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Aiolos promotes T_H17 differentiation by directly silencing *Il2* expression

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Abstract

CD4⁺ interleukin 17 (IL-17)-producing helper T cells (T_H17 cells) are instrumental in the immune response to pathogens. However, an overactive T_H17 response results in tissue inflammation and autoimmunity, and therefore it is important to identify the molecular mechanisms that control the development of T_H17 cells. IL-2 suppresses such development, but how IL-2 production is actively suppressed during T_H17 differentiation is not understood. Here we report that under T_H17-polarizing conditions, the transcription factors STAT3 and AhR upregulated the expression of Aiolos, a member of the Ikaros family of transcription factors. Using Aiolos-deficient mice, we demonstrated that Aiolos silenced the *Il2* locus, promoting T_H17 differentiation *in vitro* and *in vivo*. Thus, we have identified a module in the transcriptional program of T_H17 cells that actively limits IL-2 production and promotes their differentiation.

Cells of the T_H17 subset of helper T cells are characterized by the production of interleukin 17 (IL-17), IL-17F, IL-21 and IL-22 and have emerged as a subset of effector CD4⁺ T cells with an important role in the control of specific pathogens as well as in the development of autoimmune diseases¹. The activation of T cells in the presence of IL-6 (ref. 2) or IL-21 (ref. 3) and transforming growth factor-β1 (TGF-β1) promotes T_H17 differentiation by transcription factor STAT3-dependent mechanisms⁴, whereas IL-21 (ref. 3) and IL-23 (ref. 5) expand T_H17 cell populations and stabilize the phenotype of T_H17 cells. The signals initiated in T cells by cytokine receptors induce and activate specific transcription factors that control the transcriptional program of T_H17 cells. T_H17 differentiation is driven by the transcription factors RORγt⁶ and RORα⁷. Other transcription factors, such as c-Maf and

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AUTHOR CONTRIBUTIONS

H.J., F.J.Q., M.N. and M.R. did *in vitro* and *in vivo* experiments; M.N., A.Y., E.J.B., D.K., C.Z. and S.X. did *in vitro* experiments; K.G. and J.S. provided reagents; F.J.Q. wrote the manuscript; and V.K.K. and F.J.Q. supervised the study and edited the manuscript.

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AhR, also participate in T_H17 differentiation. AhR is known to control the expression of IL-21 and IL-22 and has an important role in T_H17 differentiation *in vivo* and *in vitro*^{8–10}.

Dysregulated population expansion of T_H17 cells has been linked to the development of tissue inflammation and autoimmunity; thus, several mechanisms are in place to control the generation and activity of T_H17 cells¹. Although IL-2 and IL-21 belong to the same family of cytokines, they have opposing effects on T_H17 differentiation: IL-21 acts as a growth factor and supports the population expansion of T_H17 cells, whereas IL-2 inhibits T_H17 differentiation^{11–15}. IL-2 limits T_H17 differentiation *in vitro* and *in vivo* by interfering with IL-6-dependent signaling events. IL-2 downregulates expression of the IL-6 receptor¹³ and also triggers the replacement of STAT3 with STAT5 on target DNA-binding sites in the *Il17a-Il17f* locus and in other genes required for T_H17 differentiation^{12,15} and thus it interferes with the T_H17 transcriptional program. Therefore, for T_H17 differentiation to proceed unabated, IL-2 expression must be actively downregulated. The uptake of IL-2 by regulatory T cells (T_{reg} cells) that express the transcription factor Foxp3 promotes T_H17 differentiation *in vivo* and *in vitro*^{11,14}. It is conceivable that in addition to the removal of IL-2 by cell-extrinsic mechanisms, cell-intrinsic mechanisms that actively limit the production of IL-2 are in place to promote T_H17 differentiation.

Here we report significant upregulation of Aiolos, a member of the Ikaros family of transcription factors, during T_H17 differentiation by a mechanism that involved STAT3 and AhR. Moreover, Aiolos bound to and silenced the *Il2* locus, suppressing the production of IL-2 and promoting T_H17 differentiation *in vitro* and *in vivo*. Thus, our results identify a transcriptional module that actively limits the production of IL-2 during T_H17 differentiation to promote the development of T_H17 cells.

RESULTS

Expression of Aiolos by T_H17 cells

Our analysis of the transcriptional programs of CD4⁺ T cell subsets showed that T_H17 cells expressed Aiolos (encoded by *Ikzf3*; data not shown). We found that naive CD4⁺CD25[−]CD62L⁺CD44^{lo} T cells differentiated *in vitro* into T helper type 1 (T_H1) or T_H2 cells had modest expression of *Ikzf3*, but *Ikzf3* expression was substantially upregulated in T_H17 cells differentiated with TGF-β1 plus IL-6 (Fig. 1a). We did not detect upregulation of genes encoding other members of the Ikaros family of transcription factors, including Ikaros itself, Helios, Eos and Pegasus¹⁶, in differentiating T_H17 cells (Fig. 1b).

We also detected *Ikzf3* expression in Foxp3⁺ T_{reg} cells differentiated *in vitro* and T regulatory type 1 cells (Tr1 cells) induced with IL-27 (Supplementary Fig. 1). The role of transcription factors of the Ikaros family in the differentiation of Foxp3⁺ T_{reg} cells and Tr1 cells has been investigated^{17–20}; thus, in this study we focused on the role of Aiolos in the differentiation of T_H17 cells. We first investigated the kinetics of *Ikzf3* expression under T_H17-polarizing conditions. *Ikzf3* expression was substantially upregulated 6 h after activation in the presence of TGF-β1 and IL-6, and its expression remained very high throughout the T_H17 differentiation (Fig. 1c). Upregulation of *Ikzf3* expression preceded the induction of *Il17a* (Fig. 1c). The activation of T cells in the absence of polarizing cytokines (T_H0 conditions) did not result in substantial upregulation of *Ikzf3* expression (Fig. 1c). Together these data demonstrated that activation of naive CD4⁺ T cells under T_H17-polarizing conditions resulted in the upregulation of *Ikzf3* expression.

Control of T_H17 differentiation by Aiolos

To determine if Aiolos has a role in the differentiation of T_H17 cells, we investigated the effect of loss of Aiolos on T_H17 differentiation using naive T cells from wild-type and

Aiolos-deficient mice²¹. Naive Aiolos-deficient CD4⁺ T cells showed significant impairment in their differentiation into T_H17 cells, as shown by their lower expression of *Il17a* and other genes encoding molecules linked to the T_H17 lineage, such as *Rorc*, *Rora*, *Maf*, *Ahr*, *Il17f* and *Il21*, and diminished secretion of IL-17 into culture supernatants (Fig. 2a–c). Retrovirus-mediated overexpression of *Ikzf3* in T cells activated under non-T_H17-polarizing conditions did not result in upregulation of the expression of *Rorc* or *Il17a* (Supplementary Fig. 2), which suggested that Aiolos participated in but was not sufficient to induce T_H17 differentiation. Conversely, Aiolos-deficient CD4⁺ T cells produced more interferon- γ (IFN- γ) than wild-type cells did when activated under T_H1-polarizing conditions (Fig. 2b).

