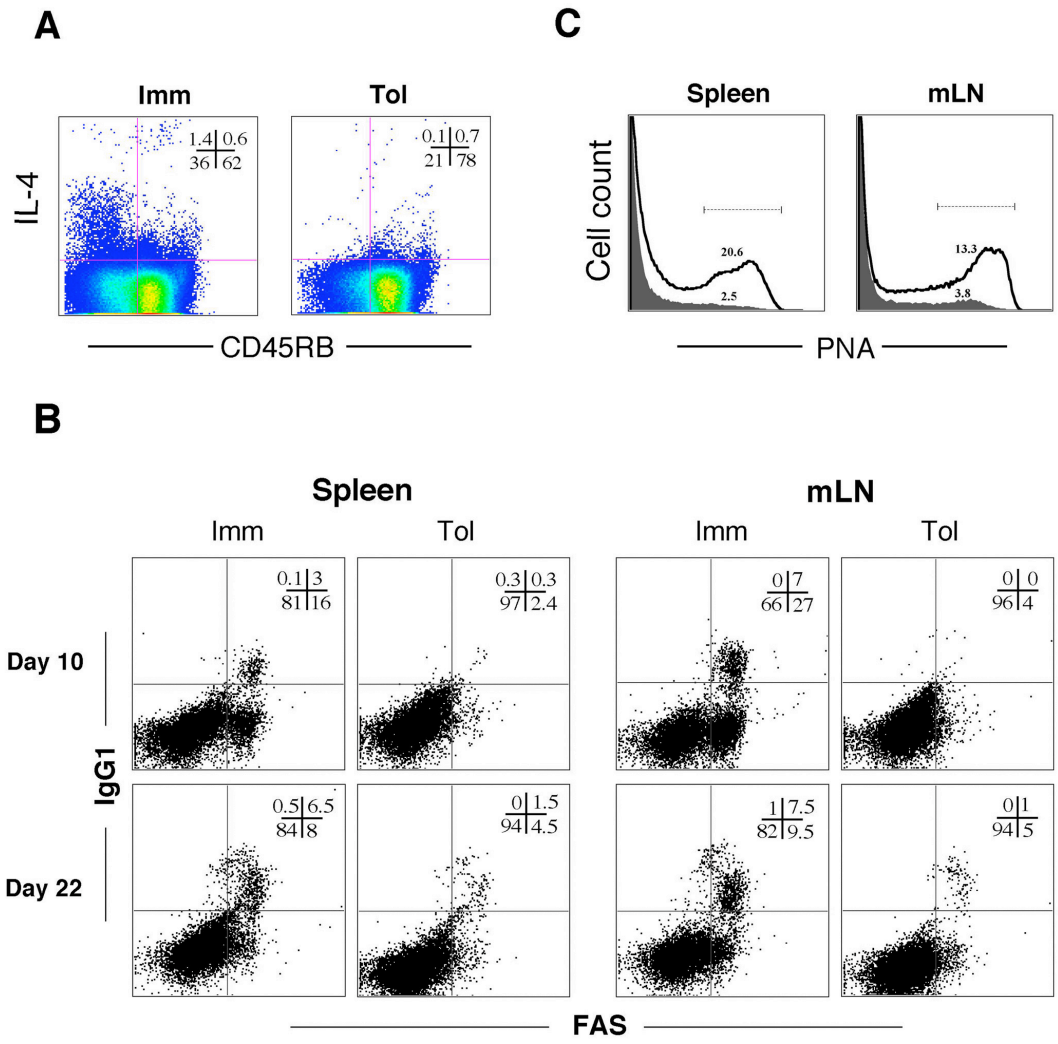
Supplementary figures



***Supplementary Figure 1*. Oral administration of OVA suppresses Th2 differentiation, Germinal Center (GC) formation and immunoglobulin class switching in spleen and lymph nodes.** This figure complements Figures 1 and 2 of the main text.

T/B monoclonal mice were administered OVA orally, and subsequently immunized with OVA-HA in alum by i.p. route (Tol group) as schematized in Figure 1A. A second group was immunized i.p. with OVA-HA in alum (Imm group). On days 10 and 22 after i.p. immunization, the spleen and mesenteric lymph nodes (mLN) cells were harvested, and single-cell suspensions were analyzed by FACS.

