Immunobiology 225 (2020) 151998

Contents lists available at ScienceDirect

Immunobiology

journal homepage: www.elsevier.com/locate/imbio

The establishment of humanized IL-4/IL-4RA mouse model by gene editing and efficacy evaluation

Peili Yan[a], Youhong Su[b], Chengzhang Shang[b], Xiaofei Zhou[b], Yi Yang[b], Wenqian An[b], Wenlin An[a], Changyuan Yu[a,**], Shihui Wang[a,c,*]

[a] College of Life Science and Technology, Beijing University of Chemical Technology, Beijing 100029, China
[b] Beijing Biocytogen, Beijing 102600, China
[c] Qinhuangdao BohaiBiological Research Institute of BUCT, No.41 of Shugu Avenue, Qinghuangdao, Hebei, 066000, PR China

ARTICLE INFO

Keywords:
Humanized IL-4
Humanized IL-4RA
Asthma, Cytokine targets
Mouse model

ABSTRACT

Asthma is a common respiratory immune disease in children and adults, and interleukin-4 (IL-4) is one of the key factors for the onset of asthma. Therefore, targeting human IL-4 and IL-4 receptor alpha (IL-4RA) has become one of the strategies for targeted therapy of cytokines. Herein, we established an animal model of asthmatic airway inflammation using double humanized IL-4/IL-4RA (hIL-4/hIL-4RA) mice, where human IL-4 and IL-4RA replaced their murine counterparts, respectively. We successfully identified the phenotype by Southern blotting, ELISA, and flow cytometry. The hIL-4/hIL-4RA mice induced by ovalbumin (OVA) exhibited several important features of asthma, such as inflammatory cell infiltration, IgE release, goblet cell hyperplasia, and Th2 cytokine secretion. Furthermore, treatment of these humanized mice with anti-human IL-4RA antibodies significantly inhibited level of these pathological indicators. Thus, hIL-4/hIL-4RA mice provide a validated preclinical mouse model to interrogate new therapeutic agents targeting this specific cytokine pathway in asthma.

1. Introduction

Asthma is a common immunological disease and is characterized by airway hyperresponsiveness (AHR), excessive mucus secretion, eosinophils infiltration, and elevated immunoglobulin E (IgE) (Lambrecht and Hammad, 2014a, 2014b). The imbalance of helper T cell 1 (Th1) / helper T cell 2 (Th2) is crucial for the pathogenesis of asthma (Kon and Kay, 1999).Th2 responses are associated with the differentiation of T lymphocytes and the recruitment of eosinophils (Deckers et al., 2003) Inhaled allergens provoke $CD4^+$ Th2 cells to produce certain cytokines (*e.g.* IL-4, 5, 13) and chemokines (*e.g.* regulated upon activation normal T cell expressed and secreted factor (RANTES), monocyte chemotactic protein-3 (MCP-3), and MCP-4) (Gavett et al., 1994). The expression of these cytokines results in the recruitment of eosinophils, mucus production, airflow obstruction, and AHR (Zavorotinskaya et al., 2003). Chemokines mainly act as supplementary of cytokines, which primarily mediate eosinophil recruitment. The cytokines IL-4 and IL-13 share many biological functions critical to the allergic response (Borish, 2010). It has been well established that IL-4 is critical for Th2 differentiation and IgE synthesis in B lymphocytes (together with IL-13). IL-5 selectively acts on eosinophils maturation, survival, and activation (Martinez and Vercelli, 2013; Steinke and Borish, 2001). IgE level in Bronchoalveolar Lavage Fluid (BALF) is closely correlated to IL-4 and IL-13 secretion. Therefore the increase of IL-4, IL-5 and IL-13 indicates the occurrence of Th2 inflammation in asthma (Lambrecht and Hammad, 2012).

Traditionally, the main clinical treatments for moderate and severe and persistent asthma include inhaled corticosteroids (ICS), leukotriene pathway inhibitors and long-acting β-agonist (LABA) (Bateman et al., 2004). Long-term management of asthma with steroid therapy may lead to severe side effects, such as infection, hypertension, hyperglycemia, obesity, aseptic necrosis of the femoral head, and nerve excitement (Yeo et al., 2017). Therefore, there is a clear and urgent need for developing safe and long-lasting therapeutics for asthma. The more recent focus on asthma treatment is to target key cytokine pathways, which are more specific and have fewer side effects than conventional treatments. Targeting IL-4 and IL-4 receptor alpha (IL-4RA) has been one of the essential strategies of cytokine-targeted therapy (Kau and Korenblat, 2014; Maes et al., 2012). IL-4RA is a shared subunit of both the IL-4 and IL-13 receptor, which is expressed on T-lymphocytes, B-lymphocytes, eosinophils, mononuclear phagocytes, endothelial cells, lung fibroblasts, bronchial epithelial cells and smooth muscle cells (Humbert

* Corresponding author at: College of Life Science and Technology, Beijing University of Chemical Technology, Beijing 100029, China.
** Corresponding author.
E-mail addresses: yucy@mail.buct.edu.cn (C. Yu), wangshihui@mail.buct.edu.cn (S. Wang).

https://doi.org/10.1016/j.imbio.2020.151998
Received 13 April 2020; Received in revised form 6 July 2020; Accepted 29 July 2020
Available online 03 August 2020

et al., 1998, August et al., 2018). Binding of IL-4RA to IL-4 could reduce the level of IL-4 in the body and exert anti-inflammatory effect in asthma patients. Thus, blocking IL-4 by IL-4RA has been deemed as an effective therapy for asthma treatment (Agache and Akdis, 2016).

