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CD3 ITAM diversity is required for optimal T cell receptor signaling and thymocyte development

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Abstract

In order for the αβ or γδT cell receptor chains (TCR) to integrate extracellular stimuli into the appropriate intracellular cellular response, they must utilize the 10 immunoreceptor tyrosine-based activation motifs (ITAMs) found within the CD3 subunits CD3γε, CD3δε, and ζζ) of the TCR signaling complex. However, it remains unclear if each specific ITAM sequence of the individual subunit (γεδζ) is required for thymocyte development or if any particular CD3 ITAM motif is sufficient. Here we show that mice utilizing a single ITAM sequence (γ, ε, δ, ζa, ζb or ζc) at each of the 10 ITAM locations exhibit a substantial reduction in thymic cellularity and limited $CD4^{-}CD8^{-}$ double negative (DN) to $CD4^{+}CD8^{+}$ (DP) maturation due to low TCR expression and signaling. Together, the data suggest that ITAM sequence diversity is required for optimal TCR signal transduction and subsequent T cell maturation.

Keywords

CD3; ITAM; signaling; thymocyte; development; TCR

INTRODUCTION

T-cell receptor (TCR) signaling not only allows peripheral T cells to respond appropriately to foreign antigen through clonal expansion and effector cytokine function, it is also essential in establishing the TCR repertoire through positive and negative selection of thymocytes. Defects in TCR signaling can lead to improper T cell selection and the establishment of autoreactive T cells or a T cell repertoire not suitable for pathogen recognition and clearance (1, 2). The TCR complex consists of either the TCRαβ or γδ heterodimers paired with the CD3γε, CD3δε and ζζ subunits. In order for the short

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cytoplasmic domains of the TCRαβ or γδ heterodimers to transduce extracellular stimuli into intracellular signaling, it must utilize the immunoreceptor tyrosine based activation motifs (ITAMs) within the CD3γε, CD3δε and ζζ dimers (3). The CD3 γ, ε and δ chain each contain a single ITAM motif while each ζ chain contains 3 distinct ITAM domains (ζa, ζb and ζc) for a total of 10 ITAMs with 6 unique sequences. However, there appears to be redundancy in the ability of the γ and δ chains to pair and assemble within the TCR complex (4, 5). This is most likely due to the fact that they have only recently been introduced into the mammalian genome by gene duplication, while the CD3ε and ζ chains are conserved and essential for TCR complex assembly and expression (6–8). Each of these CD3 subunits associates with charged residues in the transmembrane domain of the TCR to form the TCR complex (9–11). Evidence suggests that when the TCR is not engaged, the CD3ε chains are embedded in the inner leaflet of the cell membrane through charged interactions between the membrane proximal basic-rich stretch and the acidic phospholipids (12, 13). The CD3ε cytoplasmic domains become released from the plasma membrane in response to TCR ligation by the cognate peptide/MHC complex. This structural confirmation of the CD3 complex makes the intracellular domains more accessible to the Src family kinases Lck and Fyn, which engage and phosphorylate the CD3 ITAM complex and initiate the downstream signaling cascade ultimately leading to T cell survival, differentiation and effector functions (14–16). Despite the recent advances in our knowledge regarding the initiation of TCR signaling, the processes by which the CD3 chains precisely transduce subtle differences of the peptide/MHC ligands remain unclear.

