1 **Online Repository**

2 **For**

3 # Auto-antibodies to IgE and FceRI and the Natural Variability of SYK

4 # Expression in Basophils

5

6 **Methods**

7 *Materials, Buffers and Antibodies:* The following were purchased: PIPES, bovine serum
8 albumin (BSA), EGTA, EDTA, D-glucose, 2-ME (2-mercaptoethanol); RPMI 1640
9 containing 25 mM HEPES and L-glutamine (BioWhittaker, Walkersville, MD); StemCell
10 Pro media (Life Technologies, Carlsbad, CA); Percoll (Pharmacia, Piscataway, NJ);
11 Tris(hydroxymethyl)-aminomethane, Tween-20 (Bio-Rad,Hercules, CA); syk inhibitor
12 NVP-QAB205 was a gift of GlaxoSmithKline; src-family kinase inhibitor, PP1
13 (Calbiochem, San Diego, CA); PCI-32765 (MedChem Express, Monmouth Junction,
14 NJ); LY2940002 (Calbiochem, La Jolla, CA); mouse anti-human IgE Ab (6061P)
15 (Hybridoma Farms, MD); goat polyclonal anti-hIgE Ab generated and enriched as
16 described previously [1]; anti-CD32b, Ab10523 (Xencor, Inc., Moravia, CA); anti-CD32a
17 Ab, clone IV.3 (StemCell Technologies (Seattle, WA); control mouse IgG1 (Zymed, San
18 Francisco, CA); control mouse IgG2b (eBioscience, San Diego, CA); anti-IL3R-
19 phycoerythrin (BD Pharmingen, San Jose, CA); anti-mouse IgG2b-alexa647 (Molecular
20 Probes, Invitrogen, Carlsbard, CA); anti-FceRI, 15A5, and 22E7 (gift from Hoffman-
21 LaRoche); human IgG (Cappel Laboratories); anti-syk (4D10) (Santa Cruz Antibodies,
22 Santa Cruz, CA); PIPES-albumin-glucose (PAG) buffer consisted of 25 mM PIPES, 110
23 mM NaCl, 5 mM KCl, 0.1 % glucose, and 0.003 % HSA. PAGCM was PAG
24 supplemented with 1 mM $CaCl_2$ and 1 mM $MgCl_2$. Labeling with antibodies for flow
25 cytometry was conducted in PAG containing 0.25 % BSA in place of 0.003 % HSA (EB).
26 ESB is Novex electrophoresis sample buffer containing 5 % 2-mercaptoethanol. Lactic
27 acid IgE elution buffer 0.01 M lactic acid, 0.14 M NaCl, 0.005 M KCl at pH 3.9 [2,3].

28 *Preparation of FceRIalpha, IgE and HSA sepharose*

29 A flag-tagged FceRIalpha transfection plasmid was the generous gift of Dr. Robert
30 Anthony (Harvard, Boston, MA). The plasmid was used to transfect HEK cells as
31 described previously [4]. Briefly, 9 million HEK cells were transfected with 60 µg plasmid
32 in a solution of PEI (Transporter 5, Polysciences, Inc. Warrington, PA):saline (240 µl
33 stock in 3 ml saline), cultured in 2xT75 flasks (10 ml DMEM media/flask). Supernatant
34 from the flasks after 72 hours was adsorbed with anti-FLAG Ab-beads (300 µl stock
35 beads, Sigma-Aldrich, St. Louis, MO). The beads were used for adsorption experiments
36 after equilibrating into 1X PIPES buffer. IgE-sepharose was prepared by coupling 750
37 mg of JK IgE (Hybridoma Farms, Belair, MD) to 80 mg CNBr-sepharose 4B fast-flow
38 beads (Sigma-Aldrich, St. Louis, MO). HSA was coupled in a similar manner. For pilot
39 studies, test solutions of either 22E7 diluted to a concentration that was near the optimum
40 for histamine release or goat anti-IgE Ab also titrated to near its optimum for histamine
41 release were treated with different quantities of all three beads and the optimum for
42 specific adsorption chosen for subsequent experiments.

43 *Basophil purification*

44 Basophils were purified from leukopheresis packs or from blood obtained by
45 venipuncture. When used at high purity, they were purified to near homogeneity by
46 sequential application of Percoll gradients and negative selection using the basophil
47 purification kit (Stem Cell Technologies, Vancouver, BC) and columns from Miltenyi
48 Biotec (Aubum, CA) [5]. The average purity of these basophils by alcian blue staining [6]
49 was 99%. Starting viability of these cells was typically >97%. For basophils obtained
50 by venipuncture (this study was approved by the Johns Hopkins Institutional
51 Review Board), basophils were enriched on a two-step Percoll gradient [7].

