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Differential IL-7 Responses in Developing Human Thymocytes

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Abstract

IL-7 is a factor essential for mouse and human thymopoiesis. Mouse thymocytes have altered sensitivities to IL-7 at different developmental stages. CD4/CD8 double positive (DP) mouse thymocytes are shielded from the influence of IL-7 due to loss of CD127 (IL-7R α). In this study, we assessed IL-7 receptor expression and IL-7 signaling in human thymocytes. We found human DP cells to be severely limited in their ability to phosphorylate STAT-5 in response to IL-7. The relative expression levels of the IL-7-inducible proteins Bcl-2 and Mcl-1 were also lower in human DP cells, consistent with a stage-specific decrease in IL-7 responsiveness. IL-7 responses were restored in a subset of cells that matured past the DP stage. Unlike the regulation of IL-7 signaling in mouse thymocytes, loss of IL-7 signaling in human DP cells was not due to absence of CD127 but instead correlated with downregulation of CD132 (common γ chain).

Keywords

human; thymocyte; IL-7 signaling; common γ receptor; IL-7R α

1. Introduction

As thymocytes develop from early thymic progenitors (ETPs) to mature single positive T cells (SP4 or SP8), they differentially express a number of surface antigens that can be used to discriminate discreet subsets. Human and mouse thymocyte compartments have historically been discriminated using CD4 and CD8 expression as the principal markers along with numerous other antigens to further divide the subpopulations [1-3] Early thymocytes are negative for CD4 and CD8 as well as negative for T cell antigen receptors (TCR) and its signaling partner CD3. After cells mature past TCR- β selection, they express both CD4 and

CD8 (DP cells) and eventually mature into cells that are single positive for CD4 (SP4) or CD8 (SP8) and express high levels of TCR and CD3.

It is evident in the early subsets that human thymocyte development differs from that of the mouse. For example, in humans, a thymocyte subset exists that is negative for CD3 and CD8 but positive for CD4. This population is an immature intermediate single positive (ISP4) subset that is absent or very small in mice [4-6]. For this reason, CD3 or TCR expression in addition to CD4 and CD8 expression has historically been used to discriminate the least mature human thymocyte populations. These immature human thymocytes are referred to as the triple negative (TN) population (negative for CD3/TCR, CD4, and CD8) [1,7]. In mice, the least mature thymocytes are referred to as the double negative (DN) population; those that do not express CD4 or CD8. Different markers are often used for mouse cells compared to human cells to further divide the early pre- β -selection and post- β -selection thymocyte subsets [8-10]; making direct comparisons of major selection events and minor subsets between the two species difficult.

Although mice and humans have incongruous patterns of expression of multiple developmental markers, IL-7 signaling is indispensable for thymopoiesis in both of these mammalian systems. Mice genetically engineered to be deficient in expression of IL-7 or IL-7R α have severely reduced numbers of thymocytes [11,12]. Likewise, disruptions or mutations in the human IL-7 signaling pathway lead to severe combined human immunodeficiency (SCID) [13]. In SCID, lack of a functioning immune system leads to systemic infections and ultimately patient death.

Despite an absolute requirement for IL-7 signaling in both human and mouse thymocyte development, surprising differences in IL-7 receptor expression patterns occur between the two species. It has been reported that human DP thymocytes express IL-7R α [14], whereas, we and others have found mouse DP cells to lack IL-7R α [6,15]. In mice, IL-7 signaling is finely tuned and differentially regulated during thymocyte development [15,16]. Although IL-7 signaling is required for early proliferation and maturation, excess IL-7R α expression can be detrimental and leads to reduced mouse thymocyte numbers [16]. In normal mouse thymopoiesis, IL-7 signaling is shut off during the DP stage as cells audition for positive selection. This is accomplished by downregulation of IL-7R α expression as well as cell migration into the thymic cortex where little IL-7 is expressed [17]. Following positive selection, IL-7R α is re-expressed and cells then enter the IL-7-rich medulla where IL-7-mediated survival signals can be turned on [15]. It has been hypothesized that the shutoff of IL-7 signaling in the murine DP compartment is a mechanism by which death-by-neglect of thymocytes that fail to successfully express a TCR can be enforced [16]. It has not previously been established to what extent similar control of IL-7 signaling occurs in humans.

