Published in final edited form as:

Immunity. 2015 November 17; 43(5): 859–869. doi:10.1016/j.immuni.2015.09.009.

Crossreactive αβ T cell receptors are the predominant targets of thymocyte negative selection

Benjamin D. McDonald[1,2,3], **Jeffrey J. Bunker**[1,2,3], **Steven A. Erickson**[1,2], **Masatsugu Oh-Hora**[4], and **Albert Bendelac**[1,2,*]

[1]Committee on Immunology, University of Chicago, Chicago IL, 60637, USA

[2]Department of Pathology, University of Chicago, Chicago IL, 60637, USA

[4]Division of Molecular Immunology, Medical Institute of Bioregulation, Kyushu University, Fukuoka 812-8582, Japan

SUMMARY

The precise impact of thymic positive and negative selection on the T cell receptor (TCR) repertoire remains controversial. Here, we used unbiased, high-throughput cloning and retroviral expression of individual preselection TCRs to provide a direct assessment of these processes at the clonal level *in vivo*. We found that 15% of random TCRs induced signaling and directed positive (7.5%) or negative (7.5%) selection, depending on strength of signal, whereas the remaining 85% failed to induce signaling or selection. Most negatively selected TCRs exhibited promiscuous crossreactivity toward multiple other major histocompatibility complex (MHC) haplotypes. In contrast, TCRs that were positively selected or non-selected were minimally crossreactive. Negative selection of crossreactive TCRs led to clonal deletion but also recycling into intestinal $CD4^{-}CD8\beta^{-}$ intraepithelial lymphocytes (iIELs). Thus, broadly crossreactive TCRs arise at low frequency in the pre-selection repertoire but constitute the primary drivers of thymic negative selection and iIEL lineage differentiation.

INTRODUCTION

Although the importance of thymic selection in establishing a tolerant and functional T cell receptor (TCR) repertoire has been recognized for over three decades, the precise role and relative importance of positive and negative selection are still widely debated (Klein et al., 2014; Vrisekoop et al., 2014). Double positive (DP) and single positive (SP) thymocyte turnover kinetics suggest that approximately 95% of DP thymocytes fail to give SP progeny,

Corresponding Author: Albert Bendelac, abendela@bsd.uchicago.edu.
[3]Co-first author

AUTHOR CONTRIBUTIONS

B.D.M. and J.J.B. designed research, performed experiments, and analyzed data. S.A.E. helped perform experiments. M.O-H. performed some experiments with SOCE-deficient mice and provided the mice. A.B. supervised the research. B.D.M. and A.B. wrote the paper.

indicating that positively selected TCRs represent only a minor fraction of the pre-selection repertoire (Scollay et al., 1980). While an early study examining apoptosis in major histocompatibility complex (MHC) Class I and MHC class II double deficient (MHC-deficient; *B2m*$^{-/-}$*H2-Ab1*$^{-/-}$) thymuses suggests that very few thymocytes are negatively selected (Surh and Sprent, 1994), recent studies have shown that sizeable frequencies of thymocytes with elevated TCR signaling, marked by Nur77 and Helios (canonical activation markers), are rescued from cell death in mice lacking the pro-apoptotic molecule Bim, a key mediator of negative selection. (Daley et al., 2013; Stritesky et al., 2013). These and other studies also indicate that negative selection could occur in both the cortex and medulla in response to MHC ligands expressed by a variety of cell-types including dendritic cells as well as cortical and medullary epithelial cells (reviewed in (Klein et al., 2014)). Furthermore, while negative selection is traditionally envisioned as deletion of self-peptide specific T cells, it is also suggested to eliminate MHC-crossreactive TCRs that might arise as a consequence of a germline-encoded bias of TCRs for recognition of MHC (Huseby et al., 2005). Together, these findings suggest that negative selection may be more common than initially suspected and that negatively selecting TCRs may constitute a substantial fraction of selected cells. However, the relative frequencies of positive and negative selection remain unclear and global estimates of negative selection range widely between 3 and 30% of the TCR repertoire (Daley et al., 2013; Laufer et al., 1996; Merkenschlager et al., 1997; Stritesky et al., 2013; van Meerwijk et al., 1997).

Few studies have examined the pre-selection TCR repertoire. Analysis of reaggregate thymic organ cultures where MHC-deficient thymocytes are added to MHC class I and MHC class II expressing stroma reveal that 15–20% of pre-selection DP thymocytes can interact with self-MHC:peptide, but this experimental system can not distinguish between positive selection and negative selection (Merkenschlager et al., 1997). A single study has examined the preselection TCR repertoire at the clonal level by maturing MHC-deficient DP thymocytes *in vitro* with anti-CD3 and anti-CD4 antibodies to generate CD4^{+} hybridomas, and has found that only ~5% of pre-selection TCRs can react against a given MHC class II haplotype *in vitro* and that ~30% react against at least one of a panel of 8 MHC haplotypes (Zerrahn et al., 1997). These data suggest that negative selection is rare and that the pre-selection TCR repertoire is broadly MHC-reactive. Notably, this study detected few crossreactive TCRs, suggesting that crossreactivity is rare in the pre-selection repertoire and that the previously observed accumulation of crossreactive TCRs in MHC:single peptide mice is due to both impaired negative selection and extreme bias in positive selection (Barton and Rudensky, 1999; Grubin et al., 1997; Huseby et al., 2005; Sant'Angelo et al., 1997; Surh et al., 1997; Tourne et al., 1997). Importantly, an unbiased clonal assessment of the pre-selection repertoire has not been performed *in vivo* and the true frequencies of positive selection, negative selection, and MHC crossreactivity remain elusive.

Here, we have performed a high-throughput, unbiased clonal screen of random TCRαβ pairs that had not been previously submitted to selection. Over 80 random individual TCRs were expressed retrovirally in thymocytes using a conditional system for appropriate timing of expression of the TCRα chain and a mixed chimeric setting to ensure representation of a polyclonal repertoire side by side with the clonal TCR. In this system, thymocyte signaling

and development could be reliably characterized *in vivo*, allowing for a direct assessment of thymic selection processes at the clonal level and on a large scale. We found that 7.5% of pre-selection TCRs were positively selected, while 7.5% were autoreactive and negatively selected. Negative selection occurred predominantly at the DP stage and the DP-SP transition and, less frequently, at the medullary SP stage. Unlike classic MHC:peptide specific T cells, a majority of these autoreactive TCRs were crossreactive against multiple MHC haplotypes and across MHC classes. Although negative selection induced massive clonal deletion, a fraction of cells consistently escaped deletion and were recycled into intestinal intraepithelial lymphocytes. These findings reconcile previous conflicting reports by showing that negative selection is rare compared with the size of the pre-selection repertoire but nevertheless predominantly targets crossreactive TCRs. Altogether, these data have major implications for our understanding of central tolerance, highlighting MHC crossreactivity as a crucial target of negative selection and driver of innate intestinal intraepithelial lymphocyte (iIEL) lineage differentiation.

RESULTS

Thymic selection was modeled *in vivo* using a conditional retroviral TCR expression system

To facilitate the clonal analysis of TCRs from the pre-selection repertoire, we made use of mice expressing one of 4 different TCRβ transgenes (Table S1). In each of these lines, TCR diversity was restricted to the TCRα chain, allowing normal positive selection and negative selection, as judged by normal frequencies of TCR^{hi} $CD69^{+}$ $PD\text{-}1^{lo}$ thymocytes and TCR^{hi} $CD69^{+}$ $PD\text{-}1^{hi}$ thymocytes, respectively, and by normal frequencies of all $TCR\alpha\beta^{+}$ cell lineages including $CD4^{+}$ and $CD8^{+}$ T cells, regulatory T (T_{reg}) cells (TCR^{hi} $CD4^{+}$ $Foxp3^{+}$), and unconventional iIELs ($CD4^{-}$ $CD8\beta^{-}$ $TCR\alpha\beta^{+}$) (Figure S1A–D) (McDonald et al., 2014). Even when analysis was limited to Vβ-transgenic T cells expressing a given Vα family such as Vα2 or Vα8, a close approximation of the wild-type frequency of these lineages was observed. As expected, most TCRVβ and TCRVα chains examined in this study paired efficiently and could be detected at the cell surface, with the exception of the Vβ8.3/Vα2 combination (Figure S1B and data not shown) (Blackman et al., 1986).

To model the broad pre-selection repertoire, we cloned large numbers of *Trav14* (TCRVα2) and *Trav12* (TCRVα8) chains from pre-selection DP thymocytes purified from either MHC-deficient or $Trac^{-/-}$ mice. We then used a conditional retroviral system to transduce these TCRα chains into bone marrow cells of TCRVβ-transgenic *Cd4-cre* mice and subsequently reconstituted lethally irradiated CD45.1 congenic recipients (Figure 1A and Table S1). This *Cd4-cre* conditional system closely mimics the physiological kinetics and amounts of TCR expression (Figure S2A–B) (McDonald et al., 2014). In many cases, to avoid expression of endogenous Vα chains, the transduced bone marrow cells also harbored a $Trac^{-/-}$ mutation. Transduced cells could be identified by expression of vector-encoded Thy 1.1 and antibodies against Vα2 or Vα8. Finally, in all cases, control CD45.1 bone marrow cells transduced with the empty vector (lacking the Vα sequence) were coinjected to obtain mixed bone marrow chimeras where T cells expressing the TCR under study represented 1–60% of all T cells and a normal polyclonal T cell environment was preserved.

