

Regulation of CD40 Ligand Transgene Expression in Human CAR-T Cells Using FDA Approved Drugs

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ABSTRACT

Chimeric antigen receptor modified T cells (CAR-T) have shown clinical efficacy in the treatment of B cell malignancies and multiple myeloma. Several challenges restrict their application across hematologic malignancies and solid tumors, including: limited CAR-T cell expansion and persistence, tumor microenvironment-induced immunosuppression, and antigen negative tumor escape. Cluster of Differentiation 40 Ligand (CD40L), a tumor necrosis factor superfamily member transiently expressed on activated CD4 T cells, promotes dendritic cell (DC) licensing and activation through interaction with the CD40 receptor. Co-expression of engineered CD40L in CAR-T cells has the potential to reduce antigen-negative tumor escape even when native CD40L is downregulated, thereby increasing antitumor efficacy. The cytokine program associated with CD40L-mediated DC activation could also improve T cell expansion and activity. However, activation of the CD40 pathway using agonistic antibodies causes systemic immune activation that has been associated with adverse clinical events, thus limiting therapeutic application. We therefore hypothesized that precise and titratable regulation of CD40L would allow for its safe inclusion in CAR-T cell therapy, thus empowering the next generation of potent cellular immunotherapies.

To enable regulation of CD40L, we applied drug responsive domain (DRD) technology which utilizes human protein domains that are inherently unstable in the cell but are reversibly stabilized when bound to FDA-approved small molecule ligands. Fusion of transgenes to a DRD confers ligand-dependent, reversible regulation to any protein of interest. We therefore fused human CD40L to a DRD derived from the *E. coli* dihydrofolate reductase (ecDHFR) which can be regulated by the clinically-approved antibiotic trimethoprim (TMP). We evaluated CD40L expression and in the absence of ligand, the CD40L-DRD fusion was expressed at very low levels in transduced T cells. Exposure to TMP increased CD40L expression in T cells in a dose-dependent manner. To test the activity of CD40L-DRD, we incubated transduced Jurkat T cells with a reporter cell line that reads out CD40 receptor activation. Addition of TMP increased CD40 activation to similar levels seen in cells constitutively expressing CD40L. To evaluate the effect of regulated CD40L expression on DC activation, monocyte derived human DCs were exposed to control or CD40L-DRD expressing T cells. After TMP treatment, the levels of inflammatory cytokines IL12, TNF α , and IFN γ were elevated in co-cultures of DC and CD40L-DRD T cells compared to co-cultures of DCs and control T cells.

To determine the effect of CD40L on CAR-T activity *in vivo*, T cells expressing CD40L and CD19-targeting CAR were infused into CD19+ Nalm6 tumor-bearing mice. Increased tumor regression was seen in mice that received T cells co-expressing CD40L with CD19-targeting CAR compared to CAR alone. Studies are underway to evaluate the effect of regulated CD40L expression on CAR-T cell anti-tumor efficacy *in vivo*.

These findings indicate that CD40L can be regulated using DRDs and FDA approved small molecule ligands, and that regulated CD40L transgene expression by human T cells promotes DC activation and increases CAR-T antitumor activity. Regulated CD40L can be applied to CAR-T therapy to enhance immunotherapy potency by increasing T cell expansion, promoting DC activation, and inducing further epitope spreading.

