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A functional recombinant human 4-1BB ligand for immune co-stimulatory therapy of cancer

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Abstract

Co-stimulatory factors hold great promise for development into novel anti-cancer bio-therapeutics. An agonist to 4-1BB is ranked number 8 by NCI on the list of 20 agents with high potential for use in treating cancer [1]. We previously reported on a recombinant murine 4-1BB ligand fusion protein that binds 4-1BB receptor on murine T cells and stimulates their proliferation in tumor-bearing mice [2]. To facilitate clinical translation, we constructed a corresponding recombinant human 4-1BB ligand fusion protein (hIg-h4-1BBLs) and demonstrated its ability to activate human T cells in vitro. Using CHO cells transformed with a plasmid co-expressing hIg-h4-1BBLs and rat glutamine synthetase, we generated a high-producing clone by sequential selection with methionine sulfoximine. hIg-h4-1BBLs was partially purified by Protein A column chromatography and characterized biochemically and functionally, using human 4-1BB binding and human T cell proliferation assays, in vitro. SDS-PAGE and Western Blot confirmed hIg-h4-1BBLs is expressed predominantly as a functionally active multimeric protein with the ability to specifically bind to cells expressing human 4-1BB receptor and induce significant T cell proliferation in vitro using both human and monkey peripheral blood mononuclear cells. hIg-h4-1BBLs can be produced in large quantities from the high-producer clone and developed as a novel immune co-stimulatory bio-therapeutic to treat, alone and in combination with other modalities, various malignant diseases in patients through T cell activation. Process development of this clinical agent has been discussed with the FDA in a pre-IND meeting and presented to the OBA in a public hearing.

Keywords

4-1BBL; T-cell activation; immunotherapy; cancer

Introduction

Cancer immunotherapy, as a potential cancer treatment strategy, is still in its early stages of clinical development. Even though antigens expressed on tumor cells can be recognized by T lymphocytes, this interaction does not necessarily lead to tumor rejection by the T cells. One underlying reason is that T cell activation is a complicated process and generally inefficient within the tumor microenvironment [3]. T Lymphocyte activation requires at least two signals, namely, T cell receptor ligation and costimulation. Without costimulation, T lymphocytes will progress to anergy or apoptotic death in the presence of T cell receptor

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ligation [4,5]. Therefore, providing the proper costimulation for T cells that have been primed upon encountering target tumor cells is essential for effective anti-cancer immune therapy.

Costimulation can be achieved by the binding of costimulatory factors to their respective receptors on T lymphocytes. Some important co-stimulatory factors belonging to the tumor necrosis factor receptor (TNFR)/TNF superfamily, including 4-1BB/4-1BB ligand (4-1BBL), CD27/CD70, CD28/B7, CD40/CD40L, OX-40/OX40L and HUEM/LIGHT [3]. An agonist to 4-1BB is ranked number 8 by NCI on the list of 20 agents with high potential for use in treating cancer [1]. 4-1BB receptor is an inducible protein mainly expressed on the surface of activated T lymphocytes and natural killer (NK) cells (DCs) [6-9]. Ligation of 4-1BB to its natural ligand or an agonistic antibody induces signal transduction in target T cells in a CD28-independent fashion, leading to activation of a cascade of signaling pathways such as NF- κ B, c-Jun NH₂-terminal kinase/stress-activated protein kinase (JNK/SAPK), and p38 [10]. Ligation of 4-1BB with its natural ligand also leads to the rescue of CD8 T cell proliferation and production of cytokines such as IL-2, IFN- γ , TNF- α in vitro and long-term survival of CD8 T cells *in vivo*, thereby enhancing and maintaining CD8 T cell-mediated anti-tumor immune responses [11]. Studies by our laboratories and others using *in vivo* administration of agonistic anti-murine 4-1BB antibodies resulted in the regression of established tumors in a number of animal models [12–14].

We have previously reported on the construction and production of a recombinant murine 4-1BB ligand protein, which is comprised of the ectodomain of murine 4-1BB ligand that is fused to mouse IgG2a (mIg-m4-1BBLs). This protein is effective in binding to murine 4-1BB receptor, activating murine T cell proliferation in vitro, and causing tumor rejection in vivo [2]. The mechanism of action involves the activation of splenic T cells and/or bone marrow (BM)-derived dendritic cells (DCs), which express murine 4-1BB receptor. When used in combination with intratumoral injection of a replication defective adenovirus expressing murine interleukin 12 (Adv.mIL-12), mIg-m4-1BBLs proved to be a potent bio-therapeutic against disseminated metastases of MCA26 colon carcinoma, resulting in tumor rejection and long-term survival in 50% of the treated animals. Mouse 4-1BB ligand however, shares only limited similarity with the human analog, and it does not bind activated human T cells. To make a clinically relevant reagent, we constructed a recombinant fusion protein comprised of the ectodomain of human 4-1BB ligand that is fused to human immunoglobulin type 4 (hIg-h4-1BBLs). In order to produce a functional protein, we chose to express the recombinant construct in mammalian cells. By taking advantage of the glutamine synthetase (GS) selection/amplification system in CHO cells, we were able to obtain a high producing clone for hIg-h4-1BBLs, which is suitable for future clinical development.