Ligand-dependent recognition of the TCR by peptide/MHC is necessary to transduce TCR signals at the DP stage of development; however, ligand-independent mechanisms can drive differentiation of DN thymocytes (17, 18). In both ligand dependent and independent TCR signaling, all four of the CD3 subunits are necessary for the transition of DN to DP and beyond, and removal of any of the CD3 chains results in a severe block at the DN stage (7, 19). Recent data indicates that the number of functional ITAMs is important in providing a sufficient TCR signal for efficient negative selection (20). Studies utilizing retroviral mediated gene transfer of mutated CD3 ITAMs into $CD3\varepsilon^{-/-}\zeta^{-/-}$ bone marrow showed that autoimmunity develops in mice when more than 3 ITAMs cannot become phosphorylated (20). Further studies revealed that ITAM number allows for distinct TCR signaling pathways that drive proliferation and cytokine production due to the ability of the CD3 ITAMs to recruit Vav1 and Notch1 (21). Additionally, the removal of the cytoplasmic domain or an entire CD3 subunit greatly diminishes early thymocytes development at the TCRβ selection checkpoint (6, 7, 19) and the number of ITAMs correlates with the efficiency of positive and negative thymocyte selection (20, 22, 23). Evidence suggests that the ITAMs contained in the individual CD3 subunits may allow for differential recruitment of adaptor molecules including ζ-associated protein of 70 kDa (ZAP-70), Grb2 and phospholipase C (PLC)-γ (24). Interestingly, the number and ITAM sequence is also important in the design of chimeric antigen receptors (CAR) that utilize the TCR ζ chain, where studies indicate that ITAM affinity for ZAP-70 can be utilized to optimize potency of CAR reactivity (25). However, it still remains to be determined whether the specific ITAM sequences found within the CD3γε, CD3δε and ζζ subunits are necessary or redundant for optimal TCR signaling, T cell development and function.

In order to address the physiological significance of each ITAM sequence from the γ, ε, δ, ζa, ζb and ζc subunits, we used TCR:CD3 retrogenic technology (26, 27) to generate mice that expressed each of the CD3 ITAM mutants. Each ITAM mutant expresses one particular ITAM sequence or 'single flavor' for all of the 10 ITAM regions within the CD3γε, CD3δε and ζζ subunits. The 'single flavor' CD3 retrogenic model allows us to assess whether there are non-redundant roles for each ITAM sequence in mediating checkpoint survival and ultimately T cell development and function in the periphery. Our data highlight the unique physiological role for individual ITAM motifs in mediating TCR signaling and thymocyte development.

MATERIALS AND METHODS

Mice

Rag1$^{-/-}$ (recombination activating gene 1) and C57BL/6J mice were obtained from Jackson Laboratories. CD3.KO mice lacking all four CD3 chains were generated by crossing *Cd3e*$^{\Delta P/\Delta P}$ and *Cd247*$^{-/-}$ mice as described earlier (6, 20, 28). Nur77^{GFP} mice (29) were obtained from Kristin Hogquist (University of Minnesota) and crossed with CD3.KO mice. All animal experiments were performed in American Association for the Accreditation of Laboratory Animal Care-accredited, specific pathogen-free, helicobacter-free facilities in the St. Jude Animal Resource Center following national, state, and institutional guidelines. Animal protocols were approved by the St. Jude Animal Care and Use Committee.

Generation of CD3 multicistronic vectors

CD3 multiscistronic constructs were generated as described previously (20) (30) (27). Briefly, 2A peptide-linked CD3 constructs were generated by recombinant PCR and cloned into pMIA, an MSCV-based retroviral vector containing an IRES-Ametrine1.1 (Addgene) cassette. Specific ITAM sequences were substituted at each CD3 subunit by recombinant PCR and subsequent CD3 mutant constructs were generated by appropriate subcloning.

Generation of retroviral producer cells

Retroviral producer cell lines were generated as previously described (20) (30) (27). Briefly, HEK-293T cells were transiently transfected with CD3 multicistronic vectors (4 μg), together with packaging and envelope vectors using TransIT LT1 transfection reagent (Mirus). The supernatant containing virus was collected and used to transduce GP+E86 cells in the presence of polybrene (6 μg/ml) every 12h for 3–4 days until viral titer greater than 10^5/ml after 24 h was obtained.

Flow cytometric analysis, intracellular staining and cell sorting

Double positive thymocytes were purified by fluorescence activated cell sorting (FACS) using mAbs against CD4 and CD8 (eBioscience). Peripheral naïve CD4$^+$ T cells were purified by FACS using mAbs against CD4, CD45RB and CD25. DN3 and DN4 thymocytes were stained with mAbs against Mac1, Ter119, Gr1, B220, panNK, CD11b, CD11c, CD4, CD8 and γδ TCR do determine lineage positive populations. For flow cytometric analysis, mAbs against the following molecules were used: CD4 (RM4-5), CD44 (IM7), CD5 (53-7.3), CD8 (53-6.7), CD25 (PC61), CD69 (H1.2F3), B220 (RA3-6B2), TCRβ (H57),

Foxp3 (FJK-16s). Flow cytometric analysis was performed using a LSRII (Becton Dickinson) and data analysis using FloJo.

