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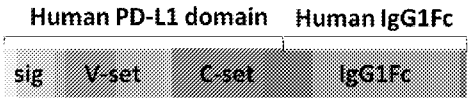
(54) **Title:** SPECIFIC BIFUNCTIONAL BY-001 (ACTIVE COMPOSITION OF HOMOMULTIMER OF CHIMERIC PROTEIN PD-L1 / FC-GAMMA1) DOWN REGULATES THE ACTIVATION OF HUMAN IMMUNE CELLS AND THE USE THEREOF

Fig. 1. Design for Chimeric Peptide of PD-1L/Fc-gamma1 (BY-001) and It's Amino Acid Sequence

A.



(57) **Abstract:** The present invention provides an active form (BY-001) of a homomultimeric chimeric protein PD-L1 / Fc-gamma1, which may have a composition of 25% dimer, 30% tetramer and 45% hexamer. BY-001 consists of two distinct functional domains at the aggregated state, one is the extracellular domain of PD-L1 and another one is the IgG1 Fc region. Accordingly, the first functional domain of PD-L1 at its aggregated state is more effective in suppressing the activity of CD3+ and CD28+ T cells; while, on the other hand, the second functional domain of FC-gamma1 at its aggregated state exerts only the suppressive effects on the immune cell bearing the Fc-gamma receptors. Thus, the features provided in present invention indicate that BY-001 regulates the functions of immune cells subsets that are responsible for the peripheral immune tolerance, which makes BY-001 potential to help restore or maintain the peripheral immune tolerance in patients with autoimmune diseases or in recipients of allografts.

WO 2020/005496 A1

Title: **Specific bifunctional BY-001 (active composition of homomultimer of chimeric protein PD-L1 / Fc-gamma1) down regulates the activation of human immune cells and the use thereof**

1. Background of the invention

The immune system normally recognizes and defends against foreign pathogens but does not respond to self-components of tissues, such intrinsic state is established and maintained precisely by the process of immune tolerance. The inflammatory damage of one or more organ systems caused by inappropriate activation of self-reactive both or either T cells and B cells is due to the loss of balance between effectors (B cells and T cells) [1, 2] and regulatory components (Treg cells and dendritic cells) of the immune system [3]. Therefore, restoration of immune tolerant state is the ultimate goal in the treatment of autoimmune diseases [4, 5].

Cell surface molecules such as CTLA-4 (cytotoxic T-lymphocyte-associated protein 4, or CD152) and PD-1 (Programmed Death-1, also known as CD279) deliver inhibitory immunoregulatory signals that are thought to be crucial to the maintenance of normal immune tolerance [6, 7] through its recognizing and binding to CD80 (B-7.1) or CD86 (B-7.2). Based on the discoveries, Bristol-Myers Squibb, a pharmaceutical company, developed a chimeric protein composed of the Fc region of the immunoglobulin IgG1 and the extracellular domain of CTLA-4 named as Abatacept (CTLA-4/Fc), thereafter, it was approved for clinic application by FDA in 2011. However, the pharmacological mechanism of Abatacept is to serve as antagonist of CD28 to interrupt the second signal, CD28 binding to B-7 for TCR activation, instead of the delivery of checkpoint signal through CTLA-4. Therefore, Abatacept interrupts the systemic cellular immune response, which, as a side effect, may cause serious infectious diseases, even cancer.

Second cell surface molecule responsible for immune checkpoint is PD-1 (Programmed cell death protein 1, also known as CD279), which is strictly expressed on thymocytes, activated T cells and pro-B cells. It has been believed that PD-1 is related biasedly to the maintenance of peripheral immune tolerance when it is associated with either of its two identified ligands: 1), Programmed Death-1 ligand 1 (PD-L1), also known as CD274 or B7-H1, broadly expressed on the hematopoietic and nonhematopoietic cells including tumor cells; 2), Programmed Death-1 ligand 2 (PD-L2), also known as CD273, or B7-DC strictly expressed on the dendric cells and macrophages. The PD-1 engagement with PD-Ls results in suppressing the activation of T cell or differentiation of mature B cell. Thus far three mechanisms have been elaborated for the pivotal role of PD-1/PD-L1 pathway in immune tolerance: (1) Induction of T cell anergy [8] through PD-L1 binding to PD-1 then activating the SHP2 to suppress the TCR/CD28 signaling; (2) suppression of B cell maturation through PD-L1 binding to PD-1 expressed on pre-B cells then activating the SHP2 to suppress the BCR signaling; [9]; (3) generation of regulatory T cells (especially type 1 (Tr1)) [10] and conversion of human TH1 into Treg cell (iTreg cell) [11]. In addition, soluble forms of PD-1 (sPD-1) and PD-L1 (sPD-L1) in peripheral blood may also be a source for the regulation of PD-1/PD-L1 pathway in immunity [12, 13]. A perfect manifestation of such mechanisms is that certain tumor cells use PD-L1 overexpression on their cell membranes and simultaneously shed into the circulation to successfully escape immune attacks [14].

In contrary with in tumor, according to the latest findings in autoimmune diseases, the weakening or deletion of the PD-1/PD-L1 pathway in immune cells is directly related to the instability or destruction of peripheral immune tolerance [10]. Mounting evidence demonstrate that impaired PD-1/PD-L1 function plays an important role in a variety of autoimmune diseases, such as SLE (systemic lupus erythematosus) and RA (Rheumatoid Arthritis), etc. [15]. The animal model demonstrates that loss of signaling of PD-1/PD-L1 (PD-1 knockout mice) develops lupus-like autoimmune disease [16], or autoimmune dilated cardiomyopathy depending upon the genetic

background [17]. PD-L1 deficiency enhances disease progression in the experimental autoimmune encephalomyelitis [18], nonobese diabetic (NOD) model of autoimmune diabetes [19, 20]and the murine model of multiple sclerosis (MS)[21]. Tissue expression of PD-L1 mediates peripheral T cell tolerance [22].

The third cell surface molecule responsible for immune tolerance is Fc-gamma receptor type IIB (one of Fc-gammaRIIA/B, collectively known as CD32). Human Fc-gamma receptors have been identified so far are hFc-gammaRI (CD64), hFc-gammaRII (types A and B, collectively known as CD32) and hFc-gammaRIII (types A and B, collectively known as CD16). Each type of receptor exhibits distinctive tissue distribution, structure and binding specificity towards various IgG subclasses (7,8). Fc-gammaRIIB is restrict expressed on B cell, monocyte/macrophage and dendritic cell in human, while basophil, eosinophil and mast cell as well in mice. Fc-gammaRII displays low affinity for monomeric IgG but high-avidity for aggregated multimeric IgG, which are particularly important in the recognition and binding of antibody–antigen complexes during an immune response [23]. The B cell stage(s) at which Fc-gammaRIIB exerts its function as a gatekeeper of self-tolerance has recently been defined [24, 25]. The main function of Fc-gammaRIIB is to inhibit activating signals, which is achieved through co-ligation of Fc-gammaRIIB with either activating Fc-gammaRs or with the BCR by immune complexes ([26]). This leads to phosphorylation of the cytoplasmic domain ITIM of Fc-gammaRIIB by the Src-family kinase lYN. This phosphorylation event is thought to require access of Fc-gammaRIIB to sphingolipid rafts in which activating Fc-gammaRs and the BCR reside following cross-linking. Subsequent binding of SH2-domain-containing inositol phosphatases (SHIPs), in particular SHIP1, result in the dephosphorization of downstream targets and inhibition of the activating signaling cascade. There is accumulating evidence that Fc-gammaRIIB mediates its function during late stages of B cell maturation, thus representing a distal checkpoint [25]. Through the analysis of an anti-DNA knock-in model, it was established that the absence of Fc-gammaRIIB resulted in the expansion of IgG-positive plasma cells secreting autoreactive antibodies (Fukuyama et al., 2005). Here, Fc-gammaRIIB might serve as the final barrier to prevent these B cells with potentially harmful BCR specificities from maturing into plasma cells that would otherwise induce tissue pathology by secretion of large amounts of self-reactive antibodies. Another cell type where Fc-gamagRIIB may play an important role in regulating immunity and tolerance are the dendritic cells [27-29]. A number of chronic inflammatory diseases have been shown to be associated with Fc-gamma receptor genetic variants and include (but are not limited to) autoimmune pathologies, such as systemic lupus erythematosus (SLE)[30], rheumatoid arthritis[31], acute allograft rejection [32] and vascular inflammatory and thrombotic disorders, such as coronary artery stenosis, peripheral atherosclerosis and vasculitis [33, 34].

Since PD-1 is expressed in activated T cells and B cells, it is expected that the PD-L1 protein can be effectively used as a therapeutic agent that specifically targets activated immune cells to suppress the inflammatory reaction not only in an autoimmune disease but also in organ transplantation. However, Fc-gammaRIIB is expressed in dendritic cells and monocytes/macrophages, it is expected also that aggregated form of Fc-gamma1 can be used as a therapeutic agent that specifically targets these immune cells to suppress the inflammatory reaction during the autoimmunity. Because the induction and maintenance of peripheral immune tolerance are dependent on the orchestra playing by all type of those cell populations. To date a peripheral immune tolerance-based immunotherapeutic agent using an agonist to rebuild the tolerance through both pathways of PD-1/PD-L1 and Fc-gamma/Fc-gamma receptors has not yet

been developed. Therefore, there is a need to develop a therapeutic reagent that the therapeutic effect not only targeting the autoreactive T cells and B cells but also targeting the inappropriately activated monocytes/macrophages and dendritic cells in inflammatory tissues, meanwhile, such reagent will avoid the causes of antibody dependent cell-mediated cytotoxicity (ADCC) and complement dependent cytotoxicity (CDC) in vivo due to its feature of biding to the activation of Fc receptor of Fc-gammaRI. Thus, when Ig chimeric proteins are used as an agent for treating an autoimmune disease or as an agent for inducing immune tolerance in organ transplantation, they cannot play the role of inhibiting inflammatory responses, and may rather aggravate inflammation.

2. Summary of the invention

In first aspect, the invention provides a bispecific biologic (chimeric protein of PD-L1/FC-gamma1) named BY-001, which is a composition of homomultimeric forms of dimer, tetramer and Hexamer, wherein the ratio of the forms is at 25% of dimer, 30% of tetramer and 45% of Hexamer respectively.

In second aspect, current invention provides the demonstrations of bispecific function of BY-001 that aggregated PD-L1 can trigger the pathway of PD-1 to decrease the cytokine production from activated T cell line (jurkate cell expressing PD-1), and also the aggregated Fc-gamma1 shows the suppressive effect on monocyte/macrophage cell line (THP-1 cell expressing Fc-gamma1RI & II) and erythroid cell line (K562 cell expressing Fc-gamma1RII).

