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## Supplemental Information

# CAR- and TRuC-redirected regulatory T cells differ in capacity to control adaptive immunity to FVIII

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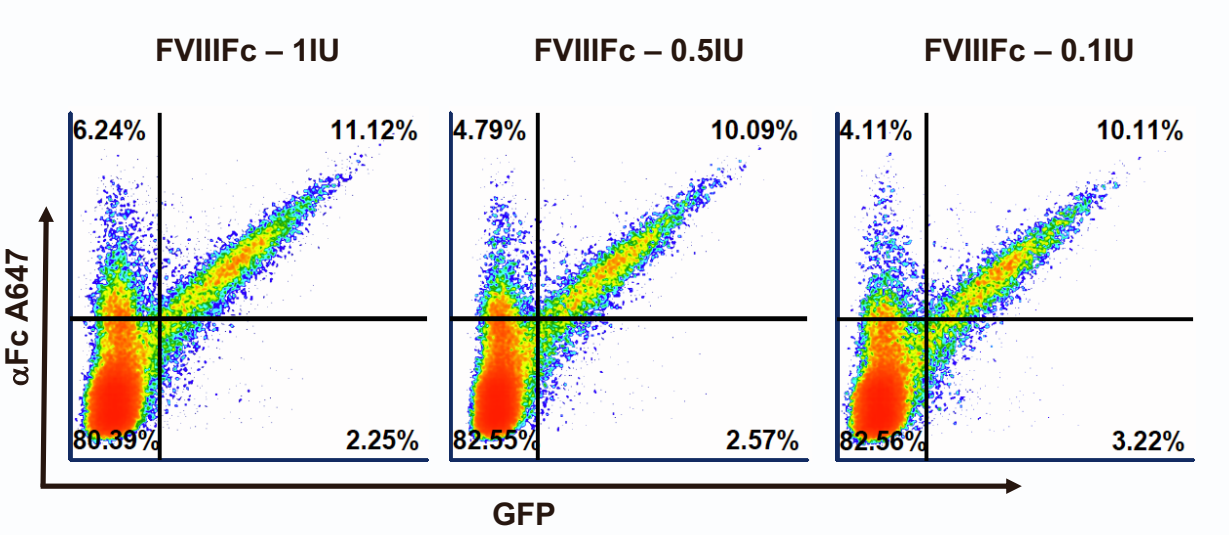
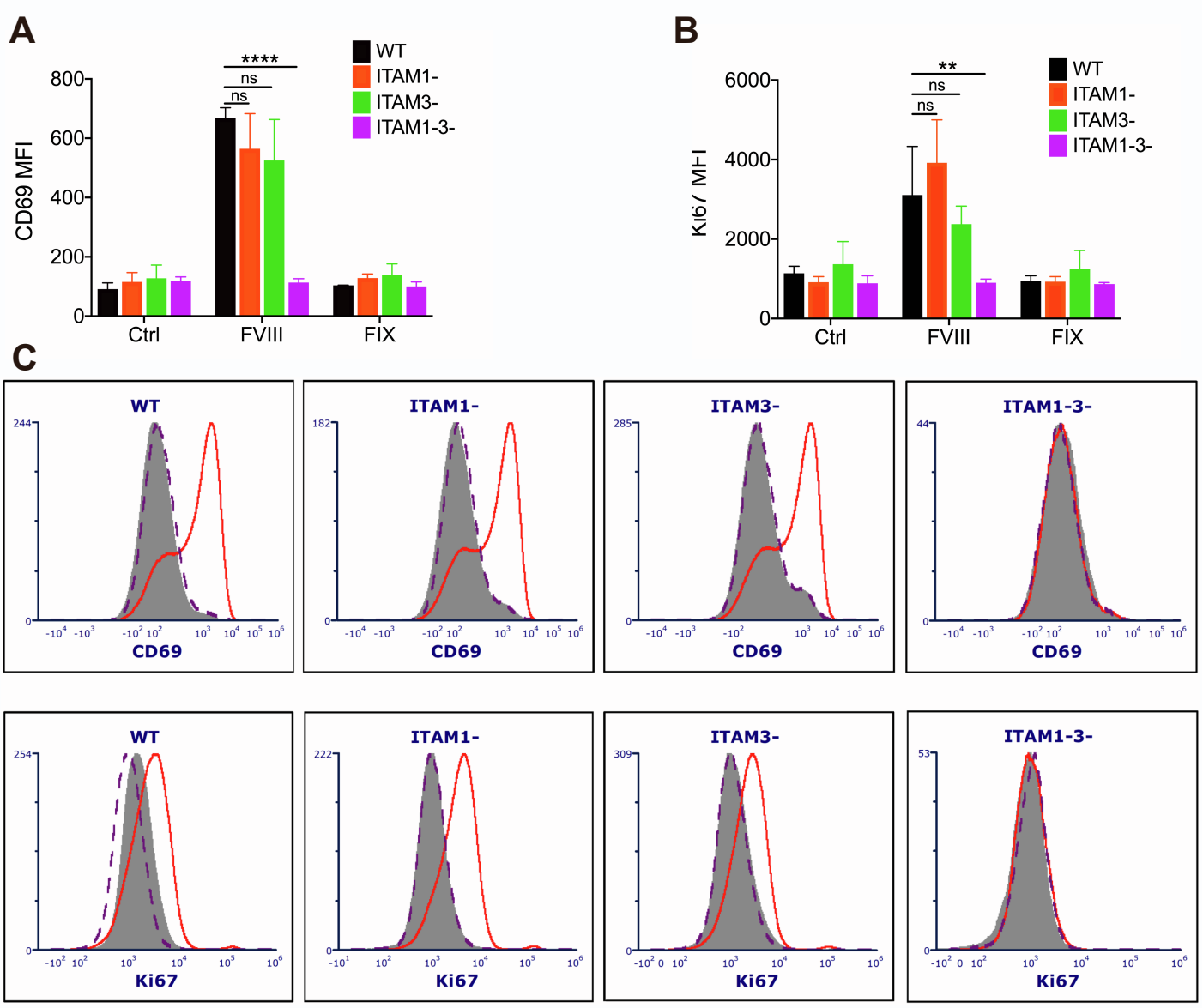
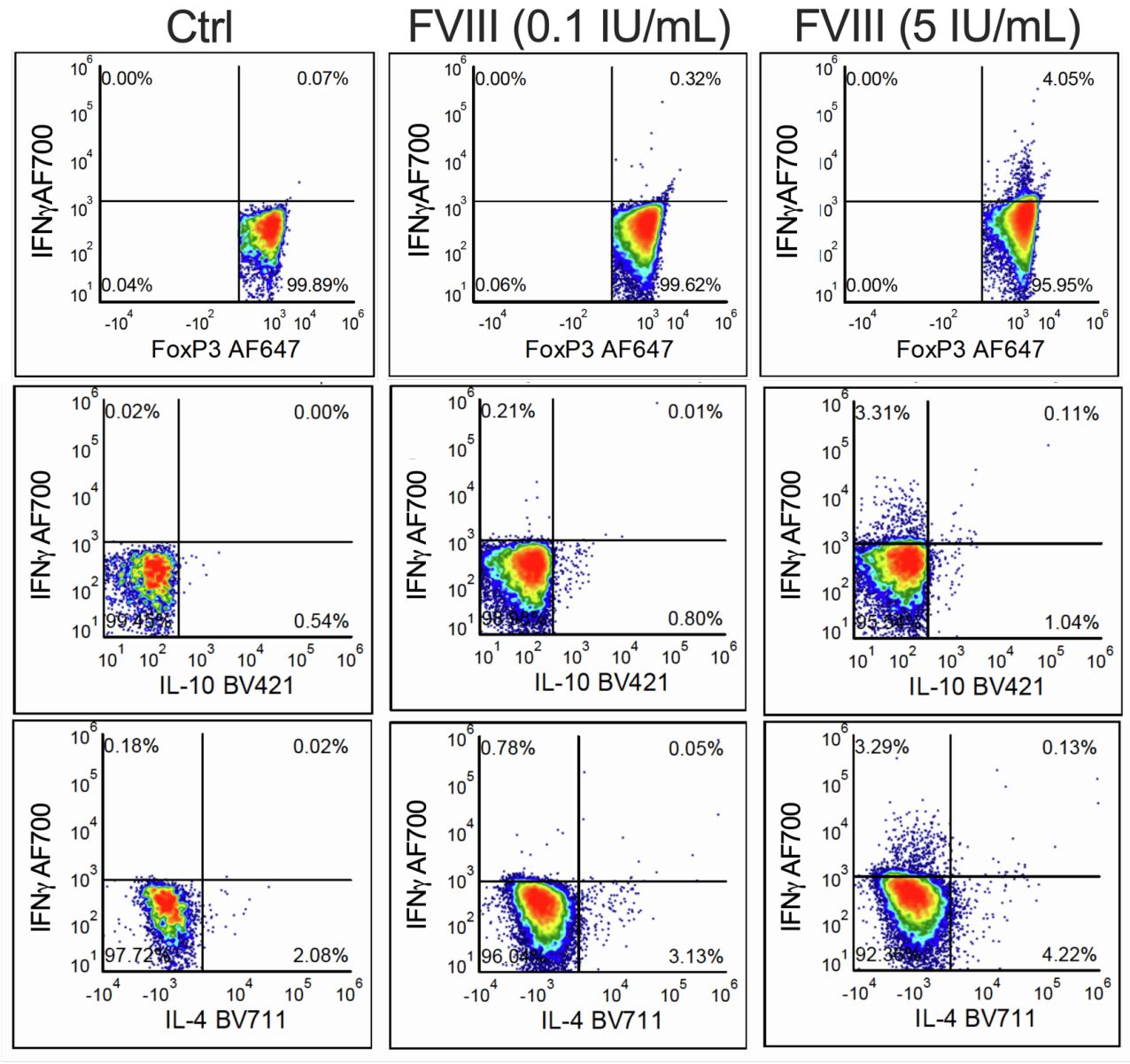


