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Title:

Combinatorial Targeting of TSLP, IL-25, and IL-33 in Type 2 Cytokine-driven Inflammation and Fibrosis

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26

27 **One Sentence Summary:** Although TSLP, IL-25, and IL-33 have emerged as important
28 initiators of type 2 immunity, combined blockade of all three mediators may be needed to treat
29 some forms of progressive type 2 cytokine driven inflammation and fibrosis.

30

31 **Abstract:** Thymic stromal lymphopoietin (TSLP), IL-25, and IL-33 are important initiators of
32 type 2-associated mucosal inflammation and immunity. However, their role in the maintenance
33 of progressive type 2 inflammation and fibrosis is much less clear. Here, using chronic models
34 of helminth infection and allergic lung inflammation, we show that collective disruption of
35 TSLP, IL-25, and IL-33 signaling suppresses chronic and progressive type 2 cytokine-driven
36 inflammation and fibrosis. In a schistosome lung granuloma model or during chronic *S. mansoni*
37 infection in the liver, individual ablation of TSLP, IL-25, or IL-33/ST2 had no impact on the
38 development of IL-4/IL-13-dependent inflammation or fibrosis. However, significant reductions
39 in granuloma-associated eosinophils, hepatic fibrosis, and IL-13-producing group 2 innate
40 lymphoid cells (ILC2s) were observed when signaling of all three mediators was simultaneously
41 disrupted. Combined blockade via mAb treatment also reduced IL-5 and IL-13 expression
42 during primary and secondary granuloma formation in the lung. In a model of chronic house dust
43 mite-induced allergic lung inflammation, combined mAb treatment did not decrease established
44 inflammation or fibrosis. TSLP/IL-33 double-knockout mice treated with anti-IL-25 mAb during
45 priming, however, displayed decreased inflammation, mucus production, and lung remodeling in
46 the chronic phase. Together, these studies reveal partially redundant roles for TSLP, IL-25, and

47 IL-33 in the maintenance of type 2 pathology and suggest that in some settings, early combined
48 targeting of these mediators is necessary to ameliorate progressive type 2-driven disease.
49
50 **Main Text:**
51
52 **Introduction**
53
54 Type 2 immunity is characterized by the production of the cytokines IL-4, IL-5, IL-9, and
55 IL-13, which play diverse roles in the immune response (*1*). In addition to suppressing the pro-
56 inflammatory activity of type 1 immune responses (*2*), type 2 immunity regulates wound healing
57 (*3*), metabolic homeostasis (*4*), and immunity to several extracellular parasites (*5*). However,
58 while the type 2 response exhibits many host protective functions, should these responses persist
59 or become dysregulated, they can contribute to the development of disease. Chronic type 2
60 cytokine production underlies diseases including allergic asthma, atopic dermatitis, allergic
61 rhinitis, ulcerative colitis, and many chronic fibroproliferative disorders (*6-9*). Therefore, a
62 better understanding of the mechanisms that regulate the initiation, maintenance and resolution
63 of type 2 immune responses could reveal novel approaches to treat a host of important human
64 diseases.
65 Three predominantly epithelial cell-derived cytokines: thymic stromal lymphopoietin
66 (TSLP), IL-25, and IL-33, have emerged as important initiators of type 2 immunity in mammals,
67 and their expression during type 2 disease in humans is widely-documented (*10-15*). These
68 alarmins are released from the epithelium and other local stromal compartments when cells are
69 damaged or stressed by allergens, pollutants or pathogens and thereby trigger the production of

70 the canonical type 2 cytokines IL-5, IL-9, and IL-13 by human and mouse cells of the innate and
71 adaptive immune system (*16, 17*). TSLP targets dendritic cells (DCs), basophils, mast cells,
72 monocytes, natural killer T cells, and type 2 innate lymphoid cells (ILC2s) (*18-21*). In humans,
73 TSLP has been shown to induce naïve human $CD4^+$ Th2 cell responses, but only in the presence
74 of DCs (*22*). IL-25 and IL-33 exhibit similar Th2-inducing activity, but rather than targeting
75 DCs, myeloid cells, and Th2 cells, they largely promote type 2 immunity by stimulating ILC2s
76 as well as basophils, mast cells, and eosinophils. IL-33 will amplify antigen-dependent and -
77 independent effector responses from both human and mouse Th2 cells (*16, 17*). One recent
78 study revealed that IL-33 can enhance TSLP and DC-mediated human Th2 memory responses *in*
79 *vitro* suggesting the alarmins could play a role in maintaining immune responses (*23*). Although
80 TSLP, IL-25, and IL-33 have all been shown to promote type 2 immunity when overexpressed in
81 mice (*10-12*), the requirement for these cytokines in the development of type 2 immunity in
82 response to allergens and helminth parasites has been more variable, with some studies
83 identifying little to no role for TSLP, IL-25, or IL-33 when targeted individually (*24-28*). This
84 variability has been attributed to the redundant and overlapping functional activities of these
85 cytokines. IL-33 and IL-25 have both been shown to induce production of IL-13 by human ILCs
86 *in vitro*, for example (*29*). However, this theory has not been systematically investigated *in vivo*,
87 nor have the combined roles of the 3 cytokines been dissected in models of chronic type 2-
88 dependent disease.
89 In the present study, we utilized both genetic- and monoclonal antibody-based strategies
90 to investigate whether bi-functional or tri-functional targeting of TSLP, IL-25, and IL-33-
91 dependent signaling more effectively controls pathogenic Th2 responses than disrupting any of
92 the pathways individually. The roles of the 3 cytokines in the initiation and maintenance of

93 primary and secondary type 2 immune responses were investigated in both acute and chronic
94 models of lung inflammation and during chronic helminth infection. These models involve
95 innate-initiated pathways as well as the development of antigen-specific T cell responses that
96 influence outcomes at later stages. A major goal was to investigate if type 2 cytokine-driven
97 inflammation and fibrosis could be ameliorated more effectively if all three epithelial cytokines
98 were targeted in combination. Moreover, in contrast to previous studies that have focused on
99 their role in the "initiation" of type 2 immunity (*30*), our studies were also designed to
100 investigate if TSLP, IL-25, and IL-33, either alone or in combination, are required for the
101 "maintenance" of established type 2-driven disease, as this is the stage where most therapeutic
102 strategies are initiated.
103
104 **Results**
105
106 **Function of IL-25 during the initiation and maintenance of type 2 inflammation**
107 We have previously shown that TSLP is not required for type 2-driven granuloma formation and
108 fibrosis induced by the eggs of the helminth parasite *Schistosoma mansoni* (*26*). Another group
109 has demonstrated that many helminths could bypass the need for TSLP in the development of
110 type 2 responses by directly modulating dendritic cell function (*28*). However, the relative
111 importance of IL-25 and IL-33 to the maintenance of established type 2-driven disease and the
112 potential redundancy of these mediators has not been assessed. Therefore, we began by
113 exploring the contribution of IL-25 in type 2-dependent inflammation and fibrosis by
114 overexpressing IL-25 in mice that were injected i.v. with live *S. mansoni* eggs. Hydrodynamic
115 delivery of an IL-25-expressing plasmid to naïve mice boosted IL-25 mRNA expression more

116 than 1000-fold in the liver (**Fig. 1A**). As observed in previous studies (*10*), corresponding
117 increases in IL-4, IL-5, and IL-13 expression were observed in both the liver and the lung (**Fig.**
118 **1A**). When the IL-25-expressing plasmid was delivered 24 hours prior to exposure to *S. mansoni*
119 eggs, the resulting granulomatous response to the eggs in the lung was exacerbated (**Fig. 1B**).
120 Indeed, granuloma volume more than doubled in the IL-25 pre-treated mice, and their lesions
121 contained many more eosinophils than control mice, which was likely due to type 2 cytokine
122 induction in the lung (**Fig. 1C**). Goblet cell hyperplasia and mucus production were also
123 augmented in lungs of mice treated with the IL-25 plasmid. The effects of IL-25 plasmid
124 administration were reduced in IL-13Rα□$^{-/-}$ mice, demonstrating that the IL-25-mediated
125 increase in type 2-associated pathology was dependent on IL-4/IL-13-mediated signaling through
126 the type II IL-4 receptor complex (**Fig. 1B**). Eosinophils accumulated following plasmid
127 administration, however, likely explained by IL-25-driven IL-5 expression (**Fig. 1C**).
128 Although these studies established that IL-25 could exacerbate type 2 cytokine-driven
129 pathology, they did not reveal whether endogenously expressed IL-25 was critical to the
130 development of granulomatous inflammation and fibrosis. To clarify the role of IL-25 in both
131 the initiation and maintenance of type 2-driven fibrosis, we used IL-25$^{-/-}$ mice in both primary
132 (like Fig. 1B) and secondary i.v. *S. mansoni* egg challenge models (*31*). In these experiments,
133 naïve or egg-sensitized IL-25$^{-/-}$ mice and wild-type littermates were challenged i.v. with live *S.*
134 *mansoni* eggs and granuloma formation was quantified on day 7 post-challenge. Neither primary
135 nor secondary granuloma formation was significantly reduced in the absence of IL-25 (**Fig. 1D**).
136 The number of granulomatous eosinophils in each group was also indistinguishable during both
137 primary and secondary challenges (**Fig. 1D, right panel**). Finally, to evaluate the requirement
138 for IL-25 in a more chronic type 2 disease setting, we exposed wild-type and IL-25$^{-/-}$ mice to *S.*

139 *mansoni* cercariae and quantified granuloma volume, tissue eosinophilia, and fibrosis in the liver
140 after 12 weeks of infection. Similar to the results in the lung (**Fig. 1D**), no significant change in
141 type 2-dependent pathology was observed in livers of IL-25$^{-/-}$ mice compared with wild-type
142 mice when chronically infected with *S. mansoni* (**Fig. 1E**).
143

144 **Role of IL-33 in type 2 inflammation and fibrosis**

145 Given that the inflammation and fibrosis induced by *S. mansoni* eggs in both the lung and liver
146 were IL-4-, IL-13-, and IL-13Rα□-dependent but did not require IL-25 or TSLP (*26*), we next
147 examined whether IL-33/ST2 receptor signaling was required in this setting. As observed in IL-
148 25$^{-/-}$ mice, mice deficient in IL-33 showed no significant reduction in either primary (**Fig. 2A**) or
149 secondary granuloma formation (**Fig. 2B**) when challenged i.v. with live *S. mansoni* eggs. In
150 both models, type 2-driven fibrosis and eosinophilia were similar in wild-type and IL-33$^{-/-}$ mice.
151 We also infected wild-type and IL-33$^{-/-}$ mice with *S. mansoni* cercariae and examined the
152 development of type 2-dependent pathology in the liver at acute (week 9) and chronic (week 12)
153 phases of infection. Although recent studies using hepatotoxic chemicals or schistosome egg-
154 driven models have suggested that IL-33 expression is critical to the development of fibrosis in
155 the liver (*32*), we observed no reduction in hepatic fibrosis in IL-33$^{-/-}$ mice at either time-point
156 (**Fig. 2C**) and picrosirius red staining of liver sections (**Fig. 2D**). The number of eosinophils in
157 the lesions and the diameter of granulomas were also similar in the absence of IL-33, confirming
158 unimpaired type 2-driven inflammation (**Fig. 2E**). The marked type 2 cytokine response that
159 normally develops in the livers of infected wild-type mice was also similarly observed in IL-33$^{-/-}$
160 mice, and in the case of IL-4 expression was even slightly increased (**Fig. 2F**), further suggesting

161 that IL-33 signaling is dispensable for the development of type 2 cytokine-driven pathology
162 during both acute and chronic *S. mansoni* infection.
163
164 **Disrupting TSLP, IL-25, and IL-33 signaling during *S. mansoni* infection**
165 To investigate whether TSLP, IL-25, and IL-33 were playing redundant roles in the maintenance
166 of type 2 cytokine-dependent granuloma formation and fibrosis, we developed strategies to
167 disrupt all three cytokine pathways simultaneously. In initial studies, $TSLP^{-/-}$ mice were crossed
168 with $IL\text{-}33^{-/-}$ mice to generate a double knockout mouse, and a highly effective neutralizing mAb
169 was introduced to the double knockout mice to block IL-25. C57BL/6 mice were infected with
170 *S. mansoni* cercariae, and the response in the absence of TSLP, IL-25, and IL-33 signaling was
171 evaluated at acute (week 9) and chronic (week 12) phases of infection. We first measured TSLP,
172 IL-25, and IL-33 gene expression and found each gene is constitutively expressed in whole liver
173 tissue at detectable levels **(Fig. S1)**. These levels of expression do not change significantly
174 during *S. mansoni* infection on a whole tissue level. In contrast to the studies in which individual
175 cytokines were targeted, we observed a small yet significant decrease in granuloma volume in
176 the triple deficient mice in the acute phase (**Fig. 3A**). This was also accompanied by a 25-30%
177 decrease in hepatic fibrosis (**Fig. 3B**) and a small yet significant decrease in the number of
178 granuloma-associated eosinophils (**Fig. 3C**). Interestingly, the decrease in pathology observed at
179 week 9 was associated with a significant decrease in the frequency of IL-13-producing type 2
180 innate lymphoid cells (ILC2s) in the mesenteric lymph nodes (MLNs) (**Fig. 3D**), which is
181 consistent with the ILC2-promoting activity of IL-25 and IL-33 (*33*). The frequency of ILC2s in
182 the liver, however, was not significantly different between the two groups (**Fig. 3D, right**
183 **panel**). Total leukocyte numbers were similar in the liver tissue and MLNs of both cohorts.

