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Supplemental Information

Two Allergen Model Reveals Complex Relationship Between IgE Cross-Linking and Degranulation

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Inventory of Supplemental Information

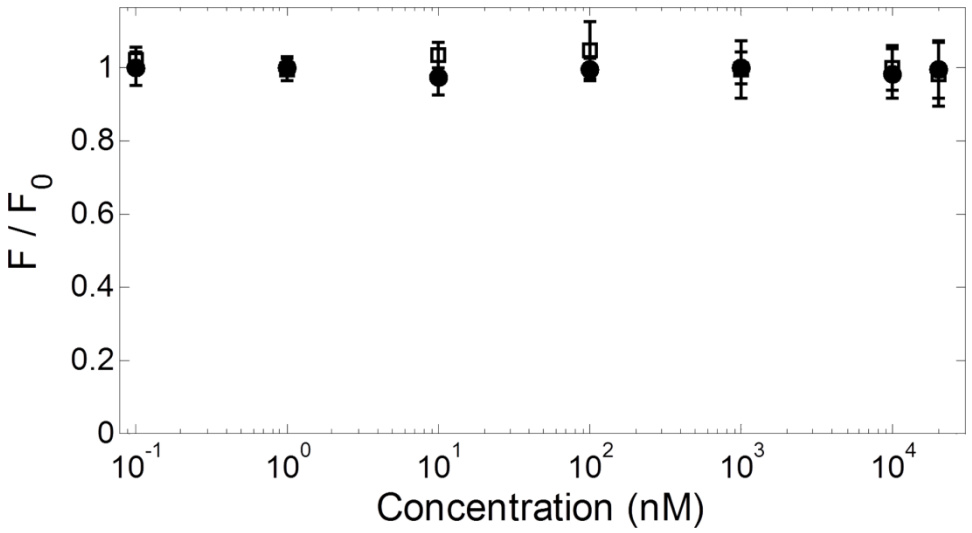

Figure S1, related to Figure 1B: As a control experiment it was confirmed that dansyl does not interact with IgE^{DNP} and similarily that DNP does not interact with IgE^{dansyl}. Dansyl (●) or DNP (□) was titrated into a 96 well plate containing a 200 µL solution of 10 nM IgE^{DNP} or IgE^{dansyl} respectively and binding was observed by monitoring the fluorescence quenching of the tryptophan residues in the IgE protein caused by either dansyl or DNP. The error bars represent the mean ± SD of triplicate experiments.

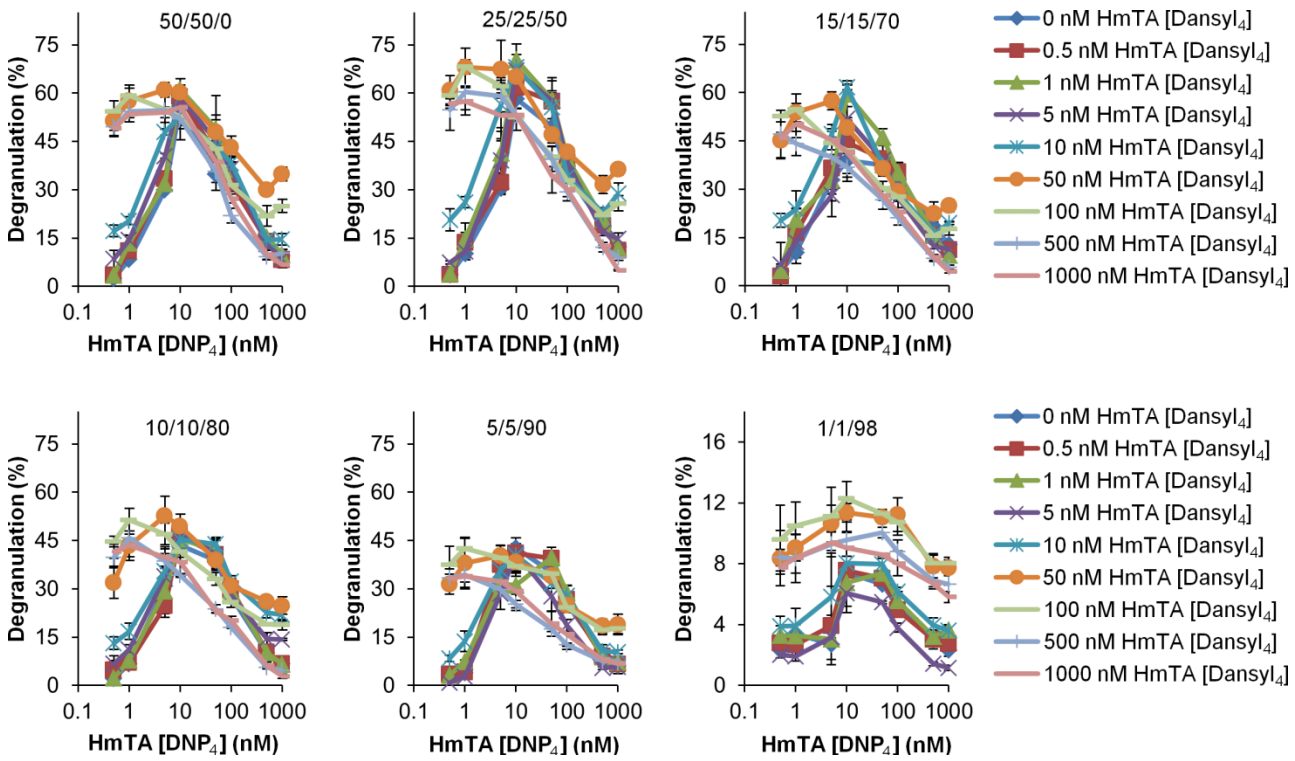

Figure S2, related to Figure 3: RBL cells were primed with mixtures of IgEs (50/50/0, 25/25/50, 15/15/70, 10/10/80, 5/5/90, and 1/1/98 IgE^{DNP}/IgE^{dansyl}/$IgE^{cyclin\ A}$) at a saturating total IgE concentration of 1 µg/mL. The cells were washed and then exposed to mixtures of HmTA [DNP_4] with HmTA [$dansyl_4$] ranging from 0.5 to 1000 nM concentration of each allergen. Data represents the means ± SD of triplicate experiments.

