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Expression and splicing of Ikaros family members in murine and human thymocytes

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Abstract

The Ikaros family of transcription factors includes five highly homologous members that can homodimerize or heterodimerize in any combination. Dimerization is essential for their ability to bind DNA and function as transcription factors. Previous studies showed that eliminating the function of the entire family blocks lymphocyte development while deletion of individual family members has relatively minor defects. These data indicate that multiple family members function during T cell development, so we examined the changes in expression of each family member as thymocytes progressed from the CD4⁻CD8⁻ double negative (DN) to the CD4⁺CD8⁺ double positive (DP) developmental stage. Further, we compared the expression of each family member in murine and human thymocytes. In both species, Ikaros and Aiolos mRNA levels increased as thymocytes progressed through the DN to DP transition, but the corresponding increases in protein levels were only observed in mice. Further, Ikaros and Aiolos underwent extensive alternative splicing in mice, whereas only Ikaros was extensively spliced in humans. Helios mRNA and protein levels decreased during murine T cell development, but increased during human T cell development. These differences in the expression and splicing of Ikaros family members between human and murine thymocytes strongly suggest that the Ikaros family of transcription factors regulates murine and human T cell development differently, although the similarities across Ikaros family members may allow different proteins to fulfill similar functions.

Keywords

T cell development; Ikaros; Aiolos; Helios; human; mice

1. Introduction

T cell development in the thymus is a highly ordered process that includes intermittent periods of proliferation and selection with the purpose of producing a diverse repertoire of T

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cells. Early T cell progenitors entering the thymus lack CD4 and CD8 expression and are called CD4⁻CD8⁻ double negative (DN) thymocytes. In mice, DN thymocytes can be subdivided into at least four major populations (DN1-4) based on their expression of CD44 and CD25 (Godfrey et al., 1993). After the DN stage, murine thymocytes express CD8 to become immature single positive (ISP) CD8⁺ thymocytes before expressing CD4 to become CD4⁺CD8⁺ double positive (DP) thymocytes (Nikolic-Zugic and Bevan, 1988). In humans, DN thymocytes are commonly divided into three subsets (DN1-3) based on their expression of CD34, CD38, and CD1a (Dik et al., 2005; Terstappen et al., 1992; Terstappen et al., 1991). After the DN3 stage, human thymocytes express CD4 to become ISP CD4⁺ thymocytes and then CD8 to become DP thymocytes (Takeuchi et al., 1993).

During early T cell development, thymocytes progress through a series of checkpoints. The first major checkpoints involve restricting the lineage potential of the cells to the T cell lineage and then to the $\alpha\beta$ T cell lineage. Once committed to the $\alpha\beta$ T cell lineage, thymocytes progress through the next major checkpoint, which occurs when TCR β is expressed for the first time. TCR β protein can be first detected in murine DN3 thymocytes (Hoffman et al., 1996) and human ISP thymocytes (Taghon et al., 2009). The next major checkpoint occurs following expression of TCR α protein, which happens for the first time in DP thymocytes in mice and humans.

The Ikaros family of transcription factors is required for T cell development (Cortes et al., 1999; Schmitt et al., 2002). The five family members (Ikaros, Helios, Aiolos, Eos, and Pegasus) share two zinc finger domains that are highly homologous across the family members. The N-terminal domain includes the DNA-binding domain, and the C-terminal domain mediates dimerization. Each family member can homodimerize or heterodimerize with any other family member, and dimerization is required for high affinity DNA binding and transcriptional activity (Hahm et al., 1998; Kelley et al., 1998; Morgan et al., 1997; Perdomo et al., 2000; Sun et al., 1996). Because of the extensive dimerization that can occur among family members, it is important to consider the entire Ikaros family as a whole. A change in the expression of one family member has the potential to alter the functionality of the entire family.

Adding complexity to the study of the Ikaros family, each family member can undergo extensive alternative splicing that results in the deletion of zinc fingers in the DNA binding domain. Deletion of one or two zinc fingers can change the DNA sequences recognized by the Ikaros dimer (Molnar and Georgopoulos, 1994; Schjerven et al., 2013a; Sun et al., 1996). Deletion of three or more zinc fingers in the DNA binding motif results in a dominant-negative variant that can dimerize with other family members, but is unable to bind DNA (Sun et al., 1996).

The importance of the Ikaros family in hematopoiesis was demonstrated in mice expressing the dominant-negative isoform of Ikaros; these mice lacked T, B, and NK cells as well as their precursors (Georgopoulos et al., 1994). In contrast, mice lacking the Ikaros dimerization domain, which preserves the function of the other family members, had impaired B cell and fetal T cell development, but only mild deficits in postnatal T cell development (Wang et al., 1996). The differences in the phenotypes observed in these two

mouse lines demonstrate that multiple Ikaros family members are critical for T cell development. To determine which family members might be required for T cell development, we analyzed how mRNA levels, protein levels, and alternative splicing of Ikaros family members change during early T cell development. In addition, we provide the first data directly comparing expression and splicing patterns of Ikaros family members in murine and human thymocytes.

2. Materials and Methods

2.1. Antibodies

The anti-mouse antibodies, anti-TER119-FITC, anti-CD4 FITC, anti-CD24-PE, anti-CD44-PE-Cy7, and anti-CD25-APCCy7, were purchased from eBioscience (San Diego, CA, USA). Anti-CD8-FITC, anti-CD8-AF647, and anti-CD4-Pacific Blue were purchased from BD Biosciences (San Jose, CA, USA). The anti-human antibodies, anti-CD1a-PerCP-Cy5.5, anti-CD1a-PECy5, anti-CD3-APCCy7, anti-CD4-Pacific Blue, anti-CD7-FITC, anti-CD8-BV785, anti-CD8-FITC, anti-CD34-PE, anti-CD34-PECy7, and anti-CD38-AF700 were purchased from Biolegend (San Diego, CA). Anti-Aiolos-PE, anti-Eos-PE, and anti-Helios-AF647 were purchased from e-Bioscience. Anti-Mouse IgG1 κ -PE and anti-Ikaros-PE were purchased from BD Biosciences. The anti-Armenian Hamster IgG-AF647 control was purchased from Biolegend.

