CD19-specific Chimeric Antigen Receptor (CAR) regulatory T cells demonstrate suppression of B cell mechanisms of action *in vitro*

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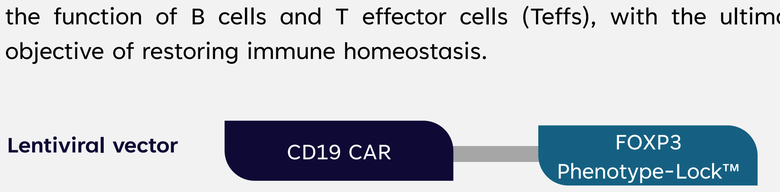
1. CD19 CAR Tregs for the modulation of B cells in autoimmune diseases

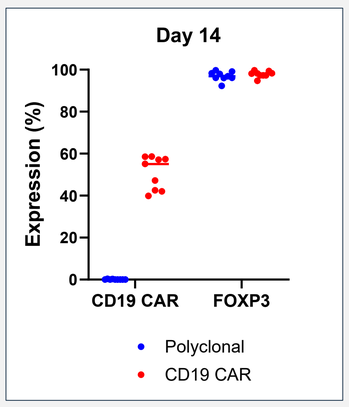
B cells play a central role in the aetiology of numerous autoimmune diseases, including Rheumatoid arthritis (RA) and Systemic sclerosis (SSc), alongside other immune cell compartments such as autoreactive T cells. B cells employ a variety of mechanisms which promote the autoimmune response, like the production of autoantibodies and antigen presentation to T cells. The suppression of these mechanisms would be beneficial in the treatment of autoimmune diseases such as RA and SSc. Regulatory T cells (Tregs) are potent modulators of the immune response with an important role in restraining autoimmunity. The mechanisms through which Tregs mediate this effect are broad and includes both contact dependent and contact independent modalities.

In diseases in which multiple immune cells contribute to pathogenesis, depletion of B cells by CAR T cells may be insufficient for complete efficacy. Therefore, a product that could modulate B cell functions that contribute to pathogenesis yet also regulate surrounding immune cell types, would be a promising strategy to resolve complex immune responses in diseases such as RA or SSc.

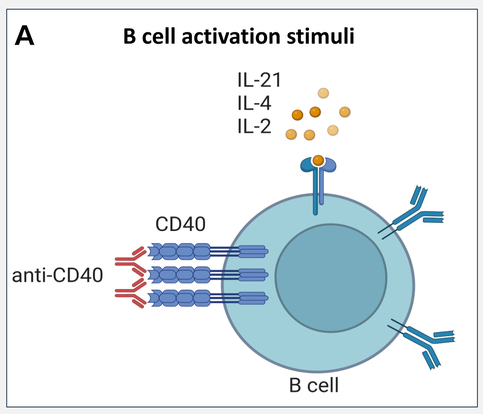
Aims

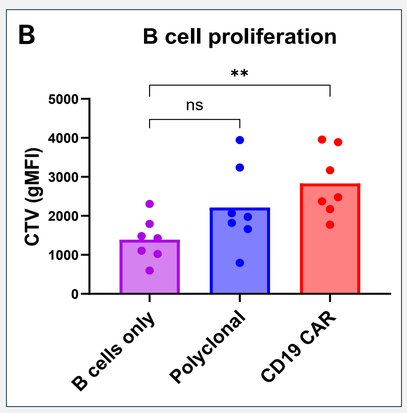
- Generate CD19-specific CAR Tregs (CD19 CAR Tregs) that also constitutively express FOXP3.
- Validate the specificity and potency of CD19 CAR Tregs in modulating the function of B cells and T effector cells (Teffs), with the ultimate objective of restoring immune homeostasis.