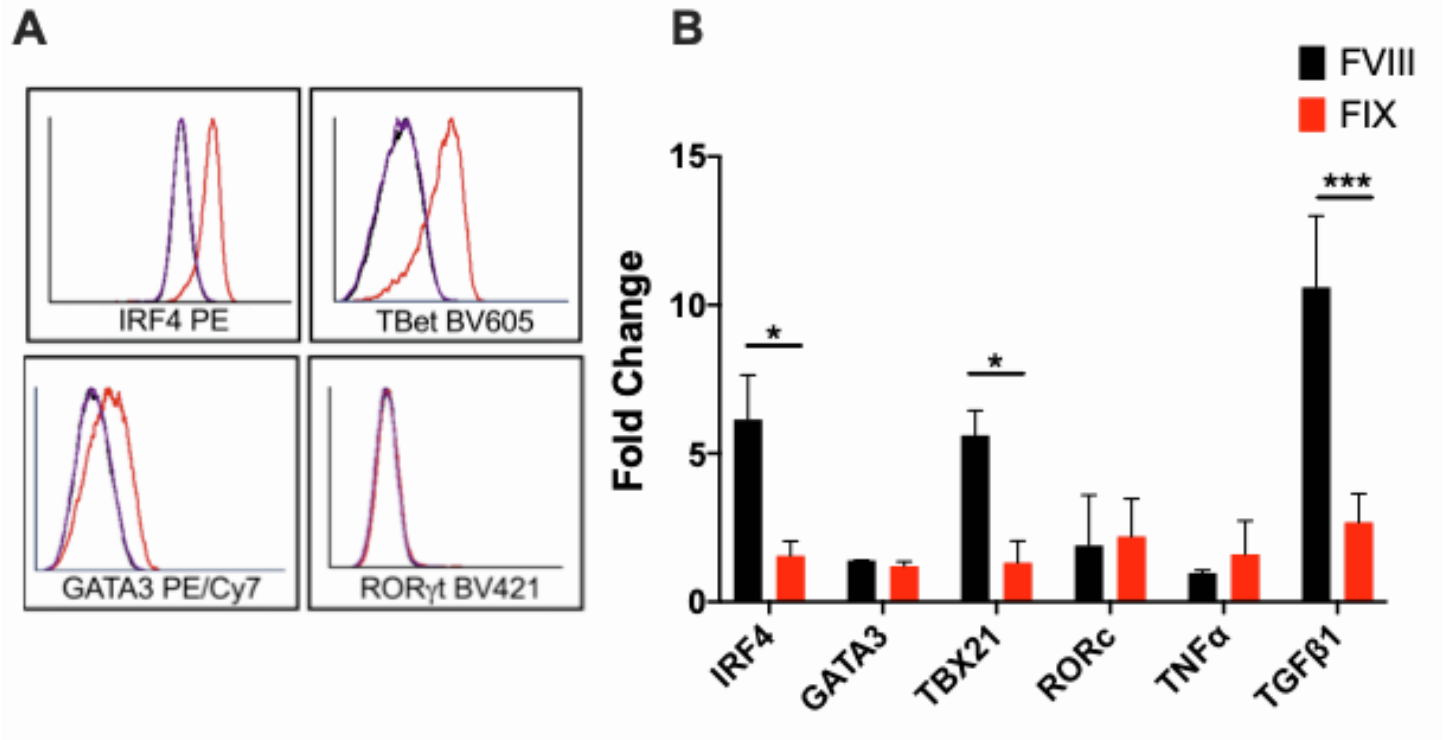

**Supplementary Figure 1. FVIII CAR binds antigen with high sensitivity.** Transduced ($GFP^+$) WT CAR Treg cells were incubated with 1, 0.5 or 0.1 IU of FVIIIFc for 20 minutes at RT, following which percentage of $GFP^+$ cells that bound FVIIIFc was detected by αFc conjugated to AF647 by flow cytometry.