2.2. FACS-purification of murine thymocytes

Wild-type C57BL/6 mice were housed under specific pathogen-free conditions and used between the ages of 3–5 weeks. All experiments were performed in compliance with the University of Kansas Medical Center Institutional Animal Care and Use Committee. To obtain DN thymocytes, single cell suspensions of murine thymocytes were immunodepleted using magnetic beads conjugated to anti-CD4 and anti-CD8 (BD Biosciences). The remaining DN cells were labeled with anti-TER119, anti-CD4, anti-CD8, anti-CD25, and anti-CD44. Using a BD FACS Aria IIIu (BD Biosciences), cells were gated on TER119⁺CD4⁺CD8⁺ thymocytes and DN1, DN2, DN3, and DN4 populations were FACS-purified according to CD44 and CD25 expression.

To collect ISP thymocytes, total thymocytes were depleted using magnetic beads conjugated to anti-CD4. Remaining cells were labeled with anti-CD4, anti-CD8, and anti-CD24, and CD8⁺CD24⁺ ISP cells were FACS-purified. To obtain DP thymocytes, single cell suspensions of total murine thymocytes were labeled with anti-CD4 and anti-CD8, and were FACS-purified.

2.3. FACS-purification of human thymocytes

After obtaining consent from the parent or guardian, human thymus samples were obtained from children (0 – 18 years) that underwent corrective surgery at Children's Mercy Hospital (Kansas City, MO) for congenital cardiac defects. Tissue samples were obtained in compliance with the Institutional Review Boards at Children's Mercy Hospital and the University of Kansas Medical Center.

To obtain DN and ISP thymocytes, single cell suspensions of total thymocytes were immunodepleted using magnetic beads conjugated to anti-CD8 and anti-CD3 (BD Biosciences). The remaining cells were labeled with anti-CD1a, anti-CD3, anti-CD4, anti-CD8, anti-CD34, and anti-CD38. CD4⁺CD8⁻ DN cells were gated on CD3⁻CD34⁺ thymocytes. DN2 and DN3 populations were FACS-purified using a FACS Aria IIIu (BD Biosciences) according to their expression of CD1a and CD38. For the ISP population, cells were gated on CD4⁺CD8⁻ thymocytes and CD3⁻ ISP cells were FACS-purified. For DP thymocytes, single cell suspensions of human thymocytes were labeled with anti-CD4 and anti-CD8 and CD4⁺CD8⁺ thymocytes were FACS-purified.

2.4. Quantitative RT-PCR (qRT-PCR)

Total mRNA was isolated from FACS-purified thymocytes using the RNeasy Mini Kit or RNeasy Micro Kit (Qiagen, Valenica, CA), depending on total cell count. Isolated mRNA was converted to cDNA using the TaqMan[®] High Capacity RNA-to-cDNA[™] kit (Applied Biosystems, Foster City, CA). For qRT-PCR, 2 μ L of cDNA was amplified using TaqMan[®] Gene Expression Assays for the Ikaros family members or GAPDH housekeeping gene (Ikaros: Mm01187878_m1, Hs00958473_m1; Helios: Mm00496108_m1; Aiolos: mM01306721_m1, Hs00232635_m1; Eos: Mm00496114_m1, Hs00223842_m1; Pegasus: Mm00731061_s1, Hs00223846_m1; GAPDH: Mm99999915_g1, Hs03929097_g1; Applied Biosystems), and was performed using a 7500 Fast Real-Time PCR System (Applied Biosystems). Relative expression levels of Ikaros family members were calculated relative to GAPDH using a relative quantification study in the 7500 Fast System Software. At least five independent experiments were performed for each subset, and in each experiment the samples were run in triplicate and averaged. Statistical significance was determined using the Log₂(Fold Change), or – Ct.

2.5. Western blot analysis

Cell lysates prepared from 2–3 $\times$ 10⁵ cells of each murine thymocyte population or 4–5 $\times$ 10⁵ cells of each human thymocyte population were separated by SDS-PAGE, transferred to nitrocellulose and probed with antibodies against murine Ikaros, murine Aiolos, murine p38 MAPK, human Ikaros, human Aiolos, or human p38 MAPK (all purchased from Santa Cruz Biotechnology, Inc., Dallas, TX). Bands were visualized using horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Inc.) and Pierce[™] ECL Western Blotting Substrate (Life Technologies, Grand Island, NY), and detected using an ImageQuant LAS-4000 gel imager (GE Healthcare Systems, Pittsburgh, PA). Protein levels of Ikaros and Aiolos were measured relative to p38 MAPK levels using the total densitometry for all protein isoforms as measured with MultiGauge Software (FujiFilm).

2.6. Intracellular staining

Intracellular staining was performed using the FoxP3/Transcription Factor Staining Buffer Set (eBioscience) according to the manufacturer's instructions. Single cell suspensions of murine thymocytes were labeled on their surface using anti-CD3, anti-CD4, anti-CD8, anti-CD24, anti-CD25, anti-CD44, and anti-TCR β . Cells were washed three times with PBS before addition of the Fixation/Permeabilization working solution. Cells were washed twice with 1X Permeabilization Buffer before labeling with anti-Helios, anti-Eos, or isotype

control. Single cell suspensions of human thymocytes were first labeled on their surface with anti-CD1a, anti-CD3, anti-CD4, anti-CD8, anti-CD34, and anti-CD38. After washing and fixation/permeabilization as described, cells were intracellularly labeled with either anti-Ikaros, anti-Aiolos, anti-Helios, anti-Eos, or isotype control antibodies. Cells were analyzed using a BD LSR II (BD Biosciences), and data were analyzed with BD FACSDiva software (BD Biosciences) and FlowJo (TreeStar, Inc., Ashland, OR). Fold change was measured as the ratio of geometric fluorescence intensity between each Ikaros family member and the corresponding isotype control labeled cells.

