ThymUS in times of stress

The Global Thymus Network convened virtually for ThymUS 2020 on 3–4 November 2020. Participants shared recent advances in thymus biology and enjoyed a reprieve from 2020 stressors.

The thymus produces T cells that protect us from invaders and that can be recruited for cancer immunotherapy, all the while tolerating our own healthy tissues. Many mysteries remain as to how the thymus accomplishes this feat. A far-flung community of scientists gathered virtually on 3–4 November to discuss cutting-edge thymic research at ThymUS 2020. The conference was organized by Nancy Manley, Marcel van den Brink, David Wiest and Juan Carlos Zúñiga-Pflücker and exclusively featured trainees and junior faculty. The meeting provided a welcome respite from months of COVID-related scientific isolation.

The thymus comprises developing T lymphocytes (thymocytes) as well as cells that support their development, including thymic epithelial cells, other immune cells (B cells, dendritic cells (DCs) and macrophages) and stromal mesenchymal and endothelial cells[1]. Research on the thymus has great potential for therapeutic application. Many labs seek to understand age-related thymic involution and peripheral immunosenescence, a potential factor in the susceptibility of the elderly to cancer and infectious diseases, such as COVID-19. The new research presented during ThymUS 2020 included advances in our understanding of unconventional T cell development, the effects of thymic atrophy and the factors contributing to regeneration, as well as the landscape of human T cell development and the underlying causes of human disease.

Jonathan Sprent delivered the Charlie Surh Memorial Keynote at ThymUS 2020. Photographs of Jonathan Sprent and Charlie Surh individually (left and right, respectively) and together (center bottom). Charlie and Juan Carlos Zúñiga-Pflücker, who introduced the keynote, are pictured together at ICI/IUIS Melbourne 2016 (center top). Photographs were provided by Jonathan Sprent.

Early thymocyte development

The most efficient T cell progenitor populations in the thymus have multilineage potential, indicating that they are derived from multipotent precursors in the bone marrow, but the identity of the precursors that migrate to the thymus has remained elusive. Marieke Lavaert (Ghent University) used single-cell transcriptional profiling of human CD34$^+$ thymocytes to characterize early T cell lineage commitment[2]. Integration of these findings with datasets interrogating CD34$^+$ progenitors in the bone marrow and peripheral blood identified two putative thymic-seeding progenitors, suggesting two parallel developmental pathways. Assessment of developmental potential in silico and ex vivo established that both candidate T cell progenitors were capable of producing T cells, but one also possessed significant plasmacytoid DC potential. The results provided new insight into what have so far been relatively poorly characterized populations in humans. As thymocytes progress from the multipotent early T cell precursor (ETP) stage, they lose non-T cell lineage potential and commit to the T cell fate. Boyoung Shin (California Institute of Technology) probed the roles of the transcription factors RUNX1 and RUNX3 during fate commitment in mice[3]. Unlike in mature T cells, RUNX1 and RUNX3 were found to act redundantly in early T cell precursors. Despite stable expression, Runx factors preferentially regulate dynamically changing genes essential for commitment by switching their genome-wide binding and interactions with cofactors.

Development of conventional αβ T cells

After successful pre-TCR signaling (β selection), αβ thymocytes progress to the CD4$^+$CD8$^+$ (double positive (DP)) stage to undertake TCRα chain rearrangement and positive selection. TCR signaling during positive selection must fall within a specific range: signaling is required to rescue developing thymocytes from cell death (positive selection), but excessive signaling can also result in cell death (negative selection)[4]. Linking differences in TCR signaling to distinct transcriptional outcomes underlying different fates has been challenging, but preliminary studies from Fernando Grigera (University of California, San Diego) investigating TCR-induced *Id3* expression indicate significant progress. Single-molecule imaging revealed differences in *Id3* transcriptional bursting

and decay after stimulation with strong and weak agonists. Grigera further observed that ID3-deficient thymocytes undergo cell death when signaled by either weak or strong ligands. These results indicate that ID3 promotes the survival of signaled thymocytes and further suggest that TCR signal strength is measured by the *cis*-regulatory elements controlling *Id3* transcription.

