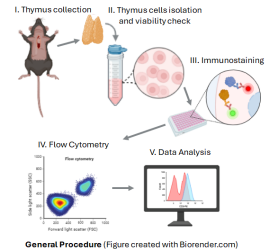


Nov 10, 2025

Multicolor Flow Cytometry Approach for Analysis of Stromal and Thymocyte Cell Populations in the Mouse Thymus

DOI

<https://dx.doi.org/10.17504/protocols.io.n2bvjem6bgk5/v1>



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Protocol Citation: Tamara Menendez Medina*, Zaima Mazorra Herrera*, Cinthia Mariel Olexen, Gisela Maria Suarez Formigo 2025. Multicolor Flow Cytometry Approach for Analysis of Stromal and Thymocyte Cell Populations in the Mouse Thymus. **protocols.io** <https://dx.doi.org/10.17504/protocols.io.n2bvjem6bgk5/v1>

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Protocol status: Working

We use this protocol and it's working

Created: November 06, 2025

Last Modified: November 10, 2025

Protocol Integer ID: 231663

Keywords: Mice thymus, Thymus flow cytometry, Thymocytes, Thymus epithelial cells , thymus stromal cell, thymocyte cell populations in the mouse thymus, mature thymocyte, characterization of thymocyte, thymocyte cell population, thymocytes development, selecting thymocyte, positive selection of thymocyte, developing thymocyte, regarding thymocyte, thymic epithelial cell, thymocyte, cell fate decisions in the thymus, late stage of thymocytes differentiation, thymus organogenesis, later stages of thymocyte development, thymocytes differentiation, thymocyte development, necessary support for thymocyte development, sp thymocyte, thymic cell population, dn cells within the thymus, indispensable for thymus organogenesis, impaired thymocyte differentiation, consistent with impaired thymocyte differentiation, thymic medulla, thymus medulla, influence on thymocyte outcome, stromal cell component, stromal cell, thymocyte outcome, hallmark features of thymic aging, associated thymic involution, functional thymus, lymphocyte develop

Abstract

The thymus is a primary lymphoid organ which contains stromal cell components that provide the necessary support for thymocyte development and maturation. Thymus stromal cells include, among others, thymic epithelial cells (TEC), endothelial cells, mesenchymal/fibroblast cells, and hematopoietic populations such as dendritic cells, B cells, and macrophages. TECs are particularly critical through all the stages of T cell development, contributing to the recruitment of hematopoietic precursors from the bone marrow, directing lineage specification, supporting proliferative expansion, and orchestrating both positive and negative selection processes (Cepeda & Griffith, 2018) (Han & Zúñiga-Pflücker, 2021).

Anatomically, the thymus is organized into two distinct regions: the cortex in the outer zone, which contains the cortical TECs (cTECs) and the medulla in the inner zone, containing the medullary TECs (mTECs). cTECs are primarily involved in the positive selection of thymocytes. In contrast, mTECs participate in the negative selection, promoting central tolerance and supporting the development of Foxp3⁺ regulatory T cells and natural killer T cells (Han & Zúñiga-Pflücker, 2021) (Nitta & Suzuki, 2016).

A young and fully functional thymus is essential for generating a pool of diverse naïve T cells, which supports robust immune responses. However, during aging, the thymus undergoes progressive involution characterized by structural and molecular modifications. These age-associated changes lead to a marked decline in the production of newly generated naïve T cells. In compensation, a homeostatic expansion of existing T cell populations occurs, resulting in reduced diversity within the peripheral T cell repertoire. Functionally, this adaptation contributes to diminished responsiveness to pathogens, increased susceptibility to autoimmune conditions, and impaired tumor surveillance (Liang et al., 2022) (Han & Zúñiga-Pflücker, 2021).

