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Costimulatory molecule ICOS plays a critical role in the development of T_H-17 and follicular T-helper cells by regulating c-Maf expression and IL-21 production

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Abstract

The inducible costimulatory molecule (ICOS) has been suggested to play an important role in the development of interleukin 17 (IL-17)-producing T helper cells (T_H-17 cells) and of follicular helper cells (T_{FH} cells), specialized helper T cells ($CD4^+CXCR5^+ICOS^{high}$) required for antibody class switching and germinal center formation. Here we show that ICOS, while not essential for the differentiation of T_H-17 cells, was critical for maintaining effector-memory T_H-17 cells as ICOS-deficient mice demonstrated a defect in the expansion of T_H-17 cells after IL-23 stimulation. In addition, we found that T_{FH} cells produced IL-17 and that ICOS-deficient mice demonstrated a reduced frequency of T_{FH} with a defect in IL-17 production. Both T_H-17 and T_{FH} cells showed increased expression of the transcription factor c-Maf—normally associated with T_H2 cells— and that loss of c-Maf results in a defect in IL-21 production, and consequently a defect in the maintenance of IL-23R expression and expansion of T_H-17 and T_{FH} cells. These data suggest that c-Maf induced by ICOS regulates IL-21 production that, in turn, regulates expansion of T_H-17 cells and T_{FH} cells.

INTRODUCTION

Interleukine 17-producing T helper cells (T_H-17 cells) have been recently described as functionally new CD4 helper T cell subset that plays an important role in the pathogenesis of many organ-specific autoimmune diseases in animal models and an increase in IL-17 expression has been associated with human autoimmune diseases including multiple sclerosis[1,2], rheumatoid arthritis[3] and psoriasis[4]. Furthermore, IL-17-producing T cells with specificity for myelin antigen have been shown to be more efficient than T_H1 cells at transferring autoimmunity in experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis[5]. There is great interest in understanding the regulation of T_H-17 differentiation. IL-6 and TGF-β are sufficient for differentiating naïve CD4 T cells into IL-17 producing T cells in vitro, and these cytokines are required for T_H-17 differentiation in vivo

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as well[6,7]. Another crucial factor for the development of pathogenic T_H-17 cells is IL-23 since IL-23p19 deficient mice, which show a defect in T_H-17 development and are resistant to EAE[5,8]. Initial studies on T_H-17 differentiation, using total $CD4^+$T cells including effector-memory T cell populations, were interpreted to suggest that IL-23 was a differentiation factor for T $_H$-17 cells[5,8,9] However, it is now appreciated that naïve $CD4^+$ T cells do not express IL-23R and that IL-23 alone is not capable of driving T_H-17 cell differentiation of naïve T cells[7]. Instead, IL-23 acts on previously differentiated T_H-17 cell to expand, stabilize and maintain the T_H-17 phenotype[5,6,10]

In addition to IL-6, IL-21, a member of the IL-2 cytokine family, has been shown to be involved in T_H-17 differentiation; in combination with TGF-β, IL-21 induces expression of IL-17, transcription factor RORγt and the IL-23 receptor (IL-23R) in naïve $CD4^+$ T cells. In vivo, IL-21 can generate T_H-17 cells even in IL-6-deficient mice, and both IL-21-deficient and IL-21R-deficient mice are defective in generating T_H-17 cells. Furthermore, once they have developed, T_H-17 cells secrete high amounts of IL-21, which then acts to amplify the T_H-17 response in an autocrine fashion[11,12].

However, T_H-17 cells are not the only T cells that produce IL-21. Another type of $CD4^+$ T helper cell, T follicular helper cells (T_{FH} cells), also produces high amounts of this cytokine[13-14] The T cell-dependent B cell response occurs within particular structures in the secondary lymphoid organs, the germinal centers (GC). T_{FH} cells provide cognate help to B cells in the GC dynamic microenvironment that results in somatic hypermutation, class switch recombination and selection of high-affinity B cells[15,16]. T_{FH} cells have been shown to be distinct from conventional T helper cells (e.g., T_H1 and T_H2 cells) and were first characterized based on expression of the chemokine receptor CXCR5. After antigen activation, T_{FH} cells begin to express CXCR5 while downregulating expression of CCR7, which enables the cells to respond to the follicle-associated chemokine CXCL13. $CXCR5^+CD4^+$ T_{FH} cells then migrate to follicles where they support GC formation and B cell responses. T_{FH} cells express other activation markers as well, including CD69, CD95, CD40L, and inducible T cell costimulator (ICOS), which has been implicated in T_{FH} cell development [17,18]

Concerning the functional role of ICOS for development of T helper cells besides T_{FH} cells, previous work has suggested that ICOS is also essential for T_H-17 cell development[9]. ICOS is a member of the CD28 family of coreceptor molecules that is induced on T cells after activation[19]; its ligand ICOS-L (B7h, GL50, LICOS, B7RP-1) is constitutively expressed at low levels on B cells, macrophages and dendritic cells, and ICOS-L expression is further upregulated upon activation of these cells. Although ICOS- and ICOS-L-deficient mice have defects in $CD4^+$ T helper cell responses, it is unclear where ICOS acts in T_H-17 cell developmental pathway and if ICOS affects the differentiation, amplification or maintenance and/or stabilization of T_H-17 cells.

As mentioned above, ICOS also plays a role in controlling T_{FH} cell development and function. Both ICOS-deficient and ICOS-L-deficient mice have defects in humoral immunity characterized by decreased levels of IgE and IgG1 in the serum and defects in antibody class switching and in GC formation[20,21]. In addition, patients who have a total defect in ICOS expression, $ICOS^{null}$ patients, exhibit a profound defect in B cell maturation and immunoglobulin isotype switching[22,23]; such ICOS deficiency is associated with an impaired development of $CXCR5^+$ T_{FH} cells and humoral immune responses both in humans and mice[24,25]. Both T_{FH} and T_H-17 cells produce IL-21, a cytokine required for the amplification of T_H-17 cells. Here we examined the relationship between T_H-17 and T_{FH} cells with the aim of shedding new light on the role of ICOS in these T helper cells. Although initial work suggested that ICOS was critical for T_H-17 differentiation[9], this study preceded our current understanding of the steps involved in T_H-17 cell differentiation[30].

In the study here we demonstrate that T_H-17 cell differentiation from naive $CD4^+$ T cells did not require ICOS, instead ICOS was critical for T_H-17 maintenance and function by regulating IL-21 production, which contributes to the expression and the maintenance of IL-23R. Furthermore, we found that both T_H-17 cells and T_{FH} cells express high relative levels of the transcription factor c-Maf and that loss of c-Maf results in a defect in IL-21 production and expansion of T_H-17 and T_{FH} cells, similar phenotypes to those produced by loss of ICOS. Thus, c-Maf induced by ICOS regulates IL-21 production that, in turn, regulates expansion of T_H-17 cells and T_{FH} cells.

RESULTS

ICOS is not crucial for T_H-17 differentiation

ICOS is important for T cell effector function, especially for the development of T_H2 responses[26,27] and it was recently suggested that ICOS also plays a role in the differentiation of Th17 cells[9]. To reassess a role for ICOS in the primary differentiation of T_H-17 cells, we sorted naïve $CD4^+CD62L^{high}$ T cells from wild-type or ICOS-deficient (*Icos*$^{-/-}$) B6 mice and differentiated them *in vitro* in the presence of IL-6 and TGF-β. After 5 days of culture in these conditions, we observed a similar frequency of IL-17 secreting cells in *Icos*$^{-/-}$ and wild-type $CD4^+$T cell cultures (Fig. 1a) and similar amounts of IL-17 cytokine production as detected by ELISA (Fig. 1b). These results indicate that ICOS is not required for primary T_H-17 differentiation *in vitro.*

As T_H-17 cells have been shown to be the major pathogenic population in the induction of EAE and other autoimmune diseases[5,28,29], we next studied the role of ICOS in driving T_H-17 differentiation during the course of EAE. We analyzed the priming of T cells following immunization of wild-type and *Icos*$^{-/-}$ mice with MOG_{35-55} peptide emulsified in CFA. Draining lymph nodes (dLN), spleen and central nervous system (CNS; brain and spinal cord) of the animals were harvested during the priming phase at day 10 post-immunization, before any clinical signs of disease (Fig. 2). Intracellular cytokine staining showed an equivalent frequency of $CD4^+$ splenocytes that produced IL-17 or IFN-γ in wild-type and *Icos*$^{-/-}$ mice. Interestingly, $CD4^+$ T cells in the dLN of *Icos*$^{-/-}$ mice showed an even higher production of IL-17 compared to wild-type mice (5% versus 2.5%) but the percentage of IFN-γ producing CD4 T cells was not significantly different between the two strains. Furthermore, when we analyzed the $CD4^+$ T cells infiltrating the CNS, we noticed that there were more $CD4^+$ T cells producing IL-17 in *Icos*$^{-/-}$ mice as compared to WT mice (56% versus 36%) (Fig. 2a). However, this increased frequency in T_H-17 cells did not persist throughout the course of disease. When we analyzed the mice after the first signs of disease or at the peak of the disease, the percentage of IL-17 cells is about the same in wild-type and *Icos*$^{-/-}$ mice (data not shown).

