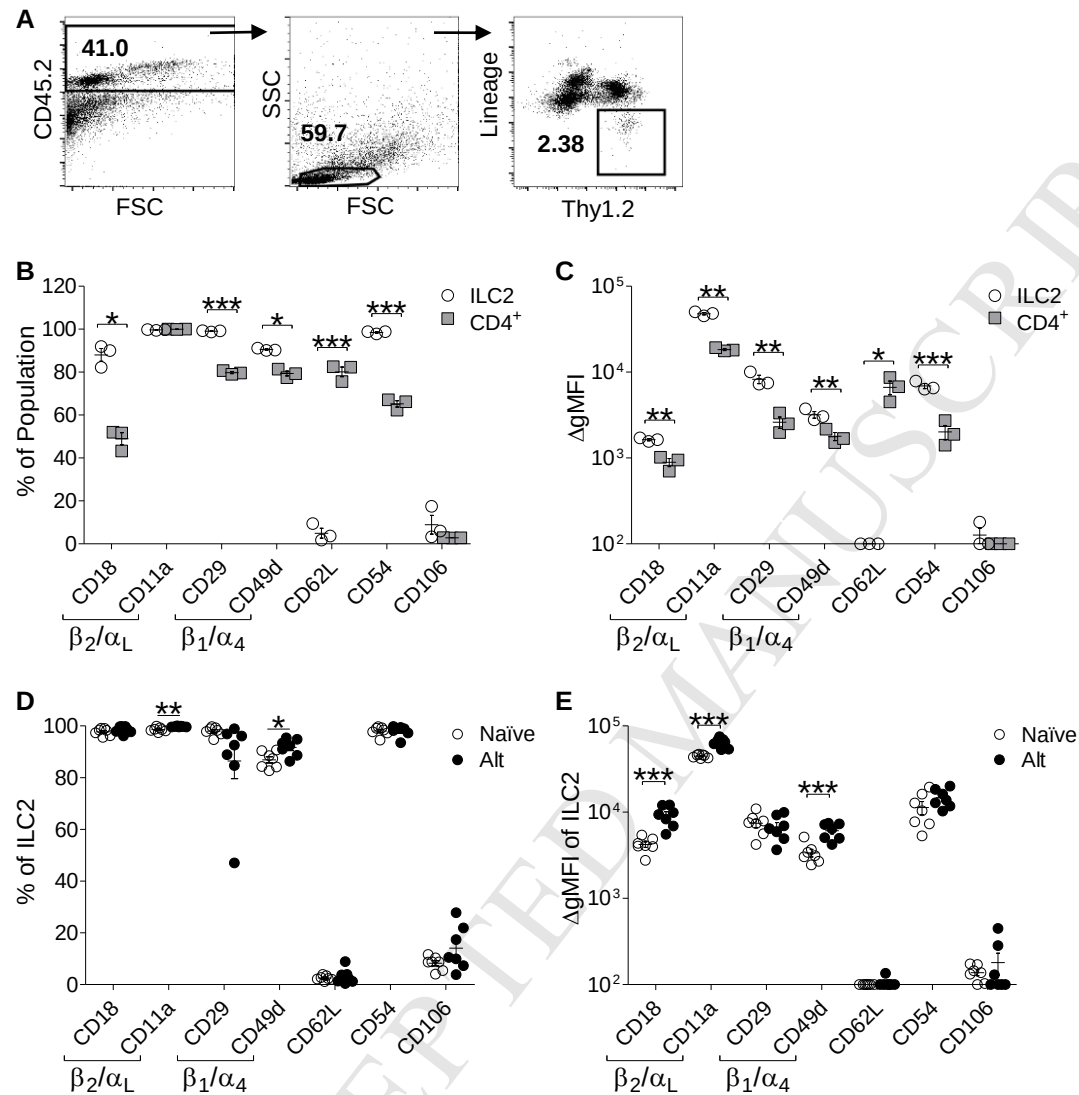
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