

Title: Human monoclonal antibodies to Ara h 2 inhibit allergen-induced, IgE-mediated cell activation

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To the Editor:

Peanut oral immunotherapy (OIT) induces specific antibodies (IgG1, IgG4 and IgA) that are hallmarks of a successful response to allergen specific immunotherapy (1, 2). Ara h 2 and Ara h 6 are the two most potent peanut allergens for IgE-mediated activation of mast cells (3-5). Overall, these two allergens are 59% homologous, with 75% homology in the α -helical regions and up to 95% homology in IgE binding linear sequences [7]. Ara h 2-specific memory B cells were isolated from three patients undergoing peanut OIT using flow cytometric sorting of allergen-binding B cells. The heavy and light chain variable regions from single-sorted specific B cells were amplified and expressed as IgG1 monoclonal antibodies (mAbs) (6). Sixteen unique mAb were analyzed. The serum pool contained sera from 10 peanut allergic donors (see eMethods).

The aim of this study was to characterize these mAb by measuring cross-reactivity to Ara h 2 and Ara h 6, determining if these mAbs bind to linear or conformational epitopes and testing if the mAbs are capable of inhibiting IgE binding to Ara h 2 in a competitive ELISA and of inhibiting IgE/IgE ϵ R1 cross-linking in an RBL SX-38 cell release assay.

For the assays of cross-reactivity, microtiter wells were coated with either purified native Ara h 2 or Ara h 6. The mAbs (50 ng/ml) were then added, followed by HRP-conjugated anti-human IgG antibody. All Ara h 2-specific mAbs bind both Ara h 2 and Ara h 6 to varying degrees (Fig. 1A). To determine if the binding-specificity of mAbs is dependent upon the structural integrity of the allergen,

we performed competitive ELISAs assay, in which binding of mAb to wells coated with native Ara h 2 or Ara h 6 was inhibited by prior incubation of the mAb with either native or reduced/alkylated (r/a) Ara h 2 and Ara h 6. The unfolded state of the linearized allergens was confirmed by CD spectra (Fig. E1). Two patterns emerged. For two mAbs, M6 and M7, the binding to both native Ara h 2 and Ara h 6 was completely inhibited by linearized forms of Ara h 2 and Ara h 6, respectively (M6, Fig. E2 and E3; M7, Fig. 1B), indicating that these two antibodies recognize linear epitopes of Ara h 2 and Ara h 6. Of note, the linearized Ara h 2 has much higher inhibitory effect than the native Ara h 2, suggesting that the linear epitope recognized is actually more exposed and therefore accessible in the linearized antigen. In contrast, the binding of all other mAbs to Ara h 2 and Ara h 6 were inhibited by native Ara h 2 and Ara h 6 much more than by linearized allergens (M33, Fig. 1B and the other mAb, Fig. E2 and E3), indicating that they recognize conformational epitopes of Ara h 2 and Ara h 6.

We next tested the ability of these mAb to inhibit binding of Ara h 2 to IgE from our serum pool. Inhibitory ELISAs revealed that binding of IgE to Ara h 2 was inhibited to varying degrees by all tested mAbs but not by a control antibody (recombinant human IgG1 Kappa mAb from Bio-Rad, Hercules, CA) (Fig. E4). Because IgE binding to allergens in competitive ELISA reveals little information about their capacity to interfere with cross-linking of receptor-bound IgE on effector cells, we then addressed the functional impact of mAbs in inhibiting IgE/FcεR1 cross-linking using RBL SX-38 cells, an *in vitro* model of the type 1 allergic reaction. RBL SX-38 cells were sensitized with IgE from a pool of 10 peanut allergic patients and challenged by Ara h 2 or crude peanut extract with/without mAbs.

Similar to the data from the inhibitory ELISA, IgE-specific mediator release was variably inhibited by allergen specific mAbs with mAb M6 and M7 being the most effective (Fig. 2A). We then examined the effect of combining either M6 or M7 (linear epitopes) with M33 or M39 (conformational epitopes) and found up to 80% inhibition of IgE/FcεR1 cross-linking by Ara h 2 (Fig. 2B). Finally, we tested the ability of our mAb to inhibit IgE/FcεR1 cross-linking by crude peanut extract (CPE). A combination of M7 and M39 or M6 and M33 were able to inhibit IgE/FcεR1 cross-linking by CPE by 60%. These results demonstrate that, although we did not find a single mAb

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sufficient to greatly inhibit IgE/FcεR1 cross-linking, a combination of 2 mAbs can be more effective. Some combinations do not show additivity, likely due to steric hinderence. Overall, there is a consistent finding that it is more difficult to block cellular activation in cell release assay than it is to block binding of IgE in ELISA. Furthermore, there was no correlation between the inhibition of IgE binding and the inhibition of cellular activation. These findings are compatible with a study using heterobivalent inhibitors which demonstrated that inhibition of every epitope-IgE interaction on an allergen is not required for complete inhibition of mast cell degranulation (7). A separate study showed that a short epitope region can be recognized by multiple antibodies, and suggested that each antibody may be able to contribute to blocking activity (8). Finally, a recent study reported that a combination of two monoclonal IgGs directed against the immunodominant cat allergen, Fel d 1, block Fel d 1 binding to cat-specific polyclonal IgE *in vitro* and prevents mast cell degranulation *in vivo* (9).

These IgG monoclonal antibodies could inhibit FcεRI-mediated activation by either of two independent, but not mutually exclusive mechanisms. One is to block binding of allergen to FcεRI-bound IgE and another is to engage the inhibitory Fc receptor (FcγRIIb; CD32). In order to determine whether the IgG-mediated inhibition of IgE/FcεR1 cross-linking acted through FcγRIIb mechanism, we added anti-CD32 antibody (2μg/ml; FUN2; known to block activity of FcγRIIb) to the cell release assays. Inhibition of cell activation by mAb was not decreased by the presence of the anti-CD32 antibody (Fig. E5). This finding effectively excludes a role of FcγRII in the observed inhibitory effect of these mAb and implies that the observed inhibitory effect is likely due to blocking of IgE binding to allergen. Confirmatory studies were conducted with basophils from normal donors sensitized with IgE from peanut allergic subjects and basophils from peanut allergic donors (Fig. E6 and E7). A dose response with mAb, M39 is shown in Figure E8.

In conclusion, our studies indicate that both linear and conformational epitopes are likely to be the major targets of Ara h 2-specific B cells isolated from patients who undergoing OIT. The IgG1 monoclonal antibodies derived from Ara h 2-specific B cells and characterized here, are capable of inhibiting the binding of specific IgE to the allergen and of inhibiting IgE/FcεR1 cross-linking by

both Ara h 2 and CPE in an *in vitro* assay of mast cell activation. To our knowledge, this is the first study to demonstrate the functional impact of human peanut-specific monoclonal antibodies on IgE/FcεR1 cross-linking.

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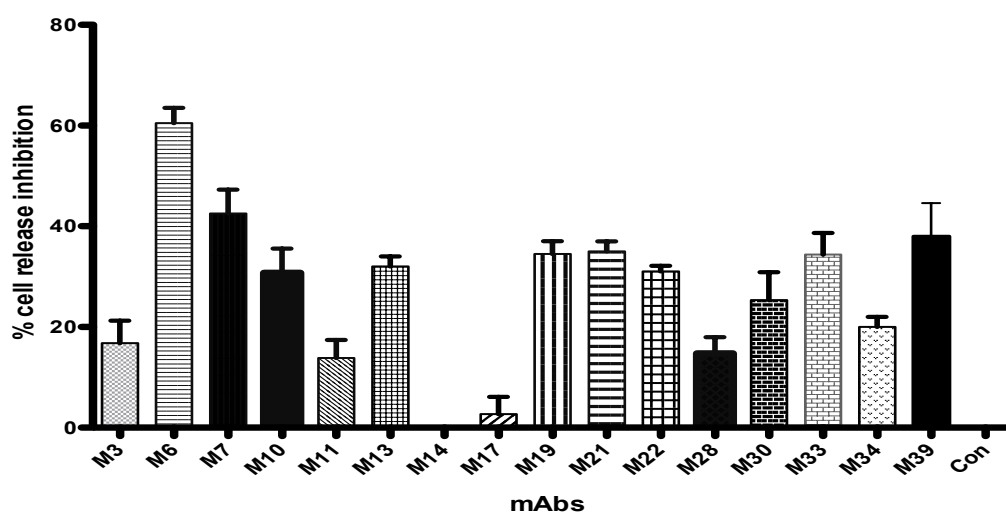
Figure Legends

Fig 1. Ara h 2 specific monoclonal antibodies (M3-M39) have cross-reactivity to Ara h 6 and mainly recognize conformational epitopes. **(A).** mAb bind to Ara h 2 and Ara h 6. **(B)** Most of these mAb bind native Ara h 2 better than they bind reduced/alkylated (r/a) Ara h 2. mAb M6 and M7 are distinct in that they bind equally to native and r/a Ara h 2. **(C).** Competitive inhibition of allergen binding by specific monoclonal antibodies to native Ara h 2 and Ara h 6 by native or r/a allergens. Results were expressed as B/B₀. B₀ and B indicate the binding to each specific allergen in the absence (B₀) or presence of inhibitor (B) in variety concentration.

Fig 2. Inhibition of IgE binding and IgE/FcεR1 by allergen specific monoclonal antibodies. RBL SX-38 cells were passively sensitized with IgE from a pool of 10 peanut allergic sera. The sensitized cells are triggered with Ara h 2 or a mixture of Ara h 2 (2ng/ml) and antibodies (each at 40ng/ml) **(A and B)** or with CPE or a mixture of CPE (40ng/ml) and antibodies (200ng/ml) **(C).** The concentration was chosen to give maximum inhibition.

“Con” is a control mAb (see text).