Materials and Methods

Plasmid construction

A bicistronic expression plasmid of the human fusion protein hIg-h4-1BBLs and rat glutamine synthetase (rGS) was constructed based on the plasmid pIRES (BD Bioscience) using overlapping PCR strategy as previously described [2]. Briefly, each fragment was obtained by PCR or RT-PCR, then three overlapping PCR reactions were performed using the PCR products as templates. The intron/signal peptide sequence was based on our previous paper [2]. The IgG4 DNA sequence was obtained by RT-PCR with template mRNA from the IgG4 isotype human hybridoma cell line VLN3G2 (ATCC, Manassas, VA). RNA extracted from human peripheral blood cells was used as the template to get the 4-1BB ligand fragment. The final PCR product containing the leader sequence, IgG4 hinge region, and the h4-1BBLs encoding sequence together was cloned into cloning site A of pIRES-

rGS, which has rGS cDNA inserted in the multiple cloning site B. rGS cDNA was generated in our lab by RT-PCR from Buffalo rat liver mRNA.

Cells and culture conditions

CHO-K1 cells were obtained from American Type Cell Collection (ATCC, Virginia) and cultured in Ham's F-12 medium (Mediatech, Manassas, VA) supplemented with 10% fetal bovine serum (Hyclone, Utah). Transfected cells were placed under selective pressure in glutamine-free DMEM (SAFC, Lenexa, Kansas) supplemented with dialyzed FBS (Hyclone, Utah), GS supplements (SAFC, Lenexa, Kansas) and 25 μ M methionine sulfoximine (MSX) (Sigma-Aldrich, St. Louis, MO). CHO-S-SFM II (Invitrogen, Carlsbad, CA) was used for generation of conditioned medium in serum-free medium to facilitate ELISA analysis and purification.

Transformation of CHO cells and selection of high yielding transformants

The linearized plasmid expressing hIg-h41BBLs and rGS was transfected into CHO cells using standard procedures for the PolyFect transfection reagent (Qiagen, Valencia, CA). The cells were refed with glutamine-free medium immediately prior to transfection. At 24-hours post-transfection, the cells were put under selection by refeeding the dishes with glutamine-free medium containing 25 μ M MSX. Following transfection, clones were expanded to confluence and cultured in CHO-S-SFMII medium for three days to generate supernatant samples for analysis by human IgG ELISA. One of the high producing clones was chosen for a second round of selection under varying concentrations of MSX (300, 350, 400, 450, and 500 μ M). Ten 24-well plates were seeded and incubated overnight in medium containing 25 μ M MSX. The next day and at day 8, two plates were refed at each MSX concentration. Starting at day 15, the plates were observed for the presence of growing clones. The clones were passaged and ELISA samples were generated as above. A single clone expressing hIg-h4-1BBLs at approximately 200 mg/L was chosen for generation of protein for all subsequent experiments.

ELISA assays

Supernatant samples were collected three days after refeeding with CHO-S-SFMII for analysis by anti-human IgG ELISA (Bethyl Laboratories, Montgomery TX), using additional reagents from the ELISA Starter Accessory Kit (Bethyl Laboratories), and performed according to manufacturer's instructions. All steps were performed at room temperature and plates were washed five times between each step. Microtiter plates were coated with affinity purified human IgG coating antibody (A80-104A), which is goat anti-human IgG-Fc antibody (Bethyl Laboratories) for one hour and blocked with 50 mM Tris, 0.14 M NaCl, 1% BSA, pH 8.0 for 30 minutes. Standards ranging from 7.8 to 1000 ng/ml and supernatant samples were incubated for 60 minutes. HRP Detection Antibody (A80-104P) (Bethyl Laboratories) was added for 60 minutes. TMB substrate was added and the plates were incubated in the dark for 15 minutes followed by addition of 0.18 M H₂SO₄. Absorbance was measured on a Spectra MAX250 plate reader (Molecular Devices, Sunnyvale CA) using Softmax Pro software at 450nm.