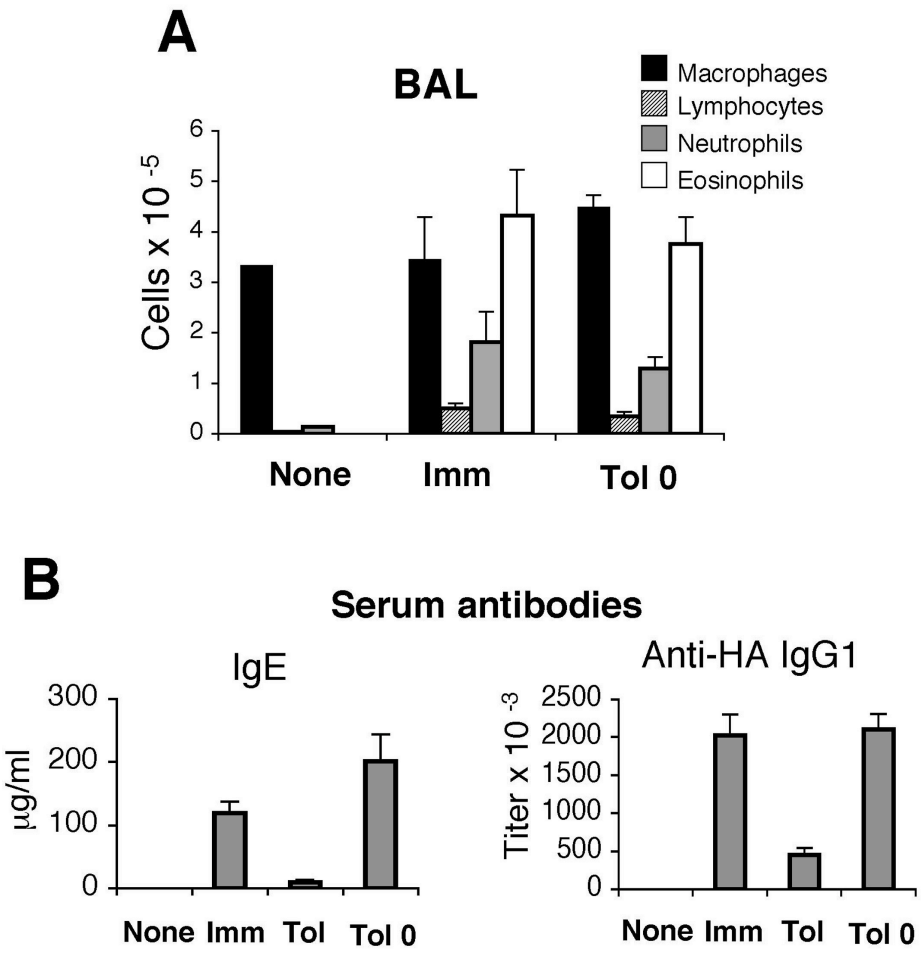
A. IL-4 production by OVA-specific T cells 10 days after immunization. mLN cells from T/B monoclonal mice from Tol and Imm groups were analyzed for the production of IL-4 by intracellular staining. Cells were stimulated in vitro with OVA peptide for 4 hours and processed as described in the Methods section. The cells were then stained with anti-CD4

and anti-CD45RB antibodies, fixed, permeabilized and incubated with anti-IL-4 antibodies for intracellular IL-4 detection. The plots are representative of 3 mice per group.

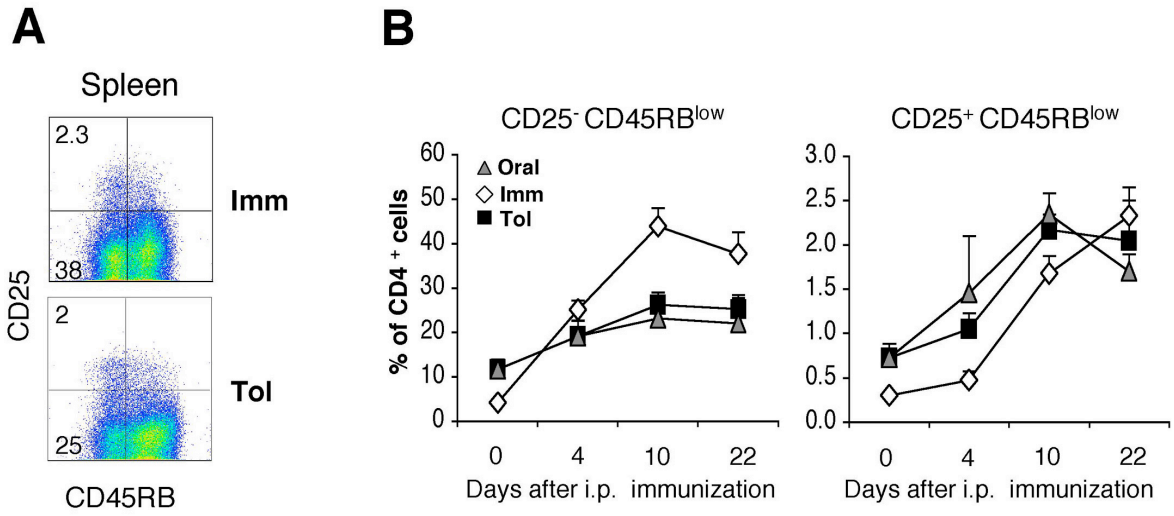
B&C. To examine GC formation and class switching, cells were stained with antibodies to B220, IgG1, and FAS, and with the lectin PNA. PNA binds to B cells located exclusively in germinal centers [1]. FAS expression is highly upregulated in GC cells [2, 3].

B. Representative FACS plots of $B220^+$ gated lymphocytes. FAS^+IgG1^- and FAS^+IgG1^+ B lymphocytes are evident on days 10 and 22 days after i.p. immunization in the Imm group. In contrast, the development of FAS^+ GC cells and switching to IgG1 in the GC was greatly impaired in the tolerant mice (Tol).