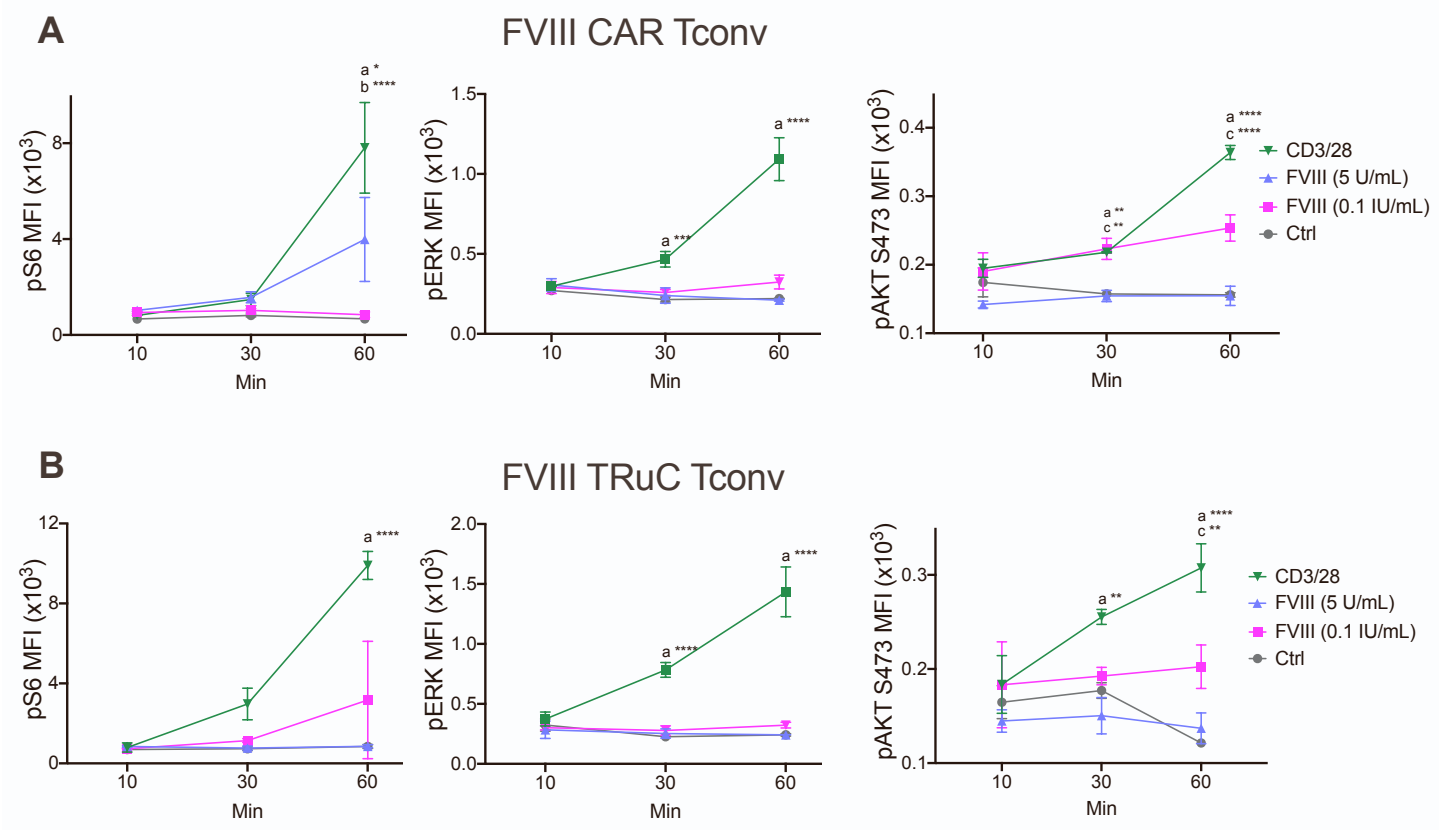

**Supplementary Figure 2. ITAM1 or ITAM3 mutations do not affect CAR Treg activation.** BDD-FVIII stimulation of WT, ITAM1$^-$ or ITAM3$^-$ FVIII CAR transduced Treg cells for 48h does not affect upregulation of **A)** CD69 or **B)** Ki67 *in vitro,* whereas double ITAM1$^-$3$^-$ mutation deactivates CD69 and Ki67 expression. Bar graphs of Tregs transduced with WT, ITAM1$^-$, ITAM3$^-$ or ITAM1$^-$ITAM3$^-$ mutated FVIII CARs. **C)** Representative histogram plots showing MFI for CD69 and Ki67 in WT, ITAM1$^-$, ITAM3$^-$ or ITAM1$^-$3$^-$ mutated FVIII CAR Tregs. Data points are averages ± SEM. * P<0.05, ** P<0.01, *** P<0.001, **** p<0.0001 by 2-way ANOVA with Dunnett's multiple comparisons test.



