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Published:

— *without international search report and to be republished upon receipt of that report*

BTNL2 FUSION PROTEIN FOR T-CELL ACTIVITY MODULATION

STATEMENT AS TO FEDERALLY SPONSORED RESEARCH

This invention was made in part with U.S. government support under Grant No. AI050746 awarded by The National Institutes of Health. The government therefore has certain rights in the invention.

FIELD

This invention relates to the fields of molecular biology, medicine and pharmacology.

BACKGROUND

This background is provided solely for the benefit of the reader in understanding the invention herein. It is not intended, nor is it to be construed, as prior art to the current invention.

BTNL2, a member of the butyrophilin family of proteins, was initially identified by Stammers in 2000 (Stammers, M., et al., Immunogenetics, 2000, 51:373-382. It was characterized as a member of the B7 superfamily of proteins. Subsequently, it was implicated in the autoimmune diseases sarcoidosis (Valentonyte, R., et al., Nature Genet., 2005, 37:357-364), myositis (Price, P., et al., Tissue Antigens, 2004, 64:575-580), rheumatoid arthritis, systemic lupus erythematosus, type 1 diabetes (Orozco, G., et al., Human Immunol. (2005) 66(12):1235-41) and multiple sclerosis (Traherne, J. A., et al., Human Mol. Genet., 2006, 15(1):155-61). Its function with regard to these diseases, however, was unknown until now.

What would be a useful advance in the art would be knowledge of the function of BTNL2 and the use of that knowledge to manipulate the activity of the protein to treat and/or prevent diseases or disorders, the pathology of which is dependent on a biological pathway at least in part modulated by BTNL2. The current invention provides the function of BTNL2, methods of modulating its function to treat or prevent the aforesaid diseases or disorders and novel BTNL2-derived compounds exhibiting enhanced activity.

SUMMARY

Thus, in one aspect, the current invention relates to a fusion protein comprising all or a biologically active fragment of human BTNL2 polypeptide.

In an aspect of this invention, the biologically active fragment comprises an extracellular domain of BTNL2.

In an aspect of this invention, the fusion protein further comprises human IgG1, that is, the fusion protein is BTNL2-Ig.

In an aspect of this invention, the fusion protein comprises the amino acid sequence shown in SEQ ID 1.

An aspect of this invention is a nucleic acid sequence that codes for the BTNL2 fusion protein.

An aspect of this invention is a method of treating or preventing a disorder characterized by abnormal T cell activity comprising administering to a patient in need thereof an agent that modulates the signaling activity of BTNL2.

In an aspect of this invention in the above method the agent increases the signaling activity of BTNL2.

In an aspect of this invention the agent is the above-referenced BTNL2-Ig fusion protein.

In an aspect of this invention, the disorder that can be treated or prevented by administering an agent that modulates the signaling activity of BTNL2 is selected from the group consisting of cancer, tissue or organ transplant rejection, allergy, autoimmune disease or immunosuppressive disorder.

In an aspect of this invention, the autoimmune disease is selected from the group consisting of Crohn's disease, Stevens-Johnson Syndrome, systemic lupus erythematosus, aplastic anemia, myositis, polychondritis, idiopathic thrombocytopenia, sarcoidosis, Sjogren's syndrome, uveitis, lung fibrosis, rheumatoid arthritis, myasthenia gravis, psoriasis, cirrhosis, aplastic anemia, encephalopathy, multiple sclerosis, scleroderma and chronic hepatitis.

In an aspect of this invention, the agent decreases the signaling activity of BTNL2.

In an aspect of this invention, the agent is a BTNL2-specific antibody.

DETAILED DESCRIPTION

Brief description of the figures

Figure 1A shows the amino acid sequence of human BTNL2 (hBTNL2) and mouse BTNL2 (mBTNL2).

Figure 1B shows the phylogenetic placement of BTNL2 within the B7/butrophilin superfamily of proteins.

Figure 1C is a comparison of the genomic organization of hBTNL2 and mBTNL2.

Figure 2 shows the expression of mBTNL2 mRNA in mouse tissues. Real-time PCR analysis was conducted using primers specific for the transmembrane and intracellular regions of BTNL2. Tissues harvested from a B6 mouse were analyzed for BTNL2 expression and the expression level in thymus was set at 1 (Fig. 2A). CD4⁺, CD8⁺ T cells and B cells purified from spleen and lymph nodes by AutoMACS sorting (90-95% purity), bone marrow-derived dendritic cells and peritoneal macrophages were used in the analysis. The expression level by CD4⁺ T cells was set at 1 (Fig. 2B).

Figure 3 shows the expression of mBTNL2 receptor in mouse lymphocytes. BTNL2-Ig fusion protein was biotinylated to stain B cells before and after LPS treatment, and CD4⁺ and CD8⁺ T cells before and after activation with ConA, which was revealed by streptavidin-PE. Histogram analysis was performed on gated B cells (Fig. 3A) and wild type, CD28⁻ or ICOS-deficient, CD4⁺ or CD8⁺ T cells. The lighter line represents human IgG1 isotype control staining; the darker line, BTNL2-Ig staining (Fig. 3B). ConA-activated splenocytes were preincubated with 20-fold excess of B7.1-Ig before stained with biotinylated BTNL2-Ig (Fig. 3C). 293 cells were transfected with a PD-1 expression vector, and subsequently stained with biotinylated PD-L1-Ig or BTNL2-Ig (Fig. 3D).

Figure 4 shows the inhibition of T-cell proliferation by mBTNL2-Ig. Purified CD4⁺ T cells were treated for 3 days and ³H-thymidine uptake was examined. T cells were activated with 1 µg/ml plate-bound anti-CD3 and anti-CD28 in the presence of indicated doses of BTNL2-Ig or control human IgG (hIg) (Fig. 4A). T cells were also activated with indicated doses of plate-bound anti-CD3 and 5 µg/ml of BTNL2-Ig or hIg for 24 hours before measuring activation induced cell death (Fig. 4B). T cells were activated with anti-CD3 and anti-CD28 as above in the presence

of 10 µg/ml plate-bound or soluble BTNL2-Ig or control hlg. Proliferation of T cells treated with hlg was set at 100% (Fig. 4C). T cell proliferation after activation with indicated doses of anti-CD3 with or without 1 µg/ml CD28 and in the presence of 10 µg/ml hlg, BTNL2-Ig, B7S1-Ig or B7H3-Ig is shown in Fig. 4D.

Figure 5 shows the regulation of IL-2 by mBTNL2-Ig. CD4⁺ T cells were activated with different doses of plate-bound anti-CD3 in the presence of 10 µg/ml of BTNL2-Ig or hlg. IL-2 production was measured at 24 hours after ELISA (Fig. 5A). CD4⁺ T cells were activated with 2 µg/ml of anti-CD3 and 1 µg/ml of anti-CD28 in the presence of different doses of BTNL2-Ig, and IL-2 production was examined by ELISA (Fig. 5B). CD4⁺ T cells were treated with 1 µg/ml anti-CD3 in the presence of 10 µg/ml hlg, BTNL2-Ig, B7S1-Ig, or B7H3-Ig with or without IL-2 (100u/ml) and/or 1 µg/ml of anti-CD28 for three days and ³H-thymidine uptake was measured (Fig. 5C).