2.7. Nested PCR

Isolated total mRNA was reverse transcribed to cDNA with AMV RT (Promega, Madison, WI) before amplification with Taq DNA Polymerase (Fisher Scientific, Pittsburgh, PA). Primary PCR reactions were performed using cloning primers specific to the 5' UTR and 3' UTR (Tables 1 and 2). Nested PCR reactions were then performed with primers that spanned all possible exon pairs (Tables 1 and 2). PCR products were run on a 2% agarose gel that was then stained with ethidium bromide and washed with water before visualization using an ImageQuant LAS-4000 gel imager (GE Healthcare Systems).

2.8. Helios variant cloning

Isolated total human mRNA was reverse transcribed and amplified with the AccuScript PFUUltra II RT-PCR Kit (Agilent Technologies, Inc., Santa Clara, CA) using the cloning primers listed for Helios in Table 1. PCR products were cut with EcoRI and XhoI (Promega), and purified from an agarose gel using the QIAquick Gel Extraction Kit (Qiagen), and cloned into the MIGR1 vector (Pear et al., 1998). cDNA was collected from positively transformed colonies using the IBI High Speed Plasmid Mini Kit (Midwest Scientific, Valley Park, MO) and sequenced by GENEWIZ, Inc. (South Plainfield, NJ).

2.8. Statistics

For most figures, statistics were performed using the one-way ANOVA with a Tukey posthoc test, and significance was defined as $p < 0.05$. For statistical analysis of the mRNA levels of Ikaros family members relative to Ikaros, the two-way ANOVA was used with a Bonferroni posthoc test, and significance was defined as $p < 0.05$.

3. Results

3.1. mRNA levels of Ikaros family members during early murine T cell development

We FACS-purified murine DN2, DN3, DN4, ISP, and DP thymocytes and analyzed the relative mRNA levels of each Ikaros family member in each cell population using qRT-PCR (Fig. 1A). Of the five family members, Aiolos mRNA levels increased most dramatically as thymocytes progressed from the DN2 to the DP stage; Aiolos mRNA levels in DP thymocytes were 27-fold higher than in DN3 thymocytes. Ikaros mRNA levels also increased during early T cell development and were 3.0-fold higher in DP thymocytes than in DN3 thymocytes. Pegasus mRNA levels were 1.6-fold higher in DN2 thymocytes than DN3 thymocytes, but increased to 4.1-fold higher in DP thymocytes than DN3 cells.

By contrast, Helios and Eos mRNA levels decreased as thymocytes progressed from the DN2 stage to the DP stage. Helios mRNA levels were 2.6-fold higher in DN2 thymocytes than DN3 cells and were similar in the DN3, DN4, ISP, and DP populations. Eos mRNA levels were comparable in DN2 and DN3 thymocytes, but were 10-fold higher in DN3 thymocytes than DP cells.

For the calculations in Fig. 1A, the mRNA levels of each Ikaros family member were first normalized to that of GAPDH before comparing cell populations. GAPDH can be induced in mature T cells (Sabath et al., 1990), so it is unclear whether this is an appropriate control gene for these assays. In addition, because Ikaros family members dimerize with each other, the ratio of family members is an important parameter. Hence, we directly compared the mRNA levels of each Ikaros family member to that of Ikaros itself (Fig. 1B). Ikaros was the predominant mRNA species in DN2 thymocytes; Ikaros mRNA levels were 7.3-fold higher than Helios, 14-fold higher than Pegasus, 15-fold higher than Eos, and 30-fold higher than Aiolos ($p < 0.01$ for all family members compared to Ikaros). As thymocytes progressed through development, their Aiolos mRNA levels increased to such an extent that Aiolos mRNA levels were comparable to Ikaros levels in DN4, ISP, and DP thymocytes. Pegasus, Helios, and Eos mRNA levels remained significantly lower than Ikaros in all murine thymocyte populations tested ($p < 0.001$). Relative to Ikaros mRNA levels, Eos mRNA levels decreased throughout early T cell development; Eos mRNA levels at the DP stage were 380-fold lower than those of Ikaros ($p < 0.001$ for DP compared to DN2). Helios and Pegasus mRNA levels did not change significantly relative to Ikaros. These data suggest that the mRNA levels of some Ikaros family member are regulated independently. In addition, Ikaros and Aiolos are the predominant family members expressed in murine thymocytes, but the other family members are also present, particularly in early stages of T cell development.

3.2 Protein levels of Ikaros family members during murine T cell development

To determine whether the Ikaros family protein levels correlated with their mRNA levels, we analyzed Ikaros, Aiolos, Helios, and Eos protein levels (Fig. 2). According to the western blot analysis and consistent with previous reports (Morgan et al., 1997), Ikaros and Aiolos protein levels were 2.5-fold and 11-fold higher in DP thymocytes than DN3 thymocytes, respectively (Fig. 2A). Helios protein levels were assessed by intracellular staining and flow cytometry (Fig. 2B) and found to be 2.3-fold higher in DN2 thymocytes than in DN3 thymocytes. Helios protein levels transiently increased in DN4 thymocytes before decreasing to levels just above the isotype control in DP thymocytes. Eos protein levels were highest among DN2 thymocytes and decreased as the cells progressed to the DP stage.

