Supporting Information

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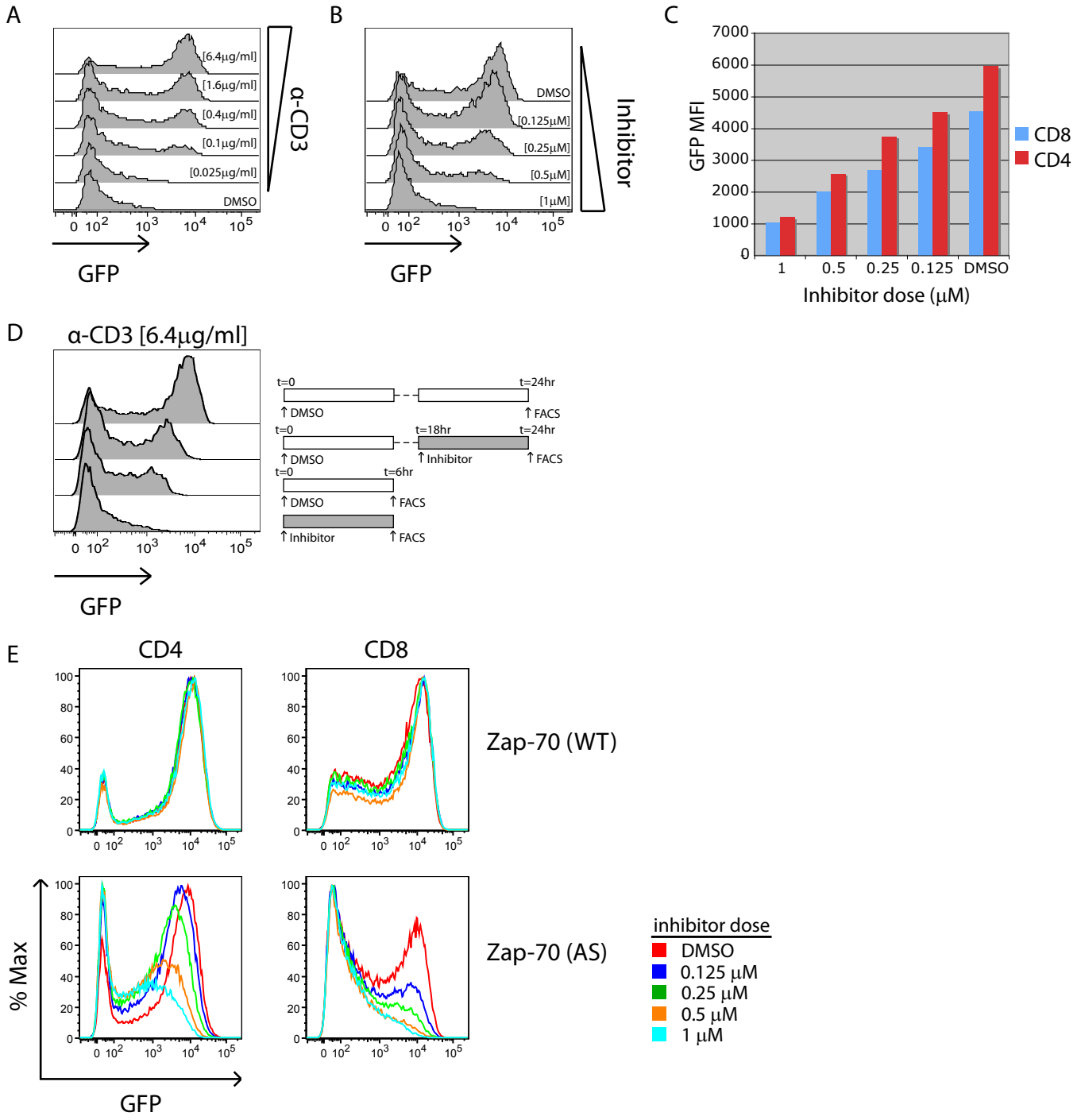

Fig. S1. Titration and detection of T-cell receptor (TCR) signaling in CD8$^+$ T cells. CD8$^+$ T cells from "analog-sensitive" zeta-chain-associated protein kinase 70 kDa (Zap70) mutant-Nur77-eGFP mice [hereafter referred to as Zap70(AS)–GFP] as in Fig. 1 were stimulated overnight with (*A*) varying doses of plate-bound anti-CD3 or with (*B*) a fixed dose of anti-CD3 (6.4 μg/mL) and either vehicle (DMSO) or the indicated concentrations of the Zap70(AS) inhibitor HXJ42. (*C*) Graph shows the mean fluorescence intensity of GFP for the conditions shown in *B* and in Fig. 1*B*. (*D*) CD8$^+$ Zap70(AS)–GFP T cells