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Supplementary Materials for

IL-10–producing Tfh cells accumulate with age and link inflammation with age-related immune suppression

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This PDF file includes:

Supplementary information

Fig S1

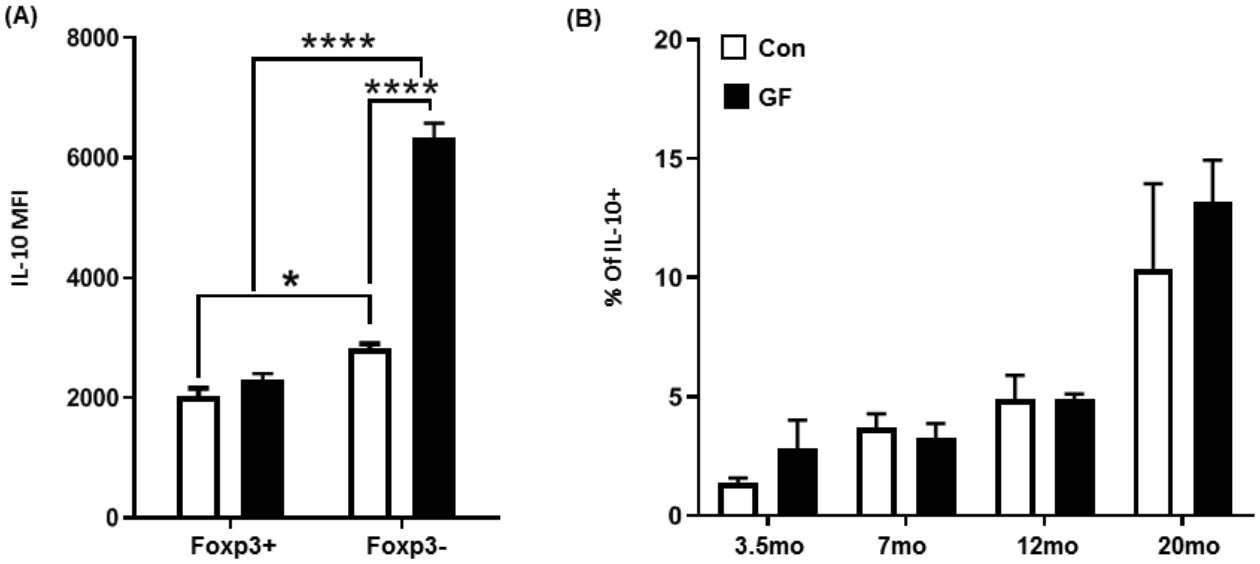

Fig. S1. Increased IL-10 MFI in aged FoxP3$^-$ CD4$^+$ T cells. (A) Splenocytes from young (1.5months, n=4) and aged (23months, n=4) C57BL/6 mice were stimulated with (P+I), stained with Abs against TCRβ, CD8, Foxp3 and IL-10 and analyzed by flow cytometry. Graph shows the mean level of IL-10 in Foxp3$^+$ IL-10$^+$ and Foxp3$^-$ IL-10$^+$ cells (mean±SEM). *p ≤ 0.05 ****p ≤ 0.0001, two-way ANOVA Sidak's multiple comparisons test. (B) **Accrual of IL-10-producing CD4$^+$ FoxP3$^-$ T cells occurs in germ-free mice.** Splenocytes from microbiota-replete conventionally-housed (Con) (n=2-4/group) or germ free (GF) (n=2-5/group) mice were stimulated with (P+I), stained with Abs against TCRβ, CD8, Foxp3 and IL-10 and analyzed by flow cytometry. Graph shows frequency of Foxp3$^-$ cells that are IL-10$^+$ (mean±SEM). Data are pooled from 3 indepndent experiments.