SDS-PAGE, Western blot and deglycosylation analysis

The purified protein (10 μ g/lane) was separated on duplicate 10% SDS-PAGE gels in the presence or absence of 2-mercaptoethanol (2-ME). One gel was stained with Coomassie Brilliant Blue to visualize the protein and the other gel was transferred to Hybond-P membrane for Western Blotting. The membrane was exposed to mouse anti-human 4-1BB ligand (Immatics, Germany) at a dilution of 1:2000 in PBS containing 0.1% Tween-20 overnight at 4°C. Following four PBS washes, the membrane was reacted with HRP-

conjugated anti-mouse for one hour at room temperature. Positive bands were visualized using the ECL Western blotting system (GE Healthcare Bioscience, Piscataway, NJ). The enzymatic method of deglycosylation was done with kit GK80110 (Prozyme, Hayward, CA). Quadruplicate aliquots of purified protein (20 μ g) were diluted to 50 μ l by adding the appropriate volume of deionized water. 10 μ l of 5 $\times$ incubation buffer and 2.5 μ l of denaturation solution were added to each tube and the tubes were mixed gently. At this time, the tubes were heated at 100°C for 5 minutes, then cooled to room temperature. 2.5 μ l of detergent solution was added to each of the tubes and the tubes were mixed gently. Then 1 μ l each of N-Glycanase, Sialidase A and O-Glycanase was added to the appropriate tubes; the first replicate tube received no enzymes, the second tube received only N-glycanase, the third tube N-glycanase and Sialidase, and the fourth tube received all three (N-Glycanase, Sialidase A and O-Glycanase). The tubes were incubated for 24 hours at 37°C. At the end of the incubation period, 45 μ l of Laemmli sample buffer without 2-ME was added to each tube, the tubes were boiled for 5min, and then 35 μ l samples were run on an SDS-PAGE gel.

Binding Assay to human 4-1BB expressing cells in vitro

To evaluate binding specificity of hIg-h4-1BBLs, CHO cells or h4-1BB transformed CHO cells (5×10^5 cells) were incubated for 45 minutes in the presence of purified hIg-h4-1BBLs (30 μ g/ml) or control human serum (1:100 dilution) and then stained with goat anti-human IgG-FITC. The results were analyzed by FACS.

Proliferation assays in vitro

Freshly isolated PBMC (5×10^4 cells/well) from humans and non-human primates were cultured in 96-well plates precoated with anti-human OKT3 (1 μ g/ml). Then either hIg-h4-1BBLs (10 μ g/ml) or human Ig control (10 μ g/ml) was added to each well. On day six, the cultures were pulsed with [3 H]-thymidine for 12 hours before harvesting. The effector cells from rhesus and cynomolgus monkeys were also compared in the assay to identify a relevant non-human primate species that would be suitable for future pharmacology and toxicity studies.

Results

Recombinant plasmid Construction and Sequence Analysis

A bicistronic plasmid expressing the human fusion protein hIg-h4-1BBLs and rat glutamine synthetase was constructed and is presented schematically in Figure 1A. The recombinant hIg-h4-1BBLs protein is comprised of a signal peptide derived from rat preproinsulin/hIL-2, the hinge region of human immunoglobulin and the ectodomain of human 4-1BB ligand, and its expected structure is depicted schematically in Figure 1B. The primary structure of the final fusion protein expression cassette was confirmed by direct sequencing (Figure 2A). The only inconsistencies between our sequence and the expected sequence based on Genbank data were sites within the IgG4 region that were also present in the primary RT-PCR product, suggesting that these substitutions are polymorphisms in the human IgG4 gene. The major differences between the IgG isotypes are in the hinge region [15], with both IgG1 and IgG4 having two cysteines that are available for covalent interaction between the H-chains. Formation of intrachain disulfide bridges that interfere with the ability to form stable interchain disulfide bonds between the two H-chains has been reported in monoclonal antibodies comprised of human IgG4, but not human IgG1, sequences [16, 17]. This is due to the specific amino acid sequence in the core hinge region. As illustrated in Figure 2C, the relevant region of our recombinant construct is identical to human IgG1 (CPPC) rather than human IgG4 (CPSC), suggesting that the recombinant protein will likely be able to form stable covalent bonds between the two H-chains.

