iScience, Volume *26*

Supplemental information

Oral tolerance to systemic vaccination remains intact without RORγt expression in regulatory T cells

Nicole B. Potchen, Andrew M.F. Johnson, Kevin Hager, Jessica Graham, Phuong Van, Katelyn H. Lyn-Kew, Lakshmi Warrier, Irene Cruz Talavera, Jennifer M. Lund, and James G. Kublin

Figure S1

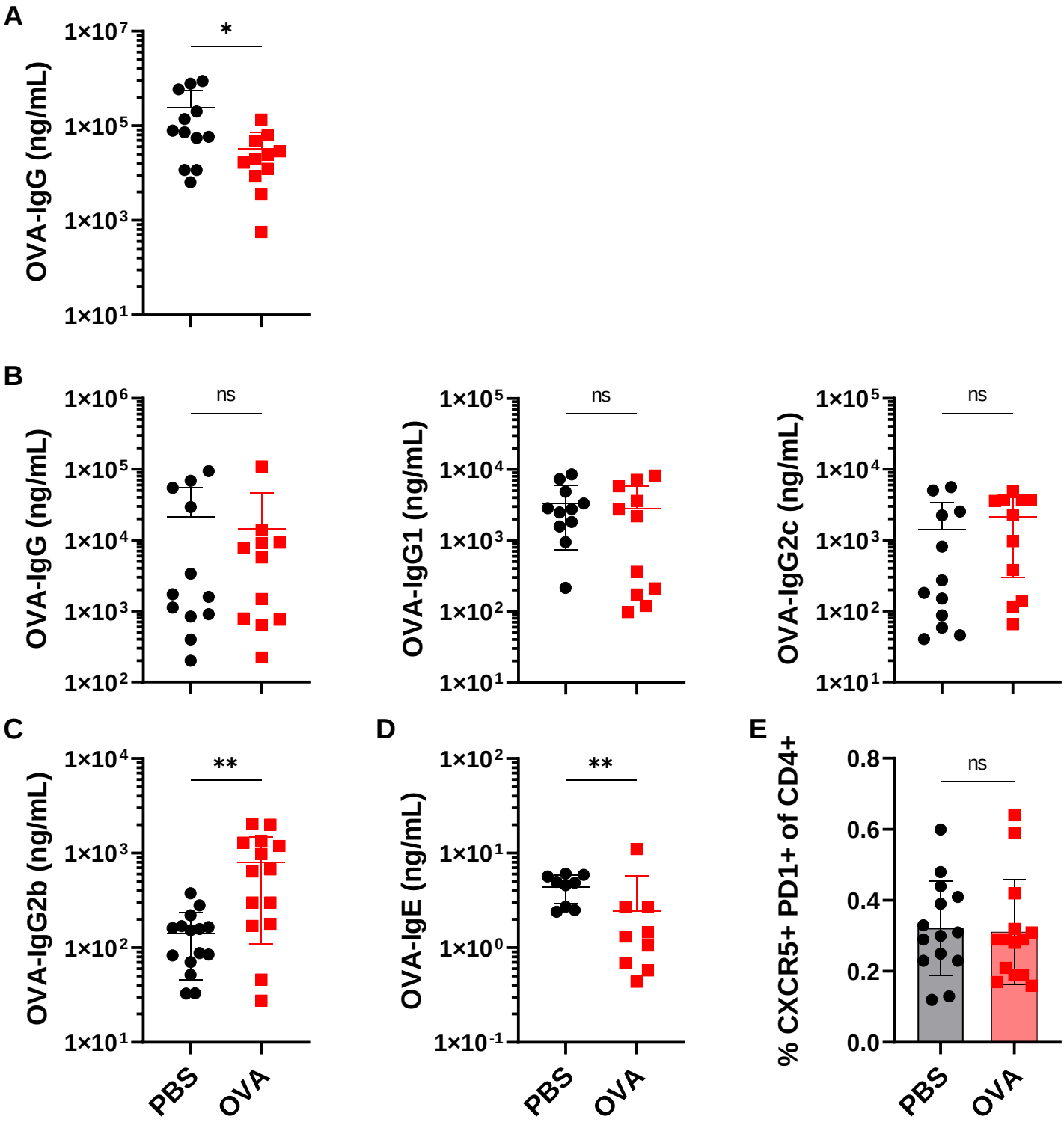

Figure S1 – Oral administration of antigen prior to vaccination with alum: IgG, Day 14, other isotypes, and T-follicular helper cells. Related to Figure 1.

Analysis of antibody titers and cellular responses for experiments described in Figure 1. **A)** Endpoint OVA-IgG titers. **B)** OVA-IgG, IgG1 and IgG2c at Day 14 following immunization after oral antigen pre-exposure. **C)** Endpoint OVA-IgG2b titers. **D)** Endpoint OVA-IgE titers. **E)** T_{FH} cells from spleens of mice, pre-gated on lymphocytes, singlets, live, CD3+, CD45+, CD4+, CD8- prior to CXCR5+ PD-1 gating (representative plot in Fig. S7). Data pooled from 3 independent experiments. Unpaired t tests were used for statistical analysis.

