

# 1 **The neonatal microenvironment programs conventional and intestinal** 2 **Tbet<sup>+</sup> $\gamma\delta$ T17 cells through the transcription factor STAT5**

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14

## 15 **Summary**

16 Interleukin(IL)-17-producing ROR $\gamma$ t<sup>+</sup>  $\gamma\delta$  T ( $\gamma\delta$ T17) cells develop in the embryonic thymus and  
17 participate in type 3 immune responses. Herein we show that  $\gamma\delta$ T17 cells rapidly proliferate within  
18 neonatal lymph nodes and gut, where upon entry they uniquely upregulate Tbet and co-express IL-  
19 17, IL-22 and interferon(IFN)  $\gamma$  in a STAT3 and retinoic acid dependent manner. Neonatal  
20 expansion was halted in mice conditionally deficient in STAT5 and its loss resulted in  $\gamma\delta$ T17 cell  
21 depletion from all adult organs. Hyperactive STAT5 mutant mice showed that the STAT5A  
22 homologue had a dominant role over STAT5B in promoting  $\gamma\delta$ T17 cell expansion and  
23 downregulating gut-associated Tbet. In contrast, STAT5B preferentially expanded IFN $\gamma$ -producing  
24  $\gamma\delta$  populations. Importantly, mice lacking  $\gamma\delta$ T17 cells due to STAT5 deficiency displayed a  
25 profound resistance to experimental autoimmune encephalomyelitis. Our data identify for the first  
26 time STAT5 as a key molecular checkpoint allowing  $\gamma\delta$ T17 cells to pass through a critical neonatal  
27 developmental window to acquire tissue-specific characteristics essential for infection and  
28 autoimmunity.

## 29 Introduction

30 Interleukin(IL)-17 producing gamma delta ( $\gamma\delta$ ) T cells ( $\gamma\delta$ T17) are one of the major type 3 innate  
31 lymphocytes in the mouse, occupying the skin and most mucosal surfaces as well as secondary  
32 lymphoid organs. Their ability to constitutively produce IL-17 and to respond rapidly to cytokines  
33 like IL-7, IL-23 and IL-1 $\beta$  renders them as a critical part of innate immunity to infections (Cho et al.,  
34 2010; Conti et al., 2014) but also makes them highly pathogenic in a number of inflammatory  
35 models (Bekiaris et al., 2013; Michel et al., 2012; Sutton et al., 2009). Thus, both experimental  
36 autoimmune encephalomyelitis (EAE) and imiquimod (IMQ)-induced psoriasis require the  
37 presence of functional  $\gamma\delta$ T17 cells (Bekiaris et al., 2013; Sutton et al., 2009). Similarly, tumor  
38 models have shown that  $\gamma\delta$ T17 cells can have either protective (Wu et al., 2014) or pathogenic  
39 (Coffelt et al., 2015) roles depending on the nature of the cancer. In humans, although a unique  
40 innate  $\gamma\delta$ T17 cell population has not been yet characterized, many groups have identified IL-17-  
41 producing  $\gamma\delta$  T cells in association with various disease states (Cai et al., 2011; Wu et al., 2014).

42 Genetic murine studies have shown that  $\gamma\delta$ T17 cells develop in the embryonic thymus in a  
43 step-wise fashion, initially involving escape of epithelial selection (Turchinovich and Hayday,  
44 2011), followed by the upregulation of a number of transcription factors, such as ROR $\gamma$ t, SOX13  
45 and cMAF, that regulate lineage commitment, specification and functional maturation (Malhotra et  
46 al., 2013; Zuberbuehler et al., 2019). Although T cell receptor (TCR) signaling is necessary for  
47  $\gamma\delta$ T17 cell development (Wencker et al., 2014), experimental evidence based on hypomorphic  
48 CD3 mice and anti-CD3/TCR antibody administration suggested that only weak TCR signals are  
49 required (Munoz-Ruiz et al., 2016; Sumaria et al., 2017). In addition, a recent study showed that  
50 TCR signaling is not important for lineage specification but for transition into the early immature  
51 stage (Spidale et al., 2018).  $\gamma\delta$ T17 cell generation is restricted to the embryonic and neonatal  
52 thymus (Haas et al., 2012) with the bone marrow displaying low capacity to produce these cells  
53 (Cai et al., 2014).

54 STAT transcription factors act downstream of cytokine and growth factor receptors to regulate  
55 a plethora of key biological processes, including lymphocyte development and function (Stark and  
56 Darnell, 2012). STAT5 is encoded by two genes, *Stat5a* and *Stat5b*, giving rise to two highly

57 homologous proteins with largely overlapping functions in mediating transcription of target genes,  
58 although STAT5B has a more dominant role in lymphoid cells as well as in cancer progression (de  
59 Araujo ED, 2019; Villarino et al., 2016). Mice deficient in both STAT5A and STAT5B have  
60 increased perinatal mortality and lack or display a severe reduction in many lymphocytic  
61 populations, such as  $\alpha\beta$  T,  $\gamma\delta$  T, regulatory CD4<sup>+</sup> T cells (Treg), natural killer (NK) T, NK and also  
62 B cells (Hoelbl et al., 2006; Yao et al., 2006; Yao et al., 2007). Although mice deficient in either  
63 STAT5A or STAT5B show hampered development and function of a few major lymphocyte  
64 subsets, their phenotype is milder than the combined loss of the two isoforms (Imada et al., 1998;  
65 Villarino et al., 2016; Villarino et al., 2017). However, there is currently no mechanistic data  
66 regarding their individual contribution in  $\gamma\delta$  T cell biology. One of the suggested mechanisms for  
67 the dependence of developing  $\gamma\delta$  T cell progenitors on STAT5 is its ability to induce TCR $\gamma$   
68 rearrangements, due to three highly interspecies conserved inverted repeat STAT5 consensus  
69 sites within the *TCR $\gamma$*  locus (Wagatsuma et al., 2015).

70 In humans, *STAT5*-associated loss of function mutations are predominantly restricted to  
71 STAT5B and these culminate in growth failure, due to impaired growth hormone receptor signaling,  
72 as well as immunodeficiency, metabolic dysregulation and autoimmune disorders as a result of  
73 Treg deficiency (Cohen et al., 2006; Hwa, 2016; Kanai et al., 2012). In contrast, *STAT5B* gain of  
74 function (GOF) mutations strongly correlate with mature T cell neoplasms (Pham et al., 2018) and  
75 have also been found in patients with neutrophilia or eosinophilia (Cross et al., 2019; Ma et al.,  
76 2017). In particular, the recurrent N642H GOF missense mutation within the Src homology 2 (SH2)  
77 domain of STAT5B results in enhanced and prolonged tyrosine phosphorylation (pY) in response  
78 to low doses of cytokines or growth factors, and is associated with poorer patient prognosis and  
79 increased risk of relapse (Bandapalli et al., 2014; Pham et al., 2018; Rajala et al., 2013).  
80 Interestingly, *STAT5B* GOF mutations are relatively frequent in aggressive  $\gamma\delta$  T cell lymphoma  
81 subtypes, such as hepatosplenic T cell lymphoma (Nicolae et al., 2014), monomorphic  
82 epitheliotropic intestinal T cell lymphoma (Kucuk et al., 2015; Nairismagi et al., 2016) and primary  
83 cutaneous  $\gamma\delta$  T cell lymphoma (Kucuk et al., 2015). Notably, approximately 20% of identified  
84 N642H mutations occur in  $\gamma\delta$  T cell derived lymphomas (de Araujo ED, 2019).

85 Herein, we show that STAT5 is critically required for the progression and expansion of  $\gamma\delta$ T17  
86 cells through neonatal life in the intestine and periphery. We provide evidence that intestinal  $\gamma\delta$ T17  
87 cells upregulate Tbet upon entry into the lamina propria after birth and co-express the cytokines IL-  
88 17, IL-22 and IFN $\gamma$  in a mechanism dependent on STAT3 and retinoic acid. Furthermore, loss of  
89  $\gamma\delta$ T17 cells due to STAT5 deficiency results in resistance to experimental autoimmune  
90 encephalomyelitis in adult mice. Importantly, we show that STAT5A promotes  $\gamma\delta$ T17 cell  
91 expansion and downregulates intestinal Tbet favoring a type 17 program, whereas STAT5B favors  
92 IFN $\gamma$ -producing  $\gamma\delta$  populations and increases intestinal Tbet expression. Collectively, our data  
93 suggest that neonatal life is a critical window of development and tissue specification for  $\gamma\delta$ T17  
94 cells, and that this process is tightly regulated by STAT5.

95

## 96 **Results**

### 97 **STAT5 regulates the neonatal expansion of $\gamma\delta$ T17 cells**

98 In order to test the importance of STAT5 in ROR $\gamma$ t expressing  $\gamma\delta$  T cells, we crossed ROR $\gamma$ t-Cre  
99 mice (Eberl and Littman, 2004) with mice floxed for both STAT5a and STAT5b (ROR $\gamma$ t<sup>CRE</sup>-  
100 STAT5<sup>F/F</sup>) (Cui et al., 2004) and analyzed the numbers of LN and skin  $\gamma\delta$ T17 cells. We found that  
101 compared to littermate controls (Cre<sup>-</sup>), ROR $\gamma$ t<sup>CRE</sup>-STAT5<sup>F/F</sup> mice (Cre<sup>+</sup>) contained severely reduced  
102 numbers of  $\gamma\delta$ T17 cells defined phenotypically as CD27<sup>-</sup>CD44<sup>+</sup> in the LN and CCR6<sup>+</sup>CD3<sup>+</sup> in the  
103 skin (Fig. 1A-B). This was confirmed by the near complete lack of IL-17-expressing  $\gamma\delta$  T cells in  
104 the LN (Fig. 1C). Deficiency in STAT5 equally affected both V $\gamma$ 4<sup>+</sup> and V $\gamma$ 4<sup>-</sup> subsets of  $\gamma\delta$ T17 cells  
105 (not shown). Interestingly, ROR $\gamma$ t<sup>CRE</sup>-STAT5<sup>F/F</sup> mice had a concomitant increase in IFN $\gamma$ -  
106 expressing  $\gamma\delta$  T cells (Fig. 1C). In ROR $\gamma$ t<sup>CRE</sup>-STAT5<sup>F/F</sup> mice, deletion of STAT5 in CD4<sup>+</sup> and CD8<sup>+</sup>  
107 T cells was not complete (Fig. S1A). Insufficient deletion in the  $\alpha\beta$  T cell compartment using  
108 ROR $\gamma$ t<sup>CRE</sup> deleter mice has also been demonstrated by others (Guo et al., 2014). Consequently,  
109 we did not observe differences in the numbers of TCR $\beta$ <sup>+</sup>CD4<sup>+</sup>CCR6<sup>+</sup> cells, which are enriched for  
110 T-helper-17 (Th17) cells (Fig S1B), or in the frequency of IFN $\gamma$ -producing CD4<sup>+</sup> T cells (Fig S1B).  
111 Surprisingly, and also in agreement with previous observations (Laurence et al., 2007), the  
112 percentage of IL-17A-producing CD4<sup>+</sup> T cells was higher even when STAT5 was only partially

