Published in final edited form as:

Immunol Res. 2016 April ; 64(2): 565–575. doi:10.1007/s12026-015-8754-x.

Ikaros, Helios, and Aiolos protein levels increase in human thymocytes after β selection

Julie L. Mitchell*, Amara Seng*, and Thomas M. Yankee*,¹

*Department of Microbiology, Molecular Genetics, and Immunology, University of Kansas Medical Center, Kansas City, KS 66160

Abstract

In human T cell development, the mechanisms that regulate cell fate decisions after TCR β expression remain unclear. We defined the stages of T cell development that flank TCR β expression and found distinct patterns of human T cell development. In half the subjects, T cell development progressed from the CD4⁻CD8⁻ double negative (DN) stage to the CD4⁺CD8⁺ double positive (DP) stage through an immature single positive (ISP) CD4⁺ intermediate. However, in some patients, CD4 and CD8 were expressed simultaneously and the ISP population was small. In each group of patients, CD3⁻ ISP and DP thymocytes were subdivided into ISP1, ISP2, DP1, DP2, DP3, DP4, and DP5 developmental stages according to their expression of CD28, CD44, CD1a, CD7, CD45RO, and CD38. The ISP2, DP2, and DP3 thymocyte populations proliferated more robustly than ISP1 and DP1 and expressed markers consistent with TCR β expression. After the DP3 stage, proliferation returned to baseline levels. We then analyzed protein levels of Ikaros, Helios, and Aiolos, the three Ikaros family members most abundantly expressed in human thymocytes. Ikaros and Helios expression increased transiently at the ISP2, DP2, and DP3 populations. Aiolos expression also increased at the ISP2, DP2, and DP3 stages, but its expression remained elevated throughout the DP4 and DP5 stages. In summary, we propose a model of human T cell development that reflects the asynchronous nature of TCR β expression and we define the subpopulations of thymocytes that are highly proliferative and express Ikaros family members.

Keywords

Human; thymus; Ikaros; T cell development; ISP thymocytes; DP thymocytes

Introduction

Human T cell precursors enter the thymus at a developmental stage in which the cells lack CD4 and CD8 expression and are called CD4⁻CD8⁻ double negative (DN) thymocytes. The

¹Corresponding author: Dr. Thomas M. Yankee, Department of Microbiology, Molecular Genetics, and Immunology, University of Kansas Medical Center, 3901 Rainbow Blvd, 3025 WHW – MS 3029, Kansas City, KS 66160, Tel: 913-588-2507, Fax: 913-588-7295, ty Yankee@kumc.edu.

Ethical approval: All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent: Informed consent was obtained from all individual participants included in the study.

DN thymocyte population can be subdivided into three subsets: DN1 (CD34⁺CD38⁻CD1a⁻), DN2 (CD34⁺CD38⁺CD1a⁻), and DN3 (CD34⁺CD38⁺CD1a⁺). After the DN3 stage, the cells express CD4 and CD8 to become CD4⁺CD8⁺ double positive (DP) thymocytes. Finally, the cells mature into single positive (SP) CD4⁺ or SP CD8⁺ thymocytes and exit the thymus.

Rearrangement of the *TCRD* and *TCRG* genes occurs during the DN stages of human development, while *TCRA* rearrangement occurs in the DP stage [1]. Between the rearrangement of *TCRG* and *TCRA* genomic loci, V(D)J recombination at the *TCRB* locus occurs and T cell receptor β chain (TCR β) protein is expressed. Rearrangement of the *TCRB* genomic locus has been reported to begin as early as the DN3 stage, but TCR β protein has not been detected until the immature single positive (ISP) developmental stage, the stage between the DN and DP populations [1–5]. However, there are TCR β ⁻ DP thymocytes [2, 3, 5], indicating that the exact timing of β selection remains unclear.

In mice, successful expression of TCR β leads to proliferation, survival, and differentiation [6–8], processes that must be tightly regulated to balance the expansion of TCR β ⁺ thymocytes with the prevention of leukemia. A family of transcription factors that is likely to regulate β selection is the Ikaros family. The Ikaros family consists of five family members (Ikaros, Helios, Aiolos, Eos, and Pegasus) that share a common structure with two zinc finger domains, an N-terminal DNA binding domain and a C-terminal dimerization domain. Protein dimerization is required for high affinity DNA binding. The dimerization domain allows for the binding of any family member to any other family member, creating the potential for a mixture of homodimers and heterodimers within the same cell [9–13]. Though the different family members have similar DNA recognition sites [9, 11], some family members are more potent transcriptional activators than others [10, 11].

Ikaros family functionality is critical for lymphocyte development as expression of dominant-negative Ikaros can block murine B and T cell development [14]. Further, during murine B cell development, signals from the pre-B cell receptor drive Ikaros and Aiolos expression, resulting in loss of expression of the surrogate light chain and withdrawal from the cell cycle [11, 15, 16].

Based on these results, we postulated that the expression of Ikaros family members may correlate with TCR β expression during human T cell development. Ikaros, Helios, and Aiolos mRNA levels increase as human thymocytes progress from the DN to the DP developmental stage [17]. However, despite large increases in mRNA levels, only subtle changes in protein levels were observed. To investigate this observation in more detail, we first defined the human thymocyte subsets in which TCR β is expressed and in which cell proliferation occurs. We then correlated these observations with changes in Ikaros, Helios, and Aiolos protein levels.

