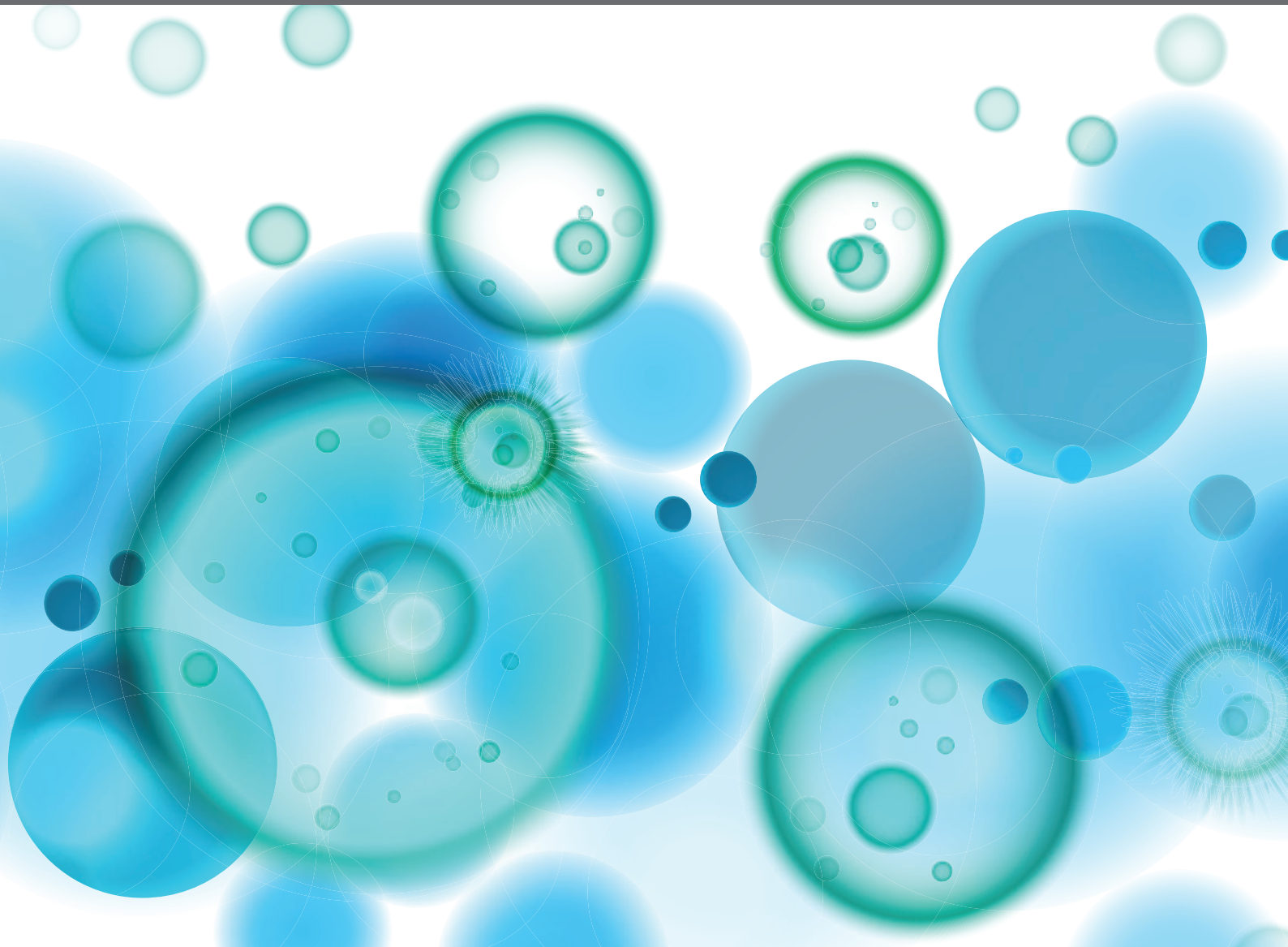


NEW INSIGHTS INTO THYMIC FUNCTIONS DURING STRESS, AGING, AND IN DISEASE SETTINGS

EDITED BY: Nicolai Stanislas van Oers, Dong-Ming Su, Ann Chidgey and
Jarrod Dudakov

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NEW INSIGHTS INTO THYMIC FUNCTIONS DURING STRESS, AGING, AND IN DISEASE SETTINGS

Topic Editors:

Nicolai Stanislas van Oers, University of Texas Southwestern Medical Center,
United States

Dong-Ming Su, University of North Texas Health Science Center, United States

Ann Chidgey, Monash University, Australia

Jarrold Dudakov, Fred Hutchinson Cancer Research Center, United States

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Table of Contents

- 05 Editorial: New Insights Into Thymic Functions During Stress, Aging, and in Disease Settings**
Nicolai S. C. van Oers, Dong-Ming Su, Ann P. Chidgey and Jarrod Dudakov
- 07 Gender Disparity Impacts on Thymus Aging and LHRH Receptor Antagonist-Induced Thymic Reconstitution Following Chemotherapeutic Damage**
Michael Ly Hun, Kahlia Wong, Josephine Rahma Gunawan, Abdulaziz Alsharif, Kylie Quinn and Ann P. Chidgey
- 27 Thymic B Cells Promote Germinal Center-Like Structures and the Expansion of Follicular Helper T Cells in Lupus-Prone Mice**
Yessia Hidalgo, Sarah Núñez, Maria Jose Fuenzalida, Felipe Flores-Santibáñez, Pablo J. Sáez, Jessica Dorner, Ana-Maria Lennon-Dumenil, Victor Martínez, Emmanuel Zorn, Mario Roseblatt, Daniela Sauma and Maria Rosa Bono
- 43 Thymic Function Associated With Cancer Development, Relapse, and Antitumor Immunity – A Mini-Review**
Weikan Wang, Rachel Thomas, Olga Sizova and Dong-Ming Su
- 52 Molecular Insights Into the Causes of Human Thymic Hypoplasia With Animal Models**
Pratibha Bhalla, Christian A. Wysocki and Nicolai S. C. van Oers
- 66 Generation and Regeneration of Thymic Epithelial Cells**
Abdullah S. Alawam, Graham Anderson and Beth Lucas
- 81 Postnatal Involution and Counter-Involution of the Thymus**
Jennifer E. Cowan, Yousuke Takahama, Avinash Bhandoola and Izumi Ohigashi
- 92 Impaired Thymic Output Can Be Related to the Low Immune Reconstitution and T Cell Repertoire Disturbances in Relapsing Visceral Leishmaniasis Associated HIV/AIDS Patients**
Maria Luciana Silva-Freitas, Gabriela Corrêa-Castro, Glaucia Fernandes Cota, Carmem Giacoia-Gripp, Ana Rabello, Juliana Teixeira Dutra, Zilton Farias Meira de Vasconcelos, Wilson Savino, Alda Maria Da-Cruz and Joanna Reis Santos-Oliveira
- 106 Human Thymic Involution and Aging in Humanized Mice**
Qing-Yue Tong, Jue-Chao Zhang, Jing-Long Guo, Yang Li, Li-Yu Yao, Xue Wang, Yong-Guang Yang and Li-Guang Sun
- 115 Phosphate Transporter Profiles in Murine and Human Thymi Identify Thymocytes at Distinct Stages of Differentiation**
Alice Machado, Marie Pouzolles, Sarah Gailhac, Vanessa Fritz, Marco Craveiro, Uriel López-Sánchez, Taisuke Kondo, Francesca Pala, Marita Bosticardo, Luigi D. Notarangelo, Vincent Petit, Naomi Taylor and Valérie S. Zimmermann
- 131 Evaluating Thymic Function After Human Hematopoietic Stem Cell Transplantation in the Personalized Medicine Era**
Ahmed Gaballa, Emmanuel Clave, Michael Uhlin, Antoine Toubert and Lucas C. M. Arruda

- 140** *Conventional and Computational Flow Cytometry Analyses Reveal Sustained Human Intrathymic T Cell Development From Birth Until Puberty*
Marieke Lavaert, Brecht Valcke, Bart Vandekerckhove, Georges Leclercq, Kai Ling Liang and Tom Taghon
- 153** *Administration of Amyloid Precursor Protein Gene Deleted Mouse ESC-Derived Thymic Epithelial Progenitors Attenuates Alzheimer's Pathology*
Jin Zhao, Min Su, Yujun Lin, Haiyan Liu, Zhixu He and Laijun Lai
- 169** *When the Damage is Done: Injury and Repair in Thymus Function*
Sinéad Kinsella and Jarrod A. Dudakov
- 181** *T-Cell Immunodeficiencies With Congenital Alterations of Thymic Development: Genes Implicated and Differential Immunological and Clinical Features*
Giuliana Giardino, Carla Borzacchiello, Martina De Luca, Roberta Romano, Rosaria Prencipe, Emilia Cirillo and Claudio Pignata
- 192** *Thymic Engraftment by in vitro-Derived Progenitor T Cells in Young and Aged Mice*
Jastaranpreet Singh, Mahmood Mohtashami, Graham Anderson and Juan Carlos Zúñiga-Pflücker
- 207** *The Thymus in Chagas Disease: Molecular Interactions Involved in Abnormal T-Cell Migration and Differentiation*
Ana Rosa Pérez, Juliana de Meis, Maria Cecilia Rodriguez-Galan and Wilson Savino



Editorial: New Insights Into Thymic Functions During Stress, Aging, and in Disease Settings

Nicolai S. C. van Oers^{1,2,3*}, Dong-Ming Su⁴, Ann P. Chidgey⁵ and Jarrod Dudakov⁶

¹ Department of Immunology, The University of Texas Southwestern Medical Center, Dallas, TX, United States, ² Department of Pediatrics, The University of Texas Southwestern Medical Center, Dallas, TX, United States, ³ Department of Microbiology, The University of Texas Southwestern Medical Center, Dallas, TX, United States, ⁴ Microbiology, Immunology and Genetics, University of North Texas Health Science Center, Fort Worth, TX, United States, ⁵ Department of Anatomy and Developmental Biology, Monash University, Melbourne, VIC, Australia, ⁶ Fred Hutchinson Cancer Research Center and the University of Washington, Seattle, WA, United States

Keywords: thymus, thymic tissue regeneration, thymic involution, severe combined immunodeficiency, tissue regeneration, FoxN1 gene, thymic epithelial cell (TEC)

Editorial on the Research Topic

New Insights Into Thymic Functions During Stress, Aging, and in Disease Settings

The thymus is a bi-lobed lymphoid organ localized above the heart whose primary function is to foster the development of the T cells of the adaptive immune system. T cells are a critical component of the cellular immune system, helping B cells produce antibodies, releasing cytokines to orchestrate effective immune responses, and killing infected cells and/or neoplasm/tumor cells. The recognition of an infected cell or tumor cell, and the ability to support B cell secretion of antibodies requires T cells express a cell surface receptor, termed the T cell receptor (TCR). This receptor selectively binds to peptides complexed to major histocompatibility complex antigens (MHC class I and II). MHC class I molecules are present on all nucleated cells in the body, while MHC class II is restricted to antigen presenting cells. In the thymus, thymocytes rearrange DNA loci comprising the genes encoding the TCR subunits. Using a process termed VDJ recombination, each thymocyte expresses a unique TCR with exclusive recognition specificities. However, only those thymocytes expressing TCRs that can engage self-peptides/self MHC complexes present on thymic epithelial cells (TECs) are “selected” to form the peripheral T cell repertoire. This involves processes coined positive and negative selection, with the ensuing repertoire of T cells capable of recognizing pathogen- or distinct tumor- derived peptides presented on self-MHC molecules without overt reactivity to self-peptides. The critical role of the thymus in this process is best exemplified with infants born with mutations in genes required for the formation of the thymus (22q11.2 deletion syndrome or DiGeorge). No thymus results in no T cells, leading to a severe combined immunodeficiency. A second excellent example pertains to the spontaneously arising nude mouse, which lacks a thymus and hair due to mutations in the *Forkhead Box N1* transcription factor. This transcription factor supports the development of TECs, the controllers of T cell fate, and defective TECs means no T cells.

Yet, as late as 1960, the thymus was a scientific conundrum. Dramatic variations in its size in humans and diverse species had been documented for over two millennia. Severe clinical repercussions based on these size differences ensued due to a complete lack of knowledge about its functions, which was not revealed until demonstrated in pivotal experiments performed by Jacques Miller using thymectomy and skin grafting. These experiments established the thymus as the site of T cell development. For this, Miller was awarded the Lasker Prize in Biomedicine in 1919 (1). Between the 1890s–1950s, an enlarged thymic tissue noted in newborns and infants was proposed causal to asthma and crib death (thymicolymphaticus) (2). This unsubstantiated pathological connection led to tens of thousands of infants receiving “therapeutic”

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National Institutes of Health (NIH),
United States

*Correspondence:

Nicolai S. C. van Oers
nicolai.vanoers@utsouthwestern.edu

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radiation treatments of 0.2–2 Gy to wipe out the thymus (3). Such an ill-conceived therapy resulted in >10,000 children developing thyroid cancers. A subsequent and less damaging treatment to reduce thymus size involved steroid injections. While not as severely marred as with the high radiation doses, the thymus could again be rendered hypoplastic. These therapies have revealed the extreme stress sensitivity of the thymus, but also its remarkable capacity for repair. In a normal setting, thymic function reaches its natural peak during the neonatal and pre-adolescent period, thereafter the thymus begins to decrease in size and activity. This process, termed thymic involution, results in significant loss in the production of T cells although some T cell development continues throughout adult life. This dynamic process, both with chronic age-related declines and acute atrophy caused by steroids or cytoreductive chemotherapy, can have profound negative impacts in the efficacy of adaptive immunity. Today, understanding how to maintain the function of the thymus throughout life, particularly after damage, is the driving goal of researchers.

In the series of articles under the title “New Insights into thymic functions during stress, aging, and in disease settings” in this issue of *Frontiers in Immunology*, novel insights and findings by leading scientists in the field of thymus biology are presented. The articles include two reviews outlining recent discoveries into the mechanisms required for the formation and specification of the thymus within the 3rd pharyngeal pouch during embryogenesis (Bhalla et al.; Giardino et al.). The contributions of key transcription factors such as *T Box Transcription Factor 1 (TBX1)* and *FOXP1* in the patterning of the pharyngeal apparatus and the subsequent demarcation of the thymus anlage are discussed in conjunction with the differentiation and expansion of thymic epithelial cells. The formation of the two different populations of epithelial cells, cortical and medullary TECs and their selective roles in supporting and educating thymocytes to form the repertoire of T cells, which is unique to each individual, is described, revealing new subsets of these cells in the process (Alawam et al.).

As is well-recognized currently, in addition to aging, the thymus undergoes a transient and sometimes massive reduction in overall size and cellularity following infections, corticosteroid production through the hypothalamus-pituitary-adrenal axis or via injections, and chemotherapy treatments. The impact on thymocytes and stromal cell populations are presented herein (Gaballa et al.; Kinsella and Dudakov). Such thymic hypoplasia is widespread in the population, and the different conditions leading to hypoplasia/aplasia of the thymus are presented in a combination of research and review articles in this series (Cowan et al.; Gaballa et al.; Pérez et al.;

Silva-Freitas et al.) For example, thymic hypoplasia is evident in pregnant women and infants born prematurely. The natural aging process causes a gradual and “permanent” hypoplasia of the thymus and this involves both loss of developing T cells and TEC subsets, with a greater impact evident in males than females, at least until middle-age (Hun et al.). The epithelial tissue within the thymus undergoes adipogenesis and epithelial to mesenchymal transitions (Lavaert et al.). Given these varied and damaging processes, improving and/or restoring thymopoiesis, from infants to the elderly, requires new therapeutic strategies. Several innovative approaches covered in this series include use of pro-T cells to improve T cell formation in the thymus (Singh et al.) and the use of specific cytokines to restore TEC functions (Kinsella and Dudakov). Humanized mouse models and comparative mouse-human studies offer additional understanding and methodologies for improving thymus functions (Machado et al.; Tong et al.).

The emergence of checkpoint blockade immunotherapy to treat cancer patients has consequences as well, since the incidence of autoimmune complications with such a patient cohort increases dramatically due to the antibody treatments (Wang et al.). While the thymus is usually considered in the context of T cell development, there is evidence of some B cell development in normal and clinical settings. Two pronounced conditions, the autoimmune diseases, Systemic Lupus Erythematosus and Myasthenia Gravis, have substantial levels of B cell development/expansion (Hidalgo et al.). In the case of Myasthenia Gravis, thymectomy is actually done for patients to eliminate the autoreactive B cells in the thymus, improving their clinical outcomes. Alzheimer's Disease is another clinical condition where mouse models have established that intrathymic injections of TECs, derived from stem cells, can actually improve cognitive functions (Zhao et al.). In summary, we have come a long way from purposely destroying the thymus to focusing on its restoration and regeneration. These are nicely collated in our *Frontiers in Immunology* Topic.

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All authors participated in the editorial reviews of the manuscripts. All authors participated in the writing and editing of the editorial article.

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Gender Disparity Impacts on Thymus Aging and LHRH Receptor Antagonist-Induced Thymic Reconstitution Following Chemotherapeutic Damage

Michael Ly Hun^{1†}, Kahlia Wong^{1†}, Josephine Rahma Gunawan^{1†}, Abdulaziz Alsharif^{1†}, Kylie Quinn² and Ann P. Chidgey^{1*}

¹ Thymus Development, Ageing and T Cell Regeneration Laboratory, Department of Anatomy and Developmental Biology, Biomedicine Discovery Institute, Monash University Clayton, Melbourne, VIC, Australia, ² Quinn Laboratory, Translational Immunology and Nanotechnology Research Program, School of Health and Biomedical Research, RMIT University, Melbourne, VIC, Australia

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Edited by:

Avinash Bhandoola,
National Institutes of Health (NIH),
United States

Reviewed by:

Claude Perreault,
Université de Montréal, Canada
Graham Anderson,
University of Birmingham,
United Kingdom

*Correspondence:

Ann P. Chidgey
ann.chidgey@monash.edu

[†]These authors have contributed
equally to this work

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One of the main consequences of thymus aging is the decrease in naïve T cell output. This condition accelerates at the onset of puberty, and presents as a major clinical complication for cancer patients who require cytotoxic therapy. Specifically, the extensive use of chemotherapeutics, such as cyclophosphamide, in such treatments damage thymic structure and eliminate the existing naïve T cell repertoire. The resulting immunodeficiency can lead to increased incidence of opportunistic infections, tumor growth relapse and/or autoimmune diseases, particularly in older patients. Thus, strategies aimed at rejuvenating the aged thymus following chemotherapeutic damage are required. Previous studies have revealed that sex hormone deprivation in male mice is capable of regenerating the thymic microenvironment following chemotherapy treatment, however, further investigation is crucial to identify gender-based differences, and the molecular mechanisms involved during thymus regeneration. Through phenotypic analyses, we identified gender-specific alterations in thymocytes and thymic epithelial cell (TEC) subsets from the onset of puberty. By middle-age, females presented with a higher number of thymocytes in comparison to males, yet a decrease in their Aire⁺ medullary TEC/thymocyte ratio was observed. This reduction could be associated with an increased risk of autoimmune disease in middle-aged women. Given the concurrent increase in female Aire⁺ cTEC/thymocyte ratio, we proposed that there may be an impediment in Aire⁺ mTEC^{hi} differentiation, and Aire⁺ cTEC^{hi} as its upstream precursor. The regenerative effects of LHRH receptor antagonist, degarelix, on TEC subsets was also less pronounced in middle-aged females compared to males, possibly due to slower progression of thymic involution in the former, which presented with greater TEC^{hi} proportions. Furthermore, following cyclophosphamide treatment, degarelix enhanced thymocyte and mature TEC subset recovery, with faster recovery kinetics observed in females. These events were found to involve both reactivation and proliferation of thymic epithelial progenitor cells. Taken together, the findings from this study portray

a relationship between gender disparity and thymus aging, and highlight the potential benefits of LHRH receptor antagonist treatment for thymic regeneration. Further research is required, however, to determine how gender may impact on the mechanisms underpinning these events.

Keywords: thymus, thymic epithelial cell, aging, gender, sex hormone deprivation, luteinizing hormone-releasing hormone, chemotherapy, regeneration

INTRODUCTION

Systemic chemotherapy regimens are commonly used to eradicate malignant tumors, but do so at the cost of harming other normal rapidly dividing cells. These include cells found within hair follicles, mucous membranes and the hematopoietic compartment. Such treatment regimens result in patient susceptibility to opportunistic infections, a consequence of immunodeficiency from T lymphocyte depletion and delayed repopulation (1). Given the increased risk of morbidity and mortality in aged cancer patients, it is clear that prompt immune reconstitution is paramount.

Cyclophosphamide is an alkylating agent that disrupts DNA replication to prevent cell proliferation (2). This commonly used chemotherapy reagent, like others, causes the thymus to transiently involute (3). In conjunction with the adverse effects of age-related thymic atrophy, the stromal damage induced by chemotherapy acts as an impediment to T cell reconstitution (4). Specifically, it has been shown that cyclophosphamide treatment in young male mice depleted the mature autoimmune regulator (Aire)⁺ medullary (m) thymic epithelial cell (TEC) population, which is important for central tolerance (5). This event presents the possibility of autoimmune reactive T cell escape (6). Furthermore, within the first couple of years following chemotherapy, peripheral T cell recovery in adult patients has been shown to result from clonal expansion of resistant memory T cells, rather than repopulating naïve T cells, that were later found to be susceptible to apoptosis (7).

Age-related thymic atrophy influences the kinetics of naïve T cell recovery following cytoablative damage. This degenerative process impedes naïve T cell output, with the functional epithelial compartment of the thymus progressively replaced by adipose (8, 9). These effects are most pronounced from the onset of puberty, where the increased production of sex steroids and sudden drop in thymocyte number parallels a proportional loss of mature mTECs (10). Moreover, the direct effects of sex hormone receptor activation has been demonstrated, with a sudden decline in TEC number and deterioration of naïve T cell output observed following testosterone treatment (11, 12).

Despite the importance of mature mTECs for self-tolerance induction, their age-related decline is less consequential during healthy normal life. Postnatal, single lineage mTEC progenitors can somewhat maintain the mature mTEC compartment (13), whilst Foxp3⁺ T regulatory cells compensate for the escape of potentially autoreactive T cells (14). However, following thymic damage and peripheral T cell loss, such as from multiple-dose chemotherapy and irradiation regimens, older patients with an

atrophied thymus have limited capacity to regenerate naïve T cells. This results in a severely restricted range of antigen specificities in the patient's T cell repertoire, and hence increased morbidity and mortality. Thus, *de novo* reconstitution of a diverse naïve T cell pool would be optimal for robust and sustained immune recovery.

The increased quiescence of a bipotent thymic epithelial progenitor cell (TEPC) subpopulation from the onset of puberty (10) possibly underlies the greater reliance on single lineage mTEC^{lo} precursors to maintain the postnatal mature mTEC^{hi} compartment (13). It is therefore possible that thymus recovery can potentially be achieved through the reactivation of adult TEPCs (15–18). Moreover, the age-induced alterations in thymic follistatin (Fst) and bone morphogenetic protein 4 (Bmp4) production have been proposed to play a role in the reduced differentiation of thymic epithelial progenitors into their downstream mature TEC counterparts via inhibition of activin A signaling (10). As such, modulation of this and other signaling cascades may also prove beneficial to ultimately achieve T cell reconstitution.

Sex hormone deprivation (SHD) via reversible chemical castration has been considered as a possible clinical strategy for thymus regeneration in immunodeficient patients (12). The most notable agents for chemical castration are gonadotropin-releasing hormone (GnRH) or luteinizing hormone releasing hormone (LHRH) analogs. These agents are commonly used in the treatment of breast cancer and metastatic prostate cancer, with clinical studies assessing immune regeneration in prostate cancer patients demonstrating improved T cell responses (19–21). In many pre-clinical studies, SHD has been shown to transiently reverse immune aging, rejuvenating both the B and T lymphocyte arms of adaptive immunity (11, 19, 22–26). A numerical increase in bone marrow-derived early thymic progenitors (ETPs), B lymphocytes and lineage-negative Sca-1⁺ c-kit⁺ (LSK) cells was observed with SHD following chemotherapy and hematopoietic stem cell transplantation. Furthermore, SHD via LHRH receptor agonist treatment has been shown to significantly augment thymic recovery (22, 27), with LHRH receptor antagonism promoting thymopoiesis more rapidly than their agonist counterparts, and facilitating increased *Delta-like 4* (*Dll4*) expression in cortical (c) TECs (12). Since LHRH receptor antagonists circumvent the initial spike in sex steroid production caused by LHRH receptor agonists, which may cause further thymic damage, they represent a potentially superior approach when considering immune regeneration. Whether or not LHRH analog-induced regeneration of the aged thymus provides adequate self-tolerance mechanisms has been questioned (28), however, a more recent publication suggests

regeneration of the mTEC compartment does occur at least in the middle-aged thymus (10). Moreover, the majority of pre-clinical research into thymic involution and sex steroid inhibition-induced thymic reactivation has been conducted in male mice. It is hence clear that further investigation is required to identify whether gender disparity exists in the loss of TEC compartments during aging and to determine whether similar mechanisms govern thymus regeneration in females.

Here, we build upon our previous research to investigate the role of postnatal bipotent TEPCs in thymus aging and damage recovery (10, 18). Specifically, we examine middle-aged female and male mice for associations between sexual dimorphism and TEC loss with aging, endogenous TEC regeneration following single dose cyclophosphamide treatment, and potential for enhanced thymic restoration following administration of the LHRH receptor antagonist, degarelix.

MATERIALS AND METHODS

Animals

C57BL/6J mice (pre-pubertal, 4-week-old; post-pubertal, 7-week-old; and middle-aged, 7–12-month-old) were obtained from Monash Animal Research Platform and housed at Animal Research Laboratory (Monash University, Australia). Mice were maintained in a controlled environment with a standard diet and water *ad libitum*. All experiments were conducted according to Australian National Health and Medical Research Council Guidelines of Animals Used for Scientific Purposes (2008), and were approved by Monash University Animal Ethics Committee (SOBSA/ADB/2015/039).

LHRH Receptor Antagonist and Chemotherapy Treatment

For all experiments, the final day of treatment was designated as day 0 (D0). Mice were treated with degarelix (Firmagon®; LHRH receptor-antagonist) at a dose of 78 µg/g, injected subcutaneously 48 h prior (D-2) to allow time for estrogen and testosterone production to reach castrate levels by D0. Cyclophosphamide (Endoxan®) in mouse tonicity phosphate-buffered saline (PBS) was injected intraperitoneally over two consecutive days at 0.1 mg/g/day (D-1 and D0) to simulate its clinical application. Mice were subsequently analyzed following euthanasia through CO₂ asphyxiation at indicated time points.

Enzymatic Digestion of Thymic Tissue

Following thoracotomy, thymi were collected in RPMI medium 1640 (Gibco, U.S.A.), and each thymus cleaned of connective tissue. Thymi were then snipped with fine scissors, and enzymatically digested using 0.02% (w/v) DNase I and 0.0185% (w/v) Liberase Thermolysin Medium (Roche, Germany) in RPMI medium 1640, for 15 min at 37°C (29). Thymic fragments were gently agitated using a wide-bore pipette tip and allowed to settle before collection of the supernatant. Fluorescence-activated cell sorting (FACS) buffer (0.1% BSA and 5 mM EDTA in PBS), was added to neutralize enzymatic activity. The remaining fragments were then digested with fresh enzyme, and the cycle repeated until completion. Smaller pipette tips were used for agitation as

digestion progressed. Lastly, filtration was performed on pooled thymic fractions through a 100 µm nylon mesh, followed by centrifugation at 500 *g*_{max} for 3 min at 4°C. Cell pellets were resuspended in FACS buffer, and cell counts acquired using a Z2 Coulter Counter (Beckman Coulter, U.S.A.).

Preparation of Lymph Nodes for T Cell Analysis

Lymph nodes (bilateral brachial and inguinal) were dissected and mechanically digested using two frosted glass slides in FACS buffer to create a single cell suspension. Cells were filtered through a 100 µm nylon mesh before counting using a Z2 Coulter Counter. A Z2 Coulter Counter was then used to determine total and viable cell numbers prior to immunostaining for flow cytometric analysis.

Flow Cytometric Analysis

Cells were resuspended in primary antibody cocktail at a concentration of 1×10^6 cells per 10 µl (minimum 20 µl), and incubated in the absence of light for 15 min at 4°C. Unbound antibodies were removed by washing with FACS buffer. Following centrifugation at 500 *g*_{max} for 3 min, cell pellets were stained with secondary antibody (where appropriate) for 15 min at 4°C. Stained samples were washed and resuspended in FACS buffer after centrifugation, then filtered into round-bottom polystyrene tubes. Lastly, propidium iodide (PI; Sigma Aldrich, U.S.A.) was added into each sample tube to exclude dead cells for live stain analyzes.

Intracellular staining was performed to identify cell proliferation (Ki-67), Aire, and Foxp3 expression in TEC and/or T cell subpopulations. Cells previously stained with extracellular markers were fixed using Cytofix™ buffer (eBioscience, U.S.A.) for 30 min at 4°C, according to the manufacturer's instructions. Samples were subsequently washed with Perm-wash buffer (BD Biosciences, U.S.A.), centrifuged at 500 *g*_{max} for 3 min, and stained with intracellular antibodies or their isotype controls for 30 min at 4°C. Stained cells were washed, resuspended in FACS buffer following centrifugation, and transferred into round-bottom tubes for flow cytometric analysis (29).

Stained cell samples were acquired using a BD FACSCanto™ II flow cytometer (BD Bioscience, U.S.A.). Parameter, voltage and compensation settings were established using BD FACSDiva v.6 software (BD Bioscience, U.S.A.). Data were analyzed using FlowLogic™ v700.1A (Inivai Technologies, Australia).

Antibodies used for immunofluorescent staining are listed below (Table 1).

Thymic Stromal Cell Isolation

To enrich for CD45⁺ thymic stromal cells, anti-mouse CD45 MicroBeads (Miltenyi Biotec, Germany) were added to pooled thymic digests (5 µl beads + 95 µl FACS buffer per 1×10^7 cells) and gently rotated for 20 min at 4°C. Samples were washed with FACS buffer and centrifuged at 300 *g*_{max} for 10 min to remove unbound magnetic beads, then resuspended in FACS buffer at a concentration of 0.5×10^8 cells/ml. Isolation of CD45⁺ fractions was achieved using the “Deplete” function of an AutoMACS Pro Separator (Miltenyi Biotec, Germany). The

TABLE 1 | Antibodies utilized for immunofluorescent staining—flow cytometric analysis.

Antibody	Marker	Host/isotype/clone	Supplier
CD45	Thymocyte	Rat IgG2b, κ 30-F11	BD Biosciences, U.S.A.
TER119	Erythrocyte	Rat IgG2b, κ TER-119	BD Biosciences, U.S.A.
EpCAM	Epithelial	Rat IgG2a, κ G8.8	Biologend, U.S.A.
UEA-1	Medullary TEC	Lectin	Vector Labs, U.S.A.
Ly-51	Cortical TEC	Rat IgG2a, κ 6C3	BD Biosciences, U.S.A.
MHCII	TEC maturity	Rat IgG2b, κ M5/114.15.2	Biologend, U.S.A.
α 6-integrin	TEPC	Rat IgG2a, κ GoH3	Biologend, U.S.A.
Sca-1	TEPC	Rat IgG2a, κ D7	eBioscience, U.S.A.
Ki-67	Proliferation	Mouse IgG1, κ B56	BD Biosciences, U.S.A.
Aire	Autoimmune regulator	Rat IgG2c, κ 5H12	Invitrogen, U.S.A.
Streptavidin	Biotin	Protein	BD Biosciences, U.S.A.
CD44	Activated T-cell	Rat IgG2b, κ IM7	eBioscience, U.S.A.
CD25		Rat OFA IgG1, λ PC61	BD Biosciences, U.S.A.
CD117		Rat IgG2b, κ 2B8	eBioscience, U.S.A.
CD3e	Thymocyte	Ar hamster IgG1, κ 145-2C11	BD Biosciences, U.S.A.
CD4	Helper T-cell	Rat IgG2b, κ RM4-5	BD Biosciences, U.S.A.
CD8	cytotoxic T-cell	Rat LOU IgG2a, κ 53-6.7	BD Biosciences, U.S.A.
CD19		Rat LEW IgG2a, κ 1D3	BD Biosciences, U.S.A.
CD11b		Rat IgG2b, κ M1/70	BD Biosciences, U.S.A.
CD11c		Ar Hamster IgG1, λ 2 HL3	BD Biosciences, U.S.A.
Gr-1/Ly-6G		Rat IgG2b, κ RB6-8C5	BD Biosciences, U.S.A.
CD45R/B220	B-cell	Rat IgG2a, κ RA3-6B2	BD Biosciences, U.S.A.
NK1.1		Mouse IgG2a, κ PK136	BD Biosciences, U.S.A.
H-2kb		Mouse BALB/c IgG2a, κ AF6-88.5	BD Biosciences, U.S.A.
CD62L	Naïve T-cell	Rat IgG2a, κ MEL-14	eBioscience, U.S.A.
Foxp3		Rat IgG2a, κ FJK-16s	eBioscience, U.S.A.
TCR β	T-cell	Ar Hamster IgG2, λ 1 H57-597	BD Biosciences, U.S.A.
CD49d		Rat IgG2b, κ R1-2	Biologend, U.S.A.
CD122		Rat SD IgG2b, κ TM- β 1	BD Biosciences, U.S.A.

purified thymic stromal cells were then incubated in RBC lysis buffer for 2 min at 37°C and resuspended in FACS buffer for immunofluorescent staining (as described in Thymic Stromal Cell Isolation). Stained CD45⁺ thymic stromal cell subsets were subsequently sorted with a BD InfluxTM I cell sorter (BD Bioscience, U.S.A.) at FlowCore (Monash University, Australia). Sorted cells were collected in RPMI medium 1640 containing 30% (v/v) fetal bovine serum (FBS) (29).

TABLE 2 | Antibodies utilized for immunofluorescent staining—immunocytochemistry.

Antibody	Marker	Host/isotype/clone	Supplier
β 5t	Cortical	Rabbit IgG	MBL International, U.S.A.
Keratin-14	Medullary TEC	Chicken IgY, Poly9060	Biologend, U.S.A.

3D TEPC Cultures

Purified TEPCs ($\sim 1 \times 10^4$) acquired from cell sorting were co-cultured with 2×10^5 irradiated mouse embryonic fibroblasts (MEFs) in 50% growth-factor-reduced Matrigel[®] (BD Biosciences, U.S.A.), placed into 24-well 0.4 μ m transwell inserts (Millipore, Merck, U.S.A.) and incubated with TEC media, as previously described (18). Media was changed every 48 h. Following a seven-day incubation at 37°C in a hypoxic environment (5% O₂, 10% CO₂) (30), colony number was determined using an optical (Zeiss Primo Vert, Germany) or multicolor confocal (Leica DMI8, Germany; Monash Micro Imaging) microscope. Colony forming efficiency (CFE%) was determined by dividing the number of colonies formed by the number of seeded TEPCs $\times$ 100%.

Immunocytochemistry of 3D Cultured TEPC Colonies

Transwell inserts were washed twice with PBS and fixed with 4% (w/v) paraformaldehyde (PFA) in PBS for 20 min at room temperature (RT). Antigen retrieval was performed by submerging inserts in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween 20, pH 6.0 in PBS) for 30 min at 95°C. Samples were then washed with washing buffer (0.1% Triton-X in PBS) for 2 h, and blocked with 1% BSA in washing buffer for another hour at RT. Colonies were stained with primary and secondary antibody cocktails (Table 2) for 2 h each at RT in the dark; samples were washed twice (5 min each) with washing buffer in between primary and secondary steps. Lastly, nuclear staining with DAPI was performed for 15 min, followed by two further washes. Transwell insert membranes were cut with a scalpel and placed on a coverslip. The membrane was gently removed from the stained colonies before application of a glass slide with fluorescence mounting media (Dako[®], U.S.A.). Images were acquired using a confocal fluorescence microscope (Nikon Eclipse Ti, U.S.A.; Monash Micro Imaging) and analyzed with Fiji-ImageJ software v.2.0.0.

RT-qPCR

The RNAqueousTM Micro Kit (Ambion, U.S.A.) was used to isolate total RNA as per manufacturer's instructions. Briefly, FACS-purified CD45⁺ thymic stromal cell subsets were lysed immediately after sorting (RNAqueousTM Lysis Solution). The cell lysate was mixed with 100% ethanol, and transferred into a silica-based filter which binds RNA. Total RNA was extracted following column purification, sample elution, and DNase treatment, with its concentration and purity measured using a NanoDrop (ThermoFisher, U.S.A.). First-strand cDNA synthesis was achieved using the Superscript III reverse transcriptase kit (Invitrogen, U.S.A.). Total RNA was denatured in conjunction

with oligo(dT)₂₀ (50 μ M) primer and dNTP mix for 5 min at 65°C. Samples were immediately placed on ice for 2 min to allow for primer-RNA adherence. The Superscript III reverse transcriptase master mix was added and the reactions run for 50 min at 50°C. Following inactivation for 5 min at 85°C, RNase H was added for 20 min at 37°C, to remove mRNA whilst leaving cDNA template for subsequent RT-qPCR.

RT-qPCR reactions were performed using SYBR Green Supermix-UDG (Invitrogen, Australia), pre-validated primer sequences (Fst and Bmp4; Qiagen, Germany) and cDNA template, in a Corbett Rotor-Gene 3000 (Corbett Research, Australia). The expression of target genes was then analyzed with Rotor-Gene software version 6.1 (Qiagen, U.S.A), relative to GAPDH using the 2 $\Delta\Delta$ Ct method.

Serum Analysis for FSH and LH

Whole blood samples were withdrawn via cardiac puncture with thoracotomy using a 26G needle, and collected in a 1.5 ml microcentrifuge tube (31). This procedure was performed immediately after euthanasia. Blood samples were allowed to clot for at least 30 min at RT, then centrifuged at 1,000–2,000 g for 10 min at 4°C. The supernatant (blood sera) was transferred into a new microcentrifuge tube and stored at –80°C. Follicular stimulating hormone (FSH) and luteinizing hormone (LH) levels were later examined via radioimmunoassay.

Statistical Analysis

All data were analyzed using GraphPad Prism v7.0 software. Independent One- or Two-Way ANOVA tests were run, with the appropriate *post-hoc* *t*-test performed for parametric tests. A *p*-value of <0.05 was considered statistically significant. Results are presented as mean + SEM, unless otherwise specified.

RESULTS

Phenotypic Differences in Female and Male TEC Subsets With Aging

Comparative immunophenotypic analyzes of TEC subpopulations were performed in 4-week (pre-pubertal), 7-week (post-pubertal), and 8-month-old (middle-aged) C57BL/6J mice, to examine for age- and gender-related differences. Using multiparameter flow cytometry, thymocytes (CD45⁺ EpCAM[–]) and TECs (CD45[–] EpCAM⁺) were divided after gating on viable (PI[–]) cells (**Figure 1A**). In addition, MHCII was used to broadly divide TECs into TEC^{lo} and mature (TEC^{hi}) subpopulations, with cortical (cTEC, UEA-1[–]) and medullary (mTEC, UEA-1⁺) TECs separated by UEA-1 (29). The proliferative capacity of TEC subsets was also assessed using Ki-67.

Total thymocyte and TEC numbers were found to be similar between 4-week-old female and male mice (**Figures 1B,C**). By 7-weeks, a drastic decline in thymocytes was observed in both females and males, with males demonstrating a further reduction by 8-months in contrast to female thymocytes which did not decrease significantly. Reduction in overall TEC number was not evident until middle-age. However, proportional differences in female and male TEC subsets were evident from 7-weeks of age (**Figure 1D**).

A recent study has demonstrated the vast numerical underestimation of TEC subsets associated with enzymatic digestion (32); therefore, we assessed the changes in TEC subsets based on proportional alterations. Gender-associated phenotypic divergence was observed in all age groups, with females exhibiting greater overall cTEC^{hi} and mTEC^{hi} proportions compared to males by middle-age (**Figure 1E**). In contrast, females accumulated less cTEC^{lo} compared to males by 8-months and males maintained a higher proportion of mTEC^{lo} throughout aging. These findings suggest overall better maintenance of TEC differentiation in females during aging and implicate a greater impediment in mTEC^{lo} to mTEC^{hi} differentiation in males compared to females by middle-age.

Analysis of proliferative capacity in TEC subsets revealed a significant reduction in the proportion of Ki-67⁺ cTEC^{lo} by 8-months in females, with a transient decrease observed in males at 7-weeks (**Figure 1F**). Female cTEC^{hi} presented with substantially more proliferating cells in comparison to males across all ages which, in addition to better maintenance of cTEC^{lo} to cTEC^{hi} differentiation, may contribute to the higher proportion of cTEC^{hi} evident in females. A decline in proliferation was only evident by middle-age rather than 7-weeks found in males. Furthermore, a transient reduction in mTEC^{lo} proliferation was seen in post-pubertal males. No significant changes in Ki-67⁺ mTEC^{hi} proportion were found with age for both genders. Collectively, these findings indicate gender-specific proliferative patterns that may be impacted by the differential influences of androgens and estrogens.

Since it has been well established that medullary expression of Aire is essential for robust central tolerance [reviewed in (14)], we therefore investigated the impact of aging and gender on Aire⁺ mTECs (**Figures 1G,H**). A proportional and numerical reduction was evident between 4- and 7-weeks of age in female and male mice, albeit not significant numerically in females until 8-months. By middle-age, females were found to have a significantly higher proportion of Aire⁺ mTECs than males despite the absence of numerical differences. Given the latter observation, it is clear that there is a reduced Aire⁺ mTEC/thymocyte ratio in middle-aged female mice, which may potentially contribute to reduced efficiency of self-tolerance mechanisms. Since a small population of cTEC^{hi} marked by Ly51 and β 5t but not K14 or UEA1, expresses intermediate levels of Aire (33), we also analyzed Aire⁺ cTECs (**Figures 1I,J**), and revealed a similar proportional decline as Aire⁺ mTECs with age and gender, however, no numerical reduction in females was observed. The reduction in male Aire⁺ cTEC number was only significant at 8-months. The concurrent increase in Aire⁺ cTEC/thymocyte ratio of middle-aged female mice with decrease in Aire⁺ mTEC/thymocyte ratio suggests a relationship may exist between these Aire⁺ TEC subpopulations.

Gender Differences in TEPC Maintenance and Differentiation With Aging

We have previously demonstrated the existence and function of bipotent postnatal TEPCs in the cTEC^{lo} compartment (10, 18). Given the gender-based differences in cTEC^{lo} and other TEC subsets, we conducted in-depth analyzes of the progenitors within, based on Sca-1 and α 6-integrin (α 6) expression.

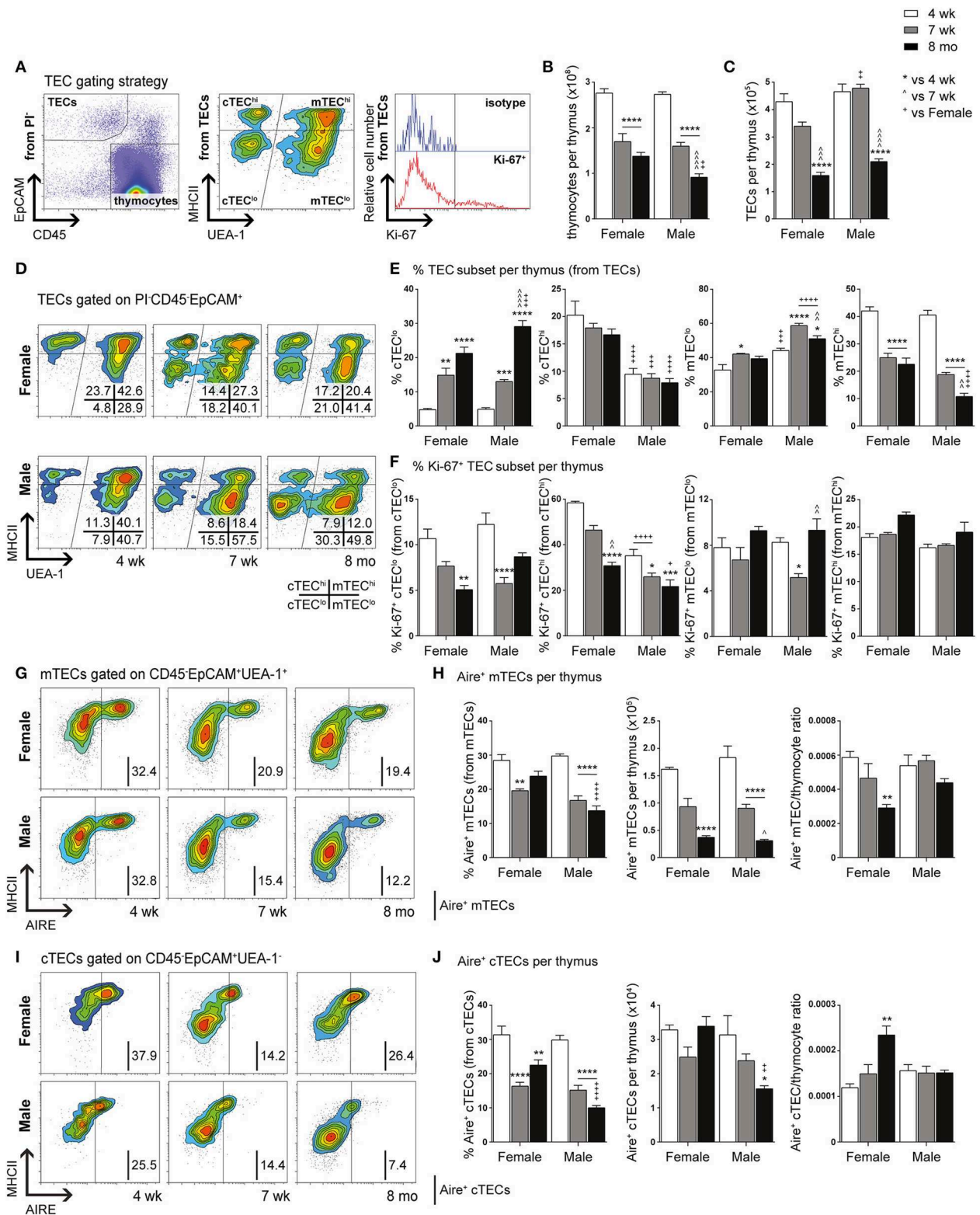


FIGURE 1 | Phenotypic analysis of TEC subsets in female and male mice during aging. **(A)** Gating strategy identifying thymocytes and TECs in 4-week-, 7-week-, and 8-month-old female and male C57BL/6J mice. UEA-1 and MHCII were used to further segregate TECs into cTEC^{lo}, cTEC^{hi}, mTEC^{lo}, and mTEC^{hi} subpopulations. Proliferative cells were identified based on fluorescence exceeding Ki-67 isotype levels. **(B)** Total thymocyte cellularity. **(C)** Total TEC cellularity. **(D)** Representative contour plots depicting proportional changes in TEC subsets in relation to gender with aging. **(E)** Proportion of cTEC^{lo}, cTEC^{hi}, mTEC^{lo}, and mTEC^{hi} subpopulations. *(Continued)*

FIGURE 1 | (F) Proportion of Ki-67⁺ cells within TEC subsets. **(G)** Representative contour plots depicting proportional changes in Aire⁺ mTEC subpopulations in relation to gender with aging. **(H)** Proportion and number of Aire⁺ mTECs per thymus, and Aire⁺ mTEC/thymocyte ratio. **(I)** Representative contour plots depicting proportional changes in Aire⁺ cTEC subpopulations in relation to gender with aging. **(J)** Proportion and number of Aire⁺ cTECs per thymus, and Aire⁺ cTEC/thymocyte ratio. Data presented as mean + SEM ($n \geq 3$). * vs. 4 wk, ^ vs. 7 wk, + vs. Female (age matched). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ordinary two-way ANOVA with Tukey's multiple comparisons.

cTEC^{lo} was delineated into $\alpha 6^{\text{hi}}$ Sca-1^{hi} (TEPCs), Sca-1^{int} and Sca-1^{lo} groups, with reduced Sca-1 expression associated with differentiation into single lineage cTEC precursors that generate the Sca-1^{lo} cTEC^{hi} subset (10). FACS analyzes demonstrated similar trends between female and male mice with aging (Figures 2A,B). The proportion of TEPCs increased significantly between 4-weeks and 7-weeks of age in both females and males, with no further change by 8-months. Sca-1^{int} cTEC^{lo} proportions were instead transiently reduced at 7-weeks, with an accumulation in males by 8-months that surpassed females. In contrast, Sca-1^{lo} cTEC^{lo} progressively decreased with age in both genders, with higher proportions observed in middle-aged females than males, suggesting continued TEPC differentiation through Sca-1^{int} in females. Our proliferative studies revealed an increase in the proportion of Ki-67⁺ TEPCs in males beyond female levels by 8-months (Figure 2C), which may partially explain the higher proportion of cTEC^{lo} observed with aging in males. No significant changes in female Ki-67⁺ TEPC proportions were found. Ki-67⁺Sca-1^{int} cTEC^{lo} proportions also remained unchanged with age. Whilst Ki-67⁺ Sca-1^{lo} cTEC^{lo} was found to decrease by 7-weeks in both genders, middle-aged male proportions later increased to levels that exceeded their female counterparts. Together, these results suggest an accumulation of TEPCs in mice, with gender disparity evident in the levels of proliferation of Sca-1^{lo} cTEC^{lo} progenitors for maintenance of the cTEC^{hi} lineage.

Delineation of the mTEC^{lo} subset into $\alpha 6^{\text{hi}}$ Sca-1^{hi}, Sca-1^{int}, and Sca-1^{lo} groups revealed similar proportional trends to the cTEC^{lo} compartment during aging (Figures 2D,E). We have previously proposed that the $\alpha 6^{\text{hi}}$ Sca-1^{hi} TEPC population differentiates toward either the mature Sca-1^{lo} cTEC^{hi} phenotype, or into $\alpha 6^{\text{hi}}$ Sca-1^{hi} mTEC^{lo} single lineage precursors that eventually give rise to Sca-1^{lo} mTEC^{hi}. The increase in $\alpha 6^{\text{hi}}$ Sca-1^{hi} mTEC^{lo} by 8-months of age was more evident in females, in parallel to a decrease in Sca-1^{int} mTEC^{lo}. Sca-1^{lo} mTEC^{lo} also decreased with age by 8-months in females, but occurred progressively from 7-weeks in males. Proliferative studies revealed an increase in the proportion of Ki-67⁺ $\alpha 6^{\text{hi}}$ Sca-1^{hi} and Sca-1^{int} mTEC^{lo} in females by 8-months (Figure 2F), with the latter potentially acting to support the maintenance of the Sca-1^{int} mTEC^{lo} subpopulation. The proportion of Ki-67⁺ Sca-1^{lo} mTEC^{lo} did not change significantly during aging in either gender. These data collectively implicate an association between medullary precursors and gender disparity, with reduced differentiation of Sca-1^{hi} to Sca-1^{lo} mTEC^{lo} underpinning the loss of Sca-1^{lo} mTEC^{hi} during aging.

Diminution of TEPC Self-Renewal Capacity Following Puberty

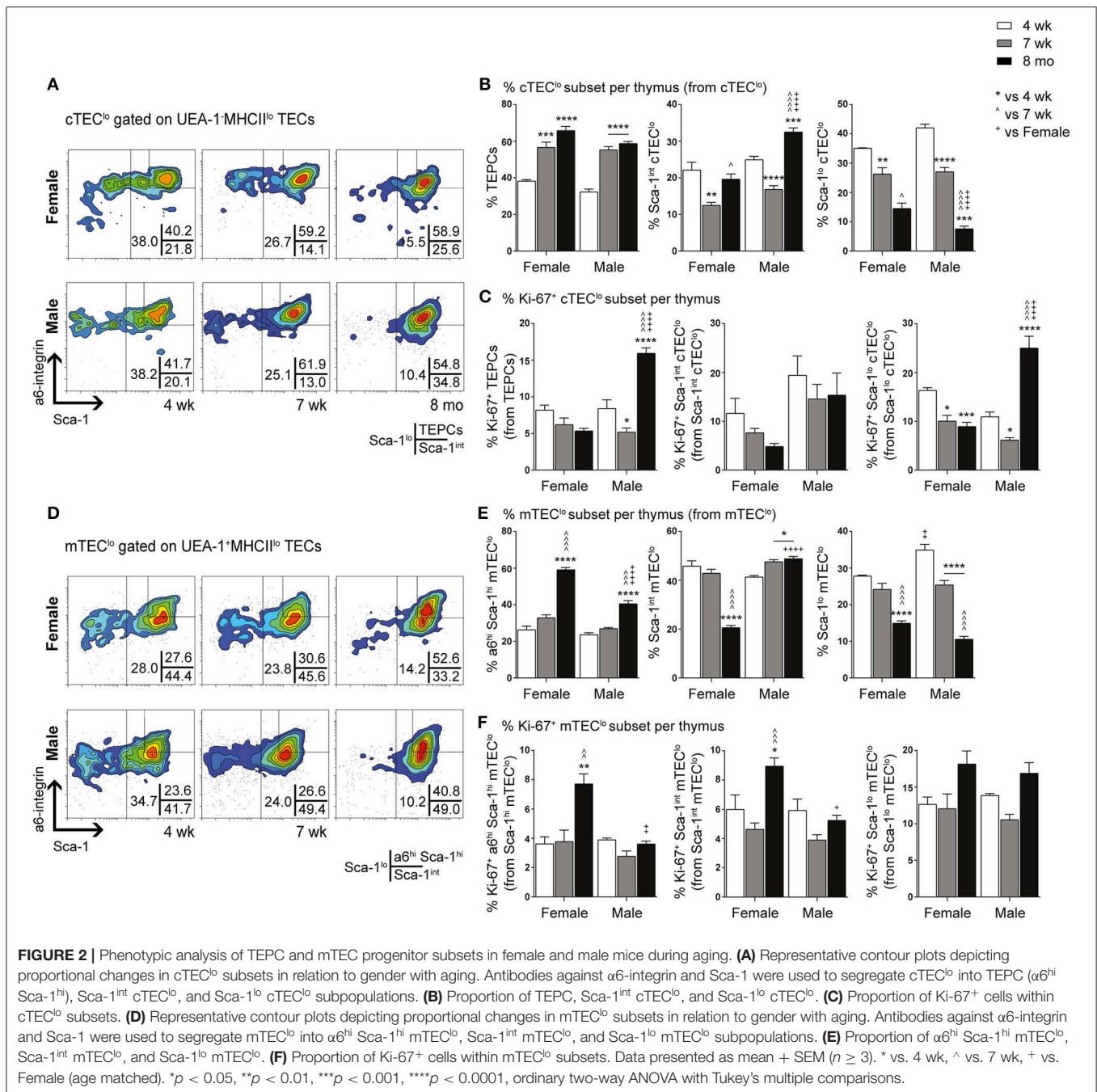
The ability of purified TEPC to self-renew and differentiate into both cTEC and mTEC lineages was investigated through

a previously described *in vitro* 3D clonogenic assay (18). The equivalent Sca-1^{hi} population in mTEC^{lo} has been shown to have no bipotent or clonogenic capacity (17, 18). We assessed the CFE% of female and male TEPCs to determine if their ability to self-renew was lost with age (Figure 3A). Following 3D co-culture of purified TEPCs with MEFs for seven days, we found a decline in CFE% from 4- to 7-week-old TEPCs, with no further reduction thereafter. This suggests attenuation of TEPC function from the onset of puberty in both genders. Gender disparity was not observed, possibly due to TEPC exclusion from their native *in vivo* environment. Immunocytochemistry was also performed on colonies generated from 4-week and 8-month old female and male purified TEPCs (Figures 3B,C), to confirm progenitor bipotency and potential age- and gender-associated differences. Colonies were stained with $\beta 5t$, K14, and DAPI, to identify cortical, medullary and nuclear regions, respectively (10). Although a higher proportion of K14⁺ colonies was observed in 4-week-old female TEPC cultures in comparison to males, no notable difference was seen with age in relation to the prevalence of colonies exhibiting K14 staining (Figure 3B). Several colonies with heterogeneous phenotypes were identified, which were segregated into K14⁺ ($\beta 5t^{+}/K14^{+}$; cTEC), and differentiating K14⁺ ($\beta 5t^{+}/K14^{+}$) groups (Figure 3C). These findings further validate that the $\alpha 6^{\text{hi}}$ Sca-1^{hi} cTEC^{lo} population contains bipotent TEPCs in both genders.

Alterations in the Fst-activinA-Bmp4 axis has previously been shown to be one of the mechanisms underlying thymic involution following puberty in male mice (10). Specifically, an increase in TEC expression of Fst, a potent antagonist of activin A, and non-TEC expression of Bmp4 was evident soon after the onset of puberty. These findings prompted us to investigate whether a similar mechanism occurred in females. Comparison of Fst and Bmp4 expression by RT-qPCR analyzes between 4-weeks (pre-puberty) and 7-weeks (post-puberty) of age, showed a substantial increase in Fst expression in cTEC and mTEC^{lo} subsets, and a 4-fold increase in Bmp4 expression by non-TECs (Figure 3D). These results implicate a similar role of TGF- β superfamily molecules in females and males, which is associated with the loss of TEC differentiation following the onset of puberty.

Gender Disparity in Middle-Aged Mice Following LHRH Receptor Antagonist Treatment

The effect of SHD on thymus regeneration in aging male mice has been widely published (10, 12, 19). The majority of these studies utilized either surgical or chemical castration with a LHRH agonist to block sex steroid production. However, a more recent publication used the LHRH receptor antagonist, degarelix, to rapidly reduce testosterone levels without the initial flare associated with agonists (12). Here, we evaluated the effects of degarelix on TEC regeneration in 8-month-old female and male



mice. Mice were given a subcutaneous injection of degarelix (78 μg/g) 48 h prior, so that sex steroids reached castrate levels by day 0 (D0), and multiparameter flow cytometry analysis of the thymus performed at several time points thereafter (D4, D7, D10, D14, and D28).

Compared to 8-month old untreated (UT) females, a substantial increase in total thymocyte number was evident at D7, D10, and D28 (**Figure 4A**). Eight-month old males instead demonstrated a significant increase in thymocyte number from D10 onwards. An ascending, transient trend in TEC number

was also evident in females following treatment, with a notable difference at D10 before returning to UT levels (**Figure 4B**). In contrast, male TEC numbers were significantly reduced, with recovery to UT levels observed only by D14. This initial TEC loss was surprising since degarelix is described as a LHRH receptor antagonist, and suggests some initial LHRH receptor stimulation in males. Although no statistical difference in the proportions of major TEC subsets was seen in females following degarelix treatment (**Figure 4C**), a gradual decline in male cTEC^{lo} proportions was observed, with mTEC^{hi} proportions

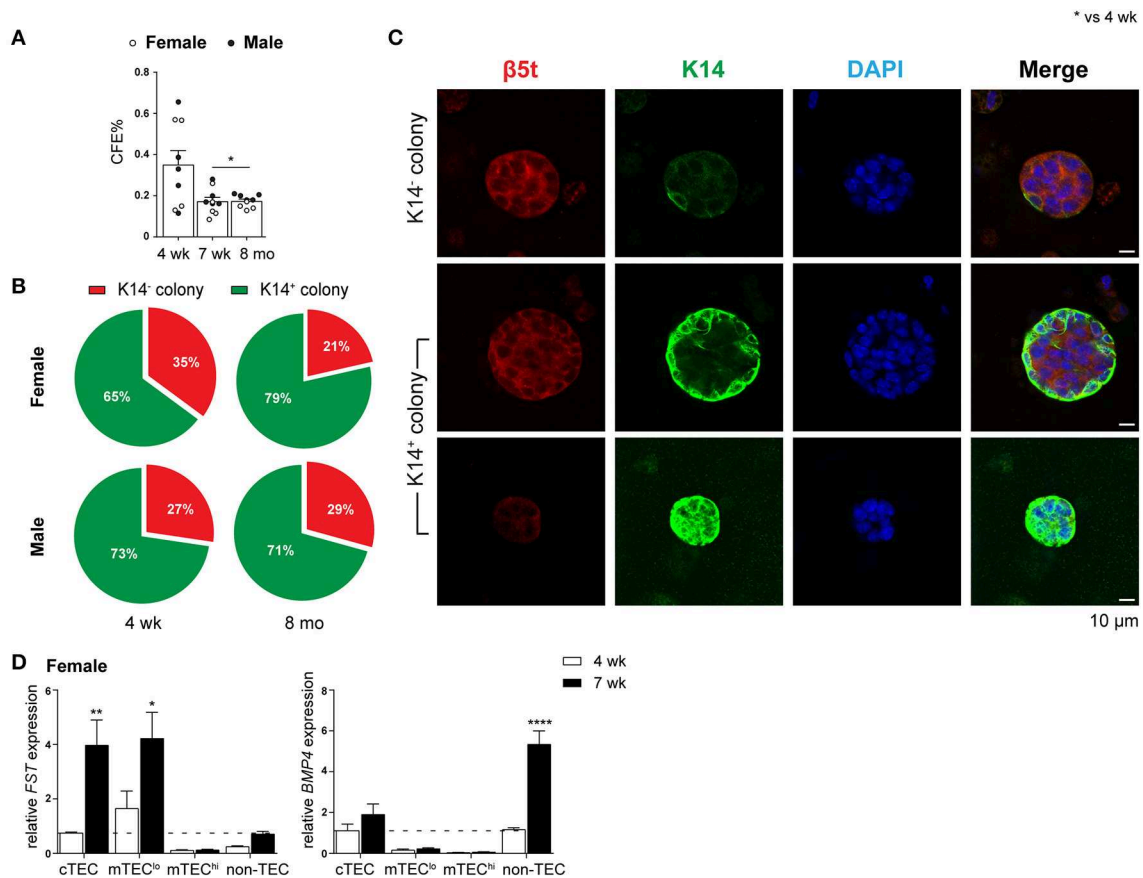


FIGURE 3 | Evaluation of self-renewal and bipotency in female and male TEPC with aging using *in vitro* 3D co-cultures. **(A)** Colony forming efficiency (CFE%) of 4-week-, 7-week-, and 8-month-old TEPCs at D7. Each symbol represents one individual well. **(B)** Proportion of K14⁻ and K14⁺ colonies. **(C)** Representative immunofluorescent images of D7 colonies from 4-week-old female TEPC co-cultures. Cortical (β 5t; red) and medullary regions (K14; green) from K14⁻ and K14⁺ colonies are shown, together with nuclear staining (DAPI; blue). Scale bar = 10 μ m. **(D)** Relative Fst and Bmp4 expression in cTEC, mTEC^{lo}, mTEC^{hi}, and non-TEC subsets from 4-week- and 7-week-old female mice; normalized to 4-week-old cTEC. Data presented as mean + SEM ($n \geq 3$). * vs. 4 wk. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$, ordinary one- or two-way ANOVA with Tukey's multiple comparisons.

surpassing UT levels by D10, following trends seen with surgical castration (10).

We further analyzed the Sca-1⁺ subsets within the cTEC^{lo} population, to investigate whether degarelix treatment triggered TEPC reactivation (Figure 4D). Consistent with reactivation of TEPC, male TEPC proportions were reduced at D4, corresponding with an increase in Sca-1^{int} cTEC^{lo} proportions. This trend was also evident in females, although attenuated and occurred only at D7. Moreover, it aligns with the transient increase in female thymocyte numbers seen at D7 and D10. The Sca-1^{lo} cTEC^{lo} subset did not demonstrate considerable change after degarelix treatment. Analysis of Sca-1⁺ mTEC^{lo} subpopulations revealed a transient reduction in female Sca-1^{lo} mTEC^{lo} at D7, and accumulation of the α 6^{hi} Sca-1^{hi} subset at D28 (Figure 4E). Male Sca-1⁺ mTEC^{lo} subpopulation analyzes revealed a similar profile to the Sca-1⁺ subsets within the cTEC^{lo} population, with a reduction of the α 6^{hi} Sca-1^{hi} subset from D4 and concurrent increase in Sca-1^{int} mTEC^{lo}. Collectively, these findings suggest that although the increase in female thymocyte production occurs in a homeostatic manner with no

major changes in TEC subset proportions, male thymopoiesis and mTEC^{hi} generation is instead enhanced following degarelix treatment conceivably via mobilization of both TEPC and mTEC^{lo} progenitor populations.

SHD-Enhanced Recovery of Thymocytes and TECs Following Cy Damage

The cytoablative effects of chemotherapy on the thymus have been previously investigated in young male mice (5, 34). We report herein, gender-related phenotypic differences in the TEC compartment of middle-aged mice following chemotherapy damage, and examined the extent to which SHD could enhance the kinetics of thymus regeneration in females compared to males.

Eight-month-old mice were chemically castrated with degarelix at D-2, followed by an intraperitoneal injection of cyclophosphamide (Cy) at D-1 and D0 (hereafter referred to as the Cy + Deg treatment group); an identical dose of Cy alone was also administered to a control group (Figure S1A). Thymus cell populations were subsequently analyzed via flow cytometry

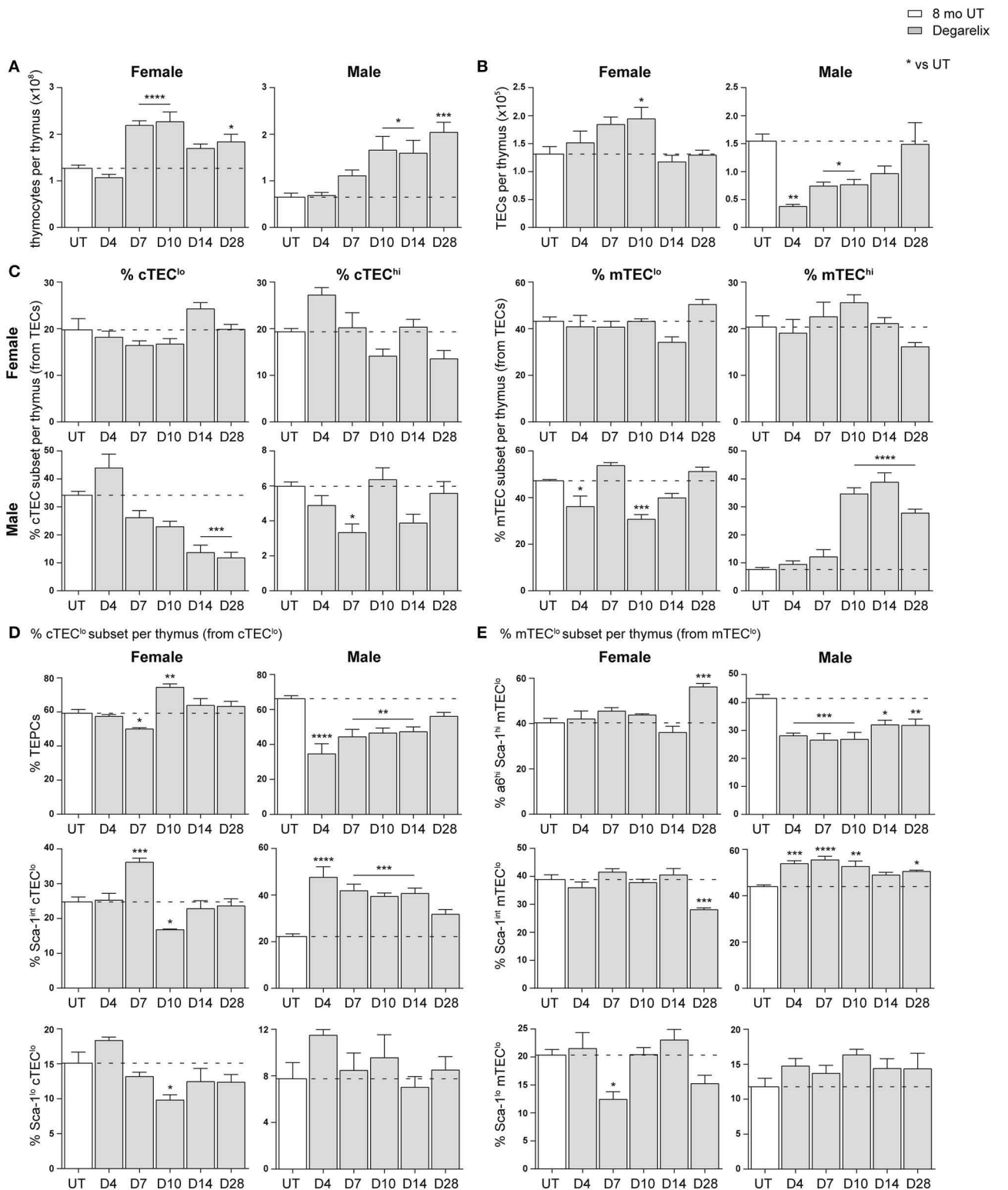


FIGURE 4 | Phenotypic analysis of TEC subsets in middle-aged female and male mice following degarelix treatment. **(A)** Total thymocyte cellularity in 8-month-old female and male mice following degarelix treatment. **(B)** Total TEC cellularity. **(C)** Proportion of cTEC^{lo}, cTEC^{hi}, mTEC^{lo}, and mTEC^{hi} subpopulations. **(D)** Proportion of TEPC, Sca-1^{int} cTEC^{lo}, and Sca-1^{lo} cTEC^{lo}. **(E)** Proportion of αβ^{hi} Sca-1^{hi} mTEC^{lo}, Sca-1^{int} mTEC^{lo}, and Sca-1^{lo} mTEC^{lo}. Data presented as mean + SEM ($n \geq 3$). * vs. UT. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ordinary one-way ANOVA with Dunnett's multiple comparisons.

at D4, D7, D10, D14, and D28 after the last day of Cy injection. Assessment of estrogen and progesterone production was also performed for females at each time point by measuring LH and FSH, respectively (**Figure S1B**). Testosterone levels have been previously shown to reach castrate levels within 24 h of degarelix treatment (12). The fluctuating LH and FSH levels in UT controls suggest a normal oestrous cycle. As expected, degarelix inhibited the secretion of LH and FSH from the anterior pituitary. Serum LH concentrations were depleted to castration levels until at least D28, with an average of $< \sim 0.166$ ng/ml whilst FSH was persistently suppressed at an average of ~ 1.7 ng/ml across all time points.

A dramatic reduction in thymocyte number was evident from D4 in both female and male Cy groups compared to UT controls (**Figure 5A**). Endogenous regeneration to UT levels in this group was only achieved by D28 in females and earlier in males, which had a lower base number. Cy + Deg treatment enhanced the kinetics of thymocyte recovery, showing statistical significance from D10 and reaching similar levels in both females and males. Interestingly, thymocyte number in males regenerated beyond UT levels as early as D10, whereas females achieved the same result at D28. By D28, thymocyte numbers were increased to ~ 1.6 -fold UT levels in females, and ~ 3.9 -fold UT levels in males. These findings suggest androgens have a more suppressive effect than estrogens on thymopoiesis. Surprisingly, the loss of thymocytes from Cy treatment in females did not coincide with significant differences in total TEC number, and only minor changes were observed with Cy + Deg treatment (**Figure 5B**). Loss in total TEC number was, however, observed in males following Cy treatment; perhaps related to a greater reliance on proliferation to maintain TEC numbers, with degarelix having no beneficial effect in the regeneration of total TEC number.

Further examination of TEC subsets however, revealed a significant regeneration of mature TEC subsets (**Figures 5C,D, Figure S1C**). Using Ly-51⁺ expression to define cTECs from mTECs (Ly-51⁻), we found the mTEC^{hi} subset to be the most affected by Cy in both genders, as evidenced by its dramatic proportional loss at D4 (**Figure 5D**). Complete endogenous recovery of mTEC^{hi} was apparent between D14 and D28. Whilst there was no proportional loss in cTEC^{hi} cells at D4, Cy damage did induce endogenous mobilization of cTEC^{lo} at D10 in females. This was coincident with enhanced cTEC^{hi} proportions at D10 and D14 before returning to UT levels at D28. In contrast, the male cTEC^{lo} subset demonstrated a persistent proportional increase immediately following Cy damage compared to UT levels. Notably, the reduced mTEC^{lo} proportion observed from D10 suggests that, in conjunction with TEPC reactivation, male thymic regeneration may involve a higher degree of mTEC^{lo} differentiation for mTEC^{hi} recovery post-Cy damage compared to females.

Degarelix treatment enhanced mTEC^{hi} recovery post-Cy damage, reaching significance by D7 in females and D14 in males (**Figure 5D**). Significant cTEC^{lo} mobilization was evident from D7 to D14 in females, returning to UT levels by D28. Cy + Deg treatment in females also prompted an initial wave of cTEC^{hi} expansion at D4. This expansion implicates sequential recovery of cTEC^{hi} and mTEC^{hi}, possibly in parallel with the

recovery of thymocyte populations. Mobilization of the cTEC^{lo} compartment was also observed in Cy + Deg treated males, which was demonstrated by reduced cTEC^{lo} proportions from D10 compared to Cy alone. Interestingly, Cy + Deg treatment also prompted an initial wave of cTEC^{hi} expansion, albeit delayed at D7–10 compared to females. This event provides insight on the mechanisms behind SHD-induced thymopoiesis. The early reduction in male mTEC^{lo} proportions suggests that overall enhancement of gender-specific endogenous repair accompanies the common thymic regenerative events induced by sex steroid deprivation.

Due to the pronounced changes in male mTEC subpopulations with Cy + Deg treatment, we examined for enhancements in their peripheral T cell pool. Total splenocyte numbers were enhanced with degarelix by D28 (data not shown), albeit no notable differences within splenic subsets were observed. We detected higher peripheral T cell numbers within brachial and inguinal lymph nodes (**Figure S2A**). This increase was evident in naïve (CD62L⁺ CD44^{lo}) CD4⁺ and CD8⁺ T cell subpopulations from D14, with enhancement also seen in central memory (CM, CD62L⁺ CD44^{hi}) and effector memory (EM, CD62L⁻ CD44^{hi}) cell numbers. Notably, EM cells reached young levels by D14 (data not shown). Ki-67 analyzes indicated involvement of homeostatic expansion of these populations from D10 (**Figure S2B**). Enhanced T regulatory (Treg, CD4⁺ CD25⁺ Foxp3⁺) cell recovery with degarelix treatment was also detected from D14, with virtual memory (VM) cells (CD8⁺ CD122^{hi} CD44^{hi} CD49d⁻) exhibiting homeostatic expansion in a lymphopenic environment (35) and clearly responding to Cy damage by D10 (**Figure S2C**). Additionally, bone marrow analyzes showed no enhancement in hematopoietic stem cell (HSC) numbers with degarelix treatment prior to D14, with no alterations in lymphoid-primed multipotent progenitors (LMPPs) and common lymphoid progenitors (CLPs) (data not shown). As the spike in thymocyte numbers occurs at D10, it is likely that the early changes induced by degarelix treatment are due to thymus-intrinsic mechanisms.

Ki-67 expression studies revealed an increase in mTEC^{hi} proliferation at D4 post-Cy damage in both genders (**Figure 5E**). This finding may reflect an immediate endogenous proliferative response to the dramatic loss of mTEC^{hi} with Cy treatment. Given that Cy + Deg treatment produced a small differential increase in Ki-67⁺ mTEC^{hi} at D14, it is unlikely that the significant regeneration of the mTEC^{hi} subset with degarelix was mostly due to enhanced proliferation of existing cells. For both genders, the cTEC compartments demonstrated significant reductions in Ki-67 expressing cells at D4 due to Cy, with subsequent endogenous recovery enhanced with degarelix treatment to beyond UT levels at D14. Although a similar trend was observed in the mTEC^{lo} subset of females, the male mTEC^{lo} subpopulation instead demonstrated no decline in proliferative capacity at D4 post-Cy damage. This may also reflect an immediate proliferative response in male mTEC^{lo}, supporting the increased reliance on existing mTEC^{lo} progenitors to replenish the mTEC^{hi} population at this age, compared to females. This finding further alludes to the differences between female and male TEC maintenance.

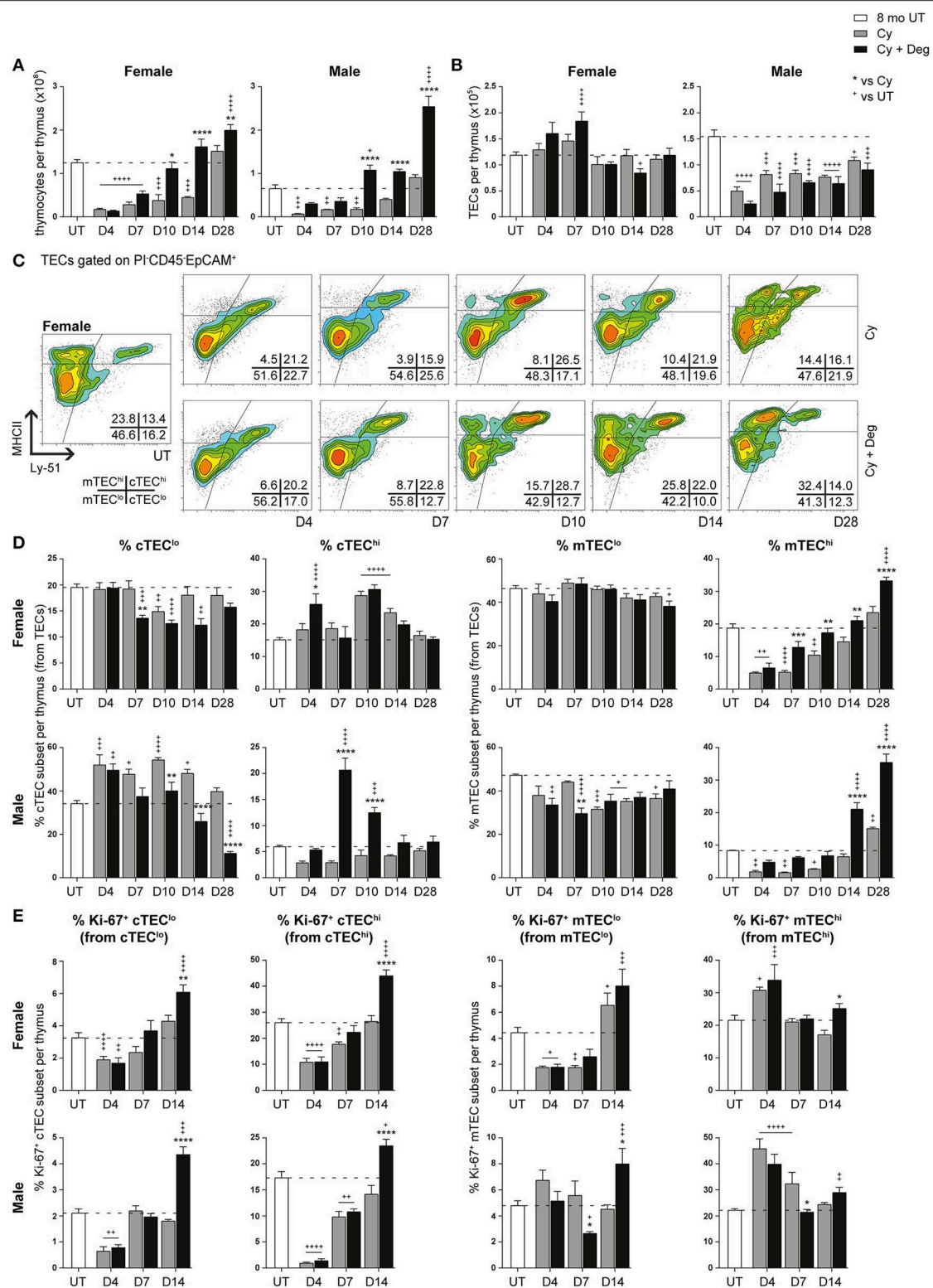
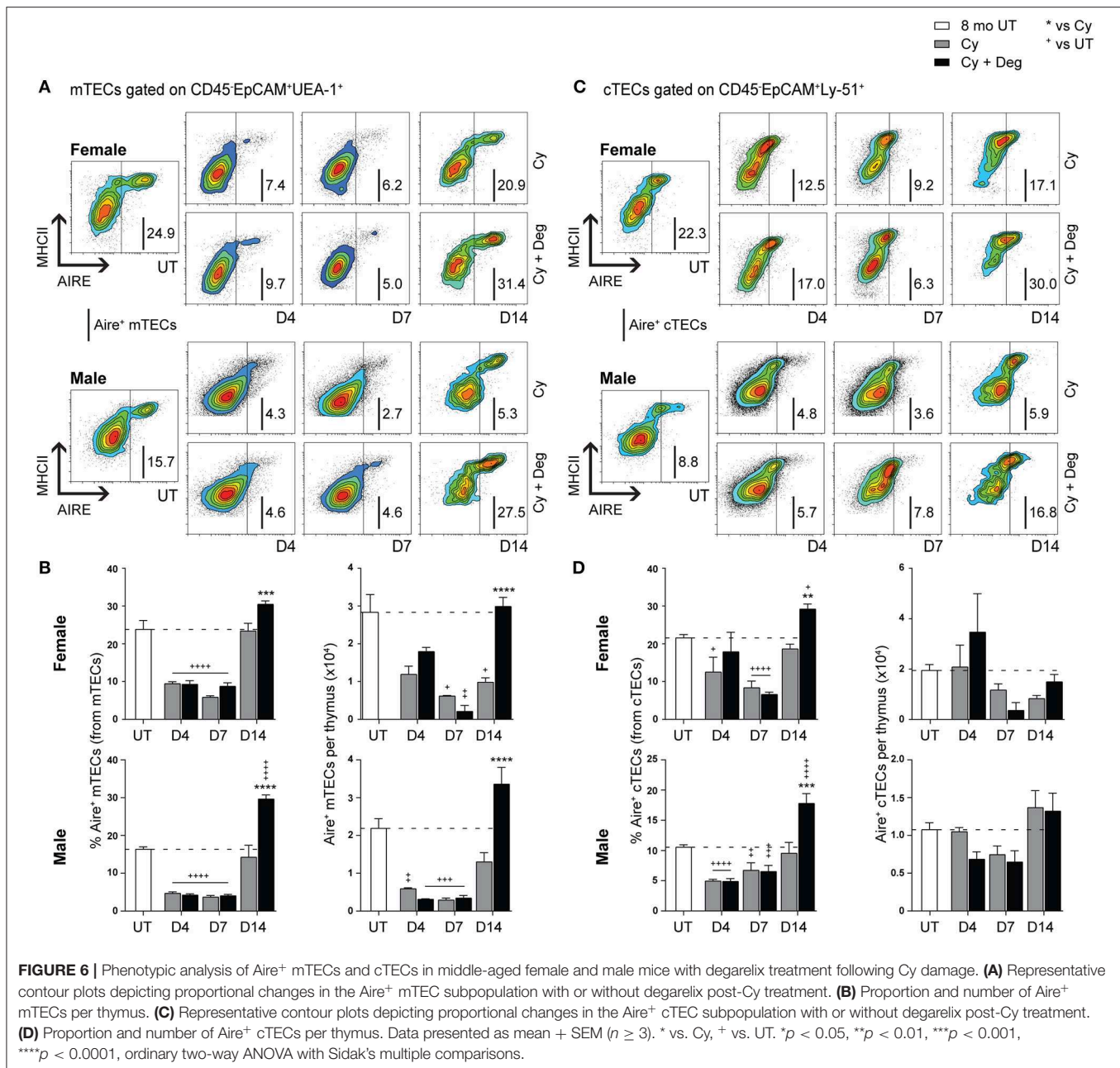


FIGURE 5 | Phenotypic analysis of TEC subsets in middle-aged female and male mice with degarelix treatment following Cy damage. **(A)** Total thymocyte cellularity in 8-month-old female and male mice with or without degarelix post-Cy treatment. **(B)** Total TEC cellularity. **(C)** Representative contour plots depicting proportional changes in female TEC subsets with or without degarelix post-Cy treatment. MHCII and Ly-51 were used to segregate TECs into cTEC^{lo}, cTEC^{hi}, mTEC^{lo}, and mTEC^{hi} subpopulations. **(D)** Proportion of cTEC^{lo}, cTEC^{hi}, mTEC^{lo}, and mTEC^{hi} subpopulations. **(E)** Proportion of Ki-67⁺ cells within TEC subsets. Data presented as mean + SEM ($n \geq 3$). * vs. Cy, + vs. UT. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ordinary two-way ANOVA with Sidak's multiple comparisons.



SHD-Enhanced Recovery of Aire⁺ mTEC Following Cy Damage

Due to the enhanced recovery of thymocytes with degarelix treatment post-Cy damage, we examined Aire expression within mTECs to determine whether self-tolerance mechanisms were in place. Our analyses revealed a loss of Aire⁺ mTEC after Cy, and enhanced recovery with degarelix treatment in both genders (Figures 6A,B). A proportional and numerical loss of Aire⁺ mTEC due to Cy damage was seen, with restoration to UT levels through endogenous repair by D14 (Figure 6B). Treatment with degarelix resulted in similar recovery kinetics in both males and females, however, Aire⁺ mTEC proportions and numbers at D14 were found to be at least 2-fold

higher than Cy alone groups. These data suggest maintenance of central tolerance, although further Aire-dependent tissue-restricted antigen (TRA) expression studies would be required to confirm this. Interestingly, no significant numerical changes were observed in Aire⁺ cTECs following Cy and Cy + Deg treatments, despite a proportional increase at D14 in both genders (Figures 6C,D).

SHD-Enhanced Recovery of TEPCs Following Cy Damage

Given the absence of pronounced proliferative changes in the mTEC^{hi} subset with degarelix-induced regeneration, we proposed that the increase in mTEC^{hi} originated from

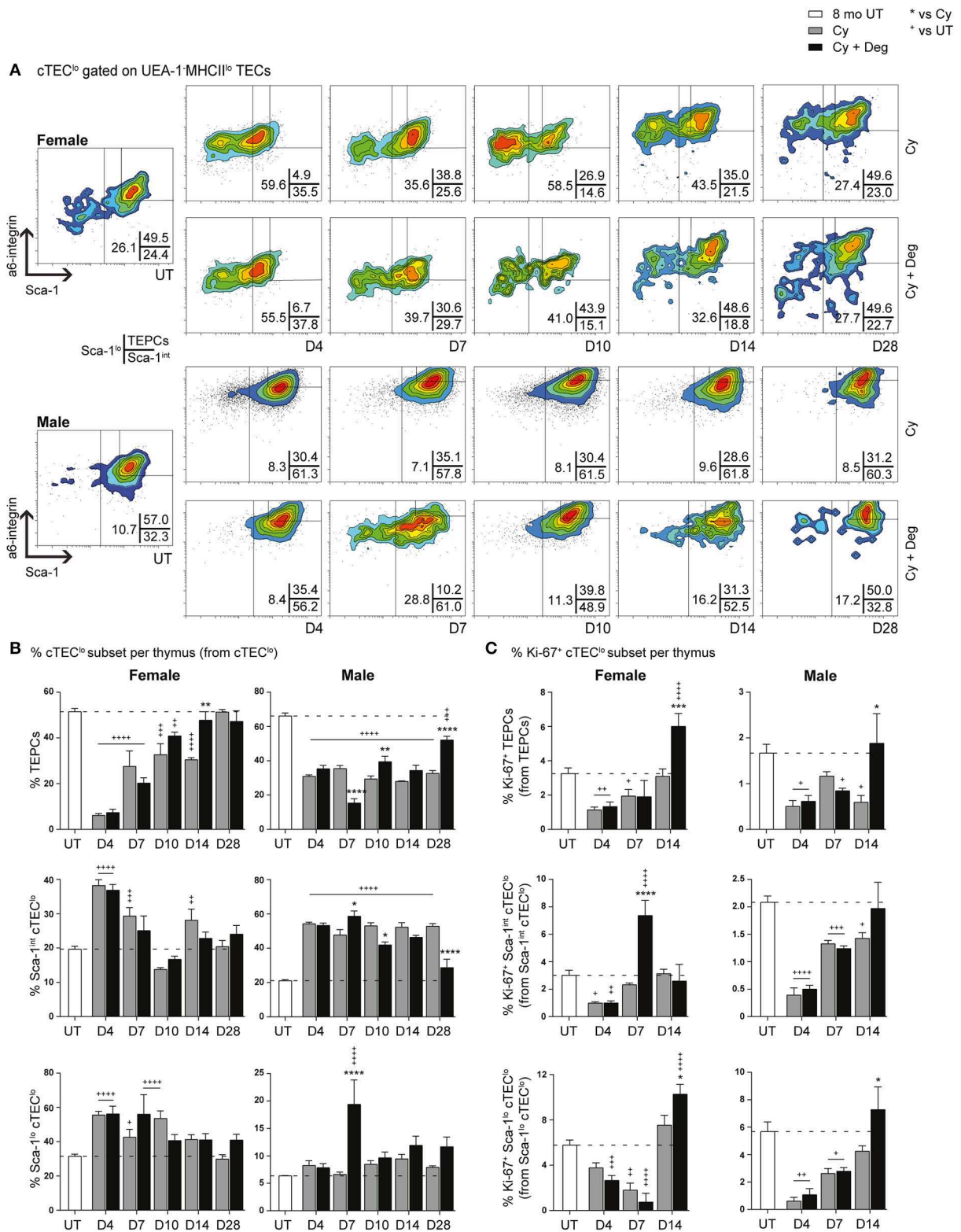


FIGURE 7 | Phenotypic analysis of TEPC subsets in middle-aged female and male mice with degarelix treatment following Cy damage. **(A)** Representative contour plots depicting proportional changes in cTEC^{lo} subsets with or without degarelix post-Cy treatment. Antibodies against α6-integrin and Sca-1 were used to divide cTEC^{lo} into TEPC (α6^{hi} Sca-1^{hi}), Sca-1^{int} cTEC^{lo}, and Sca-1^{lo} cTEC^{lo} subpopulations. **(B)** Proportion of TEPC, Sca-1^{int} cTEC^{lo}, and Sca-1^{lo} cTEC^{lo}. **(C)** Proportion of Ki-67⁺ cells within cTEC^{lo} subsets. Data presented as mean + SEM (*n* ≥ 3). * vs. Cy, + vs. UT. **p* < 0.05, ***p* < 0.01, ****p* < 0.001, *****p* < 0.0001, ordinary two-way ANOVA with Sidak's multiple comparisons.

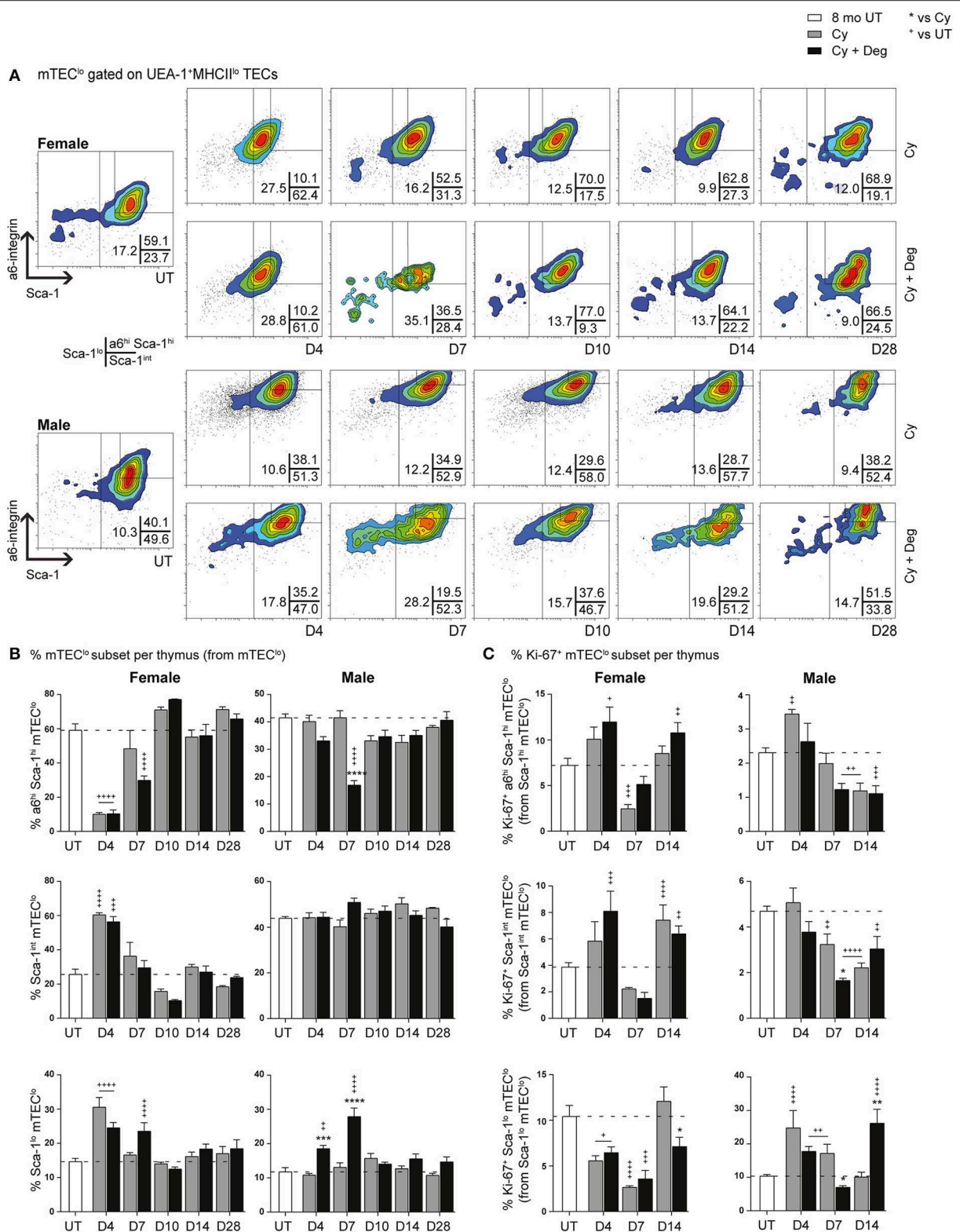


FIGURE 8 | Phenotypic analysis of mTEC progenitor subsets in middle-aged female and male mice with degarelix treatment following Cy damage. **(A)** Representative contour plots depicting proportional changes in mTEC^{lo} subsets with or without degarelix post-Cy treatment. Antibodies against α6-integrin and Sca-1 were used to segregate mTEC^{lo} into α6^{hi} Sca-1^{hi} mTEC^{lo}, Sca-1^{int} mTEC^{lo}, and Sca-1^{lo} mTEC^{lo} subpopulations. **(B)** Proportion of α6^{hi} Sca-1^{hi} mTEC^{lo}, Sca-1^{int} mTEC^{lo}, and Sca-1^{lo} mTEC^{lo}. **(C)** Proportion of Ki-67⁺ cells within mTEC^{lo} subsets. Data presented as mean + SEM ($n \geq 3$). * vs. Cy, + vs. UT. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ordinary two-way ANOVA with Sidak's multiple comparisons.

differentiation of progenitor cell populations; therefore, we investigated proportional changes in the $cTEC^{lo}$ subset where bipotent TEPCs reside (Figures 7A,B). A substantial reduction of TEPCs was evident at D4 for Cy and Cy + Deg treated females and males compared to UT controls (Figure 7B), with concurrent significant increases in $Sca-1^{int} cTEC^{lo}$ and $Sca-1^{lo} cTEC^{lo}$ proportions. These trends suggest an early endogenous response to Cy-induced TEC loss through immediate TEPC mobilization, but does not rule out direct damage to TEPCs. Thereafter, gradual TEPC renewal was evident in both treatment groups and genders, with a higher TEPC proportion observed in Cy + Deg at D14 in females and D10 in males when compared to Cy alone controls. TEPC homeostasis returned in females by D28, however males did not achieve this result even with degarelix treatment. Interestingly, degarelix treatment appeared to induce a transient reduction in male TEPC proportion at D7, which occurred simultaneously with an increase in $Sca-1^{lo} cTEC^{lo}$ proportion; a dramatic surge in $cTEC^{hi}$ was also observed at this time (Figure 5D). This finding supports our proposal of sequential TEC recovery of $cTEC^{hi}$ and $mTEC^{hi}$. Moreover, the enhanced TEPC renewal with degarelix treatment from D14 in females appears to be associated with proliferation, as evidenced by increased Ki-67 expression (Figure 7C). Transient proliferation in Cy + Deg females was also evident in $Sca-1^{int} cTEC^{lo}$ and $Sca-1^{lo} cTEC^{lo}$ populations at D7 and D14, respectively. No transient enhancement in proliferation was observed in these subsets in Cy + Deg males. From these data, we propose that Cy damage induces immediate endogenous TEPC reactivation and differentiation in middle-aged mice, with degarelix enhancing TEPC renewal at D14 by promoting proliferation.

SHD-Enhanced Differentiation and Proliferation Within $mTEC^{lo}$ Subsets Following Cy Damage

Since single lineage medullary precursors originating from TEPCs give rise to $mTEC^{hi}$, we investigated for shifts within the $mTEC^{lo}$ subset (Figures 8A,B). In both groups, a substantial reduction of $\alpha 6^{hi} Sca-1^{hi} mTEC^{lo}$ in parallel with an increase in $Sca-1^{int} mTEC^{lo}$ and $Sca-1^{lo} mTEC^{lo}$ populations was evident for females at D4, suggesting similar mobilization events observed with $cTEC^{lo}$ populations were involved in endogenous recovery (Figure 8B). Although this trend was absent in males, a transient increase in $Sca-1^{lo} mTEC^{lo}$ proportion was however detected at D4 and D7 in degarelix groups, with a reduced $\alpha 6^{hi} Sca-1^{hi} mTEC^{lo}$ proportion seen at D7. These data suggest a gender-disparate mobilization of $\alpha 6^{hi} Sca-1^{hi} mTEC^{lo}$, which may explain the accelerated recovery of the $mTEC^{hi}$ subset in Cy + Deg treated females in terms of proportional increases in $mTEC^{hi}$, reaching 20% by D7–10 in females but males not achieving this level until D14. Our proliferative analyzes showed a loss of $Ki-67^{+} Sca-1^{lo} mTEC^{lo}$ at D4 following Cy treatment in females, however the equivalent population had increased in males, supporting immediate endogenous proliferation of existing $Sca-1^{lo} mTEC^{lo}$ precursors for $mTEC^{hi}$ regeneration (Figure 8C). This was enhanced at D14 with degarelix treatment

which may relate to replenishment of this population. Together, our data implicates differentiation, rather than proliferation, as the driving force of endogenous medullary repair in females, with males engaging both mechanisms for $mTEC^{hi}$ recovery.

DISCUSSION

Progressive physiological impediment of naïve T cell generation can result in severe clinical complications following chemotherapy treatment. These conditions increase the incidence of opportunistic infections, which result in increased patient morbidity and mortality. Hence, a clear need for immune reconstitution strategies is required. Here, we examined one promising approach to restore aged-thymic function—the administration of LHRH-analogs. Given the lack of research conducted in females, we initially investigated the mechanisms behind age-related thymic involution in relation to murine sexual dimorphism. As our findings indicated phenotypic differences between females and males, we subsequently examined the impact of LHRH receptor antagonist, degarelix, on thymic recovery following Cy damage in middle-age mice, to determine whether gender disparity was apparent.

Through flow cytometric analyzes, we performed a comparative phenotypic assessment of thymic cell subpopulations in pre-pubertal, post-pubertal, and middle-aged female and male mice. We found the age-related decline in thymocyte number to be more pronounced in males, and suggest that this disparity may result from estrogen- (36) and androgen-specific mechanisms of action (37), which impact on thymocytes and/or thymic stromal cell populations. Despite greater proportions of $cTEC^{hi}$ and $mTEC^{hi}$ subpopulations in females at middle-age, which suggests better maintenance of TEC differentiation compared to males, a female-exclusive reduction in $Aire^{+} mTEC$ /thymocyte ratio was observed. Their lower $Aire^{+} mTEC$ /thymocyte ratio may be associated with an imbalance in central tolerance or Treg cell development that may contribute to the increased clinical risk of autoimmunity seen in female patients (38). Together with the age-associated increase in female $Aire^{+} cTEC$ /thymocyte ratio, we propose that there is an impediment in $Aire^{+} mTEC^{hi}$ differentiation, and that $Aire^{+} cTEC^{hi}$ may be its upstream precursor. Although localized at the cortico-medullary junction (33), which allows for prompt transition into the medulla following differentiation, the $Aire^{+} cTEC^{hi}$ population has previously been shown through ectopic studies to be incapable of ameliorating autoimmune pathology alone, and does not induce TRA genes (39). As these ectopic studies utilized $\beta 5t/Aire$ -transgenic mice that exclusively express $Aire$ in the cortex, further research is required to determine whether $Aire^{+} cTEC^{hi}$ are quiescent upstream precursors of $Aire^{+} mTEC^{hi}$. Their association with receptor activator of nuclear factor κB (RANK) signaling, which has been demonstrated to regulate $Aire$ expression in $mTECs$ (40, 41), also warrants investigation.

Our data reveal greater impairment in male TEC^{lo} to TEC^{hi} differentiation with aging than females. The pronounced increase in male $mTEC^{lo}$ proportion following puberty, together with a

reduction in Ki-67, suggests that there is a gender-related block in mTEC^{lo} to mTEC^{hi} differentiation. Moreover, the proportion of male cTEC^{lo} significantly exceeds that of females by middle-age, hinting at more severe impediment in TEPC differentiation. A potential underlying mechanism for these events was recently reported (10), and relates to a 6-fold increase in male cTEC and mTEC^{lo} Fst expression post-puberty, and its antagonistic relationship to activin A and Bmp4 signaling. Following our assessment of these TGF- β superfamily members in pre- and post-pubertal females, we revealed a 4-fold increase in female cTEC and mTEC^{lo} subsets, which suggests a similar but less profound role to males in the impediment of differentiation through inhibition of activin A signaling. The post-pubertal increase in Bmp4 expression by supporting stromal cells was, however, greater in females, and implicates better progenitor maintenance. Its receptor, Bmpr2, is primarily expressed on cTEC^{lo} and mTEC^{lo} progenitors (42), and was proposed to have a role in maintaining progenitor populations at the expense of differentiation (10). However, Bmp4 induced self-renewal of progenitors may also have a role in thymus regeneration following damage, as demonstrated following irradiation (43).

We have previously proposed that the bipotent $\alpha 6^{\text{hi}}$ Sca-1^{hi} TEPC population differentiates toward a single lineage cTEC precursor expressing low levels of Sca-1, which in turn differentiates into the mature Sca-1^{lo} cTEC^{hi} phenotype, as well as into Sca-1^{hi} mTEC^{lo} single lineage precursors (10). Despite no obvious differences in TEPC colony phenotype and CFE with gender, our *in vitro* 3D co-culture studies suggest that there is an immediate impairment in self-renewal capacity following puberty. This finding somewhat correlates with a previous study that demonstrated reduced TEC CFE with aging (44), albeit no immediate post-pubertal attenuation was observed. As the interpretation of *in vitro* results is influenced by elements within the culture systems themselves, in conjunction with the lack of sex steroids in such systems, supporting *in vivo* studies will be required to truly elucidate whether gender differences in TEPC function exist throughout aging. Nonetheless, our phenotypic assessments identified an accumulation of TEPCs following puberty in both males and females, which presented with a reduction in downstream Sca-1^{lo} cTEC^{lo} that was more pronounced in males by middle-age. Given the increased proliferation of Sca-1^{lo} cTEC^{lo} in middle-aged males, and the accumulation of Sca-1^{int} cTEC^{lo}, we suggest that there may be a block in male Sca-1^{int} cTEC^{lo} differentiation. Hence, these data indicate better maintenance of TEPC function in middle-aged females. Analyses conducted with the assumption that our $\alpha 6^{\text{hi}}$ Sca-1^{hi} phenotype can also identify mTEC^{lo} precursors revealed gender disparity in relation to the maintenance of these immature medullary subpopulations by middle-age, yet no differences between female and male Sca-1^{lo} mTEC^{lo} proportions were observed. Not surprisingly, $\alpha 6^{\text{hi}}$ Sca-1^{hi} mTEC^{lo} do not fall within the mTEC-II phenotype which express Aire and other mature mTEC markers (45). Further research is, however, required to establish the link between $\alpha 6^{\text{hi}}$ Sca-1^{hi} mTEC^{lo} and previously identified postnatal mTEC progenitor phenotypes such as stage-specific embryonic antigen-1 (SSEA-1)⁺ Claudin (Cld)3,4^{hi} TECs (46, 47).

Given that gender disparity was observed in our aging studies, we compared the regenerative effect of LHRH receptor-antagonist, degarelix, on the middle-aged female thymus to their male counterparts. Transient reversal of thymic involution was observed in females between D7 and D10 post-degarelix treatment, with negligible changes in TEC subset proportions suggesting progenitor activation followed by homeostatic TEC maintenance. This theory is further supported by the decreased proportion of TEPCs at D7 and subsequent increase by D10, which implicates brief progenitor reactivation that coincided with the expansion of thymocytes. Conversely, degarelix treatment of middle-aged males resulted in delayed thymic regeneration, but expansion of thymocyte cellularity was sustained until at least D28. The pronounced, progressive reduction in cTEC^{lo}, in conjunction with increased mTEC^{hi} from D10, suggest persistent differentiation into mature TEC subsets which is exclusive to males. Decreased TEPC and $\alpha 6^{\text{hi}}$ Sca-1^{hi} mTEC^{lo} proportions until at least D14 further supports this notion. Together, these findings implicate degarelix as having a more prominent effect on male TEC subsets and/or that androgens may be more detrimental to T cell generation than estrogens. While alterations in the mobilization of bone marrow precursors can also partially explain our observations (48), our peripheral organ analyses on middle-aged male mice did not identify an early enhancement in the provision of BM precursors with degarelix treatment (data not shown). Hence, these events are likely to be thymus-intrinsic.

Degarelix treatment was found to enhance the kinetics of thymocyte recovery in Cy-treated middle-aged mice, albeit no gender-based differences and no significant improvement in TEC number were observed. Thymocyte numbers surpassed untreated levels in females by D28 and D10 in males, indicating that androgens may have a more suppressive effect than estrogens on thymopoiesis. This enhancement in thymocyte recovery was also reported in a recent study (12), which demonstrated return to untreated levels by D42 in middle-aged male mice following irradiation. Although there is a lack of data regarding degarelix treatment following irradiation prior to this time point, it is likely that an association exists between the kinetics of thymocyte recovery and the type of cytoablative injury inflicted. Such differences are bound to have clinical implications, and warrant further investigation. Interestingly, total TEC number was exclusively reduced in male animals after Cy treatment. This reduction did not recover back to UT levels by D28, and hints at the possibility of an imbalance in Aire⁺ mTEC/thymocyte ratio, and hence an impediment in central tolerance. The ratio at D14 in males (data not shown) revealed that this was unlikely the case in degarelix treated mice, with early recovery of mTEC^{hi} proportions observed. Our TEC subset analyses demonstrated transient cTEC^{lo} mobilization with degarelix in females at D7, while mobilization in males was detected from D10. These trends were consistent with degarelix alone data. The enhanced, yet transient, recovery of cTEC^{hi} observed at D4 in females, and D7–10 in males, implicate faster kinetics of recovery in the former. Moreover, both males and females demonstrated improved mTEC^{hi} recovery. This augmentation was detected earlier in females, which further

supports their better recovery kinetics. Since the alterations in mTEC subpopulations were more pronounced in males with Cy + Deg treatment, we conducted analyses with regards to their peripheral T cell pool. Enhanced T cell recovery was observed in lymph nodes, resulting predominantly from proliferation. This enhancement is consistent with previous reports (49, 50), which also demonstrated an absence of immunosuppressive effects with LHRH receptor-antagonist treatment. Increased Treg and VM cell subpopulations also contributed to early peripheral immune reconstitution (51, 52).

Through our proliferative studies, it appeared that degarelix contributed to thymic repair predominantly by augmenting cTEC proliferation by D14, with males demonstrating a more pronounced proportional increase in Ki-67⁺ cTEC^{lo} than females. This disparity likely relates to the persistent mobilization of cTEC^{lo} in middle-aged males, which would in turn require replenishment. The increased proportion of mTEC^{hi} was likely a consequence of progenitor differentiation, rather than proliferation. These data are supported by previous findings, which showed that adult mTEC maintenance and regeneration occurs through $\beta 5t^{-}$ lineage-restricted cells (13). Whether alterations in medullary stromal signaling to the cortex contribute vastly to this process (53) is yet to be investigated. Examination of TEPCs revealed a prominent difference between males and females treated with degarelix after Cy damage. Whilst degarelix enhanced TEPC recovery in females to UT levels by D14, recovery of the progenitor pool was not achieved in males despite enhanced TEPC proliferation at D14 in both genders. This may be due to continued mTEC^{hi} production beyond D28, or possibly indicate TEPC senescence. Analyses at later time points will be required to determine whether male TEPCs eventually return to UT levels. Furthermore, the absence of decline in male $\alpha 6^{hi}$ Sca-1^{hi} mTEC^{lo} at D4 implicates gender disparity in the maintenance of medullary progenitors, which would subsequently impact on the recovery of the mTEC^{hi} subset.

This study details, for the first time, the relationship between sexual dimorphism and TEC aging from the pre-pubertal stages of life to middle-age. We revealed a potential imbalance in central tolerance that may explain the increased incidence of autoimmunity in middle-aged females. A higher Sca-1^{lo} cTEC^{lo} proportion was, however, seen in females, which appears to be associated with better maintenance of differentiation compared to males in this age group. We also demonstrated that degarelix was more effective for thymic regeneration in middle-aged males, and possibly relies on both progenitor reactivation and proliferation. Furthermore, the enhanced recovery of TEC^{hi} subsets in females treated with degarelix and Cy precedes the recovery in males and implicates faster kinetics of recovery. This likely relates to better maintenance of progenitor function in middle-aged females. Taken together, these findings stress the relevance of sexual dimorphism in adaptive immunity, and

suggest a plausible benefit to analyzing naïve T cell output in prostate and breast cancer patients treated with LHRH-analogs. Investigations into the potential negative impact of multiple-dose chemotherapy on the SHD-reactivated thymus are also required, to ascertain whether alterations in cancer treatment protocols could be beneficial.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by the Monash University Animal Ethics Committee.

