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RESEARCH PAPER

Rapid Screen of IL-5/IL-5Rα Blocking Antibodies in the HEK293-IL-5Rα-CSF2RB Transfected Cell Line

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Abstract Interleukin-5 (IL-5) binding to interleukin-5 receptor subunit alpha (IL-5Rα) increases the number of eosinophils and enhances eosinophil activity. This leads to eosinophil tissue infiltration and damage to the lungs, ultimately resulting in exacerbation of asthma. Antibodies that block IL-5 binding to IL-5Rα are thought to play an important role in advanced asthma. Currently, key methods used to screen for targeted drugs are Surface Plasmon Resonance which is costly and anti-proliferation assays which are tedious and have a low signal-to-noise ratio. Here we describe a Fluorescence Activated Cell Sorting (FACS) assay, based on human embryonic kidney (HEK)-293 cells with stable expression of IL-5Rα and the cytokine receptor common subunit beta (CSF2RB). Cells co-expressing IL-5Rα and CSF2RB had a 16% increase in the ability to bind IL-5 compared to cells expressing only IL-5Rα. The optimal concentration of IL-5 for the FACS assay was 0.1 μg/mL. The established FACS was used to screen anti IL-5 nanobodies and hybridoma supernatants for candidate antibodies that block the IL-5/IL-5α interaction. When compared to anti-proliferation assays, this method saved up to 90% of the assay time, offering the advantage of rapidity and accuracy *in vitro*. The assay described here provides a novel approach for rapid screening of IL-5/IL-5Rα blocking antibodies *in vitro* to accelerate the development of drugs for asthma.

Keywords: asthma, interleukin-5/interleukin-5 receptor subunit alpha, cytokine receptor common subunit beta, fluorescence activated cell sorting, blocking antibody

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1. Introduction

Asthma remains a major global public health problem with most asthma cases typically presenting as adult asthma (4.3%), clinical asthma (4.5%) and wheezing (8.6%) [1]. These types of asthma are characterised by acute episodes of increased respiratory symptoms referred to as exacerbations. The primary inflammatory pathway in severe asthma is the "type 2" or "T2" immune pathway. In addition to the T-helper 2 (Th2) inflammatory pathway, cells other than Th2 cells, including type 2 innate lymphocytes (ILC2), are also involved. Interleukin-5 (IL-5) promotes the activation and proliferation of eosinophils where peripheral blood eosinophilia and lung mucosal eosinophil infiltration are hallmarks of bronchial asthma [2,3]. The major sources of IL-5 in the pathogenesis of asthma are the Th2 and ILC2 cells [4]. IL-5 specifically binds to the interleukin-5 receptor subunit alpha (IL-5Rα), increasing the number of eosinophils and biological activity of eosinophils, leading to inflammation and tissue damage which in turn increases IL-5 expression, ultimately leading to exacerbation of asthma. Therefore, IL-5 produced by eosinophils is a driver of eosinophil growth and differentiation [5,6], it also is one of the most selective and potent activators of various eosinophil cellular functions

[7]. Thus, IL-5 is an ideal therapeutic target for eosinophil-related diseases such as asthma [8].

Antibodies that block IL-5 binding to IL-5Rα are thought to play a major role in advancing asthma treatment by reducing airway hyper-responsiveness and eosinophil infiltration. IL-5R is composed of the IL-5-specific α-subunit (IL-5Rα, CD125) and the signaling β-subunit (CSF2RB, CD131) shared by IL-3 and GM-CSF receptors [7]. The molecular weights of the α and β subunits are 60 kDa and 130 kDa, respectively, and IL-5 specifically binds to the α subunit [9]. The Pro^{352} Pro^{353} Val^{354} Pro^{355} (ppvp) motif and carboxyl-terminal (DC3) region of IL-5Rα play distinct roles in B-cell proliferation and differentiation [10]. Structural analysis of IL-5 and IL-5Rα revealed that although there are two equivalent receptor binding sites, the homodimeric IL-5 can only bind to one receptor molecule [11]. IL-5 is an important cytokine that causes allergic inflammation, humanized antibodies targeting IL-5/IL-5Rα are approved for the treatment of eosinophilic phenotype severe asthma [12]. To determine the efficacy and safety of IL-5 targeted therapy, it is important to assess the cellular systemic response to drugs targeting IL-5 or IL-5Rα [7]. Anti-IL5 monoclonal antibodies may be beneficial for asthmatics taking chronic oral steroids that mask eosinophilia [13]. Antibody-based drugs that target IL-5 or IL-5Rα which have been successfully marketed include Benralizumab [14], Mepolizumab and Reslizumab [4], all of which are administered intravenously. With the development of inhalation drug delivery methods, inhaled drugs may be a new alternative for severe asthma. Delivering the drugs directly into the lungs has the potential for rapid onset of action, reduced systemic exposure, lower dosage and needle-free administration [15]. Nebulized cetuximab, administered by inhalation with the mesh nebulizer AeronebPro™, limits tumor growth and reduces systemic side effects in mice [16]. Symptoms in the lungs and lymph nodes following inhalation of anti-thymic stromal lymphopoietin single domain antibodies (dAbs) were considered secondary to the immunogenic response to a human protein and were considered non-adverse [17]. Therefore, rapid screening of various IL-5/IL-5Rα blocking antibodies or small molecules could accelerate the development of inhaled drugs for the treatment of asthma.