Materials and Methods

Antibodies

The anti-human antibodies, anti-CD1a-PerCP-Cy5.5, anti-CD1a-PE-Cy5, anti-CD3–Allophycocyanin (APC)-Cy7, anti-CD4-Pacific Blue, anti-CD4-PE-CF594, anti-CD7-FITC, anti-CD7-APC, anti-CD8-Brilliant Violet (BV) 785, anti-CD28-Pacific Blue, anti-CD34-BV605, anti-CD34-PE, anti-CD38-Alexa Fluor (AF) 700, anti-CD44-PE-Cy7, and anti-TCR $\gamma\delta$ -FITC were purchased from Biolegend (San Diego, CA). Anti-Helios-AF647 was purchased from e-Biosciences (San Diego, CA), and Mouse IgG1 κ -PE control, anti-Ikaros-PE, and anti-Aiolos-PE were purchased from BD Biosciences (San Jose, CA). The Armenian Hamster IgG-AF647 control was purchased from Biolegend.

Human thymocyte labeling

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. Deidentified tissue samples void of any clinical data were obtained in compliance with the Institutional Review Boards at our institutions.

Single cell suspensions of human thymocytes were labeled on their surface with anti-CD1a, anti-CD3, anti-CD4, anti-CD7, anti-CD8, anti-CD28, anti-CD34, anti-CD38, anti-CD44, anti-CD45RO, and anti-TCR $\gamma\delta$, as previously described [18]. Cells were analyzed using a BD LSR II (BD Biosciences), and data was analyzed with BD FACSDiva software (BD Biosciences).

For labeling with 4',6'-diamidino-2-phenylindole (DAPI) staining, cells were surface labeled as indicated. After washing, cells were fixed in PBS with 2% paraformaldehyde and incubated over night at 4°C. Cells were washed and resuspended in PBS with 0.2% Tween and 1 μ g/ml DAPI and analyzed, as previously described [19].

For intracellular staining, cells were first surface stained as indicated, washed, and incubated in Foxp3/Transcription Factor Staining Buffer Set (Affymetrix/eBioscience, San Diego, CA), according to the manufacturer's instructions. Permeabilized cells were labeled with anti-Helios and either anti-Ikaros or anti-Aiolos, or isotype control antibodies before analyzing using the BD LSR II. Data were analyzed using FlowJo (TreeStar, Inc., Ashland, OR). Relative expression of Ikaros, Helios, and Aiolos in each cell population was defined as the ratio of geometric mean fluorescence intensity of each Ikaros family member and the corresponding isotype control.

Statistical analysis

The Student *t* test was performed for experiments in which two groups were compared. For comparisons across groups, the repeated measure ANOVA (Fig. 4) or one-way ANOVA (Fig. 5) analyses with Tukey posthoc tests were performed using GraphPad Prism (GraphPad Software, Inc, La Jolla, CA), and significance was defined as $p < 0.05$.

Results

The DN to DP transition in human thymocytes occurs in two patterns

Before we can analyze the expression patterns of Ikaros, Helios, and Aiolos at β selection, we must first define the CD3⁻ thymocyte subsets that express TCR β . Because TCR β protein can be first detected in the ISP CD4⁺ population [1–5], we determined the percentage of SP CD4⁺ thymocytes that lacked surface CD3 expression (Fig. 1A). Based on the analysis of 27 patients, we identified two groups of patients. The percentage of CD4⁺ cells that were CD3⁻ was $22\% \pm 2.0\%$ in Group 1 ($n = 14$) and $3.6\% \pm 0.53\%$ in Group 2 ($n = 13$).

To further explore the differences in T cell development between the groups, we gated on CD3⁻ thymocytes and re-analyzed CD4 and CD8 expression (Fig. 1B). In group 1 samples, DN, ISP CD4⁺, and DP thymocytes were readily detected. However, few ISP CD4⁺ thymocytes were observed in group 2 patients. In two patients, there appeared to be an ISP CD8⁺ population (called Group 3 in Fig. 1B). Because only two of the 27 patients were in group 3, the data from these individuals were excluded from further analysis. To quantify the difference in the developmental patterns between groups 1 and 2, we calculated the ratio of CD3⁻ DN cells to CD3⁻ ISP CD4⁺ cells. This ratio was 0.88 ± 0.13 in Group 1 patients and 6.4 ± 1.7 in Group 2 patients ($p < 0.01$).

In summary, the up-regulation of CD4 and CD8 expression observed during the transition from the DN to DP developmental stage can occur in different sequences in different individuals. In some cases, CD4 is expressed first, resulting in an ISP CD4⁺ population. In other cases, CD4 and CD8 expression occurs simultaneously. In rare cases, CD8 is expressed before CD4, resulting in an ISP CD8⁺ population.

ISP thymocytes can be subdivided based on CD44, CD1a, and CD28 expression

Next, we used CD44, CD1a, and CD28 to subdivide the ISP CD4⁺ cells thymocytes. In both patient groups, CD28 expression correlated with the expression of CD44 and CD1a, so the ISP population could be subdivided into CD44⁺CD1a⁺CD28⁻ cells (called ISP1 cells) and CD44⁺⁺CD1a⁺⁺CD28⁺ cells (ISP2 cells) (Fig. 2A). Because CD28 has been shown to correlate with TCR β expression [2], we propose that ISP1 thymocytes lack TCR β protein and ISP2 thymocytes express TCR β . The percentages of ISP CD4⁺ cells that were ISP1 were $71\% \pm 4.2\%$ in Group 1 patients and $47\% \pm 6.8\%$ in Group 2 patients ($p < 0.01$) (Fig. 2B).