Fig S2

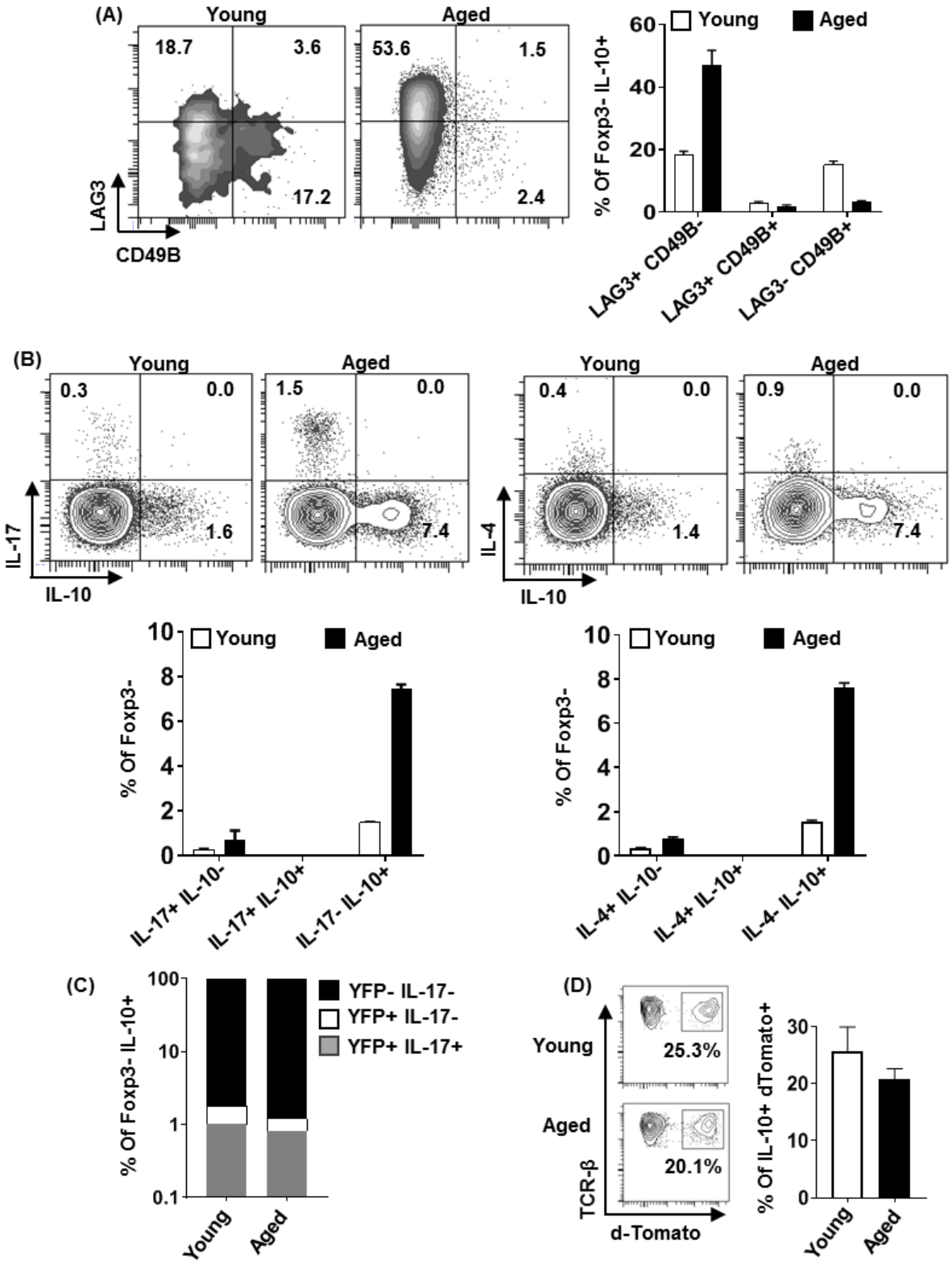
(A)
Young
Aged
18.7
3.6
17.2
53.6
1.5
2.4
LAG3
CD49B
Young
Aged
% Of Foxp3- IL-10+
LAG3+ CD49B-
LAG3+ CD49B+
LAG3- CD49B+
(B)
Young
Aged
0.3
0.0
1.6
1.5
0.0
7.4
IL-17
IL-10
0.4
0.0
1.4
0.9
0.0
7.4
IL-4
IL-10
% Of Foxp3-
IL-17+ IL-10-
IL-17+ IL-10+
IL-17- IL-10+
IL-4+ IL-10-
IL-4+ IL-10+
IL-4- IL-10+
(C)
% Of Foxp3- IL-10+
YFP- IL-17-
YFP+ IL-17-
YFP+ IL-17+
(D)
Young
Aged
25.3%
20.1%
TCR-β
d-Tomato
% Of IL-10+ dTomato+

