Allergen-specific basophil suppression associated with clinical tolerance in patients with milk allergy

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Background: Children with milk allergy who tolerate heat-denatured milk (HM) have less severe reactions and outgrow the condition earlier than those who react to HM, which might be related to differences in IgE-dependent effector cell function.

Objective: We sought to apply a novel assay to test the hypothesis that HM-tolerant children have suppressed IgE-mediated basophil responses.

Methods: Allergic, HM-tolerant, outgrown, or control subjects were defined based on oral food challenges. Whole blood cells were stimulated *in vitro* with a range of milk allergen doses in the presence or absence of autologous serum or with dilutions of autologous serum. Activated basophils were identified by means of flow cytometry as $CD63^{bright}CD123^{+}CD203c^{+}HLA\text{-}DR^{-}CD41a^{-}$.

Results: HM-tolerant subjects' basophils were significantly less responsive to milk allergen stimulation at all doses than were basophils from HM-reactive (allergic) individuals. In the absence of autologous serum, HM-tolerant subjects' basophils were significantly more reactive at low allergen concentrations. To a lesser extent, autologous serum also inhibited IL-3– and anti-IgE–induced, but not N-formyl-methionyl-leucyl-phenylalanine–induced, responses. The allergen-specific responsiveness of HM-tolerant subjects' basophils increased with dilution of autologous serum with normal pooled serum.

Conclusion: Children with milk allergy with a favorable prognosis have evidence of extrinsically suppressed allergen-specific effector cell reactivity. (J Allergy Clin Immunol 2009;123:789-794.)

Key words: *Milk allergy, basophils, basophil activation test, oral tolerance, cow's milk allergy*

Abbreviations used
fMLP: N-formyl-methionyl-leucyl-phenylalanine
HM: Heat-denatured milk
PI3K: Phosphoinositide 3-kinase

Basophils are among the least abundant populations of circulating leukocytes, but by virtue of their sensitization with allergen-specific IgE, they represent a significant effector population in allergic pathogenesis. Basophils are known to be an early and abundant source of T_H2 cytokines and other mediators of T_H2 inflammation.[1] There is also growing recognition of their capacity to modulate adaptive immunity.[2-5]

We are interested in better understanding the mechanisms of IgE-mediated hypersensitivity and its regulation in the context of food allergy and oral tolerance and in identifying biomarkers of immune tolerance that might be useful for prognosis and immunotherapy monitoring.

Because basophils are readily accessible for study, they have been attractive targets both for investigating basic mechanisms of type I allergy and for the development of novel diagnostic tools. Several groups, including our own, have developed flow cytometric approaches for assessing the activation status of these cells.[6-9] For pediatric studies in particular, there is a need for approaches that minimize the amount of patient sample that is required for any particular assay, and therefore we have focused on methods that allow us to assay basophil activation without enriching those cells from larger volumes of blood.

Here we report the novel application of a direct basophil activation test to determine whether patients with milk allergy who tolerate heat-denatured milk (HM) products also have significantly less milk allergen-induced reactivity *in vitro*. We demonstrate that the difference in basophil responsiveness is partially due to inhibition by an autologous factor present in serum, which we hypothesize to be allergen-specific IgG.

METHODS

Subjects

Fifty-five subjects were recruited from a larger clinical study on the natural history of milk allergy and were characterized based on the results of open food challenges as allergic (reactive to all forms of milk products, n = 13), heated cow's milk (HM) tolerant (n = 32), or outgrown (n = 10) subjects. Control subjects not allergic to milk were recruited from a separate study of egg allergy (n = 13).[10] Blood samples from HM-tolerant subjects were obtained at the time of the initial baseline challenge (9/32), as well as after introduction of an HM-containing diet (n = 23/32). All research protocols were approved by the Mount Sinai Institutional Review Board, and informed consent was obtained for all subjects. Allergen-specific levels and skin test data were obtained as previously described.[10]

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Reagents

Milk antigen was prepared from nonfat dried milk (Upstate/Chemicon International, Temecula, Calif) diluted in PBS. RPMI 1640 with glutamine and N-formyl-methionyl-leucyl-phenylalanine (fMLP) were purchased from Fisher Scientific (Pittsburgh, Pa). Recombinant human IL-3 was obtained from R&D Systems (Minneapolis, Minn). Polyclonal anti-IgE antibody was from Bethyl Laboratories (Montgomery, Tex). EDTA was obtained from Promega Corp (Madison, Wis). FACS lysing solution was obtained from BD Biosciences (San Jose, Calif).

Antibodies

The following mAbs were used: fluorescein isothiocyanate–conjugated anti-human CD63 (clone H5C6, mouse IgG1; BD Biosciences), phycoerythrin-conjugated anti-human CD203c (clone 97A6, mouse IgG1; Serotec, Oxford, United Kingdom), phycoerythrin-cyanin 5–conjugated anti-human CD123 (clone 9F5, mouse IgG1; BD Biosciences), allophycocyanin–conjugated anti-human CD41a (clone HIP8, mouse IgG1; BD Biosciences), and phycoerythrin-cyanin 7–conjugated anti-human HLA-DR (clone L243, mouse IgG2a; BD Biosciences).

Basophil activation

Whole blood aliquots (250 μL) were incubated with equal volumes of basophil stimulation buffer alone (RPMI plus IL-3 at 2 ng/mL; IL-3 alone control) or with the addition of milk antigens at serial 10-fold dilutions (from 3×10^{1} to 3×10^{-4} μg/mL total protein), anti-IgE antibody (0.5 μg/mL, positive control), fMLP (1 μmol/L, IgE-independent positive control), or RPMI alone (negative control) at 37°C for 30 minutes. The reaction was stopped with 50 μL of cold PBS plus 20 mmol/L EDTA. Cells were then stained for expression of CD63, CD123, CD203c, CD41a, and HLA-DR at 4°C in the dark for 30 minutes. After incubation, cells were washed with PBS plus 0.5% BSA plus 2 mmol/L EDTA. Red cells were then lysed by adding 4 mL of FACS Lysing Solution to each sample for 15 minutes. Basophil activation was assessed by means of flow cytometry (see Fig E1 in this article's Online Repository at www.jacionline.org for gating example).

IgG depletion

Plasma (1 mL) was removed from whole blood aliquots (2 mL), with 650 μL used for IgG depletion and 350 μL used for mock treatment. Protein A beads (650 μL, Fisher Scientific) were washed with RPMI and subsequently incubated with plasma at room temperature on a rocker for an hour. The sample was then spun down, and the supernatant was removed. Cells (100 μL) were then incubated with 100 μL of serum (mock treated or IgG depleted) and 200 μL of either basophil stimulation buffer (RPMI plus IL-3 at 2 ng/mL, negative control), anti-IgE antibody (0.5 μg/mL, positive control), or milk antigen (3 μg/mL total protein) at 37°C for 30 minutes. Reactions were stopped and processed for flow cytometry as above.

Flow cytometry

Samples were analyzed on a BD LSRII flow cytometer. Single-color compensation samples were prepared by using anti-mouse immunoglobulin beads (Bangs Laboratories, Fishers, Ind). Fluorescence data were acquired and autocompensated on a modified LSR-II configured for 7-color parameters by using FACS Diva software (version 4.0, BD Biosciences). As shown in Fig E1, basophils were identified as $CD123^{+}CD203c^{+}HLA\text{-}DR^{-}CD41a^{-}$. A minimum of 500 $CD123^{+}CD203c^{+}HLA\text{-}DR^{-}CD41a^{-}$ events (ie, basophils) were recorded for each condition or the sample was excluded.

Statistical analyses

Offline analysis of cytometric data was performed with FlowJo version 8.1 (Tree Star, Inc, Ashland, Ore). Graphic display and statistical analyses were performed with R analysis 2.5.0 (www.R-project.org). For determining the significance of differences in CD63 percentage between groups across various antigen stimulation concentrations (Fig 1), 2-way ANOVA was used. For other between-group and before/after serum-depletion comparisons, the unpaired or paired Wilcoxon rank sum test was used, as noted in the figure legends. Complete analysis script and raw data tables are available in this article's Online Repository at www.jacionline.org. Nonresponders were defined as individuals with less than 10% CD63 upregulation in response to either allergen or anti-IgE control.

RESULTS

Allergic donors' basophils are more reactive to milk allergen than HM-tolerant donors' basophils. In total 68 subjects were studied: the allergic (n = 13), outgrown (n = 10), HM-tolerant (n = 32), and non–milk allergic control (n = 13) groups. The median ages of each group were as follows: allergic group, 9 years (range, 3.6-16.5 years); HM-tolerant group, 7.7 years (range, 2.8-16.3 years); outgrown group, 6.9 years (4.8-10.4 years); control group, 5.7 years (1.8-13.4 years). Twenty-three percent (16/68) of these subjects exhibited the previously described nonresponder phenotype to IgE cross-linking[11] and were eliminated from subsequent analyses. There was no significant difference in the frequency of nonresponders between groups (see complete data and analysis script in this article's Online Repository at www.jacionline.org).

Basophils from allergic subjects who were reactive to extensively heated cow's milk protein (HM group) were significantly more reactive than basophils from the HM-tolerant group across the range of allergen dilutions (Fig 1; $P < .001$). Peak responses were observed for both the allergic (median, 66.8 [25%-75%, 51.6-71.3]) and HM-tolerant (median, 15.9 [25%-75%, 4.5-22.1]) groups at the 1×10^{2}–fold dilution (dose E2, approximately 1 μg/mL). Control subjects' basophils were not reactive, and only a few outgrown subjects' basophils were weakly reactive. HM-tolerant subjects' basophils were significantly more reactive to milk allergen than those of either the control ($P < .001$) or outgrown ($P < .001$) groups (Fig 1). Consistent with the findings for the entire study population,[10] the same trend between these clinical groups existed for milk-specific serum IgE levels and skin prick test wheal size; however, there is less overlap between groups as measured by basophil response than by skin test wheal size (see Fig E2 in this article's Online Repository at www.jacionline.org). In fact, basophil activation appears to correspond more strongly with allergen-specific IgE levels, particularly within the outgrown group, who were more likely to have persistent skin test reactivity (see Fig E3 in this article's Online Repository at www.jacionline.org).