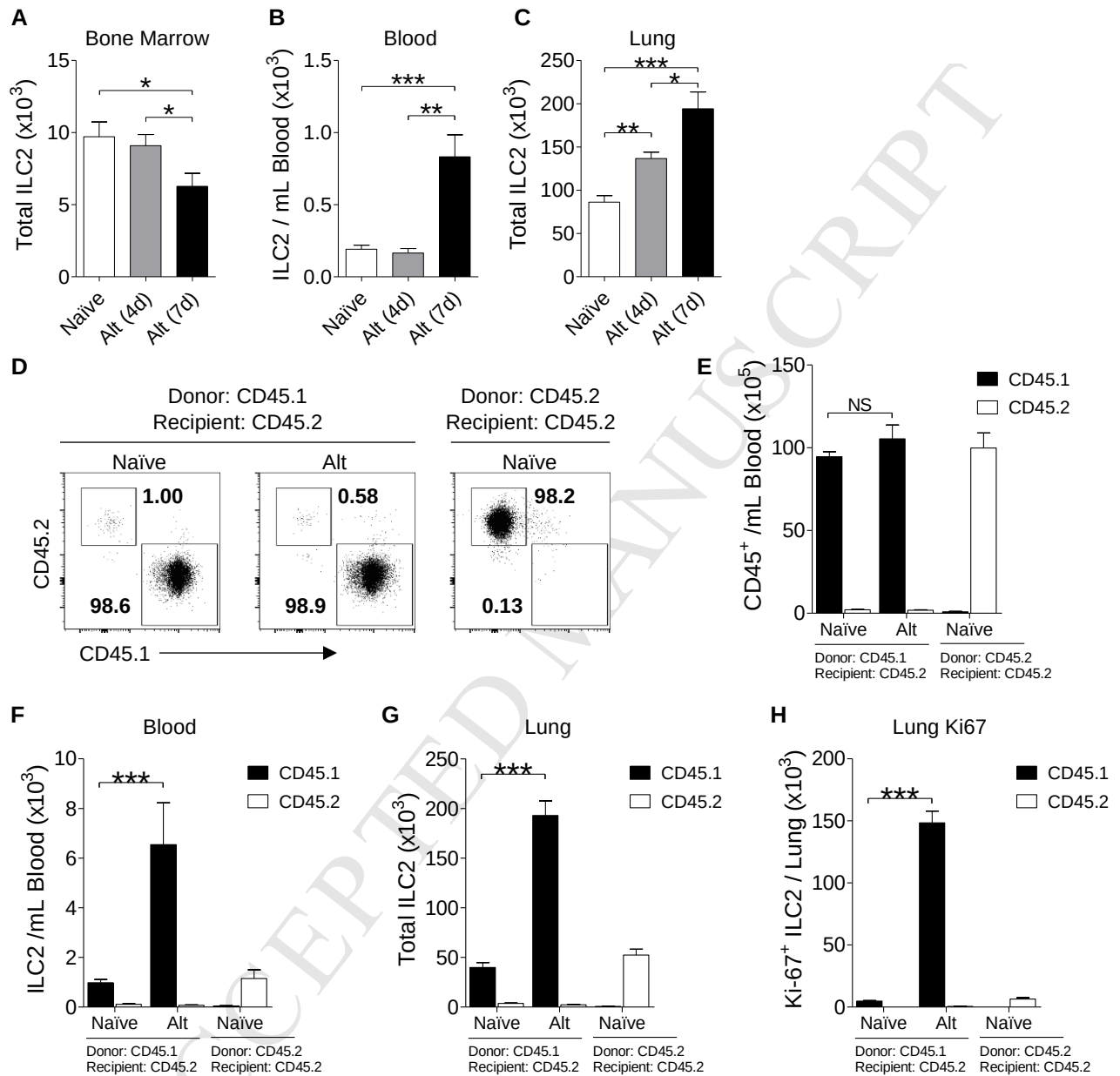


