약학석사학위논문

Immunomodulatory Effect of Monoclonal Antibodies against Human Immune-checkpoint Molecule, VISTA

인간 면역 관문 분자인 VISTA 에 대해 면역 조절 효과를 보이는 단일 클론 항체 제작 및 검증

2019 년 2 월

서울대학교 융합과학기술대학원

분자의학 및 바이오제약학과 분자의학 및 바이오제약전공

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지도교수 신 영 기

이 논문을 약학석사 학위논문으로 제출함

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안준형의 석사학위논문을 인준함

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위 원 _____ (인)

ABSTRACT

Immunomodulatory Effect of Monoclonal Antibodies against Human Immune-checkpoint Molecule, VISTA

Junhyeong An

Department of Molecular Medicine and Biopharmaceutical Sciences,

Graduate School of Convergence Science and Technology,

and College of Pharmacy, Seoul National University

Immunotherapy uses the patient's immune system, unlike conventional anticancer and autoimmune disease treatments. It is characterized by less side effects and higher expected survival rate, which is attracting much more attention as a next-generation therapy. Immunotherapy can be classified into activating immunotherapy, which promotes the function of immune cells and suppressive immunotherapy, which is used to treat autoimmune diseases and GVHD by inhibiting excessive immune cell function. The molecules related with immunotherapy are immune-checkpoint molecules that regulate the immune system. In addition to PD-1/PD-L1 and CTLA-

4, which are widely known as typical immune-checkpoint molecules, VISTA is also a T cell co-inhibitory molecule. This molecule controls the activation signal so that the immune system is not damaged when the T cell signal is overactive. Because these molecules are closely related to the function of immune cells, they can inhibit excessive immune activity. In addition to studies on anticancer treatments, there are many studies that can be used to treat autoimmune diseases and GVHD. It is a principle that induces suppressive immunotherapy by further stimulating the inhibitory checkpoint molecules. Therefore, VISTA was selected as a target for the treatment of various immune-related diseases, and the drug form was selected as a monoclonal antibody. The main purpose of this study was to examine the efficacy of the monoclonal antibody candidates obtained from the screening in terms of checkpoint blockade and stimulation.

In this study, monoclonal antibody candidates binding to the extracellular domain of human VISTA were firstly obtained from the human synthetic scFv library by the phage display technique. Thereafter, four candidates were identified by competitive enzyme-linked immunosorbent assay (ELISA) and flow cytometry with the ability to selectively bind to the antigen instead of the ligand. The experiment was conducted to verify the characteristics of four monoclonal antibodies. Antibodies were produced in transient expression systems and purified by affinity chromatography. To examine the antigen binding ability, flow cytometry and western blotting were performed. Through competitive inhibition ELISA, the IC_{50} value was determined to compete with the ligand. Inhibition of VISTA-VSIG-3

interaction and the binding ability was compared between the clones. As a result, it was determined that the developed antibodies have high productivity and purification efficiency. This demonstrates the high possibility of industrialization. In addition, the ability to specifically bind to the target antigen and to block the signal better in competition with the ligand was also excellent. *In vitro* $CD4^+$ T cell assays compared the concentration of cytokine (IFN-γ) with quantitative ELISA, whether the function of the activated T cell is inhibited or not under the co-existence of the corresponding ligand and antibodies.

Therefore, the monoclonal antibodies produced in this study showed a specific response to human VISTA and VISTA-dependent immunosuppressive effect in culturing with $CD4^+$ T cells, thus confirming the possibility of suppressive immunotherapy. This suggests that T cells that are excessively activated may be suppressed and used as an autoimmune disease and GVHD treatment agent in which the function of the immune system is higher than the normal range. We will evaluate the efficacy and the dose of the drug through further animal experiments to evaluate the possibility of future antibody treatment.

Key words : Immunotherapy, Immune-checkpoint molecule, VISTA, Therapeutic antibody, Suppressive Immunotherapy, Autoimmune disease

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LIST OF ABBREVIATIONS

GVHD	Graft-versus-host disease
CD	Cluster of differentiation
GITR	Glucocorticoid-induced TNFR-related protein
TNF	Tumor necrosis factor
ICOS	Inducible T-cell Costimulator
PD-1	Programmed cell death protein 1
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
IDO	Indoleamine-pyrrole 2,3-dioxygenase
LAG-3	Lymphocyte-activation gene 3
TIM-3	T-cell immunoglobulin and mucin-domain containing-3
VISTA	V-domain Ig suppressor of T cell activation
IFN-α	Interferon-alpha
pDC	Plasmacytoid dendritic cell
NET	Neutrophil extracellular trap
a.a.	Amino acid
PD-1H	Programmed death-1 homolog
PD-L1	Programmed death-ligand 1
VSIG-3	V-Set and Immunoglobulin domain containing 3

IGSF11	Immunoglobulin superfamily member 11
MDSC	Myeloid-derived suppressor cell
APC	Antigen presenting cell
TIGHT	T cell immunoreceptor with Ig and ITIM domains
NIH	National Institutes of Health
DMEM	Dulbecco Modified Eagle Medium
FBS	Fetal Bovine Serum
RPMI	Roswell Park Memorial Institute
Fc	Fragment crystallizable
PBS	Phosphate-buffered saline
TEA	Triethylamine
scFv	Single chain fragment variable
HA	Hemagglutinin
IPTG	Isopropyl β-D-1-thiogalactopyranoside
TES	Tris/EDTA/Saline
IgG	Immunoglobulin gamma
ELISA	Enzyme-linked immunosorbent assay
TBS	Tris-buffered saline
RT	Room temperature
HRP	Horseradish peroxidase
TMB	3,3',5,5'-Tetramethylbenzidine

BSA	Bovine serum albumin
FACS	Fluorescence-activated cell sorting
CDR	Complementarity-determining region
V_H	Heavy chain variable
V_L	Light chain variable
FITC	Fluorescein isothiocyanate
RIPA	Radioimmunoprecipitation assay
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycoltetraacetic acid
SDS	Sodium dodecyl sulfate
PMSF	Phenylmethylsulfonyl fluoride
PAGE	Polyacrylamide gel electrophoresis
PVDF	Polyvinylidene fluoride
ECL	Enhanced chemiluminescence
DPBS	Dulbecco's phosphate-buffered saline
PBMC	Peripheral blood mononuclear cell
IFN-γ	Interferon-gamma
MFI	Mean fluorescence intensity
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
IC_{50}	The half maximal inhibitory concentration
Fab	Fragment antigen-binding

INTRODUCTION

1. Immunotherapy

Immunotherapy is the next generation novel therapeutic strategy that takes point and uses the patient's immune system directly (1). Immunity must be regulated normally to maintain the body's homeostasis, and in this sense also called the concept of immune balance (2). Immunotherapy can be divided into two groups: activating immunotherapy, which aims to improve the immune function of the patient, and suppressive immunotherapy, which is used in the treatment of autoimmune diseases, GVHD, etc., by suppressing excessively activated immune responses (2-4). Unlike the conventional chemotherapy and radiation therapy methods, since it regulates the function of the immune system in the body, the side effects are minimized and the treatment efficiency is enhanced (5).

2. Immune-checkpoint molecules and Immunotherapy

Immune-checkpoint molecules are typical targets for immunotherapy. These are important because they act as regulators of the immune system. It mainly carries out a self-tolerance mechanism to prevent undue immune responses that kill cells indiscriminately. From the viewpoint of immunotherapy, immune-checkpoint

molecules are closely related to the mechanism of immune evasion of cancer (5, 6).

Immune-checkpoint molecules are divided into two groups: stimulatory checkpoint molecules and inhibitory checkpoint molecules. They act as cellular mediators in the immunosuppression environment derived from cancer and exhibit immune function (7). Stimulatory checkpoint molecules play an important role in enhancing immune function such as T cell effector function. These include CD27, CD40, CD137, GITR, and OX40, a kind of tumor necrosis factor (TNF) receptor superfamily, and CD28 and ICOS, which belong to the B7-CD28 superfamily (8-10). Inhibitory checkpoint molecules downregulate the immune response as opposed to the former. These molecules are widely known as PD-1, including CTLA-4, IDO, LAG-3, and TIM-3 (11, 12). VISTA also belongs to this category, and in recent years, its functions and roles have been actively studied. The various molecules corresponding to the two classes are shown in [Figure 1] (6, 7, 13).

As we have seen, immune-checkpoint molecules play a key role in regulating immunity, and they have been attracting much more attention as a target of immunotherapy. Among them, a method of controlling the inhibitory checkpoint molecule has been carried out in many studies (2, 14, 15) [Figure 2]. The first is the checkpoint blockade viewpoint. If the inhibitory checkpoint molecules are blocked, the malignant tumor cell can be removed by activated immune cells. This function is mainly caused by activated cytotoxic T cells. Protective antibodies are produced by plasma cells, and the pathogen is removed by neutrophil (2, 16-20). The second is the viewpoint of checkpoint stimulation. This is an approach mainly used in

diseases where excessive immunity occurs. Stimulation of inhibitory checkpoint molecules that suppression of immune cell function is further enhanced. Production of autoantibody, IFN-α by pDC is reduced and NETosis is also reduced (2). As such, it is associated with various immune-related diseases from cancer to autoimmune diseases (4, 17). Therefore, it is very popular to develop therapeutic methods targeting immune-checkpoint molecules nowadays.

3. VISTA, a negative checkpoint molecule

VISTA (Gene *VSIR*, *Homo sapiens*) is an abbreviation of V domain immunoglobulin suppressor of T cell activation and has many other names [Table 1]. It is a single-pass type I transmembrane protein and has a size of about 50 kDa (5, 21-23) [Table 1]. It is composed of signal peptide up to 1-32 a.a., extracellular domain up to 33-194 a.a., helical transmembrane up to 195-215 a.a. and cytoplasmic domain up to 216-311 a.a. (5, 23-25). The VISTA protein sequence is highly conserved with approximately 80% identity between murine and human (5, 23, 24). Because VISTA belongs to the B7 family, there is structural similarity with ligands and receptors of the B7 family. This is the reason why VISTA is called PD-1H (PD-1 Homologue) because of its high homology with PD-L1 (5, 23, 26-29). This suggests that evolutionary systems are close to each other. The expression of VISTA in the hematopoietic cell lineage is mainly expressed in the mature myeloid cell lineage system, and less in naïve T cell and activated T_{reg} cell. Expression is highly

expressed in $CD11b^{Hi}$ myeloid cell lineages such as granulocytes, macrophages, monocytes, myeloid dendritic cells, and intermediate expression in $CD11b^{Int}$ dendritic cells, $CD4^+$ and $CD8^+$ T cells. B cells, but have no expression of VISTA (5, 24, 25, 30) [Table 2].

A lot of questions remain about VISTA (21, 23, 24, 31). VISTA's partner is also not clearly identified, so it is assumed that VISTA performs self-signaling through homotypic interaction or functions through contact with other receptors. Among them, a molecule called VSIG-3 (IGSF11) has been known to date as a novel ligand of VISTA (32, 33). However, additional research is needed to determine which signaling pathway suppresses T cells.

Currently, VISTA regulates various immune cell responses in cancer, inflammatory, and autoimmune diseases and has been actively researched as a treatment for these immune-related diseases (5, 34, 35). It has a great effect on the function of $CD4^+$, $Foxp3^+$ $CD4^+$ T_{reg}, TCR_{gd} T cell, $CD8^+$ T cell, myeloid dendritic cell, and macrophage (31). This is the reason that we selected VISTA as a drug target in this study.

First, it is an aspect of anticancer treatment. Tumor cells express an inhibitory checkpoint molecule that prevents immune cells from acting and causes immune evasion. If these inhibitory checkpoint molecules are blocked to elicit effector functions of activated T cells, anticancer treatments can be used to prevent immune evasion of tumors. In the case of VISTA, it is known that various cells such as $CD4^+$, $CD8^+$ T cell, myeloid dendritic cell and MDSC mediate cell cytotoxicity through

various cytokines and thus function as anticancer efficacy (6, 7, 13, 23, 36, 37). This is in line with activating immunotherapy. Second, it is the viewpoint of autoimmune diseases and GVHD treatment (25, 35, 38, 39). The inhibitory checkpoint molecules play a role in suppressing excessive immune cell activity. In the condition of this immune response, inhibition signal is further enhanced by binding to the inhibitory checkpoint molecule can function as a suppressive immunotherapy. It is known that VISTA is related to autoimmune diseases such as lupus, autoimmune encephalomyelitis and these diseases are deepened in mice knocking out VISTA from the past researches (25, 35, 38, 39). In addition, there are studies that show that monoclonal antibody that specifically recognizes VISTA is a possible treatment for acute GVHD (27, 38, 40).

4. The conceptual design of immunotherapy agent as a monoclonal antibody

In this study, we developed an immunotherapeutic agent with immunomodulatory effect. Immunotherapy can be divided into two groups: activating immunotherapy, which improves immune cell function and shows anticancer effect, and suppressive immunotherapy, which suppresses excessive immune function and is effective for autoimmune diseases and GVHD treatment (2-4, 6). VISTA is a molecule capable of both immunotherapy of these two aspects. VISTA acts as a different pathway from PD-1/PD-L1, CTLA-4 and is known for having a dual role that functions as a

checkpoint ligand and receptor (20, 23, 40, 41). We would like to produce monoclonal antibodies targeting human VISTA to such an extent and potential as an immunomodulator. The form of drug is a monoclonal antibody, which is equivalent to immunotherapy for the inhibitory checkpoint described above (2, 5) [Figure 3].

First, from the viewpoint of checkpoint blockade, the developed monoclonal antibody binds to human VISTA and immune evasion by tumor cells does not occur [Figure 3A]. Therefore, T cell activation status is maintained, cell cytotoxicity effect by cytokine and T cell proliferation are activated, and eventually malignant/tumor cell removal is performed. Second, from the viewpoint of checkpoint stimulation, the developed monoclonal antibody interacts with VISTA and strengthens the signal of VISTA which inhibits T cell activation [Figure 3B]. Excessive immune responses due to excessive T cell activity will be suppressed, and proliferation and cytokine production ability of T cell will be decreased, and immunity returns to normal status.

In this study, monoclonal antibodies were generated and the two possibilities associated with immunotherapy were opened for immunomodulatory effect.

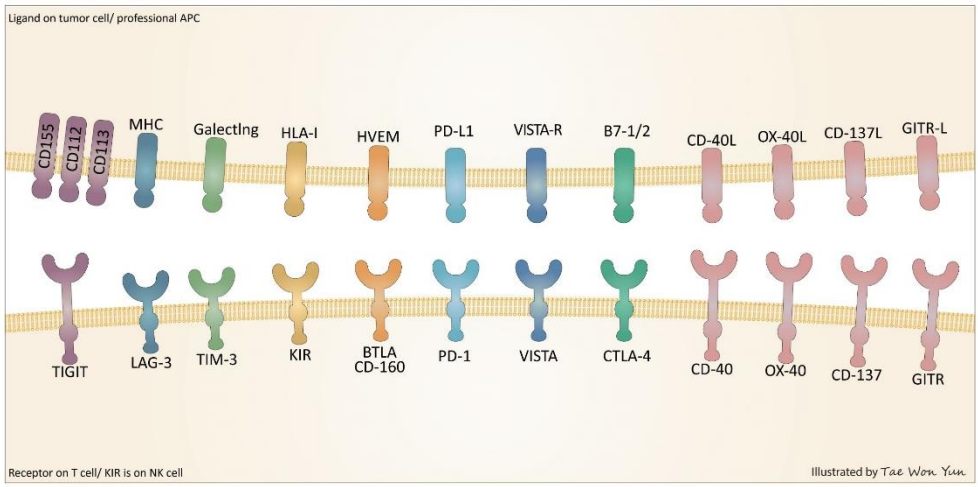


Figure 1. Variety of Immune-checkpoint Molecules.

The relationship between molecules expressed in Tumor cells or APCs and molecules expressed on T cell surfaces was shown. T-cell co-inhibitory molecules from TIGHT molecules to CTLA-4 molecules and T-cell co-stimulatory molecules from CD-40 to GITR. VISTA molecule (dark blue-colored), which is closely related to this study, is shown in the middle of the figure.

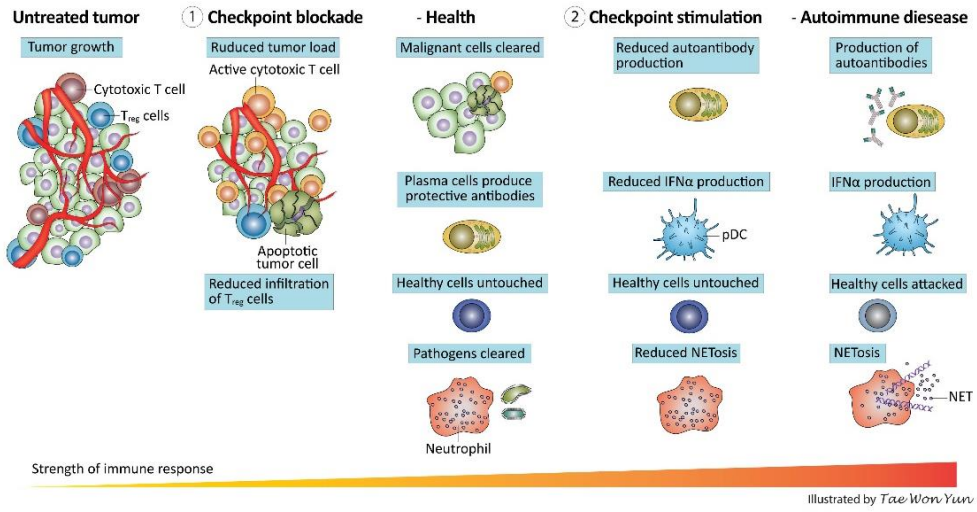


Figure 2. Immune Balance : Checkpoint Blockade and Stimulation.

There are two treatments that target the inhibitory checkpoint molecule. One is a checkpoint blockade and the other is a checkpoint stimulation. From the viewpoint of '① Checkpoint blockade', block the inhibitory checkpoint molecule to prevent immune evasion of tumor cells. It is a method to be used for anticancer treatment. From the viewpoint of '② Checkpoint stimulation', the function of the inhibitory checkpoint molecule is further enhanced to further inhibit immune cells. This leads to the down-regulation of immune cell functions and is a method used for the treatment of autoimmune diseases and GVHD.

○ **V-domain Ig-containing suppressor of T-cell activation** (**VISTA**, Entrez: 64115) = C10orf54 = Differentiation of ESC-1 (Dies1) = Platelet receptor Gi24 precursor = PD-1 homolog (PD-1H) = Death Domain 1α (DD1α) = Stress Induces Secreted Protein 1 (SISP1)
○ A type I transmembrane protein (50 kDa) belongs to the Ig superfamily

Table 1. Various Names of VISTA.

VISTA is called by various names. This is summarized in the upper part of the table. The structural features and sizes of VISTA protein are summarized in the lower part of the table.

Cell Type		Surface VISTA expression (Human)
$CD11b^{Hi}$ myeloid cells	Granulocytes Monocytes Macrophages Myeloid dendritic cells (DCs)	+++
$CD11b^{Int}$ myeloid DCs		++
$CD11c^{+}$ DCs		+
$CD14^{+}$ monocytes		+
$CD14^{+}$ neutrophils		+
$CD56^{lo}$ NK cells		+
TCRγδ T cells		+
$CD4^{+}$ naïve T cells		+
$CD4^{+}$ $Foxp3^{+}$ regulatory T cells (Tregs)		+
$CD8^{+}$ naïve T cells		+
B cells		-
Peritoneal macrophages		N/D

Table 2. VISTA Expression Levels in Various Human Cell Types.