In summary, Ikaros and Aiolos mRNA and protein levels increased as murine thymocytes progressed from the DN2 to the DP developmental stage. By contrast, Helios and Eos mRNA and protein levels decreased, except for a transient increase in Helios protein levels at the DN4 stage.

3.3 Splice variants of Ikaros and Aiolos in murine thymocytes

When cell lysates were probed with anti-Ikaros, three bands were detected (Fig. 2A), suggesting the presence of alternative splice variants. To identify the bands, we performed

nested RT-PCR using mRNA isolated from FACS-purified murine DN3, DN4, ISP, and DP thymocytes (Fig. 3). Using the nested PCR primers to amplify exons 2 through 7, bands corresponding to the molecular weights of full-length Ikaros (Ik-Full), Ikaros lacking exon 3 (Ik- 3), Ikaros lacking exons 5 and 6 (Ik- 5/6), and Ikaros lacking exons 3 and 5 (Ik- 3/5) were detected. To verify the identity of these splice variants, we performed nested PCR using primers that amplified each possible pair of exons; an example of the data generated by this approach is shown in Fig. 3C. Based on the nested PCR and the predicted molecular weights, Ik-Full, Ik- 5/6, and Ik- 3 are the most likely splice variants detected by western blot (Fig. 2A). These data are consistent with previous reports showing that Ik- 3 is a prominent splice variant in murine thymocytes (Hahm et al., 1994; Molnar and Georgopoulos, 1994)

The splice variants detected for Ikaros at the protein and mRNA levels were similar in all thymocyte populations tested. In addition, the proportion of total Ikaros represented by each splice variant was comparable in each population. Ik- 3/5 was readily detected by nested PCR, but the protein product was not observed. The polyclonal anti-Ikaros antibodies used were raised against an unknown C-terminal epitope and the antibodies could recognize Ik- 3 and Ik- 5/6, so it is unlikely that the lack of Ik- 3/5 detection is due to lack of antibody recognition.

For Aiolos, two bands were detected by western blot. The ratio of the two bands changed from being nearly equivalent in DN4 thymocytes to being predominantly full-length in DP thymocytes. To identify the two bands, we performed nested PCR, using the same strategy as for Ikaros (Fig. 3D). While the splice variants detected at the protein level were consistent across thymocyte populations, the mRNA species detected were highly variable between thymocyte populations and between mice. Based on the molecular weights of the protein bands and the splice variants most consistently detected at the mRNA level, the most likely Aiolos isoforms observed at the protein level are full-length Aiolos and Ai- 5/6. In each mouse analyzed, Ai- 5/6 mRNA was detected in DN thymocytes and the band decreased in intensity in ISP and DP thymocytes. This observation is consistent with the protein data showing that, as a percentage of total Aiolos, Ai- 5/6 is more prevalent in DN4 thymocytes than DP thymocytes.

Similarly, we analyzed mRNA splicing of Helios and found two mRNA species consistently present across the populations (Fig. 3E). These mRNA variants were full-length and an isoform that lacks a portion of the third exon, called Hel- 3b. For Pegasus, only full-length transcripts were detected (Fig. 3F).

To summarize, Ikaros and Aiolos expression increased at the protein and mRNA levels as murine thymocytes progressed from the DN2 to the DP developmental stage. Three Ikaros and two Aiolos splice variants were detected at the protein level for each thymocyte population analyzed, although the ratio of the two Aiolos isoforms shifted as thymocytes progressed through development. For both Ikaros and Aiolos, multiple splice variants, including dominant negative splice variants, were detected at the mRNA level, but not the protein level, indicating that the mRNA was not translated, the antibodies could not detect

the splice variant, or the splice variants were expressed at levels below the limits of detection. Splicing of Helios and Pegasus was more consistent across cell populations.

3.4 mRNA levels of Ikaros family members during early human T cell development

mRNA was isolated from FACS-purified human DN2, DN3, ISP, and DP thymocytes and subjected to qRT-PCR. Ikaros, Aiolos, Helios, and Pegasus mRNA levels increased as thymocytes progressed from the DN2 developmental stage to the DP stage, while Eos mRNA levels remained steady (Fig. 4A). As in mice, the most dramatic change detected was in Aiolos mRNA levels, which were 18-fold higher in DP thymocytes than DN2 thymocytes. Ikaros and Pegasus mRNA levels in DP thymocytes were 4.4-fold and 2.5-fold than in DN2 thymocytes, respectively. In contrast to murine thymocytes, in which Helios mRNA levels decreased during early T cell development, Helios mRNA levels increased in human thymocytes. Helios mRNA levels in DN3 thymocytes were 2.3-fold higher than in DN2 thymocytes and 10-fold higher in DP thymocytes than DN2 thymocytes.

We also compared the mRNA levels of each Ikaros family member to that of Ikaros (Fig. 4B). Ikaros mRNA was the most abundant family member expressed in all populations except the DP population. As thymocytes matured into the DP stage, the relative quantity of Helios increased from 9.0-fold less than Ikaros ($p < 0.05$) to 3.9-fold less than Ikaros and the difference between Ikaros and Helios mRNA levels at the DP stage was not statistically significant. Similarly, Aiolos mRNA levels increased from 20-fold less than Ikaros to 5.2-fold less than Ikaros and the change in the Ikaros to Aiolos ratio was statistically significant ($p < 0.05$). Conversely, Eos mRNA levels decreased from 14-fold less than Ikaros to 52-fold less than Ikaros, and Pegasus mRNA decreased from 3,500-fold less than Ikaros to more than 10,000-fold less than Ikaros. These data indicate that Ikaros, Aiolos, and Helios are significant contributors to the total quantity of Ikaros family mRNA, but Eos may also function in human thymocytes, particularly in DN cells.