Positively selected αβ lineage DP thymocytes undergo specification to form the helper CD4$^+$ and cytotoxic CD8$^+$ T cell lineages. The transcription factor ThPOK is necessary to impose the CD4$^+$ helper fate on TCR-signaled thymocytes, but neither the factors that initiate ThPOK expression nor the mechanism of its action are understood. To identify factors that promote CD4$^+$ versus CD8$^+$ lineage divergence, Laura Chopp (National Cancer Institute) integrated single-cell RNA sequencing (scRNA-seq) and scATAC–seq analysis of mouse thymocytes spanning the transition from uncommitted CD4$^+$CD8$^+$ to mature CD4$^+$ and CD8$^+$ cells[5]. Interrogation of gene regulatory networks identified new candidate regulators of ThPOK. Chopp further compared mouse- to human-lineage programming and demonstrated significant conservation of CD4$^+$ and regulatory T (T_{reg}) cells and agonist-selected lineage gene programs but, interestingly, CD8$^+$ lineage gene programs were less conserved.

Induction of self-tolerance

The factors influencing the fate of high-affinity TCRs, either toward clonal deletion or T_{reg} cell specification, are incompletely understood[4]. Masashi Watanabe (National Cancer Institute) studied the roles of the B7–CD28 costimulatory axis using thymic antigen-presenting cells (APCs) with conditional deficiency for B7 and mice carrying targeted mutations in CD28 (ref. [6]). Whereas B7-dependent, self-antigen-specific clonal deletion was not impacted by B7 deficiency in any single APC population, T_{reg} cell development significantly decreased with the absence of B7 on dendritic cells, highlighting the distinct contributions of APCs to the two fates. Additionally, distinct CD28 intracellular cytoplasmic tail motifs were required for clonal deletion versus T_{reg} cell development. Tom Sidwell (California Institute of Technology) studied the developmental lineages of thymic T_{reg} cells when the transcription factor BACH2 was experimentally ablated. Loss of BACH2 enhanced the TCR- and IRF4-dependent selection of CD25$^+$Foxp3$^-$ T_{reg} cell precursors but attenuated the generation of CD25$^-$Foxp3$^+$ T_{reg} cell precursors, demonstrating the two developmental pathways have discrete transcriptional and stimulation requirements.

Elise Breed (University of Minnesota) identified a new subset of medullary SIRPα$^+$ conventional dendritic cells (cDC2s) that express CD301b. When compared to other thymic DC subsets, SIRPα$^+$CD301b$^+$ cDC2s possessed strong transcriptional signatures for MHC class II antigen processing, and ablation of this population resulted in decreased clonal deletion. Interestingly, this population was dependent on signaling via the interleukin (IL) receptor IL-4Rα, with a significant population reduction in natural killer T (NKT)-cell-deficient mice, indicating that the acquisition of self-tolerance by adaptive T cells is influenced by cytokine production from innate-like T cells.

Development of unconventional T cells

Pre-TCR signals are critical to proceed to the DP thymocyte stage. Greet Verstichel (The La Jolla Institute for Immunology) showed distinct roles for the pre-Tα isoforms in thymic selection. In sharp contrast to pre-Tα deficiency, which greatly impairs the development of αβ T cells, expression of only the short isoform of pre-Tα significantly impaired the development of conventional αβ T cells but not of innate-like T cells, including DN T cells, invariant NKT cells and Eomes$^+$CD8$^+$ T cells. These results suggest that selection signals during the pre-TCR stage, prior to αβTCR expression, influence the development of conventional versus innate-like T lineages.