Expression of VISTA in various immune cells and relative difference in expression level were shown. Expression is high in $CD11b^{Hi}$ myeloid cell lineages. Also, in $CD11b^{Int}$ myeloid dendritic cells and various T cell lineages show slight expression level. However, no expression of VISTA is observed in B cells.

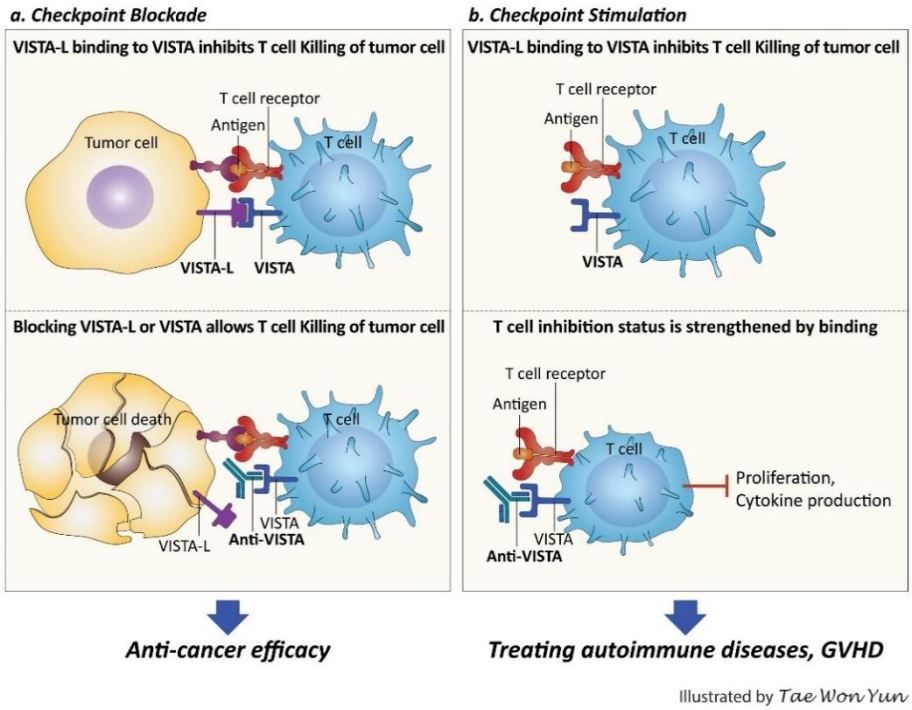


Figure 3. Conceptual Design of Monoclonal Antibody.

After selecting the form of the drug as a monoclonal antibody, the conceptual design related to VISTA was shown above. From the viewpoint of the checkpoint blockade, the produced monoclonal antibody is bound to VISTA and the immune evasion of the tumor cell does not occur. This means that the activity of the immune cells is maintained and the anticancer efficacy of killing the tumor cells is remained. From the viewpoint of checkpoint stimulation, the antibody interacts with VISTA to further enhance T cell inhibition and reduce excessive immunity. This is a method that can be used for the treatment of autoimmune disease and GVHD due to decreased cytokine production and T cell proliferation.

MATERIALS AND METHODS

Cell Culture and Cell Lines

In this study, we had to make a stable cell line expressing human VISTA. Cell was used for mouse fibroblast cell that is NIH/3T3. We selected NIH/3T3 cell for making human VISTA stable cell line. NIH/3T3 was cultured in Dulbecco's high glucose modified Eagle's medium (DMEM, Hyclone, Logan, USA).

For *in vitro* $CD4^+$ T cell assay, we used human cryopreserved peripheral blood mononuclear cells (PBMC, Zen-Bio Inc., North California, USA). Total $CD4^+$ T cells were isolated using a CD4 MicroBeads (Miltenyi Biotec, California, USA) by magnetic-activated cell sorting. $CD4^+$ T cells were cultured in Roswell Park Memorial Institute-1640 medium (RPMI-1640, Hyclone, Logan, USA).

Cell culture mediums were all supplemented with 10% fetal bovine serum (FBS, Hyclone, Logan, USA) and 1% penicillin/streptomycin (Hyclone, Logan, USA).

All cells were incubated in a humidified atmosphere of 5% CO_2 incubator at 37 ℃. The detailed information about cell line is summarized in [Table 3].

scFv Candidates Screening

Phage Display

Recombinant human VISTA Fc protein (R&D Systems, Minneapolis, USA) was used as antigen for phage display screening. OPAL and OPALS human synthetic scFv library were used for screening clones binding to the human VISTA extracellular domain. Antigen was coated in immunotubes (Nalge Nunc, New York, USA) at a 15 µg and incubated at 37 °C, over-night for binding (1st panning). The immunotube and phage library were blocked with 3% skim milk in 0.05% PBS-T (Phosphate-Buffered Saline containing Polysorbate 20) solution. Blocked phage libraries were moved into the antigen-coated immunotubes and incubate for 1 hour. After incubation, wash 3 times with 0.05% PBS-T solution. Phages were eluted with 100 mM TEA (Triethylamine) buffer for 9 minutes and neutralized with 1 M Tris-HCl, pH 8.0 buffer solution. Eluted phages were amplified by infecting in *E.coli* (Input titer serial dilution) and grown on solid LA plates at 37 °C, overnight to check the output titer. Next day, add 60% glycerol into scrapped-off phage to make output stock. Remained output was added into SB (+ampicillin) badge and incubate at 37 °C. When selected phages reached $O.D._{600}$ value of 0.5-0.7, rescue with helper phage (VCSM13) and then the same cycle was repeated 3 more times (Total 4 times panning. For antigenic amounts, 7.5 µg was used in the second-to-fourth step. Washing step was performed 5 times in the 2nd, 10 times in the 3rd and 4th Steps).

Expression of scFv hits tagged hemagglutinin (HA)

After 4th panning, gained *E.coli* (ER2537 strain for OPAL library, TG1 strain for OPALS library) infected with selected phage was injected in 150 μL SB (+ampicillin) in each well of 96-well plate. These single colonies were cultured at 37 ℃ in a shaking incubator (TAITEC, Nishikata, Japan) until it turned blurry. After incubation, add 1 M IPTG (the final concentration is 1 mM) and incubate overnight at 30 ℃ in a shaking incubator (IPTG induction step). Next day, centrifuge the plate at 3,000 rpm for 15 minutes at 4 ℃. Remove supernatant and resuspend the pellet with 1X TES buffer for 5 minutes at 37 ℃. Lyse pellets by osmotic shock by adding 0.2X TES buffer. Incubate at 37 ℃ in a shaking incubator for 5 minutes and incubate at 4 ℃ for 1 hour. After 1 hour, centrifuge the plate at 3,000 rpm for 15 minutes at 4 ℃. Move the supernatant to a new 96-well plate. The periplasmic extract containing single chain fragment variables tagged with hemagglutinin was obtained.

Verification of signals through ELISA screening

Three plates are required for this experiment. One is a non-coated plate for negative control 1, another is a human IgG-coated plate for negative control 2, the other is an antigen-coated plate for sample. Recombinant human VISTA Fc protein (R&D Systems, Minneapolis, USA) was used as antigen. Coat 30 ng antigen with PBS in each well of 96-well half-area ELISA plate (Corning, New York, USA) and incubate overnight at 4 ℃. Next day, wash the antigen-coated plate with 150 μL 0.05% TBS-T (Tris-Buffered Saline containing Polysorbate 20) for 3 times. Block the plate

with 150 μL 3% skim milk in 0.05% TBS-T for 1 hour at RT (Room temperature). The periplasmic extract containing single chain fragment variables tagged with hemagglutinin were blocked in a new plate with 6% skim milk in TBS-T while ELISA plate was blocked. After blocking, 30 μL the periplasmic extract solution was added into the antigen-coated plate and incubate for 1 hour at RT. After incubation, wash the plate 3 times with 150 μL 0.05% TBS-T. Add 30 uL of anti-Hemagglutinin horseradish peroxidase antibody in 3% skim milk in 0.05% TBS-T and incubate for 1 hour at RT. Wash the plate 3 times with 150 μL 0.05% TBS-T and add 30 μL 3, 3', 5, 5'-tetramethylbenzidine (TMB) solution for 2 minutes at RT to visualize the reaction. The reaction was terminated by adding 30 μL 1 N H_2SO_4. Optical density (O.D.) was measured the wavelength at 450 nm by using microplate reader (TECAN, Männedorf, Switcherland).

Binding ELISA for Finding Ligand

In the case of VISTA, the 96-well half-area ELISA plate (Corning, New York, USA) was coated with recombinant human VISTA Fc protein (200 ng/well, R&D Systems, Minneapolis, USA) or recombinant human VSIG-3 Fc protein (200 ng/well, R&D Systems, Minneapolis, USA) diluted in PBS and incubate for overnight at 4 ℃. Next day, wash the plate 3 times with 150 μL 0.05% PBS-T (Phosphate-Buffered Saline containing Polysorbate 20) and blocked with 180 μL 3% skim milk in 0.05% PBS-T for 1 hour at RT (Room temperature), and wash 3 times. VISTA Fc, biotin

labeled protein (BPS Bioscience, California, USA) was added into each well of the plate with various concentration range (From 31.25 to 2000 ng/well) and incubated for 4 hours at 37 ℃. After incubation, wash the plate 3 times with 150 μL 0.05% PBS-T and blocked with 180 μL 3% skim milk in 0.05% PBS-T for 1 hour at RT. After blocking, add 30 μL horseradish peroxidase (HRP)-conjugated streptavidin into each well with 3% skim milk in 0.05% PBS-T for 1 hour at RT. The plate was washed with 150 μL 0.05% PBS-T 3 times and blocked with 3% skim milk in 0.05% PBS-T for 10 minutes at RT. Add 30 μL 3, 3', 5, 5'-tetramethylbenzidine (TMB) solution and incubate until the color changes. The reaction was stopped by adding 30 μL 1 N H_2SO_4. Optical density (O.D.) was measured the wavelength at 450 nm by using microplate reader (TECAN, Männedorf, Switcherland).

Competitive ELISA for Finding scFv Candidates

In the case of VISTA, master plate made from 3rd and 4th panning of phage display is used for the plate of scFv candidates. Single colonies from each master plate were added into SB (+ampicillin) badge and incubated in a shaking incubator at 37 ℃ (TAITEC, Nishikata, Japan) until it turned blurry. After incubation, add 1 M IPTG (the final concentration is 1 mM) and incubate overnight at 30 ℃ in a shaking incubator (IPTG induction). Recombinant human VSIG-3 Fc protein (200 ng/well, R&D Systems, Minneapolis, USA) was used as an antigen and coated in an 96-well half-area ELISA plate (Corning, New York, USA). Next day, centrifuge the plate at 3,000

rpm for 15 minutes at 4 ℃. Remove supernatant and resuspend the pellet with 1X TES buffer for 5 minutes at 37 ℃. Lyse pellets by osmotic shock by adding 0.2X TES buffer. Incubate in a shaking incubator at 37 ℃ for 5 minutes and incubate at 4 ℃ for 1 hour. After 1 hour, centrifuge the plate at 3,000 rpm for 15 minutes at 4 ℃. Move the supernatant containing scFv candidate to a new 96-well plate and block with 6% bovine serum albumin (BSA) for 1 hour. After blocking, the antigen-coated plate was washed with 150 μL 0.05% PBS-T 3 times and blocked with 3% BSA in 0.05% PBS-T for 1 hour at RT. Meanwhile, scFv candidate solution and VISTA Fc, biotin labeled protein (2000 ng, BPS Bioscience, California, USA) were co-incubated into each well of new 96-well plate for 1 hour at RT. After incubation, 30 μL mixed solution was added into each well of the plate and incubated for 4 hours at 37 ℃. After incubation, wash the plate 3 times with 150 μL 0.05% PBS-T and blocked with 180 μL 3% skim milk in 0.05% PBS-T for 1 hour at RT. After blocking, add 30 μL horseradish peroxidase (HRP)-conjugated streptavidin into each well with 3% skim milk in 0.05% PBS-T for 1 hour at RT. The plate was washed with 150 μL 0.05% PBS-T 3 times and blocked with 3% skim milk in 0.05% PBS-T for 10 minutes at RT. Add 30 μL 3, 3', 5, 5'-tetramethylbenzidine (TMB) solution and incubate until the color changes. The reaction was stopped by adding 30 μL 0.5 N H_2SO_4. Optical density (O.D.) was measured the wavelength at 450 nm by using microplate reader (TECAN, Männedorf, Switcherland).

Making Stable Cell Lines

In the case of VISTA, we made stable cell lines expressing human VISTA in NIH/3T3, mouse fibroblast cell line. Human VISTA gene (Sino Biological Inc., Beijing, China) is used for gene construct and obtained from transformed DH5α competent *E.coli* (Enzynomics, Daejeon, Korea) by using Midi-prep. Gene was transfected into NIH/3T3 cells and confirmed by flow cytometry to express the human VISTA protein in the pooled state 2-3 days later. Thereafter, 125 to 2000 cells were seeded on 100 mm culture plates (Corning, New York, USA) by serial dilutions to obtain a single colony. Once a single colony is formed, transfer each colony to a 96-well culture plate (Corning, New York, USA) using a colony cylinder (Sigma-Aldrich, St. Louis, USA). Scaling-up was performed from 96-well culture plate to 24-well, from 24-well culture plate to 60 mm culture plate (Corning, New York, USA), and each single colony was confirmed by flow cytometry whether it expresses human VISTA protein on the cell surface or not. We selected only the clones whose expression was confirmed, and made the cell stock to finish making the stable cell line.

Using IntelliCyt® iQue Screener for Finding scFv Candidates

In the case of VISTA, master plate made from 3rd and 4th panning of phage display is used for plate of scFv candidates. Single colonies from each master

plate was added into SB (+ampicillin) badge and incubated in a shaking incubator at 37 ℃ (TAITEC, Nishikata, Japan) until it turned blurry. After incubation, add 1 M IPTG (the final concentration is 1 mM) and incubate overnight at 30 ℃ in a shaking incubator (IPTG induction). Next day, centrifuge the plate at 3,000 rpm for 15 minutes at 4 ℃. Remove supernatant and resuspend the pellet with 40 µL of 1X TES buffer for 5 minutes at 37 ℃ in a shaking incubator. Lyse pellets by osmotic shock by adding 0.2X TES buffer. Incubate in a shaking incubator at 37 ℃ for 5 minutes and incubate at 4 ℃ for 1 hour. After 1 hour, centrifuge the plate at 3,000 rpm for 15 minutes at 4 ℃. Move the supernatant containing scFv candidate to a new 96-well plate and block with 0.2% bovine serum albumin (BSA) in PBS for 1 hour.

NIH/3T3 cells were used as a negative cell line, and NIH/3T3-VISTA #13 cells were used as a positive cell line. We seeded negative cell lines in the negative sample area and positive cell lines in the positive sample area to a concentration of 1.0 x 10^6 cells/mL as designed in the 96-well round-bottom culture plate. Centrifuge the plate at 3,000 rpm for 5 minutes at 4 ℃. Wash the plate with 150 µL FACS buffer (0.1% BSA/DPBS, 0.22 µm filtered). Centrifuge the plate at 3,000 rpm for 5 minutes at 4 ℃ and aspirate each well. Add 1st solution (scFv leads 50 µL + FACS buffer 50 µL) and incubate the plate for 1 hour at 4 ℃. After incubation, centrifuge the plate at 3,000 rpm for 5 minutes at 4 ℃ and wash with 150 µL FACS buffer for 2 times. Then, add 2nd antibody, diluted in 100 µL FACS buffer and incubate for 1 hour at 4 ℃. After incubation, centrifuge the plate at

3,000 rpm for 5 minutes at 4 ℃ and wash with 150 µL FACS buffer for 2 times. Then, add 50 µL FACS buffer and analyzed by IntelliCyt® iQue Screener, high-throughput flow cytometry device (Sartorius, Göttingen, Germany).

Candidates Sequencing and Finding CDR Regions

First, phagemids were recovered from each *E.coli* infected with the selected phage using the Mini-prep. Kit (Nucleogen, Siheung, Korea). Candidates selected from the ELISA and flow cytometry in screening steps were sequenced (Bionics, Seoul, Korea) and CDR sequences were decoded.

Whole IgG conversion, Expression and Purification

Four clones of scFv candidates were converted to fully human IgG form. V_H genes from scFv candidates were inserted into a pcDNA™3.3-TOPO expression vector (ThermoFisher Scientific, Waltham, USA) with constant region of human IgG1 heavy chain. V_L genes from scFv candidates were inserted into a pOptiVEC™-TOPO expression vector (ThermoFisher Scientific, Waltham, USA) with constant region of human IgG1 light chain by cloning. Each integrated plasmid was amplified by Midi-prep. (Macherey Nagel ,Germany).

To produce full-form of anti-human VISTA antibodies, constant region of the heavy and light chain-integrated plasmids were co-transfected into the Expi293™

cell line (ThermoFisher Scientific, Waltham, USA) following the manufacturer's instructions and incubate these cell lines for 7 days at 37 ℃ in a humidified atmosphere of 8% CO_2 shaking incubator.

These culture soup from Expi293™ transient expression system were filtered with Corning™ Disposable Vacuum Filter/Storage Systems (0.2 μm, Corning, New York, USA). Four clones of monoclonal antibodies were purified using MabSelect SuRe™ rprotein A agarose-based resin (GE Healthcare, Chicago, USA). Resin-bound antibodies were eluted by 0.1 M glycine solution (pH 3.0) and neutralized with 1 M Tris-HCl solution (pH 8.0). Purified and concentrated antibody solutions were measured their concentration by using Cedex Bio device (Roche, Basel, Switzerland).

Flow Cytometry Analysis

To determine the expression levels of human VISTA on the cell surface and the binding abilities of converted form of 4 clones of antibodies, NIH/3T3 cells and NIH/3T3-VISTA cells were harvested using enzyme-free, PBS-based cell dissociation buffer (ThermoFisher Scientific, Waltham, USA) by incubating 10-15 min at 37 ℃ (the cell confluency was about 70-80%). Cells were washed with cold FACS buffer (0.1% BSA in DPBS) by centrifuging at 3,000 rpm for 5 minutes at 4 ℃. 4D2, 4F2, 4G3, and 4H2 clones were incubated in cold FACS buffer for 1 hour at 4 ℃. After 1 hour incubation, cells were washed with cold FACS buffer for three times, as described above. Then the secondary antibody, goat-anti-human fluorescein

isothiocyanate (FITC)-conjugated (Jackson Laboratory, Bar harbor, ME, USA), was also incubated in cold FACS buffer for 1 hour at 4 ℃. After that, cells were washed with cold FACS buffer for three times, as described above. Flow cytometry data was acquired in BD FACSCalibur (BD Biosciences, New Jersey, USA) and analyzed using CellQuest™ Pro program.

Western Blot Analysis

NIH/3T3 cells were used for negative control, and NIH/3T3-VISTA #13 cells were used for positive control. These cells were harvested and homogenized with lysis buffer containing cold radioimmunoprecipitation assay (RIPA) buffer (BIOSESANG, Seongnam, Korea), including 150mM Sodium chloride, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 50mM Tris-HCl, pH 7.5, and 2mM EDTA, protease inhibitor cocktail (Roche, Basel, Switzerland), and phosphatase inhibitor cocktail (Roche, Basel, Switzerland).

The resuspended cells were incubated on ice and vortex 3 times every 10 minutes for 30 minutes (4 times suspension during 5 seconds for 1 time on the ice). After lysis, centrifuge at 15,000 rpm for 15 minutes at 4 ℃. Take the cell supernatant. Some parts of supernatants were used for protein quantification through BCA assay (ThermoFisher Scientific, Waltham, USA) and some were reduced using 5X sample loading buffer (BIOSESANG, Seongnam, Korea) and boiled for 10 minutes for complete denaturation of proteins.

