

TNF Inhibition with Small-molecule Agents Results in Amelioration of Inflammation Similar to that of Anti-TNF Biologics While Preserving TNFR2 Signaling and Maintaining a Suppressive Regulatory T cell Phenotype

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Background

- TNF plays a critical role in inflammatory diseases such as rheumatoid arthritis, psoriasis, ulcerative colitis and Crohn's disease.
- TNF blocking biologics attenuate disease but downregulate both pro-inflammatory TNFR1 and anti-inflammatory TNFR2-mediated signaling, and cause *de novo* inflammation occurrences, termed paradoxical adverse events.
- A TNF small molecule inhibitor that blocks pro-inflammatory TNFR1 signaling but spares beneficial TNFR2 signaling may circumvent the shortcomings of biologic drugs.
- Here we present data using TNF small molecule inhibitors on TNF-mediated inflammatory signaling, inhibition of TNFR1 vs TNFR2 signaling, Treg cell activity and control of murine arthritis.

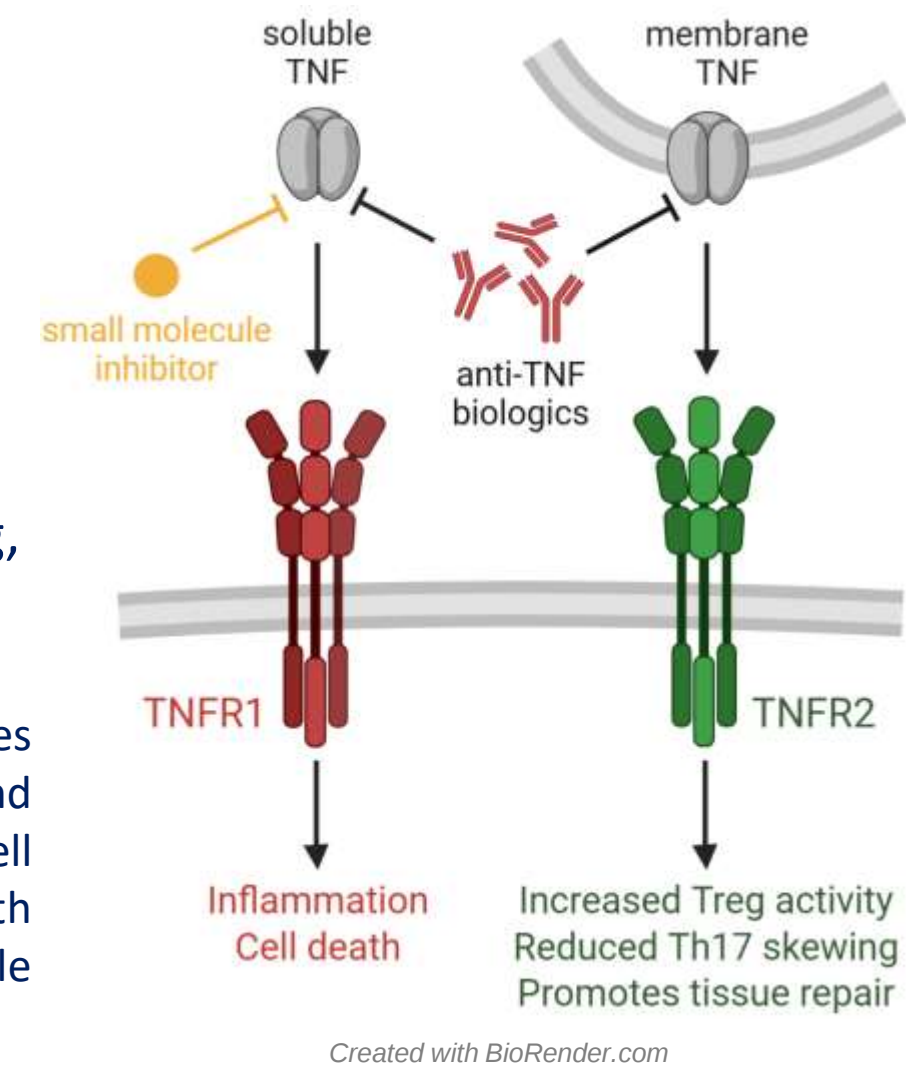


Figure 1. Soluble TNF (sTNF) activates TNFR1 which mediates inflammation and cell death. In contrast, membrane-bound TNF (mTNF) activates TNFR2 to promote Treg activity, T cell homeostasis and tissue repair. Anti-TNF biologics block both soluble and membrane TNF signaling whereas small molecule inhibitors block only soluble TNF.

