Allergen-induced airway inflammation and Th2 cell development requires Blimp-1

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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 intrinsically represses 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; Johnston et al., 2009; Neumann et al., 2014; Parish et al., 2014).
69 Furthermore, we previously found that overexpression of Blimp-1 could lead to cell

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

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

Results

Blimp-1 in T cells promotes allergic airway inflammation

Blimp-1 controls effector T cell responses and constrains autoimmunity(Crotty et al., 2010; Poholek et al., 2016). We reasoned that during a house dust mite (HDM) induced model of allergic lung disease, the absence of Blimp-1 in T cells would drive increased 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). These
115 results were surprising as prior examination of Blimp-1 in T cells has resulted in

increased Th2 effector T cell responses in a Th2 model (Poholek et al., 2016)and in vitro, Blimp-1 is not required for Th2 cell differentiation (Fig S1B)(Cimmino et al., 2008). In contrast to the lungs, Blimp-1^{CD4Cre} mice had increased Th1 and Th17 cells in the mLN, but Th2 cells (GATA3^{+} IL-13^{+}) were largely absent, constituting less than 1% of all CD4^{+} T cells (Fig S1C)(Bao and Reinhardt, 2015). Consistent with previous publications, Tregs were increased in Blimp-1^{CD4Cre} mice, likely due to Blimp-1's role in repressing IL-2, and Tregs capability to expand in conditions with excess IL-2 (Fig 1C,D)(Cretney et al., 2011; D'Cruz and Klein, 2005; Fontenot et al., 2005; Malek et al., 2002; Martins et al., 2008; Webster et al., 2009). Blimp-1 is well known to be a critical driver of IL-10, and indeed Blimp-1^{CD4Cre} mice were specifically lacking IL-10 production from T cells (Fig 1E,F, S1C)(Kallies et al., 2006; Martins et al., 2006; Neumann et al., 2014; Parish et al., 2014). Bcl6 and Blimp-1 are transcriptional repressors known to regulate one another in both T and B cells, thus we explored the levels of Bcl6 in CD4^{+} T cells(Crotty et al., 2010). Blimp-1^{CD4Cre} mice had increased expression of Bcl6 in both the lung and mLN (Fig 1E,F, S1C), suggesting that the absence of Blimp-1 in T cells resulted in a concomitant increase in Bcl6. Together, these data suggest that Blimp-1 plays a surprising and critical role in driving the differentiation of Th2 cells in response to inhaled allergens that is necessary for resulting inflammation in the lung tissue.

IgE responses are Blimp-1 independent

A hallmark of type2 driven allergic responses is the presence of high levels of circulating IgE. In contrast to the decrease in type2 immunity observed in the lungs of Blimp-1^{CD4Cre} animals (reduced Th2 cells and eosinophils in BAL), IgE levels were unchanged (Fig

139 2A). Isotype-switch to IgE is dependent on IL-4(Kopf et al., 1993). However, similar to
140 loss of Th2 cells in the lung, IL-4+ T cells in the mLN was significantly reduced in Blimp-
141 1^{CD4Cre} animals (Fig 2B,C). Antibody responses are largely driven by T follicular cells
142 (T_{FH}), and Blimp-1 antagonizes (T_{FH}) responses. Thus, we reasoned that T_{FH} cell
143 responses would be intact in Blimp-1^{CD4Cre} and thus capable of driving robust IgE
144 responses. IL-4 can be produced by both Th2 and T_{FH} cells(Zhu, 2015), therefore, we
145 determined the percentage IL-4^+ cells in both subsets (Th2, GATA3^+ and $T_{FH,}$ GATA3^-)
146 of cells in the mLNs of control and Blimp-1^{CD4Cre} animals. Despite total IL-4+ cells being
147 decreased in mLNs of Blimp-1^{CD4Cre} animals, analysis of IL-4+ cells within the T_{FH}
148 population (GATA3^-) was similar to controls (Fig 2D,E). In contrast, IL-4^+ cells within the
149 Th2 population (GATA3^+) were significantly reduced (Fig 2D,E). As expected,
150 expression of Bcl6 was higher in T_{FH} cells (GATA3^-) than Th2 cells (GATA3^+) of control
151 animals, but still reduced compared to Blimp-1^{CD4Cre} animals (Fig S2A,B). Indeed, IL-4+
152 cells from control animals comprised both Th2 (GATA3+ IL-13+) and T_{FH} (Bcl6+
153 CXCR5+) cells (Fig 2F). Blimp-1^{CD4Cre} animals, however, had similar IL-4+ T_{FH} cell
154 percentages but Th2 cells were absent (Fig 2F). These data suggest that although
155 Blimp-1 is a master regulator of Th2 cell development in the lungs, it is not required for
156 IL-4^+ T_{FH} cell development and subsequent type2 mediated IgE responses. Thus, there
157 are unique molecular pathways that can drive type2 humoral immunity for IgE
158 production (IL-4-producing T_{FH} cells) distinct from those that drive eosinophil recruitment
159 to the lung (IL-13 and IL-5 producing Th2 cells), and Blimp-1 is a major regulator that
160 molecularly dissects these two independent pathways.
161

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

We next sought to determine if Blimp-1 was expressed by all CD4 T cell subsets in the lung during the course of allergic airway inflammation, or if expression was limited to Th2 cells. To assess Blimp-1 expression, we induced allergic inflammation in Blimp-1 yellow fluorescent protein (YFP) reporter mice, which reliably tracks Blimp-1 transcription by YFP (Rutishauser et al., 2009). We assessed each CD4 T cell subset and Blimp-1 expression within each subset at 4 timepoints; day 6 and day 10 which are during and after priming, day 18, after the first challenge and day 23 after the second challenge. Th1, Th2, Th17 and Treg cells were present at each timepoint in the lung in naïve unimmunized animals and after HDM challenges (Fig S3A). When looking at the percentage of Blimp-1 YFP+ cells among each subset we found that all subsets initially upregulated Blimp-1 but that Th2 cells did so to greater degree compared to Th1 and Th17 cells. (Fig 3A-B, S3B). Tregs have been reported to express high levels of Blimp-1 in effector tissues, thus as a positive control we assessed Blimp-1 in Tregs and found nearly all Tregs expressed Blimp-1 after HDM (Fig S3C)(Cretney et al., 2011). Blimp-1 continued to increase in Th2, Treg and Th17 populations, while the percentage of Blimp-1+ cells in Th1 cells remained fairly constant over time after the initial increase from day 0 to day 5 (Fig S3B).

It was possible that the increase in Tregs (Fig 1C,D) might be responsible for suppressing Th2 cells. Therefore, we wondered if the expression of Blimp-1 was required intrinsically in T cells to drive Th2 cells or if an extrinsic mechanism such as Treg mediated control may be at play. To test the intrinsic function of Blimp-1, we

generated mixed bone marrow (BM) chimeras in Rag-deficient hosts where 50% of donor BM was from congenically marked B6 Control mice (Thy1.1) and 50% from Blimp-1^{CD4Cre} (Thy1.2). 6 weeks post reconstitution, allergic lung inflammation was induced with HDM as previously described (Fig S3D). Although irradiated hosts received equal percentages of donor BM, Blimp-1^{CD4Cre} BM reconstituted at greater than 50%, supporting its role in negatively regulating T cell survival (Poholek et al., 2016) (Fig S3E). Nevertheless, Th2 cells were significantly reduced in Blimp-1^{CD4Cre} T cells after HDM-induced allergic lung inflammation (Fig 3C,D). Tregs however were equivalently derived from Control or Blimp-1^{CD4Cre} BM, suggesting that Blimp-1 is intrinsically required in Th2 cells to promote Th2 cell differentiation and that increases in Tregs was not responsible for this effect. The reduction in Th2 cells was significant enough to result in a slight, but significant decrease in eosinophils in the BAL compared to animals that only received control BM (Fig 3E). Blimp-1^{CD4Cre} T cells (Thy1.2+) had significant decreases in IL-10 and increases in Bcl6 compared to control cells in the same animal, consistent with Blimp-1's function in driving IL-10 and repressing Bcl6 (Fig 3C,D). Taken together these data suggest that the mechanism of Blimp-1 mediated Th2 cell differentiation is cell intrinsic.

STAT3 is required for Blimp-1 expression in T cells

We next explored the upstream drivers of Blimp-1 in Th2 cells in order to understand how Blimp-1 might be controlling Th2 cell differentiation. Our previous examination of Th2 cells found that STAT3, but not STAT6, was a critical regulator of Blimp-1(Poholek et al., 2016).Furthermore, STAT3 can support Th2 cell differentiation in some contexts

208 (Stritesky et al., 2011). Therefore, we wondered whether STAT3 could be promoting
209 Th2 cell development through upregulation of Blimp-1. To explore the role of STAT3, we
210 used T cell specific STAT3 deficient animals (STAT3$^{f/f}$ CD4-Cre, referred to as
211 STAT3^{CD4Cre}) and assessed T cell subsets in the lung as well as the expression of
212 Blimp-1. After immunization with HDM, STAT3^{CD4Cre} lung tissue had reduced
213 lymphocytic infiltration as assessed by PAS staining and there was a significant
214 reduction in eosinophils found in the BAL (Fig 4A,B). Consistent with STAT3's role in
215 driving Th17 differentiation(Zhou et al., 2007), STAT3^{CD4Cre} had a complete absence of
216 Th17 cells in the lungs (Fig 4C). Intriguingly, we found a significant reduction in Th2
217 cells, similar to our results in Blimp-1^{CD4Cre}, confirming previous studies that STAT3
218 could support Th2 cell development in the context of allergic lung inflammation
219 (Stritesky et al., 2011) (Fig 4C). In contrast, Th1 cell percentages were similar in control
220 and STAT3^{CD4Cre} mice. To determine if STAT3 was required for Blimp-1 expression, we
221 assessed Blimp-1 protein expression in effector T cells. In agreement with our previous
222 study, Blimp-1 protein was significantly reduced in the absence of STAT3, suggesting
223 that STAT3 is required for Blimp-1 expression and subsequent Th2 differentiation in
224 allergic lung inflammation (Fig 4D). To confirm the loss of Blimp-1 in the absence of
225 STAT3, we crossed the STAT3^{CD4Cre} to Blimp-1 YFP reporter mice. Consistent with
226 STAT3^{CD4Cre} animals, Th2 cells were specifically reduced in STAT3^{CD4Cre} Blimp-1 YFP
227 after HDM (Fig 4E). STAT3^{CD4Cre} Blimp-1 YFP also had no Blimp-1 expression
228 (assessed by YFP) in Th1 or Th2 cells compared to control Blimp-1 YFP mice (Fig 4F).
229 Although Th1 cells also lacked Blimp-1, it did not affect Th1 cell percentages in the lung,
230 suggesting that Blimp-1 and STAT3 are not necessary for Th1 cell differentiation in

response to allergens. These data confirm the critical role of STAT3 in driving Blimp-1 in Th2 cells in vivo in an allergic lung inflammation model. These data are in contrast to our previously published work (Poholek et al., 2016) where Blimp-1 limits the percentage of Th2 cells in a worm antigen model, suggesting there is a context-dependent function for Blimp-1 in Th2 cells depending on the antigen or route of immunization.

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

We next sought to determine what environmental factors were driving Blimp-1 expression in Th2 cells in HDM induced lung inflammation. We focused our analysis on known STAT3-activating cytokines. As IL-6 has previously been shown to support Th2 cells in allergic lung disease (Doganci et al., 2005) and is a strong STAT3 activator, we first tested whether IL-6 signaling on T cells was required for Blimp-1 induction and Th2 cell formation in this model. Using IL-6Rα deficient T cells (IL-6R$\alpha^{f/f}$ CD4-Cre, referred to as IL-6Rα^{CD4Cre}) we found no significant difference in Th2 cells in the lung after HDM, however Th17 cells in the lung were absent, in line with the well-known role of IL-6 to drive Th17 development via STAT3 (Fig 5A)**.** Importantly, Blimp-1 levels were unchanged in effector (CD44^{+}) T cells (Fig 5B), suggesting IL-6 was not required to drive Blimp-1 or Th2 cells in response to HDM.