By 12 weeks post-infection, the decrease in IL-13-producing ILC2s in MLNs observed at week 9 was no longer significant (**Fig. 3D, left panel**), and while there was a modest but consistent decrease in pathology at week 9, granuloma volume and fibrosis became indistinguishable between WT and DKO + αIL-25-treated mice (**Figs. 3A-C**). Indeed, both groups of mice displayed a striking increase in IL-13-dependent fibrosis by week 12 as determined by both hydroxyproline assay (**Fig. 3B**) and picrosirius red staining (**Fig. 3E**). In addition, while the frequency of IL-13-producing ILC2s was lower in the MLN at week 9 (**Fig. 3D**), a marked increase in IL-4- and IL-13-producing CD4$^+$ T cells was observed at the same time point in the granulomatous livers of the DKO + αIL-25-treated mice (**Fig. 3F**). Antigen-specific CD4$^+$ Th2 cell cytokine production likely compensated for the transient decrease in ILC2s, thus explaining the unimpaired development of IL-13-dependent fibrosis in triple deficient mice by week 12.

Disrupting TSLP, IL-25, and IL-33 signaling during acute granuloma formation

After considering the transient nature of immune control affected by disrupting the three mediators during *S. mansoni* infection, we hypothesized that the effect of blocking all three cytokines would be more apparent when applied to a more acute model where the cytokines are blocked from the onset of injury. Primary and secondary lung granuloma models were employed for these studies because they provide simple and short-term systems to dissect the importance of TSLP, IL-25, and IL-33 during both the sensitization and maintenance phases of a type 2 cytokine-driven inflammatory response (*31*). Groups were treated with either isotype control antibodies or with αTSLP, αIL-25□□□□□□αST2 (IL-33 receptor) monoclonal neutralizing antibodies for the entire length of the experiments. The pathological effects of TSLP, IL-25, and

207 IL-33 have been directly linked to the enhanced production of IL-4, IL-5, and IL-13 by
208 downstream target cells such as $CD4^+$ Th2 cells, ILC2s, and other innate lymphocytes (*33, 34*),
209 and much of the pathology that results from the persistent activation of type 2 immunity has been
210 attributed to IL-4/IL-13-mediated signaling through the IL-4 receptor (*35*). Therefore, we used
211 IL-4R Γ -deficient mice as positive controls.

212 Surprisingly, as observed in previous lung granuloma studies where TSLP, IL-25, and IL-
213 33 were targeted individually, the combined blockade of all three cytokines had no significant
214 impact on the volume of the lesions in mice undergoing either primary (**Fig. 4A**) or secondary
215 (**Fig. 4B**) granuloma formation. In marked contrast, the lesions in IL-4R$\alpha^{-/-}$ mice were about
216 50% smaller than those in isotype control treated mice (**Figs. 4A-B**). The triple blockade did
217 lead to a >80% reduction in the number of granuloma-associated eosinophils during primary
218 granuloma formation (**Fig. 4A, right panel and tissue sections**). Macrophages and primarily
219 lymphocytes comprised the granulomas in the absence of eosinophils. Nevertheless, the
220 eosinophil deficit in the triple blockade mice was completely corrected when the mice were
221 undergoing a secondary challenge (**Fig. 4B, right panel and tissue sections**). IL-4R$\alpha^{-/-}$ mice, in
222 contrast, displayed a near complete absence of eosinophils following both primary and secondary
223 challenges.

224 Although the effects of the triple blockade on egg-induced pathology were minimal, there
225 were notable changes in cytokine expression in the lungs. Triple blockade mice displayed
226 significant reductions in IL-4, IL-5, and IL-13 expression in the lung during primary granuloma
227 formation (**Fig. 4C**) and in IL-5 and IL-13 during secondary granuloma formation (**Fig. 4D**). It
228 is worth noting that while these measurements imply a significant reduction in the type 2
229 cytokines following triple blockade, they were expressed at significantly higher levels than that

230 in IL-4Rα–deficient mice. Interestingly, changes in expression of two eosinophilic chemokines,
231 *Ccl5* and *Ccl11,* do not explain the eosinophil phenotype we observed. *Ccl5* and *Ccl11* were not
232 affected by the triple blockade during the primary response (**Fig. 4C**) although both chemokines
233 were reduced in the triple blockade mice during a secondary response (**Fig. 4D**). Rather, the
234 pattern of *Il5* gene expression likely explains why granuloma eosinophilia is reduced by the
235 triple blockade during primary granuloma formation and is restored during secondary granuloma
236 formation. The reduced *Il5* expression in triple blockade mice during primary granuloma
237 formation was on par with expression observed in IL-4Rα -deficient mice. During secondary
238 granuloma formation, *Il5* expression was reduced by the triple blockade, but it was still
239 expressed at significantly higher levels than in IL-4Rα –deficient mice.
240 As seen in many type 2 cytokine-driven diseases, we observed increased *Il33*, *Tslp*, and
241 *Il25* gene expression in the lungs of wild-type mice in the primary granuloma model (**Fig. 4C**).
242 While gene expression of *Il33* and *Tslp* increased in the lungs of mice undergoing secondary
243 granuloma formation, *Il25* was expressed at baseline levels during the secondary response (**Fig.**
244 **4D**). The increase was IL-4Rα-dependent as *Il33* and *Tslp* expression diminished to baseline
245 levels in IL-4Rα$^{-/-}$ mice. We hypothesize the low alarmin expression in IL-4Rα$^{-/-}$ mice is due to
246 decreased inflammation-driven injury in these mice.
247 Together, our studies with *S. mansoni* demonstrated that TSLP, IL-25, and IL-33 play
248 redundant roles in the maintenance of chronic type 2 immunity. More importantly, targeting all
249 three cytokines simultaneously from the initiation of primary or secondary granuloma formation
250 reduced type 2 cytokine production but offered little protection from egg-induced pathology.
251
252 **Efficacy of TSLP, IL-25, and ST2 blockade on established chronic allergy**

253 With evidence that the combined blockade of TSLP, IL-25, and IL-33 signaling had a significant
254 impact on type 2 cytokine expression, we hypothesized that the triple blockade might ameliorate
255 type 2-mediated pathology in a different disease model. We sought to investigate a model that
256 primarily targets epithelial cells, the predominant source of TSLP, IL-25, and IL-33, to
257 determine whether the maintenance of type 2 immunity induced via mucosal epithelial injury
258 was more dependent on the targeted cytokines. We chose to test the effects of administering
259 single, double, and triple mAb blockades to a model of house dust mite (HDM)-induced allergic
260 inflammation entering its chronic stage. Genes for all three alarmins are expressed at steady
261 state in the lung, and HDM induces expression of each of the alarmins with complementary
262 kinetics **(Fig. S2)**. *Il33* was upregulated acutely and at chronic stages of allergic disease. *Tslp*
263 was only upregulated in the initial hours after first HDM exposure, and *Il25* was upregulated
264 only at chronic time-points. BALB/c mice were chronically challenged i.n. with HDM on days
265 0, 7, and 14 and then received eight additional doses spread over a total of 45 days. Beginning
266 three weeks after the initiation of the allergic response, separate groups of HDM-treated mice
267 were administered doses of anti-ST2, anti-TSLP, anti-IL-25 every 3 to 4 days in various
268 combinations to achieve single, double, or triple blockades. Additional control groups received
269 either saline or isotype control antibodies with or without HDM. On day 46, all mice were
270 analyzed. As expected, in the lungs of isotype-treated control mice, chronic HDM exposure
271 resulted in a marked increase in inflammatory cells in the lung (**Fig. 5A**) and nearly a two-fold
272 increase in collagen content (**Fig. 5B**), confirming extensive lung remodeling and fibrosis.
273 Surprisingly however, none of the single, double, or triple blockade combinations led to a
274 significant decrease in inflammation or fibrosis in the sensitized mice. When the triple blockade
275 mice were analyzed more closely, we also observed little to no change in the type 2 cytokine

276 response in the lung (**Fig. 5C**), and the total number of leukocytes in the BAL and lung appeared
277 indistinguishable between the triple blockade and isotype control treated mice (**Fig. 5D**). We
278 did, however, observe a significant decrease in the percentage of eosinophils in the lung but not
279 in the BAL (**Fig. 5E**).
280
281 **Disrupting TSLP, IL-33, and IL-25 signaling during initiation and maintenance of type 2-**
282 **driven chronic allergy**
283 The failure of the triple blockade to protect against type 2-driven pathology when applied to
284 established allergy further suggested that TSLP, IL-25, and IL-33 are not critical for the
285 maintenance of chronic type 2 driven allergic lung inflammation. To test whether disrupting
286 signaling of the three cytokines during the initiation of type 2 cytokine-driven allergic lung
287 inflammation provides a benefit, in a final series of experiments, IL-33/TSLP DKO mice were
288 treated with anti-IL-25 during the entire course of chronic HDM exposure. Here, the deficient
289 mice displayed marked and significant decreases in fibrosis when compared with control HDM
290 mice on day 46 (**Fig. 6A**). Although peribronchial and perivascular inflammation in the lung
291 was similar in both groups, we observed a marked decrease in endarteritis and mucus staining in
292 the lumen of the deficient mice (**Fig. 6B**). In addition, the total number of BAL cells (**Fig. 6C**),
293 and the number of eosinophils in the BAL (**Fig. 6D**) and lung (**Fig. 6E**) were reduced. The
294 decrease in inflammatory eosinophils was also accompanied by a marked and highly significant
295 reduction in IL-4, IL-5, and IL-13 production in the lung (**Fig. 6F**) and IL-13 and IL-5 were also
296 significantly decreased in the BAL (**Fig. 6G**). We observed similar results using anti-ST2, anti-
297 TSLP, anti-IL-25 neutralizing antibodies in wild-type mice during the entire course of chronic
298 HDM exposure (**Fig. S3**).

299

300 **Discussion**

301

302 Although TSLP, IL-25, and IL-33 have each been identified as important initiators of
303 type 2 immunity, their role in the maintenance of progressive type 2 disease was much less clear.
304 In the present study, using chronic models of helminth infection and type 2 cytokine-driven lung
305 inflammation, we found that tri-functional targeting of TSLP, IL-25, and IL-33 was more
306 efficacious than blocking any one of the mediators alone. This conclusion is strengthened since
307 we made the observations using mice on both C57BL/6 and BALB/c backgrounds. In a
308 schistosome lung granuloma model or during chronic *S. mansoni* infection in the liver, selective
309 ablation of TSLP, IL-25, or IL-33/ST2 had little to no impact on the development of IL-4/IL-13-
310 dependent inflammation or fibrosis. Nevertheless, we observed modest albeit significant
311 reductions in egg-induced inflammation in the liver when signaling of all three mediators was
312 disrupted simultaneously. The reduction in inflammation in the schistosome infection model was
313 also accompanied by a small yet significant decrease in the number of granuloma-associated
314 eosinophils, a 25-30% decrease in hepatic fibrosis, and a significant reduction in the number of
315 IL-13-producing ILC2s in the mesenteric lymph nodes. The deficient mice also displayed
316 reduced expression of IL-5 and IL-13 during primary and secondary granuloma formation in the
317 lung. Furthermore, when signaling of all three mediators was disrupted in a model of chronic
318 HDM-induced allergic lung inflammation, inflammation, mucus production, and lung
319 remodeling were decreased. Together, these studies revealed redundant roles for TSLP, IL-25,
320 and IL-33 in the maintenance of these type 2-associated pathologies and suggest that aggressive

321 tri-functional targeting of these mediators may more effectively ameliorate progressive type 2-
322 driven disease.

323 Previous studies identified critical roles for TSLP, IL-25, and IL-33 in type 2 immunity to
324 some helminth parasites (*36-43*). However, the majority of these studies have focused on
325 *Nippostrongylus brasiliensis* infection, in which expulsion of the nematode parasite is delayed or
326 accelerated by relatively minor changes in type 2 immunity. Our initial studies focused on the
327 schistosome lung granuloma and *S. mansoni* infection models because these models provide
328 robust systems to dissect the role of TSLP, IL-25, and IL-33 during both the initiation and
329 maintenance phases of type 2-driven inflammation (*31*). As reported previously with TSLP (*26*),
330 we observed little to no role for IL-25 or IL-33 in IL-4/IL-13-dependent granuloma formation in
331 the lung. A recent study found modest decreases in acute inflammation in the absence of IL-25
332 (*44*), but in our studies, IL-25 or IL-33 deficiency alone had no discernable impact on the
333 development of type 2 immunity or type 2-dependent pathology, even during the initiation of a
334 primary granulomatous response. A similar outcome was observed in the liver following acute
335 and chronic infection with *S. mansoni*, suggesting that TSLP, IL-25, and IL-33 were either not
336 required or were possibly playing redundant roles (*26, 28, 38, 45*). Importantly, although we
337 found little to no role for TSLP, IL-25, or IL-33/ST2 when each mediator was ablated
338 individually, we observed significant reductions in type 2 inflammation and fibrosis in the liver
339 when all three mediators were targeted simultaneously, confirming their overlapping activities in
340 response to significant damage during acute schistosomiasis. It is possible the degree of damage
341 from parasites and other environmental triggers may impact the redundancy of the alarmins.
342 Also, schistosome egg antigens have been identified that are capable of directly activating type 2
343 responses by modulating dendritic cell function(*46, 47*). Basophil- and autocrine T cell-derived

