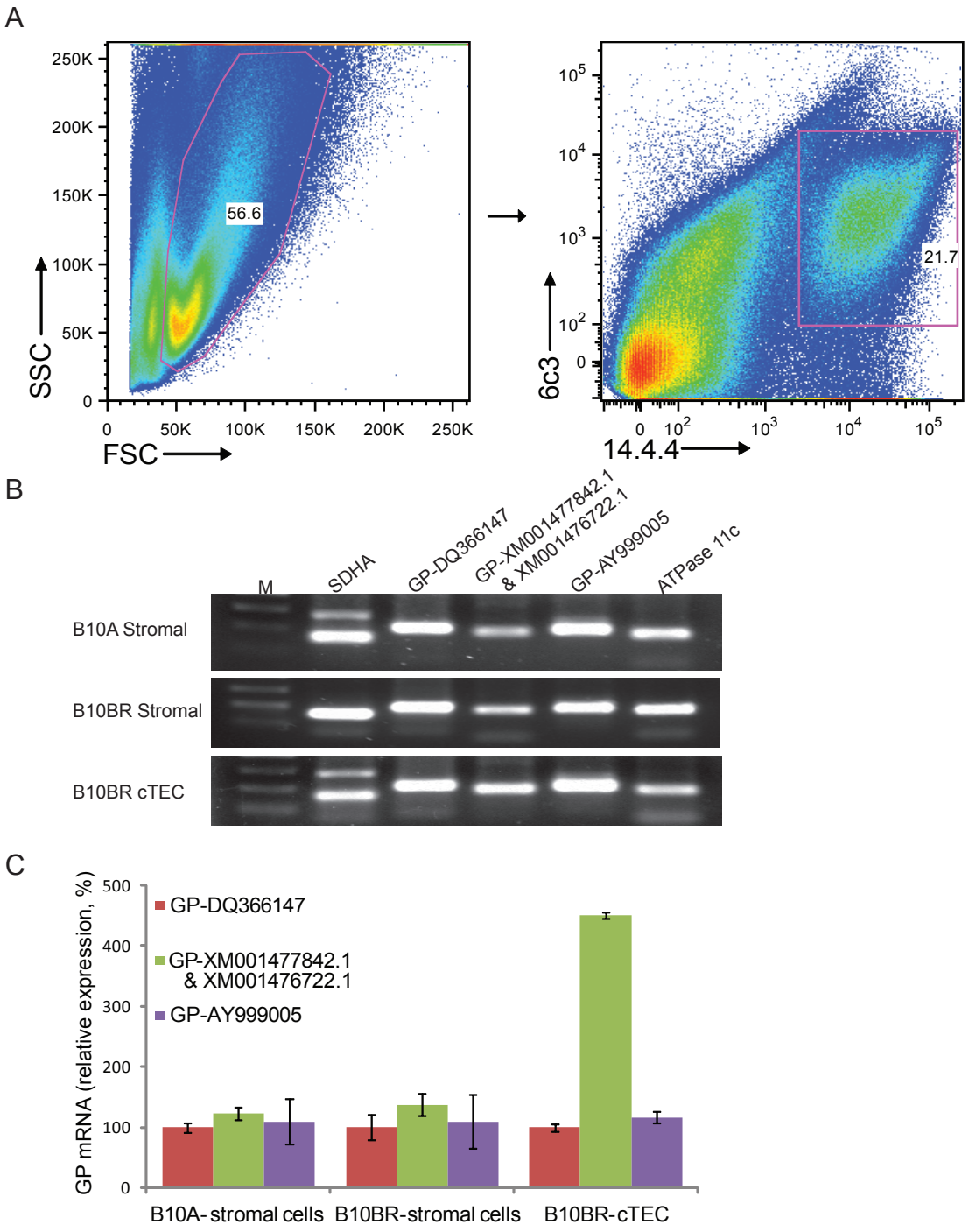
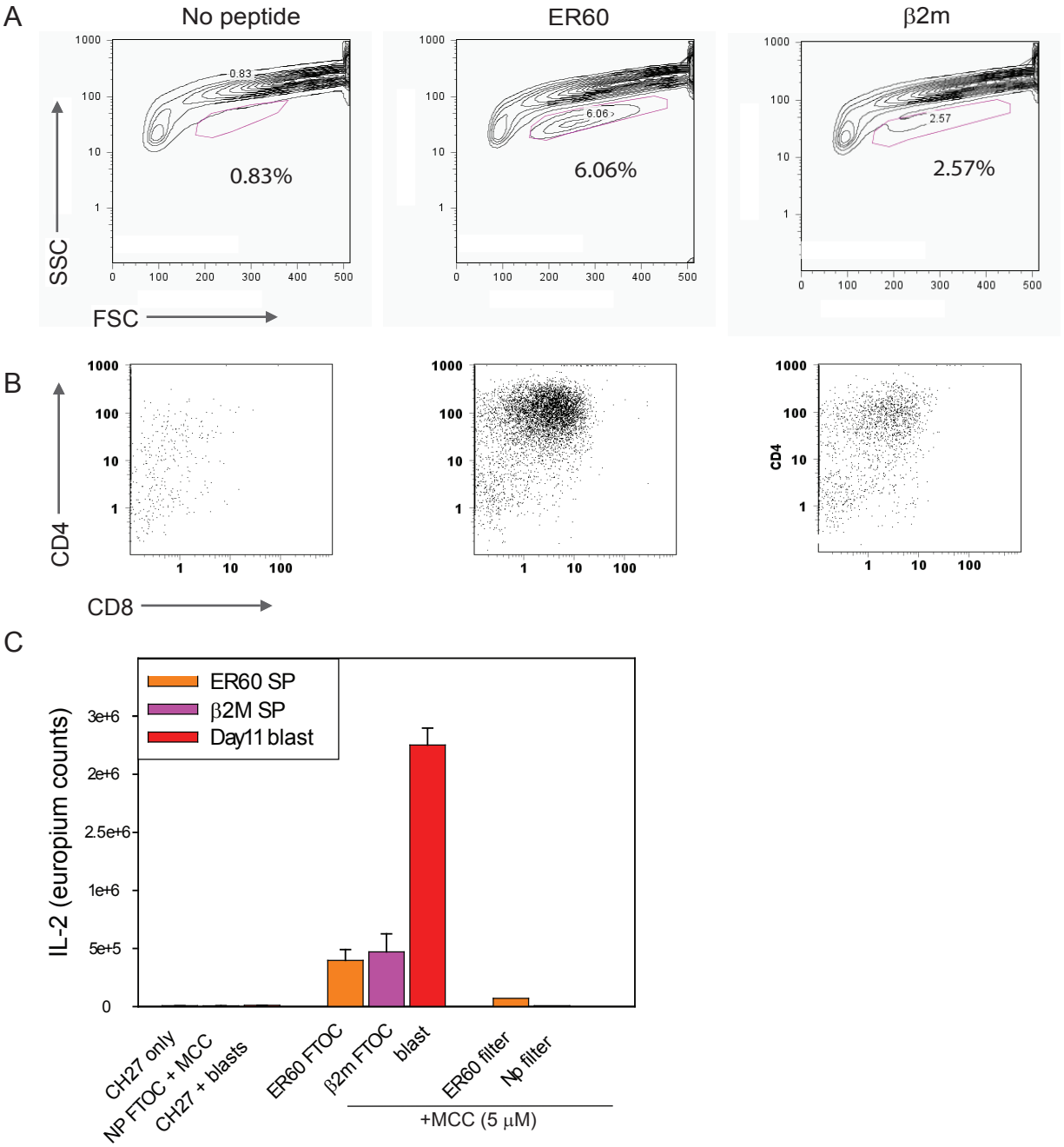
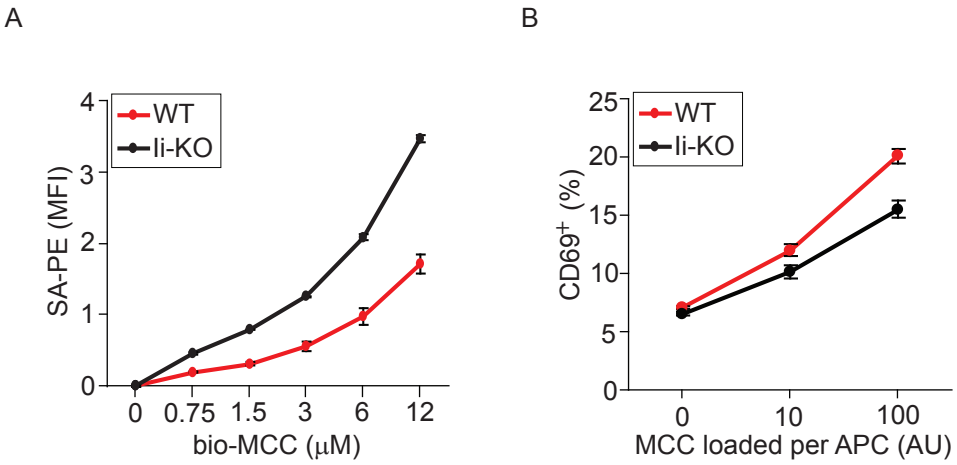


Supplementary Figure 1. GP expression in the thymus. (A) FACS profiles of the sorted cortical thymic epithelial cells used for RT-PCR analysis. Stromal cells were obtained by collagenase-D treatment of B10.A or B10.BR thymi and subsequent depletion using anti-Thy1.1 Dynal beads. $6C3^{+}14.4.4(I\text{-}E^{k})^{+}$ cTECs were sorted according to the depicted gating scheme. **(B)** RT-PCR to detect transcripts of the four genes predicted