The study of the thymic microenvironment and its influence on thymocyte outcome during aging provides valuable insights into the structural and functional changes that occur with senescence. One of the hallmark features of thymic aging is the decline in TEC cellularity, affecting both cTECs and mTECs subsets. TECs can be phenotypically identified with the pan-epithelial marker CD326 (EpCAM) within the CD45⁻ thymic cell population. Further discrimination between TEC subsets could be achieved using the CD205 marker. cTECs subset could be characterized as CD45-CD326⁺CD205⁺, whereas CD45-CD326⁺CD205⁻ cells correspond to mTECs (Jain & Gray, 2014). Aging is also associated with a reduction in MHC class II expression on TECs, reflecting a decline in TEC antigen-presenting ability (Gray et al., 2006). Within the TEC population, two subgroups, MHCII^{low} and MHCII^{high}, can be distinguished based on the expression levels of MHC class II molecules (Jain & Gray, 2014) (Xing & Hogquist, 2014). A key molecular regulator of TEC function is the transcription factor FoxN1, which is indispensable for thymus organogenesis and TEC maintenance in adults. FoxN1 expression declines progressively with age, contributing to thymic involution and reduced naïve T cell output (Rode et al., 2015). Overexpression of FoxN1 has been shown to mitigate age-related thymic changes, restoring thymic mass, architecture, and the naïve T cell population in the spleen (Zook et al., 2011).

Thymic medulla, besides containing mTECs, is populated by hematopoietic antigen presenting cells (APCs) including dendritic cells, B cells, and macrophages. Through expression and presentation of self-antigens, these APCs contribute to the process of shaping the normal T cell repertoire by negatively selecting thymocytes recognizing self-antigens. Senescence studies have demonstrated age-associated declines in APCs function and cell counts (Ki et al., 2014) (Cepeda & Griffith, 2018) (Wedemeyer & Griffith, 2025) (Van Dommelen et al., 2010).

Thymocytes development starts from the double negative (DN) to simple positive (SP) T cells. At first, T cells are CD4-CD8⁻ (DN), in the second stage they are double positive CD4⁺CD8⁺ (DP) and finally CD4⁺CD8⁻ or

CD4-CD8⁺ (SP) in the third stage. After positive selection, SP CD4⁺ or CD8⁺ T cells migrate to the thymus medulla to go through negative selection mediated by medullar TECs and APCs, where autoreactive SP T cells are deleted by apoptosis. This process of thymopoiesis results in population of peripheral blood and lymphoid sites with naive CD4⁺ and CD8⁺T cells and CD4⁺ regulatory T cells (Tregs) (Liang et al., 2022).

Early T-lineage progenitors (ETPs) frequency declines with aging, and their potential ability to reconstitute the thymus is also reduced (Min et al., 2005). Both DN and DP population cell numbers are significantly modified with aging. DN subset frequency increases in aged mice compared with young, whereas the percentage of DP (CD4⁺CD8⁺) subpopulations significantly diminishes with age. Additionally, thymic aging is concomitant with the abnormal accumulation of CD3⁺ DN cells within the thymus (Aw et al., 2010). Aging also interferes with later stages of thymocyte development. DP and SP thymocytes in aged mice display deregulated CD3 expression, which may lead to attenuated TCR-dependent stimulation (Aw et al., 2010). Consistent with impaired thymocyte differentiation in the aged thymus, T-cell receptor excision circles (TRECs) within the thymus also significantly decline with aging in mice and humans (Ortman et al., 2002).

Regarding thymocytes, the member of the high mobility group (HMG)-Box transcription factors TOX-1 has been identified as a mediator of thymic T cell expansion and the development of lymphoid inducer (Scott et al., 2019). The pattern of TOX expression in the thymus is highly regulated during the positive selection process. Downregulation of TOX-1 is observed in a later stage of positive selection, as cells take on a more mature SP phenotype (Wilkinson et al., 2002). Data about TOX expression in murine thymus during aging is scarce. Recently, it was published that Recent Thymic Emigrants (RTE) are distinguished by the expression of SOX4, IKZF2, and TOX and CD38 protein in humans. These cells significantly decrease with age (Bohacova et al., 2024). Interestingly, some TOX members have been related to T cell exhaustion in the periphery. In mice, TOX is required for the epigenetic remodeling and survival of exhausted CD8⁺ T cells in the chronic infection context (Yao et al., 2019). In contrast, a recent publication in human settings showed that TOX expression is related to anti-viral effective T cells (Sekine et al., 2020).