344 IL-4 may also be sufficient to initiate and maintain type 2 responses(*48, 49*). Therefore, alarmins
345 may not be critical to the activation or maintenance of all type-2 cytokine driven inflammatory
346 responses.
347 The type 2 response is a critical driver of wound repair pathways (*1*). However when
348 type 2 cytokine production persists or becomes dysregulated, it can lead to the development of
349 pathological fibrosis (*3*). Consequently, because of their type 2-inducing activity, there has been
350 a great deal of interest in understanding the roles of TSLP, IL-25, and IL-33 in progressive
351 fibrosis, with numerous studies identifying increased production of these cytokines in various
352 fibrotic diseases (*50-54*). Many recent studies have shown that when overexpressed in mice,
353 TSLP, IL-25, and IL-33 induce fibrosis in multiple tissues. For example, IL-25 was shown to
354 promote lung remodeling in a model of house dust mite induced allergic airway disease and
355 indirectly induced pulmonary fibrosis by stimulating the production of IL-13 from ILC2s (*44,*
356 *54*). Transgenic overexpression of IL-33 has also been shown to promote IL-13-dependent
357 cutaneous fibrosis (*55*), ILC2-mediated hepatic fibrosis (*32*), and bleomycin-induced pulmonary
358 fibrosis in mice (*56*). Transgene-induced expression of TSLP has also been shown to induce
359 pulmonary fibrosis in the lung by upregulating type 2 cytokine expression (*52*). Nevertheless,
360 evidence that these epithelial-derived alarmins are critical to the development of Th2-associated
361 fibrosis in a natural model of fibrosis was lacking prior to this study. Our studies with the
362 schistosome lung granuloma and infection models show quite unequivocally that IL-13-
363 dependent fibrosis can develop in the lung and liver independently of TSLP, IL-25, and IL-33.
364 We did, however, observe a significant decrease in fibrosis when all three mediators were
365 targeted simultaneously, with the reduction in fibrosis associated with a significant decrease in
366 IL-13 producing ILC2s. Surprisingly, at more chronic time points following infection with *S.*

367 *mansoni* the early reduction in fibrosis and ILC2 activity appeared to be compensated for by an
368 increased $CD4^+$ T cell-derived IL-13 response, suggesting that TSLP, IL-25, IL-33 and ILC2s
369 may not be critical to the maintenance of established and progressive fibrosis once the adaptive
370 immune response has taken over. The relative involvement of an adaptive antigen-specific
371 response may therefore be important in determining the relative contribution of these innate
372 pathways to chronic disease. Regardless, these data further emphasize the potential benefit of
373 early combinatorial targeting of TSLP, IL-25, and IL-33 in the treatment of type 2-driven
374 disease.

375 Because epithelial cells are a major source of TSLP, IL-25, and IL-33 and schistosome
376 eggs primarily damage the endothelium, it is possible that these cytokines are less important to
377 the development of type 2 pathology in schistosomiasis. Therefore, in a final series of
378 experiments, we utilized a chronic model of HDM-induced allergic lung inflammation to explore
379 the combined roles of TSLP, IL-25, and IL-33 in a disease where the epithelium is the primary
380 target. Here, in contrast to the lung granuloma studies in which a mAb triple blockade
381 administered from initial egg challenge had little impact on type 2 pathology, disrupting TSLP,
382 IL-25, and IL-33 signaling from first allergen exposure had a significant suppressive effect on
383 the development of fibrosis, endarteritis, and mucus deposition in the lumen. The number of
384 inflammatory cells in the BAL was also reduced, as were the number of eosinophils in the BAL
385 and lung, with the reduction in eosinophils consistent with a recent study exploring the roles of
386 TSLP, IL-25, and IL-33 in a model of chitin-induced lung inflammation (*57*). We also observed
387 marked and highly significant reductions in IL-5 and IL-13 production in the lung and BAL
388 fluid. When the combined mAb blockade of TSLP, IL-25, and IL-33 was applied to a model of
389 established allergic lung inflammation, the marked protective effects were almost completely

390 lost, however, suggesting that TSLP, IL-33, and IL-25 are either not required for the
391 maintenance of an established antigen-specific type 2 response or that earlier intervention with
392 TSLP, IL-33, and IL-25 antagonists is needed.

393 Although TSLP, IL-33, and IL-25 were all initially identified as critical drivers of type 2
394 immunity (*10, 12, 50*), several subsequent studies have illustrated that type 2 immunity can
395 develop independently of these cytokines (*24-26, 28*). The results from our experiments suggest
396 that much of the data in the latter studies are likely explained by the overlapping activities of
397 TSLP, IL-33, and IL-25. Our data also suggest the three alarmins may be dispensable for the
398 maintenance of type 2 immunity and chronic type 2-associated pathology because continued
399 exposure to complex antigens like schistosome eggs or house dust mite allergen generates a
400 potent and sustained adaptive $CD4^+$ type 2 response that can supplant the requirement for
401 alarmins and innate lymphocytes. A recent double-blind, placebo-controlled study of AMG 157,
402 a neutralizing anti-human TSLP mAb, showed that TSLP blockade could reduce allergen-
403 induced bronchoconstriction and eosinophilia (*58*). Whether targeting TSLP alone would show
404 clinical benefit in moderate-severe asthma, however, could not be discerned from this small
405 study tested on allergic individuals with near-normal baseline lung function.

406 Differences in perturbations of epithelium and other stromal cells may dictate the relative
407 contribution of the three alarmins, and further studies with different animal models of allergy
408 (e.g. allergen dosing, variety, airway hypersensitivity) will be important before large-scale
409 human studies are considered. The cost and time required for chronic models prevented us from
410 testing all combinations of single, double, and triple blockades in every model. Notably, the
411 triple blockade with mAbs from the start of allergic disease is effective, but its impact was not
412 identical to congenital knockouts by all measures. Although all three antibodies were confirmed

413 to exhibit highly effective neutralizing activity, it is possible that incomplete target coverage with
414 the antibodies might in part explain these differences as well as the minimal efficacy of treating
415 mice with established allergic disease. It is also possible that intracrine alarmin signaling such as
416 IL-33-mediated activation of NF-κB contributes to these small differences. In any case, antibody
417 target coverage should be carefully evaluated in any future study in humans. Chronic human
418 disease is likely maintained by a complex assortment of signals combined with sporadic
419 exposure to specific antigen, and a better understanding of the hierarchy of these cues will help
420 to clarify the relative contributions of TSLP, IL-33, and IL-25, as well as ILC2s. Our data
421 suggest that a strategy that simultaneously suppresses more than one of these alarmins from the
422 early phase of the disease may be required to effectively target type 2 cytokine-driven disease.
423
424 **Materials and Methods**
425
426 **Study Design**
427 Our primary objective was to investigate the effects of ablating IL-33, TSLP, and IL-25 signaling
428 on chronic type 2 inflammation and fibrosis. To do this, we developed strategies to disrupt the
429 signaling of the cytokines in mouse models of progressive type 2 immune-related pathology. No
430 statistical methods were used to predetermine sample size. Group sample size was chosen using
431 records of variance in past experiments, and variance is similar between groups being
432 statistically compared. Samples or data points were excluded only in the case of a technical
433 equipment or human error that caused a sample to be poorly controlled for. Mice or samples
434 were randomly assigned to experimental groups or processing orders. Group allocation was
435 blinded for all mouse work, when possible (e.g. administration of proteins, schistosomes, or

436 allergens, sample quantification and analysis, pathology scoring). The ARRIVE guidelines in
437 the EQUATOR Network library were followed for this report.
438
439 **Animals**
440 The National Institute of Allergy and Infectious Diseases Division of Intramural Research
441 Animal Care and Use Program, as part of the National Institutes of Health Intramural Research
442 Program, approved all of the experimental procedures (protocol "LPD 16E"). The Program
443 complies with all applicable provisions of the Animal Welfare Act
444 (http://www.aphis.usda.gov/animal_welfare/downloads/awa/awa.pdf) and other federal statutes
445 and regulations relating to animals. IL-33$^{-/-}$ and IL-33/TSLP double knockout mice on a
446 C57BL/6 background were provided by Amgen Inc. C57BL/6, BALB/c, and IL-4Rα$^{-/-}$ mice
447 were obtained from Taconic Farms Inc. IL-25$^{-/-}$ mice were obtained from Regeneron
448 Pharmaceuticals, Inc. Male and female mice between the ages of 6 weeks and 12 weeks were
449 used randomly to begin experimental models because of limited availability, and no sex-specific
450 differences were observed. Groups in individual experiments were sex-matched and age-
451 matched. All animals were housed under specific pathogen-free conditions at the National
452 Institutes of Health in an American Association for the Accreditation of Laboratory Animal
453 Care-approved facility.
454
455 **Parasite infection**
456 Mice were infected percutaneously via the tail with 35 cercaria from a Puerto Rican strain of
457 *Schistosoma mansoni* (NMRI) obtained from infected *Biomphalaria glabrata* snails (Biomedical
458 Research Institute). 35 cercaria infection in wild-type mice leads to substantial disease and liver

459 fibrosis but low mortality through the chronic phase of infection. Mice were perfused at the time
460 of euthanasia to determine worm and tissue egg burdens as described previously (*59*).
461
462 **Chronic house dust mite-induced allergy**
463 Mice anesthetized with isoflurane were challenged intranasally with 200 µg of house dust mite
464 (HDM) in 30µl saline on days 0, 7, and 14 followed by eight additional 50 µg doses in 30µl
465 saline spread over a total of 45 days. Lungs were harvested on day 46.
466
467 **Schistosome egg-induced lung granuloma models**
468 For the primary lung granuloma model, 5000 live *S. mansoni* eggs (Biomedical Research
469 Institute) in saline were injected intravenously into mice on day 0. Lungs were harvested on day
470 7 for analysis. For the secondary lung granuloma model, 5000 *S. mansoni* eggs were also
471 injected intraperitoneally on day 0. Mice were injected intravenously with 5000 live eggs
472 containing mature embryos again on day 14 before lungs were harvested on day 21.
473
474 **Hydrodynamic delivery of IL-25**
475 Mice were injected intravenously with 10µg of a mammalian expression plasmid coding for
476 murine IL-25 in 2ml of warm saline (*60*).
477
478 **Triple block of IL-33, TSLP, and IL-25 with monoclonal antibodies**
479 Anti-mouse ST2 (*61*), anti-mouse TSLP (*38*), and anti-mouse IL-25 (*62*) monoclonal antibodies
480 were generated and selected by Amgen Inc. after extensive *in vitro* and *in vivo* testing.
481 Previously unpublished tests for the efficacy of anti-TSLP included a bone marrow-derived

dendritic cell bioassay measuring the inhibition of TSLP-induced CCL17/TARC production and an assay measuring inhibition of TSLP-induced proliferation of a pro-B cell line stably transduced with murine TSLP receptor. Neutralization of IL-33, TSLP, and IL-25 signaling was achieved by administering 250µg of these antibodies, respectively, via intraperitoneal injection twice-weekly. To properly control for the neutralizing antibodies, groups administered single and double blocks also received 250µg mouse IgG1 in the absence of anti-ST2 or anti-IL-25, and 250 O g rat IgG1 in the absence of anti-TSLP.

Histopathology

Liver or lung tissue was fixed in Bouin-Hollande solution, embedded in paraffin for sectioning, and stained (Histopath of America) with Wright's Giemsa (*S. mansoni* models), hematoxylin and eosin, or Masson's trichrome (allergy model) for analysis of inflammation, picrosirius red or Masson's trichrome for fibrosis analysis, or Periodic acid-Schiff (PAS) stain for analysis of mucus production. A scale of 1 to 4 (4 being the highest) was used for scoring. A blinded pathologist measured the diameter of approximately 30 granulomas and quantified granulomatous eosinophils in Giemsa-stained sections of each sample with granulomatous pathology. Images were scanned with an Aperio ScanScope (Leica Biosystems).

Fibrosis assay

Hydroxyproline was measured as a surrogate for collagen content. A known weight of liver or lung tissue was hydrolyzed in 6 N HCl at 110°C for 18 h and then neutralized in 10 N NaOH before colorization. A standard curve comprised of dilutions of 1mM hydroxyproline (Sigma-Aldrich) (*63*).

505

506 **Leukocyte isolation for intracellular cytokine staining and eosinophil identification**

507 About 200 mg of lung or liver tissue was ground into a single-cell suspension through a 100-µm
508 nylon mesh. Leukocytes were separated on a 40% Percoll (Sigma-Aldrich) gradient (2000 rpm
509 for 15 min) and treated for 2 min with 1 ml ACK (ammonium chloride–potassium bicarbonate)
510 lysis buffer to lyse erythrocytes. After 3 hours of stimulation with phorbol 12-myristate 13-
511 acetate (PMA 10ng/ml), ionomycin (1µg/ml), and Brefeldin A (BFA, 10µg/ml), leukocytes were
512 fixed and permeabilized for 30 minutes (Cytofix/Cytoperm buffer; BD Biosciences) and then
513 stained for 30 minutes with antibodies for CD4 (Clone: RM4-5; eBioscience), IFN-γ (XMG1.2,
514 eBioscience), IL-4 (11B11, eBioscience), IL-5 (TRFK5, BD Pharmingen), and IL-13 (eBio13A,
515 eBioscience) diluted in the Permwash buffer (BD Biosciences). Unstimulated lung leukocyte
516 aliquots were set aside and stained for 30 minutes with anti-SiglecF. Postive SiglecF staining and
517 scatter profiling were used to identify eosinophils by flow cytometry. Leukocytes collected from
518 bronchoalveolar lavage were isolated with ACK lysis buffer, stimulated, fixed, permeabilized,
519 and stained as above. Expression of CD4, SiglecF, and the intracellular cytokines was analyzed
520 with a BD FACSCanto II flow cytometer and FlowJo v.7.6 software (Tree Star).