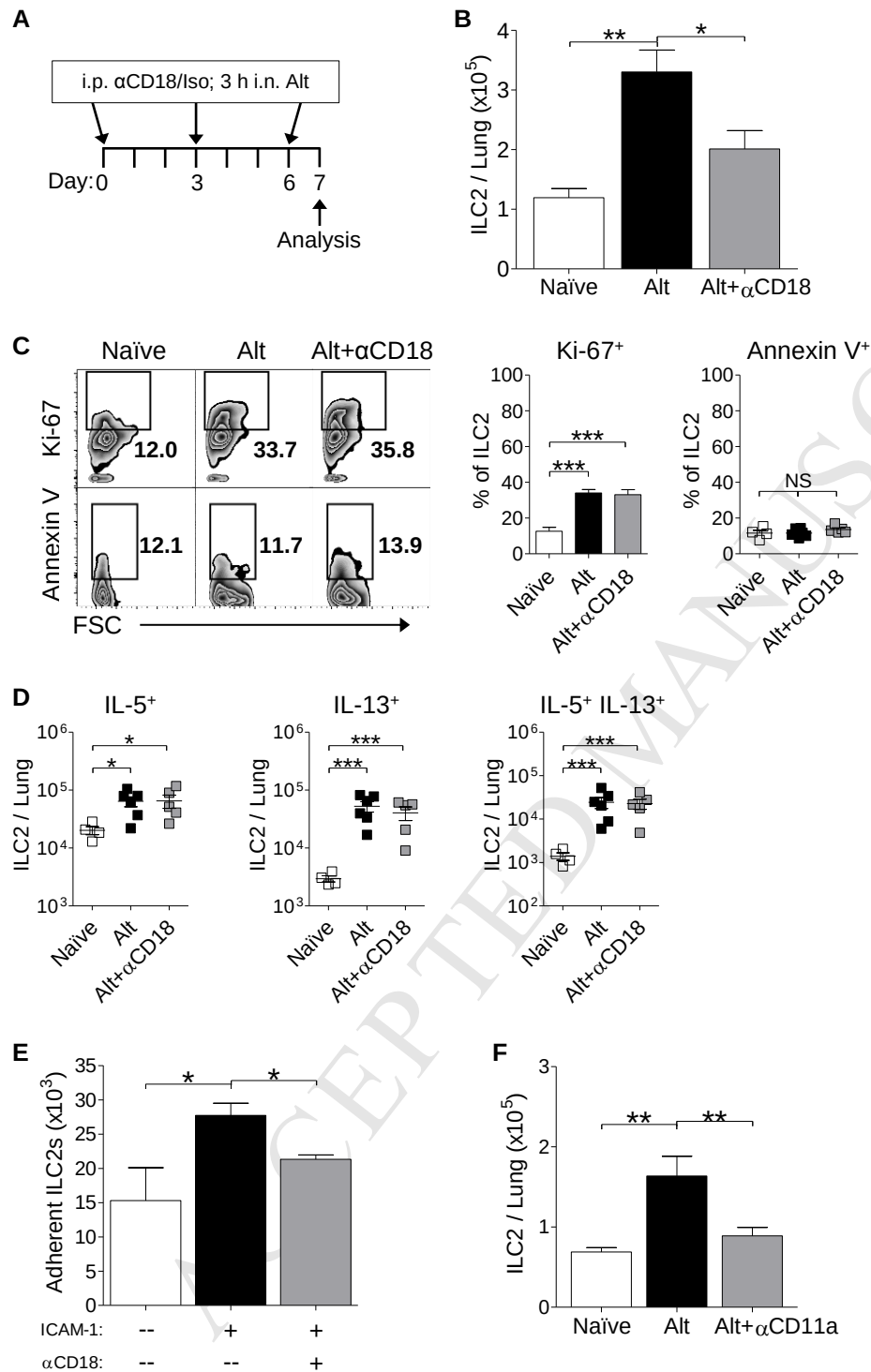
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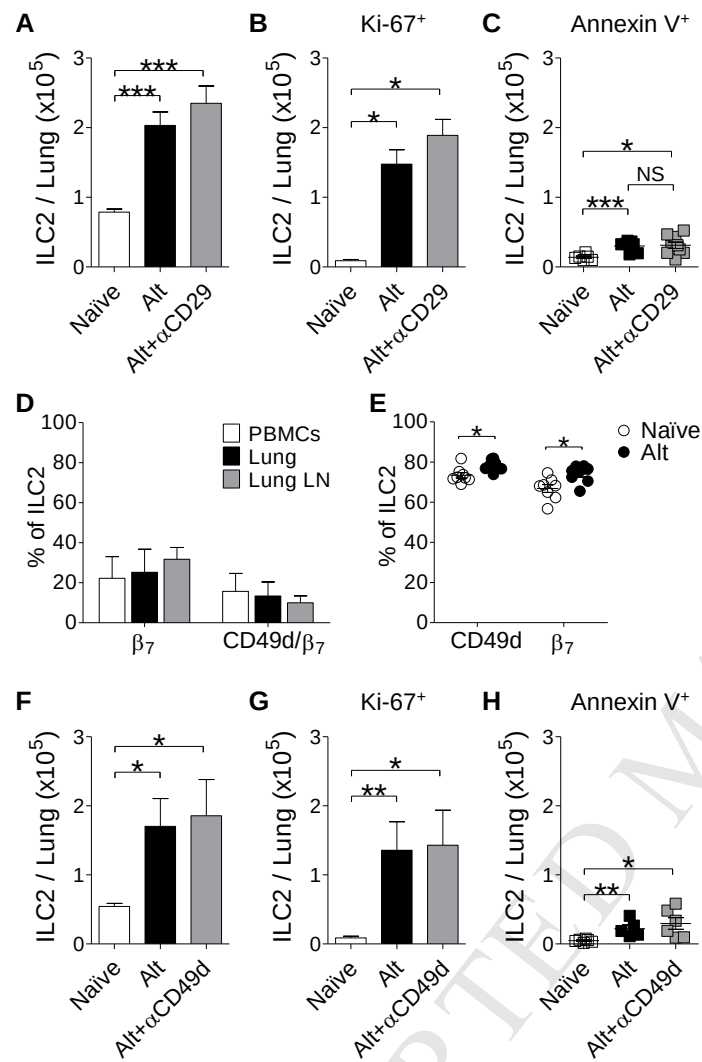