Current assays to assess IL-5/IL-5Rα blocking include the murine chronic B cell leukemia (BCL_1) proliferation assay [18-20], reporter gene detection of TF-1 cell proliferation based on the IL-5-IL-5R-STAT5 pathway [21], TF-1 (hIL-5 dependent) cell proliferation assay [22], the Time-Resolved Fluorescence Assays (TRF) assay where IL-5 in B cell differentiation activity was measured by the production of IgM in BCL_1 [10,23,24], and IL-5–induced proliferation of CTLL-2 cells [2]. These methods are generally time-consuming (48 h-72 h), expensive and require purified antibodies to detect blocking. Hence, they are not conducive to the rapid screening of candidate antibodies in the early stages of drug screens. In this study, a stably transfected HEK293 cell line over-expressing IL-5Rα and CSF2RB (HEK293-IL-5Rα(D7)-CSF2RB(5D10)) was constructed. Rapid screening of hybridoma supernatant and nanobodies against IL-5 were successfully demonstrated with an optimized concentration of IL-5 in a Fluorescence Activated Cell Sorting (FACS) assay. Our results demonstrate that HEK293-IL-5Rα(D7)-CSF2RB(5D10) can be used for rapid screening of IL-5/IL-5Rα blocking antibodies, accelerating the antibody-targeting IL-5/IL-5Rα development process.

2. Materials and Methods

2.1. Cell lines and reagents

Cell lines (HEK293, HEK293T, TF-1) were purchased from Procell Life Science & Technology Co. Ltd. HEK293 and HEK293T cells were cultured in DMEM medium (D6429-500; Sigma) supplemented with 1% Penicillin/Streptomycin (PWL062; Meilunbio) and 10% fetal bovine serum (FBS, C2810-0500; VivaCell). TF-1 was cultured in RPMI 1640 medium (R0883-500; Sigma) supplemented with 10% FBS and 2 ng/mL rhGM-CSF. ExpiCHO-S™ (A29127; Gibco) [25] was purchased from Thermofisher and cultured in ExpiCHO™ expression medium (A2910001; Gibco). The 6-well cell culture plates and T25 flasks were purchased from Guangzhou Jet Bio-Filtration Co. Ltd. Lipofectamine3000 (L3000001) and FACS staining buffer (00-4222-26) were purchased from Thermofisher. RIPA Lysis Buffer (P0013B), Geneticin (G418), Puromycin Dihydrochloride and Polybrene (Hexadimethrine Bromide) were purchased from Beyotime. Hieff Canace® Gold High Fidelity DNA Polymerase (10148ES10) was purchased from Yeasen Biotechnology. Allophycocyanin (APC)-labeled anti-CD131 antibody (10516-MM01-A) was purchased from SinoBiological Inc. Phycoerythrin-labeled anti-CD125 antibody was purchased from BD PharMingen. Benralizumab [26] analogue was expressed in Expi293F with a murine IgG1 kappa constant region (Table S1, Fig. S1). Mepolizumab, Benralizumab and IL-5-Fc were provided by Sanyou Bio Inc. The vectors pcDNA3.4 (+), pspax2, pMD2.G, pLvx-puro were obtained from Addgene. HRP-conjugated mouse anti-6×His tag monoclonal antibody (HRP-66005) and DYKDDDDK tag Polyclonal antibody (Binds to FLAG® tag epitope, 20543-1-AP) were purchased from Proteintech Group.

2.2. Cloning of the human IL-5Rα gene coding sequence

The 1,260 bp DNA sequence encoding the IL-5Rα (uniprot: Q01344) was codon-optimized for humans and synthesized by Azenta Life Sciences, Flag and 6×His tags were added to the N-terminus and C-terminus of IL-5Rα, respectively. The synthesized DNA product was cloned into pcDNA3.4 (+). The plasmid pcDNA3.4-Flag-IL-5Rα-6×His was transformed into *Escherichia coli* JM108 competent cells. When required, plasmid with low endotoxin (< 1 EU/mg) content with 90% of DNA in supercoiled confirmation was extracted using the Endotoxin-Free Plasmid Maxiprep Kit (Qiagen).

2.3. Construction of human IL-5Rα stably transfected cell line

HEK293 cells were seeded at 0.2-0.4 × 10^6 viable cells/mL in a 6-well plate and grown to reach 60-70% confluence before transfection. The plasmid was transfected into HEK293 cells using Lipofectamine3000 transfection reagent (DNA:Lipofectamine3000 = 1:3). After 36h post-transfection, G418 was added to cells at a final concentration of 1mg/mL. The G418 (final concentration 1mg/mL) selective medium was changed every two days until non-transfected cells were all dead (after 12-14 days of selection [27] where cell confluence was less than 40%). The surviving cells are henceforth referred to as HEK293-IL-5Rα.

2.4. CSF2RB (CD131) gene amplification and lentiviral plasmid construction

Total RNA was extracted from TF-1 cells (expressing CSF2RB protein) and reverse transcribed into cDNA. Specific primers (Table 1) were used to amplify the two gene segments of CSF2RB (P1 and P2) respectively and the complete CSF2RB gene was amplified by overlap PCR (CD131 primers) and cloned into the pLVX-Puro vector. The resultant plasmid, pLVX-CSF2RB, was transformed into Stable3 chemically competent cells. Positive colonies were identified by colony PCR and sequencing.

2.5. Construction of human IL-5Rα-CSF2RB stably transfected cell line

The plasmids (psPAX2:pMD2.G:pLVX-CSF2RB = 1:1:2) were transferred into HEK293T cells using Lipofectamine3000 transfection reagent. The cell medium was changed 6 h after transfection; the virus supernatant was collected at 72 h and filtered with a 0.45 μm filter membrane. HEK293-IL-5Rα (D7) cells were cultured up to 20% confluency in 6-well plates following which virus supernatant and Polybrene (1,000×) were added to the cells [28]. After incubating the cells at 37°C for 24 h, the culture medium containing virus and Polybrene was removed and replaced with fresh DMEM containing 10% FBS. After 48 h, 2 μg/mL Puromycin Dihydrochloride was added to the cells until uninfected HEK293-IL-5Rα (D7) cells were completely dead (after 3-4 days of selection). The surviving cells were named HEK293-D7-CSF2RB.

2.6. Isolation of stable transfected cell clones

Cells were diluted to 1 cell/well in selective medium (IL-5Rα: medium containing 500 μg/mL of G418; HEK293-D7-CSF2RB: medium containing 1 mg/mL of G418 and 2 μg/mL Puromycin Dihydrochloride). After 10-15 days, single clones were selected, expanded, identified and analysed.

