Small-Molecule Inhibitors of TNF Reduce Pro-inflammatory Disease Activity while Preserving TNFR2 Signaling, Which Maintains Regulatory T Cell Activity and Prevents Aberrant T Cell Cytokine Production

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EULAR Congress
June 3-6th, 2026
Poster ID POS0973

Background

- Tumor necrosis factor (TNF) plays a critical role in inflammation and anti-TNF biologics are effective in treating inflammatory diseases such as rheumatoid arthritis, psoriasis, and inflammatory bowel disease.
- TNF is initially expressed as a membrane-bound precursor that is subsequently cleaved by the metalloprotease TACE (ADAM17) to release its soluble form.
- Soluble TNF (sTNF) preferentially activates pro-inflammatory TNFR1 signaling while TNFR2 requires membrane TNF (mTNF) for efficient downstream signaling.
- Herein, we demonstrate that small-molecule TNF inhibitors block sTNF/TNFR1 signaling while preserving beneficial mTNF/TNFR2 signaling that is blocked by conventional biologic antagonists.

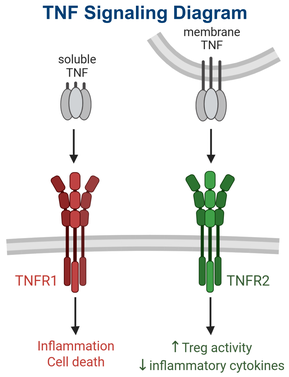

Figure 1. Soluble TNF activates TNFR1 which mediates inflammation and cell death. Membrane-bound TNF activates TNFR2 to promote Treg activity and prevent pathogenic cytokine production.

Methods

- HEK293 cells stably expressing an NF-κB-SEAP reporter or K562 cells transiently transfected with an NF-κB luciferase reporter plasmid were used to evaluate TNF receptor activity. TNFR1^{KO} K562 cells were generated using CRISPR.
- HeLa cells were engineered to express a non-cleavable form of TNF (mTNF) and cultured with reporter cells or isolated human regulatory T cells (Treg) for 4-36 hours.
- Primary human vascular endothelial cells (HUVEC) were cultured in presence of small-molecule inhibitors of TNF or adalimumab in presence of 0.125 ng/ml TNF for 24 hours. Cell supernatants were quantified for IL-8 using Meso Scale Discovery (MSD) assays.
- 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.
- For murine arthritis, anti-collagen antibodies were purchased from Chondrex or chicken collagen II in complete or incomplete Freund's adjuvant was purchased from Hooke laboratories.
- Cell culture experiments were performed with example 6 from Pat. Appl. WO 2018/197503 A1. Murine experiments were performed with an in-house synthesized small-molecule inhibitor.

Results

Small-Molecule TNF Inhibition Fully Blocks Soluble TNF-Driven Activity

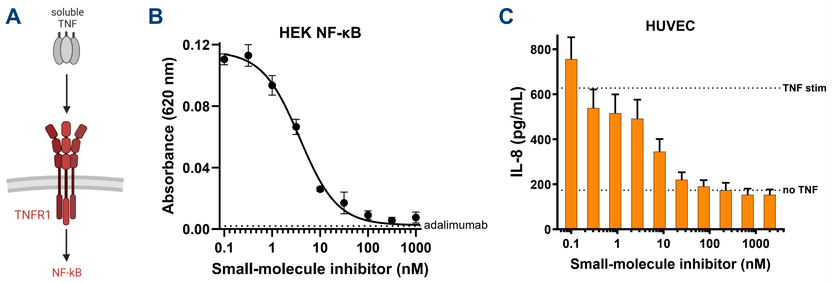

Figure 2. (A) Model of soluble TNF signaling through TNFR1 to activate NF-κB. **(B)** NF-κB reporter HEK293 cells were incubated with 0.125 ng/mL of TNF and increasing concentrations of small-molecule inhibitor, reporter activity was measured by luminescence. **(C)** Secreted IL-8 levels were measured in HUVEC media at 24 hours post incubation with soluble TNF and increasing concentrations of small-molecule inhibitor. N=4 donors