By combinatorial pairing of the 4 Vβ transgenes with these random Vα2 and Vα8 chains, we obtained 81 pre-selection TCRs (Table S1).

Most TCRs cloned from the pre-selection pool fail to be selected

As expected, we found TCRs that fell into 3 main categories. Many TCRs were developmentally arrested at the DP stage with a lack of apparent selection. These TCRs represented 69 of 81 pre-selection TCRs, corresponding to 85% of the pre-selection repertoire. These non-selected TCRs characteristically induced a uniform DP^{hi} phenotype, without expression of markers of TCR signaling such as CD69 or PD-1, low amounts of CD5, and an absence of mature peripheral T cells. One prototypical example is given in Figure 1B, as Vβ7Vα8-82, and a summary shown in Figure 2A (top) and Figure 2C–D.

Other TCRs (n=6; 7.5% of the preselection repertoire) gave a typical pattern of positive selection, with induction of CD69 at the DP stage, upregulation of CD5, and generation of either $CD4^+$ or $CD8^+$ SP thymocytes expressing the CCR7 chemokine receptor associated with migration into the medulla. These cells expressed variable but generally low amounts of PD-1. This positive selection pattern is exemplified in Figure 1B, by Vβ8.2Vα8-80 and Vβ3Vα8-82, which gave rise to $CD4^+$ and $CD8^+$ lineage T cells, respectively, and is summarized in Figure 2A (top) and Figure 2C–D.

Finally, we detected TCRs inducing negative selection (n=6; 7.5% of the preselection repertoire). All of these TCRs gave a $CD5^{hi}$ $CD69^{hi}$ DP^{lo} phenotype associated with very high amounts of PD-1. They generated very few splenic T cells, which were mostly of the $CD4^-CD8\beta^{lo/-}$ phenotype, but abundant unconventional iIEL with a similar $CD4^-CD8\beta^{lo/-}$ phenotype (Figure 1B, Vβ8.2Vα8-72 and data not shown). These data confirm and extend results from our previous study of iIEL and DP^{lo} $PD\text{-}1^{hi}$ TCRs (McDonald et al., 2014). Notably, the negatively selected thymocytes did not express CCR7, which is normally induced by positive selection and directs migration to the medulla, suggesting that they had received negative selection signals in the cortex (Figure 1B).

The phenotypes of all 81 pre-selection TCRs are summarized in Figure 2A (top) and C–D.

Positive and negative selection occur at similar frequencies

We found that an equal frequency of pre-selection TCRs underwent positive selection or negative selection. As only a small fraction of pre-selection TCRs (15%) were selected, there were only 12 such TCRs in our pool of 81 pre-selection TCRs. Therefore, to increase the size of this panel and further probe the relative frequencies of positive selection and negative selection, we purified post-selection $CD69^+$ DP thymocytes from Vβ7 or Vβ8.2 transgenic mice and cloned their *Trav14* and *Trav12* chains. The post-selection (DP $CD69^+$) phenotype represents cortical thymocytes that have recently received a selection signal, either for positive selection or negative selection. Retroviral expression of 10 of these TCRα chains in corresponding Vβ-transgenics revealed that 5 TCRs induced negative selection, whereas 5 induced positive selection (Figure 2A, bottom). Thus, positive selection and negative selection appear to occur at equal frequencies among selected TCRs.

One of these post-selection (DP CD69^{+})-derived TCRs, Vβ8.2Vα2-132, induced a pattern suggestive of medullary rather than cortical stage negative selection, based on expression of CCR7 and CD69 in SP CD8 thymocytes, along with Helios (data not shown), and lack of peripheral T cells (Figure 2B). This pattern of late negative selection, which has been reported previously (Daley et al., 2013), appeared to represent a relative minority of total negative selection (1 out of 12).

A compilation of outcomes for all pre-selection- and post-selection (DP CD69^{+})-derived TCRs is presented in Figure 2A, C–E. Altogether, the results indicate that 85% of TCRs failed to be selected, whereas 7.5% were positively selected and 7.5% were negatively selected.

Several observations and additional controls suggest that our method of TCR expression faithfully reports the fate of individual TCRs. First, our previously published experiments using a similar expression system have shown that, in all cases, TCRs extracted from peripheral T cell lineages faithfully reproduced their lineage of origin upon transgenic expression (McDonald et al., 2014). Second, of 11 TCRs cloned from post-selection (DP CD69^{+}) thymocytes, which represent thymocytes that have been signaled by MHC ligands *in vivo*, a great majority (10 of 11) induced a pattern of positive selection or negative selection in the conditional retrogenic chimeras (Figure 2A bottom and Table S1), as expected. Third, negative selection TCRs tended to be associated with a smaller thymus, highlighting their deletional phenotype (Figure 2E). Finally, we found that up to 38% of the TCRα chains that induced positive selection in our study could also be found among a reference pool of 500 sequences independently derived from two TCRVβ-only transgenic spleens. In contrast, only 2% of the non-selected TCR sequences and 0% of the negative selection TCR sequences could be found in this reference pool (Figure 2F).

The negative selection repertoire is enriched in MHC multi-reactive TCRs

To assess the MHC reactivity of individual TCRs, we purified unsignaled DP thymocytes from RV chimeras based on their CD69^{-} PD-1^{-} DP phenotype and cultured them with stimulator cells expressing different MHC haplotypes, including mixtures of allogeneic thymocytes and splenocytes (expressing both MHC I and II) as well as a panel of SV40-transformed fibroblasts (expressing only MHC I) from different strains. In this system, TCRs were tested at the relevant DP differentiation stage together with both coreceptors, ensuring physiologically relevant responses against both classes of MHC. As shown in Figure 3A, the non-selected Vβ8.2Vα8-75 TCR showed no induction of CD69 and PD-1 above background represented by B6 MHC-deficient stimulators. In contrast, the negatively selected TCR Vβ8.2Vα2-20 reacted strongly against many different stimulators, including H2^{b,d,k,r,u,q}, a very unusual pattern of multi-crossreactivity similar to the one described in MHC:single peptide mice (Huseby et al., 2005). We confirmed the reactivity of Vβ8.2Vα2-20 against the ‘d’ haplotype by expressing this TCR directly on the B10.D2 background. As shown in Figure 3B, Vβ8.2Vα2-20 expressing thymocytes were arrested at a DP^{lo} PD-1^{hi} stage and gave rise to only rare DN splenocytes and to unconventional CD4^{-}CD8β^{-} iIEL in both B6 and B10.D2 backgrounds, a pattern typical of negative selection TCRs.

To further assess the relationship between MHC crossreactivity and T cell fate, we extended our *in vitro* analysis to a total of 83 TCRs drawn predominantly from the non-selected, positive selection, and negative selection panels described previously, but also from other populations as described in Table S1. Notably, we found that most but not all negative selection TCRs exhibited a pattern of crossreactivity similar to Vβ8.2Vα2-20 (Figure 4A), with a total of 61 reactivities for 21 TCRs. In contrast, none of the 15 positive selection TCRs exhibited the cross-reactive pattern, and only a total of 7 MHC reactivities were detected (Figure 4B). Likewise, out of 47 non-selected TCRs, only 12 MHC reactivities were detected (Figure 4C). Thus, there is a massive enrichment of MHC crossreactive TCRs in the negative selection pool (Figure 4D). Importantly, however, 6 out of the 21 negative selection TCRs did not show a crossreactive pattern, suggesting that they might represent classical MHC:peptide-specific autoreactive TCRs.

We further determined whether these crossreactive TCRs could react against both MHC I and MHC II in the C57BL/6 background by using MHC I-, MHC II- and MHC I and II-deficient B6 stimulators *in vitro* or by expressing TCRs in corresponding MHC I-, MHC II- and MHC I and II-deficient mice *in vivo*. In addition to recognizing multiple MHC haplotypes, one third of negative selection TCRs also exhibited dual reactivity to MHC class I and class II, as summarized in Figure 5D. Representative examples are shown *in vitro* (Figure 5A) with Vβ8.2Vα2-107 responding to MHC I, Vβ8.2Vα2-110 to MHC II and Vβ7Vα2-U4 responding to both classes of MHC. This *in vitro* reactivity was further validated *in vivo* for a subset of TCRs where self MHC-reactivity could be confirmed by characteristic abundance of $CD69^{+}$ DP^{lo} $PD\text{-}1^{hi}$ thymocytes (Figure 5B–C). Thus, the negative selection repertoire is uniquely enriched in TCRs that exhibit prominent alloreactivity as well as MHC class I and II dual reactivity.

