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1 **Pulmonary environmental cues drive group 2 innate lymphoid cell dynamics in mice**

2 **and humans**

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One Sentence Summary

Collagen-I and CCL8-CCR8 axis control the motility of ILC2 cells in the inflamed lung

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Abstract

Group 2 innate lymphoid cells (ILC2s) are enriched in mucosal tissues (e.g. lung) and respond to epithelial cell-derived cytokines initiating type-2 inflammation. During inflammation, ILC2 numbers are increased in the lung. However, the mechanisms controlling ILC2 trafficking and motility within inflamed lungs remain unclear and are crucial for understanding ILC2 function in pulmonary immunity. Using several approaches, including lung intravital microscopy, we demonstrate that pulmonary ILC2s are highly-dynamic, exhibit amoeboid-like movement and aggregate in the lung peribronchial and perivascular spaces. They express distinct chemokine receptors, including CCR8, and actively home to CCL8 deposits located around the airway epithelium. Within lung tissue, ILC2s were particularly motile in extracellular matrix-enriched regions. We show that collagen-I drives ILC2 to dramatically change their morphology by remodeling their actin cytoskeleton to promote environmental exploration critical for regulating eosinophilic inflammation. Our study provides previously unappreciated insights into ILC2-migratory patterns during inflammation and highlights the importance of environmental guidance cues in the lung in controlling ILC2 dynamics.

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Introduction

Innate lymphoid cells (ILC), are an emerging family of immune cells that originate from fetal liver and adult bone marrow (BM) progenitors (*1, 2*). Prenatally, ILC progenitors occupy peripheral sites from mid to late stages of fetal development (*3*), and are detectable in peripheral tissues by embryonic (E) day 15.5 in the mouse (*4*). Although ILC derive from common lymphoid progenitors, they lack specific antigen receptors and lymphoid cell lineage markers, but display diverse effector functions, analogous to T cells (*5, 6*). Based on distinct lineage-determining transcription factors, ILC segregate into three different subsets – ILC1, ILC2 and ILC3 (*7-9*). Each ILC subset mirrors a T helper cell subset via the production of T helper cell signature cytokines- IFN-γ (Th1/ILC1), IL-13 (Th2/ILC2) and IL-17 (Th17/ILC3) which act to combat infection by intracellular pathogens, helminths, and extracellular pathogens respectively (*3*). Anatomically, different ILC subsets are resident in particular barrier and non-barrier sites (*3*), including the BM, skin, secondary lymphoid organs, peripheral blood and non-lymphoid tissues. ILC3 are vital for the development of adaptive immune organs in utero, however after birth thymic ILC3 are replaced by ILC2 suggesting that these cells play an important role in the thymic microenivronment (*10*). Among non-lymphoid tissues, ILC2 are enriched in mucosal sites including the lung and small intestine, where they contribute to local tissue immunoregulation, repair and homeostasis (*11, 12*). In the mouse lung, ILC2 predominate (*13*), and rapidly expand during the first week of life (*14, 15*), populating collagen-rich structures associated with medium-sized blood vessels and airways (*14*). In a steady state, long term maintenance of ILC2 in peripheral tissues, including the lung, is mainly supported by self-renewal of proliferative local tissue resident progenitor populations (*4, 15-18*). Recent evidence suggests that ICAM-1 supports ILC2 development and function during lung inflammation (*19*). However, in T helper type-2 (Th2) cell induced lung inflammation, ILC2 exit the BM (*19, 20*) and concurrently numbers are increased in the blood and lung (*21-24*). Human and mouse ILC2 express β2 integrins and these have been proposed to be involved in cell trafficking since blocking β2 integrins results in reduced ILC2

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82   numbers in the lung following allergen challenge (*22*). In addition, recent studies have

83   identified a distinct pre-ILC2 population originating in the gut that migrates to the lung and

84   other distal sites giving rise to inflammatory ILC2 (iILC2) that provide protection during worm

85   infection (*15*). Within the lung, interaction of ILC2 with other immune cell populations is a

86   critical factor for shaping type 2 inflammation (*25*).

87

88   Several factors, including the epithelial cell-derived cytokines IL-33, IL-25 and thymic stromal

89   lymphopoietin (TSLP), contribute to ILC2 activation and function (*26*). Upon activation, ILC2

90   produce cytokines, including IL-4, IL-5, IL-13, colony stimulating factor 2 (CSF2; GM-CSF),

91   and the epidermal growth factor family member amphiregulin (Areg) (*13, 27-29*). Lipid

92   mediators, including the arachidonic acid metabolites leukotriene D4 (LTD_4), prostaglandin D2

93   (PGD_2) (*30, 31*) and sphingosine 1-phosphate (S1P) (*15*) serve as potent regulators of ILC2

94   activation, accumulation and function. In contrast PGE2 and PGI2 supress ILC2 function,

95   inhibiting GATA-3, IL-5 and IL-13 expression and decreasing proliferation (*32, 33*). We have

96   previously demonstrated that epithelial cell-derived transforming growth factor (TGF)-β1 is

97   critical for ILC2 activation and significantly enhances airway ILC2 chemoactivity and

98   movement *in vitro* (*34*). Thus, ILC2 activation, behaviour and function are regulated by a wide

99   variety of factors.

100

101   Tissue environments critically control optimal immune responses, coordinating timely and

102   proportionate recruitment, motility, migration, chemotaxis, positioning, and cell–cell interaction

103   of leukocytes within inflamed tissues (*35*). Factors, including lipids, cytokines and homing

104   receptors (HRs) incorporating chemokine receptors (*3*) controlling migratory patterns of ILC2

105   in the bone marrow (BM), spleen, gut, mesenteric lymph nodes (*36*) and skin (*37, 38*) have

106   been identified. Lung ILC2 express high levels of β1 and β2 integrins, and use β2 integrins

107   selectively to migrate from the BM to the lung after intranasal (i.n.) allergen (*Alternaria*

108   *alternata*) challenge (*39*). Organ specific imprinting confers differential gene expression

109   patterns on tissue resident pulmonary ILC2 (*40*). However, ILC2 motility within the lung during

110   inflammation remains unstudied, and little is known regarding the signals that might regulate

111   it. Studies investigating T cell motility in the lung have described a combination of cell-intrinsic

112   signals and physical guidance cues coupled with biochemical signals provided by the

113   microenvironment in driving cell movement (*41*). However, the environmental guidance

114   signals controlling ILC2 dynamic behaviour in the lung remain poorly defined. Here using IL13-

115   eGFP mice (*28*), combined with several imaging approaches, including lung intravital

116   microscopy, we have documented that ILC2 exhibit amoeboid-like movement in the

117   peribronchial and perivascular space after IL-33 induced lung inflammation. We identified

118   specific molecules that communicate between the inflamed pulmonary environment and ILC2,

119   uncovering the major environmental factors from which ILC2 interpret locomotory cues within

120   the lung.

121

122   **Results**

123

124   **The number of ILC2 rapidly increases in the peribronchial / perivascular region after**

125   **rIL-33 treatment.**

126   Under homeostatic conditions, ILC2 exist in relatively low numbers in the lung, but are rapidly

127   enriched after recombinant IL-33 (rIL-33) or rIL-25 induced inflammation (*20-24, 39*),

128   promoting tissue repair during inflammation and regulating immune homeostasis. In this

129   respect, ILC2 are thought to occupy a specific niche in the lung in keeping with their putative

130   role as tissue resident sentinel cells (*14*). However, whether this locational niche is shared

131   with CD4$^+$ T cells remains unknown. Using *IL13-eGFP* mice, we investigated ILC2 numbers

132   in various sites in the lung both at homeostasis and after acute exposure to either the fungal

133   allergen *Alternaria alternata* (Alt) or rIL-33. **We first quantified GFP$^+$CD45$^+$ cells among live**

134   **lymphocytes that were either lineage positive versus lineage negative cells (fig. S1A).**

135   **ILC2 were further defined by pre-gating on live (determined using a fixable live dead**

136   **dye), GFP$^+$CD3$^-$NKp46$^-$ cells that were lineage (TCRβ, TCRγδ, CD5, CD19, CD11b,**

137 **CD11c, FCεR1, GR-1, F4/80, and TER-119) negative cells and co-expressed CD90.2,**

138 **KLRG-1, CD127 and intracellular cytokine IL-13 (fig. S1B).** As expected, in control mice,

139 ILC2 were present in very low numbers in the airways, lung tissue and lung draining lymph

140 nodes (mediastinal lymph node) (Fig. 1, A to C) as well as the blood, bone marrow, spleen,

141 inguinal and mesenteric lymph nodes (fig. S2). However, during inflammation induced by i.n.

142 administration of rIL-33 or Alt, ILC2 numbers significantly increased in the bronchoalveolar

143 lavage fluid BAL (Fig. 1A), lung tissue (Fig. 1B) and the lung draining lymph nodes (Fig. 1C)

144 as well as after rIL-33 treatment in the blood and bone marrow but not spleen, inguinal and

145 mesenteric lymph nodes (fig. S2). To confirm that we were not excluding unstimulated ILC2

146 (IL-13$^-$/GFP$^-$) in our gating, we additionally re-evaluated ILC2 numbers by gating on CD45$^+$lin$^-$

147 NKp46$^-$CD3$^-$ cells and analysed the frequency of GATA-3$^+$ (a faithful transcription factor to

148 define all ILC2s) cells (fig. S3A). Here we used balb/c mice instead of IL-13 eGFP mice as the

149 GFP signal was significantly lost following intra-nuclear staining for GATA-3. Balb/c mice were

150 challenged with PBS, Alt or rIL-33 and lungs, BAL and blood ILC2s were evaluated by gating

151 on GATA-3$^+$ ILC2s. Our data suggests that ILC2s in mock treated mice are still very low in

152 frequency compared to Alt and rIL-33 treatment (fig. S3B and C) and expressed significantly

153 lower IL-13 and IL-5 (fig. S3D). Furthermore, by phenotyping GATA-3$^+$ ILC2s for extracellular

154 markers, our data suggests that each of the surface markers is altered as per the type of

155 treatment as shown by the percent expression of each marker (fig. S3E-H) further highlighting

156 the plasticity of ILC2s in inflammation.

157

158 Similarly, imaging precision cut lung slices (PCLS) from rIL-33 treated IL13-eGFP mice,

159 revealed substantialy more IL-13$^+$GFP$^+$ cells (fig. S4A) which largely accumulated around

160 large airways and large blood vessels and to a lesser extent around alveolar capillaries (fig.

161 S4B). To establish that the IL-13$^+$GFP$^+$ cells observed after rIL-33 treatment were pre-

162 dominantly ILC2, we evaluated the proportion of GFP$^+$ cells that were CD45$^+$lin^{neg}CD4$^-$ and

163 expressed ILC2 defining markers (CD90.2, CD127, KLRG1,CD25 and co-expressed

164 intracellular IL-13). Our data showed that 98.86% of observed GFP$^+$ cells were ILC2, and only

165 1.14% of GFP$^+$ cells were CD4 T cells (fig. S4C). The source of IL-13 by ILC2 versus CD4$^+$ T

166 cells after 1 week of rIL-33 administration was further quantified (fig. S4D).

167 Since a minor proportion of CD4$^+$ T cells produced IL-13 in our lung inflammation model, we

168 included a CD4-T cell antibody to precisely evaluate the effects of rIL-33 administration on

169 numbers and phenotype of CD4$^-$GFP$^+$ ILC2 in PCLS studies. Our PCLS imaging revealed that

170 rIL-33 treatment induced a significant increase in GFP$^+$ ILC2 numbers compared to Alt or mock

171 treated mice (Fig. 1D). rIL-33 treatment induced a 6-fold greater ILC2 number compared to Alt

172 treatment (Fig. 1,D and E), consistent with the number of ILC2 detected by flow cytometry

173 (Fig. 1, A to C) and with a shared locational distribution in the inflamed lung tissue (fig. S4A).

174 Interestingly GFP$^+$ cells surrounding the bronchioles were significantly greater than the GFP+

175 cells in the alveolar tissue (fig. S4B). The majority of the GFP$^+$ cells were IL-13 producing ILC2

176 (fig. S4, C and D) and hence represented IL-13$^+$ activated ILC2, referred from here on as ILC2

177 for simplicity. Higher magnification views of the peribronchial space bordering the airways

178 revealed an intimate association of some ILC2 with the airway epithelium and that ILC2 shared

179 a locational niche with CD4$^+$ T cells (Fig. 1F, depicting two representative images from a lung

180 slice). Overall, we demonstrate that rIL-33 treatment significantly increased the number of lung

181 ILC2 compared to Alt or PBS treatment and ILC2 accumulate around the airways of inflamed

182 lungs.

183

184 **IL-33 stimulation induces ILC2 motility around blood vessels and airways.**

185 Intranasal administration of either rIL-33 or Alt induces robust inflammation in the lung (*42,*

186 *43*). A hallmark of acute tissue inflammation is leukocyte recruitment. ILC2 are relatively rare

187 in the lung compared to other leukocytes under homeostatic conditions but accumulate rapidly

188 after rIL-33 induced inflammation (Fig. 1). We have previously demonstrated that the epithelial

189 cell derived TGF-β induced after rIL-33 treatment promoted a high degree of ILC2 motility *in*

190 *vitro* (*34*).