2.7. Western blotting (WB) detection of IL-5Rα protein expression in stably transfected cell lines

Cell lysates were prepared with RIPA lysis buffer according to the manufacturer's instructions. After centrifugation at 12,000 × *g* 4°C for 10 min, the supernatants were separated by 4-12% SDS-PAGE. The separated proteins were transferred onto a PVDF membrane and detected with a HRP-conjugated mouse anti-6×His tag monoclonal antibody.

2.8. Flow cytometry analysis of HEK293-IL-5Rα and HEK293-IL-5Rα(D7)-CSF2RB cells

Approximately 10^5 cells were washed twice with 500 μL FACS staining buffer. Both cells were separately incubated with antibody (dilute antibody according to instructions) at 4°C in the dark. After 30 min of incubation, cells were washed twice in FACS staining buffer and resuspended in FACS staining buffer before analyzing on a BD FACSAriaIII.

2.9. Rapid screening of murine hybridoma antibodies and nanobodies with IL-5/IL-5Rα blocking activity

Anti-IL-5 nanobodies were screened with a phage display-

Table 1. PCR primers of CSF2RB

Primer	5'-3' sequence
P1-CD131-F	AGATCTCGAGCTCAAGCTTCGAATTCCCGCCGCCACCATGGTGCTGGCCCAGGGGCTGCT
P1-CD131-R	GCCTGTCTGGTTGGAATGAGAAGTA
P2-CD131-F	TACTTCTCATTCCAACCAGACAGGC
P2-CD131-R	CGGTAGAATTATCTAGAGTCGCGGCCGCTCAACACACCTCCCCAGGCTTGTTGAC
CD131-F	AGATCTCGAGCTCAAGCTTCGAATTCC
CD131-R	CGGTAGAATTATCTAGAGTCGCGGCCG

based approach using IL-5 alpaca immune libraries and natural libraries (Shenzhen KangTi Technology Co., Ltd). The selected nanobodies' sequences were synthesized with the Fc tag at the C-terminal and ligated into the pcDNA3.4 vector for secretory expression in ExpiCHO-S™ cells. The expressed nanobodies were purified on HiTrap Protein A HP (Cytiva, 17040201) (Fig. S2). Meanwhile, anti-IL-5 murine hybridoma antibodies were obtained from hybridoma cells produced by fusing spleen cells from IL-5 immunized mice BALB/c with myeloma cells.

The FACS assay was used to detect murine antibodies in hybridoma supernatants and purified alpaca nanobodies that bind positively to IL-5 and block the IL-5/IL-5Rα interaction. IL-5 Fc protein was diluted to 0.2 μg/mL with FACS staining buffer. Hybridoma supernatants or nanobodies were diluted with FACS staining buffer and mixed with diluted IL-5-Fc in a ratio of 1:1 at 4°C for 1 h (3 parallels for each sample). Before adding the mixtures to the cells, approximately 10^5 HEK293-IL-5Rα (D7)-CSF2RB (5D10) cells were washed twice with 500 μL FACS staining buffer. The cells were resuspended in 100 μL of the mixture and incubated at 4°C for 60 min. The cells were then washed twice with 500 μL FACS staining buffer and resuspended in 200 μL FACS staining buffer. Anti-human Fc-FITC conjugated antibody was added and incubated at 4°C for 30 min in the dark. The cells were washed twice with FACS staining buffer and suspended for FACS analysis using a BD Flow Cytometer.

3. Results

3.1. WB analysis of IL-5Rα expression on HEK293 cells

To develop a cell-based blocking assay of IL-5/IL-5Rα antibodies, liposome was used to transfer the recombinant expression vector pcDNA3.4-Flag-IL-5Rα-6×His into HEK293 cells to obtain a stable cell line expressing IL-5Rα. IL-5Rα expression in HEK293-IL-5Rα cells was analyzed by WB with HRP-labeled 6×His antibody. Untransfected HEK293 cells served as the negative control. B8, D7, G4 and E11 were clones derived from single HEK293-IL-5Rα cells. Lysates of these four clones were subjected to detection by anti-6×His tag as shown in Fig. 1. Although four monoclonal cells were isolated from the G418 screened clone pools, only clones D7 and G4 successfully expressed IL-5Rα protein, while clones B8 and E11 did not detect IL-5Rα expression.

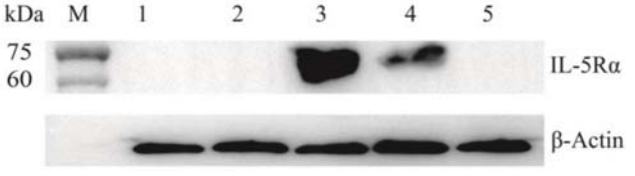

Fig. 1. WB analysis of the stable IL-5Rα cell line. Lane 1: negative control (HEK293), Lanes 2-5: B8, D7, G4, E11 cloned from HEK293-IL-5Rα. WB: Western blotting, IL-5Rα: interleukin-5 receptor subunit alpha.

3.2. FACS analysis the IL-5Rα expression and activity of HEK293-IL-5Rα

To further detect the IL-5Rα expression and activity of the four clones (B8, D7, G4, E11), 5×10^4 cells of the four clones and specific antibodies were used for FACS analysis. DYKDDDDK tag polyclonal antibody was used to detect the expression of the N-terminal Flag tag of IL-5α in the four clones (Fig. 2A). Among them, D7 and G4 were detected the N-terminal FLAG tag of IL-5Rα protein in HEK293. An anti-IL-5Rα antibody (Benralizumab analogue) and IL-5-Fc were used to detect the expression of IL-5Rα in all four clones (Fig. 2B and 2C). The results showed that both D7 and G4 cloned cells expressed IL-5Rα which could bind to IL-5, indicating that IL-5Rα had biological activity expressed on the surface of HEK293 cells. The IL-5Rα expression level of D7 was relatively higher than that of G4 (Fig. 2, Table 2). Based on these results, the D7 clone was selected for the construction of the CSF2RB stable cell line.