To further characterize the ISP1 and ISP2 populations, we analyzed CD7 and CD45RO expression (Fig. 2C). CD45RO expression increases as thymocytes progress toward the DP developmental stage [20]. In group 1 patients, $93\% \pm 1.0\%$ of ISP1 cells and $72\% \pm 4.8\%$ of ISP2 cells were CD7⁺⁺CD45RO⁻. The remaining ISP thymocytes were CD7⁺CD45RO⁺. In group 2 patients, nearly all ISP1 and ISP2 thymocytes were CD7⁺⁺CD45RO⁻.

These data suggest that ISP thymocytes progress from CD7⁺⁺CD45RO⁻ to CD7⁺CD45RO⁺ as they progress through development. To further test the developmental sequence of ISP1 and ISP2 thymocytes, we analyzed the expression of CD28, CD44, CD1a, CD28, CD7, and CD45RO on DN3 thymocytes, the stage immediately preceding the ISP stage (Fig. 2D).

DN3 thymocytes from both groups of patients are uniformly CD44⁺CD1a⁺CD28⁻CD7⁺⁺CD45RO⁻, similar to ISP1 CD7⁺⁺CD45RO⁻ thymocytes.

CD3⁻ DP thymocytes can be divided into five subsets

Our analysis of CD7 and CD45RO expression on the ISP population suggested that the CD7⁺CD45RO⁺ ISP population is the final stage before the DP population. To test this hypothesis, we analyzed CD7 and CD45RO expression on CD3⁻ and CD3^{lo} DP thymocytes from patients in group 1 and group 2 (Fig. 3A). In both groups, 85% ± 1.9% of CD3⁻ DP thymocytes were CD7⁺CD45RO⁺. Among CD3^{lo} DP thymocytes, which represent the developmental stage after CD3⁻ DP thymocytes, 95% ± 1.3% of the cells were CD7⁺CD45RO⁺.

Because a small percentage of DP thymocytes lack TCRβ expression [3], we further divided the CD3⁻ DP subpopulations to define the subsets that express or lack TCRβ. As with the ISP1 and ISP2 thymocytes, we used CD44, CD1a, and CD28 to label CD7⁺⁺CD45RO⁻ CD3⁻ DP thymocytes (Fig. 3B). We called CD44⁺CD1a⁺CD28⁻CD7⁺⁺CD45RO⁻ DP thymocytes DP1 thymocytes and CD44⁺⁺CD1a⁺⁺CD28⁺CD7⁺⁺CD45RO⁻ DP thymocytes DP2 thymocytes, as shown in Fig. 3B.

Next, we used CD38 and CD45RO to subdivide the CD7⁺CD45RO⁺CD3⁻ DP populations from each group (Fig. 3C, plot iii). As a comparison, we performed parallel analysis on DP1 and DP2 thymocytes (Fig. 3C, plot i). DP1 and DP2 thymocytes were uniformly CD38⁺, but DP2 thymocytes expressed slightly higher CD45RO levels than DP1. Two populations of CD7⁺CD45RO⁺ thymocytes (plot iii) could be detected, one that is similar to DP2 thymocytes (CD38⁺CD45RO^{lo}) and one with increased expression of CD38 and CD45RO (CD38^{hi}CD45RO^{hi}). We propose that the DP2 thymocytes mature into CD38⁺CD45RO^{lo} thymocytes. To determine whether this is a uniform population, we analyzed CD7 and CD44 expression. DP1 and DP2 had similar and high levels of CD7 and DP2 thymocytes expressed higher levels of CD44 than DP1 cells (Fig. 3C, plot ii). CD38⁺CD45RO^{lo}CD3⁻ DP thymocytes could be divided into CD44^{hi}CD7^{hi} and CD44^{lo}CD7^{lo} thymocytes (Fig. 3B, plot iv). The CD44^{hi}CD7^{hi} thymocytes resembled DP2 thymocytes, so we called this subset DP3. We called the CD44^{lo}CD7^{lo} cells DP4. In support of the conclusion that DP3 precedes DP4, the CD38^{hi}CD45RO^{hi} thymocytes from Fig. 3B, plot iii were mostly CD44^{lo}CD7^{lo} and are called DP5.

For each subpopulation of CD3⁻ DP thymocytes, we analyzed CD28 and CD1a expression (Fig. 3D). As thymocytes progressed from DP1 to DP5, CD1a expression increased and remained elevated. However, CD28 expression was transient and followed a similar pattern as CD44; surface protein levels were higher at the DP2 and DP3 stages than the other populations.

In summary, we defined five subsets of CD3⁻ DP thymocytes and placed them into developmental sequence by comparing the expression of a set of surface markers with that of the developmental stages immediately preceding and following. We then calculated the percentage of CD3⁻ DP thymocytes represented by each subset (Fig. 3E). Unlike ISP thymocytes in which there was a difference in the percentage of ISP1 and ISP2 subsets

between the two groups of patients, there were no statistically significant differences between patient groups observed in the percentages of CD3⁺ DP thymocytes within each of the five subsets (data not shown). Of the CD45RO⁺CD3⁺ DP thymocytes, the cells were equally distributed among the DP3, DP4, and DP5 developmental stages.

