Video Article

Examination of Thymic Positive and Negative Selection by Flow Cytometry

Qian Hu*[1], Stephanie A. Nicol*[1], Alexander Y.W. Suen*[1], Troy A. Baldwin[1]

[1]Department of Medical Microbiology and Immunology, University of Alberta

*These authors contributed equally

Correspondence to: Troy A. Baldwin at tbaldwin@ualberta.ca

URL: https://www.jove.com/video/4269
DOI: doi:10.3791/4269

Keywords: Immunology, Issue 68, Medicine, Cellular Biology, Anatomy, Physiology, Thymus, T cell, negative selection, positive selection, autoimmunity, flow cytometry

Date Published: 10/8/2012

Citation: Hu, Q., Nicol, S.A., Suen, A.Y., Baldwin, T.A. Examination of Thymic Positive and Negative Selection by Flow Cytometry. *J. Vis. Exp.* (68), e4269, doi:10.3791/4269 (2012).

Abstract

A healthy immune system requires that T cells respond to foreign antigens while remaining tolerant to self-antigens. Random rearrangement of the T cell receptor (TCR) α and β loci generates a T cell repertoire with vast diversity in antigen specificity, both to self and foreign. Selection of the repertoire during development in the thymus is critical for generating safe and useful T cells. Defects in thymic selection contribute to the development of autoimmune and immunodeficiency disorders[1-4].

T cell progenitors enter the thymus as double negative (DN) thymocytes that do not express CD4 or CD8 co-receptors. Expression of the αβTCR and both co-receptors occurs at the double positive (DP) stage. Interaction of the αβTCR with self-peptide-MHC (pMHC) presented by thymic cells determines the fate of the DP thymocyte. High affinity interactions lead to negative selection and elimination of self-reactive thymocytes. Low affinity interactions result in positive selection and development of CD4 or CD8 single positive (SP) T cells capable of recognizing foreign antigens presented by self-MHC[5].

Positive selection can be studied in mice with a polyclonal (wildtype) TCR repertoire by observing the generation of mature T cells. However, they are not ideal for the study of negative selection, which involves deletion of small antigen-specific populations. Many model systems have been used to study negative selection but vary in their ability to recapitulate physiological events[6]. For example, *in vitro* stimulation of thymocytes lacks the thymic environment that is intimately involved in selection, while administration of exogenous antigen can lead to non-specific deletion of thymocytes[7-9]. Currently, the best tools for studying *in vivo* negative selection are mice that express a transgenic TCR specific for endogenous self-antigen. However, many classical TCR transgenic models are characterized by premature expression of the transgenic TCRα chain at the DN stage, resulting in premature negative selection. Our lab has developed the HY^{cd4} model, in which the transgenic HY TCRα is conditionally expressed at the DP stage, allowing negative selection to occur during the DP to SP transition as occurs in wildtype mice[10].

Here, we describe a flow cytometry-based protocol to examine thymic positive and negative selection in the HY^{cd4} mouse model. While negative selection in HY^{cd4} mice is highly physiological, these methods can also be applied to other TCR transgenic models. We will also present general strategies for analyzing positive selection in a polyclonal repertoire applicable to any genetically manipulated mice.

Video Link

The video component of this article can be found at https://www.jove.com/video/4269/

Protocol

Refer to **Figure 1** for an overall scheme of the experimental protocol.

1. Dissection

1. Place sterile steel mesh screen into 60 x 15 mm Petri dish. One unit is needed per tissue sample.
2. Add 5 ml of Hank's balanced salt solution (HBSS) to each dish. Keep dishes on ice.
3. Euthanize mice with CO_2.
4. Secure mouse to dissection surface, ventral side facing up. Spray mouse with 70% ethanol for sterilization and to ensure that the fur is matted down.
5. Using surgical scissors, begin dissection by making a ventral incision in the abdominal skin just above the genitalia. Extend the incision upwards to the chin.
6. From the midline, extend the incision down along all limbs. Pull back the loose skin and pin it down to the dissection surface.
7. Harvest the thymus:
    1. Lift the bottom tip of the sternum to make an incision.

2. Avoiding the liver, cut the diaphragm to detach the rib cage and then cut the rib cage to each side. Cut the ribcage upwards on each side, taking care to avoid the lungs and heart.
3. Gently pull the ribcage back with forceps. The thymus is a white, bilobed organ located above the heart. Using the flat edge of the forceps to grasp the bottom of the lobes, gently pull the thymus and place it on the mesh screen.

8. Harvest the spleen:
   1. The spleen is a red, surfboard-shaped organ located on the left side of the mouse's abdominal cavity, below the liver.
   2. Make an incision into the abdominal cavity. Gently pull out the spleen with one pair of forceps, using the second pair to tease away connective tissue. Place the spleen on a separate mesh screen.