113 deleted (Fig. S1B). Finally, to determine whether the defect we observed in ROR $\gamma$ t<sup>CRE</sup>-STAT5<sup>F/F</sup>  
114 mice was intrinsic to the  $\gamma\delta$ T17 population we generated mixed bone marrow chimeras using  
115 CD45.1<sup>+</sup> wild-type and Cre<sup>+</sup> CD45.2<sup>+</sup> donors and analyzed lymph nodes and skin 12 weeks later.  
116 We found that by comparison to wild-type, ROR $\gamma$ t<sup>CRE</sup>-STAT5<sup>F/F</sup> BM failed to generate  $\gamma\delta$ T17 cells  
117 suggesting that the STAT5-associated defect is cell intrinsic (Fig. S2A). It is noteworthy that in the  
118 skin we could not detect any  $\gamma\delta$ T17 cells originating from ROR $\gamma$ t<sup>CRE</sup>-STAT5<sup>F/F</sup> BM (Fig. S2A).

119 We next investigated whether reduced  $\gamma\delta$ T17 cell numbers in the absence of STAT5 were due  
120 to a developmental defect. We therefore examined newborn thymi from ROR $\gamma$ t<sup>CRE</sup>-STAT5<sup>F/F</sup> and  
121 littermate control mice and found no differences in  $\gamma\delta$ T17 cellularity (Fig. 1D) or IL-17A expression  
122 (Fig. S2B). Expression of both *Stat5a* and *Stat5b* was significantly lower in ROR $\gamma$ t<sup>CRE</sup>-STAT5<sup>F/F</sup>  
123  $\gamma\delta$ T17 cells sorted from new born thymi compared to Cre<sup>-</sup> controls or CD27<sup>+</sup>  $\gamma\delta$  T cells (Fig. S2C).  
124 This suggested that the major impact of STAT5 occurs extrathymically. We thus analyzed neonatal  
125 mice and found a significant decrease in LN  $\gamma\delta$ T17 cell numbers in 7 and 14 day old mice (Fig.  
126 1E). Assessment of proliferation by Ki67 staining showed that  $\gamma\delta$ T17 cells in ROR $\gamma$ t<sup>CRE</sup>-STAT5<sup>F/F</sup>  
127 neonatal mice displayed reduced turnover compared to controls (Fig. 1F). Furthermore, expression  
128 of the anti-apoptotic STAT5 target gene product BCL2 (Tripathi et al., 2010; Yao et al., 2006) was  
129 reduced in neonatal STAT5-deficient  $\gamma\delta$ T17 cells (Fig. S2D), suggesting impaired survival.  
130 Collectively, we demonstrate that STAT5 is important for the turnover and survival of  $\gamma\delta$ T17 cells  
131 during neonatal and adult life.

132

### 133 **Differential regulation of $\gamma\delta$ T17 and CD27<sup>+</sup> $\gamma\delta$ T cells by STAT5A and STAT5B**

134 Since deficiency in STAT5 resulted in near complete loss of  $\gamma\delta$ T17 cells, we next examined the  
135 influence of hyperactive STAT5 expression. We utilized two established models of STAT5  
136 hyperactivity whereby the *Vav1* promoter drives the expression of (a) high or low copies of the  
137 hyperactive S710F STAT5A mutant (Maurer et al., 2019; Onishi et al., 1998), or (b) human wild-  
138 type (WT) or the hyperactive N642H STAT5B mutant (Pham et al., 2018). Constitutively high levels  
139 of hyperactive STAT5A resulted in very high numbers of  $\gamma\delta$ T17 cells in LN, but hyperactivation of  
140 STAT5A had a considerably smaller impact on CD27<sup>+</sup>  $\gamma\delta$  T cells (Fig. 2A-B). In contrast,

141 constitutive expression of hyperactive STAT5B increased the numbers of CD27<sup>+</sup>  $\gamma\delta$  T but had a  
142 smaller effect on  $\gamma\delta$ T17 cells (Fig. 2A-B). When we analyzed cytokine expression we found that  
143 IFN $\gamma$  was only induced by hyperactive STAT5B (Fig. S2E), whereas IL-17A could be induced at  
144 high levels both by hyperactive STAT5A as well as WT STAT5B expression (Fig. 2C). However,  
145 hyperactive STAT5B did not induce IL-17A expression (Fig. 2C).

146 Similar to the LN, skin  $\gamma\delta$ T17 cell numbers were greatly enhanced by hyperactive STAT5A  
147 whereas STAT5B had a milder impact (Fig. 2D-E). With the exception of V $\gamma$ 5<sup>+</sup> dendritic epidermal  
148 T cells, CCR6<sup>+</sup>  $\gamma\delta$ T17 cells are the only  $\gamma\delta$  population in the skin. However, mice expressing  
149 hyperactive STAT5B, and to a lesser extent mice expressing hyperactive STAT5A, contained  
150 CCR6<sup>-</sup>  $\gamma\delta$  T cells that were either V $\gamma$ 4<sup>+</sup> or V $\gamma$ 4<sup>-</sup> (Fig. 2D-F). Collectively, our data pinpoint towards  
151 a dominant role of STAT5A in supporting  $\gamma\delta$ T17 cells in LN and skin, with STAT5B supporting  
152 mainly IFN $\gamma$ -producing and CCR6<sup>-</sup>  $\gamma\delta$  T cells.

153

#### 154 **ROR $\gamma$ t<sup>CRE</sup>-STAT5<sup>F/F</sup> mice are resistant to experimental autoimmune encephalomyelitis**

155  $\gamma\delta$ T17 cells have been implicated in the pathogenesis of experimental autoimmune  
156 encephalomyelitis (EAE) (Petermann et al., 2010; Sutton et al., 2009) and we therefore  
157 investigated how well ROR $\gamma$ t<sup>CRE</sup>-STAT5<sup>F/F</sup> mice responded to MOG (myelin oligodendrocyte  
158 glycoprotein)-induced EAE. We found that compared to littermate controls, ROR $\gamma$ t<sup>CRE</sup>-STAT5<sup>F/F</sup>  
159 mice were resistant to EAE symptoms (Fig. 3A). This correlated with significantly reduced  $\gamma\delta$ T17  
160 cells in the LN and brain at days 11 and 21 after immunization (Fig. 3B-D). As expected,  $\gamma\delta$  T cell  
161 associated IL-17A production was significantly reduced at all time points in mice lacking STAT5  
162 (Fig. 3E). Although it has been recently suggested that inflammatory conditions during EAE can *de*  
163 *novo* regenerate  $\gamma\delta$ T17 cells (Papotto et al., 2017a), our data suggest that in the absence of  
164 STAT5,  $\gamma\delta$ T17 cell regeneration cannot occur.

165 It has been shown that in addition to their direct contribution to EAE pathogenesis,  $\gamma\delta$ T17 cells  
166 are required for optimal Th17 responses (Sutton et al., 2009). We therefore interrogated the CD4<sup>+</sup>  
167 T cell response in the LN and brain of ROR $\gamma$ t<sup>CRE</sup>-STAT5<sup>F/F</sup> and littermate control mice during EAE.  
168 We found that the numbers and cytokine production of CD4<sup>+</sup> T cells were not affected in the LN

169 (Fig. S1C-D), which may be a reflection of the levels of STAT5 still detectable in these cells (Fig.  
170 S1A). As expected from the clinical score and the lack of pro-inflammatory  $\gamma\delta$ T17 cells, there was  
171 a profound reduction in CD4<sup>+</sup> T cell numbers within the brain of ROR $\gamma$ t<sup>CRE</sup>-STAT5<sup>F/F</sup> mice (Fig. 3F).  
172 Collectively, our data show that loss of  $\gamma\delta$ T17 cells due to STAT5 deficiency is associated with  
173 dramatically reduced inflammatory responses in the EAE model.

174

### 175 **Intestinal lamina propria $\gamma\delta$ T17 cells express Tbet and require STAT3 and retinoic acid for** 176 **cytokine production**

177 Besides the skin and peripheral lymphoid tissues,  $\gamma\delta$  T cells with type 3 functionality have been  
178 described in the mucosa such as the lung and gut (Sheridan et al., 2013; Sutherland et al., 2014).  
179 We therefore wanted to test whether STAT5 regulated  $\gamma\delta$ T17 cells specifically in the intestinal  
180 lamina propria (LP). In order to avoid potential differences in  $\gamma\delta$ T17 surface markers in the gut, we  
181 stained small intestinal and colonic LP (sLP and cLP respectively) for ROR $\gamma$ t and Tbet and  
182 compared this to peripheral LNs. Surprisingly, we found that many ROR $\gamma$ t<sup>+</sup>  $\gamma\delta$  T cells in the gut co-  
183 expressed Tbet (Fig. 4A). We additionally confirmed the presence of ROR $\gamma$ t<sup>+</sup>Tbet<sup>+</sup>  $\gamma\delta$  T cells by  
184 generating double transgenic mice reporting GFP and AmCyan under control of the promoters for  
185 ROR $\gamma$ t and Tbet, respectively (Fig S3A). By transcription factor staining analysis we found that the  
186 ROR $\gamma$ t<sup>+</sup>Tbet<sup>+</sup>  $\gamma\delta$  T cell population was more prevalent in the ileum and proximal colon (Fig. 4B),  
187 which contrasted with the distribution of ROR $\gamma$ t<sup>-</sup>Tbet<sup>+</sup>  $\gamma\delta$  T cells in the same locations (Fig. S3B).  
188 In order to investigate which factors regulate the expression of Tbet, we analyzed mice deficient in  
189 Toll-like receptor and IL-12 signaling as well as mice depleted of their intestinal microbial flora. We  
190 found that expression of Tbet was independent of the microbiota (Fig. S3C), MyD88, TRIF and IL-  
191 12 signaling (Fig. S3D).

