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Macaque Multimeric Soluble CD40 Ligand and GITR Ligand Constructs Are Immunostimulatory Molecules In Vitro[∇]

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CD40 ligand (CD40L) and GITR ligand (glucocorticoid-induced tumor necrosis factor receptor-related protein ligand [GITRL]) are tumor necrosis factor superfamily molecules that can be used as vaccine adjuvants. In a previous human immunodeficiency virus (HIV) DNA vaccine study in mice, we found that plasmids expressing multimeric soluble forms of trimeric CD40L (i.e., many trimers) were stronger activators of CD8⁺ T-cell responses than were single-trimer soluble forms or the natural membrane-bound molecule. This report describes similar multimeric soluble molecules that were constructed for studies in macaques. Both two-trimer and four-trimer forms of macaque CD40L were active in B-cell proliferation assays using macaque and human cells. With human cells, four-trimer macaque GITRL costimulated CD4⁺ T-cell proliferation and abrogated the immunosuppressive effects of CD4⁺ CD25⁺ regulatory T cells on a mixed leukocyte reaction. These molecular adjuvants provide new tools for vaccine development in the simian immunodeficiency virus system and other macaque models.

Adjuvants can play an important role in the response to vaccination. This is particularly true of DNA vaccines, where the inclusion of cytokine or chemokine molecules has a significant effect on vaccine-induced immune responses (1, 3, 7, 18). The tumor necrosis factor superfamily (TNFSF) contains many immunologically active proteins that have significant potential as molecular adjuvants (5, 16, 19, 41). One of the most immunologically important TNFSF proteins is CD40 ligand (CD40L, also called CD154 or TNFSF5) (26), which is important for activating dendritic cells to induce CD8⁺ T-cell responses (4, 27, 29, 32), especially CD8⁺ T-cell memory responses (13).

While a soluble form of human CD40L (22) was found to be active in a phase I clinical trial for cancer (39), it would be useful to test additional forms of this molecule. In particular, it has been reported that a single-trimer form of soluble CD40L is much less active than multimeric soluble forms of this molecule (10, 11). We recently studied plasmids with three different forms of CD40L (one trimer of CD40L, two trimers of CD40L, and four trimers of CD40L) as adjuvants for a human immunodeficiency virus (HIV) DNA vaccine in mice. The results showed that vaccine-induced CD8⁺ T-cell responses were directly related to the valence of the number of trimers in the CD40L molecular adjuvant (4 > 2 > 1) (35).

Another immunostimulatory TNFSF molecule is glucocorticoid-induced TNF family-related receptor (GITR) ligand

(GITRL, also called TNFSF18), which is the activator of GITR. GITRL plays an important role in reversing the immunosuppressive effects of CD4⁺ CD25⁺ regulatory T cells (T_{reg} cells) which constitutively express GITR on their surface (28). In addition, GITRL is a costimulatory signal for antigen-responsive T cells (14, 36). In our previous mouse study, we found that surfactant protein D (SP-D)-GITRL was also effective as a molecular adjuvant for an HIV DNA vaccine and augmented CD4⁺ T-cell proliferative responses, CD8⁺ T-cell responses, and antibody responses (35).

Our previous results for murine immunization suggested that these molecular adjuvants could be useful in a DNA vaccine against HIV (35). To advance this molecular design to the simian immunodeficiency virus (SIV)/macaque model of HIV infection and macaque models of other human diseases, multimeric soluble CD40L and GITRL were constructed using macaque sequences (*Macaca mulatta*). As scaffolds for the two-trimer and four-trimer proteins, Acrp30 (adiponectin) and pulmonary surfactant protein D were used as previously described. In addition, the full-length coding sequence of macaque GITRL was determined. Using recombinant DNA techniques, three plasmids were produced: pmacAcrp30-CD40L (two trimers of CD40L), pmacSP-D-CD40L (four trimers of CD40L), and pmacSP-D-GITRL (four trimers of GITRL). The transfection of these plasmids into 293T cells led to the secretion of molecules of the expected size and immunoreactivity. macAcrp30-CD40L and macSP-D-CD40L were highly active in a human B-cell proliferation system. In addition, macSP-D-GITRL was active as a costimulatory molecule for human CD4⁺ T-cell proliferative responses and reversed the immunosuppressive effects of CD4⁺ CD25⁺ regulatory T cells

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TABLE 1. Oligonucleotide primers for the reverse transcription and amplification of mRNA coding sequences for macaque SP-D, Acrp30, and GITRL^a

Primer	Sequence	Source and accession no.	Location
macSP-D FWD	5'-GCTGCTAGCATGCTGCTCTTCTCCTCTCTGC-3'	GenBank NM_003019	172–194
macSP-D REV	5'-GACTTCCTGGCCAAACTCCTGG-3'	Trace MQAD1078F16.b1	170–191
macSP-D Nested REV	5'-GCTGTCTAGACCAAGCACTGCCCTACCCACC-3'	Trace MQAD1078F16.b1	193–214
macAcrp30 FWD	5'-GCTGCTAGCATGTTGCTGGGAGCTGTTCTACTGC-3'	GenBank AF404407	1–24
macAcrp30 REV	5'-TCCTAACTGTACTGAAAGCCTTTAATTGAC-3'	GenBank NM_004797	891–863
macAcrp30 Nested REV	5'-GCTGTCTAGAGACTTTGGGGCTGTTTGGCCTG-3'	GenBank NM_004797	865–844
macGITRL FWD	5'-CAGCTCTTATTTCTCCAAAAATGTGTTTGAGC-3'	Trace RHJHK27TF	650–681
macGITRL REV	5'-CCACCCACTGGCACCTCTG-3'	GenBank BV209630	456–438
macGITRL Nested FWD	5'-GCTGCTAGCATGTGTTTGGAGCCACTTGGAAAATATGCC-3'	Trace RHJHK27TF	670–698
macGITRL Nested REV	5'-GCTGTCTAGACCTCTGCATGTGCTGAAGGGAAT-3'	GenBank BV209630	444–421

^a Where possible, primer sequence was obtained from annotated macaque mRNA sequence data. Alternately, primers were designed from unannotated sequence data available from the NCBI macaque trace sequence archive (Trace) or annotated sequence for the human orthologs.

in a human mixed leukocyte reaction (MLR) assay. These new macaque molecular constructs should be useful for macaque studies of vaccines against SIV and other infectious pathogens.