52 *Reaction conditions*

53 For experiments requiring longer incubations (functional and expression level studies),
54 the culture conditions were RPMI-1640 media containing 0.03% human serum albumin
55 (HSA), gentamycin (100 µg/ml) and supplemented to 1 mM $CaCl_2$. When cells were
56 obtained by venipuncture and enriched by two-step Percoll gradients, the cell culture
57 density was 2 million total cells/ml of medium.

58 For some experiments, a portion of the endogenous IgE was removed by treating the cells
59 with lactic acid buffer (on ice for 6 seconds with buffer before adding 1.5 ml EB to
60 neutralize the pH) [3]. Histamine release was measured from supernatants of cells
61 stimulated in PAGCM buffer. Histamine was measured by automated fluorimetry [8]. For
62 desensitization experiments, there were two phases to the reaction, ±stimulation with
63 6061P (anti-IgE Ab) in the absence of extracellular calcium (+50 µM EDTA) for 90
64 minutes followed by re-stimulation with anti-IgE Ab or serum for 45 minutes in 1 mM
65 Ca^{++}.

66 *Definition of Positivity*

67 As the ABO-compatibility issue and the initial screening of sera were explored, a
68 dataset of histamine results from the 25% sera-challenge of recipient basophils was
69 generated. A simple algorithm for iterating towards a threshold in the presence of
70 sparse true signals was used [9]. All of the results (responses to 25% serum) were
71 first averaged, label as A1. This average (A1) was naturally skewed by the presence
72 of eventual positives. Using A1 as a threshold, the dataset was re-averaged by
73 including only results *less* than A1, label this average as A2. Under the assumption

74 that A2 represented a close approximation of the true background signal, the

75 standard deviation (SD2) of this distribution was also calculated and a threshold of

76 positivity set as A2 + 1.96*SD2 ($p>0.975$ of the assumed Gaussian distribution). This

77 value was 6% (note that this is above spontaneous release in the histamine release

78 calculation).

79 *Flow cytometry*

80 With some noted exceptions, flow cytometry was performed on cells fixed in 2%

81 paraformaldehyde for 20 minutes at room temperature before blocking with 4%BSA-PBS

82 overnight. If the basophils were highly purified, there was no need for selection

83 antibodies but if only enriched, gating for basophils was based on forward-side scatter

84 gates and labeling with anti-FcεRI antibody [10]. The monoclonal antibodies 22E7 (a gift

85 of Hoffman-LaRoche) or CRA-1 (eBiosciences, San Diego, CA) (both antibodies specific

86 for FceRIalpha) were used interchangeably (depending on which isotype mouse antibody

87 was needed) to label FceRI. Isotype control antibodies or specific antibodies were

88 incubated with basophils in EB buffer for 30 minutes prior to a secondary incubation with

89 labeled secondary antibodies for 30 minutes, followed by washing and analysis. If the

90 measurement of SYK were the target of the assay, the SYK-selective antibody 4D10 was

91 used in a protocol previously published [11]. If CD32a receptors were the target for labeling,

92 the protocol, described previously [12], included 20 μg/ml Ab10523 (anti-FcgRIIb/CD32b)

93 because clone IV.3 (anti- FcgRIIa/CD32a) could bind to FcgRIIb/CD32b through its Fc

94 region. If the FcgRIIb/CD32b receptors were the target, the 'control' labeling included

95 20 μg/ml Ab10523 (anti-FcgRIIb/CD32b, see above) as well as 1 μg/ml Ab10523-

96 alexa647 while detection of FcgRIIb/CD32b included only Ab10523-alexa647. Since
97 Ab10523 (anti-FcgRIIb/CD32b) doesn't bind to FcgRIIb/CD32b through its Fc region,
98 no blocking of FcgRIIa/CD32a was necessary. In general, mean net fluorescence is
99 reported. Calibration beads were used to ensure the flow cytometer was producing similar
100 results between daily and weekly measurements.

101 *Western blotting*

102 Pelleted cells were lysed in 20 µl of hot ESB, the tube placed in boiling water for 5
103 minutes and the samples stored at -80˚C until electrophoresis. Samples were run in a 15
104 well 8% tris-glycine gel with molecular weight markers. After transblotting to
105 nitrocellulose, membranes were blocked with 4% BSA. Equal lane loading was
106 confirmed by blotting with anti-p85a antibody. We have used this method in variety of
107 different studies and have found that basophils do not change their p85 levels during IgE-
108 mediated stimulation for short or long periods [13].