192 In agreement with their innate nature, intestinal  $\gamma\delta$ T17 cells produced IL-17A, IL-22 and IFN $\gamma$   
193 as early as 7 days after birth (Fig. 4C), indicating a functional  $\gamma\delta$ T17 population that has acquired  
194 the ability to produce IFN $\gamma$  at steady-state. Using ROR $\gamma$ t<sup>CRE</sup>-STAT3<sup>F/F</sup> mice, we showed that  
195 production of both IL-17A and IL-22 was STAT3-dependent (Fig. 4D-E and S4A-B). One of the key  
196 factors regulating IFN $\gamma$ -expressing cells is retinoic acid (RA) (Brown et al., 2015). We therefore

197 interrogated mice possessing a RA receptor (RAR) dominant negative (RARdn) transgene, which  
198 prevents active RAR $\alpha$  signaling (Rajaii et al., 2008) in ROR $\gamma$ t-expressing cells (ROR $\gamma$ t<sup>CRE-</sup>  
199 RARdn<sup>F/F</sup> mice). We found that loss of RA signaling was associated with reduced overall  
200 expression of IFN $\gamma$  (Fig. 5A and S4C) as well as reduced frequency of IL-17A<sup>+</sup>IFN $\gamma$ <sup>+</sup> and IL-  
201 22<sup>+</sup>IFN $\gamma$ <sup>+</sup> cells (Fig. 5B and S4D). In contrast, deficiency in RA signaling resulted in significantly  
202 increased frequency of IL-17A<sup>+</sup>IFN $\gamma$ <sup>-</sup> and IL-22<sup>+</sup>IFN $\gamma$ <sup>-</sup> Tbet<sup>+</sup>  $\gamma$  $\delta$ T17 cells in the colon and small  
203 intestine (Fig. 5B and S4D). Collectively, this data indicates that lamina propria Tbet<sup>+</sup>  $\gamma$  $\delta$ T17 cells  
204 are innate cells that can co-produce IL-17, IL-22 and IFN $\gamma$ , and that their cytokine expression  
205 profile is regulated by STAT3 and RA.

206

### 207 **STAT5 regulates Tbet expression and determines the progression of intestinal $\gamma$ $\delta$ T17 cells** 208 **through neonatal development**

209 Following the identification of a distinct gut-specific  $\gamma$  $\delta$ T17 population, we aimed to understand  
210 their dependence on STAT5. Similar to LNs, ROR $\gamma$ t-expressing  $\gamma$  $\delta$  T cells, irrespective of Tbet,  
211 were drastically and significantly reduced from the sLP and cLP of ROR $\gamma$ t<sup>CRE-</sup>STAT5<sup>F/F</sup> mice (Fig.  
212 6A-B). Analysis of GOF STAT5A and STAT5B mice revealed that hyperactive STAT5A  
213 downregulated Tbet in ROR $\gamma$ t<sup>+</sup> cLP  $\gamma$  $\delta$  T cells, whereas hyperactive STAT5B enhanced it (Fig. 6C)  
214 suggesting a YIN/YANG regulation in ROR $\gamma$ t<sup>+</sup> cLP  $\gamma$  $\delta$  T cells by STAT5A versus STAT5B.  
215 Hyperactive STAT5A preferentially expanded ROR $\gamma$ t<sup>+</sup> cells in the gut whereas hyperactive  
216 STAT5B favored Tbet-expressing  $\gamma$  $\delta$  T cells irrespective of whether they expressed ROR $\gamma$ t or not  
217 (Fig. 6D-F).

218 Next, we sought to determine whether STAT5 also regulated ROR $\gamma$ t<sup>+</sup>Tbet<sup>+</sup>  $\gamma$  $\delta$  T cells  
219 neonatally. We therefore analyzed neonatal gut at different time points and found that 1-2 days  
220 after birth  $\gamma$  $\delta$  T cells in the colon and small intestine expressed either ROR $\gamma$ t or Tbet but not both  
221 (Fig. 7A-C and S5A-C). Tbet was induced in ROR $\gamma$ t-expressing cells at day 4 and stabilized to  
222 adult levels within the first week of life (Fig. 7A-B and S5A-B). Expression of Tbet at neonatal day 4  
223 coincided with a rapid increase in cell proliferation, which was blunted in the absence of STAT5  
224 (Fig. 7D and S5D). ROR $\gamma$ t<sup>CRE-</sup>STAT5<sup>F/F</sup> mice did not upregulate Tbet and failed to sustain a



225 ROR $\gamma$ t<sup>+</sup>  $\gamma\delta$  T cell population after birth (Fig. 7A-C and S5A-C). However, despite their functional  
226 presence in the neonatal gut, ROR $\gamma$ t-expressing  $\gamma\delta$  T cells were not necessary for protection  
227 against early life infection with the attaching and effacing bacterium *Citrobacter rodentium* (Fig.  
228 S6A-C).

229 Collectively, our data demonstrate that during neonatal life STAT5 acts as a molecular  
230 checkpoint to promote proliferation of intestinal  $\gamma\delta$ T17 cells. Moreover, our data reveal an  
231 interesting balance between STAT5A and STAT5B, which appear to have opposing roles in the  
232 regulation of Tbet expression, thereby differentially coordinating tissue specificity of  $\gamma\delta$ T17 cells.

233

## 234 **Discussion**

235 In the present study we demonstrate that STAT5 is a critical regulator of IL-17-producing  $\gamma\delta$  T cells  
236 in the periphery, skin and gut. STAT5 was necessary during neonatal life in order to sustain  
237 proliferation and survival of  $\gamma\delta$ T17 cells. Transgenic reconstitution of hyperactive STAT5 variants  
238 showed that STAT5A preferentially sustains  $\gamma\delta$ T17 whereas STAT5B promotes IFN $\gamma$ -producing  $\gamma\delta$   
239 T cells. Physiologically, hampered development of  $\gamma\delta$ T17 cells due to STAT5 loss resulted in near  
240 complete resistance to EAE pathology and prevented Th17 cells from infiltrating the brain.  
241 Furthermore, we discovered that intestinal lamina propria  $\gamma\delta$ T17 cells co-express the type 1  
242 transcription factor Tbet and can produce IL-17, IL-22 and IFN $\gamma$  in a STAT3- and RA-dependent  
243 mechanism. Intestinal  $\gamma\delta$ T17 cells upregulate Tbet during the first week of life and are strictly  
244 STAT5-dependent for their neonatal development. Furthermore, expression of Tbet is under the  
245 antagonistic control of STAT5A and STAT5B.

246 STAT5 is a major signaling component downstream of many cytokine and growth factor  
247 receptors and is therefore involved in the development of lymphocyte lineages (Rani and Murphy,  
248 2016). Hence, both mice and humans with STAT5-associated deficiencies are severely  
249 immunocompromized (Imada et al., 1998; Kofoed et al., 2003). T cells of the  $\gamma\delta$  lineage are  
250 reduced in the thymus and lymphoid tissues of full STAT5-deficient mice (Hoelbl et al., 2006), and  
251 this has been attributed to a failure to successfully rearrange the TCR early during embryonic  
252 development (Wagatsuma et al., 2015). However, our data show that  $\gamma\delta$ T17 cells require STAT5

253 signaling to expand and survive after they exit the thymus. This suggests that  $\gamma\delta$  T cell subsets rely  
254 on STAT5 during different steps of their development and differentiation, presumably reflecting  
255 cytokine niches within the local microenvironment.

256 Detailed molecular and phenotypic studies utilizing mice deficient in either STAT5A or  
257 STAT5B have shown that despite their many commonalities, particularly at the genome-wide level,  
258 the two STAT5 gene products can display cell-specific functions (Villarino et al., 2016; Villarino et  
259 al., 2017). Thus, in CD4<sup>+</sup> T cells STAT5B has a dominant role in orchestrating differentiation and  
260 function. In this regard, our data show that both STAT5A and STAT5B can have dominant and  
261 differential roles in  $\gamma\delta$  T cells depending on the specific subset. Whereas STAT5A regulated almost  
262 exclusively  $\gamma\delta$ T17 cells and downregulated intestinal Tbet expression, STAT5B had a prevailing  
263 effect on IFN $\gamma$ -expressing  $\gamma\delta$  populations. This suggests that unlike in CD4<sup>+</sup> helper and innate  
264 lymphoid cells, STAT5A and STAT5B display significant, differential regulatory functions in  $\gamma\delta$  T  
265 cell subsets, and further pinpoints to the distinct molecular, functional and developmental  
266 requirements of  $\gamma\delta$ T17 compared to non-IL-17-producing subsets. The unique roles that we  
267 uncovered herein for STAT5A and STAT5B suggest that they display cell-specific functions and  
268 can have context-dependent, non-redundant roles in generating robust immune responses. The  
269 genetic and cellular tools that we used herein will be crucial to illuminate the specific biological  
270 functions of these two highly species-conserved proteins that play indispensable roles in infection,  
271 cancer and autoimmunity.

272 Although  $\gamma\delta$ T17 cells develop in the embryonic thymus, previous reports suggested that they  
273 populate the skin and LNs after birth (Cai et al., 2014). Findings herein indicate that the neonatal  
274 period is critical for  $\gamma\delta$ T17 cells to populate lymphoid and non-lymphoid tissues. A critical time  
275 window of opportunity has been suggested to exist during neonatal life when, upon exposure to  
276 microbiota, the immune system matures and develops cellular and humoral immunity (Al Nabhani  
277 et al., 2019; Torow and Hornef, 2017). The upregulation of Tbet in murine intestinal  $\gamma\delta$ T17 cells  
278 within days after birth and its independence on the microbiota and TLR signals suggests  
279 alternative neonatal factors such as lactation, which is predominantly STAT5A-controlled  
280 (Haricharan and Li, 2014). Neonatal-specific cytokine milieus that activate STAT5 may also

281 regulate Tbet expression in the developing gut. In this regard, hyperactive STAT5A downregulated  
282 Tbet whereas high STAT5B activity induced it, although whether this was direct or indirect through  
283 regulation of cell fate transcriptional regulators remains to be studied. Nevertheless, the  
284 identification of Tbet-expressing  $\gamma\delta$ T17 cells at steady-state indicates a form of plasticity within this  
285 lineage that is regulated post-thymically and in a tissue-specific manner. The importance of Tbet in  
286  $\gamma\delta$ T17 cells is currently unknown. However, the co-expression of IFN $\gamma$  suggests that acquisition of  
287 type 1 transcriptional and functional traits may give an advantage over infection, similar to innate  
288 lymphoid cells and Th1-transitioning Th17 cells.