In contrast to conventional αβ T cells, selection of Vγ5Vδ1$^+$ dendritic epidermal T cells (DETCs)—a subtype of γδ T cells—is dependent on Skint1 expression by medullary thymic epithelial cells (mTECs)[7]. Based on the requirement for butyrophilin heterodimerization in γδ T cell selection[8], Leticia Monin (Francis Crick Institute) investigated whether Skint1-mediated DETC selection required other Skint family members. As with Skint1 deficiency, Skint2-deficient mice failed to produce DETCs, and reaggregate cultures with thymic epithelial cells (RTOCs) demonstrated that Skint1 and Skint2 must be coexpressed on the same cell for selection. Site-directed mutagenesis demonstrated that residues in the Skint1 and Skint2 CFG face domain are required for selection, providing further evidence that this process imitates butyrophilin selection.

In mice, DP thymocytes do not express classical MHC molecules but do express MHC class Ib molecules that select innate-like cells such as NKT and MAIT cells, raising the question of whether selection on DP thymocytes induces innate-like effector function[9]. Hristo Georgiev (University of Minnesota) forced classical MHC I expression on DP thymocytes, resulting in selection of MHC I–restricted PLZF$^+$ innate-like lymphocytes. Interestingly, these mice possessed fewer NKT cells. The results indicate that downregulation of MHC I on DP thymocytes in mice is important for selecting the correct numbers of NKT cells[9].

Mouse γδ T cells are classified by specific TCR usage and distinct effector functions[9]. To determine whether human γδ T cells have effector programs similar to those in mice, Likai Tan (Hannover Medical School) analyzed γδ T cells from human newborn cord blood and adult peripheral blood by scRNA-seq and scTCR-seq. Whereas naive γδ T cells contained diverse TCRs, those with distinct effector programs were dominated by specific TCR sequences, such as Vγ9Vδ2, that were present in all donors. Vγ9Vδ2$^+$ effector cells were transcriptionally similar to innate-like NKT cell subsets and coexpressed PLZF with either of the transcription factors Tbet or RORγt[9].

Programming of γδ T cell lineages for distinct effector functions is poorly understood. TCRγδ signal strength results in graded inhibition of E protein through differential upregulation of ID3. Alejandra Contreras (Fox Chase Cancer Center) found that E protein activity regulates expression of the long noncoding RNA Gm15417 in developing γδ T cells. Loss of Gm15417 enhanced the production of mature γδ T cells and shifted their effector function, converting IFN-γ-producing cells into IL-17 producers. These results could indicate that effector functions are imprinted by differences in TCR signaling, as is seen in other innate-like T cells, with weaker TCR signaling resulting in low Gm15417 expression and acquisition of IL-17 effector function[10]. Edward Chen (University of Toronto, Sunnybrook Research Institute) used an RBPJ-inducible system[11] in conjunction with models that generate graded TCR signal strength to differentiate the influence of Notch and TCR signaling on γδ T cell effector function. IL-4-producing cells required strong TCR and Notch signaling for development, whereas IL-17 producers developed when precursors received weak TCR signaling. These findings highlight factors relevant to the acquisition of effector functions by γδ and NKT cells, suggesting a common innate-like T cell program influenced by TCR signaling and environmental cues. How differences in TCR and Notch signaling arise during the

normal ontogeny of γδ T cells remains to be determined.

Ligands for T cell selection

A fundamental question yet to be answered is how peptides presented on cortical TECs (cTECs) shape the T cell repertoire. In all jawed vertebrates examined, cTECs express a unique proteasomal subunit (β5t)[12]. To ascertain the influence of β5t on CD8 TCR selection, Melina Frankzeskakis (National Cancer Institute) identified TCRα sequences in mature CD8$^+$ T cells specific to mice possessing or lacking β5t and generated mice transgenic for these TCRα sequences. Interestingly, TCRα sequences from mice possessing β5t developed more efficiently in the presence of β5t, whereas sequences from mice lacking β5t developed more efficiently in the absence of β5t. The results indicate that the influence of β5t on presented peptides is sufficient to substantially alter the identity of selected TCRs.