Figure 6 shows the inhibition of T-cell receptor signaling pathways by mBTNL2-Ig. DO11.10 hybridoma was transfected with luciferase reporter constructs for each individual signaling pathway, and stimulated with 1 µg/ml of plate bound anti-CD3 in the presence of 10 µg/ml BTNL2-Ig or hlg for 4 hours prior to luciferase activity measurement.

Discussion

It is understood that use of the singular throughout this application including the claims includes the plural and *visa versa* unless expressly stated otherwise. That is, "a" and "the" are to be construed as referring to one or more of whatever the word modifies. Non-limiting examples are: "an agent" which is understood to include one such agent, two such agents or, under the right circumstances, as determined by those skilled in the treatment of diseased tissues, even more such agents unless it is expressly stated or is unambiguously obvious from the context that such is not intended. Likewise, "a biologically active fragment" refers to a single fragment or a mixture of two or more fragments unless, again, it is expressly stated or absolutely obvious from the context that such is not intended.

As used herein a "fusion protein" refers to a BTNL2 polypeptide, fragment or functional variant thereof operatively linked to a non-BTNL2 polypeptide. While the BTNL2 polypeptide can be the entire BTNL2 polypeptide or any biologically active fragment thereof, preferably at present the BTNL2 portion of the fusion protein is an extra-cellular domain of BTNL2. The non-BTNL2 polypeptide can be operatively

linked to either the C-terminus or the N-terminus, preferable at present to the N-terminus of the BTNL2 extra-cellular fragment.

As used herein, "T cell" refers to both CD4 and CD8 T cells and further refers to both T helper type 1 and T helper type 2 cells. By "abnormal T cell activity" is meant a quantitative increase or decrease in T cell proliferation and/or functionalization that is detrimental to the health and well-being of a patient afflicted with the abnormal T cell activity.

As used herein, a "nucleic acid sequence" refers to DNA, RNA and analogs of either generated using natural nucleotide derivatives and/or analogs. By natural nucleotide is meant a nucleotide that occurs in nature. Derivatives of natural nucleotides the chemical structure of which has been altered by the addition of atoms of functional groups, the deletion of atoms or functional groups or the substitution of atoms or functional groups with different species. Analogs of natural nucleotides refers to semi- or totally synthetic compounds that have been or are shown to mimic the biochemistry of the natural nucleotide. The nucleic acid sequence may be single or double-stranded. Preferably at present a nucleic acid sequence of this invention is double-stranded DNA.

As used herein "treating" a patient refers to the administration to the patient of a therapeutically effective amount of an agent of this invention, wherein a therapeutically effective amount refers to that amount that has a modulatory effect on T cells that results in a beneficial effect on the health and well-being of a patient including, but not limited to: (1) curing the T cell-related disorder; (2) slowing the progress of the T cell-related disorder; (3) causing the T cell-related disorder to retrogress; or, (4) alleviating one or more symptoms of the T cell-related disorder.

As used herein, "preventing" a disorder in a patient refers to administration to a patient known or suspected of being particularly susceptible to a T cell-related disorder an amount of a therapeutic agent that has a prophylactic beneficial effect on the health and well-being of the patient. A prophylactic beneficial effect on the health and well-being of a patient includes, but is not limited to: (1) preventing or delaying on-set of the T cell-related disorder in the first place; (2) maintaining a T cell-related disorder at a retrogressed level once such level has been achieved by a therapeutically effective amount of an agent, which may be the same as or different from the agent used in a prophylactically effective amount; or, (3) preventing or

delaying recurrence of the T cell- related disorder after a course of treatment with a therapeutically effective amount of an agent, which may be the same as or different from the agent used in a prophylactically effective amount, has concluded.

As used herein, "modulating" the signaling activity of BTNL2 refers to either increasing the activity of the polypeptide, which would have the effect of down-regulating the proliferation and functionalization of T cells or decreasing the activity of the polypeptide, which would have the opposite effect, i.e, up-regulating the proliferation and functionalization of T cells.

T cell-related diseases or disorders that would be expected to be susceptible to treatment or prevention by modulation of BTNL2 signaling activity include, but are not limited, to cancer, tissue or organ transplant rejection, autoimmune diseases, allergies and immunosuppressive disorders.

Examples, without limitation of autoimmune disorders that would be expected to be susceptible to treatment or prevention by modulation of BTNL2 signaling activity include, but are not limited to, Crohn's disease, Stevens-Johnson Syndrome, systemic lupus erythematosus, aplastic anemia, myositis, polychondritis, idiopathic thrombocytopenia, sarcoidosis, Sjogren's syndrome, uveitis, lung fibrosis, rheumatoid arthritis, myasthenia gravis, psoriasis, cirrhosis, aplastic anemia, encephalopathy, multiple sclerosis, scleroderma and chronic hepatitis.

Suitable routes of administration of an agent of this invention include, without limitation, oral, rectal, transmucosal, intramuscular, subcutaneous, intramedullary, intrathecal, direct intraventricular, intravenous, intravitreal, intraperitoneal, intranasal, aural or intraocular.

A presently preferred agent for decreasing the signaling activity of BTNL2 is an antibody specific to the polypeptide. As used herein, an "antibody" refers to an immunoglobulin molecule and immunologically active portions thereof. For example, without limitation, molecules that contain an antigen binding site such as Fab and F(ab')₂ fragments, single chain antibodies, intracellular antibodies, scFv, Fd, etc. may be used. Preferably the antibody binds substantially specifically to BTNL2. The antibody may be monoclonal or polyclonal. A monoclonal antibody (mAb) refers to a population of antibody molecules that contain one antigen binding site capable of immuno-reacting with a particular epitope of an antigen. A polyclonal antibody refers to a population of antibody molecules that contain multiple antigen binding sites.

The preparation of compound-specific monoclonal antibodies and of polyclonal antibodies is well-known in the art and the skilled artisan will be able to prepare such without difficulty based on the disclosures herein. Thus, no further description of appropriate antibodies or their preparation is necessary herein.

The route of administration will dictate the composition/formulation of the agent used. The following is a brief, non-limiting discussion of pharmaceutical compositions that, under appropriate circumstances, may be useful with the agents of this invention.

An agent of the present invention can be administered as such to a patient or can be administered in pharmaceutical compositions comprising one or more suitable excipient(s). Techniques for formulation of drugs for use with various methods of administration can be found in *Remington's Pharmacological Sciences*, Mack Publishing Co., Easton, PA, latest edition. The formulations and techniques discussed in Remington relate primarily to use with human patients; however, they may readily be modified for use with non-human patients by techniques well-known to those skilled in veterinary medicine.

Pharmaceutical compositions of the present invention may be manufactured by conventional means well-known in the art, for example, without limitation, using a variety of well-known mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes. The compositions may be formulated in conjunction with one or more physiologically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active agent into preparations which can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen.

For example, for injection, including, without limitation, intravenous, intramuscular and subcutaneous injection, the agent of this invention may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks' solution, Ringer's solution, physiological saline buffer or polar solvents including, without limitation, N-methyl-2-pyrrolidone, 2-pyrrolidone, other pyrrolidones, N,N-dimethylacetamide, N,N-dimethylformamide, dimethylsulfoxide, acetone and glycerol formal.

For transmucosal administration, penetrants appropriate to the barrier to be permeated may be used in the formulation. Such penetrants are generally known in the art.