We also compared the antigen specific responses of wild-type and *Icos*$^{-/-}$ $CD4^+$ T cells to MOG_{35-55} peptide. For this we immunized wild-type and *Icos*$^{-/-}$ mice with MOG_{35-55} peptide emulsified in CFA, harvested the draining lymph nodes 5 days after immunization and cultured the cells in the presence of the immunizing peptide. In these conditions, lymph node cells from the *Icos*$^{-/-}$ mice showed a higher proliferation *in vitro* in response to the immunizing antigen than the cells from wild-type mice (Fig. 2b) and produced more IL-17, less IL-10, but the same amount of IFN-γ, as detected by cytokine ELISA or intracellular cytokine staining (Fig. 2c,d). This difference in the priming of T cells is associated with a slight, but a significant increase in severity of EAE in *Icos*$^{-/-}$ mice (Fig. 2e). There were no differences in the incidence of the disease but the mean maximum disease score was higher in *Icos*$^{-/-}$ mice as compared to wild-type mice (3.42 ± 0.92 versus 2.5 ± 0.53) (Table 1). Taken together, these data suggest that co-stimulation of $CD4^+$ T cells by ICOS is not required for the differentiation or priming of T_H-17 cells or for the development of acute EAE. These results are consistent with previously published results, where *Icos*$^{-/-}$ mice were shown to have a more severe form of EAE[20] that

was associated with decreased IL-10 and increased IL-17 production during the acute phase of the disease.

Because T_H-17 cells have been shown to be constitutively present in the intestinal lamina propria[30], we analyzed the presence of IL-17-producing T cells in the gut of *Icos*$^{-/-}$ mice. We found considerable variability in the frequency of IL-17-producing cells in the gut of *Icos*$^{-/-}$ and wild-type mice and there were no significant differences in the frequency of T_H-17 cells in the gut of these mice. Thus, ICOS may not be the critical regulator of T_H-17 cells in the gut (Supplementary Fig. 1a).

IL-17 expression in T_{FH} cells

Because IL-21 is a cytokine associated with both T_{FH} and T_H-17 cells, we examined whether there is a functional relationship between these two CD4$^+$ T helper cell subsets. To address this question, we immunized C57BL/6 wild-type mice with MOG_{35-55} peptide emulsified in CFA. At day 6 we harvested the dLN and spleen and sorted the cells based on the expression of CD4, CXCR5 and ICOS. Subsequently, we stimulated the cells for 4 hours with PMA-ionomycin and compared the expression of T_H-17 cell-associated molecules such as IL-21, IL-17 and IL-23R in T_{FH} cells (CD4$^+$ICOS^{high}CXCR5$^+$) and non-T_{FH} cells (CD4$^+$CXCR5$^-$) by RT-PCR. Consistent with what has been previously reported[31], we detected a higher IL-21 expression in T_{FH} cells compared to non-T_{FH} cells. Additionally, compared to non-T_{FH} cells, we could also detect higher expression of IL-17 and IL-23R in T_{FH} cells derived either from lymph node or spleen (Fig. 3a). Furthermore, *in vitro* expansion of T cells from naive mice, by stimulation with anti-CD3 and IL-23, resulted in a higher proportion of IL-17-producing cells among T_{FH} cells (5 fold) compared to the CD4$^+$CXCR5$^-$ (non-T_{FH} cell) fraction (8% versus 2%), whereas these cell populations contained similar percentages of IFN-γ producing cells (Fig. 3b).

The above data showed that T_{FH} cells in wild-type mice are enriched in the expression of IL-17 production. However, one interpretation of the data is that higher expression of IL-17 in T_{FH} cells compared to that in CD4$^+$CXCR5$^-$ non-T_{FH} cells could simply be due to the extent of activation of the cells. Indeed, T_{FH} cells consist of uniformly activated CD4$^+$ cells, whereas CD4$^+$CXCR5$^-$ T cells consist of a mixture of activated and non-activated T cells. To resolve this issue, we sorted three different T cell populations from dLN and spleen of immunized mice: CD4$^+$ICOS^{hi}CXCR5$^+$ T_{FH} cells, activated CD4$^+$ICOS^{hi}CXCR5$^-$ and non-activated CD4$^+$ICOS^{lo}CXCR5$^-$ T cells, and then compared the expression of IL-21, IL-17 and IL-23R in these cell fractions. As anticipated, the expression of IL-17, IL-21 and IL-23R in non-activated CD4$^+$ T cells (CD4$^+$ICOS^{lo}CXCR5$^-$) was low but when we compared the level of expression in activated CD4$^+$ ICOS^{hi} T cells and T_{FH} cells, we detected higher expression of IL-21, IL-17 and IL-23R in the CD4$^+$ICOS^{hi}CXCR5$^+$ T_{FH} cells compared to the activated ICOS^{hi} effector T cells (Fig. 3c). We then expanded the numbers of the three populations of CD4$^+$ T cells *in vitro* by stimulating them with IL-12 or IL-23 for 4 days and then we measured cytokines produced by the cells. Under these conditions, CD4$^+$ICOS^{hi}CXCR5$^+$ T_{FH} cells produced about 2-fold more IL-17 compared to the activated CD4$^+$ICOS^{hi}CXCR5$^-$ T cells, whereas when expanded in presence of IL-12 all three cell populations expressed equivalent amounts of IFN-γ (Fig. 3d). These data show that the heterogeneous population of T_{FH} is enriched in IL-17 producing cells that express high level of IL-23R and that are capable of producing substantial amounts of IL-17 in response to IL-23.

Development of IL-17-secreting T_{FH} requires ICOS

As ICOS has been shown to be crucial for the development of T_{FH} cells, IL-21 production and GC formation both in mice[24,25] and humans[23], we examined the impact of the lack of ICOS in the generation of IL-17-secreting T_{FH} cells. We immunized wild-type and *Icos*$^{-/-}$ mice with

MOG_{35-55} peptide emulsified in CFA and at day 6 sorted T_{FH} cells based on the expression of CD4 and CXCR5 (Fig 4a). As reported earlier[25,32], we observed a profound decrease in the number of $CD4^+CXCR5^+$ cells in the dLN and the spleen of *Icos*$^{-/-}$ mice, though some $CD4^+CXCR5^+$ cells were clearly present (Fig 4a.). When we compared the expression of IL-21 and IL-17 in the T_{FH} cells in wild-type mice and the few $CD4^+CXCR5^+$ cells in the *Icos*$^{-/-}$ mice, the latter cells showed substantially decreased IL-21 and IL-17 production, even when adjusted for numbers (Fig. 4b,c).

Because T_{FH} cells are characterized by the expression of ICOS and CXCR5, it is not possible to sort T_{FH} cells from the *Icos*$^{-/-}$ mice on the basis of a $CXCR5^+ICOS^{hi}$ phenotype. Therefore to clearly address the role of ICOS-ICOSL pathway on the development of IL-17 producing T_{FH} cells, we also analyzed the production of IL-17 and IL-21 in T_{FH} cells ($CD4^+ICOS^{hi}CXCR5^+$) derived from the *Icosl*$^{-/-}$ (ICOS-L-deficient) mice. Consistent with the *Icos*$^{-/-}$ data, T_{FH} cells derived from the *Icosl*$^{-/-}$ mice showed a defect in the production of IL-21 and IL-17 (Supplementary Fig. 1b).

We also compared the capacity of the T_{FH} cells from wild-type and *Icos*$^{-/-}$ mice to respond to IL-23. We sorted $CD4^+CXCR5^+$ T cells from immunized wild-type and *Icos*$^{-/-}$ mice and restimulated them with antigen presenting cells (APCs), anti-CD3 and IL-23 for 4 days. We then stained the $CD4^+CXCR5^+$ T cells for intracellular expression of IL-17 and IFN-γ. Compared to T_{FH} cells from wild-type mice, TFH cell from *Icos*$^{-/-}$ mice produced much less IL-17 but more IFN-γ (Fig. 4d). Finally, because the data indicated that ICOS was required for IL-17 production by T_{FH} cells, we measured IL-23R expression by RT-PCR in sorted T_{FH} cells from dLN and spleen from wild-type and *Icos*$^{-/-}$ mice to determine the importance of IL-23R for IL-17 production. Indeed, we found a profound decrease in IL-23R expression in *Icos*$^{-/-}$ T_{FH} cells (Fig. 4e). We also observed a similar defect in IL-23R expression in T_{FH} ($CD4^+ICOS^{hi}CXCR5^+$) cells from ICOS-L-deficient mice (Supplementary Fig.1b). These data show that ICOS is important for the generation of the IL-17-producing T_{FH} cells, but not IFN-γ-producing T cells, and that T_{FH} cells are compromised in *Icos*$^{-/-}$ mice.

