Ikaros Sets the Potential for Th17 Lineage Gene Expression through Effects on Chromatin State in Early T Cell Development

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Background: Ikaros regulates early hematopoietic cell differentiation.

Results: Th17 cell differentiation is impaired in Ikaros−/− cells because of repressive and irreversible chromatin marks within lineage-determining genes.

Conclusion: Ikaros acts to integrate Th17 polarizing signals, activating essential genes and repressing genes that limit differentiation.

Significance: Ikaros plays a critical role in shaping CD4+ T cell fates in the periphery in response to acute activation signals.

Th17 cells are important effectors of immunity to extracellular pathogens, particularly at mucosal surfaces, but they can also contribute to pathologic tissue inflammation and autoimmunity. Defining the multitude of factors that influence their development is therefore of paramount importance. Our previous studies using Ikaros−/− CD4+ T cells implicated Ikaros in Th1 versus Th2 lineage decisions. Here we demonstrate that Ikaros also regulates Th17 differentiation through its ability to promote expression of multiple Th17 lineage-determining genes, including Ahr, Runx1, Rorc, Il17a, and Il22. Ikaros exerts its influence on the chromatin remodeling of these loci at two distinct stages in CD4+ T helper cell development. In naïve cells, Ikaros is required to limit repressive chromatin modifications at these gene loci, thus maintaining the potential for expression of the Th17 gene program. Subsequently, Ikaros is essential for the acquisition of permissive histone marks in response to Th17 polarizing signals. Additionally, Ikaros represses the expression of genes that limit Th17 development, including Foxp3 and Tbx21. These data define new targets of the action of Ikaros and indicate that Ikaros plays a critical role in CD4+ T cell differentiation by integrating specific cytokine cues and directing epigenetic modifications that facilitate activation or repression of relevant genes that drive T cell lineage choice.

Following initial antigen encounter, a single naïve CD4+ T cell can undergo alternative differentiation fates that result in functionally distinct T helper cell subsets. These unique programs of development are dictated by cytokines expressed in the local microenvironment by innate immune cells in response to microbial signals (for review see Refs. 1 and 2). This paradigm was defined over 20 years ago when it was discovered that IL-12 elicits the differentiation of IFNγ-producing Th1 cells, which facilitate the clearance of intracellular bacteria and viruses, whereas IL-4 drives the development of IL-4-secreting Th2 cells that mediate extracellular pathogen destruction (3). Subsequent studies have revealed the existence of additional CD4+ T cell differentiation pathways, including those that give rise to Th17 cells. Originally defined by the expression of IL-17A, Th17 cells also express IL-17F, IL-21, and IL-22 (reviewed in Ref. 4), are essential for defense against both extracellular and intracellular bacteria and fungal infections, but can also contribute to chronic inflammation associated with autoimmunity and tumor progression (5–7). Th17 cells are derived in vitro by culture of naïve CD4+ T cells in IL-6 and TGFβ, although the proinflammatory cytokines IL-1β and IL-21, together with TGFβ, promote their development as well (8, 9). In the absence of proinflammatory signals, TGFβ alone drives the development of T regulatory (Treg)2 cells, which play a critical role in down-regulating immune responses after pathogen clearance (10, 11).

The signal transduction pathways that promote Th17-specific programs of gene expression are only partially defined. Differentiation is initiated by the convergence of signals that lead to the expression of retinoic acid receptor γ-t (RORγt), a transcription factor considered a “master regulator” of the Th17 lineage (12, 13). Among these are signals downstream of antigen-induced TCR activation that induce expression of the transcription factors Irf4 and Runx1 (14, 15). IL-6 activates STAT3, which together with Runx1 and Irf4 promotes RORγt transcription (15–18). TGFβ signaling through SMAD2 also contributes to Runx1 induction as well as to expression of the ligand-activated transcription factor aryl hydrocarbon receptor (AhR), which is directly implicated in I17a and Il22 transcription (9, 15, 19, 20). These early Th17 lineage-programming sig-
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nals lead to the later expression of IL-21, which promotes sustained STAT3 activation and enhanced Th17 differentiation (21), and the IL-23 receptor (IL-23R), which promotes Th17 survival (22). AhR expression also influences Treg cell development by inducing another master regulator, Foxp3 (19). Foxp3, in turn, can directly interact with and inhibit RORγt activity, whereas IL-6 inhibits Foxp3 expression (23). The opposing actions of TGFβ and IL-6 illustrate their important cross-regulatory role in Th17 and T regulatory cell development.