For oral administration, the agents can be formulated with pharmaceutically acceptable carriers well-known in the art. Such carriers enable the agents of the invention to be formulated as tablets, pills, lozenges, dragees, capsules, liquids, gels, syrups, pastes, slurries, solutions, suspensions, concentrated solutions and suspensions for diluting in the drinking water of a patient, premixes for dilution in the feed of a patient, and the like, for oral ingestion by a patient. Pharmaceutical preparations for oral use can be made using a solid excipient, optionally grinding the resulting mixture, and processing the mixture of granules, after adding other suitable auxiliaries if desired, to obtain tablets or dragee cores. Useful excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol, cellulose preparations such as, for example, maize starch, wheat starch, rice starch and potato starch and other materials such as gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethylcellulose, sodium carboxymethylcellulose, and/or polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as cross-linked polyvinyl pyrrolidone, agar, or alginic acid. A salt such as sodium alginate may also be used.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical compositions that can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules can contain the active ingredients in admixture with a filler such as lactose, a binder such as starch, and/or a lubricant such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. Stabilizers may be added in these formulations, also.

For administration by inhalation, the agents of the present invention can conveniently be delivered in the form of an aerosol spray using a pressurized pack or a nebulizer and a suitable propellant, e.g., without limitation, dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane or carbon dioxide. In the case of a pressurized aerosol, the dosage unit may be controlled by providing a valve to deliver a metered amount. Capsules and cartridges of, for example, gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The agents may also be formulated for parenteral administration, including, without limitation, bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multi-dose containers. Useful compositions include, without limitation, suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain adjuncts such as suspending, stabilizing and/or dispersing agents. Pharmaceutical compositions for parenteral administration include aqueous solutions of a water soluble form, such as, without limitation, a salt, of the active compound. Additionally, suspensions of the active compounds may be prepared in a lipophilic vehicle. Suitable lipophilic vehicles include fatty oils such as sesame oil, synthetic fatty acid esters such as ethyl oleate and triglycerides, or materials such as liposomes. Aqueous injection suspensions may contain substances that increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Optionally, the suspension may also contain suitable stabilizers and/or agents that increase the solubility of the compounds to allow for the preparation of highly concentrated solutions. Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile, pyrogen-free water, before use.

The agents may also be formulated in rectal compositions such as suppositories or retention enemas, using, e.g., conventional suppository bases such as cocoa butter or other glycerides.

In addition to the formulations described previously, the agents may also be formulated as depot preparations. Such long acting formulations may be administered by implantation (for example, subcutaneously or intramuscularly) or by intramuscular injection. An agent of this invention may be formulated for this route of administration with suitable polymeric or hydrophobic materials (for instance, in an

emulsion with a pharmacologically acceptable oil), with ion exchange resins, or as a sparingly soluble derivative such as, without limitation, a sparingly soluble salt.

Other delivery systems for relatively hydrophobic pharmaceutical agents may be employed. Liposomes and emulsions are well-known examples of delivery vehicles or carriers for hydrophobic drugs. In addition, organic solvents such as dimethylsulfoxide may be used, although often at the risk of greater toxicity.

Additionally, the agents may be delivered using a sustained-release system, such as semi-permeable matrices of solid hydrophobic polymers containing the therapeutic agent. Various sustained-release materials have been established and are well known by those skilled in the art. Sustained-release capsules may, depending on their chemical nature, release the compounds for a few days to over 100 days. Depending on the chemical nature and the biological stability of the particular compound, additional stabilization strategies may be employed.

Pharmaceutical compositions useful herein also may comprise solid or gel phase carriers or excipients. Examples of such carriers or excipients include, but are not limited to, calcium carbonate, calcium phosphate, various sugars, starches, cellulose derivatives, gelatin, and polymers such as polyethylene glycols.

As used herein, a "patient" refers to any living entity that is susceptible to a T cell-related disorder. Presently preferred patients include mammals such as, without limitation, dogs, cats, horses, cows, pigs, rabbits, goats and sheep. Most preferably, "patient" refers to a human being.

The present invention is related to the well-established regulation of T cell activity, that is, T cell proliferation and functionalization. T cell activation requires two signals. Both are generally provided by antigen-presenting cells (APC) to resting T lymphocytes. The first signal is provided through the T cell receptor on APCs following recognition of an antigen presented by the major histocompatibility complex (MHC). It is this signal that renders the immune response antigen-specific. The second signal, which is non-specific, is termed "co-stimulation." Co-stimulation regulates both adaptive immunity and innate immunity by inducing T cells to proliferate and become functional. If only the first signal is received, T-cells attempt to activate but become non-responsive, anergic or they die. The result is down-regulation of the immune response. Thus, co-stimulatory entities have become prime targets for treatment or prevention of immune-related diseases and disorders

such as, without limitation, tissue or organ transplant, cancer, infectious diseases, autoimmunity, inflammation, atherosclerosis, rheumatoid arthritis and allergies. A prominent member of the family of co-stimulatory molecules is the B7 superfamily of proteins.

For example, B7.1 and B7.2, which are known to engage CD28 on naive T cells, are highly up-regulated on antigen-presenting cells (APC) by infectious agents. Mice deficient in CD28 or both B7.1 and B7.2 were found to exhibit severe impairments in CD4 T-cell activation and function. A second receptor for B7, CTLA4, is induced on activated T-cells. Mice deficient in CTLA4 died at the neonatal stage due to massive T-cell activation and infiltration into tissues, indicating a crucial role of CTLA4 in immune tolerance. B7h/B7RP-1 binds to ICOS, the third member of the CD28 family on activated T-cells. Analysis of mice deficient in ICOS or its ligand, B7h, revealed that this pathway regulates T-cell activation, differentiation and effector function. PD-1, an inhibitory receptor expressed on activated B- and T-cells, binds to the B7 family members PD-L1 (B7H1) and PD-L2 (B7-DC). The spontaneous autoimmunity seen in PD-1 deficient mice indicates its critical function in immune tolerance. B7-H3 is widely expressed in both lymphoid and nonlymphoid tissues, with a putative receptor expressed on activated but not naive T-cells. B7-H3 has been reported to be a positive co-stimulator in humans but studies in mice suggest that B7-H3 may serve as a negative regulator in T cell activation. B7S1, B7x and B7-H4 work through a putative receptor on activated T-cells to inhibit proliferation and IL-2 production. Due to their substantial homology with the B7 co-stimulatory proteins, the butyrophilins are considered members of the B7 superfamily.

Butyrophilin is a milk protein important in regulating secretion of milk droplets. LoucsLink annotates 15 butyrophilin-like genes in mice and 16 in humans. Butyrophilin proteins typically have a signal peptide comprising an IgV-like and an IgC-like domain, a transmembrane domain and a cytoplasmic domain. In addition, butyrophilins often possess a heptad repeat, a seven amino acid sequence encoded by a single exon. Many butyrophilin molecules also contain in their cytoplasmic region a B30.2 domain, comprising around 170 amino acids found also in TRIM proteins and stonutoxin. While the precise function of B30.2 domain in the

butyrophilins that include the domain is unclear; it has reported that TRIM (TRIMpartite Motif) alpha protein interacts with human HIV via its B30.2 domain.

Due to the fact that the IgV-like domains of B7.1 and B7.2 are more closely aligned with the IgV-like domain of butyrophilin than to any other sequence, it has been proposed that B7 and the butyrophilins might have evolved from a common ancestral gene to compose a subfamily within the Ig superfamily. A relatively new addition to the B7/butyrophilin superfamily is BTNL2.