Generation of high-producer cell line and purification of hIg-h4-1BBLs

The bicistronic plasmid expressing hIg-h4-1BBLs and rat GS was transfected into CHO cells and multiple cell clones were selected by including MSX in the culture medium. This expression/amplification system has been used by others to produce FDA-approved clinical reagents [18]. The supernatants were collected from confluent monolayers, three days after refeeding serum-free medium, for analysis by anti-human IgG ELISA. The high producing clones (>50mg/L) were carried for additional passages to demonstrate the production stability of each clone (Table 1A). Following the second round of MSX selection a single clone expressing the recombinant protein at a level of approximately 200 mg/L was identified. Owing to the presence of the human IgG hinge region in the fusion protein, hIg-h4-1BBLs can be purified using standard methods for monoclonal antibody production, namely protein A column chromatography. Confluent cultures were refed with CHO-S-SFM II medium and incubated for an additional three days, at which point the conditioned medium was collected and clarified by centrifugation and filtration. Clarified culture supernatants were purified by column chromatography on a POROS A 20µm pre-packed column (Applied Biosystems 1–5022) (Foster City, CA), in which protein A is conjugated to 20 micron particles. The conditioned medium was clarified by centrifugation and filtered through a 0.2µm membrane. The clarified conditioned medium is loaded onto a column equilibrated with 0.1M Glycine-NaOH, pH 8.6/ 0.15M NaCl, then washed with the same buffer and eluted with 0.1M Citric Acid at pH 2.5. The final recovery by this purification method is approximately 50% as indicated in Table 1B.

Biochemical characterization

The partially purified recombinant protein was analyzed by SDS-PAGE in the presence of 2-mercaptoethanol (2-ME) to demonstrate that the recombinant protein was produced as a 47 Kd band in the gel, and a Western blot was performed to show that the band contained h4-1BBLs (Figure 3A). The predominant band under denaturing conditions in the absence of 2-ME was >100Kd, which indicated that the hIg-h4-1BBLs was present in a multimeric form. As can be seen from Figure 3B, hIg-h4-1BBLs is naturally glycosylated when expressed by the stably transformed CHO cell line. The recombinant fusion protein's molecular weight is 47kd (lane 1) and the apparent molecular weight is reduced to about 45Kd after N-glycanase treatment (lane 2), suggesting the presence of N-linked glycosylation. Computer analysis of the protein sequence for the fusion protein using the software NetNGlyc 1.0 predicted the potential presence of four potential N-glycosylation sites in the human fusion protein. Additional treatment with Sialidase A or O-glycanase (lanes 3 and 4, respectively) did not cause a decrease in the apparent molecular weight of the fusion protein, indicating that there is no O-linked glycosylation even though the fusion protein has 2 potential O-linked glycosylation sites.

Functional characterization

First, we demonstrated that hIg-h4-1BBLs exhibited specific binding to human 4-1BB receptor-expressing CHO cells (Figure 4A). The hIg-h4-1BBLs protein bound to the CHO-h4-1BB cells (76%) at a significantly higher frequency than the human serum control (4.5%), and did not bind to human 4-1BB receptor-negative CHO cells ($p < 0.01$, t test). We further demonstrated that hIg-h4-1BBLs could also bind to OKT3-activated human T cells in vitro (>90%), but not the isotype control (0.04%) (Fig. 4B). Collectively, the results indicate that the recombinant protein is able to bind specifically to mammalian cells expressing the h4-1BB receptor. We then tested if the recombinant hIg-h4-1BBLs was also able to cause significant proliferation of healthy human peripheral blood mononuclear cells (PBMCs) following stimulation by anti-OKT3. As can be seen from the results in figure 5, the recombinant fusion protein induced a substantially enhanced proliferation of human PBMCs compared to control Ig ($p < 0.001$, t test). In the absence of anti-OKT3 stimulation,

hIg-h4-1BBLs could not induce human T cell proliferation (Fig. 5, “PBL”), confirming that this protein functions as a costimulatory molecule for T cell activation.

Proliferation of PBMCs from non-human primates in vitro

Since the recombinant hIg-h4-1BBLs protein will be developed as a novel biotherapeutic for cancer treatment in future clinical translational trials, we tested whether it would exhibit stimulatory activity in the PBMCs from two non-human primate species in order to identify one that would be suitable for testing potential toxicities of the trial agent. PBMCs were isolated from the whole blood of the non-human primate species and tested for proliferative activity following treatment with hIg-h4-1BBLs in conjunction with anti-OKT3 stimulation (Fig. 6). hIg-h4-1BBLs stimulated non-human primate PBMC proliferation in conjunction with anti-OKT3 stimulation (Rhesus, $p < 0.01$; Cynomolgus, $p < 0.05$, by Anova and t test), and the results indicate that both species are suitable for use in preclinical pharmacological and toxicological studies.

