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Original article

Construction and stable gene expression of AGR2xPD1 bi-specific antibody that enhances attachment between *T*-Cells and lung tumor cells, suppress tumor cell migration and promoting CD8 expression in cytotoxic *T*-cells

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ABSTRACT

There has been a substantial and consistent rise in the number of clinical trials to develop advanced and potent bispecific antibodies (BsAb) over the past two decades with multiple targets to improve the efficacy or tissue specificity of monoclonal antibodies (mAb) treatment for diseases with multiple determining factors or widely-expressed targets. In this study, we designed and synthesized BsAb AGR2xPD1 targeting extracellular AGR2, a paracrine signal, and PD1, an immune checkpoint protein. Our design is intended to use AGR2 binding to guide PD1 targeting for AGR2$^+$cancer. We used this construction to produce AGR2xPD1 BsAb by generating clonally selected stable 293F cell line with high expression. Applying this BsAb in a T cell-Tumor cell co-culture system showed that targeting both PD1 and AGR2 with this BsAb induces the attachment of TALL-104 (CD8$^+$ *T*-lymphocytes) cells onto co-cultured H460 AGR2$^+$ Lung tumor cells and significantly reduces migration of H460 cells. *T*-cell expression of CD8 and IFNγ is also synergistically enhanced by the AGR2xPD1 BsAb treatment in the AGR2$^+$H460 co-culture system. These effects are significantly reduced with AGR2 expression negative WI38 cells. Our results demonstrate that the AGR2xPD1 BsAb could be a potential therapeutic agent to provide better solid tumor targeting and synergetic efficacy for treating AGR2+ cancer by blocking AGR2 paracrine signaling to reduce tumor survival, and redirecting cytotoxic *T*-cells into AGR2+ cancer cells.

1. Introduction

Therapeutic antibodies are biopharmaceuticals that trigger a biological response against a specific antigen, which recognizes and binds to the antigen to activate or inhibit a series of biological processes such as apoptosis or *T*-cell proliferation for blocking cancer cell growth or triggering the immune system (Khatib & Salla 2022, Sibéril et al 2007). Therapeutic antibodies exert various natural functions such as antibody-dependent cell-mediated cytotoxicity (ADCC), neutralization, or complement-dependent cytotoxicity (CDC) (Suzuki et al 2015). Therapeutic antibodies are widely used to treat various diseases. In cancer treatment, therapeutic antibodies are used broadly because of their higher efficacy

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and lower adverse effects than conventional therapy (Lu et al 2020). However, research interest has been growing in BsAb due to the potential of higher efficacy and specificity over monoclonal antibodies (Ma et al 2021). BsAb could treat diseases through multiple pharmacological pathways by targeting two different antigens simultaneously.

Commercial practice commonly uses transient expressions to harvest mAb (Zhang & Shen 2012). However, in our research laboratory, the most effective and reliable way to harvest antibodies from the culture media has been to generate stable cell lines. Therefore, many BsAb formats are being proposed (Fan et al 2015). However, for proof of BsAb targeting concepts, the most effective structure is the IgG-scFv appended format for its simplicity in efficiently obtaining experimental antibodies (Orcutt et al 2010) (Cao et al 2018). Therefore, we constructed an expression AGR2 antibody IgG(H)-PD1 scFv plasmid with anti-PD1 scFv appended to the IgG heavy chain of AGR2 antibody 18A4HU (BPHA) to test the effectiveness in tumor cell elimination in this combination. Although the plasmid can be constructed through the attachment of scFv to either the heavy chain or the light chain, our evaluation suggested that the attachment of scFv to the heavy chain is more stable than the light chain.

Within the Protein Disulphide Isomerase (PDI) family, the Anterior Gradient (AGR) family of proteins has a crucial role in cancer progression and other diseases (Guo et al 2017, Moidu et al 2020). In the AGR family, AGR2 is the most studied protein (Higa et al 2011, Liu et al 2019). AGR2 usually resides within ER for its proteostasis and protein folding functions; it also localizes in other cellular compartments, plasma membrane, cytoplasm, and extracellular environment (Fessart et al 2021, Moidu et al 2020). We have reported the role of extracellular AGR2 as paracrine signaling in tumor microenvironment (TME) development (Mangukiya et al 2019, Merugu et al 2021b). AGR2 overexpression leads to enhanced cancer cell proliferation, metastasis, and survival, promoting tumor progression and drug resistance (Li et al 2015, Pohler et al 2004). High levels of AGR2 are reported in the interstitial fluid of AGR2-positive tumors (Guo et al 2017) and AGR2 antibody is enriched in AGR2-positive solid tumors within its interstitial fluid (Negi et al 2019).

PD1 is a 55 kDa transmembrane protein containing 288 amino acids with an extracellular *N*-terminal domain. PD1 is a surface receptor expressed on *T*-cells, NK cells, dendritic cells, macrophages, and monocytes; it is highly expressed on tumor-specific *T*-cells (Ahmadzadeh et al 2009). PD1 suppresses innate and adaptive immune responses (Han et al 2020). PD-L1 is the ligand of the PD1 receptor highly expressed in tumor cells. PD1/PD-L1 crosstalk forms an immune checkpoint that has a crucial role in tumor immunosuppression by inhibiting *T*-cell-mediated immune response. Thus, tumor cells escape from immune surveillance and proliferate abnormally (Arrieta et al 2017). Antibodies against PD1 and PD-L1 have been successfully used in treating various tumors (Adachi et al 2022, Kato et al 2019, Sugiyama et al 2020). However, targeting the solid tumor by PD1 antibodies has limited success, partially caused by limited tumor penetration.

In this report, we describe the construction, production, and *in vitro* activities of the AGR2xPD1 BsAb to enhance the efficacy of both anti-PD1 and anti-AGR2 treatment of solid tumors though AGR2-guided antibody enrichment in AGR2-positive tumors. We show that this combined targeting promoted *T*-cell/Tumor cell interaction, increased tumor cell death, and *T*-cell activation. We further suggest that these enhanced activities against AGR2-positive tumor cells result from the guided targeting to AGR2-positive tumor cells and the blocking of AGR2 paracrine signaling to reduce tumor survival. The most effective mechanism is the redirection of effector cells (cytotoxic *T*-cells) to the target cells (AGR2-positive cancer cells). The simultaneously targeting AGR2 and PD1 brings the AGR2-positive cancer cells and PD1-positive *T*-cells in close contact to facilitate recognizing, targeting, and attacking the cancer cells by cytotoxic $CD8^+$ *T*-cells.

2. Materials and methods

2.1. Cell lines and culture media

293F (#CRL-1573), TALL-104 (#CRL-11386), H460 (#HTB-177), and WI38 (#CCL-75) cells were purchased from ATCC (ATCC, VA, USA). H460 cells were cultured in DMEM (Gibco, #12100–046), WI38 cells were cultured in MEM (Gibco, #415000–34), and 293F and TALL-104 cells were cultured in RPMI-1640 (Gibco, #31800–022) containing 10 % FBS (Clark, #FB15015) and 1 % penicillin–streptomycin (Solarbio, #P1400). Cells were maintained in a humidified incubator (5 % CO_2 and 37 °C).

2.2. Plasmid construction for generation of AGR2xPD1 BsAb

18A4HU plasmid (for AGR2 mAb) was constructed previously in our lab (Guo et al 2016), and that plasmid was used to construct BPHA (for AGRxPD1 BsAb) plasmid. The anti-PD1 sequence was constructed and joined with 18A4HU antibody sequence with a linker under EF-1a promoter, which was performed by overlap extension PCR with a DNA polymerase kit (KOD, Tyobo, China). The AmpR promoter controlled the ampicillin resistance (AmpR) coding sequence. Puromycin resistance (PuroR) coding sequence and EGFP coding sequence were both controlled by the same Cytomegalovirus (CMV) promoter. Both coding sequence is for the expression in mammalian cells. The puromycin coding sequence is for the selecting and generating of the stable cell line. Vector and all sequences were designed and constructed by using snapgene software. The newly constructed BPHA plasmid was transformed into *E. coli* (DH5α) competent cells (Tiangen Biotech, Beijing, #CB101). All constructs were analyzed and confirmed by DNA sequencing.

