Investigational CD25 x CTLA-4 Bispecific DART® Molecule for Depletion of Tumor Infiltrating Tregs via an Enhanced Fc-dependent Effector Mechanism

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Abstract

Introduction: Tregs are suppressive cells whose presence in the tumor microenvironment (TME) has been correlated with tumor progression and may be a major impediment in the effectiveness of tumor immunotherapy. Removal of Tregs has been shown to induce cancer regression in animal models; however, selective depletion of tumor-infiltrating Tregs (TI-Tregs) is challenging as they share phenotypic markers with Tregs in healthy tissues as well as with tumor-infiltrating effector cells. To overcome cross-reactivity between these T cell subsets and achieve enhanced targeting of TI-Tregs, we employed a bispecific strategy to simultaneously target two cell surface antigens (CD25 and CTLA-4) with overlapping, high level expression on TI-Tregs. Furthermore, we incorporated Fc engineering to enable target-cell depletion via enhanced Fc-mediated effector function.

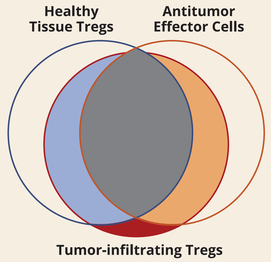
Methods: CD25 x CTLA-4 DART proteins were constructed based on CD25 and CTLA-4 mAbs and incorporating an Fc domain with enhanced binding to the activating Fc-gamma RIIIa receptor (CD16A) and reduced binding to the inhibitory Fc-gamma RIIb receptor (CD32B) Fc_{enh}, or control null Fc devoid of FcR interactions Fc_{null}. Depletion of *FoxP3*-positive cells ex vivo was monitored by flow cytometry. Immunomodulatory effects of the DART proteins were evaluated in cultured PBMC or dissociated tumor cells in various in vitro immune assays, including mixed lymphocytes reaction (MLR).

Results: Consistent with their design, we observed increased binding of CD25 x CTLA-4 DART molecules to cells when both target antigens were engaged compared to single antigen engagement. The bispecific DART molecule depleted in vitro induced *FoxP3*$^+$ CD4$^+$ TI-like Tregs cells by an Fc-dependent mechanism, with minimal effect on the viability of *FoxP3*-negative T cells. A molecule bearing a null Fc was ineffective. The extent of *FoxP3*$^+$ T-cell depletion by the Fc-enhanced CD25 x CTLA-4 DART molecule was greater than that exhibited by the combination of its monovalent components, with the depleting activity requiring both arms of the bispecific molecule. The DART molecule had only minor effect on the capacity of effector T cells to secrete IL-2 and interferon-gamma upon restimulation or to exert cytotoxic function. In contrast the combination of CD25 and CTLA-4 mAbs reduced the ability of effector T cells to produce cytokines in a restimulation assay. An increased response in the presence of the Fc-enhanced CD25 x CTLA-4 DART molecule was also observed in various immune assays, consistent with Treg suppression and preservation of T-cell effector function.

Conclusions: A CD25 x CTLA-4 DART molecule was effective in depleting activated *FoxP3*$^+$ CD4$^+$ Tregs in vitro, with reduced effects on resting Tregs or activated *FoxP3*$^-$ effector cells. Targeting CD25 and CTLA-4 in a bispecific molecular format with enhanced Fc-dependent effector function may be suitable to specifically deplete TI-Tregs.

Background

Dual-antigen Targeting for TI-Tregs Depletion



Challenge:

- TI-Tregs express many genes shared by antitumor effector cells as well as Tregs from healthy tissues. Depletion of these other cells is not feasible
- Few cell surface proteins uniquely associated with TI-Tregs; however, many are expressed only by a small fraction of TI-Tregs

Solution:

- Use bispecific reagent to target **two** cell surface **proteins,** preferentially **co-expressed** by TI-Tregs

CD25 and CTLA-4 Expression in TME

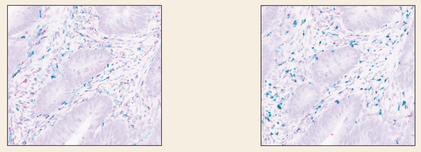


Figure 1a: Expression of *FoxP3* **(red)** and CD25 or CTLA-4 **(blue)** mRNA in colorectal cancer was analyzed by fluorescent in-situ hybridization (RNAscope™).