191

192 However, to date, dynamic movement of ILC2 has not been visualized in the lung. Since lung

193 ILC2 are relatively rare at homeostasis (Fig. 1, B, D and E, fig. S3), we evaluated ILC2

194 movement in the inflamed lungs of *IL13-eGFP* mice. Imaging live PCLS from rIL-33 treated

195 *IL13-eGFP* mice showed that ILC2 were motile in the peribronchial and perivascular space

196 (Fig. 2A; movie S1). Closer examination of the lung tissue surrounding the blood vessel

197 revealed that the majority of ILC2 exhibited 'amoeboid-like' exploratory movement (Fig. 2, B

198 and C; movies S2 and S3A) with pseudopodia and bleb-like processes in the extravascular

199 lung tissue surrounding the blood vessel, while a lesser proportion of ILC2 exhibited

200 'oscillatory' blebbing activity with little actual movement away from their start position (Fig. 2,

201 B and C; movies S2 and S3B). Similar to oscillatory ILC2, CD4$^+$ T cells (cyan) did not appear

202 to displace from their point of origin in the lung tissue (Fig. 2B; movie S2). Cell tracking of ILC2

203 and CD4$^+$ T cells (Fig. 2, D and E) revealed that in PCLS from rIL-33-treated mice, ILC2 track

204 speed (Fig. 2F), track length (Fig. 2G) and track displacement (Fig. 2H) was significantly

205 higher than that of CD4$^+$ T cells. Interestingly, ILC2 from Alt treated mouse lungs moved more

206 slowly, with shorter tracks, compared to rIL-33-treated mice (Fig. 3, A and B). In addition, rIL-

207 33 treatment induced increased ILC2 track speed (Fig. 3C), track length (Fig. 3D) and track

208 displacement (Fig. 3 E) compared to Alt treatment. Since rIL33 promoted greater ILC2

209 movement than Alt treatment, we focused on rIL-33 induced changes on ILC2 motility

210 thereafter. Since PCLS do not recapitulate the forces exerted by blood-flow and breathing *in*

211 *vivo*, we also performed lung intravital microscopy in live mice using a similar strategy to

212 previous work (*44*). We found that consistent with our data in live PCLS (Fig. 1, D to F, Fig. 2,

213 A and B), ILC2 were enriched in the extravascular tissue close to larger blood vessels and

214 exhibited amoeboid-like movement within the lung tissue (Fig. 3F, movie S4A and 4B).

215

216 In summary, our results demonstrate that ILC2s are highly dynamic in the lung after treatment

217 with either rIL-33 or allergen, where they exhibit amoeboid-like movement and travel faster

218 over greater distances than CD4$^+$ T cells.

219

ILC2s utilize distinct chemotactic pathways to home to inflammatory sites in the lung.

We have shown that within the lung, ILC2s display a specific distribution pattern and movement behaviour distinct from T cells. However, the factors that regulate ILC2 dynamics are not well understood. Since ILC subsets have key features analogous to T cell populations, it has been suggested that molecular pathways which control motility, migration and tissue homing are common to ILC subsets and T cells, but this has not yet been proven. Therefore, we examined the pattern of chemokine receptor expression on ILC populations (defined as CD45$^+$GFP$^+$Lin^{neg}KLRG1$^+$CD127$^+$CD25^{var}) isolated from the lungs of rIL-33 treated mice (or PBS treated controls) (fig. S5A and B). Surprisingly, despite the high degree of resemblance between ILCs and T cells, many of the prototypic chemokine receptors found on T cell subsets were absent on ILC, including CCR3, CCR4 (Th2), CCR6 (Th17), and CCR5 and CXCR3 (Th1). Notably, ILC did express both CCR1 and CCR8, which are associated with type 2 cytokine production by T cells in allergic contexts (*45*).

Therefore, we examined expression of CCR1, 4 and 8 specifically on IL-13$^+$ ILC2, finding that the majority of IL-13$^+$ ILC2 express CCR1 (>50%) and/or CCR8 (>90%) and that the expression of these chemokine receptors was higher in rIL-33 treated mice than in PBS treated controls (Fig. 4A). In contrast to T cells, we found that CCR4 expression was not associated with a type 2 phenotype in ILC with only 10% of IL-13$^+$ ILC also expressing CCR4. Furthermore, *in vivo* activation of ILC2 with rIL-33 did not alter CCR4 expression (Fig. 4A). Interestingly, we also found significantly greater levels of CCL8 protein, a ligand for CCR8 in mice (*46*) in the airways (Fig. 4B) and lungs (Fig. 4C) of rIL-33 treated mice compared to PBS treated control mice. We additionally evaluated other CCR8 ligands (CCL1) and CCR1 ligands (RANTES) (fig. S5C and D) in lungs of mice exposed to rIL-33 compared to control treated mice. PCLS and intravital imaging demonstrated that ILC2 accumulate in the peribronchial and perivascular space and are highly motile in rIL-33 treated IL13-eGFP mice (Fig. 2, A to C and Fig. 3F). Hence, we determined whether we could identify CCL8 chemokine deposits in areas of ILC2 movement in live PCLS of rIL-33 treated IL13-eGFP mice. CCL8 was strongly

248 expressed in the peribronchial space where ILC2 accumulate after rIL-33 treatment, at

249 significantly greater levels than basal CCL8 expression in control mice (Fig. 4, D and E) with

250 airway macrophages being the major producer for CCL8 in our model (fig. S5E).

251

252 To examine the migration of ILC2 to chemotactic agents we sorted human ILC2 from

253 peripheral blood (CD45^{+}, Lin^{neg}, CD161^{+}, CD127^{+}, CRTH2^{+} and C-Kit [variable]-) then cultured with

254 recombinant IL-2, IL-7 and IL-33 for at least 4 weeks (resulting in >99% ILC2). These ILC2s

255 expressed GATA-3 and IL-13 (fig. S6A) and produced IL-13 (fig. S6B). Among the three

256 cytokines required for ILC2 maintenance, our results suggest that IL-7 signalling in ILC2 was

257 critical for their survival, proliferation and cytokine production as demonstrated by viability (fig.

258 S6C) GATA-3 expression (fig. S6D), Ki67 staining (fig. S6E) and IL-5 production (fig. S6F).

259 We additionally compared differences in motility between ILC2 starved of IL-2 vs IL-33

260 compared to ILC2 that received all three cytokines. After 12h of live *ex vivo* imaging ILC2 track

261 speed (fig. S6G) remained comparable (movie S5A, B and C). ILC2 that were starved of IL-7

262 could not be included in this set up as absence of IL-7 significantly reduced ILC2 viability (fig.

263 S6C). Our findings are consistent with the literature and reemphasize the importance of IL-7

264 signalling being critical for the maintenance of ILC2 and other IL-7R^{+} lymphocyte populations

265 (*47*).

266

267 The potential of *in vitro* cultured ILC2 to migrate to a known ILC2 chemotactic factor - the

268 eicosanoid PGD_2 (*48*), was examined using a modified Boyden chamber chemotaxis assay

269 (Fig. 4F). As expected, all human ILC2 lines tested migrated to PGD_2 and peak chemotaxis

270 occurred at a concentration of 100nM PGD_2 consistent with previous studies (*48*). Since we

271 found CCL8 in the BAL and CCL8 deposits in the lung of rIL-33 treated mice (Fig. 4, D and

272 E), we queried whether CCL8 could induce the migration of human ILC2. Using the same

273 modified Boyden chamber assay, we found that CCL8 also induced human ILC2 chemotaxis

274 in a dose-dependent manner, peaking at 10nM CCL8 (Fig. 4G). We next compared migration

275   of ILC2 to factors thought to influence ILC2 movement. After determining the optimum

276   concentrations for each factor, we found that although PGD$_2$ was the most efficacious inducer

277   of ILC2 migration, TGF-β, IL-33 and CCL8 all promoted comparable levels of migration (Fig.

278   4H). Taken together, these data identify specific chemokine receptor expression in ILC2 and

279   show the corresponding ligand availability in areas of ILC2 accumulation. In order to confirm

280   that CCL8-CCR8 axis plays an important functional role in ILC2 migration in vivo, we

281   administered a blocking antibody against CCR8 or isotype Ab as a control and simultaneously

282   treated mice with rIL-33. Our live PCLS data showed that blocking CCR8 significantly reduced

283   ILC2 accumulation in the peribronchial region compared to control mice (Fig. 4, I and J) and

284   ILC2 migration was impaired after CCR8 receptor blocking (movie S6, A and B) with shorter

285   tracks depicted as track overlay between treatments (Fig. 4K) and reduced track length (Fig.

286   4L), track speed (Fig. 4M) and track displacement (Fig. 4N). Closer analysis of ILC2 from each

287   of these treatments revealed that significantly less of the ILC2 in rIL-33 treated mice receiving

288   the anti-CCR8 blocking antibody expressed IL-13$^+$IL-5$^+$ (Fig. 4O) and those cells that did

289   express the type 2 cytokines had significantly less IL-13 and IL-5 as determined by the mean

290   fluorescence intensity (MFI) (Fig. 4P-R). Overall, our data suggests that the CCL8/CCR8

291   pathway plays an essential role in inducing ILC2 accumulation, activation and movement

292   within the lung during rIL-33 induced inflammation.

293

294   **Extracellular matrix proteins, collagen-I, collagen-IV and fibronectin, promote**

295   **increased ILC2 motility**

296   In addition to chemokines, structural guidance cues such as extra cellular matrix (ECM)

297   proteins have been shown to facilitate movement of immune cells, including T cells (*41*). In

298   chronically inflamed lung tissue, as observed during asthma, aberrant ECM expression from

299   tissue-remodelling can further influence immune cell activation and survival, thereby altering

300   inflammatory immune responses (*49*). Therefore, we next examined whether ECM proteins

301   influenced ILC2 movement. Fibronectin, collagen-I, III (fibrillar collagens) and collagen-IV

302   (basement membrane collagen) are located around large airways and blood vessels and are

12

303 altered during lung inflammation (*50*). Proteoglycans such as versican and tenascin C are

304 changed during remodelling in various lung disorders, including asthma and chronic

305 obstructive pulmonary disease (COPD) (*51*).

306 Therefore, the influence of collagens-I, -III and -IV, fibronectin, and proteoglycans, versican

307 and tenascin C, on ILC2 movement was examined in a simplified 2D cell migration assay.

308 Human ILC2 were cultured on these substrates and cell movement tracked over 12h *in vitro*

309 by using an automated live cell imaging system using culture media with or without heat-

310 inactivated fetal bovine serum (FBS) as controls. In contrast to serum-free media, collagen-I,

311 fibronectin and collagen-IV significantly enhanced dynamics of ILC2, as shown in the cell

312 tracks and speeds (Fig. 5, A to C, movie S7, A to E). As expected, FBS supplemented media

313 significantly enhanced cell movement (Fig. 5, A to C and movie S7B). In contrast to collagen-

314 I, fibronectin and collagen-IV, which showed strong effects on ILC2 dynamics, collagen-III,

315 versican and tenascin C showed minimal influence on ILC2 movement (fig. S7A). Collagen

316 fibres also produce contrast in two-photon microscopy by generating a strong second

317 harmonic signal in tissue. Second harmonic generation (SHG) imaging suggested that the

318 area around bronchioles and associated vessels had a strong fibrillar collagen component in

319 both IL33-treated and control PCLS (Fig. 5D). We noticed a qualitative difference in the SHG

320 signal with the collagen fibres in perivascular / peribroncheal areas of rIL-33 treated mice

321 appearing 'spikier' than those in PBS treated control mice. Therefore, we analysed the

322 geometry of the SHG images of fibrillar collagen in the perivascular / peribronchial area using

323 a grey level co-occurrence matrix (GLCM) technique to quantify and compare the SHG signal

324 (*52, 53*). Initially we plotted correlation versus comparison distance. This analysis compares

325 the organisation of the extracellular matrix by considering how far it is possible to travel in a

326 straight line away from a start point and observe the same intensity (grey level) between pairs

327 of pixels. Images containing features that are generally organised in long straight lines will

328 therefore have higher levels of intensity correlation over longer comparison distances and

329 images that display structures more randomly orientated will lose this correlation at shorter

330 comparison distances producing decays with steeper slopes. As can be seen from the data

(Fig. 5E) GLCM SHG analysis of the perivascular / peribronchial area from IL-33 treated mice gave steeper decays than the controls, indicating a more disordered arrangement of shorter fibres. We also plotted homogeneity versus comparison distance. This parameter considers the distribution of grey levels at different comparison distances across the image. Therefore, images that have a texture less consistent with long fibrillar structures will have steeper decays in homogeneity with comparison distance. These data (Fig. 5, D and E) also highlighted contrast between the two conditions.

In addition to collagen, fibronectin expression was also evaluated by staining for fibronectin in live PCLS from PBS or rIL-33 treated mice. Our results showed that fibronectin expression is enriched in the peribronchial region of rIL-33 treated mice (Fig. 5F). Collectively, our data indicate that collagen-I, -IV and fibronectin influence ILC2 speed *in vitro*. Furthermore, collagen and fibronectin are modified in areas where ILC2 accumulate during rIL-33 induced inflammation.

Collagen-I enhances ILC2 actin cytoskeletal remodelling and polarity

ECM protein 'tracks' can define the path of motile cells through the tissue (*54*). We observed that ILC2 exhibited amoeboid-like movement after rIL-33 induced inflammation (Fig. 2 and 3 F). Amoeboid cell movement involves induction of a simple polarized shape, dynamic pseudopod protrusion and retraction, flexible oscillatory shape changes, and rapid low-affinity crawling (*55*). Our results showed that ECM proteins collagen-I, fibronectin and collagen-IV promoted increased human ILC2 motility in a 2D migration assay. Therefore, we next tested whether each ECM protein directly influenced changes in ILC2 phenotype, cytokine production and shape change in the same 2D environment. We found that ILC2 GATA-3 expression (fig. S7B) and IL-5 production (Fig. S7C) remained comparable after incubation over collagen-I or collagen-IV. However collagen-I selectively induced the most pronounced ILC2 shape change with characteristically elongated ILC2 cell bodies visible (Fig. 6, A and B). In amoeboid movement, force generation (protrusion and contraction) and force transduction (adhesion), are controlled by actomyosin cytoskeletal remodelling (*56*). We imaged f-actin localisation and

359   quantified ILC2 shape after 12h on the different ECM. ILC2 seeded on collagen-I were highly

360   polarised with areas of f-actin accumulation (Fig. 6B) and had significantly greater cell area

361   and perimeter (Fig. 6, C and D). Taken together these data indicate that collagen-I influences

362   ILC2 shape towards the pro-migratory exploratory phenotype we described *in vivo* (Fig. 2 and

363   3 F).

364

365   **Blocking collagen fibrillogenesis *in vivo* increases ILC2 dynamics and reduces**

366   **eosinophil accumulation in the inflamed lung.**

367   We have previously demonstrated that epithelial cell derived TGF-β1, induced after rIL-33

368   treatment, can drive ILC2 movement (*34*). Furthermore, our results show that collagen-I

369   significantly alters ILC2 shape by elongating the cell body (Fig. 6). TGF-β has been shown to

370   enhance collagen-I and III mRNA expression during remodeling and blocking collagen

371   deposition restores baseline collagen levels (*57*). We observed changes to collagen geometry

372   in the peribronchial space where ILC2 are highly dynamic after rIL-33 treatment (Fig. 5, D and

373   E). Hence, we next investigated whether we could extend our findings from the 2D assays

374   previously described in Fig 6 into *in vivo* 3D tissue by blocking newly synthesized collagen

375   and measuring ILC2 movement after rIL-33 induced inflammation. The fibrillar structure of

376   type 1 collagen morphology is stabilized by inter and intra molecular crosslinks initiated by

377   lysyl oxidases whose expression can be inhibited by β-amino propionitrile (BAPN) (*57*).