The rate of proliferation peaks during the ISP2 and DP2 stages

Expression of TCR β triggers robust proliferation in murine thymocytes [6, 21]. We used cell cycle analysis to determine which human DN, ISP, and DP thymocyte subsets were actively proliferating (Fig. 4). On average, fewer than 9% of DN2, DN3, and ISP1 thymocytes were in the S, G2, or M phase of the cell cycle. Likewise, 10% $\pm$ 3.4% of the DP1 thymocytes were in the S, G2, or M phase of the cell cycle. By contrast, 25% $\pm$ 4.5% of the ISP2 thymocytes were in the S, G2, or M phase of the cell cycle ($p < 0.001$, compared to ISP1). Further, 37% $\pm$ 6.6% and 38% $\pm$ 7.3% of the DP2 and DP3 thymocytes, respectively, were in the S, G2, or M phase of the cell cycle ($p < 0.01$, DP2 and DP3 thymocytes compared to DN, ISP1, and DP1 thymocytes).

Compared to the DP2 and DP3 populations, the percentages of thymocytes in the S, G2, or M phase were lower in the DP4 and DP5 stages; 15% $\pm$ 5.1% of DP4 thymocytes and 8.5% $\pm$ 1.5% of DP5 thymocytes were in the S, G2, or M phase of the cell cycle ($p < 0.01$, DP2 and DP3 thymocytes compared to DP4 and DP5 thymocytes). These data support the model in which TCR β expression is an asynchronous process with some cells expressing TCR β and entering the cell cycle during the ISP stage and some cells expressing TCR β and entering the cell cycle during the DP stage. Further, these data suggest that proliferation is reduced in the populations immediately prior to surface CD3 expression. The stepwise reduction in the rate of proliferation seen in the DP3, DP4, and DP5 populations further supports our model that these stages represent sequential developmental stages.

TCR β expression correlates with an increase in Ikaros, Helios, and Aiolos protein levels

Next, we used intracellular staining and flow cytometry to monitor the protein levels of Ikaros, Helios, and Aiolos in the CD3⁺ thymocyte populations. The protein levels of Ikaros, Helios, and Aiolos were higher in the ISP2 population, as compared to the ISP1 population. Helios protein levels increased 2.8-fold at this stage ($p < 0.05$), Aiolos increased 2.0-fold ($p < 0.05$), and Ikaros increased 1.9-fold ($p < 0.01$). Similarly, Ikaros protein levels in DP2 thymocytes were 1.9-fold higher than in DP1 cells ($p < 0.05$) and Aiolos protein levels were 1.8-fold higher in DP2 cells than DP1 cells ($p < 0.05$). Helios protein levels also trended higher in DP2 thymocytes than DP1, although this difference did not reach statistical significance until the DP3 stage ($p < 0.001$, DP3 vs DP1). As thymocytes continued to mature, only Aiolos protein levels remained elevated. By contrast, Helios levels were 3.1-fold higher in DP3 thymocytes than DP5 cells ($p < 0.001$) and Ikaros levels were 1.8-fold higher in DP3 thymocytes than DP5 cells ($p < 0.05$), indicating that Ikaros and Helios undergo a transient increase in protein levels when TCR β is expressed.

Discussion

The data presented here highlight the asynchronous manner by which human thymocytes express TCR β protein. Using CD28 as a marker of TCR β expression [2], we show that expression of TCR β correlates with increases in the expression of CD1a, CD44, Ikaros, Helios, and Aiolos, but does not strictly correlate with CD4 and CD8 expression. Importantly, we identified CD1a and CD44 as markers that delineated populations of TCR β ⁻ and TCR β ⁺ cells in both the ISP and DP stages within the same thymus (Fig. 2A and 3B). Greater percentages of CD1a⁺⁺CD44⁺⁺CD28⁺ ISP2 and DP2 cells were in the S, G2, or M phase of the cell cycle than CD1a⁺CD44⁺CD28⁻ ISP1 and DP1 cells (Fig. 4), supporting the model that ISP2 and DP2 thymocytes express TCR β . ISP2 and DP2 thymocytes also had higher expression of Ikaros, Helios, and Aiolos protein levels, as compared to ISP1 and DP1 cells.

Based on our analysis, we propose the model of human T cell development shown in Fig. 6. Because we are focusing on cells that lack surface TCR $\alpha\beta$ or TCR $\gamma\delta$ expression, we gated on CD3⁻ live events before analyzing the remaining markers. In developing the model, several assumptions were made. Firstly, as thymocytes progress through the developmental stages, it is more likely that protein levels of surface markers change subtly than drastically. Secondly, once TCR β is expressed, it is highly unlikely that it is lost. Lastly, TCR β ⁺ thymocytes proliferate at a greater rate than TCR β ⁻ cells, at least until rearrangement of the *TCRA* locus commences.