Transient transfection of HEK-293T cells

Transient transfection of HEK-293T cells was performed as previously described (31) with some modifications. HEK-293T cells were incubated in 6 well plates at 2×10^5/well overnight at 37°C. TCRαβ (2A linked) plasmid (1 μg) and indicated titrated CD3 plasmid (1 μg, 0.25 μg, 0.06 μg). Cells were harvested 40 h after transfection and stained with TCRβ mAbs for flow cytometry analysis.

T cell proliferation assay

$CD4^+CD25^-$ T cells were sorted as described above then plated at 0.2×10^6 cell per well in a round bottom 96 well plate with titrating amounts of plate bound anti-CD3 (2C11) and constant amount of soluble anti-CD28 (0.5μg/ml). After 48 hours, 3H was added to all wells and harvested 12hrs later.

Retroviral-mediated stem cell gene transfer

Retroviral transduction of murine bone marrow cells was performed as described earlier (15, 20, 27, 30). Bone marrow cells (4×10^6 per mouse) were injected via the tail vein into irradiated (500rads) *Rag1*$^{-/-}$ recipient mice. Retrogenic mice were analyzed 4–6 weeks after bone marrow transplant.

Statistical Analysis

Unless otherwise described, all analysis was performed using Prism 5, GraphPad Software. All statistical analysis is specified in figure legends.

RESULTS and DISCUSSION

CD3 ITAM diversity is required for efficient DN3-DN4 transition

Six CD3 ITAM ‘single flavor’ mutants CD3[ITAM-γ], CD3[ITAM-ε], CD3[ITAM-δ], CD3[ITAM-ζa], CD3[ITAM-ζb] and CD3[ITAM-ζc]) were generated to determine the role of ITAM diversity in modulating TCR expression and thymocyte development (Supplemental Fig. 1A). Using retroviral stem-cell mediated gene delivery, we expressed modified CD3 chains in CD3.KO mice (*Cd3e*$^{\Delta P/\Delta P}$:*Cd247*$^{-/-}$ mice lack expression of all four CD3 chains, herein referred to as CD3.KO (6, 15, 20, 28)). Each of the 10 ITAMs were replaced by a single ITAM sequence from either γ, ε, δ, ζa, ζb and ζc chains.

First, we wanted to determine if utilizing a single ITAM sequence for each CD3 chain impacted the assembly and cell surface expression of the TCR:CD3 complex in vitro. All CD3 ITAM mutants were able to pair with the αβTCR subunits and express TCR on the cell surface of 293T cells but with varying efficiency (Supplemental Fig. 1B). Notably, TCR expression on cells that express CD3[ITAM-γ] and CD3[ITAM-δ] single flavor ITAM mutants was approximately 50% of that found on cells expressing WT CD3 ITAMs. However, its unclear to what extent TCR expression on 293T cells can be extrapolated to thymocytes.

We next generated CD3 retrogenic mice (27, 32) to determine the affect of individual CD3 ITAMs in modulating thymocyte development. Thymocyte development not only requires successful α and β TCR chain rearrangement and pairing but also optimal TCR:CD3 complex expression and signaling. The first developmental checkpoint occurs at the DN3-DN4 transition when TCRβ is first expressed on the cell surface along with the pre-TCRα chain (pTα), while the second checkpoint at the $CD4^+CD8^+$ double positive (DP) stage coincides with positive and negative selection (33). Analysis of ITAM mutant thymi revealed a striking decrease in cellularity for all ITAM mutants (Fig. 1A). This result was not attributed to a significant difference in transduction efficiency as determined by ametrine fluorescent protein reporter expression within the B cell compartment (Supplemental Fig. 1C). Flow cytometric analysis of thymocyte subpopulations from ITAM mutant mice revealed a substantial impact on early thymocyte development. Indeed, there was a significant increase in the fraction of DN thymocytes that coincided with a reduction in DP thymocytes in all ITAM mutant mice (Fig. 1B). In particular, there was a significant increase in the percentage of the DN3 ($CD25^+CD44^-$) population compared with a dramatic loss of the DN4 ($CD25^-CD44^-$) population indicating a block at this critical maturation checkpoint. As a result, the DN3:DN4 ratio was significantly increased in all mutants with a dramatic loss in the DN4 cell compartment (Supplemental Fig. 1D). Overall, our data indicate that a combination of diverse ITAM motif sequences are necessary for proper T cell development, and limiting this diversity to a single ITAM sequence for all CD3 subunits negatively may impact pre-TCR signaling and early thymocyte survival leading to a partial block at the first developmental checkpoint.

