SUPPLEMENTAL MATERIAL

Gottschalk et al., http://www.jem.org/cgi/content/full/jem.20091999/DC1

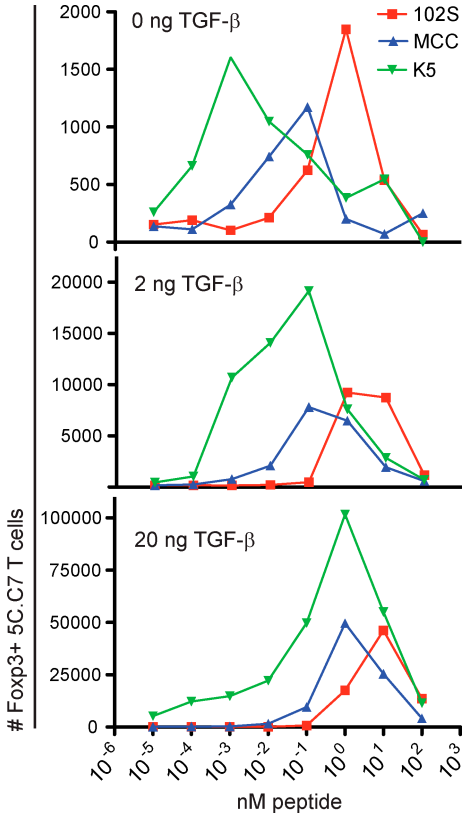

Figure S1. TGF-β raises the maximum TCR stimulation conducive for in vitro Foxp3 induction. 5C.C7 RAG2$^{-/-}$ LN cells were cultured with irradiated T cell–depleted splenocytes in the presence of IL-2 and the indicated peptide, with and without TGF-β. After 4 d, the absolute number of Foxp3-positive 5C.C7 in each condition was determined. Data are representative of at least two independent experiments.