To characterize the relevance of Aiolos to T_H17 differentiation during the course of an immune response, we studied the *in vivo* population expansion of T_H17 cells after immunization. We immunized naive wild-type and Aiolos-deficient mice with myelin oligodendrocyte peptide (amino acids 35–55 (MOG(35–55)) emulsified in complete Freund's adjuvant and, 7 d later, assessed the ability of lymph node cells to proliferate in response to MOG(35–55) and produce IFN- γ and IL-17. Aiolos-deficient mice had a slightly lower recall proliferative response to MOG(35–55) (Fig. 2d) and a significantly lower frequency of T_H17 cells immediately after isolation, concomitant with a greater proportion of T_H1 cells (Fig. 2e).

To analyze the pathogenicity of Aiolos-deficient T_H17 cells, we used a model of passive transfer of experimental autoimmune encephalomyelitis (EAE). We immunized wild-type and Aiolos-deficient mice with MOG(35–55) and reactivated lymphocytes from the mice *in vitro* with MOG(35–55) in the presence of IL-12 or IL-23 to favor the population expansion of pathogenic T_H1 cells or T_H17 cells, respectively. Aiolos-deficient T cells reactivated under T_H17-favoring conditions had significantly less ability to induce EAE after transfer into host mice deficient in recombination-activating gene 2 (*Rag2*^{−/−} mice; Fig. 2f). Conversely, Aiolos-deficient T cells reactivated under T_H1-favoring conditions showed significantly more encephalitogenic activity (Fig. 2g). Thus, Aiolos controlled the differentiation of encephalitogenic T cells. Together these data indicated that Aiolos participated in the differentiation of T_H17 cells and limited the generation of T_H1 cells *in vitro* and *in vivo*.

Aiolos limits IL-2 production in T_H17 cells

IL-2 limits the differentiation of T_H17 cells *in vitro* and *in vivo*^{11–15}. Several members of the Ikaros family have been shown to control the production of IL-2 in mouse T cells¹⁸ and human T cells^{17,22}. Thus, we examined the expression of *Il2* during T_H17 differentiation. After an initial peak, the expression of *Il2* was silenced in T_H17 cells but not in T_H0 cells (Fig. 3a). The arrest in *Il2* transcription in T_H17 cells coincided with the upregulation of *Il17a* (Fig. 3a), *Rorc* and *Rora* (Supplementary Fig. 3). Conversely, the continued production of *Il2* in T_H0 cells was concomitant with the increase in expression of *Ifng* (Fig. 3a).

Given the inhibitory effects of IL-2 on T_H17 differentiation and the reported inhibitory effects of Ikaros proteins on the production of IL-2 by T cells^{17,18,22}, we determined whether Aiolos promoted T_H17 differentiation by inhibiting the production of IL-2. Aiolos-deficient T cells activated under T_H17-polarizing conditions produced more IL-2 than did wild-type T cells activated in the same way (Fig. 3b–d), which correlated with less production of IL-17 by Aiolos-deficient cells (Fig. 3d). IL-2 induces expression of the α -chain of the IL-2 receptor (CD25), presumably as a part of a positive feedback loop²³. Accordingly, we found upregulation of *Il2ra* in Aiolos-deficient T cells activated under T_H17-polarizing conditions (Supplementary Fig. 3b,c). Thus, Aiolos-deficient T cells produced more IL-2 when activated under T_H17-polarizing conditions, and the autocrine

effects of IL-2 upregulated expression of the high-affinity IL-2 receptor, which made these cells more sensitive to the biological effects of IL-2. We also observed more production of IL-2 (Fig. 3c) and a greater frequency of IL-2⁺ T cells (Supplementary Fig. 4a,b) among naive Aiolos-deficient CD4⁺ T cells activated under T_H1-polarizing conditions than among wild-type CD4⁺ T cells activated under T_H1-polarizing conditions. In addition, Aiolos-deficient CD4⁺ T cells activated under T_H1-polarizing conditions had higher expression of *Tbx21* (which encodes T-bet) and CD25 than wild-type cells activated under T_H1-polarizing conditions had (Supplementary Fig. 4c–e). Together these data suggested that Aiolos negatively regulated the production of IL-2.

To determine whether the dysregulated production of IL-2 in Aiolos deficient CD4⁺ T cells under T_H17-polarizing conditions was responsible for the diminished differentiation of these cells along the T_H17 pathway, we investigated the differentiation of wild-type and Aiolos-deficient naive T cells under T_H17 conditions in the presence of blocking antibody to IL-2 or isotype-matched control antibody. The antibody to IL-2 abrogated the deficit in the production of T_H17 cells by Aiolos-deficient T cells (Fig. 3e), whereas a blocking antibody to IFN- γ had no substantial effect on the T_H17 differentiation of Aiolos-deficient cells (Supplementary Fig. 3d). Those observations were in agreement with the lack of upregulation of *Tbx21* in Aiolos-deficient T cells activated under T_H17-polarizing conditions (Fig. 2c) and demonstrated that the diminished differentiation of Aiolos-deficient T cells into T_H17 cells *in vitro* did not result from the inhibitory effects of IFN- γ on T_H17 differentiation^{24,25}. Antibody to IL-2 abrogated the enhanced production of IFN- γ and the upregulation of *Tbx21* and *Il2ra* in Aiolos-deficient T cells activated under T_H1-polarizing conditions (Supplementary Fig. 4f–h). Together these data demonstrated that Aiolos promoted T_H17 differentiation by limiting the production of IL-2.

Silencing of the *Il2* promoter by Aiolos in T_H17 cells

Members of the Ikaros family of transcription factors regulate IL-2 expression in mouse T cells¹⁸ and human T cells^{17,22} by interacting with the locus encoding IL-2 and triggering epigenetic changes that limit its accessibility to other transcription factors. Thus, we searched the mouse *Il2* promoter for Aiolos-binding sites and found a putative Aiolos-binding element 13 base pairs upstream of the transcription start site in the *Il2* promoter (Fig. 4a). To determine whether Aiolos bound to its putative target sequence in T_H17 cells, we used electrophoretic mobility-shift assay (EMSA) with a double-stranded oligonucleotide composed of the Aiolos-binding element in the *Il2* promoter to probe nuclear extracts from T_H17, T_H1 or T_H0 cells differentiated *in vitro*. Nuclear extracts of T_H17 cells bound the putative Aiolos-binding site, but those of T_H0 or T_H1 cells did not (Fig. 4b). The interaction between the radiolabeled oligonucleotide and the nuclear extracts of T_H17 cells was lower after preincubation with antibody to Aiolos but not after preincubation with control antibody to tubulin (Fig. 4b). Antibodies to proteins of the Ikaros family disrupt the oligomeric form responsible for DNA binding in EMSA²⁶, which suggests that Aiolos in the nuclear extracts of T_H17 cells interacted with its consensus binding element in the *Il2* promoter. In addition, chromatin immuno-precipitation (ChIP) assays showed that Aiolos interacted with its binding site in the *Il2* promoter in T_H17 cells but not in T_H1 or T_H0 cells (Fig. 4c). Thus, Aiolos interacted with the *Il2* promoter during T_H17 differentiation.

