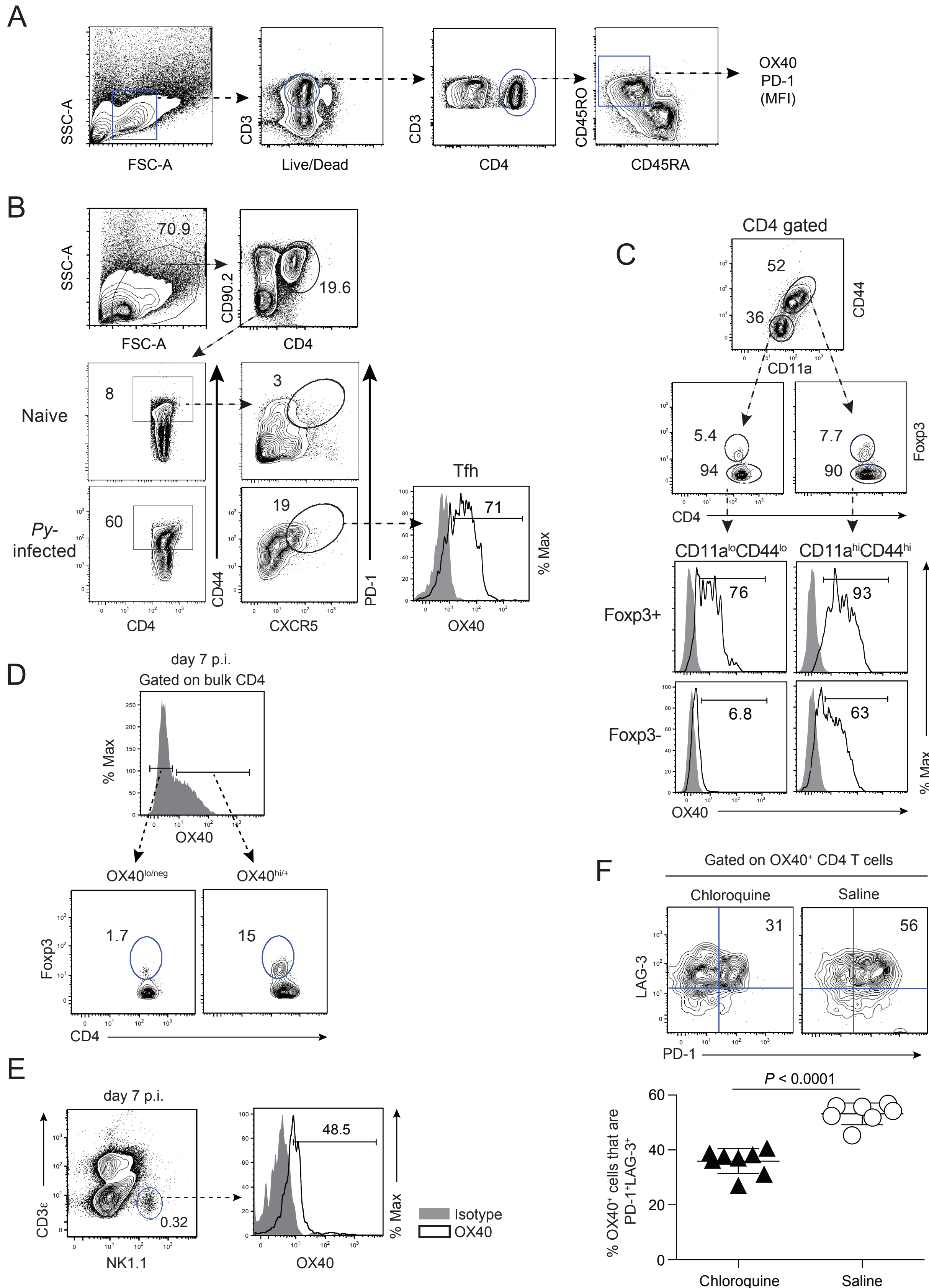
Supplemental Information

PD-1 co-inhibitory and OX40 co-stimulatory crosstalk regulates helper T cell differentiation and anti-*Plasmodium* humoral immunity

Ryan A. Zander, Nyamekye Obeng-Adjei, Jenna J. Guthmiller, Divine I. Kulu, Jun Li, Aissata Ongoiba, Boubacar Traore, Peter D. Crompton and Noah S. Butler

Supplemental Figure S1. Zander et al. 2015



Supplemental Figure Legends

Figure S1, Related to Figure 1. OX40 is expressed on human and murine effector CD4 T cells and murine Foxp3+ Treg, and NK cell subsets following blood stage Plasmodium infection. (**A**) Flow cytometric gating strategy used to identify and immunophenotype circulating antigen-experienced CD4 T cells in *P. falciparum*-infected children. (B-F) C57BL/6 mice were infected with 10^6 *P. yoelii*-infected RBCs and splenocytes were isolated at the indicated time points.

(**B**) Representative plots showing the fraction of Tfh ($CD4^{+}CD44^{hi}CXCR5^{hi}PD\text{-}1^{hi}$) cells expressing OX40 on day 20 p.i.

(**C**) Representative dot plots depicting Foxp3 expression in activated ($CD11a^{hi}CD44^{hi}$) and non-activated ($CD11a^{lo}CD44^{lo}$) $CD4^{+}$ T cells (top) and subsequent OX40 expression in $Foxp3^{+}$ and $Foxp3^{-}$ activated and non-activated T cells (bottom) on day 14 p.i.

(**D**) Representative histogram displaying OX40-expression on bulk $CD4^{+}$ T cells and subsequent Foxp3 expression among $OX40^{lo}$ (left) and $OX40^{hi}$ (right) CD4 T cell subsets on day 7 p.i.