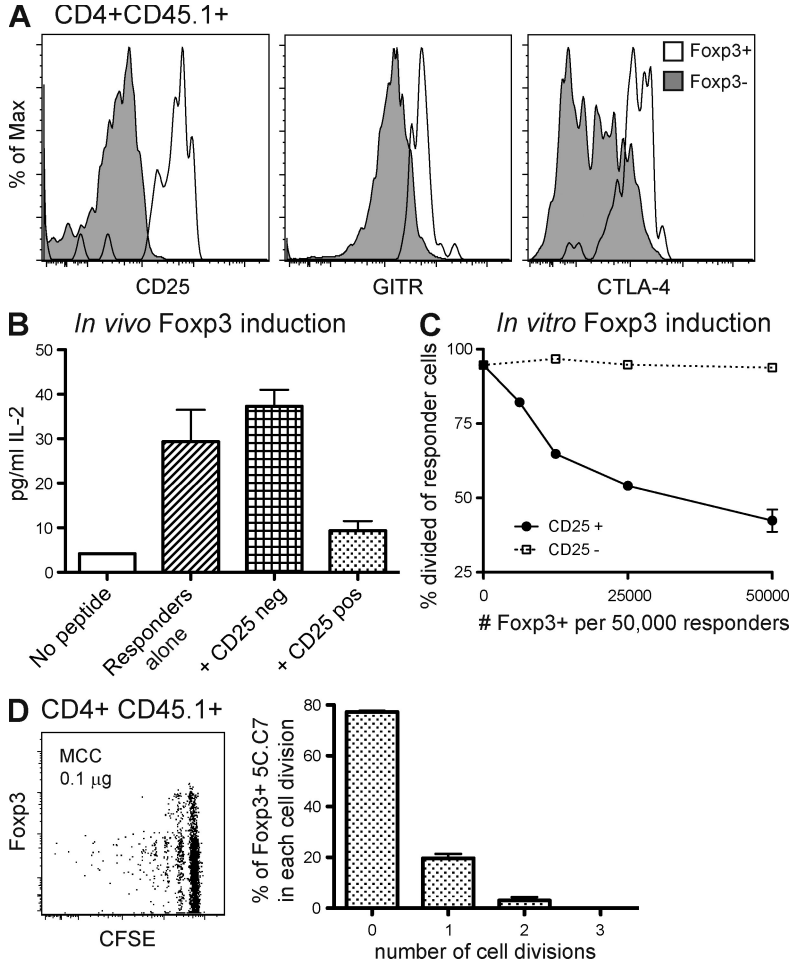

Figure S2. Foxp3⁺ 5C.C7 display characteristics of T reg cells. (A) Surface expression of CD25 and GITR, and total expression of CTLA-4, was measured on Foxp3-positive and -negative 5C.C7 8 d after injection of 0.1 µg of MCC. (B) To assess suppression by in vivo induced Foxp3⁺ 5C.C7, adoptively transferred CD25-positive and -negative CD45.1⁺ 5C.C7 T cells were sorted by FACS 2 wk after 0.1 µg MCC injection and co-cultured at a one-to-one ratio with naive 5C.C7 responders. 72 h later, supernatant IL-2 levels were measured by cytokine bead array (BD). (C) Division of CFSE-labeled 5C.C7 responders was suppressed by addition of in vitro induced Foxp3⁺ 5C.C7. Cells were induced by culturing for 4 d with 10 ng/ml TGF-β, 100 U/ml IL-2, 1 nM MCC, and T cell–depleted splenocytes and were separated into CD25-positive and -negative compartments using magnetic beads (Miltenyi Biotec). (D) 5C.C7 T cells show an inverse correlation between proliferation and Foxp3 induction. Naive 5C.C7 RAG2$^{-/-}$ CD45.1 T cells were adoptively transferred into B10.A recipients and stimulated in vivo by injecting 0.1 µg MCC. The frequency of converted cells in each division was calculated from CFSE profiles at day 6. $n = 2$. Error bars represent SEM. The ex vivo suppressor assay in B was completed with CD25⁺ 5C.C7 pooled from 10 mice, with duplicate wells. All other data are representative of at least two independent experiments.

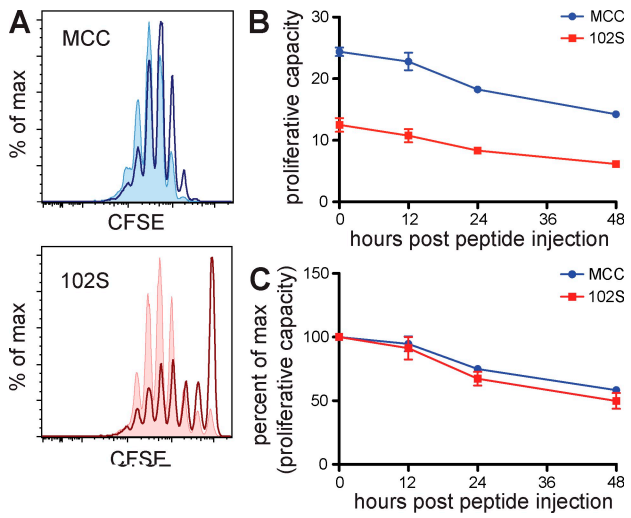

Figure S3. MCC and 102S peptides are cleared at comparable rates, in vivo, based on decreased proliferative capacity of responding T cells over time. 10 µg of either MCC or 102S was injected i.v. into B10.A recipients 0, 12, 24, and 48 h before adoptive transfer of CFSE 5C.C7 T cells. The proliferative capacity of the responding 5C.C7 was used as a readout of how much peptide was available at the indicated times after injection. (A) Representative CFSE profiles of 0 and 48 h, shaded and bold, respectively. Proliferative capacity (B) was normalized to the maximum for each peptide, at 0 h, to account for the difference in ligand strength (C). Data in B and C are pooled from two independent experiments. Error bars represent SEM.