378   Following rIL-33 treatment, we administered BAPN i.p. Strikingly, mice that received rIL-33

379   along with the collagen crosslinking inhibitor showed longer tracks depicted as individual

380   tracks (Fig. 7A) and track overlay between treatments (Fig. 7B). Cell track quantification

381   revealed that ILC2 from lungs of mice that were rIL-33 treated along with BAPN displayed

382   increased ILC2 track speed (Fig. 7C), track length (Fig. 7D) and track displacement (Fig. 7E)

383   compared to rIL-33 treated mice suggestive of a reduced interaction with the ECM.

384   Although addition of BAPN altered ILC2 motility in rIL-33 treated mice, ILC numbers only

385   slightly reduced in the lungs (Fig. 7F) with comparable ILC2 numbers in the BAL (Fig. 7G) and

386   blood (Fig. 7H). We additionally tested if BAPN directly activated ILC2 by evaluating cytokine

387   production by ex vivo sorted ILC2 from rIL-33 treated IL-13 eGFP mice. Our results showed

388   that within the time frame of 36 h post incubation, IL-5 production by ILC2 remained

389   unchanged after incubation with collagen-I + BAPN or collagen-I alone (fig. S8A). Interestingly

390   addition, of rCCL8 to the cultures significantly increased IL-5 production (fig. S8A). We next

391   evaluated if blocking collagen fibrillogenesis in rIL-33 treated mice impacted on the

392   inflammatory infiltrate in the lungs and altered lung function. rIL-33 + BAPN treated mice had

393   unchanged lung function (Fig. 8A) and comparable numbers of airway macrophages (Fig. 8B),

394   dendritic cells (Fig. 8C), neutrophils (Fig. 8D) compared to mice treated with rIL-33 alone.

395   Interestingly, blocking collagen fibrillogenesis in rIL-33 treated mice significantly reduced the

396   frequency of eosinophils (Fig. 8E) and lung CCL24 levels (Fig. 8F) while eosinophil motility

397   remained unaltered compared to mice treated with rIL33 treated alone (movie S8, A and B)

398   and further quantified by track length (fig. S8B) and track speed (fig. S8C). We finally

399   compared eosinophil accumulation in the peribronchial region of the lungs where ILC2

400   accumulate and found significantly lower number of eosinophils as demonstrated by congo

401   red staining (Fig. 8G and H).

402   Therefore, blocking newly synthesized collagen during rIL-33 treatment significantly changed

403   ILC2 motility and the ensuing eosinophil accumulation. These data indicate that ILC2

404   interactions with the ECM may impact their function and contribution to eosinophilic

405   inflammation.

406

407   **Discussion:**

408   Immune cell recruitment in inflammation involves emigration of activated leukocytes from the

409   vasculature across inflamed endothelium into the tissue space through activation of specific

410   effector programs (*58*). The ensuing response must be robust enough to control infection or

411   repair damage but sufficiently restrained so as to prevent excessive tissue damage and

412   pathology. Mucosal surfaces, such as the lung, constantly encounter potentially harmful

413   threats in the form of microbes, allergens and pollutants that can damage lung tissue and

414   impair normal physiological lung function. Group 2 innate lymphoid cells (ILC2) are central in

415 orchestrating type-2 immunity (*28*) and promoting tissue repair (*13*). We have previously

416 shown that during lung inflammation, *in vivo* and *in vitro*, TGF-β expression influences ILC2

417 movement but the mechanisms which promote ILC2 migration are not well understood (*34*).

418 Hence a more detailed spatio-temporal analysis of ILC2 induction and dynamics in the lung is

419 of great importance to develop a thorough appreciation of ILC2 behaviour and function, and

420 their interaction with other cells during type 2 inflammatory responses. Since ILC2 are a rare

421 immune cell population in the lung at homeostasis (*28*), visualizing ILC2 by intravital imaging

422 in the lungs of living animals has been challenging. Delivery of rIL-33 in mice drives type-2

423 immunity and ILC2 expand robustly and produce large amounts of IL-13 (*28, 34*) even in the

424 absence of T and B cells (*42*). In this report, by utilizing a model of rIL-33 or Alt driven lung

425 inflammation in IL-13eGFP knock-in mice, combined with sophisticated lung imaging, we

426 demonstrate for the first time that ILC2 are highly dynamic in the lung *in vivo*. Following an

427 acute inflammatory exposure, ILC2 numbers in the lung increased substantially by around 30

428 (Alt) - 100 (rIL-33) fold respectively. Simultaneously, our results show an incremental increase

429 in the number of ILC2 in both the lung draining LN and circulation after Alt or rIL-33 induced

430 inflammation. A recent study reported that administration of rIL-33 in mice promotes the egress

431 of ILC2 progenitors (ILC2Ps) from the bone marrow to peripheral tissues while mice lacking

432 IL-33 signalling had a significant accumulation of ILC2Ps in the bone marrow (*59*). This

433 suggests that the rapid expansion in ILC2 numbers induced in the lung in our model of rIL-33

434 inflammation could result as a consequence of recruitment of ILC2Ps from the BM rather than

435 *in situ* proliferation of a small steady state resident precursor. In addition, there was no

436 increase in ILC2 numbers in the spleen or non-lung draining lymph nodes indicating that

437 intranasal rIL-33 signals for ILC precursors in the bone marrow to traffic to the lungs. Our

438 imaging studies also depict ILC2 accumulation in the extra-vascular tissue surrounding large

439 blood vessels again indicating that these cells are perhaps likely to be recent emigrants from

440 the BM.

441

442   In the lung, we found that ILC2 mainly accumulate in the peribronchial / perivascular area and

443   to a lesser extent in the parenchyma. The location of ILC2 in the peribronchial area is

444   consistent with their postulated role in promoting epithelial repair. In a previous study, we

445   showed that ILC2 in the airway lumen are primed to respond to epithelial cell derived TGF-β

446   induced after rIL-33 administration, as a result of high cell surface expression of TGF-βRII,

447   and that epithelial cell derived TGF-β enhanced the migratory activity of ILC2 (*34*). Hence a

448   critical molecular communication exists between ILC2 and bronchial epithelial cells to promote

449   ILC2 movement during lung inflammation and could explain why ILC2 home to the

450   peribronchial region of the lung tissue.

451

452   By using live lung slices, we demonstrated an amoeboid pattern of movement of lung ILC2

453   after rIL-33 treatment. This observation was recapitulated in the lungs of live intact mice using

454   lung intravital microscopy of rIL-33 treated IL13eGFP mice. We found that similar to our data

455   in live lung slices, ILC2 were enriched in the lung tissue surrounding larger blood vessels

456   rather than the alveolar capillaries and characteristically move in an amoeboid manner during

457   locomotion. However, despite their functional and phenotypic similarities to CD4^{+} T cells,

458   ILC2 differed markedly in their migratory behaviour moving faster and to greater distances

459   than CD4^{+} T cells in the inflamed lung. The physical proximity of motile ILC2 to epithelial

460   cells further supports the notion that ILC2 may directly influence epithelial cell function and

461   vice versa and may not be restricted to communication via secreted cytokines but could

462   result from physical receptor-ligand interactions. It is known that motility patterns and

463   distances travelled by various leukocyte subpopulations differ (*60*). In our study, we find that

464   lung ILC2 exhibit amoeboid pattern of movement travelling distances in the range of 100-

465   200μm perhaps displaying 'searching' or 'exploratory' behaviour, consistent with their

466   sentinel role at mucosal sites (*61*). Taken together, our data suggests that lung ILC2 may

467   exhibit motility patterns similar to innate immune cells. This is consistent with findings in the

468   skin, where dermal ILC2 patrol their environment with an average speed similar to that of

469   dermal dendritic cells (*38*). This scanning behaviour of ILC2 may be suggestive of their need

470   to detect local alarmins or danger signals by damaged epithelial cells in the lung after an

471   inflammatory insult. Furthermore, the migration of lung ILC2 in close association with bronchial

472   epithelial cells seems likely to ensure that factors secreted by the epithelium, such as TGF-β,

473   can rapidly engage the ILC2. It presumably also ensures that signalling in the reverse

474   direction, from the ILC2 to the epithelial cells, for promoting epithelial repair can take place

475   efficiently.

476

477   Factors driving leukocyte motility include the distribution of adhesion molecules on leukocytes,

478   as well as chemotactic signals. It has been well established that lung ILC2 express both β1

479   and β2 integrins at homeostasis and after Alt-induced inflammation (*39*). Furthermore, ILCs

480   share many of the key features analogous to T cell populations, for example ILC2 and T cells

481   both use the homing receptor CCR7 to actively migrate to lymphoid tissues (*3*). In addition, it

482   has been shown recently that inflammatory iILC2, originating from precursors in the intestinal

483   lamina propria express high levels of CCR9 and use sphingosine 1-phosphate receptors to

484   exit the intestine into the lymph and move to distant sites like the lung where they display

485   effector activity (*15*). Interestingly, progenitors to iILC2 do not exist in the lung and are not

486   induced after inflammation induced via the intra nasal route (*15*). Hence, the chemokine

487   migratory programs of ILC2 generated in the lung after intranasal instilled mediator driven

488   inflammation have remained elusive until now and very little is understood about the process

489   by which ILC2 are attracted to sites of immune insult in the lungs. At homeostasis, we found

490   that CCR8 and CCR1 was expressed by the majority of murine lung ILC2, in contrast to other

491   T cell subsets. However, following rIL-33 treatment, the percentage of CCR8 and CCR1

492   expressing ILC2 further increased.

493

494   In addition, TGF-β acts as an ILC2 chemo-activator enhancing murine ILC2 migration in a

495   non-directional manner. Furthermore, both PGD$_2$ and to a much lesser extent IL-33 have been

496   shown to be chemotactic for human skin- and blood-derived ILC2 (*48*). Interestingly, we show

497   for the first time that in addition to PGD2, CCL8 is chemotactic for ILC2 with nanomolar

498   potency. We found that CCL8 deposits were strongly expressed in the peribronchial space

499   where murine lung ILC2 accumulate. This could be attributed to the CCL8–CCR8 ligand-

500   receptor axis which has been shown to be crucial in localizing pathogenic Th2 cells to the site

501   of eosinophilic inflammation and in driving the allergic process in the skin (*62*). To further

502   explore this hypothesis, we blocked CCR8 receptor in rIL-33 treated mice and analysed effects

503   on ILC2 motility. Our data strongly suggest that signalling via CCR8 is critical for ILC2 cytokine

504   (IL-13 and IL-5) production as well as motility of IL-13$^+$ activated ILC2. A variety of resident

505   pulmonary cells secrete CCL8, the CCR8 ligand in mice. We find that lung airway

506   macrophages are the pre-dominant cellular source of CCL8 in rIL-33 treated mice. Our data

507   suggest a previously undescribed model where macrophage derived CCL8 acts **to increase**

508   **ILC2 accumulation** to areas proximal to epithelial cells.

509

510   Overall, our results provide insights into the independent and distinct migration pathways ILC2

511   utilize in contrast to T cells. Extracellular matrix proteins such as collagens are well-known to

512   interact with leukocytes by directly binding to cell surface receptors (*63*). In this respect,

513   changes to subepithelial collagen and extracellular matrix protein deposition are important

514   pathophysiological components of airway remodeling in Th2 driven lung inflammation. We find

515   strong fibrillar collagen deposition around bronchioles and associated vessels in both rIL33-

516   treated and control PCLS where lung ILC2 accumulate. Interestingly rIL-33 treated mice

517   showed quantitative differences in both correlation and homogeneity parameters of fibrillar

518   collagen geometry compared to control mice. rIL-33 induced epithelial TGF-β is chemoactive

519   for ILC2 and administration of TGF-β in mice increases collagen-I mRNA expression in mouse

520   lungs (*49, 57*). This suggests that altered collagen distribution as a result of rIL-33 induced

521   inflammation may influence ILC2 migratory programs. In line with this, our *in vitro* 2D migration

522   assays indicate that collagen-I exclusively alters ILC2 morphology with enhanced cell spread

523   and elongation of cell body. Hence *in vivo*, collagen-I fibres may support a more polarized

524   ILC2 morphology, by increasing ILC2 traction, reducing ILC2 speed and allowing for longer

525   dwell times in specific sites of inflammation in the lung. Cross-links are essential for the

526   mechanical stability of collagen and have been shown to control T cell mobility (*64*). To test

527   the overall contribution of newly synthesized collagen on ILC2 dynamics, we used beta-

528   aminopropionitrile (BAPN) to block the formation of new collagen cross-links by inhibiting the

529   enzyme lysyloxidase. Interestingly, blockade of collagen fibrillogenesis significantly increased

530   ILC2 speed and travel distance after rIL-33 treatment. Hence suggesting that reduced collagen

531   stability, resulting as a consequence of blocking new collagen fibrillogenesis may disrupt a

532   controlled receptor-ligand interaction of ILC2 with collagen-I fibres, increasing overall

533   dynamics of ILC2. Intriguingly, although collagen-I blocking increased ILC2 movement, rIL-33

534   induced eosinophilia was significantly reduced in the lungs of these mice. We have previously

535   reported that after house dust mite treatment in mice, chemoactive epithelial derived TGF-β is

536   essential for ILC2 activation and critically controls all hallmarks features of allergic responses

537   including airway hyperresponsiveness and eosinophilia (*34*). Inferring from this knowledge our

538   data suggests that although the initial cytokine driven instruction from structural cells initiates

539   ILC2 movement, components of the inflamed lung environment namely, CCL8-CCR8

540   signaling and collagen-I are critical for regulating controlled **ILC2 accumulation** and dynamics

541   imperative for ensuring robust type-2 inflammation. Hence although TGF-β serves as a

542   chemokinetic mediator, CCL8 induced after rIL-33 treatment is an important chemotactic

543   signal that mediates ILC2 motility and cytokine production via CCR8 receptor. Furthermore,

544   the difference in interfibrillar geometry of collagen fibrils alters ILC2 migratory patterns and the

545   dwell time of ILC2 with collagen-fibrils is a critical factor towards ILC2 behaviour and function

546   in type-2 inflammation. Thus combined environmental signals from damaged lung tissue act

547   in concert to mechanistically drive ILC2 migratory patterns in the inflamed lung.

