Extended Experimental Procedures

Animals

C57BL/6 (B6), $\beta 2m^{KO}$, $RAG2^{KO}$, Lck^{KO}, Fyn^{KO}, $TCR\alpha^{KO}$ and $CD4^{KO}$ mice were purchased from The Jackson Laboratories (Bar Harbor, ME). B10.A mice were obtained from NCI-Frederick, MD. $CD8\alpha^{KO}$and $I\text{-}A\beta b^{KO}$ mice were bred in our own animal colony, as were AND TCR transgenic mice (Kaye et al., 1989), P14 TCR transgenic mice (Pircher et al., 1989), and hBcl-2 transgenic mice (Linette et al., 1996). CD4 transgenic mice expressing full length 444 or tailless 44T transgenic CD4 proteins were generated as previously described (Van Laethem et al., 2007), as were $CD155^{KO}$ mice (Maier et al., 2007). Animal care was in accordance with National Institutes of Health (NIH) guidelines.

New transgenic mouse strains constructed for this study were generated by cloning gene-specific cDNAs into the human CD2 transgenic vector to achieve T cell specific expression. For wildtype and mutant Lck transgenes, full-length Lck cDNA was cloned from purified B6 αβT cells in which residues C20 and C23 were both mutated to alanine using the QuikChange XL site-directed mutagenesis kit (Stratagene, Torrey Pines, CA). cDNAs encoding Lck^{wt} and Lck^{mut} proteins were each cloned into the transgenic vector and injected into B6 oocytes. For TCR transgenes, full-length cDNAs encoding the rearranged TCRα and TCRβ chain of each TCR (A11, B12A, B12F) were separately cloned into TCRα and TCRβ vectors that were then injected together into B6 oocytes. For A11 TCR transgenes with CDR2 mutations, residues Y48 and E54 in the cDNA encoding the A11 TCRβ chain were individually mutated to alanine, cloned into transgenic vectors, and injected together with the A11 TCRα transgenic vector into B6

oocytes. At least two independent founder lines for each transgene were generated and analyzed.

Antibodies

MAbs with the following specificities were used in the present study: CD4 (GK1.5 or RM4.4), TCRβ (H57-957), CD5 (53-7.3), CD8α (53-6.7), CD69 (H1.2F3), Vβ3 (KJ25), Vβ8 (F23.1), CD24 (M1-69), CD62L (MEL-14), CD44 (IM7), CD45 (30-F11) were obtained from BD Biosciences (San Jose, CA); CD155 (TX56), CCR7 (4B12), EpCAM (G8.8), I-A/I-E (M5/114.15.2) were from eBioscience (San Diego, CA); UEA-1 was from Vector Laboratories (Burlingame, CA); phosphotyrosine (4G10) was obtained from Millipore (Billerica, MA); anti-ZAP70 (clone 29) from BD Transduction Laboratories, Lck (3A5 mouse mAb) from Santa Cruz Biotechnology; and TCRζ (H146-968 , and serum 551).

Flow cytometry and electronic cell sorting

Cells were analyzed on a LSRII or LSRFortessa (BD Biosciences) and dead cells were excluded by forward light-scatter and propidium-iodide uptake. Electronic sorting was performed using an ARIAII.

Calcium mobilization

Thymocytes were loaded with the calcium sensitive dye Indo-1 (1.8 mM) at 31^{o}C, coated at 4^{o}C with biotinylated mAbs specific for TCRβ and CD4 (GK1.5), labeled with fluorescent anti-CD4 (RM4.4) and anti-CD69. Two minutes prior to stimulation, cells

were warmed and applied to the flow cytometer where this temperature was maintained throughout the analysis. Antibody crosslinking was induced with avidin (4μg/ml).

Immunoprecipitations and immunoblotting

Fresh thymocytes were solubilized in lysis buffer containing 1% Nonidet P-40, 10mM Tris-HCl, pH 7.2; 140mM NaCl; 2mM EDTA, 1mM NaF, 1mM orthovanadate and protease inhibitors. Immunoprecipitates were resolved by SDS-PAGE under reducing conditions with the exception of anti-TCR immunoprecipitations performed to detect associated Lck proteins which were resolved by SDS-PAGE under non-reducing conditions. After transfer, nitrocellulose membranes were incubated with specific antibody followed by incubation with either secondary antibody or protein A that was conjugated to horseradish peroxidase. Reactivity was revealed by enhanced chemiluminescence (Western Lightning ECL, PerkinElmer).