The activation and/or expression of Th lineage-specifying transcription factors are downstream of the polarizing cytokine signals (1, 2). These factors promote activation of CD4+ subset-specific genes and silence those genes associated with alternative cell fates, in part by facilitating epigenetic changes in the chromatin. However, it is still not known how unique Th-promoting signals are integrated to drive such alternative differentiation programs. The previously described chromatin-modifying activities of Ikaros suggest it may play an important role in this process. Ikaros is encoded by the Ikzf1 gene and comprises a family of sequence-specific DNA binding factors generated by alternative splicing that contribute to the normal development of most hematopoietic cell lineages (24). Ikaros can act as both a transcriptional activator and a transcriptional repressor, in part because of its ability to associate with chromatin remodeling complexes such as SWI/SNF and NuRD (nucleosome remodeling and deacetylase) (25–30).

We and others have previously analyzed peripheral naive CD4+ T cells isolated from a genetically engineered Ikaros−/− mouse (31) and demonstrated that Ikaros has an activating role in the regulation of the Th2 cytokine gene loci (Il4, Il5, and Il13) and Il10 in CD4+ T cells (32, 33). Ikaros−/− CD4+ T cells cultured under Th2 polarizing conditions exhibit defects in Th2 cytokine production and default to a Th1-like phenotype, producing large amounts of IFNγ. Ikaros directly associates with several regulatory elements within both the Th2 cytokine gene locus as well as the Ifng locus in differentiated Th2 cells. Histone hypoacetylation is observed at the Il4, Il5, and Il13 loci in naive and Th2 polarized Ikaros−/− cells, consistent with compromised Th2 gene expression. Thomas et al. (34) showed that Ikaros binds to the Tbx21 promoter and represses T-bet expression in differentiating Th2 cells, providing a molecular explanation for the default to the Th1 pathway in Ikaros−/− cells.

The effects of Ikaros on CD4+ T cell differentiation, together with its critical role as a repressor of IL-2 gene expression in the generation of CD4+ T cell anergy induction (35), illustrate Ikaros’ influence in dictating peripheral T cell development and effector phenotype and suggests the possibility that Ikaros plays a similar role in other CD4 T cell fate decisions. In the current study, we asked whether Ikaros also influences Th17 development. We show that Ikaros is required for faithful Th17 differentiation in vitro and acts at two distinct stages in development to regulate the chromatin state of multiple lineage-specifying genes. During early T cell development prior to exposure to specific differentiation signals, Ikaros prevents the acquisition of epigenetic changes that silence Th17 lineage-determining genes. Ikaros also acts to integrate Th17 polarizing signals that result in activating histone modifications to these genes and allow gene expression. Despite its profound effects on antigen-induced Th17 cell development from naive peripheral T cells, Ikaros does not exert profound effects on natural Th17 cell development in the thymus.

EXPERIMENTAL PROCEDURES

Mice—Ikaros−/− mice (B6 × Sv129) were originally derived by K. Georgopoulos (Massachusetts General Hospital, Charlestown, MA) and were the kind gift of Dr. Susan Winandy, Boston University. Ikaros−/− mice contain a deletion in exon 7 of Ikzf1, resulting in unstable message and no detectable levels of Ikaros protein (31, 36). Wild type and Ikaros−/− mice generated from heterozygous breeding pairs were housed in pathogen-free conditions at the animal care facilities of Northwestern University. Mice were used at 3–5 weeks of age.