2.3. Transfection and stable cell line generation

293F cells were seeded in two 10 cm dishes. Cell confluency was maintained at about 50 % in both dishes. One dish of the 293F cells was used for transfection of the BPHA plasmid, and another dish was used for transfection of the EGFP plasmid as control. Transfection was carried out by using polyethylenimine (PEI) (Polysciences, Warrington, PA) according to the instruction. The plasmid and PEI ratio is 1:3; 12 μg plasmid and 36 μg PEI were diluted separately with DMEM up to 500 μl each. And then, diluted PEI and plasmid were mixed up gently by pipetting and incubated at room temperature for 15 min to allow to form polyplex. Then, the polyplex of PEI and plasmid was added to the pre-washed fresh 293F cells and 4 ml pre-warmed serum and antibiotic-free DMEM to the same 10 cm dish, incubated at 37 °C for 4–6 h. After incubation, the medium was replaced with fresh complete DMEM containing 10 % FBS, and the following day, the green fluorescence of the transfected cells was checked under the fluorescence microscope. After 48 h, replace the medium with a complete a DMEM containing 10 % FBS and 3 μg/ml puromycin (Solarbio, China, #P8230).

2.4. Slot blot assay

A slot blot assay was performed to optimize different clones' binding affinity for antibodies secreted by clones. The secretion and antibody expression is varied in different clones. Specific antigen was loaded onto the gel and transferred to the membrane by

electrophoresis. After blocking the membrane with 5 % non-fat milk, was fitted with 13 slotted rectangular-shaped plates. Different cultured mediums (Previously collected in 1.5 ml tubes) for respective clones were added to each slot which was used as primary antibody. After overnight incubation, the membrane was washed thrice with TNET and incubated with secondary antibody for 1 h, then after washing, scanned the membrane.

2.5. Purification of BsAb by protein G beads

After generation of stable cell line and optimization of different clones by slot blot analysis, cultured the clones in a bulk amount that highly expressed antibody. After a day of culturing the cells, washed the cells thoroughly with sterile PBS and added serum-free RPMI with 4 μg/ml puromycin. After 48 h, the supernatant was aseptically diluted with binding buffer in a 1:1 ratio. Next, prepared 1 ml protein G beads (GE Healthcare) in a suitable column, and equilibrated the beads with pre-adjusted pH 7.2 binding buffer. Then, the diluted sample (medium + binding buffer) was added to the beads and collected the flow-through in a beaker kept on ice. After passing all the samples through beads, wash the beads properly with binding buffer. Then added elution buffer to the beads, each time adding 1 ml elution buffer up to 7 ml and collected passing through elution buffer in seven different 1.5 ml tubes labeled E1-E7 respectively. Initially checked the concentration of antibodies by using G250 reagents (Solarbio, China). Next, eluted samples were mixed and concentrated by ultracentrifugation using an ultra-15 centrifugal filter (Millipore). Finally, we measured the concentration of antibodies by Bradford assay and SDS-PAGE.

2.6. Quality checking and affinity binding of BsAb

2.6.1. Bradford assay and SDS-PAGE

After concentrating the final antibody samples, measure the concentration by Bradford assay. We added 20 μl of sample and different dilutions of BSA into 96 wells plates. BSA was used as standard. Then, 200 μl of G250 reagent was added to each respective well and analyzed the absorbance in the wavelength of 595 nm using a U.V. spectrophotometer. The concentration of the antibody sample was measured by the standard curve of concentration vS absorbance. The concentration of the antibody was measured by Bradford assay and SDS-PAGE. The size of the light chain and heavy chain was verified by SDS-PAGE and western blot.

2.6.2. Western blot

Purified BsAb was tested for affinity binding by western blot analysis. Loaded HIS-AGR2, GST-PD1, and BSA (as a negative control) samples into three repetitive wells for the incubation with three different antibodies (18A4HU mAb, PD1 mAb, and AGR2xPD1 BsAb). Proteins were separated by SDS-PAGE and transferred onto a nitrocellulose membrane. Blocked the membrane for an hour in 5 % non-fat milk, and after blocking, washed the membrane properly using TNET and cut the membrane into three pieces. Then, each part of the membrane was incubated with the respective primary antibody, i.e., 18A4HU mAb, PD1 mAb, and AGR2xPD1 BsAb, for 2 h, followed by incubation with a secondary antibody against human for 1 h.

2.7. In vitro biological activity

2.7.1. T-cell culture and activation

TALL-104 cells were grown in complete RPMI-1640 (10 % FBS and 1 % penicillin and streptomycin) with Interleukin 2 (25 ng/ml, Sinobiological, #P60568-1). They were activated by incubation with Ionomycin (2.5 μM, Beyotime, #S1672) and Phorbol myristate acetate (10 ng/ml Beyotime, #S1819) simultaneously for 2 h in 5 % CO_2 and air at 37 °C. After the incubation, cells were washed once with PBS.

2.7.2. Scratch wound-healing assay

H460 cells were seeded in a six-well plate and grown in DMEM with 10 % FBS to about 90 % confluence and starved for 24 h by culturing in a serum-free DMEM. Wounds were created by a sterile yellow tip in the cell monolayers in each well. Cells were washed with PBS to wash out the detached cells. Then added serum-free DMEM and captured images (0 h). Discarded the DMEM and activated TALL cells in serum-free RPMI-1640 were added on top of the H460 cells at an E/T ratio (effective cells to target cells) of 5:1. After 1 h, cells were treated with 18A4HU mAb alone (5 μg/ml), PD1 mAb alone (5 μg/ml), 18A4HU mAb + PD1 mAb (2.5 μg/ml each), and AGR2xPD1 BsAb (5 μg/ml) respectively. After 48 h, the TALL cells were collected in respective tubes (*used these TALL cells to perform immunofluorescence to evaluate $CD8^+$ expression*), and washed each well twice with PBS to wash out the remaining TALL cells and added fresh serum-free RPMI-1640 to each well and captured images.

2.7.3. Immunofluorescence

Collected TALL cells were placed on glass slides performed by cytospin. Cells were fixed with 4 % paraformaldehyde for 20 min, incubated with 0.1 % Triton-X for 5 min, and blocked with 5 % BSA in PBS for 1 h. Cells were incubated with CD8 and IFNγ primary antibodies respectively (1:200) (Abclonal, China) and then incubated in Dylight 594 secondary antibody (1:1000) against rabbit, with actin tracker TRITC phalloidin (FAK100, Millipore, USA). The nucleus was counterstained with DAPI (Solarbio, China). Coverslips were mounted on a slide, and images were captured using a confocal microscope.

2.7.4. Attachment of TALL cells onto H460 tumor cells

H460 cells were seeded in a 12 wells plate and grown in DMEM with 10 % FBS. After 24 h, discarded the media and added activated TALL cells suspension in RPMI-1640 on top of the H460 cells (E/T: 5:1). After one-hour cells were treated with 18A4HU mAb alone (5 μg/ml), PD1 mAb alone (5 μg/ml), 18A4HU mAb + PD1 mAb (2.5 μg/ml each), and AGR2xPD1 BsAb (5 μg/ml) respectively. Images were captured after 0 h, 24 h, and 48 h. Then, discarded the media and washed twice with PBS to wash out the remaining unbound TALL cells, and added fresh serum-free RPMI-1640 to each well and captured images.

2.8. Statistical analysis

Statistical analyses were undertaken with GraphPad prism software. As appropriate, groups were compared with unpaired two-tailed student's *t*-test. Statistical significance was defined as a level of $p<0.05$ (*, $p < 0.05$ and **, $p < 0.01$, and ***, $p < 0.001$).

