Microbiology Honors Senior Thesis

# Impact of B cell receptor diversity on thymic output

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## A B S T R A C T

The development of T cells and B cells are believed to be independent. However, it has been shown that B cells and/or immunoglobulin promote T cell diversity. How contractions of the B cell repertoire may influence T cell development is not known. A number of mechanisms have been suggested. They include: changes in thymocyte selection, a decrease or increase in survival and/or proliferation of T cells, and changes in thymic output. To determine how B cells affect thymic output, we enumerated recent thymic emigrants (RTE) in age-matched wild-type mice and with age matched mice possessing dramatically contracted BCR repertoires (HOM-QM). To this end, we isolated T cell receptor excision circles (TRECs) by a real-time PCR assay from DNA isolated by a phenol chloroform method from spleen or from thymus. We found decreased TRECs in HOM-QM mice over time suggesting that BCR repertoire contraction accelerates the decrease in thymic out put with age. These results suggest that BCR repertoire contraction accelerates thymic involution. Our results also show elevated number of TRECs in mice with contracted BCR repertoires compared to wild-type mice of the same age. This result suggests that decreases in BCR diversity cause thymus adaptive responses which may include an increase in recombination, decreased cell death, or decreased proliferation of recently formed clones. Further experiments using methods such as the incorporation of bromodeoxyuridine (BrdU) as a means of measuring RTEs may elucidate the mechanism by which a contraction in the BCR repertoire impacts T cell development.

# BACKGROUND AND INTRODUCTION

A great deal is known about the structure, development, diversity, and function of B and T cells, yet much remains to be studied about the interaction between these two sets of lymphocytes in development as well as in their function. The goal of this project is to understand whether and how B cells might influence T cell development and diversity. It has been thought that T cell development and function is largely independent of B cells.[1] However, B and T cell interactions have long been recognized for the generation of adaptive immune responses. T cells stimulate B cells to produce antigen specific antibodies.[2-4] These responses are called "T cell-dependent B cell responses." [5-7] Conversely, B cells can present antigen to T cells and help regulate T cell responses.[8] Despite these functional interactions, B cells and T cells are usually thought to develop independently and to occupy distinct anatomic and physiologic niches.

The individual compartmentalization of T cells and B cells has been supported by some experimental evidence. It has been shown that T cells develop in B cell deficient mice.[9] Individuals with X-linked agammaglobulinaemia (a type of B cell deficiency in humans) have T cells,[10] reject allografts and exhibit T cell dependent arthritis.[11,12] In 1995, Epstein et al.[13] reported that B cell deficient mice (μMT) lacking the IgM transmembrane domain encoding exon, rejected skin allografts with similar kinetics as B cell sufficient mice. These findings suggested that B cells are not required for mounting cellular immune responses. A few years later it was shown that μMT mice possessed IgA positive B-cells and therefore were not completely B-cell deficient. [14] This finding motivated new studies exploring the relationship of B cells during T cell development.

B cell deficient mice exhibit defective cellular immune responses to a variety of pathogens including: *Salmonella*,[15] *Francisella tularensis,* [16] *Plasmodium* infection in mice,[17] *Chlamydia trachomatis,*[18] *Leishmania major*,[19] corona virus,[20] and lymphocytic chroiomeningitis virus.[21] Since B cells

promote normal lymphoid organ generation, [22,23] it remains unclear whether deficiencies in T cell responses are due to the absence of B cells or rather an effect of abnormal lymphoid organs. Nonetheless, effective cellular immune responses against the previously mentioned pathogens depend on B cells. The discovery of impaired delayed-type hypersensitivity responses in patients with X-linked agammaglobulinaemia[11] suggested that B cells and/or immunoglobulin promote T cell responses.

Over the past two decades, a number of studies have suggested a role for B cells in the development of T cells in the thymus. B cells are present in the thymus, mostly in the cortical/medullary junction, and compose about 1% of total thymocytes. [24-26] B cells resident in the thymus can be differentiated from those cells that reside in the spleen because they express less MHC class II and are CD5+, IgM+, B220, and Mac-1+.[27] These thymic B cells may be required for the negative selection of immature thymocytes.[28-30] Fukuba et al.[28] found that thymic B cells contributed to newborn tolerance to superantigens and Andreu-Sanchez et al.[26] found that thymic B cells were the first B cells to produce IgG following birth, thus discovering the functions of thymic B cells in adaptive immunity.

To determine whether B cells and or immunoglobulin contribute to the development of T cells, studies conducted in this laboratory used mouse strains with varying degrees of B cell deficiency and/or immunoglobulin repertoire contraction. In mice with monoclonal T cells and B cells, Keshavarzi et al.[31] showed that T cell development in the thymus depended on B cells because in the absence of B cells, T cells underwent negative selection. The authors proposed that peptides derived from the monoclonal immunoglobulin competed with the endogenous self-peptides thus rescuing the transgenic T cells from negative selection.[31,32] Mice with contracted B cell repertoires had fewer CD4+ cells in the periphery.[33] These findings supported the concept of B cell dependent T cell development.

During early development, T cell progenitors in the thymus assemble their T cell receptor genes by random recombination of gene segments. Following gene rearrangement (V(D)J recombination), T cells undergo positive and negative selection with the assistance of thymic epithelial cells along with myeloid-derived antigen presenting cells (APCs). Only T cells that bind self-MHC and self peptide with intermediate strength will survive. The number of distinct T cells bearing distinct T cell receptors (the T cell repertoire) that mature in the thymus is the result of V(D)J recombination coupled to selection. Upon successful maturation, T cells exit the thymus and reside within the periphery. [34] Therefore, T cell diversity is established in the thymus. Whereas peripheral diversity reflects the efflux of T cells produced by the thymus and the rates of proliferation and death of peripheral T cells.

To determine whether B cells and or immunoglobulin contribute to T cell diversity, Joao et al.[32] measured T cell receptor diversity in B cell deficient mice or in mice with contracted B cell repertoires. It was shown that mice lacking B cells had increased T cell death, but higher levels of thymocyte proliferation.[32] Quasi-monoclonal (QM) mice, characterized by a dramatically reduced B cell diversity due to the replacement of the JH loci with an already pre-rearranged variable region, [35] had markedly contracted T cell repertoires.[32] This finding demonstrated that diverse B cell repertoires are essential for the development of diverse T cell repertoires. Joao et al.[32] proposed that B cells along with immunoglobulins may contribute to T cell repertoire diversity via the presentation of specific peptides to developing T cells. In agreement with this idea, inhibition of a particular immunoglobulin produced a distorted T cell selection for the specific immunoglobulin peptide.[36] Therefore, it appears that in establishing a diverse T cell repertoire, B cells are significant contributors.

How the thymus compensates for decreased selection of diverse T cells in mice with contracted T cell repertoires is not known. The overall goal of this work was to determine how thymic output varies as a function of B cell repertoire contractions, since a reduction in BCR diversity leads to a reduction in

TCR diversity. To determine thymic output, we measured T cell excision circles (TRECs), which are by products of TCR gene rearrangement and thought to persist in recent thymic emigrants.

In 1998, using chickens as a model organism, Kong et al.[37] demonstrated there were byproducts of TCR gene rearrangement (i.e. TRECs) that existed for a prolonged period of time in mature T cells. TRECs do not replicate and undergo dilution upon clonal proliferation of T cells.[38] Therefore, these episomal DNA segments indirectly measure thymic output. Before the discovery of TRECs, thymic output was difficult or impossible to properly evaluate. Douek et al.[39] measured human signal joint T cell receptor excision circles (sjTRECs) by real-time PCR (Figure 1 Geenen et al. 2003),[40] and used this information to estimate thymic output. This assay is now used in clinical applications for diagnosis of immunodeficiencies such as Severe Combined Immunodeficiency (SCID)[41] or experimental models for studying effect on the immune system including age[42] or autoimmunity such as Graft-versus-Host Disease.[43]