Materials and Methods

- HEK293 expressing a NF- κ B-SEAP reporter and K562 cells transfected with an NF- κ B luciferase reporter plasmid DNA were used; membrane TNF expressing HeLa cells (mTNF HeLa) were used and TNFR1 KO K562 cells were generated using CRISPR. Reporter cells were cultured with mTNF-expressing HeLa for 4-72 hours.
- Tregs were isolated using the CD4⁺ CD127^{low} CD25⁺ Treg isolation kit from Stem Cell Technologies and cultured with mTNF-expressing cells for 36 hours. Cells were collected for flow cytometry and cytokines were measured in cell supernatants by cytokine bead array.
- Anti-collagen antibodies for the murine arthritis model were purchased from Chondrex. Cytokines in plasma or paw homogenate were quantified using Meso Scale Discovery assays.

Results

TNF Small Molecule Inhibitor Displays a Concentration-Dependent Reduction in Soluble TNF-Stimulated NF- κ B Activation

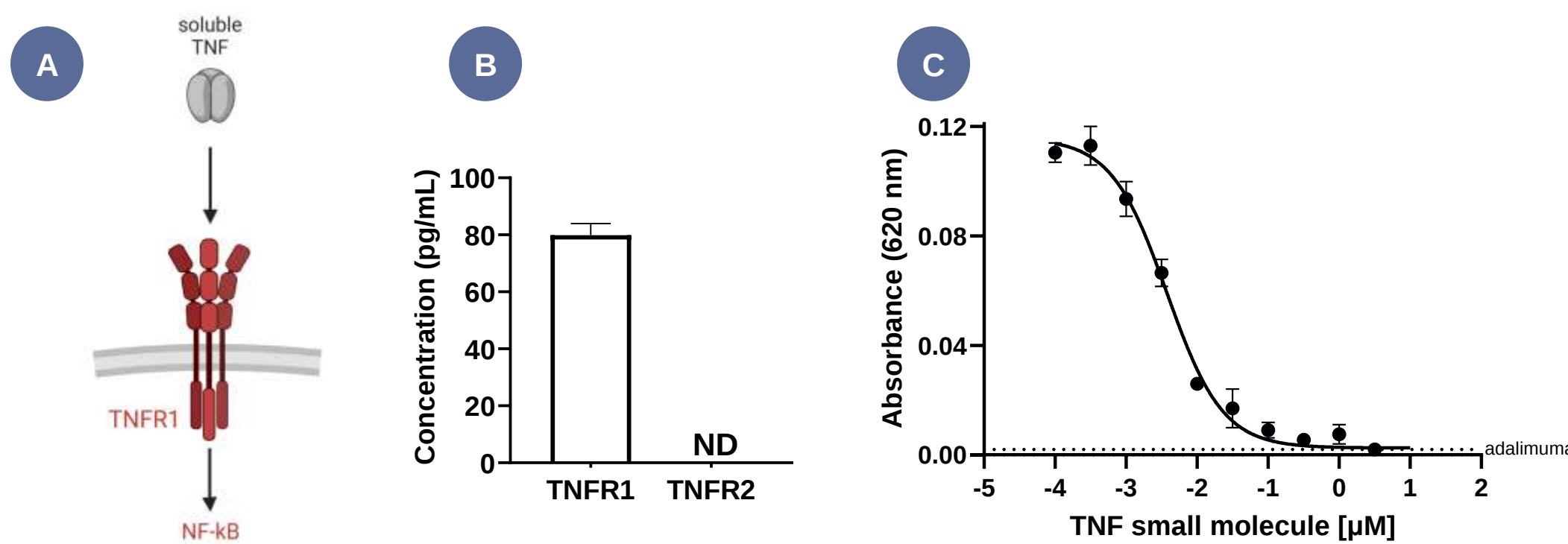


Figure 2. (A) Model of TNFR1 activation by soluble TNF. (B) HEK293 cells exclusively express TNFR1 and TNFR2 protein was not detected (ND). (C) TNF small molecule inhibitor suppressed soluble TNF-stimulated NF- κ B activation in a concentration-dependent manner in reporter HEK293 cells.

TNF Small-Molecule Inhibition Suppresses Soluble but not Membrane TNF-Stimulated NF- κ B Activation