In third aspect, the present invention also demonstrates that the functional features of both the domains PD-L1 and Fc-gamma1 in BY-001 fully inherit their characteristics in their parental proteins that have been confirmed in vitro and in vivo that published in highly recognized scientific journals. Therefore, an obvious perspective emerges that the BY-001 of the present invention is potential for a pharmaceutical composition for treating an autoimmune disease caused by abnormal regulation of an immune response or preventing the rejection of allografts.

3. Disclosure

3.1. Technical Problem and Solution

An object of the present invention is to provide BY-001 (a composition of bifunctional homomultimeric forms chimeric protein PD-L1/Fc-gamma1) comprises the forms of dimer, tetramer and Hexamer at the ratio of 25%, 30% and 45%, wherein monomer form of chimeric protein PD-L1/ Fc-gamma1 consists of two distinct domains. The amino acids of cysteine in the region of immunoglobulin Fc-gamma1 are kept in order to form the multimeric PD-L1/Fc-gamma1, such homomultimeric form provides the aggregated Fc-gamma1 (BY-001) for binding to Fc-gammaRIIB instead of Fc-gammaRI. Another object of the present invention is to provide the aggregated form (BY-001) of extracellular domain of PD-L1 protein, which shows an excellent regulating effect on activity of T cells. Third object of the present invention is to provide the aggregated form of Fc-gamma1 region, which binds only to Fc-gamma1RIIA and B and suppresses the production of pro-inflammatory factors in monocyte/macrophages and pre-erythrocytes.

3.1. Advantageous Effects

BY-001 of the present invention is a composition of chimeric protein PD-L1/Fc-gamma1 in the forms of dimer, tetramer and Hexamer. Such aggregated state of the chimeric protein PD-L1/Fc-gamma1 displays higher regulatory efficacy to PD1 and Fc-gammaRII. Therefore, it is more potential to regulate the activities of T cells including Th cells, cytotoxic-T cells and B cells during the active stage of autoimmunity, as well as to regulate the activities of monocytes/macrophages, dendritic cells and iTreg cells to restore and maintain the peripheral immune tolerance. Thereafter, the BY-001 of the present invention is potential for a pharmaceutical composition for treating an autoimmune disease caused by abnormal regulation of an immune response or preventing the rejection of allografts.

4. Description of Drawing

Fig. 1. Design for Chimeric Peptide of PD-1L/Fc-gamma1 (BY-001) and Its Amino Acid Sequence

A. Schematic diagram for the design of chimeric protein PD-L1/Fc-gmma1 consisting of extracellular portion of human PD-L1 and constant region of human IgG1 Fc. Please be noted:

1). Cysteines in V-set, C-set and IgG1Fc regions are kept for the -S-S- "disulfide" bond to form the advanced structure forms.

2). PD-L1/FC1 is 449 amino acid in total

3). The signal peptide of PD-L1 comprises amino acids 1-18, which will be digested when the protein secrets into culture medium. Therefore, the purified PD-L1/Fc-gamma1 of current invention contains 431 amino acids in total.

4). immunoglobulin V like region (V-set, 19~127 amino-acids, exon-2)

5). immunoglobulin constant like region (C-set, 133~220 amino-acids, exon 3)

6). human IgG1 heavy chain constant domain (C region, 221~449) is the region of Fc-gamma1.

B. The AA sequence of PD-L1 contained in chimeric protein PD-L1/Fc-gamma1. The sequence highlighted in yellow is the part of signal sequence for leading the protein toward the secretory pathway, which is cut away as the protein released to medium. The sequence highlighted in shadow is the region of Fc-gamma1.

Fig. 2. Visualized Protein of BY-001 in SDS-Page Gel and Western-Blot DNA fragment encoding amino acid sequence of PD-L1 stated in (fig.1. B) was inserted into vector of pFUSE-hIgG1-Fc-gamma1purchased from INVIVOGEN USA, 10515 Vista Sorrento Pkwy, San Diego, CA 92121, USA. The DNA of pFuse-PD-L1/Fc-gamma1 was transfected with 293 cells. Monofinity A Column Purification (GenScript, 860 Centennial Avenue, Piscataway, NJ 08854) was utilized to purify the BY-001 from the supernatants of 293 cell culture. The molecular wieght of expected protein in monomeric form is about 60 kilodalton (KD). Westen blot system was used for the analysis of protein product. Reducing and nonreducing condition (without dithiothreitol (DTT) for the former, with DTT for the latter) were applied for breaking up the disulfide bounds in advanced structure of BY-001.

A. SDS-page gel, Coommassie blue staining. Lane M1: Protein Marker Cat. No. 3452, Lane 1: BY-001 at Reducing condition (monomer), Lane 2: BY-001 at non-reducing condition (no monomer, but dimer, tetramer and Hexamer).

B. Western blot probed with Primary antibody: Goat Anti-Human IgG-HRP. Lane M2: Protein Marker, GenScript, Cat. No. M00521, Lane P: Human IgG1, Kappa (Sigma, Cat.No. I5154) as a positive control. Lane 1, monomer of PD-l1/Fc-gamma1 band at nonreducing condition; Lane 2, multimeric forms of BY-001, dimer, tetramer and hexamer.

C. Western blot probed with Human PD-L1/B7-H1 Antibody (R&D, Cat.No.AF156). Lane M2: Protein Marker, GenScript, Cat. No. M00521; Lane 1, monomer of PD-l1/Fc-gamma1 band at nonreducing condition; Lane 2, multimeric forms of BY-001, dimer, tetramer and hexamer.

D. Diagrams of the BY-001, cartoons of D, E and F are the graphic view for illustration of BY-001 at forms of dimer, tetramer and hexamer.

Fig. 3. BY-001 suppresses IL-2 production from Jurkate cell line Jurkate (from ATCC, USA) is a T cell line generated from human T-cell leukemia. CD3, CD28 and PD-1 are molecules expressed on

the cell surface. OKT3 is an antibody against CD3, and its binding and cross-linking will activate T cells. CD28 is a receptor for co-stimulatory molecule B7 family. The association of anti-CD28 with CD28 will greatly enhance T cell activation based on OKT3 stimulation in vitro. IL-2 production is one of the biomarkers commonly to indicate T cell activation. The goal of this experiment is to validate and demonstrate the function of BY-001 in regulation of T cell activation in vitro.

5ug/ml of OKT3 (Anti-human CD3, Clone OKT3, BD bioscience) was coated onto 96-well, U bottom micro-plate for overnight at 4°C. Cell suspension of Jurkate cells at 3 million/ml is in RPMI164 medium with 10% FBS.

A. 100ul of Cell suspension jurkate at 3 million/ml was added into each well hereafter, BY-001 was added into wells according to designed. The plate was kept in incubator at 37°C and 5% CO_2 overnight. The culture supernatants were harvested from wells for the measurement of IL-2 production. Human IL-2 High Sensitivity ELISA Kit (eBioscience, 1030 Vienna, Austria) was used for the measurement of IL-2. The results indicate that BY-001 inhibits the IL-2 production effectively at the starting concentration (0.1ug/ml) of BY-001. CD3 signal along (without the CD28 second signal) the inhibition was 87.5% at concentration of 0.1ug/ml of BY-001, such inhibition was persistent from the concentrations of 0.1ug/ml to 1ug/ml.

B. Anti-human CD28 (BD bioscience, Clone CD28.2) mixed at 2ug/ml into same jurkate cell suspension solution as used in (A). The plate was incubated at 37°C and 5% CO_2 for overnight. The culture supernatants were harvested from wells for the measurement of IL-2 production by following the method in (A). The results indicate that TCR signal with the help of co-stimulator signal make Jurkate cell produces higher level of IL-2 (from 30pg/ml at OKT3 along to 60pg/ml at OKT3 + anti-CD28), however it makes BY-001 inhibit IL-2 production as a gradient decent pattern at the concentration (0.5 to 2 ug/ml), the inhibitory rate become to be 13.1% at 0.5ug/ml, 43% at 1.0ug/ml to 67.6% at 2ug/ml of BY-001.

Fig. 4. BY-001 suppresses IL-2 production from PBMC human primary cells The PBMC was obtained from healthy human donor. PBMC are cells containing only mononuclear in their cell. Among the PBMC, CD3 is expressed on all T cells, while CD3 and CD28 double positive T cells account for the majority of all T cells. B7 family and PD-1 are expressed on most of the PBMC cell surface. Fc receptor is expressed on dendritic cell, monocyte, etc. The goal of this experiment is to validate and demonstrate that BY-001 functions as a mediator in regulation of T cell activation in human primary cell, by which to indicate the great opportunity of success in clinical trial.

The 96-well plate was coated with 3ug/ml of OKT3 at 4°C for overnight. 100ul of Jurkate cell solution at 3 million/ml in the presence or no presence of Anti-human CD28 at 2ug/ml were added into wells. The plate was kept in incubator at 37°C and 5% CO_2 for overnight, supernatants were collected from the wells for IL-2 detection with Human IL-2 High Sensitivity ELISA Kit.

A. CD3 signal along (without the CD28 second signal) the inhibition was 90.8% at concentration of 0.25ug/ml of BY-001, the inhibition is persistent at concentrations of BY-001 from 0.25 to 4 ug/ml.

B. CD3 signal together with the CD28 second signal the inhibition pattern became to be gradient decent from the inhibitory rate 17.5% at 0.25ug/ml to 74.1% at 4ug/ml of BY-001.

Fig. 5 BY-001 regulates BY-001 regulates IL-4 and TGF-betta production from K562 cell K562 cell (from ATCC, USA) is pre-erythrocyte generated from human myelogenous leukemia (of the erythroleukemia type) in which no CD3, CD28 and PD-1 molecules are expressed. However, it was reported that only Fc-gamma1 receptor II are expressed on its cell surface but no Fc-gamma1 receptor I. Therefore, only aggregated Fc-gamma1 are capable to binding to and triggering the Fc-gamma1/Fc-Gamma1RII pathway. To determine the function of aggreged Fc-gamma1 of BY-001 we measured the regulation of IL-4 and TGF-betta production in K562 cells. Apparently, the signaling transferred in this demonstration is suppressive.

A. The basal level of IL-4 production in K562 is high (84pg/ml). Addition of BY-00-1 according to designed into the cells culture decreases the IL-4 level. The inhibition is sufficient at concentration of 4 ug/ml of BY-001, which indicates that aggregated Fc-gamma1 bind to Fc-gamma1/Fc-Gamma1RII and trigger the inhibitory pathway.

B. The basal level of TGF-betta production in K562 is high (1250pg/ml). Addition of BY-00-1 according to designed into the cells culture decreases the TGF-betta level. The inhibition is sufficient at concentration of 2 and 4 ug/ml of BY-001, which indicates that aggregated Fc-gamma1 bind to Fc-gamma1/Fc-Gamma1RII and trigger the inhibitory pathway.