AUTHOR CONTRIBUTIONS

AC conceptualized and designed the study and analyzed/interpreted data. MH, KW, JG, AA, and KQ performed experiments and/or data analysis. AC, MH, KW, JG, and AA contributed to drafting the original manuscript. All authors critically evaluated the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2020.00302/full#supplementary-material>

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Thymic B Cells Promote Germinal Center-Like Structures and the Expansion of Follicular Helper T Cells in Lupus-Prone Mice

Yessia Hidalgo^{1,2}, Sarah Núñez³, Maria Jose Fuenzalida^{1,3}, Felipe Flores-Santibáñez¹, Pablo J. Sáez⁴, Jessica Dorner⁵, Ana-Maria Lennon-Dumenil⁴, Victor Martínez⁵, Emmanuel Zorn⁶, Mario Roseblatt^{1,3,7}, Daniela Sauma^{1*} and Maria Rosa Bono^{1*}

¹ Departamento de Biología, Facultad de Ciencias, Universidad de Chile, Santiago, Chile, ² Cells for Cells-Consorcio Regenero, Facultad de Medicina, Universidad de los Andes, Santiago, Chile, ³ Fundación Ciencia & Vida, Santiago, Chile, ⁴ INSERM U932, Institut Curie, Centre de Recherche, PSL Research University, Paris, France, ⁵ FAVET-INBIOGEN, Faculty of Veterinary Sciences, University of Chile, Santiago, Chile, ⁶ Department of Medicine, Columbia Center for Translational Immunology, Columbia University Medical Center, New York, NY, United States, ⁷ Facultad de Ciencias de la Vida, Universidad Andres Bello, Santiago, Chile

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Nicolai Stanislas Van Oers,
University of Texas Southwestern
Medical Center, United States

Reviewed by:

Takeshi Nitta,
The University of Tokyo, Japan
Katsuto Hozumi,
Tokai University, Japan

*Correspondence:

Daniela Sauma
dsauma@uchile.cl
Maria Rosa Bono
mrbono@uchile.cl

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Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by the activation of autoreactive T and B cells, autoantibody production, and immune complex deposition in various organs. Previous evidence showed abnormal accumulation of B cells in the thymus of lupus-prone mice, but the role of this population in the progression of the disease remains mostly undefined. Here we analyzed the spatial distribution, function, and properties of this thymic B cell population in the BWF1 murine model of SLE. We found that in diseased animals, thymic B cells proliferate, and cluster in structures that resemble ectopic germinal centers. Moreover, we detected antibody-secreting cells in the thymus of diseased-BWF1 mice that produce anti-dsDNA IgG autoantibodies. We also found that thymic B cells from diseased-BWF1 mice induced the differentiation of thymocytes to follicular helper T cells (T_{FH}). These data suggest that the accumulation of B cells in the thymus of BWF1 mice results in the formation of germinal center-like structures and the expansion of a T_{FH} population, which may, in turn, activate and differentiate B cells into autoreactive plasma cells. Therefore, the thymus emerges as an important niche that supports the maintenance of the pathogenic humoral response in the development of murine SLE.

Keywords: systemic lupus erythematosus, thymic B cells, germinal center, plasma cells, follicular helper T cells

INTRODUCTION

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by a set of clinical abnormalities ranging from mild symptoms such as malaise, arthritis, or dermatitis to more severe manifestations such as renal disease or compromise of the central nervous system. At the immunological level, SLE patients exhibit hyperactivity of T and B cells against self-antigens that leads to the secretion of autoantibodies against nuclear components such as DNA, RNA, histones, ribonucleoproteins, among others. Autoantibodies bind antigens and form immune complexes that deposit in the skin, joints, kidneys, heart, and central nervous system, generating inflammation and damage (1, 2).

The thymus is a primary lymphoid organ whose main function is the induction of immune self-tolerance to prevent autoimmunity. This organ is dedicated to T cell generation and maturation, a function that usually declines with age and can be severely compromised in autoimmune diseases (3). It has been described that patients with autoimmune conditions such as myasthenia gravis exhibit alterations in the thymic structure and its cellular components (4–6). One of the important alterations in the thymus of patients with myasthenia gravis is the increase of autoreactive B cells. In the literature, there have been reports of patients with other autoimmune diseases, such as ulcerative colitis and systemic lupus erythematosus that present abnormalities in the structure of the thymus (7–9). However, it is unknown if these changes contribute to the disease.

B cells are a scarce population in the thymic medulla of both, healthy humans and mice (~0.3% of total cells) where they are thought to function as antigen-presenting cells for thymic selection (10–13). We have previously shown that throughout normal aging, the human thymus accumulates at perivascular spaces memory B cells and plasma cells that generate antibodies with antiviral reactivity (14). Remarkably, a subset of myasthenia gravis patients has been diagnosed with thymic follicular hyperplasia which encompasses a considerable expansion of B cells and the formation of germinal centers in the thymus (15, 16). Myasthenia gravis patients often go through thymectomy, a procedure that shows an overall improved clinical outcome, highlighting the contribution of thymic abnormalities to the production of autoantibodies against the acetylcholine receptor and severity of the disease (17–19). Recent evidence suggests that infiltration of B cells to the thymus and thymic stroma destruction precedes type 1 diabetes development in NOD mice (20), supporting the idea that B cell infiltration to the thymus may be a common event in several autoimmune diseases. Alterations in the thymus structure have also been observed in rheumatoid arthritis and SLE, but the contribution of these abnormalities to these diseases are poorly understood (7, 21, 22). Among patients with SLE, between 1.5 and 2% develop thymomas and undergo thymectomy as treatment. In contrast to myasthenia gravis patients, this procedure on SLE progression has no clear health benefits (5, 9, 23).

Studies using BWF1 mice, a murine model of SLE, showed an increase in B cell frequency in the thymus of diseased mice compared to those that have not yet developed the disease (24, 25). In these studies, the authors highlight that the B1/B2 ratio in the thymus is higher than in spleen and blood. They showed that the B1 cell population migrates abnormally to the thymus due to high expression of the CXCR5 chemokine receptor, and aberrant high expression of CXCL13 (B lymphocyte chemoattractant, BLC) by myeloid dendritic cells present in the thymus. However, this study does not address the functional relevance of abnormal B cell numbers within the thymus and their contribution to SLE.

The converging evidence of B cells and plasma cells accumulation in the thymus during aging and particularly in autoimmune diseases prompted us to hypothesize that this lymphoid organ may become a specialized niche for B cells and plasma cells in the context of SLE development. To address this question, we characterized the B cell population of the thymus

of BWF1 mice during the autoimmune humoral response. Here we show that upon the onset of the disease, the thymus structure becomes highly disorganized, exhibiting an increasing number of B cells that accumulate into structures that resemble ectopic germinal centers. Accordingly, we observed the presence of antibody-secreting plasma cells, a fraction of which produces anti-dsDNA autoantibodies. Noticeably, we further found that thymic B cells from diseased BWF1 mice induce the activation and differentiation of CD4⁺ thymocytes to follicular helper T cells. Altogether these data suggest a positive feedback loop, where thymic B cells induce the differentiation of follicular helper T cells that in turn promote the differentiation of autoreactive plasma cells in the thymus.

MATERIALS AND METHODS

Mice

Female lupus-prone [NZB × NZW]F1 (BWF1) mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and maintained at the animal facility of Fundacion Ciencia & Vida. Animal work was carried out under the institutional regulations of the Fundacion Ciencia & Vida and was approved locally by the ethical review committee of the Facultad de Ciencias, Universidad de Chile. Disease incidence and severity was monitored by measuring proteinuria using a standard semi-quantitative Combur Test N (Roche Diagnostics, Germany) and an ELISA to determine antibody titers to double-stranded DNA (dsDNA). To detect early autoimmune disease, proteinuria was measured monthly during the first 5 months of age and every week after that. In this work, we used 3 and 5 months old BWF1 female mice as young mice which still do not develop autoimmune disease. Diseased mice were 9 months old in average, presented severe proteinuria (i.e., ≥500 mg/dL protein) and high levels of plasmatic antibody titers against double-stranded DNA. In all cases, age-matched [NZW × BALB/c]F1 female mice were used as non-autoimmune controls.

Flow Cytometry and t-SNE

Cell surface staining was performed in ice-cold PBS with 2% fetal bovine serum (FBS) for 30 min in the presence of Fcγ R blocking antibody (CD16/32). Viability dye eFluor 780 reagent (eBioscience) or propidium iodide (PI) were used for live/dead cells discrimination. Monoclonal antibodies (mAbs) against mouse CD8 (53-6.7) FITC, CD138 (281-2) PE or BV421, CD45R/B220 (RA3-6B2) APC or PE-Cy7, CD19 (6D5) FITC, APC or eFluor 780, CD44 (IM7) APC, CD69 (H1.2F3) PE, CD83 (Michel-19) FITC, CD86 (GL1) FITC, IgM (RMM-1) PE-Cy7, purified CD16/32 (93), CXCR5 (L138D7) PE, Ki-67 (11F6) Alexa fluor 488, OX40L (RM134L) Alexa fluor 647, Blimp-1 (5E7) PE, Bcl-6 (1G191E/A8) Alexa fluor 647, and IgG-HRP (polyclonal) were purchased from BioLegend (San Diego, CA, USA). mAbs against mouse IgD (11-26c.2a) FITC, CD5 (53-7.3) PE-Cy7, CD21/35 (4E3) PE, GL7 (GL7) eFluor 660, CD11c (N418) PE, CD62L (MEL-14) FITC, CD25 (PC61.5) APC, CD8 (53-6.7) APC-eFluor 780, CD103 (2E7) FITC, CD279/PD-1 (J43) FITC, and Foxp3 (FJK-16s) PE-Cy7 were purchased from eBioscience (San Diego, CA, USA). mAbs against mouse I-Ad FITC (AMS-

32.1), were purchased from BD Pharmingen (San Diego, CA, USA). Intracellular staining for Foxp3 and Bcl-6 was performed after cell surface staining using the Foxp3/Transcription Factor Staining Buffer Set (eBioscience) following the manufacturer's instructions. Flow cytometry was conducted on a FACSCanto II flow cytometer (BD Biosciences) or FACS Aria III (BD Biosciences) and data analysis was performed using the FlowJo software version 8 (Tree Star, Inc., Ashland, OR, USA).

For t-SNE, data was acquired in a FACS Aria III (BD Biosciences) and the analysis was performed using the Rtsne package in R software. Cells were pre-gated in FlowJo v10 (Tree Star) on single cells, live (PI negative), CD45+, CD3+, and CD4+/CD8-. After gating, 15,000 cells from both control and BWF1 mice were used as input for the tSNE analysis and the parameters were set to 1,000 iterations, theta 0.5, learning rate 200 and perplexity 30.

ELISpot

Millipore® MAIP54510 96-well-plates were activated for 2 min with 50 µl/well of 70% ethanol and washed five times with deionized water. Plates were coated with 15 µg/ml of capture antibody anti-mouse IgG or dsDNA at 10 µg/ml and incubated overnight at 4°C. The plates were pre-treated with 10 µg/ml of methyl-BSA for 3 h at 37°C to evaluate reactivity against dsDNA. Subsequently, the plates were washed with PBS and blocked with RPMI medium supplemented with 10% FBS. The number of viable cells was carefully determined and plated in triplicates. After incubation at 37°C, 5% CO₂ for 22 h, the plates were washed five times with PBS and added 0.5 µg/ml of biotinylated goat anti-mouse IgG and incubated for 2 h at room temperature. Then plates were washed five times with PBS, and avidin-enzyme conjugated to HRP (eBioscience) was added and incubated for 1 h at room temperature. After washing the plates five times with PBS, 3-amino-9-ethyl carbazole (AEC) substrate was added and incubated at 37°C for 30 min. The plates were washed with bidistilled water and dry uncovered for 3 h at 37°C. Plates were read using an ELISpot reader AELVIS and the software Eli.Analysis ELISpot Analysis Software V6.0.

Confocal Microscopy

Thymi were extracted from diseased BWF1, and age-matched control animals, thymus lobes were imbibed in RPMI + 10% FBS + 5% low melting point agarose (Invitrogen) solution. Once the agar solidified at room temperature, slices of 400 µm were obtained in a PELCO® 102 vibratome. The slices were fixed with 3.7% paraformaldehyde for 20 min at room temperature and stained for 30 min at 37°C with the following antibodies: CD4 (RM4-5) PE, CD8 (53 6.7) FITC, and CD19 (1D3) APC. After that, slices were washed with PBS, and placed on a slide with ProLong Gold antifade mounting medium (Invitrogen) and covered with a coverslip. Thymic slices were analyzed in the Zeiss LSM 710 confocal microscope, and the images analyzed with ImageJ.

Immunohistochemistry

Thymi were extracted from diseased BWF1, and age-matched control animals, and frozen at -80°C for 24 h in OCT

compound. Six micrometer cryostat sections were obtained, air dried and fixed in cold acetone for 15 min. Sections were then incubated with a single drop of peroxidase blocker for 7 min at room temperature, washed with PBS and incubated for 1 h with blocking solution (PBS + 1% BSA + 10% goat serum). Then, sections were incubated overnight at 4°C with anti-mouse B220 or anti-mouse cytokeratin 5. After that, the sections were incubated for 1 h with HRP-coupled secondary antibody, washed with PBS and incubated with DAB (3'-Diaminobenzidine) for 4–8 min. Finally, sections were stained with hematoxylin and dehydrated to be mounting and visualized in the Olympus BX51 microscope.

Coculture of Follicular Helper T Cells With B Cells

Follicular helper T cells (T_{FH}) and B cells of thymus and spleen were isolated by cell sorting from diseased BWF1 mice. T_{FH} were stained with antibodies to CD4, CD8, PD-1, CXCR5, and selected as CD4⁺CD8⁻PD-1⁺CXCR5⁺. B cells were purified as CD19⁺CD5⁻/int. CD4⁺ T cells that do not express markers of T_{FH} (PD-1⁻CXCR5⁻) were used as control. Dead cells were discarded using PI staining during the sorting. B cells were stained with CellTrace Violet (Invitrogen) according to the manufacturer's instructions and cocultured with T_{FH} at a 5:1 ratio (50,000 B cells and 10,000 T_{FH} per well). Cocultures experiments were maintained for 5 days at 37°C and 5% CO₂. Subsequently, cells were recovered, and live B cells analyzed for GL-7 expression and dilution of CellTrace Violet stain by flow cytometry.

Coculture of B Cells With Thymocytes to Assess T_{FH} Differentiation

Thymic B cells from diseased BWF1 and age-matched control mice were isolated by cell sorting (CD19⁺CD5⁻/intCD11c⁻) while thymocytes (of 3 m-control mice) were isolated as I-Ad negative cells (to deplete antigen presenting cells). Dead cells were discarded using PI staining during the sorting. After sorting, thymocytes were stained with CellTrace Violet (Invitrogen) according to the manufacturer's instructions and cocultured with B cells at a 10:1 ratio (100,000 thymocytes and 10,000 B cells) in presence of rmIL-7 (6 ng/ml-eBioscience). The cells were cultured in RPMI medium supplemented with 10% FBS, 0.055 µM 2-mercaptoethanol (Gibco), and 0.5 µg/ml Fungizone (Gibco), in U-bottom 96-well-plate (Falcon®). In some cases, a blocking antibody against OX40L (BioLegend, clone RM134L, 10 µg/ml) was used during the co-cultures. Coculture was maintained for 5 days at 37°C and 5% CO₂. Subsequently, cells were recovered, and thymocytes analyzed for T_{FH} phenotype (CD4⁺CD8⁻PD-1⁺CXCR5⁺) in a PI negative gate by flow cytometry.

In vitro B Cell Activation

Total thymic cells were activated at 2 × 10⁶ cells/ml with anti-mouse CD40 at 1.5 µg/ml and anti-mouse IgM at 5 µg/ml for 5 days at 37°C and 5% CO₂ to evaluate OX40L expression on B cells by FACS. On the other hand, total thymic cells

were activated at 2×10^6 cells/ml with LPS at $2 \mu\text{g/ml}$ for 3 days at 37°C and 5% CO_2 to evaluate Blimp-1 expression on B cells after fixation permeabilization with the corresponding buffer.

RNA-Sequencing

RNA extraction from thymic B cells was carried out on 0.6×10^6 thymic B cells isolated by cell sorting ($\text{CD19}^+\text{CD5}^-/\text{intCD11c}^-$) recovered directly in 0.5 ml of TRIzol reagent (Life Technologies). Quantification of RNA was performed using specific fluorometry with the Qubit RNA quantification assay (Life Technologies). RNA integrity was assessed using an RNA Quality Measurement Number (RQN) of Fragment analyzer with the High Sensitivity RNA Analysis Kit (Advanced Analytical Technologies), were used RNA samples with RQN values >8.2 . Sequencing libraries were prepared using the KAPA Stranded mRNA-Seq kit according to the manufacturer's protocol (Illumina). The length of the libraries was determined by capillary electrophoresis using the Standard Sensitivity NGS Fragment Analysis kit (Advanced Analytical Technologies). Libraries were quantified using the KAPA Library Quantification Kit (Kappa Biosystem) using the Eco PCR system (Illumina), following manufacturer's protocol. Libraries were sequenced on a Miseq platform (Illumina) using a v3 150 kit with 2×75 bp paired-end. Samples were subsequently analyzed using R/Bioconductor, and the DESeq2 procedure was used to normalize the data. Differentially expressed genes were identified using an adjusted p -value cut-off of 0.05 and a fold change of at least 1.5.

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE147359 (<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE147359>).

Apoptosis Assay

Cells isolated from the thymus of BWF1 diseased and age-matched control mice were stained with $1 \mu\text{l}$ of Annexin V FITC (BioLegend) and propidium Iodide at $1 \mu\text{g/ml}$ in $100 \mu\text{l}$ of binding buffer (HEPES 10 mM, NaCl 140 mM, CaCl_2 2.5 mM) by incubating for 15 min at room temperature. Staining was stopped by adding $200 \mu\text{l}$ of binding buffer, and the cells were analyzed by flow cytometry.

Statistical Analysis

Statistical analysis was performed with the GraphPad Prism program V6 (GraphPad Software, San Diego, CA, USA). The data were compared using a Student's t -test after verification of normal distribution. Mann Whitney test was used when the data did not adjust to a normal distribution. Wilcoxon signed-rank test was used to compare data with hypothetical value. $P < 0.05$ were considered significant.

RESULTS

Increased B Cell Numbers in the Thymus of Diseased-BWF1 Mice Correlates With an Abnormal Thymic Structure

We and others have reported that the frequency of B cells in the thymus increases significantly during normal aging and in several autoimmune diseases (4, 14, 20, 26). We aimed at studying the dynamics of B cell accumulation in the thymus of BWF1 lupus-prone mice during the development of the autoimmune response. For this, we analyzed the thymus of BWF1 mice at different stages preceding (3 and 5 months old) and after the onset of the disease (9 months old in average) and compared the results to age-matched controls (NZWxBALB/c)F1 mice. We observed a higher than 20-fold increase in B cell frequency and a significant 6-fold increase in absolute B cell numbers in the thymus of diseased-BWF1 mice compared with age-matched control mice (**Figures 1A,B**). A modest but significant increase in the frequency of B cells in the thymus of 5 months old BWF1 mice compared to 3 months old BWF1 mice suggest that the frequency of B cells present in the thymus increases before the onset of proteinuria.

Histological examination of the thymus of diseased-BWF1 and age-matched control mice revealed remarkable alterations in the structure of the thymus at the onset of the disease (**Figure 1C**). Fluorescent co-staining of CD4, CD8, and CD19 confirmed that the thymus of diseased mice is characterized by the presence of large B cell clusters and the absence of $\text{CD4}^+\text{CD8}^+$ double-positive (DP) thymocytes (**Figure 1C**) which are normally found in the cortex (27). In diseased mice (9 months old on average), there was a reduction of the cortex areas and large numbers of B cells (B220^+) clustered into structures reminiscent of germinal centers. In contrast, in age-matched control mice, we observed small numbers of B cells disseminated within the medulla (**Figure 1C**), as previously reported (11). Next, we analyzed the expression of the germinal center marker PNA on thymic B cells (**Figure 1D**). Although we found no statistical difference in the frequency of PNA^+ B cells between age-matched control and diseased BWF1 mice, the absolute number of PNA^+ B cells increase 5-fold in diseased BWF1 mice compared to control mice (**Figure 1E**). We also found an increase in the absolute number, but not in the frequency, of Ki67^+ B cells in the thymus of diseased BWF1 mice (**Figures 1F,G**). Since we do not observe an increase in the percentage of Ki67^+ B cells, our data suggest that the expansion of B cells in the thymus of autoimmune mice may be due to an increase in the migration of B cells from the periphery. Interestingly, we found that in diseased mice there was an expansion of non-epithelial perivascular spaces (PVS) (cytokeratin-5 $^-$) where most B220^+ B cells clustered (**Figure 1C**, **Supplementary Figure 1**). Altogether these results demonstrate that at the onset of SLE, the thymus of BWF1 mice undergoes remarkable changes in terms of structure and B lymphocyte content, with the appearance of ectopic germinal center-like structures.

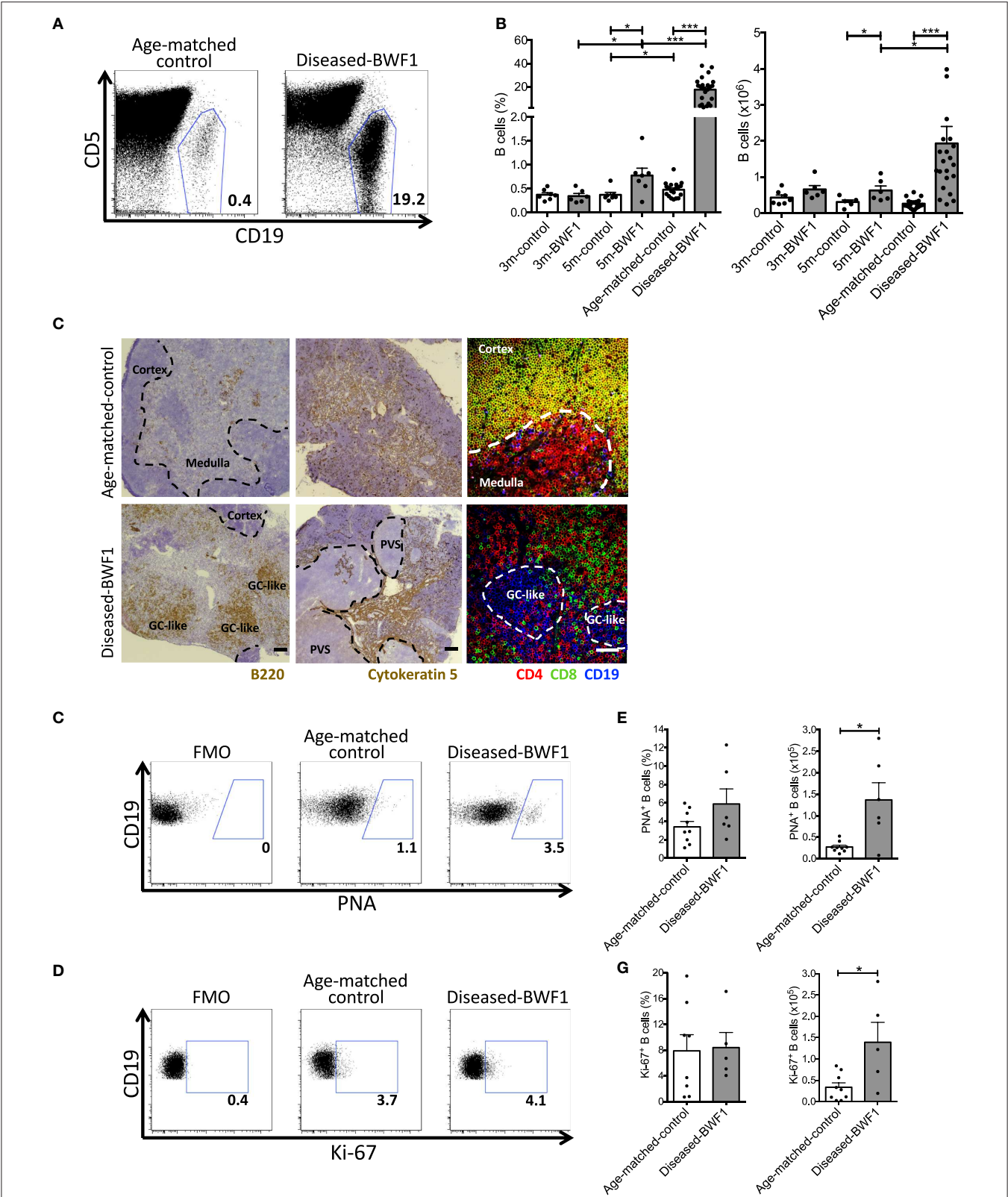


FIGURE 1 | Diseased-BWF1 mice present proliferating thymic B cells that cluster in germinal center-like structures. **(A)** Representative FACS analysis of CD19⁺ cells in the thymus of diseased-BWF1 and age-matched-control mice. CD5 was used to exclude T cells. **(B)** Thymic B cell frequency (left) and absolute number (right) in (Continued)

FIGURE 1 | BWF1 mice at different ages prior (3 and 5 months old) and after the onset of the disease and age-matched control mice. B cells were analyzed as CD19⁺CD11c⁻CD5^{-int} cells. Each dot represents one mouse ($n = 6$ –20 mice per group). Student's *t*-test, * $p \leq 0.05$; *** $p \leq 0.001$. **(C)** Representative light microscopy images of B220 (left panel) and cytokeratin 5 (middle panel) staining of thymic tissue from diseased-BWF1 and age-matched-control mice. Scale bar: 200 μ m. PVS: perivascular spaces. GC-like: germinal center-like structures. The right panel shows representative confocal microscopy images of CD4⁺ T cells (red), CD8⁺ T cells (green), and CD19⁺ B cells (blue) in thymic tissue of BWF1-disease and age-matched control mice. Scale bar: 50 μ m. **(D)** Flow cytometry plots of PNA expression in thymic B cells (CD19⁺CD11c⁻CD5^{-int} gate) from diseased-BWF1 and age-matched-control mice. **(E)** Frequency and absolute number of PNA⁺ B cells in the thymus of diseased-BWF1 and age-matched-control mice. Mann-Whitney test, $p \leq 0.05$. **(F)** Flow cytometry plots of Ki-67 expression in thymic B cells from diseased-BWF1 and age-matched-control mice **(G)** Frequency and absolute number of Ki-67⁺ B cells (CD19⁺CD11c⁻CD5^{-int} gate) in the thymus of diseased-BWF1 and age-matched-control mice. Student's *t*-test, $p \leq 0.05$. Data represent 3–4 independent experiments.

The Thymus of Diseased-BWF1 Mice Harbors IgG Anti-dsDNA Antibody-Secreting Plasma Cells

Present evidence indicates that germinal center formation depends on the activation of antigen-specific B cells by cognate T cells leading to the formation of antibody-secreting plasma cells and memory B cells (28, 29). The distribution of B cells in germinal center-like structures in the thymus of diseased-BWF1 mice suggests that they may be locally activated and differentiated into memory B cells or plasma cells. We next characterized the thymic B cells by analyzing their expression of differentiation markers. Analysis of isotype switched (IgM⁻IgD⁻) memory B cells in diseased mice did not show a significant difference compared to control mice, whereas diseased mice present a significant increase in naïve B cells (IgM⁺IgD⁺) compared to control mice (**Supplementary Figure 2**). These data indicate that a substantial fraction of B cells accumulating in the thymus of diseased mice rather display a naïve than a memory phenotype.

Interestingly, the analysis of thymic plasma cells revealed a significant increase in the percentage and absolute number of plasma cells (B220^{int}CD138⁺) in the thymus of diseased-BWF1 mice compared to age-matched control animals (**Figures 2A,B**). These results are consistent with a higher percentage of Blimp-1⁺ B cells, a transcription factor driving the differentiation of B cells to plasma cells (30, 31), in diseased mice compared to control mice (**Figures 2C,D**). To investigate the presence of functional plasma cells and their specificity, we enumerated antibody-secreting cells (ASC) by ELISpot. These experiments revealed that the thymi from diseased-BWF1 mice contain significantly higher numbers of IgG ASC (>5 times) compared to those from age-matched control mice (**Figures 2E,F**). When compared to IgG production from other organs known to harbor ASC such as bone marrow and spleen, we found a comparable number of spots of IgG ASC between the thymus and bone marrow in diseased-BWF1 mice (**Figure 2F**).

Of note, the thymus of diseased-BWF1 mice contained few IgM ASC, and we could not detect anti-dsDNA ASC of the IgM class (**Supplementary Figure 3**). Thus, the majority of thymic plasma cells in diseased BWF1 mice have gone through isotype switching secreting mainly IgG antibodies, some of which are specific of dsDNA. In summary, our results show that autoimmune BWF1 mice have proliferating B cells in germinal center-like structures within the thymus, which most likely

support the differentiation of B cells into anti-dsDNA IgG-secreting plasma cells. Thymic ASC might, therefore, contribute in a significant way to the pool of secreted IgG autoantibodies found in autoimmune mice.

Thymic B Cells From Diseased-BWF1 Mice Express Genes Associated to Cell Survival

Our data show that the B cells found in the thymus of diseased mice are distinct from normal resident thymic B cells in terms of abundance, localization, proliferation, and antibody secretion. To gain insights into the mechanisms that lead to the accumulation of this peculiar B cell population as well as into their function(s), we analyzed their transcriptomic profile using RNAseq. We identified 337 upregulated genes and 492 downregulated genes in thymic B cells from diseased-BWF1 mice compared to age-matched control mice (**Figure 3A**, **Supplementary Tables 1, 2**). Among the upregulated genes, several were related to B cell survival and development including *Upf1* and *Naip2* (**Figure 3B**). Also, *CD24a*, a negative regulator of early pre-B cell differentiation in the bone marrow was likewise upregulated in diseased mice (32–35). Among the genes that were downregulated in thymic B cells from diseased-BWF1 mice, we found *Hif1a*, *Blk*, and *Btnd2a2a*, whose low expression has been associated with the induction or development of several autoimmune diseases such as collagen-induced arthritis, experimental autoimmune encephalomyelitis and SLE (36–39). The normalized counts of these genes are shown in **Figure 3C**. To confirm that thymic B cells obtained from diseased BWF1 mice have enhanced survival compared to B cells from age-matched controls, we assessed live B cells through Annexin V/PI assay. As shown in **Figures 3D,E**, the live fraction of thymic B cells was significantly higher in diseased-BWF1 mice, suggesting that the thymus of diseased-BWF1 mice provides a niche that supports the survival of B cells.

The Thymus of Diseased-BWF1 Mice Harbor Functional Follicular Helper T Cells

In addition to the abnormal thymic structure and accumulation of B cells, characterization of the T cell thymic compartment of diseased BWF1 mice showed a significant reduction of CD4⁺CD8⁺ double-positive (DP) thymocytes compared to 3 and 5 month-old BWF1 mice (**Figure 4**), which is consistent with these animals exhibiting a smaller cortex, as observed by histology (**Figure 1C**). Along with the decrease of DP cells,

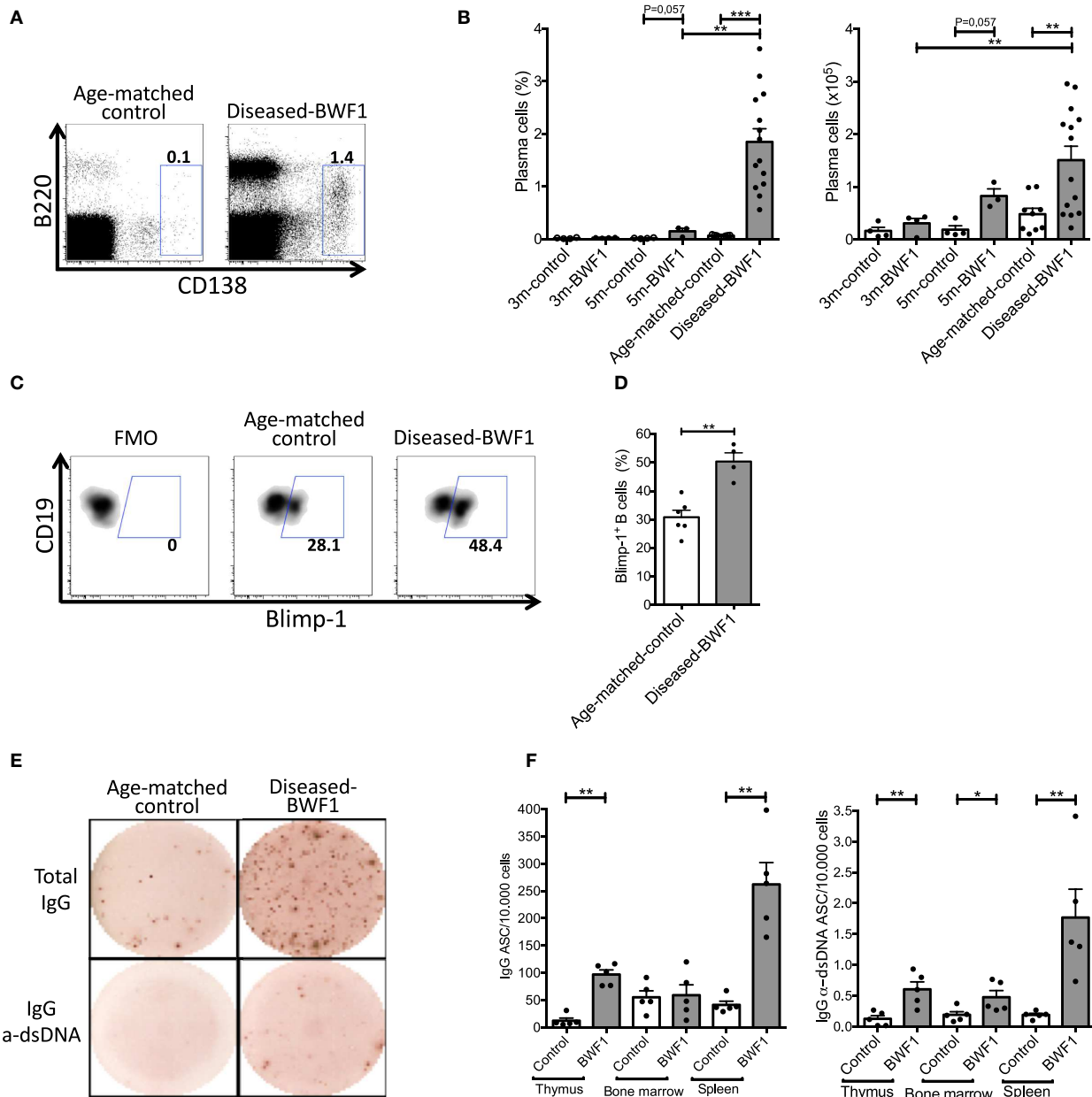


FIGURE 2 | The thymus of diseased-BWF1 mice harbors IgG anti-dsDNA antibody-secreting plasma cells. **(A)** Flow cytometry plots of B220^{int}CD138⁺ plasma cells gated on live thymic cells of diseased-BWF1 and age-matched-control mice. **(B)** Frequency (left) and absolute number (right) of plasma cells (B220^{int}CD138⁺) in BWF1 mice and age-matched controls at different stages prior and after the onset of the disease. Each dot represents one mouse ($n = 3$ –14 mice). **(C)** Flow cytometry plots and **(D)** frequency of Blimp-1⁺ thymic B cells from diseased-BWF1 and age-matched control mice after activation with LPS. Each dot represents one mouse ($n = 4$ –6 mice). Mann-Whitney's, $^{**}p \leq 0.01$. **(E)** Representative ELISPOT of total and anti-dsDNA IgG antibody-secreting cells (ASC) present in the thymus of diseased-BWF1 and age-matched control mice. **(F)** Quantification of total and anti-dsDNA IgG antibody-secreting cells in the thymus, bone marrow and spleen from diseased-BWF1 and age-matched control mice. Each dot represents one mouse ($n = 5$ mice). Student's t -test, $^{*}p \leq 0.05$; $^{**}p \leq 0.01$; $^{***}p \leq 0.001$.

we found an increase in the frequency of CD4⁺CD8⁺ double-negative cells (DN) and both CD4⁺ and CD8⁺ single-positive (SP) thymocytes compared to 3 and 5 month-old BWF1 mice and control mice (**Figure 4**). These results indicate that during

the onset of the disease, the thymus of BWF1 autoimmune mice suffers significant changes in its T cell composition in addition to the accumulation of B cells. CD69 expression on DP cells was only transiently decreased in 3 months-old BWF1

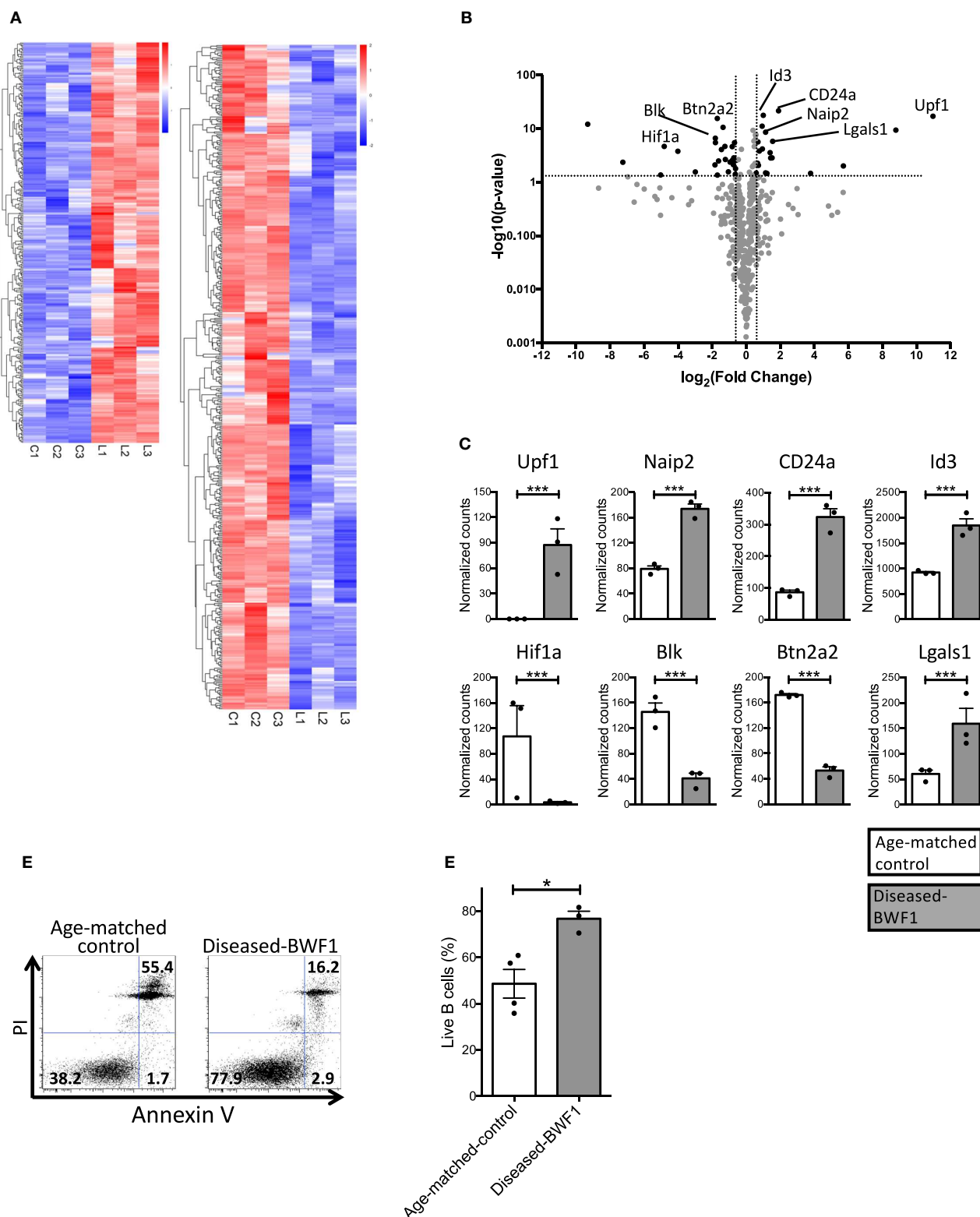


FIGURE 3 | Thymic B cells from diseased-BWF1 mice present a transcriptional profile associated to cell survival. **(A)** RNAseq transcriptome analysis of thymic B cells from diseased-BWF1 and age-matched control mice. Heatmap shows genes that are upregulated (red) and downregulated (blue) with at least 1.5-fold change and adjusted $p < 0.05$ (detailed list of genes is provided in **Supplementary Tables 1, 2**). **(B)** Volcano plot of selected genes related to apoptosis and B cell development. **(C)** RNA-seq normalized counts for selected genes. White bars: age-matched control mice; gray bars: diseased BWF1 mice. **(D)** Flow cytometric analysis of live and apoptotic B cells as assessed by Annexin V and PI in cells from thymus of diseased-BWF1 mice and age-matched control mice. **(E)** Frequency of live thymic B cells (Annexin V⁻ PI⁻) from diseased-BWF1 mice and age-matched control mice. Mann-Whitney's t -test, * $p \leq 0.05$, *** $p \leq 0.001$.

mice, previous to developing proteinuria, suggesting that the process of positive selection is not altered during the disease (**Supplementary Figure 4**).

Interestingly, transcriptomic profiling of thymic B cells from diseased BWF1 mice further revealed the increased expression of *Id3* and *Lgals1* (**Figures 3B,C**), two genes known to support the maintenance of germinal center B cells and humoral immune response (40–42). Therefore, we investigated whether the frequency and number of follicular helper T (T_{FH}) cells were enhanced in the thymus of diseased-BWF1 mice. tSNE analysis on $CD4^+$ T cells allowed us to dissect the composition of the naive ($CD44^{lo}$) and antigen experienced ($CD44^{hi}$) T cell thymic compartments. As shown in **Figure 5A**, **Supplementary Figures 5A,B**, we observed an abundance of antigen-experienced $CD44^{hi}$ T cells in the thymus from diseased-BWF1 mice compared to age-matched control mice. The increase in antigen experienced T cells in diseased mice is concomitant to a reduction in the immature and mature naive T cells (**Supplementary Figures 6A,B**). Among thymic antigen-experienced $CD4^+$ T cells found in diseased-BWF1, we detected a variety of different subsets such as memory tissue-resident $CD103^+$, some of which also express *CD69*. We also observed an increase in the frequency of regulatory T cells in diseased mice (**Supplementary Figures 6A,B**). Moreover, tSNE analysis revealed the appearance in diseased mice of a subset with T_{FH} phenotype co-expressing *PD-1* and *CXCR5*, which was absent in age-matched controls (**Figure 5A**). Further analysis revealed that the percentage and absolute numbers of $PD-1^+CXCR5^+$ increased dramatically in the thymus of diseased BWF1 mice compared to the thymus of age-matched control mice (**Figures 5B,C**). These thymus-residing $PD-1^+CXCR5^+$ cells express high levels of *Bcl-6*, a transcription factor that specifies T_{FH} program (**Supplementary Figure 7**). These results suggest that the presence of B cells and GC-like structures in the thymus of diseased BWF1 mice may be associated to the appearance of T_{FH} cells.

Similar to the role of T_{FH} cells in B-cell maturation during normal immune responses, results from animal models of SLE as well as from patients with this disease indicates that T_{FH} cells are required for autoantibody production (43, 44). To evaluate if T_{FH} present in the thymus of diseased mice could drive B cell activation and proliferation, we performed *in vitro* co-culture assays with sorted thymic T_{FH} and B cells from diseased BWF1 mice (45). Thymic T_{FH} from diseased BWF1 mice induced the proliferation of activated $GL7^+$ B cells to a similar level as splenic T_{FH} obtained from BWF1 mice (**Figure 5D**). Of note, non- T_{FH} $CD4^+$ T cells from either thymus or spleen were unable to induce proliferation of activated B cells (**Figure 5D**). These results indicate that T_{FH} present in the thymus of autoimmune mice are functional and possibly contribute to the activation and expansion of thymic B cells in diseased BWF1 mice.

Thymic B Cells From Diseased BWF1 Mice Induce the Differentiation of Follicular Helper T Cells

It is known that B cells support T_{FH} cell differentiation via OX40L in the spleen (46, 47). This evidence prompted

us to investigate whether thymic B cells could promote the differentiation of thymic T_{FH} cells. Accordingly, we found an increase in the frequency of thymic B cells expressing OX40L (2-fold) in diseased mice compared to age-matched controls (**Figures 6A,B**). Additionally, we did not find any differences in the expression of co-stimulatory molecules (*CD83*, *CD86*, and *CD40*) and the antigen presenting molecule (*I-Ad*) between B cells from diseased BWF1 mice and age-matched-control mice (**Supplementary Figure 8**). To demonstrate that thymic B cells favor the development of T_{FH} cells, we carried out co-culturing experiments of thymus B cells and thymocytes from control animals in the presence of IL-7. We observed that thymic B cells from diseased mice generate a more significant percentage of T_{FH} cells ($PD-1^+CXCR5^+$) than thymic B cells from age-matched control mice (5.2% with BWF1 B cells vs. 1.3% with control B cells) (**Figures 6C,D**). Splenic B cells were also capable of inducing the differentiation of thymocytes to T_{FH} (**Supplementary Figure 9**). Of note, thymic B cells from diseased-BWF1 mice induced a higher proliferation of $CD4^+SP$ thymocytes than B cells from age-matched control mice (**Figure 6E**) but there were no differences in *CD25* expression between the different conditions (data not shown). To evaluate whether OX40L is important to support the T_{FH} differentiation we performed the culture of thymic B cells from diseased-BWF1 mice with thymocytes in presence of an OX40L blocking antibody and we found a reduction on T_{FH} differentiation and proliferation of the $CD4^+SP$ thymocytes (**Supplementary Figure 10**). These results suggest that thymic B cells from diseased mice support the differentiation of thymocytes into T_{FH} cells through OX40L.

DISCUSSION AND CONCLUSION

SLE is a chronic autoimmune disease of unknown etiology characterized by the formation of immune complexes, which are deposited in tissues causing inflammation. In SLE, both T and B cells are overactivated and recognize autoantigens related to nuclear proteins. Although the presence of B cells in the thymus in BWF1 mice, a murine model of SLE, has already been demonstrated (24, 25), the development of this population during the progression of this autoimmune disease remains mostly unexplored. Using the BWF1 mice, here we report an increase in the number and frequency of B cells, plasma cells, and follicular helper T cells (T_{FH}) in the thymus of lupus diseased mice. Moreover, our data provide evidence that these B cells proliferate and cluster in ectopic germinal centers within the perivascular space (PVS) of the thymus. Additionally, thymic cells obtained from diseased mice produce IgG anti-dsDNA antibodies demonstrating the presence of autoantibody-secreting cells. Finally, we demonstrate that thymic B cells from diseased-BWF1 mice favor the differentiation of T_{FH} , which may, in turn, promote the activation and differentiation of B cells into autoreactive plasma cells in the thymus.

Previous studies have shown the presence of B cells in the thymus, which has been attributed a role of antigen-presenting cells involved in the negative selection of T cells (10–12). However, we demonstrated that the thymus of diseased mice loses the classical structure defined by the functional separation

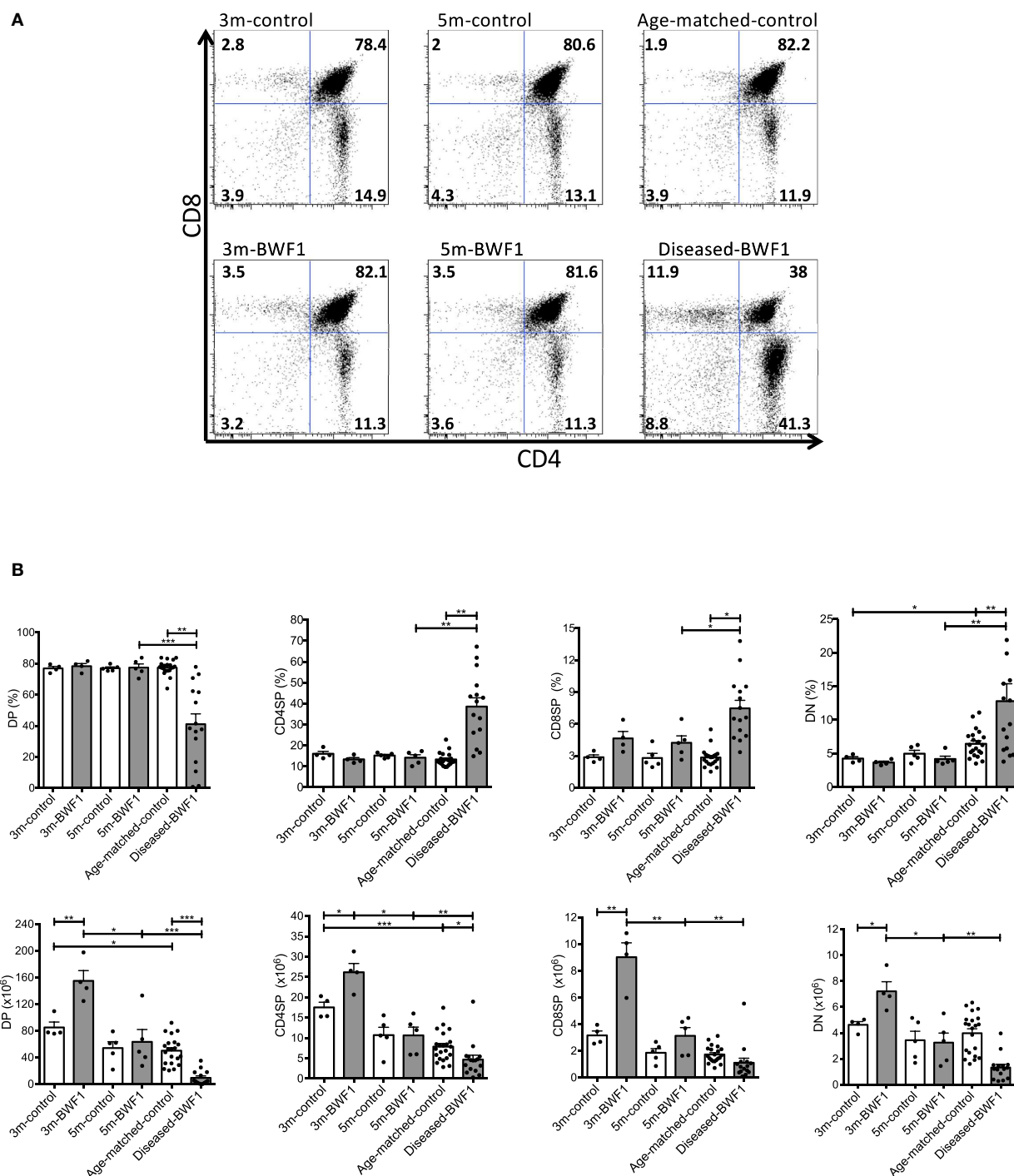


FIGURE 4 | Subpopulations of thymocytes are altered in diseased-BW1 mice. **(A)** Representative FACS analysis of thymic DP, CD4SP, CD8SP, and DN populations in BW1 mice at different ages prior and after the onset of the disease and age-matched control mice. The cells were analyzed in a I-Ad⁻ gate ($n = 4-15$ mice per group). **(B)** Summary of the frequency (up) and the absolute number of thymocyte populations (down). Each dot represents one mouse. Student's *t*-test, * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

in cortex and medulla, where the processes of selection of the T cells occur. This observation led us to investigate whether these B cells might be involved in different processes independent of antigen presentation and negative selection of T cells. In this line,

Pinto and collaborators have reported that thymic B cells produce autoantibodies that attack the thymic stroma, an event that precedes the development of type 1 diabetes (20). Moreover, our previous results demonstrated that the human thymus, as it ages

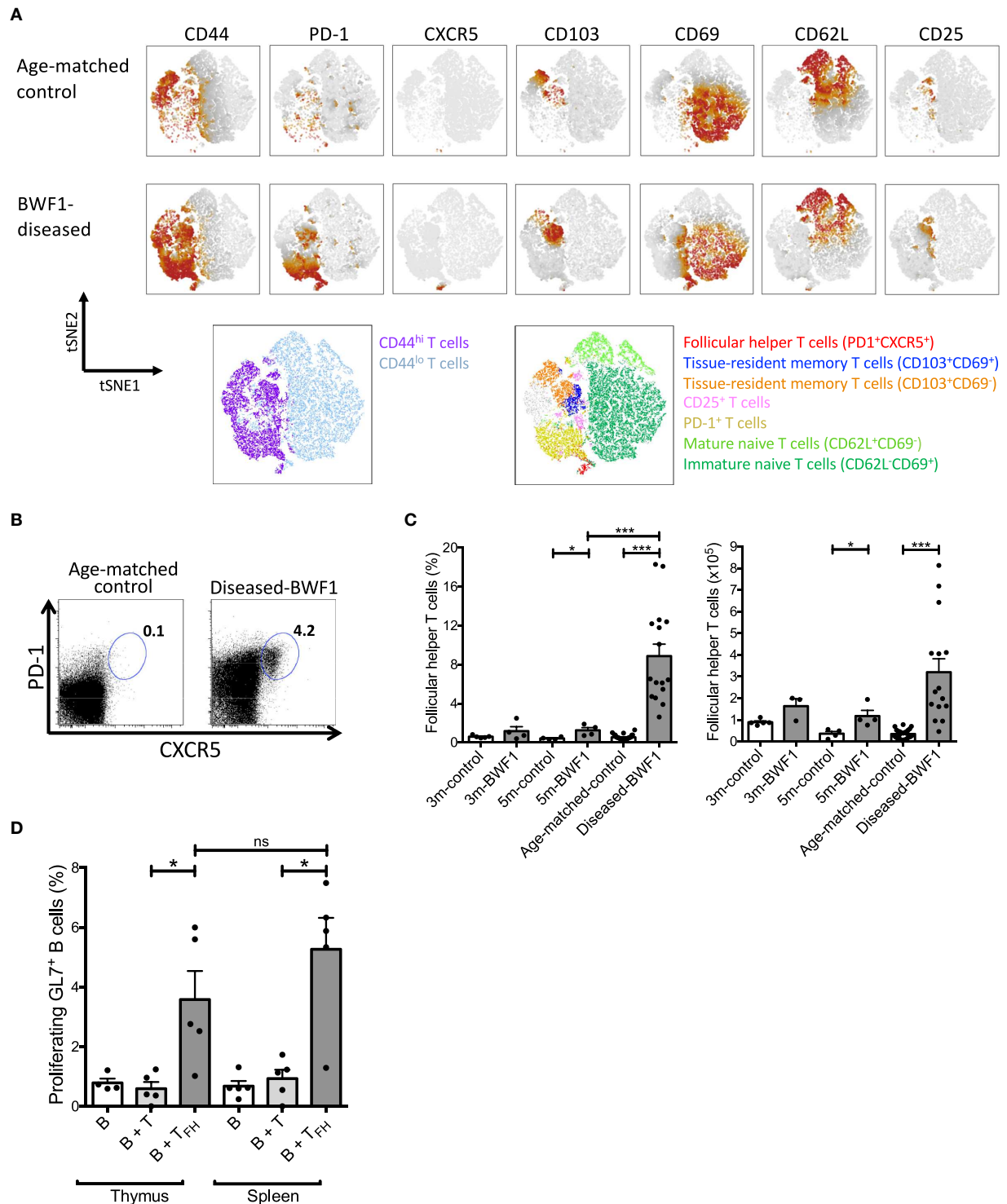


FIGURE 5 | Functional follicular helper T cells increase in the thymus of diseased-BWF1 mice. **(A)** tSNE analysis of CD4SP (CD4⁺CD8⁻ gate) cells of diseased-BWF1 and age-matched-control mice. Bottom panel shows populations colored by manual gating. **(B)** Flow cytometry plots of PD-1 and CXCR5 expression in CD4SP cells from the thymus of diseased-BWF1 mice and age-matched-control. **(C)** Frequency and absolute number of follicular T helper cells (PD-1⁺CXCR5⁺) in BWF1 mice at different ages prior and after the onset of the disease and age-matched control mice. Each dot represents one mouse ($n = 4-16$ mice). Student's *t*-test, $*p \leq 0.05$; $***p \leq 0.001$. **(D)** Percentage of proliferating thymic GL7⁺ B cells (CD19⁺) after coculture with either PD-1⁺CXCR5⁺ thymic follicular helper T cells (B+T_{FH}) or thymic PD-1⁻CXCR5⁻ non-follicular CD4⁺ T cells (B+T) isolated from diseased-BWF1 mice. The same experiments were performed with cells from spleen. Each dot represents one mouse ($n = 4-5$ mice). Student's *t*-test, $*p \leq 0.05$.

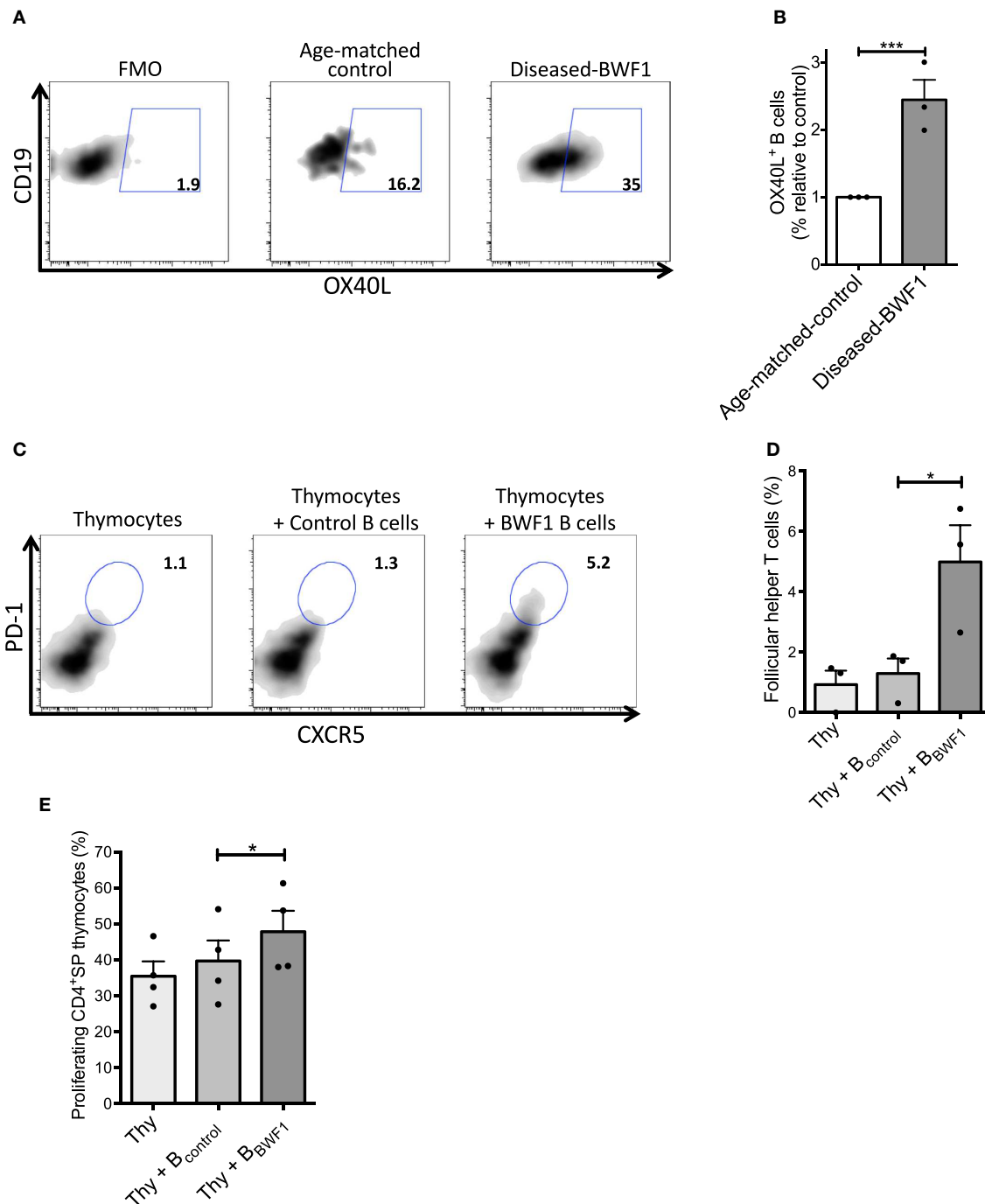


FIGURE 6 | Thymic B cells favor the expansion of follicular helper T cells in diseased-BWF1 mice. **(A)** Flow cytometry plots of OX40L expression in thymic B cells from diseased-BWF1 and age-matched-control mice following 5-days activation with anti-IgM and anti-CD40. **(B)** Frequency of thymic OX40L⁺ B cells from diseased-BWF1 relative to OX40L⁺ B cells from age-matched-control mice. Data represent three independent experiments. Wilcoxon signed rank's test, *** $p \leq 0.001$. **(C)** Flow cytometry plots and **(D)** frequency of PD-1⁺CXCR5⁺ follicular helper T cells (in a CD4⁺CD8⁻ gate) 5 days after co-culture of thymocytes (Thy) with thymic B cells from diseased-BWF1 (Thy+B_{BWF1}) or age-matched control mice (Thy+B_{control}), in presence of IL-7 (6 ng/ml). Data shows representative results of three independent experiments. **(E)** Proliferation of CD4SP thymocytes assessed by cell-trace violet dilution after co-culture of control thymocytes (Thy) with thymic B cells from diseased-BWF1 (Thy+B_{BWF1}) or age-matched control mice (Thy+B_{control}). Data represent the results of three independent experiments. * $p \leq 0.05$.

may provide a niche for viral-specific plasma cells (14). The novel data presented here showing the presence of ectopic germinal centers, auto-antibody secreting plasma cells, and T_{FH} cells

strongly argue in favor of the idea that during an autoimmune response, the thymus may acquire a new function as a niche suitable for the development of a humoral immune response.

An important finding presented here is that during the development of the autoimmune response, there is a significant increase in the frequency of B cells present in the thymus of BWF1 mice. This is not only a consequence of enrichment of B cells due to the reduction of double-positive thymocytes since as we report, there is a 6-fold increase in the absolute number of thymic B cells in diseased-BWF1 mice. An unresolved question that arises from this work is the origin of the B cells that accumulate in the thymus of diseased-BWF1 mice. Adoptive transfer experiments with splenic B cells as well as experiments with parabionts have shown that migration of peripheral B cells to the thymus in steady-state conditions does not contribute significantly to the pool of thymic B cells (11, 48, 49). However, under inflammatory conditions such as systemic LPS treatment, *Candida albicans* or *Trypanosoma cruzi* infection, it was demonstrated that mature B and T cells could efficiently migrate to the thymus (50). Thus, an intriguing possibility is that during chronic inflammation in autoimmune BWF1 mice, B cells from the periphery may continuously migrate to the thymus where they survive, proliferate, and differentiate into plasma cells.

The central role of the CXCL13 chemokine (B lymphocyte chemoattractant or BLC) in the recruitment of B cells to the thymus has already been established (51). Using a murine model of myasthenia gravis, Weiss et al. demonstrated that although thymic overexpression of CXCL13 under steady-state condition does not induce B cell recruitment to the thymus, under inflammatory conditions such as after immunization with Poly (I:C), CXCL13 overexpression enhanced B cell migration to this organ (52). In the murine model of SLE, the group of Matsushima demonstrated that dendritic cells in the thymus of BWF1 mice produce CXCL13 which attracts B cells to this organ during the development of the disease (24). The same group further explored this possibility and showed that when B cells are injected intravenously, they can enter the thymic PVS and the medulla of aged BWF1 mice (25). Thus, CXCL13 production in the thymus under inflammatory conditions may be sufficient to drive B cell migration to this organ.

Further evidence of B cell lymphopoiesis within the thymus was previously reported by Perera et al. where the authors use the Rag2-GFP reporter mice and demonstrate that B cells can develop from precursors within the thymus (11). Thus, it may be possible that during the autoimmune response, B cell lymphopoiesis within the thymus might be enhanced or there might be an increase in the survival of B cells in this organ. Whether B cells come from the periphery or are differentiated *in situ*, our RNAseq data supports the idea that within the autoimmune thymus, B cells might be exposed to an altered environment that effectively boosts their proliferation and survival. Thus, in any possible scenario, the accumulation of B cells may be favored by remarkable changes in the thymic niche during the autoimmune response supporting B cell survival and/or differentiation.

Several studies support a role of IL-7 and Delta like 4 (Dll4)-Notch signaling pathways in regulating lymphocyte development in the thymus. The group of El-Kassar has demonstrated that transgenic mice that overexpress IL-7 show a dysregulation in

thymocyte populations and an increase of B cell populations due to an induction of B-lymphopoiesis in the thymus. Interestingly, the treatment with IL-7 blocking antibodies reduces B cell populations in this organ (53). On the other hand, Billiard and collaborators have shown that anti-Dll4 treatment reduces thymocyte populations favoring the expansion of mature B cells in the thymus (54). Accordingly, it would be interesting to study IL-7 levels and Dll4 expression in the thymus of diseased-BWF1 mice in order to evaluate the contribution of these pathways in the aberrant cellular composition we observe in these mice.

The germinal center is a structure typically developed in secondary lymphoid organs where antigen-specific B cells receive the proper differentiation signals from T_{FH}, proliferate, and undergo somatic hypermutation. Germinal centers form in the center of the B cell follicles of secondary lymphoid organs, interspersed within a network of stromal cells known as follicular dendritic cells (FDCs) (55, 56). The presence of ectopic germinal centers has been widely reported in the thymus of patients with myasthenia gravis, an autoimmune disease characterized by the presence of anti-acetylcholine receptor autoantibodies. In this disease, the development of thymic follicular hyperplasia is frequently observed, with the presence of ectopic germinal centers, characterized by the presence of T_{FH} cells, B cells, and FDCs (57, 58). Our own unpublished results show that the thymus of BWF1 diseased mice present CD21/CD35+ cells within the CD45- compartment, however, we cannot rule out that these cells are B cells with lower CD45 staining or a subset of thymic epithelial cells that express CD21/CD35 as self-antigens. Although here we present significant evidence that the thymus of BWF1 diseased mice also develops ectopic germinal centers (B cell organization in the tissue, presence of T_{FH} cells and plasma cells), further studies should elucidate if these structures resemble canonical lymph node germinal centers and demonstrate that thymic B cells undergo somatic hypermutation locally.

Using a different murine model of SLE, the B6.*Sle16* lupus-prone mice, it was reported that B cells support the generation of T_{FH} through OX40L expression (46). In that report, the authors demonstrate that the ablation of OX40L expression, specifically in B cells, results in the reduction of T_{FH} and a significant decrease in the autoimmune response in these mice. In agreement with a role of OX40L in the induction of T_{FH}, we also show that in the diseased-BWF1 mice, there is an increase in the frequency of T_{FH} along with an increase in thymic B cells that express OX40L compared to control mice. Moreover, we demonstrate that only thymic B cells from diseased-BWF1 mice have the capacity to induce the differentiation of thymocytes to T_{FH} in an OX40L dependent fashion. Thus, our results recapitulate the role of OX40/OX40L interactions in the generation of the germinal center reaction observed in secondary lymphoid organs in lupus-prone mice reported by Cortini and collaborators (46). This leads us to propose that the germinal center reaction found in the thymus of lupus-prone mice is directly responsible for the generation of the auto-antibody secreting plasma cells in this organ rather than being the result of plasma cells arriving from the periphery. Activation of autoreactive B cells and differentiation into autoantibody producing plasma cells in germinal centers within the thymus

may be favored by the interaction with T_{FH} . Finally, thymic B cells may also induce the differentiation of $CD4^+$ T cells to T_{FH} , generating a positive feedback loop that sustains the humoral immune response within the thymus.

Interestingly, thymic morphological and functional alterations observed in the BWF1 and SLE patients have been described in several other autoimmune diseases including *myasthenia gravis*, type 1 diabetes, Sjogren's syndrome and ulcerative colitis (5, 20). Despite the distinct pathophysiological features, all these alterations have in common chronic inflammation, which may be hijacking the normal thymic function of T cell repertoire selection to establish a niche that sustains the humoral immune response. Studies on thymectomized BWF1 mice could give some insight into the role of the thymus as a source of autoantibodies and its real contribution to the development of the disease.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE147359.

ETHICS STATEMENT

The animal study was reviewed and approved by Comité de Bioética de Fundación Ciencia y Vida and CICUA from Universidad de Chile.

AUTHOR CONTRIBUTIONS

YH designed the study, performed experiments, analyzed the data, and wrote the manuscript. SN, MF, FF-S, PS, VM, and JD performed experiments and analyzed the data. MB, DS, SN, MR, EZ, and AL-D designed the study, analyzed the data, and wrote the manuscript. All authors critically read the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2020.00696/full#supplementary-material>

Supplementary Figure 1 | Thymic B cells from diseased-BWF1 mice are localized in perivascular spaces. Representative images of thymic tissue from diseased-BWF1 stained with B220 (left panel) and cytokeratin 5 (right panel).

Supplementary Figure 2 | Subpopulations of B cells are altered in diseased-BWF1 mice. **(A)** IgD and IgM expression in thymic B cells ($CD19^+GL7^-$) from diseased-BWF1 and age-matched-control mice. **(B)** Summary of the frequency (up) and absolute number (down) of the IgM^-IgD^+ , IgM^+IgD^+ , and IgM^+IgD^- B cells populations. $**p \leq 0.01$, $***p \leq 0.001$.

Supplementary Figure 3 | Total IgM and IgM anti-dsDNA antibodies in thymus of diseased-BWF1 mice. ELISPOT analysis was performed with total cells obtained from the thymus of diseased-BWF1 and age-matched control mice. Representative experiment of two independent experiments.

Supplementary Figure 4 | CD69 expression in DP thymocytes from diseased BWF1 and control mice. **(A)** Representative histogram of CD69 expression in DP thymocytes from diseased-BWF1 mice and age-matched-control mice. The analysis was performed in a $CD4^+CD8^+$ (DP) gate. **(B)** Summary of CD69 expression in DP thymocytes of BWF1 mice at different ages prior and after the onset of the disease and age-matched control mice. MFI: Median fluorescence intense. **(C)** Summary of the frequency of $CD69^+$ DP thymocytes from BWF1 mice at different ages prior and after the onset of the disease and age-matched control mice. Each dot represents one mouse ($n = 3-6$ mice per group). Student's *t*-test, $***p \leq 0.001$.

Supplementary Figure 5 | Diseased-BWF1 mice present an increase in the frequency of antigen-experienced $CD44^{hi}$ T cells in the thymus. Analysis of the thymic antigen-experienced T cells, immature, and mature naïve T cell population in BWF1 mice at different ages prior and after the onset of the disease and age-matched control mice **(A)** Representative example of $CD44$ and $CD62L$ expression in thymocytes from diseased-BWF1 and age-matched control mice. Analysis was carried out in a $CD4SP$ ($CD4^+CD8^-$) gate. **(B)** Frequency of thymic antigen-experienced T cell populations ($CD44^{hi}$), immature ($CD44^{lo}CD62L^-$), and mature ($CD44^{lo}CD62L^+$) T cells in BWF1 mice at different ages prior and after the onset of the disease and age-matched control mice. $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$.

Supplementary Figure 6 | Diseased-BWF1 mice present an increase in the frequency but not in the absolute number of regulatory T cells in the thymus. FACS analysis of regulatory T cells in BWF1 mice at different ages prior and after the onset of the disease and age-matched control mice. **(A)** Representative example of Foxp3 and CD25 expression in thymocytes $CD4^+SP$ ($CD4^+CD8^-$) from diseased-BWF1 and age-matched control mice. **(B)** Frequency (left) and absolute number (right) of regulatory T cells in BWF1 mice at different ages prior and after the onset of the disease and age-matched control mice. Student's *t*-test, $*p \leq 0.05$; $***p \leq 0.001$.

Supplementary figure 7 | Thymic follicular helper T cells from diseased-BWF1 express Bcl-6 transcription factor. **(A)** Flow cytometry plots of $PD-1^+CXCR5^+$ T follicular helper cells (T_{FH}) and Non- T_{FH} ($PD-1^-CXCR5^-$) of diseased-BWF1 mice (left). Analysis was carried out in a $CD4SP$ ($CD4^+CD8^-$) gate. Analysis of Bcl-6 expression in T_{FH} and Non- T_{FH} from thymus of diseased-BWF1 mice (right). **(B)** Summary of Bcl-6 expression in two independent experiments. MFI, Median fluorescence intense.

Supplementary Figure 8 | Thymic B cells from diseased-BWF1 and age-matched control mice express similar levels of co-stimulation and antigen presentation molecules. The expression of CD83, CD86, CD40, and I-Ad in thymic B cells of diseased-BWF1 and age-matched-control mice was assessed by FACS in a $CD19^+CD11c^-$ gate.

Supplementary Figure 9 | Splenic B cells induce follicular helper T differentiation from thymocytes. Frequency of $PD-1^+CXCR5^+$ follicular helper T cells (in a $CD4^+CD8^-$ gate) 5 days after co-culture of thymocytes (from 3 m-control mice) with splenic B cells from diseased-BWF1 or age-matched control mice, in presence of IL-7 (6 ng/ml).

Supplementary Figure 10 | Thymic B cells from diseased-BWF1 mice favor the expansion of follicular helper T cells in an OX40L-dependent manner. **(A)** Flow cytometry plots of $PD-1^+CXCR5^+$ follicular helper T cells (in a $CD4^+CD8^-$ gate) 5 days after co-culture. Thymocytes (from 3 m-control) were cultured with thymic B cells from diseased-BWF1 in presence of IL-7 (6 ng/ml) in all conditions and in the presence or absence of an α OX40L blocking antibody (clone RM134L, 10 μ g/mL). **(B)** Proliferation of $CD4^+SP$ populations 5 days after co-culture with thymic B cells as assessed by cell trace violet dilution.

Supplementary Table 1 | RNAseq. List of genes upregulated in thymic B cells from diseased-BWF1 compared to thymic B cells from age-matched control mice. The genes in the list were selected with at least 1.5-fold change and p -value < 0.05.

Supplementary Table 2 | RNA-seq. Genes downregulated in thymic B cells from diseased-BWF1 compared to thymic B cells from age-matched control mice. The genes in the list were selected with at least 1.5-fold change and p value < 0.05.

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Thymic Function Associated With Cancer Development, Relapse, and Antitumor Immunity – A Mini-Review

Weikan Wang¹, Rachel Thomas¹, Olga Sizova² and Dong-Ming Su^{3*}

¹ Cell Biology, Immunology, and Microbiology Graduate Program, Graduate School of Biomedical Sciences, University of North Texas Health Science Center, Fort Worth, TX, United States, ² Department of Hematopoietic Biology and Malignancy, The University of Texas MD Anderson Cancer Center, Houston, TX, United States, ³ Department of Microbiology, Immunology, and Genetics, University of North Texas Health Science Center, Fort Worth, TX, United States

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*Correspondence:

Dong-Ming Su
dong-ming.su@unthsc.edu

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The thymus is the central lymphoid organ for T cell development, a cradle of T cells, and for central tolerance establishment, an educator of T cells, maintaining homeostatic cellular immunity. T cell immunity is critical to control cancer occurrence, relapse, and antitumor immunity. Evidence on how aberrant thymic function influences cancer remains largely insufficient, however, there has been recent progress. For example, the involuted thymus results in reduced output of naïve T cells and a restricted T cell receptor (TCR) repertoire, inducing immunosenescence and potentially dampening immune surveillance of neoplasia. In addition, the involuted thymus relatively enhances regulatory T (Treg) cell generation. This coupled with age-related accumulation of Treg cells in the periphery, potentially provides a supportive microenvironment for tumors to escape T cell-mediated antitumor responses. Furthermore, acute thymic involution from chemotherapy can create a tumor reservoir, resulting from an inflammatory microenvironment in the thymus, which is suitable for disseminated tumor cells to hide, survive chemotherapy, and become dormant. This may eventually result in cancer metastatic relapse. On the other hand, if thymic involution is wisely taken advantage of, it may be potentially beneficial to antitumor immunity, since the involuted thymus increases output of self-reactive T cells, which may recognize certain tumor-associated self-antigens and enhance antitumor immunity, as demonstrated through depletion of autoimmune regulator (AIRE) gene in the thymus. Herein, we briefly review recent research progression regarding how altered thymic function modifies T cell immunity against tumors.

Keywords: thymic involution, negative selection and regulatory T (Treg) cell generation, cancer immunity, tumor microenvironment, tumor reservoir

Abbreviations: AIRE, autoimmune regulator; ERK, extracellular signal-regulated kinases; MAPK, mitogen-activated protein kinases; MHC-II, major histocompatibility complex class-II; mTECs, medullary TECs; PD-1, programmed cell death protein 1; PD-L1, programmed death-ligand 1; pTreg, peripheral Treg cells; SA-T, senescence-associated T cells; TAA, tumor-associated antigen; Tcon, conventional T cells; TCR, T cell receptor; TECs, thymic epithelial cells; T_{eff}, T effector cells; Treg, regulatory T cells; TSA, tumor-specific antigen; tTreg, thymic regulatory T cells.

INTRODUCTION

T cells are key players in cell-mediated antitumor immunity (1–4) as they have a diverse TCR repertoire specifically recognizing tremendous numbers of tumor neo-antigens, termed TSAs (5, 6), resulting from genomic mutations or viral infection. They can directly kill malignant cells in cytotoxic manners (1, 7, 8) and interact with other tumor-infiltrating immune cells (9) influencing immune surveillance. T cells are thymus-derived, heterogeneous lymphocytes, mainly including $\alpha\beta$ -TCR CD4⁺/CD8⁺ and $\gamma\delta$ -TCR T cells (10). As $\alpha\beta$ -TCR T cells are the most abundant and comprehensively studied sub-population involved in antitumor immunity, we focus on this population.

The thymus mediates T cell development and the signals received by thymocytes from thymic stromal cells, primarily TECs, determine thymocyte fate. For example, Notch ligands expressed by TECs provide continuous Notch signals to thymocytes to decide each stage of T-lineage development (11, 12). Interleukin (IL)-7 is a second indispensable factor produced by TECs for the survival, proliferation and differentiation in early stages of T cell development (13, 14). After the completion of TCR rearrangement, the development and differentiation of T cells depend on the interaction between TCR and major histocompatibility complex (MHC)/self-antigens. This interaction leads to establishment of central tolerance via negative selection and regulatory T (Treg) cell selection (15–17). Thymic involution induced by primary TEC defects affects this signaling by impacting lymphostromal interactions. The process of T cell development in the thymus is complex, but there are several important checkpoints that decide successful establishment of immune surveillance and antitumor immunity: (a) $\alpha\beta$ -TCR rearrangement to acquire various specificities of antigen recognition; (b) positive selection to achieve MHC restriction; and (c) negative selection/Treg cell generation to establish central tolerance to self (13, 17).

Thymic involution resulting from primary TEC defects occurs in the age-related phenotype, and not only reduces output of naïve T cells (18, 19), but also perturbs the interactions between MHC-II/self-peptide complexes on mTECs and TCRs on thymocytes, thereby altering TCR signaling strength, which impairs thymic negative selection and relatively enhances CD4⁺ thymic Treg (tTreg) cell generation (20, 21). These changes could lead to declined tumor immune surveillance, potentially attributed to a reduced capacity to recognize neo-antigens and deplete neoplasia. On the other hand, deliberately increasing release of self-reactive conventional T (Tcon) cells that are able to recognize tumor-borne self-antigens could enhance antitumor immunity (22–24). In addition, during aging, the involuted thymus generates relatively increased polyclonal tTreg cells (20), which, coupled with accumulated peripheral Treg (pTreg) cells (25, 26), may infiltrate to tumor mass and establish a microenvironment that suppresses both CD8⁺ and CD4⁺ T cell-mediated antitumor immunity, facilitating tumor cell survival (16, 27, 28). This could be related to the higher cancer incidence observed in the elderly (29).

Further, tumor-bearing individuals could be afflicted with cancer-related contributors of acute thymic involution, including

(a) increased apoptosis of TECs and thymocytes (30–34) and obstruction of thymocyte maturation (32, 35, 36); and/or (b) chemotherapy-induced non-malignant thymic cellular apoptosis and senescence response (37–39). These will further disrupt antitumor immunity by disrupting T cell development and creating a tumor reservoir in the involuted thymus, allowing for tumor cell dormancy and eventually metastatic relapse (37, 38).

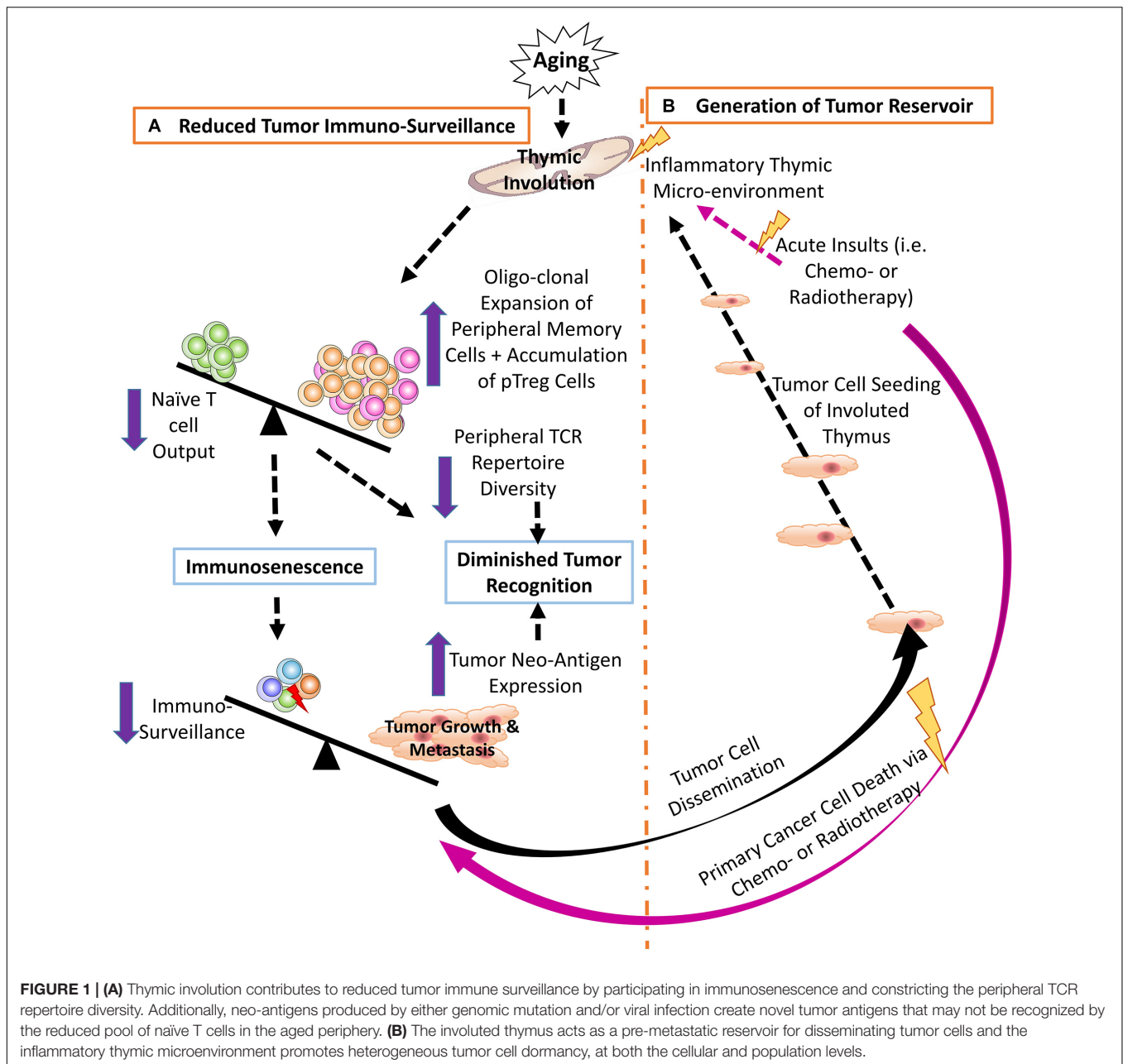
Therefore, thymic conditions impacting T cell immunity are critical issues underlying the high risk for late-life tumor development and the effectiveness (or lack thereof) of antitumor immunotherapy. Revealing the relationship between thymic conditions and T cell-mediated antitumor immunity may facilitate further studies in tumor immunology.

THYMIC INVOLUTION IS ASSOCIATED WITH DECLINED T CELL-MEDIATED IMMUNE SURVEILLANCE OF TUMORS

Tumor immune surveillance is an interaction between tumor development and antitumor immunity. The process of tumor immunoediting has three phases: elimination, equilibrium, and escape (40, 41). Elimination is an effective process of immune recognition via antigen-specific identification, and responsiveness to remove neoplasia. However, if T cells are senescent and/or tumor cells evolve into less targetable variants by genetic mutation or epigenetic modifications, the adaptive immune system might only restrain tumor growth, reaching a state of equilibrium. As this process continues it results in the selection of tumor cell variants that are resistant to antitumor response, ushering in the escape phase (40, 41).

T cell immunosenescence is largely attributed to reduced output of naïve T cells from the aged, involuted thymus (18, 42–44), resulting in increased oligoclonal expansion of peripheral memory T cells (45, 46), thereby, restricting TCR repertoire diversity (47, 48). This hampers T cell ability to recognize tumor neo-antigens, resulting from high frequency of somatic mutations in proto-oncogenes and tumor suppressors in tumor cells, and/or from viral antigens produced by virus-induced cancers. These abnormal proteins are called TSAs (5), which are regarded by T cells as foreign antigens. Normally, the T effector (Teff) cell population can recognize tremendous numbers of tumor antigens (5, 6), while the senescent T cell population, with a reduced TCR repertoire diversity, might overlook these antigens. Therefore, one of the potential mechanisms of the reduced cancer immune surveillance is a compromised TCR repertoire generated first by the involuted thymus and exacerbated by age-related peripheral memory cell expansion, which neglects to recognize certain TSAs and fails to eliminate tumors (47, 49, 50) (**Figure 1A**).

Recent studies identified several senescent T cell markers: PD-1 and CD153 in murine senescence-associated T (SA-T) cells (51–53). Previously, our knowledge was limited to T cell secondary signaling molecule CD28, which is reduced or absent in senescent T cells. CD28^{neg} “exhausted” peripheral T cells are accumulated in aged humans (54, 55). These T cells not



only lose responsiveness to co-stimulation (56), but also are involved in chronic inflammation (57). The PD-1⁺CD153⁺ senescent T cells in mice also exhibit impaired TCR-mediated proliferation and defective IL-2 production, and are biased toward the secretion of pro-inflammatory cytokines, such as IFN- γ (45). It is not clear, however, whether increased PD-1 is directly involved in senescent T cell dysfunction. The generation of SA-T cells is generally attributed to thymic involution and the aged environment (53).

There are two major immunosuppressive mechanisms blocking antitumor immunity: the intrinsic PD-1—PD-L1 axis and the extrinsic Treg—Teff axis (58). A recent finding showed that senescent T cells express increased PD-1 (51–53, 59).

This, coupled with increased PD-L1 on tumor cells (60), could lead to an enhanced PD-1/PD-L1 signaling (61), in which the interaction between PD-1/PD-L1 provides a possibility for the energy, exhaustion, and apoptosis of tumor-reactive T cells (62), thereby, reducing cancer immune surveillance associated with senescent T cells (63). We will discuss the Treg—Teff axis in the following section.

Taken together, thymic involution, immunosenescence, and the declined TCR repertoire diversity, coupled with increased age-related genomic mutations in somatic cells and increased PD-1 expression on senescent T cells in the elderly, contribute to compromised immune surveillance of tumors and the higher late-life tumor incidence.

RELATIVELY ENHANCED tTreg CELL GENERATION IN THE INVOLUTED THYMUS, COUPLED WITH ACCUMULATION OF pTreg CELLS, POTENTIALLY SUPPRESS ANTITUMOR IMMUNITY

Thymic involution not only reduces naïve T cell output, but also relatively enhances tTreg generation as displayed by an increased ratio of tTreg versus tTcon in the aged, involuted thymus (20). The basic mechanism is potentially due to altered TCR signaling strength, which may skew CD4⁺ single positive thymocytes from negative selection to Treg cell generation in the involuted thymus (43). Strong TCR signaling strength, generated by interactions between MHC-II/self-peptide complexes and self-reactive TCRs, induce clonal depletion by negative selection, while intermediate TCR signaling strength induces thymocyte differentiation into CD4⁺FoxP3⁺ tTreg cells (17, 64, 65). MHC-II/self-peptide complexes expressed by mTECs are reduced due to mTEC decline in the involuted thymus, resulting in weakened interactions (20, 43, 66). Thus, some self-reactive T clones, which should be negatively selected with strong signaling, survive and differentiate into tTreg cells due to intermediate signaling (20). In addition, such skewing of thymocytes from clonal depletion to Treg generation could modify the TCR repertoire of Treg cells to include certain self-antigens that are also expressed by tumors, enabling these Treg cells to suppress antitumor immunity.

In light of the age-related accumulation of pTreg cells in the periphery (25) and the aging-related enhancement of FoxP3 expression (67), the underlying mechanisms may not be simply due to relatively enhanced tTreg cell generation in the involuted thymus, but also potentially due to declined activation of pro-apoptotic *BIM* gene (*Bcl2* homology-3, *BH3-only*) (68) via increased methylation (68, 69) during aging. *BIM* should be activated after each immune reaction (after infection or inflammation, etc.) in order to deplete excess immune cells and return the expanded immune cell numbers to normal levels (70). However, with age, *BIM* activation in T cells is declined and homeostatic immune rebalance is hindered, resulting in an accumulation of “exhausted” senescent T cells and pTreg cells (25, 26, 71, 72). In addition, conversion of effector memory cells into memory Treg cells might occur in aged people (73). These all increase the pTreg pool (25, 74, 75).

Although Treg cells maintain immunological tolerance by suppressing excessive or aberrant immune responses mediated by Teff cells (76–78), they are opponents of antitumor immunity (79, 80) via their highly immunosuppressive functions against CD8⁺ cytotoxic T lymphocytes (CTLs) (27, 81, 82). Our current understanding is that Treg cells primarily infiltrate the tumor mass and execute suppressive function (77, 83, 84). Generally, T cell infiltration into the tumor mass correlates to tumor antigen expression. If the cancer mass expresses few neo-antigens, then greater numbers of Treg cells infiltrate to form a Treg-dominant tumor microenvironment; whereas, if the cancer mass expresses abundant neo-antigens, fewer Treg cells infiltrate, and more

effector cells including CD8⁺ T cells can be primed and expand in the tumor tissues (16, 85, 86). Tumor-infiltrating Treg cells are thought to be recruited from the preexisting thymus-derived Treg population, including autoimmune regulator gene (*Aire*)-dependent TAA-specific Treg cells (87–89), rather than from peripherally induced tumor-specific Teff cells. Therefore, central tolerance is implicated in impaired antitumor responses.

Removal of Treg cells (with monoclonal antibodies, such as anti-CD25 (90), or other means) enhances T cell antitumor responses (15, 16, 91). However, anti-CD25 antibodies potentially eliminate activated Teff cells, expressing CD25 (92). Targeted functional inactivation of Treg cells based on constitutively expressed molecules including CTLA-4, GITR, TLR8 and OX40 (93–97) is a better means to nullify Treg cell function without decreasing Treg cell numbers from surrounding Teff cells (15), nor effecting Teff cell numbers. That is why anti-CTLA-4 (98) can serve as another immune checkpoint inhibitor to reduce Treg cell activation and be used for tumor immunotherapy (99).

Although direct evidence is still lacking about whether increased tTreg cells play a role in suppressing antitumor immunity, 80 – 95% of pTreg cells are derived from thymic generated tTreg cells bearing a thymic imprint (17, 64). Therefore, relative enhancement of tTreg cell generation resulting from thymic involution is a risk factor for suppressing antitumor immunity that ought not be overlooked.

THE INVOLUTED THYMUS PLAYS A ROLE AS A TUMOR RESERVOIR BY INDUCING TUMOR DORMANCY AND INCREASING THE RISK FOR EVENTUAL METASTATIC RELAPSE

Metastatic relapse occurs when the same type of cancer recurs at a distant location (100) several years after removal of the primary tumor and adjuvant chemotherapy (101, 102), and this results mainly from chemo-resistance obtained by cancer cells in an inflammatory microenvironment (37, 38). Relapse, an immense clinical challenge, is responsible for 90% of cancer-associated mortality (103, 104). It means that cancer cells may still exist for a silent period after the primary treatment. Tumor pre-metastatic niches or reservoirs permit these silent cancer cells to hide and acquire chemo-resistance. Recently, several organs, such as the perivascular space of blood vessels in the lung and liver (105, 106) and bone marrow (107, 108), have been determined to be such reservoirs. We (37) and others (38) found that the involuted thymus is another tumor reservoir that allows for silent primary tumor cells to find safe-harbor.

Cancer cells circulating in the blood stream (109, 110) enter the thymus creating a heterogeneous environment, including malignant cancer cells and non-malignant thymic cells (TECs and thymocytes). Since the thymus contains mostly immature T cells and possesses semi-immune privilege, the cancer cells cannot be thoroughly eradicated by immune surveillance (37). In addition, the thymus is sensitive to many insults that cause involution. One of strongest insults is chemotherapy. In addition

to killing cancer cells, systemic chemotherapy also results in non-malignant cell death and/or senescence due to DNA damage (111, 112), which produces an inflammatory microenvironment. This induces chemo-resistant dormancy in the sojourning cancer cells (38, 105, 113, 114) (**Figure 1B**). Dormancy occurs at two levels (101, 108): (a) at the single-cell level, in which the dormant cancer cells exist in a quiescent state of G0 – G1 arrest (101), with increased *MAPK p38* and decreased *ERK*, (conventional dormancy); and (b) at the population level, in which cancer cell proliferation is balanced by apoptosis (dynamic dormancy) resulting in an overall unchanged total cancer cell number (115), i.e., immune equilibrium (116, 117).

Our research found that thymic-sojourning disseminated solid tumor cells show a heterogeneous dormancy phenotype, some being quiescent with features of conventional dormancy, such as increased ratio of p38/ERK (activation of p38 and inhibition of ERK), inducing tumor growth arrest (113, 118, 119), while some either propagate or undergo apoptosis with features of dynamic dormancy (37). Together, chemotherapy-induced acute thymic involution provides a chemo-resistant microenvironment for tumor dormancy, creating a pre-metastatic reservoir. Although the distinct dormancy mechanism underlying the heterogeneity of dormant tumor cells (being quiescent and dynamic) needs further investigation, these observations provide a new therapeutic target for preventing cancer relapse and metastasis.

POTENTIAL THERAPEUTIC STRATEGIES BY MODIFYING THYMIC FUNCTIONS

Since cancer is derived from self-tissues, pathogenic tumor cells are oftentimes carrying “self”-antigens, i.e., TAAs, and can be recognized by most self-reactive T_H cells that are deleted by negative selection in the thymus. Thus, this has led several groups to posit that disruption of central tolerance might further the ability of the T cell compartment to combat cancers (87, 120–122). In this regard, most of the recent studies focus on targeting *Aire*-expressing mTECs in the thymus.

Medullary TECs highly express *Aire*, allowing them to promiscuously present self-antigens to self-reactive T clones during negative selection for central tolerance establishment (13, 21, 123). Though the full scope of this process remains to be elucidated, it is readily accepted that *Aire* deficiency facilitates increased self-reactive T cell release enhancing immunity to certain cancers. One recent technique targets mTECs specifically via anti-RANK-Ligand treatments, which transiently deplete *Aire*-expressing mTECs (22, 121, 124). Because the anti-RANK-Ligand reagent is already FDA-approved, albeit for osteoporosis (125), it has potential to be easily translated to cancer patients. This strategy is also promising because the depletion is brief, with mTECs normally replenished within 2 weeks (126, 127) and full recovery observed 10 weeks after cessation of anti-RANK-Ligand treatment (22). This tactic was tested in animal models of melanoma, since several of the melanoma antigens, including gp100 and TRP-1, are controlled by *Aire* (23, 122) and up-regulated in melanoma cells (122). Importantly, many of these

studies used anti-RANK-Ligand in combination with peripheral therapies, such as checkpoint inhibitors, demonstrating greatly improved outcome in comparison to peripheral treatment alone. However, it is obvious that central therapy alone is not sufficient for tumor immunotherapy (121).

One caveat to this type of strategy is the recent finding that other transcriptional regulators are implicated in promiscuous self-antigen expression in the thymus, for example, forebrain embryonic zinc fingerlike protein 2 (*Fezf2*) (128). There are not many reports on what *Fezf2* disruption would accomplish in regards to heightened TAA targeting as observed with the above *Aire*-targeting studies. There is evidence that *Fezf2* is independent of the RANK/CD40/*Aire* axis which implies that an anti-RANK-Ligand therapy may not be as effective for disrupting *Fezf2*-dependent self-antigen expression (129).

The obvious risk for disruption of central tolerance is increased incidence of autoimmunity (130, 131), which is one of the underlying players in inflammaging in the elderly (66). This is clearly seen in patients who have mutations in *AIRE* (132) and has been recently demonstrated in mice who lack *Fezf2* (128). Another challenge to strategies that manipulate central tolerance is that some TAAs are not under the control of *Aire*, such as TRP-2 (122), and some may be under the regulation of factors that have yet to be identified.

Additionally, we know that tumor antigens not only include TAAs (“self”-antigens), but also TSAs (“foreign”/neo-antigens), which are recognized by T cells as foreign antigens (133, 134). Therefore, deletion of *Aire* expression cannot induce antitumor immunity to non-*Aire*-controlled TAA-bearing tumors nor for TSA-bearing tumors. This limits the scope of cancers that would benefit from such a strategy, and also supports studies that use combinative central and peripheral immunotherapies. Finally, it is important to also take age-related peripheral changes into account, as many other age-related changes may offset the potential benefits of such central tolerance manipulation therapies. Therefore, several potential avenues of research remain for this type of cancer immunotherapy.

CONCLUSION AND OUTSTANDING QUESTIONS

We have briefly reviewed some of the potential impacts of thymic involution (chronic age-related or acute chemotherapy-induced) on cancer and attempt to pave the way for further studies in tumor immunology. Since cancer and thymic atrophy are both associated with age, there is potential for a deeper connection. For instance, it is interesting to consider that most cancers develop in older adults, long after thymic involution has progressed. Since thymic involution is associated with declined mTEC cellularity and *Aire* expression in mTECs (66, 135), it raises the question of why there is not a natural increase in antitumor immunity in the elderly due to the defects in negative selection in the aged thymus. In addition, chemotherapy also induces TEC-impaired thymic involution (37) and declined *Aire* expression in tumor-bearing mice treated with doxorubicin (our unpublished observation). Why, then, do we not see enhanced antitumor T cell

generation? Further, estrogen has recently been identified as a repressor of *Aire* (136, 137), possibly explaining the sex-related tendencies for higher autoimmune disease incidence in women. Does this correlate with a lower incidence for development of certain TAA-expressing cancers in post-menopausal women? In addition, whether we can manipulate thymic function to better target tumor-infiltrating Treg cells by weakening tTreg generation or harness newly generated Teff cells to home to the tumor is in need of further study. Finally, since the tumor microenvironment exerts such strong immunosuppressive signals, how can immunotherapies be tailored to overcome those

signals in a tumor-specific manner without breaking peripheral tolerance completely. Moreover, many important questions remain in our understanding of the crosstalk of aging, cancer, and the impacts of thymic involution on late-life cancers.

AUTHOR CONTRIBUTIONS

D-MS: conceptualization and supervision. WW, RT, and D-MS: writing the original draft. OS: provide assistances. RT: visualization and proofreading.

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Molecular Insights Into the Causes of Human Thymic Hypoplasia With Animal Models

Pratibha Bhalla¹, Christian A. Wysocki² and Nicolai S. C. van Oers^{1,2,3*}

¹ Department of Immunology, The University of Texas Southwestern Medical Center, Dallas, TX, United States, ² Department of Pediatrics, The University of Texas Southwestern Medical Center, Dallas, TX, United States, ³ Department of Microbiology, The University of Texas Southwestern Medical Center, Dallas, TX, United States

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Ichiro Taniuchi,
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Melanie Vacchio,
National Institutes of Health (NIH),
United States

*Correspondence:

Nicolai S. C. van Oers
nicolai.vanoers@utsouthwestern.edu

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22q11.2 deletion syndrome (DiGeorge), CHARGE syndrome, Nude/SCID and otofaciocervical syndrome type 2 (OTFC2) are distinct clinical conditions in humans that can result in hypoplasia and occasionally, aplasia of the thymus. Thymic hypoplasia/aplasia is first suggested by absence or significantly reduced numbers of recent thymic emigrants, revealed in standard-of-care newborn screens for T cell receptor excision circles (TRECs). Subsequent clinical assessments will often indicate whether genetic mutations are causal to the low T cell output from the thymus. However, the molecular mechanisms leading to the thymic hypoplasia/aplasia in diverse human syndromes are not fully understood, partly because the problems of the thymus originate during embryogenesis. Rodent and Zebrafish models of these clinical syndromes have been used to better define the underlying basis of the clinical presentations. Results from these animal models are uncovering contributions of different cell types in the specification, differentiation, and expansion of the thymus. Cell populations such as epithelial cells, mesenchymal cells, endothelial cells, and thymocytes are variably affected depending on the human syndrome responsible for the thymic hypoplasia. In the current review, findings from the diverse animal models will be described in relation to the clinical phenotypes. Importantly, these results are suggesting new strategies for regenerating thymic tissue in patients with distinct congenital disorders.

Keywords: thymus development, thymic hypoplasia, TECs, mesenchymal cells, 22q11.2 deletion syndrome, *PAX1*, *FOXN1*, *CHD7*

INTRODUCTION

Thymic hypoplasia is a common transient condition seen in newborns, particularly for premature babies (1, 2). A short-lived hypoplasia of the thymus can occur at any age due to infections, diverse forms of stress, pregnancy, alcoholism, malnutrition, and radiation exposure (3–5). In the elderly, a severe and everlasting involution of the thymic tissue is a well-recognized consequence of the aging process (6, 7). There are several genetic disorders in humans that result in permanent hypoplasia or occasional aplasia of the thymus evident at birth. These genetic disorders often lead to severe combined immunodeficiency (SCID) (8). The mutations can be monogenic or multigenic, impacting either the patterning of the thymic anlage, the thymic stromal cell populations, and/or the developing thymocytes. The stromal cell populations include mesenchymal cells, TECs and endothelial cells. Clinical conditions known to impact these

stromal cell populations are chromosome 22q11.2 deletion syndrome (22q11.2del), also referred to as DiGeorge syndrome, Coloboma-heart defects-atresia choanae-retardation of growth-genital abnormalities-ear (CHARGE) syndrome arising from mutations in *Chromodomain Helicase DNA Binding Protein 7* (*CHD7*), Nude/SCID due to autosomal recessive mutations in *Forkhead Box N1* (*FOXN1*), otofaciocervical syndrome type 2 (OTFCS2) due to mutations in *PAX1*, as well as mutations in *TBX1* (located within the chromosome 22q11.2 locus) and *TBX2* (Table 1) (9–17). Hypoplasia/aplasia of the thymus can also arise in a developing fetus via teratogen exposures; diabetic- or retinoic acid- induced embryopathies (18–21). In the current review, the genetic mutations that affect the stromal cell populations needed for the formation and/or function of the thymus are described. Since these mutations often influence the specification of the thymus during embryogenesis, detailed mechanistic insights have come from mouse, rat and even zebrafish models.

REVIEW ARTICLE

Overview of Murine Thymus Development During Embryogenesis

The thymus and parathyroid glands develop from the 3rd pharyngeal pouch (PP), a temporary embryonic structure that begins as an evagination of endothelial cells from the gut tube between e9.5–10.5 (Figures 1A,B) (23). The formation of the 3rd PP requires several transcription factors including *Paired box gene* family members, *Sin oculis homolog 1* (*Six1*), and *Eyes absent 1* (*Eya1*) (23–25). As the 3rd PP forms, an endothelial layer within this region is surrounded by an area of neural crest-derived mesenchymal cells. Ectodermal in origin, these mesenchymal cells secrete bone morphogenic protein 4 (*bmp4*) and bone morphogenic protein 2 (*bmp2*) to support the patterning of the 3rd PP (26). The targeted deletion of *bmp4* in neural crest cells leads to a reduced contraction of the mesenchymal cells in the 3rd PP (26). This results in morphogenesis defects of both the thymus and parathyroid domains, which are delineated by the expression of *Forkhead box n1* (*Foxn1*) and *Glia cells missing 2* (*Gcm2*), respectively. The demarcation of the thymus domain by *bmp4* is balanced by *Sonic hedgehog* (*Shh*), which establishes the dorsal parathyroid region (27). Interestingly, the initial specification of the thymus and parathyroid regions can occur in the absence of neural crest cells, which are lacking in *spotch* mutant mice, which have mutations in the *Paired box gene 3* (*Pax3*) transcription factor (28). *Paired box gene 1* (*Pax1*) is a related family member also involved in the development of the thymic anlage (15, 29). A *Pax1* deficiency in mice leads to mild hypoplasia of the thymus (29, 30). Interestingly, *PAX1* autosomal recessive mutations in humans leads to a more severe hypoplasia of the thymus (13, 15, 17).

With regards to the stromal cell populations, the neural crest-derived mesenchymal cells have at least three distinct roles in the development of the thymic tissue. First, these cells form the thymic capsule and vasculature, establishing the overall structure of the thymus. Noteworthy, the mechanical removal of the mesenchymal capsule using e12.5 fetal thymic lobes renders the tissue hypoplastic (31–33). Yet, the development

and proportions of thymocytes subsets are normal in these mesenchymal-depleted hypoplastic tissues, revealing intact TEC functions in the setting of their reduced numbers. Second, the mesenchymal cells enable the expanding thymic lobes to detach from the pharynx between e11.5–12.5, with each lobe from the right and left 3rd PP pairing and descending into the mediastinum. This process requires both *Pax3* and *Homeobox a3* (*Hoxa3*) transcription factors, with the targeted deletion of *Hoxa3* in neural crest cells resulting in smaller sized thymic lobes remaining attached to the pharynx (28, 34). Third, the mesenchymal cells support thymic epithelial cell (TECs) expansion and differentiation. This involves a combination of ligands and growth factors produced by mesenchymal cells; *bmp4*, *bmp2*, fibroblast and insulin growth factors, *wnt* proteins, and retinoic acid (32, 33, 35–38). Cross-talk between the mesenchymal cells and TECs facilitates thymic tissue expansion, differentiation of TECs into cortical and medullary subsets and recruitment of hematopoietic thymic seeding progenitors (39, 40). The hematopoietic progenitors arrive in timed waves, with the first cells appearing prior to the vascularization of the thymic tissue (41, 42). Following tissue vascularization and remodeling of the epithelia into a 3-dimensional meshwork, the thymic seeding progenitors enter through the cortical-medullary junction (41). These progenitor cells, through a process of cell-cell interactions with TECs, develop into thymocytes. Consistent with the theme of cross-communication among the various cell types in the thymus, ligands expressed by the thymocytes further support the differentiation and expansion of TECs. For example, immature thymocytes are needed for the proper expansion of cTECs during late stages of embryogenesis (43). The cortical TECs positively select T cells expressing the correct T cell receptor (Tcr) specificity for self-peptides embedded by major histocompatibility molecules (44–46). In addition, the emergence of mature SP thymocytes enhances mTEC differentiation and proliferation by releasing epidermal growth factor (Egf) and lymphotoxin and expressing CD40L and RANKL (47–49). The mTECs ensure deletion of potentially autoreactive T cells and enable T regulatory cell selection (44–46). Of note, there are some distinctions between mouse and human thymic tissue specification during embryogenesis (25). Differing contributions of *Pax1* and *Pax9* is one such example, as detailed in the section on otofaciocervical syndrome type 2 (17). In addition, unlike mice, both humans and rats express MHC class II on developing thymocytes and these cells can support the selection and maturation of CD4 single positive cells (50–52). Several articles in the current series “new insights into thymic functions during stress, aging, and in disease settings” as well as other reviews have provided detailed information about the development and contribution of TECs in thymopoiesis (53, 54). The current review will focus on the TECs and other stromal cell types affected by selected clinical disorders.

22q11.2 Deletion Syndrome (DiGeorge Syndrome)

Chromosome 22q11.2 deletion syndrome (22q11.2del; OMIM #188400) is a common human disorder (frequency of 1/4000), resulting in variable and complex congenital malformations (8, 55–58). The congenital defects can include thymic hypoplasia,

TABLE 1 | Stromal cell intrinsic causes of thymic hypoplasia (T^{-/-}B⁺NK⁺) from specific human clinical disorders.

Disease name	Frequency in the population	Genes Affected (# SNPs in ClinVar database ^a)	Thymic hypoplasia (% of patients)	Mouse models	Stromal cell populations affected
22q11.2 deletion syndrome	1 in 4000	> 105 genes [46 coding, 7 miRNAs, 12 lncRNAs, 2 snoRNAs, rest are pseudogenes]	60–70% <1% with aplasia of the thymus	Chromosome 16 ortholog deletions, <i>Tbx1</i> targeted mice	Stromal cells (mesenchymal, endothelial, epithelial)
CHARGE syndrome	1 in 8500–10,000	<i>CHD7</i> (SNPs = 973)	50%	<i>Chd7</i> knockout and knock-in lines	Neural crest cells (mesenchyme)-TECs
Nude/SCID ^b and SCID	Rare	<i>FOXN1</i> (SNPs = 126)	90%	<i>Foxn1</i> knockout and knock-in lines	cTECs and mTECs
Otofaciocervical syndrome	Rare	<i>PAX1</i> (SNPs = 29)	100%	<i>Undulated</i> series and <i>Pax1</i> knockout lines	Endodermally-derived epithelial cells
22q11.2-like cardiovascular and skeletal disorder	Rare	<i>TBX2</i> (SNPs = 25)	100%	<i>Tbx2</i> knockout mice	Stromal cells
Maternal diabetes	3–9% of pregnancies	Multiple genes e.g., <i>CybpA1</i>	18% of those needing thymic transplant	Gestational and pre-gestational diabetes	Stromal (mesenchymal, epithelial, endothelial)
Fetal retinoid syndrome	5–20% malformation rates in live births	Multiple genes e.g., <i>Tbx1</i> , <i>Tbx2</i> , <i>Bmp4</i> , <i>Foxn1</i>	Unknown	Retinoic acid injections Enzyme KO mice	Stromal (mesenchymal, epithelial, endothelial)

^asingle nucleotide polymorphisms = SNPs, reported in ClinVar database.