3.3. Construction and detection of CSF2RB stably transfected cell line based on HEK293-D7

To improve the binding ability of IL-5 to clone D7 cells to reduce the use of IL-5-Fc in FACS blocking assay, the CSF2RB gene was transferred into D7 cells. The TF-1 cells were analyzed by FACS using the APC-conjugated anti-CD131 antibody to confirm the expression of CSF2RB protein on the surface of TF-1 cells, the results showed higher expression of the CSF2RB protein in the TF-1 cells (Fig. 3A). Therefore, TF-1 cells were selected for CSF2RB gene amplification. Total RNA was extracted from TF-1 cells (Fig. 3B) and reverse transcribed. The CSF2RB gene was amplified by overlap PCR (Fig. 3C) and ligated into the plasmid pLvx-puro (digested by *BamH* I and *Not* I) by seamless cloning technology. After lentivirus infection and

Table 2. Mean fluorescence intensity value of the FACS analysis

Cell line	Anti-Flag tag	Benralizumab analogue	IL-5-Fc
HEK293	117	142	150
B8	136	124	170
D7	2,273	1,012	419
G4	356	489	316
E11	133	122	122

FACS: Fluorescence Activated Cell Sorting, IL-5: Interleukin-5.

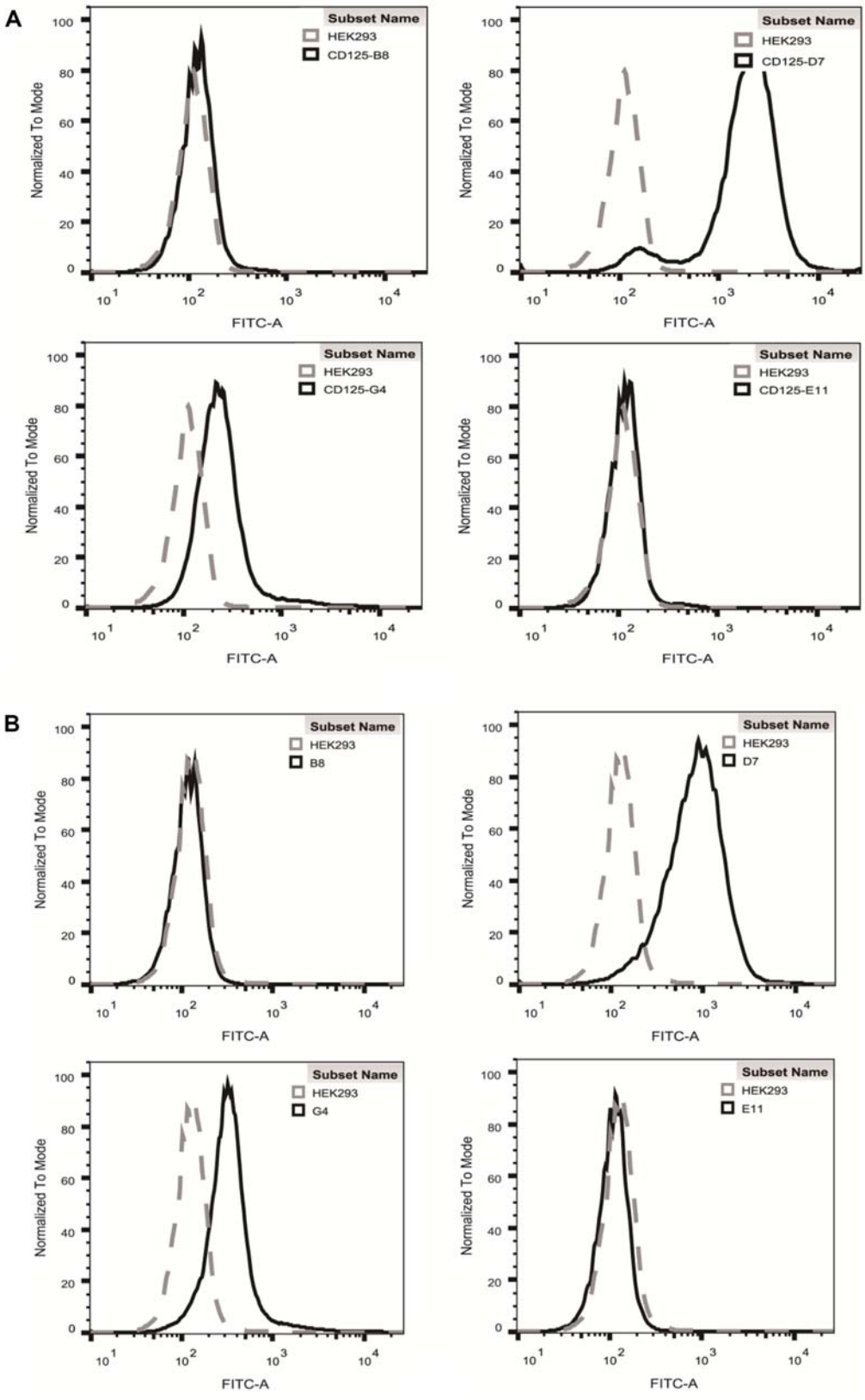

Fig. 2. FACS assay after subcloning of HEK293-IL-5Rα cells. (A) B8, D7, G4 and E11 cells were screened with the anti-Flag antibody. (B) Antibodies secreted by B8, D7, G4 and E11 cells were detected with IL-5Rα specific antibody Benralizumab analogue. (C) B8, D7, G4 and E11 clones were assayed for their ability to bind IL-5. FACS: Fluorescence Activated Cell Sorting, IL-5Rα: interleukin-5 receptor subunit alpha.