MATERIALS AND METHODS

Gene cloning. Macaque lung and adipose tissue were obtained under a protocol approved by the Institutional Animal Care and Use Committee of the National Cancer Institute. These tissues were dissected, placed in a 50-ml tube containing 25 ml RNA Later (QIAGEN, Valencia, CA), and stored at -20°C until use. Lung mRNA was extracted using the RNeasy Midi kit (QIAGEN), and adipose tissue mRNA was extracted using the RNeasy Lipid Tissue Midi kit (QIAGEN). Gene-specific cDNA was synthesized from 100 pg of extracted RNA using the RobusT I reverse transcriptase PCR (RT-PCR) kit (Finnzymes, Espoo, Finland). RNase inhibitor (RNasin Plus; Promega, Madison, WI) (0.5 μl) was included in the RT-PCR mix. Gene-specific primers were developed using sequence data from annotated macaque mRNA sequences, mRNA sequences from human orthologs, or unannotated sequences from the ongoing *Macaca mulatta* genome project. Forward and reverse primers for the RT-PCR are detailed in Table 1. The primer sets for reverse transcription and initial PCR amplification included macSP-D FWD and macSP-D REV for SP-D, macAcrp30 FWD and macAcrp30 REV for Acrp30, and macGITRL FWD and macGITRL REV for GITRL. The resulting PCR products were diluted 2,000-fold and amplified by nested or heminested PCR using PfuUltra Hot Start Master mix (Stratagene, La Jolla, CA). Primer sets are outlined in Table 1 and included the following: macSP-D FWD and macSP-D Nested REV for SP-D (heminested PCR), macAcrp30 FWD and macAcrp30 Nested REV for Acrp30 (heminested PCR), and macGITRL Nested FWD and macGITRL Nested REV for GITRL (nested PCR). The resulting PCR products were cloned in the pTOPO4 plasmid vector (Invitrogen, San Diego, CA), and at least three independent clones were sequenced on both strands for each gene construct. The macaque Acrp30 sequence was identical to a previous macaque clone (GenBank accession number AF404407) (12). To align macaque amino acid sequences to their human and mouse orthologs, the following protein sequences were used for comparison: those for human SP-D (GenBank accession number CAA46152) (20), mouse SP-D (GenBank accession number AAA92021) (23), human GITRL (GenBank accession number AAD22634) (8), and the mouse GITRL allele protein from strain CBA/Ca (GenBank accession number NP_899247) (15).

Recombinant DNA constructs. Plasmids encoding macaque SP-D, Acrp30, and GITRL (see above) and macaque CD40L (previously cloned by the Resource for Nonhuman Primate Immune Reagents [http://pathology.emory.edu/villinger/index.htm] [GenBank accession number AF344859]) were diluted 1,000-fold and used as templates for the construction of recombinant CD40L and GITRL genes. The expression vector for all constructs was pVAX1 (Invitrogen), a kanamycin-resistant vector that meets the FDA's Points To Consider for DNA vaccines (33). To construct the plasmid for a two-trimer soluble form of macaque CD40L (pmacAcrp30-CD40L), overlapping PCR primers were used to fuse macaque Acrp30 with the extracellular domain of macaque CD40L. The resulting sequence contained the corresponding macaque amino acid sequence to the murine version of Acrp30-CD40L (35). A two-amino-acid linker was placed at the fusion junction (11), and only the TNF-like portion of CD40L (without the membrane-proximal stalk) was included. The amino acid sequence around the

fusion junction was KGEPGE/LQ/GDQNPQIA, where the N-terminal portion is from macaque Acrp30 (amino acids 1 to 106 of GenBank sequence AAK92202), LQ (DNA sequence CTC CAG) is the linker, and the C-terminal portion is the TNF-like domain of macaque CD40L (amino acids 116 to 261 of GenBank sequence AAK37541).

To construct the plasmid coding for a four-trimer soluble form of macaque CD40L (pmacSP-D-CD40L), overlapping PCR primers were used to fuse the N-terminal collagen-like domain of SP-D to the entire extracellular domain of macaque CD40L, including the membrane-proximal stalk. The resulting sequence was comparable to the murine SP-D-CD40L sequence (35). The amino acid sequence around the junction between SP-D and macaque CD40L was KVELFPNG/HRRLDKIE, where the N-terminal portion is from SP-D (amino acids 1 to 257 of GenBank sequence ABE68875) and the C-terminal portion is the extracellular sequence of macaque CD40L (amino acids 48 to 261 of GenBank sequence AAK37541).

To construct the plasmid coding for a four-trimer soluble form of macaque GITRL (pmacSP-D-GITRL), overlapping PCR primers were used to fuse the collagen-rich domain of SP-D to the entire extracellular domain of macaque GITRL. The amino acid sequence around the junction between SP-D and GITRL was KVELFPNG/LQLETAKE, where the N-terminal portion is from SP-D (amino acids 1 to 257 of GenBank sequence ABE68875) and the C-terminal portion is the extracellular sequence of macaque GITRL (amino acids 49 to 177 of GenBank sequence ABE68876).