^bsevere combined immunodeficiency = SCID.

outflow track problems of the heart, hypoparathyroidism, dysmorphic facial features, and/or other midline organ involvement (Table 1). Additional complications for children with 22q11.2del include developmental delay, and over time, neurological problems such as schizophrenia and autism, malignancy, and/or autoimmunity (57–61). Most individuals with 22q11.2del have a 3 Mb microdeletion on chromosome 22, resulting in a hemizygoty of nearly 106 genes (8, 58, 60, 62, 63). A smaller, nested deletion of 1.5 Mb creates a haploinsufficiency of 30 genes, which occurs in 5–8% of 22q11.2del patients (8, 58, 60).

Thymic hypoplasia is reported for 60–70% of individuals with 22q11.2del (56, 58, 64). Due to their thymic hypoplasia, 22q11.2del patients have an average 5-fold reduction in the number of T cell receptor excision circles (TRECs) compared to matched controls, with TRECs measuring the circulating naïve T cells emerging from the thymus (56, 65, 66). In rare cases a patient with 22q11.2del may have complete thymic aplasia, resulting in near-complete absence of autologous T cells, defined by <50 naïve CD3⁺ T cells per microliter of peripheral blood (14). An effective clinical treatment option for such a patient is an allogeneic thymic tissue transplant, first depleted of thymocytes prior to the placement of small fragments of this tissue within the quadriceps muscles (67–70). The thymic stromal tissue consists of TECs, mesenchymal cells, and endothelial cells (71). Upon transplant, the stromal tissue recruits host-derived hematopoietic cells that mature into thymocytes (70, 72). A remarkable feature of this thymus transplantation procedure is the successful selection of TCR-expressing T cells recognizing peptides presented by host (recipient) antigen-presenting cells (12, 70, 72, 73). However, the processes of both positive

and negative selection and that of MHC restriction of the developing T cells are not completely understood in these thymic tissue transplants. The positive selection of host T cells in a donor thymus MHC (HLA) background could be caused by recipient-derived epithelial progenitor cells (74). Alternatively, the developing human thymocytes could promote positive selection as these cells express MHC class II molecules (50). When MHC class II is forcibly expressed on murine thymocytes, such cells can now positively select CD4 T cells (51, 52). The thymocyte-selected CD4 single positive cells formed in these mouse models are different than conventional CD4 T cells (75). They express the promyelocytic leukemia zinc finger protein (plzf) and produce both gamma-interferon and IL-4, reflecting more innate-like responses (75). The thymic transplants for 22q11.2del patients can additionally enable T regulatory cell development (69, 76). In normal thymopoiesis, these Tregs develop through interactions with medullary TECs. Negative selection is similarly not well-understood following thymus tissue grafting, with the developing T cells tolerant to both the donor and host MHC (76). It is likely that host dendritic cells along with donor mTECs tolerizing/eliminating any developing T cells targeting either host and donor peptide-MHC complexes (53, 68, 76).

Not all 22q11.2del patients who have a severe hypoplasia of the thymus are grafted with an allogeneic thymus (77, 78). Thus, matched sibling and sometimes unrelated bone marrow transplants have been successfully used to treat 22q11.2del patients who have a severe thymic hypoplasia (limited TRECs) (77–81). The recipient 22q11.2del patients have normal T cell functions and humoral immunity, suggesting T cell reconstitution. However, the majority of the donor T cells have a

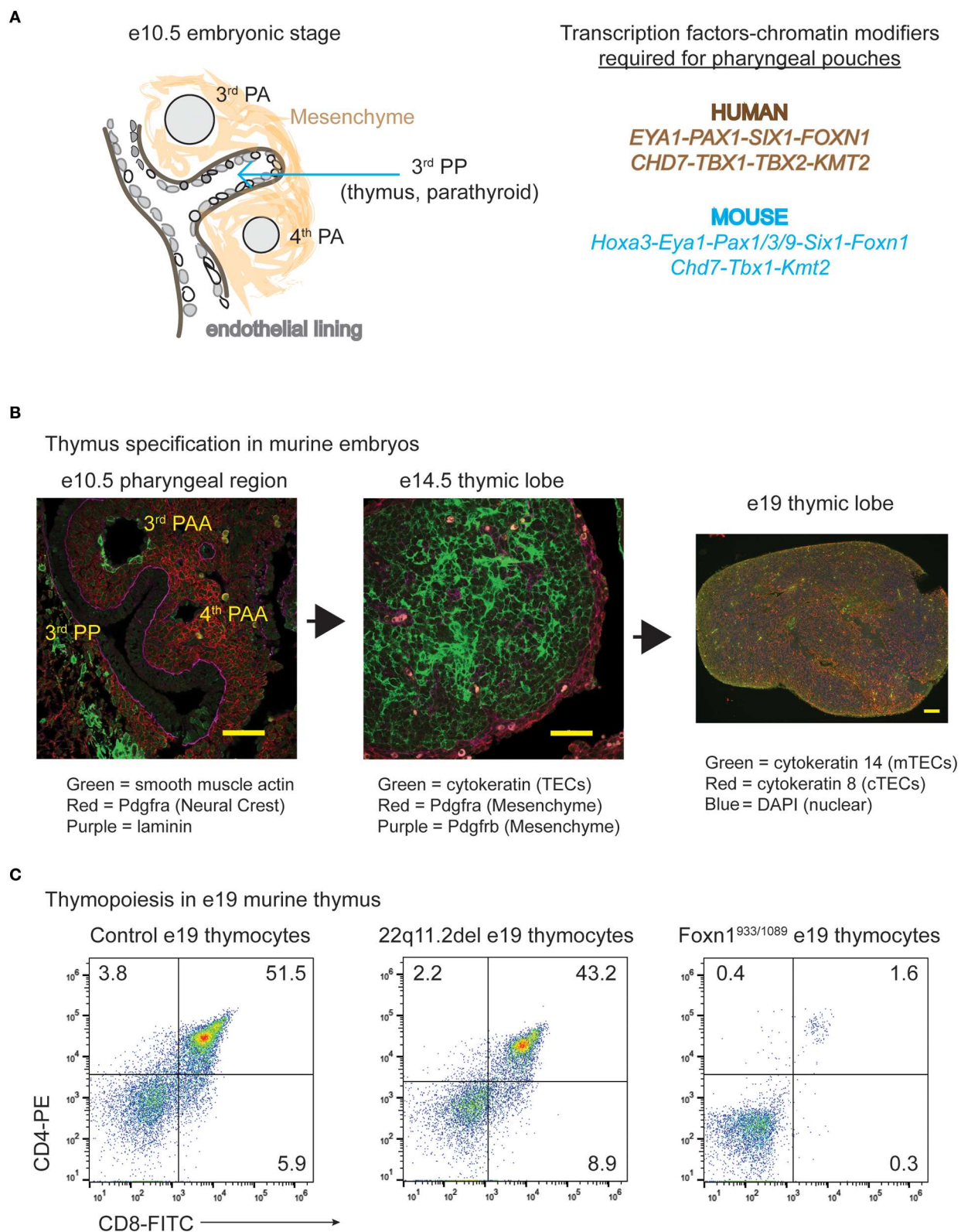


FIGURE 1 | The specification and expansion of the thymus during embryogenesis in normal and disease states. **(A)** Cartoon diagram illustrating the development process of the thymus along with the various transcription factors and gene products required. The genes that have roles in the specification of the human pharyngeal (Continued)

FIGURE 1 | apparatus that affects the 3rd pharyngeal pouch (thymus and parathyroid) are shown in brown, while those confirmed importance for these processes in mice are in blue. **(B)** Transverse tissue sections or intact thymic lobes were isolated from normal embryos at the indicated ages of gestation. The transverse sections or whole mounts of the tissue were prepared for immunohistochemistry and H&E staining. Antibodies against vascular smooth muscle, pdgfr- α (alpha) marking the mesenchymal cells and thymic capsule, pdgfr- β (beta) delineating mesenchymal cells and the vasculature, cytokeratin (TECs) and laminin were used, with the colors indicated below the image. **(C)** Thymocyte subset distributions present in e19–19.5 embryonic thymuses from control C57BL/6 mice, those modeling 22q11.2 deletion syndrome (*Tbx1*^{neo2/neo2}) and those with compound heterozygous mutations in *Foxn1* (*Foxn1*^{933/1089}) are shown. The *Foxn1* mutations genocopy that identified in a human patient (22). Both control and 22q11.2del thymuses have similar distributions of CD4 and CD8 thymocyte subset percentages, suggesting normal TEC functions. The *Foxn1* mutant mice are blocked at the CD4⁺CD8⁺ subset, indicating a severe TEC dysfunction.

memory phenotype and a limited TCR repertoire (77, 78). In the short term, there is no difference reported in the mortality for the patients receiving a thymic tissue vs. those with a bone marrow transplant (80, 81). This conclusion will require a long-term longitudinal study comparing infection and survival rates with a larger cohort. However, the lack of naïve T cell development in the bone marrow recipients is of clinical concern for 22q11.2del patients and as described in subsequent sections, individuals with *FOXN1* and *PAX1* mutations (82).

An important take-home message from the clinical approaches to treat 22q11.2del patients is that the deletion primarily impacts the stromal cells of the thymus. Yet, which stromal cell type(s) is affected by 22q11.2del remains unknown. One group has analyzed thymuses isolated from 22q11.2del patients, available since this tissue is often removed to allow surgical access to the heart (83). The most distinguishing feature of the thymuses from 22q11.2del patients is its smaller size compared to age-matched control tissues (83). Thymopoiesis appears normal, as the percentage of CD4⁺CD8⁺ (DN), CD4⁺CD8⁺ (DP), and CD4⁺CD8⁺, and CD4⁺CD8⁺ (SP) thymocyte subsets in the hypoplastic tissues is similar to that seen with control samples. The medullary region does appear smaller in the 22q11.2del samples, although the levels of a key gene expressed in medullary TECs, *Autoimmune regulator* (*AIRE*), is not statistically different from controls (83). Yet, the number of thymic CD4⁺Foxp3⁺ T regulatory cells (Tregs) is diminished in the hypoplastic lobes and these cells have less suppressive capabilities compared to controls (83). It remains unknown why this difference exists but may explain the higher prevalence of autoimmune cytopenias in the 22q11.2del cohort (56, 84, 85). The number of these CD4⁺ Tregs is also decreased in peripheral tissues, but this arises from the generalized T cell lymphopenia affecting most T cell subsets in the setting of 22q11.2del (11, 85–89).

The congenital hypoplasia/aplasia of the thymus caused by 22q11.2del occurs during the patterning of the pharyngeal apparatus in embryos (58, 90–92). This is best revealed in mice, as comparative analyses between normal embryos and those obtained from 22q11.2del mouse models suggest patterning defects of the pharyngeal pouches and arches (61, 90, 91, 93–95). The 22q11.2del mouse lines were initially developed with orthologous deletions on murine chromosome 16 to identify genes causal to the congenital malformations (Table 2). This led to the realization that the principal cause of the congenital defects was linked to a haploinsufficiency of the *T-box Transcription Factor 1* (*TBX1*) (90, 91, 93, 94, 96). *TBX1* interacts with members of the Histone-lysine N-methyltransferase (KMT2)-family,

activating the low level transcription of over 2,000 genes (97). Interestingly, the penetrance and severity of the congenital malformations due to a haploinsufficiency of *TBX1* varies considerably in the mouse models, which recapitulates the wide range of differences among individual 22q11.2del patients (Table 2). Emerging evidence suggests this variability is due to a combination of genetic and epigenetic regulators, both within and outside chromosome 22q11.2, which influence all the clinical phenotypes of 22q11.2del (8, 98, 99).

In the mouse models, haploinsufficiency of *Tbx1* is generally not very penetrant in eliciting hypoplasia/aplasia of the thymus (90, 91, 94, 96, 100). By comparing embryos expressing varying levels of *Tbx1*, expression of this transcription factor at or below 35% normal values results in a more frequent and damaging thymic hypoplasia (101). Thymic hypoplasia resulting from the reduced levels of *Tbx1* are likely caused by developmental abnormalities in the pharyngeal region. However, the studies published to date have not concentrated on the 3rd PP. What is noticeably different are the 4th pharyngeal arches (PA), adjacent to the 3rd PPs, which are absent or developmentally delayed between day e9.5–11.5 of embryogenesis (Figure 1B) (101, 102). This impacts the patterning of the structures originating from the right and left 4th PA, causing a displaced right subclavian artery and interrupted aortic arch type B, respectively. Both cardiac presentations are common clinical phenotypes of human 22q11.2del (102). *Tbx1* is specifically expressed in the regions comprising the pharyngeal arches as well as in the endothelial layer that juxtaposes the developing parathyroid (103). It is not expressed in the thymic anlage, suggesting that *Tbx1* haploinsufficiency does not directly impact TECs, consistent with the observations that enforced expression of *Tbx1* within the 3rd PP actually represses TEC development (103). A plausible explanation for the thymic hypoplasia in 22q11.2del is that reduced levels of *Tbx1* in the pharyngeal region impact the neural crest-derived mesenchymal cells that surround the 3rd PP. The importance of these mesenchymal cells and other cell types has been more clearly revealed in *Tbx1*-null embryos. An immunohistochemical analysis of these embryos reveals an abnormal distribution of proteins involved in the formation of the extracellular matrix, cell adhesions, and cell-cell contact (vinculin, paxillin and collagen) (104). Changes in the expression patterns of these proteins affects the NCC-derived mesenchyme along with the epithelial cells in the second heart field (104). Such results strongly suggest that the NCC-derived mesenchymal cells surrounding the 3rd PP may also have abnormal mesenchymal and endothelial cell distributions required for the proper patterning of the 3rd PP.

TABLE 2 | Mouse models of human clinical disorders leading to hypoplasia or aplasia of the thymus.

Genetic mutation	Impact on the thymus	Alopecia/nail cornification	Stage of developmental block in thymopoiesis	Mechanistic insights
Mouse models of 22q11.2 deletion syndrome				
LgDel	Rare mild hypoplasia	None	None	30 genes, including <i>Tbx1</i> are haploinsufficient causing partially penetrant cardiac anomalies, minimal effect on thymus
Df (16)A	Rare mild hypoplasia	None	None	
Df1/+	Rare mild hypoplasia	None	None	Early embryonic lethal
<i>Tbx1</i> ^{-/-}	Aplasia	None	DN1 ^a	
<i>Tbx1</i> ^{neo2/+} and <i>Tbx1</i> ^{neo/+}	Mild hypoplasia	None	None	50 and 70% normal <i>Tbx1</i> levels, respectively, enabling a gene dosage analysis and showing <i>Tbx1</i> key to congenital abnormalities
<i>Tbx1</i> ^{+/-}	Mild hypoplasia	None	None	
<i>Tbx1</i> ^{neo2/neo2}	Severe hypoplasia	None	None	<i>Tbx1</i> haploinsufficient (see above with Df and Lg series)
				35% normal <i>Tbx1</i> levels leads to more penetrant and severe congenital malformations
Mouse models of CHARGE syndrome: <i>Chd7</i> mutations created				
<i>Chd7</i> point mutations	Not reported	None	None	Affects the cardiac tissue and malformations of the ear canal
<i>Chd7</i> 7 ENU ^b mutations	Not reported	None	None	
<i>Chd7</i> gene trap	Not reported	None	None	12 distinct mutations matching human mutations. Phenotypes mimic human disorder
<i>Chd7</i> gene knockout		None	None	
<i>Chd7</i> ^{+/-xk} gene trap	Hypoplasia	None	None	Three mutations (exons 1, 4, and 34) with effects as with ENU
				Exon 2 targeted, clinical phenotypes as with humans
				11% embryos affected, small and ectopic location of the thymus
Mouse models of SCID and Nude/SCID: <i>Foxn1</i> mutant mice generated				
<i>Foxn1</i> nu/nu	Aplastic thymus	YES	DN1 ^a	Required for TEC development, differentiation
<i>Foxn1</i> ΔExon3	Hypoplastic thymus	NO	DN1 ^a	
<i>iFoxn1</i> Δ7,8	Hypoplastic thymus	NO	DN2-DN3 ^a	Regulates epithelial cells in the skin and nail beds
<i>Foxn1</i> ^{933/1089}	Severe hypoplasia of the thymus	NO	DN1 ^a	
<i>Foxn1</i> ^{1089/1089}	Hypoplastic thymus	NO	DP ^a	Required for TECs
				Normal hair and nail beds
				Inducible deletion of <i>Foxn1</i> causes a loss of thymic structure, reduced T cell output
				Required for TEC development, differentiation. Normal hair and nail beds
				5 amino acid region required for DP ^a to SP ^a . Normal hair and nail beds
Mouse models of Otofaciocervical syndrome: <i>Pax1</i> mutations				
<i>Undulated</i>	Mild hypoplasia	NO	None	Gly to Ser mutation causes reduced DNA binding activity. Affects the patterning of the thymic anlage
<i>Undulated short</i>	Mild hypoplasia	NO	None	
<i>Undulated extensive</i>	Mild hypoplasia	NO	None	125 kb region is deleted, including <i>Pax1</i> coding region. Affects the expression of a long non-coding RNA, which is lost while <i>Nkx2.2</i> is increased
<i>Pax1</i>	Mild hypoplasia	NO	None	
				Last exon of <i>Pax1</i> deleted. Affects the patterning of the thymic anlage
				Complete <i>Pax1</i> knockout. Affects the patterning of the thymic anlage

^aDevelopmental stages of thymopoiesis: DN subset is CD4⁻CD8⁻; DN1, CD44⁺CD25⁻; DN2, CD44⁺CD25⁺; DN3, CD44⁻CD25⁺; DP, CD4⁺CD8⁺ thymocyte subset; SP, CD4⁺CD8⁻ and CD4⁻CD8⁺ single positive subsets.

^bN-ethyl-N-nitrosourea = ENU.

This is likely what causes a size restriction on the developing thymus. In one mouse model of 22q11.2del (*Tbx1*^{neo2/neo2}), the embryonic thymus is size restricted yet still supports normal T cell development (Table 2, Figure 1C). This indicates that the TECs are functional, matching the phenotype noted in the hypoplastic thymic lobes from 22q11.2del patients (83). In summary, mouse models of 22q11.2del strongly suggest that the initial developmental problems leading to thymic hypoplasia are coupled to mesenchymal cell defects. As the mesenchymal cells provide critical support functions for TECs, the consequence is reduced TEC expansion. Comparing 22q11.2del with other

human clinical syndromes further supports this notion, as described next.

Charge Syndrome Due to Chromodomain Helicase DNA Binding Protein 7 Mutation

Coloboma-heart defects-atresia choanae-retardation of growth-genital abnormalities-ear abnormalities (CHARGE) is a multi-syndromic congenital disease (11, 105, 106). Approximately 90% of CHARGE patients have mutations in *Chromodomain Helicase DNA Binding Protein 7* (*CHD7*) (OMIM# 0214800) (107). *CHD7* is an ATP-dependent nucleosome remodeling

factor, regulating chromatin accessibility and consequently, gene expression (108). *CHD7* also positively regulates ribosomal RNA biogenesis in the nucleolus (109). Affecting an estimated 1 in every 10,000 humans, 953 mutations have so far been discovered in *CHD7* (ClinVar database). These include missense, non-sense, deletion, splicing, and frame-shift mutations, resulting in a loss-of-function of varying severity depending on the location and/or effect of the mutation on the protein (105, 110). Patients with the CHARGE syndrome have immune system problems that contribute to their recurrent infections; otitis media, sinusitis, upper airway infections, pneumonia, and/or sepsis (106, 111). These infections are most often attributed to malformations of the craniofacial region, the upper respiratory tract, and the 7th cranial nerve (facial innervation). Of note, the first descriptions of CHARGE suggested that the infectious issues were of low incidence (105). More recent reports reveal that immune system complications are far more prevalent, with developmental problems of the thymus additionally reported as causal to the increased susceptibility to infections (106, 112). An immunological assessment of 59 CHARGE patients revealed that about 50% had a $T^{-/lo}B^{+}NK^{+}$ phenotype (106). Immunoglobulin levels and subclasses were normal in most of these CHARGE patients. The absolute numbers of B cells, including memory cells, were very similar to that in controls. The low T cell numbers were a consequence of a thymic dysfunction as TREC levels for these patients were reduced relative to normal controls. Chart reviews for 36 CHARGE patients who had cardiac surgeries revealed 16 of 36 had a hypoplasia or aplasia of the thymus (106). The prevalence of the thymic hypoplasia may be higher in embryos, as a small/absent thymus was noted in seven of 10 CHARGE fetuses described in one study (113).

Chd7 is required for the formation of the multipotent migratory neural crest cells that migrate throughout the body, establishing the bone, cartilage, peripheral nervous system, and cardiac structures (114). To understand the role of *Chd7* in CHARGE, especially given the varied congenital problems that can arise, various mouse models have been developed (115–118). The mouse models include those generated with gene-trapped ES cell lines, N-ethyl-N-nitrosourea (ENU) mutagenesis, targeted mutations in *Chd7*, and various floxed alleles of the gene (Table 2). Embryological analyses indicate that *Chd7* is expressed in the pharyngeal region, including the 3rd PP, the 4th PA, and the 1st PP, the latter forming the auditory tube and middle ear canal (119). As early as e10.5, the 4th PA is malformed or absent in 50% of the *Chd7* mutant embryos, resulting in an interrupted aortic arch type B and displaced right subclavian artery, just as with 22q11.2del (90). While most of the studies did not focus on thymus abnormalities, one group did report on this tissue. In this study, about 11% of e14.5 *Chd7*^{+/xk} embryos were found to have irregularly shaped thymic lobes, smaller and more oblong in appearance along with some ectopic positioning (119). *Chd7* is expressed in the surrounding mesenchyme and at higher levels in TECs, suggesting that the *Chd7* mutations impact both neural crest-derived mesenchymal cells and TECs (115). Interestingly, modulation of retinoic acid (RA) levels *in utero* can limit the severity of the phenotypes resulting from the *Chd7* mutations (120, 121). The clinical phenotypes due to retinoic acid

embryopathies, including hypoplasia of the thymus, are discussed in a later section.

Complementing the murine models, Zebrafish studies have provided additional insights into how *Chd7* impacts thymic tissue specification. One technology commonly used in Zebrafish is a gene knockdown approach with morpholino oligonucleotides (MOs), creating morphants that have a block in transcript expression. *Chd7* morphants have a disrupted morphogenesis of the 3rd PP, with the migration and function of neural crest cells (NCCs) in this area impaired (122). Both *bmp4* and *bmp2* levels are significantly diminished in the *chd7* morphants, again revealing the importance of these soluble proteins in establishing the thymus and parathyroid domains. At later developmental time points, the *Chd7* knockdown impairs the formation of the thymic capsule and vasculature. This is coupled with a reduced formation/expansion of the TECs that may involve impaired differentiation of the endothelial layer. Finally, the TECs have a substantial loss of *Foxn1* expression, providing a mechanistic basis for the hypoplasia due to TEC abnormalities (122). In summary, the *Chd7* knockdown impacts the NCC-derived mesenchymal cells along with the TECs, which suggests that CHARGE affects more stromal cell populations than 22q11.2del.

Nude/SCID and SCID Phenotypes Linked to *FOXN1* Mutations

Autosomal recessive mutations in the *Forkhead Box N1* (*FOXN1*) transcription factor cause a $T^{-}B^{+}NK^{+}$ SCID phenotype due to a thymic aplasia as well as alopecia universalis and nail plate dystrophy (OMIM #601705) (123–127). Three distinct autosomal recessive mutations in *FOXN1* have been reported for 10 patients to date, and these mutations result in a complete loss of protein function, impacting TECs and skin epithelial cells. Patients with compound heterozygous mutations in *FOXN1* have also been reported with an atypical phenotype, a thymic hypoplasia without the co-presenting alopecia and nail dystrophy (22). With the increasing number of infants noted to have low TRECs, the subsequent use of exome sequencing for them has uncovered many individuals with single allelic mutations in *FOXN1* (22, 128). While such affected individuals will likely recover normal T cell numbers as one allele remains functional, it is unclear what impact such single allelic mutations will have on T cell output later in life (129). To date, about 131 distinct mutations in human *FOXN1* have been reported, and while many are benign, there are >20 that have either complete or partial loss of function consequences (ClinVar database). The best clinical treatment option for patients with autosomal recessive or specific compound heterozygous mutations that contribute to a loss-of-function for *FOXN1* is a thymic tissue transplant (12). Yet, while bone marrow transplants have also been undertaken for such patients, the underlying defect lies with the TECs of the thymus (22). Paralleling the clinical findings with 22q11.2del, a thymic tissue transplant is the best option as this directly resolves the TEC anomalies.

In the thymus, *Foxn1* is the master transcriptional regulator of TEC development, supporting the differentiation of both cortical and medullary TEC subsets (45, 130–132). These TEC subsets are critical for establishing the repertoire of TCR-expressing T cells that are selected to recognize but not

respond to self-peptide/MHC complexes (44, 45, 131). Foxn1 is a 648 amino acid long transcription factor that contains DNA binding and transactivation domains, both required for protein function (133, 134). The DNA binding domain of Foxn1 comprises three alpha helices, three beta sheets, and two loops (wings) (130, 135). The 3rd helix and the 2nd winged segment interact with the major and minor grooves of DNA, respectively (130, 135). The DNA binding sequence bound by Foxn1 is GAa/cGC, present in about 500 target genes (132). The genes regulated by Foxn1 include keratins, keratin-associated proteins, cytokeratins, thymo-proteasome components, and cell surface proteins (132, 136). These proteins are important for both cortical and medullary TEC functions along with the extrusion of the hair shaft through the dermal layers of the skin and for nail bed formation (132, 137, 138). In many of the promoter/enhancer elements bound by Foxn1, there are CREB and Tp63 binding sites, suggesting cooperative gene regulation by multiple transcription factors (132).

Mouse and rat models have greatly aided in delineating the functions of Foxn1. First and foremost was a spontaneously arising mutant mouse line, discovered in 1966, with a pronounced nude phenotype (*nu/nu*). Almost three decades passed before the mapping of the *nu/nu* allele to autosomal recessive mutations in Foxn1 (130, 134). The *nu* mutation results in a single base pair deletion in exon 3, causing a frameshift and almost no protein expression (130). The mice lack fur, whiskers, and nails (130, 139, 140). The thymus in the *nu/nu* mouse is a small cystic tissue that is unable to support TEC and consequently, thymocyte development (23, 141). Such nude mice are commonly referred to as Nude/SCID given their combined lack of fur and T⁺B⁺NK⁺ immune profile. An analysis of embryos from these mice show that Foxn1 is not required for the initial specification of the thymic region within the 3rd PP, but rather for the vascularization of this tissue along with TEC differentiation/expansion (142, 143). Nude rats (*rnuN*, *rnu*) and cats (*nu/nu*) with autosomal recessive mutations in Foxn1 have similarly been described, with the first nude rat actually found in 1953, prior to the mouse reports (130, 144–146). While the *nu* and *rnuN* mutations prevent translation of the DNA binding and transactivation domains, much like the autosomal recessive FOXN1 mutations in humans, *rnu* rats carry a mutation within exon 8, which creates a stop codon. This leads to the expression of a truncated protein (amino acids 1–473) lacking the transactivation domain. Characterizing this region revealed several aspartic acid residues essential for protein function (133). In an unrelated study, the introduction of a truncated Foxn1 construct, wherein only exon 3 is deleted, blocks TEC development/expansion while allowing for hair extrusion and nail formation (147). It remains unclear how this occurs as both DNA binding and transactivation domains remain intact. In a separate cohort of mice developed to genocopy the compound heterozygous FOXN1 mutations identified in an infant, the mice (Foxn1^{933/1089}) had T⁺B⁺NK⁺ immune profile with normal hair growth and nail extensions (22). Unlike 22q11.2del and CHARGE, these FOXN1 mutations directly impact TEC development, causing a loss of both DP and SP thymocytes (Figure 1C). One of the mutations in FOXN1

(FOXN1¹⁰⁸⁹) causes a loss of 5 amino acids at the very end of the DNA binding domain (p.W363C with a 5 amino acid loss). Knock-in mice harboring this mutation on both alleles (Foxn1^{1089/1089}) have a selective block in thymopoiesis at the DP stage, with hair follicles and nails appearing normal (22). This 5-amino acid sequence is highly conserved with Foxn4, an ancestral ortholog of Foxn1 (148). Interestingly, the cephalochordate species (lancelets) lack a thymus, and have a divergent sequence within this 5-amino acid stretch (22, 148). This suggests this small sequence is important for the expansion of DP thymocytes and their positive selection into CD4⁺ and CD8⁺ subsets (22). There is a 2nd patient described with distinct compound heterozygous mutations in FOXN1 (FOXN1^{1288/1465}). In functional assays, one of the mutations (Foxn1¹⁴⁶⁵) leads to a p.R489fsX61 truncation of the protein, resulting in 18% normal transcriptional activity (22). This mutation prevents the translation of the transactivation domain, revealing a requirement for this region to maintain normal TEC functions. Of note, an increasing number of single allelic FOXN1 mutations are being reported for patients initially presenting with low TRECs (22, 128). The subsequent characterization of these novel mutations will likely reveal the basis for the differential functions of FOXN1 in TEC subsets vs. skin epithelial cells. Of note, one research group has identified a cis-regulatory element (RE) in the 1st intron of Foxn1, the targeting of which reduces TEC numbers and functions without any impact on skin epithelial cells (149). This RE is a target of the Foxn1 DNA binding domain, revealing a positive autoregulatory loop (150). The possibility exists that human patients may contain such intronic FOXN1 mutations, but these have not been reported to date as whole genome sequencing, which is not commonly done, would be required to uncover them.

Post-natally, Foxn1 needs to be continuously expressed in TECs to maintain normal T cell output from the thymus (132). Thus, the inducible deletion of Foxn1 in adult mice reduces thymic cellularity, and impacts the expansion of the DN1–DN4 subsets of thymocytes (132). In “old” mice, Foxn1 levels in the thymus are reduced significantly, which partly explains the tissue involution (151–153). Restoring Foxn1 in the aged thymus significantly improves thymic cellularity and T cell output (152–155). Taken together, the numerous human reports regarding single allelic mutations in FOXN1 and the diverse mouse models are beginning to reveal key regulatory features of this critical transcription factor needed for T cell output throughout life.

Otofaciocervical Syndrome Type 2 (OTFCS2) and PAX1 Mutations

Loss-of-function mutations in PAX1 lead to skeletal defects along with thymic hypoplasia in patients, the latter contributing to the T^{−/lo}B⁺NK⁺ phenotype (13, 15, 17). Four such patients received bone marrow transplants (prior to identification of the PAX1 homozygous mutations) in an attempt to correct their SCID presentations. Notably, the bone marrow transplants were unable to restore T cell development [reviewed in (17)]. The T cells, characterized in the patients after their bone marrow transplants, were of donor origin and exhibited a memory phenotype. Such findings are consistent with the current knowledge that

PAX1 regulates the patterning of the pharyngeal region, thereby impacting the stromal cell populations that would not be corrected by a bone marrow transplant.

Using embryos isolated from pregnant mice, *Pax1* transcripts are evident in the four pharyngeal pouches as early as e10.5, and become confined to mesenchymal condensations as embryogenesis progresses (29). This transcription factor is present in the 3rd PP endoderm and is subsequently detected in a subset of TECs during embryogenesis (29). Its expression is retained in the adult thymus. The deletion of *Pax1* results in a marginal hypoplasia of the thymus (29, 30). This was reported in the *undulated* series of mouse lines that had varying mutations within *Pax1* or with surrounding regulatory elements. These mice were initially described in the 1940's due to their kinked tails and vertebral deformities (Table 2) (29, 156). All *undulated* mutants have a smaller thymus about 2/3rd normal size (29, 30). Interestingly, only in the context of a *Hoxa3* haploinsufficiency does the thymus in the *Pax1* mutant lines exhibit a more severe hypoplasia, with the two lobes ectopically positioned (30). The mild thymus phenotypes in the mouse models comprising various *Pax1* mutations sharply contrast the severe hypoplasia in humans with *PAX1* autosomal recessive mutations. One possible explanation is a compensatory contribution by murine *Pax9* when *Pax1* is lacking. *Pax9* overlaps in expression with *Pax1* in the endodermal-derived epithelium of the pharyngeal pouches (157). In mice, a complete deficiency of *Pax9* causes a thymic aplasia and a lack of teeth, while in humans, autosomal recessive mutations in *PAX9* cause selective tooth agenesis (158, 159).

Clinical Conditions During Pregnancy Leading to a Thymic Hypoplasia/Aplasia

Maternal diabetes and systemic use of retinoic acid (RA) derivatives during pregnancy can cause long-term thymic hypoplasia in newborns (18–21). What's more, gestational diabetes leads to congenital malformations in the developing fetus which overlap with those noted in individuals with 22q11.2del; hypoplasia/aplasia of the thymus, cardiac outflow tract defects, and hypoparathyroidism (160–163). Estimates suggest that 18% of infants who required a thymic tissue transplant due to an aplasia of this tissue, and did not have 22q11.2del, were born to mothers who had maternal diabetes (72). In spite of the obvious overlap in clinical presentations between 22q11.2del and diabetic embryopathy, it remains unknown how blood sugar dysregulation affects the pharyngeal apparatus.

In rodent studies, the induction of diabetes in pregnant mice and rats causes thymic hypoplasia along with intrauterine growth impairment (164). While intrauterine growth delay will certainly contribute to thymic hypoplasia, there is some evidence that the hypoplasia can result from patterning defects within the pharyngeal apparatus. As gestational diabetes in rodent models is difficult to regulate, the use of a pregestational diabetes mouse model has revealed that retinoic acid production is dysregulated in the developing embryos. Thus, pregestational diabetes reduces the expression of *Cytochrome P450 family 26 subfamily A member 1* (*Cyp26a1*), an enzyme that catabolizes retinoic acid (RA) in the caudal region (tailbud) of developing embryos (165). RA is a derivative of Vitamin A, which functions as a natural morphogen

regulating the patterning of the 3rd PP along with the 4th PA (166–170). Both reductions and elevations in RA can lead to hypoplasia of the thymus along with the other congenital malformations that overlap remarkably with 22q11.2del and CHARGE phenotypes (8). While the levels of *Cyb26a1* or related family members within the pharyngeal region were not assessed in the pregestational diabetes model, their losses would increase RA, which could cause the problems of the thymus. Consistent with this, injecting high levels of RA in pregnant mice at e9.5 results in the formation of a hypoplastic/aplastic thymus, examined at e11.5–e12.5 (21). It is known that high levels of RA can impair the migration of the NCCs in the region surrounding the 3rd PP (21). Moreover, high levels of RA can reduce the expression of *Pax1* within the 3rd PP and *Tbx1* throughout the pharyngeal apparatus (171–173). RA likewise represses *Bmp4* activity, impacting thymic tissue specification and development by ultimately reducing the levels of *Foxn1* (174). These changes have some similarity to that described for embryos developing in the setting of *Chd7* mutations.

The second medical condition that can lead to permanent hypo- or aplasia of the thymus in newborns is exposure to elevated levels of RA during pregnancy. Drugs such as tretinoin or isotretinoin are retinoids prescribed to patients to both reduce the severity of their acne and smoothen the skin. However, if taken during pregnancy, the higher levels of RA can trigger 22q11.2-like congenital malformations in the developing embryos (18, 72, 160–162, 175, 176). The mechanism for this hypoplasia is a combination of *Tbx1*, *Pax1*, and *Foxn1* suppression, as described in the preceding sections of this review (Figure 1A). The profound damage caused by RA has led to a generalized warning from the FDA for women to avoid treatments with RA derivatives during pregnancy.

CONCLUSION

A number of clinical conditions impact the specification of the thymus during embryogenesis. Interestingly, those that affect the stromal cell populations have overlapping phenotypes, revealing that many of the affected genes function in related developmental pathways. 22q11.2del appears to impact one of the earlier stromal cell types involved in this process, the NCC-derived mesenchymal cells. These cells regulate the patterning and formation of the thymic anlage. CHARGE affects mesenchymal cells, endothelial cells and the TECs, while *FOXN1* mutations selectively affect the TECs. It is becoming obvious that the three stromal cell types have considerable cross-talk to coordinate the formation and expansion of the thymus. A balanced interplay among all three is essential for the normal specification and expansion of the thymic tissue. Variations in the functions of any one of these stromal cell populations will impact the other, which likely explains the overlapping clinical phenotypes noted among affected individuals.

ETHICS STATEMENT

Animal work described in this article has been approved and conducted under the oversight of the UT Southwestern

Institutional Animal Care and Use Committee (APN numbers: 2015-101163 and 2015-101247).

AUTHOR CONTRIBUTIONS

PB and NO generated the figures. PB, CW, and NO wrote and modified the manuscript.

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Generation and Regeneration of Thymic Epithelial Cells

Abdullah S. Alawam, Graham Anderson* and Beth Lucas*

Institute for Immunology and Immunotherapy, University of Birmingham, Birmingham, United Kingdom

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Edited by:

Nicolai Stanislas Van Oers,
University of Texas Southwestern
Medical Center, United States

Reviewed by:

Nancy Ruth Manley,
University of Georgia, United States
Hyun Park,
National Cancer Institute (NCI),
United States

*Correspondence:

Graham Anderson
g.anderson@bham.ac.uk
Beth Lucas
b.lucas.1@bham.ac.uk

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The thymus is unique in its ability to support the maturation of phenotypically and functionally distinct T cell sub-lineages. Through its combined production of MHC-restricted conventional CD4⁺ and CD8⁺, and Foxp3⁺ regulatory T cells, as well as non-conventional CD1d-restricted iNKT cells and invariant $\gamma\delta$ T cells, the thymus represents an important orchestrator of immune system development and control. It is now clear that thymus function is largely determined by the availability of stromal microenvironments. These specialized areas emerge during thymus organogenesis and are maintained throughout life. They are formed from both epithelial and mesenchymal components, and collectively they support a stepwise program of thymocyte development. Of these stromal cells, cortical, and medullary thymic epithelial cells represent functional components of thymic microenvironments in both the cortex and medulla. Importantly, a key feature of thymus function is that levels of T cell production are not constant throughout life. Here, multiple physiological factors including aging, stress and pregnancy can have either short- or long-term detrimental impact on rates of thymus function. Here, we summarize our current understanding of the development and function of thymic epithelial cells, and relate this to strategies to protect and/or restore thymic epithelial cell function for therapeutic benefit.

Keywords: thymus, thymic epithelial cell, thymic atrophy, regeneration, bone marrow transplant, immune reconstitution

INTRODUCTION

While the bone marrow represents a major site of hemopoiesis, including hemopoietic stem cell development and maintenance as well as B-cell development, the generation of $\alpha\beta$ T cells relies upon the exit of lymphoid progenitors from the bone marrow and their entry into the thymus. Here, blood-borne thymus colonizing cells undergo a complex differentiation program that includes lineage restriction, proliferation, and T cell receptor gene rearrangement and selection. This results in the generation of a pool of self-tolerant $\alpha\beta$ TCR-expressing CD4⁺ and CD8⁺ mature thymocytes that then leave the thymus and form the peripheral T cell pool (1–3). Critically, intrathymic T cell development is a non-cell autonomous process and requires continual input from highly heterogeneous stromal cell populations that collectively form intrathymic microenvironments. Of particular importance is that such microenvironments contain both cortical and medullary regions, each consisting of, and defined by, specialized epithelial cells with differing roles (4, 5). Cortical thymic epithelial cells (cTEC) are responsible for driving immature CD4⁺CD8⁺ lymphoid progenitors toward the T cell lineage, and the subsequent positive selection of CD4⁺CD8⁺ thymocytes. In addition, and following developmental stages in the cortex, thymocytes migrate into medullary thymic areas, with medullary thymic epithelial cells (mTEC) attracting positively

selected thymocytes and providing environmental cues which aid in self-tolerance mechanisms that include deletion of autoreactive thymocytes via negative selection and sublineage divergence for the generation of Foxp3⁺CD4⁺ T-Regulatory (T-Reg) cells (6, 7). Based on this, current models of intrathymic $\alpha\beta$ T cell development center around a step-wise process in which sequential interactions with cTEC and then mTEC generate and shape the $\alpha\beta$ T cell pool. Importantly, in addition to generating conventional and Foxp3⁺ T-Reg that express diverse TCR repertoires, the thymus also supports the development of innate-like T cell subsets that can be defined by their expression of restricted TCR repertoires. In the embryonic period, examples of this are the serial waves of invariant $\gamma\delta$ -cells that seed specific peripheral tissues, while the post-natal generation of CD1d-restricted invariant NKT cells demonstrates how the thymus supports the development of multiple T cell types throughout life (8).

While TEC populations are known to be key regulators of these distinct T cell development programs, recent studies have uncovered significant new TEC heterogeneity that must be considered in relation to our understanding of microenvironmental control of T cell development (9–11). Significantly, therapeutic interventions can also be detrimental to thymus function (12, 13). Such clinical treatments include ablative preconditioning used in the treatment of cancer, which then impairs T cell mediated immune reconstitution following bone marrow transplantation. Consequently, studying thymic epithelial cells in both health and disease states is important to understand how thymus function is controlled, and how it might be manipulated for therapeutic benefit. Recently, important advances have been made in understanding the biology of thymic epithelium, including the developmental pathways that give rise to distinct cortical and medullary epithelial lineages. Furthermore, there is progress in how newly identified heterogeneity in thymic epithelium may map to functional specialization in thymic microenvironments.

LINEAGE SPECIFIC THYMIC EPITHELIAL CELLS

cTEC and cTEC Heterogeneity

The thymus cortex, and in particular the cTEC that reside there, play multiple key roles in T-cell development. These events include pre-TCR mediated transition of CD4⁺CD8⁺ precursors to the CD4⁺CD8⁺ stage, and the positive selection of CD4⁺CD8⁺ cells expressing $\alpha\beta$ TCRs capable of MHC recognition. Interestingly, interactions between CXCR4 and its ligand CXCL12, a chemokine expressed by cTEC, have been reported to play a role in the regulation of both these events (Table 1). For example, CXCR4–CXCL12 interactions regulate the intrathymic positioning of T cell progenitors (14) while the maturation of pre-TCR expressing CD4⁺CD8⁺ progenitors requires CXCR4–CXCL12 to act in concert with Notch signaling in order to drive β -selection (15, 16). For later stages of thymocyte development, CXCL12 has recently been reported to act as a cortex retention factor for CD4⁺CD8⁺ thymocytes,

which may enable cells to stay within the thymic cortex in order to undergo correct maturational events, including positive selection (17). Interestingly, of relevance to these studies that indicate the importance of CXCL12–CXCR4, is analysis of the expression and distribution pattern of CXCL12. Thus, analysis of CXCL12^{dsRed} knockin mice showed that CXCL12 is expressed by Ly51⁺ cTEC, and is distributed throughout the thymic cortex microenvironment (18). As such, it is not currently clear how such a broad expression pattern of CXCL12 might relate to the possibility that particular regions of the thymus cortex are specialized to support specific maturational events. Moreover, it is also important to note that deletion of CXCR4 expression using CD4^{cre}, which selectively targets CD4⁺CD8⁺ thymocytes and their downstream products, did not alter thymocyte development, nor the intrathymic positioning of CD4⁺CD8⁺, CD4⁺, and CD8⁺ thymocytes (18). Thus, at stages downstream of pre-TCR mediated events, it is currently still unclear to what extent CXCR4 plays an important role. Perhaps relevant to this, CCR9 is an additional chemokine receptor expressed by CD4⁺CD8⁺ thymocytes which, via interplay with plexinD1-semaphorin3E interactions, has been reported to play a role in the intrathymic positioning of thymocytes (19). Whether such findings collectively indicate potential functional redundancy and/or hierarchy in chemokine receptors and ligands that regulate the cortex residency of CD4⁺CD8⁺ requires further investigation.

Regarding the ability of cTEC to support MHC class I mediated positive selection of CD8⁺ T cells, processing and presentation of peptides associated with MHC-I molecules requires proteasomal degradation. The thymoproteasome is a unique type of proteasome expressed specifically by cTECs, the catalytic subunit of which is β 5t (Table 1). Mice deficient in β 5t have reduced positive selection of CD8⁺ thymocytes (20). cTEC restricted expression of β 5t is also seen within human TECs, interestingly however analysis of humans carrying mutations within PSMB11 has not revealed any adverse effects (21). Nevertheless, β 5t is a defining feature of cTEC that directly underpins at least part of their functional specialization for positive selection. Importantly, while β 5t expression by cTEC in the adult thymus is an important defining feature of cTEC functionality, it is also expressed by immature TEC progenitors. This is perhaps most notable in analysis of the embryonic thymus, where fate mapping of β 5t expressing cells showed that mTEC, including the Aire⁺ subset, are derived from β 5t-expressing cells (22). Thus, while β 5t expression is unique to TEC, and underpins cTEC function, its expression is not exclusive to mature cTEC. Moreover, expression of β 5t by TEC progenitors is also relevant to understanding functional heterogeneity in pathways of TEC development. For example, it is not clear whether β 5t⁺ progenitors are capable of cTEC functions such as positive selection prior to their transition toward the mTEC lineage. In this scenario, the embryonic thymus may harbor TEC progenitors that go through serial progression development (23), in which β 5t⁺ TEC first function as cTEC, and then differentiate further toward mTEC. Alternatively, β 5t⁺ progenitors may not possess functional capabilities of cTEC, and represent functionally immature cells that serve as a source of

TABLE 1 | Differential gene expression in cTEC and mTEC subsets.

Molecule	Gene Name	Expression pattern	Reported functions in thymus	References
CD205	<i>Ly75</i>	Broad within cTEC	Apoptotic cell clearance	(105)
$\beta 5t$	<i>Psmb11</i>	Broad within cTEC, also TEC progenitors	Thymoproteasome component, CD8 positive selection	(31, 71, 106)
PRSS16	<i>Prss16</i>	cTEC	Thymus specific serine protease, CD4 positive selection	(107)
Delta like 4	<i>Dll4</i>	cTEC	Notch ligand, regulator of T-cell commitment and β -selection	(108)
CXCL12	<i>Cxcl12</i>	Broad within cTEC	Chemokine ligand for CXCR4, regulation of β -selection	(14–18)
CCL25	<i>Ccl25</i>	cTEC and mTEC	Chemokine ligand for CCR9, recruitment and positioning of T-cell progenitors, regulator of CD4 ⁺ CD8 ⁺ thymocyte migration	(95, 109, 110)
CCRL1	<i>Ackr4</i>	cTEC and mTEC	Atypical chemokine receptor, scavenging receptor for CCL19, CCL21, CCL25	(111, 112)
LT β R	<i>Ltbr</i>	cTEC and mTEC	Ligand for lymphotoxin and light, regulator of mTEC development and thymic endothelium development. No known role in cTEC	(34, 45, 96, 97, 113, 114)
Aire	<i>Aire</i>	mTEC ^{hi}	Tissue restricted antigen expression, tolerance	(115, 116)
Fezf2	<i>Fezf2</i>	mTEC ^{hi} and mTEC ^{lo}	Tissue restricted antigen expression, tolerance	(44, 45)
RANK	<i>Tnfrsf11a</i>	mTEC ^{hi} and mTEC ^{lo} , mTEC progenitors	mTEC development	(32, 33, 38, 40, 41, 117)
OPG	<i>Tnfrsf11b</i>	mTEC ^{hi}	Negative regulator of mTEC	(33, 39, 41)
IL25	<i>Il25</i>	Thymic tuft cells	Regulation of intrathymic ILC and iNKT-cells	(9, 11)
IL15	<i>Il15</i>	mTEC ^{lo}	IL15 transpresentation, regulation of iNKT-cells	(59, 118)
IL15R α	<i>Il15ra</i>	mTEC ^{lo}	IL15 transpresentation, regulation of iNKT-cells	(59)
IL7	<i>Il7</i>	cTEC and mTEC in adult thymus, TEC progenitors in embryonic thymus	T-cell progenitor proliferation	(70)
CCL21	<i>Ccl21a</i>	mTEC	Chemokine ligand for CCR7, regulator of cortex to medulla migration of SP thymocytes	(37, 42)
Relb	<i>Relb</i>	mTEC	mTEC progenitor development	(32, 119, 120)
SCF	<i>Kltkl</i>	cTEC	Maintenance of T cell progenitors	(121, 122)

LT β R, Lymphotoxin beta Receptor; Aire, Autoimmune Regulator; RANK, Receptor Activator of Nuclear Factor κ B; OPG, Osteoprotegerin; ILC, Innate Lymphoid Cell; iNKT, invariant Natural Killer T Cell; SP, Single Positive; SCF, Stem Cell Factor.

functionally competent cTEC and mTEC. Whatever the case, expression of $\beta 5t$ by both TEC progenitors and mature cTEC makes it difficult to directly define and discriminate between these cell types, particularly in the embryonic thymus.

Although the properties of the thymus cortex are becoming increasingly well-defined, functional heterogeneity within cTEC is still relatively poorly understood. Efforts have been made to investigate the cTEC population, and heterogeneity has been suggested using Sca-1 and $\alpha 6$ -integrin, with Sca-1 positive cells expressing high levels of MHCII (20). Some of the difficulties in investigating the cTEC population is likely due at least to the relative low yield of cells obtained from the thymus compared to mTEC, making cTEC a difficult population to study. However, a recent study bypassed this problem by analyzing cTEC in mice in which CyclinD1 is overexpressed by K5 expressing cells. Although TEC were found at ~ 100 -times greater frequency, the thymus from these mice had a normal structure, and supported a normal program of T cell development. Mass spectrometry proteomics and single cell RNA sequencing confirmed cTEC specific expression of Cathepsin L, TSPP, and $\beta 5t$, and mTEC-specific expression of Cathepsin S, CD40, and Aire (24). These mice could prove to be a useful platform for further interrogation of the cTEC compartment.

In relation to functional heterogeneity within cTEC, thymic nurse cells (TNC) are large epithelial cell complexes in which single cTECs encase viable thymocytes. This is a unique feature of cTEC, and ~ 10 –15% of cTEC form such complexes; each containing between four and eight DP thymocytes (24). TNC are absent from the embryonic thymus, and are only detectable from 5 days post-birth; perhaps a reflection of a bias toward mature cTEC rather than cTEC-like progenitors. In addition, cTECs that form TNC have increased expression of CD205, CXCL12, TGF β , TSSP, and VCAM-1, compared to cTEC that are not part of TNC structures (25). Interestingly, thymocytes contained within TNC are enriched for those that have undergone secondary TCR α rearrangements, suggesting they may provide an environment then enables efficient positive selection. The presence of such epithelial-thymocyte complexes results in an estimated 20% of RNA isolated from total cTECs reflecting gene expression by enclosed DP thymocytes (24). This can be seen in single cell RNA sequencing of cTECs where newborn and adult cTECs appear to be contaminated with DP thymocytes (26). Recent single cell RNA sequencing analysis has begun to describe some heterogeneity within cTEC beyond TNC. For example, Bornstein et al. (9) found two clusters within cTEC with differential expression of genes including *Dll4*.

Whether these cTEC differ in their functional capacity has not yet been tested.

Interestingly, constitutive autophagy is a feature of TECs, which contributes to the processing and presentation of MHCII associated peptides. Comparison of this process in cTEC and mTEC using GFP-LC3 transgenic mice to allow the detection of autophagosomes, showed that cTEC exhibit a higher frequency of autophagy-positive cells compared to mTECs (27). The function of this TEC specific feature is not clear, with conflicting reports in the literature. An initial study used *Atg5*^{-/-} embryonic thymus lobes, grafted into nude mice, and showed that host mice generated symptoms of systemic autoimmunity (27). This was challenged by a later study, using targeted deletion of *Atg7* in TECs using a K14^{Cre} mouse line. These mice showed an absence of autoimmunity, even when aged (28). Cross comparisons are difficult between both studies due to the differing methods used to delete gene expression from TECs and analyze autoimmunity, thereby highlighting an area of research in need of further clarification.

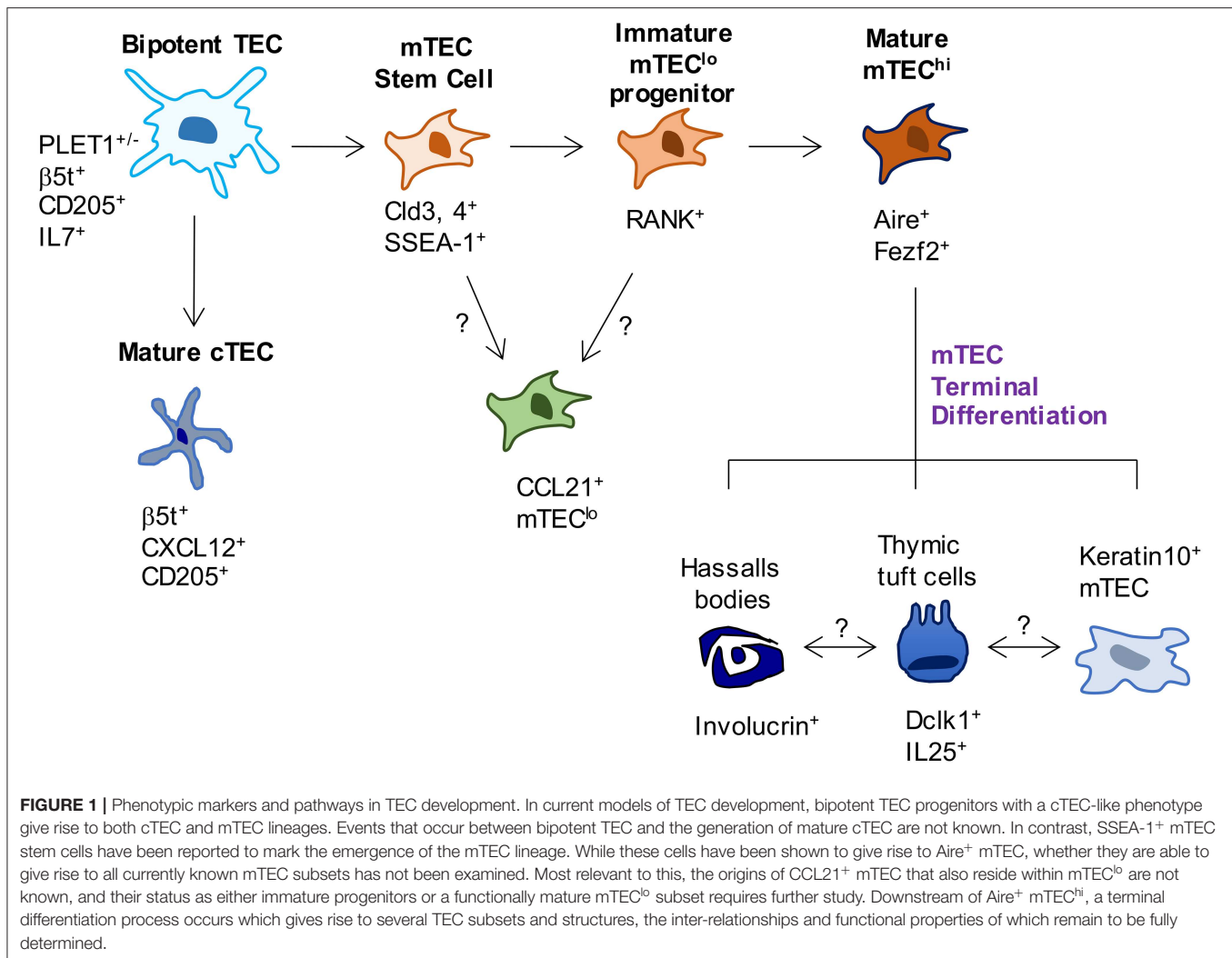
mTEC Stem Cells

As TEC development involves the formation of distinct cTEC and mTEC populations (**Figure 1**), several studies have examined early timepoints in the development of these sublineages that are downstream of bipotent progenitors. For example, TEC expression of the tight junction components, Claudin-3 and Claudin-4 (*Cld3*, 4) has been reported to mark the emergence of the mTEC lineage, with cells expressing these markers giving rise to Aire⁺ mTEC (29). Moreover, a small population of TEC which co-express *Cld3*, 4 along with the stem cell marker SSEA-1 have been termed mTEC stem cells due to their self-renewal capabilities and their ability to give rise to mTEC but not cTEC. mTEC stem cells have been further characterized by low expression of $\beta 5t$ and CD205, and high expression of RANK and LT β R, and although they are capable of producing downstream mTEC populations in adult thymus, they do so with greatly reduced efficiency compared to in the embryonic thymus (30, 31). Despite expressing very low levels of $\beta 5t$ protein, fate-mapping experiments show these cells have a history of $\beta 5t$ expression, in keeping with them being downstream of $\beta 5t$ ⁺ TEC progenitors (31). Interestingly, mice deficient in the master TEC transcription factor *Foxn1* have normal frequencies of mTEC stem cells (32), suggesting that the defects in TEC development present in nude mice is downstream of these cells. Importantly, Baik et al. (32) also clarified the function of the TNFRSF member Relb during stages of mTEC development. Although *Relb*^{-/-} mice had unaltered numbers of mTEC stem cells, using RANK^{Venus} reporter mice, it was shown that in the absence of Relb, *Cld3*, 4⁺ mTEC fail to upregulate RANK expression. The importance of RANK signaling for the mTEC compartment is clear from the large reduction in mTEC, including Aire⁺ mTEC in *Rankl*^{-/-} mice (33). Although the generation of mTEC stem cells does not require *Foxn1* or Relb, it has been shown at least in neonatal mice, to be partially dependent on LT β R, as mTEC stem cell frequencies are reduced in neonatal K14^{Cre}LT β R^{flxed} mice where targeted deletion of LT β R by TEC has been achieved (34). Despite the clear progress that has been made within this field, complex questions

remain, which is due at least in part to a wide variety of markers used to identify TEC populations, as well as differing *in vivo* and *in vitro* methods used to assess their lineage potential. Further work is needed to build a more complete profile of relationships between mature TEC compartments and TEC progenitors, and the developmental requirements of each.

Immature mTEC Progenitors

In order to gain a better understanding of complexity within TEC populations, recent studies have interrogated the mTEC population using single cell RNA sequencing. One such study sorted total “unselected” mTECs, in addition to mTEC expressing specific Tissue Restricted Antigens (TRAs), namely Tspan8 and GP2 protein. To determine the likely developmental progression (10), clustering, and pseudotime trajectory analysis was performed on the single cell RNA sequencing data obtained from these populations. In agreement with other studies, this study highlighted a distinct population of mTEC phenotypically resembling jTECs (35) through their expression of *Pdpn* and lack of expression of Aire. Importantly, such cells were also defined by expression of the chemokine *Ccl21a*, that plays an important role in the recruitment of positively selected thymocytes into the medulla (**Table 1**). However, it is important to note that not all *Ccl21* expressing mTEC appear to have high *Pdpn* expression (9). Interestingly, predicative analysis by Dhalla et al. (10) suggested CCL21⁺Pdpn⁺ immature mTEC follow a maturation pathway whereby they upregulate Aire expression, followed by expression of TRAs along with high levels of CD80 and CD86. Consistent with this, the gene signature associated with CCL21⁺ mTEC-I are present within the thymus at E14.5 whereas the genes relating to Aire⁺ mTEC-II are not (9). More recent studies examining the developmental pathway of TEC development have used trajectory analysis of large data sets. Such analysis was performed on clusters of jTEC, mTEC^{lo}, and mTEC^{hi}, identified from single cell RNA sequencing data and supported the previously described immature phenotype of jTEC, and suggested they were most likely to become mTEC^{hi} before downregulating markers associated with maturation to become mTEC^{lo} (36). While these studies provide important new information on mTEC heterogeneity, it is not fully clear whether CCL21-expressing mTEC, that typically lie within the MHCII^{lo}CD80^{lo} (mTEC^{lo}) compartment represent directly progenitors of later mTEC stages, including mTEC^{hi}. Indeed, although immature mTEC progenitors are known to reside within the bulk mTEC^{lo} compartment, the expression of CCL21 by some of these cells suggests that they are already functionally mature (37), and so could be defined as a mature mTEC subset. Perhaps relevant to this, at least in the embryonic thymus, mTEC progenitors that are able to give rise to Aire⁺ mTEC^{hi} can be defined by their expression of RANK (38, 39) (**Table 1**). Indeed, in both embryonic and adult thymus, RANK itself is a key functional regulator of the maturation of mTEC progenitors into more mature mTEC^{hi} (33, 38–40). Importantly, while RANK expression has relevance to the study of mTEC progenitors, the nature of embryonic mTEC^{lo} progenitors, and their full developmental potential, remains poorly understood. For example, it is not currently known whether RANK⁺



progenitors also express CCL21, a chemokine that is expressed by at least some mTEC (37) or whether RANK⁺ progenitors can give rise to CCL21⁺ mTEC. Moreover, analysis of RANK^{Venus} reporter mice has shown that patterns of RANK expression in the adult thymus are complex, with multiple subsets of mTEC^{lo} and mTEC^{hi}, including CCL21⁺ cells and Aire⁺ cells, demonstrating heterogeneity with regard to RANK expression (41). Thus, while it is clear that RANK is expressed by at least some mTEC progenitors, it is not known whether such progenitors have the potential to generate all mTEC subsets. Moreover, RANK may also be expressed by, and operate on, mTEC at other developmental stages. The recent generation of CCL21^{tdTomato} reporter mice (42) together with availability of RANK^{Venus} mice (41) offers a possible way to generate new dual mTEC reporter mouse strains to examine new precursor-product relationships within the mTEC lineage.

MHCII^{hi}CD80^{hi} mTEC^{hi} and Thymic Tolerance

Clear heterogeneity within mTEC exists, however segregation into subsets based on phenotype, developmental requirements

and function can make conclusions and cross comparisons challenging. Routinely, mTECs are broadly subdivided into mTEC^{lo} and mTEC^{hi} according to their levels of MHCII and CD80. Perhaps the most defining feature used to discriminate populations within mTEC^{hi} is expression of the autoimmune regulator (Aire). The transcription regulator Aire is required for efficient promiscuous gene expression of TRAs by mTEC, which is vital for the deletion of self-reactive thymocytes. This contributes to the multi-organ autoimmunity in Aire deficient mice and Aire deficient (autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy) patients. The function of Aire exceeds TRA expression. For example, Aire regulates the expression of XCL1 in mTEC; a chemokine important for the medullary localization of thymic DC and Treg generation. Mice deficient in XCL1 exhibit symptoms of autoimmunity, suggesting that Aire promotes central tolerance via multiple mechanisms (43).

Not all promiscuous gene expression is dependent on Aire, as TRA expression is evident in Aire^{-/-} mice. The transcription factor Fezf2, also expressed by mTEC, has been reported to be required for the expression of some Aire-independent genes

(44). Parallel to Aire, *Fezf2* expression is observed within the mTEC^{hi} population, and moreover immunofluorescent staining reveals co-expression of both Aire and *Fezf2* by the same cell (44, 45). Such mTEC^{hi} that express high levels of Aire, *Fezf2*, and molecules associated with antigen presentation, were also described in a recent single cell RNA dataset and termed mTEC-II (9). Interestingly, co-expression of Aire and *Fezf2* can be seen in human mTEC (44, 46), and its expression by Aire⁺ mTEC may suggest that similar mechanisms underpin central tolerance in mouse and man. Importantly, *Fezf2* expression has also been observed within mTEC^{lo} (38), suggesting TRA expression is not restricted to mTEC^{hi} cells.

mTEC Terminal Differentiation and Post-Aire mTEC: Involucrin⁺ Cells

Aire⁺ mature mTEC were once considered to be at the final stages of their maturation, with Aire expression indicative of subsequent apoptosis (47). However, several lines of evidence now show Aire⁺ mTEC can continue their development beyond phases of Aire expression, to become TEC expressing markers typical of terminally differentiated keratinocytes (48, 49). These cells form distinct structures within the thymus medulla termed Hassall's corpuscles, and can be identified based on their expression of keratin-10 and involucrin. TEC with this phenotype are likely to be downstream of Aire⁺ mTEC based on the ontogenetic appearance of both subsets. Aire⁺ mTEC appear first during ontogeny, and are dependent on RANKL provision by DP thymocytes. Subsequently a population of involucrin⁺ mTEC becomes visible, which require LT α expression by positively selected thymocytes for their development (50). This is supported from direct analysis of TEC developmental pathways and fate mapping experiments, which showed that Aire⁺ cells can proceed to become Aire⁻ cells with lower levels of MHCII (49). Further analysis of post-Aire mTEC shows they lack expression of CCL21 (51), making them distinct from other populations of MHCII^{lo} mTEC.

Although the role of Hassall's corpuscles remains elusive, further characterization of the phenotype and function of these cells has been achieved in the human thymus, where these medullary structures are much more prominent. In addition to keratin-10 and involucrin, Hassall's corpuscles in human thymus express filaggrin (52), and thymic stromal lymphopoietin (TSLP) (53). Whilst filaggrin expression within the murine medulla has been demonstrated (54), whether this specifically marks post-Aire mTEC is unclear. Expression of TSLP by Hassall's corpuscles in the human thymus has been shown to induce expression of markers associated with DC activation, which was needed to generate thymic T-Reg (53). This finding was followed up in mice, where TSLP-ZsG reporter lines were used to describe an enrichment of involucrin gene expression within reporter⁺ mTEC (55), however whether there is a role for TSLP expression by terminally differentiated mTEC in the generation of T-Reg in mouse thymus is unknown.

mTEC Terminal Differentiation and Post-Aire mTEC: Thymic Tuft Cells

A combination of fate mapping experiments and single cell RNA sequencing analysis from two independent groups suggests there are two main populations of post-Aire mTEC (9, 11). One population is the Keratin-10⁺ involucrin⁺ mTEC discussed above, whereas the other is a distinct population of TEC which resemble tuft cells that have been described at mucosal sites. Tuft cells are a type of chemosensory epithelial cell, most studied for their role in controlling helminth infection via activation of ILC2. Comparison of cells isolated from different tissues showed that tuft cells from the thymus had the greatest number of differentially expressed genes compared to tuft cells from other sites (56). Despite these differences, tuft cells from intestinal and thymic tuft cells share similarities, e.g., expression of IL25, *Trmp5*, *Dcl1*, and IL17RB.

The functions of these newly defined TEC are yet to be thoroughly explored. Unlike intestinal tuft cells, thymic tuft cells express high levels of MHCII (11) perhaps indicating an active role in antigen presentation and thymic selection. Interestingly however, evidence from Miller et al. (11) indicated a role for thymic tuft cells in central tolerance, through their expression of the tuft cell specific gene IL25. Thus, transplantation of tuft cell deficient *Pou2f3*^{-/-} thymic lobes into nude mice, resulted in the generation of anti-IL25 antibodies upon immunization, suggesting that tuft cells may act as an important source of antigen within the thymus (11). Perhaps also relevant to possible functional significance of thymic tuft cells it is interesting to note that in the gut, activation of tuft cells can be mediated by the microbial metabolite succinate. However, expression of the succinate receptor *Sucnr1* is higher in small intestinal tuft cells compared to thymic tuft cells (56), and it is currently unknown whether thymic tuft cells undergo activation, and if so, how this might occur. Initial reports also suggest thymic tuft cells are capable of regulating innate immune networks within the thymus. *Pou2f3* is the master regulator of tuft cell development, and as such, *Pou2f3*^{-/-} mice have been used to begin to determine the role of thymic tuft cells, and both thymic ILC and iNKT cells have been examined in tuft cell deficient mice. Bornstein et al. (9) proposed that due to the restricted thymic expression of IL25 by tuft cells, hematopoietic cells expressing IL25R may be dysregulated. In keeping with this, increased frequencies of ILC2 were present in the thymus of *Pou2f3*^{-/-} mice, however whether this is linked to absence of IL25 is not known. Interestingly, analysis of ILC subsets during thymus ontogeny revealed dynamic changes in their makeup. For example, while ILC3 are dominant in the embryonic thymus, ILC2 dominate post-natally (57). The reasons for this developmental switch in intrathymic ILC frequency is not clear. However, it is interesting to note that like ILC2, tuft cells also emerge post-natally, which together with the localization of both cell types within the medulla, may further emphasize a potentially important link between these cell types. If thymic tuft cells are regulators of ILC2, any increase in the latter in tuft cell deficient *Pou2f3*^{-/-} mice would indicate that tuft cell products may act to limit ILC2 proliferation and or survival. While

such factors remain unknown, as is the functional relevance of tuft cell control of ILC2 availability, it is perhaps important to note that ILC2 represent an intrathymic source of IL13 (57), a cytokine ligand for the type 2 IL4R that has been shown to be an important regulator of thymus emigration (58). Whether tuft cells limit ILC2-derived IL13 availability that then influences rates of thymus exit, has not been addressed. In addition to tuft cell-ILC interactions, Miller et al. (11) also examined *Pou2f3*^{-/-} mice to assess the potential impact of tuft cells on iNKT cells that represent a non-conventional $\alpha\beta$ T-cell lineage that is restricted to the non-polymorphic MHC class I like molecule CD1d. In line with a requirement for mTEC in NKT-cell development (59), *Pou2f3*^{-/-} mice were reported to have decreased frequencies of Tbet⁺ NKT1, PLZF⁺ NKT2, and Ror γ t⁺ NKT17 within the thymus (11). Interestingly, a reduced frequency of Treg progenitors was also seen in the thymus of both *Pou2f3*^{-/-} mice and iNKT-cell deficient *Cd1d*^{-/-} mice (60). The cellular and molecular interactions that connect tuft cells and iNKT-cells to the intrathymic development of Treg requires further investigation. Finally, studies demonstrating links between tuft cells and iNKT-cells are important as they indicate the importance of mTEC heterogeneity extends beyond its influence on conventional $\alpha\beta$ T-cell development in the thymus. How tuft cells control distinct subsets of iNKT-cells is currently not known. Given the patterns of IL25R expression by iNKT-subsets (61), and the selective intrathymic production of IL25 by tuft cells, one possibility that requires further examination is that tuft cells directly regulate at least some iNKT subsets, including NKT1 and NKT17 cells that express IL25R, via their provision of IL25. Whether tuft cells and/or additional mTEC subsets have the ability to influence individual iNKT subsets also requires further investigation.

Significantly, while there is evidence for the existence of DCLK1⁺ tuft cells within the human thymus (9), whether human and mouse thymic tuft cells express a similar array of receptors and secreted factors is currently unknown. This will be important to consider when the functions of thymic tuft cells are more fully understood. While immunofluorescence staining of mouse thymus sections showed that both Keratin 10⁺ mTEC and tuft cells are in close proximity to one another (11), the developmental relationships between the two populations is not clear. Although both populations can transit through an Aire-expressing stage, fate mapping experiments showed this isn't a feature of all tuft cells (11). Moreover, the requirement for Aire in their development may differ, for example, *Aire*^{-/-} mice show significantly reduced frequencies of Keratin-10⁺ mTEC (11, 51), whereas tuft cells are present in equivalent numbers in *Aire*^{-/-} mice (11). The initial description of thymic tuft cells proposed Hipk2, an Aire binding partner, to be a molecular regulator of this population, and the generation of Foxn1^{Cre}Hipk2^{flxed} mice revealed reduced frequencies of thymic tuft cells (11), however the mechanism behind this is unknown. An additional regulator of Keratin-10⁺ mTEC development is LT α from positively selected thymocytes, as these terminally differentiated mTEC were found in reduced frequencies in *Lta*^{-/-}, *Ltrb*^{-/-}, and *Zap70*^{-/-} mice (50). Whether lymphotoxin signaling is also a

regulator of tuft cell development in the mTEC lineage is yet to be determined.

COMMON ORIGINS OF cTEC AND mTEC

Bipotent TEC Progenitors

Despite the differing roles of cTEC and mTEC in the adult thymus, their development begins in the embryonic thymus from a common bipotent precursor that gives rise to both lineages. Initial experiments using purified populations of TEC and antibodies against MTS20 and MTS24 showed that both cTEC and mTEC are generated from Placenta expressed transcript-1⁺ (PLET1⁺) TEC (62, 63). However, at this time it was unclear whether a bipotent progenitor or individual cTEC and mTEC-restricted precursors were contained within this fraction. Direct evidence was subsequently demonstrated for the existence of a bipotent TEC progenitor in the embryonic thymus. This was shown by the microinjection of a single EpCAM1⁺ YFP⁺ cell into a non-YFP embryonic thymus, which was then grafted under the kidney capsule of a wildtype (WT) mouse. These grafts contained both Ly51⁺ cTEC, and Keratin-5⁺ mTEC, each anatomically segregated into distinct compartments, thus demonstrating that one cell can produce both TEC lineages (64). Similar conclusions were drawn from an independent study published at the same time that used mice in which a mutant allele of Foxn1 could be reverted to wildtype function in single cells at post-natal stages. Following spontaneous induction of Foxn1 gene expression in a single cell, mice were able to generate thymic tissue containing both cTEC and mTEC providing evidence that bipotent TEC progenitors are also present within the post-natal thymus (65). Although both of these studies highlighted the existence of a bipotent TEC progenitor, the phenotype of such cells remains elusive, despite attempts of further characterization. Using reaggregate thymic organ cultures (ROTC) with purified populations of TEC, Rossi et al. (66) confirmed bipotent TEC progenitor potential by MTS24⁺ TEC, but in addition showed equivalent capabilities within the MTS24⁻ fraction. These findings support the notion that bipotent TEC progenitors express PLET-1, but shows additional progenitors are also present within the embryonic thymus which lack PLET-1 expression. The bipotent potential of PLET-1 expressing TEC isolated from adult thymus has been also assessed by grafting ROTC into WT mice. These grafts showed that UEA-I⁻Ly51⁺PLET-1⁺ cells with high expression of MHCII can give rise to both cTEC and mTEC, and continue to do so up to 9 months later (67).

Beyond these original descriptions of bipotent TEC progenitors, several studies have searched for evidence that supports the existence of bipotent TEC progenitors in the post-natal and adult thymus. For example, studies using long-term BrdU retention (indicative of a quiescent state) in adult TECs revealed MHCII^{lo} α 6⁺Sca-1⁺ cells at the corticomedullary junction (CMJ) could generate both cTEC and mTEC lineages in reaggregate grafting experiments (68). However, it is not clear whether bipotent TEC or lineage-specific precursors were contained within this fraction, and their low expression

of MHCII makes this population distinct from the PLET-1⁺ cells described previously (67). In the embryo, further characterization of bipotent TEC progenitors has been possible, and studies have collectively described a cTEC-like phenotype of such cells using a variety of methods. For example, embryonic mTEC were shown to arise from TEC expressing markers commonly associated with cTEC, e.g., CD205 (69) and IL7 (70). In addition, Ohigashi et al. (22) fate mapped β 5t expression; the proteasome subunit expressed by cTEC but not mTEC, and showed that cells with a history of β 5t expression later bore hallmark features of mTEC, including Aire expression. In a subsequent study using inducible β 5tCre GFP mice to fate-map cells at post-natal stages, Ohigashi et al. (31) showed <5% of mTEC were labeled when mice were treated with doxycycline after 1 week of age, whereas doxycycline treatment from E0 labeled ~80% of mTEC. These results indicate that post-natal mTEC are derived from cells which express β 5t embryonically. The shared expression of several markers e.g., CD205, Ly51, β 5t, between cTEC and bipotent TEC progenitors makes the respective populations difficult to distinguish. However recent data from embryonic and adult TEC suggests differences now exist, and RNA sequencing analysis shows that embryonic TEC are enriched for genes involved in cell cycling and have a downregulation of genes involved in antigen presentation (9, 26). Such studies may support future approaches to identify differentially expressed genes that help to define and isolate TEC and TEC progenitor subsets.

Additional studies have provided information regarding the anatomical positioning of TEC progenitors in the post-natal thymus. In agreement with the description of an early cTEC phenotype of TEC progenitors, a combination of inducible fate mapping and confetti mice showed clusters of post- β 5t expressing TEC that were concentrated at the CMJ (jTEC), which progressed to become mTEC (71). In addition, single cell RNA sequencing datasets show populations that resemble the immature jTECs described here (9, 10). Combined, this data supports a model of serial progression, whereby bipotent precursors acquire traits usually associated with cTEC, before bearing hallmark features of mTEC. Interestingly, cells within the adult thymus resembling stem cells have been described, with Ucar et al. (72) identifying cells capable of forming spheroid colonies typical of cells with stem cell properties. Such colonies, termed thymospheres, lacked expression of EpCAM1 and Foxn1, and were shown to have the capacity to generate both cTEC and mTEC. A more recent study revisited this issue, including the nature of thymosphere forming cells, and showed using a combination of fate mapping mouse strains that thymosphere forming cells are not of epithelial cell origin. Instead, they show by fate-mapping Wnt1^{Cre+} cells, that thymosphere forming cells are neural crest derived, and such structures can incorporate bystander TECs, thereby producing the results seen in the initial study (73). As such, the possible presence and identity of TEC populations with clonal and self-renewing properties remains unclear, and further studies are required to examine the earliest stages in embryonic and post-natal TEC development that give rise to the continual generation of cTEC and mTEC lineages.

FACTORS AFFECTING RATES OF THYMUS FUNCTION

Both chronic and acute damage to the thymus have detrimental effects on its ability to support T cell development. In particular, changes that take place within thymic epithelial microenvironments result in a reduction in T cell production, and such events can take place in several ways. For example, both age-related thymic involution and therapeutic cytoablative treatments erode TEC microenvironments which then impair rates of thymocyte development. Importantly however, regeneration processes can occur within the thymus, and several efforts have been made to understand this process and to harness it for therapeutic benefit.

Age Related Thymus Atrophy

Natural thymic involution, that occurs as a result of aging, significantly reduces rates of thymic function across the life course. Analysis of recent thymic emigrants as a measure of thymic function in Rag2GFP mice highlights the constant decline in *de novo* T cell production during the first 5–6 months of life (74). Unfortunately, identification of newly produced T cells in humans is more challenging, and currently relies on the PCR quantitation of circular DNA by-products of TCR gene rearrangements termed T Cell Receptor Excision Circles (TRECs), in conjunction with surface markers including CD31 and CD103. Such analysis shows that similar to mice, thymus function in humans also declines with age (75–78), resulting in a pool of peripheral T cells in the elderly which is dominated by clonally expanded cells (79). This impacts on the ability of an aged immune system to respond to challenge such as infection and vaccination.

Age associated thymic atrophy has been well-studied in mice, and attempts have been made to understand the mechanisms behind this phenomenon. The recruitment of T cell precursors into the thymus occurs via blood vessels at the CMJ, and expression of key molecules, e.g., P-selectin and CCL25 are important in this recruitment process (80). Initial reports suggested that expression of these factors is unaffected in aged mice (81), however a more recent publication showed reduced expression of CCL25 in the thymus from aged mice (82). Despite these discrepancies, recruitment of progenitor cells into the thymus does not appear to be the causative factor behind age related thymus involution, as the ability of an aged thymus to recruit intravenous-injected lymphoid progenitors is equivalent to a young thymus (81). Moreover, intrathymic injection of T cell precursors into young and aged thymi show reduced T cell development within the aged thymus (83). Combined, this data suggests that recruitment of progenitors may not be a simple explanation for the limited thymopoiesis in aged mice, but rather that other environmental factors within the thymus likely influence T cell development and thymus cellularity.

Interestingly, analysis of the stromal compartment by immunofluorescence in aged mice showed a progressive loss of both CD205⁺ cTEC and UEA-1⁺ mTEC, resulting in epithelial “free” areas (81, 84, 85). This loss of TEC in aged mice is perhaps caused by reduced levels of proliferation and increased apoptosis

(47, 81). Moreover, aged thymi show increased expression of phosphorylated H2AX and p53 binding protein; markers of DNA damage and cellular senescence (84), which could account for the reduced thymus function seen with increasing age. The mechanisms that control TEC proliferation in aged mice is not fully understood, however it is interesting to note that whilst 95% of embryonic TECs express Foxn1, the frequency of Foxn1 expressing TECs decreases post-natally (86). In keeping with reduced Foxn1 expression in thymi from older mice, cTEC from older mice show reduced expression of Foxn1 target genes, e.g., *Dll4*, *Kitl*, *Cxcl12*, all of which are important for early stages of T cell development (82). Genetic alterations to Foxn1 in various mouse lines have been used in attempts to understand how Foxn1 may impact age related thymus involution. For example, overexpression of Foxn1 in young mice results in delayed thymus involution (87), whereas reduced Foxn1 expression in young mice causes premature thymus involution (88). In addition, regeneration of thymus function in aged mice has been possible via the upregulation of Foxn1 expression by TEC (82). Interestingly, such studies show Foxn1 aids TEC maintenance and recovery by inducing proliferation of TEC subsets, including MHCII^{lo} TEC that are known to include TEC progenitors.

Proliferation of TEC is required for normal thymus growth during ontogeny, and this high rate of TEC proliferation during early stages of organogenesis is dependent on the transcription factor Myc. Importantly, Myc expression by TEC is high in the embryo but undergoes subsequent downregulation which correlates with age. This important process limits the extent to which the thymus can grow, and therefore may contribute to age-related thymus involution. Cowan et al. (26) induced transgenic expression of Myc by TEC, which maintained a fetal gene signature in adult TEC, and caused excessive TEC proliferation and thymus hyperplasia in adult mice, suggesting this could be a mechanism by which to reverse age related thymic atrophy.

Recovery of Thymus Function During Immune Reconstitution

Cytoablative therapies such as chemotherapy and radiotherapy, that are often used in conjunction with bone marrow transplant (BMT), cause apoptosis of radiosensitive cells including thymocytes and TEC (89). As a result, there is a diminished capacity for the generation of newly produced naïve T cells. The recovery of T cells in the periphery occurs via two mechanisms; homeostatic expansion of T cells contained within the graft, and the export of naïve T cells from the thymus (Figure 2). The type of conditioning regime along with patient age, is likely to fine-tune the mechanism by which the peripheral T cell pool is restored (90, 91). As in homeostatic conditions, the production of new T cells requires the recruitment of T cell progenitors to the thymus. Thymus reconstitution following BMT appears to be limited by the number of progenitor cells available within the circulation, as a positive correlation is seen between numbers of BM cells administered, and the frequency of donor-derived DP thymocytes (92, 93). For these reasons, most studies have examined the process of thymus homing following BMT.

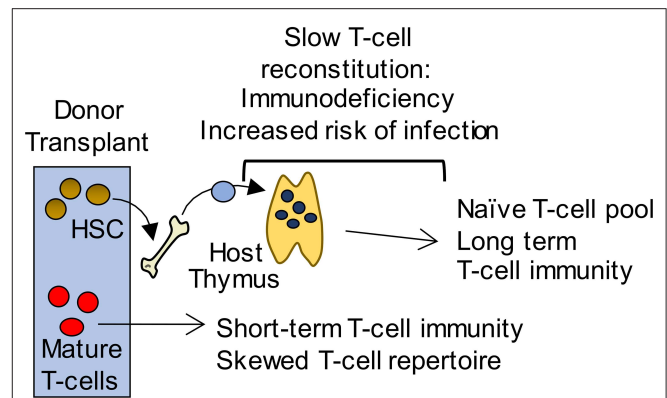


FIGURE 2 | Long-term T-cell immunity following BMT is thymus dependent. Initially, short term T-cell mediated immunity is provided by a donor derived mature T-cell pool with a limited T-cell receptor repertoire that is unable to mount effective immune responses to pathogens. In contrast, a thymus dependent pathway gives rise to an antigenically diverse naïve T-cell pool that provides long term protection. However, this pathway requires graft-derived progenitors to colonize the thymus and undergo T-cell development. As a consequence, T-cell reconstitution is slow and leaves patients immunodeficient and at potentially fatal risk of infection.

Thymic epithelial cells, including both cTEC and mTEC are reduced following irradiation in mouse models indicating the radiosensitive nature of these cells (89, 94). Although numbers of TEC are reduced, the ability of such cells to produce chemokines that are important T cell progenitor recruitment, such as CCL19, CCL21 and CCL25 is maintained following irradiation (89). In contrast, endothelial cells, which are important for the recruitment of T cell progenitors, appear to be radioresistant. Interestingly, the presentation of CCL25 by thymic endothelium is transiently disrupted following irradiation which is proposed to limit thymus reconstitution, as pre-treatment of bone marrow cells with CCL25 caused increased T cell progenitor entry to the thymus following irradiation. This is in keeping with the requirement of CCR7 and CCR9 for T cell progenitor recruitment to the thymus during steady state conditions (95), which is mirrored at long-term time points following BMT where CCR7/CCR9 double-deficient bone marrow cells were shown to contribute very minimally to the pool of DP thymocytes. In contrast to this, early thymus reconstitution following BMT does not require CCR7 and CCR9, as T cell progenitors deficient in both chemokine receptors generate DP thymocytes to an equivalent ability to WT cells in a competitive bone marrow chimera model (92). Combined, these results indicate a transient period of time soon after BMT in which thymus settling occurs independently of CCR7 and CCR9. Furthermore, additional regulators of thymus homing in the steady state and following BMT have been identified, including PSGL-1, the ligand for P-Selectin (80, 92), and lymphotoxin beta receptor (LTβR) (96). Within the thymus, LTβR is expressed by TEC, thymic mesenchyme and thymic endothelial cells, and its expression by endothelial cells is required for thymus homing during homeostatic conditions. This was illustrated by reduced frequencies of early thymic progenitors (ETP) in thymi from

germline *Ltbr*^{-/-} and *Tek*^{Cre}*LTβR*^{floxex} mice (96, 97). Moreover, the importance of LTβR for thymus homing during BMT has been demonstrated by stimulating LTβR using an agonistic antibody during the time of BMT. This resulted in increased thymus and peripheral T cell reconstitution, suggesting that boosting thymus homing via this mechanism can favorably impact the peripheral T cell pool, and could thus pose a potential therapeutic target (96).

The impact of reduced thymus homing following irradiation is long-lived, as mice irradiated using a sublethal dose at 2 months of age, show reduced frequencies of ETP 16 months later (12). This study also showed unaltered expression of CCL25 following irradiation but did not assess CCL25 presentation by endothelial cells. Instead, Xiao et al. (12) propose that long-term effects are seen within LSK that reside within the BM. Quantitation of this population in irradiated mice revealed a decrease in the percentage and number of LSK 7 months post-irradiation, suggesting that reduced numbers of LSK are responsible for reduced ETP rather than reduced recruitment of these cells to the thymus. Importantly, this study showed that despite the reduction in ETP seen following BMT, there was a compensatory proliferation of DN3 thymocytes, and as such thymus cellularity was unaffected. Contradictory to this, Zhang et al. (89) used irradiation which was targeted to the upper or lower half of mice. Such treatment revealed an impact on donor-derived T cell development only when the upper half of the body was exposed to irradiation. Although this study didn't specifically target only the thymus with their method of irradiation, the results would suggest that damage to the thymus is the biggest driver in limiting T cell reconstitution following BMT.

APPROACHES TO ENHANCE THYMUS RECOVERY

IL22 and BMP4

While mechanisms that regulate thymic regeneration following damage remain unclear, several studies have provided some understanding of factors that may regulate this process. Following depletion of DP thymocytes, intrathymic IL-22 levels were found to increase, suggesting a link to mechanisms of endogenous recovery. In support of this, total body irradiation (TBI) of IL-22 deficient mice resulted in defective thymus regeneration compared to WT mice. Furthermore, when irradiation was targeted to the thymus, an increase in IL-22 was also observed suggesting a direct intrathymic recovery mechanism. Interestingly, IL-22 levels were recorded at the highest level when the thymus had the smallest cellularity, suggesting an inverse relationship between levels of IL-22 and thymic size. Production of IL-22 following damage was attributed to radioresistant thymic LTi/ILC3 which were present in increased numbers following thymic insult. Interestingly, irradiated RORγt deficient mice that lack LTi/ILC3 did not increase their levels of intrathymic IL-22 after damage, suggesting a need for RORγt-dependent cells,

including LTi/ILC3, during thymus recovery following damage. Importantly, IL-22 is expressed by TEC, and IL-22 increased thymus cellularity due to increased proliferation of TEC and increased frequencies of all developing thymocyte subsets. These positive effects of IL-22 on thymus regeneration is limited to damage, as steady state mice treated with IL-22 showed no increase in total thymus cellularity (98). It is important to note that in this study, following TBI, LTi/ILC3 cells also upregulated RANKL expression, a molecule which has since been implicated in thymus regeneration. In another study (99), and following irradiation of WT mice, CD45⁺ cells upregulated RANKL expression compared to non-irradiated controls. Further analysis showed that RANKL expression was upregulated by radioresistant host LTi/ILC3 and CD4⁺ cells. Although LTi/ILC3 cell numbers in the thymus are low, their expression of RANKL was significantly higher than CD4⁺ SP thymocytes. To confirm the role of RANKL in thymus regeneration, WT mice were subjected to TBI, followed by neutralization of RANKL via antibody blocking that resulted in impaired TEC recovery. In a subsequent experiment, mice subjected to TBI and administered with exogenous RANKL showed significant increase in TECs compared to control mice. Here, exogenous RANKL treatment resulted in increased Ki67 expression by both cTEC and mTEC, indicative of increased proliferation, as well as reduced expression of pro-apoptotic genes. Interestingly, and perhaps in line with the potential importance of additional TNF Receptor superfamily members in thymus regeneration, a role for lymphotoxin signaling was also suggested, as stimulation by RANKL caused induction of LTα expression by LTi/ILC3 cells. Moreover, LTα deficient mice had reduced TEC recovery post-BMT compared to WT hosts, suggesting a mechanism of TEC recovery via LTα upregulation mediated by RANKL (99).

In addition to the potential of IL22, a recent study highlighted the involvement of bone morphogenic protein 4 (Bmp4) in thymus recovery following damage (100). Mice that were subjected to TBI show an upregulation of intrathymic Bmp4 levels suggesting this pathway may also be involved in thymus regeneration. In line with this, inhibition of Bmp4 by a pan BMP inhibitor prior to TBI caused an impairment in the thymus recovery mechanism. Bmp4 is expressed by multiple stromal cell types within the thymus, including fibroblasts and endothelial cells. Analysis of Bmp4 expression by qPCR on sorted populations of stromal cells following TBI revealed that Bmp4 expression was upregulated only by endothelial cells. Ex vivo expansion of EC that were transplanted post-TBI resulted in an increased TEC population, specifically cTEC, and qPCR analysis of cTEC showed an increase in *Foxn1* levels as well as the *Foxn1* target genes *Dll4*, *Kitl*, and *Cxcl12*, thus implicating Bmp4 by endothelial cells to initiate thymus recovery. Moreover, tamoxifen induced deletion of Bmp4 in endothelial cells prevented thymus recovery following TBI. Similar to the radioresistance of ILC3/LTi post-TBI, endothelial cells are also proposed to be radioresistant, as their frequencies remained unchanged in the thymus post-TBI.

Keratinocyte Growth Factor

Thymic GVHD targets the thymic microenvironment and impairs thymopoiesis. However, studies have shown that thymic GVHD can be abrogated by keratinocyte growth factor (KGF) treatment prior to transplant (94, 101–103). In a model of GVHD, mice were transplanted with allogeneic splenocytes and treated with KGF for a period of 3 days prior to and after transplant. Control mice which did not receive KGF treatment showed a reduction in thymus weight and cellularity as a result of thymic GVHD. However, treatment with KGF inhibited the induction of thymic GVHD. Furthermore, overall percentage of donor T cells, specifically CD8⁺ T cells, infiltrating the thymus was shown to be reduced following KGF treatment. Despite this, absolute numbers of cells were not reduced suggesting that abrogation of thymic GVHD by KGF treatment was not due to decreased infiltration of donor transplanted T cells. Analysis of T cell development showed a loss of DP thymocytes in mice with GVHD, which was restored with KGF treatment. Although KGF treatment was able to protect the thymus from GVHD, it did not prevent donor T cell infiltration into the spleen which resulted in acute GVHD. The receptor for KGF (FGFR2IIIb) is expressed by TECs, thus the thymic microenvironment was analyzed post-GVHD induction. Thymic cortex/medulla organization was found to be severely disrupted, however organization was maintained following KGF treatment suggesting KGF acts on TEC to protect thymic microenvironments and subsequently promote thymopoiesis (101).

Other studies have also assessed the impact of KGF treatment in a mouse models that are aimed to mimic clinical settings. Here, mice were pre-conditioned with both irradiation and cyclophosphamide then reconstituted with MHC-mismatched bone marrow. Strikingly, KGF treatment allowed for sustained increased numbers of thymocytes for at least 3 months. In addition, KGF treatment increased frequencies of peripheral donor derived naive T cells, suggesting increased thymic output rather than peripheral T cell expansion (102). Furthermore, when KGF deficient (*Fgf7*^{-/-}) mice were sub-lethally irradiated to dissect the role of endogenous KGF on thymus regeneration, they displayed significant reductions in all thymocyte subsets. In addition, *Fgf7*^{-/-} hosts that received allogeneic or syngeneic BM showed impaired regeneration of thymus as well as reduced peripheral donor and host T cells compared to WT hosts, suggesting host KGF is necessary to mediate thymus regeneration post-BMT (103). Similarly, Kelly et al. (94) studied effects of combined treatment of KGF and the p53 inhibitor Pifithrin-β (PFT-β) on thymus recovery. Lethally irradiated mice were reconstituted with T cell depleted BM and treated with KGF, PFT-β, or KGF and PFT-β. Analysis of the TEC compartment 2 weeks post-BMT showed improved thymus recovery in mice receiving combined treatment compared to either KGF or PFT-β treatment alone. Interestingly, TECs co-staining with Ly51 and Keratin5 were seen following combined treatment with KGF and PFT-β suggesting bipotent progenitors may aid in the observed TEC regeneration. Importantly, intrathymic biotin labeling, as a means to measure thymic output, showed that

combined treatment was able to improve thymic export (94). In relation to effects of KGF in humans, attempts to restore T cell numbers in relapse-remitting multiple sclerosis (RRMS) patients following antibody mediated-lymphocyte depletion, KGF treatment, given as palifermin, was shown to reduce thymopoiesis as T cell output was measured by naïve T cell count, RTE and TRECs. Strikingly, reduced thymic output was recorded 1 month post-palifermin administration, as numbers of naïve CD4⁺ were reduced compared to the placebo group. In addition, frequencies of RTE were reduced following treatment with palifermin up to 6 months later. CD4⁺ effector memory cells were increased post-palifermin suggesting decrease in TCR repertoire (104). Despite improved thymopoiesis in murine models, KGF treatment in clinical trials has not improved T cell reconstitution, suggesting different requirements for KGF mediated TEC recovery in humans.

CONCLUSIONS

The importance of thymic epithelial microenvironments for T cell development is well-established. Despite this, we still lack a clear understanding of how TEC populations are established during development, and how they change during the life-course. Importantly, we still do not have a clear picture of the changes that take place in TEC populations in response to thymus damage, and how they are restored either naturally or following therapeutic intervention. As such, understanding the cellular makeup of TEC subsets is a key initial step to gain a clearer view of TEC biology, and also inform and improve approaches to manipulate TEC microenvironments with the longer-term goal of boosting immune system recovery. Relevant to this, several studies have now reported previously unrecognized heterogeneity within TEC compartments, and single cell RNA sequencing approaches represent a powerful approach to initially describe new TEC populations. A key goal of future studies will be to examine the potential functional importance of these newly described subsets, and to place them in a developmental sequence that will provide a detailed roadmap of stages in TEC development. For example, the mTEC^{lo} population, that constitutes the majority of mTEC in the adult thymus, is now known to contain multiple populations that include progenitors of mTEC^{hi}, CCL21⁺ mTEC, and stages that represent post-mTEC^{hi} cells. Thus, and in contrast to initial thoughts, mTEC^{lo} do not simply represent ‘immature mTEC’. Approaches that enable the isolation and study of individual mTEC^{lo} subsets will be needed to understand the functional importance of this diversity. Relevant to this, gaining a better understanding of the functional properties of recently described thymus tuft cells, that reside within mTEC^{lo} and represent mTEC developmental stages that occur beyond the mTEC^{hi} stage, may be important in revealing how the thymus medulla is able to support the development of multiple T cell lineages that include conventional αβT-cells, Foxp3⁺ T-Reg and CD1d-restricted iNKT cells.

Finally, and as previously noted, while it is clear that TEC recovery occurs in damage settings that include bone

marrow transplantation, it is not known whether such recovery involves a complete restoration of all TEC subsets that are now known to exist. Indeed, it is not known whether therapeutic treatments such as IL22, RANKL and KGF impart their effects via TEC progenitors, or via individual or multiple stages in the TEC developmental program. Again, understanding the relationships between newly described TEC populations, and identification of the factors that control their development, survival and expansion will be an important step in optimizing approaches to target TEC populations for therapeutic benefit.

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AUTHOR CONTRIBUTIONS

AA and BL reviewed the literature. AA, BL, and GA wrote and edited the manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Postnatal Involution and Counter-Involution of the Thymus

Jennifer E. Cowan^{1*}, Yousuke Takahama², Avinash Bhandoola¹ and Izumi Ohigashi^{3*}

¹ Laboratory of Genome Integrity, Center for Cancer Research, National Cancer Institute, National Institutes of Health, Bethesda, MD, United States, ² Experimental Immunology Branch, National Cancer Institute, National Institutes of Health, Bethesda, MD, United States, ³ Division of Experimental Immunology, Institute of Advanced Medical Sciences, University of Tokushima, Tokushima, Japan

Thymus involution occurs in all vertebrates. It is thought to impact on immune responses in the aged, and in other clinical circumstances such as bone marrow transplantation. Determinants of thymus growth and size are beginning to be identified. Ectopic expression of factors like cyclin D1 and Myc in thymic epithelial cells (TEC)s results in considerable increase in thymus size. These models provide useful experimental tools that allow thymus function to be understood. In future, understanding TEC-specific controllers of growth will provide new approaches to thymus regeneration.

Keywords: thymus, Myc, cyclin D1, growth, involution, aging

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Edited by:

Ann Chidgey,
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Adrian Liston,
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Biotechnology, Belgium
Daniel Gray,
Walter and Eliza Hall Institute of
Medical Research, Australia

*Correspondence:

Jennifer E. Cowan
jennifer.cowan@nih.gov
Izumi Ohigashi
ohigashi@genome.tokushima-u.ac.jp

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INTRODUCTION

The thymus is the primary site of T cell development. It is a specialized environment that fosters the production of a diverse T cell repertoire, allowing T cells to recognize and eliminate foreign antigen but remain tolerant to self. The thymic epithelial cells (TEC)s educate developing T cells, where cortical (c)TECs select for those expressing functional receptors, whilst medullary (m)TECs deplete those with potential specificity to the body's own cells (1). TECs provide essential signals to T cell precursors that drive their migration, differentiation, proliferation, and survival. Correspondingly, developing thymocytes provide signals to drive TEC differentiation and organization. This process is termed thymic crosstalk (1, 2). Impaired TEC development results in severe T cell immunodeficiency (3–5).

THYMIC INVOLUTION

The thymus is the first organ to undergo aged-related involution and at an accelerated rate relative to other tissue (6). The process of thymic involution is evolutionarily conserved in all vertebrates (7). The organ undergoes rapid growth during development, peaks in size around adolescence and begins to decline with age; with the initiation of involution beginning as early as birth and no later than the onset of puberty in humans and mice (6). This thymic regression includes reductions in thymic mass, loss of thymic structure, and disorganization to thymic architecture, consequently resulting in diminished thymocyte numbers and reduced naïve T cell output (6, 8, 9). In addition to chronic age-related involution, the thymus can undergo acute atrophy under conditions of physiological stress, such as infection (10), pregnancy (11, 12), and cancer treatments (13).

Stressed-induced thymic involution results in decreased naïve T cell output and compromised host immunity, and is commonly reversible, with recovery of size and function after the insult is removed (6, 14, 15). This acute involution involves rapid reductions in proliferation and enhanced apoptosis of developing thymocytes. These impairments in developing T cells can be a direct effect of infection on thymocytes (16). However, acute thymic involution can also be a consequence of the

infectious agent on TEC, which in turn drives thymocyte death (16, 17). An example of the latter is seen with synthetic dsRNA Poly(I:C) treatment, which mimics a viral infection and rapidly triggers thymic involution in mice. This acute thymic atrophy is mediated by type I IFN responses, that do not act directly on the T cell precursors (17).

Signals between TEC and thymocytes are bi-directional, with defects and alterations in the haemopoietic component of the thymus also having dramatic consequences on TEC development and maintenance. The importance of lymphoid-epithelial cell interactions for thymic architecture was established in mice lacking mature T cells, that present with severely impaired mTEC development (18). Early stage blocks in T cell development, such as in human CD3 ϵ transgenic mice, show severely disrupted thymic architecture and defective cTEC and mTEC development (19), whilst mice with later stage defects in the TCR complex, such as genetic disruptions to the TCR α gene, Rag-1 deficiency or ZAP-70, have severely abrogated mTEC generation (19–21). Interesting, successful reconstitution in the adult thymus does not require thymic crosstalk in the fetal period. Thus, adult human CD3 ϵ transgenic mice receiving WT fetal liver transplants had increased thymic size and restored thymic architecture and function (22). As thymocyte-derived signals are so essential in the establishment and maintenance of the thymic microenvironment, a role for both lymphoid cells and epithelial cells needs to be considered in the process of stressed-induced and chronic age-related involution.

Many agents have been identified to be involved in acute stress-induced thymic atrophy, including proinflammatory cytokines, steroids, and hormones. Inhibition of such agents can prevent this acute involution (14). The drivers of chronic age-related involution remain less clear, and it is unknown if the same cellular and molecular mechanisms underlie both chronic and acute involution (6). Sex hormones, increased after puberty, facilitate the chronic involution process, and castration of old mice can successfully albeit transiently restore both thymic size and function (23). Moreover, castration of young adult mice resulted in enhanced thymic regeneration following bone marrow transplantation (24). Sex steroid ablation can also enhance thymic function in humans. Prostate cancer patients undergoing sex steroid ablation therapy present with significant increases in numbers of naïve T cells as a consequence of enhanced thymic function and T cell export (24). Although it is still poorly understood how sex steroids drive age-related thymic involution, atrophy is at least partially attributed to increased sex steroids at puberty (25).

IMMUNOLOGICAL CONSEQUENCES OF THYMIC INVOLUTION

Although aging results in widespread immunodeficiency, the direct consequences of age-related thymic involution on impaired immune function in the elderly is poorly understood. Old people are more susceptible to infection, and infections in the elderly often have higher severity compared to the young (8). Furthermore, the elderly mount poorer responses to

vaccines (26) and show increased incidence of cancers (27). These phenomena are suggested to be, at least in part, a consequence of declining numbers and diversity of naïve T cells emerging from the aged thymus, which in turn contributes to the shift toward memory phenotypes in the periphery (8, 26, 27).

Age-related thymic involution in mouse limits the numbers of recent thymic emigrants in the periphery (28). Moreover, in humans, using T-cell receptor excision circles (TREC) to measure thymic output, generation of new T cells was minimal in the elderly, with the frequency of TREC declining steadily with age (29, 30), although the precise age of when this reduction is initiated remains unclear (31). This was confirmed in a study that acquired thymic samples in addition to peripheral blood (32). Elderly individuals displayed a wide range of thymic functionality, measured by the frequency of double positive thymocytes (the higher the frequency of DP, the higher the functionality). When thymic function was compared to percentages of naïve T cells, reduced thymus function correlated well with decreased naïve T cell numbers. Moreover, this comparison identified a strong relationship between the low functioning thymi and the contraction of naïve CD8 T cells specifically. These studies also revealed a relationship between thymic function and the dynamics of peripheral naïve T cells, where reduced thymus function was associated with shortened telomere length and aberrant activation and proliferation status in naïve CD8 T cells (32).

There are examples of viruses that aged mice are more susceptible to, as a consequence of age-related alteration to T cell responses. These include West Nile Virus, where old mice have reduced rates of survival compared to young. This increased susceptibility is a consequence of age-related defects in T cell immunity, involving both CD8 and CD4 T cell responses (33). Influenza has also been identified as an age-sensitive virus. It has been demonstrated that compromised protective immunity in aging against influenza can be a consequence of the age-associated decline in CD8 T cell repertoire diversity, resulting in “holes” in the repertoire that may impair the ability of T cells to control the virus (34).

Rejuvenation of the involuted thymus restores peripheral T cell function in aged mice and humans (24, 35). Many agents have been identified to reverse thymic atrophy, although their effects are only transient. Those include interleukin-7, sex steroids, growth hormones, and keratinocyte growth factor (KGF) (36). Sex steroid ablation can also improve immune responses. The level of restoration, however, following castration is limited by age, with enhancement of thymic function following the procedure lost between 9 and 18 months. Nine-month-old castrated males when challenged with Influenza A display restored numbers of virus specific cytotoxic CD8 T cells to levels comparable to young mice, and improved viral clearance in the lung (37). In contrast, 24-month-old castrated mice did not display restored numbers of virus-specific CD8 T cells to young levels, yet still had observed reductions in lung viral titers (37). Thus, age-associated defects in T cell immunity may, at least in part, be attributed to the diminished naïve T cell repertoires, and thymic regeneration by sex steroid ablation can restore this deficiency and improve viral immune

responses, but in an age-limited manner. Moreover, sex steroid ablation must be having additional T-cell independent effects that improve viral clearance (37). Interestingly, a recent publication has suggested that restoration of thymic size in an aging host using sex steroid ablation or KGF does not confer protection to the host against West Nile Virus (38). Thus, modest increases to thymic size in aging hosts might not be sufficient to improve immune protection. The complete contribution of thymic involution to age-associated defects in T cell immunity remains to be determined.

Overall the immunological consequences of age-related thymic involution on the peripheral T cell pool potentially leaves the elderly with compromised protective immunity against pathogens. Thus, a better understanding of the molecular and cellular mechanisms underlining the causes of age-related thymic involution should offer benefits to the aging population in multiple clinical settings. It also needs to be considered, however, that age associated immune system defects may also be due to the aging of the hematopoietic cells, and not all a consequence of reduced thymic function. The general physiological consequences of aging to the host also needs to be considered when exploring the attributes of compromised protection against infectious agents in the elderly.

THE ROLE OF TECs IN THYMIC INVOLUTION

It has been reported that the transfer of bone marrow cells or early T cell progenitors isolated from young mice does not restore thymic function in aged recipients, whereas the transplantation of fetal thymi into aged hosts exhibits thymocyte development equivalent to a young host. Thus, indicating that thymic stromal cells, rather than hematopoietic cells, drive thymic involution (15, 39–41). The reduction in size of the aged thymus is accompanied with reduced TEC numbers (9). Postnatal reductions in expression of the Forkhead box N1 (Foxn1) transcription factor is sufficient to accelerate age related thymic involution (42, 43). Moreover, targeted overexpression of Foxn1 in adult TECs can attenuate the involution process and delay the decline in naïve T cells observed in the aging host (35). Foxn1 expression is diminished in aged stroma (44), suggesting a relationship between the decline of Foxn1 expression and age-associated thymic involution.

It has been demonstrated that genetic ablation of cTECs alone has profound effects on thymic size, and severely impaired thymocyte development (45). This complements a recent publication that explored the morphological changes in TECs with age and suggested dramatic changes to cTEC structure alone reduce thymic size (46). The authors genetically labeled TECs using conditional confetti mice to facilitate the visualization of individual TECs in cortical or medullary regions. First it was established that cTECs in the thymus of young mice have unique morphology, with extensive networks of projections estimated to engulf over 100 lymphocytes within them. Second, these cTEC projections contracted in the thymus

of 12-month mice, in contrast to mTEC morphology, that is unaltered with age. As the authors suggest, this dramatic alteration to cTEC morphology may contribute to thymic involution, instead of or in addition to increased cTEC death or decreased rates of cTEC proliferation with age (46). This morphological characterization of adult cTECs further offers a possible explanation as to why conventional enzymatic digestion methods used for cTEC isolation dramatically underestimate their cellularity in the postnatal thymus (47).

In addition to morphological changes with age in cTECs, their expression of Catalase, an antioxidative enzyme, is lower compared to mTECs and lymphocytes, which results in their increased susceptibility to oxidative damage (48). Indeed, the morphological alteration of cTECs is greatest in the sub-capsular cortex, which is the region of intense proliferation activity of lymphocytes, and thus of intense metabolism (46).

Transcriptional profiling of TECs in young and aged thymi has revealed dynamic gene profiles during the initial involution process in both cTECs and mTECs, aiding the understanding of the mechanisms that govern the decline of TEC numbers with age. A transcriptional hallmark of the initiation of involution in TECs was the downregulation of genes involved in cell cycle, specifically diminished E2F3 activity, suggesting possible reductions to cell cycle progression in all aging TECs (49). The same aging-associated transcriptional changes are seen in mTECs during the initial phase of thymic involution, from 2 to 10 weeks of age (50). Furthermore, gene expression profiles of cTECs display greater alterations during involution and regeneration, than those of both their mTEC counterparts and developing T cells (23). The dynamic changes in cTEC transcriptomes following castration mediated thymic regeneration revealed that genes upregulated in expression during thymic regrowth included those involved in cell morphology, cell adhesion, and cytoskeleton remodeling (46).

The underlying mechanisms of the age-related decline in TEC numbers and function that mediate thymic atrophy remain ambiguous, although many changes to TEC biology throughout life have been documented. The pivotal role of TECs in the process of thymic atrophy identify them as an attractive therapeutic target to counter thymic involution (15). Although the morphological, metabolic and transcriptional alterations with age are more prevalent in cTECs, the significance of this has yet to be determined. One major constraint of conclusively identifying a role for cTECs in age-associated thymic involution is the limitation in the numbers of cTECs able to be isolated from the adult thymus. We hope the enlarged thymus models discussed in this review will provide useful experimental tools to isolate greater numbers of adult cTECs to perform more detailed analysis of the changes that occur in this population with age. In addition, the newly refined genetic models described below will facilitate the ectopic expression of Myc specifically in cTEC or mTEC subsets (51). This could potentially determine whether such targeted manipulation is sufficient to reverse involution and force thymus growth.