The role of cathepsin L in MHC II–mediated positive selection was studied by Elisabetta Petrozziello (Ludwig–Maximilian University) using a series of TCR-transgenic mice. TEC-specific deficiency in cathepsin L prevented the development of mature CD4$^+$ T cells in MHC II–restricted TCR-transgenic mice and, furthermore, reduced TCR diversity in normal mice, indicating that cathepsin L provides selecting peptide ligands for MHC II molecules. MHC II antigen processing and presentation utilizes the endosomal–lysosomal trafficking system for peptide exchange and surface presentation. Luke Postoak (Vanderbilt University) found that CD4$^+$ T cell selection was significantly reduced when TECs lacked Vps34, as visualized by the failure of several MHC II–restricted, but not MHC I–restricted, TCR-transgenic lines to develop into mature T cells. Vps34 is implicated in autophagy as well as in vesicle fusion, but TCR-transgenic lines that failed to develop in the context of TEC-specific Vps34 deletion were able to develop in the absence of *Atg5*, an essential autophagy gene. The results indicate an important role for Vps34 and vesicle fusion in TEC-mediated CD4$^+$ T cell selection.

Generation of an MHC-restricted TCR repertoire

Two competing theories attempt to address how αβ T cells are MHC restricted. The germline theory posits that αβTCRs are evolutionarily biased to recognize MHC molecules[13]. By contrast, the selection theory proposes that MHC restriction is imposed by thymic selection. Because T cells develop in QUAD mice lacking MHC I and MHC II and CD4 and CD8 coreceptors, this indicates that MHC-independent αβTCR specificities are generated by V(D)J rearrangement. Marco Craveiro (National Cancer Institute) generated mice transgenic for TCRβ sequences from both MHC-dependent and independent T cells and assessed their development in QUAD mice as well as in normal mice. Both TCRβ sequences were able to pair with endogenous TCRα chains to support the development of MHC-dependent T cells in wild-type mice and MHC-independent T cells in QUAD mice, indicating that MHC restriction is not imposed by the TCRβ sequence.

TCR–MHC interactions almost invariably occur in a 'canonical' orientation, but a population of influenza-specific TRBV17$^+$CD8$^+$ T cells has been shown to 'reverse dock' on peptide–MHC I[14]. Pirooz Zareie (Monash University) used these TCRs to investigate the drivers underpinning the canonical docking modality. Reverse docking TCRs were prevalent in the naive influenza-specific repertoire, but, unlike conventional T cells, reverse-docked T cells failed to expand during influenza challenge. This functional defect was independent of TCR affinity but was attributable to the failure of CD3– and CD8–Lck to correctly colocalize in reverse-docked TCRs, raising the question of how these cells are selected in the thymus.

Thymic function at the extreme ends of life

Thymus size and function change dramatically with age as well as in response to stressors[15]. Whereas the chemokine CCL21a produced by mTECs is important for the migration of maturing thymocytes from the cortex to the medulla[16], Kieran James (University of Birmingham) found a new role for this chemokine in thymic emigration. Thymic loss of CCL21a, but not CCL19, decreased the numbers of thymic emigrants in neonatal mice, with a corresponding accumulation of mature T cells within the thymus. Interestingly, immunohistochemistry indicated that CCL21a is bound by heparan-sulfate-expressing perivascular mesenchyme and pericytes that surround blood vessels at the cortico–medullary junction, thereby enabling its accumulation at sites of emigration.

Age-associated thymic involution is associated with a decrease in naive peripheral T cells, potentially impairing immune responses[17]. Sam Palmer (University of Oxford) presented mathematical models that show a correlation between the incidence of several cancers and infectious diseases and decreased thymic output. Both disease risk and thymic output are described by exponential functions that double and halve every 16 years, respectively[18]. Interestingly, this correlation also holds true for COVID-19 susceptibility[19].