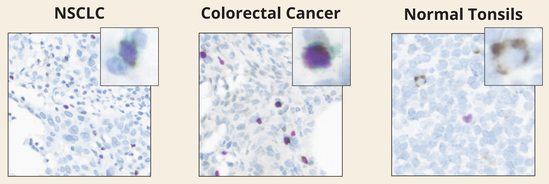


Figure 1b: Co-expression of *FoxP3* **(purple)**, CD25 **(light blue)**, and CTLA-4 **(brown)** analyzed by multiplex IHC in lung carcinoma, colorectal cancer or normal tonsils.

CD25/CTLA-4 Co-Expression in Tumor Tregs

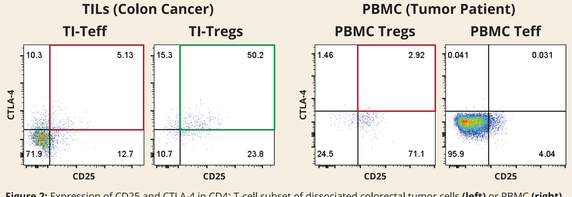
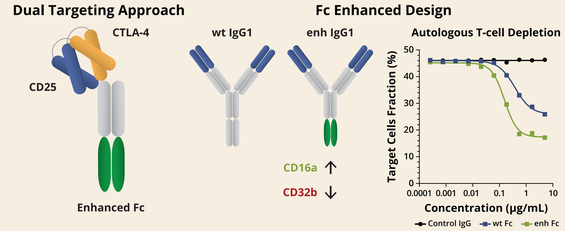


Figure 2: Expression of CD25 and CTLA-4 in CD4$^+$ T-cell subset of dissociated colorectal tumor cells **(left)** or PBMC **(right)** obtained from tumor patient. Gated on viable, EpCAM$^-$CD45$^+$CD3$^+$CD4$^+$ T effectors vs Tregs were separated by expression of *FoxP3* (observed in 15% and 2% of CD4$^+$ T cells in dissociated tumor cells and PBMC, respectively).

Results

CD25 x CTLA-4 DART Molecule with Enhanced IgG1 Fc



Schematic representation of bivalent bispecific CD25 x CTLA-4 DART molecule featuring IgG1-based Fc domain with enhanced ADCC potential.

Figure 3: Depletion of CD4$^+$ T cells in in vitro cultured PBMC treated with OKT4 mAbs with wildtype IgG1 Fc **(blue)** or IgG1 Fc engineered to enhance ADCC **(green)**. Fraction of target cells measured by FACS after 24 hours incubation.

Bispecific Binding of CD25 x CTLA-4 DART Molecule

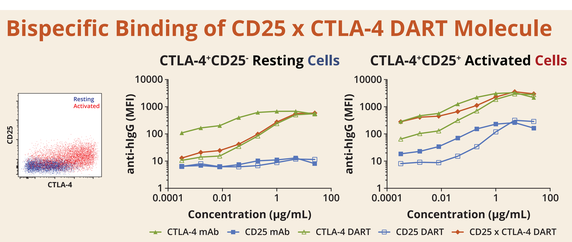


Figure 4 Left: Induction of CD25 and upregulation of CTLA-4 in cells activated by PMA + Ionomycin in Jurkat/CTLA-4 cells. **Right:** Binding of bispecific CD25 x CTLA-4 DART molecule or monospecific CD25 (CD25 x Neg), CTLA-4 (CTLA-4 x Neg) DART molecules and control mAbs to in vitro activated or resting CD25 + CTLA-4 cells measured by FACS.