to contain the GP peptide sequence. The two KIAA1466 orthologs (XM_001476722.1 and XM_001477842.1) are predicted to be 98% identical and could not be distinguished by this assay, so primers were designed to detect both transcripts. Succinate dehydrogenase complex subunit A (SDHA) is included as an internal control. M, molecular weight marker. **(C)** Quantitative PCR to measure mRNA encoding these four loci using the same primer sets as in (B).

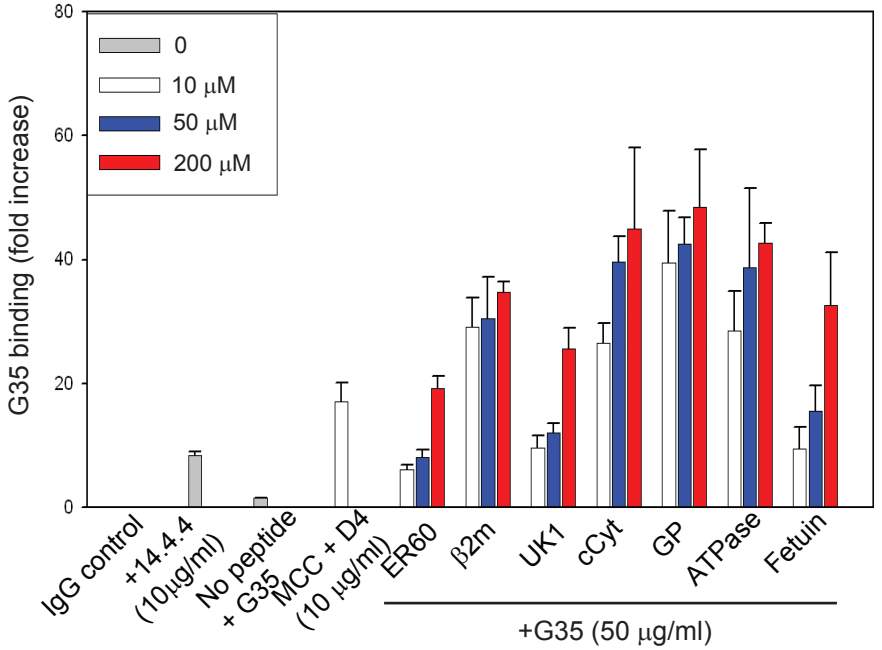


Supplementary Figure 2. Weak positively selecting peptides generate functional mature T cells. E16 5C.C7 Ii-KO FTOC were incubated for 7 days with the indicated peptides, then harvested and stimulated for 4 days with irradiated CH27 cells loaded with 5 μM MCC peptide. Cells were then analyzed by flow cytometry, and supernatants analyzed by ELISA. **(A)** Forward and side scatter profiles. Numbers indicate the percentage of live lymphocytes. **(B)** CD4 versus CD8 profiles of live cells gated in (A). **(C)** IL-2 in supernatants was measured by ELISA. Pre-stimulated wild-type 5C.C7 T cells (blasts) served as a positive control.

