The neonatal microenvironment programs conventional and intestinal Tbet⁺ γδT17 cells through the transcription factor STAT5

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Summary

Interleukin(IL)-17-producing RORγt⁺ γδ T (γδT17) cells develop in the embryonic thymus and participate in type 3 immune responses. Herein we show that γδT17 cells rapidly proliferate within neonatal lymph nodes and gut, where upon entry they uniquely upregulate Tbet and co-express IL-17, IL-22 and interferon(IFN) γ in a STAT3 and retinoic acid dependent manner. Neonatal expansion was halted in mice conditionally deficient in STAT5 and its loss resulted in γδT17 cell depletion from all adult organs. Hyperactive STAT5 mutant mice showed that the STAT5A homologue had a dominant role over STAT5B in promoting γδT17 cell expansion and downregulating gut-associated Tbet. In contrast, STAT5B preferentially expanded IFNγ-producing γδ populations. Importantly, mice lacking γδT17 cells due to STAT5 deficiency displayed a profound resistance to experimental autoimmune encephalomyelitis. Our data identify for the first time STAT5 as a key molecular checkpoint allowing γδT17 cells to pass through a critical neonatal developmental window to acquire tissue-specific characteristics essential for infection and 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 β 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 γ 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

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

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

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

Results

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

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

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

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

Differential regulation of $\gamma\delta\text{T17}$ and CD27^+ $\gamma\delta$ T cells by STAT5A and STAT5B

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

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

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

ROR γ t^{CRE}-STAT5^{F/F} mice are resistant to experimental autoimmune encephalomyelitis

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

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

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

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

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

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

interrogated mice possessing a RA receptor (RAR) dominant negative (RARdn) transgene, which prevents active RAR α signaling (Rajaii et al., 2008) in ROR γ t-expressing cells (ROR γ t^{CRE}-RARdn^{F/F} mice). We found that loss of RA signaling was associated with reduced overall expression of IFN γ (Fig. 5A and S4C) as well as reduced frequency of IL-17A⁺IFN γ ⁺ and IL-22⁺IFN γ ⁺ cells (Fig. 5B and S4D). In contrast, deficiency in RA signaling resulted in significantly increased frequency of IL-17A⁺IFN γ ⁻ and IL-22⁺IFN γ ⁻ Tbet⁺ γ δ T17 cells in the colon and small intestine (Fig. 5B and S4D). Collectively, this data indicates that lamina propria Tbet⁺ γ δ T17 cells are innate cells that can co-produce IL-17, IL-22 and IFN γ , and that their cytokine expression profile is regulated by STAT3 and RA.

STAT5 regulates Tbet expression and determines the progression of intestinal γ δ T17 cells through neonatal development

Following the identification of a distinct gut-specific γ δ T17 population, we aimed to understand their dependence on STAT5. Similar to LNs, ROR γ t-expressing γ δ T cells, irrespective of Tbet, were drastically and significantly reduced from the sLP and cLP of ROR γ t^{CRE}-STAT5^{F/F} mice (Fig. 6A-B). Analysis of GOF STAT5A and STAT5B mice revealed that hyperactive STAT5A downregulated Tbet in ROR γ t⁺ cLP γ δ T cells, whereas hyperactive STAT5B enhanced it (Fig. 6C) suggesting a YIN/YANG regulation in ROR γ t⁺ cLP γ δ T cells by STAT5A versus STAT5B. Hyperactive STAT5A preferentially expanded ROR γ t⁺ cells in the gut whereas hyperactive STAT5B favored Tbet-expressing γ δ T cells irrespective of whether they expressed ROR γ t or not (Fig. 6D-F).

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

225 ROR γ ^t $\gamma\delta$ T cell population after birth (Fig. 7A-C and S5A-C). However, despite their functional
226 presence in the neonatal gut, ROR γ ^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 γ -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 γ 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

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

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

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

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

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

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

References

Al Nabhani, Z., Dulauroy, S., Marques, R., Cousu, C., Al Bounny, S., Dejardin, F., Sparwasser, T., Berard, M., Cerf-Bensussan, N., and Eberl, G. (2019). A Weaning Reaction to Microbiota Is Required for Resistance to Immunopathologies in the Adult. *Immunity*.