548

549   ILC2 are defined by the combination of several extracellular and intracellular markers. Here

550   we use multicolour flow cytometry of cell suspensions from dissociated lung tissue to identify

551   and quantify these cells, but this approach loses spatiotemporal information by

552 definition. Therefore, we used a combination of imaging using IL13-GFP reporter mice in

553 combination with a handful of markers and dyes and detailed multicolour flow cytometry

554 together to mitigate potential misidentification of the cells. However, in an ideal experiment

555 we would be able to image tens of markers simultaneously. Newer approaches (for eg:

556 imaging mass cytometry) are starting to address this, but none allow live organism / cell /

557 tissue imaging to directly address the spatiotemporal dynamics of these populations, a

558 significant challenge in the field.

559
560 Overall, using highly sophisticated imaging techniques, we reveal how ILC2 migrate within

561 an inflamed tissue, interact with adjacent cells and lung environmental factors during a type-

562 2 inflammatory response. Specifically, we show that lung ILC2 are highly motile *in vivo* and

563 exhibit an exploratory amoeboid pattern of movement during inflammation. Additionally, we

564 identify signature chemokine receptors employed by ILC2 to home to inflammatory sites in the

565 lung. Our data provide novel insights into the mechanism by which diverse lung environmental

566 factors combine to control ILC2 migration and provide direct evidence for collagen-I in

567 exclusively regulating ILC2 shape and polarity, thereby influencing the outcome of an

568 inflammatory response. Imaging these dynamic cellular events in real-time in the lungs of

569 living animals has provided an important visual platform that could be employed for further

570 interrogation of ILC2 and other immune cell functions in the context of allergen or pathogen

571 driven Th2 inflammation.

572

573
574 **Materials and Methods**

575

576 **Mice**

577 Adult female IL13-eGFP (kind gift from A.N. McKenzie, MRC Laboratory of Molecular Biology,

578 Cambridge) (*28*) were used for experiments between 7 and 10 weeks of age. Mice were

579 housed in specific-pathogen-free conditions and given food and water ad libitum. All

580 procedures were conducted in accordance to the institutional guidelines and under the

approval of our Home Office project licence (granted under the Animals (Scientific Procedures) Act 1986). Mice were administered 10µg (*Alternaria alternata*) extract (Greer Laboratories) in 25µl of PBS intranasally 3 times a week for 1 week or PBS alone as controls. Alternatively, carrier-free recombinant murine rIL-33 (1µg per dose in 25µl PBS) (eBioscience) or PBS was administered 3 times a week for 1 week. Mice were culled 24hrs after the final cytokine or allergen dose.

Live Precision Cut Lung Slices

Ex vivo cultured precision-cut lung slices (PCLS) provided a 3D cell culture model to image ILC2 dynamics within the lung microenvironment and was adapted and modified from a previously described protocol (*65*). Briefly, prior to slicing, lungs were inflated with 0.8ml volume of 2% low melting agarose (Thermofisher) prepared in PBS. Following inflation lungs were carefully dissected out and placed in HBSS containing 2.5% HEPES (GIBCO, Life Technologies). The intact left superior lung lobe was excised and 200µm transverse sections were prepared from the upper 1mm region of the left superior lung lobe using a Compresstome VF-300 vibrating microtome (Precisionary Instruments). PCLS were incubated in complete RPMI (10% FCS, 2 mM L-glutamine, 100U/ml penicillin/streptomycin) (GIBCO, Life Technologies) for 15mins at 37°C to wash off excess agarose surrounding the tissue. Antibodies were prepared in complete DMEM solution and lung explants were incubated in staining solution for 1.5hrs at 37°C prior to imaging. Time lapse videos depicting ILC2 movement were generated over an hour using an inverted laser-scanning confocal microscope (SP5, Leica microsystems). Videos were quantified using Imaris version 8.1 (BitPlane, Oxford Instruments) and ILC2 track speed, length and displacement were quantified using Motilitylab software.

Table S1. Antibodies used for imaging of PCLS

Antibody	Clone	Fluorochrome	Target species	Manufacturer
EPCAM	G8.8	PE	Mouse	Biolegend

| CD4 | RM4-5 | BV421 | Mouse | Biolegend |
| CD31 | 390 | Alexa-647 | Mouse | Biolegend |

607

Intravital microscopy

Mouse lung intravital microscopy was performed as previously described (*44, 66*). Briefly, anaesthetised and mechanically ventilated mice were placed on a heat-mat in a right lateral position. A small custom-built circular suction chamber was surgically inserted using a small incision between the ribs of the mice for imaging on an upright laser-scanning confocal microscope with Hybrid detectors (Leica SP5). Gentle suction was applied via the vacuum port to bring the cover slip contained in the chamber into contact with the lung and stabilise the tissue. Time-lapse videos depicting ILC2 movement were generated using 488, 54 and 633nm laser lines through a 25x 0.95 N.A. long working distance water immersion objective.

Fixed Cell / PCLS imaging

Phalloidin stained ILC and fixed PCLS from IL-33 reported mice were imaged using a Zeiss LSM 880 Airyscan NLO system. DAPI was excited using a 405nm diode laser, Phalloidin AlexaFlour488 was excited at 488nm using an argon-ion laser and detected using a sub-Airy detector 32-element array (Airyscan) and processed using 'auto' 3D Airyscan processing options. Airyscan z-stacks were acquired and Imaris (Bitplane) was used to visualise each cell. SHG signals (produced at a central wavelength of 475nm) were collected from fixed PCLS using a Coherent Chameleon Discovery femtosecond pulsed laser tuned to 950 nm and collected using a non-descanned GaAsP detector with 450±50nm detection filter. A 25x 1.0 N.A. zeiss long working distance water immersion objective was used to collect all images.

SHG Analysis

50μm z-stacks were imaged over a region of 425.1μm by 425.1, with at least three stacks /mouse (n = 6 mice / condition). GLCM was performed similarly to previous studies (*67, 68*)

24

633   Briefly, Image J (NIH) and the Norwegian University of Life Sciences (UMB) GLCM plugin was

634   used for the texture analysis, modified to run automatically through the four directions, for each

635   of the 100 comparison distances. A maximum projection image was automatically thresholded

636   to produce a mask that was then applied to remove background noise bias in the GLCM

637   analysis by selecting only the collagen SHG signal. The masked image was then passed to

638   the modified GLCM texture plugin. The data files produced were processed using an ImageJ

639   macro to output both the mean and individual values for each texture parameter for each

640   image. These were then imported into Prism (GraphPad), where exponential decay models

641   were fitted to the data.

642

643   **Cell isolation (BAL, Lung and lung dLN)**

644   The airways were washed three times with 400µl of PBS and bronchoalveolar lavage fluid was

645   collected. Following centrifugation of the BAL fluid supernatants were stored at -80°C for

646   further analysis and cells were resuspended in 500µl of complete RPMI media (10% FCS,

647   2mM L-glutamine, 100U/ml penicillin/streptomycin) (GIBCO, Life Technologies). For lung cell

648   isolation, the left lung lobes were cut into small pieces and digested in complete media

649   supplemented with 0.15mg/ml collagenase (Type D; Roche Diagnostics) and 25µg/ml DNase

650   (Type 1; Roche Diagnostics) for 1h at 37°C. Lung homogenate obtained was then filtered

651   through a 70µm sieve (BD Bioscience), washed, and resuspended in 1ml of complete media.

652   Erylysis was performed on 200µl whole blood and leukocytes were washed twice and then

653   then resuspended in 1ml of complete media.

654

655   **Flow Cytometry and intracellular staining**

656   For phenotyping ILC2 in IL-13 eGFP mice by flow cytometry, ILC2 were defined as live, singlet,

657   lymphoid, GFP$^+$CD45$^+$ lineage^{neg} (Lineage cocktail: TCRβ, TCRγδ, CD3e, CD5, CD19,

658   CD11b, CD11c, FCεR1, GR-1, F4/80, NKp46 and TER-119) CD3$^-$ NKp46$^-$,

659   CD90.2$^+$/CD127$^+$/KLRG1$^+$/CD25^{var} cells with positive intracellular IL-13 and IL-5 staining.

660

For intracellular cytokine staining, cells were stimulated with PMA (Sigma-Aldrich) and ionomycin (EMD Chemicals) in the presence of Brefeldin A (Sigma-Aldrich) and incubated for 4h at 37°C. Following stimulation, cells were washed and incubated for 20min with anti-CD16/32 (BD Pharmingen) prior to staining for extracellular antigens in 5% FCS/1% BSA in PBS for 30min at 4°C. All antibodies were purchased from eBioscience (table 2) with the exception of CD31, EpCAM, CD4 (Biolegend) also shown in table 1. Following staining for extracellular markers, cells were fixed with IC fixation buffer (E-Bioscience) for 10 mins at room temperature. Cells were then washed and stained for intracellular antigens using the Intracellular Fixation & Permeabilization Buffer Set (E Bioscience) or Foxp3 / Transcription Factor Staining Buffer Set (E-Bioscience) for GATA-3 staining.

Analysis was performed with LSRFortessa III and cell sorting on FACSAria III (BD Biosciences).

Table S2. Antibodies used for flow cytometry
.

Antibody	Clone	Fluorochrome	Target species	Manufacturer	
TCR-β	H57-597	APC	Mouse	E Bioscience	
TCR-γδ	ebio GL3	APC	Mouse	E Bioscience	
TER-119	TER-119	APC	Mouse	E Bioscience	
CD19	ebio ID3	APC	Mouse	E Bioscience	
CD11b	M1/70	APC	Mouse	E Bioscience	
CD5	53-7.3	APC	Mouse	E Bioscience	
F4/80	BM8	APC	Mouse	E Bioscience	
FCεR1	MAR1	APC	Mouse	E Bioscience	
GR-1	RB6-8C5	APC	Mouse	E Bioscience	
CD11c	N418	APC	Mouse	E Bioscience	
NKp46	29A1.4	PE-Dazzle	Mouse	E Bioscience	

26

CD45	30-F11	PerCP Cy5.5	Mouse	E Bioscience	
CD3e	145-2C11	APC-Cy7	Mouse	E Bioscience	
KLRG-1	2F1/KLRG1	PE-Cy7	Mouse	E Bioscience	
CD90.2	53-2.1	BV605	Mouse	E Bioscience	
CD25	PC61	BV510	Mouse	E Bioscience	
CD127	A7R34	BV711	Mouse	E Bioscience	
IL-13	ebio 13A	PE	Mouse	E Bioscience	
IL-5	TRFK5	BV421	Mouse	E Bioscience	
GATA-3	TWAJ	PerCP-eFluor 710	Mouse	E Bioscience	

676

677

678 **Lung histology and staining**
679
680 Paraffin embedded lung sections (4-micron thickness) were stained with haematoxylin and

681 Congo red-dyes. Images were taken under polarised light using an upright dry 40x objective.

682 The number of eosinophils in the peribronchial space were quantified and corrected for the

683 surface area scored.

684

685 **Human ILC2 bulk cultures**

686 ILC2 were enriched from whole blood using RosetteSepTM Human ILC2 Enrichment Kit

687 (STEMCELL Technologies) and further sorted by FACs using CD45^{+} Lineage^{neg} (CD1a, CD3,

688 CD4, CD5, CD8, CD11c, CD14, CD16, CD19, CD20, CD34, FcγRI and CD123) (Biolegend),

689 CD161^{+}, CD127^{+}, CRTH2^{+} and C-Kit[var] (Biolegend). ILC2 were bulk cultured in IL-2, IL-7

690 (5ng/ml) and IL-33 (10ng/ml) (eBioscience) and cytokines and medium were replenished

691 every 3-4 days. For investigations involving humans ILC2 isolation, an informed consent was

692 obtained and the nature and possible consequences of the studies were explained to all

693 donors.

694

695 **Table S3. Antibodies used for sorting human ILC2**

696

Antibody	Clone	Fluorochrome	Target species	Manufacturer
CD11c	3.9	FITC	Human	Biolegend
CD123	6H6	FITC	Human	Biolegend
CD14	63D3	FITC	Human	Biolegend
CD16	B73.1	FITC	Human	Biolegend
CD161	HP-3G10	PECy7	Human	Biolegend
CD19	HIB19	FITC	Human	Biolegend
CD1a	HI149	FITC	Human	Biolegend
CD20	2H7	FITC	Human	Biolegend
CD3	OKT3	FITC	Human	Biolegend
CD34	581	FITC	Human	Biolegend
CD4	OKT4	FITC	Human	Biolegend
CD5	L17F12	FITC	Human	Biolegend
CD127	A019D5	PerCP5.5	Human	Biolegend
CRTH2	BM16	BV421	Human	Biolegend
CD45	H130	BV605	Human	Biolegend

697

698 **CCR8 antibody blocking and rCCL8 treatment**

699 CCR8 neutralisation studies were performed similar to previous reports (*69*). IL-13eGFP and

700 balb/c mice were i.p. injected with 5μg purified anti-mouse CD198 (CCR8) antibody, (Clone:

701 SA214G2) or purified Rat IgG2b, κ Isotype Ctrl Antibody two days prior to first dose of rIL-33

702 treatment and then combined with each of the three rIL-33 doses. Mice were analysed 24h

703 post the third dose. Alternately 10μg Recombinant Mouse CCL8 (MCP-2) (Biolegend) was

704 administered i.n. at the time of the three rIL-33 doses.

705

β-aminoproprionitrile (BAPN) treatment

BAPN treatment was carried out similar to previous reports (*70*). IL-13 eGFP or balb/c mice were i.p. injected with β-aminoproprionitrile (BAPN) (100 mg/kg, Sigma) dissolved in PBS and administered in 200 µl volume on day -1 and then daily for a week over the course of rIL-33 treatment.

Measurement of Airway hyperesponsiveness

Airway resistance in mice was evaluated adopting the flexiVent small animal ventilator (SciReq) as previously described (29) Prior to procedure mice were anesthetized with a combination of pentobarbital sodium (50 mg/kg, administered intraperitoneally) and ketamine (100 mg/kg, administered intramuscularly), tracheostomized, and connected to the flexiVent ventilator using a blunt-ended 19-gauge needle. The mice were ventilated by maintaining an average breathing frequency of 150 breaths/min, tidal volume of 10 ml/kg body weight and positive end-expiratory pressure of about 2 cm H2O. Differences in resistance to increasing concentrations of nebulized methacholine (3- 100 mg/ml) were analysed from the snap-shot perturbation measurements. The data was then plotted using multiple linear regression to the single-compartment model in the following form: pressure = resistance × flow + elastance × volume + fitting constant.