Fig. S2. Characterization of aged IL-10^{+} FoxP3^{-} CD4^{+} T cells. (A) Splenocytes from young (1.5months, n=8) and aged (18months, n=8) C57BL/6 mice were stimulated with (P+I), stained with Abs against TCRβ, CD8, CD49b, LAG3 and IL-10, and analyzed by flow cytometry. The plots and graph show the frequency of indicated subsets in Foxp3^{-} IL-10^{+} cells (mean±SEM). (B) Splenocytes from young (1.5months, n =4) and aged (18months, n =4) mice were stimulated as above, stained with Abs against TCRβ, CD8, IL-10, IL-17, IL-4 and analyzed by flow cytometry. The plots and graph show the frequency of indicated subsets in Foxp3^{-} cells (mean±SEM). (C) Spleen cells from young (2months, n=5) and aged (12months, n=5) IL-17A^{cre} Rosa26 ^{YFP} (R26YFP) mice were stimulated as above, stained with Abs against TCRβ, CD8, IL-10, IL-17 and analyzed by flow cytometry. Bar graph shows the frequency of cells producing IL-17 (IL-17+YFP+, gray), exTh17 (IL-17-YFP+, white) or those that never produced IL- 17 (IL-17-YFP-, black) within the total Foxp3- IL-10+ cells. (D) Spleen cells from young (2months, n=3) and aged (13months, n=4) and Foxp3^{Cre}Rosa26^{dTomato} mice were stimulated with (P+I), stained with Abs against TCRβ, CD8, IL-10 and Foxp3, and analyzed by flow cytometry. Plots and bar graph show the frequency of exTreg cells (Foxp3^{-} IL-10^{+} $^{dTomato+}$) within the total Foxp3- IL-10+ cells. Data are pooled from two independent experiments.