Figure S2

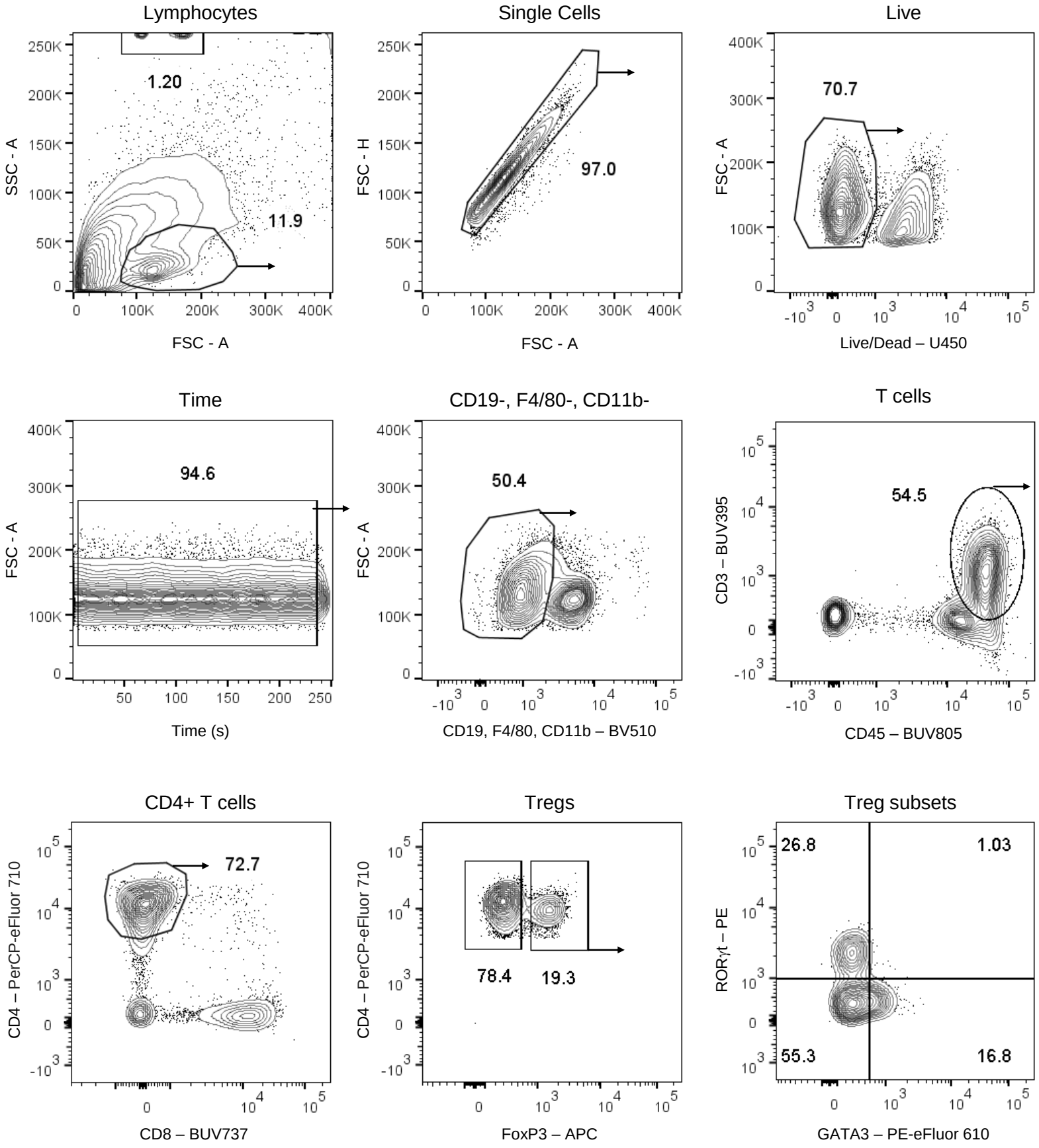

Figure S2 – Flow cytometry gating. Related to Figure 1.

Gating strategy used to identify CD4+ T cells and FoxP3+ Treg subsets. Beads to assess total cell counts are also gated in the first panel (FSC-A v SSC-A).

