OMIP 073: Analysis of human thymocyte development with a 14-color flow cytometry panel

Sarah-Jolan Bremer1 | Laura Glau1 | Christina Gehbauer1 | Annika Boxnick1 | Daniel Biermann2 | Jörg Siegmar Sachweh2 | Eva Tolosa1 | Anna Gieras1

1Department of Immunology, University Medical Center Hamburg-Eppendorf, Hamburg, Germany
2Surgery for Congenital Heart Disease, University Heart & Vascular Center Hamburg, University Medical Center Hamburg-Eppendorf, Hamburg, Germany

Abstract
This panel was designed for the identification and detailed characterization of the different developmental steps of human thymocytes. We optimized the panel for fresh tissue in order to provide an unbiased analysis of T cell development. Accurate selection of antibodies and precise gating allow us to phenotype 14 major stages of human thymocyte development and illustrate the trajectories of T cell development from early thymic progenitors (ETP) to mature T cells that are ready to populate the periphery. The panel identifies ETPs, T-lineage-committed cells (TC), CD34-positive immature single-positive CD4 cells (ISP4 CD34+), CD34-negative immature single-positive CD4 cells (ISP4 CD34-), CD45-low early double-positive cells (EDP CD45low), CD45-high early double-positive cells (EDP CD45high), late double-positive cells (LDP), single-positive CD4 cells (SP4), single-positive CD8 cells (SP8), ready-to-egress single-positive CD4 cells (rSP4), ready-to-egress single-positive CD8 cells (rSP8), T γδ cells (Tγδ), T regulatory cells (Treg), and ready-to-egress T regulatory cells (rTreg). To highlight important checkpoints during T cell development, we added antibodies relevant for specific developmental steps to the panel. These include CD1a to define TCs, CD28 as a marker for β-selection and CD69 in combination with CD45RA to determine the maturation stage of thymocytes shortly before they become ready to egress the thymus and colonize the periphery. Moreover, Annexin V, as a marker for apoptosis, provides valuable extra information concerning the apoptotic death of thymocytes. Currently, we use this panel to identify aberrations in T cell development in health and disease.

KEYWORDS
apoptosis, flow cytometry, human, T cell development, thymocytes, thymus

1 BACKGROUND

The thymus plays an essential role in establishing a functional adaptive immune system by providing the microenvironment for T cell development (1, 2). A detailed understanding of the developmental steps and cell populations in the human thymus will increase our knowledge about the origin of immune deficiencies, autoimmunity, or hematological diseases. Although T cell development has been characterized in depth in mice (3, 4), there is still some knowledge lacking concerning the development in humans, especially in the early stages.
of development. Recently published investigations on single-cell RNA sequencing and CD marker expression of thymocytes give valuable information about the heterogeneity of thymocytes and their molecular landscape (5–9). To make research results on human T cell development comparable, a consensus should be found on classification and characteristics for human thymocyte populations. We aimed to design a panel (Table 1) that allows for easy and, at the same time, unmistakable definition of thymocyte subpopulations (Figure 1(A)), to give an overview of the developmental pathway (Figure 1(B)), and to provide reference values for the defined developmental stages (Figure 1(C)).

We first determined the live cell population using live/dead staining and Annexin V (for used antibodies see Table 2). By binding to phosphatidylserine, Annexin V detects not only necrotic, but also apoptotic cells (10). Since we have observed that thymocytes undergo apoptosis upon exposure to stressful stimuli like glucocorticoids or freezing, staining with Annexin V is a powerful tool to ensure the analysis of only live cells. The possibility of staining for Annexin V-binding adds our panel suitable for the determination of apoptotic thymocytes in in vitro assays.