SDS-PAGE was done using acrylamide gels. Wet transfer was performed and the proteins were transferred to activated polyvinylidene difluoride (PVDF) membranes (Bio-rad, Hercules, California, USA). Membranes were blocked with 5% skim milk and incubated for over-night at 4 ℃ with primary antibodies (4 clones of antibody candidates, normal human IgG for negative control, commercial VISTA antibody for positive control). Next day, after incubation, wash membranes three times with 0.05% TBS-T (Tris-Buffered Saline containing Polysorbate 20) every 10 minutes and incubate for 1 hour at RT with horseradish peroxidase (HRP)-conjugated secondary antibodies, respectively. After that, wash membranes three times with 0.05% TBS-T every 10 minutes and signals were detected using enhanced chemiluminescence (ECL) solution (Bio-rad, Hercules, California, USA) in a dark room.

Competitive Inhibition ELISA

The 96-well half-area ELISA plate (Corning, New York, USA) was coated with recombinant human VSIG-3 Fc protein (200 ng/well, R&D Systems, Minneapolis, USA) diluted in PBS and incubate for overnight at 4 ℃. Next day, wash the plate 3 times with 150 μL 0.05% PBS-T (Phosphate-Buffered Saline containing Polysorbate 20) and blocked with 180 μL 3% skim milk in 0.05% PBS-T for 1 hour at RT (Room temperature), and wash 3 times for same way. While blocking the antigen-coated plate, 4 clones of antibody candidates with various concentration

range (From 0.005 to 1000 nM) and VISTA Fc, biotin labeled protein (1000 ng, BPS Bioscience, California, USA) were co-incubated into each well of a new 96-well plate for 1 hour at RT. After incubation, 30 μL mixed solution was added into each well of the plate and incubated for 4 hours at 37 ℃ with slow shaking. After incubation, wash the plate 3 times with 150 μL 0.05% PBS-T and blocked with 180 μL 3% skim milk in 0.05% PBS-T for 10 minutes at RT. After blocking, add 30 μL horseradish peroxidase (HRP)-conjugated streptavidin into each well with 3% skim milk in 0.05% PBS-T for 1 hour at RT. The plate was washed with 150 μL 0.05% PBS-T 3 times and blocked with 3% skim milk in 0.05% PBS-T for 10 minutes at RT. Add 30 μL 3, 3', 5, 5'-tetramethylbenzidine (TMB) solution and incubate until the color changes. The reaction was stopped by adding 30 μL 0.5 N H_2SO_4. Optical density (O.D.) was measured the wavelength at 450 nm by using microplate reader (TECAN, Männedorf, Switcherland).

In vitro CD4$^+$ T Cell Assay and Quantitative Human IFN-γ ELISA

For *in vitro* plate-bound CD4$^+$ T cell assay, 96-well flat-bottom culture plates (SPL Life Sciences, Pocheon, Korea) were coated overnight with anti-human CD3 [OKT3] (Abcam, Cambridge, United Kingdom) at 2.5 μg/mL mixed together with recombinant human VISTA-Fc protein (R&D Systems, Minneapolis, USA) at 3.0 μg/mL in DPBS at 4℃ overnight. Wells were washed three times with DPBS before

adding $CD4^+$ T cells. VISTA protein was not added to the wells to confirm T cell activation, and VISTA protein was added to the wells to confirm T cell inhibition. The wells to be tested for efficacy of monoclonal antibodies we produced.

To isolate $CD4^+$ T cells, we used human cryopreserved peripheral blood mononuclear cells (PBMC, Zen-Bio Inc., North California, USA). Total $CD4^+$ T cells were isolated using a CD4 MicroBeads (Miltenyi Biotec, California, USA) by magnetic-activated cell sorting.

Isolated $CD4^+$ T cells were added at 2 x 10^5 cells per well in complete RPMI media (RPMI 1640, 10% heat-inactivated FBS, 1% penicillin/streptomycin). Four clones of monoclonal antibodies (4D2, 4F2, 4G3, and 4H2) were added to final concentrations of 4, 20, and 100 nM in the wells to be tested for efficacy of monoclonal antibodies we produced. After incubation for 5 days in a humidified atmosphere of 5% CO_2 incubator at 37 ℃, the concentration of IFN-γ in the cell soup was measured by quantitative ELISA.

In the case of ELISA for measuring the concentration of IFN-γ, a human IFN-γ quantitative ELISA kit (R&D Systems, Minneapolis, USA) was purchased and used. Dilute the Capture Antibody to the working concentration in PBS with 100 uL and coat a 96-well plate overnight at room temperature. Next day, wash the plate 3 times with 400 μL wash buffer (0.05% PBS-T in PBS, pH 7.2-7.4) and blocked with 300 μL blocking buffer (1% bovine serum albumin in PBS, pH 7.2-7.4) for 1 hour at room temperature. After blocking, wash 3 times. Add 100 μL of cell soup or standards in blocking buffer to appropriate well and incubate 2 hours at room

temperature. After incubation, wash the plate 3 times with 300 µL wash buffer. Then, add 100 µL of the detection antibody, diluted in blocking buffer, to each well and incubate 2 hours at room temperature. After incubation, wash the plate 3 times with 300 µL wash buffer. Add 100 µL of the streptavidin-HRP, diluted in blocking buffer, to each well and incubate for 20 minutes at room temperature. The plate was washed with 300 µL wash buffer 3 times and add 100 µL of substrate solution to each well. Incubate for 20 minutes at room temperature. The reaction was stopped by adding 50 µL 2 N H_2SO_4. Optical density (O.D.) was measured the wavelength at 450 nm by using microplate reader (TECAN, Männedorf, Switcherland).

Cell	Organism	Tissue type	Morphology	Culture Media
NIH/3T3	*Mus musculus* , mouse	Embryo	Fibroblast	DMEM, 10% FBS, 1% abs
NIH/3T3-hVISTA #13	*Mus musculus* , mouse	Embryo	Fibroblast	DMEM, 10% FBS, 1% abs, and Hygromycin B 0.5 mg/mL

Table 3. The List of Cell Lines.

A stable cell line expressing human VISTA was constructed and used in experiments related to VISTA. A mouse fibroblast cell called NIH/3T3 was used as a parental cell line. The human VISTA gene was transfected into the parental cell line. Cell line name, organism, tissue type, morphology, and culture media are shown in the table.

RESULTS

Screening by phage display and identification of scFvs binding to human VISTA

Screening was performed using phage display technique to obtain a monoclonal antibody clones showing binding affinity to human VISTA. Phage library containing human synthetic scFv was used and phage display was performed using recombinant human VISTA protein as an antigen [Figure 4A]. Antigen was coated on immunotubes and 4 cycles were repeated to screen for clones with high signal for antigen. The ratio of the input to the output increased to the second panning step, then dropped for the third time, and rises for the fourth time [Figure 4B and 4C]. As a result of ELISA with all the clones obtained from the 3rd and 4th panning, 34 clones showing high signal could be selected and confirmed through repeated experiments [Figure 4D]. 34 clones were selected because sequencing analysis showed that there were identical clones and that the same clones were replaced with one representative clone. Thus, it was confirmed that the selected 34 clones have different sequences, and that there is no mutation in the common frame region of the library and only the CDR regions are different.

VSIG-3 is a putative ligand of VISTA

In the case of VISTA, there is no officially confirmed binding partner to date. VISTA is known to have a dual role both as a ligand and a receptor. It is known that among many molecules, a molecule called VSIG-3 is known for a putative ligand of VISTA. In this study, we were supposed to conduct a competition assay with a binding partner to select monoclonal antibody clones with better binding ability than VISTA binding partner. Therefore, we examined the binding pattern between VISTA and VISTA or between VISTA and VSIG-3 through ELISA. Experiments were performed by coating recombinant human VSIG-3 or VISTA protein on a 96-well plate and treating VISTA-biotin tagged protein by concentration [Figure 5A]. As a result, there was no binding pattern in the interaction between VISTA and VISTA protein, but a binding pattern was observed in the interaction between VISTA and VSIG-3 protein [Figure 5B and 5C].

Finding scFv clones by competing assays

After selecting VSIG-3 as a putative ligand for VISTA, competitive ELISA and competitive high-throughput flow cytometry were performed to select clones with higher binding affinity to human VISTA in competition with ligand. We then selected clones that met both conditions.

Competitive ELISA

Thirty-four clones showing high signal to the recombinant human VISTA protein obtained from phage display were selected [Figure 6A]. VSIG-3, a putative ligand of VISTA, was coated on a 96-well plate. Thereafter, 34 clones were competed with VISTA-biotin-tagged proteins and clones showing lower signal comparison to positive control (Only ligand treated) were selected [Figure 6B]. As a result, it was confirmed that 7 clones among 34 clones had a competitive effect against the putative ligand [Figure 6C]. This result reflects a decreasing in the signal that binds to VISTA when VSIG-3 and the monoclonal antibody clone compete to show binding affinity. The seven selected clones were able to bind to recombinant human VISTA better by blocking the interaction between VISTA and VSIG-3, showing up to 59.6% competitiveness [Figure 6D].

Competitive High-throughput Flow Cytometry

The previous competitive ELISA experiments used recombinant proteins, which have limitations the ability to accurately represent the native form of the antigen. Therefore, the experiment was conducted to select the clone having better binding ability even in the native form state than the putative ligand of VISTA. A stable cell line expressing human VISTA native form protein was prepared and used for the experiment. We used NIH/3T3 as a parental cell line. It was confirmed that human VISTA was expressed in stable cell lines by flow cytometry [Figure 7A and 7B].

We selected 34 clones that showed high signal through phage display and experimented with high-throughput flow cytometry device to confirm that they bind to native form human VISTA better than competition with ligand. Flow cytometry analysis was performed on a 96-well plate using NIH/3T3 as a negative cell line and NIH/3T3-VISTA #13 as a positive cell line [Figure 8A]. The isotype control of antibodies recognizing HA tagged to scFv was also analyzed to confirm that the peak shift results were reliable. When analyzed using the instrument, the sample to be analyzed appears as a blue region, a positive control (confirmation of human VISTA expression) as a green region, and a negative control (clone without binding affinity, 4H9) as a red region [Figure 8B]. Cross-contamination between samples did not occur. The flow chart also showed that the analysis results using this instrument were reliable [Figure 8C]. Performing flow cytometry, counting the number of cells used in the actual analysis was analyzed [Figure 8D]. Gating the live cell region, the positive and negative regions was performed and it increased the reliability of the assay [Figure 8E]. When the stable cell line is properly expressed by using the native human VISTA, it is confirmed that there is no problem in the analysis of the stable human cell line [Figure 8F]. As a result, 9 of 34 clones showed higher binding affinity to the native form human VISTA in competition with putative ligand VSIG-3 [Figure 8G].

Through competitive ELISA and high-throughput flow cytometry experiments, 4 clones (4D2, 4F2, 4G3, and 4H2) were found that satisfied both two experimental conditions. 3 clones with response in the ELISA but no response in the flow

cytometry and 5 clones with no response in the ELISA but reactive in the flow cytometry were identified [Figure 9A]. Sequencing analysis of four clones satisfying both two experimental conditions revealed that the CDR regions were different from each other and were defined as different scFv clones [Figure 9B]. Four clones were converted from scFv to human IgG form and their characteristics and efficacy were evaluated.

Generation and characterization of human VISTA monoclonal antibodies

Since the clone obtained from the phage display is in the form of scFv, constant regions of the heavy and the light chain should be inserted in order to construct fully human monoclonal antibodies. The heavy and light chain portions of the scFv were then cloned into the pcDNA™ 3.3-TOPO® vector containing constant region of the heavy chain and the pOptiVEC™-TOPO® vector containing constant region of the light chain, respectively [Figure 10A]. After the conversion into fully human IgG form, it was confirmed by sequencing that there was no change in sequence and that each constant region was correctly inserted (Data not shown). After incubation for 7 days with transient expression system, antibodies were purified using protein A beads by affinity chromatography [Figure 10A]. As a result, the productivities of the four clones were 36.5-78 mg/L [Figure 10B] and the purification efficiencies were as high as 87% [Figure 10C]. The formation of 50 kDa heavy chain and 25 kDa light

chain was confirmed by SDS-PAGE gel detection [Figure 10D]. As a result, each band was properly detected in the reducing condition and proved it. Human IgG was used as a control sample. SDS-PAGE gel detection was also used to confirm the loss of each clone in the purification step [Figure 10E]. All four clones were found to specifically obtain antibody-form proteins only at the elution step.

Identification of binding abilities of human VISTA monoclonal antibodies to native and non-native form human VISTA

Determining binding abilities to the native form antigen (Flow cytometry)

Four clones (4D2, 4F2, 4G3, and 4H2) were confirmed to have binding affinity in the native form human VISTA by competitive high-throughput flow cytometry in the form of scFv, but they had to confirmed by flow cytometry to show the same binding affinity after whole IgG conversion. NIH/3T3, the parental cell line for the negative cell line, and NIH/3T3-VISTA #13, the stable cell line we produced for the positive cell line, were used. As a result, none of the four clones showed any binding affinity to the negative cell line but all four clones showed a peak shift in the stable cell line expressing human VISTA, indicating binding affinity to the corresponding antigen [Figure 11A]. As a control group, goat-anti-human FITC conjugated antibody (Secondary antibody)-treated group was used. In addition, using human IgG, no binding affinity was observed.

Targeting ability was demonstrated as a ratio of binding affinities in negative cell line versus positive cell line. These were calculated as the ratio of MFI value in each analysis. As a result, the secondary antibody and human IgG sample showed a targeting ability of 1, indicating no difference in binding affinity between the two cell lines [Figure 11B]. However, the binding affinity of the four clones in the positive cell line was much higher than that of the negative cell line, confirming that the targeting ability was excellent in all four clones [Figure 11B]. The ability to bind to the antigen was found to be superior in order of 4H2, 4D2, 4F2, and 4G3 [Figure 11B].

Determining binding abilities to the non-native form antigen (Western blot)

After the four monoclonal antibody clones were found to have binding affinity to the native form human VISTA by flow cytometry, we also confirmed the binding affinity in the non-native form human VISTA via western blot. Similarly, NIH/3T3 was used as a negative cell line and NIH/3T3-VISTA #13 was used as a positive cell line. Protein was quantitatively assayed and loaded into each well by the amount corresponding to 20 μg of protein, and western blotting was performed. Human IgG was used as a negative antibody, and it was confirmed that there was no binding affinity to non-native human VISTA. A commercial human VISTA detection antibody was used for a positive antibody and showed a specific binding affinity to non-native human VISTA [Figure 12]. However, the four monoclonal antibody clones we produced did not show any binding affinities to non-native human VISTA

[Figure 12]. These results indicate that the four antibodies we produced are not recognizable in the non-native form and only recognize the native form human VISTA, specifically. Detection of one of the housekeeping genes, GAPDH, confirmed that the same amount of sample was loaded [Figure 12].

Inhibition of VISTA and VSIG-3 interaction by competing human VISTA monoclonal antibodies with ligand of VISTA

When selecting candidates for monoclonal antibodies, we have selected a clone with a greater binding affinity to VISTA through competition with its ligand. The IC_{50} value was determined by competitive inhibition ELISA to determine the ability of the antibody-form clone to interfere with the interaction between VISTA and VSIG-3, the putative ligand. Similar to the previous ELISA, recombinant human VSIG-3 protein was coated on a 96-well plate. The VISTA-biotin tagged protein and each of the four clones were treated by concentration to perform ELISA [Figure 13A]. The IC_{50} values for the 4D2, 4F2, 4G3 and 4H2 clones were 51.37, 78.14, 48.14, and 32.55 nM, respectively, at concentrations ranging from 0.005 to 1000 nM [Figure 13B].

The VISTA-dependent immunosuppressive effect of the human VISTA monoclonal antibodies

Identifying the role of VISTA as a ligand or a receptor

VISTA is a negative checkpoint molecule that regulates T cell activation and immune response. In the case of VISTA, counterpart molecule (binding partner) is not surely known until now, and T cell inhibition by VISTA is known to occur when VISTA acts on ligand or receptor in T cell functional studies. *In vitro* T cell assays were performed to assess the efficacies of the four clones of antibodies, and we examined the T cell inhibition of VISTA as a ligand and receptor. The scheme for this is shown in [Figure 14]. In the case of VISTA acting as a ligand, VISTA is expressed in tumor cell/APC or T cell and binds with unknown receptor on T cell surface to transmit T cell inhibition signal [Figure 14 'VISTA']. In the case of VISTA acting as a receptor, VISTA is present in T cell and unknown ligand is present in tumor cell/APC to transmit T cell inhibition signal [Figure 14 'VISTA-R']. In this study, we assumed the unknown ligand of VISTA as VSIG-3.

T cell inhibition occurred more when VISTA acted as a ligand.

The 96-well plates were coated with recombinant VISTA or VSIG-3 protein, which provides T cell inhibition signal, and anti-CD3 antibody [OKT3], which provide T cell activation signal was also coated. Thereafter, only $CD4^+$ T cells were isolated from human PBMC and cultured in a coated 96-well plate for 5 days. The

amount of IFN-γ, a typical T cell activation marker secreted by T cell, was measured by quantitative ELISA [Figure 15A and 15B]. In the absence of the activation signal, $CD4^+$ T cell did not secrete IFN-γ but secreted IFN-γ only when there was an activation signal. The amount of IFN-γ secreted by $CD4^+$ T cell was reduced by about 30% compared with the activation status when VSIG-3 was treated at four concentrations (1.25, 2.5, 5.0, and 10.0 μg/mL). The inhibition was saturated from 2.5 μg/mL and no further enhanced inhibition progress was made [Figure 15A].

However, in the case of VISTA, it was confirmed that the T cell inhibition was further suppressed by increasing the concentration of VISTA when treated with four concentrations (1.25, 2.5, 5.0, and 10.0 μg/mL). This pattern of inhibition was greater than that of VSIG-3, and up to 70% of T cell inhibition was observed [Figure 15B]. These results suggest that T cell inhibition occurs significantly when unknown receptor is present in T cell and VISTA acts as a ligand.

4 monoclonal antibody clones have VISTA-dependent immunosuppressive effect

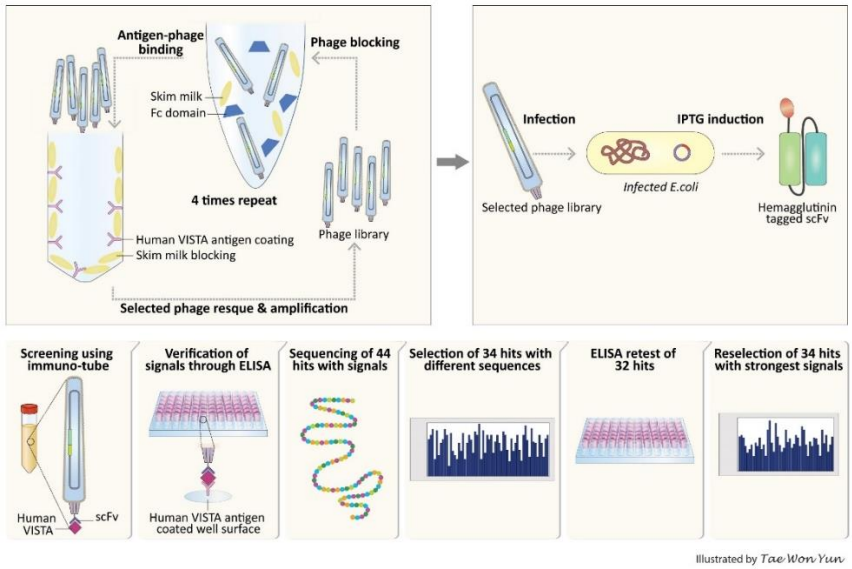
In the previous experiments, T cell activation conditions were selected as anti-CD3 antibody [OKT3] 2.5 μg/mL and T cell inhibition conditions were selected VISTA 3.0 μg/mL [Figure 15A and 15B]. First, we examined whether the produced antibodies could show their efficacies exclusively on activated T cells or not. T cell activation signals were provided and four monoclonal antibodies were treated at final concentrations of 4, 20, and 100 nM. After 5 days, cell soups were harvested in each

well and concentration of IFN-γ was measured by quantitative ELISA. As a result, all four clones did not lead to any significant changes, such as further enhancing T cell activation or leading to further enhanced T cell inhibition [Figure 15C]. Human IgG was used as a control group.