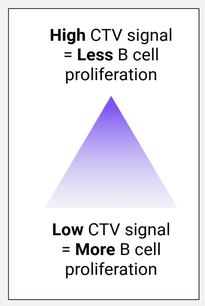




2. CD19 CAR Tregs significantly suppress the proliferation of activated B cells

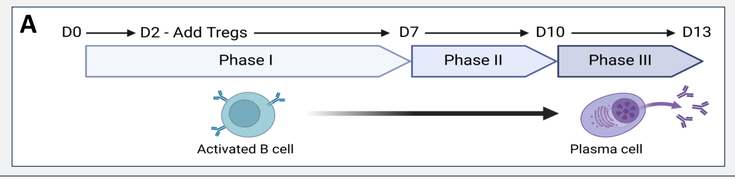


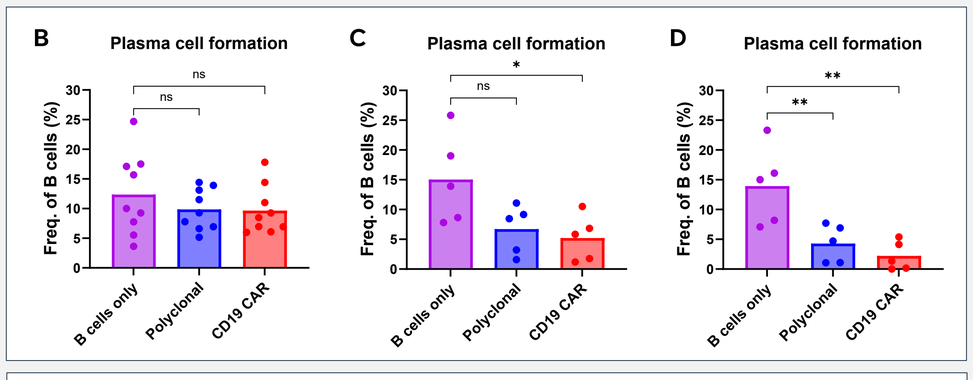


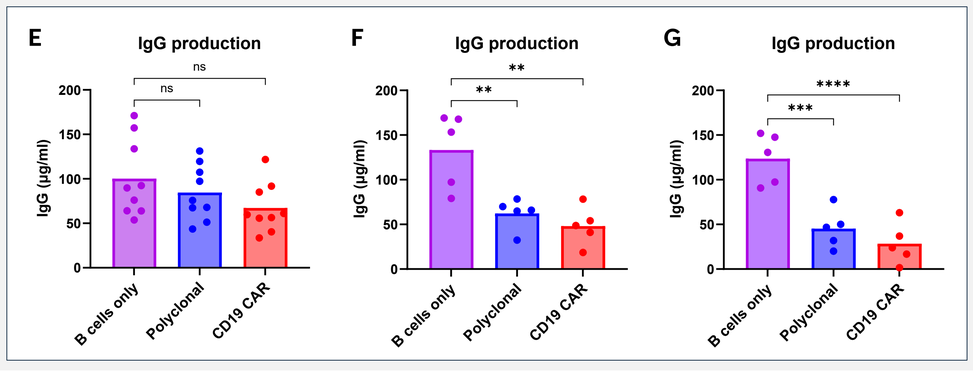


A) Schematic of stimuli used (anti-CD40, IL-21, IL-4 and IL-2) to induce B cell activation and proliferation. **B)** B cells were labelled with CellTrace™ Violet (CTV) proliferation dye and cultured in media containing B cell activating stimuli either alone, in the presence of donor-matched polyclonal Tregs or donor-matched CD19 CAR Tregs at a Treg:B cell ratio of 4:1. On day 6 post co-culture set up, B cells were analysed by flow cytometry to observe the geometric mean fluorescence intensity (gMFI) of CTV. Statistics: one-way ANOVA with Dunnett's multiple comparisons tests. n = 7.