Figure 1: CD40 Ligand can help to overcome challenges of adoptive cell therapies

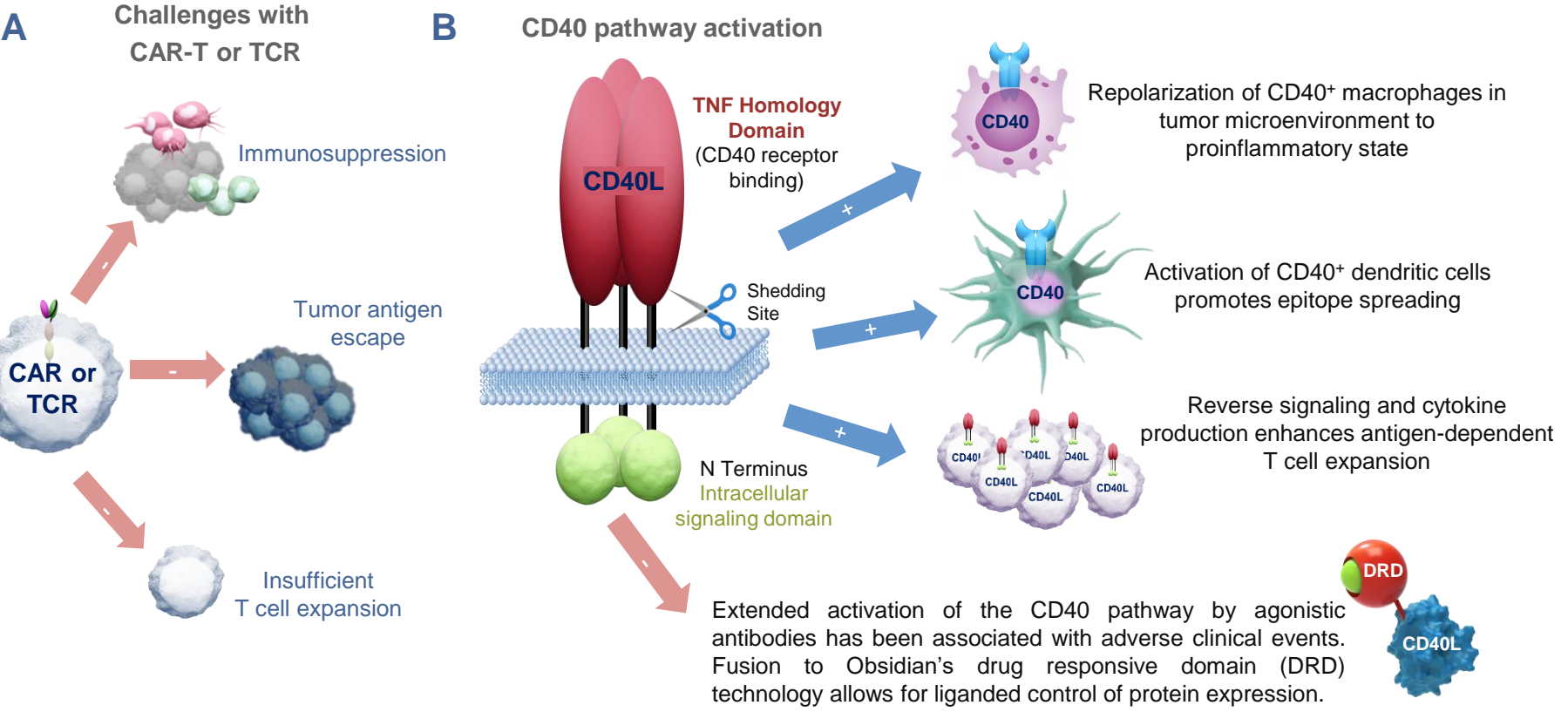


Figure 2: Engineered CD40L expression on T cells activates dendritic cells *in vivo*