(**E**) Representative dot plot (left) and histogram (right) showing OX40 expression on splenic natural killer cells ($CD3^{-}NK1.1^{+}$) on day 7 p.i.

(**F**) Representative plots (top) and summary data (bottom) showing coordinate expression of PD-1 and LAG-3 on $OX40^{+}$ CD4 T cells on day 14 p.i., seven days after administration of saline or suboptimal chloroquine. Data in B-F are representative of at least 3 independent experiments.

Supplemental Figure S2. Zander et al. 2015

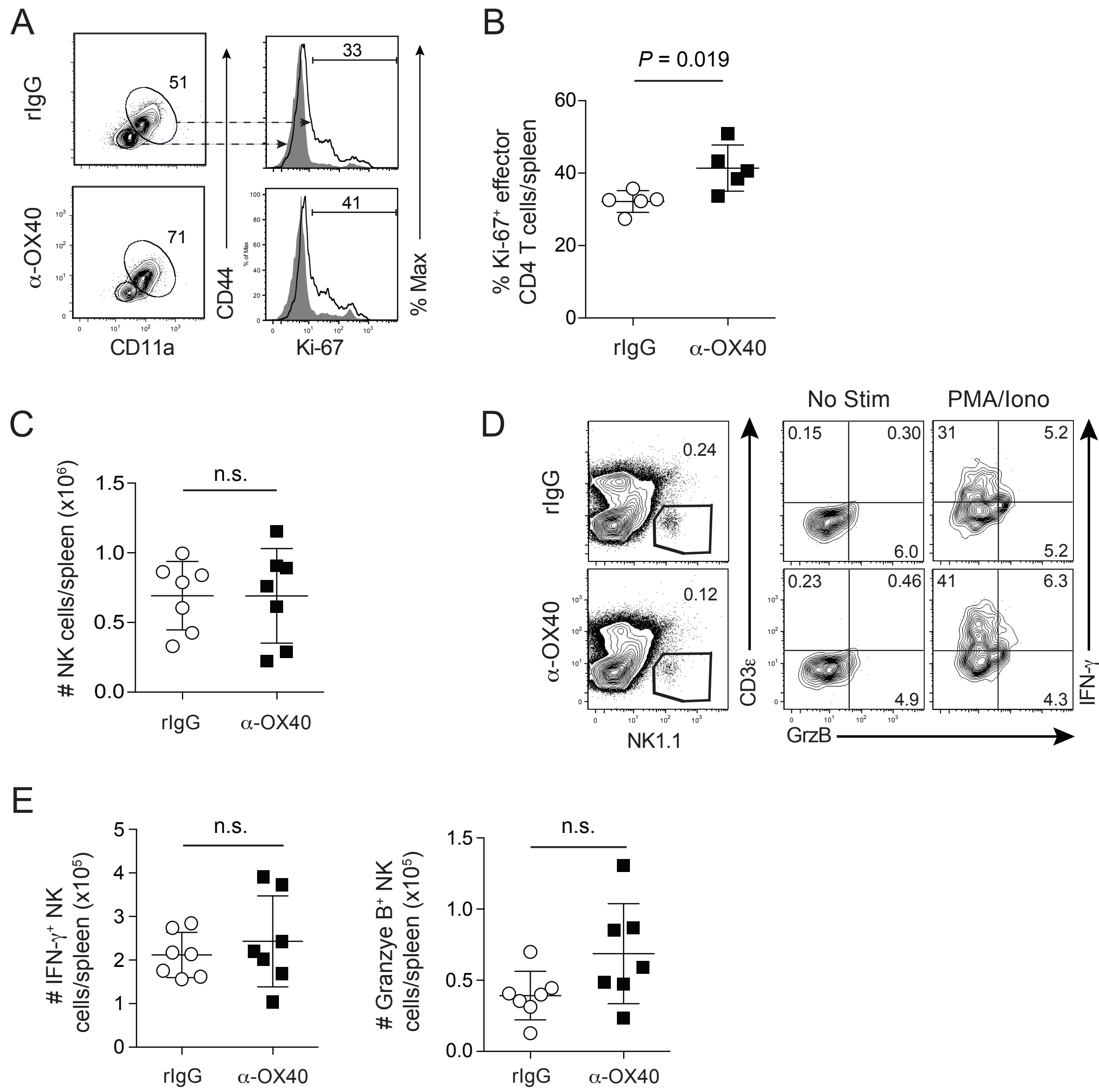


Figure S2, Related to Figure 2. Agonistic α-OX40 during *P. yoelii* infection increases the proportion of proliferating effector $CD4^+$ T cells, but does not affect the number or function of splenic NK cells. (A-E). C57BL/6 mice were infected with 10^6 *P. yoelii*-infected RBCs and treated with rat IgG or α-OX40 on days 7 and 10 p.i.

(**A**) Representative flow plots (left) and histograms (right) displaying the frequency of parasite-specific ($CD49d^{hi}CD11a^{hi}$) $CD4^+$ T cells that are positive for the nuclear proliferation antigen, Ki67, on day 14 p.i. in rat IgG (top) and α-OX40-treated (bottom) mice.

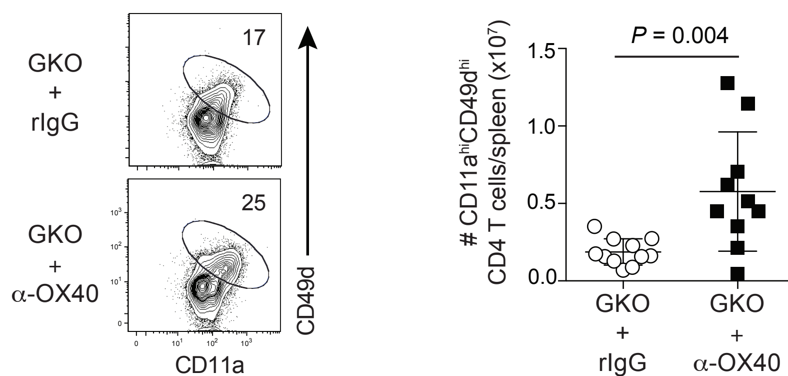
(**B**) Cumulative data (mean ± SD) display the fraction of activated $CD4^+$ T cells expressing Ki67 on day 14 p.i. Data are from 5 mice per group and were analyzed using an unpaired Student's t test.