A



B

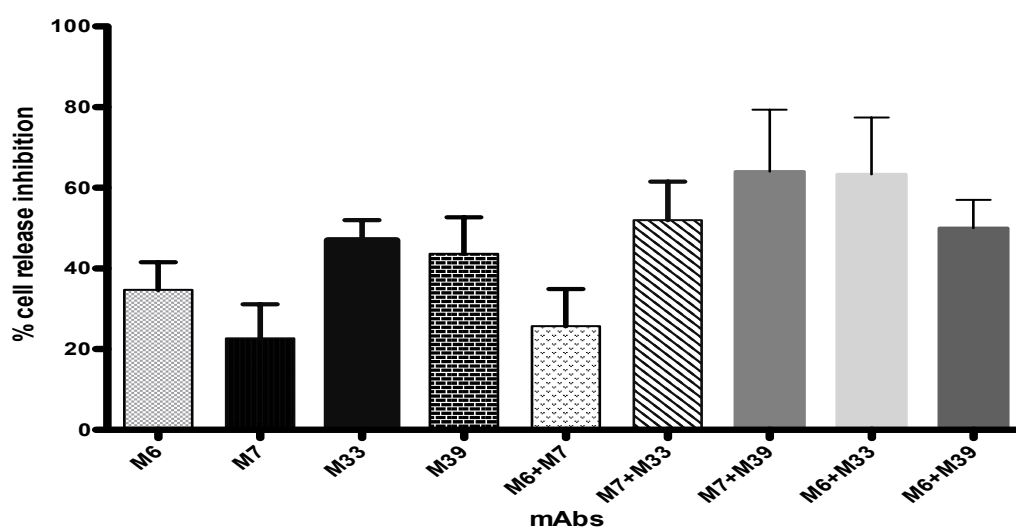
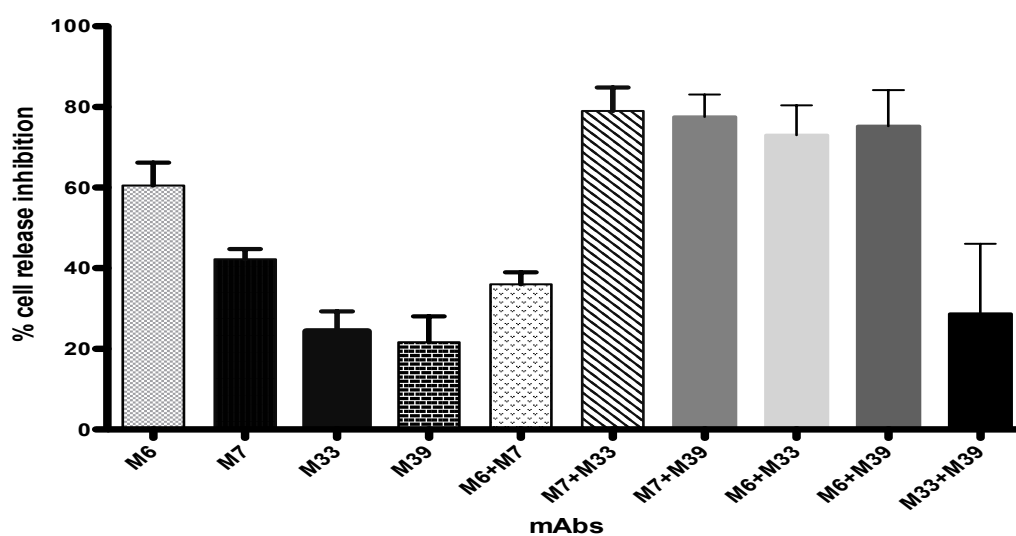
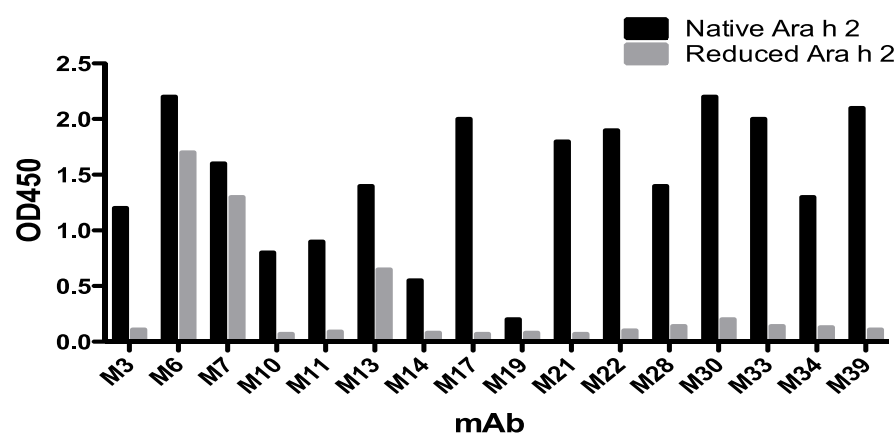
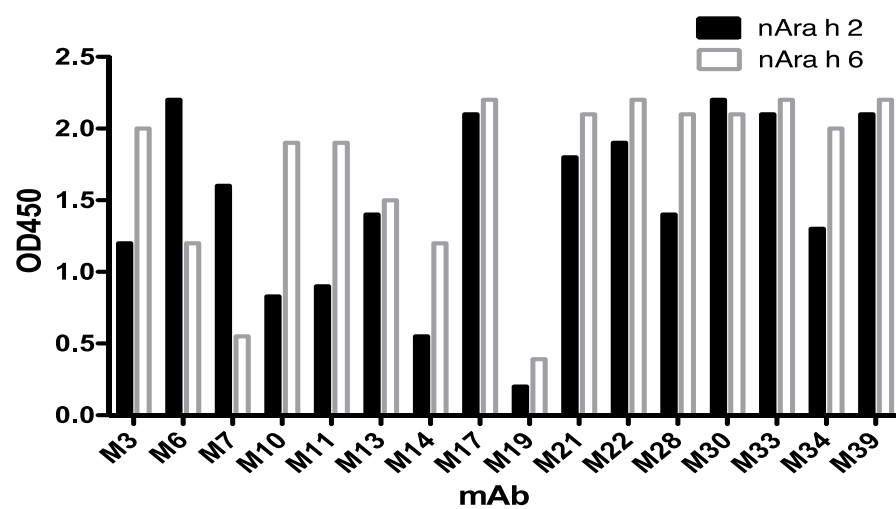


