BLOCKADE OF INTERLEUKIN-33 ACTIVITIES BY RECOMBINANT INTERLEUKIN-33 TRAP FC PROTEIN WOULD BE A NOVEL THERAPEUTIC STRATEGY IN ALLERGIC ASTHMA

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SUMMARY

The majority of autoimmune and allergic diseases are associated with abnormal expression of interleukin (IL)-33, a member of the IL-1 family of cytokines, that function dually as a proinflammatory cytokine and a transcriptional factor. We created an IL-33 inhibitor called "IL-33 Trap Fc" constructed by fusion of an Fc fragment of human immunoglobulin G1 and two distinct extracellular part receptors involved in interacting with IL-33, IL-1 receptors accessory protein, and IL-33 receptor. IL-33 Trap Fc was expressed by two systems, mammalian HEK293 cells and *Pichia pastoris* yeast. We found that these recombinant proteins were expressed as a glycoprotein and perhaps in dimeric form. IL-33 Trap Fc from HEK293 and *P. pastoris* suppressed the activity of IL-33 *in vitro* culture conditions. The glycosylation of IL-33 Trap expressed by *P. pastoris* yeast was more intensive and heterogeneous than the counterpart protein expressed from HEK293 cells. That is maybe one reason leading to a substantial decrease in the activity of IL-33 Trap Fc from *P. pastoris* compared with that from HEK293 cells. We also demonstrated that IL-33 Trap Fc expressed from HEK293 cells had therapeutic effects in ovalbumin-induced asthma mouse model. These data collectively suggested that IL-33 Trap Fc potently blocks IL-33 *in vitro* and *in vivo*, which may be a novel therapeutic strategy for IL-33-mediated allergic diseases.

Keywords: interleukin-33, interleukin-33 receptor, HEK293, *P. pastoris*, allergic asthma mouse model

INTRODUCTION

Interleukin 33 (IL-33), a member of the IL-1 cytokine family including IL-1β, IL-1α, IL-18, IL-1Ra (IL-1 receptor antagonist), is mainly found by epithelial and endothelial cells (Schmitz *et al.,* 2005). IL-33 plays a vital role in the activation of immune cells in inflammatory conditions. IL-33 directly induced Th2 cells and B cells to upregulate the expression of proinflammatory cytokines IL-4, IL-5, IL-13, and IgM antibodies (Schmitz *et al.,* 2005; Komai-Koma *et al.,* 2011). It has been shown that IL-33 induced Th2 type cytokines expression in dendritic cells to support naïve CD4+ T cells (Rank *et al.,* 2009). The function of IL-33 in IL-8 production, granule release reaction, and cell survival of eosinophil has also been reported (Schmitz *et al.,* 2005, Cherry *et al.,* 2008). In mast cells and basophil, IL-33 induced maturation, survival, adhesion, IL-8, IL-13, IL-4, IL-6, histamine production (Allakhverdi *et al.,* 2007; Iikura *et al.,* 2007; Schneider *et al.,* 2009). In addition, IL-33 facilitated neutrophil migration preventing the spread of infection (Alves-Filho *et al.,* 2010). IL-33 also activated

Th2-type immune responses that drove macrophage differentiation and augmented TNFα levels in lipopolysaccharide-induced macrophages (Espinassous *et al.,* 2009; Kurowska-Stolarska *et al.,* 2009).

Upregulation of IL-33 has been repeatedly demonstrated in atopic diseases such as allergic asthma and atopic dermatitis. The levels of IL-33 were augmented in both lesional and non-lesional skin of atopic dermatitis patients (Pushparaj *et al.,* 2009). *In vivo* murine studies and the analysis of patient samples further support the increased concentrations of IL-33 contributing to skin fibrosis (Yanaba *et al.,* 2011; Rankin *et al.,* 2010). In contrast, ST2-deficient transgenic mice showed dermatitis pathological features less than wild-type control mice (Hueber *et al.,* 2011). In addition, it has been indicated an association between IL-33 and inflammatory bowel disease. The elevated IL-33 level has been illustrated in fibroblasts and intestinal epithelial cells of patients with ulcerative colitis and Crohn's disease (Beltran *et al.* 2009, Carriere *et al.,* 2007, Pastorelli *et al.* 2010). IL-33 also promoted the proliferation of intestinal epithelial cells and stimulated trans-membrane migration of eosinophil and neutrophil from the bloodstream to the intestinal mucosa in mice (Schmitz *et al.,* 2005). The acute symptoms in experimental models for inflammatory bowel disease in mice were associated with increased IL-33 levels (Pastorelli *et al.,* 2010). These results collectively suggested that IL-33 contributed to the pathogenesis of many diseases, especially autoimmune and allergic diseases. Inhibiting IL-33 activity may be a novel therapeutic modality for these diseases.

IL-33 receptor (IL-33R), also called IL-1-receptor-like 1 (IL1RL1) and commonly known as suppression of tumorigenicity 2 (ST2), was initially discovered in 1989 (Tominaga *et al.,* 1989). IL-33 triggered an inflammatory cascade by binding to its receptor IL-33R, facilitating the co-receptor IL-1 receptor accessory (IL-1RAcP) to form the heterotrimeric signaling complex (Ali *et al.,* 2007). Apart from IL-33-blocking small molecules, inhibition of IL-33 and its receptors with monoclonal antibodies is the most common therapeutic approach targeting the IL-33/ST2 axis of allergic inflammatory disorders, and several clinical trials are now underway (Li *et al.,* 2018; Fursov *et al.,* 2016; Ohta *et al.,* 2017). Antibodies against the extracellular region of IL-33R attenuated the infiltration of acidophilic granulocytes and IL-4, IL-5, and IgE production, consequently inhibiting the pulmonary mucosa immune response in asthma mouse models (Coyle *et al.,* 1999; Honing *et al.,* 1998; Kearley *et al.,* 2009). Similarly, treatment with anti-IL33R antibody dramatically reduced allergy-related symptoms and osteoarthritis in collagen-induced rheumatoid mouse models (Palmer *et al.,* 2009). Other results demonstrated that degradation of allergic asthma cytokines, including IgE, acidophilic granulocytes, lymphocytes, IL-4, IL-5, and IL-13, was noted in animal models' bronchial mucus treated anti-IL33 antibodies (Liu *et al.,* 2009). However, the side effects of immune responses such as serum sickness and acute anaphylaxis may occur after monoclonal antibody treatment (Hansel *et al.,* 2010; Liu *et al.* 2014). Soluble IL-33R (sIL-33R), which competed with IL-33R on the cell membrane in interacting with IL-33, may provide a great solution to block IL-33 activities. When asthma mice were treated with sIL-33R, levels of IL-4, IL-5, and IL-13 were significantly suppressed (Hayakawa *et al.,* 2007). Similar effects on arthritis mouse models were obtained using sIL-33R and Fc IgG1 domain fusion protein (Palmer *et al.,* 2009). These results suggested that IL-33 inhibitory molecules, particularly anti-IL33 antibodies and sIL33R, can be potential candidates in the treatment of IL-33-related diseases.

