Supplementary Material

Intrinsic CD4+ T cell sensitivity and response to a pathogen are set and sustained by avidity for thymic and peripheral complexes of self peptide and MHC

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Supplementary Figure 1 Phenotyping and in vivo proliferative responses of mature LLO56 and LLO118 T cells. (a) In vivo proliferation of CFSE-labeled LLO56 and LLO118 T cells in response to Listeria at 2, 4, and 6 days post-infection. The presented FACS plots are representative of 6-7 mice analyzed over four experiments. (b) Expression of signaling and costimulatory molecules on LLO56 and LLO118 T cells. The distribution of CD5 expression for polyclonal B6 CD4+ T cells is included in the CD5 histogram for reference. Data are representative of at least three mice from at least three experiments.
Supplementary Figure 2 Validation of LLO56 and LLO118 signaling results. (a) Immunoblot analysis of ERK phosphorylation kinetics of PMA-stimulated LLO56 and LLO118 CD4+ T cells, representative of two experiments. (b) Confirmation of p21 band in 4G10 blots of unstimulated CD4+ whole cell lysates as phospho-TCRζ using rabbit polyclonal anti-ζ serum 777. 4G10 and anti-ζ serum staining was performed on the same blot. LLO56 was used for this validation as it gave the most easily detectable p21 band. Data are representative of at least three experiments.
**Supplementary Figure 3** CD5<sup>hi</sup> B6 CD4<sup>+</sup> and CD8<sup>+</sup> T cells are more responsive to stimulation than are their CD5<sup>lo</sup> counterparts. (a) Primary (top) and graphed (bottom) data from IL-2 capture assay analysis of CD4<sup>+</sup> and CD8<sup>+</sup> T cells stimulated with αCD3 + αCD28. Cells were gated into four equal fractions based on CD5 expression (Q1 through Q4, from lowest to highest CD5 expression). (b) Comparison of CD5 expression on stimulated and unstimulated CD4<sup>+</sup> and CD8<sup>+</sup> T cells in IL-2 capture (left), intracellular IL-2 (middle), and ERK phosphorylation (right) assays. (c) Overlays of B6 CD4<sup>+</sup> and CD8<sup>+</sup> T cells pre-sorted into CD5 fractions (Q1 through Q4) by flow cytometry, then analyzed for PMA + ionomycin-induced IL-2 (top) and PMA-induced phospho-ERK responses (3 minute stimulation, bottom). Data are representative of two or three experiments. Bar graphs depict means ± SEM, with statistical analyses done using unpaired two-tailed Student’s t tests. *P < 0.01 and **P < 0.001.
**Supplementary Figure 4** Ectopic expression of the SCN4B and SCN5A voltage-gated sodium channel genes confers self-reactivity to CD4^+ T cells in proportion to CD5 expression. (a) Gating strategy for the experiments, showing identification of transfected VGSC^+ T cells. (b) CD69 upregulation response of VGSC^+ LLO56, LLO118 and B6 CD4^+ T cells when cultured with or without B6 APCs, or with APCs pretreated with anti I-A^b. Representative primary data (left) and compiled data (right) are presented. (c) Comparison of CD69 responses of untransfected (SCN5A-SCN4B^-), singly-transfected (SCN5A-SCN4B^+), and doubly-transfected VGSC^+ T cells (SCN5A^+SCN4B^+), in the presence of B6 APCs (without I-A^b blockade). Bars depict means ± SEM. For cells cultured with and without APCs, results from eight cultures over three experiments (LLO56 and LLO118), or six cultures over two experiments (B6 CD4^+) were compiled for the graph in panel (b); for blocking studies with anti-I-A^b, 3 or 4 cultures over two experiments were compiled. Statistical analysis was done using a two-tailed Mann-Whitney test. *P < 0.001.
Supplementary Figure 5 Detailed analysis of selection and activation responses in thymocytes with transgenic expression of a TCR. (a) Identification of post-selection (TCRhiCD69+ thymocytes) from total viable LLO56 and LLO118 thymocytes, representative of at least three experiments. (b) Frequencies of NK (CD3+NK1.1+), NKT (CD3+NK1.1+), and γδ T cells (GL3+) among DN thymocytes. Data are representative of analyses of three mice from two experiments. (c) Gradual emergence of LLO56 and LLO118 responses during the DP to CD4SP thymocyte transition (top plots, labeled 1 through 5; DN thymocytes gated out for clarity). PMA + ionomycin-induced IL-2 responses (bottom left, numbers indicate % IL-2+ cells) and ERK phosphorylation after 3 minute PMA stimulation (bottom right, red and blue numbers indicate LLO56 and LLO118 pERK MFI, respectively) are presented. Data are representative of at least three experiments. (d) PMA + ionomycin-induced intracellular IL-2 responses of CD4+ T cells transgenically expressing the AND TCR on H2b or H2k MHC haplotype backgrounds, representative of three experiments. (e) Analysis of cell survival-associated markers among LLO56 and LLO118 thymocyte subsets and mature CD4+ T cells, representative of two or three experiments. Bar graphs depict means ± SEM, with statistical analyses done using unpaired two-tailed Student's t tests. *P < 0.0001.
Supplementary Figure 6 Loss of the IL-2 responses of LLO56 and LLO118 T cells in adoptive-transfer experiments tracks with deprivation of self peptide-MHC class II. (a) Cell surface phenotype of LLO56 and LLO118 T cells following 4-day transfer to B6 or MHC II-deficient recipients, representative of at least three experiments. (b) Ex vivo analysis of PMA + ionomycin-induced IL-2 responses of LLO56 and LLO118 T cells following 4-day transfer to TCR Cα-deficient (LLO56 n = 8, LLO118 n = 6) or H-2M-deficient (LLO56 n = 11, LLO118 n = 10) recipients. Data are compilations of four experiments. (c) Ex vivo analysis of PMA + ionomycin-induced IL-2 responses of freshly isolated LLO56 T cells (n = 4) or LLO56 T cells transferred to H-2M-deficient recipients for the indicated periods of time (Day 1 n = 8, Day 2 n = 6, Day 4 n = 11). (d) Schematic of experiment testing effect of self-pMHC withdrawal on LLO56 and LLO118 T cell response to Listeria in vivo. For (b) and (c), each data point comprising the bar graphs is the % IL-2+ LLO T cells from a single recipient. Bar graphs depict means ± SEM, with statistical analyses done using unpaired two-tailed Student’s t tests. NS, not significant and *P < 0.0001.