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Research Article

Camelid derived anti IgE nanoantibodies block Th2 response in induced acute allergic lung inflammation of BALB/c mice

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Abstract

During allergic inflammation, mast cells play an important role via IgE dependent mechanisms. Following allergen exposure, onset of the initial signals is Th2 cytokine dependent. This subsequently results in the augmentation of B cell activation for sequestering IgE, which binds with the FcεR on the surface of the mast cells. It initiates the degranulation of pre-packaged pro-inflammatory mediators responsible for most of the downstream clinical symptoms associated with acute allergic lung inflammation. Cross linking of antigen and the mast cell via IgE is well documented. Past efforts targeted IgE–mediated blocking pathways by humanized IgG1 monoclonal antibody (Omalizumab) were well defined. Generation of anti-IgE nanobody from Indian dromedarius camel is a first-in-class novel approach to reduce hypersensitivity against ovalbumin challenged allergic asthma in mice model. In this study, ovalbumin was intratracheally administered, resulting in an elevated amount of IgE in Balb/c mice. This study reveals that anti-IgE nanoantibody synthesis suppresses IgE production by 2.36 folds after nanobody therapy followed by 1.7 folds reduction in goblet cell metaplasia in the lung tissues. Other hallmarks of airway inflammation, like bronchoconstriction, are also reduced by the nanobody. This study demonstrates that the generation of an anti-IgE nanobody from Indian dromedarius camel presents a pioneering and effective approach, significantly suppressing IgE, indicating its potential as a first-in-class therapeutic strategy with broader implications for mitigating allergic inflammation.

Keywords: Airway hyperresponsiveness, Allergy, Immunoglobulin E, Nanobody, Variable heavy chain antibody

Highlights

- Evidence of the epidemiological finding shows a strong relationship between immunoglobulin E (IgE) antibodies and cytokine activation in the allergic onset reaction.
- Small size, high affinity towards antigen, stability, solubility and very low immunogenicity leads to prefer usage of single chain variable region (scFv)/ nanobody.
- Camelid derived anti-IgE nanobody treated mice exhibited a 2.5-fold reduction in non-invasive plethysmograph lung function test, indicating relief from allergic phenotype and epithelial damage, highlighting the crucial role of the IgE blocker.
- Elevated IgE levels, a hallmark of airway inflammation, were reduced 2-fold with camelid anti-IgE nanobody therapy, supporting the prevention of mast cell degranulation and bronchial lumen blockage, suggesting potential relief for severe allergic inflammation.
- Camelid derived recombinant IgG2 fusion protein can be an alternative immune therapy against airway inflammatory IgE.

INTRODUCTION

Allergic asthma is clinically characterized by symptoms like bronchoconstriction, chest tightness, increased mucus secretion, cough, and wheezing. Allergen induced lung inflammation includes Th2 derived cytokines, and increased number of eosinophils which are involved in developing the disease (George *et al*., 2016). Allergic lung inflammation involves various immune cells (macrophages, mast cells, monocytes) for the cascade of inflammation. Epidemiology of allergic asthma shows a strong relationship between immunoglobulin E (IgE) antibodies or total IgE and asthma (Burrows *et al*., 1989; Sears *et al*., 1991; Sporik *et al*., 1995; Sunyer *et al*., 1995). In airway inflammation, effector cells like mast cells and basophils activate receptors like FcεR and FcεRII, which bind the IgE antibodies. Affinity between serum IgE and their FcεRI receptors and their expression

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on the antigen presenting cells increases in asthma patients (Hollowaj *et al*., 2001). Membrane-bound IgE cross-links FcεRI on mast cells and/or basophils and thereby releases histamine, platelet-activating factors for which early allergic sensation appears (Rosenwasser *et al*., 2005; Stone *et al*., 2010).