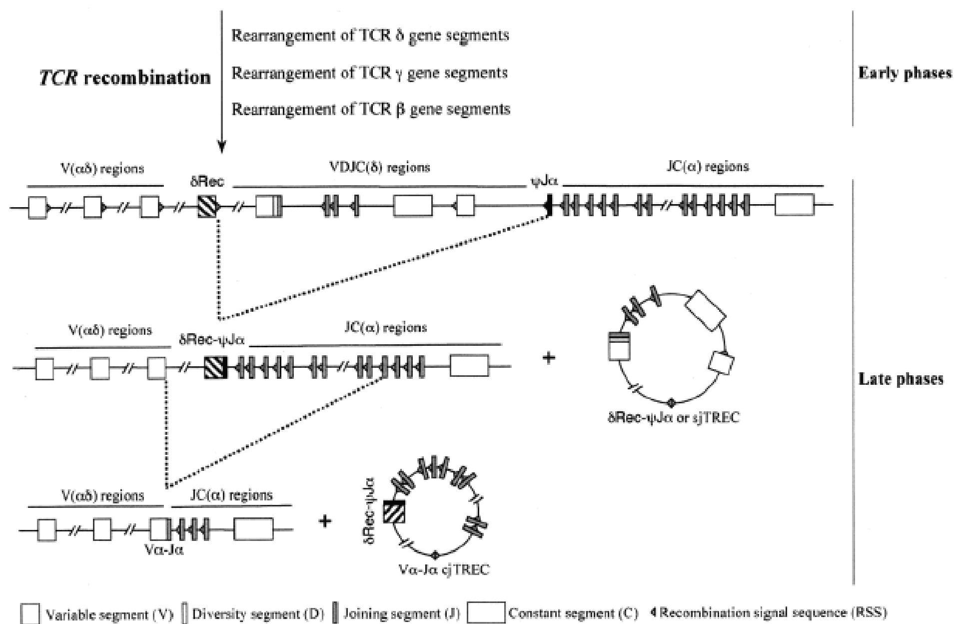

Figure 1. **From Geenen et al.[40] shows *TCRD* deletion (recombination involving δRec-ψJα gene segments) followed by subsequent *TCRA* rearrangement.** This rearrangement happens in approximately 70% of alpha/beta T cells.[44] The signal-joint T cell receptor excision circle (sjTREC) is the first to be produced due to δRec-ψJα rearrangement and is used in our assay as the marker for thymic output. The coding-joint TREC containing the δRec-ψJα gene segments is produced following *TCRAV* and *TRCAJ* recombination.

Since T cells recognize target antigen via their T cell receptors, successful gene rearrangement is imperative for a normal T cell repertoire constitution and the generation of TRECs. The majority of peripheral T cells have TCRα and TCRβ chains (~90-95%) whereas the other subset of T cells (γδ T cells) express TCRγ and TCRδ chains.[45] The diverse and specific antigen binding domains of T cells are encoded by different gene segments that undergo recombination generating the combinational diversity of TCR. TCRβ and TCRδ segments include variable (V), diversity (D) and joining (J) sequences; whereas TCRα and TCRγ contain only variable (V) and joining (J) gene segments. Recombination involves the joining of different V(D)J segments which are flanked at the ends by recombination signal sequences (RSS) – DNA segments composed of a conserved heptamer sequence next to a nonamer sequence and separated by a spacer of either 12 or 23 base pairs in length (signifying either 1 or 2 twists in the DNA double helix).[46] RSS with different spacer lengths successfully join and recombine (the 12/23 rule).[47] During recombination D-J rearrangement occurs first followed by V-DJ rearrangement. Conserved heptamer (CACAGTG) and nonamer (ACAAAAACC) sequences are recognized by proteins called recombination activation gene 1 and 2 (RAG 1, RAG2), which cut the DNA ultimately resulting in a hairpin loop and an excised portion of the signal ends, both of which are ligated.[48] The signal joints remain stable within the cell as excised episomal segments of DNA (TRECs). The average TREC content can be reported in TREC numbers per mg of tissue or more clinically applicable – number of TRECS per cell or TREC per ml (using peripheral blood samples). TREC per ml allows a better and more accurate clinical measurement following stem cell transplantation or other significant cellular proliferation

events,[49] however for our purposes TREC per cell was used in order to compare different thymic outputs across varying strains of mice.

Generation of sjTREC occurs during *TCRA* gene rearrangement encompassing the deletion of *TCRD* by cutting out the δRec and ψJα gene segments (Figure 1 Geenen et al. 2003),[40] which flank most of the *TCRD* gene segments. [50] Each newly differentiated αβ T cell can have up to two sjTRECs if rearrangement took place in both alleles and given the T cell did not undergo clonal expansion. Following the deletion of the TCRD gene, the thymocyte permanently commits to the TCRαβ subset. A coding-joint TREC (cjTREC) is produced after α rearrangement; however, these coding joints contain δRec-ψJα and may still reside on the dysfunctional *TCRAD* allele. The *TCRA* locus also contains many V and J gene segments accounting for a large number of possible combinations. TCR rearrangements including *TCRD*, *TCRG*, and *TCRB* also occur, yet these rearrangements happen much earlier than *TCRD* deletion and *TCRA* recombination. *TCRD*, *TCRG*, and *TCRB* excision circles are not ideal targets because thymocytes proliferate during the early double positive stage of T cell maturation, diluting these TRECs. [51,52] Thus, sjTRECs are better molecular targets for measuring thymocyte maturation.[39] The sjTRECs are even clearly visible on Southern blots of both thymocytes and peripheral blood cells,[53] providing evidence of their abundance in the periphery and thymus.

Although there are a number of ways to detect TRECs,[39,54-56] real-time PCR (RT-PCR) provides the most accurate, highly sensitive, and rapid method of quantification of a particular amplified TREC sequence following each PCR cycle. Sempowski et al.[42] created a real-time PCR assay utilizing murine sjTRECs (mTRECs) which have the δRec and ψJα gene segments conserved. Mice have three δRec regions (called δRec1, δRec2, δRec3).[57] However, δRec1 rearranges with ψJα with the highest frequency[58,59] and therefore this particular target was used for the murine TREC assay. There may be an underestimate of recent thymic emigrants when using this assay due to the calculation of only one of three TCRA gene

rearrangements,[44] however utilizing this specific murine sjTREC allows for the best approximation of total thymic output.

It is important to note that TRECs in the periphery are not a direct measurement of recent thymic emigrants (RTEs). Rather real-time PCR quantification of TRECs provides a calculation of the reciprocal function of homeostatic T cell proliferation. When analyzing TREC data it is important to take account peripheral T cell proliferation and T cell death, which are factors that can skew total TREC numbers.[51] Nonetheless, a real-time PCR TREC assay provides the best currently available method to estimate thymic output.

For our real-time PCR assay we reported TRECs per cell number. This was accomplished by utilizing a housekeeping gene with a particular sequence present twice in each mouse (one housekeeping gene sequence copy per chromosome). Therefore, for each RT-PCR TREC experiment conducted, the same samples were run with a probe for the specific housekeeping gene, which provided an accurate cell count of a particular sample allowing for the calculation of TRECs per cell number**.**

TREC content decreases with age in both humans[39,60-64] and mice.[42] Thymic output does play a role in this reduction, however thymic output alone may not completely explain such a dramatic decrease in TRECs with age. T cell proliferation, compensating for reductions in thymic output may dilute TREC numbers.[54,65] Therefore, whenever using TREC assays as a means of measuring thymic output, it is important to control for age during experimental calculations.

Although mice possess a number of differences in their immunologic makeup and function from humans,[66] murine models remain heavily relied on as *in vivo* immunologic systems that can be easily manipulated. Particularly effective is the use of reverse genetics or the direct and known manipulation of particular genes in order to study the effect on function.

To probe the impact of contraction of immunoglobulin repertoire in thymic output, we used genetically engineered mice – HOM-QM, RAG, and C57Bl/6. The HOM-QM mice are based on QM mice, which were created by gene targeted replacement of JH elements with an already successfully rearranged IgH variable region encoded by VH17.2.25 ($V_{H17.2.25}$ $D_{sp2.3}$ $J_{H4}$) from a 4-hydroxy-3-nitrophenylacetate (NP) specific hybridoma.[67] This new allele, termed the QM heavy chain allele, was modified on both alleles in the HOM-QM mouse. Utilizing an anti-idiotypic (id) antibody for the specific NP antibodies, the QM antibody expression of peripheral B cells can be identified.[68] More than 98% of B cells in HOM-QM mice were idiotype-positive (Unpublished Results), therefore HOM-QM mice possess a very contracted B cell repertoire and Ig compartment. This can be explained by the modification of both heavy chain alleles, which decreases the chances for selecting diverse Ig that may arise as a result of VH gene replacement.[68] Consistent with the involvement of B cells in the diversification of thymocytes, HOM-QM mice possess a contracted TCR. Unlike B cell deficient mice, HOM-QM mice possess normal lymphoid tissue, which makes them ideal models to study the effects of contracted TCR and BCR. The RAG mice used in the experiment were deficient in both T and B cells,[69,70] due to a mutation in the necessary recombination activation gene (RAG-1 ). These mice were chosen to serve as a negative control since they could not generate TRECs due to unsuccessful V(D)J recombination. Finally C57BL/6J mice were used as the wild type strain of mice (positive control) to compare TREC level with the HOM-QM mice.