In Vitro CD4+ T Helper Cell Differentiation—CD4+ T cells were isolated from wild type or Ikaros−/− mouse spleenocytes using anti-CD4 MACS beads (Miltenyi Biotec) per the manufacturer’s instructions. In some experiments, naive CD62L−, CD4+ T cells were isolated using the CD4, CD62L isolation kit (Miltenyi Biotec). Cells were plated in wells coated with 2 μg/ml anti-CD3 and 5 μg/ml anti-CD28 in medium containing 5 μg/ml anti-IFNγ, 5 μg/ml anti-IL-4, 20 ng/ml IL-6, 3 ng/ml TGF-β, and 20 ng/ml IL-23 for 3 days. They were then cultured in the same medium in the absence of anti-CD3 and anti-CD28 for an additional 2 days. On the fifth day, cells were restimulated with PMA (20 ng/ml) and ionomycin (1 μg/ml) for 5 h with brefeldin A added after 3 h. To generate regulatory T cells, CD4+ T cells were plated in 2 μg/ml anti-CD3, 5 μg/ml of anti-CD28, and 3 ng/ml of TGFβ for 3 days prior to removal from anti-CD3/CD28 co-stimulation.

Analysis of Thymic Natural Th17 Cells—Single cell suspensions were isolated from the thymi of 3–5-week-old wild type and Ikaros−/− littermate mice prior to flow cytometric analysis. To assess IL-17 production, cells were incubated for 5 h with PMA and ionomycin before analysis.

Intracellular Cytokine Staining and Flow Cytometry—Prior to analysis, cells were stained with following fluorochrome-conjugated antibodies: IL-17A-PE 9 (17B7), IL-17A-APC (17B7), IFNγ-FITC (XMG1.2), IL-4-APC (11B11), RORγt-PE (AFKJS-9), Foxp3-PE (NRRF-30), CD25-APC (PC61.5), IL-6Rα-PE (D7715A7), CD44-FITC (IM7) (all Ebiology), CD8 PE (53–67), CD4 Pacific Blue (GK1.5), CD45 PerCP (30-F11) (all BioLegend), TCRβ (H57–597), and pSTAT3-PE (4/P-STAT3) (both from Pharmingen/BD). The cells were analyzed by flow cytometry. Positive and negative cell gates were defined based on results with specific isotype control antibodies or fluorescence minus one.

Retroviral Infection—CD4+ T cells were isolated from the spleens of wild type mice as described and plated in the presence of 2 μg/ml anti-CD3, 5 μg/ml anti-CD28, 5 μg/ml anti-IFNγ, and 5 μg/ml anti-IL-4 for 24 h before infection with the control pMSCV-IRES-H-2Kb-retroviral vector or pMSCV-Ikzf1-7-IRES-H-2Kb as previously described (37). After spinfection for 2.5 h at 32 °C, 1,400 rpm, Th17-skewing cytokines were added as detailed above, and cells were cultured for an additional 5 days prior to restimulation with PMA and ionomycin for 5 h. The cells were gated on the CD4+ H-2Kb+ population
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FIGURE 1. Ikaros regulates Th17 development. A and B, CD4+ T cells (representative data from analysis of nine WT and eight Ikaros−/− mice) (A) or CD4+CD62L+ T cells (representative data from analysis of three WT and three Ikaros−/− mice) (B) were isolated from the spleens of naïve wild type (Ik+/-) and Ikaros−/− (Ik−/−) mice and subjected to differentiation under Th17 polarizing conditions for 5 days. Cells were restimulated with PMA and ionomycin for 5 h prior to analysis of IL-17A, IFNγ, and IL-4 production by flow cytometry. The cells were gated on viable CD4+ or CD4+CD62L+ cells as indicated. C, percentage of IL-17A-producing cells compiled from results of multiple independent wild type and Ikaros−/− differentiation cultures represented as individual points. D, qRT-PCR analysis of Il17a mRNA expression in cells differentiated and restimulated as described above. E, qRT-PCR expression analysis of mRNA specifying additional Th17 effector molecules in differentiated T cell cultures. The data in D and E are expressed as the means of separate qRT-PCR analyses values normalized to Hprt expression ± S.D. the using RNA isolated from six (in D) or three (in E) independent differentiation cultures. Each cDNA-primer set was run in triplicate within an experiment.

for intracellular staining analysis. For mRNA analyses, infected cells were isolated using anti-H-2Kk+ MACS beads (Miltenyi Biotec), prior to restimulation.