Animal models have been established that enable the elucidation of the pathological and physiological mechanisms underlying the disease and preclinical testing of new drugs (Zosky and Sly, 2017). For the induction of asthmatic airway inflammation, mice are sensitized to various antigens, including ovalbumin, house dust mite (HDM), and cockroach. After sensitization, inhalation of the allergen induces the expression of type-2 cytokines in the lungs, resulting in antigen-specific IgE, lung eosinophilia, and AHR (Zosky et al., 2004; Fattouh et al., 2005; Fan and Mustafa, 2016).

However, there is currently no method to directly assess the *in vivo* efficacy and toxicity of antibodies that target the human IL-4 or IL-4RA in mice since such anti-human antibodies don't cross react with their murine counterparts. To circumvent this limitation, we established an animal model where human IL-4 and human IL-4RA replaced the murine counterparts. We show in the present study that this humanized mouse strain allows investigating the efficacy and toxicity of anti-human IL-4RAantibodies. This humanized mouse model will facilitate development of new therapies based on IL-4 and IL-4RA.

2. Materials and methods

2.1. Generation of humanized IL-4/IL-4RA mice

2.1.1. Generation of humanized IL-4 mice (hIL-4 mice)

On the C57BL/6 J genetic background, a contiguous mouse genomic sequence of approximately 6.3 kb at the endogenous mouse IL-4 locus, including exon1 starting from the ATG initiation codon through exon4 ending in the TAG stop codon, was deleted and replaced with approximately 8.5 kb of human IL-4 genomic sequence comprising exon1 starting from the ATG initiation codon through exon4 ending in the TAG stop codon of human IL-4 gene.

The hIL-4 targeting vector was designed to contain homology regions, human DNA, Frt-flanked Neo resistance cassette and diphtheria toxin A (DTA) cassette (Fig. S1A). The mouse and human genomic DNA fragments from relevant BAC clone were cloned to the vector pL451 (Beijing Biocytogen Co., Ltd, China) to form an intermediate vector, which contained Frt-flanked Neo resistance cassette. The BAC clones from C57BL/6 J mouse genomic BAC library were modified with PmlI (hIL-4) intermediate vector fragment by homologous recombination. These modified BACs were used to construct the targeting vectors. The targeting vector contains a diphtheria toxin A (DTA) cassette. The DTA gene was used for negative selection of clones with random integration.

The targeting vectors were linearized before they were transfected into C57BL/6 J embryonic stem (ES) cells (Beijing Biocytogen Co., Ltd, China) by electroporation respectively. The G418-resistant ES clones were screened for homologous recombination by PCR. Correctly targeted clones were confirmed by Southern blot analysis with probes. Confirmed clones were injected into Balb/c blastocysts and implanted into pseudopregnant females to generate chimeric mice. Chimeric mice were bred to Flp mice to obtain F1 mice carrying the recombined allele containing the deleted-neo allele.

2.1.2. Generation of humanized IL-4RA mice (hIL-4RA mice)

In mouse IL-4RA locus, the genomic sequence from exon4 to exon7 was deleted and replaced with approximately 10.5 kb of human IL-4RA genomic sequence comprising exon4 starting from the Q30 codon (CAG) through exon7 ending to the S216 codon (AGC) of the human IL-4RA gene (seen in Fig. S2A).

The method for generation of hIL-4RA mice was similar to that for hIL-4 mice described above, except that the BAC clones from C57BL/6 J mouse genomic BAC library were modified with ClaI (IL-4RA)-digested intermediate vector fragment by homologous recombination.

2.1.3. Generation of humanized IL-4/IL-4RA mice

The humanized IL-4/IL-4RA (hIL-4/hIL-4RA) double knock-in mice were generated by crossing hIL-4 mice and hIL-4RA mice.

2.2. Phenotypic analyses of humanized IL-4/IL-4RA mice

2.2.1. Analysis of human IL-4 protein and IL-4RA protein expression

Humanized IL-4/IL-4RA mice and C57BL/6 mice (as wild-type mice) respectively received one dose of intraperitoneal injection of 7.5 μg mCD3 antibody. Sera were prepared and spleen was collected 1.5 h after the injection. According to the manufacturer's instructions, the expression of IL-4 protein in the serum of hIL-4/hIL-4RA mice and wild-type (WT) mice was detected using a species-specific ELISA kit (Biolegend, USA). Flow cytometry was performed to detect the expression of IL-4RA protein in spleen cells of hIL-4/hIL-4RA mice and WT mice.

2.2.2. Flow cytometry

Immune cells in the spleen were analyzed by flow cytometry and stained with antibodies. Splenocytes were prepared using ACK lysis buffer (Beyotime, China) to remove red blood cells (RBCs) and were incubated in the dark for 10 min at 4 °C in the presence of a mixture of live/dead stains (Biolegend, USA) and anti-mCD16/32 (Biolegend, USA) to block nonspecific antibody. After that, the cells were subjected to dark for 30 min at 4 °C in the presence of a mixture of appropriate fluorescently labeled monoclonal antibodies. The cells were washed with PBS and then suspended in the fluorescent-activated cell sorting (FACS) staining buffer (PBS containing 2 % FBS).