We looked at the change in the case of the presence of T cell inhibition signal. The 96-well plate was coated with 2.5 μg/mL of anti-CD3 antibody [OKT3] as a T cell activation signal and 3.0 μg/mL of VISTA as a T cell inhibition signal. After that, $CD4^+$ T cells isolated from human PBMC were treated and four monoclonal antibody clones were also treated to final concentrations of 4, 20, and 100 nM. As a result, T cell inhibition was further enhanced with increasing concentrations of antibodies in all 4 clones under the corresponding experimental conditions. This was confirmed by a gradual decreasing in the amount of secreted IFN-γ [Figure 15D]. The human IgG used as a control group did not show this pattern even when the antibody concentration increased, suggesting that the four monoclonal antibodies specifically affect T cell inhibition.

Based on the results of the two experiments, four antibodies were found to exhibit a VISTA-dependent immunosuppressive effect.

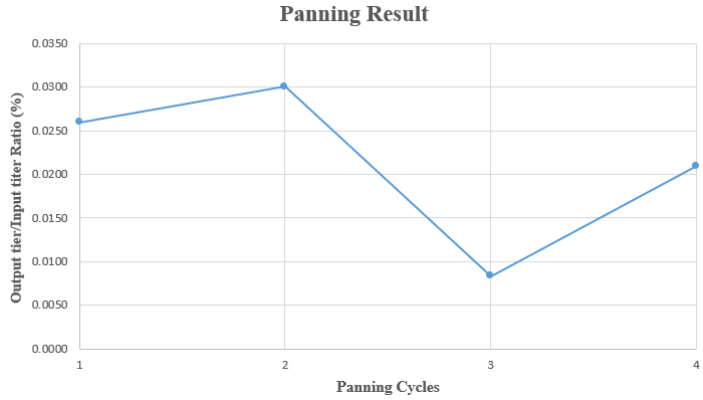
A.



B.

	1st	2nd	3rd	4th
Human scFv Library	OPALS			
Antigen (rhVISTA-Fc)	15 μg	7.5 μg	7.5 μg	7.5 μg
Blocking	6% & 3% skim milk	6% & 3% skim milk	6% & 3% skim milk	6% & 3% skim milk
Wash	3 times	5 times	10 times	10 times
Input titer	$1x10^{13}$	$1.23x10^{12}$	$4.8x10^{12}$	$3.2x10^{10}$
Output titer	$2.6x10^{9}$	$3.7x10^{8}$	$4x10^{8}$	$6.7x10^{6}$

C.



D.

1st Plate

Ag/hIgG	1	2	3	4	5	6	7	8	9	10	11	12
A	36.482	43.956	33.367	1.000	22.458	24.827	27.266	1.735	1.200	20.456	0.937	1.191
B	1.091	18.011	26.745	31.642	0.952	1.404	21.937	1.130	29.766	20.680	25.677	22.306
C	33.879	39.848	33.300	21.315	1.052	1.065	1.058	17.000	26.361	1.030	26.508	27.557
D	1.298	37.558	1.511	34.682	19.000	1.114	38.264	1.102	1.492	27.889	18.347	1.103
E	1.140	1.070	31.556	1.065	26.383	20.581	26.365	18.146	21.722	26.831	16.043	0.988
F	1.228	28.638	24.685	0.936	1.123	1.043	21.667	22.116	0.918	15.900	18.971	1.000
G	1.150	34.537	24.258	23.739	1.031	0.828	26.759	20.052	1.049	0.986	24.750	0.970
H	32.111	42.000	1.106	1.354	0.969	1.000	18.728	22.456	0.908	1.013	26.579	1.000

2nd Plate

Ag/hIgG	1	2	3	4	5	6	7	8	9	10	11	12
A	1.1600	0.9718	0.9857	0.8721	1.0930	0.9492	1.0122	0.2689	0.9908	1.0200	1.0481	0.9524
B	0.9595	1.0152	0.9861	1.1250	1.1000	1.1757	1.0435	1.0256	0.5172	0.9310	1.0225	0.8519
C	0.8523	0.7010	0.9342	0.8642	0.9709	1.0267	0.9545	0.8736	0.9479	1.0105	0.8611	0.7634
D	0.7653	1.1299	0.8764	1.1286	0.9462	1.0303	0.9059	0.8889	1.0235	0.9505	1.0333	0.7252
E	0.8642	1.0000	1.1646	1.0541	0.9623	0.8929	0.7398	0.9259	1.0575	1.7981	1.0330	0.8547
F	0.8764	1.0933	1.0130	1.0769	0.9706	0.9457	15.7742	1.3247	1.3824	0.9901	1.0106	1.0000
G	0.9000	1.0811	1.0959	0.8795	1.0326	1.3333	0.6850	1.4156	0.8205	1.0606	1.0632	0.9608
H	0.9740	1.1600	1.0000	0.7708	0.9901	0.9756	1.1228	1.4091	0.9706	1.0196	1.0825	1.0396

3rd Plate

Ag/hIgG	1	2	3	4	5	6	7	8	9	10	11	12
A	0.9136	1.0299	1.1548	1.0735	0.9583	0.8889	0.9778	0.7143	1.0110	0.9364	1.0430	1.0000
B	1.1333	1.1333	0.8352	0.9250	0.9375	1.0390	0.9239	1.0000	0.9238	0.8627	0.6720	0.7299
C	0.8987	1.9600	1.3133	1.0429	1.2021	0.9605	0.8738	1.1538	0.9778	0.9903	0.9082	0.7500
D	1.0617	1.2262	1.0986	1.0694	1.0860	0.9444	0.9877	1.0000	0.9888	0.9794	0.9570	0.8000
E	1.5581	1.3117	0.9651	0.9744	0.9451	1.0400	0.9684	1.1169	1.0889	0.9906	1.0568	0.9684
F	1.2619	1.0449	0.8989	1.0263	1.0202	0.9375	0.9770	0.8462	1.0263	0.8629	0.9238	0.9320
G	0.9024	1.0556	21.9342	1.1351	1.0404	1.0366	1.1136	0.8351	1.3021	0.9712	0.9674	1.0000
H	0.4000	0.7629	0.9189	1.0972	1.0101	1.0370	1.1607	1.2718	1.0208	1.0777	1.0426	3.5294

Figure 4. Screening by Phage Display using Recombinant Human VISTA Protein as an Antigen.

(A) This is a schematic figure showing the overall scFv clone screening process from phage display to ELISA. After ELISA, 34 clones were selected. **(B)** This is a table summarized the panning results up to the 4^{th} step. Used human synthetic scFv library name, the amount of used antigen, blocking and washing steps, and the input and output titer values are demonstrated. **(C)** It is a graph that shows the ratio of input to output ratio to each panning step. **(D)** This table shows the signal of antigen (recombinant human VISTA Fc protein) versus control (human IgG) obtained from each panning step.

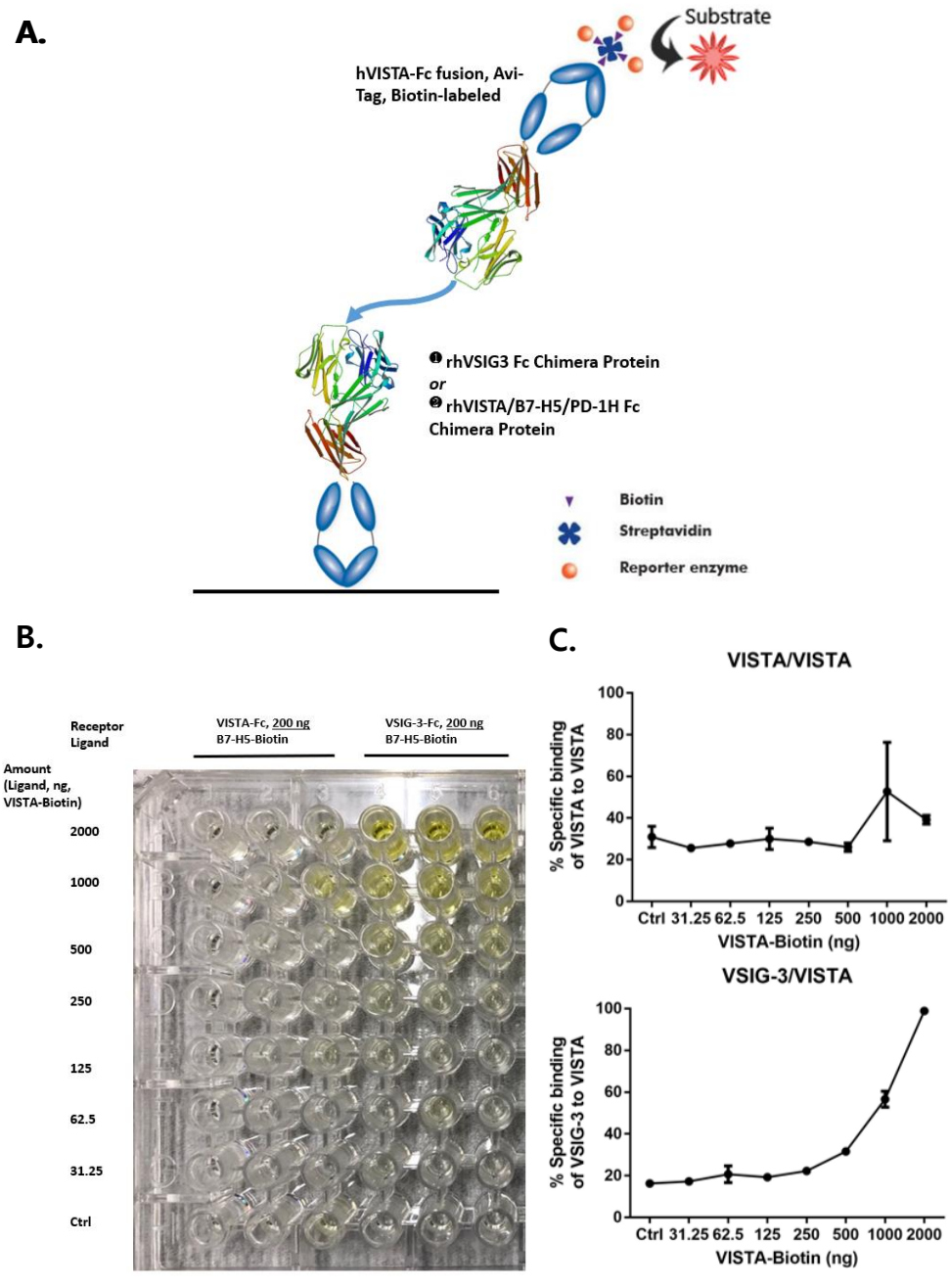

A.
Substrate
hVISTA-Fc fusion, Avi-Tag, Biotin-labeled
❶ rhVSIG3 Fc Chimera Protein
or
❷ rhVISTA/B7-H5/PD-1H Fc Chimera Protein
Biotin
Streptavidin
Reporter enzyme
B.
Receptor Ligand
VISTA-Fc, 200 ng B7-H5-Biotin
VSIG-3-Fc, 200 ng B7-H5-Biotin
Amount (Ligand, ng, VISTA-Biotin)
2000
1000
500
250
125
62.5
31.25
Ctrl
C.
VISTA/VISTA
% Specific binding of VISTA to VISTA
Ctrl 31.25 62.5 125 250 500 1000 2000
VISTA-Biotin (ng)
VSIG-3/VISTA
% Specific binding of VSIG-3 to VISTA
Ctrl 31.25 62.5 125 250 500 1000 2000
VISTA-Biotin (ng)

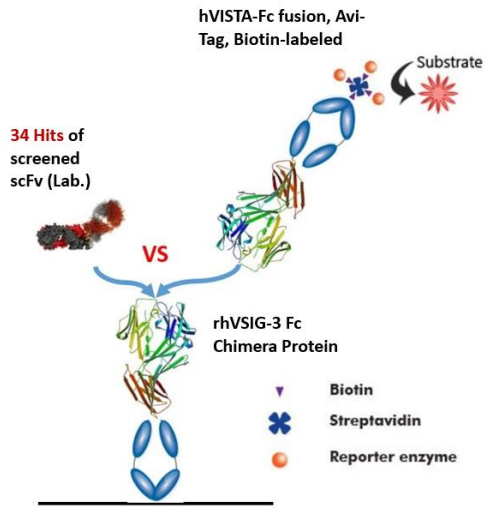
Figure 5. Binding ELISA of VISTA for Finding Ligand and Binding Pattern.

(A) This is a schematic figure of binding ELISA experiment. Recombinant human VISTA or VSIG-3 protein was coated on a 96-well half-area ELISA plate. **(B)** This is a picture of a 96-well plate showing ELISA results. **(C)** As a result, no binding pattern could be observed between VISTA-VISTA interaction. However, the binding pattern of VSIG-3-VISTA interaction was found with increasing VISTA-biotin tagged protein concentration. Taken together, VSIG-3 was selected as the putative ligand of VISTA.

A.

Only those with an Ag/hIgG Ratio value of 2 or higher are selected.		
scFv Antibody Library	**Clone**	**Ag/hIgG Ratio**
OPALS	4A1	36.482
	4A2	43.956
	4A3	33.367
	4A5	22.458
	4B3	26.745
	4B7	21.937
	4B9	29.766
	4B11	25.677
	4B12	22.306
	4C3	33.300
	4C9	26.361
	4C12	27.557
	4D2	37.558
	4D4	34.682
	4D7	38.264
	4D10	27.889
	4E3	31.556
	4E5	26.383
	4E9	21.722
	4E10	26.831
	4E11	16.043
	4F2	28.638
	4F3	24.685
	4F10	15.900
	4G2	34.537
	4G3	24.258
	4G7	26.759
	4G11	24.750
	4H2	42.000
	4H7	18.728
	4H11	26.579
	3F7	15.7742
	3G3	21.9342
	3H12	3.5294
Negative Control (OPALS)	4H9	0.908
Total 34 leads, 1 clone of Negative Control		

B.



C.

10 min					
VISTA	VSIG-3 200 ng, VISTA 2000 ng				
	1	2	3	4	5
A	4A1	4A2	4A3	4A5	4B3
B	4B7	4B9	4B11	**4B12**	4C3
C	4C9	4C12	***4D2***	4D4	4D7
D	4D10	4E3	4E5	4E9	4E10
E	4E11	***4F2***	***4F3***	4F10	4G2
F	***4G3***	4G7	***4G11***	**4H2**	4H7
G	4H11	3F7	3G3	3H12	
H	P.C	4H9	P.C	P.C	Blank

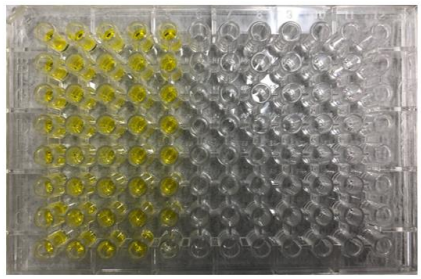


Plate #1 (Non-Shaking)					
VISTA	VSIG-3 200 ng, VISTA 2000 ng				
	1	2	3	4	5
A	1.3730	1.0800	1.0260	1.1490	1.4290
B	1.3680	1.2640	1.3140	**1.0180**	1.3160
C	1.3780	1.2290	***0.9230***	1.2710	1.3850
D	1.0620	1.2690	1.1860	1.3400	1.4490
E	1.4190	***0.6180***	***0.6700***	1.4420	1.3760
F	***0.7030***	1.3970	***0.9430***	**1.0510**	1.4650
G	1.5730	1.2680	1.3180	1.4210	0.8000
H	1.2110	1.3140	1.3270	1.3280	0.1560

10 min					
VISTA	VSIG-3 200 ng, VISTA 2000 ng				
	1	2	3	4	5
A	4A1	4A2	4A3	4A5	4B3
B	4B7	4B9	4B11	**4B12**	4C3
C	4C9	4C12	***4D2***	4D4	4D7
D	4D10	4E3	4E5	4E9	4E10
E	4E11	***4F2***	***4F3***	4F10	4G2
F	***4G3***	4G7	***4G11***	**4H2**	4H7
G	4H11	3F7	3G3	3H12	
H	P.C	4H9	Blank		

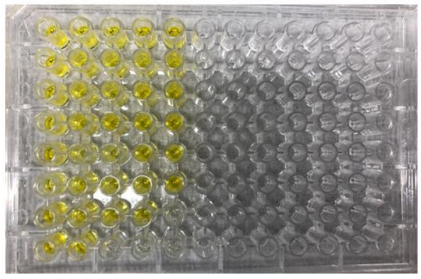


Plate #1 (Non-Shaking)					
VISTA	VSIG-3 200 ng, VISTA 2000 ng				
	1	2	3	4	5
A	1.5010	1.3890	1.2120	1.3950	1.6340
B	1.6220	1.3820	1.6230	**0.9720**	1.5260
C	1.5830	1.5350	***1.0520***	1.5350	1.5230
D	1.3410	1.5690	1.8290	1.4520	1.6450
E	1.6790	***0.6010***	***0.6660***	1.3980	1.2290
F	***0.7910***	1.4670	***0.9790***	***1.0120***	1.8050
G	1.6990	1.4820	1.4410	1.2010	0.1200
H	1.6900	1.4260	0.0890	0.1080	0.1260

D.

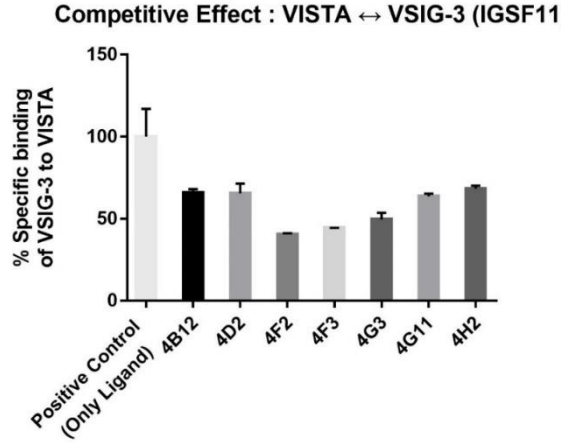
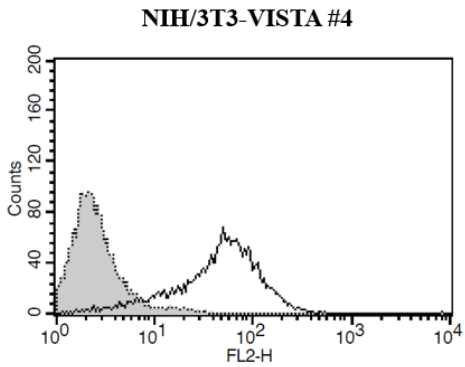
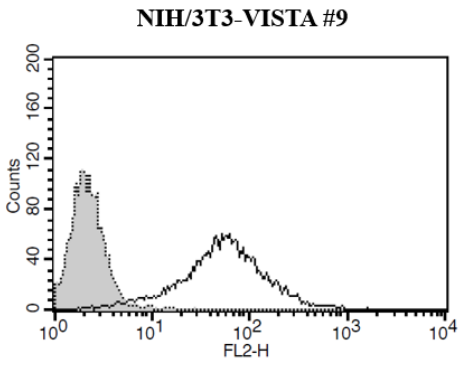


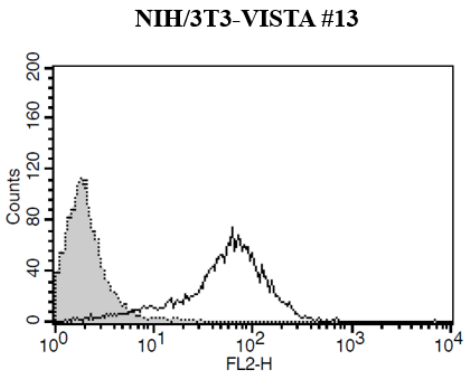
Figure 6. Competitive ELISA of VISTA for Finding scFv Candidates.