C. The diagram for BY-001 binding to and triggering the Fc-gammaRII, thereafter to deliver the suppressive signal. ITAM stands for immunoreceptor tyrosine-based activation motif in which the tyrosine residues are phosphorylated upon the binding between Fc-gamma1 and Fc-gamma1RII. While ITIM stands for the immunoreceptor tyrosine-based inhibitory motif in which the tyrosine residues are phosphorylated, thereafter the phosphatase SHIP1 or 2 is activated to dephosphorylate the phosphorylated tyrosine in ITAM of Fc-gammaRIIA or C via the crosslinking of bound Fc-gamma1s of BY-001.

Fig. 6. BY-001 regulates the IL-4 and TGF-betta production from THP-1 cells THP-1 cell (from ATCC, USA) is THP-1 is a human monocytic cell line derived from acute monocytic leukemia in which no CD3, CD28 and PD-1 molecules are expressed. However, it was reported that both Fc-gamma1 receptor I and II are expressed on its cell surface. To date, it has been believed that Fc region of monomeric antibody is high affinity to Fc-gamma1RI but with very low avidity to Fc-gammaRII. In contrast, the Fc portion of antigen-antibody complex binds to Fc-gammaRII instead of Fc-gammaRI. Therefore, to determine the efficacy of aggregated Fc-gamma1 of BY-001 in binding to and triggering the Fc-gamma1/Fc-gamma1RII pathway, we measured the regulation of IL-4 and TGF-betta production in THP-1 cells. Apparently, the signaling transferred in this demonstration is suppressive, which indicates also that the aggregated Fc-gamma1 of BY-001 triggers the inhibitory pathway of Fc-gamma/Fc-gammaRIIB.

A. The basal level of IL-4 production in THP-1 is high (86pg/ml). Addition of BY-00-1 according to designed into the cells culture decreases the IL-4 level. The inhibitions are sufficient at concentration of 2 and 4 ug/ml of BY-001, which indicates that aggregated Fc-gamma1 bind to Fc-gamma1/Fc-Gamma1RII and trigger the inhibitory pathway only.

B. The basal level of TGF-betta production in THP-1 is high (485pg/ml). Addition of BY-00-1 according to designed into the cells culture decreases the TGF-betta level. The inhibition is

observed at concentration of 2 and 4 ug/ml of BY-001, which indicates that aggregated Fc-gamma1 bind to Fc-gamma1/Fc-Gamma1RII and trigger the inhibitory pathway only.
C. The diagram for BY-001 binding to and triggering the Fc-gammaRII, thereafter to deliver the suppressive signal. ITAM stands for immunoreceptor tyrosine-based activation motif in which the tyrosine residues are phosphorylated upon the binding between Fc-gamma1 and Fc-gamma1RII. While ITIM stands for the immunoreceptor tyrosine-based inhibitory motif in which the tyrosine residues are phosphorylated, thereafter the phosphatase SHIP1 or 2 is activated to dephosphorylate the phosphorylated tyrosine in ITAM of Fc-gammaRIIA or C via the crosslinking of bound Fc-gamma1s of BY-001. Apparently, the Fc-gammaRI has no effect on the inhibition, which is benefited for BY-001, hereinafter, in dealing with autoimmunity.

Fig. 7 Advanced structural difference between BY-001 and antigen-antibody complex specific antibodies associate with antigen with electrostatic attraction and hydrophobic bonds or hydrogen bonds, which are separated easily in SDS page gel of protein electrophoresis. While the PD-L1/Fc-gamma1 of present invention bound together with disulfide bound (S-S) without the component of antigen, and the S-S bound only can be broken up under the of reducing condition (by adding DTT) in SDS page gel of protein electrophoresis.
A. The diagram is the dimer of BY-001 bound together by S-S bound
B. The diagram is the tetramer of BY-001 bound together by S-S bound
C. the diagram is the hexamer of BY-001 bound together by S-S bound
D. the diagram is the antigen-antibody complex

Fig. 8 Expected mechanism for BY-001 to rebuild the peripheral immune tolerance Genetic alterations (gene mutation or polymorphism) may cause the individual difficult to maintain the peripheral tolerance of immunity. The dendritic cells, macrophages, T cells are become to be very fragile to resist the alteration of microenvironment, then easily to be activated inappropriately. The outcome is the generation of auto-cytotoxic T cell against self-tissues. The addition of BY-001 will directly reduce the activity of cytotoxic T cells by which to induce the auto-cytotoxic T cell anergy, on the other hand, BY-001 will also regulate the activities of Th cell, Treg cells and dendritic cells, macrophages which is critical to rebuilt peripheral immune tolerance.

5. Detailed Description of the Invention

5.1. Definitions: Prior to describing the invention in more detail the following definitions are provided. Unless otherwise stated, all technical and scientific terms used herein have the same meanings as commonly understood by one of ordinary skill in the art to which this invention belongs. Any methods and materials with similar or equivalent function to those described herein can be used in the practice or testing of the present invention. Methods, devices, and materials suitable for such uses are now described. All publications cited herein are incorporated herein by reference in their entirety for the purpose of describing and disclosing the methodologies, reagents, and tools reported in the publications that might be used in connection with the invention.

In accordance with the object of the present invention, in one aspect, there is provided a fusion protein comprising the extracellular domain of PD-L1 protein or a fragment thereof and a human immunoglobulin Fc-gamma1 region ("PD-L1/Fc-gamma1 chimeric protein," "PD-L1/Fc-gamma1 protein," "PD-L1 chimeric protein" or "chimeric protein'). The extracellular domain of PD-L1 may be a polypeptide comprising an immunoglobulin V (V-set) like domain of PD-L1 and an immunoglobulin C (C-set) like domain of PD-L1. The extracellular domain of PD-L1 is a domain exposed outside a cell membrane, and may be a polypeptide comprising the amino acids at positions 19 to 220 of sequence of Fig. 1 B of current invention. In addition, the extracellular domain of PD-L1 or a fragment thereof may be of human origin. In addition, the extracellular domain of PD-L1 may have about 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more homology to the polypeptide sequence consisting of the amino acids at positions 19 to 220 of sequence of Fig.1B. In addition, Human IgG1 heavy chain constant domain (C region, 221~449) of the sequence of Fig1B is the region of Fc-gamma1. All the Cysteines in V-set and C-set and human immunoglobulin Fc-gamma1 region of PD-L1/Fc-gamma1 thereof are kept in current invention for the -S-S- "disulfide" bond to form the advanced structure.

As used herein, the term "BY-001" refers to a composition of homomultimeric forms of bifunctional chimeric protein PD-L1/ Fc-gamma1 at the ratio of 25% of dimer, 30% of tetramer and 45% of Hexamer.

As used herein, the term "immune cell" includes cells that are of hematopoietic origin and that play a role in the immune response, such as B cells, T cells, dendritic cells, natural killer cells, monocytes and macrophages, etc.

As used herein, the term "T cell" includes CD4+ Th1 and Th2 cells, CD3+ and CD8+ cytotoxic T cells including self-reactive T cells, CD25+ and FOX3+ Treg cells and inducible Treg cells (iTreg).

As used herein, the term "B cells" refers to the B cell potential to producing the antibodies including the self-reactive antibodies.

As used herein, the term "aggregated PD-L1" refers to the extracellular portion of PD-L1 of human or mouse origin at the aggregated state thereof.

As used herein, the term "aggregated Fc-gamma1" refers to the regions of hinge, CH2 and CH3 of human IgG1 heavy chain of human or mouse origin at the aggregated state thereof, in addition, any peptide combined to the regions of hinge, CH2 and CH3 of human IgG1 heavy chain of human or mouse origin at the aggregated state thereof is include.

As used herein, the term "inhibitory signal" refers to a signal transmitted via an inhibitory receptor molecule on an immune cell. Such as CTLA-4, PD-1 and Fc-gammaRIIB.

5.2. Mode for Invention

Hereinafter, the invention is explained in accordance with the examples to further illustrate the application without limiting its scope.

In accordance with the object of the present invention, first structure of protein in one aspect, there is provided bifunctional chimeric protein in first structure of protein comprising a first function domain, the extracellular domain of PD-L1 protein or a fragment thereof and a second function domain, the human immunoglobulin Fc-gamma1 region. The bifunctional chimeric protein of current invention is stated as “PD-L1/Fc-gamma1 chimeric protein” or “PD-L1/Fc-gamma1 protein” or “PD-L1 chimeric protein” or “chimeric protein”. The extracellular domain of PD-L1 may be a polypeptide comprising an immunoglobulin V (V-set) like domain of PD-L1 and an immunoglobulin C (C-set) like domain of PD-L1. The extracellular domain of PD-L1 is a domain exposed outside a cell membrane, and may be a polypeptide comprising the amino acids at positions 19 to 220 of sequence of Fig. 1 B of current invention. In addition, the extracellular domain of PD-L1 or a fragment thereof may be of human origin. In addition, the extracellular domain of PD-L1 may have about 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more homology to the polypeptide sequence consisting of the amino acids at positions 19 to 220 of sequence of Fig.1B. In addition, Human IgG1 heavy chain constant domain (C region, 221~449) of the sequence of Fig1B is the region of Fc-gamma1.

The present invention provided, advanced structure of protein in another aspect, is bifunctional chimeric protein in advanced structure of protein comprising a composition of homomultimer of dimer, tetramer and hexamer of PD-L1/Fc-gamma1 chimeric protein (BY-001). All the Cysteines in V-set and C-set and human immunoglobulin Fc-gamma1 region of PD-L1/Fc-gamma1 thereof are kept in current invention for the -S-S- “disulfide” bond to form the advanced structure. Hence, once the PD-L1 is fused to second domain components Fc-gamma1, it is a complex naturally composed in dimer, tetramer and Hexamer, in fact PD-L1 dimer, tetramer and Hexamer are formed, on the other hand, Fc-gamma1 dimer, tetramer and Hexamer are formed also, for example as presented in Fig.2 of current invention.

To date, the subsets of immune cells of activated T cells, B cells and dendritic cells are related to the autoimmunity and rejection of allografts, while monocytes/macrophages are one of the key subsets related to inflammatory reaction, particularly to the chronic inflammation. It has highly recognized that the cell signaling pathways PD-1/PD-L1 and Fc-gammaRII/Fc-gamma are crucial in the regulation of functions of the subsets of immune cells mentioned above.

Under physiological condition, PD-1 is expressed in activated T cells and B cells and is bound and triggered by monomeric PD-L1, thereafter, the PD-1/PD-L1 pathway delivers the cell signaling of immune checkpoint. Thus PD-1/PD-L1 axis is one of the pathways that exert the key roles in the establishing and maintenance of immune tolerance. The provided in current invention is the PD-L1 in chimeric protein at its aggregated state and is expected to act on the PD-1 signaling axis.