3. Results

3.1. ***Plasmid construction for generation of target-guided immune checkpoint blocking BsAb*** *(AGR2xPD1)*

The humanized 18A4HU mAb (agtuzumab) targeting AGR2 was developed in our lab (Guo et al 2016). It has been shown to significantly inhibit lung cancer *in vitro* and *in vivo* and is enriched in the interstitium, or interstitial space, of AGR2-positive xenograft tumors (Guo et al 2016) (Negi et al 2019)**.** Pembrolizumab is a humanized monoclonal antibody targeting the immune checkpoint protein PD1. It is widely used in immunotherapy against

many cancer types, such as melanoma, lung cancer, head and neck, Hodgkin lymphoma, and stomach cancer. However, as a second-line treatment, the response rate for pembrolizumab in late-stage non-small cell lung cancer (NSCLC) patients is about 20 % and about 45 % with over 50 % PDL1 expression score (Dang et al 2016). In this study, we developed an appended BsAb in IgG(H)-scFv format, with the PD1-targeting scFv from pembrolizumab appended to the IgG heavy chain of AGR2 targeting 18A4HU to improve the efficacy of both mAbs (Fig. 1). In this design, the transcription of BsAb is driven by the EF-1a promoter while the translations between the light chain and the heavy chain with the appended scFv are separated by an internal ribosomal entry site sequence (IRES) (Fig. 1A top left) The vector backbone with an EGFP-puromycin fusion protein expression unit is also shown (Fig. 1A bottom left). The complete circular plasmid construct is shown in (Fig. 1A right). The expected AGR2xPD1 product is illustrated in (Fig. 1B).

3.2. *General strategy* ***of efficient generation of high-quality BsAb through the clonal selection of cell lines with high-level expression of high-quality AGR2xPD1 BsAb***

Although recombinant antibody production by transient expression is prevalent, we had a better experience using clonal-selected stable expression cells for the consistency in quality and quantity of recombinant antibody production. High-quality plasmid DNA was isolated from 4 bacterial colonies (Fig. 2A) and fully sequenced before transfecting into 293F cells according to the clonal-expression evaluation and antibody purification strategy outlined as a flow chart in (Fig. 2B). First, transfection efficiencies were monitored according to the percentage of cells expressing EGFP (Fig. 2C). After subsequent puromycin selection, the colones with enriched EGFP cells are independently purified for multiple rounds (Fig. 2D) and selected according to their growth, EGF expression, and antibody production. The clonal cells with best antibody productions were selected and expanded and used in AGR2xPD1 BsAb production.

3.3. Clonal selection and quality assessment of clones

After the generation of the puromycin-resistant cells, these cells were pooled and digested as single cells. To generate single-cell clones, the cells were diluted in DMEM medium, and 100 to 1000 cells were seeded in 10 cm dishes for a week or until enough single-cell colones were visible (64 to 128 cells) and could be picked under the microscope for EGFP expression and purity, and each of the clones was seeded in a single well of 96-well plates. Cloned cells in each well were cultured for 7 days then separately digested and diluted to seed 10 cm dishes to form new colones of the same lineage. This selection cycle was repeated to the 6th generation to obtain relatively pure clones. These clones were expanded and grown for 48 or 72 h in various media to test the production of antibodies against AGR2 secreted in the media (Fig. 3A & 3B). Our results showed that clone 2A7 secreted a higher amount of antibody at 48 h in both RPMI and Gibco 293 freestyle medium, and clone 4B secreted a higher amount of antibody at 72 h in RPMI and Gibco 293 freestyle medium. Therefore, we selected clone 2A7 with 48 h growth in RPMI medium to purify AGR2xPD1 BsAb.

To directly compare the antibodies produced by various clones for quantity and structural integrity, several clones were cultured without serum for 48 h, and the conditioned medium was collected for further testing. The conditioned media were centrifuged, and the supernatant samples from six different clones (8F, 8E, 8D, 6B, 2A7, and 4B) were loaded on SDS-PAGE for western analysis with goat anti-human IgG. We found that all of the clones produced AGR2xPD1 BsAb, heavy and light chains with expected sizes (Fig. 3C). The growth of all of the respective clones (8F, 8E, 8D, 6B, 2A7, and 4B) were also shown to estimate cell numbers for antibody production efficiency (Fig. 3D).

3.4. Expression diversity of EGFP tag and BsAb in subclones

During clone purification cycles, we experienced increased dissociation of EGFP expression and BsAb production. This is possibly the result of separating the EGFP-puromycin fusion transcription

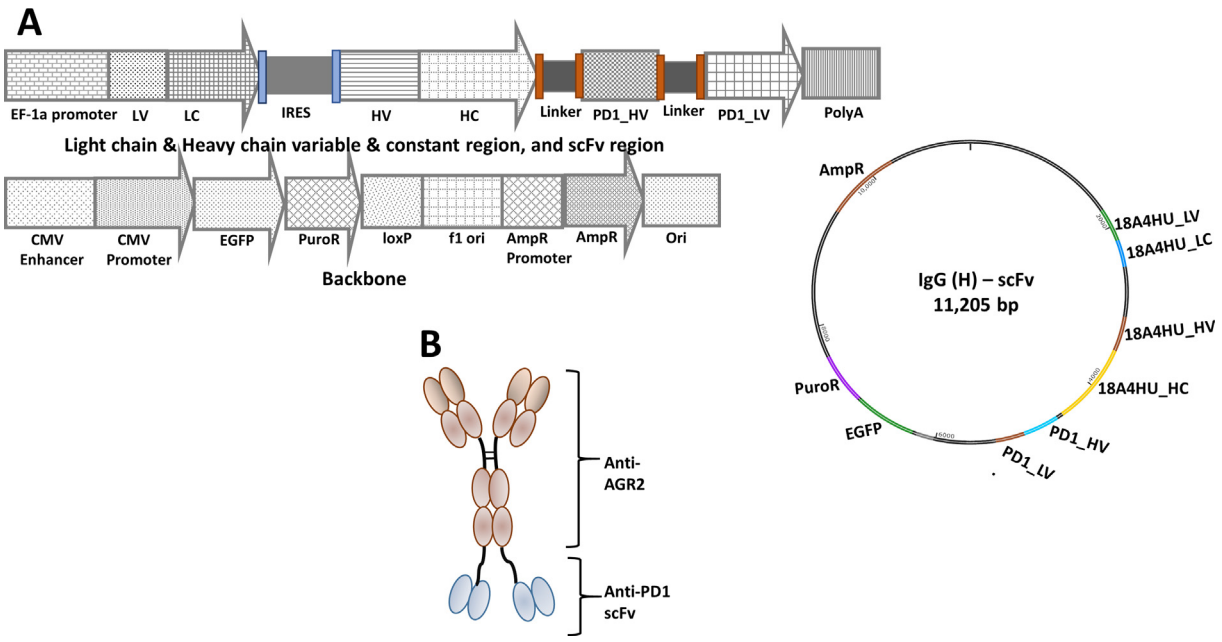

Fig. 1. Construction of plasmid for the generation of AGR2xPD1 BsAb. (A) BPHA plasmid map for the generation of AGR2xPD1 BsAb. (B) Schematic representation of AGR2xPD1 scFv BsAb structure.