TECs MEDATE CHANGES TO THYMIC SIZE THROUGHOUT LIFE

In addition to controlling thymic atrophy, TECs regulate all changes in thymic size observed throughout life. This includes the dramatic increase seen during mouse embryogenesis, when the thymus doubles in size daily, and the continued expansion, although at a reduced rate, until 4 weeks of age, when thymic cellularity peaks. TECs also maintain this maximal size until around 8 weeks of age, when involution is initiated (9). Defects in TEC development and numbers results in diminished thymic size (52). Moreover, dramatic expansion in TEC numbers in adulthood results in simultaneous expansion in the numbers of developing lymphocytes and severe thymic hyperplasia (51, 53–55). Correspondingly, and as mentioned previously, postnatal disruption of TECs triggers accelerated thymic atrophy (42, 43). Furthermore, restricting the *Foxn1* dependent TEC progenitors results in reduced thymic size during the initial formation of the thymus, and this persists into the adult period (56).

The rapid expansion in thymic size and TEC number during fetal and neonatal life is supported by TECs having unique properties that distinguish them from postnatal populations. This includes higher rates of proliferation (9, 51) and ribosomal biogenesis (51). Additionally, fetal TECs are functionally distinct compared to their adult counterparts. This includes their ability to undergo successful engraftment following intra-thymic injection into an adult host, a property lost after birth (57). In addition, fetal TECs have an exclusive ability to support development of fetal waves of gamma delta T cells (58). Moreover, it has also been suggested that TEC progenitors in the fetal and postnatal thymus differ. TECs in the adult thymus have a turnover rate of ~2 weeks (9), yet their putative self-renewal capacity and precursor-product relationships remain unclear (1, 2). A common bipotent TEC progenitor has been identified at E13.5 that can give rise to both cTECs and mTECs (59, 60). During initial thymus formation, TEC progenitors can first acquire some cTEC specific markers, before differentiating into the mTEC lineage (2, 61), but mTEC lineage restricted progenitors have also been identified in the fetal thymic environment (62). Corresponding progenitor populations in the adult thymus remain to be determined (2).

Myc ACTIVITY IN TECs IS LIMITING FOR THYMIC SIZE AND FUNCTION

Understanding the transcriptional controllers of fetal TECs and how they support the rapid expansion in thymic size during embryogenesis offers insight into mechanisms underlying thymic function and regeneration. Recently, transcriptional assessment of TECs through development at a single cell resolution has revealed distinct transcriptional programs of TECs at specific stages of life (51, 63–65). This included the identification of a fetal specific program comprising high levels of expression of Myc target genes, including cell cycle genes and genes

involved in ribosomal biogenesis. Such genes displayed declining expression in TECs through fetal development, coordinated with reduced expression of Myc protein. Transcript levels corresponded with declining rates of cell proliferation and correlates of ribosomal biogenesis in TECs through development. It was hypothesized that this high Myc activity in fetal TECs drives the expansion in TEC numbers and consequently thymic size during early development (**Figure 1A**) (51). It has previously been reported that mice with conditional removal of Myc in TECs present with small thymi in adulthood, as a consequence of reduced rates of proliferation and decreased TEC numbers (66). Consistently, enhancing Myc expression in TECs resulted in a severe increase in TEC number and dramatic thymic hyperplasia in adulthood (**Figure 1B**). This continued Myc expression in TECs conferred expression of a fetal-specific transcriptional program, including high levels of expression of genes involved in cell cycle and ribosomal biogenesis (51). Interestingly, this ribosomal signature was unique to the Myc model, as another transgenic large thymus model, driven by cell cycle gene cyclin D1, did not present with increased transcripts of genes involved ribosomal biogenesis (51, 67).

The dramatic thymic growth conferred by the Myc transgene did not jeopardize function, similar to previous reports with cyclin D1 transgenic mice (53, 54), and such enlarged thymi also produced increased numbers of recent thymic emigrants. Furthermore, this continued Myc expression preserved the ability of TECs to engraft following intrathymic injection into adulthood. Myc overexpression did not need to be maintained throughout life to increase thymic size, with inducible expression of Myc in adult and aged TECs being sufficient to similarly promote thymic growth (**Figure 1C**). Although the decline in Myc activity was most striking between fetal and adult TECs, the same reductions, but at much more modest rates, could be detected transcriptionally between adult and aged cTEC populations (51). Thus, this decline in Myc activity in TECs could underlie the reduction in thymic cellularity observed during involution.

Collectively, these results identify Myc as a major regulator of a fetal specific transcriptional program of TECs. They establish a role for Myc activity in rapid thymic growth during development; provide evidence Myc expression can confer at least one functional distinct property of fetal TECs into adulthood and furthermore, confirm that Myc activity is limiting for thymic size and function (51). Upstream controllers of Myc in TECs remain to be identified and are logical candidates in modulating thymic function and regeneration.

In addition to unique transcriptional profiles of fetal TECs, the adult TECs were highly enriched for genes involved in antigen processing and presentation, along with regulation of the actin cytoskeleton and the lysosomes (51). Our results suggest an adult specific transcriptional program of TECs supports the maintenance of a functional thymus in adulthood, complementing and extending previous work (49, 63, 64).

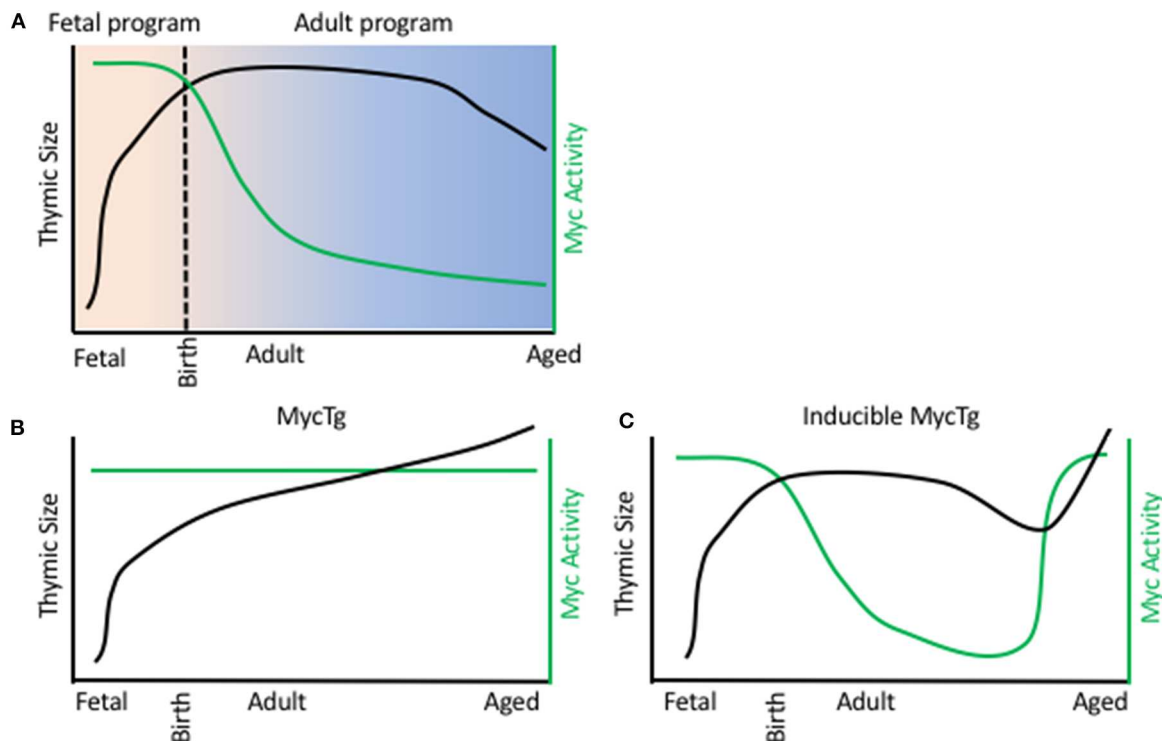


FIGURE 1 | Myc activity in TEC limits thymic size. **(A)** Thymic size (black line) and Myc activity in TECs (green line) during a WT mouse life span. X axis represents age, from day of fertilization. B/C. Thymic size (black line) and Myc activity in TECs (green line) during a FoxN1 MycTg mouse **(B)** or an inducible MycTg mouse **(C)** life span.

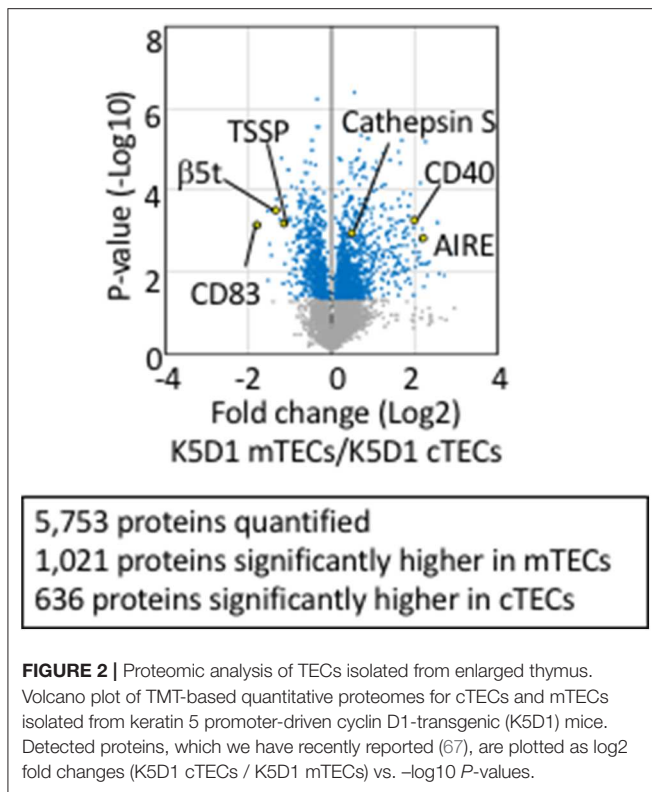
CYCLIN D1 OVEREXPRESSION IN THYMIC EPITHELIAL CELLS ENLARGES FUNCTIONAL THYMUS

Cyclin family molecules are key regulators for cell cycle progression that control the number of cells and the size of organs. Cyclin proteins associate and activate cyclin-dependent kinases (CDKs), serine/threonine kinases, important for the progression of cell cycle (68). Approximately 30 members of cyclin family proteins, defined by the cyclin box domain, are known in human and mice (69, 70). Among the cyclin family proteins, cyclin A, cyclin B, cyclin D, and cyclin E are canonical cyclins, which interact with cell-cycle-related CDKs and regulate cell division (71, 72). These canonical cyclins regulate distinct phases of cell cycle; cyclin A regulates S and G2 phases, cyclin B regulates M phase, cyclin D regulates G1 phase, and cyclin E regulates G1 and S phases (73–76).

Cyclin D1 is a member of cyclin D family, along with cyclin D2 and cyclin D3. Overexpression of cyclin D1 in cells results in a rapid progression from G1 to S phase, whereas inhibition of cyclin D1 prevents the entry into S phase (77). The overexpression of cyclin D1 in mouse increases the incidence of carcinoma in the mammary gland and the liver (78, 79). Correlation between elevated expression of cyclin D1 and various cancers and their poor prognosis is also noted in human (80, 81) and mouse (82).

To investigate the role of cyclin D1 in epithelial cells, Robles et al. generated transgenic mice in that cyclin D1, encoded by human *CCND1* gene, was driven by bovine keratin 5 promoter (53). Keratin 5 is primarily expressed in basal keratinocytes of the skin epidermis. It was found that these keratin 5-driven cyclin D1-transgenic (K5D1) mice exhibit epidermal hyperplasia in the skin (53). The authors also found that the thymus in K5D1 mice exhibit severe hyperplasia, which is apparent by 14 weeks of age when age-associated thymic involution is detectable in control mice, whereas the thymus in K5D1 mice at 2 weeks of age is comparable in size to that in control mice (53). In the thymus, TEC progenitors and mTECs highly express keratin 5 (54, 83), and both the cortical and medullary regions are enlarged in the thymus of K5D1 mice (53, 54).

The enlarged thymuses in K5D1 mice contain ~100-fold larger numbers of TECs compared with control C57BL/6 (B6) thymuses, and the cellularity of thymocytes in K5D1 mice reaches to ~50-fold larger numbers of that in B6 mice (67, 84). In K5D1 mice, the cellularity of cTECs is elevated similarly to that of mTECs, although keratin 5 is predominantly detectable in mTECs but not cTECs (67). It is possible that the increase in the number of cTECs is at least in part due to cyclin D1-mediated promotion of cell cycle in keratin 5-expressing TEC progenitors (54). It is additionally possible that cell cycle progression within cTEC



compartment is promoted in K5D1 mice, since a remarkably elevated amount of cyclin D1 transcripts are detected in K5D1 cTECs (67). The signals provided by developing thymocytes play an important role in the development of cTECs (19, 83). Indeed, hCD3 ϵ transgenic mice, in which early thymocyte development is primarily defective, do not exhibit thymic hyperplasia even when they are crossed with K5D1 mice to overexpress cyclin D1 in TECs (54). Thus, developing thymocytes appear to contribute to the increase in TEC cellularity in K5D1 mice.

K5D1 thymuses support thymocyte development similar to B6 thymuses, as the proportion of developing thymocytes subsets, including CD4 $^{-}$ CD8 $^{-}$, CD4 $^{+}$ CD8 $^{+}$, CD4 $^{+}$ CD8 $^{-}$, and CD4 $^{-}$ CD8 $^{+}$ thymocytes in K5D1 mice is almost comparable to that in B6 mice (67). However, mature CD4 $^{-}$ or CD8 $^{-}$ single positive thymocytes identified as CD69 $^{-}$ Qa2 $^{+}$ are accumulated in the K5D1 thymus (84). This accumulation is possibly due to unelevated amount of sphingosine-1-phosphate (S1P), produced by unelevated number of endothelial cells in the K5D1 thymus, because S1P plays an essential role in promoting thymic egress of mature thymocytes (85). Indeed, in contrast to thymocytes, which are $\sim$ 50-fold-elevated in cell number, the increase of splenic T cell number in K5D1 mice is only 2- to 3-fold of that in B6 mice (67, 84). In addition to the possible limitation in the machinery for thymic egress of mature thymocytes, limited availability of cytokines, such as Interleukin-7 (IL-7), to maintain peripheral T cells, may also limit the cellularity of the peripheral T cell pool in K5D1 mice (86).

THYMIC EPITHELIAL CELLS IN CYCLIN D1-MEDIATED ENLARGED THYMUS ARE FUNCTIONALLY POTENT

The function of the thymus to produce self-protective T cells and to instill their self-tolerance is chiefly mediated by cTECs and mTECs, respectively. The capability of thymocyte development in K5D1 mice suggests the functional equivalence between K5D1 TECs and B6 TECs. cTECs uniquely express β 5t-containing thymoproteasome important for optimal production of immunocompetent CD8 $^{+}$ T cells (87–89), whereas Aire expressed in mTECs plays a role for the establishment of self-tolerance in T cells (90). The enlarged K5D1 thymuses contain β 5t $^{+}$ cTECs in the cortex and Aire $^{+}$ mTECs in the medulla (67). It was shown that β 5t deficiency in K5D1 mice results in the impaired generation of CD8 $^{+}$ T cells in the thymus, and the loss of Aire in mTECs causes autoimmune inflammation in various tissues, including the retinas and salivary glands, in K5D1 mice (67). T cells generated in the K5D1 thymus show a proliferative response to allogenic stimulation and are unresponsive to synergic cells (67). T cells that express TCR specific for a male specific H-Y antigen are positively selected in the thymus of female K5D1 mice, whereas those T cells are negatively selected in the thymus of male K5D1 mice (54). Thus, cTECs and mTECs in the enlarged K5D1 thymus are functionally competent to produce immunocompetent yet self-tolerant T cells.

PROTEOMIC PROFILING OF THYMIC EPITHELIAL CELLS ISOLATED FROM CYCLIN D1 ENLARGED THYMUS

Isolation of cTECs and mTECs generally involves enzymatic digestion of thymic tissues. Although the thymus in one postnatal B6 mouse contains more than 1×10^6 cTECs and more than 1×10^6 mTECs, the number of either cTECs or mTECs isolated from one B6 mouse is typically $< 1 \times 10^4$ cells (46, 47). However, a large number of cells (typically more than 5×10^5 cells per sample) are required for current mass spectrometry-based technology of proteomic analysis, unlike transcriptomic analysis, which can be carried out from small number of cells. Using the enlarged thymus in K5D1 mice, it is possible to isolate $\sim 2 \times 10^5$ cTECs and $\sim 2 \times 10^5$ mTECs from one mouse (67). These relatively large numbers of cTECs and mTECs allow unbiased proteomic analysis.

cTECs and mTECs isolated from K5D1 mice are qualified for proteomic analysis, because RNA-sequencing-based transcriptomic profiles of cTECs and mTECs are highly similar between K5D1 mice and B6 mice (67). For example, functionally relevant genes in mTECs, including *Aire*, *Ccl21a*, and *Tnfrsf11a* (encoding RANK), as well as Aire-dependent and Aire-independent tissue-restricted self-antigen genes are highly detected in mTECs in an indistinguishable manner between K5D1 mTECs and B6 mTECs (67). Similarly, functionally relevant genes in cTECs, including *Dll4* (encoding DLL4), *Psmbl1* (encoding β 5t), *Prss16* (encoding thymus-specific serine

protease or TSSP), and *Ctsl* (encoding cathepsin L), are highly detected in cTECs in an indistinguishable manner between K5D1 cTECs and B6 cTECs (67). Only a minor difference in transcriptomic profiles between K5D1 TECs and B6 TECs is the overexpression of cyclin D1 and cell-cycle-related genes (67). Another minor difference detected between K5D1 TECs and B6 TECs is the difference in the number of CD4⁺CD8⁺ thymocytes tightly associated with isolated cTECs (67). cTECs form multicellular complexes that a single cTEC completely envelops a variety number of CD4⁺CD8⁺ thymocytes (46, 91). These multicellular complexes include thymic nurse cells, which entirely encapsulate CD4⁺CD8⁺ thymocytes (91) and so cannot be fully dissociated from encapsulated CD4⁺CD8⁺ thymocytes by current technology for cTEC isolation (67). The number of enclosed DP thymocytes per one isolated cTEC is smaller in K5D1 mice than B6 mice, so that transcriptomic profiles of K5D1 cTECs is less affected than those in B6 cTECs by the signals derived from CD4⁺CD8⁺ thymocytes (67). Nevertheless, cTECs and mTECs isolated from K5D1 mice are not only functionally potent to produce immunocompetent and self-tolerant T cells but also are highly similar in transcriptomic profiles, so that cTECs and mTECs isolated from K5D1 mice are largely qualified for proteomic profiling to better understand the biology of cTECs and mTECs.

Tandem mass tag (TMT)-based mass spectrometry analysis is a powerful technology for unbiased proteomics that enables multiplex analysis of relative quantification of proteins (92). It successfully quantified 5,753 protein species in total in cTECs and mTECs isolated from K5D1 mice by the TMT-based quantitative proteomic analysis (67). Similar to the transcriptomic profiles, proteomic profiles show a sharp contrast between cTECs and mTECs; 636 proteins, including β 5t, TSSP, and CD83, which are known to play an important role in cTECs, are significantly ($p < 0.05$) higher in cTECs than mTECs, whereas 1,021 proteins, including Aire, cathepsin S, and CD40, which are known to play an important role in mTECs, are significantly ($p < 0.05$) higher in mTECs than cTECs (Figure 2). It was noticed that secretory proteins, including cytokines and chemokines, are removed from isolated cTECs and mTECs during cell isolation procedures and are undetectable in the proteomic profiles (67).

These proteomic profiles for cTECs and mTECs have led to integrated analysis of proteomic and transcriptomic profiles in cTECs or mTECs (67). Approximately 70% of molecules detected in proteomic analysis of K5D1 TECs are detected in transcriptomic profiles of B6 TECs, whereas ~30% of molecules detected in transcriptomic profiles of B6 TECs are detected in proteomic analysis of K5D1 TECs (67). These overlapped molecules include many molecules previously reported in TECs and include molecules such as DCLK1, Avil, and Trmp5, expressed by thymic tuft cells, a recently described subpopulation of mTECs (64, 65, 67). Newly identified molecules include cathepsin D and calpain 1, abundantly expressed in cTECs, and cathepsin C, cathepsin H, and cathepsin Z, abundant in mTECs (67). These proteases may play an important role in processing self-antigens in cTECs and mTECs and in enabling TCR repertoire selection in the thymus, in addition to previously described β 5t, TSSP, and cathepsin L in cTECs,

and cathepsin S in mTECs. The integrated analyses of K5D1 TECs further reveal that genetic loss of β 5t specifically alters the amount of individual proteasomal components in cTECs but minimally affects proteomic and transcriptomic profiles in cTECs (67).

It is interesting to note that proteomic profiles and transcriptomic profiles do not provide a proportional correlation between the amount of proteins and mRNAs in cTECs and mTECs (67). It is possible that post-transcriptional turnover of transcripts and post-translational turnover of proteins may account for the disagreement between the abundance of mRNAs and proteins (67). Thus, quantitative proteomic profiling of TECs isolated from K5D1 mice has revealed a previously unknown platform for further exploring molecular biology of TECs.

Propagation of mobilized cell lines that maintain functionally relevant molecular expression profiles has not been successful for cTECs or mTECs, so that the enlarged thymus provides a useful source of large numbers of freshly isolated cTECs and mTECs, not only for proteomic analysis but also for other analyses, including metabolomic analysis and other biochemical analyses. It is certainly important to identify MHC-associated self-peptides presented by cTECs and mTECs, which induce positive and negative selection of thymocytes to form an immunocompetent yet self-tolerant TCR repertoire.

WHY DOES THE THYMUS INVOLUTE?

Although the immunological consequences of thymic involution have potential disadvantages to the elderly, thymic involution may have evolved for the benefit of the aging host. However, such benefits may be less favorable given current lifespans. One hypothesis is that reduced thymic activity would protect against autoimmunity (93). Seemingly at odds with this idea, accelerated thymic involution in young mice can disrupt central immune tolerance, by perturbed negative selection resulting in the release of autoreactive T cells into the periphery (94, 95). However, experimental systems could conceivably differ from naturally occurring thymic involution. Separately, it is known that some T cells in elderly mice that express markers of self-recognition are selectively retained during aging (96, 97). This could offer an explanation as to why the elderly have a predisposition to certain autoimmune conditions (94, 97), that could be mitigated by thymic involution. Overall, considerably more work is needed to determine whether the elderly are indeed at increased risk for autoimmunity, and whether reduced thymic activity may have any protective function.

It has also been suggested the involution process may conserve energy. The majority of developing thymocytes die during the stringent selection process in the thymus, where over 90% are estimated to undergo cell death (98), and the long-lived peripheral T cells established in early life can undergo homeostatic proliferation to maintain their numbers (99). Thus, energy may be best diverged into other biological processes after the T cell pool is established in early life (6).

Another possibility is that the reduced numbers of naïve T cells may also reduce the incidence of leukemias (6). Others have

suggested the diminished numbers of recent thymic emigrants from the involuting thymus forces enhanced selection of the peripheral T cell repertoire and conserves the persistence of long-lived memory T cells in the periphery which are favorable to the host in old age (99, 100). If such theories are correct and involution is beneficial to the host, approaches to restore thymic size and function in old people become questionable. Therefore, a better understanding of whether thymic involution is favorable to the host will establish if thymic regeneration would be beneficial as a clinical approach.

CONCLUSIONS AND PERSPECTIVES

T cell-mediated immunity is essential throughout life. However, the thymus, where T cells are generated, involutes rapidly in early life. Postnatal thymic involution is attributable to TECs rather than thymocytes. In this review, we summarize recent findings that the decline in a fetal-specific transcriptional program of TECs controls the size of the postnatal thymus and that cTEC morphology is altered during postnatal thymic involution. These findings provide novel insights into molecular and cellular mechanisms in TECs that control thymus size during involution. Thymic involution results in reduced thymocyte development and reduced numbers of naïve T cells and so is predicted to result in immunological disadvantages, including increased incidence in infectious disease and delayed T cell reconstitution after hematopoietic stem cell transplantation (101). Therefore, prevention of thymic involution and regeneration of thymic

function is likely to be useful for the maintenance and improvement of T-cell-mediated immune homeostasis. On the other hand, the increase in the number of peripheral T cells is limited in mice that carry an enlarged thymus, suggesting that merely enhancing the number of TECs and the size of the thymus is insufficient to expand the peripheral T cell pool.

In this review, we also summarize how the development of large thymus models are useful tools for understanding TEC biology. This includes the versatility of enlarged K5D1 thymi for biochemical analysis, including proteomic profiling of TECs. Using such useful tools, we can examine whether functional senescence is induced in enlarged thymi and how counter-involution in aged mice may impact health. Moreover, such models could give novel targets for manipulation to prevent or reverse thymus atrophy.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Impaired Thymic Output Can Be Related to the Low Immune Reconstitution and T Cell Repertoire Disturbances in Relapsing Visceral Leishmaniasis Associated HIV/AIDS Patients

Maria Luciana Silva-Freitas¹, Gabriela Corrêa-Castro^{1,2}, Glaucia Fernandes Cota³, Carmem Giacoia-Gripp⁴, Ana Rabello³, Juliana Teixeira Dutra⁵, Zilton Farias Meira de Vasconcelos⁵, Wilson Savino^{6,7,8}, Alda Maria Da-Cruz^{1,7,8,9*} and Joanna Reis Santos-Oliveira^{1,2,7*}

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*Correspondence:

Alda Maria Da-Cruz
alda@ioc.fiocruz.br
Joanna Reis Santos-Oliveira
joanna.oliveira@ifrrj.edu.br

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¹ Laboratório Interdisciplinar de Pesquisas Médicas, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz (FIOCRUZ), Rio de Janeiro, Brazil, ² Núcleo de Ciências Biomédicas Aplicadas, Instituto Federal de Educação, Ciência e Tecnologia Do Rio de Janeiro (IFRJ), Rio de Janeiro, Brazil, ³ Centro de Referência em Leishmanioses, Instituto René Rachou, Fundação Oswaldo Cruz (FIOCRUZ), Belo Horizonte, Brazil, ⁴ Laboratório de AIDS e Imunologia Molecular, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz (FIOCRUZ), Rio de Janeiro, Brazil, ⁵ Laboratório de Alta Complexidade, Instituto Nacional de Saúde da Mulher, da Criança e Do Adolescente Fernandes Figueira (IFF), Fundação Oswaldo Cruz (FIOCRUZ), Rio de Janeiro, Brazil, ⁶ Laboratory on Thymus Research, Oswaldo Cruz Institute, Oswaldo Cruz Foundation (FIOCRUZ), Rio de Janeiro, Brazil, ⁷ National Institute of Science and Technology on Neuroimmunomodulation, Oswaldo Cruz Institute, Oswaldo Cruz Foundation (FIOCRUZ), Rio de Janeiro, Brazil, ⁸ Rede de Pesquisas em Saúde Do Estado Do Rio de Janeiro/FAPERJ, Rio de Janeiro, Brazil, ⁹ Disciplina de Parasitologia/DMIP, Faculdade de Ciências Médicas, Universidade do Estado do Rio de Janeiro (UERJ), Rio de Janeiro, Brazil

Background: Visceral leishmaniasis/HIV-co-infected patients (VL/HIV) accounts for around 8% of VL reported cases in Brazil. Relapses of *Leishmania* infection after anti-leishmanial treatment constitute a great challenge in the clinical practice because of the disease severity and drug resistance. We have shown that non-relapsing-VL/HIV (NR-) evolved with increase of CD4⁺ T-cell counts and reduction of activated CD4⁺ and CD8⁺ T cells after anti-leishmanial treatment. This immune profile was not observed in relapsing-VL/HIV patients (R-), indicating a more severe immunological compromising degree. Elevated activation status may be related to a deficient immune reconstitution and could help to explain the frequent relapses in VL/HIV co-infection. Our aim was to evaluate if this gain of T cells was related to changes in the peripheral TCRVβ repertoire and inflammatory status, as well as the possible thymus involvement in the replenishment of these newly formed T lymphocytes.

Methods: VL/HIV patients, grouped into non-relapsing (NR- = 6) and relapsing (R- = 12) were evaluated from the active phase up to 12 months post-treatment (mpt). HIV-infected patients (non-VL) and healthy subjects (HS) were included. The TCRVβ repertoire was evaluated ex vivo by flow cytometry, whereas the plasmatic cytokine levels were assessed by Luminex assay. To evaluate the thymic output, DNA was extracted from PBMCs for TCR rearrangement excision circles (TREC) quantification by qPCR.

Results: VL/HIV cases presented an altered mobilization profile (expansions or retractions) of the TCRV β families when compared to HS independent of the follow-up phase ($p < 0.05$). TCRV β repertoire on CD4 $^{+}$ T-cells was more homogeneous in the NR-VL/HIV cases, but heterogeneous on CD8 $^{+}$ T-cells, since different V β -families were mobilized. NR-VL/HIV had the inflammatory pattern reduced after 6 mpt. Importantly, VL/HIV patients showed number of TREC copies lower than controls during all follow-up. An increase of recent thymic emigrants was observed in NR-VL/HIV individuals at 10 mpt compared to R- patients ($p < 0.01$), who maintained lower TREC contents than the HIV controls.

Conclusions: VL/HIV patients that maintain the thymic function, thus generating new T-cells, seem able to replenish the T lymphocyte compartment with effector cells, then enabling parasite control.

Keywords: visceral leishmaniasis/HIV-1 co-infection, thymic output, TCRV β repertoire, relapses, immune response

INTRODUCTION

Visceral leishmaniasis (VL) is a neglected tropical disease associated with poverty, being a public health issue in endemic countries, mainly in tropical and subtropical regions (1, 2). Most of VL cases in the Americas occurs in Brazil, where around 4,000 new cases are reported annually (1–3). An increasing number of HIV-associated VL (VL/HIV) cases has been identified since 2001 reaching 7.8% of the whole VL cases reported in 2017 (3). Noteworthy, VL/HIV patients frequently fail to respond successfully to treatment, exhibiting a high rate of drug toxicity, relapses and mortality (1, 2, 4).

VL/HIV patients evolve with an intense immunosuppression and, paradoxically, potentiated cellular activation, despite antiretroviral therapy (ART) and clinical remission of VL (2, 5). We previously demonstrated that *Leishmania* infection was the main co-factor associated with the immune activation state in HIV-infected individuals (5). Allied to this, elevated levels of lipopolysaccharide (LPS) pointed out that microbial products from the gut lumen translocation, could also be involved in the exacerbated pro-inflammatory status of VL alone (6) and VL/HIV co-infected patients (2, 7). Plasma inflammatory cytokines levels, as well as soluble molecules associated with inflammation such as d-dimers, neopterin, soluble CD163 and leptin levels have been described as important predictors of severity and death in VL (8–12).

We showed that relapsing-VL/HIV patients maintained low CD4 $^{+}$ T lymphocyte counts, higher percentages of activated CD4 $^{+}$ and CD8 $^{+}$ T cells, sCD14 and anti-*Leishmania* IgG3, even 12 months after anti-leishmanial treatment, whereas this immune profile was reverted in non-relapsing-VL/HIV patients after anti-*Leishmania* treatment (13). This indicates intense activation leading to an exhaustion of the immune response to the parasite, which may contribute to the frequent VL relapses or even faster disease progression (2, 14).

Continuous cellular activation induces immunosenescence and exhaustion of primary immune resources. As a consequence, decreased generation of new T cells and lower peripheral

T-cell repertoire diversity takes place (15–17). In this respect, it is relevant to point out that the thymus of HIV patients is also affected, compromising both the lymphoid and microenvironmental compartments of the organ (18). Thymic capacity of exporting mature T lymphocytes can be ascertained by quantifying the T-cell receptor excision circles (TRECs). These circles are generated intrathymically during the somatic gene rearrangement process that generates the T-cell receptor (TCR) and are unique of naive T $\alpha\beta$ cells, allowing the identification in the periphery of the so-called recent thymic emigrants (RTEs) (19–21).

In a second vein, HIV-positive patients evolve with disturbances in the generation of the T lymphocyte receptor V β repertoire (TCRV β) (17), which can potentially compromise the effector responsiveness to a variety of antigens, including *Leishmania*. In this context, disorders of the TCRV β repertoire have been related to the immunopathogenesis of several diseases, such as cancer (22); rheumatoid arthritis (23); hematological comorbidities (24); Chagas disease (25); cutaneous leishmaniasis (26, 27); and HIV/AIDS (28, 29). HIV-positive patients with a restrict mono-oligoclonal TCRV β repertoire profile present a rapid AIDS-progression, suggesting that the T-cell repertoire disturbances do influence the HIV/AIDS prognosis (28) and of its association with other infections, such as Epstein Barr virus (30). Moreover, after ART, a change in the profile of the TCRV β repertoire is observed since, not only newly V β families appear in the periphery, but also others are positive or negatively mobilized (29, 31).

We recently showed that VL-HIV co-infected patients with satisfactory clinical evolution and no recurrence of VL (non-relapsing) presented increased content of the circulating CD4 $^{+}$ T cell pool, indicating that they still have the ability to replenish the peripheral T cell compartment. This was not observed in relapsing VL-HIV patients, suggesting a sort of burnout of T cell sources (32). The main source of these T cells, peripheral lymphoid organs or thymus, can be affected by both *Leishmania* and HIV infection (18, 33, 34). Accordingly, it is conceivable that the compromising degree of one of these compartments may

be related to a deficient immune response. These features may contribute to the lack of parasite control which in turn could explain the frequent relapses in VL/HIV (13, 14). Thus, the aim of this study was to evaluate if the gain of T cells observed in NR-VL/HIV patients after anti-*Leishmania* treatment (13) was related to changes in the mobilization profile of the peripheral TCRV β repertoire, and whether the thymus is involved in the replenishment of newly T cells, especially in non-relapsing VL/HIV co-infected patients.

MATERIALS AND METHODS

Casuistic of the Study and Ethical Aspects

Eighteen VL/HIV co-infected patients were recruited from Hospital Eduardo de Menezes, Belo Horizonte, Brazil, being prospectively followed from February 2011 up to March 2013. The same patient cohort was previously evaluated by Silva-Freitas et al. (13) and those studies revealed differences in cellular activation and a senescent phenotype. Briefly, these patients were grouped in relapsing VL/HIV (R-VL/HIV; $n = 12$) e non-relapsing VL/HIV (NR-VL/HIV; $n = 6$) respectively, according to the occurrence or not of VL relapse episodes throughout life. Clinical aspects, diagnosis, treatment and ethical aspects were previously reported (13). Parasitological tests (direct exam and culture) from bone marrow aspirates were used to confirm the VL diagnosis in all patients. At that time, the first line treatment recommended by the Brazilian Ministry of Health for VL/HIV patients was amphotericin B deoxycholate for 4 weeks (35). After treatment, secondary prophylaxis with amphotericin B was offered every 2 weeks to those VL/HIV patients that maintained absolute CD4 $^{+}$ T lymphocyte counts below 350 cells/mm 3 (36).

The immunological parameters were evaluated in the following monitoring periods: active phase, early post-treatment, 6 and 10 (for TREC assessment) and 12 months post-treatment (mpt). Patients infected only with HIV as well as healthy subjects (HS) were included as controls. This study was approved by ethical review boards of Hospital Eduardo de Menezes and of the Oswaldo Cruz Foundation (René Rachou and Oswaldo Cruz Institutes).

Evaluation of TCRV β Repertoire Levels by Flow Cytometry

To evaluate the V β repertoire of T lymphocytes we used the IOTest $^{\text{®}}$ Beta Mark kit (Beckman-Coulter, Fullerton, CA, EUA), which includes specific monoclonal antibodies for 24 V β chains belonging to 19 out of 26 V β families known. The following 24 V β families were evaluated: V β 1, 2, 3, 4, 5.1, 5.2, 5.3, 7.1, 7.2, 8, 9, 11, 12, 13.1, 13.2, 13.6, 14, 16, 17, 18, 20, 21.3, 22, 23. In this kit, three V β different chains were simultaneously analyzed in a single dotplot, being labeled with the following fluorochromes: FITC or PE or with the FITC-PE combination. Therefore, this flow cytometry protocol allowed to analyze the expression of V β chains in the subpopulations of CD4 $^{+}$ and CD8 $^{+}$ T lymphocytes using anti-CD4 PercP (Peridinin-chlorophyll proteins) and anti-CD8 APC (allophycocyanin) monoclonal antibodies. The gate of these subpopulations was defined within the CD3 $^{+}$ T lymphocyte compartment, in a tube containing anti-CD4 PercP, anti-CD8

APC and anti-CD3 FITC monoclonal antibodies. From this lymphocyte subpopulations (CD4 $^{+}$ or CD8 $^{+}$ T cells), a dotplot was created to define the percentages of cells expressing such V β family. Peripheral blood mononuclear cells (PBMCs) obtained of the Ficoll-Hypaque gradient centrifugation were used to *ex vivo* immunophenotyping and all samples were acquired by a FACSCalibur $^{\text{®}}$ device (BD Biosciences, San Jose, CA, USA). For each sample, 20,000 events were acquired within the lymphocyte gate. The cytometry analyses were performed using the Cell Quest Pro $^{\text{TM}}$ software (BD Biosciences, San Jose, CA, USA).

Quantification of Cytokine Levels

A multiplex assay was performed to quantify the serum levels of the following cytokines: IFN- γ , TNF, IL-1 β , IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12, IL-13, IL-17, MCP-1, and MIP-1 β . Cytokine contents were calculated by Luminex technology (Bio-Plex Workstation; Bio-Rad Laboratories, USA). Data analysis was performed using the software provided by the manufacturer (Bio-Rad Laboratories, USA). Recombinant cytokines were used to establish standard curves and the sensitivity of the assay. Results were expressed as Median Fluorescence Intensity (MFI) (37). The MFI of the last point of each standard curve was used to determine the detection limit of each cytokine.

Quantification of T Cell Receptor Excision Circles (TRECs) by qPCR

PBMCs previously cryopreserved were thawed and the DNA was extracted directly from the cells using the QIAamp DNA Blood Mini kit, following the manufacturer's instructions (Qiagen, Manchester, UK). The DNA was extracted of an initial concentration of cells which ranged from 1 to 5 million PBMC/mL. After extraction, the eluted DNA was quantified through of NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). TRECs were quantified by real-time quantitative Polymerase Chain Reaction (qPCR), according to the principles of the test established by Douek et al. (19). Briefly, 2 μ L of extracted DNA was added in MicroAmp $^{\text{®}}$ Optical 96-well reaction plate (Applied Biosystems $^{\text{®}}$) with 3 μ L of TREC reaction mix, which consisted in: miliQ water; TREC primers: Forward (5'-CAC ATC CCT TTC AAC CAT GCT-3') and Reverse (5'-GCC AGC TGC AGG GTT TAG G-3'); probe TREC (6-FAM-ACA CCT CTG GTT TTT GTA AAG GTG CCC ACT-39-TAMRA); and the 2x TaqMan Universal Master Mix II enzyme (Applied Biosystems $^{\text{®}}$). Reaction final volume was 5 μ L/well. To perform TREC quantification, a plasmid containing TREC sequence cloned, kindly provided by Dr. Daniel Douek (Vaccine Research Center, NIH, USA) was serially diluted from 10 6 to 10 $^{-1}$ TREC copies/ μ L and used as standard curve amplified in parallel in each experiment. To control the quality of the assay and the integrity of the extracted DNA, the RNaseP endogenous constitutive gene was quantified in all samples. Moreover, in order to normalize the copy number of the target gene (TREC/ μ L) by the number of total cells contained in each sample, we used a standard curve of RNaseP that was serially diluted from 10 6 up to 10 1 PBMC/ μ L. Each sample was run in duplicate and the qPCR assay was performed in the 7,500 Real-Time PCR System equipment (Life Technologies).

The analysis was performed in the 7,500 software v2.3 (Life Technologies) and the final result was expressed by 10^6 cells, as TREC copies/ 10^6 PBMC.

Statistical Analysis

The statistical analyses were performed using GraphPad Prism software (version 6.0, San Diego, CA, USA). For comparisons between VL/HIV co-infected patients and control groups, we used non-parametric tests: Mann Whitney when two groups were analyzed; ANOVA (Kruskal–Wallis) and Dunns post-test when three or more groups were simultaneously compared. Parametric test (Wilcoxon) was applied when the same patient was compared in his/her different phases of follow-up. Differences were considered statistically significant when the p value was ≤ 0.05 . Heatmap analyses were performed to evaluate the differential expression patterns of TCRV β repertoire on T cell subpopulations in VL/HIV patients. For this, the online software Heat mapper[®] (Wishart Research Group at the University of Alberta) was applied to draw a heatmap from a spreadsheet containing the expression index of each TCRV β family for each patient evaluated, using the following formula: % Y-V β family expression of the X-patient in a given follow-up period divided by average of the % Y-V β family expression in the healthy controls. The clustering method used for analysis was the average linkage, and the distance measurement method applied was Euclidean.

RESULTS

Mobilization of the CD4⁺ and CD8⁺ T-Cell V β Repertoire After anti-*Leishmania* Treatment in Non-relapsing and Relapsing Visceral Leishmaniasis/HIV Co-infected Patients

As we previously demonstrated (13), NR-VL/HIV and R-VL/HIV patients presented very low levels of CD4⁺ T cells during active phase of VL (Table 1). However, at 6 mpt a significant increase in this population was observed in NR-VL/HIV patients but not in R-VL/HIV, remaining until 12 mpt. Although CD8⁺ T lymphocyte counts also increased in the NR-VL/HIV group after treatment, no significant differences were found throughout the follow-up when the patients were evaluated individually (Table 1). This indicates that the mobilization occurs mainly in the CD4⁺ T cell pool. Since NR-VL/HIV patients had a higher degree of immune reconstitution in comparison to R-VL/HIV cases, we investigated whether this T cell input was associated with newly TCRV β repertoire diversity or even differentially mobilized.

Then, we evaluated the overall TCRV β mobilization profile of VL/HIV groups in comparison to HS. Considering both NR-VL/HIV and R-VL/HIV patients, in all clinical phases, the TCRV β repertoire was significantly altered (higher or lower expression) in CD4⁺ T cells (70.8%; 17 out of 24 families) (Table 2) and in CD8⁺ T cells (50%, 12 out of 24 families) (Table 3). However, CD4⁺ T cells V β families were mobilized differently in NR-VL/HIV and R-VL/HIV groups (Table 2). In terms of CD8⁺ T cells, the NR-VL/HIV group

TABLE 1 | T cell absolute counts of non-relapsing and relapsing visceral leishmaniasis/HIV (VL/HIV) co-infected patients.

VL/HIV group	Follow-up phase	CD4 ⁺ cells/mm ³ Median [IQR]	CD8 ⁺ cells/mm ³ Median [IQR]	n
Non-relapsing	Active VL	86.5 [66.3–120.5]	364 [238.5–1,032]	6
	Early post-treatment	166.5 [120.3–263.8]	499 [386.8–961.8]	6
	6 mpt	297 [216.8–478.5]*	766.5 [428.8–1,872]	6
	12 mpt	350 [284.5–659.5]**	806 [651.5–1,408]	6
Relapsing	Active VL	116 [51.8–185]	418 [226.8–881]	12
	Early post-treatment	189.5 [96–257.8]	556 [390.3–1,305]	12
	6 mpt	132 [76–203]	425 [309–705]	11
	12 mpt	126 [39.25–232.3]	327.5 [218–1,476]	10

VL/HIV, patients with visceral leishmaniasis co-infected by HIV; mpt, months post-treatment; IQR, interquartile range; *Significant difference between non-relapsing (NR-) and relapsing (R-) VL/HIV patients at 6 mpt; **significant difference between non-relapsing (NR-) and relapsing (R-) VL/HIV patients at 12 mpt; n, number of patients in each follow-up phase.

maintained the expression of several families, whereas R-VL/HIV, not only mobilized few families but also their use was reduced in relation to healthy subjects. Nine families were simultaneously altered in both T cell subsets (V β 18, V β 5.1, V β 20, V β 5.2, V β 23, V β 11, V β 14, V β 4, V β 7.2). Two out of the nine families showed a similar mobilization profile in the T cell subsets: V β 23 and V β 7.2 (Figures 1C,E, 2D,E). Interestingly, the V β 7.2 family was significantly less expressed (below 1%) in both T cells in NR-VL/HIV and R-VL/HIV patients but in different time points of the follow-up (Figures 1E, 2E and Supplementary Figures 1A–D), suggesting a restriction of this family in VL/HIV patients.

A further analysis was to compare the TCRV β mobilization in the active phase with the different phases after therapy. In relation to CD4⁺ T cells, three families were increased in NR-VL/HIV patients during the active phase, in comparison to HS: V β 5.1, V β 13.6 and V β 1 but only V β 5.1 and V β 13.6 families remained significantly more expressed after treatment (Table 2; Figure 1F and Supplementary Figures 1A, 2). By contrast, among R-VL/HIV patients, six families were more elevated: V β 18, V β 13.6, V β 23, V β 22, V β 13.2 e V β 4 (Table 2; Figures 1C,D,F and Supplementary Figure 1A). All these families, except V β 22, remained significantly higher immediately after anti-*Leishmania* treatment (Table 2; Figures 1C,D,F and Supplementary Figure 2). Four families displayed punctual changes in the TCRV β mobilization: higher percentages of V β 3 (throughout the follow-up; Figure 1A) and decreased expression levels of V β 23 (12 mpt; Figure 1C) in NR-VL/HIV, decreased representation of V β 18 in R-VL/HIV (12 mpt; Figure 1D) and decreased expression of V β 9 in both groups (12 mpt; Figure 1B).

In order to provide an individual qualitative overview of the V β disturbances we used the heatmap strategy to represent the fold change of the percentages of each TCRV β family presented by each VL/HIV patient in all phases of follow-up.

TABLE 2 | Differences in the CD4⁺ T-cell V β repertoire mobilization profile of non-relapsing and relapsing visceral leishmaniasis/HIV (VL/HIV) co-infected patients.

	Non-relapsing (NR-VL/HIV)				Relapsing (R-VL/HIV)			
	Active	Early post-treat	6 mpt	12 mpt	Active	Early post-treat	6 mpt	12 mpt
V β families	5.3							
7.1								
3								
9								
17								
16								
18								
5.1								
20								
13.1								
13.6								
8								
5.2								
2								
12								
23								
1								
21.3								
11								
22								
14								
13.2								
4								
7.2								

*V β families up-expressed (■) or down-expressed (■) in CD4⁺ T cells in comparison to healthy subjects. VL/HIV (patients with visceral leishmaniasis co-infected by HIV); Active (VL active phase); Early post-treat (immediately after anti-Leishmania post-treatment); 6 mpt (six months post-treatment); 12 mpt (12 months post-treatment); mpt (months post treatment).

The CD4⁺ TCRV β repertoire analysis by heatmap confirmed that, although the V β 9 family was highly mobilized during the active phase in NR-VL/HIV (4 out of 5 patients), it was reduced in both groups of co-infected patients after the anti-*Leishmania* treatment (Figures 1B, 3A; $p < 0.05$). The V β 18 family also presented increased expression levels during the active phase and early after treatment against *Leishmania* among NR-VL/HIV patients (4 out of 5 patients), decreasing in the 6 and 12 mpt (Figure 3A), although such a decrease was not statistically significant (Figure 1B). This same pattern was observed in relation to V β 22, V β 23, and V β 18 families, among the R-VL/HIV patients, where 5 out of 8 patients in the VL active phase mobilized these families (Figure 3A). At 12 mpt, the heatmap analysis confirmed the reduction in the expression levels of the V β 13.1 (4 out of 5 patients) and V β 23 families (all patients) among NR-VL/HIV group (Figure 3A). Also, it is important to mention V β 3 family, whose mobilization tended to be higher among the majority of NR-VL/HIV patients throughout the follow-up (Figure 3A).

TABLE 3 | Differences in the CD8⁺ T-cell V β repertoire mobilization profile of non-relapsing and relapsing visceral leishmaniasis/HIV (VL/HIV) co-infected patients.

	Non-relapsing (NR-VL/HIV)				Relapsing (R-VL/HIV)			
	Active	Early post-treat	6 mpt	12 mpt	Active	Early post-treat	6 mpt	12 mpt
V β families	5.3							
7.1								
3								
9								
17								
16								
18								
5.1								
20								
13.1								
13.6								
8								
5.2								
2								
12								
23								
1								
21.3								
11								
22								
14								
13.2								
4								
7.2								

*V β families up-expressed (■) or down-expressed (■) in CD8⁺ T cells in comparison to healthy subjects. VL/HIV (patients with visceral leishmaniasis co-infected by HIV); Active (VL active phase); Early post-treat (immediately after anti-Leishmania post-treatment); 6 mpt (6 months post-treatment); 12 mpt (12 months post-treatment); mpt (months post treatment).

Concerning CD8⁺ T-cells, the V β 18 and V β 3 families were more mobilized (V β 3 above 5%) in NR-VL/HIV patients during the active phase and after anti-*Leishmania* treatment when compared to HS (Table 3; Figures 2A,B and Supplementary Figure 1C). Although the R-VL/HIV group had lower percentages of V β 3 family on CD8⁺ T cells in comparison to the NR- group, this family was the only one mobilized above 5% among R-VL/HIV patients at 12 mpt (Figure 2A and Supplementary Figure 1D). The representation of V β 18 family on CD8⁺ T cells significantly decreased in NR-VL/HIV patients at 12 mpt in relation to active and post-treatment (Figure 2B), showing a different pattern of the R-VL/HIV group (Figure 2B). Among the R-VL/HIV group, only the V β 23 on CD8⁺ T cells was more expressed during the VL active phase (Table 3; Figure 2D and Supplementary Figure 1A). In the NR-VL/HIV group, the mobilization percentages of this family significantly reduced at 12 mpt, in comparison to the previous phases (Figure 2D). Curiously, the percentages of CD8⁺ T cells expressing V β 2

remained lower in both NR-VL/HIV and R-VL/HIV groups during all the follow-up period (Table 3; Figure 2C) in relation to HS, indicating a possible mobilization profile characteristic of the VL/HIV association in this subpopulation.

Noteworthy, practically no significant change in the TCRV β mobilization profile was seen in CD8⁺ T cells from relapsing patients when compared to HS (Table 3; Figure 2 and Supplementary Figure 3). This may be due to the fact that many TCRV β families have been differently mobilized in each clinical phase for each patient, suggesting that the CD8⁺ TCRV β repertoire in R-VL/HIV patients is more heterogeneous, and may be related to a reduced ability of parasite control.

Again, the heatmap analysis of the CD8⁺ TCRV β repertoire (Figure 3B) reinforced our previous observations, such as the high use of V β 18 by 3 out of 5 NR-VL/HIV patients and only one out of 8 R-VL/HIV cases (Figure 3B) during the active VL. This family reduced in all NR-VL/HIV patients at 6 and 12 mpt. The V β 23 family mobilization was also expressively reduced in all NR-VL/HIV patients at 12 mpt, with a trend to be more used among R-VL/HIV patients mainly in the VL active phase (4 out of 8). Finally, the levels of V β 3 family were not only elevated during all the clinical follow-up of the NR-VL/HIV (3 out of 5 patients), but also tended to be more expressed in this group in comparison to R-VL/HIV patients (Figures 2A, 3B). Overall, an extremely heterogeneous pattern of CD8⁺ TCRV β repertoire in the R-VL/HIV cases was detected, with numerous differences in the individual mobilization profile.

Decrease of Pro-inflammatory Cytokine Levels in Non-relapsing VL/HIV Co-infected Patients

Considering that NR-VL/HIV and R-VL/HIV patients differed in their T cell reconstitution profile (CD4⁺ T cell counts and TCRV β repertoire), especially after 6 mpt, we investigated its potential impact upon the pro- and anti-inflammatory cytokine status of these patients. During the active phase and early post-treatment, NR-VL/HIV and R-VL/HIV patients exhibited similar levels of IL-8 and TNF (Figures 4A,B). Nevertheless, in NR-VL/HIV patients there was a significant decrease in the levels of these cytokines, from 6 mpt compared to the early phases. By contrast, R-VL/HIV patients kept or even augmented IL-8 and TNF levels, which were significantly higher than those observed in NR-VL/HIV cases (Figures 4A,B).

Interestingly, we also observed a significant decrease in IL-10 serum levels, in NR-VL/HIV patients at 6 and 12 mpt, in comparison to the VL active phase (Figure 4E). This finding may be associated to the fact of the parasite load in this group have become undetectable after anti-*Leishmania* treatment (Figure 4F). Moreover, we found a negative correlation between the IL-10 levels in the active phase of VL and the total number of VL episodes ($r = -0.65$, $p < 0.05$) (see Supplementary Figure 4H).

For the other cytokines, the same pattern was observed: the majority of the NR-VL/HIV patients showed a tendency to reduce IFN- γ and IL-6 (Figures 4C,D), as well as IL-2, IL-17, CCL4, IL-13, and IL-4 levels (see

Supplementary Figures 4B,C,E–G) at 6 and 12 mpt in relation to the active phase and immediately after the anti-*Leishmania* treatment. On the other hand, among the R-VL/HIV patients, IFN- γ and IL-6 (Figures 4C,D), as well as IL-1 β , CCL2, CCL4, and IL-4 (see Supplementary Figures 4A,D,E,G) levels tended to remain elevated or even higher at 6 and 12 mpt in comparison to those found in the early periods of clinical follow-up.

Thymic Output May Contribute to the Replenishment of T Cells in Non-relapsing VL/HIV Patients

As we previously found that both NR-VL/HIV and R-VL/HIV individuals exhibit elevated percentages of senescent T cells (13), but different degree of immune reconstitution, inflammatory profile and mobilization of V β families, we questioned whether the thymic compartment could be contributing to this differential immune status. As depicted in Figure 5A, the numbers of TREC copies per million of PBMCs were lower in the VL/HIV co-infected patients during active phase (1.54 TRECs; IQR: 0.99–2.94 TRECs) and early post-treatment (0.99 TRECs; IQR: 0.34–1.62 TRECs) when compared to HIV mono-infected or healthy subjects (HIV: 3.71 TRECs; IQR: 1.74–17.37 TRECs; and HS: 42.08 TRECs; IQR: 15.41–116.6 TRECs). Nevertheless, a tendency to increase ($p=0.06$) was observed among co-infected patients at 10 mpt (2.91 TRECs; IQR: 1.13–11.99), as compared to the initial phases of the follow-up (Figure 5A). When these patients were split into relapsing (R-VL/HIV) and non-relapsing (NR-VL/HIV), it was observed that the augment in TRECs at 10 mpt was due to a significant increase in NR-VL/HIV patients in comparison to R-VL/HIV (Figure 5B). The median of TREC copies in PBMCs seen in NR-VL/HIV patients were higher than those observed in the HIV mono-infected individuals and some of NR-VL/HIV patients showed data rather close to the median of HS (Figure 5B, dashed line). By contrast, R-VL/HIV patients maintained low TREC copies during all the follow-up period, and below the medians of the control group (Figure 5B).

The significant increase in the numbers of TREC copies in NR-VL/HIV patients at 10 mpt (Figure 5B) may reflect their ability to replenish the pool of peripheral CD4⁺ T cells, as we showed in the Table 1.

Importantly, the TREC copy numbers per million PBMCs at 10 mpt negatively correlated ($p < 0.05$, $r = -0.545$) with the number of relapses observed in VL/HIV patients in this same period of follow-up, as seen in Figure 5C, again suggesting that the impairment of the thymic output may influence the occurrence of VL relapses.

DISCUSSION

Relapses in VL/HIV patients are a challenge for clinicians since it is, not only a frequent event, but also difficult to manage because of the scarce therapeutic options. A negative correlation between CD4⁺ T cell counts and cellular activation levels in VL/HIV co-infected patients was previously demonstrated (13). Corroborating with these results, relapsing-VL/HIV patients whose activation levels were elevated during 12 months of the

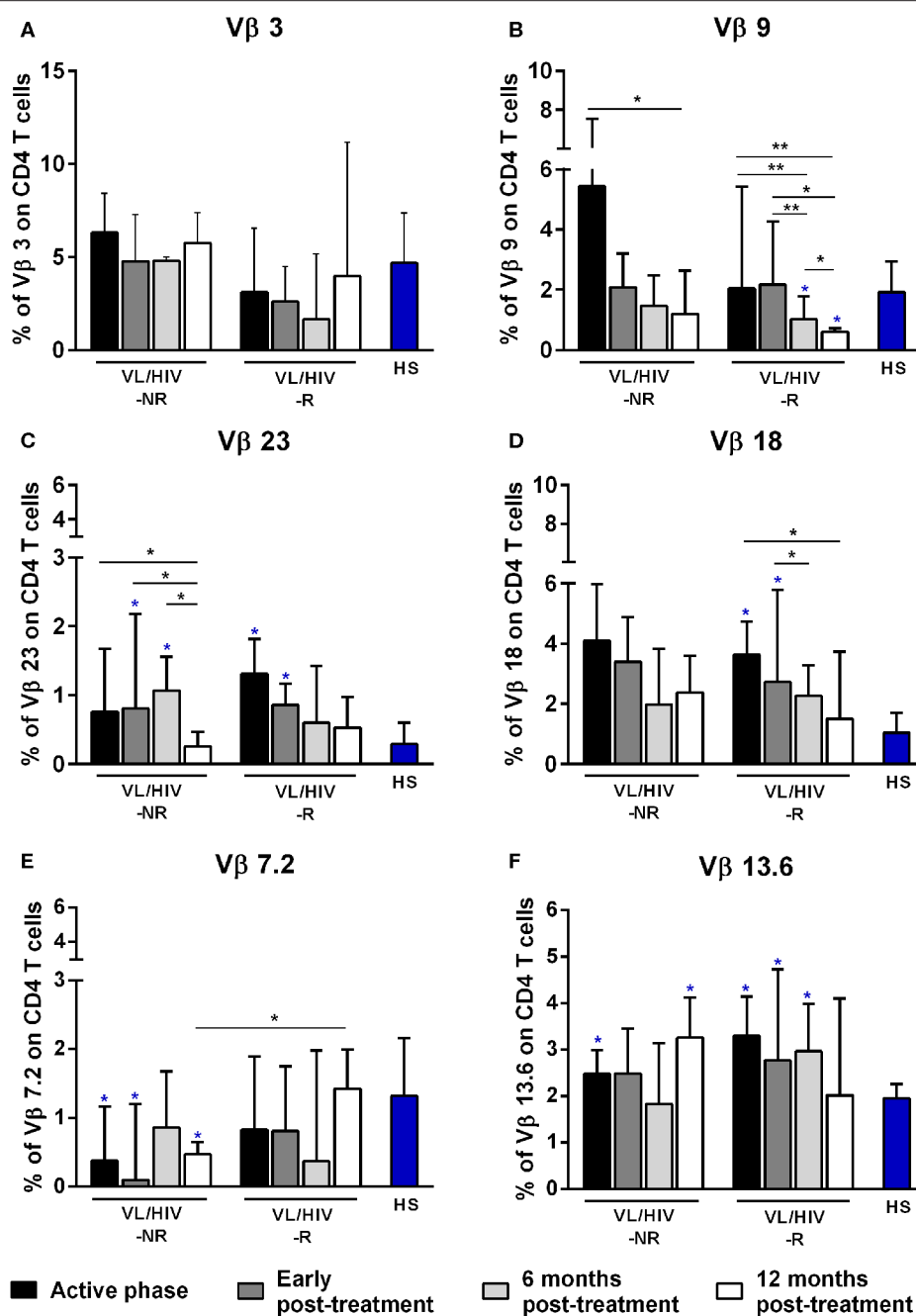


FIGURE 1 | Percentages of the Vβ families on CD4⁺ T lymphocytes among non-relapsing (NR) and relapsing (R) visceral leishmaniasis/HIV (VL/HIV) co-infected patients. The mobilization percentages of Vβ3 (A), Vβ9 (B), Vβ23 (C), Vβ18 (D), Vβ7.2 (E), and Vβ13.6 (F) in NR-VL/HIV co-infected patients in comparison to R-VL/HIV group. The blue asterisk represents the statistical differences in relation to healthy subjects (HS). The column bar represents the median values with interquartile range. * $p < 0.05$ ** $p < 0.005$.

clinical follow-up also maintained low CD4⁺ T-lymphocyte counts. On the other hand, non-relapsing-VL/HIV presented a gain of CD4⁺ T cells, but not of CD8⁺ T cells. Therefore, we decided to investigate the quality of the T cells circulating in the periphery, in terms of diversity and mobilization profile of Vβ families, cytokine status, and the probable origin of T cells.

Disturbances in the TCRVβ repertoire diversity are observed in HIV monoinfected patients at the acute phase of the infection (28, 29, 31, 38), perpetuating through chronic infection (31). Herein, we showed that VL/HIV co-infected patients also suffered significant disturbances in the expansion or retraction of several TCRVβ-families, mainly during active and VL

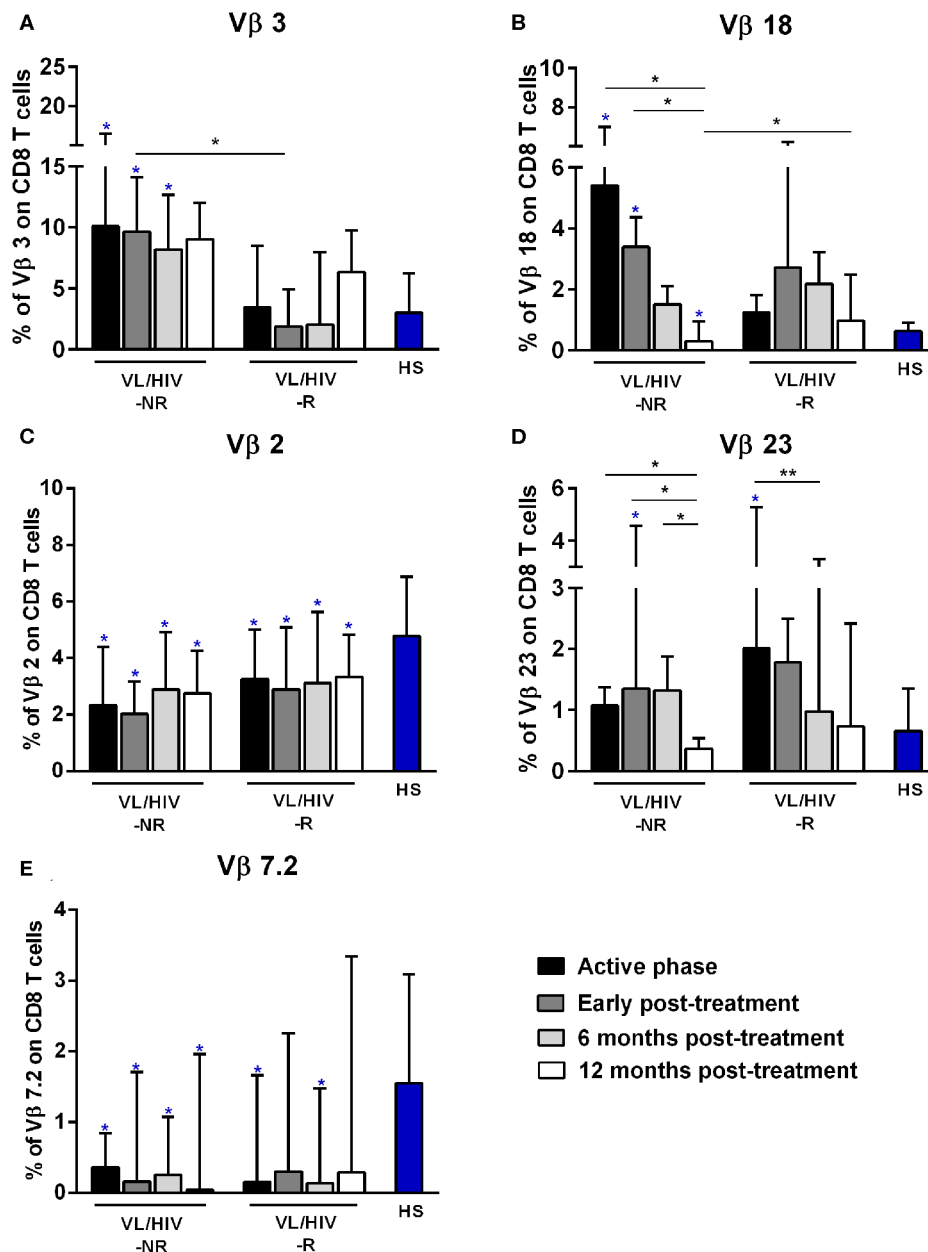
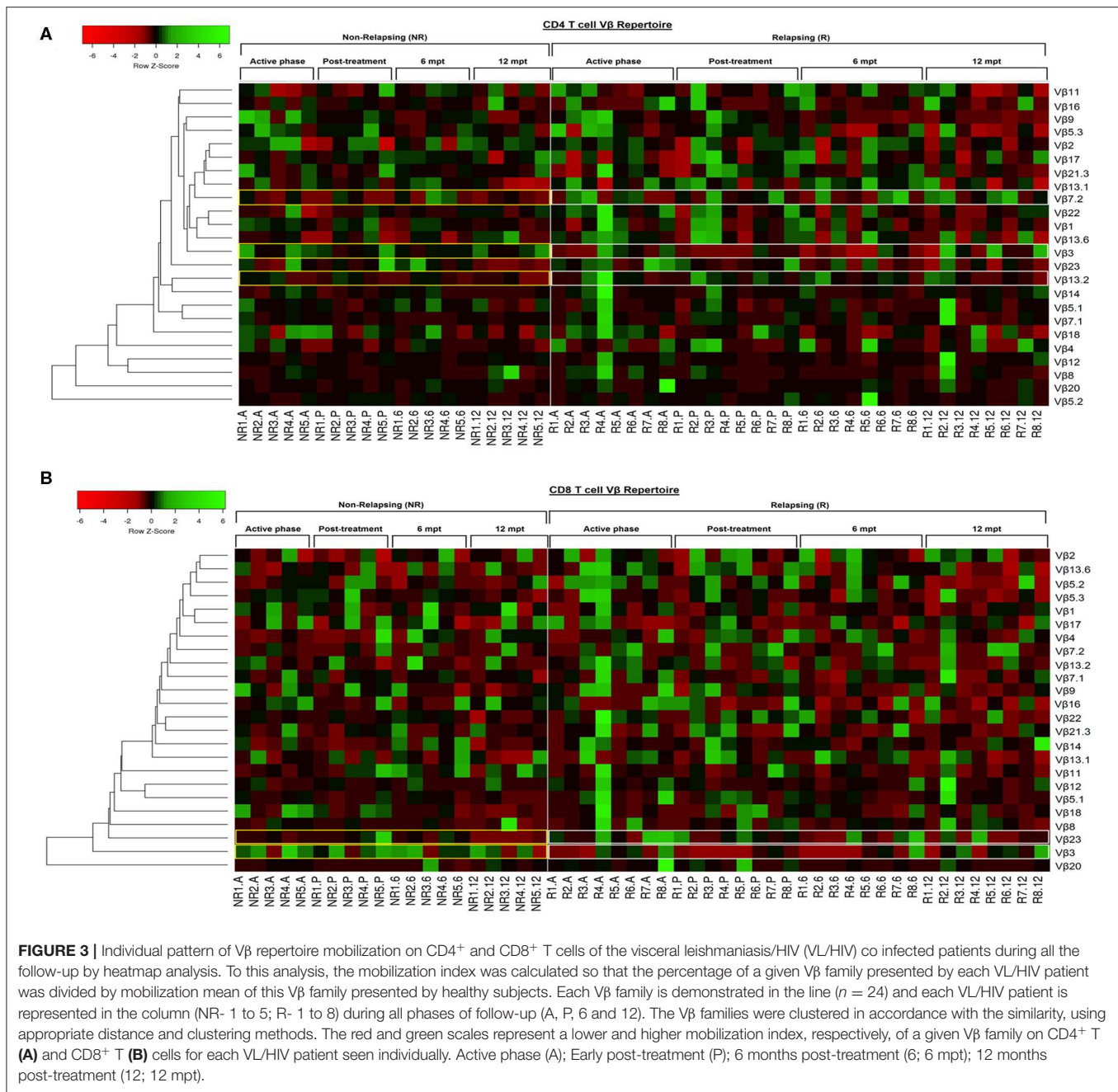


FIGURE 2 | Percentages of the V β families on CD8⁺ T lymphocytes among non-relapsing (NR) and relapsing (R) visceral leishmaniasis/HIV (VL/HIV) co-infected patients. The mobilization percentages of V β 3 (A), V β 18 (B), V β 2 (C), V β 23 (D), and V β 7.2 (E) in NR-VL/HIV co-infected patients in comparison to R-VL/HIV patients. The blue asterisk represents the statistical differences in relation to healthy subjects (HS). The column bar represents the median values with interquartile range. * $p < 0.05$ ** $p < 0.005$.

post-treatment phases, regardless of these are relapsing or non-relapsing. In terms of TCRV β on CD4⁺ T cells, NR-VL/HIV and R-VL/HIV groups mobilized different V β families when compared to healthy individuals. However, to the TCRV β on CD8⁺ T cells it is interesting to note that the NR-VL/HIV patients mobilized several families either to a greater or lesser expression, whereas R-VL/HIV group mobilized few families; most of them being down expressed in comparison to the HS.

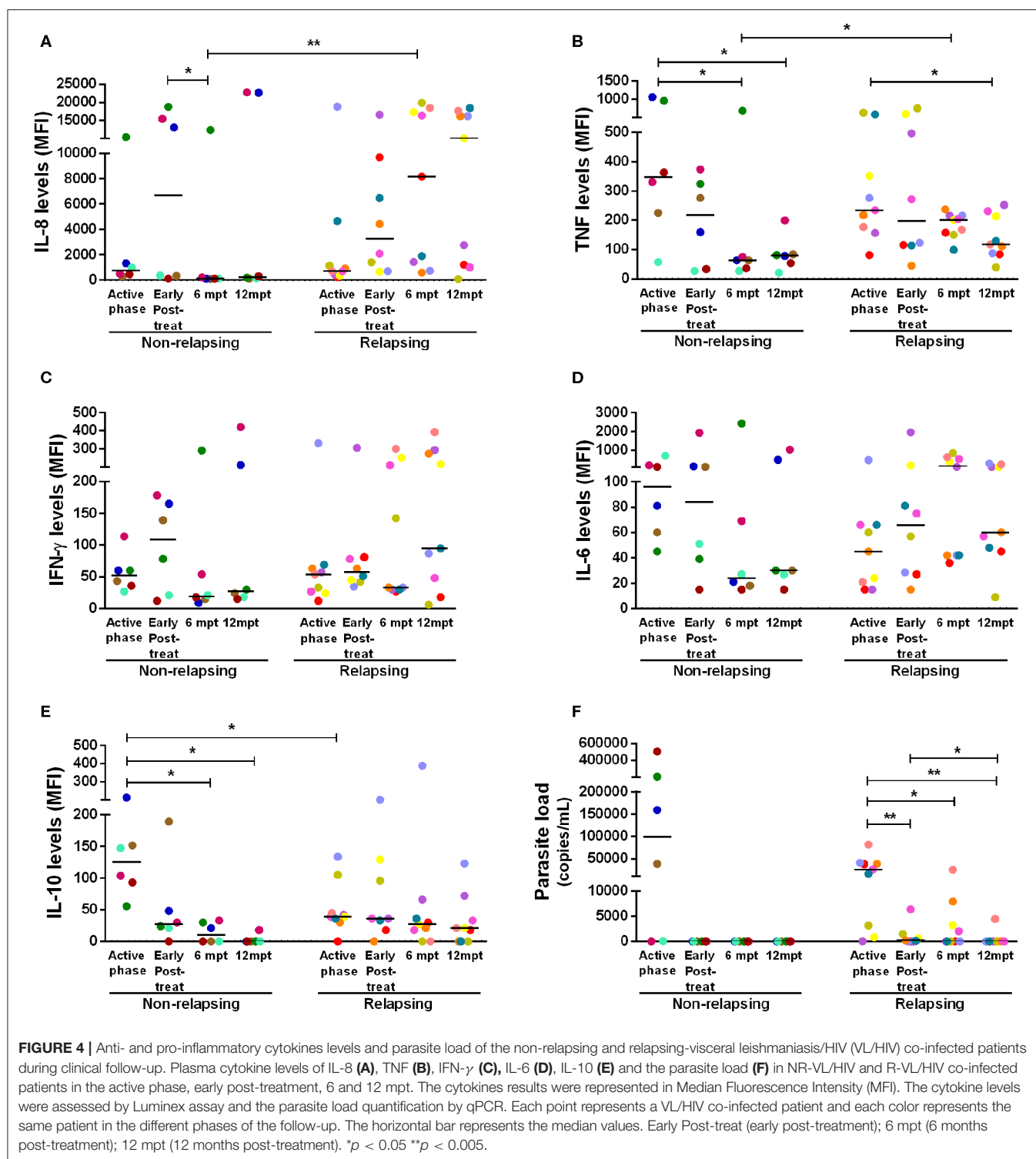
In this context, the analysis of these disturbances by heatmap suggested a more homogeneous TCRV β repertoire on CD4⁺ T-cells, especially in the NR-VL/HIV cases. Differently, the TCRV β repertoire on CD8⁺ T-cells is extremely heterogeneous when assessed individually, since different V β -families were mobilized, through expansions or retractions. These differential alterations were more intense among R-VL/HIV patients. Indeed, in HIV-1 infection, major disturbances have been described in the



CD8⁺ TCRV β repertoire, regardless of the clinical status, CD8⁺ and CD4⁺ T-cell counts, as well as viral load (39). On the other hand, the CD4⁺ TCRV β repertoire appears to be severely disturbed when there are low CD4⁺ T cells counts and high HIV viremia (39). Considering that CD8⁺ T-cells are an important subpopulation for parasite control, we could infer that the intense disturbance on the CD8⁺ T-cell repertoire may be related to the predisposition to VL relapses.

Although a characteristic TCRV β mobilization profile was not seen in neither R-VL/HIV nor NR-VL/HIV cases, it is noteworthy the fact that three V β families were significantly

retracted at 12 mpt of the NR-VL/HIV patients in relation to the early stages of the follow-up (V β 9 on CD4⁺ T-cells; V β 18 on CD8⁺ T-cells; V β 23 in both CD8⁺ and CD4⁺ T-cells, $p < 0.05$). Of these, only two (V β 9 on CD4⁺ T-cells; V β 18 on CD8⁺) were highly mobilized in the active phase of this group. Similarly, R-VL/HIV patients also presented a reduction in the mobilization of some V β families (V β 9 and V β 18 on CD4⁺ and V β 23 on CD8⁺ T cells, $p < 0.05$) in relation to the active phase. Moreover, the V β 3 family was less mobilized in CD4⁺ and CD8⁺ T-cells in R-VL/HIV patients throughout the clinical follow-up, although it is a V β family that is usually



increased in Brazilian HIV-positive patients, with or without ART (29). These retractions, regardless of whether it is R or NR, could be related to the return to baseline state of expression of V β families or even to a severe clonal exhaustion process. Considering the continuous *Leishmania* stimulation in the scenario of VL/HIV co-infection, it is plausible that the

exhaustion of primary immune resources may influence the effector immune response and therefore the occurrence of relapse episodes. Herein, we showed that there are changes in the TCRV β mobilization profile of co-infected patients, especially during the clinical follow-up. Even so, the association between the mobilization of these families and parasitic control could

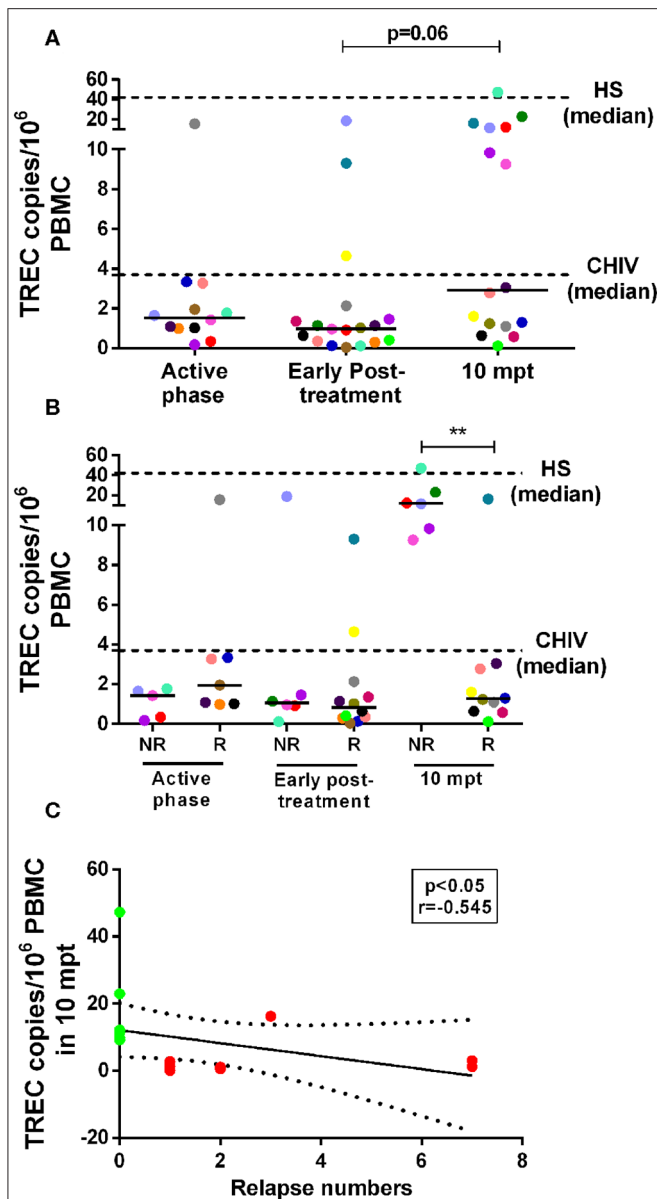


FIGURE 5 | Number of T cell receptor excision circles (TREC) copies/10⁶ PBMC in visceral leishmaniasis/HIV (VL/HIV) co-infected patients during the prospective follow-up and its correlation with number of VL relapses. TREC copy numbers in the VL/HIV-co-infected patients (A) and TRECS copy numbers in non-relapsing-VL/HIV (NR-) and relapsing-VL/HIV (R-) group (B) during the clinical follow-up. The number of TREC copies was evaluated from 1 to 5 × 10⁶ cells/mL obtained of the peripheral blood of all VL/HIV co-infected patients in the active, early post-treatment and 10 mpt phases, as well as HIV mono-infected patients (CHIV) and healthy subjects (HS). Negative correlation (C) between the number of TREC copies at 10 mpt and the total number of relapses presented by VL/HIV patients (Spearman correlation, $r = -0.545$; $p < 0.05$). The green and red colors represent the NR-VL/HIV and R-patients, respectively (C). The dashed lines represent the median of TREC copies/10⁶ PBMC of the HIV mono-infected patients and HS (A,B). Each point represents a patient and each color represents the same patient in the different phases of follow-up. The horizontal bar represents the median values. 10 mpt (10 months post-treatment). ** $p < 0.005$.

not be addressed in this study. New approaches based on other study designs and using specific stimulations may clarify these questions.

It is important to stress that cytofluorometry is not informative for evaluating the specificity of a given TCR repertoire or defining clonotypes. Herein, this strategy aimed at determining if the percentages of T cells using a given V β family differed between patients and controls and for evaluating the dynamics of mobilizations in terms of peripheral frequency, especially after an immunological reconstitution.

In addition to the gain in CD4⁺T lymphocyte numbers, the reduction of the T-cell activation status was markedly observed in VL/HIV patients who achieved clinical remission after anti-leishmanial therapy (13). Then, a possible relationship between the improvement of the immune status along with the replenishment of T lymphocytes pool (CD4⁺ T cell gain and TCRV β repertoire) on the profile of systemic inflammatory cytokine, was addressed. We found a tendency of the NR-VL/HIV group to reduce the plasmatic pro- and anti-inflammatory cytokine levels throughout the clinical follow-up. In this respect, low levels of IL-10 in the active phase of the disease in R-VL/HIV group may contribute to the maintenance of an activated and inflammatory immune status, due to the lack of regulatory action of this cytokine, as suggested by negative correlation observed between this parameter and the VL relapse numbers. A cytokine storm has been associated with the severity of VL alone (9), with the worsening of the immune status and progression to AIDS in HIV-positive individuals (40). Moreover, it has been associated with the presence of *Leishmania* in VL/HIV patients (7). As in a vicious circle, this inflammatory status may contribute to maintaining the high levels of cellular activation that, in turn, continuously compromises the general immune status of relapsing-VL/HIV patients, generating exhaustion and peripheral senescence, thus compromising central immune functions.

Despite the reduction of inflammatory status in NR-VL/HIV patients, the high proportion of peripheral senescent T cells previously verified (13) indicates that these patients present an impairment of the T cell proliferative capacity. This raised the question on the origin of the newly-formed T cells detected in the circulation of NR-VL/HIV patients, which prompted us to investigate a putative thymus participation in this process. Herein, for co-infected patients, the TREC copy numbers were low during the VL active phase, suggesting a thymic functional impairment in the renewal of the T-cell pool, despite ART use and low or undetectable viral load in the majority of patients (13). In fact, it is well-described that thymic functionality is compromised in HIV-infected patients, especially in those without ART (20, 21, 32, 41, 42), which is in keeping with the fact that the virus has the ability to infect or at least to affect thymic stromal cells, T-lymphocyte progenitors and thymocytes, resulting in lower production of new T-cells with consequent impairment of the immune reconstitution (43–45). However, TREC copies in VL/HIV co-infected patients were even lower than those seen in HIV-solely infected patients. This fact points out that not only

residual HIV, but also *Leishmania* can be contributing to the severely impaired thymic function in VL/HIV patients. Previous studies have shown that *Leishmania* can affect T-cell progenitors in the bone marrow and the thymic microenvironment (46–48), which may also favor the deficient thymic output under conditions of VL. Additionally, as previously described, the parasite potentiates the immune activation degree (5, 49), mainly in R-VL/HIV cases (13), which in turn may lead to thymic dysfunction.

It is noteworthy that such thymic output deficiency seems to be more severe in those patients presenting VL relapses, since they maintained low TREC levels during all clinical setpoints of the follow-up, whereas non-relapsing-VL/HIV patients recovered these values at 10 mpt. Considering that TREC copy numbers were accompanied by maintenance of low absolute CD4⁺ T-cell counts in relapsing-VL/HIV and correlated negatively with the number of relapses, we suggest that the thymic impairment favors the loss of parasitic control and the recurrences in visceral leishmaniasis. Future quantification of TREC copies in sorted CD4⁺ and CD8⁺ T-cells would be useful to define if the CD4⁺ T-cell counts recovery among non-relapsing cases is related to increased TREC copy numbers in this subpopulation.

To the best of our knowledge no other study has reported an evaluation of thymic output in VL/HIV co-infected patients. In this scenario, along with TCRV β repertoire disturbances and intense inflammatory status, it is expected that relapsing-VL/HIV patients present a qualitative deficit in the effector cellular immune response, which in turn may predispose to VL relapses. This set of factors may culminate in a higher susceptibility to VL relapses among VL/HIV co-infected patients who have a deficient immune system *per se* and that, along with each relapse, become increasingly unable to control the parasite.

In conclusion, our findings indicate that impaired thymic output is related to the low immune reconstitution and T cell repertoire disturbances in relapsing visceral leishmaniasis associated HIV/AIDS patients.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Hospital Eduardo de Menezes and Oswaldo Cruz Foundation (René Rachou and Oswaldo Cruz Institutes). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

MS-F, GC-C, JS-O, and AD-C: formal analysis, investigation, methodology, organized the database, and wrote the draft of the manuscript. JS-O and AD-C: conceptualization, funding acquisition and project administration. WS: formal analysis and

critically revised the manuscript for intellectual content. GC: recruitment and clinical follow-up of the patients. JT and ZV: methodology and organized the database of TREC. CG-G and AR: writing—review and editing of the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2020.00953/full#supplementary-material>

Supplementary Figure 1 | Percentages of T-cell receptor V β families among non-relapsing (NR) and relapsing (R) visceral leishmaniasis/HIV (VL/HIV) co-infected patients during the VL active phase and 12 months post treatment. Mobilization levels of the 24 V β families in NR-VL/HIV and R-VL/HIV co-infected patients in the active phase (**A,C**) and 12 mpt (**B,D**). This evaluation was performed in accordance with the literature to indicate the families that were less (<1%) and more (>5%) mobilized by CD4⁺ (**A,B**) and CD8⁺ (**C,D**) T-cells V β repertoire. These mobilization limits (1% and 5%) were represented by dashed lines. The asterisks point to the significant differences for higher (blue) and lower (red) mobilization in the VL/HIV groups in relation to healthy subjects (HS). The column bar represents the median values with interquartile range. 12 mpt (12 months post-treatment). **p* < 0.05.

Supplementary Figure 2 | Mobilization profile of CD4⁺ TCRV β repertoire presented by non-relapsing (NR) and relapsing (R) visceral leishmaniasis/HIV (VL/HIV) co-infected patients during clinical follow-up. The percentages of the 19 out of 24 V β families that makes up the CD4⁺ T-cell repertoire were evaluated in the VL/HIV co-infected patients during clinical follow-up and in healthy subjects (HS). The column bar represents the median values with interquartile range. The blue asterisk represents the difference statistical in relation to HS. **p* < 0.05.

Supplementary Figure 3 | Mobilization profile of CD8⁺ TCRV β repertoire presented by non-relapsing (NR) and relapsing (R) visceral leishmaniasis/HIV

(VL/HIV) co-infected patients during clinical follow-up. The percentages of the 19 out of 24 V β families that makes up the CD8⁺ T-cell repertoire were evaluated in the VL/HIV co-infected patients during clinical follow-up and in healthy subjects (HS). The blue asterisk represents the difference statistical in relation to HS. The column bar represents the median values with interquartile range. * $p < 0.05$, ** $p < 0.005$.

Supplementary Figure 4 | Anti- and pro-inflammatory cytokines and chemokines levels of the visceral leishmaniasis/HIV (VL/HIV) co-infected patients during clinical follow-up. Levels of IL-1 β (A), IL-2 (B), IL-17 (C), CCL2 (MCP-1)

(D), CCL4 (MIP-1 β) (E), IL-13 (F), and IL-4 (G) in NR-VL/HIV and R-VL/HIV co-infected patients throughout clinical follow-up. (H) Negative correlation between the IL-10 levels during VL active phase and the total number of VL episodes in the VL/HIV patients (NR- and R- groups). The measure of these soluble factors was performed by Luminex assay. The results were represented as Median Fluorescence Intensity (MFI). Each point represents a VL/HIV co-infected patient and each color represents the same patient in the different phases of the follow-up. The horizontal bar represents the median values. Early Post-treat (early post-treatment); 6 mpt (6 months post-treatment); 12 mpt (12 months post-treatment). * $p < 0.05$ ** $p < 0.005$.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Human Thymic Involution and Aging in Humanized Mice

Qing-Yue Tong^{1,2}, Jue-Chao Zhang^{1,2}, Jing-Long Guo^{1,2}, Yang Li^{1,2}, Li-Yu Yao¹,
Xue Wang^{1,2}, Yong-Guang Yang^{1,2,3} and Li-Guang Sun^{1,2*}

¹ Key Laboratory of Organ Regeneration & Transplantation of the Ministry of Education, The First Hospital of Jilin University, Changchun, China, ² National-local Joint Engineering Laboratory of Animal Models for Human Diseases, Changchun, China, ³ International Center of Future Science, Jilin University, Changchun, China

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Yong Fan,
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United States
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University of Connecticut,
United States

*Correspondence:

Li-Guang Sun
sunliguang1717@vip.163.com

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Thymic involution is an important factor leading to the aging of the immune system. Most of what we know regarding thymic aging comes from mouse models, and the nature of the thymic aging process in humans remains largely unexplored due to the lack of a model system that permits longitudinal studies of human thymic involution. In this study, we sought to explore the potential to examine human thymic involution in humanized mice, constructed by transplantation of fetal human thymus and CD34⁺ hematopoietic stem/progenitor cells into immunodeficient mice. In these humanized mice, the human thymic graft first underwent acute recoverable involution caused presumably by transplantation stress, followed by an age-related chronic form of involution. Although both the early recoverable and later age-related thymic involution were associated with a decrease in thymic epithelial cells and recent thymic emigrants, only the latter was associated with an increase in adipose tissue mass in the thymus. Furthermore, human thymic grafts showed a dramatic reduction in *FOXP1* and *AIRE* expression by 10 weeks post-transplantation. This study indicates that human thymus retains its intrinsic mechanisms of aging and susceptibility to stress-induced involution when transplanted into immunodeficient mice, offering a potentially useful *in vivo* model to study human thymic involution and to test therapeutic interventions.

Keywords: thymus involution, aging, human, humanized mouse, recent thymic emigrants

HIGHLIGHTS

- Human thymus/HSPC-grafted hu-mice show robust thymopoiesis and T cell development.
- Human thymus is susceptible to stress-induced involution in hu-mice.
- Human thymus retains its intrinsic mechanisms of aging in hu-mice.

INTRODUCTION

Aging is a continuous process that is associated with increased susceptibility to infection, autoimmunity, and cancer (1, 2). The thymus gland is an essential lymphoid organ responsible for the production of T cells (3, 4). Thymic involution, or the shrinking of the thymus with age, is common in all species possessing a thymus. However, most of what we know about thymic aging is based on mouse studies, which is unlikely to be identical to humans. It has been shown that naïve T cells from young and aged mice comparable amounts of T-cell receptor excision circles (TREC), whereas the TREC content of naïve human T cells is high in neonates and declines with age

(5). Another difference is that thymic output maintains naïve T cell populations in mice, whereas human T cells may divide in the periphery without losing their naïve phenotype as currently defined (6). Thus, mouse studies may offer limited insights into the process and underlying mechanisms of human thymic aging.

Previous snapshot studies of human thymic tissues suggest that the human thymus grows from birth to 2–3 years of age, followed by involution throughout the period of adolescence (4), and a weight decrease of several fold by the age of 50–60 years (7). Although these snapshot studies provide some insight into the aging of the human thymus, the nature of this process in humans is still largely unexplored due to the lack of a model system that permits longitudinal studies of human thymic involution. Transplantation of human fetal thymic tissue (under renal capsule) and fetal liver-derived CD34⁺ cells (i.v.) achieves efficient human thymopoiesis and T cell development in immunodeficient mice (8–10). The reconstituted mice showed sustained repopulation with multilineages of human lymphohematopoietic cells, including T, B and dendritic cells, and the formation of secondary lymphoid organs. The engrafted human thymus was found to consist of human thymocytes with a normal phenotypic distribution of double negative, double positive, CD4 single positive, and CD8 single positive cell populations (9). Here, we sought to understand the involution and aging of human thymus in this humanized mouse (hu-mice) model. We found that human thymus in hu-mice undergoes both stress-induced and age-related thymic involution, suggesting that the hu-mouse model may be useful for understanding human thymic involution and testing therapeutic interventions.

MATERIALS AND METHODS

Mice and Human Samples

NOD.CB17-*Prkdc*^{scid}/NcrCrl (NOD/SCID) mice were purchased from Beijing Vital River Laboratory Animal Technology Co. Ltd., and were housed in a specific pathogen-free micro-isolator environment and used in experiments at ~5 weeks of age. Discarded human fetal tissues with gestational age of 17 to 20 weeks and human blood samples were obtained with informed consent at the First Hospital of Jilin University. Protocols involved in the use of human tissues and animals were reviewed and approved by the Institutional Review Board and Institutional Animal Care and Use Committee of the First Hospital of Jilin University, and all of the experiments were performed in accordance with the protocols.

Construction of Humanized Mice

Hu-mice were constructed by transplantation of human fetal tissues (~1 mm³ in size, under renal capsule) and fetal liver-derived CD34⁺ cells (i.v.; 3×10^5 /mouse) from the same fetal donor into 2 Gy-irradiated NOD/SCID mice as previously described (9, 11).

Flow Cytometric Analysis

Human immune cell reconstitution in hu-mice was analyzed by flow cytometry (FCM) using various combinations of the following monoclonal antibodies: anti-human CD45, CD3, CD4, CD8, CD45RA, CD45RO, CD69, CCR7, CD31 (all purchased from Biolegend, San Diego, CA, USA); and anti-mouse CD45 (BD Pharmingen) and Ter119 (Biolegend, San Diego, CA, USA). Peripheral blood was collected from tail vein into heparinized tubes, and mononuclear cells were purified by density gradient centrifugation with Histopaque-1077 (Sigma-Aldrich, St. Louis, MO, USA). FCM analysis was performed on a FACS Fortessa (BD Biosciences). Dead cells were excluded from the analysis by gating out lower forward scatter and high propidium iodide-retaining cells. Data analysis was performed using FlowJo 10.3 software.

Immunofluorescence and Histological Analysis

Human thymic grafts were embedded in tissue freezing medium (OCT compound-embedding medium for frozen specimens; Miles Laboratories, Elkhart, IN, USA) and immediately frozen in liquid nitrogen and then stored at -80°C . Cryosections (3.5 μm) were prepared and fixed in cold acetone for 20 min. The sections were stained with Alexa Fluor 647 anti-human CD326 (EpCAM) antibody (clone 9C4, 1:200; Biolegend, San Diego, CA, USA), followed by DAPI staining. The slides were imaged and processed using a fluorescence microscope. To evaluate fat deposition, thymic sections were stained with Oil Red O (for visualizing fat deposition) and hematoxylin. Images of the sections were collected using a light microscope (Olympus Corporation) from four different fields at $\times 100$ magnification. Image Pro Plus software was used to analyze the integrated optical density (IOD) of the Oil Red O-stained areas.

TABLE 1 | Quantitative real-time PCR primer sequences.

Genes	Forward (5'-3')	Reverse (5'-3')
<i>FOXP1</i>	TCCCTCACTCACTGACTTCG	GTGGCATCGAAGATGATGTC
<i>AIRE</i>	GATGACCTGGAGTCCCTTCT	CTCATCAGAGCTGCATGTCC
<i>EpCAM</i>	AATCGTCAATGCCAGTGTACTT	TCTCATCGCAGTCAGGA TCATAA
<i>SJ TREC</i>	CAC ATC CCT TTC AAC CAT GCT	TGC AGG TGC CTA TGC ATC A
<i>TRAC</i>	TGG CCT AAC CCT GAT CCT CTT	GGA TTT AGA GTC TCT CAG CTG GTA CAC

TABLE 2 | Quantitative real-time PCR probe sequences.

Genes	Sequences (5'-3')
<i>SJ TREC</i> probe	FAM-ACA CCT CTG GTT TTT GTA AAG GTG CCC ACT TAMRA
<i>TRAC</i> probe	FAM-TCC CAC AGA TAT CCA GAA CCC TGA CCTAMRA

Real-Time PCR

Total RNA was extracted with Trizol (Invitrogen, Waltham, MA, USA), and cDNA was synthesized using TransScript First-Strand cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China). Quantitative real-time PCR was performed using a SYBR Green Kit (TransGen Biotech) with a StepOnePlus Real-Time PCR System (Applied Biosystems, Inc., Carlsbad, CA, USA). Quantitative real-time PCR primer sequences used in this study are shown in **Table 1** (12, 13). Reactions were performed in triplicate in three separate experiments. Relative gene expression was normalized to EpCAM.

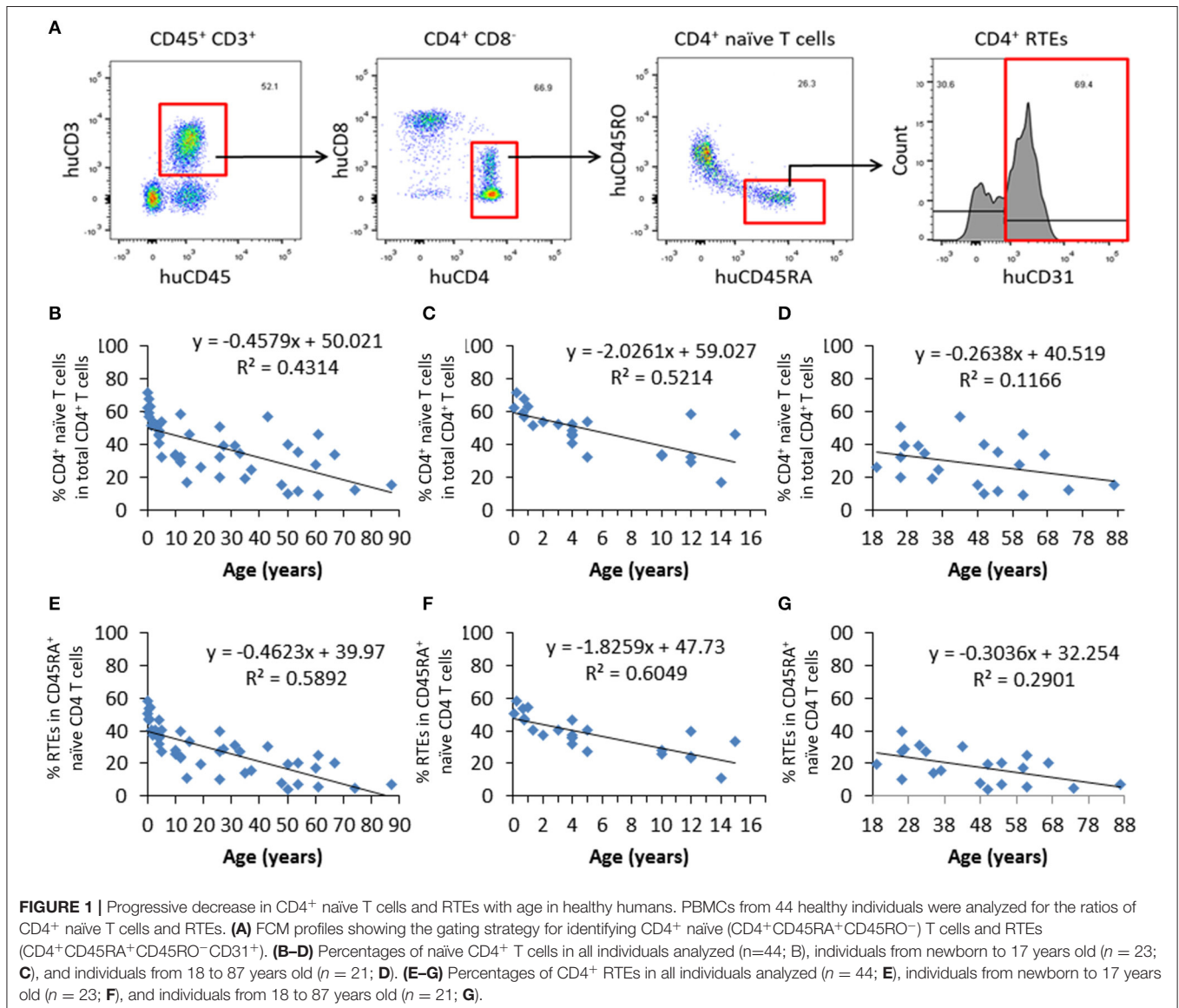
Real-Time PCR for Relative TREC Expression

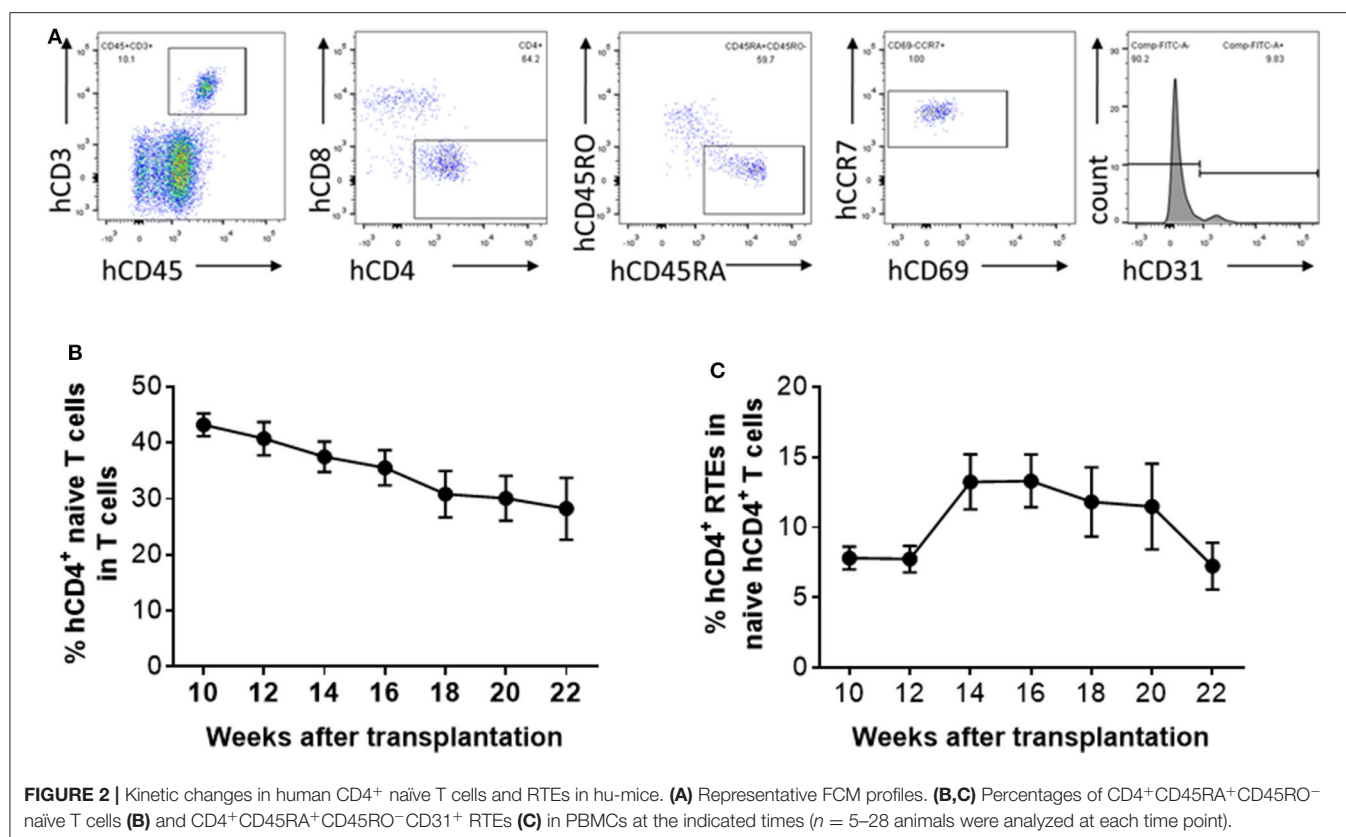
DNA was purified from human PBMCs and humanized mouse PBMCs and spleen cells using the QIAamp DNA

Blood Mini Kit according the manufacturer's instructions (Qiagen, Hilden, Germany). Quantitative real-time PCR was performed using AceQ Universal U+ Probe Master Mix V2 (Vazyme Biotech) with a StepOnePlus Real-Time PCR System (Applied Biosystems, Inc., Carlsbad, CA, USA). Quantitative real-time PCR primer and probe sequences used in this study are shown in **Tables 1, 2**. Reactions were performed in triplicate in three separate experiments. Relative gene expression was normalized to TRAC. (14)

Statistical Analysis

All data are presented as mean $\pm$ S.D and statistical significances between two groups were calculated using unpaired, non-parametric, two-tailed Student's *t*-test. Differences with a *P*-values of < 0.05 were considered statistically significant.





Statistical analysis was performed using GraphPad Prism 5 software and Microsoft Office Excel 2013.

RESULTS

Age-Associated Decrease in CD4⁺ naïve T Cells and Recent Thymic Emigrants in Healthy Humans

Blood samples from 44 healthy individuals (age 31 days to 87 years) were analyzed for the ratios of CD4⁺ naïve T cells and recent thymic emigrants (RTEs) by flow cytometry (FCM), in which CD4⁺ naïve T cells and RTEs were identified as CD4⁺CD45RA⁺CD45RO⁻ and CD4⁺CD45RA⁺CD45RO⁻CD31⁺, respectively (Figure 1A). We found that there was an age-associated decline in CD4⁺ naïve T cells (Figure 1B), while such tendency was not detected in the levels of total CD4⁺ T cells (Figure S1). Consistent with previous studies (15), the slope of decline was sharper for the individuals up to the end of puberty (from 1 month to 15 years old) than those 19–87 years old (Figures 1C,D). Similar to CD4⁺ naïve T cells, CD4⁺ RTEs also showed a progressive age-associated decrease (Figure 1E), with a much sharper decline for individuals before the end of puberty (Figures 1F,G). For the levels of both naïve T cells and RTEs, the coefficient of correlation with age was significantly higher for individuals aged from 1 month to 15 years old than those 19–87 years old ($p < 0.0001$ and $p < 0.0001$ for native T cells and RTEs, respectively). These

data are in agreement with previous reports (16, 17) confirming that CD4⁺ naïve T cells and CD4⁺ RTEs are adequate measures of thymic output potential (Figure 1).

Kinetic Changes in Human CD4⁺ naïve T Cells and RTEs in Humanized Mice

Peripheral blood was collected from hu-mice at 10, 12, 14, 16, 18, 20, and 22 weeks after human thymus and CD34⁺ cell transplantation, and analyzed for human CD4⁺ naïve T cells and CD4⁺ RTEs (Figure 2A). The percentage of CD4⁺ naïve T cells showed a relatively steady decline during the observation period of 22 weeks (Figure 2B). However, the kinetics of the naïve T cell levels were not coincident with the kinetics of the RTE levels. The percentage of CD4⁺ RTEs in T cells was low until 12 weeks, and then increased by nearly 2-fold between 12 and 14 weeks (Figure 2C). CD4⁺ RTE levels remained similar between 14 and 16 weeks, and declined progressively thereafter (Figure 2C). The low percentage of CD4⁺ RTEs early after humanization may reflect the recovery process of the transplanted thymic tissue. However, RTE levels in naïve CD4⁺ T cells in hu-mice were surprisingly low in general compared to those of humans (Figure 1). It has been reported that human T cells can divide in the periphery without losing their naïve phenotype (6). Thus, although the lower RTE levels in hu-mice may be due to inefficient T cell output of the human thymic graft, it may also be attributed to increased homeostatic proliferation of naïve human T cells in hu-mice.

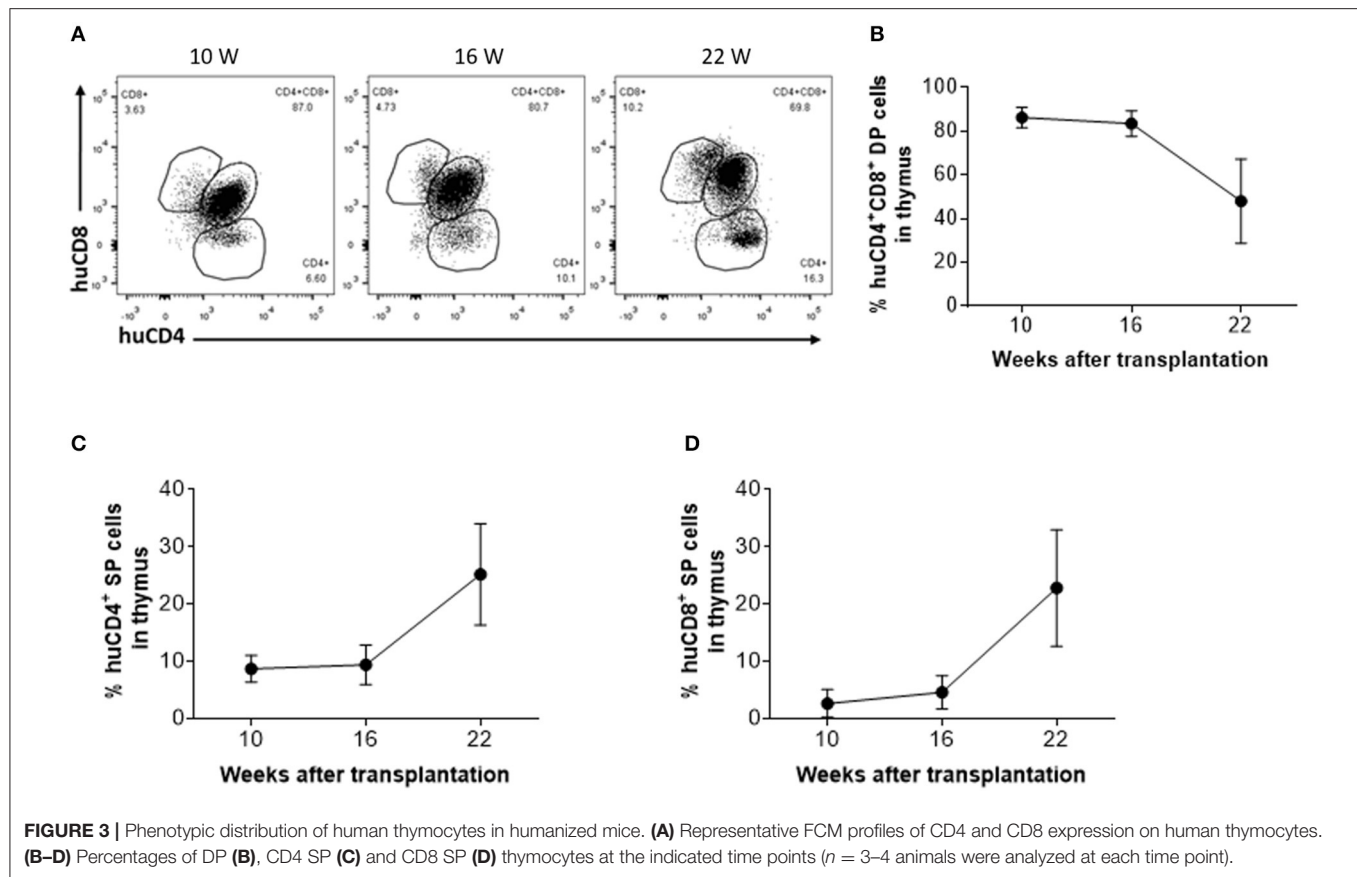


FIGURE 3 | Phenotypic distribution of human thymocytes in humanized mice. **(A)** Representative FCM profiles of CD4 and CD8 expression on human thymocytes. **(B–D)** Percentages of DP **(B)**, CD4 SP **(C)** and CD8 SP **(D)** thymocytes at the indicated time points ($n = 3–4$ animals were analyzed at each time point).