Results

Small-Molecule TNF Inhibition Fully Blocks Soluble TNF Signaling But Does Not Block Membrane TNF Signaling in TNFR1/TNFR2 Dual-Expressing Cells

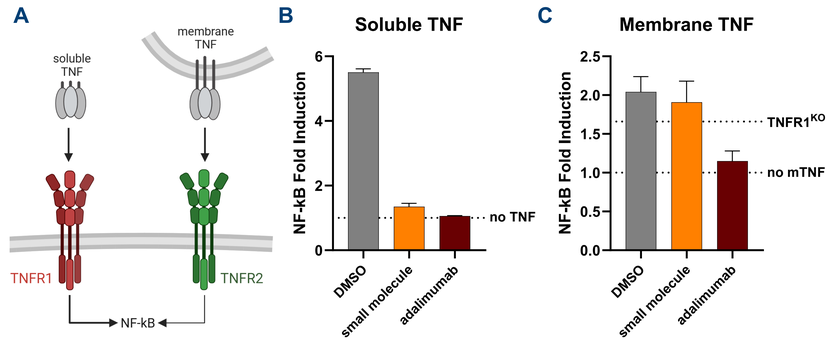

Figure 3. (A) Model of NF-kB activation by soluble or membrane TNF. **(B)** Both a small-molecule TNF inhibitor and adalimumab inhibit soluble TNF activation of NF-κB in HEK293 cells. **(C)** Adalimumab, but not the small-molecule TNF inhibitor, suppressed membrane TNF-stimulated NF-κB activation through TNFR2 in K562 cells. TNFR1 was blocked using an anti-TNFR1 antibody.

IL-2 Signaling Drives Expression of TNFR2 on Human T Helper Cells and is Abundantly Expressed on Highly Suppressive Regulatory T Cells

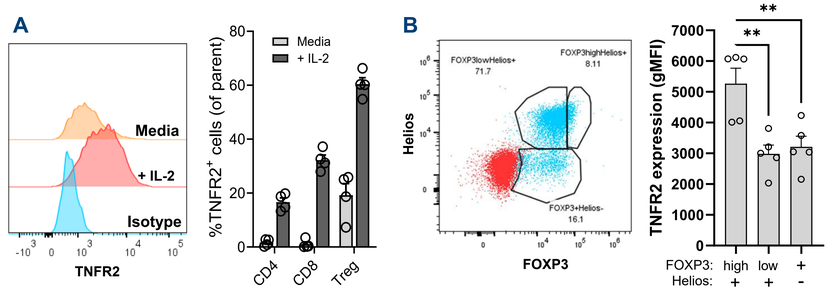

Figure 4. (A) Total T cells were isolated from PBMC and cultured for 48 hours with or without IL-2 prior to surface TNFR2 analysis by flow cytometry. A greater percentage of T regulatory cells express surface TNFR2 compared to CD4$^+$ and CD8$^+$ T cells. **(B)** TNFR2 expression was evaluated on freshly isolated healthy human Tregs. The highly suppressive Foxp3^{High};Helios$^+$ Tregs showed the most abundant TNFR2 expression. N=4 human donors, **p<0.01

Membrane TNF-Signaling through TNFR2 Enhances Treg Activity Which is Blocked by an Anti-TNF Biologic but Not a Small-Molecule Inhibitor