Researchers have shown various potential therapeutic strategies to inhibit the development and progression of asthma. Alternative therapy using anti-human IgE monoclonal antibodies (anti huIgE MCA) has been found to successfully prevent histamine release and binding IgE *in vitro* (Presta *et al.*, 1994; Rabe *et al.*, 1998). Humanized anti-IgE antibody (Omalizumab) has been shown to reduce the circulating free IgE (less than 50 ng/mL) (Boulet *et al.*, 1997). Common adverse reactions may happen during omalizumab injection. Pain and itching at the injection site, as well as the development of parasitic infestations, have been reported. Some patients had laryngeal oedema and bacterial sinusitis even after 23rd dose of omalizumab injection (Yalcin *et al*., 2013). Membrane bound IgE is most promising for immunologists because they can generate isotype specific IgE responses. It is reported that CεmX monoclonal antibody can control IgE production by targeting mIgE^{+} B cells (Jiun-Bo *et al*., 2010). Based on this, Qualizumab has been developed. Camelid antibody (nanobody) has been developed against human serum albumin (WO2012175400A1) for therapeutic and prophylactic application in allergic asthma and rhinitis diseases (Bruno *et al.*, 2012). These nanobodies have been validated under clinical trials, mainly phase I. Single domain antibodies or nanobodies are composed of two heavy chains of conventional antibodies only and are, therefore, referred to as heavy chain antibodies (HCAbs) (Ghassabeh *et al*., 2010). They, however, lack light chains and CH1 domain. These heavy chain antibodies contain a single variable domain (VHH) and two constant domains (CH_2 and CH_3) (Deffar *et al*., 2009).

The aim of this study is to develop single domain nanobody from Indian camel (*Camelus dromedarius*) against ovalbumin specific serum IgE and to determine its therapeutic effects on ovalbumin induced acute allergic asthma in Balb/c mice. We investigated airway inflammation, associated with Th2 responses, expression of membrane bound IgE, histopathological changes and circulating basophils and eosinophils marker in inflammation cascade.

MATERIALS AND METHODS

Preparation of immunogen

For the immunogen preparation, mice were categorized into two distinct groups (n=23 for each group). a. Control, b. Ova.

The mice from Ova group were sensitized with intraperitoneal administration of 100 g chicken egg ovalbumin (ova), in a complex with Al $(OH)_3$, on day 0 (Shakeri *et al*., 2017; Banerjee *et al*., 2009), followed by intratracheal challenge with 250 g ova on day 8. Further, 125 g ova was intratracheally challenged on days 15, 18 and 21 (Banerjee *et al*., 2009). After sacrifice on day 22, murine blood (800 L/mice) was collected in normal Eppendorf tubes. After one hour (allow to clot), blood was centrifuged at 4000 rpm for 30 min. Straw colored serum (first allowed to clot at room temperature and then centrifuge) was separated from the blood cells (we separated serum because our aim was to isolate serum IgE) and stored at -20°C. Total immunoglobulins were pooled (The salt fractionation involved an ammonium sulfate cut at 55%, followed by overnight dialysis at a low temperature) by the overnight dialysis and concentration of the IgE was estimated by standard protocol (Banerjee *et al.*, 2009) from Ova treated groupand control (Placebo).

Estimation of serum IgE from blood serum

Concentration of ova specific serum IgE was determined using 96 micro titre plates which were coated with 50 g/mL ovalbumin. Protocol was followed by data sheet from BD Biosciences. Murine serum sample was diluted and added to the ovalbumin (Paul *et al*., 2019). Biotin labeled rat anti-mouse IgE was used (BD Biosciences) followed by avidin – HRP. Finally, TMB substrate was used and intensity of colour developed was measured by ELISA plate reader at 405 nm. Standard graph was prepared by purified IgE (BD Biosciences).

Immunization protocol for nanobody production

Three years old male camel breed (Mewari) was immunized [NRCC/ PME/ 6(141)/ 2000-Tech/ Vol II]. On day 0, 100 g of mice origin total immunoglobulin (Khaled *et al*., 2015) was mixed with 1 mL PBS and 1 mL of Freund (Complete followed by incomplete) adjuvant (Sigma). Higher dose was applied every seven days (around 20 times camel was immunized) Immunization schedule is presented in Supplementary Fig. 1. On day 7, 150 µg of immunoglobulin complex was injected subcutaneously. After 105 days, blood was collected from immunized and naïve animal. Finally, ouchterlony double diffusion assay was performed to observe immunogenic response (Supplementary Fig. 2).

Construction of the phage display Libraries

Total RNA was isolated from camel blood collected with anticoagulant (Sodium heparin salt; 40 IU/mL) followed by cDNA synthesis. First PCR was performed

using Forward primer (5′-GTCCTGGCTGCT CTTCTACAAGG-3′) and (5′-GGTACGTGCTGTTGAACTGTTCC-3′) (Supplementary Fig. 3). Second PCR was performed with addition of VHH Forward (5′-GATGTGCAGCTGCAGGAGTCTGGRGGAGG-3′) and reverse (5′ -GGACTAGTGCGGCCGCTGGAGACGGTGACCTGGGT-3′) primers (Supplementary Fig. 4) (Ghassabeh *et al.*, 2010). Purified second PCR product was ligated with pGEM-T Easy vector (Promega). This plasmid was double digested by Pst1 and Not1 restriction enzymes, and the product was further ligated to pHEN4 phagemid vector (Ghassabeh *et al*., 2010) (Supplementary Fig. 5). TG1 *E. coli* bacteria were used for the transformation. Positive clone was confirmed by colony PCR using GIII universal primer (Supplementary Fig. 6).

