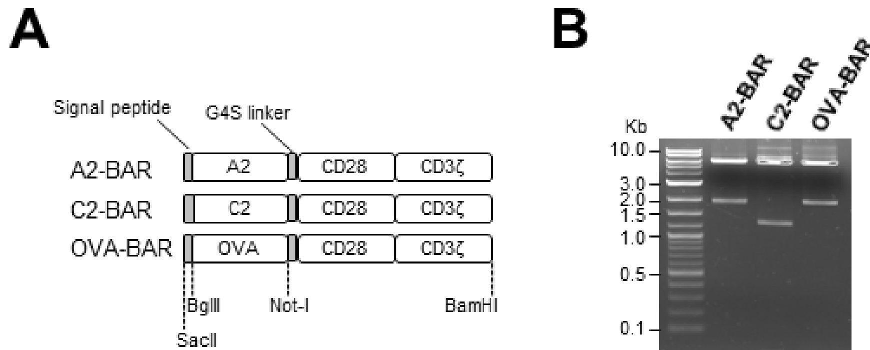
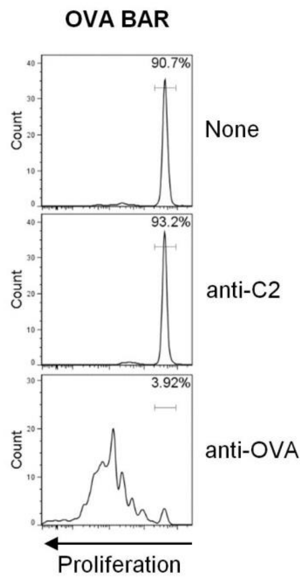
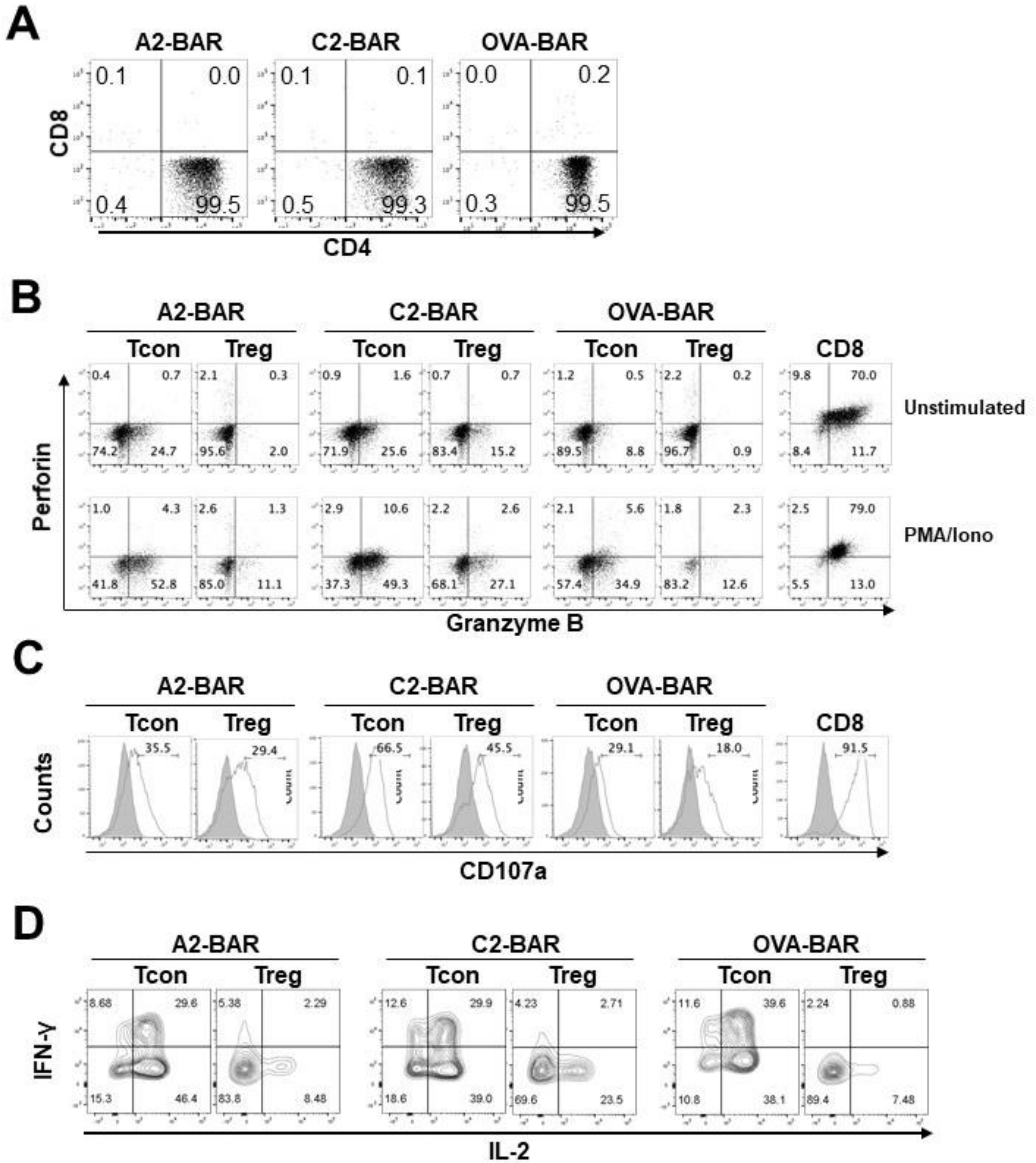
Supplemental Materials



Supplemental Figure 1. Construction of A2-, C2-, and the control OVA-BARs. (**A**) Schematic illustration for the BAR constructs. The cDNA sequences for a BAR were arranged in the following order: antigen-CD28-CD3ζ from N- to C-terminal. A G4S linker was placed between the antigen and CD28. Convenient restriction sites were placed flanking the antigen domain and the whole transgene. (**B**) Restriction analysis of the BAR transgene inserts. The BAR retroviral transfer plasmids were digested with restriction enzymes SacII and XhoI, and analyzed by electrophoresis on a 1% agarose gel. The expected size for the A2-BAR, C2-BAR and OVA-BAR inserts were 1898, 1274, and 1952bp, respectively.

