

# Enhancing Responses to DNA Vaccination Using Plasmids for Soluble, Multimeric CD40L Ligand and GITR Ligand

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## ABSTRACT

**Background:** The effectiveness of HIV DNA vaccines can be improved by the addition of immunostimulants. One of the most powerful endogenous immunostimulatory molecules is CD40 ligand (CD40L, CD154), a trimeric tumor necrosis factor (TNF) superfamily ligand expressed on the surface of activated CD4+ T cells that has been difficult to use as a solubilized protein.

**Methods:** Based on *in vitro* studies, it was hypothesized that multimerization (i.e., many trimers) would be needed to utilize soluble CD40L in a vaccine formulation. Consequently, three kinds of CD40L plasmids were tested in mice: pTr-CD40L, a 1-trimer-soluble CD40L containing an isoleucine zipper; pAcrp30-CD40L, a 2-trimer CD40L produced as a fusion protein with the body of Acrp30, a spontaneously multimerizing collectin; and pSP-D-CD40L, a 4-trimer CD40L produced as a fusion protein with the body of pulmonary surfactant protein D (SP-D), another collectin. Antigen-specific splenic CD8+ immune responses were measured using CTL assays, IFN- $\gamma$  ELISpot, and major histocompatibility class I tetramers.

**Results:** When combined with a plasmid for secreted, codon-optimized Gag (pScGag) and co-injected into mice intramuscularly three times at two-week intervals, 1-trimer soluble CD40L had almost no adjuvant effects but the 2- and 4-trimer soluble CD40L molecules led to strong CD8+ T-cell responses in direct proportion to their valence (4 > 2 > 1). No toxicity was appreciated for any of the constructs. Similar results were found using an antigen plasmid for Env. However, no version of CD40L significantly augmented antigen-specific proliferative responses by splenocytes or enhanced antibody production. Consequently, glucocorticoid-induced TNF receptor ligand (GITRL), another TNF superfamily ligand that co-stimulates CD4+ T cells and abrogates the immunosuppressive activity of CD4+CD25+ regulatory T cells, was tested as a 4-trimer plasmid, pSP-D-GITRL. This molecule not only stimulated CD8+ T-cell responses but also stimulated antigen-specific proliferative responses and antibody production.

**Conclusions:** These results indicate that fusions between immunostimulatory TNF superfamilies and collectins can significantly enhance responses to HIV DNA vaccines and act as a novel vaccine adjuvant.

## INTRODUCTION

When used alone, antigen-encoding DNA vaccines seldom elicit useful immune responses in humans [1]. However, the addition of cytokine or chemokine coding sequences can significantly improve the strength of DNA vaccines [2,3]. CD40L is especially attractive as a molecular adjuvant because it is the principal endogenous activator of dendritic cells (DCs) and the molecular embodiment of CD4+ T cell "help" [4]. After CD40L stimulation, DCs are "licensed" to cross-present antigen to CD8+ T cells. In the absence of CD40L stimulation, DCs are unable to generate long-lived memory CD8+ T cell responses [5].

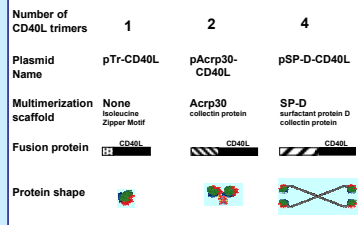
Despite the recognized importance of CD40L (over 6,000 papers in PubMed refer to CD40L or CD40), it has proved difficult to utilize this molecule for vaccines. As a member of the TNF superfamily (TNFSF) of ligands, CD40L is produced as a trimeric, Type II, membrane protein. Plasmids encoding full-length membrane CD40L usually do not augment immune responses to DNA vaccines. Consequently, a number of studies have utilized a soluble, 1-trimer form of CD40L ("sCD40L") that was originally produced by Immunex. However, full CD40 stimulation requires the clustering of receptors in the plane of the membrane, which can only be achieved by multimeric forms of CD40L.

In this project, CD40L has been expressed as a soluble multimeric molecule. In a DNA vaccine approach, 2- and 4-trimer multimers of CD40L were much more effective adjuvants than 1-trimer CD40L as judged by CD8+ T cell responses in mice. At the same time, these CD40L-adjuvanted DNA vaccines failed to generate significant CD4+ T cell responses or antibody responses. Thus, DNA vaccines containing soluble multimeric CD40L are "CD8-focused" – a unique vaccine response profile that may be especially valuable in the context of HIV infection [6].