521

522 **Leukocyte isolation from liver and mesenteric lymph node for ILC2 staining**

523 Liver or lymph node tissue was ground into a single-cell suspension through a 100-µm nylon
524 mesh, and hepatic leukocytes required further separation using a 40% Percoll gradient and ACK
525 lysis as described above. Leukocyte samples from both tissues were stimulated, fixed, and
526 permeabilized as described above. Then they were stained for 30 minutes with antibodies for
527 CD16/32 (Clone: 2.4G2, BDBiosciences), CD4 (RM4-5, eBioscience), IL-13 (eBio13A,

528 eBioscience), ST2 (DJ8, MD Biosciences), and ICOS (C398.4A, Biolegend) diluted in
529 Permwash buffer (BD Biosciences). Expression of the surface markers and intracellular IL-13
530 was analyzed with a BD FACSCanto II flow cytometer and FlowJo v.7.6 software (Tree Star).
531
532 **RNA isolation and quantitative real-time PCR**
533 Lung or liver tissue was homogenized in TRIzol Reagent (Life Technologies) using Precellys 24
534 (Bertin Technologies). Total RNA was extracted from the homogenate by addition of chloroform
535 followed by the recommendations of the MagMax-96 Total RNA Isolation Kit (Life
536 Technologies). RNA was then reverse transcribed using SuperScript II Reverse Transcriptase
537 (Life Technologies). Real-time RT-PCR was performed on an ABI Prism 7900HT Sequence
538 Detection System (Applied Biosystems). Quantities of mRNA expressed by a particular gene
539 were determined using Power SYBR Green PCR Master Mix (Applied Biosystems), normalized
540 to ribosomal protein, large, P2 (RPLP2) mRNA levels in each sample, and then articulated as a
541 relative increase or decrease compared with mRNA levels expressed by the same gene in naive
542 controls. Primers were designed using Primer Express software (version 2.0; Applied
543 Biosystems). Forward and reverse primer sequences are listed in Table S1.
544
545 **Bronchoalveolar lavage, cell differential determination, and ELISA**
546 1 ml of ice-cold PBS supplemented with 5mM EDTA was injected through the trachea into the
547 lungs and aspirated using a syringe. ~$1x10^5$ cells were spun for 5 mins with a Shandon Cytospin
548 3 centrifuge (Thermo Scientific) onto a slide before being fixed with methanol and stained with
549 Diff-Quik (Boehringer) to identify leukocyte cell-types. Levels of IL-4, IL-5, and IL-13 in the

550 undiluted BAL were quantified using a Luminex-based multiplex assay according to
551 manufacturer's protocol (EMDMillipore).
552

553 **Statistical analysis**

554 All data were analyzed with Prism (Version 5; GraphPad). Data sets were compared with a two-
555 tailed t-test, and differences were considered significant if *P* values were less than 0.05. A
556 Welch's correction was used when an F-test comparing variances had a *P* value of less than 0.05.
557

558 **Supplementary Materials**

559 Fig. S1. Alarmin gene expression in the liver.
560 Fig. S2. Kinetics of alarmin gene expression in chronic HDM model.
561 Fig. S3. Neutralizing all three alarmins with mAbs during initiation and maintenance of type 2-
562 driven allergy reduces inflammation and fibrosis.
563 Table S1. qPCR Primer Sequences.
564

565 **References**

566

567 1. W. C. Gause, T. A. Wynn, J. E. Allen, Type 2 immunity and wound healing: evolutionary
568 refinement of adaptive immunity by helminths. *Nature reviews. Immunology* **13**, 607-614
569 (2013).
570 2. A. K. Abbas, K. M. Murphy, A. Sher, Functional diversity of helper T lymphocytes.
571 *Nature* **383**, 787-793 (1996).

572 3. T. A. Wynn, Fibrotic disease and the T(H)1/T(H)2 paradigm. *Nature reviews.*
573 *Immunology* **4**, 583-594 (2004).
574 4. A. Chawla, K. D. Nguyen, Y. P. Goh, Macrophage-mediated inflammation in metabolic
575 disease. *Nature reviews. Immunology* **11**, 738-749 (2011).
576 5. J. E. Allen, R. M. Maizels, Diversity and dialogue in immunity to helminths. *Nature*
577 *reviews. Immunology* **11**, 375-388 (2011).
578 6. H. Y. Kim, R. H. DeKruyff, D. T. Umetsu, The many paths to asthma: phenotype shaped
579 by innate and adaptive immunity. *Nature immunology* **11**, 577-584 (2010).
580 7. G. Bouma, W. Strober, The immunological and genetic basis of inflammatory bowel
581 disease. *Nature reviews. Immunology* **3**, 521-533 (2003).
582 8. F. Y. Liew, N. I. Pitman, I. B. McInnes, Disease-associated functions of IL-33: the new
583 kid in the IL-1 family. *Nature reviews. Immunology* **10**, 103-110 (2010).
584 9. T. A. Wynn, T. R. Ramalingam, Mechanisms of fibrosis: therapeutic translation for
585 fibrotic disease. *Nature medicine* **18**, 1028-1040 (2012).
586 10. M. M. Fort, J. Cheung, D. Yen, J. Li, S. M. Zurawski, S. Lo, S. Menon, T. Clifford, B.
587 Hunte, R. Lesley, T. Muchamuel, S. D. Hurst, G. Zurawski, M. W. Leach, D. M.
588 Gorman, D. M. Rennick, IL-25 induces IL-4, IL-5, and IL-13 and Th2-associated
589 pathologies in vivo. *Immunity* **15**, 985-995 (2001).
590 11. V. Soumelis, P. A. Reche, H. Kanzler, W. Yuan, G. Edward, B. Homey, M. Gilliet, S.
591 Ho, S. Antonenko, A. Lauerma, K. Smith, D. Gorman, S. Zurawski, J. Abrams, S.
592 Menon, T. McClanahan, R. de Waal-Malefyt Rd, F. Bazan, R. A. Kastelein, Y. J. Liu,
593 Human epithelial cells trigger dendritic cell mediated allergic inflammation by producing
594 TSLP. *Nature immunology* **3**, 673-680 (2002).

595 12. J. Schmitz, A. Owyang, E. Oldham, Y. Song, E. Murphy, T. K. McClanahan, G.
596 Zurawski, M. Moshrefi, J. Qin, X. Li, D. M. Gorman, J. F. Bazan, R. A. Kastelein, IL-33,
597 an interleukin-1-like cytokine that signals via the IL-1 receptor-related protein ST2 and
598 induces T helper type 2-associated cytokines. *Immunity* **23**, 479-490 (2005).
599 13. C. A. Christianson, N. P. Goplen, I. Zafar, C. Irvin, J. T. Good, Jr., D. R. Rollins, B.
600 Gorentla, W. Liu, M. M. Gorska, H. Chu, R. J. Martin, R. Alam, Persistence of asthma
601 requires multiple feedback circuits involving type 2 innate lymphoid cells and IL-33. *J*
602 *Allergy Clin Immunol* **136**, 59-68 e14 (2015).
603 14. A. Shikotra, D. F. Choy, C. M. Ohri, E. Doran, C. Butler, B. Hargadon, M. Shelley, A. R.
604 Abbas, C. D. Austin, J. Jackman, L. C. Wu, L. G. Heaney, J. R. Arron, P. Bradding,
605 Increased expression of immunoreactive thymic stromal lymphopoietin in patients with
606 severe asthma. *J Allergy Clin Immunol* **129**, 104-111 e101-109 (2012).
607 15. D. Cheng, Z. Xue, L. Yi, H. Shi, K. Zhang, X. Huo, L. R. Bonser, J. Zhao, Y. Xu, D. J.
608 Erle, G. Zhen, Epithelial interleukin-25 is a key mediator in Th2-high, corticosteroid-
609 responsive asthma. *American journal of respiratory and critical care medicine* **190**, 639-
610 648 (2014).
611 16. P. Licona-Limon, L. K. Kim, N. W. Palm, R. A. Flavell, TH2, allergy and group 2 innate
612 lymphoid cells. *Nature immunology* **14**, 536-542 (2013).
613 17. M. D. Smithgall, M. R. Comeau, B. R. Yoon, D. Kaufman, R. Armitage, D. E. Smith, IL-
614 33 amplifies both Th1- and Th2-type responses through its activity on human basophils,
615 allergen-reactive Th2 cells, iNKT and NK cells. *Int Immunol* **20**, 1019-1030 (2008).
616 18. M. R. Comeau, S. F. Ziegler, The influence of TSLP on the allergic response. *Mucosal*
617 *immunology* **3**, 138-147 (2010).

618 19. J. Mjosberg, J. Bernink, K. Golebski, J. J. Karrich, C. P. Peters, B. Blom, A. A. te Velde,
619 W. J. Fokkens, C. M. van Drunen, H. Spits, The transcription factor GATA3 is essential
620 for the function of human type 2 innate lymphoid cells. *Immunity* **37**, 649-659 (2012).
621 20. T. Y. Halim, R. H. Krauss, A. C. Sun, F. Takei, Lung natural helper cells are a critical
622 source of Th2 cell-type cytokines in protease allergen-induced airway inflammation.
623 *Immunity* **36**, 451-463 (2012).
624 21. B. S. Kim, M. C. Siracusa, S. A. Saenz, M. Noti, L. A. Monticelli, G. F. Sonnenberg, M.
625 R. Hepworth, A. S. Van Voorhees, M. R. Comeau, D. Artis, TSLP elicits IL-33-
626 independent innate lymphoid cell responses to promote skin inflammation. *Science*
627 *translational medicine* **5**, 170ra116 (2013).
628 22. N. Lu, Y. H. Wang, Y. H. Wang, K. Arima, S. Hanabuchi, Y. J. Liu, TSLP and IL-7 use
629 two different mechanisms to regulate human CD4+ T cell homeostasis. *J Exp Med* **206**,
630 2111-2119 (2009).
631 23. N. Murakami-Satsutani, T. Ito, T. Nakanishi, N. Inagaki, A. Tanaka, P. T. Vien, K.
632 Kibata, M. Inaba, S. Nomura, IL-33 promotes the induction and maintenance of Th2
633 immune responses by enhancing the function of OX40 ligand. *Allergol Int* **63**, 443-455
634 (2014).
635 24. M. A. Willart, K. Deswarte, P. Pouliot, H. Braun, R. Beyaert, B. N. Lambrecht, H.
636 Hammad, Interleukin-1alpha controls allergic sensitization to inhaled house dust mite via
637 the epithelial release of GM-CSF and IL-33. *J Exp Med* **209**, 1505-1517 (2012).
638 25. S. Jang, S. Morris, N. W. Lukacs, TSLP promotes induction of Th2 differentiation but is
639 not necessary during established allergen-induced pulmonary disease. *PloS one* **8**, e56433
640 (2013).

641 26. T. R. Ramalingam, J. T. Pesce, M. M. Mentink-Kane, S. Madala, A. W. Cheever, M. R.
642 Comeau, S. F. Ziegler, T. A. Wynn, Regulation of helminth-induced Th2 responses by
643 thymic stromal lymphopoietin. *J Immunol* **182**, 6452-6459 (2009).
644 27. K. Oboki, T. Ohno, N. Kajiwara, K. Arae, H. Morita, A. Ishii, A. Nambu, T. Abe, H.
645 Kiyonari, K. Matsumoto, K. Sudo, K. Okumura, H. Saito, S. Nakae, IL-33 is a crucial
646 amplifier of innate rather than acquired immunity. *Proc Natl Acad Sci U S A* **107**, 18581-
647 18586 (2010).
648 28. J. C. Massacand, R. C. Stettler, R. Meier, N. E. Humphreys, R. K. Grencis, B. J.
649 Marsland, N. L. Harris, Helminth products bypass the need for TSLP in Th2 immune
650 responses by directly modulating dendritic cell function. *Proc Natl Acad Sci U S A* **106**,
651 13968-13973 (2009).
652 29. J. M. Mjosberg, S. Trifari, N. K. Crellin, C. P. Peters, C. M. van Drunen, B. Piet, W. J.
653 Fokkens, T. Cupedo, H. Spits, Human IL-25- and IL-33-responsive type 2 innate
654 lymphoid cells are defined by expression of CRTH2 and CD161. *Nature immunology* **12**,
655 1055-1062 (2011).
656 30. S. A. Saenz, B. C. Taylor, D. Artis, Welcome to the neighborhood: epithelial cell-derived
657 cytokines license innate and adaptive immune responses at mucosal sites. *Immunological*
658 *reviews* **226**, 172-190 (2008).
659 31. T. A. Wynn, I. Eltoum, I. P. Oswald, A. W. Cheever, A. Sher, Endogenous interleukin 12
660 (IL-12) regulates granuloma formation induced by eggs of Schistosoma mansoni and
661 exogenous IL-12 both inhibits and prophylactically immunizes against egg pathology. *J*
662 *Exp Med* **179**, 1551-1561 (1994).

