Blimp-1 is essential for Th2 cell development and allergic asthma

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Summary: The transcriptional repressor Blimp-1 acts via a pro-inflammatory IL-10-STAT3 axis as a critical positive regulator of Th2 cells in the lung in response to allergens driving pathophysiology associated with asthma disease.

Abstract

A Th2 immune response is central to allergic airway inflammation, which afflicts millions worldwide. However, the mechanisms that augment GATA3 expression in an antigen-

primed developing Th2 cell are not well understood. Here, we describe an unexpected role for Blimp-1, a transcriptional repressor that constrains autoimmunity, as an upstream promoter of GATA3 expression that is critical for Th2 cell development in the lung, but dispensable for T_{FH} function and IgE production. Mechanistically, Blimp-1 acts through Bcl6, which is necessary to drive GATA3 expression. Surprisingly, the anti-inflammatory cytokine IL-10, but not the pro-inflammatory cytokines IL-6 or IL-21, is required via STAT3 activation to upregulate Blimp-1 and promote Th2 cell development. These data reveal a hitherto unappreciated role for an IL-10-STAT3-Blimp-1 circuit as an initiator of an inflammatory Th2 response in the lung to allergens. Thus, Blimp-1 in a context-dependent fashion can drive inflammation by promoting rather than terminating effector T cell responses.

Introduction

Asthma is a complex, chronic inflammatory disease of the airways. House dust mite (HDM) is a major indoor allergen that is globally ubiquitous in living environments and is capable of inducing allergic lung inflammatory diseases (Calderón et al., 2015). Immune cell infiltration, including eosinophils and IgE-mediated sensitization are hallmarks of allergic airway disease, which is primarily driven by strong type2 cytokine responses (such as IL-4, IL-5 and IL-13) predominantly produced by activated $CD4^+$ T cells of a Th2 phenotype(Lambrecht and Hammad, 2015; Licona-Limón et al., 2013; Pascual and Peters, 2005; Ray and Cohn, 1999; Zhang et al., 1999). Th2 cells are differentiated following the activation of naive $CD4^+$ T cells in the presence of IL-4, and the master transcription factor GATA3(Kopf et al., 1993; Zhang et al., 1997; Zheng and Flavell,

47 1997). However, the signals that support this process in vivo are still not well
48 understood (Lambrecht and Hammad, 2015; Pulendran et al., 2010). Several IL-4
49 secreting cells have been proposed to promote Th2 cell development such as NKT
50 cells, basophils, or early-activated CD4 T cells(Croft and Swain, 1995; Seder et al.,
51 1991; Yoshimoto et al., 1995). However, there is evidence that IL-4-independent Th2
52 cell differentiation can occur, suggesting additional cytokines may play an important role
53 in initiating or supporting Th2 cell differentiation in response to allergens (Dent et al.,
54 1998; Oliphant et al., 2011; Ouyang et al., 2000; Stritesky et al., 2011). As evidence,
55 both STAT3 signaling and cytokines such as thymic stromal lymphopoietin (TSLP) can
56 promote Th2 cell differentiation (Rochman et al., 2018; Stritesky et al., 2011). Thus,
57 additional regulators of Th2 cells beyond the IL-4-STAT6-GATA3 circuit play a role in
58 type2 immune responses.

59 B lymphocyte-induced maturation protein-1 (Blimp-1) is a transcriptional repressor
60 required for plasma cell development and function (Minnich et al., 2016; Turner et al.,
61 1994). However, Blimp-1 also has important functions in T cells to regulate effector
62 responses (Crotty et al., 2010; Fu et al., 2017). Conditional deletion of Blimp-1 in T cells
63 causes spontaneous accumulation of effector T cells and systemic autoimmunity,
64 suggesting that Blimp-1 constrains T cell-mediated autoimmunity (Kallies et al., 2006;
65 Martins et al., 2006). In CD4 T cells, Blimp-1 can repress Bcl6 to antagonize T follicular
66 helper cell (T_{FH}) differentiation, control IL-10 expression in effector (Th1 and Th17) and
67 regulatory T cells (Tregs), and regulate the differentiation and function of effector T cells
68 (Cretney et al., 2011; Heinemann et al., 2014; Johnston et al., 2009; Neumann et al.,
69 2014; Parish et al., 2014). Furthermore, we previously found that overexpression of

70 Blimp-1 could lead to cell death, suggesting Blimp-1 also controls effector responses by
71 limiting effector cell numbers directly (Poholek et al., 2016).

72 Our previous studies showed that disrupting Blimp-1 in T cells increased Th2 responses
73 in a worm antigen model. Therefore, we hypothesized that T cell specific deficiency of
74 Blimp-1 in an allergic airway inflammation model would lead to increased expansion of
75 effector cells and more severe disease due to increased Th2 responses. Unexpectedly,
76 we found that T cell specific Blimp-1 deficiency protected mice from the development of
77 allergic lung inflammation and Th2 cells in the lung were severely reduced. STAT3 via
78 IL-10 was required for Blimp-1 expression and Th2 cell development in this model,
79 suggesting IL-10 may play an unexpected role in supporting Th2 cell differentiation.
80 Mechanistically, our data supports an intrinsic role for Blimp-1 mediated repression of
81 Bcl6, which in turn can repress GATA3. Thus, Blimp-1 indirectly supports Th2
82 differentiation by promoting GATA3 expression. These data identify a new context-
83 dependent role for Blimp-1 in T cells that is essential for the full development of allergic
84 lung disease, highlighting a previously unappreciated pathway with potential therapeutic
85 targets for the treatment of asthma disease.

86

87 **Results**

88 *Blimp-1 in T cells promotes allergic airway inflammation*

89 Blimp-1 controls effector T cell responses and constrains autoimmunity (Crotty et al.,
90 2010; Poholek et al., 2016). We reasoned that during a house dust mite (HDM) induced
91 model of allergic lung disease, the absence of Blimp-1 in T cells would drive increased
92 effector T cells and exacerbate disease. To test this, we compared mice in which Blimp-

93 1 was conditionally deleted in all T cells (Blimp-1$^{f/f}$ x CD4-cre, referred to as Blimp-
94 1^{CD4Cre}) to Cre-negative littermate controls (Blimp-1$^{f/f}$). Allergic inflammation in the
95 airways was induced by repeated intranasal (i.n.) administration of HDM antigen for 10
96 days (priming) followed by two re-challenges of 2-3 days each after rests of 3-4 days
97 (Fig S1A). As expected, histological assessment of lung tissue following HDM
98 administration of control animals revealed substantial lymphocyte infiltration to the
99 tissue and mucus in the airways, indicating a robust allergic inflammatory response to
100 HDM had occurred (Fig 1A). In addition, neutrophils, eosinophils, lymphocytes and
101 monocytes were readily found in the bronchiolar lavage fluid (BAL) of control animals
102 (Fig 1B). Contrary to our hypothesis, we found that Blimp-1^{CD4Cre} animals had no signs
103 of inflammation based on histological analysis of lung tissue (Fig 1A). Analysis of BAL
104 identified a specific loss of eosinophils, suggesting type2 immunity was impaired (Fig
105 1B). Eosinophils are recruited by Th2 cells that produce IL-5, while mucus in the
106 airways is due to the presence of IL-13^{+} cells (Erle and Sheppard, 2014; McBrien and
107 Menzies-Gow, 2017). To determine if there were alterations in the CD4^{+} T cell response
108 to HDM, we isolated lymphocytes from the lung and mediastinal lymph node (mLN) and
109 assessed CD4 T cell subsets by flow cytometry. HDM is known to induce a mixed
110 response with differentiation/expansion of Th1, Th2 and Th17 cells. We observed robust
111 increases of Th1, Th2 and Th17 cells as well as T regulatory cells (Treg) in the lungs of
112 control animals compared to unimmunized, naïve animals (Fig 1C, D). In contrast,
113 Blimp-1^{CD4Cre} animals were specifically lacking Th2 cells (GATA3^{+} IL-13^{+} or GATA3^{+} IL-
114 5^{+}) in the lung, while Th1 and Th17 cells were largely unaffected (Fig 1C,D). In contrast
115 to the lungs, Blimp-1^{CD4Cre} mice had increased Th1 and Th17 cells in the mLN, but Th2

116 cells (GATA3^{+} IL-13^{+}) were largely absent, constituting less than 1% of all CD4^{+} T cells
117 (Fig S1B)(Bao and Reinhardt, 2015). Consistent with previous publications, Tregs were
118 increased in Blimp-1^{CD4Cre} mice, likely due to Blimp-1's role in repressing IL-2, and
119 Tregs capability to expand in conditions with excess IL-2 (Fig 1C,D)(Cretney et al.,
120 2011; D'Cruz and Klein, 2005; Fontenot et al., 2005; Malek et al., 2002; Martins et al.,
121 2008; Webster et al., 2009). Blimp-1 is well known to be a critical driver of IL-10, and
122 indeed Blimp-1^{CD4Cre} mice were specifically lacking IL-10 production from T cells (Fig
123 1E,F, S1B)(Kallies et al., 2006; Martins et al., 2006; Neumann et al., 2014; Parish et al.,
124 2014). Bcl6 and Blimp-1 are transcriptional repressors known to regulate one another in
125 both T and B cells, thus we explored the levels of Bcl6 in CD4^{+} T cells(Crotty et al.,
126 2010). Blimp-1^{CD4Cre} mice had increased expression of Bcl6 in both the lung and mLN
127 (Fig 1E,F, S1B), suggesting that the absence of Blimp-1 in T cells resulted in a
128 concomitant increase in Bcl6. <u>We next assessed airway hyperreactivity (AHR), to</u>
129 <u>determine if Blimp-1 dependent loss of Th2 cells in the lung was sufficient to alter lung</u>
130 <u>function. Control mice repeatedly immunized and re-challenged with HDM had</u>
131 <u>increased AHR in response to methylcholine, however Blimp-1^{CD4Cre} were protected</u>
132 <u>from AHR and looked similar to unimmunized mice (Fig 1G). Thus, Blimp-1 in T cells</u>
133 <u>played a significant role in promoting allergic asthma in response to inhaled allergens.</u>
134
135 These results were surprising as prior examination of Blimp-1-deficient T cells had
136 increased Th2 cells in a Th2 model using a worm antigen (Poholek et al., 2016) and in
137 vitro, Blimp-1 is not required for Th2 cell differentiation (Fig S1C)(Cimmino et al., 2008).
138 <u>To determine if the requirement for Blimp-1 to drive Th2 cells was specific to HDM or to</u>

139 a lung-specific pathway in response to inhaled allergens, we intranasally immunized
140 Control and Blimp-1^{CD4Cre} mice with soluble egg antigen (SEA) derived from
141 *Schistosoma mansoni* worms. In our previous study (Poholek et al., 2016), we found
142 subcutaneous injection of SEA in Blimp-1^{CD4Cre} mice resulted in a modest increase in
143 Th2 responses. In contrast, intranasal immunization of SEA resulted in a Blimp-1-
144 dependent requirement for Th2 generation in the lung, similar to our results with HDM
145 (Fig S1D,E). These data suggest that Blimp-1 plays a surprising and critical role in
146 driving a lung-specific pathway driving the differentiation of Th2 cells in response to
147 inhaled antigens but may constrain effector Th2 responses when antigens are primed
148 subcutaneously. These data suggest Blimp-1 can have contrasting functions in T cell
149 differentiation and function depending on the route of immunization and location of T
150 cell priming, which has not previously been appreciated.
151
152 *IgE responses are Blimp-1 independent*
153 A hallmark of type2 driven allergic responses is the presence of high levels of circulating
154 IgE. In contrast to the decrease in type2 immunity observed in the lungs of Blimp-1^{CD4Cre}
155 animals (reduced Th2 cells and eosinophils in BAL), IgE levels were unchanged (Fig
156 2A). Isotype-switch to IgE is dependent on IL-4(Kopf et al., 1993). However, similar to
157 loss of Th2 cells in the lung, IL-4^{+} T cells in the mLN was significantly reduced in Blimp-
158 1^{CD4Cre} animals (Fig 2B,C). Antibody responses are largely driven by T follicular cells
159 (T_{FH}), and Blimp-1 antagonizes (T_{FH}) responses. Thus, we reasoned that T_{FH} cell
160 responses would be intact in Blimp-1^{CD4Cre} and thus capable of driving robust IgE
161 responses. IL-4 can be produced by both Th2 and T_{FH} cells(Zhu, 2015), therefore, we

162 determined the percentage IL-4$^+$ cells in both subsets (Th2, GATA3$^+$ and $T_{FH,}$ GATA3$^-$)
163 of cells in the mLNs of control and Blimp-1^{CD4Cre} animals. Despite total IL-4+ cells being
164 decreased in mLNs of Blimp-1^{CD4Cre} animals, analysis of IL-4+ cells within the T_{FH}
165 population (GATA3$^-$) was similar to controls (Fig 2D,E). In contrast, IL-4$^+$ cells within the
166 Th2 population (GATA3$^+$) were significantly reduced (Fig 2D,E). As expected,
167 expression of Bcl6 was higher in T_{FH} cells (GATA3$^-$) than Th2 cells (GATA3$^+$) of control
168 animals, but still reduced compared to Blimp-1^{CD4Cre} animals (Fig S2A,B). Indeed, IL-4+
169 cells from control animals comprised both Th2 (GATA3+ IL-13+) and T_{FH} (Bcl6+
170 CXCR5+) cells (Fig 2F). Blimp-1^{CD4Cre} animals, however, had similar IL-4+ T_{FH} cell
171 percentages but Th2 cells were absent (Fig 2F,G). These data suggest that although
172 Blimp-1 is a master regulator of Th2 cell development in the lungs, it is not required for
173 IL-4$^+$ T_{FH} cell development and subsequent type2 mediated IgE responses. Thus, there
174 are unique molecular pathways that can drive type2 humoral immunity for IgE
175 production (IL-4-producing T_{FH} cells) distinct from those that drive eosinophil recruitment
176 to the lung (IL-13 and IL-5 producing Th2 cells), and Blimp-1 is a major regulator that
177 molecularly dissects these two independent pathways.
178
179 *Blimp-1 is intrinsically required in T cells to promote Th2 cells*
180 We next sought to determine if Blimp-1 was expressed by all CD4 T cell subsets in the
181 lung during the course of allergic airway inflammation, or if expression was limited to
182 Th2 cells. To assess Blimp-1 expression, we induced allergic inflammation in Blimp-1
183 yellow fluorescent protein (YFP) reporter mice, which reliably tracks Blimp-1
184 transcription by YFP (Rutishauser et al., 2009). We assessed each CD4 T cell subset