We previously demonstrated that B cell deficiencies lead to contractions in T cell repertoire.[32] Why this occurred and to what extent B cell diversity could influence T cell development were the central topics explored in this current project. Since the positive selection of T cells depends on TCR recognition of diverse self-peptides presented in context of self-MHC,[71] a possible link between antibodies produced by diverse B cells could influence T cell repertoire development. To study to which

degree B cells impact T cell thymic output and how a greatly reduced T cell repertoire impacts thymic output , we compared the frequency and rate of production of recent thymic emigrants in HOM-QM mice, C57BL/6J mice (positive control), and RAG mice (negative control). Since murine thymic function is inversely correlated with age[39], we determined thymic output at intervals of 5, 12, and 20 weeks of age. Five mice of each type were analyzed to account for variation. Because we feared that commercial DNA isolation kits could compromise the isolation of small pieces of DNA as sjTREC; we developed a novel procedure derived from older techniques of small viral DNA isolation using phenol-chloroform-isoamyl.[72] A real time PCR assay using a custom FAM probe and a plasmid specific for murine T cell receptor exicision circles (developed by Sempowski , Duke University 2001) was used to quantify recent thymic emigrants. Based on the findings of these experiments we should be able to conclude whether diverse B cells can promote or delay thymic output and to what extent thymic output serves as a mechanism in maintaining and promoting a diverse T cell repertoire.

## MATERIALS AND METHODS

*Murine sample preparation:*

Female C57BL/6J mice (ages 5, 12, and 20 weeks) and B6.129S7-Rag1tm1Mom/J were obtained from The Jackson Laboratory (Bar Harbor, ME). HOM-QM mice were specifically bred in the laboratory. All mice were housed in the specific pathogen free facility located in the Biological Science Research Building (BSRB) at the University of Michigan. All procedures and handling of mice were done in accordance to UCUCA set guidelines and regulations for animal care and use.

After euthanasia with $CO_2$ gas, the thymus and spleen of mice were removed and stored on ice in separate tubes containing DPBS solution (HyClone Laboratories, South Logan, UT). Tissues were mashed through a 70µm Falcon cell strainer screen (Becton Dikinson, Franklin Lakes, NJ) using a $3cm^3$ syringe plunger (Becton Dickinson). Samples were centrifuged for 5min at 1200RPM and the eluent discarded. Following centrifugation, 1ml of ACK Lysing Buffer (Lonza, Walkersville, MD) was added to each sample pellet for 5 minutes to allow red blood cell lysis. Following lysis, the cells were resuspended in DPBS solution and 10µl of each sample was added to 90µl of tryptophan blue for cell count using a hemocytometer (Hausser Scientific, Horsham, PA). One million cells of each sample were set aside for Flow Cytometry analysis while the rest of the sample was used for DNA isolation.

*Flow* cytometry for cell sorting and phenotypic analysis

Suspensions of cells from spleen and thymus containing around one million cells were stained with three different antibodies from BD Pharmingen. Antibodies used included: FITC-conjugated anti-CD3 (mAb 17A2), PE-conjugated anti-CD4 (mAb H129.19), and APC-conjugated CD8 (mAb 53-6.7). FACS analysis was performed using the FACSCanto II(Becton Dickinson Biosciences). Sample cells were analyzed for CD3, CD4, and CD8 presentation between the different strains of mice – HOM-QM, C57BL/6J, and RAG.

*TREC DNA Isolation Procedure*

Cells set aside after tissue extraction and cell count were suspended in DPBS solution to produce a homogenous solution of $10^9$ cells/ml. An alkaline sodium dodecyl sulfate (SDS) buffer (containing 50mM NaCl, 2mM EDTA, 1% solution sodium dodecyl sulfate and at pH 12.4) was added to each sample (1ml per $10^7$ cells) in order to lyse the cells. Following cell lysis, 10-15ml of each sample was poured into a tube (if needed the sample was pipetted or cut with scissors to separate). The samples were vortexed for 2 minutes to shear the DNA (formed liquid film on walls). Next, samples were incubated at 30$^o$C for 30min for alkali-induced DNA strand separation. After incubation 0.05 volume of 1M Tris-hydrochloride (pH 7.1) was added (while swirling) to obtain a final pH of 8.5 to 9.0. Once the pH was stabilized, 0.2 volume of 3M NaCl and 0.02 volume of proteinase K solution were added to each sample. These mixtures were then incubated at 37$^o$C for 30min allowing partial degradation of cellular proteins. The lysate was then chilled to 20$^o$C and an equal volume of phenol-chloroform-isoamyl was added and mixed by inverting. Up to 12mls of sample was added to Phase Lock Gel (PLG) 15mL light tubes (5 PRIME, Gaithersburg, MD). The PLG tubes were centrifuged for 5 minutes at 2500 RPM, which allowed for separation of the top aqueous phase containing the target DNA from the phenol-chloroform-isoamyl along with other denatured proteins. Following aqueous layer removal, 0.5 volumes of 7.5M $NH_4Ac$ and 2.5 volumes of absolute ethanol (stored at -20$^o$C) was added to the sample, mixed, and stored overnight at -20$^o$C. The precipitate was recovered by centrifuging at 5 degrees, 12000 x g for 20minutes (wash step was repeated). Following the wash, the pellet was dried in a SpeedVac (Savant/Thermo Scientific) to ensure removal of any remaining contaminants. The pellet was resuspended in RNase/DNase free water and 1uL was used on the NanoDrop Spectrophotometer (Thermo Scientific) to calculate yield and $OD_{260}/OD_{280}$ readings.

*Mouse sjTREC Plasmid DNA Amplification*

Amplification of sjTREC DNA was accomplished by inserting mouse sjTREC plasmid DNA (obtained from Sempowski, Duke University 2010) into growing *E. coli* for DNA amplification. For plasmid transformation LB (Lennox LBroth) agar plates containing 30mg/ml of kanamycin were made and 0.5µl of sjTREC plasmid DNA was added to chemically competent *E.coli* - One Shot Top 10 Cells (Invitrogen). The cells were incubated on ice for 30 minutes followed by heat shocking at $42^{o}$C for exactly 30 seconds. After briefly icing the cells, 250µl of sterile SOC media (Invitrogen) was added and shook horizontally (225 RPM) for one hour at $37^{o}$C. Then 200µl of the cell media was spread on kanamycin containing LB plates and incubated overnight at $37^{o}$C. Following incubation, isogenic colonies were selected and inoculated into 2ml of starting broth (30µg/ml kanamycin LB broth). The broth was then grown for 8 hours at $37^{o}$C with shaking. The starter broth was then added into a large (500ml) kanamycin containing (30µg/ml) LB broth and grown overnight at $37^{o}$C with shaking. The 500ml bacterial culture was harvested by centrifugation at 5,000 x g for 10 minutes (care was taken when decanting the supernatant and proper measures were taken to ensure all antibiotic resistant bacteria was properly killed in bleach). Once a bacterial pellet was obtained, the plasmid DNA was purified using the GenElute HP Plasmid Maxi Prep Kit (Sigma) and the corresponding purification procedure. Using the NanoDrop Spectrophotometer (Thermo Scientific) the $OD_{260}/OD_{280}$ readings were obtained to ensure purity and concentration. Lastly, the purified sjTREC DNA was distributed into aliquots and stored at -$20^{o}$C.

*Creating Standard Curves with Amplified sjTREC DNA*

To measure TREC levels, standard samples based on copy numbers of sjTREC plasmid DNA and wildtype(C57BL/6J) mouse DNA (for the *Tfrc* housekeeping gene) were created. Standard samples with the gene of interest present at 300,000 copies, 30,000 copies, 3,000 copies, 300 copies, and 30 copies

per 5µl were created via serial dilution in order to make standard curves following RT-PCR. Standard samples were put into aliquots (to ensure use only once after thawing) and stored at -80$^{o}$C. Extreme care and caution were taken when handling plasmid DNA to ensure no contamination or exposure to contaminated surfaces or objects.