Fig S3

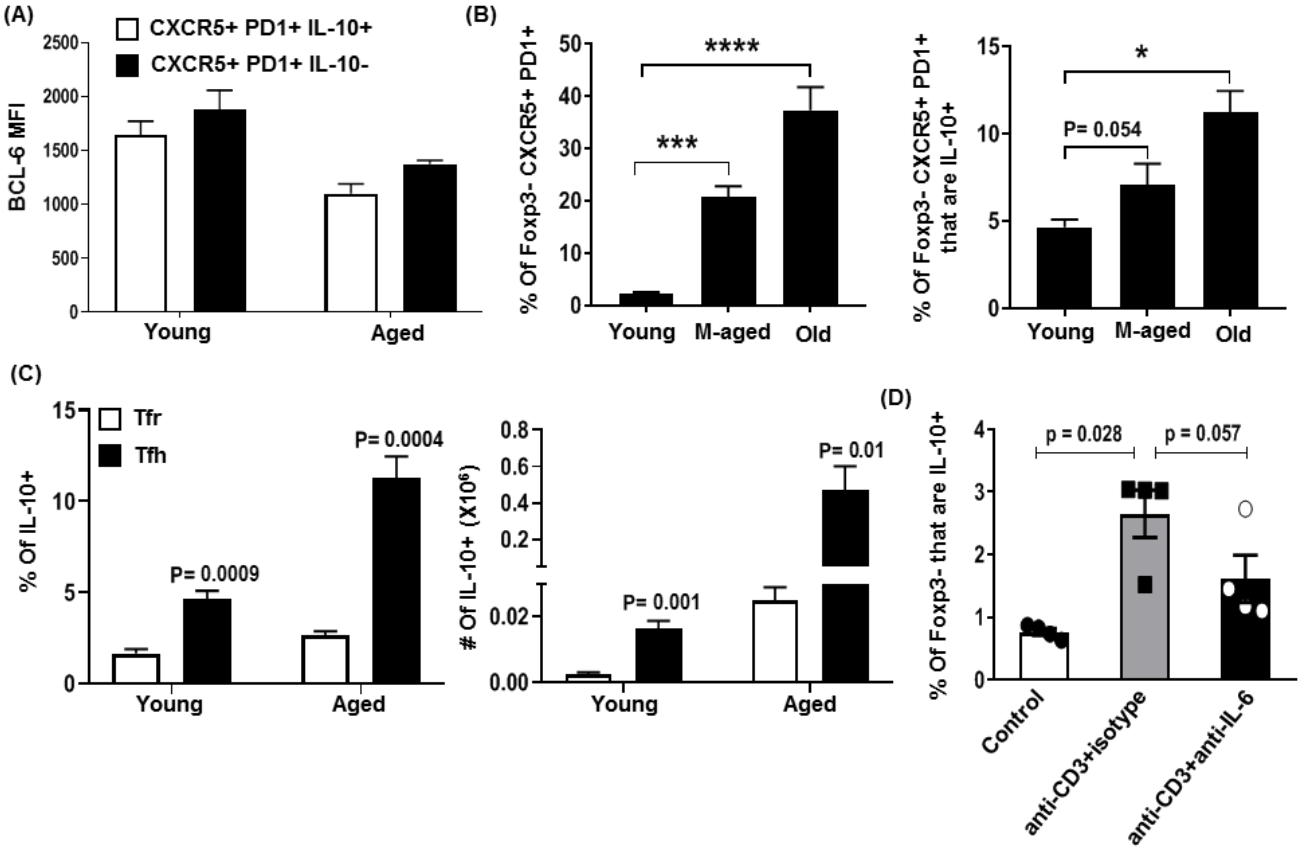

Fig. S3. Progressive accrual of Tfh10 cells with age. (A) Splenocytes from young (2months, n=4) and aged (21months, n=4) C57BL/6 mice were stimulated with P+I and stained with Ab against TCRß, CD8, CXCR5, PD1, Foxp3, BCL6 and IL-10. The representative bar graph shows the level of expression of BCL6 with age in cells that are $CXCR5^+$ $PD1^+$ $IL\text{-}10^-$ and $CXCR5^+$ $PD1^+$ $IL\text{-}10^+$ (mean±SEM). (B) Splenocytes from young (2months, n=4), middle age (15.5months, n=8) and old (18.5months, n=4) C57BL/6 mice were stimulated as above and stained with Ab against TCRß, CD8, CXCR5, PD1, Foxp3 and IL-10. The representative bar graphs show the frequency of $Foxp3^-$ that $CXCR5^+$ $PD1^+$ and those that produce IL-10 (mean±SEM). Data pooled from two independent experiments. *p ≤0.05, ***p ≤0.001, ****p ≤0.0001, one-way ANOVA. (C) Splenocytes from young (2months, n=4),

and aged (18months, n=4/group) C57BL/6 mice were stimulated as above and stained with Ab against TCRß, CD8, CXCR5, PD1, Foxp3 and IL-10. The representative bar graphs show the frequency and number IL-10-producing Tfh ($Foxp3^-$ $CXCR5^+$ $PD1^+$) and IL-10-producing Tfr $Foxp3^+$ $CXCR5^+$ $PD1^+$ (mean±SEM), multiple t tests. (D) **Anti-IL-6 antibody is functional and prevents anti-CD3 driven Tr1 accrual *in vivo*.** Young C57BL/6 mice (2months, n=4/group) were injected with anti-CD3, control IgG or anti-IL-6 twice at time 0 and 40hrs. Mice were sacrificed 4hrs after second injection. Splenocytes were stimulated with P+I and stained with Ab against TCRß, CD8, Foxp3 and IL-10. The representative bar graph shows the frequency of $Foxp3^-$ cells that produce IL-10 (mean±SEM), Student's t-test.