Western blots. 293T cells were transiently transfected with the plasmid constructs using Lipofectamine 2000 (Invitrogen). Forty-eight hours later, supernatants were centrifuged and filtered. Antibodies for biotinylated mouse anti-human CD40L (clone 24-31; Ancell, Bayport, MN) or mouse anti-human GITRL (clone BAM-6943; R&D Systems, Minneapolis, MN) were bound to streptavidin-coated magnetic beads (Dyna Beads, LLC, Brown Deer, WI) and used to pull down their respective proteins. The beads were then loaded onto 10% sodium dodecyl sulfate-polyacrylamide gels, electrophoresed, and blotted onto Immobilon-P membranes (Millipore, Billerica, MA). The membranes were blocked and then probed with goat anti-human CD40L (R&D Systems) or goat anti-human GITRL (R&D Systems), followed by horseradish peroxidase-conjugated anti-goat antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). The signal was developed onto X-ray film using chemiluminescence.

Human and macaque subjects. Studies using macaque blood cells were conducted under a protocol approved by the Emory University Institutional Animal Care and Use Committee. Studies using human blood were conducted under a protocol approved by the University of California, San Diego Human Research Protections Program.

Human B-cell proliferation assay. Peripheral blood mononuclear cells (PBMC) from healthy donors were isolated on Ficoll-Hypaque gradients (Amersham Biosciences, Piscataway, NJ). B lymphocytes were isolated by positive selection using anti-CD19 immunomagnetic beads (MACS B-cell isolation kit II; Miltenyi Biotec, Auburn, CA), following the manufacturer's instructions. The purity of the preparation was checked by flow cytometry analysis and was $>98\%$ in all experiments (data not shown). A total of 5×10^4 cells/well in a 96-well plate were incubated in RPMI 1640 supplemented with 10% autologous human serum, and 50 $\mu\text{g}/\text{ml}$ gentamicin sulfate (R10 medium). Supernatant from transiently transfected 293T cells was added to wells to a final volume of 200 μl . Transfection supernatants included macaque SP-D-CD40L, macaque Acrp30-CD40L,

human SP-D-CD40L, mutant T147N human SP-D-CD40L, and mock-transfected cells for comparison. CD40L concentrations were determined by an enzyme-linked immunosorbent assay, and an equivalent amount of immunoreactive protein was added to each well to yield a final concentration of approximately 2 $\mu\text{g/ml}$. After 4 days, wells were pulsed with 1 μCi of [^3H]thymidine and harvested onto glass filters 18 h later. Radioactive incorporation was measured by ^3H scintillation counting.

Macaque B-cell proliferation assay. PBMC were isolated from normal healthy macaques, resuspended at 10^7 cells/ml in phosphate-buffered saline (PBS) containing 3 μM carboxyfluorescein diacetate-succinimidyl ester (CFSE), and incubated at room temperature for 5 min. Then 10 ml of PBS containing 5% fetal bovine serum (PBS-5% FBS) was added to quench the reaction, and cells were washed with PBS containing 5% FBS and then resuspended at 10^7 cells/ml in RPMI 1640 containing 10% FBS.

Supernatants from transfected 293T cell cultures were prepared as described above except using Dulbecco's minimal essential medium containing 10% FBS. Three supernatants were tested: mock (supernatant from untransfected 293T cells), empty vector (pVAX1), and pmacSP-D-CD40L. One hundred microliters of supernatant was added to 1×10^6 CFSE-labeled PBMC in a total volume of 600 μl . Pokeweed mitogen was used as a positive control. Cultures were incubated for 72 h, and then the cells were washed with PBS-2% FBS and stained for flow cytometry. The following antibodies were used: phycoerythrin-conjugated anti-CD4, peridinin chlorophyll *a* protein-conjugated anti-CD8, and allophycocyanin-conjugated anti-CD20 (all from BD Pharmingen, San Diego, CA). Following staining, the cells were washed with PBS-2% FBS and fixed with 200 μl of 1% formaldehyde. Flow cytometry was performed using a FACSCalibur (BD Biosciences), and the data were analyzed using the FlowJo software package (Tree Star, Inc., Ashland, OR).

Human CD4⁺ T-cell proliferation assays. Anti-CD3 antibody-coated 96-well plates were prepared as described previously (34). Briefly, goat anti-mouse immunoglobulin G antibody (Jackson ImmunoResearch) was diluted 1:100 in 50 mM carbonate buffer (pH 9.5) and incubated at 37°C for 90 min on non-tissue-culture-treated 96-well plates (100 μl per well). Plates were washed twice with PBS and blocked with PBS containing 2% human serum for 30 min at 37°C. Anti-CD3 monoclonal antibody (MAB) (R&D Systems) was added at a concentration ranging from 0 to 100 ng/ml, and plates were incubated overnight at 4°C. The next day, human PBMC were isolated on a Ficoll-Hypaque gradient (Amersham Biosciences). The CD4⁺ lymphocyte population was isolated using a Dynal CD4⁺ negative selection kit (Dynal Biotech, Brown Deer, WI). Cells were resuspended at 2×10^6 cells/ml in R10 medium. Unbound anti-CD3 antibody was removed from the plates, and 100 μl of CD4⁺ T cells was added to each well. Protein samples were then added in a volume of 100 μl . Plates were incubated at 37°C in 5% CO₂. After 4 days, 1 μCi of [^3H]thymidine was added, and cells were harvested 18 h later. Radioactive incorporation was measured by ^3H scintillation counting.

Human CD4⁺ CD25⁺ regulatory T-cell inhibition assay. Plates (96 wells per plate) coated with anti-CD3 MAB (R&D Systems) were prepared as described above at an antibody concentration of 10 ng/ml, and plates were incubated overnight at 4°C. The next day, PBMC from two subjects were isolated on Ficoll-Hypaque gradients (Amersham Biosciences).

Allogeneic stimulator cells for a mixed leukocyte reaction were prepared from PBMC from subject 1 and depleted of T cells using fluorescein isothiocyanate (FITC)-conjugated anti-human CD3 antibody (BD Pharmingen) and MACS anti-FITC microbeads (Miltenyi Biotec). These T-cell-depleted PBMC were irradiated with 4,000 rads and resuspended at 1×10^6 cells/ml in complete medium (RPMI 1640 containing 10% human serum and 20 $\mu\text{g/ml}$ gentamicin sulfate).