Transcription factors of the Ikaros family control the epigenetic status of their target genes^{18,22,27}. Chromatin modifications have an important role in the regulation of gene expression; the presence of acetylated histone H3 (H3ac) and acetylated histone H4 (H4ac), and of histone H3 trimethylated at Lys4 (H3K4me3), has been linked to the active transcription of genes, whereas the presence of H3K9me3 or H3K27me3 is associated with the repression of gene expression¹⁴. Thus, to investigate the molecular basis of the

suppression of *Il2* expression by Aiolos in T_H17 cells, we analyzed the abundance of H3K4me3, H3K9me3, H3K27me3, H3ac and H4ac in the *Il2* promoter of wild-type and Aiolos-deficient T cells polarized *in vitro* into T_H17 cells. Aiolos-deficient T_H17 cells had less H3K9me3 and H3K27me3 (Fig. 4d) but more H3K4me3 and more H3ac and H4ac (Fig. 4e) than did wild-type T_H17 cells. Thus, in Aiolos-deficient T_H17 cells, the *Il2* promoter was characterized by epigenetic modifications associated with active gene expression.

To determine the functional consequences of the interaction of Aiolos with the *Il2* promoter, we transfected reporter constructs containing the firefly luciferase gene under the control of the *IL2* promoter into human Jurkat T cells. Transfection of this luciferase reporter together with a construct encoding Aiolos resulted in substantial and dose-dependent repression of the transcriptional activity of the *IL2* promoter (Fig. 4f). Transfection of expression vectors for Aiolos into Jurkat cells also induced inhibition of expression of endogenous *IL2* (data not shown). Together these data demonstrated that Aiolos induced epigenetic modifications that silenced *Il2* expression in T_H17 cells.

Control of Aiolos expression by AhR and STAT3 in T_H17 cells

Activation of STAT3 initiates T_H17 differentiation in response to polarizing cytokines⁴. Detailed kinetic analysis of gene expression showed that upregulation of *Stat3*, *Rorc* and *Ahr* preceded the expression of *Ikzf3* during T_H17 differentiation (Fig. 5a). We detected phosphorylation of STAT3, which is known to control its own transcription as well as the expression of *Rorc* and *Ahr* in T_H17 cells^{4,28,29}, as early as 5 min after the initiation of T_H17 differentiation (Supplementary Fig. 5). Genome-wide ChIP analyses have reported interaction of STAT proteins with the *Ikzf3* locus³⁰. Moreover, the transcription factor AhR is important in the development of T_H17 cells^{8,10}. Thus, to investigate the mechanisms that control Aiolos expression in T_H17 cells, we searched for STAT3- and AhR-binding sites in the mouse *Ikzf3* promoter and found three putative STAT3-responsive elements and three putative AhR-binding sites (Fig. 5b). Whole-genome ChIP analysis of RORγt followed by sequencing did not show enrichment in the binding of RORγt to the *Ikzf3* promoter (data not shown).

ChIP assays showed interaction of AhR (Fig. 5c) and STAT3 (Fig. 5d) with their binding sites in the *Ikzf3* promoter in T_H17 cells but not in T_H0 cells (used as a control) or when we used isotype-matched control antibodies. To determine the functional consequences of the interaction of AhR and STAT3 with the *Ikzf3* promoter in T_H17 cells, we assessed at the effect of AhR and STAT3 on transactivation of the *IKZF3* promoter in luciferase reporter assays. Transfection of a reporter containing the firefly luciferase gene under the control of the *IKZF3* promoter together with a construct encoding AhR or constitutively activated STAT3 into Jurkat T cells resulted in substantial transactivation of the *IKZF3* promoter (Fig. 5e).

Retrovirus-mediated overexpression of AhR in activated T cells resulted in small but significant upregulation of *Ikzf3* expression and concomitantly lower *Il2* expression ($P < 0.05$; Supplementary Fig. 6), which suggested that additional signaling pathways were needed to make the *Ikzf3* locus accessible to AhR during T_H17 differentiation. To further investigate the role of AhR in the control of *Ikzf3* expression, we used the AhR-specific ligand FICZ, which activates AhR in T_H17 cells^{10,31}. Activation of AhR with FICZ during T_H17 differentiation resulted in significant upregulation of *Ikzf3* expression (Fig. 5f). Conversely, inhibition of AhR signaling with the specific inhibitor CH-223191 led to significantly lower *Ikzf3* expression during T_H17 differentiation (Fig. 5f). We obtained similar results with Jurkat cells activated in the presence of FICZ or CH-223191 (data not shown). Together these data demonstrated that STAT3 and AhR regulated *Ikzf3* expression in T_H17 cells.

Aiolos controls the generation of IL-17-producing memory T cells

IL-2 has an important role in the phenotype and abundance of memory T cells³². Because our data showed that Aiolos limited the generation of T_H17 cells *in vivo* by silencing *Ii2* expression, we investigated the role of Aiolos in the generation of T_H17 cells under physiological conditions. We found a significantly greater frequency and quantity of CD62L^{lo}CD44^{hi} CD4⁺ memory T cells in Aiolos-deficient mice than in wild-type mice (Fig. 6a–c), which suggested that the population expansion and/or survival of memory T cells was enhanced in Aiolos-deficient mice. Moreover, Aiolos-deficient mice had a significantly lower frequency of IL-17⁺ CD4⁺ memory T cells (Fig. 6d,e) and lower RORγt expression in those cells (Fig. 6f) than did wild-type mice, concomitant with a greater frequency of IFN-γ-producing memory T_H1 cells and higher expression of *Tbx21* in Aiolos-deficient mice than in wild-type mice (Fig. 6d–f). Thus, Aiolos limited the primary differentiation of T_H17 cells and their incorporation into the pool of CD4⁺ memory T cells under physiological conditions.

Induction of colitis by Aiolos-deficient T cells

T_H1 cells³³ and T_H17 cells³⁴ are polarized in the gut in response to signals provided by the commensal flora. IFN-γ-producing T_H1 cells have a pathogenic role in the induction of colitis after transfer into *Rag2*^{−/−} mice, but T_H17 cells do not³⁵. IL-2 limits the differentiation of T_H17 cells in the gut^{12,15}; thus, we investigated the role of Aiolos in the differentiation of gut T_H17 cells. We transferred naive wild-type or Aiolos-deficient CD4⁺ T cells into *Rag2*^{−/−} mice and analyzed the production of IL-17 and IFN-γ in recipient mice 1 month after that transfer. T cells isolated from the mesenteric lymph nodes of mice reconstituted with Aiolos-deficient T cells produced less IL-17 than did those from mice reconstituted with wild-type cells (Fig. 7a). Consistent with our *in vitro* data, the diminished production of IL-17 was concomitant with enhanced production of IFN-γ and IL-2 (Fig. 7a), which led to a significantly higher ratio of IFN-γ⁺ cells to IL-17⁺ cells among CD4⁺ T cells (Fig. 7a,b).