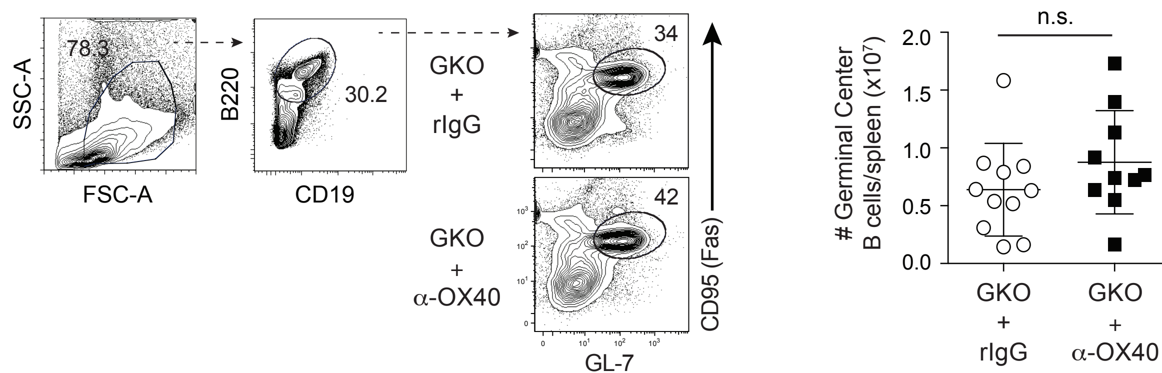
(**C**) Cumulative data (mean ± SD) showing the total number of splenic NK cells on day 14 p.i. in rat IgG and α-OX40 treated mice.

(**D**) Representative flow plots showing the frequency of splenic NK cells in IgG-treated (top) and α-OX40-treated mice (bottom) on day 14 p.i. and their functional production of IFN-γ and Granzyme B after ex vivo stimulation with PMA and ionomycin.

(**E**) Cumulative data (mean ± SD) showing the total number of splenic NK cells expressing IFN-γ (left) or Granzyme B (right) on day 14 p.i. Data in C-E are representative of 2 independent experiments.

Supplemental Figure S3. Zander et al. 2015





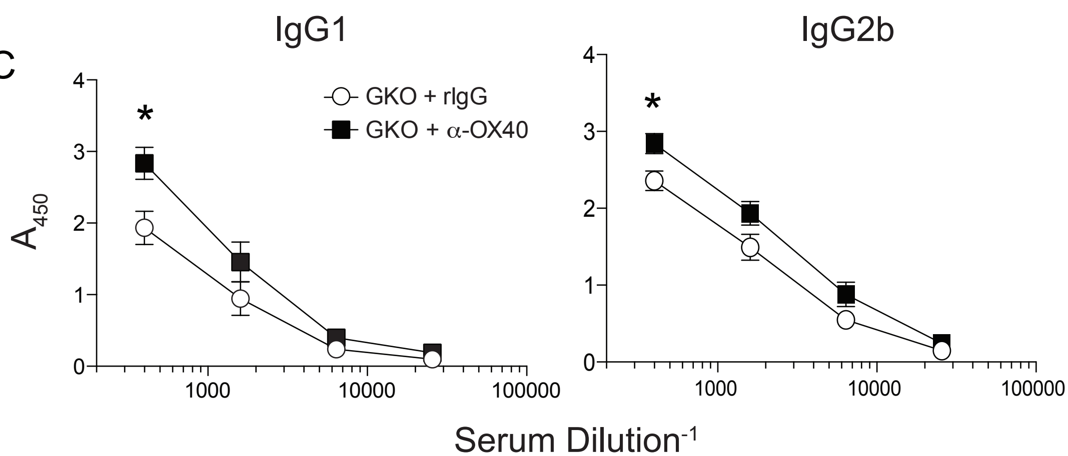


Figure S3, Related to Figure 3. Agonistic α-OX40 enhances $MSP1_{19}$-specific IgG1 and IgG2b production in the absence of IFN-γ. (A-C). Interferon-gamma knockout (GKO) mice were infected with 10^6 *P. yoelii*-infected RBCs and administered rat IgG or α-OX40 on days 7 and 10 p.i.

(**A**) Representative flow plots (left) and cumulative data (right) displaying the frequency and total numbers of parasite-specific ($CD49d^{hi}CD11a^{hi}$) $CD4^+$ T cells from rat IgG and α-OX40-treated GKO mice on day 22 p.i.

(**B**) Representative flow plots (left) and cumulative data (right) showing the frequency and total numbers of splenic germinal center ($CD95^+GL\text{-}7^+CD19^+$) B cells from rat IgG and α-OX40 treated GKO mice on day 22 p.i.

(**C**) Cumulative data showing the relative titers of $MSP1_{19}$-specific IgG1 (left) and IgG2b (right) on day 20 p.i. in GKO mice treated with rat IgG (n=5) or α-OX40 (n=5). Data (mean ± SD) in A-B were analyzed using unpaired Student's t tests (*$P < 0.05$). Data (mean ± SEM) in C were analyzed using multiple t tests while correcting for multiple comparisons using the Holm-Sidak method. Data in A-C are representative of 3 independent experiments.

