Molecular engineering of a therapeutic antibody for Blo t 5–induced allergic asthma

To the Editor:

Epidemiological studies have established a causal link between sensitization to house dust mite (HDM) allergens and the development of allergic asthma. Despite the coexistence of the 2 major dust mite species in the tropics and dual sensitization profiles in atopic individuals, IgE responses targeting Blomia tropicalis have limited cross-reactivity with Dermatophagoides pteronyssinus.1 Among all known allergens of B tropicalis, Blo t 5 is the principal sensitizing allergen in tropical countries. Blo t 5 has a high allergenic potency with more than 70% of IgE reactivity in subjects with asthma1–3 and 96.7% in children with asthma.2 As such, Blo t 5 is a potential target for therapeutic approaches in B tropicalis–mediated allergic asthma. Previous attempts at neutralizing allergens have been made using allergen-specific polyclonal IgG antibodies. In this study, we sought to explore the potential of using engineered allergen-specific mAbs as a novel treatment modality for HDM-driven allergic asthma.

To facilitate this, we first isolated an mAb with high affinity for Blo t 5 (see Fig E1, B, in this article’s Online Repository at www.jacionline.org). Epitope mapping studies with western and immuno-dot blot revealed that this mAb binds a linear epitope on an exposed surface of the Blo t 5 protein (Fig E1, C–E). Furthermore, our antibody targets a region of Blo t 5 previously implicated as a conformational IgE-binding epitope.5 Moreover, a dose-dependent inhibition of Blo t 5–specific IgE binding from allergic patients’ sera was observed when coincubated with increasing concentrations of anti–Blo t 5 mAb (Fig 1, A). This implies that our mAb may be binding the Blo t 5 antigen in a way that occludes the binding site of Blo t 5–specific IgE in patients’ serum by steric hindrance or distorting the Blo t 5 protein conformation such that Blo t 5–specific IgE in patients’ sera can no longer bind.

We then engendered chimeric variants in which the murine constant region (Fc) of the antibody was replaced with the constant domains of human IgG1, IgG1-LALA, and IgG2-S228P (Fig 1, B). Integrities of the expressed antibodies were verified by SDS-PAGE under reducing and nonreducing conditions. The chimeric antibodies were assembled with a heavy and light chain (Fig 1, C) and were correctly folded into full-length IgGs as depicted by bands at 150 kDa and larger on a nonreducing gel (Fig 1, D). Binding specificities for Blo t 5 were retained despite the molecular manipulations, with comparable affinities in the nM range as the murine IgG1 antibody (Fig 1, E).

The chimeric anti–Blo t 5 mAbs were tested for their ability to inhibit allergen-induced human basophil activation. Expression of CD63 was upregulated on stimulation with Blo t extract and anti-IgE (Fig 1, F) and close to 60% of this response was inhibited in the presence of the chimeric antibodies. The extent of inhibition was the strongest and weakest for the chimeric IgG2-S228P and IgG1 variant, respectively (Fig 1, G). In concordance with the widely documented anti-inflammatory properties of IgG4-blocking antibodies elicited by successful allergen-specific immunotherapy (SIT),5 our data intimate that engineered IgG4 antibodies can function as antagonists in allergic diseases by impairing basophilic activity.

We next examined the in vivo effects of anti–Blo t 5 mAb treatment in a murine model of Blo t 5–induced severe allergic asthma. As opposed to those generated via ovalbumin sensitization, more relevant animal models of HDM-induced allergic asthma are now well-established. We assessed the therapeutic potential of our anti–Blo t 5 mAb in a murine asthma model involving the passive transfer of Blo t 5–specific T(2) cells to naive mice and subsequently challenging the mice intranasally with Blo t 5 protein. The mice exhibited an asthmatic phenotype characterized by pulmonary eosinophilia, increases in serum IgE levels, and mucus hypersecretion.7

The murine anti–Blo t 5 mAb was administered intravenously at 100 μg per mouse on days 1 and 13 (Fig 2, A). Histologic analyses were performed to investigate the impact of mAb treatment on the structural integrity of asthmatic lungs (Fig 2, B and C). Hematoxylin and eosin staining revealed that mAb administration significantly decreased the overall inflammatory status of the airways by ameliorating epithelial thickening and inflammatory cell infiltration. In addition, goblet cell hyperplasia, mucus hypersecretion, and mucus plugging as denoted by the black arrows were majorly alleviated with mAb treatment. Last, the deposition of collagen fibers (denoted by blue staining) is indicative of airway remodeling and fibrosis. Treatment with anti–Blo t 5 mAb attenuated the extent of airway remodeling as highlighted by the reduced collagen deposits around the airway. Overall, anti–Blo t 5 mAb treatment notably restored the lung architecture in mice with asthma.