Fig. 1



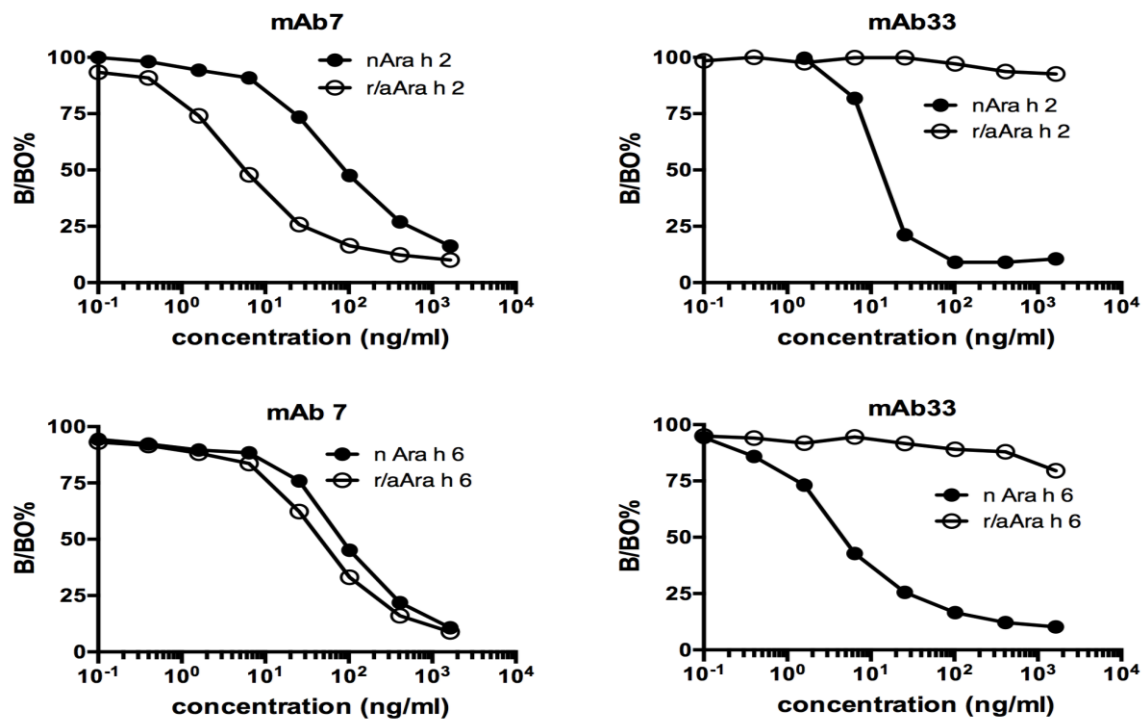
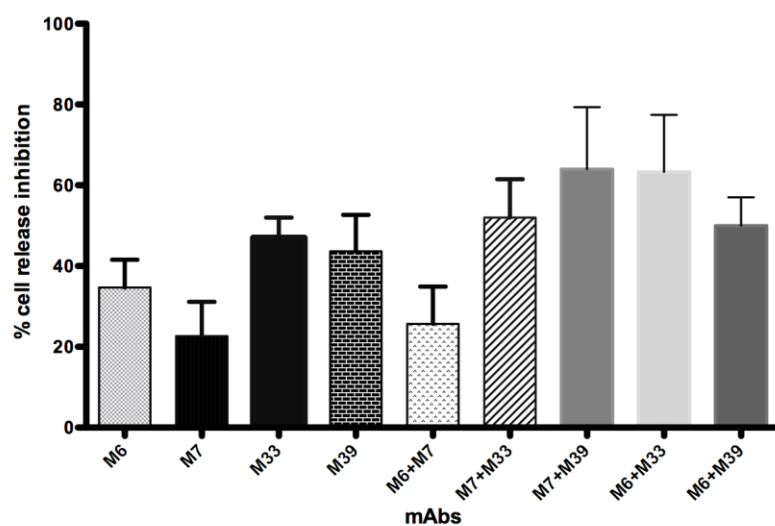
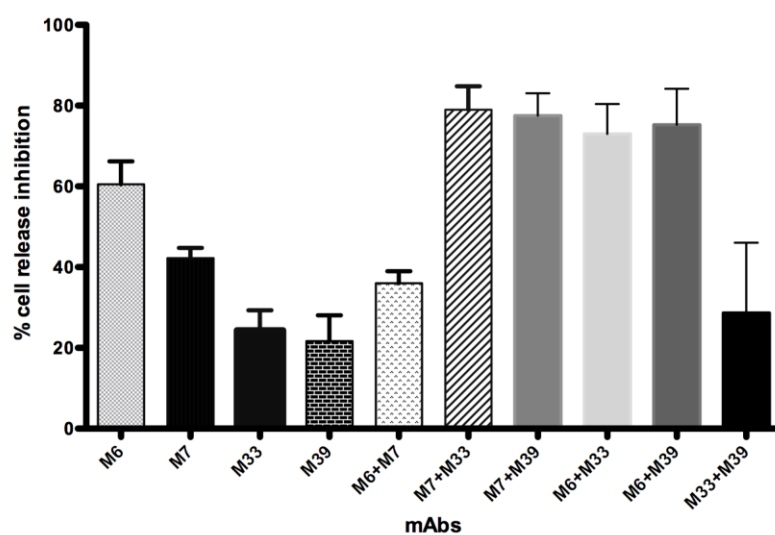
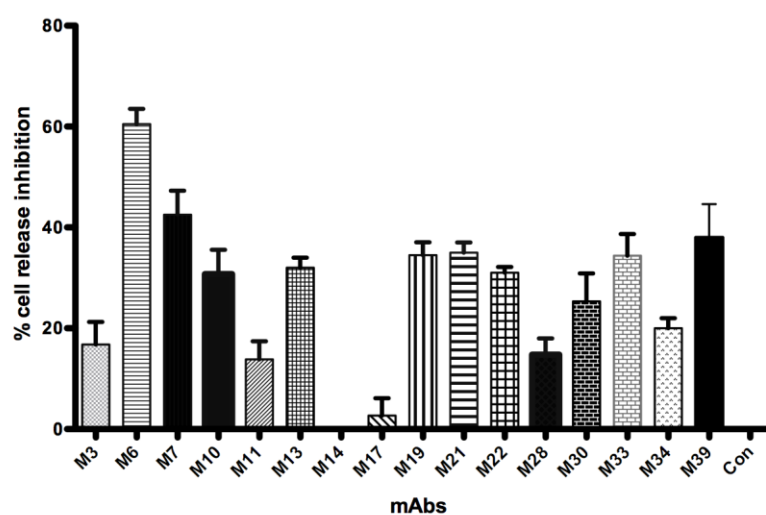
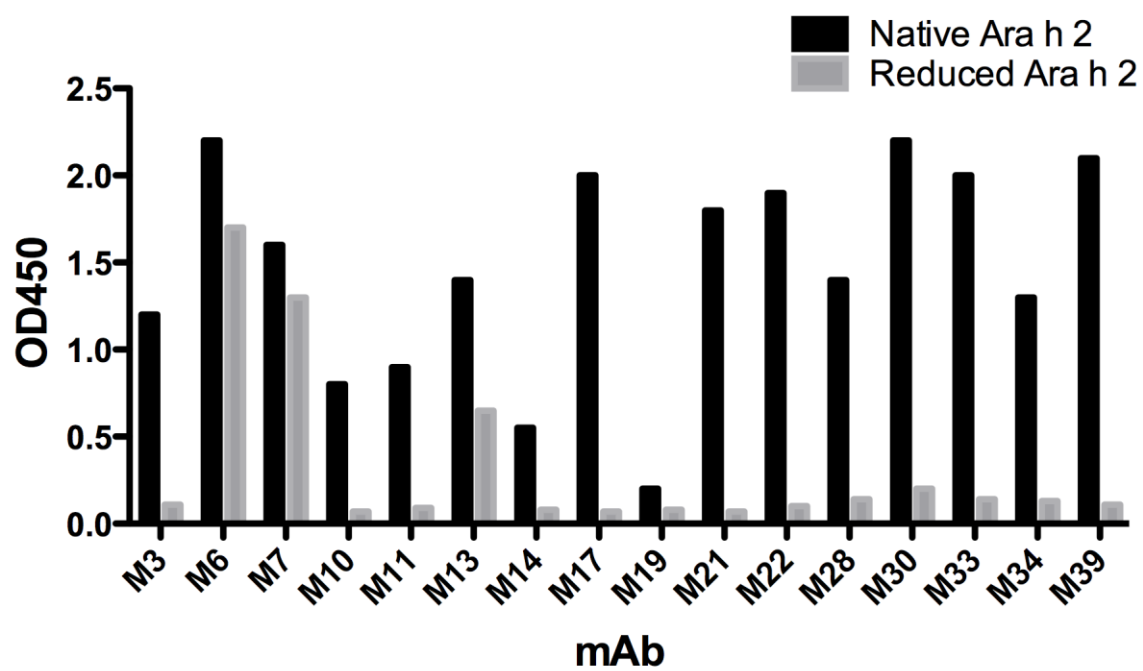
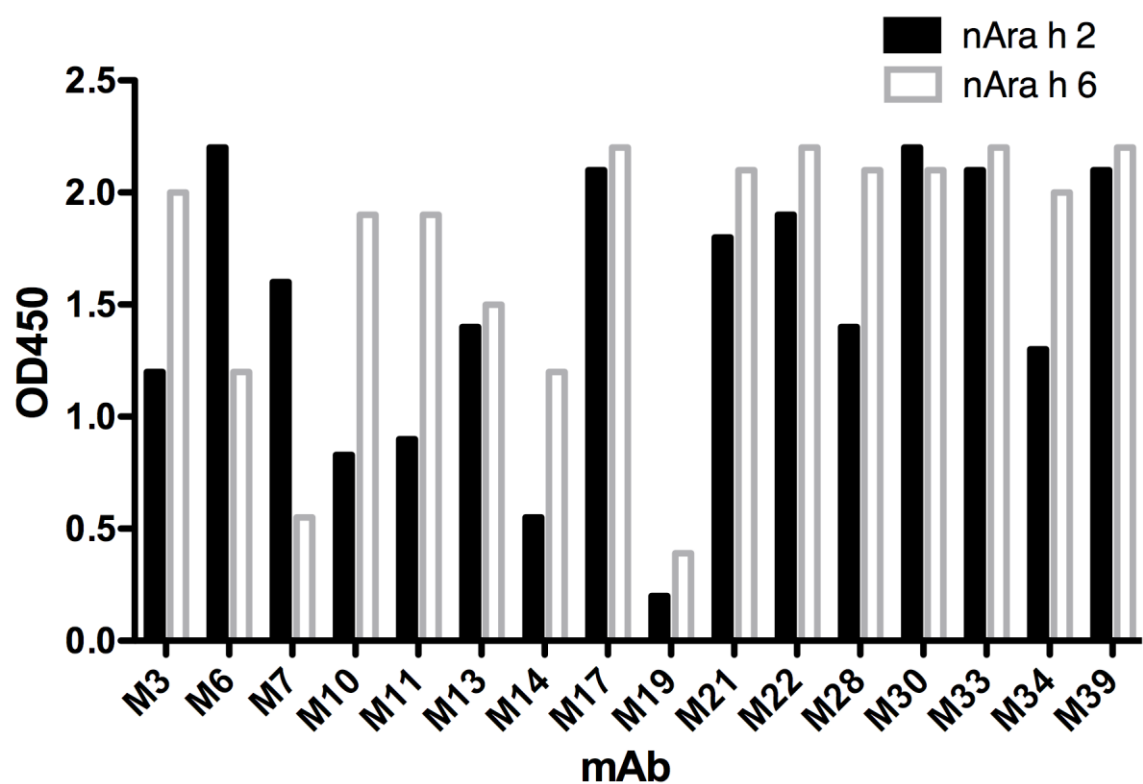
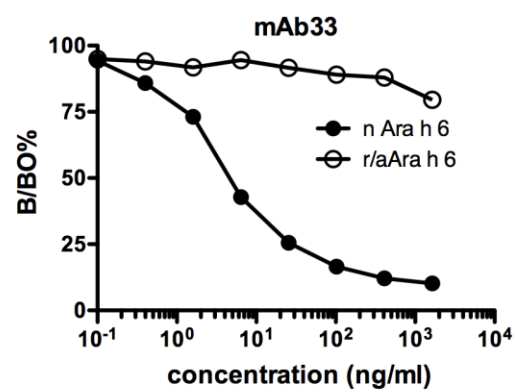
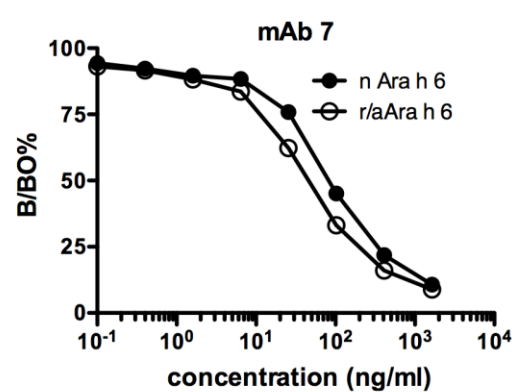
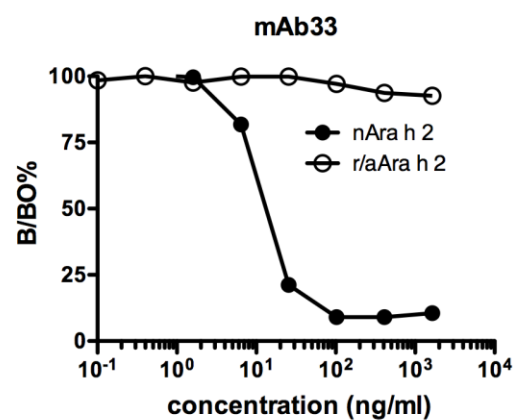
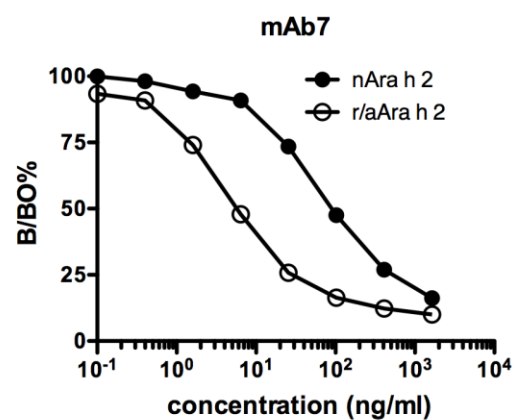


