Online Methods

Mice

C57BL/6J, C57BL/6-FoxP3-GFP (B6.Cg-Foxp3tm1Mal/J), *Tcra*$^{-/-}$ (B6.129S2-Tcratm1Mom/J), *Add2*$^{-/-}$ (B6.129S4-*Add2*^{tm1Llp}/LlpJ) [51], *Padi4*$^{-/-}$ (B6.Cg-*Padi4*$^{tm1.1Kmow}$/J) [52] mice were purchased from The Jackson Laboratory (Bar Harbor, ME). YAe62β mice have been previously described [53]. For flow cytometric analysis mice both male and female mice were analyzed between 1-8 weeks of age. For deep sequencing experiments, male and female mice were used at 2 and 8 weeks of age. Bone marrow chimeric mice were generated as previously described [53], and analyzed at 6-8 weeks post transfer. Mice expressing FoxP3-GFP and the YAe62 TCRβ chain, and all mouse sub-lines were maintained in a specific pathogen-free environment in accordance with institutional guidelines in the Animal Care Facility at the University of Massachusetts Medical School.

Antibodies

anti-TCRβ (clone H57-597) cat# 109220; anti-CD62L (clone MEL-14) cat# 104424, anti-PD-1 (clone 29F.1A12) cat# 135210, anti-CD25 (clone PC61) cat# 102038, anti-Nrp1 (clone 3E12) cat# 145211, anti-H-2 K^b (clone AF6-88.5) cat# 116515, anti-GITR (clone DTA-1) cat# 126318, anti-FR4 (clone 12A5) cat# 125009, anti-CD73 (clone TY/11.8) cat# 127223, anti-XCR1 (clone ZET) cat# 119505, anti-CD5 (clone 53-7.3) cat# 100624 Biolegend; anti-CD4 (clone GK1.5) cat# 552051, anti-mVα2 (clone B20.1) cat# 562944, anti-CD44 (clone IM7) cat# 560780, anti-CTLA4 (clone UC10-4F10-11) cat# 553720, anti-CD11c (clone HL3) cat# 562454, anti-CD11b (clone M1/70) cat# 557657, anti-CD23 (clone B3B4) cat# 553139 BD Pharmingen; anti-CD8 (clone 5H10) cat# MCD0817 ThermoFisher; anti-B220 (clone RA3-6B2), anti-Foxp3 (clone FJK-16s), anti-CD69 (clone H1.2F3), anti-F4/80 (clone BM8) cat# 25-4801-82, anti-MHCII (clone M5/114) cat# 50-112-9473, anti-IgM (clone eB121-15F9) cat# 11-5890-82, anti-CD24 (clone M1/69) cat# 47-0242-82, anti-CD19 (clone 1D3) cat# 48-0193-82 eBioscience.

Cell surface and intracellular staining and tetramer enrichment

Murine thymocytes, lymph node cells and splenocytes from 1-8 weeks old mice were stained with the following cell antibodies: anti-TCRβ (H57-597), anti-CD62L (MEL-14), anti-PD-1 (29F.1A12), anti-CD25 (PC61), anti-Nrp1 (3E12), anti-H-2 K^b (AF6-88.5), anti-GITR (DTA-1), anti-FR4 (12A5), anti-CD73 (TY/11.8) Biolegend; anti-CD4 (GK1.5), anti-mVα2 (B20.1), anti-CD44 (IM7), anti-CTLA4 (UC10-4F10-11) BD Pharmingen; anti-CD8 (5H10) ThermoFisher; anti-B220 (RA3-6B2), anti-Foxp3 (FJK-16s), anti-CD69 (H1.2F3) eBioscience. All antibodies were used at a 1:300 dilution unless otherwise stated. Tetramer staining was carried out in the presence of 50nM dasatinib (Sigma), cells were incubated with dasatinib for 30min at 37c, then incubated for 2hrs with 10μg/mL MHCII tetramer at 37c with additional antibodies added for the last 30min. For intracellular CTLA-4 levels, cells were fixed and permeabilized using the FoxP3 Transcription factor staining buffer set (eBiosciences) at 4c following the manufactures protocol, and subsequently stained overnight with anti-CTLA-4 (1:200 dilution) and anti-Foxp3 (1:200 dilution) at 4c. Analysis of flow cytometric data was performed using FlowJo version 9.9.6 (TreeStar). For analyses of IA^b-Add2 specific T cells using tetramer enrichment, thymocytes and splenocytes were incubated with 10ug/ml IA^b-Add2 tetramer coupled to PE for 60min at 22c, then column enriched using anti-PE MACS beads (Miltenyi Biotech) following established protocols [54]. An example of tetramer analyses is shown in Supplementary Fig. 7.

Thymocyte, T cell and APC sorting

Thymocytes and splenocytes were from freshly isolated from TCRC$\alpha^{+/-}$.Foxp3-GFP and TCRC$\alpha^{+/-}$.YAe62b.Foxp3-GFP mice, and sorted for thymic CD4SP and splenic CD4 T_{conv} cells (TCRβ^+, CD4$^+$, CD8^{neg}, CD25^{neg}, Foxp3-GFP^{neg}, B220^{neg}) cells, and thymic and splenic tT$_{reg}$ cells (TCRβ^+, CD4$^+$, CD8^{neg}, Foxp3-GFP$^+$, CD8^{neg}, B220^{neg}) (FACS Aria, BD Biosciences). In some experiments, thymocytes and splenocytes populations of were also sorted using IA^b-Padi4 tetramer$^+$ staining. For DC and macrophage subset isolation, spleens were minced, and digested with 2mg/ml Collagenase D (Roche), 20µg/ml DNAse 1 (Roche) in RPMI media supplemented with 10% FCS and 0.1 M EDTA fro 30min at room temp. Following digestion, splenic slurry was filtered through a 70µm mesh filter, washed and centrifuged over a 15%/12% (w/v) OptiPrep density gradient for 15 min at room temp. and low density cells isolated for flow cytometry sorting. Splenic APC subsets were sorted based on CD19^{neg} TCRβ^{neg} and the following cell surface markers; cDC1: MHCII$^+$ CD11c$^+$ XCR1$^+$ CD11b^{neg}; cDC2: MHCII$^+$ CD11c$^+$ XCR1^{neg} CD11b^{low}; Macs: CD11b^{hi} F4/80$^+$ CD11c^{neg}. All B cells were sorted by CD19$^+$ TCR^{neg}, then CD5^{hi} B cells; remaining CD5^{lo} B cells were gated for CD24^{hi} Transitional B cells, while CD24^{lo} cells were divided into Follicular B cells, CD23$^+$ IgM$^+$ and Marginal zone B cells, CD23^{lo} IgM^{hi}. Thymic APC subsets were enriched by staining with MHCII APC and anti-APC MACS beads (Miltenyi) and subsequently flow sorted using based on CD19^{neg} TCRβ^{neg} and the following cell surface markers; pDC: CD11c^{lo} B 220$^+$; cDC CD11c$^+$ XCR1$^+$ and CD11c$^+$ XCR1^{neg}. Thymic B cells were sorted by CD19$^+$ B220$^+$ TCR^{neg}. All sorted T cell populations were greater than 99% pure, while APC subsets were greater than 98% pure.All sorted T cell populations were greater than 99% pure, while APC subsets were greater than 98% pure.

TCR cloning and generation of T cell transfectomas

Thymic CD4SP and tT$_{reg}$ cells were sorted from 2 week old TCRC$\alpha^{+/-}$.Foxp3-GFP and TCRC$\alpha^{+/-}$.YAe62β.Foxp3-GFP mice. For thymocytes that express the YAe62β chain, cells were directly sorted into Trizol (Invitrogen), RNA was isolated by precipitation with RNase free glycogen (Invitrogen) following the manufactures protocol. cDNA was prepared using oligo-dT's (Promega) and Omniscript RT kit (Qiagen). cDNA was PCR amplified for rearranged TCRα chains using a set of PCR primers corresponding to Vα 2, 4, 8 and 11 gene families. Amplified rearranged TCR Vα genes were PCR cloned as a fusion with the TCR Cα region into an MSCV based retroviral expression vector. Plasmids were sequenced and only novel TCRs without amplification errors were further studied. For thymocytes that express polyclonal TCRβ chains, sorted cells were cultured in 24 well plates coated with αCD3/αCD28 and 20U/ml of rIL-2 for six days, and converted into hybridomas as previously described [53]. TCRβ chain gene family use was determined by flow cytometry analyses. To clone TCRα and TCRβ chains, RNA was isolated from T cell hybridomas, converted to cDNA, and TCRα and TCRβ chains were PCR amplified using a set of degenerated PCR primers corresponding to all mouse Vα and identified Vβ gene family. Amplified products were cloned as fusions with the TCR Cα or TCR Cβ region into an MSCV based retroviral expression vector.

