Multimeric forms of CD40 ligand (CD40L), 4-1BB ligand (4-1BBL), OX40 ligand (OX40L), CD27L/CD70, and other TNFSFs for cancer immunotherapy

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Abstract

The TNF SuperFamily (TNFSF) of ligands include 19 molecules some of which play key roles in the immune system. All of these ligands are produced as trimeric Type II membrane molecules that can be released from the cell surface as single trimers. However, more than a decade of research has shown that the receptors for TNFSFs on responding cells require clustering in order to signal. Thus, single trimers are poor stimuli because they cannot produce the necessary clustering. Likewise, studies of agonist anti-TNFSF receptor antibodies (anti-CD40, anti-4-1BB, anti-DR5, etc.) have shown that these antibodies must be mounted via their Fc portion to FcRs on an adjacent cell in order to cluster and signal these receptors. This means that agonistic antibodies to TNFSF receptors only function in microenvironments where there is a directly adjacent FcR-bearing cell. However, we have solved the receptor clustering problem by creating fusion proteins that contain many TNFSF trimers. As scaffolds for these molecules, we used surfactant protein D (SP-D) to make molecules with 4 trimeric arms ("UltraLigands™") and ACRP30 (A Complement-Related Protein 30 kD or adiponectin) to make molecules with 2 trimeric arms ("MegaLigands™"). Numerous papers from independent labs have reported the ability of UltraCD40L™ and MegaCD40L™ to activate human and mouse cells. UltraOX40L™and MegaOX40L™ have also been studied and most recently Ultra4-1BBL™ has been produced. We are applying these unique materials in the following ways: (1) Using MegaCD40L™ or UltraCD40L™ plus IL-4, human B cells have been grown from small amounts of blood to serve as highly efficient APCs for expanding antigen-specific CD8+ T cells in vitro; (2) Mega4-1BBL™ or Ultra4-1BBL™ are being used to activate TCR-stimulated CD8+ cytotoxic T cells in vitro; and (3) MegaOX40L™ or UltraOX40L™ are being used to stimulate CD4+ T cells in vitro. These cell-free proteins can be used as practical reagents to simplify and improve the generation of immune cells for adoptive immunotherapy.

Receptor clustering is needed for CD40 activation

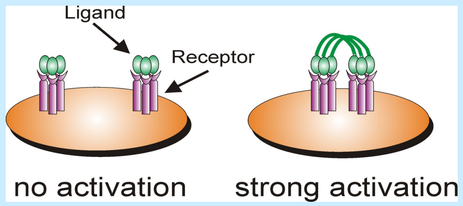


Fig. 1. Receptor clustering is needed for TNFRSF activation. CD40, 4-1BB (CD137), GITR, CD27, DR3, DR5, and Fas are some of the TNFSF receptors known to require clustering in order to trigger downstream signaling. Clustering can be achieved by an antibody mounted on FcRs on an adjacent cell or, as shown in this poster, by a many-trimer form of their TNFSF ligand.

Molecular design of 2- and 4-trimer TNFSF ligands

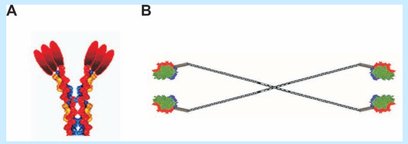


Fig. 2. Designing many-trimer forms of TNFSF ligands. The extracellular domain (ECD) of a TNFSF ligand can be genetically fused to one of two protein scaffolds, Acrp30 (Panel A) or surfactant protein D (SP-D) (Panel B). Following expression of the protein in CHO cells, 2-trimer or 4-trimer proteins are produced. The 2-trimer Acrp30-TNFSF ligand is also called a Mega-Ligand™, whereas the 4-trimer SP-D-TNFSF ligand is also called an Ultra-Ligand™. Commercially available proteins include MegaCD40L™, MegaOX40L™, MegaAPRIL™, MegaTNF™, and MegaFasL™.

SP-D-CD40L + IL-4 stimulates the massive proliferation of cultured human B cells for use as APCs