To identify other possible STAT3-driven cytokines we performed whole-tissue qPCR of both lung and mLN and found IL-10, IL-21, and IL-27 were all upregulated in response to HDM, particularly in the mLN (Fig 5C). IL-21 is known to play a critical role in Th2 cell

generation in HDM induced allergic lung inflammation, however the mechanism is unclear(Coquet et al., 2015). Furthermore IL-21 is known to drive Blimp-1 in B cells (Ozaki et al., 2004). Using IL-21R deficient mice (IL-21R$^{-/-}$) we found a significant reduction in eosinophils in the lung consistent with previous reports (Coquet et al., 2015)(Fig 6A). We also found a significant reduction in Th2 cells in the lung and mLN (Fig 6B, Fig S4A). Surprisingly, although IL-21R$^{-/-}$ displayed a loss of Th2 cells similar to Blimp-1^{CD4Cre} and STAT3^{CD4Cre}, Blimp-1 levels were similar in effector T cells in both control and IL-21R$^{-/-}$ animals (Fig 6C, Fig S4B). We wondered if IL-21 could play an important role in driving Blimp-1 only in Th2 cells, and the absence of Th2 cells limited our ability to detect this. To test this, we treated Blimp-1 YFP mice with anti-IL-21R antibody (or control IgG antibody) during the course of HDM-induced allergic lung inflammation and tracked Blimp-1 expression by YFP. Similar to IL-21R$^{-/-}$ mice, Th2 cells were significantly reduced in the lung of anti-IL-21R treated mice (Fig 6D). However, Blimp-1 levels were intact in the few remaining Th2 cells found (Fig 6E). While Blimp-1 levels were slightly reduced in Th1 cells, this had no effect on Th1 cell development (Fig 6D-E). These data suggest that while IL-21 is critical for Th2 cell development and eosinophil recruitment during HDM-induced allergic lung inflammation, the role of IL-21 in Th2 cell formation is independent of the function of Blimp-1 in driving Th2 cells.

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

Our previous studies using worm-derived antigens (soluble egg antigens from *S. Mansoni*) demonstrated that IL-10 is a critical factor driving Blimp-1 expression in Th2

277 cells(Poholek et al., 2016). Although IL-10 is thought to be anti-inflammatory in allergic
278 responses, we nonetheless explored if IL-10 might also be a driver of Blimp-1 in HDM-
279 induced allergic lung inflammation. To test this, we treated Blimp-1 YFP mice with anti-
280 IL10R antibody or IgG control during HDM immunization. Surprisingly, we found a
281 consistent reduction in eosinophils recruited to the lungs (Fig 7A), and a concomitant
282 decrease in Th2 cells in the lung (Fig 7B,C). Th1 cells were increased, while Th17 and
283 Treg cells were unaffected (Fig 7C, data not shown). Intriguingly, Blimp-1 was
284 specifically and significantly reduced in Th2 cells but unaffected in Th1 cells (Fig 7D,E),
285 suggesting IL-10 was an important regulator of Blimp-1 in Th2 cells in the context of
286 allergic antigen stimulation. As Blimp-1 is an intrinsic regulator of IL-10 expression (Fig
287 3D), we wondered if IL-10 expression was also affected by loss of Blimp-1. Within the
288 total effector ($CD44^+$) T cell population, IL-10 levels were unchanged (Fig S5A),
289 however in Th2 cells, expression of IL-10 shifted from a Blimp-1+ population in IgG
290 treated control mice, to a Blimp-1 negative population in anti-IL-10R treated mice (Fig
291 7F,G). These data suggest that Blimp-1 expression may not be continually required for
292 IL-10 expression in T cells. To address the possibility that IL-10 and IL-21 act in a
293 redundant fashion to regulate Blimp-1 expression we performed double antibody
294 blockade of both IL-10R and IL-21R in Blimp-1 YFP animals. Blockade of IL-10R and IL-
295 21R together did not have a synergistic effect on Blimp-1 expression, suggesting the
296 effects of IL-10 and IL-21 are independent of one another (Fig S5B,C). Taken together,
297 these data suggest that IL-10 is an important regulator of Blimp-1 in T cells supporting
298 Th2 cells in the lung during allergic inflammation. These data are somewhat counter-
299 intuitive as IL-10 is typically thought of as an anti-inflammatory cytokine and previous

studies implicated a role for IL-10 in suppressing Th2 cells (Coomes et al., 2016). However, our data clearly point to an additional role of IL-10 in promoting inflammation of the lung via Th2 cells and recruitment of eosinophils to the lung.

Blimp-1 supports GATA3 expression and Th2 development through suppression of Bcl6

We next sought to understand how Blimp-1 was functioning to control Th2 cell differentiation. Since GATA3 is the master regulator of Th2 cell development (Zhang et al., 1997; Zheng and Flavell, 1997), we determined more specifically the expression of master regulators in T cell subsets in the absence of Blimp-1. Indeed, although T-bet levels were unchanged, the loss of Blimp-1 led to a specific and robust decrease in GATA3 (Fig 8A,B). Bcl6 is a well-known repressor of GATA3 (Kusam et al., 2003; Sawant et al., 2012), and Blimp-1 deficient T cells had a robust increase in Bcl6 expression (Fig 1F). We hypothesized that in wildtype cells, Blimp-1 may be suppressing Bcl6 to promote GATA3 expression, but that in Blimp-1^{CD4Cre} animals, increased expression of Bcl6 would suppress GATA3 and Th2 cell development. To test this, we induced allergic lung inflammation in mice that had a T cell specific deficiency in both Blimp-1 and Bcl6 (Blimp-1$^{f/f}$ Bcl6$^{f/f}$ CD4Cre^{+}, referred to as doubleKO^{CD4Cre})(Xie et al., 2017). Control (Bcl6$^{f/f}$ or Blimp-1$^{f/f}$, CD4Cre^{-}), Bcl6-deficient (Bcl6$^{f/f}$ CD4Cre^{+}, Bcl6^{CD4Cre}), Blimp-1^{CD4Cre} or doubleKO^{CD4Cre} mice were immunized with HDM and assessed for lung inflammation as well as development of T cell subsets in the lung and mLN. As before, examination of lung tissue found a consistent reduction in inflammation in Blimp-1^{CD4Cre} lungs compared to Control or Bcl6^{CD4Cre} (Fig 9A). doubleKO^{CD4Cre} mice had lymphocyte infiltration and mucus that was similar to controls, suggesting

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

Discussion:

346 In this study, we have uncovered a previously unappreciated role for Blimp-1 in driving
347 Th2 cell development in the context of an allergen induced lung inflammation model.
348 House dust mite antigen is a widely used model of allergic lung inflammation owing to
349 its physiologic relevance to human health as dust mites are a well-known allergic trigger
350 in many individuals with asthma disease(Thomas, 2016). Based on our previous study,
351 we expected Blimp-1 to suppress inflammatory effector responses to HDM. On the
352 contrary, we uncovered an unexpected role for Blimp-1 in the specific control of Th2 cell
353 differentiation, which has not been previously demonstrated in *in vitro* assays or our
354 prior *in vivo* Th2 model (Cimmino et al., 2008; Poholek et al., 2016). Previous studies
355 exploring the role of Blimp-1 in T cells have elucidated many functions for Blimp-1 that
356 collectively could be described as controlling effector functions of T cells that mediate
357 disease states. In CD8 T cell responses to viral infection, Blimp-1 supports effector cell
358 differentiation while repressing memory cells (Kallies et al., 2009; Rutishauser et al.,
359 2009; Shin et al., 2009). Blimp-1 also plays a critical role in supporting IL-10 expression
360 in Tregs and effector CD4 T cells, the absence of which can have important
361 consequences for controlling infections by pathogens such as Toxoplasma or chronic
362 viral infection (Cretney et al., 2011; Neumann et al., 2014; Parish et al., 2014). Blimp-1
363 also controls differentiation of an inflammatory Th17 cell that produces IFNγ and GM-
364 CSF and subsequent autoimmune disease in an EAE model (Jain et al., 2016). In each
365 of these systems, Blimp-1 primarily controls effector properties of the T cells; supporting
366 cytokines such as IL-10, IFNγ, and GM-CSF, suggesting that Blimp-1 is part of a cell
367 program to further drive a terminal effector differentiation state. Finally Blimp-1 is known
368 to repress the differentiation of T_{FH} cells(Johnston et al., 2009; Nurieva et al., 2009).

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

372 Th2 cells are primary producers of several important type2 cytokines that drive
373 allergic lung disease, including IL-4, IL-5, and IL-13(Licona-Limón et al., 2013; Ray and
374 Cohn, 1999). Each of these cytokines has a unique function in asthma disease, but
375 collectively they enforce several hallmarks of asthma, including IgE production,
376 recruitment of eosinophils and mucus production in the airways. Intriguingly, we found
377 that Blimp-1 controlled only GATA3+ Th2 cells but was not required for all IL-4+
378 producing cells. T_{FH} cells are a critical population of effector T cells that is superior at
379 initiating and supporting B cell responses such as IgE(Crotty, 2011). Furthermore, they
380 can express IL-4, which is required for isotype switch to IgE(Zhu, 2015). Although the
381 loss of Blimp-1 did not result in an increase in T_{FH} cells after HDM, as it has in other
382 systems, IL-4^{+} T_{FH} cells were intact, resulting in normal IgE responses to HDM despite
383 the absence of Th2 cells. These results suggest that the pathways that drive T_{FH} and
384 Th2 cells in response to allergens can be molecularly dissected, and that Blimp-1 is a
385 major regulator distinguishing these two inflammatory cell types. This may enable
386 therapeutic targeting of these populations distinctly, and supports further exploring the
387 upstream pathways of each cell population.