Real-time PCR analysis has revealed that BTNL2 expression is found not only in lymphoid organs but also in non-lymphoid organs as well, most abundantly in the intestine. Abundant expression of BTNL2 mRNA in intestine suggests that the protein has an important function in the regulation of mucosal immunity or tolerance in addition to its demonstrated involvement with lymphoid tissue-initiated immune responses. With regard to the latter, it has been shown that mutations of BTNL2 (sometimes referred to in papers as BTL-II), has been clearly implicated in certain human autoimmune diseases. For example, it has been demonstrated that a truncating splice site mutation in BTNL2 disrupts its membrane localization and this disruption has been associated with the autoimmune disease sarcoidosis, which is characterized by increased inflammatory activity of macrophages and CD4+ helper T cells.

While the apparent involvement of BTNL2 in autoimmune disease was quite clearly established by the above observations, it was not known exactly what the regulatory function of the protein was that was being disrupted so as to permit occurrence of the associated diseases. As noted above, and described below, it has now been revealed that BTNL2 is a down-regulator of T-cell proliferation and activation. (Nguyen, T, et al., J. Immunology, 2006, 176:7354-60). During the course of the work that led to this observation, it was discovered that a derivative of BTL2, a fusion protein of BTNL2 with human IgG, BTNL2-Ig, which is a compound of this invention, was surprisingly an even more potent down-regulator of T cell proliferation than BTNL2 itself and thus would be expected to provide a powerful therapeutic agent against disorders associated with disabled BTNL2 or simply over-active T cells. Further, while the protein itself would provide a therapeutic effect, it would also be expected that the chimeric gene expressing the protein, stably transfected into affected cells, would likewise have a beneficial effect and as such is also an

embodiment of this invention.

Characteristics of mouse BTNL2 molecule. Full-length murine BTNL2 cDNA was amplified from a spleen cDNA library and sequenced. Predicted mouse BTNL2 protein has a distinct protein structure when compared with B7 family members. It has four extracellular Ig domains, consisting of two IgV-IgC pairs (IgVa-IgCa and IgVb-IgCb), unlike most B7s, which have two. Human and monkey B7-H3 uniquely have a two-Ig and a four-Ig isoforms and the latter is predominantly expressed in most tissues. The Ig domains of BTNL2 have conserved cysteines and the DxGxYxC motif in the two IgV-like domains (Fig. 1A). Phylogenetic analysis of BTNL2 shows it to be more similar to the butyrophilin family members than to B7 family molecules (Fig. 1B). This is further affirmed by the presence of a heptad sequence, characteristic for many butyrophilins, which is located between the two pairs of IgV-IgC domains (Fig. 1C). Unlike many other butyrophilins such as BT3.1, BTNL2 does not have a B30.2 domain in the intracellular region.

Overall, human and mouse BTNL2 proteins are 63% identical (Fig. 1A). It would be predicted, however, that human and mouse BTNL2 should function similarly because the IgV domain of B7 family member mediates receptor binding. The NCBI database established all the intron/exon boundaries for mouse BTNL2 gene. As with all B7 members, each domain is encoded by a separate exon (Fig. 1C). In mice, five other butyrophilin-like proteins are found within this same region; however, these five genes are not conserved in humans (Fig. 1C).

Expression of BTNL2 mRNA. To examine BTNL2 expression, two pairs of PCR primers that span either the third/fourth Ig domains or the transmembrane/intracellular coding regions of BTNL2 gene were used. RT-PCR analysis revealed that BTNL2 is widely expressed in tissues. To compare the relative expression level of BTNL2 in different tissues, real-time PCR analysis was used. BTNL2 mRNA was found expressed in non-lymphoid tissues, most abundantly in intestine, and at reduced levels in lung and stomach (Fig. 2A). In addition, BTNL2 mRNA was also expressed in lymphoid organs, thymus, spleen and lymph nodes. PCR analysis was performed on purified immune cells resulting in the observation that BTNL2 was expressed in T cells, B cells, and macrophages (Fig. 2B).

Expression of a putative receptor for BTNL2. A soluble BTNL2-Ig fusion protein domain of human IgG1 was prepared to examine the function of BTNL2.

BTNL2-Ig was purified by protein A, biotinylated, and used to determine putative receptors for the molecule. A BTNL2 receptor, bound by BTNL2-Ig, was found constitutively expressed on splenic B220+ B cells (Fig. 3A). B cells appeared to express a higher level of the receptor after LPS stimulation for two days. Bone marrow dendritic cells, bone marrow macrophages, peritoneal macrophage, granulocytes and NK cells were not significantly bound by BTNL2-Ig. On the other hand, ConA-activated T cells but not un-stimulated T cells were also significantly stained by BTNL2-Ig (Fig. 3B). Staining of BTNL2-Ig was also observed in activated but not resting DO 11.1 O H hybridoma cells.

B7 family members signal through members of the CD28 superfamily. While CD28 is expressed by naive T cells, CTLA4, ICOS and PD-1 are expressed by activated T cells. BTNL2 receptor expression on both T and B cells is similar to PD-1, which is expressed on activated T and B cells. BTNL2-Ig binds to T cells from CD28- or ICOS-deficient mice after ConA activation (Fig. 3B). B7.1-Ig did not block binding of BTNL2-Ig to activated T cells (Fig. 3C), suggesting that BTNL2 does not bind to CD28 or CTLA4. In addition, BTNL2-Ig did not stain PD-1 transfected 293 cells, whereas PDL1-Ig did (Fig. 3D). All these indicate that a putative receptor for BTNL2 is distinct from CD28, CTLA4, ICOS and PD-1.

Immobilized BTNL2-1g inhibits T cell activation. Although BTNL2 receptor is expressed on B cells, it does not appear to affect B cell proliferation in contrast to PD-1. BTNL2-Ig, when given either in solution or plate bound, did not affect B cell proliferation when these cells were activated by either LPS or the combination of anti-IgM and anti-CD40. BTNL2-Ig did not induce B cell proliferation by itself, which also indicates that there is no significant endotoxin contamination in the protein preparation herein.

In T cells, the receptor for BTNL2 is up-regulated upon activation. When CD4+ T cells were activated with anti-CD3 and anti-CD28, a dose-dependent inhibition of proliferation was observed in the presence of plate-bound BTNL2-Ig compared to human IgG control (Fig. 4A). Activation-induced cell death was not increased by BTNL2-Ig treatment (Fig. 4B). When BTNL2-Ig was given in soluble form instead of plate bound, it did not inhibit anti-CD3 and anti-CD28 induced proliferation of CD4+ T cells (Fig. 4C). This implies that BTNL2, like PD-L1, inhibits

proximal TCR (T cell receptor) signaling events and that BTNL2 receptor must be in proximity of the TCR in order to achieve this inhibition.

Two new B7 family members, B7-H3 and B7S1, were recently reported to inhibit mouse T cell proliferation. The activity of BTNL2-Ig, B7-H3-Ig and B7S1-Ig were compared. In the absence of CD28 co-stimulation, BTNL2-Ig, B7S1-Ig, or B7-H3-Ig all effectively inhibited anti-CD3 stimulated T cell proliferation (Fig. 4D). When CD28 co-stimulation was provided by anti-CD28, BTNL2-Ig inhibited T cell proliferation more strongly than B7-H3-Ig but less potently than B7S1-Ig (Fig. 4D).

