Using the TNF Superfamily Ligands (TNFSF) as Many-Trimer Multimers for Cancer Immunotherapy

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Summary: strong immunity using multi-trimer forms of TNFSF ligands

- Ligands in the TNF Superfamily (TNFSFs) are some of the best “gas pedals” for anti-tumor immunity
- Receptor clustering is essential for signaling by CD40, OX40, 4-1BB, and GITR
- “Agonistic” anti-TNFSF receptors antibodies can delete suppressive Tregs, whereas multi-trimer TNFSFs lacking Fc are pure agonists
- Multi-trimer CD40L is directly cytotoxic to ovarian and breast cancer cells while also stimulating CD8+ T cell responses (“one-two punch”)
- Multi-trimer OX40L lacking Fc may have advantages in therapy
- Multi-trimer 4-1BBL can be used to stimulate CD8+ T cells and expand NK cells in vitro – useful for generating TILs and CARTs
19 Members of the TNF SuperFamily (TNFSFs)

- 19 distinct proteins make up the TNFSFs
- Each can be proteolytically cleaved from the cell membrane
- But the soluble 1-trimer proteins are largely inactive

Early mistake: thinking 1 trimer is enough
Receptor clustering is required for activation of many TNFSF receptors.
“Agonistic” anti-receptor antibodies cluster receptors, but cause cell depletion

**Forward Effect:** for stimulation, agonistic antibodies must be arrayed by Fc receptors on an adjacent cell

**Depletion Effect:** antibodies also lead to ADCC and phagocytosis of Tregs and effector cells

Our solution: multi-trimer ligands that are independent of FcRs

Covalently linked TNFSF trimers cause receptor clustering and cell activation without requiring FcRs.
MegaLigand™ Platform

- 2-trimer MegaLigands are fusion proteins between a TNFSF ligand and the Acrp30 scaffold protein
- Acrp30 naturally circulates in plasma at 5-10 μg/ml
- cGMP production and Phase I study completed for MegaFasL™ (APO010) in cancer
UltraLigand™ Platform

- 4-trimer UltraLigands are fusion proteins between a TNFSF ligand and the Surfactant Protein D (SPD) scaffold protein
- SPD is a self-assembling multimerizing lung protein with 4 trimeric, collagen-like “arms”
- UltraLigands are rugged – their activity is stable at RT for >1 month and survives >10 cycles of freeze-thaw
Part I

Multi-trimer CD40L
CD40L activates dendritic cells to generate CD8+ T cells.

- **Exogenous CD40L** activates DCs.
- **Endogenous CD40L on CD4+ T cells.**

Once formed, CD8+ killer T cells move to targets.
CD40L - activator of dendritic cells

Panel A: DCs cultured with normal fibroblasts

Panel B: DCs cultured with fibroblasts expressing membrane CD40L

CD40L as a vaccine adjuvant

- To examine the effect of valency on adjuvant activity, 1-trimer, 2-trimer, and 4-trimer DNA constructs were tested in a DNA vaccine.
- 1-trimer CD40L contained the same isoleucine “zipper” as the original Immunex/Amgen product ("sCD40LT").
Adjuvant effects of Multi-Trimer CD40L

- A plasmid for HIV Gag antigen (pScGag) was mixed with 1-, 2-, and 4-trimer CD40L plasmids
- 4-trimer SP-D-CD40L was superior for inducing CD8+ T cell responses measured by ELISSPOT (above) and cytotoxicity assay (not shown)

Intratumoral DNA treatment of B16F10 melanoma

- Combination of UltraCD40L™ (SP-D-CD40L) DNA with TLR agonists cured established melanoma
- Mice survived for > 1 year tumor-free

Intratumoral UltraCD40L™ and UltraGITRL™ plasmid DNA cures A20 lymphoma in mice

After A20 B cell lymphomas reached > 4 mm in diameter, they were injected every other day X 5 with plasmid DNA (arrows) encoding multimeric UltraCD40L (SP-D-CD40L), natural membrane CD40L (MemCD40L), or multimeric UltraGITRL (SP-D-GITRL). UltraCD40L and UltraGITRL, but not membrane CD40L, were very effective treatments for this tumor.
Ovarian cancer cells, but not normal ovarian cells, express the CD40 receptor.