- Bandapalli, O.R., Schuessele, S., Kunz, J.B., Rausch, T., Stutz, A.M., Tal, N., Geron, I., Gershman, N., Izraeli, S., Eilers, J., *et al.* (2014). The activating STAT5B N642H mutation is a common abnormality in pediatric T-cell acute lymphoblastic leukemia and confers a higher risk of relapse. *Haematologica* 99, e188-192.
- Bekiaris, V., Sedy, J.R., Macauley, M.G., Rhode-Kurnow, A., and Ware, C.F. (2013). The Inhibitory Receptor BTLA Controls gammadelta T Cell Homeostasis and Inflammatory Responses. *Immunity* 39, 1082-1094.
- Brown, C.C., Esterhazy, D., Sarde, A., London, M., Pullabhatla, V., Osma-Garcia, I., Al-Bader, R., Ortiz, C., Elgueta, R., Arno, M., *et al.* (2015). Retinoic acid is essential for Th1 cell lineage stability and prevents transition to a Th17 cell program. *Immunity* 42, 499-511.
- Cai, Y., Shen, X., Ding, C., Qi, C., Li, K., Li, X., Jala, V.R., Zhang, H.G., Wang, T., Zheng, J., and Yan, J. (2011). Pivotal role of dermal IL-17-producing gammadelta T cells in skin inflammation. *Immunity* 35, 596-610.
- Cai, Y., Xue, F., Fleming, C., Yang, J., Ding, C., Ma, Y., Liu, M., Zhang, H.G., Zheng, J., Xiong, N., and Yan, J. (2014). Differential developmental requirement and peripheral regulation for dermal Vgamma4 and Vgamma6T17 cells in health and inflammation. *Nature communications* 5, 3986.
- Cho, J.S., Pietras, E.M., Garcia, N.C., Ramos, R.I., Farzam, D.M., Monroe, H.R., Magorien, J.E., Blauvelt, A., Kolls, J.K., Cheung, A.L., *et al.* (2010). IL-17 is essential for host defense against cutaneous *Staphylococcus aureus* infection in mice. *J Clin Invest* 120, 1762-1773.
- Coffelt, S.B., Kersten, K., Doornebal, C.W., Weiden, J., Vrijland, K., Hau, C.S., Verstegen, N.J.M., Ciampricotti, M., Hawinkels, L., Jonkers, J., and de Visser, K.E. (2015). IL-17-producing gammadelta T cells and neutrophils conspire to promote breast cancer metastasis. *Nature* 522, 345-348.
- Cohen, A.C., Nadeau, K.C., Tu, W., Hwa, V., Dionis, K., Bezrodnik, L., Teper, A., Gaillard, M., Heinrich, J., Krensky, A.M., *et al.* (2006). Cutting edge: Decreased accumulation and regulatory function of CD4⁺ CD25(high) T cells in human STAT5b deficiency. *J Immunol* 177, 2770-2774.
- Conti, H.R., Peterson, A.C., Brane, L., Huppler, A.R., Hernandez-Santos, N., Whibley, N., Garg, A.V., Simpson-Abelson, M.R., Gibson, G.A., Mamo, A.J., *et al.* (2014). Oral-resident natural Th17 cells and gammadelta T cells control opportunistic *Candida albicans* infections. *J Exp Med* 211, 2075-2084.
- Cross, N.C.P., Hoade, Y., Tapper, W.J., Carreno-Tarragona, G., Fanelli, T., Jawhar, M., Naumann, N., Pieniak, I., Lubke, J., Ali, S., *et al.* (2019). Recurrent activating STAT5B N642H mutation in myeloid neoplasms with eosinophilia. *Leukemia* 33, 415-425.
- Cui, Y., Riedlinger, G., Miyoshi, K., Tang, W., Li, C., Deng, C.X., Robinson, G.W., and Hennighausen, L. (2004). Inactivation of Stat5 in mouse mammary epithelium during pregnancy reveals distinct functions in cell proliferation, survival, and differentiation. *Mol Cell Biol* 24, 8037-8047.
- de Araujo ED, E.F., Neubauer HA, Meneksedag-Erol D, Manaswiyoungkul P, Eram MS, Seo H, Qadree AK, Israelian J, Orlova A, Suske T, Pham HTT, Boersma A, Tangermann S, Kenner L, Rüllicke T, Dong A, Ravichandran M, Brown PJ, Audette GF, Rauscher S, Dhe-Paganon S, Moriggl R, Gunning PT (2019). Structural and Functional Consequences of the STAT5BN642H Driver Mutation. *Nature communications*.
- Eberl, G., and Littman, D.R. (2004). Thymic origin of intestinal alphabeta T cells revealed by fate mapping of RORgammat⁺ cells. *Science* 305, 248-251.