Thymus size is controlled by Myc activity in TECs[20], which was utilized by Jennifer Cowan (National Cancer Institute) to assess *Toxoplasma gondii*–associated mortality in models of Myc-enhanced thymic function. Enhanced thymus cellularity and function enabled peripheral naive T cell populations to be maintained in aged mice and prevented age-associated mortality caused by *Toxoplasma gondii*. Whereas Cowan's work highlighted the importance of thymic output in preventing immunosenescence, Sandip Ashok Sonar (University of Arizona) emphasized the role of lymph nodes in maintaining the naive T cell population. Longitudinal assessment of inducibly-labeled recent thymic emigrants demonstrated that the presence of naive T cells in secondary lymphoid organs decreases with age, but the age at which the decrease was observed varied between lymph nodes at different anatomical sites.

Thymus regeneration

Thymic regeneration after damage was revealed to be influenced by diverse cell types within the thymus, including endothelial cells, DP thymocytes and eosinophils. Lorenz Jahn (Sloan Kettering Institute) evaluated the kinetics of irradiation-mediated acute thymic atrophy and regeneration with age. Regeneration began to slow by 18 months of age. scRNA-seq analysis identified a putative ETP-niche-forming subset of CXCL12^{hi}SCF^{hi} endothelial cells that declined with age, correlating with slower ETP recovery in this model system. Sinead Kinsella (Fred Hutchinson Cancer Research Center) discovered that apoptotic DP thymocytes inhibit thymic regeneration through TAM receptors, which detect apoptotic cell membrane components, on DCs and endothelial cells. Blocking TAM receptors increased the production of IL-23 and BMP4, which are important for regeneration. After irradiation-induced damage, however, metabolic alterations in DP cells resulted in pyroptotic instead of apoptotic cell death, releasing damage-associated molecular patterns (DAMPs), including ATP, which stimulated thymic regeneration and TEC recovery. These results indicate that alterations in thymocyte metabolism after damage support thymic recovery. Emilie Cosway (University of Birmingham) identified a role for eosinophils in regeneration after irradiation, with thymic recovery failing

in eosinophil-deficient mice. Secretion of eotaxin by IL-4Rα-signaled mTECs recruited eosinophils to the thymus. Consistent with this, administration of IL-5, which boosts eosinophil recruitment and function, enhanced thymic regeneration.

From bedside to bench

DiGeorge syndrome is caused by a chromosomal deletion at 22q11.2 and is characterized by thymic hypoplasia and lymphopenia[21], but the mechanism is unknown. New findings from Pratibha Bhalla (University of Texas, Southwestern) identified mesenchymal cells as the source of the defect in the *Tbx1*$^{neo2/neo2}$ mouse model of this disease. By reaggregating sorted populations of thymocytes, epithelial cells and mesenchymal cells from embryonic wild-type or mutant mice in RTOCs, Bhalla demonstrated that replacement of mutant mesenchyme with normal mesenchyme enables functional reaggregation and thymocyte development. Consistent with this, scRNA-seq of embryonic mutant and wild-type thymuses identified alterations in mutant mesenchymal subsets that could account for the formation of a hypoplastic thymus.

Formation of the thymus from the third pharyngeal pouch requires the transcription factor Foxn1. Typically, clinical presentation of Foxn1-mediated deficiencies in T cell production occurs with homozygous gene disruption[22]. However, Ioanna Rota (University of Oxford) characterized a new human Foxn1 mutation that causes thymic hypoplasia and lymphopenia in individuals who are heterozygous. Thymic size was similarly reduced in heterozygous mutant mice. Molecular characterization of mutant Foxn1 supported its dominant-negative effects, differentiating it from other loss-of-function mutations that have been characterized[23].

Rag1 or *Rag2* gene defects can cause disease ranging from severe combined immunodeficiency (SCID, RAG deficiency) to mild immune dysregulation with autoimmunity (*Rag* hypomorphic mutations)[24]. Functional development and maintenance of the mTEC compartment is reliant on multiple signals from developing single positive (SP) thymocytes, and RAG-deficient mice have pronounced defects in mTEC development. Francesca Pala (National Institute of Allergy and Infectious Diseases) used scRNA-seq to characterize the impacts of hypomorphic *Rag1* alleles on TECs. The thymus in RAG1 hypomorphic mice was dominated by cTECs, with very few mTECs, reminiscent of the neonatal TEC compartment. The cTEC compartment contained unique cell populations not present in wild-type mice. These results suggest that aberrant T cell development and autoimmunity due to hypomorphic RAG1 may be mediated by altered cTEC function in addition to the reduced abundance of mTECs.