Aging of Human Thymic Grafts in Humanized Mice

To determine whether thymic aging is responsible for the observed decline in CD4⁺ RTE levels 16 weeks after thymic transplantation (**Figure 2C**), human thymic grafts were harvested 10, 16, and 22 weeks after transplantation and analyzed for thymopoiesis and involution (**Figure 3A**). Although a significant population of CD4⁺CD8⁺ double-positive (DP) thymocytes was detected in the human thymic grafts, a significant decline in the ratio of DP thymocytes, which was associated with an increase in CD4 single positive (SP) and CD8 SP thymocytes, was clearly detected between 16 and 22 weeks (**Figures 3B–D**).

The observed decline in DP thymocytes suggests an age-associated decrease in the function of the human thymic grafts in hu-mice. To confirm this, we next measured the number of EpCAM-positive thymic epithelial cells (TECs) in the human thymic grafts. Immunofluorescence staining revealed that EpCAM-positive TECs increased by ~3-fold between 10 and 16 weeks, and then declined progressively (by over 20-fold between 16 and 22 weeks; **Figure 4A**). In line with this observation, RT-qPCR analysis revealed a similar increase in EpCAM expression between weeks 10 and 16 and a substantial decline between weeks 16 and 22 (**Figure S2**). The early increase in TECs presumably reflects the recovery process of the human thymic graft. However, the later decrease in TECs is likely to be the consequence of thymic involution.

We also measured changes in adipose tissue mass, another indicator of age-related thymic involution (18). Oil Red O staining revealed a progressive increase in lipid-laden cells. Lipid-laden cells were not detected or barely detectable in the human thymic grafts at weeks 10 and 16, but a dramatic increase (over 10-fold) was detected at week 22 (**Figure 4B**). Together, our data suggest that significant age-related involution of human thymus occurred 16 weeks after transplantation.

Finally, real-time PCR was performed to measure expression of transcriptional factor forkhead box protein N1 (Foxn1) and autoimmune regulator (Aire) in the human thymic grafts. We found that *FOXN1* expression in the human thymic grafts harvested at week 10 was considerably lower than in the original human fetal thymus (prior to transplantation into mice; **Figure 5A** and **Figure S3A**). These results suggest that rapid aging or involution occurred following thymic transplantation into mice, and/or that the mouse xenogeneic environment may not provide optimal conditions to support human thymic epithelial cell (TEC) function. A further decrease in *FOXN1* expression was seen between 10 and 16 weeks (**Figure 5A**), likely reflecting an age-related thymic involution. Like *FOXN1*, there was a marked loss of *AIRE* expression in human thymic grafts by 10 weeks after transplantation, and *AIRE* expression remained low throughout the observation period of 22 weeks (**Figure 5B** and **Figure S3B**).

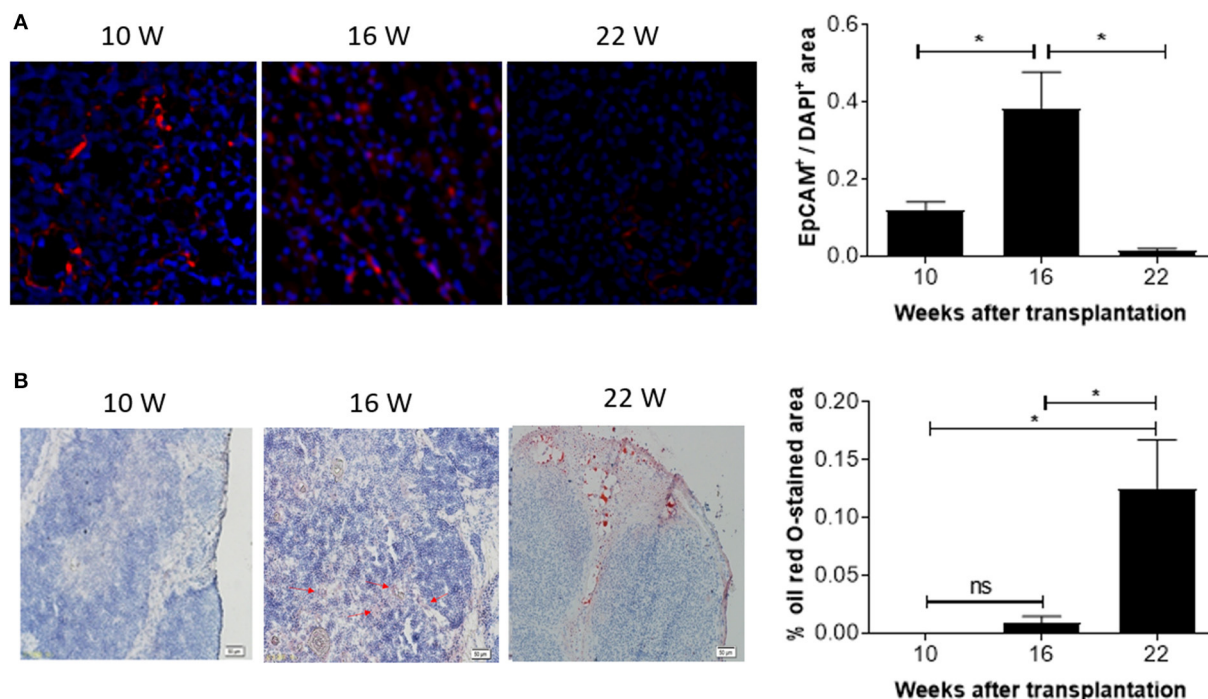


FIGURE 4 | Kinetic changes in EpCAM⁺ TECs and fat-deposition in human thymic grafts from hu-mice. Tissue sections of human thymic grafts prepared from hu-mice at weeks 10, 16, and 22 were stained with anti-EpCAM antibody and DAPI (**A**; $n = 3-6$ at each time point) or with Oil Red O and Hematoxylin (**B**; $n = 4-5$ animals at each time point). (**A**) Images of representative samples (left) and ratios of EpCAM⁺ to DAPI⁺ areas (right). (**B**) Images of representative samples (left) and percentages of Oil Red O-stained areas (right). Data are presented as mean ± SEM. * $p < 0.05$.

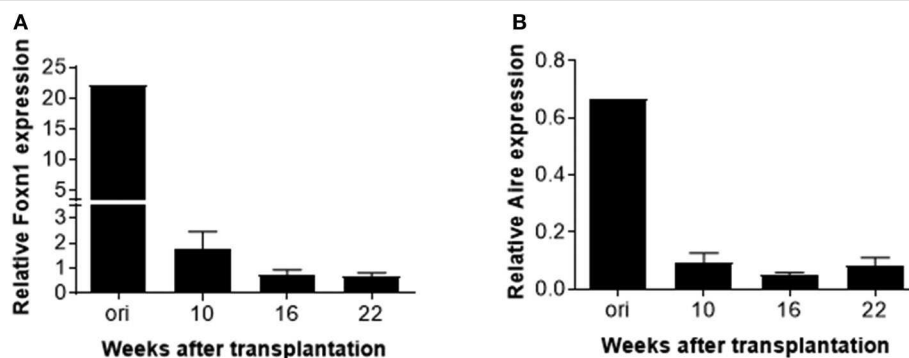


FIGURE 5 | *AIRE* and *FOXP1* expression in TECs in human thymic grafts. Human thymic grafts prepared from hu-mice at weeks 10, 16 and 22 were analyzed for *FOXP1*, *AIRE*, and EpCAM gene expression by real-time RT-PCR analysis. Relative expression levels (normalized to EpCAM) of *FOXP1* (**A**) and *AIRE* (**B**) genes shown as the mean ± SEM ($n = 3-4$ animals were analyzed at each time point).

Relative TREC Expression Between Human and Humanized Mice

TRECs are stable episomal, non-replicative DNA circles generated during T-cell receptor gene rearrangement in developing T-lymphocytes in the thymus. Therefore, TRECs are a marker for recently formed T-lymphocytes (19). We measured relative expression of TRECs in human (in different age ranges)

and hu-mice (10, 16, and 22 weeks after transplantation). In line with previous reports (20, 21), human PBMCs showed a clear age-dependent decline in TRECs (**Figure 6A**). In hu-mice, relative TREC expression showed a moderate increase from week 10 to week 16, followed by a significant decline at week 22 (**Figure 6B**), suggesting an age-associated decrease of thymic output after 16 weeks.

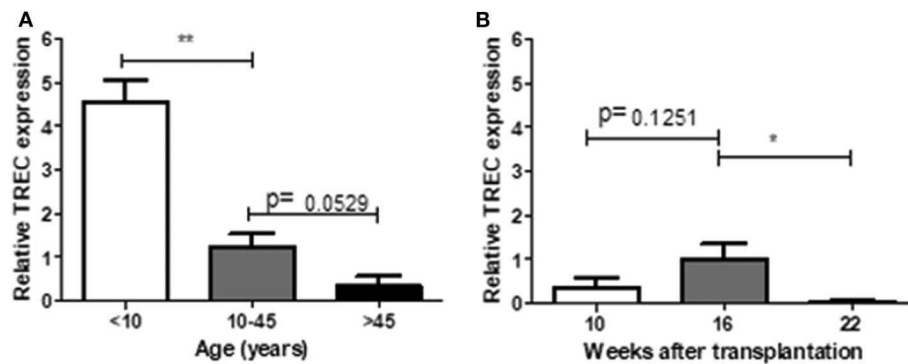


FIGURE 6 | Relative TREC expression between human and humanized mice. PBMCs prepared from 14 healthy individuals **(A)** and hu-mice at 10th, 16th, and 22nd week **(B)** were analyzed for the relative TREC expression by real-time PCR. Relative expression levels (normalized to TRAC) shown as the mean ± SEM ($n = 3-7$ human samples were analyzed at each age range, $n = 3-4$ animals were analyzed at each time point). Data are presented as mean ± SEM. * $p < 0.05$ and ** $p < 0.01$.

DISCUSSION

The thymus is a primary lymphoid organ where T cells are generated. Thymic involution, or the shrinking of the thymus with age, is an important factor that inhibits thymic output, leading to immune defects in vertebrates. Although thymic atrophy or involution has been extensively investigated in animal models, in humans the process and its underlying mechanisms remain relatively unknown, largely due to the lack of an *in vivo* model for longitudinal studies. The lack of a suitable *in vivo* model is also a bottleneck in developing interventions to treat thymic involution. In this study, we explored the potential to study human thymic involution in hu-mice constructed by transplantation of human thymic tissue and hematopoietic stem/progenitor cells into immunodeficient mice.

Thymic involution with age is a chronic process that results in progressive reduction in thymic output, leading to degeneration of adaptive immunity in aged individuals. In addition to age-related involution, the thymus may undergo acute (recoverable) atrophy under certain stress conditions, such as infection, pregnancy, and chemotherapy. In hu-mice, we found that human thymus first undergoes acute recoverable involution, followed by an age-related chronic form of involution. The first thymic involution was likely caused by transplantation stress, and recovered by week 14. Although the early recoverable atrophy (likely induced by transplantation stress) and the later age-related thymic involution were both associated with a decrease in TECs and RTEs, only the latter was associated with an increase in adipose tissue mass in the thymus. This is consistent with previous reports in mice that age-related thymic involution is closely associated with adipocyte expansion in the thymus (22).

FOXN1 appears in the sixth week of gestation in humans, serving as a key regulator of TECs development and differentiation in the fetal and adult thymus (23). Snapshot analysis of thymic tissues revealed that thymic FOXN1 transcription correlates with age, with a sharp decline after adolescence (23). Mouse studies revealed that the frequency of FOXN1⁺ TECs declines rapidly within a few weeks after birth and remains constant up to 2 years of age, while FOXN1

downregulation continues on a per-cell basis through the end of life (24). AIRE is another key transcription factor; it is expressed in mTECs at the final maturation stage and also shows downregulation with age (25, 26). We found that the expression levels of both FOXN1 and AIRE decreased substantially by 10 weeks after transplantation, when compared to the original fetal thymic tissue. Since FOXN1⁺ and AIRE⁺ TECs are highly sensitive to toxic or damaging insults (24, 27), both transplantation stress and aging factors may contribute to the observed early drop in FOXN1 and AIRE expression in the human thymic grafts. We acknowledge that this study did not investigate whether changes in these transcription factors are different among different types of TECs.

We noted that thymic output, as determined by measuring RTEs, is considerably lower in hu-mice compared to humans. It has been reported that human T cells can divide in the periphery without losing their naïve phenotype (6). Thus, although the lower RTE levels in hu-mice may be due to inefficient T cell output of the human thymic graft in the mouse environment, it may also be attributed to an increase in homeostatic proliferation of naïve human T cells in hu-mice. Regardless, further analysis of the thymic grafts indicated that the frequency of RTEs is correlated with thymic function, and thus can be used as an indicator of thymic involution. TREC is another measure of thymic function. It can be used to estimate thymic activity in peripheral blood because intrathymic and peripheral TREC values are correlated (20). We measure relative TREC expression of hu-mice at different time points after transplantation. Relative TREC expression was correlated the ratio of RTEs, with a moderate increase from week 10 to week 16, followed by a significant decrease at week 22. These results suggest that the human thymus went through a recovery process until 16 weeks after transplanted into the mice and began to involute thereafter.

In conclusion, this study suggests that human thymus in hu-mice undergoes both stress-induced acute thymic involution and age-related chronic thymic involution. We acknowledge that the hu-mouse host environment is not identical to that of a human. However, previous studies from our group and others have confirmed that human thymic grafts remain functional in

this hu-mouse model (8, 9, 28). Furthermore, the current study provides a proof-of-principle, that human thymic grafts retain their intrinsic mechanisms of thymic aging and susceptibility to stress-induced acute involution. Thus, this hu-mouse model offers a potentially useful *in vivo* system for understanding human thymic involution and testing therapeutic interventions.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Medical Ethics Committee of the First Hospital of Jilin University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin. The animal study was reviewed and approved by Institutional Animal Care and Use Committee of the First Hospital of Jilin University.

AUTHOR CONTRIBUTIONS

Q-YT, J-CZ, J-LG, and YL performed experiments and analyzed data. L-YY, XW, and Y-GY discussed the data and critically reviewed the manuscript. Q-YT and L-GS conceptualized the project and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2020.01399/full#supplementary-material>

Figure S1 | CD4+ T cells are unchanged with age in healthy humans. PBMCs from 44 healthy individuals were analyzed for the ratios of CD4+ T cells.

Figure S2 | EpCAM expression in human thymic grafts. Human thymic grafts prepared from hu-mice at weeks 10, 16, and 22 were analyzed for EpCAM gene expression by real-time RT-PCR analysis. Relative expression levels (normalized to β -actin) of EpCAM gene shown as the mean $\pm$ SEM ($n = 3$ –4 animals were analyzed at each time point).

Figure S3 | AIRE and FOXP1 expression in human thymic grafts. Human thymic grafts prepared from hu-mice at weeks 10, 16, and 22 were analyzed for AIRE and FOXP1 gene expression by real-time RT-PCR analysis. Relative expression levels (normalized to β -actin) of FOXP1 (A) and AIRE (B) genes shown as the mean $\pm$ SEM ($n = 3$ –4 animals were analyzed at each time point).

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Phosphate Transporter Profiles in Murine and Human Thymi Identify Thymocytes at Distinct Stages of Differentiation

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Ann Chidgey,
Monash University, Australia

Reviewed by:

Masahiro Ono,
Imperial College London,
United Kingdom
Anna Furmanski,
University of Bedfordshire,
United Kingdom

*Correspondence:

Naomi Taylor
taylor4@mail.nih.gov
Valérie S. Zimmermann
valerie.zimmermann@igmm.cnrs.fr

[†]These authors have contributed
equally to this work

[‡]These authors share
senior authorship

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Alice Machado^{1,2†}, Marie Pouzolles^{1†}, Sarah Gailhac², Vanessa Fritz², Marco Craveiro²,
Uriel López-Sánchez², Taisuke Kondo¹, Francesca Pala³, Marita Bosticardo³,
Luigi D. Notarangelo³, Vincent Petit⁴, Naomi Taylor^{1,2*‡} and Valérie S. Zimmermann^{1,2*‡}

¹ Pediatric Oncology Branch, Center for Cancer Research, National Cancer Institute, National Institutes of Health (NIH), Bethesda, MD, United States, ² Institut de Génétique Moléculaire de Montpellier, University of Montpellier, CNRS, Montpellier, France, ³ Laboratory of Clinical Immunology and Microbiology, Division of Intramural Research, National Institute of Allergy and Infectious Diseases, NIH, Bethesda, MD, United States, ⁴ Metafora-Biosystems, Paris, France

Thymocyte differentiation is dependent on the availability and transport of metabolites in the thymus niche. As expression of metabolite transporters is a rate-limiting step in nutrient utilization, cell surface transporter levels generally reflect the cell's metabolic state. The GLUT1 glucose transporter is upregulated on actively dividing thymocytes, identifying thymocytes with an increased metabolism. However, it is not clear whether transporters of essential elements such as phosphate are modulated during thymocyte differentiation. While PIT1 and PIT2 are both phosphate transporters in the SLC20 family, we show here that they exhibit distinct expression profiles on both murine and human thymocytes. PIT2 expression distinguishes thymocytes with high metabolic activity, identifying immature murine double negative (CD4⁻CD8⁻) DN3b and DN4 thymocyte blasts as well as immature single positive (ISP) CD8 thymocytes. Notably, the absence of PIT2 expression on RAG2-deficient thymocytes, blocked at the DN3a stage, strongly suggests that high PIT2 expression is restricted to thymocytes having undergone a productive TCR β rearrangement at the DN3a/DN3b transition. Similarly, in the human thymus, PIT2 was upregulated on early post- β selection CD4⁺ISP and TCR $\alpha\beta$ ⁻CD4^{hi}DP thymocytes co-expressing the CD71 transferrin receptor, a marker of metabolic activity. In marked contrast, expression of the PIT1 phosphate importer was detected on mature CD3⁺ murine and human thymocytes. Notably, PIT1 expression on CD3⁺DN thymocytes was identified as a biomarker of an aging thymus, increasing from $8.4 \pm 1.5\%$ to $42.4 \pm 9.4\%$ by 1 year of age ($p < 0.0001$). We identified these cells as TCR $\gamma\delta$ and, most significantly, NKT, representing $77 \pm 9\%$ of PIT1⁺DN thymocytes by 1 year of age ($p < 0.001$). Thus, metabolic activity and thymic aging are associated with distinct expression profiles of the PIT1 and PIT2 phosphate transporters.

Keywords: thymus, phosphate transporters, glucose transporters, metabolism, human, mice, aging

INTRODUCTION

The thymus is critical for the differentiation of T lymphocytes, promoting the generation of a pool of functionally competent T cells that provide protection against pathogens and tumors while maintaining self-tolerance. T cell differentiation in the thymus arises from progenitor cells that are derived from bone marrow hematopoietic stem cells (HSC) [reviewed in (1–5)]. Once progenitor cells enter into the thymus, the thymic environment generally results in their acquisition of a short-lived T cell precursor phenotype [as has been previously shown for common lymphocyte progenitors; (6, 7)]. Signals mediated through Notch1 (8), IL-7R (8), stem cell factor receptor (SCFR) (9) and CXCR4 (10) regulate the survival and proliferation of early T cell progenitors prior to the β -selection checkpoint. β -selection allows the differentiation of only those precursor T cells with productive, in-frame rearrangements of the TCR β locus. In mice, β -selection occurs at a precise stage, within CD4⁺CD8⁺ double negative (DN) 3 (CD25⁺CD44⁺) thymocytes (9) whereas in humans, this step occurs in CD4⁺ intermediate single positive (ISP) cells as well as in double positive (DP) CD4⁺CD8 α ⁺CD8 β ⁺ thymocytes (11, 12). This TCR rearrangement results in a proliferative burst of murine as well as human thymocytes (13, 14), requiring an increased metabolism that is dependent on PI3K signaling downstream of Notch, IL-7, CXCR4 and the TCR (15–21).

Our understanding of the metabolic changes that regulate T cell differentiation and proliferation has generally focused on the roles of sugars, amino acids and fatty acids (22–24). However, it is clear that oxygen tension and pH balance, as well as minerals, vitamins, and electrolytes also participate to the metabolic crosstalk that occurs during T cell development. Indeed, the uptake of calcium (25–27) and iron (28) have long been known to be critical for T cell differentiation and more recently, potassium has been shown to regulate the effector function of T lymphocytes (29, 30).

The metabolic needs of proliferating cells are generally procured by an augmented entry of nutrients into the cells. Cell surface transporter expression is a rate-limiting step in nutrient entry and the induction of glucose, glutamine and other amino transporters are required for optimal T cell proliferation and effector function (31–36). In the context of differentiation within the thymus, we and others have demonstrated an upregulation of the GLUT1 glucose transporter on metabolically active murine as well as human thymocytes (14, 37, 38). Critically, the absence of thymic GLUT1 has been shown to result in a 60–70% loss of thymocytes (37). Interestingly though, the SLC1A5 glutamine transporter does not appear to be required for murine thymocyte differentiation (31–36, 39), possibly due to a redundancy with other glutamine transporters. However, the transferrin receptor, mediating iron delivery into differentiating thymocytes via transferrin, distinguishes metabolically active thymocytes and is required for thymocyte differentiation (14, 28, 40). Thus, several metabolite transporters play critical roles in the potential of an early thymocyte progenitor to differentiate to a mature T lymphocyte.

Notably though, the role of mineral transporters in T cell differentiation have not been extensively studied. Phosphorous is the sixth most abundant element in the human body and its anion phosphate is the most abundant, accounting for 1% of total body weight (41, 42). Humans take up approximately 16 mg/kg of phosphate per day from their diet via Na(+)-dependent SLC34 transporters that are expressed in the kidney and small intestine (42, 43). Transport into other cell types is regulated by the Na(+)-dependent PiT1/SLC20A1 and PiT2/SLC20A2 transporters (44, 45). Furthermore, more recently, an inorganic phosphate exporter, XPR1/SLC53A1 has also been identified (46). PiT1 and PiT2 share 60% sequence homology and in addition to a high affinity for P(i) (47, 48), they serve as retroviral receptors for the gibbon ape leukemia virus and koala endogenous retrovirus (49, 50) as well as the amphotropic murine leukemia virus (51, 52), respectively. PiT1 and PiT2 expression as well as their heterodimerization have been shown to be modulated by phosphate concentrations, at least in certain cell types (46, 53, 54).

While phosphate uptake by PiT1 and PiT2 are essential for bone homeostasis (42, 43), it is now clear that PiT1 and PiT2 play critical roles in multiple cell types. PiT1 is critical for survival as its deletion results in embryonic lethality at E12.5 due to severe anemia (55) while mutations in both PiT2 and XPR1 are associated with primary familial brain calcification (Fahr's disease) (56–59). Conditional deletions of PiT1 have revealed an important role for this transporter in cell proliferation and development (60–63), erythroid and B cell differentiation (64, 65) as well as inflammation and wound healing (66, 67). However, the thymic expression profiles of neither the PiT1 nor the PiT2 phosphate transporter are known. We hypothesized that the PiT1 and PiT2 transporters would display differential expression profiles, potentially allowing the identification of thymocytes with distinct maturation states. Here, we identify PiT2 as a marker of metabolically active thymocytes in both the murine and human thymus. In contrast, PiT1 distinguishes a CD3⁺DN subset of DN thymocytes that increases with age. We identify the majority of these cells as NKT thymocytes, thereby serving as a potential marker of age-related thymic atrophy. Thus, phosphate transporter expression identifies distinct thymocyte subsets, correlating murine and human thymocyte differentiation and identifying thymus populations that change as a function of age.

METHODS AND MATERIALS

Mice and Cell Lines

C57Bl/6 mice were purchased from Charles River and maintained under specific pathogen-free conditions in the IGMM animal facility (Montpellier, France) or the NCI animal facility (Bethesda, MD). *Rag2*^{−/−} mice as well as Pmel-1 (B6.Cg-Thy1a/Cy Tg(TcraTcrb)8Rest/J) mice on a C57Bl/6 background were purchased from Jackson Laboratories. Unless indicated, mice were between 4 and 8 weeks of age. In indicated experiments, mice were 2 weeks, 8 weeks, or 1 year of age. All experiments were approved by the local animal facility

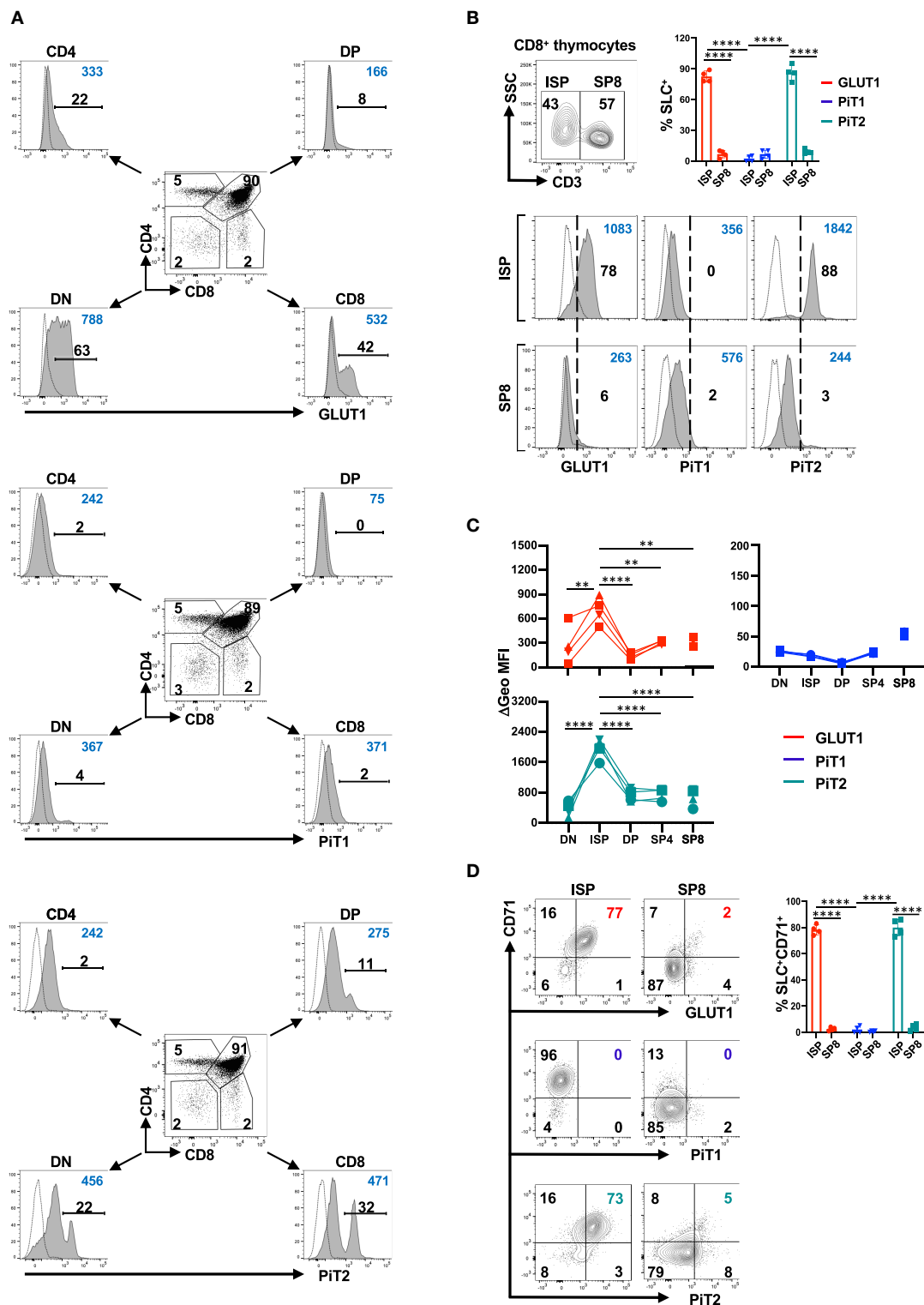


FIGURE 1 | The PiT2 phosphate transporter is co-expressed with GLUT1 and CD71 transporters in the murine thymus. **(A)** Surface expression of the GLUT1 glucose transporter as well as PiT1 and PiT2 phosphate transporters were evaluated on murine thymocytes as a function of their CD4/CD8 profiles. Mean fluorescence intensity (MFI) on double negative (DN), double positive (DP), and single positive (SP) CD4 and CD8 thymocytes is shown for each transporter. Specific staining is shown in gray histograms and non-specific FMO staining is shown as dotted line histograms. The percentages of positively stained cells (black) and geometric MFIs (blue) in each histogram are indicated. Data are representative of one of eight individual thymi. **(B)** The distribution of CD3⁺CD8⁺ intermediate single positive (ISP) (Continued)

FIGURE 1 | thymocytes and mature CD3⁺ SP8 thymocytes within the CD8⁺ thymocyte gate was evaluated as a function of CD3 staining and a representative dot plot is presented (top). GLUT1, PiT1 and PiT2 staining within the ISP and SP8 gates are presented relative to non-specific staining and percent staining (black) as well as geometric MFIs (blue) are indicated in each histogram (bottom). Data are representative of one of eight individual thymi and quantification of transporter (solute carrier, SLC) expression in ISP8 and SP8 thymocytes is shown. **(C)** Delta geometric MFIs of GLUT1 (red), PiT1 (blue) and PiT2 (green) staining in DN, ISP, DP, SP4, and SP8 subsets are presented for four individual thymi. **(D)** The phenotype of ISP and SP8 thymocytes was evaluated as a function of GLUT1, PiT1, and PiT2 transporters and the CD71 transferrin receptor and the percentages of cells in the different quadrants are indicated (left panel). Quantification of the percentages of solute carrier (SLC) ⁺ cells co-expressing CD71 in ISP and SP8 thymocytes are presented ($n = 4$, right panel). ** $p < 0.01$; **** $p < 0.0001$.

institutional review boards. Animal care and experiments were performed in accordance with National Institutes of Health (NIH) and French national guidelines.

HAP1 cells, harboring a near-haploid genome, were derived from the chronic myelogenous leukemia (CML) cell line KBM-7 (68), and a gene edited HAP1 line with a deletion of SLC20A2/PiT2 was obtained from Horizon Discovery, as described (69). BxPC3, a pancreatic cancer cell line obtained from the ATCC, was used for shRNA-mediated knockdown of PiT1, as previously described (61).

Thymocyte Preparation

Murine thymi were removed after sacrifice. Human thymi were removed during corrective cardiac surgery of pediatric patients aged 4 months–7 years at La Timone Hospital or from the pathology department of the Children's National Medical Center in Washington, DC following cardiothoracic surgery from children with congenital heart disease, as the thymic tissue is routinely removed and discarded to gain adequate exposure of the retrosternal operative field. Use of these thymus samples for this study was determined to be exempt from review by the NIH Institutional Review Board in accordance with the guidelines issued by the Office of Human Research Protections. All tissues were processed after isolation. Tissue was transferred to a sterile 10 mm² tissue culture dish. Single cell thymocyte suspensions were generated by physical disruption of tissue and filtration through 70 μ m nylon screens.

Flow Cytometry

Murine thymocytes were stained with the following directly conjugated mAbs; CD3, CD25, CD8, CD71, c-Kit, CD44, CD11b, CD19, Ter119, Gr1, PD1, CD4, TCR $\gamma\delta$ and NK1.1 (from Becton Dickinson, BioLegend or eBiosciences). Human thymocytes were stained with the following directly conjugated mAbs; CD8 α , CD4, CD33, CD19, CD56, GlyA, TCR $\alpha\beta$, and CD71. Cells that were not thymocytes were eliminated with a dump including mAbs against CD19, Gr1, CD11b, and Ter119 for murine samples and CD19, CD33, and GlyA for human samples. Soluble ligands derived from the receptor binding domains (RBDs) of the HTLV, koala endogenous retrovirus (Ko-RBD) and mouse amphotropic-MLV (A-RBD) retrovirus were used to detect expression of their respective receptors; GLUT1, PiT1, and PiT2, as previously described (46, 70, 71) (Metafora biosystems). Stained cells were analyzed by flow cytometry (FACS-Canto II or LSR II-Fortessa, Becton Dickinson, San Jose, CA) and $1\text{--}2 \times 10^6$ events/sample were routinely acquired. The gating strategies for human and murine thymocytes are shown in **Supplementary Figure 1**. When indicated, molecules

of equivalent soluble fluorochrome (MESF) were evaluated by Quantum MESF beads (Bang Laboratories, Fisher Indiana). Delta geometric mean fluorescence intensity (dGeo MFI) was calculated as the Geometric MFI of specific staining minus the Geometric MFI of the FMO. Data analyses were performed using Diva (BD Biosciences), and FlowJo Mac v.10.6.2 software (Tree Star).

Statistical Analyses

Data were analyzed using GraphPad software version 8 (Graph Pad Prism, La Jolla, CA) and p -values were calculated using unpaired t -tests and one- or two-way ANOVA (Tukey's multiple comparison test), as indicated. P -values for comparisons of all conditions in the different figure panels are presented in the figure legends.

RESULTS

Surface Expression of the GLUT1 and PiT2 Transporters Characterizes ISP Murine Thymocytes

GLUT1 has previously been shown to be expressed on metabolically active murine and human thymocytes, with cell surface levels exhibiting significant differences as compared to mRNA or even intracellular protein levels (14, 37). This is critical as it is the rapid translocation of solute carriers from intracellular stores to the cell surface that reflects the cell's response to extracellular stimuli; this has been extensively described for the insulin-mediated induction of GLUT1/GLUT4 to the cell surface within minutes of stimulation (72, 73). However, measurements of the cell surface expression of multipass transmembrane proteins such as SLC2A1/GLUT1 and the phosphate importers (PiT) have been hindered by a paucity of reliable antibodies, due to sequence conservation and poor immunogenicity of extracellular loops (74). Here, we utilized tagged receptor binding domain (RBD) fusion proteins from the HTLV (H2-RBD), Koala endogenous retrovirus (Ko-RBD) and mouse amphotropic MLV retrovirus (A-RBD) to specifically detect expression of GLUT1, PiT1, and PiT2, respectively, as previously shown (46, 69–71, 74–77). The specificity of H2-RBD binding to GLUT1 has previously been reported (74, 76) and the specificity of Ko-RBD and A-RBD to PiT1 and PiT2, respectively, were evaluated as a function of shRNA-mediated knockdown and CRISPR gene editing (**Supplementary Figure 2**) (69).

Within the murine thymus, analyses of immature DN, DP, and single positive CD4 and CD8 thymocytes revealed the presence of subpopulations of GLUT1⁺ and PiT2⁺ cells within the DN and CD8⁺ thymocyte gates (**Figure 1A**). In contrast, distinct subsets

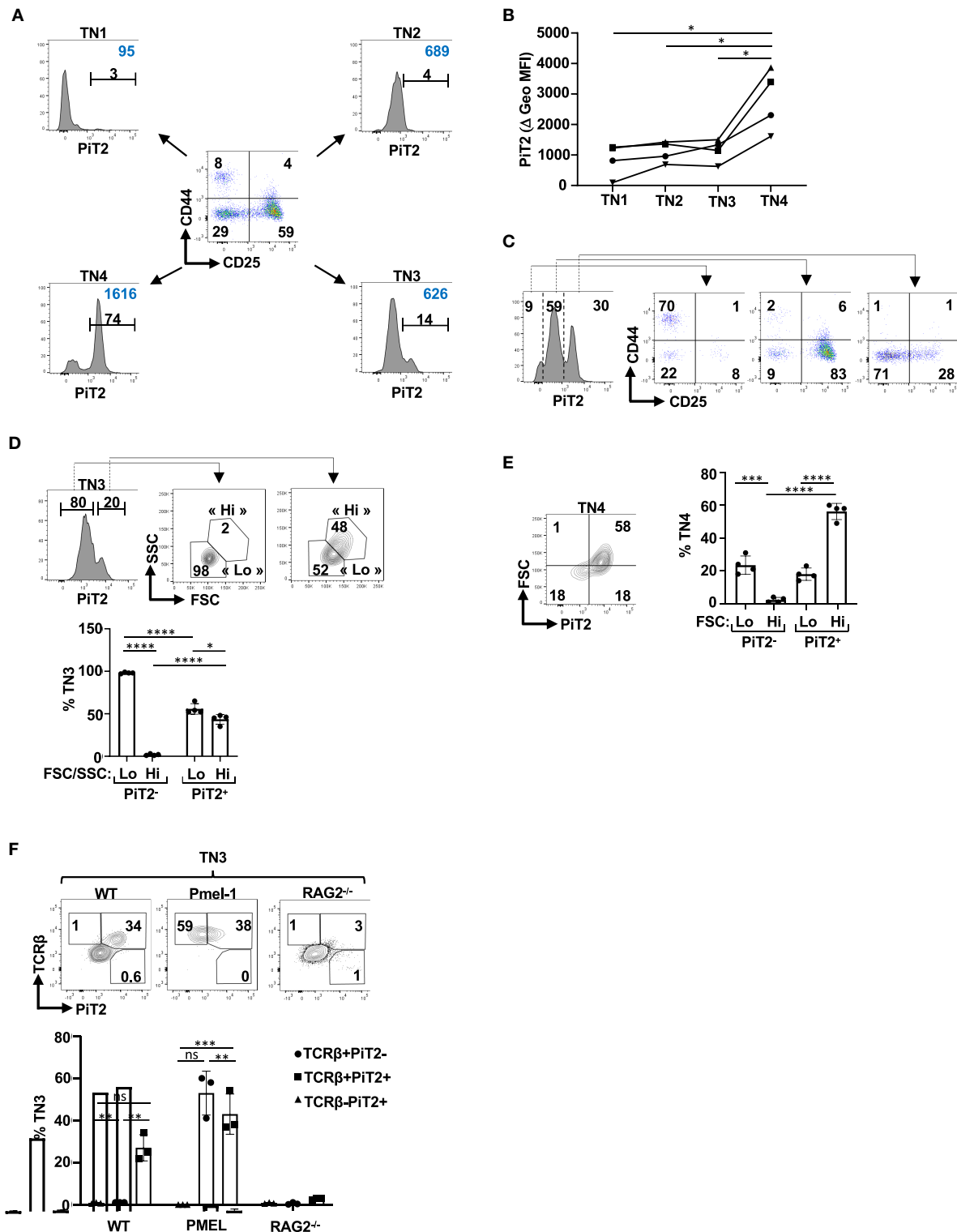


FIGURE 2 | β selection of TN thymocytes results in the induction of the Pit2 phosphate transporter. **(A)** Profiles of TN thymocytes were evaluated as a function of their CD44/CD25 profiles and a representative dot plot showing the TN1 (CD44⁺CD25⁻), TN2 (CD44⁺CD25⁺), TN3 (CD44⁻CD25⁺), and TN4 (CD44⁻CD25⁻) subsets is presented. Histograms of Pit2 expression in each TN subset is shown. The percentages of positively staining cells (black) and geometric MFI (blue) are presented. **(B)** Pit2 staining in TN1, TN2, TN3 and TN4 thymocytes from four individual thymi are presented as a function of their geometric MFI. **(C)** CD44/CD25 profiles of TN thymocytes distinguished as a function of their Pit2 levels are presented. **(D)** TN3 thymocytes were distinguished on the basis of Pit2 expression and FSC/SSC

(Continued)

FIGURE 2 | profiles of PiT2-negative and PiT2⁺ cells are presented. The percentages of FSC/SSC-lo and FSC/SSC-hi subsets, used to distinguish TN3a and TN3b thymocytes, are presented (top). Quantification of the percentages of cells within the FSC/SSC-lo and FSC/SSC-hi gates are presented for PiT2-negative and PiT2-positive TN3 thymocytes ($n = 4$, bottom). **(E)** Expression of PiT2 in TN4 thymocytes was evaluated as a function of FSC and a representative plot is presented (right). Quantification of the percentages of FSC-lo and FSC-hi TN4 thymocytes are shown as a function of PiT2 expression ($n = 4$, right). **(F)** Representative histograms showing PiT2 staining on TN3 thymocytes from WT, Pmel-1 TCR transgenic and *Rag2*^{-/-} thymocytes are shown as a function of TCR β expression (top panels). Quantification of the percentages of PiT2⁺ cells in the TCR β ⁻ and TCR β ⁺ TN3 subsets are shown for WT, Pmel-1, and *Rag2*^{-/-} mice. Statistical differences were evaluated by a 2-tailed unpaired *t*-test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

of PiT1⁺ cells were not easily detected (**Figure 1A**). To evaluate the identity of the GLUT1⁺ and PiT2⁺ CD8 thymocyte subset, we assessed whether these transporters were expressed in the immature CD3⁻ population or the mature CD3⁺ population. As shown in **Figure 1B**, the vast majority of GLUT1⁺ as well as PiT2⁺ cells were immature ISP thymocytes while PiT1 expression on CD8 thymocytes was not detected ($p < 0.0001$). Moreover, the geometric mean of GLUT1 and PiT2 expression decreased significantly between ISP8 and SP8 thymocytes, from 699 ± 167 to 330 ± 24 and 1970 ± 277 to 680 ± 237 , respectively ($p = 0.003$ and $p < 0.0001$, respectively, **Figures 1B,C**). Expression of the CD71 transferrin receptor is often a marker of a cell's metabolic activity and it has been shown to be co-expressed with GLUT1 in the human thymus (14, 28). We therefore evaluated transporter profiles as a function of CD71 expression in ISP8 as compared to SP8 thymocytes. Notably, CD71 expression was largely confined to the ISP subset and was co-expressed by both GLUT1⁺ and PiT2⁺ thymocytes ($78 \pm 4\%$ vs. $3 \pm 1\%$, $p < 0.0001$ and $80 \pm 6\%$ vs. $4 \pm 2\%$, $p < 0.0001$, respectively; **Figure 1D**). In contrast, PiT1 was not detected on the CD71⁺ISP subset ($2 \pm 2\%$). Together, these results demonstrate a strong association of GLUT1 and PiT2 on metabolically active murine ISP thymocytes (**Figure 1D**).

While GLUT1⁺ and PiT2⁺ subsets were not clearly discerned in the CD4 gate, we specifically evaluated regulatory T cell (Treg) thymocytes in the Foxp3⁺CD4⁺ subset. Approximately 50% of Foxp3⁺ thymocytes were CD25⁻ as compared to CD25⁺, representing immature and mature Treg subsets, respectively (78). Interestingly, while similar percentages of CD25⁻Foxp3⁺ and CD25⁺Foxp3⁺ thymocytes expressed GLUT1 ($25 \pm 8\%$ of CD25⁻ and $29 \pm 7\%$ of CD25⁺, respectively), PiT2 expression was significantly higher in CD25⁺ Treg ($20 \pm 7\%$ vs. $39 \pm 10\%$, $p < 0.01$, **Supplementary Figure 3**). The significance of PiT2 expression on CD25⁺Foxp3⁺ thymocytes remains to be determined.

PiT2 Expression Distinguishes Metabolically Active TN Thymocyte Subsets

The differentiation of CD4⁻CD8⁻CD3⁻ (TN) thymocytes has been historically divided into four subsets on the basis of CD44 and CD25 expression, with TN1, TN2, TN3 and TN4 subsets defined as CD44⁺CD25⁻, CD44⁺CD25⁺, CD44⁻CD25⁺ and CD44⁻CD25⁻, respectively (1, 2, 4). Given the heterogeneity of PiT2 expression in the DN thymocyte subsets (**Figure 1A**), we specifically evaluated PiT2 levels in each of the TN subsets. As shown in **Figure 2A**, PiT2 levels were heterogeneous even within specific TN subsets but high levels were detected mainly

within TN3 and TN4 subsets, evaluated as a function of percent positively stained thymocytes and the MFI of staining. Indeed, MFI increased significantly between TN3 and TN4 thymocytes ($p < 0.05$, **Figure 2B**); the vast majority of TN1 thymocytes were PiT2-negative, whereas TN3 and TN4 thymocytes were detected in both the PiT2-intermediate and PiT2-high gates (**Figure 2C**).

β -selection, the first checkpoint in thymocyte development, occurs at the TN3 stage. Only TN3 thymocytes expressing a functional pre-TCR proliferate and progress to the DP stage of thymocyte differentiation (13, 79). Those TN3 and TN4 cells that have undergone a productive TCR β gene rearrangement have been historically distinguished from the majority of TN3/TN4 cells with random TCR β gene rearrangements by their size, monitored as a function of forward and side scatters (FSC/SSC) (13). Furthermore, TN3/TN4 thymocytes are activated through PI3K/Akt signaling (15, 17, 20, 21) resulting in the induction of metabolic transporters such as the GLUT1 glucose transporter (14) as well as chemokine receptors such as CXCR4 (14, 21). We therefore evaluated whether expression of PiT2 in TN thymocytes allows a discrimination of β -selection. Notably, $98 \pm 1\%$ PiT2-negative TN3 thymocytes were characterized as FSC/SSC-lo and $<2\%$ as FSC/SSC-hi ($p < 0.0001$, **Figure 2D**). Thus, PiT2 negative cells appear to represent a TN3a profile (FSC-lo). Interestingly though, within the PiT2⁺ gate, TN3 thymocytes exhibited both FSC/SSC-lo and FSC/SSC-hi profiles, with $56 \pm 6\%$ and $43 \pm 4\%$, respectively. Furthermore, within the TN4 gate, PiT2 distinguished FSC-lo and FSC-hi cells. Within the PiT2-negative TN4 subset, only $2 \pm 1\%$ were FSC-hi while similarly to the TN3 population, PiT2⁺ TN4 thymocytes were both FSC-lo and FSC-hi ($18 \pm 4\%$ and $56 \pm 5\%$, respectively, **Figure 2E**). Thus, even though the percentages of PiT2⁺ cells that are FSC-hi are significantly higher than those that are FSC-lo ($p < 0.0001$), the presence of an FSC-lo subset suggests PiT2 expression might allow for a more rigorous identification of TN3 and TN4 thymocyte subsets that have undergone TCR β gene rearrangement. Specifically, PiT2 expression may be a marker of those TN thymocytes that have responded to TCR/CXCR4 signaling.

To directly address this point, we evaluated PiT2 profiles within the TN subset of WT, RAG2-deficient and Pmel-1 thymi. In the absence of RAG2, thymocytes are blocked at the TN3 stage of differentiation as they are not able to rearrange a functional pre-TCR (80, 81) while Pmel-1 thymocytes, harboring a transgenic TCR against the gp100 melanoma antigen, do not need to undergo TCR rearrangement for their selection (82) (**Supplementary Figure 4**). Importantly, while a delineated peak of PiT2⁺TCR β ⁺ TN3 thymocytes was detected in WT thymi ($27 \pm 6\%$), this was not the case in RAG2-deficient thymi

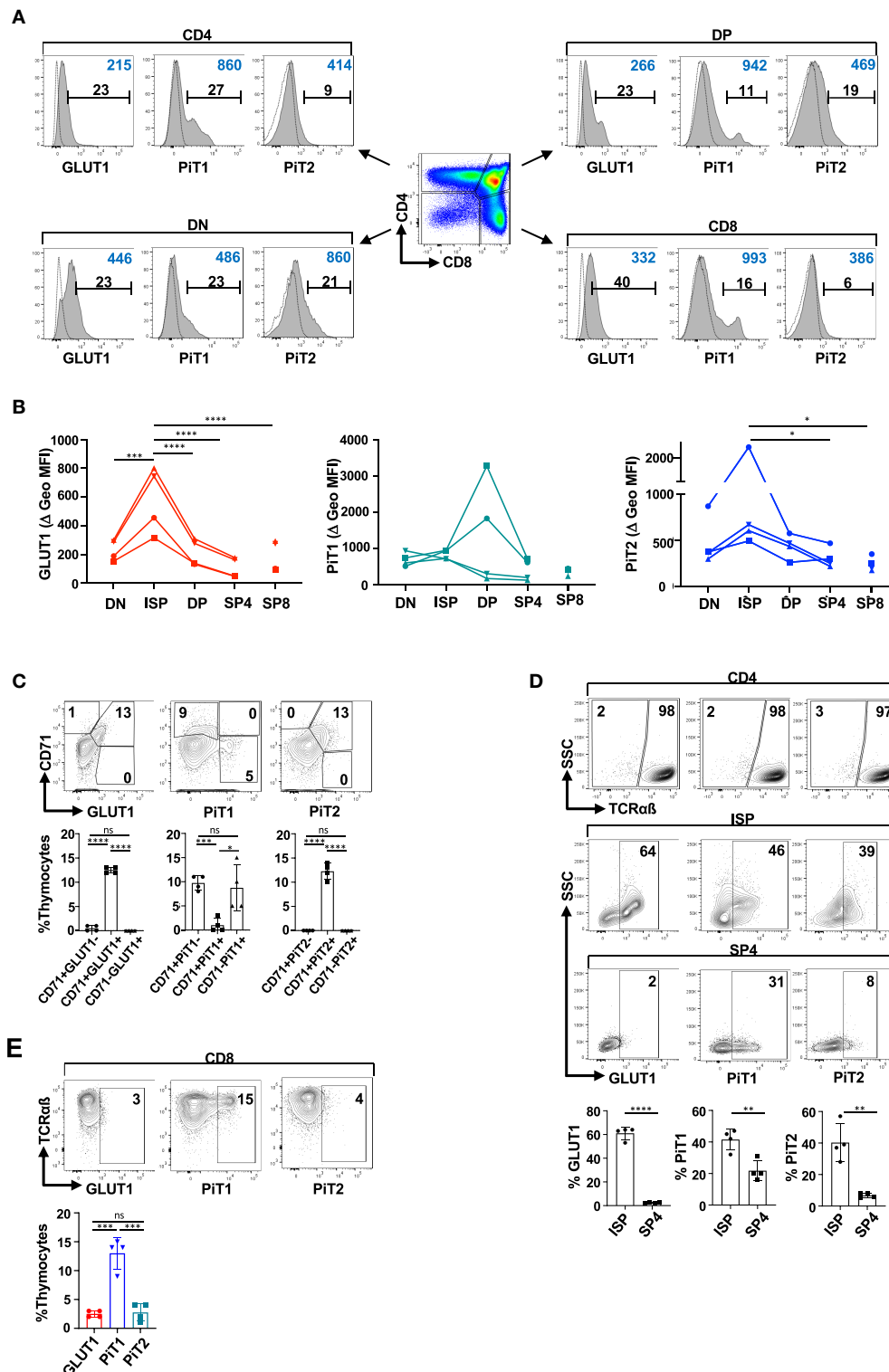


FIGURE 3 | PIT2 expression identifies metabolically active TCR $\alpha\beta^-$ thymocytes in the human thymus. **(A)** Surface expression of GLUT1, PIT1, and PIT2 transporters was evaluated on freshly isolated human thymocytes and representative histograms are shown as a function of their CD4/CD8 profiles. Specific staining is shown in gray histograms and non-specific FMO staining is shown as dotted line histograms. The percentages of positively stained cells (black) and geometric MFIs (blue) in each histogram are indicated. **(B)** Delta geometric MFIs of GLUT1, PIT1, and PIT2 staining in DN, DP, ISP, SP4, and SP8 subsets are presented from four analyses. **(C)** Expression of GLUT1, PIT1, and PIT2 were evaluated as a function of the CD71 transferrin receptor and representative dot plots are presented (top). The relative (Continued)

FIGURE 3 | percentages of thymocytes co-expressing the indicated transporter are quantified and means $\pm$ SD are shown (bottom). **(D)** Immature CD4⁺ ISPs and mature SP4 thymocytes were differentiated on the basis of TCR $\alpha\beta$ expression (top). Expression of GLUT1, PiT1, and PiT2 within TCR $\alpha\beta$ ⁺ ISP and TCR $\alpha\beta$ ⁺ SP4 thymocytes are presented (middle plots). Quantification of transporters within ISP and SP4 subsets are shown (bottom). **(E)** GLUT1, PiT1, and PiT2 expression were evaluated in SP8 thymocytes as a function of TCR $\alpha\beta$ expression. Representative dot plots are presented. Statistical differences were evaluated by a 2-tailed unpaired *t*-test. **p* < 0.05; ***p* < 0.01; ****p* < 0.001; *****p* < 0.0001.

($3 \pm 1\%$, $p < 0.0001$; **Figure 2F**). Furthermore, in the Pmel-1 thymus where all TN3 thymocytes were TCRb⁺, a similar percentage of thymocytes as in WT mice expressed the PiT2 phosphate importer (**Figure 2F**). Conversely, PiT2 expression was not detected on TCRb⁺ TN3 thymocytes in either WT or RAG2^{-/-} mice (**Figure 2F**). Collectively, these data reveal the importance of the PiT2 phosphate transporter in identifying TCRb⁺ TN3 cells that have undergone a productive TCR β gene rearrangement.

PiT1 and PiT2 Expression Profiles Characterize Distinct Subsets of Human Thymocytes

T cell differentiation in both mouse and humans occurs in the thymus, proceeding through discrete developmental stages [reviewed in (83)]. While many of the same markers have been used to characterize murine and human differentiation, including CD4 and CD8, some differ and the ISP stage in humans is characterized as CD3⁻CD4⁺CD8⁻ (as compared to CD3⁻CD4⁻CD8⁺ in mice) (83). It was therefore important to determine whether the profiles of the PiT1 and PiT2 phosphate transporters in human thymocytes were similar to that detected in mice. Interestingly, while PiT1 was detected in only very low levels in the murine thymus, equivalent percentages of human thymocytes expressed surface GLUT1, PiT1, and PiT2 transporters ($11 \pm 1\%$, $10 \pm 2\%$, and $10 \pm 2\%$, respectively; **Supplementary Figure 5**). However, it is important to note that the profiles of expression on DN, DP, SP4, and SP8 thymocyte subsets was distinct (**Figure 3A**).

GLUT1 and PiT2 profiles were similar in the murine thymus, reflecting metabolically active thymocytes that had undergone a productive TCR β gene rearrangement (**Figure 2**). Indeed, the MFI of GLUT1 and PiT2 staining was significantly higher on ISPs than on other subsets, decreasing from 579 ± 232 to 109 ± 71 and 1103 ± 828 to 306 ± 111 between ISP4 and SP4, respectively ($p > 0.0001$ and $p > 0.05$, **Figure 3B**). Thus, we assessed whether GLUT1 and PiT2 expression in the human thymus was associated with expression of the CD71 transferrin receptor, as in the murine thymus (**Figure 1**). Indeed, as shown in **Figure 3C**, almost all CD71⁺ thymocytes were GLUT1⁺ and PiT2⁺, representing $13 \pm 1\%$ and $12 \pm 2\%$ of total thymocytes, respectively (only $0.5 \pm 0.6\%$ and 0% of all CD71⁺ thymocytes were GLUT1⁻ or PiT2⁻, respectively). Interestingly though, only $1 \pm 2\%$ of PiT1⁺ thymocytes co-expressed CD71 while $9 \pm 5\%$ of PiT1⁺ thymocytes were CD71-negative (**Figure 3C**). Furthermore, within the CD4SP population, GLUT1⁺ and PiT2⁺ cells were almost exclusively within the ISP thymocyte subset ($61 \pm 5\%$ and $40 \pm 7\%$) as compared to the mature SP4 subset ($3 \pm 1\%$ and $7 \pm 2\%$, respectively; $p < 0.0001$ and

$p < 0.01$, **Figure 3D**). Thus, similarly to the murine thymus, both GLUT1 and PiT2 expression are detected on metabolically active thymocyte subsets that have been signaled following TCR β gene rearrangement.

In contrast with GLUT1 and PiT2, PiT1 expression was detected on mature SP4 as well as SP8 thymocytes. Within the SP8 thymocyte subset, $13 \pm 3\%$ of TCR^{Hi}SP8 thymocytes were characterized by high surface PiT1 expression, and conversely, $<3\%$ of these cells expressed GLUT1 or PiT2 ($3 \pm 1\%$ and $3 \pm 2\%$, respectively, **Figure 3E**). These data point to important similarities in metabolite transporter expression in human thymocytes, especially as concerns those transporters that function as biomarkers for metabolic activity. However, there are also differences in transporter profiles between the species, especially as regards PiT1 levels, that remain to be evaluated.

NKT Thymocytes Expressing the PiT1 Transporter Are a Biomarker of Thymic Aging

Thymic function declines with age, due to changes in the thymic environment itself as well as to a decrease in the entry of BM-derived precursors that support thymopoiesis (84, 85). As such, we evaluated the impact of age on thymocyte subsets and more specifically, on the expression of phosphate transporters on these subsets. As previously shown (86–88), thymocyte numbers (**Figure 4A**) as well as c-Kit⁺ early thymic precursors decreased with age, diminishing from $1.7 \pm 0.2\%$ to $0.7 \pm 0.3\%$ between 2 weeks and 1 year of age ($p < 0.0001$; **Figure 4B**).

Given the decreased thymopoiesis in aging mice, we hypothesized that phosphate transporter profiles in the thymus would change with age. While we did not detect significant changes in the overall percentages of PiT2⁺ thymocytes as a function of age (**Figure 4C**), the percentages of PiT2 expression in the mature CD3⁺ subset was decreased by 1 year of age (from $10 \pm 3\%$ to $5 \pm 2\%$, $p < 0.01$, **Figure 4C**). However, in marked contrast, the percentages of PiT1 expression increased significantly with age, from $0.1 \pm 0.03\%$ to $0.5 \pm 0.1\%$ ($p < 0.0001$; **Figure 4D**). Moreover, at all ages, the expression of PiT1 was significantly higher in CD3⁺ subset as compared to the CD3⁻ subset, with a difference of 75 ± 19 to 12 ± 4 at 1 year of age ($p < 0.0001$, **Figure 4D**). Within the CD3⁺PiT1⁺ thymocyte subset we evaluated the percentages of DN, DP and SP thymocytes and found that the percentages of PiT1⁺CD3⁺ thymocytes that were DN increased significantly over time, from $14.1 \pm 2.8\%$ to $48.9 \pm 1.8\%$, respectively ($p < 0.0001$; **Figure 4E**). Thus, increased PiT1 expression on CD3⁺ thymocytes is a marker of thymic aging.

We next specifically monitored PiT1 expression in the CD3⁺DN thymocyte gate and found that with age, the

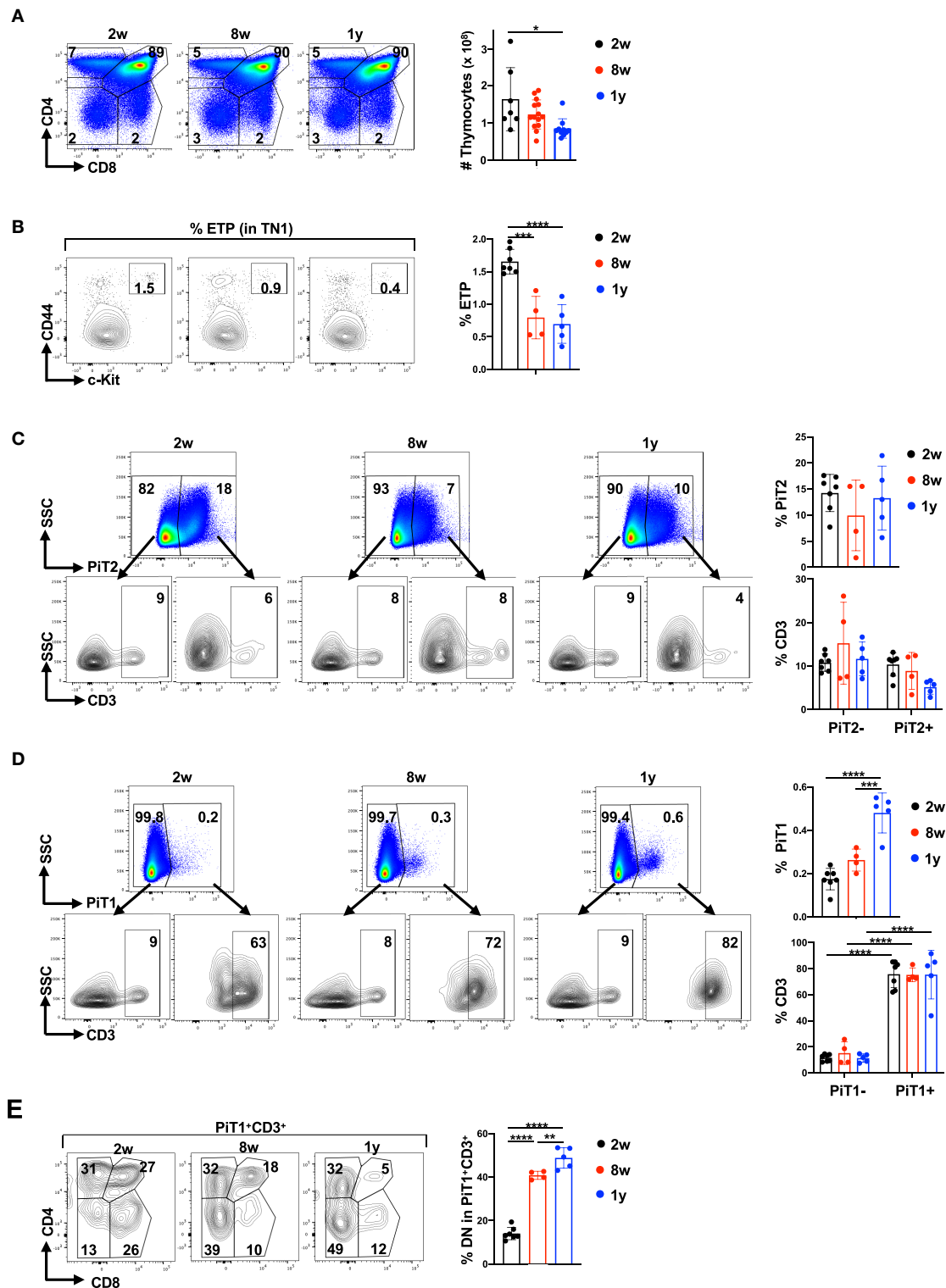


FIGURE 4 | PIT1 but not PIT2 expression increases in CD3⁺DN thymocytes as a function of age. **(A)** Total thymocyte numbers were evaluated in mice at 2 weeks (2w), 8 weeks (8w), and 1 year (1y) of age. Representative CD4/CD8 profiles (top left) and quantification of total thymocytes (top right) are presented ($n = 7-14$ per age group). **(B)** The percentages of early thymic progenitors (ETP) within the TN1 gate were evaluated by CD44 and c-Kit expression and representative dot plots (left) as well as quantifications (right) are presented. **(C)** The percentages of thymocytes expressing PIT2 were evaluated as a function of age and representative plots are presented (top). The percentages of CD3⁺ thymocytes within the PIT2⁻ and PIT2⁺ subsets are presented (bottom) and quantifications are shown ($n = 4-7$ per age group). **(D)** Representative plots (top) and quantifications (bottom) are presented. The percentages of CD3⁺ thymocytes within the PIT1⁻ and PIT1⁺ subsets are presented (bottom) and quantifications are shown ($n = 4-7$ per age group). **(E)** Representative plots (top) and quantifications (bottom) are presented. The percentages of CD3⁺ thymocytes within the PIT1⁺CD3⁺ and PIT1⁺CD3⁻ subsets are presented (bottom) and quantifications are shown ($n = 4-7$ per age group). (Continued)

FIGURE 4 | group, left). **(D)** The percentages of thymocytes expressing PIT1 were evaluated as a function of age (top) and CD3⁺ and CD3⁺ subsets representative plots (bottom) as well as quantifications (right) are presented. **(E)** The phenotype of PIT1⁺CD3⁺ thymocytes was evaluated as a function of their CD4/CD8 profiles (left) and the percentages of DN thymocytes within the PIT1⁺CD3⁺ subset is quantified for the different age groups (right). Statistical differences were evaluated by a one-way ANOVA test. **p* < 0.05; ***p* < 0.01; ****p* < 0.001; *****p* < 0.0001.

percentages of PIT1⁺ thymocytes increased massively—from $8.4 \pm 1.5\%$ at 2 weeks to $22.6 \pm 5.6\%$ at 8 weeks and further increasing to $42.4 \pm 9.4\%$ at 1 year (*p* < 0.0001; **Figure 5A**). To further characterize these PIT1⁺CD3⁺DN thymocytes, we monitored expression of the CD25, CD71, PD1, and CD44 markers. Notably, PIT1⁺CD3⁺DN thymocytes were CD25-negative and were not likely to be metabolically active as they did not express the CD71 transferrin receptor (**Figure 5B** and **Supplementary Figure 6**). Indeed, in accord with a reduced metabolism in the aging thymus, the percentage of PIT1⁺CD3⁺DN expressed CD71 decreased from $26 \pm 11\%$ to $9 \pm 9\%$ between 2 weeks and 1 year of age (*p* < 0.05, **Supplementary Figure 6**). Furthermore, these cells are unlikely to represent autoreactive thymocytes that have not undergone clonal deletion because this subset of CD3⁺DN thymocytes has been shown to be PD1⁺ (89) and the PIT1⁺CD3⁺DN subset did not express detectable levels of PD1 (**Figure 5B**). Importantly, all thymocytes in the PIT1⁺CD3⁺DN subset co-expressed CD44, increasing from $6 \pm 1\%$ to $38 \pm 11\%$ between 2 weeks and 1 year of age (*p* < 0.0001, **Figure 5B**).

CD44 expression is a marker in CD3⁺DN thymocytes of both $\gamma\delta$ and NKT thymocytes (90–94). The percentages of thymocytes harboring a $\gamma\delta$ TCR was not altered with age ($5.5 \pm 1.4\%$) but the percentages of PIT1⁺ TCR $\gamma\delta$ thymocytes did increase, from $2.8 \pm 1.6\%$ to $8.4 \pm 3.5\%$ between 2 weeks and 1 year of age (*p* = 0.004; **Figure 5C**). Importantly though, PIT1 expression was detected on <15% of all $\gamma\delta$ thymocytes. We therefore evaluated the evolution of NKT cells and found that they were significantly augmented in 1 year old mice, increasing from $4 \pm 0.4\%$ to $13 \pm 8\%$ (*p* < 0.05, **Figure 5D**). Moreover, despite the overall decrease in thymocyte numbers (**Figure 4A**), the number of PIT1⁺NKT increased from 3×10^5 to 8×10^5 (*p* < 0.05, **Figure 5D**) and NKT cells did not express either GLUT1 or PIT2 (**Supplementary Figure 7A**). We therefore evaluated whether NKT thymocytes accounted for a significant percentage of PIT1⁺DN thymocytes—while they accounted for $43 \pm 11\%$ of this subset at 8 weeks of age, the percentage increased to $77 \pm 9\%$ by 1 year of age (*p* < 0.001, **Figure 5E**). Thus, while markers of autoreactivity and metabolic activity decreased with age, PIT1 expression on $\gamma\delta$ thymocytes and more notably on NKT cells appears to serve as phenotypic biomarkers of an aging thymus.

DISCUSSION

Metabolite transporters of the SLC superfamily comprise more than 400 genes, regulating the uptake of nutrients, vitamins, neurotransmitters, elements and ions [reviewed in (95)]. As such, they are likely to be critical components of all cell fate decisions, governing survival, proliferation, differentiation and

function. While the impact of SLCs that transport nutrients such as glucose and amino acids have been extensively evaluated in T cell differentiation and function (35–37, 39, 96, 97), studies of anion-transporting SLCs have been more limited. Moreover, it is critical to evaluate cell surface expression of metabolite transporters as their induction is often regulated by translocation from intracellular compartments rather than by increased transcription and/or translation (98–100). Here, we show that PIT1/SLC20A1 and PIT2/SLC20A2, SLCs that have been characterized as ubiquitous “housekeeping” phosphate importers, are only expressed at the cell surface of a small percentage of thymocytes. Moreover, the two importers exhibit distinct cell surface expression profiles. In contrast with PIT1, PIT2 was detected on thymocytes with high metabolic activity, concomitant with expression of the GLUT1 and CD71 transporters as well as high FSC/SSC profiles. This profile, in both the murine and human thymus, identified immature thymocytes that had undergone a productive TCR β rearrangement. Indeed, in the absence of RAG2, PIT2 expression was not upregulated, and its induction in TCR β ⁺ DN thymocytes shows that it serves as a biomarker of the DN3b/DN4 switch. Expression of PIT1, on the other hand, increased with age, exhibiting a significant increase on CD3⁺ DN thymocytes. We identified these cells to be PIT1⁺CD3⁺NK1.1⁺, revealing the association of PIT1⁺NKT thymocytes with thymic aging.

While we did not evaluate the impact of dynamic changes in PIT1 and PIT2 levels on phosphate uptake, it is important to note that these importers can regulate cell function and differentiation in a phosphate uptake-independent manner. Specifically, *Pit1*-null MEFs do not exhibit altered uptake of phosphate (60) and conditional deletion of PIT1, while significantly affecting erythroid and B cell differentiation in mice, does not decrease phosphate uptake in these cells (65). Moreover, while decreased PIT1 expression in tumor cells results in attenuated proliferation and tumor growth, these effects are independent of phosphate transport activity (61). That being said, depletion of extracellular phosphate is associated with an induction of both PIT1 and PIT2 on transformed cells (57) and it is therefore interesting to speculate that intrathymic phosphate levels decrease upon thymic involution, as a function of age. In that regard, intrathymic phosphate levels may themselves impact thymic involution—deletion of the *Klotho* gene, mediating the role of FGF-23 in the control of phosphate (101), has been found to result in premature thymic aging (102).

While the mechanisms via which PIT1 impacts on cell cycle and hematopoietic lineage differentiation have still not been fully elucidated, it appears that the ERK1/2 pathway is involved in this process. Specifically, increases in extracellular phosphate have been shown to induce ERK1/2 signaling and upregulation of cyclin D1 (103, 104). While ERK1/2 phosphorylation has

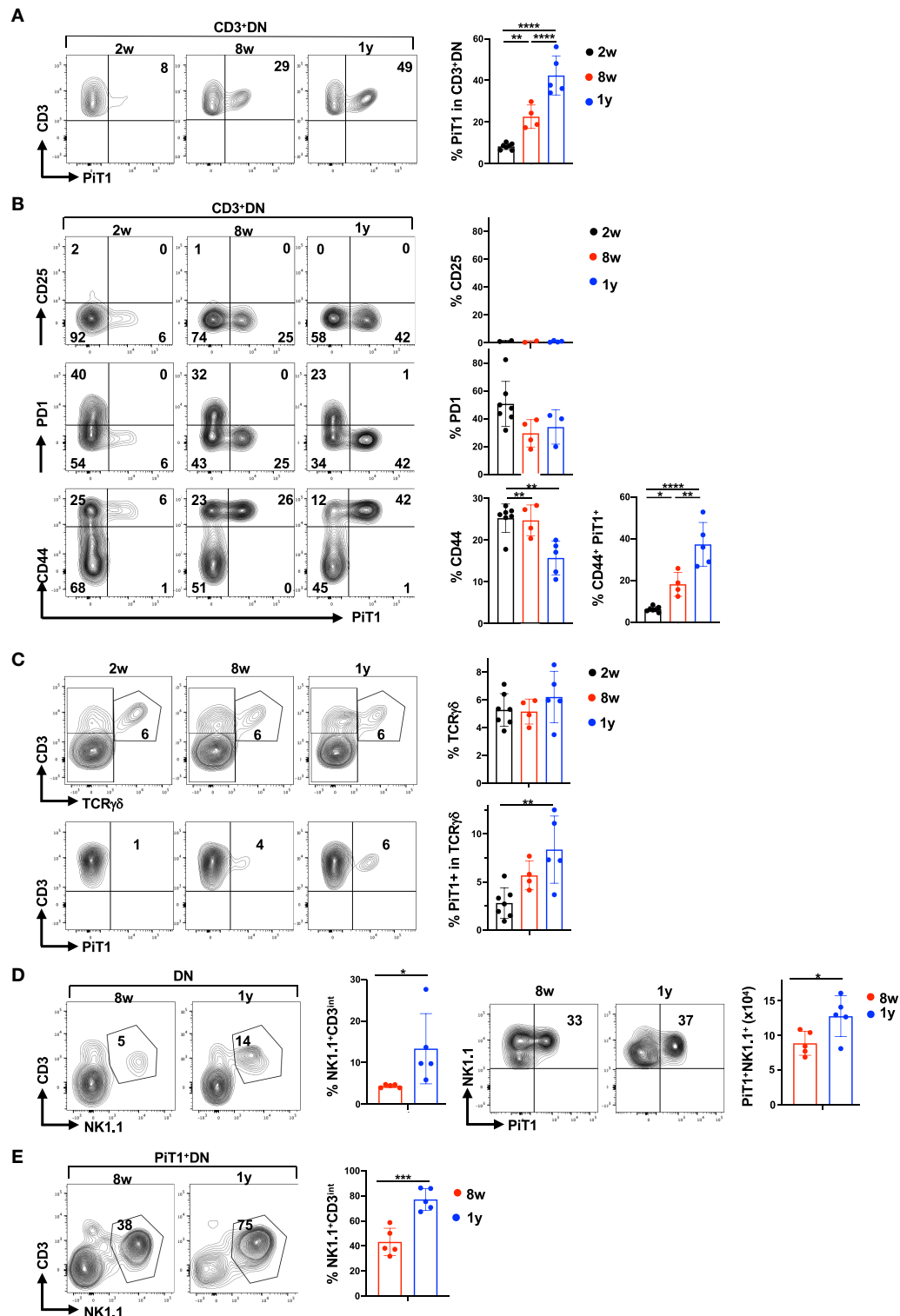


FIGURE 5 | Thymic aging is associated with a significant increase in PIT1⁺ NKT thymocytes. **(A)** PIT1 expression in CD3⁺DN thymocytes is shown as a function of age (left) and quantifications in the different age groups are presented (right). **(B)** PIT1⁺CD3⁺DN thymocytes were evaluated as a function of CD25, CD71, PD1, and CD44 expression and representative profiles are presented (left). Quantifications of each staining profile in the different age groups are shown (right). **(C)** TCRγδ thymocytes were evaluated in the DN population by CD3/TCRγδ staining (top) and the percentages of PIT1 expressing TCRγδ T cells are presented (bottom). Quantifications are presented at the right. **(D)** NKT and NK thymocytes were evaluated in the DN population as a function of their CD3/NK1.1 staining profiles and representative plots are presented at the right. **(E)** PIT1⁺DN thymocytes were evaluated as a function of their CD3/NK1.1 staining profiles and representative plots are presented at the right. *(Continued)*

FIGURE 5 | 8 weeks and 1 year are shown (left). Expression of PiT1 in NK1.1⁺ thymocytes are presented (left) and quantifications in 8 weeks and 1 year old mice are shown ($n = 5$). **(E)** The percentages of NKT thymocytes within the PiT1⁺DN population are shown as a function of CD3/NK1.1 staining (left) and quantifications for 8 weeks and 1 year mice are shown ($n = 5$, right). Statistical differences were evaluated by a one-way ANOVA test. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

been linked to phosphate uptake, more recent data suggest that activation of this pathway is mediated by a phosphate-regulated heterodimerization of PiT1 and PiT2, independently of phosphate uptake. Indeed, deficient ERK1/2 phosphorylation in PiT-1 or PiT2-depleted cells was rescued by transport-deficient PiT mutants (53). Thus, the impact of phosphate sensing by PiT1 and PiT2, can potentially modulate thymocyte differentiation in a manner that is independent of phosphate uptake. Moreover, as robust ERK activation has been shown to be associated with thymocyte death whereas a low/brief ERK activation is associated with positive selection (105–107), it will be of much interest to study the impact of PiT2 on ERK activation in thymocytes and determine its potential role in positive selection.

Our finding that PiT1 expression profiles are altered in the aging thymus adds to our understanding of thymic involution and alterations in thymocyte subsets as a function of age. The percentage of CD3⁺DN cells expressing the PiT1 transporter increased by 5-fold between 2 weeks and 1 year of age. One subset of CD3⁺DN thymocytes represents DP thymocytes with an autoreactive TCR wherein strong TCR signaling directs them to a CD3⁺CD44⁺PD1⁺DN stage of differentiation (89). However, consistent with a decreased differentiation and metabolism in older mice, the percentages of these CD3⁺DN thymocytes expressing PD1 or the CD71 transferrin receptor was significantly diminished and they did not represent the PiT1⁺ subset. Interestingly though, ~50% of PD1⁺CD44⁺CD25⁺CD3⁺DN thymocytes, a subset that was previously shown to accumulate in the thymus of older mice (85, 108–111), exhibited high levels of PiT1 by 1 year of age. CD44⁺CD3⁺DN thymocytes can represent $\gamma\delta$ or NKT thymocytes (90–94) and as such, we evaluated both these subsets. While $\gamma\delta$ thymocytes did not increase with age, NKT cells were significantly augmented. This NKT subset was specific to the CD3⁺DN population as expression of NK1.1 on CD3^{int}CD4SP thymocytes was low ($6 \pm 5\%$), did not change with age, and did not exhibit high PiT1 expression (**Supplementary Figure 7B**). The significance of high PiT1 levels in this CD3⁺DN thymocyte subset is at present unknown but it will be of much interest to determine whether PiT1 expression on $\gamma\delta$ or NKT thymocytes alters their function and/or can serve as a biomarker of thymic aging. Furthermore, the profile of PiT1 expression in responses to thymic insults such as irradiation and chemotherapy, resulting in alterations in the extracellular thymus niche, is not known. Indeed, the impact of toxic insults, and conversely, thymic regeneration strategies such as IL-22 or chemical castration (84, 112, 113), may alter the dynamic expression profiles of PiT1 as well as PiT2.

Differences in the profiles of the PiT1 and PiT2 transporters in the murine and human thymus suggest that they play distinct roles in thymocyte differentiation and proliferation. Conditional loss of PiT1, albeit with suboptimal deletion of the floxed allele,

did not inhibit murine thymocyte differentiation (65). These data are consistent with the low-level expression of PiT1 that we detected in the thymi of young mice, and especially in immature thymocytes. In contrast with homozygous PiT1 deletion which results in embryonic lethality (55), mice with deleted PiT2 are viable and develop brain calcifications (56). The pathophysiology of these mice resembles the pathology of patients with primary familial brain calcification (PFBC). Indeed, 40% of patients with this neurodegenerative disease harbor mutations in PiT2 (58). While thymocyte differentiation has not been evaluated in these mice, the high level of PiT2 in immature thymocytes progressing through the β -selection checkpoint suggests that thymocyte differentiation would be negatively affected, as detected in mice with a conditional deletion of GLUT1 (37). In support of a potential role for PiT2 in early thymus differentiation, deletion of genes that alter thymocyte metabolism, such as apoptosis-inducing factor (AIF) (114) and SdhD (115), have resulted in a block in thymocyte differentiation, at the DN3/DN4 transition. Finally, it will be of interest to determine whether patients with PFBC exhibit decreased thymocyte differentiation, evaluated as a function of TRECs, especially given the association in several genetic immunodeficiencies with CNS involvement (116).

Our data highlight distinct profiles of PiT1 and PiT2 in murine and human thymus, revealing developmental specificities in the expression of these phosphate transporters. Furthermore, increased PiT1 levels on CD3⁺DN thymocytes, identified in majority as NKT thymocytes, was found to be a biomarker of thymi of >1 year of age. Our study also identifies PiT2/SLC20A2 as a member of the family of metabolite transporters that characterizes immature thymocytes with high metabolic activity and the upregulation of PiT2 at the beta-selection checkpoint is conserved between mouse and man. The list of metabolite transporters that are induced at the beta-selection checkpoint, now comprising PiT2, GLUT1 and CD71, is likely to grow—pointing to an extensive metabolic crosstalk regulating the proliferation and differentiation of immature thymocytes.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal studies were reviewed and approved by the Languedoc-Rousillon Animal Care Committee, Montpellier, France and the NCI Animal Care Committee, Bethesda, MD.

AUTHOR CONTRIBUTIONS

VZ and NT conceived and supervised the study. AM, MP, SG, VF, MC, VZ, and NT were involved in study design. AM, MP, SG, VF, MC, UL-S, VP, and VZ performed experiments. TK, FP, MB, and LN contributed to sample preparation and quality control. AM, MP, VZ, and NT wrote the manuscript and all authors critically reviewed the manuscript. All authors participated in data analysis and discussions.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2020.01562/full#supplementary-material>

Supplementary Figure 1 | Gating strategies for evaluation of murine and human thymocyte subsets. **(A)** Gating strategy for murine thymocytes showing FSC/SSC profiles, followed by SSC-W/SSC-H, FSC-W/FSC-H and Live-Dead analysis. Non-T lineage thymocyte subsets were eliminated by a << Dump >> staining with anti-CD11b, -CD19, -Ter119, and -Gr1 mAbs. CD4/CD8 profiles as well as

profiling of DN thymocytes and CD8⁺ thymocytes (CD3-ISP and CD3⁺ SP8) are shown. **(B)** Profiles for live freshly isolated CD4-selected T cells. For evaluation of naïve and memory CD4 T cells, CD45RA/CD45RO profiles are presented. **(B)** Gating strategy for human thymocytes showing FSC/SSC profiles, followed by SSC-W/SSC-H, FSC-W/FSC-H and Live-Dead analysis. Representative CD4/CD8 profiles and evaluation of CD4⁺ thymocytes (CD3-ISP and CD3⁺ SP4) are shown. The percentages of cells in each gate are indicated.

Supplementary Figure 2 | Specificity of retroviral envelope binding domain binding to PIT1 and PIT2 phosphate transporters. **(A)** BxPC3 cells were transduced with a PIT1 specific shRNA and PIT1 expression in parental and shRNA-transduced cells was monitored with the Koala retroviral envelope receptor binding domain (Ko-RBD, Metafora Biosystems). A representative histogram with non-specific binding in gray, parental in blue and shRNA knockdown in red is shown (left) and quantification from three independent experiments was evaluated by molecules of equivalent soluble fluorochrome (MESF). **(B)** The specificity of the amphotropic MLV RBD for evaluation of PIT2 expression was evaluated as previously reported (69), monitoring binding of the amphi-delta SU construct (A-RBD) in the parental haploid HAP1 cell line as well as following CRISPR-mediated knockout of PIT2 (HAP1-DPIT2). Representative histograms showing non-specific (line) and specific (gray) staining are presented. Note that there is an auto-fluorescence in HAP1-DPIT2 cells relative to the non-specific staining.

Supplementary Figure 3 | GLUT1 and PIT1 expression profiles on Foxp3⁺ thymocytes. Expression of GLUT1 and PIT2 was evaluated in CD25⁻Foxp3⁺ and CD25⁺Foxp3⁺ thymocytes (left) and representative histograms are shown (middle). The percentages of cells in each gate are indicated. Quantification of GLUT1 and PIT2 detection in the CD25⁻Foxp3⁺ and CD25⁺Foxp3⁺ subsets are presented ($n = 5$, left).

Supplementary Figure 4 | Profiles of TN thymocytes in WT, Pmel-1 and RAG2^{-/-} mice. Expression profiles of TN thymocytes from WT, Pmel-1 and RAG2^{-/-} mice, distinguishing TN1, TN2, TN3, and TN4 thymocytes as a function of CD44⁺CD25⁻, CD44⁺CD25⁺, CD44⁻CD25⁺, and CD44⁻CD25⁻ staining, are presented.

Supplementary Figure 5 | Detection of GLUT1, PIT1, and PIT2 on human thymocytes. The detection of GLUT1, PIT1, and PIT2 on human thymocytes was evaluated and representative histograms, indicating the percentages of positively stained cells, are shown (left). Quantification of percentages with horizontal lines presenting means $\pm$ SD are presented ($n = 4$ from three independent thymi, representative of five independent thymus specimens; right).

Supplementary Figure 6 | Lack of CD71 transferrin receptor expression on PIT1⁺CD3⁺DN thymocytes. Expression of PIT1 and CD71 was evaluated on CD3⁺DN thymocytes from 2 weeks, 8 weeks, and 1yo mice. Representative dot plots are presented (left) and quantification of CD71 expression in the different age groups is shown (right).

Supplementary Figure 7 | PIT1 but not GLUT1 or PIT2 is expressed on NK1.1⁺ thymocytes. **(A)** Expression of GLUT1, PIT1, and PIT2 were evaluated within NK1.1⁺ thymocytes in the DN gate and representative histograms as well as percent positively staining cells are shown (left). Quantification of GLUT1, PIT1, and PIT2 in NK1.1⁺ thymocytes is shown with horizontal lines presenting means $\pm$ SD ($n = 5$, right). **(B)** CD4⁺ thymocytes were evaluated as a function of intermediate and high CD3 expression at 8 weeks and 1 year of age (left plots), and NK1.1/PIT1 staining profiles were evaluated in both subsets (middle plots). Quantification of NK1.1 staining within the CD3^{int}CD4⁺ thymocyte subset is shown ($n = 5$, right).

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Conflict of Interest: VP and NT are inventors on patents describing the use of RBD ligands but NT no longer has any patent rights. VP is the co-founder of METAFORA-biosystems, a start-up company that focuses on metabolite transporters under physiological and pathological conditions.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Evaluating Thymic Function After Human Hematopoietic Stem Cell Transplantation in the Personalized Medicine Era

Ahmed Gaballa¹, Emmanuel Clave^{2,3}, Michael Uhlin^{1,4,5}, Antoine Toubert^{2,3} and Lucas C. M. Arruda^{1*}

¹ Department of Clinical Science, Intervention and Technology, Karolinska Institutet, Stockholm, Sweden, ² INSERM UMR-1160, Institut de Recherche Saint-Louis, Hôpital Saint-Louis APHP, Paris, France, ³ Université de Paris, Paris, France, ⁴ Department of Applied Physics, Science for Life Laboratory, Royal Institute of Technology, Stockholm, Sweden, ⁵ Department of Clinical Immunology and Transfusion Medicine, Karolinska University Hospital, Stockholm, Sweden

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*Correspondence:

Lucas C. M. Arruda
lucas.arruda@ki.se

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Hematopoietic stem cell transplantation (HSCT) is an effective treatment option for several malignant and non-malignant hematological diseases. The clinical outcome of this procedure relies to a large extent on optimal recovery of adaptive immunity. In this regard, the thymus plays a central role as the primary site for *de novo* generation of functional, diverse, and immunocompetent T-lymphocytes. The thymus is exquisitely sensitive to several insults during HSCT, including conditioning drugs, corticosteroids, infections, and graft-vs.-host disease. Impaired thymic recovery has been clearly associated with increased risk of opportunistic infections and poor clinical outcomes in HSCT recipients. Therefore, better understanding of thymic function can provide valuable information for improving HSCT outcomes. Recent data have shown that, besides gender and age, a specific single-nucleotide polymorphism affects thymopoiesis and may also influence thymic output post-HSCT, suggesting that the time of precision medicine of thymic function has arrived. Here, we review the current knowledge about thymic role in HSCT and the recent work of genetic control of human thymopoiesis. We also discuss different transplant-related factors that have been associated with impaired thymic recovery and the use of T-cell receptor excision circles (TREC) to assess thymic output, including its clinical significance. Finally, we present therapeutic strategies that could boost thymic recovery post-HSCT.

Keywords: T-cells, thymus, hematopoietic stem cell transplantation, TREC, immune reconstitution, thymic function

INTRODUCTION

Hematopoietic stem cell transplantation (HSCT) represent the earliest form of stem cell therapy and has been used over six decades as treatment for several malignant and non-malignant blood conditions (1). Its clinical outcome relies on a successful immune recovery, particularly an optimal T-cell reconstitution. Following HSCT, innate immune cells recover in the initial weeks to months post-HSCT, while the T-cell pool recovery is accomplished through thymic-independent homeostatic proliferation of donor-derived mature T-cells in the 1st year (2). Thereafter, the thymic-dependent *de novo* generation of naïve T-cells from donor hematopoietic stem cells (HSCs)

play an essential role for immune restoring in a process that can take years and is responsible for the full restoration of TCR specificities (3). The appropriate reconstitution of the T-cell compartment is of utmost importance not only to fight opportunistic pathogens but also to promote tumor control. A prolonged post-transplant immunodeficiency is still a hurdle associated with increased infection, secondary malignancies, relapse, and high mortality rates (3). The key role played by T cells have been highlighted by the efficacy of donor lymphocyte infusions (DLI) in controlling disease relapse and by several works on T-cell reconstitution (4, 5). T-cell reconstitution, as result of thymic rebound post-transplant, is therefore strictly related to HSCT success and will be the focus of this review.

T-CELL RECONSTITUTION POST-HSCT

The T-cell compartment recovery after transplantation occurs by two temporally and spatially distinct pathways. In the 1st weeks and months post-transplant, donor-derived, and remaining T cells not depleted by the conditioning regimen undergo peripheral expansion, comprising a thymic-independent T-cell reconstitution (**Figures 1A,B**) (6). This mechanism of homeostatic proliferation, also called lymphopenia-induced proliferation, is dependent of homeostatic cytokines such as IL-2, IL-7, and IL-15 (7–9). This results in the preferential expansion of CD8+ memory T cells, a subpopulation more responsive to cytokines due to previous antigen experience (such as cytomegalovirus, CMV) (10). Although thymic-independent pathway serves as a short track for rapid replenishment of the virtually empty T-cell pool, the extent to which it contributes to protect against infection is rather limited due to skewness of the TCR repertoire (11, 12). Additionally, T cells undergoing intense homeostatic proliferation are dysfunctional (13), present short telomeres (14, 15), and are more prone to activation-induced cell death (13). This altogether contributes to an incomplete T-cell reconstitution associated with high incidence of infections post-HSCT.

A complete reconstitution of the T-cell compartment depends on the thymic rebound and consequent *de novo* production of naïve T cells by the recipient thymus. Thymic-generated T cells undergo TCR rearrangement and stringent selection steps, resulting in a self-tolerant, highly diverse repertoire of polyfunctional T cells (3) (**Figure 1C**). This is supported by the seeding of the thymus with lymphoid progenitors arising from donor HSCs in constant maturation in the recipient's bone marrow (BM) (16). Within the thymus, T-cell progenitors undergo multi-differentiation steps and phenotypic changes, ultimately leading to the generation of a diverse repertoire of self-tolerant naïve T cells. These steps entail interactions between T-cell precursors (thymocytes), cortical thymic epithelial cells (cTECs), and stromal cells such as medullary TECs and dendritic cells (17, 18). This mechanism is tightly regulated and lead to the generation of naïve and MHC-restricted T-cells (CD4+, CD8+), with a non-self-antigen-specific TCR (19). Consequently, there is long-term immune recovery and complete T-cell reconstitution

with high TCR specificities to several antigens that underlies infection control.

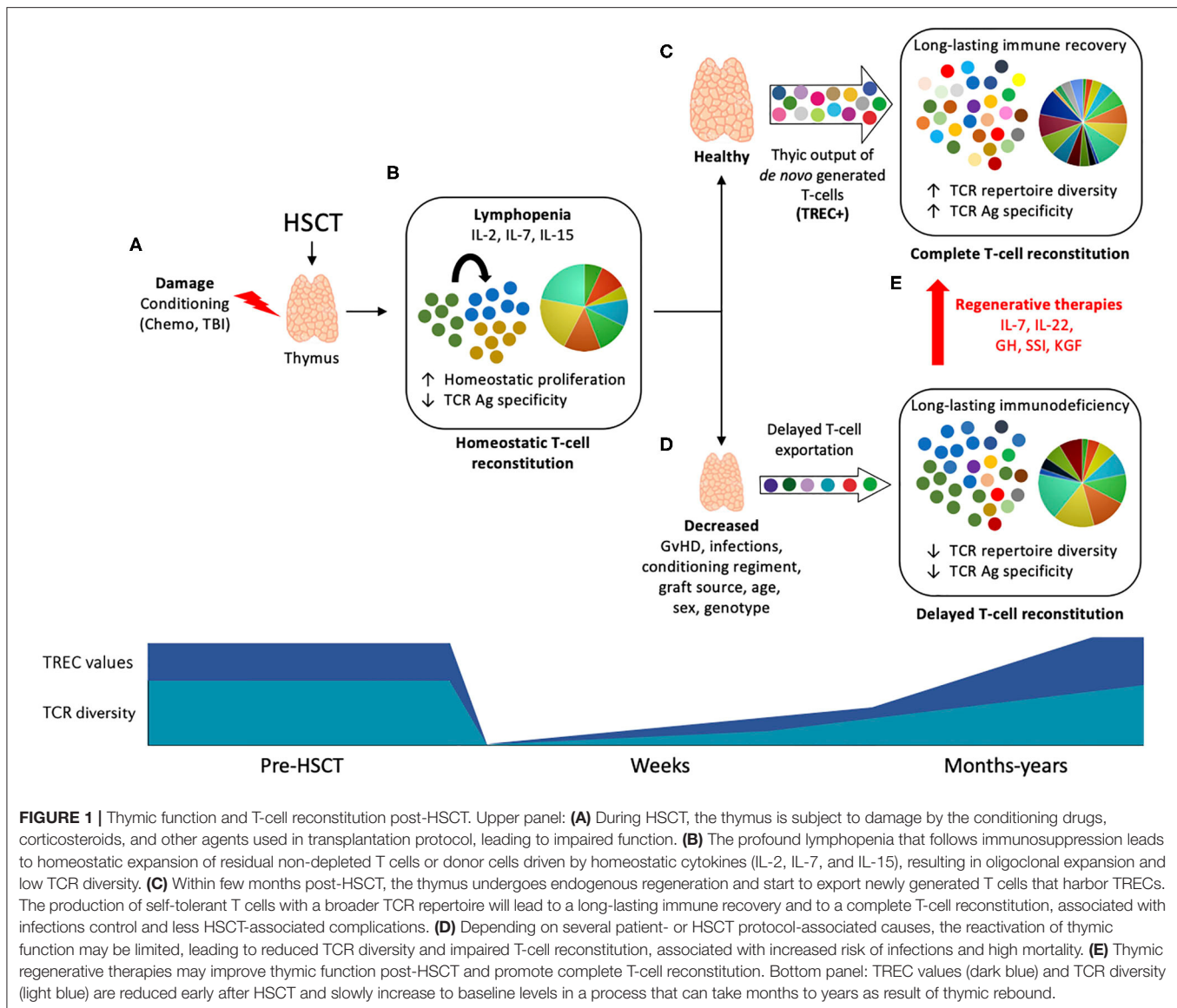
The thymus is a sensitive organ and suffer considerable damage during most HSCT protocols by the conditioning regimen and corticoids, and also post-transplant by infections and GvHD, leading to incomplete T-cell reconstitution and increased risk of infection and mortality (**Figures 1A,D**) (2). Regenerative therapies are under investigation to avoid thymic injury and to boost thymic output when appropriate (**Figure 1E**).

SURROGATE MARKERS OF THYMIC OUTPUT: ADVANCES AND LIMITATIONS

Understanding how the thymus contributes to T-cell reconstitution following HSCT has received considerable attention over the past years. Several efforts have been conducted to monitor thymic output, including assessment of thymic mass using computed tomography (20) and identifying recent thymic emigrants (RTE) using several phenotypic markers of naïve T cells (CD31+, CD45RA+) (21). Nevertheless, these methods either have provided semiquantitative estimates or were limited by their inability to distinguish between RTEs and long-lived naïve T cells (22, 23). TCR excision circles (TRECs) are stable episomal circular DNA fragments generated as by-products of TCR genes rearrangement and are exported from the thymus to the periphery within RTEs. Since the TCR δ locus is inserted within the TCR α locus, recombination of TCR α entails deletion of the TCR δ segment at a specific site that is common for ~70% of thymocytes, resulting in δ Rec- Ψ J α signal joint TRECs (sjTREC) and coding joint (cjTREC) (24, 25) (**Figure 2A**). sjTREC values reflect the thymic output of newly generated T cells and present a strong positive correlation with naïve CD4+, CD8+, and regulatory T cells (26). Douek et al. (25) initially introduced TRECs as a reliable surrogate marker for thymic output in the context of HIV infection.

As result of the progress in molecular techniques, besides the advancement in HSCT and the growing interest in the thymic role post-HSCT, sjTREC quantification has become feasible for many researchers and has been extensively utilized for monitoring RTE in several studies (3, 27–31). However, it is of utmost importance to take into account that in the lymphopenic setting post-HSCT, the increase or decrease in sjTREC levels does not necessarily correlate to thymic output alone (32). In fact, sjTREC levels can be influenced by other external factors such as longevity and apoptosis of naïve T cells or degradation of the sjTREC itself (33). To take into account the issue of peripheral T-cell proliferation, Ki67 staining within naïve CD4+ T-cells together with TREC quantification has been proposed to more accurately model thymic output (34).

Given the potential limitation of using sjTREC alone, a Canadian-French group developed a novel method that depends on simultaneous quantification of TRECs generated at two different thymopoietic checkpoints. β TREC is produced during D β J β rearrangement at the DN3 stage and sjTREC generated at the DP stage (35). The estimation of β TREC provides valuable information about intra-thymic proliferation that occurs between



TCR β - and TCR α -chain rearrangements (36) (Figure 2A). Furthermore, the sj/ β TREC ratio is not influenced by the dilution effect of peripheral proliferation (37). Despite the advantages of this method, it is labor intensive and time consuming which has limited its wide use. To overcome this, other researchers have developed more simplified methods for quantification of β TREC (36, 37).

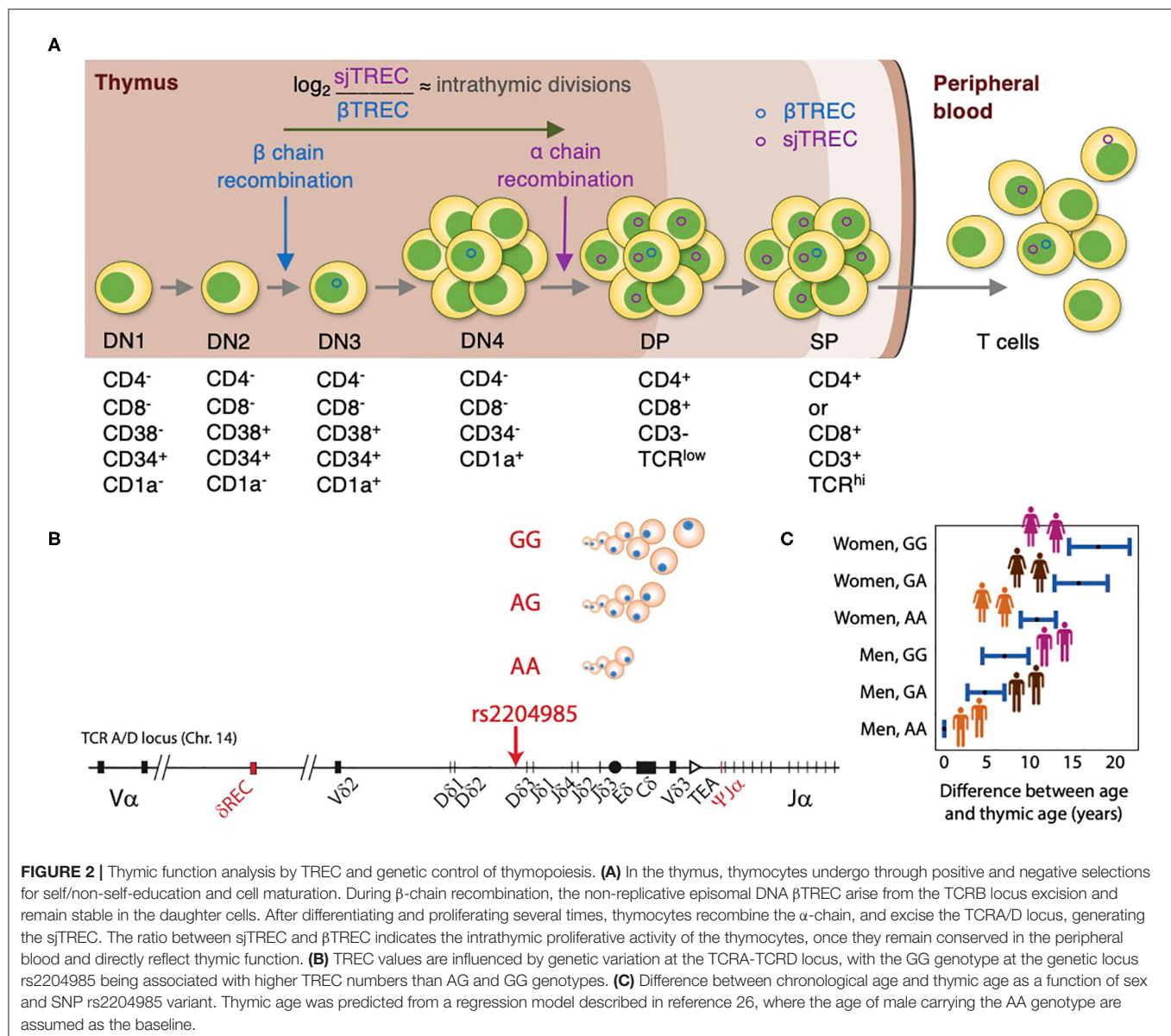
FACTORS AFFECTING THYMIC FUNCTION POST-HSCT

Restoration of the normal T-cell repertoire post-HSCT is a slow and long-term process that depends on the regenerative capacity of the thymus. The over-time exportation of TREC + RTEs results in an increased TCR repertoire diversity, that does not reach baseline values until months or even years post-HSCT

(Figure 1). Several parameters have been shown to influence thymic recovery following transplantation. Some of them are general, like the age, gender, and the genetic variation; while some others are transplant-specific such as conditioning, GvHD, and graft source. Here, we will briefly discuss some of these factors.

General Age

Age-related thymic atrophy/involution is a physiologic process that has been described even before revealing the immunological function of the thymus itself. Thymic function reaches its peak by the 1st year of life and gradually declines thereafter (16). It has been postulated that thymic mass decreases by an annual rate of 3% until middle age, and subsequently by 1% per year (38). In the same way, recent data have described that sjTREC values present a decrease of about 4–5% per year (26), resulting in the subsequent reduction in of naïve T-cell counts. During



involution, adipose tissue gradually replaces thymic stromal cells resulting in shrinkage in its size and progressive reduction in thymopoiesis, as shown in mice (39). This leads to the involution of thymic epithelial space and reduction of number of thymocytes due the increase of thymopoiesis-suppressive cytokines (40, 41). The reduction in IL-7 (42), impaired TCR β -chain rearrangement (43), alterations in hormones and growth factors, and changes in thymic niche have also been suggested as possible underlying mechanisms in rats and humans (44, 45). Of note, age-related involution does not lead to a complete loss of thymic function as residual thymic output can still be retained even in advanced ages, albeit significantly reduced (46). On this matter, age is an independent risk factor related to thymic function impairment in HSCT (47), with the thymic rebound post-HSCT shown to be reduced in elderly as compared to young adults and resulting in reduced naïve T-cells production (48).

Impaired immune reconstitution post-HSCT correlates with increased morbidity and mortality caused by infection and relapse (3, 49). Thus, is critical the development of strategies that enhance thymic output and immune reconstitution, particularly in elderly patients.

Sex

Apart from age, thymic output has also been linked to gender. Age-related thymic involution is higher in males compared to females (50) and testosterone treatment results in decreased thymic output (51). This was confirmed by two large population studies where the women presented 66–86% higher sjTREC values than men of all age ranges (26). It is therefore expected that women would have a better outcome post-HSCT with regards to infections and relapse due to the higher thymic function than men. In fact, recent data from a large cohort of

around 12,000 patients have shown that recipient gender is an important prognostic factor independent of donor gender, with male recipients having inferior survival compared to females regardless of donor gender (52). This might partly be associated with the higher thymic function in females. Although a female recipient is beneficial, female donors to a male recipient has been shown to be deleterious, and a higher transplant-related mortality for male recipients of female allografts compared with other recipient-donor sex combinations was initially reported (53). Such observations led the European Group for Blood and Marrow Transplantation to include the female-to-male HSCT as a risk score, making male donors a safer choice for transplantation (54). This remark can result from the reduced thymic function in males, but it may also be due to the allogeneic response of female donor T cells toward minor histocompatibility antigens from male recipient (52).

Genetic Factors

Genetic background can also be implicated in determining the thymic function and the rate of thymic involution. In this regard, Clave et al. (26) have assessed the impact of 5,699,237 common single-nucleotide polymorphisms (SNPs) on sjTREC levels in a genome-wide association study of 1,000 patients from a *Milieu Intérieur* cohort. This revealed for the first time a common genetic variant (rs2204985) in a 25-kb region within the *TCRA-TCRD* locus in the intergenic D δ 2–D δ 3 segments affecting thymic output (**Figure 2B**). This was further replicated in an independent cohort. In both cohorts, a 43 and 44% increase of sjTREC values in GG homozygotes was observed as compared to AA homozygotes, respectively (26). The impact of this SNP was further validated in immunodeficient mice transplanted with human donor HSCs carrying the GG, GA, or AA genotype. The GG genotype was associated with a higher sjTREC and TCR diversity compared to mice transplanted with AG and AA donor genotype. Thymic age based on TREC output was then predicted from a regression model taking into account age, sex, and this genetic variation. Accordingly, the thymic function of a 40-year-old female with a GG genotype would be the same as a 21.5-year-old male with the AA genotype (**Figure 2C**). This study has introduced a new concept of thymic age which accounts for age, gender, and genotype (26). Their results highlight the need for personalized medicine and can be of great significance particularly in donor selection for HSCT settings. Further studies are required to reveal the clinical relevance of this SNP post-HSCT.

Transplant-Related GvHD

GvHD is a common complication post-HSCT. Although the skin, liver, gastrointestinal tract, and lung are the classical primary targeted organs, accumulating evidences also suggest the damage on the hematopoietic system (55, 56). Using sj/ β TREC quantifications, Clave et al. have shown a significant reduction in thymic output in patients with acute GvHD (aGvHD). However, this effect was transient in young patients, suggesting that aGvHD-induced insults are reversible in <25 years old patients and depend on the regenerative capacity of the thymus

(57). Consistent with their findings, we showed that sjTREC levels were not affected by aGvHD in a long-term follow-up study (58). Conversely, chronic GvHD was associated with decreased TREC levels regardless of disease resolution (58–60), suggesting a permanent irreversible insult. Divergent GvHD prophylaxis regimens are employed in different centers and have been suggested as possible underlying cause or long-term reduced thymic function. We then studied in a prospective randomized trial, the effect of different GvHD prophylaxis (cyclosporine/methotrexate vs. tacrolimus/sirolimus) on TREC levels post-HSCT. Results indicated no difference between the two arms of the study at any time point (60).

Conditioning Regimen

HSCT is preceded by cytoreductive conditioning regimens, with the aims of reducing malignant burden, avoiding graft rejection, and enhancing engraftment (61). The severity of toxicities associated with the conditioning varies according to the intensity of conditioning protocol used during HSCT. In contrast to reduced intensity conditioning (RIC), myeloablative conditioning (MAC) is associated with higher toxicity and organ damage (61, 62). Thus, it is reasonable to assume that patients receiving MAC are more prone to impaired thymopoiesis. Surprisingly, reports from several groups were inconsistent; while some studies showed rapid reconstitution in RIC recipients (63, 64), no difference or even delayed T-cell reconstitution in RIC recipients was shown by others (57, 65, 66). This controversy can be justified by realizing that RIC is mainly indicated for elderly and patients with co-morbidities. Additionally, the combination of ATG and/or DLI with RIC regimen in some works can jeopardize the real effect of mild conditioning on thymic output. Randomized trials comparing the impact of different conditioning regimens on the thymic function is warranted.

Graft Source

HSCs source has also been shown to impact TREC values following HSCT. However, whether peripheral blood or BM is favorable for better thymic recovery is still elusive. We have earlier shown increased TREC levels in BM recipients early post-HSCT (60, 67). Furthermore, in a recent retrospective study, we assessed TREC levels in 63 recipients after a median of 12 years post-HSCT. We found that TREC levels were higher in BM graft recipients, suggesting a beneficial role in the long-term (58). Despite other studies have not shown a significant association between TREC and stem cell graft source (59, 68), a higher engraftment and supportive thymic function is expected in BM grafts, which contain mesenchymal stromal cells and dendritic cells that can possibly engraft in the host after HSCT and support hematopoiesis.

CLINICAL SIGNIFICANCE OF MONITORING THYMIC FUNCTION IN HSCT SETTING

Monitoring thymic output in HSCT patients have significantly improved our understanding main issues related to HSCT and has allowed researchers to identify factors affecting

thymic recovery post-HSCT. The association between pre-transplantation TREC values and survival established thymic function to be a reliable predictor for morbidity and mortality (3, 69). Additionally, the clearance of CMV viremia and survival after umbilical cord blood (UCB) depends on a successful reconstitution of thymopoiesis (70, 71). In this regard, the monitoring of TREC levels in 331 samples from 158 allogeneic HSCT patients showed a strong correlation between low TREC levels and opportunistic infection in the first 6 months post-HSCT (72). Moreover, low TREC levels post-HSCT has been associated with higher incidence of relapse (49). Similarly, results by Wils et al. (73) indicated a significant reduction in incidence of severe infections and lower risk of non-relapse mortality in patients who showed effective thymic recovery early post-HSCT. In children undergoing T-cell depleted haploidentical HSCT, Clave et al. (74) showed that the incidence of leukemia relapse was found to be higher in patients who had undetectable β TREC and low sjTREC levels post-HSCT. The same group has also reported similar findings in children who underwent UCB-HSCT (75). In a retrospective study, we earlier showed increased OS and decreased transplantation-related mortality in patient who had higher TREC levels at 3 months post-HSCT (67). In another study, we found high TREC levels post-HSCT to be associated with improved survival and decreased relapse incidence in leukemia patients (76). Corroborating with these results, Torlen et al. (60) reported reduced transplantation-related mortality and increased 5-year OS in patients with high TREC levels in the first 6 months post-transplantation. Of note, the thymic rebound post-transplantation is associated with a favorable clinical response to autologous HSCT in autoimmune disease patients as well (71).