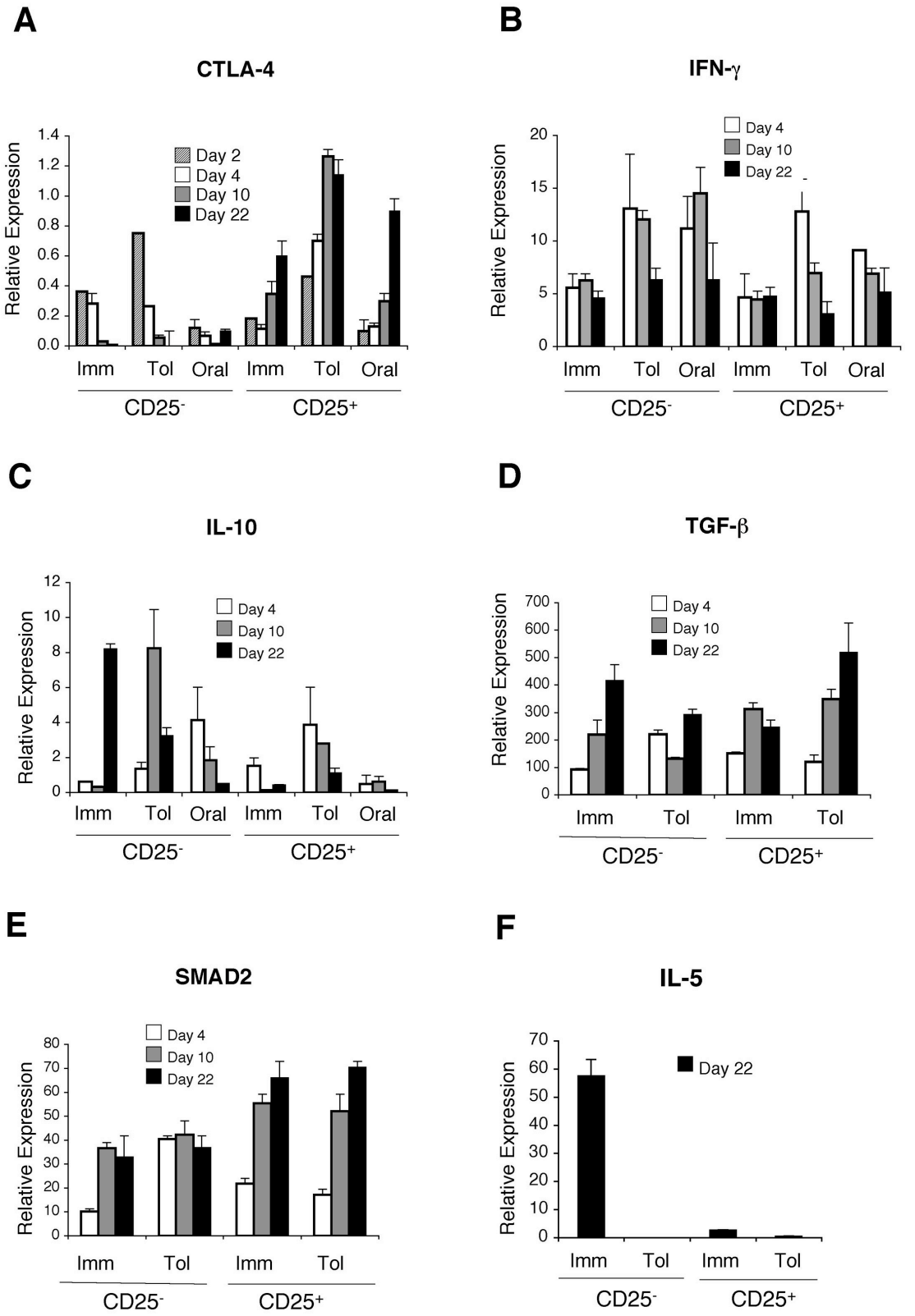
C. The suppression of GC formation was also evident by the reduced numbers of PNA+ cells in the tolerant mice. The figure shows FACS histograms of $B220^+$ gated cells. Shadow: Tol group; Open: Imm group.



***Supplementary Figure 2*. The effect of concomitant OVA feeding and immunization on antigen-induced airway inflammation and antibody production in T/B monoclonal mice.**
T/B monoclonal mice received a solution of 1% OVA dissolved in the drinking water for 5 consecutive days. Half of the mice were immunized with OVA-HA in alum by i.p. route **on the same day** of the initiation of the OVA treatment (Tol0 group). The other half was immunized 2 days after the end of the oral treatment, as schematized in Figure 1A of the manuscript (Tol group). A third group of mice received OVA-HA i.p. immunization without oral exposure (Imm.). The three groups of mice were challenged intranasally (i.n.) with 10 µg of OVA-HA. The mice were analyzed for lung inflammation and antibody production one day after the second i.n. challenge. Untreated T/B monoclonal were included as control (None group).
A. BAL cells were collected and differential counts were performed on stained cytospin preparations. Results are mean and SEM of five mice per group.
B. Sera from None, Imm, Tol and Tol0 groups were collected on day 22 of immunization and Ab production was quantified by ELISA. Results are expressed as mean ± SEM.
The results show that to establish oral tolerance in the T/B monoclonal mice the oral administration of OVA needs to precede the i.p. immunization.