- 366 Guo, X., Qiu, J., Tu, T., Yang, X., Deng, L., Anders, R.A., Zhou, L., and Fu, Y.X. (2014). Induction
367 of innate lymphoid cell-derived interleukin-22 by the transcription factor STAT3 mediates protection
368 against intestinal infection. *Immunity* 40, 25-39.
- 369
- 370 Haas, J.D., Ravens, S., Duber, S., Sandrock, I., Oberdorfer, L., Kashani, E., Chennupati, V.,
371 Fohse, L., Naumann, R., Weiss, S., *et al.* (2012). Development of interleukin-17-producing
372 gammadelta T cells is restricted to a functional embryonic wave. *Immunity* 37, 48-59.
- 373
- 374 Haricharan, S., and Li, Y. (2014). STAT signaling in mammary gland differentiation, cell survival
375 and tumorigenesis. *Mol Cell Endocrinol* 382, 560-569.
- 376
- 377 Hirano, M., Guo, P., McCurley, N., Schorpp, M., Das, S., Boehm, T., and Cooper, M.D. (2013).
378 Evolutionary implications of a third lymphocyte lineage in lampreys. *Nature* 501, 435-438.
- 379
- 380 Hoelbl, A., Kovacic, B., Kerenyi, M.A., Simma, O., Warsch, W., Cui, Y., Beug, H., Hennighausen,
381 L., Moriggl, R., and Sexl, V. (2006). Clarifying the role of Stat5 in lymphoid development and
382 Abelson-induced transformation. *Blood* 107, 4898-4906.
- 383
- 384 Hwa, V. (2016). STAT5B deficiency: Impacts on human growth and immunity. *Growth Horm IGF*
385 *Res* 28, 16-20.
- 386
- 387 Imada, K., Bloom, E.T., Nakajima, H., Horvath-Arcidiacono, J.A., Udy, G.B., Davey, H.W., and
388 Leonard, W.J. (1998). Stat5b is essential for natural killer cell-mediated proliferation and cytolytic
389 activity. *J Exp Med* 188, 2067-2074.
- 390
- 391 Kanai, T., Jenks, J., and Nadeau, K.C. (2012). The STAT5b Pathway Defect and Autoimmunity.
392 *Frontiers in immunology* 3, 234.
- 393
- 394 Kofoed, E.M., Hwa, V., Little, B., Woods, K.A., Buckway, C.K., Tsubaki, J., Pratt, K.L., Bezrodnik,
395 L., Jasper, H., Tepper, A., *et al.* (2003). Growth hormone insensitivity associated with a STAT5b
396 mutation. *N Engl J Med* 349, 1139-1147.
- 397
- 398 Kucuk, C., Jiang, B., Hu, X., Zhang, W., Chan, J.K., Xiao, W., Lack, N., Alkan, C., Williams, J.C.,
399 Avery, K.N., *et al.* (2015). Activating mutations of STAT5B and STAT3 in lymphomas derived from
400 gammadelta-T or NK cells. *Nature communications* 6, 6025.
- 401
- 402 Laurence, A., Tato, C.M., Davidson, T.S., Kanno, Y., Chen, Z., Yao, Z., Blank, R.B., Meylan, F.,
403 Siegel, R., Hennighausen, L., *et al.* (2007). Interleukin-2 signaling via STAT5 constrains T helper
404 17 cell generation. *Immunity* 26, 371-381.
- 405
- 406 Lochner, M., Peduto, L., Cherrier, M., Sawa, S., Langa, F., Varona, R., Riethmacher, D., Si-Tahar,
407 M., Di Santo, J.P., and Eberl, G. (2008). In vivo equilibrium of proinflammatory IL-17+ and
408 regulatory IL-10+ Foxp3+ RORgamma t+ T cells. *J Exp Med* 205, 1381-1393.
- 409
- 410 Ma, C.A., Xi, L., Cauff, B., DeZure, A., Freeman, A.F., Hambleton, S., Kleiner, G., Leahy, T.R.,
411 O'Sullivan, M., Makiya, M., *et al.* (2017). Somatic STAT5b gain-of-function mutations in early onset
412 nonclonal eosinophilia, urticaria, dermatitis, and diarrhea. *Blood* 129, 650-653.
- 413
- 414 Malhotra, N., Narayan, K., Cho, O.H., Sylvia, K.E., Yin, C., Melichar, H., Rashighi, M., Lefebvre, V.,
415 Harris, J.E., Berg, L.J., *et al.* (2013). A network of high-mobility group box transcription factors
416 programs innate interleukin-17 production. *Immunity* 38, 681-693.
- 417
- 418 Maurer, B., Nivarthi, H., Wingelhofer, B., Pham, H.T.T., Schleiderer, M., Suske, T., Grausenburger,
419 R., Schiefer, A.I., Prchal-Murphy, M., Chen, D., *et al.* (2019). High activation of STAT5A drives
420 peripheral T-cell lymphoma and leukemia. *Haematologica*.

