

# Enhanced vaccine and anti-tumor immunity using GITR ligand (GITRL) or extracellular ATP (ATPe)

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## ABSTRACT

**Background:** GITR signaling obviates immunosuppression by CD4+CD25+ regulatory T cells (Tregs). The natural ligand of GITR is GITRL, a TNF superfamily ligand. Extracellular ATP (ATPe) downregulates Tregs by two mechanisms: it activates macrophages and DCs to secrete IL-1 $\beta$ , an anti-Treg cytokine; and it is selectively toxic to Tregs. **Methods:** DNA vaccines encoding either HIV-1 Gag or the MSP-1 19kDa blood stage antigen of *Plasmodium yoelii* were studied in mice. Plasmids for novel 4-trimer secreted forms of GITRL or CD40L were added as adjuvants. After the vaccination, the mice were either analyzed for T cell and antibody responses or challenged with *P. yoelii*-parasitized red blood cells to induce malaria. For tumor immunotherapy studies, established A20 lymphoma or B16F10 melanoma tumors were injected intratumorally with plasmid DNA encoding 4-trimer soluble GITRL or CD40L. In some experiments, the tumors were also injected with CpG (TLR9) and poly(I:C) (TLR3) with or without ATPe. **Results:** As an adjuvant, GITRL was about half as effective as CD40L in promoting CD8+ T cell responses. However, GITRL was stronger than CD40L at enhancing CD4+ T cell proliferation and antibody responses to the vaccine antigen. As a malaria vaccine adjuvant, GITRL led to strong protection from malaria-induced death in mice. When used for tumor immunotherapy, injections either GITRL or CD40L cured A20 lymphoma tumors. For B16F10 melanoma tumors, cure required the quadruple combination of CD40L + CpG + poly(I:C) + ATPe. **Conclusions:** 4-trimer soluble GITRL and CD40L are strongly active and can be used as vaccine adjuvants and for tumor immunotherapy.

## BACKGROUND

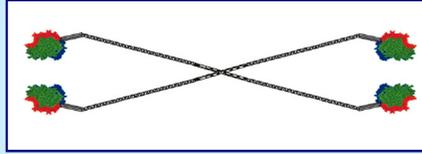
Receptor clustering is important for signaling from TNFR-II, Fas, CD40, 4-1BB, TRAIL-R2 (DR5), TAC1, and probably also GITR. As a result, these receptors are preferentially stimulated by the membrane form of their corresponding ligand or, in the case of BAFF-R, a 60-mer multimeric soluble complex. Similarly, 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 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 Acrp30 (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.



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## MOLECULAR DESIGN



**Figure 1. Construction of 4-trimer, multimeric soluble GITRL and CD40L.** 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 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. To date, multimeric fusion proteins of GITRL, CD40L, RANKL, OX40-L, 4-1BBL, CD70, TRAIL, BAFF, and APRIL have been produced in this manner.

## 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*.

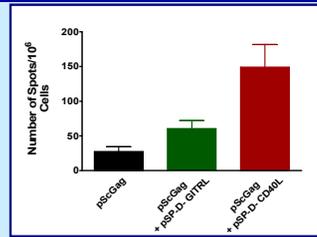
**TNFSF plasmids:** pSP-D-CD40L and pSP-D-GITRL encoded 4-trimer soluble CD40L and GITRL respectively. pMemCD40L encoded full-length, transmembrane CD40L.

**Mouse vaccinations:** BALB/c mice were injected i.m. in both quadriceps every other week X 3 with a combination of antigen plasmid (80  $\mu$ g of either pScGag or pMSP1) plus 4-trimer TNFSF plasmid (20  $\mu$ g of either pSP-D-GITRL, pSP-D-GD40L, or control empty vector).

**Immunoassays:** Two weeks after the last vaccination, splenic CTLs were analyzed by IFN- $\gamma$  ELISPOT using P815 stimulator cells pulsed with the H-2Kd peptide, AMQMLKETI.

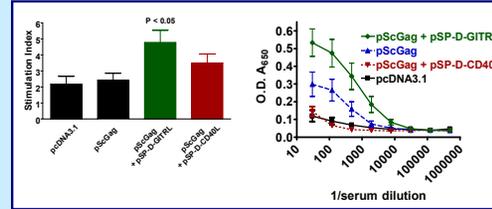
**Malaria challenge:** BALB/c mice were injected i.p. with 2 X 10<sup>4</sup> *P. yoelii*-infected RBCs.