Negative selection at the DP stage requires store-operated calcium entry

DP^{lo} $PD\text{-}1^{hi}$ thymocytes have been previously suggested to undergo negative selection based on higher induction of Nur77, Helios, and Egr2 downstream of TCR signaling, caspase 3 activation, annexin-V staining and rescue by Bim-deficiency or Bcl-xL overexpression. However, while recent experiments suggest that store-operated calcium entry (SOCE) is required for the generation of unconventional iIELs along with the other agonist-signaled NKT and Treg cell lineages (Oh-Hora et al., 2013), the requirement of SOCE for the generation of $TCR\beta^{hi}$ DP^{lo} $PD\text{-}1^{hi}$ thymocytes undergoing negative selection, which are the thymic precursors to iIELs, has not been directly tested. By analysis of mice with conditional deletion of Stim1 and Stim2 in hematopoietic cells, we found that the $TCR\beta^{hi}$ DP^{lo} $PD\text{-}1^{hi}$ phenotype was absolutely dependent on SOCE, even in mixed WT: *Vav-icre* $Stim1^{fl/fl}Stim2^{fl/fl}$ chimeras, establishing the cell-intrinsic re-quirement of SOCE for negative selection at the DP stage (Figure 6A–C). In contrast, as previously reported, positive selection of conventional $TCR\beta^{hi}$ $PD\text{-}1^{lo}$ $CD4^{+}$ and $CD8^{+}$ T cells was unperturbed (Figure 6A–C). Furthermore, upon stimulation of CD69- DP thymocytes with anti-TCRβ antibody and dendritic cells *in vitro*, SOCE was also required for efficient downregulation of CD4 and CD8 as well as for expression of PD-1 (Figure 6D). Thus, these results further establish the peculiar agonist signaling pathway required for negative selection of self-

reactive DP thymocytes and explain the absence of iIELs in Stim1 and 2 double-deficient mice.

DISCUSSION

Here, we took advantage of an improved retroviral method of expression of TCRs to perform a large-scale study of preselection TCRs at the clonal level *in vivo* in conditions that preserved physiological aspects of thymic development, such as normal kinetics and amounts of TCR expression, as well as a polyclonal environment. By determining both the level of signaling and the corresponding fate of thymocytes for individual TCRs, we derived a direct, unbiased estimate of the rates at which thymocytes undergo positive selection (7.5%) and negative selection (7.5%). Furthermore, by performing a detailed analysis of the pattern of MHC reactivity for large numbers of random TCRs corresponding to pre- and post-selection stages, we resolved a longstanding controversy regarding the true impact of negative selection on the TCR repertoire and the frequency of crossreactive TCRs.

TCRs showing a highly unusual pattern of MHC crossreactivity have been occasionally reported in mouse and human T cell clones, but were consistently found at high frequency in mice expressing a single MHC:peptide complex (Huseby et al., 2005; Logunova et al., 2005). These crossreactive TCRs lacked the degree of specificity for peptide and MHC that is the hallmark of most classical antigen-specific T cells. Instead, they had promiscuous specificity for various MHC and peptide ligands. One group hypothesized that crossreactive TCRs illustrated the inherent, germline-encoded bias of TCRs for binding MHC molecules and predicted that they represent a large fraction of the pre-selection repertoire (Huseby et al., 2005). In this view, a critical function of negative selection, in addition to self-tolerance, was to create an MHC- and peptide-specific repertoire by removing crossreactive TCRs. Contrasting with this view, a study of $CD4^+$ hybridomas derived from MHC-deficient preselection thymocytes reported that crossreactivity was very rare and occurred to a similar extent in the pre-selection and MHC-selected repertoires (Zerrahn et al., 1997). This study implies that the high frequency of crossreactive TCRs reported in MHC:single peptide mice must have resulted from their aberrant accumulation due to both biased positive selection and impaired negative selection. Here, by focusing our study on a large number of pre-selection TCRs, as well as a large group of random TCRs inducing negative selection, we found that crossreactivity was very infrequent among pre-selection TCRs overall, whereas an overwhelming majority (71%) of TCRs associated with negative selection were crossreactive. Furthermore, the crossreactive TCRs could be observed in multiple Vα/Vβ combinations. These results reconcile the two opposing models by showing that, as shown in the preselection hybridoma panel study (Zerrahn et al., 1997), the frequency of crossreactive TCRs is indeed very low in the pre-selection repertoire, whereas, as suggested from the MHC:single peptide studies (Huseby et al., 2005), crossreactive TCRs can be found at high frequency among normal unbiased negative selection thymocytes. Although our results were established across 4 different Vβ transgenic chain, including Vβ which is overrepresented in the natural repertoire, and 2 different families of Vα, we cannot exclude the possibility that certain Vβ or Vα may be more or less biased towards crossreactivity, a subject of further studies.

Our finding that positive selection and negative selection occurred at a similar rate implies that half of the TCRs being signaled in the thymic environment are autoreactive, emphasizing the major impact of thymic negative selection in shaping the TCR repertoire. However, this impact seems mostly due to the high relative frequency of crossreactive TCRs rather than classical MHC:peptide specificity. In addition, TCRs sharing the same TCRβ chain and differing only by a few amino acid residues in their CDR3 α regions could nevertheless exhibit drastically different patterns of thymic selection and MHC reactivity, consistent with the proposition that the germline-encoded TCR bias for MHC is encoded in the CDR1 and CDR2 regions, whereas hy-pervariable CDR3 loops determine the outcome of allele-specific selection (Garcia et al., 2009).

The finding that negative selection thymocytes predominantly express crossreactive TCRs is also consistent with the fact that signaling for negative selection seems to be predominantly induced in the cortex or at the junction between cortex and medulla, as indicated by the DP^{lo} $PD\text{-}1^{hi}$ $CCR7^{-}$ phenotype of a great majority of thymocytes undergoing negative selection. Indeed, a promiscuous self-reactive TCR is likely to be detected early by dendritic cells or epithelial cells in the cortex whereas, in contrast, specific recognition of tissue-restricted antigens is more likely to operate in the medulla. In that respect, although this study did not address the pattern of MHC-and peptide-specificity of natural Treg cells, it is interesting to consider that differences might exist between Aire-dependent TCRs, which are expected to be MHC- and peptide-specific, and other Treg cells, for which promiscuous recognition of self-ligands might be advantageous. Future experiments should be designed to answer this important question.

While there have been various estimates of the frequency of negative selection induced by antigen recognition in the medulla, most studies have relied on indirect assessments or transgenic systems. Our results suggest that relatively few TCRs undergo negative selection at that late stage of differentiation, although it remains possible that, similar to reports on Treg cell differentiation (Bautista et al., 2009; Leung et al., 2009), cell-extrinsic factors might limit medullary negative selection against a particular antigen.

Finally, it is noteworthy to consider that negative selection is not synonymous with clonal deletion, as a fraction of negative selection TCRs are recycled into $CD4^{-}CD8\beta^{-}$ iIELs (McDonald et al., 2014; Pobezinsky et al., 2012). We previously found that TCRs cloned from these iIELs invariably give rise to DP^{lo} $PD\text{-}1^{hi}$ thymocytes bearing the hallmarks of elevated TCR signaling, and conversely, TCRs cloned from DP^{lo} $PD\text{-}1^{hi}$ thymocytes give rise exclusively to $CD4^{-}CD8\beta^{-}$ iIELs. Here, we further extended these tight correlations with random pre-selection TCRs, and formally demonstrated a selective requirement for agonist signaling in the formation of DP^{lo} $PD\text{-}1^{hi}$ thymocytes, as shown by their selective dependence on SOCE proteins Stim1 and Stim2, similar to NKT and Treg cell thymocytes (Oh-Hora et al., 2013). Because the iIEL TCRs are broadly crossreactive, we speculate that, similar to NK receptors, they might function like innate-like receptors sensing MHC ligands rather than specific peptides in the intestinal environment. Conversely, the finding that crossreactive TCRs represent a prominent population of autoreactive TCRs warrants close consideration of their role in autoimmune diseases.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6, BALB/c, C3H, B10.D2 (B10.D2-Hc^{1} H2^{d} H2-T18^{c}/nSnJ), B10.BR (B10.BR-H2^{k2} H2-T18^{a}/SgSnJJrep), *Trac*$^{-/-}$ (B6.129S2-*Tcra*^{tm1Mom}/J), *B2m*$^{-/-}$ (B6.129P2-*B2m*^{tm1Unc}), and CD45.1 (B6.SJL-Ptprca Pep3b/BoyJ) mice were from Jackson Laboratories. *Cd4-cre* (B6.Cg-Tg(*Cd4-cre*)1Cwi), *H2-Kb*$^{-/-}$ *H2-Db*$^{-/-}$ (B6.129P2-*H2-K*^{btm1} *H2-D*^{btm1}), *H2-Ab1*$^{-/-}$ (B6.SJL(129)-Ptprca/BoyAiTac *H2-Ab1*^{tm1Gru}), and *B2m*$^{-/-}$*H2-Ab1*$^{-/-}$ (MHC-deficient) (B6.129-*H2-Ab1*^{tm1Gru} *B2m*^{tm1Jae} N17) mice were from Taconic. TCR Vβ3, Vβ7, Vβ8.2, Vβ8.3, Vβ7 U1 Tg^{cond}, and Vβ7 U4 Tg^{cond} transgenic mice were previously described and maintained in our colony (Malchow et al., 2013; McDonald et al., 2014; Savage et al., 2011; Savage et al., 2008). *Vav-icre Stim1*$^{fl/fl}$ *Stim2*$^{fl/fl}$ mice were previously described and maintained at Kyushu University (Oh-Hora et al., 2013). All mice were maintained in a specific pathogen-free environment at either the University of Chicago or Kyushu University and experiments were performed in accordance with the guidelines of the Institutional Animal Care and Use Committees.

