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## **Fate mapping of interleukin 17-producing T cells in inflammatory responses**

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## ABSTRACT

We describe a reporter mouse strain designed to fate-map cells that have activated IL-17A. Here we show that T<sub>H</sub>17 cells show distinct plasticity in different inflammatory settings. Chronic inflammatory conditions in EAE caused a switch to alternative cytokines in T<sub>H</sub>17 cells, whereas acute cutaneous infection with *Candida albicans*, did not result in deviation of T<sub>H</sub>17 to alternative cytokine production, although IL-17A production was shut off in the course of the infection. During development of EAE, IFN- $\gamma$  and other pro-inflammatory cytokines in the spinal cord were produced almost exclusively by 'ex-T<sub>H</sub>17' cells whose conversion was driven by IL-23. Thus, this model allows relating the actual functional fate of effector T cells to T<sub>H</sub>17 developmental origin irrespective of IL-17 expression.

## INTRODUCTION

IL-17-producing T<sub>H</sub>17 cells, were classified as a new effector CD4<sup>+</sup> T cells subset on the basis of being independent of the transcription factors GATA-3 and T-bet that together with the marker cytokines interferon  $\gamma$  (IFN- $\gamma$ ) and IL-4 define T<sub>H</sub>1 and T<sub>H</sub>2 cells respectively<sup>1,2</sup>. The identification of IL-6 and TGF- $\beta$  as differentiation factors<sup>3</sup> as well as ROR $\gamma$ t and ROR $\alpha$  as lineage-defining transcription factors<sup>4,5</sup> finalized acceptance of T<sub>H</sub>17 as a separate subset. Nevertheless, it was clear early on that T<sub>H</sub>17 cells displayed considerable plasticity and readily acquired the capacity to produce IFN- $\gamma$  in addition to IL-17 production or completely shut off IL-17 production *in vitro*<sup>6-10</sup>, explaining the initial erroneous assumption that these cells may have diverged from a common T<sub>H</sub>1 precursor<sup>11</sup>. While extensive plasticity was attributed to *in vitro*-generated T<sub>H</sub>17 cells, it was suggested that T<sub>H</sub>17 cells developing *in vivo* retain their phenotype<sup>12</sup>. As many additional stimuli influence T<sub>H</sub>17 differentiation, including cytokines as well as environmental factors acting through the aryl hydrocarbon receptor (reviewed in<sup>13</sup>), it is conceivable that the requirements for full effector differentiation of T<sub>H</sub>17 cells are not met *in vitro*. Thus, it would be advantageous to analyse more closely T<sub>H</sub>17 cells that have developed *in vivo* to determine whether plasticity is also detectable under these conditions. We therefore decided to generate a T<sub>H</sub>17 reporter system that would allow not only identification, but also fate mapping of these cells *in vivo*. To trace noninvasively cells expressing IL-17, we inserted a *Cre* recombinase into the *Il17a* locus (*Il17a*<sup>Cre</sup>) and crossed these mice to *Rosa26*-enhanced yellow fluorescence protein (*R26R*<sup>eYFP</sup>) reporter mice. In this mouse strain the fluorescent reporter would permanently label *Il17*<sup>Cre</sup> cells,

thus allowing identification of cells that switched on the IL-17 program as well as alternative effector programs that might have been activated.

Here we show that eYFP expression was induced exclusively under T<sub>H</sub>17 conditions and gradually increased during effector differentiation *in vivo* and terminally differentiated effector cells all co-expressed IL-17 and eYFP. However, T<sub>H</sub>17 cells rapidly lost IL-17A expression in the course of inflammatory immune responses *in vivo*. Fate determination of eYFP<sup>+</sup> ‘ex-T<sub>H</sub>17’ cells under different inflammatory conditions revealed distinct capacities for alternative fates depending on whether the inflammatory conditions were chronic or acute and rapidly resolved. For instance, in the course of experimental autoimmune encephalomyelitis (EAE) induction eYFP<sup>+</sup> cells resident in the inflamed spinal cord did not express IL-17A protein anymore and a considerable proportion of instead produced IFN- $\gamma$ . In contrast, in acute cutaneous infection with *Candida albicans*, IL-17A production also was shut down, but there was no deviation of eYFP<sup>+</sup> cells to IFN- $\gamma$  production. In this model eYFP<sup>+</sup> IL-17<sup>-</sup> cells appeared quiescent with respect to cytokine production and rapidly decayed.

This suggests that the fate decisions of T<sub>H</sub>17 cells are shaped by different inflammatory conditions *in vivo* allowing distinct patterns of plasticity. Whereas pathogenicity in chronic inflammatory conditions is linked to the expression of additional pro-inflammatory cytokines, clearance of an infection that results in resolution creates an anti-inflammatory environment that precludes T<sub>H</sub>17 plasticity and the adoption of alternative cytokines.

## RESULTS

### Generation of IL-17A fate reporter mouse

To obtain an IL-17A-specific reporter that would allow tracing of *Il17a* expressing cells we generated a ‘knockin’ mouse strain bearing Cre recombinase in the *Il17a* gene locus (*Il17a*<sup>Cre</sup>, **Supplementary Fig.1**). Cre activity was monitored by breeding with R26R<sup>eYFP</sup> reporter mice, thus allowing the visualization of cells that had activated the IL-17 program irrespective of current production of this cytokine. Analysis of tissues from non-immune reporter mice revealed eYFP<sup>+</sup> cells in haematopoietic cells of spleen and small intestine lamina propria in line with previously described distribution of IL-17A-producing T cells both of CD4<sup>+</sup> and  $\gamma\delta$  T cell lineage<sup>4</sup>. Also a CD4<sup>-</sup>CD8<sup>-</sup> T cell receptor-positive NKT population in the spleen was marked by eYFP, but no reporter expression in NKp46<sup>+</sup> cells was detected (**Supplementary Fig.2a**). A CD45<sup>+</sup> population of lineage-negative cells containing lymphoid tissue-inducer-like (LTi) cells (reviewed in <sup>14</sup>) some of which expressed CD4<sup>+</sup> was also found marked by eYFP (**Supplementary Fig.2b**). In the skin a sizeable fraction of  $\gamma\delta$  T cells, but very few CD4<sup>+</sup> T cells, were eYFP<sup>+</sup>, (**Supplementary Fig.2c**).

*In vitro* stimulation of FACS purified naïve CD4<sup>+</sup> T cells under T<sub>H</sub>17 conditions generated a population of T<sub>H</sub>17 cells that were detectable by intracellular staining for IL-17A as well as eYFP expression. There was no induction of eYFP under conditions that led to T<sub>H</sub>1, T<sub>H</sub>2, T<sub>H</sub>9 or iTreg polarization (**Fig.1a**). Intracellular IL-17 expression without eYFP expression was exaggerated following restimulation with PdBU-ionomycin, which may induce early commitment to IL-17 production before full effector

status is achieved. In contrast anti-CD3 stimulation showed a higher concordance between IL-17 and YFP expression (**Supplementary Fig.3**).

To investigate whether this discrepancy was caused by aberrant expression of eYFP from the recombined  $R26R^{eYFP}$  locus, we performed PCR from genomic DNA isolated from purified  $eYFP^- CD4^+ IL-17^+$  T cells (**Fig.1b gate 1**),  $eYFP IL-17A^- CD4^+$  T cells (**Fig.1b gate2**) as well as  $eYFP^+ CD4^+$  T cells (**Fig.1b gate 3**). Analysis of the  $R26R26^{eYFP}$  locus showed that  $eYFP^- IL-17A^+$  cells had not undergone Cre-mediated recombination (gate 1) similar to cells that had not switched on IL-17A production at all (gate 2) and in marked contrast to  $eYFP^+$  cells in gate 3. Since the amount of intracellular IL-17A protein was lower in  $T_H17$  cells that failed to express eYFP, it suggests that levels of IL-17A transcription and therefore protein concentrations of Cre-recombinase were below a critical threshold to induce recombination (**Fig.1c top panel**). In line with this assumption, intracellular staining for Cre protein was much lower in  $eYFP^- IL-17$ -producing cells compared with  $IL-17^+ eYFP^+$  cells (**Fig.1c bottom panel**). About 50% of PdbU-ionomycin-stimulated  $T_H17$  cells isolated from naive mice co-expressed eYFP and IL-17A *ex vivo*, whereas more than 70% of  $\gamma\delta$  T cells showed co-expression of IL-17A and eYFP. Neither  $CD4^+$  nor  $\gamma\delta eYFP^+$  cells isolated from naive mice expressed IFN- $\gamma$  (**Fig. 1d,e**). Thus, IL-17A is faithfully marked by eYFP once  $T_H17$  cells have acquired full effector function.

### ***In vivo* kinetics of eYFP and IL-17 expression**

To evaluate the kinetics of eYFP reporter expression and the stability of IL-17 cytokine expression *in vivo*, mice were immunized with myelin oligodendrocyte glycoprotein (MOG) peptide in complete Freund's adjuvant (CFA) to induce experimental autoimmune encephalomyelitis (EAE), a T<sub>H</sub>17-dependent chronic autoimmune disease. IL-17A protein and eYFP expression in CD4<sup>+</sup> T cells and  $\gamma\delta$  T cells isolated from draining lymph nodes of MOG-CFA immunized mice are shown (**Fig.2a and Supplementary.Fig.4a**). In both T cell subsets the percentage of double IL-17A eYFP expressing cells increased over time, with co-expression more pronounced and complete in  $\gamma\delta$  T cells than in T<sub>H</sub>17 cells. Absolute numbers of eYFP<sup>+</sup> CD4<sup>+</sup> T<sub>H</sub>17 cells increased over time, whereas eYFP<sup>+</sup>  $\gamma\delta$  T cells, although becoming the dominant population within the total  $\gamma\delta$  T cell pool, did not increase in absolute numbers (**Fig.2b**). In the spinal cord of mice that succumbed to disease the vast majority of IL-17A-expressing T cells also expressed eYFP (**Fig.2c,d and Supplementary Fig.4b**), but  $\gamma\delta$  T cells represented only a minority of infiltrating T cells. Staining for Ki-67, a cellular marker for proliferation showed eYFP<sup>+</sup> T<sub>H</sub>17 cells from the draining lymph node exhibited an increased proliferation rate compared to eYFP<sup>-</sup> cells at day 12 (**Fig.2e**). In contrast basal proliferation in non-immune mice (day 0) and proliferation in the spinal cord were similar in eYFP<sup>-</sup> and eYFP<sup>+</sup> cells.

