Generation of FVIII-Specific Chimeric Antigen Receptor (CAR) Tregs to Suppress Inhibitor Development in Hemophilia A Mice

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Introduction

- Hemophilia A is an X-linked clotting factor disorder in which patients have a deficiency in coagulation factor VIII (FVIII). Current treatment is based on FVIII replacement therapy; however, 20-30% of patients develop inhibitory anti-FVIII antibodies (inhibitors), preventing the FVIII treatment from working.
- Regulatory T cells (Tregs) are immunosuppressants expressing the biomarkers CD4, FoxP3, and CD25. FoxP3 is the transcription factor regulating the development and function of Tregs. Consequently, the role of Tregs of the $CD4^+CD25^+FoxP3^+$ phenotype in tolerance to coagulation factors has emerged, as they can suppress both the B and T cells involved in inhibitor formation.
- Antigen specific Tregs were generated by engineering and retrovirally transducing a chimeric antigen receptor (CAR) molecule with specificity to human FVIII. CARs are recombinant receptors that provide both antigen-binding and T-cell activating functions by redirecting immune reactivity toward a chosen antigen. Antigen recognition via the single chain antibody variable regions triggers signaling via the CD28 and CD3ζ co-stimulatory domains, which induces CAR-Treg activation and proliferation, without the need for MHC restriction.
- Antigen recognition and signaling by CAR-Tregs may also result in activation-induced cell death (AICD) of the transduced cells. Thus, the goal of this investigation is to not only test if FVIII specific CAR-Tregs reverse inhibitor formation in a mouse model of hemophilia A, but to also find a balance between antigen specific activation and AICD of the CAR-Treg.

Methods

- A single chain variable fragment (ScFv) from a human immunoglobulin directed against human FVIII was generated and fused to the CD3 and CD28 signaling domains of a 2nd generation CAR, then packaged into a retroviral system, pMys-IRES-GFP (which allows the delivery of genes to mammalian cells). CD3/CD28 bead activated $CD4^+$ T cells were then retrovirally transduced to generate FVIII CAR-Tregs (stimulation of CD3 and CD28 is required for Treg expansion).
- Binding of FVIII-CAR Tregs to Fc-FVIII was tested using an A647 conjugated Fc antibody. To test for activation of the FVIII-CAR Tregs, upregulation of the CD69 activation marker was assessed 24 hours after stimulation with Fc-FVIII.
- To find a balance between antigen specific activation and AICD of the CAR-Treg, we made a series of mutations in the Immunoreceptor Tyrosine-based Activation Motifs of the CD3ζ domain, ITAM-1 and ITAM-3, by site-directed mutagenesis.
- We tested for prevention of inhibitory antibody formation by adoptively transferred CAR Tregs in mice. Inhibitors were generated by once weekly injections of FVIII, and inhibitory antibody titers in treated and untreated mice will be tested by ELISA and the Bethesda assay.

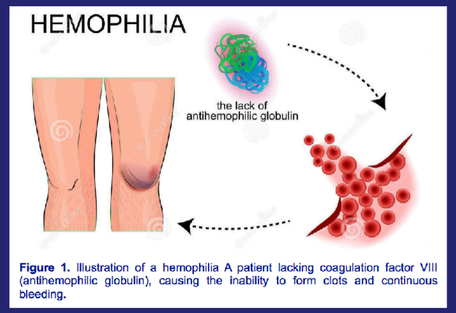

Figure 1. Illustration of a hemophilia A patient lacking coagulation factor VIII (antihemophilic globulin), causing the inability to form clots and continuous bleeding.

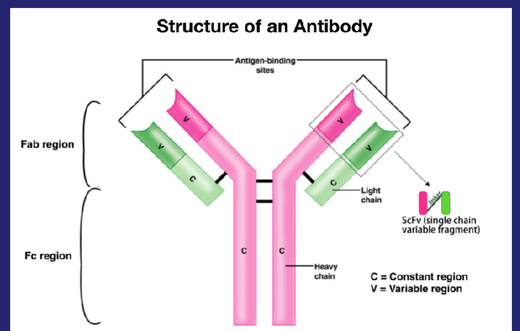

Figure 2. An antibody is a protein used by the immune system to neutralize pathogens. Fab is the antigen-binding fragment region. Fc is the fragment crystallizable tail region that interacts with cell surface (Fc) receptors and complement proteins, allowing activation of the immune system. An ScFv is a fusion protein of the variable regions of the heavy and light chains, connected by a linker. It is used for antigen-binding domains of artificial T cell receptors.