Based on these assumptions, we found three patterns of CD4 and CD8 expression in CD3⁻ thymocytes (Fig. 1B): the canonical pattern in which CD4 is expressed prior to CD8, creating an ISP CD4⁺ population (Group 1); a pattern in which CD4 and CD8 are expressed simultaneously (Group 2); and a third pattern that appeared in two patients in which CD8 is expressed prior to CD4 (Group 3). While it might be expected that individual cells would take random paths from the DN to the DP stage, it was unexpected that most cells in an individual followed one of the three patterns. Because the tissue samples were collected without clinical data, we are unable to determine whether the age of the patient, comorbidities, or pre-surgery medications might influence the developmental pattern [22–24]. The implications of these developmental patterns on physiology or pathophysiology are unknown; however, these observations imply that DN3 thymocytes could differentiate directly into ISP1 or DP1 thymocytes, as shown in Fig. 6, or an ISP CD8⁺ population that is not shown in the figure.

Regardless of the pathway that thymocytes follow during the DN to DP transition, TCR β ⁻ and TCR β ⁺ subsets of ISP and DP thymocytes could be detected in each individual. ISP1 and DP1 cells appear to be pre- β selection cells based on their lack of CD28 expression (Figs. 2A and 3B) and low proliferation rate (Fig. 4). The presence of TCR β ⁻ DP thymocytes suggests that remodeling of the CD8 locus is not dependent on pre-TCR signals, as it is in murine thymocytes [25]. Further, these data suggest that ISP1 cells could express TCR β protein and differentiate into ISP2 cells. The newly formed ISP2 thymocytes likely differentiate into either DP2 or DP3 cells, depending on whether the cells upregulate CD45RO before or after CD8.

If TCR β expression fails to occur during the ISP CD4⁺ stage, the cells likely differentiate into DP1 thymocytes. DP1 cells may retain the potential to successfully rearrange their *TCRB* locus, express TCR β protein, and differentiate into DP2 thymocytes. However, if DP1 thymocytes fail to express TCR β , the cells likely undergo apoptosis.

As in murine T cell development, TCR β expression in human thymocytes triggers robust proliferation, as seen by a greater percentage of CD3⁻ DP thymocytes in the S, G2, or M phase of the cell cycle than DN thymocytes [4, 5, 26]. We extend these observations by showing which subpopulations of ISP and DP thymocytes are the most rapidly dividing (Fig. 4). Specifically, more than 20% of ISP2, DP2, and DP3 thymocytes were in the S, G2, or M phase of the cell cycle, as compared to less than 10% of ISP1 and DP1 thymocytes. Proliferation was most robust in the DP2 and DP3 stages and then declined in the DP4 and DP5 stages, the final stages prior to the expression of TCR α and surface TCR. This is analogous to the proliferation that occurs after TCR β expression in murine thymocytes. The first murine thymocytes that express TCR β proliferate more robustly than TCR β ⁺ thymocytes at later stages [19, 27, 28]. At the DP5 stage, thymocytes decrease their expression of CD44 and increase their expression of CD38 (Fig. 3C). CD38 is first expressed at the DN2 stage, the stage in which rearrangement of the *TCRD* and *TCRG* loci occurs [1]. This suggests that *CD38* transcription may be regulated via similar mechanisms as RAG-1 or RAG-2. In chronic lymphocytic leukemia, CD38 expression is regulated by the E-box factor, E2A [29]. E2A binds the RAG enhancer regulatory element in developing murine B cells [30] and human cancer cell lines [31]. Thus, the increase in CD38 expression on DP5 cells may correlate with the onset of *TCRA* rearrangement.

With this detailed image of the developmental stages that surround TCR β expression, we are now able to examine changes in the protein levels of Ikaros, Helios, and Aiolos at this critical point in human T cell development. It was previously shown that the mRNA levels of each of these molecules increased dramatically as human thymocytes progressed from the DN to the DP stages [17]. However, previous data also showed that protein levels in total DP thymocytes were only slightly elevated, as compared to DN thymocytes. Using flow cytometry, we are now able to subdivide the ISP and DP populations and detect transient changes in protein expression that occur as thymocytes mature.

Protein levels of Ikaros, Helios, and Aiolos increased when thymocytes expressed TCR β and proliferated (Fig. 5). The relative increase in Helios expression was greater than that of Ikaros and Aiolos, suggesting that the composition of Ikaros family dimers changes when thymocytes express TCR β . More Ikaros family dimers likely contain Helios in ISP2, DP2, and DP3 thymocytes than ISP1 and DP1 cells. The other significant difference among family members is that the increase in Ikaros and Helios protein levels was transient; protein levels of these two family members were lower in DP4 and DP5 thymocytes than DP2 and DP3 thymocytes. By contrast, Aiolos protein remained elevated in DP4 and DP5 thymocytes. This observation indicates that more Ikaros family dimers contain Aiolos in DP4 and DP5 thymocytes than previous developmental stages.

The importance of small changes in the expression of Ikaros family members was demonstrated in a study in which transgenic mice were generated that express low levels of

Helios [32]. Expressing ten-fold lower levels of Helios than Ikaros resulted in B cell lymphoma. The mechanism by which changes in Ikaros family dimers influence gene transcription is unknown. Because of the high degree of homology within the DNA binding motifs of each family member, it is likely that all family members can bind the same DNA sequences with similar affinities. However, the relative potency of each family member is different. For example, Aiolos is a more potent transcriptional activator than Ikaros [11].