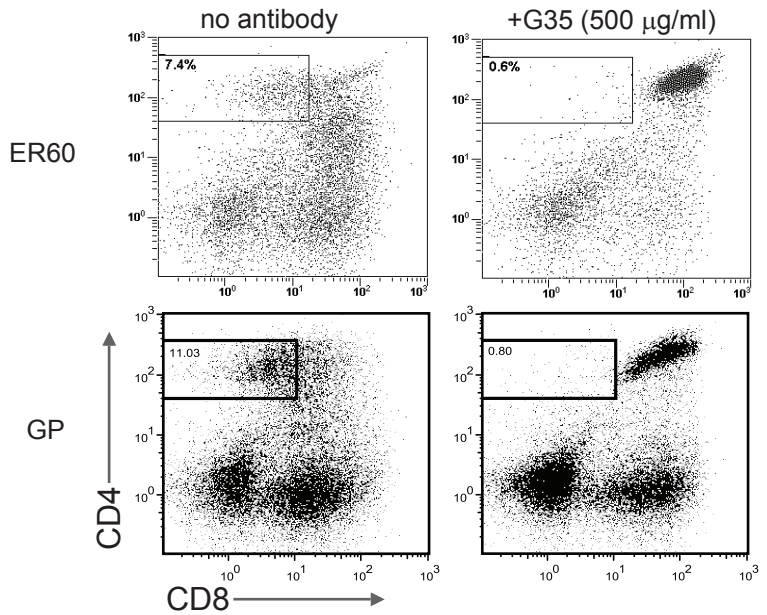


Supplementary Figure 3. Invariant chain-dependent peptides contribute to 5C.C7 T cell activation. (A) T -cell-depleted B10.BR or Ii-KO splenocytes were incubated with the indicated concentrations of biotinylated MCC, then washed extensively and stained with streptavidin-PE. **(B)** Wild-type and Ii-KO splenocytes were loaded with equal amounts of MCC, and were then used as APCs to stimulate naïve 5C.C7 T cells. The percentage of $CD69^+$ T cells after 4 hours was determined by flow cytometry.

A



B



Supplementary Figure 4. G35 binds to GP-I-E^k and blocks GP-I-E^k-induced positive selection. (A) CHO cells expressing GPI-linked I-E^k were incubated overnight with the indicated peptides, then washed and incubated 2 hours with 50 μg/ml G35 antibody, or with 10 μg/ml 14.4.4 (as a positive control for I-E^k expression and detection), or 10 μg/ml D4 (as a positive control for MCC loading and detection). G35 binding was detected using HRP-labeled rat anti-mouse IgG, and the fold increase over background is given for the various peptide-I-Ek complexes. D4 binding to MCC-I-E^k was used as a positive control . **(B)** E16 5C.C7 Ii-KO FTOC were incubated with 500 μM ER60 or GP peptide for 5 days, with or without addition of 500 μg/ml G35 antibody. Positive selection was assessed by flow cytometric analysis of CD4 and CD8 expression.