As responder cells for an MLR, the CD4⁺ lymphocyte population from subject 2 was isolated using a Dynal CD4⁺ negative selection kit (Dynal Biotech). To separate the CD4⁺ CD25⁺ and CD4⁺ CD25⁻ populations, CD4⁺ cells were incubated with a 1:20 dilution of FITC-conjugated anti-CD25 antibody (BD Pharmingen), and the two populations were separated using MACS anti-FITC microbeads (Miltenyi Biotec), following the manufacturer's instructions. Both CD4⁺ T-cell populations were resuspended at 4×10^5 cells/ml in complete medium. Wells received 50 μl of irradiated T-cell-depleted PBMC and either 25 μl of CD4⁺ CD25⁻ cells plus 25 μl of medium, or 25 μl each of CD4⁺ CD25⁻ and CD4⁺ CD25⁺ cells. One hundred microliters of 293T cell supernatant was added to each well. Cells were incubated at 37°C in 5% CO₂. After 4 days, 1 μCi of [^3H]thymidine was added, and cells were harvested 18 h later. Radioactive incorporation was measured by ^3H scintillation counting.

Nucleotide sequence accession numbers. Novel macaque sequence data were submitted to the NCBI for SP-D (GenBank accession number DQ457096) and GITRL (GenBank accession number DQ457097).

RESULTS

Cloning of macaque proteins. In order to develop recombinant macaque-specific TNFSF ligand molecules, total mRNA was extracted from macaque lung and adipose tissue. The entire regions coding for macaque Acrp30, SP-D, and GITRL were isolated by RT-PCR and cloned into the pTOPO4 plasmid vector. The nucleic acid sequence for the Acrp30 coding region was identical to the previously reported macaque sequence (GenBank accession number AF404407) (12). The macaque SP-D amino acid sequence was found to be 95% identical to the human sequence (GenBank accession number CAA46152) and 74% identical to the mouse sequence (GenBank accession number AAA92021) (Fig. 1A). Similarly, the macaque GITRL amino acid sequence was found to be 97% identical to the human sequence (GenBank accession number AAD22634) and 48% identical to the mouse sequence (GenBank accession number NP_899247) (Fig. 1B).

Construction and expression of soluble macSP-D-CD40L, macAcrp30-CD40L, and macSP-D-GITRL proteins. The macaque Acrp30, SP-D, and GITRL genes, along with a previously cloned macaque CD40L gene (37), were used as templates to construct recombinant proteins macSP-D-CD40L, macAcrp30-CD40L, and macSP-D-GITRL (Fig. 2). Overlap PCR was used to create these fusion proteins as described in Materials and Methods, and the final constructs were cloned into the pVAX1 expression vector.

To prepare protein from these plasmids, 293T cells were transiently transfected and their supernatants were collected 48 h later. A two-step Western blot procedure was used where the supernatants were precipitated using neutralizing monoclonal antibodies that recognize correctly folded human orthologs of these proteins. Polyacrylamide gel electrophoresis separation was performed after the proteins were boiled in the presence of the reducing agent beta-mercaptoethanol, and single bands were seen at positions that corresponded to the molecular masses of the respective monomers (Fig. 3). These bands were absent from control pVAX1-transfected 293T cell supernatants. These results indicate that the plasmids express the expected proteins.

macSP-D-CD40L and macAcrp30-CD40L proteins induced human and macaque B-cell proliferation in vitro. To determine whether macSP-D-CD40L and macAcrp30-CD40L are biologically active, a B-cell proliferation assay was used. Immunomagnetically isolated CD19⁺ human B cells were incubated in the presence of the recombinant CD40L proteins, and interleukin 4 (IL-4) was also included as a proliferation cofactor in a parallel experiment (38). As shown in Fig. 4, the multimeric, soluble macaque CD40L proteins significantly increased human B-cell proliferation compared to medium alone. This proliferation was observed both in the presence and absence of IL-4, giving a *P* value of <0.01 for macSP-D-CD40L (with or without IL-4) compared to medium alone. For macaque Acrp30-CD40L protein, there was significant activity compared to medium alone with *P* values of <0.01 (without IL-4) and <0.05 (with IL-4). Human SP-D-CD40L showed a further threefold increase in proliferation above the macaque protein, which was abrogated by the mutation of CD40L at residue T147 (17). To confirm that CD40L was the active moiety producing B-cell activation, proteins were incubated