Supplemental Figure S4. Zander et al. 2015

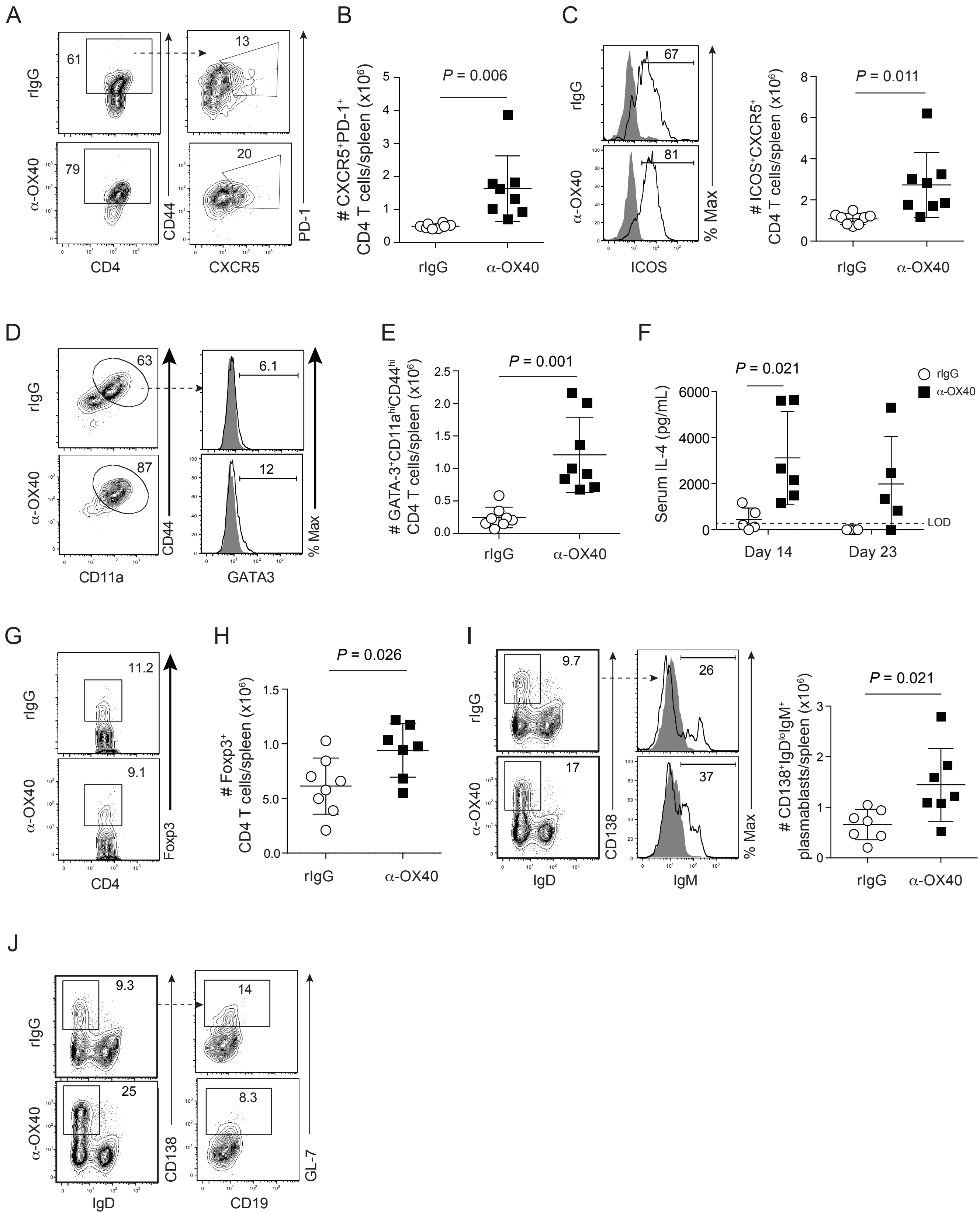


Figure S4, Related to Figure 4. T follicular helper (Tfh) and antibody responses are maintained during *P. yoelii* infection in C57BL/6 mice treated with agonistic α-OX40. (A-J). C57BL/6 mice were infected with 10^6 *P. yoelii*-infected RBCs and administered rat IgG or α-OX40 on days 7 and 10 p.i.

(**A-B**) Representative flow plots (A) and cumulative data (B) showing the fraction and total number of splenic Tfh (PD-1^+CXCR5^+CD44^+CD4^+) cells on day 14 p.i. in rat IgG and α-OX40 treated mice.

(**C**) Representative flow plots (left) and cumulative data showing the total number (right) of CXCR5^+CD44^+ splenic CD4^+ T cells expressing ICOS on day 14 p.i. from rat IgG and α-OX40-treated mice.

(**D-E**) Representative dot plots (D) and cumulative data (E) of GATA-3 expression among splenic CD11a^{hi}CD44d^{hi}CD4^+ T cells from rat IgG (top) and α-OX40-treated mice (bottom).

(**F**) Serum IL-4 concentration in rIgG and α-OX40-treated mice on days 14 and 23 p.i. as measured by ELISA.

(**G-H**) Representative plots (G) and cumulative data (H) depicting the fraction and total numbers of CD4+ T cells expressing Foxp3 on day 14 p.i. in the spleens of rat IgG (top) and α-OX40-treated mice (bottom).

(**I**) Representative flow plots (left) showing IgM expression among splenic (CD138^+IgD^-CD19^+B$220^{+)}$ plasmablasts on day 14 p.i. from rat IgG (top) and α-OX40 (bottom) treated mice. Cumulative data (mean ± SD) (right) showing the total number of IgM^+ splenic plasmablasts in rat IgG and α-OX40-treated mice.