Michel, M.L., Pang, D.J., Haque, S.F., Potocnik, A.J., Pennington, D.J., and Hayday, A.C. (2012). Interleukin 7 (IL-7) selectively promotes mouse and human IL-17-producing gammadelta cells. *Proc Natl Acad Sci U S A* 109, 17549-17554.

Munoz-Ruiz, M., Ribot, J.C., Grosso, A.R., Goncalves-Sousa, N., Pamplona, A., Pennington, D.J., Regueiro, J.R., Fernandez-Malave, E., and Silva-Santos, B. (2016). TCR signal strength controls thymic differentiation of discrete proinflammatory gammadelta T cell subsets. *Nat Immunol* 17, 721-727.

Nairismagi, M.L., Tan, J., Lim, J.Q., Nagarajan, S., Ng, C.C., Rajasegaran, V., Huang, D., Lim, W.K., Laurensia, Y., Wijaya, G.C., *et al.* (2016). JAK-STAT and G-protein-coupled receptor signaling pathways are frequently altered in epitheliotropic intestinal T-cell lymphoma. *Leukemia* 30, 1311-1319.

Nicolae, A., Xi, L., Pittaluga, S., Abdullaev, Z., Pack, S.D., Chen, J., Waldmann, T.A., Jaffe, E.S., and Raffeld, M. (2014). Frequent STAT5B mutations in gammadelta hepatosplenic T-cell lymphomas. *Leukemia* 28, 2244-2248.

Onishi, M., Nosaka, T., Misawa, K., Mui, A.L., Gorman, D., McMahon, M., Miyajima, A., and Kitamura, T. (1998). Identification and characterization of a constitutively active STAT5 mutant that promotes cell proliferation. *Mol Cell Biol* 18, 3871-3879.

Papotto, P.H., Goncalves-Sousa, N., Schmolka, N., Iseppon, A., Mensurado, S., Stockinger, B., Ribot, J.C., and Silva-Santos, B. (2017a). IL-23 drives differentiation of peripheral gammadelta17 T cells from adult bone marrow-derived precursors. *EMBO Rep*.

Papotto, P.H., Ribot, J.C., and Silva-Santos, B. (2017b). IL-17+ gammadelta T cells as kick-starters of inflammation. *Nat Immunol* 18, 604-611.