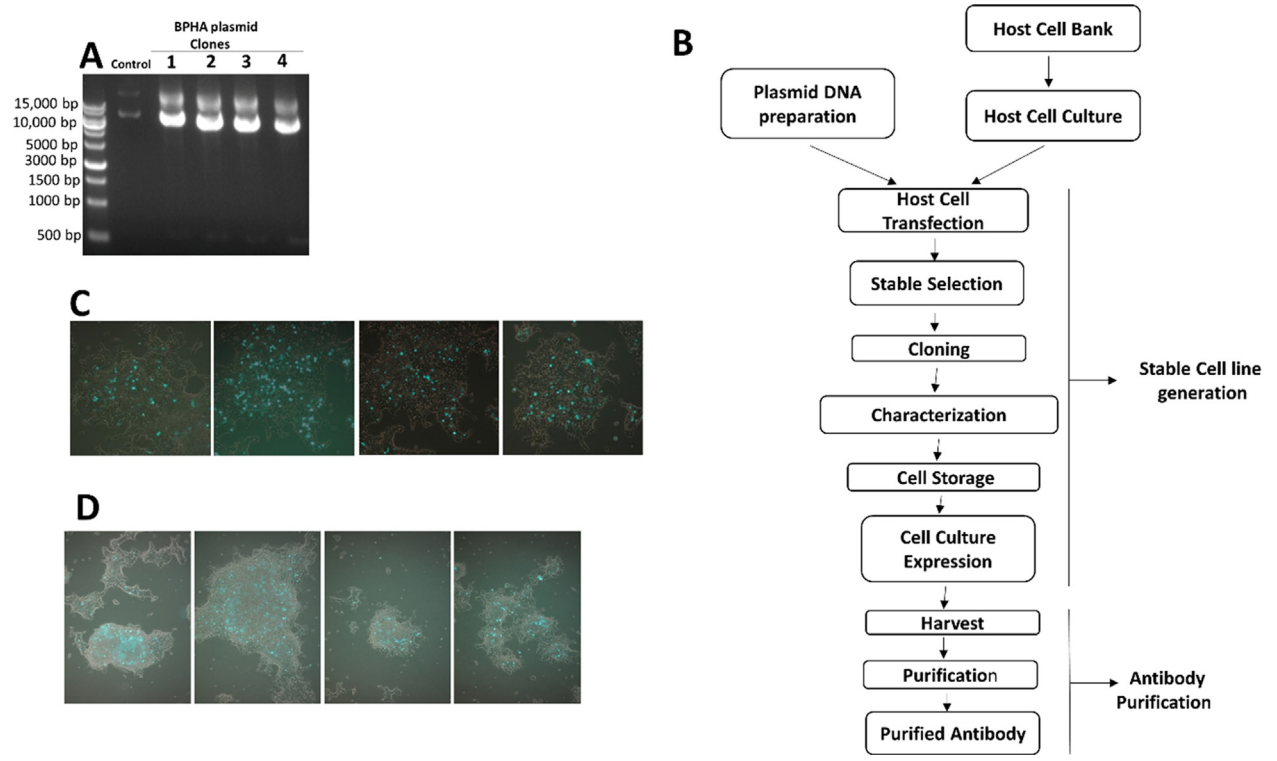

Fig. 2. Transfection and stable cell line generation: (A) Agarose gel electrophoresis showing BPHA plasmid was used to generate AGR2xPD1 BsAb. (B) Flow chart for stable cell line preparation for generation and purification of AGR2xPD1 BsAb. (C) Cells expressing EGFP after 48 h transfection with BPHA plasmid into 293F cells. (D) Enriched EGFP cells were independently cultured for multiple rounds.

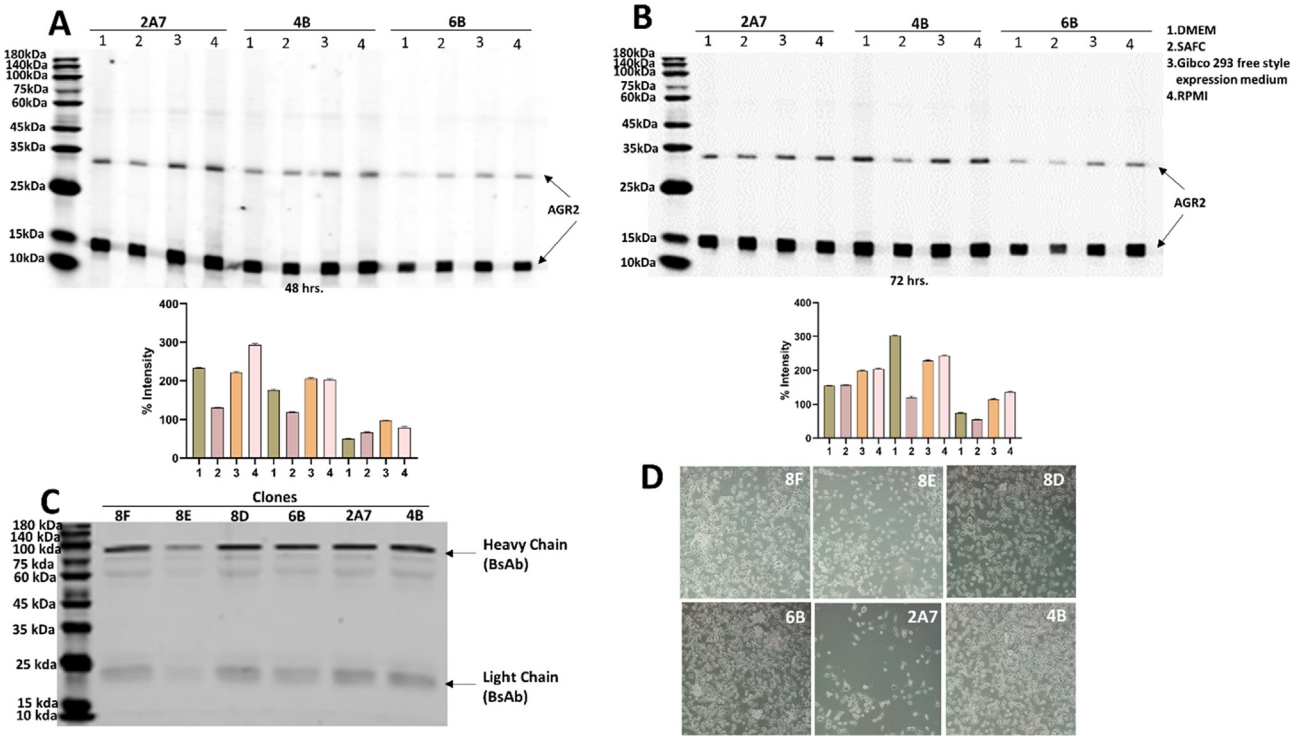

Fig. 3. Growth optimization and quality assessment of clones. (A & B) Optimization of antibody secretion of three clones 2A7, 4B, and 6B in two different time points, 48 h (A) and 72 h (B), using four different media DMEM, SAFC, Gibco 293 freestyle expression medium, and RPMI 1640, evaluated by slot blot assay. It was shown that 2A7 clone secreted a high amount of antibody at 48 h in RPMI and Gibco 293 freestyle medium, and 4B clones secreted a high amount of antibody at 72 h in RPMI and Gibco 293 freestyle medium. Finally we selected 2A7 clones for further process. Additionally we used 48 h incubation period and RPMI 1640 to purify AGR2xPD1 BsAb. Band intensity was measured and analyzed by Image J, and Graphpad prism software. (C) Size verification of secreted antibody AGR2xPD1 BsAb was assessed by western blot analysis using the culture supernatant of different clones. All of the clones showed appended heavy chain and normal light chain of the antibody with expected sizes. (D) Images showing the growth state of cells from respective clones.

unit in the vector and the BsAb transcription insert unit of the construct during stable integration and subsequent recombination processes. Therefore, using green fluorescence as a surrogate marker is not enough to assess BsAb production and targeting affinity for further subcloning. In addition to the green fluorescence marker, we also evaluated the binding affinity of the BsAb produced by different 2A7 subclones against one of its targets, AGR2, by western blot analysis.

The 2A7 subclones were produced as described before with several rounds of subcloning to identify better BsAb producers (Fig. 3). The subclones of 2A7 were cultured for 48 h in a serum-free medium and were first checked for fluorescence. We found that all of the clones expressed various levels of green fluorescence (Fig. 4A). We further evaluated the supernatants and cell lysates of six different subclones as primary antibody to detect AGR2 by western blot using goat anti-human IgG antibody as the secondary antibody (Fig. 4B). Our results showed that subclones 1, 2, and 8 were the better clones for BsAb production (Fig. 4B). Therefore, we decided to use these clones for the purification of AGR2xPD1 BsAb in bulk amounts. Subclones 1, 2, and 8 were further expanded to generate stable, productive cell lines.