Autologous serum from HM-tolerant subjects inhibited allergen-specific responses. Whole blood cells from each subject were divided and washed and then resuspended in either autologous plasma or RPMI before stimulation to evaluate whether the observed differences in basophil responsiveness between study subject groups reflected intrinsic or extrinsic features of those cells. HM-tolerant basophil responses across the antigen dose range were enhanced in the absence of serum, and that trend reached statistical significance at lower allergen concentrations (Fig 2; $P < .01$ at 300 pg/mL). A similar trend was observed in the outgrown group (see Fig E4, *A*, in this article's Online Repository at www.jacionline.org), although it did not reach statistical significance. Representative examples of individual allergen dose-response curves in the absence or presence of autologous serum are shown in Fig E5 (available in this article's Online Repository at www.jacionline.org). In contrast, allergic donors' basophils were not more reactive to milk allergen in the absence of autologous serum protein (Fig E4, *A*), suggesting either the specific presence of an inhibitory factor in the serum of HM-tolerant

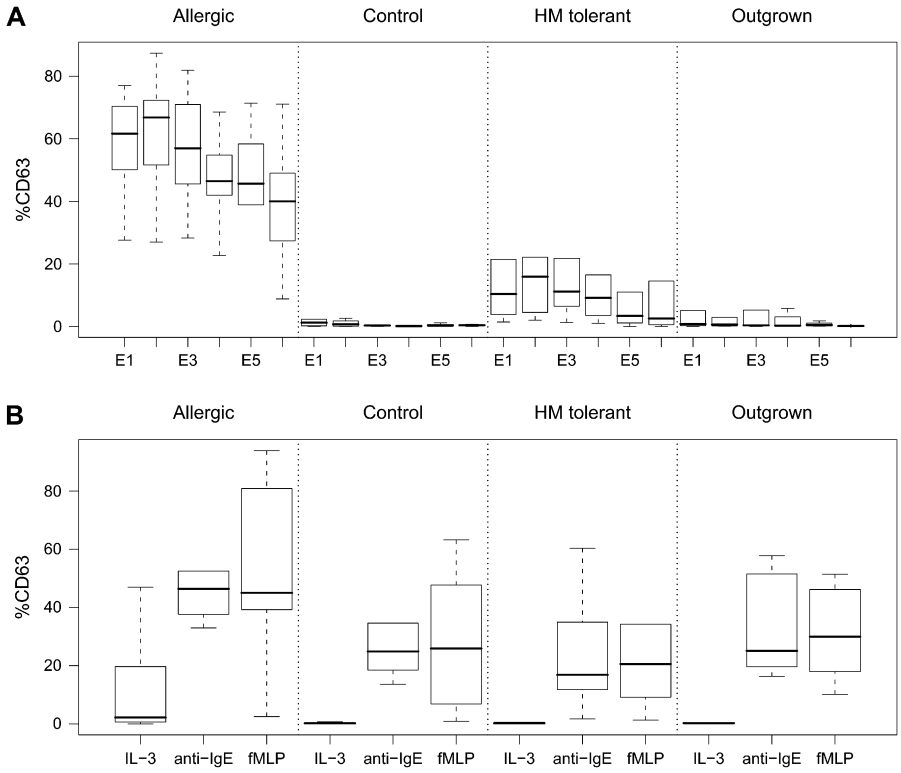

FIG 1. Allergen-specific and nonspecific basophil reactivity by patient group. **A**, Percentage of basophils that are $CD63^{bright}$ cells after 30 minutes' stimulation with 10-fold serial dilutions of milk protein (*E1-E6* corresponds to 3×10^{1} to 3×10^{-4} μg/mL). **B**, Percentage of basophils that are $CD63^{bright}$ cells after 30 minutes' stimulation with IL-3 alone, polyclonal anti-IgE (1 μg/mL), or fMLP (1 μmol/L). The allergic (n = 13), control (n = 13), HM-tolerant (n = 32), and outgrown (n = 10) groups are shown. *Box plots* represent the median, 25th and 75th percentile, and range.

and outgrown groups or specific sensitivity to suppression by a nonspecific serum factor.

There was also enhancement of nonspecific basophil reactivity in the HM-tolerant group in the absence of autologous serum; stimulation with either IL-3 alone ($P < .01$) or anti-IgE ($P < .05$) induced stronger responses in the absence of serum (see Fig E4, *B*). This effect was specific to IL-3 and anti-IgE; there was no enhanced responsiveness measured in the RPMI (not shown) or fMLP stimulation (see Fig E4, *B*).

To specifically address the role of IgG, in a subset of subjects we resuspended washed cells in 10% autologous serum that had been either mock treated (beads alone) or affinity depleted of IgG by using protein A beads. These cells were then stimulated with IL-3 alone, milk antigen (dose E3, 0.1 μg/mL), or anti-IgE. Total serum IgG was depleted by approximately 60% to 80%, as determined by means of SDS-PAGE (data not shown). Basophil responsiveness to milk allergen showed a trend toward enhancement in IgG-depleted serum (n = 6, $P = .078$); however, the effect was nonspecific because both IL-3–induced ($P < .05$) and anti-IgE–induced ($P = .078$) responses also tended to be enhanced (see Fig E6 in this article's Online Repository at www.jacionline.org).

Based on these findings, we hypothesized that polyclonal IgG had a generally inhibitory effect on phosphoinositide 3-kinase (PI3K)–dependent signaling in basophils (see the Discussion section).[12,13] Therefore to further investigate whether there was an allergen-specific or general inhibition factor of basophil activation present in the sera from HM-tolerant individuals, we conducted dilution experiments comparing allergen-induced activation with anti-IgE activation of whole blood samples serially diluted with normal human serum to maintain physiologic concentrations of total IgG. Basophil responses to the same concentration of allergen (3×10^{-1} μg/mL) increased dramatically with increasing dilution of autologous serum, whereas there was no consistent change in the anti-IgE response (Fig 3). However, heterologous serum from HM-tolerant donors was unable to suppress basophil responses of Allergic donors (data not shown).

Active ingestion of milk allergen is associated with basophil hyporesponsiveness. Subjects for this study were recruited cross-sectionally from a larger study.[10] Some HM-tolerant subjects were evaluated at the time of their initial challenge before the introduction of baked milk products into their diet (n = 9), whereas some were evaluated 3 or more months after the inclusion of HM products (n = 23). Subjects in the parent study who ingested baked milk products for at least 3 months had decreased skin test reactivity and milk-specific IgE levels and increased casein-specific IgG4 levels over time.[10]

We compared basophil reactivity within the HM-tolerant subjects with respect to their diet. Basophils from HM-tolerant subjects including milk in their diets were less reactive on *in vitro* stimulation with milk protein, especially at low concentrations (approximately ≤10 ng/mL, Fig 4). There was also a trend toward lower specific IgE levels, higher casein-specific IgG4 levels, and lower skin test reactivity in the subjects ingesting milk, although these differences did not reach statistical significance in this small subset (see Fig E7 in this article's Online Repository at www.jacionline.org). Basophil responses to IL-3 alone and anti-IgE

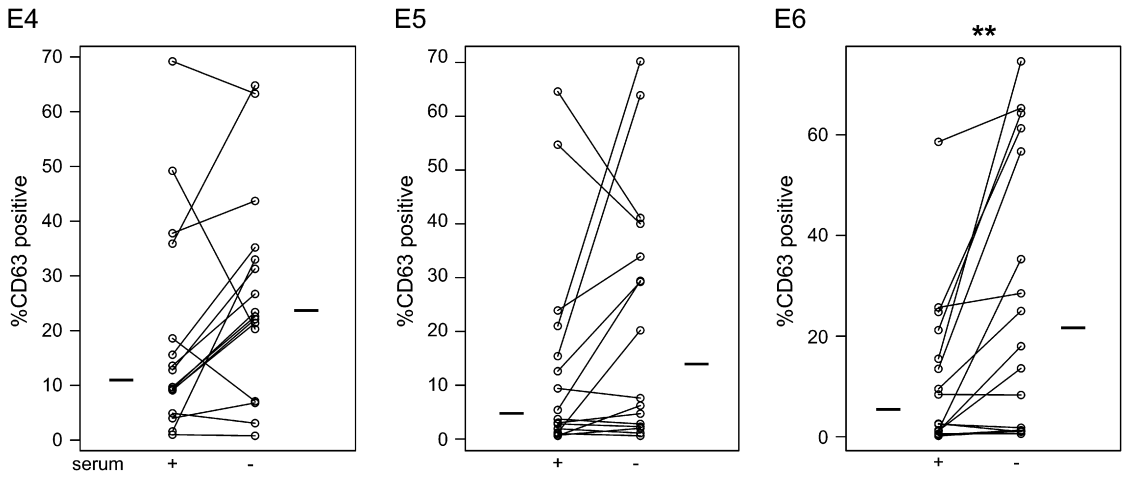

FIG 2. Enhancement of HM-tolerant basophil responses to milk allergen in the absence of autologous serum. *Panels E4* to *E6* represent the percentage of activated basophils ($CD63^{bright}$) after 30 minutes' stimulation with 10-fold serial dilutions of milk protein (E4-E6 correspond to 3×10^{-2} to 3×10^{-4} μg/mL) in the presence *(+)* or absence *(–)* of autologous serum. *Bars* indicate the median. $^{**}P < .01$.