Petermann, F., Rothhammer, V., Claussen, M.C., Haas, J.D., Blanco, L.R., Heink, S., Prinz, I., Hemmer, B., Kuchroo, V.K., Oukka, M., and Korn, T. (2010). gammadelta T cells enhance autoimmunity by restraining regulatory T cell responses via an interleukin-23-dependent mechanism. *Immunity* 33, 351-363.

Pham, H.T.T., Maurer, B., Prchal-Murphy, M., Grausenburger, R., Grundschober, E., Javaheri, T., Nivarthi, H., Boersma, A., Kolbe, T., Elabd, M., *et al.* (2018). STAT5BN642H is a driver mutation for T cell neoplasia. *J Clin Invest* 128, 387-401.

Rajaii, F., Bitzer, Z.T., Xu, Q., and Sockanathan, S. (2008). Expression of the dominant negative retinoid receptor, RAR403, alters telencephalic progenitor proliferation, survival, and cell fate specification. *Dev Biol* 316, 371-382.

Rajala, H.L., Eldfors, S., Kuusanmaki, H., van Adrichem, A.J., Olson, T., Lagstrom, S., Andersson, E.I., Jerez, A., Clemente, M.J., Yan, Y., *et al.* (2013). Discovery of somatic STAT5b mutations in large granular lymphocytic leukemia. *Blood* 121, 4541-4550.

Rani, A., and Murphy, J.J. (2016). STAT5 in Cancer and Immunity. *J Interferon Cytokine Res* 36, 226-237.

Sagaidak, S., Taibi, A., Wen, B., and Comelli, E.M. (2016). Development of a real-time PCR assay for quantification of *Citrobacter rodentium*. *J Microbiol Methods* 126, 76-77.

Sandrock, I., Reinhardt, A., Ravens, S., Binz, C., Wilharm, A., Martins, J., Oberdorfer, L., Tan, L., Lienenklaus, S., Zhang, B., *et al.* (2018). Genetic models reveal origin, persistence and non-redundant functions of IL-17-producing gammadelta T cells. *J Exp Med* 215, 3006-3018.