289 Animal models have linked  $\gamma\delta$ T17 cells to immune responses during inflammation, infection  
290 and cancer where they can be either protective or pathogenic (Papotto et al., 2017b). In the  
291 imiquimod model of psoriasis,  $\gamma\delta$ T17 cells are important to drive disease; however, their  
292 pathogenic role can be redundant and compensated by other inflammatory cells (Sandrock et al.,  
293 2018). In the EAE mouse model,  $\gamma\delta$ T17 cells have also been shown to contribute to pathogenicity  
294 (Petermann et al., 2010; Sutton et al., 2009). Herein, we provide evidence that  $\gamma\delta$ T17 cells are  
295 necessary and non-redundant for full development of EAE symptoms and for the mobilization of  
296 Th17 cells to the brain. Despite their absence from all major organs from neonatal life onwards,  
297 other innate inflammatory cells could not compensate for their absence.  $\gamma\delta$  T cells have co-evolved  
298 alongside  $\alpha\beta$  T and B cells (Hirano et al., 2013), however their function has diversified and was  
299 imprinted by the specific tissue cues present in different locations. It is thus not surprising that the  
300 immunological response of different  $\gamma\delta$  T cell subsets will vary and will be essential or redundant  
301 depending on the inflammatory or infectious context.

302 In summary, we provide evidence that the neonatal microenvironment, acting in synergy with  
303 tissue-specific and STAT5-driven molecular cues, regulates the development, functional  
304 maturation and immunological importance of  $\gamma\delta$ T17 cells.

305

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547

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556

557 **Author Contributions:** V.B. designed the study, oversaw all work, performed experiments and  
558 wrote the manuscript. D.K. designed and performed experiments, analyzed data and helped write  
559 the manuscript. J.R. and R.A. designed and performed experiments, analyzed data and helped  
560 write the manuscript. H.N., T.S., B.M. and R.M. generated the STAT5 GOF mutant strains,  
561 performed experiments, analyzed data and helped write the manuscript. E.C. and A.T. performed  
562 and analyzed experiments.

563

564 **Competing interests:** The authors declare no competing interests.

565

566

567



568 **Methods**

569 **Mice**

570 All animal breeding and experiments were performed in house at DTU and only after approval from  
571 the Danish Animal Experiments Inspectorate. ROR $\gamma$ <sup>CRE</sup> and ROR $\gamma$ <sup>GFP</sup> mice (Lochner et al., 2008)  
572 were provided by Professor Gerard Eberl and subsequently bred in house. STAT5<sup>F/F</sup> mice were  
573 generated as previously described (Cui et al., 2004) and bred in house. STAT3<sup>F/F</sup> mice were  
574 purchased from The Jackson Laboratory and bred in house. RAR $\alpha$ <sup>F/F</sup> mice were generated as  
575 previously described (Rajaii et al., 2008), were provided by Professor William W. Agace, (Lund  
576 University, Sweden) and bred in house. Intestinal tissue from IL12<sup>-/-</sup> mice (JAX stock #002692 )  
577 was provided by Professor William W. Agace, (Lund University, Sweden). GOF STAT5a and  
578 STAT5b mice were generated as previously described (Maurer et al., 2019; Pham et al., 2018) and  
579 were bred at the University of Veterinary Medicine Vienna (Vienna, Austria). Frozen sperm  
580 samples from Tbet-AmCyan mice (Yu et al., 2015) were provided by Professor Jinfang Zhu  
581 (NIH/NIAID, MD ) and after re-derivation mice bred in house. Intestinal tissue from mice deficient in  
582 TRIF and MyD88 was provided by Dr Katharina Lahl (DTU, Denmark).

583

584 **Cell culture media and buffers**

585 All cell culture and single cell suspensions were prepared using RPMI 1640 (Invitrogen)  
586 supplemented with 10% heat inactivated fetal bovine serum (FBS)(Gibco), 20mM Hepes pH 7.4  
587 (Gibco), 50  $\mu$ M 2-mercaptoethanol, 2 mM L-glutamine (Gibco) and 10,000 U/ mL penicillin-  
588 streptomycin (Gibco). 10x Hank's balanced salt solution (HBSS)(Gibco) was diluted to 1x with  
589 sterile nuclease-free water and supplemented with 15 mM HEPES pH 7.4 (Gibco) to prepare  
590 HBSS-HEPES while it was supplemented with 2mM EDTA, 15mM HEPES, 50 $\mu$ g/mL Gentamycin  
591 and 2% FBS to prepare HBSS-EDTA. Isotonic Percoll was prepared by mixing 90%v/v of Percoll  
592 (GE healthcare) with 9%v/v 10x HBSS and 1%v/v 1M HEPES pH 7.4. Isotonic Percoll was  
593 subsequently diluted with HBSS-EDTA to the desired concentration. FACS buffer was prepared by  
594 mixing 3% heat inactivated FBS with DPBS (Gibco).

595

596 **Isolation of lymphocytes from lymph nodes (LNs), thymus, skin, small intestine and colon**

597 LNs were dissected, cleared off fat and crushed against a 70µm cell strainer to prepare single cell  
598 suspensions. Cell suspensions were then washed and filtered through a 40µm cell strainer. Cells  
599 were counted and  $2.5 \times 10^6$  cells were used for staining of surface antigens and flow cytometry  
600 analysis.

601 Thymus lobes from 1-day old pups were dissected and dissociated in supplemented RPMI  
602 using dissection microscope to prepare single cell suspension. The cell suspensions were filtered  
603 through a 40µm cell strainer and stained for surface antigens under sterile conditions before FACS  
604 sorting.

605 Skin lymphocytes were prepared from ears as follows: first, the dorsal and ventral sides of the  
606 ears were mechanically separated, they were subsequently cut into small pieces followed by  
607 enzymatic digestion with 0.25mg/ml collagenase IV, 0.166mg/ml hyaluronidase and 0.1mg/ml  
608 DNase I (all enzymes from Sigma-Aldrich) in supplemented RPMI for 1 hour at 37°C with constant  
609 stirring at 700 rpm. Undigested tissue was crushed against a 70µm cell strainer to prepare a single  
610 cell suspension. After washing, the cell pellet was re-suspended and filtered through a 40µm cell  
611 strainer to remove tissue debris and used for flow cytometry staining.

612 Small intestines and colons were dissected from adult mice and were HBSS-HEPES to  
613 remove intestinal contents. Fat and Peyer's patches were removed before and then the tissues  
614 were open longitudinally and cut into small pieces of approximately 2-3 cm. Chopped tissue was  
615 washed 4 times (2 alternate cycles of 10 and 15 min each ) using 15 mL of HBSS-EDTA buffer at  
616 37°C in a shaking incubator. Tissue pieces were then digested using 0.3mg Liberase TM (Roche)  
617 and 0.15 mg of DNase (Sigma Aldrich) per preparation in 5mL supplemented RPMI for 40 minutes  
618 on the magnetic stirrer at 37°C. The resulting cell suspensions were filtered through 70µm cell  
619 strainers, collected in complete RPMI and subsequently pelleted by centrifugation. The cell pellets  
620 were then re-suspended in 5 mL 40% Percoll, layered on 4 mL of 70% Percoll, and centrifuged at  
621 20 °C and  $800 \times g$  for 20 min with deceleration set to 0. Cells from the interphase were collected,  
622 washed once and then re-suspended in supplemented RPMI. For neonatal gut samples, cell

623 suspensions, following digestions, were filtered through 70µm cell strainers and were then used  
624 directly.

625

### 626 **Experimental Autoimmune Encephalomyelitis**

627 EAE was induced by sub-cutaneous injection of 50µg of MOG35-55 peptide in CFA, while 2 ng  
628 pertussis toxin were intra-peritoneally (i.p.) injected on the day of immunization and 2 days later.

629 From day 11 after immunization and until day 21, mice were weighed and scored for clinical signs  
630 as follows: 0: no symptoms; 1: tail paralysis; 1.5: impaired righting reflex; 2: paralysis of one hind  
631 limb; 2.5: paralysis of both hind limbs; 3: paralysis of one fore limb; 3.5: paralysis of one fore limb  
632 and weak second for limb; 4: total limb paralysis.

633 Mice were euthanized at days 11 or 21 after immunization mice and were perfused with PBS.  
634 LN cells were isolated as described above. Brain tissue was mechanically minced and passed  
635 through a 70µm cell strainer to obtain a single cell suspension. Lymphocytes were separated using  
636 density gradient centrifugation with 47% Percoll (GE Healthcare), layered on 4 mL of 70% Percoll,  
637 and centrifuged at 20 °C and 900 × g for 30 min with deceleration set to 0.

638

### 639 ***In vitro* stimulation of lymphocytes**

640 For LN lymphocytes, 10<sup>7</sup> cells were cultured for 3.5 hours in the presence of 50ng/ml PMA (Sigma)  
641 ,750ng/ml ionomycin (Sigma) and 1µL /mL BD Golgistop (containing monensin). Single cell  
642 suspensions from intestinal lamina propria were stimulated with 40 ng/ml of IL-23 (R&D Systems)  
643 for 3 hours followed by 50ng/ml PMA, 750ng/ml ionomycin and 1µL /mL BD Golgistop for an  
644 additional 3 hours. After 6 hours, cells were harvested and washed with PBS and used for flow  
645 cytometry staining. FoxP3 transcription factor staining kit (eBiosciences)

646

### 647 **Flow Cytometry**

648 Cells were harvested by centrifugation at 400 g for 5 minutes at 4°C followed by staining with  
649 fixable viability stain (BD Horizon FVS700) for 10 minutes on ice in PBS. Subsequently, surface  
650 antigens were stained in FACS buffer for 30 minutes on ice. For cytokine staining, cells were then

651 fixed and permeabilized by incubation in BD Fix/Perm solution for 15 minutes at room temperature  
652 followed by washing once in BD Perm/Wash solution. Intracellular cytokines were stained in BD  
653 Perm/Wash for 15 minutes at room temperature. For transcription factor staining, following surface  
654 staining, the cells were fixed using the Fixation/Permeabilization buffer in BD Transcription Factor  
655 kit for 45 minutes at 4°C. Transcription factors were stained in permeabilization buffer from the  
656 same kit for 45 minutes at 4°C. Conversely, for combined transcription factor and cytokine staining,  
657 after surface staining, the cells were fixed using the Fixation/Permeabilization buffer in FoxP3  
658 transcription factor staining kit (eBiosciences) for 1 hour at 4°C. Cytokines and transcription factors  
659 were then stained in the permeabilization buffer from the same kit following the manufacturer's  
660 guidelines.