BOOSTING THYMIC OUTPUT POST-HSCT

Despite the thymus can spontaneously restore its function post-HSCT, depending on recipient's age or repeated insults suffered during transplantation protocol, thymic regeneration might be impaired for long periods. Enhancing thymic function leading to efficient T-cell reconstitution might be a promising route for future HSCT. In this regard, several strategies have been investigated yet so far only few have been successfully translated to the clinic (**Figure 1E**) (2).

Growth hormone (GH) is one of the somatotrophic hormones with a pivotal role in hematopoiesis. In a prospective randomized trial, Napolitano et al. investigated the impact of daily subcutaneous injections of rGH for 6 months on thymic function of HIV-infected patients. They demonstrated increased thymic output and TREC levels in treated patients (77). Corroborating with these findings, Hansen et al. (78) reported in a randomized placebo-controlled trial increase in thymic indices in HIV group treated by low dose rGH. Although GH treatment has been used in children undergoing HSCT to treat post-radiation growth disorders (79, 80), its role in immune reconstitution has not so far been well-investigated.

The observation of rapid decline of thymic function with onset of puberty has suggested a role of sex hormones in thymic

involution and sex steroid inhibition (SSI) as a feasible strategy to restore immune competence in immunodeficient individuals (81). In fact, SSI surgically or pharmacologically has been associated with improved thymic function following HSCT in humans (82, 83), indicating that this approach can be used to boost thymic regeneration.

Keratinocyte growth factor (KGF) is produced by thymocytes and other stromal cells in the thymus and act inducing the expansion of epithelial cells. Several studies showed that administration of KGF alone or in combination with androgen blockade before HSCT is associated with improved regenerative capacity of the thymus and efficient T-cell reconstitution post-transplantation (84). Additionally, it has been shown that KGF protects TECs from the radiation-, conditioning-, and GvHD-induced damage in murine models (85–87), but its effects in humans are still elusive.

In addition to the above described strategies, several studies on murine models highlighted the role of IL-7, IL-21, IL-22, and zinc in restoring normal thymopoiesis (42, 88–93). In fact, rIL-7 administration after human allogeneic T-cell depleted HSCT demonstrated an increase in T-cell recovery and increased TCR diversity, but no significant increase on thymic output was reported (94). Additionally, the advances in thymic organoids bioengineering technologies can provide a novel solution in the future (95, 96).

CONCLUDING REMARKS

The understanding of thymic function on HSCT outcomes have evolved tremendously in the last decades. Recently, the role of recipient age and sex and donor genotyping has been unveiled as important factors associated with HSCT response, together with GvHD, conditioning regimen, and graft source. The remarkable finding that a donor genetic SNP affects strongly the recipient thymic output open a new era of graft selection (26), especially in cases when the recipient would benefit from an improved thymic function. Additionally, the recently published thymus cell atlas paved the way for a better understanding of human T-cell development and may impact HSCT research in the near future (97). The challenge for the next decades will be to translate these advances into a better donor selection and how to identify the proper recipient that should be treated with thymic boosters and which one to be used.

AUTHOR CONTRIBUTIONS

AG, EC, MU, AT, and LA prepared the figures, wrote the manuscript, and revised the final version. All authors contributed to the article and approved the submitted version.

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Conventional and Computational Flow Cytometry Analyses Reveal Sustained Human Intrathymic T Cell Development From Birth Until Puberty

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Ann Chidgey,
Monash University, Australia

Reviewed by:

Naomi Taylor,
National Institutes of Health (NIH),
United States
Lauren Ehrlich,
University of Texas at Austin,
United States

*Correspondence:

Kai Ling Liang
kailing.liang@UGent.be
Tom Taghon
tom.taghon@UGent.be

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Marieke Lavaert, Brecht Valcke, Bart Vandekerckhove, Georges Leclercq, Kai Ling Liang* and Tom Taghon*

Department of Diagnostic Sciences, Faculty of Medicine and Health Sciences, Ghent University, Ghent, Belgium

The thymus is the organ where subsets of mature T cells are generated which subsequently egress to function as central mediators in the immune system. While continuously generating T cells even into adulthood, the thymus does undergo involution during life. This is characterized by an initial rapid decrease in thymic cellularity during early life and by a second age-dependent decline in adulthood. The thymic cellularity of neonates remains low during the first month after birth and the tissue reaches a maximum in cellularity at 6 months of age. In order to study the effect that this first phase of thymic involution has on thymic immune subset frequencies, we performed multi-color flow cytometry on thymic samples collected from birth to 14 years of age. In consideration of the inherent limitations posed by conventional flow cytometry analysis, we established a novel computational analysis pipeline that is adapted from single-cell transcriptome sequencing data analysis. This allowed us to overcome technical effects by batch correction, analyze multiple samples simultaneously, limit computational cost by subsampling, and to rely on KNN-graphs for graph-based clustering. As a result, we successfully identified rare, distinct and gradually developing immune subsets within the human thymus tissues. Although the thymus undergoes early involution from infancy onwards, our data suggests that this does not affect human T-cell development as we did not observe significant alterations in the proportions of T-lineage developmental intermediates from birth to puberty. Thus, in addition to providing an interesting novel strategy to analyze conventional flow cytometry data for the thymus, our work shows that the early phase of human thymic involution mainly limits the overall T cell output since no obvious changes in thymocyte subsets could be observed.

Keywords: human thymopoiesis, aging, computational flow cytometry, puberty, thymic involution

INTRODUCTION

The thymus is the organ where bone marrow-derived thymic progenitors undergo stepwise developmental changes to generate multiple distinct subsets of mature T cells. Subsequently, these naïve T cells egress from the thymus to peripheral lymphoid organs and function as central mediators of the immune system. Thymic involution is an evolutionary process that is conserved in almost all vertebrates (1). This involves regression of the thymus with disruption of the structural integrity that is important to support T-cell development. As a result, the T cell output decreases and this is reflected by a reduction in thymic cellularity and overall size of the organ. Based on the kinetics of thymic involution, two phases of thymic regression have been proposed (2). The first phase involves a rapid decrease in thymic cellularity during early life. In contrast to that, the second phase begins in adulthood in which thymic involution proceeds age-dependently at a steady state. At this stage, age-related declines in T cell output are contributed by both the progressive involuting thymic stromal compartment and intrinsic developmental defects in myeloid-biased thymic progenitors which originate from the aging bone marrow (3–8). Ultimately, thymic involution restricts the diversity of the peripheral T cell repertoire and leads to deterioration of the immune system in the elderly.

Studies in the mouse system have been crucial to advance our understanding of thymic involution. In this species, T cell development after birth is a continuous process that leads to the build-up of thymocytes until the onset of puberty. Following that, the first phase of murine thymic involution begins at 4–7 weeks of age when thymic cellularity decreases sharply (9–11). Indeed, the rise in sex steroid levels during puberty has an established causative role in thymic involution (12). In contrast to the mouse, studies examining the human thymus revealed that human T cell development is a dynamic process that fluctuates before the puberty period (13, 14). Within 3–4 weeks after birth, the thymic cellularity of neonates remains low. Thereafter, human T cell development raises, and the thymus of infants reaches its maximum cellularity at 6 months of age (14). The early onset of human thymic involution prior to puberty is corroborated by the morphological alterations found in the thymus from 1 year of age (15). Nevertheless, Weerkamp and colleagues found that the cellular composition of the thymus, in consideration of the different subsets of developing thymocytes, remains similar from 6 months to 8 years of age. Hence, involution in the thymus of infants does not impede its ability to sustain T-cell development in early childhood. Sex steroid ablation has been shown to improve human thymic and hence immune regeneration (16, 17). However, the physiological changes of human thymic composition from birth until the onset of puberty, which is around the age of 11.5 years old, remains unclear (18). To address this, we performed multi-color flow cytometry on thymic samples collected from birth to 14 years of age in order to examine changes in population distribution at critical developmental stages of human T cell development. In addition, we also studied age-associated changes in mature T and non-T lineage populations that reside in the thymus. In acknowledgment of the inherent limitations posed by

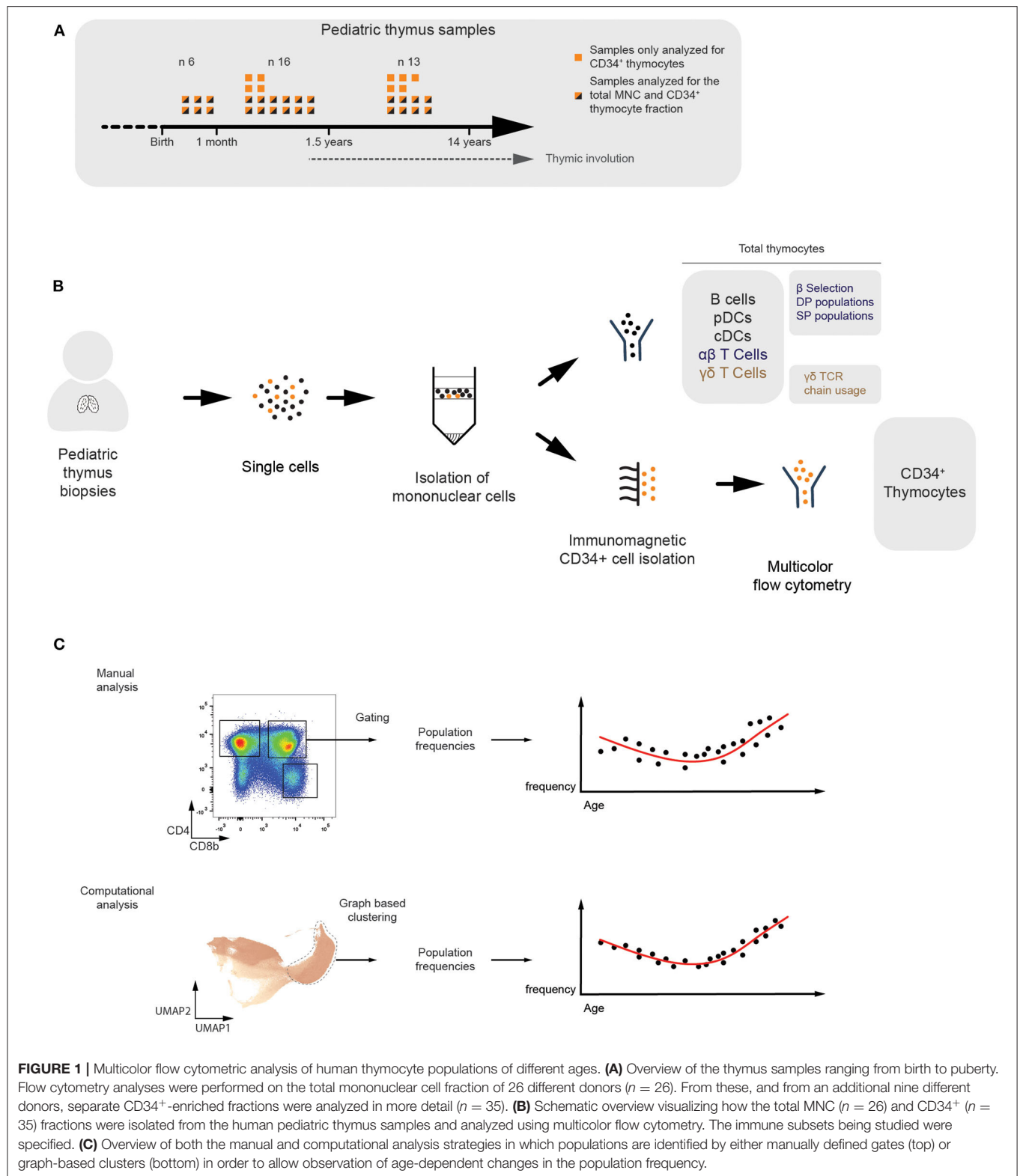
conventional flow cytometry analysis, we implemented a novel computational analysis pipeline that is adapted from single-cell transcriptome sequencing data analysis to present our data.

RESULTS

The Human Postnatal Thymus Is Characterized by An Increased Frequency in B Cells Following the Onset of Thymic Involution

To study the age-dependent fluctuations in the frequencies of thymic immune cells, we collected 35 human thymus samples ranging from birth to 14 years of age. The CD34⁺ thymocyte fraction was isolated from all the samples and the total mononuclear cell fraction (MNC) was studied from 26 of them (Figure 1A). Multicolor flow cytometry was applied to identify different subsets of thymic progenitors present in the CD34⁺-enriched fractions. For the MNC fractions, we immunophenotyped developing and mature T-lineage subsets, spanning both the $\alpha\beta$ and $\gamma\delta$ branches, and mature non T-lineage cells (Figure 1B). The flow cytometry data generated from these samples was analyzed using manually defined gates. In addition, we applied a computational approach, based on established single cell RNAseq pipelines (19), which consists of two steps. First, in order to improve computational throughput, compensated and biexponentially transformed data is subsampled and pooled, either in a random manner or by using geometric sketching (20) which allows for enrichment of rare subpopulations, or these subsampling methods can be combined as was done in all the following analyses. Subsequently, a k-nearest neighbor (KNN) graph was constructed and Leiden clustering was performed (21) to allow identification of populations within the data, which in turn were visualized using an UMAP manifold (22) (Figure 1C).

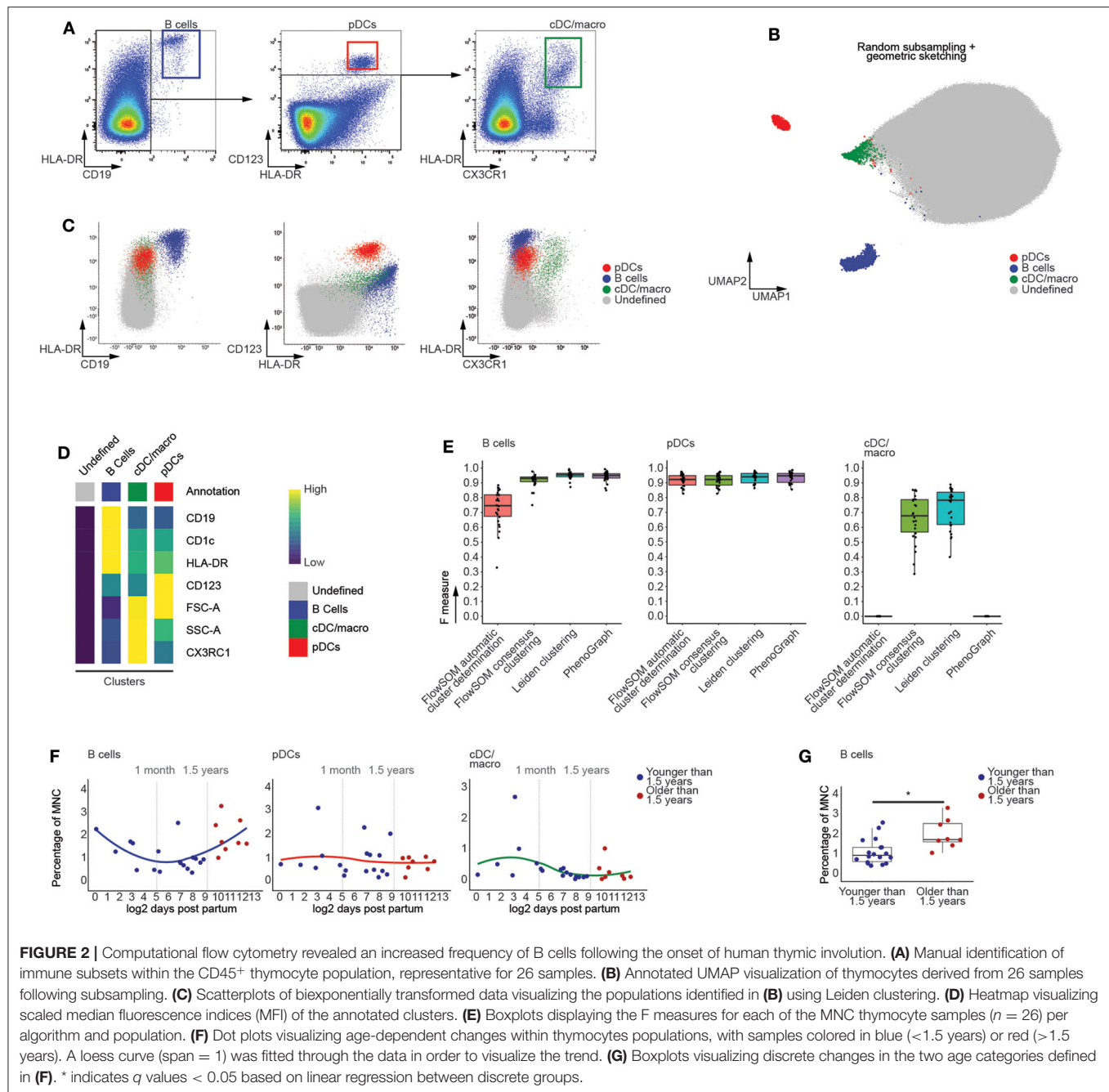
Using manually defined gates, we examined the CD45⁺ fraction of human thymic MNC in order to identify populations of CD19⁺HLA-DR⁺ B cells, HLA-DR⁺CD123⁺ plasmacytoid dendritic cells (pDCs) and HLA-DR⁺CX3CR1⁺ conventional dendritic cell and macrophages (cDC/macro) (Figure 2A) and HLA-DR⁺CD14⁺ monocytes (Figure S1A) (23, 24). These populations were used to assess the performance of our graph-based clustering approach, which appeared to successfully identify the distinct populations (Figure 2B and Figure S1B) and which recapitulated the phenotype of manually identified mature non T-lineage subsets based on both the biexponentially transformed scatterplots (Figure 2C and Figure S1C) and the median fluorescent indices (MFIs) of each of the annotated clusters (Figure 2D). Further assessment of our methods performance was done by calculating F measures for each population, taking both the precision and recall of the manually defined populations into account. This also allowed comparisons with well-established state-of-the-art methods such as PhenoGraph and FlowSOM (25, 26), of which the latter can both automatically detect an optimal number of clusters or return a specific number of clusters (Figure 2E and Figure S1D). This comparison revealed that our approach either is equally or even more performant than FlowSOM or PhenoGraph. In addition,



the frequencies of these manually defined populations were not affected by the initial subsampling of the data (Figure S1E), which was also the case for the populations that we cover later (Figure S2). This suggests that our computational approach was

not only able to identify relevant immune subsets, but also allows assessment of age-dependent changes in population frequencies.

As the onset of thymic involution occurs after 1 year of age (15), we categorized the samples in two groups, being younger



or older than 1.5 years of age, and used linear regression to test for changes in population frequency. This approach revealed that the population of computationally identified B cells significantly increases in frequency after 1.5 years of age (Figures 2F,G), consistent with earlier reports on increased B cell numbers in the aging thymus (27) which validates our choice to generate two groups based on this age. In contrast, the myeloid populations appeared largely unaffected by aging prior to puberty (Figures 2F,G and Figure S3), in agreement with recently published findings (28). Together, these findings not only validate the quality of our data, but also demonstrate the

capability of our approach to robustly identify rare populations, with frequencies as low as 0.2%, within the generated flow cytometric data.

Developing CD34⁺ Human Thymocytes Frequencies Are Unaffected by Physiological Growth Leading To Puberty

During human life, the thymus is continuously seeded by bone marrow derived multipotent progenitor cells. These CD34⁺ precursors lack high expression of CD7 and can

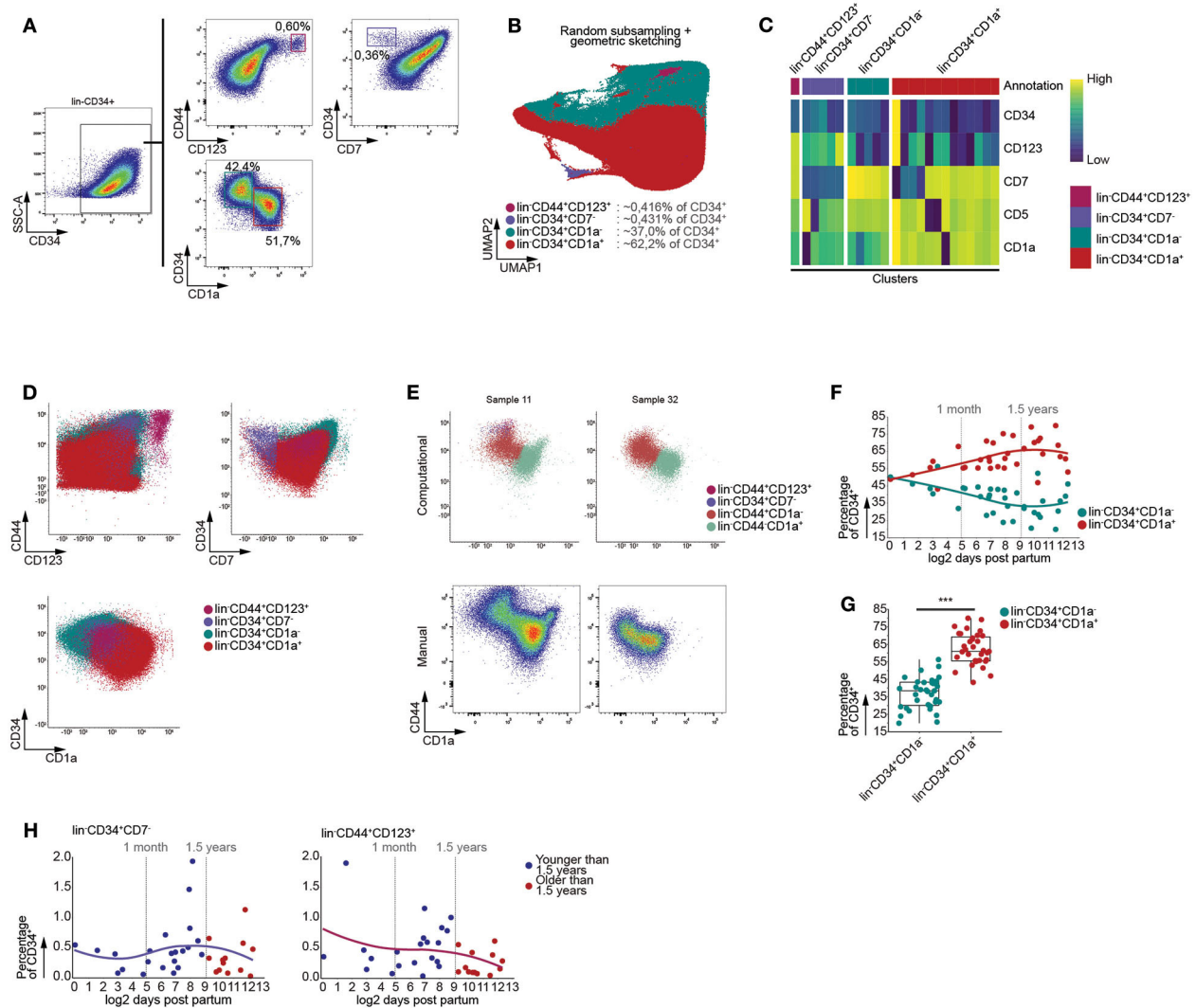
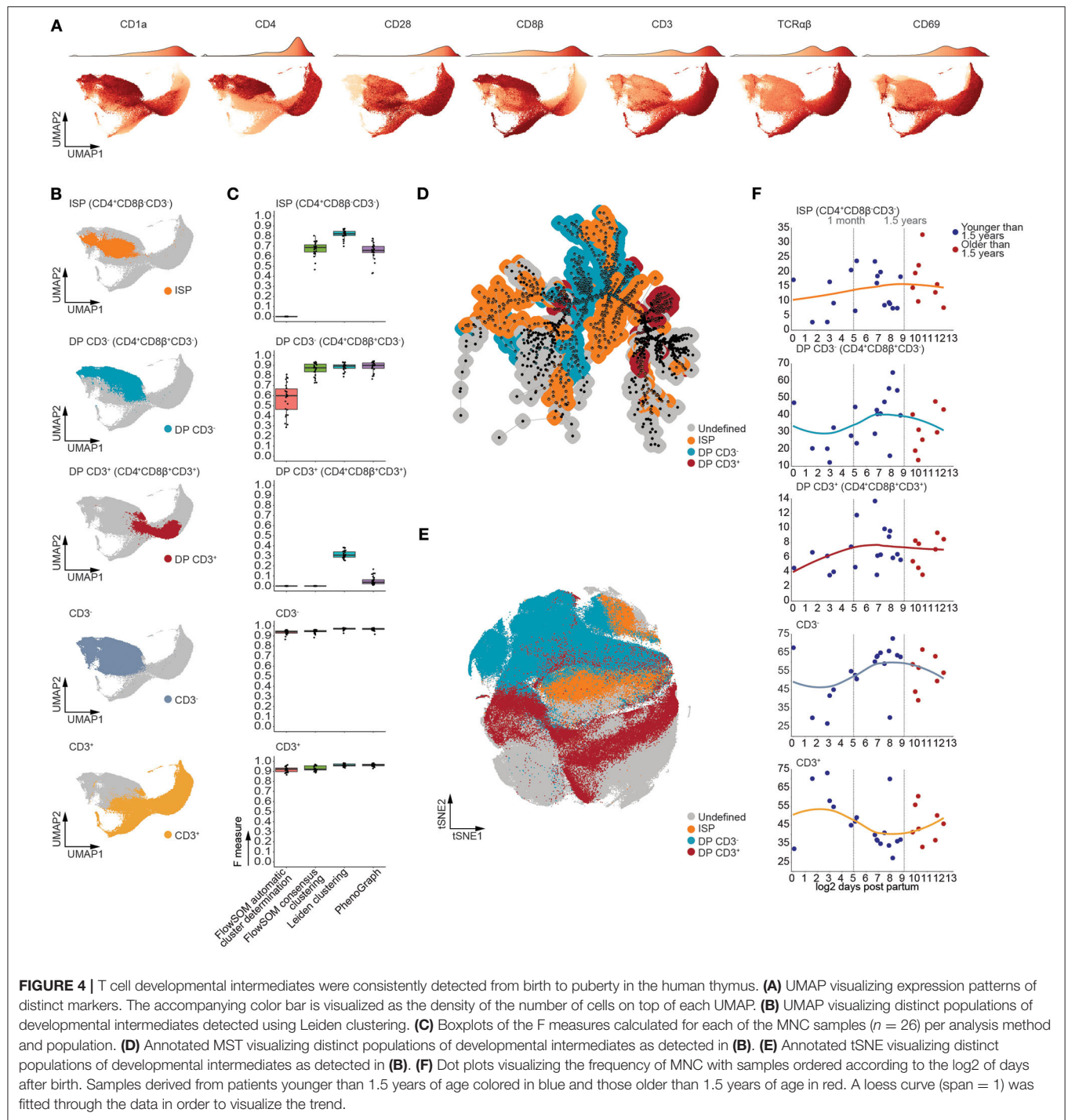


FIGURE 3 | Graph based clustering allows identification of gradual developmental changes within CD34⁺ thymocytes. **(A)** Schematic overview of immune subset identification using manual gating, representative for 35 samples. Scatterplots visualize biexponentially transformed data. **(B)** Annotated UMAP visualization of subsampled, both randomly and by geometric sketching, CD34 enriched thymocytes, derived from 35 samples. **(C)** Heatmap visualizing the scaled MFIs of each cluster grouped according to the annotated population. **(D)** Scatterplots of biexponentially transformed data visualizing the annotated populations allowing comparison between the manual gating (**A**) and computationally defined populations. **(E)** Visualization of biexponentially transformed data displaying computationally defined populations based on CD44 expression (Top), highlighting difficulties with defining manual gates (Bottom). **(F)** Dot plots visualizing population frequencies with samples ordered according to log₂ transformed age. Observations are colored according to subset corresponding to either the lin⁻CD34⁺CD1a⁻ or lin⁻CD34⁺CD1a⁺. A loess curve (span = 1) was fitted through the data in order to visualize the trend. **(G)** Boxplots visualizing changes in frequency of MNC between the lin⁻CD34⁺CD1a⁻ or lin⁻CD34⁺CD1a⁺. Statistical significance denoted by *** indicates q values < 0.0001 based on a paired t-test between discrete groups. **(H)** Dot plots visualizing the frequency of MNC with samples ordered according to the log₂ of days after birth. Samples derived from patients younger than 1.5 years of age colored in blue and those older than 1.5 years of age in red. A loess curve (span = 1) was fitted through the data in order to visualize the trend.

give rise to T cells and to a lesser extent pDCs (29–31). In order to assess age-dependent effects on these initial stages of T cell development, we enriched the CD34⁺ thymocytes and performed multi-color flow cytometry. Using manual gating, we were able to identify an immature CD3⁻CD4⁻CD19⁻CD56⁻(lin⁻)CD7⁻CD34⁺ progenitor population, both the uncommitted lin⁻CD34⁺CD1a⁻ and T-lineage committed lin⁻CD34⁺CD1a⁺ thymocyte populations,

and lin⁻CD44⁺CD123⁺ DC progenitors (Figure 3A) (30, 32, 33).

We were able to recover these populations from the lin⁻CD34⁺ thymocytes fraction following exclusion of CD44 from the data, as it follows a similar expression pattern to CD34 and has been inversely associated with T-lineage commitment (34), and performing batch correction with linear regression, prior to generating a KNN-graph and clustering (Figures 3B–D).



This indicated that our approach was capable of identifying highly distinct and rare subpopulations, as well as more subtle changes within relatively homogenous populations. Calculating F measures for each population further confirmed our approach to be capable of faithfully recovering these populations and to be equally as performant or outperform well established algorithms (**Figure S4A**), such as FlowSOM and PhenoGraph.

Due to the nature of our computational approach, which allowed for both the simultaneous analysis of multiple samples and batch correction using linear regression, we were able to consistently identify particular populations despite inter-sample variation. In case of our data, incorporation of CD44 while excluding CD34 prior to KNN-graph generation allowed us to robustly isolate the $\text{lin}^- \text{CD44}^+ \text{CD1a}^-$ and $\text{lin}^- \text{CD44}^- \text{CD1a}^+$

populations (**Figure 3E**), regardless of clear differences between samples with respect to the expression level of specific cell surface markers.

As an increase in B cell frequency could be observed from 1.5 years of age onwards, following the onset of thymic involution, we assessed whether or not this was the case for the subpopulations we identified within the CD34⁺ fraction of human thymocytes. However, no significant age-dependent changes within the lin⁻CD34⁺CD1a⁻, lin⁻CD34⁺CD1a⁺ (**Figure S4B**), lin⁻CD44⁺CD1a⁻ and lin⁻CD44⁻CD1a⁺ (**Figure S4C**) thymocyte populations could be observed using linear regression. These findings were confirmed as we were able to observe significant differences in the proportions of lin⁻CD34⁺CD1a⁻ and lin⁻CD34⁺CD1a⁺ (**Figures 3F,G**), and lin⁻CD44⁺CD1a⁻ and lin⁻CD44⁻CD1a⁺ thymocytes (**Figures S4D,E**), regardless of age. Similarly, the lin⁻CD44⁺CD7⁻ and lin⁻CD44⁺CD123⁺ populations displayed no significant age-dependent differences despite the onset of thymic involution (**Figure 3H**). Using these data, we were able to validate that our computational approach is capable of identifying both highly distinct and gradually transitioning populations in a manner that disregards inter-sample variation, enabling the identification of age-dependent effects or lack thereof following the onset of thymic involution.

Thymic Involution Does Not Affect Major Developmental Intermediates During Thymopoiesis

As our computational approach allows for robust identification of developmental intermediates, we generated a UMAP manifold visualizing all major stages of TCRαβ T-cell development (**Figure 4A**), with the exception of CD34⁺ progenitors, which constitute ~1% of the human postnatal thymus. Using multiple rounds of Leiden clustering, we isolated the CD4⁺CD8β⁻CD3⁻ immature single positive (ISP), the CD4⁺CD8β⁺CD3⁻ and CD4⁺CD8β⁺CD3⁺ double positive, and the total CD3⁻ and CD3⁺ populations (**Figure 4B** and **Figure S5A**). Despite the complexity of this developmental trajectory, this approach was able to faithfully recapitulate these populations (**Figure S5B**). One exception to this was the double positive CD3⁻ (CD4⁺CD8β⁺CD3⁻) population (**Figure S5C**) which contained a minor fraction of CD4⁻CD8β⁺CD3⁻ cells, randomly varying between 0.25 and 2.5% of MNC (**Figure S5D**) and which could be observed by both manual or computational analysis (**Figure S5E**). Nevertheless, for the main subsets, these findings were further confirmed by calculating F measures, which not only validated our approach but also revealed our method to be equally or more performant compared to FlowSOM and PhenoGraph (**Figure 4C**), in line with our previous observations. Moreover, visual inspection of the t-distributed Stochastic Neighbor Embedding (tSNE) and minimal spanning tree (MST), used to visualize PhenoGraph and FlowSOM, respectively, revealed the developmental intermediates to be scattered (**Figures 4D,E**) rather than being closely grouped as in our UMAP visualization (**Figure 4B**) (35). In line with our findings that the onset of thymic involution does not affect the

frequencies the CD34⁺ thymocyte subpopulations, no significant age-dependent changes in population frequencies could be observed following 1.5 years of age using linear regression in **Figure 4F**, suggesting that the onset of thymic involution does not initially affect the frequency of major developmental intermediates during human TCRαβ T cell development.

The Human Thymus Maintains Generation of Mature αβ and γδ T Cells From Birth To Puberty

Following expression of a functional αβ TCR, developing CD3⁺ thymocytes bifurcate toward either the single positive (SP) CD4⁺ or SP CD8β⁺ branch (**Figure 5A**). Maturation of these SP populations, from an immature naïve to a mature naïve phenotype, is marked by a decrease in CD1a expression prior to thymic egression (36, 37). Using Leiden clustering, we distinguished these SP populations (**Figure 5B**) and also identified developmental intermediates within the SP CD8β⁺ thymocytes, being the SP CD8β⁺CD1a^{hi} and SP CD8β⁺CD1a^{lo} subsets (**Figure 5C**). In order to circumvent differences in clustering between both branches of αβ T cells, logistic regression was used to infer these mature SP CD8β⁺ populations onto the SP CD4⁺ branch, allowing accurate identification of the SP CD4⁺CD1a^{hi} and SP CD4⁺CD1a^{lo} developmental intermediates (**Figure 5C** and **Figure S6A**). These populations were then validated using F measures, revealing high correspondence with their manually defined counterparts, and allowing us to confirm our approach to be equally or more performant than FlowSOM and PhenoGraph (**Figure S6B**). Moreover, in line with our earlier analysis of the major T-lineage developmental stages, both the MST and tSNE visualizations used for FlowSOM or PhenoGraph were not capable of tightly arranging these populations in contrast to the UMAP manifold used here (**Figures S6C,D**) (35).

In agreement with the developmental intermediates precluding bifurcation of the αβ T cells, linear regression revealed no significant changes in population frequency following the onset of thymic involution (**Figure 5D**). In addition, examination of the total SP population frequencies did not reveal significant differences prior to and after 1.5 years of age (**Figure S6E**).

Prior to the expression of a functional αβ TCR, a minor fraction of thymocytes branch away from the αβ T-lineage to give rise to γδ T cells, which constitute approximately 2% of all thymocytes regardless of age (**Figure S6F**). This minor population of T cells is characterized by limited variation in their TCR chain usage compared to αβ T cells. Consequently, we stained MNC with antibodies directed at the Vδ1, Vδ2, and Vγ9 TCR chains and assessed age-dependent effects in these corresponding γδ T cell subpopulations. As this panel was optimized to assess variation within these rare γδ T cells, examination of the entire MNC fraction was not possible, necessitating isolation of CD3⁺TCRγδ⁺ cells using manual gating (**Figure 5E**). Leiden clustering robustly identified populations of TCRγδ⁺Vγ9⁺Vδ1⁺Vδ2⁻, TCRγδ⁺Vγ9⁺Vδ1⁻Vδ2⁺, TCRγδ⁺Vγ9⁻Vδ1⁺Vδ2⁻, TCRγδ⁺Vγ9⁻Vδ1⁻Vδ2⁺ and TCRγδ⁺Vγ9⁻Vδ1⁻Vδ2⁺

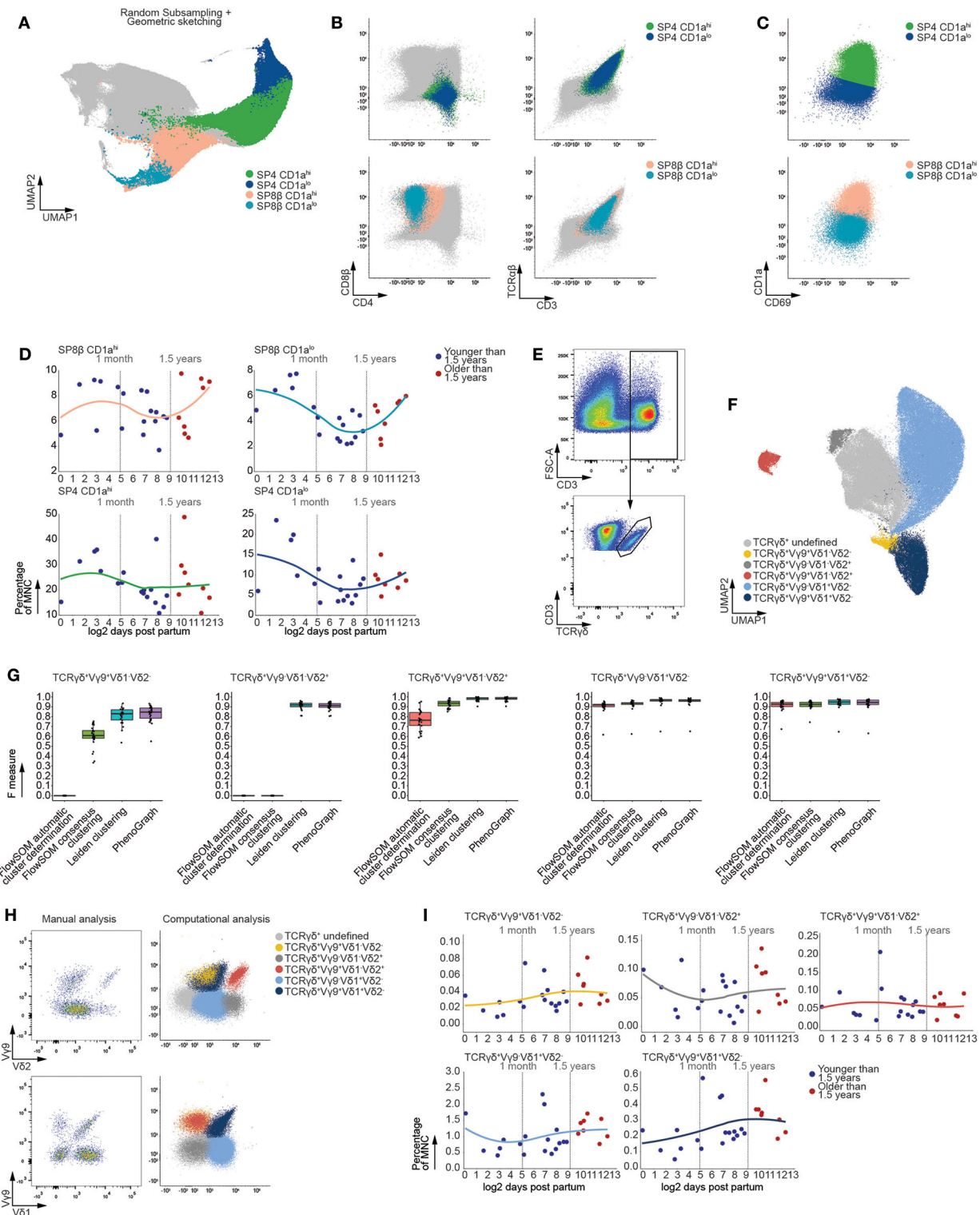


FIGURE 5 | The frequencies of mature T cells populations remain stable following thymic involution. **(A)** Fully annotated UMAP visualizing subpopulations of mature SP CD4⁺ and SP CD8β⁺ T cells detected using Leiden clustering. **(B)** Scatterplots of biexponentially transformed data visualizing the populations detected in **(A)** (Continued)

FIGURE 5 | using relevant markers. **(C)** Scatterplots of biexponentially transformed data visualizing subpopulations of either SP CD8 β ⁺ or SP CD4⁺ T cells based on decreased expression of CD1a. **(D)** Dot plots visualizing the frequency of MNC with samples ordered according to the log2 of days after birth. Samples derived from patients younger than 1.5 years of age colored in blue and those older than 1.5 years of age in red. A loess curve (span = 1) was fitted through the data in order to visualize the trend. **(E)** Scatterplots of biexponentially transformed data visualizing manual gating used to isolate CD3⁺TCR $\gamma\delta$ ⁺ T cells, representative for 26 samples. **(F)** Fully annotated UMAP visualizing subpopulations of mature CD3⁺TCR $\gamma\delta$ ⁺ T cells ($n = 26$) based on variable expression of V δ 1, V δ 2, and V γ 9 TCR chain expression detected using Leiden clustering. **(G)** Boxplots visualizing the F measures for each of the MNC samples ($n = 26$) per analysis method and population. **(H)** Scatterplots visualizing biexponentially transformed data covering the CD3⁺TCR $\gamma\delta$ ⁺ subpopulations obtained from a single sample representative for 26 samples (left) and CD3⁺TCR $\gamma\delta$ ⁺ subpopulations obtained from the simultaneous analysis of 26 samples (right). **(I)** Dot plots visualizing the frequency of MNC with samples ordered according to the log2 of days after birth. Samples derived from patients younger than 1.5 years of age colored in blue and those older than 1.5 years of age in red. A loess curve (span = 1) was fitted through the data in order to visualize the trend.

thymocytes within the CD3⁺TCR $\gamma\delta$ ⁺ cells (**Figure 5F**), which corresponded well to the manually detected populations and proved our approach to be equally or more performant compared to current algorithms (**Figure 5G**). Moreover, our approach allowed for enrichment of rare populations as clustering was performed on all 26 samples simultaneously (**Figure 5H**), which is often hampered during manual gating. Despite the robust detection of these $\gamma\delta$ T cell subpopulations, no significant age-dependent effects could be observed within each subpopulation following the onset of thymic involution (**Figure 5I**). This lack of age-dependent effects within populations that constitute both the mature $\alpha\beta$ and $\gamma\delta$ T cell further confirmed the lack of observed variation in frequency within the developing T-lineage cells.

DISCUSSION

The lack in improved therapies that are directed at the recovery of T cell development following hematopoietic stem and progenitor cell transfer in the case of immunodeficiencies or malignancies can, in part, be attributed to the limited understanding of how aging affects this biological process. In order to study these age-dependent effects, we collected the MNC fraction from 26 thymic biopsies and the CD34⁺ fraction from an additional nine thymic biopsies, ranging from birth to puberty, obtained from patients undergoing cardiac surgery. Using multi-color flow cytometry, we assessed the frequencies of T-lineage developmental intermediates within the CD34⁺ fraction up until the SP CD4⁺ and SP CD8 β ⁺ cells, as well as the frequencies of $\gamma\delta$ T cells and thymic non T-lineage subsets, which fulfill a supportive role in maintaining thymic homeostasis. Analysis of these data was done computationally by subsampling the data prior to performing consecutive rounds of Leiden clustering, thereby allowing simultaneous isolation of these populations in all samples, while circumventing batch effects. We were able to validate the performance of our computational pipeline using these flow cytometric datasets. In turn, this allowed assessment of age-dependent effects on immune subsets within the human thymus, revealing the human thymus to consistently generate both $\alpha\beta$ and $\gamma\delta$ T cells and their developmental intermediates despite thymic involution. Moreover, cell types involved in maintenance of thymic homeostasis did not display significant variation, except for B cells which increase in frequency as previously reported (27).

The increasing complexity of data obtained by biologists has necessitated the development of advanced analysis methods. In the field of flow cytometry, this has resulted in the generation

of algorithms such as flowSOM (26), which implements self-organizing maps to reduce the number of observations and effectively decrease computational costs, and PhenoGraph (25), which approximates the data using KNN-graphs and subsequently clusters using the Louvain algorithm allowing the analysis of thousands of individual cells. Recently, single cell RNAseq has become increasingly popular, spurring the development of even more efficient clustering methods such as the Leiden clustering (21), dimensionality reduction techniques such as UMAP (22) and subsampling methods that preserve the topology of the original data, such as geometric sketching (20). Here, we combined these methods to perform an initial subsampling of the flow cytometric data, effectively reducing the computational cost, followed by the generation of a KNN-graph and Leiden clustering. The initial subsampling was validated using manually defined subpopulations, demonstrating that neither random subsampling nor geometric sketching negatively influences the approximation of population-based frequencies, confirming the validity of this initial step which is essential to reduce computational costs. Future implementation of these methods will depend on the research question at hand as random subsampling does not alter population frequencies, thereby resulting in a slightly more reliable approximation of population frequencies while being limited in the recovery of rare populations. This contrasts with geometric sketching that enriches for rare populations. By implementing both methods for validating Leiden clustering, we were able to recover highly distinct, yet rare subpopulations of non T-lineage populations that recapitulated the manually identified subsets. In addition, Leiden clustering was capable of isolating developmental intermediates within the CD34⁺ thymocyte fraction and the MNC fraction, as well as subsets of mature $\alpha\beta$ and $\gamma\delta$ T cells in the MNC fraction. This graph-based clustering approach appeared to be advantageous to overcome biological differences, demonstrating robust identification of for instance lin[−]CD44⁺CD1a[−] and lin[−]CD44[−]CD1a⁺ populations despite clear inter-sample variation. Moreover, batch effects, attributable to technical variations, could be overcome by implementing linear regression, effectively enabling simultaneous analysis of all CD34⁺ samples. In conclusion, our analysis pipeline can overcome technical effects by enabling batch correction, allowing the simultaneous analysis of multiple samples, while limiting the computational cost by subsampling and relying on KNN-graphs that open the possibility of graph-based clustering which enables the robust identification of both distinct and gradually developing immune subsets.

The human thymus has been reported to undergo involution in two phases, with the first one characterized by a decrease in cellularity from 6 months onwards and already undergoing histological changes following the first year of childhood. Therefore, we assessed age-dependent variation within populations frequencies of non T-lineage cells which revealed an increase in the B cell frequency following the onset of thymic involution and which was previously reported to be involved in thymic immune surveillance during aging (27). These findings did not only validate our data quality, despite the modest statistical power, but also revealed a biologically relevant timepoint within our samples age range which coincided with the previously reported histological changes. Rather than describing potential fluctuations within our data, as previously done (14), we assessed whether the onset of thymic involution influences thymocyte population frequencies by defining discrete groups based on the increase in B cell frequency. This effectively disregarded fluctuations in population frequencies attributable to childbirth within the first month of life. In contrast to B cells, myeloid population frequencies remained stable regardless of age, in line with earlier findings based on scRNAseq data (28) and consistent with their role in antigen presentation and maintenance of thymic homeostasis. Indeed, detailed analysis of the immature thymocyte populations within the CD34⁺ fraction did not reveal age-dependent fluctuations in their corresponding frequencies. In contrast, both the lin⁻CD34⁺CD1a⁻ and lin⁻CD34⁺CD1a⁺, and the lin⁻CD44⁺CD1a⁻ and lin⁻CD44⁺CD1a⁺ populations were found to be significantly different from each other regardless of their age, suggesting that aging does not affect the distribution of committed CD34⁺ progenitors. In addition, assessment of the MNC fraction did not reveal significant changes in population frequencies of T cell developmental intermediates, despite the fact that the human thymus is characterized by changes in structural integrity from the age of 1 year and onwards. Furthermore, no age-dependent changes could be observed within the mature $\alpha\beta$ and $\gamma\delta$ T cells subpopulations. Together, even though the human thymus undergoes structural changes during childhood and is characterized by a decrease in cellularity, our findings suggest that this does not result in a disruption or significant disturbance in the production of T cells, nor in changes in the kinetics or survival/proliferation of specific developmental intermediates. This may therefore imply that therapeutic efforts, directed at the recovery of T cells in case of immunocompromised children, should not only be focused on the T cells themselves, but also on their microenvironment which possibly limits their numbers due to niche availability.

METHODS

Isolation of MNC and CD34⁺ Progenitors From Postnatal Thymus

Thymus from patients undergoing cardiac surgery were obtained and used according to and with the approval of the Medical Ethical Commission of Ghent University Hospital (Belgium). The thymus tissue was mechanically disrupted to obtain a single cell suspension. After overnight incubation at 4°C, MNC

were derived from the cell suspension using a Lymphoprep density gradient and cryopreserved. For flow cytometry analysis of CD34⁺ thymocytes, frozen MNC fractions derived from all samples were thawed for CD34 enrichment using magnetic activated cell sorting (Miltenyi Biotec) according to the instructions of the manufacturer prior to staining. For flow cytometry analysis of developmental intermediates of T-lineage, mature T and non-T lineage populations, frozen MNC fractions from 26 samples were thawed for direct staining.

These samples were stained for flow cytometric analysis using the following antibodies: anti-CD34 PerCP-eFluor710 (Cat# 46-0349-42, RRID:AB_2016673), anti-CD4 FITC (Cat# 11-0042-82, RRID:AB_464896), anti-CD7 Alexafluor700 (Cat# 561603, RRID:AB_10898348), anti-CD3-APC (Cat# 300411, RRID:AB_314065), anti-CD14-APC (Cat# 130-110-576, RRID:AB_2655048), anti-CD19-APC (Cat# 17-0193-82, RRID:AB_1659676), anti-CD56-APC (Cat# 341025, RRID:AB_400558), anti-CD123 PE-Cy7 (Cat# 306009, RRID:AB_493577), anti-CD44 PE (Cat# 12-0441-82, RRID:AB_465664), anti-CD5 AmCyan (Cat# 555350, RRID:AB_395754), anti-CD1a Pacific Blue (Cat# 48-0019-42, RRID:AB_1907358), anti-CD45 PerCP-Cy5.5 (Cat# 304027, RRID:AB_1236444), anti-CD19 FITC (Cat# 302205, RRID:AB_314235), anti-HLA-DR APC-Cy7 (Cat# 327017, RRID:AB_2566388), anti-CD1c AlexaFluor700 (Cat# 331529, RRID:AB_2563656), anti-CLEC9A APC (Cat# 353805, RRID:AB_2565518), anti-CX3CR1 PE (Cat# 355703, RRID:AB_2561680), anti-CD14 FITC (Cat# 367115, RRID:AB_2571928), anti-CD16 PE (Cat# 561313, RRID:AB_10643606), anti-CD11c Pacific Blue (MHCD11C28, RRID:AB_10375450), Anti-CD28 PerCP-Cy5.5 (Cat# 302921, RRID:AB_2073719), anti-CD69 FITC (Cat# 310903, RRID:AB_314838), anti-CD3 APC-Cy7 (Cat# 300425, RRID:AB_830754), anti-CD27 AlexaFluor700 (Cat# 356415, RRID:AB_2562515), anti-TCR $\alpha\beta$ APC (Cat# 306717, RRID:AB_10612747), anti-CD25 PE-Cy7 (Cat# 25-0259-42, RRID:AB_1257140), anti-CD8 β PE (Cat# 641057, RRID:AB_1645747), anti-CD4 Amcyan (Cat# 300501, RRID:AB_314069), anti-CD1a Pacific Blue (Cat# 48-0019-42, RRID:AB_1907358), anti-CD34 PerCP (Cat# 343519, RRID:AB_1937270), anti-V δ 1 APC (Cat# 130-119-145, RRID:AB_2733450), anti-V δ 2 PE-Cy7 (Cat# 130-111-129, RRID:AB_2653979), anti-TCR $\gamma\delta$ PE (Cat# 130-109-357, RRID:AB_2654033), anti-V γ 9 Pacific Blue (Cat# 130-107-486, RRID:AB_2653945).

Data Preprocessing

Flow cytometric data was exported and loaded into FlowJo, where the data underwent compensation and biexponential transformation. Using manually defined gates, doublets (FSC-A vs. FSC-W and SSC-A vs. SSC-W), dead cells and debris (FSC-A vs. SSC-A) were excluded from the analysis. For the immunophenotyping of CD34⁺ thymocytes, CD3⁺CD14⁺CD19⁺CD56⁺ lineage cells were also excluded from the analysis. In the case of monocyte and macrophage staining, CD11c⁺CD123⁺ lineage cells were excluded. In addition, all the non T-lineage mature cells were gated for CD45 expression.

From the flowjo workspaces, these preprocessing gates were isolated using the flowWorkspace library in R. Subsequently, each exported fcs file was loaded in R using the read FCS function from the FlowCore library and unwanted cells, based on either gating or because these cells were out the detection limit, were removed. Next, using functions from the FlowCore library, samples were compensated using the compensation function and biexponentially transformed using the logicleTransform function which uses fixed parameters in contrast to the biexponential transformation in FlowJo. For each antibody panel all samples were combined in a Single Cell Experiment object. Finally, z-scores were calculated from these transformed data, allowing the usage of FSC-A and SSC-A during subsampling and KNN-graph generation. For these subsequent steps, specific markers were selected from each panel based on their performance. From the panel used to stain CD34⁺ thymocytes, the CD34, CD44, CD7, CD5, and CD123 markers were retained; from the monocyte/macrophage panel, the CD14, CD16, and HLA-DR markers, in addition to FSC-A and SSC-A parameters were retained; from the B cell and DC panel, the CX3RC1, HLA-DR, CD19, CD1c, and CD123 markers, in addition to the FSC-A and SSC-A parameters were retained; from the $\gamma\delta$ T cell panel, the TCR $\gamma\delta$, V γ 9, V δ 1, and V δ 2 markers, in addition to the FSC-A and SSC-A parameters were retained; and from the $\alpha\beta$ T cell panel, the CD4, CD8 β , TCR $\alpha\beta$, CD3, CD69, CD1a, and CD28 markers were retained.

In case batch correction was performed, the lmFit and residuals.MArrayLM functions from the limma package (38) were used. In order to improve computational efficiency, each sample needed to be subsampled. This was done in both a randomized manner using the sample function and by running the geometric sketching function (20) directly on the transformed data in R using the reticulate package, which enables usage of python modules in R.

For visualization purposes, the data was transformed using the scale_x_flowJo_biexp and scale_y_flowJo_biexp functions from the ggcyto library to allow comparisons between populations derived from manual analysis and computational analysis.

Data Analysis

These subsampled data were used to generate a UMAP (22) using the uwot library in R. This was done by feeding the z-scores directly in the umap function which ran with the ret_nn and ret_model variables enabled and with “fgraph” specified for the ret_extra variable. The resulting UMAP was used to visualize the data while the connectivities matrix was used for clustering. In order to perform Leiden clustering, the connectivities were used to generate a directed igraph object using reticulate and subsequently used in the find_partition function from the leidenalg module while specifying the RBConfigurationVertexPartition function as partition_type in R using reticulate.

Benchmarking

The performance of our method was benchmarked using well established methods, such as FlowSOM and PhenoGraph. The

FlowSOM pipeline was run using a symmetric 1225 node self-organizing map (SOM). Metaclusters were detected using the MetaClustering function, specifying the method variable as metaClustering_consensus, allowing automatic cluster detection, and the metaClustering_consensus function. For both clustering approaches a maximum of 50 clusters was requested. In order to visualize the FlowSOM results each node was given equal size using the UpdateNodeSize function and plotted using the PlotPies function.

PhenoGraph was run using standard settings. Data visualization was achieved by generating a tSNE without preprocessing the data by principal component analysis.

The performance each method was compared by calculating the F measure for each sample using the manual gating as a reference. This was done using the ConfusionMatrix function, specifying the mode as “prec_recall,” from the caret package.

Calculation of Frequencies

From the data subsampled using geometric sketching, each sample was subdivided in 10 equal bins. Using the MiniBatchKmeans function from the sklearn module, these bins were then clustered into groups of approximately 500 cells. Using these clusters as a reference, the sampling bias resulting from geometric sketching was calculated. The coefficient of determination (R^2) used to compare frequencies prior to and after subsampling was calculated using the stat_poly_eq function from the ggpmisc library.

Inference of Populations Using Logistic Regression

In order to transfer the subpopulations identified within the SP CD8 β ⁺, logistic regression was performed using the LogisticRegression function from the sklearn python module in R using reticulate. The best parameters were assessed using the GridSearchCV function from the sklearn python module in R using reticulate. Following classification of the SP CD4⁺ using logistic regression, the accuracy defined as $\frac{TP+TN}{TP+TN+FN+FP}$, sensitivity $\frac{TP}{TP+FN}$ and specificity $\frac{TN}{TN+FP}$ were calculated.

Statistical Analysis

Differences between discrete age groups were tested using linear regression where the day on which the samples were acquired on the flow cytometer was taken into account as a confounding variable. Differences between populations were tested using a paired *t*-test. The *p*-values were corrected for multiple testing using the p.adjust function.

Data Visualization

All data was visualized using the ggplot2 library. Loess curves and boxplots were also generated using the ggplot2 library.

Software

Data analysis was done using R (version 3.6.3) and python (version 3.7.3), while manual gating was done using FlowJo (version 10.0.7).

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

Thymus from patients undergoing cardiac surgery were obtained and used according to and with the approval of the Medical Ethical Commission of Ghent University Hospital (Belgium).

AUTHOR CONTRIBUTIONS

BV performed experiments. ML performed bioinformatics analysis, designed the study, and wrote the paper. KL designed the study, wrote the paper, and supervised the study. TT designed the study, wrote the paper, supervised the study, and acquired funding. BV and GL provided expertise. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2020.01659/full#supplementary-material>

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Administration of Amyloid Precursor Protein Gene Deleted Mouse ESC-Derived Thymic Epithelial Progenitors Attenuates Alzheimer's Pathology

Jin Zhao^{1,2}, Min Su³, Yujun Lin³, Haiyan Liu³, Zhixu He^{2,4*} and Laijun Lai^{3,5*}

¹ Guizhou Provincial Key Laboratory for Regenerative Medicine, Tissue Engineering and Stem Cell Research Center, Department of Immunology, School of Basic Medical Sciences, Guizhou Medical University, Guiyang, China, ² Key Laboratory of Adult Stem Cell Translational Research, Chinese Academy of Medical Sciences, Guiyang, China, ³ Department of Allied Health Sciences, University of Connecticut, Storrs, CT, United States, ⁴ Department of Pediatrics, Affiliated Hospital of Zunyi Medical University, Zunyi, China, ⁵ University of Connecticut Stem Cell Institute, University of Connecticut, Storrs, CT, United States

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Edited by:

Nicolai Stanislas Van Oers,
University of Texas Southwestern
Medical Center, United States

Reviewed by:

Liqi Li,
National Institutes of Health (NIH),
United States
Dong-Ming Su,
University of North Texas Health
Science Center, United States

*Correspondence:

Zhixu He
hzx@gmc.edu.cn
Laijun Lai
laijun.lai@uconn.edu

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Alzheimer's disease (AD) is a devastating neurodegenerative disorder and the most common cause of dementia in older adults. Although amyloid-beta (A β) plaque deposition and chronic neuroinflammation in the central nervous system (CNS) contribute to AD pathology, neither A β plaque removal nor anti-inflammatory therapy has shown much clinical success, suggesting that the combinational therapies for the disease-causative factors may be needed for amelioration. Recent data also suggest that systemic immunity in AD should be boosted, rather than suppressed, to drive an immune-dependent cascade needed for A β clearance and brain repair. Thymic epithelial cells (TECs) not only play a critical role in supporting T cell development but also mediate the deletion of autoreactive T cells by expressing autoantigens. We have reported that embryonic stem cells (ESCs) can be selectively induced to differentiate into thymic epithelial progenitors (TEPs) *in vitro* that further develop into TECs *in vivo* to support T cell development. We show here that transplantation of mouse ESC (mESC)-TEPs into AD mice reduced cerebral A β plaque load and improved cognitive performance, in correlation with an increased number of T cells, enhanced choroid plexus (CP) gateway activity, and increased number of macrophages in the brain. Furthermore, transplantation of the amyloid precursor protein (APP) gene deleted mESC-TEPs (APP^{-/-}) results in more effective reduction of AD pathology as compared to wild-type (APP^{+/+}) mESC-TEPs. This is associated with the generation of A β -specific T cells, which leads to an increase of anti-A β antibody (Ab)-producing B cells in the spleen and enhanced levels of anti-A β antibodies in the serum, as well as an increase of A β phagocytosing macrophages in the CNS. Our results suggest that transplantation of APP^{-/-} human ESC- or induced pluripotent stem cell (iPSC)-derived TEPs may provide a new tool to mitigate AD in patients.

Keywords: Alzheimer's disease, amyloid-beta, thymic epithelial cells, embryonic stem cells, amyloid precursor protein, T cells

INTRODUCTION

Alzheimer's disease (AD) is a devastating age-related neurodegenerative disorder, affecting over 34 million people worldwide (1). AD is characterized by progressive loss of memory and cognitive functions (1–5). The cognitive decline in AD is associated with hallmark protein aggregates, amyloid-beta ($A\beta$) plaques and neurofibrillary tangles, which are accompanied by neuroinflammation, and synaptic and neuronal loss (1–5). $A\beta$ plaques play a central role in the pathogenesis of AD and are generated from proteolytic cleavage of amyloid precursor protein (APP) (6, 7). $A\beta$ can accelerate neuronal cell death and neuronal tangle formation, affect synaptic function adversely and eventually cause neuron loss (2–4, 8). The accumulated $A\beta$ plaques and neuroinflammation have led to numerous attempts over the years to treat AD, either by removing the $A\beta$ plaques, or by systemic anti-inflammatory drug administration to arrest brain inflammation. However, the drugs tested thus far for AD have largely failed in the clinic (4, 9–12). These failures suggest that, although removal of $A\beta$ plaques may be important, this approach alone is not enough to arrest or reverse cognitive loss. Furthermore, recent data also suggest that mitigating neuroinflammation in AD necessitates stimulation, rather than suppression of the immune system, to drive an immune-dependent cascade needed for the $A\beta$ clearance and brain repair (4, 13, 14). It has been shown that anti-inflammation is an active mechanism mediated by recruitment of circulating immune cells to sites of brain pathology (15–17). In addition, systemic immune deficiency is associated with cognitive dysfunction (18) and accelerated AD pathology (19, 20).

T cells are the major component of the immune system. Multiple lines of evidence have suggested that T cells play an important role in the CNS maintenance and repair. For example, systemic T cell deficiency is associated with increased neuronal loss in animal models of CNS injury or AD (14, 19, 21). Systemic T cells not only participate in CNS repair, but are also needed for life-long brain plasticity (18, 22, 23). Both T cells and monocyte-derived macrophages recognizing brain antigens are required for coping with and helping heal brain damage during central nervous system (CNS) injuries (24–29). T cells present in the periphery play an important role in adaptive–innate immunity cross-talk and help in CNS repair (16, 19, 20). Furthermore, it has been suggested that autoreactive T cells that recognize CNS-specific antigens augment the recruitment of monocyte-derived macrophages to the brain (14, 26).

The thymus is the primary organ for T cell generation. It, however, undergoes age-dependent thymic involution, resulting

in decreased numbers of T cells in the elderly. This reduction has direct etiological linkages with many diseases (30–34), including acceleration of the development and progression of AD (19). T cell development in the thymus depends on the thymic microenvironment, in which thymic epithelial cells (TECs) are the major component (35–41). However, TECs undergo both qualitative and quantitative loss over time, which is believed to be the major factor responsible for age-dependent thymic involution (30–34). It is well-known that embryonic stem cells (ESCs) have the dual ability to propagate indefinitely *in vitro* in an undifferentiated state and to differentiate into many types of cells (42). We have reported that ESCs can be selectively induced to generate TEPs *in vitro* (43–46). When transplanted into young or old mice, the ESC-TEPs further develop into TECs, reconstitute the normal thymic architecture, and promote T cell generation, resulting in increased number of functional T cell in the periphery (43–46).

We hypothesized that AD aged mice and patients have a very severe defect in the thymic microenvironment and that transplantation of ESC-TEPs into AD mice would rejuvenate the aged thymic microenvironment, leading to an increased number of functional T cell in the periphery, resulting in attenuated AD pathology. It is well-known that TECs, especially medullary TECs (mTECs), are involved in the deletion of autoreactive T cells. We have demonstrated that transplantation of ESC-TEPs expressing disease-causative self-antigen results in the deletion of the antigen-specific autoreactive T cells (47, 48). Our hypothesis further proposes that transplantation of APP gene-deleted ESC-TEPs would lead to the generation of $A\beta$ -specific autoreactive T cells that could help the production of other $A\beta$ -specific immune cells to clear the $A\beta$ plaques in the CNS.

We show here that transplantation of APP gene deleted ($APP^{-/-}$) or their wild-type ($APP^{+/+}$) mouse ESC (mESCs)-derived-TEPs results in enhanced thymopoiesis, increased T cell number, especially IFN- γ -producing cells, in the periphery, enhanced choroid plexus (CP) gateway activity, and enhanced recruitment of macrophages into the brain. Consequently, these mice have reduced $A\beta$ deposits in the brain and improved cognitive performance. Furthermore, transplantation of $APP^{-/-}$ mESC-TEPs has a greater effect than that of $APP^{+/+}$ mESC-TEPs in clearance of $A\beta$ deposits in the CNS and reversal of cognitive decline. This is related to the generation of $A\beta$ -specific T cells, increased numbers of anti- $A\beta$ antibody (Ab)-producing B cells in the spleen, increased levels of anti- $A\beta$ Ab in the serum, and enhanced function of macrophages to phagocytose $A\beta$ in the brain. Our results suggest that human ESC (hESC)- or induced pluripotent stem cell (iPSC)-derived TEPs, especially $APP^{-/-}$ hESC or iPSC-TEPs, may serve as a novel tool to modify AD pathology.

MATERIALS AND METHODS

Mice

3xTg-AD, APP/PS1, C57BL/6 (B6) mice were purchased from Jackson Laboratory. The mice were used in accordance with a protocol approved by the Institutional Animal Care and Use Committee of the University of Connecticut.

Abbreviations: AD, Alzheimer's disease; $A\beta$, amyloid-beta; CNS, central nervous system; TECs, thymic epithelial cells; ESCs, embryonic stem cells; TEPs, thymic epithelial progenitors; CP, choroid plexus; APP, amyloid precursor protein; Ab, antibody; iPSCs, induced pluripotent stem cell; CRISPR, clustered Regularly Interspaced Short Palindromic Repeats; Cas9, CRISPR-associated protein; qRT-PCR, real-time qualitative RT-PCR; K5, keratin 5; K8, keratin 8; NOR, Novel object recognition; sA β , soluble $A\beta$ protein; i.t., intrathymically; WT, wild-type; Ctrl, control cell; DG, dentate gyrus; GFAP, glial fibrillary acid protein; icam1, intercellular adhesion molecule 1; ccl2, chemokine C-C motif ligand 2; vcam1, vascular cell adhesion molecule 1; cxcl10, C-X-C motif chemokine 10; SRA1, scavenger receptor A.

Cell Culture

B6 mESC line (from Cyagen, Santa Clara, CA) were cultured in ESGRO Complete Plus Serum-free Clonal Grade Medium with GSK3 β inhibitor supplement (Millipore, Temecula, CA). For TEP differentiation, mESCs were first induced to differentiate into definitive endoderm, and then TEPs in the presence of BMP-4, FGF 7, FGF10, and EGF, as well as rFOXN1 and rHOXA3 protein as we previously described (43).

Genome Editing

The APP gene in mESCs was knocked out by the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated protein (Cas9) genome editing. B6 mESCs were transfected with APP-specific double nickase plasmids or control double nickase plasmids (from Santa Cruz Biotechnology). The cells were screened to obtain APP^{-/-} and APP^{+/+} mESCs. The information of the plasmids and gRNA sequences are shown in **Supplemental Figure 1**.

Intrathymic Injection

Mice were anesthetized and injected with 5×10^4 cells in 10–20 μ l PBS into the thymus posterior to the upper sternum using a 26–28 gauge needle as described (49).

Reverse Transcription Polymerase Chain Reaction (RT-PCR) and Real-Time Qualitative RT-PCR (qRT-PCR)

Total RNA was extracted from tissues or cells using a Nucleo Spin RNA II kit (Macherey-Nagel, Düren, Germany). The RNA was converted into complementary DNA using High Capacity cDNA Reverse Transcription Kit (Invitrogen, USA). RT-PCR was performed with GoTaq[®] Green Master Mix (Promega, USA). qRT-PCR was performed with the Power SYBR green master mix (Applied Biosystems, UK) using the 7500 real-time PCR system (Applied Biosystems, UK). The primer sequences are shown in **Supplemental Table 1**.

Western Blot Analysis

GFP⁺ mESC-TECs were purified from the thymocytes using a magnetic-activated cell sorter immunomagnetic separation system (Mitenyi Biotec). The cells were collected and lysed. Equal amounts of denatured proteins were loaded onto a 4–12% Bis-Tris gel (Invitrogen, Carlsbad, CA), electrophoresed and transferred onto a PVDF membrane (Invitrogen). The membranes were blocked with 5% nonfat milk in TBST (mixture of Tris-Buffered Saline and Tween 20), and incubated with anti-mouse APP monoclonal antibody (Invitrogen) at 4 degree overnight. The membranes were then incubated with goat anti-mouse IgG HRP-conjugated secondary antibody and developed with a SuperSignal West Pico chemiluminescence substrate (Thermo Scientific, Rockford, IL).

Immunohistochemistry

The brain tissues were incubated in a fixative solution, embedded in OCT medium, snap frozen, and subsequently cut into 6 μ m sections. The cultured cells were incubated with primary antibodies. The following primary antibodies were used: mouse

anti-A β (clone 6E10,) and rabbit anti-GFAP (Biolegend, USA). After washing, the sections were incubated with fluorochrome-conjugated secondary antibody, counterstained with 4', 6'-diamidino-2-phenylindole (DAPI) and observed under a Nikon A1R Spectral Confocal microscope (Nikon, Kanagawa, Japan). To quantify the staining intensity, total cells and background fluorescence intensity were measured using ImageJ software (NIH, USA), and the intensity of specific staining was calculated as described (4).

Flow Cytometry Analysis

To analyze TECs, the thymi were incubated at 37°C in 0.01 (w/v) liberase (Roche, Nutley NJ) and 0.02% (w/v) DNase I (Roche) with regular and gentle agitation as described (50). A single-cell suspension of tissues was stained with fluorochrome-conjugated antibodies directly or indirectly as described (51). For intracellular staining, the cells were first permeabilized with a BD Cytfix/Cytoperm solution for 20 min at 4°C. The following antibodies were used: CD4, CD8, EpCAM1, CD45, CD11b, F4/80, IFN γ , Ly6c, and SRA1 (BioLegend, San Diego, CA, or ThermoFisher Scientific), Keratin (K) 5 (Covance, Dallas, TX), and K8 (US Biological, Salem, MA). The samples were analyzed on an LSRFortessa X-20 Cell Analyzer (BD Biosciences). Data analysis was performed using FlowJo software (Ashland, OR).

T Cell Proliferation Assay

Splenocytes were stained with 5 μ M CFSE (ThermoFisher Scientific) for 15 min. at 37°C. The cells were then cultured in a 96-well flat-bottom plate in the presence of plate-bound A β 40 or A β 42 (Anaspec, USA) and anti-CD3 antibody for 3 days. The cells were then stained with anti-PE labeled-CD4 and APC labeled-CD8 antibodies and analyzed for CFSE levels by T cells using flow cytometry.

ELISA Assay for Anti-A β 40 or Anti-A β 42 Antibody

A β 40 or A β 42 (Anaspec, USA) was coated on 96-well microplates overnight at 4°C, then blocked with blocking buffer (2% BSA+5% goat serum in PBS) for 2 h at room temperature. The serum samples diluted into 1:1000 were added to the plates and incubated 2 h at room temperature. After washing, HRP-conjugated goat anti-mouse IgG (Biolegend) was added to the plates and incubated for 1 h. The reaction was developed by TMB substrate (Thermo Scientific, USA) and stopped with 0.1 N HCl. The microplate was read at 450 nm under a microplate reader (Bio-Tek, ELX800, USA). The antibody concentrations were calculated using a standard curve generated with known concentrations of anti-A β antibody.

Soluble A β Protein (sA β) Isolation and Quantification

Brain parenchyma was dissected, snap-frozen and kept at –75°C until homogenization. The samples were homogenized, and the supernatants were collected and detected for the concentrations of A β _{1–40} and A β _{1–42} by ELISA as described (13).

B Cell ELISpot Assay

MultiScreen-IP plates (Millipore, Billerica, MA) were washed with 70% ethanol, rinsed three times with PBS, coated with A β 40 (4 μ g/ml) or A β 42 (4 μ g/ml) at 4°C overnight. The plates were blocked with blocking buffer (2% BSA in RPMI medium). 1×10^4 splenocytes were added into the plates and incubated for 48 h. The plates were washed 6 times with 0.25% Tween 20 (Sigma, USA) in PBS, incubated with HRP-conjugated goat anti-mouse IgG (H+L) (Biolegend) for 1 h, developed with a DAB Peroxidase Substrate Kit (Vector, USA), and counted for ELISpots (52).

Amyloid Phagocytosis Assay

HiLyte Fluor 647 Beta-Amyloid (1–42) (Anaspec) was resuspended in Tris/EDTA (pH 8.2) at 20 mM and then incubated in the dark for 3 days at 37°C to promote aggregation. Macrophages in suspension were pretreated in low serum medium as described (53). The HiLyte Fluor 647 Beta-Amyloid was added and incubated for 5 h. Cells were stained with macrophage markers; amyloid phagocytosis by the macrophages was determined by flow cytometry (53).

Barnes Maze

Barnes Maze was conducted as previously described (54, 55). Briefly, each mouse was placed in the center of the maze and subjected to aversive stimuli. Mice were trained 4 training trials per day for 5 days, and a probe test was performed 24 h after the last training trial. The latency and number of errors were recorded for the training trial and probe test.

Novel Object Recognition (NOR) Test

A NOR test was conducted as previously described (54–56). Briefly, mice were trained by allowing them to explore two identical objects placed at opposite ends of the arena for 10 min. 24 h later, mice were tested with one copy of the familiar object and one novel object of similar dimensions for 3 min. The time spent on exploring and sniffing of each object was recorded. The NOR index represents the percentage of time mice spent exploring the novel object.

Statistical Analysis

P-values were based on the two-sided Student's *T*-test. A confidence level above 95% ($p < 0.05$) was determined to be significant.

RESULTS

1. Deletion of the APP gene in mESCs to generate APP^{-/-} mESCs and generation of TEPs from APP^{-/-} and APP^{+/+} mESCs *in vitro*.

Since A β is produced from proteolytic cleavage of APP, we deleted the APP gene in mESCs using CRISPR and Cas9 genome editing. B6 mESCs were transfected with APP-specific double nickase plasmids that contain the APP-specific-guide RNAs, and the Cas9 nuclease and GFP genes. The cells were screened in puromycin to

obtain APP^{-/-} mESCs. The gene deletion was confirmed by RT-PCR with one of primers spanning the gRNA region (Figure 1A). The cells that were transfected with control double nickase plasmids containing non-targeting scrambled gRNA, Cas9, and GFP genes were used as a control (APP^{+/+} mESCs).

Both APP^{-/-} and APP^{+/+} mESCs were positive for alkaline phosphatase (AP) activity, indicating that the mESCs were in an undifferentiated state (Figure 1B). We then induced the APP^{-/-} and APP^{+/+} mESCs to differentiate into TEPs *in vitro* following our protocol (43). After the differentiation, both APP^{-/-} and APP^{+/+} mESC-derived cells contained comparable numbers of EpCAM1 positive cells that co-expressed K5 and K8, a phenotype of TEPs (Figure 1C). We purified EpCAM1⁺ TEPs from APP^{-/-} and APP^{+/+} mESC-derived cells and injected an equal number of the TEPs into the thymus of syngeneic mice. Two months later, the mESC-TEPs generated comparable numbers of GFP⁺ mESC-derived TECs that accounted for 51–58% of total TECs.

Western blot analysis showed that purified GFP⁺ APP^{+/+} mESC-TECs expressed APP protein, whereas GFP⁺ APP^{-/-} mESC-TECs did not (Figure 1D and Supplemental Figure 2). Together, these results indicate that the APP gene has been deleted in the APP^{-/-} mESCs, and that the deletion does not affect the differentiation ability of mESCs into TEPs and TECs.

2. Both APP^{-/-} and APP^{+/+} mESC-TEP-transplanted AD mice have an improved cognitive performance, and APP^{-/-} mESC-TEP-transplanted mice perform better than APP^{+/+} mESC-TEP-transplanted mice.

To determine whether transplantation of APP^{-/-} and APP^{+/+} mESC-TEPs affects cognitive performance in AD mice, 3XTg-AD mice aged 12 months, an age of advanced cerebral pathology, were injected intrathymically (i.t.) with APP^{-/-} or APP^{+/+} mESC-TEPs. APP^{-/-} or APP^{+/+} mESC-derived EpCAM1⁻ non-TEPs (control cells) were used as controls. Two months later, the mice were evaluated for spatial learning and memory. It has been reported that the Barnes maze, a hippocampal-dependent spatial task (57, 58), is the most sensitive test for detecting cognitive deficits in 3XTg-AD mice (59). We found that both APP^{-/-} and APP^{+/+} mESC-TEP-treated mice had significantly greater Barnes maze learning curves than control cell-treated mice (Figure 2A). Furthermore, APP^{-/-} mESC-TEP-treated mice performed better than APP^{+/+} mESC-TEP-treated mice, almost reaching the performance level observed in wild-type (WT) non-AD mice (Figure 2A). Since there were no significant differences between APP^{-/-} and APP^{+/+} mESC-EpCAM1⁻ control cell-transplanted mice in all of the results in this paper (data not shown), we pooled the data from the two groups and named this group as control cell (Ctrl)-transplanted mice.

Both APP^{-/-} and APP^{+/+} mESC-TEP-treated mice also had decreased latency to find the target zone during the probe trial conducted 24 h after the final training session (Figure 2B), indicating an improved memory performance. In addition, the number of errors committed in the APP^{-/-} or APP^{+/+} mESC-TEP-treated mice was also significantly reduced, as compared to control cell-treated mice (Figure 2C).

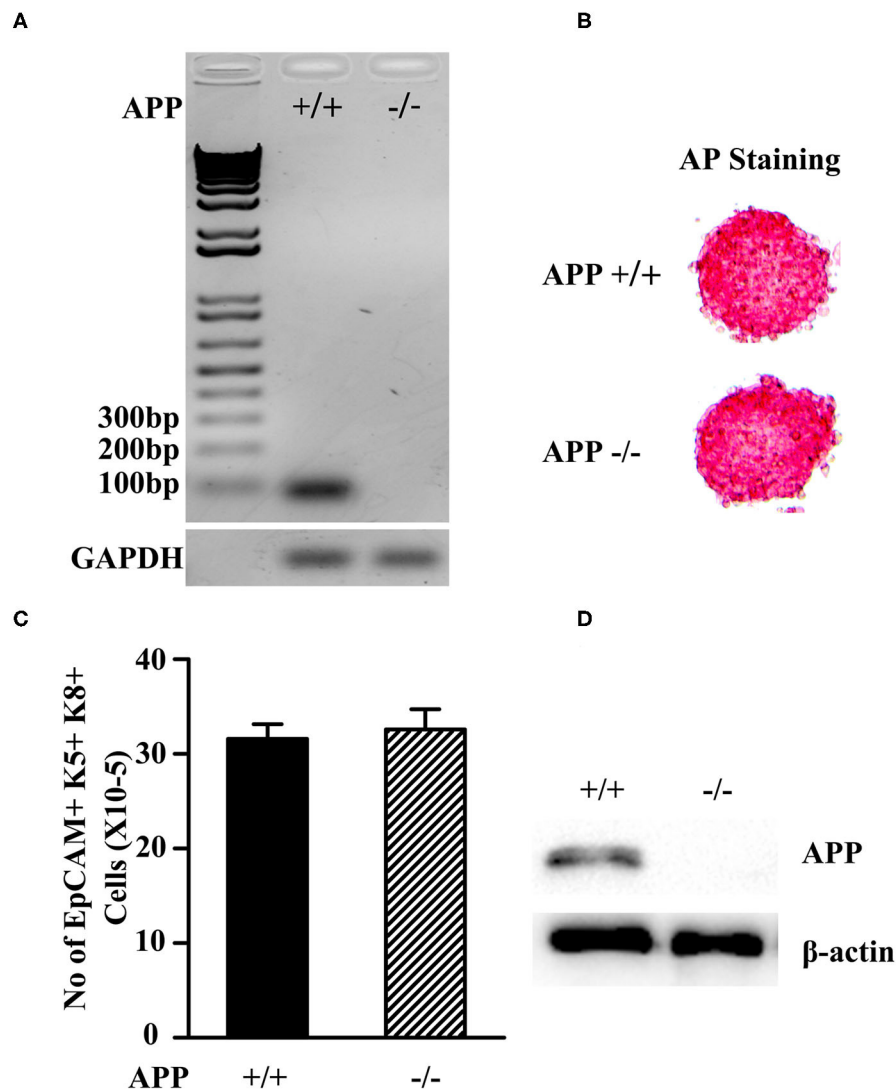


FIGURE 1 | Characterization of APP^{-/-} and APP^{+/+} mESCs. **(A)** The expression of the APP mRNA in APP^{-/-} and APP^{+/+} mESCs was measured by RT-PCR. **(B)** APP^{-/-} and APP^{+/+} mESCs were assessed for the expression of the pluripotent marker AP. **(C)** APP^{-/-} and APP^{+/+} mESCs were induced to differentiate into TEPs *in vitro*. The number of mESC-derived TEPs (EpCAM1⁺K5⁺K8⁺) were analyzed by flow cytometry. **(D)** APP^{-/-} and APP^{+/+} mESC-EpCAM1⁺ TEPs were injected into the thymus of syngeneic C57BL/6 mice. Two months later, GFP⁺ mESC-TECs were purified from the thymus, and analyzed for the expression of APP protein by Western blot. The data are presented from 3 independent experiments.

The NOR test is to study learning and memory in rodents based on their spontaneous tendency to have more interactions with a novel than with a familiar object (60). NOR is a more cortically-dependent novel object recognition preference task (57, 58). In agreement with the results in the Barnes maze task, APP^{-/-} or APP^{+/+} mESC-TEP-treated mice performed significantly better than control cell-treated mice (**Figure 2D**). In all of these studies (**Figures 2A–D**), APP^{-/-} mESC-TEP-treated mice performed significantly better than APP^{+/+} mESC-TEP-treated mice. Together, our data suggest that transplantation of APP^{-/-} or APP^{+/+} mESC-TEPs results in improved spatial learning and memory in 3xTg-AD mice and that transplantation

of APP^{-/-} mESC-TEPs demonstrates greater effectiveness than APP^{+/+} mESC-TEPs.

- Both APP^{-/-} and APP^{+/+} mESC-TEP-transplanted AD mice have reduced AD pathology with greater reduction in APP^{-/-} mESC-TEP-transplanted mice.

We then determined whether transplantation of APP^{-/-} or APP^{+/+} mESC-TEPs leads to improved AD pathology. After the Barnes maze and NOR tests (**Figure 2**), the brains were harvested and immunohistochemical analysis performed. We found that both APP^{-/-} and APP^{+/+} mESC-TEP-transplanted 3XTg-AD mice had a reduced cerebral Aβ plaque

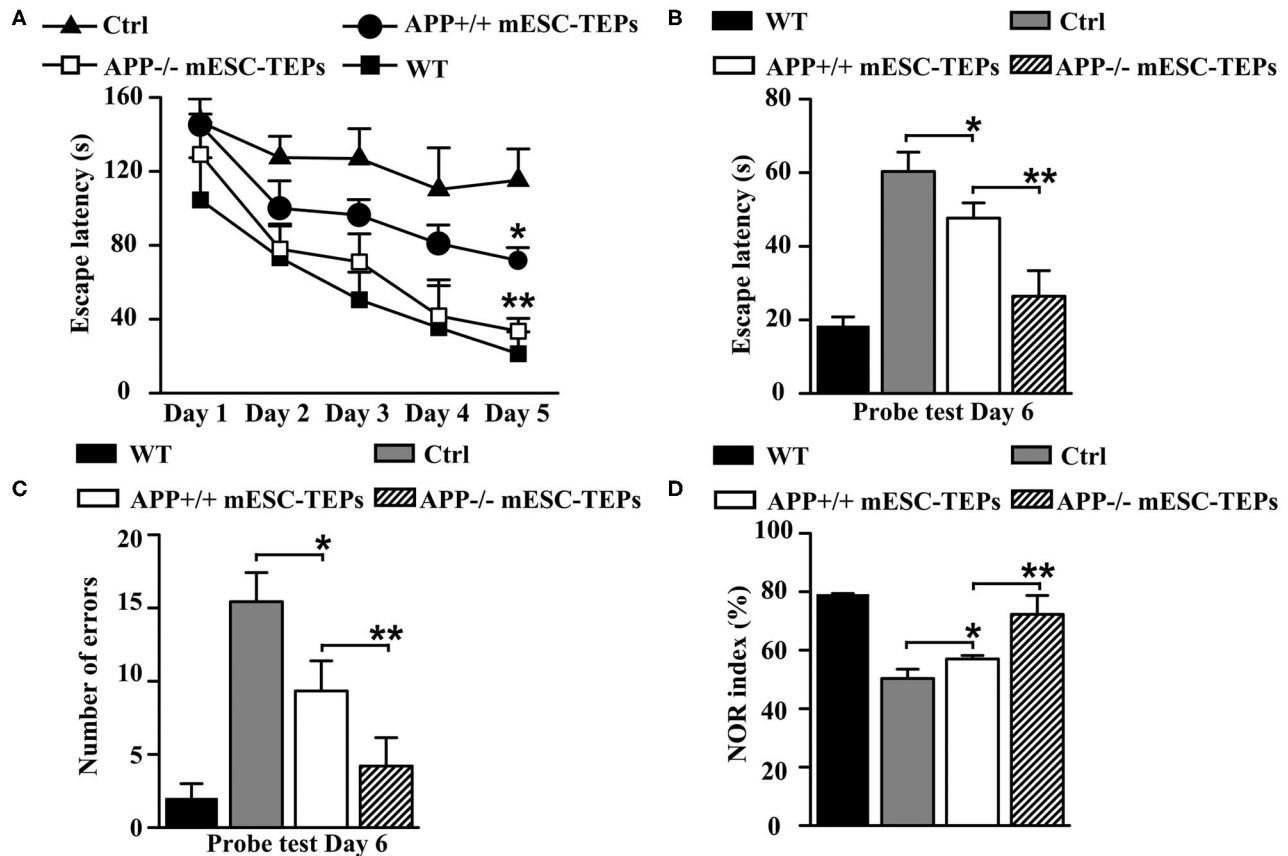


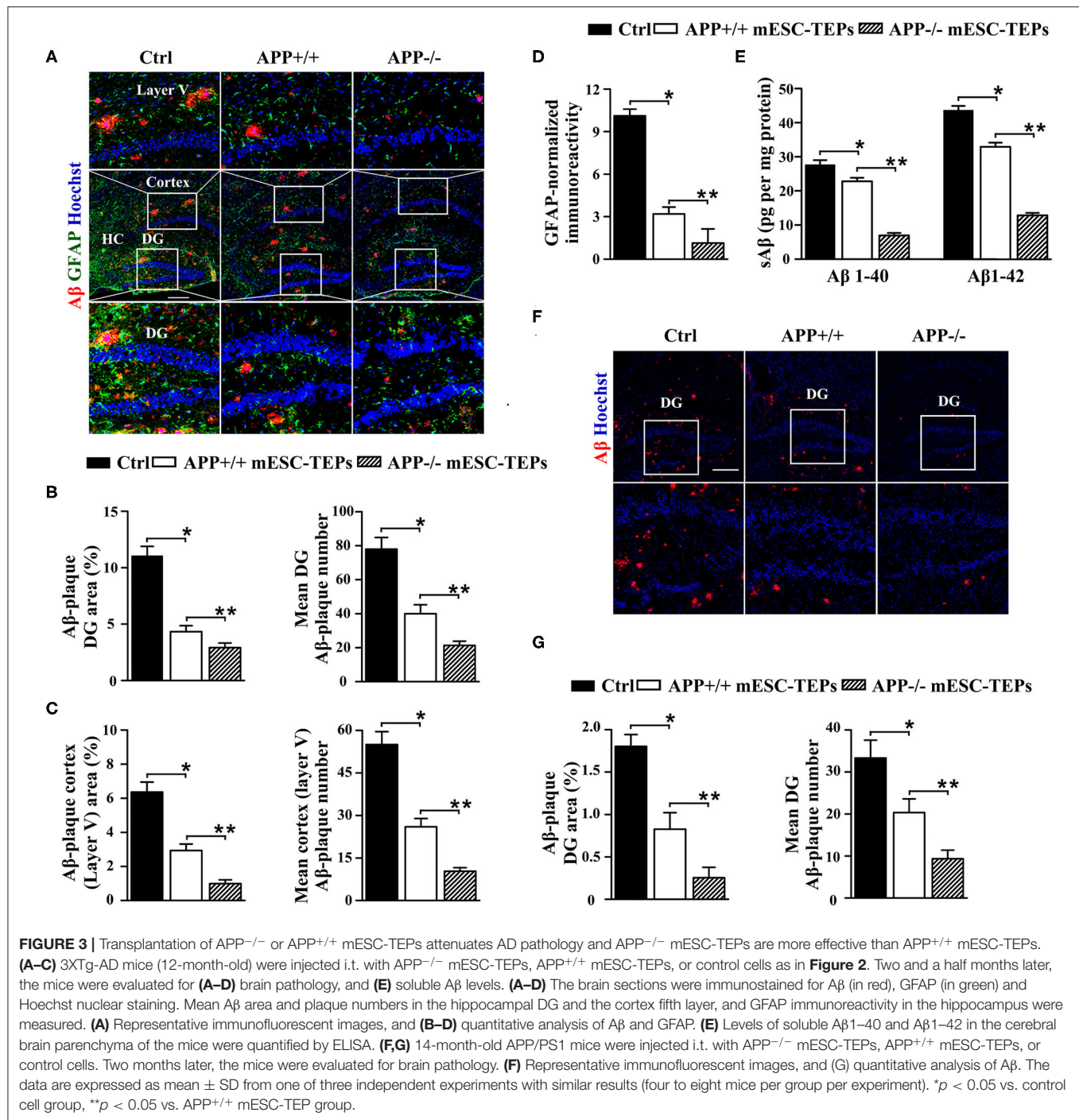
FIGURE 2 | Transplantation of APP^{-/-} and APP^{+/+} mESC-TEPs improves cognitive performance in AD mice and APP^{-/-} mESC-TEPs are more effective than APP^{+/+} mESC-TEPs. **(A–D)** 3XTg-AD mice (12-month-old) were injected i.t. with APP^{-/-} EpCAM1⁺ mESC-TEPs, APP^{+/+} EpCAM1⁺ mESC-TEPs, or control cells (5×10^4 cells/mouse). Age-matched untreated WT mice were also used as controls. Two months later, the mice were evaluated for cognitive performance by Barnes Maze and Object Recognition tests. **(A)** The escape latency during the training period, and **(B,C)** the escape latency and the number of errors committed during the probe trial are shown. **(D)** NOR index was determined as the time spent interacting with the novel object divided by the total time of exploration during the testing phase. The data are expressed as mean $\pm$ SD from one of three independent experiments with similar results (four to eight mice per group in each experiment). * $p < 0.05$ vs. control cell group, ** $p < 0.05$ vs. APP^{+/+} mESC-TEP group.

load in the hippocampus, specifically in the dentate gyrus (DG) and in the cerebral cortex (layer V) (Figures 3A–C), areas showing robust A β -plaque pathology in AD mice. Comparatively, APP^{-/-} mESC-TEP-transplanted mice had the greater reduction in A β -plaque load (Figures 3A–C). Astrogliosis, as assessed by glial fibrillary acid protein (GFAP) immunoreactivity, was also reduced in APP^{-/-} and APP^{+/+} mESC-TEP-treated mice, as compared to control cell-treated mice (Figures 3A,D). Again, APP^{-/-} mESC-TEP-transplanted mice had, by comparison, a greater reduction in GFAP immunoreactivity (Figures 3A,D).

Since impaired synaptic plasticity and memory deficits in AD are associated with elevated cerebral soluble A β 1-40/A β 1-42 (sA β) levels (61), we then measured sA β levels in the AD mice by ELISA. Consistent with the immunohistochemical results, both APP^{-/-} and APP^{+/+}

mESC-TEP-treated mice had reduced cerebral sA β , as compared to control cell-treated mice (Figure 3E). Transplantation of APP^{-/-} mESC-TEPs demonstrated the greater reduction (Figure 3E).

We likewise examined the effect of mESC-TEPs in another AD model, APP/PS1 mice, which develop A β -plaque pathology at a more advanced age than do 3XTg-AD mice. Transplantation of APP^{-/-} or APP^{+/+} mESC-TEPs reduced hippocampal A β plaque load, as compared to control cell-treated mice, and APP^{-/-} mESC-TEP-treated mice had more A β plaque load reduction than APP^{+/+} mESC-TEP-treated mice (Figures 3F,G). Taken together, our results suggest that transplantation of APP^{-/-} or APP^{+/+} mESC-TEPs into AD mice results in clearance of A β plaques and reversal of cognitive decline, and APP^{-/-} mESC-TEP-treated mice perform better than APP^{+/+} mESC-TEP-treated mice.

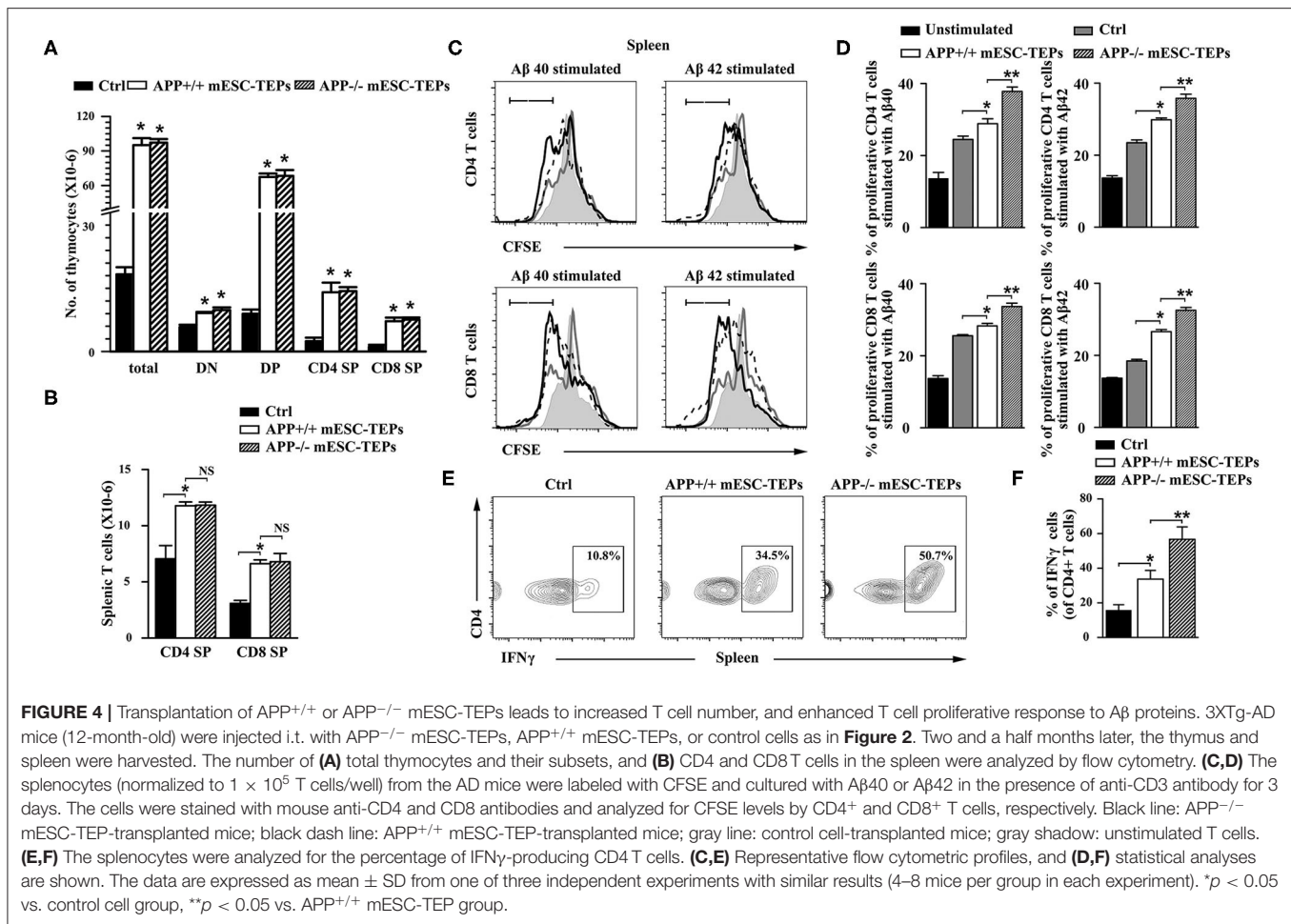


4. Both APP^{-/-} and APP^{+/+} mESC-TEP-transplanted AD mice have increased T cell numbers, and APP^{-/-} mESC-TEP-transplanted mice have enhanced T cell proliferation in response to Aβ stimulation.

We have previously demonstrated that transplantation of mESC-TEPs results in enhanced thymopoiesis and increased T cell numbers in the spleen (43–45). Consistent with the previous reports, APP^{+/+} or APP^{-/-} mESC-TEP-transplanted mice had

increased numbers of thymocytes in the thymus and T cells in the spleen compared to control cell-treated mice. The number of T cells in the thymus and the spleen between APP^{+/+} and APP^{-/-} mESC-TEP-transplanted mice were not significantly different (Figures 4A,B).

Thymocytes can be divided into four major subsets: CD4 and CD8 double negative (DN), double positive (DP), CD4 single positive (SP), and CD8 SP thymocytes. DN thymocytes

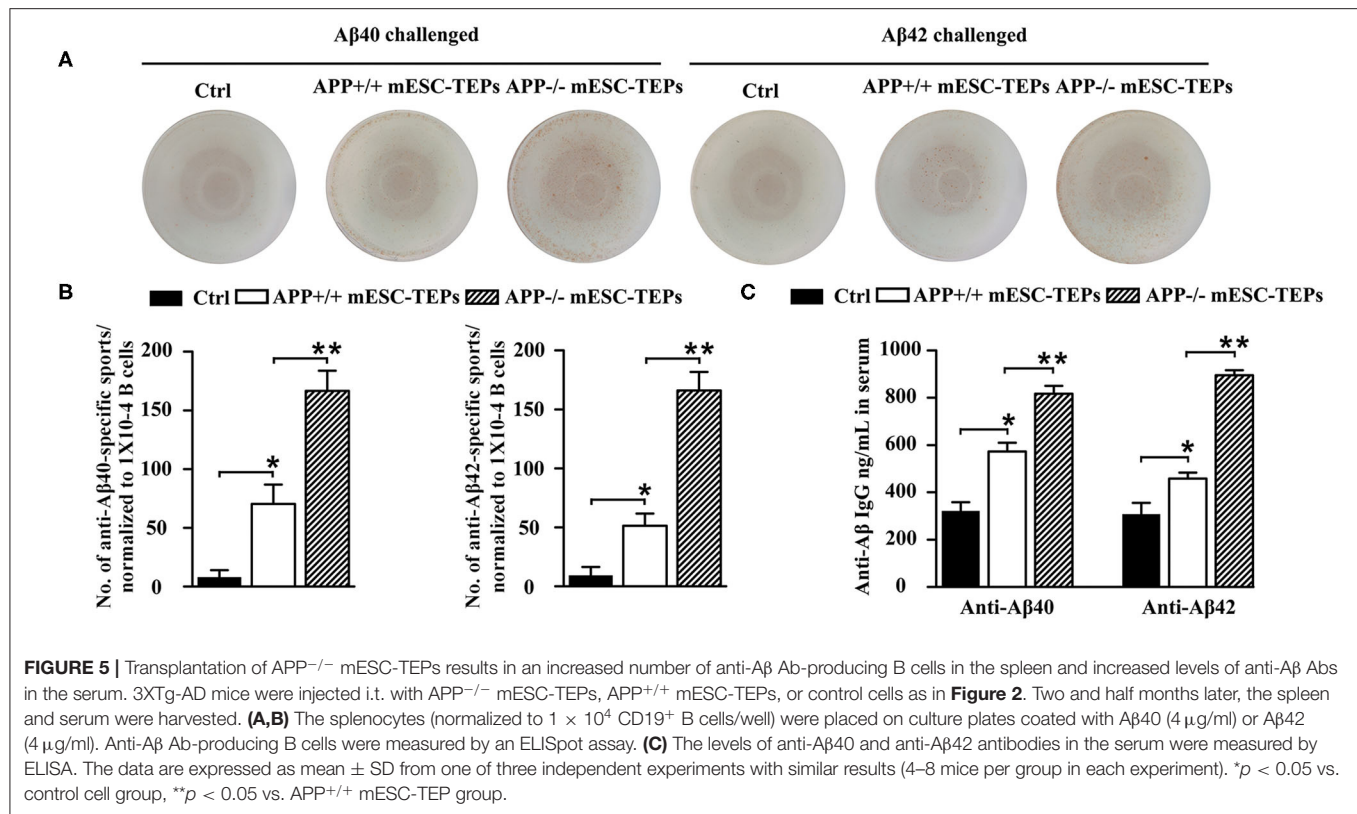


can be further divided into DN1 to DN4 subsets based on the expression of CD44 and CD25. APP^{+/+} or APP^{-/-} mESC-TEP-transplanted mice had decreased percentages of DN subsets (Supplemental Figure 3), suggesting improved thymocyte development. Because APP^{+/+} or APP^{-/-} mESC-TEP-transplanted mice had increased numbers of total thymocytes, and numbers of each thymocyte subsets in these mice were higher than those in control cell-treated mice (Figure 4A and Supplemental Figure 3). We also analyzed the percentage and number of regulatory T cells (Tregs) in the thymus and the spleen. Although the percentages of Tregs were not significant different among the groups, the number of Tregs in APP^{+/+} or APP^{-/-} mESC-TEP-transplanted AD mice was higher than WT or control cell-transplanted AD mice (Supplemental Figures 3, 4). Similarly, the percentages of CD11c⁺ dendritic cells (DCs) and CD19⁺ B cells in the spleen were not significant different among the groups (Supplemental Figure 4).

We then determined the proliferation of the splenic T cells in response to Aβ40 and Aβ42 protein stimulation in the presence of anti-CD3 antibody *in vitro*. The proliferation of CD4 and CD8 T cells from APP^{+/+} mESC-TEP-transplanted mice was slightly higher than that from control cell-transplanted

mice (Figures 4C,D), which may be due to enhanced T cell function after transplantation of mESC-TEPs. Furthermore, the proliferation of both CD4 and CD8 T cells from APP^{-/-} mESC-TEP-transplanted mice was significantly higher than that from APP^{+/+} mESC-TEP-transplanted mice (Figures 4C,D). The latter results suggest that Aβ-specific autoreactive T cells might not be deleted in the thymus of APP^{-/-} mESC-TEP-transplanted mice, leading to presence of Aβ-specific autoreactive T cells in the periphery, resulting in greater proliferative response. Of note, although the proliferation of both CD4 and CD8 T cells in response to anti-CD3 antibody alone (without Aβ40 or Aβ42 peptide) from APP^{+/+} or APP^{-/-} mESC-TEP-transplanted mice was greater than that from control cell-transplanted mice, there was no significant difference between APP^{+/+} and APP^{-/-} mESC-TEP-transplanted groups (data not shown).

It has been reported that PD-1 blockade reduced AD pathology involves an IFNγ-dependent immunological response (4). We then analyzed IFNγ-producing T cells in the spleen and found that the percentage of IFNγ-producing CD4 T cells in APP^{+/+} or APP^{-/-} mESC-TEP-transplanted AD mice was significantly higher than that in control cell-treated mice (Figures 4E,F). This is consistent with our previous reports that transplantation of mESC-TEPs leads to the generation



of functional T cells including enhanced production of IFN γ (44, 45). Compared with APP^{+/+} mESC-TEP-transplanted mice, the percentage of IFN γ -producing CD4⁺T cells in APP^{-/-} mESC-TEP-transplanted mice showed a larger increase, which is probably due to enhanced anti-A β autoimmunity in APP^{-/-} mESC-TEP-transplanted AD mice. Furthermore, more IFN γ was detected in the supernatant of cultured splenocytes from APP^{-/-} mESC-TEP-transplanted AD mice in response to A β 40 or A β 42 protein stimulation (Supplemental Figure 5).

5. APP^{-/-} mESC-TEP-transplanted mice have an increased number of anti-A β Ab-producing B cells in the spleen and increased level of anti-A β Ab in the serum.

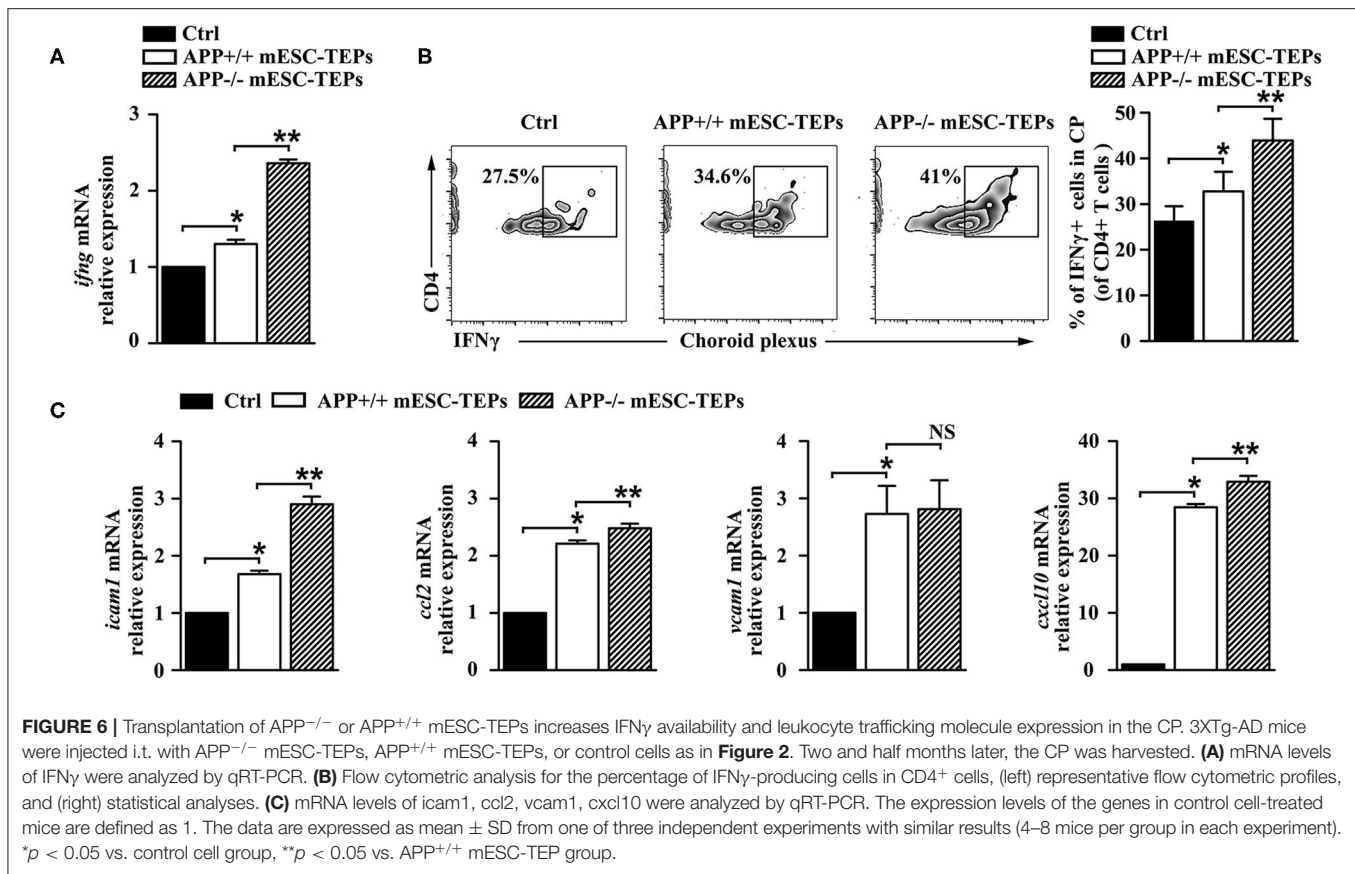
It is well-known that T cells can help B cell functions. We then determined whether the enhanced T cell proliferation to A β stimulation in mESC-TEP-transplanted AD mice led to increased production of anti-A β Ab-producing B cells. We used A β 40 and A β 42 as antigens for an ELISpot assay to measure anti-A β Ab-producing B cells in the spleen. The number of anti-A β Ab-producing B cells in APP^{+/+} mESC-TEP-transplanted mice was higher than that in control cell-transplanted mice, while the number of anti-A β Ab-producing B cells in APP^{-/-} mESC-TEP-transplanted mice was higher than that in APP^{+/+} mESC-TEP-transplanted mice (Figures 5A,B).

We also analyzed the levels of anti-A β Abs in the serum. Consistent with the ELISpot results, the levels of both anti-A β 40 and anti-A β 42 antibodies in the serum of APP^{+/+} mESC-TEP-treated mice were higher than those in control cell-treated

mice, and the levels of these antibodies in APP^{-/-} mESC-TEP-treated mice were significantly higher than those in APP^{+/+} mESC-TEP-treated mice (Figure 5C). The results suggest that increased T cell number in APP^{+/+} or APP^{-/-} mESC-TEP-treated mice, especially A β -specific T cells in APP^{-/-} mESC-TEP-treated mice, help to generate anti-A β Ab-producing B cells that secrete anti-A β Abs into the serum.