(**J**) Representative flow plots showing the fraction of splenic (CD138^+IgD^-CD19^+B$220^{+)}$ plasmablasts that express GL-7 on day 14 p.i. from rat IgG (top) and α-OX40-treated (bottom) mice.

Cumulative data (mean ± SD) in (B-I) are pooled from two independent experiments. Data in A-J are representative of at least 3 independent experiments and were analyzed using unpaired Student's t tests.

Supplemental Figure S5. Zander et al. 2015

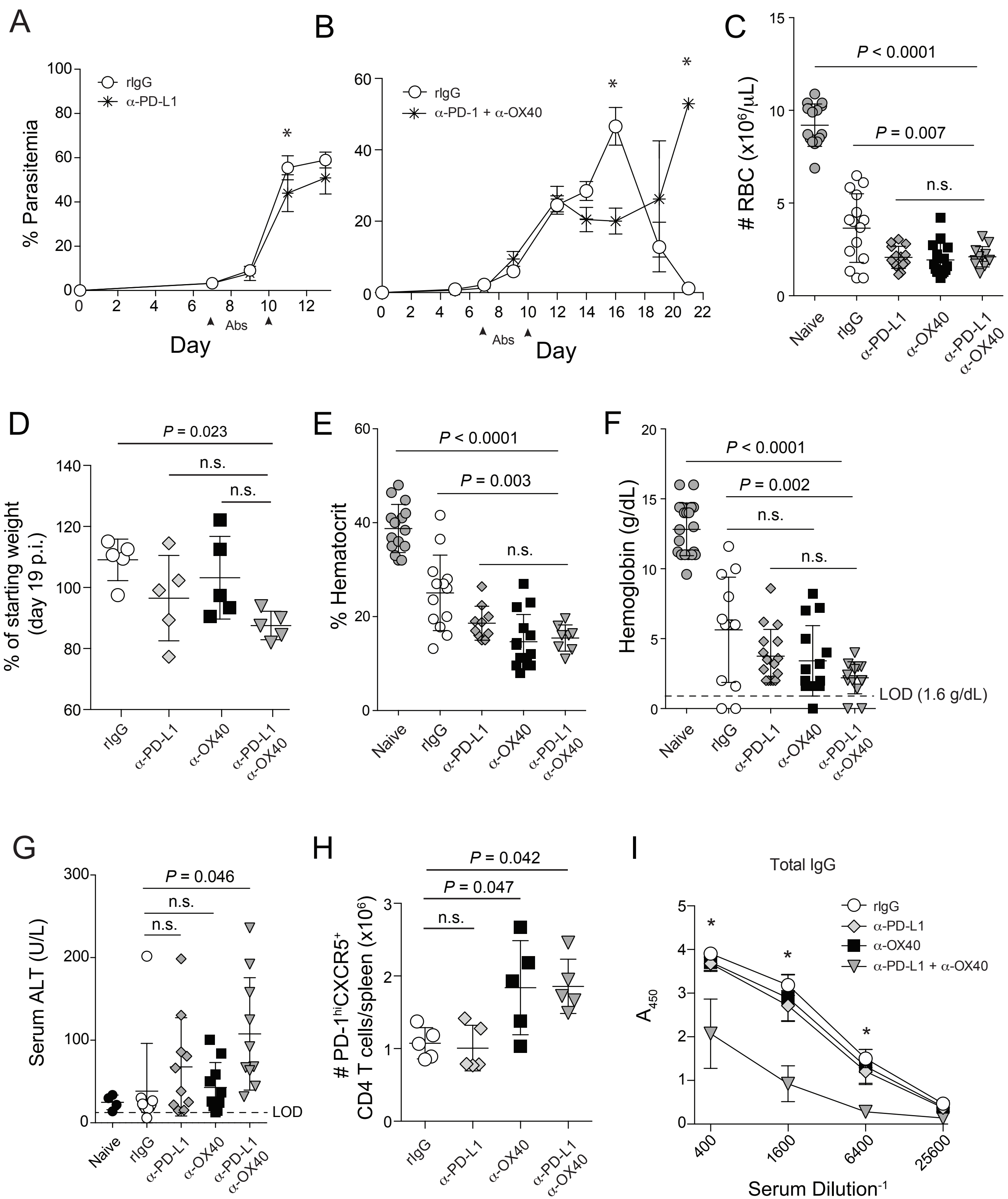


Figure S5, Related to Figure 5. Simultaneous stimulation of OX40 and blockade of PD-1/PD-L1 interactions numerically expands and enhances the function of parasite-specific effector CD4 T cells but decreases parasite-specific humoral immunity. (A-I). C57BL/6 mice were infected with 10^6 *P. yoelii*-infected RBCs and treated with either rat IgG, α-PD-L1, α-OX40, α-PD-L1 + α-OX40, or α-PD-1 + α-OX40 on days 7 and 10 p.i.

(**A-B**) Parasite growth kinetics in mice treated with either control IgG or α-PD-L1 (A), and in mice treated with either control IgG or α-PD-1 + α-OX40 mAbs (B). Data (mean ± SEM) in (A,B) are from 5 mice/group (* $P < 0.05$).

(**C**) Total RBC counts in experimental groups of mice on day 20 p.i. Data (mean ± SD) are pooled from 2 independent experiments.

(**D**) Relative weight changes (mean ± SD) in experimental groups of mice between day 0 and day 20 p.i.

(**E-F**) Hematocrit (E) and hemoglobin (F) were measured in whole blood collected from experimental mice on day 20 p.i. Data (mean ± SD) are pooled from 2 independent experiments.

(**G**) Serum alanine transaminase (ALT) levels in sera collected from experimental mice. Data (mean ± SD) are pooled from 2 independent experiments.