(A) The antigen/control ratios of the 34 clones obtained from the phage display are summarized. 34 clones were selected for antigen/control ratio of 2 or more. As a negative control, 4H9 clone which does not show any binding affinity to antigen was used. **(B)** This is a schematic figure of competitive ELISA experiment. Recombinant human VSIG-3 protein was coated on a 96-well half-area ELISA plate. VISTA-biotin tagged protein and each scFv clone competed. As a result, clones with reduced signal were selected. **(C)** Design of plate used in experiments, substrate solution (TMB solution) treatment time, and picture of a 96-well plate showing ELISA results are shown. **(D)** This is a graph summarizing the experimental results. Seven clones were selected that had a signal lower than that of a positive control (treating only ligand).

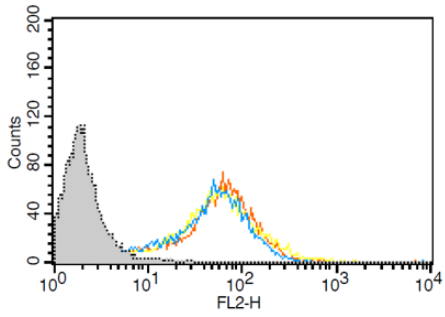
A.







B.



Key	Name	Parameter	Gate
	NIH3T3-VISTA_4_VISTA.008	FL2-H	G2
	NIH3T3-VISTA_9_VISTA.006	FL2-H	G2
	NIH3T3-VISTA_13_VISTA.012	FL2-H	G2
	NIH3T3-VISTA_4_Mock.007	FL2-H	G2

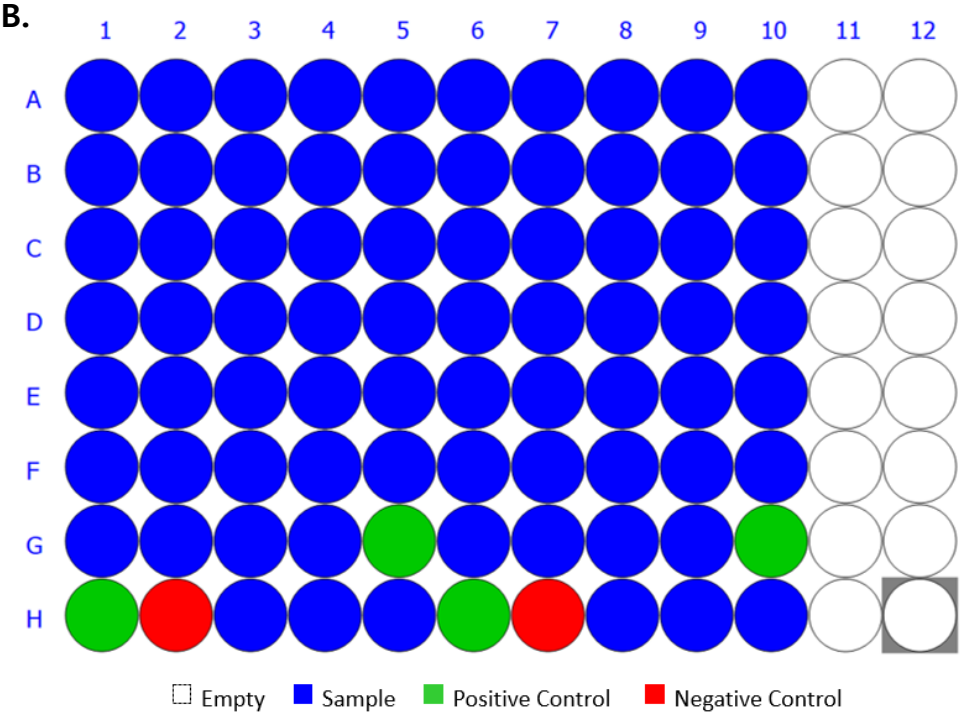
Figure 7. Making Stable Cell Lines of VISTA.

(A) Three stable cell lines expressing human VISTA were examined by flow cytometry to determine the expression level. The human VISTA gene was transfected into the NIH/3T3 cell line to produce a stable cell line. As a control group, a group treated only with secondary antibody was used. **(B)** Three stable cell lines are shown in one histogram.

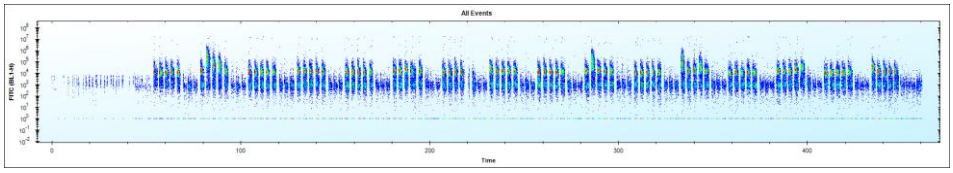
A.

	Negative Cell Line (NIH/3T3)					Positive Cell Line (NIH/3T3-VISTA #13)						
VISTA	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	4A1	4A2	4A3	4A5	4B3	4A1	4A2	4A3	4A5	4B3		
B	4B7	4B9	4B11	4B12	4C3	4B7	4B9	4B11	4B12	4C3		
C	4C9	4C12	4D2	4D4	4D7	4C9	4C12	4D2	4D4	4D7		
D	4D10	4E3	4E5	4E9	4E10	4D10	4E3	4E5	4E9	4E10		
E	4E11	4F2	4F3	4F10	4G2	4E11	4F2	4F3	4F10	4G2		
F	4G3	4G7	4G11	4H2	4H7	4G3	4G7	4G11	4H2	4H7		
G	4H11	3F7	3G3	3H12	P.C (VISTA-PE)	4H11	3F7	3G3	3H12	P.C (VISTA-PE)		
H	P.C (VISTA-PE)	4H9 (N.C)	FITC-Ctrl (HA-FITC)	PE-Ctrl (Iso-PE)	Mock	P.C (VISTA-PE)	4H9 (N.C)	FITC-Ctrl (HA-FITC)	PE-Ctrl (Iso-PE)	Mock		

B.

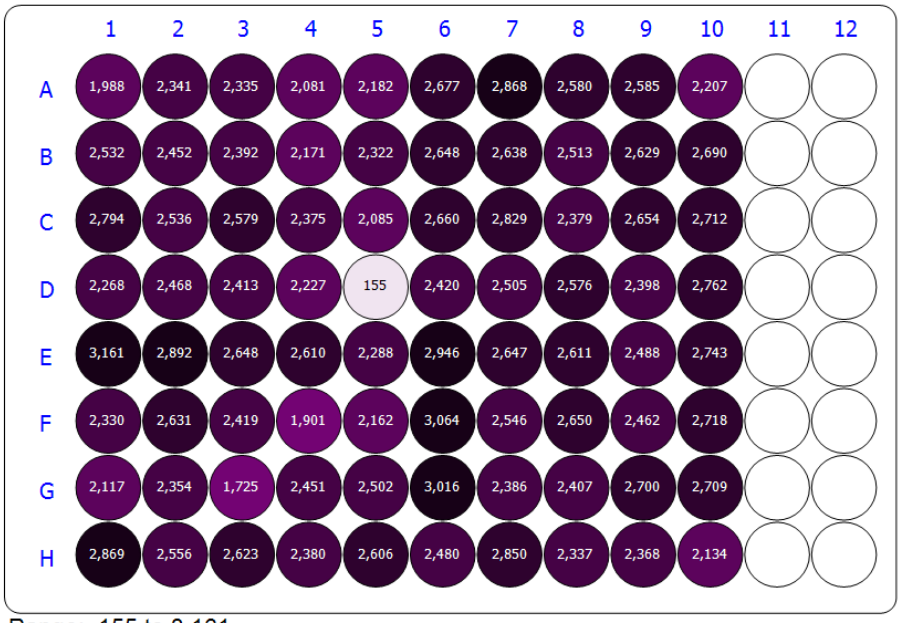


C.



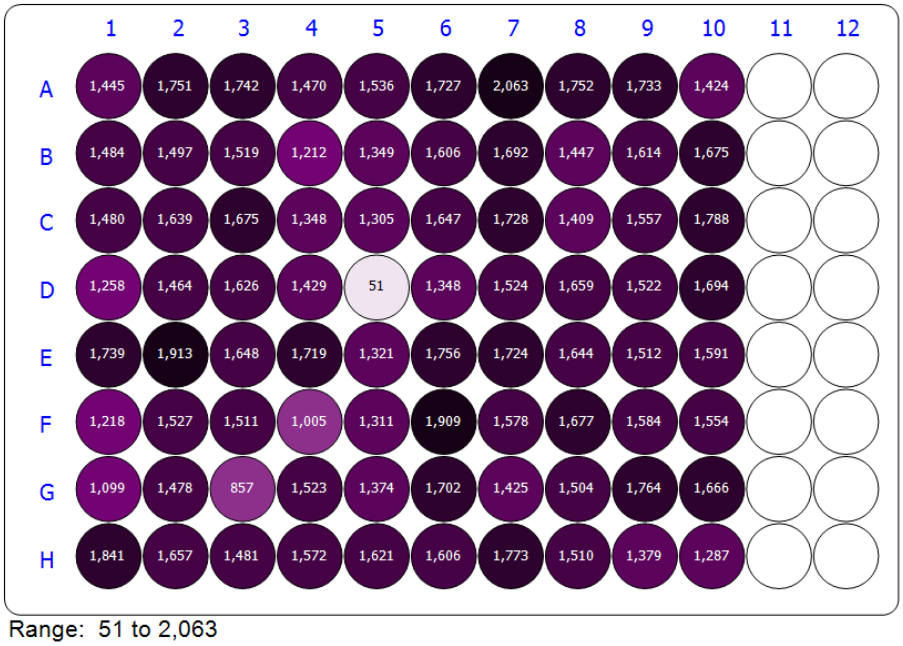
D.

Count of All Events



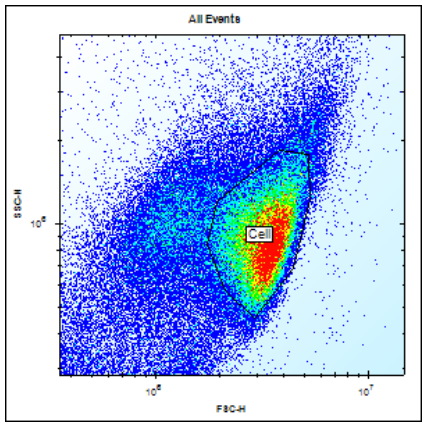
Range: 155 to 3,161

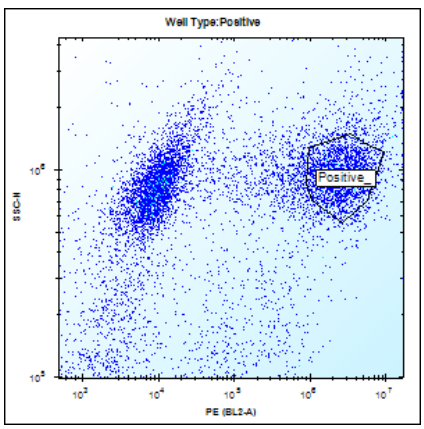
Count of Cell

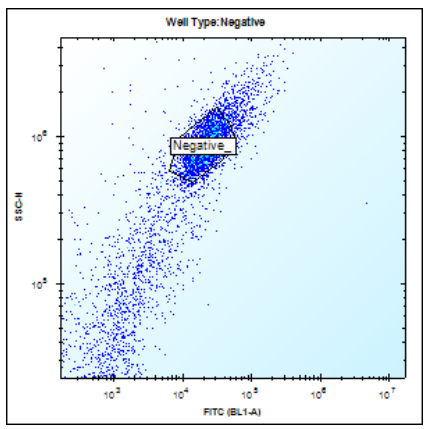


Range: 51 to 2,063

E.



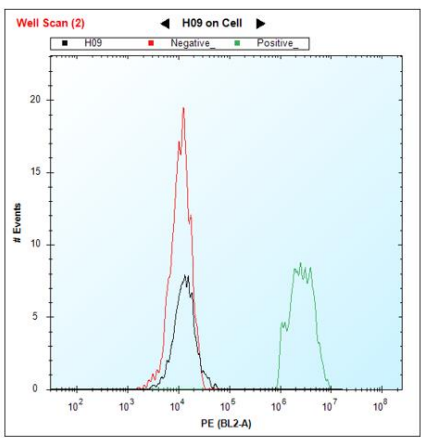


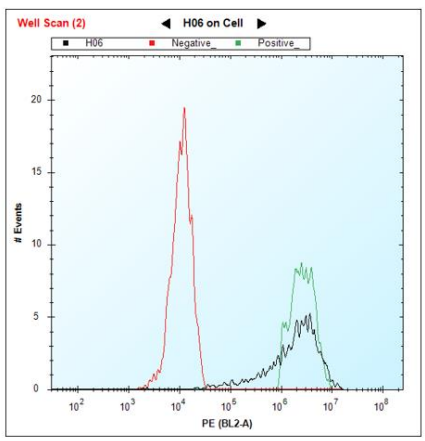


F.

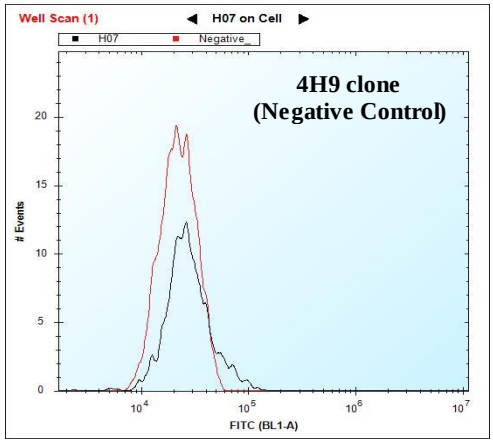
Negative Cell Line (NIH/3T3)

Positive Cell Line (NIH/3T3-VISTA #13)





Negative Control Positive Control Sample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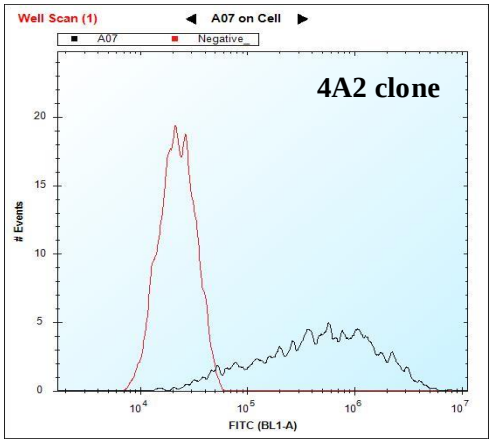
H07 on Cell
H07
Negative_
4H9 clone
(Negative Control)
Events
FITC (BL1-A)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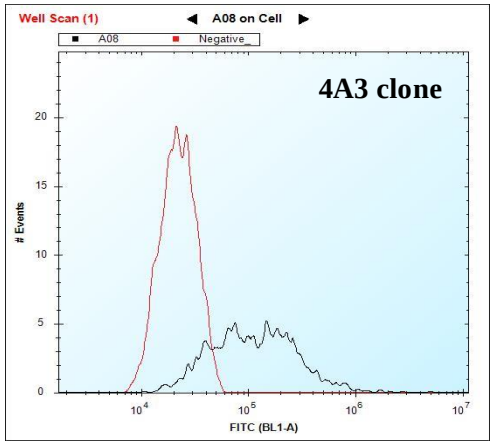
A07
Negative_
4A2 clone
Events
FITC (BL1-A)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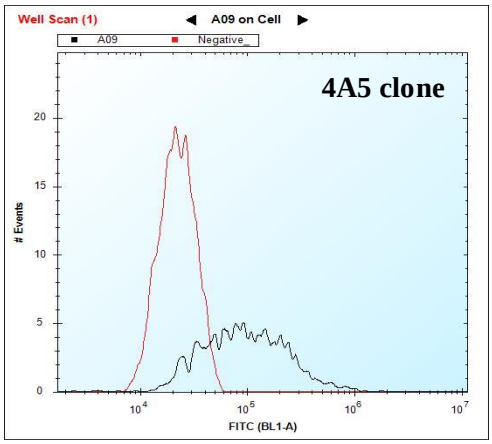
A08
Negative_
4A3 clone
Events
FITC (BL1-A)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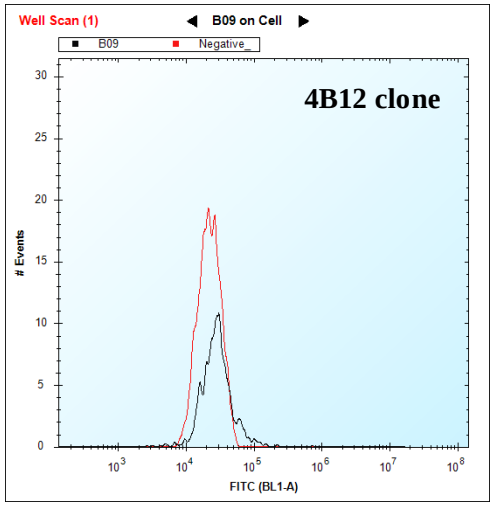
A09 on Cell
A09
Negative_
4A5 clone
Events
FITC (BL1-A)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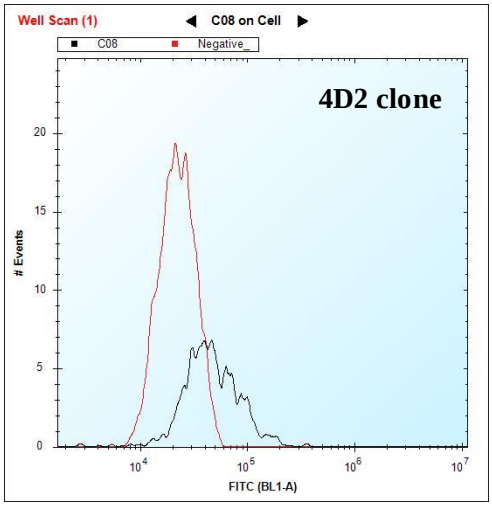
B09
Negative_
4B12 clone
Events
FITC (BL1-A)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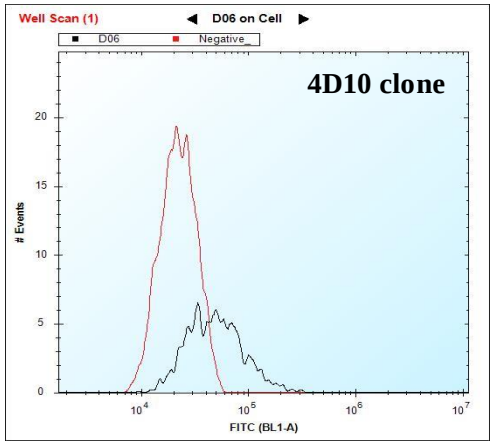
C08 on Cell
C08
Negative_
4D2 clone
Events
FITC (BL1-A)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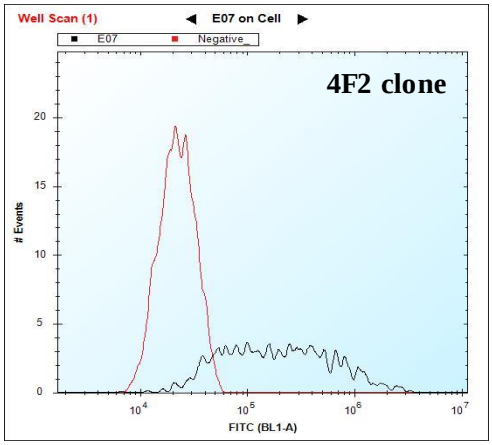
D06
Negative
4D10 clone
Events
FITC (BL1-A)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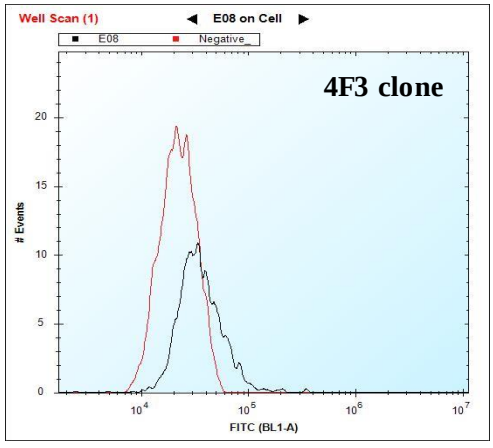
E07
Negative
4F2 clone
Events
FITC (BL1-A)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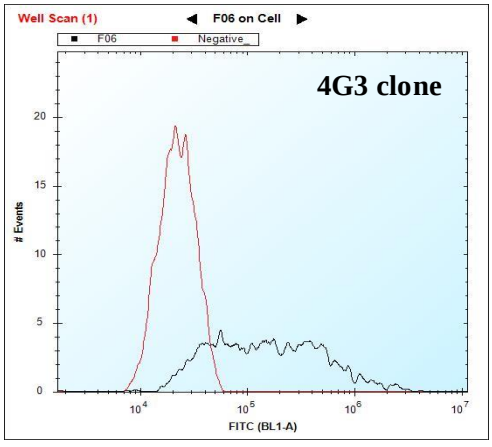
E08 on Cell
E08
Negative
4F3 clone
Events
FITC (BL1-A)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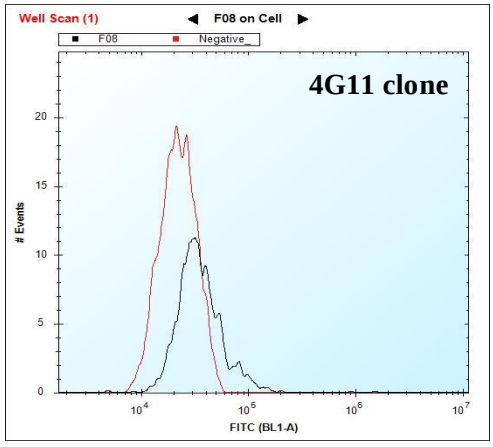
F06
Negative
4G3 clone
Events
FITC (BL1-A)