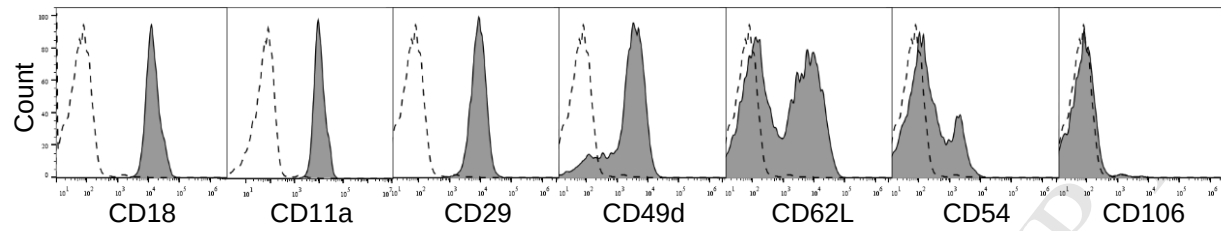
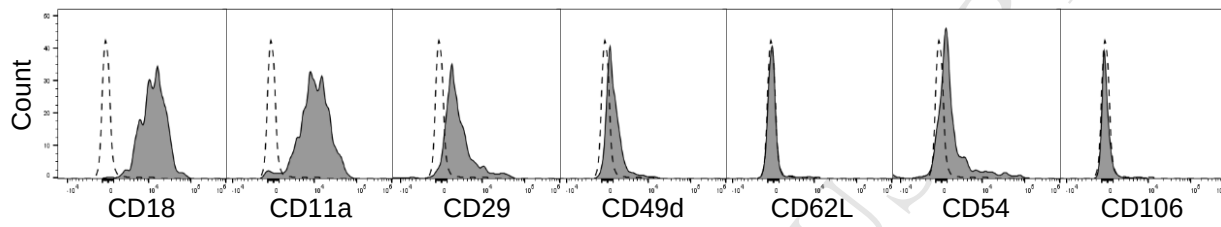
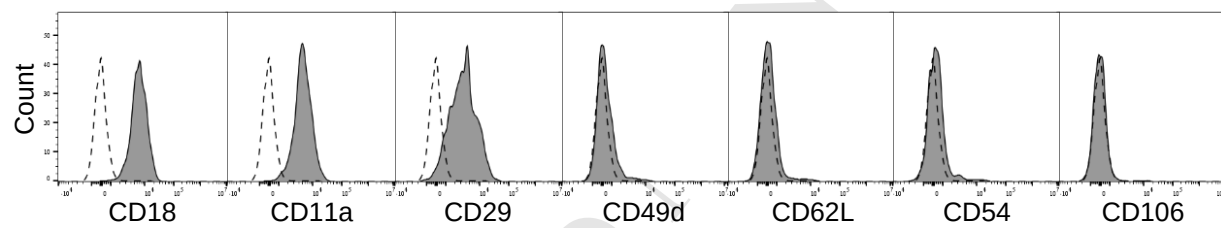
Figure 5 (single column)