- 476 Sheridan, B.S., Romagnoli, P.A., Pham, Q.M., Fu, H.H., Alonzo, F., 3rd, Schubert, W.D., Freitag,
477 N.E., and Lefrancois, L. (2013). gammadelta T cells exhibit multifunctional and protective memory
478 in intestinal tissues. *Immunity* 39, 184-195.
- 479
- 480 Spidale, N.A., Sylvia, K., Narayan, K., Miu, B., Frascoli, M., Melichar, H.J., Zhihao, W., Kisielow, J.,
481 Palin, A., Serwold, T., *et al.* (2018). Interleukin-17-Producing gammadelta T Cells Originate from
482 SOX13(+) Progenitors that Are Independent of gammadeltaTCR Signaling. *Immunity* 49, 857-872
483 e855.
- 484
- 485 Stark, G.R., and Darnell, J.E., Jr. (2012). The JAK-STAT pathway at twenty. *Immunity* 36, 503-
486 514.
- 487
- 488 Sumaria, N., Grandjean, C.L., Silva-Santos, B., and Pennington, D.J. (2017). Strong
489 TCRgammadelta Signaling Prohibits Thymic Development of IL-17A-Secreting gammadelta T
490 Cells. *Cell reports* 19, 2469-2476.
- 491
- 492 Sutherland, T.E., Logan, N., Ruckerl, D., Humbles, A.A., Allan, S.M., Papayannopoulos, V.,
493 Stockinger, B., Maizels, R.M., and Allen, J.E. (2014). Chitinase-like proteins promote IL-17-
494 mediated neutrophilia in a tradeoff between nematode killing and host damage. *Nat Immunol* 15,
495 1116-1125.
- 496
- 497 Sutton, C.E., Lalor, S.J., Sweeney, C.M., Brereton, C.F., Lavelle, E.C., and Mills, K.H. (2009).
498 Interleukin-1 and IL-23 induce innate IL-17 production from gammadelta T cells, amplifying Th17
499 responses and autoimmunity. *Immunity* 31, 331-341.
- 500
- 501 Torow, N., and Hornef, M.W. (2017). The Neonatal Window of Opportunity: Setting the Stage for
502 Life-Long Host-Microbial Interaction and Immune Homeostasis. *J Immunol* 198, 557-563.
- 503
- 504 Tripathi, P., Kurtulus, S., Wojciechowski, S., Sholl, A., Hoebe, K., Morris, S.C., Finkelman, F.D.,
505 Grimes, H.L., and Hildeman, D.A. (2010). STAT5 is critical to maintain effector CD8+ T cell
506 responses. *J Immunol* 185, 2116-2124.
- 507
- 508 Turchinovich, G., and Hayday, A.C. (2011). Skint-1 identifies a common molecular mechanism for
509 the development of interferon-gamma-secreting versus interleukin-17-secreting gammadelta T
510 cells. *Immunity* 35, 59-68.
- 511
- 512 Villarino, A., Laurence, A., Robinson, G.W., Bonelli, M., Dema, B., Afzali, B., Shih, H.Y., Sun,
513 H.W., Brooks, S.R., Hennighausen, L., *et al.* (2016). Signal transducer and activator of
514 transcription 5 (STAT5) paralog dose governs T cell effector and regulatory functions. *eLife* 5.
- 515
- 516 Villarino, A.V., Sciume, G., Davis, F.P., Iwata, S., Zitti, B., Robinson, G.W., Hennighausen, L.,
517 Kanno, Y., and O'Shea, J.J. (2017). Subset- and tissue-defined STAT5 thresholds control
518 homeostasis and function of innate lymphoid cells. *J Exp Med* 214, 2999-3014.
- 519
- 520 Wagatsuma, K., Tani-ichi, S., Liang, B., Shitara, S., Ishihara, K., Abe, M., Miyachi, H., Kitano, S.,
521 Hara, T., Nanno, M., *et al.* (2015). STAT5 Orchestrates Local Epigenetic Changes for Chromatin
522 Accessibility and Rearrangements by Direct Binding to the TCRgamma Locus. *J Immunol* 195,
523 1804-1814.
- 524
- 525 Wencker, M., Turchinovich, G., Di Marco Barros, R., Deban, L., Jandke, A., Cope, A., and Hayday,
526 A.C. (2014). Innate-like T cells straddle innate and adaptive immunity by altering antigen-receptor
527 responsiveness. *Nat Immunol* 15, 80-87.
- 528
- 529 Wu, P., Wu, D., Ni, C., Ye, J., Chen, W., Hu, G., Wang, Z., Wang, C., Zhang, Z., Xia, W., *et al.*
530 (2014). gammadeltaT17 cells promote the accumulation and expansion of myeloid-derived
531 suppressor cells in human colorectal cancer. *Immunity* 40, 785-800.

Yao, Z., Cui, Y., Watford, W.T., Bream, J.H., Yamaoka, K., Hissong, B.D., Li, D., Durum, S.K., Jiang, Q., Bhandoola, A., *et al.* (2006). Stat5a/b are essential for normal lymphoid development and differentiation. *Proc Natl Acad Sci U S A* 103, 1000-1005.

Yao, Z., Kanno, Y., Kerenyi, M., Stephens, G., Durant, L., Watford, W.T., Laurence, A., Robinson, G.W., Shevach, E.M., Moriggl, R., *et al.* (2007). Nonredundant roles for Stat5a/b in directly regulating Foxp3. *Blood* 109, 4368-4375.

Yu, F., Sharma, S., Edwards, J., Feigenbaum, L., and Zhu, J. (2015). Dynamic expression of transcription factors T-bet and GATA-3 by regulatory T cells maintains immunotolerance. *Nat Immunol* 16, 197-206.

Zuberbuehler, M.K., Parker, M.E., Wheaton, J.D., Espinosa, J.R., Salzler, H.R., Park, E., and Ciofani, M. (2019). The transcription factor c-Maf is essential for the commitment of IL-17-producing gammadelta T cells. *Nat Immunol* 20, 73-85.