Different markers related to thymocytes development, survival, and migration have been studied in murine settings. In this case, L-selectin (CD62L) is a cell adhesion molecule that is expressed on most circulating leukocytes. The expression of CD62L is increased in the late stage of thymocytes differentiation and allows mature thymocytes to enter from circulation into lymph nodes (Weinreich & Hogquist, 2008).

Another important marker related to thymus development is the CD127 molecule (IL-7Ra chain). IL-7 augments survival of TN and SP thymocytes and is also essential for $\gamma\delta$ T cells and thymic DC development (Lynch et al., 2009). Lymphocyte development is severely impaired in mice lacking IL-7 or IL7 receptor (IL-7R) or treated with anti-IL-7 antibodies (Peschon et al., 1994). In both syngeneic and allogeneic mouse models, administration of IL-7 leads to enhanced T-cell reconstitution through increased thymic T-cell development and homeostatic proliferation, as well as decreased T-cell apoptosis but increased T-cell function (Alpdogan et al., 2003).

Regulatory T-cells (Tregs) were identified as sentinels of the immune response, keeping aberrant/exaggerated immune reactions in balance. After CD4 lineage commitment, some CD4 single-positive (SP) thymocytes, upon TCR stimulation, can develop into CD25⁺Foxp3⁺ Treg cells. The absolute number of Tregs in the thymus decreases with age, reflecting the reduction in cellularity that accompanies age-associated thymic involution (Chiu et al., 2007).

Costimulatory and coinhibitory molecules affect cell fate decisions in the thymus by enhancing or dampening signaling through the TCR. Studies have shown that PD-1 is expressed by developing thymocytes (Nishimura et al., 1996). A recent publication has reported that PD-1 limits the production of IL-2 within the thymus, thereby restraining the development of Tregs (Caruso et al., 2024).

The present protocol describes the development of flow cytometry panels tailored for the identification and characterization of thymocytes and thymus stromal cells. The panels could be useful to compare young and aged mice in senescence studies, offering valuable insights into age-associated changes in thymic architecture and immune function.

Attachments



[Figure 1 Thymocytes....](#)

571KB



[Figure 2 Stromal cel...](#)

306KB

Materials

Biological species

- C57BL/6J mice (The Jackson Laboratories)

