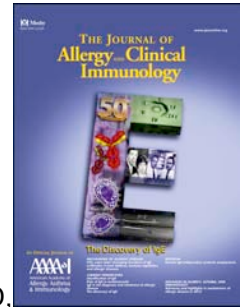


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Molecular engineering of a therapeutic antibody for Blo t 5-induced allergic asthma.

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To the Editor:

Epidemiological studies have established a causal link between sensitization to HDM allergens and the development of allergic asthma. Despite the co-existence of the two major dust-mite species in the tropics and dual sensitization profiles in atopic individuals, IgE responses targeting *B. tropicalis* have limited cross-reactivity with *D. pteronyssinus*¹. Amongst 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 asthmatic subjects¹⁻³ and 96.7% in asthmatic children⁴. 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 utilizing engineered allergen-specific monoclonal antibodies as a novel treatment modality for HDM-driven allergic asthma.

To facilitate this, we first isolated a monoclonal antibody with high affinity for Blo t 5 (**Supplementary Fig 1, B**). 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 (**Supplementary Fig 1, C-E**). Interestingly, our antibody targets a region of Blo t 5 previously implicated as a conformational IgE binding epitope⁵. Moreover, a dose-dependent inhibition of Blo t 5-specific IgE binding from allergic patients' sera was observed when co-incubated 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 IgG4-S228P (**Fig 1, B**). Integrities of the expressed antibodies were verified by SDS-PAGE under reducing and non-reducing 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 150kDa and larger on a non-reducing 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 upon 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 IgG4-S228P and IgG1 variant, respectively (**Fig 1, G**). In concordance with the widely documented anti-inflammatory

properties of IgG4 blocking antibodies elicited by successful SIT⁶, our data intimates 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_H2 cells to naive mice and subsequently challenging the mice intra-nasally with Blo t 5 protein. The mice exhibited an asthmatic phenotype characterized by pulmonary eosinophilia, increases in serum IgE levels and mucus hypersecretion⁷.

The murine anti-Blo t 5 mAb was administered intravenously at 100ug per mouse on days 1 and 13 (**Fig 2, A**). Histological analyses were performed to investigate the impact of mAb treatment on the structural integrity of asthmatic lungs (**Fig 2, B and C**). H&E 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 hyper-secretion and mucus plugging as denoted by the black arrows, were majorly alleviated with mAb treatment. Lastly, the deposition of collagen fibres (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 asthmatic mice.

Treatment with our mAb significantly reduced the total immune cell count and the infiltration of eosinophils into the airways but did not impact upon neutrophilia (**Fig 2, D**). Analysis of cytokines in the BALF revealed a suppression in eotaxin levels with no changes in other key TH2 cytokines (**Supplementary Fig 2**). Interleukin-5 was shown to prime eosinophils to chemoattractants whereas eotaxin was responsible for directing the actual migration to the site of inflammation⁸. The recruitment of eosinophils to specific tissues and their subsequent activity within these sites might be IL-5-independent⁹. 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.

Serological effects of anti-Blo t 5 mAb administration in severely allergic asthmatic mice were also analyzed by ELISA. A five-fold reduction in total IgE levels was observed in the sera from mice treated with anti-Blo t 5 mAb compared to 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 Forced

Expiratory Volumes (FEVs) (**Fig 2, F**) and a decrease in respiratory rate (**Supplementary Fig 3**) when the mice were treated with anti-Blo t 5 mAb. Collectively, the anti-allergic effects of anti-Blo t 5 mAb treatment resulted in a major improvement in asthmatic symptoms (**Video E1**).

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 monoclonal antibody treatment in a murine model of severe allergic asthma driven by a clinically relevant and dominant allergen instead of the traditional OVA-induced asthma. It is also important to recognize that murine asthmatic models may not reflect the spectrum of immunological and physiological 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 employed in this study and anti-Blo t 5 mAb administration was capable of ameliorating this phenomenon.

Lastly, we have utilized chimeric antibodies to highlight the potential of utilizing allergen-specific IgG4 antibodies in inhibiting basophil activation. Whilst 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 employed when anti-TNF α is utilized for chronic inflammatory diseases. Taken together, this work has important implications on the current understanding of allergic asthma and may impact upon future design of targeted therapeutics for an allergic disease lacking an optimal treatment regime.