Discussion

It is well known that without costimulatory signaling, activated T cells will progress to anergy and even apoptosis. Our previous study showed that a costimulatory signal was important for induction of a strong anti-tumor immune response in animal models [2]. To induce a T-cell mediated antitumor immune response, we tested the efficacy of ligation of the co-stimulatory molecule 4-1BB, which is a receptor expressed in activated CD4+ and CD8+ T-cells. An agonist to 4-1BB is ranked number 8 on the NCI list of agents with high potential for use in treating cancer [1]. Activation of the 4-1BB receptor is accomplished by binding to 4-1BB ligand, a naturally occurring protein expressed by antigen presenting cells, which results in activation of the NF- κ B, c-Jun NH₂-terminal kinase/stress-activated protein kinase (JNK/SAPK) and p38 signaling pathways in T cells [19]. 4-1BB ligation can also rescue activated T cells from activation-induced cell death and lead to expansion and long-term survival of CD8+ T cells in vivo [20].

We have previously reported that intratumoral injection of Adv.mIL12 resulted in the production of high mIL12 concentrations within the tumor microenvironment without elevated systemic levels and induced an effective antitumor response mediated mainly by natural killer cells. We found that intratumoral injection of an adenovirus vector expressing the membrane-bound form of murine 4-1BB ligand (Adv.m4-1BBLm), together with an adenovirus vector expressing murine interleukin-12 (Adv.mIL12) in mice with metastatic breast cancer in the liver significantly enhanced the long-term survival of the treated animals when compared to either treatment alone. Following the combination treatment, surviving animals were able to reject a subsequent challenge of parental tumor cells implanted at a distant site. In vitro assays showed that both NK and CTL anti-tumoral activity were induced following the combination treatment, and in vivo lymphocyte subset depletion studies confirmed that both NK and CTL immune cells were required to reject a subsequent challenge of parental tumor cells implanted at a distant site.

One potential limitation of intratumoral delivery of Adv.m4-1BB-Lm is that transduced tumor cells are relatively poor antigen presenting cells with impaired ability to prime naive T cells in secondary lymphoid organs in vivo. We therefore tested a recombinant murine 4-1BB soluble ligand fusion protein (mIg-m4-1BBLs) in murine models of metastatic breast and colon cancer in the liver, and found that the fusion protein was also effective in producing long-term survival when combined with intratumoral Adv.mIL12 injection in ~50% of mice with advanced tumors. Inspired by these results, we constructed a recombinant human 4-1BB ligand fusion protein, hIg-h4-1BBLs, for future clinical development. This is the first report on the production and characterization of a functional

human 4-1BB ligand, which contains the extracellular domain of the natural ligand for human 4-1BB receptor and a portion of human IgG4.

Naturally, 4-1BB ligand is expressed on the surface of professional antigen presenting cells such as activated macrophages, dendritic cells and B cells [3], and it has been reported that cross-linking to its cognate receptor expressed on T cells is essential for its biological function. Although it exists naturally as a trimer, dimers are also effective while the monomers are not active [21]. Taking this into consideration, we designed a human 4-1BB ligand fusion protein consisting of the extracellular 4-1BB ligand domain for binding, and the hinge-CH2-CH3 of human immunoglobulin, which occurs naturally in the dimeric form, to generate a dimeric 4-1BB ligand molecule. The hinge-CH2-CH3 of human IgG also proves useful for purification by protein A/G column chromatography and increases the stability of this protein in blood. Preliminary biochemical characterization of the recombinant protein however, indicated that it might exist in a multimeric form and its exact molecular structure will need to be determined by further analyses.

The selection of the appropriate isotype of immunoglobulin was based on the complement activation activity [22]. The IgG4 isotype has no complement activation function and was chosen for our recombinant fusion protein to avoid complement-activated killing of bound cells. However, human IgG4 has been reported to form intrachain disulfide bonds that interfere with the ability to form stable interchain disulfide bonds between the heavy chains in the hinge region and exchange of IgG half molecules can occur in vivo [15]. In our recombinant construct, the amino acid sequence of the relevant portion of the IgG molecule that is the determining factor in this intrachain disulfide bond formation is mutated and is identical to that of IgG1. Thus the IgG4 hinge region in the recombinant protein is expected to behave like IgG1 in this regard and form a stable interchain disulfide bond between the heavy chains in the hinge region, which will have no complement activation function and form a stable dimer in vivo, making it optimal for clinical development.