Experimental Procedures

Fluorescence Quenching Binding Assay

The binding constants of the monovalent haptens to the respective IgEs were determined using a fluorescence quenching assay. Briefly, DNP and dansyl quench the fluorescence from the IgE tryptophan residues, occurring at 335 nm, only when the two molecules are in proximity to each other (~10 nm). The monovalent haptens were titrated into a 96 well plate containing a 200 µL solution of 10 nM IgE^{DNP}, IgE^{dansyl}, or $IgE^{cyclin\ A}$ in PBS. All experiments were repeated in at least triplicate.

Synthesis of the tetravalent and bivalent synthetic allergens

All molecules were synthesized using standard Fmoc chemistry on a solid support. Fmoc-protected amino acids were activated with HBTU and DIEA in DMF for 3 min and coupling was monitored with Kaiser tests. Fmoc-protecting groups were removed by exposure to 20% piperidine in DMF. The synthetic allergens were synthesized using multiple lysine derivatives to achieve branching, whereas *N*-

Fmoc-amido-dPEG8-acid was used to provide the EG_8 linkers. The syntheses of the synthetic allergens were identical except for the conjugation of the different haptens. The synthetic strategy was as follows: Boc-Lys(Fmoc)-OH was conjugated to NovaPEG Rink Amide resin. After removal of the Fmoc group a second Boc-Lys(Fmoc)-OH group was added to the molecule and Fmoc was removed. Next Fmoc-Lys(ivDde)-OH was conjugated to the molecule. After removal of the Fmoc group, Fmoc-Lys(Fmoc)-OH was conjugated to the ligand to facilitate branching. Both Fmoc groups were removed simultaneously providing two primary amines for the conjugation of the ethylene glycol linkers. After removing the Fmoc groups on the ethylene glycol linkers, Boc-Lys(Fmoc)-OH was conjugated to each chain followed by the removal of the Fmoc groups. This provided two primary amines for the conjugation of the first set of haptens, DNP, dansyl or acetyl. The next step was the removal of the orthogonal protecting group, ivDde, from the previously conjugated lysine residue using 2% hydrazine in DMF. The same branching process was repeated by sequentially conjugating Fmoc-Lys(Fmoc)-OH, *N*-Fmoc-amindo-dPEG8-acid, and Boc-Lys(Fmoc)-OH to the molecule, thus providing two additional primary amines for the conjugation of the second set of haptens, dansyl or DNP. Conjugation of the haptens was achieved using: dansyl chloride [5-(dimethylamino)naphthalene-1-sulfonyl chloride] for dansyl, 1-fluoro-2,4-dinitrobenzene for DNP and acetic anhydride to create the acetyl group. Following synthesis all molecules were cleaved from the solid support using 2×92:4:4 TFA (trifluoroacetic acid)/H_2O/TIS for 30 min and purified using RP-HPLC with a semi-preparative Zorbax C18 column (9.4 mm×250 mm), using linear solvent gradients of 2.5% min^{-1} increments in acetonitrile at a 4.0 ml/min flow rate. We monitored the column eluent with a diode array detector allowing a spectrum from 200 to 400 nm to be analyzed. The purified products were characterized using a Bruker microTOF II mass spectrometer. The purity of all synthesized ligands was estimated to be >97% by an analytical injection using the above described HPLC with a Zorbax C18 analytical column (4.6 mm×150 mm). The calculated exact mass of HmTA [DNP_4] ($C_{154}H_{267}N_{31}O_{61}$) was 3526.8744 Da; found 3527.9013 Da. The calculated exact mass of HmTA [$dansyl_4$] ($C_{178}H_{303}N_{27}O_{53}S_4$) was 3795.0727 Da; found 3796.0856 Da. The calculated exact mass of HmBA [DNP_2] ($C_{166}H_{267}N_{27}O_{55}$) was 3278.8926 Da; found 3279.8939 Da.

Degranulation Assays

RBL cells and IgE^{DNP} were kindly provided by Dr. Wilson (University of New Mexico). IgE^{dansyl} (clone 27-74) and $IgE^{cyclin\ A}$ (clone BF683) were purchased from BD Biosciences. For the degranulation assays the cells were plated at 0.5×10^6 cells/ml in a 96-well plate and were incubated for 24 h followed by a 2 h incubation with the indicated IgE antibody mixtures. The cells were washed immediately before the experiments and were stimulated with the indicated concentrations of the synthetic allergens. Degranulation was detected spectroscopically by measuring the activity of the granule-stored enzyme *β*-hexosaminidase secreted into the supernatant on the substrate *p*-nitrophenyl-*N*-actyl-*β*-*O*glucosamine. All degranulation assays were repeated at least in triplicate. In all of the experiments the total IgE concentration was kept constant at 1 μg/ml.