185 and Blimp-1 expression within each subset at 4 timepoints; day 6 and day 10 which are
186 during and after priming, day 18, after the first challenge and day 23 after the second
187 challenge. Th1, Th2, Th17 and Treg cells were present at each timepoint in the lung in
188 naïve unimmunized animals and after HDM challenges (Fig S3A). When looking at the
189 percentage of Blimp-1 YFP+ cells among each subset we found that all subsets initially
190 upregulated Blimp-1 but that Th2 cells did so to greater degree compared to Th1 and
191 Th17 cells (Fig 3A-B, S3B). Tregs have been reported to express high levels of Blimp-1
192 in effector tissues, thus as a positive control we assessed Blimp-1 in Tregs and found
193 nearly all Tregs expressed Blimp-1 after HDM (Fig S3C)(Cretney et al., 2011). Blimp-1
194 continued to increase in Th2, Treg and Th17 populations, while the percentage of
195 Blimp-1+ cells in Th1 cells remained fairly constant over time after the initial increase
196 from day 0 to day 5 (Fig S3B).
197
198 It was possible that the increase in Tregs (Fig 1C,D) might be responsible for
199 suppressing Th2 cells. Therefore, we wondered if the expression of Blimp-1 was
200 required intrinsically in T cells to drive Th2 cells or if an extrinsic mechanism such as
201 Treg mediated control may be at play. To test the intrinsic function of Blimp-1, we
202 generated mixed bone marrow (BM) chimeras in Rag-deficient hosts where 50% of
203 donor BM was from congenically marked B6 Control mice (Thy1.1) and 50% from
204 Blimp-1^{CD4Cre} (Thy1.2). 6 weeks post reconstitution, allergic lung inflammation was
205 induced with HDM as previously described (Fig S3D). Although irradiated hosts
206 received equal percentages of donor BM, Blimp-1^{CD4Cre} BM reconstituted at greater than
207 50%, supporting its role in negatively regulating T cell survival (Poholek et al., 2016)

208 (Fig S3E). Nevertheless, Th2 cells were significantly reduced in Blimp-1^{CD4Cre} T cells
209 after HDM-induced allergic lung inflammation (Fig 3C,D). Tregs however were
210 equivalently derived from Control or Blimp-1^{CD4Cre} BM, suggesting that Blimp-1 is
211 intrinsically required in Th2 cells to promote Th2 cell differentiation and that increases in
212 Tregs was not responsible for this effect. The reduction in Th2 cells was significant
213 enough to result in a slight, but significant decrease in eosinophils in the BAL compared
214 to animals that only received control BM (Fig 3E). Blimp-1^{CD4Cre} T cells (Thy1.2+) had
215 significant decreases in IL-10 and increases in Bcl6 compared to control cells in the
216 same animal, consistent with Blimp-1's function in driving IL-10 and repressing Bcl6 (Fig
217 3C,D). Taken together these data suggest that the mechanism of Blimp-1 mediated Th2
218 cell differentiation is cell intrinsic.
219
220 *STAT3 is required for Blimp-1 expression in T cells*
221 We next explored the upstream drivers of Blimp-1. Our previous examination of Th2
222 cells found that STAT3, but not STAT6, was a critical regulator of Blimp-1. In vitro,
223 STAT3 can bind directly to the *Prdm1* locus when stimulated with a strong STAT3-
224 activating cytokine such as IL-10 (Poholek et al., 2016). Furthermore, STAT3 can
225 support Th2 cell differentiation in murine allergic lung models (Stritesky et al., 2011).
226 Therefore, we wondered whether STAT3 could be promoting Th2 cell development in
227 our HDM-induced allergic model through upregulation of Blimp-1. To explore the role of
228 STAT3, we used T cell specific STAT3 deficient animals (STAT3$^{f/f}$ CD4-Cre, referred to
229 as STAT3^{CD4Cre}) and assessed T cell subsets in the lung. After immunization with HDM,
230 STAT3^{CD4Cre} lung tissue had reduced lymphocytic infiltration as assessed by PAS

231 staining and there was a significant reduction in eosinophils found in the BAL (Fig
232 4A,B). Consistent with STAT3's role in driving Th17 differentiation(Zhou et al., 2007),
233 STAT3^{CD4Cre} had a complete absence of Th17 cells in the lungs (Fig 4C). Intriguingly,
234 we found a significant reduction in Th2 cells, similar to our results in Blimp-1^{CD4Cre},
235 confirming previous studies that STAT3 could support Th2 cell development in the
236 context of allergic lung inflammation (Stritesky et al., 2011) (Fig 4C). In contrast, Th1
237 and Treg cell percentages were similar in control and STAT3^{CD4Cre} mice. IL-10 was
238 reduced in effector T cells (FoxP3-), consistent with a role for STAT3 in driving IL-10
239 expression, in contrast IL-10 was unaffected in STAT3-deficient Tregs (Fig 4D)
240 (Stumhofer et al., 2007). Thus, Treg percentages and effector function appeared intact
241 in the absence of STAT3 expression in T cells. To determine if STAT3 was required for
242 Blimp-1 expression, we assessed Blimp-1 protein expression in effector T cells. In
243 agreement with our previous study, Blimp-1 protein was significantly reduced in the
244 absence of STAT3, suggesting that STAT3 is required for Blimp-1 expression and
245 subsequent Th2 differentiation in allergic lung inflammation (Fig 4E). To determine if all
246 T cells required STAT3 for Blimp-1 expression or just Th2 cells, we crossed the
247 STAT3^{CD4Cre} to Blimp-1 YFP reporter mice. Consistent with STAT3^{CD4Cre} animals, Th2
248 cells were specifically reduced in STAT3^{CD4Cre} Blimp-1 YFP after HDM (Fig 4F).
249 STAT3^{CD4Cre} Blimp-1 YFP mice had significantly reduced Blimp-1 expression (assessed
250 by YFP) in Th1, Th2 cells and Treg cells compared to control Blimp-1 YFP mice (Fig
251 4G). Although Th1 cells also lacked Blimp-1, it did not affect Th1 cell percentages in the
252 lung, suggesting that Blimp-1 and STAT3 are not necessary for Th1 cell differentiation

253 in response to allergens. Taken together, these data confirm the critical role of intrinsic
254 STAT3 in driving Blimp-1 in Th2 cells in vivo in an allergic lung inflammation model.
255
256 *IL-6 and IL-21 are not required for expression of Blimp-1 in Th2 cells*
257 We next sought to determine what environmental factors were driving Blimp-1
258 expression in Th2 cells in HDM induced lung inflammation. We focused our analysis on
259 known STAT3-activating cytokines. As IL-6 has previously been shown to support Th2
260 cells in allergic lung disease (Doganci et al., 2005) and is a strong STAT3 activator, we
261 first tested whether IL-6 signaling on T cells was required for Blimp-1 induction and Th2
262 cell formation in this model. Using IL-6Rα deficient T cells (IL-6R$\alpha^{f/f}$ CD4-Cre, referred
263 to as IL-6Rα^{CD4Cre}) we found no significant difference in Th2 cells in the lung after HDM,
264 however Th17 cells in the lung were absent, in line with the well-known role of IL-6 to
265 drive Th17 development via STAT3 (Fig 5A). Importantly, Blimp-1 levels were
266 unchanged in effector (CD44^{+}) T cells (Fig 5B), suggesting IL-6 was not required to
267 drive Blimp-1 or Th2 cells in response to HDM.
268
269 To identify other possible STAT3-driven cytokines we performed whole-tissue qPCR of
270 both lung and mLN and found IL-10, IL-21, and IL-27 were all upregulated in response
271 to HDM, particularly in the mLN (Fig S4A). IL-21 is known to play a critical role in Th2
272 cell generation in HDM induced allergic lung inflammation, however the mechanism is
273 unclear(Coquet et al., 2015). Furthermore IL-21 is known to drive Blimp-1 in B cells
274 (Ozaki et al., 2004). Using IL-21R deficient mice (IL-21R$^{-/-}$) we found a significant
275 reduction in eosinophils in the lung consistent with previous reports (Coquet et al.,

276 2015)(Fig 5C). We also found a significant reduction in Th2 cells in the lung and mLN
277 (Fig 5D, Fig S4B). Surprisingly, although IL-21R$^{-/-}$ displayed a loss of Th2 cells similar to
278 Blimp-1^{CD4Cre} and STAT3^{CD4Cre}, Blimp-1 levels were similar in effector T cells in both
279 control and IL-21R$^{-/-}$ animals (Fig 5E, Fig S4C). We wondered if IL-21 could play an
280 important role in driving Blimp-1 only in Th2 cells, and the absence of Th2 cells limited
281 our ability to detect this. To test this, we treated Blimp-1 YFP mice with anti-IL-21R
282 antibody (or control IgG antibody) during the course of HDM-induced allergic lung
283 inflammation and tracked Blimp-1 expression by YFP. Similar to IL-21R$^{-/-}$ mice, Th2
284 cells were significantly reduced in the lung of anti-IL-21R treated mice (Fig 5F).
285 However, Blimp-1 levels were intact in the few remaining Th2 cells found (Fig 5G).
286 While Blimp-1 levels were slightly reduced in Th1 cells, this had no effect on Th1 cell
287 development (Fig 5F,G). These data suggest that while IL-21 is critical for Th2 cell
288 development and eosinophil recruitment during HDM-induced allergic lung inflammation
289 as has previously been shown, the role of IL-21 in Th2 cell formation is independent of
290 the function of Blimp-1 in driving Th2 cells(Coquet et al., 2015).
291
292 *IL-10 is required for Blimp-1 expression in Th2 cells*
293 Our previous studies using worm-derived antigens demonstrated that IL-10 is a critical
294 factor driving Blimp-1 expression in Th2 cells(Poholek et al., 2016). Although IL-10 is
295 generally thought to be anti-inflammatory, there have been contradictory findings in
296 allergic asthma, with both anti-inflammatory and pro-Th2 roles described (Bandukwala
297 et al., 2007; Coomes et al., 2016; Hammad et al., 2001; Igietseme et al., 2000; Laouini
298 et al., 2003; Pulendran et al., 2010; Wilson et al., 2007). To test if IL-10 might be a

299 driver of Blimp-1 in HDM-induced allergic lung inflammation, we treated Blimp-1 YFP
300 mice with anti-IL10R antibody or IgG control during HDM immunization. Surprisingly, we
301 found a consistent reduction in eosinophils recruited to the lungs (Fig 6A), and a
302 concomitant decrease in Th2 cells in the lung (Fig 6B,C). Th1 cells were increased,
303 while Th17 and Treg cells were unaffected (Fig 6C, data not shown). Intriguingly, Blimp-
304 1 was specifically and significantly reduced in Th2 cells but unaffected in Th1 cells (Fig
305 6D,E), suggesting IL-10 was an important regulator of Blimp-1 in Th2 cells in the context
306 of allergic antigen stimulation. As Blimp-1 is an intrinsic regulator of IL-10 expression
307 (Fig 3D), we wondered if IL-10 was also affected by loss of Blimp-1. Within the total
308 effector ($CD44^+$) T cell population, the percentage of $IL\text{-}10^+$ cells was unchanged,
309 however the total amount of IL-10 per cell was slightly reduced (MFI) (Fig S5A).
310 Specifically within Th2 cells, expression of IL-10 shifted from a Blimp-1+ population in
311 IgG treated control mice, to a Blimp-1 negative population in anti-IL-10R treated mice
312 (Fig 6F,G). These data suggest that Blimp-1 expression may not be continually required
313 for IL-10 expression in T cells. To address the possibility that IL-10 and IL-21 act in a
314 redundant fashion to regulate Blimp-1 expression we performed double antibody
315 blockade of both IL-10R and IL-21R in Blimp-1 YFP animals. Blockade of IL-10R and IL-
316 21R together did not have a synergistic effect on Blimp-1 expression, suggesting the
317 effects of IL-10 and IL-21 are independent of one another (Fig S5B,C).
318
319 <u>IL-10 is a pleotropic cytokine that acts on many cell types, thus it was unclear if</u>
320 <u>blockade of IL-10 signaling in vivo acted by directly inhibiting signaling to T cells, or if it</u>
321 <u>was through an accessory cell such as dendritic cells. To assess the role of IL-10R on T</u>

322 cells directly, we generated mice lacking IL-10Rα on T cells (IL-10Rα$^{f/f}$ crossed to CD4-
323 Cre, herein referred to as IL-10Rα^{CD4Cre}). In contrast to published studies showing IL-10
324 signaling on T cells was required to suppress Th2 responses (Coomes et al., 2016), we
325 found that loss of IL-10Rα on T cells led to a reduction in Th2 cells in the lung, while
326 Th1 cells were elevated (Fig 7A). Th17 and Treg cells were unchanged compared to
327 controls. In addition, Blimp-1 was significantly reduced in effector T cells lacking IL-
328 10Rα (Fig 7B). The reduction in Th2 cells lead to a slight decrease in eosinophils in the
329 lung (Fig 7C). Similar to our results in Blimp-1^{CD4Cre} mice, Bcl6 was elevated (Fig 7D). In
330 contrast, IL-10 levels were subtly increased in effector T cells, despite decreases in
331 Blimp-1 (Fig 7D). These data were highly similar to our results with IL-10R blocking
332 antibody, suggesting IL-10 signaling during responses to HDM directly to T cells leads
333 to Blimp-1 upregulation and Th2 cell differentiation, but is not sufficient to alter overall
334 IL-10 production from T cells (Fig S5A). Finally, to determine if IL-10 signaling was
335 required intrinsically on T cells, we generated 50:50 mixed BM chimeras of IL-10Rα-
336 intact and -deficient BM (Control (CD45.1):Control (CD45.2) or Control (CD45.1):IL-
337 10Ra^{CD4Cre}(CD45.2)) and assessed T cell responses in the lung after HDM inhalation.
338 Th2 cells were reproducibly lower when derived from IL-10Rα^{CD4Cre} BM than from
339 Control BM, whereas Th1, Th17 and Treg cells were similar or increased (Fig S5D,E).
340 Taken together, these data suggest that IL-10 is an important driver of Blimp-1 in T cells
341 supporting Th2 cells in the lung during allergic inflammation in response to inhaled
342 allergens. These data are consistent with a pro-inflammatory role for IL-10 that
343 promotes Th2 cells similar to prior studies showing a pro-Th2 role for IL-10 in allergic

344 asthma (Bandukwala et al., 2007; Hammad et al., 2001; Igietseme et al., 2000; Laouini
345 et al., 2003; Pulendran et al., 2010; Williams et al., 2013).
346
347 *Blimp-1 supports GATA3 expression and Th2 development through suppression of Bcl6*
348 We next sought to understand how Blimp-1 was functioning to control Th2 cell
349 differentiation. Since GATA3 is the master regulator of Th2 cell development (Zhang et
350 al., 1997; Zheng and Flavell, 1997), we determined more specifically the expression of
351 master regulators in T cell subsets in the absence of Blimp-1. The loss of Blimp-1 led to
352 a specific and robust decrease in GATA3, while T-bet was unaffected and Rorγt was
353 increased (Fig 8A, B). Bcl6 is a well-known repressor of GATA3 (Kusam et al., 2003;
354 Sawant et al., 2012), and Blimp-1 deficient T cells had a robust increase in Bcl6
355 expression (Fig 1F). We hypothesized that in wildtype cells, Blimp-1 may be
356 suppressing Bcl6 to promote GATA3 expression, but that in Blimp-1^{CD4Cre} animals,
357 increased expression of Bcl6 would suppress GATA3 and Th2 cell development. To test
358 this, we induced allergic lung inflammation in mice that had a T cell specific deficiency in
359 both Blimp-1 and Bcl6 (Blimp-1$^{f/f}$ Bcl6$^{f/f}$ CD4Cre^{+}, referred to as doubleKO^{CD4Cre})(Xie et
360 al., 2017). Control (Bcl6$^{f/f}$ or Blimp-1$^{f/f}$, CD4Cre^{-}), Bcl6-deficient (Bcl6$^{f/f}$ CD4Cre^{+},
361 Bcl6^{CD4Cre}), Blimp-1^{CD4Cre} or doubleKO^{CD4Cre} mice were immunized with HDM and
362 assessed for lung inflammation as well as development of T cell subsets in the lung and
363 mLN. As before, examination of lung tissue found a consistent reduction in inflammation
364 in Blimp-1^{CD4Cre} lungs compared to Control or Bcl6^{CD4Cre} (Fig 9A). DoubleKO^{CD4Cre} mice
365 had lymphocyte infiltration and mucus that was similar to controls, suggesting
366 inflammation was present when Bcl6 and Blimp-1 were both absent in T cells (Fig 9A).