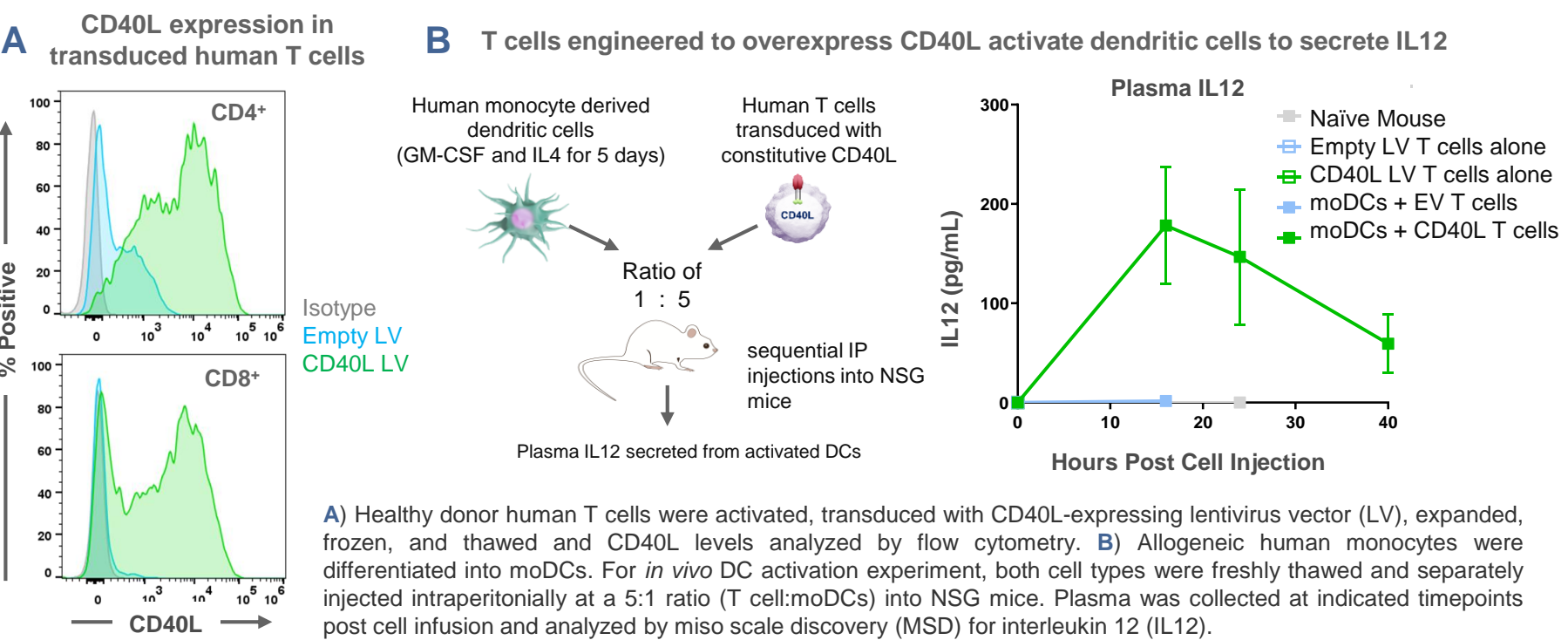


Figure 3: CD40L expression enhances CD19-targeted CAR-T cell anti-tumor efficacy *in vivo*

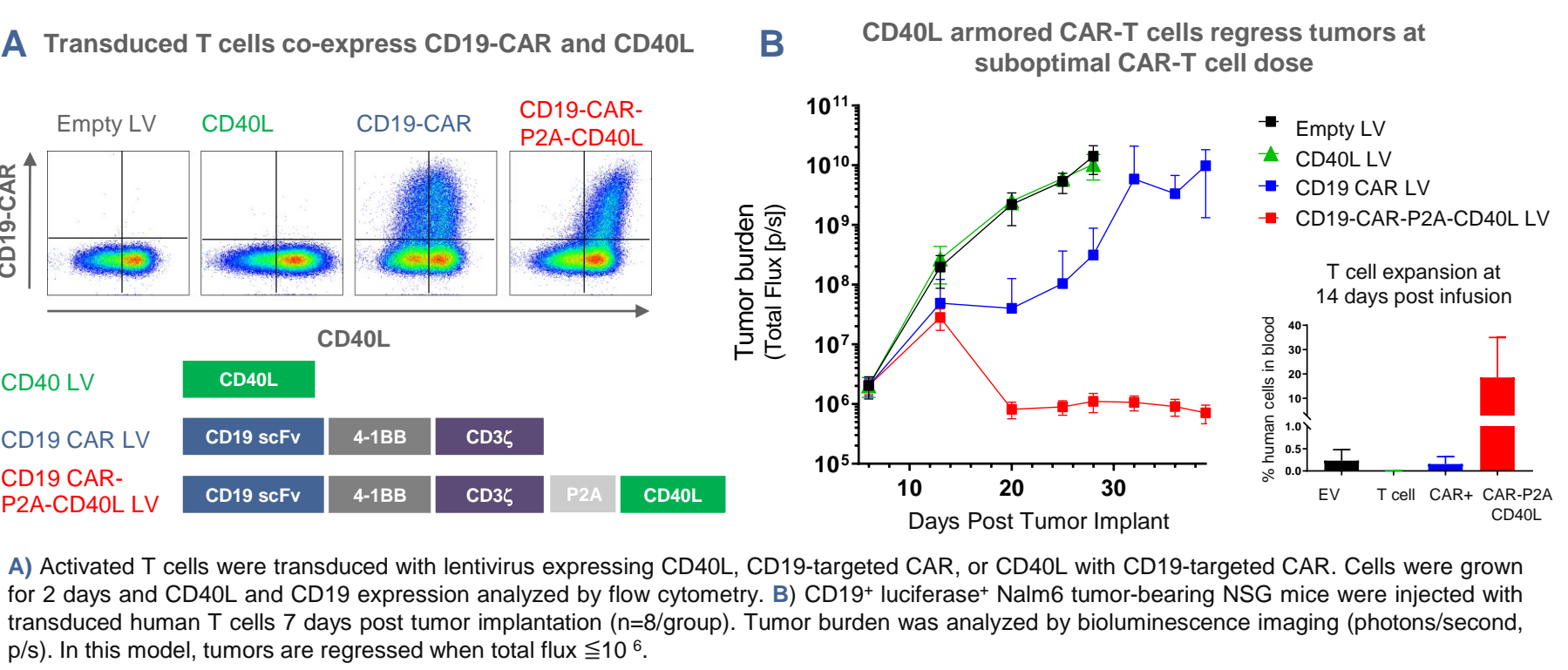


Figure 4: Addition of a drug responsive domain (DRD) allows for regulation of protein expression using FDA approved small molecules