were stimulated for varying durations of inhibitor treatment, and GFP induction expression was then assessed by flow cytometry. (*E*) WT Nur77–GFP (*Upper*) and Zap70(AS)–GFP (*Lower*) T cells were stimulated for 20 h with plate-bound anti-CD3 in the presence of varying concentrations of HXJ42. GFP fluorescence was assessed by flow cytometry and the data indicate no off-target effect of HXJ42 on WT CD4$^+$ or CD8$^+$ T cells. Data are representative of at least three independent experiments.

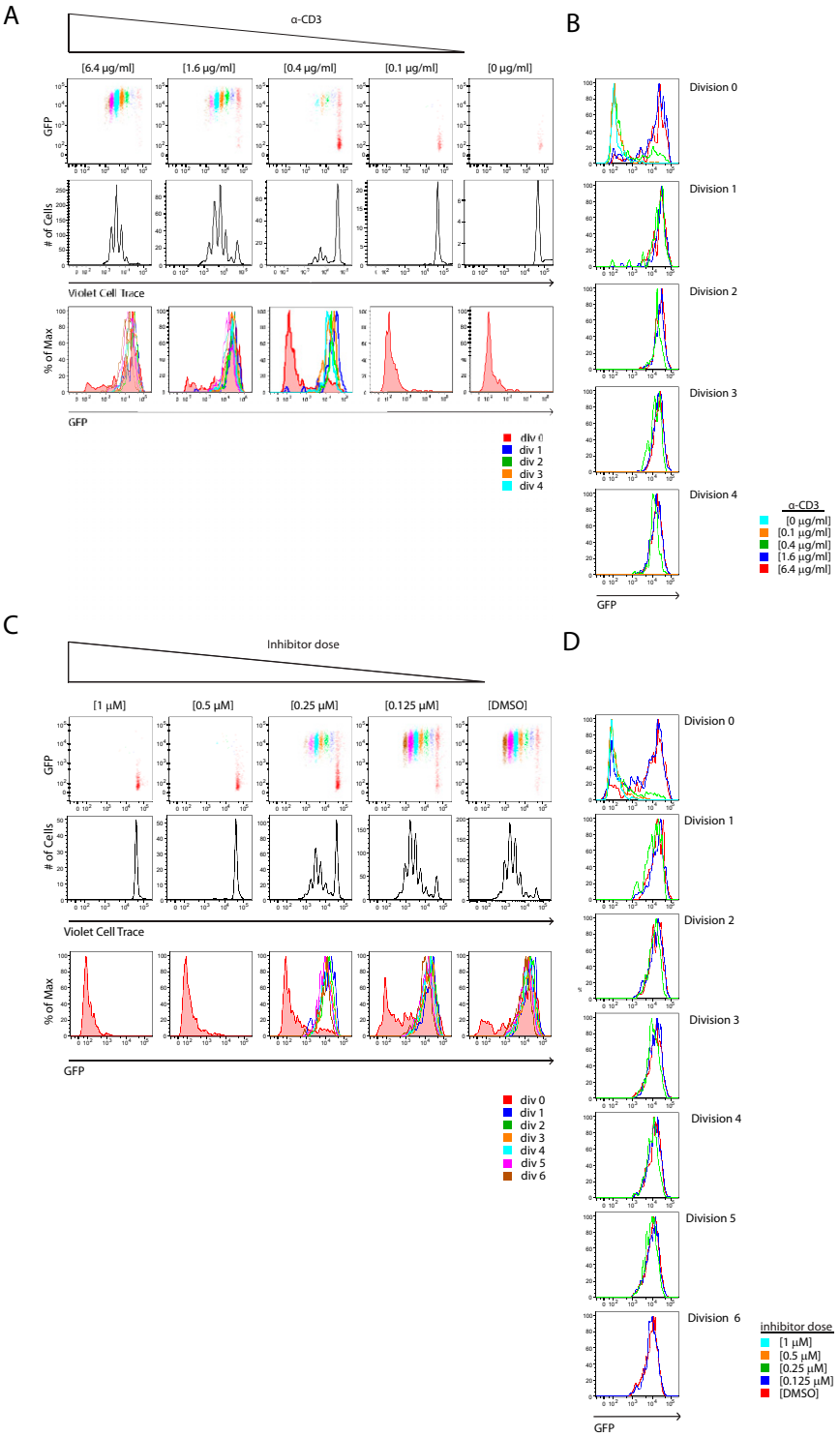

Fig. S2. TCR signaling threshold in CD8$^+$ T cells. Nur77–GFP CD8$^+$ T cells were loaded with violet cell trace dye and stimulated with (*A* and *B*) varying doses of plate-bound anti-CD3 for 