In 2003, Economides *et al.* published for the first time a model of IL-1 Trap and IL-4 Trap created by fused two different cytokine receptors extracellular domain involved in the interaction process of cytokines, IL-1R or IL-4R and IL-1R AcP, respectively, followed by the constant region of human IgG. Their data showed that these cytokine traps resulted in a 100-fold

increase in affinity to their ligands, IL-1 or IL-4. These cytokine traps also effectively neutralized cytokine activity in murine asthma models (Economides *et al.*, 2003). Holgado *et al.* have recently generated a monomeric recombinant protein named IL33 Trap by binding the extracellular region of IL-33R at the N-terminal end and its co-receptor IL-1R AcP at the C-terminal end of the molecule. The combination of IL-33R and IL-1R AcP further augmented binding affinity to IL-33 than single forms with soluble ST2; consequently, IL33 Trap dramatically inhibited the IL-33- induced immune pathway in both *in vitro* and *in vivo* acute allergic airway mouse models (Holgado *et al.,* 2019).

In this study, we created and investigated the IL33-inhibitory activity of the recombinant IL-33 Trap Fc protein expressed from mammalian HEK293 cells and *Pichia pastoris* yeast *in vitro* and *in vivo* models. IL-33 Trap Fc is a three-part hybrid protein consisting of the extracellular region of mouse IL-1R AcP, the extracellular region of mouse IL-33R, and the human Fc IgG1 region. The fusion Fc region to the C- terminal of IL-33 Trap Fc is expected to perform IgG dimeric structures, enhancing stability and biological activities of the recombinant proteins.

MATERIALS AND METHODS

Cells, agents, and mice

HEK293 cells kindly provided by Prof. T.D.H Ho (University of Science – Vietnam National University Ho Chi Minh City, Vietnam) were cultured in DMEM medium (Thermo Fisher Scientific, Fremont, CA, USA) complemented with 10% heat-inactivated fetal bovine serum (Merck, Darmstadt, Germany) and 2 mM L-glutamine (Thermo Fisher Scientific, Fremont, CA, USA) at 37°C, 10% CO2. Mouse lymphoma EL-4 cells, obtained from the American Type Culture Collection (ATCC), were cultured in RPMI 1640 medium (Merck, Darmstadt, Germany) plus 5% fetal bovine serum and 2 mM L-glutamine at 37°C, 5% CO2.

Yeast cells *Pichia pastoris* GS115 (*his4*) were from Thermo Fisher Scientific. Plasmids pFL-mIL33R full encoding length of mouse IL-33R, and plasmid pmsIL1RAcP-Fc encoding full length of mouse IL-1RAcP and Fc IgG1 domain are kind gifts from Prof. M.U. Martin (Justus Liebig University, Giessen, Germany). Plasmid pETDuet-mIL33 encoding mouse IL-33 kindly provided by Prof. W. Falk (University of Regensburg, Regensburg, Germany). Swiss mice (4- to 6-week-old female) were purchased from Pasteur Institute in Ho Chi Minh City (Ho Chi Minh City, Vietnam). Cells were negative for mycoplasma.

Construction of plasmids encoding IL-33 Trap Fc expressed by HEK293 cells and *P. pastoris* yeast

cDNA encoding extracellular region of the mouse IL-33R (Pro18 – Arg332) was amplified by PCR using a pair of primers mIL33R-P18s (5' gcaagctagcccccatgtatttgacagttacgg 3') and mIL33R-R332as (5'-aaagcggccgctcggtgatcaattggttgtttc-3') on the template plasmid pFL-mIL33R. cDNA encoding extracellular region of the mouse IL-1RAcP (S21 – V347) was amplified by PCR using a pair of primers msIL1RAcP- S21s (5' gtataagctttcggagcgctgtgatgactggg 3') and msIL1RAcP- V347as (5' attagctaggccaccttggcagcctgctcagc 3') on the template plasmid pmsIL1RAcP-Fc. The PCR product was cloned into a Signal pIgplus vector containing human Fc IgG1 via restriction sites to generate a recombinant pmIL-33 Trap Fc -Cell vector.

For constructing a recombinant plasmid expressed in *P. pastoris* yeast, cDNA encoding the extracellular region of the mouse IL-1RAcP (Ser21 – Val347) was amplified by PCR using a pair of primers IL1RAcP-Ser21s (5' tacgtatcggagcgctgtgatgactggg 3') and IL1RAcP-Val347as (5' aacctaggcaccttggcagcctgctcagc 3'). The amplified PCR product was cloned into yeast expression vector pmsIL33R-Fc-Yeast, a recombinant pPIC9K containing IL33R fused hIgG1 constructed from our previous research

[Nguyen *et al.* 2015], via restriction sites to generate pmIL-33 Trap Fc -Yeast recombinant vector.

All the constructs were confirmed by DNA sequencing analysis.

Transient expression of IL-33 Trap Fc from HEK293 cells

HEK293 cells were transiently transfected by expressing plasmid pIL-33 Trap Fc -Cell using polyethyleneimine (PEI). Seventy - two hours after transfection, culture supernatant of HEK293 cells was harvested. The recombinant HEK293 – derived IL-33 Trap Fc in the supernatant was purified with protein A sepharose bead (BD Biosciences, San Jose, CA, USA) and stored at -80ºC for further experiments.

Generation of *P. pastoris* clone stably expressing IL-33 Trap Fc protein

P. pastoris GS115 yeast was transfected using electroporation with pmIL-33 Trap Fc - Yeast vector linearized by *Sal*I (NEB, Ipswich, MA, USA). The yeast transformants were selected as the manufacturer's instruction. In brief, yeast colonies growing on minimal dextrose (MD) medium plate were picked up and transferred onto yeast extract – peptone – dextrose (YPD) agar plates containing geneticin G418 (Thermo Fisher Scientific, Fremont, CA, USA) with increasing concentrations of 1, 2, 4 and 6 mg/ml. Survived clones at the highest antibiotic concentration were selected for PCR using a pair of primers IL1RAcP-Ser21s and IL1RAcP-Val347 to confirm the presence of the target gene in the cells. For the expression of IL-33 Trap Fc from yeast, a single colony of the yeast on YPD agar plus G418 (4 mg/ml) plate was inoculated into 25 ml of buffered glycerol-complex medium (BMGY) and cultured at 28°C, 250 rpm until the cultures reach OD600nm of 3.0 – 4.0. The cells were then harvested by centrifugation at 1500xg for 5 minutes at room temperature. The cell pellet was re-suspended in 50 ml of buffered methanol-complex medium (BMMY) to OD600nm of 1.0. The cultures were maintained in a shaking incubator at 20°C, 250 rpm, and supplemented daily with methanol to a final concentration of 1%. After 72 hour-induction with methanol, yeast cells were removed from the culture supernatant by centrifugation at 4,000×g for 10 minutes at 4ºC. The *P. pastoris* clone stably expressing IL-33 Trap Fc at the highest level was chosen and used throughout the study.