Chromatin Immunoprecipitation—Histone modification and Ikaros binding assays were performed using ChIP, per the manufacturer’s instructions. Briefly, isolated CD4+ T cells were cultured under Th17-polarizing conditions for 3 days. The cells were treated with 1% formaldehyde, and nuclear lysates were prepared using EMD Millipore (Billerica, MA) ChIP assay kit. Sonicated lysates were precleared with protein G beads (for anti-Ikaros IPs) or protein A beads (for anti-H3 IPs) (EMD Millipore) for 1 h at room temperature prior to immunoprecipitation (overnight at 4 °C) with anti-H3K4me3, anti-H3K27me2 (Upstate Biotechnology), or Ikaros (M20; Santa Cruz Biotechnology). Immunoprecipitated DNA was purified and used as a template for quantitative PCR amplification with primers as described (supplemental Table S1). For H3K4me3 ChIP assays, DNA-protein complexes were also subjected to immunoprecipitation with an isotype control IgG antibody. For Ikaros ChIP assays, negative controls include quantitative real time (qRT)-PCR amplification with primers complementary to sequences within the respective gene loci that neither contain nor are proximal to any consensus Ikaros binding sequences.

cDNA Isolation and qRT-PCR—mRNA isolation was performed using the SV total RNA isolation kit (Promega, Madison, WI). mRNA was reverse transcribed into cDNA using the SuperScript III reverse transcriptase (Invitrogen). For qRT-PCR assays, DNA amplification was measured by nonspecific SYBR Green incorporation in an IQ5 thermal cycler (Bio-Rad). Relative template levels were calculated using the 2−ΔΔCt method, with Hprt1 as reference gene.

Statistics—Statistical comparisons were performed using Student’s unpaired t test or Tukey’s multiple comparisons test and analyzed using Prism 6 (GraphPad Software, San Diego, CA).

RESULTS

Ikaros Promotes Th17 Differentiation—Previous studies have implicated Ikaros in regulating CD4+ cell fate and effector function (32–35), but its role in Th17 differentiation has not been assessed. We first examined the consequences of Ikaros deficiency on IL-17A production in differentiated Th17 cells. Splenic CD4+ (Fig. 1A) or CD4+CD62L+ T cells (Fig. 1B) from naïve wild type and Ikaros−/− mice were cultured under Th17-polarizing conditions for 5 days. The proportion of IL-17A+ cells observed in Ikaros−/− cultures was reduced compared with wild type cultures. This difference was consistently observed in multiple experiments (Fig. 1C). Similar to wild type cells, Ikaros−/− T cells did not express appreciable IL-4 and only low levels of IFNγ.

Although IL-17A is often used to define the Th17 phenotype, it is only one of the prototypical cytokines expressed by these cells. The relative expression of Il17a, Il17f, Il21, Il22, and Il23r...
(p19 subunit) mRNA was compared in differentiated wild type and Ikaros−/− T cells using qRT-PCR. These experiments revealed that expression of all Th17 lineage genes assessed was consistently lower in Ikaros−/− cells (Fig. 1, D and E). Il9 and Il10 expression was also reduced, whereas Il6 was increased in Ikaros−/− T cells. Thus, Ikaros not only regulates IL-17A but is also implicated in the control of multiple genes associated with Th17 development.

Blockade of Ikaros Activity Inhibits IL-17 Production in Differentiated Wild Type Cells—Early T cell development in the thymus is impaired Ikaros−/− mice (38, 39) and could explain defects in expression of Th17-associated cytokines. To assess this possibility, we determined the effect of ectopic expression of Ikaros-7 (Ik-7), a dominant-negative Ikaros isoform (37), in wild type cells cultured under Th17 conditions. Purified naive CD4+ T cells were activated by anti-CD3 and anti-CD28 under neutral conditions for 24 h, infected with the MSCV-IK7-IRES-H2Kk retrovirus or a control MSCV-IRES-H2Kk, cultured for an additional 5 days under Th17-polarizing conditions and analyzed. Enforced expression of Ik7 resulted in decreased IL-17A protein (Fig. 2, A and B) and mRNA (Fig. 2C) compared with cells infected with the control virus. The expression of other Th17-associated genes, including Il17f, Il21, Il9, Il10, and Il23r, was also inhibited (Fig. 2D). This ability of Ik7 transduction to mimic the Ikaros−/− phenotype supports a role for Ikaros in faithful Th17 development and demonstrates that the altered Th17 differentiation of Ikaros−/− cells is not merely the consequence of inherent developmental defects in naive Ikaros−/− CD4+ cells. Notably, Il22 expression was not affected by Ik7 expression in wild type cells (Fig. 2D).