4 d, or (*C* and *D*) with a fixed concentration of plate-bound anti-CD3 (6.4 μg/mL) and the indicated concentrations of HXJ42, as in Fig. 2. Cells were subsequently stained for T-cell markers and (*A* and C) assessed for dye dilution and GFP fluorescence in combination (*Top Row*), dye dilution alone (*Middle Row*), or GFP fluorescence alone (*Bottom Row*). Individual cell divisions are color-coded (*Top* and *Bottom Rows*). (*B* and *D*) Histograms show GFP expression by cells in each cell division that have been treated with varying concentrations of anti-CD3 or HXJ42. Data are representative of at least three independent experiments.

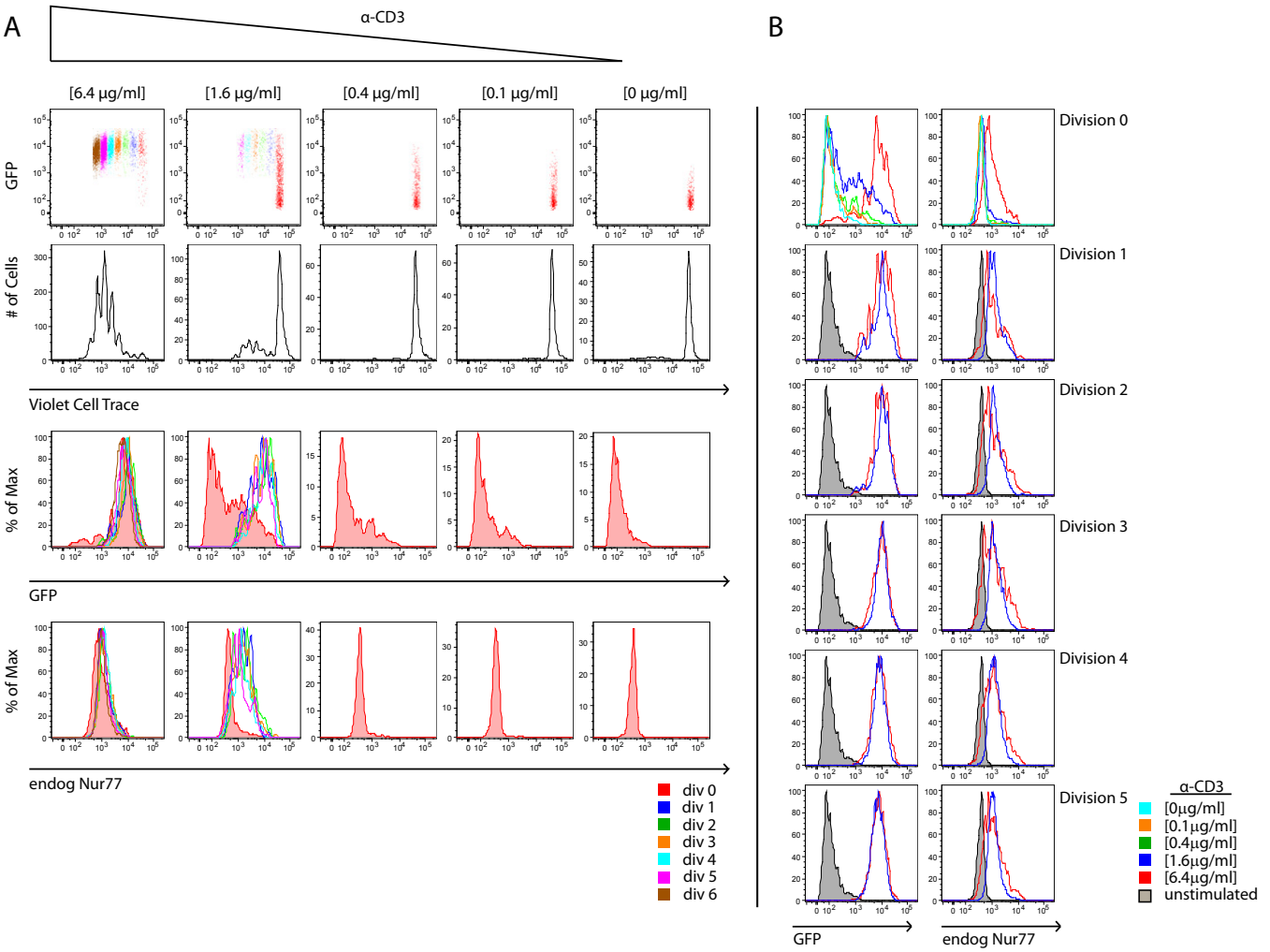