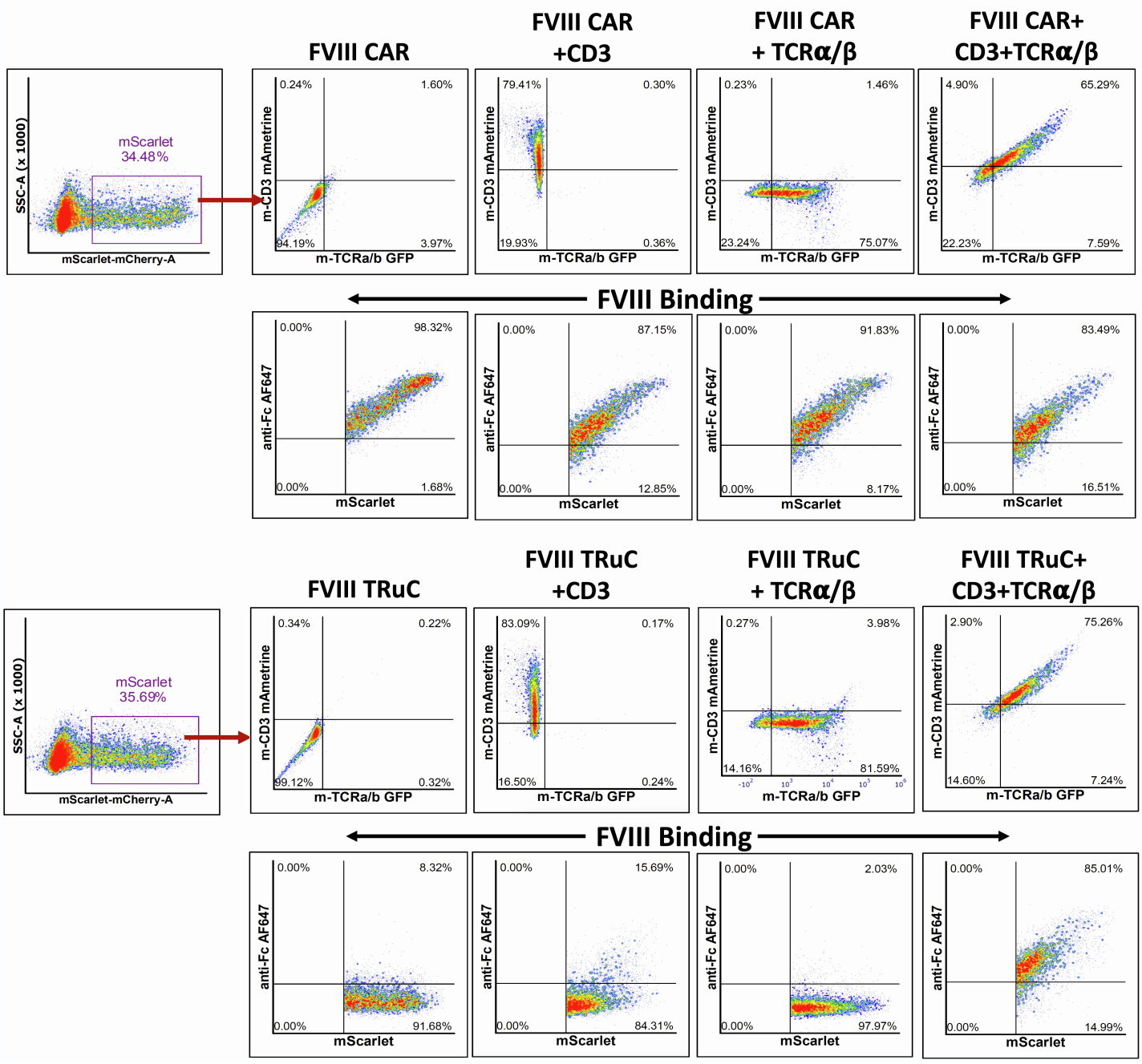

**Supplementary Figure 3. CAR Tregs exhibit heterogenous cytokine release.** Representative intracellular cytokine staining plots of BDD-FVIII stimulated CAR Tregs *in vitro,* depicting co-expression of FoxP3 and IFNγ, as well as a heterogenous population of IL-4, IL-10 and IFNγ secreting cells.