3. CD19 CAR Tregs impair plasma cell generation and reduce IgG production

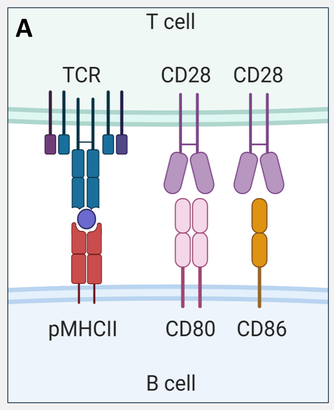


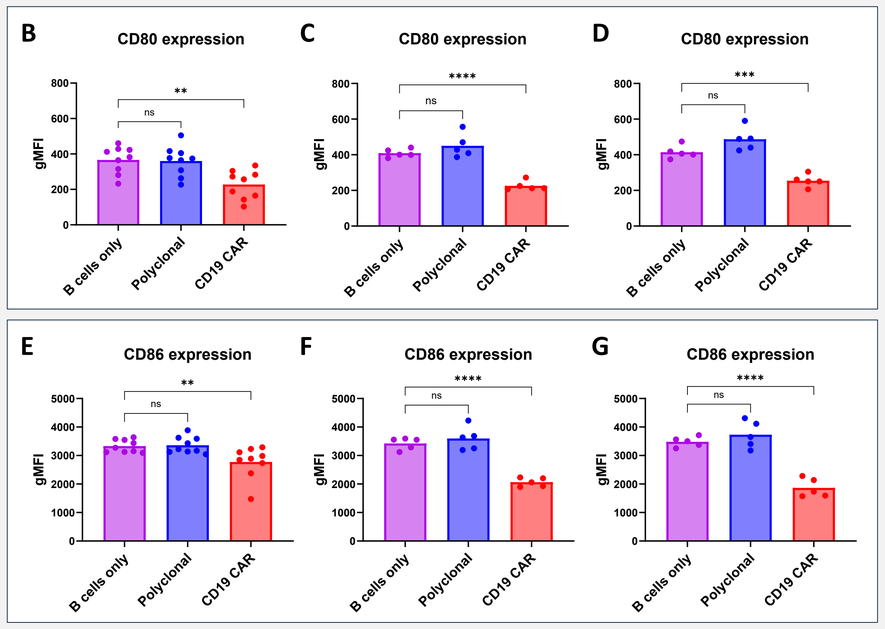




A) Schematic of the three-phase "Cheng protocol" (Cheng *et al.* (2022) doi: 10.1038/s41467-022-33787-8) used to activate and differentiate B cells into antibody producing plasma cells. **B-G)** B cells were cultured in media containing B cell activating stimuli either alone, in the presence of donor-matched polyclonal Tregs or donor-matched CD19 CAR Tregs at Treg:B cell ratios of 1:1 (**B** and **E**), 4:1 (**C** and **F**) and 8:1 (**D** and **G**). On day 13 post initiation of the Cheng protocol, the B cells were analysed by flow cytometry to distinguish the proportion of plasma cells ($CD38^+CD138^+$) (**B-D**). Supernatant from day 13 was analysed by ELISA of the production of total IgG (**E-G**). Statistics: one-way ANOVA with Dunnett's multiple comparisons tests. n = 5-9.

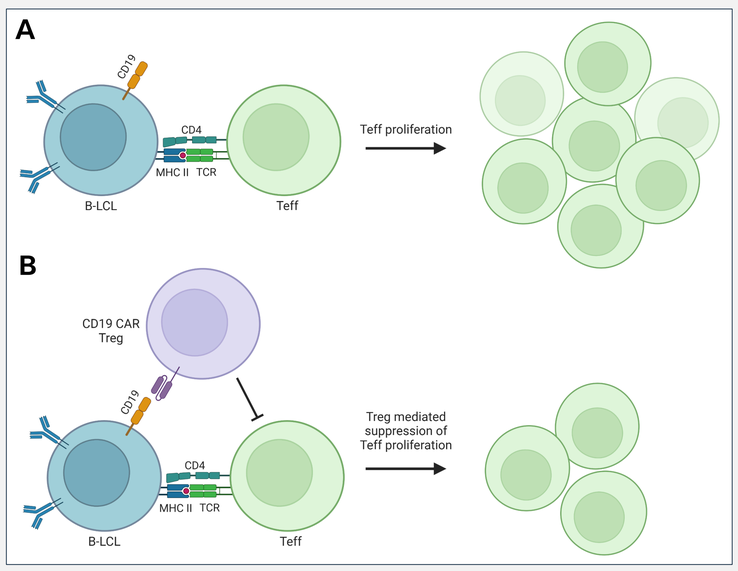
4. CD19 CAR Tregs reduce expression of CD80 and CD86 co-stimulatory molecules on the surface of B cells