Chemotaxis Assay

Assays of chemotactic responsiveness were carried out as previously described using 96-well ChemoTx® plates with 5µm pores (Neuroprobe). Migrating cells were detected by the use of CellTiterGlo® Dye (Promega) and resulting luminescence measured using a TopCount scintillation counter (PerkinElmer). In all experiments, each data point was assayed in duplicate. Data are reported as Chemotactic Indices, defined as the migratory response to a particular stimulus divided by the migratory response to media alone.

Analysis of ILC2 dynamics on ECM proteins

Human ILC2 were obtained by sorting and differentiating ILC2 from peripheral blood. ILC2 lines were seeded on tissue culture plates coated with 10% FBS, 50µg/ml of fibronectin, collagen-I, and -IV. Human ILC2 dynamics was imaged on the JuLI imaging system for a period of 12h. ILC2 dynamics quantified as changes in speed and displacement were quantified using Image J software.

Statistical analysis

All data were analyzed using Graph Pad Prism 7. Time series videos were analysed and cell tracks were quantified by Imaris software. Tracks were imported to Prism for quantifying track length and track speed. Additionally tracks were imported into MotilityLab software to display tracks from centroid and differences in mean square displacement. All line graphs and bar charts are expressed as mean ±SD and data analyzed with non-parametric Mann-Whitney U test where significance was defined as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ and, $****p < 0.0001$.

Supplementary Materials

Figure S1 Gating strategy for identification of ILC2 populations, related to Fig. 1 A-C.

Figure S2. Quantification of ILC2 in different tissues, related to Fig. 1 A-C.

Figure S3. Gating strategy for identification of ILC2 populations based on GATA-3 expression, related to Fig. 1 A-C.

Figure S4. Distribution of ILC2 and $CD4^+$ T cells in rIL33 treated mice lungs, related to Fig. 1 D and F.

Figure S5. Chemokine receptor expression on ILC, related to Fig 4. (A-B).

Figure S6. Phenotype and IL-13 production by human ILC2 lines, related to figures 4 and 5.

Figure S7. ILC2 motility on extracellular matrix proteins, collagen-III and proteoglycans Versican and tenascin-C, related to figure 5A-C.

760   Figure S8. BAPN fails to affect ILC2 cytokine production and eosinophil movement, related to

761   figure 8G.

762   Movie S1. Time-lapse imaging of rIL-33 treated mouse lung PCLS showing ILC2 movement

763   and location, related to Fig. 2 A.

764   Movie S2. Time-lapse imaging of rIL-33 treated mouse lung PCLS depicting differences in

765   movement of independent ILC2, related to Fig. 2 B.

766   Movie S3. Time-lapse imaging of rIL-33 treated mouse lung PCLS depicting high power videos

767   differences in movement of independent ILC2, related to Fig. 2 C.

768   Movie S4. Intra-vital imaging of rIL-33 treated mouse lung depicting amoeboid ILC2

769   movement, related to Fig. 2 H.

770   Movie S5. Influence of ILC2 survival and cytokines on ILC2 motility, related to figure 5A-C.

771   Movie S6. Time-lapse imaging of lung PCLS from rIL-33 treated mouse treated with anti-CCR8

772   Ab or Isotype Ab, related to Fig. 4I.

773   Movie S7. Collagen-I induced ILC2 shape change and elongation of cell body, related to Fig.

774   5 A.

775   Movie S8. Time-lapse imaging of a lung PCLS assessing the effect of BAPN on rIL-33 treated

776   mouse lung eosinophil movement, related to Fig. 8G.

777

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980
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992

997

998   **Author Contributions**

999   C.M.L. conceived the idea and directed the study. L.M.C. provided expertise in imaging

1000   techniques. J.E.P. provided expert advice on chemotaxis assays. M. B. H. and M.F.K provided

1001   expert advice on intravital imaging. F.P., L.D., L.G.G., L.M.C. and C.M.L. wrote the manuscript.

1002   F.P. L.D. designed the experiments and F.P., L.D., L.J.E., L.M.C., J.V., R.O. performed

1003   experiments. F.P., E.J.M., L.M.C and L.D. analysed the data. F.P., E.J.M., L.M.C. and L.D.

1004   generated figures. R.O. maintained and genotyped the mouse lines.

1005

1006   **Competing Interests**

1007   The authors declare that they have no competing interest.

1008   **Main text figure legends:**

1009   **Fig 1. The number of ILC2 rapidly increase in the peribronchial / perivascular region**

1010   **after rIL-33 treatment**. IL13-eGFP mice were treated with 3 doses of rIL-33 (1µg per dose),

1011   Alt (10µg) or PBS (25µl) over 1 week and culled 24h after the final dose. The frequency of

1012   ILC2 (GFP$^+$CD45$^+$Lin^{neg}CD3$^-$NKp46$^-$CD127$^+$CD90.2$^+$KLRG1$^+$CD25^{var}IL-13$^+$IL-5$^+$) in the **(A)**

1013   airways (BAL fluid), **(B)** lung and **(C)** lung draining lymph nodes (mediastinal). Live viable

1014   precision cut lung slices of 200µm thickness were obtained and stained for CD31 (Magenta,

1015   the lung structure and blood vessels), CD4 (cyan, T cells, orange arrow), EpCAM (Red, to

1016 visualise bronchial epithelium) and GFP (ILC2, white arrow). Images of 1024μm x 1024μm

1017 field of view (FOV) were taken under a 20x objective using an inverted confocal microscope.

1018 **(D)** Images showing ILC2 (GFP$^+$CD4$^-$) CD4+ T cells (CD4+GFP-) location in PBS, rIL-33 and

1019 Alt treated mice, scale bar, 150 μm. **(E)** Number of ILC2 (GFP$^+$CD4$^-$) in lung sections per FOV

1020 taken under a 10x objective. **(F)** Schematic illustration of the lung depicting the anatomical

1021 location in the lung where precision cut lung slices were prepared. Representative images

1022 show two regions of the lung slice from a rIL-33 treated mouse showing distribution of ILC2

1023 and CD4+ T cells, scale bar 150 μm. n = 4 mice per group (Mock(PBS)), n= 6 mice per group

1024 (Alt or rIL-33 treatment). Data representative of 4 experiments. *p < 0.05, **p < 0.01, ***p <

1025 0.001 and, **** p < 0.0001.

1026

1027 **Fig 2. rIL-33 stimulation induces ILC2 motility around blood vessels and airways.** IL13-

1028 eGFP mice were treated with 3 doses of rIL-33 (1μg per dose), over 1 week and culled 24h

1029 after the final dose. Live viable precision cut lung slices (PCLS) of 200μm thickness were

1030 obtained and stained for CD31 (Magenta, the lung structure and blood vessels), CD4 (cyan,

1031 T cells, orange arrow), EpCAM (Red, to visualise bronchial epithelium) and GFP (ILC2, white

1032 arrow), and time-lapse video taken (1024μm x 1024μm field of view (FOV), 45 min duration

1033 under a 20x objective using an inverted confocal microscope) **(A)** Static image depicting the

1034 location of ILC2 and CD4$^+$ T cells, scale bar 100 μm. **(B)** Zoomed in section of the blood vessel

1035 in figure 2A, scale bar 20 μm. **(C)** High power images of boxed cells in figure 2B showing

1036 differences in pattern of cell movement (oscillatory vs amoeboid movement). ILC2 and CD4$^+$

1037 T cells dynamics were tracked and plotted as **(D)** individual tracks or **(E)** tracks commencing

1038 from centroid and overlaid. **(F)** Track speed, **(G)** track length and **(H)** track displacement were

1039 quantified. Representative images shown in **(A-C)** are from rIL-33 treated mice, where n = 6

1040 mice per treatment (3 slices per mouse were imaged). For **(F-H)** in box and whiskers plots,

1041   each dot represents an individual cell. Data are representative from 4 experiments where n =

1042   6 mice per treatment. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ and, **** $p < 0.0001$.

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1044   **Fig 3. rIL-33 stimulation induces ILC2 motility around blood vessels and airways.** IL13-

1045   eGFP mice were treated with 3 doses of rIL-33 (1µg per dose) or Alt (10µg) over 1 week. Live

1046   precision cut lung slices were obtained and ILC2 dynamics were compared between the two

1047   and the differences were plotted as **(A)** individual tracks and **(B)** tracks commencing from

1048   centroid and overlaid. Differences in tracks between treatments were quantified as **(C)** track

1049   speed, **(D)** track length and **(E)** track displacement. Intravital microscopy (IVM) was performed

1050   in live IL-13eGFP mice after rIL-33 treatment (one 512µm x 512µm field of view (FOV) in a 1-

1051   hour-duration video). **(F)** Static images of different frames captured during the course of the

1052   video depicting amoeboid shape changes of ILC2 at separate time-points, scale bar 20 µm. n

1053   ≥ 4 mice per group. Data representative of 4 experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

1054   and, $****$ $p < 0.0001$. Quantifications from **(A-E)** are representative of 4 experiments, where

1055   n = 6 mice per treatment (3 slices per mouse were imaged). For **(F)** IVM images are

1056   representative of 6 individual IL-33 treated mice. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and, **** p

1057 < 0.0001.

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1059   **Fig 4. ILC2 utilize distinct chemotactic pathways to home to inflammatory sites in the**

1060   **lung**. IL13-eGFP mice were treated with 3 doses of rIL-33 (1µg per dose) or PBS (25µl), over

1061   1 week and culled 24h after the final dose. **(A)** The percentage of murine ILC2

1062   ($CD45^{+}Lin^{neg}NKp46^{-}CD3^{-}$) expressing CCR1, CCR4 and CCR8. CCL8 levels in murine **(B)**

1063   BAL and **(C)** lung. **(D)** Location of CCL8 expression and ILC2 and **(E)** quantified CCL8

1064   deposits in PCLS stained for CD31 (Magenta, the lung structure and blood vessels), CCL8

1065   (cyan, yellow arrow), EpCAM (Red, to visualise bronchial epithelium) and GFP (ILC2, white

1066   arrow), images of 1024µm x 1024µm FOV, scale bar 150 µm. Human ILC2 lines were

1067   generated and migration to varying concentrations of **(F)** PGD_2 and **(G)** CCL8 were

determined. **(H)** Peak migratory responses of a human ILC2 cell line to IL-25, TGF-β, rIL-33, CCL8 and PGD$_2$. IL13-eGFP mice treated with rIL-33 were also treated with 5µg purified anti-mouse CCR8 antibody i.p., rCCL8 i.n. or an isotype control and PCLS obtained and stained. **(I)** Localisation of ILC2 in live PCLS. **(J)** Number of ILC2 per FOV under 10x objective. Time-lapse imaging of 45 min duration was performed and ILC2 **(K)** track from centroid, **(L)** track length and **(M)** track speed and **(N)** track displacement were quantified. In box and whiskers graphs each data point represents an individual cell. Balb/c mice treated with rIL-33 were further treated with rCCL8, αCCR8 or Isotype control antibody. **(O)** Percentage of IL-13$^+$IL-5$^+$ ILC2 (CD45$^+$lin$^-$NKp46$^-$CD3$^-$GATA-3$^+$). **(P)** Representation Histogram of MFI of IL-13 and IL-5 and quantification of MFI for **(Q)** IL-13 and **(R)** IL-5 from GATA+ ILC2. For panels **A-E** n ≥ 4 mice per group. Data representative of 4 experiments. For panels **F-H** n = 3 individual donors. Data representative of 3 experiments. For panels **I-R**, n = 5 mice per group. Data representative of 2 experiments *p < 0.05, **p < 0.01, ***p < 0.001 and,**** p < 0.0001.

Fig 5. Extracellular matrix proteins, collagen-IV and fibronectin, promote increased ILC2 motility. Human ILC2 lines were seeded on tissue culture plates coated with either 10% FBS, fibronectin, collagen-I, collagen-IV or serum free coating (control) for 24h. Cell movement was imaged via the JuLI imaging system and plotted as **(A)** individual tracks, **(B)** track speed dot plots and **(C)** track speed spider plot. IL13-eGFP mice were treated with 3 doses of rIL-33 (1µg per dose) or PBS (25µl), over 1 week and culled 24h after the final dose PCLS obtained. **(D)** SHG imaging of PCLS revealing collagen fibres, representative maximum intensity projections, scale bar 50µm. **(E)** GLCM analysis of SHG imaging. **(F)** Images of Fibronectin expression and localisation. PCLS stained for CD31 (Magenta, the lung structure and blood vessels), Fibronectin (cyan, yellow arrow), EpCAM (Red, to visualise bronchial epithelium) and GFP (ILC2, white arrow) and images of 1024µm x 1024µm field of view (FOV) were taken, scale bar 150µm. For panels **A-C,** n = 3 donors (in triplicate). Data representative of 3

1095   experiments. For panels **D-F** n = 6 (in triplicate). ∗p < 0.05, ∗∗p < 0.01, ∗∗∗p < 0.001 and,∗∗∗∗,

1096   **** P < 0.0001.

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1099   **Fig 6. Collagen-I enhances ILC2 actin cytoskeletal remodelling and polarity.** Human ILC2

1100   lines were seeded on tissue culture plates coated with either 10% FBS, fibronectin, collagen-

1101   I, collagen-IV or serum free coating (control) and imaged after 12 hours. **(A)** Bright field images

1102   depicting change in shape. **(B)** Actin remodelling following staining with Phalloidin (green) and

1103   DAPI (cyan) and imaging using Airyscan detection (maximum intensity projections), scale bar

1104   5μm. **(C)** Cell area. **(D)** Cell perimeter. n = 3 donors. Data representative of 2 experiments. ***

1105 P < 0.001.

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1107   **Fig 7. Blocking collagen fibrillogenesis *in vivo* increases ILC2 dynamics in the inflamed**

1108   **lung.** IL13-eGFP mice treated with rIL-33 were further treated with (BAPN) along with controls

1109   were culled 24 hours after the final dose. ILC2 dynamics from live PCLS were plotted as either

1110   **(A)** individual tracks or **(B)** tracks commencing from centroid and overlaid**.** Differences in

1111   tracks between treatments were quantified as **(C)** track speed, **(D)** track length and **(E)** track

1112   displacement. Total ILC2 () in **(F)** Lungs, **(G)** BAL and **(H)** Blood were enumerated. For panels

1113   **A-E** n ≥ 4 mice per group. Data representative of 4 experiments. For panels **F-H** n = 6 mice

1114   per group. Data representative of 2 experiments. * P < 0.05, ** P < 0.01, *** P < 0.001, **** P

1115   < 0.0001.