Well Scan (1)
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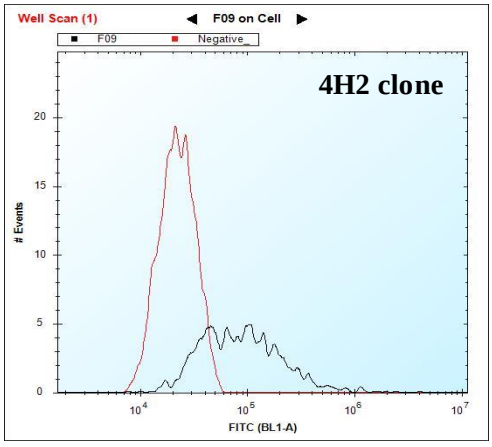
F08
Negative
4G11 clone
Events
FITC (BL1-A)


Well Scan (1)
F09 on Cell
F09
Negative
4H2 clone
Events
FITC (BL1-A)

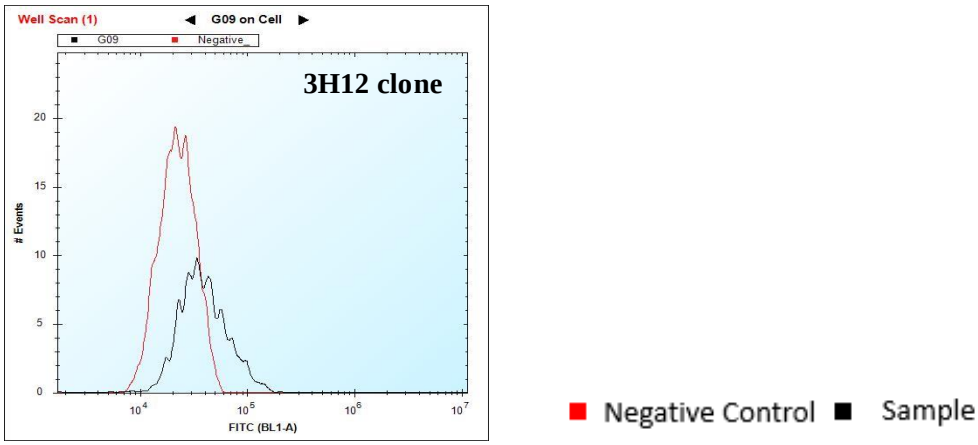


Figure 8. Finding scFv Candidates by using IntelliCyt® iQue Screener.

(A) It is a plate design figure for competitive high-throughput flow cytometry experiment using IntelliCyt® iQue screener. NIH/3T3 cells that do not express human VISTA were used as negative cell lines, and NIH/3T3-VISTA #13 cells expressing human VISTA were used as positive cell lines. We used 34 clones selected by phage display and 4H9 clone which does not show binding affinity to VISTA as a negative sample. As a positive control, PE conjugated antibody capable of detecting human VISTA was used. Isotype control analysis was performed using HA-FITC and PE-conjugated control antibodies, respectively. **(B)** This is a schematic design figure in the analysis program. The sample area to be analyzed is shown with blue, positive control group is shown with green, and negative control group is shown with red.

(C) It is a flow chart showing 'Time-All events'. An air gap is injected between the samples. This confirms no cross-contamination.

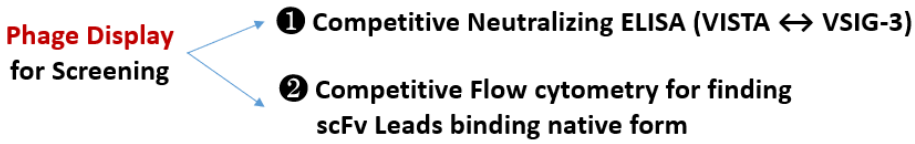
(D) The above figure shows the total number of cells used in the analysis, and the figure below shows the number of cells analyzed in the gating region.

(E) This is the dot plot showing the whole cell. It is a flow chart that gating only the positive region and gating only the negative region, respectively.

(F) This is the result of comparing the stable cell line expressing human VISTA actually expresses human VISTA in comparison with the negative sample.

(G) Among the 34 clones, we were able to select 9 clones that bind better to VISTA through competition with VSIG-3, the putative ligand of VISTA. These are detected by peak shift.

A.

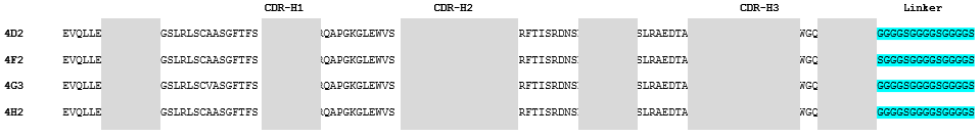


	VISTA
Competition* O, Cell Binding** O	4D2, 4F2, 4G3, 4H2
Competition O, Cell Binding X	4B12, 4F3, 4G11
Competition X, Cell Binding O	4A2, 4A3, 4A5, 4D10, 3H12

* : Neutralizing ability against VISTA ↔ VSIG interaction
** : Binding ability against VISTA ↔ VSIG interaction

B.

<Heavy Chain>



<Light Chain>

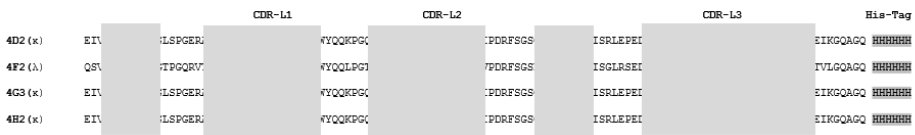
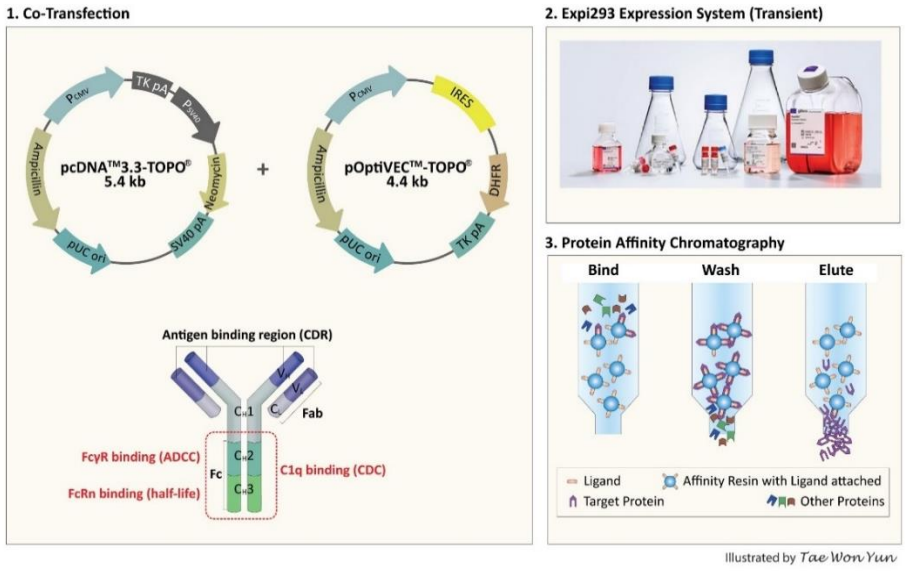


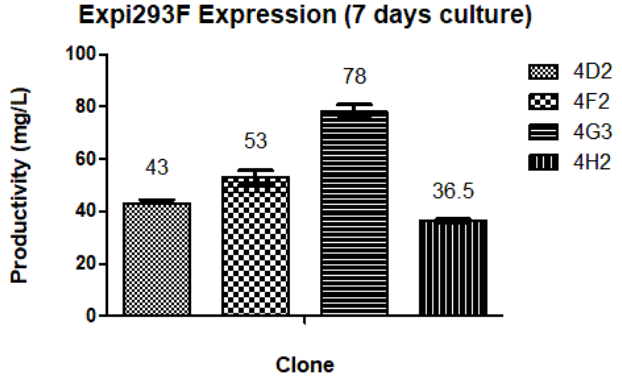
Figure 9. Identification of CDR Regions of Selected 4 scFv Clones.

(A) The results obtained by competitive ELISA and high-throughput flow cytometry in 34 clones obtained from phage display are summarized in the table. 'Competition' means clones capable of inhibiting the interaction between VISTA and VSIG-3 (a putative ligand of VISTA in this study). The meaning of 'cell binding' means clones that inhibit the interaction between VISTA and VSIG-3 and more closely bind to VISTA. Finally, 4 clones, 4D2, 4F2, 4G3, and 4H2, were selected. **(B)** This is the sequencing analysis results for four scFv clones. It was confirmed that the CDR regions were different from each other, and that there was no variation in the frame region sequences (some sequences are not disclosed because of security).

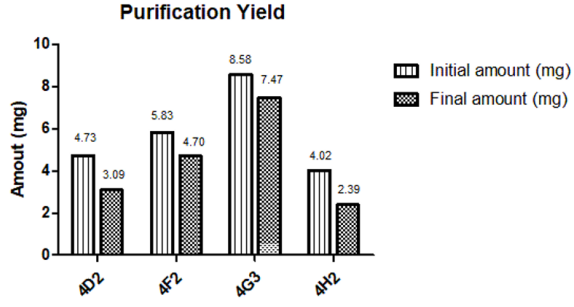
A.



B.

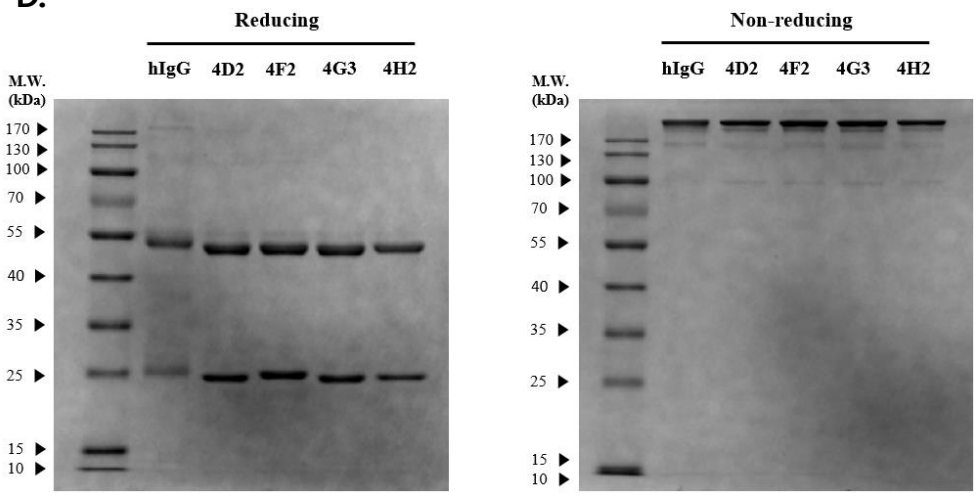


C.



Target	Clone	Initial amount (mg)	Final amount (mg)	Yield (%)	Loss amount (mg)	Loss (%)
VISTA	4D2	4.73	3.09	*65.33*	1.64	34.67
	4F2	5.83	4.70	*80.62*	1.13	19.38
	4G3	8.58	7.47	*87.06*	1.11	12.94
	4H2	4.02	2.39	*59.45*	1.63	40.55

D.


Reducing
hIgG
4D2
4F2
4G3
4H2
M.W. (kDa)
170
130
100
70
55
40
35
25
15
10
Non-reducing
hIgG
4D2
4F2
4G3
4H2
M.W. (kDa)
170
130
100
70
55
40
35
25
15
10

E.

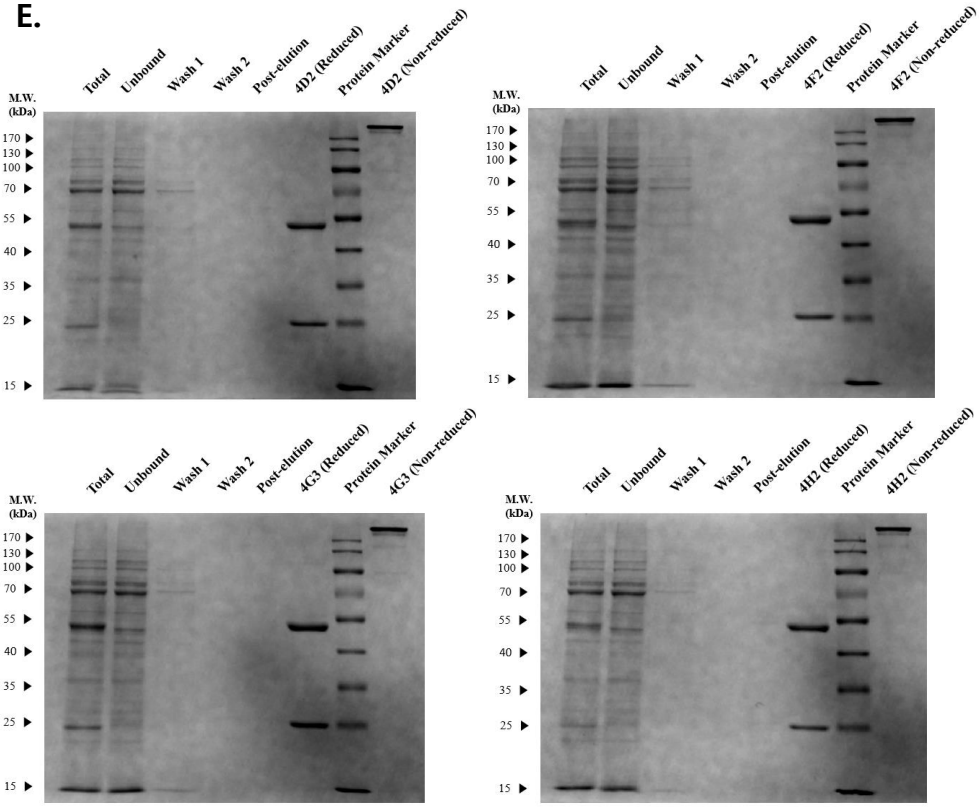
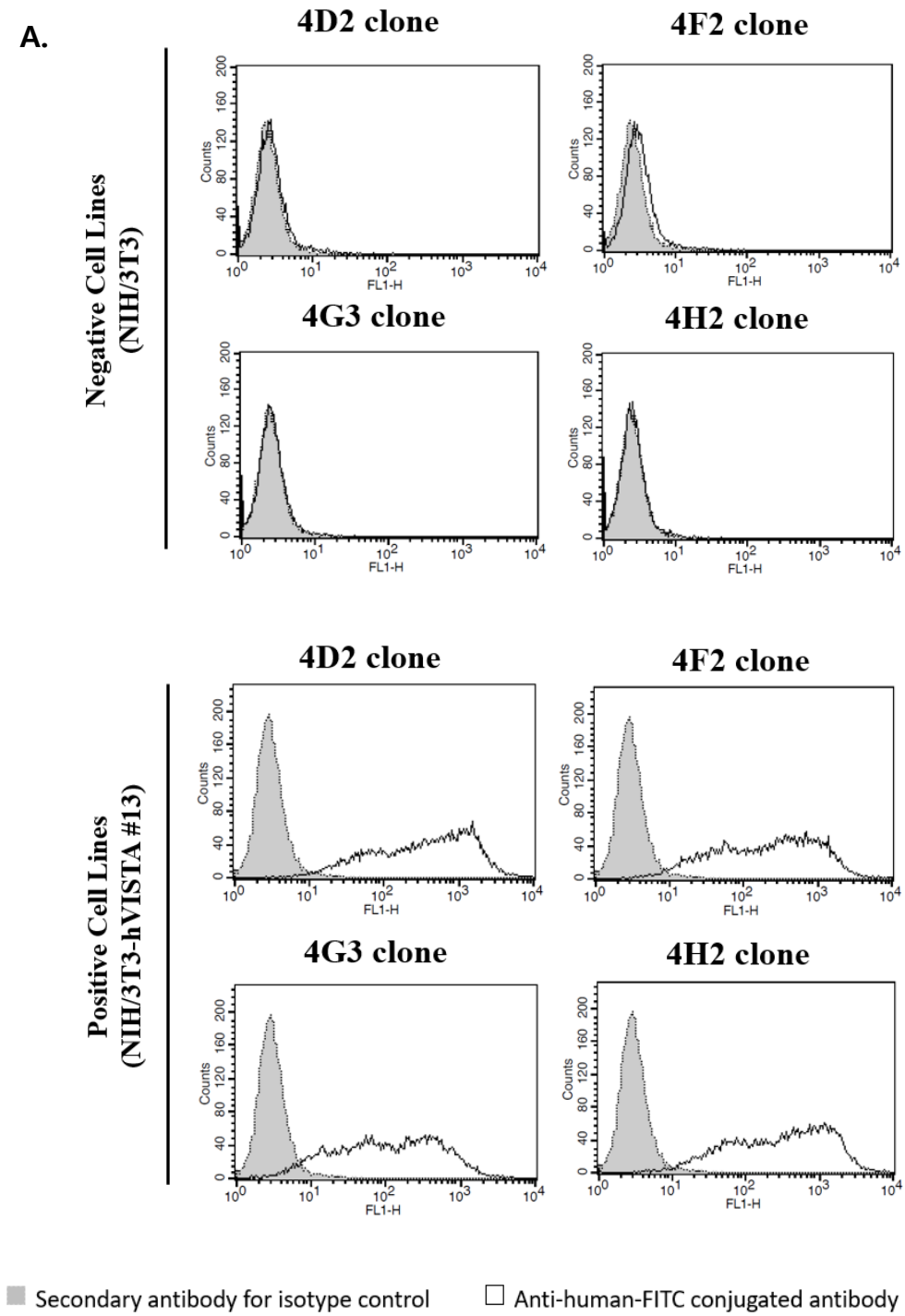

Total
Unbound
Wash 1
Wash 2
Post-elution
4D2 (Reduced)
Protein Marker
4D2 (Non-reduced)
M.W. (kDa)
170
130
100
70
55
40
35
25
15
Total
Unbound
Wash 1
Wash 2
Post-elution
4F2 (Reduced)
Protein Marker
4F2 (Non-reduced)
M.W. (kDa)
170
130
100
70
55
40
35
25
15
Total
Unbound
Wash 1
Wash 2
Post-elution
4G3 (Reduced)
Protein Marker
4G3 (Non-reduced)
M.W. (kDa)
170
130
100
70
55
40
35
25
15
Total
Unbound
Wash 1
Wash 2
Post-elution
4H2 (Reduced)
Protein Marker
4H2 (Non-reduced)
M.W. (kDa)
170
130
100
70
55
40
35
25
15

Figure 10. Whole IgG conversion, Expression and Purification.