To validate the function of PD-L1 at its aggregated forms as stated (Fig. 2) in current invention, in one embodiment as presented in Fig.3 and Fig.4 of current invention, the present invention provides validation that PD-1 on immune cells is activated by the aggregated form of PD-L1 (BY-

001), thereafter to deliver the cell signaling of immune checkpoint. The bifunctional chimeric protein PD-L1/Fc-gamma1 comprising a first domain PD-L1 that binds to and triggers PD-1. For example, as shown in current invention, BY-001 inhibits the IL-2 production from the Jurkate cells (Fig.3) and human PBMCs of healthy donor (Fig. 4) after the treatment with either OKT3 antibody or CKT3 antibody + CD28 antibody. OKT3 antibody along or together with CD28 antibody as the co-stimulator are commonly used to trigger TCR signaling for the activation of CD3+ T cells. IL-2 production is one of the biomarkers commonly to indicate T cell activation. It has commonly believed that inflammatory reaction in autoimmunity or rejection of allografts are caused by inappropriate activation of the self-reactive T cells and self-reactive B cells due to the peripheral immune tolerance breakdown [1-3]). The current methods in the treatment of autoimmune diseases or protection of allografts are to suppress the activity of the mentioned self-reactive T cells and self-reactive B cells [5, 35]. To date, it has believed that cell surface receptor of PD-1 is expressed only in activated T cells and B cells [35]. The association between PD-1 and PD-L1 induces the activated T anergy cell or B cell anergy or apoptosis, or alternatively the association induces the differentiation of Treg cell from Th cell [36]. Therefore, the current invention provided homomultimeric forms of PD-L1/Fc-gamma1 (BY-001), a novel concept of soluble chimeric proteins, is potential to treat certain immune and inflammatory disorders in one dimension.

According to the ability to activate or suppress the immune response Fc-gamma receptors are divided in two classes: hFc-gammaRI, hFc-gammaRIIA and hFc-gammaRIIIA are activating receptors via the cytoplasmic ITAM (immunoreceptor tyrosine-based activation motif), whereas hFc-gammaRIIB is suppressing receptor via the cytoplasmic ITIM (immunoreceptor tyrosine-based inhibitory motif) [37]. Fc-gammaRI is a high-affinity receptor that binds to only monomeric IgG and is expressed constitutively in dendritic cells, monocytes and macrophages in both human and mice[38]. In contrast to Fc-gammaRI, the other three types express in most of the immune cell populations, except the B cells, and exhibit affinity to Fc-gamma of IgGs in antigen-antibody complex, among them Fc-gammaRIIB exerts the suppressive signaling through its binding with one Fc-gamma and crosslinking others Fc-gammaRIIA with others Fc-gamma in antigen-antibody complex. Furthermore, Fc-gammaRIIB is expressed on B cell and appears to function in a B cell-autonomous manner to regulate autoreactive cells in the periphery. There have been extensively reported that Fc-gammaRII plays key role in autoimmunity and inflammatory disorders. Fc-gammaRII/Fc-gamma axis dominates the activation and chemo-cytokines production profile of dendritic cells and monocytes/macrophages, which is critical for the process of inflammation, and also is the key role in maintenance of immune tolerance. The provided in current invention is the Fc-gamma1 in chimeric protein at its aggregated state and is expected to act on the Fc-gammaRII signaling axis.

To validate the function of Fc-gamma1 at its aggregated forms as stated (Fig. 2) in current invention, in another embodiment, as a novel concept of soluble chimeric proteins, presented in Fig. 5 and Fig. 6 of current invention, the present invention provides validation that Fc-gammaRIIB on immune cells is activated by the aggregated form of Fc-gamma1 (BY-001), thereafter to deliver the suppressive cell signaling to immune cells. In current invention, the artificially aggregated form PD-L1/Fc-gamma1 (BY-001) in tetramer and hexamer are distinct from the Fc form in Ag-Ab complex in structure according to diagram in Fig. 7 of current invention. As shown in current

invention, for example, BY-001 inhibits the IL-4 and TGF-betta production from the K562 cells and THP-1 cells. It was reported that K562 cells express only Fc-gamma receptor II [39], while THP-1 cells express both Fc-gamma receptor I and II on the cell surface[40]. Apparently, the activating receptor of Fc-gammaRI on THP-1 cell didn't influence the suppressive pathway of Fc-gammaRIIB/Fc-gamma1. The machinery of suppressive pathway may comprise Fc-gamma1 bound with Fc-gammaRIIB and others Fc-gamma1 in multimeric form crosslinked the Fc-gammaRIIA or RIIC, thereafter, the inhibitory signaling is delivered and the activity of the cells are decreased through the pathway.

Collectively, BY-001 exerts dual functions that in one hand binds to and triggers the PD-1 pathway through multimeric PD-L1, and on the other hand binds to Fc-gamma1RIIB through multimeric Fc-gamma1 to trigger the suppressive signaling into the cells. Thereafter, BY-001 is capable to regulate the subsets of immune cells activated T cells and B cells, meanwhile to regulate the subsets of dendritic cell and monocytes/macrophages, the immune cell populations tightly relate to autoimmunity and protection of allografts. Therefore, BY-001 is potential to treat certain immune and inflammatory disorders. In the setting of autoimmune, alloimmune and inflammatory diseases, the homomultimeric forms of chimeric protein of this invention can reduce autoimmune, alloimmune and inflammatory manifestations by one or more mechanisms. For example, the PD-L1 extracellular domain in homomultimeric forms of chimeric protein of this invention can bind to the PD-1 on immune cell, such as an activated T cell or pre-B cell; while the second domain in homomultimeric forms of chimeric protein binds to and cross-links the FC receptors that express on dendritic cell and macrophage and other immune cell bearing Fc-gammaRII or RIII, etc.. Through these binding and cross-linking events, the receptors PD-1 for the first domain trigger the T cell anergy, repress the B cell maturation, differentiation of Treg cells and regulatory dendritic cells; the FC receptors for second domain of the chimeric protein may inhibit the activated dendritic cell and macrophage in the inflammatory location. All these events are critical to induce and maintain the immune tolerance of the body.

In addition, once the FC-gamma1 contained in chimeric protein anchored to FC receptor on cell surface of dendritic cell and macrophage, now membrane-anchored, therefore, the chimeric protein of the present invention may mediate its activity by spanning two neighboring cells, and thereby establish an immune tolerant microenvironment at inflammatory site. Alternatively, for example, a PD-L1 containing chimeric protein can bind to the B7-1 costimulator on an antigen-presenting cell, thereby interfering with its costimulatory, immune-activating function.

As indicated in schematic diagram of Fig. 8, BY-001 is potential for the treatment of autoimmune diseases and protection of allografts. For example, type 1 diabetes (T1D, Systemic Lupus Erythematosus (SLE), Rheumatoid Arthritis (RA) and inflammatory bowel disease (IBD) (including ulcerative colitis (UC) and Crohn's disease (CD)), multiple sclerosis (MS), scleroderma, and other diseases and disorders, such as PV (pemphigus vulgaris), psoriasis, atopic dermatitis, celiac disease, Chronic Obstructive Lung disease, Hashimoto's thyroiditis, Graves' disease (thyroid), Sjogren's syndrome, Guillain-Barre syndrome, Goodpasture's syndrome, Addison's disease, Wegener's granulomatosis, primary biliary sclerosis, sclerosing cholangitis, autoimmune hepatitis, polymyalgia rheumatica. Raynaud's phenomenon, temporal arteritis, giant cell arteritis, autoimmune hemolytic anemia, pernicious anemia, polyarteritis nodosa. Behcet's disease, primary

bilary cirrhosis, uveitis, myocarditis, rheumatic fever, ankylosing spondylitis, glomerulenephritis, sarcoidosis, dermatomyositis, myasthenia gravis, polymyositis, alopecia areata, and vitilgo.

9. References:

1. Davidson, A. and B. Diamond, *Autoimmune diseases.* N Engl J Med, 2001. **345**(5): p. 340-50.
2. Goding, J.W., *Autoimmune diseases.* N Engl J Med, 2001. **345**(23): p. 1707-8.
3. Bluestone, J.A., *Mechanisms of tolerance.* Immunol Rev, 2011. **241**(1): p. 5-19.
4. Smilek, D.E., M.R. Ehlers, and G.T. Nepom, *Restoring the balance: immunotherapeutic combinations for autoimmune disease.* Dis Model Mech, 2014. **7**(5): p. 503-13.
5. Brackmann, H.H., et al., *Immune tolerance induction: What have we learned over time?* Haemophilia, 2018. **24 Suppl 3**: p. 3-14.
6. Boonman, Z.F., et al., *Maintenance of immune tolerance depends on normal tissue homeostasis.* J Immunol, 2005. **175**(7): p. 4247-54.
7. Schep, S.J., et al., *Review of immune tolerance induction in hemophilia A.* Blood Rev, 2018. **32**(4): p. 326-338.
8. Bardhan, K., T. Anagnostou, and V.A. Boussiotis, *The PD1:PD-L1/2 Pathway from Discovery to Clinical Implementation.* Front Immunol, 2016. **7**: p. 550.
9. Thibult, M.L., et al., *PD-1 is a novel regulator of human B-cell activation.* Int Immunol, 2013. **25**(2): p. 129-37.
10. Francisco, L.M., P.T. Sage, and A.H. Sharpe, *The PD-1 pathway in tolerance and autoimmunity.* Immunol Rev, 2010. **236**: p. 219-42.
11. Amarnath, S., et al., *The PDL1-PD1 axis converts human TH1 cells into regulatory T cells.* Sci Transl Med, 2011. **3**(111): p. 111ra120.
12. Zhu, X. and J. Lang, *Soluble PD-1 and PD-L1: predictive and prognostic significance in cancer.* Oncotarget, 2017. **8**(57): p. 97671-97682.
13. Li, Y., et al., *Role of soluble programmed death-1 (sPD-1) and sPD-ligand 1 in patients with cystic echinococcosis.* Exp Ther Med, 2016. **11**(1): p. 251-256.
14. Alsaab, H.O., et al., *PD-1 and PD-L1 Checkpoint Signaling Inhibition for Cancer Immunotherapy: Mechanism, Combinations, and Clinical Outcome.* Front Pharmacol, 2017. **8**: p. 561.
15. Zamani, M.R., et al., *PD-1/PD-L and autoimmunity: A growing relationship.* Cell Immunol, 2016. **310**: p. 27-41.
16. Liao, W., et al., *The Systemic Activation of Programmed Death 1-PD-L1 Axis Protects Systemic Lupus Erythematosus Model from Nephritis.* Am J Nephrol, 2017. **46**(5): p. 371-379.
17. Nishimura, H., et al., *Autoimmune dilated cardiomyopathy in PD-1 receptor-deficient mice.* Science, 2001. **291**(5502): p. 319-22.
18. Dinesh, R.K., B.H. Hahn, and R.P. Singh, *PD-1, gender, and autoimmunity.* Autoimmun Rev, 2010. **9**(8): p. 583-7.
19. Wang, C.J., et al., *Protective role of programmed death 1 ligand 1 (PD-L1)in nonobese diabetic mice: the paradox in transgenic models.* Diabetes, 2008. **57**(7): p. 1861-9.
20. Martinov, T., et al., *PD-1 pathway-mediated regulation of islet-specific CD4(+) T cell subsets in autoimmune diabetes.* Immunoendocrinology (Houst), 2016. **3**.
21. Constantinescu, C.S., et al., *Experimental autoimmune encephalomyelitis (EAE) as a model for multiple sclerosis (MS).* Br J Pharmacol, 2011. **164**(4): p. 1079-106.
22. Keir, M.E., et al., *Tissue expression of PD-L1 mediates peripheral T cell tolerance.* J Exp Med, 2006. **203**(4): p. 883-95.