1116

1117   **Fig 8. Blocking collagen fibrillogenesis reduces eosinophil accumulation in the**

1118   **inflamed lung.** Balb/c mice were treated with rIL-33 or PBS with or without BAPN. **(A)** Airway

1119   responsiveness to methacholine. Lung **(B)** alveolar macrophages (AM), **(C)** dendtitic cells

1120   (DC), **(D)** neutrophils and **(E)** were enumerated by flow cytometry. **(F)** CCL24 protein in lung

1121   tissue. **(G)** Congo red stained eosinophils (blue arrow) in lung histological samples. scale bar

1122   50μm **H** Quantification of eosinophil from histological samples. For panels **A-E** n ≥ 6 mice per

1123   group. Data representative of 2 experiments. For panels **G and H** n ≥ 4 mice per group. Data

1124   representative of 2 experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$.

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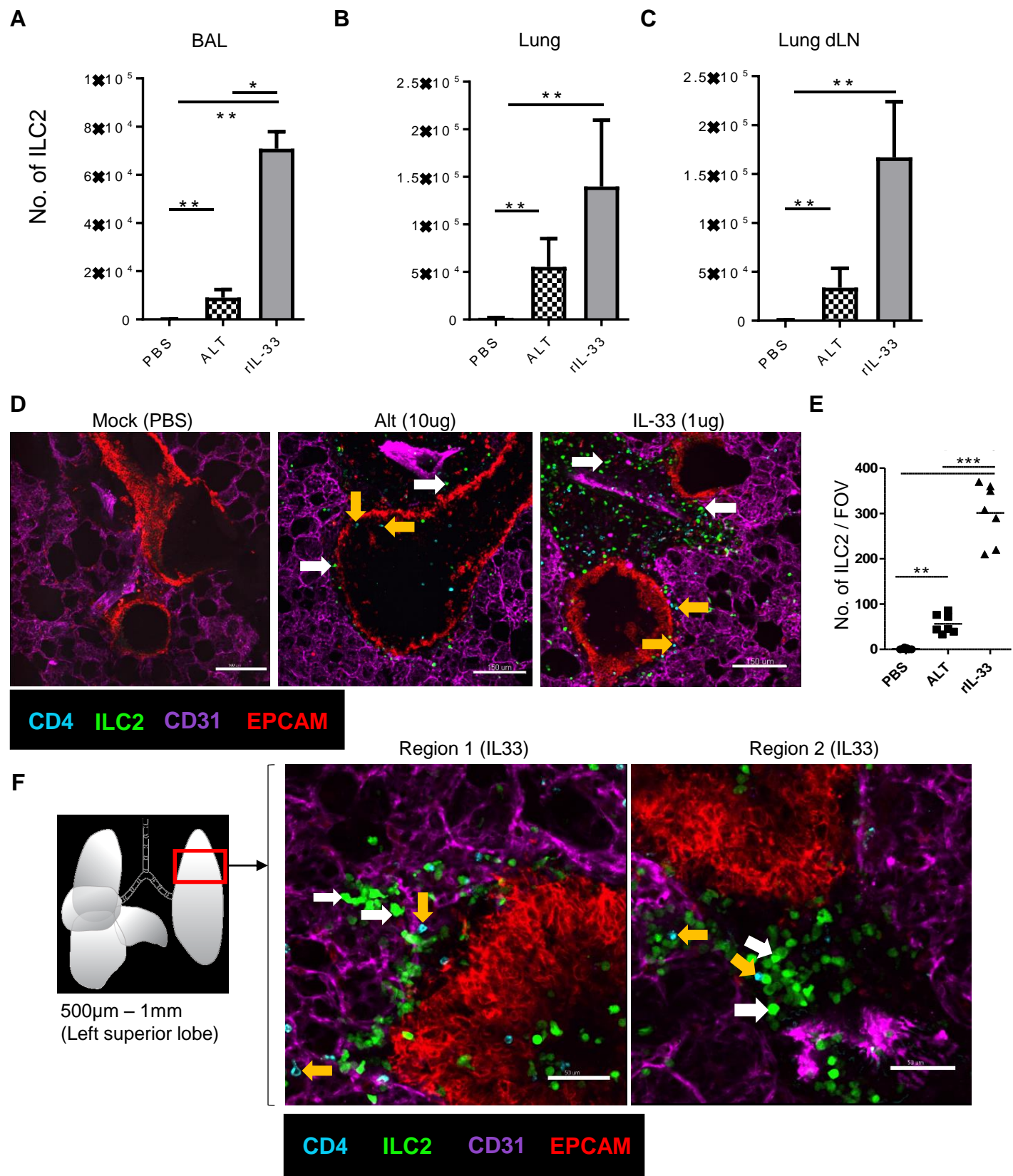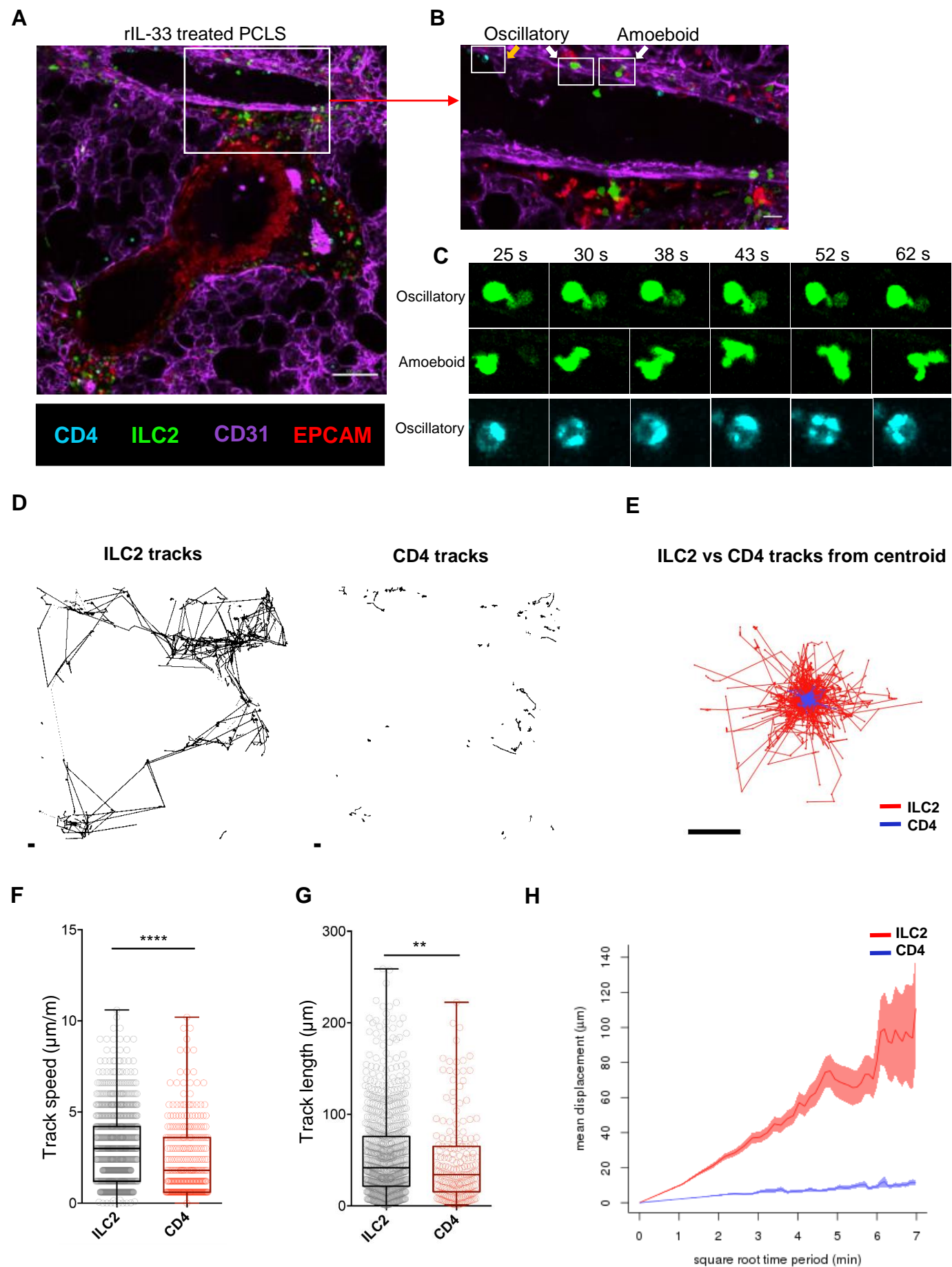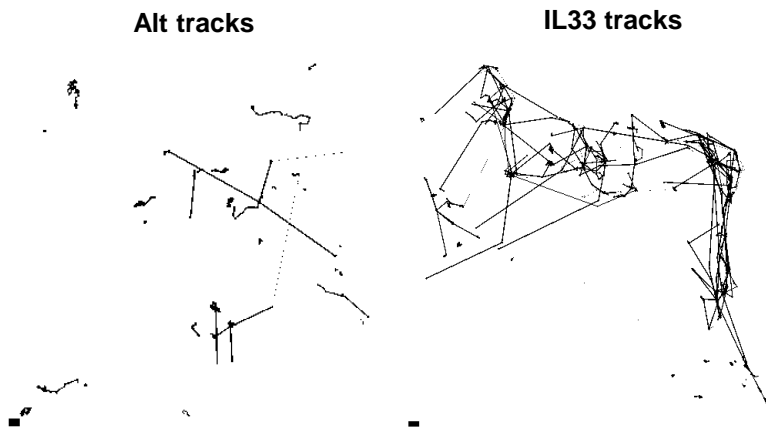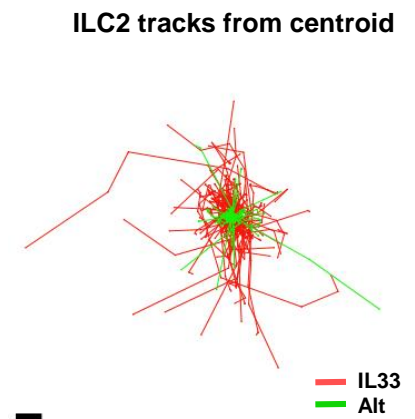1149
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Figure 1

A BAL

B Lung

C Lung dLN

D

Mock (PBS)　　Alt (10ug)　　IL-33 (1ug)

CD4　ILC2　CD31　EPCAM

E

F

500μm – 1mm
(Left superior lobe)

Region 1 (IL33)　　Region 2 (IL33)

CD4　ILC2　CD31　EPCAM

Figure 2

A

rIL-33 treated PCLS

| CD4 | ILC2 | CD31 | EPCAM |

B

Oscillatory   Amoeboid

C

	25 s	30 s	38 s	43 s	52 s	62 s
Oscillatory						
Amoeboid						
Oscillatory						

D

ILC2 tracks

CD4 tracks

E

ILC2 vs CD4 tracks from centroid

— ILC2
— CD4

F

Track speed (µm/m)

****

ILC2   CD4

G

Track length (µm)

**

ILC2   CD4

H

mean displacement (µm)

— ILC2
— CD4

square root time period (min)

Figure 3

A

Alt tracks

IL33 tracks

B

ILC2 tracks from centroid

IL33
Alt

C

Track speed (µm/m)

****

ALT   rIL-33

D

Track length (µm)

****

ALT   IL-33

E

IL33
Alt

mean displacement (µm)

square root time period (min)

F

L-IVM (rIL33 treated)

Snapshots of amoeboid movement

5.38 s   5.51 s   5.71 s   6.85 s

3.35 s   4.01 s   6.3 s   9.2 s

CD31   ILC2

Figure 4

A CCR1, CCR4 and CCR8 Murine ILC2s

B BAL CCL8

C Lung CCL8

D Mock / rIL-33
CCL8 ILC2 CD31 EPCAM

E CCL8 deposits

F PGD₂

G CCL8

H Peak chemotactic response

I rIL-33 + Iso / rIL-33 + αCCR8
ILC2 CD31 EPCAM

J ILC2 number

K rIL-33 + Iso / rIL-33 + αCCR8

L Track length

M Track speed

N Track displacement

O IL-13⁺/IL-5⁺ ILC2

P Gated on GATA⁺ ILC2

Q MFI IL-13

R MFI-IL-5

Figure 5

A

Serum free	10% FBS	Fibronectin	Collagen I	Collagen-IV

B

Track speed (μm/min)

****
****
****

Serum free · 10% FBS · Fibronectin · Collagen - I · Collagen-IV

C

Serum free
10% FBS
Fibronectin
Collagen-I
Collagen-IV

D

Collagen SHG

Mock

IL-33

E

Correlation / a.u. ± SEM

mock
rIL-33

Comparison distance / μm

Homogeneity / a.u. ± SEM

Comparison distance / μm

F

Fibronectin

Mock

IL-33

Fibronectin
ILC2
CD31
EPCAM

Figure 6

A

| Control | 10% FBS | Fibronectin | Collagen I | Collagen IV |

B

DAPI Phalloidin

C

ILC2 area

***

Cell area (um^2)

Serum free, 10% FBS, FBN, Coll I, Coll IV

D

ILC2 perimeter

***

Cell perimeter (um)

Serum free, 10% FBS, FBN, Coll I, Coll IV

Figure 7

A

rIL-33

rIL-33 + BAPN

B

ILC2 tracks from centroid

— rIL-33
— rIL-33 + BAPN

C

Track speed (µm/min)

***

rIL-33   rIL-33 + BAPN

D

Track length (µm)

****

rIL-33   rIL-33 + BAPN

E

Mean displacement (µm)

— rIL-33
— rIL-33 + BAPN

Square root time period (min)