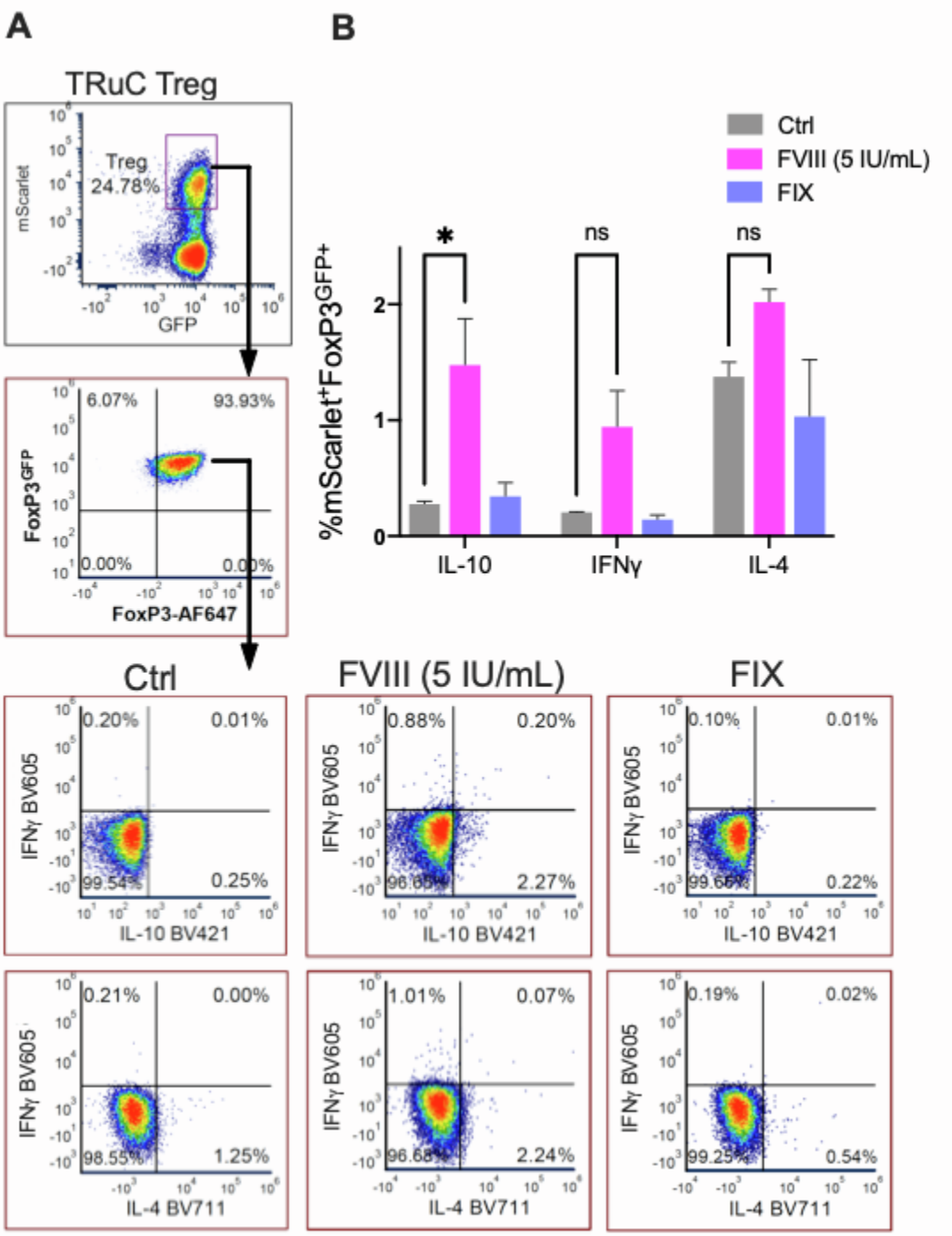

**Supplementary Figure 4. Heterogenous transcription factor expression by FVIII CAR Tregs. A)** Representative histogram plots showing upregulation of transcription factors IRF4, TBet, GATA3, but not RORγt by BDD-FVIII stimulated CAR Tregs (red histograms) as compared to unstimulated controls (purple histogram). **B)** mRNA expression levels for IRF4, GATA3, TBX21, RORc, TNFα and TGFβ1 in BDD-FVIII or FIX stimulated FVIII CAR Tregs as determined by real time RT-PCR. Fold upregulation in mRNA expression is normalized to the housekeeping gene GAPDH and to unstimulated control. Data points are averages ± SEM. * P<0.05, ** P<0.01, *** P<0.001 by 2-way ANOVA with Sidak's multiple comparisons test.