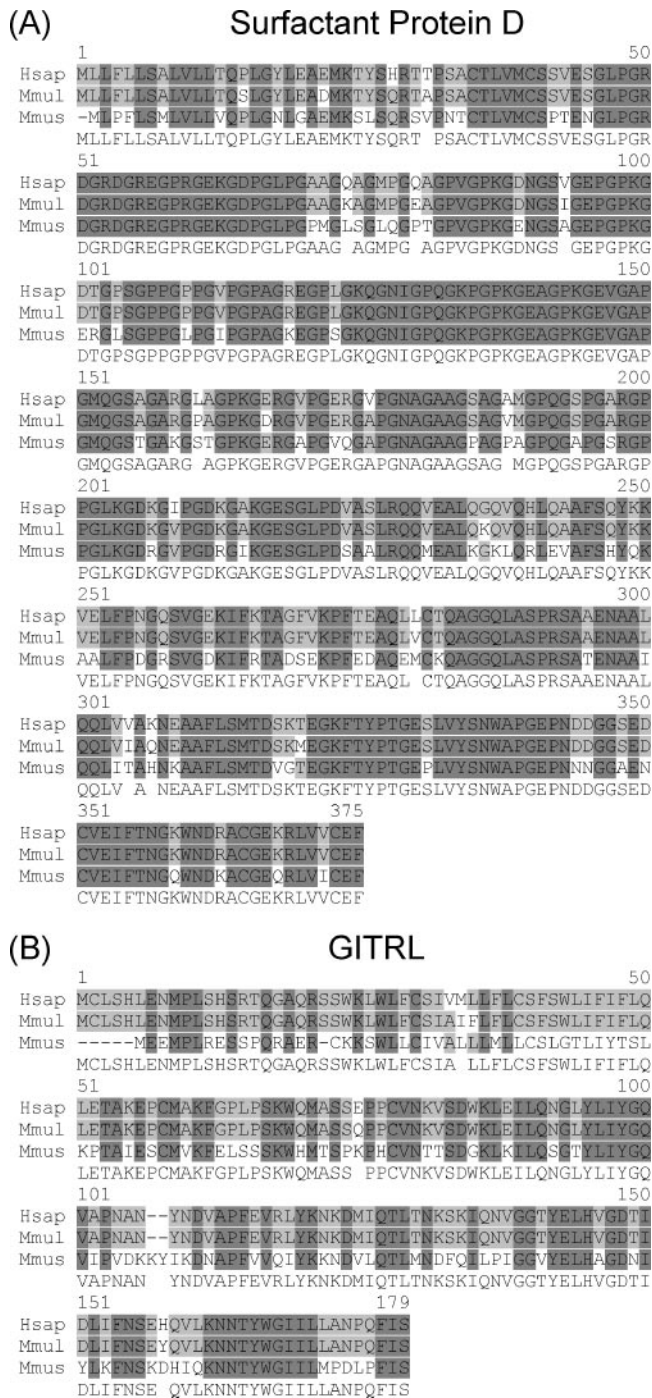


FIG. 1. Comparison of human, macaque, and mouse GITRL and surfactant protein D protein sequences. Sequence data from cloned macaque cDNA were compared to the known sequences of human and mouse genes. (A) Alignment of *Homo sapiens* (Hsap), *Macaca mulatta* (Mmul), and *Mus musculus* (Mmus) surfactant protein D amino acid sequences. (B) Alignment of *Homo sapiens*, *Macaca mulatta*, and *Mus musculus* GITRL amino acid sequences. Identity across all three species is highlighted with dark gray shading, and pairwise identity is highlighted with light gray shading. The consensus sequence is shown underneath each alignment. Gaps (dashes) were introduced to maximize alignment.

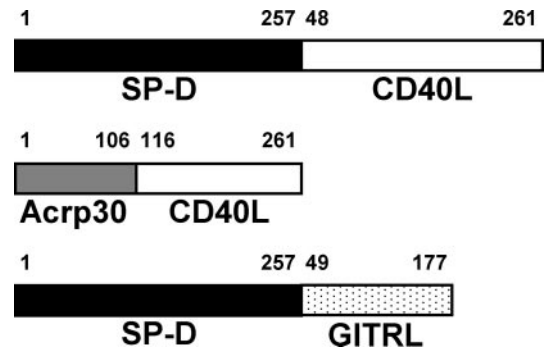


FIG. 2. Recombinant DNA constructs for pmacSP-D-CD40L, pmacAcrp30-CD40L, and pmacSP-D-GITRL. On the basis of the *in vivo* activities of mouse versions of the plasmids encoding SP-D-CD40L, Acrp30-CD40L, and SP-D-GITRL (35), similar constructs were made using the macaque orthologs. The numbers shown correspond to the amino acid residues of each component in the construct. For the macaque Acrp30-CD40L recombinant, a linker of lysine-glutamine was inserted between the two genes. SP-D-CD40L contained the full extracellular domain of CD40L, including the membrane-proximal stalk, while the Acrp30-CD40L gene encoded only the CD40L head group following the design of Holler et al. (11).

with anti-human CD40L MAb prior to incubation with B cells, and no significant activity was observed. Consistent with a protein-mediated response, boiling reduced the proliferation-stimulating capacity of the supernatants.

To confirm that macSP-D-CD40L is active on macaque cells, macaque PBMC were cultured with supernatants from mock-transfected, empty vector control-transfected, or pmacSP-D-CD40L-transfected 293T cells. As judged by CFSE dilution

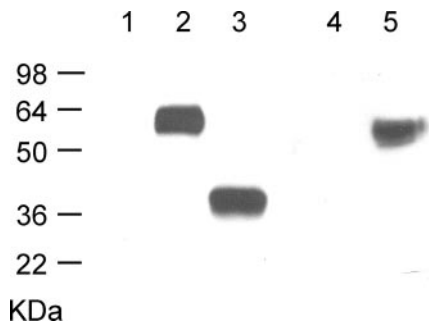


FIG. 3. Expression and secretion of macaque SP-D-CD40L, Acrp30-CD40L, and SP-D-GITRL from plasmid-transfected 293T cells. The plasmids described in the legend to Fig. 2 were transfected into 293T cells. The empty vector pVAX1 was used as a control transfection. A human CD40L-specific or human GITRL-specific monoclonal antibody was used to pull down macSP-D-CD40L and macAcrp30-CD40L or macSP-D-GITRL from their respective transfection supernatants. The immunoprecipitated proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted with polyclonal anti-human CD40L or anti-human GITRL antibodies. Molecular mass standards (in kilodaltons) were run for each Western blot and are combined into this montage image where lanes 1 to 3 were immunoblotted with antibody for human CD40L and lanes 4 and 5 were immunoblotted with antibody for human GITRL. Lanes 1 and 4 contain negative-control supernatants from pVAX1-transfected 293T cells. Lane 2 contains pmacSP-D-CD40L transfection supernatant, lane 3 contains pmacAcrp30-CD40L transfection supernatant, and lane 5 contains pmacSP-D-GITRL transfection supernatant.