In this study, expression of the IL-7 receptor chains (CD127 and CD132) and IL-7 responsiveness of human thymocyte subsets were measured. Despite marked differences in IL-7 receptor expression compared to mice, human thymocyte subsets were similar to mouse thymocytes in IL-7 responses. Human DP cells showed greatly diminished responses to IL-7 compared to the other compartments, as measured by STAT-5 phosphorylation which correlated with downregulation of CD132 in this subset. Additionally, expression of Bcl-2 and Mcl-1 (IL-7-induced proteins) correlated with IL-7 sensitivity, suggesting loss of IL-7 signaling occurs *in vivo* at the DP stage of human thymocyte development.

2. Subjects and Methods

2.1 Thymocyte isolation

Human thymus tissue was obtained during pediatric cardiac surgeries at Saint Francis Hospital in Tulsa, OK and The Children's Hospital at OU Medical Center, Oklahoma City, OK, from

patients aged 1 week to 3 years (mean age = 18.75 months). This was done in accordance with protocols approved by the Institutional Review Boards of both the University of Oklahoma and Saint Francis Hospital. Only freshly isolated thymocyte samples were used for the experiments in this report. This was necessary due to reported preferential death of early human thymocyte subsets in short term (4°C) or long term storage (-80°C or liquid nitrogen) [14]. Single cell thymocyte suspensions were generated by forceful disruption of thymuses with 3-ml syringe plungers through 70 micron nylon screens. Thymocyte manipulations were conducted in complete tumor medium (CTM) [18].

2.2 Antibodies and Flow Cytometry

Human thymocyte subpopulations were discriminated using FITC-coupled anti-CD3 mAb (Clone S4.1, Invitrogen/Caltag, Carlsbad, CA), Pacific Blue coupled anti-CD4 mAb (Clone RPA-T4, Biolegend, San Diego, CA), APC/Cy7-coupled anti-CD8 mAb (Clone RPA-T8, Biolegend) and when sorting, a PE-coupled non-T lineage cocktail which included: CD11c (Clone 3.9, eBioscience San Diego, CA), CD14 (Clone 61D3, eBioscience), CD19 (Clone H1B19, eBioscience), CD56 (Clone MEM188, eBioscience), and CD235a, glycophorin A (Clone H1R2, eBioscience). IL-7R α expression was assessed using PE-labeled mAb specific for CD127 (Clone 40131, R&D Systems, Minneapolis, MN) and PE-coupled mouse IgG₁ (Clone 11711, BD Pharmingen, San Jose, CA) was used as an isotype control. Common γ receptor expression was assessed using PE-labeled mAb specific for CD132 (Clone TUGh4, Biolegend) and a PE-coupled rat IgG2b, κ (Biolegend) was used as an isotype control. Cells were analyzed using an LSR II Special Order System flow cytometer (BD Biosciences, San Jose, CA)

2.3 Intracellular pSTAT-5 staining

Fresh human thymocytes were sorted using a Dako MoFlo cell sorter (Carpinteria, CA). Before treatment with cytokine, thymocyte populations were incubated at 37°C for 20 min in warm S-MEM (Invitrogen, Carlsbad, CA). The cells were then incubated in either S-MEM alone or with 25 ng/ml IL-7 (R&D Systems) for an additional 20 min. At the end of the incubation, cells were fixed and stained for intracellular pSTAT-5 (Tyr⁶⁹⁴) (BD Biosciences) as previously described [16,19].

2.4 Western blotting

Thymocyte subsets were sort purified and NP-40 whole cell lysates were generated from untreated and cytokine treated (25 ng/ml IL-7 or IFN- γ) samples [15,19] and then Western blotted as previously described [18,20] using primary antibodies specific to phosphorylated STAT-5 (Cell Signaling; rabbit polyclonal against human), Mcl-1 (Biovision, Mountain View, CA; rabbit polyclonal against human) Bcl-2 (Santa Cruz Biotechnology, Inc; mouse monoclonal against human) and β -actin (Santa Cruz Biotechnology, Inc; goat polyclonal against human). HRP-conjugated secondary antibodies were polyclonal goat anti-rabbit IgG (KPL, Inc. Gaithersburg, MD), goat anti-mouse IgG (Santa Cruz Biotechnology, Inc.), and donkey anti-goat IgG (Santa Cruz Biotechnology, Inc.).