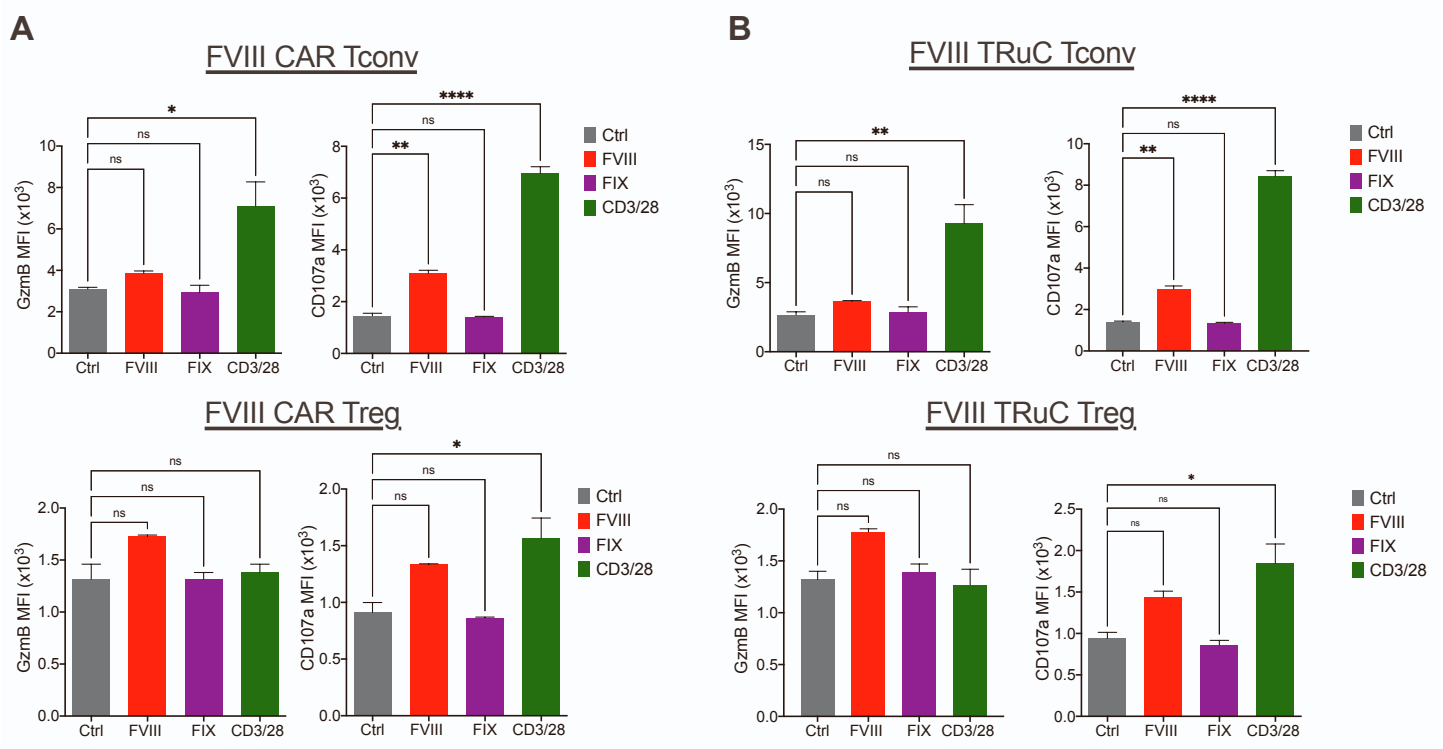

**Supplementary Figure 5. TCR triggering of CAR or TRuC engineered Tconv cells elicits a strong signaling response.** Phospho-flow cytometry of FVIII CAR Tconv and FVIII TRuC Tconv cells for estimation of pS6, pERK, pAKT (S473) at indicated times following stimulation with anti-CD3/28 microbeads, high dose (5 IU/mL) BDD-FVIII, low dose (0.1 IU/mL) BDD-FVIII, or unstimulated controls. Data points are averages ± SEM. * P<0.05, ** P<0.01, *** P<0.001, **** p<0.0001 by 2-way ANOVA with Tukey's multiple comparison test. Groups with significant differences are annotated **a:** anti-CD3/28 vs control, **b:** FVIII (5 IU/mL) vs control, **c:** FVIII (0.1 IU/mL) vs control.


FVIII CAR
FVIII CAR +CD3
FVIII CAR + TCRα/β
FVIII CAR+ CD3+TCRα/β
SSC-A (x 1000)
mScarlet-mCherry-A
mScarlet 34.48%
m-CD3 mAmetrine
m-TCRa/b GFP
0.24%
1.60%
94.19%
3.97%
79.41%
0.30%
19.93%
0.36%
0.23%
1.46%
23.24%
75.07%
4.90%
65.29%
22.23%
7.59%
FVIII Binding
anti-Fc AF647
mScarlet
0.00%
98.32%
0.00%
1.68%
0.00%
87.15%
0.00%
12.85%
0.00%
91.83%
0.00%
8.17%
0.00%
83.49%
0.00%
16.51%
FVIII TRuC
FVIII TRuC +CD3
FVIII TRuC + TCRα/β
FVIII TRuC+ CD3+TCRα/β
mScarlet 35.69%
0.34%
0.22%
99.12%
0.32%
83.09%
0.17%
16.50%
0.24%
0.27%
3.98%
14.16%
81.59%
2.90%
75.26%
14.60%
7.24%
0.00%
8.32%
0.00%
91.68%
0.00%
15.69%
0.00%
84.31%
0.00%
2.03%
0.00%
97.97%
0.00%
85.01%
0.00%
14.99%

**Supplementary Figure 6. Surface expression of FVIII TRuC is dependent on incorporation into the TCR-CD3 complex.