In order to obtain large quantities of protein using a mammalian expression system, a marker that can be used for selection and amplification is necessary. The use of the GS gene as an amplifiable selectable marker was first reported by Bebbington in 1992 [23]. Myeloma cell transfectants expressing both glutamine synthetase (GS) and the antibody fragment were successfully selected in glutamine free medium and high expression cell clones making chimeric antibody were amplified in medium containing the glutamine synthetase inhibitor, methionine sulfoximine (MSX). In addition to GS, dihydrofolate reductase (DHFR) is also often used for amplification. As myeloid cells have endogenous DHFR activity, a high level of inhibitor i.e. methotrexate (MTX) is needed. The most popular cell lines used for production of recombinant proteins and monoclonal antibodies for clinical use are Chinese hamster ovary (CHO) cells, baby hamster kidney, and mouse myeloma cell (NS0), as well as human embryonic kidney cells (HEK-293). CHO have been successfully employed for the production of many FDA-approved monoclonal antibody therapeutic drugs [18]. It has been reported that the CHO expression system works quite efficiently in producing large quantities of protein [24].

More importantly, the protein obtained by this method demonstrated functional activity in vitro using monkey and human peripheral blood cells. Concerns regarding low yield when using mammalian expression systems are addressed by incorporating the GS selection system. In our hands, the yield from our CHO clone is approximately 200mg/L, which is comparable to monoclonal antibody production in animal ascites. In order to produce large quantities of protein for clinical use, we have adapted the high expressing clone to suspension culture, which is more suitable for large-scale cGMP production. Overall, this high producing h4-1BBLs cell clone will prove to be an invaluable resource for future

translation of our preclinical bench work to clinical trials in cancer patients by immune co-stimulatory therapy.

We have already held pre-IND discussions with reviewers at the FDA regarding the recombinant fusion protein and our proposed combination clinical trial. Their only concern regarding the structure of the protein was related to reports in the literature of in vivo exchange of heavy and light chain pairs, resulting in the generation of bi-specific antibodies. We have addressed that concern by demonstrating that the relevant region of our recombinant protein is identical to IgG1, rather than IgG4. There only other concerns regarding the recombinant protein were regarding the incorporation of additional steps into the purification process, which has been undertaken by the RAID program of NCI. We also submitted our preliminary data and proposed clinical trial protocol to the Office of Biotechnology Activities (OBA) and presented our protocol at a public hearing of the Recombinant DNA Advisory Committee (RAC), which did not express any concerns regarding the structure of hIg-h4-1BBLs. Thus the recombinant protein product will be suitable for future development as a powerful immune co-stimulatory agent in the treatment of multiple cancer types in humans.

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Figure 1.

Panel A, a schematic representation of the expression cassette of the recombinant hIg-h4-1BBLs fusion protein. An expression cassette of hIg-h4-1BBLs is inserted 5' to the ECMV internal ribosome entry site (IRES) in the pIRES plasmid from BD Biosciences. The rat glutamine synthetase (rGS) cDNA is inserted 3' to the IRES, which provides a selection marker for amplification in transfected mammalian cells. **Panel B, a schematic representation of the hIg-h4-1BBLs cDNA and its corresponding protein structure.** The recombinant construct contains a hybrid signal peptide from rat preproinsulin/human interleukin 2, followed by 228 amino acids of hlgG4 that includes the hinge-CH₂-CH₃ region of immunoglobulin that will enable the resulting protein molecule to dimerize, and 204 amino acids of h4-1BB ligand that is comprised exclusively of its extracellular domain.

