Tumor-targeted checkpoint inhibitors for reactivation of tumor-infiltrating lymphocytes

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BACKGROUND

Despite their clinical benefit, immune checkpoint inhibitors (ICIs) are known to induce toxic effects (e.g., autoimmune responses) by inadvertent but on-target immune activation. To minimize such toxicity, we are developing ICIs targeted to patients' tumors, with the goal of selectively activating tumor-infiltrating lymphocytes (TILs). Here, we present TILsRx-001, a tetravalent protein consisting of two chains connected via Fragment crystallizable (Fc) domains with knob-in-hole mutations. Each N-terminus carries a human CD80 extracellular domain (ECD) variant with increased affinity for PD-L1 due to two point mutations (V68M and E160R), and each C-terminus contains an affinity-enhanced anti-B7-H3 scFv for tumor targeting (**Figure 1**).

METHODS

TILsRx-001 was characterized by surface plasmon resonance (SPR), flow cytometry, and a cell-based bioassay measuring TILsRx-001's potential to bind to target cells expressing B7-H3, anti-CD3, and PD-L1, and to antagonize PD-1 signaling in co-cultured "effector" cells, reflected by increased luciferase activity.

For the bioassay, CHO-K1 cells engineered to express both PD-L1 and an agonistic CD3 antibody in scFv format are cocultured with Jurkat-derived reporter cells expressing human PD-1 protein and NFAT-RE-induced luciferase [WO2025098364A1] (GS-J2B/PD-1). Binding of PD-L1 to PD-1 on the GS-J2B/PD-1 reporter cells, in the absence of treatment, reduces the luciferase signal (**Figure 1A**). In contrast, treatment with an immune checkpoint inhibitor, such as an anti-PD1 antibody, increases the luciferase signal. Similarly, transient transfection of the engineered CHO-K1 cells with an expression plasmid encoding a tumor-associated antigen (TAA) (such as B7-H3) and treatment with a TAA-targeted "TILsRx" multi-specific binding protein carrying CD80 extracellular domains also blocks the PD-L1:PD-1 interaction and consequently increases the luciferase signal (**Figure 1B**).

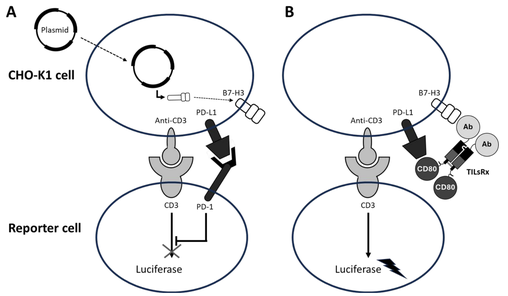


Figure 1. Principle of reporter bioassay to assess TILsRx interference with PD-L1:PD-1 interaction. A, without TILsRx; **B**, with TILsRx.

RESULTS

All design features of TILsRx-001 tested were confirmed. Target cell binding was demonstrated using flow cytometry. With recombinant proteins as analytes, TILsRx-001 bound B7-H3, PD-L1, CD28, and CTLA-4 with affinities (KD) of 206 nM, 165 nM, 1.95 μM, and 456 pM, respectively. In comparison, a wild-type TILsRx construct identical to TILsRx-001, except that it lacks the V68M/E160R mutation, bound PD-L1, CD28, and CTLA-4 with affinities (KD) of 8.12 μM, 2.67 μM, and 848 pM, respectively. Moreover, TILsRx-001 strongly antagonized the immune inhibitory effect of PD-L1 on cells co-expressing PD-L1 and B7-H3, demonstrating its potential as a cancer cell-targeted ICI for the PD-1:PD-L1 checkpoint. This effect was not apparent with soluble CD80 variants (CD80-Fc) with or without a mutation (D60E/E160R) strongly increasing the affinity of CD80-Fc for PD-L1) (**Figure 4**), suggesting 1), that target cell-binding is a requirement, and 2), that the effect occurs in cis.

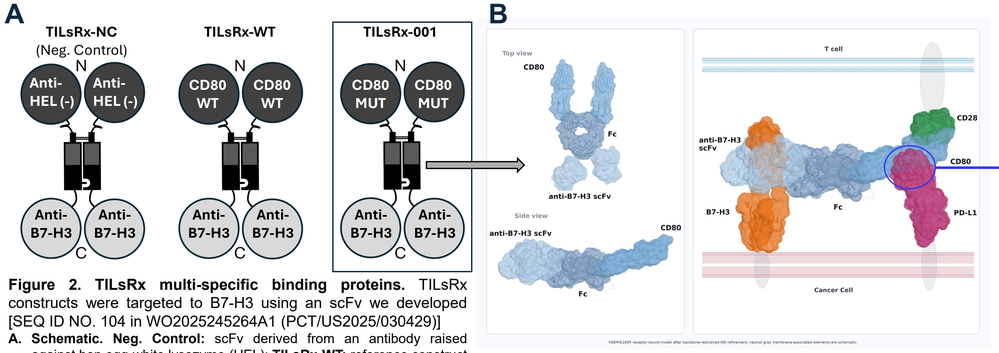


Figure 2. TILsRx multi-specific binding proteins. TILsRx constructs were targeted to B7-H3 using an scFv we developed [SEQ ID NO. 104 in WO2025245264A1 (PCT/US2025/030429)]

A. Schematic. Neg. Control: scFv derived from an antibody raised against hen egg-white lysozyme (HEL); **TILsRx-WT:** reference construct with wild-type (WT) CD80 ECD; **TILsRx-001:** CD80 ECD with V68M/E160R double mutation.