CD34+ hematopoietic stem and progenitor cells migrate from the bone marrow to the thymus, where they undergo specialized processes of maturation and selection (11). These early thymic progenitors (ETP) can be defined as CD34+CD45RA+CD1a− and can give rise to different immune cell types. Upon Notch signaling, T cell-specific genes like CD7 are upregulated (5, 12, 13). The progenitors are T-lineage-committed (TC) when they express CD1a, and the development of other lineages such as B cells, NK cells, or DCs is inhibited (1, 14). Rearrangement of the T cell receptor (TCR) takes place within a CD7+ subset, and at this stage, CD4 is upregulated and CD34 downregulated (15). Therefore, the immature single positive stage (ISP4) can be divided into a CD4+CD34+ and a CD4+CD34− population. Moreover, we discovered a population of early double-positive (EDP) cells expressing CD4 and CD8 that still express CD7, intermediate levels of CD34 and show low-level expression of CD45 (EDP CD45low). These findings indicate that ISP4 thymocytes do not necessarily completely lose CD34 expression or gain high levels of CD45 before becoming double-positive.

Expression of a functional TCR β chain that will pair with a pre-TCR α chain parallels development of progenitors into CD4+ CD8+ (double-positive) cells. TCR α rearrangement paves the way for a functional TCR αβ–CD3 complex that can be primarily seen in the double-positive population (1). The double-positive cells can be further subdivided into a CD4+CD8− CD3− (early double-positive [EDP]) and a CD4+ CD8+ CD3+ (late double-positive [LDP]) population. CD28 expression correlates with the expression of TCR β chain and provides information on β-selection at different developmental stages (16). Rearrangement of the TCR γ and TCR δ loci occurs even before the TCR β chain is recombined, and thymocytes keep their γδ potential from the TC stage onwards for an elongated period (17). Cells with functionally rearranged TCR γ and TCR δ chains become CD3+ T γδ cells (18). Thymocytes undergo tightly regulated selection processes to gain a broad but self-tolerant TCR repertoire (19, 20). Thymocytes expressing a TCR that does not recognize self-MHC-peptides die by neglect, while thymocytes expressing a TCR with an excessive affinity for self-MHC-peptides are considered to be potentially autoreactive and undergo negative selection. Cells with a low to moderate affinity are positively selected (19). Positively selected thymocytes differentiate into CD8+ cytotoxic T cells or CD4+ helper T cells during selection processes, depending on their specificity of the clonal TCR to MHC class I or MHC class II molecules, respectively (21). Cells with a higher TCR signal strength are likely to become T regulatory cells (Treg) characterized by the expression of CD25 and FOXP3 (22). Recent findings suggest that Treg-commitment takes place already at the DP stage (23). In order to leave their thymic environment and migrate to the periphery, single-positive CD4 (SP4), single-positive CD8 (SP8), or Treg cells need to lose their retention marker CD69 and upregulate CD45RA (2). Finally, T cells that are ready to egress from the thymus can be identified by a CD3+ CD45RA+ CD69− phenotype.

The average percentages of the 14 subpopulations are depicted in Figure 1(C) (see also Online Table 7). DPs constitute the largest subpopulation with more than three-quarters of all thymocytes. Here, EDPs are more than twice the number of LDPs. The more immature CD45low compartment is dominated by ISP4 CD34+ cells. The largest subpopulation within the more mature CD45high compartment is SP4, followed by SP8, Treg and T γδ cells.

To visualize the co-expression of all markers on each cell simultaneously, we performed the dimensionality reduction algorithm UMAP (Uniform Manifold Approximation and Projection) (24) on the live, CD45+ cell population (Figure 1(D)). The UMAP overlay plot (Figure 1(E)) shows all thymocyte subpopulations segregated according to the gating strategy (Figure 1(A)).