Fig. S3. Correlation between endogenous Nur77 expression and GFP reporter in vitro. CD4$^+$ T cells from Nur77–GFP reporter mice were loaded with violet cell trace dilutional dye and stimulated as described in Fig. 2*A*. After 4 d, cells were subsequently assessed for (*A*) GFP fluorescence and dye dilution, GFP alone, or endogenous Nur77 expression by cell division. (*B*) Histograms show GFP fluorescence (*Left*) and endogenous Nur77 staining (*Right*) for cells within the indicated cell division. Data are representative of at least three independent experiments.

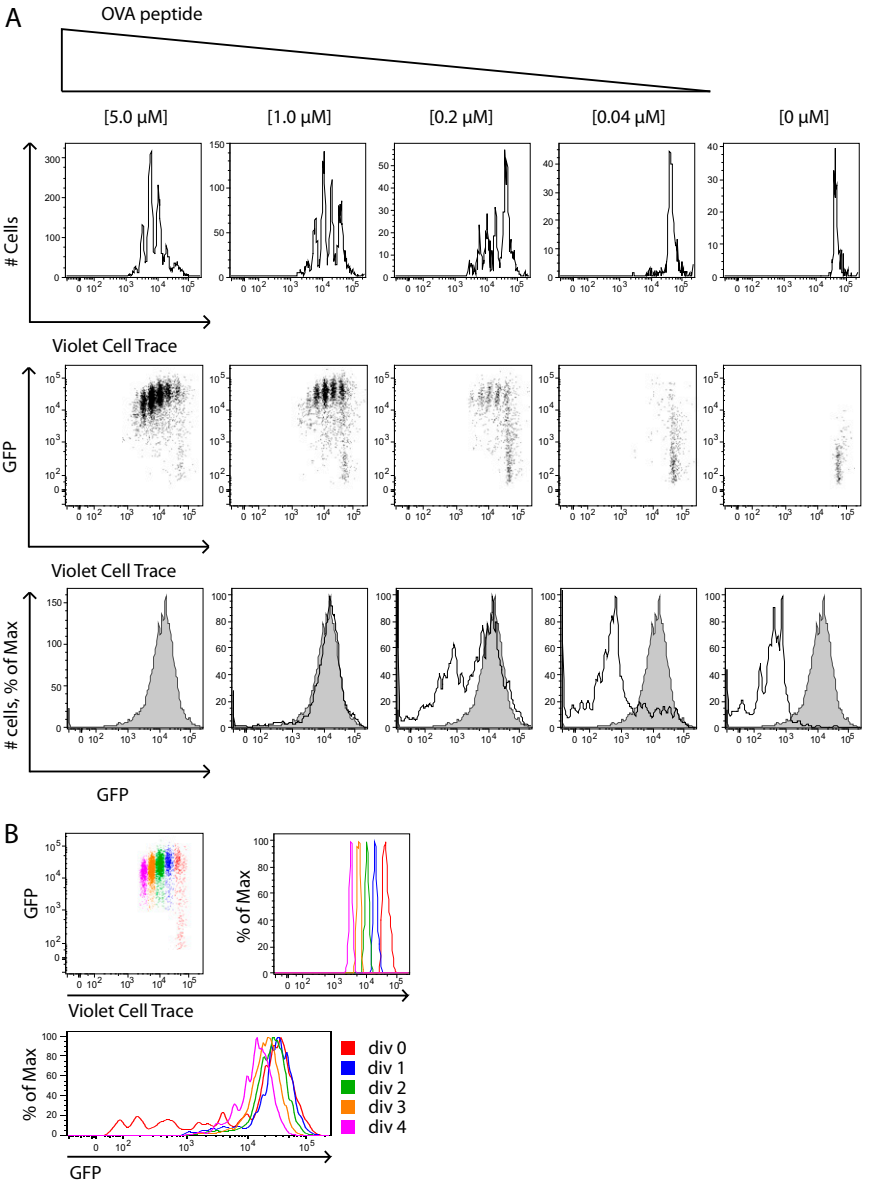

Fig. S4. TCR signaling threshold in CD4$^+$ T cells stimulated with peptide MHC (pMHC) in vitro. (*A*) Total lymph node cells from OT2 transgenic mice harboring the Nur77–GFP reporter (OT2–GFP mice) were loaded with violet cell trace dye and stimulated with varying doses of ovalbumin (OVA) peptide for 4 d. CD4$^+$ cells were assessed for dye dilution alone (*Top*) or in combination with GFP fluorescence (*Middle*) by flow cytometry. (*Bottom*) GFP expression in the entire population of T cells (not gated by individual cell division). The gray shaded histogram in each panel is a reference population receiving maximum stimulation (5 μM OVA). (*B*) Individual cell divisions from the 5 μM OVA dose above are color coded (*Upper*). Overlaid histograms (*Lower*) compare GFP fluorescence by cells within each color-coded cell division. Data are representative of at least three independent experiments.