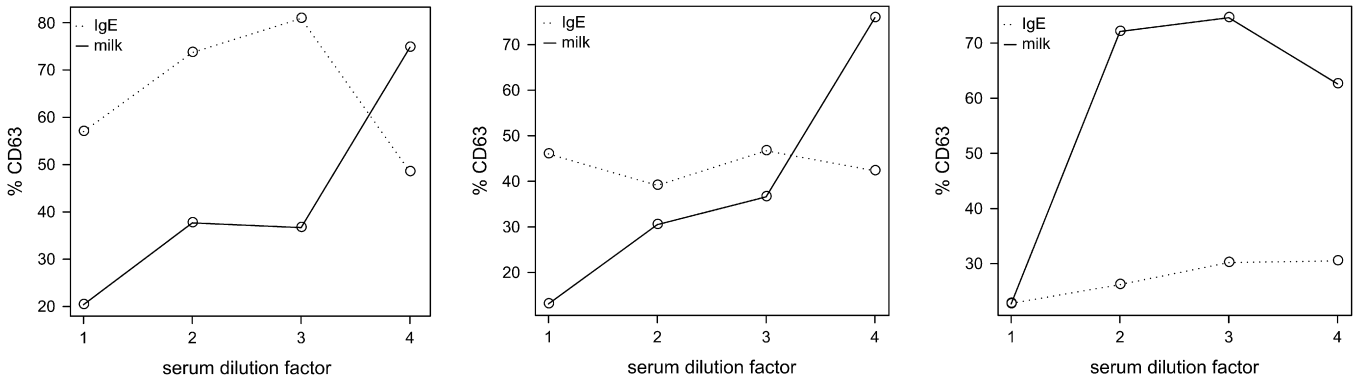

FIG 3. Serum inhibition of allergen-specific basophil activation is dose dependent. Panels show 3 representative individual donors' basophil activations to stimulation with milk allergen (3×10^{-2} μg/mL, *solid line*) over a 4-fold range of autologous serum diluted with normal human AB serum. The *broken line* indicates the basophil activation to polyclonal anti-IgE control.

were lower in children actively ingesting milk, suggesting that there might be a degree of intrinsic basophil suppression caused by chronic allergen exposure (ie, desensitization), whereas fMLP responses were no different between diet groups (see Fig E8 in this article's Online Repository at www.jacionline.org).

DISCUSSION

IgE-mediated milk allergy is naturally outgrown by most affected children.[14-16] The mechanism of this naturally acquired tolerance are unknown, as is its relationship to the normal oral tolerance of individuals who have no history of milk allergy or to tolerance that might be induced by oral or sublingual immunotherapy.

A recent study found that children with milk allergy who are able to tolerate extensively heated (baked) milk might outgrow their allergies sooner and are less clinically sensitive than those who are reactive to heated milk.[10] Here we show that basophils from these individuals, directly stimulated in a whole blood assay, are less reactive to milk allergen, in part because of a serum inhibitory factor.

Allergen-specific IgG has been associated with both natural and immunotherapy-induced tolerance, and we hypothesize that it might be associated with the inhibition observed in these experiments. We hypothesized that allergen-specific IgG should inhibit allergen-specific, but not general, anti-IgE stimulation. Indeed, in serum dilution experiments we were able to demonstrate a preferential effect on basophil activation by allergen compared with anti-IgE (Fig 3).

Allergen specificity is also suggested by the greater effect of serum inhibition at low antigen doses (Fig 2 and see Fig E3), at which we speculate specific IgG might be in excess of antigen. High specific IgG/allergen ratios would favor both competitive inhibition of IgE binding (ie, blocking), as well as formation of antibody-antigen complexes with sufficient avidity to interact with inhibitory FcγRIIb. The observation that HM-tolerant individuals ingesting milk have both lower basophil reactivity and higher specific IgG4 levels appears to support the hypothesis of allergen-specific IgG as the serum inhibitor.

It is interesting to note, however, that allergic donors' basophils tend to be generally more reactive as well (Fig 1, *B*) and that both

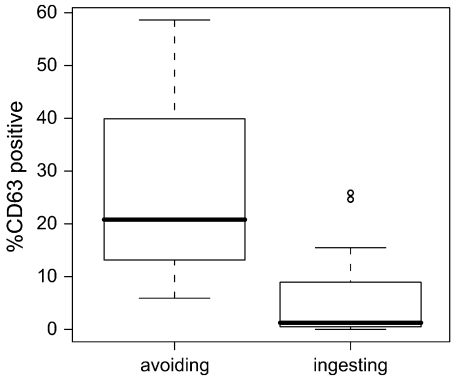

FIG 4. Ingestion of dietary milk antigen is associated with less milk-induced basophil activation. Percentage of activated basophils ($CD63^{bright}$) after 30 minutes' stimulation with milk protein at 3×10^{-4} μg/mL is shown. *Box plots* represent the median, 25th and 75th percentile, and range, and *circles* represent outliers. $P < .01$.

serum depletion and IgG depletion of HM-tolerant samples enhances both the IL-3– and anti-IgE–induced basophil response (see Figs E4, *B*, and E6). We found that serum IgG has a broad inhibitory activity because affinity depletion nonspecifically enhanced basophil responsiveness (see Fig E6). It is well established that specific IgG, by means of coaggregation of FcγRIIb and FcεRI, can potently inhibit basophil activation.[17] In addition, FcγRIIb signaling and activation of the phosphatase, SHIP-1, can inhibit PI3K-dependent pathways, generally even in the absence of direct coligation.[18] Both IL-3 and FcεRI basophil activation signal through PI3K,[19] whereas fMLP does not.[13] Furthermore, monomeric IgG (specifically monomeric Fc IgG fragments) has been demonstrated to have a FcγRIIb-dependent inhibitory function in macrophages.[12] We hypothesize that monomeric IgG is signaling in this way in basophils and accounts for the nonspecific inhibitory activity observed.

Serum dilution experiments with normal human serum to maintain polyclonal IgG concentration did confirm the presence of an allergen-specific inhibition in HM-tolerant individuals (Fig 3). We hypothesized that this is allergen-specific IgG. We were unable, however, to inhibit allergic donors' basophils using pooled heterologous serum from HM-tolerant subjects despite the presence of allergen-specific IgG (not shown). It might be that distinct epitope specificity accounts for the lack of cross-inhibition. We have previously observed significant heterogeneity of epitope recognition even within restricted epitope-rich regions of a small food allergen.[20] If this does account for the lack of heterologous inhibition, this would suggest that competitive blocking, rather than engagement of inhibitory FcγRIIb, predominates in these patients, at least in this *in vitro* assay. Alternatively, HM-tolerant subject's basophils might be more susceptible to the serum inhibitor, irrespective of whether it is specific IgG. Future experiments to affinity deplete allergen-specific IgG may help to clarify this question.

We did find evidence of intrinsic basophil suppression associated with antigen exposure. Allergen and anti-IgE, but not fMLP, responses were all reduced in subjects with exposure to dietary antigen (see Fig E8). We hypothesize that this is the result of antigen-induced desensitization, a state of non–antigen-specific but pathway-specific (ie, FcεRI-mediated) suppression of responsiveness induced by prior stimulation[21-23] that occurs because of *in vivo* antigen exposure. Response to IL-3 alone was also less with dietary antigen exposure (see Fig E8). Activation by IL-3 alone has been documented in a subset of patients (termed hyperreleasable) who are also hyperresponsive to IgE-cross-linking and other secretagogues.[24] We are not aware of evidence that FcεRI-mediated desensitization affects IL-3 responsiveness, and the mechanism of desensitization is unclear. However, downregulation of the protein tyrosine kinase Syk has been implicated,[25] and this is a known to be upregulated by IL-3.

IgE-sensitized basophils are the largest population of allergen-specific leukocytes in peripheral blood, and there is a growing appreciation of the effector role they play, as well as their capacity for modulating adaptive immunity.[3,4,26] Because basophils have a short half-life and the turnover of mast cell–bound IgE is slow, basophil reactivity might correlate more closely with changes in the allergen-specific immune response than mast cell reactivity. Basophil and mast cell reactivity are both suppressed by dietary antigen, which correlates with higher specific IgG4 levels (see Fig E7). However, basophils might be disproportionately affected by intrinsic antigen-induced desensitization because of their normal compartmentalization in the peripheral blood, where they are likely to encounter higher levels of antigen than skin mast cells after ingestion. It is not known whether or to what degree basophils participate in the *in vivo* effector response to food allergens, but it has been found that patients with food allergy with chronic exposure to the offending antigen have increased spontaneous histamine release and increased constitutive expression of basophil activation markers.[7,27] Although increased plasma histamine levels are commonly seen during food-induced anaphylactic reactions, tryptase levels are rarely found to be increased, suggesting active participation by non–mast cell, histamine-secreting cells, such as the basophil.[28]

We have shown here that it is possible to monitor allergen-specific basophil responses from pediatric subjects with the use of flow cytometry to identify and phenotype basophils from stimulated, unfractionated peripheral blood cells. Basophil reactivity in this patient population was strikingly distinct between HM-tolerant and HM-reactive subjects, raising the possibility that this assay might be clinically useful to discriminate between these groups. Because reactivity to HM has been associated with a higher risk of reactions requiring epinephrine,[10] basophil reactivity might also be a useful prognostic test for identifying individuals with greater clinical sensitivity. We are now using this assay as a research tool to assess both naturally occurring and immunotherapy-induced changes; however, controlled food challenges remain the gold standard for determining clinical reactivity.

Clinical implications: Assessment of allergen-specific basophil activation might be a useful research tool for identifying patients who will tolerate HM products and for monitoring the acquisition of immune tolerance in patients with a history of milk allergy.

REFERENCES

1. Devouassoux G, Foster B, Scott LM, Metcalfe DD, Prussin C. Frequency and characterization of antigen-specific IL-4- and IL-13- producing basophils and T cells in peripheral blood of healthy and asthmatic subjects. J Allergy Clin Immunol 1999; 104:811-9.
2. Lim LHK, Burdick MM, Hudson SA, Mustafa FB, Konstantopoulos K, Bochner BS. Stimulation of human endothelium with IL-3 induces selective basophil accumulation in vitro. J Immunol 2006;176:5346-53.
3. Mack M, Schneider MA, Moll C, Cihak J, Brühl H, Ellwart JW, et al. Identification of antigen-capturing cells as basophils. J Immunol 2005;174:735-41.