Helios expression is different in murine and human thymocytes [17]. Specifically, Helios mRNA levels decrease as murine thymocytes progress from the DN to the DP stage while Helios mRNA levels increase in human thymocytes. Thus, it is difficult to draw conclusions regarding the function of Ikaros family members using data from the opposite species. However, loss of Ikaros in murine thymocytes obviates the need for TCR β expression for differentiation into DP thymocytes, even though Ikaros could not drive proliferation after TCR β expression [33]. In human thymocytes, Ikaros, Helios, and Aiolos were most abundantly expressed in the thymocyte populations that were most proliferative (Figs. 4 and 5), suggesting that the Ikaros family might promote proliferation in human TCR β ⁺ thymocytes. The decrease in Ikaros and Helios coupled with sustained Aiolos expression seen in DP4 and DP5 thymocytes might lead to the slowing of cell cycle progression and rearranging of the *TCRA* locus. More experiments are necessary to determine the function of Ikaros in human thymocytes.

Consistent with a role for Ikaros family members in pre-TCR-mediated proliferation and differentiation is the observation that pre-BCR-mediated Ikaros and Aiolos expression during murine B cell development induces exit from the cell cycle [15, 16, 34, 35]. Further, exogenous expression of Ikaros in a pre-B cell line could halt cell division [35]. In addition, expression of Ikaros in an Ikaros-deficient DN3-like murine thymoma cell line can induce differentiation and expression of TCR α [36, 37]. This differentiation is independent of TCR β expression as loss of Ikaros expression on a RAG-1^{-/-} background induced differentiation to the DP stage and expression of TCR α germline transcripts [33].

In summary, we defined the stages of human T cell development flanking the expression of TCR β to a greater level of detail than has been previously reported. Further, we show that Ikaros, Helios, and Aiolos protein levels fluctuate during this critical time in T cell development.

References

1. Dik WA, Pike-Overzet K, Weerkamp F, et al. New insights on human T cell development by quantitative T cell receptor gene rearrangement studies and gene expression profiling. *J Exp Med*. 2005; 201:1715–23. [PubMed: 15928199]
2. Taghon T, Van de Walle I, De Smet G, De Smedt M, Leclercq G, Vandekerckhove B, Plum J. Notch signaling is required for proliferation but not for differentiation at a well-defined beta-selection checkpoint during human T-cell development. *Blood*. 2009; 113:3254–63. [PubMed: 18948571]
3. Carrasco YR, Trigueros C, Ramiro AR, de Yebenes VG, Toribio ML. Beta-selection is associated with the onset of CD8beta chain expression on CD4(+)CD8alphaalpha(+) pre-T cells during human intrathymic development. *Blood*. 1999; 94:3491–8. [PubMed: 10552959]

4. Blom B, Verschuren MC, Heemskerk MH, et al. TCR gene rearrangements and expression of the pre-T cell receptor complex during human T-cell differentiation. *Blood*. 1999; 93:3033–43. [PubMed: 10216100]
5. Joachims ML, Chain JL, Hooker SW, Knott-Craig CJ, Thompson LF. Human alpha beta and gamma delta thymocyte development: TCR gene rearrangements, intracellular TCR beta expression, and gamma delta developmental potential--differences between men and mice. *J Immunol*. 2006; 176:1543–52. [PubMed: 16424183]
6. Michie AM, Zuniga-Pflucker JC. Regulation of thymocyte differentiation: pre-TCR signals and beta-selection. *Semin Immunol*. 2002; 14:311–23. [PubMed: 12220932]
7. Aifantis I, Gounari F, Scorrano L, Borowski C, von Boehmer H. Constitutive pre-TCR signaling promotes differentiation through Ca²⁺ mobilization and activation of NF-kappaB and NFAT. *Nat Immunol*. 2001; 2:403–9. [PubMed: 11323693]
8. Fehling HJ, Krotkova A, Saint-Ruf C, von Boehmer H. Crucial role of the pre-T-cell receptor alpha gene in development of alpha beta but not gamma delta T cells. *Nature*. 1995; 375:795–8. [PubMed: 7596413]
9. Hahm K, Cobb BS, McCarty AS, et al. Helios, a T cell-restricted Ikaros family member that quantitatively associates with Ikaros at centromeric heterochromatin. *Genes Dev*. 1998; 12:782–96. [PubMed: 9512513]
10. Kelley CM, Ikeda T, Koipally J, Avitahl N, Wu L, Georgopoulos K, Morgan BA. Helios, a novel dimerization partner of Ikaros expressed in the earliest hematopoietic progenitors. *Curr Biol*. 1998; 8:508–15. [PubMed: 9560339]
11. Morgan B, Sun L, Avitahl N, et al. Aiolos, a lymphoid restricted transcription factor that interacts with Ikaros to regulate lymphocyte differentiation. *EMBO J*. 1997; 16:2004–13. [PubMed: 9155026]
12. Perdomo J, Holmes M, Chong B, Crossley M. Eos and pegasus, two members of the Ikaros family of proteins with distinct DNA binding activities. *J Biol Chem*. 2000; 275:38347–54. [PubMed: 10978333]
13. Sun L, Liu A, Georgopoulos K. Zinc finger-mediated protein interactions modulate Ikaros activity, a molecular control of lymphocyte development. *EMBO J*. 1996; 15:5358–69. [PubMed: 8895580]
14. Cortes M, Wong E, Koipally J, Georgopoulos K. Control of lymphocyte development by the Ikaros gene family. *Curr Opin Immunol*. 1999; 11:167–71. [PubMed: 10322160]
15. Ma S, Pathak S, Trinh L, Lu R. Interferon regulatory factors 4 and 8 induce the expression of Ikaros and Aiolos to down-regulate pre-B-cell receptor and promote cell-cycle withdrawal in pre-B-cell development. *Blood*. 2008; 111:1396–403. [PubMed: 17971486]
16. Thompson EC, Cobb BS, Sabbattini P, et al. Ikaros DNA-binding proteins as integral components of B cell developmental-stage-specific regulatory circuits. *Immunity*. 2007; 26:335–44. [PubMed: 17363301]
17. Mitchell JL, Seng A, Yankee TM. Expression and splicing of Ikaros family members in murine and human thymocytes. *Molecular immunology*. 2015 in revision.
18. Xiong J, Parker BL, Dalheimer SL, Yankee TM. Interleukin-7 supports survival of T-cell receptor-beta-expressing CD4(–) CD8(–) double-negative thymocytes. *Immunology*. 2013; 138:382–91. [PubMed: 23215679]
19. Zeng L, Dalheimer SL, Yankee TM. Gads^{–/–} mice reveal functionally distinct subsets of TCRbeta + CD4-CD8– double-negative thymocytes. *J Immunol*. 2007; 179:1013–21. [PubMed: 17617593]
20. Fujii Y, Okumura M, Inada K, Nakahara K, Matsuda H. CD45 isoform expression during T cell development in the thymus. *Eur J Immunol*. 1992; 22:1843–50. [PubMed: 1378021]
21. von Boehmer H, Aifantis I, Feinberg J, et al. Pleiotropic changes controlled by the pre-T-cell receptor. *Curr Opin Immunol*. 1999; 11:135–42. [PubMed: 10322152]
22. Varas A, Jimenez E, Sacedon R, Rodriguez-Mahou M, Maroto E, Zapata AG, Vicente A. Analysis of the human neonatal thymus: evidence for a transient thymic involution. *J Immunol*. 2000; 164:6260–7. [PubMed: 10843679]
23. Weerkamp F, de Haas EF, Naber BA, Comans-Bitter WM, Bogers AJ, van Dongen JJ, Staal FJ. Age-related changes in the cellular composition of the thymus in children. *J Allergy Clin Immunol*. 2005; 115:834–40. [PubMed: 15806007]