To assess the relevance of silencing of the *Ii2* locus by Aiolos in the context of inflammation, we studied the development of colitis in *Rag2*^{−/−} mice given adoptive transfer of CD4⁺CD25[−]CD45RB^{hi} T cells. In this model, the transferred cells differentiate into T_H1 cells and T_H17 cells and drive the development of inflammatory colitis. IL-2 is colitogenic in this experimental model³⁶, and increases in the ratio of T_H1 cells to T_H17 cells³⁷ have been linked to a more-severe wasting disease. We adoptively transferred purified CD4⁺CD25[−]CD45RB^{hi} T cells from wild-type or Aiolos-deficient mice into *Rag2*^{−/−} mice. Recipients of Aiolos-deficient T cells developed a more-aggressive wasting disease than that of recipients of wild-type T cells, as shown by their accelerated weight loss (Fig. 7c,d), shorter colons (Fig. 7e) and inflammatory histological scores (Fig. 7f). The greater colitogenic activity of Aiolos-deficient T cells was linked to more production of IFN-γ and a higher ratio of IFN-γ⁺ cells to IL-17⁺ cells in the CD3⁺CD4⁺ T cell compartment of the lamina propria (Fig. 7g,h) and less production of IL-17 by gut T cells (Fig. 7g). We found no significant difference between recipients of wild-type or Aiolos-deficient T cells in their production of IL-22 or in the Foxp3⁺ T_{reg} cell or IL-10⁺ T cell compartments (Supplementary Fig. 7a–c). To determine whether the differences in disease induction were dose dependent, we transferred fewer purified CD4⁺ CD25[−] CD45RB^{hi} T cells from wild-type or Aiolos-deficient mice (1 × 10⁵ cells per mouse) into *Rag2*^{−/−} mice. Even under these experimental conditions, in which the recipient mice required more time to develop wasting disease, the Aiolos-deficient T cells induced a more-severe disease than did the wild-type T cells. Together these data showed that Aiolos controlled the ratio of T_H1 cells to T_H17 cells *in vivo* and, consequently, the development of tissue inflammation.

DISCUSSION

IL-2 has been identified as a negative regulator of T_H17 differentiation *in vivo* and *in vitro*¹². In this study, we have identified the transcription factor Aiolos as an active inhibitor of IL-2 production and thus a promoting factor for T_H17 differentiation. We found that under T_H17-polarizing conditions, AhR together with STAT3 induced Aiolos expression. Aiolos then bound to the *Il2* promoter and induced chromatin modifications that resulted in *Il2* silencing. Aiolos-deficient naive CD4⁺ T cells produced more IL-2 and showed impaired differentiation into T_H17 cells, which was reversed by blockade of IL-2 function. Thus, Aiolos promoted T_H17 differentiation by actively silencing *Il2* transcription under T_H17-polarizing conditions. In conclusion, we have identified a module in the transcriptional program of T_H17 cells that limits the autocrine inhibitory effects of IL-2 and thereby promotes T_H17 differentiation.

In addition to finding Aiolos expression in T_H17 cells, we detected Aiolos expression in Foxp3⁺ T_{reg} cells and IL-27-induced Tr1 cells. Because IL-27 suppresses IL-2 production in Tr1 cells³⁸, our data would suggest that this is most probably achieved by the induction of Aiolos in these cells. Similarly, there is strict control of the production of IL-2 by T_{reg} cells such that they are entirely dependent on exogenous IL-2 for population expansion. Aiolos¹⁷ and Eos¹⁸, another member of the Ikaros family, participate in the control of endogenous IL-2 production in these cells.

IL-2 is a potent endogenous T cell-driven inhibitor of T_H17 differentiation, and IL-2 concentrations must be controlled to promote T_H17-type immunity^{12,13,15}. *In vivo*, the availability of IL-2 (and consequently T_H17 differentiation) can be regulated by control of the production of IL-2 and/or its consumption. Foxp3⁺ T_{reg} cells have been shown to promote T_H17 differentiation *in vivo* and *in vitro* by consuming IL-2 (refs. 11,14). Our data have demonstrated that under T_H17-polarizing conditions, the production of IL-2 was limited by a T_H17 cell-intrinsic mechanism mediated by Aiolos. Thus, the silencing of *Il2* expression by Aiolos complemented the consumption of IL-2 by Foxp3⁺ T_{reg} cells to promote the differentiation of T_H17 cells during the course of an immune response. Moreover, the silencing of *Il2* expression in T_H17 cells by Aiolos might be complemented by other mechanisms that operate in T cells to control the production of IL-2. One such mechanism is the inhibition of activation of the transcription factor NF-κB and *Il2* expression by the transcription factors Foxo1 and Foxo3a, which have high expression in resting T cells and whose expression is partially controlled by STAT3 in activated T cells³⁹.

IL-2 and its receptor have been genetically linked to many human and mouse autoimmune diseases. The development of type 1 diabetes in mice of the nonobese diabetic (NOD) strain shows linkage to the *Idd3* locus, which includes *Il2* (ref. 40). NOD mice have less IL-2, an observation initially linked to diminished function of Foxp3⁺ T_{reg} cells⁴⁰. However, the lower expression of IL-2 in NOD mice is correlated with more circulating T_H17 cells⁴¹, which suggests that an imbalance in the production of IL-2 and its control over T_H17 differentiation, in addition to its effects on Foxp3⁺ T_{reg} cells, has important consequences for the development of autoimmunity. Thus, *in vivo*, mechanisms that regulate *Il2* expression might influence the development of autoimmunity at different levels. First, they can control the activity⁴⁰ and homeostasis⁴² of Foxp3⁺ T_{reg} cells. Second, they can regulate the generation of T_H17 cells and alter the balance between T_H1 cells and T_H17 cells^{12,13,15,39} (as shown here). Third, initial alterations in the balance between T_H1 cells and T_H17 cells might be amplified by the inhibitory effects of IFN-γ and IL-17 on T_H17 differentiation²⁴ and T_H1 differentiation³⁵, respectively.

IL-21 belongs to the IL-2 family of cytokines; however, IL-2 and IL-21 have opposite effects on T_H17 cells: IL-2 inhibits the differentiation of T_H17 cells¹², whereas IL-21 promotes the generation and population expansion of T_H17 cells³. The *Il2* and *Il21* loci are in close proximity and are inherited as a haplotype on chromosome 3 in mice and chromosome 4 in humans; thus, it has been proposed that they might be regulated by competition for alternative use of regulatory elements⁴³. We did not find binding sites for Ikaros proteins in the *Il21* promoter (data not shown). On the surface, such findings might suggest that members of the Ikaros family of transcription factors do not regulate *Il21* expression. However, AhR, which has high expression by T_H17 cells^{10,31}, transactivates both the *Il2* promoter⁴⁴ and the *Il21* promoter⁴⁵. Therefore, it is possible that by restricting the accessibility of target DNA sequences in the *Il2* promoter of T_H17 cells, Aiolos directs AhR to induce the expression of *Il21* but not of *Il2* in T_H17 cells.

In conclusion, we found that Aiolos participated in a transcriptional module in T_H17 cells that limited the autocrine inhibitory effects of IL-2 on T_H17 differentiation. This T_H17 cell-intrinsic mechanism of the regulation of IL-2 complemented the consumption of IL-2 by Foxp3⁺ T_{reg} cells to promote T_H17 differentiation *in vivo* and *in vitro*. As T_H17 cells have an important role in the immune response to infection and autoimmune disorders¹, characterization of the molecular mechanisms that control the development of T_H17 cells might provide new targets for the therapeutic manipulation of effector T cell responses.

ONLINE METHODS

Animals

Aiolos-deficient mice²¹, *Rag2*^{-/-} mice, ROR γ t-deficient mice and C57BL/6 mice were kept in a conventional, pathogen-free facility at the Harvard Institutes of Medicine. All experiments were carried out in accordance with guidelines prescribed by the Institutional Animal Care and Use Committee of Harvard Medical School.