4. Sokol C, Barton G, Farr A, Medzhitov R. A mechanism for the initiation of allergen-induced T helper type 2 responses. Nat Immunol 2008;9:310-8.
5. Denzel A, Maus UA, Gomez MR, Moll C, Niedermeier M, Winter C, et al. Basophils enhance immunological memory responses. Nat Immunol 2008;9: 733-42.
6. Ebo DG, Hagendorens MM, Schuerwegh AJ, Beirens LMN, Bridts CH, Clerck LSD, et al. Flow-assisted quantification of in vitro activated basophils in the diagnosis of wasp venom allergy and follow-up of wasp venom immunotherapy. Cytometry 2007;72B:196-203.
7. Shreffler WG. Evaluation of basophil activation in food allergy: present and future applications. Curr Opin Allergy Clinical Immunol 2006;6:226-33.
8. de Weck AL, Sanz ML, Gamboa PM, Aberer W, Bienvenu J, Blanca M, et al. Diagnostic tests based on human basophils: more potentials and perspectives than pitfalls. Int Arch Allergy Immunol 2008;146:177-89.
9. Ocmant A, Peignois Y, Mulier S, Hanssens L, Michils A, Schandené L. Flow cytometry for basophil activation markers: the measurement of CD203c up-regulation is as reliable as CD63 expression in the diagnosis of cat allergy. J Immunol Methods 2007;320:40-8.
10. Nowak-Wegrzyn A, Bloom KA, Sicherer SH, Shreffler WG, Noone S, Wanich N, et al. Tolerance to extensively heated milk in children with cow's milk allergy. J Allergy Clin Immunol 2008;122:342-7.
11. Lichtenstein LM, MacGlashan DW. The concept of basophil releasability. J Allergy Clin Immunol 1986;77:291-4.
12. Samuelsson A, Towers TL, Ravetch JV. Anti-inflammatory activity of IVIG mediated through the inhibitory Fc receptor. Science 2001;291:484-6.
13. Knol EF, Koenderman L, Mul FP, Verhoeven AJ, Roos D. Differential activation of human basophils by anti-IgE and formyl-methionyl-leucyl-phenylalanine. Indications for protein kinase C-dependent and -independent activation pathways. Eur J Immunol 1991;21:881-5.
14. Høst A. Primary and secondary dietary prevention. Pediatric allergy and immunology: official publication of the European Society of Pediatric Allergy and Immunology. Pediatr Allergy Immunol 2001;12(suppl 14):78-84.
15. Skripak JM, Matsui EC, Mudd K, Wood RA. The natural history of IgE-mediated cow's milk allergy. J Allergy Clin Immunol 2007;120:1172-7.
16. Wood RA. The natural history of food allergy. Pediatrics 2003;111(suppl):1631-7.
17. Kepley CL, Cambier JC, Morel PA, Lujan D, Ortega E, Wilson BS, et al. Negative regulation of FcepsilonRI signaling by FcgammaRII costimulation in human blood basophils. J Allergy Clin Immunol 2000;106:337-48.
18. Brauweiler AM, Cambier JC. Fc gamma RIIB activation leads to inhibition of signalling by independently ligated receptors. Biochem Soc Trans 2003;31: 281-5.
19. Hauswirth AW, Sonneck K, Florian S, Krauth MT, Bohm A, Sperr WR, et al. Interleukin-3 promotes the expression of E-NPP3/CD203C on human blood basophils in healthy subjects and in patients with birch pollen allergy. Int J Immunopathol Pharmacol 2007;20:267-78.
20. Shreffler WG, Lencer DA, Bardina L, Sampson HA. IgE and IgG4 epitope mapping by microarray immunoassay reveals the diversity of immune response to the peanut allergen, Ara h 2. J Allergy Clin Immunol 2005;116:893-9.
21. Macglashan D, Miura K. Loss of syk kinase during IgE-mediated stimulation of human basophils. J Allergy Clin Immunol 2004;114:1317-24.
22. MacGlashan DW, Lichtenstein LM. The transition from specific to nonspecific desensitization in human basophils. J Immunol 1981;127:2410-4.
23. Macglashan D, Vilariño N. Nonspecific desensitization, functional memory, and the characteristics of SHIP phosphorylation following IgE-mediated stimulation of human basophils. J Immunol 2006;177:1040-51.
24. MacDonald SM, Vonakis BM. Association of the Src homology 2 domain-containing inositol 5′ phosphatase (SHIP) to releasability in human basophils. Mol Immunol 2002;38:1323-7.
25. Kepley CL. Antigen-induced reduction in mast cell and basophil functional responses due to reduced Syk protein levels. Int Arch Allergy Immunol 2005; 138:29-39.
26. Denzel A, Maus UA, Gomez MR, Moll C, Niedermeier M, Winter C, et al. Basophils enhance immunological memory responses. Nat Immunol 2008;9: 733-42.
27. Sampson HA, Broadbent KR, Bernhisel-Broadbent J. Spontaneous release of histamine from basophils and histamine-releasing factor in patients with atopic dermatitis and food hypersensitivity. N Engl J Med 1989;321:228-32.
28. Sampson HA. Food anaphylaxis. Br Med Bull 2000;56:925-35.

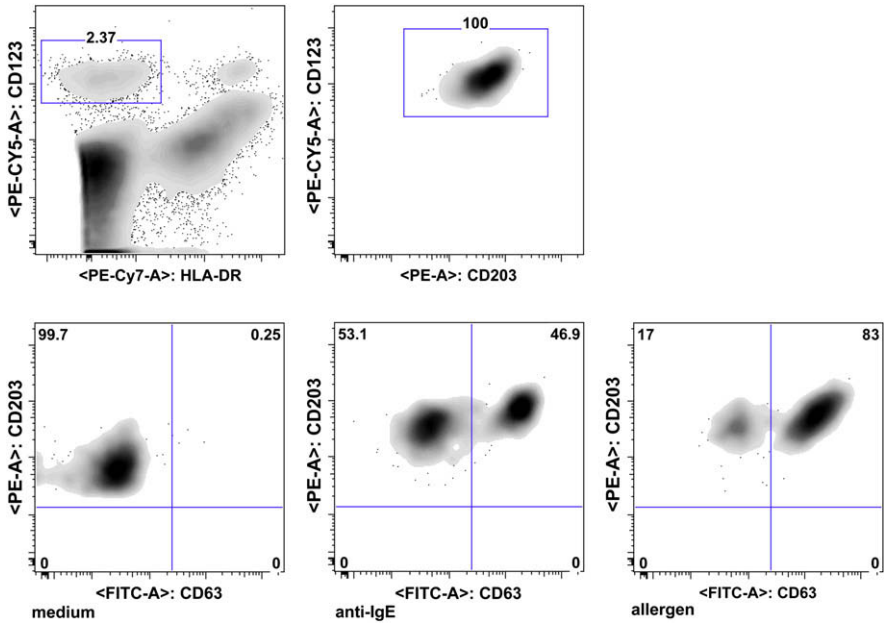

FIG E1. Gating strategy and representative data for the basophil activation assay. Basophils were activated in whole blood for 30 minutes and identified by means of flow cytometry as $CD123^{+}HLA\text{-}DR^{-}$ *(top left)*, lineage-negative (not shown), and $CD203c^{+}$ *(top right)* cells. Basophil activation was assessed based on expression of CD63 and reported as percentage of $CD63^{bright}$ cells because its expression after activation was bimodal. Shown is expression of CD203c and CD63 in medium alone *(bottom left)*, anti-IgE *(bottom middle)*, and milk allergen *(bottom right)*. *PE*, Phycoerythrin; *CY5*, cyanin 5; *Cy7*, cyanin 7; *FITC*, fluorescein isothiocyanate.

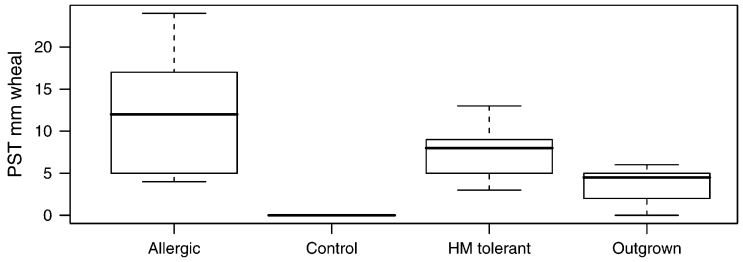

FIG E2. Skin prick test *(PST)* reactivity (in millimeters of wheal response) to milk allergen by patient group. Allergic subjects (n = 13) were clinically reactive to HM. Nonallergic control subjects (n = 13) had no history of milk allergy. HM-tolerant subjects (n = 32) were not clinically reactive to HM. Outgrown subjects (n = 10) were fully tolerant at baseline but had a confirmed history of milk allergy.

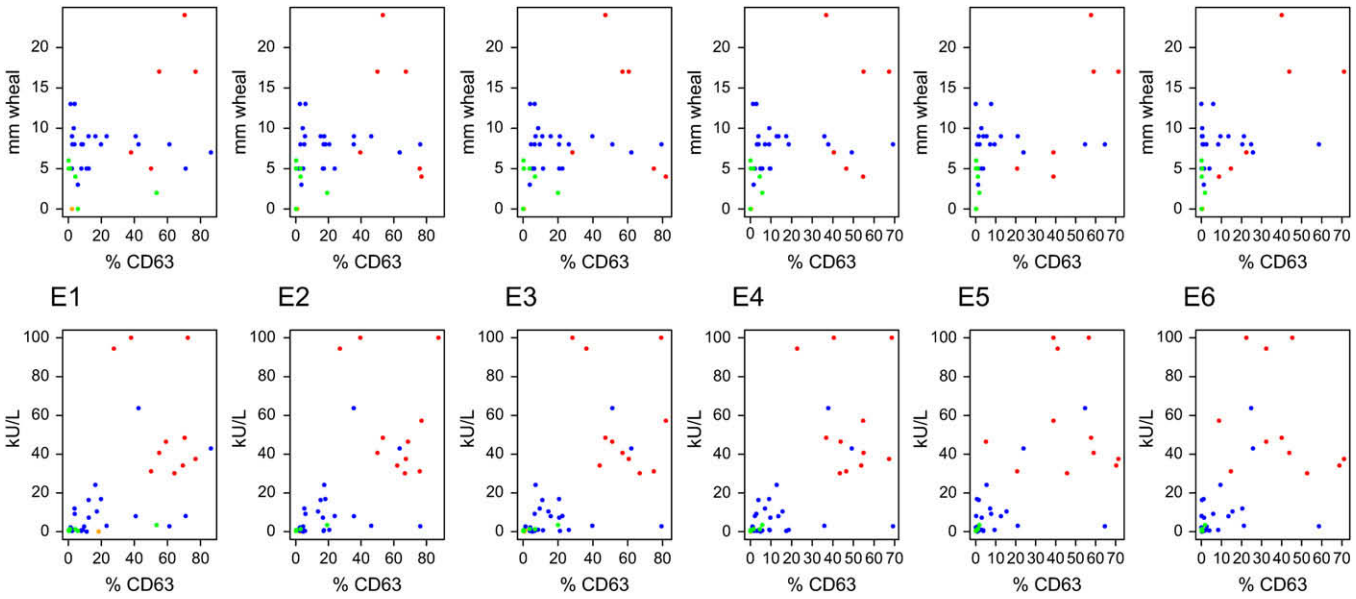

FIG E3. Relationship of basophil activation to skin test responses and specific IgE levels. Basophil response is plotted for each allergen dose *(E1-E6)* as a percentage of $CD63^{bright}$ cells against skin test wheal responses to milk extract in millimeters *(top row)* or milk-specific serum IgE levels (in kilounits per liter, *bottom row*). Allergic subjects are plotted in *red*, HM-tolerant subjects in *blue*, outgrown subjects in *green*, and control subjects in *yellow*.