Figure E1**A. Peripheral Blood****B. Lung****C. Lung Lymph Nodes**

 Isotype control
 Adhesion molecule

CD nomenclature:	Alternative name:
CD18	Integrin β_2
CD11a	Integrin α_L
CD29	Integrin β_1
CD49d	Integrin α_4
CD62L	L-selectin
CD54	ICAM-1
CD106	VCAM-1

Figure E2

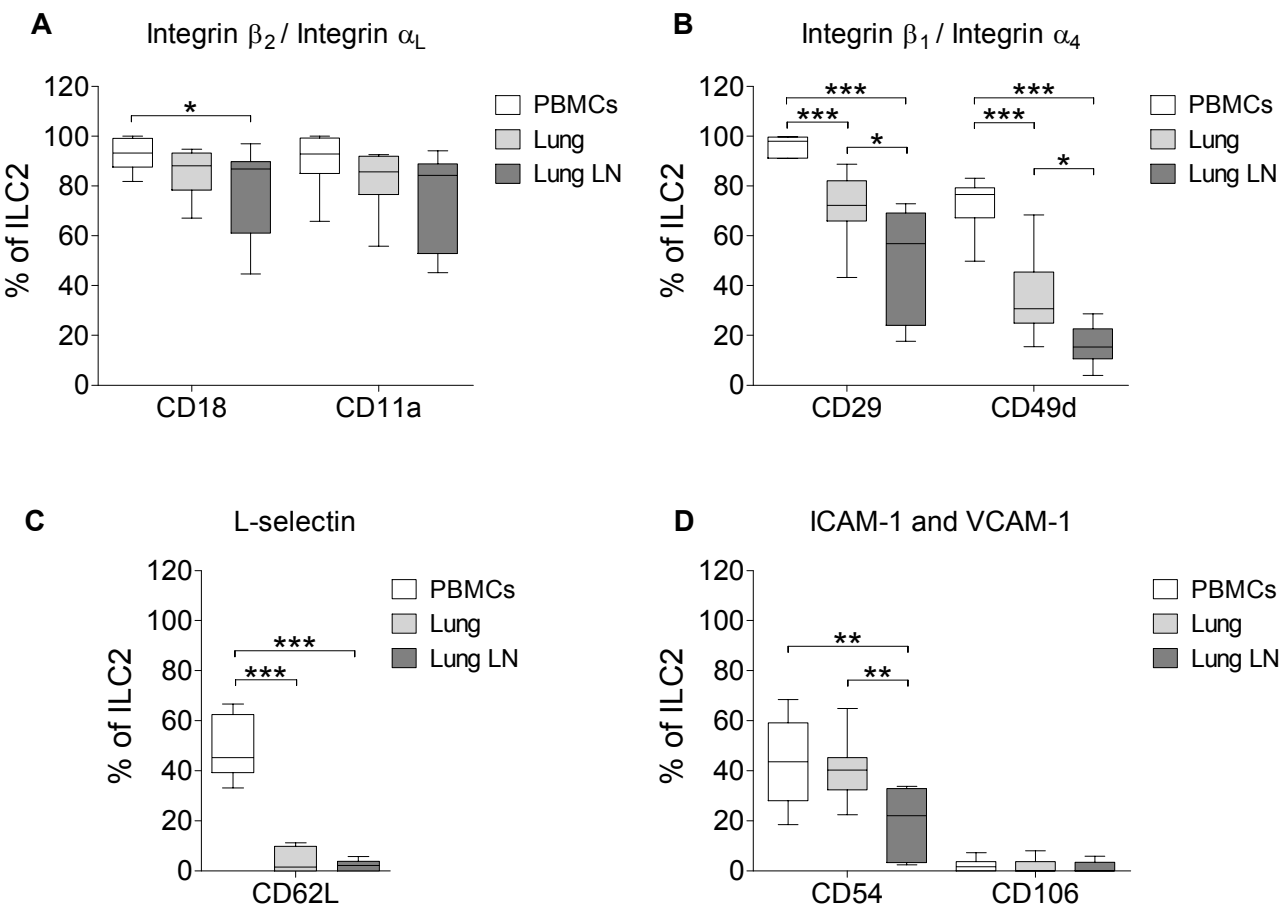
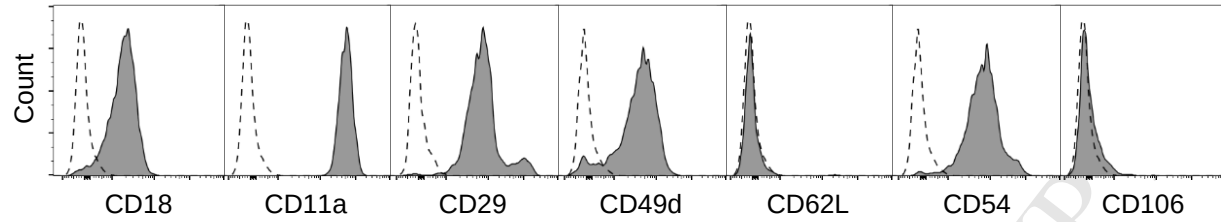
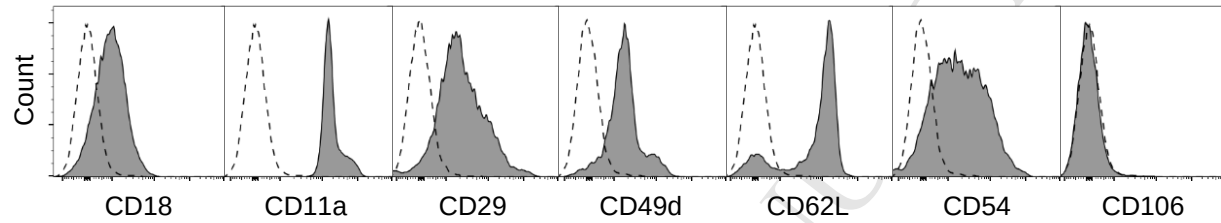