109 *ABO compatibility:*

110 All of our donors that supplied basophils for the bioassay of sera-induced histamine
111 release were typed for the AB and Rh antigens. For these same donors, sera was also
112 obtained. The donors and sera were tested for histamine release by stimulating a
113 "recipient" (Percoll-separated basophils, see above) basophil with 25% serum from
114 either an AB/Rh matched donor or with a mis-matched AB/Rh donor. For a series of
115 24 donor sera (from non-CIU patients) first tested on a 3 O+ basophils for whom
116 optimal IgE-mediated histamine release averaged 28%, no sera were found to
117 induce histamine release. For a series of 20 sera that were tested on deliberately

118 mismatched “recipient” basophils (e.g., A+ on B+, etc.), only 1 serum induced modest
119 release (5-10%). Further exploration of this one example showed that it would
120 induce release only when paired with one particular recipient basophil. Type
121 matching or mismatching could be done without release except for the one
122 particular “recipient” donor. During the course of additional experiments, one
123 additional donor was found with this characteristic. Therefore, AB/Rh matching
124 appears irrelevant to whether sera induce release but in approximately 5% of sera
125 tested, there could be some release induced that could be considered idiosyncratic
126 to specific pairings of “recipient” basophils and serum. Despite the appearance of no
127 problem with AB/Rh matching, for the remainder of the experiments in which
128 serum was tested on “recipient” basophils, these basophils were chosen to be O+ or
129 O-.

130 **Results**

131 *Difference in positivity between general and basopenic groups*

132 There were three subjects that appeared in both the general CSU group and in the
133 subsequently screened CSU group with basopenia. Statistically, the two groups are
134 therefore not independent. Removal of the 3 basopenic subjects from the general
135 group (and retaining them in the basopenic group) results in a chi-squared of 6.26,
136 p=0.011, i.e., a difference in frequency between groups but performing the analysis
137 by removing them from the basopenic group (and retaining them in the general
138 group) leads to no difference in frequency (p=0.20).

139 *Drug sensitivity*

140 The first assay tested the effects of four pharmacological inhibitors known to inhibit
141 enzymes involved in IgE-mediated secretion. Previous studies have identified agents
142 that operate on signal transduction steps that are relatively specific for the IgE-
143 mediated signaling cascade but not effective for GTP-binding protein-dependent
144 receptors such as fMLPR. Four points in the early IgE-mediated cascade were
145 chosen, the src-family kinases, SYK, BTK, and PI3 kinase (likely delta in human
146 basophils). The relevant drugs were PP1 or PP2 (src-family kinases, both tested),
147 NVP-QAB205 (SYK inhibitor), PCI-32765 (BTK inhibitor) and LY294002 (PI3K
148 inhibitor). Each has received detailed study for their behavior on human basophils
149 [14-16]. Figure E2 shows a schematic of where these drugs are thought to act in the
150 IgE-signaling pathway.

151 For this assay, we discovered that the presence of serum could shift the IC50 of the
152 drugs being used as indicators of an IgE-mediated reaction. This was most notable
153 for PP1 or PP2, two src-family kinase inhibitors. For these inhibitors, for reasons not
154 explored, serum almost completely eliminated the drugs' effectiveness. Figure E3
155 summarizes the results for all four drugs with and without serum. Therefore, results
156 with PP1 or PP2 were not included in subsequent testing. Serum shifted the IC50 of
157 NVP-QAB205 (SYK inhibitor) by 2-3 fold, LY294002 (PI3K inhibitor) by 6-fold and
158 PCI-32765 (BTK inhibitor) by only a minor degree. It wasn't known whether cells
159 were experiencing lower intracellular levels of the drugs (e.g., due to metabolism or
160 binding to sera proteins) or whether a property of the cellular response to the
161 action of the drugs was different in the presence of serum. Because the mechanisms
162 were unclear, for subsequent experiments in the presence of serum, the drug

163 concentrations were conservatively kept near the expected IC50 concentrations
164 determined from previous studies. But this conservative approach led to results
165 where the ability to inhibit 6061P/anti-IgE Ab-induced release was more variable
166 due to the serum effect and so the method to analyze the results across different
167 experiments was to normalize the extent of inhibition based on how effectively
168 6061P/anti-IgE Ab-induced release was inhibited in the presence of a non-releasing
169 serum (see methods). Figure E4A plots the similarity index for the 13 multiply
170 positive sera. Four of the sera induced release that was unresponsive to these 3
171 drugs.