A DNA sequence of hlg-h41BBLs

ATGGCCCTGTGGATCGACAGGATGCAACTCCTGCTTGCATTGCACTAAGCTTTGCACTTGT
 CACAAACAGITCCAAATATGGTCCCCATGCCCA^{CG}TGCCAGCACCTGA^{AC}TCTGGGG
 GGAC^{CG}TCACTTCTCT^{CT}TCCCCCAAAACCAAGGACAC^{CT}CATGATCTCCCGGACCC
 CTGAGGTCACGTGCGTGGTGGTGGACGTGAGCCAGGAAGACCCGAGGTCCAGTTCAACT
 GGTACGTGGATGGCGTGGAGGTGCATAATGCCAAGACAAAGCCGCGGAGGACAGTTCA
 ACAGCACGTACCGTGTGGTCAGCGTCTCACCGTCTGCACAGGACTGGCTGAACGGCA
 AGGAGTACAAGTGAAGGTCTCAACAAAG^{CC}TCCCAG^{CC}CCATCGAGAAACCATCTC
 CAAAGCCAAAGGGCAGCCCCGAGA^{AC}CACAGGTGTACACCTGCCCCATCC^{CG}GAGGA
 GATGACCAAGAACAGGTGACGCTGACCTGCCTGGTCAAAGGCTTCTAT^{CC}CAGCGACATC
 GCCGTGGAGTGGGAGAGCAATGGGCAGCCGAGAACTACAAGACACGCTCCCGTG
 CTGGACTCCGACGCGCTCTTCTCTATAGCA^{AG}CT^{CA}CCGTGGACAAGACAGGTGGC
 AG^{CA}AGGGGA^{CG}TCTCTCATGCTCCGTGATGCATGAGGCTGCAACAACACTACAC^{CA}
 GAAGAGCTCTCCCTGTCTCTGGGTAAG^{CC}TGCCCTGGGCGGTGTCGGGGCTCGCGC
 CTCGCCCGGCTCCCGGCCAGCCGAGACTCCCGGAGGTCCTCGAGCTTTCCGCCGAGC
 ATCCGCCCGGCTCTTGGACCTGCGGCAGGGCATGTTTGGCGAGTGGTGGCCAAATG
 TTCTGCTGATCGATGGGCCCTGAGCTGGTACAGTGACCCAGGCTGGCAGGCGTGTCC
 TGACGGGGGCTGAGCTACAAAGAGGACACGAAGGAGCTGGTGGTGGCCAGGCTGGA
 GTCTACTATGTCTTCTTCAACTAGAGCTGCGGCGGTGGTGGCGGCGAGGCTCAGGC
 TCCGTTTCACTTGGCTGCACCTGCAGCCACTGCGCTCTGCTGCTGGGCGCGCCCTG
 GCTTTGACCGTGGACCTGCCACCCGCTCTCCGAGGCTCGGAACCTCGGCTTCGGTTTC
 CAGGGCCGCTTGGTGCACCTGAGTGCCGCGCAGCGCTGGGCGTCCATCTTCACACTGAG
 GCCAGGGCACGCCATGCTGGCAGCTTACCCAGGGCGCCACAGTCTTGGGACTCTCCGG
 GTGACCCCGAAATCCAGCCGACTCCCTTACCAGGTCGGAATAA

B Amino acid sequence of hlg-h4-1BBLs

MAIWIDRMQLLSIALSLALVTNSSKYGPPCP^PCPAPE^LLLGGPS
 VFLFPPKPKDTLMISRTPEVTCVVVDVSDQEDPEVQFNWYVDGV
 EVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCKVSN
 KALP^{API}EKTISKAKGQPREPQVYTLPPS^{RE}EEMTKNQVSLTCLV
 KGFYPSDIAVEWESNGQPENNYKTTTPVLDSDGSFFLY^{SL}TLTV
 DKSRWQ^QGNVFCSCVMHEALHNNHYTKSLSLSLGKACPWAVS
 GARASPGSAASPR^LREGPELSPDDPAGLLDLROGMFAQLVAQN
 VLLIDGPLSWYSDPGLAGVSLTGGLSYKEDTKELVVAKAGVYYV
 FFQLELRVYVAGEGSGSVSLALHLQLR^{SA}AGAAALALTVDLPP
 ASSEARN^SAFGFQGRLLHLSAG^QRLGVHLHTEARARHAWQLTQ
 GATVLLGLFRVTP^EIPAGLPSRSE

C

Human IgG1:	CDKTH ^T CP ^P PC ^P PAPE
Recombinant construct:	SKYGP ^P CP ^P PC ^P PAPE
Human IgG4:	SKYGP ^P CP ^P SC ^P PAPE

Figure 2.

Panel A, coding sequence of the hlg-h4-1BBLs fusion protein. The underlined nucleotides at the 5' end encode the hybrid signal peptide and the underlined sequences at the 3' end are those encoding the extracellular domain of human 4-1BB ligand. The nucleotides in between are those of human IgG4. The red letters in IgG4 sequence indicate differences from the corresponding sequence in Gene Bank. **Panel B, the corresponding amino acid sequence of the recombinant fusion protein.** The recombinant protein has a total of 457 amino acids, which is comprised of a signal peptide of 24 amino acids, followed by 228 amino acids of human IgG4 and 204 amino acids of human 4-1BB ligand. The red letters indicate amino acid substitutions from the corresponding protein in the Gene Bank database (AAI1020). **Panel C, the amino acid sequences of human IgG1, the recombinant construct and human IgG4.** The critical residue in position 228 (EU-numbering convention) is S in human IgG4, which is capable of forming intra-chain disulfide bonds. The corresponding residue in human IgG1 is P, which does not form intra-chain disulfide bonds. The corresponding residue in the recombinant protein construct is also a P (red).