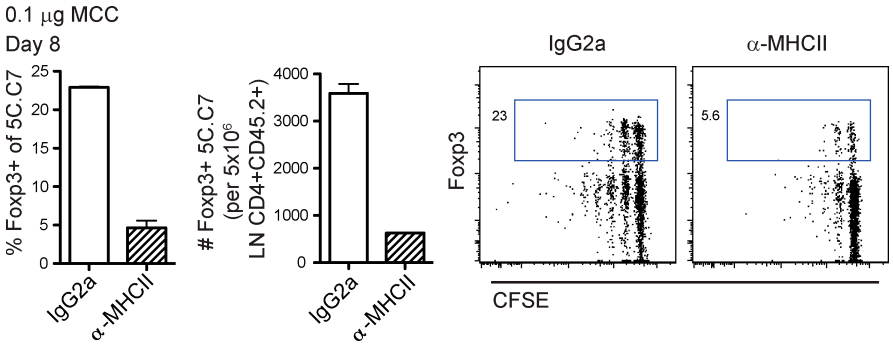

Figure S4. Injection of antibody to MHCII does not enhance Foxp3 induction by the optimal dose of MCC. CFSE-labeled 5C.C7 were transferred into B10.A recipients, which were subsequently injected i.v. with 0.1 µg of MCC. 10 h later, recipients received injection of antibody to MHCII or control IgG2a antibody. The number and percentage of 5C.C7 expressing Foxp3 were assessed 8 d after peptide injection. Error bars depict the SD of two to three mice per group, adjacent to representative dot plots showing the percentage of Foxp3+ cells. These data are representative of at least three independent experiments.

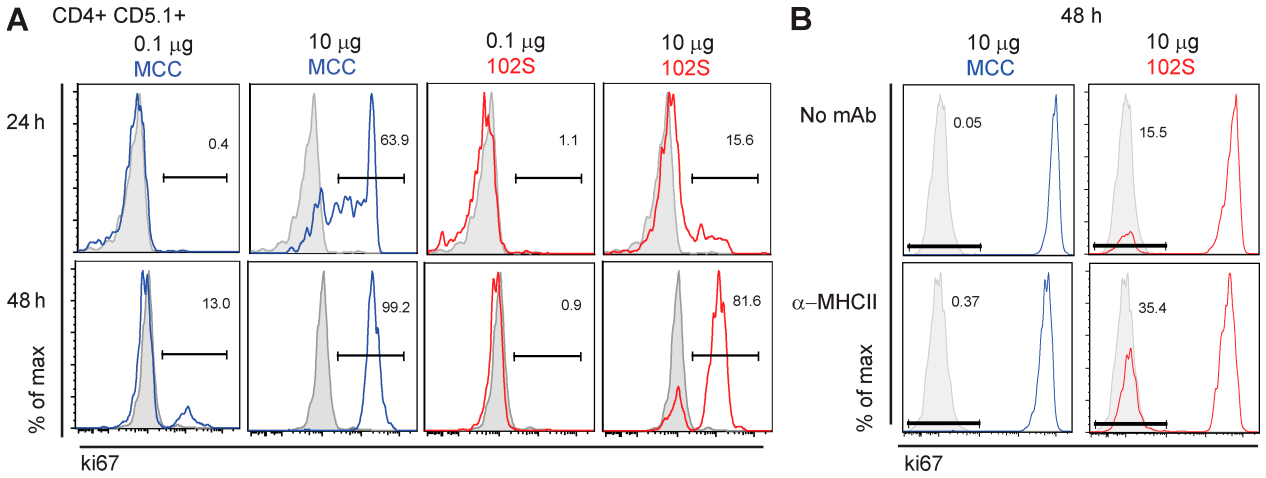

Figure S5. 102S-stimulated cells proliferate with a temporal delay, compared with the same dose of strong agonist MCC. (A) Cell cycle status of adoptively transferred RAG2$^{-/-}$ CD45.1 5C.C7 T cells was assessed based on expression of the proliferation marker ki67, 1 and 2 d after injection of the indicated peptide. (B) The frequency of ki67^{-} cells 2 d after peptide injection was used to determine the effect of blocking MHCII on cell cycle entry in 102S- and MCC-stimulated cells. Histograms are gated on CD4^{+}CD45.1^{+} cells and are pooled from two mice. The percentage of cells that are positive or negative for ki67 are shown on the histograms. Shaded histograms represent 5C.C7 cells from mice that did not receive peptide. Data are representative of three independent experiments.