Figure S3

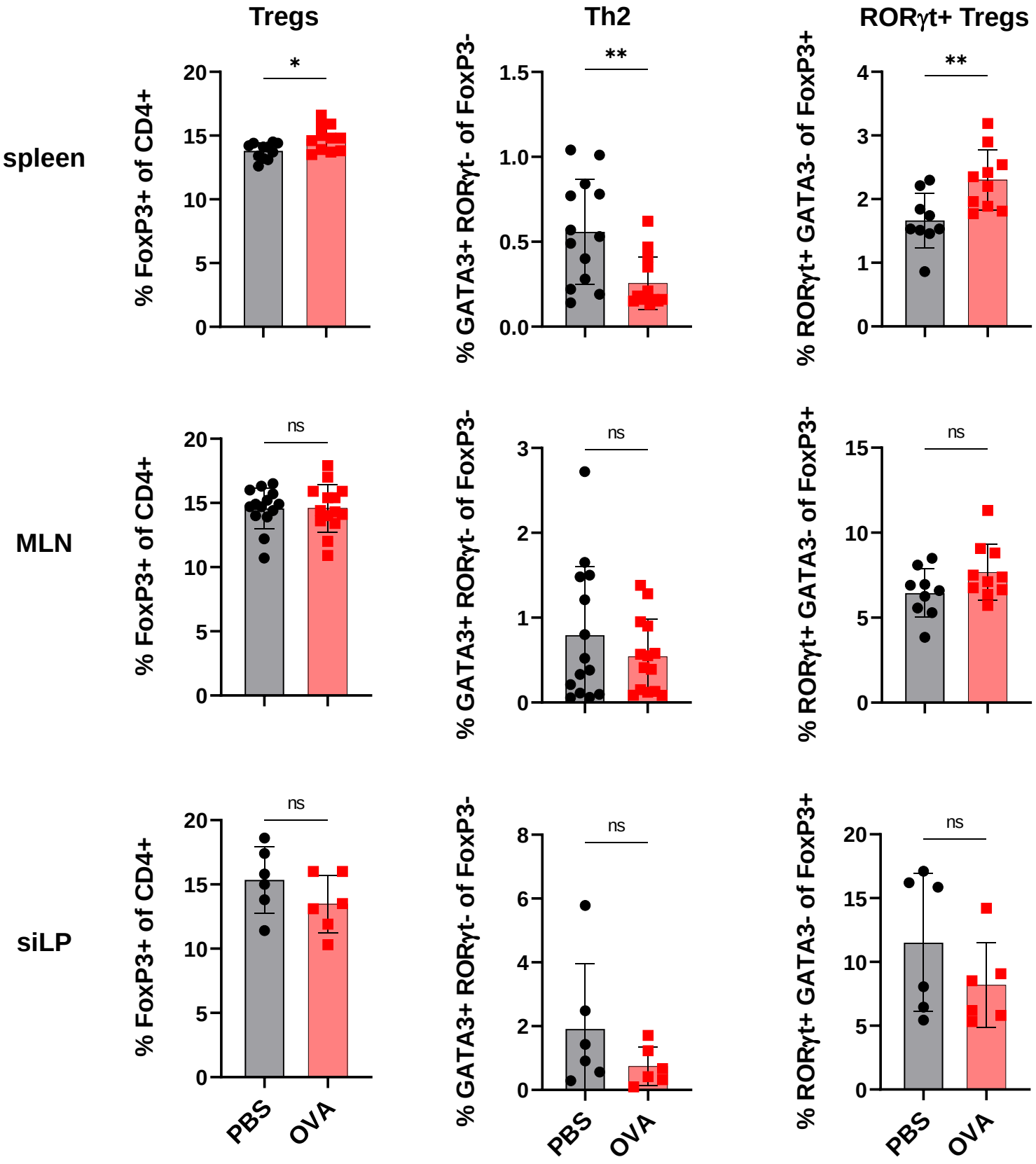

Figure S3 – Oral exposure to antigen prior to vaccination with alum: CD4+ responses. Related to Figure 1.

CD4+ frequencies of indicated subset in spleen, MLN, and siLP for experiment defined in Figure 1A. Gated using strategy outlined in Fig. S2. Data pooled from 3 independent experiments. Unpaired t tests were used for statistical analysis.