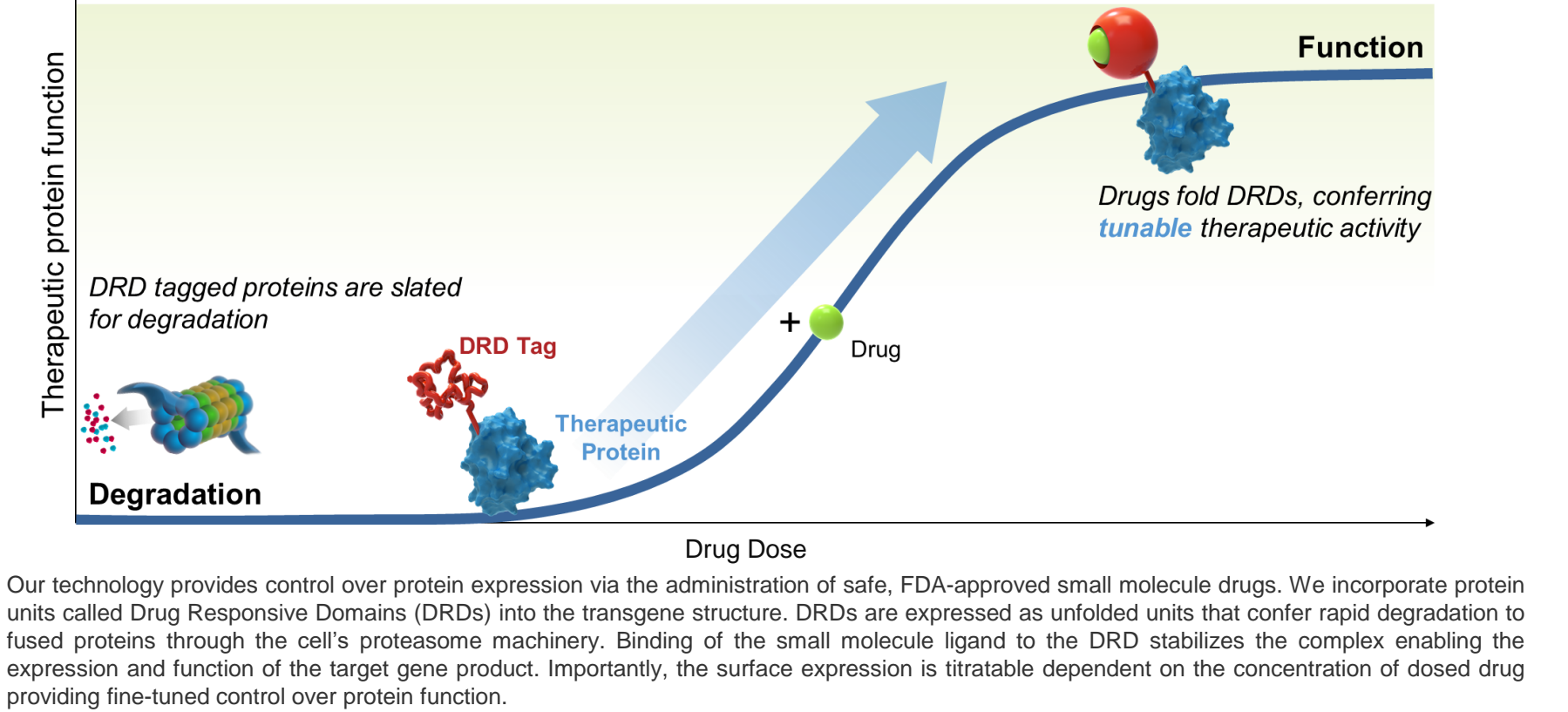
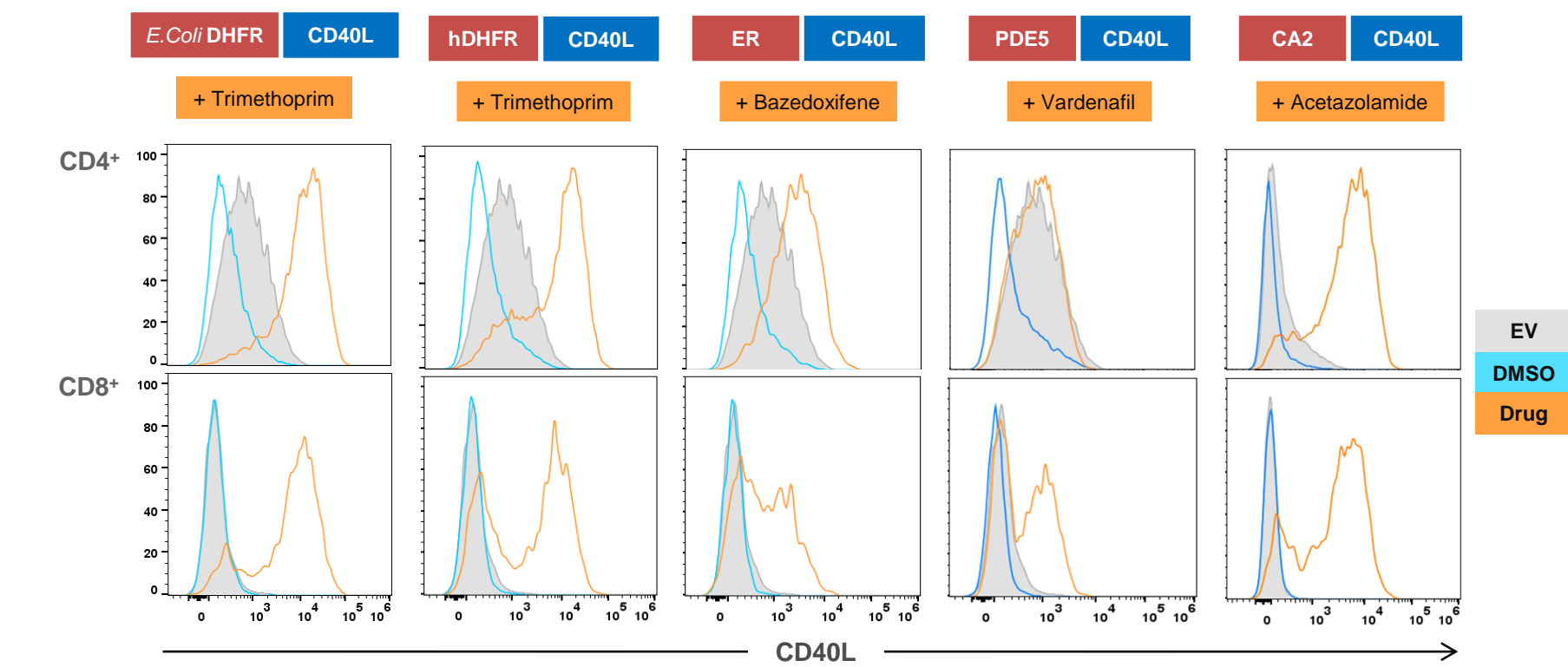