Reagents and solutions

- RPMI 1640 Medium GlutaMAX
- Fetal Bovine Serum, heat inactivated
- Bovine serum albumin solution 30%
- Phosphate Buffered Saline (PBS)
- Penicillin-Streptomycin for cell culture
- PBS/ Azide: PBS pH7.2 with Sodium Azide 0.01%
- FACS buffer: 1% BSA in PBS
- sRPMI: RPMI 1640 culture medium supplemented with 10% Fetal bovine serum and 1% Penicillin/Streptomycin
- Trypan blue solution
- PerFix-nc kit (Beckman Coulter, catalogue number B10825).
- OptiLyse C, No-Wash Lysing Solution (Beckman-Coulter, catalogue number A11894).
- LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Invitrogen, catalogue number L34965).
- TruStain FcX PLUS (anti-mouse CD16/32) antibody (clone S17011E) (Biolegend, catalogue number 156603).
- Rat anti-mouse, PE-Texas Red/ CD45 monoclonal antibody (Clone 30-F11) (Invitrogen, catalogue number MCD4517).
- Rat anti-mouse, PE/CD205 (DEC-205) monoclonal antibody (clone NLDC-145) (Biolegend, catalogue number 138213).
- Rat anti-mouse, eFluor 450/ CD326 (EpCAM) monoclonal antibody (clone G8.8) (Invitrogen, eBioscience, catalogue number 48-5791-82).
- Rat anti-mouse, APC/IA-IE (MHC class II) monoclonal antibody (clone M5/114.15.2) (BioLegend, catalogue number: 107614).
- Rabbit anti-human (reactivity to Human, Mouse and Rat), unconjugated FOXN1 polyclonal antibody (Bioss, catalogue number BS-6970R).
- Goat anti-rabbit, Alexa Fluor 488-IgG H&L (Abcam, catalogue number ab150077).
- Rat anti-mouse Brilliant Violet 785/ CD3 monoclonal antibody (clone 17A2) (Biolegend, catalogue number 100232)
- Rat anti-mouse PCY7/CD62L monoclonal antibody (clone MEL-14) (Invitrogen, catalogue number 25-0621-82)
- Armenian hamster anti-mouse eFluor450/ PD-1 monoclonal antibody (clone J43) (eBioscience, catalogue number 48-9985-82)
- Rat anti-mouse PE/CD25 monoclonal antibody (clone PC61.5) (Invitrogen, catalogue number 12-0251-82)
- Rat anti-mouse Qdot605/ CD4 monoclonal antibody (clone RM4-5) (Invitrogen, catalogue number Q10092)
- Rat anti-mouse BV650/ CD8 monoclonal antibody (clone 53-6.7) (Biolegend, catalogue number 100742)
- Rat anti-mouse CD127-AF700 monoclonal antibody (clone A7R34) (eBioscience, catalogue number 56-1271-82)
- Rat anti-mouse eF660/ Tox1 monoclonal antibody (clone TXRX10) (Invitrogen, catalogue number 50650282)
- Rat anti-mouse AF488/ FoxP3 monoclonal antibody (clone FJK-16s) (Invitrogen, catalogue number 53577382)

Laboratory supplies

- Curved and straight forceps
- Dissection scissors
- Pippete micro tip with filter, sterile (20 μ L, 200 μ L and 1000 μ L)
- Disposable CultureTubes, Non-Sterile, 12 $\times$ 75 mm tubes, Polypropylene, 5 mL
- Strainer Cap for FlowTubes with 35 μ m strainer mesh
- 15 mL Conical Sterile Polypropylene Centrifuge Tubes
- 50 mL Conical Sterile Polypropylene Centrifuge Tubes
- 1.5 mL microcentrifuge tubes
- 96-well U-bottom cell culture plate
- 70 μ m Cell Strainer for 50ml Tube
- 0.22 μ m syringe filter
- Biohazard bags 30 mL sterile plastic containers
- 3-mL sterile syringe
- Serological pipettes 5ml

Equipment

- Adjustable micropipettes
- Serological pipette controller
- Refrigerated Centrifuge with rotor for 1.5 to 2 mL microtubes
- Refrigerated Centrifuge with rotors for 15- and 50-mL conical tubes
- Centrifuge with rotor for ELISA plates
- 4°C laboratory Refrigerator
- -20°C Laboratory Freezer
- -80°C Laboratory Freezer
- Water bath
- Vortex Mixer
- Biosafety cabinet, Class II, Type A2
- Flow Cytometer (Beckman Coulter DxFLEX)

Software

- FlowJo (BD Biosciences, Version 10)

I- Thymus collection

- 1 Euthanize mice in accordance with national regulations on laboratory animal welfare, in accordance with internationally accepted standards for the care and use of laboratory animals, and after approval of the corresponding local ethic committees. For the present protocol mice were anesthetized with an intraperitoneal injection of ketamine (120 mg/kg) and xylazine (16 mg/kg), a dosage commonly used for deep anesthesia prior to terminal procedures in rodent models (Cossarizza et al., 2021). Once unresponsiveness was confirmed, euthanasia was performed by decapitation (Leary et al., 2020). The procedure was carried out by a trained veterinary physician and following national and institutional animal care guidelines.