Selection and purification for antigen specific single domain antibody (VHHs)

M9 Minimal media was used to propagate the positive clone of the transformed bacteria. Infection was carried out by M13KO7 helper phage for phage display (Ghassabeh *et al*., 2010). Specific antigen was coated on Maxisorp microtitre plates, and three rounds of panning were performed for phage enrichment (Supplementary Fig. 7). Specificity was confirmed by phage titration. VHH containing colonies were grown in 24 well plates to express the protein followed by standard ELISA protocol. Anti HA antibody was used to determine the expressed protein. Absorbance was read at 405 nm. After selection of the final clone, VHH fragments were cloned into pHEN6 vector in WK6 *E. coli* bacteria, followed by 1 mM IPTG induction. This recombinant protein was tagged with 6XHis and then passed through the Nickel column. Purification was carried out based on the affinity chromatography. Purified protein was further run on 14% SDS gel to confirm the size, i.e. 15 kDa. Periplasmic fraction was obtained by osmotic shock. Protein purification was carried out by Ni-NTA column and confirmed by western blot using anti-His antibody.

***In vivo* neutralizing capacity**

Mice: Female BALB/c mice of 6-8 weeks weighing 20-25 g were categorized into four groups (n= 5 for each group): A. Control, B. Ova, C. Ova+ anti-mouse ova specific immunoglobulin CAb, D. Ova+ anti-mouse placebo immunoglobulin CAb.

Induction of chronic allergic asthma and therapy: Induction of the allergic hyperresponsiveness in mice was done by ovalbumin challenge, as mentioned before (Banerjee *et al*., 2009). The control group were treated with only saline water, whereas the ova group, ova along with ova specific IgE and ova along with disease free placebo IgE were sensitized along with therapy, 100 µg of purified nanobody was injected intravenously (i.v) through tail region after 30 min of each ovalbumin challenge. On day 22, mice were sacrificed.

Measurement of lung function: Mice were challenged with increasing doses of methacholine 5mg/mL, 10 mg/mL, 15 mg/mL, and 20 mg/mL. Whole-body non-invasive plethysmograph was used to measure the airway hyperresponsiveness to methacholine 24 hrs after the last ovalbumin challenge. Degree of bronchoconstriction expressed as enhanced pause (Penh) was measured after each challenge for 4 min time duration (Banerjee *et al*., 2009).

Preparation of bronchoalveolar lavage fluid (BALF): BALF was collected in ice cold PBS from both lungs. Total cells were counted using Trypan blue, and differential cell count was performed using Wright-Giemsa stain under 10X and 40X magnification (Paul *et al*., 2018).

Lung histology: For histology, lung tissues were harvested and dehydrated followed by embedding in paraffin. Then 5 µm sections were cut, and visualized by hematoxylin and eosin staining. Alcian blue staining was performed to observe mucus around the pulmonary blood vessels (Banerjee *et al*., 2009).

Ova-specific serum IgE: To determine serum IgE, a standard IgE graph was plotted according to data sheet. Ovalbumin was added for coating in 96 well microtitre plates. After blocking, serum sample containing IgE was added and incubated. Biotin-tagged anti-mouse IgE was used, and finally, Avidin-HRP (1:1000 dilution) was added. TMB substrate was used for the colour development. Absorbance was read at 405 nm (Paul *et al*., 2019).

Colony forming unit-cell (CFU-c) assay: Progenitor cells were detected in the culture using semi solid media known as CFU-c assay. Cells were restricted to one or two lineages. Methyl cellulose is the semi solid gelling agent in which progenitor cells were incorporated and have self-renewal capacity. Cells were incubated in 5% CO_2 humidified chamber. Clonogenic potential was counted after 7 days under microscopic view (Paul *et al*., 2018).

Statistical analysis

Data from the present study were analyzed using GraphPad Prism 6. All values are represented as Mean±SEM, and p-values less than 0.05 are considered statistically significant. Statistical significance has been calculated by student ‘t’- test.