388 Multiple STATs including STAT3, STAT4 and STAT5 have been implicated in
389 driving Blimp-1 in effector T cells(Jain et al., 2016; Neumann et al., 2014; Xin et al.,
390 2016). In line with previous reports, we have found a central role for STAT3 in
391 upregulating expression of Blimp-1 (Jain et al., 2016; Poholek et al., 2016). Although we

392 had previously found that IL-10 was required for Blimp-1 expression in Th2 cells, we did
393 not initially explore IL-10 in this context for a few reasons. First, there are several well-
394 known STAT3 activating cytokines such as IL-6, IL-21, and IL-27, and several of these
395 had already been implicated in supporting Th2 cells in allergic lung disease
396 models(Coquet et al., 2015; Doganci et al., 2005; Leonard and O'Shea, 1998; Licona-
397 Limón et al., 2013; Rincon et al., 1997). Secondly, IL-10 generally is thought to act as
398 an anti-inflammatory cytokine and previous work had suggested IL-10 suppresses Th2
399 responses in an HDM driven model(Coomes et al., 2016; Tournoy et al., 2000).
400 However, we were surprised to find that neither IL-6 nor IL-21 signaling to T cells had
401 any significant function on Blimp-1 expression in T cells. Rather, IL-10 seems to play a
402 predominant role in regulating Blimp-1 in particular in Th2 cells. We initially
403 hypothesized that perhaps IL-21 initiated Blimp-1 expression while IL-10 maintained
404 expression, yet double antibody blockades of IL-21R and IL-10R did not reveal a
405 synergistic effect in regulating Th2 or Blimp-1 expression. IL-21 clearly plays a critical
406 role in supporting Th2 cells in HDM induced allergic lung disease but its mechanism is
407 independent of Blimp-1 (Coquet et al., 2015). There may be other combinatorial effects
408 of STAT3 activating cytokines that support Blimp-1 expression, which requires further
409 study.
410 Our studies on IL-10 blockade highlights the potentially critical contribution of IL-
411 10/IL-10R signaling in promoting Th2 cell differentiation in response to allergens. IL-10
412 is classically thought of as an anti-inflammatory cytokine(Moore et al., 2001). Therefore,
413 the role of IL-10 in promoting Blimp-1 expression in Th2 cells and subsequent Th2 cell
414 development may seem at first counterintuitive. One recent study analyzed the

415 responses of IL-10Rα$^{-/-}$ CD4+ T cells during HDM-induced allergic lung inflammation
416 and found that IL-10 acts directly on T cells to suppress Th2 cells(Coomes et al., 2016).
417 In contrast, we found that antibody blockade of IL-10R results in reduced Th2 cells and
418 a significant loss of Blimp-1 expression within the Th2 compartment. There are several
419 reports suggesting a STAT6- and IL-4-independent mechanism exists to drive Th2
420 cells(Dent et al., 1998; Ouyang et al., 2000). Furthermore, IL-10 from IRF4^{+} DCs, the
421 main Th2-driving DC population, has been implicated in supporting Th2 cells during
422 allergic responses(Cook and MacDonald, 2016; Gao et al., 2013; Krishnaswamy et al.,
423 2017; Kumamoto et al., 2013; Williams et al., 2013).Given that many cells can secrete
424 IL-10, it will be interesting to determine the pertinent cell types involved in this
425 process(Gabrysova et al., 2014). We postulate that dendritic cells can secrete IL-10
426 after sensing allergens such as HDM, and then promote Blimp-1 expression and
427 subsequent Th2 cell development through STAT3 activation. IL-10 can also act in a
428 paracrine or autocrine manner from T cells to further drive Blimp-1 expression and IL-10
429 expression, which may further support Th2 cells. However, it is likely additional factors
430 may act in concert with IL-10 to support Blimp-1, such as TSLP, which can directly limit
431 Bcl6 via STAT5(Rochman et al., 2018).
432 Blimp-1 mainly acts as a transcriptional repressor by recruiting histone
433 methyltransferases such as G9a which results in downregulation of gene
434 expression(Gyory et al., 2004). Recent studies have identified Blimp-1 binding near the
435 IL-10 gene, suggesting Blimp-1 may also have a role as an activator of gene
436 expression(Minnich et al., 2016). However, our study is more in line with an indirect
437 mechanism of activation whereby Blimp-1 represses Bcl6 which represses IL-

438 10(Hollister et al., 2013; Xie et al., 2017). Loss of Bcl6 and Blimp-1 together restored IL-
439 10 expression in T cells, and blockade of IL-10R signaling resulted in robust IL-10^{+}
440 Blimp-1^{-} populations, suggesting T cells can make IL-10 in the absence of Blimp-1. We
441 postulate that there may be more than one way for Blimp-1 to control gene expression
442 of IL-10, however our studies clearly point to a more complex mechanism beyond
443 Blimp-1 directly activating the IL-10 gene, at least in CD4 effector cells responding to
444 allergens in the lung. More studies carefully exploring Blimp-1 and IL-10 expression in T
445 cells are required to fully understand this complex interaction.
446 As Blimp-1 has effects in almost every T cell subset, it was important to
447 determine the intrinsic function of Blimp-1 in Th2 cells in this system. As Th2-specific
448 deletion of Blimp-1 is challenging, we opted to use mixed bone marrow chimeras, which
449 supported an intrinsic role for Blimp-1. Mechanistically this was due to the critical
450 function of Bcl6 in limiting GATA3 expression. Although its known that Bcl6 is an
451 important repressor of GATA3(Kusam et al., 2003; Sawant et al., 2012), it has not been
452 appreciated that Blimp-1 plays an important role in suppressing Bcl6 in Th2 cells.
453 Although STAT5 can also directly suppress Bcl6, our data suggest that this may be
454 insufficient in some contexts, and that STAT3 driven Blimp-1 is also required to limit
455 Bcl6 and indirectly support GATA3 and subsequent Th2 cell differentiation(Johnston et
456 al., 2012; Oestreich et al., 2012). Further studies that explore the complex interplay of
457 STAT3 and STAT5 activating cytokines in the control of Blimp-1, Bcl6 and GATA3 in
458 Th2 cells may reveal exactly how Th2 cells differentiate in response to allergens, which
459 has long been not fully understood.

In summary, our study exploring the T cell intrinsic role of Blimp-1 in HDM induced allergic airway inflammation has uncovered several findings relevant to the differentiation of Th2 cells in allergen induced lung inflammation. We found an important role of STAT3 in promoting Blimp-1 expression and subsequent Th2 cell differentiation. We additionally determined a potential pro-inflammatory role of IL-10 in driving Blimp-1 expression and promoting inflammation of lung via Th2 cells. Finally, we have described a new role for Blimp-1 in Th2 cell differentiation by repressing Bcl6 and indirectly supporting GATA3 expression. Taken together, our study elucidates a new context-dependent role for Blimp-1 in T cells that promotes, rather than constrains, allergen-induced airway disease.

Materials and Methods

Mice

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

All *in vivo* experiments were performed independently two to three times, with at least 4 mice per group.

Chemicals and Reagents

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

House dust mite (HDM) model

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

Bronchoalveolar lavage fluid (BAL)

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

Histology

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

Flow cytometry

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

brefeldin A and monensin for 1.5 hours and processed for staining as above. Cells were collected using a 5 laser BD Fortessa and analyzed with FlowJo.

Bone marrow chimeras

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

Antibody blockade

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

ELISA

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

Quantitative polymerase chain reaction

Total RNA was isolated from lung tissue and mediastinal draining lymph node using TRIzol Reagent (Thermo Fisher Scientific). About 1 µg RNA was converted into cDNA using qScript cDNA Synthesis Kit (Quanta BioSciences). Quantitative real-time PCR

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

In vitro T cell polarization

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

Statistical Analysis

For statistical analysis, unpaired Mann Whitney t test, one-way Anova or Kruskil-Wallis test was performed using GraphPad Prism (version 8.02, GraphPad), unless otherwise specified to calculate statistical significance (reported as means ± SD). A p value of 0.05 was considered significant.

Supplemental material

Fig. S1 shows HDM-induced allergic airway model, in vitro differentiation assays, and mLN data related to Fig 1. Fig S2 shows Bcl6 expression levels in mLN T_{FH} and Th2

cells. Fig S3 shows T cell subsets and Blimp-1 expression over time in HDM-induced allergic airway inflammation, and bone marrow chimera experimental setup and chimerism results. Fig S4 shows mLN data from IL-21R deficient mice related to Fig 6. Fig S5 shows total IL-10 expression levels after blockade and double blockade experiments related to Figure 7.

Author Contributions:

Conceptualization, K.H., A.R. and A.C.P; Methodology, S.K., A.R., K.H., and A.C.P; Investigation and Validation, K.H.,A.H., S.K., and A.C.P; Formal Analysis, K.H., A.H., S.K., and A.C.P; Resources, M.M.X., A.L.D., and A.R.; Writing - Original Draft, K.H., and A.C.P; Writing – Review & Editing; K.H., A.R. and A.C.P.; Supervision, A.R. and A.C.P., Funding Acquisition, A.C.P.

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Abbreviations:

BAL: bronchiolar lavage fluid

BM: bone marrow

HDM: house dust mite

T_{FH}: T follicular helper

YFP: yellow fluorescent protein

Figure Legends:

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

Analysis of lung tissue isolated from control (Blimp-1$^{f/f}$ CD4Cre^{-}) or Blimp-1^{CD4Cre} (Blimp-1$^{f/f}$ CD4Cre^{+}) animals i.n. immunized with HDM. A) PAS staining B) Percent and absolute number of infiltrating inflammatory cells in the BAL. C,E) Flow analysis and D,F) Percent of indicated subsets of T cells isolated from lungs gated on Live, CD4^{+} TCRβ^{+}. Data are pooled from 2-3 experiments with 8-13 total mice per group, mean $\pm$ SD. Kruskal-Wallis One-way ANOVA(D) or Mann-Whitney t-test (B,F) *p<0.05 **p<0.01 ***p<0.001 ****p<0.0001

Figure 2: IgE responses are Blimp-1 independent

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

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

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

827 Blimp YFP+ in each subset in unimmunized naïve animals C) Flow analysis and D)
828 Percentages of T cell subsets, IL-10 and Bcl6 expression isolated from lungs of mixed
829 BM chimeras gated on Live $CD4^+$ $TCR\beta^+$ (Tregs) and $FoxP3^-$ (Th2) and Thy1.1
830 (Control); or Thy1.2 (Blimp-1^{CD4Cre}) after HDM. E) Eosinophil percentages in BAL of
831 Control BM chimeras (Control only or Blimp-1^{CD4Cre} only) or mixed BM chimeras. Data
832 are pooled from 2 experiments with 14 mixed BM chimeras and 1-3 control only BM
833 chimera mice per group, mean ± SD. Wilcoxon matched-pairs signed rank test (D)
834 Mann-Whitney t-test (E). *p<0.05 **p<0.01 ***p<0.001 ****p<0.0001
835
836 <u>Figure 4: STAT3 is required for Blimp-1 expression in T cells</u>
837 Analysis of lung tissue isolated from control ($STAT3^{f/f}$ $CD4Cre^-$) or $STAT3^{CD4Cre}$
838 ($STAT3^{f/f}$ $CD4Cre^+$) animals i.n. immunized with HDM. A) PAS staining B) Percent of
839 eosinophils in the BAL. C) Percent of T cell subsets in lung gated on Live, $CD4^+TCR\beta^+$.
840 D) Percent of Blimp-1^+ effector T cells ($CD4^+TCR\beta^+FoxP3^-CD44^+$) in mLN and
841 representative histogram. Data are pooled from 2 experiments with 8 mice total per
842 group, mean ± SD. E) Percent of Th1 ($IFN\gamma^+$, T-bet^+) and Th2 (IL-13^+ $GATA3^+$) cells
843 gated on $CD4^+TCR\beta^+FoxP3^-$, and F) Blimp-1 YFP+ cells within Th1 and Th2 cells in the
844 lung after HDM. Data are pooled from 2 experiments with 6-8 total mice per group,
845 mean ± SD. Mann-Whitney t-test. *p<0.05 **p<0.01 ***p<0.001 ****p<0.0001
846
847 <u>Figure 5: IL-6 signaling in T cells is not required for Th2 cells in the lung.</u>
848 Analysis of lung tissue isolated from control (IL-$6R\alpha a^{f/f}$ $CD4Cre^-$) or $IL6R\alpha^{CD4Cre}$ (IL-
849 $6R\alpha^{f/f}$ $CD4Cre^+$) animals i.n. immunized with HDM. A) Percent of indicated subsets of T

850 cells isolated from lungs gated on Live, $CD4^+$ $TCR\beta^+$ $FoxP3^-$ (non-Treg) or $FoxP3^+$

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

852 are pooled from 2 experiments with 10 total mice per group, mean ± SD. Mann-Whitney

853 t-test. *p<0.05 **p<0.01 ***p<0.001 ****p<0.0001 C) qPCR of indicated cytokines in

854 lungs and mLN of unimmunized or HDM-immunized animals.