The following PCR primers were used:

Vα1 GGGAATTCTCCSTACACATCAGAGACTC
Vα2 GGGGGGAGATCTCGAGCCACCATGGACACGATCCTGACAGCA
Vα4 GGGGGGAGATCTCGAGCCACCATGGACTCTTCTCCAGGC

Vα8 GGGGGGAGATCTCGAGCCACCATGCGTCCTGTCACCTGCTCAGTTCTTGTGCTC
Vα11 GGGGGGGAGATCTCGAGCCACCATGCAGAGGAACCTGGGAGCT
TCR Cα CTGGTACACAGCAGGTTCCGGATTCTGGATGT

Vβ2 GGGGGGGAATTCGTCGACGAGCCACCATGTGGCAGTTTTGCATT
Vβ4 GGGGGGGAATTCGTCGACGAGCCACCATGGGCTCCATTTTCCTC
Vβ8 GGGGGGGAATTCGTCGACGAGCCACCATGGGCTCCAGACTCTTC
Vβ11 GGGGGGGAATTCGTCGACGAGCCACCATGGCCCCTAGGCTCCTT
TCR Cβ - CTTGGGTGGAGTCACATTTCTCAGATCTTC

For T cell transfectoma generation, TCRα or TCRβ chains were cloned into MSCV based vectors with an IRES element containing resistance genes for puromycin or neomycin, respectively. Retroviral vectors were co-transfected with pCLEco accessory plasmid into the Phoenix packaging line via Lipofectamine 2000 (Life Technologies, Carlsbad, CA). TCR constructs were transduced into $CD4^+$ T cell hybridoma 5KC-73.8.20 that lacks endogenous TCRα or TCRβ chains by retroviral spinfection (1000 x g, 2hrs). T cells were selected in 1mg/mL of neomycin or 0.8μg/mL of puromycin for 1 week and stained with anti-TCRβ antibody (H57-597) to ensure equivalent levels of TCR expression.

T cell transfectoma stimulation with APC and peptide library screen
In vitro splenocyte reactivity was determined by incubating 10^5 T cells transfectomas with 5 x 10^5 splenocytes from 6-10 week old naïve C57BL/6 mice or mice that had received 25μg LPS (Sigma) and 100μg anti-CD40 (clone FGK-4.5, Bio-X-cell) i.p. 5 days prior. Supernatants from cultures were screened for IL-2 content using an HT-2 cell based assay bioassay [35]. Analyses of APC subset specific reactivity was determined by culturing 10^5 T cells transfectomas with titrating numbers of cDC1, cDC2, macrophages, and B cell subsets isolated from 6-10 week old naïve C57BL/6 mice or mice that had received LPS and anti-CD40.

To construct an IA^b self peptide library, we curated mass spectrometry approaches that assessed the IA^b immunopeptidome of the spleen, LN, thymus, and in LN that drain sites of inflammation in C57BL/6 mice [55-60]. We further accounted for the combined effects of variability in protease cleavage sites and the ability of MHC-II molecules to present peptides with N- and C-terminal extensions. These characteristics of MHC-II peptide presentation allow nested sets of the same self-peptide core sequence to be presented [61]. To limit duplicate self-peptide cores within the library, we synthesized only the largest possible peptide fragment, or when the nested species spanned >18 amino acids, multiple peptide fragments that had an overlap of at least 10 amino acids. This approach reduced the size of the library to ~1750 unique self-peptides (Supplementary Dataset).

To identify peptide ligands recognized by tT_{reg} TCRs, 10^5 T cell transfectomas were incubated with 3×10^4 of IA^{b+} $B7.1^+$ $ICAM-1^+$ fibroblasts [35] and 37μg/ml of a mixture of 20 individual peptides (AALabs, San Diego CA) for 24 hrs and analyzed for IL-2 production. Positive responses in the primary screen were confirmed by culturing T cell transfectomas and fibroblasts with titrating amounts of individual soluble peptides. EC_{50} values were calculated by fitting to a log (agonist) vs response (three parameters) curve (GraphPad, Prism).

TCR clonotype analysis of thymic CD4SP, tT_{reg} cells and peripheral tT_{reg} cells
Thymic CD4SP and tT_{reg} cells, and splenic $CD4^+$ T_{conv} and $CD4^+$ tT_{reg} cell populations were sorted from 3 replicate groups (3 mice per group) of TCRCα$^{+/-}$. YAe62β.Foxp3-GFP

mice to a 98% purity (FACS Aria, BD Biosciences). RNA was isolated using Trizol and precipitated with RNase free glycogen (Invitrogen) following the manufactures protocol. cDNA was prepared using oligo-dT's (Promega) and Omniscript RT kit (Qiagen). cDNA was amplified with 20 rounds PCR with generic

Vα2 primer (5'-CCCTGGGGAAGGCCCTGCTCTCCTGATA-3') and

TCR Cα primer (5'- GGTACACAGCAGGTTCTGGGTTCTGGATG-3').

1/10th volume of the first round PCR was amplified with an additional 20 rounds of PCR using barcoded primers, for post sequence identification of originating T cell population, containing Illunima PE read primer and P5/7 regions, respectively. The resulting 300bp fragment was gel purified (Gene Clean II, MP Biomedicals) and sequenced on a MiSeq using a single read 250bp run (Illumina). Sequence data sets were parsed by barcode using the script fastq-multx and clonotypes for each population were tabulated using TCRklass [62].

For comparing 2wk and 8wk thymic T_{reg} frequencies, clonotype counts were summed across replicate datasets. In addition, the counting of each clonotype contained a pseudocount of 1 to act as a buffer when certain clonotypes are not observed in the dataset due to finite sampling of the population (false negatives) [35]. Frequencies for 103 $Vα2^+$ thymic clonotypes observed in the 2 week old or 8 week old thymic T_{reg} datasets were derived from the proportion among the total repertoire of the corresponding age. Padi4-reactive clonal frequencies were determined by averaging Vα2 clonotypes frequencies among 3 replicate deep sequence datasets. For pie charts, the clonal distribution of Padi4-reactive TCRs was determined as a relative proportion of the average frequency for the 5287, 6235, 5290, 4699, 4738, 5292, 6239, 6236, 6256, 6237, 6238 clonotypes.

Statistical analysis
Experimental results were analyzed for significance using one-way ANOVA Tukey multiple comparisons test; unpaired 2-tailed t test; unpaired 2-tailed t test with Welch's correction; non-parametric Dunn's multiple comparison test; one-way ANOVA Sidak's multiple comparison test; Kruskal-Wallis and Dunn's multiple comparisons test. Correlation plots are based on probable cure fit using Akaike's Information Criteria. Specific test to evaluate significance is described in the figure legends. Statistical analyses were performed using Prism v7.04 (Graphpad software). P-values ≤ 0.05 were considered significant (*$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, ****$p \leq 0.0001$) P values > 0.05; non-significant (ns)

Production of soluble and membrane bound MHC-peptide and TCR proteins
Soluble and biotinylated pMHC used for SPR, tetramer production and crystallography was produced using the baculovirus expression system and purified as previously described [27, 53]. Soluble TCRs used for surface plasmon resonance experiments and crystallography were expressed in Escherichia coli and refolded as described previously [53]. The TCRs produced in E. coli were murine TCR Vα and TCR Vβ domains fused to the human Cα and Cβ domains carrying an engineered disulfide bond to improve folding yield and protein stability. TCRα and TCRβ chains were expressed as inclusion bodies in the BL21 (DE3) strain of E. coli (EMD Millipore, Darmstadt, Germany). Soluble TCRs were produced by refolding mixtures of denatured TCRα and TCRβ chains, isolated from inclusion bodies. The refolded TCRs were purified further by size exclusion and ion exchange chromatography.

Surface plasmon resonance

Equilibrium affinity and binding kinetics for TCRs binding to IA^b-Padi4 and IA^b-Add2 were obtained by surface plasmon resonance on a BIAcore 3000 instrument (BIAcore AB, Uppsala, Sweden). Measurements of Padi4-specific TCRs engaging IA^b-Add2, and the Add2 TCR binding IA^b-Padi4 were used as a negative control. pMHC complexes were coupled to the sensor surface via a biotin–streptavidin linkage, with the streptavidin amine coupled to a CM5 sensor chip. For equilibrium affinity experiments, ~2000–8000 resonance units (RU) of soluble biotinylated pMHC were captured on the chip surface by NeutrAvidin (Thermo Fisher). Experiments were performed at 25°C in 10 mM HEPES, 150 mM NaCl, 3 mM EDTA, and 0.005% surfactant P20 at pH 7.4. Soluble TCR was injected for 30-120 sec at a flow rate of 5 uL/min at concentrations ranging from 0.5 – 150 μM. Data points were collected at 0.4-s intervals and identical injections over a flow cell containing an irrelevant MHC-I complex were subtracted. The data were analyzed with BIAeval v4.1 and Prism v7.04 (GraphPad) software and equilibrium binding affinity (K_D) was determined using a 1:1 Langmuir binding model. For kinetic experiments, 500-1000 RU of soluble pMHC was captured on the NeutrAvidin-coupled CM5 chip. TCRs were injected at a flow rate of 95 uL/min for 15-30 s with a dissociation time of 180 s between each sample. TCR concentrations ranged from 0.25 – 40 μM. Data points were collected at 0.2 s intervals and reference cell adjustments were made as described above. Kinetic data were analyzed with BiaEval software. The dissociation rate (k_{off}) was determined by fitting the dissociation phase of the curve with a 1:1 Langmuir dissociation model. The half life ($t_{1/2}$) and association rate (k_{on}) were determined from the calculated K_D and k_d values. Values for the calculated confinement time, k_a, were done as described using a k_{on} rebinding threshold of 60,000 1/M*s [27].