2. Cell Preparation

1. Using a plunger from a 3 ml syringe, grind organs into the mesh screen until only connective tissue and fat remains. Rinse mesh with HBSS from the dish three times. Use a new plunger for each tissue sample.
   1. Other options for homogenizing tissue can be used in place of this method.
2. Count cells using a hemocytometer.
3. Pellet cells by centrifugation at 335 x g for 5 min at 4 °C.
4. Resuspend splenocytes in 500 µl of ACK lysis buffer (0.15 M NH_4Cl, 10 mM $KHC0_3$, 0.1 mM Na_2EDTA, pH 7.2) for 10 min at room temperature. Resuspend thymocytes at 20 x 10^6 cells/ml in FACS buffer (PBS, 1% FCS, 0.02% sodium azide) and set aside on ice.
5. Return splenocytes to isotonicity by adding 5 ml of HBSS. Pellet splenocytes by centrifugation and resuspend at 20 x 10^6 cells/ml in FACS buffer. If cells need to remain sterile, you may use sterile RPMI + 10% FCS.

3. Staining Cells for Flow Cytometry

The purpose of this protocol is to outline strategies for the analysis of positive and negative selection using wildtype (WT) and HY^{cd4} mice. For general considerations regarding flow cytometry experimental design, acquisition, and analysis, we refer readers to an excellent review by Tung *et al.*[11]

1. Aliquot 4 x 10^6 thymocytes per flow cytometry sample to each well of a 96-well plate, as well as 1 x 10^6 WT splenocytes per compensation control. If using transgenic mice, you should include a WT mouse as a control in your experiment.
2. Block Fc receptors by incubating cells with anti-CD16/32 (clone 2.4G2) for 10 min on ice.
3. Spin plate at 335 x g at 4 °C for 5 min. Remove lid and dispel liquid from wells by flicking the plate once, face down into a sink. Resuspend each well in 200 µl of FACS buffer. Repeat the wash.
4. Prepare antibody cocktails as listed below, using the optimal concentration of each antibody based on dilution in 200 µl of FACS buffer per well. The optimal concentration of each antibody is defined as the lowest concentration needed to give the largest separation of positive and negative populations. If there is no negative population for a given antibody, an isotype antibody should be included. The total volume per well can be scaled down (*e.g.* to 100 µl per well) if desired.
   1. WT thymus: anti-TCRβ, anti-CD4, anti-CD8, anti-CD69 or anti-CD5, anti-CD24
   2. HY^{cd4} thymus: anti-HY TCR (T3.70), anti-CD4, anti-CD8, anti-CD69 or anti- CD5, anti-CD24
      To obtain better separation of $CD69^{lo}$ and $CD69^{hi}$ populations in thymocytes, it is recommended that a biotinylated anti-CD69 primary antibody be used, followed by a secondary stain containing fluorochrome-conjugated streptavidin. We use the same strategy for CD5 staining.
5. Incubate cells with 200 µl of antibody cocktail in FACS buffer for 30 min on ice in the dark.
6. Concurrent with antibody cocktail staining, stain each compensation control with a single fluorochrome-conjugated antibody. Ideally, compensation staining involves the same antibodies as used in the antibody cocktail. However, staining for each individual antigen may not be feasible for larger experiments when many antigens are being assayed. Therefore, staining for either anti-CD4 or anti-CD8 is recommended due to high expression of these antigens, or the use of compensation beads. Stain in FACS buffer for 30 min on ice in the dark. Leave one compensation control unstained.
7. Wash cells twice with FACS buffer.
8. Resuspend cells in FACS buffer and transfer to FACS tubes.
9. Acquire samples on flow cytometer. Include acquisition of FSC-A and FSC-W to allow for doublet discrimination.

4. Analysis of Flow Cytometry Data - Non-TCR Transgenic Mice

We use FlowJo for flow cytometry data analysis. Refer to **Figure 2A** for the gating strategy for non-TCR transgenic mice.

1. Using FSC-A by SSC, electronically gate on the "lymphocyte" population.
2. Within the "lymphocyte" population, use FSC-A by FSC-W to electronically gate the $FSC\text{-}W^{lo}$ population to exclude cell doublets and aggregrates. This is the "singlet" gate.
3. Using the events in the "singlet" gate, plot CD8 by CD4. Draw gates for DN, DP^{dull}, DP^{bright}, $CD4^+CD8^{lo}$, CD4SP and CD8SP populations as depicted in **Figure 2B.**
4. Under the CD4SP and CD8SP gates, create TCRβ by CD24 plots. Use the quadrant gating tool to draw gates as depicted in **Figure 2C.**
5. Create a new plot from the "singlet" gate: TCRβ by CD69. Draw gates A, B, C, D as depicted in **Figure 2D.**
6. Create another plot from the "singlet" gate: TCRβ by CD5. Draw gates i, ii, iii, iv as depicted in **Figure 2E.** This is another strategy for examining positive selection.
7. Apply DN, DP^{dull}, DP^{bright}, $CD4^{int}$, CD4SP and CD8SP gates from under "singlet" to gates i, ii, iii, iv, A, B, C, D (**Figure 2D, E**).