Cell Isolation

Single cell suspensions of thymocytes and splenocytes were obtained by mechanical disruption of the tissue through a 70 μm cell strainer. Small intestines were excised from mice and fat, Pey-er's patches, and luminal contents removed. The intestine was opened longitudinally and cut into 1 cm pieces. Pieces were stirred with a magnetic stirrer for 2 hours in RPMI-10% FCS and then purified by running over a glass-wool column (Fisher Science France) followed by centrifugation on 40% percoll (Sigma) to further remove epithelial cells and debris.

Retrovirus Production, Infection, and Chimera Generation

Trav14 and *Trav12* chains were PCR-cloned from cDNA derived from FACS-sorted preselection (DP CD69^{-}), or selected (CD4^{+} CD69^{+}, DP CD69^{+}, total CD69^{+}, or DP^{lo} PD-1^{hi}) thymocytes as listed in Table S1. The *Trac* chain genes were inserted into a conditional retroviral vector, pMGflThy1.1, as described below (McDonald et al., 2014; Turner et al., 2010). Recom-bined *Trav14* or *Trav12* gene segments and the *Trac* constant region were PCR amplified using Q5 DNA polymerase (New England Biolabs) with 5% DMSO. PCR products were gel purified using a QIAquick Gel Extraction Kit (Qiagen). Purified *Trav14* or *Trav12* and TRAC products were assembled together with NotI and AgeI digested pMGflThy1.1 using Gibson Assembly Master Mix (New England Biolabs). Assembled product was transformed into XL10 Gold ultra-competent cells (Agilent) and plasmids carrying the correct insert were purified using the HiSpeed Midi Prep kit (Qiagen). Plasmid preparations were sequenced to verify the TCR insert. Constructs were transfected into Plat-E packaging cells using lipofectamine (Life Technologies) (Morita et al., 2000). Harvested retroviral supernatant was filtered through a 0.45 μm filter and frozen in a dry ice and ethanol bath. TCR Vβ3, Vβ7, Vβ8.2, or Vβ8.3 transgenic *Cd4-cre* mice (*Trac*$^{-/-}$ or *Trac*$^{+/+}$) were injected with 150 mg kg^{-1} 5-Fluorouracil (APP Pharmaceuticals) three days prior to bone marrow harvest. After harvest, bone marrow was cultured for two days in X-Vivo 10 medium (Lonza) supplemented with 15% FCS, 1% penicillin and streptomycin, 100

ng mL^{-1} mouse SCF, 10 ng mL^{-1} mouse IL-3, and 20 ng mL^{-1} human IL-6 (all from Biolegend). Stimulated cells were infected with TCRα-encoding retrovirus in the presence of 4 μg mL^{-1} polybrene (EMD Millipore) by centrifugation at 800×g for 90 minutes at 30°C. After 20–24 hours of additional culture in medium as above, bone marrow cells were stained with an antibody against mouse thy 1.1, and MACS-enriched (Miltenyi Biotec). In order to enable side-by-side comparison between a particular TCRα chain and a polyclonal wild-type repertoire, wild-type CD45.1 mice were treated as above, but instead infected with empty (lacking TCRα-coding sequences) pMGflThy1.1 and MACS enriched. For experiments designed to confirm TCR multire-activity, wild-type B10.D2 or B10.BR mice were treated as above, but instead infected with empty pMGfl4 (lacking TCR-α and encoding a signaling-deficient human CD4 protein) and MACS enriched. Recipient mice were lethally irradiated with 1000 rads from a gamma cell 40 irradiator with a cesium source. At least 4 hours after irradiation, recipients were injected with a 1:1 mixture of WT to TCR transduced Vβ-transgenic bone marrow. Chimeric mice were analyzed 4–6 weeks post reconstitution. We confirmed proper expression of the transduced TCRα chains by staining thymocytes with antibody specific for Vα2 or Vα8.

Antibodies and Flow Cytometry

Purified cell populations were incubated with Fc Block (Biolegend Or BD) prior to FACS staining. Fluorochrome or biotin conjugated monoclonal antibodies (clone in parentheses) against mouse CCR7 (4B12), CD4 (GK1.5 or RM4–5), CD5 (53–7.3), CD8α (53–6.7), CD8β (53–5.8), CD45.1 (A20), CD45.2 (104), CD69 (H1.2F3), Foxp3 (FJK16s), PD-1 (29F.1A12), TCRβ (H57–597), Thy1.1 (OX-7), Vα2 (B20.1), Vα8 (KT50), Vβ3 (KJ25), Vβ7 (TR310), Vβ8.2 (KJ16–133.18), Vβ8.3 (8C1), and human CD4 (OKT4) were purchased from Biolegend, eBioscience, or BD Biosciences. CCR7 staining was done at 37°C prior to the addition of other antibodies per the manufacturer's instructions (eBioscience). Samples were analyzed on an LSRII (Becton Dickinson) or sorted on a FACSAria (Becton Dickinson) with doublet exclusion and DAPI staining to remove dead cells when possible. Data was analyzed using FlowJo (Tree Star).

TCR Sequencing

Sorted cell populations were frozen in Trizol (Life Technologies) prior to use. RNA was isolated using an RNeasy mini kit (Qiagen) and cDNA synthesized using SuperScript III (Life Technologies). TCR $Vα2^+$ TCRs were amplified using forward primer: 5'-ATGGACAAGATCCTGACAGCA-3' and reverse primer: 5'-TCAACTGGACCACAGCCTCAGC-3'. TCR $Vα8^+$ TCRs were amplified with the same reverse primer as above, but with a pair of forward primers: 5'-ATGAACATGCGTCCTGACACCT-3' and 5'- ATGAACATGCGTCCTGTCACCT-3'.

PCR products were cloned into Zero Blunt TOPO (Life Technologies), transduced into XL-10 Gold Ultracompetent Cells (Agilent Technologies), and sequenced. TCR sequences were analyzed using IMGT (Lefranc et al., 2009). Only in-frame TCR rearrangements were selected for retroviral expression. To determine the overlap between TCRs scored as positive selection, negative selection, or non-selected and those found among wild-type

splenocytes, we obtained at least 350 *Trav14* and *Trav12* sequences derived from sorted $CD4^+$ or $CD8^+$ T cells from pools of 2 Vβ7 and 2 Vβ8.2 transgenic mice.

Thymocyte Stimulation Cultures

Thymocytes from RV chimeras were depleted of $CD69^+$ and $PD\text{-}1^+$ cells by AutoMACS to enrich for unsignaled DP thymocytes and 1×10^5 were cultured with either 2×10^4 MHC class I-expressing SV-40 transformed fibroblasts derived from mice of 8 different MHC haplotypes or with 5×10^5 cells (2.5×10^5 thymocytes + 2.5×10^5 splenocyte) obtained from various mouse strains as listed in the text and figures (Knowles et al., 1979). All cultures were incubated for 18–24 hours in RPMI-10% FCS at 37°C prior to analysis. After incubation, cultures were harvested and CD69 and PD-1 expression were examined on cells pre-gated for expression of thy 1.1 (transduced cells) and the RV-encoded TCR.

For experiments involving stimulation of WT or SOCE-deficient thymocytes, 5×10^5 DP CD69-cells were sorted and co-cultured with 5×10^4 DCs isolated as previously described (Inaba et al., 2009) in RPMI-10% FCS at 37°C for 18 hours in wells with or without plate-bound anti-TCRβ antibody at 3 μg mL^{-1}.

Statistical Analysis

Statistical analysis was performed in Prism (Graph Pad Software) using the unpaired t test or Mann-Whitney test. If the groups that were compared had significantly different variances (P<05 by F test), Welch's correction was applied. **P*<0.05; ***P*<0.01; ****P*<0.001.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

We thank P. Savage for providing Vβ3 and Vβ8.3 transgenic mice; A. Chervonsky for SV-40 transformed fibroblast cell lines; and D. Leclerc and M. Olson for cell sorting. B.D.M. and J.J.B. were supported by an NIH Medical Scientist Training Program grant T32GM007281. Research was supported by NIH RO1 AI038339 and by Digestive Diseases Research Center of Excellence P30DK42086.