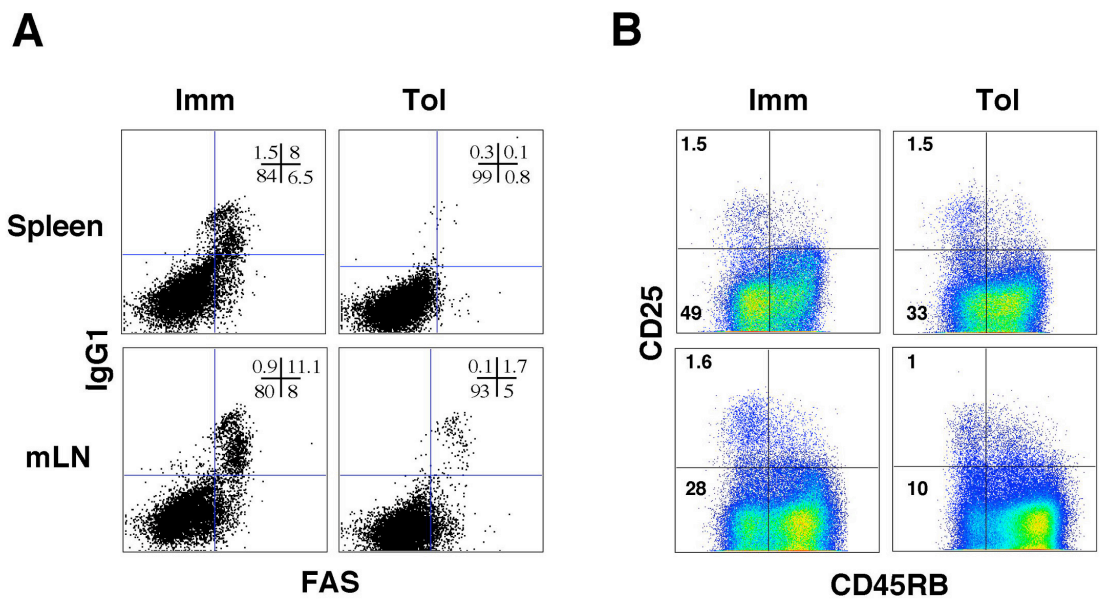


***Supplementary Figure 3*. Effect of previous OVA-feeding on Treg and Teffector/memory development.** This figure complements the data in Figure 4 of the manuscript. The expression of CD25 and CD45RB by OVA-specific T cells from the Tol (OVA fed + OVA-HA immunized), Imm (OVA-HA immunized) and Oral (OVA-fed) groups (as schematized in Figure 1A of the manuscript, except that these mice did not receive nasal challenge) were analyzed after i.p. immunization. Spleen cells were stained with antibodies recognizing CD25, CD45RB, and CD4, and analyzed by FACS.
A. Representative dot plots of gated $CD4^+$ spleen cells from the Tol and Imm groups at day 22. Similar staining and quadrant gating was used to determine the percentage of $CD25^-CD45RB^{low}$ (Teff/mem phenotype) and $CD25^+CD45RB^{low}$ (Treg phenotype) cells in the experimental groups at various times after immunization (see B).
B. Kinetics of appearance of $CD25^-CD45RB^{low}$ (Teff/mem) and $CD25^+CD45RB^{low}$ (Treg) cells. On days 0 (right before immunization), and days 4, 10 and 22 after immunization, spleen cells from Tol (closed squares), Imm (open diamonds) and Oral (closed triangles) groups were analyzed for the presence of CD4 cells with Teff/mem or Treg phenotype as described above. The results show mean and STD of 5-15 mice per group per time point.