Ikaros Regulates the Expression of Lineage-determining Transcription Factors—Given the decreased expression of multiple Th17-associated cytokine genes, we asked whether Ikaros also influences expression of Th17 lineage-determining transcription factors. As shown in Fig. 3 (A–C), RORγt protein and mRNA was reduced in the absence of Ikaros, as was Ahr and Runx1 gene expression, whereas Ifi4 and Def6 were unaffected. Consistent with the study by Thomas et al. (34), expression of Tbx21, which encodes T-bet, a factor that is essential for Th1 development (40) but limits Th17 differentiation (41), was increased as well.

Ectopic expression of Ik-7 in wild type cells results in a recapitulation of the expression profile of Ikaros−/− cells in that Rorc expression is reduced, Tbx21 expression is increased, but there is no effect on the expression of Ifi4 or Def6 (Fig. 3D). Surprisingly, despite the significantly diminished expression of Ahr and Runx1 expression in Ikaros−/− cells, Ik7 transduction had minimal effects on expression of these genes.

Ikaros−/− T Cells Do Not Default to the Treg Lineage under the Influence of Th17 Differentiation Signals—Our previous studies revealed that Ikaros−/− CD4+ T cells differentiated under Th2-polarizing conditions not only fail to express normal amounts of IL-4 but show aberrant increased production of IFNγ (32). These data indicate that during normal IL-4-induced Th2 development, Ikaros suppresses the Th1 developmental program, in part through effects on Tbx21, and activates Th2 lineage genes. The close developmental relationship between Th17 and Treg cells, mediated by common TGFβ signaling pathways (42), suggests that Ikaros exerts a similar influence in dictating Th17-Treg lineage choice. Thus, we next examined the expression of Foxp3, which is essential for development of Treg cells (43) but also limits Th17 development (23).

Increased expression of Foxp3 mRNA and protein is observed in Ikaros−/− T cells differentiated under Th17-polarizing conditions (Fig. 4, A and B). Transduction of Ik7 also results in enhanced expression in differentiated wild type cells (Fig. 4C). Notably, differentiation under Treg signals results in strong Foxp3 induction in 70–80% of wild type cells, but not Ikaros−/− CD4+ T cells (Fig. 4D). Taken together, these data...
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Ikaros is essential for normal Th17 as well as inducible Treg cell development and can exert opposing effects on the same gene target in response to distinct differentiation signals.

Ikaros Regulates the Chromatin State of Th17 Lineage-determining Genes

Ikaros regulates gene expression, in part by regulating chromatin modifications within target gene loci (25–30). We performed RVista analysis of the \( \text{Il17a} \), \( \text{Rorc} \), \( \text{Runx1} \), and \( \text{Ahr} \) loci to identify potential Ikaros binding sites (see Fig. 6A). As observed within the \( \text{Il4} \) gene locus (32), a constellation of consensus Ikaros and STAT sites are clustered throughout these loci.

Histone 3 lysine 4 trimethylation (H3K4me3) is a chromatin modification associated with poised or actively transcribed genes (44), whereas histone 3 lysine 27 dimethylation (H3K27me2) marks silenced or inactive genes (44–48). Basal and differentiation-induced H3K4me3 and H3K27me2 chromatin modifications were evaluated by ChIP at several of these consensus Ikaros sites. These include the \( \text{Il17a} \) promoter and sites within CNS-2, a conserved noncoding sequence previously implicated in IL-17 transcription (49); the \( \text{Rorc} \) promoter, as well as two sites, at /H110012.7 and /H110015.0 in the \( \text{Rorc} \) gene, that are modified in Th17 cells (50); and the proximal promoter regions of the \( \text{Ahr} \) and \( \text{Runx1} \) genes.