** FVIII CAR and FVIII TRuC plasmids were either singly transfected into HEK-293 cells, or co-transfected with the murine CD3δγεζ (CD3) plasmid with mAmetrine tag, murine TCR alpha/beta encoding plasmid (TCRα/β) with GFP tag, or both plasmids, CD3+TCRα/β to form the TCR-CD3 complex, ($GFP^+mAmetrine^+$). FVIII CAR or FVIII TRuC ($mScarlet^+$) surface expression was confirmed by incubation with 1IU FVIIIFc, for 20 minutes at RT, following which the percentage of $mScarlet^+$ cells that bound FVIIIFc was detected by αFc conjugated to AF647 by flow cytometry. FVIII CAR surface expression was independent of expression of CD3, TCR, or the TCR-CD3 complex. However, FVIII TRuC surface expression was dependent on co-expression of CD3 and TCRα/β, indicating its incorporation into the CD3-TCR complex.




**Supplementary Figure 7. FVIII TRuC Tregs exhibit controlled cytokine expression. A)** Intracellular cytokine staining of FVIII TRuC transduced Tregs stimulated with BDD-FVIII (5IU/mL), an irrelevant antigen, FIX or left unstimulated (Ctrl) for 36h in vitro. Representative dot plots to assess whether IFNγ and IL-10 or IFNγ and IL-4 are co-expressed. **B)** Bar graphs for the same. Data points are averages ± SEM. * P<0.05, ** P<0.01, *** P<0.001 by 1-way ANOVA with Tukey's multiple comparisons for **(B).**




**Supplementary Figure 8. FVIII specific CAR or TRuC Tregs do not upregulate cytotoxic markers *in vitro*.** Upregulation of Granzyme B and CD107a in **(A)** FVIII stimulated CAR Tconv cells or CAR Tregs and **(B)** FVIII stimulated TRuC Tconv cells or TRuC Tregs. Mock stimulated cells, cells stimulated with an irrelevant antigen or anti-CD3/28 bead stimulated cells serve as controls. Data points are averages ± SEM. * P<0.05, ** P<0.01, *** P<0.001 by 1-way ANOVA with Tukey's multiple comparisons.