855

856 <u>Figure 6: IL-21 is not required for Blimp-1 mediated Th2 cell differentiation</u>

857 Analysis of lungs isolated from IL-21R-intact or IL-21R-deficient animals (A-C), or

858 control (IgG) or anti-IL-21R treated animals (D-E) i.n. immunized with HDM. A) Percent

859 of eosinophils in the BAL. B) Percent of Th1 ($IFN\gamma^+$, T-bet^+) and Th2 (IL-13^+ $GATA3^+$)

860 cells in lung gated on Live, $CD4^+TCR\beta^+FoxP3^-$. C) Expression of Blimp-1 and Bcl6 in

861 lungs in T cells gated on Live, $CD4^+TCR\beta^+FoxP3^-$. Data are pooled from 3 experiments

862 with 9-10 mice total per group, mean ± SD, Mann-Whitney t-test. D) Percent of Th1

863 ($IFN\gamma^+$, T-bet^+) and Th2 (IL-13^+ $GATA3^+$) cells in lung gated on Live, $CD4^+TCR\beta^+FoxP3^-$

864 and E) Blimp-1 YFP^+ cells within Th1 and Th2 cells in the lung after HDM. Data are

865 pooled from 2 experiments with 6-7 mice total per group, mean ± SD, Mann-Whitney t-

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

867

868 <u>Figure 7: IL-10 is required for Blimp-1 expression in Th2 cells</u>

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

870 with HDM. A) Percent of eosinophils in the BAL. B) Flow analysis of Th1 and Th2 cells

871 in lung. C) Percent of Th1 and Th2 cells in lungs gated on Live, $CD4^+TCR\beta^+FoxP3^-$. D)

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

873 Flow analysis and G) Percent of IL-10$^+$ and Blimp-1 YFP$^+$ expression in Th1 and Th2
874 cells in lungs. Data are pooled from 3 experiments with 11-12 mice total per group,
875 mean± SD. Mann-Whitney t-test. *p<0.05 **p<0.01 ***p<0.001 ****p<0.0001
876
877 Figure 8: Transcription factor expression in effector T cells in the absence of Blimp-1.
878 A) Percent of effector cells expressing the transcription factors shown isolated from lung
879 after HDM. Gated on Live, CD4$^+$ TCRβ$^+$ FoxP3$^-$ CD44$^+$. B) Histograms of data from A
880 comparing naïve, Control effector (CD44$^+$) or Blimp-1^{CD4Cre} effector (CD44$^+$) T cells.
881 Data are pooled from 2-3 experiments with 8-10 total mice per group. Mann Whitney t-
882 test. *p<0.05 **p<0.01 ***p<0.001 ****p<0.0001
883
884 Figure 9: Blimp-1 supports Th2 development through suppression of Bcl6
885 Analysis of lungs isolated from Control, Bcl6^{CD4Cre}(Bcl6$^{f/f}$ CD4Cre$^+$), Blimp-1^{CD4Cre}(Blimp-
886 1$^{f/f}$ CD4Cre$^+$), or doubleKO^{CD4Cre}(Bcl6$^{f/f}$ Blimp-1$^{f/f}$ CD4Cre$^+$) animals i.n. immunized with
887 HDM. A) PAS staining B) Percent of eosinophils in the BAL C) Percent of Th1(IFNγ$^+$, T-
888 bet$^+$), Th2 (IL-13$^+$ GATA3$^+$) and Treg (FoxP3$^+$) cells isolated from lungs gated on Live,
889 CD4$^+$TCRβ$^+$ (Treg), and FoxP3$^-$ (non-Treg). D) Total IgE in serum. E) Percent of IL-10$^+$
890 cells in effector (CD4$^+$TCRβ$^+$FoxP3$^-$, left) or Treg (CD4$^+$TCRβ$^+$FoxP3$^+$, right) cells in
891 lung. Data are pooled from 3 experiments with 7-17 total mice per group, mean± SD.
892 Kruskal-Wallis One-way ANOVA. *p<0.05 **p<0.01 ***p<0.001 ****p<0.0001
893
894 Figure S1: *In vitro* T cell differentiation, and T cell subsets in mLN after HDM

895 A) Schematic for HDM-induced allergic lung inflammation model used throughout this
896 study. B) Flow staining of Th1 (IFNγ^+, T-bet$^+$) and Th2 (IL-13$^+$ GATA3$^+$) in vitro
897 polarization of T cells isolated from Control or Blimp-1^{CD4Cre} animals. C) Percent of
898 indicated subsets of T cells isolated from mLN gated on Live, CD4$^+$ TCRβ^+. Data are
899 pooled from 2-3 experiments with 8-13 total mice per group, mean ± SD. Mann-Whitney
900 t-test *p<0.05 **p<0.01 ***p<0.001 ****p<0.0001
901
902 Figure S2: Bcl6 expression in T_{FH} and Th2 cells in the mLN.
903 A) Expression of Bcl6 in indicated populations. Geometric Mean shown in top right
904 corner of each track. B) Geometric Mean of Bcl6 in indicated samples. Wilcoxon
905 matched-pairs signed rank test. *p<0.05 **p<0.01 ***p<0.001 ****p<0.0001
906
907 Figure S3: Blimp-1 YFP expression over time and BM chimera reconstitution.
908 A) Absolute number of CD4 T cell subsets during the course of HDM-induced allergic
909 lung inflammation model. B) Absolute number of Blimp-1 YFP$^+$ cells of each CD4 T cell
910 subset shown in A. C) Flow analysis and Fold change of Blimp-1 YFP$^+$ FoxP3$^+$ Treg
911 cells in the lung of naïve unimmunized animals and after HDM. Fold change calculated
912 as percent of Blimp-1 YFP$^+$ Tregs after HDM over average of Blimp-1 YFP$^+$ Treg cells in
913 unimmunized mice. D) Schematic of mixed bone marrow chimera generation E)
914 Reconstitution 4-6 weeks post irradiation and injection of donor marrow isolated from
915 blood (4 weeks) or lungs post HDM-induced lung inflammation. Flow cytometry and
916 percent of Thy1.1$^+$ (Control) and Thy1.2$^+$ (Blimp-1^{CD4Cre}) in both tissues shown.
917 Wilcoxon matched-pairs signed rank test. *p<0.05 **p<0.01 ***p<0.001 ****p<0.0001

918

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

920 A) Percent of Th1 (IFNγ^+, T-bet$^+$) and Th2 (IL-13$^+$ GATA3$^+$) cells in mLN gated on Live,

921 CD4$^+$TCRβ^+FoxP3$^-$. B) Expression of Blimp-1 and Bcl6 in mLN in T cells gated on Live,

922 CD4$^+$TCRβ^+FoxP3$^-$. Data are pooled from 3 experiments with 9-10 mice total per group,

923 mean $\pm$ SD, Mann-Whitney t-test. *p<0.05 **p<0.01 ***p<0.001 ****p<0.0001

924

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

926 A) Percent of IL-10 expressing cells isolated from the lung gated on Live, CD4$^+$ TCRβ^+

927 FoxP3$^-$ CD44$^+$. B) Percent of Th1 (IFNγ^+, T-bet$^+$) and Th2 (IL-13$^+$ GATA3$^+$) cells in the

928 lung isolated from Blimp-1 YFP animals treated as Control (IgG) or treated with anti-IL-

929 10R, anti-IL21R, or both anti-IL-10R and anti-IL21R for the duration of the HDM-induced

930 allergic lung inflammation model. C) Percent of Blimp-1 YFP$^+$ Th1 and Th2 cells in B.