Fig S4

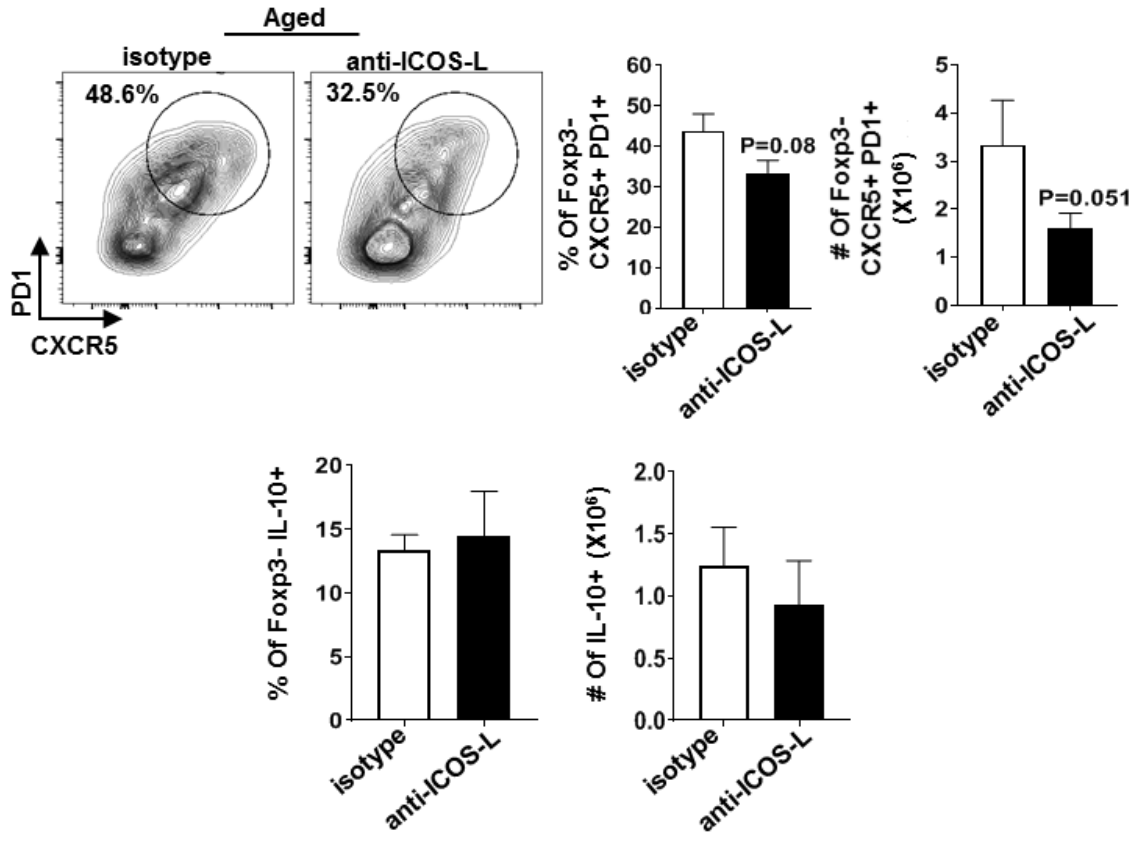

Fig. S4. ICOS neutralization reduces Tfh cells, but not Tfh10 cells. Aged (16months, n=6) C57BL/6 mice were treated with isotype control or α-ICOSL blocking antibody (16months, n=5) on day 0, 3, 6, 9 and sacrificed on day 12. Splenocytes were stimulated with (P+I), stained with Ab against TCRß, CD4, CD8, CXCR5, PD1, Foxp3 and IL-10 and analyzed by flow cytometry. The representative plots and bar graphs show the frequency of $Foxp3^-$ that $CXCR5^+$ $PD1^+$ and the frequency of $Foxp3^-$ that are $IL\text{-}10^+$ (mean±SEM), Student's t-test.