*Quantification of sjTREC via Real-Time PCR*

The Mastercycler ep realplex real-time PCR system (Eppendorf) was used for all real-time PCR samples run. TREC DNA from mouse samples was aliquoted and 100ng was used in each Eppendorf plate well. The forward and reverse primers targeting murine δRec-ψJα excision circles for sjTREC quantification were obtained from Sempowski (Duke University 2010). The forward primer, which is upstream of ψJα segment, had the following sequence: CAT TGC CTT TGA ACC AAG CTG. The reverse primer, which is downstream of the δRec1 segment, had the following sequence: TTA TGC ACA GGG TGC AGG TG. A fluorescent probe was created for RT-PCR: FAM - CAG GGC AGG TTT TTG TAA AGG TGC TCA CTT - QSY (Applied Biosystems). The mouse *Tfrc* gene (Taqman Copy Number Reference Assay, Applied Biosystems) was chosen as the housekeeping gene in order to quantify cell number in mouse DNA samples. Since the *Tfrc* sequence is present twice in mice (once on each allele) when calculating cell numbers, *Tfrc* total numbers were dived by two. When running the RT-PCR, each sample was run in triplicate. Standards of either sjTREC plasmid DNA for TREC samples or C57BL/6J mouse DNA for *Tfrc* samples were run in order to create a standard curve. Alongside all plates that were made a non-template control was run in one of the wells. Real-time PCR conducted for sjTREC DNA samples contained: 12.5µl of 2X Taqman Universal PCR Master Mix (No AmpErase UNG, Applied Biosystems), 1.5µl of 5µM forward and 5µM reverse primer mix, 0.05µl of 100µM FAM-QSY probe, 5.95µl nucleotide free and nuclease-free $H_2O$ in each well. While real-time PCR conducted for *Tfrc* samples contained: 12.5µl of 2X Taqman Universal PCR Master Mix (No AmpErase UNG), 1.25µl 20X *Tfrc* Mouse Taqman

Copy Number Reference Assay, 6.25µl nucleotide free and nuclease-free $H_2O$ in each well. 5µl of sample (containing 100ng of mouse DNA) was added to each well. Real-time PCR cycler conditions were set for 95$^oC$ for 10 minutes (initial heating and activation) followed by 40 cycles of 95$^oC$ for 15 seconds (denaturation) and 60$^oC$ for 1 minute (combined primer/probe annealing and elongation). Following completion of real-time PCR, data standard curves were established and analysis was performed establishing the total sjTREC per cell count.

## R E S U L T S

*Phenol-chloroform vs. QIAamp Mini Kit murine DNA isolation Methods*

Because analysis of TREC relies on isolation of scant quantities of DNA, the reliability and efficiency of isolation could influence results. Accordingly, we sought to optimize the procedure, testing the commonly used commercial isolation kit against the classical phenol-chloroform method. A comparison of C57Bl/6 spleen and thymus spectrophotometry values using the QIAamp MiniKit (n = 9) and phenol-chloroform (n = 6) DNA isolation technique was conducted (Table 1A and 1B). These results indicate relatively similar ($p < .05$) purity readings as indicated by the $OD_{260}/OD_{280}$ and $OD_{260}/OD_{230}$ values, indicating both methods are comparably reliable. However, a statistically significant difference ($p < .05$) was found in ng/µl recovered and total yield. This finding was replicated in the comparison of HOM-QM (Table 1A and 1B) spleen and thymus spectrophotometry values (QIAamp MiniKit method – spleen n = 5, thymus n = 4; phenol-chloroform – n = 5). The difference in yields between the two methods were particularly significant due to the implication that the isolated DNA can be compromised and provide skewed data when performing future experiments utilizing these products. Although the phenol-chloroform DNA isolation technique had large variation between the different samples (Figure 2), the total yield was significantly higher than the yield obtained by the QIAamp Minikit for every sample tested ($p < 0.05$, three of the four comparisons had p values less than 0.01). Therefore, the phenol-chloroform DNA isolation method provides an equally reliable and much more efficient way to isolate smaller DNA segments such as those used for TREC quantification.

*Total T cell numbers in C57BL/6J and HOM-QM mice*

We next asked which T cell populations in the thymus and spleen might contribute to TREC. To address this question, FACS analysis was conducted for samples used in RT-PCR analysis. Figure 3 shows representative results obtained for thymocytes and splenocytes from 12 week old C57BL/6J and 12

week old HOM-QM mice. This example demonstrates how different subpopulations were separated in order to calculate total single positive (SP) T cell subsets of CD4+ and CD8+ in the thymus (Figure 3 A and B) along with CD3+CD4+ and CD3+CD8+ cells in the periphery (Figure 3 C and D) of mice. Figure 3 shows only one representative FACS example of either a C57BL/6J or HOM-QM strain, yet these results typify analysis seen for the majority of animals studied.

Comparing FACS data (n = 2 for each mouse per age group), we found that age matched HOM-QM and C57BL/6J mice have statistically similar numbers of CD4-CD8- double negative, CD4+CD8+, double positive; CD4+ and CD8+ single positive thymocytes (Figure 4 A and B). However, the number of single positive thymocytes decreases significantly between 5 weeks and 12 weeks of age in C57BL/6J and HOM-QM mice, perhaps owing to thymic atrophy. Total numbers of T cells in the periphery of C57BL/6J and HOM-QM mice were similar in aged-matched mice (Figure 4 C and D). These results indicate that even though HOM-QM mice exhibit decreased BCR repertoire and ultimately decreased TCR repertoire, they maintain a normal number of T cells within the peripheral and thymic environments. Whether there is an altered mechanism of proliferation, recombination, or cell death remains to be answered.

*sjTREC quantification assay using real-time PCR*

To determine whether contraction of the B cell repertoire impairs generation of new T cells by the thymus, we enumerated recent thymic emigrants by measuring TRECs in the spleens of C57BL/6J, HOM-QM, and RAG mice (as a negative control). Because the number of TRECS measured in the periphery reflects both thymic production and survival of recent thymic emigrants we also measured TRECS in thymocytes to establish the upper limit of TRECS reflecting thymic emigration.

To enumerate recent thymic emigrants we modified a real-time PCR assay from Sempowski et al.[42] This assay enumerates TRECS by amplifying sjTREC with forward and reverse-specific primers.[42] To

determine the number of genomes in each reaction we amplified the transferring receptor (*Tfrc*) housekeeping gene. Standard curves were obtained by serial dilutions of a sjTREC DNA plasmid (kindly provided by Dr. Sempowski) (Figure 5). To calculate the number of genomes, the number of copies determined from the cycle threshold (CT) value, was divided by two to allow for two copies of *Tfrc* per chromosome pair. The lower limit of detection of the assay was 30 copies of DNA and the upper limit was 30,000 copies of sjTRECs or *Tfrc*. Using 100ng of DNA allowed detection of sjTREC CT values for all samples except for samples obtained from RAG-deficient mice. This was expected since RAG-deficient mice do not rearrange T cell receptor genes and therefore should not contain sjTRECS.

*Age related differences in sjTRECs in the periphery and thymus*

We asked first whether levels of sjTREC vary with age. To address this question we performed real-time PCR for sjTREC using samples from C57BL/6J and HOM-QM mice (n = 6 for each) obtained at 5 weeks, 12 weeks and 20 weeks of age(Figures 6 A-D). The peripheral TREC levels in both C57BL/6J and HOM-QM did not appear to change significantly with age (Figures 6 C and D). The number of TRECs per 10,000 cells appears to decrease in HOM-QM mice at 12 and 20 weeks compared to the number obtained at 5 weeks. This apparent decrease entirely reflected results of two samples of the 5 week old HOM-QM with extremely high TREC number. To determine whether contraction of the B cell repertoire in HOM-QM mice may lead to decreased peripheral TRECs than what would normally be expected, more samples must be analyzed.

Because of thymic atrophy over time, one might expect TREC levels to decrease between 5 weeks of age and 20 weeks of age. Our results however reveal no statistically significant change in thymic TRECs in C57BL/6J mice (Figure 6A). Rather, TREC levels appear to increase slightly in the 20 week C57BL/6J mice. This apparent increase however reflects entirely the unusually high levels of TREC detected in two samples. These TREC levels are much greater than other values. More C57BL/6J thymic

TREC values would have to be analyzed in order to conclude whether thymic TRECS decrease with age. Our results show that the number of TREC decreases with age in HOM-QM mice. Figure 6B shows a statistically significant decrease in the number of ($p < 0.01$) between samples obtained from 12 old week and 5 week old mice. This decrease may reflect thymic atrophy leading to reduced thymocyte recombination and T cell generation or alternatively an increase in proliferation of thymocytes that recently recombined their TCR. These results suggest that thymic involution might occur more rapidly in HOM-QM mice than in C57/BL6 mice and raise the possibility that the defective BCR repertoire in HOM-QM mice disturbs thymic function, as we previously showed, and hastens thymic decline, as we now propose.