Inhibition of T cell proliferation by B7S1 or B7-H3 is functionally associated with reduced IL-2 production. It was thus desirable to determine whether BTNL2 likewise inhibits IL-2 expression by activated T cells. It was found that plate-bound BTNL2-Ig moderately reduced IL-2 production when CD4⁺ T cells were activated with different dose of anti-CD3 alone (Fig. 5A). A modest but dose-dependent reduction of IL-2 production by BTNL2-Ig was also observed when CD4⁺ T cells were activated with anti-CD3 together with anti-CD28 (Fig. 5B). BTNL2-Ig suppressed T cell proliferation more potently than it effected IL-2 production.

Whether inhibition of T cell proliferation by BTNL2-Ig could be restored by the addition of exogenous IL-2 was also examined. In the absence of CD28 co-stimulation, inhibition of proliferation by BTNL2-Ig was the least responsive to IL-2 whereas proliferation of T cells treated with B7S1-Ig and B7-H3-Ig was sharply increased by IL-2 (Fig. 5C). Inhibition of proliferation by BTNL2 is thus in part but not solely through reduction of IL-2 production and the mechanism of inhibition by BTNL2 may differ from that of B7-H3 and B7S1. On the other hand, CD28 co-stimulation greatly increased proliferation in B7-H3-treated T cells, less so in BTNL2-Ig-treated cells, and had no effect on cells stimulated with B7S1-Ig (Fig. 5C). IL-2 and CD28 co-stimulation synergistically enhanced T cell proliferation in the presence of B7-H3-Ig or BTNL2-Ig but not with B7S1-Ig (Fig. 5C).

DO11.10 hybridoma (DO11.10H) has been used to dissect TCR and co-receptor signaling mechanisms. It was found that DO11.10H expressed a receptor for BTNL2 upon activation, and BTNL2-Ig partially inhibited IL-2 production and activation-induced cell death in DO 11.10H cells. Thus, BTNL2 transfected with DO11.10H having luciferase reporters for AP-1, NFAT, and NF- κ B pathways was examined. BTNL2-Ig treatment resulted in at least 50% reduction of anti-CD3-

mediated activation of AP-1, NFAT, and NF- κ B (Fig. 6). Inhibition of all three general pathways suggests that BTNL2, like other negative B7-like co-stimulators, inhibits proximal TCR signaling events.

The compound of this invention, BTNL2-Ig, potently inhibits T cell proliferation, but only modestly affects IL-2 production. Addition of exogenous IL-2 only moderately improved the proliferation of T cells treated with BTNL2-Ig. This appears unique compared to T cells treated with B7-H3 or B7S1 fusion proteins. Moreover, inhibition of T cell proliferation by B7-H3-Ig or BTNL2-Ig, but not by B7S1-Ig, could be reversed by anti-CD28 co-stimulation. Although qualitative difference here maybe due to nature of the recombinant fusion proteins, the differential response by T cells treated with different molecules to anti-CD28 and IL-2 suggests distinct intracellular signaling mechanisms whereby the receptors for these molecules function.

Examples

Cloning and sequence analysis of BTNL2. A mouse spleen cDNA library obtained from Dr. Michael Bevan's laboratory (University of Washington, Seattle) was used to clone full-length BTNL2 using PCR primers (forward, ATGGTGGATTGCCCACGGTATAG, and reverse, TCATCTGAGCCTCTCATCAGAAG). The full-length cDNA of BTNL2 was cloned by high fidelity DNA polymerase. The PCR products were directly sequenced multiple times. The sequence results were then compared with existing sequence in the NCBI database: the polymorphic differences are shown in Figure 1A. Sequence analysis revealed four polymorphic differences, shown in bold, from the existing sequence in NCBI database. The predicted mouse BTNL2 amino acid sequence was aligned with human BTNL2 sequence. The “[” symbol mark the intron/exon border, and leader and transmembrane domains are underlined. An asterisk (*) denotes identity, colon (:) denotes conservation of strong groups, and a period (.) denotes conservation of weak groups between mouse and human sequences.

Comparison of BTNL2 amino acid sequence with other B7 and butyrophilin family members was performed using CLUSTAL W. Full-length protein sequences of murine B7 family members and butyrophilins were used for phylogenetic analysis with the exception of human BT3.1, which does not have a murine homologue. The result of the phylogenic analysis is shown in Fig. 1B. The location of the intron/exon

boundary of the BTNL2 gene was identified using the NCBI databases. Transmembrane prediction was performed with TMHMM Server v. 2.0 (<http://www.cbs.dtu.dk/services/TMHMM-2.0>) and the leader sequence was predicted with SignalP Y2.0.b2 (<http://www.cbs.dtu.dk/services/SignalP-2.0/#submission>).

Real-time PCR analysis. Primers spanning the third/fourth Ig domains [forward TTCACAATGCCAGAACTTCG (983 to 1002 nt), reverse TTCCATCTCTGTCCCTCCAC (1179 to 1198 nt)] and the transmembrane/intracellular coding sequences [forward CTCTGGGCCAGGAGAAAAC (1319 to 1337 nt), reverse TGAGCCTCTCATCAGAAGGAA (1520 to 1540 nt)] were used to analyze the expression of BTNL2 mRNA. cDNA samples were made from tissues of B6 mice and were subjected to real-time PCR analysis using SYBR Green Supermix according to the manufacturer's instructions (Bio-Rid, Hercules, CA) with the following modification: denaturation for 30s at 95 °C, annealing for 20s at 60 °C and extension for 30s at 72 °C with fluorescence detection at 60 °C after each cycle. The results were obtained and analyzed with iCyclere iQ real-time detection software (Bio-Rad, Hercules, CA).

Production of BTNL2-Ig protein. The sequence coding the extracellular portion of BTNL2 protein (amino acid 26 to 457) was PCR amplified and subcloned into DES-hlg vector consisting of an insect expression plasmid pMT/Bip/V5-HisA (Invitrogen, San Diego, CA) backbone and a human IgG1 Fc tag containing the dimerization sequence (14). The PCR primers, with added restriction sites, were: forward AGATCTCACCCAGATGACTTCAGAGTGGTC (73 to 96 nt) and reverse ACTAGTTATCTTGGAGTCTGAGAGAGGGAAAC (1343 to 1368 nt). The BTNL2-Ig expression vector was stably transfected into *Drosophila* S2 cells and induced to secrete BTNL2-Ig fusion protein upon CuSO₄ treatment. BTNL2-Ig fusion protein was further purified by a Protein A column. SDS-PAGE and Coomassie Blue staining were used to analyze the protein preparation, which showed only one major band at the predicted molecular weight.

Flow cytometry analysis. BTNL2-Ig and a control human IgG were biotinylated with Sulfo-NHS-LC-Biotin (Pierce) and used in conjunction with anti-CD4-FITC (L3T4), CD8-Percy5.5 (Ly-2), CD11b-PE (3A33), CD11c-PE (HL3) and

B220-FITC (RA3-6B2) antibodies (BD Pharmingen) for analysis of various populations of immune cells by flow cytometry. Total splenocytes were activated with 5 µg/ml of LPS for 2 days prior to B cell analysis. Total splenocytes were activated with 2.5 µg/ml of ConA for 2 days prior to T cell analysis. For B7.1-Ig blocking experiment, splenocytes were preincubated with 20-fold excess of B7.1-Ig before stained with biotinylated BTNL2-Ig. 293T cells were transfected with a PD-1 expression vector using calcium phosphate, and subsequently stained with biotinylated PD-L1-Ig or BTNL2-Ig. For activation-induced cell death assay, CD4⁺ T cells were activated with indicated doses of plate-bound anti-CD3 and 5 µg/ml of BTNL2-Ig or hIg for 24 hours before stained with propidium iodide (PI) followed by flow cytometry analysis. The percentage of cells stained positive for PI was determined as an estimate of apoptosis, and plotted against anti-CD3 concentration.