F

Lung ILC2

No. of ILC2

Mock   Mock + BAPN   rIL-33   rIL-33 + BAPN

G

BAL ILC2

No. of ILC2

Mock   Mock + BAPN   rIL-33   rIL-33 + BAPN

H

Blood ILC2

No. of ILC2

Mock   Mock + BAPN   rIL-33   rIL-33 + BAPN

Figure 8

Fig. S1. Gating strategy for identification of ILC2 populations, related to Fig. 1 A-C. IL13-eGFP mice treated with 3 doses or rIL-33 (1μg per dose) over 1 week and culled 24h after the final dose. Cells were pre-gated on lymphoid, live (determined using a fixable live dead dye), lineage and frequencies of GFP+CD45+ cells among lineage positive versus lineage negative cells was evaluated in (A). (B) ILC2 were defined by pre-gating on lymphoid, live (determined using a fixable live dead dye), GFP+CD3-NKp46- cells that were lineage (TCRβ, TCRγδ, CD5, CD19, CD11b, CD11c, FCεR1, GR-1, F4/80, and TER-119) negative cells and co-expressed intracellular IL-13 and surface expression of CD90.2, KLRG-1, CD127 as shown in histogram plots.
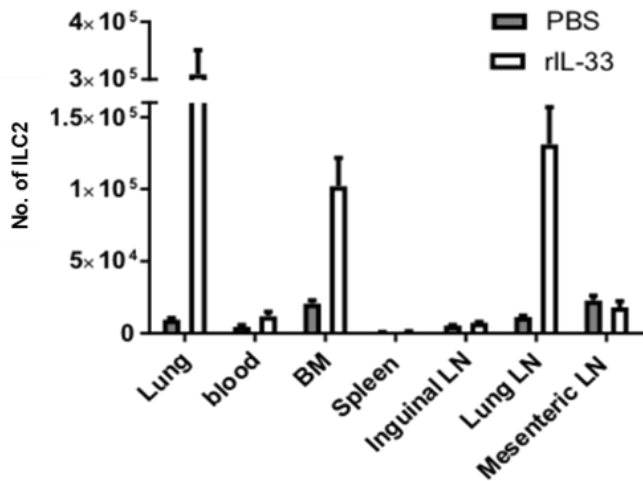
Fig. S2. Quantification of ILC2 in different tissues, related to Fig. 1 A-C. Il13-eGFP mice treated with 3 doses of rIL-33 (1μg per dose) or PBS (25μl) over 1 week and culled 24h after the final dose. ILC2 number in lungs, blood, bone marrow (BM), Spleen, Inguinal lymph node (LN), lung draining LN and mesenteric LN. n ≥ 6 mice per group. Data representative of 2 experiments.
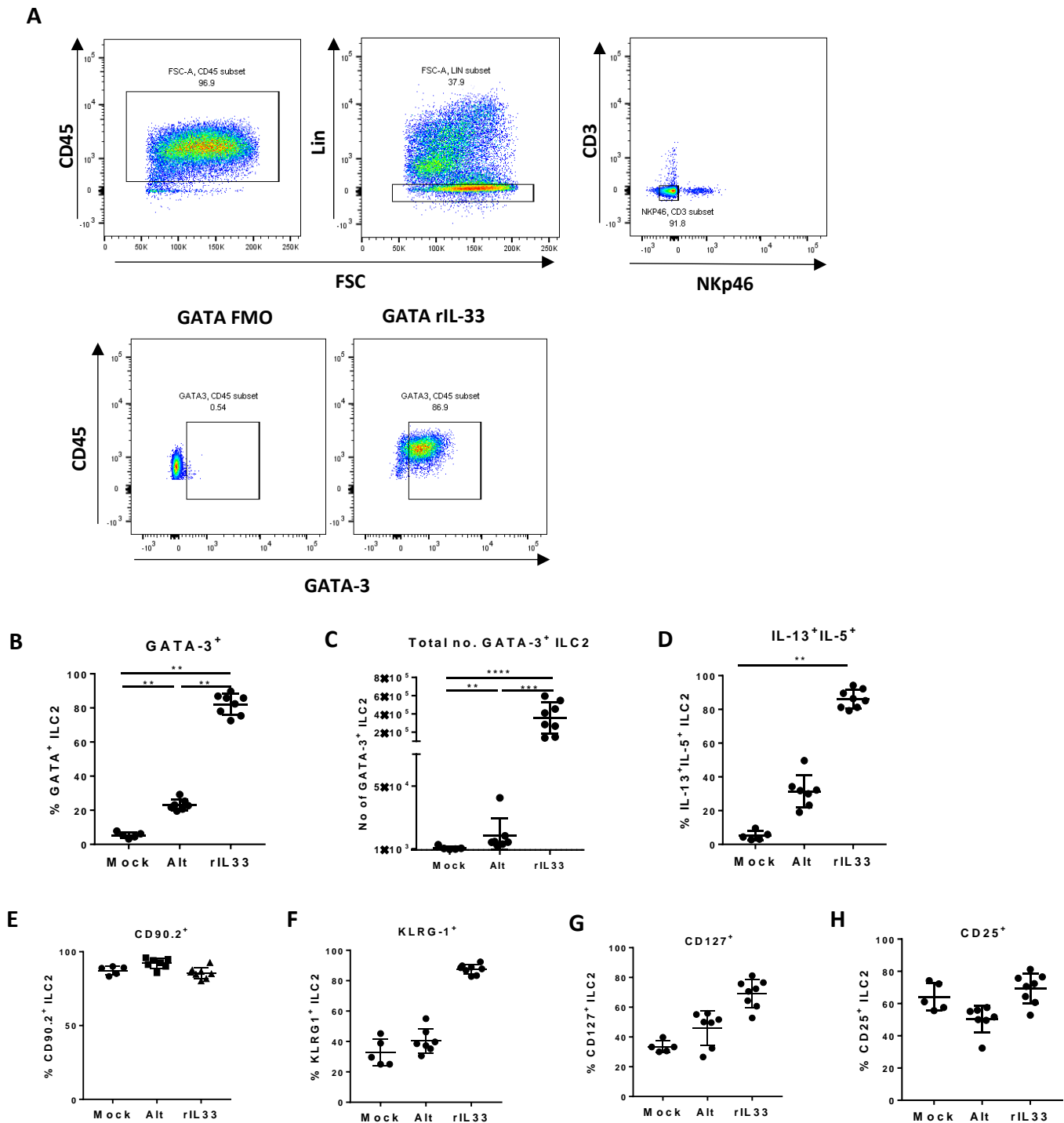
**Fig. S3. Gating strategy for identification of ILC2 populations based on GATA-3 expression, related to Fig. 1 A-C.** ILC2 were identified by gating on CD45^{+} cells that were lineage neg (lin^{-}) (Lineage cocktail: TCRβ, TCRγδ, CD5, CD19, CD11b, CD11c, FCεR1, GR-1, F4/80, and TER-119) lymphocytes that were additionally CD3e^{-} and NKp46^{-} and expressed GATA-3 as shown in **(A)**. **(B)** shows % of GATA-3^{+} ILC2 in lungs of mock, Alt and IL-33 treated mice which were further quantified in number as shown in **(C)** and co-expressed IL-13 and IL-5 **(D)**. ILC2 were further evaluated for percent expression of CD90.2 **(E)**, KLRG-1**(F)**, CD127 **(G)** and CD25 **(H).** Data shown for are from one of 4 independent experiments with a total of n ≥ 6 mice per group, * $P < 0.05$, **** $P < 0.01$,*** $P < 0.001$ and **** $P < 0.0001$**.