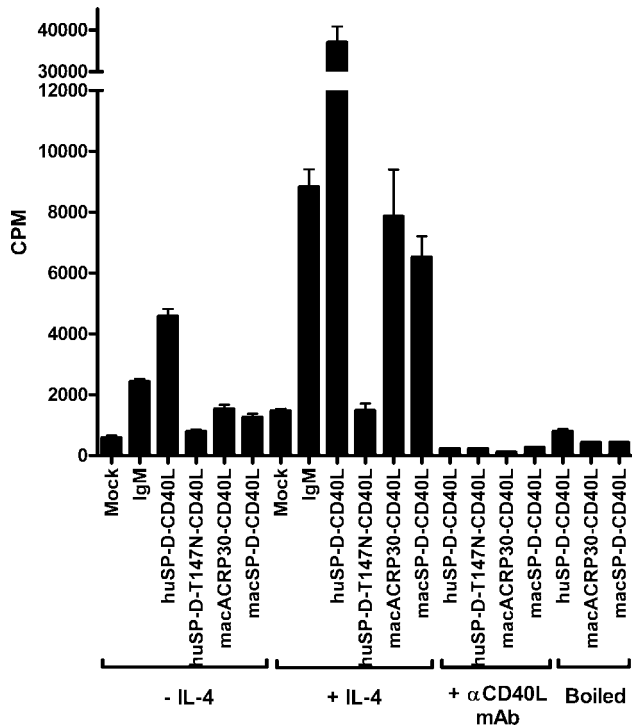


FIG. 4. Macaque SP-D-CD40L and Acrp30-CD40L induced human B-cell proliferation in vitro. CD19⁺ B cells were isolated from human PBMC and incubated for 5 days with supernatant from transfected 293T cells with (+) or without (-) 10 ng/ml human IL-4. B cells were incubated with 10 μg/ml immunoglobulin M (IgM) as a positive control. Mock-transfected 293T cell supernatant was used as a negative control. For selected samples, 2 μl of anti-human CD40L antibody (α CD40L mAb) was added to 100 μl of supernatant and incubated for 20 min at room temperature prior to use. Other supernatant samples were boiled for 10 min and cooled on ice for 5 min before being added. The level of proliferation was determined by [³H]thymidine incorporation, as measured by scintillation counting of cells harvested onto glass-fiber filters. Data are plotted as the means (plus SEMs [error bars]) of triplicate wells. huSP-D-CD40L, human SP-D-CD40L.

72 h later, macSP-D-CD40L induced cell division in 3.6% of CD20⁺ macaque B cells even in the absence of IL-4 (Fig. 5).

Macaque SP-D-GITRL protein costimulated human CD4⁺ T cells in vitro. GITR, the receptor for GITRL, is present on the surfaces of both effector (T_{eff}) and T_{reg} CD4⁺ T cells (31).

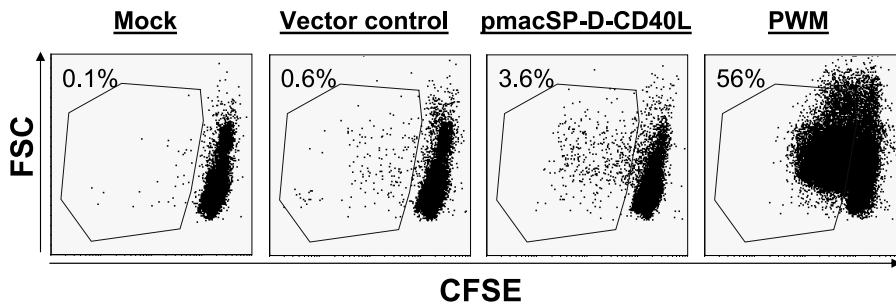


FIG. 5. Macaque SP-D-CD40L induced macaque B-cell proliferation in vitro. Macaque CFSE-labeled PBMC were cultured for 72 h in supernatants prepared from 293T cells that had either been mock transfected (mock) or transfected with empty vector (vector control) or pmacSP-D-CD40L. Pokeweed mitogen (PWM) was used as a positive control. Cells were stained for CD4, CD8, and CD20, and the CD4⁻ CD8⁻ CD20⁺ population was scored as B cells. Numbers on the graphs represent the frequencies of CFSE-negative cells as a percentage of total CD20⁺ cells. FSC, forward scatter.

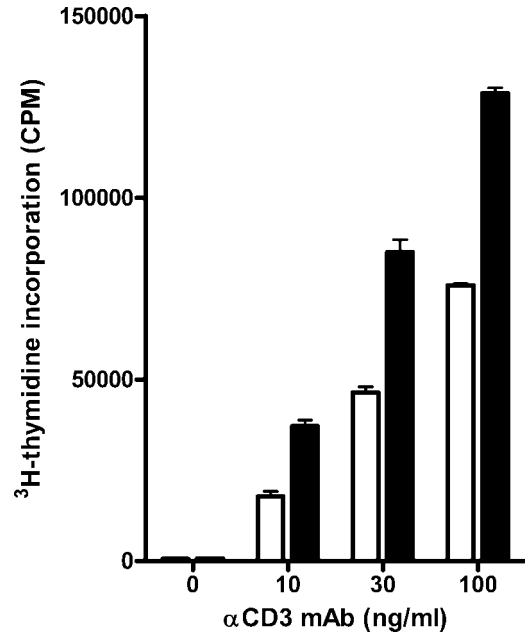


FIG. 6. Macaque SP-D-GITRL costimulated the proliferation of human CD3-activated CD4⁺ T cells. Supernatants from 293T cells transfected with plasmids expressing either β-galactosidase (LacZ) or macaque SP-D-GITRL were used as a source of test proteins. Human CD4⁺ T cells were added to wells coated with increasing concentrations of anti-CD3 antibody (αCD3 mAb) along with the test proteins. The level of proliferation was determined by [³H]thymidine incorporation, as measured by scintillation counting of cells harvested onto glass-fiber filters. The open bars represent cells treated with control LacZ 293T cell supernatant, and the filled bars represent cells treated with macaque SP-D-GITRL-containing 293T cell supernatant. Data are plotted as the means (plus SEMs [error bars]) of triplicate wells.