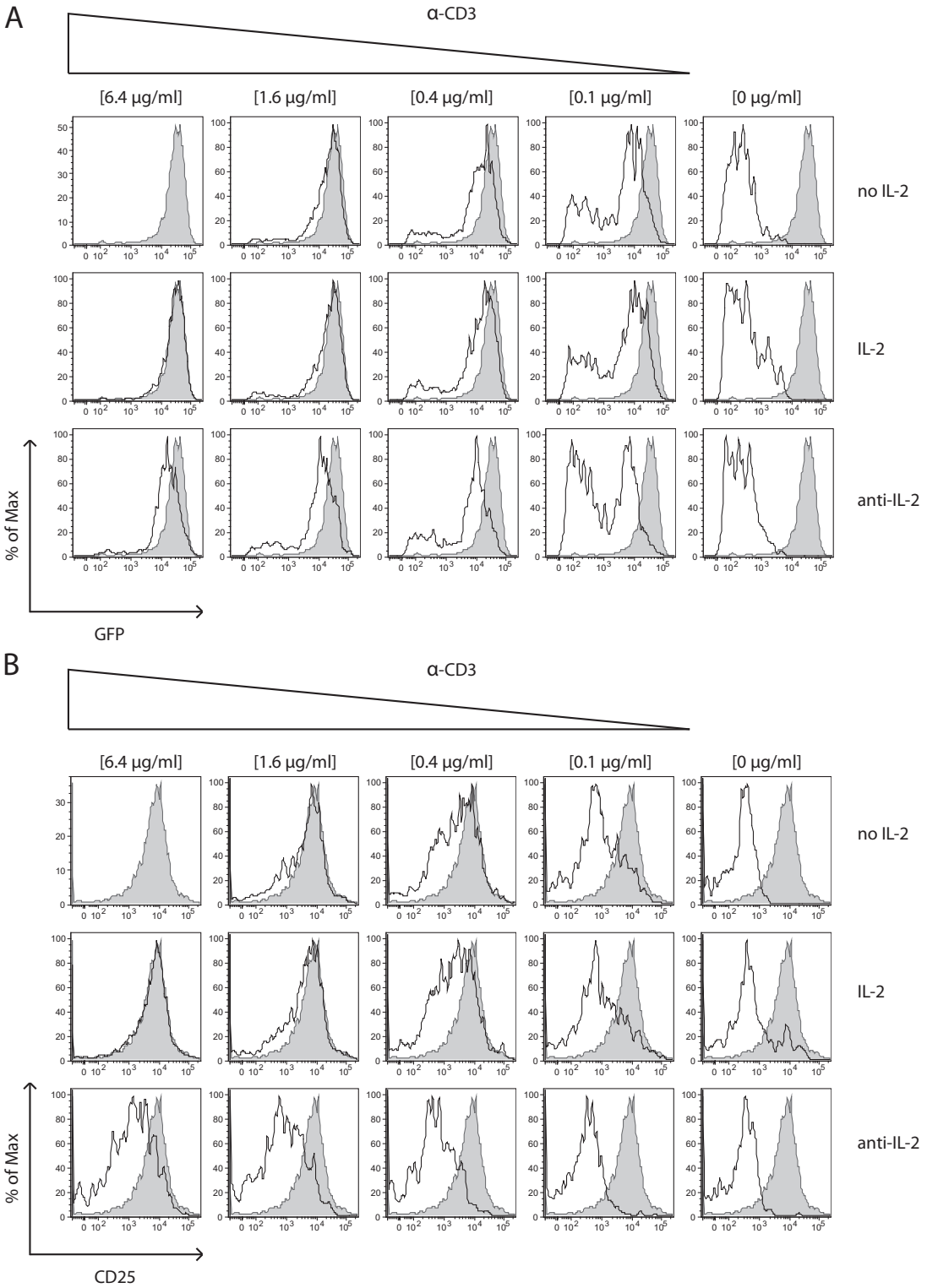

Fig. S5. TCR signaling threshold is dissociated from CD25 expression by anti–IL-2 antibody treatment. (*A*) GFP and (*B*) CD25 expression on samples displayed in Fig. 5. All panels represent total live cells, not gated by cell division as in Fig. 5. The gray shaded histogram overlayed for reference on each panel represents cells stimulated with 6.4 μg/mL anti-CD3 without exogenous IL-2 or anti–IL-2 added. Data in this figure are representative of at least three independent experiments.