The antibodies against mouse cell-surface molecules were listed as follows (purchased from Biolegend, USA) (against mouse targets unless otherwise noted): anti-CD3-Alexa Fluor 488 (clone 17A2, USA), -CD19-APC-Cy7 (clone 6D5, USA), -CD4-BV421 (clone GK1.5, USA), -CD8-BV711 (clone 53−6.7, USA), -CD11c-BV605(clone N418, USA), -CD11b-PE (clone M1/70, USA), -NK1.1-PE-Cy7 (clone PK136, USA), -F4/80-FITC (clone 8M8, USA), -Gr1-APC (clone RB6−8C5, USA), -human IL-4RA -PE antibodies (clone G077F6, USA).

For staining of intracellular cytokine, cells were fixed and permeabilized in Zombie NIR™Fixable Viability Kit (BioLegend,USA), and then stained with Foxp-APC (clone FJK-16 s, USA). After that, cells were subjected to multicolor flow cytometry with Attune NxT (Thermo Fisher Scientific, USA) and analyzed using FlowJo 10.

2.2.3. IgE induction and measurement

Splenocytes were prepared from hIL-4/hIL-4RA mice and WT mice. Primary B cells were isolated and purified from splenocytes by negative selection using CD3ε MicroBead Kit (Miltenyi Biotec, Germany) to remove T cells from the sample according to the manufacturer's instructions. Briefly, splenocytes were washed with mac buffer, added with CD3e-Biontin (5 μL/10^7 cells), incubated at 4 °C for 10 min, and neutralized with mac buffer. The sample was washed with mac buffer, added with Anti-Biotin microbead (10 μL/10^7 cells), incubated at 4 °C for 15 min, and neutralized with mac buffer. Then the sample was washed with mac buffer and loaded in LD column. The separated B cells were counted and verified by flow cytometry. B cells (4×10^6/mL) were cultured in RPMI 1640 (Gibco, UK) supplemented with 10 % FBS, 1% penicillin and streptomycin and recultured with 50 ng/mL lipopolysaccharide (LPS) alone or 50 ng/mL LPS add with 50 ng/mL IL-4. The culturing supernatant was collected on day 6 for quantification of total IgE by ELISA (Amirzargar et al., 2009).

2.3. In vivo efficacy evaluation of anti-human IL-4RA antibody

2.3.1. Induction of allergic asthma and treatment

Humanized IL-4/IL-4RA mice (4–5 week-old females, n = 7–8) were randomized into four groups: the phosphate-buffered saline (PBS) group (denoted as PBS), the OVA group (denoted as OVA), the OVA +

isotype antibody group (denoted as Isotype), and the OVA + positive drug group (denoted as Positive drug). Mice in OVA-containing groups (OVA, Isotype, and Positive drug groups) were injected intraperitoneally with 40 μg of OVA (Grade V, Sigma-Aldrich, USA) and 2.0 mg of aluminum hydroxide (sigma-Aldrich, USA) in 200 μL of PBS for sensitization on days 0, 7, and 14. Then these mice were challenged by nebulization to 2% OVA (Grade V, sigma -Aldrich, USA) for 30 min on days 21, 22, 23, 24, and 25. Mice in the PBS group were sensitized and challenged with PBS alone. On day 26, mice in all groups were sacrificed and various tissues were collected for analysis. The Isotype antibody group and the Positive drug were administered i.p. with 25 mg/kg of human IgG4 isotype control antibody (CrownBio, China) or anti-human IL-4RA monoclonal antibody (Beijing Biocytogen Co., Ltd, China) on days 20 and 23.

2.3.2. Serum collection and analysis

Twenty-four hours after the last challenge with OVA or PBS, whole blood was collected from hIL-4/hIL-4RA mice and serum was prepared. OVA-specific IgE and mouse total IgE levels in serum were determined using commercial ELISA kits (Biolegend, USA) respectively, according to the manufacturer's instructions.

2.3.3. Bronchoalveolar lavage collection and analysis

Twenty-four hours after the last challenge with OVA or PBS, mice were sacrificed immediately by pentobarbital sodium injection. The trachea was cannulated with 18 G needle, and then the lung was lavaged with 0.5 mL sterile PBS thrice repeatedly. The fluid was centrifuged at 400 × g for 7 min at 4 °C. The cell pellet was suspended immediately in 25 μL FACS staining buffer and incubated in the dark for 10 min at 4 °C in the presence of a mixture of live/dead stains (Biolegend, USA) and anti-mCD16/32 (Biolegend, USA) to block non-specific antibody. After that, the cells were subjected to dark for 30 min at 4 °C in the presence of a mixture of appropriate fluorescently labeled monoclonal antibodies. The following antibodies against mouse cell-surface molecules were used: anti-CD45-FITC (Biolegend, clone 30-F11, USA), -CD11c-PE/Cy7 (eBioscience, clone N418, USA), -CD11b-V450 (BD, clone M1/70, USA), -Ly6G-AF700 (Biolegend, clone 1A8, USA), -SiglecF-PE (BD, clone E50 − 2440, USA). The cells were washed with PBS and then suspended in FACS staining buffer. The cells was subjected to multicolor flow cytometry with Attune NxT (Thermo Fisher Scientific, USA), and analyzed using FlowJo 10.