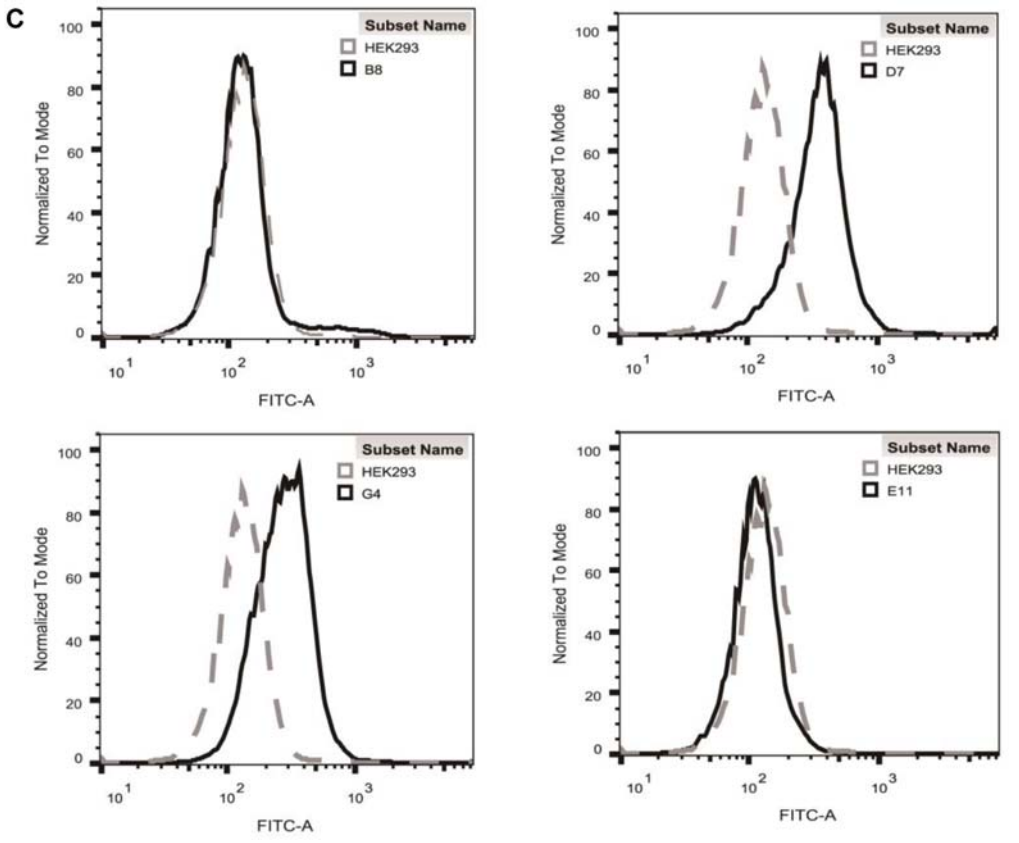

Fig. 2. Continued.

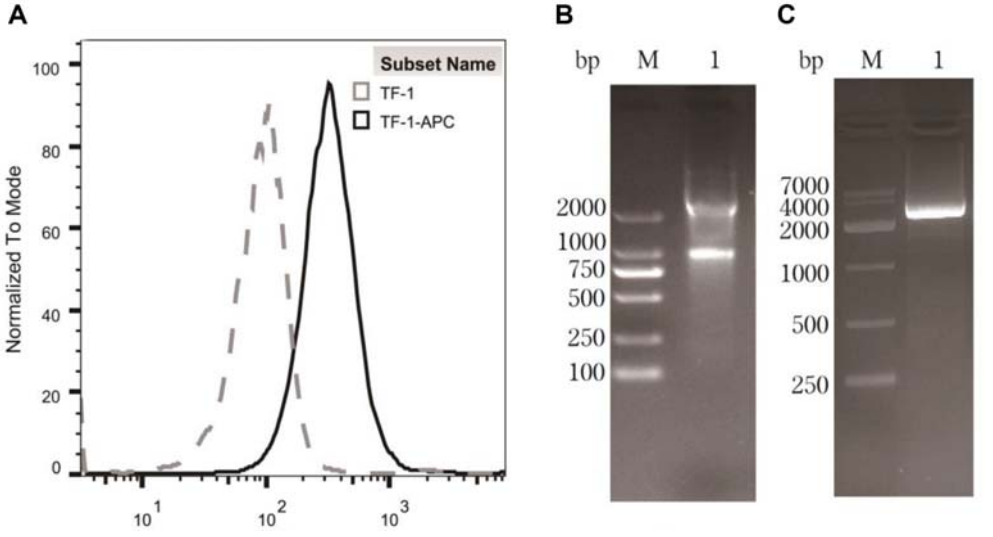

Fig. 3. CSF2RB gene amplification. (A) FACS assay detected the overexpression of CSF2RB in TF-1 cells. (B) Total RNA extraction from TF-1 cells. Lane M: DNA marker, Lane 1: total RNA of TF-1 cells. (C) PCR amplification of CSF2RB. Lane M: DNA marker, Lane 1: amplified product for CSF2RB. FACS: Fluorescence Activated Cell Sorting, APC: allophycocyanin.

puromycin screening, APC-anti CD131 mAb was used to screen the clone pools by FACS detection. High expression of CSF2RB protein was observed in HEK293-IL-5Rα (D7)-CSF2RB clone pools (results not shown). Monoclonal cells of HEK293-D7-CSF2RB were isolated and designated D7-1E5, D7-2D2 and D7-5D10. The single cell clones were