Purification of IL-33 Trap from the culture supernatant

The recombinant IL-33 Trap Fc protein in HEK293- and *P. pastoris*- culture supernatants were purified by immunoprecipitation using protein A sepharose. Sepharose beads were mixed with culture supernatant at a concentration of 2 µl/ml for 16 hours at 4ºC. The beads were washed with cold PBS to eliminate unbound proteins. The bound proteins were eluted in 100 mM glycine buffer (pH 2.0). The eluted protein solution was neutralized by 1.5 M Tris- HCl buffer (pH 8.8) and stored at -80ºC.

SDS-PAGE and Western blotting

Protein samples were dissolved in Laemmli buffer with or without β-mercaptoethanol at 95°C for 10 min and analyzed by electrophoresis in 10% polyacrylamide gels. Protein bands in gels were stained by coomassie blue or were transferred onto the nitrocellulose membrane. Recombinant IL-33 Trap Fc protein on the membrane was detected by hybridizing with horseradish peroxidase (HRP)-conjugated goat anti- huIgG1 Fc antibody (Merck).

In vitro interaction of mIL-33 and IL-33 Trap

The mix of 30 µg of biotin-tagged mouse IL-33 (biotin-mIL33) and 10 µg of IL-33 Trap Fc were mixed in 1 ml of PBS containing 2% BSA and incubated at 37ºC for 1h. IL-33 Trap Fc in the mixture was pulled down by protein A sepharose beads. The pulled-down proteins were analyzed by Western blotting using HRP-conjugated goat anti-IgG1 Fc antibody or HRP-conjugated Streptavidin (GE Healthcare, NY, USA).

Deglycosylation of recombinant IL-33 Trap

The mix of 10 µg of recombinant HEK293- or yeast-derived IL-33 Trap Fc was denatured in 20 µl of 1X glycoprotein denaturing buffer (NEB, Massachusetts, USA) at 100°C for 10 minutes. After that, 5 µl of 10X G7 reaction buffer, 5 µl of 10% NP40, 15 µl of H2O, and 5 µl of enzyme peptide: N-glycosylase F (NEB) 200 U/µl were added to the reaction, following incubated at 37°C for 3 hours. The deglycosylated and undeglycosylated IL-33 Trap Fc were analyzed by Western blotting using HRP-conjugated anti-huIgG1 Fc antibody.

Investigation of IL-33 Trap Fc on EL-4 cell model

EL-4 cells were stimulated for 8 hours with 10 ng/ml of recombinant mouse IL-33 (rmIL-33) protein (Thermo Fisher Scientific) and 0.5 µM calcium ionophore A23187 (Merck) with or without IL-33 Trap Fc at various concentrations for 30 minutes at 37°C. For control, the IL-33 unstimulated EL-4 cells were also treated with the same concentrations of IL-33 Trap. After incubation, the expression level of mouse IL-2 (mIL-2) in EL-4 culture supernatants was determined by ELISA kit (BD Biosciences) according to the manufacturer's instructions. The IL-33 inhibitory activity of IL-33 Trap Fc was identified by the amount of IL-2 produced from EL-4 cells under stimulation of IL-33.

Investigation of IL-33 Trap Fc activity on OVA-induced asthma mouse model

Mice were sensitized by intraperitoneal injection with 60 µg of ovalbumin (Merck) and 10 mg of aluminum hydroxide gel (Merck) in saline on day 1 and day 7. On days 14, 15, and 16, mice were challenged twice (4 hours interval) daily by nasal administration with 100 µl of 1% OVA in saline using an ultrasonic nebulizer (Omron Corp., Tokyo, Japan). Along with the sensitization and aeroallergen challenge, from day 0 to day 14, mice were injected at intervals of 2 days with IL-33 Trap at tested doses. On days 14, 15, 16, the mice were injected with IL-33 Trap Fc 30 minutes before OVA nasal administration. The mice were sacrificed 24 hours after the last aeroallergen challenge, and the bronchoalveolar lavage (BAL) fluid was obtained for further analyses. Briefly, BAL fluids were centrifuged to separate supernatant and cell fractions. The IL-5 concentration in supernatants of BAL fluids was quantified using an IL-5 ELISA kit (R&D Biosystems, Minnesota, USA). The cell fractions from BAL fluids were stained by FITC conjugated anti-CD45 antibody (BD Biosciences) and processed to FACS analysis to determine the CD45+ cell density. The animal experiments were approved by the animal experiment and welfare committee of the Biotechnology Center of HCM city and were performed according to the guideline on animal experiments.

Statistical analysis

The data are represented as means of (at least) three independent experiments ± SD. The data were analyzed by the ANOVA test using GraphPad Prism (GraphPad, CA, USA) software. A value of $p < 0.05$ was considered to be significant.

RESULTS

Expression, purification, and characterization of recombinant IL-33 Trap Fc protein from HEK293 and *Pichia pastoris*