8. Calculate the number of cells in a particular subset by multiplying organ cellularity by the frequency of each subsequent gate until you reach your target population. For example, the number of TCRβ^{hi}CD69- CD8SP thymocytes would be determined by **thymus cellularity x %TCRbhi CD69- (fraction D) x %CD8SP.**
    1. Counting already takes into account the live and dead cells so do not include the "lymphocyte" gate in your calculations.

5. Analysis of Flow Cytometry Data - TCR Transgenic Mice

Refer to **Figure 3A** for the gating strategy for TCR transgenic mice.

1. Follow steps 4.1 and 4.2 as in the previous section.
2. Under the "singlet" gate, create a T3.70 by SSC plot and gate on the T3.70^{+} cell population to analyze antigen-specific T cells (**Figure 3B**). In some circumstances, it may be of interest to compare this population to the non-antigen specific (T3.70-) T cell population.
3. Within the T3.70^{+} population, draw DN, DP, CD4SP and CD8SP gates (**Figure 3C**).
    1. For WT control samples, draw these gates under the “singlet” gate or create a T3.70- gate as there will be very few T3.70^{+} cells.
4. Under the CD8SP gate, create a T3.70 by CD24 plot. Use the quadrant gating tool to divide cells into four populations (**Figure 3F**).
5. Create an overlaid histogram depicting CD69 expression in the DP compartment of WT mice and T3.70^{+} DP compartment of HY^{cd4} mice (**Figure 4A**). Repeat to examine CD5 expression (**Figure 4B**).
6. Calculate the absolute number of cells in each subset of interest as in 4.8.

Representative Results

In physiological TCR transgenic models and WT mice, positive selection begins at the DP^{bright} stage before moving into the DP^{dull} stage after antigen encounter. DP^{dull} thymocytes then enter a transitional CD4^{+}CD8^{lo} stage before becoming CD4SP or CD8SP thymocytes (**Figure 2B**). Mature SP thymocytes are characterized by high TCR expression and loss of CD24 (**Figure 2C**). While the CD8 by CD4 profile can reveal defects in positive selection, examining TCRβ by CD69 or CD5 can provide further insight into where the defect lies. Both CD69 and CD5 are upregulated after TCR stimulation, with stronger interactions driving higher expression of these markers.

On the TCRβ by CD69 plot, gate A (TCRβ^{lo}CD69-) represents a population of pre-selection DP thymocytes, gate B (TCRβ^{int}CD69^{+}) represents a transitional population directly after TCR engagement, gate C (TCRβ^{hi}CD69^{+}) represents the population of cells directly post-positive selection, and gate D (TCRβ^{hi}CD69^{-}) represents a more mature population of cells (**Figure 2D)**. In a WT mouse, gate A consists of DP^{bright} cells, while gate B primarily consists of DP^{bright} with some DP^{dull} and CD4^{+}CD8^{lo} cells. Gate C primarily consists of DP^{dull}, CD4^{+}CD8^{lo} and CD4SP cells, whereas gate D primarily consists of CD4SP and CD8SP. Absence of populations B and C may be indicative of impaired positive selection. Changes in the ratio of CD4SP to CD8SP within population D may suggest alterations in lineage commitment. The loss of populations C and D may reflect survival issues following positive selection.

Examining TCRβ by CD5 is another strategy to identify pre- and post-positive selection populations. Population i (TCRβ^{lo}CD5^{lo}) represents pre-selection DP thymocytes and population ii (TCRβ^{lo}CD5^{int}) are cells initiating positive selection (**Figure 2E**). These populations consist primarily of DPbright thymocytes. Population iii (TCRβ^{int}CD5^{hi}) represents thymocytes in the process of undergoing positive selection and consist primarily of DP^{dull} and CD4^{+}CD8^{lo} thymocytes. Population iv (TCRβ^{hi}CD5^{hi}) consists primarily of post-positive selection SP thymocytes. Impaired generation of populations ii and iii suggests defective positive selection. Changes in the ratio of CD4SP to CD8SP within population iv despite normal preceding populations may suggest alterations in lineage commitment. An absence of population iv may indicate decreased survival following positive selection. For example, in TOX$^{-/-}$ mice, defective CD4SP differentiation leads to a drastic reduction in populations iv, C and D while positive selection remains intact[12]. True defects in positive selection can be seen with *Bcl11b* inactivation during the DP stage, which leads to loss of populations B, C and D[13,14]. RasGRP1-deficient mice have an earlier defect in positive selection, resulting in the presence of only population A (unpublished data).