Figure E3**A. ILC2****B. CD4⁺Thy1.2⁺ cells**

 Isotype control
  Adhesion molecule

CD nomenclature:	Alternative name:
CD18	Integrin β_2
CD11a	Integrin α_L
CD29	Integrin β_1
CD49d	Integrin α_4
CD62L	L-selectin
CD54	ICAM-1
CD106	VCAM-1

Figure E4

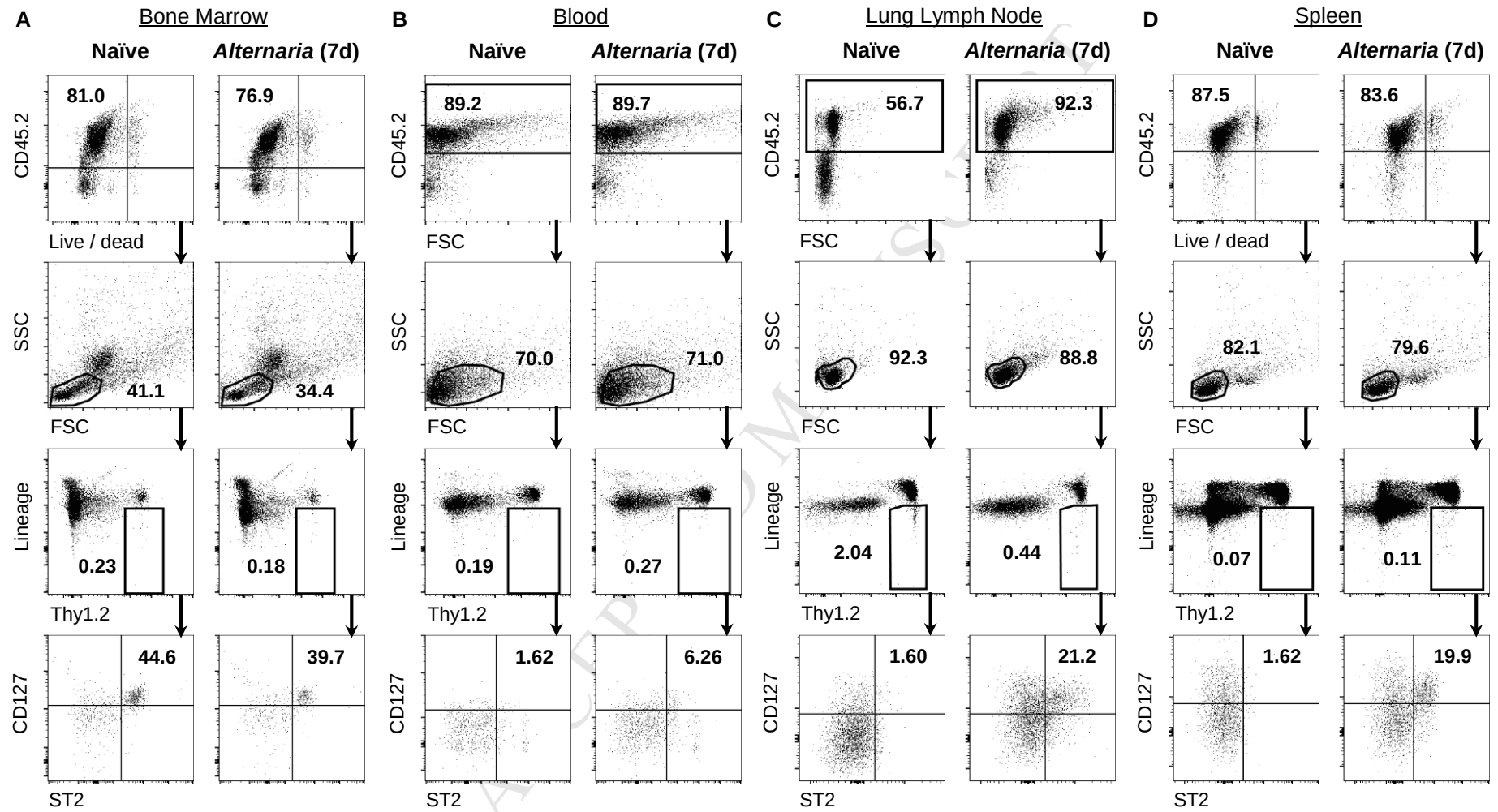


Figure E5

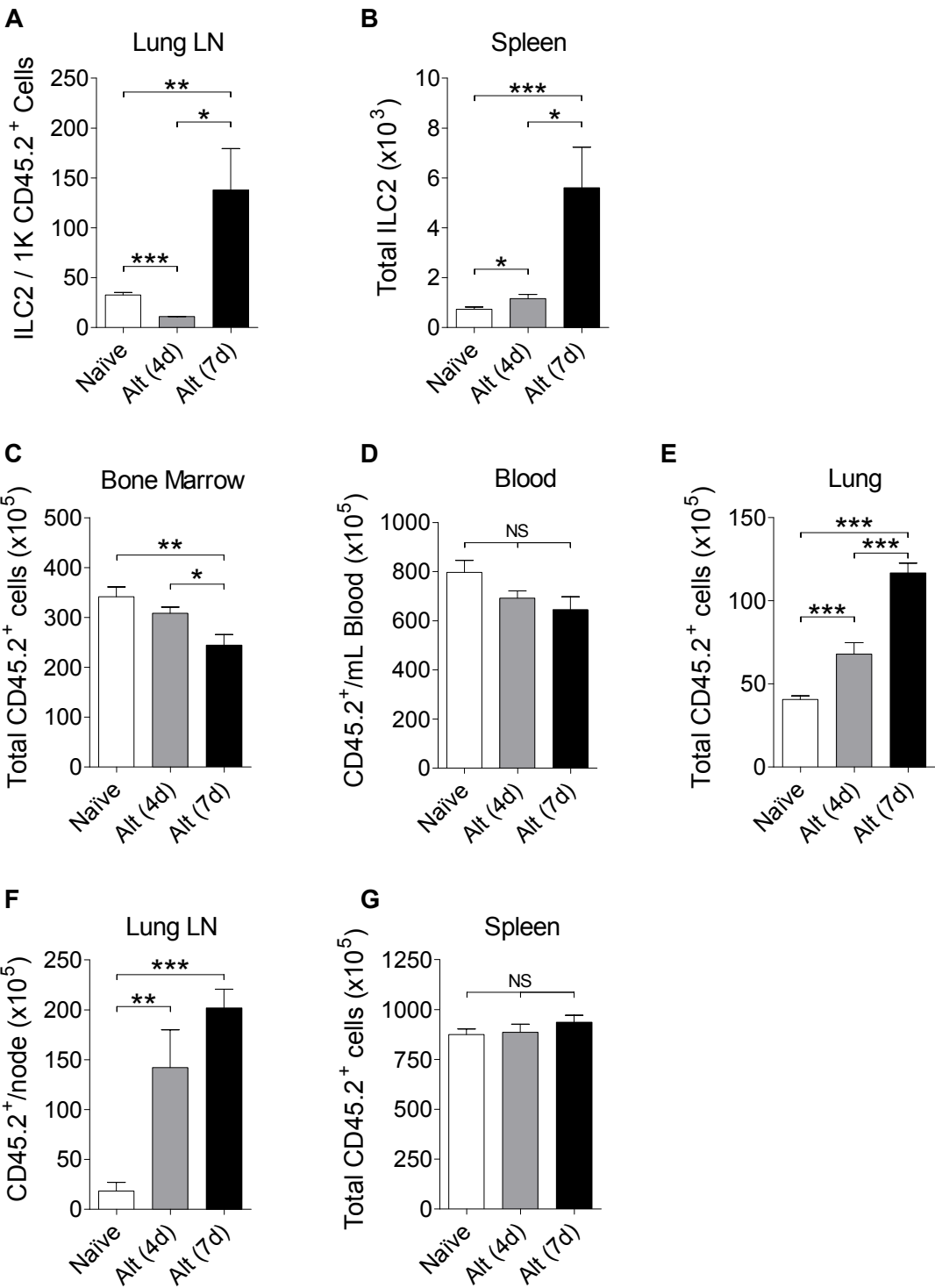


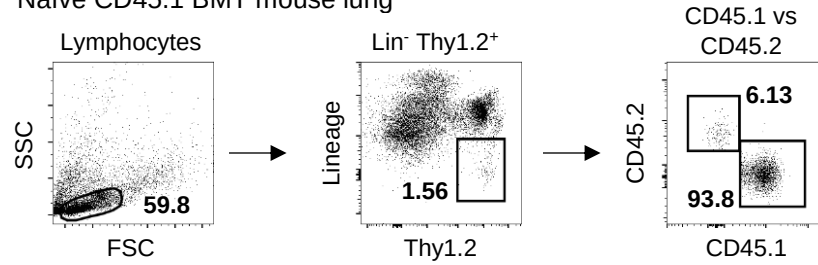
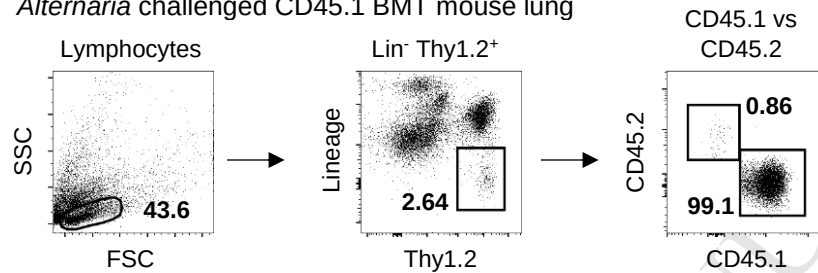