Fig. 2







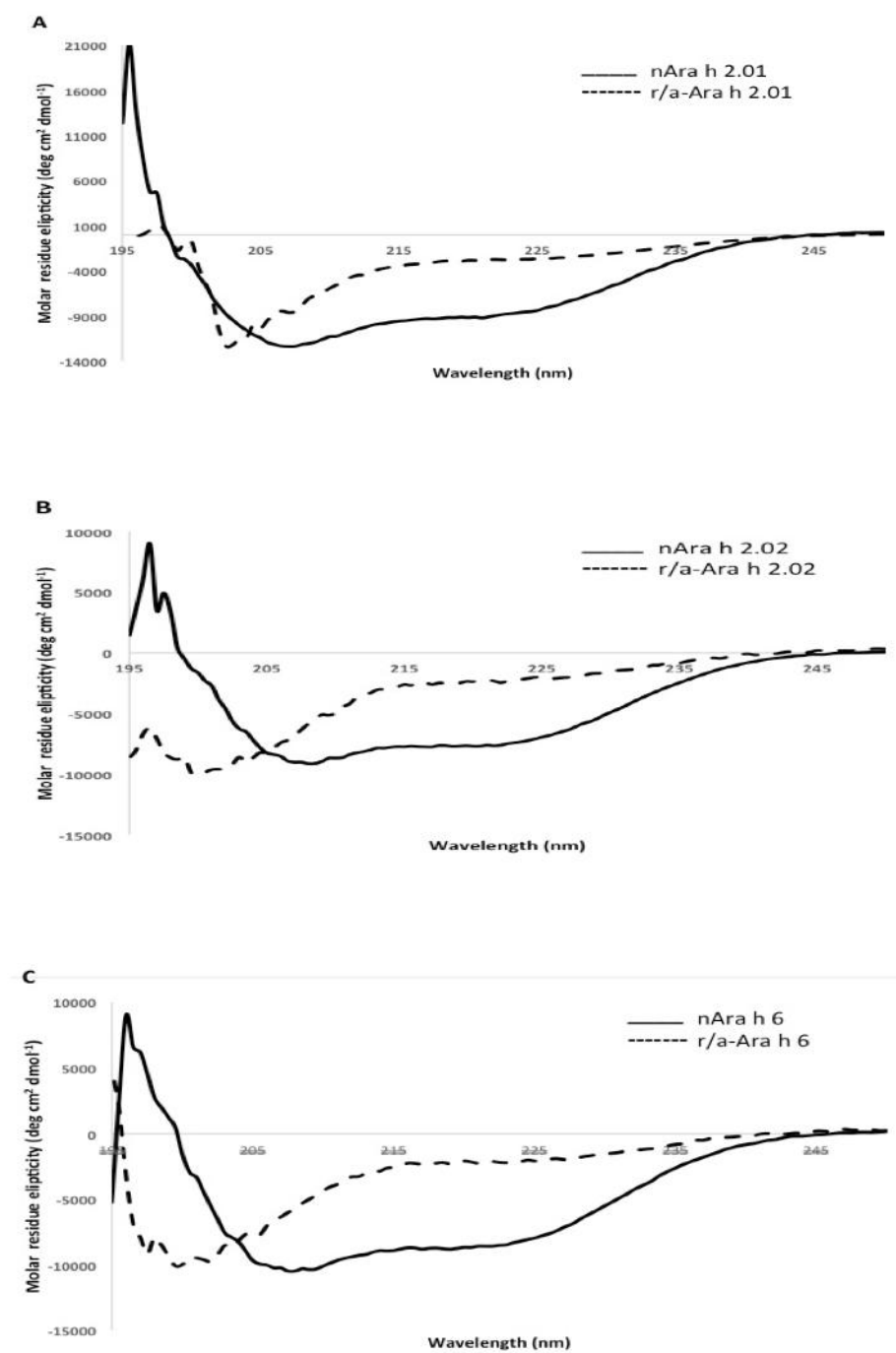
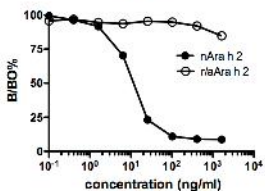
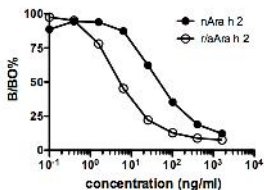


Fig. E1

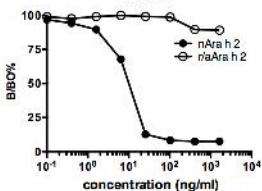
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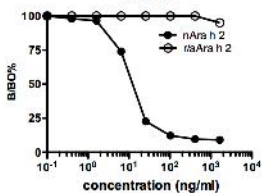
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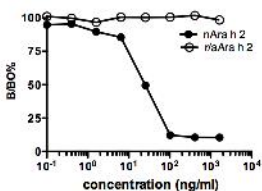
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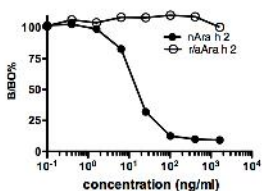
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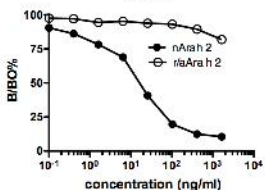
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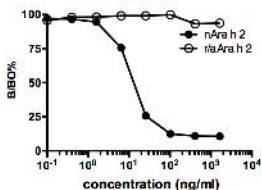
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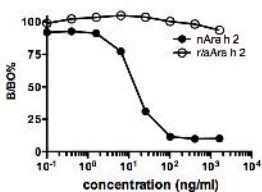
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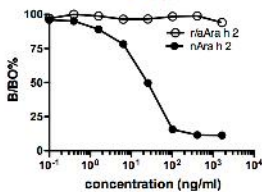
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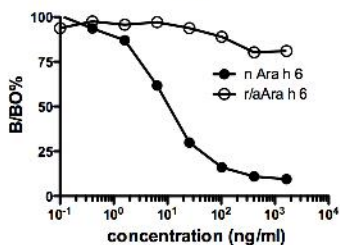
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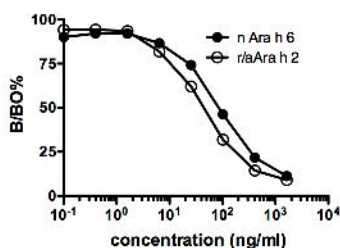
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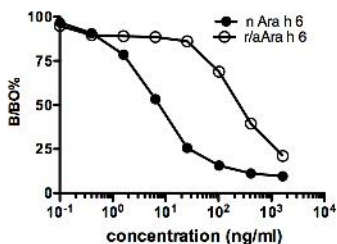
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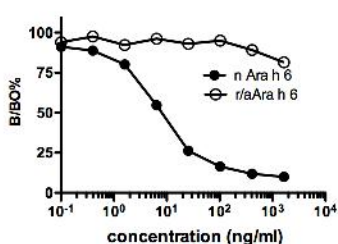
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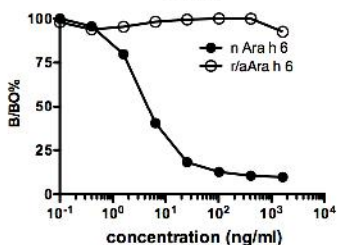
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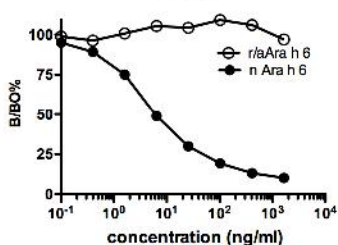
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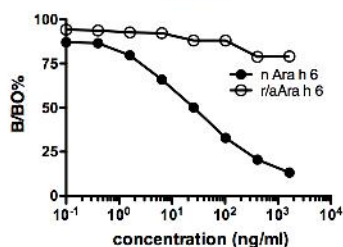
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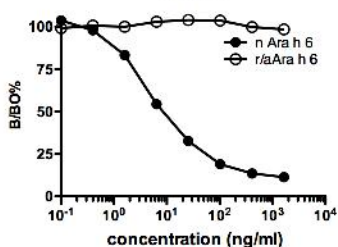
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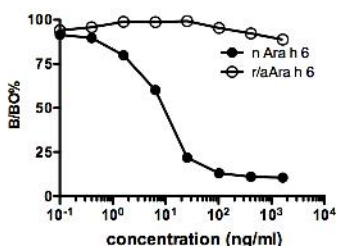
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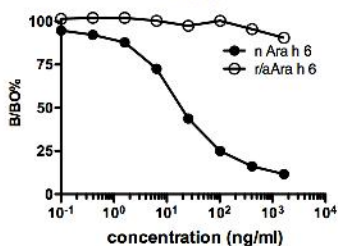
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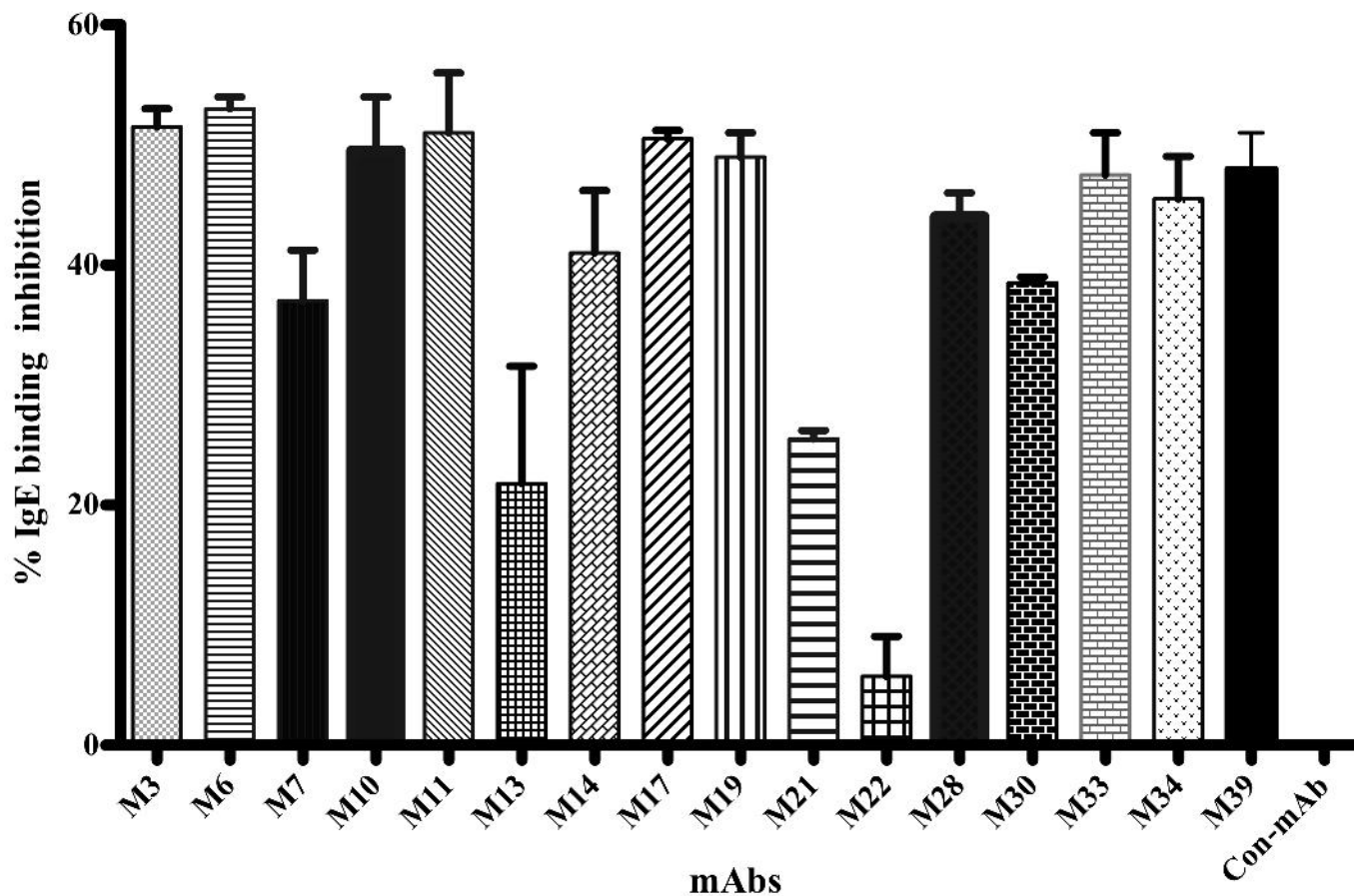