172 A second assay determined whether desensitization of the IgE-mediated pathway
173 also inhibited the ability of the serum to induce release. Parenthetically, we
174 discovered that the inclusion of serum sometimes slowed the rate of desensitization
175 of FceRI. For this series, basophils were stimulated with 6061P/anti-IgE Ab in the
176 absence of extracellular calcium for 1 hour (or no 6061P/anti-IgE Ab to act as the
177 non-desensitized control), extracellular calcium returned to the reaction and the
178 cells further stimulated with 6061P/anti-IgE Ab or serum at 25% final
179 concentration. The control re-challenge included 6061P/anti-IgE Ab in negative
180 serum or PAGCM. As before, the useful metric was to compare the desensitization
181 inhibition of the serum response to the desensitized inhibition to 6061P/anti-IgE Ab.
182 If 6061P/anti-IgE Ab induced desensitization of serum to an extent similar
183 6061P/anti-IgE Ab-induced desensitization to 6061P/anti-IgE Ab, the similarity
184 index was near 1.0. Figure E4B plots these results for the 13 multiply positive sera.
185 As found for the drug testing, the same 4 sera did not pass the similarity test.

186 The third assay was a natural variation of the NVP-QAB205 (SYK inhibitor)
187 experiments, taking advantage of basophil donors whose expression level of SYK is
188 so poor that IgE-mediated histamine release is near zero [3, 17]. There is little reason
189 to expect an FceRI-dependent process or a SYK dependent process, to induce release
190 from these donors' basophils. Prior to this experimental series, we noted that some
191 of the 13 multiply positive sera were not found to induce strong release. If release
192 induced with these sera from a non-releaser were poor, it would be difficult to know
193 whether the release was expected or appropriately suppressed. Therefore, the
194 calculation of similarity was based on the expectations for release with each of the
195 13 sera. This was the last series of studies done and at this point, there were 4-6
196 independent measurements of these 13 sera to induce release from strongly
197 releasing basophil donors. An average of the multiple measurements for a given
198 serum could be calculated and the histamine release from the non-releaser
199 calculated relative to the expectation. Therefore, a value of near zero would be
200 expected for sera that operated through SYK. Several non-releasing donors were
201 used for this test and those sera causing release were repeated. With this test there
202 were only 5 sera that continued to pass the similarity metric (see figure E4C).

203 *Adsorption of sera*

204 To verify that these sera likely contained an antibody to IgE or FceRI, the sera were
205 adsorbed with either IgE- or FceRIalpha-beads. The online repository describes the
206 coupling of sepharose beads to HSA, IgE or FceRIalpha. Three sera with sufficient
207 titers were adsorbed with a 12.5% v/v quantity of each of these beads for 2 hours at

208 4˚C. The bead supernatants were used to stimulate releasing basophils (as above).
209 For positive controls, a solution of anti-FceRIalpha (22E7) monoclonal antibody or
210 anti-IgE (goat) polyclonal antibody (diluted into negative serum) at concentrations
211 that produce optimal and slightly suboptimal histamine release were also adsorbed
212 with the beads. These controls provide a context for the adsorption results with
213 positive sera (not shown in figure E5, IgE-beads did not adsorb 22E7 and alpha-
214 beads did not adsorb anti-IgE Ab). In all 4 instances tested (1 was excluded because
215 the response was relatively poor for this serum), either IgE- (1 instance) or
216 FceRIalpha- (3 instances) beads inhibited the ability of sera to induce release. Two
217 of the anti-FceRIalpha positive sera appeared to also be partially adsorbed with IgE
218 beads and might therefore also be an anti-IgE Ab (figure E5).

219 In an additional test, basophils were treated with lactic acid buffer to dissociate a
220 significant fraction of their surface IgE antibody or incubated with additional IgE in
221 an attempt to fully occupy any non-occupied receptors. One set of cells remained
222 untreated. All 3 sets were stimulated with the 5 sera. This test examines whether
223 the ant-FceRIalpha antibodies were sensitive to the occupancy of FceRI. The anti-
224 IgE-like serum (MM) was inhibited by dissociation. However, each of the anti-
225 FceRIalpha-like sera were sensitive to occupancy in that dissociating the
226 endogenous IgE enhanced their ability to induce release and increasing occupancy
227 with sensitization decreased the response to the serum. The one serum that was not
228 examined in the immuno-adsorption study also was sensitive to occupancy, i.e.,
229 serum-induced release was enhanced following IgE dissociation.

230 *IL-3 effects on sera-induced histamine release*

231 None of the non-CIU sera induced replicable release; this included sera from
232 subjects classified as non-releasers. An additional test was performed on sera from
233 non-releasers and releasers. Basophils were cultured for 24 hours with 10 ng/ml IL-
234 3 to markedly enhance their sensitivity to secretagogues. Surprisingly, all sera
235 induced histamine release but there was no difference between the two categories
236 of donor (releaser vs. non-releaser)(figure E6B, figure E6A shows the release
237 distributions for the basophils, stimulated with an optimal concentration of
238 6061P/anti-IgE Ab of serum source subjects). In addition to the challenge with sera,
239 the effect of the BTK inhibitor PCI-32765 on each response was tested. None of the
240 responses were inhibited (ratio of response+inhibitor/response-inhibitor =
241 1.03±0.07)(data not shown). In addition, the test was also preformed on basophils
242 for which the endogenous IgE was dissociated prior to culture and challenge. There
243 were no differences in the responses to the sera and no differences between
244 releasers and non-releasers (data not shown).