3.5. Purification of BsAb directly from the supernatant of stable cells and western blot confirmation against recombinant AGR2 and PD1

Stable cells were cultured in bulk after 48 h of culture, collected the supernatant, and filtered through a 0.45 μM filter to remove the suspended particles and cell debris. To maintain the pH, the filtered supernatant was diluted with binding buffer at a 1:1 ratio. Then, the diluted supernatant was passed through protein G beads. Protein G binds with the Fc region of the heavy chain of the antibody. Subsequently, the bound antibody was eluted using the elution buffer. Different samples were collected during various stages of the purification process, and performed western blot to detect antibodies in different samples (Fig. 5A). After the purification process, the size of the heavy chain and light chain was verified by western blot analysis and SDS-PAGE (Fig. 5B & C).

Affinity bindings of BsAb to its targets were evaluated by western blot analysis. HIS-tagged AGR2 was purified in our lab. AGR2 and PD1 proteins were loaded separately onto the gel and transferred to the PVDF membrane in three duplicates with BSA loaded as a negative control. It was shown in (Fig. 5D) that AGR2xPD1 BsAb binds simultaneously against both target AGR2 and PD1, whereas 18A4 mAb binds against only AGR2 and PD1 mAb binds against only PD1. This result proved that BsAb specifically binds with two different antigens, AGR2 and PD1.

3.6. AGR2xPD1 BsAb induces attachment of TALL cells onto H460 tumor cells

To elucidate the effect of AGR2xPD1 BsAb treatment in a TALL and H460 co-culture, phase-contrast images of co-cultured TALL and H460 cells were investigated at 0 h, 24 h, and 48 h after AGR2xPD1 BsAb treatment, to evaluate the attachment of TALL cells to H460 cells, images were also taken 48 h after washing the co-culture twice to remove unbound TALL cells. We found that after 24 h and 48 h, TALL cells' attachment onto H460 tumor cells in the AGR2xPD1 BsAb treated group was significantly higher compared to that of 18A4HU mAb, PD1 mAb, and a combination of 18A4HU mAb and PD1 mAb treated group. In comparison, with AGR2xPD1 BsAb treatment, minimal co-cultured TALL cells attach to WI38 cells, a human lung cell line not expressing AGR2 (Fig. 6A). In addition, after washing out of unbound TALL cells, we found minimal attachment of TALL cells onto H460 in 18A4HU mAb, PD1 mAb, and a combination of 18A4HU mAb and PD1 mAb treated group, while significant TALL-H460 attachment was found in AGR2xPD1 BsAb treated group but not for the same treatment in co-cultured TALL and AGR2 negative WI38 cells (Fig. 6B) indicating

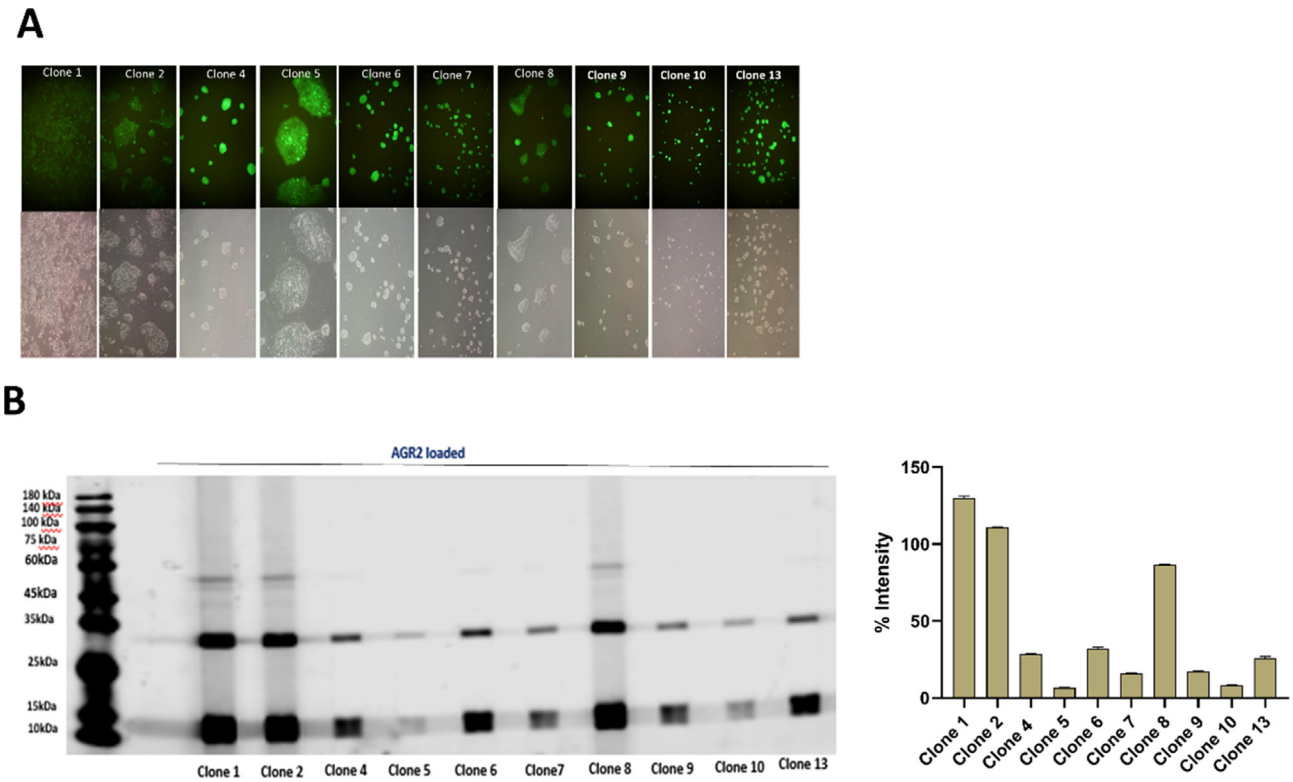

Fig. 4. Selection of subclones based on affinity binding against AGR2 (A) Selected clones expressing green fluorescence. (B) Supernatants of different subclones were evaluated based on affinity binding against AGR2 by slot blot western assay. It was detected that clone 1, clone 2, and clone 8 secreted higher amounts of antibodies, and these three clones were selected for further process. Band intensity was measured and analyzed by Image J, and GraphPad prism software.