As negative selection involves deletion of small antigen-specific populations, defects in this process are best observed using TCR transgenic mice. In HY^{cd4} mice, thymocytes expressing the transgenic HY TCR can be detected with the monoclonal antibody T3.70 (**Figure 3B**). The HY TCR recognizes the male-specific HY antigen presented within MHC class I D^{b}. Thus, HY^{cd4} male (M) mice undergo negative selection of HY TCR^{+} thymocytes, as indicated by a reduction in T3.70^{+} D^{P} thymocyte numbers (**Figure 3D**) and a more dramatic decrease in T3.70^{+} CD8SP thymocyte numbers (**Figure 3C, E**). In contrast, HY^{cd4} female (F) mice undergo positive selection to generate T3.70^{+} CD8SP T cells. By its strictest definition, negative selection prevents the generation of mature self-reactive thymocytes. Thus, while a reduction in D^{P} thymocyte numbers is indicative of negative selection, the absence of antigen-specific SP thymocytes is the most accurate measure. Further examination of the few T3.70^{+} CD8SP thymocytes in HY^{cd4} M mice reveals that most are immature (CD24^{hi}), while most T3.70^{+} CD8SP thymocytes in the HY^{cd4} F have reached maturity (CD24^{lo}) (**Figure 3F**). This provides further support that negative selection occurs in HY^{cd4} M mice.

One caveat of TCR transgenic models is that the transgenic TCR is expressed highly throughout thymocyte development. As a result, it is difficult to further characterize positive selection by identifying populations based on TCR and CD69/CD5 expression. However, CD69 and CD5 expression do correlate with the strength of TCR signalling. In WT mice, most DP thymocytes do not undergo positive or negative selection, which requires TCR engagement of pMHC, and thus do not upregulate CD69 or CD5. HY^{cd4} F mice have a large population of T3.70^{+} DP thymocytes that undergo positive selection, as indicated by an increase in the CD69^{+} population compared to WT (**Figure 4A**). Negative selection involves a higher affinity TCR stimulus than positive selection; this is indicated by higher expression of CD69 on T3.70^{+} DP thymocytes in HY^{cd4} M mice, resulting in a shift of the histogram peak to the right. Similar trends are seen with CD5 expression (**Figure 4B**). When analyzing thymic selection in genetically manipulated mice, examining CD69 or CD5 expression can determine whether the defect lies with TCR signalling or a downstream outcome of TCR stimulation.

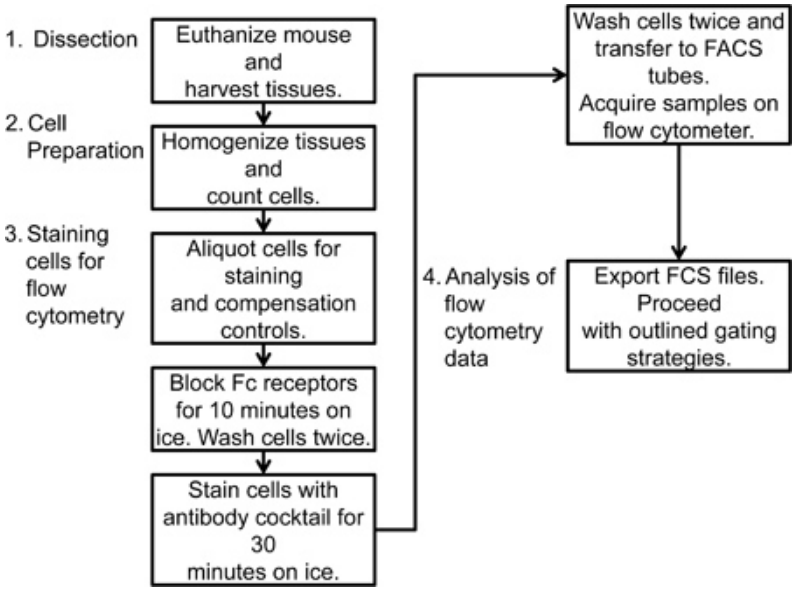

Figure 1. Overall scheme of the experimental protocol.