Note: Maintain sterile technique throughout all the procedure to prevent contamination. Always follow institutional safety guidelines when handling biological materials.

- 2 Position the mouse on its back and secure each limb to the dissection board. Spray with 70% ethanol to sterilize the mouse body.
- 3 Open the thoracic cavity with scissors, carefully exposing the heart and the thymus without cutting major blood vessels.
- 4 Extract the thymus gently using forceps, removing surrounding fat and connective tissue.
- 5 Transfer the thymus to a plastic container pre-filled with sRPMI medium and wash twice with sRPMI. Keep the organ in sRPMI at 4 °C until processing.

II- Thymus cell isolation and preparation for flow cytometry

- 6 Place a 70 µm cell strainer on top of a 50-mL tube. Place the thymus onto the strainer and gently press it through using the plunger of a 3-mL syringe. Rinse with sRPMI in between the mashing to facilitate dissociation. Cells will pass through the strainer, while clumps and debris are retained. At the end, rinse the strainer with sRPMI, adding up to a total volume of 10 mL.
- 7 Centrifuge the cell suspension at 300 x g for 5 minutes at 2-8°C. Discard the supernatant and gently dissociate the cell pellet in the remaining liquid. Add 5 mL of sRPMI to the resuspended cells.
- 8 Perform cell count and viability assessment using Trypan blue exclusion staining. Prepare a 1:1 dilution of the cell suspension with Trypan blue. Load the diluted sample

onto a hemocytometer, count viable (unstained) and non-viable (blue-stained) cells. Calculate the total cell count and viability percentage.

Note: Before starting the immunostaining, all antibodies should be titrated to determine the optimal working concentration. For each antibody prepare additional control samples: no stain, single antibody staining, multi-antibody staining, and fluorescence minus one (FMO) staining controls. Fluorochrome combinations should be carefully selected based on the instrument and available lasers. Consultation with flow cytometry core personnel is strongly recommended to minimize spectral overlap. Fluorochrome combinations used in the present protocol were optimized for use on cytometers with at least three lasers: blue (488 nm), red (640 nm), and violet (405 nm).

III- Immunostaining

9 **LIVE/DEAD staining protocol**

- 9.1 Centrifuge the cells at 300 x g for 5 minutes. Discard the supernatant and resuspend the cell pellet with PBS at a concentration of 10^7 cells/mL.
- 9.2 Transfer the cells to a 96-well U-bottom plate, dispensing 200 μ L per well (2×10^6 cells per well). Add 50 μ L PBS to each well. Centrifuge at 300 x g for 5 minutes and discard the supernatant.
- 9.3 Repeat the wash by adding 250 μ L of PBS to each well, centrifuging and discarding the supernatant.
- 9.4 Resuspend the cell pellet in 200 μ L of a 1:100 dilution (in PBS) of the Live Aqua Dead-Fixable Cell Staining Reagent (and previously prepared in DMSO following the manufacturer recommendations).
- 9.5 Incubate the cells for 30 minutes at 4°C, protected from light.
- 9.6 Add 50 μ L PBS, centrifuge, and discard the supernatant.
- 9.7 Perform a final wash with 250 μ L of PBS, centrifuge, and discard the supernatant.

10 **Fc receptor blocking**

- 10.1 Resuspend cell pellets in 100 μ L of PBS/ Azide solution.
- 10.2 Add 0.5 μ g (1 μ L) of TruStain FcX™ PLUS (anti-mouse CD16/32) antibody per well following manufacturer instructions.
- 10.3 Incubate for 10 minutes at 4°C protected from light.
- 10.4 Wash the cells by adding 150 μ L of FACS buffer, centrifuge, and discard the supernatant.
- 10.5 Repeat the wash with 250 μ L of FACS buffer, centrifuge, and discard the supernatant.
- 10.6 Proceed to stain the thymocytes and the thymic stromal cell populations separately.