24. Murphy M, Epstein LB. Down syndrome (trisomy 21) thymuses have a decreased proportion of cells expressing high levels of TCR alpha, beta and CD3. A possible mechanism for diminished T cell function in Down syndrome. *Clin Immunol Immunopathol.* 1990; 55:453–67. [PubMed: 1692775]
25. Harker N, Garefalaki A, Menzel U, Ktistaki E, Naito T, Georgopoulos K, Kioussis D. Pre-TCR signaling and CD8 gene bivalent chromatin resolution during thymocyte development. *J Immunol.* 2011; 186:6368–77. [PubMed: 21515796]
26. Ramiro AR, Trigueros C, Marquez C, San Millan JL, Toribio ML. Regulation of pre-T cell receptor (pT alpha-TCR beta) gene expression during human thymic development. *J Exp Med.* 1996; 184:519–30. [PubMed: 8760805]
27. Penit C, Lucas B, Vasseur F. Cell expansion and growth arrest phases during the transition from precursor (CD4–8–) to immature (CD4+8+) thymocytes in normal and genetically modified mice. *J Immunol.* 1995; 154:5103–13. [PubMed: 7730616]
28. Webb LM, Vigorito E, Wymann MP, Hirsch E, Turner M. Cutting edge: T cell development requires the combined activities of the p110gamma and p110delta catalytic isoforms of phosphatidylinositol 3-kinase. *J Immunol.* 2005; 175:2783–7. [PubMed: 16116162]
29. Saborit-Villarroya I, Vaisitti T, Rossi D, D'Arena G, Gaidano G, Malavasi F, Deaglio S. E2A is a transcriptional regulator of CD38 expression in chronic lymphocytic leukemia. *Leukemia.* 2011; 25:479–88. [PubMed: 21212793]
30. Hsu LY, Luring J, Liang HE, Greenbaum S, Cado D, Zhuang Y, Schlissel MS. A conserved transcriptional enhancer regulates RAG gene expression in developing B cells. *Immunity.* 2003; 19:105–17. [PubMed: 12871643]
31. Chen Z, Xiao Y, Zhang J, et al. Transcription factors E2A, FOXO1 and FOXP1 regulate recombination activating gene expression in cancer cells. *PLoS One.* 2011; 6:e20475. [PubMed: 21655267]
32. Dovat S, Montecino-Rodriguez E, Schuman V, Teitell MA, Dorshkind K, Smale ST. Transgenic expression of Helios in B lineage cells alters B cell properties and promotes lymphomagenesis. *J Immunol.* 2005; 175:3508–15. [PubMed: 16148093]
33. Winandy S, Wu L, Wang JH, Georgopoulos K. Pre-T cell receptor (TCR) and TCR-controlled checkpoints in T cell differentiation are set by Ikaros. *J Exp Med.* 1999; 190:1039–48. [PubMed: 10523602]
34. Mandal M, Powers SE, Ochiai K, Georgopoulos K, Kee BL, Singh H, Clark MR. Ras orchestrates exit from the cell cycle and light-chain recombination during early B cell development. *Nat Immunol.* 2009; 10:1110–7. [PubMed: 19734904]
35. Ferreiros Vidal I, Carroll T, Taylor B, et al. Genome-wide identification of Ikaros targets elucidates its contribution to mouse B cell lineage specification and pre-B cell differentiation. *Blood.* 2013
36. Collins B, Clambey ET, Scott-Browne J, White J, Marrack P, Hagman J, Kappler JW. Ikaros promotes rearrangement of TCR alpha genes in an Ikaros null thymoma cell line. *Eur J Immunol.* 2013; 43:521–32. [PubMed: 23172374]
37. Kathrein KL, Lorenz R, Innes AM, Griffiths E, Winandy S. Ikaros induces quiescence and T-cell differentiation in a leukemia cell line. *Mol Cell Biol.* 2005; 25:1645–54. [PubMed: 15713624]