2.5 Data analysis

Flow cytometric data were analyzed for Table 1 using FACSDiva software (BD Biosciences). The histogram overlays shown in Fig. 1 were generated using FloJo software (Tree Star, Inc., Ashland OR). Paired student t-tests were performed using GraphPad Prism software in order to generate the *p* values for the comparisons described in the Results (section 3.1).

3. Results

3.1 IL-7R α signaling is inhibited in DP thymocytes (Fig. 1, 2)

Human thymocytes were stained with mAbs to CD3, CD4, CD8, CD127 (IL-7R α), and CD132 (common γ receptor), as well as assessed for responses to IL-7 as described in the Subjects and Methods. Populations were defined as follows: TN (CD3⁻, CD4⁻, CD8⁻), ISP4 (CD3⁻, CD8⁻, CD4⁺), DP^{CD3lo} (CD3^{-lo}, CD4⁺, CD8⁺), DP^{CD3hi} (CD3⁺, CD4⁺, CD8⁺), SP4 (CD4⁺, CD8⁻, CD3⁺), SP8 (CD8⁺, CD4⁻, CD3⁺). As shown in a representative thymus in Figure 1, all of the subsets of human thymocytes expressed IL-7R α however; there was a marked loss of the common γ chain in the DP compartments. This result is in contrast to the expression pattern found in mice, where DP cells do not express IL-7R α yet retain expression of the common γ chain, albeit at lower levels [19].

To assess IL-7 responsiveness, human thymocyte subsets were sort purified and incubated with or without IL-7 and subjected to intracellular staining for pSTAT-5 as described in the methods section. Similar to previous findings in mice [15,16,19], human thymocytes downregulated pSTAT-5 responses to IL-7 during the DP stage of development, where thymocytes are auditioning for positive selection (Fig. 1). This result was consistent in thymocyte assays from 7 different human subjects (Table 1) as well as 3 additional subjects whose thymuses were assayed by Western blotting (Fig. 2). The ability to phosphorylate STAT-5 in response to IL-7 was restored in a subset of the cells that had undergone positive selection (SP compartments). The SP4 compartment had a larger percentage of cells that had regained responsiveness to IL-7 compared to the SP8 compartment. This result was consistent across the 7 patients shown in Table 1 ($p < 0.0001$).

As is evident in Table 1, the percentages of responsive cells in multiple subpopulations varied in the thymuses from the individuals that were studied, however, the loss of responsiveness and the loss of CD132 as cells transitioned from ISP to DP^{CD3-} was consistent and statistically significant ($p = 0.0058$ for change in pSTAT-5 and $p = 0.0052$ for change in CD132). The restoration of pSTAT-5 responses to IL-7 and the increased expression of CD132 in the SP compartments compared to the DP^{CD3-} cells were also statistically significant changes ($p < 0.001$ for SP4 and SP8 for both changes).

Further evidence that IL-7 responses are downregulated in human DP thymocytes is shown in Fig. 2. Sort purified subsets were incubated in serum free media, IL-7, or IFN- γ (negative control for STAT-5 signaling) then cells lysed and STAT-5 phosphorylation assessed by Western blotting. Similar to the intracellular flow cytometric experiments, loss of IL-7 responsiveness in the DP cells and restoration of responsiveness at the SP4 and SP8 stages were clearly evident.

3.2 Expression of IL-7-induced proteins is reduced in DP thymocytes (Fig. 3)

In an attempt to address whether DP cells experience IL-7 signaling *in vivo*, we sorted cells from the TN, ISP4, DP^{CD3lo}, DP^{CD3hi}, SP4, and SP8 populations and assessed expression of the survival proteins Bcl-2 and Mcl-1, both of which are known to be induced by IL-7 [21-23]. As predicted, losses of Bcl-2 and Mcl-1 expression were evident in the DP thymocytes (Fig. 3). These results suggest that IL-7 signaling is inhibited *in vivo* at the DP stage of development of human thymocytes.