11 Thymocytes staining

- 11.1 After the Fc receptor blocking step, add 50 μ L of FACS buffer containing appropriately diluted (based on previous titrations) primary, fluorescent-conjugated antibodies to each well for surface staining: PE-TxRed/ CD45, Brilliant Violet 785/ CD3, PCY7/ CD62L, eFluor450/ PD-1, PE/ CD25, Qdot605/ CD4, BV650/ CD8, AF700/CD127. Incubate mixes for 15- 30 min at room temperature protected from light.
- 11.2 Add 250 μ L of PBS to each well, transfer the contents to 5 mL flow cytometry tubes, add an additional 750 μ L of PBS to each tube (to complete 1 mL final volume) following instructions from the PerFix-nc kit (Beckman Coulter), centrifuge at 300 x g for 5 minutes, and discard the supernatant.
- 11.3 Resuspend cell pellets in 100 μ L of PBS.
- 11.4 Add 10 μ L of the Fixative Reagent (Reagent 1 of the PerFix-nc kit).
- 11.5 Vortex and incubate for 15 minutes at room temperature (18 – 25 °C).

- 11.6 Vortex and add 300 μ L of the Permeabilizing Reagent (Reagent 2 of the PerFix-nc kit) to each tube and vortex.
- 11.7 Add fluorophore-conjugated antibodies for intracellular staining: eF660/ Tox1 and AF488/ FoxP3 at adequate concentration each (based on previous titration). Vortex and incubate for 15 – 30 min at room temperature.
- 11.8 Add 3 ml of Final 1X Reagent (prepared by diluting the 10X concentrated Final Solution, Reagent 3 of the PerFix-nc kit, with type II water), vortex.
- 11.9 Centrifuge at 300 x g for 5 minutes and resuspend cell pellets in 250 μ L of Optilyse C.
- 11.10 Filter all samples through 70 μ m cell strainers immediately before acquisition on the Dx Flex Flow cytometer.

12 **Thymic Stromal Cells staining**

- 12.1 After the Fc receptor blocking step, add 50 μ L of FACS buffer containing appropriately diluted (based on previous titrations) primary, fluorescent-conjugated antibodies to each well for surface staining: PE-Texas Red/ CD45, PE/ CD205, eFluorTM 450/ CD326 (EpCAM) and APC/ IA-IE (MHCII). Incubate mixtures for 15- 30 min at room temperature protected from light.
- 12.2 Add 250 μ L of PBS to each well, transfer the contents to 5 mL flow cytometry tubes, add an additional 750 μ L of PBS to each tube (to complete 1 mL final volume) following instructions from the PerFix-nc kit (Beckman Coulter), centrifuge at 300 x g for 5 minutes, and discard the supernatant. Resuspend cell pellets in 100 μ L of PBS.
- 12.3 Add 10 μ L of the Fixative Reagent (Reagent 1 of the PerFix-nc kit).
- 12.4 Vortex and incubate for 15 minutes at room temperature (18 – 25 $^{\circ}$ C).
- 12.5 Vortex and add 300 μ L of the Permeabilizing Reagent (Reagent 2 of the PerFix-nc kit) to each tube and vortex.
- 12.6 Add the unconjugated FoxN1 antibody at an adequate concentration (based on a previous titration). Vortex and incubate for 15 – 30 min at room temperature.

- 12.7 Add the secondary antibody, anti-rabbit-AF488 conjugate following the manufacturer recommendations, and incubate 20 minutes at room temperature.
- 12.8 Add 3 ml of Final 1X Reagent (prepared by diluting the 10X concentrated Final Solution, Reagent 3 of the PerFix-nc kit, with type II water), vortex.
- 12.9 Centrifuge at 300 x g for 5 minutes and resuspend cell pellets in 250 uL of Optilyse C.
- 12.10 Filter all samples through 70 µm cell strainers immediately before acquisition on the Dx Flex Flow cytometer.