Treatment with our mAb significantly reduced the total immune cell count and the infiltration of eosinophils into the airways but did not have an impact on neutrophilia (Fig 2, D). Analysis of cytokines in the bronchoalveolar lavage fluid revealed a suppression in eotaxin levels with no changes in other key T(2) cytokines (see Fig E2 in this article’s Online Repository at www.jacionline.org). IL-5 was shown to prime eosinophils to chemoattractants, whereas eotaxin was responsible for directing the actual migration to the site of inflammation.8 The recruitment of eosinophils to specific tissues and their subsequent activity within these sites might be IL-5–independent.9 It is therefore highly plausible that the diminished eosinophilia observed in mAb-treated mice was an effect of reduced chemotaxis to the airways due to lower levels of eotaxin present.

Serologic effects of anti–Blo t 5 mAb administration in severely allergic mice with asthma were also analyzed by ELISA. A 5-fold reduction in total IgE levels was observed in the sera from mice treated with anti–Blo t 5 mAb compared with untreated mice, and mice treated with an isotype control antibody. Mice treated with anti–Blo t 5 mAb also had approximately 50% lower Blo t 5–specific IgE levels (Fig 2, E). Furthermore, key lung function parameters indicate an increase in the FEVs (Fig 2, F) and a decrease in respiratory rate (see Fig E3 in this article’s Online Repository at www.jacionline.org) when the mice were treated with anti–Blo t 5 mAb. Collectively, the antiallergic

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FIG 1. Molecular engineering of anti–Blo t 5 and application of chimeric variants in basophil activation. A, Inhibition of Blo t 5–specific IgE binding in patients’ sera by anti–Blo t 5 murine IgG1. B, Chimeric variants generated and used in this study. C, Purified chimeric antibodies on reducing SDS-PAGE gel. D, Purified chimeric antibodies on nonreducing SDS-PAGE gel. E, Affinity ELISA of purified anti–Blo t 5 chimeric antibodies. F, Representative flow plots illustrating basophil activation test and antagonistic capacity of chimeric anti–Blo t 5 mAbs. G, Percentage inhibition of basophil activation by chimeric antibodies. *P < .05. **P < .01. ***P < .0001.
FIG 2. In vivo study of anti–Blo t 5 mAb. A, Schematic diagram depicting experimental design involving intravenous injection of Blo t 5–specific TH2 cells, intranasal challenges with Blo t 5 protein (black arrows), and antibody treatment. B, Representative images of histochemical stainings of mouse lung sections. C, Scoring of extent of inflammation and mucus. D, Total and differential immune cell counts in the bronchoalveolar lavage fluid. E, Serum total and Blo t 5–specific IgE levels. F, FEV measurements. *P < .05. **P < .01. ***P < .0001.
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In summary, we have engineered an allergen-specific mAb targeting Blo t 5, the major allergen of the HDM species B. tropicalis, and showed that this mAb is capable of antagonizing Blo t 5–specific IgE binding in patients’ sera. More critically, we have demonstrated, for the first time, the protective effects of an allergen-specific mAb treatment in a murine model of severe allergic asthma driven by a clinically relevant and dominant allergen instead of the traditional ovalbumin-induced asthma. It is also important to recognize that murine models with asthma may not reflect the spectrum of immunologic and physiologic changes that underlie the pathology of established asthma in humans. However, airway remodeling, an important hallmark of chronic asthma that is often missing in animal models, was prominent in the mouse model used in this study and anti–Blo t 5 mAb administration was capable of ameliorating this phenomenon.