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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 γ ^{CRE} and ROR γ ^{GFP} mice (Lochner et al., 2008)
 572 were provided by Professor Gerard Eberl and subsequently bred in house. STAT5^{F/F} mice were
 573 generated as previously described (Cui et al., 2004) and bred in house. STAT3^{F/F} mice were
 574 purchased from The Jackson Laboratory and bred in house. RAR α ^{F/F} 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^{-/-} 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 μ 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 μ 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 2.5×10^6 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

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

Experimental Autoimmune Encephalomyelitis

EAE was induced by sub-cutaneous injection of 50µg of MOG35-55 peptide in CFA, while 2 ng pertussis toxin were intra-peritoneally (i.p.) injected on the day of immunization and 2 days later. From day 11 after immunization and until day 21, mice were weighed and scored for clinical signs as follows: 0: no symptoms; 1: tail paralysis; 1.5: impaired righting reflex; 2: paralysis of one hind limb; 2.5: paralysis of both hind limbs; 3: paralysis of one fore limb; 3.5: paralysis of one fore limb and weak second for limb; 4: total limb paralysis.

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

***In vitro* stimulation of lymphocytes**

For LN lymphocytes, 10⁷ cells were cultured for 3.5 hours in the presence of 50ng/ml PMA (Sigma), 750ng/ml ionomycin (Sigma) and 1µL /mL BD Golgistop (containing monensin). Single cell suspensions from intestinal lamina propria were stimulated with 40 ng/ml of IL-23 (R&D Systems) for 3 hours followed by 50ng/ml PMA, 750ng/ml ionomycin and 1µL /mL BD Golgistop for an additional 3 hours. After 6 hours, cells were harvested and washed with PBS and used for flow cytometry staining. FoxP3 transcription factor staining kit (eBiosciences)

Flow Cytometry

Cells were harvested by centrifugation at 400 g for 5 minutes at 4°C followed by staining with fixable viability stain (BD Horizon FVS700) for 10 minutes on ice in PBS. Subsequently, surface 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, 1×10^6 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⁺CD45.2⁺ mice were lethally irradiated (900 rad). Next day they were injected
681 intravenously with 10×10⁶ cells of whole bone marrow cells from CD45.1⁺ wild-type and CD45.2⁺
682 RORγt^{CRE}-STAT5^{F/F} 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 5×10⁶ 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

Figures and Legends

Figure 1

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

Flow cytometric analysis of γδ T cells in RORγt^{CRE}-STAT5^{F/F} (Cre⁺) and littermate control mice (Cre⁻). In graphs each symbol represents a mouse and line the median. **p < 0.01, ***p < 0.001, ****p < 0.0001 using Mann-Whitney test.

(A) Expression of CD27 and CD44 in order to identify CD27⁺CD44⁺ γδT17 cells in the LN.

Numbers indicate percent of CD27⁺CD44⁺ within the γδ T cell compartment.

(B) Numbers of γδT17 cells in the LN (staining as in A) and skin. In the skin, γδT17 cells were identified as CD45⁺CD3^{Low}Vγ5⁺TCRβ⁺TCRγδ⁺CCR6⁺.

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

(D) Numbers of CD27⁺CD44⁺ γδT17 cells in day 2 and day 7 old thymi.

(E) Numbers of CD27⁺CD44⁺ γδT17 cells in day 7 and day 14 old LN.

730 (F) Frequency of Ki67⁺RORγt⁺ or Ki67⁺CCR6⁺ γδT17 cells within the CD44⁺TCRγδ⁺ 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⁺ $\gamma\delta$ 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^{Low}) or
 738 high (STAT5AS710F^{High}) 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⁻CD44⁺ γδT17 cells in the LN.
743 Numbers indicate percent of CD27⁻CD44⁺ or CD27⁺CD44⁻ within the γδ T cell compartment.

744 (B) Numbers of γδT17 (staining as in A) and CD27⁺ cells in the LN (** in WT denotes difference by
745 comparison to STAT5BN642H).

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

747 (D) Expression of CCR6 and Vγ4 in skin γδT17 cells (staining as in Fig. 1B). Numbers indicate
748 percent of CCR6⁺ or CCR6⁻ cells within the γδ T cell compartment.

