

Brief Definitive Report**Identification of a Human OX-40 Ligand, a Costimulator of CD4⁺ T Cells with Homology to Tumor Necrosis Factor**

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*From the Department of Pathology, Stanford University School of Medicine, Stanford, California 94305; and *Becton Dickinson Immunocytometry Systems, San Jose, California 95131***Summary**

The human OX-40 cell surface antigen is a CD4⁺ T cell activation marker that acts as a costimulatory receptor and is a member of the nerve growth factor receptor/tumor necrosis factor (TNF) receptor family. Using a soluble form of the receptor, the extracellular region fused with human immunoglobulin Fc, we expression cloned the human OX-40 ligand cDNA from a library derived from an activated B lymphoblastoid cell line MSAB. The encoded protein is identified as gp34, a type II transmembrane antigen previously known to be expressed only by human T cell lymphotropic virus 1-infected cells. We describe gp34 as a new member of the TNF family, and find that the recombinant ligand expressed in COS cells costimulates phorbol myristate acetate, phytohemagglutinin, and anti-CD3-induced CD4⁺ T cell proliferation.

The human 106/OX-40 antigen is a 50-kd cell surface glycoprotein expressed primarily on activated CD4⁺ T cells (1–4, and Godfrey, W. R., D. Buck, F. F. Fagnoni, M. A. Harara, and E. G. Engleman, manuscript submitted for publication). The antigen is not expressed on freshly isolated PBMC or on purified subpopulations of PBMC, including B cells, monocytes, dendritic cells, CD4⁺, or CD8⁺ T cells. Few tumor cell lines have been shown to express this antigen, and those that are positive are dim with the exception of certain HTLV-I-infected T cell lines. The cDNA encoding the human OX-40 antigen has been cloned (3, 4, and Godfrey, W. R. et al., manuscript submitted for publication) and shown to be a member of the nerve growth factor receptor (NGFR)¹/TNFR gene family (5–7), which also includes the fas/APO-1, CD40, CD27, CD30, and 4-1BB/ILA antigens. The known functions of the members of this family include the regulation of growth control, apoptosis, and differentiation (7).

The activation and growth of CD4⁺ T cells is controlled by contextual costimulatory signals in addition to ligation of the antigen receptor (8, 9). It is becoming increasingly clear that there are multiple pathways in addition to the B7/CD28 interaction that can mediate costimulation (10, 11). However, relatively little is known about the molecular identity, tissue distribution, and signalling mechanisms of these other pathways. It is interesting to note that several members of the NGFR/TNFR family have been shown to act as costimulatory receptors, including CD27 (12), CD30 (13), 4-1BB (14, 15), and fas (16). We have recently demonstrated

that the human 106/OX-40 antigen can also act as a costimulatory receptor for CD4⁺ T cells, by cross-linking the antigen with either soluble or immobilized L106 antibody (Godfrey, W. R. et al., manuscript submitted for publication).

Putative ligands have been described for all the reported NGFR/TNFR family members, except for the OX-40 antigens (7). The reported ligands have been shown to constitute a family of molecules related to TNF, and can exist in membrane-bound forms. The identity of the natural human OX-40 ligand thus became a high priority in the attempt to better understand the function of the 106/human OX-40 receptor. Here we report the generation of a soluble receptor construct, the detection of ligand on activated EBV-transformed B cells, the identification of a human OX-40 ligand as gp34, and the characterization of the recombinant ligand as a costimulator of CD4⁺ T cell proliferation.

Materials and Methods

mAbs. The mAbs OKT3 (CD3), leu2a (CD8), 63D3 (CD14), leu11a (CD16), and L243 (HLA-DR) were prepared and purified in this laboratory. mAb L106-PE conjugate was produced by Becton Dickinson Immunocytometry Systems (San Jose, CA).

Peripheral Blood Cells, Cell Lines, and Activation. PBMC were isolated from healthy volunteer donors by using Ficoll-Hypaque density gradients. T cells were further purified by rosetting with neuraminidase-treated SRBC. B cells were further purified by passage of the nonrosetting cells over a Percoll gradient and collection of the high density fraction, followed by depletion of CD3 (OKT3),

CD16 (leu11a), and CD14 (63D3) positive cells using immunomagnetic beads (Dynal, Inc., Great Neck, NY). Monocytes were prepared by passage of PBMC over a Percoll gradient and recovery of the low density fraction. The cell line MSAB, an EBV-transformed B cell line, was originally derived at Cetus Corp. (Berkeley, CA). THP-1, a monocytic cell line, was obtained from American Type Culture Collection (Rockville, MD). The MT-2 and HUT-102 cell lines were obtained from the laboratory of Dr. Steven Fount of Stanford University. B cells, T cells, MSAB, and THP-1 were activated for 3 d with 5 ng/ml PMA (Sigma Chemical Co., St. Louis, MO) and 500 ng/ml ionomycin (Calbiochem-Novabiochem Corp., La Jolla, CA).