931 Data are pooled from 2-3 experiments with 6-10 total mice per group. Kruskal-Wallis

932 One-way ANOVA. *p<0.05 **p<0.01 ***p<0.001 ****p<0.0001

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

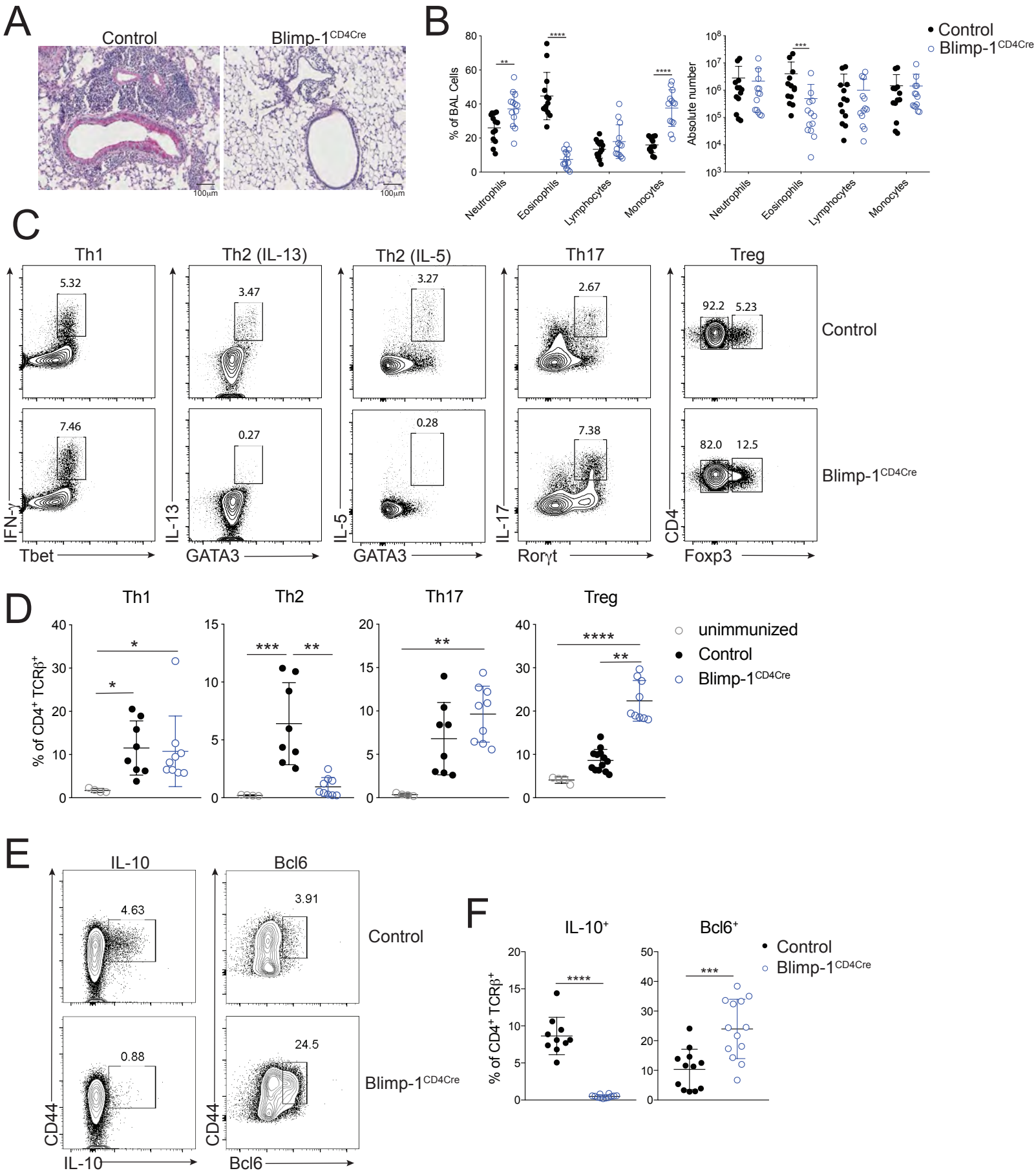

Figure 2: IgE responses are Blimp-1 independent

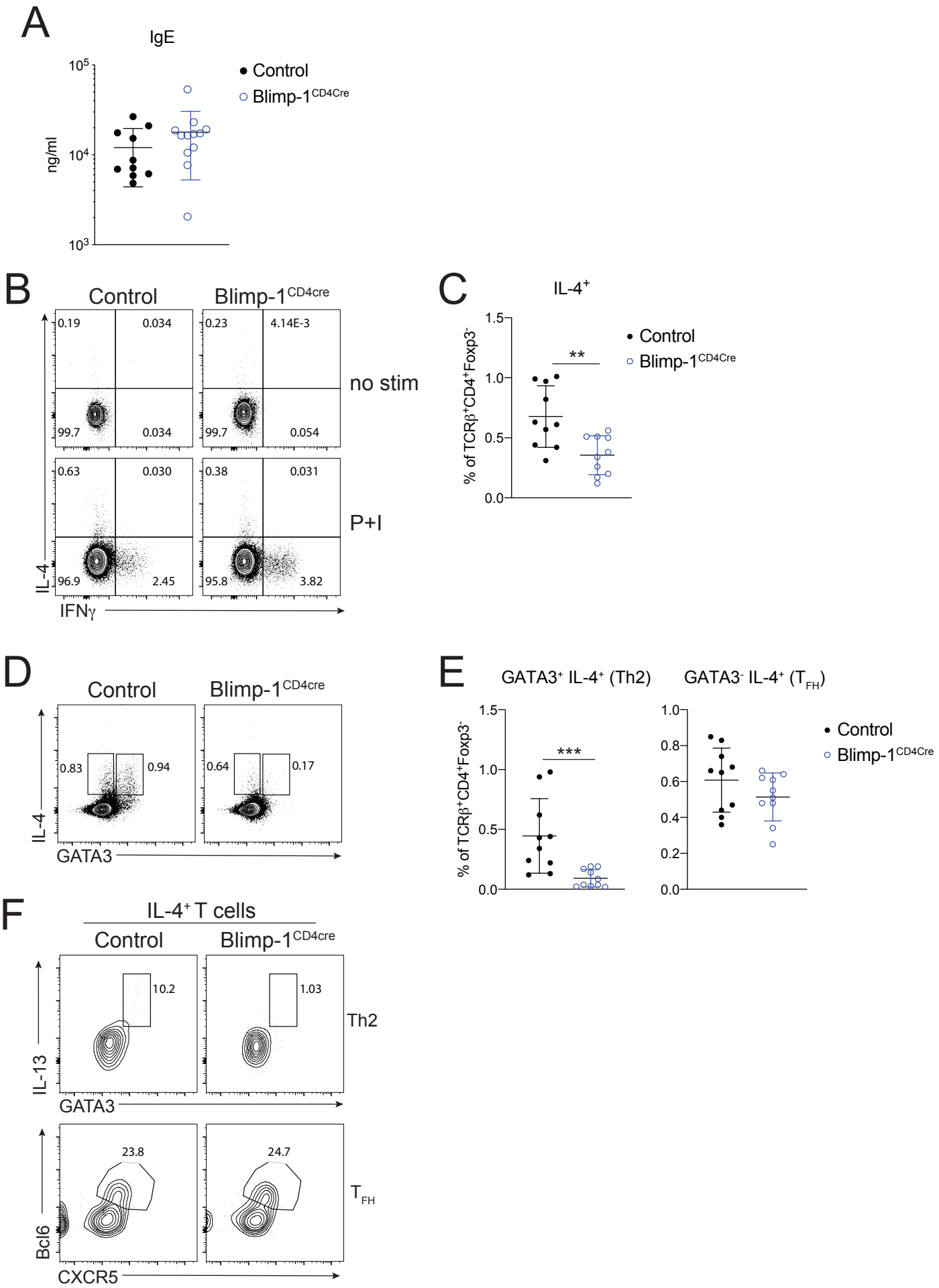

Figure 3: Blimp-1 is intrinsically required in T cells to promote Th2 cells

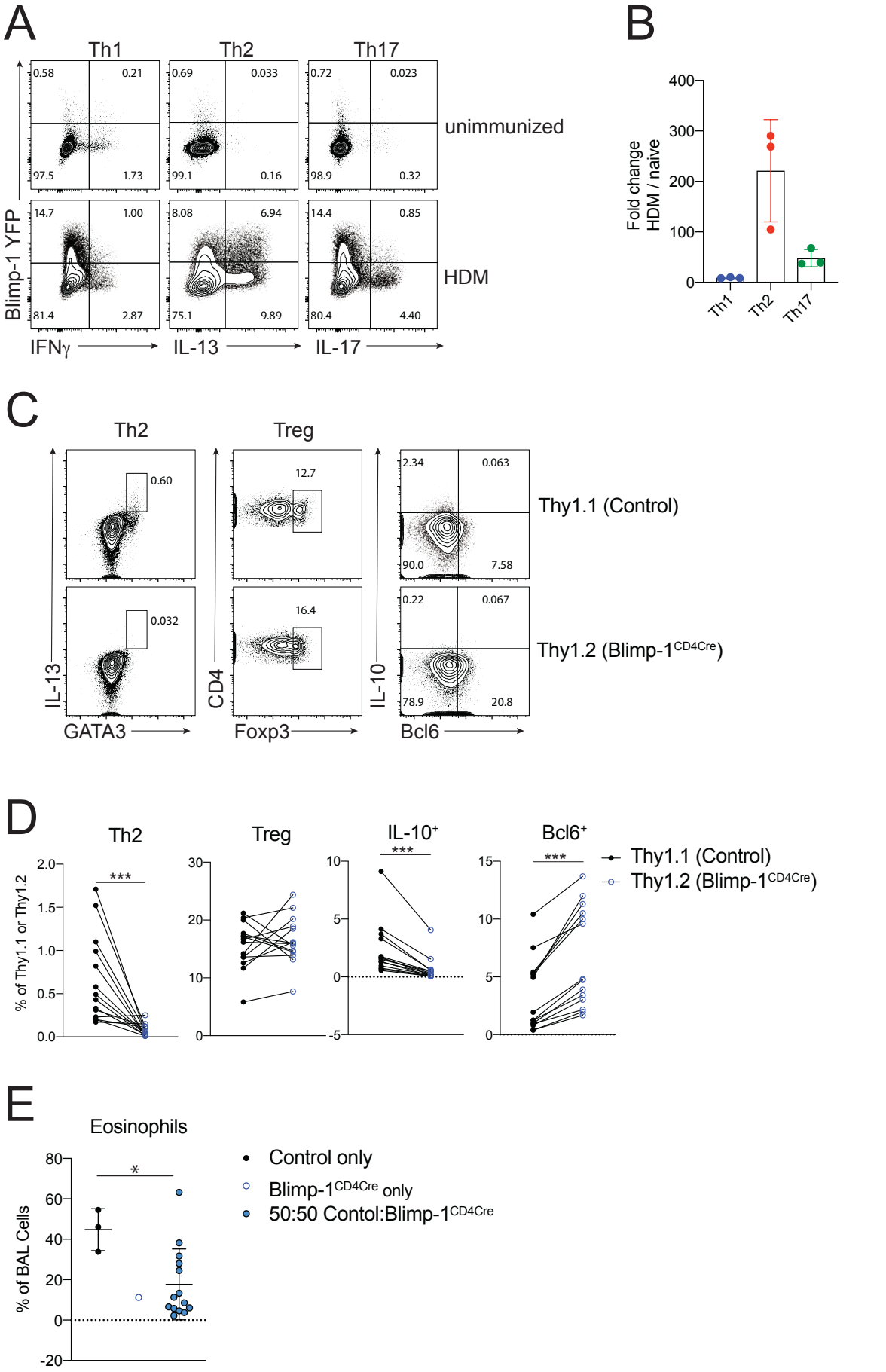

Figure 4: STAT3 is required for Blimp-1 expression in T cells

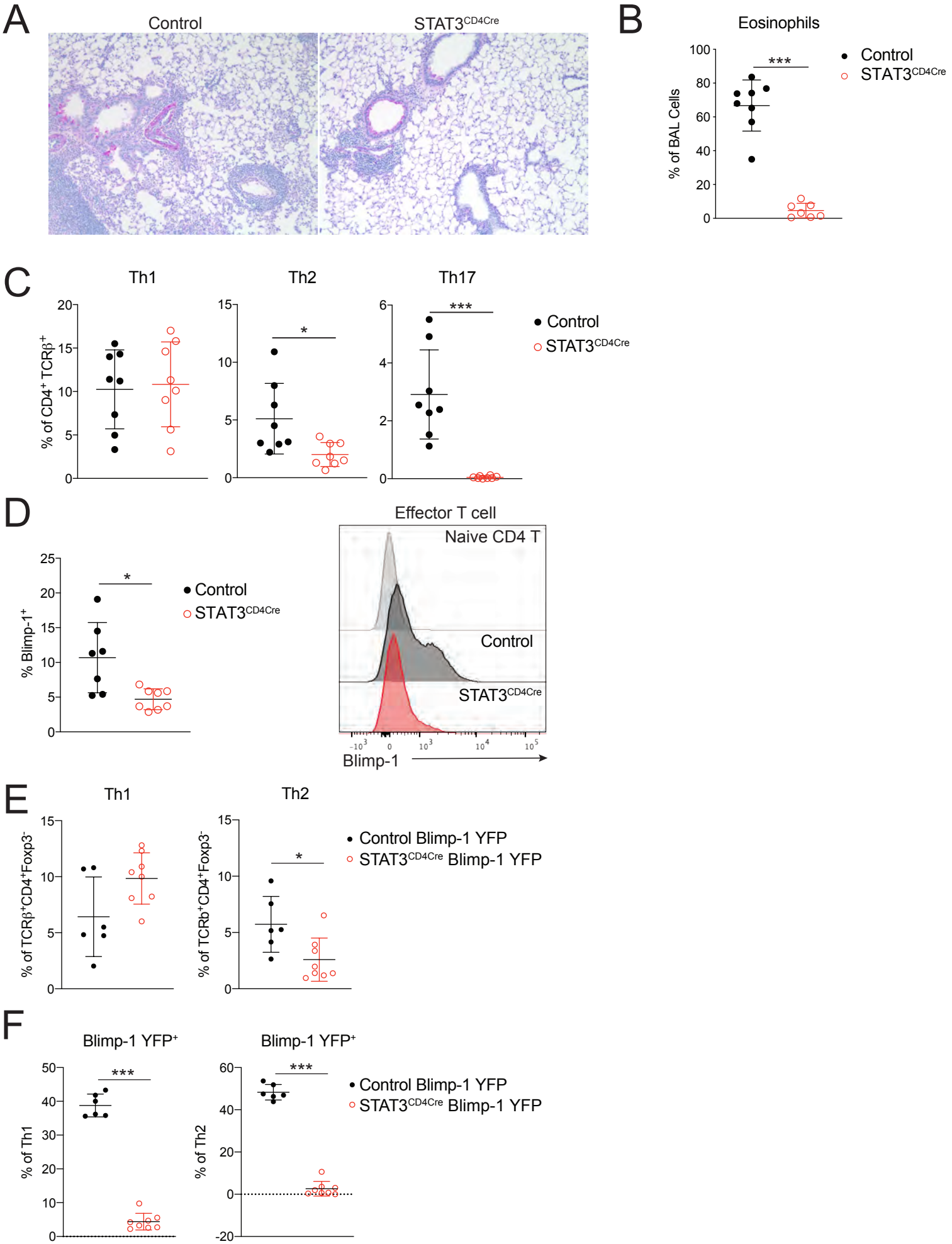

Figure 5: IL-6 signaling in T cells is not required for Th2 cells in the lung.