749 (E) Numbers of CCR6⁺ and CCR6⁻ cells identified in D (* in STATBBN642H denotes significant
750 difference by comparison to WT). (F) Ratio of CCR6⁺ over CCR6⁻ cells in WT compared to
751 STAT5AS710F^{High} (5A) and STAT5BN642H (5B) mice.

752

753

754 **Figure 3**

755

756 **Figure 3. RORγt^{CRE}-STAT5^{F/F} mice are resistant to EAE**

757 Disease progression and flow cytometric analysis of γδ and CD4⁺ T cells in RORγt^{CRE}-STAT5^{F/F}
 758 (Cre⁺) and littermate control mice (Cre⁻) that had been previously immunized with 50 μg 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±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 γδT17 cells in the LN (staining as in Fig. 1A) of unimmunized controls (EAE⁻)
 766 and at 11 (B) and 21 (C) days after immunization.

767 (D) Numbers of γδT17 cells in the brain (identified as CD45⁺CD3⁺TCRβ⁻TCRγδ⁺CD44⁺) at days 11
 768 and 21 after immunization.

769 (E) Expression of IL-17A within the LN γδ T cell compartment of unimmunized controls (EAE⁻) and
 770 at 11 and 21 days after immunization. (F) Numbers of CD4⁺CD44⁺CD3⁺TCRβ⁺ 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 $ROR\gamma t^{Cre}-STAT3^{F/F}$ (Cre^+) and
 775 littermate control mice (Cre^-). 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 γ t and Tbet within the $\gamma\delta$ T cell compartment of the LN, sLP and cLP.
779 Numbers indicate percent of ROR γ t and Tbet expression.

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

782 (C) Expression of ROR γ t and IL-17A, ROR γ t and IL-22, or IL-17A and IFN γ 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 γ (bottom) in ROR γ t^{CRE}-STAT3^{F/F} (Cre⁺)
785 and littermate control mice (Cre⁻). Numbers indicate percent of positive expression.

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

Figure 5

Figure 5. Retinoic acid receptor signaling regulates IFNγ production in intestinal Tbet⁺ γδT17 cells

Flow cytometric analysis of cytokine expression in colonic γδ T cells in RORγt^{CRE}-RARdn^{F/F} (Cre⁺) and littermate control mice (Cre⁻) following IL-23 stimulation. In graphs, each symbol represents a mouse and line the median. *p < 0.05, **p < 0.01, ***p < 0.001 using Mann-Whitney test.

(A) Expression of CD44 and IFNγ (dot plots) and frequency of IFNγ⁺ γδ T cells (graph) in RORγt^{CRE}-RARdn^{F/F} (Cre⁺) and littermate control mice (Cre⁻).

(B) Expression of IL-17A and IFNγ (top dot plots) or IL-22 and IFNγ (bottom dot plots) with graphical representation of the frequency of IL-17A⁺IFNγ⁺ and IL-17A⁺IFNγ⁻ or IL-22⁺IFNγ⁺ and IL-22⁺IFNγ⁻ γδ T cells in RORγt^{CRE}-RARdn^{F/F} (Cre⁺) and littermate control mice (Cre⁻).

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^{CRE}-STAT5^{F/F} (Cre⁺) and littermate control
806 mice (Cre⁻) 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 γ t⁺Tbet⁺ and ROR γ t⁺Tbet⁻ 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 γ t⁺
814 cLP $\gamma\delta$ T cells from WT, STAT5AS710F^{High} (5a) or STAT5BN642H (5b) mice as described in
815 Figure 2. In graph, colors indicate two different experiments.

816 (D) Numbers of ROR γ t⁺ (E), ROR γ t⁺Tbet⁺ (F) and ROR γ t⁻Tbet⁺ $\gamma\delta$ 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⁺ γδT17 cells**

821 Flow cytometric analysis of colonic γδ T cells in RORyt^{CRE}-STAT5^{F/F} (Cre⁺) and littermate control
822 mice (Cre⁻) 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 RORyt and Tbet within the γδ T cell compartment of cLP at the indicated days
826 after birth. Numbers indicate percent of RORyt and Tbet expression.

827 (B) Frequency of cLP RORyt⁺Tbet⁺ γδ T cells at the indicated days after birth.

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

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

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