*Comparison of sjTRECs between C57BL/6J and HOM-QM mice*

We next asked whether the number of sjTREC in C57BL/6J differs from the number of sjTREC in HOM-QM mice at each age. The comparison is shown in Figure 7. The Student's *t*-test was used as tool to explore differences. Due to high variation especially in the HOM-QM 5 week samples, the only statistically significant difference ($p < 0.01$) was between the thymic TRECs of 12 week old C57BL/6J and HOM-QM mice (Figure 7B). This result in agreement with the concept that thymic TRECs decline abnormally quickly in HOM-QM mice. Still, firm conclusion will require analysis of larger numbers of mice. By analyzing more mice, it can be determined if a large variation exists in the 5 week HOM-QM thymic TRECs, 20 week C57BL/6J thymic TRECs, and 5 week HOM-QM peripheral TRECs or whether there can be significant differences between these samples.

To determine whether levels of TREC in thymus and spleen might be related, a linear regression analysis was performed for peripheral and thymic TRECS for C57BL/6J and HOM-QM mice at age of 12 weeks (Figure 8). The analysis might shed light on basic questions about T cell development and maintenance of the T cell compartment. Results shown in figure 8 indicate that the number of TRECS in

the periphery of HOM mice varies directly with the number of TRECS measured in the thymus of the same mice ($r^2$=0.7244) while such a relationship is less evident in C57BL/6 mice ($r^2$=0.07547). In one interpretation, these results suggest that the number of TRECS in the periphery of HOM-QM mice reflect mostly thymic output while in the C57BL/6 mice the number of TRECS measured in the periphery depends on thymic output along with cellular proliferation.

Although thymic TRECs in the HOM-QM mice exceed thymic TRECs in C57BL/6 mice, the number of peripheral TREC is similar in both strains of mice. Therefore, the kinetics of recombination in HOM-QM may exceed the kinetics of recombination in C57BL/6, or the extent of proliferation in C57BL/6 might exceed that in HOM-QM or the rate of cell death in HOM-QM might exceed that in C57BL/6. The similarity peripheral TREC levels may indicate relatively similar levels of thymic output between C57BL/6J and HOM-QM mice. Alternatively the similar peripheral levels despite higher TREC count in the thymus in HOM-QM mice (assuming this constitutes higher thymic output) could reflect higher levels of peripheral cell proliferation compensating for reduced TCR repertoire diversity. More experiments are needed to distinguish between these possibilities.

Table 1A QIAamp DNA Minikit isolation method

| | BL/6 spleen | BL/6 thymus | HOM-QM spleen | HOM-QM thymus |
|---|---|---|---|---|
| **260/280 Avgs.** | 1.80 | 1.80 | 1.82 | 1.84 |
| **260/230 Avgs.** | 1.86 | 2.11 | 2.22 | 2.15 |
| **ng/µl** | 121.09 | 66.44 | 80.82 | 69.90 |
| **total µg Avgs.** | 48.44 | 26.58 | 25.19 | 14.54 |

Table 1B Phenol chloroform DNA isolation method

| | BL/6 spleen | BL/6 thymus | HOM-QM spleen | HOM-QM thymus |
|---|---|---|---|---|
| **260/280 Avgs.** | 1.79 | 1.78 | 1.82 | 1.81 |
| **260/230 Avgs.** | 2.10 | 2.02 | 2.39 | 2.24 |
| **ng/µl** | 432.24* | 400.10* | 436.25* | 493.60* |
| **total µg Avgs.** | 343.72* | 320.03* | 373.93* | 423.81* |

Table 1. **Comparison of mean measurements from NanoDrop spectrophotometry results using commercialized DNA isolation kits (A) and phenol chloroform technique (B).** Values representing $OD_{260}/OD_{280}$ and $OD_{260}/OD_{230}$ purity readings are relatively similar ($p > .05$) between the two methods and across both types of mice - C57BL/6J and HOM-QM. Differences appear in concentration (ng/µl) and total yield (µg). Isolation of DNA by the phenol chloroform method (B) yielded significantly higher concentration of DNA than isolation by the QIAamp DNA MiniKit (B). *, $p < 0.05$

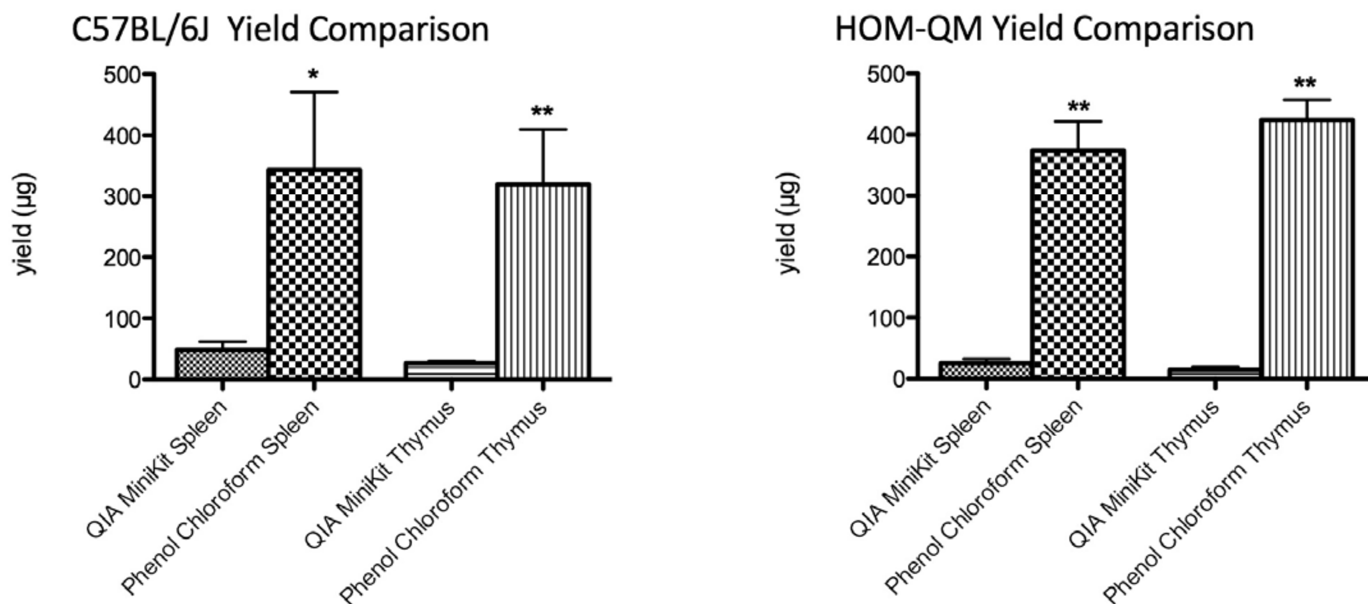


Figure 2. **Comparison of total DNA yield from C57BL/6J and HOM-QM mice using two different procedures.** Following extraction of spleen and thymus from C57BL/6J and HOM-QM mice, two different methods were used in preparing isolates of DNA. Although there were significant levels of variation using the phenol chloroform technique, in all cases the QIA MiniKit method produced significantly lower yields of DNA ($p < .05$). Data represent mean DNA yield ± SEM (*$p < 0.05$, **$p < 0.01$)