Figure S4

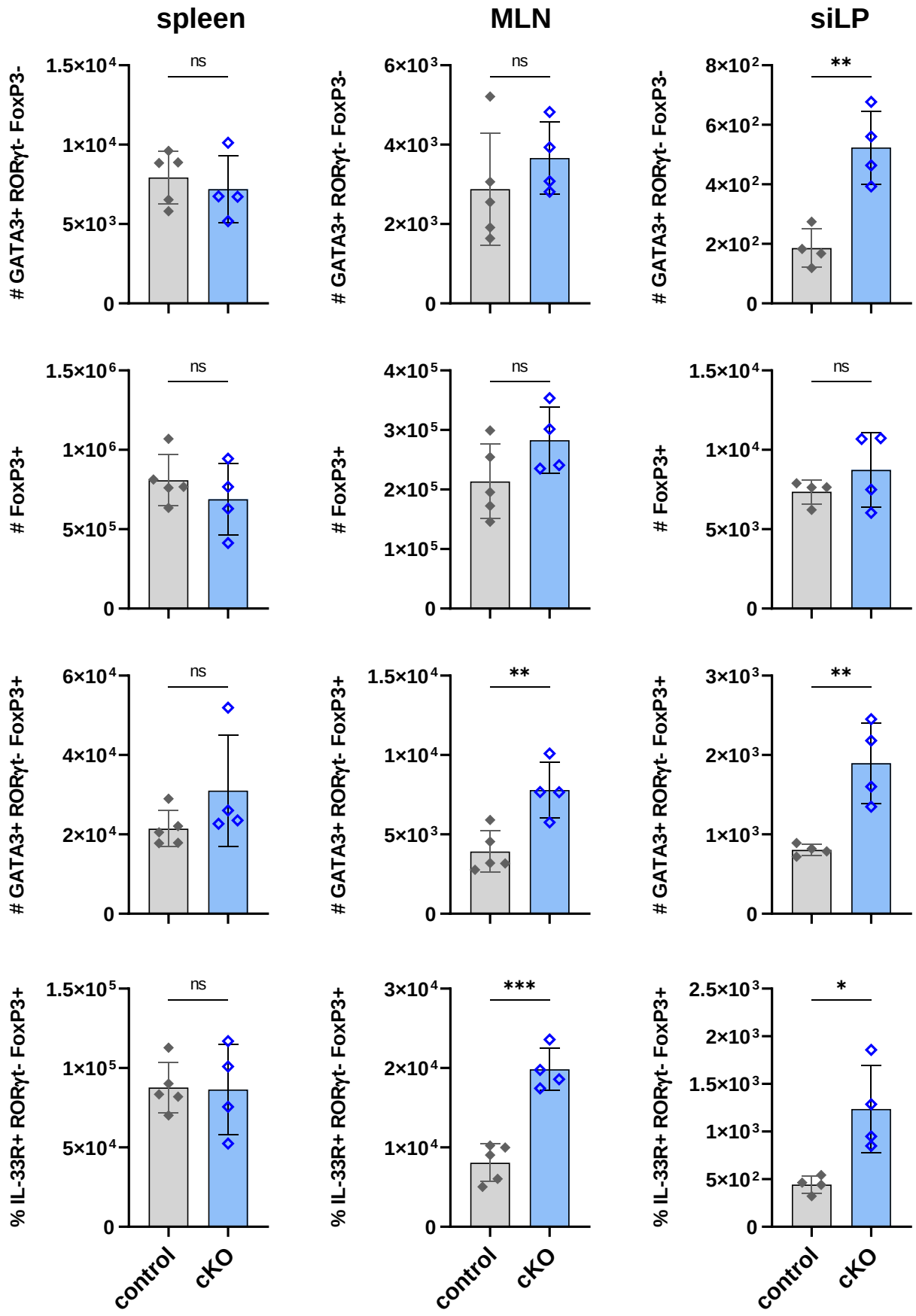

Figure S4 – Counts of Tregs, GATA3+ Tconv and Tregs, and IL-33R+ Tregs from Rorc$^{fl/fl}$ FoxP3^{cre} cKO and control mice. Related to Figure 2.

Total cell counts of indicated Treg or Treg subset from Rorc$^{+/+}$ FoxP3^{cre} (control, gray) or Rorc$^{fl/fl}$ FoxP3^{cre} (cKO, blue) mice in spleen, MLN or siLP at steady-state. Data representative of 3 independent experiments. Unpaired t tests were used for statistical analysis.