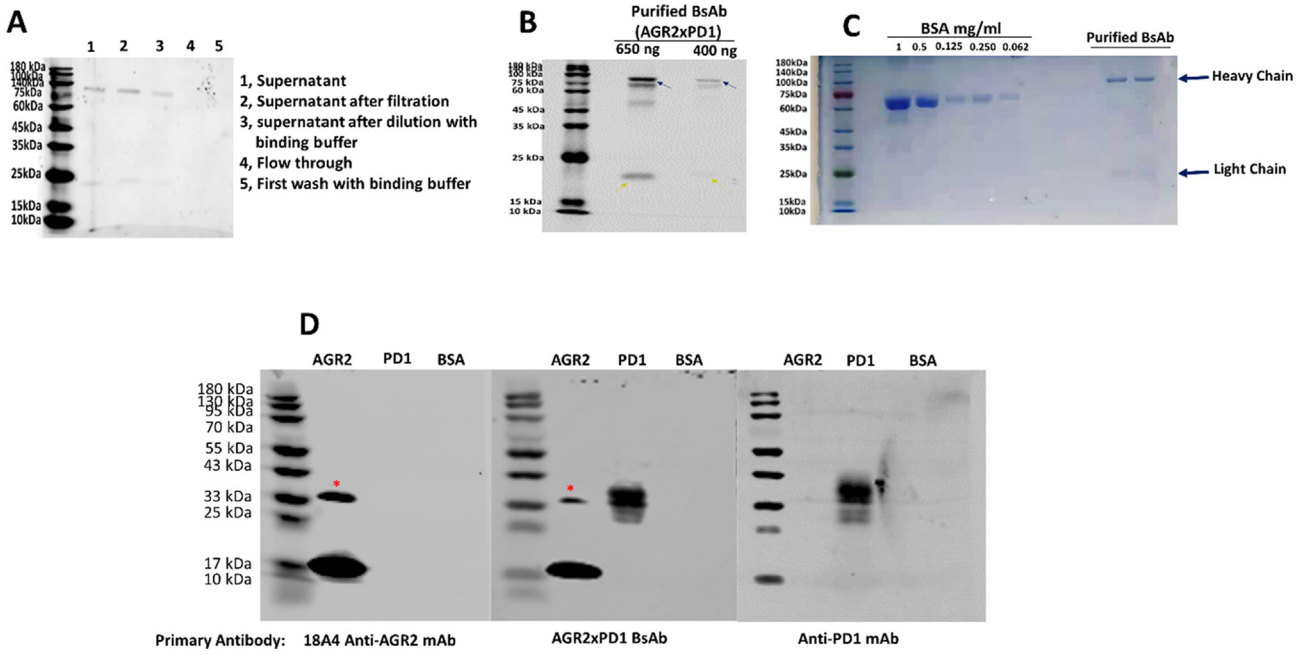

Fig. 5. Purification of AGR2xPD1 BsAb and affinity checking (A) Purification of AGR2xPD1 BsAb samples were taken in different stages of the purification process and performed western blot for the detection of antibodies in different samples. (B&C) Size verification of purified BsAb by western Blot and SDS-PAGE. (D) Affinity binding of AGR2xPD1 BsAb against AGR2 and PD1, compared with 18A4 mAb and PD1 mAb. AGR2xPD1 BsAb selectively and simultaneously binds with AGR2 and PD1. **Note:** Asterisk (*) indicating the AGR2 dimer.

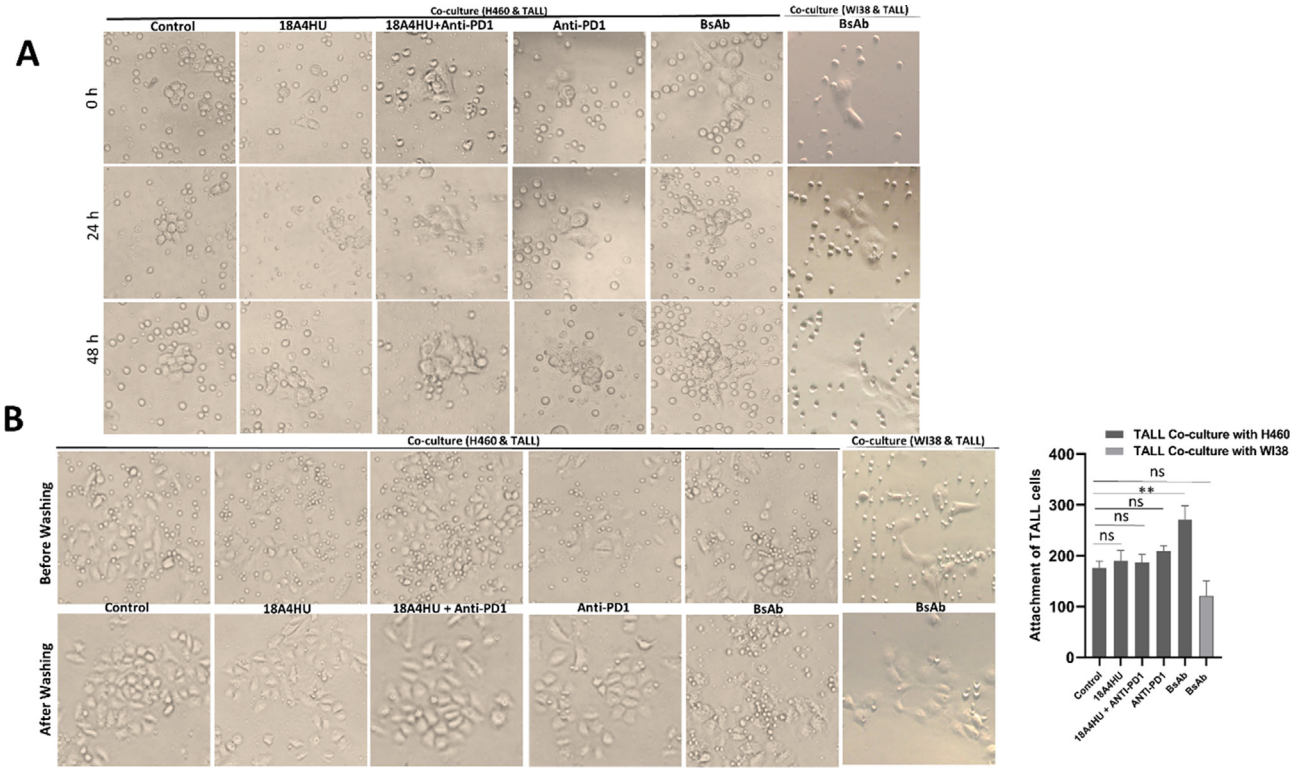

Fig. 6. AGR2xPD1 BsAb induces attachment of TALL cells onto H460 tumor cells. H460 and TALL cells were co-cultured. These co-cultures were treated with the following antibodies: control (PBS), 18A4HU mAb alone (5 μg/ml), the combination of 18A4HU mAb and PD1 mAb (2.5 μg/ml each), PD1 mAb alone (5 μg/ml), and AGR2xPD1 BsAb (5 μg/ml). WI38, lung fibroblast cells, which do not express AGR2, co-cultured with TALL and, treated with AGR2xPD1 BsAb (5 μg/ml) found no significant attachment. (A) H460 and TALL cells were co-cultured and treated with different antibodies. Images were taken after 0 h, 24 h, and 48 h. Found significant attachment of TALL cells onto H460 after 48 h of treatment compared to other treated groups. (B) After 48 h, washed twice to remove unbound TALL cells, and images were taken before and after washing. It was found that the attachment of TALL cells onto H460 was significant.

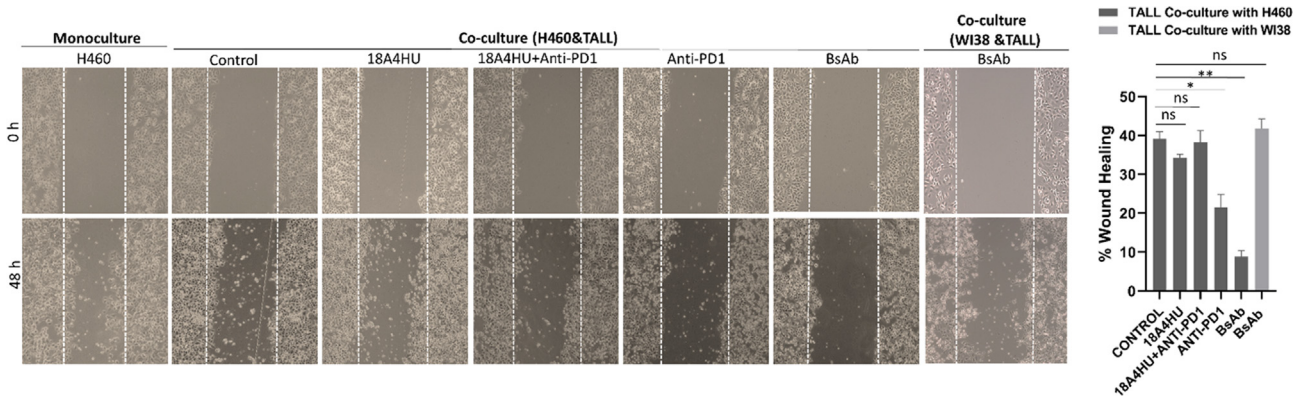

Fig. 7. AGR2xPD1 BsAb inhibits the migration of H460 tumor cells when co-cultured with TALL cells. H460 and TALL cells (Monoculture and co-culture) were seeded. These co-cultures were undertaken in the presence of the following antibodies: control (PBS), 18A4HU mAb alone (5 μg/ml), the combination of 18A4HU mAb and PD1 mAb (2.5 μg/ml each), PD1 mAb alone (5 μg/ml), and AGR2xPD1 BsAb (5 μg/ml). A wound was caused, and different treatments were added to the co-culture wells. Images were captured at 0 h and 48 h. Scratch wound healing assay to evaluate the migration of H460, % wound healing was significantly lowered in PD1 mAb treated group, and highly significant in AGR2xPD1 BsAb treated group. WI38, lung fibroblast cells, which do not express AGR2, co-cultured with TALL and, treated with AGR2xPD1 BsAb (5 μg/ml) found no abrogated migration. The percentage of wound healing is calculated by $(D_0 - D_{48}) \times 100/D_0$. Whereas D_0 = mean distance of wound at 0 h and D_{48} = mean distance of wound at 48 h.