A sizeable fraction of the eYFP<sup>+</sup> T<sub>H</sub>17 cells in the draining lymph nodes were already negative for IL-17A early after immunization whereas most  $\gamma\delta$  T cells remained double positive for eYFP and IL-17A (**Fig.2a**). Similarly, in the spinal cord of mice with disease



(around day 15) only a fraction of the CD4<sup>+</sup> eYFP<sup>+</sup> cells still expressed IL-17A protein, whereas most  $\gamma\delta$  T cells remained IL-17<sup>+</sup> (**Fig.2c**).

### **IFN- $\gamma$ expression by eYFP<sup>+</sup> ‘ex-T<sub>H</sub>17’ cells in EAE**

Other reports have shown the presence of IL-17A and IFN- $\gamma$  double producers in inflamed tissues<sup>4,15</sup>, but their developmental origin has remained unclear. We therefore tested the source of IFN- $\gamma$  producers in lymph nodes and spinal cord of MOG-CFA immunized mice. At day 3, CD4<sup>+</sup> T cells in the draining lymph nodes expressed little IFN- $\gamma$  and originated exclusively from eYFP<sup>-</sup> T<sub>H</sub>1 cells. A higher proportion of  $\gamma\delta$  T cells expressed IFN- $\gamma$ , again mostly from the eYFP<sup>-</sup> fraction (**Fig.3a left panels**). IFN- $\gamma$  expression increased over time primarily in eYFP<sup>+</sup> CD4<sup>+</sup> T cells, whereas IFN- $\gamma$  expression decreased in  $\gamma\delta$  T cells (**Fig.3a middle and right panels and Supplementary Fig.4a**). We compared the effect of restimulation by PdBU-ionomycin with antigen-specific restimulation using MOG peptide or anti-CD3 restimulation and observed that antigen-specific re-stimulation of cells from draining lymph nodes resulted in much higher concordance between IL-17 expression and eYFP expression (representative FACS plots in **Supplementary Fig.5a**). In the spinal cord, half of the eYFP<sup>+</sup> CD4<sup>+</sup> T cells switched off IL-17A expression and virtually all IFN- $\gamma$  in CD4<sup>+</sup> T cells was derived from eYFP<sup>+</sup> ‘ex-T<sub>H</sub>17’ cells. In contrast, eYFP<sup>+</sup>  $\gamma\delta$  T cells remained IL-17A<sup>+</sup> and did not switch to IFN- $\gamma$  expression (**Fig.3c and Supplementary Fig.5b**). Restimulation with MOG peptide compared with PdBU-ionomycin (**Fig.3d**) confirmed that MOG -specific IFN- $\gamma$  was mostly derived from ‘ex-T<sub>H</sub>17’ cells with only a minute fraction of eYFP<sup>-</sup> T<sub>H</sub>1 cells contributing to expression of this cytokine. There was no switch of eYFP<sup>+</sup> cells to

Foxp3 expression showing that eYFP<sup>+</sup> cells that cease production of IL-17 do not deviate towards a regulatory phenotype (**Supplementary Fig.5c**). Thus, our data show that under chronic inflammatory conditions in EAE, T<sub>H</sub>17 cells rapidly switch to production of IFN- $\gamma$  and represent the major source of this cytokine.

### **CD4<sup>+</sup> T cell cytokine production during EAE**

We next analysed both eYFP<sup>+</sup> and eYFP<sup>-</sup> CD4<sup>+</sup> T cells isolated from lymph nodes 6 days after MOG-CFA immunization and from spinal cord (day 15) to evaluate expression of additional cytokines. FACS plots and bar graphs are shown (**Fig 4 and Supplementary Fig 6**).

The majority of eYFP<sup>+</sup> cells from lymph nodes of non-immunized mice expressed IL-17 without any IFN- $\gamma$  and also showed some co-expression of GM-CSF, TNF, IL-2, IL-17F but very little IL-22 (**Fig.4a top panels**). A proportion of eYFP<sup>+</sup> cells acquired IFN- $\gamma$  expression at day 6 after immunization. These cells mostly co-expressed IL-17A and exhibited substantially increased expression of GM-CSF, IL-2 and IL-22. All IL-17<sup>+</sup> cells co-expressed TNF at that stage, whereas expression of IL-17F remained at baseline levels (**Fig.4a middle panels**). In the spinal cord IL-17A expression was reduced and although co-expression of IL-17A with IFN- $\gamma$ , GM-CSF and TNF was still evident, a sizeable proportions of cell produced the latter cytokines in the absence of IL-17A expression. Interestingly there was no IL-17F expression in the spinal cord population and greatly reduced proportions of IL-22-producing cells (**Fig.4a bottom panel**). With the exception of TNF and IL-2, the eYFP<sup>-</sup> fraction showed low expression of all cytokines (**Fig.4b**).

Measurement of cytokine protein concentrations from sorted, restimulated eYFP<sup>+</sup> and eYFP<sup>-</sup> cells confirmed predominant cytokine secretion from the eYFP<sup>+</sup> population. IL-17A, IFN- $\gamma$ , GM-CSF and TNF were about 10-fold higher in the spinal cord compared to draining lymph nodes despite the lower expression profiles seen by intracellular staining (**Fig.4c**). The progressive development of eYFP<sup>+</sup> cells from predominantly IFN- $\gamma$ <sup>-</sup> IL-17A-secreting cells towards IFN- $\gamma$ <sup>+</sup> IL-17A<sup>+</sup> cells and finally to IFN- $\gamma$  expression alone suggests alternative cell fate decisions are adopted during effector cell development. Thus, pro-inflammatory cytokines produced by effector cells in the spinal cord were almost exclusively derived from ‘ex-T<sub>H</sub>17’ cells with no apparent contribution by T<sub>H</sub>1 cells.

### **Transcriptional changes in ‘ex-T<sub>H</sub>17’ cells**

The eYFP<sup>+</sup> population was heterogeneous consisting of both IL-17A<sup>+</sup> IFN- $\gamma$ <sup>-</sup> and IL-17A<sup>-</sup> IFN- $\gamma$ <sup>+</sup> cells. To distinguish these populations and to unequivocally prove that the source of IFN- $\gamma$  was a cell with characteristics of a T<sub>H</sub>17 rather than a T<sub>H</sub>1 program, we used an adoptive transfer model. Because loss of IL-17A protein expression is accompanied by loss of CCR6 expression, we FACS purified CD4<sup>+</sup> CCR6<sup>+</sup> eYFP<sup>+</sup> cells from draining lymph nodes of mice immunized four days previously with MOG-CFA. To optimize the cell yield and adoptive transfer we used reporter mice that expressed the 2D2 transgenic T cell receptor specific for MOG<sup>16</sup>. We then transferred 1 x 10<sup>4</sup> CCR6<sup>+</sup> eYFP<sup>+</sup> CD4<sup>+</sup> T cells into RAG-1-deficient mice that were immunized with MOG-CFA to mimic the chronic inflammation of EAE. At the same time we performed single cell PCR from the starting inoculum of CCR6<sup>+</sup> eYFP<sup>+</sup> CD4<sup>+</sup> T cells to determine ongoing

transcription of *Il17a* and *Ifng* (**Supplementary Table 1**). About 30% of the adoptively transferred eYFP<sup>+</sup> T<sub>H</sub>17 cells produced IFN- $\gamma$ , in the lymph nodes compared to 60% in the spinal cord (**Fig.5a**). Single cells RT-PCR confirmed the majority of cells expressed *Il17a* and little *Ifng* at the time of transfer ( **Supplementary Table 1**).