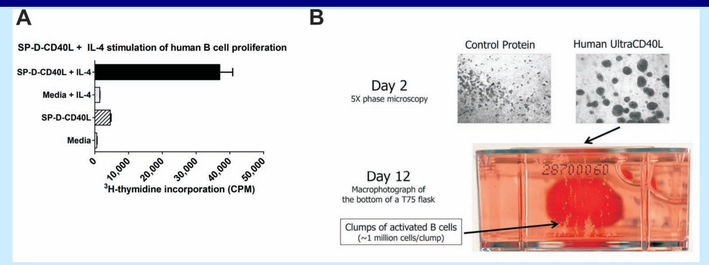


Fig. 3. Stimulation of human B cell proliferation by SP-D-CD40L (UltraCD40L) + IL-4. B cells were purified from PBMCs using anti-CD19 immunomagnetic beads (Miltenyi). The cells were cultured in 96-well plates at 2 X 10^5 cells/ml in RPMI 1640, 10% FBS, and 10 ng/ml IL-4. Panel A: Four days later, the cells were pulsed with ^{3}H-Tdr and proliferation measured as thymidine incorporation. Panel B: Visualization of growing CD40-B cells. When grown in SP-D-CD40L-containing media, B cells expressed adhesion molecules and formed multicellular clumps that were visible to the unaided eye by day 12. Here, a 75 cm^2 flask was cultured vertically and the photograph was taken looking upward. The B cells continue to proliferate so long as SP-D-CD40L is added every 3-4 days. These CD40-B cells can be cryopreserved and thawed for later use.

SP-D-CD40L activates B cells to express APC-related surface proteins

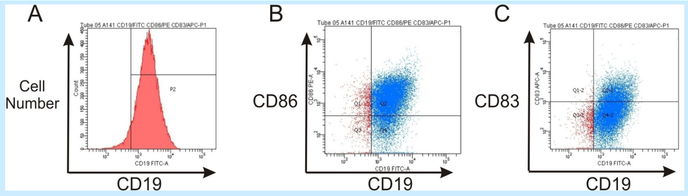
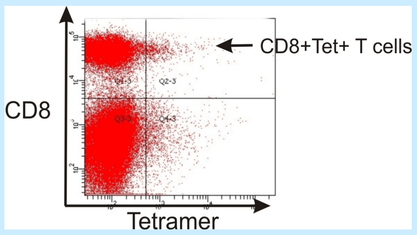


Fig.4. Activation markers expressed by CD40L-stimulated B cells. Panel A Cultures like that shown in Fig. 3 contain about 94-97% of cells that are positive for the CD19 B cell marker. Panel B: The majority of CD19+ cells are also CD86+ (75% in the example shown). Panel C: Many CD19+ B cells are also positive for the dendritic cell and longevity marker, CD83 (about 5.5% in the example shown). Not shown: These B cells are also >95% positive for CD54, CD80, MHC-I, and MHC-II.

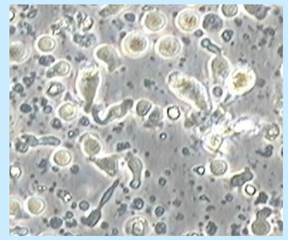
Generation of CD8+ T cells using peptide-pulsed CD40-B cells

Fig. 5. Peptide-pulsed CD40-B cells are APCs for CD8+ T cells. Autologous HLA-A2.1 CD40-B cells were treated with mitomycin and pulsed with CMV pp65 antigen (NLV peptide). As APCs, these cells generated antigen-specific CD8+ T cells in 2 weeks that were recognized by NLV/A2.1- tetramer staining.



Generation of cytotoxic CD8+ T cells using peptide-pulsed CD40-B cells

Fig. 6. CD40-B cells as APCs for CD8+ T cells. Autologous B cells grown with SP-D-CD40L were pulsed with peptide antigen and used to stimulate naïve CD8+ T cells. After 2 weeks, the activated T cells (characterized by podocytes) had killed most of the B cells in the cultures (only their apoptotic debris remains). By cytotoxicity assay, CD8+ T cells could be generated against both "easy" antigens like CMV pp65 as well as very difficult antigens like WT1 peptide.



Uses for many-trimer TNFSF ligands

Adoptive Immunotherapy:

UltraCD40L™ generated CD40-B cells pulsed with tumor antigens as stimulators for adoptive CD8+ T cell therapy.

Ultra4-1BBL™ to grow highly active TILs.

***In Vivo* Immunotherapy:**

MegaCD40L™ as a cancer vaccine adjuvant.

MegaCD40L™ to treat CD40+ carcinomas (ovarian cancer, breast cancer, head and neck cancers, etc.) that are directly killed by CD40 stimulation.

MegaOX40L™ immunostimulator for cancer therapy.

UltraGITRL™ immunostimulator for cancer therapy.

MegaTRAIL™ for anti-tumor effects.

Conclusions

- **Many-trimer multimers enable the production of strongly active TNFSF ligands.**
- **In some cases, multimeric ligands may be superior to agonistic anti-TNFRSF antibodies.**
- **In collaboration with partners around the word, numerous uses have been found for MegaCD40L™ and UltraCD40L™.**

Acknowledgements

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For publications, see www.multimericbio.com

Patent protection for multimeric TNFSFs (MegaLigands™ and UltraLigands ™) is provided by US 7,300,774B1, US 7,332,298B2, EP01246925B1, US 2009/0081157A1, and related counterparts worldwide.