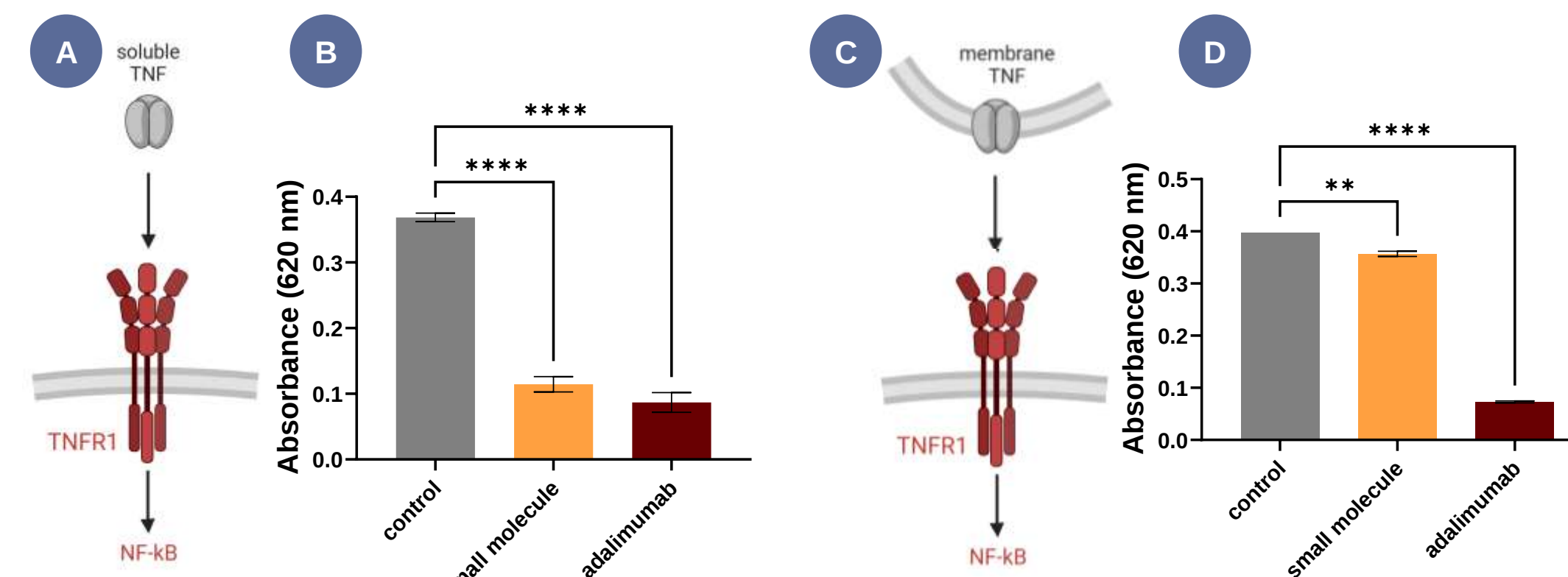


Figure 3. (A) Model of TNFR1 activation by soluble TNF. (B) Both TNF small molecule inhibitor and anti-TNF biologic Adalimumab inhibit soluble TNF activation of NF- κ B in HEK293 cells. (C) Model of TNFR1 activation by membrane TNF. (D) Adalimumab but not TNF small molecule inhibitor provides near complete suppression of membrane TNF signaling using mTNF HeLa co-culture with HEK293 cells.

TNF Small-Molecule Inhibition Suppresses Soluble TNF-Stimulated IL-8 Secretion from Primary Human Endothelial Cells