Fig S5

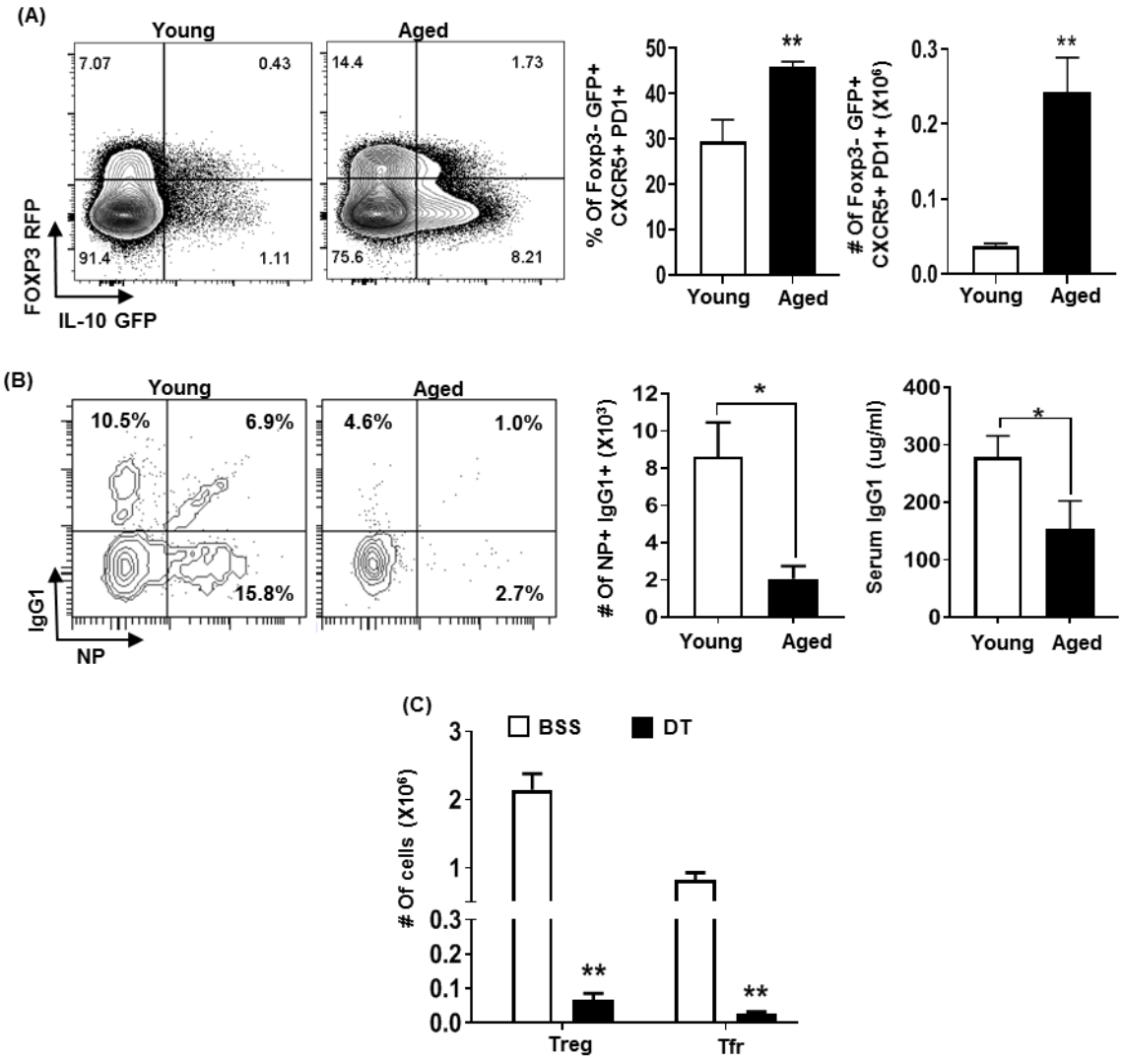

Fig. S5. Normal accrual of Tfh10 cells occurs in IL-10 VertX-FoxP3-RFP mice. (A)Young (3.5months, n=5) and aged (18months, n=5) IL-10GFP FOXP3RFP dual reporter mice were immunized i.p. with influenza nucleoprotein in alum (50µg/monthsuse). Mice were sacrificed eight days later, and spleen cells were stained with Abs against CD4, cells were assessed for IL-10 (GFP fluorescence) and Foxp3 (RFP fluorescence). Plots show gating strategy and graphs show the frequency and number of $CD4^+$ $FOXP3^-$ RFP^- $IL\text{-}10^+$ GFP^+ that are $CXCR5^+$ $PD1^+$ cells. Data pooled from two independent experiments. **Aged mice have decreased antibody response to NP-KLH and efficiency of deletion of Treg and Tfr in FoxP3-DTR**

mice. (B) Young (2months, n=6) and aged (18months, n=5) mice were immunized with NP-KLH in Alum and sacrificed 20 days later. Splenocytes were stained with Abs against CD19, B220, GL7 and Fas and analyzed by flow cytometry. Representative plots identifying GC B cells NP-specific as Fas^{hi} $GL7^{hi}$ that are $IgG1^{+}$ NP^{+}. Graphs show the frequency and the total number of splenic B cells that are $IgG1^{+}$ NP^{+} (mean±SEM), as well as serum levels of immunoglobulin specific for NP (IgG1) of young vs old mice obtained 20 days after immunization (mean±SEM). (C) Aged (20months, n≥4/group) Foxp3-DTR C57BL/6 mice were immunized with NP-KLH in alum (100ug). Mice were treated with Diphtheria Toxin (DT) or BSS on day -1, 2, 5 and 8. All mice were sacrificed on day10. Representative graph shows the total number of $Foxp3^{+}$ Treg cells and $Foxp3^{+}$ $CXCR5^{+}$ $PD1^{+}$ Tfr cells (mean±SEM). *p ≤ 0.05, **p ≤ 0.01, Student's t-test.