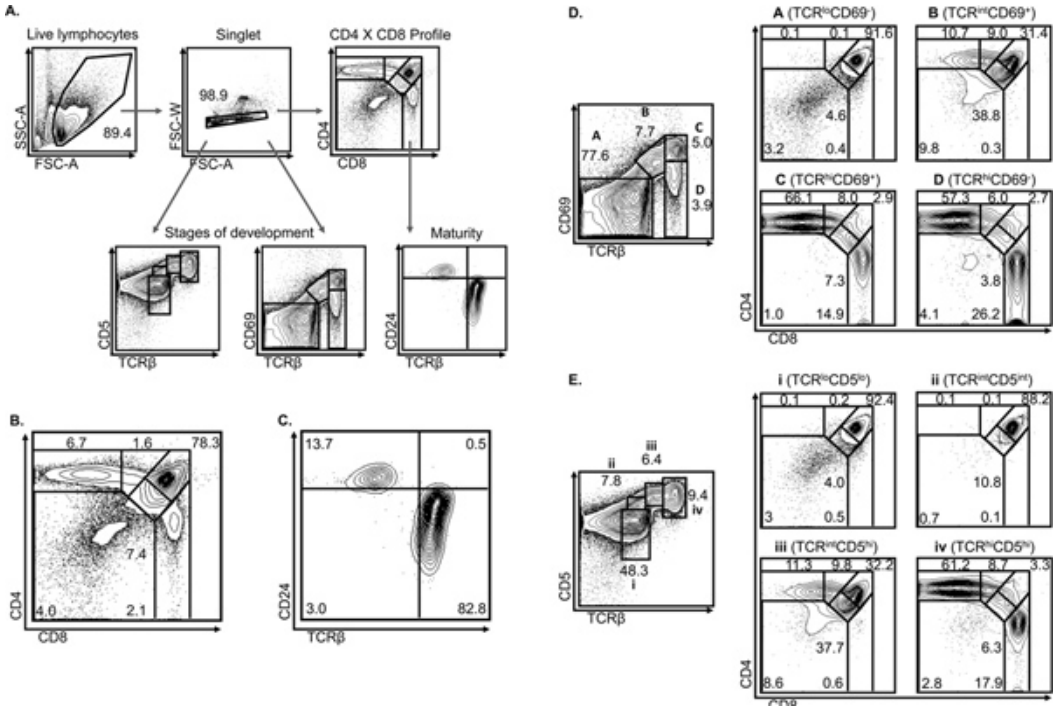

Figure 2. Analyzing thymic selection in non-TCR transgenic mice. (A) Gating strategy for analyzing thymic selection in non-TCR transgenic mice. (B) CD8 by CD4 profile of a WT thymus. (C) Examining the maturity of SP thymocytes by TCRβ and CD24 expression. (D) Stages of positive selection can be distinguished by CD69 by TCRβ expression, along with CD8 and CD4 profiling in each subset. (E) TCRβ by CD5 is another strategy that can be used to separate developmental stages. Click here to view larger figure.

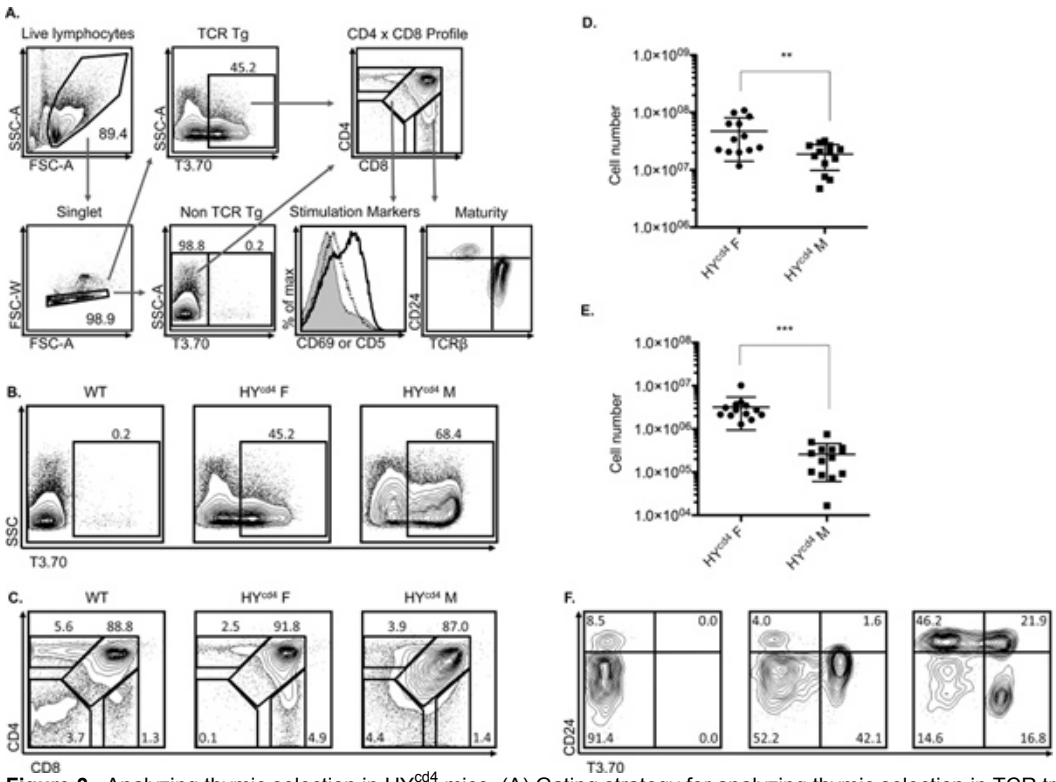

Figure 3. Analyzing thymic selection in HY^{cd4} mice. (A) Gating strategy for analyzing thymic selection in TCR transgenic mice. (B) The frequency of thymocytes expressing the HY TCR (T3.70^{+}) in WT, HY^{cd4} F and HY^{cd4} M mice. (C) CD8 by CD4 profiles of the T3.70^{+} population in indicated mice. In addition to frequencies, the absolute number of T3.70^{+} DP (D) and especially T3.70^{+} CD8SP (E) thymocytes are important indicators of negative selection. (F) Examining the maturity of CD8SP thymocytes in indicated mice. Click here to view larger figure.