ITAM diversity is necessary for optimal TCR expression and signaling

We next determined whether a single ITAM sequence could impact the level of TCR expression after β chain rearrangement and positive of selection. For all ITAM mutants except CD3[ITAM-ζc, TCR expression on both CD4 and CD8 SP thymocytes was significantly reduced (Fig. 2A and Supplemental Fig. 1E). Interestingly, TCR expression found on CD3[ITAM-ζc] CD4SP was only slightly reduced, while CD3[ITAM-ζc] CD8SP TCR expression was similar to that of WT cells. Due to the low TCR expression found on all of the ITAM mutants, it is conceivable aberrant TCR signaling caused downregulation of the TCR complex. To visualize intracellular TCR signaling, we utilized the $Nur77^{GFP}$ reporter mouse strain crossed onto the CD3.KO line. $Nur77^{GFP}$ reporter mice have been shown to provide a faithful readout for endogenous TCR signal strength (29). Interestingly, $Nur77^{GFP}$ expression found in CD4 and CD8 SP of most ITAM mutants was significantly lower (Fig. 2B and Supplemental Fig. 1F) indicating attenuated TCR signaling. The only exceptions were CD3[ITAM-ζc] for CD4SP, as well as, CD3[ITAM-ζa], CD3[ITAM-ζb] and CD3[ITAM-ζc] for CD8SP. Strong TCR signaling causes CD5 upregulation and in turn, TCR down-modulation (34). Consistent with the reduced $Nur77^{GFP}$ expression observed, CD5 expression was also significantly lower on CD3[ITAM-ε], CD3[ITAM-γ], CD3[ITAM-δ] and CD3[ITAM-ζa] CD4 SP cells while only CD3[ITAM-ε] and CD3[ITAM-δ] CD8 SP cell had reduced CD5 expression, indicating perturbed TCR signaling (Fig. 2C and Supplemental Fig. 1G). The kenetic signaling model of $CD4^+$ and $CD8^+$ T cell lineage commitment suggests that a cessation of TCR/CD3 signaling during positive selection of $CD4^+CD8^-$ intermediates directs cells to differentiate into CD8SP T cells (35, 36).

Therefore, it is likely that single ITAM sequence usage impairs TCR signaling (as indicted by altered CD5 and Nur77 expression) thus directing a higher percentage of DP thymocytes into the $CD8^+$ T cell lineage. Taken together, these data suggest that CD3 ITAM diversity is necessary for TCR expression and subsequent TCR signaling in CD4 SP thymocytes and optimal CD4 SP development, while CD8 development was less perturbed by single mutant ITAMs.

CD3 ITAM diversity is necessary for peripheral T cell signaling and proliferation