663 32. T. McHedlidze, M. Waldner, S. Zopf, J. Walker, A. L. Rankin, M. Schuchmann, D.
664 Voehringer, A. N. McKenzie, M. F. Neurath, S. Pflanz, S. Wirtz, Interleukin-33-
665 dependent innate lymphoid cells mediate hepatic fibrosis. *Immunity* **39**, 357-371 (2013).
666 33. J. A. Walker, J. L. Barlow, A. N. McKenzie, Innate lymphoid cells--how did we miss
667 them? *Nature reviews. Immunology* **13**, 75-87 (2013).
668 34. S. J. Galli, N. Borregaard, T. A. Wynn, Phenotypic and functional plasticity of cells of
669 innate immunity: macrophages, mast cells and neutrophils. *Nature immunology* **12**, 1035-
670 1044 (2011).
671 35. S. J. Van Dyken, R. M. Locksley, Interleukin-4- and interleukin-13-mediated
672 alternatively activated macrophages: roles in homeostasis and disease. *Annual review of*
673 *immunology* **31**, 317-343 (2013).
674 36. A. Zhao, J. F. Urban, Jr., R. Sun, J. Stiltz, M. Morimoto, L. Notari, K. B. Madden, Z.
675 Yang, V. Grinchuk, T. R. Ramalingam, T. A. Wynn, T. Shea-Donohue, Critical role of
676 IL-25 in nematode infection-induced alterations in intestinal function. *J Immunol* **185**,
677 6921-6929 (2010).
678 37. C. Zaph, A. E. Troy, B. C. Taylor, L. D. Berman-Booty, K. J. Guild, Y. Du, E. A. Yost,
679 A. D. Gruber, M. J. May, F. R. Greten, L. Eckmann, M. Karin, D. Artis, Epithelial-cell-
680 intrinsic IKK-beta expression regulates intestinal immune homeostasis. *Nature* **446**, 552-
681 556 (2007).
682 38. B. C. Taylor, C. Zaph, A. E. Troy, Y. Du, K. J. Guild, M. R. Comeau, D. Artis, TSLP
683 regulates intestinal immunity and inflammation in mouse models of helminth infection
684 and colitis. *J Exp Med* **206**, 655-667 (2009).

685 39. A. M. Owyang, C. Zaph, E. H. Wilson, K. J. Guild, T. McClanahan, H. R. Miller, D. J.
686 Cua, M. Goldschmidt, C. A. Hunter, R. A. Kastelein, D. Artis, Interleukin 25 regulates
687 type 2 cytokine-dependent immunity and limits chronic inflammation in the
688 gastrointestinal tract. *J Exp Med* **203**, 843-849 (2006).
689 40. D. R. Neill, S. H. Wong, A. Bellosi, R. J. Flynn, M. Daly, T. K. Langford, C. Bucks, C.
690 M. Kane, P. G. Fallon, R. Pannell, H. E. Jolin, A. N. McKenzie, Nuocytes represent a
691 new innate effector leukocyte that mediates type-2 immunity. *Nature* **464**, 1367-1370
692 (2010).
693 41. L. Y. Hung, I. P. Lewkowich, L. A. Dawson, J. Downey, Y. Yang, D. E. Smith, D. R.
694 Herbert, IL-33 drives biphasic IL-13 production for noncanonical Type 2 immunity
695 against hookworms. *Proc Natl Acad Sci U S A* **110**, 282-287 (2013).
696 42. N. E. Humphreys, D. Xu, M. R. Hepworth, F. Y. Liew, R. K. Grencis, IL-33, a potent
697 inducer of adaptive immunity to intestinal nematodes. *J Immunol* **180**, 2443-2449 (2008).
698 43. P. G. Fallon, S. J. Ballantyne, N. E. Mangan, J. L. Barlow, A. Dasvarma, D. R. Hewett,
699 A. McIlgorm, H. E. Jolin, A. N. McKenzie, Identification of an interleukin (IL)-25-
700 dependent cell population that provides IL-4, IL-5, and IL-13 at the onset of helminth
701 expulsion. *J Exp Med* **203**, 1105-1116 (2006).
702 44. E. Hams, M. E. Armstrong, J. L. Barlow, S. P. Saunders, C. Schwartz, G. Cooke, R. J.
703 Fahy, T. B. Crotty, N. Hirani, R. J. Flynn, D. Voehringer, A. N. McKenzie, S. C.
704 Donnelly, P. G. Fallon, IL-25 and type 2 innate lymphoid cells induce pulmonary
705 fibrosis. *Proc Natl Acad Sci U S A* **111**, 367-372 (2014).
706 45. Y. H. Wang, T. Ito, B. Homey, N. Watanabe, R. Martin, C. J. Barnes, B. W. McIntyre,
707 M. Gilliet, R. Kumar, Z. Yao, Y. J. Liu, Maintenance and polarization of human TH2

708 central memory T cells by thymic stromal lymphopoietin-activated dendritic cells.
709 *Immunity* **24**, 827-838 (2006).
710 46. S. Steinfelder, J. F. Andersen, J. L. Cannons, C. G. Feng, M. Joshi, D. Dwyer, P. Caspar,
711 P. L. Schwartzberg, A. Sher, D. Jankovic, The major component in schistosome eggs
712 responsible for conditioning dendritic cells for Th2 polarization is a T2 ribonuclease
713 (omega-1). *J Exp Med* **206**, 1681-1690 (2009).
714 47. G. Schramm, K. Mohrs, M. Wodrich, M. J. Doenhoff, E. J. Pearce, H. Haas, M. Mohrs,
715 Cutting edge: IPSE/alpha-1, a glycoprotein from Schistosoma mansoni eggs, induces
716 IgE-dependent, antigen-independent IL-4 production by murine basophils in vivo. *J*
717 *Immunol* **178**, 6023-6027 (2007).
718 48. N. Noben-Trauth, J. Hu-Li, W. E. Paul, IL-4 secreted from individual naive CD4+ T cells
719 acts in an autocrine manner to induce Th2 differentiation. *European journal of*
720 *immunology* **32**, 1428-1433 (2002).
721 49. D. Jankovic, M. C. Kullberg, N. Noben-Trauth, P. Caspar, W. E. Paul, A. Sher, Single
722 cell analysis reveals that IL-4 receptor/Stat6 signaling is not required for the in vivo or in
723 vitro development of CD4+ lymphocytes with a Th2 cytokine profile. *J Immunol* **164**,
724 3047-3055 (2000).
725 50. B. Zhou, M. R. Comeau, T. De Smedt, H. D. Liggitt, M. E. Dahl, D. B. Lewis, D.
726 Gyarmati, T. Aye, D. J. Campbell, S. F. Ziegler, Thymic stromal lymphopoietin as a key
727 initiator of allergic airway inflammation in mice. *Nat Immunol* **6**, 1047-1053 (2005).
728 51. K. Yanaba, A. Yoshizaki, Y. Asano, T. Kadono, S. Sato, Serum IL-33 levels are raised in
729 patients with systemic sclerosis: association with extent of skin sclerosis and severity of
730 pulmonary fibrosis. *Clinical rheumatology* **30**, 825-830 (2011).

731 52. A. Usategui, G. Criado, E. Izquierdo, M. J. Del Rey, P. E. Carreira, P. Ortiz, W. J.
732 Leonard, J. L. Pablos, A profibrotic role for thymic stromal lymphopoietin in systemic
733 sclerosis. *Annals of the rheumatic diseases* **72**, 2018-2023 (2013).
734 53. P. Santulli, M. Even, S. Chouzenoux, A. E. Millischer, B. Borghese, D. de Ziegler, F.
735 Batteux, C. Chapron, Profibrotic interleukin-33 is correlated with uterine leiomyoma
736 tumour burden. *Human reproduction* **28**, 2126-2133 (2013).
737 54. L. G. Gregory, C. P. Jones, S. A. Walker, D. Sawant, K. H. Gowers, G. A. Campbell, A.
738 N. McKenzie, C. M. Lloyd, IL-25 drives remodelling in allergic airways disease induced
739 by house dust mite. *Thorax* **68**, 82-90 (2013).
740 55. A. L. Rankin, J. B. Mumm, E. Murphy, S. Turner, N. Yu, T. K. McClanahan, P. A.
741 Bourne, R. H. Pierce, R. Kastelein, S. Pflanz, IL-33 induces IL-13-dependent cutaneous
742 fibrosis. *J Immunol* **184**, 1526-1535 (2010).
743 56. I. G. Luzina, P. Kopach, V. Lockatell, P. H. Kang, A. Nagarsekar, A. P. Burke, J. D.
744 Hasday, N. W. Todd, S. P. Atamas, Interleukin-33 potentiates bleomycin-induced lung
745 injury. *American journal of respiratory cell and molecular biology* **49**, 999-1008 (2013).
746 57. S. J. Van Dyken, A. Mohapatra, J. C. Nussbaum, A. B. Molofsky, E. E. Thornton, S. F.
747 Ziegler, A. N. McKenzie, M. F. Krummel, H. E. Liang, R. M. Locksley, Chitin activates
748 parallel immune modules that direct distinct inflammatory responses via innate lymphoid
749 type 2 and gammadelta T cells. *Immunity* **40**, 414-424 (2014).
750 58. G. M. Gauvreau, P. M. O'Byrne, L. P. Boulet, Y. Wang, D. Cockcroft, J. Bigler, J. M.
751 FitzGerald, M. Boedigheimer, B. E. Davis, C. Dias, K. S. Gorski, L. Smith, E. Bautista,
752 M. R. Comeau, R. Leigh, J. R. Parnes, Effects of an anti-TSLP antibody on allergen-

753 induced asthmatic responses. *The New England journal of medicine* **370**, 2102-2110
754 (2014).
755 59. A. W. Cheever, M. E. Williams, T. A. Wynn, F. D. Finkelman, R. A. Seder, T. M. Cox,
756 S. Hieny, P. Caspar, A. Sher, Anti-IL-4 treatment of Schistosoma mansoni-infected mice
757 inhibits development of T cells and non-B, non-T cells expressing Th2 cytokines while
758 decreasing egg-induced hepatic fibrosis. *J Immunol* **153**, 753-759 (1994).
759 60. T. Suda, D. Liu, Hydrodynamic gene delivery: its principles and applications. *Molecular*
760 *therapy : the journal of the American Society of Gene Therapy* **15**, 2063-2069 (2007).
761 61. G. Palmer, D. Talabot-Ayer, C. Lamacchia, D. Toy, C. A. Seemayer, S. Viatte, A.
762 Finckh, D. E. Smith, C. Gabay, Inhibition of interleukin-33 signaling attenuates the
763 severity of experimental arthritis. *Arthritis Rheum* **60**, 738-749 (2009).
764 62. E. A. Rickel, L. A. Siegel, B. R. Yoon, J. B. Rottman, D. G. Kugler, D. A. Swart, P. M.
765 Anders, J. E. Tocker, M. R. Comeau, A. L. Budelsky, Identification of functional roles
766 for both IL-17RB and IL-17RA in mediating IL-25-induced activities. *J Immunol* **181**,
767 4299-4310 (2008).
768 63. T. A. Wynn, L. Barron, R. W. Thompson, S. K. Madala, M. S. Wilson, A. W. Cheever,
769 T. Ramalingam, Quantitative assessment of macrophage functions in repair and fibrosis.
770 *Current protocols in immunology / edited by John E. Coligan ... [et al.]* **Chapter 14**,
771 Unit14 22 (2011).
772

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777 **Author contributions:** KMV TRR MRC DES TAW conceived and designed the experiments;
778 KMV TRR AWC LAB KMH RWT SW performed the experiments; KMV TRR LAB LB KMH
779 KNK MRC DES TAW analyzed the data; KMH analyzed the statistics; AWC ALB MRC DES
780 contributed reagents/materials/analysis tools; KMV TAW wrote the paper.
781 **Competing interests:** ALB, MRC, DES work for a for-profit company.
782 **Data and materials availability:** Genes of interest can be accessed in NCBI's GenBank with
783 the following codes: *Rplp2:* NM_026020, *Il4:* NM_021283, *Il5:* NM_010558, *Il13:*
784 NM_008355, *Ifnγ:* NM_008337, *Il25:* NM_080729, *Il13:* NM_008355, *Ccl5:* NM_013653,
785 *Ccl11:* NM_011330, *Il33:* NM_001164724, *Tslp:* NM_021367.
786

787 **Figures Legends**

788

789 **Figure 1. Ablating IL-25 offers no protection against type 2-mediated pathology.**
790 A. Quantitative PCR analysis of gene expression in lung and liver tissue from wild type
791 C57BL/6 mice seven days after hydrodynamic injection of IL-25 (*n* = 5 mice) or PBS (*n* = 2). B.
792 Histopathology analysis of livers from wild type and IL-13R Γ 1$^{-/-}$ mice seven days after *S.*
793 *mansoni* egg exposure and 8 days after hydrodynamic injection of IL-25 or PBS (*n* = 12-15 per
794 group; pooled from two independent experiments; scale bars=50μm). C. Cytokine quantification
795 from bronchoalveor lavage fluid (BALF) of mice in B (*n* = 4-5 per group). D. Histopathology
796 analysis of lungs from IL-25$^{-/-}$ mice and littermate controls 7 days after challenge with *S.*
797 *mansoni* eggs with (2°) or without priming (1°) with 5000 *S. mansoni* eggs 14 days prior to

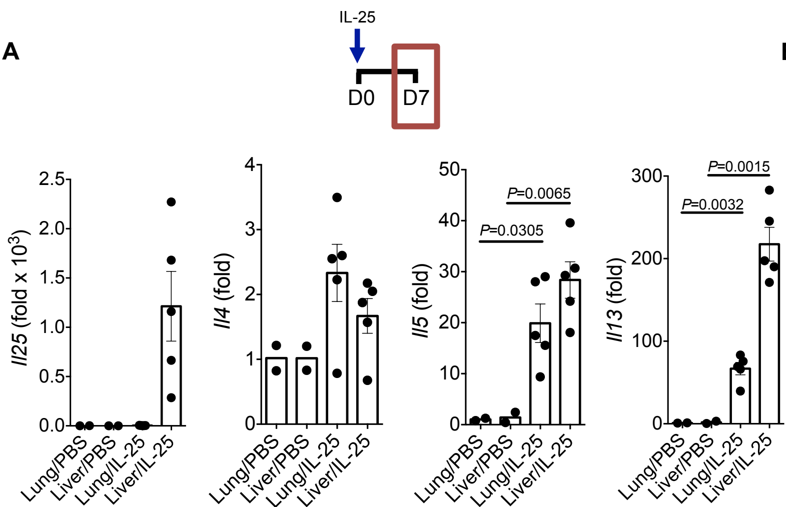
798 challenge (granuloma volume 1º: *n* = 18-23 per genotype pooled from three experiments;
799 granuloma volume 2º: *n* = 9-10 per genotype pooled from two experiments; eosinophils: *n* = 5
800 per genotype). E. Histopathology analysis and fibrosis quantificiation of livers of IL-25$^{-/-}$ mice
801 and littermate controls 12 weeks after infection with *S. mansoni* cercariae (*n* = 9 per genotype).
802 A Student's t-test was used to measure all *P* values, and *P*>0.05 except where reported. Error
803 bars represent standard error of the mean and each data point represents a value for an individual
804 mouse. Data are representative of two independent experiments unless otherwise noted.
805