Fusion Proteins and Immunofluorescence. Soluble 106 antigen was constructed as a fusion protein, which consisted of the extracellular region of the 106Ag fused to the C region of the human Ig H chain IgG1 (106-Ig). The Ig-encoding fragment of 4-1BB-Ig (17), which contains the hinge, CH2, and CH3 domains, was subcloned into the 106 cDNA-containing plasmid. The Ig fragment was released by digestion with BamHI, filling in with Klenow polymerase, and subsequent digestion with EagI. This generated a fragment with blunt and NotI compatible ends, which was ligated into a SmaI/NotI-digested 106 plasmid. Soluble fusion proteins were produced by transient transfections of COS-7 cells, and the supernatants were recovered after 7 d. Control fusion proteins were produced by parallel transfections of the CDM7B⁻ vector (containing CD5 signal-Ig) (18), and the 4-1BB-Ig vector. Cells were stained with fusion protein in the presence of 10% goat serum and 5% fetal bovine serum, and binding was visualized by staining with PE-goat anti-human IgG, γ chain specific (American Qualex Antibodies & Immunochemicals Co., La Mirada, CA). Flow cytometry was performed using a Profile II (Coulter, Hialeah, FL).

cDNA Cloning. RNA was isolated from day 3 PMA/ionomycin activated MSAB cells. cDNA was synthesized (19) using superscript reverse transcriptase (GIBCO BRL, Gaithersburg, MD) and an oligo dT primer. The blunt cDNA was ligated to nonself-complementary BstXI adaptors and passed over a Sepharose S-500 sizing column to remove unligated adaptors and small fragments. The linked cDNA was then ligated into the pcDNA-I expression vector (Invitrogen, San Diego, CA), and electroporated into *Escherichia coli* strain MC1061/P3 (20, 21).

Subconfluent COS-7 cells were electroporated (22) with the plasmid cDNA library. After 48 h of culture, cells were recovered from the plates with PBS-EDTA solution and stained with 106-Ig. The rare antigen-positive COS cells were selected on goat anti-human Ig coated panning plates (23, 24). Plasmids were recovered by alkaline lysis and precipitation, and were electroporated back into bacteria. Bacteria transformed with plasmids recovered after the second round of COS cell selection were cloned and amplified. DNA purified from individual clones was electroporated into COS cells and tested for the ability to confer expression of the 106-ligand by immunofluorescence.

The cDNA encoding the 106-ligand was subcloned into Bluescript and sequenced by the dideoxy chain termination method using fluorescent M13 primers and an ALF sequencer (Pharmacia, Piscataway, NJ).

Immunoprecipitation. Day 3 PMA/ionomycin activated MSAB cells were labeled with ¹²⁵I using lactoperoxidase. Cell lysates were prepared by extracting with 1% NP-40 in Tris/saline containing protease inhibitors. 106-Ig and 4-1BB-Ig fusion proteins were incubated with the extracts, and the immune complexes were precipitated with goat anti-human conjugated Sepharose beads. The beads were eluted with SDS/ME sample buffer, and the eluent run on a 0.1% SDS, 5–15% polyacrylamide gradient gel (25).

Costimulation Assays. PBMC were isolated from healthy volunteer donors by using Ficoll-Hypaque density gradients. CD4⁺ T cells were further purified by rosetting with neuraminidase-treated SRBC, removal of nylon wool adherent cells, and depletion of CD8 (leu2a), CD16 (leu11a), and HLA-DR (L243) expressing cells by immunomagnetic bead selection (Dynal). 10⁵ CD4⁺ T cells were cultured per well in 96-well plates in AIM-V serum-free media (GIBCO BRL) at 37°C in a 8% CO₂ humidified atmosphere. The cells were incubated in flat-bottom plates in the presence of PMA, PHA, or soluble OKT3, and fixed COS-7 cells transfected with 106-ligand or empty pcDNA-I vector. COS cells were fixed at day 2 after transfection in 1% paraformaldehyde for 7 min at 25°C. Wells were pulsed with 1 μ Ci of [³H]thymidine for the final 18 h of culture and incorporated radioactivity was measured in a scintillation counter. Representative data are shown from four experiments using different donors. All results are expressed as mean cpm \pm SEM of triplicate cultures.