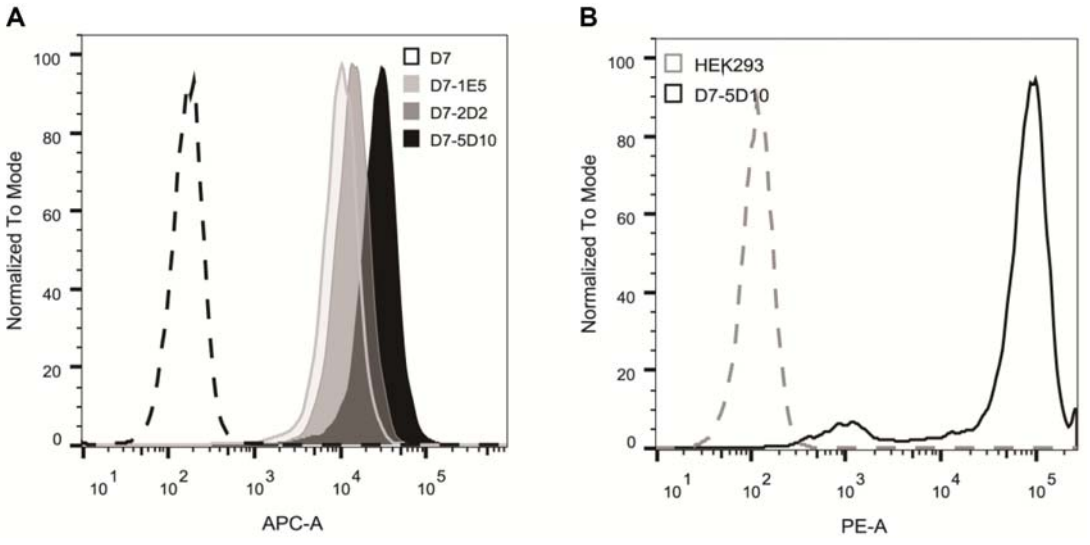

Fig. 4. FACS analysis of CSF2RB and IL-5Rα expression. (A) Detection of CSF2RB over-expression on the surface of D7-1E5, D7-2D2 and D7-5D10 using the APC-anti CD31 antibody (APC-A). (B) Detection of IL-5Rα over-expression on the surface of D7-5D10 using the PE-anti CD125 antibody (PE-A). FACS: Fluorescence Activated Cell Sorting, IL-5Rα: interleukin-5 receptor subunit alpha, APC: allophycocyanin, PE: phycoerythrin.

then subjected to FACS detection (anti-CD131). As shown in Fig. 4A, all three clones overexpressed CSF2RB compared to HEK293-D7 cells with D7-5D10 showing the highest expression of CSF2RB. Both IL-5Rα and CSF2RB proteins were co-expressed in D7-5D10 (Fig. 4). D7-5D10 co-expressing CSF2RB and IL-5Rα had increased IL-5 binding by approximately 16% (mean fluorescence intensity [MFI] of D7-5D10: 319; MFI of D7: 266; MFI of HEK293: 141) compared to D7 cells expressing only IL-5Rα (Fig. 5).

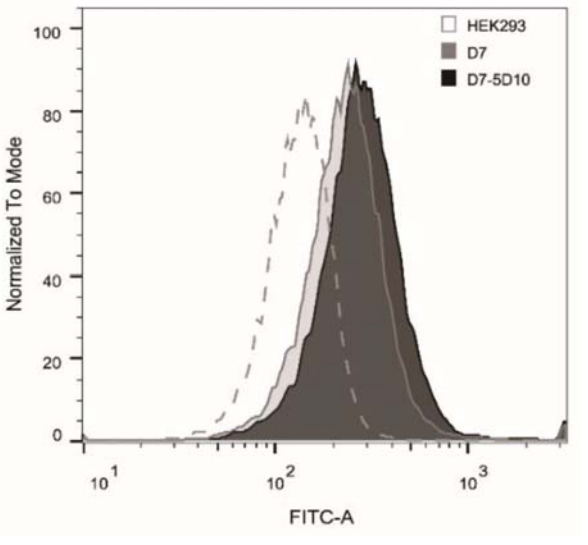

Fig. 5. Comparison of D7 (expressing IL-5Rα) and D7-5D10 (co-expressing CSF2RB and IL-5Rα) cells binding to IL-5 via IL-5-Fc. IL-5Rα: interleukin-5 receptor subunit alpha.

3.4. Optimization of the binding assay for HEK293-D7-5D10

Key experimental parameters of the IL-5 binding assay were initially optimized before assessing potential blocking of IL-5/IL-5Rα, including the use of different concentrations of IL-5. Different concentrations of IL-5-Fc, 20, 6.67, 2.22, 0.74, 0.25, 0.08, 0.027 and 0.0027 μg/mL, were added to HEK293-D7-5D10 cells (2 parallels for each concentration) and the binding was detected by FACS (IgG1 with Fc tag was used as negative control). MFI values for the different IL-5 concentrations were calculated using the FLOWJO (https://www.flowjo.com/) software. The representative plot of the binding characteristics of IL-5-Fc and HEK293-D7-5D10 cells in Fig. 6 showed that IL-5 binds strongly to

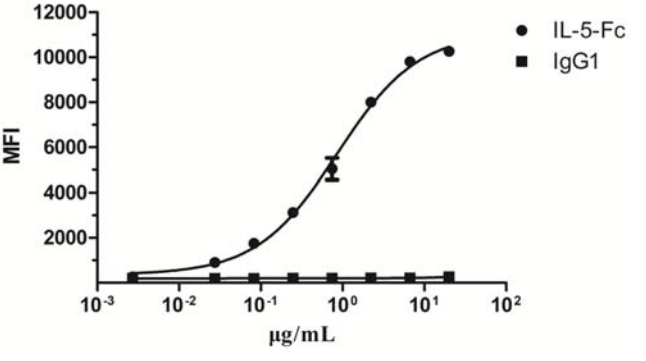

Fig. 6. Mean fluorescence intensity (MFI) measured by FACS at different concentrations of IL-5 binding to the D7-5D10 clone cells. FACS: Fluorescence Activated Cell Sorting, IL-5: Interleukin-5.