T cell proliferation and activation

To test T-cell reactivity, purified αβ LNT responder cells ($1x10^5$/well) were labeled with CFSE and co-cultured for 4d with LPS activated and irradiated splenic B stimulator cells ($2x10^5$/well). For *in vivo* proliferation, $5\text{-}10x10^6$ CFSE-labeled T cells were injected into unmanipulated lymphopenic host mice. IL-2 production by T-hybridomas were measured by standard ELISA.

Construction of mixed bone marrow chimeras

10^7 T cell-depleted bone marrow stem cells from each donor were injected into sub-lethally irradiated (6 Gy) RAG^{KO} host mice and analyzed 8-10wks later.

Thymic epithelial cell isolation. Thymi were teased apart to release all thymocytes and then treated with collagenase/DNAse/dispase for 4 sequential digestions at 37^{o}C for 10 minutes each (Gray et al., 2008). Cells were then stained with MAb specific for EpCAM, CD45, MHCII, Ly51 and UEA-1, to distinguish cortical and medullary thymic epithelial cells.

Real-time quantitative PCR. RNA was isolated from epithelial cells and thymocytes using the RNEasy protocol (Qiagen) and then reverse transcribed to cDNA by oligo(dT) priming with the SuperScriptTM III First-Strand Synthesis System (Invitrogen). Genomic DNA was removed using DNA-free kit (Ambion) and amplification of gene-specific products was done using the following TaqMan probes for the house keeping gene Rpl13a, *Rpl13A* (Mm01612986_gH) and the following murine genes: CD155, *Pvr* (Mm00493398_m1); ThPOK, *Zbtb7b* (Mm00784709_s1); CD40L, *Cd40lg* (Mm00441911_m1); T-bet, *Tbx21* (Mm00450960_m1); PLZF, *Zbtb16* (Mm01176865_m1) and Sox13, *Sox13* (Mm00488352_m1).

Distal Runx3d expression was assayed using the SYBR green detection system (Qiagen). Gene expression values were normalized to values of Rpl13a in the same sample and using the following primer sets:

Runx3d forward 5'-GCGACATGGCTTCCAACAGC-3'

Runx3d reverse 5'-CTTAGCGCGCCGCTGTTCTCGC-3'

Rpl13A forward 5’-CGAGGCATGCTGCCCCACAA-3’

Rpl13A reverse 5’-AGCAGGGACCACCATCCGCT-3’

Supplemental References

Gray, D.H., Fletcher, A.L., Hammett, M., Seach, N., Ueno, T., Young, L.F., Barbuto, J., Boyd, R.L., and Chidgey, A.P. (2008). Unbiased analysis, enrichment and purification of thymic stromal cells. J Immunol Methods *329*, 56-66.

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Linette, G.P., Li, Y., Roth, K., and Korsmeyer, S.J. (1996). Cross talk between cell death and cell cycle progression: BCL-2 regulates NFAT-mediated activation. Proc Natl Acad Sci U S A *93*, 9545-9552.

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Pircher, H., Burki, K., Lang, R., Hengartner, H., and Zinkernagel, R.M. (1989). Tolerance induction in double specific T-cell receptor transgenic mice varies with antigen. Nature *342*, 559-561.

Supplemental Figure Legends

Figure S1. Lck sequestration model of MHC restriction, Related to Figure 1. In preselection DP thymocytes, all available membrane Lck is bound to CD4 and CD8 coreceptor proteins. Consequently, only TCR that co-engage their thymic selecting ligand together with one or the other coreceptor can access coreceptor-associated Lck to initiate TCR signaling of thymic selection. Because CD4 and CD8 coreceptors bind to pMHC-II and pMHC-I complexes, respectively, only MHC-restricted TCR can signal thymic selection, resulting in generation of an exclusively MHC-restricted TCR repertoire. In contrast, MHC-independent TCR that engage non-MHC selecting ligands in the thymus are unable to signal thymic selection because they cannot access Lck. However, in the absence of coreceptors, Lck is not sequestered so that MHC-independent TCR can signal thymic selection.