Figure S5

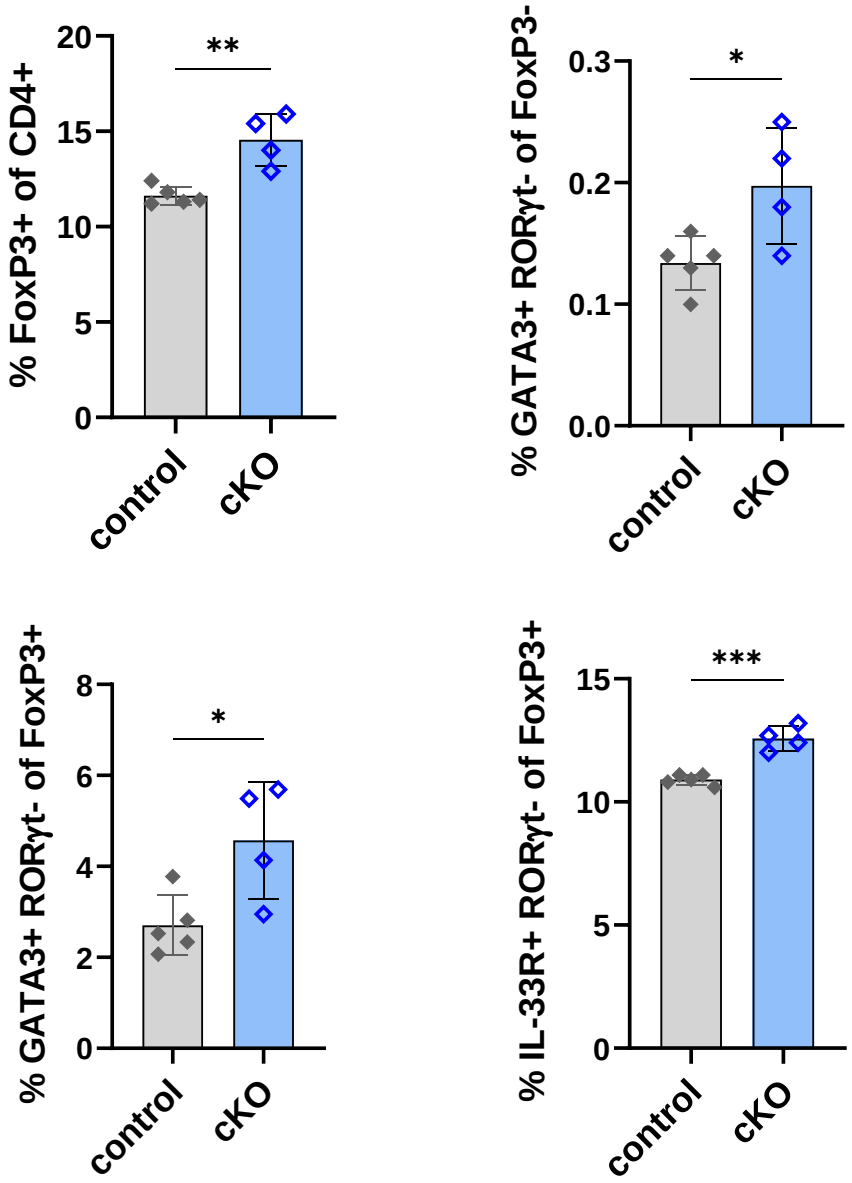

Figure S5 – Compensatory increase of GATA3+ and IL-33R+ Tregs systemically in mice lacking RORγt expression in Tregs. Related to Figure 2.

Frequencies of indicated Treg or Tconv cell from spleens of control or cKO mice. Data representative of 3 independent experiments. Unpaired t tests were used for statistical analysis.