3.5 Protein levels of Ikaros family members during human T cell development

Despite statistically significant increases in Ikaros and Aiolos mRNA levels, western blot analysis showed that Ikaros and Aiolos protein levels were similar across the thymocyte populations (Fig. 5A). However, subtle changes in protein levels were noted using intracellular staining and flow cytometry (Fig. 5B). For example, Aiolos protein levels were 1.9-fold higher in DP thymocytes than in DN2 cells. In DP thymocytes, a distinct subpopulation of cells with slightly higher Ikaros protein could be detected. Helios protein levels increased to a greater extent than Ikaros and Aiolos as thymocytes matured; Helios protein levels were 4.9-fold higher in DP thymocytes than the earlier developmental stages. Eos protein levels decreased slightly as thymocytes progressed from the DN to DP stage ($p < 0.05$). These data suggest that post-transcriptional regulation of Ikaros, Aiolos, and Eos is a critical factor in regulating protein levels, while Helios protein levels are more tightly linked to transcriptional control.

3.6 Splice variants of Ikaros and Aiolos in human thymocytes

Using western blot analysis, we detected three bands when cell lysates derived from DN2, DN3, ISP, and DP thymocytes were probed with anti-Ikaros (Fig. 5A), suggesting that

multiple splice variants of Ikaros are expressed in human thymocytes. To identify the Ikaros splice variants, we used nested PCR (Fig. 6A). The pattern of Ikaros splicing was complex and varied across thymocyte populations and between individuals. In addition to the loss of intact exons, Ikaros splice variants can include the addition of sixty intronic base pairs following exon 2 and the deletion of thirty base pairs at the 3' end of exon 6 (Sun et al., 1999a; Sun et al., 1999b; Sun et al., 1999c). These additions and deletions resulted in the complex pattern seen in the nested PCR.

The identity of each mRNA species in Fig. 6A was determined by calculating its molecular weight and verified using nested PCR primers that include each possible pair of exons, as shown in Fig. 6B. Because of the complexity of Ikaros splicing and the fact that not all mRNA splice variants are translated at detectable levels, we are unable to definitively identify the two minor splice variants of Ikaros detected on the western blot. Possible splice variants represented by the band at 55 kD are Ik- 3 or Ik- 5/6. The band at 39 kD might be Ik- 3/4/5/6.

Unlike Ikaros, only one Aiolos band could be detected by western blot and this band was full-length Aiolos (Fig. 5A). Using nested RT-PCR, we detected one sample in which Aiolos lacked exon 5 (Ai- 5), but all the other samples only contained full-length Aiolos (Fig. 6C). Helios was also subjected to alternate mRNA splicing, as determined by nested RT-PCR (Fig. 6D). The splicing pattern varied between patients and between thymocyte populations, but samples containing full-length Helios (Helios-Full), Helios lacking the terminal seventy-eight bases of exon 3 (Hel- 3b), Helios lacking exon 6 (Hel- 6), and Helios lacking part of exon 3 and all of exon 6 (Hel- 3b/6) were detected. Hel- 3b is missing the first DNA-binding zinc finger, as previously reported (Hahm et al., 1998). Hel- 6 and Hel- 3b/6 have not been previously reported, but we used high fidelity RT-PCR to amplify the mRNA from total human thymocytes and sequenced the cDNA as verification of its identity.

4. Discussion

We report the first comprehensive characterization of the expression and splicing patterns of the entire Ikaros family of transcription factors during the DN, ISP, and DP stages of murine and human T cell development. In both mice and humans, Ikaros and Aiolos mRNA levels increased as thymocytes differentiated from the DN2 to DP stages (Figs 1 and 4), but the corresponding increase in protein levels was only observed in mice (Figs. 2 and 5), suggesting that human Ikaros and Aiolos mRNA might be stored for rapid translation following a stimulus. Further, multiple Ikaros and Aiolos mRNA splice variants were detected in mice (Fig. 3), but only Ikaros underwent extensive alternative splicing in humans (Fig. 6). Another remarkable difference between mice and humans was that Helios mRNA and protein levels decreased during murine T cell development (Figs. 1 and 2), but increased during human T cell development (Figs. 4 and 5). Helios mRNA underwent alternative splicing in humans and we identified two novel splice variants (Hel- 6 and Hel- 3b/6). These differences in the expression and splicing of Ikaros family members between human and murine thymocytes indicate that the Ikaros family of transcription factors regulates murine and human T cell development differently.

The importance of small changes in expression levels among Ikaros family members was highlighted in a study by Dovat, *et. al.* (Dovat et al., 2005), who transgenically expressed Helios in murine B cells, which express very low levels of endogenous Helios (Kelley et al., 1998). Even though the transgenic expression resulted in a level of Helios that was approximately ten-fold lower than Ikaros, the mice developed B cell lymphoma characterized by increased Bcl-xL expression and B cell receptor-mediated proliferation.

The significance of changing the protein levels of any family member during T cell development is that the ratio of each family member to each other family member changes, thereby altering the composition of the Ikaros family dimers. In mice, DN2 thymocytes expressed Ikaros, Helios, and Eos protein (Fig. 2). Although the reagents to analyze Pegasus protein levels were unavailable, Pegasus mRNA was present in DN2 thymocytes at similar levels as Ikaros, Helios, and Eos (Fig. 3). Thus, the predominant dimer species present in murine DN2 thymocytes likely consist of Ikaros paired with itself and each other family member. Ikaros-Helios heterodimers have been observed in a murine thymocyte cell line (Kelley et al., 1998; Sridharan and Smale, 2007) and the homology in the zinc finger domains across family members suggest that the other heterodimer species are likely to exist. As murine thymocytes progress toward the DP developmental stage, expression of Helios and Eos declines, so the dimer species likely switch to predominantly Ikaros- and Aiolos-containing homodimers and heterodimers, which are also known to occur in murine thymocyte cell lines (Zhang et al., 2012).