Several other TNFSF ligands are also candidate molecular adjuvants. The ligand for GITR (Glucocorticoid-Induced TNF receptor-Related) is especially interesting because GITR is expressed on CD4+CD25+ regulatory T cells ("Tregs"). GITR stimulation of Tregs turns off their immunosuppressive effects and augments immune responses. In this project, a 4-trimer soluble multimeric form of GITRL was found to be a potent molecular adjuvant for CD8+ T cells, CD4+ T cells, and antibody responses.

## MOLECULAR DESIGN

Fig. 1. Construction of soluble CD40L molecules with 1-, 2-, or, 4-trimers.



Plasmids were constructed in the pcDNA3.1 expression vector. Membrane CD40L (pMemCD40L, not depicted). The full-length natural form of murine CD40L was cloned by RT-PCR from anti-CD3/anti-CD28-stimulated murine spleen cells.

- 1-trimer soluble CD40L (pTr-CD40L):** Using PCR construction, an isoleucine zipper was fused to the extracellular domain of murine CD40L (including its stalk), and cloned into the pcDNA3.1 expression vector.
- 2-trimer soluble CD40L (pAcrp30-CD40L):** The body of murine Acrp30 was fused to the extracellular domain of murine CD40L. Acrp30 is a V-shaped molecule with two trimeric arms that can present two trimeric TNFSF extracellular domains.
- 4-trimer soluble CD40L and GITRL (pSP-D-CD40L and pSP-D-GITRL):** The body of murine surfactant protein D (SP-D) was fused to the extracellular domains of murine CD40L or GITRL (including their stalks). SP-D is a plus-sign shaped molecule with four trimeric arms that can present four trimeric TNFSF extracellular domains.

## VACCINE METHODS

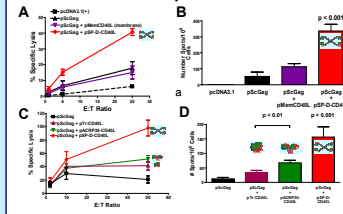
**Vaccine plasmids:** A plasmid for secreted, codon-optimized HIV Gag (pScGag) was used as the test antigen [7]. Similar results were obtained with an HIV envelope plasmid and a plasmid for the MSP-1 protein of *Plasmodium yoelii*.

**Mouse vaccinations:** BALB/c mice were injected i.m. in both quadriceps every other week X 3 with a combination of pScGag (80 µg) plus CD40L plasmid or empty control vector (20 µg).

**Immunoassays:** Two weeks after the last vaccination, serum was collected and the mice were euthanized. Splenocyte CD8+ T cell activity was determined by CTL activity and IFN- $\gamma$  ELISpot assay using peptide-pulsed P815 cells. CD4+ T cell proliferative responses to Gag (p24) protein were determined by 3H-TdR incorporation. Cytokine production was detected using IFN- $\gamma$  and IL-4 ELISAs. Antibody titer was determined by ELISA using Gag-coated plates.

## SOLUBLE, MULTIMERIC CD40L ADJUVANTS A "CD8-FOCUSED" VACCINE RESPONSE

Fig. 2. CD40L valence (1 < 2 < 4 trimers) is important for its vaccine adjuvant effects.



### CD8+ T cell Responses

**Panel A – 4-trimer soluble CD40L dramatically enhances the cytotoxic CD8+ T cell response to a DNA vaccine.** Plasmid DNA for secreted, codon-optimized Gag was the antigen plasmid. As judged by others, the addition of a plasmid for full-length, membrane CD40L (pMemCD40L) had no enhancing effects. In contrast, a plasmid for 4-trimer soluble CD40L (pSP-D-CD40L) dramatically increased the CTL response to Gag. Splenic CTLs were measured after re-stimulation for 5 days and tested for killing of P815 targets pulsed with the H-2K<sup>b</sup> immunodominant peptide, AMGLMKEITL.

**Panel B – 4-trimer soluble CD40L dramatically increases the frequency of IFN- $\gamma$ -producing CD8+ T cells generated in response to a DNA vaccine.** From the same experiment as Panel A, splenocytes were tested immediately after harvest in an overnight IFN- $\gamma$  ELISpot assay using H-2K<sup>b</sup> P815 stimulator cells pulsed with Gag peptide. Again, membrane CD40L showed little or no adjuvant activity, whereas a plasmid for 4-trimer soluble CD40L (pSP-D-CD40L) significantly increased the CD8+ T cell response.

**Panel C – The number of CD40L trimers determines the strength of the cytotoxic CD8+ T cell response to a DNA vaccine.** As judged by CTL assay, 1-trimer CD40L (pTr-CD40L) similar in design to the immunex protein, sCD40L) was a weak adjuvant for the CD8+ T cell response to a DNA vaccine. 2-trimer CD40L (pAcrp30-CD40L) was more effective, but 4-trimer CD40L (pSP-D-CD40L) was consistently the most active molecular adjuvant.