2.3.4. Histology

Lung tissue was prepared for histological analyses by fixation in 10 % buffered formalin. Subsequently, paraffin was applied for routine embedding, then sectioned with 5 μm and subjected to hematoxylin and eosin (H&E) staining. At last, these tissues were scored for mucus secretion and inflammatory cells based on this principle, which was absent (0), rare (1), mild (2), moderate (3), or severe (4) by an observer blinded. Periodic acid-schiff (PAS) protocols were served to assess airway epithelial goblet cell.

2.3.5. Quantitative real -time PCR

Lung tissues were harvested from five hIL-4/hIL-4RA mice and frozen in liquid nitrogen. Total RNA from lung tissues was extracted using Trizol reagent (TAKARA Bio Inc) according to the manufacturer's instructions. The mRNA expression of hIL-4 and mIL-13 was determined using GAPDH as the internal control. Primer annealing temperatures and number of cycling were as follows: 95 °C for 2 min, 45 cycles of 95 °C for 15 s, and 60 °C for 1 min. The primer sequences were as follows:

hIL-4 Forward, AACAGCCTCACAGAGCAGAAGAC;
hIL-4 Reverse, GTGTTCTTGGAGGCAGCAAAG;
mIL-13 Forward, CCAGGTCCACACTCCATACC;
mIL-13 Reverse, TGCCAAGATCTGTGTCTCTCC;
GAPDH Forward, ACCACAGTCCATGCCATCAC;
GAPDH Reverse, GCCTGCTTCACCACCTTCTT.

In order to calculate the efficiency of the amplification, the relative quantity of each target gene was obtained using comparative $2^{-\Delta\Delta CT}$ method.

2.4. Statistical analyses

The results were expressed as the mean ± SEM. SPSS 19 was used to perform the statistical analysis and Graph Pad Prism 6 software was used for graphical plotting of data. Comparisons of all data were made using Student's *t*-test and one-way analysis of variance (ANOVA). *P* values < 0.05 were considered statistically significant. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and **** $p < 0.0001$.

2.5. Study approval

The experimental animals were housed in the SPF barrier facility of the Animal Center of Beijing Biocytogen Co., Ltd. The experimental animals were acclimatized for 3 days before being used in experiments. All animal procedures were in accordance to IACUC guidance.

3. Results

3.1. Generation of humanized IL-4/IL-4RA mouse model and phenotypic analysis

3.1.1. Generation of humanized IL-4/IL-4RA mouse model

Humanized IL-4 mice and humanized IL-4RA mice were respectively generated by gene targeting on the C57BL/6 J genetic background and confirmed by Southern blot analyses (Fig. S1 and Fig. S2 in the supplement data). Humanized IL-4 mice and humanized IL-4RA mice were born at normal Mendelian ratios, developed normally and showed no apparent abnormalities when housed in a standard pathogen-free facility. After that, humanized IL-4/IL-4RA double knock-in mice were generated by crossing hIL-4 mice and hIL-4RA mice.

3.1.2. Human IL-4 and IL-4RA expression in humanized IL-4/IL-4RA mice

The hIL-4/hIL-4RA mice were supposed to only express human IL-4 and IL-4RA proteins, while WT mice could only express mouse IL-4 and IL-4RA proteins. So we determined the concentrations of the four proteins in hIL-4/hIL-4RA mice to verify whether the mouse model was successfully established, using the WT mice as the control. The human and mouse IL-4 proteins in the serum of hIL-4/hIL-4RA and WT mice were assayed by ELISA (Fig. 1A). As expected, mouse IL-4 was only detected in WT mice ($p < 0.001$), whereas human IL-4 was only expressed in hIL-4/hIL-4RA mice ($p < 0.001$).

The human and mouse IL-4RA proteins expressed in the splenocytes of hIL-4/hIL-4RA mice and WT mice were collected and analyzed by flow cytometry (Fig. 1B). The results showed that mouse IL-4RA was only up-regulated in wild-type mice, while human IL-4RA was only up-

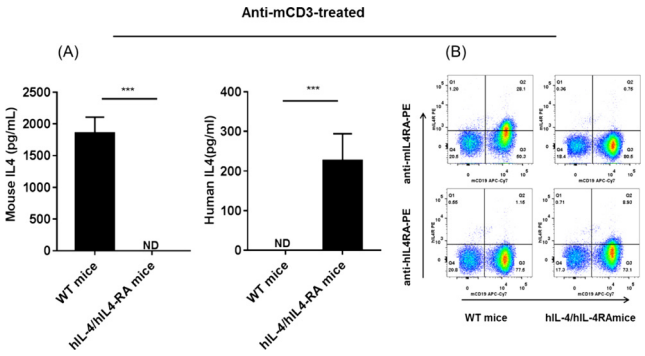

Fig. 1. Expression of IL-4 and IL-4RA proteins in hIL-4/hIL-4RA and WT mice. (A) Species-specific ELISA was used to detect IL-4 expression in the serum (n = 3). (B) Expression of IL-4RA protein in the spleen detected by flow Cytometry.