In humans, Ikaros and Aiolos protein levels remained steady during early T cell development, but Helios protein levels increased and Eos protein levels decreased (Fig. 5). Our data would predict that dimers containing Ikaros, Helios, and Eos are common in human DN2 thymocytes. As thymocytes mature into DP cells, Ikaros-Ikaros homodimers, Helios-Helios homodimers, and Ikaros-Helios heterodimers are more likely to be found. Pegasus is unlikely to be functional in human thymocytes. A small percentage of DP thymocytes expressed Eos (Fig. 5), but co-staining with other markers revealed that all Eos⁺ DP thymocytes are regulatory T cell precursors (data not shown).

The Ikaros family members are derived from duplication events originating with a common gene (John et al., 2009). Consistent with this shared origin, the family members share a high degree of homology, particularly within the DNA binding sites, which are nearly perfectly conserved across family members and between humans and mice (Hahm et al., 1998; Morgan et al., 1997). This homology means that Ikaros, Aiolos, and Helios can bind identical DNA sequences with comparable affinity, but the ability of each family member to drive transcription varies; Aiolos activity is greater than that of Ikaros, which is greater than Helios (Kelley et al., 1998; Morgan et al., 1997). The observations that different Ikaros family members vary in their transcriptional potency despite comparable DNA binding suggests that the activity of each family member is determined by their ability to bind co-transcription factors.

A protein complex that has been linked to Ikaros function is the nucleosome remodeling and histone deacetylase (NuRD) complex. Ikaros, Aiolos, and Helios are all capable of associating with the NuRD complex, but the nature of the Ikaros dimers present in the cells

can regulate which genes are bound by the NuRD complex (Kim et al., 1999; O'Neill et al., 2000; Sridharan and Smale, 2007; Zhang et al., 2012). Mi-2 β , the catalytic core protein of the NuRD complex, primarily associates with genes that promote differentiation in the presence of Ikaros and genes associated with proliferation in the absence of Ikaros (Zhang et al., 2012). The mechanism for this differential gene association is unknown, but may be related to the nature of the Ikaros dimers present in the cell.

An example of where differences in the expression of Ikaros family members may influence gene transcription is in the expression of CD4 and CD8 α . In mice, Ikaros and Mi-2 β bind the CD8 α and CD4 genetic loci. Mi-2 β binds the CD8 α locus during early T cell development and is removed from the DNA when CD8 is expressed later in development (Harker et al., 2011). During the DP stage, Mi-2 β associates with the CD4 locus and disruption of Mi-2 β expression results in the inability to optimally express CD4 (Naito et al., 2007; Williams et al., 2004). The role of Ikaros family members in Mi-2 β -mediated regulation of CD4 and CD8 transcription is unclear, but Ikaros associates with the CD8 α and CD4 loci in murine DN and DP thymocytes (Harker et al., 2011; Harker et al., 2002; Naito et al., 2007). This constitutive association of Ikaros with these loci suggests that other Ikaros family members may regulate Mi-2 β recruitment or release. The increase in Aiolos expression we observed in murine thymocytes (Fig. 2) may affect Mi-2 β binding to these loci. In support of this model, Ikaros^{+/-} Aiolos^{-/-} mice have an increase in the number of CD4⁺CD8^{-/lo} cells with an immature phenotype (Harker et al., 2002). This population resembles the CD4⁺ ISP stage of human development. Thus, the opposing expression of CD4 and CD8 observed in human and murine ISP thymocytes may be related to the fact that Aiolos protein levels increase in murine thymocytes and Helios protein levels increase in human thymocytes.

Another mechanism by which Ikaros dimer composition might regulate T cell development is through control of Notch-dependent transcription. Ikaros family members bind the same DNA consensus sequence (GGGAA) as the Notch-dependent transcriptional regulator RBP-J κ (Chari and Winandy, 2008; Geimer Le Lay et al., 2014; Kleinmann et al., 2008; Schjerven et al., 2013b; Tun et al., 1994). Based on this observation, it has been proposed that Ikaros might act as a competitive inhibitor of Notch-dependent transcription. In support of this model, murine thymocytes lacking Ikaros have elevated expression of Notch-dependent genes (Chari and Winandy, 2008; Kleinmann et al., 2008). In addition, the increase in Aiolos protein levels in murine thymocytes correlates with the developmental stage at which cells reduce their ability to respond to Notch ligation (Xiong et al., 2011).

In addition to the protein levels of Aiolos and Helios being markedly different between human and murine thymocytes, the splicing patterns of Ikaros and Aiolos were also different. In mice, three prominent variants of Ikaros were found: Ik-FL, Ik- 5/6, and Ik- 3 (Fig. 2). These variants were detected at the mRNA and protein levels and the relative quantity of each variant was comparable in each thymocyte population tested. By contrast, mRNA splicing of Ikaros in human thymocytes varied dramatically across the thymocyte populations and between patients (Fig. 6), but the major Ikaros isoform detected at the protein level was full-length. Some splice variants detected at the mRNA level in normal thymocytes had been previously reported to be aberrant splice variants in patients with acute

lymphoblastic leukemia (Asanuma et al., 2013; Klein et al., 2006; Marcais et al., 2010; Nakase et al., 2000; Nuckel et al., 2009; Ruiz et al., 2004; Sun et al., 1999a; Sun et al., 1999b; Sun et al., 1999c; Zhao et al., 2016). Our data suggest that these unusual splice variants occur naturally, although their relative abundance may be elevated in leukemia and they may be translated into protein in leukemia. For Aiolos, the opposite splicing pattern was noted; there was minimal splicing in human thymocytes (Fig. 6), but highly variable splicing in murine thymocytes (Fig. 3). This variability in splicing was detected primarily at the mRNA level and did not necessarily translate into detectable protein.