245 *Inhibitory effects on IgG*

246 The question was whether antibodies that interact with a cell surface protein (in
247 this case, IgE or FceRI) and are already bound and constrained to the cell surface,
248 are inhibited in the interaction of their Fc domains with cell surface CD32b due to
249 nonspecific IgG in the extracellular medium. Several known anti-IgE or anti-FceRI
250 antibodies were examined for the influence of CD32b on their activity and for what
251 concentrations of nsIgG antibody interfered with any interaction. We have

252 previously demonstrated that polyclonal goat anti-IgE Ab interacts with
253 FcegRIIb/CD32b and that inhibition of this interaction with a selective anti-CD32b
254 antibody (Ab10523) enhances induced histamine release. This interaction is
255 sufficiently strong to observe significant enhancement of release in the presence of
256 Ab10523 (anti- FcegRIIb/CD32b). Figure E7A shows that nsIgG can also reverse
257 interaction of goat anti-IgE with FcegRIIb/CD32b with an IC50 of approximately 2.5
258 mg/ml with full reversal (relative to Ab10523 (anti- FcegRIIb/CD32b)) not
259 apparent at 10 mg/ml. A mouse IgG2b anti-IgE Ab was examined. There have been
260 indications in previous studies that there is some interaction of mouse antibodies
261 with CD32b but as is apparent in Figure E7B, enhancement of release is modest with
262 Ab10523 (anti- FcegRIIb/CD32b). The inclusion of nsIgG also enhances release, to
263 an extent exceeding somewhat or equivalent to Ab10523 (anti- FcegRIIb/CD32b)
264 with an IC50 of 300 μg/ml. We found that 22E7, an anti-FceRIalpha antibody is also
265 only weakly enhanced with Ab10523 (anti- FcegRIIb/CD32b) (data not shown,
266 enhancement of 1.20±0.05 fold). These results suggest that interaction with
267 FcgRIIb/CD32b varies considerably between anti-IgE/Fc antibodies, with goat Ab
268 showing the strongest interaction.

269 These experiments suggested that the range of possible interaction is large but that
270 a typical human IgG interacting with CD32b might be reversible by competing with
271 nsIgG. Therefore, these results suggest that 1) nsIgG levels less than 1.5 mg/ml
272 (equivalent to 10% serum) could be tolerated if a human anti-FceRI or anti-IgE had
273 Fc binding characteristics like goat anti-IgE while 10 fold lower concentrations
274 would be needed (1% serum) if the characteristics were like mouse anti-IgEs.

275

276 **Figure Legends**

277 **Figure E1:** Serum induced histamine release distributions for the 3 categories of
278 subject. The final concentration of serum was 25%. The initial screening required
279 that a serum induce release consistently and a failure in a second screen removed
280 the serum from consideration. The sera that passed the first screening are shown as
281 pairs with those that failed the second screening as open circles and those that
282 passed the second screening as filled gray circles. The average of release of the first
283 test of eventual double positives was 43% while the average release for the first test
284 of eventual single positives was 19%. The average anti-IgE Ab-induced release of
285 the recipient basophils was 70%.

286 **Figure E2:** Cartoon of the early IgE-mediated signaling reaction to show the relative
287 position in the cascade and the drugs that inhibit each step.

288 **Figure E3:** IC50 of 4 drugs with specificity for IgE-mediated secretion with and
289 without 25% serum present. Panel A: src-family kinase inhibitor, PP1 (n=3)(filled
290 circle = no serum present, filled square = 25% serum present, applies to all panels).
291 Panel B: SYK inhibitor, NVP-QAB205 (n=1). Panel C: BTK inhibitor, PCI-32765 (n=1).
292 Panel D: PI3 kinase inhibitor, LY294002 (n=1).