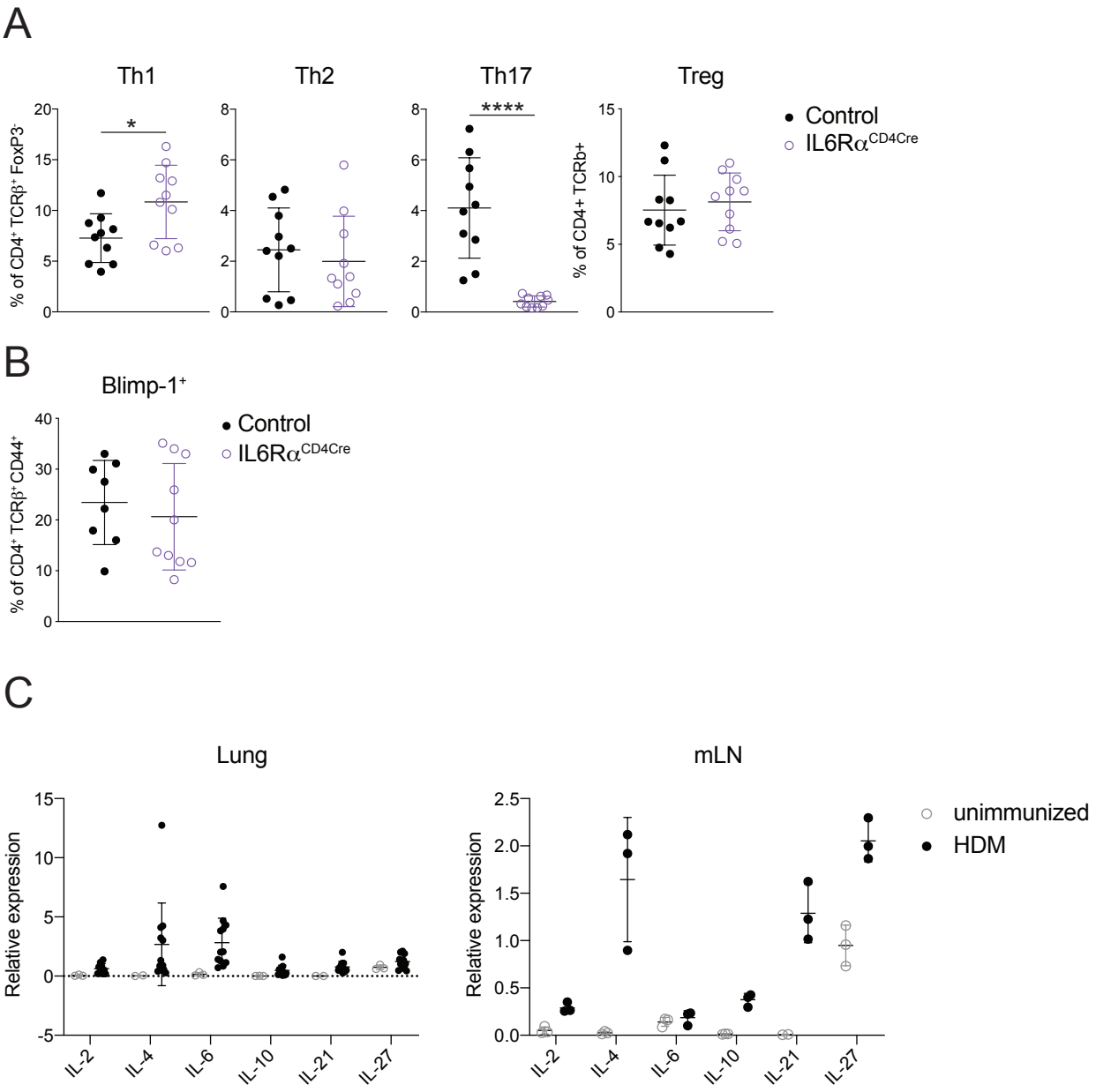

Figure 6: IL-21 is not required for expression of Blimp-1 in Th2 cells

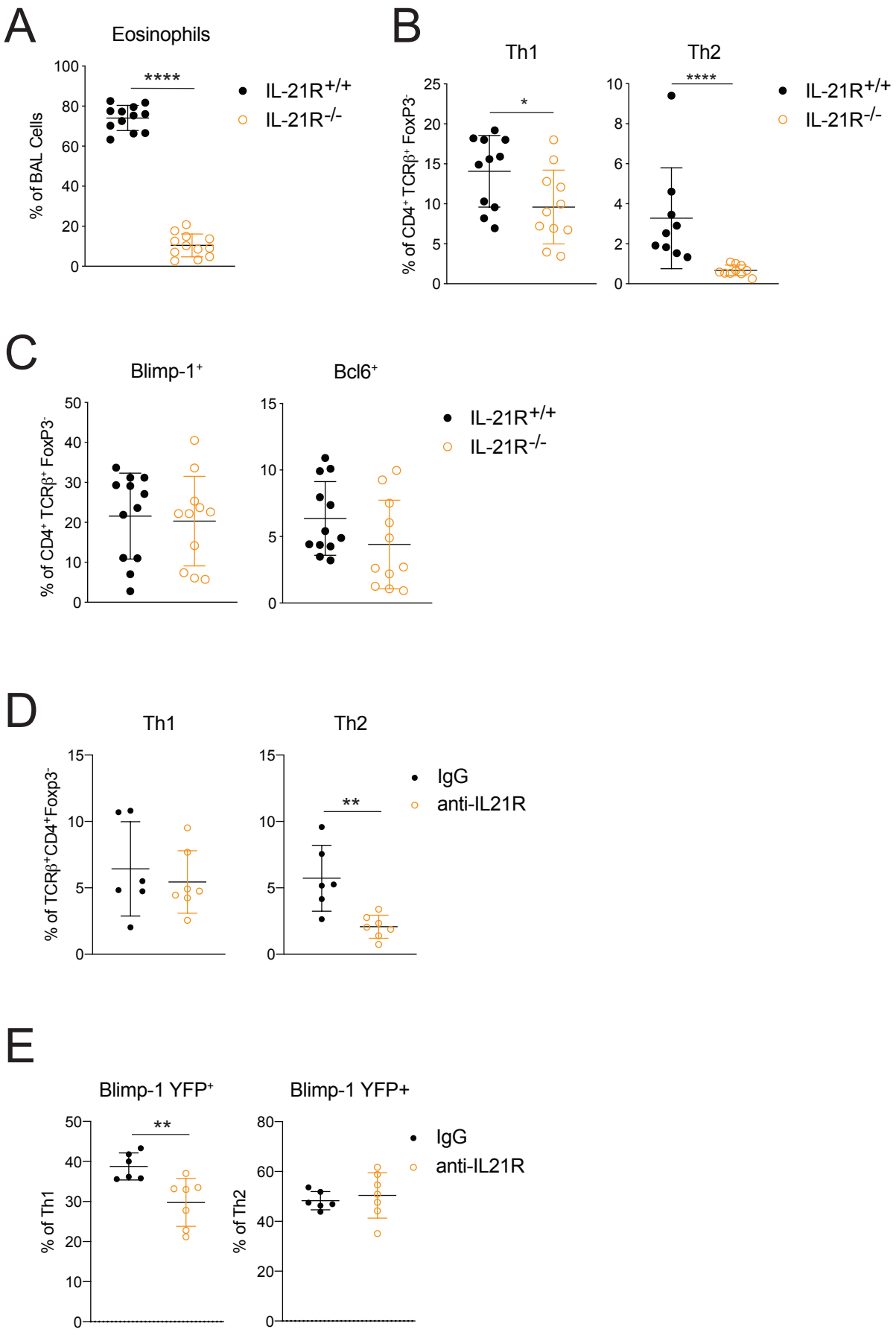

Figure 7: IL-10 is required for Blimp-1 expression in Th2 cells

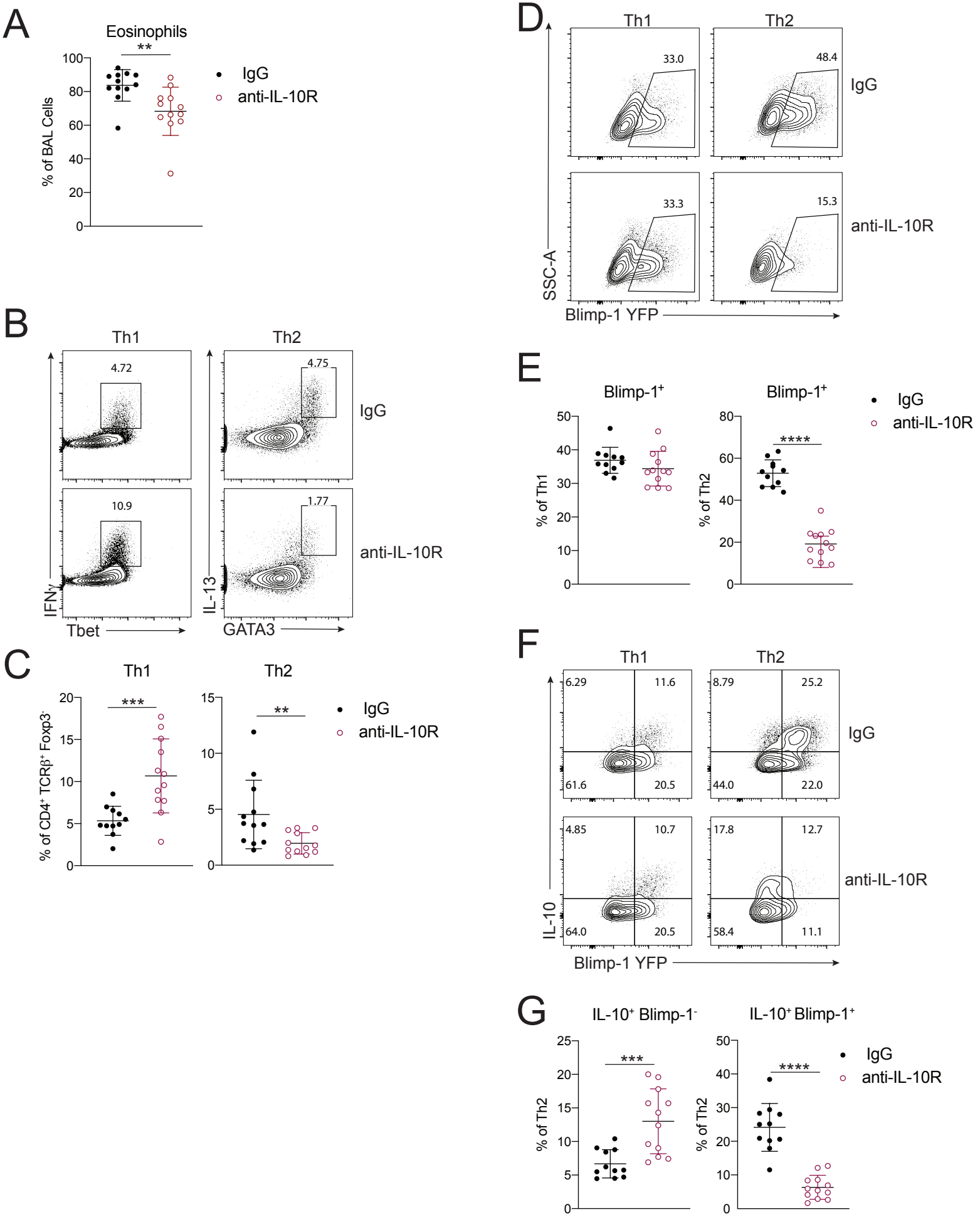

Figure 8:Transcription factor expression in effector T cells in the absence of Blimp-1