In vitro T cell differentiation

Naive CD4⁺ T cells (CD4⁺CD44^{lo}CD62L^{hi}CD25⁻) were purified by flow cytometry from the spleen and lymph nodes; the purity of isolated T cell populations routinely exceeded 98%. Naive T cells were stimulated with plate-bound anti-CD3 (2 μ g/ml; 145-2C11; Biolegend) and anti-CD28 (2 μ g/ml; PV-1; Abcam), in the presence of IL-12 (30 ng/ml) and anti-IL-4 (10 μ g/ml; C17.8; Biolegend) for the generation of T_H1 cells; IL-4 (30 ng/ml) and anti-IFN- γ (10 μ g/ml; XMG1.2; Biolegend) for the generation of T_H2 cells; or IL-6 (30 ng/ml), TGF- β 1 (3 ng/ml) and anti-IL4 and anti-IFN- γ for the generation of T_H17 cells. Mouse IL-4, IL-6, IL-12, anti-IFN- γ and anti-IL-2 (404-ML-010, 406-ML-025, 419-ML-050, MAB485 and MAB702, respectively) were from R&D Systems; TGF- β 1 (PHG9204) was from Invitrogen. FICZ (6-formylindolo[3,2-b]barbazole) was from Enzo Life Sciences and CH-223191 was from EMD Chemicals.

Quantitative PCR

RNA was extracted with RNeasy columns (Qiagen), then cDNA was prepared and used as a template for real-time PCR. Expression was normalized to *Gapdh*. All primers and probes were from Applied Biosystems (assay identifier in parentheses): *Gata3* (Mm01337569_m1), *Tbx21* (Mm00450960_m1), *Rorc* (Mm00441144_g1), *Ikzf1* (1187884), *Ikzf2* (1263584), *Ikzf3* (Mm01304161_m1), *Ikzf4* (1160002), *Ikzf5* (Mm01131648_g1), *Ifng* (Mm00801778_m1), *Il17a* (Mm00439619_m1), *Il2* (Mm00434256_m1), *Ahr* (Mm01291777_m1), *Stat3* (Mm01219775_m1), *Gapdh* (Mm99999915_g1), *RORC* (Hs01076112_m1), *IL17A* (Hs00936345_m1), *AHR* (Hs00907316_m1), *IKZF3* (Hs00232635_m1), *B2M* (4326319E) and *GAPDH* (4310884E).

Measurement of cytokines

Cytokines were measured by ELISA³¹. For intra-cellular cytokine staining, cells were stimulated for 4 h with PMA (phorbol 12-myristate 13-acetate; 50 ng/ml; Sigma), ionomycin (1 µg/ml; Sigma) and monensin (GolgiStop; 1 ml/ml; BD Biosciences). After staining of surface markers, cells were fixed and made permeable according to the manufacturer's instructions (BD Biosciences). All antibodies to cytokines were from Biolegend.

Proliferation assays

Lymph-node cells obtained from mice at day 7 after immunization were restimulated for 3 d in the presence of MOG(35–55) and incorporation of [³H]thymidine was measured as described⁹.

Plasmids

The *IL2* and *IKZF3* promoter reporters were from SwitchGear Genomics. Vectors encoding constitutively activated STAT3, AhR and Aiolos were from GeneCopoeia or Addgene.

EMSA

T_H0, T_H1 and T_H17 cells were differentiated for 48 h, then nuclear extracts were prepared and normalized for protein content with the Bradford Assay. For construction of the EMSA probe, two complementary oligonucleotides containing the Aiolos-binding site in the *IL2* promoter (5'-AAATTGC CTCCCATGCTGAAGAGCTGCCTA-3' and 5'-TAGGCAGCTCTTCAGCAT GGGAGGCAATTT-3') were annealed and radiolabeled with [γ -³²P]dATP. Nuclear extracts were incubated for 30 min at 25 °C with radiolabeled DNA probe in binding buffer (10 mM Tris, pH 7.9, 50 mM NaCl, 2 mM MgCl₂, 1 mM EDTA, 1 mM DTT, 5% glycerol (vol/vol) and 25 ng/µl poly(dI:dC)). DNA-protein complexes were fractionated by electrophoresis through a 6% nondenaturing polyacrylamide gel. For identification of binding specificity, nuclear extracts were preincubated with anti-Aiolos (8B2; Active Motif).

ChIP

Cells were crosslinked with 1% formaldehyde and lysed with 0.35 ml lysis buffer (1% SDS, 10 mM EDTA and 50 mM Tris-HCl, pH 8.1) containing 1× protease inhibitor 'cocktail' (Roche Molecular Biochemicals). Chromatin was sheared by sonication and supernatants were collected after centrifugation and diluted in buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl and 20 mM Tris-HCl, pH 8.1). Antibody (5 µg) was prebound for a minimum of 4 h to protein A and protein G Dynal magnetic beads (Invitrogen) and was washed three times with ice-cold 5% BSA in PBS, and then was added to the diluted chromatin and immunoprecipitated overnight (antibodies described below). Magnetic bead–chromatin complexes were then washed six times in RIPA buffer (50 mM HEPES, pH 7.6, 1 mM EDTA, 0.7% sodium deoxycholate, 1% Nonidet-P40 and 0.5 M LiCl), followed by three times with Tris-EDTA buffer. Immunoprecipitated chromatin was then extracted with 1% SDS, 0.1 M NaHCO₃ and heated for at least 6 h at 65 °C for reversal of the formaldehyde crosslinking. DNA fragments were purified with a QIAquick DNA purification Kit (Qiagen) and analyzed by SYBR Green real-time PCR (primers described below). The following antibodies were used for ChIP: anti-AhR (N-19; Santa Cruz Biotechnology), anti-Aiolos (ab69930; Abcam), antibody to phosphorylated STAT3 (9134; Cell Signaling Technology), antibody to acetylated H3 (06-599; Millipore), antibody to acetylated H4 (06-866; Millipore), anti-H3K4me3 (ab8580; Abcam), anti-H3K9me3 (ab8898; Abcam) and anti-H3K27me3 (6002; Abcam). The following primer pairs were used: *IL2* forward, 5'-TAAGTGTGGGCTAACCCGA-3', and reverse, 5'-CAAGGAGCACAAGTGTCATGTGA-3'; *ikzf3*-1 forward, 5'-

GGCTTGACATTCAAAAGTGGGTGCG-3', and reverse, 5'-CTGGCTGGCGGCCGCTTTAAAG-3'; ikzf3-2 forward, 5'-TCCTTCCGTGTCAGGCAATCTGGA-3', and reverse, 5'-GGGTTCCGCACCCACTTTTGAATGT-3'; ikzf3-3 forward, 5'-GTTCTGCCTTCAGTTCCTCTCCCC-3', and reverse, 5'-GAAGGTGTTGGTTCACAGAGGCGTG-3'.

Transfection and luciferase assay

Jurkat T cells were grown in DMEM supplemented with 10% FBS, transfected with TransIT-Jurkat Transfection Reagent (Mirus) and 2–5 µg plasmid and activated with PMA and ionomycin, then luciferase activity was analyzed 48 h after transfection with a dual luciferase assay kit (New England Biolabs).

Retroviral infection

Naive CD62L^{hi}CD25^{lo}CD44^{lo}CD4⁺ T cells were transduced with retroviruses as described⁴⁵ after activation with plate-bound anti-CD3 and anti-CD28.

Induction of EAE

Adoptive transfer of cells to induce EAE was done as described⁴⁵. Wild-type and Aiolos-deficient mice were immunized with 100 µg MOG(35–55) (MEVGWYRSPFSRVVHLYRNGK) in complete Freund's adjuvant. Draining lymph nodes and spleens were collected 11 d after immunization and were cultured for 3 d with MOG(35–55) (20 µg/ml) and carrier-free recombinant IL-12 (10 ng/ml; R&D Systems) or IL-23 (10 ng/ml; R&D Systems). Subsequently, 20×10^6 cells were transferred intravenously into naive *Rag2*^{-/-} mice, which were injected intraperitoneally with 200 ng pertussis toxin on days 0 and 2.