4. Discussion

In this study, we sought to determine whether the differential IL-7 signaling previously published in mouse thymocyte subsets also occurs in human thymocytes. While there has been controversy concerning the expression of IL-7R α in human thymocytes [24-26], our data

showing IL-7R α expression throughout human thymocyte development agrees with previous reports [14,24,26]. For example, in analyzing various storage methods for human thymus tissue, Young and Angel described similar trends in expression of IL-7R α across six major thymocyte subsets [14]. Taken alone, the IL-7R α expression data suggest that human thymocytes should retain responsiveness to IL-7 throughout their developmental and differentiation process. In contrast, our results show that IL-7-mediated STAT-5 signaling is largely limited to the TN, ISP4, SP4 and SP8 compartments. This is further supported by experiments reported by Napolitano et al. in which fetal human DP thymocytes were shown to have inhibited responses to IL-7, as measured by induction of Bcl-2 and effects on apoptosis and proliferation. [26] Importantly, human thymocytes in the CD3⁻ subset within the DP compartment robustly express IL-7R α yet are incapable of signaling in response to IL-7 as measured by phosphorylation of STAT-5. This loss of human DP thymocyte signaling via IL-7 differs from murine thymocyte signaling, where loss of IL-7 signaling at the DP stage is due to downregulation of murine IL-7R α expression [15,19]. Whereas, transcriptional data indicate that the other component of the IL-7R, the common γ -chain, is ubiquitously expressed among human thymocyte populations [27], the data presented in Fig. 1 clearly show that protein expression of the common γ chain is greatly diminished in the human DP populations, suggesting posttranslational mechanisms may be in place to downregulate the expression of this receptor.

Although loss of the common γ chain may explain the lack of IL-7 signaling in the DP cells, there was not as strict a correlation with SP4 and SP8 signaling. As cells matured into the SP compartments, we consistently saw a larger percentage of these cells staining positive for both receptors than we saw responding to IL-7. This indicates multiple mechanisms may be responsible for the downregulation of IL-7 signaling in the human thymus as has been shown in mouse DP cells where signaling is inhibited by SOCS proteins as well as loss of IL-7R α . [15,28] This has been further illustrated in mouse thymus where enforced IL-7R α expression in DP cells in transgenic mice failed to completely restore IL-7 signaling. [6,16] It should also be noted that loss of the common γ chain predicts that human DP cells will also be inefficient in responses to the other cytokines that share this receptor subunit, such as IL-2, IL-4, IL-9, IL-15, and IL-21. We intend to investigate the response of human thymocyte subsets to these cytokines in the future.

IL-7 signaling can be mediated by the PI-3K/AKT pathway in addition to the JAK/STAT pathway that we assessed in our experiments [29]. However, Johnson, et al. reported that more mature human thymocytes respond to IL-7 through the STAT-5 pathway and not the PI-3K/AKT pathway [30]. In their study, thymocytes were sorted into immature (CD34⁺) and mature (CD34⁻, DP and SP cells combined) compartments and assessed for IL-7 responses. Collectively, these data suggest that DP cells are not responsive to IL-7 via either pathway. In further support of this, our data in Fig. 3 show loss of Mcl-1 and Bcl-2 in the DP compartment. The expression of both of these survival proteins can be upregulated by IL-7 signaling [21-23].

Another group also identified a difference in IL-7R α expression in early human thymocytes compared to mouse thymocytes. The earliest human T cell precursors in the thymus (Lin-CD34⁺CD10⁺CD24⁻CD7⁻ cells) were shown to express IL-7R α , whereas early mouse thymic progenitors lack IL-7R α [31]. Although, numerous differences may exist between the two species, the data suggest that loss of IL-7 signaling at the DP stage is an evolutionarily conserved process that is mediated by different mechanisms in mice and humans. Knowledge regarding human thymocyte development has lagged behind that of the murine system; however, important discoveries are emerging. For example, Taghon, et al. recently defined markers that identify the human thymocyte subsets flanking the β -selection checkpoint [32].