Based on reports indicating the beneficial effects of cytokine trap protein, we hypothesized that the recombinant fusion protein consists of two extracellular IL-33 receptors and the human IgG1 Fc that inhibit the IL-33 activities by competing with intracellular membrane-bound IL-33R for interaction to IL-33 and improve plasma half-life of recombinant proteins. To evaluate the fusion protein's therapeutic potential, the recombinant IL-33 Trap Fc protein was constructed by engineering inline fusions of two IL-33 receptor extracellular domains, including IL-1RAcP and IL-33R, followed by the human IgG1 Fc. The C terminal of IL-33 Trap Fc was conjugated with a human Fc IgG1 region to support recombinant protein

purification from the cell culture medium and enhance stability and biological functions of IL-33 Trap Fc protein via inter-chain disulfide bond on its structure (Figure 1).
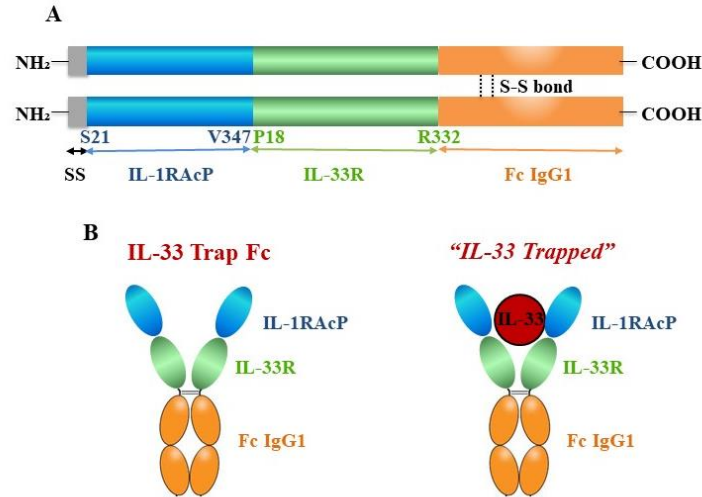

Figure 1. Schematic of the recombinant IL-33 Trap Fc protein. (A) IL-33 Trap Fc is expressed as a secreted fusion protein with an N-terminal human CD-33 secretory signal peptide (SS). Each subunit of the recombinant IL-33 Trap Fc fusion protein consisting of the extracellular region of IL-33R (P18-R332) and its co-receptor IL-1R AcP (S21-V247) is linked to human IgG1 Fc at the C-terminal end, which facilitates homodimer structure. (B) Diagram of IL-33 Trap Fc action model. The dimeric IL-33 Trap Fc protein generated by the Fc region of human IgG1. The combination of two IL-33 receptors, IL-33R and IL-1R AcP, was expected to further augment binding affinity to IL-33 than a single receptor.

We analyzed secreted protein fractions from the cell culture medium of HEK293 cells or *P. pastoris* transformed plasmids encoding the IL-33 Trap Fc gene (Figure 2A). The total concentrated supernatants and preliminarily purified proteins were obtained by 50 kDa cut-off Amicon centrifugal filters and immunoprecipitation (IP) with protein A sepharose beads, consequently. Cells transformed empty vectors were used as a negative control. There were no significant differences in the profiles of the different protein fractions between the total concentrated supernatants containing IL-33 Trap Fc proteins and negative control, especially in HEK293 cell cultures. With the IP sample of HEK293 transfected plasmid encoding IL-33 Trap Fc, apart from two bands of 25 kDa and 55 kDa, which were also observed in the negative control, there was an additional protein band with a molecular weight of around 150 kDa. The 150 kDa band could be the recombinant IL-33 Trap Fc protein synthesized and secreted by HEK293

cells. The two contaminant bands of 25 kDa and 55 kDa could be the light and heavy chains of bovine IgG in FBS, a growth supplement for *in vitro* cultivation of mammalian cells, that co-eluted by Protein A sepharose beads. For *P. pastoris*, IP results showed a high molecular weight smear in the range of 150 kDa to over 200 kDa and some protein bands with smaller sizes. These proteins did not observe in the negative control. These protein complexes could be IL-33 Trap Fc or protein fractions related to the target proteins in *P. pastoris* cultured supernatant.

We confirmed that the proteins detected in the culture supernatant of HEK293 cells and *P. pastoris* yeast were the target IL-33 Trap Fc with the Western blotting using anti-IgG1 Fc antibody (Figure 2B). While IL-33 Trap Fc secreted by HEK293 cells showed only one band with a molecular weight of 150 kDa, these proteins expressed from *P. pastoris* yeast were a smear with the various molecular weights from 150 kDa to over 200 kD and some small fractions.
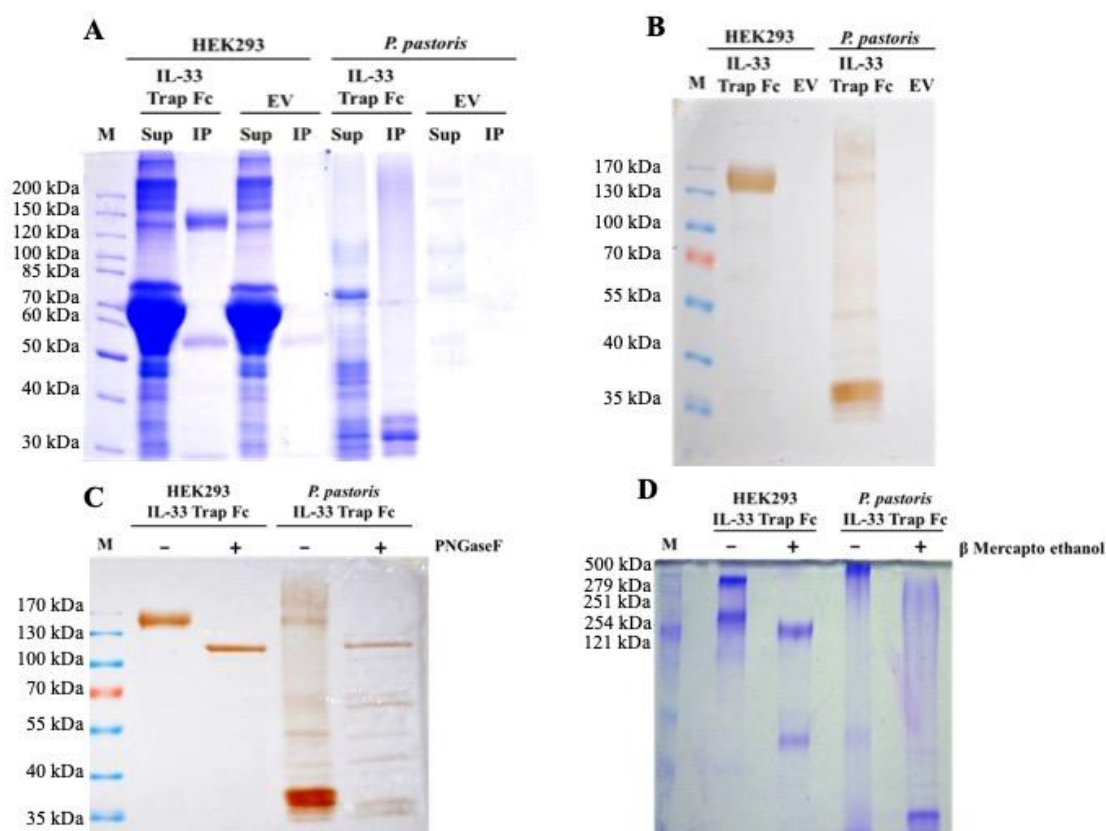
Figure 2. Expression, purification, and characterization of recombinant IL-33 Trap Fc expressed by HEK293 and *Pichia pastoris*. Cell culture supernatants of HEK293 transiently transfected with IL-33 Trap Fc encoding vector or empty vector (EV) were collected and concentrated by filtration through a 50kDa cut-off Amicon Ultra-15 centrifugal filter (Sup). IL-33 Trap Fc in 10 ml of culture supernatant was harvested with protein A sepharose by immunoprecipitation (IP). Culture supernatants of *P. pastoris* GS115 clone stably transfected with IL-33 Trap Fc encoding vector or empty vector (EV) were treated the same with HEK293 culture supernatants. (A) The concentrated supernatant samples (Sup) and the post-IP samples (IP) of HEK293 cells and *P. pastoris* yeast cultures were analyzed by SDS/PAGE with Coomassie blue staining. (B) Post-IP samples of HEK293 and *P. pastoris* were analyzed by Western blotting using HRP-conjugated anti-Fc IgG1 antibody. (C) Deglycosylated and undeglycosylated IL-33 Trap Fc were analyzed with Western blotting using HRP-conjugated anti-Fc IgG1 antibody. (D) HEK293 and *P. pastoris* – derived IL-33 Trap Fc were treated in reducing or non-reducing conditions and resolved by SDS/PAGE with Coomassie blue staining.