## SP-D-CD40L IS BETTER THAN SP-D-GITRL FOR ELICITING CD8+ T CELL RESPONSES



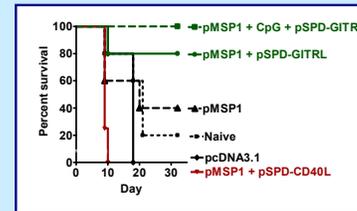
**Figure 2. Plasmid DNA for 4-trimer soluble GITRL is less active than CD40L for eliciting CD8+ T cell responses in response to vaccination.** IFN- $\gamma$  ELISPOT analysis of splenocytes shows that CD40L, as expected, is effective at "licensing" DCs for CD8+ T cell responses. GITRL, while capable of adjuvanting CD8+ T cell responses, is less potent than CD40L. These conclusions were supported by cytotoxicity assays and tetramer analyses (not shown). For details, see Stone et al [1].

## SP-D-GITRL IS BETTER THAN SP-D-CD40L FOR ELICITING CD4+ T CELL AND ANTIBODY RESPONSES



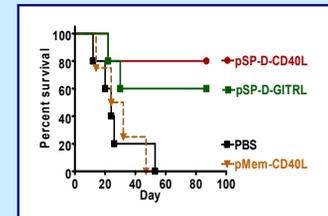
**Figure 3. Plasmid DNA for 4-trimer soluble GITRL, but not CD40L, enhanced CD4+ T cell and antibody responses to vaccination.** Mice were vaccinated as in Fig. 2. Left Panel: Proliferative responses to Gag protein are expressed as the stimulation index. Right Panel: Serum IgG responses were measured by ELISA on Gag protein-coated plates.

## SP-D-GITRL IS AN EFFECTIVE ADJUVANT FOR A MALARIA VACCINE



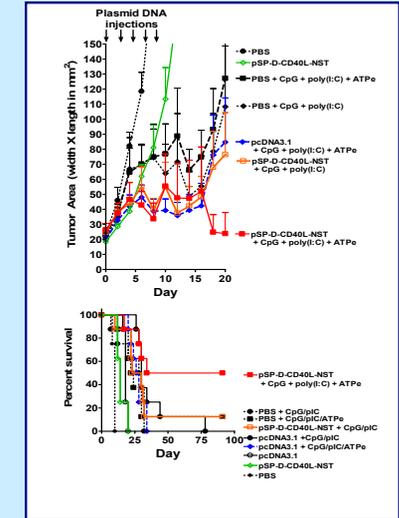
**Figure 4. Plasmid DNA for 4-trimer soluble GITRL was an effective adjuvant for a malaria vaccine.** pSP-D-GITRL combined with pMSP1 antigen plasmid elicited an enhanced anti-MSP1 antibody response (not shown). Upon challenge with merozoite-infected RBCs, there was significantly greater protection in mice vaccinated with pMSP1 + pSP-D-GITRL compared to pMSP1 alone (p = 0.04). CD40L, which has been reported to contribute to malaria-induced pathology, led to more rapid death in this experimental system.

## SP-D-GITRL IS AN EFFECTIVE IMMUNOTHERAPY FOR A20 LYMPHOMA



**Figure 5. Intratumoral injections of either 4-trimer soluble GITRL or CD40L cure mice of established lymphoma.** Mice with A20 B lymphoma tumors  $\geq$  4 mm in diameter were injected intratumorally with either pSP-D-GITRL or pSP-D-CD40L every other day X 5, resulting in long-term tumor-free survival. In contrast, transmembrane CD40L, pMem-CD40L, had no effect.

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



**Figure 6. 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 X 5 with 50  $\mu$ g control plasmid (pcDNA3.1) or pSP-D-CD40L-NST (a No-Stalk modification with deleted CD40L stalk and added I $\kappa$ B $\alpha$  signal sequence). In some cases, CpG (ODN 1018) and poly(I:C) (25  $\mu$ g each) were also injected on the day after each plasmid injection. Extracellular ATP $\gamma$ S (ATPe, 100  $\mu$ M) was included in the CpG + poly(I:C) injections to activate the inflammasome and kill Tregs. pSP-D-CD40L-NST + CpG + poly(I:C) + ATPe cured mice without evidence of toxicity or autoimmune vitiligo.

## CONCLUSIONS

- ▶ Multimeric soluble TNFSFs can be produced as highly active proteins by fusing them with the body of surfactant protein D (SP-D, Fig. 1).
- ▶ SP-D-GITRL enhanced DNA vaccines for CD8+ T cell (Fig. 2), CD4+ T cell (Fig. 3), and Ab responses (Fig. 3).
- ▶ pSP-D-GITRL adjuvanted a DNA vaccine against malaria, a disease with a strong Treg component (Fig. 4).
- ▶ pSP-D-GITRL injections into A20 lymphoma, a Treg-rich tumor, led to long-term, tumor-free survival (Fig. 5).
- ▶ Cure of established melanoma in mice can be achieved using pSP-D-CD40L-NST + CpG + poly(I:C) + extracellular ATP (ATPe) (Fig. 6) demonstrating synergy between CD40 + TLR + anti-Treg inflammasome stimulation.

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