Table 1. List of primers are used in the experiment.

Sl. No.	Name of the Oligo	Sequence 5′ to 3′	References
1	**CALL 001 -F**	GTCCTGGCTGCTCTTCTACAAGG	(Ghassabeh *et al*., 2010)
2	**CALL 002-R**	GGTACGTGCTGTTGAACTGTTCC	(Ghassabeh *et al*., 2010)
3	**PstI**	GATGTGCAGCTGCAGGAGTCTGGRGGAGG	(Ghassabeh *et al*., 2010)
4	**NotI**	GGACTAGTGCGGCCGCTGGAGACGGTG ACCTGGGT	(Ghassabeh *et al*., 2010)
5	**Universal Primer**	TCACACAGGAAACAGCTATGAC	(Ghassabeh *et al*., 2010)
6	**GIII primer**	CCACAGACAGCCCTCATAG	(Ghassabeh *et al*., 2010)
7	**BstEII**	GATGTGCAGCTGCAGGAGTCTGGRGGAGG	(Hmila *et al*., 2008)

RESULTS

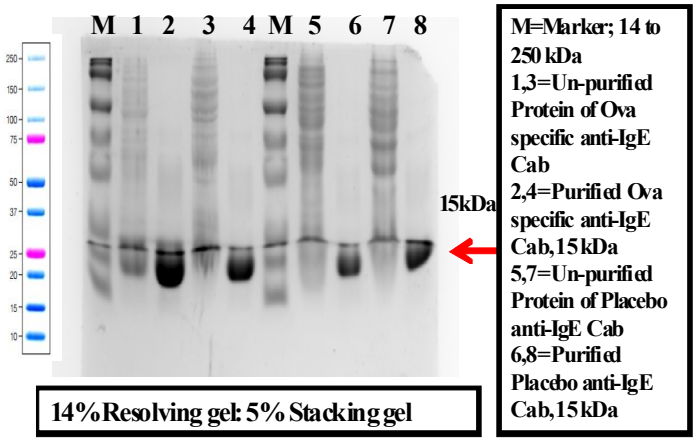
Purification and purification of nanobodies: Unpurified protein, and purified protein while unpurified protein was complex mixture of the whole bacterial protein and successful cloning shows exact expression of recombinant protein from the bacteria system (Fig. 1).

Anti-IgE nanobodies can inhibit airway hyper-responsiveness: Intratracheally exposure to ova for 21 days increased the inflammatory cell recruitment and infiltration of eosinophils, as well as increased Penh value in lung function test by Plethysmograph (Fig. 2). Anti-IgE nanobody therapy helped reduce Penh value compared to OVA treated mice. Placebo IgE nanobody treatment was non-specific and that's why reduction in airway hyperresponsiveness was not effective.

Mice were challenged with increasing doses of methacholine (5mg/mL, 10 mg/mL, 15 mg/mL and 20 mg/mL). At 5 mg/mL, mice of the ovalbumin group showed 3.2 fold increase in Penh compared to control group and after anti-IgE nanobody treatment, mice showed 2.5 fold reduction in Penh, which is very close to control group. Placebo treated group shows high Penh value that indicates nonspecific neutralization effect of antibody therapy.

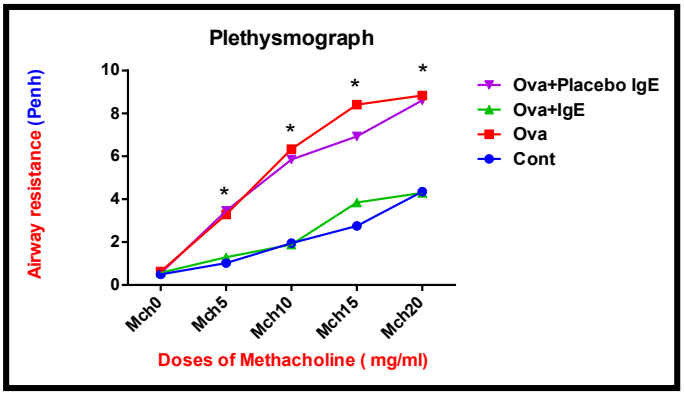
When animals were challenged with methacholine dose of 10 mg/mL, Penh value was increased by 3.24 fold in ovalbumin treated mice compared to control group, while in nanobody therapy mice, Penh value was increased 3.34 folds compared to ovalbumin treated mice.