(**H**) Cumulative data (mean ± SD) showing the total number of splenic CD4 T cells exhibiting a canonical Tfh cell surface phenotype ($PD\text{-}1^+CXCR5^+CD44^+CD4^+$) on day 14 p.i. in rat IgG, α-PD-L1, α-OX40, and α-PD-L1 + α-OX40-treated mice. Data are representative of 3 independent experiments.

(**I**) Cumulative data (mean ± SEM) showing ELISA absorbance values for $MSP1_{19}$-specific total IgG in serum collected on day 23 p.i. from *P. yoelli*-infected mice treated with either rat IgG, α-PD-L1, α-OX40, and α-PD-L1 + α-OX40. Data in I are representative of 3 independent experiments (* $P < 0.05$).

Supplemental Figure S6. Zander et al. 2015

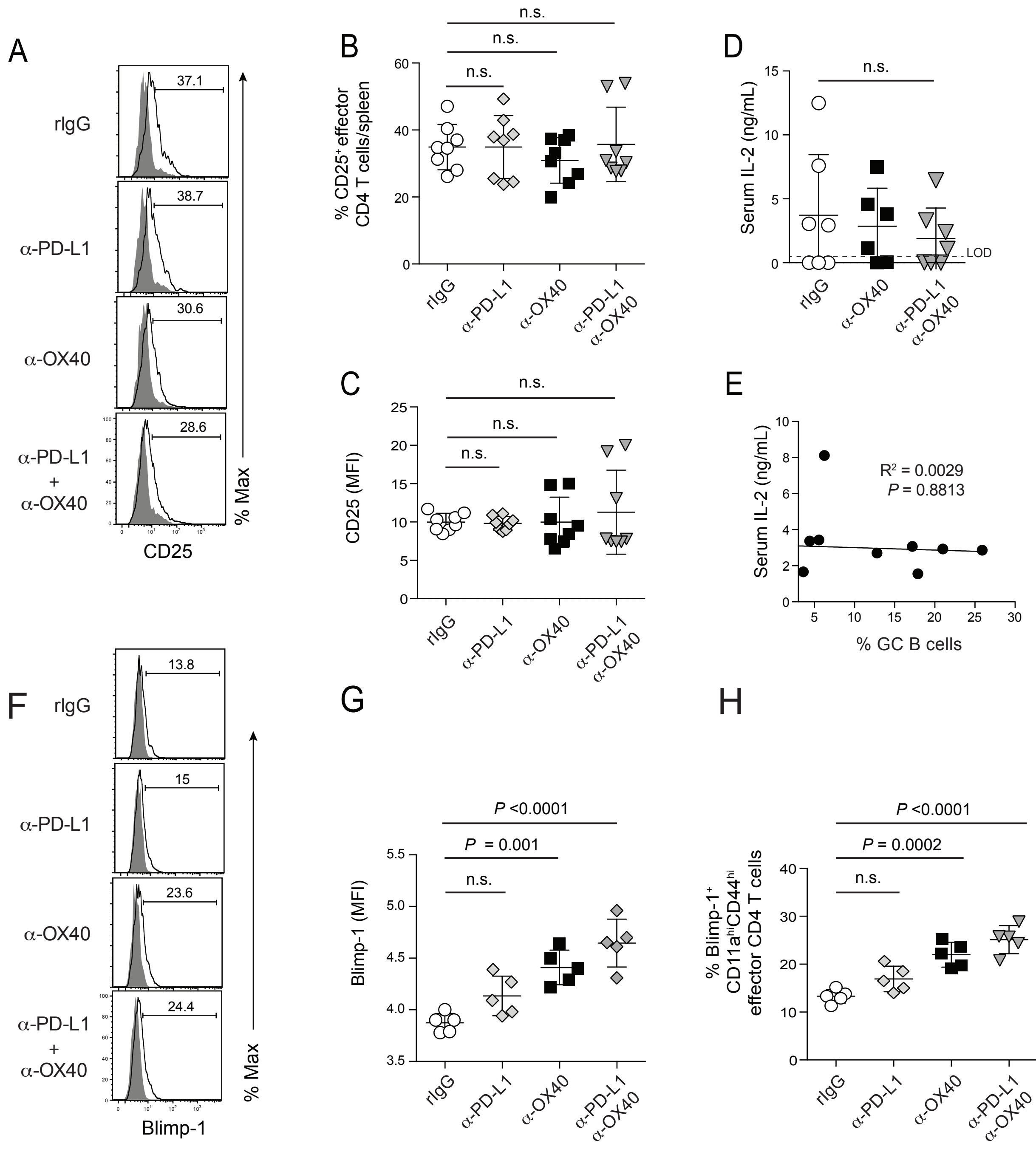


Figure S6, Related to Figure 6. The collapse of Bcl-6^{+} Tfh responses following coordinate blockade of PD-1 signaling and exogenous ligation of OX40 is not associated with increased CD25 expression on parasite-specific CD4 T cells or elevated levels of circulating IL-2. (A-H) C57BL/6 mice were infected with 10^{6} *P. yoelii*-infected RBCs and treated with rat IgG, α-PD-L1, α-OX40, or α-PD-L1 + α-OX40, on days 7 and 10 p.i.

(**A-B**) Representative histograms (A) and cumulative data (B) depicting the fraction of effector (CD49d^{hi}CD11a^{hi}) CD4+ T cells expressing CD25 on day 14 p.i. in rat IgG, α-PD-L1, α-OX40, and α-PD-L1 + α-OX40 treated mice. Data (mean ± SD) in B are pooled from 2 independent experiments, are representative of 3 independent experiments and were analyzed using one-way ANOVA while correcting for multiple comparisons via the Tukey method.