**Panel D – The number of CD40L trimers determines the frequency of IFN- $\gamma$ -producing CD8+ T cells generated in response to a DNA vaccine.** As in Panel B, an IFN- $\gamma$  ELISpot assay was used to measure freshly harvested splenocytes that produced IFN- $\gamma$  in response to MHC class I peptide. Again, the efficacy of the CD40L molecular adjuvants was directly related to their valence, 1-trimer < 2-trimer < 4-trimer.

### CD4+ T cell Responses

CD4+ T cell responses were measured as proliferation of whole spleen cells in response to exogenous protein (Gag). Consistently, no significant increase was found when any of the CD40L adjuvants were added to DNA vaccination (see Fig. 3, Panel C for an example). Also, minimal amounts of IFN- $\gamma$  or IL-4 were produced by these splenocyte cultures.

### Antibody Responses

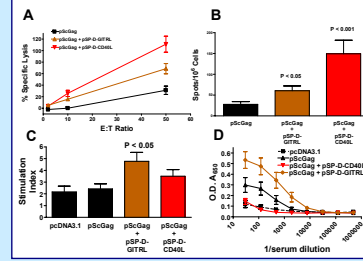
While the pScGag DNA vaccine induced modest levels of IgG antibodies, none of the soluble CD40L constructs enhanced IgG production. In fact, 4-trimer soluble CD40L (pSP-D-CD40L) suppressed anti-Gag IgG responses (see Fig. 3, Panel D, consistent with other reports that CD40 stimulation arrests plasma cell differentiation [8]).

### Lack of Toxicity

Mice appeared normal throughout the vaccination experiments. The histology of the i.m. injection sites 48 hours after vaccination showed no inflammation, and lung histology was normal at the conclusion of the experiments. Unlike many other adjuvants, spleen size and cell numbers were not increased by pSP-D-CD40L. Furthermore, when the pScGag antigen plasmid and the pSP-D-CD40L adjuvant plasmid were not mixed but instead were injected separately into opposite quadriceps, there was no vaccine response, indicating that pSP-D-CD40L (unlike TLR agonists) does not induce systemic immune activation.

## SOLUBLE, MULTIMERIC GITRL ADJUVANTS ALL LIMBS OF THE IMMUNE RESPONSE

Fig. 3. Plasmid DNA for SPD-GITRL is active as a vaccine adjuvant for CD8+ T cell, CD4+ T cell, and IgG responses.

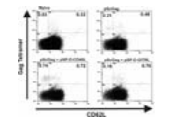


**GITRL effects on CD8+ T cell responses:** The addition of a 4-trimer soluble GITRL to DNA vaccination significantly increased CTLs (Panel A) and IFN- $\gamma$ -producing cells as measured by ELISpot (Panel B). However, these CD8+ T cell responses were not as strong as those produced using 4-trimer CD40L as a molecular adjuvant.

**GITRL effects on CD4+ T cell responses:** Unlike CD40L, 4-trimer GITRL significantly increased the proliferative response of whole spleen cells to Gag protein (Panel C). **GITRL effects on IgG responses:** Using ELISA with plates coated with Gag protein, 4-trimer GITRL significantly augmented anti-Gag IgG following DNA vaccination (Panel D).

## LONG-TERM MEMORY CD8+ T CELLS ARE GENERATED BY SOLUBLE, MULTIMERIC CD40L AND GITRL

Fig. 4. Long-lived, tetramer<sup>+</sup> CD8+ T cells measured 3 months after vaccination.



Splenocytes were taken from vaccinated mice that had been rested for 3 months, stained with PerCP-anti-CD8, FITC-anti-CD62L, and PE-H-2K<sup>b</sup> peptide tetramer, and analyzed by gating on CD8+ cells. The 4-trimer CD40L adjuvant increased both CD62L<sup>hi</sup> and CD62L<sup>lo</sup> cells, whereas GITRL was especially effective at generating CD62L<sup>hi</sup> cells (the phenotype of central memory cells).

## CONCLUSIONS

- Soluble, multimeric CD40L is a powerful molecular adjuvant for DNA vaccines (Fig. 2), and generates an unusual "CD8-focused" immune response that may be ideal for an HIV vaccine [6].
- Soluble, multimeric GITRL is a novel molecular adjuvant that generates a more well-rounded response to DNA vaccination, and enhances CD4+ T cell, CD8+ T cell, and IgG responses (Fig. 3).
- The CD8+ T cell responses induced by soluble, multimeric CD40L and GITRL are long-lived (Fig. 4), unlike the responses to many other adjuvants.
- These new molecular adjuvants may be even more effective when combined with other methods for DNA vaccination, TLR agonists, and/or boosting with viral vector vaccines.

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