661 All antibodies were used at a 1:200 dilution unless otherwise specified. Antibodies used herein  
662 were as follows: CD4-FITC (RM4-4, BD biosciences), CD19-FITC (6D5, Biolegend), TCRβ-  
663 APCeF780 (H57-597; eBioscience), TCRγδ-BV421 (GL3, BD biosciences), CD45-V500 (30-F11,  
664 BD biosciences), CD3-PECF594 (BM10-37, BD biosciences), RORγt-APC (B2D, BD biosciences),  
665 IL-17-BV786 (TC11-18H10, BD biosciences), IL-22-PE (1H8PWSR; eBioscience), T-bet-PECy7  
666 (4B10, Biolegend), IFNγ-PerCP-Cy5.5 (XMG1.2; BD biosciences), CD69-V450 and Pe-CF594  
667 (H1.2F3; BD biosciences), CCR6-Alexa Fluor 647 (140706; BD biosciences), CD27 PE-Cy7  
668 (LG.3A10; BD biosciences), CD44-V500 (1M7; BD biosciences), Ki67-BV786 (B56 ; BD  
669 biosciences 1:100)

670 To determine the level of pSTAT5,  $1 \times 10^6$  cells were fixed 100 μL with BD phosflow Lyse/Fix  
671 (diluted to 1x with water) for 10 minutes at 37°C. Subsequently, cells were washed once with FACS  
672 buffer and re-suspended in 100 μL BD phosflow perm buffer III, which was pre-chilled to -20°C,  
673 and incubated on ice for 30 minutes. Cells were then washed once with FACS buffer and stained  
674 for 30 minutes on ice in FACS buffer. Antibodies used in the staining of p-STAT5 were: CD4-FITC  
675 (GK1.5; BD Biosciences), CD8-APC 53-6.7; BD Biosciences) and pSTAT5-PEcy7 (47/Stat5  
676 pY694; BD Biosciences; 5 μL/test). Samples were acquired using BD LSR Fortessa™ and BD  
677 FACSDiva software v8.0.2.

678

679 **Bone Marrow chimera**

680 First, CD45.1<sup>+</sup>CD45.2<sup>+</sup> mice were lethally irradiated (900 rad). Next day they were injected  
681 intravenously with 10×10<sup>6</sup> cells of whole bone marrow cells from CD45.1<sup>+</sup> wild-type and CD45.2<sup>+</sup>  
682 RORγt<sup>CRE</sup>-STAT5<sup>F/F</sup> mixed at 1:1 ratio. All Chimeras were analyzed after 12-14 weeks after  
683 reconstitution.

684

685 **Antibiotics administration**

686 Pregnant female mice were treated with a cocktail of the following antibiotics: 1mg/ml Collistin, 5  
687 mg/ml Streptomycin, 1mg/ml Ampicillin and 0.5mg/ml Vancomycin (all antibiotics Sigma) in their  
688 drinking water starting three days before delivery and until weaning of pups (3 weeks after the  
689 birth). The antibiotic-containing drinking water was replaced once a week until analysis.

690

691 **Citrobacter infection**

692 *Citrobacter. rodentium* strain DBS100 (ATCC 51459; American Type Culture Collection) was  
693 purchased from ATCC and was cultured in Luria–Bertani broth overnight. CFU/ml (Dose) was  
694 determined by measuring the OD at 600 nm. Pups that were 10-12 days old were infected by oral  
695 gavage of 5×10<sup>6</sup> CFU/mouse in a volume of 50μl. At day 6 post infection, the pups were  
696 euthanized and colons and fecal samples were collected. Bacterial load in the feces was  
697 determined as described (Sagaidak et al., 2016).

698

699 **Cell sorting, RNA extraction, cDNA synthesis and Real-Time PCR**

700 Stained cells from thymi of 1-day old pups were sorted using BD ARIA FUSION™ and BD  
701 FACSDiva software v8.0.2. Target populations were sorted directly in RLT buffer (Qiagen)  
702 supplemented with 2-mercaptoethanol. Total RNA was extracted using Rneasy micro kit (Qiagen)  
703 and then used for cDNA synthesis using iScript cDNA synthesis kit (Biorad), according to the  
704 manufacturer's protocol. SsoFast EvaGreen supermix (Biorad) was used to catalyze real-time PCR  
705 reactions, which were run on CFX96 (Biorad) and analyzed using Bio-rad CFX manager software.  
706 Gene expression levels were normalized to that of beta-actin. The following primers were used:

707 *Actb*, Fwd-GGCTGTATTCCCCTCCATCG, Rev- CCAGTTGGTAACAATGCCATGT; *Stat5a*, Fwd-  
708 TCCGCAGCACCAGGTAAA, Rev- GGGATTATCCAAGTCAATAGCATC; *Stat5b*, Fwd-  
709 ACAACGGCAGCTCTCCAG, Rev-TGGGCAAACCTGAGCTTGGATC.

710

#### 711 **Data Analysis**

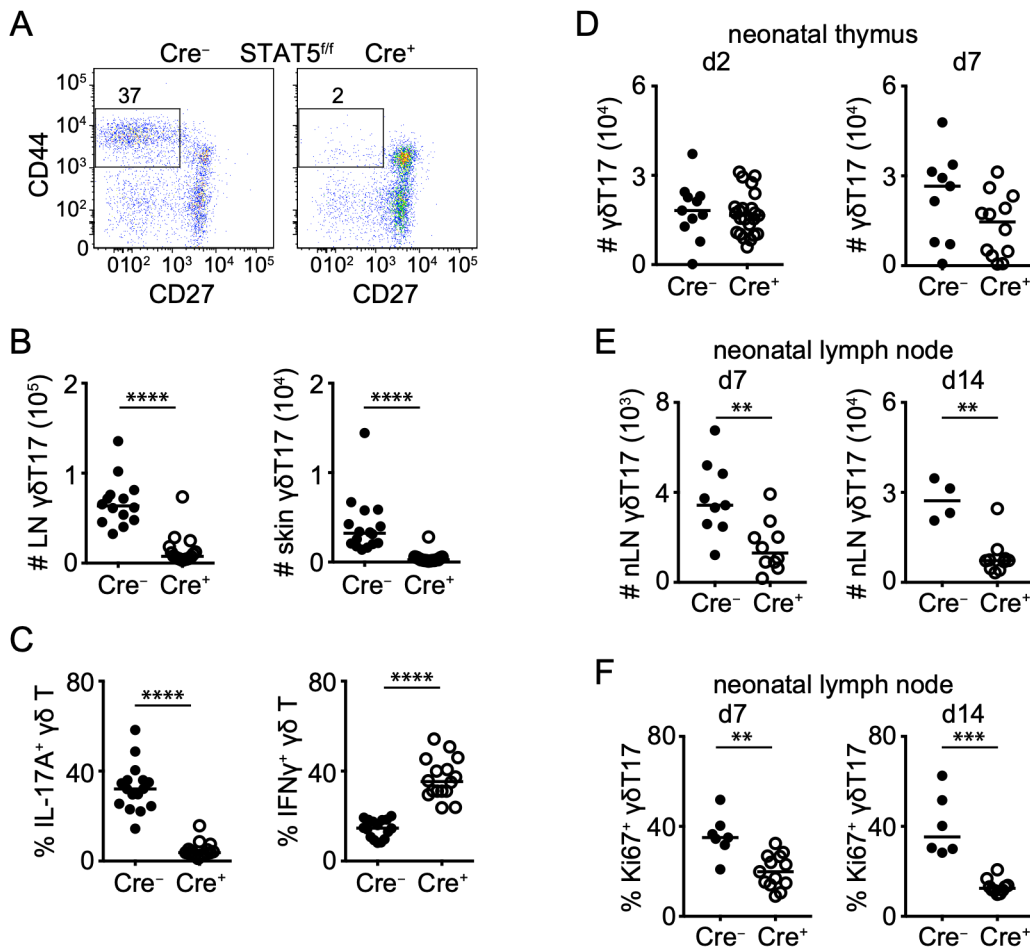
712 Flow cytometry data was analyzed using Flow Jo V 10 software. All the statistical analyses and  
713 graphs were generated using Prism v7. All statistical tests used are described in the Figure  
714 legends

715

716

717 **Figures and Legends**

718 **Figure 1**



719 **Figure 1. STAT5 is necessary for the neonatal expansion of γδT17 cells**

720 Flow cytometric analysis of γδ T cells in RORγt<sup>CRE</sup>-STAT5<sup>F/F</sup> (Cre<sup>+</sup>) and littermate control mice

721 (Cre<sup>-</sup>). In graphs each symbol represents a mouse and line the median. \*\*p < 0.01, \*\*\*p < 0.001,

722 \*\*\*\*p < 0.0001 using Mann-Whitney test.

723 (A) Expression of CD27 and CD44 in order to identify CD27<sup>-</sup>CD44<sup>+</sup> γδT17 cells in the LN.

724 Numbers indicate percent of CD27<sup>-</sup>CD44<sup>+</sup> within the γδ T cell compartment.

725 (B) Numbers of γδT17 cells in the LN (staining as in A) and skin. In the skin, γδT17 cells were

726 identified as CD45<sup>+</sup>CD3<sup>Low</sup>Vγ5<sup>+</sup>TCRβ<sup>-</sup>TCRγδ<sup>+</sup>CCR6<sup>+</sup>.

727 (C) Expression of IL-17A and IFNγ within the LN γδ T cell compartment.