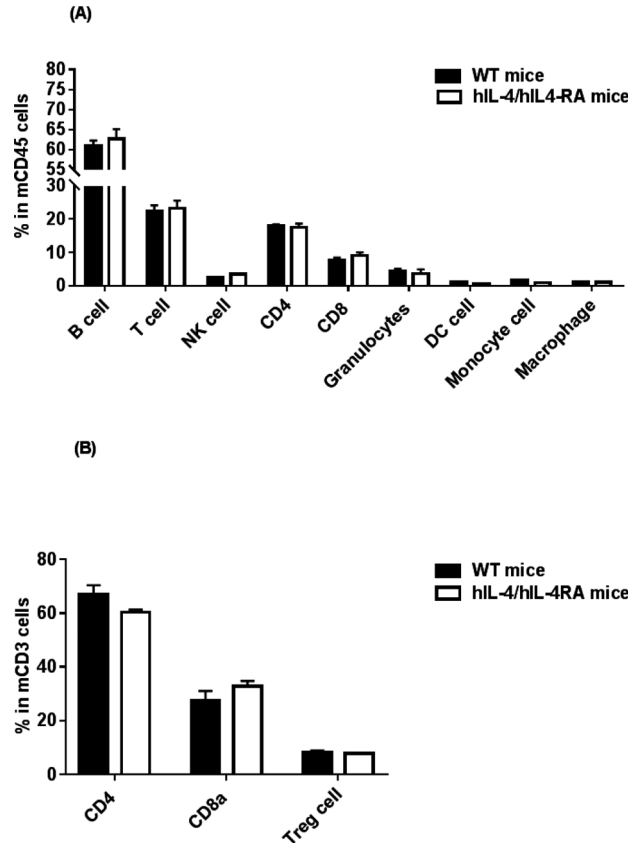

Fig. 2. Analysis of immune cells subpopulation in hIL-4/hIL-4RA and WT mice (n = 3). (A) The frequency of T cells, B cells, NK cells, Monocyte, DC cell, and macrophage cells in mCD45 cells. (B) The frequency of CD4 T cells, CD8 T cells, and Treg cells in mCD3 cells.

regulated in hIL-4/hIL-4RA mice.

These results indicated that the hIL-4/hIL-4RA mice we established could successfully express both human IL-4 and IL-4RA proteins, while the mouse IL-4 and IL-4RA proteins were undetectable.

3.1.3. Analysis of leukocytes subpopulations and T cell subpopulations

To evaluate the immune system of the hIL-4/hIL-4RA mice, we compared the leukocyte subpopulations in splenocytes of the hIL-4/hIL-4RA and WT mice by flow cytometry. As shown in Fig. 2A, the leukocyte subpopulations, including T cells, B cells, natural killer cells (NK), monocytes, dendritic cells (DC), and macrophages, in mouse cluster of differentiation cells (mCD45 cells as leukocyte mark) in hIL-4/hIL-4RA mice and WT mice were not statistically different ($p > 0.05$). A more detailed analysis of T cell subsets revealed that the percentages of $CD4^+$, $CD8^+$, and Treg cells in cluster of differentiation 3 (CD3 cells as T cell mark) were not significantly different in hIL-4/hIL-4RA mice and WT mice (Fig. 2B) ($p > 0.05$). The above results (Fig. 2) collectively proved that the differentiation development of immune cells in humanized mice were not impaired, indicating that hIL-4/hIL-4RA mice had normal immune functions.

3.1.4. IL-4 induced IgE secretion in cultured splenic B cells in vitro

In order to verify whether the signaling pathway of humanized IL-4 was blocked, Splenic B cells from hIL-4/hIL-4RA mice and WT mice isolated in *vitro* were stimulated to see whether they could normally express IgE (Fig. 3). The total IgE was undetected (< 2 ng/mL) in cultures only with LPS. The addition of hIL-4 induced the increase of IgE level to 115.33 ± 22.63 ng/mL in splenic B cells of hIL-4/hIL-4RA mice. Comparably, the addition of mIL-4 induced the increase of IgE level to 79.14 ± 9.65 ng/mL in splenic B cells of WT mice. These results

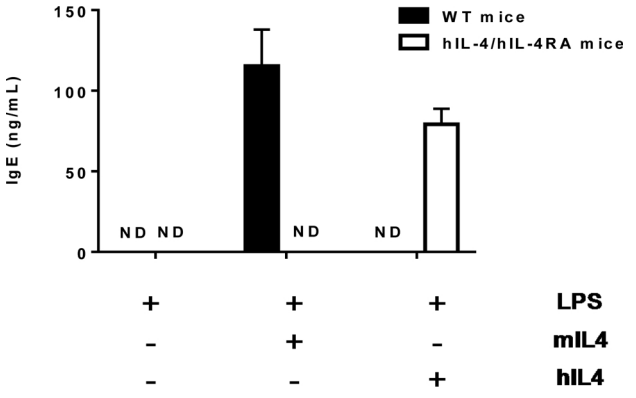

Fig. 3. Effects of LPS and LPS plus IL-4 on lgE level (n = 3).

suggested that the signaling pathway of IL-4 in hIL-4/hIL-4RA mice was not blocked (Fig. 3).