Figure S6

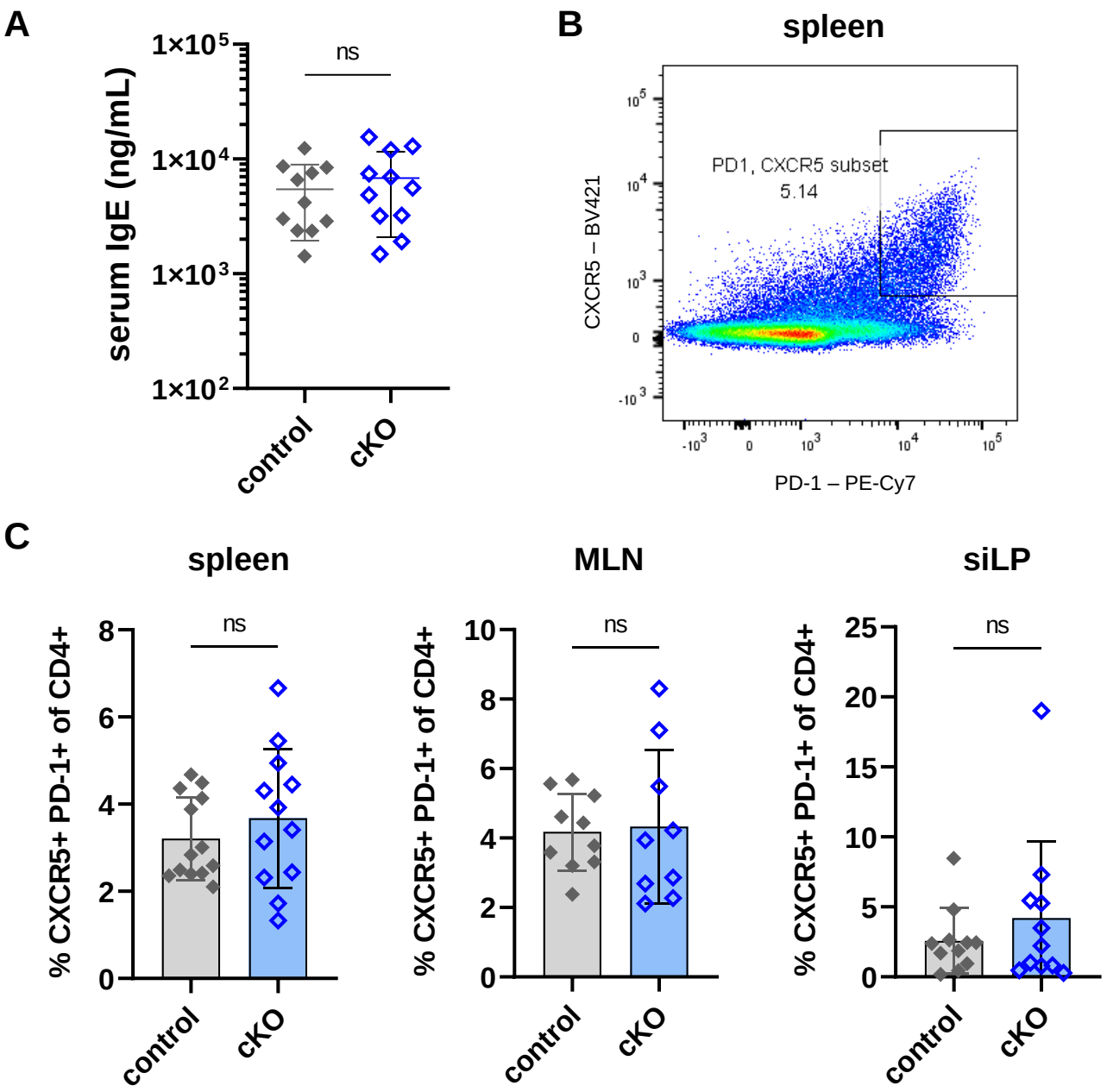

Figure S6 – Phenotyping of mice lacking RORγt expression in Tregs: IgE and T_{FH} cells. Related to Figure 2.

Analysis of $Rorc^{+/+}$ $FoxP3^{cre}$ (control, gray) or $Rorc^{fl/fl}$ $FoxP3^{cre}$ (cKO, blue) mice at steady-state. **A)** Total serum IgE titers at baseline. **B)** Representative gating of CXCR5+ PD-1+ T_{FH} cells pre-gated on lymphocytes, singlets, live, CD3+, CD45+, CD4+, CD8-. **C)** T_{FH} frequencies from indicated tissue in cKO or control mice. Data pooled from 3 independent experiments. Unpaired t tests were used for statistical analysis.