Colitis

CD4⁺ T cells were purified from the spleen and lymph nodes of wild-type and Aiolos-deficient mice by negative selection with magnetic beads (Miltenyi Biotec), and naive CD4⁺CD25^{lo}CD45RB^{hi} T cells were purified by sorting with flow cytometry. CD4⁺CD45RB^{hi}CD25^{lo} naive T cells (2×10^5) were injected into age- and sex-matched *Rag2*^{-/-} mice and weight loss was monitored.

Statistical analysis

Prism software (GraphPad Software) was used for statistical analyses. *P* values of less than 0.05 were considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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References

1. Korn T, Bettelli E, Oukka M, Kuchroo VK. IL-17 and Th17 cells. *Annu Rev Immunol.* 2009; 27:485–517. [PubMed: 19132915]
2. Bettelli E, et al. Reciprocal developmental pathways for the generation of pathogenic effector T_H17 and regulatory T cells. *Nature.* 2006; 441:235–238. [PubMed: 16648838]
3. Korn T, et al. IL-21 initiates an alternative pathway to induce proinflammatory T_H17 cells. *Nature.* 2007; 448:484–487. [PubMed: 17581588]
4. Yang XO, et al. STAT3 regulates cytokine-mediated generation of inflammatory helper T cells. *J Biol Chem.* 2007; 282:9358–9363. [PubMed: 17277312]
5. McGeachy MJ, et al. The interleukin 23 receptor is essential for the terminal differentiation of interleukin 17-producing effector T helper cells *in vivo*. *Nat Immunol.* 2009; 10:314–324. [PubMed: 19182808]
6. Ivanov II, et al. The orphan nuclear receptor ROR γ t directs the differentiation program of proinflammatory IL-17⁺ T helper cells. *Cell.* 2006; 126:1121–1133. [PubMed: 16990136]
7. Yang XO, et al. T helper 17 lineage differentiation is programmed by orphan nuclear receptors ROR α and ROR γ . *Immunity.* 2008; 28:29–39. [PubMed: 18164222]
8. Kimura A, Naka T, Nohara K, Fujii-Kuriyama Y, Kishimoto T. Aryl hydrocarbon receptor regulates Stat1 activation and participates in the development of Th17 cells. *Proc Natl Acad Sci USA.* 2008; 105:9721–9726. [PubMed: 18607004]
9. Quintana FJ, et al. Control of T_{reg} and T_H17 cell differentiation by the aryl hydrocarbon receptor. *Nature.* 2008; 453:65–71. [PubMed: 18362915]
10. Veldhoen M, et al. The aryl hydrocarbon receptor links T_H17-cell-mediated autoimmunity to environmental toxins. *Nature.* 2008; 453:106–109. [PubMed: 18362914]
11. Chen Y, et al. Foxp3⁺ regulatory T cells promote T helper 17 cell development *in vivo* through regulation of interleukin-2. *Immunity.* 2011; 34:409–421. [PubMed: 21435588]
12. Laurence A, et al. Interleukin-2 signaling via STAT5 constrains T helper 17 cell generation. *Immunity.* 2007; 26:371–381. [PubMed: 17363300]
13. Liao W, Lin JX, Wang L, Li P, Leonard WJ. Modulation of cytokine receptors by IL-2 broadly regulates differentiation into helper T cell lineages. *Nat Immunol.* 2011; 12:551–559. [PubMed: 21516110]
14. Pandiyan P, et al. CD4⁺CD25⁺Foxp3⁺ regulatory T cells promote Th17 cells *in vitro* and enhance host resistance in mouse *Candida albicans* Th17 cell infection model. *Immunity.* 2011; 34:422–434. [PubMed: 21435589]
15. Yang XP, et al. Opposing regulation of the locus encoding IL-17 through direct, reciprocal actions of STAT3 and STAT5. *Nat Immunol.* 2011; 12:247–254. [PubMed: 21278738]
16. Georgopoulos K. Haematopoietic cell-fate decisions, chromatin regulation and ikaros. *Nat Rev Immunol.* 2002; 2:162–174. [PubMed: 11913067]
17. Gandhi R, et al. Activation of the aryl hydrocarbon receptor induces human type 1 regulatory T cell-like and Foxp3⁺ regulatory T cells. *Nat Immunol.* 2010; 11:846–853. [PubMed: 20676092]
18. Pan F, et al. Eos mediates Foxp3-dependent gene silencing in CD4⁺ regulatory T cells. *Science.* 2009; 325:1142–1146. [PubMed: 19696312]
19. Thornton AM, et al. Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3⁺ T regulatory cells. *J Immunol.* 2010; 184:3433–3441. [PubMed: 20181882]
20. Umetsu SE, Winandy S. Ikaros is a regulator of Il10 expression in CD4⁺ T cells. *J Immunol.* 2009; 183:5518–5525. [PubMed: 19828627]
21. Wang JH, et al. Aiolos regulates B cell activation and maturation to effector state. *Immunity.* 1998; 9:543–553. [PubMed: 9806640]
22. Bandyopadhyay S, et al. Interleukin 2 gene transcription is regulated by Ikaros-induced changes in histone acetylation in anergic T cells. *Blood.* 2007; 109:2878–2886. [PubMed: 17148585]
23. Depper JM, et al. Interleukin 2 (IL-2) augments transcription of the IL-2 receptor gene. *Proc Natl Acad Sci USA.* 1985; 82:4230–4234. [PubMed: 2987968]