367 Consistent with this finding, we saw no difference in the recruitment of eosinophils to the
368 BAL in the doubleKO^{CD4Cre} lungs compared to control or Bcl6^{CD4Cre}, however Blimp-
369 1^{CD4Cre} mice had a consistent reduction in eosinophils as seen previously (Fig 9B). In
370 the lung, Th1 cells were increased in both Blimp-1^{CD4Cre} and doubleKO^{CD4Cre} mice
371 compared to control and Bcl6^{CD4Cre} animals (Fig 9C). Interestingly, Th2 cells were
372 increased in doubleKO^{CD4Cre} compared to Blimp-1^{CD4Cre} mice, suggesting the loss of
373 Bcl6 in Blimp-1^{CD4Cre} was sufficient to restore Th2 cell differentiation (Fig 9C). Treg cells
374 in doubleKO^{CD4Cre} were increased compared to controls, similar to Blimp-1^{CD4Cre},
375 suggesting that Blimp-1's repression of IL-2 is Bcl6-independent (Fig 9C). As expected,
376 IgE levels were dramatically reduced in Bcl6^{CD4Cre} and doubleKO^{CD4Cre} mice, likely due
377 to the loss of T_{FH} cells in the absence of Bcl6 expression (Fig 9D). Finally, we explored
378 the expression of IL-10 in both effector and Treg cells. Intriguingly, the absence of Bcl6
379 restored IL-10 expression in doubleKO^{CD4Cre}, suggesting the function of Blimp-1 in
380 driving IL-10 is via suppression of Bcl6 expression, and thus may be indirect rather than
381 direct, as previously reported (Fig 9E,F)(Minnich et al., 2016; Xie et al., 2017). This
382 effect was greater in Tregs than in effector T cells, although the amount of IL-10 per cell
383 was similar (Fig 9F). Taken together, these data indicate that Blimp-1 functions
384 intrinsically in T cells to suppress Bcl6 expression which is permissive for GATA3
385 expression, subsequent development of Th2 cells, and expression of IL-10 in T cells in
386 the context of an HDM-induced allergic lung inflammation model.
387
388 **Discussion:**

389 In this study, we have uncovered a previously unappreciated role for Blimp-1 in driving
390 lung-specific Th2 cell development in the context of an allergic asthma model. House
391 dust mite antigen is a widely used model of allergic lung inflammation owing to its
392 physiologic relevance to human health as dust mites are a well-known allergic trigger in
393 many individuals with asthma disease(Thomas, 2016). Based on our previous study, we
394 expected Blimp-1 to suppress inflammatory effector responses to HDM. On the
395 contrary, we uncovered an unexpected role for Blimp-1 in the specific control of Th2 cell
396 differentiation, which has not been previously demonstrated in *in vitro* assays or our
397 prior *in vivo* Th2 model (Cimmino et al., 2008; Poholek et al., 2016). Previous studies
398 exploring the role of Blimp-1 in T cells have elucidated many functions for Blimp-1 that
399 collectively could be described as controlling effector functions of T cells that mediate
400 disease states. In CD8 T cell responses to viral infection, Blimp-1 supports effector cell
401 differentiation while repressing memory cells (Kallies et al., 2009; Rutishauser et al.,
402 2009; Shin et al., 2009). Blimp-1 also plays a critical role in supporting IL-10 expression
403 in Tregs and effector CD4 T cells, the absence of which can have important
404 consequences for controlling infections by pathogens such as Toxoplasma or chronic
405 viral infection (Cretney et al., 2011; Neumann et al., 2014; Parish et al., 2014). Blimp-1
406 also controls differentiation of an inflammatory Th17 cell that produces IFNγ and GM-
407 CSF and subsequent autoimmune disease in an EAE model (Jain et al., 2016). In each
408 of these systems, Blimp-1 primarily controls effector properties of the T cells; supporting
409 cytokines such as IL-10, IFNγ, and GM-CSF, suggesting that Blimp-1 is part of a cell
410 program to further drive a terminal effector differentiation state. Finally, Blimp-1 is
411 known to repress the differentiation of T_{FH} cells(Johnston et al., 2009; Nurieva et al.,

412 2009). Here, for the first time, we have unexpectedly shown that Blimp-1 is essential for
413 supporting the differentiation of a lineage of helper cells, Th2 cells, suggesting Blimp-1
414 has a more complex role than previously appreciated.

415 To better understand the difference for Blimp-1 function in Th2 cell differentiation
416 between responses to inhaled (i.n.) HDM and subcutaneous (s.c.) SEA explored in our
417 prior publication, we immunized mice with SEA i.n. and HDM s.c. and tracked Th2
418 responses in the lung. We found that Blimp-1 was required for Th2 cells in the lung in
419 response to SEA (Fig S1D) while there was no effect on Th2 cells to HDM s.c. (data not
420 shown). This points to a lung-specific role for Blimp-1 in driving Th2 cells, suggesting
421 that lung-specific factors or pathways control type 2 immunity in response to inhaled
422 allergens, which may have important therapeutic implications as allergens are
423 introduced via inhalation.

424 Th2 cells are primary producers of several important type2 cytokines that drive
425 allergic lung disease, including IL-4, IL-5, and IL-13(Licona-Limón et al., 2013; Ray and
426 Cohn, 1999). Each of these cytokines has a unique function in asthma disease, but
427 collectively they enforce several hallmarks of asthma, including IgE production,
428 recruitment of eosinophils and mucus production in the airways. Intriguingly, we found
429 that Blimp-1 controlled only GATA3$^+$ Th2 cells but was not required for all IL-4$^+$
430 producing cells. T_{FH} cells are a critical population of effector T cells that is superior at
431 initiating and supporting B cell responses such as IgE(Crotty, 2011). Furthermore, they
432 can express IL-4, which is required for isotype switch to IgE(Zhu, 2015). Although the
433 loss of Blimp-1 did not result in an increase in T_{FH} cells after HDM, as it has in other
434 systems, IL-4$^+$ T_{FH} cells were intact, resulting in normal IgE responses to HDM despite

435 the absence of Th2 cells. These results suggest that the pathways that drive T_{FH} and
436 Th2 cells in response to allergens can be molecularly dissected, and that Blimp-1 is a
437 major regulator distinguishing these two inflammatory cell types. This may enable
438 therapeutic targeting of these populations distinctly and supports further exploring the
439 upstream pathways of each cell population.

440 Multiple STATs including STAT3, STAT4 and STAT5 have been implicated in
441 driving Blimp-1 in effector T cells(Jain et al., 2016; Neumann et al., 2014; Xin et al.,
442 2016). In line with previous reports, we have found a central role for STAT3 in
443 upregulating expression of Blimp-1 (Jain et al., 2016; Poholek et al., 2016). Although we
444 had previously found that IL-10 was required for Blimp-1 expression in Th2 cells, we did
445 not initially expect IL-10 to promote allergic disease based on its known anti-
446 inflammatory roles. There are several well-known STAT3 activating cytokines such as
447 IL-6, IL-21 that had already been implicated in supporting Th2 cells in allergic lung
448 disease models(Coquet et al., 2015; Doganci et al., 2005; Leonard and O'Shea, 1998;
449 Licona-Limón et al., 2013; Rincon et al., 1997). In addition, some studies had shown IL-
450 10 suppresses Th2 responses in an HDM driven model(Coomes et al., 2016; Tournoy
451 et al., 2000). However, upon finding that neither IL-6 nor IL-21 signaling to T cells had
452 any significant function on Blimp-1 expression in T cells, we pursued IL-10 as a
453 potential regulator of Blimp-1 in this context. Our studies using both IL-10R antibody
454 blockade and genetic deletion of IL-10R from T cells demonstrated a clear role for IL-10
455 acting directing on T cells to drive Blimp-1 and subsequent Th2 cells in response to
456 inhaled allergens. A previous study analyzed the responses of IL-10R$\alpha^{-/-}$ $CD4^{+}$ T cells
457 during HDM-induced allergic lung inflammation and found that IL-10 acts directly on T

458 cells to suppress Th2 cells, in contrast to our findings here (Coomes et al., 2016).We
459 believe a difference in location of antigen priming may explain this discrepancy, as our
460 immunizations were all intranasal, however in Coomes et al mice were first primed i.p.
461 with HDM in Alum followed by i.n. challenge. Together with our data using SEA i.n., this
462 suggests that the route of immunization plays a major role in driving unique Th2
463 differentiation pathways. Several reports have suggested a STAT6- and IL-4-
464 independent mechanism exists to drive Th2 cells(Dent et al., 1998; Ouyang et al.,
465 2000). Furthermore, IL-10 from IRF4^{+} DCs, the main Th2-driving DC population, has
466 been implicated in supporting Th2 cells during allergic responses(Cook and MacDonald,
467 2016; Gao et al., 2013; Krishnaswamy et al., 2017; Kumamoto et al., 2013; Williams et
468 al., 2013).Given that many cells can secrete IL-10, it will be interesting to determine the
469 pertinent cell types involved in this process(Gabrysova et al., 2014). We postulate that
470 dendritic cells can secrete IL-10 after sensing allergens such as HDM via the lung, and
471 then promote Blimp-1 expression and subsequent Th2 cell development through STAT3
472 activation. IL-10 can also act in a paracrine or autocrine manner from T cells to further
473 drive Blimp-1 expression and IL-10 expression, which may further support Th2 cells.
474 However, it is likely additional factors may act in concert with IL-10 to support Blimp-1,
475 such as TSLP, which can directly limit Bcl6 via STAT5(Rochman et al., 2018). Full
476 understanding of how a lung-specific pathway via IL-10 and Blimp-1 can promote Th2
477 cells will be of interest for future studies.
478 Blimp-1 mainly acts as a transcriptional repressor by recruiting histone
479 methyltransferases such as G9a which results in downregulation of gene
480 expression(Gyory et al., 2004). Recent studies have identified Blimp-1 binding near the

481 IL-10 gene, suggesting Blimp-1 may also have a role as an activator of gene
482 expression(Minnich et al., 2016). However, our study is more in line with an indirect
483 mechanism of activation whereby Blimp-1 represses Bcl6 which represses IL-
484 10(Hollister et al., 2013; Xie et al., 2017). Loss of Bcl6 and Blimp-1 together restored IL-
485 10 expression in T cells, and blockade of IL-10R signaling resulted in robust IL-10^{+}
486 Blimp-1^{-} populations, suggesting T cells can make IL-10 in the absence of Blimp-1. We
487 postulate that there may be more than one way for Blimp-1 to control gene expression
488 of IL-10, however our studies clearly point to a more complex mechanism beyond
489 Blimp-1 directly activating the IL-10 gene, at least in CD4 effector cells responding to
490 allergens in the lung. More studies carefully exploring Blimp-1 and IL-10 expression in T
491 cells are required to fully understand this complex interaction.
492 As Blimp-1 has effects in almost every T cell subset, it was important to
493 determine the intrinsic function of Blimp-1 in Th2 cells in this system. As Th2-specific
494 deletion of Blimp-1 is challenging, we opted to use mixed bone marrow chimeras, which
495 supported an intrinsic role for Blimp-1. Mechanistically this was due to the critical
496 function of Bcl6 in limiting GATA3 expression. Although its known that Bcl6 is an
497 important repressor of GATA3(Kusam et al., 2003; Sawant et al., 2012), it has not been
498 appreciated that Blimp-1 plays an important role in suppressing Bcl6 in Th2 cells.
499 Although STAT5 can also directly suppress Bcl6, our data suggest that this may be
500 insufficient in some contexts, and that STAT3 driven Blimp-1 is also required to limit
501 Bcl6 and indirectly support GATA3 and subsequent Th2 cell differentiation(Johnston et
502 al., 2012; Oestreich et al., 2012). Further studies that explore the complex interplay of
503 STAT3 and STAT5 activating cytokines in the control of Blimp-1, Bcl6 and GATA3 in

504 Th2 cells may reveal exactly how Th2 cells differentiate in response to allergens, which
505 has long been not fully understood.
506 In summary, our study exploring the T cell intrinsic role of Blimp-1 in HDM
507 induced allergic airway inflammation has uncovered several findings relevant to the
508 differentiation of Th2 cells in allergen induced lung inflammation. We found an important
509 role of STAT3 in promoting Blimp-1 expression and subsequent Th2 cell differentiation.
510 We additionally determined a potential pro-inflammatory role of IL-10 in driving Blimp-1
511 expression and promoting inflammation of lung via Th2 cells. Finally, we have described
512 a new role for Blimp-1 in Th2 cell differentiation by repressing Bcl6 and indirectly
513 supporting GATA3 expression. Taken together, our study elucidates a new context-
514 dependent role for Blimp-1 in T cells that promotes, rather than constrains, allergen-
515 induced airway disease.
516

517 **Materials and Methods**

518 **Mice**

519 C57BL/6NJ (005304), B6.Cg-Tg(Prdm1-EYFP)1Mnz/J (008828), Tg(Cd4-cre)1Cwi/BfluJ
520 (017336), B6.129-*Prdm1*^{tm1Clme}/J (008100), B6.129S(FVB)-*Bcl6*$^{tm1.1Dent}$/J (023727),
521 B6.129S7-*Rag1*^{tm1Mom}*/J* (002216), B6N.129-Il21r^{tm1Kopf}/J (019115), B6;SJL-*Il6ra*$^{tm1.Drew}$/J
522 (012944), B6(SJL)-*Il10ra* $^{tm1.1Tlg}$/J (028146) were purchased from Jackson Laboratories,
523 Bar Harbor, Maine. STAT3^{flox} were from D. Levy (MGI: 2384272)*(Lee et al., 2002)*
524 Animals were housed in specific pathogen–free enclosures at the John G. Rangos Sr.
525 Research Center of UPMC Children's Hospital of Pittsburgh. All experiments were
526 approved by the University of Pittsburgh Animal Care Committee and the American

527 Veterinary Medical Association. All mice were over 6 weeks of age and a mixture of
528 male and female mice were used. All *in vivo* experiments were performed independently
529 two to three times, with at least 4 mice per group.

530 **Chemicals and Reagents**

531 Flow cytometry antibodies were purchased from BDBiosciences, or ThermoFisher
532 Scientific(eBioscence). Blocking antibodies (IL-21R, IL-10R and IgG control) were
533 purchased from BioXCell. CD90 Microbeads were from Miltenyi Biotec. DNAse I, and
534 Collagenase A were purchased from Sigma-Aldrich. qPCR reagents (Trizol, qScript,
535 qPCR FastMix II) were purchased from Thermo Fisher Scientific or Quanta Biosciences.
536 IgE Elisa Kit was purchased from Thermo Fisher Scientific.