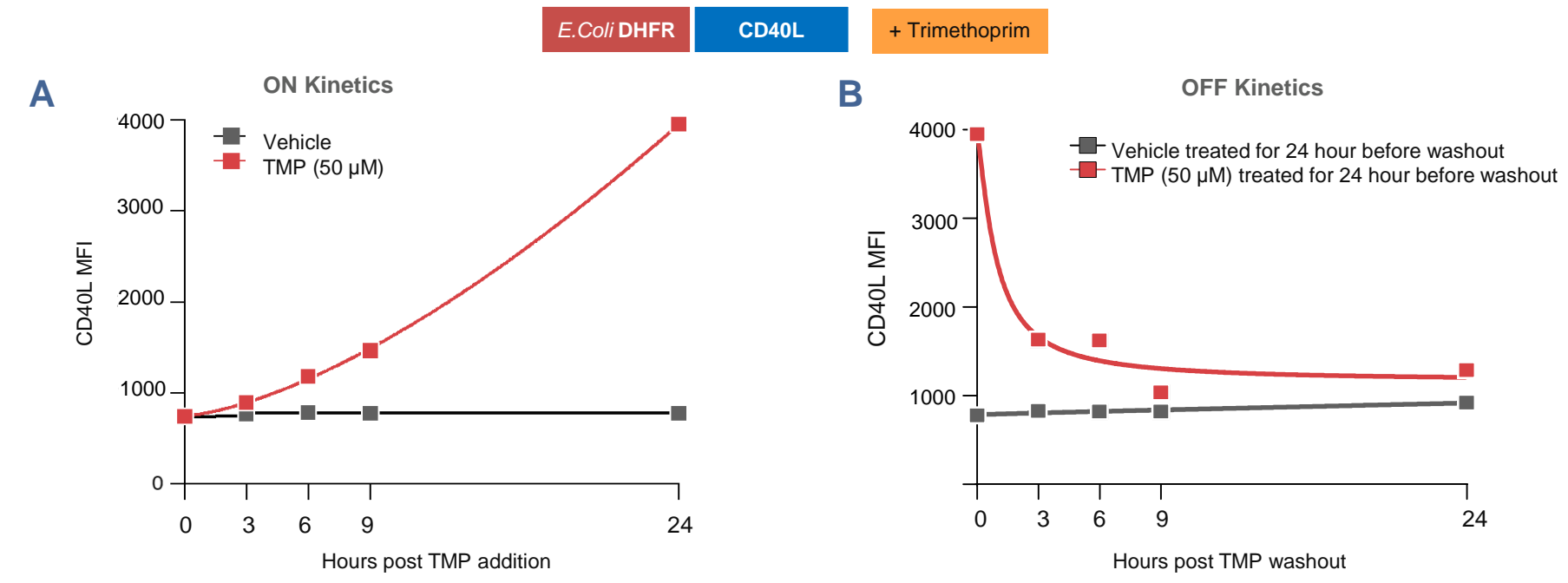