In vitro T cell assays. CD4⁺ T cells from naive C57BL/6 mice were purified by autoMACS with anti-CD4 microbeads to 90-95% purity. T cells were treated with plate-bound anti-CD3 (2 C 1 1) and/or anti-CD28 (37.51) antibodies (BD Pharmingen) in the absence or presence of a human IgG or BTNL2-Ig protein. Complete RPMI medium containing 10% FBS was used for T cell culture. IL-2 production was measured 24 hours after T cell activation, and cell proliferation measured at 72 hours with the addition of ³H-thymidine in the last 6-8 hours.

Luciferase assay. DO11.10 T cell hybridoma cells were maintained in complete RPMI1640 medium supplemented with 10% FBS (Hyclone, Logan, UT). 5x10⁶ cells were transfected with 1 µg of luciferase promoter reporter plasmids of NFAT, NF-κB or AP-1 (gifts of Dr. Richard Flavell at Yale University) and 0.25 µg of PRL-null (Promega, Madison, WI) by electroporation at 960 microfarads and 250 V as the manufacture recommended (Bio-Rad). After overnight culture, the cells were stimulated with 1 µg/ml of plate bound anti-CD3 Ab with or without human Ig fusion protein for 4 hours. Cells were lysed and luciferase activity was measured by using the DualLuciferase system as the manufacture recommended (Promega, Madison, WI).

WHAT IS CLAIMED:

1. A fusion protein comprising all or a biologically active fragment of human BTNL2 polypeptide.
2. The fusion protein of claim 1, wherein the biologically active fragment comprises an extracellular domain of BTNL2.
3. The fusion protein of claim 1, further comprising human IgG1, that is, the fusion protein is BTNL2-Ig.
4. The fusion protein of claim 1, comprising the amino acid sequence shown in SEQ ID 1.
5. A nucleic acid sequence that codes for the BTNL2 fusion protein.
6. A method of treating or preventing a disorder characterized by abnormal T cell activity comprising administering to a patient in need thereof an agent that modulates the signaling activity of BTNL2.
7. The method of claim 6, wherein the agent increases the signaling activity of BTNL2.
8. The method of claim, wherein the agent is BTNL2-Ig fusion protein.
9. The method of claim 6, wherein the disorder that can be treated or prevented by administering an agent that modulates the signaling activity of BTNL2 is selected from the group consisting of cancer, tissue or organ transplant rejection, allergy, autoimmune disease or immunosuppressive disorder.
10. The method of claim 9, wherein the autoimmune disease is selected from the group consisting of Crohn's disease, Stevens-Johnson Syndrome, systemic lupus erythematosus, aplastic anemia, myositis, polychondritis, idiopathic thrombocytopenia, sarcoidosis, Sjogren's syndrome, uveitis, lung fibrosis, rheumatoid arthritis, myasthenia gravis, psoriasis, cirrhosis, aplastic anemia, encephalopathy, multiple sclerosis, scleroderma and chronic hepatitis.
11. The method of claim 6, wherein the agent decreases the signaling activity of BTNL2.
12. The method of claim 6, wherein the agent is a BTNL2-specific antibody.

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[illegible]