Results and Discussion

To screen for human 106/OX-40-ligand, a soluble 106Ag-Ig fusion protein (106-Ig) was constructed, and produced by transient transfections of COS-7 cells and harvesting supernatants. Using indirect immunofluorescence, screening of freshly isolated PBMC revealed no staining with the 106-Ig. Trace expression was noted on staining PHA or PMA/ionomycin activated T cells and PMA/ionomycin activated B cells, with <4% positive cells (data not shown). Staining of fresh, adherent, PMA or IFN- γ activated monocytes was negative. Screening of tumor lines revealed that the EBV-transformed B cell lines, MSAB and 9037, and a Burkitt lymphoma, Jiyoye, demonstrated trace reactivity, <5% positivity. However, after activation with PMA/ionomycin, the MSAB cell line dramatically increased its expression over 3–4 d, beginning on day 2 (Fig. 1 A). Subsequent rescreening of tumor cell lines after stimulation with PMA/ionomycin revealed that the monocytic cell line, THP-1, could also express ligand after 3–4 d (Fig. 1 A). Immunoprecipitation of ¹²⁵I surface-labeled activated MSAB cells with the 106-Ig fusion protein revealed a 32-kD antigen, with a minor band at 60 kD probably representing a nonreduced dimer (Fig. 1 B).

Using a cDNA library prepared from day 3 PMA/ionomycin activated MSAB cells, 106-Ig was used to select for cDNA clones that encode the 106-ligand. After two rounds of expression, immunoselection, and bacterial transformation, individual clones were isolated. Transfection of COS-7 cells with purified plasmid DNA from 3 out of 14 clones conferred high level expression of 106-Ig binding activity. This binding activity was shown to be specific, in that neither a soluble fc fragment of Ig nor an irrelevant fusion protein, 4-1BB-Ig, bound transfectants (Fig. 2 A).

Sequencing the three 1-kb 106-ligand cDNAs revealed that all three encode the same protein. This protein was found to be identical to gp34, an antigen previously known to be expressed only by HTLV-1-infected T cells, and reported to have a region of homology with L-lactate dehydrogenase (26–28). The only difference between the 106-ligand and gp34 cDNA clones is 101 bases of additional 5' untranslated region (UTR) found in two of the 106-ligand clones. The 106-