REFERENCES

Barton GM, Rudensky AY. Requirement for diverse, low-abundance peptides in positive selection of T cells. Science. 1999; 283:67–70. [PubMed: 9872742]

Bautista JL, Lio CW, Lathrop SK, Forbush K, Liang Y, Luo J, Rudensky AY, Hsieh CS. Intraclonal competition limits the fate determination of regulatory T cells in the thymus. Nature immunology. 2009; 10:610–617. [PubMed: 19430476]

Blackman M, Yague J, Kubo R, Gay D, Coleclough C, Palmer E, Kappler J, Mar-rack P. The T cell repertoire may be biased in favor of MHC recognition. Cell. 1986; 47:349–357. [PubMed: 3490311]

Daley SR, Hu DY, Goodnow CC. Helios marks strongly autoreactive CD4+ T cells in two major waves of thymic deletion distinguished by induction of PD-1 or NF-kappaB. The Journal of experimental medicine. 2013; 210:269–285. [PubMed: 23337809]

Garcia KC, Adams JJ, Feng D, Ely LK. The molecular basis of TCR germline bias for MHC is surprisingly simple. Nature immunology. 2009; 10:143–147. [PubMed: 19148199]

Grubin CE, Kovats S, deRoos P, Rudensky AY. Deficient positive selection of CD4 T cells in mice displaying altered repertoires of MHC class II-bound self-peptides. Immunity. 1997; 7:197–208. [PubMed: 9285405]

Huseby ES, White J, Crawford F, Vass T, Becker D, Pinilla C, Marrack P, Kappler JW. How the T cell repertoire becomes peptide and MHC specific. Cell. 2005; 122:247–260. [PubMed: 16051149]

Inaba K, Swiggard WJ, Steinman RM, Romani N, Schuler G, Brinster C. Isolation of dendritic cells. Current protocols in immunology / edited by John E. Coligan … [et al.]. 2009; Chapter 3 Unit 3 7.

Klein L, Kyewski B, Allen PM, Hogquist KA. Positive and negative selection of the T cell repertoire: what thymocytes see (and don't see). Nature reviews. Immunology. 2014; 14:377–391.

Knowles BB, Koncar M, Pfizenmaier K, Solter D, Aden DP, Trinchieri G. Genetic control of the cytotoxic T cell response to SV40 tumor-associated specific antigen. Journal of immunology. 1979; 122:1798–1806.

Laufer TM, DeKoning J, Markowitz JS, Lo D, Glimcher LH. Unopposed positive selection and autoreactivity in mice expressing class II MHC only on thymic cortex. Nature. 1996; 383:81–85. [PubMed: 8779719]

Lefranc MP, Giudicelli V, Ginestoux C, Jabado-Michaloud J, Folch G, Bellahcene F, Wu Y, Gemrot E, Brochet X, Lane J, et al. IMGT, the international ImMunoGeneT-ics information system. Nucleic Acids Res. 2009; 37:D1006–D1012. [PubMed: 18978023]

Leung MW, Shen S, Lafaille JJ. TCR-dependent differentiation of thymic Foxp3+ cells is limited to small clonal sizes. The Journal of experimental medicine. 2009; 206:2121–2130. [PubMed: 19737865]

Logunova NN, Viret C, Pobezinsky LA, Miller SA, Kazansky DB, Sundberg JP, Chervonsky AV. Restricted MHC-peptide repertoire predisposes to autoimmunity. The Journal of experimental medicine. 2005; 202:73–84. [PubMed: 15998789]

Malchow S, Leventhal DS, Nishi S, Fischer BI, Shen L, Paner GP, Amit AS, Kang C, Geddes JE, Allison JP, et al. Aire-dependent thymic development of tumor-associated regulatory T cells. Science. 2013; 339:1219–1224. [PubMed: 23471412]

McDonald BD, Bunker JJ, Ishizuka IE, Jabri B, Bendelac A. Elevated T cell receptor signaling identifies a thymic precursor to the TCRalphabeta(+)CD4(−)CD8beta(−) in-traepithelial lymphocyte lineage. Immunity. 2014; 41:219–229. [PubMed: 25131532]

Merkenschlager M, Graf D, Lovatt M, Bommhardt U, Zamoyska R, Fisher AG. How many thymocytes audition for selection? The Journal of experimental medicine. 1997; 186:1149–1158. [PubMed: 9314563]

Morita S, Kojima T, Kitamura T. Plat-E: an efficient and stable system for transient packaging of retroviruses. Gene Ther. 2000; 7:1063–1066. [PubMed: 10871756]

Oh-Hora M, Komatsu N, Pishyareh M, Feske S, Hori S, Taniguchi M, Rao A, Ta-kayanagi H. Agonist-selected T cell development requires strong T cell receptor signaling and store-operated calcium entry. Immunity. 2013; 38:881–895. [PubMed: 23499491]

Pobezinsky LA, Angelov GS, Tai X, Jeurling S, Van Laethem F, Feigenbaum L, Park JH, Singer A. Clonal deletion and the fate of autoreactive thymocytes that survive negative selection. Nature immunology. 2012; 13:569–578. [PubMed: 22544394]

Sant'Angelo DB, Waterbury PG, Cohen BE, Martin WD, Van Kaer L, Hayday AC, Janeway CA Jr. The imprint of intrathymic self-peptides on the mature T cell receptor repertoire. Immunity. 1997; 7:517–524. [PubMed: 9354472]

Savage AK, Constantinides MG, Bendelac A. Promyelocytic leukemia zinc finger turns on the effector T cell program without requirement for agonist TCR signaling. Journal of immunology. 2011; 186:5801–5806.

Savage PA, Vosseller K, Kang C, Larimore K, Riedel E, Wojnoonski K, Jungbluth AA, Allison JP. Recognition of a ubiquitous self antigen by prostate cancer-infiltrating CD8+ T lymphocytes. Science. 2008; 319:215–220. [PubMed: 18187659]

Scollay RG, Butcher EC, Weissman IL. Thymus cell migration. Quantitative aspects of cellular traffic from the thymus to the periphery in mice. European journal of immunology. 1980; 10:210–218. [PubMed: 7379836]

Stritesky GL, Xing Y, Erickson JR, Kalekar LA, Wang X, Mueller DL, Jameson SC, Hogquist KA. Murine thymic selection quantified using a unique method to capture deleted T cells. Proceedings of the National Academy of Sciences of the United States of America. 2013; 110:4679–4684. [PubMed: 23487759]

Surh CD, Lee DS, Fung-Leung WP, Karlsson L, Sprent J. Thymic selection by a single MHC/peptide ligand produces a semidiverse repertoire of CD4+ T cells. Immunity. 1997; 7:209–219. [PubMed: 9285406]

Surh CD, Sprent J. T-cell apoptosis detected in situ during positive and negative selection in the thymus. Nature. 1994; 372:100–103. [PubMed: 7969401]

Tourne S, Miyazaki T, Oxenius A, Klein L, Fehr T, Kyewski B, Benoist C, Mathis D. Selection of a broad repertoire of CD4+ T cells in H-2Ma0/0 mice. Immunity. 1997; 7:187–195. [PubMed: 9285404]

Turner VM, Gardam S, Brink R. Lineage-specific transgene expression in hema-topoietic cells using a Cre-regulated retroviral vector. Journal of immunological methods. 2010; 360:162–166. [PubMed: 20600080]

van Meerwijk JP, Marguerat S, Lees RK, Germain RN, Fowlkes BJ, MacDonald HR. Quantitative impact of thymic clonal deletion on the T cell repertoire. The Journal of experimental medicine. 1997; 185:377–383. [PubMed: 9053438]

Vrisekoop N, Monteiro JP, Mandl JN, Germain RN. Revisiting thymic positive selection and the mature T cell repertoire for antigen. Immunity. 2014; 41:181–190. [PubMed: 25148022]

Zerrahn J, Held W, Raulet DH. The MHC reactivity of the T cell repertoire prior to positive and negative selection. Cell. 1997; 88:627–636. [PubMed: 9054502]