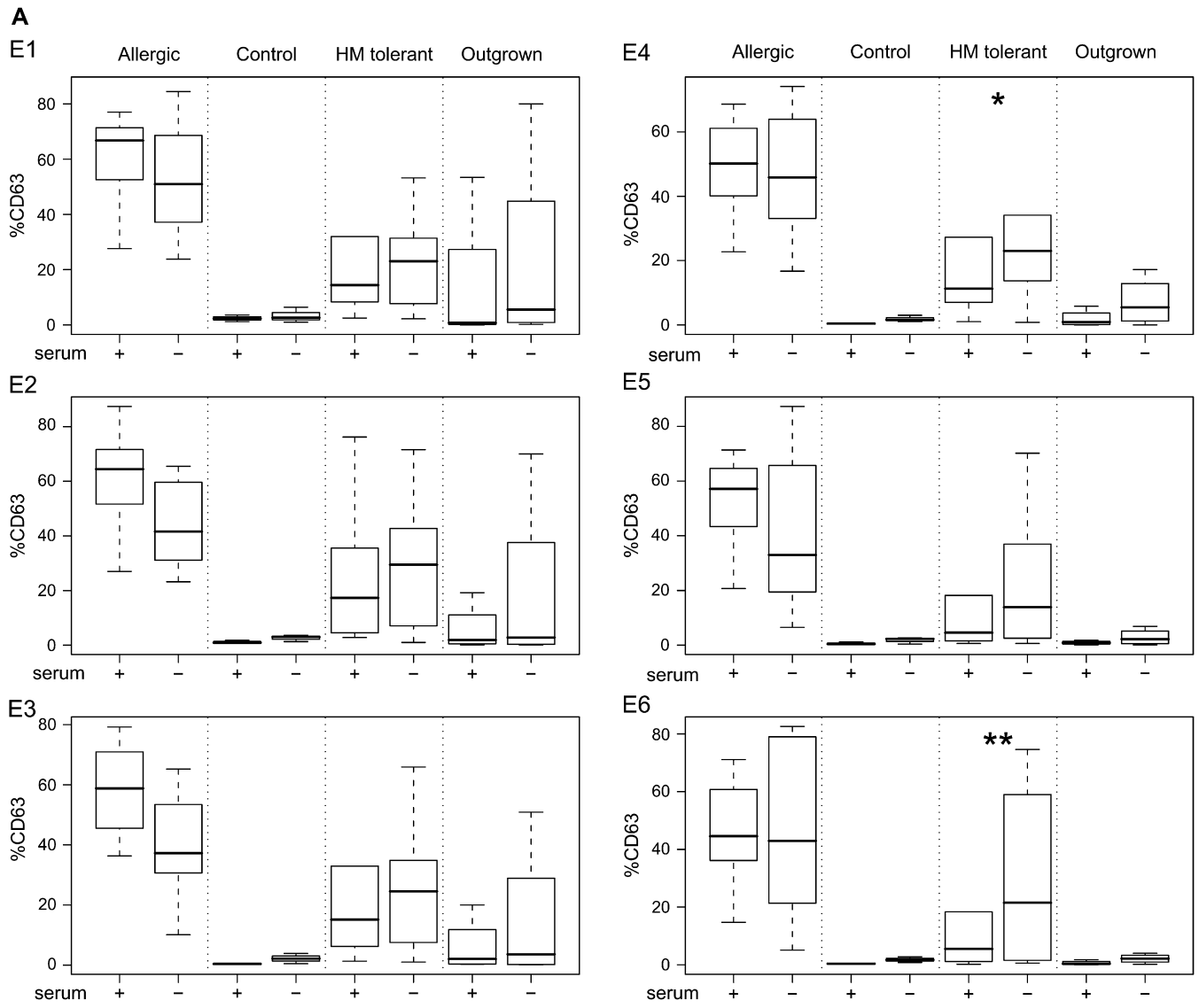

FIG E4. Control basophil activation with or without autologous serum. Distribution of percentage $CD63^{bright}$ cells by patient group and serum condition (with, +; without, –) after stimulation for 30 minutes with **A**, serial dilutions of milk antigen or **B**, IL-3 alone, anti-IgE, or fMLP is shown. Stimulation with either IL-3 alone ($P = .002466$) or anti-IgE ($P = .03186$) induced stronger responses in the absence of serum. This effect was specific to IL-3 and anti-IgE; there was no enhanced responsiveness measured in the RPMI (not shown) or fMLP stimulations. *Box plots* represent the median, 25th and 75th percentile, and range, and *circles* represent outliers.

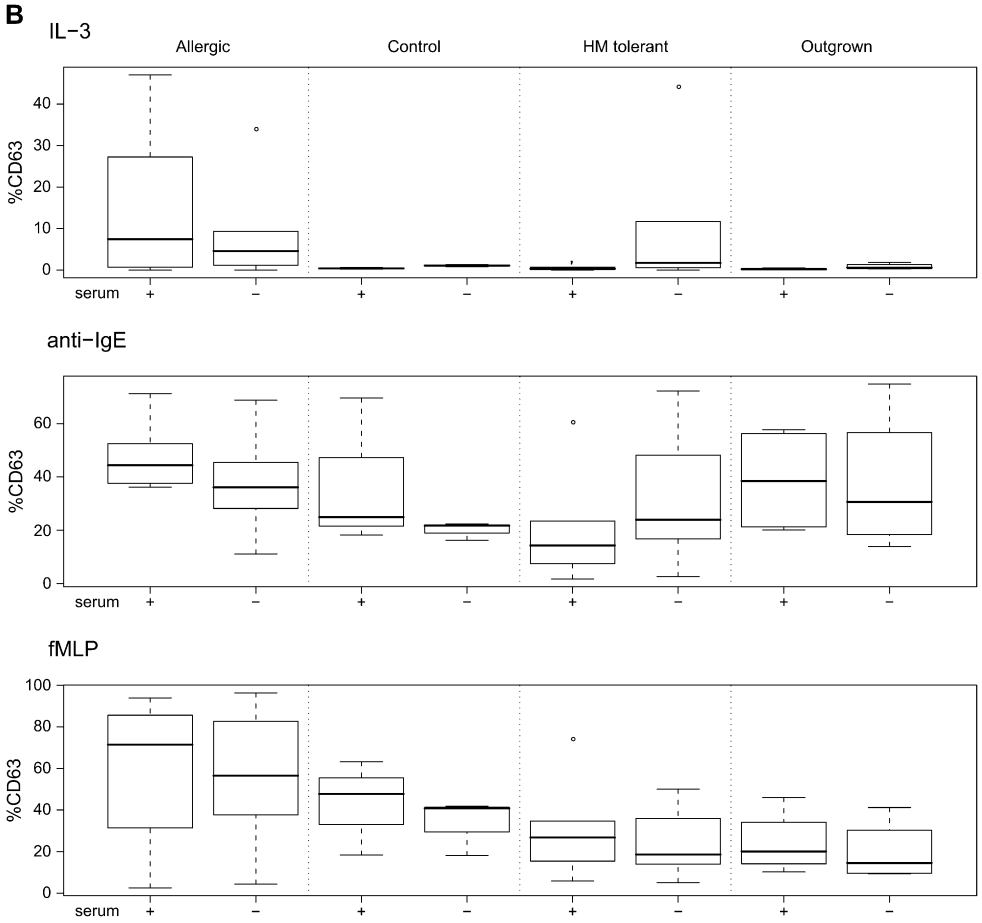

FIG E4. *(Continued)*

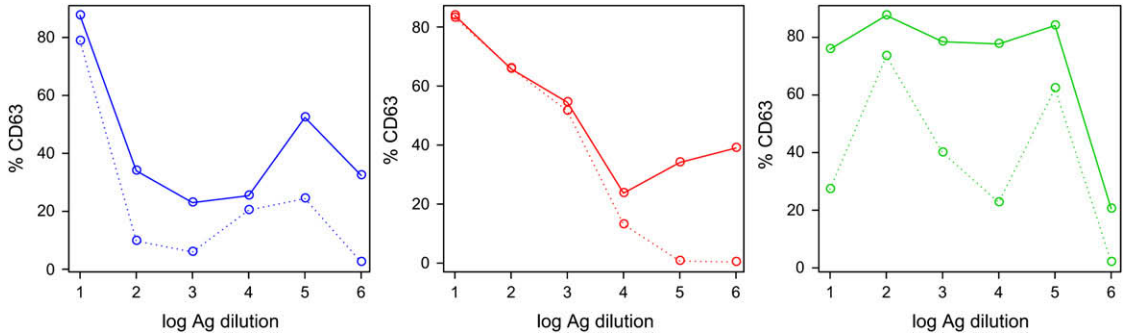

FIG E5. Basophil activation dose response. The percentage of $CD63^{bright}$ cells in response to the range of milk allergen dilutions (doses E1-E6, approximately 10^{1} to 10^{-4} μg/mL) in the presence *(solid line)* or absence *(broken line)* of autologous serum is shown.