As new details continue to emerge about human thymocyte development, it will be important to characterize which cytokines are involved in the survival, differentiation, and selection of each discreet population. Furthermore, as efforts may soon emerge to use IL-7 therapy to treat thymic atrophy in aged humans and various other clinical conditions [33,34], it is important to better understand which human thymocyte subsets are capable of responding to IL-7 and to what extent exogenous IL-7 or drugs that inhibit IL-7-dependent processes will impact T cell development.

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Fig. 1. IL-7R α and common γ receptor expression and IL-7 signaling in the TN, ISP4, DP^{CD3^{lo}}, DP^{CD3^{hi}}, SP4 and SP8 thymocyte subsets

IL-7R α and common γ receptor expression levels were evaluated on gated thymocytes by staining with antibodies to CD3, CD4, CD8, CD127, and CD132 (left panels). Open histograms represent staining with isotype control mAb and closed histograms represent IL-7R α (CD127) and common γ receptor (CD132) staining. Equivalent cell numbers from each subset were treated with (open histograms) or without (closed histograms) IL-7 and then analyzed for intracellular levels of pSTAT-5 (right panels). The data in this figure are from patient #1, detailed in Table 1.

Fig. 2. Western blotting confirms that DP thymocytes do not respond to IL-7 via the STAT-5 signaling pathway

Lysates of equivalent cell numbers from sorted subsets (TN, DP^{CD3^{lo}}, DP^{CD3^{hi}}, SP4 and SP8) were treated with IL-7, IFN- γ , or medium alone and then analyzed by Western blotting for pSTAT-5, or β -actin. The loss of pSTAT-5 response in the DP cells was seen in samples from all 3 patients for which this assay was performed.

Fig. 3. Mcl-1 and Bcl-2 protein expression is diminished in human DP thymocytes

Human thymocytes were sorted into six major subpopulations (TN, ISP4, DP^{CD3lo}, DP^{CD3hi}, SP4, and SP8). Sorted cells (500,000 cells/lane) were lysed and blotted with antibodies against Mcl-1, Bcl-2, and β -actin as described in the methods sections. Shown are three separate experiments (A, B, and C) from 3 individual patients.

Table 1

IL-7 receptor expression and IL-7 response profiles of human thymocytes.

Patient (age/gender)	TN	ISP4	CD3-DP	CD3+ DP	SP4	SP8
1 (2yr/f)	pSTAT5*	47.9	68.1	0.7	57.7	31.2
	CD127#	55.6	99.1	93.3	86.7	89.2
	CD132#	61.3	83.0	8.8	96.1	99.0
2 (6mo/m)	pSTAT5*	59.3	46.2	0.0	61.2	29.9
	CD127#	90.6	94.8	98.4	88.4	97.1
	CD132#	97.2	94.8	13.5	98.9	99.9
3 (1wk/f)	pSTAT5*	35.4	65.9	0.4	42.2	7.0
	CD127#	57.0	96.9	87.3	72.6	81.0
	CD132#	78.7	75.4	3.1	85.1	89.8
4 (5mo/f)	pSTAT5*	48.2	24.9	0.3	58.1	26.5
	CD127#	76.0	96.3	469.2	73.6	80.0
	CD132#	58.1	41.7	1.5	82.6	84.7
5 (3yr/f)	pSTAT5*	23.2	19.0	0.9	52.6	29.4
	CD127#	54.0	79.4	95.5	86.2	42.0
	pSTAT5*	41.9	17.5	0.9	55.5	30.6
6 (3yr/m)	CD127#	90.3	64.0	99.0	79.1	53.5
	pSTAT5*	50.4	16.5	0.1	52.1	17.5
	CD127#	19.2	95.5	92.7	77.8	71.1

* Numerical values represent the percent staining positive for pSTAT5 in each IL-7-treated population relative to the corresponding untreated population.

Numerical values represent the percent staining positive for surface expression of the indicated IL-7 receptor chain relative to isotype control staining.