Crystallization and data collection
Soluble TCR and pMHC were mixed in a 1:1 ratio at equimolar concentrations of 10mg/ml in 10 mM Tris pH 7.5. Crystals of the 4699, 5287, and 4738 TCRs in complex with IA^b-Padi4 were grown in 100 mM Sodium Cacodylate, 100 mM Sodium Citrate with 14% (w/v) PEG4000 pH 6.0 (4699), 14% (w/v) PEG4000 pH 5.6 (5287), or 12% (w/v) PEG4000 pH 5.8 (4738). Crystals of the 6235, 6236, and 6256 in complex with IA^b-Padi4 were grown in 100 mM Sodium Cacodylate, 100 mM Sodium Citrate, 14% (w/v) PEG4000 pH 5.8. All crystals were grown at 25°C using hanging drop vapor diffusion. Crystals were cryopreserved by transferring to crystallization buffer containing 20% glycerol/80% mother liquor and flash frozen in liquid nitrogen. X-ray diffraction data were indexed, integrated, and scaled with XDS [63]. Unit cell parameters and data collection statistics are shown in Supplementary Table 2.

Structure determination
Initial phases were obtained by molecular replacement using Phaser [64] using PDB 4P23 as the search model. One TCR and pMHC molecule per asymmetric unit were present in all six structures. Rigid body refinement followed by cycles of coordinate position, atomic displacement factor, and translation/libration/screw refinement were performed using PHENIX [65], alternated with manual model adjustments in Coot [66]. Density for the peptide, TCR variable domains, and MHC was clearly observed in composite omit maps built by the Crystallography and NMR System [67]. No density was observed for the linker (GGGGSLVPRGSGGGGS) tethering the peptide to the N-terminal end of the MHC β chain. The structure was evaluated during refinement with MolProbity [68].

Structure analysis

All six TCR:IA^b-Padi4 complexes were superimposed in PyMOL (Delano Scientific) using the pair fit function, aligning the α carbons of the MHC helices. Contacts were calculated using a 4Å cutoff in Ncont (CCP4 Suite). Buried Surface Area was determined by the PISA server from PDBe using a 1.4Å radius probe [69]. TCR crossing and incident angles were calculated as previously described [70]. PDB coordinates for other TCR:pMHC complexes used in crossing/incident angle and BSA calculations include: 1AO7, 1BD2, 1D9K, 1FO0, 1G6R, 1J8H, 1KJ2, 1LP9, 1MI5, 1MWA, 1NAM, 1OGA, 1QRN, 1QSE, 1QSF, 1U3H, 1YMM, 1ZGL, 2AK4, 2BNQ, 2BNR, 2E7L, 2F53, 2GJ6, 2IAM, 2IAN, 2NX5, 2OI9, 2OL3, 2P5E, 2P5W, 2PXY, 2VLR, 2WBJ, 2YPL, 2Z31, 3C5Z, 3C60, 3C6L, 3DXA, 3E2H, 3E3Q, 3FFC, 3GSN, 3H9S, 3HG1, 3KPR, 3KPS, 3MBE, 3MV7, 3MV8, 3MV9, 3O4L, 3O6F, 3PL6, 3PQY, 3PWP, 3QDG, 3QDJ, 3QDM, 3QEQ, 3QFJ, 3QIB, 3QIU, 3QIW, 3RDT, 3RGV, 3SJV, 3TF7, 3TFK, 3TJH, 3TPU, 3UTT, 3VXM, 3VXR, 3VXS, 3VXU, 4E41, 4FTV, 4G8G, 4G9F, 4GG6, 4GRL, 4H1L, 4JFD, 4JFE, 4JFF, 4JRX, 4JRY, 4MAY, 4MJI, 4MNQ, 4MS8, 4MVB, 4MXQ, 4N0C, 4N5E, 4NHU, 4OZF, 4OZG, 4OZH, 4OZI, 4P23, 4P2O, 4P2Q, 4P2R, 4P46, 4P4K, 4P5T, 4PRH, 4PRI, 4PRP, 4QRP, 4Y19, 4Y1A, 4Z7U, 4Z7V, 4Z7W, 5BRZ, 5BS0, 5D2L, 5D2N, 5EU6, 5HHM, 5HHO, 5JZI.
Electron density shown in Supplementary Fig. 6 was calculated from a 2Fobs-Fcalc map contoured to 1.5 sigma displayed in PyMOL.
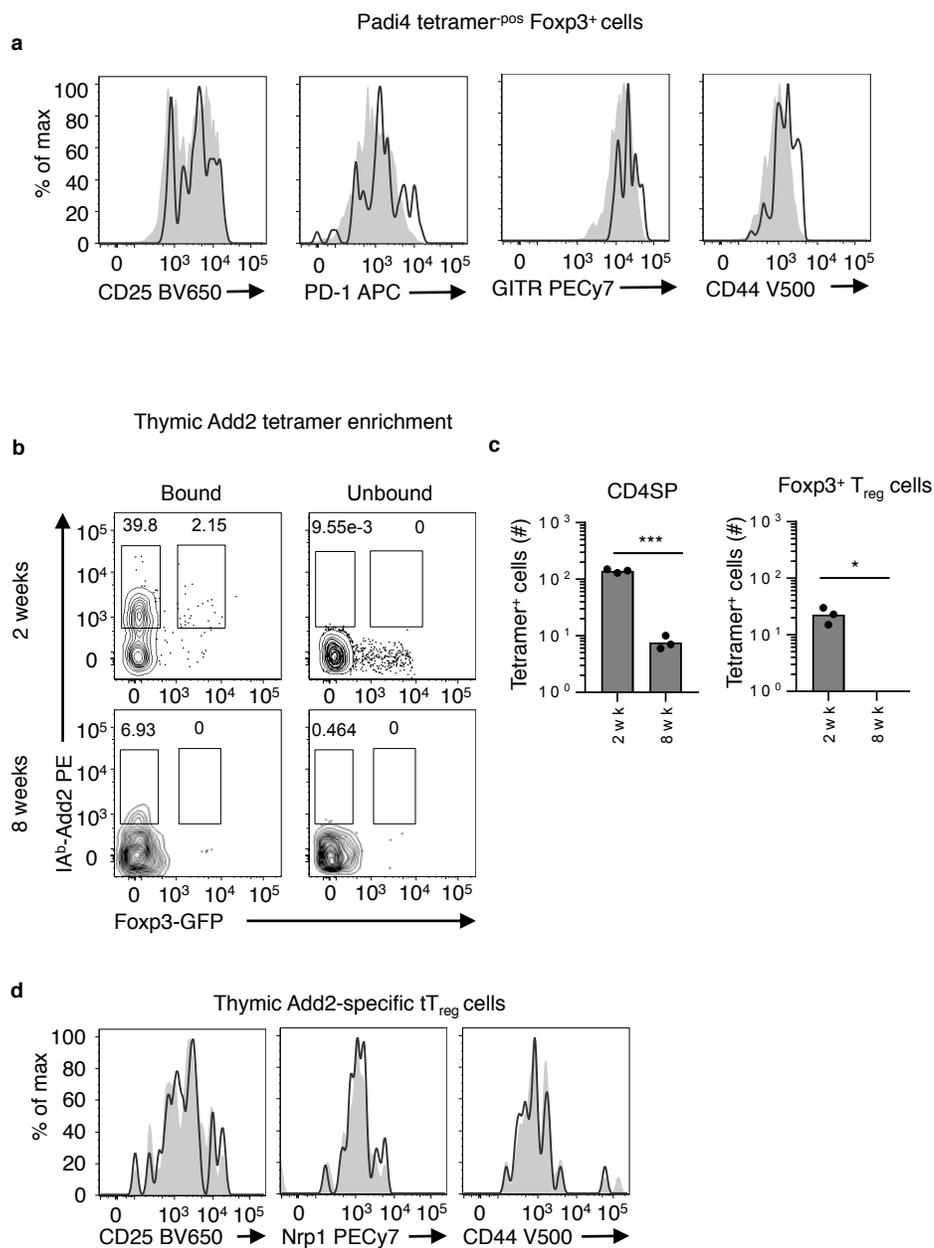
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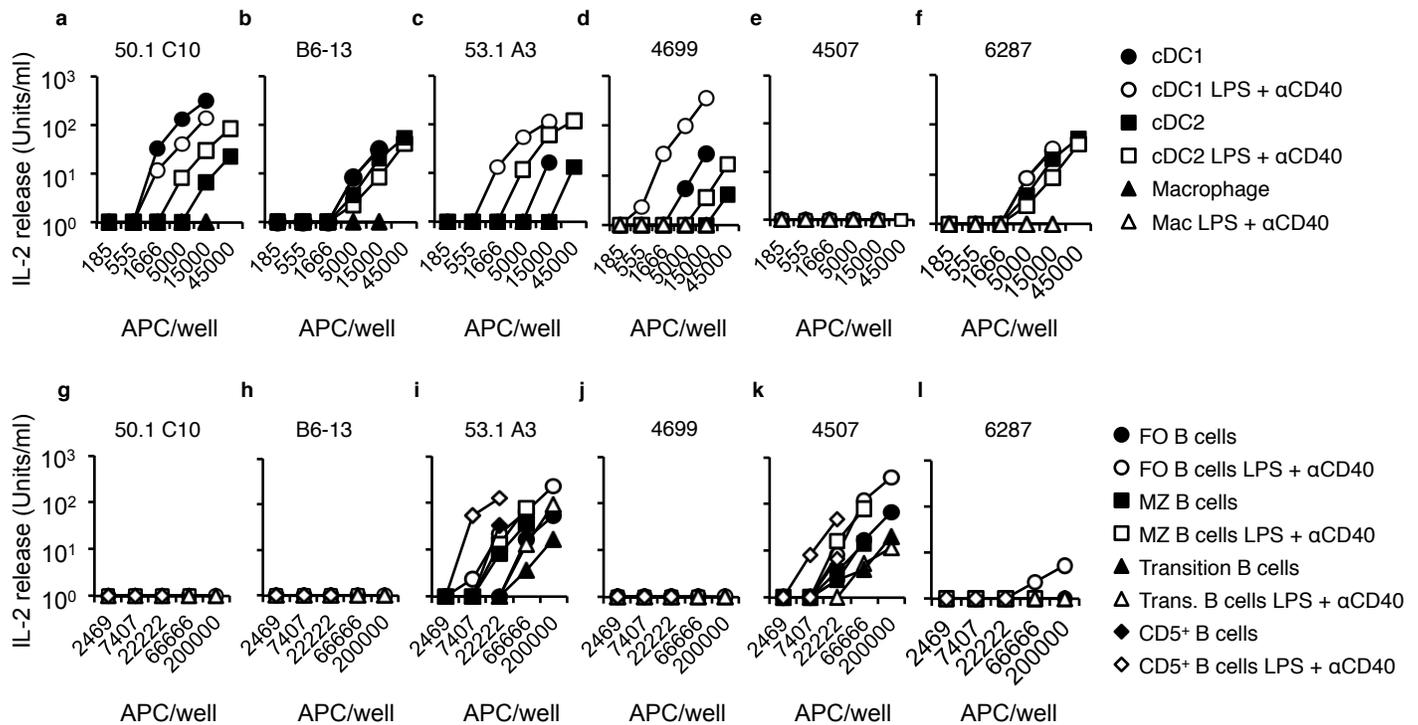
Supplementary Figure 1. T cell receptors expressed on neonate-derived tT$_{reg}$ cells can recognize steady state and inflammation-dependent self-antigens presented by dendritic cell and/or B cell subsets. (**a**) IL-2 response of B6-50.1C10, (**b**) B6-13 (**c**) 53.1.A3, (**d**) 4699, (**e**) 4507 (**f**) 4699 (**g**) 4783 tT$_{reg}$ hybridomas cultured with titrating amounts of cDC1, cDC2 and macrophages or (**g-l**) B cell subsets isolated from naïve mice (filled symbol) or mice pretreated with LPS and αCD40 (open symbol). Results are from three independent experiments with similar results.