(A) This is the schematic figure of whole IgG conversion, expression, and purification. The vector containing the constant region of the heavy chain and the light chain respectively was used for the cloning, the Expi293™ system was used for the transient expression, and the protein A beads were used for the purification by affinity chromatography. **(B)** These are the production results of the four clones for 7 days. The maximum production rate was 78 mg/L. **(C)** These are the purification efficiencies of the four clones. The maximum purification efficiency was 87%. **(D)** The results of SDS-PAGE gel detection confirmed that the antibodies were eluted properly from the eluted solution obtained after purification. As a control antibody, the size of bands was compared using human IgG. It was confirmed that the 50 kDa heavy chain and the 25 kDa light chain were normally formed in all four clones. **(E)** Each monoclonal antibody clone was purified and SDS-PAGE gel detection was performed to check for the loss of antibodies at each purification step. It was confirmed that the antibodies were specifically eluted only from the elution step in all four clones.

A.


4D2 clone
4F2 clone
4G3 clone
4H2 clone
Negative Cell Lines (NIH/3T3)
Positive Cell Lines (NIH/3T3-hVISTA #13)
Counts
FL1-H
Secondary antibody for isotype control
Anti-human-FITC conjugated antibody

B.

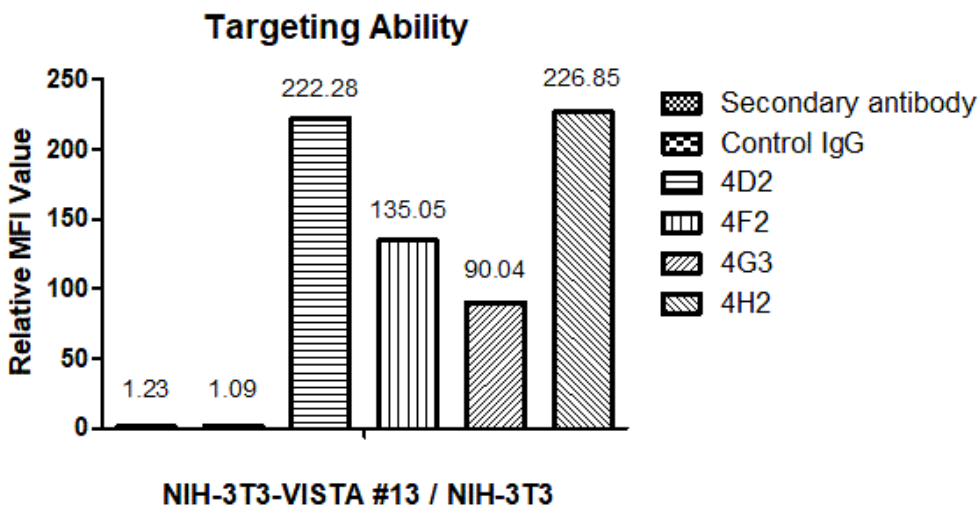


Figure 11. Targeting Ability Test by Flow Cytometry.

(A) Experiments were conducted to examine the ability of each monoclonal antibody clone to bind to native form human VISTA by flow cytometry. NIH/3T3 cells were used as negative cell lines, and NIH/3T3-VISTA #13 cells were used as positive cell lines. **(B)** The ratio between the MFI values in the negative cell line and the MFI values in the positive cell line was obtained and the relative MFI value was calculated to show the targeting ability of each clone as a numerical value. The secondary antibody group used as the isotype control group of the FACS antibody and the human IgG group used as the negative control group showed a relative MFI value of 1 indicating no significant binding ability.

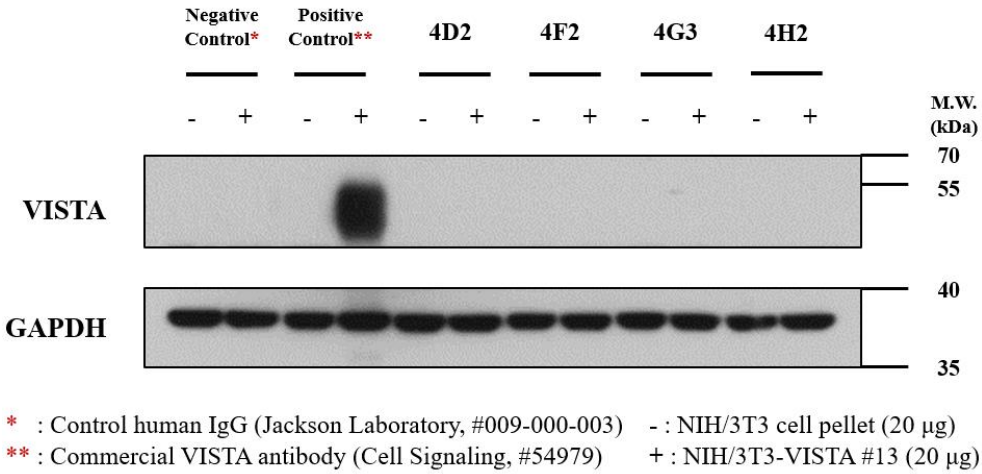
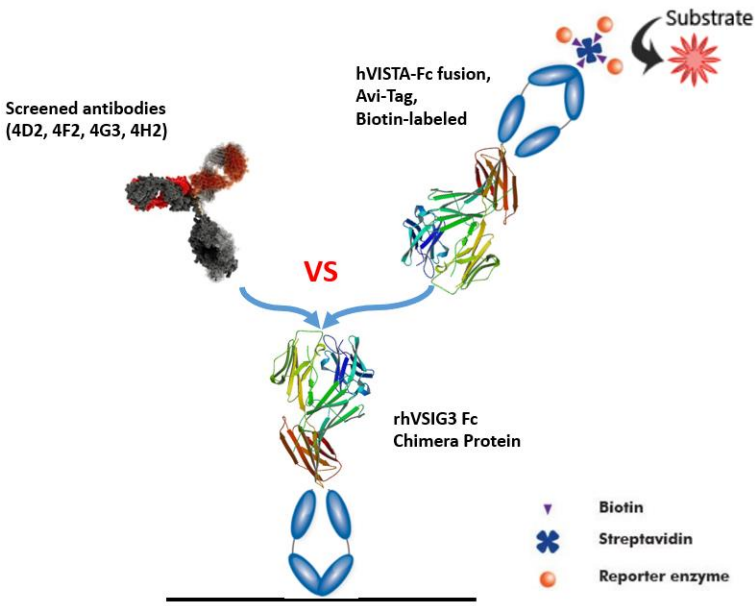


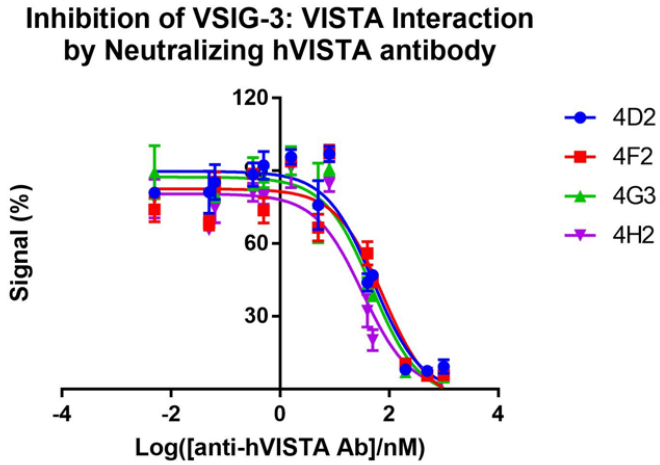
Figure 12. Western Blot Assay.

Western blot analysis was performed to determine whether the four antibody clones bind to non-native form human VISTA or not. NIH/3T3 cells were used as negative cell lines, and NIH/3T3-VISTA #13 cells were used as positive cell lines. Human IgG was used as a negative control antibody and commercial antibody was used as a positive control antibody for western blot detection. As a result, human IgG showed no binding ability and commercial antibody recognized non-native form human VISTA. The four antibodies did not detect band for VISTA and confirmed that they did not have binding affinity for non-native form human VISTA.

A.



B.



Anti-hVISTA Ab	4D2	4F2	4G3	4H2
$LogIC_{50}$	1.711	1.893	1.683	1.513
IC_{50} (nM)	51.37	78.14	48.14	32.55

Figure 13. Competitive Inhibition ELISA for Finding IC_{50}.

(A) This is a schematic figure for a competitive inhibition ELISA. The abilities to interfere with the interaction between VISTA and VSIG-3 were identified by IC_{50} by treating the four antibodies with 13 concentration ranges. **(B)** Signal inhibition curves of VISTA-VSIG-3 were obtained. The IC_{50} values of each clone are shown in the table.

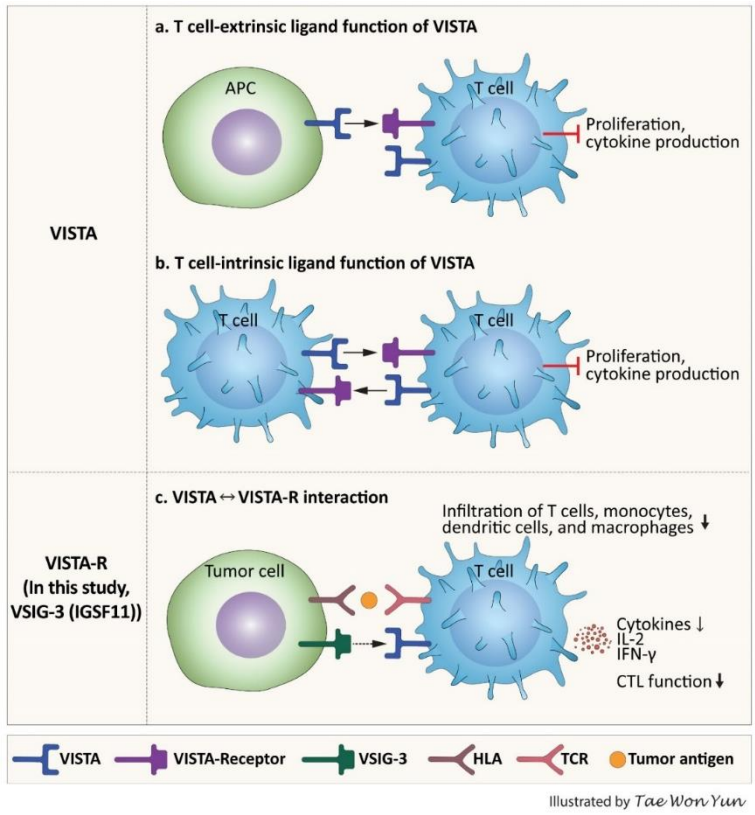
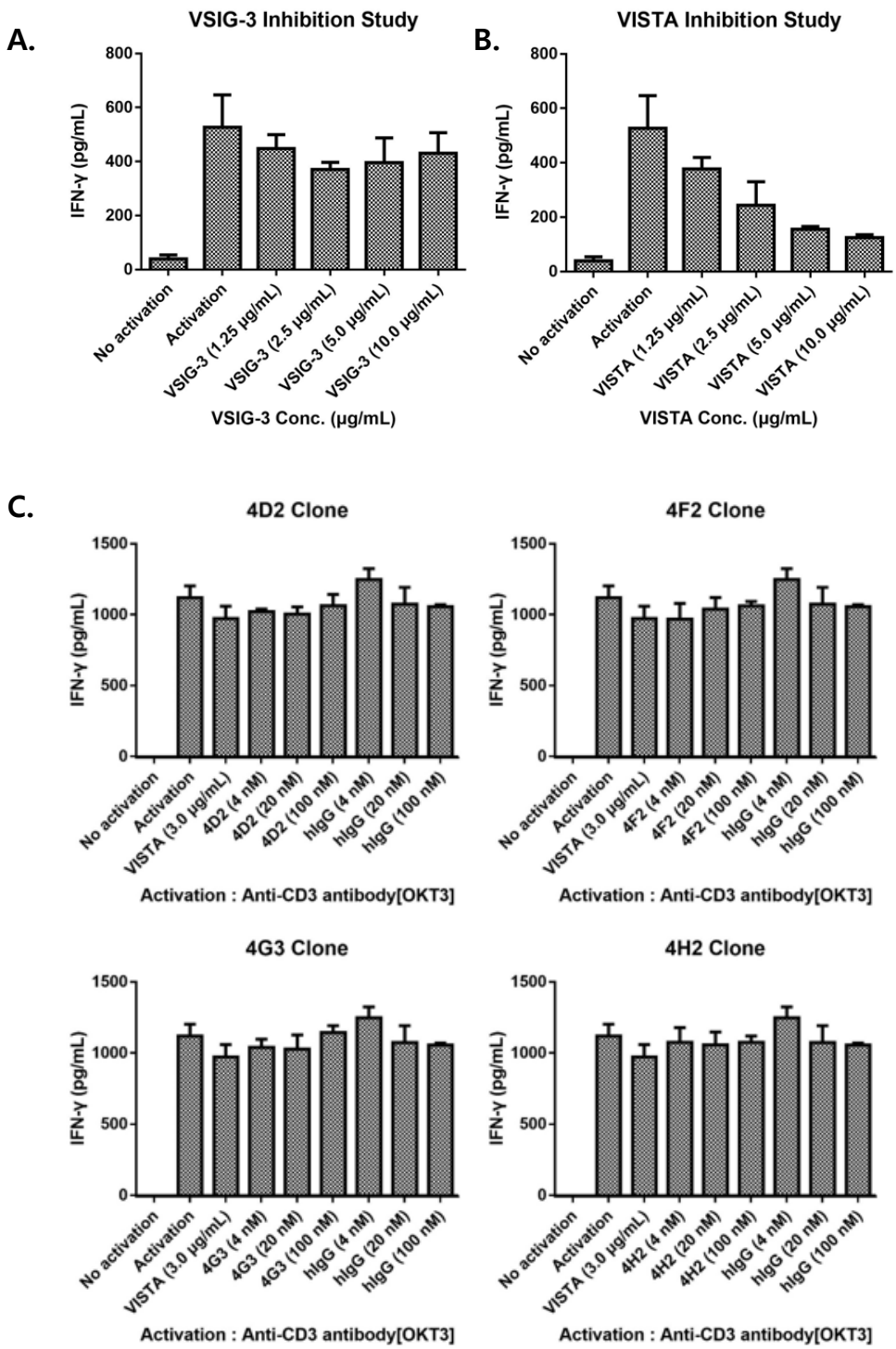


Figure 14. The Schematic Design of VISTA acting as a Ligand or a Receptor.

The schematic figure shows when VISTA acts as a ligand or a receptor to regulate T cell activation. 'a. T cell-extrinsic ligand function of VISTA' indicates that VISTA is present in APC/tumor cells and functions as a ligand. Thus, T cell inhibition signal is transmitted by binding to the unknown receptor of T cell surface. 'b. T cell-intrinsic ligand function of VISTA' is a model in which T-cell has both VISTA and unknown receptor and signals are transmitted between T cells. More research is needed on the binding partner and signal pathway of VISTA.


A.
VSIG-3 Inhibition Study
IFN-γ (pg/mL)
800
600
400
200
0
No activation
Activation
VSIG-3 (1.25 μg/mL)
VSIG-3 (2.5 μg/mL)
VSIG-3 (5.0 μg/mL)
VSIG-3 (10.0 μg/mL)
VSIG-3 Conc. (μg/mL)
B.
VISTA Inhibition Study
IFN-γ (pg/mL)
800
600
400
200
0
No activation
Activation
VISTA (1.25 μg/mL)
VISTA (2.5 μg/mL)
VISTA (5.0 μg/mL)
VISTA (10.0 μg/mL)
VISTA Conc. (μg/mL)
C.
4D2 Clone
IFN-γ (pg/mL)
1500
1000
500
0
No activation
Activation
VISTA (3.0 μg/mL)
4D2 (4 nM)
4D2 (20 nM)
4D2 (100 nM)
hIgG (4 nM)
hIgG (20 nM)
hIgG (100 nM)
Activation : Anti-CD3 antibody[OKT3]
4F2 Clone
IFN-γ (pg/mL)
1500
1000
500
0
No activation
Activation
VISTA (3.0 μg/mL)
4F2 (4 nM)
4F2 (20 nM)
4F2 (100 nM)
hIgG (4 nM)
hIgG (20 nM)
hIgG (100 nM)
Activation : Anti-CD3 antibody[OKT3]
4G3 Clone
IFN-γ (pg/mL)
1500
1000
500
0
No activation
Activation
VISTA (3.0 μg/mL)
4G3 (4 nM)
4G3 (20 nM)
4G3 (100 nM)
hIgG (4 nM)
hIgG (20 nM)
hIgG (100 nM)
Activation : Anti-CD3 antibody[OKT3]
4H2 Clone
IFN-γ (pg/mL)
1500
1000
500
0
No activation
Activation
VISTA (3.0 μg/mL)
4H2 (4 nM)
4H2 (20 nM)
4H2 (100 nM)
hIgG (4 nM)
hIgG (20 nM)
hIgG (100 nM)
Activation : Anti-CD3 antibody[OKT3]

D.