The observed molecular weight of target IL-33 Trap Fc proteins from HEK293 and *P. pastoris* was higher than the predicted protein molecular weight of 116.8 kDa calculated from the amino acid sequence. We hypothesized that the high molecular weight results from glycosylation, which added covalent attachment of sugar moieties to the polypeptide chain. This hypothesis was tested by treated IL-33 Trap Fc with the presence or absent enzyme deglycosylation PNGase F, following Western blotting analysis (Figure 2C). Mass of IL-33 Trap Fc proteins expressed from HEK293 and *P. pastoris* were returned to the theoretical size of about 116.8 kDa after deglycosylation. These results suggested that the recombinant IL-33 Trap Fc proteins were glycosylated by mammalian HEK293 cells and *P. pastoris* yeast.

Furthermore, the proteins expressed from *P. pastoris* were glycosylated more heterogeneously than the counterpart proteins expressed from HEK293 cells.

The Fc region of human IgG1 conjugated to the C-terminal of IL-33 Trap Fc was expected to form disulfide-linked dimeric proteins. We examined dimerization through disulfide bond formation in IL-33 Trap Fc structure with SDS-PAGE under reducing (with β-mercaptoethanol) and non-reducing (without β-mercaptoethanol) conditions (Figure 2D). As expected, in non-reducing conditions, IL-33 Trap Fc from HEK293 cells was visualized as a band of about 350 kDa, while it was observed at approximately 150 kDa under reducing conditions. The same phenomenon was observed with IL-33 Trap Fc expressed from *P. pastoris* yeast, which was about 500kDa in non-reducing and around 150 to over 300 kDa in the reducing conditions. These results collectively indicated that the recombinant IL-33 Trap Fc expressed from mammalian cells and yeast could be secreted into the culture supernatant in dimeric form.

Recombinant IL-33 Trap Fc expressed by HEK293 and *P. pastoris* interacted with IL-33 and inhibited the activity of IL-33

We investigated the ability to interact with biotin-conjugated mouse IL33 (bio-mIL33) of IL-33 Trap Fc protein under *in vitro* conditions with the co- IP assay (Figure 3A). A specific 30 kDa protein band was detected by SDS/PAGE Western blot analysis with biotin antibody in a reaction mixture of IL-33 Trap Fc and bio-mIL33 (IP: anti-IgG1 Fc / WB: anti-biotin). This band was not detected upon removal of bio-mIL33 from the mixture. In addition, the analysis results of a mixture of IL-33 Trap Fc and bio-mIL33 with anti-Fc IgG1 antibody (IP: anti-IgG1 Fc / WB: anti-Fc IgG1) showed a band with a molecular weight of 150 kDa or a smear of between 150-300 kDa corresponding to the size of IL-33 Trap Fc expressed by HEK293 cells or *P. pastoris* yeast. Thus, the 30 kDa protein was bio-mIL33 bound to IL-33 Trap Fc and pulled down by protein A sepharose. The nonspecific

binding of bio-mIL33 on protein A sepharose bead was excluded as a 30 kDa protein was not observed in the case of using bio-mIL33 alone. The data suggested that IL-33 Trap Fc from two expression systems, HEK293 cells and *P. pastoris* yeast, efficiently interacted with bio-mIL33. The nonspecific interaction of recombinant IL-33 Trap Fc protein to bio-mIL33 at the site of biotin molecules did not appear (data not shown).

We next examined the negative regulation of IL-33 Trap Fc with IL-33 activity in the murine lymphoma EL-4 cell line. Under the stimulation of IL-1 family cytokines, including IL-1β, IL-18, and IL-33, activated EL-4 cells generated a unique cytokine profile, including IL-2 [Ali *et al.* 2007]. EL-4 cells incubated IL-33, following treatment with or without IL-33 Trap Fc at various concentrations. The ability to inhibit IL-33 activity of IL-33 Trap Fc was assessed by the amounts of IL-2 produced by EL-4 cells by ELISA assay (Figure 3B). IL-33 Trap Fc expressed by HEK293 cells significantly reduced the production of IL-2 by EL-4 cells. At a 100-fold higher molar dose than the stimulated dose of IL-33, this recombinant protein completely inhibited IL-33 activity (p <0.001 compared with IL-33 Trap Fc- untreated cells). The counterpart recombinant protein, IL-33 Trap Fc expressed from *P. pastoris* yeast, also blocked IL-33; however, the IL-33 inhibitory effect of IL-33 Trap Fc from *P. pastoris* was significantly lower than that of the protein from HEK293 cells at the same experimental conditions (p<0.05 compared with HEK293- derived IL-33 Trap Fc - treated cells). The yeast-derived protein blocked roughly half of IL-33 action at a 100-fold greater molar dosage of IL-33 Trap Fc than the active dose IL-33.

We then compared the biological activity of HEK293-derived IL-33 Trap Fc and IL-33R Fc, a recombinant Fc IgG1-fused soluble IL33R protein generated in our previous work [Nguyen *et al.* 2015] (Figure 3C). The complete suppression of IL-33 activity was achieved when EL-4 cells were treated with IL-33R Fc concentration 1000 fold-molar higher than IL-33

(p<0.001 compared with IL-33R Fc untreated cells). At the dose 100-fold higher than IL-33, IL-33 Trap Fc completely inhibits IL-33 activity; however, IL-33R Fc only inhibited 64.2% of this cytokine's activity (p <0.001). These results showed that the combination of two distinct receptors involved in the IL-33 binding pathway, including IL1RAcP co-receptor and IL33R, significantly augmented the blocking of IL-33

activity compared to a single IL33R domain.