a

Padi4 tetramer⁻ᵖᵒˢ Foxp3⁺ cells

b

Thymic Add2 tetramer enrichment

c

d

Thymic Add2-specific tT_reg cells

Supplementary Figure 2. Phenotype and age-dependent selection of Padi4- and Add2-specific tT$_{reg}$ cells in the thymus of YAe62β.*Foxp3-GFP* mice. (**a**) Flow cytometry analyzing the expression of CD25, PD-1, GITR and CD44 on IA^b-Padi4 tetramer^{pos} Vα2$^+$CD4$^+$ tT$_{reg}$ cells in thymus of YAe62β.*Foxp3-GFP* at 2 weeks of age (black line). Gray histograms are IA^b-Padi4 tetramer^{neg} Vα2$^+$CD4$^+$ tT$_{reg}$ cells in the same mice. Data are example from three independent experiments giving similar results. (**b**) Age-dependent thymic development of Add2$_{606-621}$-specific tT$_{reg}$ cells. Flow cytometry of Vα2$^+$ CD4SP thymocytes in YAe62β.*Foxp3-GFP* mice at 2 weeks (top row) and 8 weeks of age (bottom row) following tetramer-based enrichment, stained with IA^b-Add2 tetramer. (**c**) Quantification of IA^b-Add2 tetramer^{pos} Vα2$^+$ Foxp3-GFP^{neg} CD4SP and Foxp3-GFP^{pos} Vα2$^+$ CD4SP thymocytes in YAe62β.*Foxp3-GFP* mice at different ages. (**d**) Flow cytometry analyzing the expression of CD25, NRP1, and CD44 on IA^b-Add2 tetramer^{pos} Vα2$^+$CD4$^+$CD8$^-$Foxp3-GFP^{pos} thymocytes in YAe62β.*Foxp3-Gfp* mice at 2 weeks of age (black line). (b-d) Data are from three independent experiments with similar results, bars represent the mean cell number. Gray is expression on Foxp3$^+$ tetramer negative cells in the same mice. (c) CD4SP ***P<0.001 unpaired 2-tailed t test; Foxp3$^+$ T$_{reg}$ cells *P<0.05 unpaired 2-tailed t test with Welch's correction.
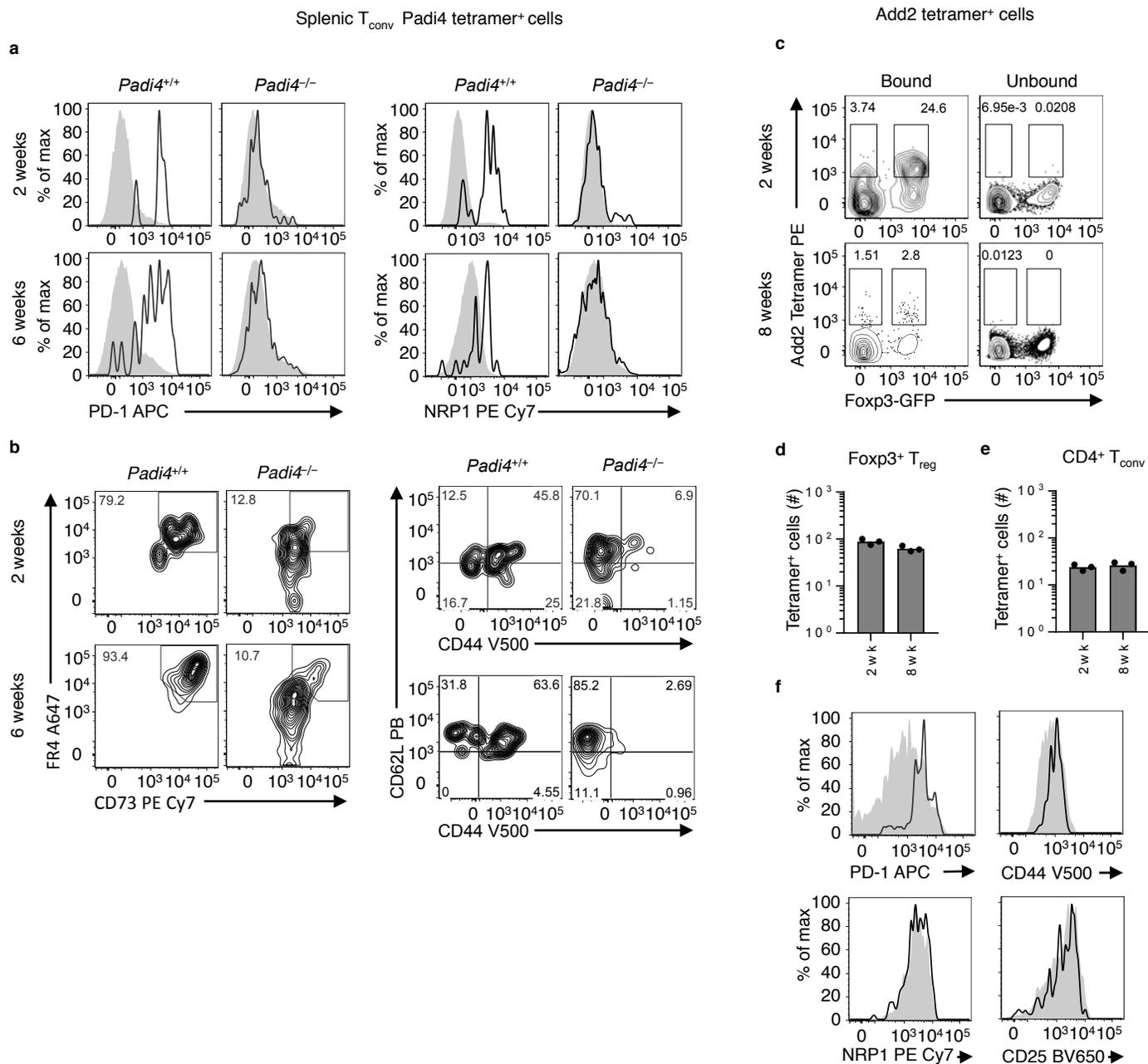
Supplementary Figure 3. Phenotype of splenic Padi4-specific CD4+ T_{conv} cells and Add2-specific tT_{reg} cells. $Padi4_{92-105}$-specific CD4+ T cells have a naïve phenotype in *Padi4−/−* mice. (**a**) Flow cytometry of IA^b-Padi4 tetramer^{pos} Foxp3-GFP^{neg} Vα2+ CD4 T cells in *Padi4+/+* and *Padi4−/−* YAe62β.