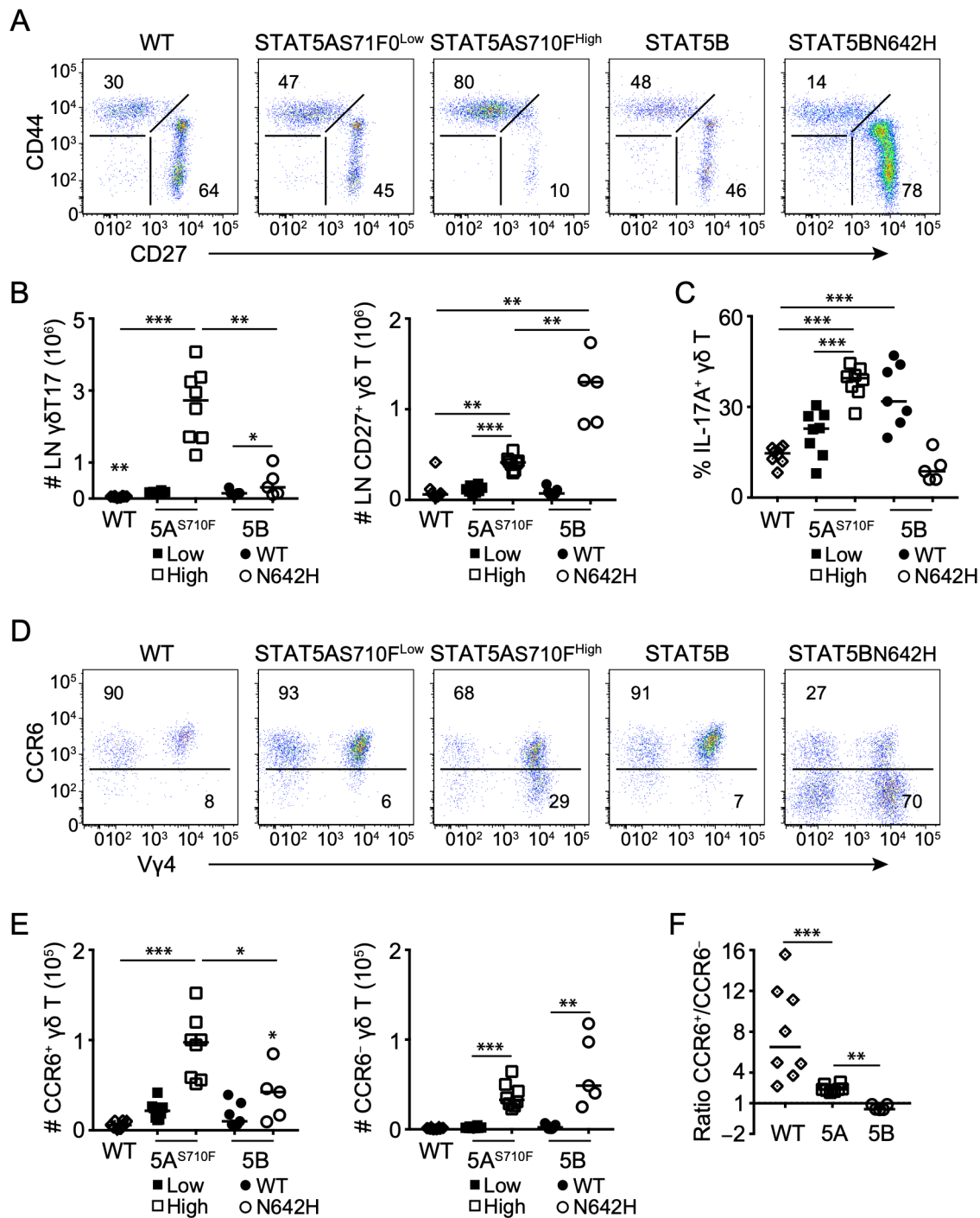
728 (D) Numbers of CD27<sup>-</sup>CD44<sup>+</sup> γδT17 cells in day 2 and day 7 old thymi.

729 (E) Numbers of CD27<sup>-</sup>CD44<sup>+</sup> γδT17 cells in day 7 and day 14 old LN.

730 (F) Frequency of Ki67<sup>+</sup>RORγt<sup>+</sup> or Ki67<sup>+</sup>CCR6<sup>+</sup> γδT17 cells within the CD44<sup>+</sup>TCRγδ<sup>+</sup> compartment  
731 in day 7 and day 14 old LN.  
732  
733



734 **Figure 2**



735 **Figure 2. Differential impact of STAT5A and STAT5B on  $\gamma\delta$ T17 and CD27<sup>+</sup>  $\gamma\delta$  T cells**

736 Flow cytometric analysis of  $\gamma\delta$  T cells in mice that are either wild-type (WT) or constitutively

737 express under the *Vav1* promoter one of the following forms of STAT5: low (STAT5AS710F<sup>Low</sup>) or

738 high (STAT5AS710F<sup>High</sup>) copy number of the hyperactive STAT5A S710F mutant, or human wild-

739 type (STAT5B) or the hyperactive N642H STAT5B mutant (STAT5BN642H). In graphs, each

740 symbol represents a mouse and line the median. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001 using Mann-  
741 Whitney test.

742 (A) Expression of CD27 and CD44 in order to identify CD27<sup>-</sup>CD44<sup>+</sup>  $\gamma\delta$ T17 cells in the LN.  
743 Numbers indicate percent of CD27<sup>-</sup>CD44<sup>+</sup> or CD27<sup>+</sup>CD44<sup>-</sup> within the  $\gamma\delta$  T cell compartment.

744 (B) Numbers of  $\gamma\delta$ T17 (staining as in A) and CD27<sup>+</sup> cells in the LN (\*\* in WT denotes difference by  
745 comparison to STAT5BN642H).

746 (C) Expression of IL-17A within the LN  $\gamma\delta$  T cell compartment.

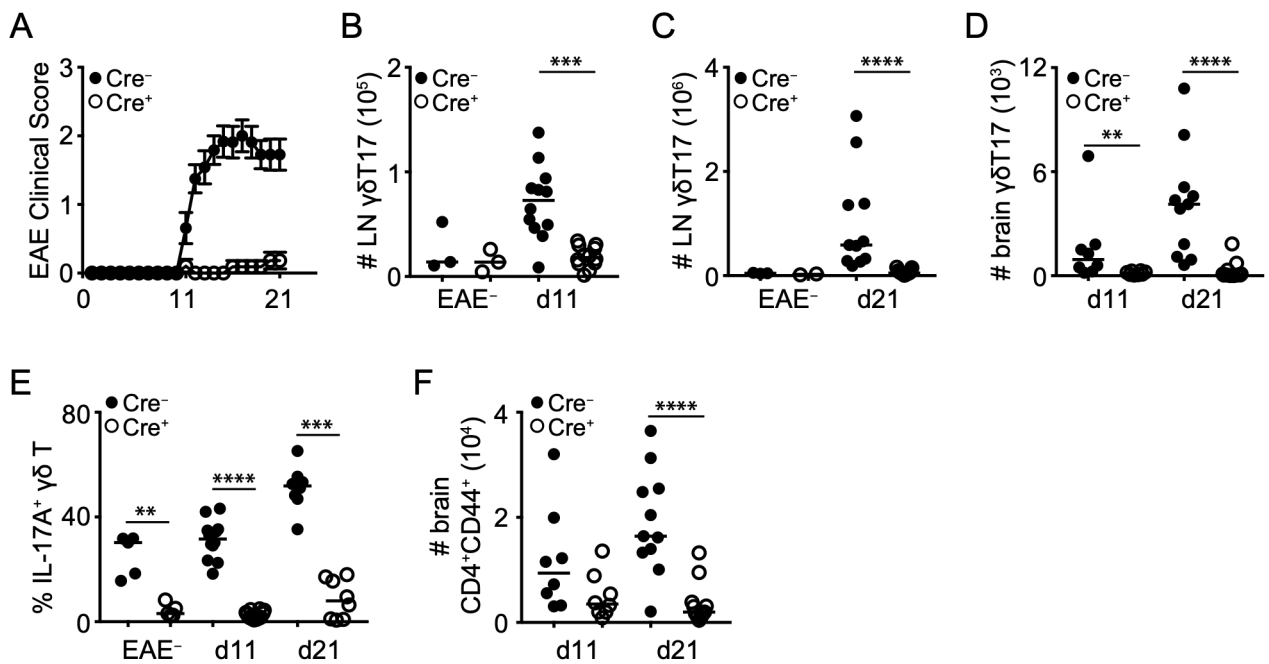
747 (D) Expression of CCR6 and V $\gamma$ 4 in skin  $\gamma\delta$ T17 cells (staining as in Fig. 1B). Numbers indicate  
748 percent of CCR6<sup>+</sup> or CCR6<sup>-</sup> cells within the  $\gamma\delta$  T cell compartment.

749 (E) Numbers of CCR6<sup>+</sup> and CCR6<sup>-</sup> cells identified in D (\* in STATBBN642H denotes significant  
750 difference by comparison to WT). (F) Ratio of CCR6<sup>+</sup> over CCR6<sup>-</sup> cells in WT compared to  
751 STAT5AS710F<sup>High</sup> (5A) and STAT5BN642H (5B) mice.

752

753

754 **Figure 3**



755

756 **Figure 3. ROR $\gamma$ t<sup>CRE</sup>-STAT5<sup>F/F</sup> mice are resistant to EAE**

757 Disease progression and flow cytometric analysis of  $\gamma\delta$  and CD4<sup>+</sup> T cells in ROR $\gamma$ t<sup>CRE</sup>-STAT5<sup>F/F</sup>  
758 (Cre<sup>+</sup>) and littermate control mice (Cre<sup>-</sup>) that had been previously immunized with 50  $\mu$ g MOG  
759 peptide in CFA and 200 ng Pertussis toxin. In graphs, each symbol represents a mouse and line  
760 the median (except in A). \*\*p < 0.01, \*\*\*p < 0.001, \*\*\*\*p < 0.0001 using Mann-Whitney test.