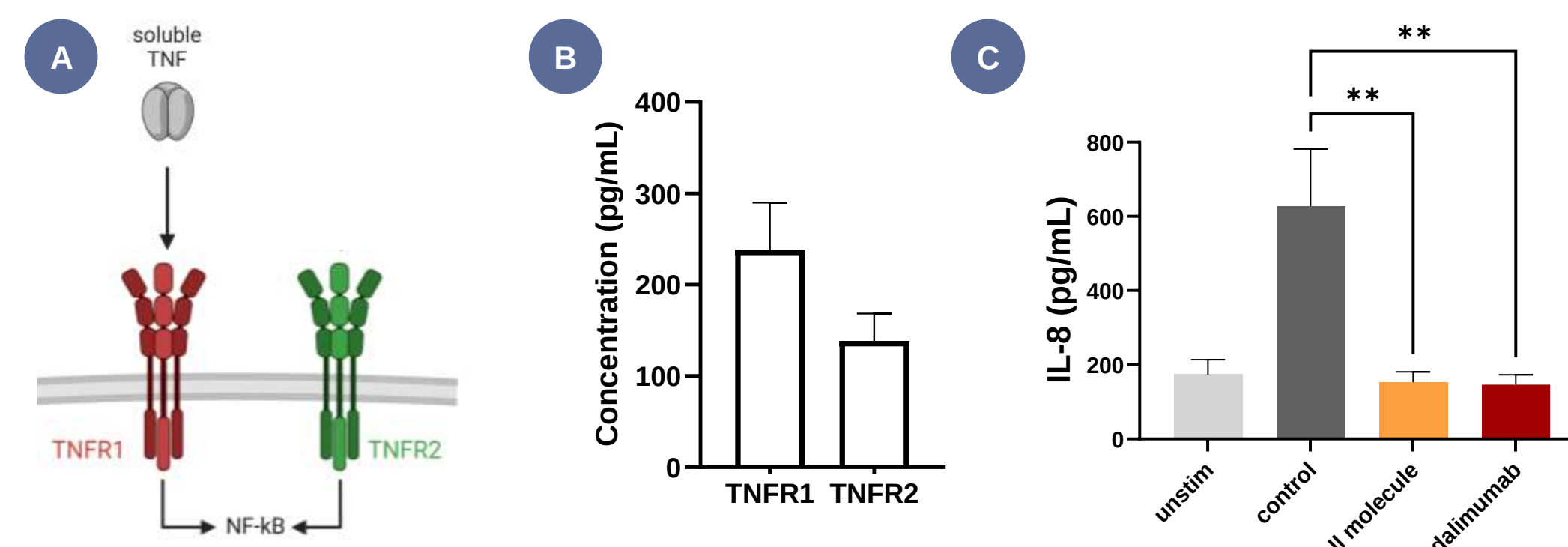


Figure 4. (A) Model of TNFR1 activation by soluble TNF in TNFR1/R2 dual expressing cells. (B) Primary HUVEC cells express both TNFR1 and TNFR2 protein. (C) Soluble TNF-stimulated IL-8 secretion is inhibited by TNF small molecule inhibitor and Adalimumab.

TNF Small Molecule Inhibitor Does Not Block TNFR2-Dependent NF- κ B Activation Stimulated by Membrane TNF

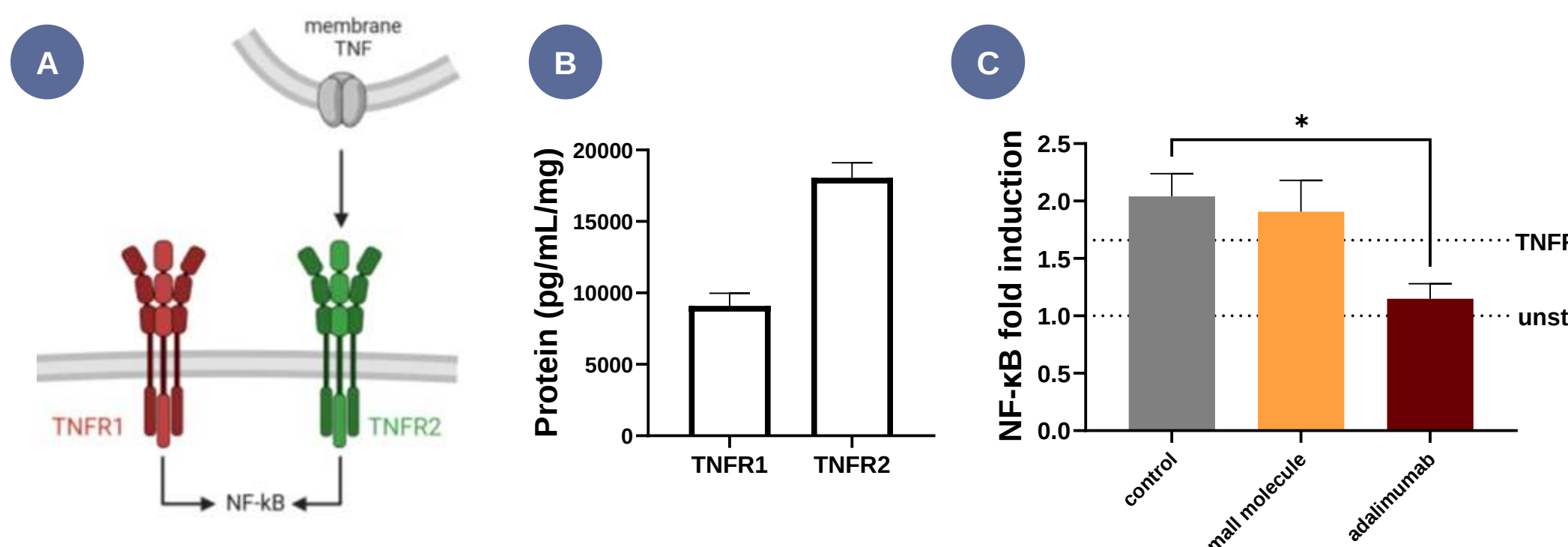


Figure 5. (A) Model of TNFR2 activation by membrane TNF. (B) K562 cells express both TNFR1 and TNFR2 protein. (C) Adalimumab but not TNF small molecule inhibitor suppressed membrane TNF-stimulated NF- κ B activation under conditions where TNFR1 was blocked using anti-TNFR1 antibody.