Fig. S4 **Distribution of ILC2 and CD4 T cells in rIL-33 treated PCLS**
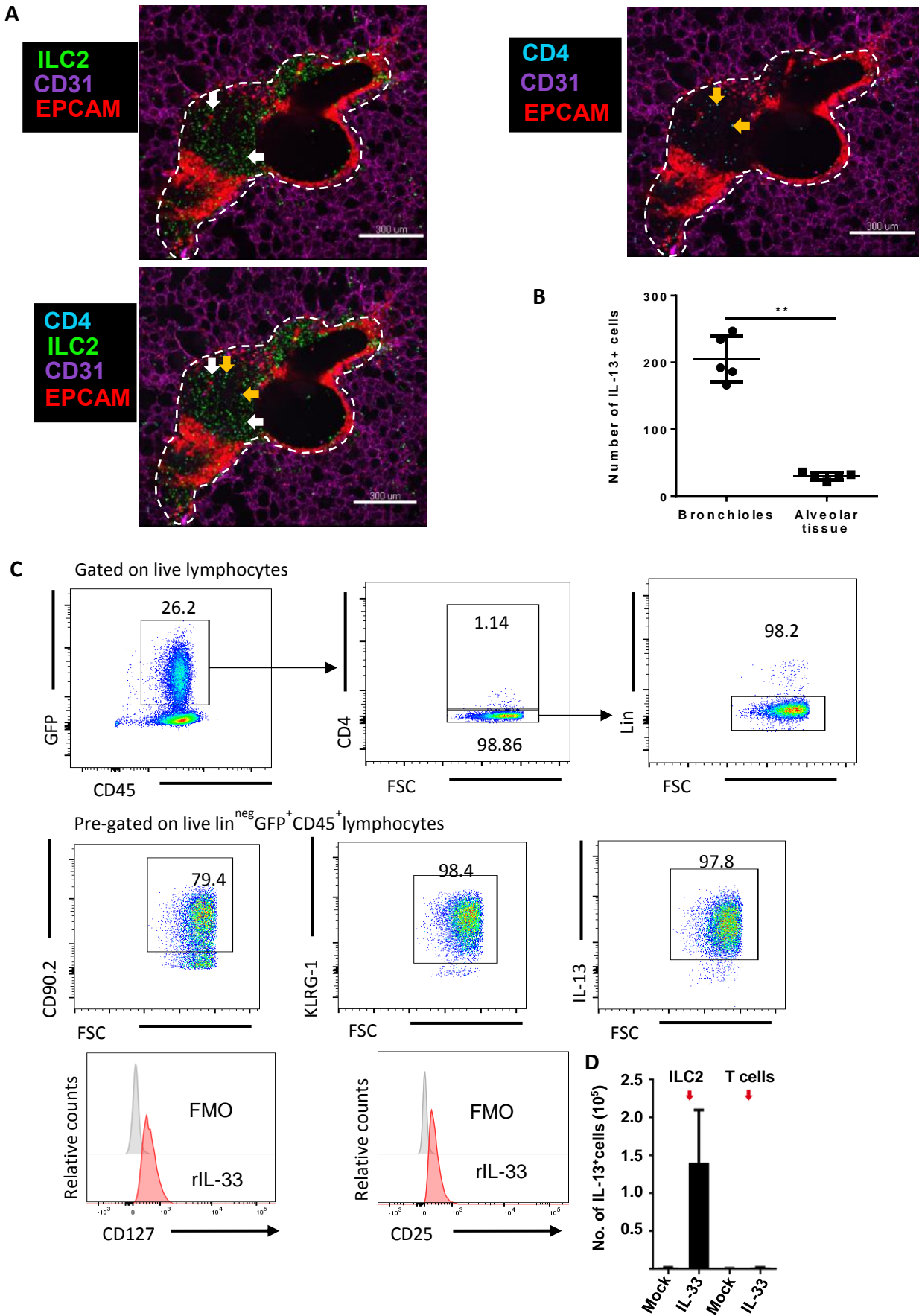
Fig. S4. Distribution of ILC2 and CD4$^+$ T cells in rIL33 treated mice lungs, related to Fig. 1 D and F. IL13-eGFP mice were treated with 3 doses of rIL-33 (1μg per dose), over 1 week and culled 24h after the final dose. **(A)** Live viable precision cut lung slices (PCLS) of 200μm thickness were obtained and stained for CD31 (Magenta, the lung structure and blood vessels), CD4 (cyan, T cells, orange arrow), EpCAM (Red, to visualise bronchial epithelium) and GFP (ILC2, white arrow). Highlighted in the insets (white dashed line) are areas of ILC2 and CD4$^+$ T cell accumulation. **(B)** Quantification of the number of IL-13$^+$GFP$^+$ cells close to large blood vessels versus alveolar capillaries. **(C)** GFP$^+$ cells were assessed for ILC2 phenotypic expression by flow cytometry. Live GFP$^+$CD45$^+$CD4$^-$Lin^{neg} cells co-expressing CD90.2, KLRG-1 and intracellular IL-13 (dot plots), with CD127 and CD25 expression **depicted** as histogram plots. **(D)** Quantification of number of IL-13 producing ILC2 versus CD4 T cells. n = 4 mice per group. Data representative of 4 experiments. * $P < 0.05$, **** $P < 0.01$,** *** $P < 0.001$ and ****$P < 0.0001$.
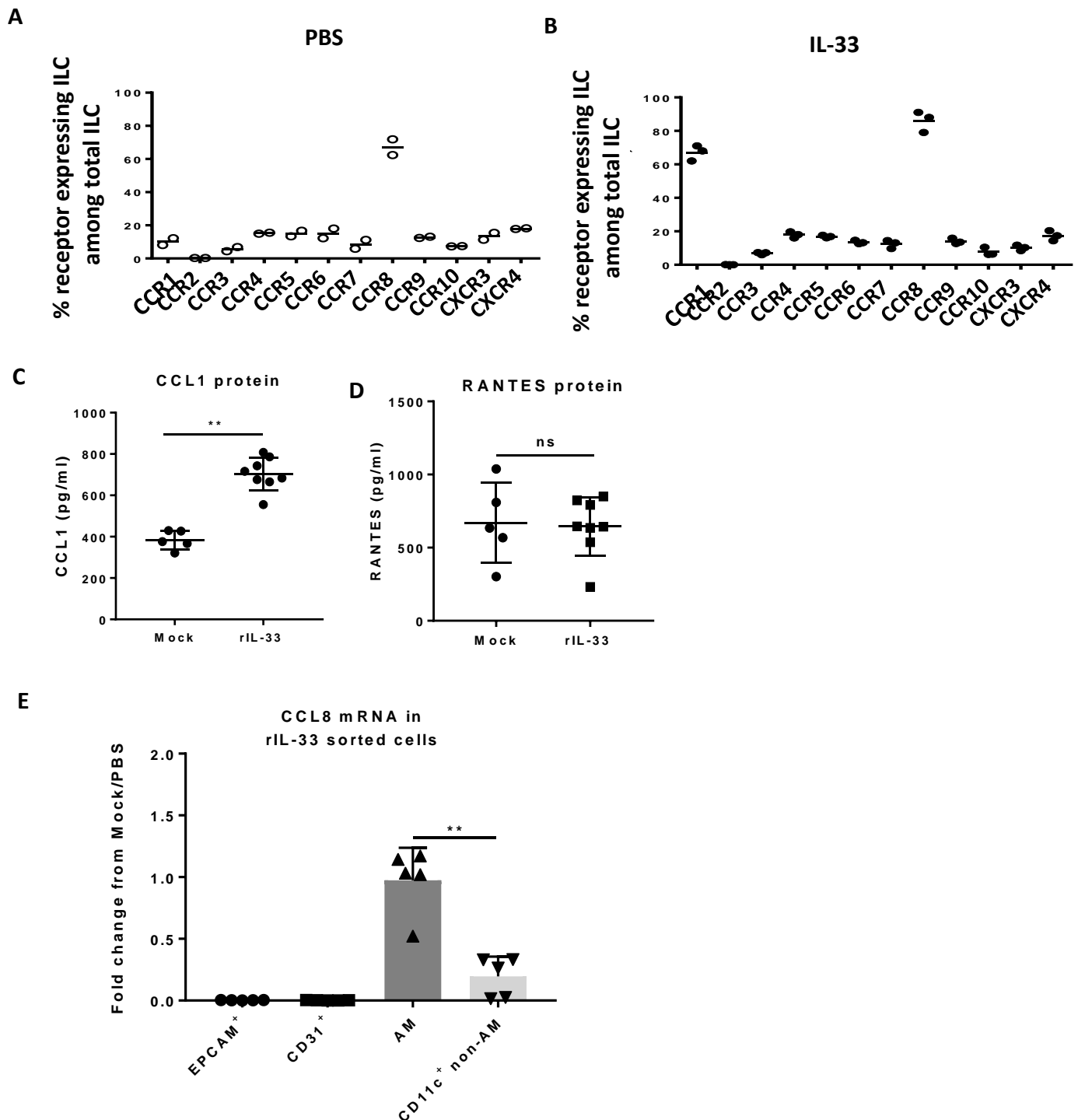
Fig. S5. Chemokine receptor expression on ILC, related to Fig 4. Mice were treated with rIL-33 (1µg, 3 times a week for 1 week) or PBS (25µl) and culled 24h after last dose. Percentage of lung ILC (GFP$^+$CD45$^+$Lin^{neg}KLRG1$^+$CD127$^+$CD90.2$^+$) expressing chemokine receptors in mice treated with **(A)** PBS or **(B)** rIL-33. Levels of **(C)** CCL1 **(D)** RANTES. **(E)** CCL8 expression levels in lung epithelial cells, endothelial cells, airway macrophages (AM) and non-AM. For panels A and B, n = 4 mice per group. Data representative of 4 experiments. For **C and D**, n = 5 mice (Mock(PBS)) and mice 6 mice (IL-33) per group and in **E**, n= 5 mice per group. Data representative of 2 experiments * P < 0.05, **__P < 0.01,__** *** P < 0.001, **** P < 0.0001.
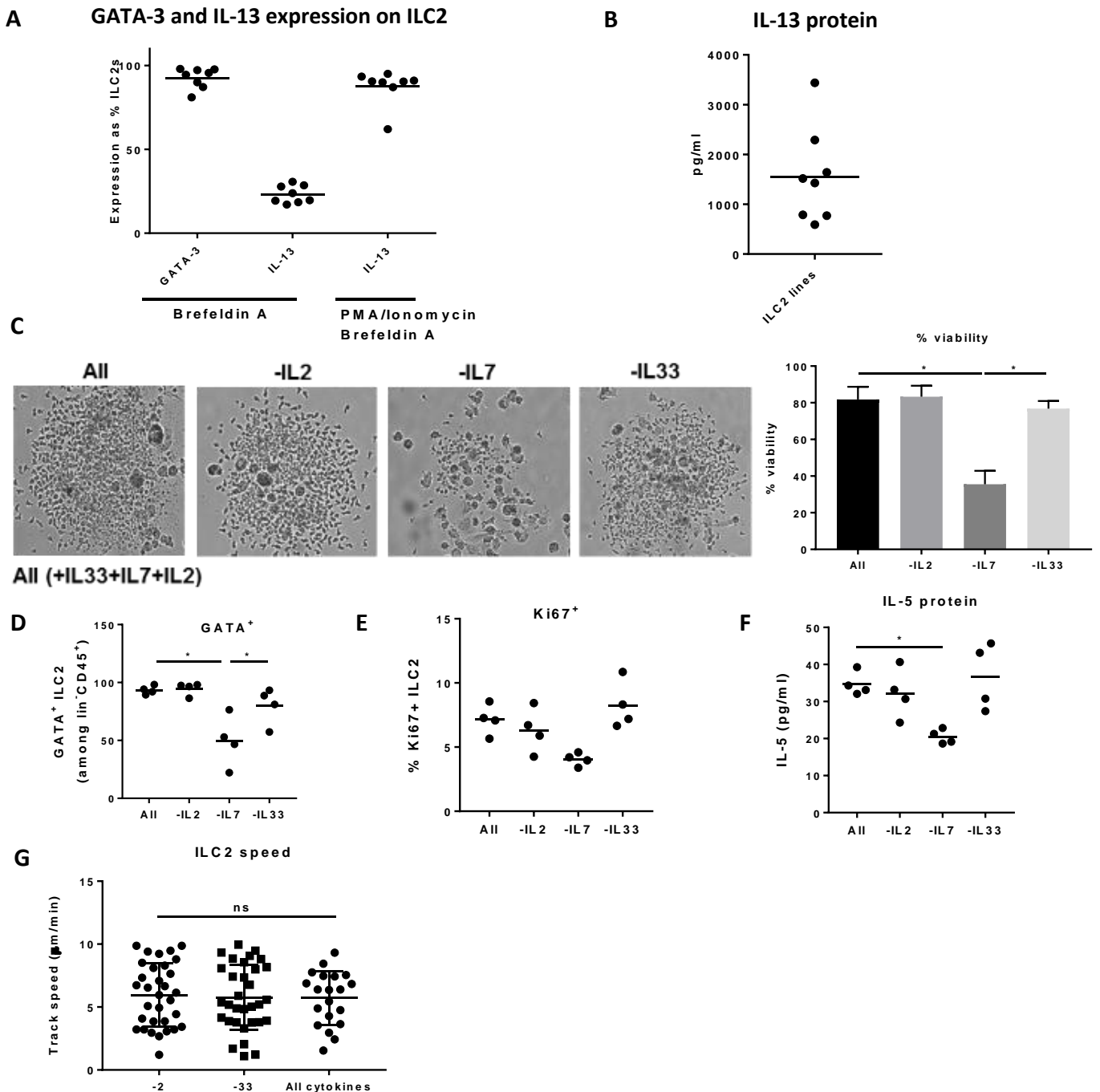
Fig. S6. Phenotype and IL-13 production by human ILC2 lines, related to figure4 and 5. Human ILC2 lines were generated. **(A)** GATA-3 and IL-13 expression by flow cytometry. **(B)** IL-13 production by ELISA as shown in. ILC2 lines were starved of either, IL-2, IL-7 or IL-33 for 72 hours and phenotype, proliferation, cytokine production and motility were compared to ILC2 that received all three cytokines. **(C)** Bright field images following starvation and quantification of viability. **(D)** GATA-3 and **(E)** Ki-67 expression assessed by flow cytometry. **(F)** IL-5 protein levels by ELISA. **(G)** track speed. n ≥ 3 donors (in triplicate). Data representative of 2 experiments. .∗p < 0.05, ∗∗p < 0.01, ∗∗∗p < 0.001 and,∗∗∗∗ p < 0.0001.
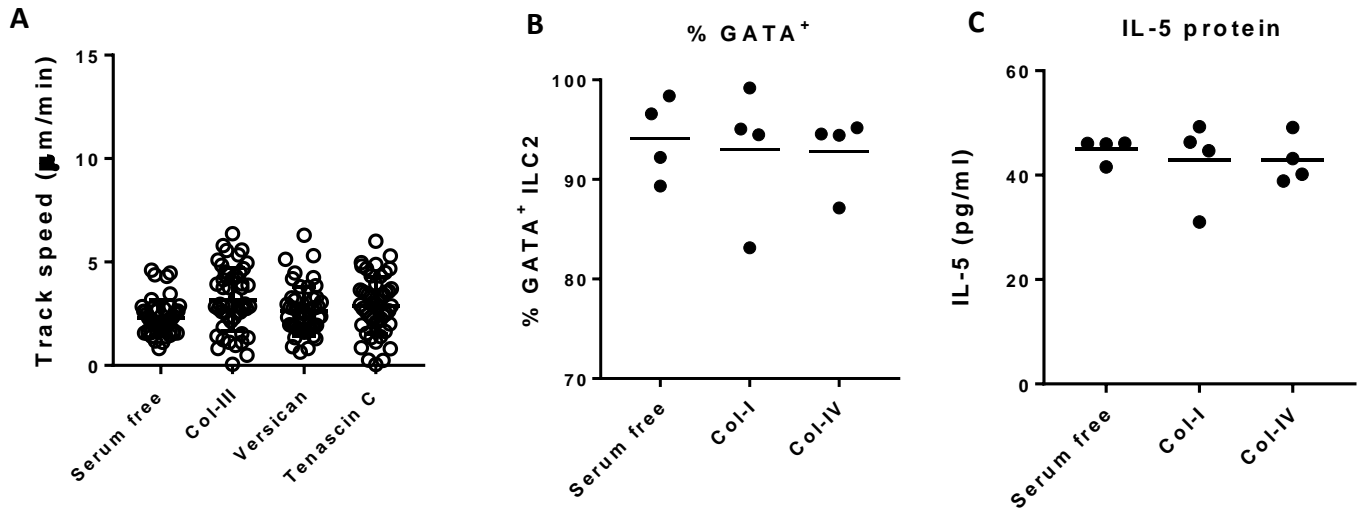
Fig. S7. ILC2 motility on extracellular matrix proteins, collagen-III and proteoglycans Versican and tenascin-C, related to figure 5A-C. **(A)** Human ILC2s seeded on tissue culture plates coated with either 10% FBS, collagen-III (col-III), versican or tenascin-C and migratory dynamics quantified as track speed. **(B)** GATA-3 expression and **(C)**IL-5 production from human ILC2 lines grown with either Collagen-I (col-I) or Collagen-IV (col-IV). Data shown are representative of 2 experiments with a total of n= 4 donor cell lines per treatment performed in triplicate.

A IL-5 protein

B

C
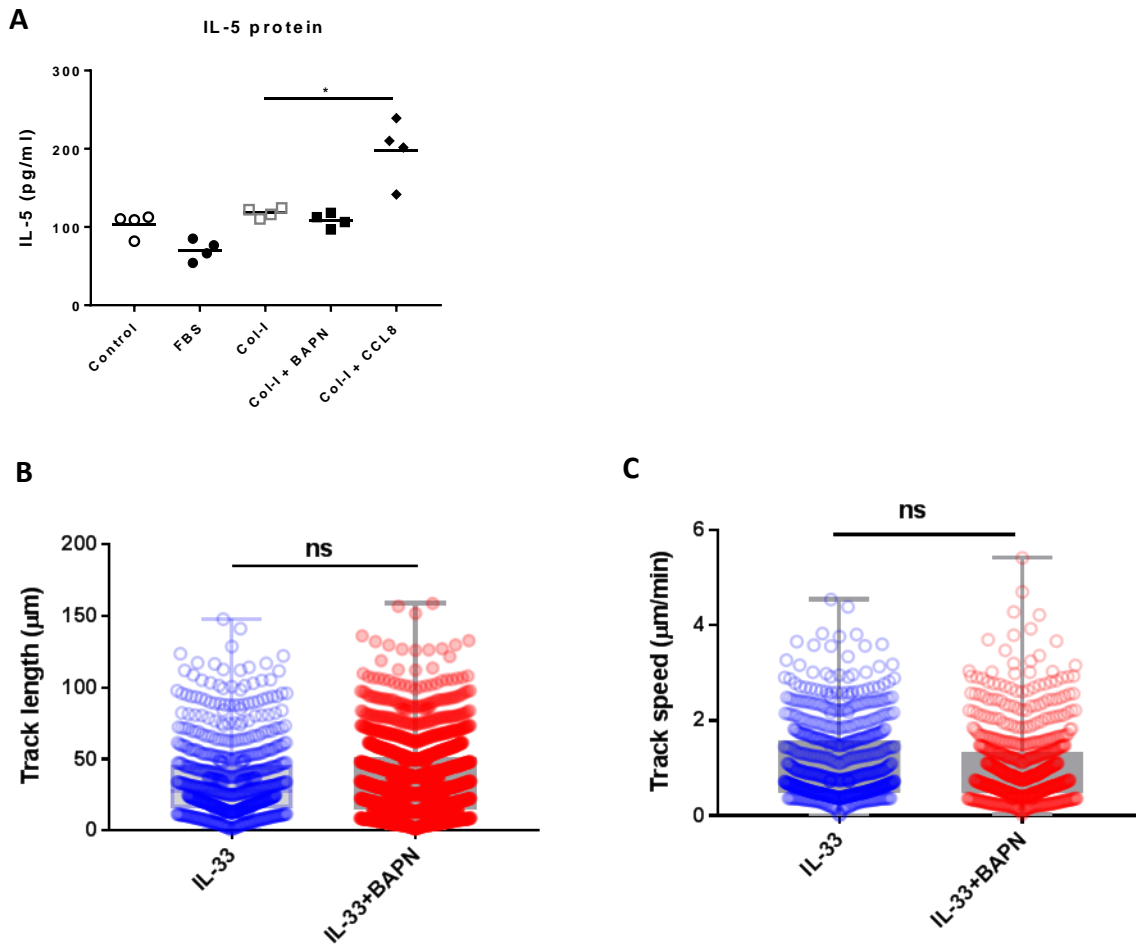
Fig. S8. BAPN fails to affect ILC2 cytokine production and eosinophil movement, related to figure 8G. Balb/c mice were treated with rIL-33 or PBS with or without BAPN. **(A)** Lung ILC2 were FACS sorted and seeded on FBS or collagen-I (col-I) in the presence or absence of BAPN or CCL8 and IL-5 production was assessed by ELISA. Live PCLS were obtained and imaged for eosinophils (Siglec-F⁺CD11c⁻) and motility was quantified by **(B)** track length and **(C)** track speed. n = 4 mice per group. Data representative of 2 experiments.