537 **House dust mite (HDM) model**

538 25 µg of LPS-low HDM (Stallergenes-Greer Pharmaceuticals) was resuspended in 25 µl
539 of sterile PBS and given intranasally to mice anesthetized with isoflurane daily for 10
540 days (priming) and re-challenged two times for 2-3 days per re-challenge with periods of
541 3-4 days rest between challenges (Fig S1A).

542 **Bronchoalveolar lavage fluid (BAL)**

543 After the final re-challenge, BAL was isolated from anesthetized mice by infusion and
544 recovery of approximately 0.9 ml of sterile PBS into the lungs. Total BAL cell numbers
545 were determined using automated cell counters (Nexcelom Bioscience LLC. USA), and
546 $4x10^5$ BAL fluid cells were cytospun onto glass slides for staining using Richard-Allan
547 Scientific™ Three-Step Stain (Thermo Fisher Scientific) following manufacture's
548 protocol. Neutrophils, eosinophils, monocytes and lymphocytes were determined out of
549 a total of 300 cells.

550 **Histology**

551 Lungs fixed in Safefix II (Fisher Scientific) were paraffin embedded, sectioned, and
552 stained for hematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) staining. The
553 pathological changes were visualized under a microscope.

554 **Flow cytometry**

555 Lungs of anesthetized mice were perfused with sterile PBS, removed and digested.
556 Briefly, the lung tissues were digested in a collagenase-DNase suspension for 45 min at
557 37 °C, then processed on a gentleMACS dissociator (Miltenyi Biotec, Bergisch
558 Gladbach, Germany) according to the manufacture's protocol. Single cell suspensions
559 were obtained by passing the dissociated tissue through a 70µm cell strainer (Fisher
560 Scientific) and washed with PBS containing 2% FBS and 0.5M EDTA. Red cells lysis
561 was performed (ACT lysing buffer, Thermo Fisher Scientific) and washed with PBS
562 containing 2% FBS. The mediastinal draining lymph node was dissociated in 10 ml of
563 complete RPMI1640 medium and passed through 40um filters. Cells were stimulated for
564 2.5 hours with phorbol 12-myristate 13-acetate (PMA) (50ng/ml) and ionomycin
565 (1µg/ml), with the addition of brefeldin A (GolgiPlug, BD) and monensin. Cells were
566 processed for staining with fluorochrome-labeled antibodies. Cells were resuspended in
567 Hanks' balanced salt solution and stained for live/dead using LIVE/DEAD Fixable Dead
568 Cell Stain and cell surface markers (see table S1 for antibodies). Cells were fixed
569 overnight with Foxp3 / Transcription Factor Staining Buffer Set (Thermo Fisher
570 Scientific) and stained with intracellular cytokine or transcription factor staining for 45
571 min. For intracellular Blimp-1 staining, cells from lung or mediastinal draining lymph
572 node were stimulated with 10 µg/ml anti-CD3/anti-CD28 or 100 µg/ml HDM for 36

573 hours, respectively, then re-stimulated with PMA and ionomycin, with the addition of
574 brefeldin A and monensin for 1.5 hours and processed for staining as above. Cells were
575 collected using a 5 laser BD Fortessa and analyzed with FlowJo.

576 **Bone marrow chimeras**

577 Bone marrow cells were isolated from femurs of mice, and T cells were depleted using
578 Thy1.1 or Thy1.2 positive selection and a magnetic separation kit (Miltenyi Biotec). Cells
579 were counted, mixed in a ratio of 50:50, and injected intravenously into Rag1$^{-/-}$-deficient
580 hosts. Rag1$^{-/-}$-deficient host mice were lethally irradiated at 750 cGy for 10 min,
581 followed by 4 hours rest, and a subsequent at 750 cGy for 10 min one day prior to
582 injection with cells. A total of 20 × 10^6 cells were injected per mouse. Four weeks after
583 reconstitution, mice were bled to check reconstitution efficiency, and 6 weeks after
584 reconstitution, they were used for HDM experiment, as described above.

585 **Antibody blockade**

586 Mice were injected intraperitoneally (i.p.) with 100 µl of sterile PBS containing 0.2 mg of
587 anti-IL-10R MAb (1B1.3A), anti-IL-21R MAb (4A9), or injected both anti-IL-10R and anti-
588 IL-21R MAbs on day 1, 3, 5, 7, 9, 15, 17, 21 during the HDM immunization as described
589 above. Control mice were injected with 0.2 mg IgG1 isotype control (TNP6A7) MAb.

590 **ELISA**

591 The total IgE in the serum was assessed by commercial ELISA kit according to the
592 manufacturer's protocol.

593 **Quantitative polymerase chain reaction**

594 Total RNA was isolated from lung tissue and mediastinal draining lymph node using
595 TRIzol Reagent (Thermo Fisher Scientific). About 1 µg RNA was converted into cDNA

596 using qScript cDNA Synthesis Kit (Quanta BioSciences). Quantitative real-time PCR
597 (qPCR) was performed on with TaqMan probes [mouse IL-2 (Mm00434256_m1),
598 mouse IL-4 (Mm00445259_m1), mouse IL-6 (Mm0046190_m1), mouse IL-10
599 (Mm01288386_m1), mouse IL-21 (Mm00517640_m1), mouse IL-27 (Mm00469294_m1)
600 and mouse β-actin (Mm02619580_g1), Thermo Fisher Scientific] and TaqMan
601 PerfeCTa® qPCR FastMix® II mix (Quanta BioSciences) and detected by a CFX96
602 Real-time PCR Detection Machine (Bio-Rad). ΔΔCt was calculated to determine the
603 relative expression normalized to β-actin.

604 **In vitro T cell polarization**

605 Naïve CD4 T cells were isolated from spleens and lymph nodes by magnetic
606 enrichment and flow sorting ($CD4^+$ $CD25^-$ $CD44^{lo}$ $CD62L^+$) and activated with plate-
607 bound anti-CD3 and anti-CD28 (10ug/ml each) for 3 days in complete RPMI
608 supplemented with: Th1, IL-12(10ng/ml), anti-IL-4(10ug/ml); Th2, IL-4(10ng/ml), anti-
609 IFNγ(10ug/ml). Cells were stimulated with PMA, ionomycin, and brefeldin A for 2 hours
610 prior to flow staining as above.

611 **Airway Hyperreactivity (AHR)**

612 We have described assessment of AHR in mice in detail previously(Raundhal et al.,
613 2015). Briefly, mice were anesthetized and subjected to the forced oscillation technique
614 for measuring AHR using a Flexivent PFT apparatus (SCIREQ). Measurements of lung
615 function were made following perturbation with increasing doses of methacholine.

616 **Statistical Analysis**

617 For statistical analysis, unpaired Mann Whitney t test, one-way Anova or Kruskil-Wallis
618 test was performed using GraphPad Prism (version 8.02, GraphPad), unless otherwise

619 specified to calculate statistical significance (reported as means ± SD). A p value of
620 0.05 was considered significant.
621
622 **Supplemental material**
623 Fig. S1: Lung-specific Blimp-1 dependent Th2 development in vivo; related to Fig 1. Fig
624 S2: Bcl6 expression levels in T_{FH} and Th2 cells in mLN; related to Fig 2. Fig S3: Blimp-1
625 expression dynamics in HDM induced allergic lung inflammation; related to Fig 3. Fig
626 S4: IL-21R signaling is required for T cell responses but not Blimp-1 in the mLN; related
627 to Figure 5. Fig S5: IL-10 and IL-21 do not cooperatively regulate Blimp-1 expression;
628 related to Figure 6,7.
629
630 **Author Contributions:**
631 Conceptualization, K.H., A.R. and A.C.P; Methodology, S.K., A.R., K.H., and A.C.P;
632 Investigation and Validation, K.H.,A.H., S.K.,S.H. and A.C.P; Formal Analysis, K.H.,
633 A.H., S.K., and A.C.P; Resources, M.M.X., A.L.D., and A.R.; Writing - Original Draft,
634 K.H., and A.C.P; Writing – Review & Editing; K.H., A.R. and A.C.P.; Supervision, A.R.
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652

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873 24.
874

875 **Abbreviations:**

876 BAL: bronchiolar lavage fluid

877 BM: bone marrow

878 HDM: house dust mite

879 T_{FH}: T follicular helper

880 YFP: yellow fluorescent protein

881

882 **Figure Legends:**

883 Figure 1: T cell expression of Blimp-1 promotes allergic lung inflammation

884 Analysis of lung tissue isolated from control (Blimp-1$^{f/f}$ CD4Cre^{-}) or Blimp-1^{CD4Cre} (Blimp-
885 1$^{f/f}$ CD4Cre^{+}) animals i.n. immunized with HDM. A) PAS staining B) Percent and
886 absolute number of infiltrating inflammatory cells in the BAL. C,E) Flow analysis and
887 D,F) Percent of indicated subsets of T cells isolated from lungs (gated on Live, CD4^{+}
888 TCRβ^{+}). Th2: Gata3^{+} IL-13^{+}. G) Assessment of airway hyperreactivity (AHR) in
889 unimmunized or HDM-immunized mice. Rn: Central airway resistance (Newtonian
890 resistance) Rrs: Respiratory system resistance. Data are pooled from 2-3 experiments
891 with 8-13 total mice per group, mean ± SD. Kruskal-Wallis One-way ANOVA(D) or
892 Mann-Whitney t-test (B,F) *p<0.05 **p<0.01 ***p<0.001 ****p<0.0001

893

894 Figure 2: IgE responses are Blimp-1 independent

895 A) Total IgE in serum from animals in Figure 1. B,D) Flow analysis and C,E) Percent of
896 IL-4, IFNγ and GATA3 expression in mLN T cells (gated on Live CD4^{+} TCRβ^{+} FoxP3^{-}).
897 P+I: PMA, ionomycin. F, G) Flow analysis and percentage of IL-4^{+} cells shown in B.
898 Gated on Live, CD4^{+} TCRβ^{+} FoxP3^{-} IL-4^{+}. Data are pooled from 2-3 experiments with 9-
899 13 total mice per group, mean ± SD. Mann-Whitney t-test. *p<0.05 **p<0.01 ***p<0.001
900 ****p<0.0001

901

902 Figure 3: Blimp-1 is intrinsically required in T cells to promote Th2 cells

903 A) Expression and B) Fold changes of Blimp-1 YFP^{+} cells in T cell subsets (gated on
904 Live, CD4^{+} TCRβ^{+} FoxP3^{-}) in lungs of naïve unimmunized mice or after HDM. Fold
905 change calculated as percent of Blimp-1 YFP^{+} in each subset after HDM over average

906 of Blimp YFP$^+$ in each subset in unimmunized naïve animals C) Flow analysis and D)
907 Percentages of T cell subsets, IL-10 and Bcl6 expression isolated from lungs of mixed
908 BM chimeras gated on Live CD4$^+$ TCRβ^+ (Tregs) and FoxP3$^-$ (Th2) and Thy1.1
909 (Control); or Thy1.2 (Blimp-1^{CD4Cre}) after HDM. E) Eosinophil percentages in BAL of
910 Control BM chimeras (Control only or Blimp-1^{CD4Cre} only) or mixed BM chimeras. Data
911 are pooled from 2 experiments with 14 mixed BM chimeras and 1-3 control only BM
912 chimera mice per group, mean ± SD. Wilcoxon matched-pairs signed rank test (D)
913 Mann-Whitney t-test (E). *p<0.05 **p<0.01 ***p<0.001 ****p<0.0001
914
915 <u>Figure 4: STAT3 is required for Blimp-1 expression in T cells</u>
916 Analysis of lung tissue isolated from control (STAT3$^{f/f}$ CD4Cre$^-$) or STAT3^{CD4Cre}
917 (STAT3$^{f/f}$ CD4Cre$^+$) animals i.n. immunized with HDM. A) PAS staining B) Percent of
918 eosinophils in the BAL. C) Percent of T cell subsets in lung (gated on Live,
919 CD4$^+$TCRβ^+). D) Percent of IL-10 producing effector T cells (FoxP3$^-$) and Tregs
920 (FoxP3$^+$). E) Percent of Blimp-1$^+$ effector T cells (gated on CD4$^+$TCRβ^+FoxP3$^-$CD44$^+$) in
921 mLN and representative histogram. Data are pooled from 2 experiments with 8 mice
922 total per group, mean ± SD. F) Percent of Th1 (IFNγ^+ T-bet$^+$) and Th2 (IL-13$^+$ GATA3$^+$)
923 cells (gated on CD4$^+$TCRβ^+FoxP3$^-$), and G) Blimp-1 YFP+ cells within Th1, Th2 and
924 Treg cells in the lung after HDM. Data are pooled from 2 experiments with 6-8 total mice
925 per group, mean ± SD. Mann-Whitney t-test. *p<0.05 **p<0.01 ***p<0.001 ****p<0.0001
926
927 <u>Figure 5: IL-6 and IL-21 are not required for Blimp-1 mediated Th2 cell differentiation.</u>

928 A) Percent of T cell subsets (gated on Live, $CD4^+$ $TCR\beta^+$ $FoxP3^-$ (non-Treg) or $FoxP3^+$

929 (Treg)) or B) Percent of Blimp-1^+ cells (gated on Live, $CD4^+$ $TCR\beta^+$ $FoxP3^-$ $CD44^+$)

930 isolated from lungs of control (IL-6$R\alpha^{f/f}$ $CD4Cre^-$) or IL6$R\alpha^{CD4Cre}$ (IL-6$R\alpha^{f/f}$ $CD4Cre^+$)

931 animals i.n. immunized with HDM. C) Percent of eosinophils in the BAL, D) Th1 (IFNγ^+,

932 T-bet$^+$) and Th2 (IL-13^+ $GATA3^+$) cells (gated on Live, $CD4^+TCR\beta^+FoxP3^-$) and E)

933 percent of Blimp-1^+ or $Bcl6^+$ (gated on Live, $CD4^+TCR\beta^+FoxP3^-$) T cells in lung isolated

934 from IL-21R-intact or IL-21R-deficient animals immunized with HDM. Data are pooled

935 from 2-3 experiments with 9-10 total mice per group, mean ± SD. F) Percent of Th1

936 (IFNγ^+ T-bet$^+$) and Th2 (IL-13^+ $GATA3^+$) cells (gated on Live, $CD4^+TCR\beta^+FoxP3^-$) and

937 G) Blimp-1 YFP^+ cells within Th1 and Th2 cells isolated from the lung from control (IgG)

938 or anti-IL-21R treated animals immunized with HDM. Data are pooled from 2

939 experiments with 6-7 mice total per group, mean ± SD, Mann-Whitney t-test. *$p<0.05$

940 **$p<0.01$ ***$p<0.001$ ****$p<0.0001$

941

942 <u>Figure 6: IL-10 is required for Blimp-1 expression in Th2 cells</u>

943 Analysis of lungs isolated control (IgG) or anti-IL10R treated animals i.n. immunized

944 with HDM. A) Percent of eosinophils in the BAL. B) Flow analysis and C) Percent of Th1

945 and Th2 cells in lungs (gated on Live, $CD4^+TCR\beta^+FoxP3^-$). D) Flow analysis and E)

946 Percent of Blimp-1 YFP^+ cells in Th1 and Th2 cells in the lungs. F) Flow analysis and G)

947 Percent of IL-10^+ and Blimp-1 YFP^+ expression in Th1 and Th2 cells in lungs. Data are

948 pooled from 3 experiments with 11-12 mice total per group, mean± SD. Mann-Whitney

949 t-test. *$p<0.05$ **$p<0.01$ ***$p<0.001$ ****$p<0.0001$

950

951 Figure 7: IL-10R is required on T cells for Blimp-1 expression and Th2 cell development

952 Analysis of lung tissue isolated from control (IL-10R$\alpha^{f/f}$ CD4Cre^{-}) or IL10Rα^{CD4Cre} (IL-

953 10R$\alpha^{f/f}$ CD4Cre^{+}) animals i.n. immunized with HDM. A) Percent of T cell subsets

954 isolated from lungs (gated on Live, CD4^{+} TCRβ^{+} FoxP3^{-} (non-Treg) or FoxP3^{+} (Treg)).