6. Transplantation of both APP^{-/-} and APP^{+/+} mESC-TEPs enhances the brain's choroid plexus (CP) activity.

The CP, the epithelial layer that forms the blood-CSF barrier, is a selective gateway for leukocyte entry to the CNS (13). AD mice have a defect in the CP gateway, as indicated by significantly lower levels of leukocyte homing and trafficking molecule expression in the CP (13). In contrast, IFN γ signaling enhances the expression of leukocyte trafficking molecules (15). Since we have demonstrated that IFN γ -producing T cells were increased in the spleen of APP^{-/-} or APP^{+/+} mESC-TEP-treated AD mice (Figure 4E), we analyzed IFN γ availability at the CP in these mice. qRT-PCR analysis revealed a higher IFN γ mRNA expression level in the CP of APP^{-/-} or APP^{+/+} mESC-TEP-transplanted AD mice, compared with control cell-treated mice (Figure 6A). Flow cytometric examination confirmed a significantly higher percentage of IFN γ -producing CD4⁺ immune cells in this compartment in APP^{-/-} or APP^{+/+} mESC-TEP-transplanted AD mice (Figure 6B). Again, the IFN γ mRNA expression levels and the percentage of IFN γ -producing CD4⁺ immune cells in the CP APP^{-/-} mESC-TEP-transplanted AD



mice were higher than those in APP^{+/+} mESC-TEP-transplanted AD mice (**Figures 6A,B**), consistent with the results for the percentage of IFN γ -producing CD4⁺ splenic T cells among the mice (**Figure 4E**).

Since increased IFN γ availability can enhance CP activity in this compartment (4, 13), we analyzed the expression of leukocyte homing and trafficking molecules, including intercellular adhesion molecule 1 (*icam1*), chemokine C-C motif ligand 2 (*ccl2*), vascular cell adhesion molecule 1 (*vcam1*), and C-X-C motif chemokine 10 (*cxcl10*) in the CP. As shown in **Figure 6C**, the mRNA expression levels of these leukocyte trafficking molecules in the CP of APP^{+/+} mESC-TEP-treated AD mice were higher than those in control cell-treated mice. The mRNA expression levels of these molecules (except *vcam1*) in the CP of APP^{-/-} mESC-TEP-treated AD mice were higher than those in APP^{+/+} mESC-TEP-treated mice (**Figure 6C**). These results suggest that administration of APP^{-/-} or APP^{+/+} mESC-TEPs results in an increased CP activity, which is likely due to the increased IFN γ availability in this compartment.

7. APP^{-/-} mESC-TEP-transplanted mice have an increased number of A β phagocytosing macrophages in the brain and the spleen.

Increased CP activity can result in recruitment of monocyte-derived macrophages to the brain to attenuate AD pathology

(4, 13). Since transplantation of APP^{-/-} or APP^{+/+} mESC-TEPs led to an increased CP activity, we analyzed whether there was an increased number of monocyte-derived macrophages in the brain. It has been shown that CD45^{hi}/CD11b⁺ cells represent a myeloid population enriched with CNS-infiltrating monocyte-derived macrophages in the brain (4, 13). We found both APP^{-/-} and APP^{+/+} mESC-TEP-transplanted AD mice had an elevated proportion of CD45^{hi}/CD11b⁺ cells in the brain, as compared to control cell-treated mice (**Figures 7A,B**). The proportion of CD45^{hi}/CD11b⁺ cells in the brain of APP^{-/-} mESC-TEP-transplanted AD mice was higher than that in APP^{+/+} mESC-TEP-transplanted mice. Furthermore, the CD45^{hi}/CD11b⁺ cells in APP^{-/-} mESC-TEP-transplanted mice had a higher percentage of lymphocyte antigen 6c (Ly6C) positive cells than those in APP^{+/+} mESC-TEP-transplanted mice (**Figure 7C**). The CD45^{hi}/CD11b⁺ cells in APP^{-/-} mESC-TEP-transplanted mice also expressed higher levels of chemokine receptor *ccr2* and scavenger receptor A (SRA1) (**Figures 7D,E**). It has been reported that Ly6C and *ccr2* are related to myeloid cell neuroprotection in AD (62), whereas SRA1 is an A β -binding scavenger receptor associated with A β -plaque clearance (63). We also analyzed the phagocytosis ability of CD45^{hi}/CD11b⁺ cells and found that the cells in APP^{-/-} mESC-TEP-transplanted mice had a higher ability to phagocytose

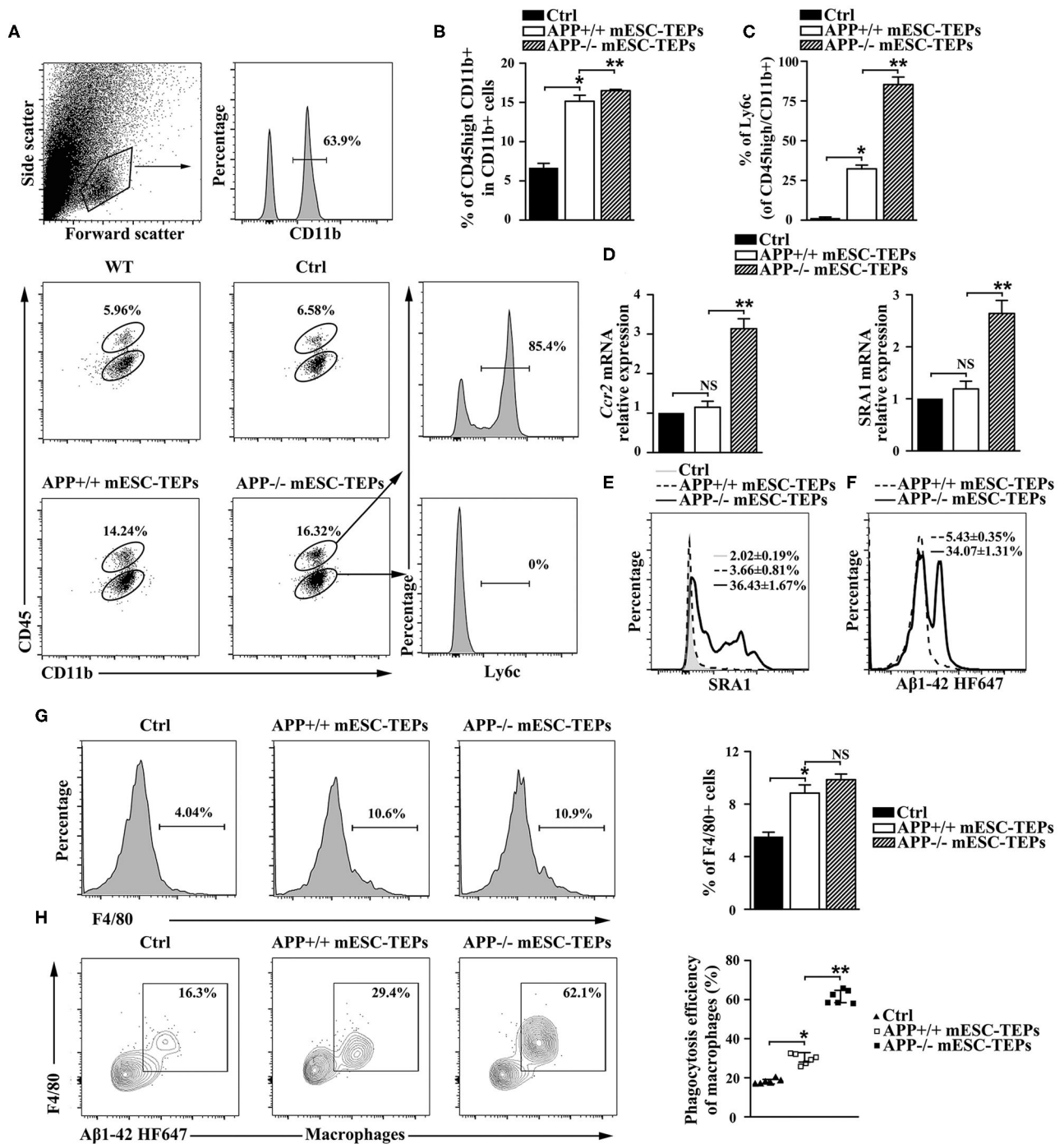


FIGURE 7 | Transplantation of APP^{-/-} mESC-TEPs results in an increased number of Aβ phagocytosing macrophages in the brain and the spleen. 3XTg-AD mice were injected i.t. with APP^{-/-} mESC-TEPs, APP^{+/+} mESC-TEPs or control cells as in **Figure 2**. Two and half months later, **(A–F)** the brain and **(G,H)** the spleen were harvested. **(A–C)** The brain was analyzed for the percentage of CD45^{hi}/CD11b⁺ cells and the expression of Ly6C by CD45^{hi} or CD45^{lo} cells. **(A)** Flow cytometry gating strategy is shown. **(B,C)** Statistical analysis of the percentages of **(B)** CD45^{hi}/CD11b⁺ cells in CD11b⁺ cells and **(C)** Ly6C⁺ in CD45^{hi}/CD11b⁺ cells. **(D,E)** The brain was analyzed for **(D)** the expression of *CCR2* and *SRA1* mRNA by qRT-PCR (the expression level of the genes in control cell-treated mice is defined as 1), and **(E)** the expression of SRA1 protein by CD45^{hi}CD11b⁺ cells using flow cytometry. **(F)** CD45^{hi}/CD11b⁺ cells were isolated from brains and analyzed for phagocytosis using HF647 Aβ42. **(G,H)** The splenocytes were analyzed for **(G)** the percentage of F4/80⁺ macrophages and **(H)** the ability of F4/80⁺ macrophages to phagocytose Aβ42. The data are expressed as mean ± SD from one of three independent experiments with similar results (4–8 mice per group in each experiment). *p < 0.05 vs. control cell group, **p < 0.05 vs. APP^{+/+} mESC-TEP group.

A β 42 than those in APP^{+/+} mESC-TEP-transplanted mice (**Figure 7F**).

We then analyzed macrophages in the spleen and found the percentages of F4/80⁺ macrophages in APP^{-/-} or APP^{+/+} mESC-TEP-transplanted AD mice were higher than that those in control cell-treated mice (**Figure 7G**). Although the percentage of F4/80⁺ macrophages in APP^{-/-} mESC-TEP-transplanted mice was slightly higher than that in APP^{+/+} mESC-TEP-transplanted mice, the difference did not reach statistical significance (**Figure 7G**). Furthermore, F4/80⁺ macrophages in APP^{+/+} mESC-TEP-transplanted mice were more able to phagocytose A β 42 than those in control cell-transplanted mice, and the macrophages in APP^{-/-} mESC-TEP-transplanted mice had a greater ability than those in APP^{+/+} mESC-TEP-transplanted mice (**Figure 7H**), in agreement with the data for the macrophage function in the brain (**Figure 7F**).

DISCUSSION

AD is hallmarked by the accumulation of A β plaques in the brain, which can adversely affect synaptic function and eventually cause neuron loss (2–4, 7, 8). The brain has been traditionally considered a site of immune privilege and exempt from systemic immune surveillance (16, 24). It is now accepted that neuro-immunological cross-talk, in which circulating immune cells enter the CNS, play an important role in brain tissue maintenance and repair, especially in pathological conditions (18, 24–26, 64–66). Like the situation in cancer immunology, onset of AD may reflect systemic immune suppression and the loss of immune surveillance (13, 67), impairing the ability to mount an immune response to fight brain pathology (13, 14, 68). For example, it has been shown that AD severity is greater in immunocompromised mice (19). In contrast, replacement of the missing adaptive immune populations, such as T cells and B cells, can dramatically reduce AD pathology (19). Boosting recruitment of monocyte-derived macrophages to sites of brain pathology also facilitates A β plaque clearance and relieves AD pathology (4, 13, 69–72). Therefore, systemic immunity in AD should be driven, rather than suppressed, to initiate an immune-dependent cascade to dissipate the A β clearance and repair the brain (4, 13, 14).

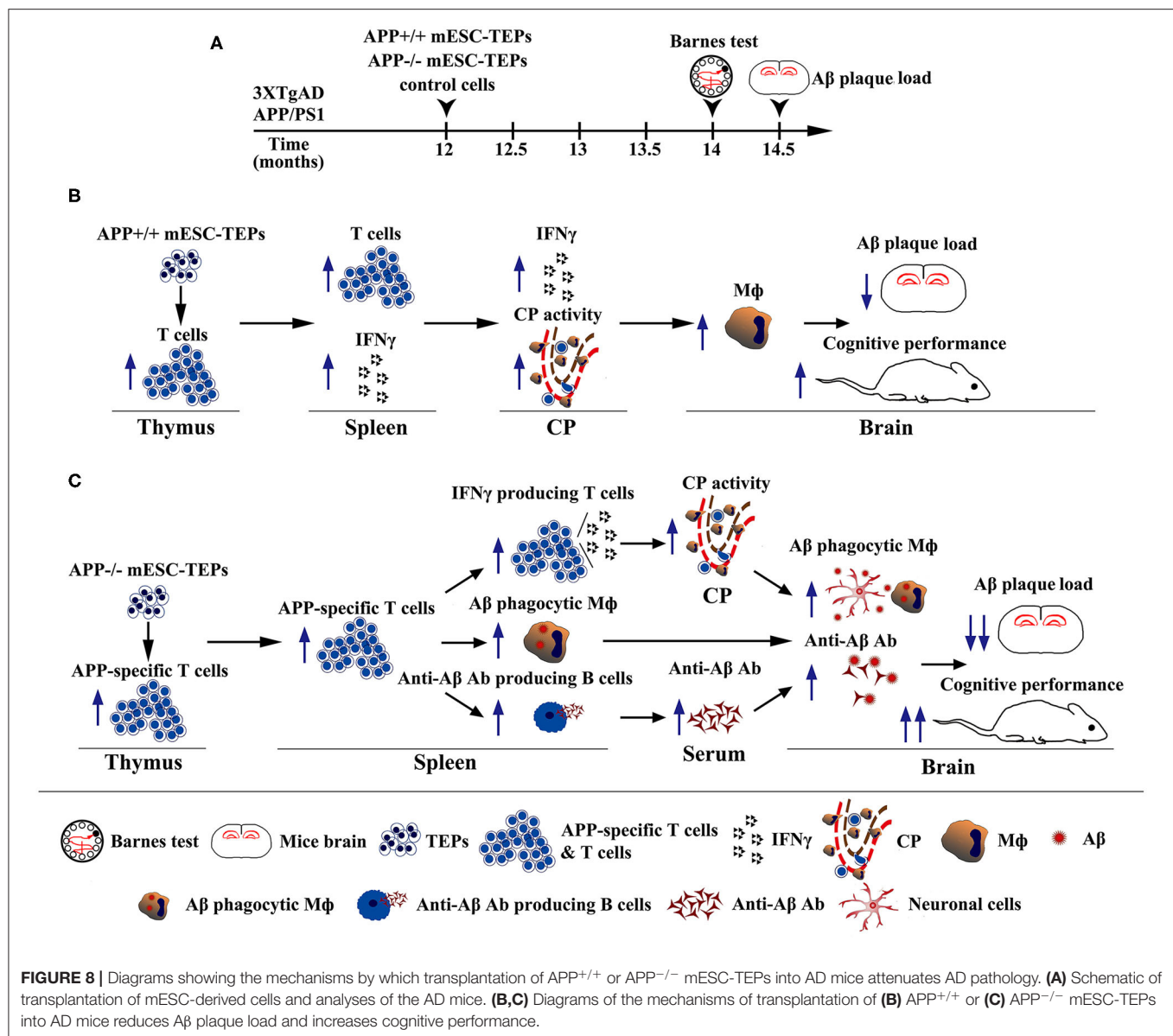
It is well-known that the thymus, the primary organ for T cell generation, undergoes a profound atrophy with age, a process termed thymic involution, resulting in decreased numbers of T cells in older adults. The reduced T cell number in older adults is likely to contribute AD development and progression. Indeed, in this study, we have shown that transplantation of either APP^{-/-} or APP^{+/+} mESC-TEPs enhances thymopoiesis that results in increased number of T cells, especially IFN γ -producing T cells in the spleen and the CP, leading to enhanced CP activity and increased number of macrophages in the brain. In addition, these mice also have an increased number of macrophages in the spleen. It has been shown that increased IFN γ availability in the CP can enhance the CP activity (4, 13). Both APP^{+/+} and APP^{-/-} mESC-TEP-transplanted mice have enhanced expression of leukocyte homing and trafficking molecules icam1, vcam1, cxcl10, and ccl2 in the CP, which may be

due to the increased IFN γ availability in this compartment. It is likely that the enhanced CP activity leads to increased migration of macrophages into the brain, resulting in an increased number of macrophages in this organ. It is also possible that increased T cell numbers in the spleen aid the macrophages, increasing their number in the spleen, likewise contributing to the increase in the brain. Although the improved thymopoiesis and an increased number of immune cells in the periphery (especially macrophages in the brain) attenuate AD pathology, they are insufficient for reduction of cerebral A β plaque load and for improving cognitive performance as indicated by the data that transplantation of APP^{+/+} mESC-TEPs is less efficient than that of APP^{-/-} mESC-TEPs.

Compared to APP^{+/+} mESC-TEP-transplanted mice, APP^{-/-} mESC-TEP-transplanted mice have increased A β -induced T cell proliferation, increased anti-A β Ab-producing B cells in the spleen and anti-A β Abs in the serum, as well as increased A β phagocytosing macrophages in the brain. Since TECs expressing self-antigens play a critical role in deleting autoreactive T cells specific to the antigens, transplantation of APP^{-/-} mESC-TEPs could result in the failure to delete A β -specific autoreactive T cells in the thymus, leading to the presence of the autoreactive T cells in the periphery. This is supported by the data that T cells from APP^{-/-} mESC-TEP-transplanted mice have increased proliferation in response to A β stimulation. The A β -specific autoreactive T cells may then help to produce anti-A β Ab-producing B cells that secrete anti-A β Abs into the serum and to produce A β phagocytosing macrophages that migrate into the brain. Together, these A β -specific immune cells and Abs reduce the AD pathology. Our results support the notion that breaking A β -specific immune tolerance is a novel target for AD immunotherapy (14).

Studies have shown that adaptive–innate immunity cross talk is important in ameliorating AD progression, in which T cells are critical (19). CD4 T cells are essential in the activation of B cells to secrete antibodies to mediate humoral immune responses (73, 74). Antibody response to an antigen requires help from the antigen-specific T cells. B cell antigen receptor usually delivers an antigen to intracellular sites where it is degraded and returned to the B cell surface as the peptide bound to MHC II molecule. The peptide:MHC II complex is recognized by the antigen-specific helper T cells, inducing the B cells to develop into antibody-secreting cells. It is possible that A β -specific autoreactive T cells generated in APP^{-/-} mESC-TEP-transplanted AD mice recognize the A β peptide:MHC II on B cells, and stimulate the B cells to proliferate and differentiate into plasma cells secreting anti-A β antibodies. Consequently, the anti-A β antibodies neutralize the toxin of A β and/or facilitate uptake of A β by macrophages by coating to A β to enhance the recognition by Fc receptors on macrophages.

CD4 T cells are also important in activating macrophages (75). Once activated, the macrophages phagocytose the related antigens. It has been shown that recruitment of circulating monocyte-derived macrophages can modify AD pathology (16, 76–78) by removing misfolded protein including A β -plaques (69, 79, 80), balancing the local inflammatory milieu (71, 80), reducing gliosis (81), and protecting synaptic structures (71, 82,



83). Since activated macrophages can cause local tissue damage (84–87), it is important that the macrophage activity is strictly regulated by antigen-specific T cells. It will be of interest to determine whether Aβ-specific autoreactive T cells in APP^{-/-} ESC-TEP-transplanted AD mice only activate the macrophages that specifically phagocytose Aβ, avoiding unnecessary local tissue damage.

In summary, we have demonstrated that transplantation of APP^{+/+} or APP^{-/-} mESC-TEPs into AD mice attenuates AD pathology, which is associated with enhanced systemic IFN γ -producing T cells and CP gateway activity with increased expression levels of leukocyte homing and trafficking molecules, as well as increased number of macrophages in the CNS. Furthermore, transplantation of APP^{-/-} mESC-TEPs has

significantly greater effectiveness. This is related to the generation of T cells reactive with Aβ, which accompanied by increased number of anti-Aβ Ab-producing B cells in the spleen and enhanced level of anti-Aβ Ab in the serum, as well as an increased number of Aβ phagocytosing macrophages in the brain (Figure 8). Our results suggest that transplantation of APP^{-/-} human ESC-TEPs or iPSC-TEPs has the potential to be used in the prevention and treatment of AD patients.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee of the University of Connecticut.

AUTHOR CONTRIBUTIONS

JZ designed experiments, performed experiments, analyzed data, and wrote the manuscript. MS performed experiments and analyzed data. YL and HL performed experiments. ZH designed experiments, analyzed data, and supervised the study. LL designed experiments, analyzed data, supervised the study and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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When the Damage Is Done: Injury and Repair in Thymus Function

Sinéad Kinsella¹ and Jarrod A. Dudakov^{1,2,3*}

¹ Program in Immunology, Clinical Research Division, Fred Hutchinson Cancer Research Center, Seattle, WA, United States,

² Immunotherapy Integrated Research Center, Fred Hutchinson Cancer Research Center, Seattle, WA, United States,

³ Department of Immunology, University of Washington, Seattle, WA, United States

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Avinash Bhandoola,
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Dong-Ming Su,
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The University of Tokyo, Japan
Jennifer Elizabeth Cowan,
National Institutes of Health (NIH),
United States

*Correspondence:

Jarrod A. Dudakov
jdudakov@fredhutch.org

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Even though the thymus is exquisitely sensitive to acute insults like infection, shock, or common cancer therapies such as cytoreductive chemo- or radiation-therapy, it also has a remarkable capacity for repair. This phenomenon of endogenous thymic regeneration has been known for longer even than its primary function to generate T cells, however, the underlying mechanisms controlling the process have been largely unstudied. Although there is likely continual thymic involution and regeneration in response to stress and infection in otherwise healthy people, acute and profound thymic damage such as that caused by common cancer cytoreductive therapies or the conditioning regimes as part of hematopoietic cell transplantation (HCT), leads to prolonged T cell deficiency; precipitating high morbidity and mortality from opportunistic infections and may even facilitate cancer relapse. Furthermore, this capacity for regeneration declines with age as a function of thymic involution; which even at steady state leads to reduced capacity to respond to new pathogens, vaccines, and immunotherapy. Consequently, there is a real clinical need for strategies that can boost thymic function and enhance T cell immunity. One approach to the development of such therapies is to exploit the processes of endogenous thymic regeneration into novel pharmacologic strategies to boost T cell reconstitution in clinical settings of immune depletion such as HCT. In this review, we will highlight recent work that has revealed the mechanisms by which the thymus is capable of repairing itself and how this knowledge is being used to develop novel therapies to boost immune function.

Keywords: endogenous thymic regeneration, immune restoration, T cell reconstitution, thymic epithelial cells, BMP4, IL-22

INTRODUCTION

Generation of a diverse but tolerant T cell repertoire, which is critical for adaptive immune function, is dependent on the development and maturation of T cell precursors in the thymus. The process of T cell development is reliant on the interactions with the stromal microenvironment, comprised of highly specialized thymic epithelial cells (TECs), endothelial cells (ECs), mesenchymal cells, dendritic cells (DCs) and macrophages. However, despite its importance for generating and maintaining T cells, thymic function is extremely sensitive to acute damage such as that caused by everyday insults like stress and infection, as well as more profound injuries such as that caused by cytoreductive therapies. Nevertheless, the thymus also has a remarkable capacity to regenerate itself from these acute injuries (1, 2), although until recently this phenomena has been largely unstudied. However, despite its crucial function, the ability of the thymus to facilitate efficient T cell generation deteriorates progressively with age

(3, 4); which considerably hampers the ability of the thymus to respond to acute insults. Age-related thymic atrophy and immunosenescence are hallmarks of immune aging and ultimately lead to a constriction of the TCR repertoire (5), decreased naïve T cells and accumulation of memory T cells in the periphery; and chronic low-grade inflammation termed “inflamm-aging,” all conferring insufficient protective responses to pathogens and neoantigens (6–8). Together, these acute and chronic thymic injuries underlie prolonged immune deficiency associated with multiple conditions including the conditioning required for hematopoietic cell transplantation (HCT) and cytoreductive cancer treatments such as radio- and chemo-therapies.

Given the poor outcomes that are associated with deficient T cell immunity, there is a clear clinical need for therapies that can boost thymic function in periods of acute injury or reverse age-related thymic involution. In this review, we will outline what we know about how the thymus is damaged during different modalities of insult and the work that has been done to develop therapeutic strategies to boost thymic function; either ensuing acute insult such as following HCT, or in aged individuals to boost responses to vaccines or immunotherapy (Figure 1).

ACUTE DAMAGE AND ENDOGENOUS REGENERATION IN THE THYMUS

Everyday Insults: Stress and Infection

Thymic involution is a routine response to acute insult incurred by multiple triggers including emotional and physical distress, malnutrition, and opportunistic bacterial and viral infections. These can be modeled using approaches such as synthetic corticosteroid treatment, such as dexamethasone (9); nutrient depletion (10); sex steroid treatment (11); and several viral and bacterial infection models. While acute thymic involution results primarily from the loss of cortical thymocytes (12, 13); in cases of chronic atrophy, such as that induced by age-related thymic decline, thymocyte loss is preceded by the loss of Foxn1+ TECs, resulting in the functional decline the TEC compartment and the initiation of age-related thymic atrophy (14, 15).

Continuous export of naïve cells from the thymus, or recent thymic emigrants (RTEs), is essential for effective immune response to acute and chronic infections (16, 17). However, most acute bacterial or viral infections result in acute thymic atrophy, largely due to intense lymphocyte depletion as a result of increased apoptosis of thymocytes and interference with thymocyte development (18–21); which can, at least partially, be attributed to the increased induction of IFN γ from activated CD8+ T cells (22) and Natural Killer (NK)-driven responses (23). Most of the studies looking at viral infection-related thymic function have concentrated on HIV, which leads to several modes of thymic dysfunction including thymic atrophy, reduced thymic output, reduced export of immature thymocytes and disruption of the thymic microenvironment (24–26). Notably, effective response to anti-retroviral therapies was found to depend on competent thymic function, with enhanced function in HIV-infected children with higher basal levels of thymic function

(27), in contrast with infected adults who have a reduced thymic output and output decreased CD4+ T cells (28, 29). Moreover, in addition to viral load, quantification of CD4+ RTE has long been known as a suitable marker for HIV disease progression, and a recent study has demonstrated the use of RTE CD4+ T cells as a marker of perinatal HIV infection in infants (30); further strengthening the link between viral infection, efficient thymic function, and therapeutic implications of thymic atrophy.

Although less well studied, bacterial infections also have negative effects on thymic function, primarily by enhancing thymocyte apoptosis. *Streptococcus suis* infection promotes thymic atrophy specifically by inducing increased activation of pro-apoptotic pathways and apoptotic cell death in thymocytes (31); while *Mycobacterium tuberculosis* infection also induces thymic atrophy (32), possibly by regulating glucocorticoid levels and in this way impact on homeostatic endocrine-immune communication (33). In fact, glucocorticoids are central to many acute forms of thymic involution (34, 35), directly inducing the apoptosis of CD4+CD8+ DP thymocytes, which preferentially express the glucocorticoid receptor (36).

Metabolic distress due to lack of nutrients, primarily glucose, leads to an attenuation of thymic function, with perturbed thymopoiesis in non-obese diabetic (NOD) mice (37), and reduced thymic atrophy with glucose supplementation in models of mitochondrial dysfunction (38).

Cytoreductive Therapies

Most therapies used in cancer treatments are cytoreductive, such as chemotherapy or radiation. One prominent example of this is that the pre-conditioning regimens required for successful HCT result in profound injury to the thymus, and, in contrast to the relatively early recovery of platelets, erythrocytes, and leukocytes involved in innate immunity, recipients of an HCT experience prolonged post-transplant deficiency in the recovery of adaptive immunity, especially T cell immunity. This delayed T cell reconstitution can last a year or more due to a delay in full recovery of function and T cell repertoire (39–41). Moreover, post-transplant T cell deficiency is associated with an increased risk of infections (39, 40, 42, 43), relapse of malignancy (44), and the development of secondary malignancies (45–50). In fact, infection and relapse account for >50% of mortality following allogeneic-HCT (allo-HCT) (51). T cell reconstitution after transplant is critically dependent on the thymus (39, 41, 47, 52–58) and thymic function pre-transplant can have a significant impact on clinical outcomes. Similarly, damage caused by cytoreductive chemotherapy results in significant thymic damage and can lead to a profoundly delayed recovery of T cells (45, 59). In mouse models of chemotherapy, in addition to almost complete depletion of thymocytes, there was also a severe depletion of TECs, most prominently MHCII^{hi} TECs (60); likely as they are the most highly proliferating TEC subset (61, 62). Specifically, genotoxic stress caused by chemotherapy leads to senescence in the thymic stromal compartment and the induction of an inflammatory environment in the thymus with endothelial cell secretion of IL-6, generating a chemoresistant niche that is cytoprotective to certain cancer cells, such as lymphoma and melanoma

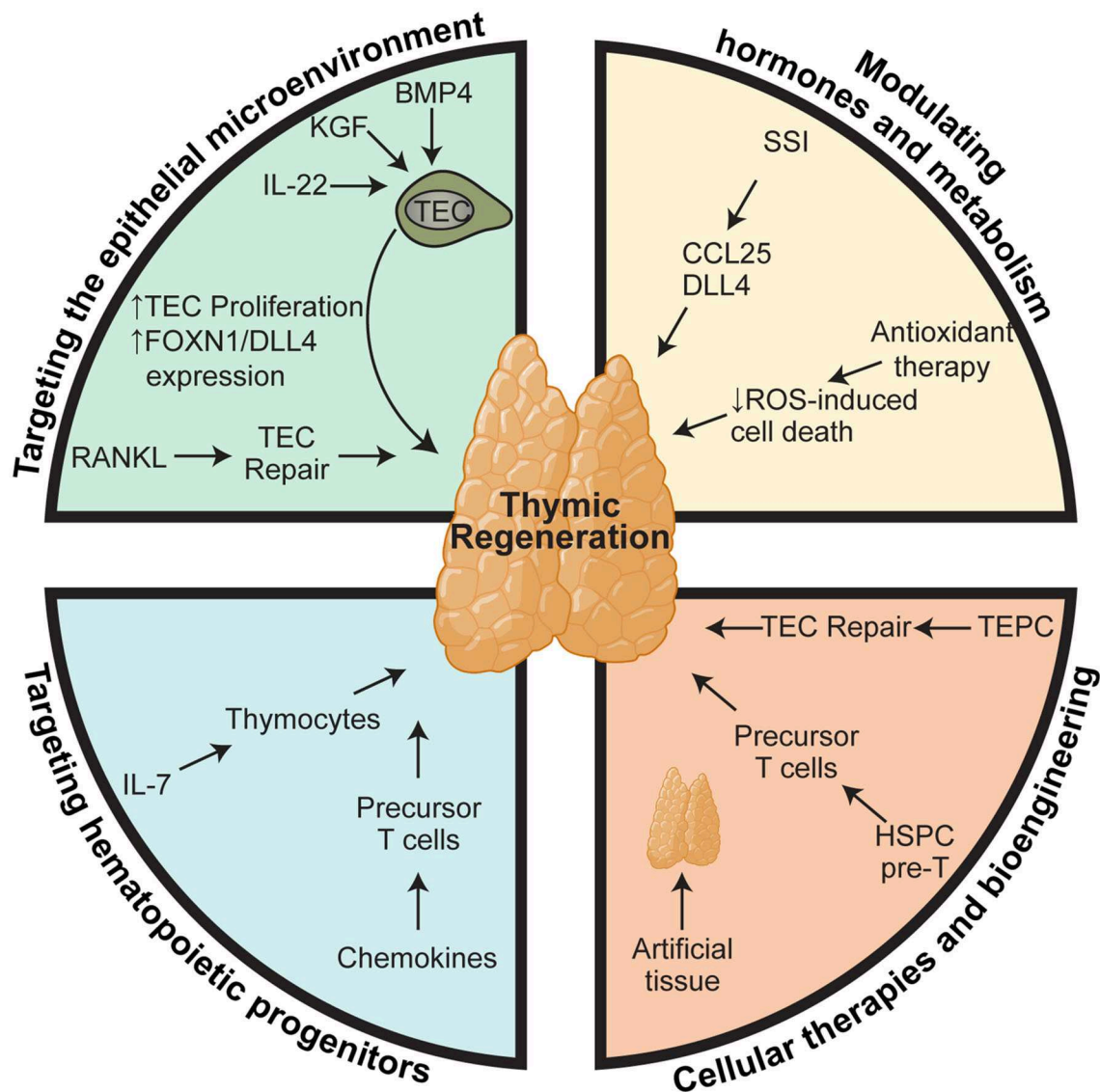


FIGURE 1 | Therapeutic approaches for boosting thymus function. Regenerative therapies to boost thymic function after acute damage or to reverse age-related involution can be broadly stratified into four subgroups based on their cellular or molecular targets: (1) targeting the epithelial microenvironment that supports thymopoiesis; (2) targeting the precursors that provide the supply of developing thymocytes; (3) modulation of hormones and metabolism; and (4) cellular therapies and bioengineering. However, within each of these therapeutic modalities there are key nexus points at which they act mechanistically. One approach relies on stimulating TEC function, such as IL-22, BMP4, KGF, RANKL, SSI, which act by either promoting survival, proliferation, differentiation, or expression of key thymopoietic molecules like DLL4 and KITL. In contrast, approaches such as administration of exogenous IL-7 and chemokine therapies target T cell precursors to promote their migration, proliferation, and differentiation directly. Similarly, many of the bioengineering approaches have sought to recapitulate these same functions such as providing TEC signals or a ready supply of T cell precursors. Elements of the figure were generated using Biorender.com.

(63, 64). Accompanying the damage caused by cytoreductive conditioning, the risk of further thymic damage caused by Graft-vs.-Host Disease (GVHD) is significant in the context of an allo-HCT. In fact, the thymus is a particularly sensitive GVHD target organ and presents pathological features even in the context of subclinical GVHD (65–67). Furthermore, there is likely a link between acute GVHD-mediated thymic damage and the formation of chronic GVHD, which may in part be a failure for tolerance induction (68–70).

STRATEGIES OF BOOSTING THYMIC FUNCTION I: TARGETING NON-HEMATOPOEITIC CELLS

Given the sensitivity of thymic function to negative stimuli, even everyday insults, a reparative capacity is crucially important for renewal of immune competence. In fact, this capacity of the thymus to regenerate itself has been known for longer than even the immunological function of the tissue was discovered

(71, 72); however, until recently the mechanisms underlying this process have been poorly understood. One approach to developing therapies to enhance thymic function has come from exploiting these pathways of endogenous regeneration. Many of these pathways that mediate endogenous regeneration have been found to be effective for exogenous regeneration in periods of acute and profound injury such as that caused by cytoreductive chemotherapy and γ -radiation. Interestingly, many of these pathways specifically target TECs to mediate regeneration.

Interleukin-22

Although the phenomenon of endogenous thymic regeneration has been known for over almost a 100 years, it was not until recently that pathways mediating this regeneration have been described. The first of these was centered around the production of Interleukin-22 (IL-22), a member of the IL-10 family that typically targets non-hematopoietic cells such as epithelial cells and fibroblasts (73). In this regenerative network, acute damage to the thymus (and specifically the depletion of thymocytes) triggers the release of Interleukin-23 (IL-23) from dendritic cells, which induces the production of IL-22 by a group 3 innate lymphoid cells (2, 74–76). Expression of IL-22R in the thymus is lacking on thymocytes but detected in both cTECs and mTECs populations (2). IL-22 acts on TECs to mediate repair but the specific molecular mechanisms are not clear. In addition to the thymus, IL-22 also has a major role in the regeneration of epithelial cells in a diverse range of tissues including gut, lung, skin, breast, and kidney (77). The IL-22 receptor is a type 2 cytokine receptor, and a heterodimer formed of two subunits: IL-10 receptor 1 (IL-10R1) and IL-22 receptor A2 (IL-22RA2) (78). IL-22 receptor binding induces intracellular inactivation of the Jak1/Tyk2 complex which further allows downstream signaling and phosphorylation of Signal Transducer and Activator of Transcription (STATs) 1, 3, and 5, with a preference for STAT3 phosphorylation (79), including in TECs (2) which is consistent with the upregulation of *Foxn1* concurrently with IL-22 in the thymus (76), and the importance of STAT3 for TEC maintenance (80). Furthermore, Ruxolitinib, a chemotherapeutic agent that inhibits Jak1 signaling also prevents thymic regeneration after injury (81).

Similar to other tissues (77), IL-22 is not required for the formation or maintenance of the thymus under steady-state physiological conditions; however, it has a key role in driving thymic regeneration after injury, by acting directly on TECs to induce survival and proliferation, potentially via regulation of *Foxn1* expression (2, 76). Of note, both the numbers of innate lymphoid cells (ILC) 3 and IL-22 levels were decreased in the thymus and gut of mice with GVHD (74, 82), suggesting that ILCs are a target of alloreactive cells and this depletion likely causes a failure to repair after damage. Due to the diverse pathophysiological roles of IL-22, and the key role in epithelial cell regeneration, modulation of the IL-22-IL22R system is an attractive therapeutic target. In fact, a clinical trial is currently underway to assess the efficacy and safety of administration of IL-22 in combination of systemic corticosteroids to limit the effects of GVHD after hematopoietic

stem cell transplantation, with secondary readouts to assess T cell reconstitution (NCT02406651).

Bone Morphogenic Protein 4

Although the role of thymic BMP4 and the endogenous BMP4R antagonist Noggin have been well-described in thymic development (83, 84), only recently has BMP4 been described as a regulator of thymic regeneration after acute injury (85). In the thymus, the source of BMP4 is fibroblasts and endothelial cells (ECs) (85). ECs are a highly radio-resistant population of cells in the thymus (85, 86) and are unique in their ability to induce BMP4 production in response to injury. Importantly, thymic expression of both *Bmpr1a* and *Bmpr2* were identified on TEC populations, with a higher expression of the non-redundant *Bmpr2* on cTECs (85); consistent with BMP4-induced expression of FOXN1 and its downstream target delta-like 4 (DLL4) specific to cTECs (85, 87). Although the importance for FOXN1 and DLL4 for the development of TECs and thymocytes, respectively, has been well studied (88, 89), recent findings have also highlighted their importance for thymic regeneration, with intrathymic concentration of DLL4 profoundly impacting on thymic size (90), and reports suggesting that the induction of FOXN1 can counteract age-associated thymic involution (91), acute damage (85), and thymic damage post-transplantation (92). While much of the role of BMP4 seems to be mediated by induction of the FOXN1/DLL4 axis, given the requirement for BMP4 in *in vitro* differentiation of TECs from multipotent progenitors (93–95), it is possible that an alternate mechanism may be by stimulating bipotent progenitors present in the adult thymus (96–99). Unfortunately, the preclinical studies assessing BMP4 have yet to successfully treat mice with recombinant protein, a therapeutic strategy has been developed that utilizes a technique of allowing for the propagation and expansion of tissue-specific ECs that can be transplanted and mediate regeneration across multiple tissues (85, 100–105). In the thymus, it was found that this therapeutic cellular strategy was dependent on the expression of BMP4 by transplanted ECs (85).

Keratinocyte Growth Factor

Keratinocyte growth factor (KGF, also known as FGF-7), is a fibroblast growth factor and acts as a mitogen targeting TECs, inducing epithelial proliferation in several organs (106–108). In the thymus, KGF is primarily produced by mature $\alpha\beta^+$ thymocytes and feeds back to facilitate the proliferation and expansion of mTECs via the activation of p53 and NF- κ B pathways (106, 108), preserving the thymic cytoarchitecture. Of note, KGF is also produced by thymic fibroblasts (108). Expression of the KGF receptor, fibroblast growth factor receptor-2 of the IIIb variant (*Fgfr2IIIb*), is limited to TECs (109), and *Fgfr2-IIIb*^{-/-} mice have defective thymopoiesis and reduced cellularity, accounted for specifically by a reduction in TECs (110). KGF modulates TEC functionality by negatively regulating the levels several gatekeepers of positive selection, such as MHC-II invariant chain (Ii), and cathepsin L (CatL) (108), and acts on TECs to produce several cytokines that act directly on thymocytes to facilitate maturation, such as bone morphogenic protein 2 (BMP2), BMP4, Wnt5b, and Wnt10b (109).

Under normal physiological conditions, KGF can enhance thymic cellularity by increasing the number of early thymic progenitors (ETPs) equating to an enhanced number of engraftment niches, and increased TEC proliferation (109). Although it was shown that KGF is not essential in uninjured conditions (110, 111), studies using *KGF^{-/-}* mice demonstrated the critical role of KGF on thymus function and immune reconstitution after insult, modeled by both syngeneic and allogeneic bone marrow transplant (112). The same study showed that exogenous administration of recombinant KGF enhanced thymopoiesis in young and middle-aged mice, and attenuated the negative effects of acute thymic injury, such as that caused by dexamethasone treatments, cyclophosphamide, and irradiation, highlighting an extremely attractive therapeutic approach to efficiently facilitating immunocompetence after damage. Additionally, exogenous KGF administration improved post-transplantation T cell reconstitution. Furthermore, pre-conditioning with KGF prior to bone marrow transplantation reduces GVHD in mouse models by protecting against epithelial injury (113). However, a recent clinical trial noted a reduction in thymopoiesis in lymphopenic patients following administration of KGF (114), highlighting that more studies need to be carried out before KGF can be used across the board as a therapeutic regulator of thymic regeneration.

RANKL

Receptor activator of nuclear factor kappa-B ligand (RANKL), a member of the Tumor necrosis factor (TNF) superfamily, is implicated in multiple physiological roles in the periphery, primarily in bone biology (115). RANKL has an essential role in the thymus as a potent inducer of epithelial cell differentiation by regulating the key mTEC transcription factor Aire (116). In this way, RANKL governs the maturation of Aire⁻ mTECs to Aire⁺ mTECs which subsequently present MHC-II peptides that drive the elimination of self-reactive T cells during negative selection (117). RANKL is non-redundant for fetal Aire⁺ mTEC development, and is produced during development by ILCs, and subsequently by subsets of thymocytes (116, 118–120); although absence of RANKL postnatally can be compensated for by other factors (121). Importantly, RANKL is increased in CD4⁺ thymocytes and ILCs after injury from the cytoreductive conditioning required prior to HCT, suggesting that RANKL plays a role in endogenous regeneration of the thymus (2, 122).

The prominent role of RANKL in mTEC biology points to the ability of RANKL to modulate thymic regeneration and output. RANKL administration shows an enhancement of thymic function after bone marrow transplantation by boosting TEC subsets, including TEC progenitor niches (122). Moreover, systemic administration of recombinant soluble RANKL (sRANKL) improved thymic medullary architecture in RANKL deficient mice (123), and transgenic mice overexpressing human sRANKL, or mice lacking the soluble RANKL receptor OPG, have an enlarged thymic medulla with increased numbers of Aire⁺ mTECs (119, 124, 125), highlighting a therapeutic platform for the use of recombinant RANKL as a therapeutic for thymus regeneration.

STRATEGIES OF BOOSTING THYMIC FUNCTION II: TARGETING HEMATOPOEITIC CELLS

Given the fact that Cell development requires the input of hematopoietic progenitors, and the fact that the supply of those progenitors is severely limited after acute injury (126, 127), one approach to promoting thymic function is to directly stimulate precursor populations; either in the BM or thymus.

Bone Marrow Progenitors

Several approaches have been attempted that seeks to improve thymic function by stimulating the function of bone marrow hematopoietic progenitors. For instance, preclinical studies have shown that administration of Flt3L can also enhance both thymic dependent and independent T cell reconstitution (128, 129). The effects of Flt3L are predominantly due to an expansion in Flt3⁺ progenitors in the BM (130). However, increases in T cell reconstitution can be at the expense of B-lymphopoiesis which is significantly declined with exogenous Flt3L administration and, in particular, its effects on the EPLM subset of BM progenitors (131, 132).

Chemokines are key regulators of thymopoiesis, facilitating thymic population and intrathymic cell migration. Importantly, as the thymus does not contain long term progenitors that would enable self-renewal, repopulation of the thymus requires continuous recruitment of T cell progenitors (133). CCL25 (with its receptor CCR9) and CCL21 (with its receptor CCR7) play an important role in thymic colonization with hematopoietic progenitors (134). Interestingly, chemokine therapy, whereby bone marrow progenitors received CCL25 and CCL21 treatment prior to transplant, rescues thymic homing of progenitors which is otherwise suppressed in irradiated mice (86).

Thymic T Cell Precursors

While there are several approaches that have been postulated that target thymic precursor cells the most prominent and developed of these is with the lymphopoietic cytokine interleukin-7 (IL-7). IL-7 has a non-redundant role as a survival molecule in lymphoid tissues in mice and humans, most importantly in the thymus where IL-7 is critical for appropriate thymocyte development. An elegant study by Shitara et al. (135) showed that specific deletion of IL-7 in TECs resulted in the profound reduction in $\alpha\beta$ and $\gamma\delta$ T cells; and mice deficient for *Il-7* have a peripheral loss of $\gamma\delta$ T cells, a significant reduction in $\alpha\beta$ T cells (136), an absence of innate lymphoid cell subsets (137), and disorganization of lymphoid tissue (138). Moreover, mice lacking *Il-7* have a reduced number of DN2 or DN3 cells (139, 140), essentially creating a thymic block and limiting the progression thymocytes to maturity.

IL-7 is produced primarily by non-hematopoietic stromal cells such as TECs and signals by binding to the heterodimeric IL-7 receptor (IL-7R), comprised of IL-7R α (also known as CD127) and the cytokine receptor γ -chain (also known as CD132), and induces an anti-apoptotic pro-survival signaling cascade via the activation of phosphoinositide 3-kinase (PI3K) and the Janus Kinase (JAK)-STAT pathway. The expression of IL-7R α

on developing thymocytes occurs in a cyclical pattern, with expression seemingly dependent on the fluctuating need for IL-7 signaling at different stages of thymocyte maturation (141), demonstrated by absence in the earliest T cell progenitors, expression at later DN stages, absence at the DP stage and re-expression in SP thymocytes.

These critical roles of IL-7 in both thymocyte development and in peripheral T cell homeostasis (142) reveal IL-7 as a strong therapeutic candidate to enhance T cell development and activation. Clear evidence exists for the therapeutic potential of IL-7 administration on thymic regeneration, centered on the beneficial effects of IL-7 on increasing progenitor T cells in the thymus and subsequently expanding circulating naïve T cells in viral infection setting (143); however, IL-7 therapy only transiently increased naïve T cells in the aged setting in rhesus macaques, with a more prominent and long lasting effect in the memory T cell compartment (144). Although recombinant IL-7 immunotherapy has had some success in clinical trials for treating septic shock (145), infection (146), and cancer remission (147), along with some early promise in the setting of HCT (148), further studies are necessary to identify a strategy for thymus-dependent IL-7 therapy.

STRATEGIES OF BOOSTING THYMIC FUNCTION III: MODULATION OF HORMONES AND METABOLISM

Given the impact of sex steroids on thymic function (149, 150), surgical or chemical ablation of sex steroids has been a well-studied means of boosting thymic function (58, 151). In fact, sex steroid inhibition (SSI) has been shown to promote thymic function in young as well as old mice, and enhances reconstitution after acute insult such as chemotherapy or HCT (60, 152–154). Furthermore, given that SSI is a standard and approved therapy for prostate cancer, thymic function has been assessed in prostate cancer and after HCT and significant improvement observed (155, 156). Although whole organismal ablation of sex steroids will understandably have systemic effects, and the specific means by which SSI improves thymic function are not yet clear, several putative mechanisms have been proposed. In particular, SSI has been shown to (1) promote lymphoid potential and overall function of hematopoietic stem and progenitor cells (2, 152, 157, 158) induce the expression of CCL25 (159), which promotes the importation of hematopoietic progenitors from the circulation (3, 134, 160) induces the expression of the Notch ligand DLL4 (90). Interestingly, KGF was not required for the beneficial effects of SSI on thymus (154), and in fact combination therapies have shown great promise, with the combined KGF administration and androgen blockage with Lupron, revealing reduced epithelial damage and enhanced T cell reconstitution after bone marrow transplant in mice (161). However, it has also been reported that regrowth of the thymus can result in an increase in autoreactive T cells in the periphery, particularly in models of castration, reflecting a lack of synergy between quality and quantity of thymopoiesis (162).

In addition to sex steroids, several other hormones and metabolic components have been implicated in thymic function and their modulation has been shown to improve thymopoiesis, particularly in the aged. Administration of the appetite stimulating hormone Ghrelin led to improved thymic cellularity and thymic output in aged mice (163), and similarly oral zinc supplementation increased thymic cellularity in aged mice (164). Targeting accumulating reactive oxygen species with antioxidants has proven to be beneficial in protecting against age-related thymic atrophy, whereby treatment with the mitochondrial antioxidant SkQ1 reduced age-associated thymic atrophy and increased the number of CD4+ and CD8+ thymocytes (165). Similarly, Leptin, a peptide hormone secreted from adipose tissue, has a protective effect on thymopoiesis in LPS-treated mice and mice that had been starved, primarily due to rescue from metabolic defects including increased corticosterone levels (10, 166).

STRATEGIES OF THYMIC REGENERATION IV: CELL THERAPIES AND BIOENGINEERING APPROACHES

Hematopoietic Precursors

In addition to the use of growth factors and hormone modulation, several groups have been working on cellular therapies that may enhance thymic function. Given that some of the delay in T cell reconstitution is due to the limited supply of BM-derived progenitors (126), in addition to the time taken for development into a naïve lymphocyte from a transplanted HSC, early studies that concentrated on providing hematopoietic cells found that lymphoid precursors isolated from donor bone marrow could be used to boost thymic function when infused into a recipient at the time of HCT, giving an early boost to T cell development (167). To overcome the limited number of hematopoietic progenitors in BM, an alternate approach of using precursor T cell populations that have been expanded using *ex vivo* culture systems that use Notch-1 stimulation of hematopoietic precursor cells has been demonstrated (168–173). Using this regimen, adoptive transfer of T cell precursors into lethally irradiated allogeneic HCT recipients caused a significant increase in thymic cellularity and chimerism, as well as enhanced peripheral T and NK cell reconstitution compared with recipients of allogeneic hematopoietic stem cells only (168, 174–178).

Thymic Epithelial Cells

In addition to the use of hematopoietic cells that can act as a boost of T cell precursors, another approach is to identify and isolate populations of thymic epithelial progenitor cells (TEPC). TEPC have been successfully isolated from fetal thymi and induced to generate a new thymus in athymic recipients (179–182), and neonatal TECs, or TECs derived from pluripotent progenitors can promote enhanced thymic function (183, 184). However, while there is evidence of a bipotent TEPC in the postnatal thymus (97–99), their capacity to self-organize as a whole organ like fetal TEPCs is limited. A TEC-like progenitor

cell appropriate for this purpose has also been generated by direct conversion of embryonic fibroblasts by induced expression of the TEC transcription factor FOXP1 (185); although the efficacy of this therapy in a regeneration setting has not been investigated.

Artificial Thymic Niches

Finally, there are also several approaches that do not rely on the endogenous thymus at all, but rather concentrate on *de novo* formation of whole organs *ex vivo* that can be transplanted into patients as required (186, 187). Although *in vivo* evidence of their efficacy is still only limited, several approaches have been used to generate artificial thymuses *ex vivo*, including decellularizing the tissue, which has been performed in several tissues including the thymus, as well as generating synthetic matrices to support T cell development (188–190). Both of these approaches would require some cellular input to generate a functional thymus; namely the thymic epithelial microenvironment would need to be recapitulated with specific factors or, more likely, cells such as TECs derived from multipotent progenitors or reprogrammed, as above. Moreover, a recent report has demonstrated the efficacy of an artificial pre-thymic niche by implanting a scaffold with the Notch ligand DLL4 that acts as an intermediary between the BM and thymus (191). Although these approaches have shown some promise in preclinical mouse studies (192), further advances need to be made before this can be a viable therapeutic option.

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CONCLUSION

Enhancing the regenerative capacity of the thymus and increasing thymic output, together with the expansion of the TCR repertoire has immensely beneficial clinical implications. Although there has been extensive progress in the development of multiple therapies targeting thymic regeneration and output, a deeper understanding of key endogenous molecular mechanisms that govern involution and regeneration of the thymus are needed to further the development of clinically translatable therapies.

AUTHOR CONTRIBUTIONS

SK and JD wrote, drafted, and edited the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: The authors have patents and patent applications around potential therapeutics to promote thymus regeneration, including some listed in this review (IL-22 and BMP4) and others as yet unpublished.

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T-Cell Immunodeficiencies With Congenital Alterations of Thymic Development: Genes Implicated and Differential Immunological and Clinical Features

Giuliana Giardino, Carla Borzacchiello[†], Martina De Luca[†], Roberta Romano, Rosaria Prencipe, Emilia Cirillo and Claudio Pignata*

Department of Translational Medical Sciences, Pediatric Section, Federico II University of Naples, Naples, Italy

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Nicolai Stanislas Van Oers,
University of Texas Southwestern
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Jennifer Elizabeth Cowan,
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United States
Naomi Taylor,
National Institutes of Health (NIH),
United States

*Correspondence:

Claudio Pignata
pignata@unina.it

[†]These authors have contributed
equally to this work

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Combined Immunodeficiencies (CID) are rare congenital disorders characterized by defective T-cell development that may be associated with B- and NK-cell deficiency. They are usually due to alterations in genes expressed in hematopoietic precursors but in few cases, they are caused by impaired thymic development. Athymia was classically associated with DiGeorge Syndrome due to *TBX1* gene haploinsufficiency. Other genes, implicated in thymic organogenesis include *FOXN1*, associated with Nude SCID syndrome, *PAX1*, associated with Otofaciocervical Syndrome type 2, and *CHD7*, one of the genes implicated in CHARGE syndrome. More recently, chromosome 2p11.2 microdeletion, causing *FOXI3* haploinsufficiency, has been identified in 5 families with impaired thymus development. In this review, we will summarize the main genetic, clinical, and immunological features related to the abovementioned gene mutations. We will also focus on different therapeutic approaches to treat SCID in these patients.

Keywords: Thymus, *FOXN1* gene, *PAX1* gene, Pax 1/9, CHARGE, *CHD7* gene, *TBX1* gene, DiGeorge anomaly

INTRODUCTION

The thymus is a primary lymphoid organ which plays a pivotal role in the development of mature T cells from immature bone marrow CD34⁺ precursors. Together with the parathyroid glands, it develops from the 3 pharyngeal pouch (PP) (1). The epithelial components of the thymus derive from the endothelial layer, while the mesenchymal capsule derives from neural crest, originated from ectoderm (1, 2). The first stage of thymic development is independent of the transcription factor forkhead box N1 (*Foxn1*) expression (3). In this phase, Paired box 1 (*Pax1*), Eyes absent homolog 1 (*Eya1*), sine oculis homeobox (*Six*), homeobox A3 (*Hoxa3*), and T-box 1 (*Tbx1*) drive the outgrowth of the thymic epithelial anlage from the 3rd PP (1, 4, 5). *Hoxa3* and *Eya1* are also implicated in the development of neural crest derived mesenchymal cells (6–8). Studies suggest that chromodomain helicase DNA-binding 7 (*Chd7*) might be implicated in the development of both neural crest cell-derived mesenchyme and pharyngeal endoderm-derived thymic epithelial cells (TECs). *Pax3* and *Hoxa3* expression in mesenchymal cells allows the detachment of the thymic lobes from the pharynx (9, 10). Thymus development in early and late stages is regulated by the interactions among various cell types. The thymus three-dimensional (3D) architecture allows a proper intercellular cross talk (3). In the first phase of organogenesis, mesenchymal cells release

bone morphogenetic protein 4 (Bmp4), Bmp2, fibroblast and insulin growth factors (Fgf, Igf), Wnt proteins, and retinoic acid supporting the differentiation of TECs into cortical (cTECs) and medullary (mTECs) subsets (7, 8, 11–14). In the second phase, Foxn1 induces the expression of chemokine (C-C motif) ligand 25 (CCL25), delta like canonical Notch ligand 4 (*Dll4*), and *Hoxa3*, allowing thymocyte recruitment and TECs differentiation in cTECs and mTECs. Mesenchymal cells are implicated in the recruitment of hematopoietic thymic seeding progenitors, as well (15, 16). Thymocytes participate to TECs differentiation process through the release of epidermal growth factor (Egf) and lymphotoxin factors (17–19). In support of this, mice with defective T-cell development show defective organization of the thymic medulla (20, 21), that is restored after stem cell transplantation (21, 22).

Bone marrow derived hematopoietic stem cells (HSCs) enter the thymus through cortico-medullary junction, where they proliferate (23). The V(D)J rearrangement of the double negative (DN) thymocytes T-cell receptor β (TCR β) gene takes place in the thymic cortex (24, 25). Membrane expression of pre-TCR complex is necessary for the expression of the co-receptors CD4 and CD8, as well as V-J rearrangement of the TCR α genomic region (26). Double positive thymocytes with a functional TCR- $\alpha\beta$ receptor capable of binding to self-MHC ligands are positively selected (27–29). This process is regulated by Prss16 and $\beta 5t$, which are expressed in cTECs (30–34). Into medulla, self-reactive thymocytes are deleted through the negative selection, a process mediated by dendritic cells and Aire-expressing mTECs (35, 36). Mutations in genes implicated in different steps of thymic development, including *FOXN1*, *PAX1*, *TBX1*, *CDH7* impair T-cell development in humans (Figure 1). Alterations of the immune system in these conditions range from an isolated reduction of T-cell count to severe combined immunodeficiency (SCID). This review is focused on definition of the role of different genes implicated in thymus development and of primary immunodeficiencies (PIDs) due to their deficiency. Moreover, therapeutic options for PIDs with congenital athymia are discussed.

GENE FUNCTION AND RELATED SYNDROME

FOXN1 Deficiency and Nude-SCID Syndrome

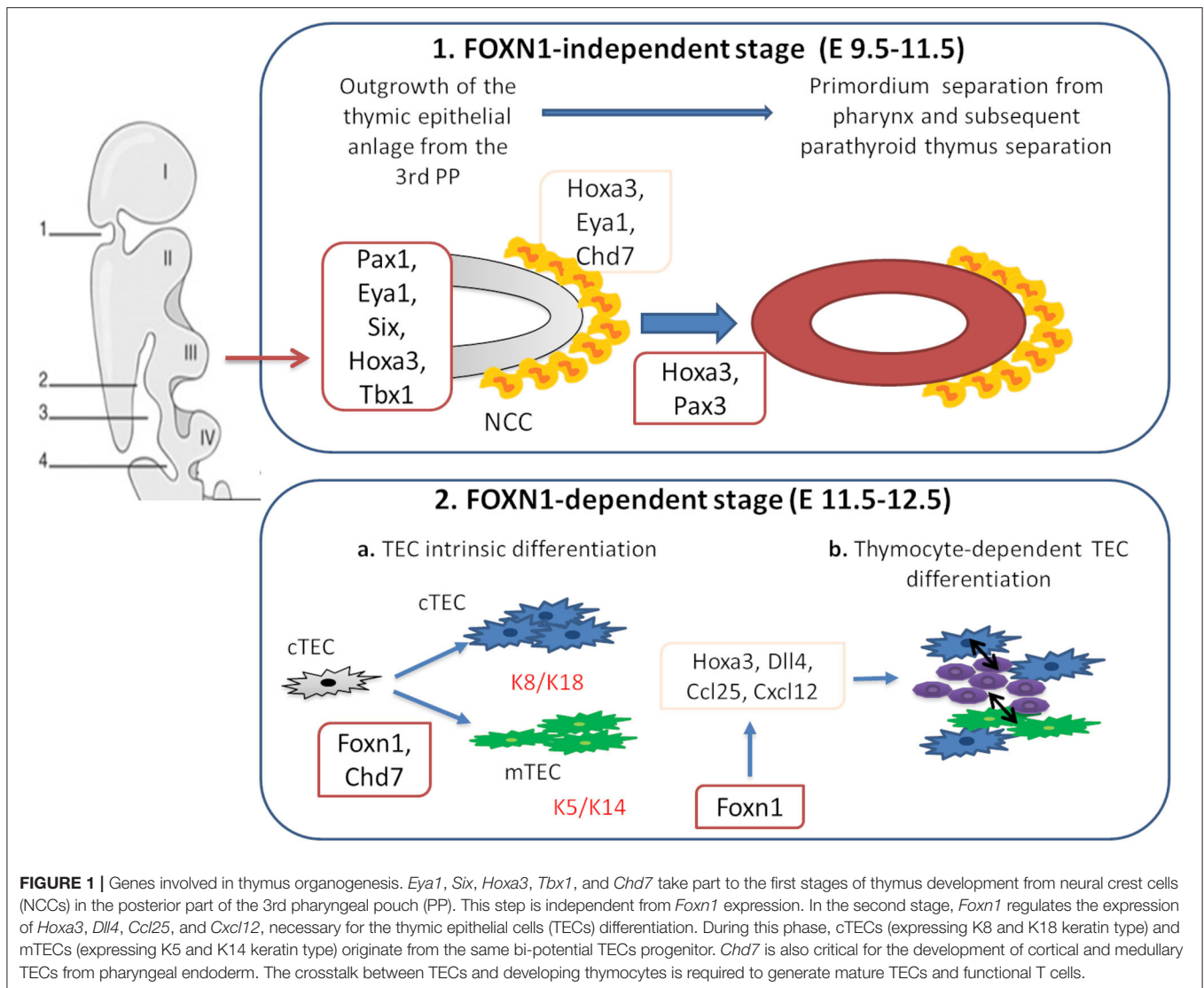
FOXN1 is located on chromosome 17q11.2 and it is composed of eight exons (30 kb) (37, 38). This gene is a member of the Fox gene family that comprises different Winged helix transcription factors implicated in development, metabolism, cancer, and aging (39). During fetal life, *FOXN1* is expressed in mesenchymal and epithelial cells including liver, lung, intestine, kidney, and urinary tract. In postnatal life, its expression is restricted to keratinocytes and TECs (40, 41). *FOXN1* is involved in development, function and maintenance of hair follicles, and TECs (Figure 1) (42–44). The expression of *FOXN1* in TECs leads to the production of chemokines, such as C-X-C motif chemokine ligand 12 (CXCL12), implicated in the recruitment of hematopoietic

progenitors and of molecules, as DLL4 notch ligand, implicated in maturation of the progenitors toward the T-cell line (Figure 1) (45–47). Studies in mice showed that Foxn1 plays a pivotal role in morphogenesis of the 3D architecture (48, 49) by inhibiting a basic morphogenetic pattern of tubulogenesis and inducing the expression of genes that drive TECs differentiation (50). *FOXN1* also regulates *Prss16* and $\beta 5t$ gene expression, implicated in positive selection (45, 51). Knockout of *Hoxa1*, *Pax1*, *Pax9*, *Eya1*, or *Six1* affects *Foxn1* expression resulting in impaired thymic function (52). *Foxn1* expression is also regulated by signals from the surrounding endothelium and mesenchyme, including Bmp4 and Wnt (53, 54).

In epidermis, *FOXN1* is expressed in keratinocytes of suprabasal layer, where these cells stop proliferating and start the terminal differentiation (55). In keratinocytes, *FOXN1* regulates the transcription of more than 50 genes, including protein kinase B and C (PKB and PKC). PKC is an inhibitor of human hair follicle growth *in vitro* (56–58). It is up-regulated in Foxn1 $^{-/-}$ mice while its activity is suppressed in mice overexpressing Foxn1 gene in which differentiation is inhibited. Studies conducted on human keratinocytes have confirmed the role of the gene in the differentiation of the epidermis (58).

Homozygous *FOXN1* mutations cause Nude SCID syndrome, first observed in two Italian sisters presenting with congenital universal alopecia, nail dystrophy, and severe T-cell immunodeficiency with rudimentary thymus (59). In the last few years, neonatal screening and next generation sequencing techniques have led to the identification of subjects with novel homozygous, compound heterozygous, and heterozygous mutations (60). Homozygous patients suffer from immunodeficiency with susceptibility to pneumonia, chronic diarrhea, candidiasis or mycobacterial infections, and Omenn syndrome (59, 61–64). Most of the heterozygous patients show nail dystrophy, usually presenting as leukonychia and minor immunological changes (60, 65, 66). Recurrent infections and atopic dermatitis are observed only in a minority of the patients (60).

Immunological features in patients with homozygous *FOXN1* mutations so far reported include reduction of T lymphocytes, particularly CD4 $^{+}$ T cells (61–64), reduction of T-cell receptor excision circles (TRECs) and naive T CD45RA $^{+}$ lymphocytes, with increase of T memory CD45RO $^{+}$ lymphocytes (62–64). In addition, an increase in the DN CD4-CD8- lymphocytes in the periphery (61–63), a reduction in CD31 $^{+}$ cells, recently emigrated from the thymus was observed in few cases (63). Proliferative response to phytohemagglutinin (PHA) is usually poor or absent and TCR repertoire is oligoclonal (59, 61–63). Although natural killer (NK) and B cells are usually numerically normal, they may be functionally compromised, with impaired production of specific antibodies. Recent studies proved that *FOXN1* haploinsufficiency, caused by *FOXN1* heterozygous mutations, may be associated with T cell-lymphopenia in infants showing low TRECs at newborn screening (60). A progressive normalization of CD4 count is observed in adulthood in heterozygous subjects while CD8 are usually persistently low (60). The increase in CD4 levels is associated to persistently low CD45RA levels suggesting a mechanism of homeostatic



proliferation. A more significant homeostatic expansion in CD4 than CD8 might explain the difference in CD4 and CD8 T cells. Alternatively, another hypothesis is that *FOXN1* dosage plays a stricter role in the expression of CD8 development genes (60).

PAX1 DEFICIENCY AND OTOFACIOCERVICAL SYNDROME TYPE 2

PAX1 is a member of a family of genes that encodes transcription factors implicated in embryogenesis in vertebrates (67). It is located on chromosome 20p11.22 and contains 5 exons (10 Kb) (67, 68). This gene is expressed in fetal mesenchymal cells in the body of intervertebral disks and plays an important role in formation of the segmented vertebral column in humans (69). In mice, it is also expressed in cochlea and has a role in hearing process (70). *PAX1* is also implicated in thymus organogenesis by contributing to the outgrowth of the thymic

epithelial anlage from the 3rd PP and to the regulation of TECs differentiation/survival balance (Figure 1) (71, 72). During pre-natal life, it is expressed in the 3rd PP from E9.5, while in the post-natal thymus it is only expressed in cTECs (72) (Figure 1). In mice, *Pax1* deficiency is associated with moderate thymic hypoplasia (72) that is more severe only when it is associated with *Hoxa3* haploinsufficiency (73). On the contrary, in humans, *PAX1* deficiency is associated with severe thymic hypoplasia, leading to SCID (74). The difference between human and mouse phenotype may be due to a compensatory contribution by murine *Pax9* when *Pax1* is lacking (75).

Otofaciocervical syndrome (OTFCS) is an autosomal dominant disorder characterized by short stature, facial dysmorphism (long face, narrow mandible), shoulder girdle abnormalities, hearing loss, and mild intellectual disability (76). Two different forms of OTFCS have been described but thymus development is only affected in OTFCS2, caused by *PAX1* mutations. Different biallelic deleterious *PAX1* variants cause

OTFCS2 and SCID, characterized by absent thymic shadow, chronic diarrhea, recurrent respiratory infections, pneumonia, and also Omenn Syndrome (74). Different severity of OTFCS2 phenotype is described in different families (70, 71, 77).

The immunological phenotype of patients with OTFCS2 was described very recently in six patients who showed T-cell lymphopenia, impaired proliferative response to mitogens, and normal levels of B and NK cells. In one of them, 98% of CD4 and CD8 T cells were CD45RO⁺ cells, while CD45RA⁺CD31⁺ cells and TRECs were undetectable. TCRV β analysis showed an oligoclonal spectrum. Lymph node biopsy of another patient showed absence of germinal centers and almost total absence of CD3⁺ T cells. Patients with Omenn Syndrome also showed eosinophilia and increased IgE levels (74).

DIGEORGE SYNDROME AND 22Q11.2 DELETION

DiGeorge syndrome (DGS) is usually associated with 3 or a 1.5 Mb *de novo* microdeletion of 22q11.2 (78, 79). In the central region of deletion maps *TBX1* gene containing 9 exons (80) and encodes a Tbx transcription factor, implicated in the regulation of nearly 2,000 genes (81, 82). It is strongly expressed in the 3rd and 4th PP endoderm and in the 4th pharyngeal arch arteries (83, 84), in the otic vesicle, vertebral column, later in tooth bud and, at a lower extent, in the brain (85). It is implicated in pharyngeal arch segmentation and outgrowth of the TECs from the 3rd PP (**Figure 1**). However, it should be noted that *Tbx1* is not expressed in the thymic anlage and thus it is not directly implicated in TECs development (86). On the contrary, *Tbx1* enforced expression within the 3rd PP represses TECs development (86). Reduced levels of *Tbx1* in 22q11.2 deletion syndrome (22q11.2DS) impair the development of neural crest-derived mesenchymal cells that surround the 3rd PP, leading to thymic hypoplasia (87). *TBX1* regulates the expression of secreted Fgfs molecules, namely *Fgf8* and *Fgf10*, implicated in the control of TECs proliferation, differentiation, migration, and survival (88). Fgf receptor IIIb (*FgfR2-IIIb*) regulates the cascade of *Hox3* paralogs transcription factors, *Pax1/Pax9*, and winged helix nude (*Whn*) (**Figure 1**) (89). Moreover, *TBX1* interferes with the ability of small mother against decapentaplegic 1 (SMAD1) to bind SMAD4, preventing effective Bmp4 signaling (82). Bmp4 also contributes to early thymus and parathyroid morphogenesis (90). Isolated *TBX1* mutations may be rarely reported in patients with DGS (91) and *TBX1* gain-of-function mutations can result in the same phenotypic spectrum of loss-of-function mutations (92).

An embryonic phenocopy of DGS with impaired thymus development can be observed because of the lack of retinoic acid during gestation (79, 93, 94). Retinoic acid is able to regulate the expression of *Tbx1* and other molecules implicated in thymus organogenesis including *Pax1*, *Pax9*, *Hoxa3*, *Fgf8*, and *Bmp4* (95). Other epigenetic factors, including maternal diabetes (93) or prenatal exposure to retinoic acid or alcohol

may also explain the alteration of thymus development in DGS (96–101). Recently, we reported on a 7-year-old DGS patient born to a mother with gestational diabetes mellitus in whom a 371 Kb-interstitial deletion of 3p12.3, involving the Zinc Finger Protein 717 (*ZNF717*), MicroRNA-1243 (*MIR-1243*), and *MIR-4273* genes was identified (102). MiRNAs are small, non-coding RNAs involved in the modulation of gene expression by targeting messenger RNAs for degradation, translational repression, or both (103). miRNA-4273 regulates the expression of *Bmp3*, a member of the transforming growth factor β superfamily, involved in thymus and kidney development (104). The expression of other miRNAs, including *MIR-185*, and *MIR-150* can be impaired in 22q11.2DS patients (105). *MIR-185* reduction increases Bruton's tyrosine kinase (*Btk*) expression leading to autoantibody production while *MIR-185* increase leads to dose-dependent T-cell lymphopenia (105, 106). Reduced *MIR-150* expression contributes to the reduction of T and B cells (107). Dysregulation of miRNA biogenesis, due to DiGeorge Critical Region Gene 8 (*DGCR8*) haploinsufficiency, is implicated in the pathogenesis of immunological, cardiac, endocrinological, and neurological phenotype (105, 108). Other genes implicated in the immune response are included in the deleted region. Alterations of Crk-like (*CRKL*), a gene encoding a 39-kDa adapter protein belonging to the Crk family, implicated in many cellular functions, including cell migration and adhesion, are associated with impaired T-cell proliferation in response to TCR triggering (109).

DGS has a prevalence of 1:4,000 newborns (79, 110). Most of these patients present with thymic and parathyroid hypoplasia, congenital heart defects, and craniofacial dysmorphisms (78, 79). Thymic development ranges from athymia in complete DGS (cDGS) to a completely normal thymus development in partial DGS (pDGS), resulting in a variable spectrum of T-cell deficiency (78, 79, 111). cDGS is reported in about 1.5% of the patients (111). Patients with DGS show a wide spectrum of T-cell alterations ranging from completely normal T-cell development to cDGS with absent thymic development (112, 113). In 22q11.2DS patients, thymus is usually small or hypoplastic. The size of the thymus does not predict the levels circulating T cells. In fact, microscopic rests of TECs may be present at aberrant locations (114). Low CD3⁺ T-cell percentage is the most common T-cell defect, followed by low CD3 number (78). CD4 and CD8 compartments are similarly affected (78). T-cell number and percentage tend to increase during the follow up starting from the first year of life (78). Naive CD4 and CD8 lymphocytes are lower in pDGS patients compared to controls independently of age and they decline more rapidly with age (78). The improvement of the lymphopenia with age is not due to a recovery of the thymic function but to the peripheral homeostatic expansion of the available T cells, as suggested by the evidence that T cells are predominantly or almost exclusively of a memory phenotype, TRECs are low and the repertoire is oligoclonal (78, 115, 116). T-cell proliferation, total immunoglobulins, and specific antibody response to vaccines are typically normal. IgM levels are often low, and some patients may show selective IgA deficiency (78). The study of the thymic architecture and

thymocyte development in thymi obtained from pediatric pDGS patients revealed a reduction of mature CD4+ and CD8+ T cell frequency, associated with reduced proportion and function of T regulatory cells (Tregs) (117).

The majority of DGS patients suffer from chronic otitis media, which correlates with primarily conductive hearing impairment (78). Most patients have increased susceptibility to mild infections and only rarely to atypical or severe infections (78). Abnormalities of PP derivatives, predisposing to bacterial colonization, more than immune defects are implicated in this susceptibility (78). Autoimmune diseases, mainly presenting as rheumatoid diseases and idiopathic thrombocytopenia purpura, are reported in ~10% of DGS patients (113, 116, 118). Predisposition to autoimmunity in DGS patients is partially explained by the mechanism of lymphopenia induced T-cell homeostatic proliferation together with the reduction of natural Tregs (nTregs) (117, 119).

CHD7 HAPLOINSUFFICIENCY AND CHARGE SYNDROME

CHARGE (coloboma, heart defects, atresia choanae, growth retardation, genital abnormalities, and ear abnormalities) syndrome is associated to haploinsufficiency in *CHD7* gene, located on chromosome 8q12 (120). *CHD7* is implicated in chromatin organization of mesenchymal cells, derived from the cephalic neural crest (121). Impaired *CHD7* expression correlates with defects in neural crest cells, cephalic mesenchyme, pharyngeal arches, brain, otic vesicle and, in the mesoderm of the developing heart, especially in the outflow tract of the heart (122, 123). *CHD7* is critical for the development of cTECs and mTECs from pharyngeal endoderm by regulating *Bmp4*, which in turn regulate *Foxn1* expression (Figure 1) (124). *Chd7* deficiency is also associated with down-regulation of *Ikaros*, Interleukin 7 receptor (*Il7r*), recombinae activating gene 1 (*Rag1*) (124). Studies suggest that *CHD7* can also regulate *TBX1* expression (74, 125).

CHARGE syndrome is characterized by different degrees of thymic alterations and even by complete thymic aplasia, resulting in combined immune deficiency (126). A wide spectrum of T-cell deficiency and isolated humoral immune deficiency may be observed in patients with CHARGE syndrome (126). They may show severe T-cell deficiency resembling SCID or, similarly to DGS, they may present transient lymphopenia, that usually normalizes over time. A close association between lymphopenia and hypocalcemia has been identified (115). Severity of the T-cell lymphopenia relates to the degree of thymic hypoplasia (127). CHARGE patients also show decreased naive CD4 and CD8 T-cells, peripheral T cells, and TRECs. Peripheral B-cell differentiation and immunoglobulin production are normal but in a 3rd of the patients an abnormal expression of IgM on class-switched memory B cells and diminished production of specific antibodies may be observed (127). Selective antibody deficiency tends to resolve spontaneously over time (127). In a recent paper, immunological features were compared between CHARGE and DGS patients.

Total lymphocyte count was slightly lower in DGS patients compared to CHARGE patients and persistent lymphopenia was more common in DGS patients than in CHARGE. IgM levels were significantly lower in DGS patients compared to CHARGE (128).

Most of the patients have increased risk of recurrent infections, including recurrent otitis media, sinusitis, conjunctivitis, dermatitis, respiratory tract infections, pneumonia, and sepsis (127). Severe or atypical infections may also be reported including recurrent oral candidiasis, recurrent severe infections, septic shock, and chronic viral infections (129). As for partial DGS also in CHARGE syndrome the frequent need of invasive operative procedures and anatomical alterations, including extensive ear, sinus, nasal and palatal malformations, altered Eustachian tube anatomy, gastroesophageal reflux, or neurological abnormalities with compromised drainage and aspiration may help explain the susceptibility to infections (127). Immune defects including impaired thymus development and subsequent humoral deficiency may also contribute to the susceptibility to infections but at a lower extent (128). Patients with CHARGE syndrome also show increased risk of atopy, reported in 65% of the patients, usually presenting as food allergy (128). Increased susceptibility to autoimmune disorders may also feature this syndrome (129). Patients with complete athymia may present with atypical SCID (Omenn-like) phenotype (130).

2P11.2 MICRODELETION AND FOXI3 HAPLOINSUFFICIENCY

A microdeletion at 2p11.2 has been identified in five families presenting with DGS features, including hypocalcaemia, asymmetric crying face, low TRECs and T-cell lymphopenia, without typical facial dysmorphism, and heart abnormalities. *FOXI3*, a member of FOX family transcription factors, implicated in development of brachial arch-derived structures was considered the candidate gene for this phenotype (131). Early in embryonic development *Foxi3* is broadly expressed in the pre-placodal ectoderm surrounding the neural plate, from which all craniofacial sensory organs derive (132, 133). Subsequently, its expression is restricted to the region from which otic and epibranchial placodes derive and finally to the ectoderm and endoderm of the pharyngeal arches. A deletion of *FOXI3* gene has been recently identified in a patient with left congenital aural atresia and ipsilateral internal carotid artery agenesis (134). *Foxi3* is also implicated in the differentiation of the epithelial cells within the epidermis as suggested by the identification of *Foxi3* heterozygous mutations in several hairless dog breeds with hair follicle and teeth defects (135) and in thymic cortico-medullary differentiation. *Foxi3* is implicated in segmentation of the pharyngeal apparatus and LOF of *Foxi3* alone or in combination with *Tbx1* LOF, leads to failure of the pharyngeal arch segmentation due to the inability of the epithelia to properly invaginate with subsequent thymic hypoplasia/aplasia (136).

The main clinical features of the different syndromes are compared in Table 1.

TABLE 1 | Comparison of the main clinical features among different congenital disorders of thymic development.

	DGS	FOXN1	OTFC2 syndrome	CHARGE syndrome
Dysmorphic features	Low ears, telecanthus, down/up slanting palpebral fissures, short philtrum, velopharyngeal insufficiency	Epicanthal folds	Ear malformations, preauricular fistulas, vertebral malformations, lacrimal ducts abnormalities, abnormal clavicles and scapulae, retrognathia, downslanting palpebral fissures, long eyelashes, blue sclerae, epicanthal folds, small nose	Ear abnormalities, coloboma, choanal atresia, cleft palate
Cutaneous alterations	–	Alopecia, nail dystrophy	–	–
Thymic alterations	Aplasia (cDGS), hypoplasia/normal (pDGS)	Aplasia (homozygous mutations), hypoplasia (heterozygous mutations)	Aplasia/hypoplasia	Aplasia/hypoplasia
Cardiopathy	Tetralogy of fallot, ventricular septal defect, type B interrupted aortic arch, truncus arteriosus, right aortic arch, aberrant right subclavian artery	–	–	Atrial septal defects, ventricular septal defect, patent ductus arteriosus
Infections	Recurrent/severe infections (cDGS) recurrent infections (pDGS)	Recurrent/severe infections (homozygous mutations), recurrent infections (heterozygous mutations)	Recurrent/severe infections	Recurrent/severe infections
Omenn syndrome	+	+	+	+
	(cDGS)	(homozygous mutations)		

DGS, DiGeorge syndrome; cDGS, complete DGS; pDGS, partial DGS; OTFC2 syndrome, Otofaciocervical syndrome type 2; CHARGE syndrome, coloboma, heart defects, atresia choanae, growth retardation, genital abnormalities, and ear abnormalities syndrome.

TREATMENT OPTIONS FOR ATHYMIC CONDITIONS

Hematopoietic stem cell transplantation (HSCT) represents the cornerstone for the treatment of SCID. However, in SCID due to genetic defects that impair development and function of the thymic epithelium, theoretically thymus transplantation would represent the most appropriate therapy. Thymic tissue is obtained from infants undergoing to median sternotomy for open heart surgery. Cultured postnatal human thymic tissue is then transplanted in thin slices into the quadriceps muscle (137). In cases with successful transplant, few months after the transplant the graft is colonized by host stem cells and is able to support normal thymopoiesis (138) leading to the development of mature naive T-cell with diverse TCRV β repertoire and able to proliferate in response to mitogens. T-cell levels in surviving patients are usually low for age but are sufficient to respond to viral, disseminated, and other infections leading to a resolution of the immunodeficiency (139). In some cases, reduced thymic output may be explained by other comorbidities, such as heart failure and hypoxia stress, that may cause hypoperfusion of the graft (139). The success of the transplant may be also limited in patients with active viral infections since the virus itself, its treatment, or both might inhibit the thymopoiesis (139). In patients with successful transplant, naive T-cells are usually detected within 6 months after the transplant (139, 140). Antibody responses

and immunoglobulin levels normalize (62) even though numbers of class-switched memory B cells may remain relatively low. The success of the transplant is not correlated with the amount of tissue transplanted, HLA matching, culture conditions or immunosuppression of the recipient (141). Immunosuppression can be used to delete reactive oligoclonal T cells and mature T cells responsible of graft-versus-host disease and graft rejection (137, 139). Overall survival in DGS is 75% and mortality is usually related to pre-transplantation morbidity, mainly viral infections, and chronic lung disease (115, 140, 142). Thymus transplantation has been recently used to treat 2 patients with *FOXN1* deficiency and both survived (62, 143, 144) while its use in athymic CHARGE and OTFC2 patients has never been reported. Autoimmune disorders, including thyroiditis and severe cytopenia, represent the most common complication after thymus transplant (139, 140).

Adoptive transfer of mature T cells from human leukocyte antigen identical siblings through bone marrow transplantation represents an alternative to thymus transplant to treat SCID in athymic patients (145). However, only post-thymic T cells engraft in this case and naive T cells do not develop. Survival after matched unrelated donor and matched sibling transplantations in cDGS were reported as being 33 and 60%, respectively (145) while in CHARGE out of six patients treated with HSCT three had graft vs. host disease and three died post-transplant (125, 130, 146, 147). Long-lasting survival patients after matched sibling donor transplantation are reported (148). Four *FOXN1* deficient

patients underwent HSCT and two survived (59, 63, 64, 149). One of them is currently well at 22 years of age (unpublished data). The study of the T cell compartment in this patient, 5 years after HSCT showed a marked reduction of CD4CD45Ra levels with normal CD8CD45Ra levels. However, TCRV β repertoire, was largely impaired in the CD8 subset (150). Six patients with OTFC2 were treated with allogeneic HSCT. T-cell reconstitution was not observed in any of the patients, despite successful engraftment in three of them. In one of the cases with successful engraftment all the T cells showed a memory (CD45RO+) phenotype, but no *de novo* generation of a polyclonal repertoire of naive T cells was observed. The remaining 2 patients showed persistent T cell lymphopenia leading to severe and recurrent infections, and death for septic shock in one patient and to severe autoimmune hemolytic anemia in the other (74). Together, these data indicate that HSCT may be unable to correct the profound T cell immunodeficiency of this disease.

CONCLUSIONS

In conclusion, in this review we described different pathways involved in thymus development and the clinical phenotypes associated with their impairment. We also summarized the outcome related to different therapeutic approaches to these disorders. We highlighted the clinical importance of the early detection of a defect in the pathways involved in T-cell development. With the recent introduction of newborn screening programs a timely identification of patients affected with defects of the T-cell development before the onset of the symptoms

is now possible, and this prompted the definitive treatment with HSCT. This has been proven effective in improving the prognosis. However, in some cases HSCT is not required for the management of infants with T-cell lymphopenia at birth, since the T-cell development tend to improve with age (prematurity, FOXP1 haploinsufficiency). In other cases, HSCT may not be curative since the defect involves thymus development. However, genome-wide association studies have shown that a large proportion of variants likely to cause human disease are located outside of the protein-coding domains, so whole genome wide approaches might lead to proper identification and thus correct treatment plans for immunodeficiency disorders resulting from aberrant expression of some of the genes discussed in the present review.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication. GG, CB, MD, EC, and RR reviewed the literature, organized and wrote the manuscript. CP wrote the manuscript and supervised all the work. RP did the picture. All authors reviewed and approved the manuscript.

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Thymic Engraftment by *in vitro*-Derived Progenitor T Cells in Young and Aged Mice

Jastaranpreet Singh¹, Mahmood Mohtashami², Graham Anderson³ and Juan Carlos Zúñiga-Pflücker^{1,2*}

¹ Department of Immunology, University of Toronto, Toronto, ON, Canada, ² Sunnybrook Research Institute, Toronto, ON, Canada, ³ Institute of Immunology and Immunotherapy, University of Birmingham, Birmingham, United Kingdom

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Nicolai Stanislas Van Oers,
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Tokushima University, Japan

*Correspondence:

Juan Carlos Zúñiga-Pflücker
jczp@sri.utoronto.ca

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T cells play a critical role in mediating antigen-specific and long-term immunity against viral and bacterial pathogens, and their development relies on the highly specialized thymic microenvironment. T cell immunodeficiency can be acquired in the form of inborn errors, or can result from perturbations to the thymus due to aging or irradiation/chemotherapy required for cancer treatment. Hematopoietic stem cell transplant (HSCT) from compatible donors is a cornerstone for the treatment of hematological malignancies and immunodeficiency. Although it can restore a functional immune system, profound impairments exist in recovery of the T cell compartment. T cells remain absent or low in number for many months after HSCT, depending on a variety of factors including the age of the recipient. While younger patients have a shorter refractory period, the prolonged T cell recovery observed in older patients can lead to a higher risk of opportunistic infections and increased predisposition to relapse. Thus, strategies for enhancing T cell recovery in aged individuals are needed to counter thymic damage induced by radiation and chemotherapy toxicities, in addition to naturally occurring age-related thymic involution. Preclinical results have shown that robust and rapid long-term thymic reconstitution can be achieved when progenitor T cells, generated *in vitro* from HSCs, are co-administered during HSCT. Progenitor T cells appear to rely on lymphostromal crosstalk via receptor activator of NF- κ B (RANK) and RANK-ligand (RANKL) interactions, creating chemokine-rich niches within the cortex and medulla that likely favor the recruitment of bone marrow-derived thymus seeding progenitors. Here, we employed preclinical mouse models to demonstrate that *in vitro*-generated progenitor T cells can effectively engraft involuted aged thymuses, which could potentially improve T cell recovery. The utility of progenitor T cells for aged recipients positions them as a promising cellular therapy for immune recovery and intrathymic repair following irradiation and chemotherapy, even in a post-involution thymus.

Keywords: T cell progenitors, T cell development, thymus regeneration, thymic hypoplasia, thymic involution, aged thymus

INTRODUCTION

T cells are essential mediators of antigen-specific, long-term adaptive immunity. The thymus is responsible for the development of self-tolerant, immunocompetent T cells, but given a lack of self-renewing cells, is continually reliant on replenishment of new T cell progenitors derived from bone marrow (BM) hematopoietic stem cells (HSCs). Subsequent maturation of T cells occurs through a series of tightly regulated and directed differentiation stages that are dependent on signals from the specialized thymic microenvironment. These processes lead to the generation of mature $CD4^+CD8^-$ and $CD4^-CD8^+$ single positive (SP) T cells that exit the thymus and seed the peripheral organs (spleen, lymph nodes), where they can subsequently encounter antigen, undergo expansion, and acquire effector or memory functions (1).

T cell immunodeficiency, or T lymphopenia, can result from perturbations to the thymic microenvironment due to age-related thymic involution, irradiation required for cancer treatment or from infections that directly target T cells (2). One of the major causes of T cell deficiency is primary immunodeficiency, which is often inherited, leading to an early disease onset. Extrinsic factors can also adversely affect the immune response. In particular, the use of chemotherapeutic agents such as cyclophosphamide prior to hematopoietic stem cell transplantation (HSCT) can cause rapid transient involution of the thymus (3–5). While these drugs are critically required for eradication of the cancer cells, they also harm healthy dividing cells, including cells of the hematopoietic compartment and thymic epithelial cells (TECs) that constitute part of the thymic microenvironment. As a result, there is a delay in T cell recovery following HSCT, and a paucity of *de novo* T cell generation from HSC-derived progenitors (5–9), which can be particularly problematic for aged patients that are concomitantly undergoing age-related thymic involution (Figure 1). The end result is dramatic changes in the T cell compartment of patients including a decline in naïve T cell output, reduced T cell diversity, and increased susceptibility to infection, autoimmune diseases and cancer (10). Therefore, altered thymic architecture is a key trigger for the deterioration of T cell-related immune function in the aged, and insight into strategies that enhance thymic function in adults is of critical importance. Here, we explore the challenges of T cell recovery and thymic regeneration following myeloablative and irradiation treatments, and leading approaches in the field to overcome these issues. We focus on recent advances that take advantage of cell-based treatments, such as progenitor T cell engraftment, for overcoming periods of immunodeficiency following HSCT, particularly in aged individuals.

T CELL RECONSTITUTION AFTER MYELOABLATIVE TREATMENTS

Allogeneic HSCT is a mainstay for the treatment of a large number of diseases of the hematopoietic system. A number of modifications to HSCT procedures, including T cell depletion, $CD34^+$ hematopoietic stem/progenitor cell selection,

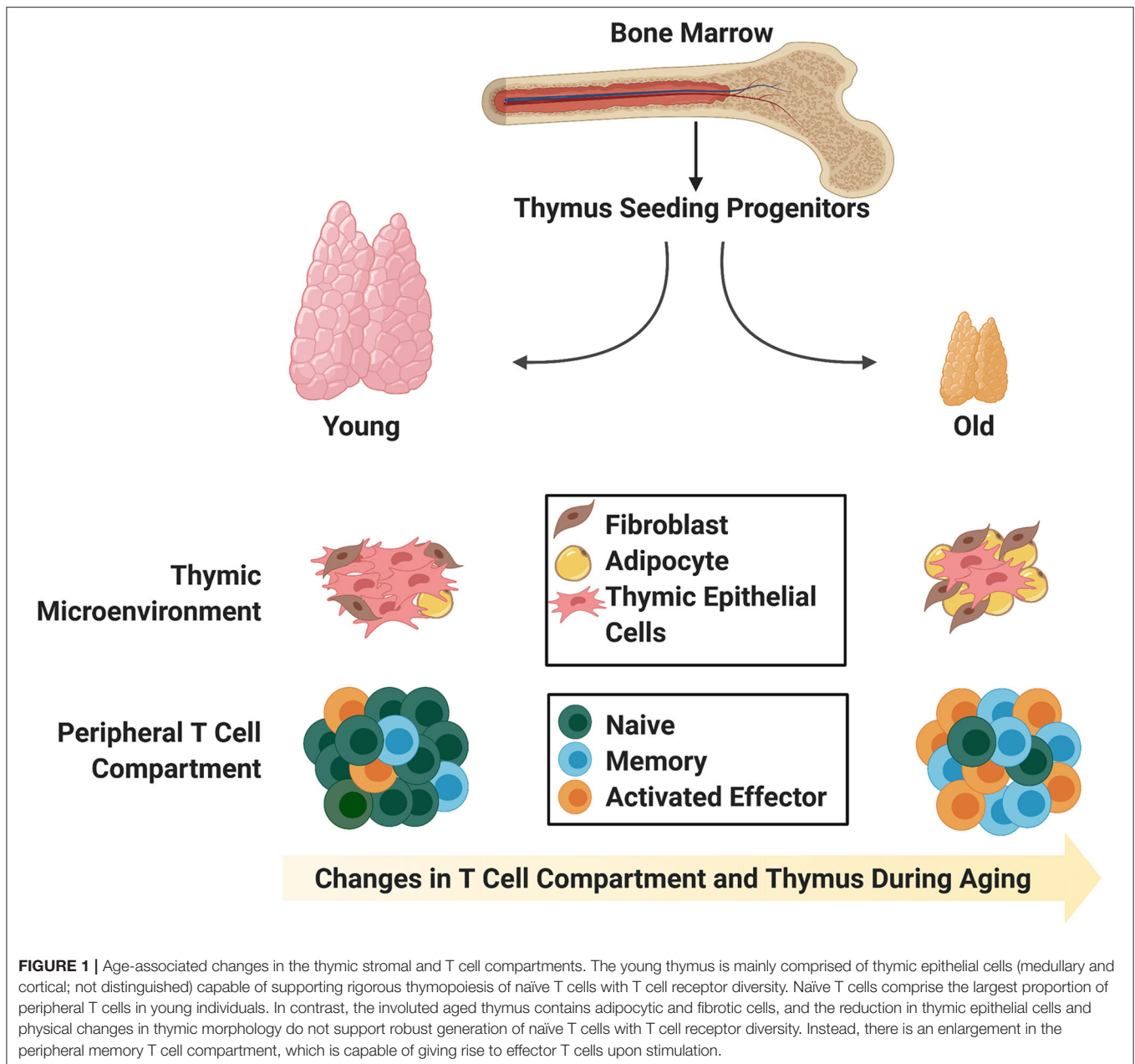
and the use of irradiation and chemotherapeutic drugs, have greatly improved post-transplant clinical outcomes (11, 12). Nevertheless, T cell repopulation post-transplantation remains a major hurdle (5). T cell recovery is often delayed by months and it may take years to fully restore normal numbers of T cells and functionality, if at all (13, 14). Furthermore, there appears to be an inverse correlation between time to post-HSCT T cell recovery and the age of the recipient (15). While it may take up to 6 months to 1 year in the young to recover T cells with a wide T cell receptor (TCR) repertoire, it may take years in adult patients to witness evidence of new T cell generation, if ever (16–18). With an increasingly aging population, there is an imminent need for a dependable way to reconstitute all blood cells, including T cells, in cancer patients that have received chemotherapy and irradiation. Otherwise, patients remain susceptible to a variety of complications that can result in mortality due to general weakened immunity that renders them vulnerable to opportunistic infections and potential cancer relapse (19).

To mitigate cancer relapse, many clinics maintain the standard practice of not depleting donor T cells from HSCT grafts. While donor T cells contained within the graft can mount anti-tumor responses against the relapsed cancer, they can also induce graft vs. host disease (GvHD) against vital organs, even in human leukocyte antigen (HLA)-matched transplants (20). The complications of GvHD are, in turn, managed by defined doses of prescribed immunosuppressants, which aim to balance the anti-tumor effects of the graft with the suppression of GvHD (21). However, this is an unlikely long-term solution for patients, as the vast majority of patients succumb to GvHD or cancer relapse due to immunosuppression within 5 years post-transplant. To this end, an extensive understanding of the phenotypic changes and microenvironmental cues facilitating the transition of HSCs in the BM to T cells in the thymus may be useful for development of strategies to improve T cell outcomes post-HSCT.

The Intrathymic Intricacies of T Cell Development

T cell precursors arise from HSCs in the adult BM (Figure 1). Thymus seeding progenitors (TSPs) arrive in the thymus in small numbers, where upon interaction with the thymic epithelial cells (TECs), give rise to early thymic progenitors (ETPs). ETPs in both human and mice differentiate through successive $CD4^-CD8^-$ double negative (DN) stages that require Notch receptor and Delta-like-4 (Dll4) ligand interactions. Targeted deletions of either *Notch1* receptor in hematopoietic cells, or *Dll4* in TECs, result in the abrogation of T cell development (22–25). Notch signaling mediates cell fate restriction, which begins within the BM TSP subset, and continues following entry into the thymus (26).

Proper stage-specific maturation of T cell precursors is dependent on thymic structure and the diverse cells present in thymic stroma. The thymus is differentiated into an outer cortical and an inner medullary region, which contain cortical and medullary TECs (cTECs and mTECs), respectively. Their maintenance, in turn, is governed by a complex interplay between lymphoid and stromal compartments within the thymus (termed



lymphostromal thymic crosstalk) (27). Studies in the postnatal mouse have demonstrated that TSPs express CCR7 and CCR9, and enter the thymus at the junction between the cortex and medulla (1). Thymic entry is governed by the chemokines CCL21 and CCL25, which are expressed primarily by mTECs and cTECs, respectively. As they differentiate, T cell precursors migrate outwards toward the subcapsular zone of the cortex in response to a CCL25 chemotactic gradient (1, 28, 29). In the subcapsular zone, the $CD4^-CD8^-$ DN thymocytes proliferate and subsequently differentiate into $CD4^+CD8^+$ DP cells that up-regulate CCR7 on their cell surface and migrate toward the medulla (mediated by CCR7 ligands, CCL19 and CCL21) where the final stages of T cell development take place (30).

These chemokine-receptor interactions constitute one of the pivotal crosstalk signals dictating strict migration of developing thymocytes through the thymus, and they are conserved in humans (31, 32).

Thymic crosstalk also induces proliferation and ensures selection of a T cell repertoire that is self-tolerant, but reactive to foreign challenges. Relatively little is known of the involvement of crosstalk in the development and function of the thymic cortex, although the notion of crosstalk is well-accepted (27). Mouse models showing a block in early T cell development ($CD3\epsilon$ transgenic mice, $CD117^{-/-}CD132^{-/-}$ double-mutant mice) lack demarcation of cortical and medullary areas, and show marked reductions in thymocyte numbers compared to wildtype

(WT) mice, implying that the early stages of T cell development are important in TEC maturation (27). Thymic crosstalk during positive and negative selection events have been better defined. Low/intermediate affinity interactions between TCR and major histocompatibility complex (MHC)/peptide complexes on TECs prevent cell death during positive selection. Positively selected thymocytes then localize to the medulla, where high affinity interactions between the TCR and MHC/peptide complexes on thymic dendritic cells result in apoptosis via negative selection of self-reactive cells (1, 28). Self-tolerance by negative selection is established through the expression of the transcription factors autoimmune regulator (*Aire*) and *Fzf2* by mTECs (33). These two independent factors are regulated by distinct signaling pathways, the RANK/CD40 pathway and the LT β R pathways, respectively, are non-redundant and promote the expression of peripheral tissue-restricted antigens (TRA) on mTECs (33, 34). T cells displaying a strong self-reactivity toward TRA are deleted.

The non-canonical nuclear factor- κ B (NF- κ B) pathway has a well-established role in thymic crosstalk during selection. Lymphotoxin- β receptor (LT β R), RANK, and CD40 are the key tumor necrosis factor (TNF) superfamily members that signal through the non-canonical NF- κ B pathway in the context of thymic crosstalk and self-tolerance (35). In mice, LT β R deletions led to defective medullary development, and a reduced or absent medulla. The reduction of *Aire*⁺ mTECs was relatively mild (35, 36), although *Fzf2*-dependent TRA expression on mTECs was drastically decreased (33). The involvement of CD40 ligand (CD40L)-CD40 interactions has also been described (35). Overexpression of CD40L in the thymus resulted in abrogated thymus cortex formation and medullary expansion, whereas analysis of CD40L-deficient mice revealed no obvious defect in medullary organization by immunohistochemistry. RANK- and RANKL-deficient mice exhibit a severe reduction in *Aire*⁺ mTECs and overall mTEC numbers, although the formation of a thymic medulla is still detectable. Furthermore, RANKL-RANK and CD40L-CD40 signaling exhibit a cooperative role in mTEC development and self-tolerance (35). Notably, the defects seen in these deficient mice do not affect *Fzf2*-restricted TRA expression and are milder compared to mutant mice deficient in the NF- κ B activation pathway such as *aly/aly* (NF- κ B-inducing kinase mutation), and RelB-deficient mice (35–37). Although the expression and function of these molecules on thymocyte and TEC subsets is well-understood in mice, this has yet to be elucidated in the human context. Furthermore, harnessing insights from these developmental processes could be important for restoring the thymus and T cell-mediated immunity in the aged following HSCT.

AGE-RELATED THYMIC INVOLUTION

Defects in both the hematopoietic and thymic stromal components contribute to decreased thymic output in aged individuals. The fundamental reason for the late or absent T cell recovery in aged patients is the natural and progressive decline of the immune system, especially of the thymus, in

a process called thymic involution. Thymic involution starts to occur before puberty, but is at its peak during puberty and the decline continues steadily with age (38). Although thymus involution in the aged was noted many years before the function of thymus was discovered (39), the evolutionary advantage of this physiological process remains a mystery. Thymic involution, which results in its several fold size reduction, is not perceptible with physical examination, but it is concomitant with physiological changes that can be measured. Multidetector computed tomography (CT) scans can detect increasing replacement of soft tissue that is mainly occupied by thymocytes and TECs by a less dense, X-ray permissive fatty tissue (40, 41). These changes impact both the thymocyte and TEC compartments of the thymus, having detrimental impacts on the establishment of T cell-based immunity in the adult.

Impairments in T Cell Development

Since the thymus requires continuous seeding by BM HSC-derived progenitors, the chance of T cell generation is further diminished since aged HSCs have a propensity to differentiate toward the myeloid fate (42) in both mice (43–47) and humans (48, 49). These alterations in HSC function inevitably affect the lymphoid compartment, and different groups have shown that aged mice not only have less ETPs, but that the ETPs also have reduced expansion and differentiation potential (50, 51). Furthermore, HSCs from old mice do not efficiently repopulate lymphoid cells in young recipients (46).

The later stages of T cell development are also affected by aging. DP and SP thymocytes in mice show reduced CD3 expression, suggesting that they may have potential defects in TCR-based stimulation (52–54). These results are supported by the observation that cells from aged mice have cell cycle defects (53). It follows that defects during development could impact the kinetics, proliferation, signaling capacity, and therefore, immunocompetence, of recent thymic emigrants (RTEs) from the aged thymus (55, 56). It has been demonstrated that the size of the peripheral T cell pool remains relatively consistent throughout life, even after thymic involution (57), so it is likely that these defects are etched into RTEs during their development within the thymus. RTEs can be detected through assessment of T cell receptor excision circles (TRECs) (58) or by cell surface markers in T cells circulating in the blood (59, 60). Studies have shown that there are lower TREC levels in elderly individuals, and these are associated with an ~80% reduction in naïve T cells (61–64) and an increase in the memory T cell compartment (**Figure 1**). The memory T cells in adults, while able to undergo proliferative responses, possess defects including skewed cytokine production (65, 66).

Taken together, there are several immunological consequences of aging including the continual decrease in naïve T cell output, a limited TCR repertoire (67) and hence, the narrowing of the diversity of foreign antigens potentially recognized by aging individuals, leading to a higher susceptibility to pathogens.

Changes in the Thymic Stromal Microenvironment

Age-induced changes within the thymic niche could likely account for many of the T cell developmental defects seen within the aged thymus. During thymic involution, there are alterations in the architecture starting at around the time of puberty when the rate of atrophy is greatest (38, 68, 69). This atrophy is concomitant with a blurred demarcation between cortical and medullary regions, down regulation of various TEC markers, and increased adiposity (70, 71) and fibrosis (72, 73) of the aged thymus (**Figure 1**). Increased adiposity, particularly in the human thymus, has been linked to an inhibition of thymic function (71). In support of this notion, one group demonstrated that induction of obesity in a mouse model accelerated thymic involution (74), and further, inhibition of adipogenesis within the thymus could ameliorate age-related thymic involution (70). The mechanism behind how adipocytes within the thymus alter T lymphopoiesis and thymic function are unclear, but likely involve the action of cytokines secreted by adipocytic cells, which aggravate, but do not cause, thymic involution (10, 71). Given the impairments in both the thymocyte and TEC compartments during age-related thymic involution, it could be expected that lymphostromal crosstalk between these two compartments is also impaired and is a likely contributing factor toward thymic involution.

HSCT in the Aging Population

Considering the typical disadvantages presented by the aged thymic microenvironment, further injury by conditioning regimens to susceptible TECs could be critically damaging to thymic engraftment following HSCT (75, 76). There is a substantial lag between the time of bone marrow engraftment following HSCT and migration of thymic seeding progenitors to the thymus even in younger patients. Preclinical studies confirmed that total body irradiation followed by HSCT results in poor engraftment in the aged thymus when compared to young (73). During this refractory period, there is an absence of crosstalk between thymic stromal and hematopoietic cells, which prolongs thymic regression. The resulting inferior stromal environment is inefficient for T cell proliferation of even young thymocytes (77). It also leads to blurred definition of boundaries between the medullary and cortical regions, suggesting inadequacies in TEC function (72). In support of this notion, the age-related decrease in Aire expression in the medulla and abnormally low negative selection of emerging thymocytes, led to increased autoreactivity and inflammation (78). Taken together, there appears to be a confluence of events that undermine the restoration of conditions for *de novo* generation of T cells within the aged thymus following HSCT. Employing strategies that aid in thymic regeneration and T cell reconstitution remains an important task.

STRATEGIES FOR ENHANCING THYMIC REGENERATION

To reverse the disadvantages posed by age-related changes, there have been wide-ranging attempts to protect the thymus from the

deleterious effects of myeloablative regimens, and preserve and strengthen thymic properties that enable T cell reconstitution. While there are many lines of evidence for the demise of the thymus with age, there is confirmation of its continued function as an essential component of immunity. Detection of TRECs has confirmed that there is a constant output of naïve T cells or RTEs that continue to migrate to the periphery (8). There is also strong evidence for recruitment of TSPs into the thymus after HSCT (73). Comparison of the young and the aging thymus has led to the discovery of a range of possible interventions that aim to improve the thymic milieu to rejuvenate the stromal cells. These include keratinocyte growth factor (KGF) administration for protecting TECs during irradiation-induced injury (79–82), and IL-22 administration for stimulation of TEC proliferation and repair (83). However, these approaches have not led to rapid, early or complete restoration of the peripheral T cell compartment after transplant, and it is difficult to delineate whether these strategies directly enhance thymic activity in the aged, or, in part, due to the promotion of hematopoiesis in the BM. Furthermore, these approaches focus solely on restoration of the thymic microenvironment, but do not address the effects on progenitor cells incoming to the thymus.

Accordingly, the most remarkable effect on thymus size and output was uncovered as a consequence of physical (84) or chemical castration in both sexes (51, 85), revealing the potential for reversal of age-related thymic atrophy. These effects may be blocked by administration of sex hormones, demonstrating the direct effect of androgens on immunity, and specifically, on thymus involution. Chemical castration involves the administration of agonists of gonadotropin-releasing hormone receptor, which eventually leads to *hypogonadism* and hence, a reduction of sex hormones, testosterone and estradiol, a treatment also known as sex steroid inhibition (SSI). SSI results in physiological changes in the thymus at the molecular level, including an increase in the chemokine CCL25 for the recruitment of CCR9-bearing progenitor cells (86, 87). Importantly, SSI also appears to directly increase DLL4 expression in TECs (88), which is typically downregulated in the aging thymus. Age-related perturbation of DLL4 expression levels appear to be restored by SSI (88), enabling effective inhibition of B cell development and promotion of mainly T cell development, but also generation of alternative lineages such as dendritic cells (89).

The effect of thymic restoration by SSI, while striking, is transient, with the mouse thymus reaching its peak within 2 weeks of treatment and returning to its involuted size after 2 weeks (68, 90). In addition, although cellularity within the thymus is temporarily restored, aged TECs, especially in the medulla, remain qualitatively different from those from a young thymus according to one study (68). Gene expression profiles of aged TECs restored by SSI largely aligned with the TECs from non-castrated control aged mice and not the young mice. Furthermore, there was limited TRA presentation by regenerated TECs, which created an imbalance in central tolerance. Despite this shortcoming, no adverse effects were observed in SSI-treated preclinical models, although there is evidence or speculation for regulatory T cells mitigating autoreactivity by cells that may have escaped central tolerance. At this point, it appears that

the protective benefits of creating more RTEs that mature in the periphery may outweigh the adverse effects of potential autoreactive T cells that escape negative selection processes (68, 91). Hence, SSI remains a viable option for treatment of the aged to generate *de novo* peripheral T cells. In this regard, when SSI treatment was given following the combination of chemical therapy and HSCT in preclinical models, thymocyte recovery was twice as efficient with SSI treatment (85).

In contrast to the clear increase in the number of thymocytes after cytoablative treatment followed by SSI, the TEC compartment of the thymus appears to increase in cell size, but not necessarily due to TEC proliferation (85, 92). Elegant immunofluorescence analyses of TECs demonstrated that morphological changes in TECs, and not *de novo* regeneration, were largely responsible for the expansion of the cortical region of the aged thymus after SSI treatment (92). Concurrently, genetic pathways associated with cell morphology exhibited the most dynamic changes in TECs during thymic regeneration.

Interestingly, the plasticity observed in the thymus is not unique to SSI treatment. In many diseased states, including lymphocytic choriomeningitis virus (LCMV) infection as an example, the thymus is diminished in size. Consistently, a dramatic reduction in thymocyte number (especially the CD4⁺CD8⁺ DP population) was observed (93). This is likely a protective measure against potential tolerization to viral antigens. The observation that the thymus is reduced to a fraction of its normal size within 9 days after LCMV infection, and the restoration to its former size 15 days after the virus is cleared, is reminiscent of the thymus' response to SSI treatment. It also suggests that the regenerative ability of the thymus is intrinsic to the thymic stroma.

While the approaches discussed so far have inherent benefits, they do not successfully address the two-pronged problem with T cell recovery after HSCT in aged populations – that is, the need for increased thymus-seeding progenitors in the host, and an intact thymic environment for provision of appropriate developmental cues for T cell development.