We next evaluated whether the defects observed in thymocyte development affect the establishment and function of peripheral T cells. Splenic and lymph node cellularity were severely reduced in all CD3 ITAM mutant groups with a significant reduction in both CD4 and CD8 ratios compared to wild type ITAMs (Fig. 3A, Fig. 3B and Supplemental Fig. 2A). The reduced TCR expression observed on SP thymocytes was also present in peripheral T cells in the lymph nodes and the spleen (Supplemental Fig. 2B and 2C). Similar to that observed in the thymus, low TCR expression found on peripheral T cells was not a result of an increase in TCR signaling as indicated by Nur77 expression (Fig. 3C and Supplemental Fig. 2D). The generation and survival of $CD4^+Foxp3^+$ regulatory T cells (T_{regs}) requires a high level of TCR recognition between the self-peptide MHC (37, 38). If TCR signaling is diminished in each ITAM mutant group, then we might observe a decrease in the peripheral $CD4^+Foxp3^+$ T_{reg} population. Indeed, many of the CD3 ITAM mutant groups showed reduced percentages of $CD4^+Foxp3^+$ T_{regs} in the lymph nodes, however, only the CD3[ITAM-ζa] mutant had reduced $CD4^+Foxp3^+$ T_{regs} in both spleen and lymph nodes (Fig. 3D). The low TCR expression found on all peripheral T cells also indicates that ITAM ‘single flavor’ T cells may not be able to proliferate in response to TCR stimulation to the same level as wild type T cells. In order to examine proliferative potential, peripheral CD4 T cells from ITAM ‘single flavor’ and WT mice were stimulated by anti-CD3 crosslinking. All ‘single flavor’ groups had significant reductions in proliferative capacity in vitro (Fig. 3E). Collectively, these data suggest that when T cells express a single ITAM sequence, there is limited TCR signaling and proliferative capacity, which may be due in part to the overall decrease in TCR expression.

The high number of ITAMs associated with the TCR:CD3 complex insures that subtle differences in affinity and avidity to peptide/MHC results in the appropriate intracellular signaling responses (39). Previous studies have shown that the number of functional ITAMs allows for the maturation of the immunological synapse and full T cell activation (21). In our current unique study, we have demonstrated that limiting the amino acid sequence of individual ITAMs to a single ITAM sequence for each of the CD3 subunit perturbs TCR signaling and the development of a polyclonal T cell compartment. Astonishingly, switching all of the ITAM sequences to either the γ, ε, δ, ζa, ζb or ζc sequence allows for varying degrees of thymocytes development. In particular, the CD3[ITAM-ζc] and CD3[ITAM-ζb] ITAM sequences saw near normal or normal cell surface TCR expression and TCR signaling for both CD4 and CD8 thymocytes. These data support previous work showing that mutations in the zeta chain ITAMs (a, b and c) alters negative selection of autoreactive T cells (20). Additionally, disrupting a particular ITAM of the ζ chain may impact further phosphorylation of the remaining ζ chains and result in an attenuated TCR signal (40, 41).

Although specific mutations of tyrosine residues within the individual ITAMs of CD3ζ (a–c) allow for qualitatively identical TCR signals (42), our studies suggest that only the expression of the CD3ζ-c ITAM mutant allows for near normal T cell development. Comparing all CD3 chains, the CD3ζ chain associates with the most molecules, which is not surprising considering the 6 ITAMs contained within the CD3ζ chains (39). Although further experimentation utilizing more physiological approaches such as ITAM mutant mice generated via transgenesis or CRISPR/Cas9 will be necessary, our data suggest that the zeta-c ITAM (and to a lesser extent, zeta-b) sequence allows for sufficient binding of adaptor molecules leading to adequate downstream TCR signaling for near normal thymocyte development and basal TCR signaling in the periphery. Nevertheless, ITAM diversity is clearly superior.