Next we induced EAE in reporter mice and isolated CD4<sup>+</sup> CCR6<sup>+</sup> eYFP<sup>+</sup> and CD4<sup>+</sup> CCR6<sup>-</sup> eYFP<sup>+</sup> cells from the spinal cord to analyse their transcriptional profiles. As shown in the FACS plots of the sorted populations (**Fig.5b**), the eYFP<sup>+</sup> CCR6<sup>+</sup> population contained the most single IL-17A producers with few double producers of IFN- $\gamma$  and IL-17A. In contrast, the eYFP<sup>+</sup> CCR6<sup>-</sup> fraction contained the majority of double IFN- $\gamma$  and IL-17A producers as well as IFN $\gamma$  single producers but few IL-17A single producers. CCR6<sup>-</sup> eYFP<sup>+</sup> cells downregulated mRNA for *Il17a* and upregulated *Ifng* consistent with the protein expression data. *Il12rb1* mRNA was expressed at equal amounts in CCR6<sup>+</sup> and CCR6<sup>-</sup> eYFP<sup>+</sup> cells, whereas only CCR6<sup>-</sup> cells upregulated IL-12-specific *Il12rb2* receptor gene expression and *T-bet*, while downregulating *Rorgt* and *Il-23r*. Similar transcriptional changes were reported previously in T<sub>H</sub>17 cells generated *in vitro* with the notable exception of IL-12R $\beta$ 2 which is not switched off *in vitro*<sup>7</sup> but is absent on IL-17A expressing cells developing *in vivo* (**Fig.5b**). Importantly, IFN- $\gamma$  producing ‘ex-T<sub>H</sub>17’ cells could be distinguished from T<sub>H</sub>1 producers of IFN- $\gamma$  by *Ahr* expression, which remained high in CCR6<sup>-</sup> eYFP<sup>+</sup> cells and by IL-1R1 expression. The latter is absent on T<sub>H</sub>1 cells in lymphoid tissue and expressed in low amounts on T<sub>H</sub>1 cells from the spinal cord. In contrast eYFP<sup>+</sup> IL-17A as well as IFN $\gamma$  expressing cells express high amounts of IL-1R1 (**Fig.5c**). To test the functional significance of IL-1R

expression, we FACS purified eYFP<sup>+</sup> and eYFP<sup>-</sup> CD4 T cells from draining lymph node and spinal cord of MOG-CFA immunized mice and stimulated the cells in the presence of IL-1 $\beta$ . This resulted in a marked enhancement of IL-17A as well as IFN- $\gamma$  production exclusively from the eYFP<sup>+</sup> fraction in agreement with the expression pattern of IL-1R (**Fig5d**). Thus, IL-1R expression could potentially be used as a marker for non-T<sub>H</sub>1 derived IFN- $\gamma$  producers.

### **Effector deviation depends on IL-23 signalling**

The importance of IL-23 signalling for T<sub>H</sub>17 effector differentiation is well-established<sup>17</sup>. Furthermore, on the basis of *in vitro* studies IL-23 is thought to contribute to functional deviation of T<sub>H</sub>17 cells<sup>8</sup>. To determine the role of IL-23 signalling in our fate reporter model we immunized IL-23p19-deficient IL-17A reporter mice with MOG-CFA. Although IL-23p19-deficient reporter mice developed a much smaller proportion of eYFP<sup>+</sup> CD4<sup>+</sup> T cells than wild-type reporter mice, the proportion of IL-17A-single producers after PdBu-ionomycin stimulation was similar (**Fig.6a representative FACS plots and bar graphs**). However, the eYFP<sup>+</sup> cells from IL-23p19-deficient reporter mice lacked IL-17A<sup>+</sup> IFN- $\gamma$ <sup>+</sup> double producers as well as IFN- $\gamma$ <sup>+</sup> single producers compared to wild-type mice. This phenotype was more pronounced following restimulation with MOG peptide, where, even in wild-type mice, the proportion of double producers in eYFP<sup>+</sup> cells was much lower than that observed after PdBu-ionomycin stimulation and completely absent in IL-23p19-deficient reporter mice (**Fig.6b**). To pinpoint the mechanistic link between IL-23 and deviation towards a T<sub>H</sub>1-like profile, we tested for T-bet expression in eYFP<sup>+</sup> and eYFP<sup>-</sup> CD4<sup>+</sup> T cells from wild-type or IL-23p19-deficient

reporter mice. Absence of IL-23 signalling in T<sub>H</sub>17 cells prevented T-bet upregulation, suggesting this transcription factor is controlled by IL-23.

Taken together these data support our initial assumption that developmental progression of effector cell differentiation proceeds via an eYFP<sup>+</sup> stage before IL-17A is switched off and alternative fates are adopted. Furthermore, they suggest that IL-23 stimulation is not only crucial for attaining full effector function as shown before, but is also necessary for double expression of IL-17A and IFN $\gamma$ , induction of T-bet and the consecutive deviation towards IFN- $\gamma$  production.

### **T<sub>H</sub>17 cell plasticity in different inflammatory conditions**

The developmental plasticity of T<sub>H</sub>17 cells towards a T<sub>H</sub>1 like phenotype has been well documented (reviewed in<sup>18</sup>), but it is unclear whether these cells can adopt other effector fates in a different inflammatory context. Reporter mice were cutaneously infected with *Candida albicans* hyphae. IL-17-producing  $\gamma\delta$  T cells and T<sub>H</sub>17 cells are important for the defence against this fungal infection, as revealed by the severe mucocutaneous infections with *Candida* in patients with defective IL-17 responses<sup>19-21</sup>. In contrast to chronic inflammation in EAE, infection with *Candida albicans* leads to an acute infection that is rapidly cleared. Infection was accompanied by infiltration of neutrophils (**Supplementary Fig.7**) and an early innate IL-17A response mediated by  $\gamma\delta$  T cells as well as a progressive involvement of T<sub>H</sub>17 cells (**Fig.8a**). By day 3 after infection the majority of both  $\gamma\delta$  T cells and CD4<sup>+</sup> T cells expressing IL-17A also expressed eYFP. However, by day 5 when *Candida* was cleared (data not shown), a large fraction of

eYFP<sup>+</sup> cells had switched off IL-17A expression and by day 9 the absolute numbers of infiltrating CD4<sup>+</sup> T cells (both eYFP<sup>+</sup> or eYFP<sup>-</sup>) and  $\gamma\delta$  T cells had drastically declined (**Fig.8b**). Candida infection also elicited a substantial IFN- $\gamma$  response by CD4<sup>+</sup> T cells, but in marked contrast to the situation we encountered in the inflamed spinal cord during EAE, virtually none of these were eYFP<sup>+</sup>, indicating their T<sub>H</sub>1 origin (**Fig.8c**). The eYFP<sup>+</sup> population that had switched off IL-17A appeared quiescent with respect to cytokine expression as neither IL-22, nor GM-CSF or IL-17F were expressed once IL-17A expression was extinguished (**Fig.8d**). qPCR analysis of CD11b<sup>+</sup> and CD11c<sup>+</sup> cells isolated from infected skin showed a strong *Il10* and relatively weak *Il23a* expression suggesting that the milieu in the surrounding skin tissue is biased towards an anti-inflammatory profile that may curtail T<sub>H</sub>17 deviation towards alternative fates (**Fig.8e**). This data suggest that in contrast to chronic inflammation, acute inflammation promotes an anti-inflammatory environment in which T<sub>H</sub>17 cells downregulate *Il17a* transcriptional activity without activating alternative cytokine loci. Thus, resolution of acute inflammation goes hand in hand with disappearance of T<sub>H</sub>17 and  $\gamma\delta$  T cells rather than a switch to alternative effector fates that we characterized during chronic inflammation.

## DISCUSSION

Lineage commitment and fate determination are fundamental biological processes that have been extensively studied in a variety of biological systems. In T cell biology these issues combined with the question of terminal differentiation, stability or plasticity have been intensively discussed and investigated in recent years. To some extent the unusual biology of the T<sub>H</sub>17 subset triggered these questions, although they are by no means restricted to T<sub>H</sub>17 cell biology and indeed are now explored in other effector T cell subsets as well (reviewed in <sup>22,23</sup>). Given the documented rapid fate changes of cultured T<sub>H</sub>17 *in vitro* or following adoptive transfer, driven by epigenetic instability at transcription factor as well as cytokine gene loci<sup>24,25</sup>, the logical choice was to generate a reporter mouse which would allow permanent tracing of cells that had activated the *Il17a* locus as one indicator implying mandatory expression of this locus as a pre-requisite for T<sub>H</sub>17 fate determination. The reporter mouse we generated is to our knowledge the first fate reporter mouse for IL-17A, as existing mice generated by BAC transgenesis so far only monitor transcriptional activity at the closely linked *Il17f* locus<sup>26</sup>. IL-17F is largely co-expressed with IL-17A in lymphoid organs and may play a special role in lung inflammation, but we found little IL-17F expression in neuronal inflammation in line with the reported absence of a phenotype for IL-17F-deficient mice in EAE (reviewed in <sup>27</sup>)

We provided experimental evidence that induction of the eYFP reporter correlated well with effector function. Our data also indicate that *in vitro* generated T<sub>H</sub>17 cells do not attain the same level of effector differentiation as seen *in vivo* during the course of



inflammatory immune responses that lead to IL-17A induction. Even the few IL-17A-expressing CD4<sup>+</sup> and  $\gamma\delta$  T cells found in lymphoid organs of mice without deliberate immunization contained a higher proportion of eYFP<sup>+</sup> cells than we detected during *in vitro* differentiation of T<sub>H</sub>17 cells. It is likely that many factors that shape T<sub>H</sub>17 differentiation *in vivo* are not adequately mimicked by *in vitro* conditions.

A recent study suggested that in contrast to T<sub>H</sub>17 cells differentiated *in vitro*, Th17 cells generated *in vivo* appeared developmentally stable<sup>12</sup>. From our study it was evident that T<sub>H</sub>17 cells activated *in vivo* also rapidly shut off IL-17 production. This process closely followed the acquisition of eYFP in *Il17a*<sup>Cre</sup> reporter mice since there was virtually no evidence for a direct progression of IL-17 to IFN- $\gamma$  producers, bypassing the eYFP<sup>+</sup> effector stage. Interestingly, analysis of T<sub>H</sub>17 development in IL-23p19-deficient reporter mice not only showed reduced acquisition of eYFP, but also lack of the double positive IL17<sup>+</sup> IFN- $\gamma$ <sup>+</sup> population that usually develops following immunization *in vivo*. This suggests that T<sub>H</sub>17 cells require prior effector cell differentiation to produce IFN- $\gamma$ . Similar observations were made previously by investigating intestinal T<sub>H</sub>17 responses in IL-23 receptor-deficient mice<sup>28</sup>. Since T-bet expression was strongly curtailed in T<sub>H</sub>17 cells from IL-23p19-deficient reporter mice, it is likely that IL-23 is upstream of T-bet and is required for its expression. IL-23 is abundant during chronic inflammation<sup>29</sup>, but is downregulated during resolution of the inflammatory response making IL-23 perhaps the major decision point for T<sub>H</sub>17 cell plasticity.