Patients with SCID are identified shortly after birth by quantifying recent thymic emigrants. Although there are many known causes of SCID, including those highlighted above, some individuals carry mutations in new genes that must be identified using next-generation sequencing (NGS)[25]. Robert Sertori (Fox Chase Cancer Center) applied a new method for rapidly validating NGS gene hits in zebrafish. T cell development is assessed after morpholino-based knockdown of the zebrafish orthologue, and the ability of a wild-type or mutant human orthologue to rescue T cell development is assessed by heat-shock-inducible re-expression. This methodology identified two new genes with impacts on T cell development, and these will be followed up in mouse models.

The creation and optimization of in vitro thymus models has received considerable interest, as they could allow the patients who are immunodeficient to be treated with in vitro–derived T cells or by thymic regeneration[26]. Stephan Ramos (University of Colorado) is forging a path toward the latter goal using patient-derived induced pluripotent stem cells (iPSCs) to generate functional isogenic thymic organoids in vitro on the basis of previous in vivo work[27]. Although iPSC-generated TECs expressed canonical TEC markers and tissue-restricted antigens, they failed to reproduce classical thymic architecture, and T cell development was discernable but inefficient. In vitro systems also allow closer examination of developmental requirements[28]. Amelie Montel-Hagen (University of California, Los Angeles) detailed the optimization of murine artificial thymic organoids (M-ATO). M-ATO-mediated T cell development followed the kinetics and classical gene expression programs of in vivo T cell development and produced polyclonal mature CD4$^+$ and CD8$^+$ αβ T cells as well as T_{reg}-like cells and γδ T cells. This system allowed for the efficient production of T-lineage progeny from single hematopoietic stem cells, thus allowing the assessment of developmental potential using single cells[29].

ThymUS 2020 concluded with the Charlie Surh Memorial Keynote presented by Jonathan Sprent (Garvan Institute of Medical Research), a giant of the thymus community who has made pioneering contributions to T cell biology throughout his career, many of them in close collaboration with the late Charlie Surh[30]. Sprent shared his personal, evolving perspective on trogocytosis from the 1970s to the present day. Trogocytosis, simply defined as cell membrane exchange, can occur between T cells and APCs. CD28–B7 (T cell–APC) interactions are required, but efficiency is enhanced by other surface receptor interactions such as TCR–MHC and LFA-1–ICAM-1. Remarkably, T cells can also absorb cell-free membranes in the form of DC-derived exosomes containing B7, peptide–MHC and costimulatory ligands, and such exosomes can result in T cell stimulation and activation[31]. The Sprent lab is capitalizing on these observations and investigating the translational potential of exosomes in cancer immunotherapy and allograft transplantation.

Conclusion

The research presented at ThymUS 2020 highlighted major advances in the field of thymus biology. Researchers are increasingly taking advantage of new single-cell transcriptomic and epigenomic technologies to better understand mouse and human T cell development and to understand thymus function in disease and during atrophy and recovery. The interest in unconventional T cells at previous meetings has continued, and progress has been made on understanding the signals directing the programming of effector function during development. New progress was made in the fields of human T cell development, mechanisms of CD4–CD8 lineage divergence, and TCR induced transcriptional programming. Finally, the impact of thymic involution and atrophy on peripheral immune function was clarified; new mechanisms contributing to thymic formation, age-related involution and regeneration were identified; and technologies to identify the impact of human mutations were refined.

Despite the virtual format, ThymUS 2020 was a resounding success and inspired us with the notable progress that has occurred, and colleagues were encouraged by plans for the Global Thymus Network to meet in person for the Kyoto T Cell Conference in October 2021. ❐

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Competing interests

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