The contributions of each individual CD3 subunit in pre-TCR and TCR signaling is still unresolved (19), although data suggests signaling molecules such as ZAP-70, PLC-γ, p85 and Grb2 may be recruited to particular subunits (24, 43, 44). Our data indicates that a specific ITAM sequence not only influences the TCR/CD3 complex formation, but also influences basal TCR signaling and TCR expression. We found that TCR expression is impacted for some but not all CD3 mutants in 293T cells but is significantly lower in thymocytes with all the ITAM mutant groups except with the CD3[ITAM-ζc] single flavor mutant. However, its not clear if reduced TCR expression in this setting is due to assembly/expression issues or the consequences of reduced TCR signaling and T cell selection. Even if it is the former, this would still be a physiologically relevant observation suggesting the CD3 ITAM diversity is required for optimal TCR expresson. These observations may also be due to the inability of the single ‘flavor’ CD3 ITAM mutants to recruit the appropriate adaptor molecules that are necessary for TCR signal transduction. Interestingly, the ζζ dimer contains 6 out of the 10 ITAMs and data suggests sequences found in the ζ chain dimer may be necessary for efficient recruitment of adaptor molecules such as ZAP-70 (45). Indeed, human patients with truncated T cell receptor ζ chain exhibit low TCR expression and develop autoimmunity (46). Additionally, ITAM deficiencies in the CD3ε and CD3γ chains in mice result in a severe block at the DN stage of development (4, 7). Mutating individual ITAMs of the γ chain specifically impairs selection of weak-affinity TCRs (22). It is also important to note that our observations are pertinent given the availability of chimeric antigen receptors (CARs) that make use of the CD3ζ chain to transduce intracellular signals and activate the antigenic cell of interest (47, 48). It may be possible that certain ITAMs with optimal affinities for Zap70 can be used to optimize TCR reactivity to target antigen with a decrease off target effects (25). By substituting all of the ITAM sequences for a single, identical ITAM sequence, our studies have helped clarify the non-redundant role of the 10 ITAM sequences found within the TCR:CD3 complex. Further studies utilizing CD3 mutant ITAM transgenic mice or CRISPR/Cas9 targeted mice will need to be performed to delineate whether a single ITAM sequence can recruit and bind the same level of adaptor molecules, such as ZAP-70, which are necessary for full transduction of the TCR signaling pathway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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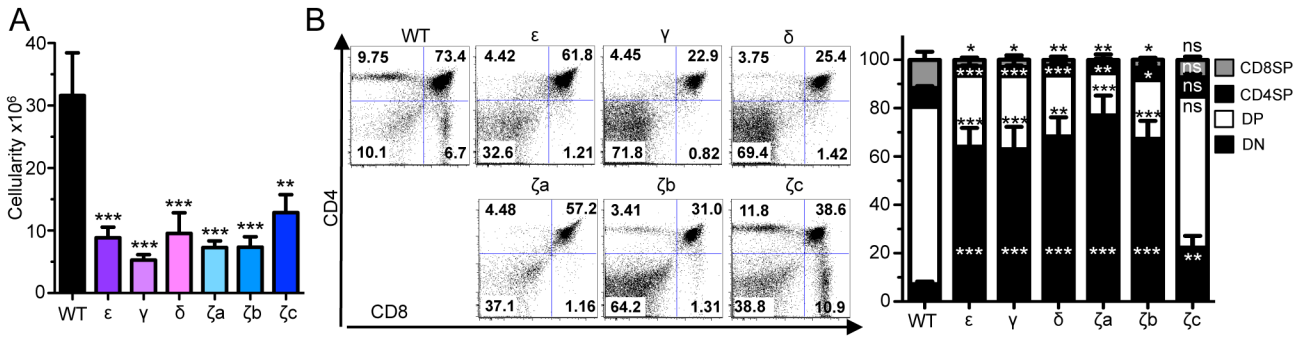

Figure 1. Mice with a single ITAM sequence for all CD3 subunits exhibit major deficiencies in thymic development

(A) *Rag1*$^{-/-}$ mice reconstituted with bone marrow utilizing a single ITAM sequence for the CD3 subunits have reduced thymic cellularity (n=13–15 mice per group, 3 independent experiments, **p<0.01 and ***p<0.001).

(B) Abnormal frequencies of CD4 SP, CD8SP, DN and DP thymocytes in mice expressing single CD3 ITAM sequences. Representative dot plots (left) and ratios (right) (n=13–15 mice per group, at least 3 separate experiments). Statistical Analysis was performed using two-tailed unpaired t-test. *p<0.05, **p<0.01 ***p<0.001.