955 B) Percent of Blimp-1^{+} cells (gated on Live, CD4^{+} TCRβ^{+} FoxP3^{-} CD44^{+}). C) Percent of

956 eosinophils in the BAL. D) Percent of Bcl6^{+} and IL-10^{+} T cells isolated from lungs (gated

957 on Live, CD4^{+} TCRβ^{+}). Data are from 1 experiment with 5 mice per group, mean± SD.

958 Mann-Whitney t-test. *p<0.05 **p<0.01 ***p<0.001 ****p<0.0001

959

960 Figure 8: Transcription factor expression in effector T cells in the absence of Blimp-1.

961 A) Percent of effector cells expressing the transcription factors shown isolated from lung

962 after HDM. Gated on Live, CD4^{+} TCRβ^{+} FoxP3^{-}. B) Histograms of data from A

963 comparing naïve, Control effector (CD44^{+}) or Blimp-1^{CD4Cre} effector (CD44^{+}) T cells.

964 Data are pooled from 2-3 experiments with 8-10 total mice per group. Mann Whitney t-

965 test. *p<0.05 **p<0.01 ***p<0.001 ****p<0.0001

966

967 Figure 9: Blimp-1 supports Th2 development through suppression of Bcl6

968 Analysis of lungs isolated from Control, Bcl6^{CD4Cre}(Bcl6$^{f/f}$ CD4Cre^{+}), Blimp-1^{CD4Cre}(Blimp-

969 1$^{f/f}$ CD4Cre^{+}), or doubleKO^{CD4Cre}(Bcl6$^{f/f}$ Blimp-1$^{f/f}$ CD4Cre^{+}) animals i.n. immunized with

970 HDM. A) PAS staining B) Percent of eosinophils in the BAL C) Percent of Th1(IFNγ^{+} T-

971 bet^{+}), Th2 (IL-13^{+} GATA3^{+}) and Treg (FoxP3^{+}) cells isolated from lungs (gated on Live,

972 CD4^{+}TCRβ^{+} (Treg), and FoxP3^{-} (non-Treg)). D) Total IgE in serum. E) Flow analysis of

973 IL-10 and FoxP3 T cells isolated from lungs of indicated mice after HDM-induced

974 allergic asthma. Gated on Live, TCRβ^+ CD4$^+$. F) Percent and Geo MFI of IL-10$^+$ cells in
975 effector (CD4$^+$TCRβ^+FoxP3$^-$) or Treg (CD4$^+$TCRβ^+FoxP3$^+$) cells in lung. Data are
976 pooled from 3 experiments with 7-17 total mice per group, mean± SD. Kruskal-Wallis
977 One-way ANOVA. *p<0.05 **p<0.01 ***p<0.001 ****p<0.0001
978
979 Figure S1: Lung-specific Blimp-1 dependent Th2 development in vivo.
980 A) Schematic for HDM-induced allergic lung inflammation model used throughout this
981 study B) Percent of indicated subsets of T cells isolated from mLN gated on Live, CD4$^+$
982 TCRβ^+. Data are pooled from 2-3 experiments with 8-13 total mice per group, mean ±
983 SD. C) Flow staining of Th1 (IFNγ^+ T-bet$^+$) and Th2 (IL-13$^+$ GATA3$^+$) in vitro polarization
984 of T cells isolated from Control or Blimp-1^{CD4Cre} animals. D) Analysis of lung tissue
985 isolated from control (Blimp-1$^{f/f}$ CD4Cre$^-$) or Blimp-1^{CD4Cre} (Blimp-1$^{f/f}$ CD4Cre$^+$) animals
986 i.n. immunized with SEA. Percent of indicated subsets of T cells isolated from lungs
987 gated on Live, CD4$^+$ TCRβ^+ FoxP3-. E) Analysis of Tregs from lungs, gated on Live,
988 CD4$^+$ TCRβ^+ FoxP3$^+$. Data are pooled from 2 experiments with 5 total mice per group,
989 mean ± SD. Mann-Whitney t-test *p<0.05 **p<0.01 ***p<0.001 ****p<0.0001
990
991 Figure S2: Bcl6 expression in T_{FH} and Th2 cells in the mLN.
992 A) Expression of Bcl6 in indicated populations. Geometric Mean shown in top right
993 corner of each track. B) Geometric Mean of Bcl6 in indicated samples. Wilcoxon
994 matched-pairs signed rank test. *p<0.05 **p<0.01 ***p<0.001 ****p<0.0001
995
996 Figure S3: Blimp-1 expression dynamics in HDM induced allergic lung inflammation.

997 A) Absolute number of CD4 T cell subsets during the course of HDM-induced allergic
998 lung inflammation model. B) Absolute number of Blimp-1 YFP$^+$ cells of each CD4 T cell
999 subset shown in A. C) Flow analysis and Fold change of Blimp-1 YFP$^+$ FoxP3$^+$ Treg
1000 cells in the lung of naïve unimmunized animals and after HDM. Fold change calculated
1001 as percent of Blimp-1 YFP$^+$ Tregs after HDM over average of Blimp-1 YFP$^+$ Treg cells in
1002 unimmunized mice. D) Schematic of mixed bone marrow chimera generation E)
1003 Reconstitution 4-6 weeks post irradiation and injection of donor marrow isolated from
1004 blood (4 weeks) or lungs post HDM-induced lung inflammation. Flow cytometry and
1005 percent of Thy1.1$^+$ (Control) and Thy1.2$^+$ (Blimp-1^{CD4Cre}) in both tissues shown.
1006 Wilcoxon matched-pairs signed rank test. *p<0.05 **p<0.01 ***p<0.001 ****p<0.0001
1007
1008 <u>Figure S4: IL-21R signaling is required for T cell responses but not Blimp-1 in the mLN.</u>
1009 A) qPCR of indicated cytokines in lungs and mLN of unimmunized or HDM-immunized
1010 animals B) Percent of Th1 (IFNγ^+ T-bet$^+$) and Th2 (IL-13$^+$ GATA3$^+$) cells in mLN gated
1011 on Live, CD4$^+$TCRβ^+FoxP3$^-$. C) Expression of Blimp-1 and Bcl6 in mLN in T cells gated
1012 on Live, CD4$^+$TCRβ^+FoxP3$^-$. Data are pooled from 3 experiments with 9-10 mice total
1013 per group, mean ± SD, Mann-Whitney t-test. *p<0.05 **p<0.01 ***p<0.001 ****p<0.0001
1014
1015 <u>Figure S5: IL-10 and IL-21 do not cooperatively regulate Blimp-1 expression.</u>
1016 A) Percent and Geo MFI of IL-10 expressing cells isolated from the lung gated on Live,
1017 CD4$^+$ TCRβ^+ FoxP3$^-$ CD44$^+$. B) Percent of Th1 (IFNγ^+ T-bet$^+$) and Th2 (IL-13$^+$ GATA3$^+$)
1018 cells in the lung isolated from Blimp-1 YFP animals treated as Control (IgG) or treated
1019 with anti-IL-10R, anti-IL21R, or both anti-IL-10R and anti-IL21R for the duration of the

1020 HDM-induced allergic lung inflammation model. C) Percent of Blimp-1 YFP^{+} Th1 and
1021 Th2 cells in B. Data are pooled from 2-3 experiments with 6-10 total mice per group.
1022 Kruskal-Wallis One-way ANOVA. *p<0.05 **p<0.01 ***p<0.001 ****p<0.0001 D)
1023 Reconstitution 4-6 weeks post irradiation and injection of donor marrow isolated from
1024 the lungs post HDM-induced lung inflammation. Percent of CD45.1^{+} (Control) and
1025 CD45.2^{+} (IL-10Rα^{CD4Cre}) are shown in both 50:50 Control:Control and 50:50 Control:IL-
1026 10Rα^{CD4Cre}. E) Percentages of T cell subsets isolated from lungs of mixed BM chimeras
1027 gated on Live CD4^{+} TCRβ^{+} FoxP3^{-} (Th1, Th2, Th17) or FoxP3+ (Treg) and
1028 CD45.1(Control); or CD45.2 (Control or IL-10Rα^{CD4Cre}) after HDM. Data are pooled from
1029 2 experiments with 5 total mice per group. Mann-Whitney t-test *p<0.05 **p<0.01
1030 ***p<0.001 ****p<0.0001