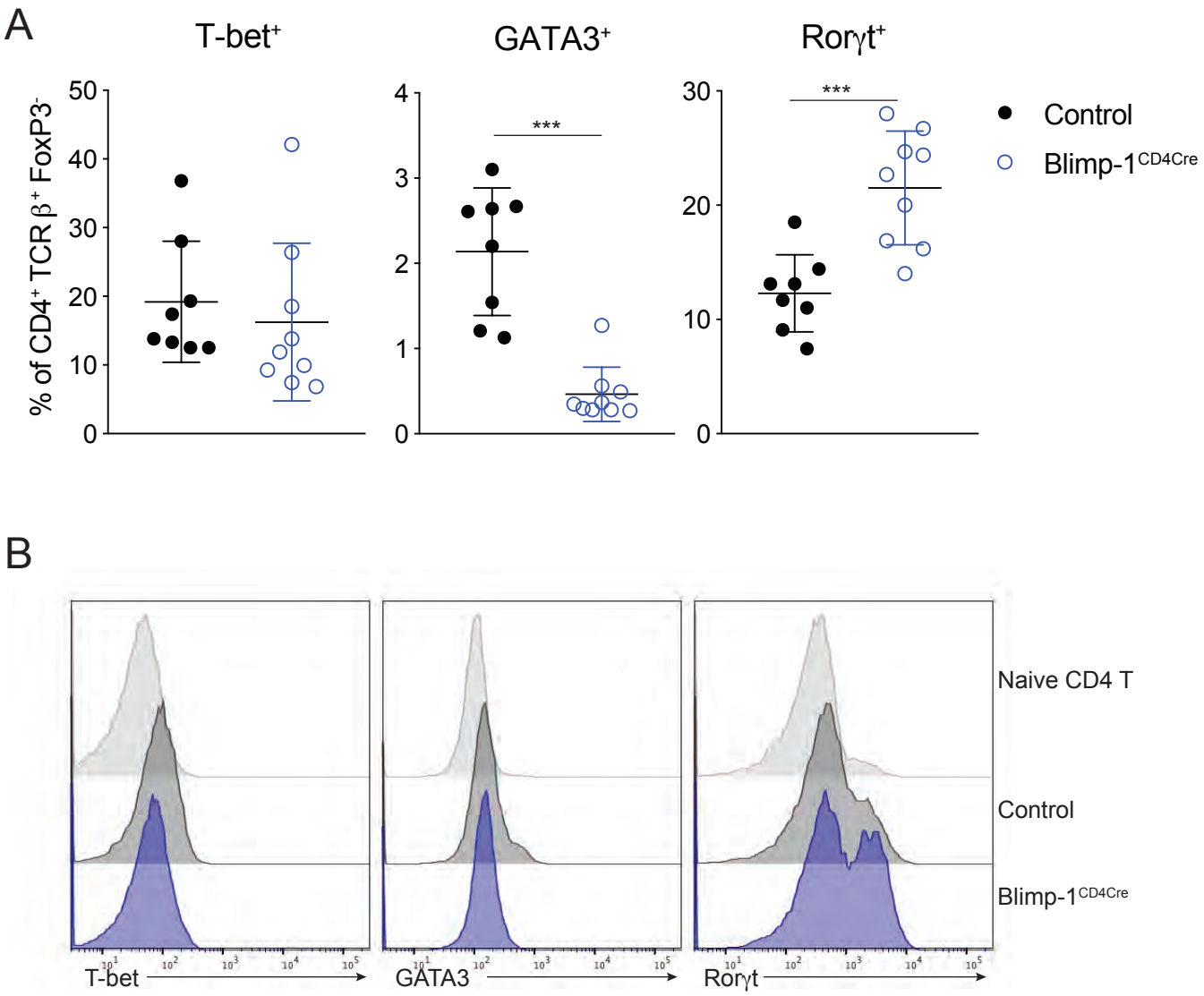

Figure 9: Blimp-1 supports Th2 development through suppression of Bcl6

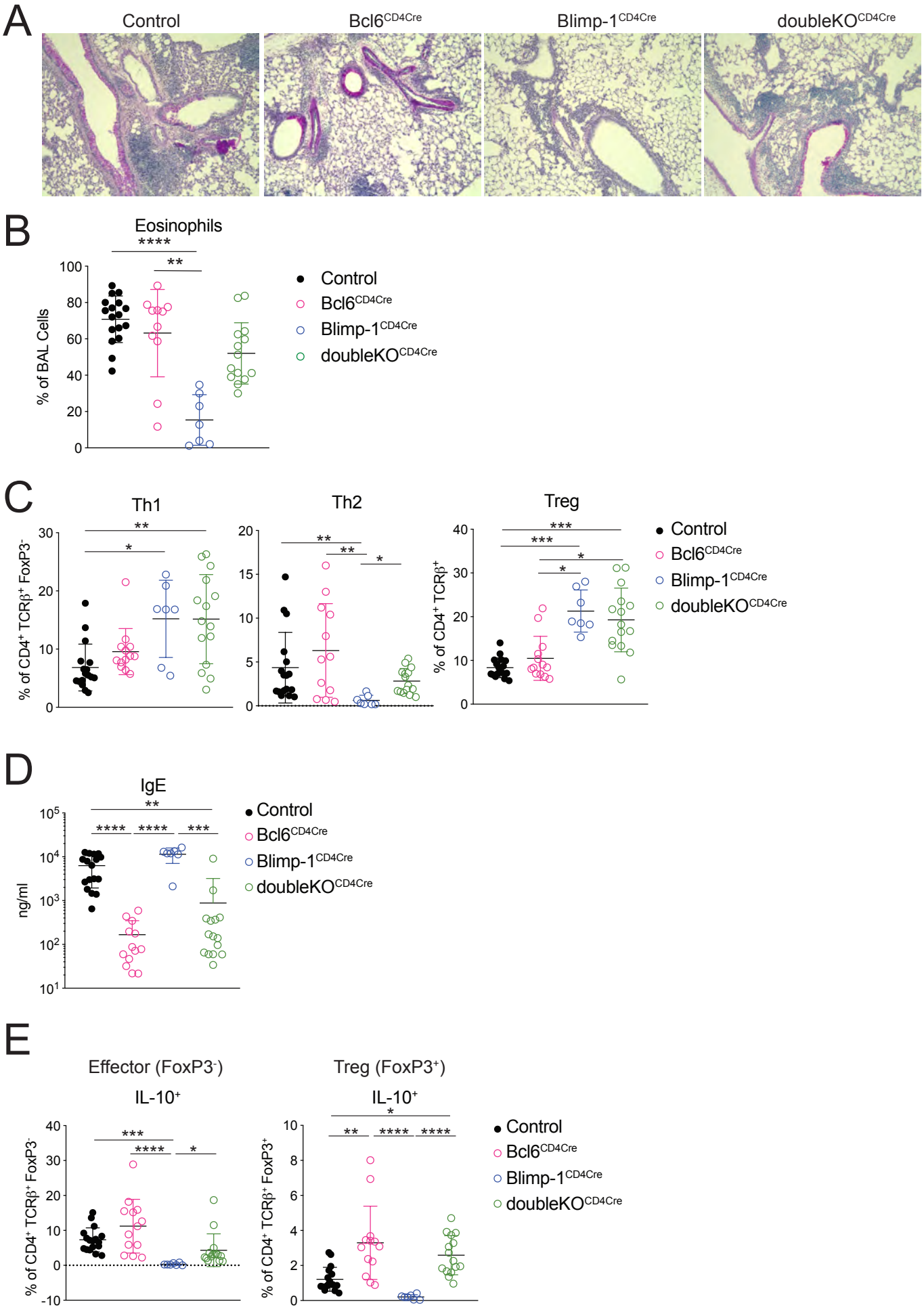

Figure S1: In vitro T cell differentiation and T cell subsets in mLN after HDM.

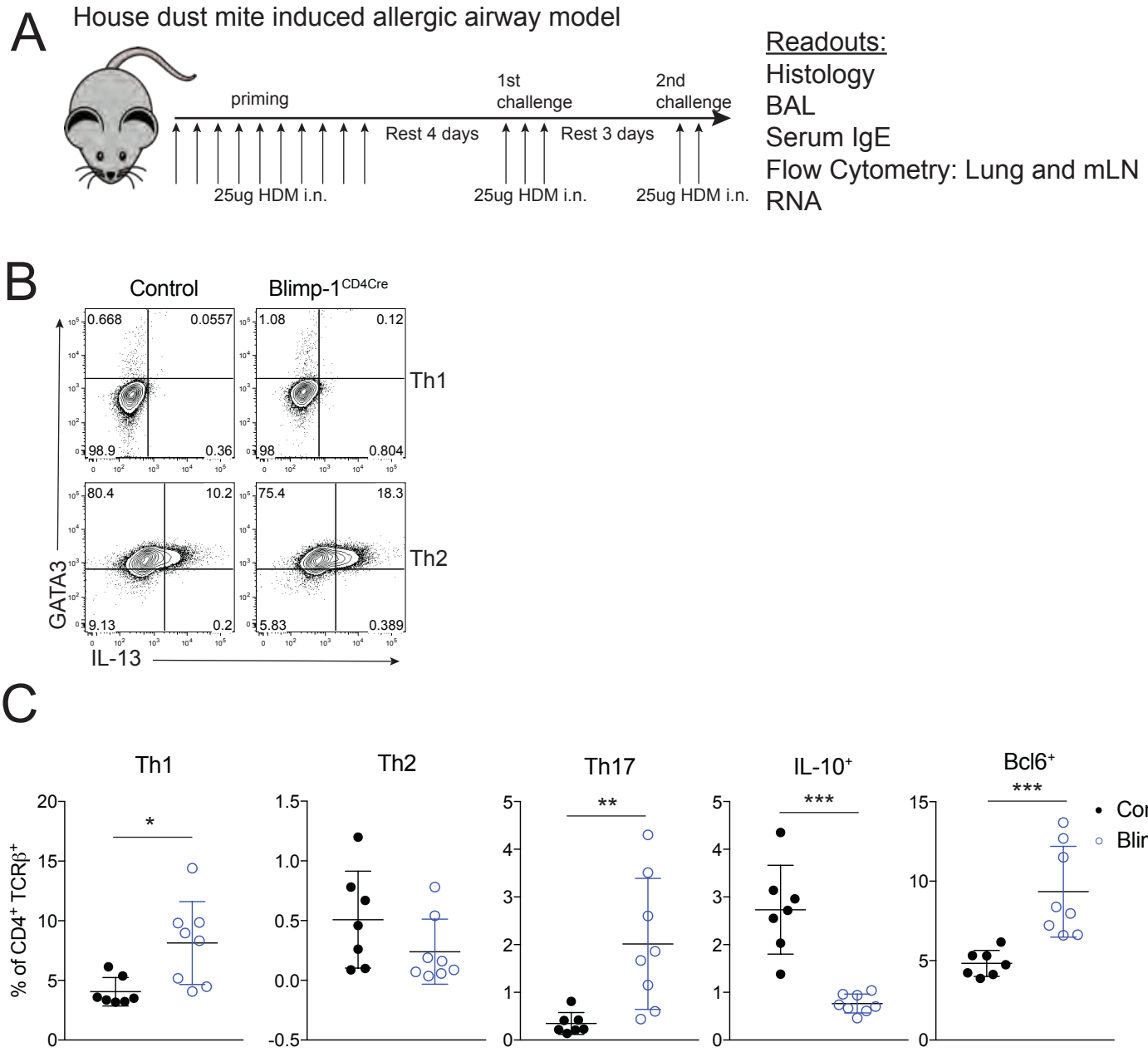

Figure S2: Related to Figure 2. Bcl6 expression in T_{FH} and Th2 cells in the mLN.