*Foxp3-Gfp* mice at 2 weeks (top row) and 6 weeks of age (bottom row) for PD-1 and Nrp1 (black line), and (**b**) FR4 and CD73, and CD44 and CD62L. Frequency and total cell numbers are shown in Fig. 4. (**c**) Flow cytometry of Vα2+ CD4+ T cells in YAe62β.*Foxp3-Gfp* mice at 2 weeks (top row) and 8 weeks of age (bottom row) following tetramer enrichment protocol, stained with IA^b-Add2 tetramer. Quantification of IA^b-Add2 tetramer^{pos} (**d**) Vα2+ Foxp3-GFP^{pos} tT_{reg} cells and (**e**) Vα2+ Foxp3-GFP^{neg} CD4 T_{conv} cells in YAe62β.*Foxp3-GFP* mice at 2 and 8 weeks age. (**f**) Flow cytometry analyzing the expression of PD-1, CD44, Nrp1 and CD25 on Vα2+ CD4+ Foxp3-GFP+ T_{reg} cells isolated from 2 week old YAe62β.*Foxp3-GFP* mice (black line). Gray histograms are IA^b-Add2 tetramer^{neg} Vα2+ CD4+ Foxp3-GFP^{pos} T_{reg} cells in the same mice. (a-f) Data from 3 independent experiments with similar results with 3 mice per group, bars represent the data mean. (d, e) NS P>0.05 ratio unpaired 2-tailed t test.
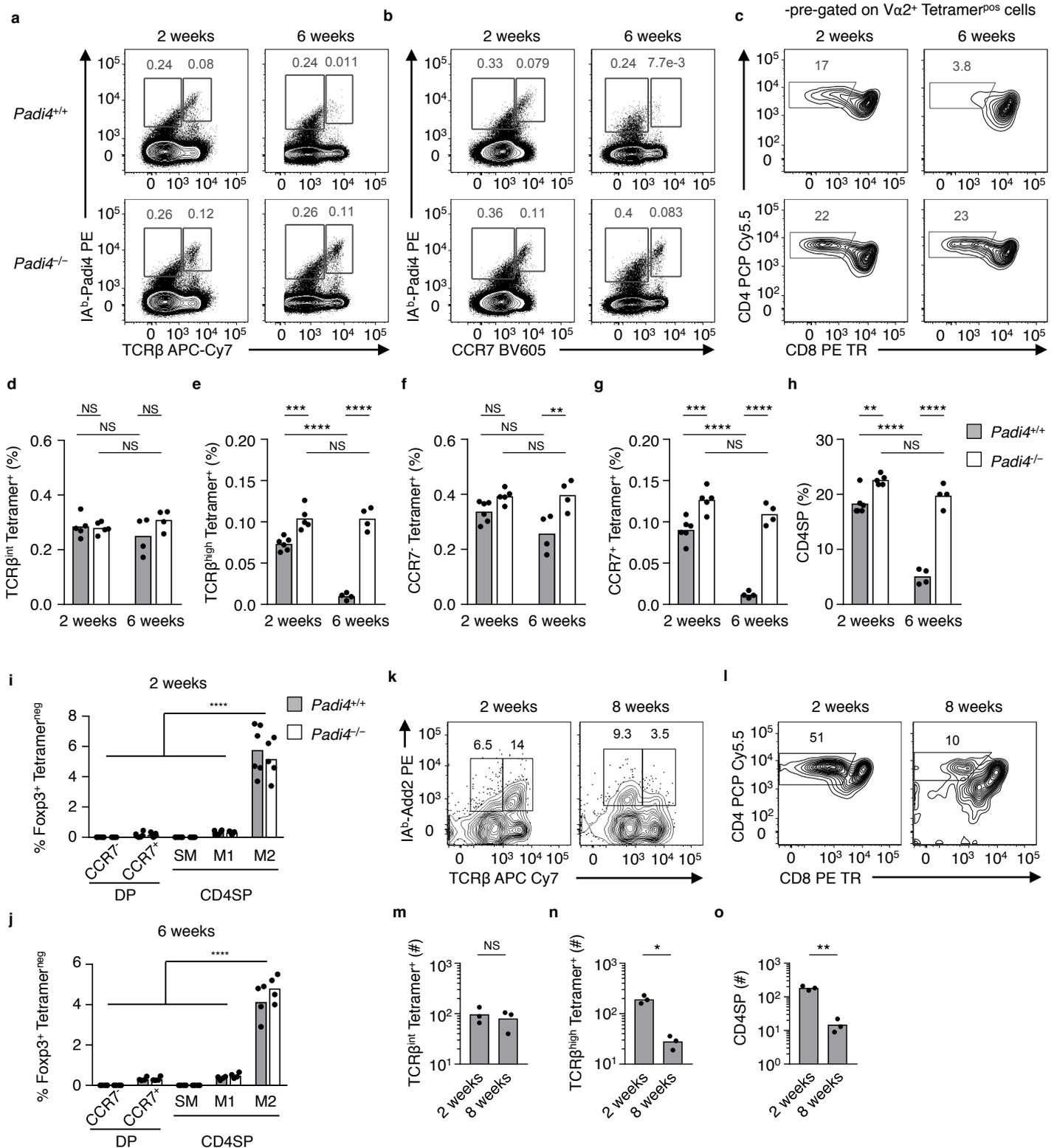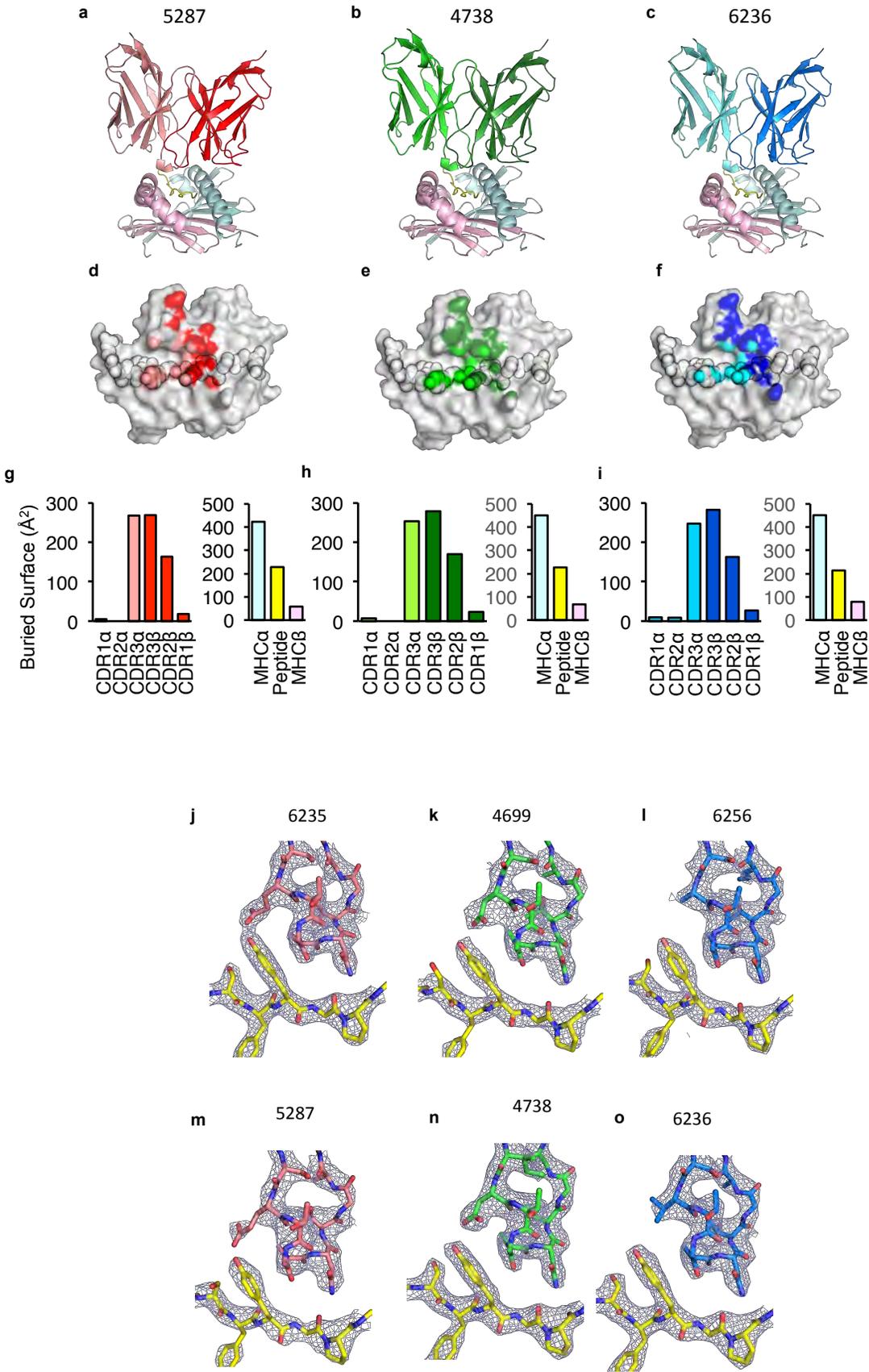
Supplementary Figure 4. Padi4- and Add2-specific thymocytes undergo negative selection at the DP to CD4SP transition in adult thymus. (**a-h**) The neonate but not adult thymus generates Padi4-tetramer[pos] TCRβ[hi], CCR7^{+} CD4SP thymocytes. (**a,b**) Flow cytometry analyses of total Vα2[+] thymocyte isolated from neonate and adult, *Padi4*^{+/+} (top row) and *Padi4*^{–/–} (bottom row) YAe62β.*Foxp3-GFP* mice, for IA[b]-Padi4 tetramer binding and (**a**) TCRβ and (**b**) CCR7 expression. (**c**) Flow cytometry analyses of total Vα2[+] IA[b]-Padi4 tetramer[pos] thymocytes for CD4 and CD8 expression. (**d-h**) Quantification of the frequency of Vα2[+] IA[b]-Padi4 tetramer[pos] thymocytes in neonatal and adult thymus that are (**d**) TCRβ[int], (**e**) TCRβ[hi], (**f**) CCR7^{neg}, (**g**) CCR7^{+} and (**h**) CD4SP. (**i,j**) Quantification of total tT[reg] development in (**i**) neonate and (**j**) adult thymus,