23. Shashidharamurthy, R., et al., *Dynamics of the interaction of human IgG subtype immune complexes with cells expressing R and H allelic forms of a low-affinity Fc gamma receptor CD32A.* J Immunol, 2009. **183**(12): p. 8216-24.
24. Pritchard, N.R. and K.G. Smith, *B cell inhibitory receptors and autoimmunity.* Immunology, 2003. **108**(3): p. 263-73.
25. Chu, S.Y., et al., *Inhibition of B cell receptor-mediated activation of primary human B cells by coengagement of CD19 and FcgammaRIIb with Fc-engineered antibodies.* Mol Immunol, 2008. **45**(15): p. 3926-33.
26. Bruhns, P. and J.L. Teillaud, *Inhibitory IgG Receptor-Expressing Cells: The Must-Have Accessory for Anti-CD40 Immunomodulatory mAb Efficacy.* Cancer Cell, 2016. **29**(6): p. 771-773.
27. Desai, D.D., et al., *Fc gamma receptor IIB on dendritic cells enforces peripheral tolerance by inhibiting effector T cell responses.* J Immunol, 2007. **178**(10): p. 6217-26.
28. Dhodapkar, K.M., et al., *Selective blockade of the inhibitory Fcgamma receptor (FcgammaRIIB) in human dendritic cells and monocytes induces a type I interferon response program.* J Exp Med, 2007. **204**(6): p. 1359-69.
29. Gordon, J.R., et al., *Regulatory dendritic cells for immunotherapy in immunologic diseases.* Front Immunol, 2014. **5**: p. 7.
30. Smith, K.G. and M.R. Clatworthy, *FcgammaRIIB in autoimmunity and infection: evolutionary and therapeutic implications.* Nat Rev Immunol, 2010. **10**(5): p. 328-43.
31. Nakamura, A. and T. Takai, *A role of FcgammaRIIB in the development of collagen-induced arthritis.* Biomed Pharmacother, 2004. **58**(5): p. 292-8.
32. Callaghan, C.J., et al., *Regulation of allograft survival by inhibitory FcgammaRIIb signaling.* J Immunol, 2012. **189**(12): p. 5694-702.
33. Watkins, A.A., et al., *IRF5 deficiency ameliorates lupus but promotes atherosclerosis and metabolic dysfunction in a mouse model of lupus-associated atherosclerosis.* J Immunol, 2015. **194**(4): p. 1467-79.
34. Sage, A.P. and Z. Mallat, *Readapting the adaptive immune response - therapeutic strategies for atherosclerosis.* Br J Pharmacol, 2017. **174**(22): p. 3926-3939.
35. Fife, B.T. and K.E. Pauken, *The role of the PD-1 pathway in autoimmunity and peripheral tolerance.* Ann N Y Acad Sci, 2011. **1217**: p. 45-59.
36. Ulges, A., et al., *Context- and Tissue-Specific Regulation of Immunity and Tolerance by Regulatory T Cells.* Adv Immunol, 2016. **132**: p. 1-46.
37. Roghanian, A., et al., *New revelations from an old receptor: Immunoregulatory functions of the inhibitory Fc gamma receptor, FcgammaRIIB (CD32B).* J Leukoc Biol, 2018.
38. Mancardi, D.A., et al., *The high-affinity human IgG receptor FcgammaRI (CD64) promotes IgG-mediated inflammation, anaphylaxis, and antitumor immunotherapy.* Blood, 2013. **121**(9): p. 1563-73.
39. Chiofalo, M.S., et al., *Subclass specificity of the Fc receptor for human IgG on K562.* Cell Immunol, 1988. **114**(2): p. 272-81.
40. Scholl, P.R., D. Ahern, and R.S. Geha, *Protein tyrosine phosphorylation induced via the IgG receptors Fc gamma Ri and Fc gamma RII in the human monocytic cell line THP-1.* J Immunol, 1992. **149**(5): p. 1751-7.

6. Claims:

6.1. An active composition (BY-001) of homomultimeric forms of chimeric protein PD-L1/Fc-gamma1 comprising the forms of dimer, tetramer and Hexamer, wherein the probable ratio of the forms is at 25% of dimer, 30% of tetramer and 45% of hexamer respectively (Fig. 2).

6.2. A form of homomultimeric chimeric protein PD-L1/Fc-gamma1 in advanced structure consisting of multiple monomer of chimeric protein PD-L1/ Fc-gamma1, wherein the monomer of chimeric protein PD-L1/ Fc-gamma1 contains two distinct function domains.

6.3. The two distinct function domains in the monomeric chimeric protein PD-L1/ Fc-gamma1 according to claim 2 consisting of the PD-L1 domain (extracellular domain of human PD-L1) and the Fc-gamma1 domain (region of human IgG1 Fc), wherein the PD-L1 domain contains the portions of immunoglobulin V like region (V-set region, 19~127 amino-acids, exon-2) and immunoglobulin constant like region (C-set region, 128~220 amino-acids, exon 3), and the Fc-gamma1 domain contains the regions of hinge, CH2 and CH3 in human IgG1 heavy chain (Fig. 1).

6.4. BY-001 (a composition of bifunctional homomultimeric forms of chimeric protein PD-L1/Fc-gamma1) according to claim 1 and 3 comprising the polypeptide PD-L1, wherein the aggregated form of PD-L1 portion bind to and trigger PD-1 (CD279) expressed on T cells.

6.5. BY-001 (a composition of bifunctional homomultimeric forms of chimeric protein PD-L1/Fc-gamma1) according to claim1 and 3 comprising the polypeptide Fc-gamma1, wherein the aggregated form of Fc-gamma1 portion bind to Fc-gammaRIIA/B and trigger the suppressive function of Fc-gammaRII expressed on monocyte/macrophages or progenitor of erythrocyte.

6.6. A method of treating $CD3^+$ $CD28^+$ immune cells comprising administering effective amount of BY-001 according to claim 4, wherein aggregated PD-L1 portion of BY-001 suppresses the activity of T cell (Fig. 3).

6.7. A method of treating human peripheral blood monocyte (hPBMC) comprising administering effective amount of BY-001 according to claim 4, wherein aggregated PD-L1 portion of BY-001 suppresses the activation of PD-1 bearing lymphocytes among the PBMC (Fig. 4).

6.8. A method of treating immune cells bearing Fc-gammaRII only comprising administering effective amount of BY-001 according to claim 4, wherein the homomultimeric Fc-gamma1 of BY-001 bind to and trigger the Fc-gammaRIIA/B on immune cells to suppress the cytokines production (Fig. 5).

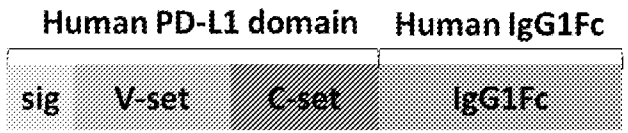
6.9. A method of treating immune cells comprising immune cells bearing Fc-gammaRI and Fc-gammaRII administering effective amount of BY-001 according to claim 4, wherein the homomultimeric Fc-gamma1 of BY-001 bind to and trigger the Fc-gammaRIIA/B on immune cells to suppress the cytokines production (Fig. 6).

6.10. The bifunctional proof of the domains contained in BY-001 according to claims 6, 7, 8 and 9 comprising the features of triggering the pathways of PD-1/PD-L1 and Fc-gamma1RIIB/Fc-gamma, wherein the features inherit the characteristics in vitro and in vivo that have been published in highly recognized scientific journals as mention in the part of background.

6.11. BY-001 according to claim 10 comprising a pharmaceutical composition for the treatment of immune diseases or protection of allografts, wherein the therapeutic potential of BY-001 is indicated

Fig. 1. Design for Chimeric Peptide of PD-1L/Fc-gamma1 (BY-001) and It's Amino Acid Sequence

A.



B.

1 MRIFAVFIFMTYWHLLNAFTVTVPKDLYVVEYGSNMTIECKFPVEKQLDLAALIVYWEMED
62 KNIIQFVHGEEDLKVQHSSYRQRARLLKDQLSLGNAALQITDVKLQDAGVYRCMISYGGAD
123 YKRITVKVNAPYNKINQRILVVDPVTSEHELTCQAEGYPKAEVIWTSSDHQVLSGKTTTTNS
185 KREEKLFNVTSTLRINTTTNEIFYCTFRRLDPEENHRSDKTHTCPPCPAPELLGGPSVFLFPP
248 KPKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSV
308 LTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTC
370 LVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSV
430 MHEALHNHYTQKSLSLSPGK 449

