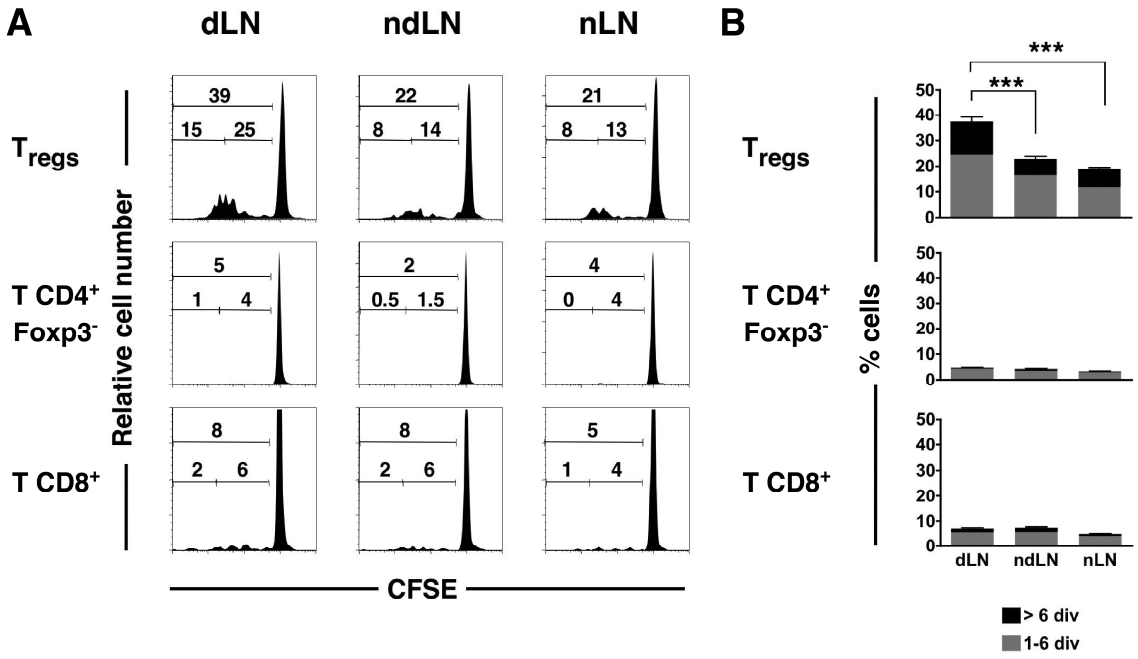
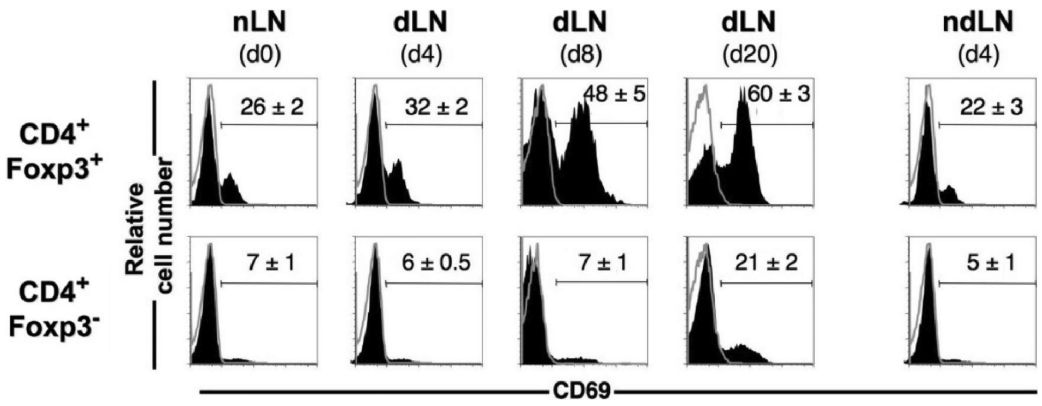
Supplementary data