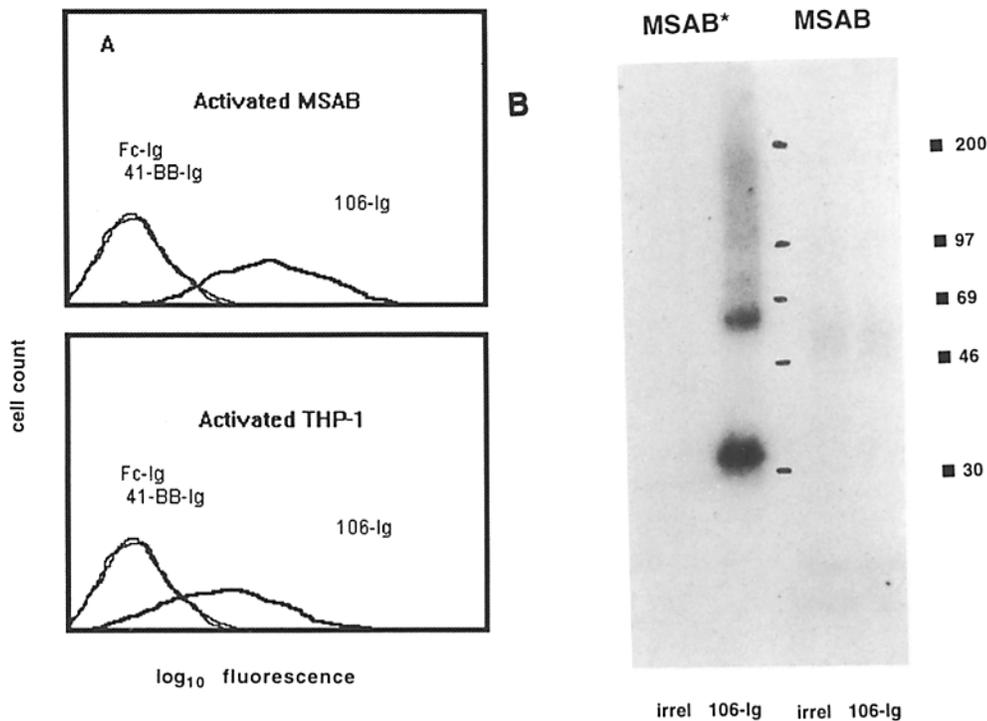


Figure 1. Expression of 106-ligand. (A) Activated MSAB and THP-1 cell lines. Cells were activated with 5 ng/ml PMA and 500 ng/ml ionomycin and stained after 72 h of culture. The cells were stained with Fc-Ig, 4-1BB-Ig, and 106-Ig, followed by PE-conjugated goat anti-human Ig. (B) Immunoprecipitation of 106-ligand. Resting and PMA/ionomycin activated MSAB cells were surface labeled with ^{125}I , and lysed in NP-40-containing buffer. Immune complexes were generated with 106-Ig or 4-1BB-Ig fusion proteins, and evaluated by SDS-PAGE on a reducing 5–15% gradient gel.

ligand/gp34 cDNA encodes a 183 amino acid (aa) polypeptide of the type II membrane configuration (Fig. 2 B). Alignment of the 133-aa extracellular domain with human TNF- α reveals 15% aa homology, and alignment with the emerging ligand family reveals primarily one core region of conservation (Fig. 2 C). There is also a remarkable conservation of the hydrophobicity or charged nature of certain aa residues. Analysis of secondary structure as predicted by the Chou and Fasman (29) algorithm reveals a propensity to form a large amount of beta structure. Also of note in the deduced mRNA, is the presence of a 58-bp AU rich region in the 3' UTR, including two AUUUA elements. Similar AU-rich motifs are frequently present in cytokine mRNAs, and have been shown to confer instability which is regulatable by cellular activation (30).

Cross-linking of the 106/OX-40 antigen on CD4⁺ T cells with recombinant 106-ligand generated costimulatory signals. COS-7 cells transfected with the 106-ligand cDNA-containing plasmid (COS/106-ligand) and were paraformaldehyde fixed and tested for their ability to costimulate proliferation. Cultures of CD4⁺ T cells activated with submitogenic doses of PMA (Fig. 3 A), soluble anti-CD3 antibody (Fig. 3 B), or submitogenic doses of PHA (Fig. 3 C), were indeed found to be costimulated by COS/106-ligand cells and not by COS cells transfected with empty vector. Moreover, soluble 106-Ig, but not 4-1BB-Ig, inhibited these responses (Fig. 3 D).

The 106-Ig fusion proteins were used to stain the HTLV-I-transformed cell lines, MT-2 and HUT-102. It is interesting to note that MT-2 cells stained brightly whereas HUT-102 cells stained minimally. In contrast, staining these cell lines with the L106 antibody revealed an inverted pattern of staining, in that MT-2 cells stained minimally and HUT-102 cells

stained brightly (Fig. 4). HTLV-I-infected cell lines represent the richest known sources of nonrecombinant 106/OX-40 antigen and 106-ligand, and the reciprocal pattern of staining suggests possible autocrine interactions.

Using a soluble receptor construct (106-Ig) we have cloned a ligand for the 106/OX-40 antigen, and found it to be identical to gp34. 106-ligand/gp34 expression is newly described as a late activation antigen on the EBV-transformed B cell line, MSAB, and on the monocytoid cell line, THP-1, after stimulation with PMA/ionomycin. By staining with 106-Ig, high level 106-ligand expression was detected on the HTLV-I-infected cell line MT-2, the cell line from which gp34 was originally identified and cloned (26). gp34 is here newly described as a member of the TNF family, and is demonstrated to mediate functional effects upon binding the 106/OX-40 antigen present on activated CD4⁺ T cells.

The *in vivo* distribution of the 106-ligand/gp34 remains unclear. Previous work with the anti-gp34 antibodies revealed no positive cell source after staining a broad panel of 106-ligand/gp34 on transformed or virally infected cell lines of three hematopoietic lineages, B cell, T cell, and monocytoid. However, staining of resting or activated peripheral blood B cells, T cells, and monocytes has yielded only trace positive staining. This extremely restricted pattern of expression of 106-ligand suggests that there are stringent requirements for the activation of the 106-ligand/gp34 enhancer in nontransformed cells. Alternatively, the 106-ligand/gp34 could be expressed primarily as a soluble molecule. It is interesting to note that the immunogen used to generate the first anti-gp34 antibody was a glycoprotein fraction derived from the supernatant of MT-2 cells (26). In addition, by dot blot we find evidence of soluble ligand in the supernatants of transfected