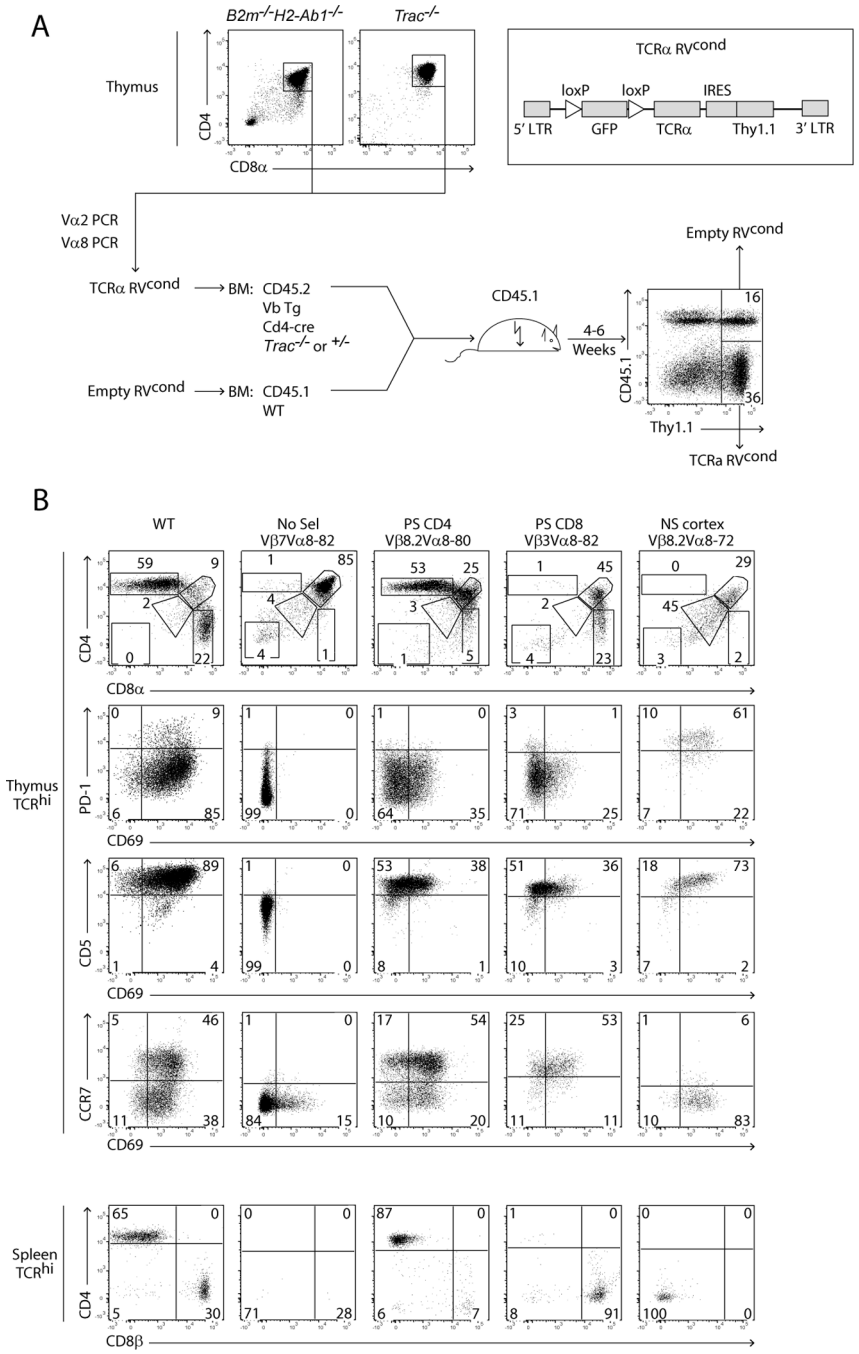

Figure 1. Expression of pre-selection TCRs

A) Pre-selection DP thymocytes were sorted from MHC-deficient or *Trac*$^{-/-}$ mice as shown, and TCRα chains were cloned and inserted into a conditional retroviral vector, as diagrammed. TCRα RV^{cond} contains a loxP-flanked GFP, the TCRα chain of interest, and an IRES-Thy1.1. Only after *Cd4-Cre*-mediated excision of the upstream GFP sequence can the TCRα chain be translated. Bone marrow from TCRVβ transgenic *Cd4-Cre* mice was transduced with TCRα RV^{cond}, and mixed with control CD45.1-congenic WT bone marrow transduced with Empty RV^{cond}, before injection into lethally irradiated, CD45.1-congenic

recipient. B) Representative flow cytometry of the thymus and spleen of mixed RV chimeras, gated as indicated, demonstrating the different types of selection outcomes observed. All plots are pre-gated on transduced (Thy1.1^{+}) CD45.2 cells. The TCR^{hi} gate was determined using antibodies specific for the relevant Vβ and Vα expressed in a given chimera. No Sel, no selection; PS, positive selection; NS, negative selection. Additional information about each TCR can be found in Table S1 and additional controls in Figures S1 and S2. Data shown are representative of more than 100 independent chimeras as detailed in subsequent figures.

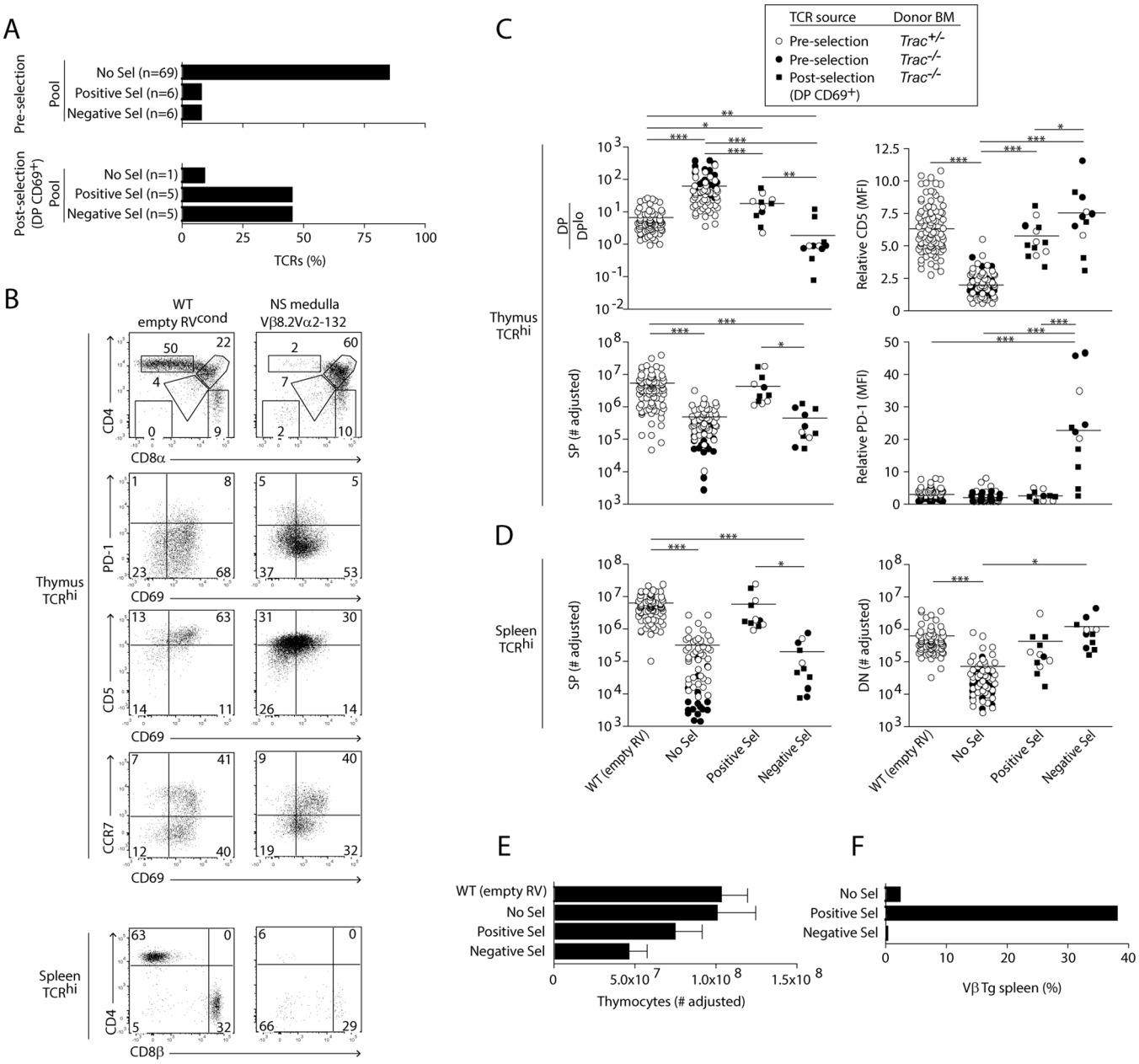

Figure 2. Frequencies of positive selection, negative selection, and No Selection outcomes for pre-selection and post-selection (DP CD69$^+$) TCRs

A) Summary of outcomes. B) Flow cytometry of thymus and spleen from a TCR identified as inducing medullary rather than cortical negative selection. C–D) Summary of all 92 pre-selection and post-selection (DP CD69$^+$) TCRs analyzed in RV chimeras. CD5 and PD-1 expression are presented relative to control amounts in CD69$^-$ DPs. Cell numbers were normalized based on the frequency of transduction in splenic non-T cells. Open and filled circles denote chimeras constructed from *Trac*$^{+/-}$ and *Trac*$^{-/-}$ bone marrow, respectively. E) The numbers of thymocytes were normalized as in C–D. F) TCRα amino acid sequences from TCRs scored as no selection, positive selection, or negative selection were compared with a reference pool of TCRα sequences derived from Vβ-only transgenic sple-nocytes, to

determine the frequency of overlap. p values were calculated by Student's t test. Data pooled from 20 independent experiments.