Our data collectively indicated that even though both IL-33 Trap Fc expressed from HEK293 cells and *P. pastoris* yeast interacted and inhibited IL-33, IL-33 Trap Fc from HEK293 cells achieved the biological activity more remarkable than that of the counterpart protein from *P. pastoris* yeast.
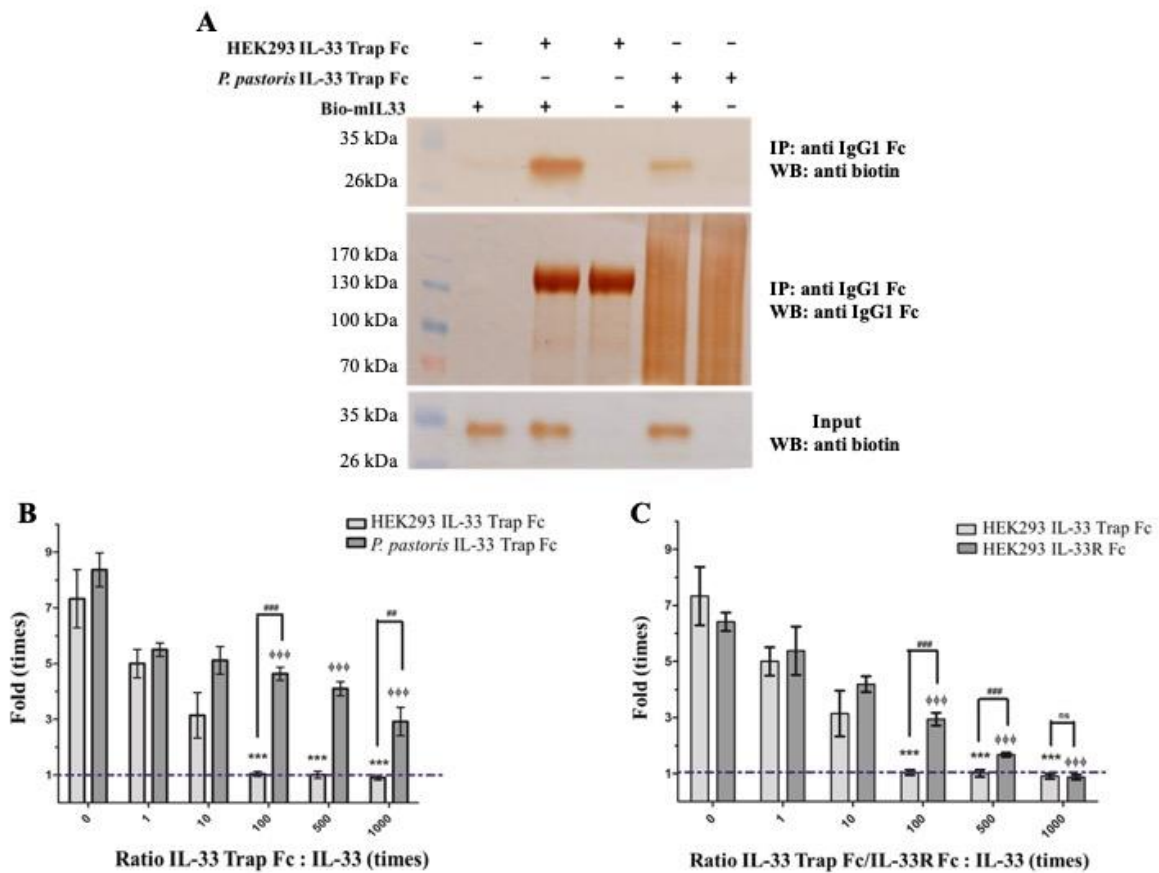

Figure 3. The *in vitro* activity of IL-33 Trap Fc expressed from HEK293 cells and *Pichia pastoris*. (A) IL-33 Trap Fc expressed from HEK293 and *P. pastoris* were incubated with biotin-mIL33 and was immunoprecipitated using protein A sepharose. Co-precipitated biotin-mIL33 was detected by Western blotting with HRP-conjugated streptavidin (upper panel). Precipitated IL-33 Trap Fc was shown by Western blotting with HRP-conjugated anti-human IgG1 antibody (middle panel). The presence of biotin-mIL33 in the input was confirmed by Western blotting with HRP-conjugated streptavidin (lower panel). (B) EL-4 cells were stimulated with 10 ng/ml of mIL-33 and 0.5 µM calcium ionophore with or without IL-33 Trap Fc from HEK293 cells or *P. pastoris* yeast; (C) IL-33 Trap Fc or IL-33R Fc from HEK293 cells. The final molar ratio between IL-33 Trap Fc or IL-33R Fc and mIL-33 were 0, 1, 10, 100, 500, and 1000 folds. Cells were incubated for 8 hours, and culture supernatants were harvested, then mIL-2 in the supernatants was evaluated by ELISA. Depicted are fold inductions of mIL-2 produced by EL-4 cells stimulated with mIL-33 to unstimulated controls. The results are presented as the means ± SD and analyzed by ANOVA test (n=6). ***p< 0.001 and ΦΦΦp< 0.001 compared with IL-33 Trap Fc or msIL33R-Fc untreated cells; ###: p< 0.001, ##: p< 0.01.

HEK293-derived IL-33 Trap Fc attenuated airway inflammation in the mouse model of allergic asthma