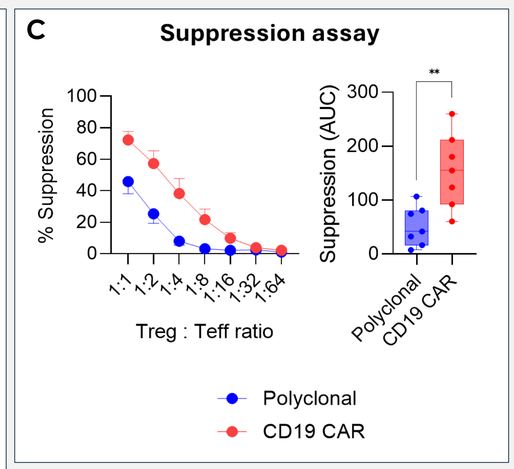




A) Schematic of the antigen presenting cell (APC) function of B cells, in which CD80/CD86 molecules on the B cells provide co-stimulation to T cells through binding with CD28. **B-G)** B cells were activated using the previously mentioned three-phase Cheng protocol (see section 3). B cells were cultured in media containing B cell activating stimuli either alone, in the presence of donor-matched polyclonal Tregs or donor-matched CD19 CAR Tregs at Treg:B cell ratios of 1:1 (**B** and **E**), 4:1 (**C** and **F**) and 8:1 (**D** and **G**). On day 13 post initiation of the Cheng protocol, the B cells were analysed by flow cytometry for the expression of CD80 (**B** to **D**) and CD86 (**E** to **G**) based on gMFI. Statistics: one-way ANOVA with Dunnett's multiple comparisons tests. n = 5-9.

5. CD19 CAR Tregs exhibit bystander suppression of Teff cells





A) Schematic of B-Lymphoblastoid Cell Line cells (B-LCLs) inducing proliferation of Teffs. **B)** Schematic of suppression assay in which CD19 CAR Tregs activated through CD19 on B-LCLs inhibit Teff proliferation via bystander suppression. **C)** Suppression assay to investigate the capacity of polyclonal Tregs and CD19 CAR Tregs to suppress Teff proliferation after stimulation with irradiated B-LCLs. Left panel shows suppression curves across serial Treg:Teff ratios (1:1–1:64); right panel shows corresponding area under the curve (AUC) values summarising overall suppressive capacity. Statistics: unpaired t-test. n = 7.

6. Conclusions

- We have shown that upon engagement of target antigen CD19 CAR Tregs can specifically modulate B cell mechanisms of action *in vitro* including:
  - B cell proliferation
  - Differentiation of primary B cells into plasma cells
  - Antibody production
  - Potential to activate T cells via antigen presentation
- We have shown evidence that upon engagement of antigen CD19 CAR Tregs mediate bystander suppression of Teffs *in vitro*.
- This work demonstrates that CD19 CAR Tregs can both dampen pathogenic properties of B cells and suppress the proliferation of activated Teffs. In the context of autoimmunity, modulation of both these cell types and others in the surrounding milieu, such as macrophages and stroma, could more broadly address the complex disease pathogenesis vs depletion of the B cell pool alone.