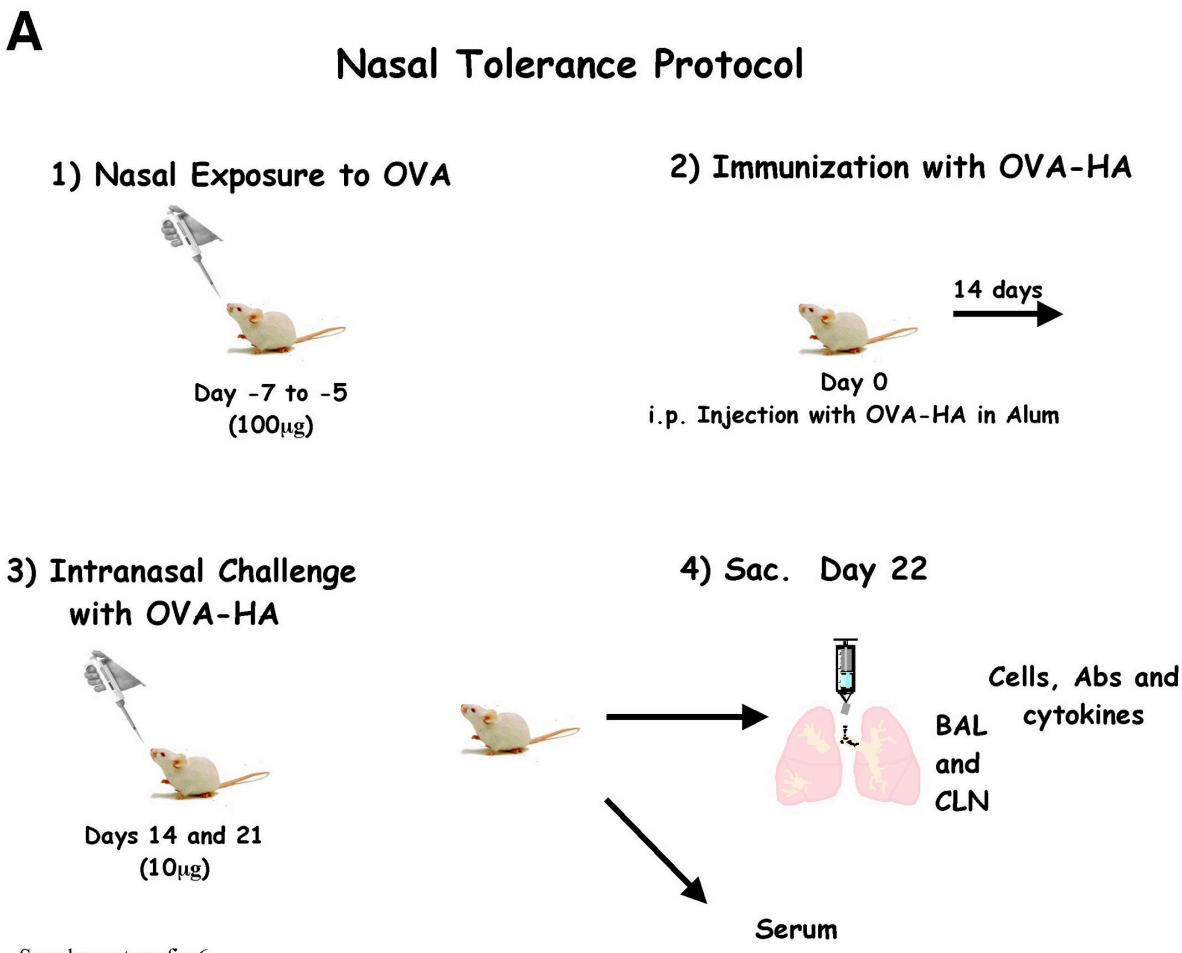


Supplementary Figure 4. **CD25$^+$ cells from immunized and tolerant groups express CTLA-4.** This figure is a complement for Figure 5 of the main text.

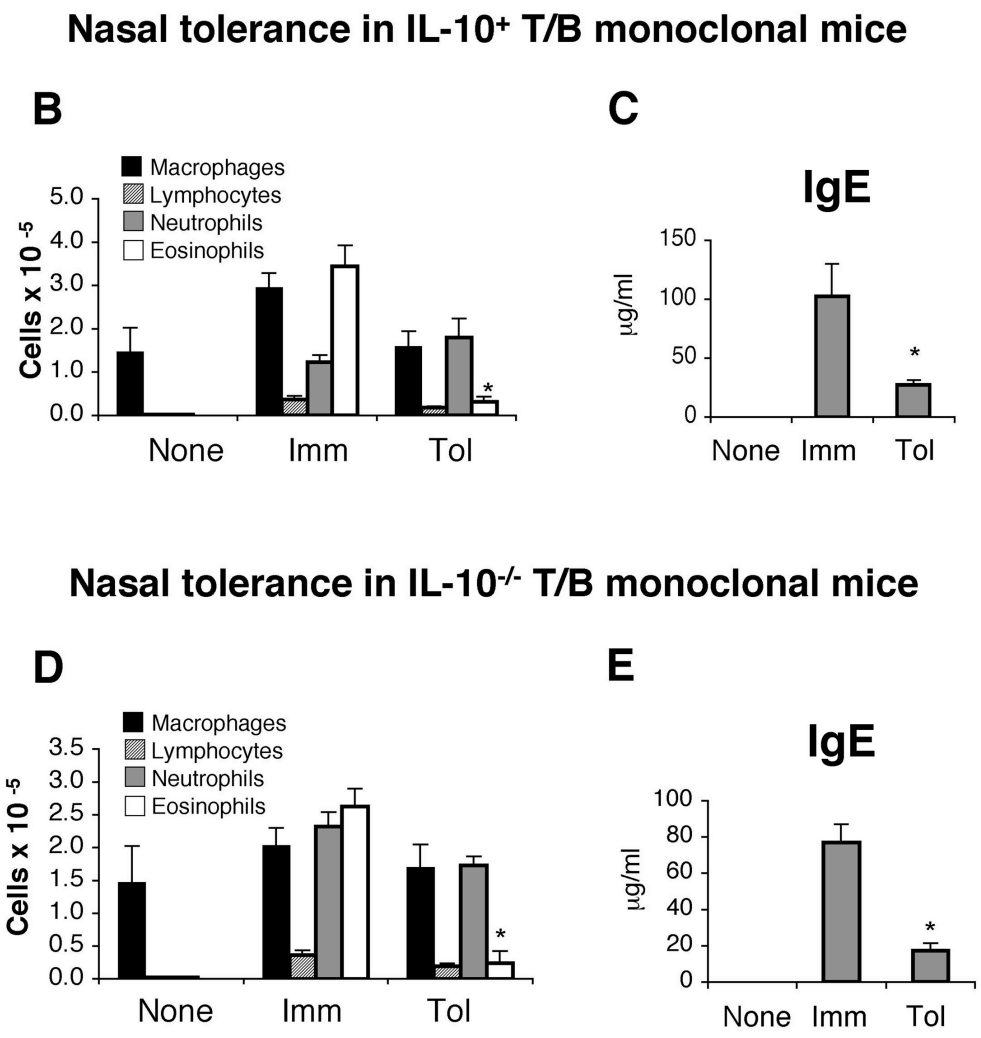
A. The expression of CTLA-4 in purified OVA-specific $CD25^+$ and $CD25^-$ T cells from Tol (OVA fed + OVA-HA immunized), Imm (OVA-HA immunized) and Oral (OVA-fed) groups was analyzed on days 2, 4, 10 and 22 after immunization. $CD25^+$ and $CD25^-$ cells were purified from pooled spleen and mLN of 3 mice per sample. Expression of Foxp3 and IL-4 was determined by real time PCR as described in the Methods section. Expression values were normalized to β-actin. The results are mean and STD of three wells per sample. The kinetics experiment is representative of three.
The expression of IFN-γ (B), IL-10 (C), TGF-β (D), SMAD2 (E) and IL-5 (F) were analyzed by real time PCR in purified OVA-specific $CD25^+$ and $CD25^-$ T cells from Tol and Imm samples at the indicated times after i.p. immunization. The results are mean and STD of three wells per sample.
For comparison, a sample of differentiated Th1 cells [4] run in the same assay yielded a value 200 fold higher for IFN-γ (mean relative expression= 2,680). A sample of Th2 cells differentiated in vitro by repeated stimulation [4] expressed about 40 fold higher IL-10 (mean relative expression= 292) than the highest value detected in the Imm or Tol samples.