B. Modeled TILsRx-001 structure bound to B7-H3, PD-L1, and CD28. Many additional conformations are expected.

C. Potential interactions between V68M/E160R and PD-L1.

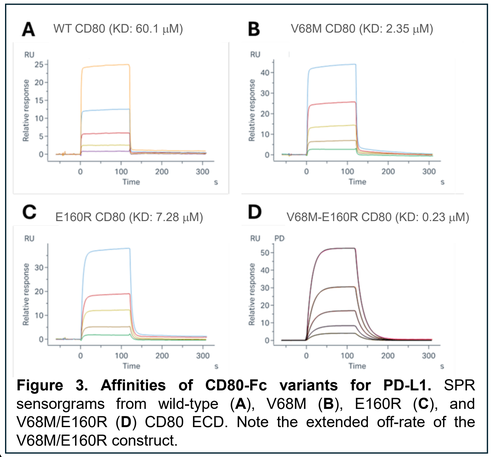


Figure 3. Affinities of CD80-Fc variants for PD-L1. SPR sensorgrams from wild-type (**A**), V68M (**B**), E160R (**C**), and V68M/E160R (**D**) CD80 ECD. Note the extended off-rate of the V68M/E160R construct.

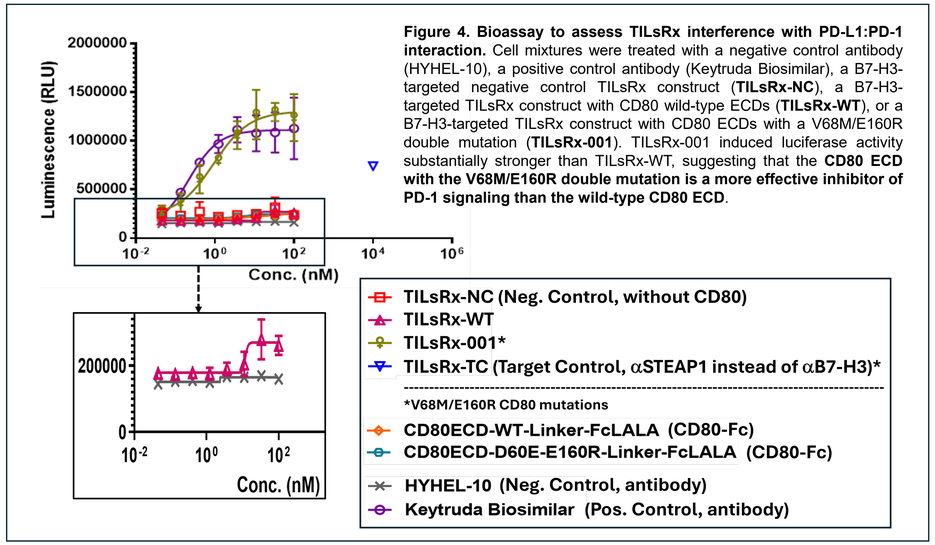


Figure 4. Bioassay to assess TILsRx interference with PD-L1:PD-1 interaction. Cell mixtures were treated with a negative control antibody (HYHEL-10), a positive control antibody (Keytruda Biosimilar), a B7-H3-targeted negative control TILsRx construct (**TILsRx-NC**), a B7-H3-targeted TILsRx construct with CD80 wild-type ECDs (**TILsRx-WT**), or a B7-H3-targeted TILsRx construct with CD80 ECDs with a V68M/E160R double mutation (**TILsRx-001**). TILsRx-001 induced luciferase activity substantially stronger than TILsRx-WT, suggesting that the **CD80 ECD with the V68M/E160R double mutation is a more effective inhibitor of PD-1 signaling than the wild-type CD80 ECD**.

Additional TILsRx Design

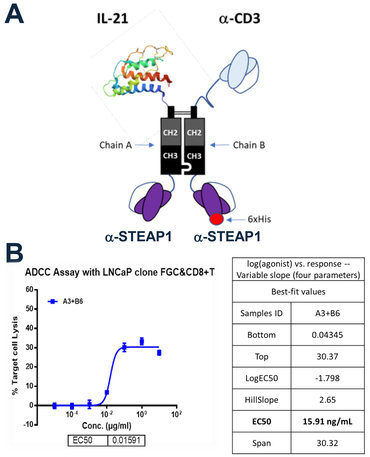


Figure 5. IL-21/CD3-TILsRx targeted to STEAP1, a prostate cancer TAA. A, Schematic. **B,** dose-response experiment with CD8+ human T cells and LNCaP clone FGC (prostate cancer) target cells.

CONCLUSIONS AND OUTLOOK

- TILsRx-001 has the potential to act as a tumor-targeted checkpoint inhibitor.
- The V68M/E160R double mutation in TILsRx-001 increased the affinity for PD-L1 49-fold.
- To fine-tune the affinities for PD-L1, CD28, and CTLA-4, additional CD80 mutations are being evaluated.
- TILsRx-001 is a clinical candidate, with preclinical efficacy, toxicity, pharmacokinetic (PK) and pharmacodynamic (PD) studies planned.