Furthermore it was evident that the eYFP<sup>+</sup> population constituted the main source of other cytokines. Notably GM-CSF which is strongly implicated in EAE<sup>30,31</sup> was produced exclusively by eYFP<sup>+</sup> T<sub>H</sub>17 and ‘ex-T<sub>H</sub>17’ cells. The propensity for a switch to IFN- $\gamma$  production was pronounced in the setting of EAE and accompanied by upregulation of IL-12R $\beta$ 2 and T-bet and downregulation of ROR $\gamma$ t, IL-23R and CCR6. Interestingly, expression of AhR was retained, thus making IFN- $\gamma$  producing ‘ex-T<sub>H</sub>17’ cells susceptible to environmental stimuli. In contrast to *in vitro* generated T<sub>H</sub>17 cells from mice or humans, which retain expression of both IL-12R chains<sup>7,32</sup>, T<sub>H</sub>17 cells developing *in vivo* did not express the IL-12R $\beta$ 2 chain (**Fig.5b**), making such cells refractory to the action of IL-12. Furthermore, stimulation of AhR, which is highly expressed on T<sub>H</sub>17 cells, leads to upregulation of both IL-12R $\beta$ 1 and IL-12R $\beta$ 2<sup>33</sup>. Recent data showed that T<sub>H</sub>2 cells, which switch off IL-12R $\beta$ 2 during differentiation *in vitro*<sup>34</sup> re-express the receptor when exposed to type I interferon or IFN- $\gamma$ <sup>35</sup>. Similar mechanisms may contribute to re-induction of IL-12R $\beta$ 2 in T<sub>H</sub>17 cells during EAE with a potential involvement of plasmacytoid dendritic cells as source of type I interferon<sup>36,37</sup>. In this respect it is also worth noting that interferon alpha receptor-dependent inhibition of intracellular osteopontin may regulate IL-27 expression and subsequent inhibition of IL-17<sup>38</sup>. The relative roles of IL-17A versus IFN- $\gamma$  in the pathology of EAE as well as multiple sclerosis in humans remain controversial with evidence both supporting a protective as well as pathogenic involvement of IFN- $\gamma$  in CNS inflammation<sup>39-42</sup>. Effector cytokines produced by ‘ex-T<sub>H</sub>17’ cells during the chronic stage may thus play different roles in the pathogenesis of autoimmune diseases.

Our data reconcile seemingly conflicting results obtained by adoptive transfer of *in vitro* generated T<sub>H</sub>17 or T<sub>H</sub>1 cells<sup>43-45,42</sup> that seemed to refute the important role of T<sub>H</sub>17 cells in various autoimmune models compared with earlier data that highlighted their crucial importance in these models (as reviewed in<sup>46</sup>). They also explain how T-bet-deficient mice are protected from EAE, which seemed at odds with an important role for T<sub>H</sub>17 cells in CNS pathology. It appears that IL-23 mediated T-bet induction resulting in IFN- $\gamma$  expression by ‘ex-T<sub>H</sub>-17’ cells is an important step in the pathophysiology of EAE, since IFN- $\gamma$  represents by far the most abundant cytokine in the spinal cord of mice with EAE. These data also explain our earlier findings that mice with transgenic expression of a dominant negative TGF $\beta$ RII are protected from EAE and contain very few T<sub>H</sub>1 cells in the spinal cord despite an overabundance of T<sub>H</sub>1 cells throughout the lymphoid organs<sup>29</sup>. Thus, IL-17-producing cells, including innate  $\gamma\delta$  T cells or NKT cells<sup>47</sup> might play an early role in recruitment of cells involved in setting up inflammatory responses, whereas the conversion to a T<sub>H</sub>1-like cytokine profile may be the major pathological driver. Interestingly, pathogenic effector cells T<sub>H</sub>17 cells must lose responsiveness to TGF- $\beta$  to allow effective T-bet expression. It is possible that this could explain the recent report of pathogenic T<sub>H</sub>17 cells that arise in the apparent absence of TGF- $\beta$  signaling<sup>48</sup>.

In contrast to the situation in EAE, a switch of IL-17A to IFN- $\gamma$  production was not evident following *Candida albicans* infection of the skin, suggesting that T<sub>H</sub>17 plasticity depends on the inflammatory milieu. Whereas immunization with MOG in CFA induces chronic inflammatory response because of the depot effect of the immunogen emulsion, *Candida* infection of the skin was rapidly cleared in wild-type mice. The infection caused

an early influx of neutrophils sustained by the IL-17A response, which was shut off in parallel with clearance of the neutrophil infiltration. At that stage the skin milieu was characterized by high expression of IL-10 and relatively modest IL-23 expression, suggesting a balance in favour of an anti-inflammatory microenvironment. The surviving eYFP<sup>+</sup>, but IL-17A<sup>-</sup> CD4 population showed minimal detectable cytokine secretion. Such a population of 'ex-T<sub>H</sub>17' cells would be invisible by any other means of detection, as IL-17A secretion was not even induced by PdBU-ionomycin stimulation. This would explain a previous report<sup>49</sup>, suggesting that T<sub>H</sub>17 cells cannot be detected in the pool of long-lived CD4<sup>+</sup> memory T cells. It remains to be seen whether surviving eYFP<sup>+</sup> cells carry memory of their initial induction and are able to re-initiate a T<sub>H</sub>17 program upon re-encounter with the original pathogen in similar inflammatory settings.

Expression of IL-1R1 on 'ex-T<sub>H</sub>17' cells could be a useful marker for identifying cells which develop through a T<sub>H</sub>17 program as neither T<sub>H</sub>1 nor T<sub>H</sub>2 cells normally express significant levels of this receptor, which has been shown to be crucial for pathogenesis of autoimmune diseases such as EAE<sup>50</sup>. Thus, our data reconcile a host of literature that identifies various pro-inflammatory cytokines, including GM-CSF, IFN $\gamma$ , IL-1 in the pathogenesis of autoimmune diseases, as they are all linked to T cells that have started their effector stage as T<sub>H</sub>17 cells.

Taken together our data suggest that fate decisions of T<sub>H</sub>17 cells are shaped by different inflammatory conditions *in vivo* allowing distinct patterns of plasticity. Thus, IL-17A fate reporter mice constitute a valuable tool for dissection of effector cytokine programs that

may have originated initially from  $T_H17$  cells and will facilitate mechanistic analysis of the inflammatory drivers that shape acquisition of the functional fate of effector T cells.

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#### **Author contributions:**

H.K., J.H.D.,H.A.,C.W. designed and performed experiments, M.V.,E.H.,Y.L.,M.T., U.M. and A.G. were involved in generating the construct, targeting ES cells and injections. A.J.P and B.S. are joint senior authors who designed the targeting construct and experiments and wrote the paper.

## FIGURE LEGENDS

### **Figure 1.** Induction of fate reporter eYFP<sup>+</sup> cells in IL-17-producing cells

(a) Naïve CD4<sup>+</sup>CD44<sup>lo</sup>CD25<sup>-</sup> T cells were cultured under T<sub>H</sub>1, T<sub>H</sub>2, T<sub>H</sub>9, T<sub>H</sub>17 or iTreg conditions for 4 days and stained for indicated intracellular cytokines. Dot plots show intracellular cytokine expression vs eYFP (b) Schematic representation of PCR primer location used for assessment of Cre-mediated recombination at the *ROSA26 eYFP* locus. Cells cultured under T<sub>H</sub>17 conditions *in vitro* were sorted according to the gates indicated and DNA from sorted populations were tested for recombination at the *ROSA26 eYFP* locus. (c) mean fluorescence intensity for IL-17A(top) and Cre (bottom) in IL-17A<sup>+</sup>eYFP<sup>-</sup> and IL-17A<sup>+</sup>eYFP<sup>+</sup> populations. Mean values and SD are shown. \*denotes *P* value 0.03, \*\* denotes *P* value 0.01 (d) LN cells from non-immune mice were stained for CD4 and γδ TCR, followed by intracellular IL-17A and IFN-γ staining. Dot plots show intracellular staining vs eYFP expression. The FACS plots are representative for three independent experiments and bar graphs showing experimental variability are shown in (e).

### **Figure 2.** Kinetics of eYFP and IL-17A expression during EAE induction

(a) Draining LN cells from MOG/CFA immunized mice were stained for CD4 and γδ TCR and assessed for eYFP and intracellular IL-17A at the indicated days after immunization. (b) Absolute numbers of eYFP<sup>+</sup> CD4 and γδ T cells in the draining LN. Data show mean + SD from three mice. (c) Expression of eYFP and IL-17A of infiltrating CD4<sup>+</sup> and γδ T cells in the spinal cord. (d) The percentage of eYFP positive

CD4<sup>+</sup> T cells or  $\gamma\delta$  T cells in the spinal cord (day 16 after EAE induction) is shown. The bars indicate mean derived from four immunized mice. Data in (a) and (c) represent three independent experiments. (e) FACS analysis for expression of the proliferation marker Ki-67 performed on gated CD4 eYFP<sup>-</sup> or eYFP<sup>+</sup> cells from lymph node (day 0 and day 12) and spinal cord (day15) with experimental variation in three independent experiments indicated by % values shown in the plots.