293 **Figure E4:** Three tests for sera similarity to an IgE-mediated response. The methods
294 section describes the metric of 'similarity' as is relevant to each test. For some of the
295 sera where results neared thresholds for exclusion, the tests were repeated (e.g.,

296 ER). Panel A: similarity index (>0.90 showing similarity to an IgE-mediated
297 response) for 3 selective inhibitors of the FceRI-mediated activation pathway. *Black*
298 *bar* = SYK inhibitor, NVP-QAB205 at 0.75 µM; *lightest grey bar*, BTK inhibitor, PCI-
299 32765 at 75 nM; *medium grey bar*, PI3K inhibitor, LY294002 at 7.5 µM. Asterisks
300 denote sera not passing this test. Panel B: similarity index for cross-desensitization
301 between a known anti-IgE Ab and sera-induced release (>0.90 showing similarity to
302 an IgE-mediated response). Asterisks denote sera not passing this test. Panel C:
303 Ability of sera to induce release from a known non-releasing basophil. The metric
304 for this test was the fraction of the expected response for each specific serum based
305 on past performance of the same serum on releasing basophils. To accommodate
306 variation, a value greater than 0.3 was considered a positive response from a non-
307 releaser basophil although a threshold of less than 0.05 would be appropriate for a
308 known aggregating anti-IgE Ab. Asterisks denote sera not passing this test.

309 **Figure E5:** Adsorption of sera with either HSA-coupled, IgE-coupled or FceRIalpha-
310 coupled sepharose beads. Basophils were challenged with stimuli that had been
311 adsorbed with the various stimuli shown. The ordinate metric is the response
312 relative to the response to as stimulus after adsorption with HSA-coupled beads.
313 The control responses (absorption with HSA-coupled beads) to anti-IgE Ab (goat
314 anti-IgE Ab), 22E7 (anti-FceRIalpha), AB, MM, AS, and DS sera was 83%, 64%, 18%,
315 36%, 42%, and 5%, respectively. The data shows averages from 3 experiments for
316 the 22E7 (anti-FceRIalpha) and anti-IgE Ab (goat anti-IgE Ab), MM and AS and n=1
317 for DS and AB.

318 **Figure E6:** Effect of overnight IL-3 (10 ng/ml) on the response of basophils to
319 releaser (R) and non-releaser (NR) sera. Panel A: Histamine release from the
320 basophils of serum donors categorized as releasers (R) or non-releasers (NR).
321 Points are the response of each donors' basophils to stimulation with anti-IgE
322 Ab/6061P at an optimal concentration (0.5 μg/ml). Horizontal bars represent the
323 averages. Panel B: sera from these basophil phenotypes was used to stimulate
324 basophils first cultured for 18 hours with IL-3 at 10 ng/ml. Points are responses to
325 each sera tested, horizontal bars represent the averages ± standard deviation.

326 **Figure E7:** Sensitivity of mouse and goat anti-IgE Abs to nonspecific IgG to block
327 CD32b. Panel A: The positive control (rightmost bar) in this protocol is the inclusion
328 of Ab10523 (anti-FcgRIIb/Cd32b) at 50 μg/ml to block the participation of
329 FcgRIIb/CD32b during stimulation with a mouse monoclonal anti-hIgE Ab. The
330 leftmost bar is the response without Ab10523 (anti-FcgRIIb/Cd32b). In-between is
331 the concentration-dependence of nonspecific hIgG to block the participation of
332 FcgRIIb/Cd32b in the response to mouse anti-IgE Ab. Panel B: similar to panel A
333 except the stimulus was goat polyclonal anti-IgE Ab.