Figure S7

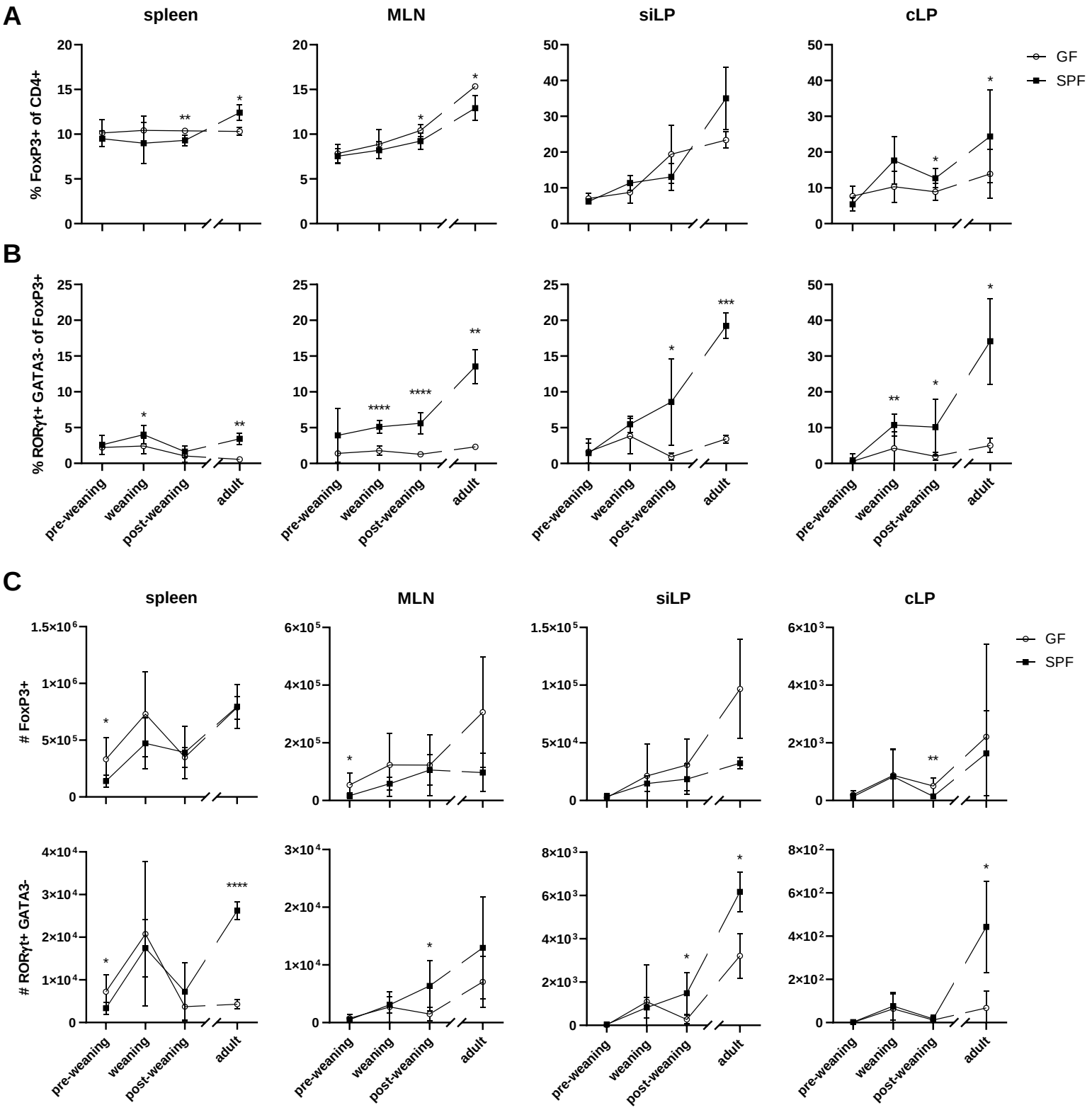

Figure S7 – GF vs SPF profiling early in life. Related to Figure 4.

A) Frequencies of FoxP3+ Tregs and **B)** RORγt+ Tregs in GF and SPF mice over time in various tissues. **C)** Counts of FoxP3+ Tregs and RORγt+ Tregs corresponding to frequencies reported in Fig. S7A. For time course experiments, data pooled from 10 independent experiments of mice at various ages. n = 5-10 for each group at each timepoint early in life and n = 3 for adult mice.

Figure S8

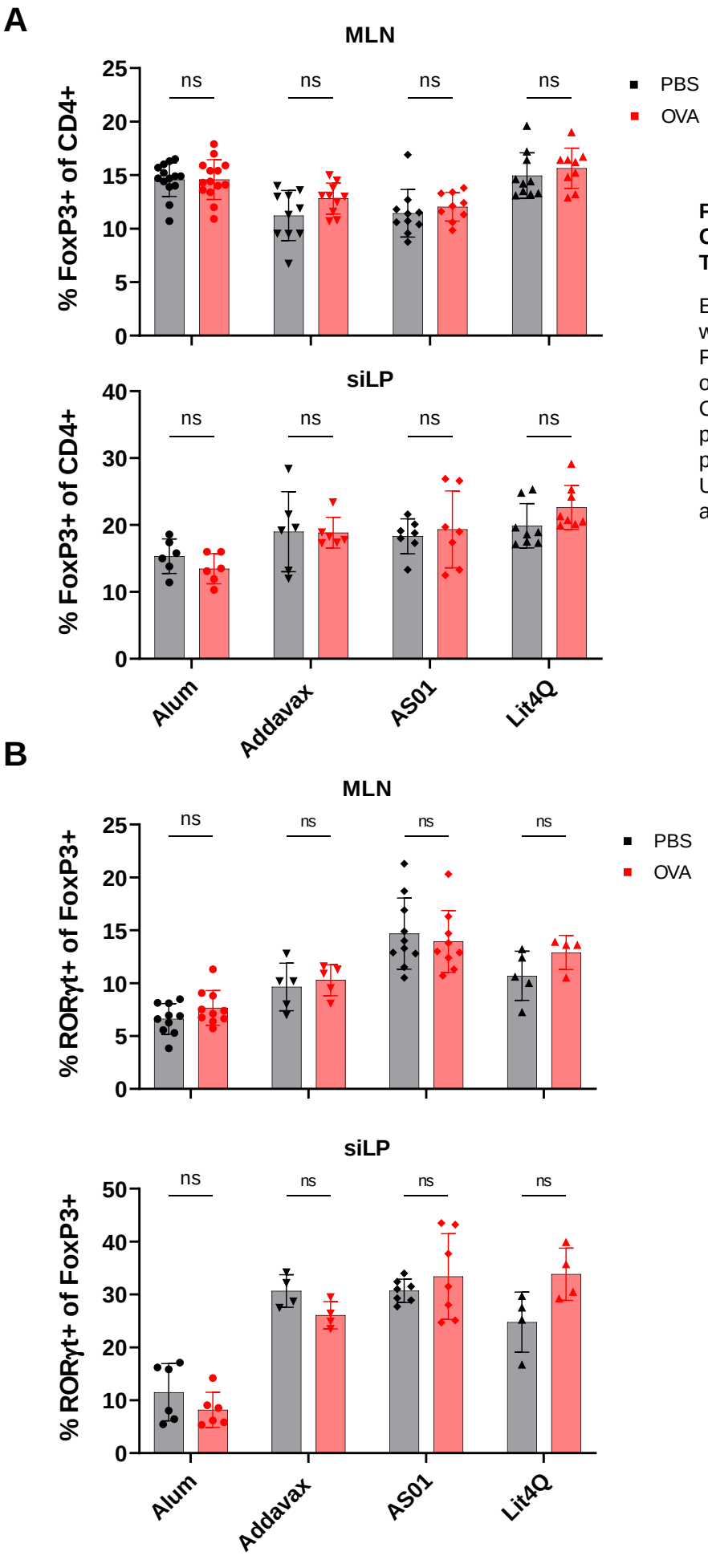

Figure S8 – No effect of oral exposure to OVA on frequencies of Tregs or RORγt+ Tregs in MLN and siLP. Related to Figure 5.