Fig. 2. Visualized Protein of BY-001 on SDS-Page Gel and Western-Blot

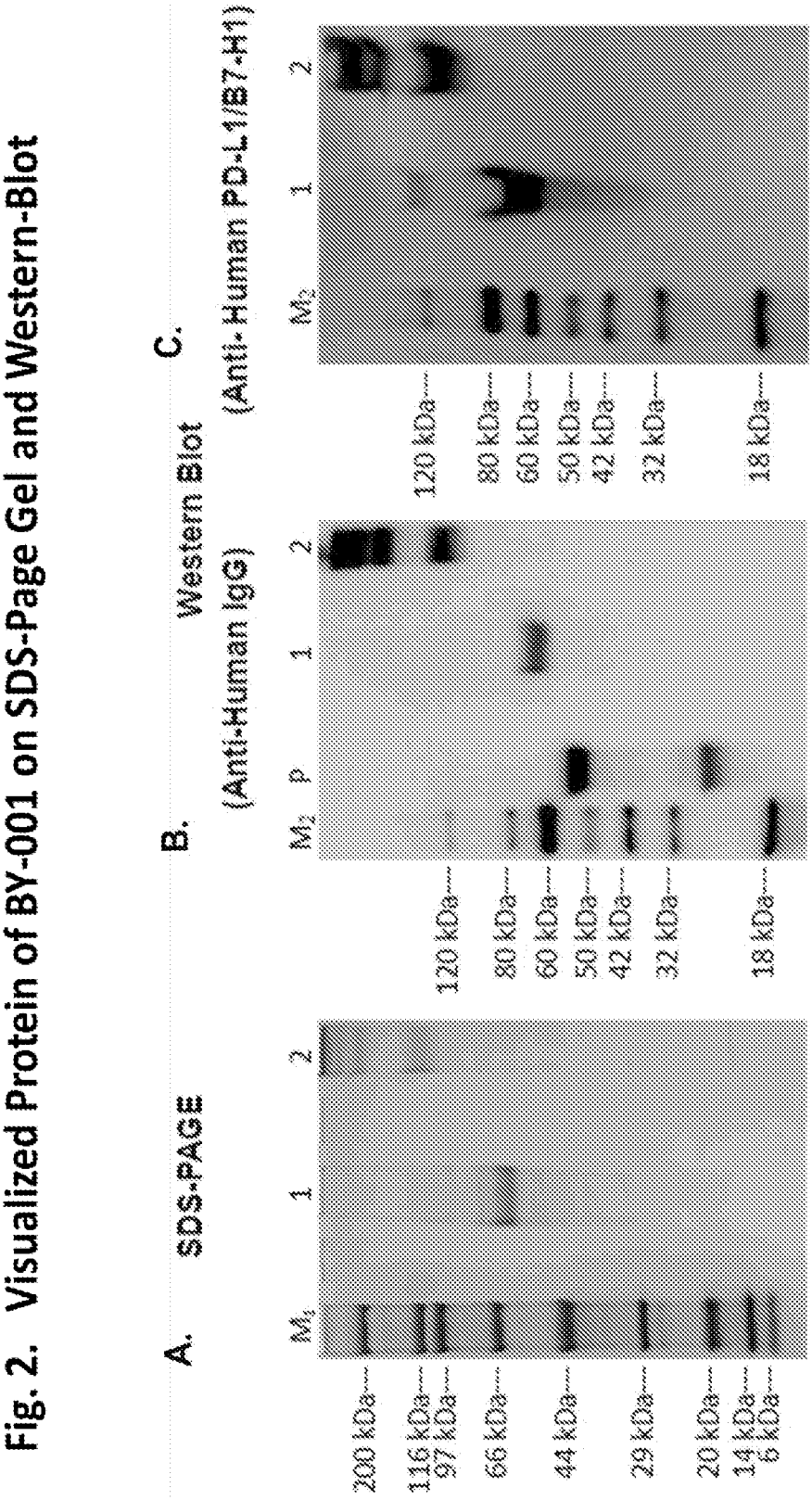


Fig. 2. Visualized Protein of BY-001 on SDS-Page Gel and Western-Blot

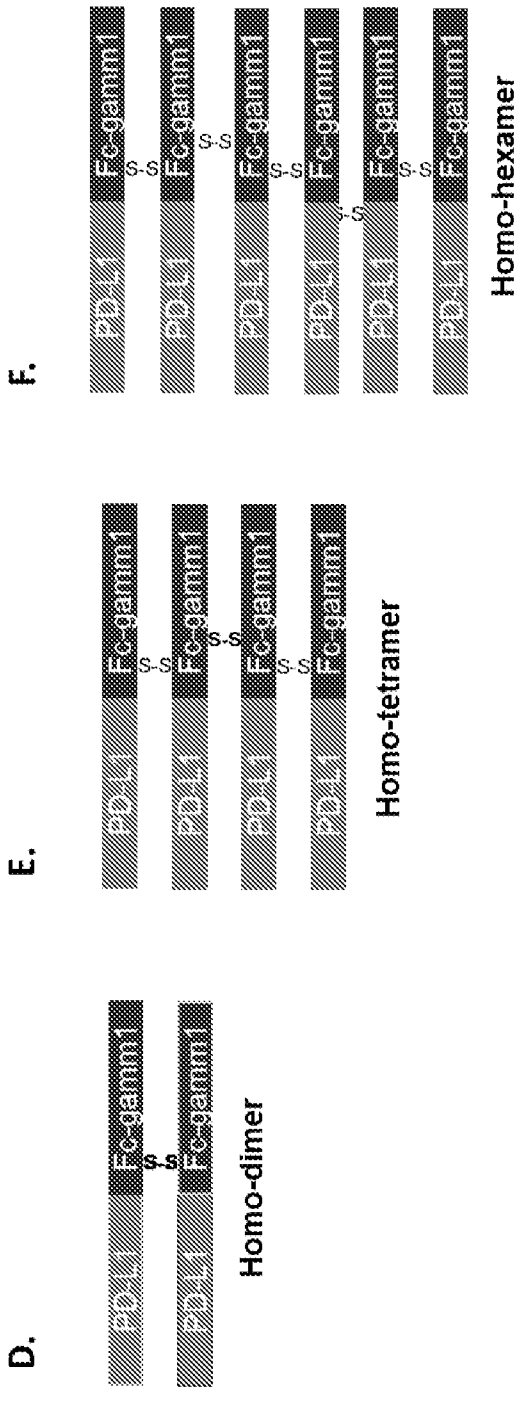


Fig. 3 BY-001 suppresses IL-2 production from Jurkate cell line

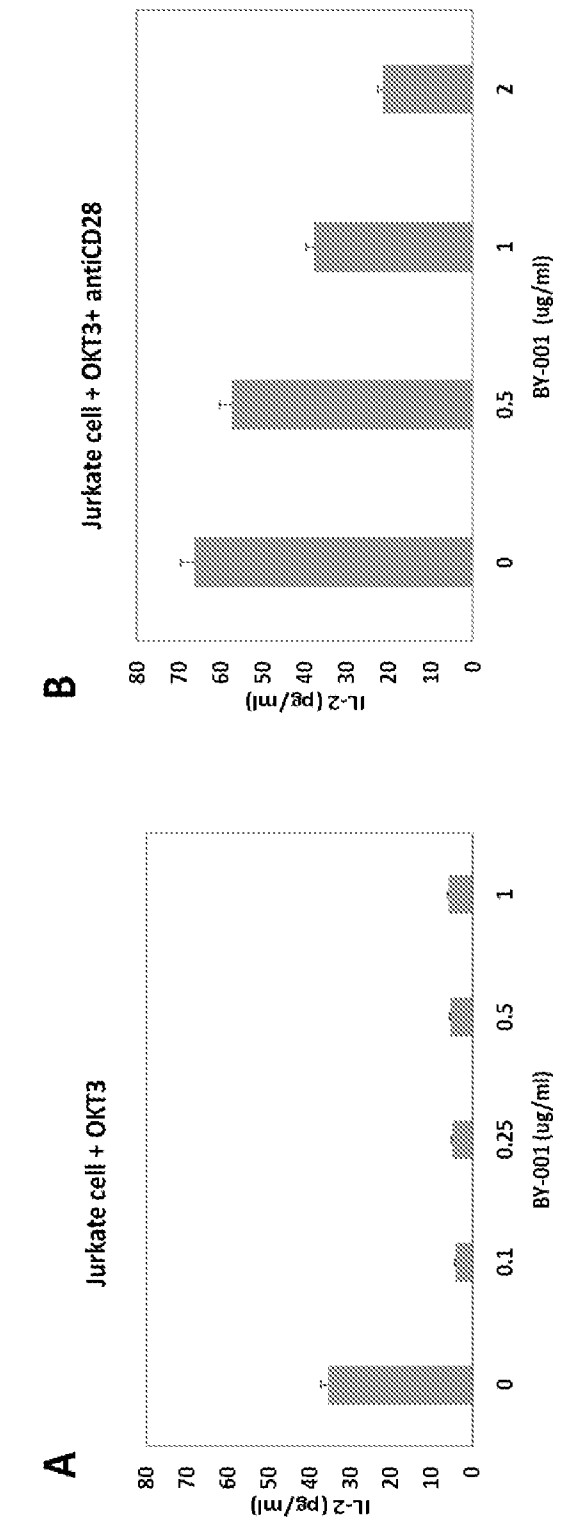


Fig. 4 BY-001 suppresses IL-2 production from PBMC human primary cells

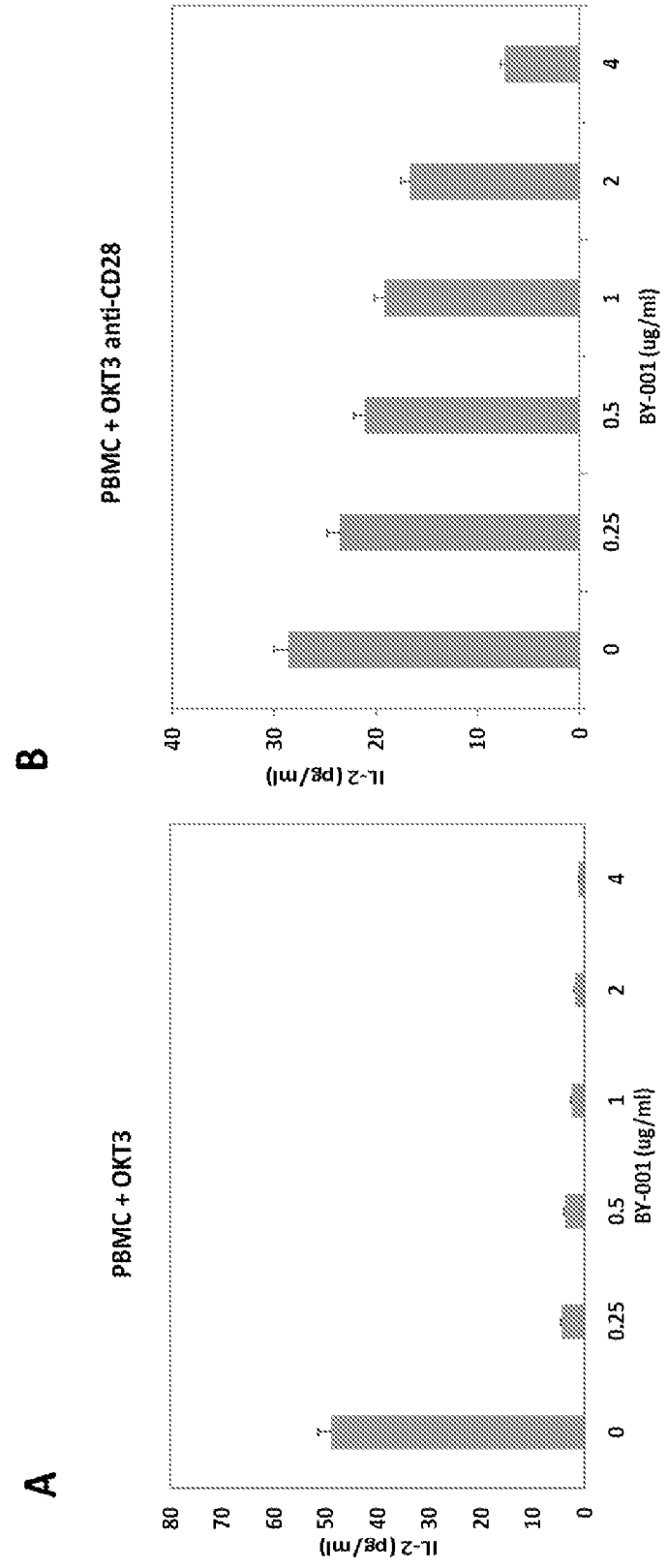