**Figure 3** IFN- $\gamma$  expression and antigen specificity in eYFP<sup>+</sup> and eYFP<sup>-</sup> CD4 T cells

(a) Draining LN cells from MOG/CFA immunized mice were stained for CD4 and  $\gamma\delta$  TCR and assessed for eYFP and intracellular IFN $\gamma$  at the indicated days after immunization. (b) bar graphs showing % of eYFP<sup>+</sup> or eYFP<sup>-</sup> CD4 T cells from draining lymph nodes at day 12 expressing cytokines following restimulation with PdBU-ionomycin (left panel) or MOG peptide (right panel). Mean values +/- SD of three individual mice are shown. (c) Representative FACS plots showing expression of eYFP and IFN- $\gamma$  of infiltrating CD4<sup>+</sup> and  $\gamma\delta$  T cells in the spinal cord. (d) bar graphs showing % of eYFP<sup>+</sup> or eYFP<sup>-</sup> CD4 T cells from spinal cord at day 15 expressing cytokines following restimulation with PdBU-ionomycin (left panel) or MOG peptide (right panel).

**Figure 4.** Cytokine expression in eYFP<sup>+</sup> and eYFP<sup>-</sup> CD4<sup>+</sup> T cells in the draining LN and spinal cord.

(a) Lymph nodes from non-immune mice (day 0) as well as draining LN and spinal cord cells 6 and 15 days after EAE induction, respectively, were stained for CD4 and intracellular cytokines as indicated. The dot plots show cytokine expression profiles in



gated eYFP<sup>+</sup> CD4<sup>+</sup> T cells **(a)** and gated eYFP<sup>-</sup> CD4<sup>+</sup> T cells in **(b)**. The data are representative for at least three independent experiments. **(c)** Cytokine concentrations measured in supernatant of 2x10<sup>4</sup> sorted eYFP<sup>+</sup> or eYFP<sup>-</sup> CD4 T cells isolated from day 6 lymph nodes or day 15 spinal cord and restimulated *in vitro* with anti-CD3/anti-CD28 for 24h. Data show mean values +/- SD of cytokines measured from three individual mice.

**Figure 5** Transcriptional changes in eYFP<sup>+</sup> CD4<sup>+</sup> T cells

**(a)** CCR6<sup>+</sup> eYFP<sup>+</sup> CD4<sup>+</sup> T cells from 2D2 IL-17-reporter mice were purified 4 days after MOG-CFA immunization and adoptively transferred into immunized Rag-deficient mice. FACS plots and bar graphs of IL-17A and IFN- $\gamma$  expression in eYFP<sup>+</sup> CD4<sup>+</sup> T cells from draining lymph nodes and spinal cord (day 16) restimulated with PdBU-ionomycin (upper panels) or MOG peptide (lower panels). Histograms show mean values for individual mice +/- SD.

**(b)** CCR6<sup>-</sup> and CCR6<sup>+</sup> eYFP<sup>+</sup> CD4<sup>+</sup> T cells from spinal cord were sorted for qPCR analysis. Representative FACS plots show expression of IL-17A and IFN- $\gamma$ . Relative gene expression in sorted (not restimulated) cells normalized to the expression of *Hprt* is shown. **(c)** CD4<sup>+</sup>eYFP<sup>-</sup>IFN- $\gamma$ <sup>+</sup>, representing T<sub>H</sub>1, (shaded gray), CD4<sup>+</sup>eYFP<sup>+</sup>IFN- $\gamma$ <sup>+</sup> (ex-T<sub>H</sub>17 -dotted line), and CD4<sup>+</sup>eYFP<sup>+</sup>IL-17A<sup>+</sup> (T<sub>H</sub>17- solid line) from draining LN and spinal cord cells 15 days after MOG-CFA immunisation were gated and assessed for IL-1R1 expression. The data represent at least three independent experiments. **(d)** Cytokine levels measured in supernatant of purified eYFP<sup>+</sup> or eYFP<sup>-</sup> CD4 T cells from draining lymph nodes or spinal cord (day 15) restimulated with anti-CD3 +/- 20 ng/ml IL-1 $\beta$  for

24 hr. Data show mean values  $\pm$  SD of cytokines from three individual mice. The data represent at least three independent experiments.

**Figure 6.** IL-23 signalling is required for acquisition of eYFP<sup>+</sup> IFN- $\gamma$ <sup>+</sup> profile

**(a)** Representative FACS plots (left) and bar graphs showing expression of IL-17A and IFN $\gamma$  in eYFP<sup>+</sup> and eYFP<sup>-</sup> CD4 T cells from day 12 draining lymph nodes of wild-type or IL-23 *p19*<sup>-/-</sup> reporter mice following restimulation with either PdBU/ionomycin or **(b)** MOG peptide. Data show mean values  $\pm$  SD of three individual mice.

**Figure .7.** T-bet expression is curtailed in the absence of IL-23

Representative FACS plots and bar graphs showing expression of eYFP and T-bet in eYFP<sup>+</sup> and eYFP<sup>-</sup> CD4 T cells from day 6 draining lymph nodes of wildtype or IL-23p19 deficient reporter mice. The bar graphs show means  $\pm$  SD of three individual mice.

**Figure 8.** Cutaneous infection with *Candida albicans*

Skin (1cm<sup>2</sup>) of IL-17A fate reporter mice infected with *Candida albicans* hyphae was analyzed at the timepoints depicted. **(a)** intracellular IL-17A and eYFP expression in CD4<sup>+</sup> and  $\gamma\delta$  T cells is shown. **(b)** absolute numbers of eYFP positive CD4<sup>+</sup> and  $\gamma\delta$  T cells and eYFP<sup>-</sup> cells in the skin at the indicated time points. Mean  $\pm$  SD values from 3-5 mice per time point are shown. **(c)** intracellular IFN- $\gamma$  and eYFP expression in CD4<sup>+</sup> T cells. Data are representative of two independent experiments **(d)** histograms showing cytokine expression in gated eYFP<sup>+</sup> IL-17<sup>+</sup> and eYFP<sup>+</sup> IL-17<sup>-</sup> CD4 T cells on day 5 after infection. The data are representative of two independent experiments. **(e)** CD11b<sup>+</sup> and

CD11c<sup>+</sup> cells were MACS sorted from the infected skin for qPCR analysis. Relative gene expression in sorted cells normalized to the expression of Hprt is shown. The bar graphs show means  $\pm$  SD of three individual mice. Data are representative of two independent experiments.

## ONLINE METHODS

**Mice.** Codon-improved Cre recombinase (iCre)<sup>51</sup> was inserted into the first exon of the *Il17a* locus in R1 embryonic stem cells by homologous recombination to express iCre under the control of the endogenous *Il17a* promoter. Details of the gene targeting and the *Il17a* targeting strategy are given in **Supplementary Fig. 1a, b**. To visualize Cre-mediated recombination, *R26R<sup>eYFP</sup>* mice<sup>52</sup> were intercrossed with *Il17a<sup>Cre</sup>* mice generating *Il17a<sup>Cre</sup> R26R<sup>eYFP</sup>* mice. This strain was further bred with *Il23a<sup>-/-</sup>* mice to obtain *Il23a<sup>-/-</sup> Il17a<sup>Cre</sup> R26R<sup>eYFP</sup>* or with 2D2 transgenic mice. All reporter mice used in this study had been backcrossed to C57BL/6 for at least six generations. RAG-1-deficient mice on B6 background were bred in our animal facility and all mice were kept under specified pathogen free conditions. All animal experiments were done according to institutional guidelines and Home Office regulations.

***In vitro* T cell differentiation and cytokine determination.** FACS purified naïve T cells (CD4<sup>+</sup>CD25<sup>-</sup>CD44<sup>lo</sup>) were cultured in Iscove's modified Dulbecco medium (IMDM, Sigma) supplemented with 5% FCS, 2x10<sup>-3</sup>M L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin and 5x10<sup>-5</sup> M mercaptoethanol (all Sigma) in the presence of anti-CD3 (1 µg/ml) and 10 µg/ml anti-CD28 (both plate bound). Cytokines for effector cell differentiation were: 3 ng/ml IL-12 for T<sub>H</sub>1, 10 ng/ml IL-4 for T<sub>H</sub>2, 5 ng/ml TGF-β for iTreg, 1 ng/ml TGF-β, 20 ng/ml IL-6 and 10 ng/ml IL-1 for T<sub>H</sub>17 and 10 ng/ml IL-4 and 1 ng/ml TGF-β for T<sub>H</sub>9. Cells were cultured for 4 days and then restimulated for 4 h with PdBU/ionomycin (both at 500 ng/ml) in the presence of brefeldin A (1 µg/ml) before

intracellular staining for cytokines. Alternatively, cells were cultured with MOG peptide (50 µg/ml) or 1 µg/ml anti-CD3 overnight with brefeldin A present for the last 5 hours. All antibodies for surface and intracellular staining were purchased from Biolegend. IL-22 was detected by staining with either monoclonal antibody AM22.3 (kindly provided by Dr. J.-C. Renauld, Brussels) or alternatively by goat anti-mouse IL-22 (Biolegend). Determination of cytokines in supernatants of FACS purified cells from lymph node or spinal cord at the adjusted concentration of  $5 \times 10^4$ /ml was done after overnight culture with plate-bound anti-CD3 and anti-CD28 in the absence or presence of IL-1 $\beta$  (20ng/ml) using FlowCytomix (Bender Medsystems).

**Preparation of tissue-resident lymphocytes.** Briefly, lymphocytes in the lamina propria were prepared by cutting the small intestine into 1 cm pieces, shaking for at 37°C in 10 ml 'IEL buffer' (PBS supplemented with 10% FCS, 1 mM pyruvate, 20 µM HEPES, 10 mM EDTA, and penicillin/streptomycin, 10 µg/ml Polymyxin B) to remove epithelial and intraepithelial cells and then digesting the remaining tissue using 1 mg/ml collagenase D (Roche) and 10 U/ml DNaseI (Sigma) at 37°C for 1 hr followed by separation on a 36.5% Percoll gradient. Lymphocytes in the spinal cord from mice with clinical score 3-4 at day 12-16 after MOG/CFA immunization were prepared by mashing the spinal cord through 70 µm mesh filter and separation on a 36.5% Percoll gradient.