GITR stimulation leads to increased proliferation of T_{eff} cells when activated in combination with a costimulatory signal through CD3 (6). In order to confirm the in vitro activity of macaque SP-D-GITRL, supernatant from pSP-D-GITRL-transfected 293T cells was mixed with human CD4⁺ T cells and placed in wells coated with increasing concentrations of anti-human CD3 antibody. Supernatant from 293T cells transfected with a vector expressing the β-galactosidase protein was used as a control. Without anti-CD3 MAb, both control and SP-D-GITRL supernatants were unable to induce proliferation (Fig. 6).

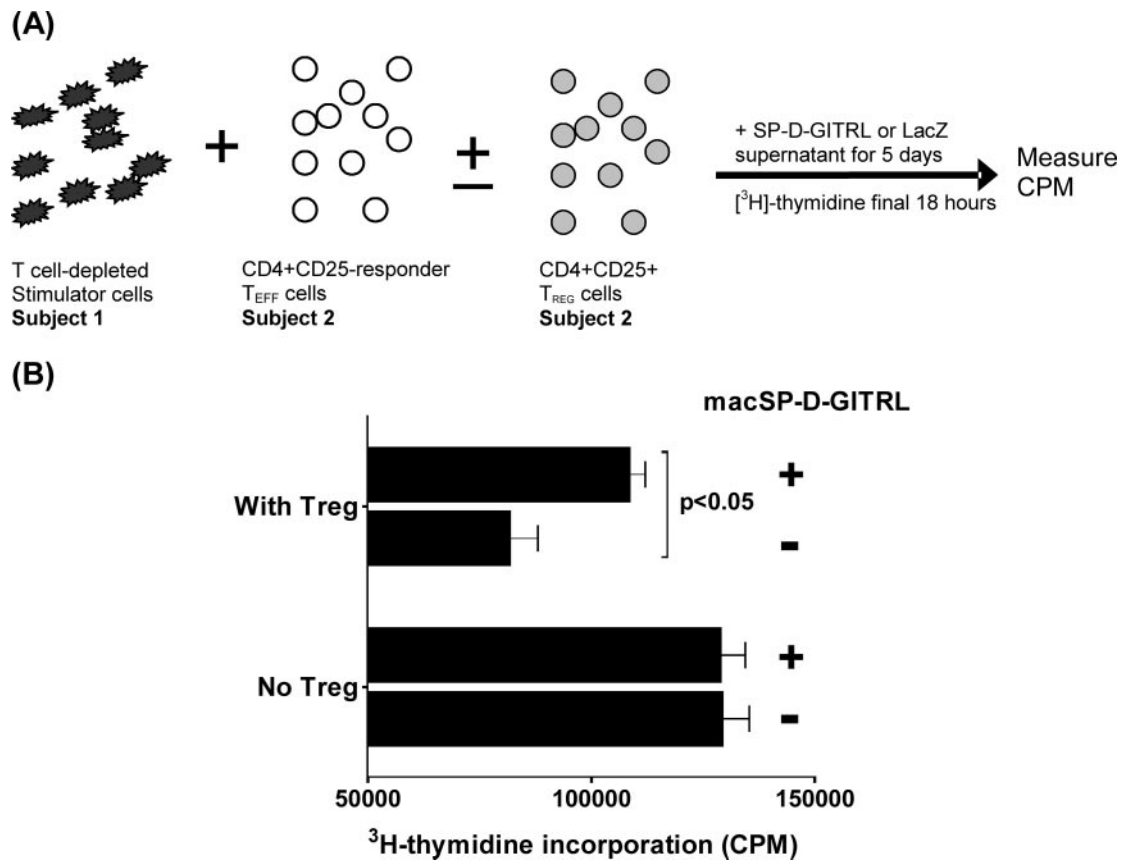


FIG. 7. Macaque SP-D-GITRL reversed human T_{reg}-cell suppression of a mixed leukocyte reaction. As MLR stimulator cells, PBMC from subject 1 were depleted of T cells and then irradiated. Responder CD4⁺ T cells were isolated from subject 2 and separated into CD25⁻ T_{eff} cells and CD25⁺ T_{reg} cells using immunomagnetic beads. To perform the assay for T_{reg}-cell immunosuppression, cultures were set up containing the irradiated allogeneic stimulator cells and T_{eff} cells with and without T_{reg} cells and cultured for 5 days on anti-CD3 antibody-coated plates with the indicated 293T cell transfection supernatants. The test supernatants were produced either by 293T cells transfected with an irrelevant β -galactosidase (LacZ) plasmid as a negative control or by 293T cells transfected with pmacSP-D-GITRL. [³H]thymidine was present for the last 18 h and used to assess proliferation of the nonirradiated CD4⁺ responder cells from subject 2. (A) Schematic diagram of the experimental protocol. (B) Proliferation of CD4⁺ responder cells in the absence or presence of CD4⁺ CD25⁺ T_{reg} cells (T_{reg}:T_{eff} = 1:1). The control 293T cell-LacZ supernatant is indicated by a minus sign. The 293T cell-macSP-D-GITRL supernatant is indicated by a plus sign. Data are plotted as the means (plus SEMs [error bars]) of triplicate wells, beginning with 50,000 cpm on the x axis to highlight the differences in proliferation.

However, anti-CD3 antibody-activated cells displayed increased proliferation, which was augmented with the addition of macaque SP-D-GITRL. When cells were costimulated with 10, 30, or 100 ng/ml anti-CD3 antibody, macaque SP-D-GITRL significantly increased CD4⁺ T-cell proliferation compared to the control ($P < 0.001$).