Figure 1: Blimp-1 in T cells promotes allergic airway inflammation

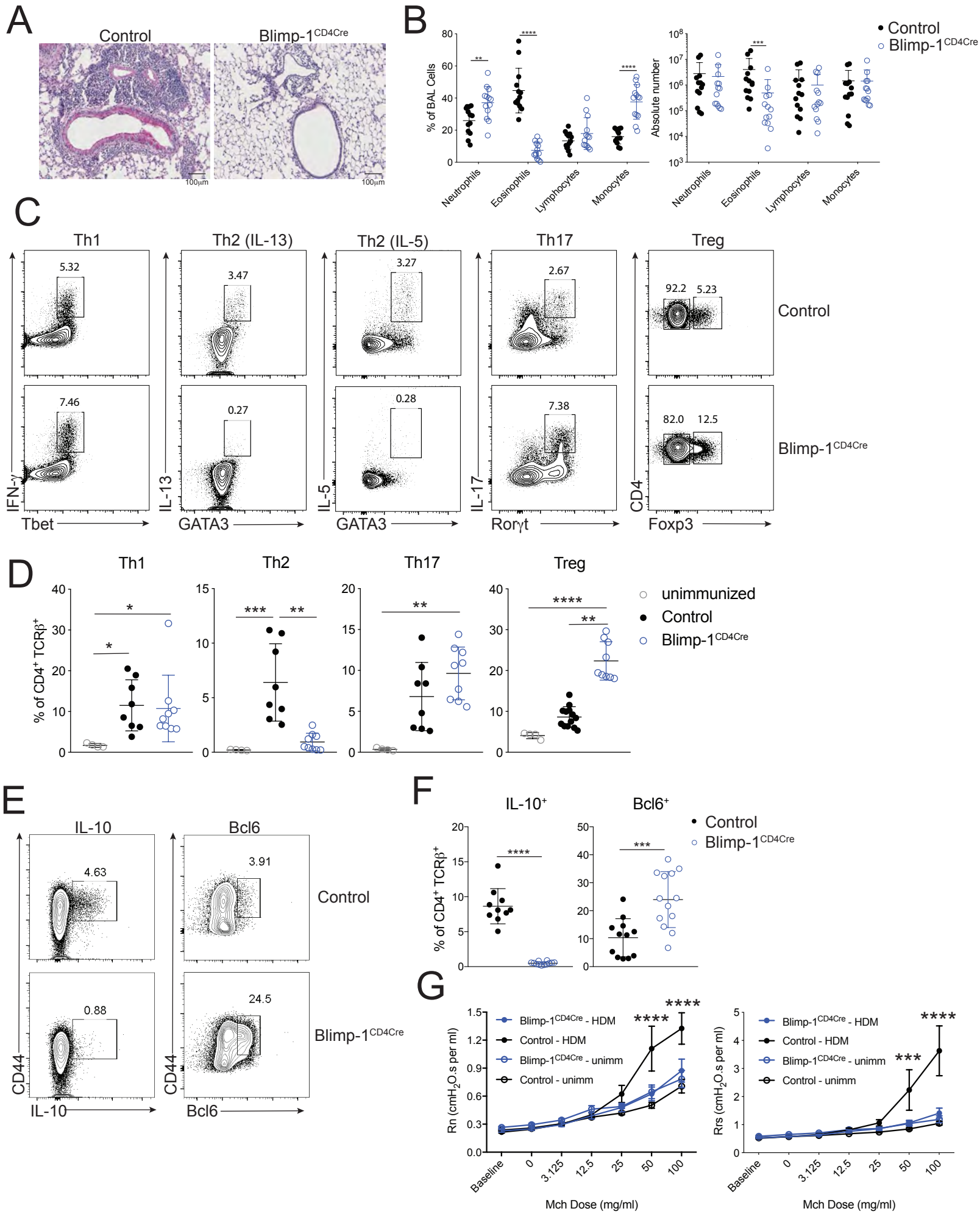

Figure 2: IgE responses are Blimp-1 independent

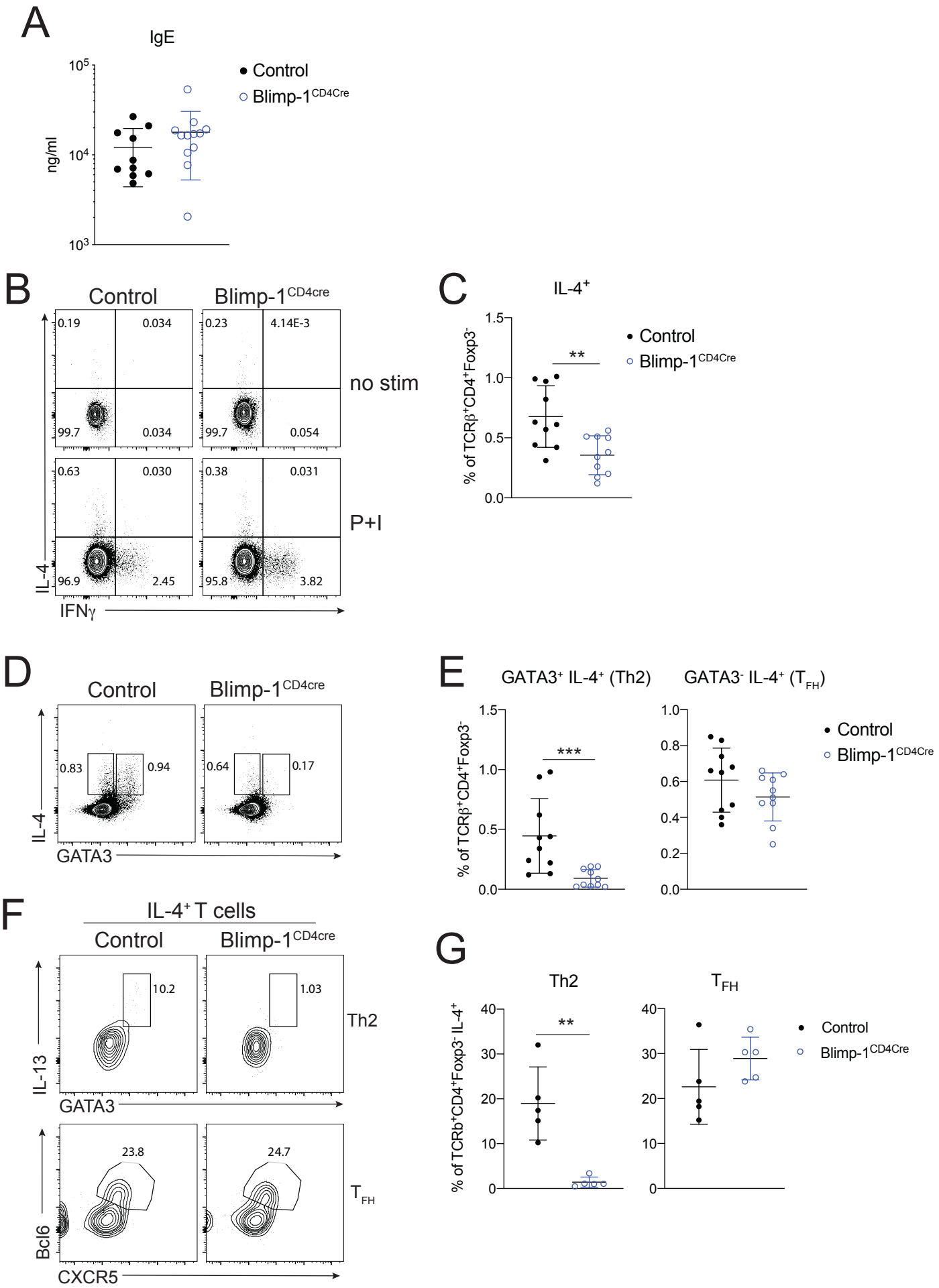

Figure 3: Blimp-1 is intrinsically required in T cells to promote Th2 cells

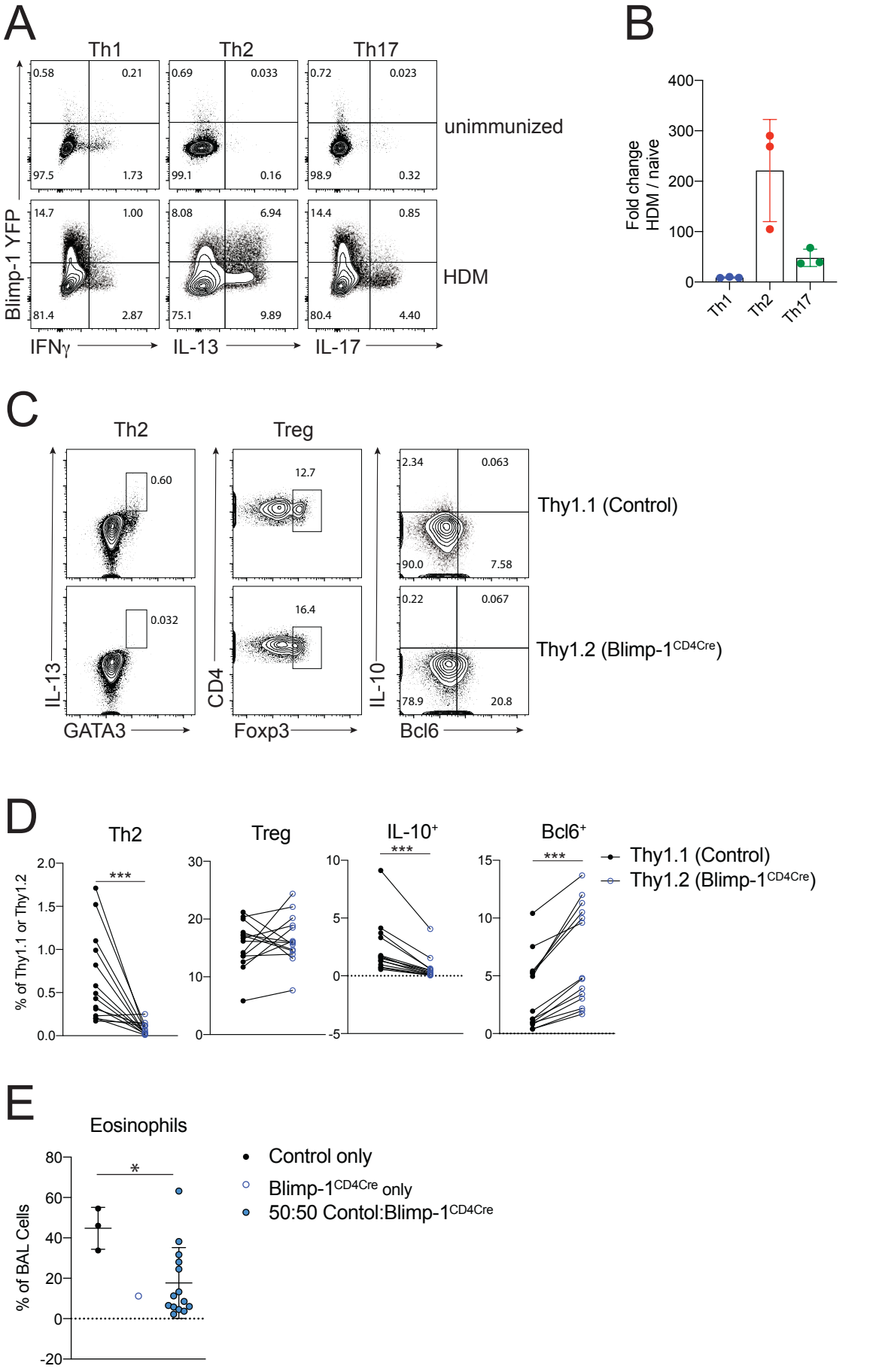

Figure 4: STAT3 is required for Blimp-1 expression in T cells

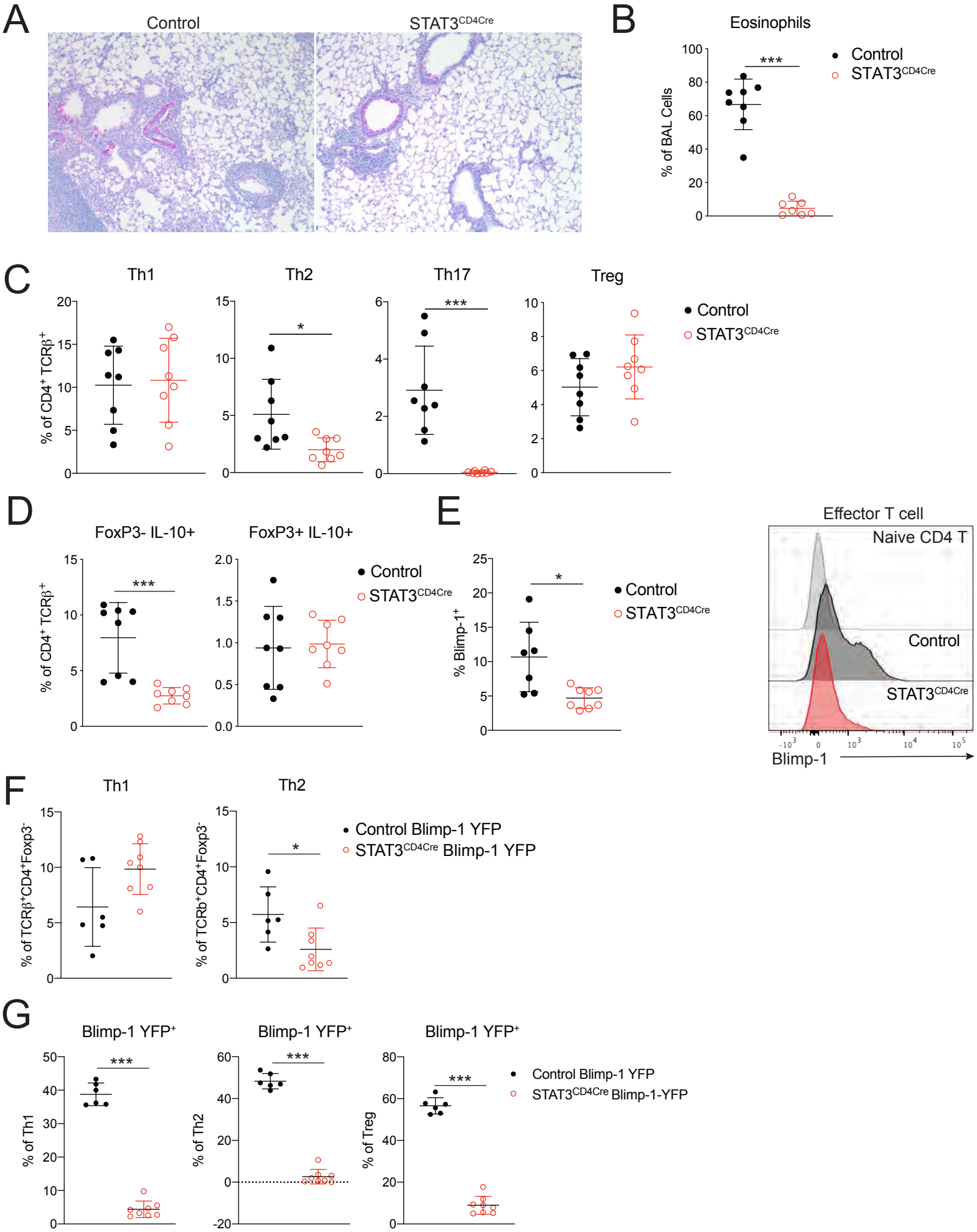

Figure 5: IL-6 and IL-21 are not required for expression of Blimp-1 in Th2 cells

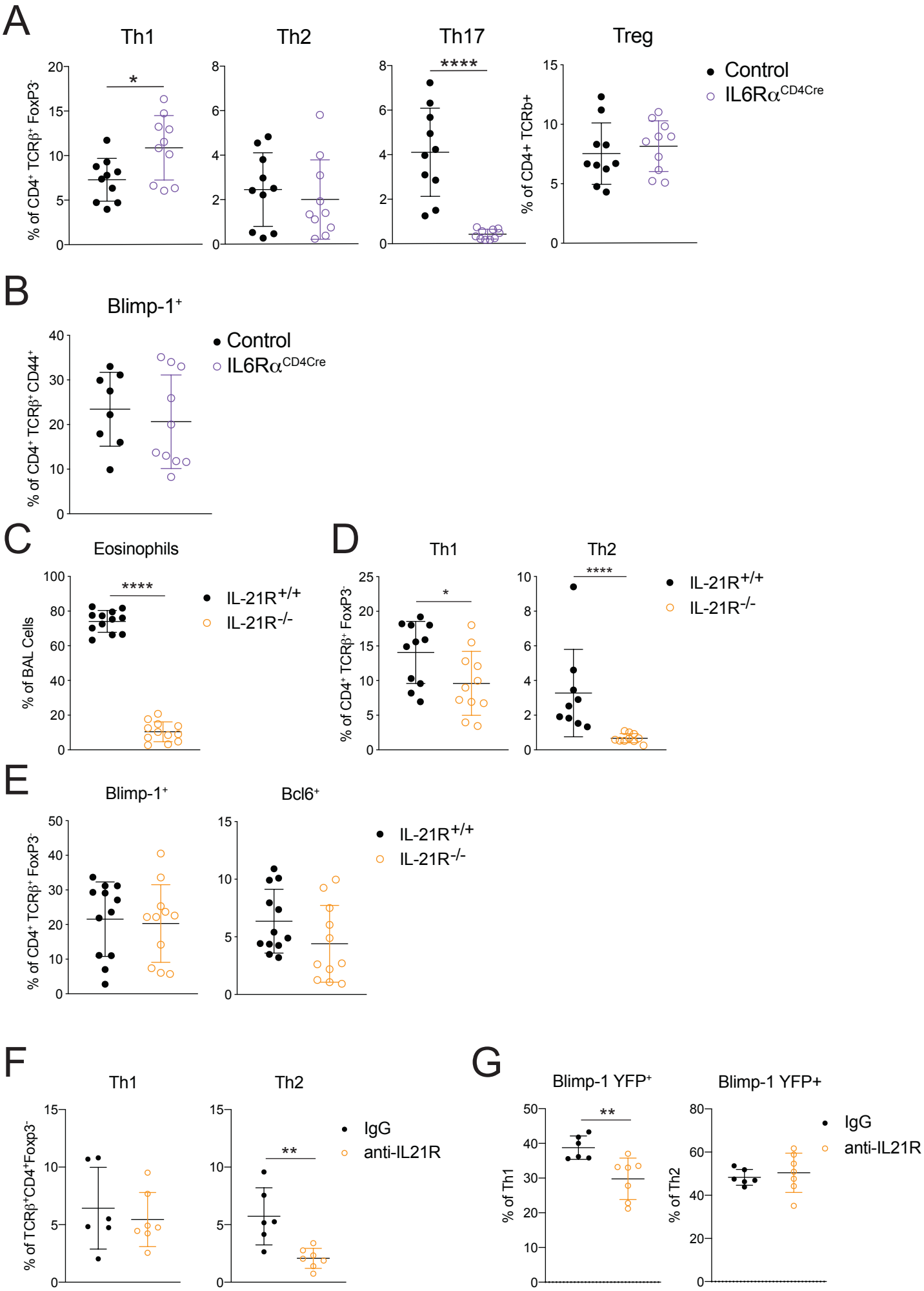

Figure 6: IL-10 is required for Blimp-1 expression in Th2 cells

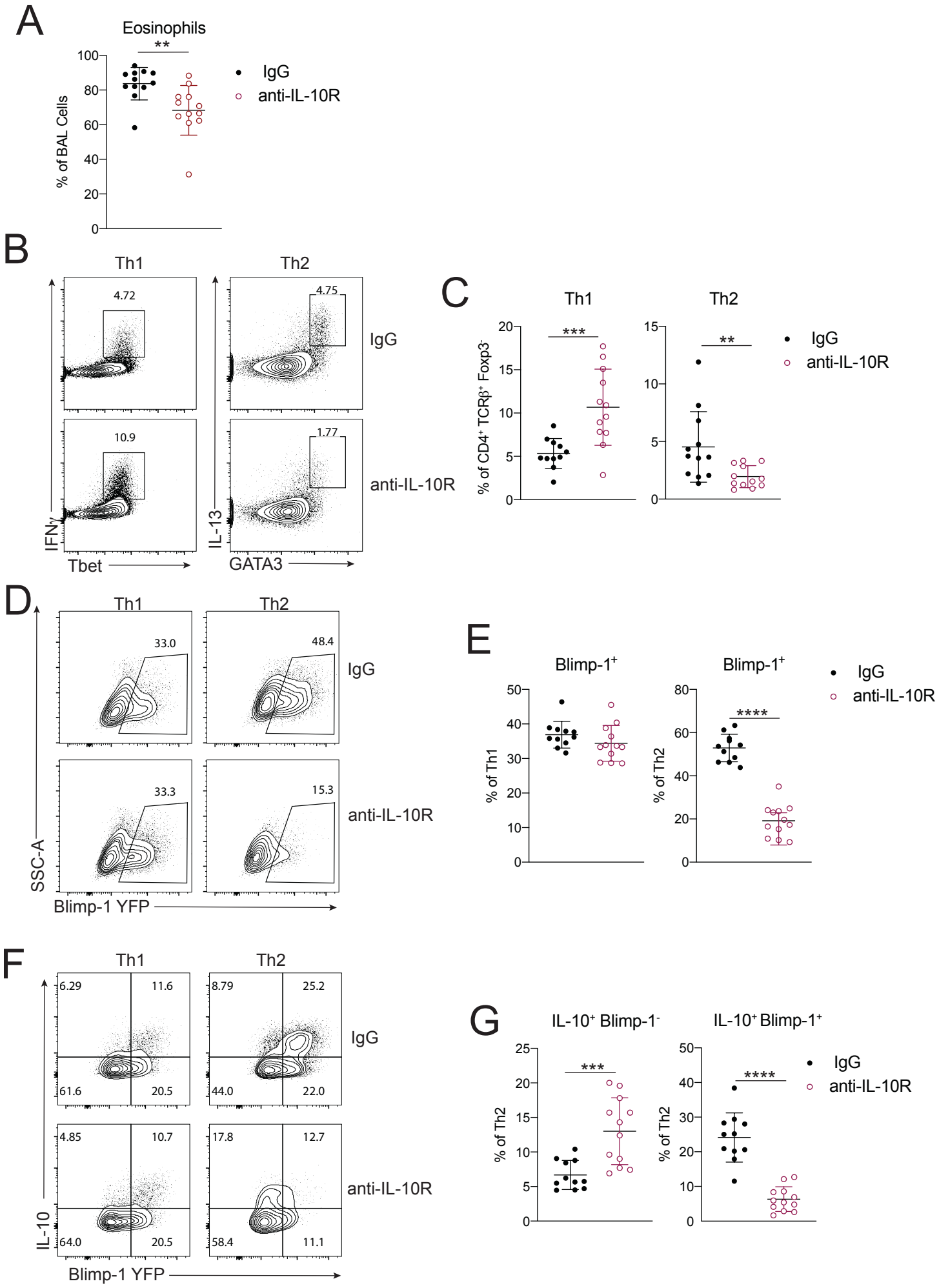

Figure 7: IL-10R is required on T cells for Blimp-1 expression and Th2 cell development in the lung