**EAE induction.** Mice were injected subcutaneously at two sites with a total of 100 µl emulsion of IFA containing 250 µg MOG peptide 35-55 and 250 µg heat-killed *Mycobacterium tuberculosis* strain H37Ra. Mice received 200 ng *Bordetella pertussis*

(Calbiochem) i.p. on the day of immunization and two days later. Clinical assessment of EAE was performed daily and clinical scores were assessed according to the following criteria: 0 = unaffected, 1 = flaccid tail, 2 = impaired righting reflex and/or gait, 3 = partial hind limb paralysis, 4 = total hind limb paralysis, 5 = total hind limb paralysis with partial fore limb paralysis. According to Home Office regulations all mice with a clinical score of 5 were culled.

**Cutaneous infection with *Candida albicans*.** *Candida albicans* strain SC5413 was obtained from the Institute of Medical Sciences, University of Aberdeen, UK. Hyphae were prepared by allowing cells to germinate in culture at 37°C, in 5% CO<sub>2</sub>, for 2 h in RPMI medium (resulting in >98% of hyphae formation). An area of 2 cm<sup>2</sup> in the ventral skin of mice was shaved and superficial scratches were made using a needle. 2 x 10<sup>5</sup> *Candida albicans* hyphae were applied on the skin surface. At the relevant time point mice were culled and 1 cm<sup>2</sup> of infected skin excised, minced and digested in IMDM containing 400 µg/ml liberase (Roche) and 1 mg/ml collagenase D (Roche) for 1 h at 37°C under continuous stirring. Cells were then collected and activated with Pdbu, ionomycin and brefeldin A for 2.5 h before intracellular cytokine staining.

**Real time PCR.** RNA was extracted from FACS purified CCR6<sup>+</sup> and CCR6<sup>-</sup> CD4 T cells isolated from spinal cord using Trizol and reverse transcribed with Omniscript (Qiagen) according to the manufacturer's protocol. The cDNA served as template for the amplification of genes of interest and a housekeeping gene (*Hprt1*) by real-time PCR, using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA) and

universal PCR Master Mix (Applied Biosystems, Warrington, UK) on a ABI-PRISM 7900 Sequence Analyzer (Applied Biosystems, Foster City, CA). Target gene expression was calculated using the comparative method for relative quantification upon normalization to *Hprt1* gene expression.