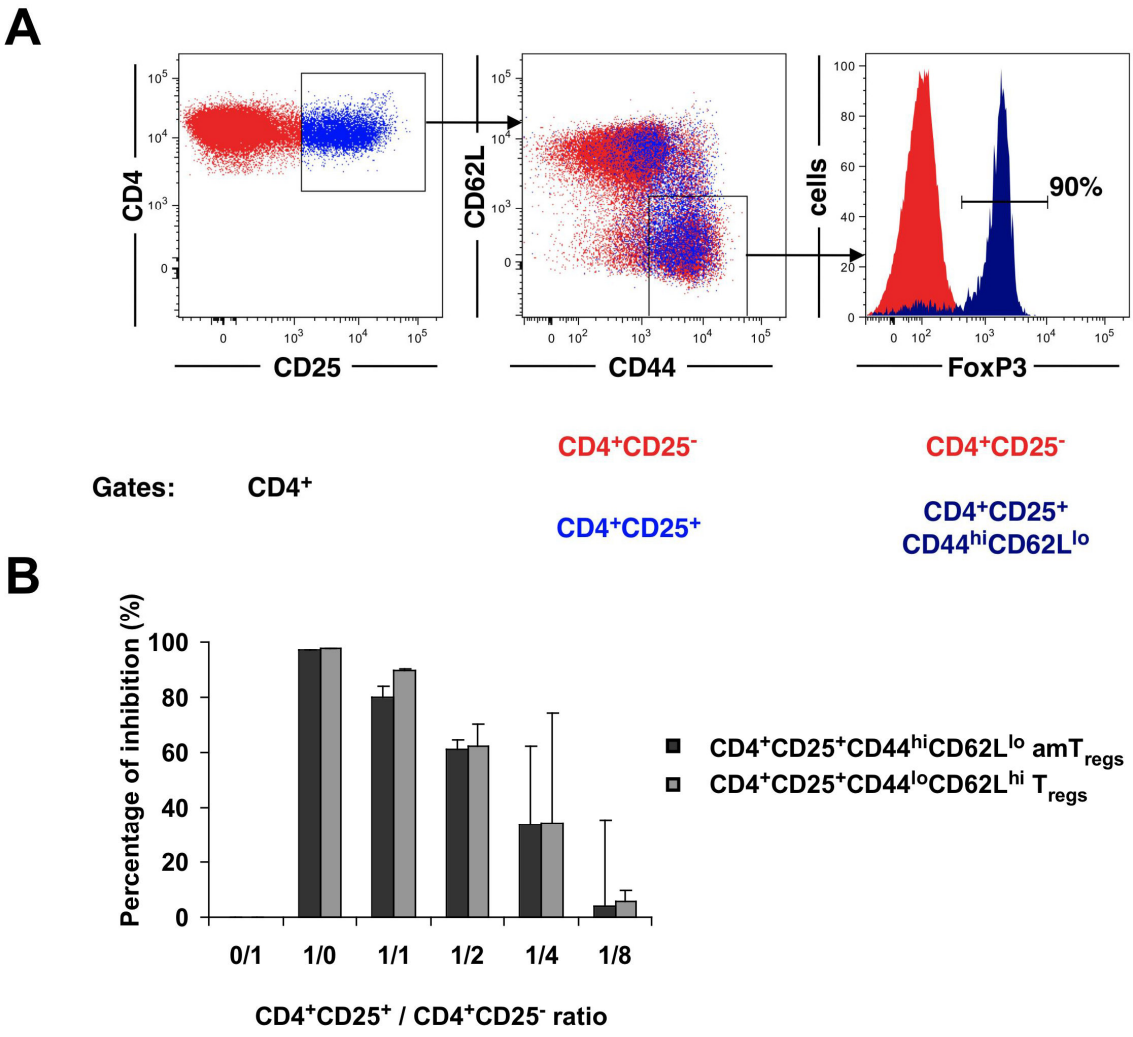
Supplementary Figure 1

Memory-like proliferation of T_{regs} in 4T1 tumor-bearing mice. One day 4T1 tumor-bearing or un-manipulated mice received CFSE-labeled Thy1.1 congenic cells from normal BALB/c mice. Division profiles of Thy1.1$^+$ donor cells were evaluated by flow cytometry in dLNs and ndLNs of tumor bearers, or in nLNs from tumor free mice, 3 days after adoptive transfer. **(A)** Percentage of cells that had undergone no (CFSE^{hi}), few (CFSE^{int}) or more than 6 divisions (CFSE$^-$) are shown in each panel. **(B)** Corresponding mean percentages of expended cells ±SD from **(A)**. CD4$^+$Foxp3$^+$ from dLNs divide significantly more than in other organs (***: $P < 0.0001$). Each panel is representative of three mice per experiment (2 experiments).