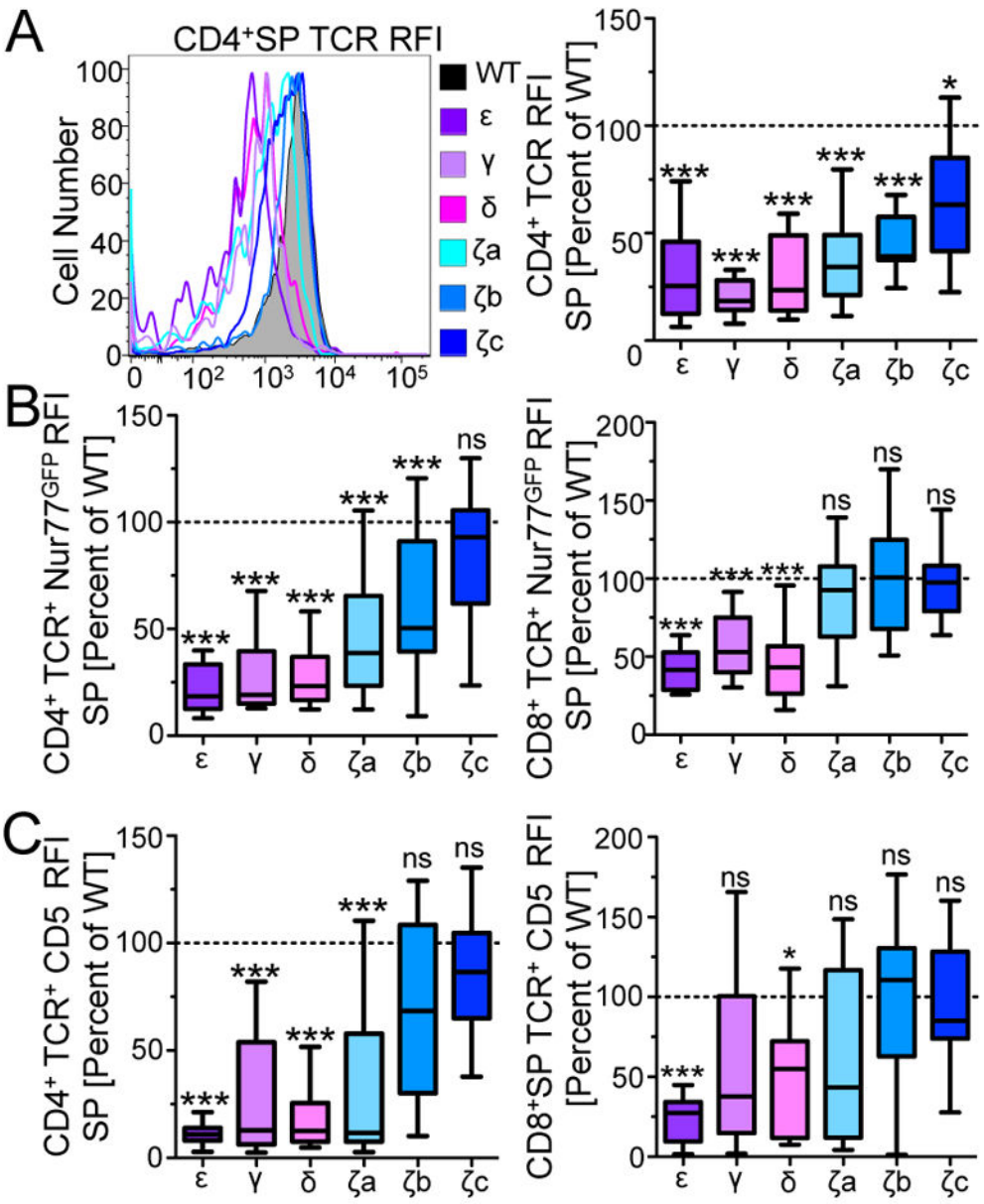

Figure 2. CD3 ITAM diversity mediates optimal TCR expression and basal TCR signaling

(A) CD3εζ deficient *Rag*$^{-/-}$ bone marrow was transduced with WT or 'single flavor' ITAM CD3. WT and 'single flavor' ITAM CD4^{+} single positive thymocytes TCR expression was measured. Representative histograms are shown (left). Expression is graphed as relative to the WT CD3 ITAM transduced control (on right, n=10–15 mice per group, 3 independent experiments).