Experimental timeline from Figure 1A was used with various adjuvants. **A)** Frequencies of FoxP3+ Tregs or **B)** RORγt+ Tregs pre-gated on lymphocytes, singlets, live, CD3+, CD45+, CD4+, CD8- of mice gavaged with OVA or PBS prior to immunization. For each adjuvant, data pooled from 2-3 independent experiments. Unpaired t tests were used for statistical analysis.

Figure S9

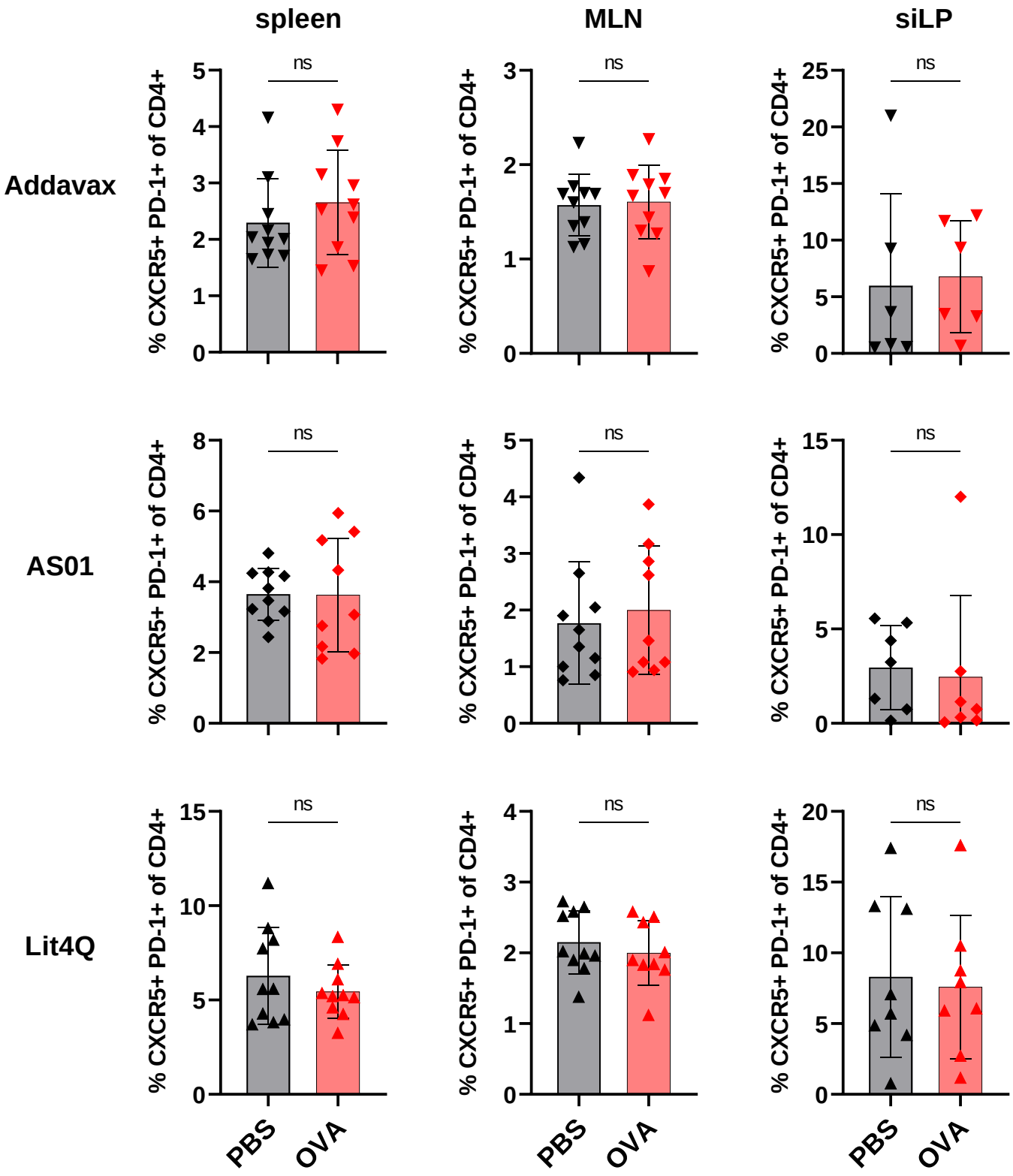

Figure S9 – T_{FH} cells are unchanged between OVA and PBS exposed groups prior to immunization with various adjuvants. Related to Figure 5.

T_{FH} frequencies from indicated tissue from indicated tissue of mice exposed to OVA or PBS prior to immunization with Addavax, AS01 or Lit4Q. of CXCR5+ PD-1+ T_{FH} cells pre-gated on lymphocytes, singlets, live, CD3+, CD45+, CD4+, CD8-. For each adjuvant, data pooled from 2 independent experiments. Unpaired t tests were used for statistical analysis.