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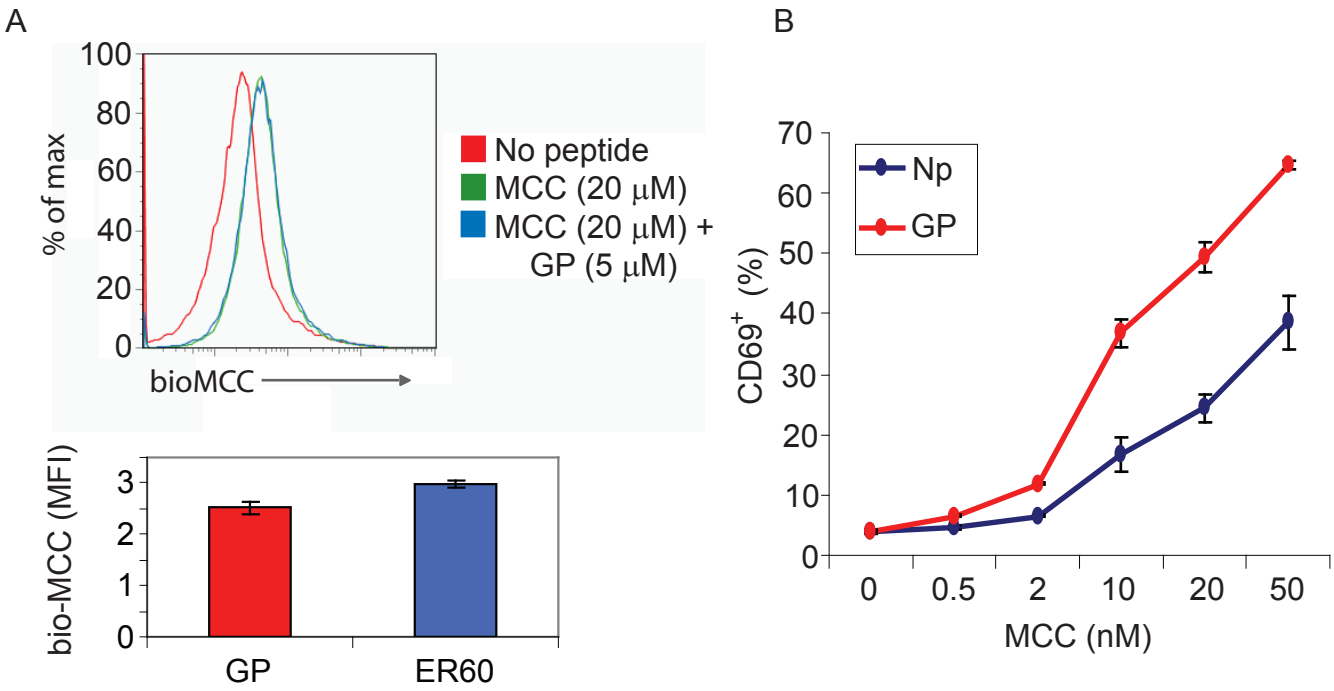
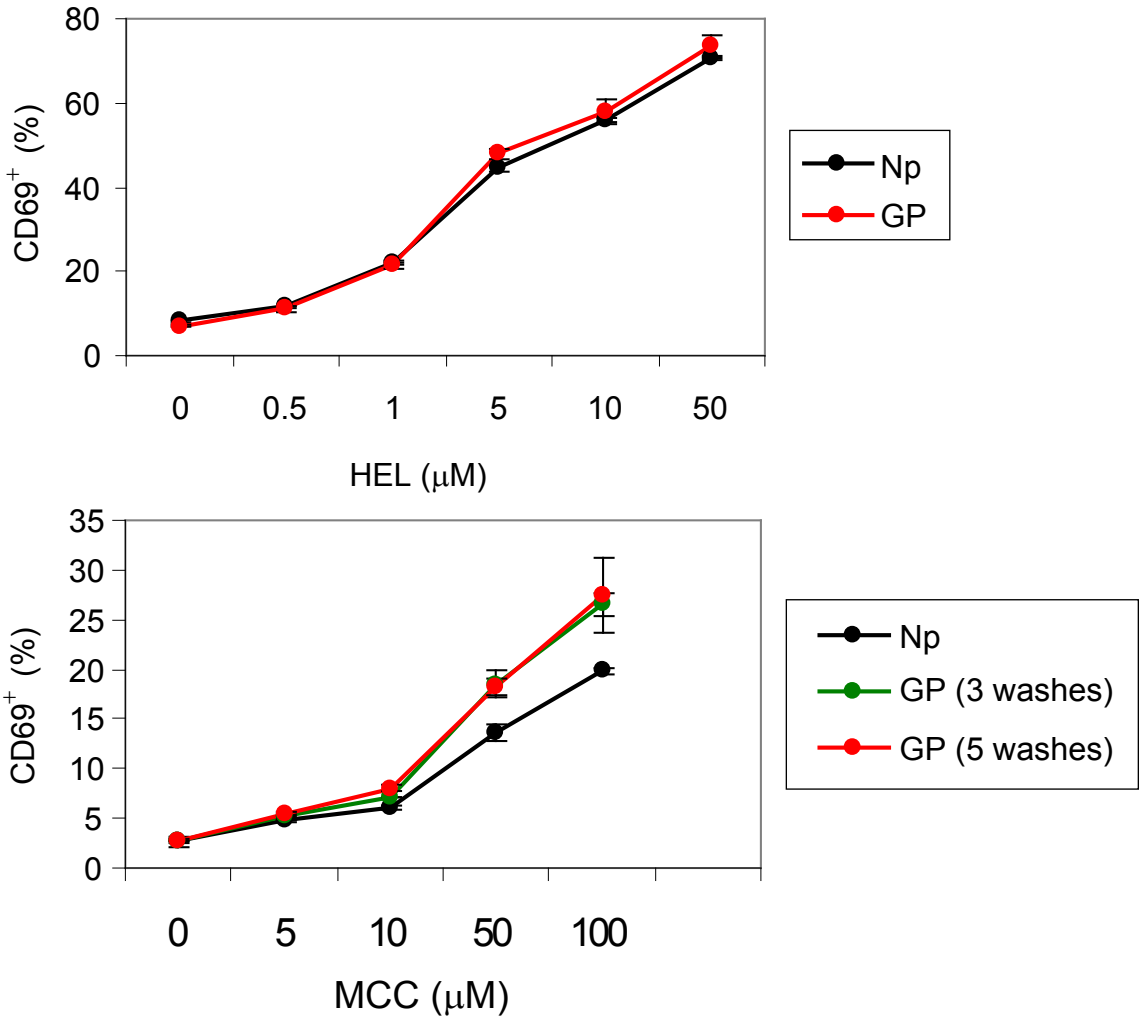
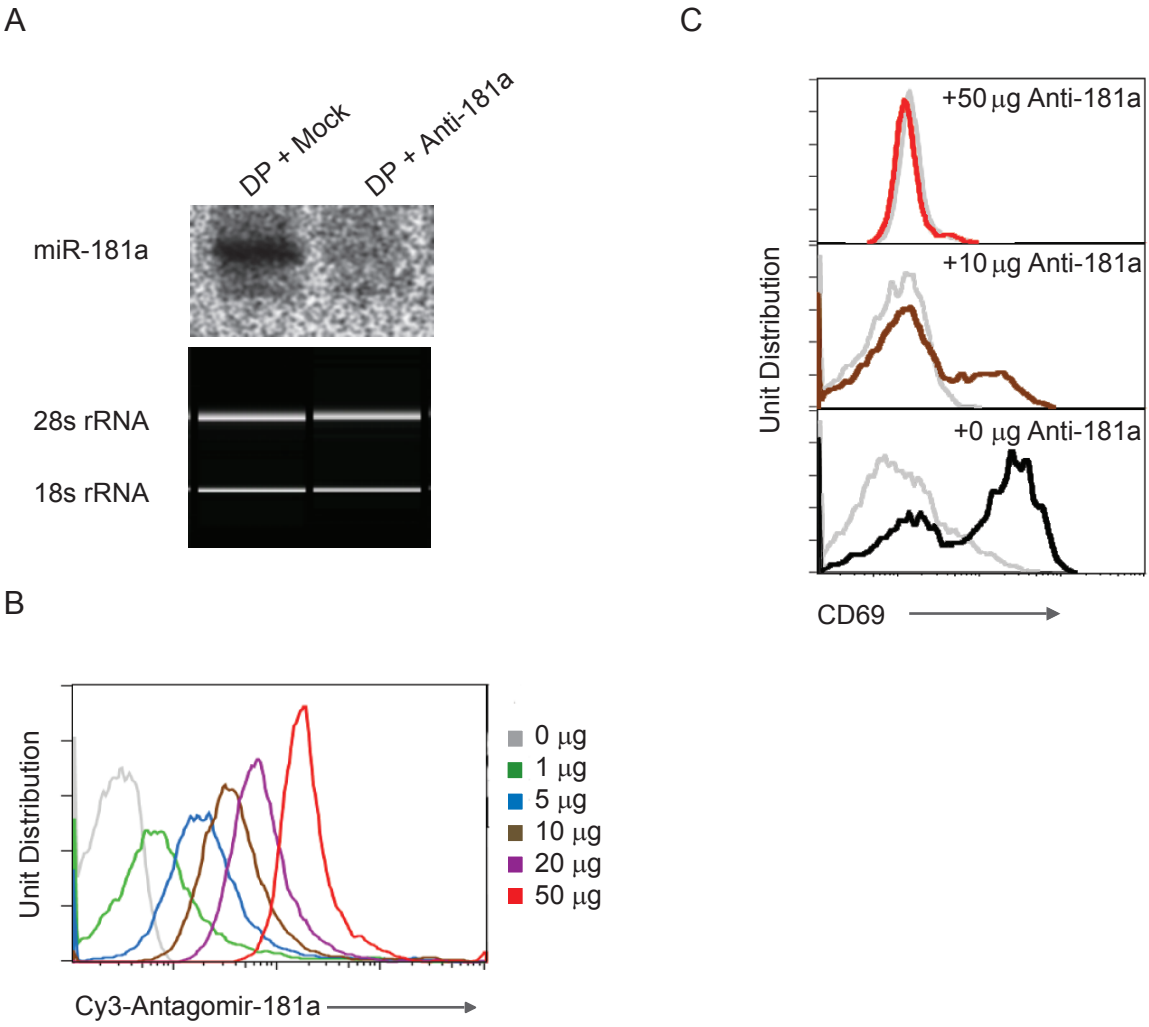


Supplementary Figure 5. GP-mediated enhancement of the 5C.C7 T cell response is not the result of enhanced MCC loading. Some peptides, such as CLIP, can catalyze the loading of exogenously added peptides into class II molecules. To make sure that this is not how GP enhances the 5C.C7 T cell response, we loaded B10.BR splenic APCs with 20 μM biotin-MCC, in the presence or absence of 5 μM GP or ER60 peptide. **(A)** Loaded MCC-I-E^k was detected using streptavidin-PE and flow cytometry. **(B)** Splenic APCs were loaded with MCC at the indicated