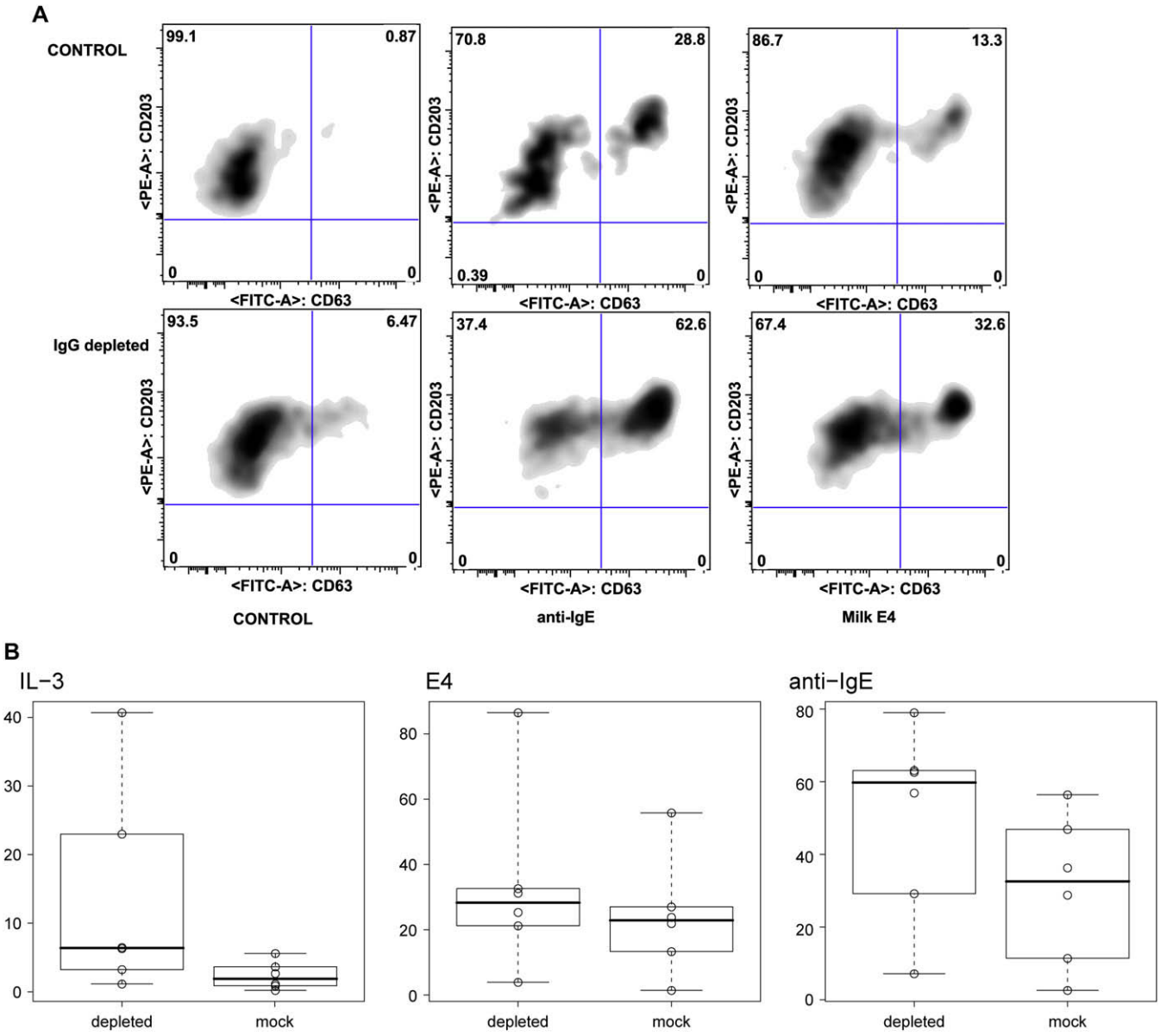

FIG E6. Polyclonal IgG inhibits basophil reactivity to IL-3 and anti-IgE. **A**, Representative example of enhanced basophil activation in serum free conditions. Density plots showing CD63 and CD203c expression on $CD123^{+}HLA\text{-}DR^{-}$ lineage-negative cells after stimulation in the presence (*top row*, control) or absence (*bottom row*, IgG depleted) of autologous serum. **B**, Summary data from 6 experiments using different HM-tolerant subjects. $P < .05$ for difference in the IL-3 response between cells stimulated in serum depleted or undepleted (mock) of IgG. *PE*, Phycoerythrin; *FITC*, fluorescein isothiocyanate.

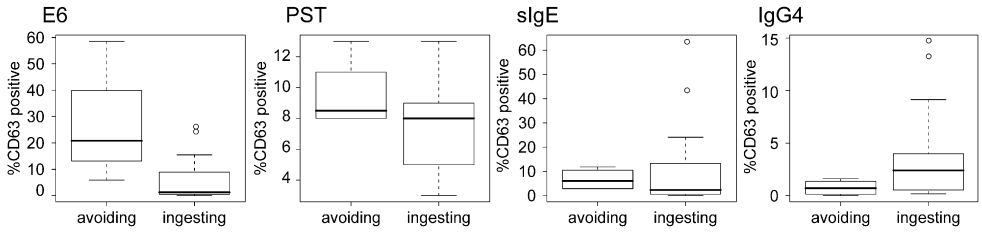

FIG E7. Relationship of diet to allergen-specific immune response. Among HM-tolerant subjects, 9 were strictly avoiding milk, and 23 had incorporated milk into the diet for 3 months or more. *Box plots* represent the median, 25th and 75th percentile, and range, and *circles* represent outliers for basophil activation at the lowers allergen concentration (dose E6, approximately 10^{-4} μg/mL), milk allergen skin test wheal *(PST)*, milk-specific serum IgE level *(sIgE)*, and milk-specific IgG4 level *(IgG4)*.