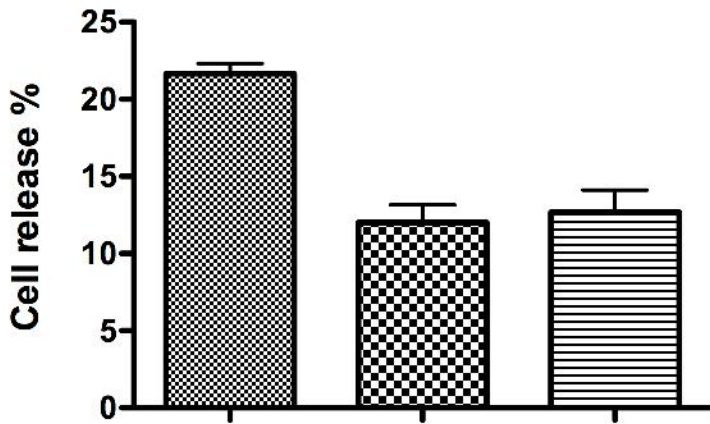
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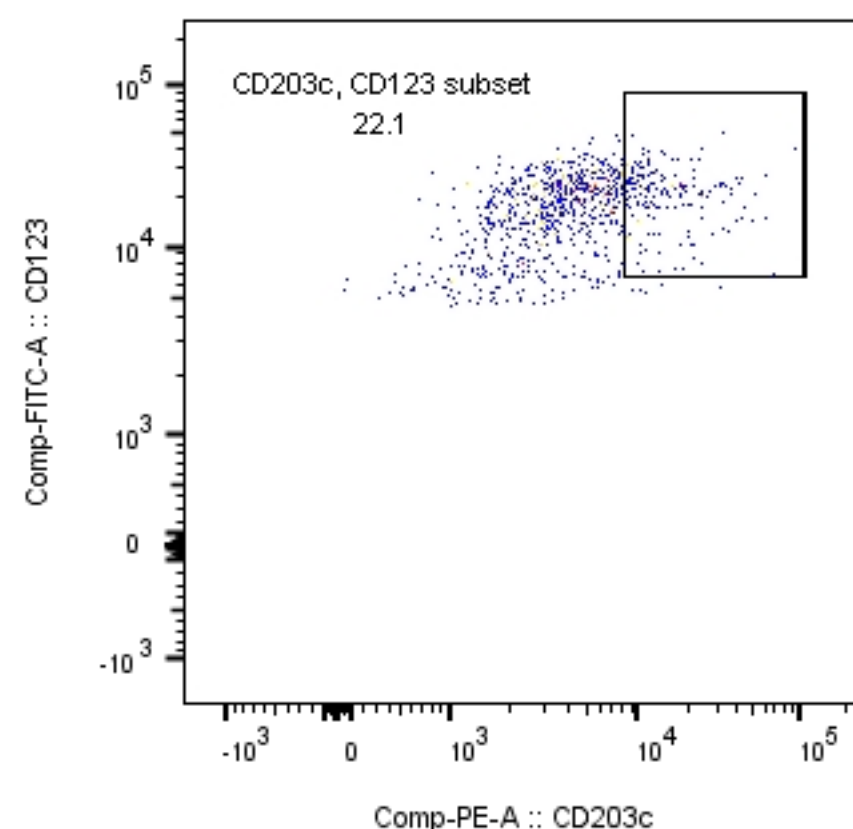
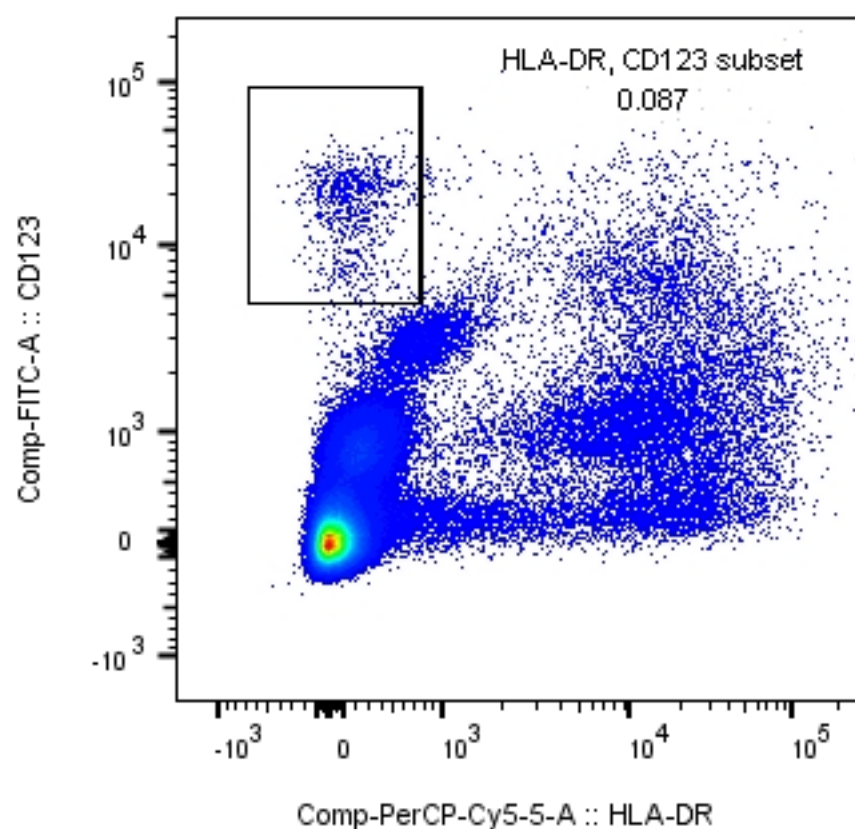
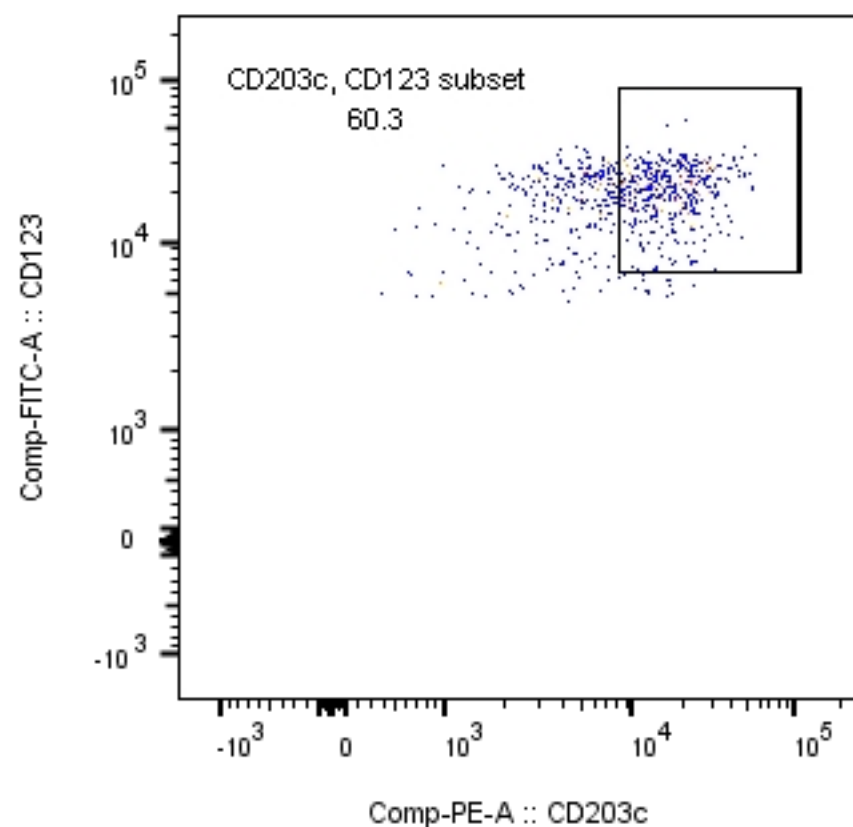
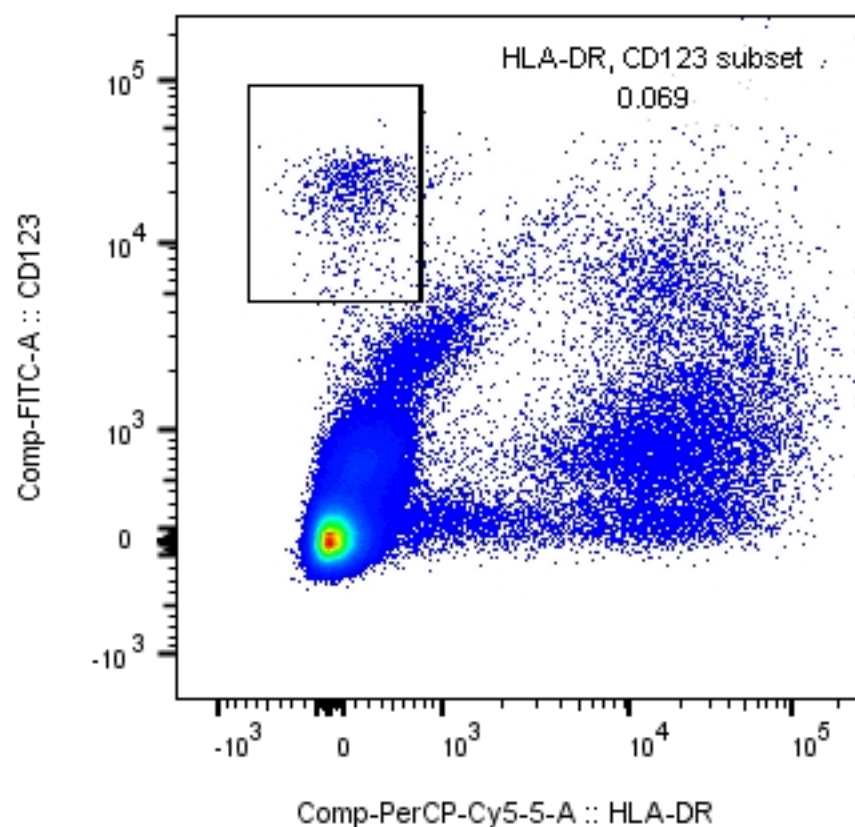
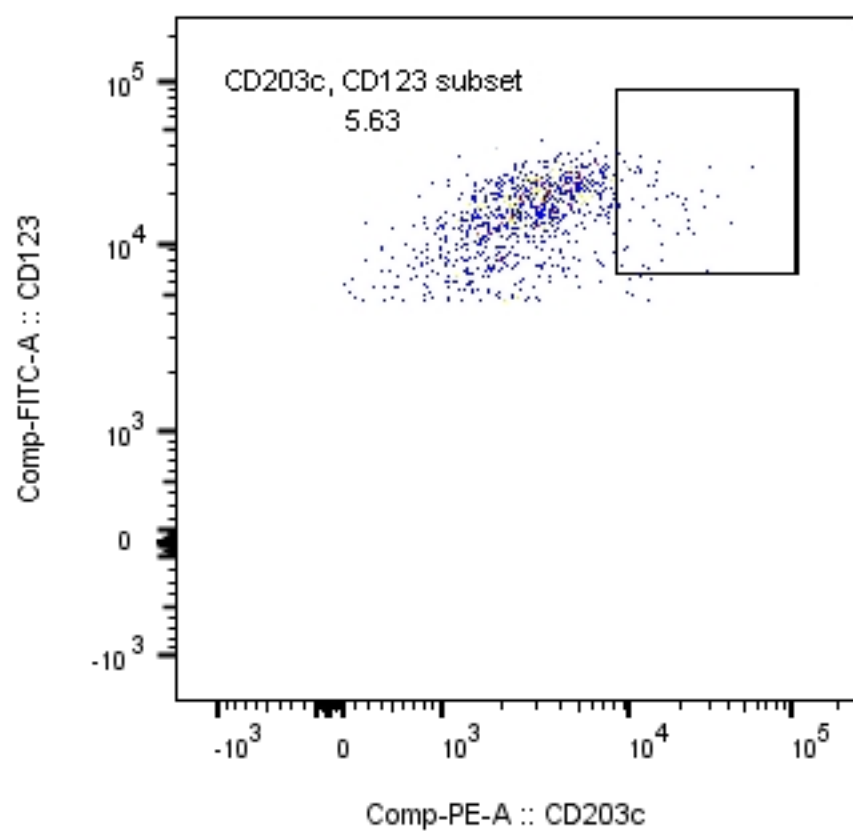
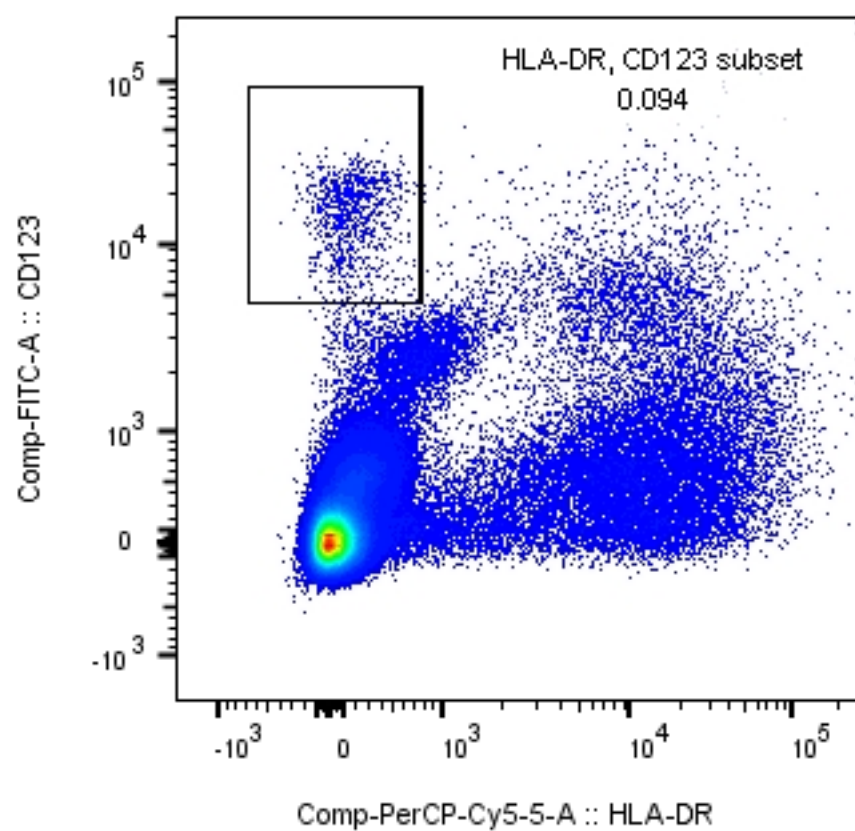