AGR2 expression is necessary for AGR2xPD1 BsAb induced TALL cell attachment.

3.7. Abrogated migration of H460 mediated by AGR2xPD1 BsAb-induced $CD8^+$ cytotoxic T-cells

We performed a wound healing assay to exhibit the inhibitory effect of AGR2xPD1 BsAb-induced $CD8^+$ cytotoxic *T*-cells against AGR2-expressing H460 cells. After 48 h of treatment, we found that migration was significantly reduced in AGR2xPD1 BsAb treated group compared to 18A4HU mAb, PD1 mAb, and the combination of 18A4HU mAb and PD1 mAb treated group (Fig. 7). Furthermore, AGR2 expression seems necessary for migration inhibition by AGR2xPD1 BsAb-induced TALL cells because the migration was not affected significantly for AGR2 negative WI38 cells by AGR2xPD1 BsAb treated TALL cells (Fig. 7 far right).

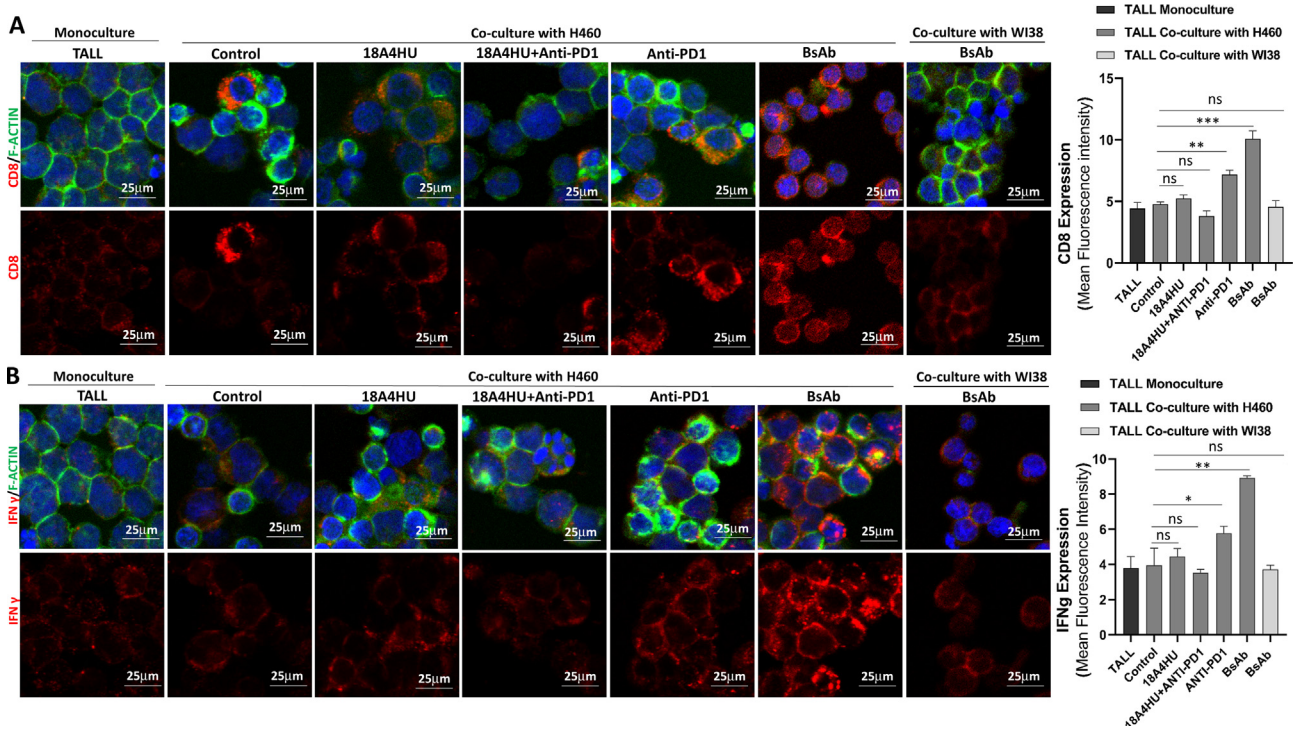

Fig. 8. BsAb induces significantly higher expression of CD8 and IFNγ in TALL cells compared to other mAbs treated groups. H460 and TALL cells (Monoculture and co-culture) were seeded. These co-cultures were undertaken in the presence of the following antibodies: control (PBS), 18A4HU mAb alone (5 μg/ml), the combination of 18A4HU mAb and PD1 mAb (2.5 μg/ml each), PD1 mAb alone (5 μg/ml), and AGR2xPD1 BsAb (5 μg/ml). After 48 h of treatment, TALL cells were collected and performed immunofluorescence to evaluate the expression of CD8 and IFNγ. WI38, lung fibroblast cells, which do not express AGR2, co-cultured with TALL and, treated with AGR2xPD1 BsAb (5 μg/ml) found no significant CD8 and IFNγ expression by co-cultured TALL cells. (A) *T*-cells expression of CD8 synergistically enhanced in co-cultures treated with AGR2xPD1 BsAb. (B) Secreted IFNγ expression by *T*-cells enhanced in co-cultures treated with AGR2xPD1 BsAb. Mean fluorescence intensity measured by Image J software, and correction of the cell fluorescence made by the following formula, corrected total cell fluorescence (CTCF) = Integrated mean fluorescence intensity – (Area of Selected Cell × Mean Fluorescence of Background readings).

3.8. T-cell expression of CD8 and IFNγ synergistically enhanced in co-cultures treated with AGR2xPD1 BsAb

After 48 h of treatment in wound healing assay, we collected co-cultured TALL cells and performed immunofluorescence to exhibit the expression of CD8 and IFNγ. We found that expression of CD8 and IFNγ is significantly higher in AGR2xPD1 BsAb treated group compared to 18A4HU mAb, PD1 mAb, and the combination of 18A4HU mAb and PD1 mAb treated group (Fig. 8). The migration of co-cultured H460 tumor cells attenuated by AGR2xPD1 BsAb-induced higher expression of $CD8^+$ cytotoxic *T*-cells. Fig. 9.

4. Discussion

AGR2 is present in the intracellular and extracellular compartments. However, AGR2 is expressed in various cancers but overexpressed in lung cancer. Extracellular AGR2 influences the tumor microenvironment and enhances angiogenesis by interacting with growth factors. Extracellular or secreted AGR2 influences fibroblast organization, elongation, and migration; besides, it has also been revealed that AGR2 secreted by cancer cells is taken up by surrounding fibroblasts. Murine antibody 18A4 developed in our lab significantly inhibits the uptake and activation of AGR2 (Merugu et al 2021a). 18A4 was humanized (18A4HU I) to overcome the immunogenicity by CDR grafting technique and de-immunization analysis. A mouse xenograft study showed the 18A4HU I effectively inhibits xenograft tumor growth (Guo et al 2016). PD1 is an immune inhibitory transmembrane receptor of the CD28 family that regulates *T*-cell activity. PD1 is expressed in *T*-cells, B-cells, monocytes, and natural killer cells. Most of the cells express PD-L1 and PD-L2. In normal conditions, PD-L1 and PD-L2 interact with the PD1 receptor to prevent lymphocyte over-activation and maintain immune tolerance. However, PD-L1 is over-expressed in several tumors, including lymphoma, melanoma, non-small-cell lung cancer, and other cancer types (Na et al 2017). Therefore, cancer cells inhibit *T*-cell signaling by employing crosstalk of PD1/PD-L1 and escape from *T*-cell-induced immune surveillance (Na et al 2017). Blocking PD1/PD-L1 interaction by mAb has been shown to restore *T*-cell activation and antitumor response (Li et al 2020). Currently, two FDA-approved therapeutic mAbs are available in the market, targeting PD1: nivolumab and pembrolizumab (Na et al 2017).