CD25 x CTLA-4 Enhances T-cell Activation in Vitro

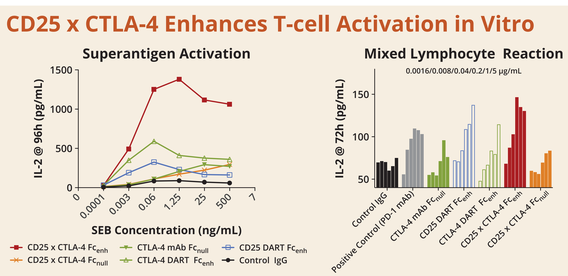
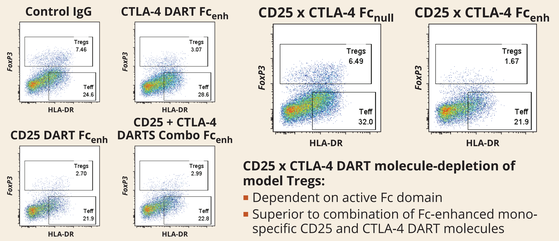


Figure 5 Left: PBMC from healthy human donors were activated in vitro by SEB in the presence of 1 µg/mL of CD25 x CTLA-4 DART molecules or parental mAbs. **Right:** CD4$^+$ cells purified from freshly isolated healthy donor PBMC co-incubated with monocyte-derived DC from another donor to initiate allogeneic activation in the presence of indicated molecules.

CD25 x CTLA-4 Depletes in Vitro-induced Treg Cells

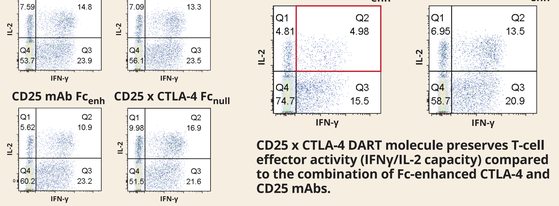


CD25 x CTLA-4 DART molecule-depletion of model Tregs:

- Dependent on active Fc domain
- Superior to combination of Fc-enhanced mono-specific CD25 and CTLA-4 DART molecules

Figure 6: PBMC from healthy human donors were activated in vitro with CD3 beads in the presence of 1 µg/mL of indicated molecules. Activation conditions were selected to recapitulate immunophenotype of TILs as monitored by FACS (data not shown). Fraction of CD4$^+$*FoxP3*$^+$ Treg cells and *FoxP3*-HLA-DR^{high} effector cells was measured by FACS after 48 hours incubation.

CD25 x CTLA-4 Preserves Effector T-cell Function



CD25 x CTLA-4 DART molecule preserves T-cell effector activity (IFNγ/IL-2 capacity) compared to the combination of Fc-enhanced CTLA-4 and CD25 mAbs.

Figure 7: PBMC from healthy human donors were activated in vitro with CD3 beads for 48 hours followed by 72 hours of incubation with 1 µg/mL of indicated molecules. Cells were then restimulated by PMA/Ionomycin in the presence of Brefeldin A and expression of IL-2 and IFN-gamma in CD3$^+$ CD4$^+$ T cells was measured by intracellular FACS staining.

CD25 x CTLA-4 DART Molecule Depletes TI-Tregs

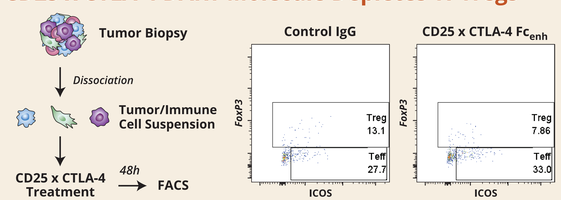


Figure 8: Dissociated colorectal tumor cells were incubated in complete media in the presence of control IgG or CD25 x CTLA-4 DART molecule at 1 µg/mL final concentration. Fraction of *FoxP3*$^+$ Treg cells was measured by FACS; gated on viable EpCAM$^-$CD45$^+$CD3$^+$CD4$^+$ T cells.

Conclusions

- Tumor-infiltrating Tregs co-express CD25 and CTLA-4
- CD25 x CTLA-4 DART molecule bearing an engineered Fc-domain mediates autologous depletion of Tumor associated Tregs and in vitro-induced Tregs
- CD25 x CTLA-4 DART molecule preserves T-cell effector function and enhances immune responses in vitro

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