**Statistical Analysis.** All statistical analysis was performed using a two-tailed Student T test.

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# Figure 1

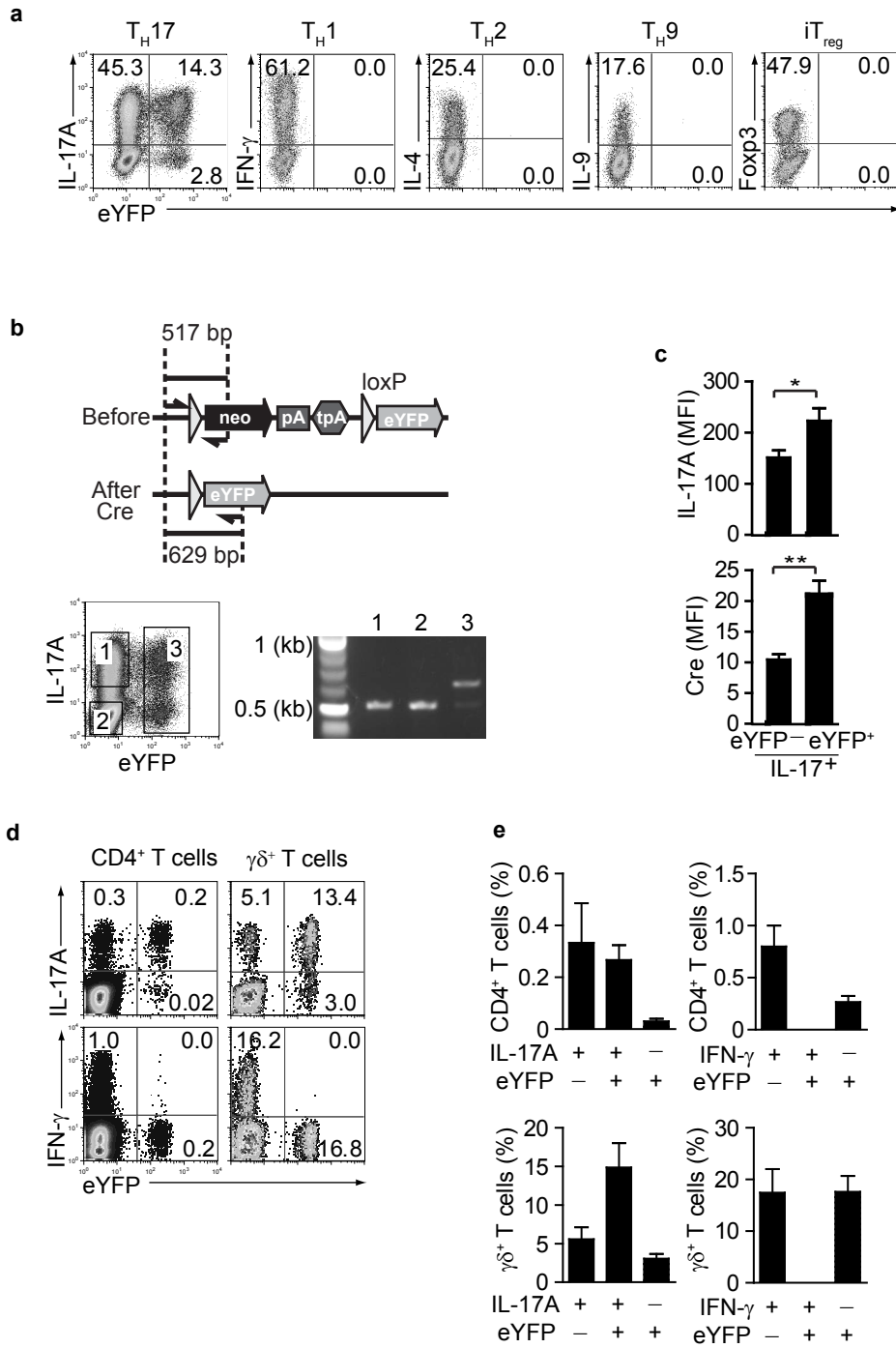


Figure 2

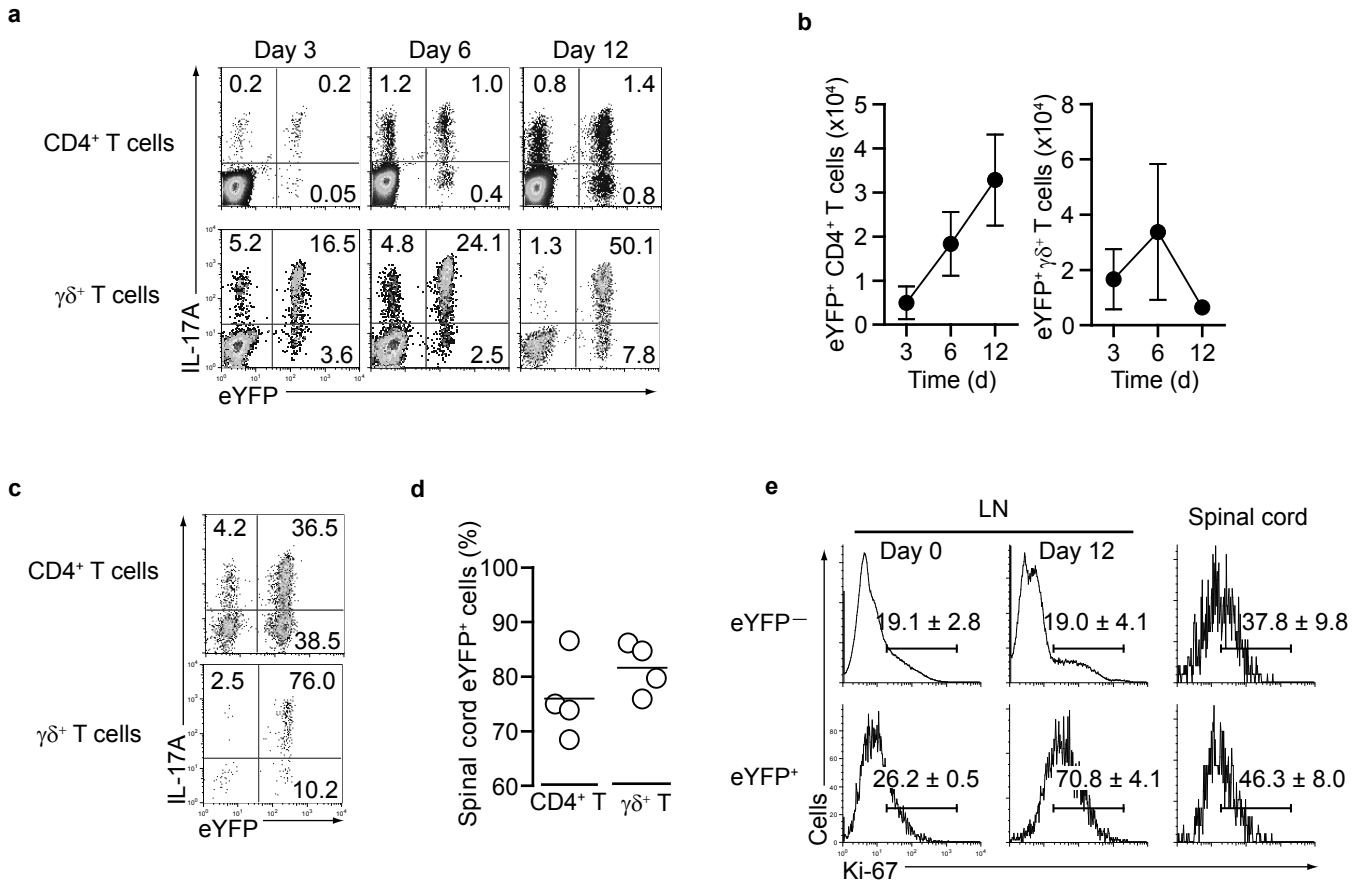
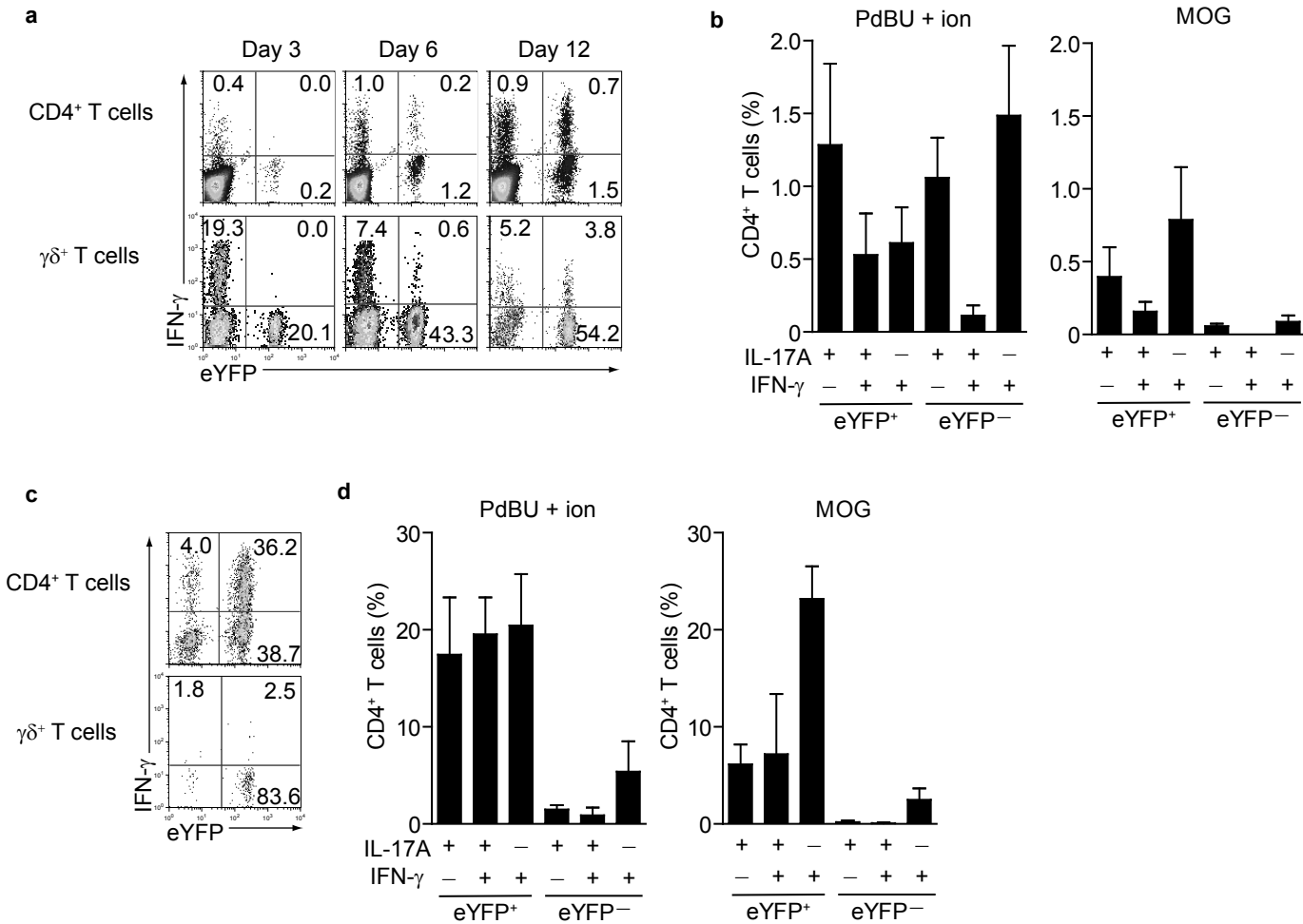
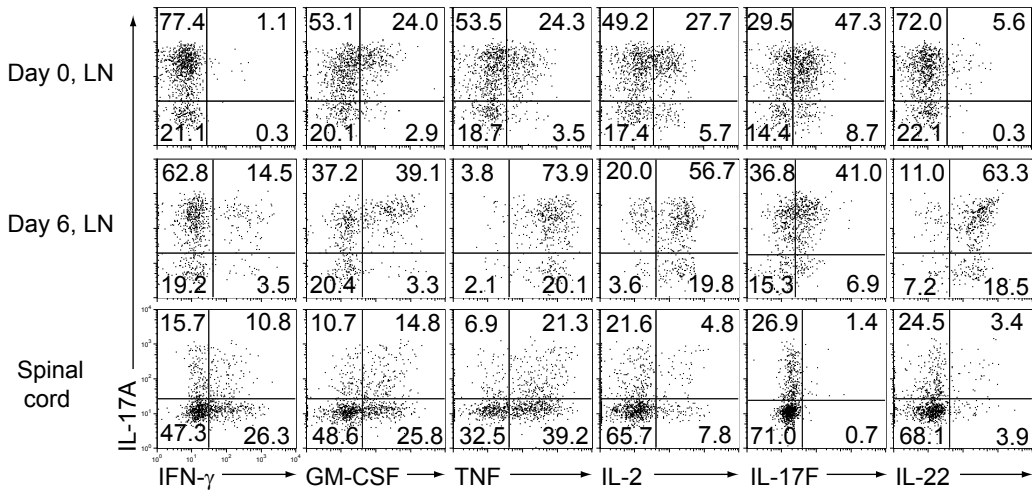