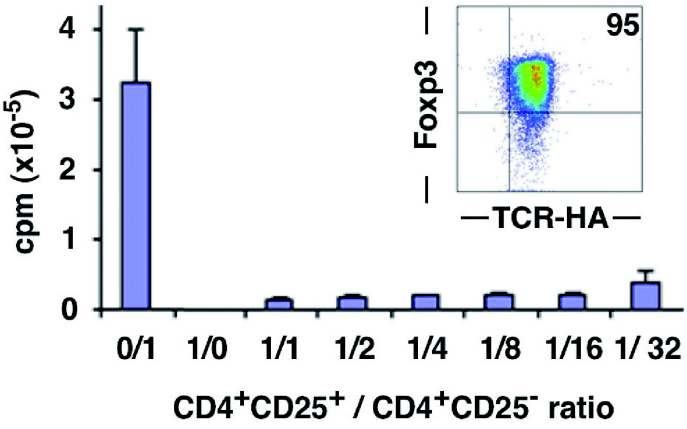
Supplementary Figure 2

Activation status of T_{regs} and T_{effs} of tumor-bearing mice assessed by CD69 expression. Levels of CD69 (Very Early Activation Antigen) expressed by $Foxp3^+$ and $Foxp3^-$ $CD4^+$ T-cells from dLNs, ndLNs or nLNs were evaluated by flow cytometry at days 4, 8 and 20 of 4T1 tumor growth. Each panel is representative of three mice per experiment. Mean percentages ±SD of $CD69^+$ cells among indicated T-cell populations are illustrated. Gray histograms represent the background staining with isotype matched irrelevant mAbs. Representative flow cytometry analyses of one of two independent experiments are shown.