The above results collectively proved that the hIL-4/hIL-4RA mice we established correctly expressed hIL-4 and hIL-4RA proteins, had normal immune system, and functional signaling pathway of humanized IL-4, indicating that the mouse model could be applied on the efficacy evaluation of anti-human IL-4RA antibody drugs.

3.2. In vivo efficacy evaluation of anti-human IL-4RA antibody

In this section, we evaluated the efficacy of anti-human IL-4RA monoclonal antibody drug by using the hIL-4/hIL-4RA mouse model through inflammatory cell counting, IgE secretion, and hematoxylin and eosin (H&E) staining.

3.2.1. Inflammatory cell counting

The BALF profile of inflammatory cells, especially eosinophils cell, is a good indicator of lung tissue inflammation during allergic asthma (Zaslona et al., 2014). We determined the numbers of eosinophils ($CD45^+$ $Siglec\ F^+$ $CD11c^-$), neutrophils ($CD45^+$ $Siglec\ F^-$ $CD11b^+$ $Ly6G^+$), and macrophages ($CD45^+$ $Siglec\ F^-$ $CD11c^+$) in BALF of hIL-4/hIL-4RA mice (Kishta et al., 2018). A marked and significant accumulation of total leukocytes, eosinophils, neutrophils, and macrophages was observed following OVA challenge in mice (Fig. 4A–D). After two doses of anti-human IL-4RA monoclonal antibody treatment, positive drug group decreased numbers of all leukocytes, eosinophils, and neutrophils, when compared with the OVA group and the isotype control group (Fig. 4 A–D). Specifically, eosinophils as a percent of CD45 in the positive control drug group also significantly decreased as compared with the OVA group ($p < 0.001$) and the isotype control group ($p < 0.001$)(Fig. 4 E).

3.2.2. Levels of OVA-specific IgE and total IgE

The OVA-specific IgE and total IgE levels are also good indicators of lung tissue inflammation during allergic asthma (Pandey, 2013). We measured the OVA-specific IgE and total IgE in the serum by ELISA (Fig. 5). Compared with the PBS control group, the serum levels of total IgE and OVA-specific IgE increased significantly in the OVA groups ($p < 0.001$). Treatment with anti-IL-4RA greatly reduced the levels of both OVA-specific IgE and serum total IgE, as opposed to OVA alone or isotype treated group ($p < 0.001$).

3.2.3. Histological analysis

One of the hallmarks of a successful asthma model is the widespread infiltration of inflammatory cells especially eosinophils cell into the alveoli, bronchioles, and surrounding blood vessels (Jang et al., 2018). The OVA group showed a marked increase of inflammatory infiltrate in bronchial and vascular regions and inflammatory secretions compared with the PBS control group, indicating the successful establishment of

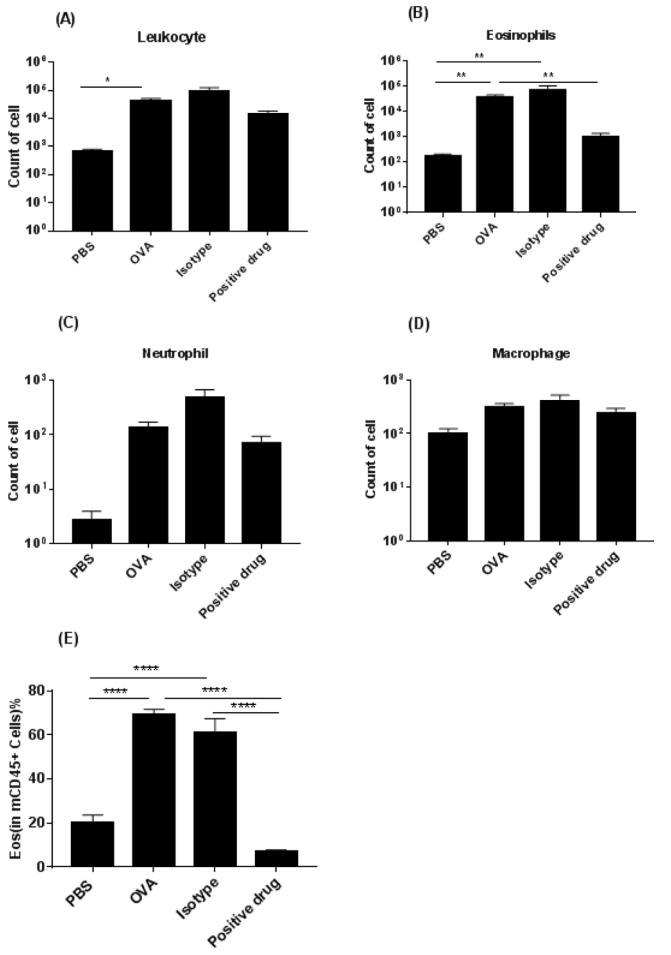

Fig. 4. Differential cell counts in bronchoalveolar lavage fluid (n = 7-8). (A-D) Numbers of leukocytes, eosinophils, neutrophils, and macrophages in the bronchoalvelar fluid. (E) The proportion of eosinophils in mCD45 cells.