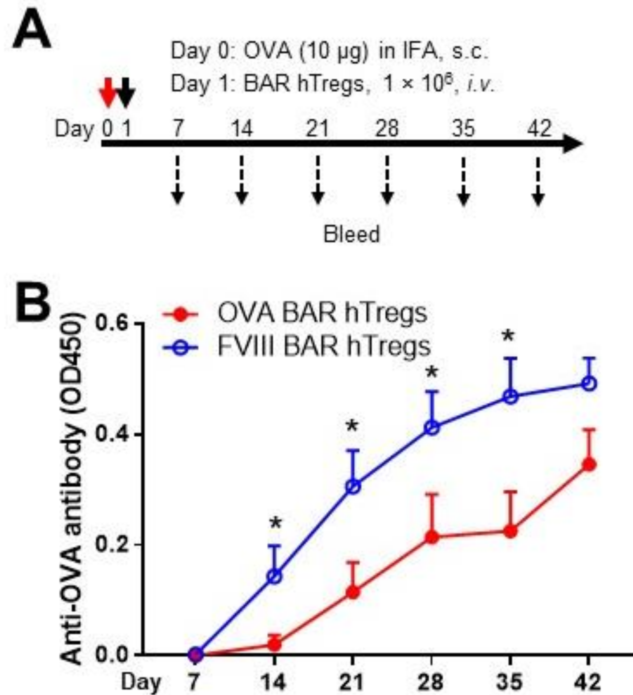


Supplemental Figure 2. OVA-BAR transduced human CD4⁺ conventional T cells proliferated to polyclonal anti-OVA rabbit IgG stimulation. The OVA-BAR transduced and rested human $CD4^+$ T cells were labeled with proliferation dye eFluor 450. Then the cells were cultured for 3 days in the presence or absence of 1 μg/ml of polyclonal anti-OVA rabbit IgG or anti-C2 mAb (clone 2C11). The proliferative response was analyzed by FACS and cells shown were gated on live and GFP^+ singlets.



Supplemental Figure 3. Cytokines expression in BAR hTregs after long-term *in vitro* expansion. (**A**) The staining of CD8 and CD4 expression in the expanded BAR Tregs. More than 99% of the cells remain CD4 positive, excluding the possibility of significant contamination from cytotoxic CD8 T cells. (**B**) Intracellular expression levels of Perforin and Granzyme B in

the expanded BAR Tregs compared to BAR Tcon as well as enriched human CD8 T cells. FACS sorted human Tregs ($CD4^{+}CD25^{hi}CD127^{low}$) and naive Tcon ($CD4^{+}CD25^{-}CD127^{+}CD45RA^{+}$) from the same donor were BAR transduced, sorted for GFP^{+} cells, and expanded for a total of 21 days *in vitro* as described in the method. The cells were cultured with protein transport inhibitor Brefeldin A and Monensin in the presence or absence of PMA / Ionomycin for 5 hrs at 37°C, followed by surface staining for CD4 and viability, and intracellular staining for Perforin and Granzyme B. The dot plots shown were gated on live $CD4^{+}$ singlets. (**C**) Staining of the degranulation marker, CD107a, in the expanded BAR hTregs, compared to BAR Tcon as well as enriched human CD8 T cells. The BAR hTregs, BAR hTcon, or human CD8 T cells were cultured with or without stimulation for 5 hrs as described above. In addition, fluorescent anti-CD107a antibody was added at the beginning of the culture. The overlapping histograms shown were gated on live singlet cells. Grey filled shows unstimulated cells, and the dotted black line shows the cells with PMA/Ionomycin stimulation. (**D**) IFN-γ and IL-2 expression in long-term expanded BAR hTregs. The BAR hTregs and BAR Tcon were prepared as described above. At the end of the *in vitro* expansion (day 22), the cells were re-stimulated with 1μg/ml soluble anti-CD3ε (clone 64.1) for 48 hrs, followed by FACS surface staining for CD4 and viability, and intracellular staining for IFN- γ and IL-2. The data shown were gated on live $CD4^{+}$ singlets.



Supplemental Figure 4. **The xenogeneic suppression of OVA-BAR hTregs on anti-OVA antibody development in E16 mice**. (A) Protocol of the *in vivo* experiment. Briefly, age-matched female E16 mice (C57Bl/6 background, n = 5) were divided into two groups. On day 0, all the mice were immunized subcutaneously with 10μg of OVA in incomplete Freund's adjuvant (IFA). On day 1, the mice received 1×10^6 of OVA-BAR hTregs or the control FVIII-BAR hTregs depending on the group. (B) Serum anti-OVA antibody levels over the time course. The antibody levels were measured by an ELISA assay, and the data were expressed as mean ± SE. Compared to FVIII-BAR hTregs, treatment with OVA-BAR hTregs significantly suppressed the anti-OVA antibody development up to 4 weeks following the immunization. $*p < 0.05$ as determined by the student's *t*-test.