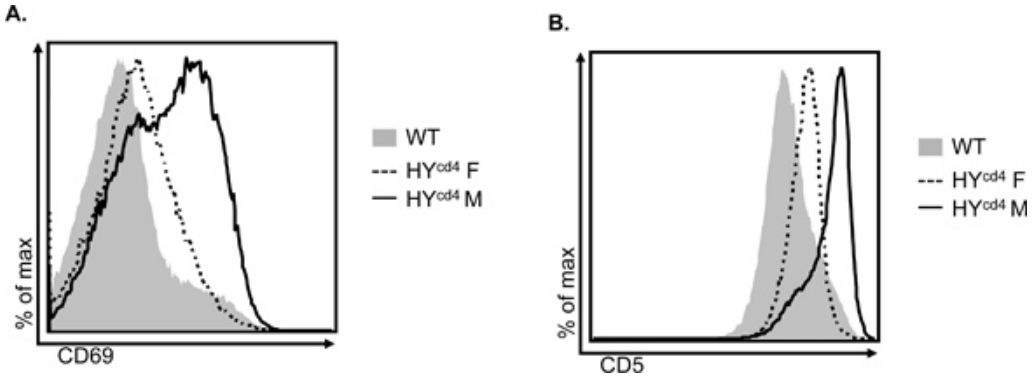

Figure 4. Expression of activation markers CD69 (A) and CD5 (B) on total DP thymocytes from WT and T3.70^{+} DP thymocytes from HY^{cd4} F and HY^{cd4} M mice.

Discussion

The protocol presented here can be used to examine positive and negative selection in non-TCR transgenic and TCR transgenic mice. This protocol describes the staining of surface antigens. For further analysis of molecular mechanisms, it is often necessary to perform intracellular staining. We use the BD Biosciences Cytofix/Cytoperm Kit for most intracellular proteins and the BD Biosciences Foxp3 Staining Kit for transcription factors. We usually acquire our samples immediately after staining. However, samples can be stored overnight in FACS Buffer with 1% formaldehyde at 4 °C in the dark. Be aware that some fluorochromes and antibodies may not be compatible with fixation.

For analysis of positive selection, we recommend using biotinylated anti-CD69 or anti-CD5 to provide better separation of negative, intermediate, and highly expressing populations (**Figure 2**). As described in the Representative Results, this gating strategy will help distinguish defects in positive selection from lineage commitment, as the two may require different follow up. In addition to generation of mature SP thymocytes, positive selection can be confirmed by upregulation of IL-7Rα and CCR7. However, these markers are expressed at low level on post-selection DP thymocytes compared to SP thymocytes[15,16]. For detailed analysis of maturation stages within the DP compartment, intracellular Zap70 expression can be measured[17]. This may be particularly useful in demonstrating that populations with similar expression of maturation markers, such as i and ii on the TCRβ x CD5 plot (**Figure 2E**), represent different stages of development.

The HY^{cd4} model has the advantages of representing both positive and negative selection, as well as physiological expression of the transgenic TCR. However, these methods can be applied to other TCR transgenic models with certain considerations. Always include an antibody against the transgenic TCR of choice to enable study of antigen-specific T cells. For some analyses, it may be of interest to compare the antigen-specific population to the non-specific population as an internal control. Studying negative selection in other TCR transgenic models requires further considerations. The stage of thymocyte development during which negative selection takes place varies among the different TCR transgenic models. HY^{cd4} thymocytes engage pMHC at the DP stage, thus analysis of the HY-specific CD8SP progeny population is the correct readout for negative selection[10,18] (**Figure 3**). If negative selection occurs during the DN to DP transition as in classical HY mice[19], the analysis of interest would be the presence or absence of antigen-specific DP thymocytes. Selection against the ubiquitous HY antigen occurs in the thymic cortex[20]. In contrast, negative selection against tissue-restricted antigens occurs in the thymic medulla[21,22]. OT-I RIP-mOva mice recapitulate this type of selection as the Ova antigen is expressed under the control of the rat insulin promoter, which is active only in the thymic medulla and pancreas[23]. Since positive selection of DP thymocytes in the thymic cortex is first required for migration into the medulla, the absence of mature ($CD24^{lo}$) OT-I TCR^{+} CD8SP thymocytes indicates successful negative selection in OT-I Rip-mOva mice[24].

While using TCR transgenic mice has the advantage of being able to study a large population of antigen-specific cells, a potential caveat is altered T cell development due to limited ligands that can mediate positive and negative selection of the transgenic TCR. In RAG-sufficient HY^{cd4} mice, another caveat is that co-expression of endogenous TCRα chains has the potential to modulate positive and negative selection on a minority of HY TCR^{+} thymocytes. This caveat is not unique to the HY^{cd4} model, but rather TCR transgenic models in general. The representative HY^{cd4} presented are derived from HY^{cd4} mice on a Rag-sufficient background. Although somewhat more complex in nature, there are many ways to study thymocyte selection in a more physiological setting of limited precursor frequency. For example, TCR transgenic and WT mixed bone marrow chimeras can be generated to reduce the antigen-specific thymocyte precursor frequency. Additionally, one can use mice that express only a transgenic TCRβ chain, such as the Vb8 chain of the HY TCR[25] or the Vb5 chain of the OT-I TCR[26] to limit precursor frequency. Because the transgenic TCRβ chain is paired with various endogenous TCRα chains, tracking of antigen-specific thymocytes requires the use of pMHC tetramers. A limitation of this method is that TCR expression is too low on DP thymocytes to allow detection with tetramers. This limits further study of the molecular mechanisms in DP thymocytes that lead to positive and negative selection. More recently, a combination of pMHC tetramers and magnetic bead enrichment has been used to enumerate small, antigen-specific populations of $CD4^{+}$ T cells in unmanipulated mice[27].