Supplementary Figure 4 cont. based on thymocytes stage of development. Data are derived from 3 independent experiments giving similar results, bars represent the data mean; n = 6 WT and *Padi4$^{-/-}$* mice at 2 at weeks old and n = 4 at 6 weeks old. (**k**) Flow cytometry analyses of total Vα2$^+$ thymocyte isolated from neonate and adult, YAe62β.*Foxp3-GFP* following IA^b-Add2 tetramer enrichment, for IA^b-Add2 tetramer binding and TCRβ expression. (**l**) Flow cytometry analyses of total Vα2$^+$ IA^b-Add2 tetramer^{pos} thymocytes for CD4 and CD8 expression. (**m-o**) Quantification of frequency of Vα2$^+$ IA^b-Add2 tetramer^{pos} thymocytes in neonatal and adult thymus that are (**m**) TCRβ^{int}, (**n**) TCRβ^{hi} and (**o**) CD4SP. Data pooled from 3 independent experiments giving similar results with 3 mice per group, bars represent the data mean. (d-j) Significance identified using a one-way ANOVA Sidak's multiple comparison test and (m,o) unpaired 2-tailed t test. ns P>0.05, *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.
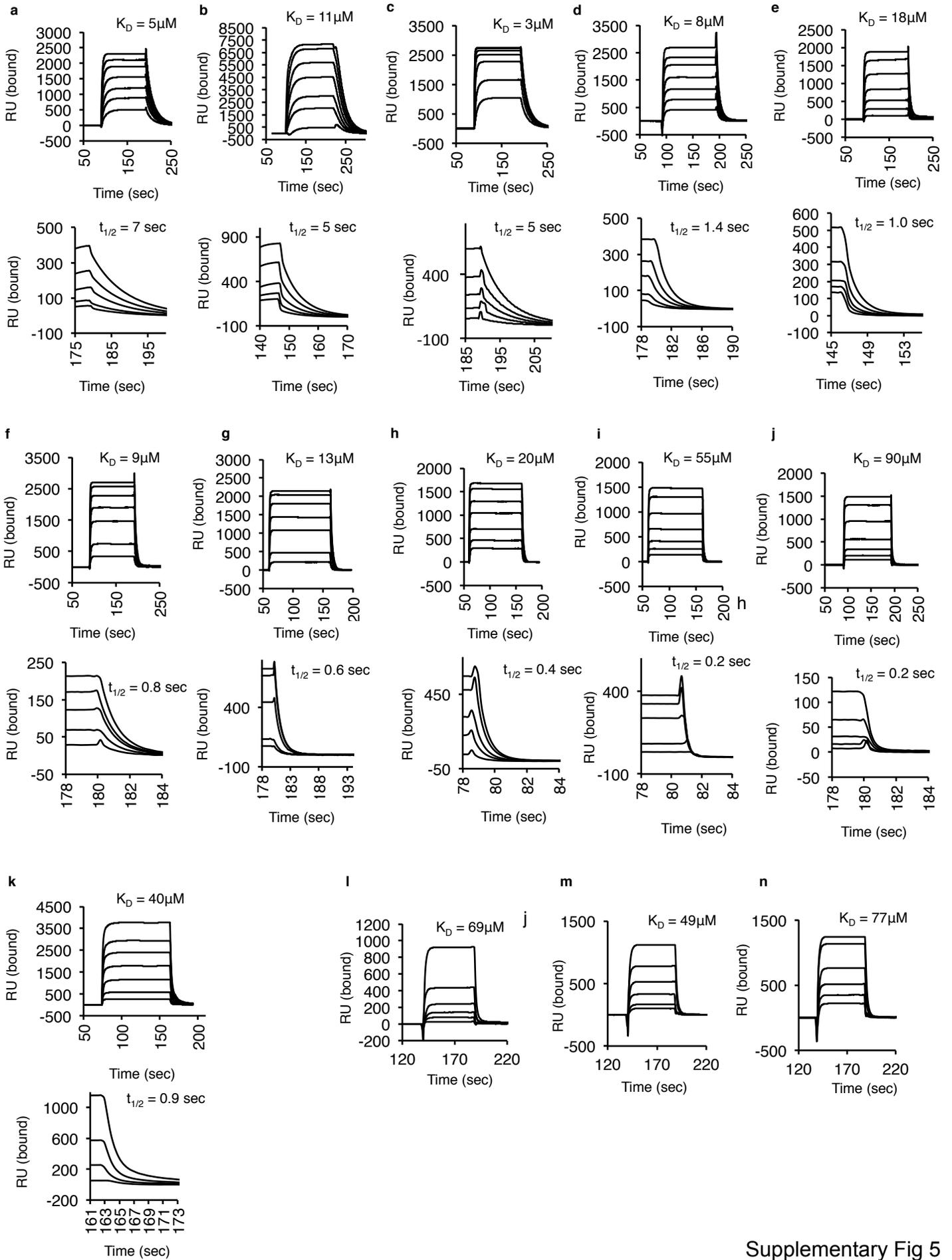
Supplementary Fig 5

Supplementary Figure 5. Surface plasmon resonance sensograms to measure the equilibrium affinity and kinetics of TCRs binding IA^b-Padi4 and Add2. (**a-j**) Surface plasmon resonance sensograms of 0.5-150µM soluble (**a**) 5287, (**b**) 5290, (**c**) 6235, (**d**) 4699, (**e**) 4738 (**f**) 6239, (**g**) 6236, (**h**) 6256, (**i**) 6237 and (**j**) 6238 TCRs binding IA^b-Padi4; equilibrium affinity (top row) and binding kinetics (bottom row). (**k**) Surface plasmon resonance sensograms of 1-150µM soluble 6287 TCR binding IA^b-Add2; equilibrium affinity (top row) and binding kinetics (bottom row). (**l-n**) Surface plasmon resonance sensograms of 5-150µM soluble (**i**) 6235, (**j**) 4699 and (**k**) 6256 TCRs binding IA^b-Padi4 P2F. Disassociation sensograms for determining $t_{1/2}$ are shown in Fig. 8. Sensograms are background subtracted from each TCR interacting with a non-cognate (IA^b-Add2 or IA^b-Padi4 ligand). Data are examples of 4 biological replicates, giving similar results.