Defective secondary T_H-17 responses of *Icos*$^{-/-}$ T cells

Since *Icos*$^{-/-}$ had no defect in T_H-17 differentiation in the presence of IL-6 plus TFG-β, we compared the responsiveness of *Icos*$^{-/-}$ and wild-type T cells to IL-23. We first cultured total $CD4^+$ T cells from unimmunized wild-type or *Icos*$^{-/-}$ mice in the presence of IL-23 to expand in vivo differentiated T_H-17 cells. During activation in the presence of IL-23, we observed a decrease in expansion of IL-17-producing cell numbers in *Icos*$^{-/-}$ mice (Fig. 5a). Since *in vivo* differentiated T_H-17 cells reside in the memory pool of T cells, we sorted the subset of effector-memory $CD62L^{lo}CD4^+$ T cells from unimmunized WT and *Icos*$^{-/-}$ mice and then stimulated the cells with IL-23. Under these conditions we found that the reduced numbers of T_H-17 cells was even more profound: *Icos*$^{-/-}$ effector-memory $CD4^+$ T cells produced 5-fold less T_H-17 cells in presence of IL-23 compared to wild-type effector-memory $CD4^+$ T cells (Fig 5a). To further study this effect of ICOS-deficiency, we differentiated naive wild-type or *Icos*$^{-/-}$ $CD4^+$ T cells with IL-6 and TGF-β *in vitro* for 5 days and then further cultured the cells in the presence of IL-23 for another 3-4 days: under these conditions, *Icos*$^{-/-}$ $CD4^+$ T cells yielded a 2- to 3-fold lower frequency of IL-17-producing T cells than did wild-type $CD4^+$ T cells (Fig. 5b). Moreover, IL-17-producing $CD4^+$ T cells in the dLN from wild-type mice immunized with MOG35-55 peptide expanded in numbers in the presence of IL-23 *ex vivo* (5-7% to ~30%), IL-17 producing $CD4^+$ T cells from *Icos*$^{-/-}$ mice did not expand further in the presence of IL-23 (Fig. 5c). Thus, these data suggest that *Icos*$^{-/-}$ cells do not have a primary defect in T_H-17 differentiation, but rather exhibit a defect in the expansion in cell numbers in response to IL-23.

We next investigated whether IL-23 unresponsiveness was a reflection of reduced expression of IL-23R or reduced production of IL-21 during T_H-17 differentiation. At the end of the differentiation, naive $CD4^+$ T cells from *Icos*$^{-/-}$ mice differentiated in the presence of IL-6 and TGF-β showed, at day 5, 50% lower IL-23R expression but only a slight decrease in the expression of the IL-12Rβ1 common chain, when compared to wild-type T_H-17 cells (Fig. 5d) whereas the percentage of IL-17-producing cells and the amount of IL-17 mRNA expression were equivalent in both cultures (data not shown). Those data were consistent with our observation that there is no defect in T_H-17 differentiation in *Icos*$^{-/-}$ T_H-17 (Fig. 1) and, furthermore, that differentiating T_H-17 cells express lower amount of IL-23R. These data suggest that costimulation of conventional T_H-17 cells through ICOS is necessary for either maximal IL-23R expression or maintenance of IL-23R expression. We also observed decreased IL-21 and IL-22 production by *Icos*$^{-/-}$ T_H-17 cells (Fig. 5e and Supplementary Fig. 1c) during the primary differentiation with IL-6 and TGF-β. Because IL-21 has been implicated not only in T_H-17 differentiation but also in IL-23R upregulation, it is possible that reduced expression of IL-23R in *Icos*$^{-/-}$ mice is secondary to reduced production of IL-21 by T cells.

ICOS regulates IL-21 production via c-Maf

To understand the mechanism by which ICOS regulates IL-21 production and differentiation of T_H-17, and potentially T_{FH}, cells, we undertook a microarray analysis of differentiated T_H-17 cells. The result showed that transcription factor c-Maf was upregulated in T_H-17 cells (data uploaded on ArrayExpress). As c-Maf has been shown to be downstream ICOS, we wondered whether c-Maf could be involved in T_H-17 differentiation by regulating IL-21 and IL-23R expression. We first differentiated naïve CD4 T cells into T_H-17 cells and observed that T_H-17 cells differentiated with IL-6 plus TGF-β or IL-21 plus TGF-β expressed c-Maf and this expression was further increased by exposure to IL-23 (Fig. 6a). Expression of c-Maf in T_H-17 cells was over 500 times higher than that of T_H1 or T_H2 cells. Similarly $CD4^+ICOS^{hi}CXCR5^+$ T_{FH} cells showed higher expression of c-Maf compared to $CD4^+ICOS^{hi}$CXCR5- and $CD4^+ICOS^{lo}$CXCR5- non-T_{FH} cells (Fig. 6a). We then measured c-Maf expression during T_H-17 differentiation in *Icos*$^{-/-}$ cells that were stimulated with wild-type APC in presence of IL-6 and TFG-β. We found that *Icos*$^{-/-}$ T_H-17 cells did not upregulate c-Maf expression, confirming the previously published observation that ICOS is crucial for c-Maf upregulation in T_H0 effector cells[35]. Because c-Maf regulation occurs 'downstream' of ICOS signaling, we tested if c-Maf-deficient cells "phenocopy" ICOS-deficient cells. For this, we differentiated naïve CD4 T cells from c-Maf-deficient mice in the presence of IL-6 plus TGF-β for 4 days and then evaluated the frequency of IL-17-secreting cells. We observed a similar frequency of IL-17-secreting c-Maf-deficient and IL-17-secreting wild-type $CD4^+$ T cells (Fig. 6b). Similar to ICOS, then, c-Maf is not required for T_H-17 differentiation.

However, we speculated that, like ICOS, c-Maf would be required for maintenance of IL-17-producing cells after they have been differentiated. To test this we restimulated the naïve CD4 T cells from c-Maf-deficient mice above, stimulated with IL-6 and TGF-β for 4 days, with IL-23 for an additional 3 days. Again, like ICOS-deficient T cells, c-Maf-deficient T cells yielded a 2 fold lower frequency of IL-17-producing T cells compared to wild-type T cells (Fig. 6c). This decrease in the frequency of $IL\text{-}17^+$ T cells in c-Maf-deficient mice was further confirmed by the production of IL-17 detected by ELISA (Fig. 6c). We also measured IL-23R expression during differentiation of c-Maf-deficient cells under T_H-17 polarizing conditions (IL-6 and TGF-03 for 4 days). At an early time-point during primary differentiation (d3) of the cells we detected the same level of IL-23R expression in c-Maf-deficient and wild-type cells; however after secondary expansion (+ IL-23 for additional 4 days), expression of IL-23R was lower in c-Maf-deficient cells which correlates with the decrease in the frequency of the T_H17 cells (Fig. 6d). Finally, because ICOS-deficient mice have a defect in IL-21 production, we also analyzed IL-21 production by c-Maf-deficient cells polarized under T_H-17 conditions.

Similar to ICOS-deficiency again, IL-21 production by c-Maf-deficient cells was reduced compared to wild-type cells (Fig. 6e). These data suggest that c-Maf is not necessary for inducing transcription of IL-23R, but more likely for maintaining IL-23R expression by inducing IL-21.

Because IL-21 has been shown to be crucial for GC formation and T_{FH} development, we also investigated whether c-Maf was involved in the development of the T_{FH} cells. For this, we immunized wild-type and c-Maf-deficient mice with TNP-OVA emulsified in CFA and analyzed the draining lymph nodes and the spleen after 7 days. We observed a profound decrease in the number of $CD4^+ICOS^{hi}CXCR5^+$ cells present in the dLN (Fig 6f) and the spleen of c-Maf-deficient mice (data not shown). Taken together, these data indicate that c-Maf participates in T_{FH} cells and T_H-17 development most likely by regulating IL-21 production.

DISCUSSION

In this study we demonstrated that conventional T_H-17 cells do not require ICOS costimulation for their differentiation or to acquire pathogenic potential to induce EAE, but rather that ICOS is necessary for the IL-23-driven expansion of already differentiated T_H17 cells. We further show that the defect in expansion of differentiated T_H-17 cells and in development of IL-17-secreting T_{FH} cells in ICOS-deficient mice is in part due to a defect in the upregulation of a transcription factor c-Maf. ICOS-deficient and c-Maf-deficient T_H-17 cells show a decrease of IL-21 production and consequently a decrease in IL-23R expression, suggesting that ICOS may be involved in regulating IL-23R expression in differentiating T_H-17 and T_{FH} cells via the transcription factor c-Maf. c-Maf induced by ICOS must regulate IL-21 production which participates to amplify T_H-17 cells and stabilizes these cells by inducing IL-23R expression. Since IL-23 is critical for expanding and maintaining IL-17 production, this highlights one of the mechanisms by which ICOS may regulate IL-21, IL-17 and T_{FH} cell development by regulating c-Maf expression.