Ovarian cancer tumors obtained at surgery were examined under the microscope. Using a labeled antibody (brown staining), the CD40 receptor was detected on the surface of all of the tumor cells.

Normal ovarian and peritoneal lining cells do not have CD40 on their surface.

S. Ghamande et al. (EA Repasky), Recombinant CD40 ligand therapy has significant antitumor effects on CD40-positive ovarian tumor xenografts grown in SCID mice and demonstrates an augmented effect with cisplatin. Cancer Res 61:7556-7562, 2001.
CD40L is directly cytotoxic for ovarian cancer cells in vivo

Surgically resected human ovarian cancer tumors were implanted into immunodeficient SCID mice. **Left Panel:** Normal ovarian cancer tissue. **Right Panel:** After treatment of mice with CD40L (75 μg of soluble protein), there was rapid death of the ovarian cancer cells (arrows and black stain for apoptotic cell death). (This experiment used sCD40LT from Immunex that has not been made for 14 years.)

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CD40L is directly cytotoxic for ovarian cancer cells in vivo

- **75% tumor reduction** with monotherapy - just 2 weekly CD40L treatments
- **99% tumor reduction** when CD40L was combined with cisplatin therapy
- This is a direct cytotoxic effect on ovarian cancer cells since SCID mice have no immune system


MegaCD40L™ Ovarian Cancer Treatment

One-two punch against ovarian cancer:

1. MegaCD40L™ kills ovarian cancer cells directly.
2. MegaCD40L™ activates “killer” T cells to clean-up any remaining cancer cells.

Intraperitoneal administration (which is standard of care for ovarian cancer) minimizes the systemic effects of MegaCD40L™.

PET-CT scan of metastatic intraperitoneal ovarian cancer
Breast cancer cells also express the CD40 receptor

In most breast cancer cases, the tumor cells express the CD40 receptor, as shown in this case of ductal carcinoma using a labeled anti-CD40 antibody (brown staining). In contrast, normal breast cells express little or no CD40.

In vitro studies showed that CD40L, but not anti-CD40 antibody, suppressed the growth of the breast cancer cell lines.

Breast cancer causes malignant pleural effusions (MPE) in ~ 7-11% of women, resulting in > 15,000 MPE cases/year in the U.S. alone¹.

Breast cancer cells express the CD40 receptor (96% by immunohistochemistry) and show slower growth or apoptosis after CD40L stimulation².

This suggests that MegaCD40L™ should be tested as a treatment for MPE in breast cancer.

Part II

Multi-Trimer OX40L
Agonistic OX40 antibody can deplete T cells

Anti-human OX40 (106-222) requires crosslinking by FcR-bearing cells. When the antibody was used at the optimal concentration for depleting immunosuppressive Tregs, it also depleted 10-15% of the desired memory CD4+ T cells.

Comparison of two forms of 2-trimer OX40L

- 3-part fusion protein (Fc/trimerizing domain/OX40L)
- Fc component interacts with FcRs
- Phase 1 clinical trial started 09/2014

- 2-part fusion protein (Acrp30/OX40L)
- No Fc component

AgonOx/MedImmune MEDI6383

Multimeric’s MegaOX40L™
Part III

UltraCD40L™ to Grow B cell APCs
Primary B cells grow indefinitely when supplied with CD40L

50 ml of blood was obtained from healthy subjects (green) or cancer patients (red) and cultured with 3T3 fibroblasts expressing membrane CD40L plus IL-4. By re-stimulating every 5 days, unlimited numbers of B cells could be generated in vitro.