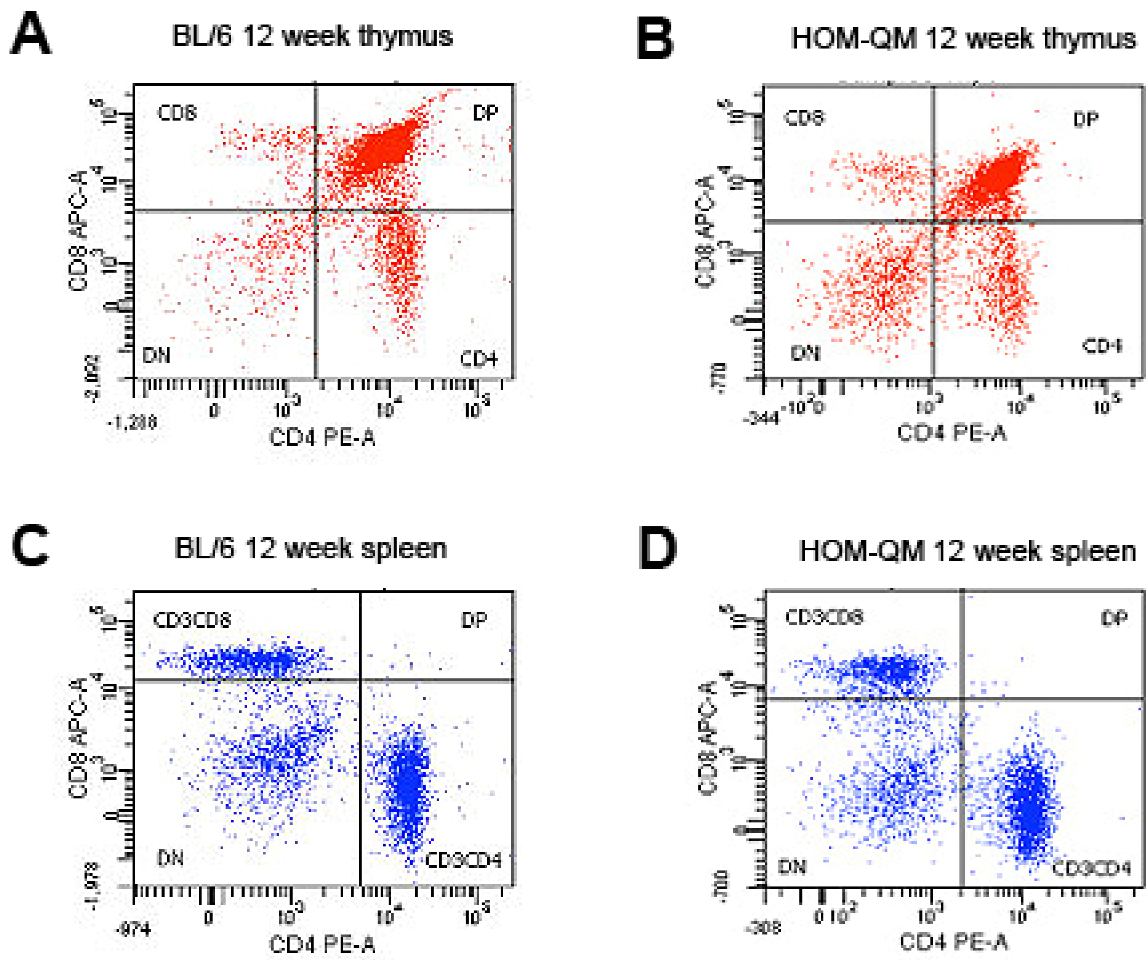


Figure 3. **Fluorescence-activated cell sorting (FACS) example comparison between 12 week old C57BL/6J and 12 week old HOM-QM mouse.** Panels A and B show a subpopulations of thymic cells distributed into quadrants representing CD8+ (top left), CD4+CD8+ (double positive, top right), CD4-CD8- (double negative, bottom left), and CD4+ (bottom right) in 12 week old C57BL/6J or 12 week old HOM-QM mice, respectively. Panels C and D show a subpopulation of CD3 spleen cells (blue) divided into quadrants representing CD3+CD8+ (top left) and CD3+CD4+ (bottom right) in the 12 week old C57BL/6J or 12 week old HOM-QM mice, respectively.

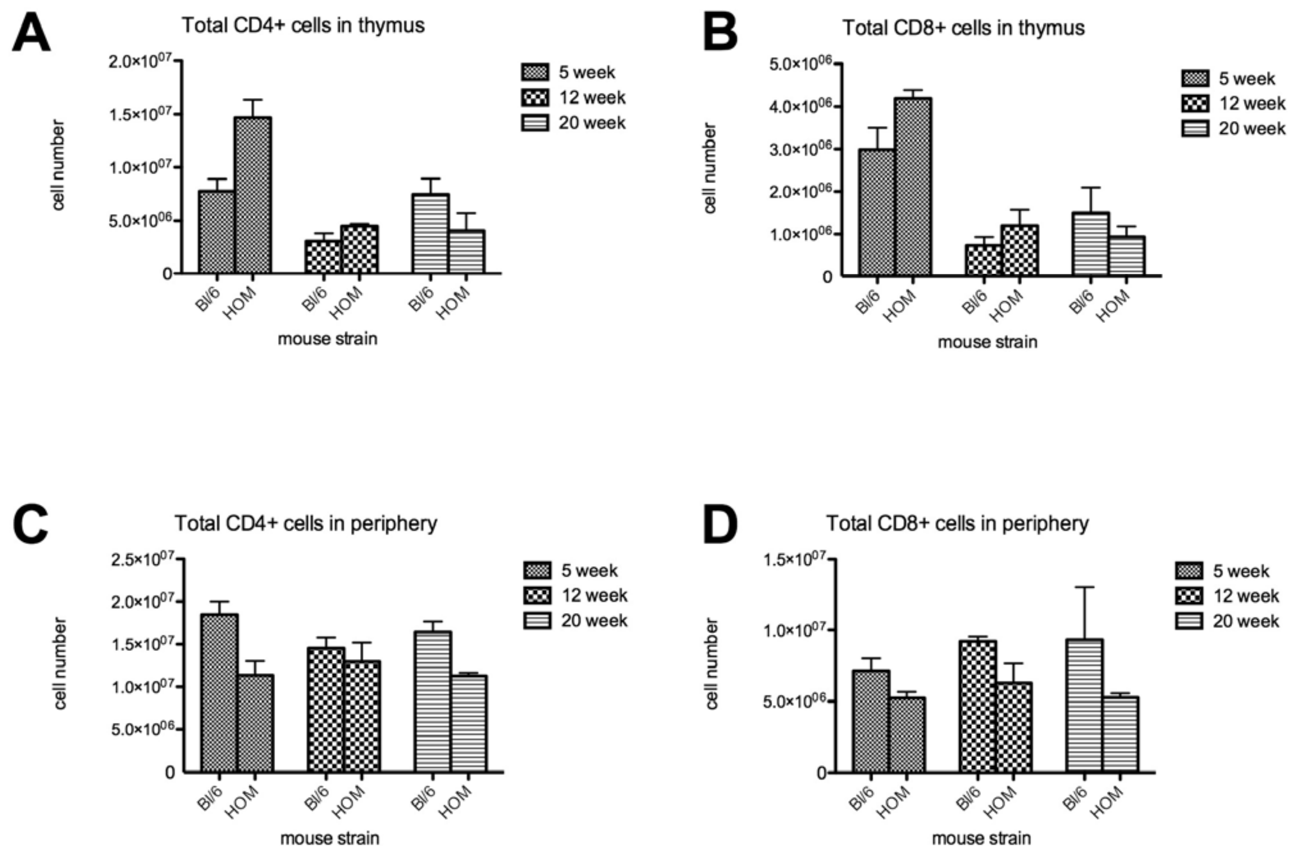


Figure 4. **Number of single positive (SP) CD4+ or CD8+ thymocytes and CD3+CD4+ or CD3+CD8+ splenocytes in age-matched C57BL/6J and HOM-QM mice.** All data was calculated using FACSCanto II and BD FACSDiva software. Panels A and B show the number of CD4+ and CD8+ single-positive thymocytes (respectively) in 5, 12, and 20 week C57BL/6J and HOM-QM mice. Panels C and D show the number of CD3+CD4+ and CD3+CD8+ T cells (respectively) in the spleens of 5, 12, and 20 week old C57BL/6J or HOM-QM mice. Data represent mean cell numbers ± SEM.

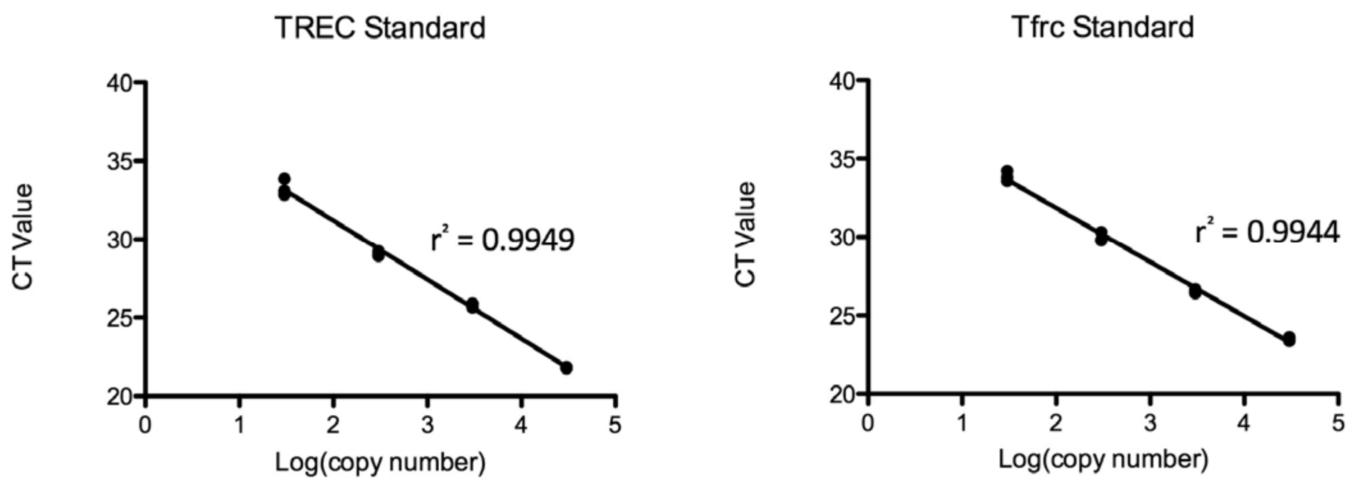


Figure 5. **Real-time -PCR standard curves for TREC and *Tfrc*.** Left Panel indicates an example of the sjTREC DNA standard curve obtained by serial dilutions of sjTREC DNA plasmid (kindly provided by Dr. Sempowski). Right Panel indicates the standard curve obtained from amplifying the transferring receptor gene (*Tfrc)* from serial dilutions of C57BL/6J mouse DNA. To create curves, PCR was performed in samples representing aliquots of 30,000, 3,000, 300, and 30 copies. All acceptable PCR runs had standard curves with an $r^2$ value ≥ 0.98.