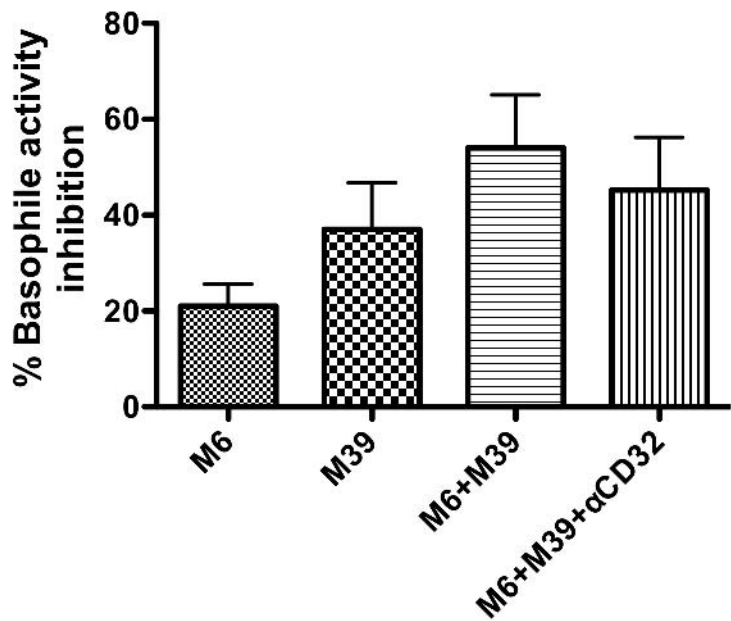
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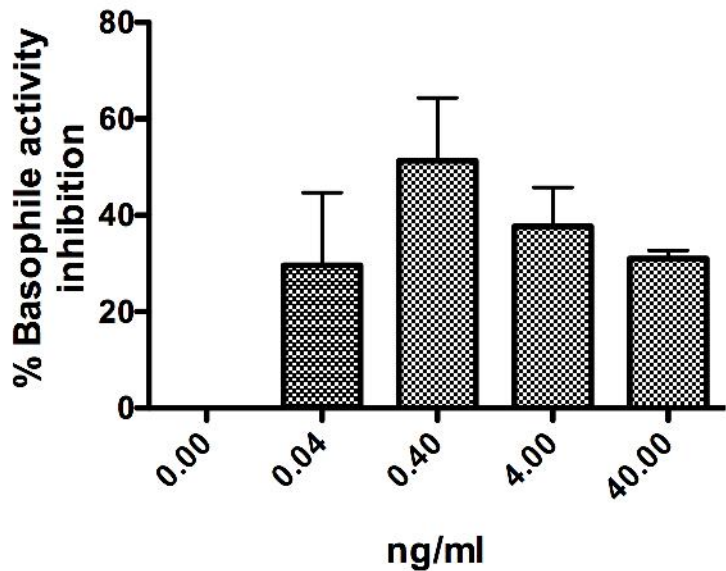












E Methods

Subjects

Subjects who contributed serum to our serum pool were enrolled as part of a clinical trial entitled, Blood Samples for the Study of Peanut, Tree Nut and Other Food Allergies, approved by the University of Colorado Denver Institutional Review Board. Details are available at <https://www.clinicaltrials.gov/ct2/show/NCT02192866>. All participants had a strong history of allergic reactions to ingestion of peanuts and had PN-sIgE >14kU/L.

Sera

The serum pool had a total IgE concentration of 200 kIU/L and peanut-specific IgE concentration of 39 kIU/L (19% specific IgE). All had high Ara h 2 and Ara h 6-sIgE (average (range) of 64 (9.8-455) and 37 (7.3-98) kU/L, respectively).

Proteins

Ara h 2 and Ara h 6 were purified (99% pure) from a saline extract of peanuts as described by Chen et al (10).

Human basophil activation analysis

100µl whole blood from non-allergic subjects with or without anti-CD32 antibody were incubated for 1h at 37°C. CPE (50 ng/ml) were incubated with or without mAbs were incubated for 1.5 h at 37°C before adding to the cells. Cells were then incubated for 20min at 37°C. Basophils were identified as CD123⁺ CD HLA-DR⁺ CD203⁺.

E legends

Fig E1. Far-UV CD spectra of native and reduced/alkylated Ara h 2 and Ara h 6. **(A)** Ara h 2.01. **(B)** Ara h 2.02. **(C)** Ara h 6. Native Ara h 2.01, Ara h 2.02 and Ara h 6 showed minima at 208 and 220 nm and a steep increase in ellipticity below 200 nm, indicating a high content of α -helices, while reduced/alkylated Ara h 2.01, Ara h 2.02 and Ara h 6 showed an unfolded structure, with β -structures and random coils.

Fig E2. Competitive inhibition of allergen-specific monoclonal antibodies to native Ara h 2 by native or reduced/alkylated allergen. Results were expressed as B/B0. B0 and B indicate the binding to specific allergen in the absence (B0) or presence of inhibitor (B) in variety concentration.

Fig. E3. Competitive inhibition of allergen specific monoclonal antibodies to native Ara h 6 by native or reduced/alkylated allergen. Results were expressed as B/B0. B0 and B indicate the binding to specific allergen in the absence (B0) or presence of inhibitor (B) in variety concentration.

Fig. E4. Inhibition of IgE binding to Ara 2 by allergen specific monoclonal antibodies. M3-M39 are specific monoclonal antibodies. Plates were coated with anti-human IgE antibodies and IgE from a pool of 10 peanut allergic sera was bound to the anti-IgE. A mixture of 3 ng/ml of biotinylated Ara h 2 plus 300 ng/ml individual mAbs was added and the plate developed with avidin-HRP. Con is a control mAb (see text).

47 **Fig. E5.** Inhibition of RBL SX-38 activation by monoclonal M6 is not affected by blocking
48 FcγRIIb. Cells were sensitized with serum with or without anti-hCD32 antibody (2μg/ml) for 3
49 hours before challenge with Arah 2 or allergen and mAb mixture.

50
51 **Fig. E6.** mAbs (M6+M39) inhibit basophil activation induced by CPE. Basophils from a non-
52 allergic donor, sensitized with IgE from a patient with peanut allergy were stimulated with PBS
53 (A) or CPE (B and C) without mAbs (B) or with mAbs (C).