Based on clinical and other studies, it has been demonstrated that therapeutic mAbs have some functional limitations, including inadequate pharmacokinetics, impaired interactions with the immune systems, and therapeutic resistance (Chames et al 2009). Numerous efforts are being attempted to overcome these limitations and develop more productive approaches, such as combination therapy and identifying new targets; however, the most effective approach is developing BsAb. It has higher binding avidity to targets and can interact with more than one surface antigen, having less therapeutic resistance. The most effective mechanism is the redirection of effector cells (Immune cells) to the target cells (Tumor) (Li et al 2021, Na et al 2017, Waite et al 2020). Therefore, we developed a novel AGR2xPD1 BsAb, which simultaneously targets AGR2 and PD1 and employs a unique approach that the PD1

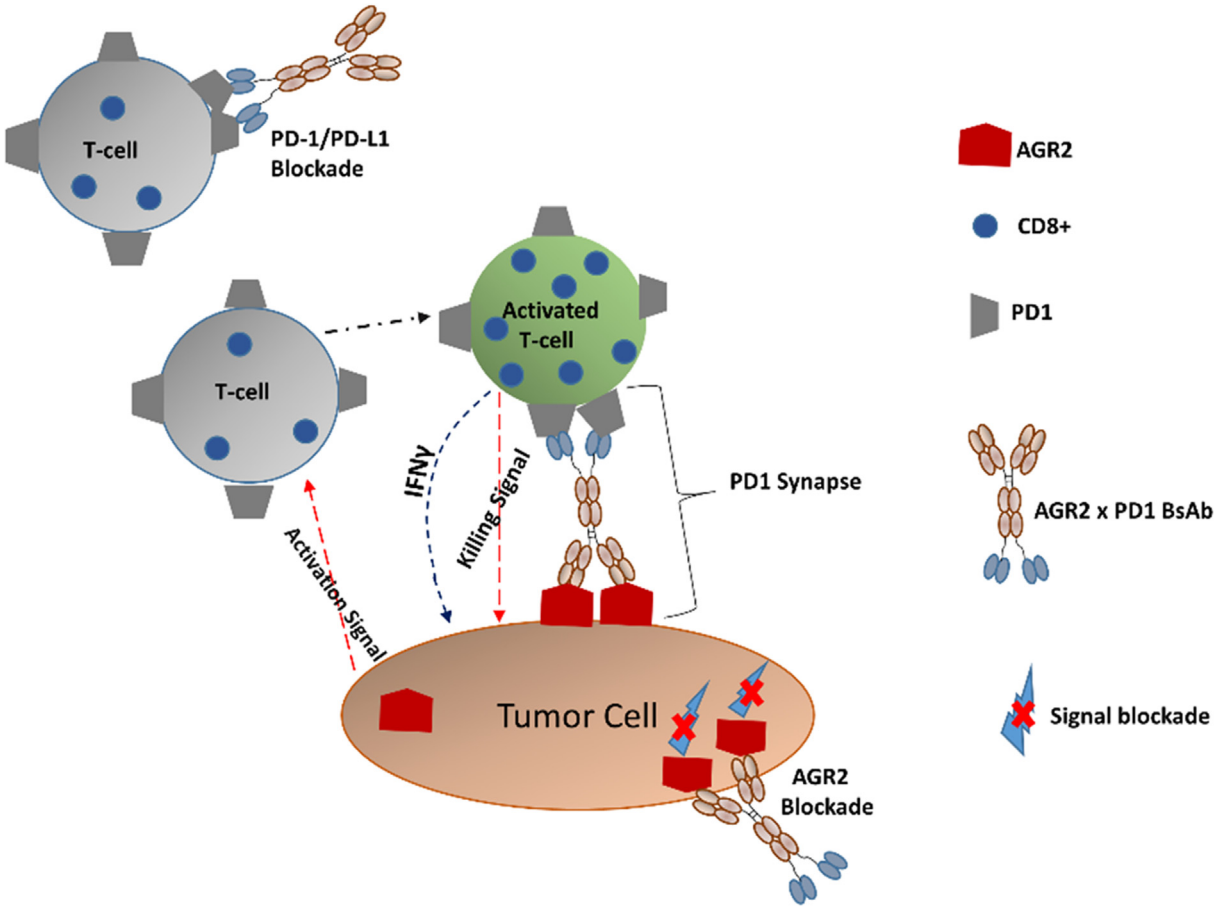

Fig. 9. Schematic representation of AGR2xPD1 BsAb-mediated tumor cell killing. AGR2xPD1 BsAb-induced numerous killing mechanisms are (1) formation of PD1 synapse leading to activation of *T*-cells and other immune cells, resulting in killing of tumor cells by cytotoxic proteins, (2) Blockade of PD1/PD-L1 crosstalk. (3) Blockade of AGR2-mediated tumor progression.

antibody is directed to the AGR2-overexpressed tumor microenvironment by the anti-AGR2 antibody. As a result, AGR2xPD1 BsAb binds with AGR2 and PD1, bringing close contact with AGR2 overexpressing cancer cells and PD1 expressing immune cells. Additionally, by targeting AGR2-overexpressing tumor cells, AGR2xPD1 BsAb could enhance the antibody localization and activate *T*-cells and other immune cells within the tumor microenvironment. We found *in vitro* study that this novel BsAb very effectively induced attachment of H460 and TALL ($CD8^+$ *T*-lymphocytes) cells attenuated migration of H460 compared to 18A4HU mAb, PD1 mAb, and the combination of 18A4HU mAb and PD1 mAb treated group. In addition, it has also been shown that AGR2xPD1 BsAb-induced higher expression of CD8 in *T*-cells compared to 18A4HU mAb, PD1 mAb, and a combination of 18A4HU mAb and PD1 mAb treated group.

Here, to develop AGR2xPD1 BsAb, we designed and constructed a plasmid containing the sequences of two mAbs PD1 and AGR2, and transfected it into the mammalian 293F cells. Therefore, we developed the stable cell line by selecting transfected 293F cells using puromycin. Stable cells were grown in bulk amounts. The supernatant was collected and diluted with binding buffer in a 1:1 ratio to maintain the neutral pH of protein G beads (McCaw et al 2014). Diluted supernatant was used to harvest and purify BsAb through protein G affinity chromatography.

5. Conclusion

This study demonstrated that the novel AGR2xPD1 BsAb, that we developed redirected *T*-cells to lung cancer cells and showed higher anti-tumor response compared to 18A4HU mAb, PD1 mAb, and the combination of 18A4HU mAb and PD1 mAb treated group. It has also shown that this novel BsAb induces significantly higher attachment of *T*-cells onto AGR2-overexpressing cancer cells. Cancer cells that proliferate by escape from *T*-cell immune surveillance through PD1/PD-L1 crosstalk, AGR2xPD1 BsAb, effectively bring close contact with AGR2-overexpressing cancer cells under *T*-cells immune surveillance, as well as induces *T*-cell immune activation by inhibiting PD1/PD-L1 interaction. These findings could provide a novel strategy for treating AGR2-mediated cancer progression. Further investigation of the effect of AGR2xPD1 BsAb *in vivo* is ongoing to better define the anti-tumor effect and to check toxicities levels.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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