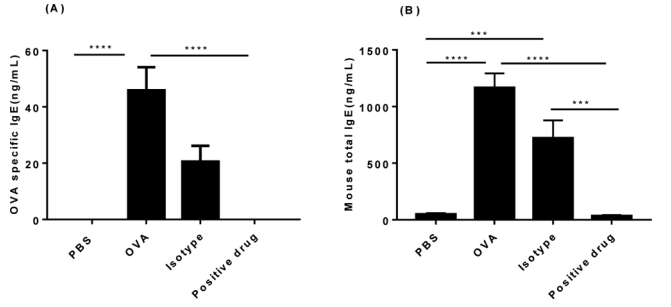

Fig. 5. Serum levels of total IgE and OVA-specific IgE (n = 7-8). (A) ELISA was used to detect the level of total IgE in the serum. (B) ELISA was used to detect the level of OVA-specific IgE.

the humanized asthma model (Fig. 6A&B). The treatment group showed a tendency of a more significant decrease in eosinophils infiltration and inflammatory secretions compared with the study group (Fig. 6B-D). The mucus score and total score of the H&E slices also showed that anti-IL-4RA treatment can reduce mucus secretion (Fig. 6E&F).

3.2.4. PAS analysis

The influences of anti-IL-4RA treatment on goblet cell hyperplasia and mucus production were assessed by PAS (Fig. 7). Goblet cell hyperplasia and mucus production was observed in the OVA group but not in the PBS control group, indicating the successful establishment of the hIL-4/hIL-4RA asthma model (Fig. 7A&B). Treatment anti-human IL-

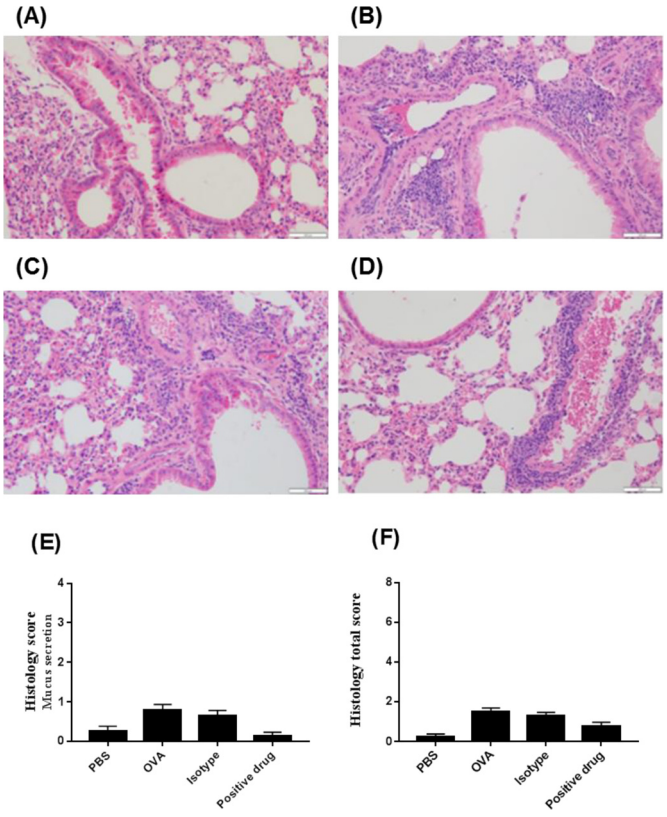

Fig. 6. Lung specimens from hIL-4/hIL-4RA mouse stained by H&E (n = 7-8). (A) Lung specimen from mouse receiving normal saline challenge (PBS group). (B) Lung specimen from mouse receiving OVA challenge. (C and D) Lung specimens from mice receiving isotype antibody and anti-human IL-4RA antibody. (E and F) The mucus score and total score of the H&E slices.

4RA antibody groups (Positive drug groups) showed a decreasing trend in goblet cell infiltration and mucus production as the dosage increased (Fig. 7C-E).

3.2.5. The mRNA Expression of hIL-4 and mIL-13

One of the hallmarks of a successful asthma model is the increase in Th2-related cytokine (IL-4 and IL-13) mRNA levels, therefore the mRNA expressions of hIL-4 and mIL-13 were determined by quantitative real -time PCR. Compared with the PBS group, the OVA group showed a statistically significant upregulation in gene expression of hIL-4 and mIL-13 ($p < 0.05$, Fig. 8), indicating that asthma model was successful. Compared with the OVA group, the positive drug groups showed a concentration-dependently down-regulation in gene expression of hIL-4 ($p < 0.05$, Fig. 8A).

At 25 mg/kg dosage, the expression of hIL-4 mRNA was suppressed to the level of the PBS group. As for mIL-13, the expression of it can also be down-regulated by the positive drug, but the results were statistically non-significant.

These results collectively indicated that the hIL-4/hIL-4RA mouse model could be applied in the preparation of asthma models and the efficacy evaluation of anti-human IL-4RA antibody drugs.

4. Discussion

Airway inflammation in asthma is a complex phenomenon driven predominantly by Th2 type cells. Hence, blocking Th2 cytokine action is a new approach in anti-asthma therapies. IL-4 plays a crucial role in mediating Th2 effects (Brusselle et al., 1994). In the present study, we have established a new genetically humanized mouse model with human IL-4 and IL-4RA replacing their mouse counterparts.