Figure 5: Five drug responsive domains regulate CD40L with FDA approved drugs



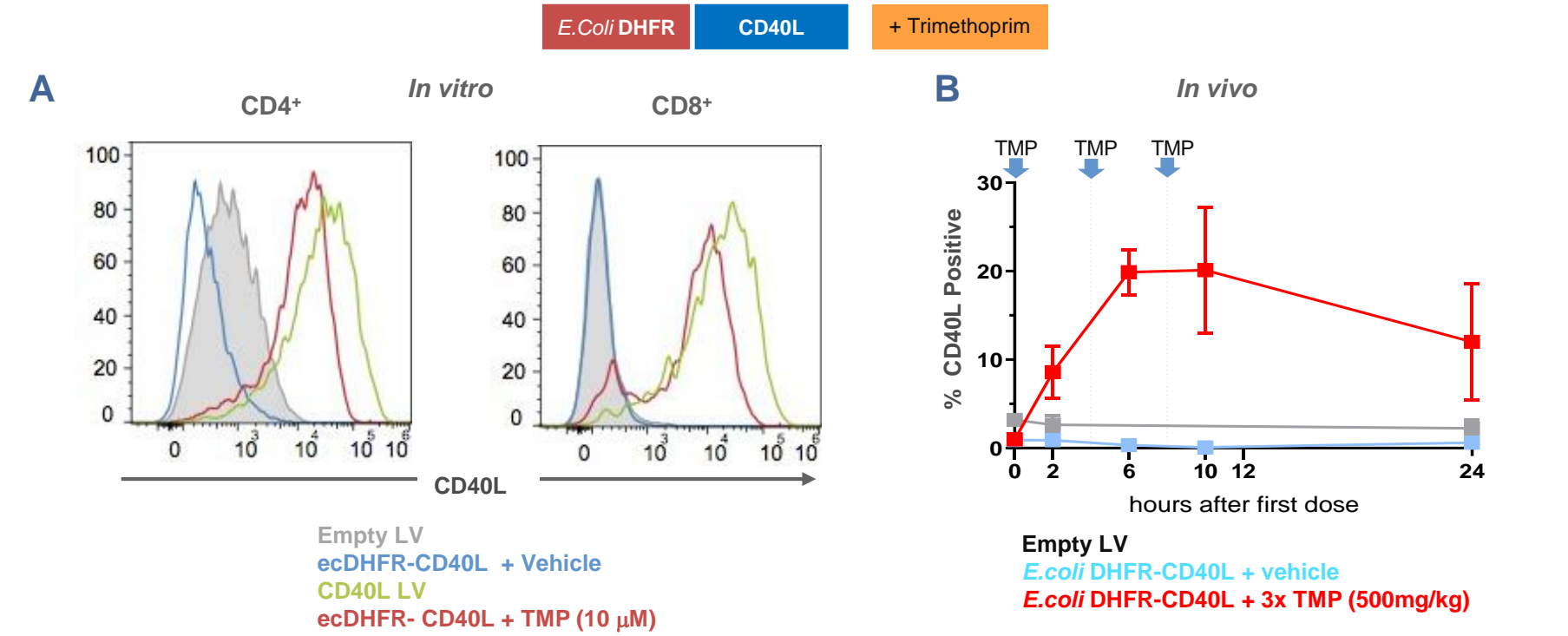
Drug responsive domains were made by mutating the proteins *E.coli* and human dihydrofolate reductase (DHFR), estrogen receptor (ER), phosphodiesterase isozyme 5 (PDE5), and carbonic anhydrase 2 (CA2). To test regulation, activated T cells were transduced with lentivirus expressing DRD regulated CD40L. Two days later, cells were treated with vehicle or ligand for 24h after which they were analyzed for CD40L surface expression. Note the endogenous expression of CD40L in activated CD4-cells (grey). Regulated expression with all destabilizing domains significantly enhanced CD40L expression beyond endogenous levels. Both *E.coli* DHFR and CA2 drug responsive domains show levels close to constitutive expression with clinically relevant ligand doses.