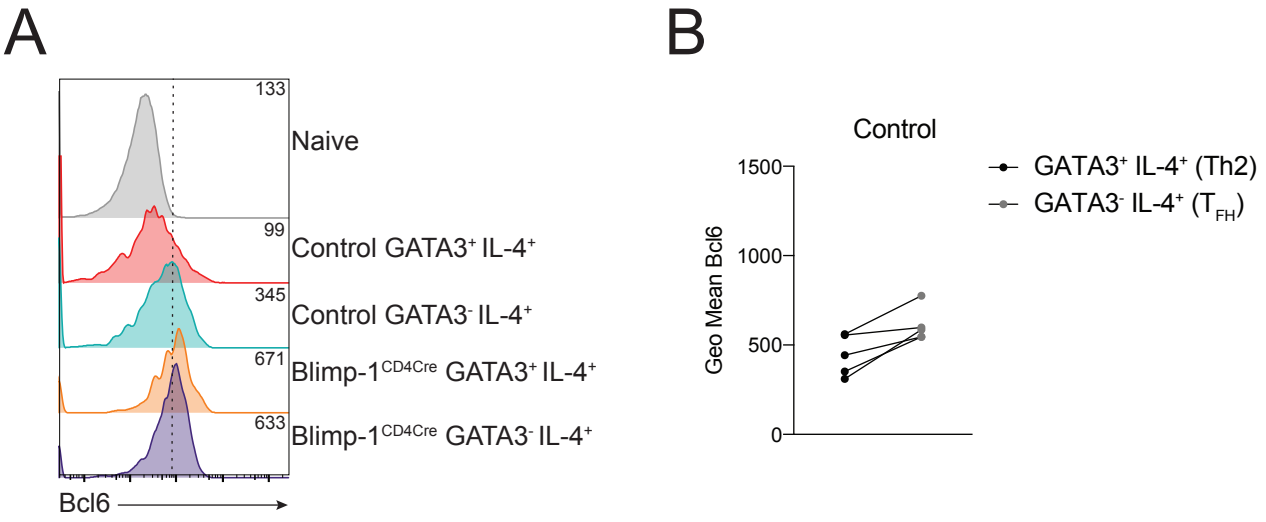

Figure S3, Related to Figure 3. Blimp-1 YFP expression over time and BM chimera recon-

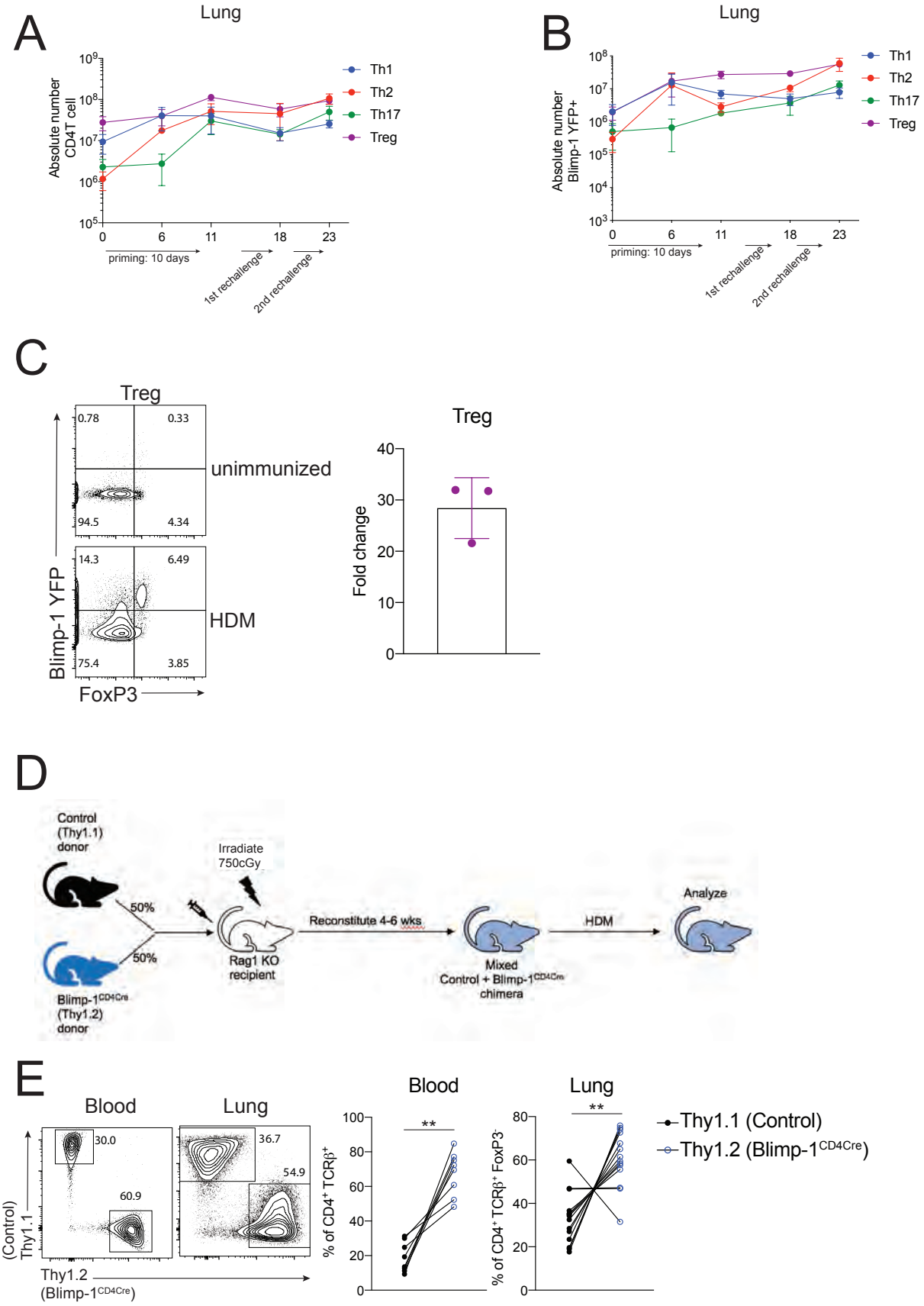

Figure S4: IL-21R signaling is required for T cell responses but not Blimp-1 in mLN

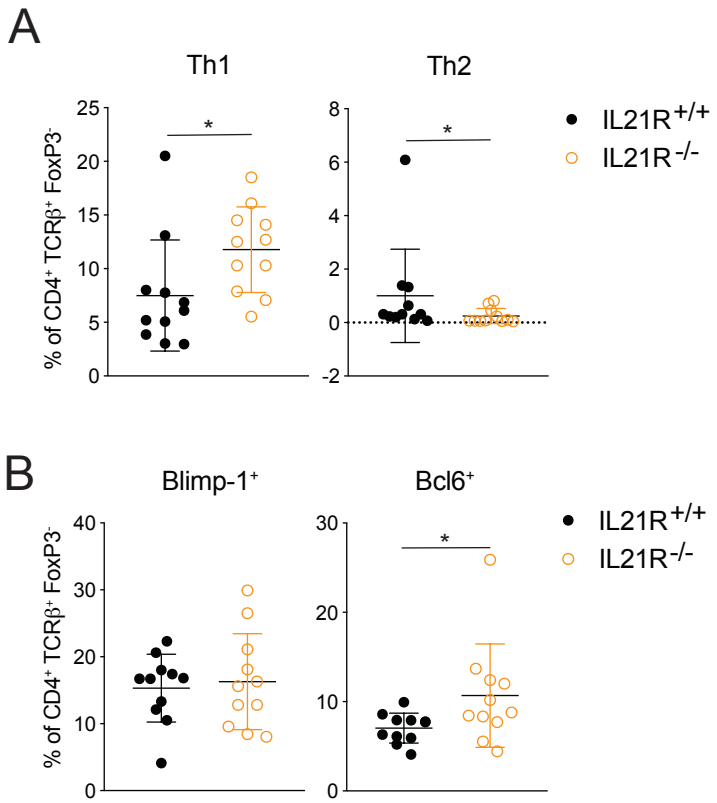
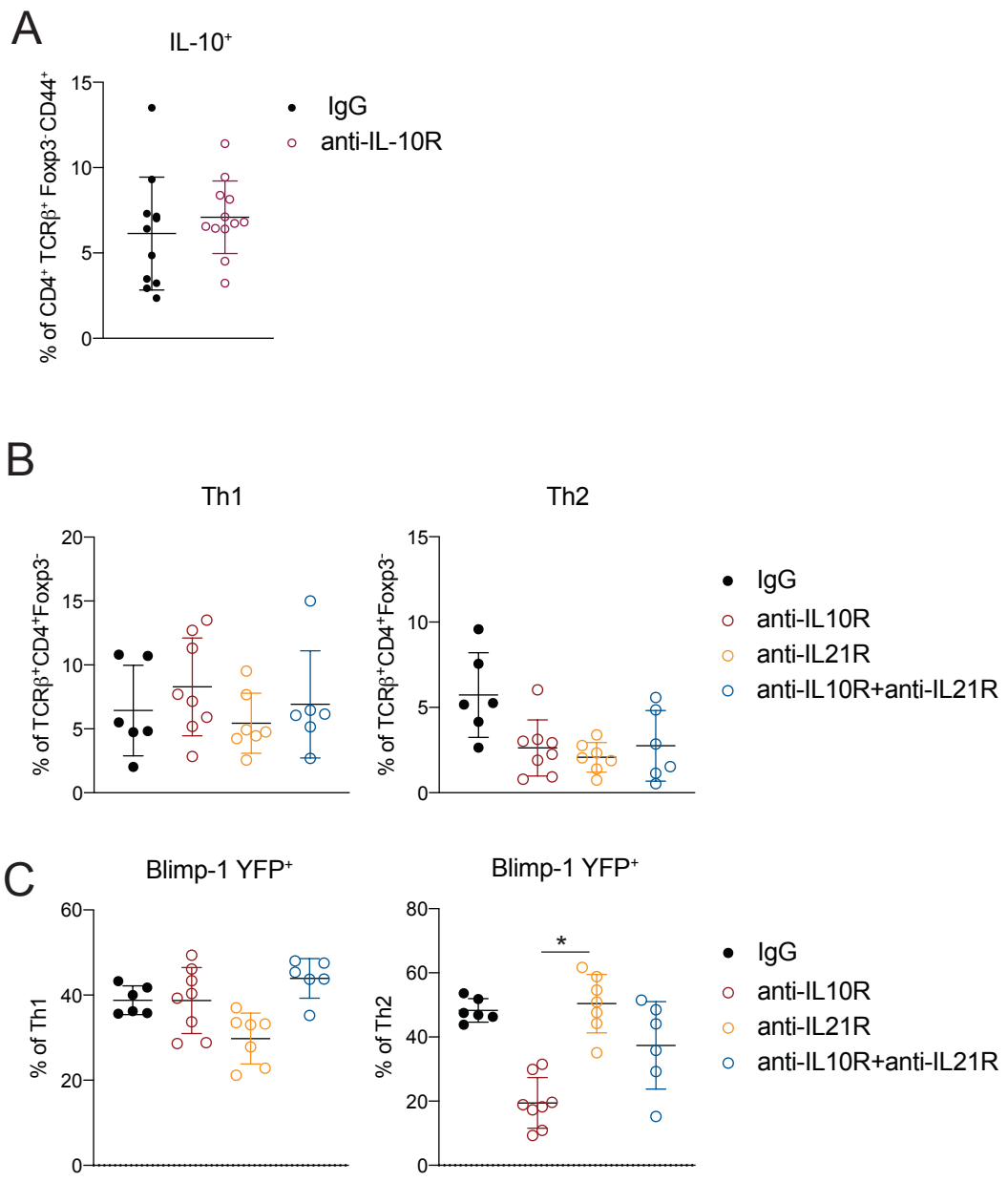
Figure S5: IL-10 and IL-21 do not cooporatively regulate Blimp-1 expression.