(**C**) Cumulative data showing the MFI of CD25 expression on parasite-specific (CD49d^{hi}CD11a^{hi}) CD4^{+} T cells from rat IgG, α-PD-L1, α-OX40, and α-PD-L1 + α-OX40 treated mice. Data (mean ± SD) are are pooled from 2 independent experiments, are representative of 3 independent experiments and were analyzed using one-way ANOVA while correcting for multiple comparisons via the Tukey method.

(**D**) IL-2 concentration in serum of rat IgG and α-OX40-treated mice on day 20 p.i as measured by ELISA. Data in (D) are pooled from 2 independent experiments and were analyzed using a one-way ANOVA while correcting for multiple comparisons via the Tukey method.

(**E**) Cumulative data depicting relationships between serum IL-2 levels and the frequency of GC B cells among all splenic B cells on day 20 p.i. in mice treated with either rat IgG, α-PD-L1, α-OX40, or both α-PD-L1 + α-OX40. Data were analyzed using linear regression.

(**F-H**) Representative histograms (F) and cumulative data (G,H) depicting the fraction of effector (CD49d^{hi}CD11a^{hi}) CD4+ T cells expressing Blimp-1 and the MFI of Blimp-1 on day 14 p.i. in rat IgG, α-PD-L1, α-OX40, and α-PD-L1 + α-OX40 treated mice. Data (mean ± SD) are representative of 2 independent experiments were analyzed using a one-way ANOVA while correcting for multiple comparisons via the Tukey method. Symbols in B-E,G,H represent individual mice.

Supplemental Figure S7. Zander et al. 2015

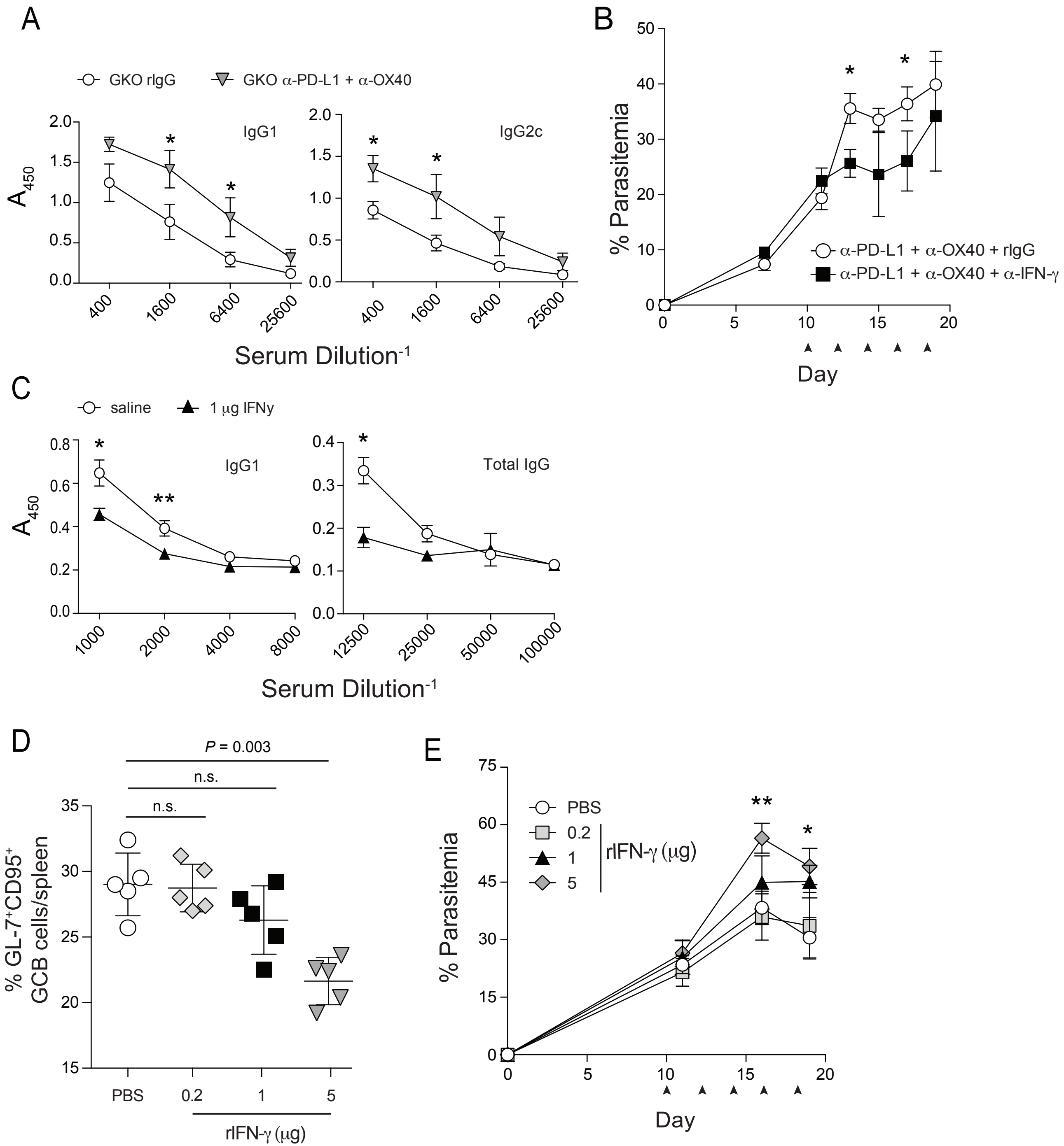


Figure S7, Related to Figure 7. IFN-γ is necessary and sufficient to limit humoral immunity during experimental *P. yoelii* malaria.

(**A**) Cumulative data (mean ± SEM) showing ELISA absorbance values for $MSP1_{19}$-specific IgG1 (left) and IgG2c (right) in serum collected on day 20 p.i. from *P. yoelii*-infected IFN-γ knockout (GKO) mice treated with either rat IgG or both α-PD-L1 + α-OX40. Data in A are from 5 mice per group, are representative of 3 independent experiments and were analyzed using multiple Student's t tests while correcting for multiple comparisons using the Holm-Sidak method (* $P < 0.05$).