Figure 6: DRD fused CD40L induces expression with rapid off kinetics after drug washout



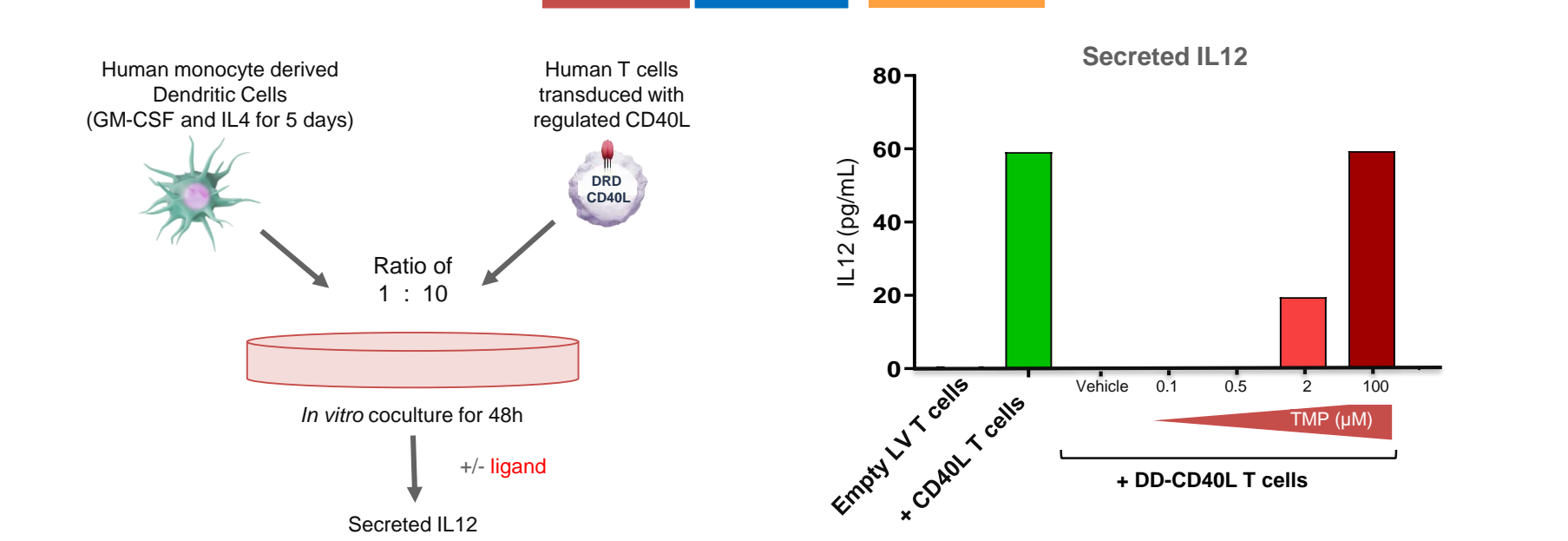
To study the kinetics of regulated surface expression, Jurkat cells were stably transduced with *E.coli* DHFR regulated CD40L. A) For ON kinetics measurements, cells were incubated with TMP for the indicated duration of time before analysis for CD40L expression by FACS. B) For OFF kinetics, cells were incubated with TMP or vehicle for 24h followed by extensive PBS washing and fixation of the cells at the indicated timepoints after washout. CD40L surface expression of the cells was determined by FACS. Interestingly, surface expression increases moderately after TMP addition which could be a consequence of the necessary trimerization for proper surface trafficking of CD40L. In contrast, after washout of TMP, cells quickly lost surface expression.