Negative selection is primarily mediated by clonal deletion of self-reactive thymocytes via the intrinsic apoptosis pathway. Apoptotic thymocytes can be detected by flow cytometry using intracellular staining for cleaved caspase-3. The BH3-only Bcl-2 family member Bim is essential for caspase-3 activation in thymocytes in response to TCR stimulation[18,28]. By crossing Bim-deficient mice to different TCR transgenic models, our lab has found that Bim is required for negative selection against ubiquitous self-antigens but not tissue-restricted antigens, indicating the existence of multiple negative selection mechanisms[18,29]. Similar approaches have implicated the orphan steroid receptor Nur77 as another key mediator of thymic negative selection[30,31]. Transcriptional analysis of DP thymocytes from HY^{cd4} mice has generated a list of genes specifically induced during positive and negative selection[32]. Applying the methods presented here to mice manipulated in these genes may provide insight into the molecular mechanisms of thymic selection.

It is ideal to follow up examination of thymic selection with an analysis of the peripheral immune compartment. However, it is often difficult to separate central and peripheral deletion. Negative selection is ultimately reflected by the absence of autoimmunity. Though they have limitations, TCR transgenic mice are a powerful and practical way to study physiological negative selection. Crossing TCR transgenic mice to mice deficient in particular genes is an effective way to investigate the molecular mechanisms of negative selection. It is important to be aware, however, that peripheral inflammation due to a gene defect can cause non-specific thymocyte deletion mediated by corticosteroids and cytokines[7-9]. In such cases, the strategy presented here can be applied to the analysis of thymi from neonatal mice before the onset of inflammation. Positive selection, on the other hand, can be studied in either TCR- or non-TCR transgenic models, saving investigators the troubles of crossbreeding. A better understanding of the molecules involved in positive and negative selection will lead to novel strategies for the diagnosis and treatment of autoimmune and immunodeficiency disorders.

Disclosures

No conflicts of interest declared.

Acknowledgements

The authors would like to thank Bing Zhang for his technical assistance. This work was funded by the Canadian Institutes for Health Research (MOP-86595). T.A.B is a CIHR New Investigator and AHFMR Scholar. Q.H. is supported by a CIHR Canada Graduate Scholarship - Doctoral and an AIHS Full-time Studentship. S.A.N. is supported by a Queen Elizabeth II Graduate Scholarship. A.Y.W.S. is supported by an NSERC Postgraduate Scholarship - Doctoral.

References

1. Liston, A., Lesage, S., Wilson, J., Peltonen, L., & Goodnow, C.C. Aire regulates negative selection of organ-specific T cells. *Nat. Immunol.* **4**, 350-354 (2003).
2. Liston, A., *et al.* Gene dosage--limiting role of Aire in thymic expression, clonal deletion, and organ-specific autoimmunity. *J. Exp. Med.* **200**, 1015-1026 (2004).
3. Hogquist, K.A., Baldwin, T.A., & Jameson, S.C. Central tolerance: learning self-control in the thymus. *Nat. Rev. Immunol.* **5**, 772-782 (2005).
4. Liston, A., Enders, A., & Siggs, O.M. Unravelling the association of partial T-cell immunodeficiency and immune dysregulation. *Nat. Rev. Immunol.* **8**, 545-558 (2008).
5. Starr, T.K., Jameson, S.C., & Hogquist, K.A. Positive and negative selection of T cells. *Annu. Rev. Immunol.* **21**, 139-176 (2003).