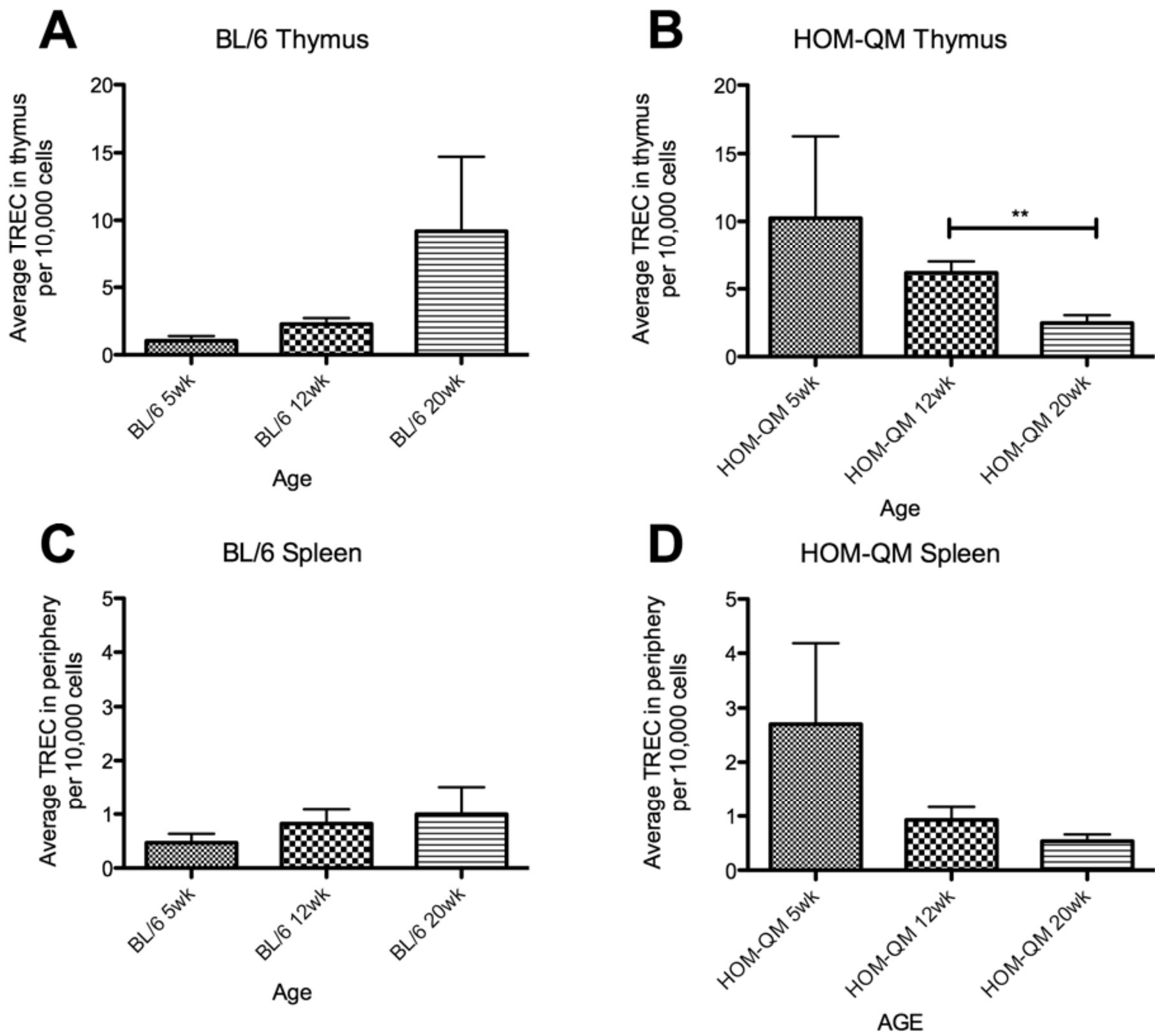


Figure 6. **Peripheral and thymic TRECs per 10,000 cells in mice ranging from 5 to 20 weeks of age.** Panels show the number of TRECs per 10,000 cells in thymocytes (A and B) or splenocytes (C and D) of C57BL/6J and HOM-QM mice. Data from Panels A-D represent mean TRECs/10,000 cells ± SEM (**$p < 0.01$)

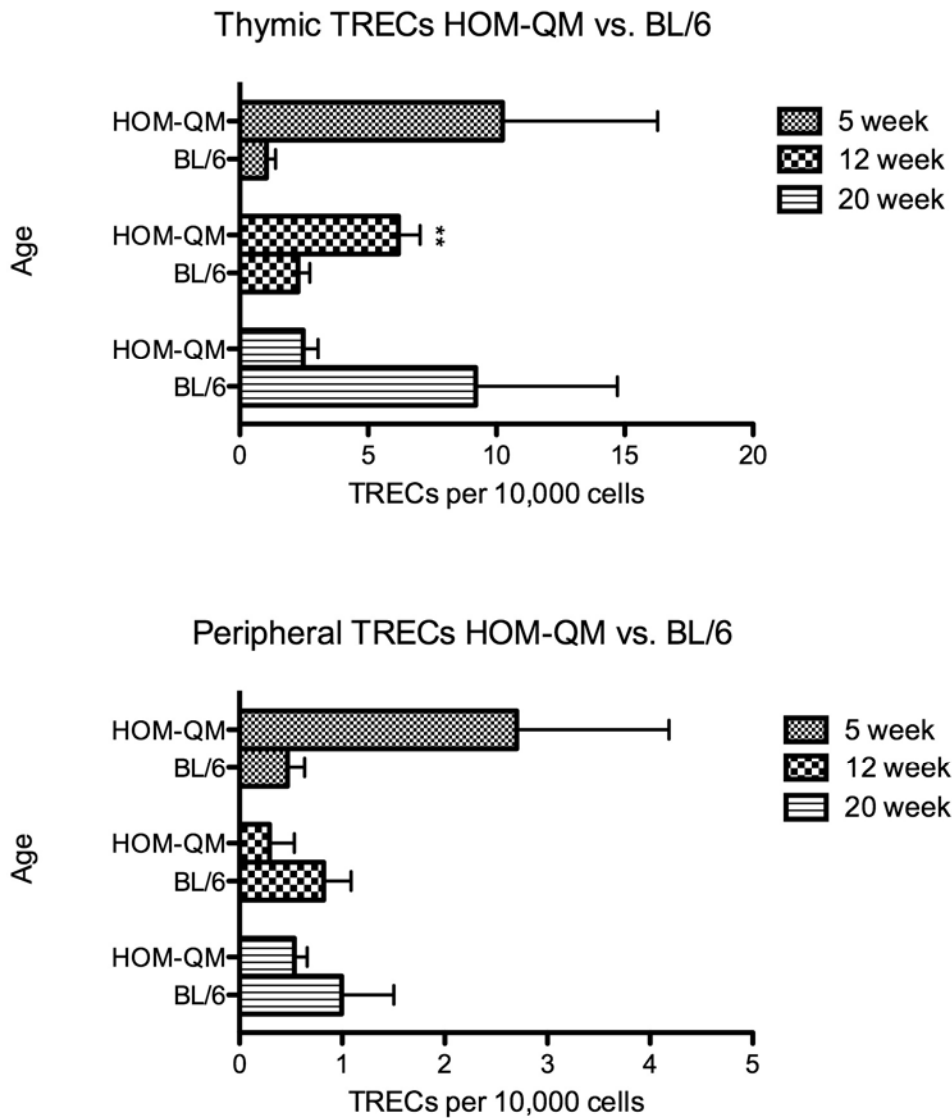


Figure 7. **Comparison of C57BL/6J and HOM-QM TRECs per 10,000 cells across different ages.** Panel A-B show thymic and peripheral TREC levels (respectively) between C57BL/6J and HOM-QM mice that were either 5, 12, or 20 weeks old upon original splenectomy or thymectomy. Data represent mean DNA yield ± SEM (**$p < 0.01$)