24. Kimura A, Naka T, Kishimoto T. IL-6-dependent and -independent pathways in the development of interleukin 17-producing T helper cells. *Proc Natl Acad Sci USA*. 2007; 104:12099–12104. [PubMed: 17623780]
25. Villarino AV, Gallo E, Abbas AK. STAT1-activating cytokines limit Th17 responses through both T-bet-dependent and -independent mechanisms. *J Immunol*. 2010; 185:6461–6471. [PubMed: 20974984]
26. Thomas RM, et al. Ikaros enforces the costimulatory requirement for IL2 gene expression and is required for anergy induction in CD4⁺ T lymphocytes. *J Immunol*. 2007; 179:7305–7315. [PubMed: 18025173]
27. Koipally J, Renold A, Kim J, Georgopoulos K. Repression by Ikaros and Aiolos is mediated through histone deacetylase complexes. *EMBO J*. 1999; 18:3090–3100. [PubMed: 10357820]
28. Durant L, et al. Diverse targets of the transcription factor STAT3 contribute to T cell pathogenicity and homeostasis. *Immunity*. 2010; 32:605–615. [PubMed: 20493732]
29. Wei L, Laurence A, Elias KM, O'Shea JJ. IL-21 is produced by Th17 cells and drives IL-17 production in a STAT3-dependent manner. *J Biol Chem*. 2007; 282:34605–34610. [PubMed: 17884812]
30. Wei L, et al. Discrete roles of STAT4 and STAT6 transcription factors in tuning epigenetic modifications and transcription during T helper cell differentiation. *Immunity*. 2010; 32:840–851. [PubMed: 20620946]
31. Quintana FJ, et al. Antigen microarrays identify unique serum autoantibody signatures in clinical and pathologic subtypes of multiple sclerosis. *Proc Natl Acad Sci USA*. 2008; 105:18889–18894. [PubMed: 19028871]
32. Doms H, Wolslegel K, Lin P, Abbas AK. Interleukin-2 enhances CD4⁺ T cell memory by promoting the generation of IL-7R α -expressing cells. *J Exp Med*. 2007; 204:547–557. [PubMed: 17312008]
33. Kühn R, Lohler J, Rennick D, Rajewsky K, Muller W. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell*. 1993; 75:263–274. [PubMed: 8402911]
34. Ivanov II, et al. Induction of intestinal Th17 cells by segmented filamentous bacteria. *Cell*. 2009; 139:485–498. [PubMed: 19836068]
35. O'Connor W Jr, et al. A protective function for interleukin 17A in T cell-mediated intestinal inflammation. *Nat Immunol*. 2009; 10:603–609. [PubMed: 19448631]
36. Kameyama K, et al. IL-2 is positively involved in the development of colitogenic CD4⁺ IL-7R α high memory T cells in chronic colitis. *Eur J Immunol*. 2010; 40:2423–2436. [PubMed: 20623548]
37. Cox JH, et al. IL-27 promotes T cell-dependent colitis through multiple mechanisms. *J Exp Med*. 2011; 208:115–123. [PubMed: 21173106]
38. Villarino AV, et al. Helper T cell IL-2 production is limited by negative feedback and STAT-dependent cytokine signals. *J Exp Med*. 2007; 204:65–71. [PubMed: 17227909]
39. Oh HM, et al. STAT3 promotes T cell survival and inhibits IL-2 production through up-regulation of class O forkhead transcription factors. *J Biol Chem*. 2011; 286:30888–30897. [PubMed: 21730069]
40. Yamanouchi J, et al. Interleukin-2 gene variation impairs regulatory T cell function and causes autoimmunity. *Nat Genet*. 2007; 39:329–337. [PubMed: 17277778]
41. Liu SM, et al. Differential IL-21 signaling in APCs leads to disparate Th17 differentiation in diabetes-susceptible NOD and diabetes-resistant NOD. Idd3 mice. *J Clin Invest*. 2011; 121:4303–4310. [PubMed: 22019586]
42. Fontenot JD, Rasmussen JP, Gavin MA, Rudensky AY. A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nat Immunol*. 2005; 6:1142–1151. [PubMed: 16227984]
43. Wilson CB, Rowell E, Sekimata M. Epigenetic control of T-helper-cell differentiation. *Nat Rev Immunol*. 2009; 9:91–105. [PubMed: 19151746]
44. Jeon MS, Esser C. The murine IL-2 promoter contains distal regulatory elements responsive to the Ah receptor, a member of the evolutionarily conserved bHLH-PAS transcription factor family. *J Immunol*. 2000; 165:6975–6983. [PubMed: 11120824]

45. Apetoh L, et al. The aryl hydrocarbon receptor interacts with c-Maf to promote the differentiation of type 1 regulatory T cells induced by IL-27. *Nat Immunol.* 2010; 11:854–861. [PubMed: 20676095]

Figure 1.

Expression of Aiolos by T_H17 cells. **(a)** Quantitative real-time PCR analysis of *Ikzf3*, *Tbx21*, *Gata3* and *Rorc* mRNA in naive CD4⁺CD44^{Lo}CD62L^{hi}CD25⁻ T cells differentiated for 48 h in T_H0, T_H1, T_H2 or T_H17 conditions, presented relative to the expression of *Gapdh* mRNA (encoding glyceraldehyde phosphate dehydrogenase). ND, not detected. **(b)** Quantitative real-time PCR analysis of mRNA encoding Ikaros (*Ikzf1*), Helios (*Ikzf2*), Aiolos (*Ikzf3*), Eos (*Ikzf4*) and Pegasus (*Ikzf5*) in naive CD4⁺ T cells differentiated for 48 h in T_H0 or T_H17 conditions (presented as in **a**). **(c)** Quantitative real-time PCR analysis of the time course of the expression of *Ikzf3* and *Il17a* mRNA in naive CD4⁺ T cells polarized into T_H0 or T_H17 cells (presented as in **a**). Data are from one of three independent experiments with similar results (error bars, s.e.m.).

Figure 2.

Aiolos controls the development of TH17 cells. (a) Quantitative real-time PCR analysis of *Il17a*, *Ifng* and *Ikzf3* mRNA in wild-type (WT) and Aiolos-deficient (KO) naive CD4⁺ T cells differentiated for 48 h in TH0 or TH17 conditions (presented as in Fig. 1a). (b) Enzyme-linked immunosorbent assay (ELISA) of IL-17 and IFN-γ in supernatants of wild-type and Aiolos-deficient naive T cells differentiated for 48 h in TH0, TH1 or TH17 conditions. (c) Quantitative real-time PCR analysis of *Rorc*, *Rora*, *Maf*, *Ahr*, *Tbx21*, *Il17a*, *Il17f* and *Il21* mRNA in wild-type and Aiolos-deficient naive CD4⁺ T cells differentiated for 48 h in TH17 conditions (presented as in Fig. 1a). (d,e) Proliferative recall response to MOG(35–55) (d) and expression of IFN-γ and IL-17 by T cells in the CD3⁺CD4⁺ fraction (e, left) 7 d after immunization of wild-type and Aiolos-deficient mice with MOG(35–55) in complete Freund's adjuvant. Right (e), ratio of IFN-γ⁺ cells to IL-17⁺ cells (IFN-γ⁺/IL-17⁺) among the cells at left. Numbers in quadrants (e, left) indicate percent cells in each throughout. **P* < 0.01, compared with wild-type T cells (Student's *t*-test). (f,g) EAE clinical scores of *Rag2*^{−/−} mice given wild-type or Aiolos-deficient CD4⁺ T cells reactivated with MOG(35–55) in the presence of IL-23 (f) or IL-12 (g). **P* < 0.01 and ***P* < 0.001, compared with mice given wild-type donor cells (two-way analysis of variance (ANOVA)). Data are representative of three experiments (a–c) or two experiments (f,g) or are from one of three independent experiments with similar results (d,e; error bars, s.e.m.).

Figure 3.

Aiolos limits IL-2 production in T_H17 cells. **(a)** Quantitative real-time PCR analysis of *Il2*, *Il17a* and *Ifng* mRNA in naive $CD4^+$ T cells differentiated for 0, 1, 2, 4, 6, 8, 10, 12, 16, 20, 24, 30, 42, 48, 50, 52, 60 or 72 h in T_H17 conditions (presented as in Fig. 1a). **(b)** Quantitative real-time PCR analysis of *Il2* mRNA in naive wild-type and Aiolo-deficient $CD4^+$ T cells differentiated into T_H17 cells with IL-6 and TGF- β 1 (presented as in Fig. 1a). **(c)** ELISA of IL-2 in supernatants of wild-type and Aiolo-deficient naive T cells differentiated for 48 h in T_H0 or T_H17 conditions. * $P < 0.05$ and ** $P < 0.01$, compared with wild-type T cells (two-way ANOVA). **(d)** Flow cytometry analysis of the expression of IL-2 and IL-17 in naive wild-type or Aiolo-deficient $CD4^+$ T cells differentiated for 48 h or 72 h in T_H17 conditions. **(e)** Flow cytometry analysis of IL-17 expression in naive wild-type and Aiolo-deficient $CD4^+$ T-cells differentiated for 72 h in T_H17 conditions without antibodies (None) or with the addition of blocking antibody to IL-2 (Anti-IL-2) or isotype-matched control antibody (IC). Numbers adjacent to outlined areas indicate percent IL-17 $^+$ CD4 $^+$ (T_H17) cells. Data are from one of two independent experiments with similar results (error bars **(b,c)**, s.e.m.).