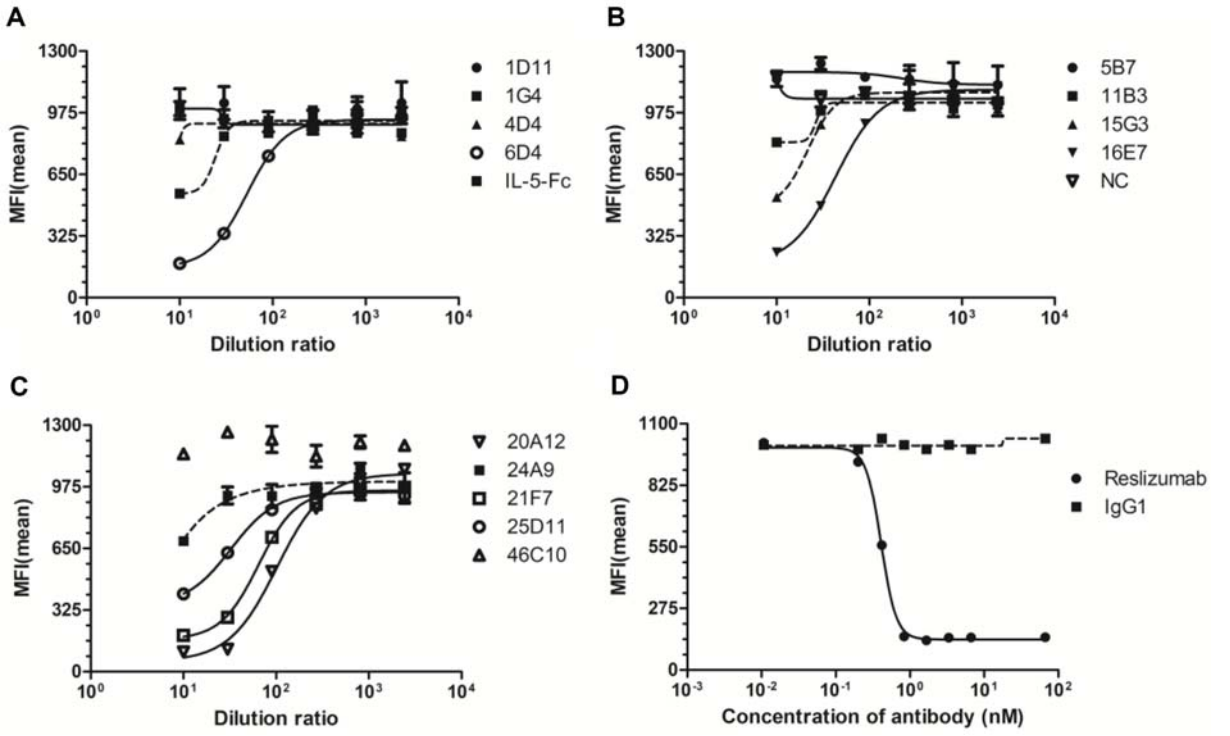

Fig. 7. FACS detection of murine hybridoma antibodies in hybridoma cell supernatants. 1D11, 1G4, 4D4, 6D4, 5B7, 11B3, 15G3, 16E7, 20A12, 24A9, 21F7, 25D11, 46C10: 13 supernatants of the parental hybridoma for FACS blocking assay, IL-5-Fc: 0.1 μg/mL IL-5-Fc without hybridoma supernatants, IgG1: negative controls. FACS: Fluorescence Activated Cell Sorting, MFI: mean fluorescence intensity, IL-5: Interleukin-5.

IL-5Rα on the surface of HEK293-D7-5D10 cells (EC_{50} = 0.859 μg/mL).

3.5. Blocking detection of hybridoma cell supernatants and nanobodies

Due to the low concentration of specific antibodies in the hybridoma supernatant (10-50 μg/mL), 0.1 μg/mL of IL-5-Fc was used in the FACS to improve the sensitivity of the blocking assay. Antibody (Reslizumab) was used to validate the feasibility of HEK293-D7-5D10 for blocking assays (Fig. 7D). To validate the blocking effect of IL-5-positive supernatants from the parental hybridoma cells, IL-5-Fc was mixed with diluted supernatants (IL-5-negative supernatants, named NC, from other hybridoma cells were used as negative control) prior to incubation with HEK293-D7-5D10. Fig. 7A, 7B, and 7C showed that with increasing dilution ratio, the blocking effect of the clones (6D4, 16E7, 20A12, 21F7, 25D11) decreased significantly and the blocking effect of the clones (1G4, 11B3, 15G3, 24A9) decreased slightly. In addition, some clones (1D11, 4D4, 5B7, 46C10 and NC) had no significant blocking effect. Subclones of the 5 parental hybridoma clones (significant blocking) were also positive for blocking (data not shown), the purified monoclonal antibodies (20A12-1, 16E7-1, 25D11A8-1) significantly inhibited the proliferation of TF-1 cells, which depend on IL-5 for proliferation (Fig. S3). To test the suitability of other types of antibodies, nanobodies were used for functional validation of cells. The IL-5 positive nanobodies were detected by FACS analysis with huNB66 (Patent No. CN112225807B) as a positive blocking control. As shown in Fig. 8, Z6 and Z8 nanobodies had no

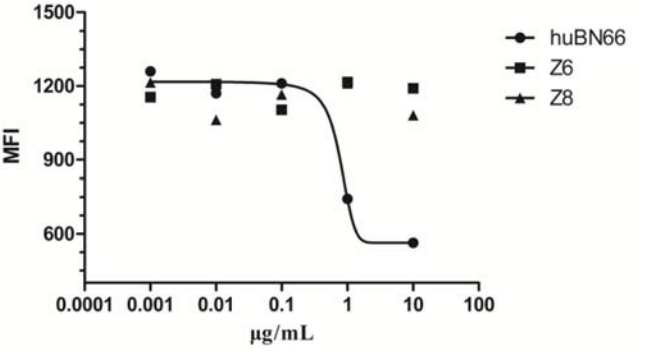

Fig. 8. FACS blocking detection of nanobodies. The nanobodies, Z6 and Z8 were tested for binding to IL-5. HuBN66 was included as the positive blocking control. FACS: Fluorescence Activated Cell Sorting, MFI: mean fluorescence intensity, IL-5: Interleukin-5.

blocking effect, whereas huNB66 showed a significant blocking effect which is consistent with the proliferation results of TF-1 mentioned in the patent (CN112225807B).