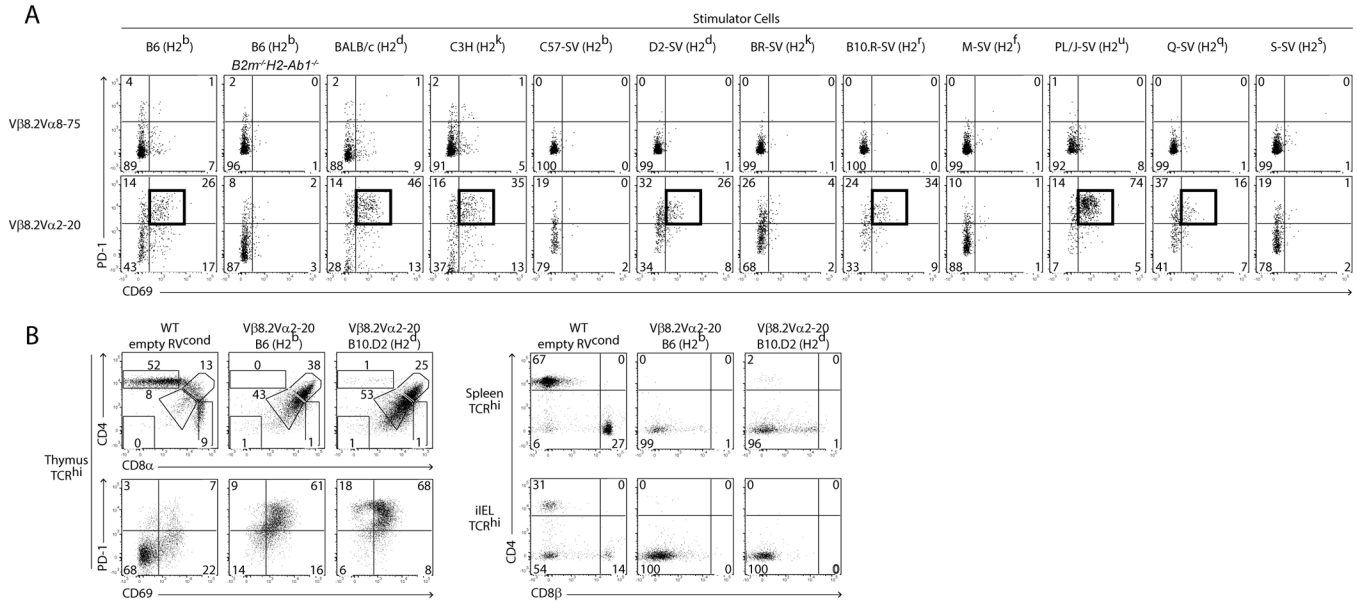

Figure 3. Identification of MHC crossreactive TCRs

A) Unsignaled CD69^{-} PD-1^{-} DP thymocytes from no selection TCR Vβ8.2Vα8-75 or negative selection TCR Vβ8.2Vα2-20 were cultured with a mixture of mouse thymocytes and splenocytes or with SV-40 transformed fibroblasts derived from indicated strains of various MHC haplotypes prior to staining for CD69 and PD-1. Black boxes highlight MHC-reactive TCRs. B) Flow cytometry of Vβ8.2Vα2-20 expressing cells in a B6 (H2^{b}) or B10.D2 (H2^{d}) backgrounds. Similar *in vivo* results were obtained with 3 out of 3 additional TCRs rective against the H2^{b} or H2^{d} backgrounds *in vitro*.

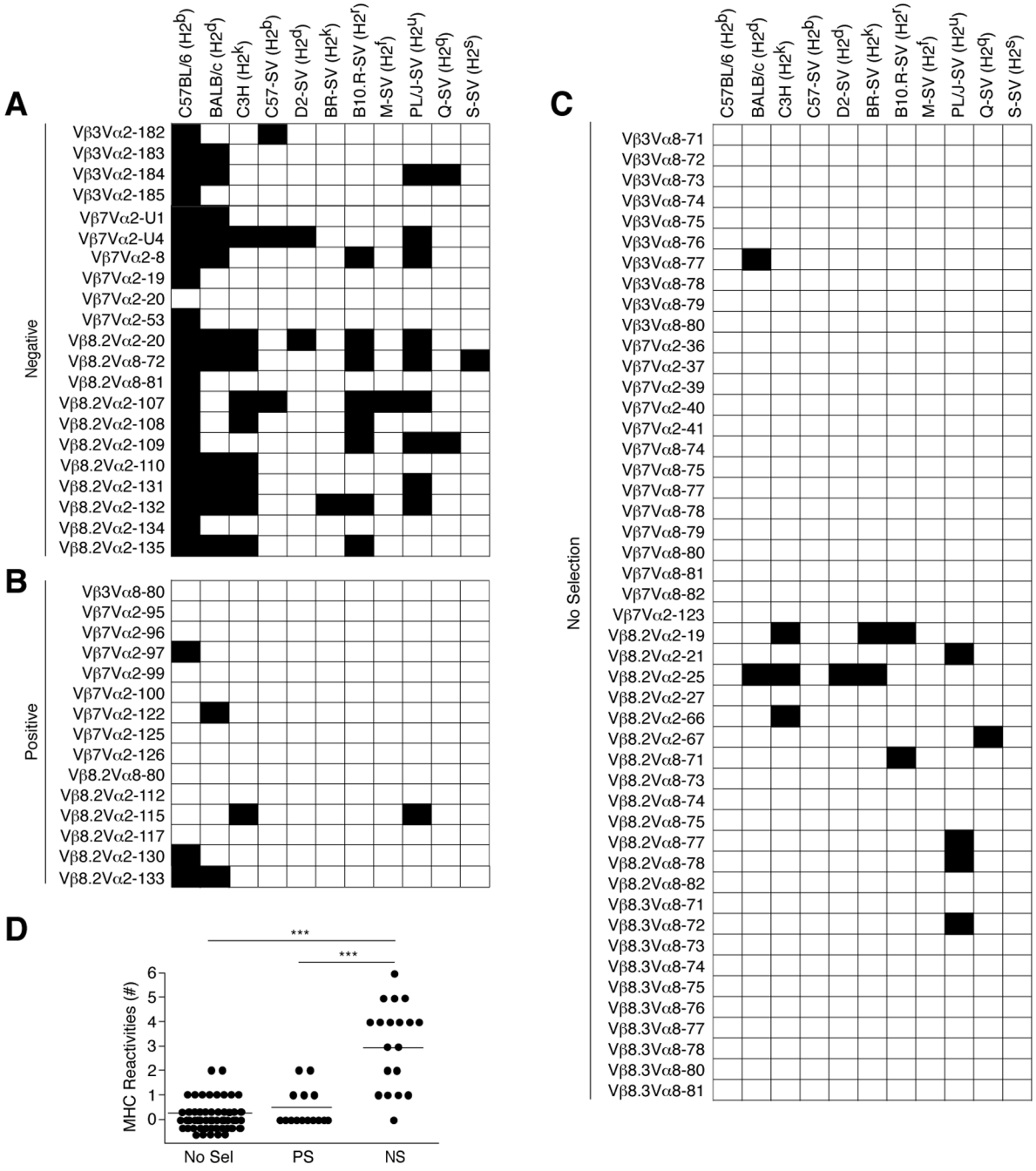

Figure 4. Most negative selection TCRs are MHC crossreactive

TCRs are listed and classified according to their negative selection (A), positive selection (B), or no selection (C) phenotype, determined as in Fig. 3A, with filled box denoting reactivity against corresponding MHC expressed by a mixture of spleen and thymic cells, or by a panel of SV40-transformed fibroblasts as in Fig. 3. MHC reactivity was defined by induction of greater than 10% increase in $CD69^{+}PD\text{-}1^{+}$ cells over MHC-deficient stimulator cells. Most TCRs were from the pre-selection pool, but some TCRs obtained from iIELs or post-selection ($CD69^{+}$) pools (as listed in Table S1) were added to increase the size of the

positive selection and negative selection TCR collections. D) Summary of MHC crossreactivity for TCRs of each group. p values were calculated by Mann-Whitney test. Data pooled from 15 independent experiments.