6. McCaughtry, T.M. & Hogquist, K.A. Central tolerance: what have we learned from mice? *Seminars in immunopathology.* **30**, 399-409 (2008).
7. Zhan, Y., *et al.* Without peripheral interference, thymic deletion is mediated in a cohort of double-positive cells without classical activation. *Proceedings of the National Academy of Sciences of the United States of America.* **100**, 1197-1202 (2003).
8. Brewer, J.A., Kanagawa, O., Sleckman, B.P., & Muglia, L.J. Thymocyte apoptosis induced by T cell activation is mediated by glucocorticoids *in vivo*. *J. Immunol.* **169**, 1837-1843 (2002).
9. Martin, S. & Bevan, M.J. Antigen-specific and nonspecific deletion of immature cortical thymocytes caused by antigen injection. *European journal of immunology.* **27**, 2726-2736 (1997).
10. Baldwin, T.A., Sandau, M.M., Jameson, S.C., & Hogquist, K.A. The timing of TCR alpha expression critically influences T cell development and selection. *J. Exp. Med.* **202**, 111-121 (2005).
11. Tung, J.W., *et al.* Modern flow cytometry: a practical approach. *Clinics in laboratory medicine.* **27**, 453-468, v (2007).
12. Aliahmad, P. & Kaye, J. Development of all CD4 T lineages requires nuclear factor TOX. *J. Exp. Med.* **205**, 245-256 (2008).
13. Kastner, P., *et al.* Bcl11b represses a mature T-cell gene expression program in immature CD4(+)CD8(+) thymocytes. *Eur. J. Immunol.* **40**, 2143-2154 (2010).
14. Albu, D.I., *et al.* BCL11B is required for positive selection and survival of double-positive thymocytes. *J. Exp. Med.* **204**, 3003-3015 (2007).
15. Van De Wiele, C.J., *et al.* Thymocytes between the beta-selection and positive selection checkpoints are nonresponsive to IL-7 as assessed by STAT-5 phosphorylation. *J. Immunol.* **172**, 4235-4244 (2004).
16. Ueno, T., *et al.* CCR7 signals are essential for cortex-medulla migration of developing thymocytes. *J. Exp. Med.* **200**, 493-505 (2004).
17. Saini, M., *et al.* Regulation of Zap70 expression during thymocyte development enables temporal separation of CD4 and CD8 repertoire selection at different signaling thresholds. *Science signaling.* **3**, ra23 (2010).
18. Hu, Q., Sader, A., Parkman, J.C., & Baldwin, T.A. Bim-mediated apoptosis is not necessary for thymic negative selection to ubiquitous self-antigens. *J. Immunol.* **183**, 7761-7767 (2009).
19. Kisielow, P., Bluthmann, H., Staerz, U.D., Steinmetz, M., & von Boehmer, H. Tolerance in T-cell-receptor transgenic mice involves deletion of nonmature CD4+8+ thymocytes. *Nature.* **333**, 742-746 (1988).
20. McCaughtry, T.M., Baldwin, T.A., Wilken, M.S., & Hogquist, K.A. Clonal deletion of thymocytes can occur in the cortex with no involvement of the medulla. *J. Exp. Med.* **205**, 2575-2584 (2008).
21. Derbinski, J., Schulte, A., Kyewski, B., & Klein, L. Promiscuous gene expression in medullary thymic epithelial cells mirrors the peripheral self. *Nat. Immunol.* **2**, 1032-1039 (2001).
22. Anderson, M.S., *et al.* Projection of an immunological self shadow within the thymus by the aire protein. *Science.* **298**, 1395-1401 (2002).
23. Kurts, C., *et al.* Constitutive class I-restricted exogenous presentation of self antigens *in vivo*. *J. Exp. Med.* **184**, 923-930 (1996).
24. Nitta, T., Nitta, S., Lei, Y., Lipp, M., & Takahama, Y. CCR7-mediated migration of developing thymocytes to the medulla is essential for negative selection to tissue-restricted antigens. *Proceedings of the National Academy of Sciences of the United States of America.* **106**, 17129-17133 (2009).
25. Bouneaud, C., Kourilsky, P., & Bousso, P. Impact of negative selection on the T cell repertoire reactive to a self-peptide: a large fraction of T cell clones escapes clonal deletion. *Immunity.* **13**, 829-840 (2000).
26. Gallegos, A.M. & Bevan, M.J. Central tolerance to tissue-specific antigens mediated by direct and indirect antigen presentation. *J. Exp. Med.* **200**, 1039-1049 (2004).
27. Moon, J.J., *et al.* Naive CD4(+) T cell frequency varies for different epitopes and predicts repertoire diversity and response magnitude. *Immunity.* **27**, 203-213 (2007).
28. Bouillet, P., *et al.* BH3-only Bcl-2 family member Bim is required for apoptosis of autoreactive thymocytes. *Nature.* **415**, 922-926 (2002).
29. Suen, A.Y. & Baldwin, T.A. Proapoptotic protein Bim is differentially required during thymic clonal deletion to ubiquitous versus tissue-restricted antigens. *Proceedings of the National Academy of Sciences of the United States of America.* (2012).
30. Calnan, B.J., Szychowski, S., Chan, F.K., Cado, D., & Winoto, A. A role for the orphan steroid receptor Nur77 in apoptosis accompanying antigen-induced negative selection. *Immunity.* **3**, 273-282 (1995).
31. Zhou, T., *et al.* Inhibition of Nur77/Nurr1 leads to inefficient clonal deletion of self-reactive T cells. *J. Exp. Med.* **183**, 1879-1892 (1996).
32. Baldwin, T.A. & Hogquist, K.A. Transcriptional analysis of clonal deletion *in vivo*. *J. Immunol.* **179**, 837-844 (2007).