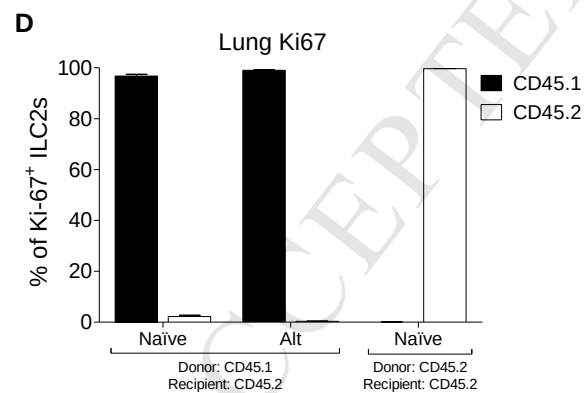
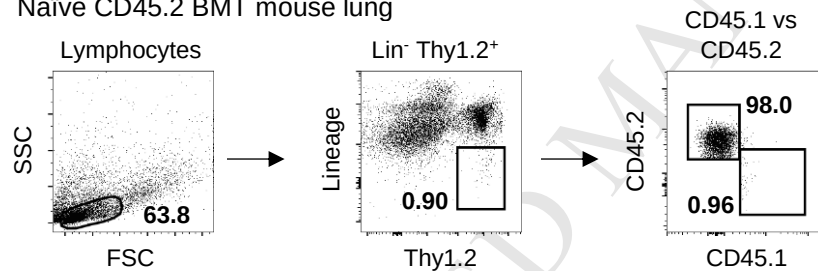
Figure E6**A** Naïve CD45.1 BMT mouse lung**B** *Alternaria* challenged CD45.1 BMT mouse lung**C** Naïve CD45.2 BMT mouse lung

Figure E7