a   5287
b   4738
c   6236

d
e
f

g
Buried Surface (Å²)
CDR1α CDR2α CDR3α CDR3β CDR2β CDR1β
MHCα Peptide MHCβ

h
CDR1α CDR2α CDR3α CDR3β CDR2β CDR1β
MHCα Peptide MHCβ

i
CDR1α CDR2α CDR3α CDR3β CDR2β CDR1β
MHCα Peptide MHCβ

j   6235
k   4699
l   6256

m   5287
n   4738
o   6236

Supplementary Figure 6

Supplementary Figure 6. Padi4-specific TCRs with long, moderate and short dwell times use conventional docking orientations on IA^b-Padi4. (**a-c**) Ribbon diagrams of (**a**) 5287 (pdb: 6MKR), (**b**) 4378 (pdb: 6MNG) and (**c**) 6236 (pdb: 6MNN) TCRs binding IA^b-Padi4. The 5287 TCR is colored red (TCRβ) and pink (TCRα); the 4378 TCR is colored dark green (TCRβ) and light green (TCRα); the 6236 TCR is colored dark blue (TCRβ) and light blue (TCRα). IA^b-Padi4 is colored cyan (IA^{b}α chain), yellow (peptide), and magenta (IA^{b}β chain). (**d-f**) Projections of the (**d**) 5287, (**e**) 4378 and (**f**) 6236 TCRs bound to IA^b-Padi4. 5287 TCRα contacts are colored pink, TCRβ contacts are colored red; 4378 TCRα contacts are colored light green, TCRβ contacts are colored dark green; 6236 TCRα contacts are colored light blue, TCRβ contacts are colored dark blue. The peptide residues are outlined in black. (**g-i**) The amount of buried surface area (BSA) of the (**g**) 5287:IA^b-Padi4, (**h**) 4378:IA^b-Padi4 and (**i**) 6236:IA^b-Padi4 complexes contributed by TCRα and TCRβ loops, and the peptide or MHC chains. Figures were made with PyMol. (**j-o**) Electron density in the (**j**) 6235, (**k**) 4699, (**l**) 6256, (**m**) 5287, (**n**) 4783 and (**o**) 6236 CDR3α and peptide region contoured at 1.5 sigma from |2Fobs-Fcalc| map with phases calculated for a model with peptide p2Y (yellow).
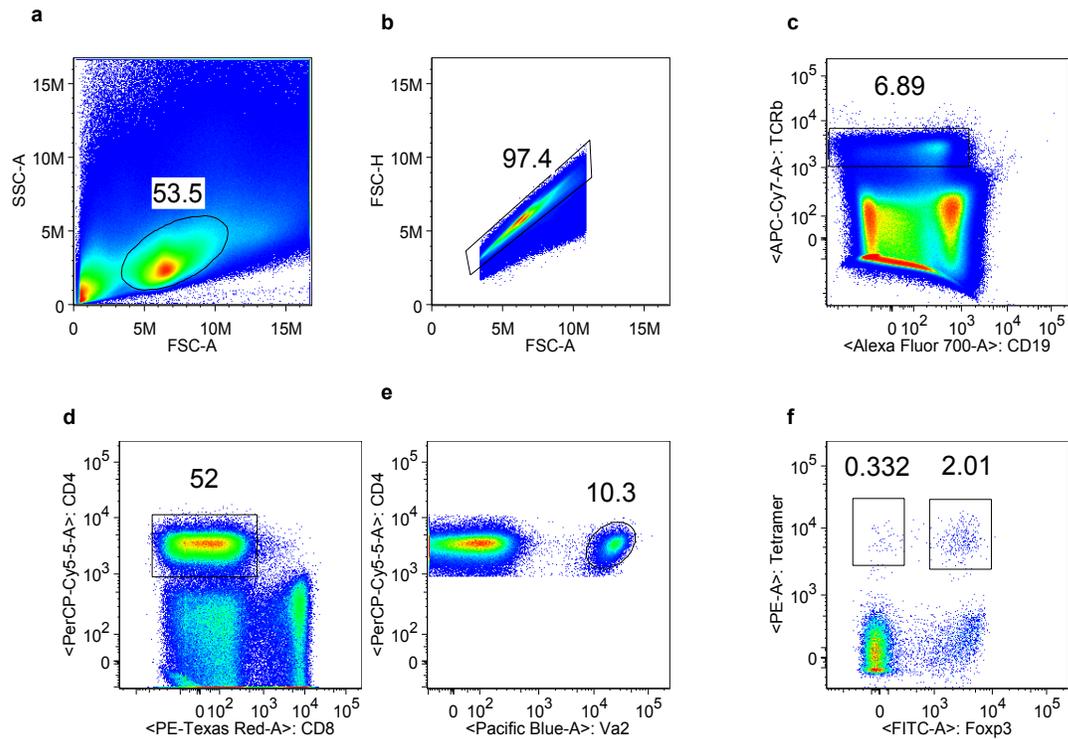
Supplementary Figure 7. Gating strategy to analyze Padi4-specific tT_{reg} cells and CD4+ T_{conv} cells in 2 week old YAe62β mice. (a) Lymphocytes were gated on forward and side scatter and (b) doublets removed by gating FSC-H by FSC-A. (c) TCR+ lymphocytes were then (d) gated for the expression of (d) CD4+ and CD8neg, and (e) the expression of Vα2. (f) Gated T cells were then analyzed for Foxp3-GFP expression, and staining with IAb-Padi4 tetramer. Subsequent phenotypic stains were then analyzed on these populations.

Supplementary Table 1. Neonatal tT$_{reg}$ TCR sequences and identified self-peptide ligand reactivity

Clone ID	TCRβ	Vβ	CDR3β	Jβ	TCRα	Vα	CDR3α	Jα	Antigen (gene)	Region	Peptide	EC$_{50}$ (nM)
4699	8	TRBV13-2	CASGDFWGDTLYFG	TRBJ2-4	2	TRAV14D-3/DV8	CAASDTGANTGKLTF	TRAJ52	Padi4	92-105	VRVSYYGPKTSPVQ	0.814
4738	8	TRBV13-2	CASGDFWGDTLYFG	TRBJ2-4	2	TRAV14D-3/DV8	CAGIDTGANTGKLTF	TRAJ52	Padi4	92-105	VRVSYYGPKTSPVQ	2.29
6287	8	TRBV13-2	CASGDFWGDTLYFG	TRBJ2-4	2	TRAV14-2	CAARATGGNNKLTF	TRAJ56	Add2	606-621	SPSKAGTKSPAVSPSK	7.26
5853	8	TRBV13-2	CASGDFWGDTLYFG	TRBJ2-4	11	TRAV4D-4	CASRNSNNRIFF	TRAJ31	Gpd2	235-250	TAARYGAATANYMEVV	35.2
5803	8	TRBV13-2	CASGDFWGDTLYFG	TRBJ2-4	8	TRAV12D-1	CALRNTGNYKYVF	TRAJ40	Gpd2	235-250	TAARYGAATANYMEVV	79.6
5915	8	TRBV13-2	CASGDFWGDTLYFG	TRBJ2-4	11	TRAV4D-3	CAARGGNTGKLIF	TRAJ37	Gpd2	235-250	TAARYGAATANYMEVV	110
4630	8	TRBV13-2	CASGDFWGDTLYFG	TRBJ2-4	2	TRAV14-2	CAASENSGTYQRF	TRAJ13	Gsn	599-614	AAYLWVGAGASEAEKT	80.4
4513	8	TRBV13-2	CASGDFWGDTLYFG	TRBJ2-4	8	TRAV12-3	CALSALNNNAPRF	TRAJ43	Kcnk5	290-304	APKDSYQTSEVFINQ	100
4748	8	TRBV13-2	CASGDFWGDTLYFG	TRBJ2-4	2	TRAV14-2	CAAANSGTYQRF	TRAJ13	Rps25	62-76	VPNYKLITPAVVSER	164
4754	8	TRBV13-2	CASGDFWGDTLYFG	TRBJ2-4	2	TRAV14-2	CAASEGNNRIFF	TRAJ31	Hist1h1c	155-171	PKKAKKPAAAAVTKKVA	189
52.1 A6	11	TRBV16	CASSFDRGQAPLF	TRBJ1-5	2	TRAV12-2	CAASPSSGQKLVF	TRAJ16	Cltb	40-53	NDPGFGAPAASQVA	191
4776	8	TRBV13-2	CASGDFWGDTLYFG	TRBJ2-4	2	TRAV14-2	CAASRAGSGGKLTL	TRAJ44	Ppp1cb	311-327	SGRPVTPPRTANPPKKR	294
4623	8	TRBV13-2	CASGDFWGDTLYFG	TRBJ2-4	2	TRAV14-1	CAAIANTGANTGKLTF	TRAJ52	Galnt4	29-44	VSTLYASPGAGGAREL	336
4753	8	TRBV13-2	CASGDFWGDTLYFG	TRBJ2-4	2	TRAV14-1	CAASANTGANTGKLTF	TRAJ52	Galnt4	29-44	VSTLYASPGAGGAREL	343
50.1 C10	8	TRBV13-2	CASGDGLGGDTQYF	TRBJ2-5	11	TRAV4D-4	CAATGTGGYKVVF	TRAJ12	Cilp	1128-1142	SAFQYLQSTPARSPA	347
3H14	8	TRBV13-2	CASGDFWGDTLYFG	TRBJ2-4	2	TRAV14D-3/DV8	CAAVATGGNNKLTF	TRAJ56	Ugcg	233-247	IAEDYFMAKAIADRG	59.7
4819	8	TRBV13-2	CASGDFWGDTLYFG	TRBJ2-4	2	TRAV14-2	CAASETGANTGKLTF	TRAJ52	Ugcg	233-247	IAEDYFMAKAIADRG	372
4820	8	TRBV13-2	CASGDFWGDTLYFG	TRBJ2-4	2	TRAV14-2	CAASITGSGGKLTL	TRAJ44	Ugcg	233-247	IAEDYFMAKAIADRG	752
4751	8	TRBV13-2	CASGDFWGDTLYFG	TRBJ2-4	2	TRAV14D-3/DV8	CAASRTGGNNKLTF	TRAJ56	Ugcg	233-247	IAEDYFMAKAIADRG	752
4787	8	TRBV13-2	CASGDFWGDTLYFG	TRBJ2-4	2	TRAV14D-3/DV8	CAASARVATGGNNKLTF	TRAJ56	Map4k1	385-400	DGSLKLVTPEGAPAPG	695
4777	8	TRBV13-2	CASGDFWGDTLYFG	TRBJ2-4	2	TRAV14-2	CAASGGADRLTF	TRAJ45	Cmip	160-174	KKIYKYKKVLSNPSR	695
4693	8	TRBV13-2	CASGDFWGDTLYFG	TRBJ2-4	8	TRAV12-3	CALSDRTNAYKVIF	TRAJ30	Eif3f	37-50	APTPAATPAASPAP	695
4507	8	TRBV13-2	CASGDFWGDTLYFG	TRBJ2-4	8	TRAV12-3	CALSDRTNTNKVVF	TRAJ34	Aldh8a1	50-65	EAAREAFPAWSSRSPQ	853
4731	8	TRBV13-2	CASGDFWGDTLYFG	TRBJ2-4	2	TRAV14D-3/DV8	CAASGGNYNQGKLIF	TRAJ23	Ldha	68-82	GSLFLKTPKIVSSKD	1150