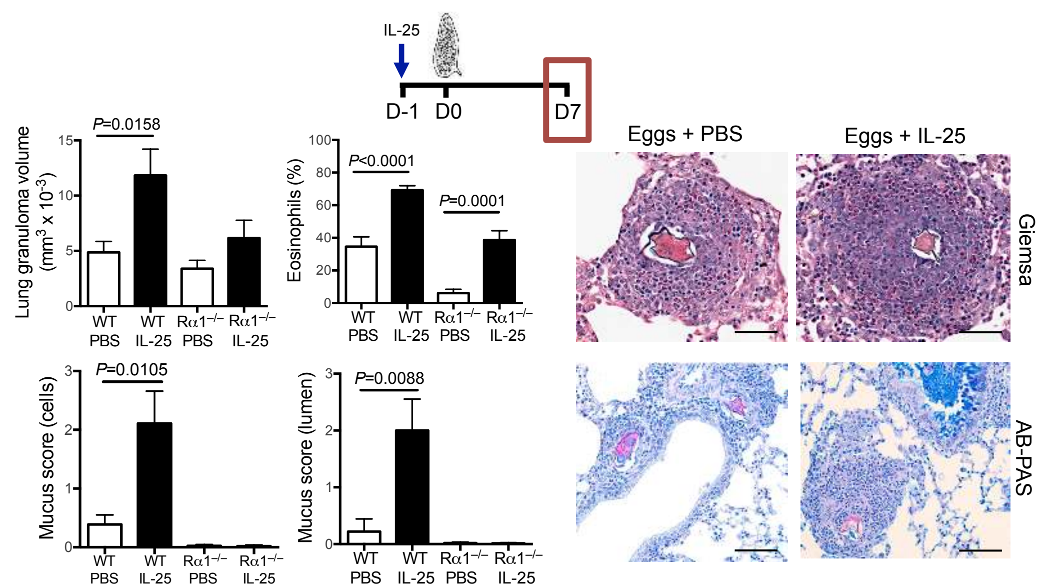
806 **Figure 2. Ablating IL-33 offers no protection against type 2-mediated pathology.**
807 A. Fibrosis quantification and histopathology analysis of lungs from wild type C57BL/6 and IL-
808 33$^{-/-}$ mice 7 days after challenge with *S. mansoni* eggs (*n* = 7-10 per genotype). B. Fibrosis
809 quantification and histopathology analysis of lungs of the same mouse strains 21 days after
810 priming with *S. mansoni* eggs and 7 days after challenge with eggs (*n* = 10 per genotype). C.
811 Fibrosis quantification of livers from the same mouse strains infected with *S. mansoni* cercariae
812 (*n* = 7-10 per genotype). D. Micrographs of representative liver tissue sections of mice in C
813 collected 9 weeks after infection and stained with picrosirius red (scale bar=100μm). E.
814 Histopathology analysis of livers from the mice in C (*n* = 7-10 per genotype). F. Intracellular
815 cytokine analysis of lymphocytes isolated from livers of mice in C nine weeks after infection
816 measured by flow cytometry (*n* = 8 per genotype). A Student's t-test was used to measure all *P*
817 values, and *P*>0.05 except where reported. Data are representative of two independent
818 experiments for each of the models.
819

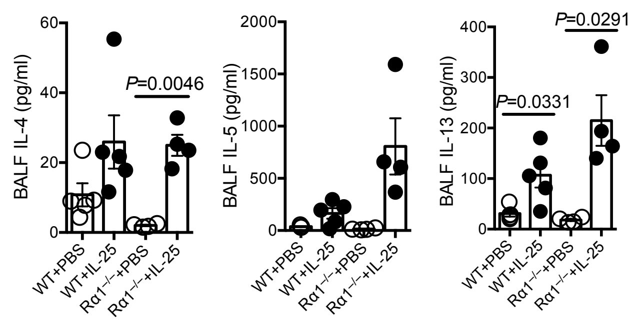
820 **Figure 3. Disruption of all three mediators simultaneously has a transient effect on Th2**
821 **pathology driven by *S. mansoni*.**
822 A. Granuloma measurement (*n* = 14-19 per group pooled from two independent experiments)
823 from livers of *S. mansoni*-infected wild type C57BL/6 mice administered isotype control
824 antibody and IL-33/TSLP double knockout (DKO) mice administered anti-IL-25. B. Fibrosis
825 quantification from livers of infected mice (*n* = 7-10 per group). C. Quantification of granuloma
826 eosinophils from livers of infected mice (*n* = 7-9 per group). D. Quantification of $CD4^{-}IL\text{-}13^{-}$
827 $ST2^{+}ICOS^{+}$ leukocytes from the mesenteric lymph nodes (MLNs; *n* = 7 per group) and livers
828 (week 9: *n* = 7-8 per group; week 12: *n* = 14-15 per group pooled from two independent
829 experiments) of infected mice by flow cytometry. E. Micrographs of representative liver tissue
830 sections of mice 12 weeks after infection and stained with picrosirius red (scale bar=500μm). F.
831 Intracellular cytokine analysis of liver lymphocytes of infected mice by flow cytometry (*n* = 14-
832 17 per genotype pooled from two experiments). A Student's t-test was used to measure all *P*
833 values, and P>0.05 except where reported. Data are representative of two independent
834 experiments.
835
836 **Figure 4. Combined TSLP, IL-25, and ST2 mAb blockade during granuloma generation**
837 **diminishes type 2 immunity but not pathology.**
838 A. Histopathology analysis of wild type BALB/c and $IL\text{-}4R^{-/-}$ mice seven days after injection
839 with *S. mansoni* eggs. Wild type egg-injected mice were either intraperitoneally (IP)
840 administered anti-ST2, anti-TSLP, and anti-IL-25, or corresponding isotype control antibodies
841 (*n* = 8-9 per group). Micrographs are of representative lung sections stained with Masson's
842 trichrome (scale bars = 50 μm). C. Quantification of gene expression in lung tissue from mice in

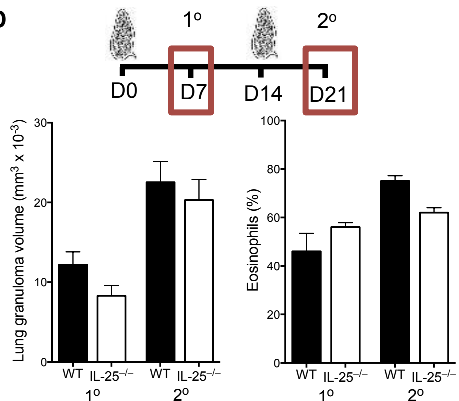
843 A assayed by qPCR and shown relative to expression in lungs of naïve BALB/c mice (*n* = 3). B.
844 Histopathology analysis of wild type BALB/c and IL-4R$^{-/-}$ mice seven days after injection with
845 *S. mansoni* eggs and 21 days after priming with *S. mansoni* eggs (Isotypes IP, Triple block IP: *n*
846 = 8-9 per group; IL-4R$^{-/-}$: *n* = 5). Wild type egg-injected mice were either intraperitoneally
847 administered anti-ST2, anti-TSLP, and anti-IL-25, or corresponding isotype control antibodies
848 for all three weeks. Micrographs are of representative lung sections stained with Masson's
849 trichrome (scale bars = 50 μm). D. Quantification of gene expression in lung tissue from mice in
850 B assayed by qPCR and compared to a different group of naïve BALB/c controls (*n* = 3). A
851 Student's t-test was used to measure all *P* values, and *P*>0.05 except where reported. Data are
852 representative of two independent experiments.
853
854 **Figure 5. Efficacy of TSLP, IL-25, and ST2 mAb blockade on established chronic allergy.**
855 Wild type BALB/c mice were sensitized and challenged intranasally with house dust mite
856 (HDM), and starting on day 21, anti-ST2, anti-TSLP, and/or anti-IL-25 were administered in
857 various combinations to different groups to achieve single, double, or triple blocks. Additional
858 control groups received only isotype control antibodies with or without HDM. To properly
859 control for the triple blockade group, groups administered single and double blocks also received
860 IgG1 in the absence of anti-ST2 or anti-IL-25, and rat IgG1 in the absence of anti-TSLP. All
861 mice were analyzed on day 46. A. Histopathology analysis of lung sections stained with
862 Masson's trichrome and scored for peribronchial and perivascular inflammation (*n* = 6-10 per
863 group pooled from two experiments). B. Quantification of fibrosis from lung tissue. C.
864 Quantification of gene expression from lung tissue measured by qPCR. D. Quantification of
865 leukocytes in the BALF and lung tissue. E. Quantification of eosinophils shown as a percentage

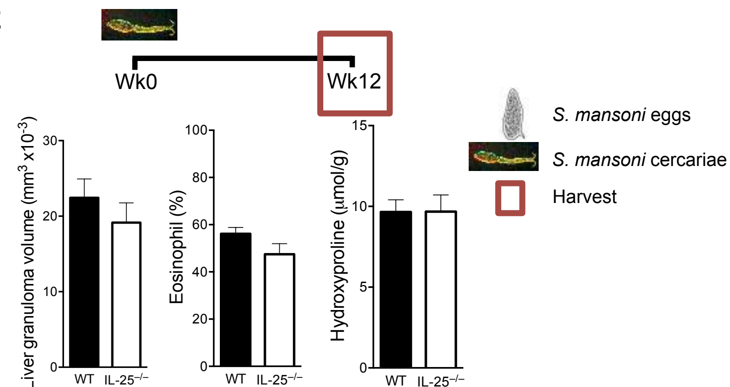
866 of total inflammatory cells in BALF and lung tissue. Student's t-test was used to measure all *P*
867 values, and *P*>0.05 except where reported.
868
869 **Figure 6. Disruption of all three mediators during initiation and maintenance of type 2-**
870 **driven chronic allergy reduces inflammation and fibrosis.**
871 Wild type C57BL/6 and IL-33/TSLP DKO mice were sensitized and challenged intranasally with
872 HDM over 45 days. DKO mice were IP administered αIL-25 (DKO+αIL-25/HDM), and HDM-
873 treated wild-type C57BL/6 mice were IP administered an IgG1 isotype control (Isotype/HDM).
874 A control group of C57BL/6 mice received intranasal saline instead of HDM and the isotype
875 (Isotype/Saline). All mice were analyzed on day 46. A. Quantification of fibrosis from lung
876 tissue (Isotype/Saline: *n* = 5; Isotype/HDM: *n* = 9; Triple block/Saline: *n* = 8). B. Histopathology
877 analysis of lung sections stained with Masson's trichrome for scoring of inflammation and AB-
878 PAS for mucus scoring. Micrographs are of representative lung sections stained with Masson's
879 trichrome (scale bars = 50 μm). C. Quantification of leukocytes in the BALF. D. BALF
880 leukocyte differential. E. Quantification of eosinophils in lung tissue. F. Intracellular cytokine
881 quantification of lung tissue lymphocytes by flow cytometry. G. Intracellular cytokine
882 quantification of BALF lymphocytes by flow cytometry. A Student's t-test was used to measure
883 all *P* values, and *P*>0.05 except where reported. Data are representative of two independent
884 experiments.