When mice were challenged with 15 mg/mL methacholine, ova treated mice showed 3 fold higher Penh value compared to control group, while nanobody brought it down 2.18 fold and



[After coomassie stain, lanes 1 and 3 show un-purified protein of Ova specific anti-IgE camelid antibody. Lanes 2 and 4 show purified Ova-specific anti-IgE camelid antibody, 15 kDa. Lanes 5 and 7 show un-purified protein of placebo anti-IgE camelid antibody. Lanes 6 and 8 show purified placebo anti-IgE camelid antibody, 15 kDa. M indicates protein marker of 10 to 250 kDa ranges.]

Fig. 1. SDS PAGE



[Mice were challenged with OVA-Al $(OH)_2$ intratracheally upto 21 days, and on 22 day, lung function was tested from each group (n=3) as described in Fig. 2. Data are represented as mean±SEM. The outcome of the data is statistically significant and was performed by the student 't' test, while comparisons were *p<0.005 for control, OVA, OVA + anti-IgE, OVA + Placebo IgE.]

Fig. 2. Lung function test by Plethysmograph

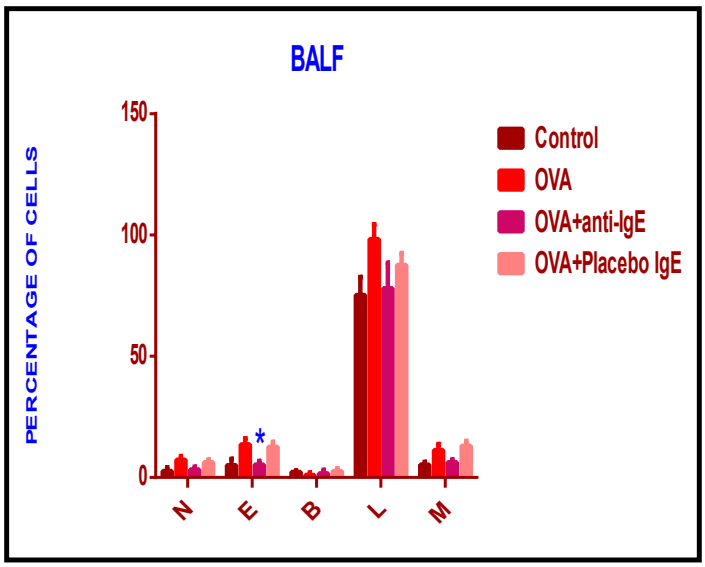
placebo IgE treated group showed no change of Penh value.

When final dose i.e. 20 mg/mL of methacholine was applied, ova group showed 2 fold higher Penh value compared to control group, and nanobody brought it down to 2 fold. Placebo IgE group showed no change of Penh value (All the data remain significant in which $p<0.05$, compared to control, ova, ova+anti-IgE and ova+placebo IgE) (Fig. 2).

Anti-IgE nanobodies reduce infiltration in BALF: In BALF, after nanobody application, neutrophil was reduced by 2.70 fold compared to ovalbumin treated sample. Eosinophils were reduced by 2.56 fold. After nanobody therapy, lymphocytes and monocytes were brought down 1.26 and 1.78 fold while placebo IgE therapy showed non-specific reaction values (Fig. 3) (*$p<0.05$, compared to ova).

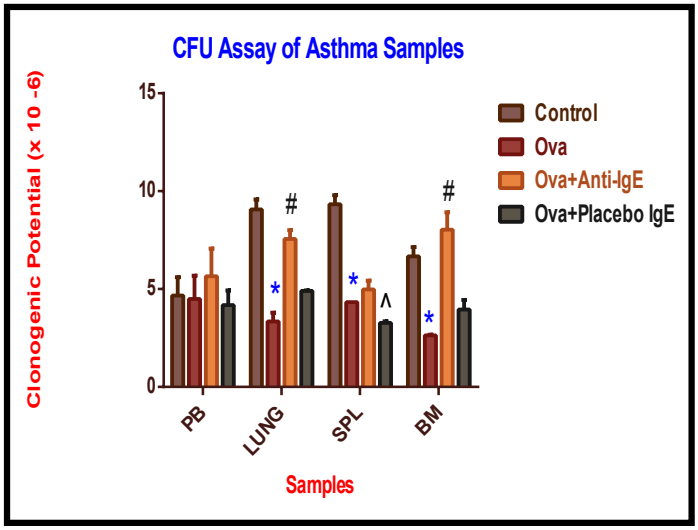
Anti-IgE nanobodies control the regenerative capacity in bone marrow and lung: Data represented the effect of anti-IgE nanobody therapy in hematopoietic pools or stem cell niches after the ovalbumin challenge (Fig. 4). In the lungs of the asthmatic mouse, its regenerative capacity was being tampered with by ovalbumin; it suppressed colony formation 2.79 fold, whereas anti-IgE nanobody helped to increase the clonogenic potential by 2.24 fold, which was statistically significant (*$p<0.05$ compared to control, # $p<0.05$ compared to Ova group). In the spleen, ovalbumin suppressed colony formation by 2.20 fold, and anti-IgE application showed no effect as such (*$p<0.05$ compared to control; ^$p<0.05$ compared to ovalbumin treated sample). In bone marrow, ovalbumin suppressed colony formation by 2.5 fold, while anti-IgE nanobody helped to regenerate the capacity of colony formation by 3 fold. Here, no significant result was found after placebo IgE application (Fig. 4) (*$p<0.05$ compared to control, # $p<0.05$ compared to Ova group).