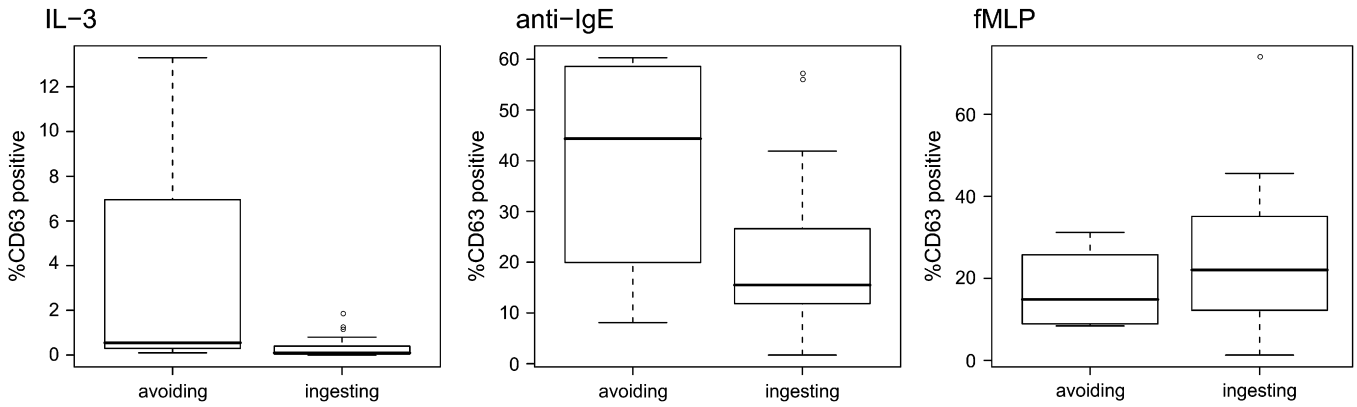

FIG E8. Relationship of diet to nonspecific basophil reactivity. Among HM-tolerant subjects, 9 were strictly avoiding milk, and 23 had incorporated milk into the diet for 3 months or more. *Box plots* represent the median, 25th and 75th percentile, and range, and *circles* represent outliers for basophil activation (percentage $CD63^{bright}$ cells) caused by IL-3 alone, anti-IgE, or fMLP.

```
# Copyright 2008, Wayne G. Shreffler. Free non-comercial use is welcome.
# This is a R analysis script for analysis of data set produced for the
manuscript, "Allergen-Specific Basophil Activation Associated with Clinical
Tolerance in Patients with Milk Allergy."


#R is by the R Foundation for Statistical Computing and is freely available
at http://www.R-project.org. R Development Core Team (2006). R: A language
and environment for statistical computing. R Foundation for Statistical
Computing, Vienna, Austria. ISBN 3-900051-07-0, URL.

# THIS WILL RUN WITHOUT MODIFICATION ON OS X but will require editing of
pipe functions and substitution of quartz functions (e.g. with 'pdf') for
use on Windows OS.
# Direct questions or suggestions about this script to the author at
wayne.shreffler@mssm.edu

current_dir <- readLines(pipe("pwd"))
download.file("http://www.iisinai.org/shreffler/milk_baso_data.zip",
"milk_baso_data.zip")
pipe("unzip 'milk_baso_data.zip'", "w")

baso <- read.csv(paste(current_dir, "/data/data_sum.csv", sep=""),
header=TRUE)

# create table for comparison of groups accross stimulants using ech
visitID as unique -- note some duplicate patients at multiple time points

# use only wash_status 'no' data
baso_washless <- subset(baso, wash_status == "n")
# exclude non-responders
baso_washless <- subset(baso_washless, Responder==1)

tmp <- data.frame(ID=baso_washless$visit_ID, group=baso_washless$STATUS,
stim=c(array("E1", length(baso_washless$E1)), array("E2",
length(baso_washless$E2)),array("E3", length(baso_washless$E3)),
array("E4", length(baso_washless$E4)), array("E5", length(baso_washless
$E5)), array("E6", length(baso_washless$E6))), CD63=c(baso_washless$E1,
baso_washless$E2, baso_washless$E3, baso_washless$E4, baso_washless$E5,
baso_washless$E6), HMCD63=c(baso_washless$HM_E1, baso_washless$HM_E2,
baso_washless$HM_E3, baso_washless$HM_E4, baso_washless$HM_E5,
baso_washless$HM_E6))

# define factors
groupf <- tmp$group
stimf <- tmp$stim
```

```
agg <- by(tmp$CD63, list(stimf, groupf), fivenum)

# plot figure 1A
quartz(,10,10);layout(matrix(c(1,2),2,1))
boxplot(agg, ylab="%CD63", cex.lab=1.4, names=array(c("E1", "E2", "E3",
"E4", "E5", "E6"),24), outline=FALSE); abline(v=c(6.5, 12.5, 18.5), lty=3)
mtext("Allergic", 3, line=1, at=3.5)
mtext("Control", 3, line=1, at=9.5)
mtext("HM tolerant", 3, line=1, at=15.5)
mtext("Outgrown", 3, line=1, at=21.5)
mtext("A", cex=4, at=-1)

# anova for HM tolerant versus Allergic
attach(subset(tmp, group == "HM tolerant" | group == "Allergic"))
model <- lm(CD63 ~ group + stim)
anova(model)
detach(subset(tmp, group == "HM tolerant" | group == "Allergic"))
# anova for HM tolerant versus Control
attach(subset(tmp, group == "HM tolerant" | group == "Control"))
model <- lm(CD63 ~ group + stim)
anova(model)
detach(subset(tmp, group == "HM tolerant" | group == "Control"))
# anova for HM tolerant versus Outgrown
attach(subset(tmp, group == "HM tolerant" | group == "Outgrown"))
model <- lm(CD63 ~ group + stim)
anova(model)
detach(subset(tmp, group == "HM tolerant" | group == "Outgrown"))


# analysis for anti-IgE, fMLP, and IL-3
tmp2 <- data.frame(ID=baso_washless$visit_ID, group=baso_washless$STATUS,
stim=c(array("anti_IgE", length(baso_washless$anti_IgE)), array("fMLP",
length(baso_washless$fMLP)), array("IL3", length(baso_washless$IL3))),
CD63=c(baso_washless$anti_IgE, baso_washless$fMLP, baso_washless$IL3))

groupf <- tmp2$group
stimf <- tmp2$stim
agg <- by(tmp2$CD63, list(stimf, groupf), fivenum)

# plot figure 1B
boxplot(agg, ylab="%CD63", cex.lab=1.4, names=array(c("IL-3", "anti-IgE",
"fMLP"),12), outline=FALSE); abline(v=c(3.5, 6.5, 9.5), lty=3)
mtext("Allergic", 3, line=1, at=2)
mtext("Control", 3, line=1, at=5)
mtext("HM tolerant", 3, line=1, at=8)
mtext("Outgrown", 3, line=1, at=11)
```

```
mtext("B", cex=4, at=-0.5)

# rank sum test for differences between HM tolerant and Allergic responses
to controls
wilcox.test(subset(tmp2, stim == "fMLP" & group == "HM tolerant")$CD63,
subset(tmp2, stim == "fMLP" & group == "Allergic")$CD63)
wilcox.test(subset(tmp2, stim == "anti_IgE" & group == "HM tolerant")$CD63,
subset(tmp2, stim == "anti_IgE" & group == "Allergic")$CD63)
wilcox.test(subset(tmp2, stim == "IL3" & group == "HM tolerant")$CD63,
subset(tmp2, stim == "IL3" & group == "Allergic")$CD63)

# analysis for washing effect using subset of data with pre and post wash
data available
baso_wash <- subset(baso, wash_done == "y")
# exclude non-responders
baso_wash <- subset(baso_wash, Responder==1)
groupf <- factor(baso_wash$STATUS)
washf <- factor(baso_wash$wash_status)
by(baso_wash$E4, list(washf,groupf), length)
agg1 <- by(baso_wash$E1, list(washf,groupf), fivenum); agg2 <- by(baso_wash
$E2, list(washf,groupf), fivenum); agg3 <- by(baso_wash$E3,
list(washf,groupf), fivenum); agg4 <- by(baso_wash$E4, list(washf,groupf),
fivenum); agg5 <- by(baso_wash$E5, list(washf,groupf), fivenum); agg6 <-
by(baso_wash$E6, list(washf,groupf), fivenum)

wash_tmp <- subset(baso_wash, STATUS == "HM tolerant")
wilcox.test(subset(wash_tmp, wash_status == "y")$E6, subset(wash_tmp,
wash_status == "n")$E6, paired=TRUE, alternative = "greater")
wilcox.test(subset(wash_tmp, wash_status == "y")$E5, subset(wash_tmp,
wash_status == "n")$E5, paired=TRUE, alternative = "greater")
wilcox.test(subset(wash_tmp, wash_status == "y")$E4, subset(wash_tmp,
wash_status == "n")$E4, paired=TRUE, alternative = "greater")

# figure 2
quartz(,10,10); layout(matrix(c(1,2,3,4,5,6),3,2))
boxplot(agg1, ylab="%CD63", cex.axis=1.5, cex.lab=1.2, names=array(c("+",
"-"),8), outline=FALSE); abline(v=c(2.5, 4.5, 6.5), lty=3); mtext("E1",
3,0.5,cex=3,at=-.2); mtext("serum", 1 ,0.75, at=0);
mtext(c("Allergic","Control","HM tolerant","Outgrown"),
3,1,at=c(1.5,3.5,5.5,7.5))
boxplot(agg2, ylab="%CD63", cex.axis=1.5, cex.lab=1.2, names=array(c("+",
"-"),8), outline=FALSE); abline(v=c(2.5, 4.5, 6.5), lty=3); mtext("E2",
3,0.5,cex=3,at=-.2); mtext("serum", 1 ,0.75, at=0)
boxplot(agg3, ylab="%CD63", cex.axis=1.5, cex.lab=1.2, names=array(c("+",
"-"),8), outline=FALSE); abline(v=c(2.5, 4.5, 6.5), lty=3); mtext("E3",
3,0.5,cex=3,at=-.2); mtext("serum", 1 ,0.75, at=0)
```

```
boxplot(agg4, ylab="%CD63", cex.axis=1.5, cex.lab=1.2, names=array(c("+",
"-"),8), outline=FALSE); abline(v=c(2.5, 4.5, 6.5), lty=3); mtext("E4",
3,0.5,cex=3,at=-.2); mtext("serum", 1 ,0.75, at=0)
mtext("*",3,-3,at=5.5,cex=2); mtext(c("Allergic","Control","HM
tolerant","Outgrown"),3,1,at=c(1.5,3.5,5.5,7.5))
boxplot(agg5, ylab="%CD63", cex.axis=1.5, cex.lab=1.2, names=array(c("+",
"-"),8), outline=FALSE); abline(v=c(2.5, 4.5, 6.5), lty=3); mtext("E5",
3,0.5,cex=3,at=-.2); mtext("serum", 1 ,0.75, at=0)
boxplot(agg6, ylab="%CD63", cex.axis=1.5, cex.lab=1.2, names=array(c("+",
"-"),8), outline=FALSE); abline(v=c(2.5, 4.5, 6.5), lty=3); mtext("E6",
3,0.5,cex=3,at=-.2); mtext("serum", 1 ,0.75, at=0)
mtext("**",3,-3,at=5.5,cex=2)

# wash effect on controls; figure E5
agg7 <- by(baso_wash$IL3, list(washf,groupf), fivenum); agg8 <-
by(baso_wash$anti_IgE, list(washf,groupf), fivenum); agg9 <- by(baso_wash
$fMLP, list(washf,groupf), fivenum)
quartz(,10,10); layout(matrix(c(1,2,3),3,1))
boxplot(agg7, ylab="%CD63", cex.axis=1.5, cex.lab=1.2, names=array(c("+",
"-"),8)); abline(v=c(2.5, 4.5, 6.5), lty=3); mtext("IL-3",
3,2,cex=2,at=0.1); mtext("serum", 1 ,0.75, at=0.5);
mtext(c("Allergic","Control","HM tolerant","Outgrown"),
3,1,at=c(1.5,3.5,5.5,7.5))
boxplot(agg8, ylab="%CD63", cex.axis=1.5, cex.lab=1.2, names=array(c("+",
"-"),8)); abline(v=c(2.5, 4.5, 6.5), lty=3); mtext("anti-IgE",
3,2,cex=2,at=0.4); mtext("serum", 1 ,0.75, at=0.5)
boxplot(agg9, ylab="%CD63", cex.axis=1.5, cex.lab=1.2, names=array(c("+",
"-"),8)); abline(v=c(2.5, 4.5, 6.5), lty=3); mtext("fMLP",
3,2,cex=2,at=0.2); mtext("serum", 1 ,0.75, at=0.5)


wilcox.test(subset(wash_tmp, wash_status == "y")$IL3, subset(wash_tmp,
wash_status == "n")$IL3, paired=TRUE, alternative = "greater")
wilcox.test(subset(wash_tmp, wash_status == "y")$anti_IgE, subset(wash_tmp,
wash_status == "n")$anti_IgE, paired=TRUE, alternative = "greater")
wilcox.test(subset(wash_tmp, wash_status == "y")$fMLP, subset(wash_tmp,
wash_status == "n")$fMLP, paired=TRUE, alternative = "greater")



# some additional data exploration comparing BAT to sIgE and PST; figure E3
quartz(,15,7); layout(matrix(c(1,2,3,4,5,6,7,8,9,10,11,12),2,6,