Human Regulatory T Cells Display High Expression of TNFR2 Compared to Other T Cell Subtypes

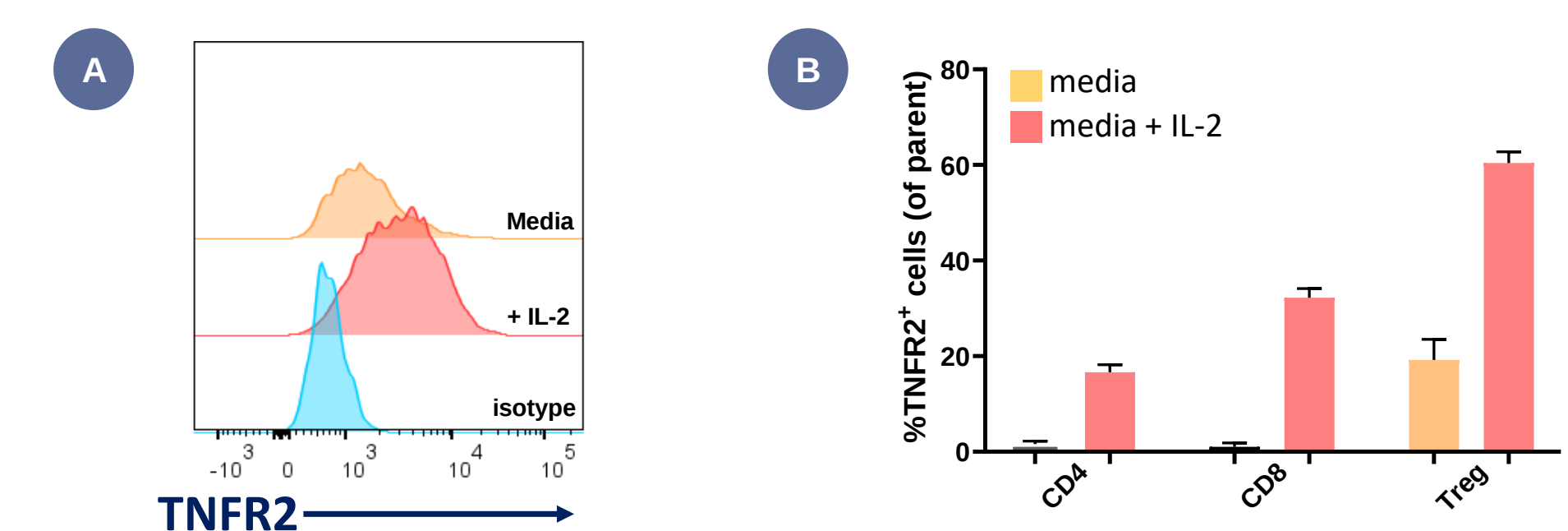


Figure 6. (A) Total T cells were isolated from PBMC and cultured for 48 hours with or without IL-2 prior to surface TNFR2 analysis by flow cytometry. (B) A greater percentage of T regulatory cells express surface TNFR2 compared to CD4⁺ and CD8⁺ T cells.

TNF Small-Molecule Inhibition Does Not Block the Immunosuppressive Phenotype of Tregs