Last, we have used chimeric antibodies to highlight the potential of using allergen-specific IgG4 antibodies in inhibiting basophil activation. Although immunogenic murine components limit the prolonged use of chimeric antibodies in clinical settings, full antibody humanization can potentially reduce the likelihood of this problem arising. This potentially mirrors the approach used when anti–TNF-α is used for chronic inflammatory diseases. Taken together, this work has important implications on the current understanding of allergic asthma and may have an impact on future design of targeted therapeutics for an allergic disease lacking an optimal treatment regime.

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METHODS

Animals
Female BALB/c mice (8-12 weeks old) used for mAb generation and male C57BL/6 (8-10 weeks old) used for asthma models were maintained under pathogen-free conditions at the Centre for Comparative Medicine. All experiments were performed in accordance with approved institutional guidelines of the Institutional Animal Care and Use Committee of the National University of Singapore, under protocol number 106/10 for hybridoma development and 015/12 for asthma models.

Generation and purification of anti-Blo t 5 mAb
Recombinant Blo t 5 protein was expressed in *Escherichia coli* strain, BL21, and was purified by size exclusion chromatography and anion exchange chromatography (GE Healthcare Life Sciences). Purified Blo t 5 protein was used to immunize female BALB/c mice intraperitoneally with Freud’s adjuvant (Sigma-Aldrich, St Louis, Mo), following which the mice were sacrificed for their spleen. Splenocytes were then fused with myeloma cell line NS1 using polyethylene glycol 8000 (PEG8000) (Sigma-Aldrich). Hybridoma clones were screened and identified for anti-Blo t 5 responses 3 weeks postfusion. Responders were subcloned by limiting dilution to obtain monoclones. Once their spleen. Splenocytes were then fused with myeloma cell line NS1 using polyethylene glycol 8000 (PEG8000) (Sigma-Aldrich). Hybridoma clones were screened and identified for anti-Blo t 5 responses 3 weeks postfusion. Responders were subcloned by limiting dilution to obtain monoclones. Once

Affinity determination by ELISA
Briefly, varying concentrations of recombinant Blo t 5 protein (125 ng/mL-2 μg/mL) were diluted in 0.1 mol sodium bicarbonate coating buffer and coated onto Maxisorp plates, overnight at room temperature. The plates were washed with PBS-0.05% Tween-20, blocked with 0.2% BSA, and incubated overnight with serial dilutions of anti-Blo t 5 mAbs. The plates were washed again and incubated with goat anti-mouse IgG conjugated with horseradish peroxidase (1:5000 vol/vol in 0.2% BSA; Thermo Scientific). Subsequently, the plates were developed with 1-Step Ultra TMB-ELISA Substrate (Thermo Fisher Scientific) and quenched with 1 mol sulphuric acid. OD was acquired using a microplate reader at 450 nm. Calculations of affinity and dissociation constants were performed as described previously.

Immunodot blot
A peptide library of the Blo t 5 protein comprising 15-mer peptides with 10 overlapping amino acids was used. Five microgram of each peptide was spotted onto preactivated polyvinylidene difluoride (PVDF) membrane and 1 μg of recombinant Blo t 5 protein was used as positive control. The membrane was air dried and blocked with 5% skimmed milk before incubating with the anti-Blo t 5 mAb (1:500 in 5% skimmed milk). The membrane was then washed thrice with PBS-0.2% Tween-20 for 7 minutes each followed by incubation with goat antinouse IgG conjugated with horseradish peroxidase (1:10,000 vol/vol in 5% skimmed milk; Thermo Scientific). Washing with PBS-0.2% Tween-20 was repeated. Finally, the membrane was developed with Western Lightning Plus Enhanced Chemiluminescence (Perkin Elmer, Waltham, Mass) and the signals on the membrane were captured on light-sensitive film in the dark room.

Measurement of total IgE and Blo t 5–specific IgE
Serum was obtained via cardiac puncture. Total IgE was measured using the Mouse IgE ELISA Set (BD Biosciences, Franklin Lakes, NJ) according to manufacturer’s instructions. To measure Blo t 5–specific IgE, sera were preabsorbed with Protein G Sepharose 4 Fast Flow beads (GE Healthcare Life Sciences) before proceeding with the measurement of IgE as described earlier.