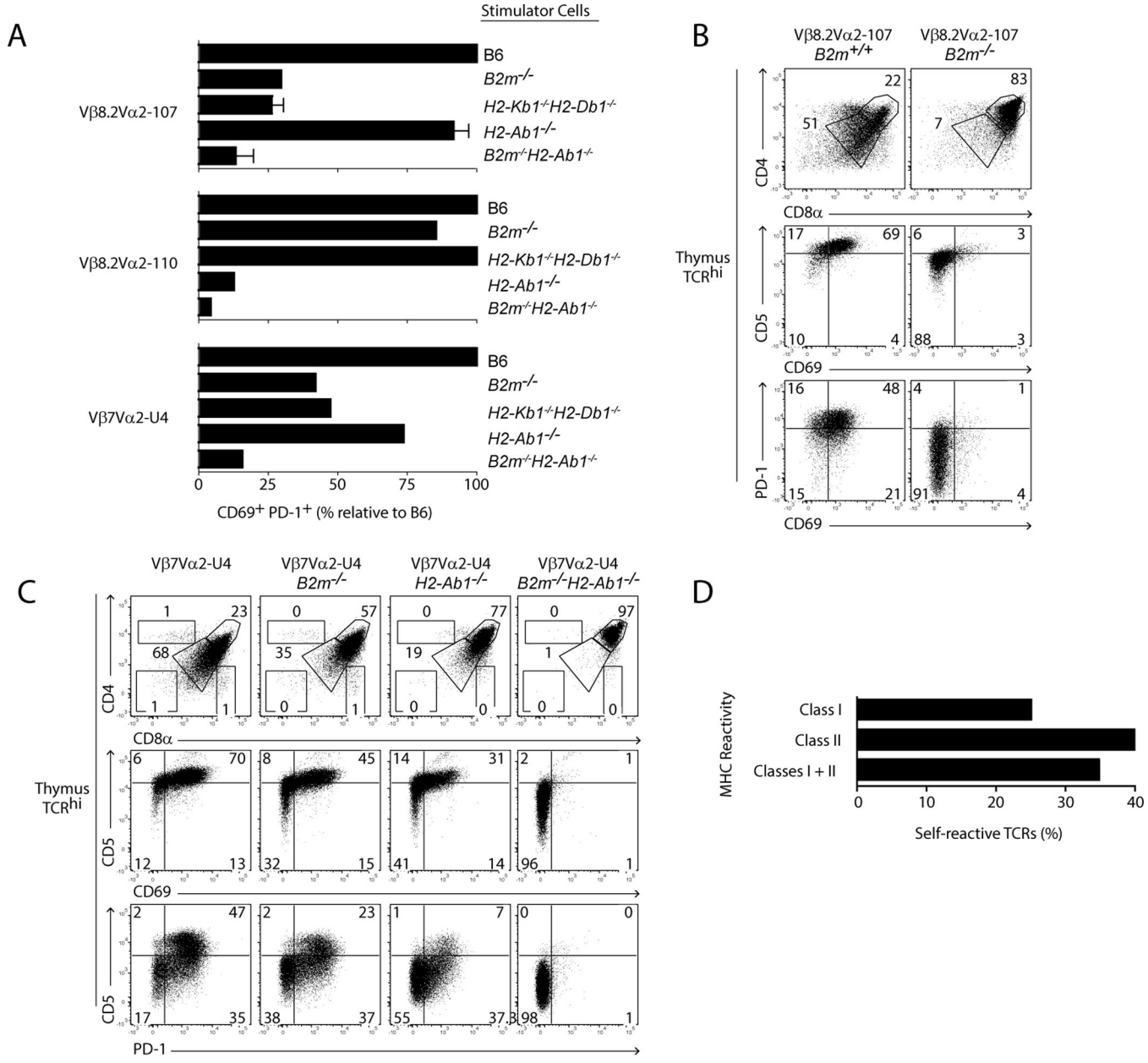

Figure 5. Crossreactive TCRs are frequently reactive against both MHC class I and II

A) Unsignaled CD69^{-} PD-1^{-} DP thymocytes purified from the indicated retrogenic mice were cultured with stimulator cells derived from either WT or various MHC-deficient mouse strains in the B6 background to determine the relative increase in CD69^{+} PD-1^{+} cells. Representative TCRs are shown. B–C) *In vivo* validation by examining thymocyte development in MHC I-deficient, MHC II-deficient, or MHC-deficient backgrounds for Vβ8.2Vα2-107 (B) and for Vβ7Vα2-U4 (C). In total, we confirmed the expected MHC reactivity *in vivo* for 4 out of 4 different TCRs. D) Summary of MHC class I and class II reactivities for 19 negative selection TCRs. Data are pooled from 8 independent experiments.

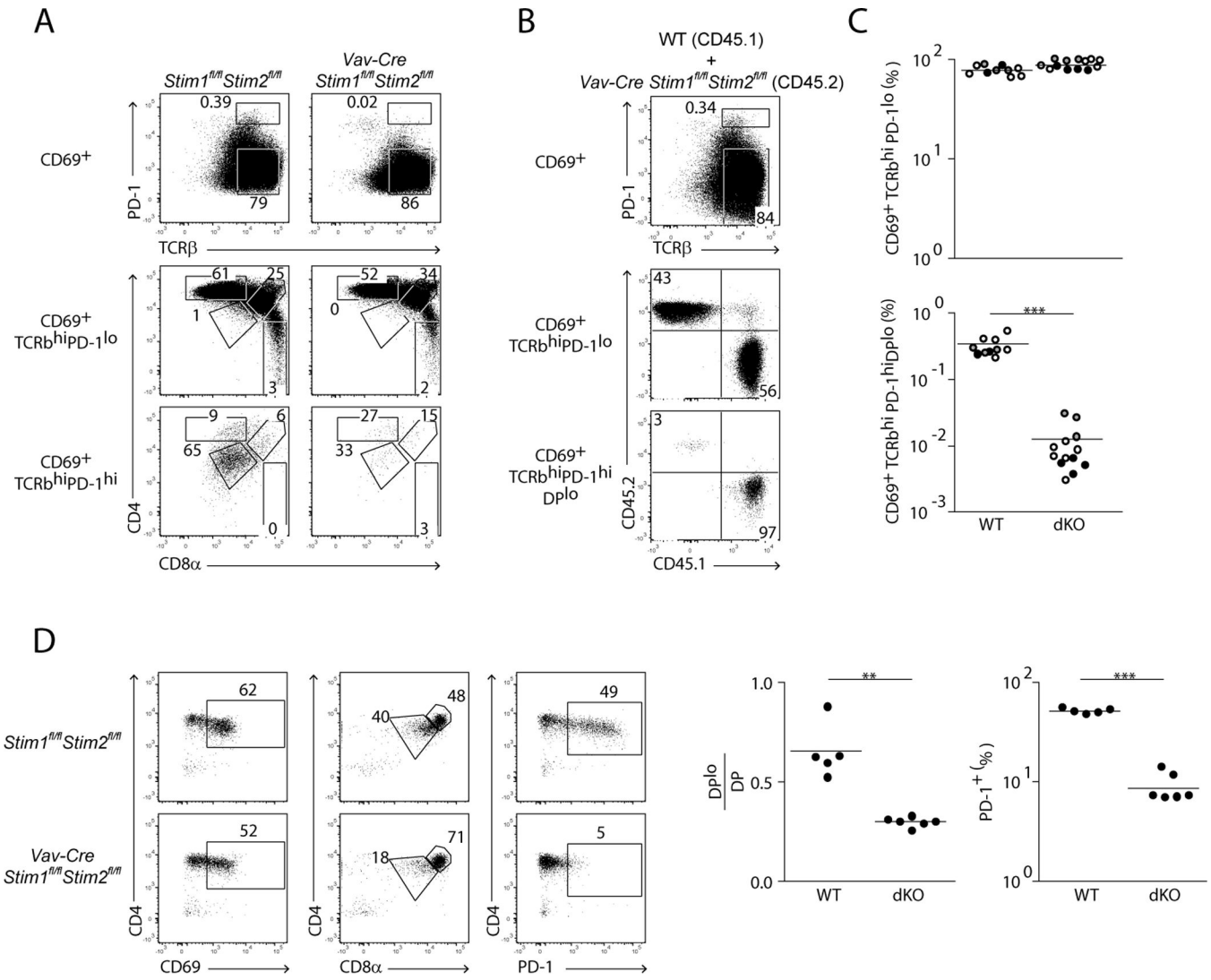

Figure 6. Cortical negative selection requires store-operated calcium entry

A) Flow cytometry analysis of thymus from *Vav-icre Stim1*$^{fl/fl}$*Stim2*$^{fl/fl}$ (dKO) and littermate control mice gated as indicated. B) Flow cytometry analysis of mixed bone marrow chimeras composed of a 1:1 mixture of WT (CD45.1) and *Vav-iCre Stim1*$^{fl/fl}$*Stim2*$^{fl/fl}$ (CD45.2) cells. C) Summary plots with data points pooled from experiments using WT and dKO mice (black circles), mixed bone marrow chimeras (gray filled circles), and non-competitive bone marrow chimeras (open circles). D) Left: Flow cytometry analysis of sorted DP CD69^{-} thymocytes from mice of the indicated genotypes stimulated for 18 hours with plate-bound anti-TCRβ + DCs. Right: Summary plots. p values were calculated by Student's t test. Data pooled from 4 experiments (C) or 2 experiments (D).