Macaque SP-D-GITRL abrogated the suppressive activity of human CD4⁺ CD25⁺ regulatory T cells. Studies of CD4⁺ CD25⁺ T_{reg} cells indicate that GITR activation abrogates T_{reg}-cell-mediated suppression (2, 31). To study the effect of macaque SP-D-GITRL on T_{reg}-cell activity, SP-D-GITRL-containing supernatant was incubated with either CD4⁺ CD25⁻ T_{eff} cells or a 1:1 mix of T_{eff} and T_{reg} CD4⁺ T-cell populations in a MLR using irradiated allogeneic stimulator cells and anti-CD3 costimulation. In the presence of control supernatant, total CD4⁺ T-cell proliferation (mean \pm standard error of the mean [SEM] of triplicate wells) was 129,235 \pm 6,140 cpm, and the addition of T_{reg} cells suppressed this to 81,665 \pm 6,423 cpm, which is 37% suppression compared to T_{eff} cells alone (Fig. 7). In the presence of macaque SP-D-GITRL, the T_{eff}-

cell proliferation values were 128,845 \pm 5,612 cpm without T_{reg} cells and 108,468 \pm 3,656 with T_{reg} cells, which is 16% suppression compared to the number obtained using T_{eff} cells alone ($P < 0.05$). Therefore, the reduction in T_{reg}-cell-mediated suppression from 37% to 16% in the presence of macSP-D-GITRL represents a 57% abrogation of T_{reg}-cell-mediated immunosuppression by this protein as gauged by this assay. Boiled macaque SP-D-GITRL supernatant was inactive, thereby ruling out any stimulatory effects from contaminants, such as lipopolysaccharide (data not shown). In a parallel experiment, SP-D-GITRL treatment of CD4⁺ CD25⁻ T cells in the absence of CD3 did not lead to increased proliferation (data not shown), consistent with the concept that GITRL is a costimulatory factor and not a complete T-cell stimulus (14).

DISCUSSION

Due to their close evolutionary relationship to humans, non-human primates have provided invaluable animal models for studying a wide variety of human clinical conditions, including

HIV infection and AIDS (9). While the human and monkey genomes are highly similar, the small differences noted between monkey and human protein sequences are often sufficient to elicit neutralizing antibodies when the human xenogenic reagents are given to monkeys (24, 25, 40). Circumventing this potential problem, this study reports three biologically active molecular adjuvants that were designed specifically for macaque vaccine studies. To this end, the nucleic acid sequences of the regions coding for macaque GITRL and SP-D were determined. Macaque SP-D was nearly identical to its human ortholog (Fig. 1A). Similarly, macaque GITRL differed in only five amino acid positions from its human ortholog (Fig. 1B). The sequence of macaque CD40L has been previously reported and is also nearly identical to its human ortholog (37). Nevertheless, the construction of fully autologous macaque proteins should avoid potential cross-species differences in biological activity and reduce or eliminate the possibility that these adjuvant molecules might elicit immune responses directed against themselves. For example, in the mouse system, we were unable to detect any antibodies against murine SP-D-CD40L when a plasmid for this protein was used in a DNA vaccine (35).

Plasmids for two forms of macaque soluble CD40L were produced (Fig. 2). pmacAcrp30-CD40L is anticipated to form a V-shaped molecule with two trimers of CD40L (11). pmacSP-D-CD40L is anticipated to form a four-armed cruciate molecule that has four trimers of CD40L (10). When these plasmids were transfected into 293T cells, proteins of the expected immunoreactivity and size were secreted as assessed by Western blotting (Fig. 3). To determine whether the 293T cell-produced proteins were active, both a human B-cell proliferation assay (30, 38) and a macaque B-cell proliferation assay were used. Both the two-trimer and four-trimer forms of macaque soluble CD40L were highly active on human B cells in the presence of IL-4 (Fig. 4), and four-trimer macaque CD40L was active on macaque B cells even in the absence of IL-4 (Fig. 5). These results support the prediction that these molecules will also be effective as dendritic cell activators and vaccine adjuvants.

It is of note, however, that the maximal proliferation of human B cells induced by macaque CD40L reagents appeared severalfold lower than that of a similar aliquot of B cells stimulated with the human CD40L reagent (Fig. 4), suggesting species-specific differences. Conversely, the use of a human CD40L trimer protein in the stimulation of macaque B cells versus human B cells required fivefold-higher levels of trimer to induce maximal proliferation of macaque B cells *in vitro*, and this proliferative activity was markedly lower than that exhibited by human B cells (data not shown).

A plasmid for a four-trimer form of macaque soluble GITRL was also produced as a fusion protein with SP-D. When this plasmid was transfected into 293T cells, a protein of the expected immunoreactivity and size was secreted as assessed by Western blotting (Fig. 3). To determine whether the 293T cell-produced protein was active, two assays were used. The first assay relied on the ability of GITR stimulation to serve as a costimulus for CD4⁺ T-cell proliferation in response to a T-cell receptor stimulus (14, 36). With human CD4⁺ T cells and immobilized anti-CD3 antibody, macSP-D-GITRL was clearly active in this costimulation assay (Fig. 6). The

second assay measured the ability of GITR stimulation to abrogate the immunosuppressive effects of CD4⁺ CD25⁺ regulatory T cells on CD4⁺ T-cell proliferation in a mixed leukocyte reaction (21, 31). With human cells from two subjects in this MLR-based assay, macSP-D-GITRL was found to be active in reversing CD4⁺ CD25⁺ regulatory T-cell immunosuppression (Fig. 7).

In conclusion, these plasmids for multimeric soluble CD40L and GITRL provide macaque reagents for use in a variety of experimental vaccine or immunotherapeutic studies. Given our recent findings that the murine forms of these molecules are strong molecular adjuvants for HIV DNA vaccination in mice, it will be interesting to determine whether the macaque forms of these plasmids can be used to augment immune responses to DNA vaccines in the SIV/macaque model as a preliminary step for their future clinical use.

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