Anti-IgE nanobodies reduce Ovalbumin specific IgE: Repeated challenge of the allergen exposure activates Th2 differentiation via various cytokine involvements. When the target organ was the lung, after activation, B cells synthesised IgE, which was the hallmark of allergic inflammation. Successfully blocking of the IgE was observed by nanobody therapeutic



[Data represents the differential count of BAL fluid after sacrifice of the mice. Initially, mice were challenged with OVA, OVA + anti-IgE, and OVA + Placebo IgE. A smear of the BAL fluid was stained with Wright Giemsa, and cells were counted under 40X in the compound light microscope. There were three different fields were observed, and the percentage was calculated by counting 100 cells. Data are represented as mean±SEM, while n=3. Student 't' test was performed, and data are statistically significant as *$p<0.005$ for control, OVA, OVA + anti-IgE, OVA + Placebo IgE.]

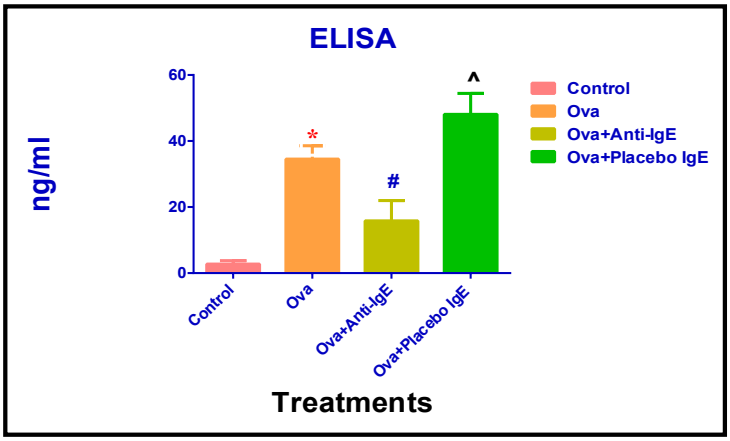
Fig. 3. Differential count of BALF



[Data are represented as regenerative capacity after OVA, OVA + anti-IgE, OVA + Placebo IgE challenge in mice. Samples were assessed from peripheral blood, lung, spleen and bone marrow. Samples were analysed from triplicate values; data are represented as mean±SEM. Student't' test was performed, and data are statistically significant as *$p<0.005$ for OVA, #$p<0.005$ for OVA + anti-IgE, ^$p<0.005$ for OVA + Placebo IgE.]