54
55 **Fig. E7.** Inhibition of human basophils by monoclonal M6+M39 (each 2ng/ml) is minimally
56 affected by blocking FcγRIIb. Basophils from non-allergic donors sensitized with IgE from a
57 patient with peanut allergy (n=4) and basophils from peanut allergic donors (n=3) were triggered
58 with CPE in the presence of M6 or M6+M39 with and without anti-hCD32. Summary data are
59 shown.

60
61 **Fig. E8. Dose response inhibition of M39.** Basophils from a peanut allergic donor were
62 stimulated with CPE in the presence of varied doses of M39.

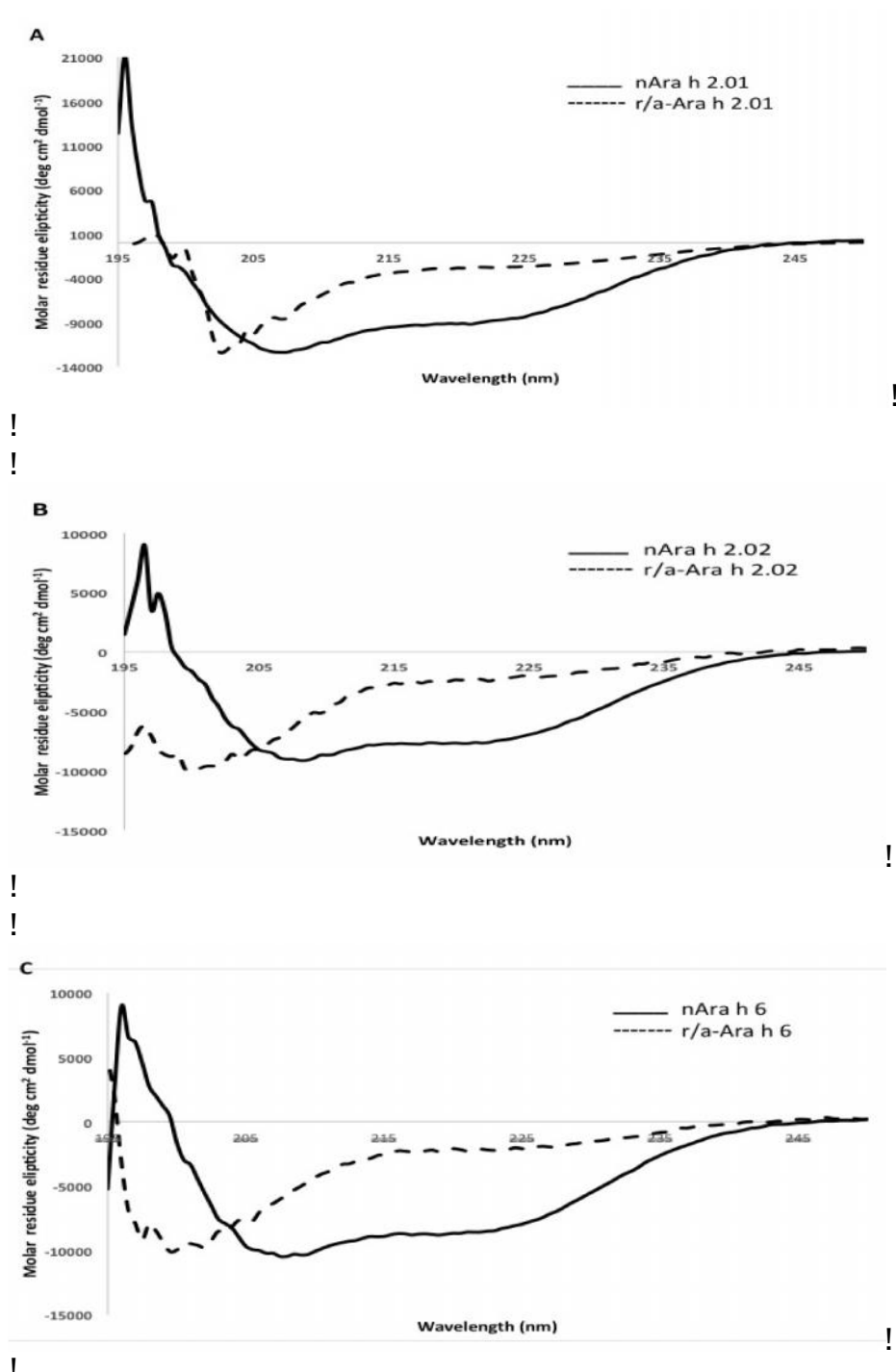


Fig. E1

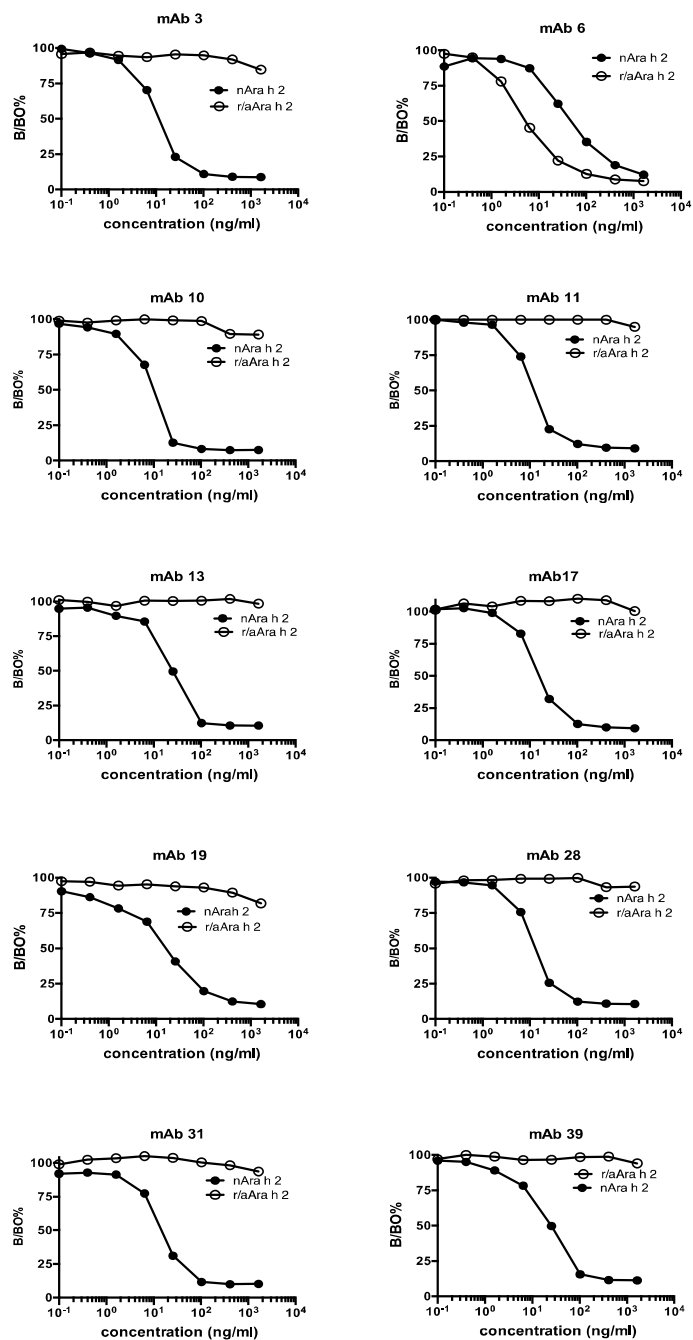