In summary, we use this 14-color panel to study the thymus of immunologically healthy children as well as children that display immunodeficiencies or have syndromes associated with immunological alterations. Since thymic tissue is taken from infants undergoing corrective cardiac surgery, this panel will be of special interest for researchers investigating the link between congenital heart diseases and alterations in the immune system.

| TABLE 1 | Summary table |
|----------|----------------|
| **Purpose** | Comprehensive immunophenotyping of thymocytes |
| Species | Human |
| Cell types | Thymocytes |
| Cross-reference | None to date |
FIGURE 1  (A) Gating strategy for a 14-color flow cytometry panel to immunophenotype human thymocytes. The figure shows a representative sample of a 9-month-old donor. (B) T cell development in the human thymus. Developmental stages are numbered according to the gating in (A). (C) Average frequencies of human thymocyte subsets (percentage of CD45+ cells) in children aged 6–12 months. The insets show the least frequent subsets: left, subpopulations before EDP CD45high stage; right, subpopulations after LDP stage (n = 8). (D and E) UMAP analysis of 78,000 exported CD45+ cells. (D) UMAP plots visualizing the expression of each individual cell surface marker. (E) UMAP overlay plot illustrating all major thymocyte subpopulations. 1 ETP, early thymic progenitors; 2 TC, T-lineage-committed cells; 3 ISP4 CD34+, CD34-positive immature single-positive CD4 cells; 4 EDP CD45low, CD45-low CD34-positive early double-positive cells; 5 (5a+5b) ISP4 CD34-, CD34-negative immature single-positive CD4 cells; 6 EDP CD45high, CD45-high CD34-negative early double-positive cells; 7 LDP, late double-positive cells; 8 (8a+8b)SP4, CD4-single-positive cells; 9 rSP4, ready-to-egress CD4-single-positive cells; 10 (10a+10b) Treg, T regulatory cells; 11 rTreg, ready-to-egress T regulatory cells; 12 (12a+12b) SP8, CD8-single-positive cells; 13 rSP8, ready-to-egress CD8-single-positive cells; 14 T γδ, T γδ cells
The study was approved by the local ethics committee and written informed consent was received from the parents (approved protocol number PV5482).

SIMILARITY TO OTHER OMIPs

None to date.

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AUTHOR CONTRIBUTIONS

Sarah-Jolan Bremer: Data curation; formal analysis; funding acquisition; project administration; validation; visualization; writing-original draft; writing-review & editing. Laura Glau: Formal analysis; software; visualization; writing-review & editing. Christina Gehbauer: Project administration; validation; writing-review & editing. Annika Boxnick: Data curation; validation; writing-review & editing. Daniel Biermann: Resources; validation; writing-review & editing. Jörg Siegmar Sachweh: Resources; validation; writing-review & editing. Eva Tolosa: Conceptualization; formal analysis; funding acquisition; methodology; project administration; resources; supervision; validation; visualization; writing-original draft; writing-review & editing. Anna Giera: Conceptualization; data curation; formal analysis; funding acquisition; project administration; supervision; validation; visualization; writing-original draft; writing-review & editing.

CONFLICT OF INTERESTS

The authors have no conflicts of interest to declare.