During human T cell development, there were marked changes in the mRNA levels of Ikaros and Aiolos that were not detected at the protein level (Figs. 4 and 5). This discrepancy between mRNA and protein levels was not observed in murine thymocytes nor was it observed for human Helios. These data indicate that the rates of synthesis or degradation of Ikaros and Aiolos proteins change during human T cell development. Recent studies have shown that the thalidomide derivatives lenalidomide and pomalidomide induce the degradation of Ikaros and Aiolos through activation of the CRBN-CRL4 E3 ubiquitin ligase (Fischer et al., 2014; Gandhi et al., 2014; Kronke et al., 2014a; Kronke et al., 2014b). Importantly, Helios is resistant to CRBN-CRL4-mediated degradation (Kronke et al., 2014b), suggesting a possible mechanism for the selective increase in Helios protein levels.

In summary, we report the first comprehensive characterization of the expression and splicing of Ikaros family members during murine and human T cell development. In both species, members of the Ikaros family change their expression at the mRNA and protein levels in manners that are expected to profoundly alter the functionality of the family. In addition, there were notable differences in the expression and splicing patterns of Ikaros family members that strongly suggest that murine and human T cell development are regulated differently. Alternatively, there may be such redundancy in the Ikaros family that different expression patterns fulfill similar functions.

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Abbreviations

DN	double negative
ISP	immature single positive
DP	double positive

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Highlights

1. We define the expression pattern of Ikaros family member in T cell development.
2. We compare the expression of Ikaros family members in humans and mice.
3. Helios expression increases during T cell development in humans, not mice.
4. Ikaros and Aiolos protein levels are controlled post-transcriptionally in humans.

Figure 1. Ikaros, Aiolos, and Pegasus mRNA levels increase during early murine T cell development

mRNA isolated from murine DN2 (CD4⁻CD8⁻CD44⁺CD25^{hi}), DN3 (CD4⁻CD8⁻CD44⁻CD25^{hi}), DN4 (CD4⁻CD8⁻CD44⁻CD25⁻), ISP (CD4⁻CD8⁺CD24⁺), and DP (CD4⁺CD8⁺) thymocyte populations was subjected to qRT-PCR. *A*) For each thymocyte population, the relative expression of each Ikaros family member was normalized to that of DN3 thymocytes and the log₂ (Fold Change) is shown. (n = 7 independent samples; **p* < 0.05, ***p* < 0.01, ****p* < 0.001 as compared to DN3) *B*) For each population, relative mRNA levels of the Ikaros family members were normalized to those of Ikaros.

Figure 2. Ikaros and Aiolos protein levels increase whereas Helios and Eos protein levels decrease during early murine T cell development

A) Cell lysates prepared from FACS-purified murine DN2, DN3, DN4, ISP, and DP thymocytes were probed with antibodies against Ikaros, Aiolos, or p38 MAPK. Using densitometry, the sums of the splice variants were normalized to the quantity of p38 MAPK. The fold change in protein level relative to DN3 is shown. Data are representative of at least five independent experiments. B–C) Murine thymocytes were surface labeled with antibodies against CD4, CD8, CD24, CD25, CD44 and TCR β before fixing, permeabilizing, and intracellular staining with either anti-Helios (dark line) or isotype control (shaded histogram) (B) or either anti-Eos (dark line) or isotype control (shaded histogram) (C). DN thymocytes were gated on surface CD24⁺TCR β [−] cells before being gated based on CD25 and CD44 expression. For each population shown, the geometric mean fluorescence intensity (GMFI) of the anti-Helios or anti-Eos staining was divided by the GMFI of the isotype control. Data are representative of six mice and three independent experiments. (* p < 0.05, ** p < 0.01, *** p < 0.001 as compared to DN3).

Figure 3. Splicing patterns of Ikaros family members during early murine T cell development
 A) The exon structure of Ikaros, Aiolos, Helios, and Eos is shown along with the location of the four N-terminal DNA binding zinc fingers and the two C-terminal zinc fingers that mediate dimerization. The outer and inner primer pairs for the nested PCR are shown. B and D) Total mRNA isolated from DN3, DN4, ISP, and DP thymocytes was amplified using the outer primers shown in (A). The PCR product was reamplified using the inner primers shown in (A). The identity of the splice variants for Ikaros (B), Aiolos (D), Helios (E), and Pegasus (F) are shown. Data shown represent two independently derived sets of thymocytes, out of four independent experiments. C) To confirm splice variants, total mRNA isolated from DN3, DN4, ISP, and DP thymocytes was amplified using the outer primers shown in (A), and the PCR product was reamplified using all possible pairs of inner primers described in Table 1. A representative gel is shown for Ikaros expression in DP thymocytes.

Figure 4. Ikaros, Helios, Aiolos, and Pegasus mRNA levels increase during early human T cell development

mRNA isolated from human DN2 (CD4⁻CD8⁻CD3⁻CD34⁺CD38⁺CD1a⁻), DN3 (CD4⁻CD8⁻CD3⁻CD34⁺CD38⁺CD1a⁺), ISP (CD4⁺CD8⁻CD3⁻), and DP (CD4⁺CD8⁺) thymocytes was subjected to qRT-PCR. **A)** For each thymocyte population, the relative expression of each Ikaros family member was normalized to that of DN2 thymocytes and the log₂ (Fold Change) is shown. (n = 5 independent samples; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ as compared to DN3) **(B)** For each population, relative mRNA levels of the Ikaros family members were normalized to that of Ikaros.