Figure 4.

Aiolos silences the *IL2* promoter in T_H17 cells. **(a)** *IL2* promoter; red indicates the Aiolos-binding site. **(b)** EMSA of the interaction of nuclear extracts of T_H0, T_H1 and T_H17 cells with a radiolabeled oligonucleotide containing the putative Aiolos-binding site in *IL2* promoter, and competition of that interaction with an antibody to Aiolos that disrupts the oligomeric form of Aiolos responsible for DNA binding in EMSA (top). Anti-tubulin serves as a control. Below, SDS-PAGE and silver staining of the nuclear extracts normalized for total protein content, to demonstrate equal loading of samples. M, molecular size markers in kilodaltons (kDa). **(c)** ChIP analysis of the interaction of Aiolos, or of proteins precipitated with isotype-matched control antibody (Ctrl), with the binding site in the *IL2* promoter in T_H17, T_H1 or T_H0 cells differentiated *in vitro*. **(d,e)** ChIP analysis of the abundance of H3K4me3, H3K9me3 and H3K27me3 **(d)** and of H3ac and H4ac **(e)** in the *IL2* promoter of wild-type and Aiolos-deficient T_H0, T_H1 and T_H17 cells, presented relative to results obtained with isotype-matched control antibody. **(f)** Luciferase activity in Jurkat T cells transfected with various concentrations (horizontal axis) of an *IL2* luciferase reporter (pIKZF3) plus a construct encoding Aiolos, then activated for 24 h with the phorbol ester PMA and ionomycin; results are presented relative to renilla luciferase activity

(cotransfected control). * $P < 0.001$ (one-way ANOVA). Data are from one of two to three independent experiments with similar results (error bars (**e–f**), s.e.m.).

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Figure 5.

AhR and STAT3 control Aiolos expression in T_H17 cells. **(a)** Quantitative real-time PCR analysis of *Rorc*, *Ahr*, *Stat3* and *Ikzf3* mRNA in naive CD4⁺ T cells differentiated for 0, 1, 2, 4, 6, 8, 10, 12, 16, 20, 24, 30, 42, 48, 50, 52, 60 or 72 h in T_H17 conditions (presented as in Fig. 1a). **(b)** STAT3-responsive elements (blue; SRE-1, SRE-2 and SRE-3) and AhR-binding sites (green; xenobiotic response elements XRE-1, XRE-2 and XRE-3) in the *Ikzf3* promoter. **(c,d)** ChIP analysis of the interaction of AhR **(c)** or STAT3 **(d)**, or proteins precipitated with an isotype-matched control antibody (ctrl), with AhR-binding sites **(c)** or STAT3-responsive elements **(d)** in the *Ikzf3* promoter in T_H17 or T_H0 cells differentiated *in vitro*. **(e)** Luciferase activity in Jurkat T cells transfected with an *IKZF3* luciferase reporter, alone (–) or with a construct encoding constitutively activated STAT3 (STAT3ca) or AhR, separately (middle) or together (STAT3ca + AhR; far right), then activated for 24 h with PMA and ionomycin (presented as in Fig. 4f). **(f)** Quantitative real-time PCR analysis of *Ikzf3* mRNA in naive CD4⁺ T cells cultured for 48 h in T_H0 conditions (T_H0) or with IL-6 and TGF-β1 alone (T_H17) or in the presence of the AhR agonist FICZ (T_H17 + FICZ) or the AhR antagonist CH-223191 (T_H17 + Inh; presented as in Fig. 1a). **P* < 0.05 (one-way ANOVA). Data are from one of two independent experiments with similar results (error bars **(c–f)**, s.e.m.).

Figure 6.

Aiolo controls the generation of IL-17-producing CD4⁺ memory T cells. **(a)** Flow cytometry of splenic CD4⁺ T cells from wild-type and Aiolo-deficient mice. Numbers adjacent to outlined areas indicate CD62L^{hi}CD44^{lo} cells (top left) or CD62L^{lo}CD44^{hi} cells (bottom right). **(b)** Frequency of CD62L^{lo}CD44^{hi} T cells in the CD3⁺CD4⁺ T cell fraction of wild-type and Aiolo-deficient mice. **P* < 0.01 (Student's *t*-test). **(c)** Total CD62L^{lo}CD44^{hi} CD4⁺ T cells in wild-type and Aiolo-deficient mice. **P* < 0.05 (Student's *t*-test). **(d,e)** Flow cytometry analysis of the expression of IL-17 and IFN-γ on splenic CD4⁺CD44^{hi}CD62L^{lo} T cells **(d)** and ratio of IFN-γ⁺ cells to IL-17⁺ cells **(e)** among CD3⁺ CD4⁺ T cells stimulated *in vitro* for 4 h with PMA and ionomycin. **P* < 0.05 (Student's *t*-test). **(f)** Quantitative real-time PCR analysis of *Rorc* and *Tbx21* mRNA in splenic CD4⁺CD44^{hi} T cells sorted by flow cytometry (presented as in Fig. 1a). Data are from one of two to three independent experiments with similar results (error bars **(b,c,e,f)**, s.e.m.).

Figure 7.

Aiolos-deficient CD4⁺CD25⁻CD45RB^{hi} T cells induce an aggressive wasting disease in *Rag2*^{-/-} recipient mice. **(a)** ELISA of IL-17, IFN-γ and IL-2 in supernatants of CD4⁺ T cells isolated from *Rag2*^{-/-} mice reconstituted with wild-type or Aiolos-deficient CD4⁺ T cells, followed by activation of the cells *in vitro* for 48 h. **(b)** Ratio of IFN-γ⁺ cells to IL-17⁺ cells among CD3⁺CD4⁺ T cells from the lamina propria of *Rag2*^{-/-} mice reconstituted as in **a**. **(c)** Change in body weight of *Rag2*^{-/-} mice that received wild-type or Aiolos-deficient CD4⁺CD25⁻CD45RB^{hi} T cells. Dashed line indicates baseline (set as 0%). **(d)** Linear-regression curves of results in **c**. Dashed lines indicate the 95% confidence intervals. **(e)** Colon lengths of *Rag2*^{-/-} mice that received wild-type or Aiolos-deficient cells, measured from the colocecal junction to the anal verge. **(f)** Quantification of pathological changes in the mice in **c**. **(g)** ELISA of IFN-γ and IL-17 in supernatants of CD4⁺ T cells isolated from *Rag2*^{-/-} mice reconstituted as in **c**, followed by activation for 48 h. **(h)** Ratio of IFN-γ⁺ cells to IL-17⁺ cells among CD3⁺CD4⁺ T cells from the lamina propria of *Rag2*^{-/-} mice reconstituted as in **c**. **P* < 0.05 and ***P* < 0.01, compared with recipients of wild-type T cells (Student's *t*-test). Data are representative of three experiments with similar results, with four to five mice per group (error bars, (a-c),(e-h) s.e.m.).