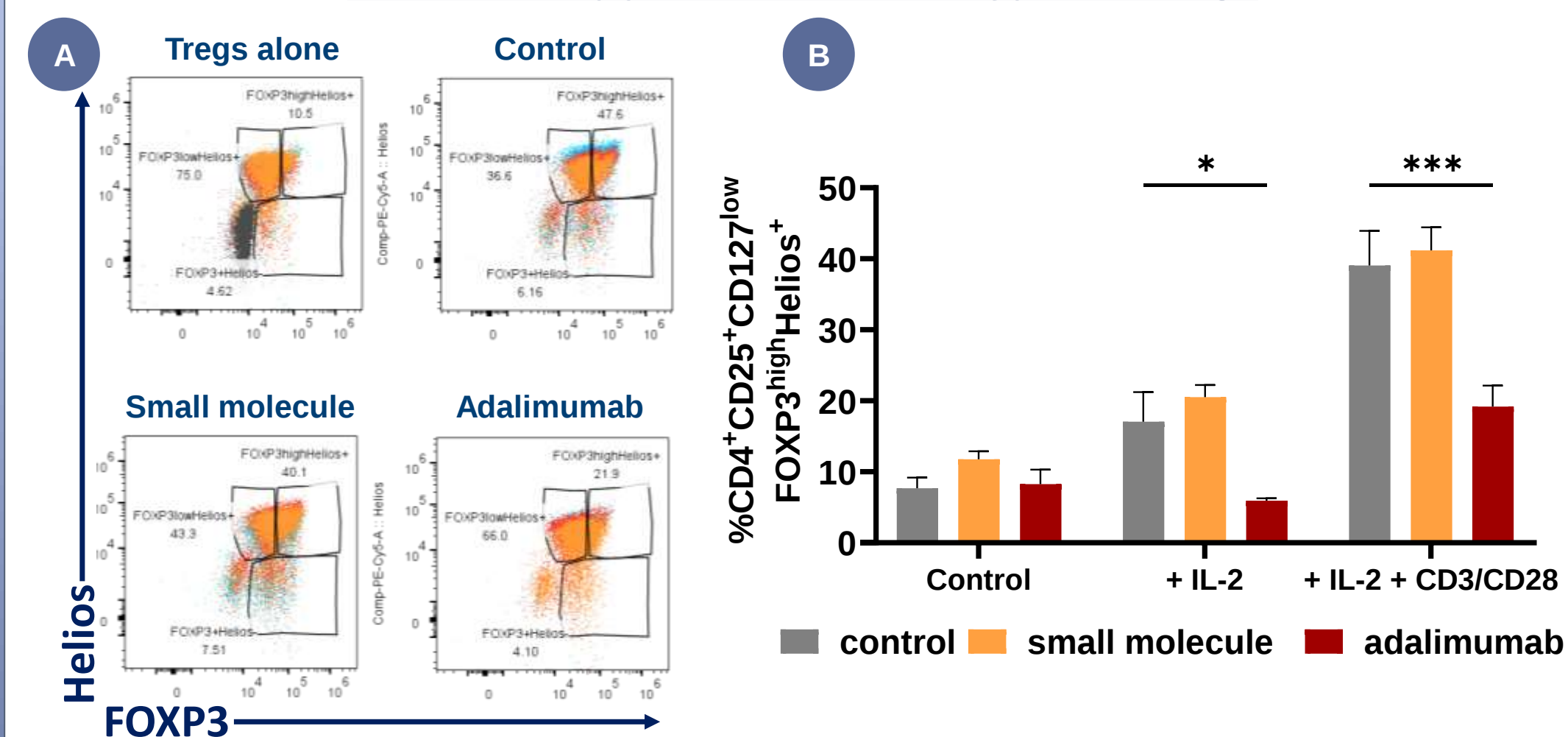


Figure 7. (A) Purified human Tregs were stimulated with IL-2 or with IL-2 and anti-CD3/CD28 and co-cultured with mTNF HeLa cells. (A) FoxP3⁺Helios⁺ Treg populations were assessed by flow cytometry under basal or activated conditions. (B) Stable immunosuppressive Tregs were preserved using TNF small molecule inhibitor but impaired by Adalimumab.

TNF Small-Molecule Inhibition Does Not Block mTNF-Driven Immunosuppressive Cytokine Production from Tregs