PROGENITOR T CELLS AND THYMIC RECONSTITUTION

We and others have shown that another promising strategy to enhance T cell reconstitution post-HSCT is the adoptive transfer of *in vitro*-derived proT cells to rapidly restore the T cell compartment and T cell mediated immunity (94–97). Zakrzewski et al. initially demonstrated that adoptive transfer of *in vitro*-derived mouse CD4⁺CD8⁺ DN cells together with HSCs led to increased T cell reconstitution in both the thymus and periphery of mice that had undergone allogeneic HSCT. More importantly, *in vitro*-derived T cells exhibited early graft vs. tumor activity in HSCT recipients (95). We extended this to humans and characterized a CD34⁺CD7⁺⁺ proT cell subset generated from umbilical cord blood (UCB)-HSC co-cultures with OP9-DL1 cells (96). This population was capable of homing to and engrafting the thymus of NOD-*scid* IL2R γ ^{null} (NSG) immunodeficient mice, demonstrating their ability to

“kickstart” the process of T cell recovery. The human proT cell population could be further fractionated based on CD5 expression, with CD5⁺ proT2-cells having enhanced thymic reconstitution capacity when placed in competition with CD5⁺ proT1-cells. Of note, human proT2-cells, when transferred together with HSCs, had the ability to facilitate long-term HSC-derived T-lymphopoiesis in a preclinical mouse model (97). Thus, the thymus-bound potential of proT cells offers several advantages for their implementation during HSCT by shortening the time a patient is left immunocompromised.

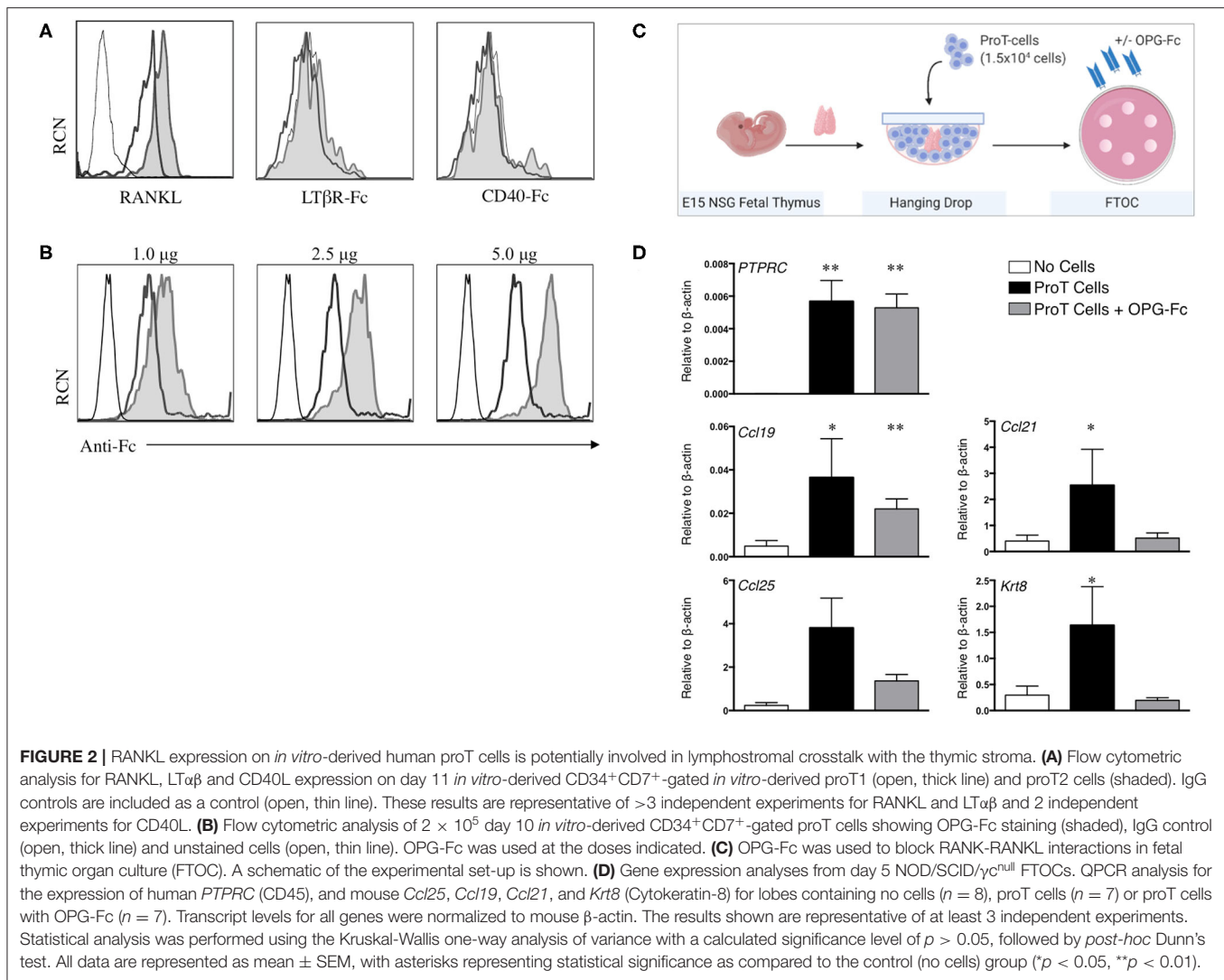
Importantly, proT cells lack a cell-surface TCR and therefore are unable to induce GvHD. Strict histocompatibility between proT cells and the host is not required (2, 98), as developing T cells within the thymus undergo selection and tolerization by the host thymus. After proT cell adoptive transfer in pre-clinical mouse models, the mature functional T cells that emerge have a broad repertoire capable of combatting both cancer and infections (95, 99), and can develop into various functional subsets (effector, helper, memory) (100), while demonstrating no autoreactivity.

A Role for ProT Cells in Lymphostromal Crosstalk

ProT cell-engrafted NSG mice also display thymic plasticity and restoration of the thymic microenvironment (97). We previously demonstrated that the highly disorganized thymic structure of NSG mice was alleviated after adoptive transfer of *in vitro*-derived human proT cells, which induced the organization of cortical and medullary environments (97). These findings were strengthened by the observed increase in transcripts for *Ccl25* and *Ccl19* within the thymuses of proT-engrafted mice. Together, these findings suggested that human proT cells could directly act on stromal elements in the NSG thymus, engage in lymphostromal crosstalk, and thus, promote the formation of chemokine-rich niches for the recruitment of CCR7- and CCR9-expressing BM-derived TSPs.

As described above, signaling through the LT β R, CD40 and RANK receptors on TECs are among the key crosstalk signals required for the maturation of the TEC compartment (35, 101). Thus, we assessed cell surface expression of ligands for these receptors (RANKL, LT α β , and CD40L) on human *in vitro*-derived proT cells (94). We previously reported the expression of RANKL on proT cells, with the proT2 subset expressing 2.5x higher levels than proT1 cells (97), and as shown in **Figure 2A**. However, we observed little to no expression of LT α β and CD40L on proT cells. These findings suggest a potential specific role for RANKL in proT cell-mediated effects in the thymus.

As our findings described so far were consistent with the known critical role for RANKL in thymus organogenesis, organization and repair (27), our next goal was to demonstrate a role for RANKL in proT cell-induced effects within the thymus. The naturally-occurring decoy receptor for RANKL, osteoprotegerin (OPG) has a high affinity for RANKL, and is produced physiologically to prevent RANK/RANKL interactions (102). We therefore utilized an OPG-Fc chimeric fusion to block RANKL on proT cells from human HSC/OP9-DL cell



co-cultures. OPG-Fc binding of proT cells was dose-dependent, with 5.0 μ g of OPG-Fc being the saturating dose for 2×10^5 cells (**Figure 2B**). Next, we investigated the effects of OPG-Fc-mediated blocking on thymic stroma using fetal thymic organ cultures (FTOC). ProT cells were incubated with saturating doses of OPG-Fc and then used to reconstitute E15 NSG fetal thymus lobes in hanging drop (**Figure 2C**). ProT-only and non-reconstituted lobes were used as controls, and all lobes were analyzed by QPCR. After 5 days in FTOC, proT-only FTOC cultures revealed an increase in transcript levels for TEC-derived chemokines including *Ccl19* and *Ccl21* compared to non-reconstituted lobes, as expected from our previous *in vivo* observations (97) (**Figure 2D**). *Ccl25* transcripts were also increased, although the results did not formally reach statistical significance. In contrast, transcript levels for these chemokines, with the exception of *Ccl19*, were not elevated in FTOC cultures containing OPG-Fc. Notably, the presence of OPG-Fc did not affect the ability of proT cells to reconstitute fetal thymic lobes as compared to proT-only FTOC, as the expression levels for the

human CD45 transcript (*PTPRC*) were comparable between the two conditions.

While DN thymocytes in mice are known to influence cortex formation, studies have demonstrated that mouse DP and SP thymocytes induce formation of the medulla and maturation of mTECs (103, 104). Specifically, positively selected thymocytes have been shown to regulate the development of the medulla through RANK/RANKL interactions (104). However, it is important to note that our studies reveal a potential role for RANKL on T cell subsets prior to positive selection.

Little is known about the role of the RANK/RANKL axis in facilitating lymphostromal crosstalk by DN thymocytes, including proT cells. In a study of mouse thymocyte subsets, Hikosaka et al. noted that DN cells express high levels of RANKL transcripts, while they fail to express LT α , LT β or CD40L (35). Developmentally, this subset is comparable to the human proT cell population. Consistently, it was also reported that a small subset of cells within the mouse cortex, a proportion of CD205⁺CD40⁺ cTEC-restricted progenitors (105) express

RANK (106). To further elaborate on the potential role of RANKL within our proT cell transfer model, we generated an immunodeficient version of the RANK Venus reporter mouse (107), referred to as RANK-Venus hSirp^αT^gRag^{-/-}γC^{null} (RV-SRG) mice. We showed that proT cells were capable of thymic engraftment in RV-SRG mice, with the emergence of CD45⁺CD7⁺CD5⁺ cells 2-weeks post-injection (**Figure 3A**). Importantly, these donor-derived cells were not yet at the DP stage, allowing us to focus on events mediated by DN cells. Analysis of the TEC compartment in control (non-injected) RV-SRG mice demonstrated a sizeable proportion of cells (11.1%) that were CD205⁺Venus⁺, which is a subset that likely contains immature, bipotent TEC progenitors (106) (**Figure 3B**). In contrast, proT cell-injected RV-SRG mice displayed less Venus⁺CD205⁺ bipotent TEC progenitors, but instead showed the appearance of more differentiated MHCII⁺ UEA-1⁺ mTECs in the Venus⁺CD205⁻ compartment (**Figure 3C**). This finding is consistent with the ability of proT cells to induce changes in the thymic microenvironment, including the formation of the medullary compartment, in immunodeficient mice (97).

The generation of the RV-SRG mouse model will enable further characterization of RANKL interactions with RANK-expressing TECs. Importantly, there is known cross-reactivity of human RANKL with mouse RANK (109), providing further support for our observations. Venus⁺ cells could be sorted to assess signaling events, such as the initiation of the downstream components in the non-canonical NFκB pathway (36). Furthermore, *in vivo* analyses, including those with OPG-Fc would be necessary to further evaluate whether RANKL-based signaling is the sole mechanism underlying thymic changes. Later time points can also be assessed to establish whether proT cells and their downstream progeny can drive a fully functional thymic microenvironment including AIRE expression on mTECs. Nevertheless, these findings are consistent with a critical role for proT cell-mediated enhancement of thymic reconstitution, and this is likely due to, in part, a RANKL-induced differentiation and reorganization of the thymic architecture, which leads to more effective recruitment of BM-derived T lymphocyte progenitors after HSCT.

ProT Cells for an Aged Thymus?

The notion that proT cells dually serve as TSPs and thymus reorganizers raises the exciting possibility that proT cells could be used as a cell-based strategy to reverse thymic involution. In **Figure 4**, we show that despite age-related thymic atrophy, mouse proT cells readily engraft the thymus of young and aged mice. ProT cells in mice are contained within the CD25-expressing DN2 and DN3 subsets, both of which have thymic engraftment capacity (110). We co-cultured CD45.1 BM-derived Lineage⁻ Sca1^{hi} cKit^{hi} (LSK) cells with OP9-DL cells for 10 days (**Figure 4A**). Hematopoietic cells from co-cultures were harvested and enriched for CD25⁺ proT cells. ProT cells were intravenously injected along with BM cells (GFP⁺ to distinguish from host) into lethally irradiated, congenic CD45.2 wild-type (WT) C57BL/6 young (3 months) and aged (18–20 months) hosts. Additionally, young and aged recipients were injected with

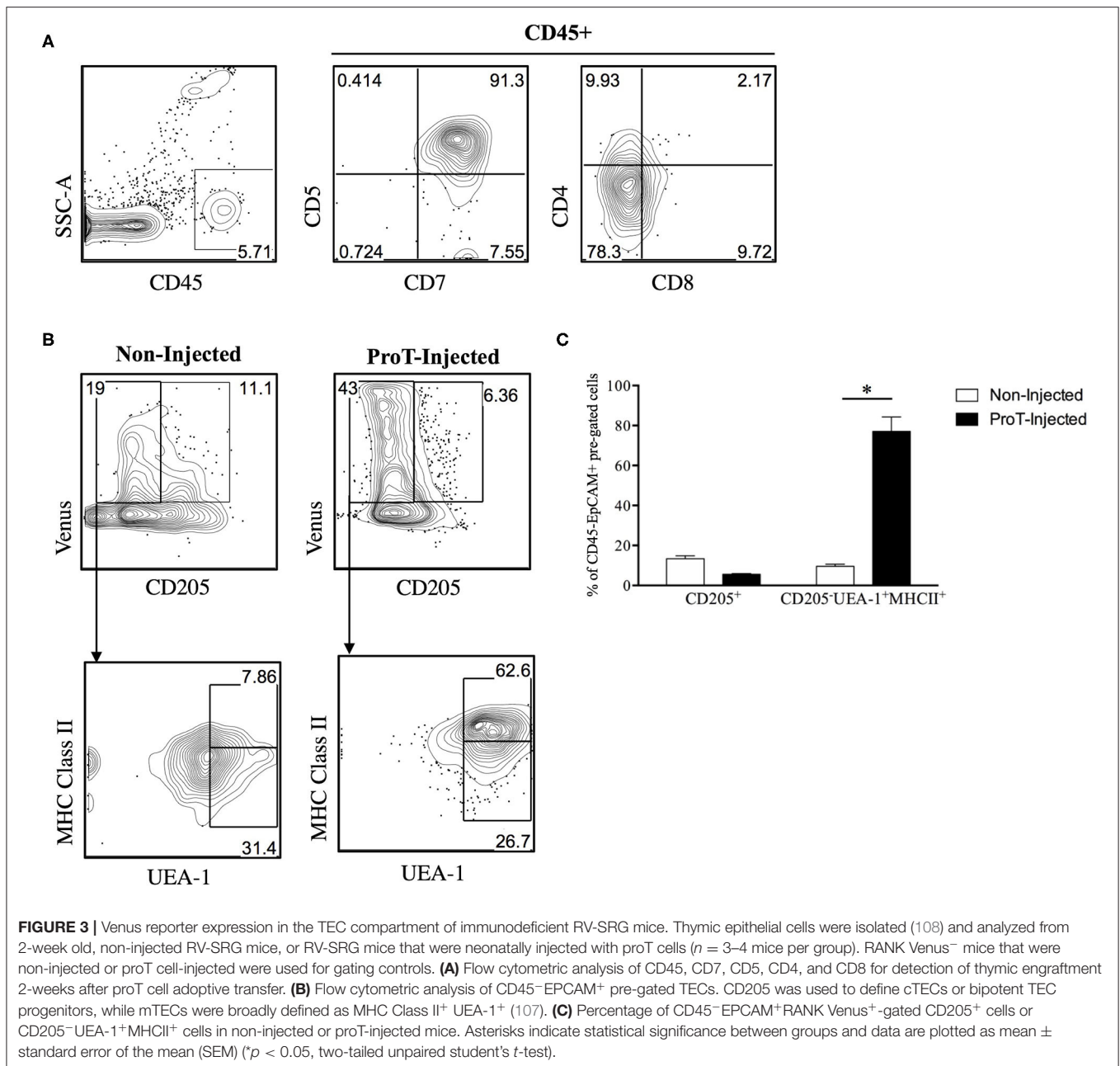
BM cells only, without proT cells as controls. On D7 and D14, thymuses were harvested and analyzed by flow cytometry.

Irrespective of the age of the host or the cell type injected, a significant portion of differentiated thymocytes was CD45.2⁺ and GFP⁻, signifying that these thymocytes were of host origin. This was consistent with previous observations, which identified radio-resistant host DN cells capable of repopulating the post-irradiation thymus due to niche availability (111, 112). On D7, we observed successful proT cell engraftment in the thymuses of both young and aged mice. However, the vast majority of thymocytes were of host origin (**Figure 4B**). Of note, the host thymocytes were at the DP, or CD4 and CD8 SP developmental stages compared to the engrafted proT cell-derived thymocytes, which were predominantly at the DN stages, suggesting that DN cells derived from engrafted proT cells were developmentally poised to mature into DP cells. Indeed, by D14, proT cell-derived thymocytes comprised the majority of cells in both aged and young mice (**Figure 4C**). ProT cells developed into mature T cells and appeared to only slightly lag developmentally compared to the host cells. Therefore, our findings revealed that under these conditions, proT cells contributed to the thymic make-up of both young and aged mice, but did not lead to accelerated thymic reconstitution.

Proportionally, small numbers of GFP⁺ cells derived from the BM graft appeared in the thymus and only by D14 (**Figure 4C**). This was despite the fact that donor GFP⁺ cells consistently made up over 70% of the cells in the BM (**Figure 4D**). Notably, while 6×10^6 proT cells were injected compared to 1×10^6 of BM cells, only up to 2% of cells in the BM were proT cell-derived. This was likely a result of the impure CD25⁺-enriched fraction, which contained traces of CD11b⁺ myeloid cells, that were injected into mice (not shown). As such, the CD45.1⁺ cells present in BM were mostly CD11b⁺. We are conducting further experiments to determine whether the contribution of GFP⁺ donor BM-derived cells to the thymus could increase with time. Since both the engrafted proT cells and the host DN cells are not self-renewing, thymus seeding progenitors from the BM would be recruited to fill the niches emptied after maturation of the proT cell graft and the host DN cells.

ProT cell engraftment was successful in both young and aged mice, although we observed a 10-fold difference in the number of thymocytes between young and aged hosts (**Figure 4E**). Considering that donor BM GFP⁺ cells contribute only up to 5% of the thymocytes, the comparison essentially measured the contribution of proT cells to thymic reconstitution. Importantly, there was an increase in the number of thymocytes with the administration of proT cells in young mice at D14, while no difference was observed in aged mice, despite favourable engraftment by proT cells. Therefore, while the capacity for engraftment of progenitors was high in both the young and the aged hosts, it is likely that the capacity of progenitors to proliferate was better accommodated in young hosts.

Our results show that the injected congenic proT cells were able to compete for the available niches to engraft the thymus of young and aged mice. The kinetics of proT cell-derived thymocytes suggests that proT cells are able to occupy these niches as early as D7, albeit at relatively small



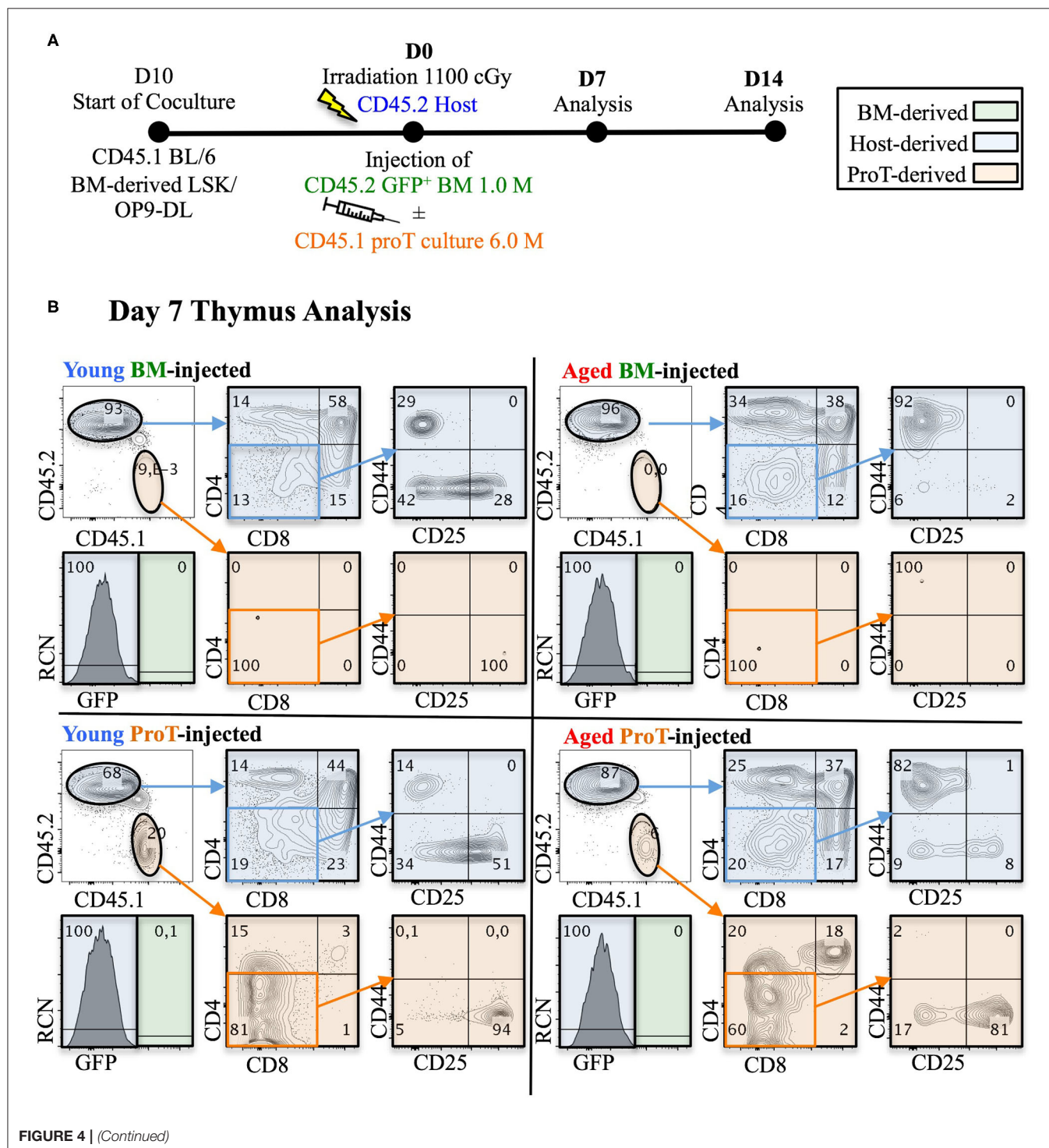
proportions. The percentage increase by D14 and increase in cell number suggests that upon reconstituting the thymus, proT cells help to restore thymic activity. It is important to note that the conditions used did not fully mimic cytoablative regimens used in the clinic, which includes successive rounds of chemotherapy (113). Successive rounds of chemotherapy could potentially clear the thymus of the radio-resistant DN that persist after irradiation. This would render the thymus more similar to the NSG thymus, where proT cells were able to accelerate thymic reconstitution when compared to injection of HSCs alone (97). Further investigation on the addition of chemical therapy in our protocol is underway to closely

follow clinical practices and determine whether proT cells “kickstart” the process of thymic reconstitution, particularly now that we have observed that proT cells can engraft involuted irradiated thymuses.

While we show success in engraftment of proT cells in aged mice, further exploration is required for adopting proT cell therapy as part of future clinical trials. Despite the increase in the number of T cells in aged mice using SSI, there is evidence that RTEs are blocked from entering the lymph nodes and functioning effectively (114). Age-related changes in the physiology of lymph nodes may render them incapable of recruiting T cells. Therefore, a disease model showing the effectiveness of the newly generated

T cells will be important to demonstrate improvements in functional immunity. In addition, mouse-derived OP9 cells are not appropriate for human or clinical use and alternative systems that are amenable to Good Manufacturing Practices (GMP) would be required. Recent progress and breakthroughs have led us from OP9-DL cells to a two-dimensional (2-D) plate-bound

matrix with DLL4 to readily generate human (CD34⁺CD7⁺) and mouse (DN CD25⁺) proT cells that are able to engraft mouse models (115, 116). The next step would be to expand the human culture system to large scale in order to generate sufficient number of proT cells to successfully engraft the thymus of patients (98).



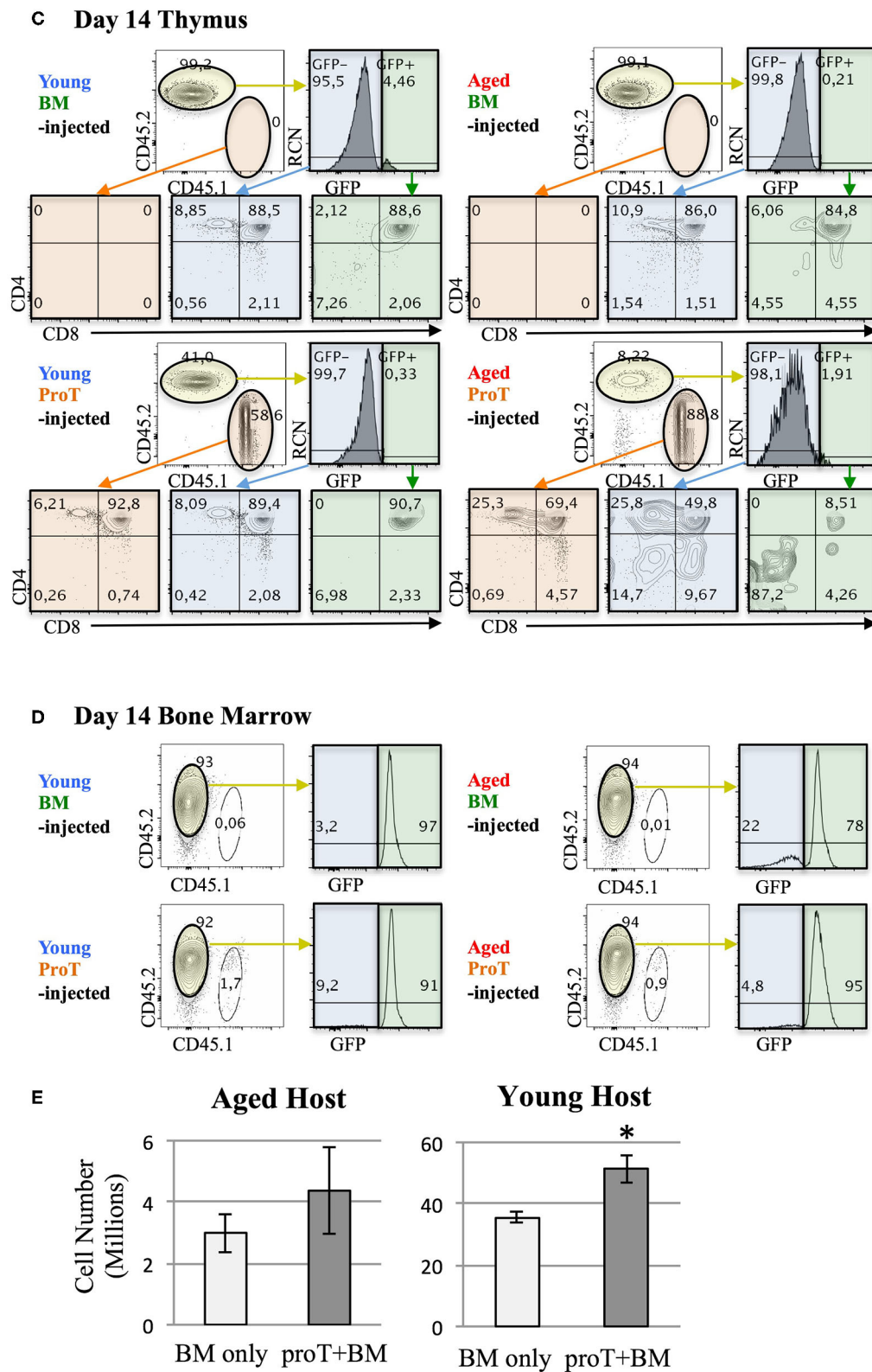


FIGURE 4 | Adoptive transfer of mouse proT cells into lethally-irradiated aged mice. A schematic for experiments is shown (A). LSK/OP9-DL co-cultures were initiated 10 days prior to injection. 18-month old (Aged) and 3-month old (Young) wild-type C57BL/6 (CD45.2) mice were irradiated at 1.025 Gy on the day of injection. (Continued)

FIGURE 4 | All irradiated mice were intravenously injected with 1×10^6 cells extracted from GFP⁺ BM. In addition to the bone marrow cells, some mice also received 6×10^6 proT cells harvested from 10 day co-culture of OP9-DL/BM LSK cells from congenic mice (CD45.1⁺) ($n = 3$ or 4 per group). Plots show the analysis of thymuses that were dissected on 7 **(B)** and 14 days **(C)** after injection. Thymocytes were stained with the appropriate cell lineage markers and analyzed by flow cytometry. Plots showing host-derived cells (CD45.2) are shaded in blue, proT cell-derived cells (CD45.1) are shaded in orange and BM graft-derived GFP⁺ cells are shaded in green. Bone marrow analysis of the corresponding mice in C show contribution of GFP⁺ BM grafts to the BM of the irradiated host on D14 **(D)**. Total thymocyte number was analyzed on D14 **(E)**. The error bars correspond to SEM (* $p < 0.05$, two-tailed unpaired student's t -test).

CONCLUSION

The targeted approaches with proT cells, cytokine and SSI treatments, all exploit different non-overlapping pathways to improve thymic engraftment and function. ProT cells appear to engage in crosstalk with TECs through RANK/RANKL interactions. Cytokine-specific interventions may activate various signaling pathways, and SSI lifts the suppression imposed by sex hormones on the thymic environment. Ideally, a combination approach where cytokines, SSI and proT cells are cooperatively administered could potentially have additive effects on reversing the damaging effects of cytoablative treatments on thymic function. In this scenario, cytokines would potentially protect TECs from chemical and radiation-induced damage, while injected proT cells could fill receptive niches made available through the morphological thymic changes induced by SSI. This combination strategy has the potential to be adapted to improve T cell-dependent immunity in aged populations.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

All animal studies were reviewed and approved by Sunnybrook Research Institute Animal Care Committee. Human UCB samples were obtained in accordance with approved guidelines established by the Research Ethics Board of Sunnybrook Health Sciences Centre.

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AUTHOR CONTRIBUTIONS

JS designed and performed RANK/RANKL experiments and *in vivo* experiments with Rank-Venus mice, analyzed the data, and wrote the manuscript. MM performed *in vivo* experiments with aged mice, analyzed the data, and wrote the manuscript. GA contributed critical reagents used in this study and provided critical experimental advice. JZ-P provided critical experimental advice and edited the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2020.01850/full#supplementary-material>

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Conflict of Interest: We have submitted a patent describing the method of producing and using Stemregenin-expanded proT cells. JZ-P is a co-founder of Notch Therapeutics.

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The Thymus in Chagas Disease: Molecular Interactions Involved in Abnormal T-Cell Migration and Differentiation

Ana Rosa Pérez^{1,2*}, Juliana de Meis^{3,4,5}, Maria Cecilia Rodriguez-Galan⁶ and Wilson Savino^{3,4,5*}

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Edited by:

Nicolai Stanislas Van Oers,
University of Texas Southwestern
Medical Center, United States

Reviewed by:

Jerry Niederkorn,
University of Texas Southwestern
Medical Center, United States
Batu Erman,
Sabanci University, Turkey

*Correspondence:

Ana Rosa Pérez
perez_anarosa@yahoo.com.ar;
perez@idicer-conicet.gob.ar
Wilson Savino
wilson.savino@fiocruz.br;
savino.w@gmail.com

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¹ Instituto de Inmunología Clínica y Experimental de Rosario, CONICET-Universidad Nacional de Rosario, Rosario, Argentina, ² Centro de Investigación y Producción de Reactivos Biológicos, Facultad de Ciencias Médicas, Universidad Nacional de Rosario, Rosario, Argentina, ³ Laboratory on Thymus Research, Oswaldo Cruz Institute, Oswaldo Cruz Foundation, Rio de Janeiro, Brazil, ⁴ National Institute of Science and Technology on Neuroimmunomodulation, Oswaldo Cruz Institute, Oswaldo Cruz Foundation, Rio de Janeiro, Brazil, ⁵ Rio de Janeiro Research Network on Neuroinflammation, Oswaldo Cruz Institute, Oswaldo Cruz Foundation, Rio de Janeiro, Brazil, ⁶ Inmunología, CIBICI CONICET, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Córdoba, Argentina

Chagas disease, caused by the protozoan parasite *T. cruzi*, is a prevalent parasitic disease in Latin America. Presently, it is spreading around the world by human migration, thus representing a new global health issue. Chronically infected individuals reveal a dissimilar disease progression: while nearly 60% remain without apparent disease for life, 30% develop life-threatening pathologies, such as chronic chagasic cardiomyopathy (CCC) or megaviscerae. Inflammation driven by parasite persistence seems to be involved in the pathophysiology of the disease. However, there is also evidence of the occurrence of autoimmune events, mainly caused by molecular mimicry and bystander activation. In experimental models of disease, is well-established that *T. cruzi* infects the thymus and causes locally profound structural and functional alterations. The hallmark is a massive loss of CD4⁺CD8⁺ double positive (DP) thymocytes, mainly triggered by increased levels of glucocorticoids, although other mechanisms seem to act simultaneously. Thymic epithelial cells (TEC) exhibited an increase in extracellular matrix deposition, which are related to thymocyte migratory alterations. Moreover, medullary TEC showed a decreased expression of AIRE and altered expression of microRNAs, which might be linked to a disrupted negative selection of the T-cell repertoire. Also, almost all stages of thymocyte development are altered, including an abnormal output of CD4[−]CD8[−] double negative (DN) and DP immature and mature cells, many of them carrying prohibited TCR-V β segments. Evidence has shown that DN and DP cells with an activated phenotype can be tracked in the blood of humans with chronic Chagas disease and also in the secondary lymphoid organs and heart of infected mice, raising new questions about the relevance of these populations in

the pathogenesis of Chagas disease and their possible link with thymic alterations and an immunoendocrine imbalance. Here, we discuss diverse molecular mechanisms underlying thymic abnormalities occurring during *T. cruzi* infection and their link with CCC, which may contribute to the design of innovative strategies to control Chagas disease pathology.

Keywords: Chagas disease, thymocyte depletion, thymic epithelial cells, gene expression, cell adhesion and migration

INTRODUCTION

Chagas disease is caused by the hemoflagellate protozoan *Trypanosoma cruzi*. Infection was initially an enzooty maintained among wild animals and *Reduviidae* family insects as vectors. The classical vectorial pathway occurs by contact with feces or urine of hematophagous triatomine bugs, which are frequent in Latin American endemic areas (1, 2). After the triatomine bite feed with blood, it usually defecates close to the bite. The parasites present in the feces then enter through the damaged skin when the person scratches the itchy bite or, through mucous membranes like ocular conjunctiva. Particularly, mucosal oral transmission has been associated with high mortality and morbidity, increased prevalence, and severity of the cardiac pathology (3–7). Moreover, parasites can be transmitted by contaminated blood transfusion, organ transplantation, and vertically. These latter types of transmission are also responsible for Chagas disease dissemination in non-endemic areas, including the USA, Europe, and Asia (8, 9). Nearly 6–7 million people in Latin America plus 1 million in the USA are infected with *T. cruzi* with 670,000 premature disability and death per year worldwide (8–10).

Human Chagas disease shows a short acute phase (2 months), a period in which parasites are numerous in blood and tissues. During this phase, *T. cruzi* can infect host skeletal muscle, heart, lymphoid cells, adipocytes, mucosal sites, neurons, glands, liver, among others. Moreover, in some target tissues, damage can persist in the chronic phase of the disease (3, 11–13). Following the acute phase, patients enter into a long latent phase, with no symptoms and scarce parasitism, which can remain silent for the rest of life. After 10–30 years, one-third of infected patients eventually develop clinical symptoms as CCC, megacolon, or megaesophagus (14). The CCC is associated with mononuclear cell infiltrate, fiber damage, fibrosis, and rare presence of parasites. The inflammatory infiltrate in CCC exhibits more CD8⁺ over CD4⁺ T cells and hearts from patients present high granzyme A expression, suggestive of cytotoxicity in the tissue (15–19).

THE THYMUS IN CHAGAS DISEASE

Since Chagas disease was described in 1909, numerous studies have been conducted on the pathogenesis of the disease and the evolution of both acute and chronic phases of infection (1, 2). However, dissection of diverse pathogenic mechanisms remains open to investigation. Upon recognition that *T. cruzi* persists in the host during the chronic phase, the hypothesis stating

that the chronic tissue damage is mediated and maintained by inflammatory reactions caused by the continuous parasite's cycles of replication was reinforced (20) and the autoimmune hypothesis of the disease (the most accepted until then) was questioned (21). However, there is profuse evidence on the occurrence of autoimmune events, mainly caused by molecular mimicry and bystander activation (22). These mechanisms are not mutually exclusive, and both likely operate conjointly. In any case, it is well-established that *T. cruzi* infects the thymus and causes locally structural and functional alterations (23). Therefore, understanding the possible implications of thymic changes in the immunopathology of this parasite infection may help to appreciate new edges of the disease.

Studies in animal models of acute Chagas disease revealed marked thymus atrophy, mainly caused by thymocyte death, as well as functional alterations, including an abnormal output of immature and mature cells (24). These data suggested that both systemic and thymic inflammation might drive to central tolerance defects, while simultaneously increase the suspicion of a thymic involvement in the development of CCC, although this issue remains uncertain. In this sense, the following questions still need to be approached:

- Can the observations made in the thymus of *T. cruzi* acutely infected mice be transposed to what happens in humans?
- Can thymic alterations persist during the chronic phase?
- Is the thymic atrophy a mere side effect secondary to an immunoendocrine imbalance?
- Which are the consequences of cellular and molecular alterations promoted by the intrathymic parasite infection?
- Which of these thymic abnormalities may contribute to CCC?

Nevertheless, studying the human thymus in the context of Chagas disease to answer these questions is not an easy task. In humans, determining the occurrence of atrophy requires non-invasive techniques, which also prevents obtaining tissue biopsies for cytometry, molecular, or microscopic studies. Thymus size can be ascertained in children at an early age by ultrasound but requires qualified operators. In adults, there is normal age-associated atrophy together with a huge increase in adipose tissue, making it still much more difficult to evaluate the organ.

Additionally, the acute phase of vector-induced infection usually remains unnoticed, which prevents thymic evaluation as it occurs. Moreover, infection mainly occurs in rural areas where it is unlikely to have adequate medical equipment and suitable personnel. Congenital cases, when detected, are rapidly

treated, preventing the tracking of putative changes in the organ size. Future non-invasive studies, including determination of T-cell excision circles, which reflect thymocyte export to the periphery, will hopefully be carried out in orally-infected symptomatic individuals, and provide clues to unravel important thymus-related issues in both children and adults undergoing Chagas disease.

Beyond all these relevant points, which remain to be unveiled, there is no doubt that in the murine model of Chagas disease profound thymic alterations occur, which may be of relevance in terms of both thymic selection and immune competition. Accordingly, we discuss below the most relevant cellular, molecular, and functional alterations observed in the thymus of infected mice and discuss their possible resemblance to the human disease.

THE THYMIC MICROENVIRONMENT IN *T. CRUZI* INFECTED MICE

During a variety of systemic infections, several changes occur in the thymus leading to the local presence of both soluble factors, cytokines, chemokines and pathogen-derived antigens that could alter not only the thymic microenvironment but also the normal T cell differentiation process, including the export of mature T lymphocyte to secondary lymphoid organs (the normal process of differentiation is summarized in **Box 1**).

A first key point regarding the thymus in experimental Chagas disease is the fact the *T. cruzi* can invade the organ. The parasite can infect thymic microenvironmental cells, including phagocytes and TEC (42–44). Medullary TEC from infected mice exhibited a significant decrease in AIRE gene expression (45), which might be related to disruption in the negative selection of the T-cell repertoire. Nevertheless, the levels of AIRE expression remain rather controversial since no differences were also found in another study (46). Although definitive evidence is lacking, differences reported may be due to the fact that in one experiment (45), measurements were done taking separated TEC, whereas in the other, the whole thymus was used as the primary source of RNA (46). Previous studies showed that GC treatment in mice transiently reduced the number of AIRE⁺mTEC (47). Thus, it is conceivable that the diminution in AIRE⁺TEC numbers during infection may be secondary to the rise in systemic glucocorticoids (GC) levels.

In vitro, the parasite invades growing TEC, modifying distinct biological features. These changes include a decrease proliferation, enhanced production of extracellular matrix proteins (ECM), and the corresponding receptor expression, leading to the consequent enhancement in the ability of TEC to adhere developing thymocytes, particularly on those cells infected by the parasite (48) (**Table 1**).

When studying the expression of microRNAs in TECs from infected animals, many of these non-coding RNAs were up- or down-regulated. The bioinformatic-based simulation revealed that various down-regulated miRNAs can result in the up-regulation of biological processes involving ECM proteins, cell adhesion, and cell migration (49). In keeping with these

BOX 1 | Intrathymic T-cell development.

In physiological conditions, several T cell lineages arise in the thymus including conventional $\alpha\beta$ T cells, $\gamma\delta$ T cells, CD4⁺FoxP3⁺ regulatory T cells (tTreg), and NKT cells. Recently, more lineages have been added to the list, including several subtypes of innate T cells (25, 26).

From the arrival of the bone marrow-derived T-cell precursors into thymus until the ultimate export of mature T cells to the periphery, a migratory journey inside the thymus takes place, involving a large number of interactions which promote the complex process of T-cell differentiation. This process necessarily depends on T-cell receptor (TCR) gene rearrangement and on the interaction of TCR with self-peptides presented by class I or class II proteins of the major histocompatibility complex (MHC) expressed by microenvironmental cells. Nevertheless, other types of interactions are also relevant including those mediated by chemokines, cytokines, lectins, lipid signalling molecules, hormones and extracellular matrix (ECM) proteins (27–30). Once bone marrow-derived T-cell precursors enter the thymus through blood vessels located at the cortico-medullary junction, they differentiate in DN thymocytes. Based on the expression of CD44 and CD25, the sequential maturation process of DN cells indicates that DN1 thymocytes develop to DN2 and DN3 thymocytes, which migrate to the subcapsular area of the thymic lobules, where they rearrange TCR- β chain-encoding-genes, express the pre-TCR receptor and proliferate. At the DN3 stage, CXCL12/CXCR4-mediated interactions contribute to cell proliferation and differentiation toward the DN4 and subsequently to the DP stage (28, 31, 32). Thymocytes that do not experience a productive TCR gene rearrangement during the DN stage die by apoptosis, whereas those expressing productive TCRs evolve to DP cells, which in turn can recognize self-peptides in the context of MHC molecules. This interaction determines the events of negative and positive selection (28, 33, 34). The intrathymic negative selection process is essential to establish self-tolerance in the T-cell repertoire, deleting thymocytes exhibiting TCRs with high-avidity for self-peptides (33). Most of the DP thymocytes die due to lack of positive selection as their TCR cannot recognize own MHC-peptide complexes on thymic microenvironmental cells (*death by neglect*) possibly through induction of apoptosis mediated by endogenous steroids (35, 36). Positively selected thymocytes become committed to CD4⁺ or CD8⁺ T lineages, depending on the class of MHC molecule with which the TCR interacts. Particularly, physiological levels of glucocorticoids (GC) have an important role in the normal thymocyte development, regulating essential process like antigen-specific selection, TCR-dependent activation, ultimately shaping the T cell repertoire [revised in (37, 38)].

Besides, among CD4⁺ T-cells, two distinct groups of cells with opposite roles differentiate within the thymus: the classical CD4⁺ T helper lymphocytes and tTregs (28, 39). Moreover, innate memory CD8⁺ T cells (T_{IM}-CD8⁺) are generated as a lineage different from conventional CD8⁺ T cells. Some reports also demonstrated that thymic selection of T_{IM}-CD8⁺ cells results from the interaction with hematopoietic cells present in the thymus and not with TEC, as is the case of conventional T cells (25, 26, 40, 41). As an overall result of these events, a vast repertoire of T cells able to react with peptides restricted by MHC molecules is generated.

Once the differentiation process is complete, mature single positive (SP) cells congregate near medulla vessels and are exported to the periphery. These recently exported cells are known as recent thymic emigrants (RTE). They are naïve T lymphocytes that migrate mainly toward the T-cell-dependent areas of secondary lymphoid organs.

findings, the expression of cell adhesion and cell migration-related molecules, including chemokines as well as ECM proteins by TEC is enhanced in thymuses from infected mice (23, 42, 50, 51), as well as after *in vitro* infection (48) with functional consequences as further discussed in detail below.

TABLE 1 | *Trypanosoma cruzi* infects mouse thymic epithelial cells (TECs)*, affecting TEC replication and adhesion of thymocytes.

	Non-infected	Infected	p-value
Thymic epithelial cell numbers**			
Total TEC numbers	7.00 ± 0.63	3.20 ± 0.33	$p < 0.02$
BrdU+ TEC numbers	56 ± 1.10	14 ± 0.40	$p < 0.02$
Association index applied to quantify thymocyte adhesion to TECs***			
	TEC/Tc(-)	TEC/Tc(+)	
	67 ± 0.9	200 ± 21.5	$p < 0.05$

*The TEC line IT-76M1 was originally developed from a primary culture of BALB/c thymic stromal cells and was kindly provided by Dr. T. Itoh (Tohoku University Medical School, Sendai, Japan). TEC cultures were allowed to adhere to culture flasks (1×10^5 cells) and were infected with *T. cruzi* 24 h later, being then washed extensively. Trypomastigote forms of the Colombian strain were applied to infect TEC cultures. After 6 h, free parasites were discarded by repeated washings, and the TEC cultures were maintained for further 48 h.

**The numbers of cultured TECs were ascertained by directly counting single-cell suspensions after detaching the cells. Additionally, the proliferative status of the infected and uninfected TEC cultures was analyzed using a BrdU incorporation assay (30 min at 37°C). The presence and counting of BrdU+ cells were determined by fluorescence microscopy.

***Infected or non-infected TEC cultures were maintained for 48 h, being then incubated with 5×10^6 thymocytes, obtained by the mechanical disruption of control thymuses, in serum-free RPMI medium for 30 min at 37°C. Floating, non-adherent thymocytes were removed and the plates were fixed in methanol and stained with Giemsa solution. The number of adhered thymocytes per at least 1,000 TECs were directly counted, and the association index (AI) was calculated as follows:

$$AI = \frac{\text{TECs with bound thymocytes} \times \text{thymocytes bound to TECs} \times 100}{\text{total TEC number total TEC number}}$$

All data showed in this table were retrieved from Farias-de-Oliveira et al. (48).

THYMOCYTE DEPLETION IN ACUTE TRYPANOSOMA CRUZI INFECTION

Thymic atrophy seems to be a common finding in various experimental infections; being however much less documented in humans (52). As mentioned before, DP thymocyte loss by apoptosis is evident during the acute phase of *T. cruzi* infection (12, 24, 42, 53, 54), and diverse molecules have been raised as candidates to enhance thymocyte death (55, 56). However, the most relevant way involved in *T. cruzi*-induced apoptosis of developing DP thymocytes seems to be related to the systemic rise of GC levels as a consequence of the host's stress response to the infection (57, 58). Regarding the *T. cruzi* infection, it has been clearly proved that complete GC ablation by adrenalectomy or the blockade of the GC receptor (GR) by RU486 administration clearly prevented DP cell loss by apoptosis (56, 57, 59).

Two main apoptotic pathways have been described: one of them is the extrinsic pathway, dependent on membrane "death receptors" and caspase 8, while the other is the intrinsic or mitochondrial pathway, dependent on Apaf-1 and caspase 9. Both pathways lead to the activation of caspase 3, which executes the programmed cell death. There is considerable evidence

that in diverse cell types, including thymocytes, GC cause apoptosis via the intrinsic pathway (60–62). These pathways were regulated by proteins from the Bcl-2 family consisting of pro- and anti-apoptotic proteins, like Bax, Bid, Bim, and Puma (61). Also, in thymocytes, GC can modulate the balance between members of this family, triggering the release of cytochrome c by mitochondria (60, 63, 64). Cytochrome c participates in the formation of the apoptosome with Apaf-1 and pro-caspase 9, leading to caspase 9 activation with consequent activation of caspase 3. These data are reinforcing by the fact that, in thymocytes, GC-driven apoptosis is prevented in mice deficient in the pro-apoptotic proteins Bak and Bax (65), Apaf-1 (63, 66) or caspase 9 (67).

Despite the fact that the exact apoptotic program initiated by GC has not been fully described in DP thymocytes, the use of specific peptide inhibitors has implicated caspases 8, 9, and 3 in this process during *T. cruzi* infection (68). These findings suggest that both intrinsic and extrinsic apoptotic pathways may be involved. However, studies performed in C57BL/6 mice deficient in both TNF-receptors (TNF-R_{1/2} double knockout mice) showed that the genetic ablation of TNF-triggered death pathways failed in preventing *T. cruzi*-induced cortical thymocyte depletion (56). Other series of studies discarded the involvement of interactions mediated by Fas/Fas-L or even perforin in triggering cell death within the thymus (55). These data strongly indicate that apoptotic pathways activated through death receptors are not involved in the *T. cruzi*-driven thymic atrophy. Yet, how can be explained the activation of caspase 8, observed by Farias de Oliveira and co-workers? (68). It is possible that caspase 9, in addition to cleaving pro-caspase 3, also activates lysosomal cathepsin B, which in turn activates caspase 8 providing an alternative route for caspase 3 through the mitochondrial pathway (64). This hypothesis remains to be tested since the release of cathepsin B has not been yet evaluated during infection with *T. cruzi* in DP thymocytes. Moreover, some findings suggest that GC can act, in addition to caspase 9, through caspase 8 (52, 58, 59, 64, 69–71). In any case, the findings summarized above strongly suggest that the mitochondrial pathway of apoptosis is triggered in DP thymocytes because of the GC rise driven by pro-inflammatory cytokines (56–58).

Other regulatory mechanisms may also influence the outcome of the GC-induced thymus atrophy in *T. cruzi* infected animals. It is known that the cellular response to GC is regulated by the availability of different GR isoforms. The GR α and GR β receptors are the most abundant (72). GR α binds GC, while GR β does not bind the hormone or transactivate target genes, acting as an inhibitor of GR α activity (72). Interestingly, *T. cruzi*-infected thymuses progressively down-regulate GR α gene expression (73). Although GR β thymic contents have not been evaluated, this process may be a compensatory mechanism conferring thymocytes greater resistance to the deleterious effects of the hormone during the acute infection in mice (73). Moreover, tissue GC availability can also be modulated by 11 β -hydroxysteroid dehydrogenases (11 β -HSD)-1 and 2. While 11 β -HSD1 increases the availability of active GC, 11 β -HSD2 converts active GC to a less active metabolite (72).

Interestingly, thymuses from *T. cruzi* infected animals, show an enhancement of 11 β -HSD1 gene expression, paralleled by increased production of functional GC by TEC, revealing an active intrathymic steroidogenic machinery (73), which synergizes the local consequences of systemic GC (74, 75).

Additionally, it has been shown that prolactin (PRL), which is enhanced intrathymically during *T. cruzi* infection, counteracts the deleterious effects of GC. Accordingly, the administration of metoclopramide (a PRL secretagogue), significantly prevented the thymic atrophy as well as the abnormal exit of DP thymocytes (73). Interestingly, the onset of thymic atrophy in *T. cruzi*-infected animals is associated with an inverse balance between the intrathymic expression of GR and PRL receptor (PRLR), possibly altering the sensitivity of DP thymocytes to GC-induced apoptosis (73). Mature CD4⁺ and CD8⁺ SP thymocytes are much more resistant to the GC proapoptotic effects than the immature DP cells (76), and this fact is clearly seen during the infection, by the increase in the intrathymic relative numbers of SP thymocytes.

Other molecules possibly involved in thymic atrophy during *T. cruzi* infection have been described, although much less studied than GC. Yet, it cannot be ruled out that they might synergize their effects. Galectin(Gal)-3 is a carbohydrate-binding protein that modulates cell death in mature and immature T cells (77). Gal-3 has a sequence in the protein core similar to Bcl-2, a well-characterized suppressor of apoptosis. Intracellularly, Gal-3, like Bcl-2, maintains mitochondrial integrity and prevents cytochrome c release, blocking cell death (77, 78). Nevertheless, in contrast to the antiapoptotic function of intracellular Gal-3, extracellular Gal-3 can directly induce thymocyte death (12, 77). In the normal murine thymus, Gal-3 is mainly restricted to the medulla, where it is produced and accumulates on the cell surface of TECs and phagocytic cells (79). Interestingly, in the thymus of *T. cruzi* infected mice, protein contents of Gal-3 are increased in the medulla, and to a lesser extent in the cortex (12). Additionally, thymocytes from infected mice showed an enhanced Gal-3 gene expression and increased cytoplasmatic protein storage (12), suggesting that the accumulation of Gal-3 may be associated with the depletion of DP thymocytes. Moreover, thymuses from Gal-3 knockout mice showed diminished cellularity than wild type mice, but the proportions of thymocyte subsets are maintained (12, 80). When infected, and despite the low thymic cellularity exhibited by Gal-3 knockout mice, DP thymocyte proportions were preserved as compared to wild-type mice (12, 80). Therefore, understanding the exact role of Gal-3, as well as other pro-apoptotic family members like Gal-1, in thymic atrophy during *T. cruzi* infection requires further studies.

It has been shown that extracellular ATP, acting via P2X7 receptors, increases DP thymocyte sensitivity to death by rising membrane permeability during the peak of *T. cruzi* acute infection (81, 82). Deprivation of intrathymic key cytokines may be also involved in the loss of thymocytes. In this regard, previous studies showed that thymocyte responses to mitogens in *T. cruzi* acutely infected mice are decreased due to compromised IL-2 secretion and high levels of IL-10 and interferon IFN- γ (24).

Lastly, some studies showed that thymic atrophy was not induced by a non-virulent strain of *T. cruzi*, suggesting that

parasite-derived virulence factors may be involved in the death of thymocytes. In keeping with this hypothesis, some studies indicate that the parasite-derived enzyme trans-sialidase may be involved in the thymic atrophy (83, 84). Despite that *T. cruzi* can infect the thymus (43, 46, 58) and release locally antigens, some studies have shown that when trans-sialidase is artificially shed to circulation, it induces thymocyte apoptosis (85). Moreover, mice treated with trans-sialidase displayed enhanced thymocyte apoptosis within the thymic nurse cell complexes, findings resembling thymic alterations in infected animals (83). Additionally, the trans-sialidase treatment *per se* was able to promote a decrease of thymocyte proliferative ratios after Concanavalin A stimulation, similar to those observed in the experimental models of *T. cruzi* infection. Interestingly, the administration of trans-sialidase neutralizing antibodies in *T. cruzi* infected mice significantly prevented thymocyte apoptosis (83). Furthermore, lactitol, a competitive inhibitor of trans-sialidase that blocks the transfer of sialyl residues by the enzyme, was able to prevent thymocyte depletion (86, 87). Strikingly, when acute infection (and the conceivable shedding of trans-sialidase) is controlled by benznidazole administration, thymus atrophy is not so evident (88).

Trans-sialidase probably acts upon CD43 (89). Nevertheless, the molecular mechanisms involved in CD43-mediated apoptosis of DP thymocytes during *T. cruzi* infection deserve a more profound investigation. However, beyond the probable direct effects caused by an active trans-sialidase on DP thymocytes, it is also conceivable that the immune response triggered against this molecule activates the HPA axis and/or the intrathymic machinery of GC synthesis, causing GC rise with consequent death of DP thymocytes. Yet, the existence of these circuitries remains to be determined.

Conjointly, these data indicate that thymic depletion of DP thymocytes in acute *T. cruzi* infection is primarily caused by GC enhancement, and perhaps this pathway can be modulated by multiple interactions involving both endogenous factors and infectious agent-derived moieties. This complex scenario is summarized in **Figure 1**.

OTHER MECHANISMS THAT COMPROMISE THYMIC CELLULARITY IN THE CONTEXT OF *T. CRUZI* INFECTION

It is well-established that, during the process of thymic atrophy, the loss of DP thymocytes is followed by a drop in absolute numbers of thymic CD4⁺FoxP3⁺ tTreg cells, as well as CD4⁺ and CD8⁺ SP thymocyte (90). Nonetheless, *T. cruzi* infection induces an increase in the proportion of tTreg cells within the thymic CD4⁺ SP T cell compartment, apparently as result of an increase in its self-renewal capacity compared to CD4⁺FoxP3⁺ T cells (90). These findings are independent of the mouse or parasite genetic strain, but can vary in intensity (90–92).

Interestingly, some findings demonstrate a negative regulatory role for CD28 in inhibiting differentiation of SP thymocytes, possibly declining thymic selection (93). In contrast, more recent findings suggest that CD28-mediated transduction signals

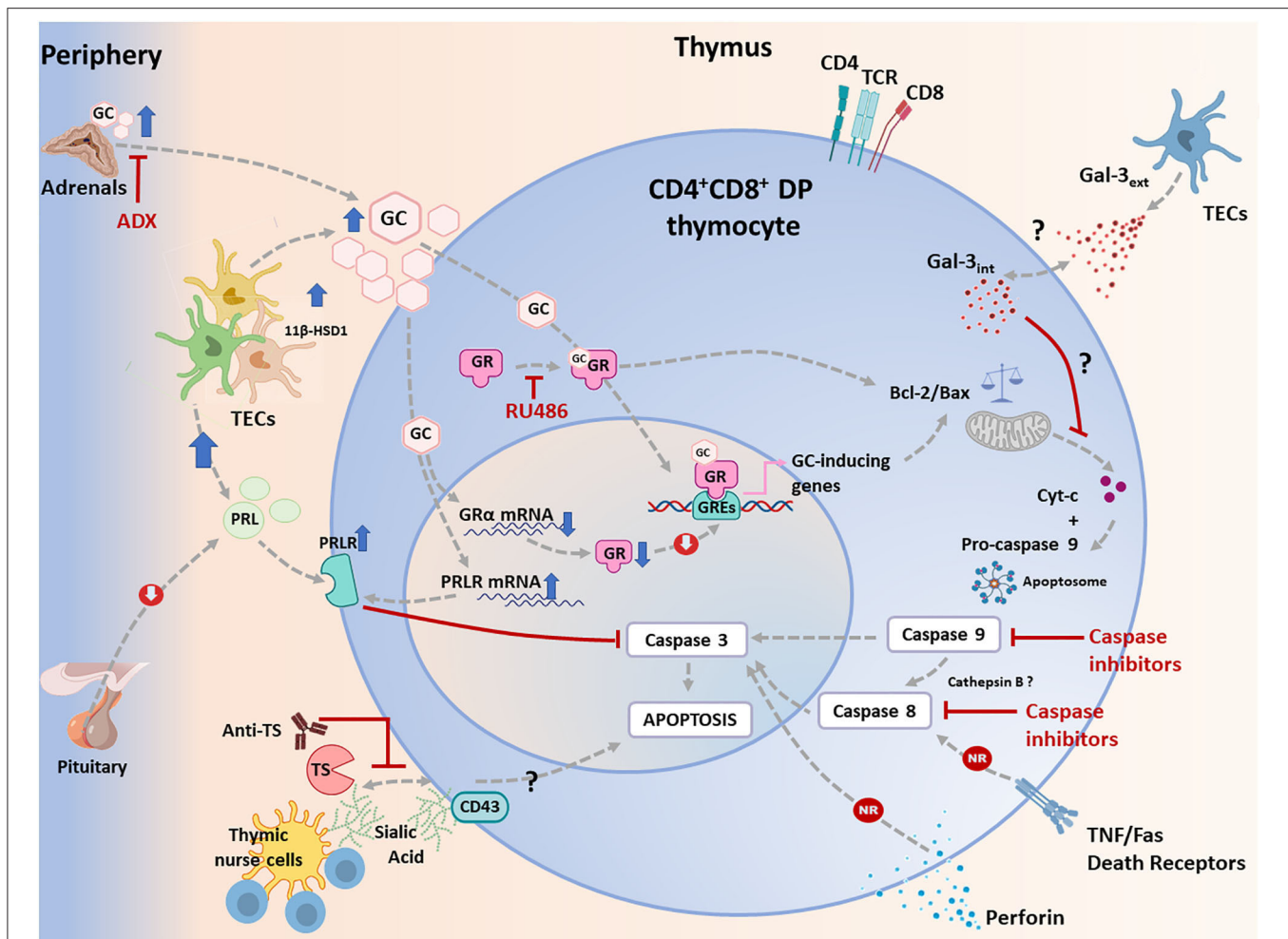


FIGURE 1 | Apoptotic signaling pathways triggered in $CD4^+CD8^+$ double positive (DP) thymocytes during *T. cruzi* infection in mice. Apoptosis can be initiated via two different routes including extrinsic and intrinsic pathways, which converge in a caspase activation cascade. The most relevant physiological way involved in *T. cruzi*-induced apoptosis of developing $CD4^+CD8^+$ DP thymocytes is linked to the systemic rise of glucocorticoid (GC) levels secondary to the host's stress response to the infection. This process is synergized by an increase in intrathymic production of GC by thymic epithelial cells (TECs), conjointly with an increased availability of induced-11 β -hydroxysteroid dehydrogenase (11 β -HSD)-1. As proved, GC-driven apoptosis is greatly prevented by adrenalectomy or by the blockade of GC receptor (GR) with RU486. In addition, GC can stimulate the intrinsic pathway either genomically or non-genomically, a process that involves proteins of the Bcl-2 family. Pro-apoptotic and anti-apoptotic proteins of the Bcl-2 family are up-and down-regulated respectively, leading to the release of cytochrome c, which then forms the apoptosome together with pro-caspase 9 and Apaf-1, leading to the activation of caspase 9. This enzyme then activates caspase 3. During *T. cruzi* infection, cytochrome c is enhanced, and caspase 9 is activated in $CD4^+CD8^+$ DP thymocytes. As shown, inhibition of caspase 9 weakens $CD4^+CD8^+$ thymocyte death. In parallel, increased systemic and intrathymic GC production leads to a diminution of GR expression, probably as a negative regulatory loop tending to control unnecessary harmful effects upon the gland. Moreover, *T. cruzi* infection induces the synthesis of prolactin (PRL) by TECs, while GC improved the expression of the PRL receptor (PRLR) in TECs. Increased PRLR signaling then counterbalances GC-induced effects, preventing caspase 3 activation. Extrinsic signals can be initiated by cell death ligands (FasL and TNF- α) and involve caspase 8, but these routes seem to be non-relevant (NR) during *T. cruzi* infection, since atrophy takes also place in TNFR1+2 double knock-out mice or Fas knock-out animals. Similar results were observed in perforin knock-out animals. Since caspase 8 is also active, a possible alternative route for its activation may involve cathepsin B, but this route deserves confirmation. Yet, it is clear that caspase 8 inhibition reduces thymocyte death during *T. cruzi* infection. Thymocyte apoptosis can also be triggered by trans-sialidase (TS), a *T. cruzi* derived enzyme, and this effect is partially blocked by anti-TS antibodies against. Trans-sialidase transfers sialic acid residues between the parasite and thymocytes, and probably acts through CD43. The subsequent steps leading to apoptosis are unknown. Other pathways described, as caused by intracellular and extracellular Gal-3, seems to influence the intrinsic apoptotic pathway.

favor $CD4^+FoxP3^+CD25^+$ tTreg development and proliferation in steady-state conditions (94, 95). Therefore, it is possible to presume that given the inflammatory environment that is established by *T. cruzi* infection, CD28-mediated co-stimulatory signals could, in part explain the relative increase of tTregs among $CD4^+$ SP cells. Nevertheless, the precise role of CD28 in tTreg

development in the thymus during *T. cruzi* infection has not been addressed yet and deserves further investigation.

In addition, tTreg differentiation from $CD4^+CD25^+Foxp3^+$ $\rightarrow$ tTreg appears to be highly dependent on IL-2 and IL-15 secretion by $CD4^+$ SP T cells located in close contact with medullary TEC (96). Both IL-2 and IL-15 trigger signaling

pathways in T-cell precursors through receptor complexes containing the common cytokine receptor γ c-chain subunit, which is involved in the activation of Stat5 proteins and Foxp3 expression (97–99). Consequently, the failure of medullary CD4⁺CD25⁺Foxp3[−] T cells to differentiate into tTreg in *T. cruzi* infected animals may be at least in part, the result of the reduced IL-2 intrathymic contents (90). Interestingly, in non-lethal models of disease, the partial thymic recovery observed after the acute phase, were linked to the increase in the numbers of IL-2R expressing cycling cells (24). Accordingly, *T. cruzi* may not only induce the depletion of cytokines that trigger the γ c-chain family of receptors, but also alter the expression of these receptors, impairing the normal tTreg development (90, 100, 101), with potential consequences in the appearance of autoimmune CD4-dependent events.

In addition, the Th1 milieu that is established during *T. cruzi* infection may severely affects TCR affinity and the strength of co-stimulatory signals. Thus, the intrathymic presence of pro-inflammatory cytokines can also modulate the selection process, affecting their thresholds of survival and death (102, 103). Previous studies carried out in *T. cruzi* infected mice knock-out for TNF-R₁₊₂, IFN- γ , iNOS, or IL-12, revealed that only IL-12 is likely involved in thymic atrophy by apoptosis and DP loss (104). Possibly, signals driven by this cytokine may affect the process of negative selection, but this issue has not been yet confirmed. In a second vein, IL-4 and IL-10 are not involved in thymocyte survival or death after infection, coinciding with data showing that the absence of both cytokines do not affect thymocyte cell populations in physiological contexts (102).

THE INTRATHYMIC T-CELL REPERTOIRE AND EXIT OF POTENTIALLY AUTOREACTIVE CELLS

The autoimmune hypothesis of Chagas disease points out to the presence of T and B autoreactive cells in the chronic phase, which may be involved in organ damage. Accordingly, it might be possible that these autoreactive cells have not completed their thymic education correctly (negative central tolerance) before their exit to the periphery. Taking in advantage the expression of endogenous superantigens (also called Minor lymphocyte stimulating -Mls- antigens) by thymic stromal cells in mice, is possible to evaluate changes in the selection process through the screening of TCR-V β repertoire (for more details, see **Box 2**) (105). Studies focused in describing the T cell repertoire in *T. cruzi* infected animals in terms of TCR-V β s rearrangements, confirmed that enhanced numbers of T cells bearing potentially autoreactive TCRs (that in physiological conditions, should have been deleted in the thymus by negative selection) were present in the secondary lymphoid compartment (106).

These studies were carried out in BALB/c mice, taking into the advantage that in these animals, thymocytes expressing the “prohibited” segments V β 5 and V β 12 are normally deleted during negative selection, whereas thymocytes bearing V β 8 are not eliminated and are found in the periphery after maturation process. The most striking observation in *T. cruzi* infected

BOX 2 | Minor lymphocyte stimulating antigens help to evaluate positive and negative selection processes in the thymus.

Minor lymphocyte stimulating (Mls) antigens are encoded by endogenous superantigens that were incorporated into the genome of distinct inbred mice from the mouse mammary tumor virus family (105, 107). It has been shown that Mls antigens expressed in the thymus induce depletion of T cells bearing Mls-reactive TCR-V β domains, allowing the exploration of the conditions for both positive and negative selection events. These studies revealed the importance of Mls and Mls-like products in the shaping of the T cell repertoire and revealed the presence in the periphery of T cell clones with “forbidden TCRs” that may have escaped from selection processes (105, 108).

Diverse mouse strains with distinct Msl phenotypes have been used to evaluate the peripheral T cell repertoire [revised in (108, 109)]. Particularly, the following mouse strains were used to evaluate the T cell repertoire after *T. cruzi* infection:

- BALB/c mice, which contain Mls^a antigens, leading to the intrathymic TCR-V β 3,5,11,12 depletion (106, 110, 111)
- C57BL/6 mice do not express antigens Mls antigens; therefore they do not deplete specific TCR-V β clones during negative selection (112).
- C3H/HeJ mice, which contain Mls^c antigens, leading to the intrathymic TCR-V β 3,5,11,12 depletion (112).
- CBA/J mice, which contain Mls^a and Mls^c antigens, leading to the intrathymic TCR-V β 3,5,6,7,8,1,9,11,12 depletion (110).

All these studies were focused on the evaluation of TCR-V β patterns in thymic or peripheral SP T cells, except one which also evaluated the TCR-V β repertoire in thymic and peripheral DP T cells (106). This particular work revealed the existence of DP T cells in the periphery that would have escaped from the thymus, bypassing the process of negative selection.

BALB/c mice was the anomalous presence in the periphery of immature and activated CD25^{high}DP cells exhibiting V β 5 and V β 12 segments. However, despite higher numbers of immature thymocytes bearing such V β segments were noticed in the thymus, they were no longer detected in the intrathymic SP stage, implying that negative selection apparently occurs. Similar data were reported by other groups (110, 111). These results also indicate that DP thymocytes bearing prohibited V β 12 segments escape from the thymus and gain the secondary lymph organs, where they further may differentiate into mature and potentially autoreactive CD4⁺ or CD8⁺ T cells (106). Supporting this idea, increasing numbers of CD8⁺ SP T cells exhibiting V β 5 and V β 12 segments were detected in the lymph nodes of BALB/c chronically infected mice (106).

Further studies showed that intrathymic key elements involved in the promotion of negative selection remain functional during the acute chagasic thymic atrophy (46). Moreover, using a transgenic system consisting in chicken egg ovalbumin (OVA)-specific T-cell receptor, it was shown that OVA administration in infected mice with thymic atrophy promoted OVA-specific thymocyte apoptosis, reinforcing the idea that a rather normal process of negative selection takes place during the infection (46).

Despite the fact that human beings have no TCR repertoire deletions compared with mice, during human Chagas disease there is also a differential expression of TCR-V β 5 T cells from infected patients in different stages of the disease, and preferential

expansion of CD4⁺Vβ5⁺ and CD8⁺ Vβ5⁺ T cells was also observed when mononuclear cells were exposed *in vitro* to parasite antigens, suggesting a possible commitment of these cells in the pathology (113). The preferential expansion of Vβ5⁺ populations occurs in both CD28⁺ and CD28⁻ CD4⁺ T cell subsets and is related to restricted sequences of CDR3 (114, 115). Interestingly, C57BL/6 mice, which do not suffer the clonal deletion of TCR-Vβ cell subsets, also expand peripherally CD8⁺ Vβ5⁺ clones during infection (112). Secondly, it was reported an increased frequency of T cells bearing Vβ7 in chronic and symptomatic patients bearing HLA haplotypes that were previously associated with susceptibility to cardiac damage (116). These data suggest that dominant antigenic epitopes may be involved in the expansion of peripheral T cells expressing specific Vβ regions. In this regard, some authors raised the bet proposing the existence of a *T. cruzi*-derived exogenous T superantigen-like moieties that may induce the development of a CD8⁺ Vβ-skewed response (112). Nevertheless, we cannot rule out that the escape of immature and potentially autoreactive T cells might shape the peripheral T cell repertoire. Reinforcing this hypothesis, we showed the presence of DP and DN cells bearing an activated phenotype in patients with chronic Chagas disease (46).

Additionally, it seems evident that the loss of tTreg cells may compromise the reposition of the Treg-peripheral pool. Such sustained alteration overtime may be partially related to the T-cell autoimmune events occurring in CCC (117, 118). It is interesting to note in this regard that even though Treg cells can develop “*de novo*” from CD4⁺ SP T cells in the periphery (pTreg cells), it have also been found an enhanced proportions of Treg cells in chronic but symptomless chagasic patients compared to those exhibiting CCC (117, 119, 120). Likely, in patients with CCC, Treg cells are not sufficient and competent to control the systemic inflammatory process and the cardiac lesions mediated by activated CD8⁺HLA-DR⁺ T cells and potentially autoimmune DP HLA-DR⁺ T cells. Unfortunately, there are no studies evaluating the tTreg and pTreg repertoire in mice and humans infected with *T. cruzi*. These studies may help to elucidate the nature of the rare self-antigens that induce Treg cell differentiation.

In addition, recent work showed that Th1 systemic response against *T. cruzi* is able to influence the intrathymic development of a particular population among CD8⁺ SP thymocytes: the “CD8⁺ innate cells or innate memory-phenotype” (T_{IM}-CD8⁺) cells (43). As mentioned in **Box 1**, these subpopulation is generated as an independent lineage different from conventional CD8⁺ T cells (25, 26, 121). However, little is known concerning their intrathymic development in normal steady-state conditions and even less after pathological situations. Nevertheless, new evidence showed that enrichment of CD8⁺ SP thymocytes with an “innate phenotype” could be seen after Th1-driven infectious processes where systemic expression of IL-12 and IL-18 is triggered. This effect was particularly observed after infection with 2 different strains of *T. cruzi*. Of note, the systemic production of these inflammatory cytokines induced high expression of both IL-4 and IL-15 in the thymus, generating an appropriate niche for the conversion of DP thymocytes (an also potentially autoimmune DP cells)

toward the innate phenotype over the conventional CD8⁺ SP pathway (43).

Taken together, these findings suggest that the release of potentially autoreactive T cells in the periphery of the immune system (**Table 2**), linked to potential abnormalities in the negative central tolerance mechanisms, might contribute to the autoimmune process found in both murine and human Chagas disease.

ABNORMAL INTRATHYMIC T-CELL MIGRATION, EXIT, AND RE-ENTRANCE FOLLOWING ACUTE *T. CRUZI* INFECTION

Cumulated evidence clearly shows that the intrathymic cell migration and both the exit and re-entry of T cells during infection are altered. These points are discussed in more depth below and schematically summarized in **Figure 2**.

Intrathymic Migratory Response to Various Cell-Migration Related Molecules

As mentioned above, thymic atrophy and DP T cells loss caused by *T. cruzi* infection in mice (**Figure 3A**) are accompanied by thymic microenvironmental changes that include altered expression of migration-related components. These changes alter thymocyte intrathymic migration possibly influencing their development and inducing alterations in the export of thymocytes. One would suspect that changes in the expression of migratory-related proteins could lead to disturbances in the thymocyte journey throughout the thymic lobule. In fact, the migratory properties of thymocytes are profoundly affected during murine *T. cruzi* infection (13, 46, 50, 51, 128–130).

A broad diversity of chemokines is constitutively expressed in defined areas inside the thymic lobules, preferentially attracting a given particular subpopulation of thymocytes expressing the corresponding receptors. In particular, chemokines such as CXCL12 and CCL4 have major roles in the intrathymic migration of developing thymocytes and thymocyte precursors. CXCL12 is produced by TEC, mainly in the subcapsular, and medullary regions and mediates the initial migratory phases attracting DN cells from the cortico-medullary junction to the subcapsular zone, where specific signals induce and regulate the earliest stages of thymocyte development. Moreover, CXCL12 is involved in the migration of DP cells and the corresponding receptor CXCR4 is highly expressed in this population (131). Interestingly, marked migration disturbances concerning this chemokine were demonstrated in the thymus during experimental infection with both *T. cruzi* strains. Thymuses from infected animals showed a CXCL12 distribution denser and not organized, likely due to the virtual disappearance of the cortical region, while the remaining DP cells increased the expression of the corresponding receptor CXCR4 (51). These findings were paralleled by an upregulation of CCL4 expression and an increased density of the respective receptor CCR5 on DP cells. A further aspect to be discussed is that CXCL12 binds and it is also presented by ECM proteins like fibronectin. In this regard, enhanced CXCL12 and fibronectin co-localization was clearly detected

TABLE 2 | Characteristics of extrathymic CD4⁺CD8⁺ double positive (DP), CD4⁺CD8[−] double negative (DN) T cells and peripheral TCR-Vβ T cell repertoire in mice and humans infected with *T. cruzi*.

Characteristics	Mice	References	Humans	References
CD4⁺CD8⁺ DP T cells				
Extrathymic appearance during the acute phase	✓	(13, 46, 50, 106, 122)	ND	
Extrathymic appearance during the chronic phase	✓	(46)	✓	(46, 123)
Expression of activation markers	✓	(46)	✓	(46, 123)
Pro-inflammatory cytokine production	✓	(46)	✓	(123)
Enhanced expression of VLAs and chemokine receptors	✓	(50)	ND	
Granzyme/Perforin expression	✓	(46)	✓	(123)
Expression of prohibited segments TCR-Vβ	✓	(50, 106)	ND	
Presence in cardiac tissue	✓	(122)	✓	(123)
CD4⁺CD8[−] DN T cells				
Extrathymic appearance during the acute phase	✓	(124, 125)	ND	
Extrathymic appearance during the chronic phase	✓	(106)	✓	(124–127)
Expression of activation markers	✓	(124, 125)	✓	(126)
Pro-inflammatory cytokine production	✓	(124)	✓	(126)
Peripheral T- cell repertoire				
Skewed TCR-Vβ expression in peripheral T cells	✓	(106)	✓	(113–116)

ND, not determined.

in thymuses from *T. cruzi* infected animals. Additionally, *ex-vivo* experiments showed that fibronectin/CXCL12 enhanced the haptotactic migration of immature thymocytes from *T. cruzi* infected mice as compared to controls, suggesting that fibronectin increases CXCL12 sequestration, thus facilitating its presentation to thymocytes and enhancing their migration toward this chemokine (51).

The ECM-driven migration is settled mainly by the interaction of thymocytes with TEC. A progressive increase in the expression of fibronectin and laminin, in both cortex and medulla of thymic lobules, was observed in *T. cruzi* infected mice. When cortical thymic nurse cells were exposed *in vitro* or *in vivo* to *T. cruzi* there was an increase in the deposition of ECM proteins, with an intensified release of DP cells from the lymphoepithelial complexes (23). In parallel, an enhancement in the expression of corresponding very late antigen(VLA)-4, VLA-5, and VLA-6 receptors on thymocytes was observed in the thymus of *T. cruzi* infected mice (42, 50).

In a second vein, the expression of TNF-α is also enhanced in the thymus during *T. cruzi* infection and *ex-vivo* studies suggest that, when TNF-α is complexed with fibronectin, it favors the migratory capacity of DP thymocytes derived from *T. cruzi* infected animals (129). Besides cytokines and chemokines, ECM components might adsorb parasite-derived antigens, contributing to the establishment and perpetuation of *in situ* reactions. In this respect, it is noteworthy that the parasite antigen cruzipain binds fibronectin in myocardial tissue possibly favoring T cell infiltration while trans-sialidase promotes fibronectin-driven thymocyte migration (130, 132).

The intrathymic journey of thymic regulatory CD4⁺Foxp3⁺ Treg cells also seems to be affected during *T. cruzi* infection. In addition to studies showing a marked loss of tTreg cells in the thymus of infected mice, an abnormal cortical localization of

Foxp3⁺ cells were detected. Strikingly, the membrane expression levels of the chemokine receptors CCR7 and CXCR4 are enhanced in this population. Notoriously, in normal and infected thymus, the Foxp3⁺ population expressed a higher proportion of both receptors compared to Foxp3[−] cells, but the membrane level of both receptors (ascertained by MFI) was diminished in Foxp3⁺ cells from infected animals. Moreover, fibronectin-driven migration of Foxp3⁺ thymocytes from infected animals was clearly diminished in *transwell* cell migration assays, associated to a diminution in VLA-4 and VLA-5 (90). These findings support the notion that tTreg cells may undergo alterations in their trafficking capacity during *T. cruzi* infection, although functional studies in this particular cell subset remain to be done.

Overall, alterations in the intrathymic expression of ECM proteins, soluble chemoattractant molecules, and cytokines, as well as changes in the levels of integrins and chemokine receptors, likely contribute to thymocyte migratory alterations observed during *T. cruzi* infection.

Alterations in Thymic Exit and Extrathymic Immature T Cells

Changes in the intrathymic migration during *T. cruzi* infection are linked to the abnormal appearance of thymus-derived immature DN and DP T lymphocytes in peripheral lymphoid organs and blood from infected hosts (13, 50, 124); features that are summarized in **Table 2**. Accordingly, this premature leakage in thymocyte developmental terms of immature cells from the thymus may also contribute to the establishment of the thymic atrophy observed during parasite infection.

As already commented herein, experimental *T. cruzi* acute infection induces a strong polyclonal T-cell activation with an over-representation of some TCR-Vβ families (112), which

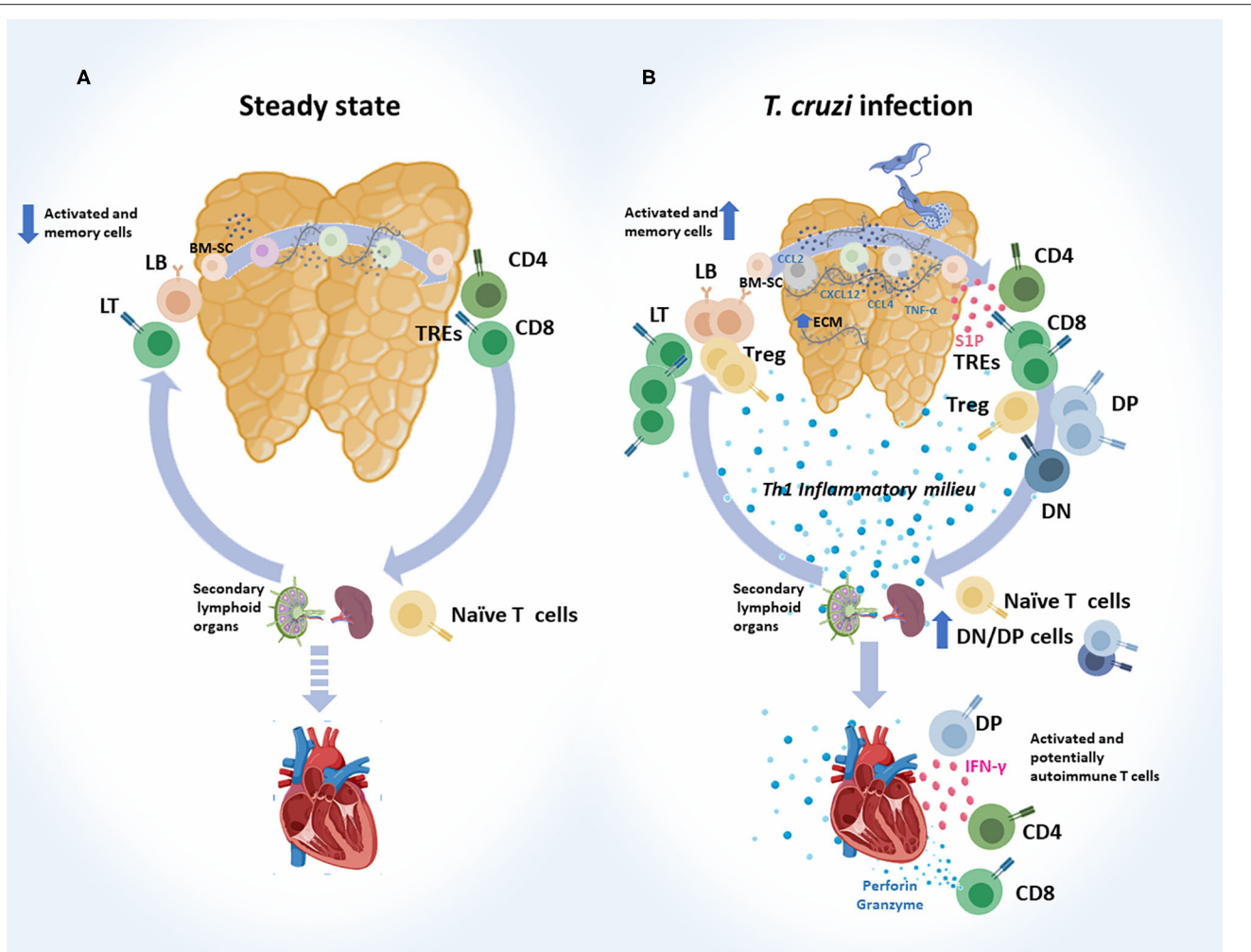


FIGURE 2 | Abnormal intrathymic T-cell migration, exit and re-entry following acute *T. cruzi* infection. **(A)** Steady state. Normally, after bone marrow-derived stem cells (BM-SC) enter into the thymus and commit to the T cell lineage, thymocytes engage a complex journey through the organ. Stromal-derived signals like chemokines and cytokines, and also the interaction with extracellular (ECM) proteins, guide the traffic and maturation of developing thymocytes (DN $\rightarrow$ DP $\rightarrow$ SP). Selected thymocytes leave the organ as recent thymic emigrants (RTEs), mainly represented by mature CD4⁺ and CD8⁺ SP T cells. These cells migrate to secondary lymphoid organs and turn into naïve T cells. When encountering their specific antigen, naïve T cells become activated and migrate to target tissues to execute their effector functions. Some of them become memory T cells, and a very low proportion of memory/activated T cells may re-enter the thymus. **(B)** *T. cruzi* infection. Migratory properties of thymocytes and thymus-derived T cells are profoundly affected during murine acute *T. cruzi* infection. Intrathymically, these alterations are linked to enhanced ECM deposition, increased levels of CCL2, CXCR4, CXCL2, and TNF- α , which have haptotactic functions. DP thymocytes and tTregs are among the most affected cell types. Once the intrathymic development process is completed, cells leave the thymus, as recent thymic emigrants RTEs guided by an enhanced S1P gradient in the bloodstream. Conjointly with CD4⁺ and CD8⁺ SP cells, progressively increased proportions of DN and DP T cells escape from the organ, probably as a consequence of thymic microenvironmental abnormalities, together with intrathymic and systemic Th1 pro-inflammatory contexts. In mice, a proportion of SP and DP cells carried prohibited TCR-V β fragments. Naïve T cells may encounter *T. cruzi*-derived parasites in secondary lymph nodes and become effector T cells. Potentially, autoimmune DN and DP T cells detected in the periphery also showed an effector-like T phenotype. Effector T cells, target heart to control parasites and at the same time diverse autoimmune events may occur. In addition, increased proportion of memory/activated T cells re-enter the thymus. DN, double negative; DP, double positive; SP, single positive.

overlap the presence of extrathymic immature DP T cells expressing potentially autoreactive TCRs (Table 2) (50, 106, 133). Although this phenomenon may be a consequence of failure in the negative selection process, the data available consistently indicate that thymopoiesis (including negative selection events) remains functional during the *T. cruzi* infection (46), reinforcing the notion that the abnormal release of immature T lymphocytes is rather related to intrathymic migratory disturbances. In normal

conditions, the signaling pathway mediated by the bioactive lipid sphingosine-1 phosphate (S1P) through its receptors (S1PRs) is responsible for the exit of mature SP cells from the thymus (46). In this regard, it was demonstrated that thymic S1P availability plays a role in the exit of immature DN cells in experimental Chagas disease (125) and probably in the escape of DP cells. At the thymic level, both S1P and the balance of enzymes involved in the S1P pathway during *T. cruzi* infection are altered,

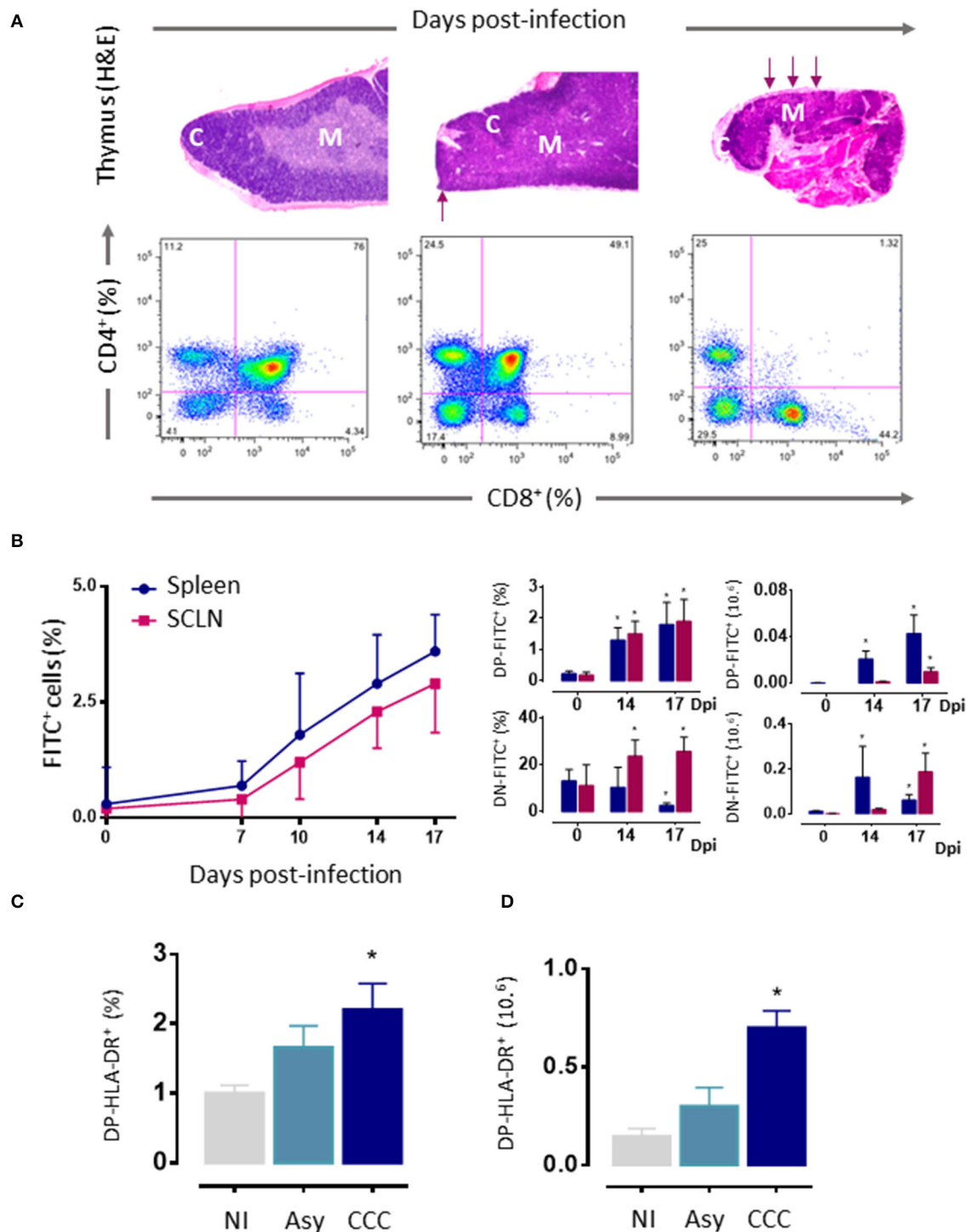


FIGURE 3 | Thymic atrophy and abnormal exit of DP cells *T. cruzi* acutely infected mice. **(A)** Upper panels. Photomicrographs showing the progressive loss of cortical DP thymocytes during *T. cruzi* infection (H&E staining, 20X). Arrows indicate zones with cortical loss. Bottom panels: Representative dot plots showing an intense loss of the CD4⁺CD8⁺ DP subset, assayed by cytofluorometry. **(B)** Increased export of lymphocytes from the thymus of *T. cruzi* infected C57BL/6 mice. Acutely infected mice received intrathymic injection of FITC and were analyzed 16 h later by flow cytometry to detect recent thymic emigrants (FITC⁺ cells) in peripheral lymphoid organs. Left graph shows how the absolute numbers of FITC⁺ RTEs progressively increased with the infection. Such increase comprises the abnormal release of immature CD4⁺CD8⁺ DP and CD4⁺CD8⁺ DN lymphocytes, whose relative and absolute numbers are progressively higher, in both subcutaneous lymph nodes and spleen (right graphs). **(C)** Circulating activated HLA-DR⁺DP cells in patients with chronic Chagas disease. DN, double negative; DP, double positive; RTEs, Recent thymic emigrants; NI, non-infected patients; Asy, asymptomatic patients without cardiopathy to demonstrated; CCC, patients with chronic chagasic cardiomyopathy; H&E hematoxylin-eosin. **(A)** Was adapted from Villa-Verde et al. (79), **(B)** from Barbosa-ferreira et al. (7); Mantuano-Barradas et al. (81), and **(C)** from Acha-Orbea et al. (107). **p* < 0.05.

showing a reduction of S1P paralleled by a downregulated gene expression of the kinase SPHK1/2 (the enzyme that forms S1P by the phosphorylation of sphingosine) and up-regulation of the phosphatase SGPL1 (the enzyme involved in the terminal breakdown of S1P) (125). Since S1P levels are not altered in the serum of infected animals, the S1P gradient (S1P in thymus < S1P serum) that is established favors the exit of the cells from the thymus. Moreover, increased membrane expression of S1PRs (particularly S1P1 and S1P3) on DN and DP thymocytes was observed following *T. cruzi* infection (125). The blockade of S1P receptors with FTY720 avoided the escape of DN to the periphery and consequently restored physiological proportions of DN thymocytes in infected mice (125), demonstrating that S1P-mediated pathways contribute to the premature exit of these cells.

In humans, extrathymic DP T cells have been described in pathological conditions such as diverse infectious diseases, autoimmune diseases, chronic inflammatory disorders, and certain lymphoblastic diseases (134–136). In *T. cruzi* infected mice, the first evidence of an aberrant thymic release of DP lymphocytes to the periphery was the observation that these cells progressively accumulated in spleen and subcutaneous lymph nodes (**Figure 3B**) (13). Some studies support the idea that such DP cells may be memory CD4⁺ helper T cells that, after activation, have acquired the ability to retain the expression of the CD8 α chain (123, 135, 137). Nevertheless, in our studies, thymocytes were previously intrathymically labeled with a fluorescent dye and 16–24 h later tracked in peripheral lymphoid organs (13), precluding the possibility that these cells can be quickly differentiated into memory cells. Actually, DP T cells can also be detected in the circulation and, in low numbers, within the heart of acutely infected mice (122). The augmented presence of these cells in peripheral organs (including the heart) may represent an accelerated recruitment of T cells from thymus as a compensatory mechanism to overcome the anergy/immunosuppression described during the acute phase of *T. cruzi* infection (138). Accordingly, these peripheral DP cells exhibit high densities of ECM and chemokine receptors (50) and an activated phenotype, showing increased IFN- γ production and cytotoxic activity (107). These data are strongly related to humans, with the presence of activated HLA-DR^{high} DP T cells in patients with the cardiac form of the disease (**Figure 3C**). Such an increase, not seen in patients in the asymptomatic form, suggests a positive correlation between the frequencies of circulating and tissue DP cells and the presence of cardiomyopathy (46, 123), by far the most important clinical consequence of *T. cruzi* infection (46, 123). Yet, the low proportion of these cells in the blood of patients makes it difficult to isolate them in a sufficient quantity for functional studies and, therefore, also hinders their clinical implication. In addition, in the mouse model, similar results were observed with peripheral DN T cells, which exhibit increased mRNA levels for TNF- α and IL-17A upon polyclonal activation (124). Moreover, $\alpha\beta$ and $\gamma\delta$ DN T cells have been also detected in the blood of patients with chronic Chagas disease (126, 127). Interestingly, while $\alpha\beta$ DN T cells from CCC patients expanded *in vitro* showing a clear pro-inflammatory profile, $\gamma\delta$ DN T cells from indeterminate patients exhibited an

anti-inflammatory profile (127). Overall, these findings suggest that DN T cells may be linked with the establishment of different clinical forms of Chagas disease.

Despite the mechanisms underlying the premature thymocyte release during the chagasic thymic atrophy remains elusive, some data suggest that parasite-derived antigens could be involved in the altered exit of immature T cells since intrathymic injection of trans-sialidase in mice enhances the abnormal release of DP cells (130). Hypothetically, trans-sialidase might also play a role in the altered release of DP cells in patients with the indeterminate or cardiac clinical forms of Chagas disease; since there was a gradual enhancement of antibody titres against trans-sialidase as the frequency of the peripheral blood DP cell subset increases (130).

In addition, abnormal thymic exit can also be associated with the immunoendocrine imbalance that takes place during *T. cruzi* infection in both mice and humans (122). In patients with chronic Chagas disease, extrathymic DP T cells positively correlated with circulating levels of TNF- α and with the cortisol/DHEAS ratio, particularly in patients having CCC (122). This raises the question of whether there is a cause/effect relationship between the immunoendocrine alterations and the proportion of extrathymic DP T cells linked to clinical progression in humans with Chagas disease. In these individuals, the establishment of a chronic inflammatory milieu may affect the activated state of DP T cells, and at the same time, the cortisol/DHEAS ratio imbalance may act as a permissive scenario to myocarditis development. In this respect, further investigation on the relationship among TNF- α , DHEAS, and the cortisol/DHEAS ratio with the phenotype and effector functions of extrathymic DP cells, may help to elucidate the contribution of these cells to the progression to CCC (122).

A further issue that remains to be explained is whether or not differences seen in the regional immunology following *T. cruzi* infection are somehow related to the exit of T cells from the thymus. It has been shown that splenomegaly and lymphadenopathies, particularly the expansion of subcutaneous lymph nodes is observed in experimental models and patients, mainly secondary to T and B cell polyclonal activation (139–142). Conversely, mesenteric lymph nodes (MLN) and Peyer's Patches are described to be largely reduced in experimental models of acute infection (42, 52, 143–145). Splenomegaly and subcutaneous lymph node hypertrophy are consequences of tissue T/B lymphocyte activation and expansion (139, 141, 146). In contrast, the MLN atrophy revealed a reduction of T and B lymphocyte numbers, as well as a decrease in IL-2, IL-4, and IL-10 production by activated T cells (54). The mechanism involved in IL-2 deprivation is this particular group of lymph nodes is not clear but can be associated to the differential distribution of Tregs, since IL-2 could be produced in normal levels and be sequestered by these cells in secondary lymphoid organs during infection (54, 147). Additionally, MLN cells from infected mice show reduced capacity to proliferate and enhanced apoptosis mediated by Fas, TNFR1/p55 and IL-4 deprivation, through caspase 9 activation (70, 148). Noticeably, apoptosis is also elevated in Peyer's Patches during acute infection (54). Overall, it seems

plausible to raise the hypothesis of a differential homing of Tregs from the thymus to the periphery; such difference being related to the expansion vs. a contraction of T and B cells in peripheral lymphoid organs. Although difficult to be settled (due to the small amounts of Tregs in the thymus), such a possibility can be experimentally tested by studying RTEs, comparing the numbers of recent thymic Treg emigrants in various lymphoid organs.

Re-entry of Mature T Cells Into the Thymus of *T. cruzi* Infected Mice

Under physiological conditions, the re-entry of mature T cells into the thymus is mainly restricted to peripheral cells that have acquired an activated phenotype or also memory cells. Yet, a non-negligible proportion of cells that recirculate back to the organ exhibit a naïve phenotype (149–151). This process has been described during the early phase of a Th1 inflammatory/infectious process. In this scenario, the cellularity of the thymus is seriously compromised, a situation that fits well with murine *T. cruzi* infection. Actually, it was demonstrated that a large number of mature peripheral B and T cells could enter the thymus in *T. cruzi*-infected mice, when atrophy is evident, compared to the number observed in non-infected mice where the thymus is intact (152).

The reasons for cell migration of both T and B cells back to the thymus have been addressed by several groups. In the case of peripheral B cells, it has been postulated their entry in the thymus would allow T-cell tolerance to immunoglobulins and to other B-cell-specific antigens (153). Others have proposed that B cells found in the thymus could participate in negative selection by acting as antigen-presenting cells (154). As for T cells, it has been suggested that the thymus can function as a repository of memory T cells (155), while others have demonstrated an important role of peripheral mature T cells in maintaining medullary epithelial cells (MEC) (156). The authors provided evidence indicating that i.v. injected T cells from lymph nodes are able to localize in the medullary region of the murine thymus and led to marked regeneration of MEC (156). Yet, such results need more undoubtful confirmation.

Furthermore, in cases of infection, the thymus might recruit immune cells specific to the invading pathogens that could ultimately lead to microbe-specific tolerance, impairing host resistance. This effect has been reported for *Mycobacterium avium* (157, 158), *Murine leukemia virus* (MLV) (159), *Lymphocytic choriomeningitis virus* (LCMV) (160) and *Hepatitis virus* (HBV) infections (161). Regarding to viral infections, it is possible that the intrathymic presence of viral proteins was responsible for inducing pathogen-specific T cell tolerance since both MLV and HBV, as well as Zika virus, can infect TECs (159, 162). As for *T. cruzi* infection, our recent work demonstrated both the presence of the parasite inside murine thymic macrophages and epithelial cells, along with a large number of activated/memory CD8⁺ CD44^{high} cells specific for one of the most immunogenic parasite antigen, the antigen TSKB20, a trans-sialidase derived epitope (43). However, new data are needed to

demonstrate if these events can trigger parasite-specific tolerance as well.

Despite several laboratories have described the phenomenon of mature peripheral cells migrating into the thymus, there is a lack of evidence that determines the mechanism by which this process occurs. Nonetheless, it has been demonstrated that T cells able to enter the thymus of *T. cruzi*-infected animals exhibit enhanced expression of CD44 and CD62L but low expression of CD69, compatible with a central memory phenotype. Interestingly, blocking CD62L with neutralizing antibodies did not affect the immigration of mature T or B into the thymus, indicating that the process is independent of this selectin (152). In a second vein, in the infected organ, the enhanced expression of CCL2 is accompanied by an increase in the number of CCR2⁺ T cells. Accordingly, the administration of irbesartan (antagonist of CCL2) diminished the entrance of T cells, suggesting that the thymic increase in the contents of the chemokine facilitates the recruitment of peripheral CCR2⁺ lymphocytes (152).

It should be noted, however, that the ability of peripheral T cells to migrate into the thymus does not seem to be restricted to those cells bearing the activated/memory phenotype. This notion is reinforced by findings showing intrathymic tTreg cells with a marked maturational profile (90), suggesting that at least, a proportion of tTreg detected in the thymus may correspond to pTreg cells that have re-entered the organ. Interestingly, the appearance of these cells is detected when the atrophy is well-established, as Hodge and colleagues proposed earlier (152). Although the functional relevance of Treg cell re-entry into the thymus of during *T. cruzi* infection is still unknown, it is possible that peripheral Tregs and T effector cells that regain the organ regulate intrathymic tTreg differentiation. In this regard, some authors suggest that the re-entry of activated pTregs cells bearing the phenotype CD62L[−]CXCR4⁺ into the thymus might inhibit IL-2-dependent differentiation of tTreg cells (151, 163). Others speculate that the re-entry of T effector cells may contribute to the induction of tolerance by promoting tTreg development, by acting as a source of IL-2 (164). Whatever the case, remaining tTregs observed during *T. cruzi* infection express increased and diminished levels of CD62L and CD184/CXCR4, respectively (149).

CONCLUDING REMARKS AND PERSPECTIVES

The most important concept emerging from the data discussed above is that *T. cruzi* infection disrupts intrathymic homeostasis, in terms of both microenvironmental and lymphoid compartments of the organ. As summarized in **Figure 4**, almost all stages of thymocyte development are altered: caspases-dependent massive apoptosis, changes in the amounts of tTreg cells, qualitative changes in the intrathymically repertoire, altered expression of cell-migration related receptors, together with an abnormal exit of immature DN and DP T cells. Whether all these changes occur in humans is to be determined, although at least

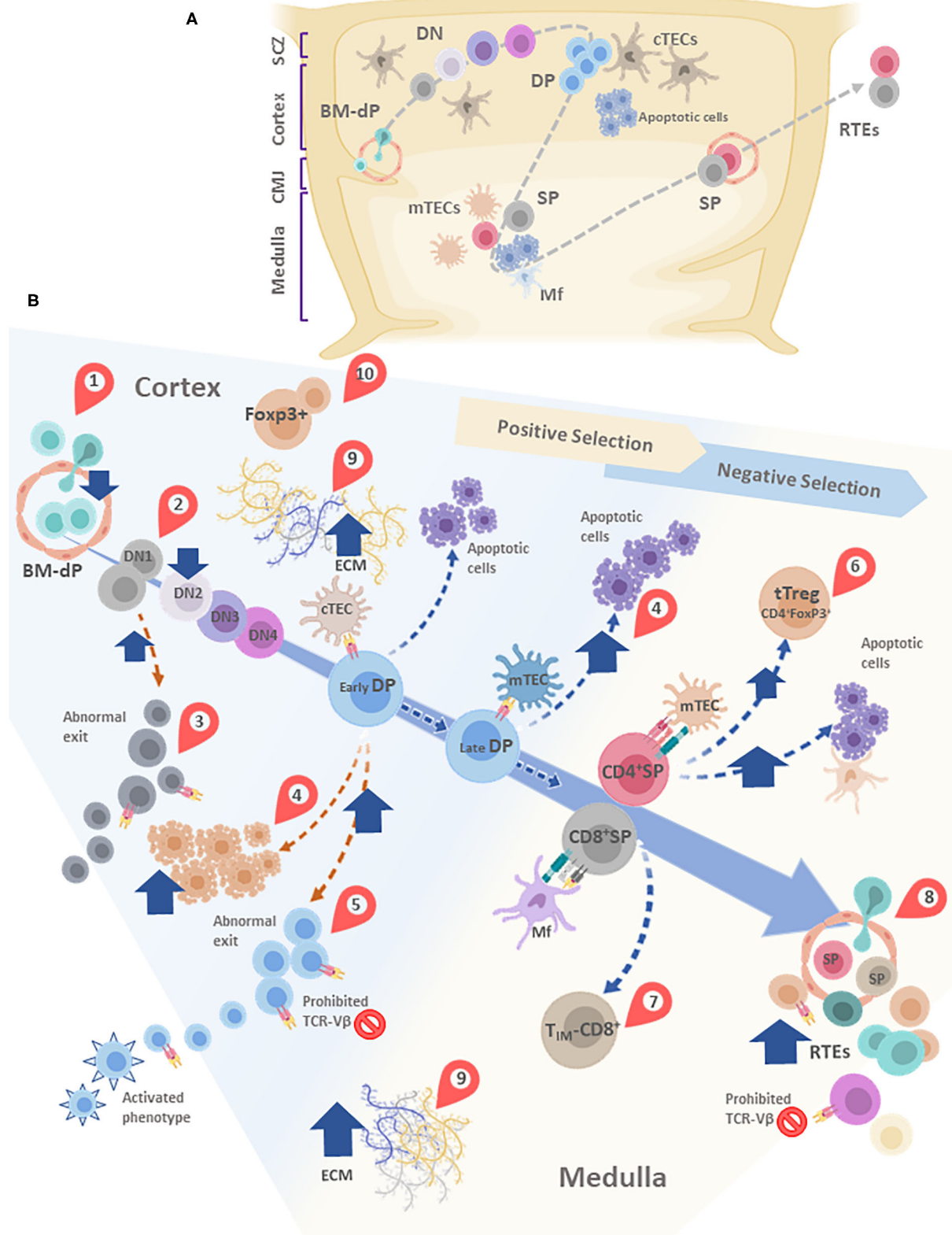


FIGURE 4 | (A) Simplified schematic model of thymocyte migration across the thymic lobule. **(B)** Conveyor belt representation of thymocyte maturation and the processes of positive and negative selection. BM-derived precursors enter through blood vessels located in the cortico-medullary junction (CMJ). The most immature
(Continued)

FIGURE 4 | thymocytes originated from these pluripotent precursors are called DN cells, since lack CD4 and CD8. DN cells go through four stages (DN1-DN4) defined by the expression of CD44 and CD25. During differentiation, DN cells migrate from the CMJ to the subcapsular zone of the thymus. Cell precursors are irreversibly committed to the T-cell lineage during the passage from DN1 to DN2 state. In this stage, cells that are successful in the rearrangement of the β -chain of the T-cell receptor (TCR) are selected for further maturation steps. After passing by the DN4 stage, survived thymocytes are converted into double-positive (DP) thymocytes by acquisition of CD4 and CD8. In this step occurs the rearrangement of the genes encoding the α -chain of the TCR. Double-positive thymocytes undergo positive and negative selection, and their destinies are determined by the interaction with the thymic epithelial cells (TECs). Once positively selected, they are able to generate single-positive (SP) cells. In this step, thymocytes move away from the cortex and enter the medulla. A large percentage of thymocytes die by neglect, due to the non-functionality of their TCRs. The remaining cells interact with different degrees of affinity with their specific antigens. A strong affinity prompts apoptosis and subsequent clonal deletion. An intermediate affinity may induce regulatory T cells. Also, T_{IM} -CD8: Innate memory CD8 cells appear. Low affinities allow their differentiation to SP cells. Finally, mature thymocytes reach the CMJ and exit to the periphery as RTEs. In most of these stages, diverse alterations are observed after *T. cruzi* infection, which are indicated in the figure by red teardrop pointers: (1) Decreased entrance of BM-dP; (2) Retention of thymocytes in the DN1 state; (3) DN abnormal escape; (4) Enhanced DP thymocyte apoptosis, mainly driven by enhanced levels of glucocorticoids. (5) DP abnormal escape, with many of them carrying prohibited TCR-V β , also exhibiting an activated phenotype; (6) tTreg accumulation among SP thymocytes; (7) Increase in T_{IM} -CD8 cells; (8) Increase in RTE proportions, with many of them carrying prohibited TCR-V β ; (9) Increased deposition of ECM molecules in both cortex and medulla, (10) Abnormal presence of FoxP3⁺ cells in the cortical area. cTEC, cortical thymic epithelial cells; mTEC, medullary thymic epithelial cells; DC, dendritic cell; Mf, macrophages; DN, double negative; DP, Double positive; SP, single positive; BM-dP, Bone marrow-derived precursors; CMJ, Corticomedullary junction; RTEs, Recent thymic emigrants; T_{IM} -CD8, Innate memory CD8⁺ cells; SCZ, Subcapsular Zone (SCZ); ECM, extracellular matrix.

abnormal DP and DN lymphocytes have been found in patients with Chagas disease.

The further knowledge on the molecular mechanisms underlying thymic abnormalities occurring during *T. cruzi* infection, as well as the consequences of the thymic abnormalities upon the peripheral immune response to the parasite, may contribute to designing innovative strategies to control Chagas disease pathology.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication. AP made figures.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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