334

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383

384

385

Figure E1

%Histamine Release

80

60

40

20

0

non-CSU

CSU

CSU
basopenic

Figure E2

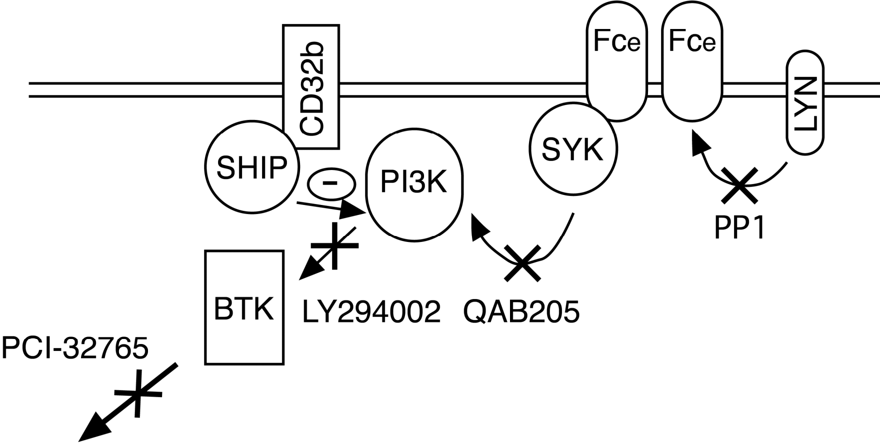


Figure E3

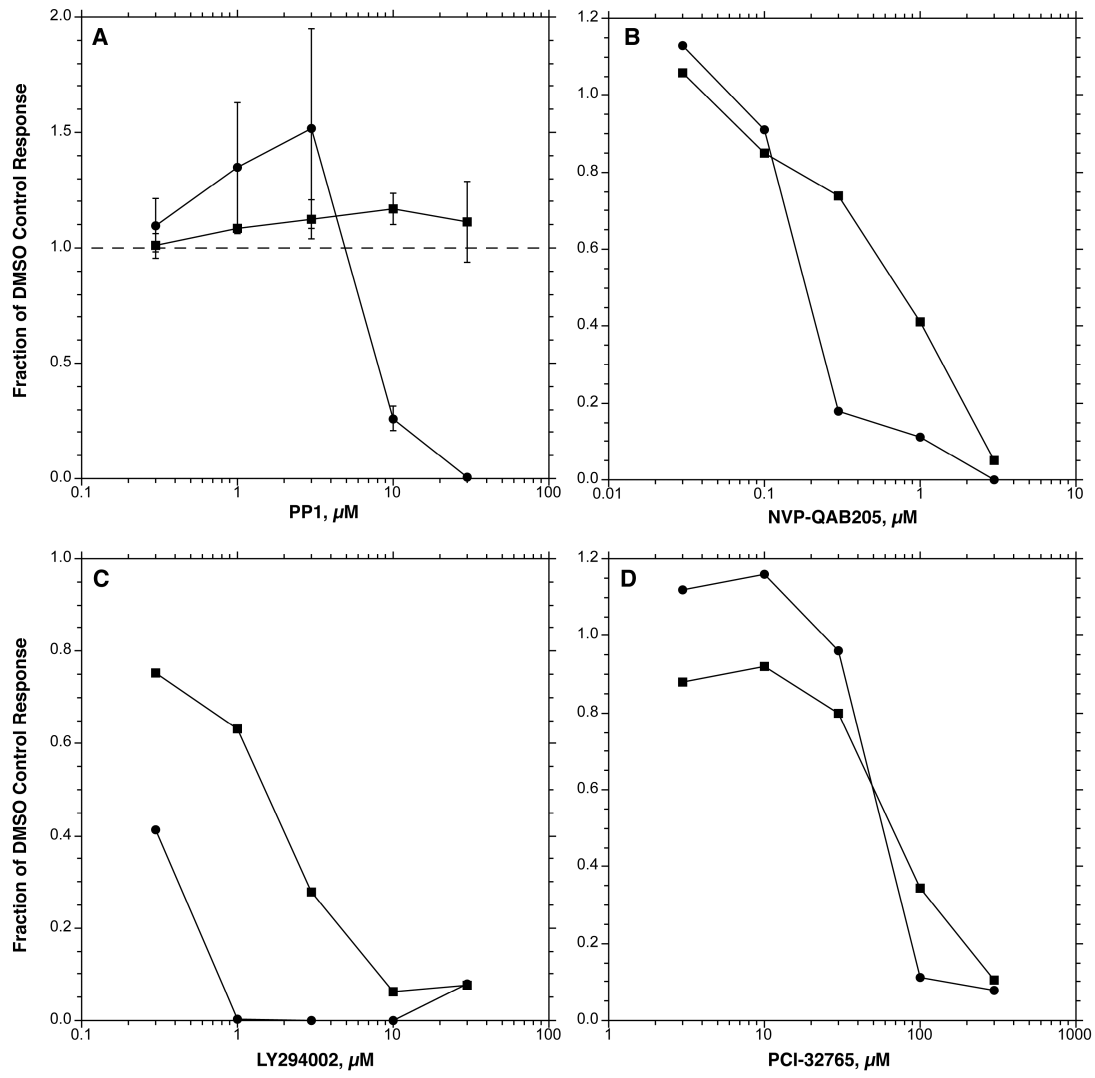
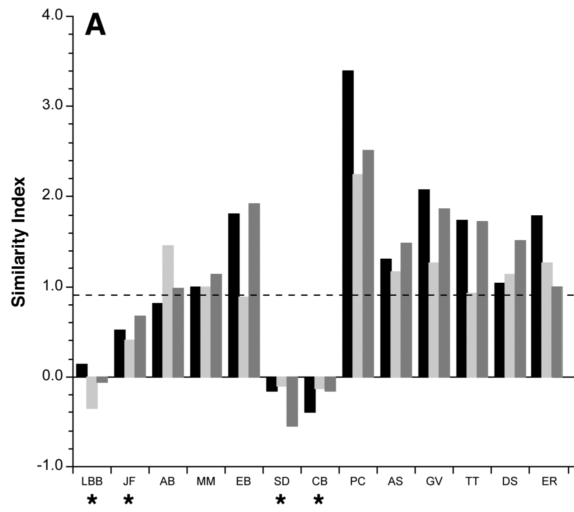
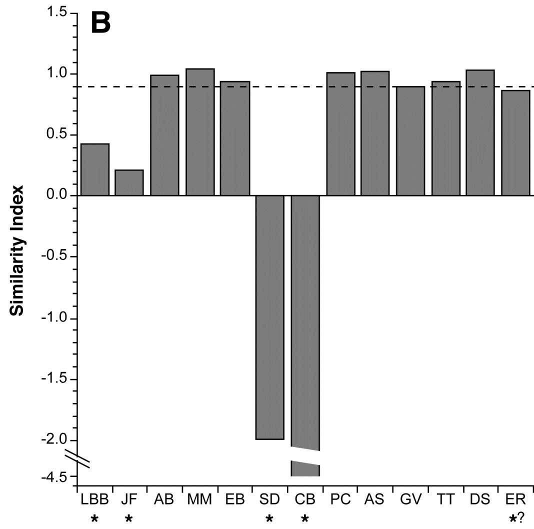