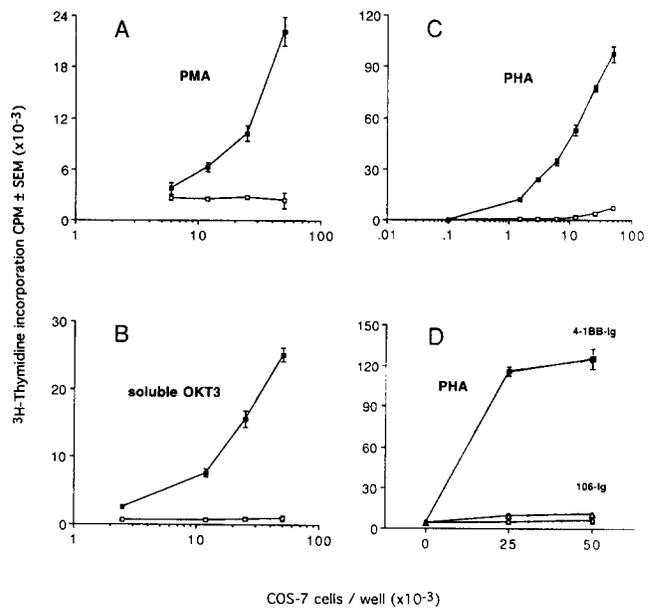
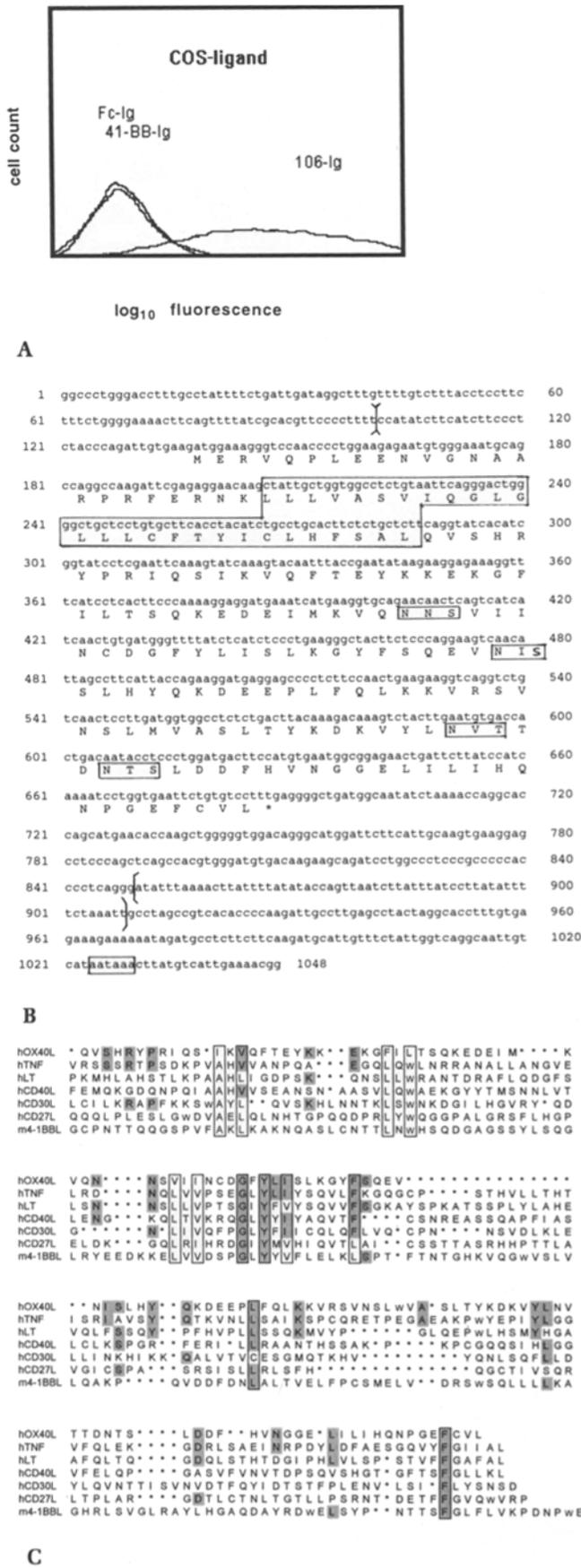


Figure 3. Recombinant 106-ligand costimulates the proliferation of CD4⁺ T cells. Proliferative response of purified CD4⁺ T cells after culture for 5 d in the presence of: (A) 2.5 ng/ml PMA and a titration of fixed COS/106-ligand (■) or COS/vector (□); (B) 2 μg/ml soluble OKT3 and a titration of fixed COS/106-ligand (■) or COS/vector (□); (C) 50 ng/ml PHA and a titration of fixed COS/106-ligand (■) or COS/vector (□); (D) 50 ng/ml PHA, 2 × 10⁴ fixed COS/106-ligand, and either 4-1BB-Ig (■) or 106-Ig (Δ) fusion proteins, or 2 × 10⁴ COS/vector (□).