Figure S2. Signaling of thymic selection by Lck^{wt} and Lck^{mut} proteins in mice containing the Bcl-2^{Tg}, Related to Figure 1.

(A) Thymocyte profiles from mice containing only Lck^{wt} or Lck^{mut} proteins. Thymus cellularity is indicated by the numbers on top of each plot which display the mean $\pm$ SE of nine mice in each group. Data are representative of 9 experiments.

(B) Numbers of γδ and αβ T cells in lymph nodes of Bcl-2^{Tg} mice containing only Lck^{wt} or Lck^{mut} proteins. Mean $\pm$ SE (n=9 mice/group).

***, $p<0.001$; ****, $p<0.0001$.

Figure S3. αβTCR and thymocytes from $Quad^{KO}$ mice, Related to Figure 3.

(A) Reactivity against MHC^{KO} stimulators of two T-hybridomas (named A11 and B12) that were derived from $Quad^{KO}Bcl\text{-}2^{Tg}$ mice. Data are expressed relative to stimulation with immobilized anti-TCRβ (1μg/ml) and represent the mean ± SE of triplicate cultures. Data are representative of 3 experiments.

(B) TCR expressed by A11 and B12 T cell hybridomas. One TCRαβ (named A11) was cloned from the A11 T-hybridoma; two TCRαβ (B12A and B12F which utilized different TCRα but the identical TCRβ) were cloned from the B12 T-hybridoma. TCR sequences were analyzed using IMGT software and summarized as displayed.

(C) CD155 is the ligand for both A11 and B12A TCR. TCR-negative 4G4 cells that had been retrovirally transduced to express A11, B12A, and B12F TCR were assessed for their reactivity against MHC^{KO} and $MHC^{KO}CD155^{KO}$ stimulator cells. Data are representative of 2 experiments.

(D) Profiles of thymocytes expressing MHC-restricted transgenic TCR in the presence and absence of MHC and coreceptors. Thymocyte profiles from wildtype mice (black lines) and $Quad^{KO}Bcl\text{-}2^{Tg}$ mice (red lines) expressing AND or P14 MHC-restricted TCR transgenes. Numbers in the CD5 profiles indicate CD5 MFI. Numbers in the CD69 and CCR7 profiles indicated the frequencies of $CD69^{+}$ and $CCR7^{+}$ thymocytes. Thymus cellularity is indicated on top of the CD4/CD8 profiles.

Figure S4. Expression of genes characteristic of innate and γδ T cells, Related to Figure 4.

Expression of *Zbtb16* mRNA which encodes PLZF and *Sox13* was determined by quantitative PCR in the indicated sorted cell populations. A11 and B12A LN T cells were from $Quad^{KO}RAG^{KO}Bcl\text{-}2^{Tg}$ host mice.

Figure S5. Thymocyte differentiation and lineage-specific gene expression stimulated by MHC-independent and MHC-restricted TCR transgenes, Related to Figure 5.

(A) Five stages of thymocyte development (annotated I through V) were defined according to CD69 and CCR7 expression as indicated. CD4 vs CD8α expression was plotted for thymocytes at each of these developmental stages. Ungated thymocyte plots are shown in extreme right panels.

(B) Expression of helper- and effector-lineage genes in LN T cells generated by A11 and B12A MHC-independent TCR in $MHC^{KO}RAG^{KO}Bcl\text{-}2^{Tg}$ mice. $CD4^+$ and $CD8^+$ LN T cells were purified by electronic sorting and assessed for expression of the indicated genes by quantitative PCR.

(C) Early Lck sequestration impairs γδT cell generation. γδ T cell numbers in the LNs of $Quad^{KO}$, 444. $Quad^{KO}$, and 44T. $Quad^{KO}$ mice. Mean $\pm$ SE (n=10 mice/group). ****, $p<0.0001$.