Figure 3.

Panel A, characterization of purified hIg-h4-1BBLs by SDS-PAGE and Western Blot.

10ug of partially purified hIg-h4-1BBLs was denatured, in the presence or absence of 2-mercaptoethanol, by boiling in 1% SDS and analyzed by SDS gel electrophoresis. Left panel: Gel picture after separation on 10% SDS-PAGE and staining with Coomassie Brilliant blue. Right panel: Western blot using a duplicate gel run under identical conditions and transferred to Hybond-P membrane. The membrane was incubated with anti-human 4-1BB ligand antibody (1:2000) followed by an HRP-conjugated secondary antibody. Detection of positive bands was carried out using the ECL western blotting system.

Panel B, SDS-PAGE of hIg-h4-1BBLs after enzymatic deglycosylation.

Partially purified hIg-h4-1BBLs was treated with glycanases and analyzed on a 10% SDS-PAGE gel. Each lane contains 20µg of denatured protein. Lane 1: Untreated control, lane 2: treated with N-glycanase only, lane 3: treated with N-glycanase and sialidase A, lane 4 treated with N-glycanase, sialidase A and O-glycanase.

Figure 4. Specific binding of hIg-h4-1BBLs to h4-1BB-expressing CHO cells and activated human T cells by FACS analysis
Panel A, binding of hIg-h4-1BBLs to h4-1BB-expressing CHO cells. CHO or h4-1BB-expressing CHO cells (5×10^5 cells) were incubated for 45 minutes in the presence of $10 \mu\text{g}/\text{ml}$ hIg-h4-1BBLs or control human serum (1:100 dilution), stained with goat anti-human IgG-FITC and subjected to FACS analysis. **Panel B, binding of hIg-h4-1BBLs to activated human T cells.** Human PBMC (5×10^5 cells) were stimulated with OKT3 ($0.25 \mu\text{g}/\text{ml}$) for 3 days. Non-stimulated PBMC (no OKT3) were incubated for 3 days as controls. The cells were incubated with commercial anti-human 4-1BB antibody (BD Cat#: 555956, $20 \mu\text{l}/\text{test}$) and two preparations of partially purified hIg-h4-1BBLs ($10 \mu\text{g}/\text{ml}$), which was followed by staining with goat anti-human IgG-FITC secondary antibody and co-staining with anti-human CD3-APC antibody.

Figure 5. Proliferation assays of Human PBMC in the presence of hIg-h4-1BBLs

Freshly isolated human PBMCs (5×10^4 cells/well) were cultured in 96-well plates and stimulated with human OKT3 (0.25 $\mu\text{g}/\text{ml}$). Control wells were set up that received only OKT3 stimulation without any hIg-h4-1BBLs or human Ig added (designated as “CT”). hIg-h4-1BBLs, or human Ig as control, were diluted in the same buffer to concentrations of 10, 2.5, 0.625, 0.312, and 0.078 $\mu\text{g}/\text{ml}$ and added in triplicate to the appropriate wells. An additional two sets of wells were set up that received PBMCs and hIg-h4-1BBLs or human Ig (10 $\mu\text{g}/\text{ml}$), without OKT3 stimulation (designated as “PBL”). On day six, the cultures were pulsed with [^3H]-thymidine for 12 hours before harvesting. The counts shown are the average of triplicate wells. Similar results have been obtained in three separate experiments.

Figure 6. Proliferation assays of normal monkey PBMC in response to hIg-h4-1BBLs
Cynomolgus and rhesus monkey PBMCs (1×10^5 cells) were cultured with medium containing 5-10 $\mu\text{g/ml}$ hIg-h4-1BBLs or control hIg in the presence or absence of anti-CD3 (0.5 $\mu\text{g/ml}$) in pre-coated 96-well plates and incubated for 96 hrs. On day four, the cultured cells were pulsed with [^3H]-thymidine for 12 h before harvesting. The results presented are averaged from triplicate wells.

Table 1a

Methionine Sulfoximine (MSX) Selection

Round of Selection	[MSX]	Number of Clones Analyzed	Number of Positive Clones	Range of [hIg] (µg/ml)
First	25 µM	202	94	0.03 – 80
Second	300–500 µM	97	97	100 – 160

Table 1b

Protein A Column Purification

	Volume	[hIg]	Total hIg	Percent Yield
Suspension Culture Supernatant	300 ± 30 ml	150 ± 30 µg/ml	40 ± 6 mg	100
Protein A Column Eluate	7 ± 2 ml	2.0 ± 0.5 mg/ml	21 ± 4 mg	52 ± 10