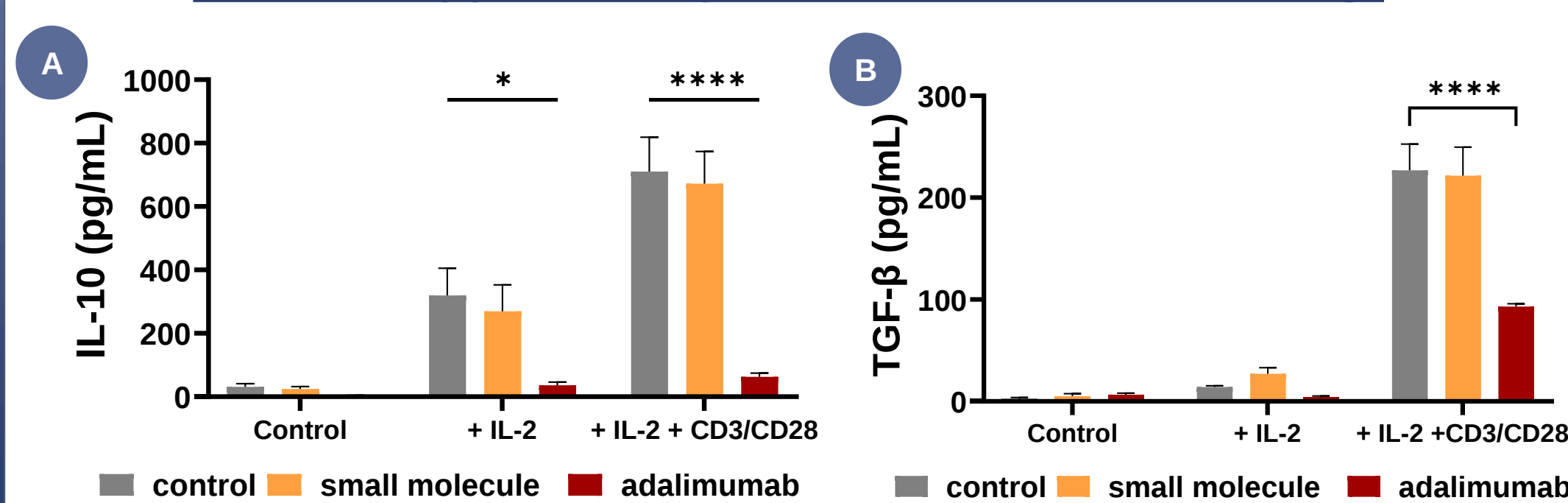


Figure 8. Purified human Tregs were stimulated with IL-2 or with IL-2 and anti-CD3/CD28 and co-cultured with mTNF HeLa cells. Secretion of (A) IL-10 and (B) TGF- β were impaired by Adalimumab but not TNF small molecule inhibitor.

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TNF Small-Molecule Inhibition Provides Equivalent Disease Control as an Anti-TNF Biologic in Murine Arthritis Model

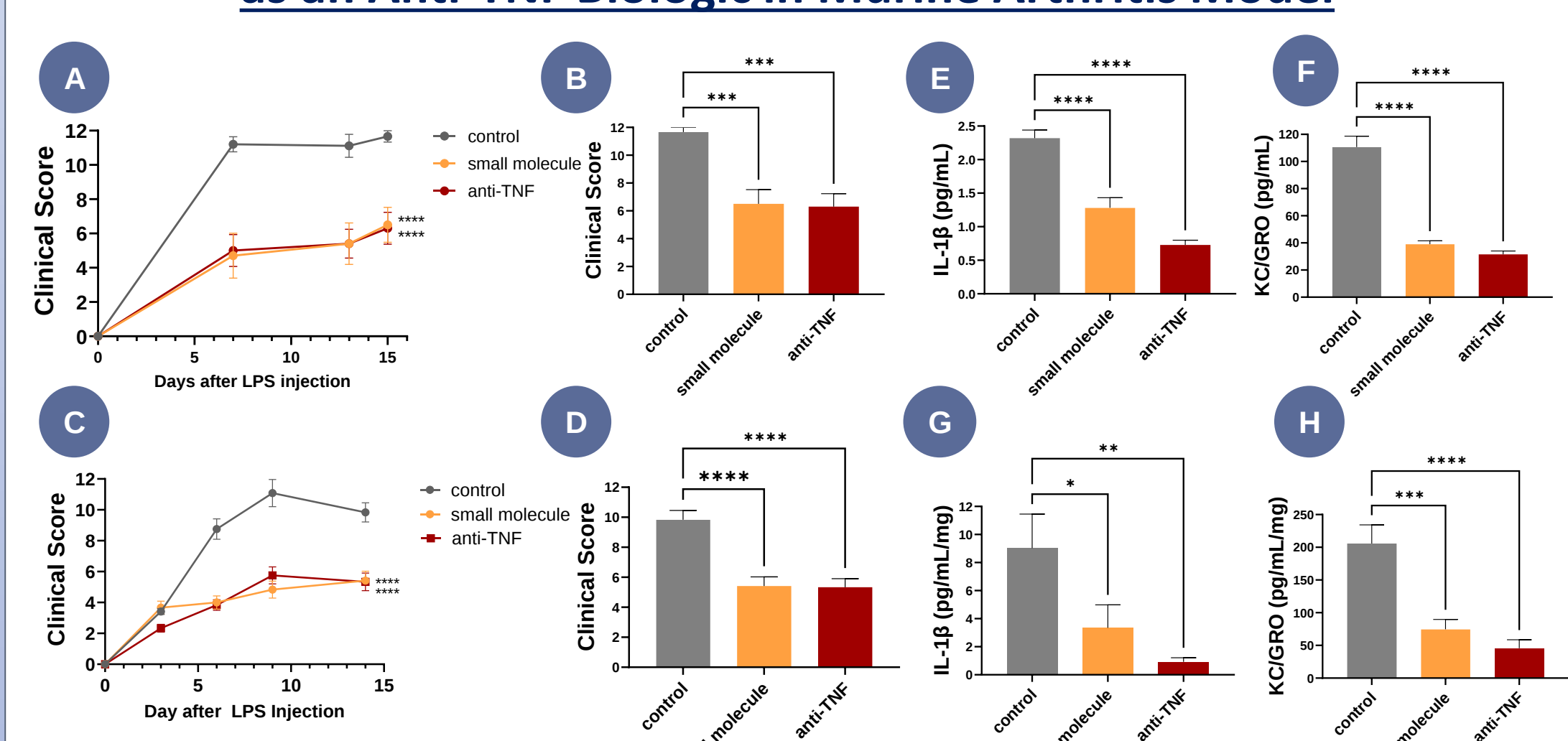


Figure 9. The Collagen antibody-induced arthritis (CAIA) model in (A-B) BALB/c and (C-D) DBA/1J female mice was used. The clinical score was assessed over the study duration (A, C) and at study end (B, D) which was equally suppressed by the TNF small molecule inhibitor and anti-TNF biologic. In BALB/c mice, IL-1 β and KC/GRO were suppressed by TNF small molecule inhibitor and anti-TNF biologic in serum (E-F) and paw tissue (G-H).