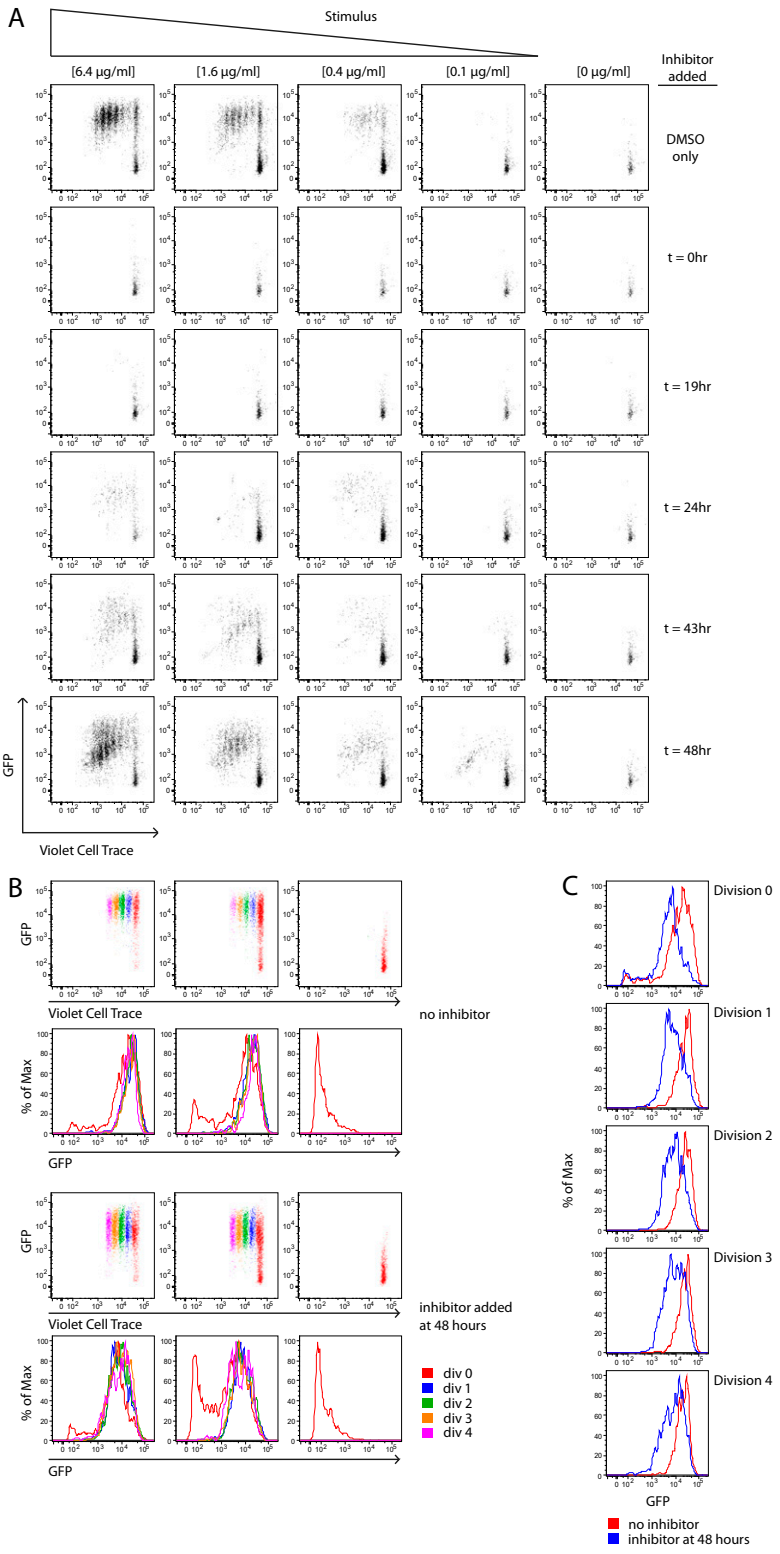

Fig. S6. Continuous TCR signaling is dispensable after 24 h for commitment of CD8$^+$ T cells to proliferation. (*A*) CD8$^+$ T cells from Zap70(AS)–GFP mice were loaded with violet cell trace dye and stimulated with varying doses of plate-bound anti-CD3 along with a fixed dose of anti-CD28 as in Fig.6. Samples were then treated with 1 μM HXJ42 or vehicle alone at various time points after initial stimulation, and all samples were then harvested at 96 h. Cells were stained for T-cell markers and assessed for GFP fluorescence. (*B*) Individual cell divisions marked by dye dilution in samples treated with vehicle alone or HXJ42 at 48 h from Fig. 6 were color coded and overlaid. (*C*) GFP expression within each cell division from Fig. 6 samples treated with vehicle or HXJ42 are overlaid. Data are representative of at least three independent experiments.