Fig. 5 BY-001 regulates IL-4 and TGF-beta production from K562 cell

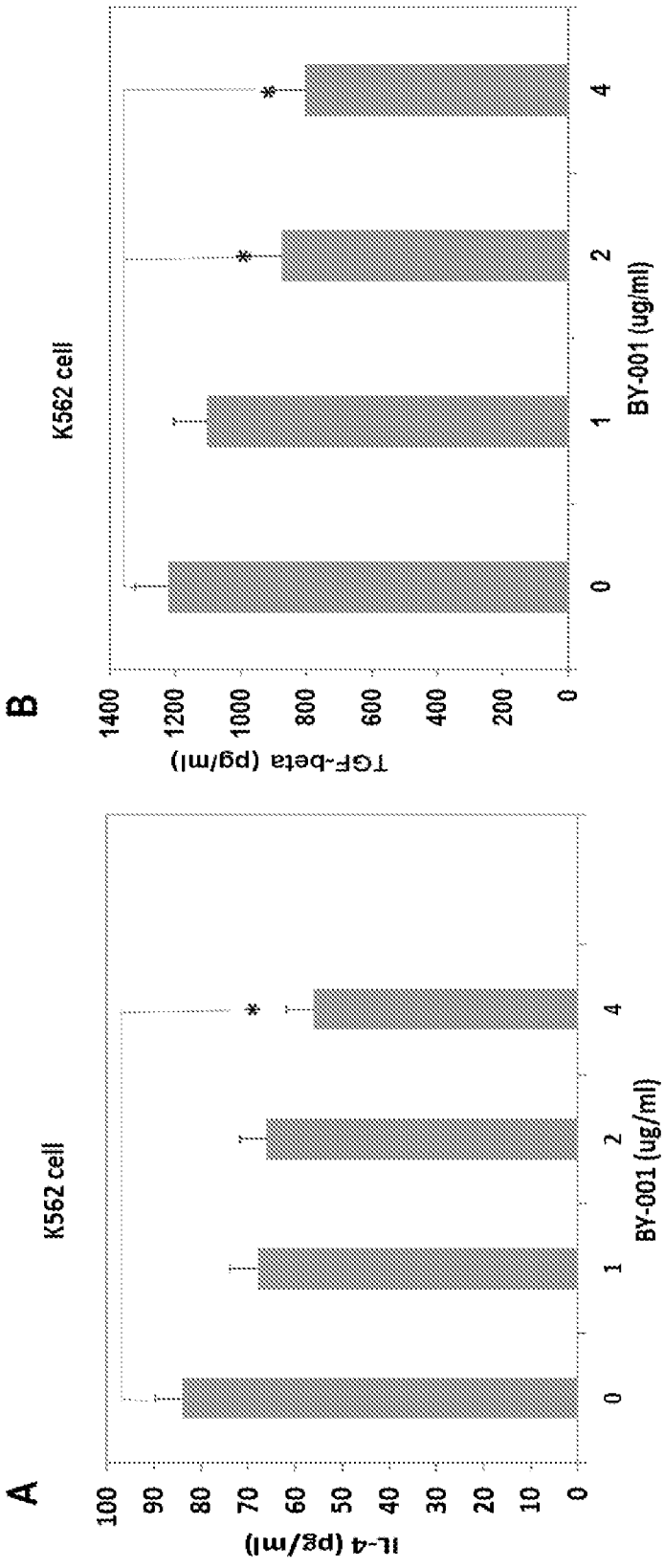


Fig. 5 BY-001 regulates IL-4 and TGF-beta production from K562 cell

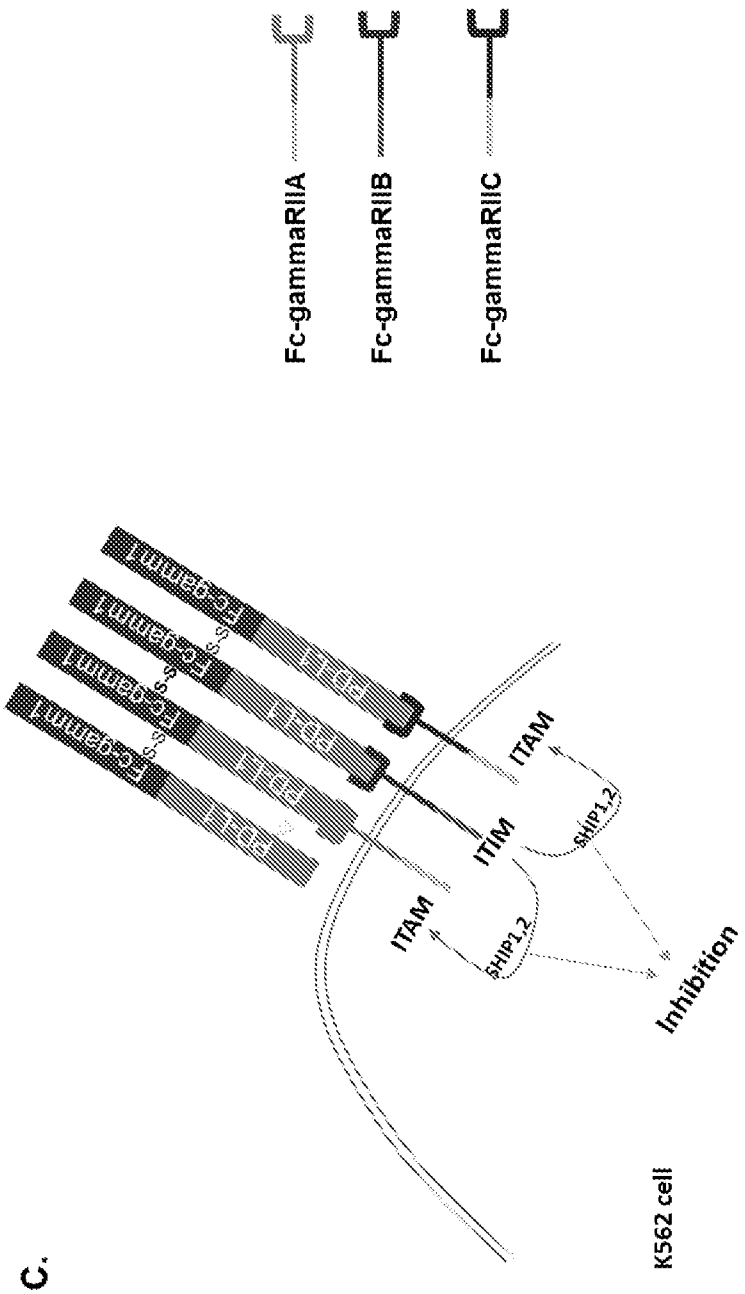


Fig. 6 BY-001 regulates IL-4 and TGF-beta production from THP-1 cell

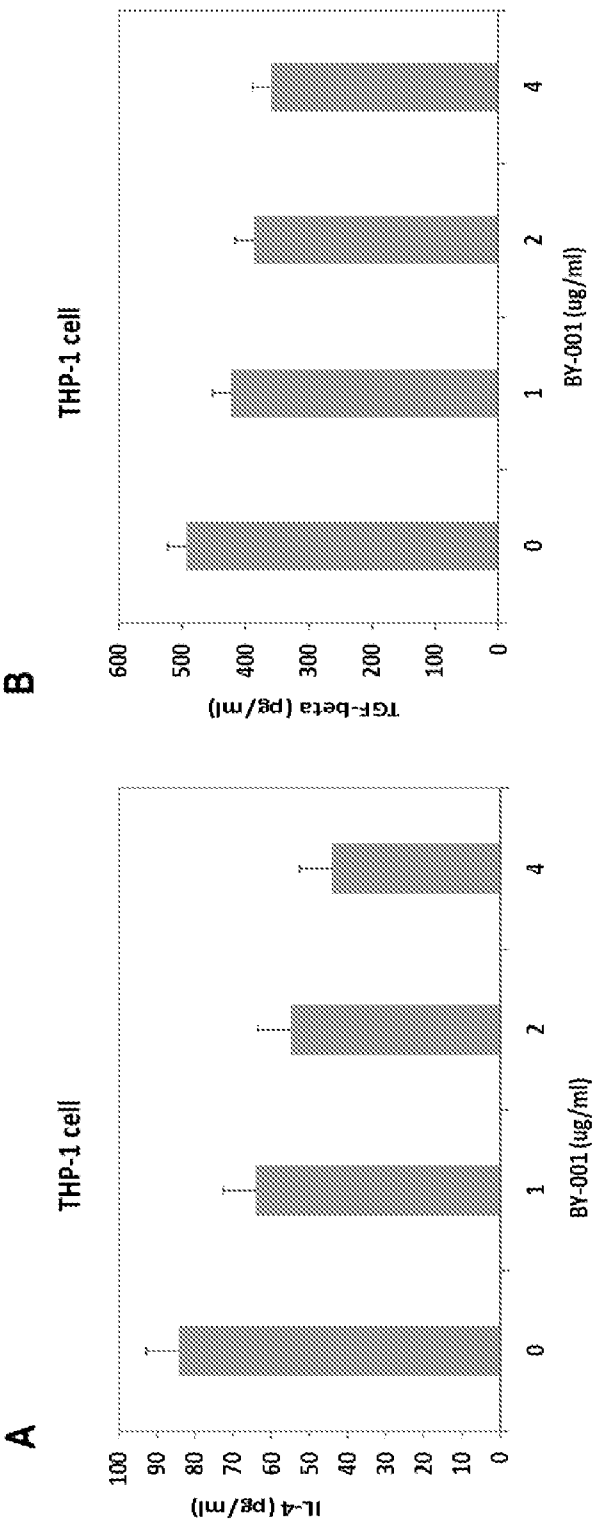


Fig. 6 BY-001 regulates IL-4 and TGF-beta production from THP-1 cell

C.