4. Discussion

Asthma is a chronic and heterogeneous airway disease characterized by respiratory symptoms such as wheezing, coughing, chest tightness and breathlessness [29]. In particular, many patients with severe asthma had an elevated type 2 (T2) phenotype characterized by eosinophilic inflammation [30]. T2-elevated asthma is characterized by the accumulation of eosinophils within the airways. IL-5 is the major biological factor affecting differentiation, growth, activation, survival and recruitment of eosinophils into the airways. IL-5 plays a central role as the most important pathogenic mediator of eosinophilic asthma and is also an important therapeutic target for biological anti-asthma therapy [29].

IL-5 is a homodimeric cytokine and binds to its specific receptor (IL-5R) located on target cells. The α chain of IL-5R (IL-5Rα) and β-chain (βc) of IL-3R and GM-CSFR together form a ternary complex to activate the JAK/STAT signaling pathway [11]. Therefore, IL-5 and IL-5Rα are ideal targets to develop new treatments for asthma. Although there are commercially available intravenous antibody drugs targeting IL-5Rα or IL-5, with the development of inhaled drugs, targeting IL-5 or IL-5Rα inhaled drugs (nanobody, modified antibody, double antibody, peptide and so on) are getting more attention. The drug discovery pipeline requires rapid screening of candidate antibodies or proteins. In our study, the IL-5Rα stably transfected cell line was constructed and shown to secrete IL-5Rα sized at ~60 kDa. The observed protein size was larger than the predicted molecular weight of 49.51 kDa, where the higher molecular weight observed for the expressed receptor molecule may be due to glycosylation or other post-translational modifications [31]. A stable IL-5Rα-CSF2RB cell line was constructed by lentivirus infection, in which the CSF2RB gene was amplified from TF-1 cDNA. Due to repetitive sequences in the CSF2RB, the two sequences of CSF2RB were amplified by segmented amplification, and the complete CSF2RB gene was obtained by overlapping amplification and followed by ligation to a lentiviral vector by seamless cloning.

It has been reported that the complex dimer protein of CSF2RB and IL-5Rα can significantly increase the affinity of IL-5Rα and IL-5 [11]. However, in this study, although IL-5Rα and CSF2RB proteins were co-expressed in HEK293 cells, co-expression did not significantly increase the binding of IL-5 compared with cells expressing IL-5Rα alone. On the one hand, this could be due to the inability of the stable transfected cell lines to form a ternary complex [32] even though they co-expressed two membrane proteins. Or the CSF2RB protein maybe already interacted with overexpressed IL-5Rα to form a ternary complex, although additional overexpression of CSF2RB protein did not increase the ability of IL-5Rα to bind to IL-5. According to the results on the EMBL-EBI website (https://www.ebi.ac.uk/), the Transcripts Per Million (TPM) of IL-5Rα in the kidney was below the cut-off value, whereas the TPM of CSF2RB was 2-3. Benralizumab analogue (constant domain of mouse IgG1, kappa, targeting IL-5Rα) and Mepolizumab (targeting IL-5) were used to validate the feasibility of the blocking assays. Both antibodies showed an obvious blocking effect. At the same time, different hybridoma supernatants (6D4, 20A12, 16E7, 25D11) were screened and shown to have a significant blocking effect. After subcloning and purification of the hybridomas, the data obtained were consistent with the TF-1 proliferation assay (results not show). This method can also be used for rapid screening of IL-5/IL-5Rα blocking antibodies targeting IL-5.

There are two methods for IL-5/IL-5Rα blockade detection: *in vitro* activity assay and *in vivo* activity assay. The *in vitro* activity assays include surface plasmon resonance (SPR) but the SPR assay is costly. The *in vivo* activity assay includes the cell proliferation assay which takes a while to complete (48-72 h), is costly and has high variability in the data with low signal-to-noise ratio [21]. In this study, the FACS-based blocking assay was cost-effective with short detection time (< 2 h) high sensitivity and potentially applicable to different types of antibodies and other applications. However, the method provides no information about biological effects and cell proliferation inhibition assays are still needed to gauge the biological effects of candidate antibodies. In this study, the stable cells IL-5R (D7)-CSF2RB (5D10) were successfully used as a rapid screen for IL-5/IL-5Rα blocking by hybridoma supernatants and nanobodies. In the future, other IL-5/IL-5Rα blocking molecules such as peptides [33], modified antibodies, *etc*. may also be rapidly screened by this FACS-based method to accelerate the whole drug development process.

5. Conclusion

In summary, we have established a stable cell line expressing IL-5Rα and a stable cell line co-expressing IL-5Rα and CSF2RB, respectively. From the FACS analysis, both the cell line expressing IL-5Rα and the cell line co-expressing IL-5Rα and CSF2RB could bind to IL-5, and the transfected cell line co-expressing IL-5Rα and CSF2RB had better ability to bind to IL-5. In the FACS blocking IL-5 and IL-5Rα binding assay, the constructed stable cell lines can be

used for hybridoma cell supernatants and nanobodies for blocking assays to accelerate the development of asthma drugs targeting IL-5.

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Author's Contributions

Designed and carried out the experiments to construct stably cell lines: Shijie Li and Fei Han; Designed and carried out the FACS assay: Shijie Li, Chang Liu, and Weiyan Dai; Protein expression and purification: Wenfeng Ke; Drafted the work and revised: Shijie Li and Eric Fordjour; Final approval of the version to be published: Shijie Li, Yongqi Chen, Yankun Yang, and Zhonghu Bai.

Ethical Statements

The authors declare there is no conflict of interest.
No human or animal subjects were involved in the study so there was no need for any ethical approval or consent.

Electronic Supplementary Material (ESM)

The online version of this article (doi: 10.1007/s12257-022-0315-2) contains supplementary material, which is available to authorized users.

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