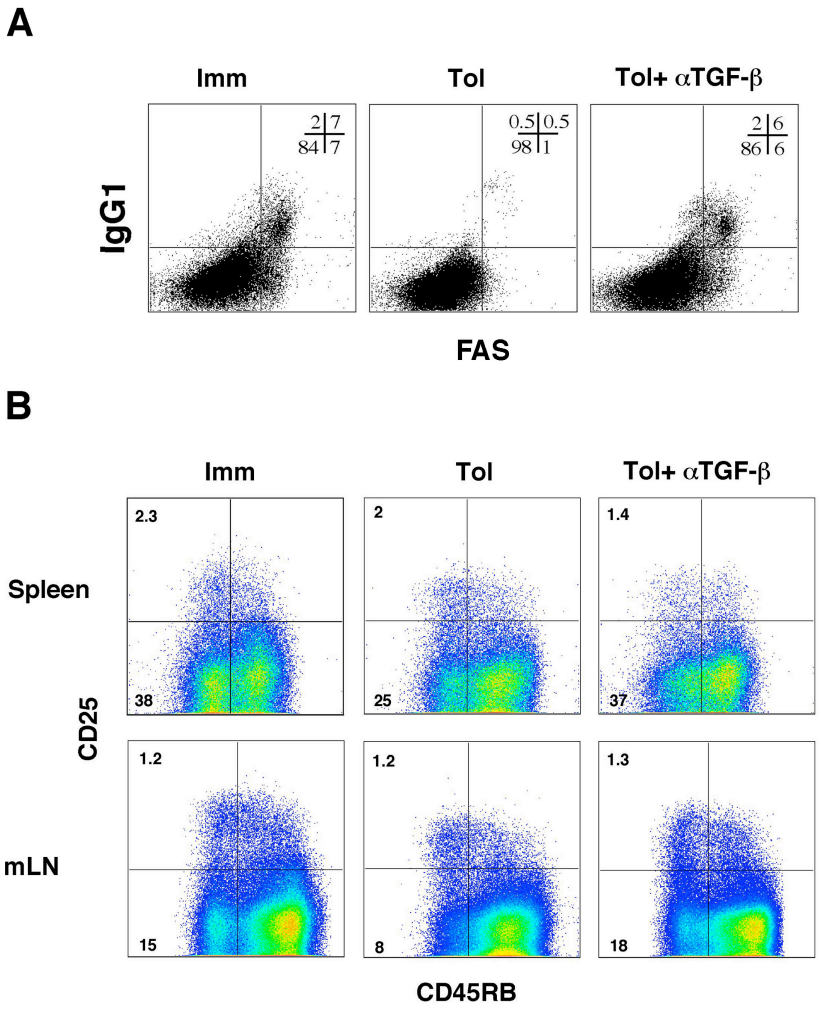
***Supplementary Figure 5*. Oral tolerance in T/B monoclonal IL-10$^{-/-}$ mice.**
This figure complements Figure 8 of the main text.
T/B monoclonal IL-10$^{-/-}$ mice were administered OVA in the drinking water, immunized i.p. and challenged i.n. as schematized in Figure 1A. Tol and Imm mice, and a control group of untreated IL10$^{-/-}$ mice (None), were analyzed on day 22 after i.p. immunization. Results of one experiment representative of three are shown.
A. FACS analysis of spleen and mLN cells 22 days after immunization shows suppression of the GC reaction (FAS^{+} cells, upper and lower right quadrants) and class switching to IgG1 (upper right quadrant) in Tol IL-10$^{-/-}$ mice. Plots show FAS and IgG1 staining of B220^{+} gated cells.
B. Decreased frequency of CD4^{+}CD45RB^{low}CD25^{-} cells in Tol IL-10$^{-/-}$ mice. Spleen and mLN cells from Tol and Imm T/B monoclonal IL-10$^{-/-}$ mice were stained with antibodies to CD4, CD25 and CD45RB. Representative dot-plots of gated CD4^{+} cells are shown. Note the decrease in the percentage of CD25^{-}CD45RB^{low} cells in the Tol samples (lower left quadrant).



Supplementary fig.6



***Supplementary Figure 6*. IL-10^{+} and IL-10$^{-/-}$ T/B monoclonal mice are susceptible to nasal tolerance.** To induce nasal tolerance, anesthetized mice were administered intranasally (i.n.) 100 µg of OVA in 50 µl of PBS (25 µl per nostril) for three consecutive days. Five days later, the mice were immunized with 100 µg of OVA-HA in alum by i.p. route. To induce airway inflammation, the mice were administered intranasally (i.n.) 10µg of OVA-HA in 50µl of PBS on days 14 and 21 after the i.p. immunization. Serum and BAL were harvested on day 22 and analyzed for antibody production and cellular inflammation (protocol schematized in panel A, previous page).
The results show first, that T/B monoclonal mice are susceptible to nasal tolerance, and second, that IL-10 is not required for nasal tolerance to asthma in this system. Nasal administration of OVA was effective in suppressing IgE production and eosinophilic lung inflammation, but did not affect IgG1 production.
B and D. Cellular composition of the BAL of IL-10^{+} (B) or IL-10$^{-/-}$ T/B monoclonal mice mice (D) 22 days after immunization. Tol: mice that received OVA i.n., OVA-HA i.p. immunization and OVA-HA i.n. challenge. Imm: mice that received OVA-HA i.p. and OVA-HA i.n. challenge. None: untreated mice. *Significant differences ($P< 0.05$) between eosinophil values of Imm and Tol groups.
C and E. IgE serum antibody levels on day 22 after i.p. immunization. C: IL-10^{+}, E: IL-10$^{-/-}$ mice.



