

# Applications of multimeric, soluble TNFSFs to vaccination and tumor immunotherapy

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## ABSTRACT

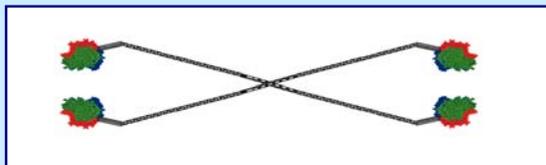
Clustering is important for full TNFSF stimulation. Clustering can be brought about by membrane TNFSF or by using a multimeric soluble form of a TNFSF. To produce the later, we fused the extracellular domains of TNFSFs with the body of surfactant protein D (SP-D) to form a molecule with four trimeric arms. Such molecules have been made for CD40L, GITRL, BAFF, RANKL, CD70, 4-1BBL, OX40L, LIGHT, and TRAIL. Since it is technically demanding to purify such large proteins, in vivo studies have been performed by injecting expression plasmids for the SP-D-TNFSF involved. Three types of biological effects have been demonstrated in mice: (1) pSP-D-CD40L is a strong adjuvant for DNA vaccines that elicit CD8+ T cells; (2) pSP-D-GITRL and especially pSP-D-BAFF are strong adjuvants for DNA vaccines that elicit antibody; (3) pSP-D-CD40L combined with TLR agonists is a strong antitumor immunostimulant when injected into established B16F10 melanoma and other tumors.

## BACKGROUND

Many receptors in the TNFR superfamily need to be clustered for full signaling activity. Such receptors include TNFR2 (p75), Fas, TRAIL-R2 (DR5), 4-1BB (CD137), BAFF-R (BR3), and CD40. As a result, these receptors are preferentially stimulated by the membrane form of their corresponding ligand or, in the case of BAFF-R, a 60-mer multimeric soluble complex. Similarly, engineered forms of soluble FasL, TRAIL, 4-1BBL, and CD40L are more active if they are either intentionally crosslinked or produced in forms that spontaneously aggregate in solution.

In order to consistently produce multimeric forms of TNFSF ligands, we and others have fused the extracellular regions of TNFSFs to the body of members of the collectin and C1q families, such as surfactant protein D (SP-D) and Acrop30 (adiponectin). These molecules have trimeric, collagen-like "arms" that are joined by a disulfide-linked "hub" resulting in molecules made entirely of autologous subunits. However, the large size of these proteins and their tendency to aggregate when concentrated has made them difficult to purify and study. Consequently, we have used DNA expression plasmids to deliver these molecules in vivo for studies of vaccination and tumor immunotherapy.

## MOLECULAR DESIGN



**Figure 1. Construction of 4-trimeric, multimeric soluble CD40L, GITRL, BAFF, RANKL, 4-1BBL, OX40L, CD70, LIGHT, and TRAIL.** Plasmids were constructed in the pcDNA3.1, pVAX1, or pCAGEN (similar to pCAGGS) expression vectors. The body of surfactant protein D (SP-D) was fused to the extracellular domains of these murine, macaque, or human TNFSF ligands. SP-D is a plus-sign shaped molecule with 4 trimeric arms that can present 4 trimeric TNFSF extracellular domains. Similar constructs were made using the ACRP30 scaffold that presents 2 trimeric TNFSF extracellular domains.

**Grant Support:** NIH R21 AI52842, University-wide AIDS Research Program, Mesothelioma Applied Research Foundation UCSD Center for AIDS Research, and the VA San Diego Healthcare System.

## DNA VACCINE METHODS

**Antigen plasmids:** The antigen plasmids encoded secreted, codon-optimized forms of either HIV-1 Gag (pScGag) or the MSP1 19 kDa malaria antigen from *Plasmodium yoelii*.

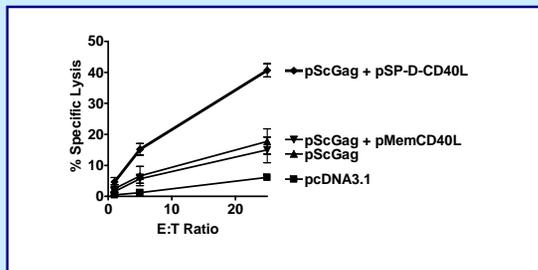
**TNFSF plasmids:** pSP-D-CD40L and pSP-D-GITRL encoded 4-trimeric soluble CD40L and GITRL respectively. pMemCD40L encoded full-length, membrane CD40L.

**Mouse vaccinations:** BALB/c mice were injected i.m. in both quadriceps every other week X 3 with a combination of antigen plasmid (80 µg of either pScGag or pMSP1) plus 4-trimeric TNFSF plasmid (20 µg of either pSP-D-CD40L, pSP-D-GITRL, or control empty vector).

**Immunoassays:** Two weeks after the last vaccination, splenic CTLs were re-stimulated for 5 days and tested for killing of P815 cells pulsed with H-2Kd immunodominant peptide, AMQMLKETI.