It was first proposed that ICOS is required for T_H-17 cell differentiation[9]H. However, since ICOS-deficient mice are more susceptible to EAE than are wild-type mice[20] and T_H-17 cells are critical for the induction of EAE, these paradoxical results raised the question at which step ICOS is required for T_H-17 development. Using ICOS-deficient mice we re-analyzed the role of ICOS in various steps of T_H-17 differentiation. In vitro, there is no defect in acute T_H-17 differentiation in *Icos*$^{-/-}$ mice. On the contrary, *in vivo*, in immunized mice there is an increase in IL-17 production and a decrease in IL-10 production during the priming phase in *Icos*$^{-/-}$ mice and this may be one of the reasons why *Icos*$^{-/-}$ mice show increased susceptibility to EAE. This observation is consistent with previous reports showing that *Icos*$^{-/-}$ mice are more susceptible to EAE and experimental autoimmune uveoretinitis[20,36]. Our studies indicate that the increased susceptibility to EAE and EAU is not due to primary defect in the generation of T_H-17 cells following immunization, but most likely due to loss of IL-10-producing T cells. Indeed, IL-10 secretion by T cells is stimulated by ICOS signaling[19,37,38] and IL-10 is a key immunoregulatory cytokine involved in the induction of T regulatory type 1 (Tr1) cells, the function of $Foxp3^+$ T_{reg} cells and the suppression of autoimmunity[39,40].

Icos$^{-/-}$ mice have been shown to have a defect in the development of T_{FH} cells and here we show that they have a defect in IL-21 production. Since IL-21 is an amplification factor for T_H-17 cells and is produced at high levels by T_{FH} cells, we examined the link between T_H-17 cells and T_{FH} cells and observed an enhanced expression of molecules associated with T_H-17 cells, including IL-17, IL-21 and IL-23R, in IL-17-producing T_{FH} cells. IL-17 has previously been associated with the development of autoantibody responses and deficiency in IL-17 or blockade of IL-17 also has been shown to decrease autoantibody responses[29,41]. A recent study further showed that IL-17 is crucial for autoreactive germinal center development in

autoimmune BXD2 mice. In that study, IL-17-producing T cells and IL-17R$^+$ B cells were found co-localized in GCs and IL-17 was shown to play a role in GC formation *in vivo*[42]. This suggests that IL-17 not only induces tissue inflammation by acting on the IL-17R expressed on the parenchymal and other tissue cells, but it could also function on B cells to promote the GC reaction. Since a fraction of CXCR5- and ICOS-expressing T_{FH} cells were IL-17-producing cells, our results suggest that the IL-17 producing cells observed by Hsu et al[42] are most likely T_{FH} cells.

The crucial role of ICOS in GC development and T_{FH} generation is well established; ICOS- or ICOS-L-deficient mice develop fewer and smaller GC after immunization, associated with impaired T cell-dependent B cell responses and impaired isotype class switching[20,21,43,44].More recently, similar impairments in B cell responses have been reported in ICOS-deficient patients[25]. Furthermore, *Sanroque* mice, which carry a disruptive mutation in roquin, a repressor of ICOS, develop severe autoimmune, lupus-like disease associated with high expression of IL-21, accumulation of CXCR5$^+$ICOS^{hi} T cells, increased formation of GC and increased production of auto-antibodies[45,46]. Whether these mice also show enhanced expression of IL-17- and T_H-17-associated genes in the T_{FH} cells has not been addressed, but our results suggest that part of the phenotype observed in the *Sanroque* mice may also be due to enhanced IL-17 production by T_H-17 and T_{FH} cells. Taken together, these observations suggest that the defects in the number of T_{FH} cells and in GCs reaction, as well as a defect in class switching, in ICOS-deficient mice and ICOS-deficient patients may partly be due to a defect in IL-21 production which acts as an autocrine growth factor to expand T_{FH} cells.

Upon antigen-specific activation and differentiation, T_H-17 cells may have several different fates: a population of differentiated T_H-17 cells become effector T_H-17 cells, downregulate CCR7, upregulate CCR6 and migrate to the target organs where they mediate their effector function[54]; some T_H-17 cells maintain the expression of CCR7, stay in the secondary lymphoid organs and become IL-17-producing central memory cells; and a population of T_H-17 cells that express high level of ICOS and IL-23R, that downregulate CCR7 and upregulate CXCR5, become part of the heterogeneous T_{FH} cell pool that migrates to follicles where they interact with B cells to induce GC formation. Our data suggest that ICOS mediates T_{FH} cell development most likely by regulating IL-21 production, and consequently could act indirectly to regulate the IL-23R expression on these cells. This raised an issue of how ICOS regulates IL-21 expression and expansion of T_H-17 cells and T_{FH} cells. Our data suggest that the transcription factor c-Maf, which is downstream of ICOS, regulates IL-21 production and subsequently IL-23R expression in developing TH-17 and TFH cells. The study by Hsu et al. found enhanced production of IL-23 by non-lymphoid cells with increased numbers of T_{FH} cells in the spleen of autoimmune BXD2 mice[42], suggesting that IL-23 may be important for expansion of IL-17$_+$ T_{FH} cells as well. Whether there is a generalized defect in T_{FH} cells in IL-23R-deficient mice or just IL-17-producing cells remains to be clarified. Nevertheless, our observations suggest a role for ICOS in regulating IL-23R expression and IL-17 production by T_H-17 cells and T_{FH} cells. Therefore, a decrease in T_{FH} and T_H-17 cell responses in ICOS-deficient might be due to a defect in c-Maf upregulation, IL-21 production and consequently a defect in upregulation of IL-23R on T_H-17 and T_{FH} cells. Whereas IL-21 and IL-23 are not crucial for initial T_H-17 differentiation, they are crucial for expansion and maintenance of differentiated T_H-17 cells. Since our data show that ICOS regulates IL-23R on differentiating T_H-17 cells, lack of IL-23R upregulation in ICOS-deficient cells might be one of the reasons why ICOS-deficient mice show a defect in memory T_H-17 responses. ICOS has previously been shown not only to costimulate T cell activation, but also induce T cell survival in other T cell subsets[47,48]. It is likely that ICOS is playing a similar role in survival of T_{FH} cells as well by regulating IL-21 production and IL-23R expression in IL-17-secreting T_{FH} cells. Taken together our data suggest that ICOS regulates the fate of differentiated IL-17-producing T cells via induction of c-Maf transcription factor that in turn induces IL-21.

METHODS

Animals and induction of EAE

ICOS-deficient (*Icos*$^{-/-}$) mice were backcrossed onto the C57BL/6 background for 10 generations. Genotyping of *Icos*$^{-/-}$ mice was performed by PCR as previously described[49]. Chimeric c-Maf-deficient (*Maf*$^{-/-}$) mice were generated in the laboratory of I-Cheng Ho by reconstituting RAG-deficient (*Rag*$^{-/-}$) Balb/c mice with fetal liver cells from c-Maf-deficient mice. All mice for experiments were 8-12 wk old. EAE was induced by injecting mice subcutaneously (into the flanks) with 100 μl of an emulsion containing 100 μg of MOG_{35-55} peptide (MEVGWYRSPFSRVVHLYRNGK) and 250 μg of *M. tuberculosis* extract H37-Ra (Difco) in Complete Freund's Adjuvant (CFA). In addition, mice received 200 ng of pertussis toxin (List biological Laboratories via Cedarlane Ltd) intraperitoneally (i.p.) on days 0 and 2. Clinical signs of EAE were assessed according to the following score: 0, no signs of disease; 1, loss of tone in the tail; 2, hind limb paresis; 3, hind limb paralysis; 4, tetraplegia; 5, moribund. Mice were kept in a conventional pathogen-free facility at the Harvard Medical School. All experiments were carried out in accordance with guidelines prescribed by the Institutional Animal Care and Use Committee (IACUC) at Harvard Medical School.

Preparation of CNS mononuclear cells

Mice were perfused through the left cardiac ventricle with cold PBS. The forebrain and cerebellum were dissected and spinal cords flushed out with PBS by hydrostatic pressure. CNS tissue was cut into pieces and digested with collagenase D (2.5 mg/ml, Roche Diagnostics) and DNaseI (1 mg/ml, Sigma) at 37 °C for 45 min. Mononuclear cells were isolated by passing the tissue through a cell strainer (70 m), followed by a percoll gradient (70%/37%) centrifugation. Mononuclear cells were removed from the interphase, washed and resuspended in culture medium for further analysis.