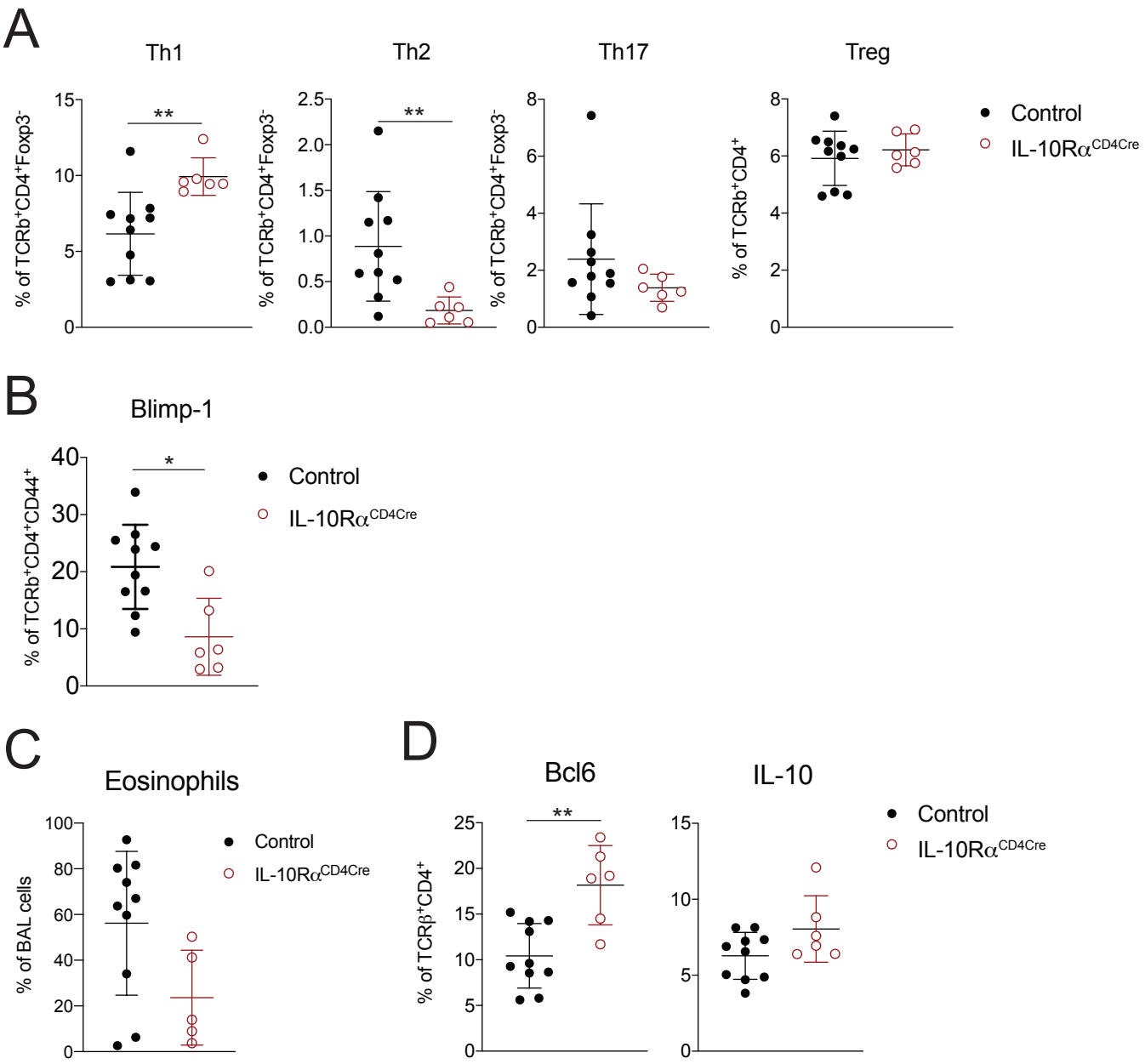

Figure 8:Transcription factor expression in effector T cells in the absence of Blimp-1

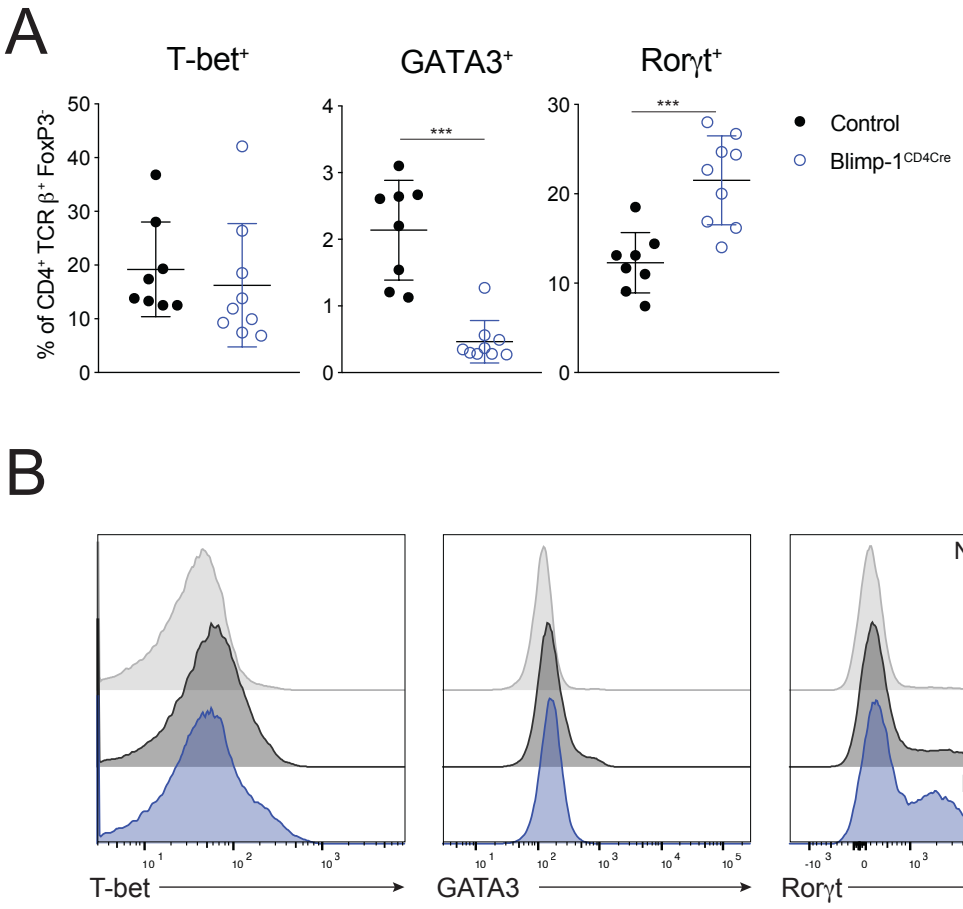

Figure 9: Blimp-1 supports Th2 development through suppression of Bcl6

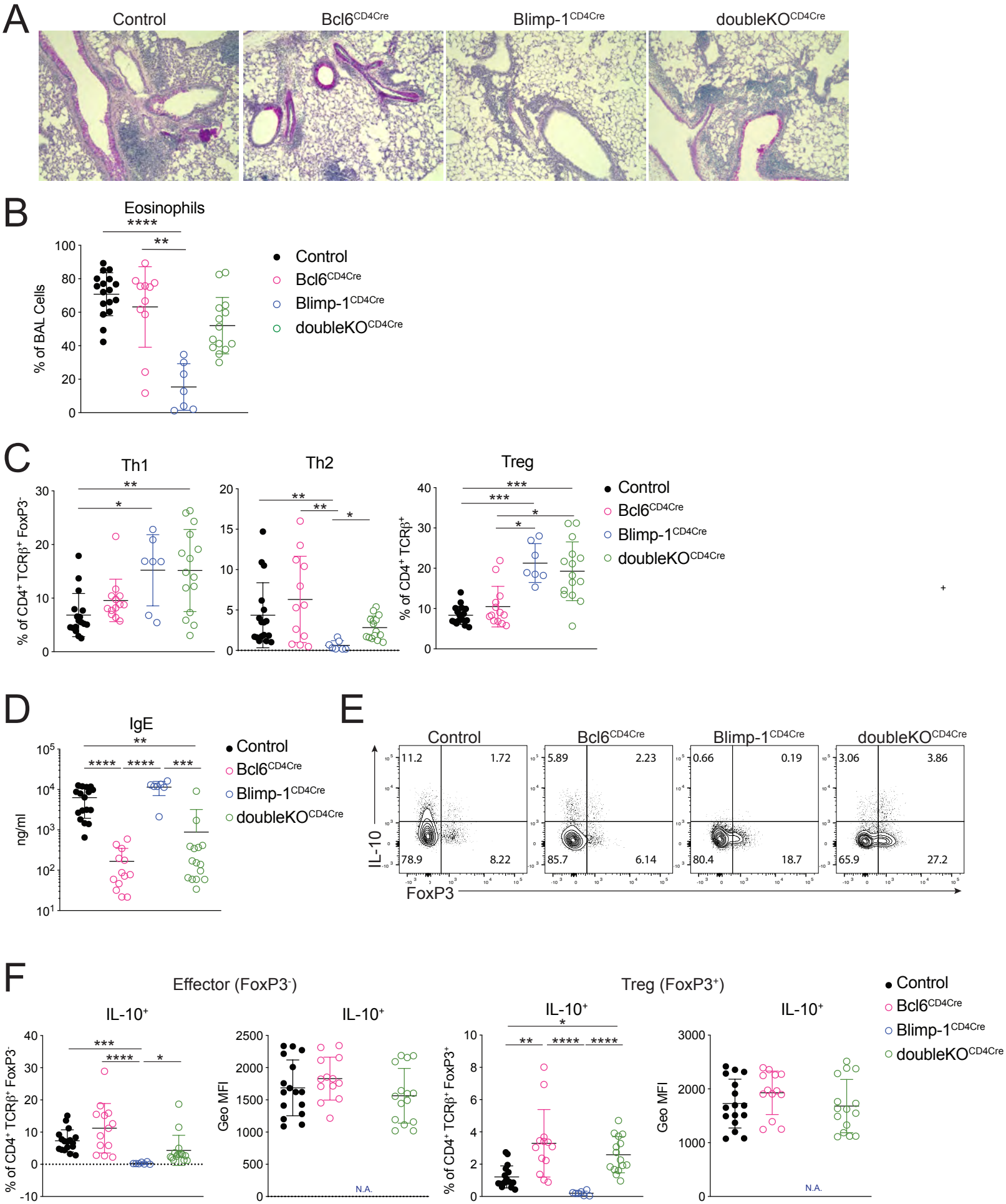

Figure S1: Lung-specific Blimp-1 dependent Th2 development in vivo.

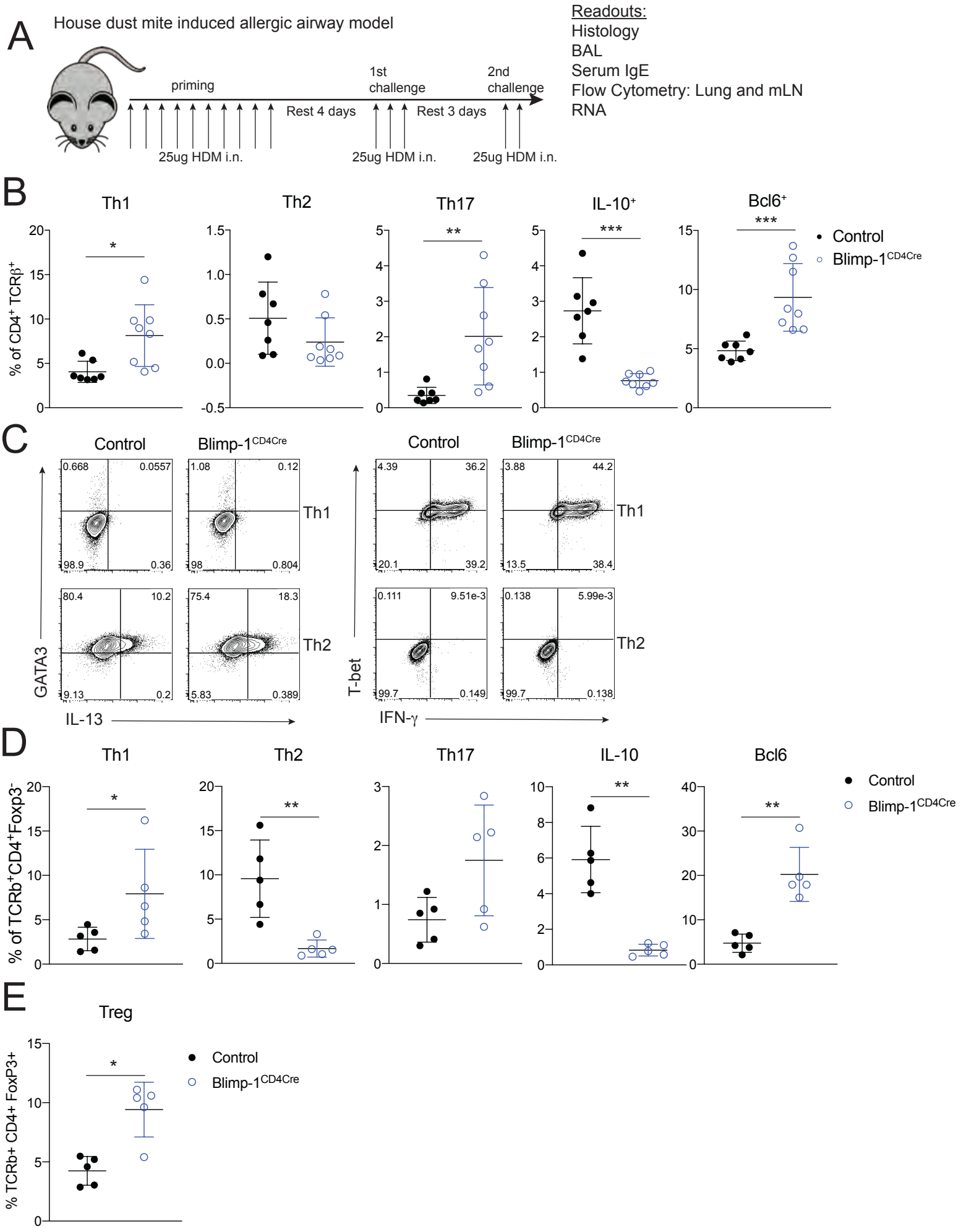

Figure S2: Bcl6 expression in T_{FH} and Th2 cells in the mLN.