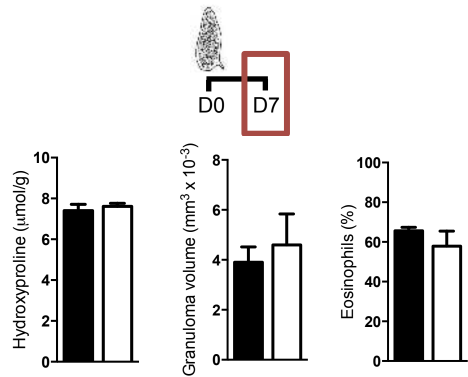

A
IL-25
D0
D7
Il25 (fold x 10^3)
Il4 (fold)
Il5 (fold)
Il13 (fold)
P=0.0305
P=0.0065
P=0.0032
P=0.0015
Lung/PBS
Liver/PBS
Lung/IL-25
Liver/IL-25


B
IL-25
D-1
D0
D7
Eggs + PBS
Eggs + IL-25
Giemsa
AB-PAS
Lung granuloma volume (mm^3 x 10^-3)
Eosinophils (%)
Mucus score (cells)
Mucus score (lumen)
P=0.0158
P<0.0001
P=0.0001
P=0.0105
P=0.0088
WT PBS
WT IL-25
Rα1−/− PBS
Rα1−/− IL-25


C
BALF IL-4 (pg/ml)
BALF IL-5 (pg/ml)
BALF IL-13 (pg/ml)
P=0.0046
P=0.0331
P=0.0291
WT+PBS
WT+IL-25
Rα1−/−+PBS
Rα1−/−+IL-25

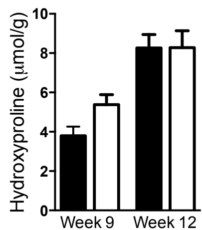

D
1º
2º
D0
D7
D14
D21
Lung granuloma volume (mm^3 x 10^-3)
Eosinophils (%)
WT
IL-25−/−

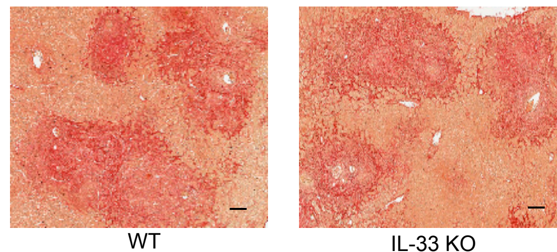

E
Wk0
Wk12
S. mansoni eggs
S. mansoni cercariae
Harvest
Liver granuloma volume (mm^3 x10^-3)
Eosinophil (%)
Hydroxyproline (μmol/g)
WT
IL-25−/−

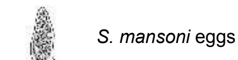


WT

IL-33 KO



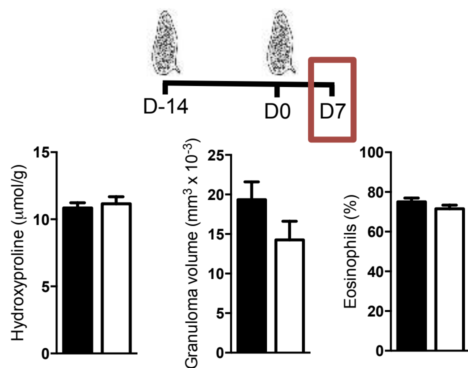


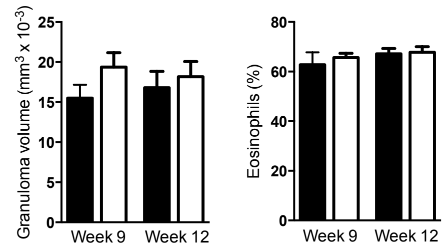


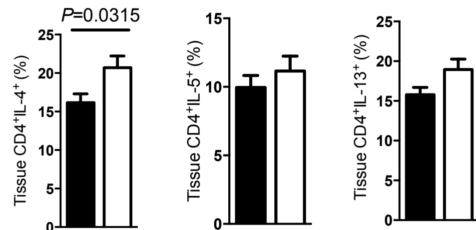
S. mansoni eggs

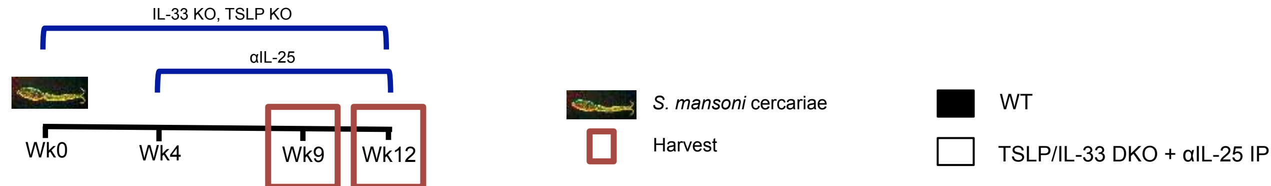
S. mansoni cercariae

Harvest









A

P=0.0153

Granuloma volume (mm^3 x 10^{-3})

30 20 10 0

Week 9 Week 12

B

P=0.008

Hydroxyproline (μmol/g)

15 10 5 0

Week 9 Week 12

C

P=0.0095

Eosinophils (%)

50 40 30 20 10 0

Week 9 Week 12

D

P=0.0006

MesLN $CD4^{-}IL13^{+}ST2^{+}ICOS^{+}$ (%)

0.20 0.15 0.10 0.05 0.00

Week 9 Week 12

Liver $CD4^{-}IL13^{+}ST2^{+}ICOS^{+}$ (%)

0.25 0.20 0.15 0.10 0.05 0.00

Week 9 Week 12

E

WT

TSLP/IL-33 DKO + αIL-25 IP

F

P<0.0001

P=0.0156

Tissue $CD4^{+}IL\text{-}4^{+}$ (%)

15 10 5 0

Week 9 Week 12

Tissue $CD4^{+}IL\text{-}5^{+}$ (%)

8 6 4 2 0

Week 9 Week 12

P=0.0042

Tissue $CD4^{+}IL\text{-}13^{+}$ (%)

20 15 10 5 0

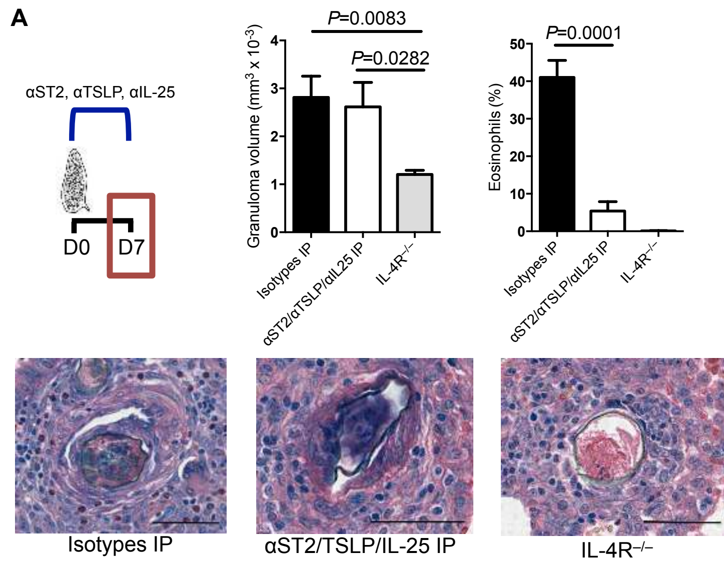
Week 9 Week 12

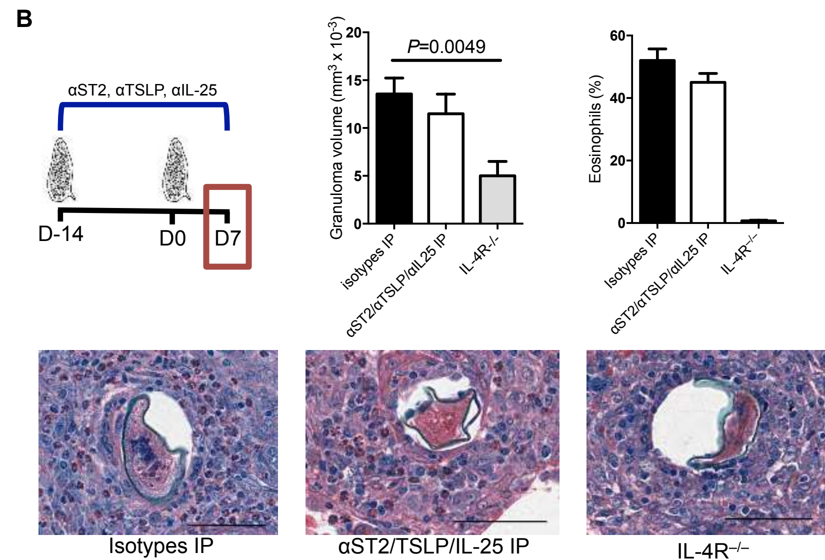
P=0.0252

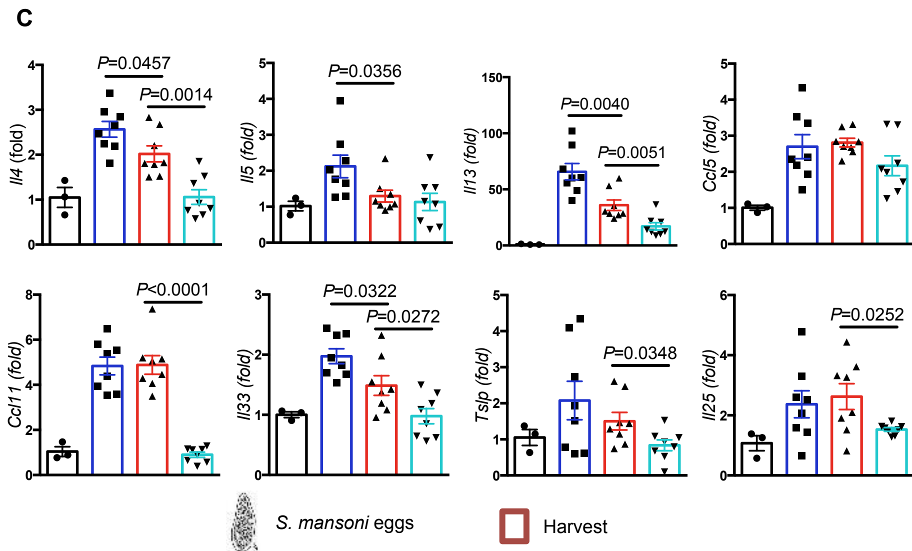
Tissue $CD4^{+}IFN\text{-}\gamma^{+}$ (%)

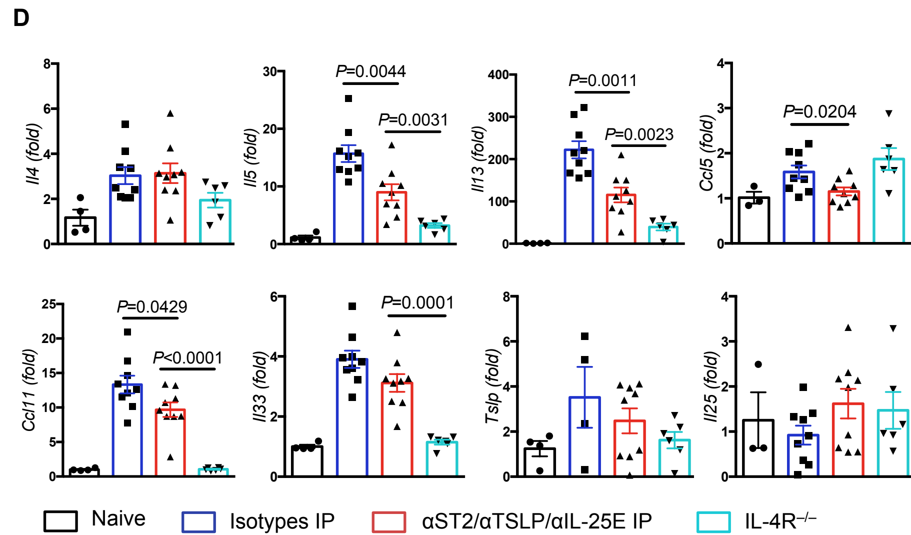
20 15 10 5 0

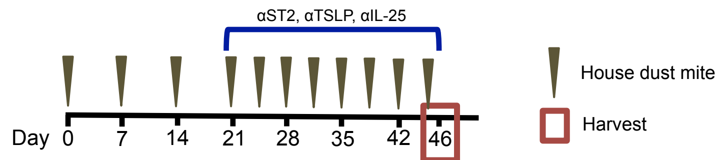
Week 9 Week 12

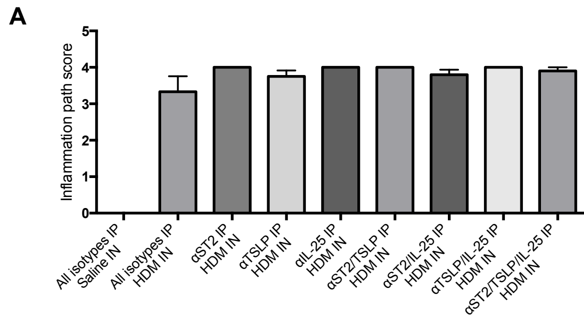

A
αST2, αTSLP, αIL-25
D0
D7
P=0.0083
P=0.0282
Granuloma volume (mm3 x 10-3)
Isotypes IP
αST2/αTSLP/αIL25 IP
IL-4R–/–
P=0.0001
Eosinophils (%)
Isotypes IP
αST2/TSLP/IL-25 IP
IL-4R–/–

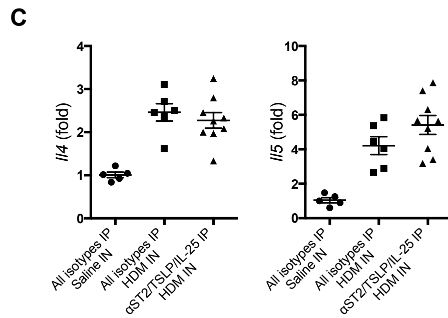

B
αST2, αTSLP, αIL-25
D-14
D0
D7
P=0.0049
Granuloma volume (mm3 x 10-3)
Eosinophils (%)
Isotypes IP
αST2/αTSLP/αIL25 IP
IL-4R-/-
Isotypes IP
αST2/TSLP/IL-25 IP
IL-4R–/–

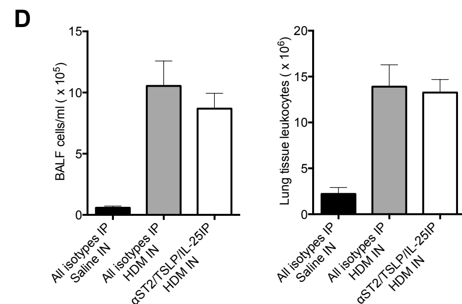

C
Il4 (fold)
P=0.0457
P=0.0014
Il5 (fold)
P=0.0356
Il13 (fold)
P=0.0040
P=0.0051
Ccl5 (fold)
Ccl11 (fold)
P<0.0001
Il33 (fold)
P=0.0322
P=0.0272
Tslp (fold)
P=0.0348
Il25 (fold)
P=0.0252
S. mansoni eggs
Harvest