Fig. 4. CFU assay

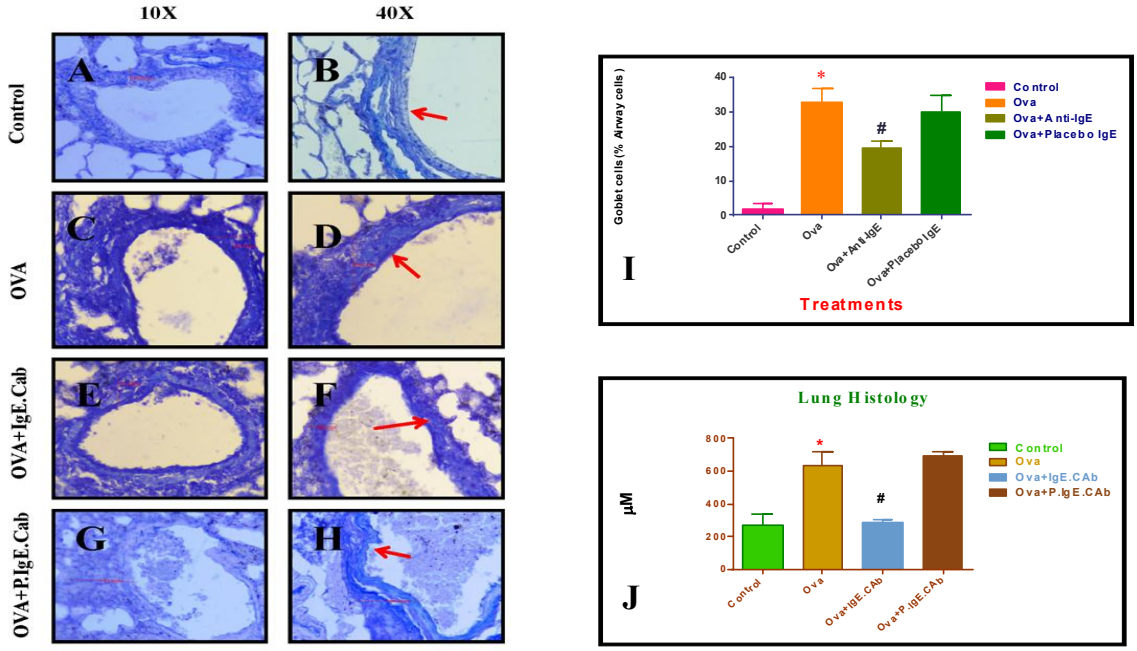
application in this study. Results showed 19 fold elevation in the amount of IgE after ovalbumin challenges, which was, very interestingly, brought down by 2 fold after intra venous application of anti-IgE nanobody, whereas placebo IgE had mismatch non-specific reaction (Fig. 5). (Significant value was accepted if $p<0.05$. *$p<0.05$ compared to control; #$p<0.05$ compared to Ova group; n=3). Significant value was analyzed by student 't' test.



[Allergic inflammation was determined by elevation of the serum IgE levels. Comparisons were assessed by control, OVA, OVA + anti-IgE and OVA + placebo IgE serum samples. Data are represented as mean±SEM (n=3); statistical significance was performed by student 't' test; comparison are *$p<0.005$ for OVA, #$p<0.005$ for OVA + anti-IgE, ^$p<0.005$ for OVA + Placebo IgE.]

Fig. 5. ELISA of serum IgE

Anti-IgE nanobodies reduce goblet cells metaplasia: Lung sections (5 µm) of control mice, Ova treated, Ova + anti-IgE treated and Ova + P. IgE treated were mounted and stained with alcian blue according to standard protocol. Ova treated mice, and Placebo IgE treated mice showed peribronchiolar and perivascular eosinophilic inflammation while anti-IgE treated mice showed reduction of eosinophilic inflammation. Measurements of goblet cells were high (19 folds increase) in Ova treated mice, whereas anti-IgE nanobody reduced them by 1.67 folds. Placebo treated IgE therapy did not show any massive change of the goblet cells metaplasia (Fig. 6.I). Measurement of thickness of the lung section was increased by 2.33-fold in Ova treated mice while thickness was reduced by 2.21 fold in anti-IgE nanobody mice. Placebo-treated IgE mice showed no significant change in thickness (Fig. 6.J).



[Anti-IgE nanaobody treatment reduces cellular infiltration are assessed histopathology. Cut sections of the tissues are stained with H&E and examined under a microscope. There are four different views that shows distinct features of asthmatic airway inflammation. **Fig. I** represents goblet cell metaplasia; data are statistically significant as *$p<0.005$ for OVA, #$p<0.005$ for OVA + anti-IgE. **Fig. J** represents thickness of the alveolar region. Thickness was measured under microscopic view in which *$p<0.005$ for OVA, #$p<0.005$ for OVA + anti-IgE. Data are statistically significant assessed by student 't' test. Thickness was measured by µM scale, n=3, and data are mean±SEM.]