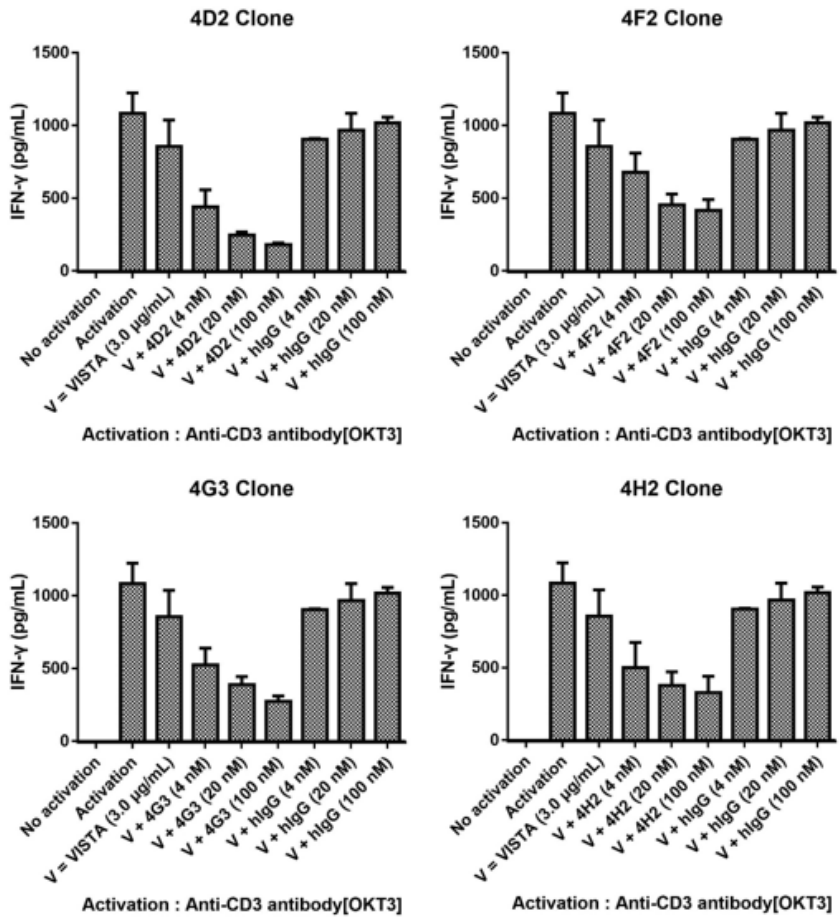


Figure 15. *In vitro* CD4⁺ T cell Assay and Quantitative Human IFN-γ ELISA.

(A) This is the result of *in vitro* CD4⁺ T cell inhibition assay performed with VSIG-3 as a putative binding partner of VISTA. About 30% inhibition was observed, and the degree of inhibition was patterned to saturation at 2.5 μg/mL of VSIG-3. **(B)** This is the result of *in vitro* CD4⁺ T cell inhibition assay when VISTA was provided as a T cell inhibition signal. As a result, a more dramatic T cell inhibition pattern was

observed than when VSIG-3 was treated. **(C)** These are the results of experiments whether four antibody clones alone can induce any T cell inhibition status in the $CD4^+$ T cell activation status or not. The T cell activation signal was provided at 2.5 μg/mL of anti-CD3 antibody [OKT3] and 3.0 μg/mL of VISTA for inhibition signal. Antibodies were treated at 4, 20, and 100 nM. As a negative control group, 4, 20, and 100 nM treatments of human IgG were used. As a result, it was confirmed that antibodies alone did not affect T cell activation and inhibition status. **(D)** These are the results of experiments to confirm the efficacies of the antibodies in the presence of T cell inhibition signal. The T cell activation signal was provided at 2.5 μg/mL of anti-CD3 antibody [OKT3] and 3.0 μg/mL of VISTA for inhibition signal. Antibodies were treated at 4, 20, and 100 nM. As a negative control group, 4, 20, and 100 nM treatments of human IgG were used. As a result, it was confirmed that $CD4^+$ T cell inhibition was further enhanced by increasing the concentration of the antibodies to be treated when the inhibition signal was provided. This pattern was observed in all four clones and no change of inhibition status was observed in the control human IgG treated group.

The $CD4^+$ T cells used in the experiments were isolated from human PBMC separated by magnetic-activated cell sorting, and IFN-γ, which is known as a typical T cell activation marker, was selected as the cytokine to check the occurrence of T cell inhibition. The concentration of IFN-γ in the cell soup obtained by culturing $CD4^+$ T cells was quantitated by ELISA.

DISCUSSION

In this study, monoclonal antibodies with an immunomodulatory effect were designed by targeting VISTA, a kind of human immune-checkpoint molecule, and their characteristics were verified. The specific antibodies of human VISTA were obtained using phage display technique and competitive ELISA and high-throughput flow cytometry were used to select clones with better binding ability to VISTA than ligand.

Immunotherapy is currently attracting attention as a next-generation therapy that can reduce the side effects of anticancer and autoimmune disease treatments and improve treatment efficiency (1-5). Immune-checkpoint molecules play a key role in regulating the immune response and are associated with immunotherapy (2, 14, 15). In 2011, Yervoy® (Therapeutic antibody targeting CTLA-4) from Bristol-Myers Squibb (BMS) was approved by the US Food and Drug Administration (FDA), creating a market for drugs targeting immune-checkpoint molecules (36, 42). After that, Opdivo® and Keytruda® (targeting PD-1) were approved in 2014, and the market will gradually expand, creating a $10 billion market in 2017 (36, 42). However, up to now, commercially available drugs targeting immune-checkpoint molecules are immune-checkpoint inhibitors that are all aimed at anticancer effects (2, 16-20). It is also associated with diseases such as autoimmune diseases and (acute) GVHD, but drugs targeting these diseases are not yet on the market and are

undergoing much research (4, 17). Immune-checkpoint molecules have many relationships in both activating immunotherapy and suppressive immunotherapy but focused more on activating immunotherapy nowadays. In this study, we decided to select an immune-checkpoint molecule as a drug target with the possibilities of immunotherapy in both two aspects, which has not been developed much yet. VISTA is a protein that meets this goal.

VISTA is an inhibitory checkpoint molecule that regulates T cell activation/inhibition status and immune response (5, 23-25, 30). It is mainly expressed in myeloid cell lineages, and it is also expressed in T cells but not in B cells (5, 24, 25, 30). In the T cell, VISTA is known to have dual roles of ligand and receptor (23, 24, 31). Its ligand and signal pathways are not yet known. VISTA is known to be non-overlapping pathways with PD-1/PD-L1, and CTLA-4. Since VISTA is an inhibitory checkpoint molecule, many studies have been conducted in terms of checkpoint blockade and stimulation. However, there is no drug that has been approved by the FDA (7).

The Checkpoint blockade view is associated with activating immunotherapy (2-4, 6). Inhibitory checkpoint molecules block the immune evasion of cancer cells to elicit the anticancer effect. Currently, there is a phase I clinical drug in Johnson & Johnson (JNJ) (7). Checkpoint stimulation is associated with suppressive immunotherapy (2-4, 6). In contrast to checkpoint blockade, there is no drugs in the clinical trials, but active research is underway to confirm that VISTA is involved in various inflammatory diseases and autoimmune diseases such as lupus, autoimmune

encephalomyelitis, acute graft-versus-host disease and psoriasis (5, 25, 28, 35, 43). We have developed monoclonal antibodies targeting human VISTA and examined the immunomodulatory effect in this study.

We screened the scFv clones using the extracellular domain of the human VISTA protein. A number of scFv clones with high signal to the human VISTA antigen were generated. Total 34 clones were selected by sequencing analysis, replacing clones with the same sequence. These 34 clones were competed with VSIG-3, a putative ligand of VISTA. Through competitive ELISA and high-throughput flow cytometry experiments, 4 clones (4D2, 4F2, 4G3, and 4H2) were selected. These are bound better to the recombinant human VISTA antigen and the native human VISTA form than VSIG-3, a putative ligand of VISTA. The selected four scFv clones were converted to fully human IgG form and examined for binding affinity. Through flow cytometry, we observed whether it binds to the native form human VISTA or not. Through western blot, we observed whether it binds to the non-native form human VISTA or not. As a result, the native form was recognized by four clones of monoclonal antibodies but not the non-native form. In scFv form, a signal was detected in the recombinant human VISTA protein, but not after conversion. We considered the following experimental reasons. From scFv to human IgG, constant regions of the heavy and the light chain were inserted, respectively. This implies that the ability to recognize the antigen has changed. Although there is no change in the CDR region, it is considered that insertion of the constant regions influenced the overall structure of the antibody. After binding specificity has been confirmed, we

tested for the ability to inhibit VISTA and VSIG-3 interactions. The IC_{50} values, which is an indicator of inhibition of interaction between VISTA and VSIG-3 in 4 clones, were measured from 32.55 to 78.14 nM.

Since VISTA is a protein associated with T cell activation/inhibition, we examined how T cell inhibition was occurred through *in vitro* T cell assay and the effect of four antibody clones on T cell status (24, 25). T cells were selected from among many VISTA-expressing cell lineages and $CD4^+$ T cells were isolated from human PBMC. IFN-γ, which is a T cell development and activation marker of T_H1 cell and macrophage was measured by quantitative ELISA (24, 25). First, because VISTA regulates T cell activation, we examined what kind of paradigm is working. The counterpart of VISTA has not been clearly identified (5). Therefore, the experiments were carried out assuming that VISTA acts as both a ligand or a receptor (21, 24, 25, 31, 35, 38, 44, 45). When VISTA acts as a ligand, it binds to the unknown receptor of T cell to transmit an inhibition signal. When acting as a receptor, it binds to the unknown ligand of APC or tumor cell to transmit a T cell inhibition signal. As a result, T cell proliferation and various cytokine production are inhibited, resulting in decreasing in overall immune functions. We conducted this study assuming the unknown ligand of VISTA as VSIG-3 (32, 33). As a result, T cell inhibition was observed in VSIG-3 but the inhibition was within the range of not more than 30%. When VSIG-3 concentration reached 2.5 μg/mL, T cell inhibition saturation was observed. This is the case when VSIG-3 is an unknown ligand of VISTA and VISTA is a receptor of VSIG-3 and transmits a T cell inhibition signal. However, when

VISTA acted as a ligand, a larger inhibition pattern was observed. T cell inhibition was further enhanced as the concentration of VISTA was increased. These results suggest that VISTA acts as a ligand rather than a receptor to provide a greater inhibitory signal to T cells (24, 25, 31). From these experiments, the T cell inhibition signal to be used for the efficacy evaluation of the antibodies was selected as VISTA 3.0 μg/mL. First, we examined the efficacy of monoclonal antibodies in T cell activation status. As a result, four clones of monoclonal antibodies did not induce any activation or inhibition status in T cells. This means that the antibodies alone do not affect the T cell effector function. We then examined the efficacy of four clones of monoclonal antibodies when T cell activation status was affected by the VISTA 3.0 μg/mL inhibition signal. As a result, T cell inhibition was further enhanced with the increase of antibody concentration in the presence of inhibition signal, unlike the cases of treatment of activation signal alone. The same inhibition patterns were observed in all four clones. Based on the results of two experiments, the monoclonal antibodies we produced do not show any efficacy alone in the T cell activation status, but it seems to be associated with VISTA-mediated inhibition signaling to inhibit T cell activation status.

The functions and ligands of VISTA are needed more research (5). We have made one hypothesis to demonstrate the *in vitro* $CD4^+$ T cell assay results. VISTA was found to elicit inhibition status when acting as a ligand rather than a receptor in T cell. This means that the VISTA which acts as a ligand meets the unknown receptor of T cell and induces T cell inhibition. Because the ligand role of VISTA is more

important, it seems that monoclonal antibodies that bind to human VISTA in activated T cell does not lead to significant changes in immune function alone. Thereafter, we mimicked the environment in which the inhibition signal is transmitted and VISTA acts as a ligand. The efficacy of the antibodies in this experiment can be explained as follows. Four anti-human VISTA antibodies (4D2, 4F2, 4G3, and 4H2) can multimerize VISTA ligand and transduce the T cell inhibition signal by interacting with the receptor on the T cell surface. Overall, the monoclonal antibodies we produced have a VISTA-dependent immunosuppressive effect. The schematic figure for this hypothesis is shown in [Figure 16].

In conclusion, we have constructed four clones of monoclonal antibodies that specifically recognize a protein called VISTA, one of the human immune-checkpoint molecules. In addition, VSIG-3 was identified as a putative ligand for VISTA, which is currently being studied by other researchers (32, 33). It was confirmed that the antibodies we produced had high productivity and purification efficiency and high industrialization potential. It also binds specifically to the target and is also capable of blocking the interaction between VISTA and VSIG-3, the putative ligand of VISTA. Therefore, we confirmed that the anti-human VISTA antibodies we produced in this study exhibits a specific response to VISTA protein and a high biological activity. It implies that these four antibodies have possibilities of future treatment of many immune-related diseases.

For further study, we are planning to conduct experiments on two aspects. One is the identification of the function of VISTA as checkpoint molecule and the other

is the aspect of monoclonal antibody production. First, we will investigate the T cell inhibition ability of VISTA in comparison with other inhibitory checkpoint molecules, CTLA-4 and PD-1, in terms of functional identification of VISTA. In addition, since the counterpart molecule of VISTA is not clearly identified, we are going to do the ligand/signal pathway identification work. Second, in the aspect of monoclonal antibody productions, 5 clones (4A2, 4A3, 4A5, 4D10 and 3H12) showing high signal in competitive high-throughput flow cytometry but not in competitive ELISA will be converted into fully human IgG form to evaluate the efficacy of these antibodies. At last, to confirm the multimerization effect, we will use a scFv/Fab form or secondary antibodies capable of multimerizing the primary antibodies.

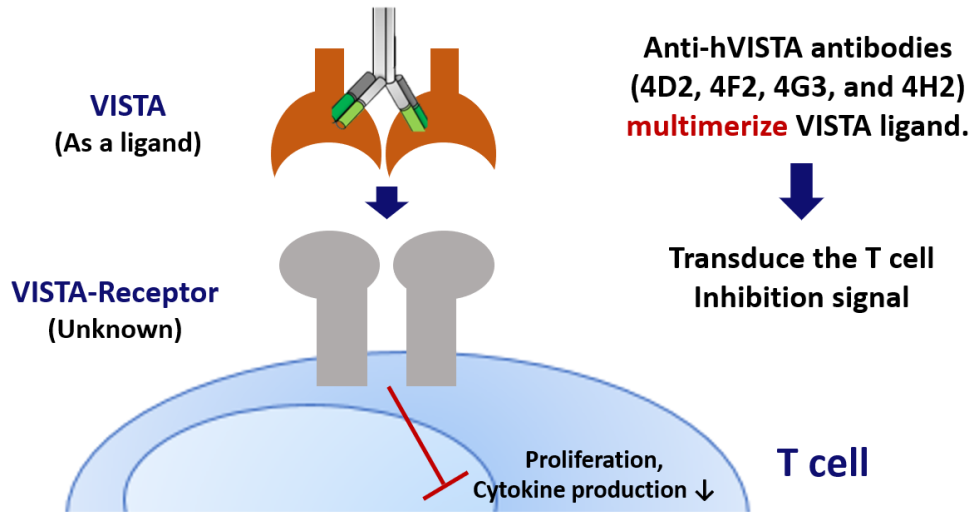


Figure 16. The Schematic Design of VISTA-dependent Immunosuppressive Effect Hypothesis.

This figure is explaining the hypothesis of experimental results. We generated anti-human VISTA antibodies (4D2, 4F2, 4G3, and 4H2 clone). These four clones further enhanced T cell inhibition. These results demonstrated that four clones of monoclonal antibodies have a VISTA-dependent immunosuppressive effect. These suggest the possibility of future treatment of immune-related diseases.

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요약(국문초록)

면역치료는 기존의 항암 및 자가면역질환 치료와 달리 환자의 면역 체계를 이용한다. 부작용이 덜하고 기대 생존율이 보다 높은 것이 특징으로 차세대 치료법으로 큰 주목을 받고 있다. 면역치료는 면역 세포의 기능을 증진시켜 항암 치료를 시도하는 활성 면역치료와 과도한 면역 세포의 기능을 억제하여 자가 면역 질환, 장기 이식 거부 반응의 치료에 사용하는 억제 면역치료로 나눌 수 있다. 이와 연관된 분자에는 면역계를 조절하는 면역관문분자를 꼽을 수 있다. 그중 대표적인 타깃으로 널리 알려진 PD-1/PD-L1, CTLA-4 외에도 VISTA가 있는데 이들과 마찬가지로 T세포 보조억제분자이다. 이 분자는 T세포의 신호가 과하게 작용할 때 체내 면역계가 손상을 받지 않게끔 활성 신호를 제어하게 된다. 이 분자들은 면역 세포의 기능과 밀접한 관련이 있기에 이들을 통해 과도한 면역 활성을 억제할 수 있다. 항암 치료에 관한 연구와 더불어 자가 면역 질환 및 장기이식 거부반응의 치료에도 사용할 수 있다는 연구가 활발히 진행되고 있다. 억제 면역관문분자를 더욱 자극하여 억제 면역치료법을 유도하는 원리이다. 따라서 이러한 특징을 보이는 VISTA를 면역 관련 질환의 치료를 위한 타깃으로

선정하고, 약물의 형태를 단일 클론 항체로 선정하였다. 그 후, 스크리닝을 통해 얻은 후보물질이 억제 면역관문분자 차폐와 자극 중 어떠한 효능을 보이는지 살펴보고 특성을 검증하는 것이 이 연구의 주된 목적이다.

본 연구는 파지 디스플레이 기법으로 인간 합성 단일 사슬 항체조각 라이브러리에서 VISTA의 세포 바깥 부분에 결합하는 후보들을 일차적으로 선별하였다. 그 후 경쟁적 억제 효소 결합 면역 침강 분석법 및 유세포 분석을 통해 리간드 대신 항원에 선택적으로 결합하는 능력이 큰 후보물질을 최종 각 4 개씩 발굴하였다. 그리고 제작한 항체의 특성 검증을 위한 실험을 진행하였다. 일시적 발현 시스템에서 항체를 생산하고 친화성 크로마토그래피법으로 정제하였다. 항원 결합 능력을 살펴보기 위해 유세포 분석 및 웨스턴 블로팅으로 확인하였다. 경쟁적 억제 효소 결합 면역 침강 분석법을 통해서는 리간드와 경쟁하며 이들의 신호전달을 얼마나 차단하는지 IC_{50} 를 구해 각 클론 간에 비교하였다. 그 결과, 개발한 항체가 생산성과 정제 효율이 좋아 산업화의 가능성이 높음을 확인하였다. 또, 타깃에 특이적으로 결합하며 리간드와의 경쟁에서 우위를 선점하는 신호 차단 능력도 우수하였다. 체외 T세포 배양을 통한 효능 평가 실험은 활성 T세포의 기능이 해당 리간드와 항체의 존재 하에 활성 상태가 억제되는지, 유지되는지를 분비되는

인터페론 감마의 농도를 정량 효소 결합 면역 침강 분석법으로 비교하였다.

따라서, 본 연구에서 제작된 단일클론항체가 VISTA에 대해 특이적인 반응을 보이고, 실제 T세포와의 배양에서 VISTA-의존적 면역억제효과를 보이므로 억제 면역치료제로서 가능성이 있음을 확인하였다. 이를 통해 과도하게 활성화된 T세포를 억제하여 면역계의 기능이 정상 범위보다 크게 올라간 자가면역질환 및 장기이식거부 반응 치료제로 이용될 수 있음을 제시한다. 추가적인 동물실험을 통해 효능 평가 및 투여 용량을 결정하여 향후 항체치료제로의 가능성을 평가하고자 한다.

주요어 : 면역치료, 면역 관문 분자, VISTA, 항체치료제, 억제 면역치료, 자가면역질환

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