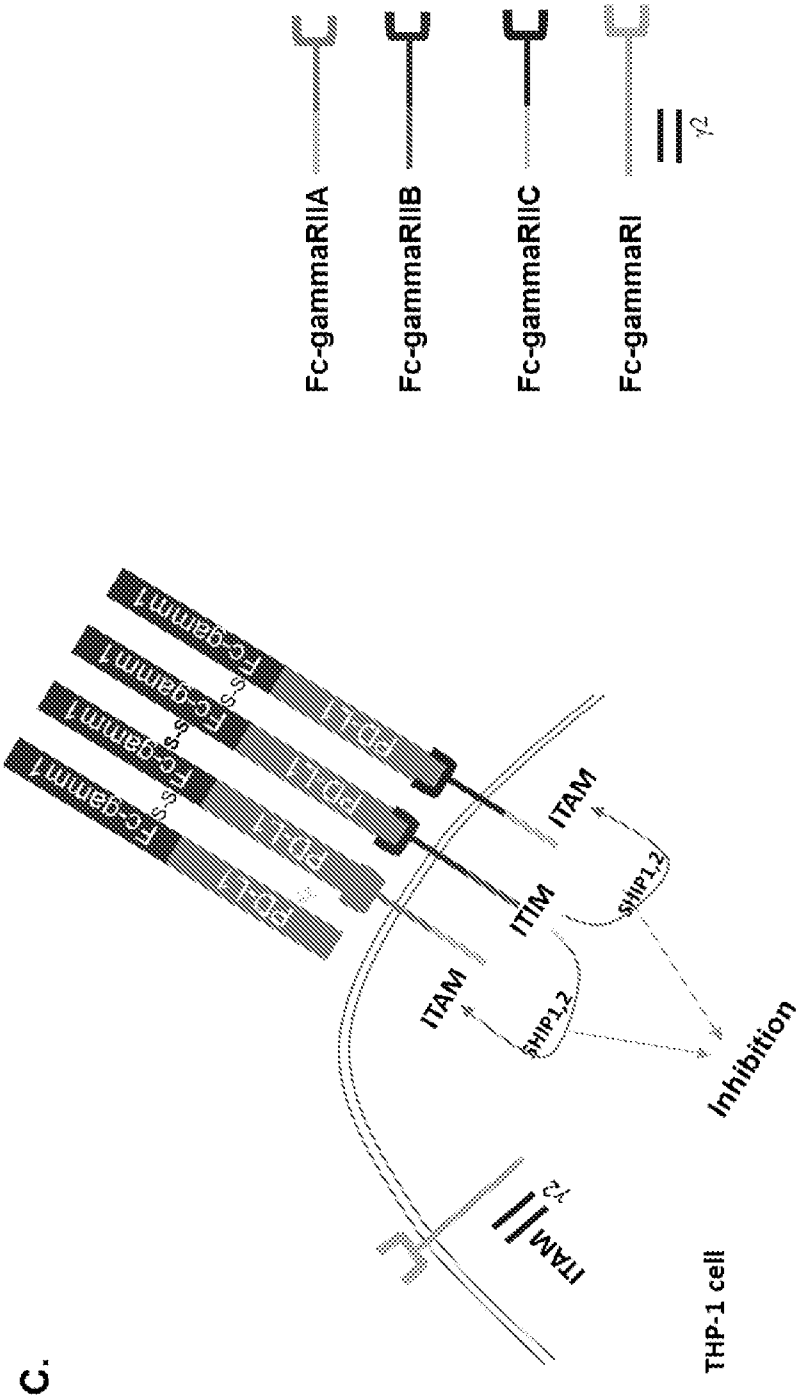


Fig. 7 Advanced structural difference between BY-001 and antigen-antibody complex

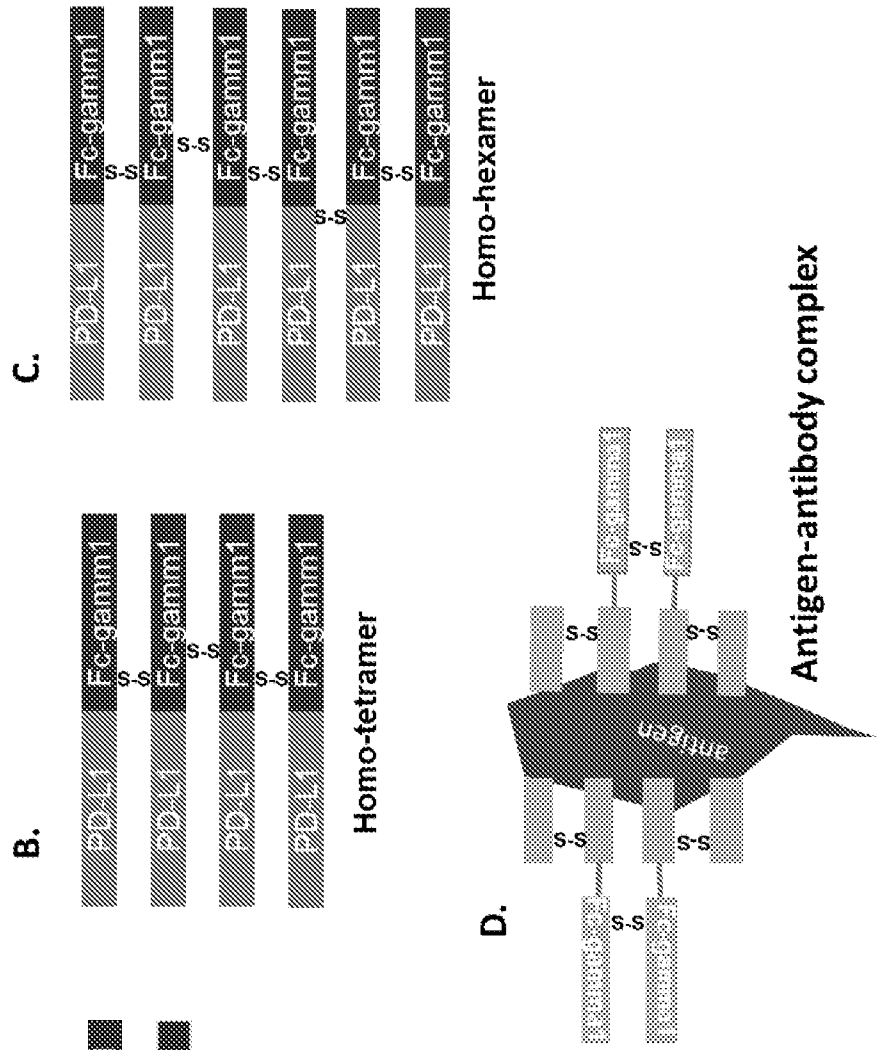
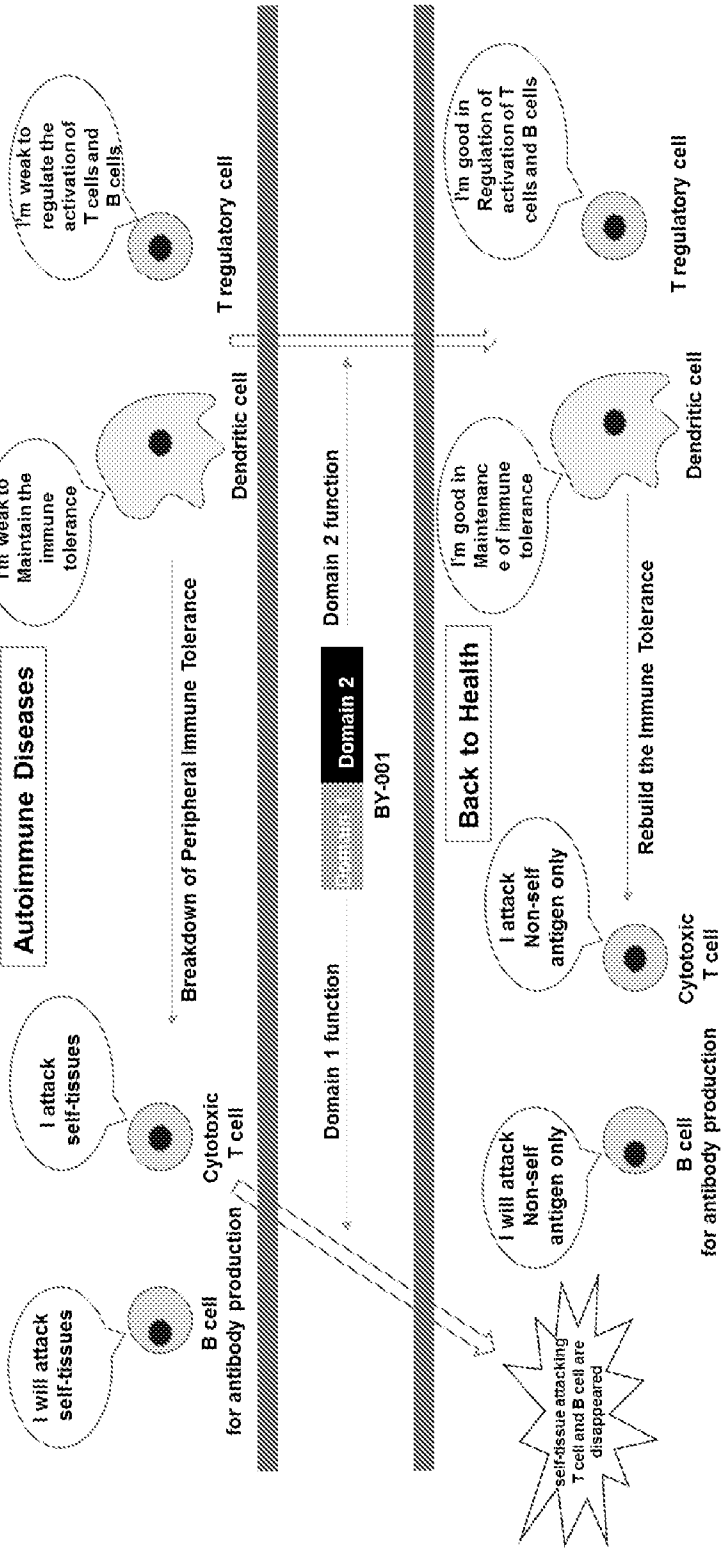


Fig. 8 Scientific Background for BY-001 Working on Autoimmunity



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2019/035818

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☒ forming part of the international application as filed:

      ☒ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

      ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

SEQ ID NOs: 1 and 2 were searched.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2019/035818

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 4-11
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2019/035818

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A61K 38/17; A61K 39/00; A61K 39/395; A61K 47/68; A61P 1/04; A61P 3/10 (2019.01)

CPC - A61K 38/00; A61K 38/17; A61K 38/177; A61K 39/0005; A61K 39/395; C07K 14/705; C07K 14/70596; C07K 19/00; C07K 2317/52; C07K 2319/00; C07K 2319/30; C12N 15/62; C12N 15/63 (2019.08)

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

USPC - 424/134.1; 424/192.1 (keyword delimited)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2017/0189476 A1 (GENEXINE, INC. et al) 06 July 2017 (06.07.2017) entire document	1-3
A	WO 2017/201131 A1 (ALBERT EINSTEIN COLLEGE OF MEDICINE, INC.) 23 November 2017 (23.11.2017) entire document	1-3
A	SONG et al. "Protective effects of Fc-fused PD-L1 on two different animal models of colitis," Gut, 05 June 2014 (05.06.2014), Vol. 64, Pgs. 260-271. entire document	1-3
A	US 2015/0203579 A1 (REGENERON PHARMACEUTICALS, INC.) 23 July 2015 (23.07.2015) entire document	1-3
A	US 2015/0361155 A1 (THOMAS JEFFERSON UNIVERSITY) 17 December 2015 (17.12.2015) entire document	1-3

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 August 2019	13 SEP 2019
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450 Facsimile No. 571-273-8300	Authorized officer Blaine R. Copenheaver PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774