Growth of primary B cells using UltraCD40L – an alternative source of APCs

SP-D-CD40L (UltraCD40L™) can be used to grow unlimited amounts of B cells for adoptive immunotherapy

- Unlimited amounts of APCs from small amounts of blood, unlike DC harvests using leukapheresis
- Antigens can be introduced by peptide pulsing or RNA transfection
- Used to grow anti-tumor or anti-viral CD8+ T cells for adoptive immunotherapy
- Can be injected systemically for anti-tumor effects
Growth of primary CD20 human B cells using UltraCD40L™ + IL-4

Day 2
5X phase microscopy

Day 12
Macrophotograph of the bottom of a T75 flask

Clumps of activated B cells (~1 million cells/clump)

Images kindly provided by Dr. John Mathison, The Scripps Research Institute
UltraCD40L-generated CD40-B cells can be used to generate CD8+ cytotoxic T cells (CTLs).

CD40-B cells cultured using UltraCD40L™ were pulsed with a peptide antigen (CMV NLV peptide) and used to expand CD8+ T cells. The photomicrograph shows the elongated CD8+ T cells binding to and killing the peptide-pulsed B cells, just as they would tumor cells.
Part IV

Ultra4-1BBL™ for T and NK Cells
**T cell costimulatory effects of 4-1BB (CD137)**


**4-1BB receptor (CD137) specifically identifies the TILs capable of killing tumor cells.** Ye Q et al. (DJ Powell), CD137 accurately identifies and enriches for naturally occurring tumor-reactive T cells in tumor. Clin Cancer Res. 20:44-55, 2014


**CART cells containing a 4-1BB signaling domain are enter tumors, have strong anti-cancer effects, and proliferate in vivo.** Song DG et al. (DJ Powell), In Vivo Persistence, Tumor Localization, and Antitumor Activity of CAR-Engineered T Cells Is Enhanced by Costimulatory Signaling through CD137 (4-1BB). Canc Res 71:4617-4627, 2011. Kalos, M et al (CH June), T cells with chimeric antigen receptors have potent antitumor effects and can establish memory in patients with advanced leukemia. Sci Translat Med 3:95ra73, 2011.
Initial 4-1BB stimulation prepares T cells for invasion into antigen-bearing tissues

**Vaccine study:** Mice were vaccinated with Ad5 vector expressing Gag with or without Ad5-SPD-4-1BBL (Ultra4-1BBL). Following two vaccinations, the mice were challenged with Vaccinia-Gag, where Vaccinia virus preferentially replicates in ovaries. When 4-1BBL was included in the initial vaccine, the resulting CD8+ T cells are highly invasive for Gag-expressing ovarian tissues. Kanavagelu SK, et al. (RS Kornbluth and GW Stone), Vaccine 30:691-702, 2012.
Ultra4-1BBL™ activates human T cells in vitro

Human PBMCs were cultured on plate-bound anti-CD3 antibody along with dilutions of human Ultra4-1BBL. IL-2 production was measured 3 days later.
Ultra4-1BBL™ is a growth factor for human NK cells in vitro

NK cells were purified from human PBMCs and stimulated with K562 cells at a 1:1 ratio. Two days later, the cultures with supplemented with IL-2 and IL-15. The culture flask on the right also received human SPD-4-1BBL (Ultra4-1BBL™) at 1 ug/ml. After 10 days, the Ultra4-1BBL flask contained 4 times as many NK cells.
Human NK cells grown with Ultra4-1BBL™ retain NK markers.

Close up of NK cells grown with human SPD-4-1BBL (Ultra4-1BBL™) showing very dense growth in culture.
Selective growth of NK cells using Ultra4-1BBL™ in vitro

NK cells are negative for the CD3 T cell marker and positive for the CD16 and CD56 markers. Starting with purified NK cells, the cells grown with human SPD-4-1BBL (Ultra4-1BBL™) remain ~90% pure NK cells.
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Contact for Further Information

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