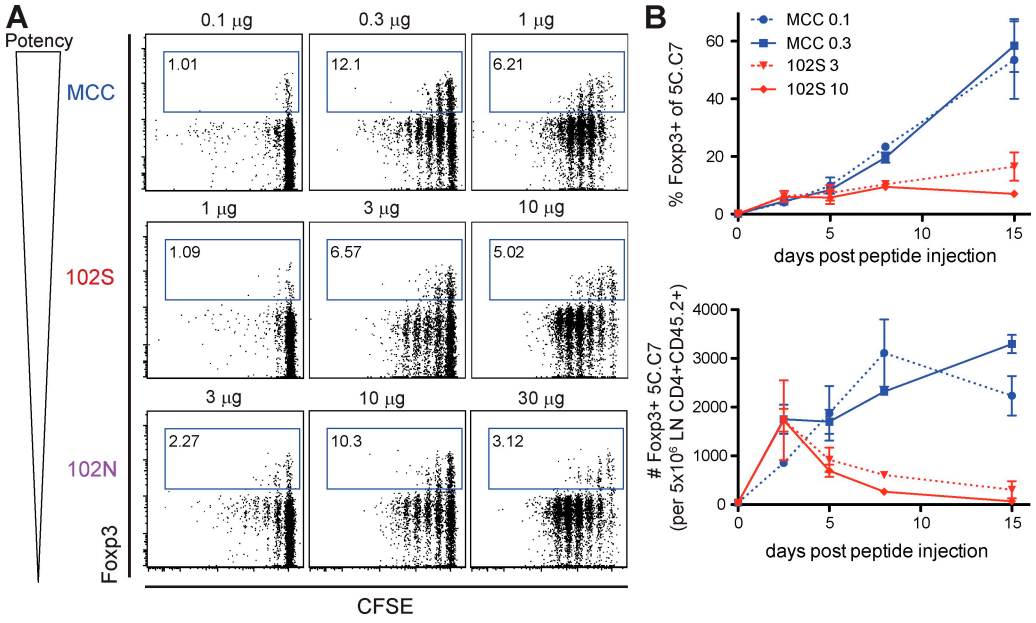

Figure S6. TCR ligand potency determines optimal dose for proliferation and initial Foxp3 induction in vivo. 5C.C7 RAG2$^{-/-}$ CD45.1 T cells were CFSE labeled, transferred into B10.A recipients, and subsequently stimulated in vivo by i.v. injection of the indicated peptide and dose. (A) Representative dot plots from 5 d after peptide injection are gated on CD4^{+}CD45.1^{+} LN cells and show the percentage of Foxp3^{+} 5C.C7. (B) Time course data shown in Fig. 6, with additional peptide doses included. $n = 2$ with SD. Data are representative of at least three independent experiments.