T cell proliferation

For proliferation assays, mice were immunized with peptide-CFA as described above. A single-cell suspension was prepared from the draining lymph nodes or spleens on day 5 after immunization. Cells were cultured in DMEM-10% FCS supplemented with 5×10^{-5} M mercaptoethanol, 1 mM sodium pyruvate, nonessential amino acids, L-glutamine, and 100 U penicillin/100 g streptomycin per ml. During the last 16 h, cells were pulsed with 1 μCi of [^{3}H]thymidine (PerkinElmer) followed by harvesting on glass fiber filters and analysis of incorporated [^{3}H]thymidine in a β-counter (1450 Microbeta, Trilux, PerkinElmer).

Intracellular cytokine staining

For intracellular cytokine staining, cells were isolated as described above and stimulated in culture medium containing phorbol 12-myristate 13-acetate (PMA, 50 ng/ml, Sigma), ionomycin (1 μg/ml, Sigma) and monensin (GolgiStop 1 μl/ml, BD Biosciences) at 37 °C, in a humidified 10% CO_2 atmosphere for 4 h. After staining of surface markers (CD4), cells were fixed and permeabilized using Cytofix-Cytoperm and Perm/Wash buffer (BD Biosciences) according to the manufacturer's instructions. All antibodies to cytokines (IFN-γ, IL-17, IL-10) including the corresponding isotype controls were obtained from BD Biosciences and used a 1:200 dilution. Cells were incubated at 4 °C for 20 min and washed twice in Perm-Wash before analysis.

Cytokine ELISA

Lymph nodes cells from immunized mice were cultured with MOG_{35-55} peptide (100μg/ml). Supernatant from the cultures were harvested 48 hours after activation and secreted cytokines were determined by Enzyme-linked ImmunoSorbent assay using purified coating and

biotinylated detection antibodies for IL-17 (R&D Systems), IFN-γ and IL-10 (BD Bioscience). Detection antibodies were measured using horseradish peroxidase-conjugated streptavidin (Endogen), developed using 3.3',5.5'-Tetramethyl-benzidine (TMB) liquid substrate system (SIGMA) and stopped with 0.5M H_2SO_4.

In vitro T cell differentiation

Spleen cells from 2D2 mice were stimulated with 50 μg/ml MOG35-55 peptide in the presence or absence of IL-23 (20 ng/ml). For differentiating naïve T cells in vitro, $CD4^+$ T cells were purified using anti-CD4 beads (Miltenyi) or further FACS sorted into naive $CD4^+CD62L^{hi}$ cells. $CD4^+$ T cells were stimulated with C57BL/6 irradiated spleen cells and 1 μg/ml of anti-CD3 (145-2C11) for 3-5 days in the presence of cytokines: human TGF-β1 (3 ng/ml), mouse IL-6 (20 ng/ml) or IL-23 (20 ng/ml) (all R&D Systems) for T_H-17 differentiation, mIL-12 (5ng/ml; BD Pharmingen) and anti-mIL-4 (10 μg/ml, clone 11B11) for T_H1 differentiation and mIL-4 (10 ng/ml; R&D system) and anti-m-IL-12 (10 μg/ml, BD Pharmingen) for T_H2 differentiation.

Real-time PCR

RNA was extracted using the RNAeasy columns (Qiagen). Complementary DNA was transcribed as recommended (Applied Biosystems) and used as template for quantitative PCR. The expression of IL-17, IL-21, IL-23R was determined using specific primers and probes (Applied Biosystems). The Taqman analysis was performed on the AB 7500 Fast System (Applied Biosystem). The gene expression was normalized to the expression of the housekeeping gene β-actin.

Microarray analysis

Mouse spleen $CD4^+CD44^{Low}CD62L^{High}$ cells were stimulated with 2ug/ml anti-CD3 (eBioscience), 2ug/ml anti-CD28 (eBioscience) in the presence (T_H-17 condition) or absence (T_H0 condition) of hTGFβ-1 (1ng/ml eBioscience) and mouse IL-6 (10ng/ml eBioscience). Cells were collected after 24 hours of culture using RNAeasy columns (Qiagen) for total RNA isolation. Isolated total RNA from T_H0 and T_H-17 conditioned cells were submitted for the microarray gene transcription comparison analysis using Affymetrix Mouse 430A 2.0 array chips.

Statistical evaluation

Statistical evaluations of cell frequency measurements were performed using the unpaired Student's *t*-test for samples with unknown and potentially disparate variances.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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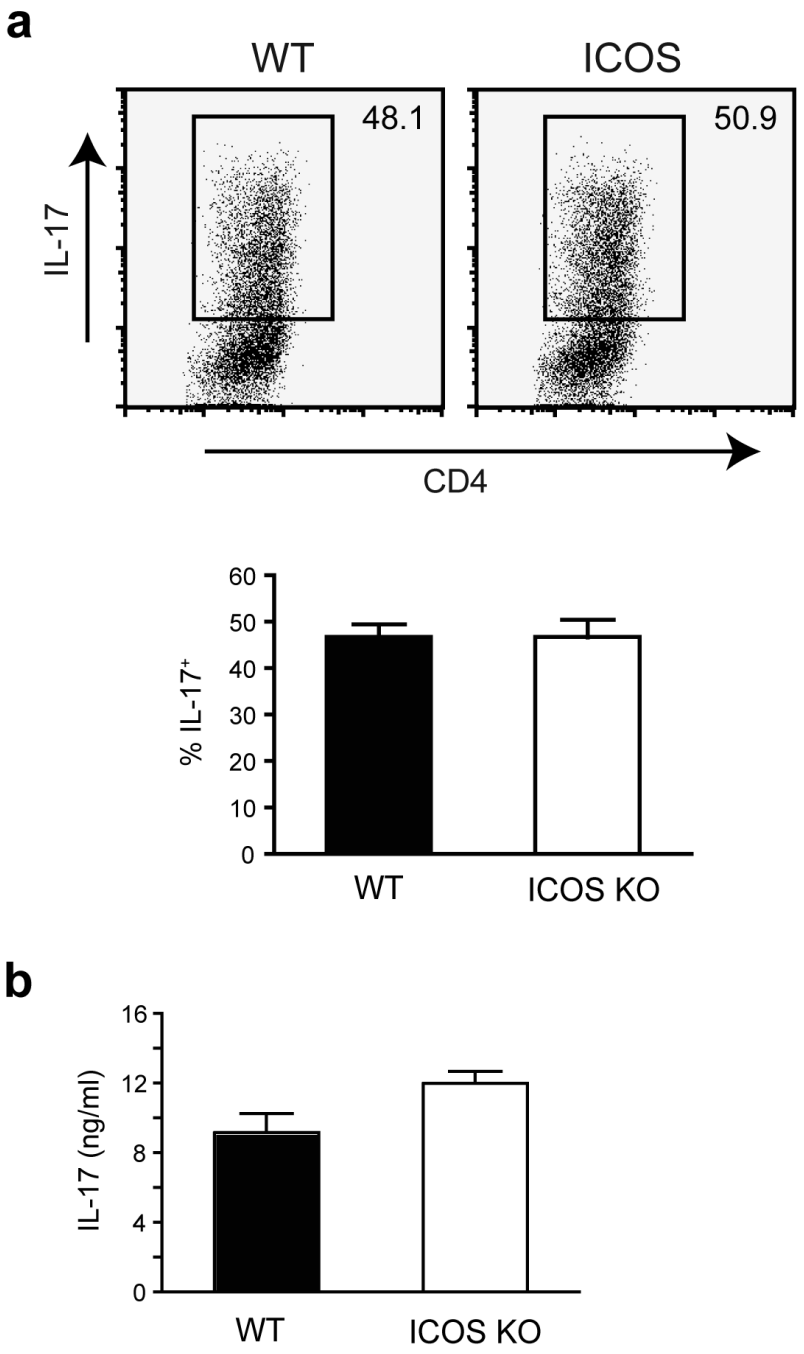

Figure 1. ICOS is not crucial for T -17 differentiation in vitro

(a,b) Naïve $CD4^+CD62L^{high}$ T cells from ICOS-deficient (*Icos*$^{-/-}$) or wild-type mice were stimulated with soluble anti-CD3, antigen-presenting cells, IL-6 and TGF-β for 5 days and then were restimulated with PMA-ionomycin. IL-17 production in $CD4^+$ cells was measured by intracellular cytokine staining (**a**) or by ELISA (**b**). Numbers adjacent to boxed areas or in quadrants indicate percent positive cells in each quadrant. Data represent the mean ± s.d. of six experiments (**a**) or one of four experiments (**b**) (mean ± s.d. of triplicate wells).