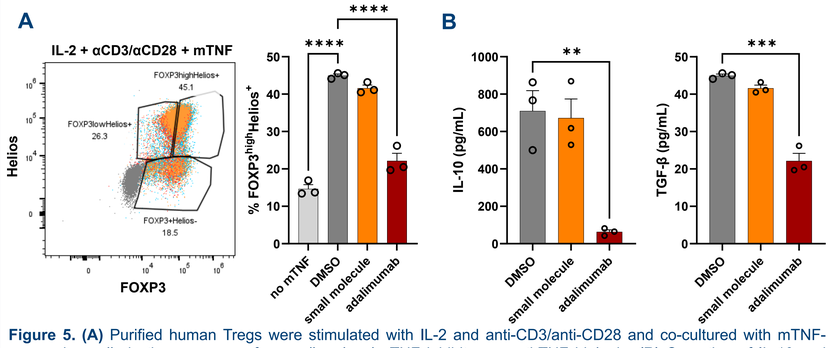

Figure 5. (A) Purified human Tregs were stimulated with IL-2 and anti-CD3/anti-CD28 and co-cultured with mTNF-expressing cells in the presence of a small-molecule TNF inhibitor or anti-TNF biologic. **(B)** Secretion of IL-10 and TGF-β was measured in supernatants by MSD. N=3 healthy human donors, **p<0.01; ***p<0.001; ****p<0.0001

Results

Small-Molecule TNF Inhibition Provides Equivalent Disease Control as a Biologic in Murine Arthritis Models and May Prevent Pathogenic T Cell Skewing

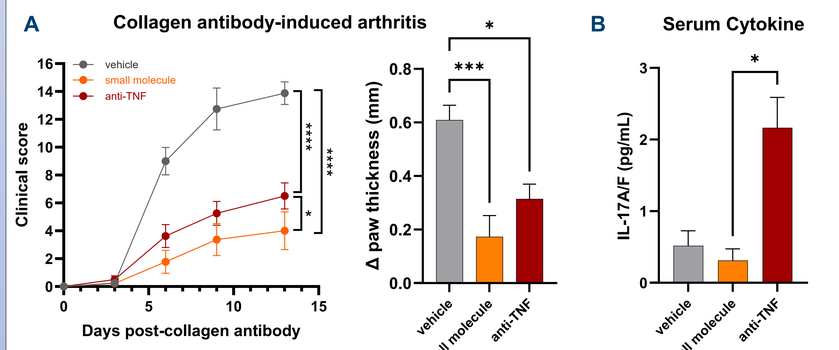

Figure 6. (A) Arthritis clinical score and paw thickness were assessed in the collagen antibody-induced arthritis (CAIA) model demonstrating that small-molecule TNF inhibition provides equivalent disease inhibition compared to an anti-TNF biologic. **(B)** Serum IL-17A/F production is not significantly changed by small-molecule inhibitor but is increased in the anti-TNF biologic group. **(C)** Clinical score and paw thickness were assessed in the collagen II-induced arthritis (CIA) model. **(D)** Paw tissue homogenates demonstrated that an anti-TNF biologic increased IL-17A/F production compared to small-molecule inhibitor and the vehicle control. *p<0.05; ***p<0.001; ****p<0.0001

Conclusions

- Small-molecule inhibitors of TNF show equivalent blockade of pro-inflammatory sTNF/TNFR1 signaling as anti-TNF biologics but do not block membrane TNF signaling through TNFR2.
- Membrane TNF signaling enhances the suppressive capacity of regulatory T cells which is not blocked with a small-molecule TNF inhibitor but is abrogated by an anti-TNF biologic.
- In a murine arthritis model, small-molecule TNF inhibition reduces disease activity as well as an anti-TNF biologic and reduces inflammatory cytokine production, potentially through TNFR2 signaling.

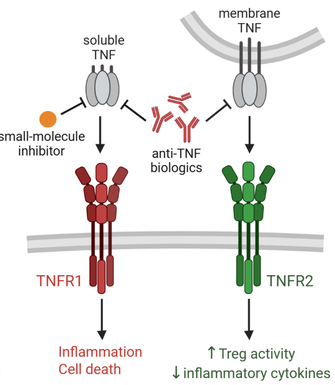

Figure 7. Anti-TNF biologics block both soluble and membrane TNF signaling whereas small-molecule inhibitors block only soluble TNF, leaving TNFR2 signaling available to promote Treg activity and suppress inflammatory cytokine production