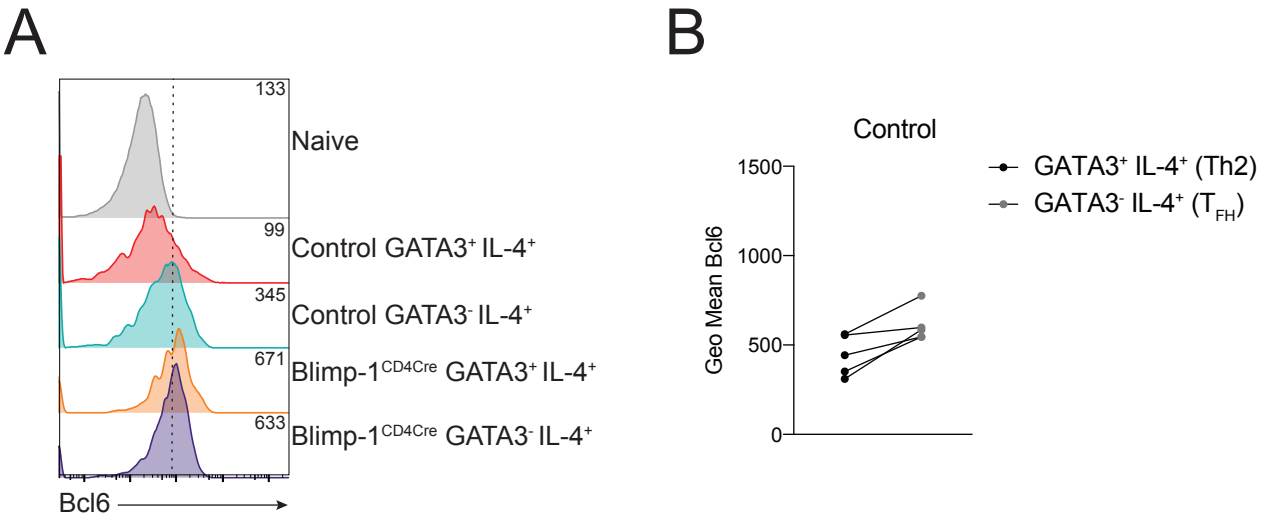

Figure S3: Blimp-1 expression dynamics in HDM induced allergic lung inflammation

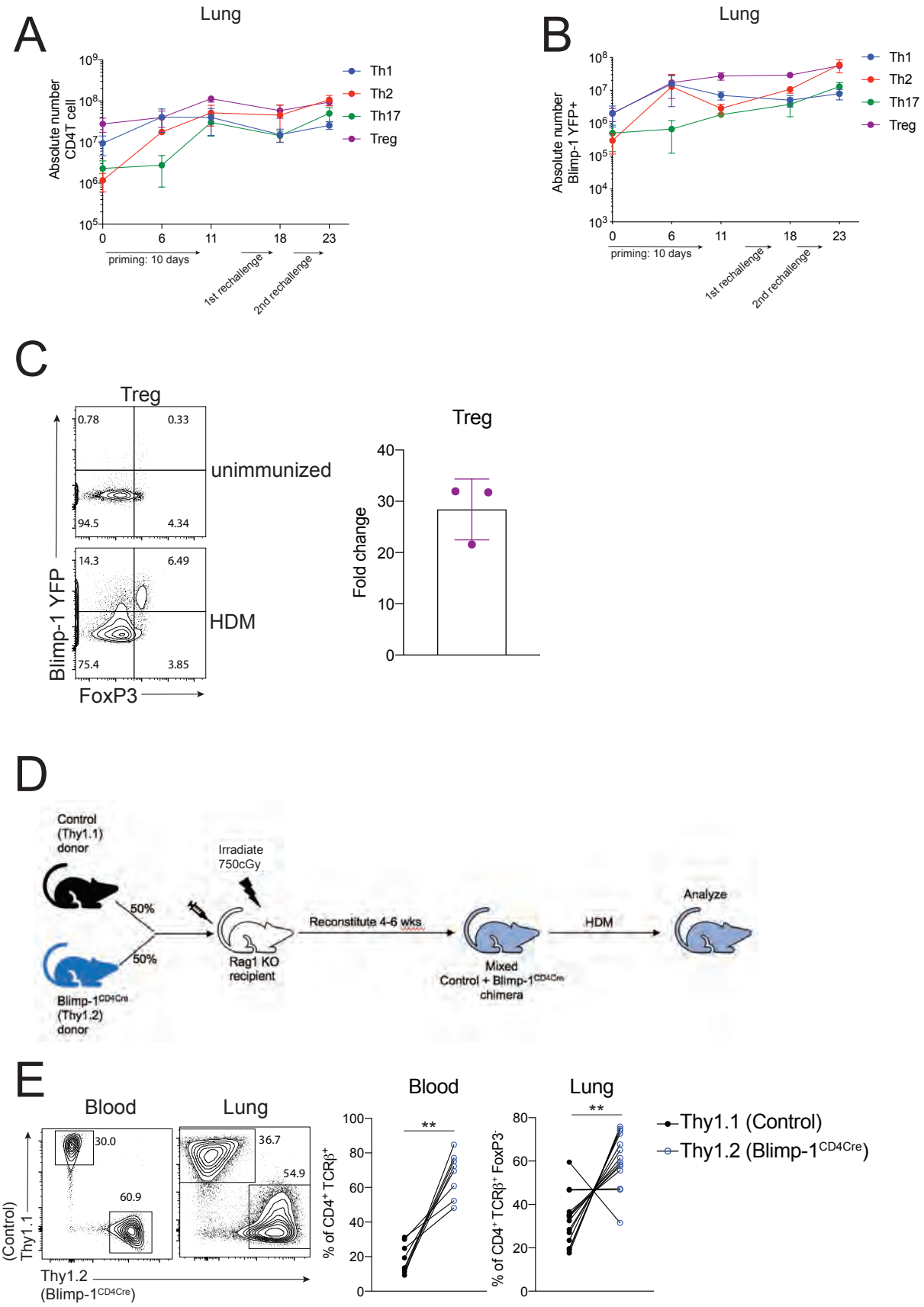

Figure S4: IL-21R signaling is required for T cell responses but not Blimp-1 in the mLN

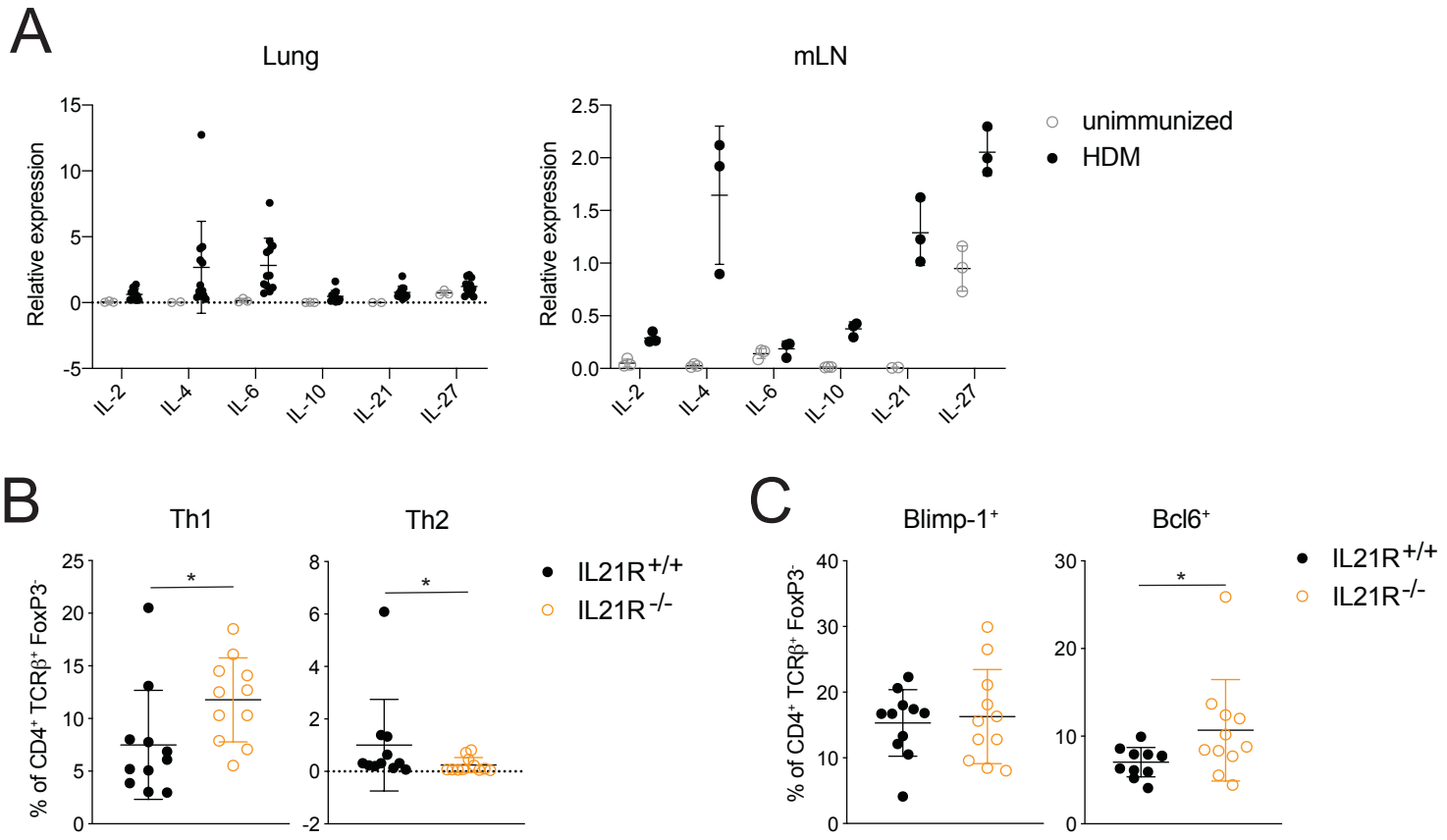
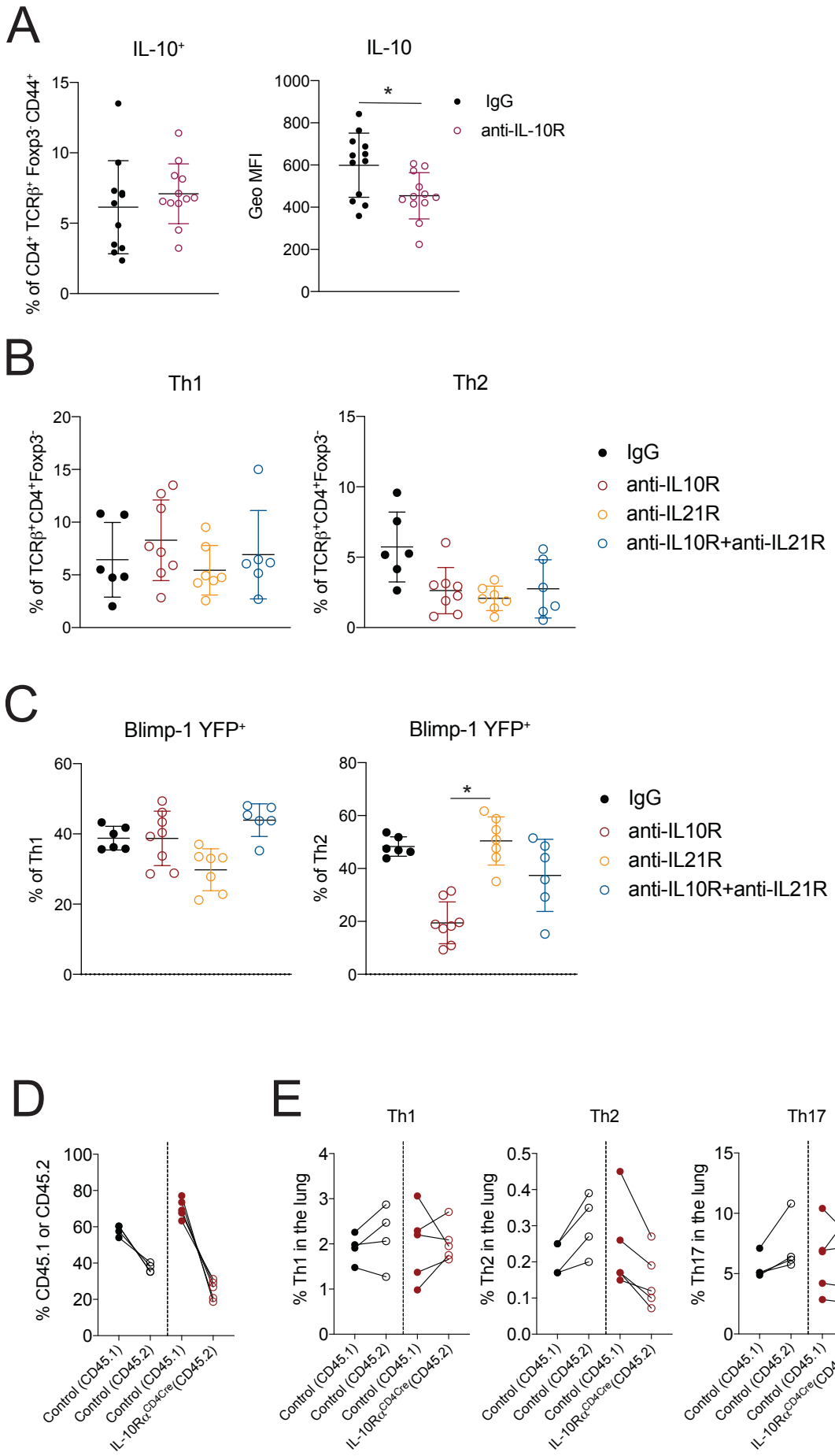
Figure S5: IL-10 and IL-21 do not cooporatively regulate Blimp-1 expression.