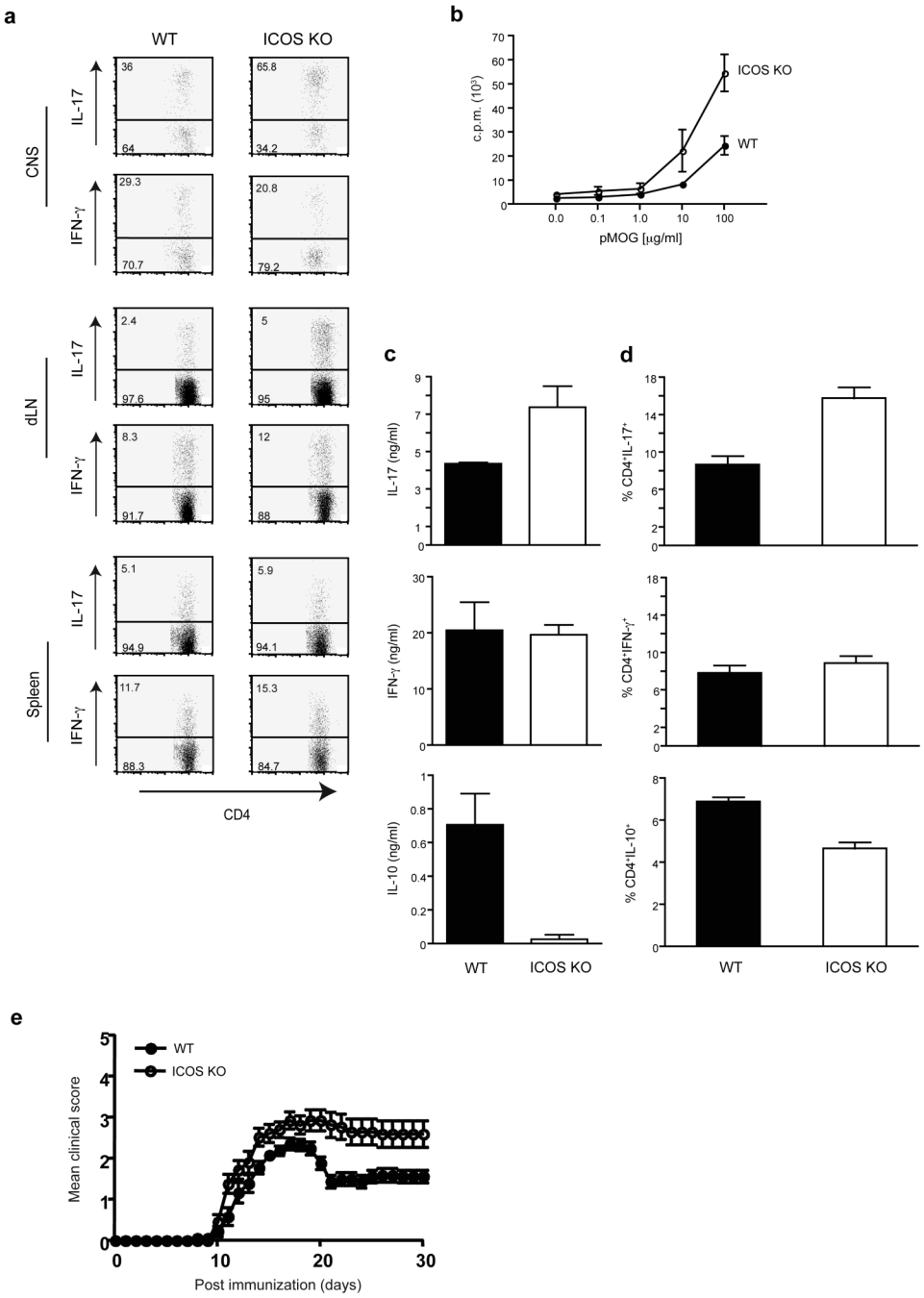

Figure 2. ICOS is not crucial for T_H-17 differentiation *in vivo* nor for induction of acute EAE

(**a-e**) ICOS-deficient (*Icos*$^{-/-}$) or wild-type mice were immunized with MOG35-55 emulsified in complete Freund's adjuvant to induce EAE. (**a**) Flow cytometry for intracellular cytokine secretion in CD4$^+$ T cells at day 10 obtained from mononuclear cells isolated from CNS, dLN and spleen activated with PMA-ionomycin. (**b-d**) Lymph node cells from immunized mice were tested for antigen-specific response: proliferation in triplicate cultures by ^{3}[H] thymidine incorporation (mean ± s.d.) (**b**), cytokine production by ELISA (day 2) (**c**) and intracellular cytokine staining by flow cytometry (day 5) (**d**). (**e**) The mean clinical EAE scores for the ICOS-deficient (dark circles) and wild-type control (open circles) groups (mean ± s.e.m.).

Numbers adjacent to boxed areas or in quadrants indicate percent positive cells in each. Data represent one of four experiments (**a**) or the mean ± s.d. of five (**b,c,d**) or four (**e**) experiments.

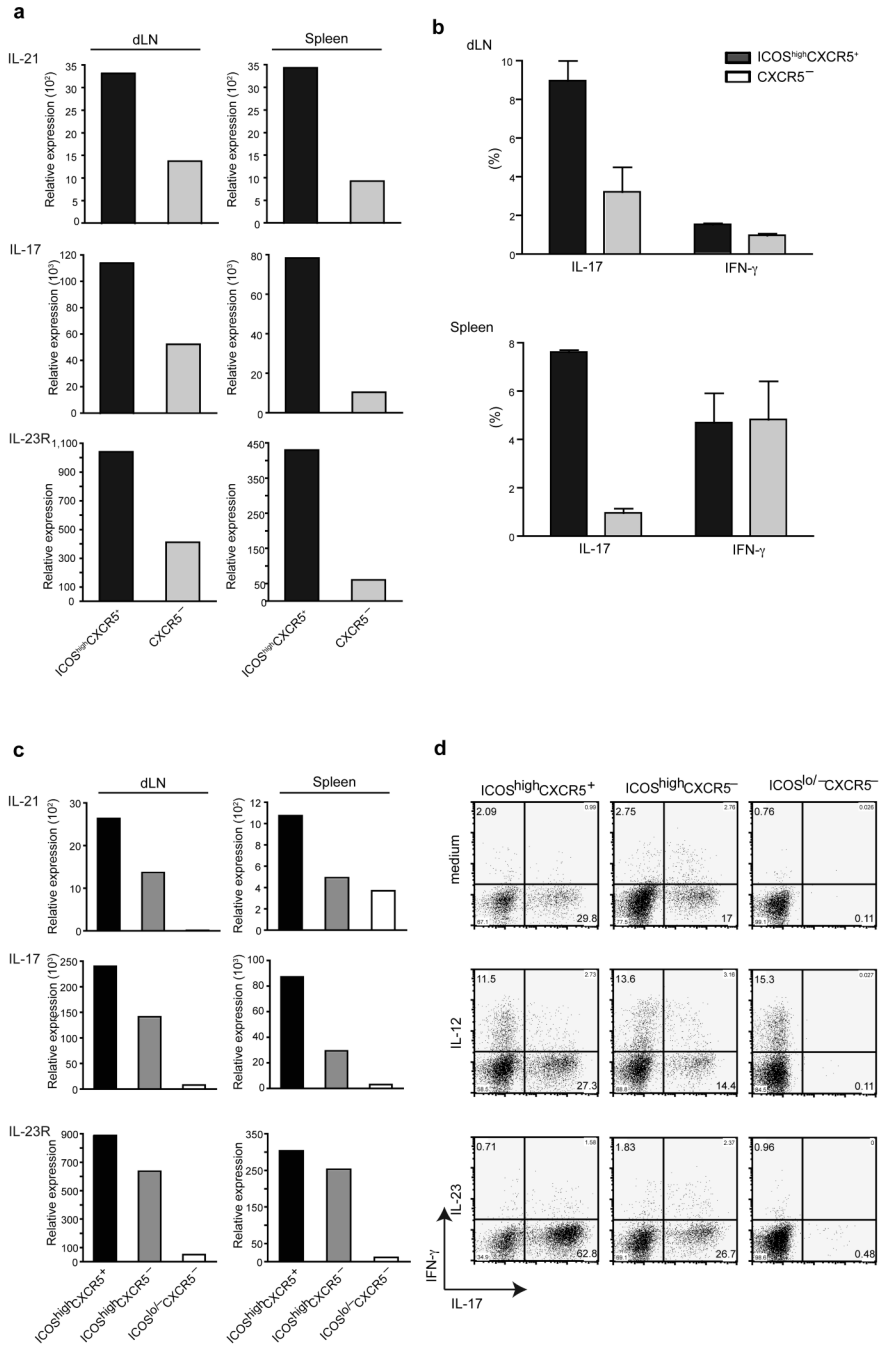

Figure 3. T_{FH} cells produce IL-17

(a-d) FACS-sorted $CD4^+ICOS^{hi}CXCR5^+$, $CD4^+ICOS^{hi}CXCR5^-$ and $CD4^+ICOS^{lo}CXCR5^-$ T cells were sorted by flow cytometry from wild-type immunized mice and then analyzed. (**a**) RT-PCR for expression of IL-21, IL-17 and IL-23R in $CD4^+ICOS^{hi}CXCR5^+$ (black histograms) and ($CD4^+CXCR5^-$) light grey histograms) T cells from draining lymph node or spleen 4 hr after restimulation of the cells in vitro with PMA/ionomycin; expression relative to β-actin. (**b**) Flow cytometry for expression of IL-17 and IFN-γ by cells in **a** stimulated instead with soluble anti-CD3, antigen-presenting cells and IL-23 for 4 days. (**c**) RT-PCR for expression of IL-21, IL-17 and IL-23R mRNA (relative to β-actin) in the indicated cells after treatment with PMA-ionomycin for 4 hours. (**d**) Flow cytometry of the indicated cells