TNF Small-Molecule Inhibition May Prevent Pathogenic CD4⁺ T Cell Skewing to Inflammatory Th1 and Th17 Phenotypes

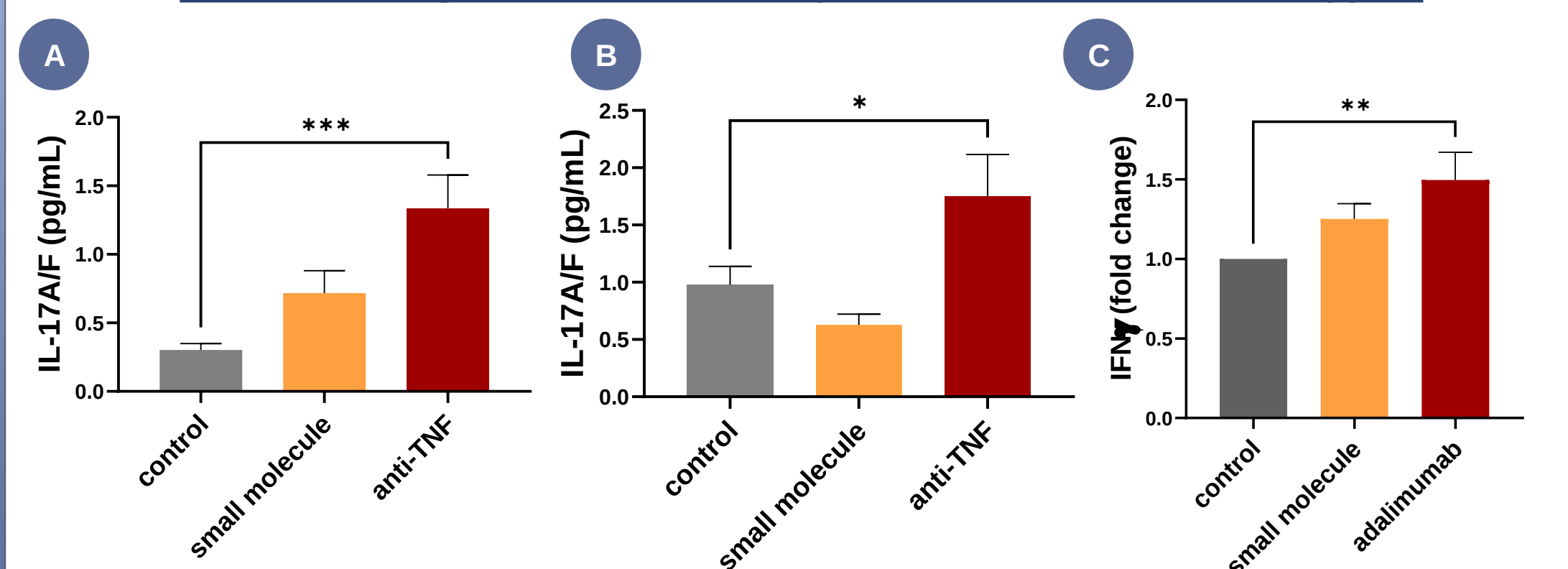


Figure 10. In the CAIA mouse model, serum IL-17A/F production is not significantly changed by TNF small molecule inhibitor treatment but increased in anti-TNF biologic treated (A) BALB/c and (B) DBA/1J mice. (C) TNF small molecule inhibitor does not enhance IFN γ production in human CD4⁺ T cells co-cultured with mTNF HeLa cells and activated with anti-CD3/CD28 antibodies, as compared to an anti-TNF biologic.

Conclusions

- TNF small-molecule inhibition suppresses pro-inflammatory soluble TNF/TNFR1 signaling equivalently to anti-TNF biologic
- The TNF small molecule inhibitor retains potentially beneficial mTNF/TNFR2 signaling
- Sparing of TNFR2 promotes human regulatory T cell stability and a suppressive phenotype
- In murine arthritis, TNF small molecule inhibitor inhibits disease activity as well as anti-TNF
- TNF small molecule inhibition may restrain Th1/Th17 skewing that is dysregulated by anti-TNF biologic

Statistics Summary. In the poster, bar heights are the mean, error bars are the SEM, and * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$, ns not statistically significant.