FIG. 1A

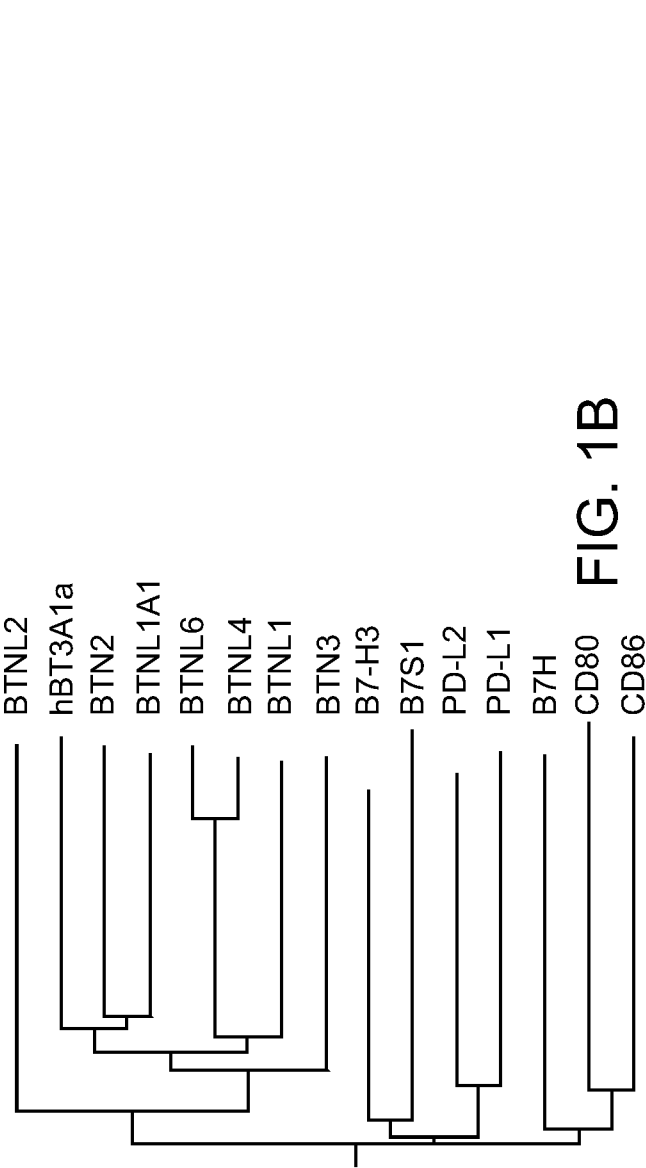


FIG. 1B

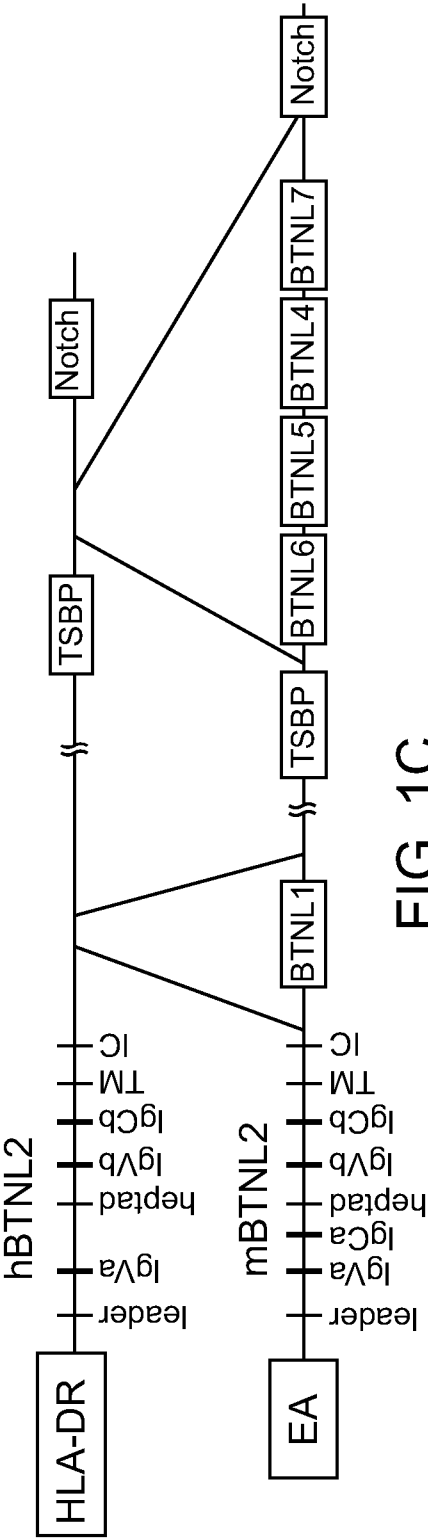


FIG. 1C

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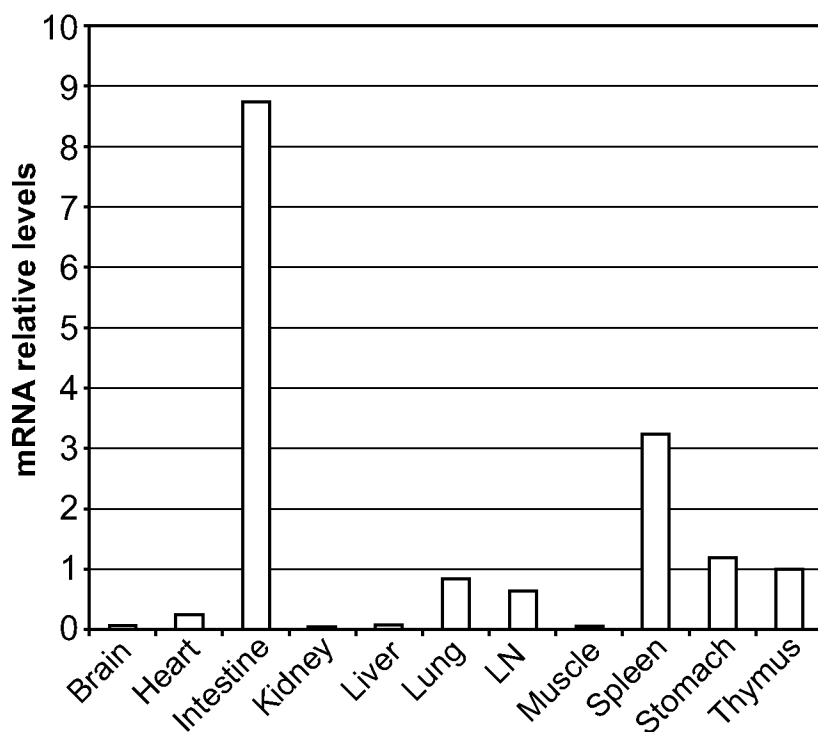