stimulated in vitro with IL-12 or IL-23 for 4 days and then restimulated with PMA-ionomycin. Numbers adjacent to boxed areas or in quadrants indicate percent positive cells in each. Data represent one of six experiments (**a**) or mean ± s.d. of three experiments (**b**).

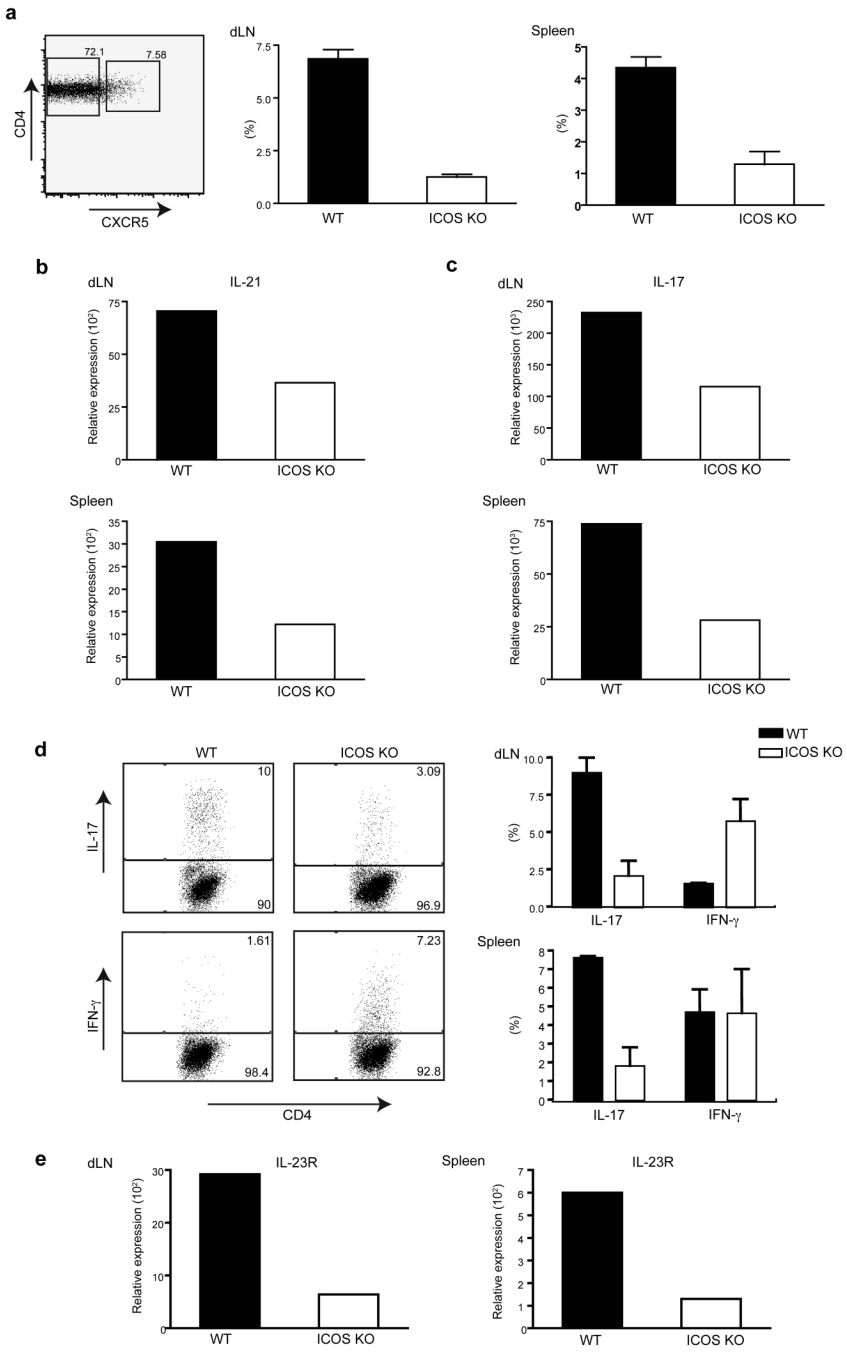

Figure 4. ICOS-deficient T_{FH} cells are defective in IL-17 production

(a-c) $CD4^+CXCR5^+$ from dLN and spleens of wild-type and ICOS-deficient immunized mice were restimulated with PMA-ionomycin for 4 hours. The percentage of $CD4^+CXCR5^+$ cells was assessed by flow cytometry (**a**) and the expression of IL-21 (**b**) and IL-17 (**c**) was assessed by quantitative PCR relative. (**d**) Flow cytometry of $CD4^+CXCR5^+$ cells from wild-type or ICOS-deficient immunized mice were stimulated with anti-CD3, antigen-presenting cells and IL-23 for 4 days and then restimulated with PMA-ionomycin and the percentage of IL-17+ and IFN-γ+ cells was measured by intracellular cytokine staining. (**e**) RT-PCR for IL-23R expression in $CD4^+CXCR5^+$ cells from dLN and spleens of wild-type and ICOS-deficient immunized mice restimulated with PMA-ionomycin for 4 hours; expression is relative to β-

actin. Numbers adjacent to boxed areas or in quadrants indicate percent positive cells in each. Data represent the mean ± s.d. of four (**a**) or three (**d**) experiments or represent one of six (**b,c**) or four (**e**) experiments.