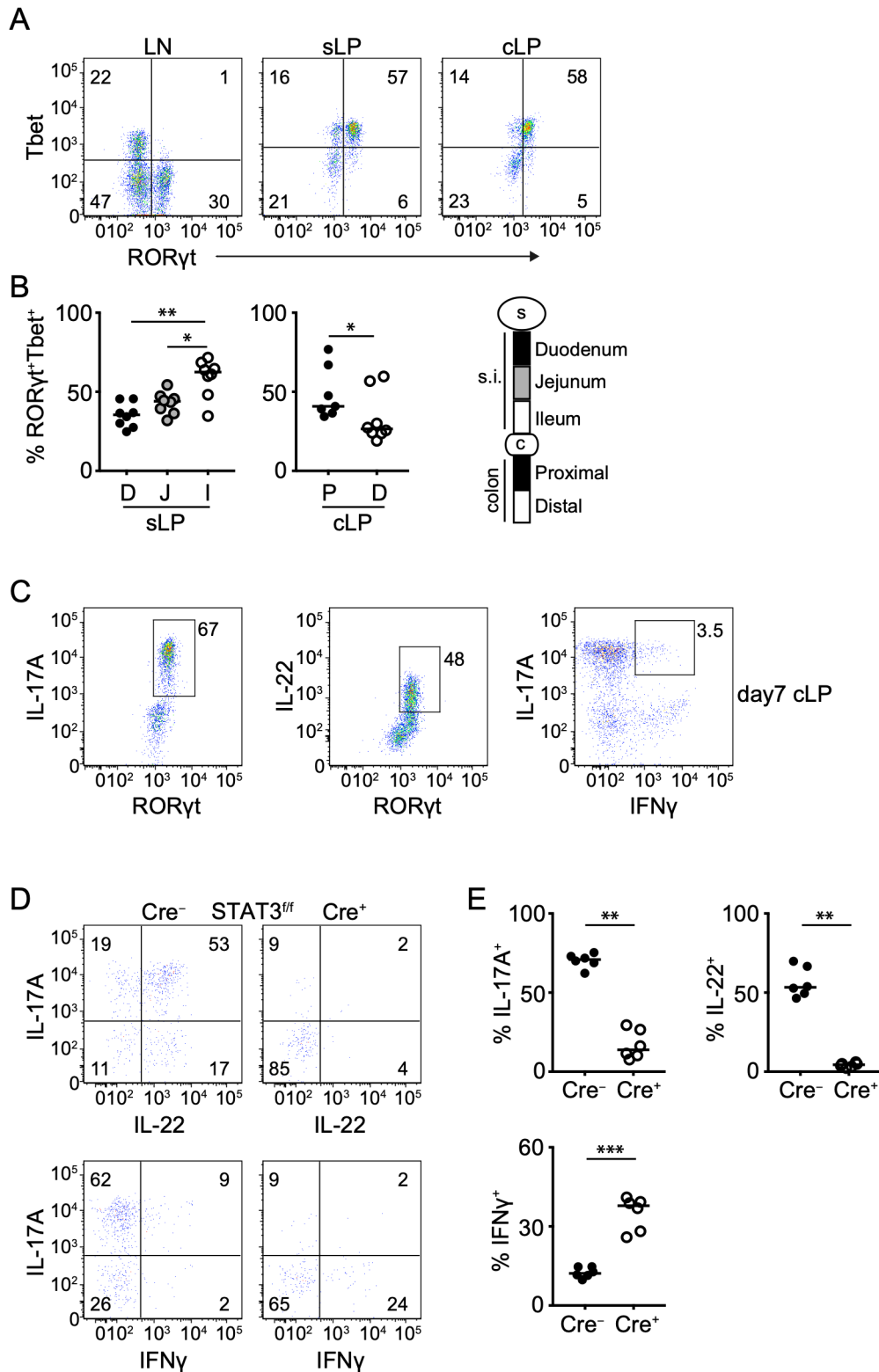
761 (A) Clinical symptoms of EAE until day 21 post immunization. Data is pool of 20 mice per genotype  
762 and shown as mean $\pm$ sem. Statistical analysis was performed using 2-way ANOVA with  
763 Bonferroni's multiple comparisons test. ANOVA p-value < 0.0001; Bonferroni's test returned  
764 significance for days 11-21 with day 11 p = 0.003 and days 12-21 p < 0.0001.

765 (B-C) Numbers of  $\gamma\delta$ T17 cells in the LN (staining as in Fig. 1A) of unimmunized controls (EAE<sup>-</sup>)  
766 and at 11 (B) and 21 (C) days after immunization.

767 (D) Numbers of  $\gamma\delta$ T17 cells in the brain (identified as CD45<sup>+</sup>CD3<sup>+</sup>TCR $\beta$ <sup>-</sup>TCR $\gamma\delta$ <sup>+</sup>CD44<sup>+</sup>) at days 11  
768 and 21 after immunization.

769 (E) Expression of IL-17A within the LN  $\gamma\delta$  T cell compartment of unimmunized controls (EAE<sup>-</sup>) and  
770 at 11 and 21 days after immunization. (F) Numbers of CD4<sup>+</sup>CD44<sup>+</sup>CD3<sup>+</sup>TCR $\beta$ <sup>+</sup> cells in the brain at  
771 days 11 and 21 after immunization.

772 **Figure 4**



773 **Figure 4. Intestinal  $\gamma\delta$ T17 cells express Tbet and require STAT3 for IL-17A and IL-22**

774 Flow cytometric analysis of LN and intestinal  $\gamma\delta$  T cells in WT or in ROR $\gamma$ t<sup>CRE</sup>-STAT3<sup>F/F</sup> (Cre<sup>+</sup>) and

775 littermate control mice (Cre<sup>-</sup>). In graphs, each symbol represents a mouse and line the median.

776 Cytokine detection was performed following IL-23 re-stimulation. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p <$   
777  $0.001$ , \*\*\*\* $p < 0.0001$  using Mann-Whitney test.

778 (A) Expression of ROR $\gamma$ t and Tbet within the  $\gamma\delta$  T cell compartment of the LN, sLP and cLP.  
779 Numbers indicate percent of ROR $\gamma$ t and Tbet expression.

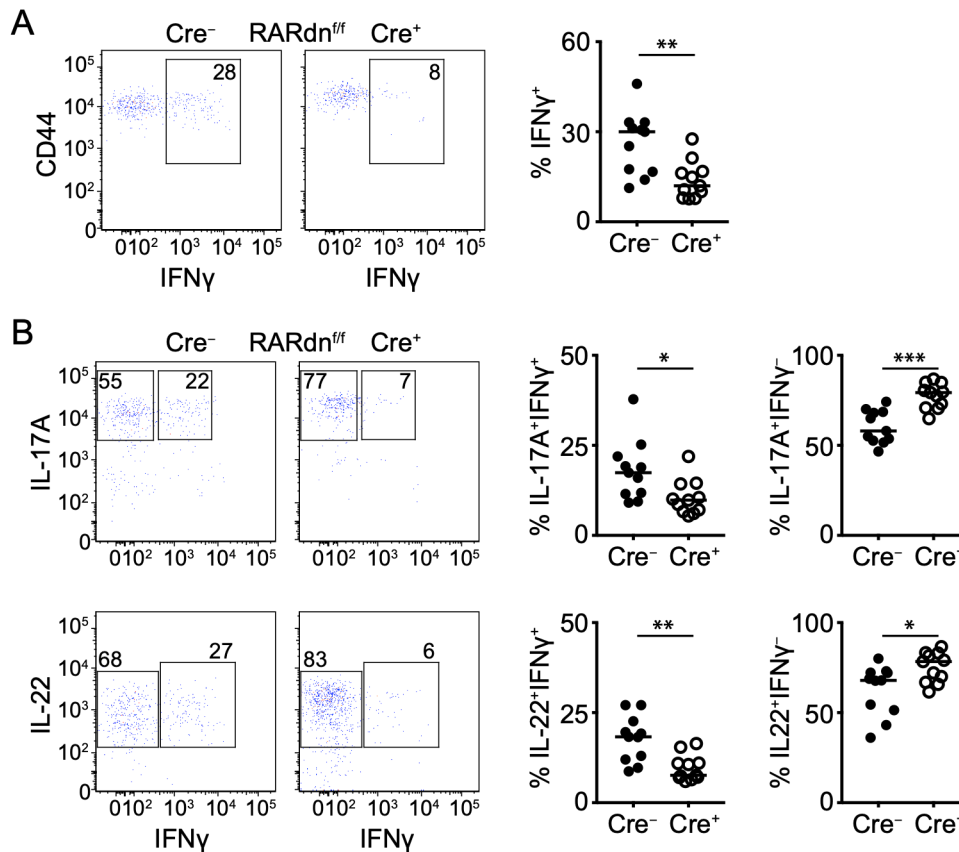
780 (B) Frequency of ROR $\gamma$ t<sup>+</sup>Tbet<sup>+</sup> cells within the  $\gamma\delta$  T cell compartment in the indicated small  
781 intestinal and colonic segments.

782 (C) Expression of ROR $\gamma$ t and IL-17A, ROR $\gamma$ t and IL-22, or IL-17A and IFN $\gamma$  in the  $\gamma\delta$  T cell  
783 compartment of cLP from day 7 old mice (representative of two experiments).

784 (D) Expression of IL-17A and IL-22 (top) or IL-17A and IFN $\gamma$  (bottom) in ROR $\gamma$ t<sup>CRE</sup>-STAT3<sup>F/F</sup> (Cre<sup>+</sup>)  
785 and littermate control mice (Cre<sup>-</sup>). Numbers indicate percent of positive expression.

786 (E) Frequency of IL-17A<sup>+</sup>, IL-22<sup>+</sup> and IFN $\gamma$ <sup>+</sup>  $\gamma\delta$  T cells in ROR $\gamma$ t<sup>CRE</sup>-STAT3<sup>F/F</sup> (Cre<sup>+</sup>) and littermate  
787 control mice (Cre<sup>-</sup>).  
788  
789

790 **Figure 5**



791 **Figure 5. Retinoic acid receptor signaling regulates IFN $\gamma$  production in intestinal Tbet<sup>+</sup>**  
 792  **$\gamma\delta$ T17 cells**

793 Flow cytometric analysis of cytokine expression in colonic  $\gamma\delta$  T cells in ROR $\gamma$ t<sup>CRE</sup>-RARdn<sup>F/F</sup> (Cre<sup>+</sup>)  
 794 and littermate control mice (Cre<sup>-</sup>) following IL-23 stimulation. In graphs, each symbol represents a  
 795 mouse and line the median. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001 using Mann-Whitney test.