(B) Nur77 expression (RFI) was calculated for CD4^{+} (left) and CD8^{+} (right) SP thymocytes. Expression is graphed as relative to the WT CD3 ITAM transduced control. (n=10–13 mice per group, 3 independent experiments).

(C) CD5 expression (MFI) was calculated for CD4^{+} (left) and CD8^{+} (right) SP thymocytes. Expression is graphed as relative to the WT CD3 ITAM transduced control. (n=10–13 mice per group, 3 independent experiments). RFI – Relative Fluorescent Intensity. Statistical

Analysis was performed using a one-sample *t*-test. ***p<0.001, *p<0.05 and ns: not significant.

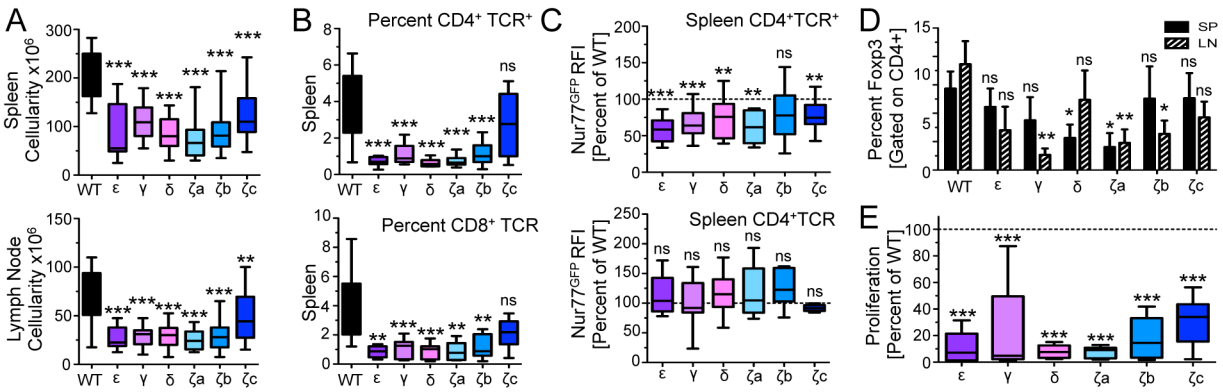

Figure 3. CD3 ITAM diversity impacts peripheral T cell and T_{reg} development

(A) Total spleen and peripheral lymph node cell numbers were calculated for each *Rag*$^{-/-}$ mouse reconstituted with single flavor ITAMs and WT CD3 ITAM transduced bone marrow. Statistical Analysis was performed using a one-sample *t*-test (n=10–13 mice per group, 3 independent experiments). ***p<0.001 and **p<0.01.

(B) Spleen CD4$^+$ and CD8$^+$ ratios and (C) Nur77 expression were calculated for each *Rag*$^{-/-}$ mouse reconstituted with single flavor ITAMs and WT CD3 ITAM transduced bone marrow. Expression is graphed as relative to the WT CD3 ITAM transduced control. Statistical Analysis was performed using a one-sample *t*-test (n=10–13 mice per group, 3 independent experiments). ***p<0.001, **p<0.01, *p<0.05 and ns: not significant.

(D) The percentages of Foxp3$^+$ CD4$^+$ T cells in single flavor ITAM retrogenic mice were analyzed 5 weeks post-bone marrow transfer. Ametrine$^+$ TCRβ$^+$ CD4$^+$ Foxp3$^+$ T cells in the spleens and lymph nodes were calculated (n=12–15 in each group). Statistical Significance: *<0.05, **<0.01, and ns: not significant, two-tailed student's t-test.

(E) The proliferation of CD4$^+$ T cells in single flavor ITAM retrogenic mice was analyzed 5 weeks post-bone marrow transfer. MACS-enriched peripheral CD4$^+$ T cells were activated in vitro with plate-bound CD3ε (1ug/ml each) for 72 hrs. The incorporation of ^{3}H were analyzed. Proliferation is graphed as relative to the WT CD3 ITAM transduced control. Compiled data from 5 experiments. Statistical Significant ***<0.001, One-Way ANOVA, Bonferroni's Multiple Comparison test.