Figure 7: Trimethoprim induces regulatable expression of DRD fused CD40L *in vivo*



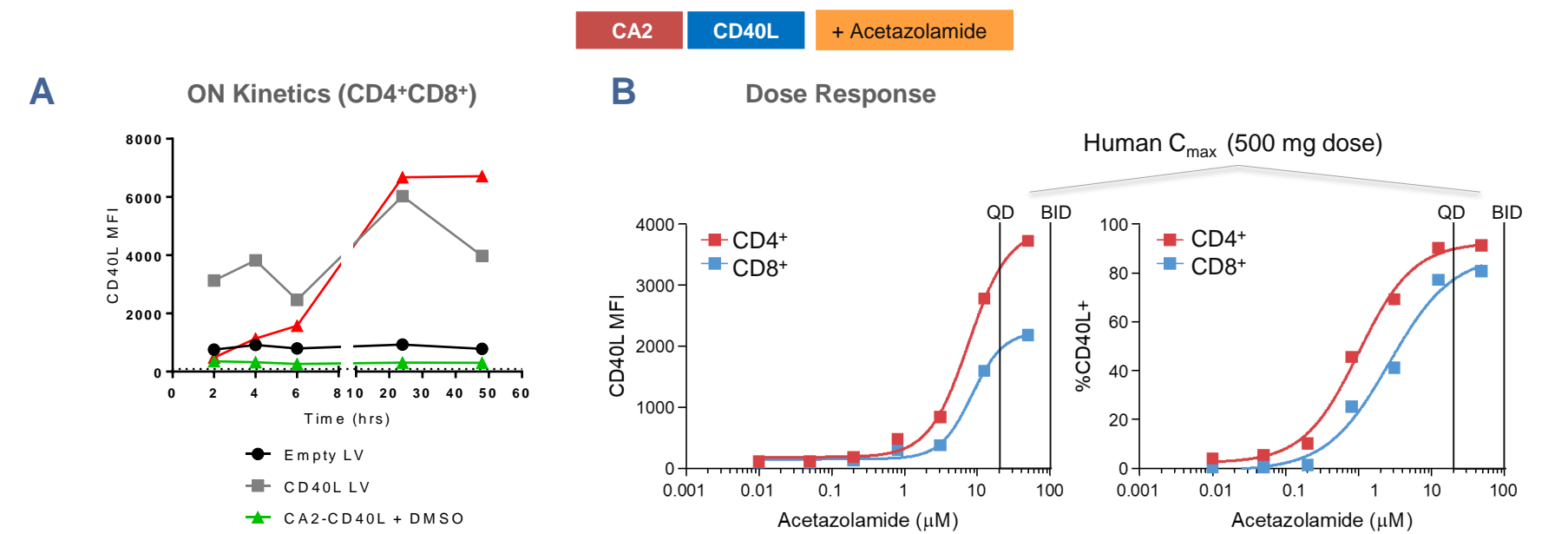
A) Pre infusion, T cells were transduced with lentivirus expressing constitutive or *E. coli* DHFR regulated CD40L. Two days later, cells were treated with vehicle or 10 μ M TMP for 24h after which they were analyzed for CD40L surface expression. B) The same T cells were expanded *in vitro* for 10 days before infusion into NSG mice (n=4/group). Two days after infusion, animals were dosed orally 3x at the indicated times with 500 mg/kg TMP (\blacktriangledown). Blood samples taken prior to dosing or 2, 6, 10 and 24 hours post-dosing were analyzed for CD40L surface expression on human T cells. Surface expression reached a maximum at 10 hours after the first dose.

Figure 8: DRD fused CD40L in T cells supports pharmacological regulation of DC activation *in vitro*



Healthy donor derived human T cells were activated, virally transduced with CD40L or ecDHFR-CD40L and expanded. In addition, allogeneic human monocytes were differentiated with IL4 and GM-CSF for 5 days before cryopreservation. Cells were cryopreserved and freshly thawed for co-culture experiment. For co-cultures both cell types were freshly thawed and incubated together at a 10:1 ratio (T cell:moDC) for 2 days before secreted IL12 was analyzed. For *E. coli* DHFR regulation, TMP was added during the co-culture period.

Figure 9: Regulation of CD40L with a human DRD from carbonic anhydrase 2



To study the kinetics of regulated surface expression, activated T cells were stably transduced with CA2 regulated CD40L. A) Cells were treated with various doses of acetazolamide (ACZ) and DMSO and fixed at the indicated timepoints. Cells were stained, and surface CD40L was measured using FACS analysis. Expression reached its peak at 24 hours with highest doses expressing higher than constitutive levels. B) Dose response curves of CA2 regulated CD40L with ACZ blotted as median fluorescent intensity or gated percent positive. The indicated area is an approximate C_{max} of ACZ concentration in the plasma of humans from various clinical studies.

SUMMARY

- Expression of constitutive CD40L in human T cells enhances CAR efficacy and dendritic cell activation *in vivo*
- Drug responsive domains enable regulation of CD40L expression using FDA-approved small molecule drugs at clinically achievable concentrations
- Regulated CD40L in T cells activate dendritic cells to produce IL12