COS-7 cells (data not shown). However, these situations represent instances of either pathological or contrived overexpression.

With only 15% aa homology to TNF, and the substitution of certain invariant aa residues described for the TNF/ligand family, the 106-ligand/gp34 exhibits the greatest divergence of the reported TNF/ligand family members. However, this family of molecules is very diverse in terms of primary sequence, and other members exhibit low percentages of aa homology with TNF as well: CD27L, -20%, CD30L, -16%, and 4-1BBL, -16%. The family of molecules is related by the predicted formation of almost exclusively beta sheet-containing secondary structures in the extracellular domain, and by analogy with TNF, they are predicted to fold into

Figure 2. Expression and sequence analysis of recombinant 106-ligand. (A) COS-7 cells were transfected with the 106-ligand cDNA or with irrelevant CD4 cDNA, and were stained with fusion protein after 48 h of culture. COS cells transfected with the irrelevant cDNA were nonreactive with all fusion proteins (data not shown). (B) Sequence of the 106-ligand cDNA. A bracket demarks the 101 bases of additional 5' UTR, the transmembrane is boxed, the four N-linked glycosylation sites are in small boxes, the 59 base AU rich region is marked by a pair of brackets, and the polyadenylation signal is noted by a small box. (C) Alignment of the extracellular domain of TNF/ligand family. Conserved residues or two nearly identical hydrophobically conserved residues among the whole family are boxed, and residues shared with 106-ligand/gp34 by TNF-α or at least two other family members are shaded. These sequence data are available from EMBL/GenBank/DBJ under accession number x79929.

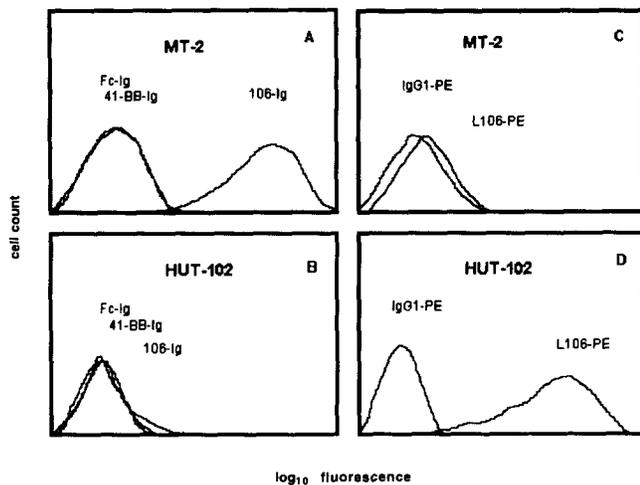


Figure 4. Expression of 106-ligand and 106/human OX-40 antigen on HTLV-I-infected cell lines. (A) MT-2 cells were stained with fusion proteins and goat anti-human Ig. (B) HUT-102 cells were stained with fusion

a beta-jellyroll conformation (6). The relationship of 106-ligand/gp34 to the previously described 70-kD mouse OX40-Ig binding protein expressed on peritoneal exudate B cells is unknown, as no cDNA for that antigen has been reported (31).

Since the initial description of the rat OX-40 antigen as a marker specific for activated CD4⁺ T cells (1), and the finding that it is a NGFR/TNFR family member (2), there has been much interest in the potential function of this molecule. Our studies demonstrate that the recombinant 106-ligand/gp34 interacts avidly with the human 106/OX-40 antigen and can induce potent costimulatory signals. Because the 106-ligand/gp34 appears as a late activation antigen, we speculate that it may function to transmit a late costimulus, and to thereby promote the survival of activated CD4⁺ T cells, and prolong the immune response at sites of active ligand expression.

proteins and goat anti-human Ig. (C) MT-2 cells were stained with L106-PE or an irrelevant IgG1-PE. (D) HUT-102 cells were stained with L106-PE or an irrelevant IgG1-PE.

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