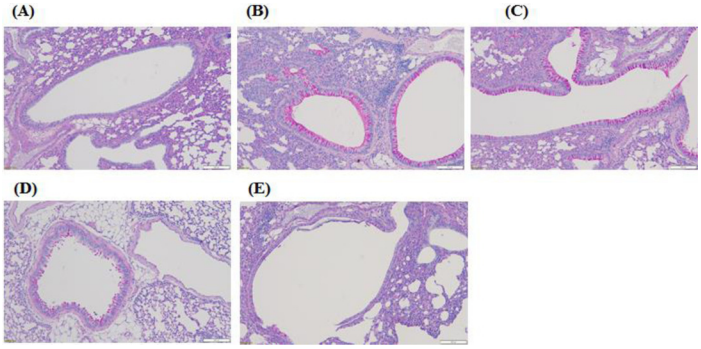

Fig. 7. Lung specimens from hIL-4/hIL-4RA mice stained by PAS (n = 5). (A) Lung specimen from mouse receiving normal saline challenge (PBS group). (B) Lung specimen from mouse receiving OVA challenge (OVA group). (C, D, E) Lung specimens from mice receiving anti-human IL-4RA antibody treatment (Positive drug group) with the dose of 3, 10, and 25 mg/kg, respectively.

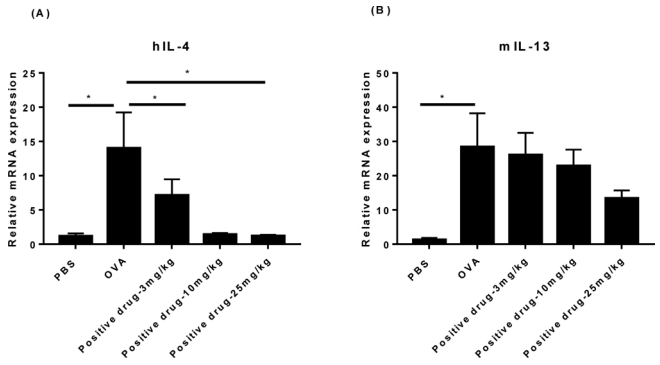

Fig. 8. Expression of hIL-4 (A) and mIL-13 (B) gene in the lung homogenate of hIL-4/hIL-4RA mice (n = 5).

Homozygotes of these double humanized mice expressed human IL-4 and human IL-4RA proteins, albeit at lower levels compared with those in wild type mice, but not the murine IL-4 and murine IL-4RA (Fig. 1). It is not clear what leads to this decreased expression of both human IL-4 and human IL-4RA as these human knockings are still under control of the native mouse IL-4 and IL-4RA gene promoters. It is conceivable, however, genomic engineering might affect non-promoter regulatory elements that impact on transcription, or the changed RNA and protein sequences might be less stable. Despite lowered expression, humanized IL-4 and IL-4RA mice were functional in terms of their responses to human IL-4 as indicated in their response to produce IgE both *in vitro* and *in vivo* and to human IL-4RA as indicated in blocking human IL-4RA with an anti-human IL-4RA antibody lowered IgE production *in vitro* (Fig. 5) and *in vivo* (Fig. 3) and reduced eosinophil and neutrophil infiltration in lung *in vivo* (Fig. 4). Similar profiles of leukocyte and T cell subpopulations between wild type and double humanized mice also demonstrate that immune cell development is not impaired in these double humanized mice (Fig. 2). These results are consistent with findings of ovalbumin induced asthma model in wild type mice (Reddy et al., 2016), and with findings of anti-IL-4 and/or anti-IL-4RA therapies in wild type mice ,(Halwani et al., 2016).

5. Conclusion

We have generated hIL-4/hIL-4RA mice by genetic engineering and established the murine OVA-induced asthma model in these mice. This model has been validated pharmacologically in *vitro* and in *vivo* with a known anti-human IL-4RA antibody in terms of IgE secretion and immune cell infiltration in lung tissues. We concluded that these hIL-4/hIL-4RA mice are a very useful tool in evaluating anti-IL-4 and anti-IL-4RA therapeutics in the preclinical setting.

Ethics approval and consent to participate

Not applicable.

Human and animal rights

The authors declare that the procedures followed were in accordance with the regulations of the responsible Clinical Research Ethics Committee and in accordance with those of the World Medical Association.

Consent for publication

Not applicable.

Authorship contributions

Peili Yan, Youhong Su, Chengzhang Shang, and Xiaofei Zhou performed experiments and analyzed data. Yi Yang and Wenqian An contributed to discussion. Changyuan Yu, Wenlin An, and Shihui Wang designed the experiments and analyzed data. Peili Yan, Chengzhang Shang, and Shihui Wang wrote the paper.

Funding

This work was supported by National Key Research and Development Project (218YFA0903000), National Natural Science Foundation of China (21606013), Shenzhen Science and Technology Project (Project number: JCYJ20180507183842516), and National Mega-project for Innovative Drugs (2019ZX09721001-007-002).

Declaration of Competing Interest

The authors declare no conflict of interest, financial or otherwise.

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