Fig. E2

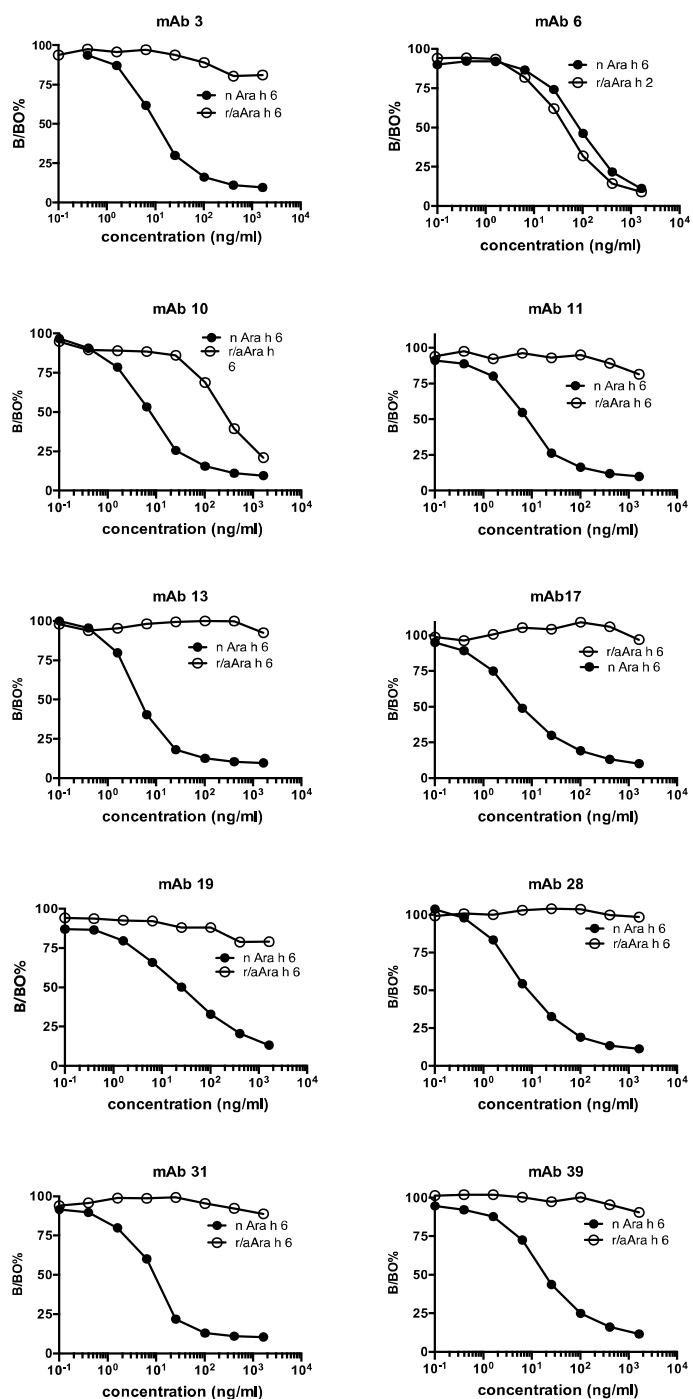


Fig. E3

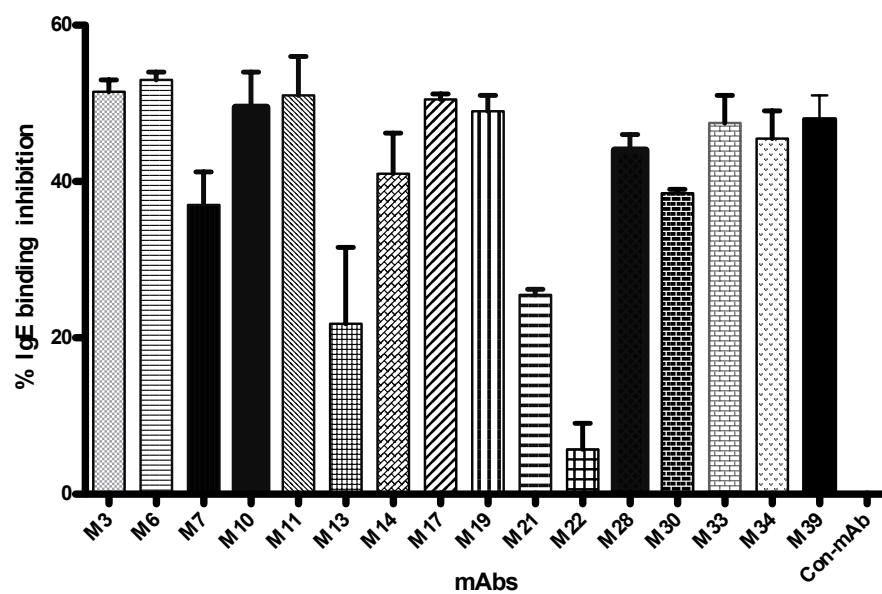


Fig. E4

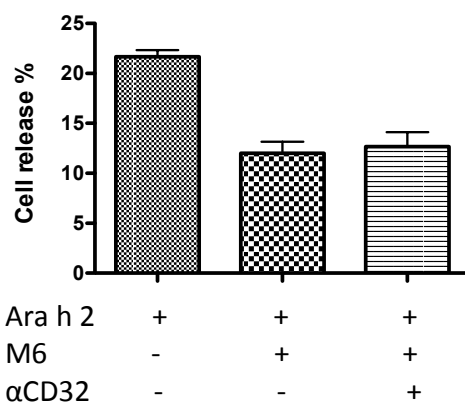
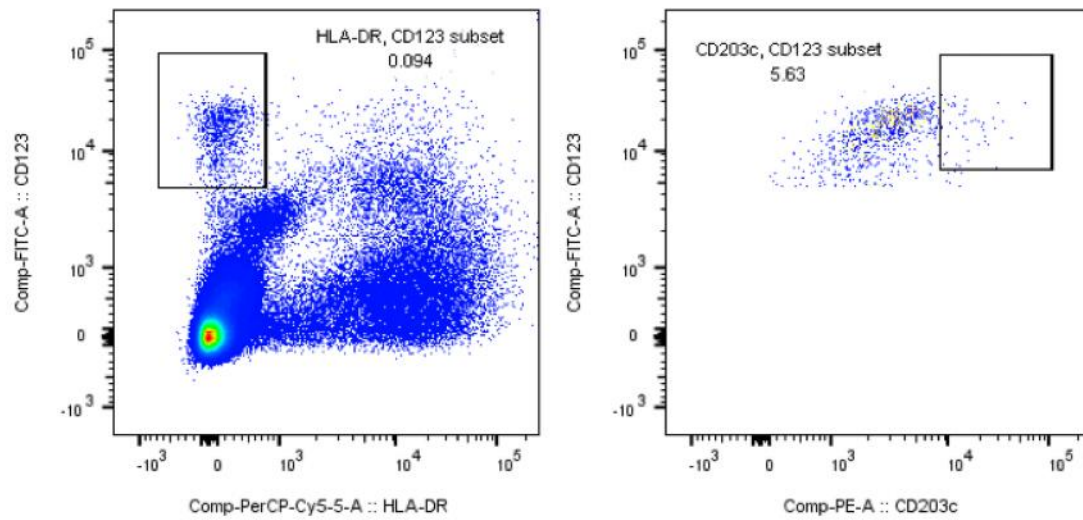


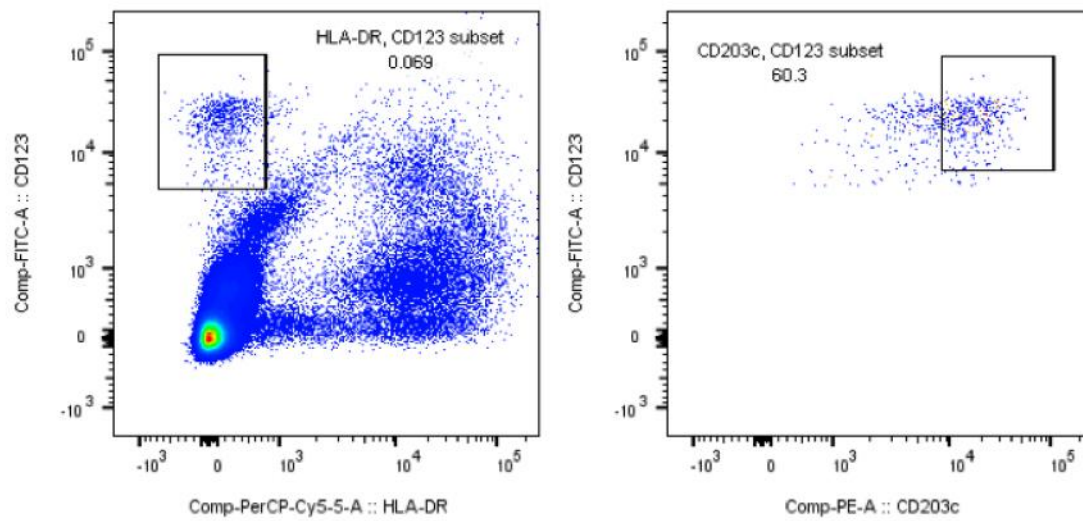
Fig. E578
E579
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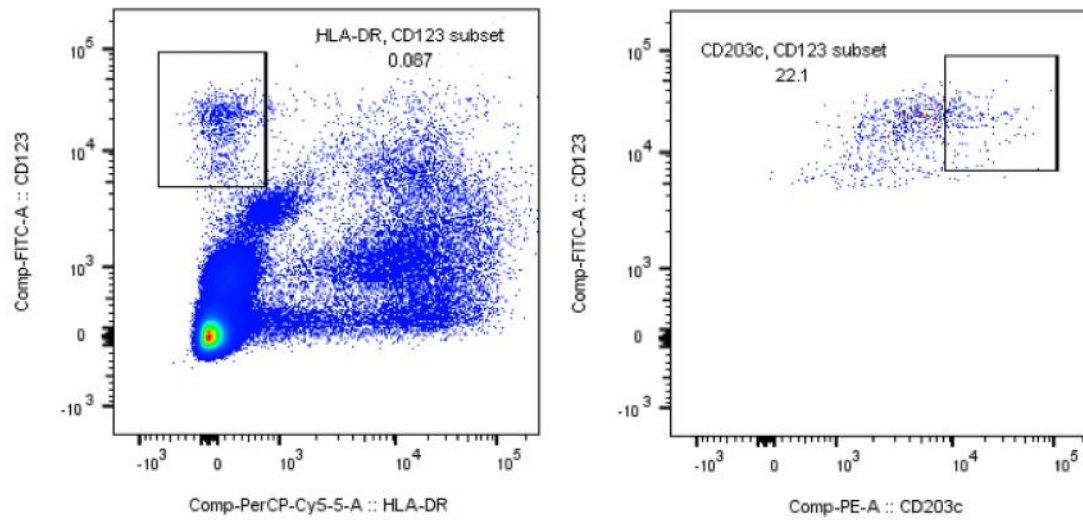
A



B



C



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Fig. 6

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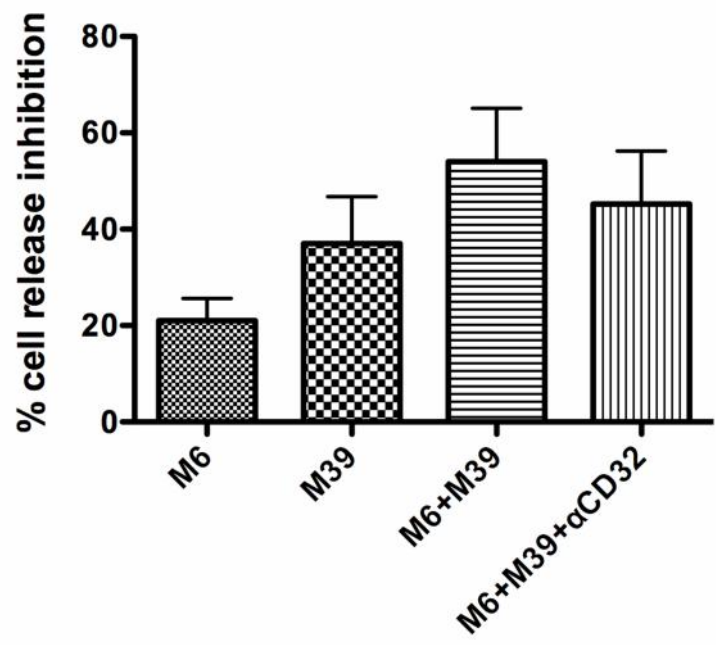
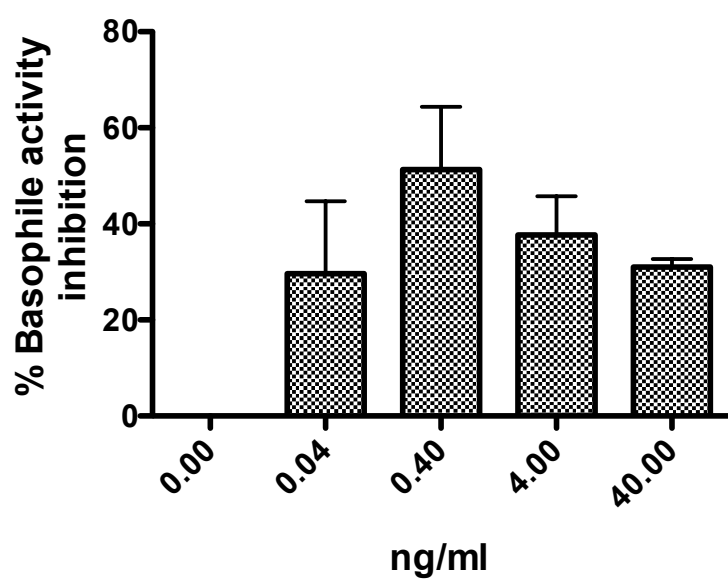


Fig. E7

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Fig. E8