FIG. 2A

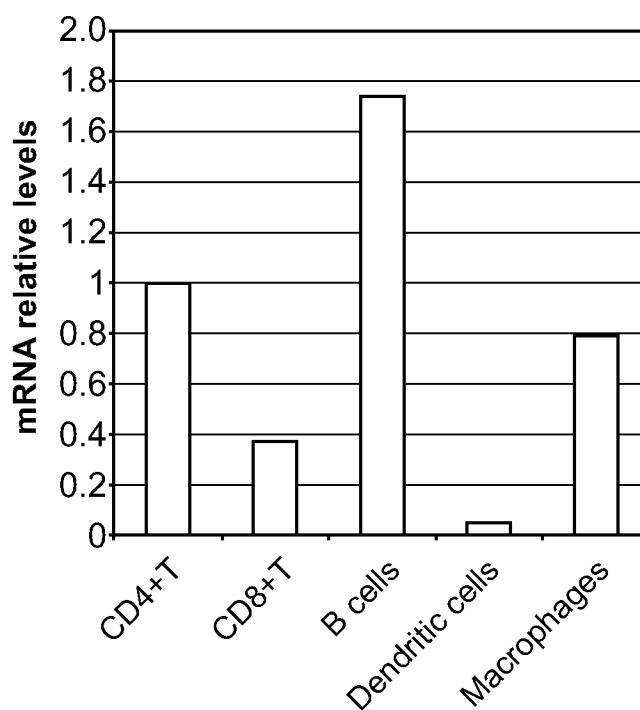


FIG. 2B

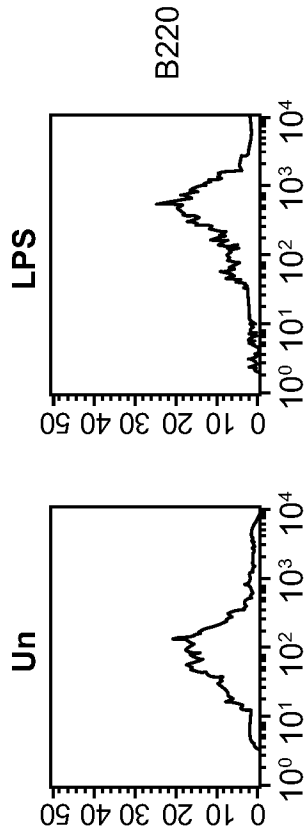


FIG. 3A

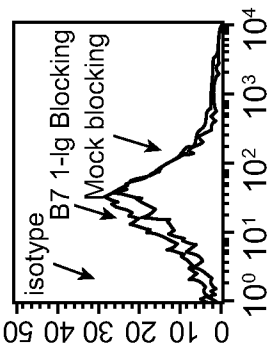


FIG. 3C

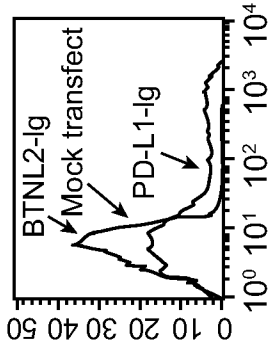


FIG. 3D

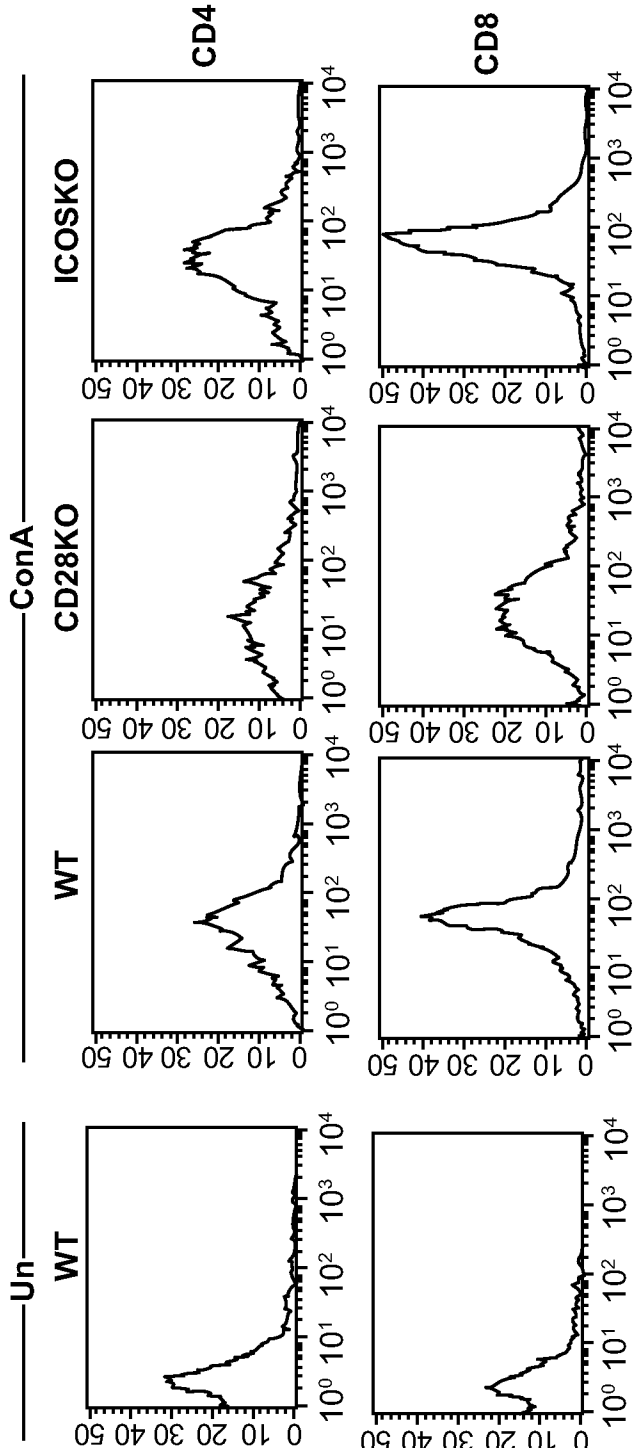


FIG. 3B

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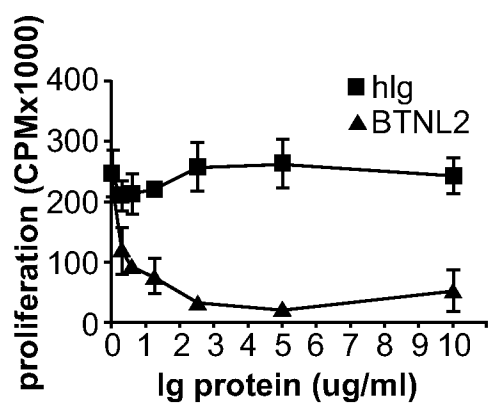


FIG. 4A

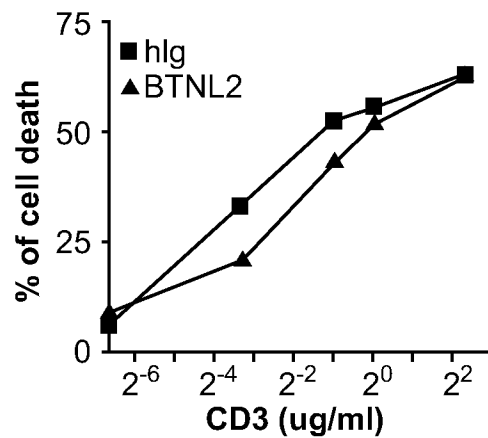


FIG. 4B

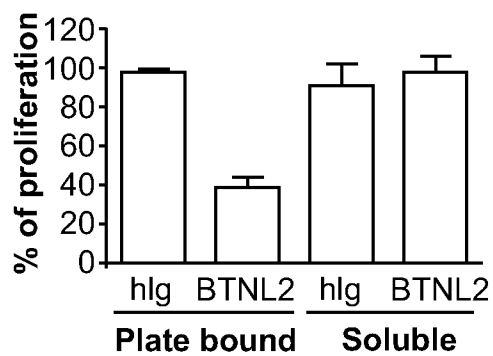


FIG. 4C

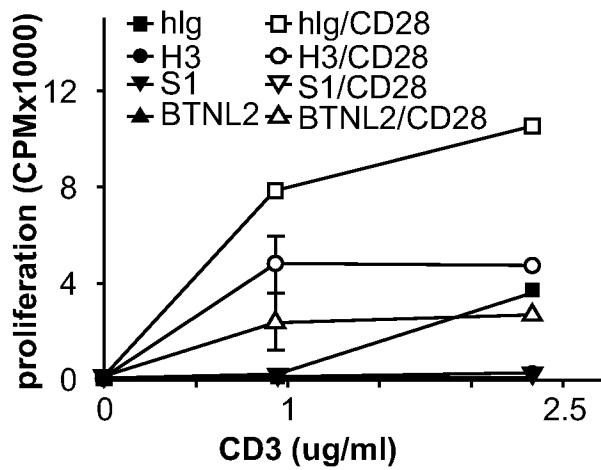


FIG. 4D

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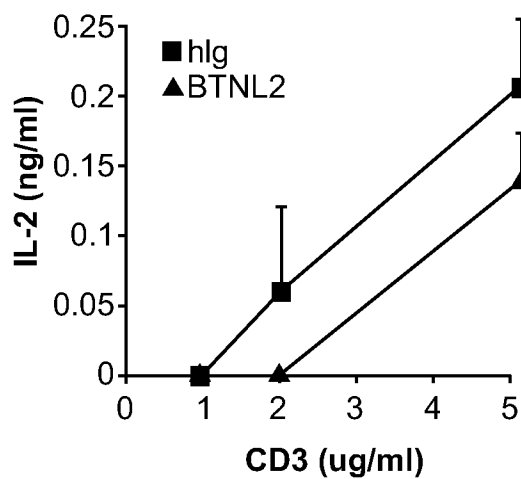


FIG. 5A

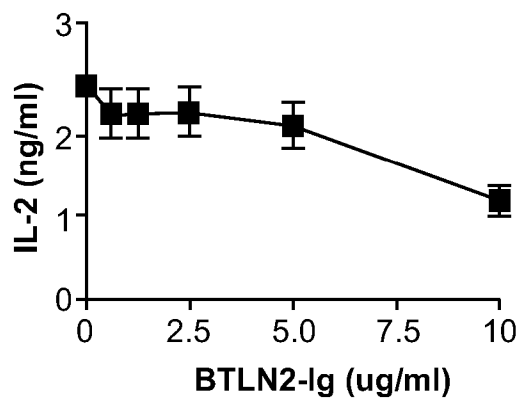


FIG. 5B

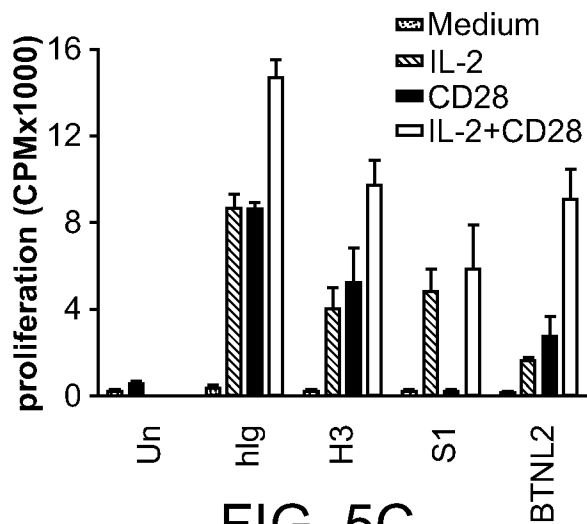


FIG. 5C