Figure 3

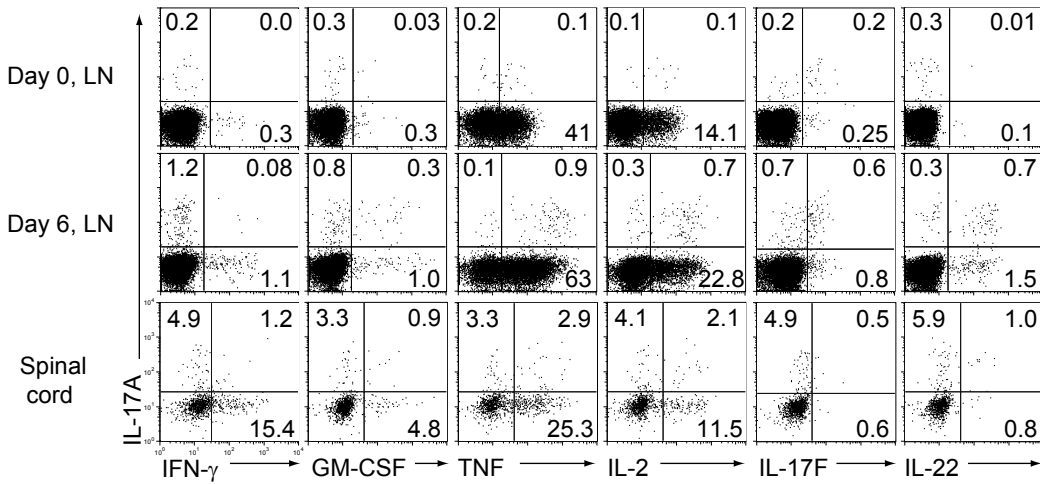


# Figure 4

**a**



**b**



**c**

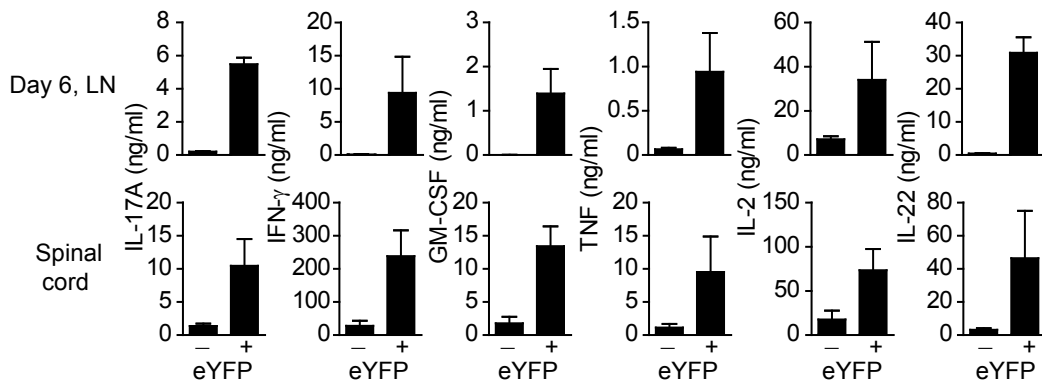


Figure 5

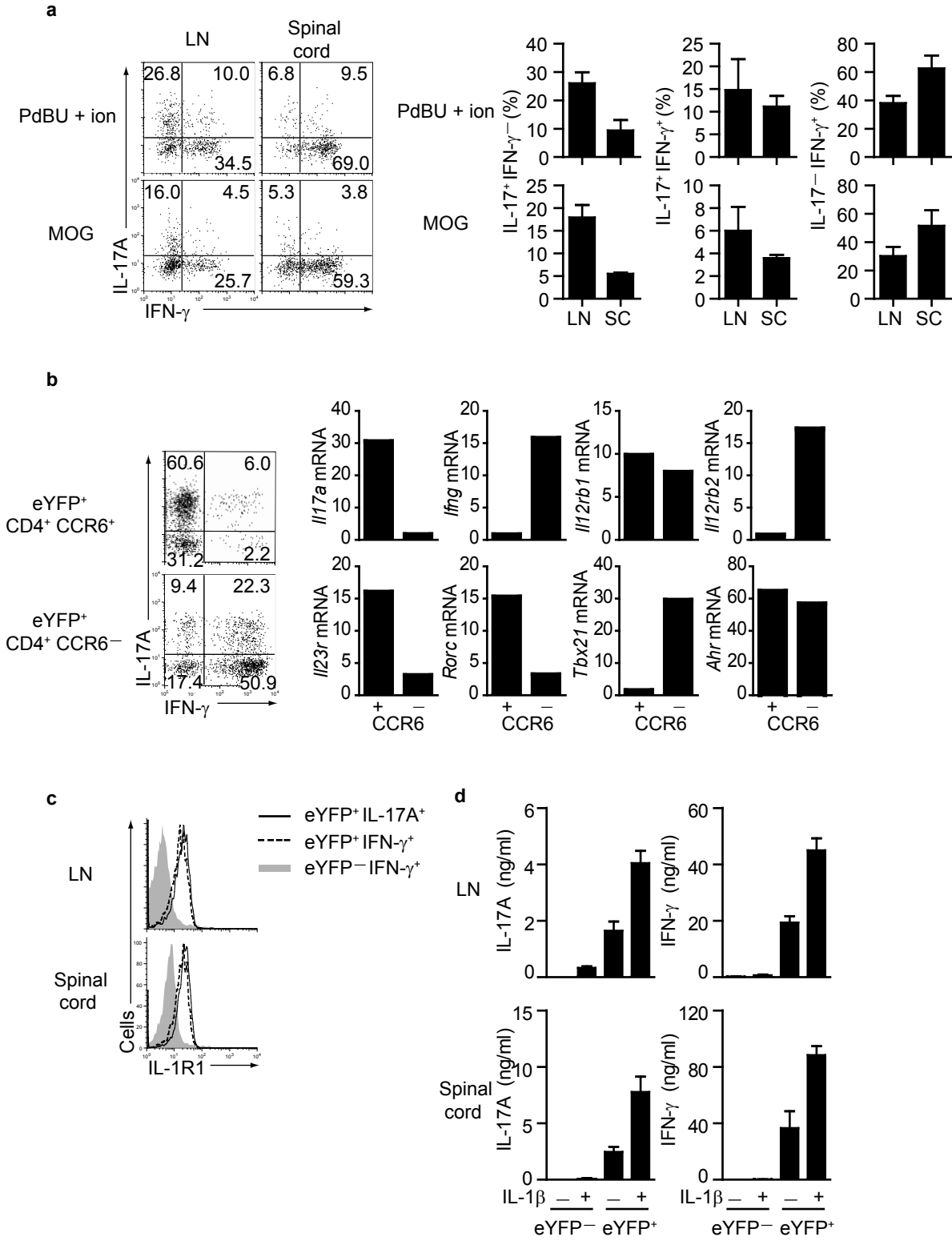
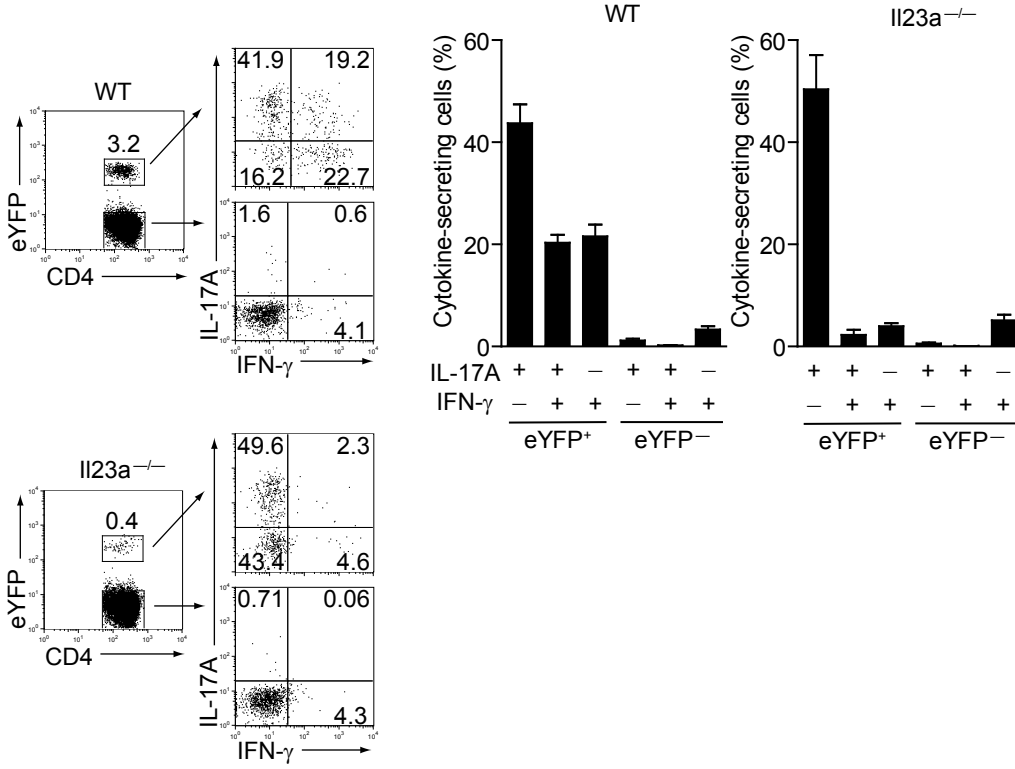


Figure 6

a



b

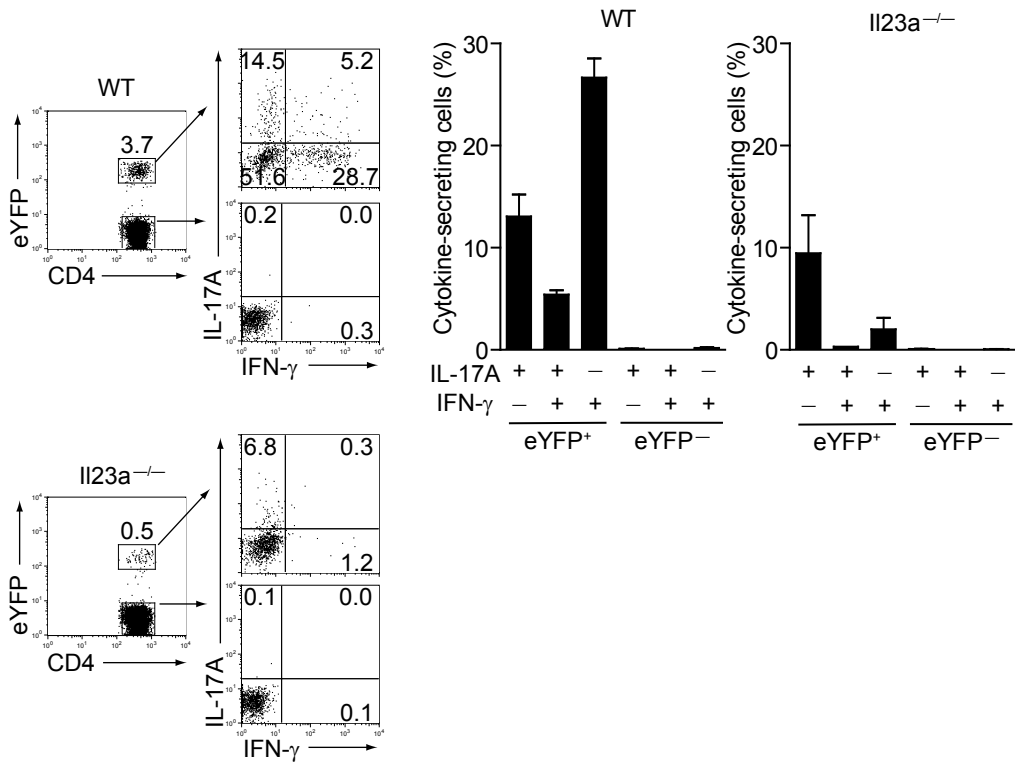




Figure 7

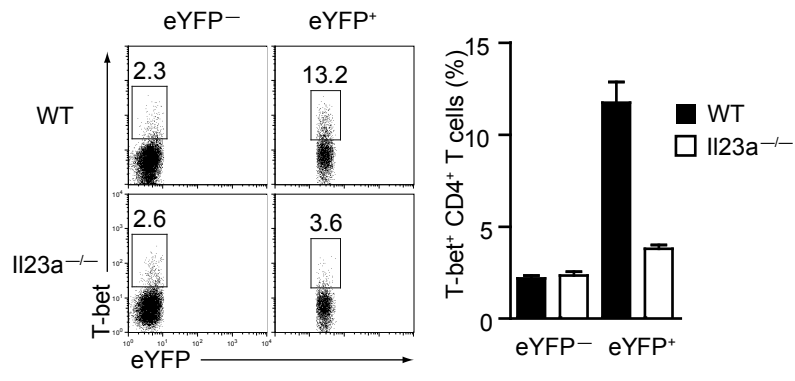


Figure 8