As shown in Fig. 5, distinct patterns of these activating and repressive modifications were detected in wild type and Ikaros \( ^{-/-} \) T cells. In naïve mice, activating H3K4me3 modifications were not detected in either wild type or Ikaros \( ^{-/-} \) CD4+ T cells (Fig. 5A). In addition, repressive H3K27me2
marks at these sites were generally unique to naïve Ikaros−/− T cells, with the exception of the Il17 promoter site, which showed H3K27me2 modifications in both wild type and Ikaros−/− T cells (Fig. 5B). This general pattern of repression in naïve cells is not unique to Th17 lineage-determining genes. Both wild type and Ikaros−/− CD4+ T cells showed H3K27me2 modifications in other Th cell lineage-determining genes including Th1-specifying Tbx21, Th2-specifying Gata3, and Treg-specifying Foxp3, but the repressive modifications in Ikaros−/− cells are consistently more pronounced.

Upon Th17 differentiation, naïve wild type T cells acquired permissive H3K4me3 marks (Fig. 5D), consistent with expression of these Th17-lineage genes. Strikingly, Ikaros−/− cells retained repressive H3K27me2 marks and failed to acquire activating H3K4me3 marks (Fig. 5E).

Ikaros Binds to the Ahr and Runx1 Promoters—The profound differences in histone marks shown above indicate that Ikaros is essential for conferring the chromatin landscape of the Ahr, Runx1, Il17a, and Rorc genes. To determine whether Ikaros directly controls expression of these genes, the ability of Ikaros to bind to these same sites was evaluated by ChIP. As shown in Fig. 6 (A and B), Ikaros binding can be detected within the Ahr and Runx1 promoters, but not at any of the sites within the Rorc or Il17a gene loci. These data suggest that Ikaros controls Th17 development by directly modulating chromatin modifications in the Ahr and Runx1 genes, which in turn control expression of Rorc and Il17a.

Similar Proportions of Natural Th17 Cells Are Observed in the Thymus of Wild Type and Ikaros−/− Mice—Ikaros−/− mice have multiple hematopoietic cell defects. These include lack of lymph node development as well as altered dendritic cell function, precluding an ability to assess the functional consequences of Ikaros deficiency on inducible Th17 cell responses to antigen in vivo (24). However, a population of natural IL-17-producing cells (nTh17) that acquire their effector function in the thymus during development prior to populating peripheral
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FIGURE 6. Ikaros binds to the Ahr and Runx1 promoters. A, Ikaros consensus binding sites revealed by RVista analysis. B, ChIP assays were used to assess Ikaros binding to indicated Ikaros consensus sites in wild type CD4+ T cells after 3 days of culture under Th17 differentiation conditions. Primers flanking relevant regions were used (supplemental Table S1). The data are expressed as percentages of total input DNA. Negative controls include qRT-PCR amplification with primers complementary to sequences that do not contain and that are not proximal to any consensus Ikaros binding sequences. The data are representative of four ChIP experiments using independent differentiation cultures.

DISCUSSION

Abundant in vitro and in vivo studies have identified a number of transcription factors that regulate Il17a expression and Th17 cell differentiation (15, 41, 51–53). These include RORγt, IRF4, Runx1, Ahr, and STAT3, each of which is required for optimal Th17 differentiation; and FoxP3, T-bet, Aiolos and STAT1, which inhibit Th17 development. We now provide evidence that Ikaros is also required for robust Th17 differentiation via its critical role in promoting the expression of several of these Th17 lineage-associated genes. Loss of Ikaros activity results in the significantly reduced expression of IL-17A as well as other hallmark effector molecules, including IL-17F, IL-21, IL-22, IL-9, IL-10, and IL-23R in IL-6 and TGFβ differentiated CD4+ T cells. The expression of genes encoding lineage-determining transcription factors such as Ahr, Runx1, and Rorc is also compromised in Ikaros−/− mice, as is the normal inhibition of Foxp3 and T-bet expression.