We evaluated the therapeutic effects of IL-33 Trap Fc expressed from HEK293 cells on the ovalbumin (OVA)-induced asthma mouse model (Figure 4). OVA-induced allergic asthma mice have asthmatic phenotypes, such as high levels of serum IgE, inflammatory cytokines, airway inflammation, histological features, airway hyperresponsiveness, which are similar to the features observed in human asthma. Following treatment with IL-33 Trap Fc from HEK293 by intrapleural injections, CD45^{+} eosinophil population in BALF OVA-induced asthma mice were analyzed by flow cytometry (Figure 4B, Figure 4C). The number of CD45^{+} eosinophils in the BALF of asthmatic mice was substantially higher than that of the normal mice used as control ($p < 0.05$). Treatment with IL-33 Trap Fc reduced the number of eosinophils in OVA-induced asthma mice in a dose-dependent manner. Specifically, an intrapleural injection of 20 µg of IL-33 Trap Fc drastically suppressed the CD45^{+} eosinophil population in the BALF ($p < 0.5$ compared to asthmatic mice). There were no differences in the number of inflammatory cells between 20 µg of IL-33 Trap Fc –treated asthmatic mice and normal groups.
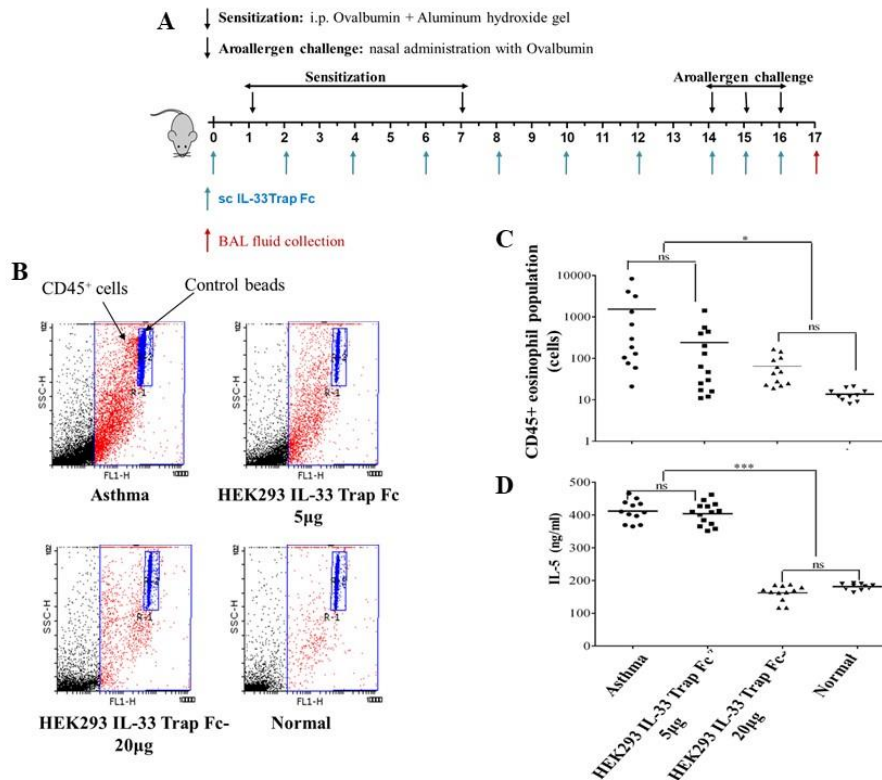

Figure 4. The therapeutic activity of IL-33 Trap Fc expressed by HEK293 on an asthma mouse model. (A) Experimental setup for IL-33 Trap Fc treatment on OVA-induced asthma mouse model. Mice (n=20) were sensitized by intraperitoneal injection with 60 µg of ovalbumin (Merck) and 10 mg of aluminum hydroxide gel in saline on day 1 and day 7. On days 14, 15, and 16, mice were challenged by nasal administration with 100 µl of 1% OVA in saline. Mice were injected at intervals of 2 days with IL-33 Trap at indicated doses from day 0 to day 14. On days 14, 15, 16, the mice were injected with IL-33 Trap Fc 30 minutes before OVA nasal administration. The mice were sacrificed 24 hours after the last aeroallergen challenge, and the BAL fluid was obtained. (B) Histogram and (C) graph the numbers of CD45+ eosinophil in BAL fluids were determined using Trucount tubes with flow cytometry. (D) IL-5 levels in BAL fluids were quantified by ELISA. The data were analyzed by ANOVA test (n=10 – 14). ***p< 0.001, *p< 0.05, ns: non-significant.

We also measured the levels of IL-5, a Th2 cytokine that promotes eosinophilic inflammation and infiltration into the airways in BALF with ELISA assay (Figure 4D). We found that consistent with our findings for the CD45$^+$ eosinophil population, IL-33 Trap Fc decreased IL-5 levels in BALF of OVA-induced asthmatic mice. The administration of 20 µg of IL-33 Trap Fc significantly decreased IL-5 levels than the OVA group (p<0.001). There were no statistically significant differences in IL5 concentrations from 20 µg of IL-33 Trap Fc–treated and untreated asthma mice. The body weight of mice remained unchanged during the experimental period, and no significant differences were observed between groups (data not shown).

These *in vivo* data collectively suggested that IL-33 Trap Fc could prevent or decrease allergic asthma symptoms in the OVA-induced allergic asthma mouse model.

DISCUSSION

We investigated the role of purified IL-33 Trap Fc proteins expressed from two different systems, mammalian HEK293 cells and *P. pastoris* yeast, in blocking IL-33 activity *in vitro* and *in vivo* models of OVA-induced allergic asthma mice. We demonstrated that IL-33 Trap Fc from these systems interacted and captured free IL-33 molecules, restricting the deleterious effects of elevated IL-33. We also proved the anti-allergic effects of IL-33 Trap Fc expressed from HEK293 cells on a murine asthma model.

IL-33 Trap Fc, engineered by linking two different extracellular ligand-binding portions of IL-33 receptors, IL-1R AcP co-receptor and IL-33R, to the Fc portion of human IgG1, could be a novel potential protein for the treatment of inflammatory and allergic diseases by binding and restricting IL-33 availability. Cytokine trap consisting of two distinct receptors was expected to be a novel therapeutic strategy that has some advantages: (1) its dimeric form having longer half-life than the monomeric form, (2) blocking activity of cytokine with higher affinity than that

measured for the natural receptors on cells [Economides *et al.* 2003]. Recently, IL-1 Trap, trade name Arcalyst, a dimeric fusion protein engineered by combination IL-1R, IL-1RAcP, and Fc IgG1 domain, was approved by FDA for treatment of cryopyrin-associated periodic syndromes (CAPS), including familial cold autoinflammatory syndrome, Muckle-Wells syndrome, and neonatal-onset multisystem inflammatory disease [Analyst FDA Approval History - Drugs.com]. Our results demonstrated that IL-33 inhibitory activity of IL-33 Trap Fc from HEK293 cells was 10 times higher than that of IL-33R fused Fc IgG1 domain (IL-33R Fc) protein. That means conjugation of the extracellular region of co-receptor, IL1R-AcP, to the IL33R-Fc is necessary to increase the interoperability and capture of IL-33, consequently enhancing the inhibitory function of IL-33 Trap Fc.