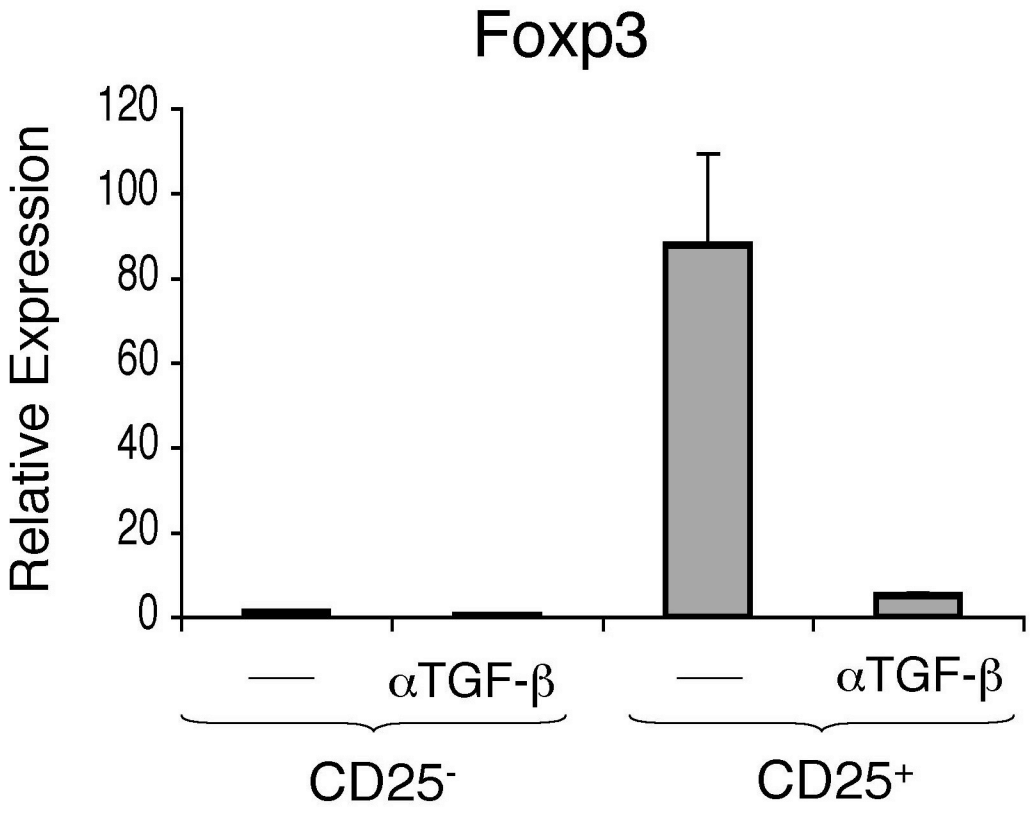
***Supplementary Figure 7*. Neutralization of TGF-β in mice of the Tol group restores germinal center development and the levels of $CD25^{-}CD45RB^{low}$ cells in lymphoid organs.**

This figure is a complement to Figure 9 of the manuscript.

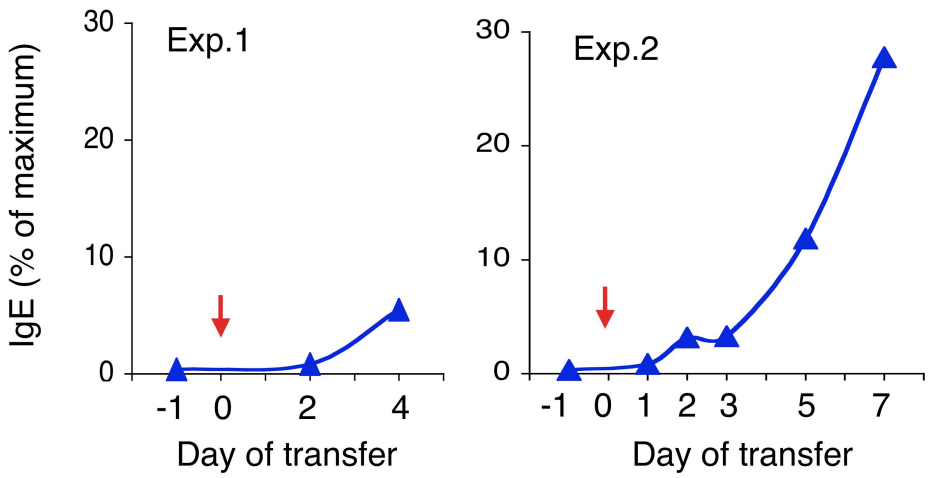
Anti-TGF-β antibodies (1D11) were administered before, during and right after oral OVA treatment (days –8, -5, -2 and 1 according to the scheme in Figure 1A). The mice were immunized with OVA-HA i.p. and challenged i.n. as indicated in Figure 1A. Spleen and mLN cells were analyzed by FACS on day 22 after i.p. immunization. The results shown are representative of three independent experiments.