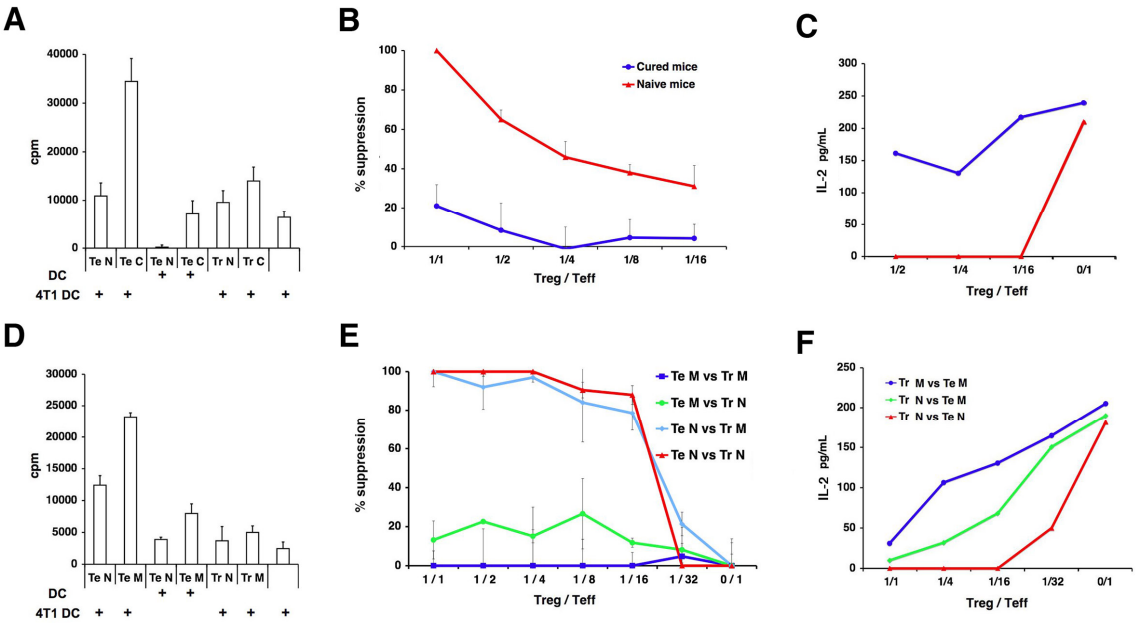
Supplementary Figure 3

$CD4^+CD25^+CD44^{hi}CD62L^{lo}$ T-cells express Foxp3 and are bona fide memory Tregs. **(A)** Flow cytometry gating strategy and Foxp3 expression of $CD4^+CD25^+CD44^{hi}CD62L^{lo}$ T cells from normal mice. **(B)** Inhibition of T-cell proliferation in vitro. 5 x 10^4 $CD4^+CD25^-$ T-cells and the indicated ratio of $CD4^+CD25^+CD44^{hi}CD62L^{lo}$ amT_{regs} (black) or $CD4^+CD25^+CD44^{lo}CD62L^{hi}$ T_{regs} (gray) were incubated with anti-CD3 and anti-CD28 mAbs for 3 days. Proliferation was measured by adding ^3H thymidine at day 3 to 4 of culture. Mean percentages of inhibition ±SEM of the proliferation are shown (triplicate cultures). Both populations of T_{regs} are equally suppressive.



Supplementary Figure 4

Peptide specific suppression assay of cultured TCR-HA Tregs. TCR-HA Tregs were cultured in vitro (43). Flow cytometry profile of the cultured $CD4^+CD25^+Foxp3^+$ TCR-HA Tregs is shown in the inserted panel. 5 x 10^4 $CD4^+CD25^-$ T-cells and the indicated ratio of $CD4^+CD25^+$ TCR-HA Tregs were incubated with 10^4 HA-loaded $CD11c^+$ DCs for 3 days. Proliferation was measured by adding ^3H thymidine at day 3 to 4 of culture. Mean cpm of suppression ±SEM are shown (triplicate cultures). In vitro cultured TCR-HA Tregs have an extremely potent suppressor activity.



Supplementary Figure 5

Antitumor activated/memory T_{effs} resist to T_{reg} suppression *in vitro*. (A-C) 5 x 10^4 $CD4^+CD25^-$ cells and the indicated ratio of $CD4^+CD25^+$ cells from the naive mice (TeN and TrN respectively) or from the mice cured of 4T1 tumors by anti CD25 treatment 6 months earlier (TeC and TrC) were incubated with $CD11c^+$ dendritic cells pulsed with 4T1 lysate. Inhibition of proliferation was measured by adding ^3H thymidine at day 3 to 4 of culture. Mean cpm **(A)** and mean percentages of suppression **(B)** observed are shown. IL-2 present in the culture supernatant was also evaluated at day 1.5 of culture using beads array assay **(C)**. **(D-F)** Resistance to suppression of 4 x 10^4 $CD4^+CD44^{hi}CD45RB^{lo}CD25^-$ cells (TeM) from the cured mice and of 4 x 10^4 $CD4^+CD44^{int}CD45RB^{int}CD25^-$ cells (TeN) from the naive mice was tested against the indicated ratio of $CD4^+CD44^{int}CD45RB^{int}CD25^+$ T_{regs} (TrN) from the naive mice or $CD4^+CD44^{hi}CD45RB^{lo}CD25^+$ T_{regs} (TrM) from the 4T1-cured mice. Culture conditions were identical to **(A)**. Results are expressed as the mean ±SEM of triplicate cultures.