TCR V and J regions defined using IMGT nomenclature.
EC$_{50}$ values are average of 3 independent experiments.

Supplementary Table 2. T cell response, affinity and kinetics of binding of Padi4 and Add2 specific TCRs

TCR	Specificity	TRAV	CDR3a	IL2 release EC$_{50}$ (nM)	K$_D$ (μM)	k$_{on}$ (1/M·s) x 10^3	t$_{1/2}$ (s)	t$_a$ (s)
5287	Padi4$_{92-105}$	14-3	CAASETGANTGKLTF	0.02 ± 0.07	5 ± 0.4	20 ± 2	7 ± 0.2	8 ± 0.3
6235	Padi4$_{92-105}$	14D-3	CAASETGANTGKLTF	0.02 ± 0.03	3 ± 1.6	69 ± 30	5 ± 0.3	8 ± 3
5290	Padi4$_{92-105}$	14-2	CAAENTGANTGKLTF	0.02 ± 0.03	11 ± 0.5	15 ± 2	5 ± 0.2	6 ± 0.2
4699	Padi4$_{92-105}$	14D-3	CAASDTGANTGKLTF	0.6 ± 0.1	8 ± 2	62 ± 5	1.4 ± 0.1	2.3 ± 0.2
4738	Padi4$_{92-105}$	14D-3	CAGIDTGANTGKLTF	1.3 ± 0.2	18 ± 1	38 ± 10	1 ± 0.2	1.4 ± 0.2
6239	Padi4$_{92-105}$	14D-3	CAASNTGANTGKLTF	0.7 ± 0.1	9 ± 1	91 ± 14	0.8 ± 0.1	1.5 ± 0.2
6236	Padi4$_{92-105}$	14D-3	CAASVTGANTGKLTF	5 ± 1	13 ± 3	77 ± 18	0.6 ± 0.1	1.0 ± 0.2
6256	Padi4$_{92-105}$	14D-3	CAASATGANTGKLTF	41 ± 12	20 ± 2	91 ± 29	0.4 ± 0.1	0.8 ± 0.2
6237	Padi4$_{92-105}$	14D-3	CAASSTGANTGKLTF	1450 ± 120	55 ± 16	58 ± 21	0.2 ± 0.1	0.3 ± 0.1
6238	Padi4$_{92-105}$	14D-3	CAASTTGANTGKLTF	290 ± 40	90 ± 21	35 ± 12	0.2 ± 0.1	0.3 ± 0.1
6287	Add2$_{606-621}$	14-2	CAARATGGNNKLTF	40 ± 5	40 ± 20	20 ± 10	0.9 ± 0.1	1.2 ± 0.2
6235	Padi4 P2F	14D-3	CAASETGANTGKLTF	n/d	69 ± 6	70 ± 35	0.2 ± 0.05	0.3 ± 0.1
4699	Padi4 P2F	14D-3	CAASDTGANTGKLTF	n/d	49 ± 2	96 ± 48	0.2 ± 0.05	0.4 ± 0.2
6256	Padi4 P2F	14D-3	CAASATGANTGKLTF	n/d	77 ± 42	76 ± 37	0.2 ± 0.05	0.4 ± 0.2

The equilibrium affinities and kinetics of binding were determined by SPR measured in 4 four independent experiments. k$_{on}$ values were estimated from the measured K$_D$ and t$_{1/2}$ values. The standard error reported for IL2 release, K$_D$ and t$_{1/2}$ is based on one standard deviation. The error reported for the k$_{on}$ represents the maximum error associated with both the K$_D$ and t$_{1/2}$ values. The confinement time (t$_a$) values were estimated from the average values of the k$_{on}$ and t$_{1/2}$, using threshold for rebinding of 100,000 1/M·s. Reported t$_a$ error was calculated from the error associated with k$_{on}$.

Supplementary Table 3. Data collection and refinement statistics for TCRs in complex with I-Ab/padi4

	4699 : IA^b-padi4	6235 : IA^b-padi4	6256 : IA^b-padi4	4738 : IA^b-padi4	6236 : IA^b-padi4	5287 : IA^b-padi4
Data collection						
Space group	C 1 2 1	C 1 2 1	C 1 2 1	C 1 2 1	C 1 2 1	C 1 2 1
Cell dimensions						
a,b,c (Å)	255.448, 73.643, 64.566	250.057, 69.03, 64.687	254.351, 73.771, 63.142	250.917 69.163 64.15	250.412 68.872 64.466	257.022, 73.761, 65.207
α,β,γ(°)	90, 90.391, 90	90, 91.51, 90	90, 90.517, 90	90 91.479 90	90 91.97 90	90, 90.56, 90
Resolution (Å)	29.4 - 3.2 (3.420-3.2)	29.67 - 2.9 (3.004 - 2.9)	28.86 - 3.1 (3.311 - 3.1)	29.66 - 2.66 (2.76 - 2.66)	29.62 - 2.83 (2.931 - 2.83)	128 – 3.35[a] 65.2 - 3.35[b] (3.47-3.35)
Total no. of observations	73637 (13700)	110563 (18582)	144955 (26928)	218095 (14311)	180794 (11859)	64231 (9693)
No. of unique observations	19627 (3564)	24697 (3978)	21458 (3884)	31505 (2074)	26309 (1817)	17652 (2542)
Multiplicity	3.8 (3.8)	4.5 (4.7)	6.8 (6.9)	6.9 (7.0)	6.9 (6.5)	3.6 (3.8)
Data completeness (%)	98 (99)	100 (100)	100 (100)	99 (91)	99 (94)	99 (100)
I/σ$_I$	6.7 (1.7)	6.8 (1.9)	11.5 (4.8)	11.6 (1.9)	11.9 (2.1)	6.8 (2.4)
R$_{merge}$	0.14 (0.69)	0.15 (0.72)	0.11 (0.31)	0.11 (0.76)	0.12 (0.87)	0.16 (0.53)
Refinement						
Non-hydrogen atoms						
Protein	5916	6133	6070	6077	6100	5982
Water	0	0	0	13	0	0
R$_{work}$/R$_{free}$	0.232/0.278	0.217/0.272	0.219/0.265	0.230/0.270	0.219/0.263	0.232/0.272
r.m.s. deviations						
Bond lengths (Å)	0.008	0.011	0.004	0.004	0.013	0.005
Bond angles (°)	1.14	0.9	0.71	0.71	1.31	0.74
Ramachandran plot (%)						
Favored	91	93	93	94	94	93
Allowed	8.9	6.3	6.2	5.5	6.1	7
Disallowed	0.13	0.26	0.38	0.26	0.26	0

Values in parentheses refer to the highest resolution shell for each dataset. [a]resolution range for data collection [b]resolution range for refinement