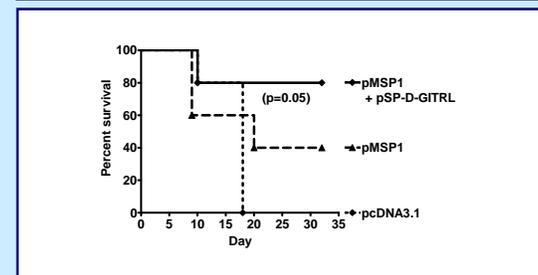
**Malaria challenge:** BALB/c mice were injected i.p. with 2 X 10<sup>4</sup> *P. yoelii*-infected RBCs.

## IN A DNA VACCINE, 4-TRIMER SOLUBLE CD40L IS A STRONG ADJUVANT FOR ANTI-HIV CD8+ T CELLS



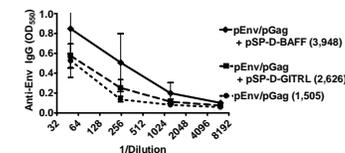
**Figure 2. Plasmid DNA for 4-trimer soluble CD40L enhanced CD8+ T cell responses.** A cytotoxicity assay of splenocytes from vaccinated mice showed that pSP-D-CD40L augmented responses against peptide-pulsed P815 target cells. pMemCD40L encodes membrane CD40L and had no effect. Similar results were seen with an IFN $\gamma$  ELISPOT assay. See Stone et al [1].

## IN A DNA VACCINE, 4-TRIMER SOLUBLE GITRL IS A STRONG ADJUVANT FOR A MALARIA VACCINE



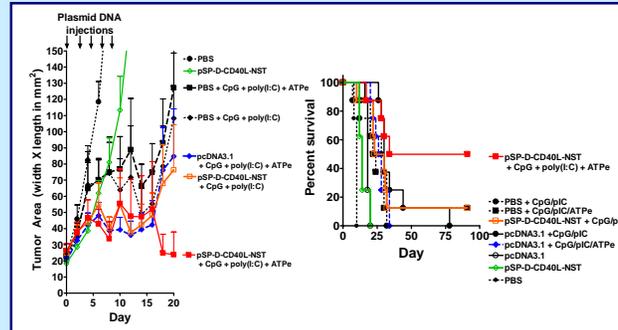
**Figure 3. Plasmid DNA for 4-trimer soluble GITRL protected mice from malaria.** pSP-D-GITRL combined with the pMSP1 antigen plasmid elicited an enhanced anti-MSP1 antibody response (not shown). Upon challenge with merozoite-infected RBCs, there was significantly greater protection in mice vaccinated with pMSP1 + pSP-D-GITRL compared to pMSP1 alone.

## IN A DNA VACCINE, 4-TRIMER SOLUBLE BAFF IS A STRONG ADJUVANT FOR ANTIBODIES TO HIV



**Figure 4. Plasmid DNA for 4-trimer soluble BAFF and GITRL enhanced antibody responses to an HIV-1 Env vaccine.** Mice immunized to have CD8+ T cell responses against HIV-1 Gag were vaccinated with DNA for Env and Gag. IgG ELISA just 1 week after vaccination is shown, with geometric mean titer (GMT) in parenthesis. pSP-D-BAFF or pSP-D-GITRL increased GMT by 2.6- and 1.7-fold respectively.

## CD40 – TLR - INFLAMMASOME SYNERGY CURES ESTABLISHED MELANOMA TUMORS



**Figure 5. Synergy between CD40 + TLR + Inflammasome stimulation for melanoma tumor treatment.** B16F10 tumors in C57BL/6 mice  $\geq 4$  mm in diameter (8/group) were injected every other day X 5 with 50 µg control plasmid (pcDNA3.1) or pSP-D-CD40L-NST (a No-Stalk modification with deleted CD40L stalk and added tPA signal sequence) in 100 µl PBS. CpG (ODN 1018) and poly(I:C), 25 µg each in 50 µl PBS, were injected on the day after each plasmid injection. Extracellular ATP $\gamma$ S (ATPe, 100 µM) was included in the CpG + poly(I:C) injections to activate the inflammasome. As shown, pSP-D-CD40L-NST + CpG + poly(I:C) + ATPe cured mice without evidence of toxicity or autoimmune vitiligo.

## CONCLUSIONS

- ▶ Fusion of the extracellular domains of TNFSF ligands to surfactant protein D (SP-D, Fig. 1) is an effective way to produce multimeric, soluble TNFSFs.
- ▶ DNA delivery of these constructs enhanced vaccines for CD8+ T cells (pSP-D-CD40L, Fig. 2) and antibodies (pSP-D-BAFF and pSP-D-GITRL, Fig. 3 & 4).
- ▶ Cure of established melanoma in mice can be achieved using pSP-D-CD40L-NST + CpG + poly(I:C) + extracellular ATP (ATPe) (Fig. 5) demonstrating synergy between CD40 + TLR + inflammasome stimulation.

[1] Stone, G.W. et al. Multimeric soluble CD40 ligand and GITRL ligand as adjuvants for HIV DNA vaccines. *J. Virol.* 80:1762-72, 2006.

[2] Stone, G.W. et al. Macaque multimeric soluble CD40 ligand and GITRL ligand constructs are immunostimulatory molecules in vitro. *Clin Vaccine Immunol.* 13:1223-30, 2006.