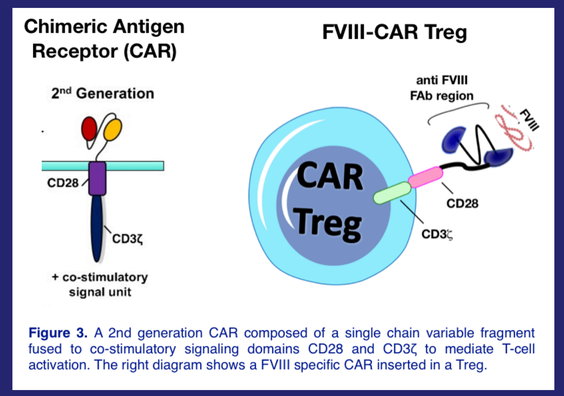

Figure 3. A 2nd generation CAR composed of a single chain variable fragment fused to co-stimulatory signaling domains CD28 and CD3ζ to mediate T-cell activation. The right diagram shows a FVIII specific CAR inserted in a Treg.

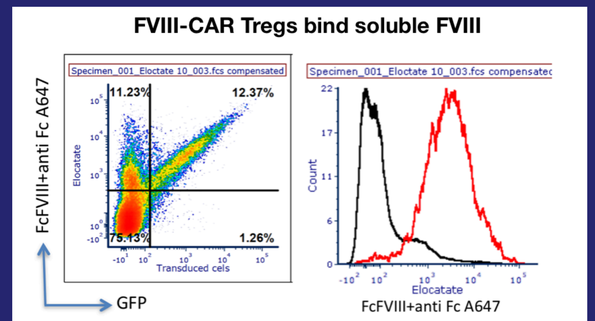

Figure 4. Binding to Fc-FVIII was tested using an A647 conjugated Fc antibody. GFP percentage indicates percentage of transduced cells. Right diagram shows that that FVIII-CAR Tregs bound recombinant Fc-FVIII (eloctate).

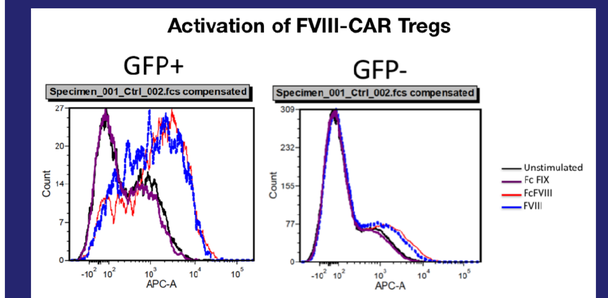

Figure 5. Illustrating that only the GFP+ (transduced) cells bind the CD69 activation marker. The Fc-FIX antigen was not activated, indicating specific binding to Fc-FVIII.

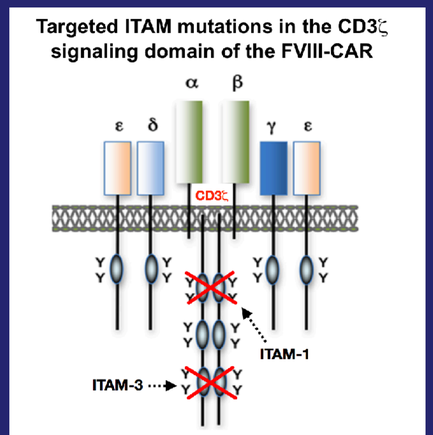

Figure 6. A series of mutations were made for the ITAMs by site-directed mutagenesis. Mutating either ITAM-1 or ITAM-3, but not both, provides optimized signaling by the CAR.

Results

- GFP percentage indicated 12.37% transduced cells. FVIII CAR-Tregs bound Fc-FVIII (Eloctate) in a specific manner, with no binding observed to control Fc-FIX.
- Stimulation for 24 hours with Fc-FVIII resulted in upregulation of the CD69 activation marker for the GFP+ transduced cells only, indicating activation of FVIII-CAR Tregs. No non-specific activation with the unrelated Fc-FIX antigen was observed.
- The native CAR, with all ITAMs of the CD3ζ chain domain left intact, lead to over-activation and some induced cell death. A crippled CAR, with both ITAM-1 and ITAM-3 mutated, did not provide sufficient activation signals. A CAR with either ITAM-1 or ITAM-3 mutated provided an optimized signaling that did not cause cell death.
  - Percent upregulation of the CD69 activation marker used to asses activation of the cells. Percent GFP loss used to assess percent cell death.
- We are currently testing for the suppression of inhibitor development in hemophilia A mice following adoptive immunotherapy with FVIII CAR-Tregs and continued weekly administrations of FVIII.

Conclusion

- We demonstrate that CAR-Tregs directed to human FVIII show antigen-specific binding, activation and proliferation. The suppressive activity of FVIII CAR-Treg on inhibitory antibody formation is now being evaluated in hemophilia A mice that are given factor replacement therapy with FVIII.
- Our results from testing mutations in the signaling domain of the FVIII-CAR should provide us with a superior cell-therapy protocol for immune tolerance to hemophilia.
- At the end of the study, we hope to establish that FVIII specific suppression by redirection of Tregs would reduce inhibitor titers to manageable levels. If successful, the approach for expression in human cells can be developed, with the goal of translation to the clinic.

Acknowledgements

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