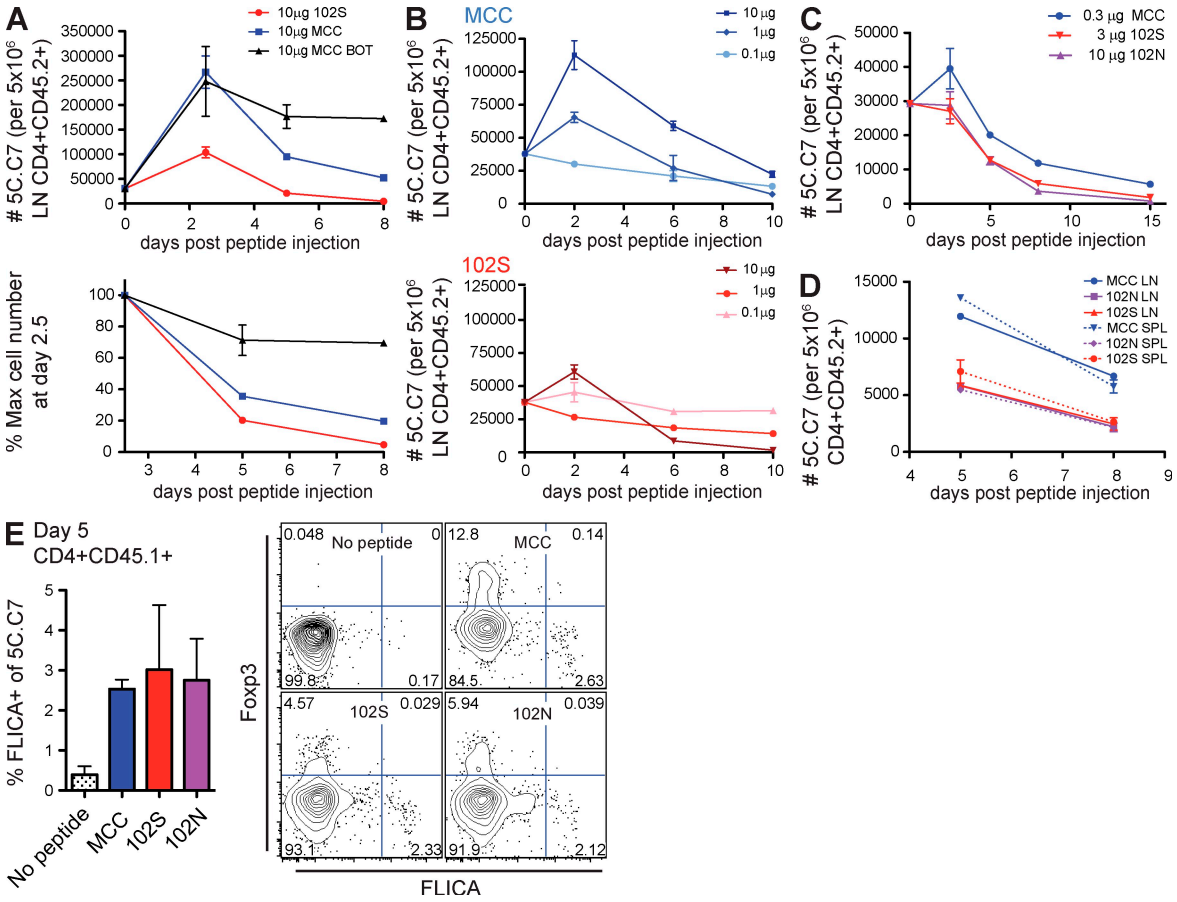

Figure S7. Intravenous injection of either MCC or 102S results in rapid dose-dependent deletion of responding 5C.C7. 10^6 adoptively transferred 5C.C7 T cells were stimulated with the indicated peptide injected i.v., unless specified BOT, which is a control injection for an effector response, at the base of the tail with peptide and LPS. (A–D) At the indicated time points after peptide injection, recipients were scarified and the number of total 5C.C7 in the LNs (A–C) or spleen (D) was determined, as normalized to the endogenous CD4+CD45.2+ compartment. In (C-D) recipients were injected with doses previously determined to give equivalent CFSE dilution and peak Foxp3 induction (0.3 μg MCC, 3 μg 102S, and 10 μg 102N). (E) At 5 d after peptide injection, Poly Caspases FLICA kit (ImmunoChemistry Technologies) was used to detect the presence of active caspases. Bar graphs show data pooled from two experiments with the SEM. Representative contour plots are gated on $CD4^+CD45.1^+$ cells showing the percentage of 5C.C7 within each quadrant. All data are representative of at least two independent experiments.

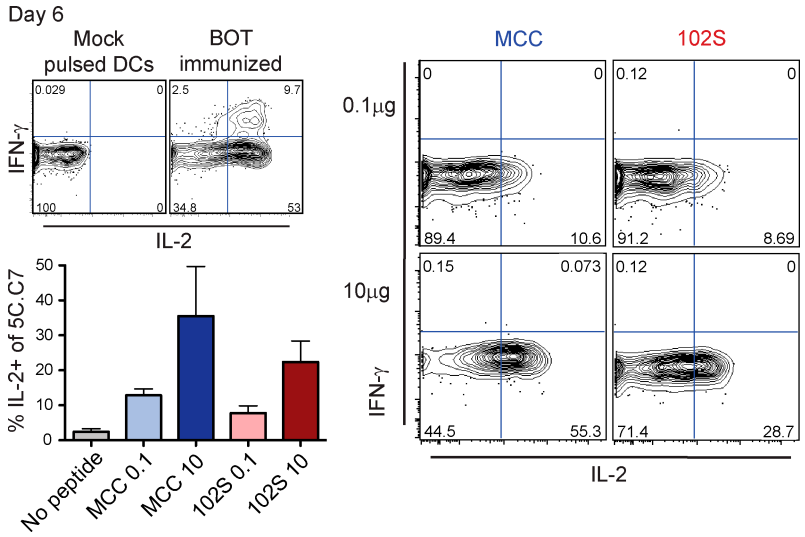

Figure S8. Intravenous injection of MCC and 102S peptides results in 5C.C7 production of IL-2, but not IFN-γ. CD4+CD45.1+ LN cells were harvested 6 d after they were stimulated in vivo by injection of the indicated peptide (injected i.v. unless indicated at the base of tail [BOT], a subcutaneous control immunization including LPS). Cells were subsequently restimulated ex vivo for 5 h with MCC-pulsed dendritic cells before intracellular cytokine staining. Bar graphs show data pooled from two independent experiments, and error bars show the SEM. Representative contour plots are gated on CD4+CD45.1+ cells and show the percentage of cells in each quadrant.