Our finding that the proportion of thymic IL-17-producing T cells in Ikaros−/− mice is similar to that of wild type mice indicates that Ikaros exerts a more profound effect on inducible Th17 cell development and further supports the idea that although overlapping, some of mechanisms regulating nTh17 cell development appear to be distinct from that of Th17 antigen-induced differentiation in the periphery (54, 55). Parallel disparities are also found in pathways that regulate nTreg and iTreg cells, where the expression of Helios, a member of the Ikaros transcription factor family, is uniquely expressed in thymic derived Treg cells (56).

Our data do not provide evidence of a direct effect of Ikaros on Rorc or Il17. However, Ikaros associates with the Ahr and Runx1 promoters, supporting the idea that these TGFβ-regulated genes are direct targets. AhR is a ligand-activated transcription factor that responds to environmental toxins as well as to endogenous ligands (reviewed in Ref. 57). Although AhR potentiates the expression of Il17, it is absolutely essential for Il22 expression (20, 58, 59). In vivo, AhR−/− mice exhibit deficient Th17 responses and less severe disease in models of auto-

FIGURE 7. Ikaros does not regulate natural Th17 cell development in the thymus. A, single cell suspensions were isolated from the thymi of 3–5-week-old wild type and Ikaros−/− littermate mice and analyzed by flow cytometry. CD4 single positive (CD4+ Il-17+) populations are gated on CD45+ TCRβ+ CD44hi cells. For assessment of IL-17 production, cells were incubated for 5 h either alone or with PMA and ionomycin, and brefeldin A was added for the last 2 h before intracellular cytokine staining. The numbers represent the percentages of cells in indicated gates. B, percentage of CD45+ TCRβ+ CD44hi CD44hi/Il-17+ cells in indicated mice. C, numbers of CD45+ TCRβ+ CD44hi/Il-17+ cells in indicated mice. Plots are representative of analysis of four individual wild type and Ikaros−/− mice. Statistics are based on Tukey’s multiple comparisons test.
immune disease, including EAE, the rodent model of multiple sclerosis, and collagen-induced arthritis (20, 60). Runx1, like Ahr, is induced by TGFβ signaling (18, 61, 62) and is required for Rorc expression. Moreover, Runx1 interacts with RORγt at the Il17a promoter to drive its transcription (15). Thus, reduced expression of Ahr and Runx1 in Ikaros-deficient T cells, which can directly affect the activity and/or production of RORγt, IL-17A and IL-22, is likely the major contributor to impaired Th17 differentiation.

When Ikaros activity is inhibited in wild type cells by Ik7, interfering with the formation of DNA-binding Ikaros protein complexes, responses to Th17-driving signals are similar to those observed in Ikaros-/- cells. Thus, deficits in Th differentiation are not due to inherent alterations in early T cell development. Surprisingly, the activation-induced expression of Ahr, Runx1, and IL22, a gene strictly dependent on AhR for its expression (20), was unaffected. This selective failure of Ik7 to inhibit some, but not all Ikaros-regulated genes, may be related to the timing of Ahr and Runx1 induction after TCR engagement and IL-6 + TGFβ activation. Alternatively, if Ikaros-DNA complexes formed at these target sites prior to expression of Ik7 in infected cells, blockade by this dominant-negative isoform may be ineffective in reversing these associations. It is notable that increases in Il17a, Il17f, Il21, Il22, Il9, Il10, Il23r, and Rorc in wild type cells are still diminished by Ik-7 transduction even in the face of apparently normal Ahr and Runx1 expression, indicating an additional and distinct role for Ikaros in promoting gene expression, independent of AhR and Runx1.

Consistent with its previously described actions, Ikaros controls Th17 gene expression via regulation of the chromatin state of multiple Th17-lineage genes. Histone modification patterns in naïve and differentiated wild type and Ikaros-/- cells reveal that Ikaros exerts its effects at two discrete stages in CD4 T cell development. In naïve CD4+ T cells, Ikaros is necessary to maintain gene expression potential by blocking the acquisition of repressive H3K27me2 marks within the Il17, Rorc, Ahr, and Runx1 genes. This implies that Ikaros acts during thymic T cell development, prior to CD4+ T cell exit to the periphery. Remarkably, once established, the repressive state associated with H3K27me2 marks in the absence of Ikaros is resistant to combined signals from the TCR, IL-6R, and TGFβR. Independently, Ikaros is required for the formation of permissive H3K4me3 modifications that are linked to active gene transcription in response to activating signals from both the TCR and cytokine receptors.