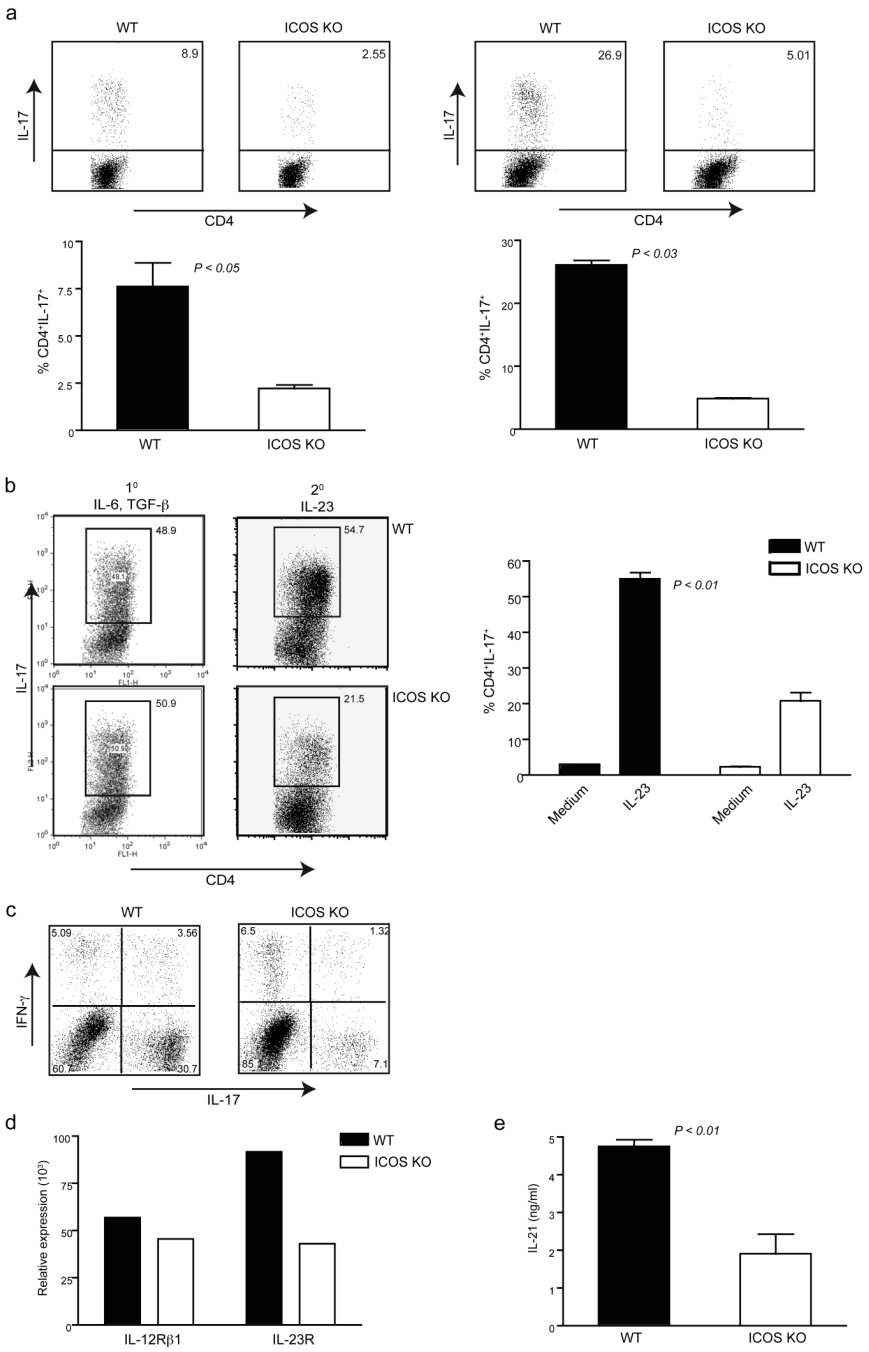

Figure 5. ICOS-deficient T cells show a defect in secondary T_H-17 responses

(**a-c**) Flow cytometry of ICOS-deficient or wild-type $CD4^+$ T cells (left panel) or $CD4^+CD62L^{lo}$ T cells (right panel) stimulated with anti-CD3, APCs, and IL-23 for 5 days and then restimulated with PMA-ionomycin (**a**); wild-type or ICOS-deficient cells differentiated for 5 days with IL-6 and TGF-β for 5 days and then restimulated with anti-CD3, APCs and IL-23 for an additional 4 days (**b**); or lymph nodes cells from immunized wild-type or ICOS-deficient mice cultured with MOG_{35-55} and IL-23 for 4 days (**c**). (**d,e**) RT-PCR expression of IL-12Rβ1 and IL-23R (**d**) and ELISA for production of IL-21 (**e**) from naïve T cells from ICOS-deficient or wild-type mice stimulated with soluble anti-CD3, APCs, IL-6 and TGF-β

for 5 days; expression to β-actin. Data represent the mean ± s.d. of four (**a,b**) experiments or represent one of three (**c**) or two (**d,e**) experiments.

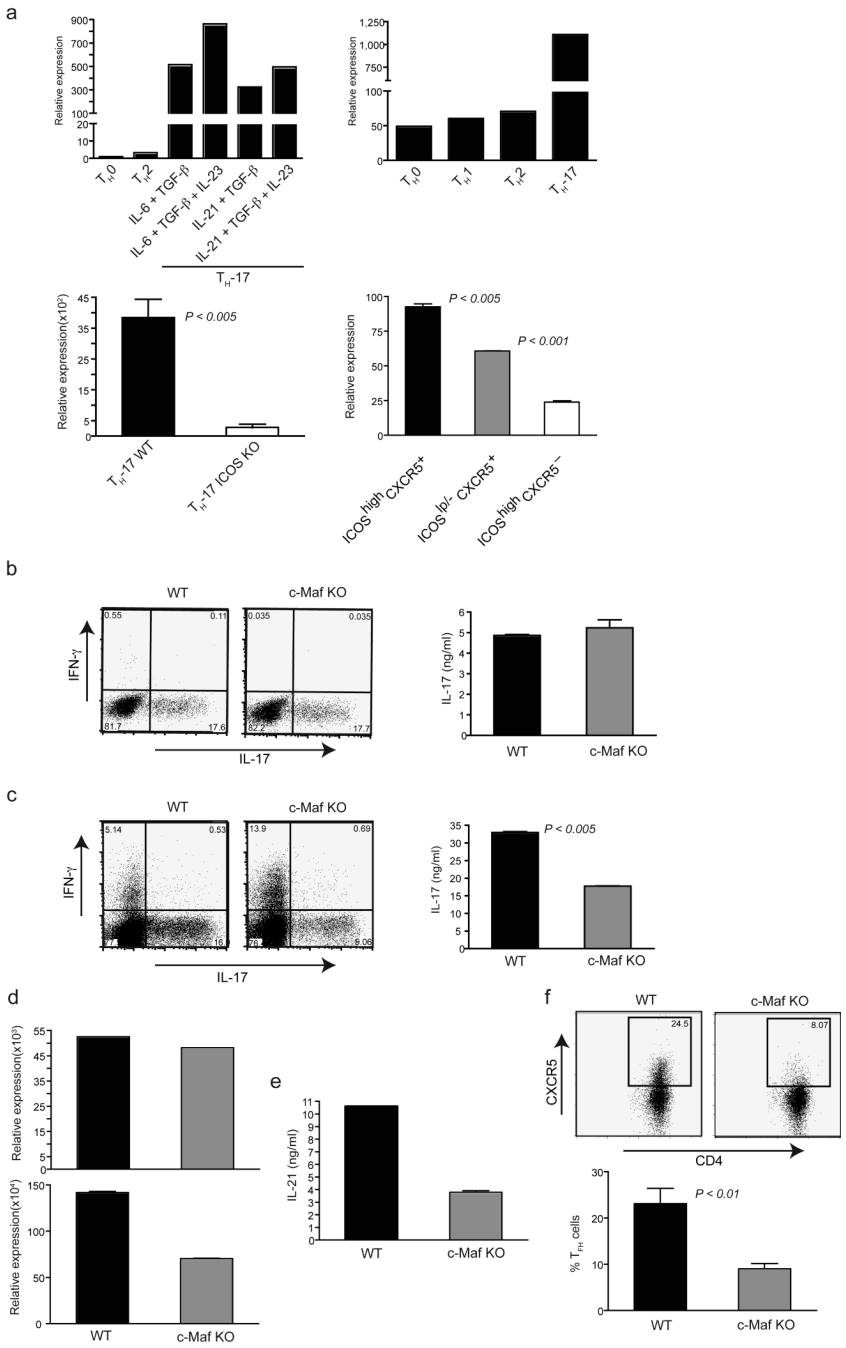

Figure 6. c-Maf in T_H-17 differentiation

(**a**) RT-PCR of c-Maf expression in $CD4^+CD62L^{high}$ T cells from wild-type mice stimulated under T_H0, T_H1, T_H2, T_H-17 conditions, or as indicated with plate bound anti-CD3 and anti-CD28 (top left) or with anti-CD3 and APCs (top right) (expression relative to GADPH); c-Maf expression in ICOS-deficient or wild-type naïve $CD4^+CD62L^{hi}$ T cells stimulated with anti-CD3, antigen-presenting cells, IL-6 and TGF-β (bottom left; expression relative to β-actin); c-Maf expression in $CD4^+ICOS^{hi}CXCR5^+$, $CD4^+ICOS^{hi}CXCR5^-$ and $CD4^+ICOS^{lo}CXCR5^-$ cells from dLN of wild-type mice immunized with MOG and restimulated *in vitro*; (bottom right; expression relative to β-actin). (**b,c**) Flow cytometry (left) and ELISA (right) for IFN-γ and IL-17 production by c-Maf-deficient or wild-type naïve $CD4^+CD62L^{hi}$ T cells stimulated

in vitro (**b**) or c-Maf-deficient or wild-type T_H-17 cells restimulated *in vitro* (**c**). (**d**) RT-PCR for IL-23R expression in T_H-17 cells day 3 of 'primary differentiation' (TGF-β + IL-6; left) or 'secondary expansion' (+IL-23, right); expression is relative to β-actin. (**e**) ELISA for IL-21 production at day 3 of primary differentiation of T_H-17. (**f**) Flow cytometry of wild-type and c-Maf-deficient mice immunized with TNP-OVA in complete Freund's adjuvant. Numbers indicate percent positive T_{FH} cells ($CD4^+CXCR5^+ICOS^+$) in each quadrant.

Table 1

EAE in wild-type and ICOS KO mice

ICOS-deficient or wild-type mice were immunized with MOG_{35-55} emulsified in complete Freund's adjuvant and then monitored for development of EAE. Statistical analysis was performed using the unpaired Student's *t*-test for samples with unknown and potentially disparate variances

Group	Incidence	Mean day of onset (mean ± s.d.)	Mean maximum score (mean ± s.d.)	Mortality
Wild-type	16/18	10.8 ± 0.93	2.5 ± 0.53*	0/18
ICOS KO	22/22	11.42 ± 1.6	3.4 ± 0.92*	5/22

* $P < 0.05$