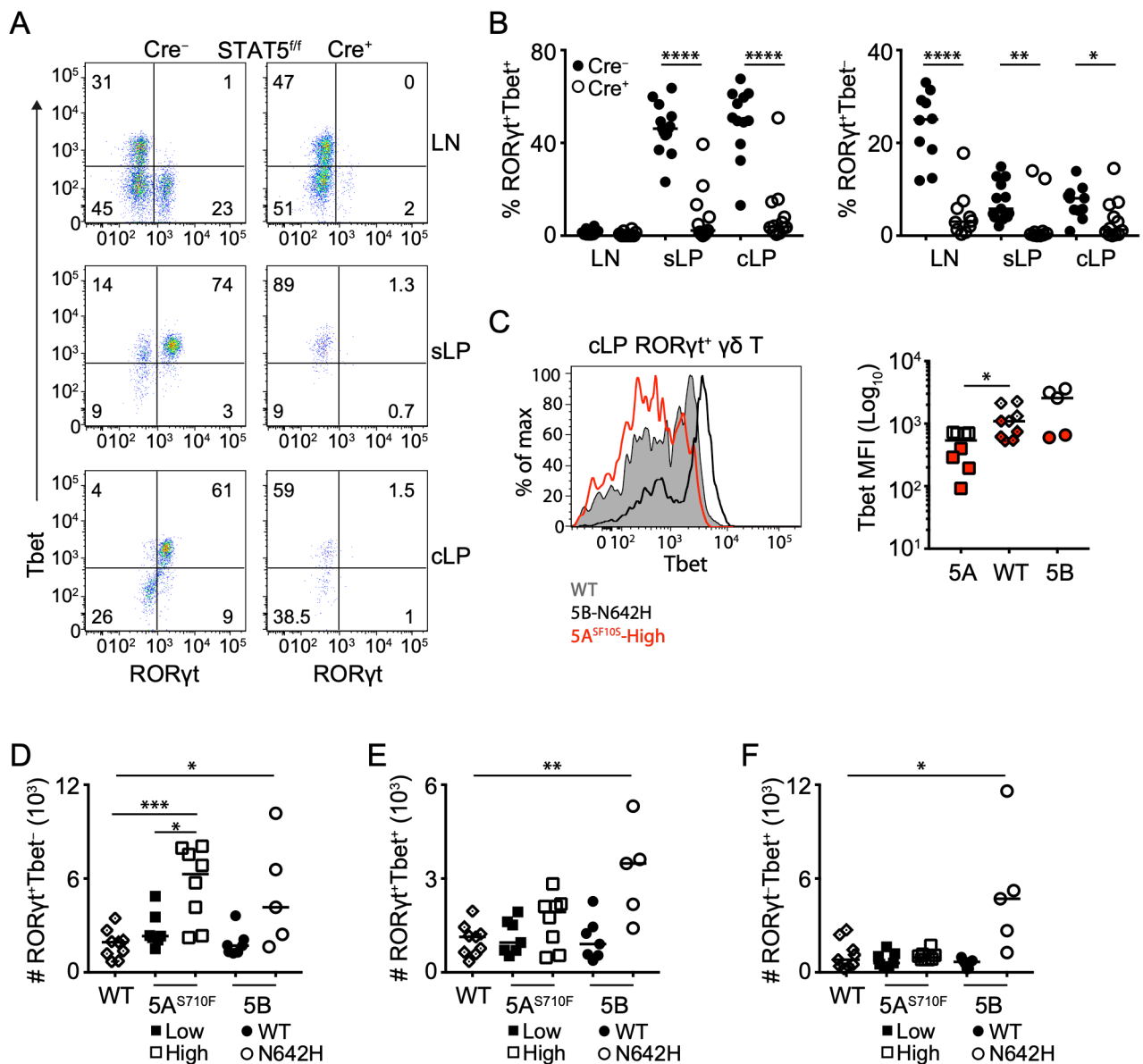
796 (A) Expression of CD44 and IFN $\gamma$  (dot plots) and frequency of IFN $\gamma$ <sup>+</sup>  $\gamma\delta$  T cells (graph) in  
 797 ROR $\gamma$ t<sup>CRE</sup>-RARdn<sup>F/F</sup> (Cre<sup>+</sup>) and littermate control mice (Cre<sup>-</sup>).

798 (B) Expression of IL-17A and IFN $\gamma$  (top dot plots) or IL-22 and IFN $\gamma$  (bottom dot plots) with  
 799 graphical representation of the frequency of IL-17A<sup>+</sup>IFN $\gamma$ <sup>+</sup> and IL-17A<sup>+</sup>IFN $\gamma$ <sup>-</sup> or IL-22<sup>+</sup>IFN $\gamma$ <sup>+</sup> and IL-  
 800 22<sup>+</sup>IFN $\gamma$ <sup>-</sup>  $\gamma\delta$  T cells in ROR $\gamma$ t<sup>CRE</sup>-RARdn<sup>F/F</sup> (Cre<sup>+</sup>) and littermate control mice (Cre<sup>-</sup>).

801

802

803 **Figure 6**



804 **Figure 6. STAT5 is a critical determinant of Tbet-expressing intestinal γδT17**

805 Flow cytometric analysis of intestinal γδ T cells in RORγt<sup>CRE</sup>-STAT5<sup>F/F</sup> (Cre<sup>+</sup>) and littermate control  
 806 mice (Cre<sup>-</sup>) in STAT5A and STAT5B hyperactive mutant mice as described in Figure 2. In graphs,  
 807 each symbol represents a mouse and line the median. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, \*\*\*\*p <  
 808 0.0001 using Mann-Whitney test.

809 (A) Expression of RORγt and Tbet within the γδ T cell compartment of the LN, sLP and cLP.

810 Numbers indicate percent of RORγt and Tbet expression.

811 (B) Frequency of ROR $\gamma$ t<sup>+</sup>Tbet<sup>+</sup> and ROR $\gamma$ t<sup>+</sup>Tbet<sup>-</sup> cells within the  $\gamma\delta$  T cell compartment in LN, sLP  
812 and cLP.

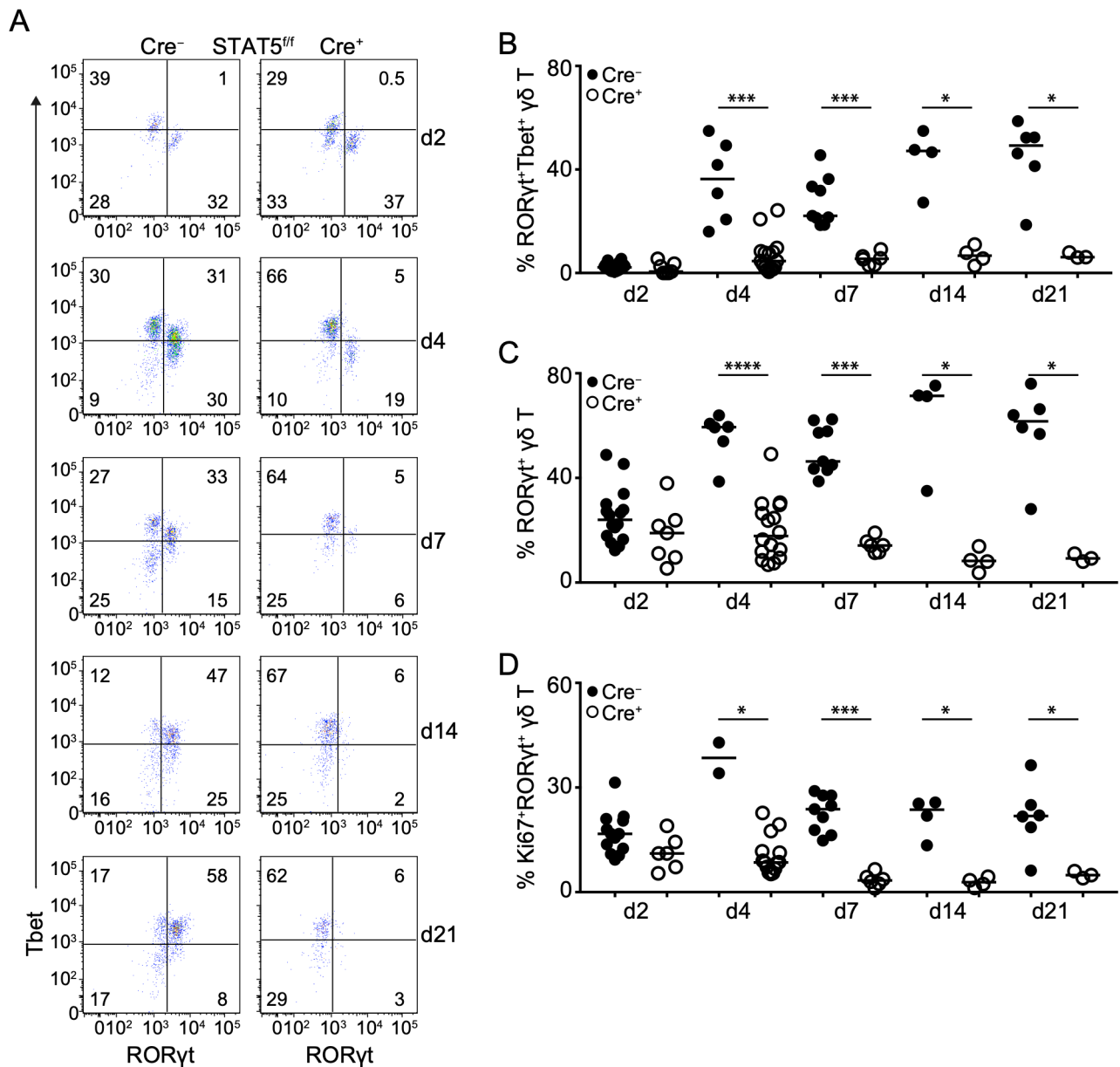
813 (C) Expression of Tbet (histogram) and Tbet mean fluorescent intensity (MFI) (graph) in ROR $\gamma$ t<sup>+</sup>  
814 cLP  $\gamma\delta$  T cells from WT, STAT5AS710F<sup>High</sup> (5a) or STAT5BN642H (5b) mice as described in  
815 Figure 2. In graph, colors indicate two different experiments.

816 (D) Numbers of ROR $\gamma$ t<sup>+</sup> (E), ROR $\gamma$ t<sup>+</sup>Tbet<sup>+</sup> (F) and ROR $\gamma$ t<sup>-</sup>Tbet<sup>+</sup>  $\gamma\delta$  T cells in the cLP of the  
817 indicated STAT5A and STAT5B hyperactive mutant mice or WT control mice.

818



819 **Figure 7**



820 **Figure 7. STAT5 regulates the neonatal fate of intestinal Tbet<sup>+</sup> γδT17 cells**

821 Flow cytometric analysis of colonic γδ T cells in RORγt<sup>CRE</sup>-STAT5<sup>F/F</sup> (Cre<sup>+</sup>) and littermate control  
 822 mice (Cre<sup>-</sup>) during neonatal ontogeny. Day of birth is counted as day(d)1. In graphs each symbol  
 823 represents a mouse and line the median. \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001, \*\*\*\*p < 0.0001 using  
 824 Mann-Whitney test.

825 (A) Expression of RORγt and Tbet within the γδ T cell compartment of cLP at the indicated days  
 826 after birth. Numbers indicate percent of RORγt and Tbet expression.

827 (B) Frequency of cLP RORγt<sup>+</sup>Tbet<sup>+</sup> γδ T cells at the indicated days after birth.

828 (C) Frequency of cLP ROR $\gamma$ t<sup>+</sup>  $\gamma$  $\delta$  T cells (including Tbet<sup>+</sup>) at the indicated days after birth.

829 (D) Frequency of cLP Ki67<sup>+</sup>ROR $\gamma$ t<sup>+</sup>  $\gamma$  $\delta$  T cells at the indicated days after birth.

830

831