Fig S6

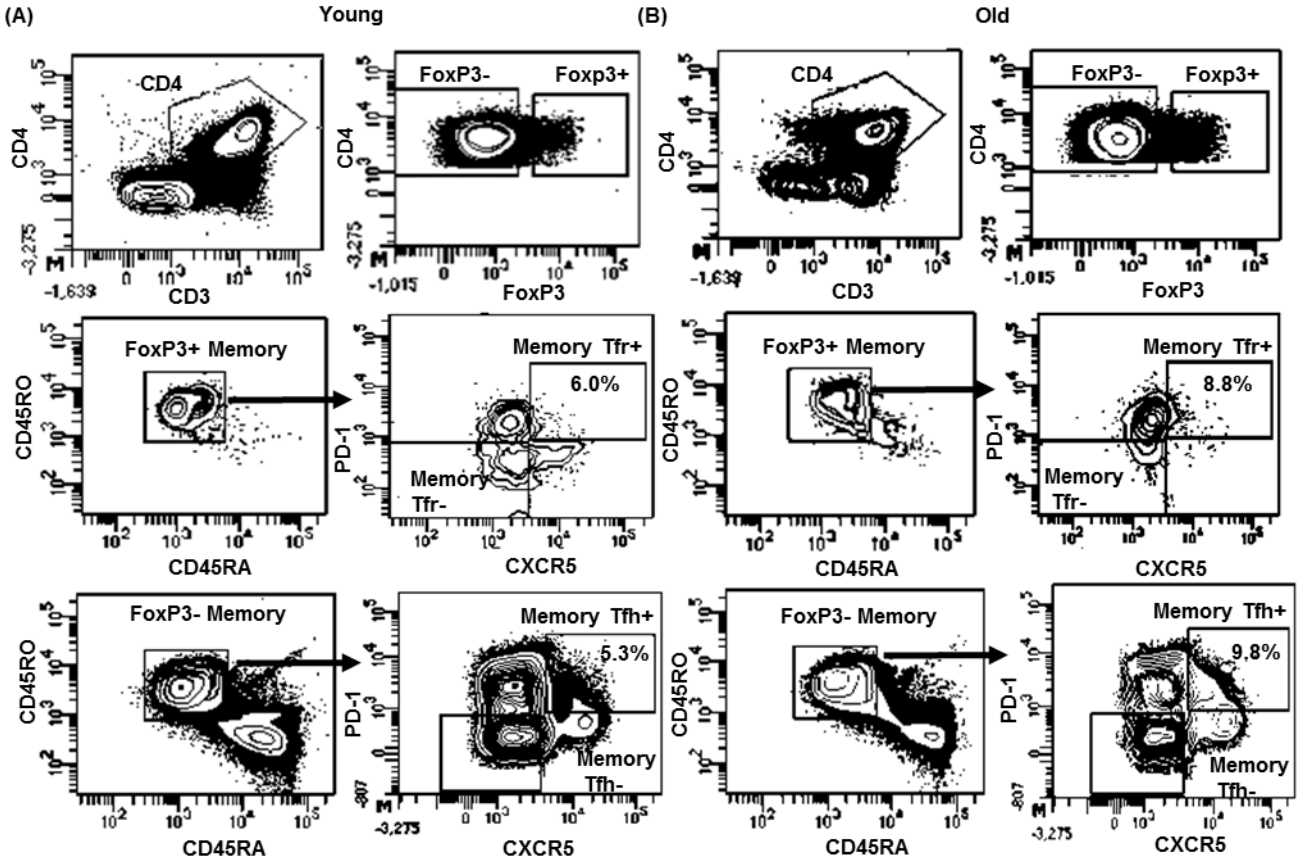

Fig. S6. Flow cytometry gating strategy to identify Tfh and Tfr in human Splenocytes.

Representative flow cytometry gating of (A) young and (B) old, human spleen single cells suspensions that were used in our cell sorting to identify Memory Tfh+($CD3^+CD4^+FoxP3^-CD45RO^+CD45RA^-PD\text{-}1^+CXCR5^+$), Memory Thf- ($CD3^+CD4^+FoxP3^-CD45RO^+CD45RA^-PD\text{-}1^-CXCR5^-$), Memory Tfr+($CD3^+CD4^+FoxP3^+CD45RO^+CD45RA^-PD\text{-}1^+CXCR5^+$), Memory Tfr-($CD3^+CD4^+FoxP3^+CD45RO^+CD45RA^-PD\text{-}1^-CXCR5^-$) cells.