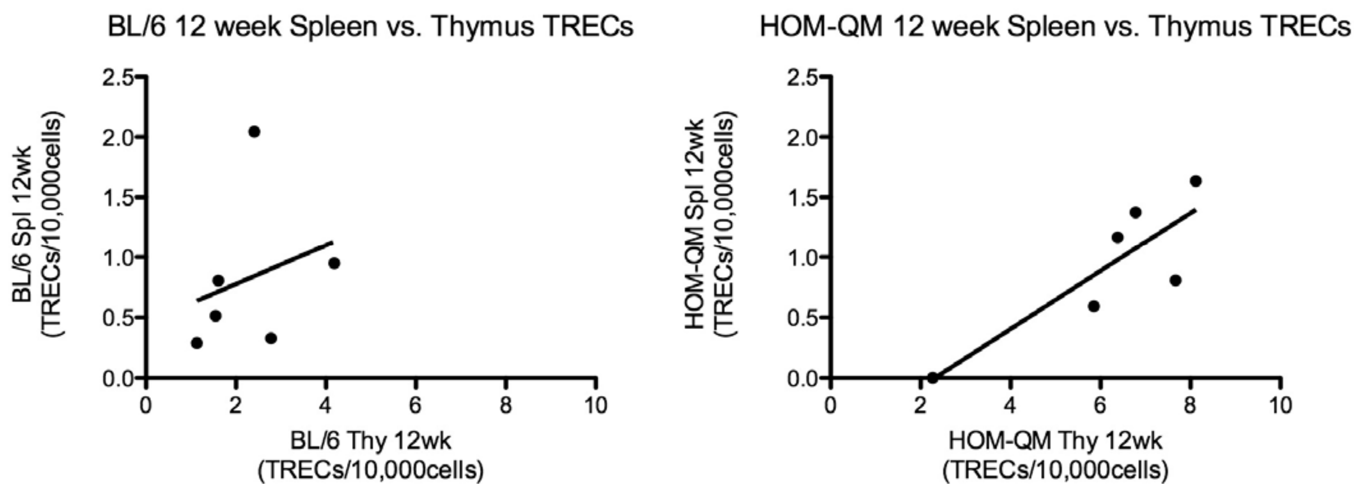


Figure 8. **Linear regression analysis of peripheral and thymic TRECs in C57BL/6J or HOM-QM mice.** Left and right panels show linear regression analysis of TRECS obtained from 12 week old C57BL/6J or HOM-QM samples (respectively) with y-axis representing peripheral TRECs per 10,000 cells and x-axis representing thymic TRECs per 10,000 cells.

# D I S C U S S I O N

Reasoning that recovery of DNA from tissue samples might limit the accuracy and reproducibility of analysis of TREC we tested several approaches to DNA isolation. We show here that a commercial DNA kit commonly used for this type of analysis fails to generate optimal samples for study. Instead, a phenol-chloroform DNA isolation technique generates greater yields of DNA than can be obtained using widely available commercialized DNA isolation kits. According to information accompanying the QIAamp DNA MiniKit, each mg of thymus or spleen tissue should produce 0.2-1.2 µg of DNA. The average C57BL/6J murine thymus and spleen weights are 46mg and 95mg, respectively.[73] Therefore, under ideal conditions the QIAamp DNA isolation kit should recover up to 55.2mg DNA from a thymus and 114mg DNA from a spleen. The phenol-chloroform technique recovers far more DNA than this amount. Our results cast doubt on the value of commercial DNA kits, which are not designed for isolating small pieces of DNA such as sjTREC. Therefore, results obtained following DNA isolated using commercial kits may not be reliable.

This research was designed to address the question of how decreases in the diversity of the BCR repertoire, might impact T cell development. To address this question, I explored the impact of BCR repertoire diversity on thymic size and thymic output, using TREC levels as a measure of newly formed thymocytes. Comparing TREC numbers in the thymus of C57BL/6J with TREC numbers in the thymus of HOM-QM mice, I found a significant difference in the way in which amounts of TREC change with age. Assuming thymic TRECs are representative measurements of thymic function; this finding suggests that a reduction in BCR repertoire may lead to higher rates of thymic involution. Due to the extremely high variation in thymic TREC levels in 5 week old HOM-QM mice, I cannot conclude whether there was a significant increase in TRECs early in mice with nearly monoclonal B cell compartments. However, if thymic TREC numbers are indeed increased early in HOM-QM mice, this may represent compensation

for profound decreases in TCR diversity. Put in another way, my result raises the possibility that rate of T cell development compensates for structural changes in the T cell compartment. Thus, T cell progenitors undergoing maturation may be proliferating less, possess increased levels of recombination, and/or encounter decreased negative selection leading to less cell death. Different assays and experiments need to be utilized in order to conclude which of these three outcomes is actually occurring in the thymic environment.

Representative samples obtained from C57BL/6J and HOM-QM mice at 12 weeks of age revealed a similar level of peripheral TRECs with a higher thymic TREC content in HOM-QM mice. This finding was interesting because it supports the idea that the thymus in HOM-QM mice may be adapting to defects in selection either by increasing V(D)J rearrangement, decreasing cell death, or decreasing expansion of recently formed clones. More experiments are needed to determine how the thymus might compensate for decreased TCR diversity.

We used a real-time PCR assay to estimate the number of recent thymic emigrants. An alternative method involves administration of bromodeoxyuridine (BrdU), a nucleotide analogue incorporated into newly synthesized DNA. Tough and Sprent[75] adopted BrdU uptake to measure recent thymic emigrants based on their observation that RTE take in lower levels of BrdU than other proliferating cells owing to the extremely fast rate of replication in the thymus prior to export. The measurement of RTE using this method might confirm our findings and help distinguish changes in T cell production from changes in recombination and proliferation.

Future experiments can also utilize MicroBeads (Miltenyi Biotec Inc., Auburn, CA) to isolate T cell populations. This would allow more accurate counts and comparisons of just T cell lymphocyte populations. To answer questions on what other mechanisms may be involved in thymic adaptation to

TCR reduction, BrdU incorporation coupled with *in vitro* 5-(and -6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) incorporation between C57BL/6J and HOM-QM mice may be used to provide further insight into whether T cell life span differs in mice with contracted BCRs. BrdU incorporation, as previously stated, can be used to label proliferating cells. CFSE incorporation can be used to study multiple successive generations of cell division due to equal division of the CFSE intracellular fluorescent label between daughter cells.[74] These sets of experiments may provide answers to the question of whether the B cell repertoire controls thymocyte proliferation and/or to survival and proliferation of peripheral T cells.

# ABBREVIATIONS

- Ig – immunoglobulin
- IgH – immunoglobulin heavy chain
- APC – antigen presenting cell
- QM – quasi-monoclonal
- CD4 – cluster of differentiation 4
- CD8 – cluster of differentiation 8
- HOM-QM – homozygous QM (quasi-monoclonal)
- RAG1/2 – recombination activating gene 1/2
- V – variable segment
- D – diversity segment
- J – joining segment
- TREC – T cell receptor excision circle
- sjTREC – signal joint T cell receptor excision circle
- cjTREC – g coding-joint T cell receptor excision circle
- mTREC – murine signal joint T cell receptor excision circle
- TCR$\alpha$ – T cell receptor alpha (also TCRA)
- TCR$\beta$ – T cell receptor beta (also TCRB)
- TCR$\gamma$ – T cell receptor gamma (also TCRG)
- TCR$\delta$ – T cell receptor delta (also TCRD)
- SCID – severe combined immunodeficiency
- id – idiotype
- FACS – fluorescence-activated cell sorting
- FITC – fluorescein isothiocyanate
- PE – Phycoerythrin
- APC – allophycocyanin
- DP – double positive T cell (contains both CD4 and CD8)
- DN – double negative T cell (does not possess CD4 nor CD8)
- SP – single positive T cell (possess either CD4 or CD8)
- RT-PCR – real-time polymerase chain reaction
- CT – cycle threshold
- *Tfrc* – transferrin receptor protein
- FAM – 6-carboxyfluorescein
- QSY – quencher
- BrdU - bromodeoxyuridine

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