Figure E4





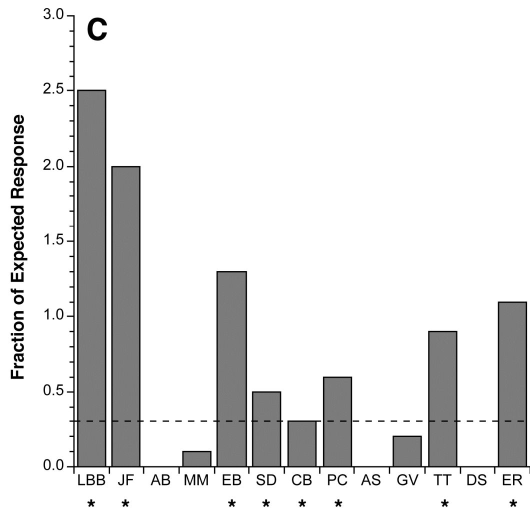


Figure E5

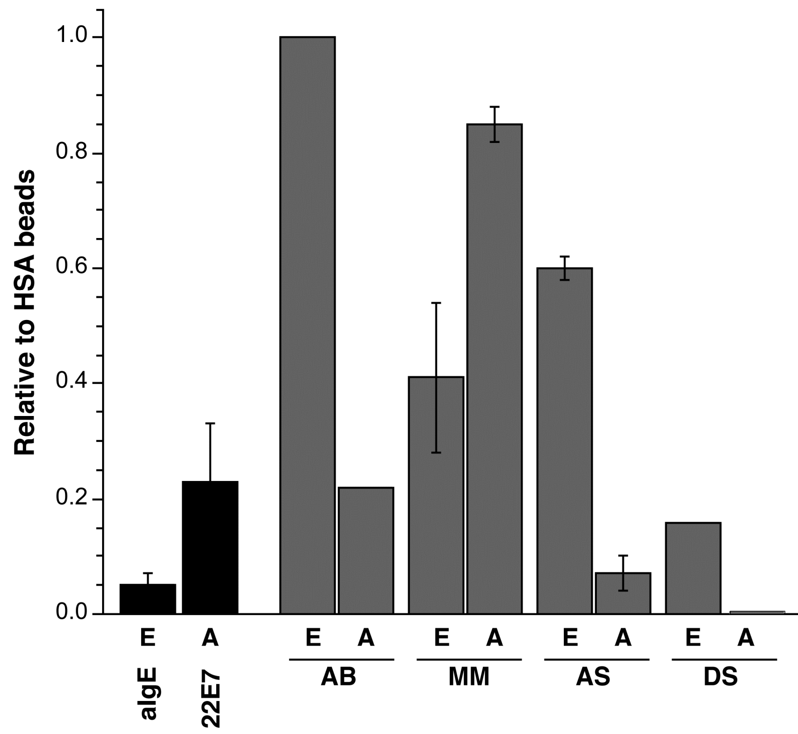


Figure E6

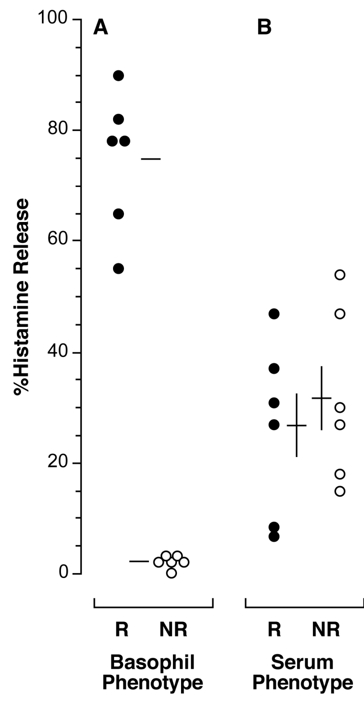


Figure E7