A. Spleen cells were harvested on day 22 after immunization and stained with antibodies to B220, IgG1 and FAS. Representative plots of $B220^{+}$ gated cells are shown. Imm and Tol groups were treated as indicated in Figure 1A of the manuscript. Mice belonging to the Tol + αTGF-β group received anti-TGF-β antibodies, as described above.

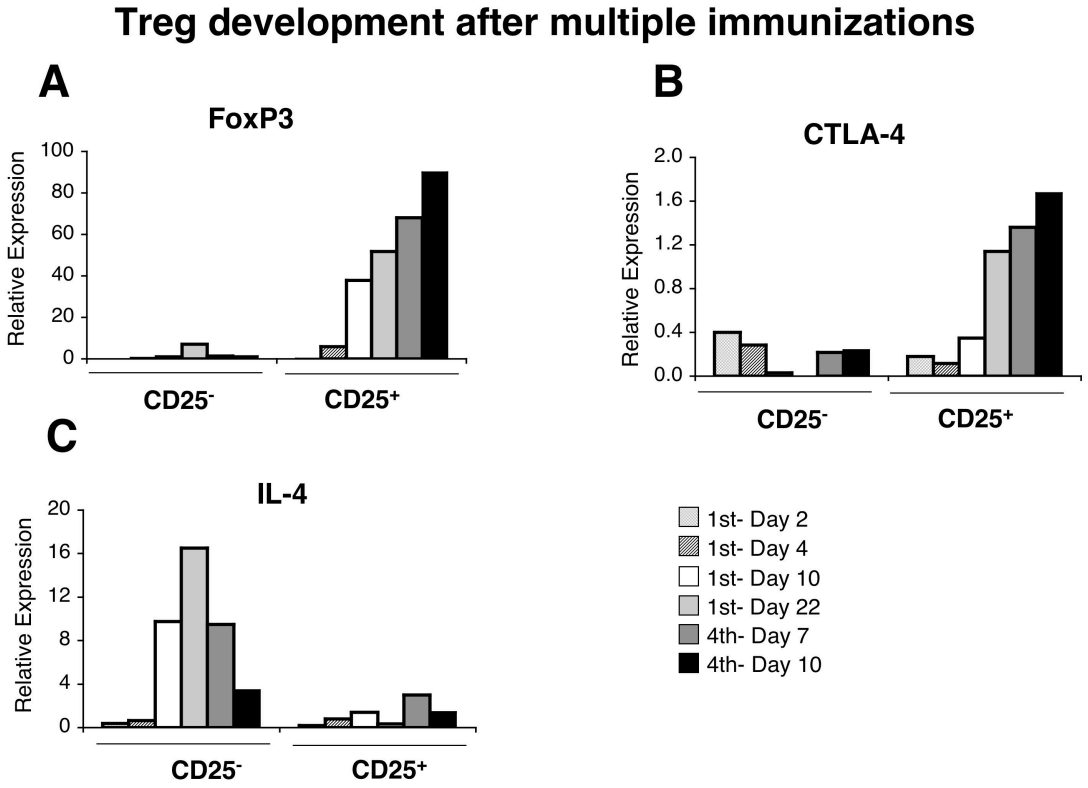
B. Increased percentage of $CD25^{-}CD45RB^{low}$ cells in spleen and mLN of Tol mice treated with anti-TGF-β antibodies. Cells harvested on day 22 were stained with antibodies to CD4, CD25 and CD45RB. The plots show representative samples of gated $CD4^{+}$ cells. Note the increase in the percentage of $CD25^{-}CD45RB^{low}$ cells in the Tol+α-TGF-β samples (lower left quadrant) compared with the Tol samples.



***Supplementary Figure 8*. Administration of anti-TGF-β antibodies suppresses Foxp3 expression in CD4$^+$CD25$^+$ spleen cells from mice administered OVA orally.**
This figure complements Figure 10B of the manuscript. T/B monoclonal mice were administered 2 mg of anti-TGF-β antibodies (αTGF-β) one day before and three days after the beginning of administration of 1% OVA in the drinking water. After 5 days of oral-OVA, CD4$^+$ splenic cells were analyzed for expression of Foxp3 by real time PCR.



***Supplementary Figure 9*. Critical time for sensitivity to suppression.**
Total spleen cells from BALB/c mice were transferred to T/B monoclonal ($2x10^7$ cells/mouse) at different times before or after i.p. immunization with OVA-HA in alum. Serum IgE levels were determined by ELISA on days 26 (Exp.1) or 19 (Exp.2) after immunization. The arrows indicate the immunization day. Results were expressed as % of IgE levels from immunized, non-transferred mice. n= 5-7 mice per group.



***Supplementary Figure 10*. Multiple immunizations of T/B monoclonal mice with OVA-HA favor Foxp3 expression over IL-4 expression.** T/B monoclonal mice were immunized with OVA-HA in alum by i.p. route four times, with approximately three weeks intervals between immunizations. At the indicated days after the primary and 4th immunization, $CD4^+CD25^-$ and $CD4^+CD25^+$ cells were purified from pooled spleen and mesenteric LNs. The expression of Foxp3, CTLA-4 and IL-4 in the samples was determined by real time PCR using β-actin for normalization.

References for the Supplementary Material

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