concentrations, then washed extensively, then loaded with 5 μM GP or no peptide (np) prior to incubation with naïve 5C.C7 T cells. Under these conditions, GP and MCC were never present in solution at the same time and thus GP cannot influence MCC loading. 5C.C7 T cell CD69 expression was measured by flow cytometry.



Supplementary Figure 6. Effect of peptide competition for loading on 5C.C7 and 3A9 responses to antigen plus GP. B10.BR splenic APCs were loaded for one hour with the indicated concentrations of either HEL or MCC, then washed extensively, loaded one hour with or without (np) 5 μM GP peptide, then washed extensively again prior to addition of 3A9 or 5C.C7 T cells. For 5C.C7 T cells, APCs were washed either 3 or 5 times after GP incubation, as indicated. CD69 expression on T cells was measured by flow cytometry.



Supplementary Figure 7. Effect of antagomir-181a on miR-181a expression and TCR sensitivity. (A) Northern blot analysis of mock-treated or antagomir-181a-treated 5C.C7 DP thymocytes (50 µg/ml antagomir-181a), using an antisense miR-181a probe. 28s and 18s ribosomal subunit RNA are shown for the same samples, as measured by Agilent RNA Analyzer, to confirm equivalent loading. **(B)** Flow cytometry profiles of 5C.C7 DP thymocytes treated with the indicated doses of a Cy3-labeled antagomir-181a. **(C)** CD69 expression on 5C.C7 DP thymocytes treated with the indicated doses of Cy3-labeled antagomir-181a in response to MCC-pulsed CH27 cells.