IV- Flow cytometry operation

- 13 The no stain sample is used to set laser voltages, the single-color staining samples are used to compensate fluorochrome emission overlap, and the FMO samples are used to provide empirical evidence that the instrument was well compensated. Instrument compensation is essential for proper interpretation of the results.
- 14 Run and record all samples, including all controls, after compensation adjustment.

V- Data Analysis

- 15 **Thymocytes gating strategy** (Cossarizza et al., 2021) (Figure 1)
 - 15.1 Thymocytes were first gated based on forward scatter (FSC-A) and side scatter (SSC-A) properties (Figure 1 Panel A).
 - 15.2 Doublets were excluded by FSC-A versus FSC-H gating and SSC-A versus SSC-H (Figure 1 Panels B and C, respectively).
 - 15.3 Viable cells were identified using the viability dye (Live Aqua, negative gate) (Figure 1 Panel D).
 - 15.4 After singlets and viable cells selection, two strategies of gating were performed. One strategy gated CD4 and CD8 expression on CD45+CD3+ population to distinguish more mature cells (Figure 1 Panel E, F and G) and the other strategy gated CD4 and CD8 expression on viable cells (Figure 1 Panel H). In both strategies, four types of

cells were defined as: Double positive DP (CD4+CD8+), Double negative DN (CD4-CD8-), Simple positive SP (CD4-CD8+ or CD4+CD8-).

- 15.5 Precursors and Thymic Tregs were distinguished on CD4+viable cells. In this case, precursors T regs were defined as FOXP3+ cells while Thymic T regs were stained as CD25+FOXP3+ T cells (Figure 1 Panel I and J, respectively).
- 15.6 The rest of the markers (TOX-1, CD62L, PD-1 and CD127) were analyzed inside DN, DP and SP populations. As examples expression of TOX-1 in DP population and PD-1 in CD3+CD8+ populations is shown (Figure 1 Panel K and L, respectively).
- 16 **Thymic stromal cells gating strategy** (Jain & Gray, 2014)(Gray et al., 2002)(Gray et al., 2008)(Rode et al., 2015)(Figure 2).
- 16.1 All thymus cells were first gated based on forward scatter (FSC-A) and side scatter (SSC-A) properties as previously described (Jain & Gray, 2014) (Gray et al., 2002) (Figure 2 Panel A).
- 16.2 Doublets were excluded by FSC-A versus FSC-H gating and SSC-A versus SSC-H (Figure 2 Panels B and C).
- 16.3 Viable cells were identified using the viability dye (Live Aqua, negative gate) (Figure 2 Panel D).
- 16.4 After singlets and viable cell selection, non-hematopoietic and hematopoietic cells were defined as CD45- and CD45+, respectively (Figure 2 Panel E).
- 16.5 Within the CD45+ subpopulation, hematopoietic APCs were identified as MHCII positive with the surface marker IA-IE (IA-IE+) (Figure 2 Panel F). Within the CD45-subpopulation, TECs were identified as CD326+ cells (Figure 2 Panel G).
- 16.6 MHCII expression into TECS was analyzed with the surface marker IA-IE, and cells were classified as MHCII(IA-IE)low or MHCII(IA-IE)high (Figure 2 Panel H).
- 16.7 FoxN1 expression into TECS was analyzed and cells were classified as FoxN1- or FoxN1+ (Figure 2 Panel I).
- 16.8 Division of TECS into subpopulations was assessed with the surface marker CD205 as CD326+CD205- (mTECs) and CD326+CD205+ (cTECs) (Figure 2 Panel J).

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Acknowledgements

We thank the staff of the animal facilities at UAEU for their support and assistance in animal handling. We also acknowledge ADSCC for technical support and resources contributing to this work.