Lung histology and scoring
PBS perfusion was performed and the lungs were fixed by inflating with 0.8 mL of 4% paraformaldehyde (Sigma-Aldrich). Lung tissues were further fixed for at least 2 days in 4% paraformaldehyde. Downstream sample processing and staining procedures were performed by the Advanced Molecular Pathology Laboratory situated at the A*Star Institute of Molecular and Cell Biology. Hematoxylin and cosin–stained lung sections were scored on cell infiltration as previously described.11 Similarly, periodic acid–Schiff–stained lung sections were quantified for airway mucus production as previously described.12

Molecular engineering of chimeric variants
Total RNA was extracted from 1 × 10^6 hybridoma cells using the High Pure RNA isolation kit (Roche, Basel, Switzerland). Extracted RNA was converted to cDNA using Superscript III First Strand cDNA Synthesis Kit (Invitrogen, Carlsbad, Calif). Variable regions of the heavy and light chains were PCR amplified with specific primers using the AmpliTaq Gold DNA polymerase (Thermo Fisher Scientific). PCR products were analyzed by DNA gel electrophoresis, bands at corresponding sizes were excised, and DNA was purified using QIAGEN Gel Extraction Kit. Purified PCR products were then cloned into pT75 plasmid vectors, which possess the human IgG constant regions (Courtesy of A/P Brenda Hanson). The IgG1-LALA variant carries a L234A and L235A double mutation that has been shown to abolish Fc receptor binding,13 whereas the IgG2-S228P variant carries a S228P mutation that reportedly eliminates the Fab arm exchange phenomenon commonly associated with IgG_2 antibodies.14,15 Constructs were subsequently transfected into suspension HEK-6E cells with polyethylenimine. Cell culture supernatants were harvested 7 days later and chimeric antibodies were purified with Protein A Sepharose Beads (GE Healthcare Life Sciences).

Basophil activation test
The basophil activation test was performed as previously described.11,10 Briefly, whole blood collected from sensitized donors was stained with Pacific-Blue Anti-human HLA-DR, APC Anti-human CD294 (CRTH2), and fluorescein isothiocyanate (FITC) Anti-human CD63 (Biolegend, San Diego, Calif), following which stained whole blood was incubated with PBS, Blo t extract only, or Blo t extract with varying concentrations of anti-Blo t 5 antibody for 20 minutes at 37°C. Activation was terminated with 3.8% EDTA and trythrocytes were lysed with ammonium chloride potassium lysis buffer. Cells were then washed, resuspended in PBS, and analyzed by flow cytometry on Fortessa FACSscan. Basophils were identified as the HLA-DR−/−/CRTH2+ population (Fig E3). A first gate was made around the lymphocyte population in the side scatter versus forward scatter (FSC) dot plot (Fig E3, A). A second gate was placed at the cell singlet population in the FSC-H versus FSC-A dot plot (Fig E3, B), followed by gating around the CRTH2+ population in the HLA-DR versus CRTH2 dot plot (Fig E3, C). Stimulation was then analyzed by the percentage of CD63+ cells in the absence/presence of the Blo t extract.

Statistical analysis
Data were expressed as means ± SEMs. All data analyses were performed with Graphpad Prism 5 software. Comparisons between 2 groups were assessed with 2-tailed unpaired Student t test and results are considered to be statistically significant when the calculated P value is less than .05.

REFERENCES


FIG E1. Epitope analysis of anti–Blo t 5 mAb. A, Purified murine anti–Blo t 5 on reducing and nonreducing SDS-PAGE gel. B, Affinity ELISA of murine anti–Blo t 5 mAb. C, Western blot of Blo t 5 protein with anti–Blo t 5 mAb under denaturing and nondenaturing (native) conditions. D, Immuno-dot blot with Blo t 5 peptide library consisting of 15-mer peptides with 10 amino acids overlap. Peptides 1 and 2 were excluded as they represented the signal peptide, which was absent in the recombinant Blo t 5 used in this study. E, Modeling by PyMOL software to visualize the position of peptide 14 on the Blo t 5 protein.
FIG E2. Cytokine and chemokine levels in the bronchoalveolar lavage fluid. A, Eotaxin levels. B, IL-4 levels. C, IL-5 levels. D, IL-13 levels. *P < .05.
FIG E4. Basophil gating strategy. **A**, SSC vs FSC, **B**, FSC-H vs FSC-A, **C**, HLA-DR Pacific Blue vs CRTH2 APC. APC, Allophycocyanin; SSC, side scatter.