Figure 5. Helios protein levels, but not Ikaros, Aiolos, or Eos protein levels, increase during early human T cell development

A) Cell lysates prepared from FACS-purified human DN2, DN3, ISP, and DP thymocytes were probed with antibodies against Ikaros, Aiolos, or p38 MAPK and quantified as described in the legend to Figure 2. Data are representative of three independent experiments. B) Human thymocytes were labeled with antibodies against CD4, CD8, CD34, CD38, and CD1a before fixing, permeabilizing, and staining with anti-Helios and either anti-Ikaros or anti-Aiolos (dark lines) or the appropriate isotype controls (shaded histogram). For each population shown, the geometric mean fluorescence intensity (GMFI) of the anti-Ikaros, anti-Aiolos, or anti-Helios staining was divided by the GMFI of the corresponding isotype control. Data were normalized to the DN2 population (* $p < 0.05$).

Figure 6. Ikaros and Helios, but not Aiolos, mRNA undergoes extensive alternative splicing in human thymocytes

Nested RT-PCR was performed on FACS-purified DN2, DN3, ISP, and DP human thymocytes to examine Ikaros (*A*), Aiolos (*C*), and Helios (*D*) alternative splicing, as described in the legend to Figure 3A. Two representative sample sets are shown from three independent experiments. *B*) A representative gel showing the results of nested RT-PCR for DN3 thymocytes using all possible pairs of inner primers shown in Table 2.

Table 1

Nested PCR primers for murine Ikaros and Aiolos

Murine	Ikaros	Aiolos	Helios	Pegasus
5' Cloning	CGCCCGAATTCACATAAACC'TGAAGAC	GCCGGAATTCGGCGACATGGAAGATATAC	ACCGGAATTCGACTATGGA AACAGAGCC	GTAGGAATTCTCTGGTGCGGTTG
Exon 2 Forward	TCAGTGACACTCCAGATGAAAGG	ACGCTCTGAATGACTACAGCTTGCC	TTGACCTCACCTCAAGCACACC	
Exon 3 Forward	TCAGGAGTTGGAGGCATTCTG	TGAAACTGCGACGTGTGCGGG	ATTGCGAGCAGCGAGGTGGC	
Exon 4 Forward	TACCCAGAAAGGCAACCTCC	AGATGCCGCTCACGGGACACCT	CAGTGCGGAGCTTCTTTACCCAG	
Exon 5 Forward	TGGATATTGTGGCCGGAGC	GCCGTACAAAGTGTGAGTTTCTGCG	ACTGGAGGAACACAAGGAACGC	
Exon 4 Reverse	GCAGGCATAGTTGCAAAAGATGG	CCCGTGAGCGGATCTCTCCTTTG	GAGAGCGTCCCCTTCTTCTACAAGC	
Exon 5 Reverse	CCAAAGTAGTTGTGGCATCGC	TCGGCAGCGTTCTCTTGCT	CTGGGCTGCTTGTAGCTTCG	
Exon 6 Reverse	CTCTTACGTTTGGCGACATTGC	TCGGCTTTGATGTGTCTTTGCCCTCC	GAGCTTCTCTATGACAGCAGGTCTC	
Exon 7 Reverse	CTCCGATGACACAGACTTGG	GGACAGACCTCGTTTCAGAAAGGCAAG	CCACCTCAGCGATTGTGCG	
3' Cloning	TATTCTCGAGTGGGTTTAGCTCAGGTGG	TGTTGACCCCTCGAGTTGAGGACAG	GAATCTCGAGGGCCTAGTGGAATGTGTGC	CCGCTCGAGAAACAATGTTGATTGTG

Table 2

Nested PCR primers for human Ikaros, Aiolos, and Helios

Human	Ikaros	Aiolos	Helios
5' Cloning	CGACGCACAAATCCACATAACCTGAG	GGCAGCGACATGGAAGATATAC	TGCACTTTGACTATGGAAACAGAGGC
Exon 1 Forward	CATGGATGCTGATGAGGGTC	CACTCAGGAGCAGTCTGTG	
Exon 2 Forward	TAAGCGATACTCCAGATGAGGG	AATGTGGACAGTGGAGAAGGC	TTGACCTCACCTCAAGCACACC
Exon 3 Forward	TCGGGAGTTGGAGGCATTCTG	GTCTCATTCGATAGTAGCAGGC	ATTGAGAGCAGCGAGGTGGC
Exon 4 Forward	GGCACATCAAGCTGCATTCC	AGAAGAGATGCGCTCACGG	CTTCCACTGTAACCAGTGTGGAGC
Exon 5 Forward	TGGATATTGTGGCCGAAGC	GAGAAGTTCCCTTGAGGAGC	ACTGGAGGAACACAAGGAACGC
Exon 3 Reverse	CCATTCATTTACAGGCACGC	TCATCTTTCCACTGGTTGGC	GGCCCAATGCAAACCATGCC
Exon 4 Reverse	AGGCGTAGTTGCAGAGGTGG	GTGAGCGCATCTCTCTTTGG	GCGTCCCTTCTTCTACAGGC
Exon 5 Reverse	CCAAGTAGTTGTGGCAGCG	CCTCAAGGGAACCTCTCTGC	CTGCGCTGCTTGTAGCTTCG
Exon 6 Reverse	GACGTTACTTGCTAGTCTGTCC	GCTAATCTGTCCAGTACGAGAGC	GAGCTTCTCTATGACAGCAGGTCTC
Exon 7 Reverse	TTGTGCAGCTGGTACATCG	ACCGTTTGACATCTCAGCC	CCACTTCAGCGATTGTGCTTGG
3' Cloning	TTGTCTGGTCCAGTCCAGTCTATGC	GAGACCAGATATTCATTCAGCAGG	GAGGAAAGGTGGGATTGTAAGTGC