Repressive marks also predominate at other genes that direct Th1, Th2, and Treg cell development in naïve Ikaros-/- T cells, although the differences between wild type and Ikaros-/- cells are not as pronounced as seen at the Ahr and Runx1 promoters. This observation suggests that the effects of Ikaros are not unique to Th17 lineage genes, but that this transcription factor exerts a more global effect in early thymic development of cells destined to become inducible T helper or T regulatory cells. Given that Th2 and Treg differentiation is compromised in Ikaros-/- cells (32–34), it is likely that Ikaros is required for overcoming base-line repression of these genes in response to skewing signals as well.

During Th17 differentiation, Ikaros also influences the expression of both Foxp3 and T-bet, factors that negatively regulate Th17 development through AhR- and Runx1-mediated effects, respectively (41, 63). Modest increases (2–2.5-fold) in Foxp3 protein and mRNA were observed in Ikaros-/- cells cultured under Th17 polarizing conditions. Foxp3 was previously demonstrated to repress Th17 differentiation by directly interacting with both Runx1 and RORγt and antagonizing their function (15, 23, 63). Foxp3 is also induced by TGFβ, and its expression is AhR-dependent (15, 19, 64, 65). We show that Ikaros normally limits Foxp3 expression under IL-6 and TGFβ culture conditions, thereby allowing unimpeded Th17 differentiation. Notably, differentiation of Ikaros-/- cells with TGFβ alone to generate Treg cells results in markedly compromised Foxp3 expression, likely because of reduced Ahr expression in Ikaros-/- cultures. These data strongly suggest that Ikaros exerts distinct effects on this gene in response to IL-6 + TGFβ compared with TGFβ alone. We also observe increases in IL-6 + TGFβ-induced Tbx21/T-bet expression in Ikaros-/- cells. Enhanced Tbet could participate in the inhibition of Th17 development in Ikaros-/- cells by interacting with Runx1, thereby blocking Rorc activation as previously described (41).

Although not assessed in this study, there are additional AhR-regulated genes that impact Th17 polarization and may be dysregulated in AhR-deficient Ikaros-/- cells. Under Th17 polarizing conditions, AhR-/- T cells show increased IL-2 production, resulting in the autocrine-mediated up-regulation of the high affinity IL-2α receptor and enhanced sensitivity to IL-2. IL-2 contributes to down-regulation of the IL-6R (66) and, through its activation of STAT5, triggers replacement of STAT3 on target binding sites within Th17 genes, blocking Th17 differentiation (67). Aiolos, which is induced under Th17 differentiation conditions, represses IL-2-dependent inhibition of Th17 development by silencing the IL2 promoter and limiting IL-2 expression (15, 53). In addition, STAT1 is activated by IL-6 and binds to the Il17 promoter, repressing its transcription (68). AhR has been shown to directly interact with STAT1, limiting its activation and ability to repress Il17a (52). AhR-deficient CD4+ T cells show abnormally sustained STAT1 activation under Th17 differentiation conditions, leading to decreased IL-17A production. Thus, we speculate that reduced AhR expression in Ikaros-/- cells may also lead to a loss of Aiolos-mediated IL-2 repression and enhanced STAT1 activation, thereby also contributing to impaired Th17 differentiation. Studies to test this hypothesis are in progress.

In summary, these studies expand our understanding of the influence that Ikaros exerts in peripheral CD4+ T helper cell lineage choice. We have defined multiple Ikaros target genes that are essential for Th17 development, most of which are induced downstream of TGFβ signaling. We speculate that Ikaros may also act to repress genes that limit Th17 development, such as Tbx21 and Foxp3, through similar epigenetic mechanisms. Together with our previous work demonstrating that Ikaros is necessary for faithful Th2 development, these data support a central role for Ikaros in integrating the unique cytokine cues that dictate CD4+ T cell lineage choices. Ikaros appears to be able to discriminate between these disparate signals to affect distinct programs of development in CD4+ T
cells. How it accomplishes this remains unknown and is an important area of future study.

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