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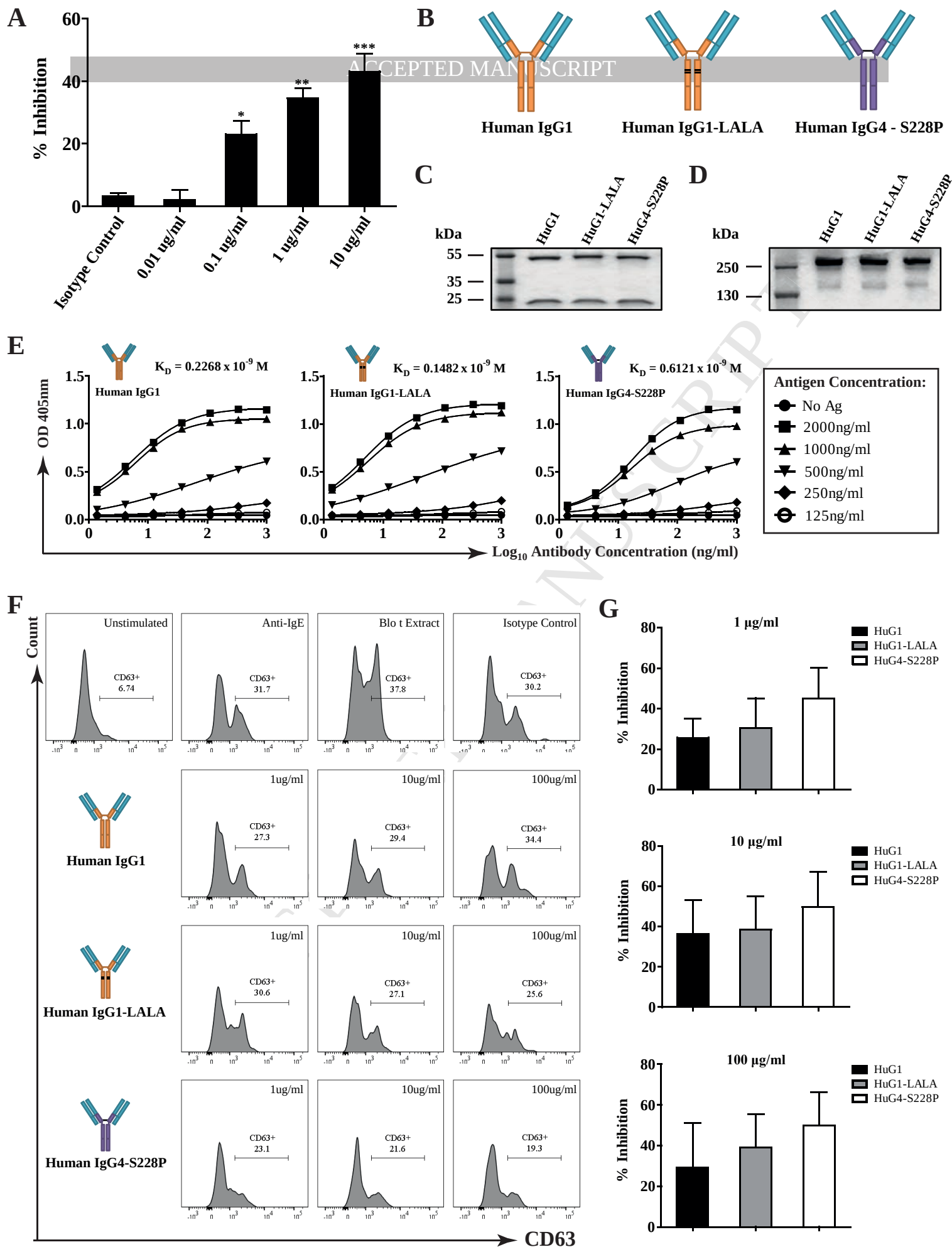
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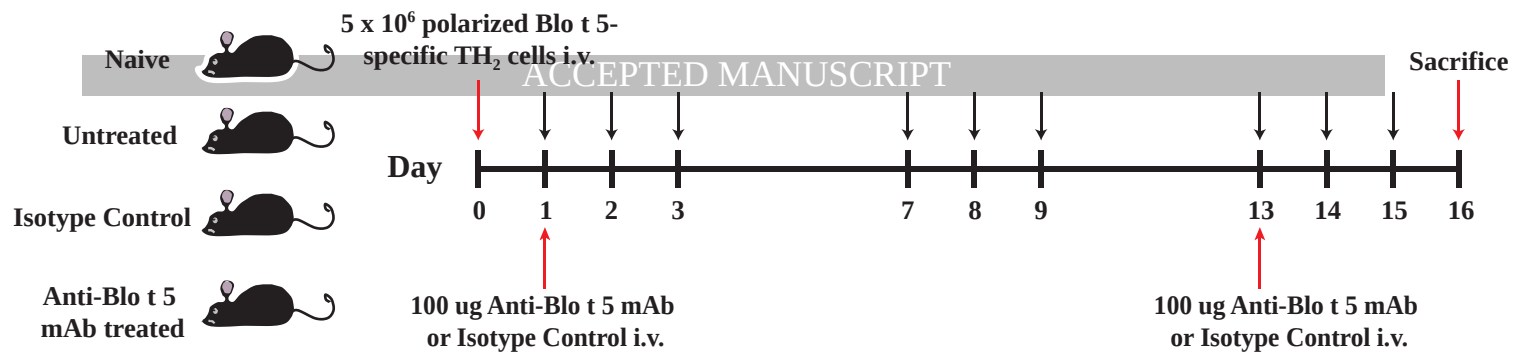
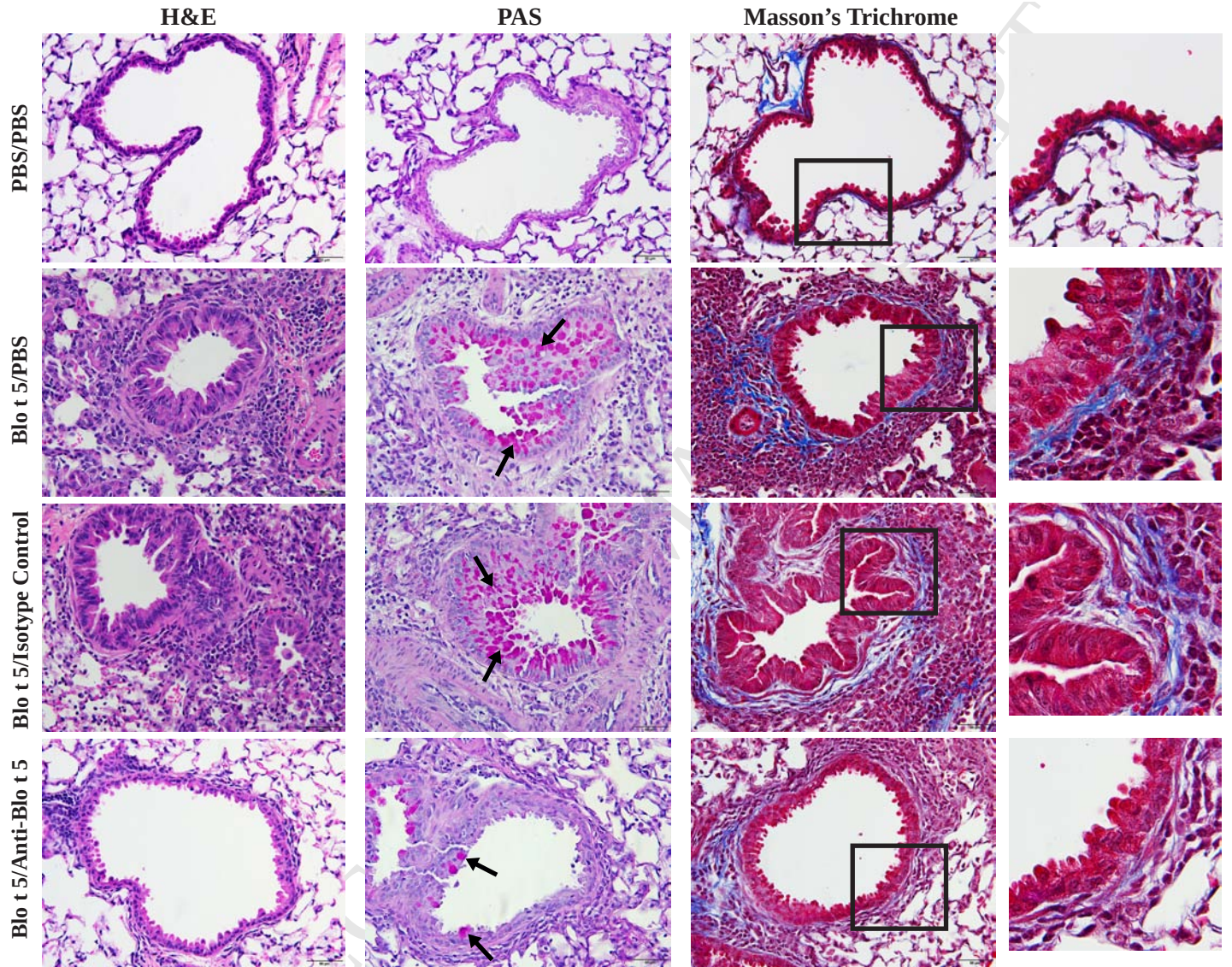
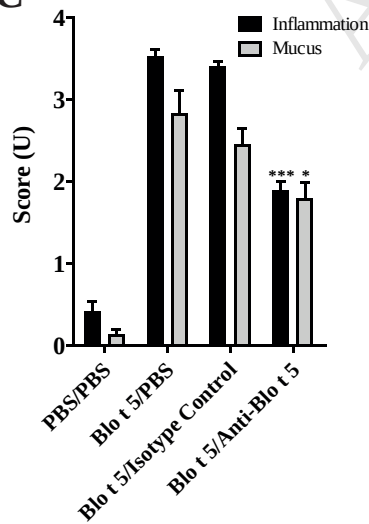
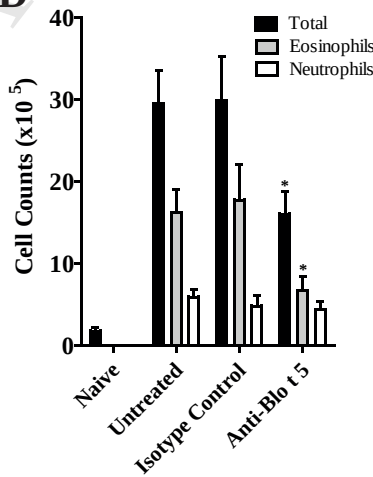
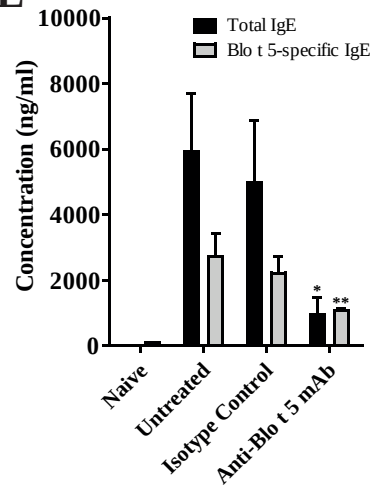
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FIGURE LEGENDS

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 non-reducing SDS-PAGE gel. **E**, Affinity ELISA of purified anti-Blo t 5 chimeric antibodies. **F**, Representative flow plots illustrating BAT and antagonistic capacity of chimeric anti-Blo t 5 mAbs. **G**, Percentage inhibition of basophil activation by chimeric antibodies. *P<0.05, **P<0.01 ***P<0.0001.

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 BALF. **E**, Serum total and Blo t 5-specific IgE levels. **F**, Forced Expiratory Volume (FEV) measurements. *P<0.05, **P<0.01 ***P<0.0001.



A**B****C****D****E****F**