Figure 1. Upregulation of CD4 and CD8 occurs in two patterns

A) TCRγδ⁻ thymocytes were analyzed for CD4 and CD8 expression (*upper panels*).

Thymocytes were gated on SP CD4⁺ cells and analyzed for CD3 and CD4 expression. The percentages of SP CD4⁺ thymocytes that were CD3⁻ were calculated. Representative dot plots are shown for a thymus with a high ISP percentage (Group 1) and low ISP percentage (Group 2). The scattergram shows the percentages of SP CD4⁺ thymocytes that were CD3⁻ along with the line dividing patients in group 1 and group 2. *B)* Total thymocytes were gated on CD3⁻ cells and expression of CD4 and CD8 expression is shown from a representative thymus from each group. The ratio of the percentages of DN to CD4⁺ SP thymocytes within the CD3⁻ population was calculated for groups 1 and 2. The lines indicate the mean ± SE of each group.

Figure 2. CD1a and CD44 expression correlate with CD28 in ISP cells

A) ISP CD4⁺ thymocytes were analyzed for CD28, CD44, and CD1a expression and divided into ISP1 and ISP2 subpopulations. B) The percentages of ISP thymocytes from each subject in groups 1 and 2 is shown. The line indicates the mean $\pm$ SE for each group. C) ISP1 and ISP2 thymocytes from group 1 and group 2 patients were analyzed for CD7 and CD45RO expression. D) DN3 thymocytes from group 1 (*left panels*) and group 2 (*right panels*) patients were analyzed for CD1a, CD44, CD28, CD7, and CD45RO expression.

Figure 3. CD3⁻ DP cells can be divided into five subpopulations

A) CD3⁻ and CD3^{lo} DP thymocytes were analyzed for CD7 and CD45RO expression. *B)* CD3⁻CD7⁺⁺CD45RO⁻ thymocytes were analyzed for CD44, CD1a, and CD28 expression and divided into DP1 (*lightly shaded cells*) and DP2 (*darkly shaded cells*) subsets. *C)* CD3⁻CD7⁺⁺CD45RO⁻ thymocytes (plots i and ii) and CD3⁻CD7⁺CD45RO⁺ thymocytes (plots iii, iv, and v) were analyzed for expression of CD38, CD45RO, CD44, and CD7, as shown. Lightly shaded cells in plots i and ii reflect the shading in panel *B*. *D)* DP1, DP2, DP3, DP4, and DP5 thymocytes (as defined in panel *C*) were analyzed for CD28 and CD1a expression. *A–D)* Representative dot blots from a patient in group 1 (*upper panels*) and a patient from group 2 (*lower panels*) are shown in each panel. *E)* The percentages of CD3⁻ DP thymocytes in each of the five subsets are shown.

Figure 4. ISP2, DP2, and DP3 cells have increased proliferation

A) Thymocytes from the indicated cell populations were analyzed for DNA content using DAPI. The percentages of cells in the S, G2, or M phase of the cell cycle are shown.

Representative data are shown as histograms. The bar graph shows the mean $\pm$ SE for five independent experiments.

Figure 5. Ikaros, Helios, and Aiolos protein levels increase with TCR β expression

A) The indicated populations of thymocytes were intracellularly stained with anti-Helios, anti-Ikaros, or anti-Aiolos (dark lines), or the appropriate isotype controls (shaded histogram). The geometric mean fluorescence intensity (GMFI) of the anti-Ikaros, anti-Helios, or anti-Aiolos was normalized to the GMFI of the isotype control for each population, and the mean $\pm$ SE fold change relative to ISP1 is shown for four independent experiments.

	DN3	ISP1	ISP2	DP1	DP2	DP3	DP4	DP5
CD4	-	+	+	+	+	+	+	+
CD8	-	-	-	+	+	+	+	+
CD1a	+	+	++	+	++	++	++	++
CD44	+	+	++	+	++	++	+	+
CD28	-	-	+	-	+	-/+	-	-
CD45RO	-	-	+	-	+	++	++	+++
CD7	++	++	++/+	++	++	++/+	+	+
CD38	+	+	+	+	+	+	+	++
Cell cycle	-	-	++	-	++	++	+	-
Ikaros	+	+	++	+	++	++	+ / ++	+
Helios	+	+	++	+	++	++	+ / ++	+
Aiolos	+	+	++	+	++	++	++	++

Figure 6.
A model of human T cell development through the CD3⁻ developmental stages.