ORCID

Anna Giera https://orcid.org/0000-0002-5147-2281

REFERENCES

1. Spits H. Development of αβ T cells in the human thymus. Nat Rev Immunol. 2002;2(10):760–72.
2. Plum J, De Smedt M, Leclercq G, Taghon T, Kerre T, Vandekerckhove B. Human intrathymic development: A selective approach. Semin Immunopathol. 2008;30(4):411–23.
3. Zühlig-Pflücker J. T-cell development made simple. Nat Rev Immunol. 2004;4(1):67–72.
4. Germain RN. T-cell development and the CD4–CD8 lineage decision. Nat Rev Immunol. 2002;2(5):309–22.
5. Lavaert M, Liang KL, Vandamme N, Park J-E, Roels J, Kowalczyk MS, et al. Integrated scRNA-Seq identifies human postnatal thymus seeding progenitors and regulatory dynamics of differentiating immature thymocytes. Immunity. 2020;52:1–17.
6. Park JE, Botting RA, Conde CD, Popescu DM, Lavaert M, Kunz DJ, et al. A cell atlas of human thymic development defines T cell repertoire formation. Science. 2020;367(6480):1–11.
7. Kalina T, Fiser K, Pérez-Andrés M, Kužilková D, Cuenca M, SAW B, et al. CD maps–dynamic profiling of CD1–CD100 surface expression on human leukocyte and lymphocyte subsets. Front Immunol. 2019;10(2434):1–15.
8. Zhou W, Yui MA, Williams BA, Yun J, Wold BJ, Cai L, et al. Single-cell analysis reveals regulatory gene expression dynamics leading to lineage commitment in early T cell development. Cell Syst. 2019;9(4):321–37.
9. Chopp LB, Gopalan V, Ciucci T, Ruchinskas A, Rae Z, Lagarde M, et al. An integrated epigenomic and transcriptomic map of mouse and human αβ T cell development. Immunity. 2020;53(6):1182–201.
10. Crowley LC, Marfell BJ, Scott AP, Waterhouse NJ. Quantitation of apoptosis and necrosis by annexin v binding, propidium iodide uptake and flow cytometry. Cold Spring Harb Protoc. 2016;11:953–7.

11. Yoganathan K, Chen ELY, Singh J, Zúñiga-Pflücker JC. T-cell development: from T-lineage specification to Intrathymic maturation. In: Passos GA, editor. Thymus Transcriptome and cell biology. London: Springer-Nature; 2019. p. 67–115.

12. Schmitt TM, Zúñiga-Pflücker JC. Induction of T cell development from hematopoietic progenitor cells by delta-like-1 in vitro. Immunity. 2002;17(6):749–56.

13. Weerkamp F, Pike-Overzet K, Staal FJT. T-sing progenitors to commit. Trends Immunol. 2006;27(3):125–31.

14. Spits H, Blom B, Jaleco AC, Weijer K, Verschuren MCM, Van Dongen JJM, et al. Early stages in the development of human T, natural killer and thymic dendritic cells. Immunol Rev. 1998;165:75–86.

15. Van de Valle I, Davids K, Taghon T. Characterization and isolation of human T cell progenitors. In: Bosselut R, Vacchio MS, editors. T-cell development. Methods and protocols. Volume 1323. New York: Springer; 2016. p. 221–37.

16. Taghon T, Van de Valle I, De Smet G, De Smedt M, Leclercq G, Vandeckerekhove B, et al. 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(14):3254–63.

17. Dik WA, Pike-Overzet K, Weerkamp F, de Ridder D, de Haas EFE, Baert MRM, 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(11):1715–23.

18. Taghon T, Rothenberg EV. Molecular mechanisms that control mouse and human TCR-αβ and TCR-γδ T cell development. Semin Immunopathol. 2008;30(4):383–98.

19. Klein L, Kyewski B, Allen PM, Hogquist KA. Positive and negative selection of the T cell repertoire: What thymocytes see (and don’t see). Nat Rev Immunol. 2014;14(6):377–91.

20. Starr TK, Jameson SC, Positive HKA. Negative selection of T cells. Annu Rev Immunol. 2003;21(1):139–76.

21. Egawa T. Regulation of CD4 and CD8 coreceptor expression and CD4 versus CD8 lineage decisions. Adv Immunol. 2015;125(1):1–40.

22. Owen DL, Sjaastad LE, Farrar MA. Regulatory T cell development in the thymus. J Immunol. 2019;203(8):2031–41.

23. Vanhanen R, Leskinen K, Mattila IP, Saavalainen P, Arstila TP. Epigenetic and transcriptional analysis supports human regulatory T cell commitment at the CD4+CD8+ thymocyte stage. Cell Immunol. 2020;347:1–7.

24. McInnes L, Healy J, Melville J. UMAP: Uniform Manifold Approximation and Projection for Dimension Reduction. 2018. p. 1–51. Available from: http://arxiv.org/abs/1802.03426.

**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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