(**B**) Parasite growth kinetics in mice treated with α-PD-L1 + α-OX40 mAbs on day 7 and 10 p.i. and subsequently treated every 2 days with 500 μg control IgG or α-IFN-γ (clone XMG1.2) from days 10 to 18 p.i. Data (mean ± SEM) in B are from 5 mice/group and represent 1 of 2 independent experiments (* $P < 0.05$).

(**C**) Cumulative data (mean ± SEM) showing ELISA absorbance values for $MSP1_{19}$-specific IgG1 (left) and total $MSP1_{19}$-specific IgG (right) in serum collected on day 20 p.i. from *P. yoelii*-infected mice treated every 3 days with either PBS or 1 μg of recombinant IFN-γ from days 7 to 16 p.i. Data are representative of 3 independent experiments (* $P < 0.05$; ** $P = 0.053$).

(**D**) *P. yoelii*-infected mice were treated every 3 days with PBS or the indicated amount of recombinant IFN-γ from days 7 to 16 p.i. On day 20 p.i., splenic GC B cells were enumerated using flow cytometric staining ($CD19^+GL\text{-}7^+CD95^+$ cells). Data (mean ± SD) are representative of 2 independent experiments.

(**E**) Parasite growth kinetics in mice treated every 3 days with PBS or the indicated amount of recombinant IFN-γ from days 7 to 16 p.i. Data (mean ± SEM) in (E) are from 5 mice/group and are representative of 2 independent experiments (* $P < 0.05$ for 5 μg vs. 0.2 and PBS and for 1 μg vs. PBS; ** $P \leq 0.01$ for 5 μg vs. 0.2 μg and PBS).

Supplementary Experimental Procedures

Human and mouse T cell staining and flow cytometry. Anti-human CD3-Alexa Flour 700 (clone OKT3), CD4-eFlour 450 (clone RPA-T4), CD45RA-APC-eFlour 780 (clone HI100), CD45RO-PE-cy7 (clone UCHL1) and OX40- PE (ACT-35) from ebioscience, and anti-human PD1- BV605 (clone EH12.1) from BD bioscience. FlowJo software (Tree Star) was used for analysis. Mouse splenocytes were subjected to red blood cell lysis, washed and subsequently stained using fluorescently labeled antibodies against mouse CD3ε (clone 145-2C11), CD4 (clone GK1.5), CD8 (clone 53-6.7), CD11a (clone M17/4), CD11b (clone M1/70), CD19 (clone 6D5), CD25 (clone PC61), B220 (clone RA3-6B2), CD44 (clone IM7), CD49d (clone R1-2), CD95 (clone Jo2), CD134 (clone OX-86), CD138 (clone 281-2), ICOS (clone 7E.17G9), PD-1 (clone RMP1-30), LAG-3 (clone eBioC9B7W), NK1.1 (clone PK136), T and B cell activation antigen (clone GL-7), F4/80 (clone BM8), IgD (clone 11-26c.2a), or IgM (clone RMM-1). Reagents were acquired from Biolegend, Tonbo, eBioscience or BD Bioscience. In some experiments, mouse cells were permeabilized with cytofix/cytoperm (BD Bioscience) followed by intracellular staining using anti-mouse IFN-γ (XMG1.2; Biolegend) and anti-IL-2 (JES6-5H4; eBioscience) or anti-granzyme B (clone GB11). For analysis of T follicular helper cells, splenocytes were incubated for 30 min at 25 °C with biotin-conjugated anti-CXCR5 (2G8; BD Bioscience) and subsequently stained for 30 min at 4 °C in the presence of fluorochrome-conjugated anti-CD4, anti-CD44, anti-PD-1, and streptavidin-PE-Cy7. T-bet (4B10), Bcl-6 (K112-91), GATA-3 (TWAJ), Blimp-1 (5E7), Ki67 (SolA15) and FoxP3 (FJK-16s) were stained after fixation and permeabilization using the FoxP3 staining buffer set (eBioscience). Data were acquired using a Stratedigm S1200Ex flow cytometer and analyzed using FlowJo software (Tree Star, Inc., Ashland OR).

MSP1$_{19}$ and Cytokine ELISA. Plates (Nunc) were coated with recombinant MSP1$_{19}$ (MR4) blocked with 2.5% BSA/5% normal goat serum and MSP1$_{19}$-specific IgG was detected in pre-diluted serum samples using HRP-conjugated goat anti-mouse-IgG, -IgG1, -IgG2b, -IgG3 or -IgM (Jackson ImmunoResearch). The SureBlue Reserve TMB Kit (KPL) was used as substrate and absorbance was analyzed with a Spectra Max 340 (Molecular Devices). For serum cytokine analyses, plates were coated with 2 μg/mL of IL-2, IL-4, or IFN-γ capture antibodies (eBioscience), blocked with 2.5% BSA/5% fetal calf serum. Serum samples were subsequently applied at a 1:5 dilution. Wells were washed, incubated with biotinylated detection antibodies (eBioscience) and developed with streptavidin-HRP at room temperature for 30 minutes before applying SureBlue Reserve TMB substrate as described above.

Serum ALT and clinical chemistries. Serum alanine transaminase was measured using a colorimetric endpoint assay kit (Bioo Scientific, Austin, TX) following the manufacturer's instructions. RBC counts, hematocrit and hemoglobin concentrations were measured from whole mouse blood with assistance from the OUHSC Department of Comparative Medicine using a ProCyte Dx Hematologic Analyzer (IDEXX Laboratories, Westbook, ME)