To our knowledge, our data demonstrated for the first time the therapeutic effects of the recombinant IL-33 Trap Fc protein on asthmatic animal models. In a previously published paper, Holgado *et al.,* showed that IL33Trap protein, a fusion protein of the extracellular region of IL-33R and its co-receptor IL-1R AcP, was further augmented the anti-inflammatory activities *in vitro* and a mouse model of acute allergic airway inflammation compared to that of soluble IL-33R (Holgado *et al.,* 2019). The difference between our IL-33 Trap Fc protein and IL33Trap created by Holgado *et al.* is conjugation Fc IgG1 region at the C terminus of IL-33 Trap Fc. The Fc IgG1 region has been engineered to prolong serum half-life and facilitate recombinant protein collection and purification from cell culture media. Fusing a recombinant protein to an IgG Fc region is a typical approach for increasing bioactivity via dimerization and extending the plasma half-life of recombinant proteins (Kontermann *et al.,* 2011; Czajkowski *et al.* 2012, Levin *et al.,* 2015; Sockolosky *et al.,* 2014; Sockolosky *et al.,* 2015). Many approved therapeutic drugs are Fc fusion proteins, such as etanercept (Enbrel®), belatacept (Nulojix®), and romiplostim (NPlate®), which are engineered by

fusion Fc regions and tumor necrosis factor (TNF) receptor 2, CTLA-4's extracellular region, and a TPO-mimetic peptide, respectively (Beck *et al.,* 2011). Therefore, our purpose fused the Fc IgG1 domain in the design of IL33Trap-Fc protein to direct the formation of a disulfide-linked dimer molecule, consequently increasing the ligand-binding affinity compared to that of the monomeric molecule. However, dimeric Fc fusion molecules could cause adverse effects on tissue permeability, nonspecific interactions to Fc receptors or Fc-associated effector functions, and low expression in host cells... We need further experiments to prove whether Fc fusion plays a vital role in expanding the anti-inflammatory activities of IL-33 Trap Fc protein.

The methylotrophic *P. pastoris* yeast was initially utilized to increase the yield and reduce the overall production cost of the fusion protein compared to the mammalian cells expression system. This yeast has been used to express several recombinant soluble receptors successfully as potential biopharmaceutical products, including soluble IL-6 receptor (Vollmer *et al.,* 1996), soluble transforming growth factor-β receptor (TGF-βR) (Glansbeek *et al.,* 1998), soluble IL-1 receptor type I [Klasing *et al.,* 2001), soluble human α7 acetylcholine receptor (Avramopoulou *et al.,* 2004), endothelial cell protein C receptor (EPCR) (Sagaseta *et al.,* 2009). We previously reported that the IL-33 inhibitory activity of the yeast-derived recombinant IL-33R Fc protein is comparable to the counterpart protein expressed from the mammalian expression system (Nguyen *et al.,* 2015). We also showed that the IL-33R Fc from *P. pastoris* were glycosylated higher than the corresponding protein produced in a mammalian expression system (Nguyen *et al.,* 2015). The glycosylation process in proteins expressed from *P. pastoris* yeast, which leads to an increase in the molecular mass of the recombinant protein, has been reported in several previous studies (Rodenburg *et al.,* 1998; Zhu *et al.,* 1999). The current study demonstrated that compared with the counterpart recombinant protein expressed from HEK293 cells, IL-33

Trap Fc expressed from *P. pastoris* was glycosylated much more intensively and heterogeneously. It implies that the recombinant protein from *P. pastoris* yeast might be glycosylated at different levels leading to the variety in its molecular weight, which may influence the reaction kinetics, solubility, serum half-life, thermal stability, *in-vivo* activity immunogenicity, and protein-protein interaction of a given recombinant protein (Demain *et al.,* 2009). Further work is needed to identify whether IL-33 Trap Fc expressed from *P. pastoris* is suitable for developing a safe, effective, and convenient therapeutic protein.

The biological activities in inhibiting IL-33 of IL-33 Trap Fc expressed from HEK293 cells and *P. pastoris* yeast was analyzed on murine lymphoma EL-4 cells. Under the stimulation of IL-33, EL4 cells synthesize and secret IL-2 into the extracellular environment. The IL-2 production from EL-4 cells is directly proportional to stimulated IL-33 levels. At the concentration of IL-33 Trap Fc 100 times higher than the concentration of IL-33, the yeast-derived recombinant protein attained 50% inhibition of IL-33 activity. Interestingly, the counterpart from mammalian cells was able to inhibit IL-33 activity at the same concentration completely. *P. pastoris* is an expression system with various advantages, such as having post-translation modifications of recombinant proteins, high production yield, simple scaling-up, and low-cost culture conditions. Unfortunately, the heavy glycosylation of *P. pastoris* yeast-derived IL-33 Trap Fc in a heterogeneous pattern may be a reason that leads to a decrease of its biological activity compared to the counterpart protein from mammalian cells.

We demonstrated the clinical feasibility of IL-33 Trap Fc protein obtained from HEK293 cells in an *in vivo* experimental model. Th2 cytokines, such as IL-4, -5, and -13, play an important role in the pathophysiology of asthma. IL-4 promoted allergic inflammation via Th2 cell differentiation, IgE synthesis, and mucus hypersecretion (Seder *et al.,* 1992; Schuijs *et al.,*

2013); IL-5 facilitated eosinophilic inflammation and infiltration into the airways (Foster *et al.,* 1996; Flood-Page *et al.,* 2003), and IL-13 triggered airway hyperresponsiveness (Finkelman *et al.,* 2010). In addition, eosinophilic infiltration in pulmonary vessels, alveolar ducts, and alveoli is common in asthma. Our result showed that IL-33 Trap Fc reduced the typical asthmatic features, including eosinophilic infiltration and airway hyperresponsiveness, with a corresponding downregulation in the levels of IL-5. A clinical trial of IL-33 Trap Fc can reveal therapeutic efficacy and possible adverse effects on normal tissues in the future.

In conclusion, we showed that two expression systems, mammalian HEK293 cells and *P. pastoris* yeast, could express and secrete recombinant IL-33 Trap Fc protein in a glycosylated and perhaps dimeric form. HEK293-derived IL-33 Trap Fc inhibited the IL-33 activity more effectively than the yeast-derived counterpart molecule *in vitro* conditions. Furthermore, the combination of two extracellular receptors, IL-1RAcP and IL-33R, enhanced the activity of this protein in blocking the activity of the IL-33 cytokine. A majority of allergic asthma has characteristic inflammation cytokines regarding the abundant expression of IL-33 and the present study suggested that IL-33 Trap Fc may be a therapeutically tractable candidate for the treatment of allergic asthma diseases.

Authors contributions: *QDN designed the study. TTTN and PHV prepared materials, conducted experiments. TTTN and QDN analyzed the data and drafted the manuscript. All authors read and approved the final manuscript.*

Conflict of interests: *The authors declare that there is no conflict of interest in this research.*

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