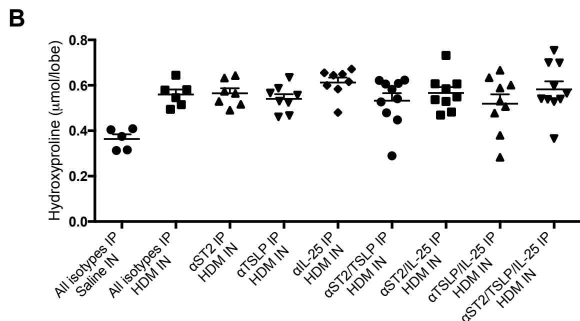

D
Il4 (fold)
Il5 (fold)
P=0.0044
P=0.0031
Il13 (fold)
P=0.0011
P=0.0023
Ccl5 (fold)
P=0.0204
Ccl11 (fold)
P=0.0429
P<0.0001
Il33 (fold)
P=0.0001
Tslp (fold)
Il25 (fold)
Naive
Isotypes IP
αST2/αTSLP/αIL-25E IP
IL-4R–/–

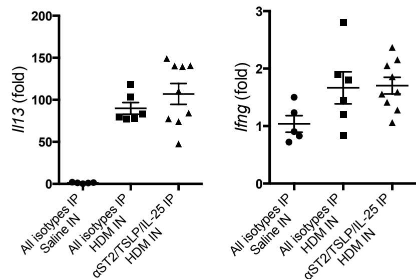

αST2, αTSLP, αIL-25
House dust mite
Harvest
Day 0 7 14 21 28 35 42 46

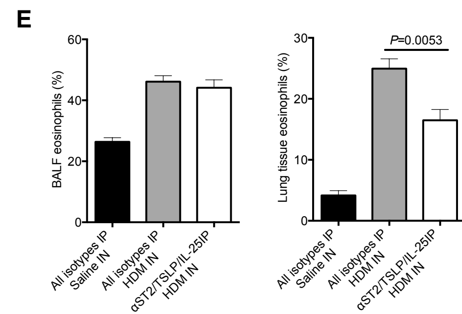

A
Inflammation path score
All isotypes IP Saline IN
All isotypes IP HDM IN
αST2 IP HDM IN
αTSLP IP HDM IN
αIL-25 IP HDM IN
αST2/TSLP IP HDM IN
αST2/IL-25 IP HDM IN
αTSLP/IL-25 IP HDM IN
αST2/TSLP/IL-25 IP HDM IN

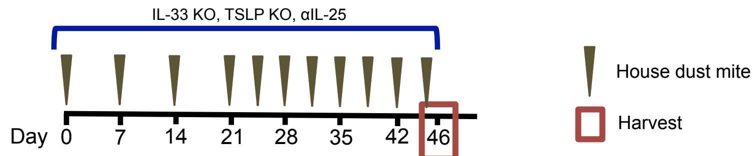

C
Il4 (fold)
Il5 (fold)
All isotypes IP Saline IN
All isotypes IP HDM IN
αST2/TSLP/IL-25 IP HDM IN


D
BALF cells/ml (x 10^5)
Lung tissue leukocytes (x 10^6)
All isotypes IP Saline IN
All isotypes IP HDM IN
αST2/TSLP/IL-25IP HDM IN


B
Hydroxyproline (μmol/lobe)
All isotypes IP Saline IN
All isotypes IP HDM IN
αST2 IP HDM IN
αTSLP IP HDM IN
αIL-25 IP HDM IN
αST2/TSLP IP HDM IN
αST2/IL-25 IP HDM IN
αTSLP/IL-25 IP HDM IN
αST2/TSLP/IL-25 IP HDM IN


Il13 (fold)
Ifng (fold)
All isotypes IP Saline IN
All isotypes IP HDM IN
αST2/TSLP/IL-25 IP HDM IN


E
BALF eosinophils (%)
Lung tissue eosinophils (%)
P=0.0053
All isotypes IP Saline IN
All isotypes IP HDM IN
αST2/TSLP/IL-25IP HDM IN



A

Hydroxyproline/lung (μmol)
P=0.0015
P=0.0207

B

Peribronchial inflammation score

Perivascular inflammation score

Endarteritis score
P<0.0001

Istotypes IP / HDM IN

C

BALF cells (x 10^4)

Mucus score (cells)

Mucus score (lumen)

TSLP/IL-33 DKO + αIL-25 IP / HDM IN

D

BALF eosinophils (x 10^4)

BALF monocytes/macrophages (x 10^4)

BALF lymphoctyes (x 10^4)

BALF neutrophils (x 10^4)

E

Lung tissue eosinophils (%)
P=0.0379

F

Lung tissue $CD4^+IL\text{-}4^+$ (%)
P=0.0009

Lung tissue $CD4^+IL\text{-}5^+$ (%)
P<0.0001

Lung tissue $CD4^+IL\text{-}13^+$ (%)
P=0.0004

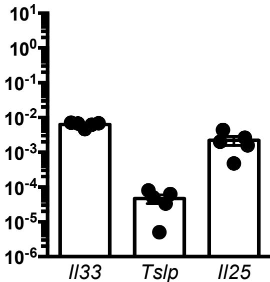
Lung tissue $CD4^+IFN\text{-}\gamma^+$ (%)

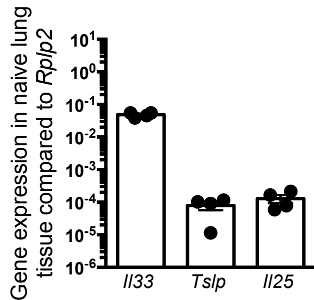
G

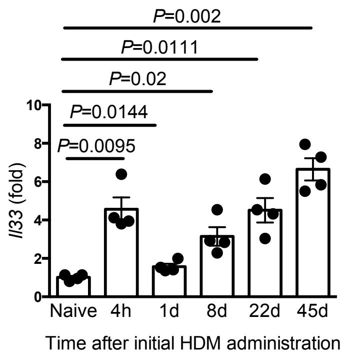
BALF $CD4^+IL\text{-}5^+$ (%)
P=0.0369

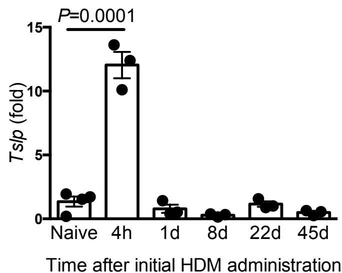
BALF $CD4^+IL\text{-}13^+$ (%)
P=0.0335

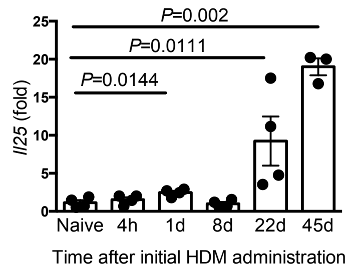
Isotypes IP / Saline IN
Isotypes IP / HDM IN
TSLP/IL-33 DKO + αIL-25 IP / HDM IN

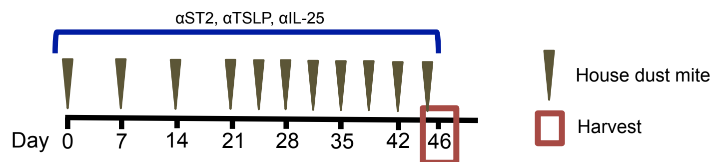

Gene expression in naive liver tissue compared to Rplp2
10^1
10^0
10^-1
10^-2
10^-3
10^-4
10^-5
10^-6
Il33
Tslp
Il25


A
Gene expression in naive lung tissue compared to Rplp2
10^1
10^0
10^-1
10^-2
10^-3
10^-4
10^-5
10^-6
Il33
Tslp
Il25

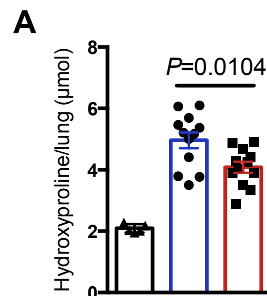

B
P=0.002
P=0.0111
P=0.02
P=0.0144
P=0.0095
Il33 (fold)
0
2
4
6
8
10
Naive
4h
1d
8d
22d
45d
Time after initial HDM administration


P=0.0001
Tslp (fold)
0
5
10
15
Naive
4h
1d
8d
22d
45d
Time after initial HDM administration

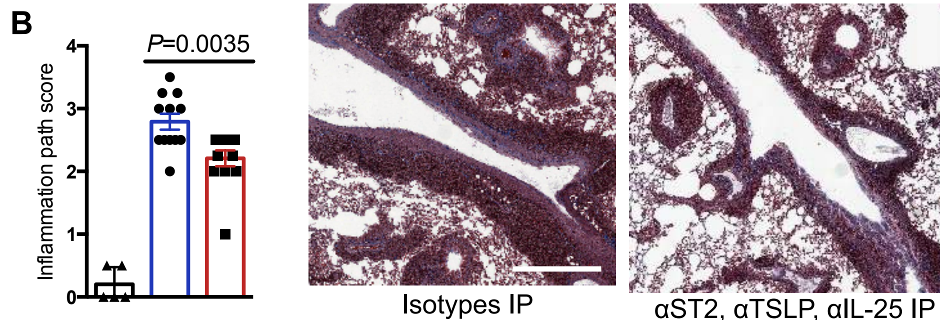

P=0.002
P=0.0111
P=0.0144
Il25 (fold)
0
5
10
15
20
25
Naive
4h
1d
8d
22d
45d
Time after initial HDM administration



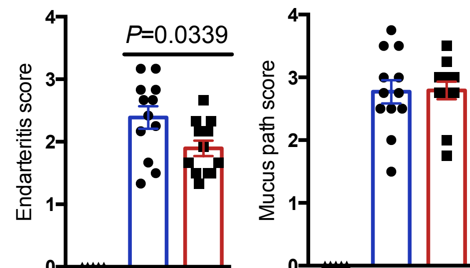
αST2, αTSLP, αIL-25
House dust mite
Harvest
Day 0 7 14 21 28 35 42 46



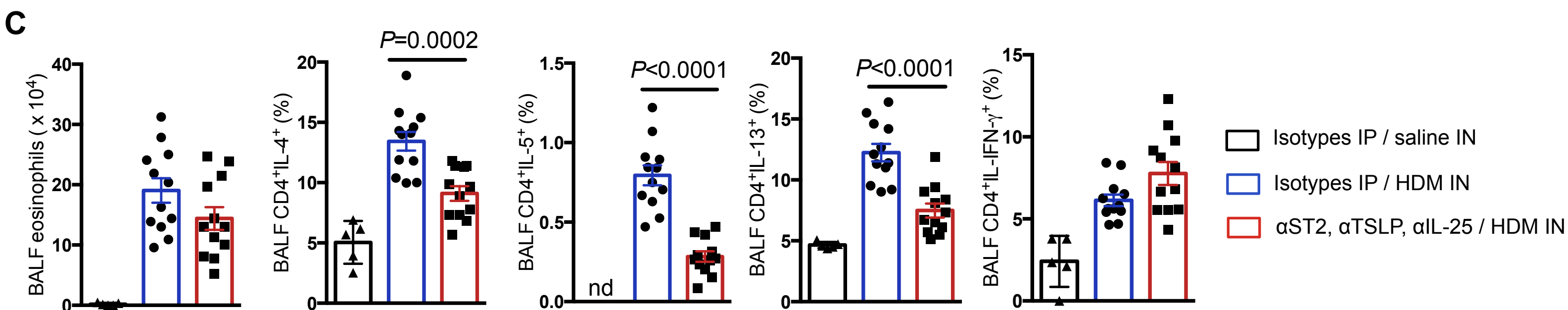
A
Hydroxyproline/lung (μmol)
P=0.0104



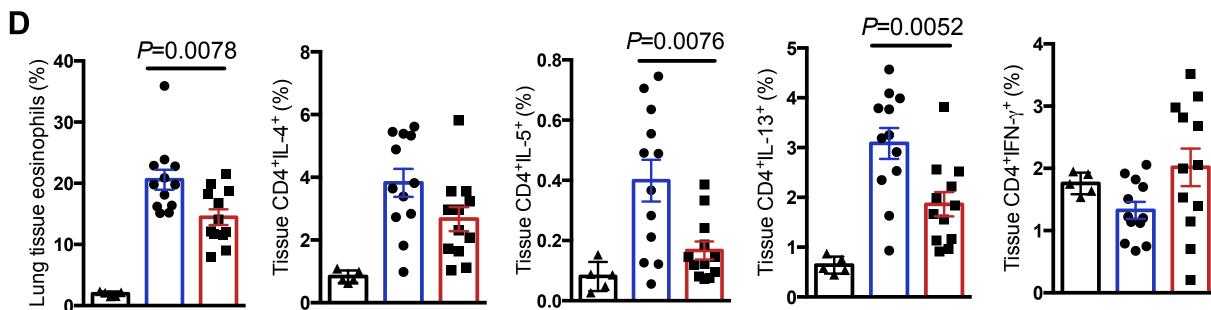
B
Inflammation path score
P=0.0035
Isotypes IP
αST2, αTSLP, αIL-25 IP



Endarteritis score
P=0.0339
Mucus path score



C
BALF eosinophils (x 10⁴)
BALF CD4⁺IL-4⁺ (%)
P=0.0002
BALF CD4⁺IL-5⁺ (%)
P<0.0001
nd
BALF CD4⁺IL-13⁺ (%)
P<0.0001
BALF CD4⁺IL-IFN-γ⁺ (%)
Isotypes IP / saline IN
Isotypes IP / HDM IN
αST2, αTSLP, αIL-25 / HDM IN



D
Lung tissue eosinophils (%)
P=0.0078
Tissue CD4⁺IL-4⁺ (%)
Tissue CD4⁺IL-5⁺ (%)
P=0.0076
Tissue CD4⁺IL-13⁺ (%)
P=0.0052
Tissue CD4⁺IFN-γ⁺ (%)