Fig. 6. Histology

DISCUSSION

Airway hyper-responsiveness and allergic disease are complex phenomenon associated with Th2 type cells. Evidences of the epidemiological finding show strong relationship between immunoglobulin E (IgE) antibodies and cytokine activation in the allergic onset reaction. As already explained by *Burrows et al.* (1989), asthma is always associated with serum IgE related reaction. In all previous studies, different therapeutic strategies have been applied to ameliorate the disease. For the relief of the disease, multiple strategies have been used – blocking different pathways, bioactive compound in phytochemicals, receptor blocking antagonistic approach and so on. In this study, we describe here camelid derived anti-IgE antibody to block the IgE mediated pathogenesis of allergic disease. It has been previously reported that a direct approach against IgE is Omalizumab, which is the first successful recombinant, humanized laboratory based anti-IgE approved drug, has successfully reduced free circulating IgE at less than 50 ng/mL (MacDonald *et al.*, 1987). Mostly, the biggest drawback of the monoclonal antibody is its large size and the inability of kidney filtration system to remove it successfully. Therefore, camelid derived recombinant IgG2 fusion protein can be an alternative therapeutic immune therapy against airway inflammatory IgE. Camelid derived aforementioned HCAbs have several advantages compared to conventional antibodies, including high affinity towards antigen, stability, solubility and very low immunogenicity. No cytotoxic effects have been reported in serum of the injected mice (Dumoulin *et al.*, 2002; Cortez-Retamozo *et al.*, 2004; Revets *et al.*, 2005; Coppieters *et al.*, 2006). In our study, after nanobody application, mice revealed 75% survival rate even though use of extreme critical dose in intracheal challenges with allergen sensitization mode. To comprehend the molecular mechanisms underlying pathophysiology and explore nanobodies as a potential immune therapy, we measured lung function using non-invasive plethysmography. Penh values of camelid IgE-treated mice showed 2.5 fold reductions compared to Ova-treated mice, and the values were close to those of the control mice. To understand the critical role of IgE blocker, plethysmograph is the ideal assay to assess the airway hyper-responsiveness. In this study, characteristic features of epithelial damage have been found in lung and have increased cell recruitment. Infiltrating eosinophils also support the development of eosinophilia in Ova-treated mice, which was reduced by 2.56 fold in BALF. Progenitor cells were damaged and irrevocably lost after allergen exposure, while camelid derived nanobody helped restore its regenerative capacity (2.24 fold increase in lung, 3.00 fold increases in bone marrow). Elevated amount of IgE is the hallmark in airway inflammation. Our results showed 19 fold overproduction of IgE, which was brought down to 2 fold by anti-IgE nanobody therapy. This data supports blocking the condition of the activated B cells and the release of IgE, ultimately preventing mast cell degranulation and blocking the bronchial lumen with mucus, proteins, cellular debris and camelid nanobody supposed to relieve hyper-responsiveness in severely allergic patients.

We assessed the goblet cells, which are nothing but pseudo-stratified columnar squamous epithelial cells. After repeated challenges by allergen, these cells remained metaplastic, filled with acidic mucin. In allergic condition, it becomes barrel shaped which is the major hallmark of the allergic reaction (Banerjee *et al.*, 2009). Under 40X and 100X magnifications, number of goblet cells are high (19 folds increase compared to control) and nanobody helped to reduce the number by around 1.67 folds. Finally, analysis of the lung section under the light microscope confirms that thickened membrane surrounds the alveolar region. Thickening layer is drastically increased 2.33 fold and it is reduced by 2.21 fold by single domain antibody application.

Due to the complexity and diverse nature of the disease, mouse model is truly representing the clinical asthma. We should focus to create the disease in a specific target rather than all features to be reproduced in a single model. When looking into potential approaches for novel therapy to ameliorate the disease, we generally focus on the specific target moiety of the pathophysiology of the disease. On that principle, we used novel format, i.e. anti-IgE recombinant fusion protein, for direct neutralizing IgE antibodies. Our findings may be explored for further knowledge and evaluation of new findings of airway inflammation in future.

Conflict of interest: There is no conflict of interest among the authors.

Author's contribution: PP: Performed the experiment and the assays, analyzed the data and wrote the manuscript; NG, SM: Performed some of the assays, analyzed the data and wrote the manuscript; SKG: Immunized the Camel and performed some of the assays; ERB: Initiated the project, designed the experiments, analyzed the data and wrote the manuscript.

Ethical approval: Animals were reared in specific pathogen-free conditions at the animal house, Department of Zoology, under the University of Calcutta. The experiment was performed as per rules given by the departmental and institutional ethical committee [885/ac/05/CPCSEA, dated 22.06.2022].

Data availability statement: The authors confirm that

the data supporting the findings of this study are available within the article and its supplementary materials. Raw data that support the findings of this study are available from the corresponding author. The corresponding author is willing to provide the raw data upon reasonable request.

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