

## Results

*The Limited Frequency of Activated CD4 T Cells Is Independent of DC-induced Activation.* To further assess whether the restriction in the frequency of activated CD4 T cells was due to inadequate conditions for antigen presentation by the APCs, which would limit the presentation or the antigen itself or the probability of interaction between the naive CD4 T cells and competent APC, we tested the frequency of activated DO11.IL-2P/GFP CD4 cells stimulated directly through their TCR using anti-CD3 and anti-CD28 mAbs. DO11.IL-2P/GFP CD4 cells were incubated for 20 h in culture plates coated with increasing amounts of anti-CD3 in the presence of anti-CD28 (Fig. S2 A). The frequency of GFP<sup>+</sup> cells increased to 13.9% of the total CD4 population with 3  $\mu$ g/ml immobilized anti-CD3 and 10  $\mu$ g/ml anti-CD28, and did not increase with higher concentration of anti-CD3. Similar limited frequencies of GFP<sup>+</sup> cells were observed upon mitogenic stimulation with PMA/ionomycin of CD4 T cells from DO11.IL-2P/GFP transgenic mice (unpublished data).

*Activated CD4 T Cells Do Not Inhibit Activation of Neighboring T Cells.* We wanted to assess the possibility that T cells activated early in the response actively inhibited their clonal partners through an APC-independent mechanism. To address this issue, DO11.IL-2/GFP T cells were activated for 24 h by plate-bound anti-CD3 and 10  $\mu$ g/ml anti-CD28 mAbs under limiting T cell precursor frequency conditions in 96-well plates and the number of GFP<sup>+</sup> cells/well was measured (Fig. S2 B). Irrespective of the density of T cells per well, including conditions with less than one T cell per well on average, the frequencies of IL-2/GFP<sup>+</sup> cells within the CD4 populations were quite consistent ( $\sim$ 10%), refuting a possible bystander inhibitory effect of early-activated T cells or of a potential T regulatory population.

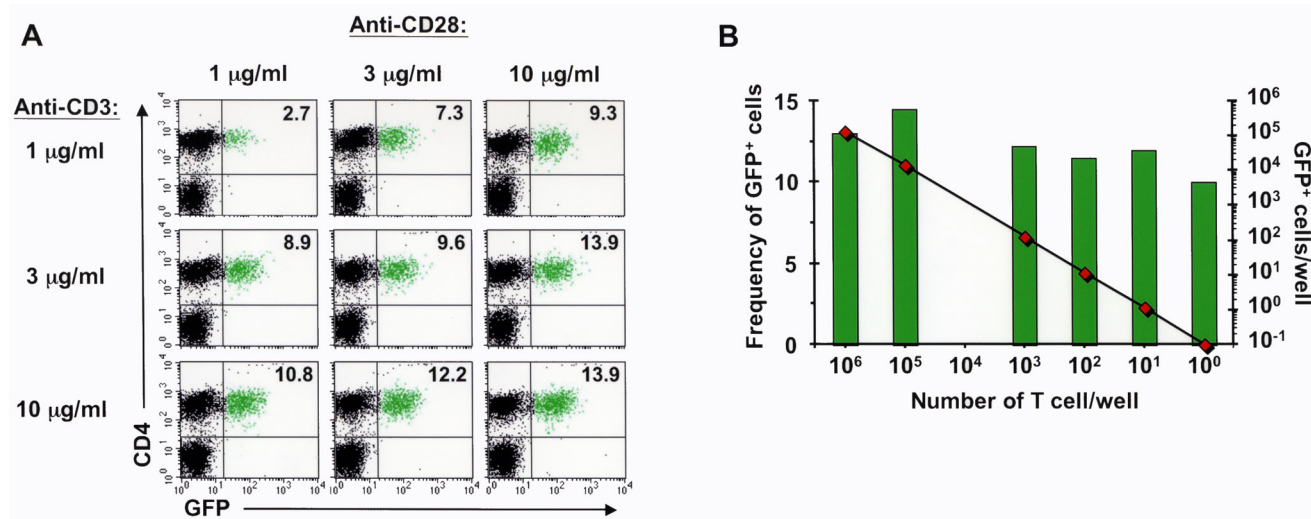


Figure S2. Limiting dilution analysis of naive DO11.IL-2P/GFP Tg T cells activated by anti-CD3/anti-CD28. (A) Stimulation of naive DO11.IL-2P/GFP Tg T cells by anti-CD3/anti-CD28. Isolated lymph node cells from DO11.IL-2P/GFP.RAG-2<sup>-/-</sup> mice were cultured in 1–10  $\mu$ g/ml anti-CD3 pre-coated wells plus 1–10  $\mu$ g/ml anti-CD28 for 24 h and analyzed by flow cytometry. The frequency of GFP<sup>+</sup> cells among the CD4<sup>+</sup> cells is indicated for each dot-plot. (B) Limiting dilution analysis of naive DO11.IL-2P/GFP Tg T cells activated by optimal anti-CD3/anti-CD28. Naive CD4<sup>+</sup> T cells isolated from DO11.IL-2P/GFP.RAG-2<sup>-/-</sup> mice were cultured in 10  $\mu$ g/ml anti-CD3 pre-coated wells plus 5  $\mu$ g/ml anti-CD28 at the indicated input cell densities per well. The frequencies of GFP<sup>+</sup> cells were determined by flow cytometric analysis (input cell numbers 10<sup>6</sup> and 10<sup>5</sup> per well) or direct counting by fluorescence microscopy (input cell numbers <10<sup>4</sup> per well) after stimulation for 20 h, and are based on minimum counts of 250 cells per condition.