byrow=TRUE))
plot(baso_washless$E1,baso_washless$PST,type="n", ylab="mm wheal", xlab="%
CD63", cex=1.5)
points(subset(baso_washless, STATUS=="Allergic")$E1,subset(baso_washless,
```

```
STATUS=="Allergic")$PST, col="red", pch=19)
points(subset(baso_washless, STATUS=="Control")$E1,subset(baso_washless,
STATUS=="Control")$PST, col="orange", pch=19)
points(subset(baso_washless, STATUS=="HM tolerant")
$E1,subset(baso_washless, STATUS=="HM tolerant")$PST, col="blue", pch=19)
points(subset(baso_washless, STATUS=="Outgrown")$E1,subset(baso_washless,
STATUS=="Outgrown")$PST, col="green", pch=19)
mtext("E1", side=1, at=0, cex=2, line =6)

plot(baso_washless$E2,baso_washless$PST,type="n", ylab="mm wheal", xlab="%
CD63", cex=1.5)
points(subset(baso_washless, STATUS=="Allergic")$E2,subset(baso_washless,
STATUS=="Allergic")$PST, col="red", pch=19)
points(subset(baso_washless, STATUS=="Control")$E2,subset(baso_washless,
STATUS=="Control")$PST, col="orange", pch=19)
points(subset(baso_washless, STATUS=="HM tolerant")
$E2,subset(baso_washless, STATUS=="HM tolerant")$PST, col="blue", pch=19)
points(subset(baso_washless, STATUS=="Outgrown")$E2,subset(baso_washless,
STATUS=="Outgrown")$PST, col="green", pch=19)
mtext("E2", side=1, at=0, cex=2, line =6)

plot(baso_washless$E3,baso_washless$PST,type="n", ylab="mm wheal", xlab="%
CD63", cex=1.5)
points(subset(baso_washless, STATUS=="Allergic")$E3,subset(baso_washless,
STATUS=="Allergic")$PST, col="red", pch=19)
points(subset(baso_washless, STATUS=="Control")$E3,subset(baso_washless,
STATUS=="Control")$PST, col="orange", pch=19)
points(subset(baso_washless, STATUS=="HM tolerant")
$E3,subset(baso_washless, STATUS=="HM tolerant")$PST, col="blue", pch=19)
points(subset(baso_washless, STATUS=="Outgrown")$E3,subset(baso_washless,
STATUS=="Outgrown")$PST, col="green", pch=19)
mtext("E3", side=1, at=0, cex=2, line =6)

plot(baso_washless$E4,baso_washless$PST,type="n", ylab="mm wheal", xlab="%
CD63", cex=1.5)
points(subset(baso_washless, STATUS=="Allergic")$E4,subset(baso_washless,
STATUS=="Allergic")$PST, col="red", pch=19)
points(subset(baso_washless, STATUS=="Control")$E4,subset(baso_washless,
STATUS=="Control")$PST, col="orange", pch=19)
points(subset(baso_washless, STATUS=="HM tolerant")
$E4,subset(baso_washless, STATUS=="HM tolerant")$PST, col="blue", pch=19)
points(subset(baso_washless, STATUS=="Outgrown")$E4,subset(baso_washless,
STATUS=="Outgrown")$PST, col="green", pch=19)
mtext("E4", side=1, at=0, cex=2, line =6)

plot(baso_washless$E5,baso_washless$PST,type="n", ylab="mm wheal", xlab="%
```

```
CD63", cex=1.5)
points(subset(baso_washless, STATUS=="Allergic")$E5,subset(baso_washless,
STATUS=="Allergic")$PST, col="red", pch=19)
points(subset(baso_washless, STATUS=="Control")$E5,subset(baso_washless,
STATUS=="Control")$PST, col="orange", pch=19)
points(subset(baso_washless, STATUS=="HM tolerant")
$E5,subset(baso_washless, STATUS=="HM tolerant")$PST, col="blue", pch=19)
points(subset(baso_washless, STATUS=="Outgrown")$E5,subset(baso_washless,
STATUS=="Outgrown")$PST, col="green", pch=19)
mtext("E5", side=1, at=0, cex=2, line =6)

plot(baso_washless$E6,baso_washless$PST,type="n", ylab="mm wheal", xlab="%
CD63", cex=1.5)
points(subset(baso_washless, STATUS=="Allergic")$E6,subset(baso_washless,
STATUS=="Allergic")$PST, col="red", pch=19)
points(subset(baso_washless, STATUS=="Control")$E6,subset(baso_washless,
STATUS=="Control")$PST, col="orange", pch=19)
points(subset(baso_washless, STATUS=="HM tolerant")
$E6,subset(baso_washless, STATUS=="HM tolerant")$PST, col="blue", pch=19)
points(subset(baso_washless, STATUS=="Outgrown")$E6,subset(baso_washless,
STATUS=="Outgrown")$PST, col="green", pch=19)
mtext("E6", side=1, at=0, cex=2, line =6)

plot(baso_washless$E1,baso_washless$sIgE,type="n", ylab="kU/L", xlab="%
CD63", cex=1.5)
points(subset(baso_washless, STATUS=="Allergic")$E1,subset(baso_washless,
STATUS=="Allergic")$sIgE, col="red", pch=19)
points(subset(baso_washless, STATUS=="Control")$E1,subset(baso_washless,
STATUS=="Control")$sIgE, col="orange", pch=19)
points(subset(baso_washless, STATUS=="HM tolerant")
$E1,subset(baso_washless, STATUS=="HM tolerant")$sIgE, col="blue", pch=19)
points(subset(baso_washless, STATUS=="Outgrown")$E1,subset(baso_washless,
STATUS=="Outgrown")$sIgE, col="green", pch=19)

plot(baso_washless$E2,baso_washless$sIgE,type="n", ylab="kU/L", xlab="%
CD63", cex=1.5)
points(subset(baso_washless, STATUS=="Allergic")$E2,subset(baso_washless,
STATUS=="Allergic")$sIgE, col="red", pch=19)
points(subset(baso_washless, STATUS=="Control")$E2,subset(baso_washless,
STATUS=="Control")$sIgE, col="orange", pch=19)
points(subset(baso_washless, STATUS=="HM tolerant")
$E2,subset(baso_washless, STATUS=="HM tolerant")$sIgE, col="blue", pch=19)
points(subset(baso_washless, STATUS=="Outgrown")$E2,subset(baso_washless,
STATUS=="Outgrown")$sIgE, col="green", pch=19)

plot(baso_washless$E3,baso_washless$sIgE,type="n", ylab="kU/L", xlab="%
```

```
CD63", cex=1.5)
points(subset(baso_washless, STATUS=="Allergic")$E3,subset(baso_washless,
STATUS=="Allergic")$sIgE, col="red", pch=19)
points(subset(baso_washless, STATUS=="Control")$E3,subset(baso_washless,
STATUS=="Control")$sIgE, col="orange", pch=19)
points(subset(baso_washless, STATUS=="HM tolerant")
$E3,subset(baso_washless, STATUS=="HM tolerant")$sIgE, col="blue", pch=19)
points(subset(baso_washless, STATUS=="Outgrown")$E3,subset(baso_washless,
STATUS=="Outgrown")$sIgE, col="green", pch=19)

plot(baso_washless$E4,baso_washless$sIgE,type="n", ylab="kU/L", xlab="%
CD63", cex=1.5)
points(subset(baso_washless, STATUS=="Allergic")$E4,subset(baso_washless,
STATUS=="Allergic")$sIgE, col="red", pch=19)
points(subset(baso_washless, STATUS=="Control")$E4,subset(baso_washless,
STATUS=="Control")$sIgE, col="orange", pch=19)
points(subset(baso_washless, STATUS=="HM tolerant")
$E4,subset(baso_washless, STATUS=="HM tolerant")$sIgE, col="blue", pch=19)
points(subset(baso_washless, STATUS=="Outgrown")$E4,subset(baso_washless,
STATUS=="Outgrown")$sIgE, col="green", pch=19)

plot(baso_washless$E5,baso_washless$sIgE,type="n", ylab="kU/L", xlab="%
CD63", cex=1.5)
points(subset(baso_washless, STATUS=="Allergic")$E5,subset(baso_washless,
STATUS=="Allergic")$sIgE, col="red", pch=19)
points(subset(baso_washless, STATUS=="Control")$E5,subset(baso_washless,
STATUS=="Control")$sIgE, col="orange", pch=19)
points(subset(baso_washless, STATUS=="HM tolerant")
$E5,subset(baso_washless, STATUS=="HM tolerant")$sIgE, col="blue", pch=19)
points(subset(baso_washless, STATUS=="Outgrown")$E5,subset(baso_washless,
STATUS=="Outgrown")$sIgE, col="green", pch=19)

plot(baso_washless$E6,baso_washless$sIgE,type="n", ylab="kU/L", xlab="%
CD63", cex=1.5)
points(subset(baso_washless, STATUS=="Allergic")$E6,subset(baso_washless,
STATUS=="Allergic")$sIgE, col="red", pch=19)
points(subset(baso_washless, STATUS=="Control")$E6,subset(baso_washless,
STATUS=="Control")$sIgE, col="orange", pch=19)
points(subset(baso_washless, STATUS=="HM tolerant")
$E6,subset(baso_washless, STATUS=="HM tolerant")$sIgE, col="blue", pch=19)
points(subset(baso_washless, STATUS=="Outgrown")$E6,subset(baso_washless,
STATUS=="Outgrown")$sIgE, col="green", pch=19)


# some additional plots, figure E2
quartz(,8,4); plot(baso_washless$PST ~ baso_washless$STATUS, ylab="PST mm
```

```
wheal", xlab="")


#Figure 5

hm <- subset(baso_washless, STATUS == "HM tolerant")
quartz(,12,4); layout(matrix(c(1:3),1,3,byrow=FALSE))
plot(hm$E4 ~ hm$current_diet, cex.lab=1.3, cex.axis=1.5, ylab="%CD63
positive", xlab=""); mtext("E4",3,1,cex=2,at=0.3)
plot(hm$E5 ~ hm$current_diet, cex.lab=1.3, cex.axis=1.5, ylab="%CD63
positive", xlab=""); mtext("E5",3,1,cex=2,at=0.3)
plot(hm$E6 ~ hm$current_diet, cex.lab=1.3, cex.axis=1.5, ylab="%CD63
positive", xlab=""); mtext("E6",3,1,cex=2,at=0.3)

#Figure E7
quartz(,12,4); layout(matrix(c(1:3),1,3,byrow=FALSE))
plot(hm$IL3 ~ hm$current_diet, cex.lab=1.3, cex.axis=1.5, ylab="%CD63
positive", xlab=""); mtext("IL-3",3,1,cex=2,at=0.4)
plot(hm$anti_IgE ~ hm$current_diet, cex.lab=1.3, cex.axis=1.5, ylab="%CD63
positive", xlab=""); mtext("anti-IgE",3,1,cex=2,at=0.6)
plot(hm$fMLP ~ hm$current_diet, cex.lab=1.3, cex.axis=1.5, ylab="%CD63
positive", xlab=""); mtext("fMLP",3,1,cex=2,at=0.5)



#Figure E6
quartz(,16,4); layout(matrix(c(1:4),1,4,byrow=FALSE))
plot(hm$E6 ~ hm$current_diet, cex.lab=1.3, cex.axis=1.5, ylab="%CD63
positive", xlab=""); mtext("E6",3,1,cex=2,at=0.4)
plot(hm$PST ~ hm$current_diet, cex.lab=1.3, cex.axis=1.5, ylab="%CD63
positive", xlab=""); mtext("PST",3,1,cex=2,at=0.3)
plot(hm$sIgE ~ hm$current_diet, cex.lab=1.3, cex.axis=1.5, ylab="%CD63
positive", xlab=""); mtext("sIgE",3,1,cex=2,at=0.3)
plot(hm$casein_g4 ~ hm$current_diet, cex.lab=1.3, cex.axis=1.5, ylab="%CD63
positive", xlab=""); mtext("IgG4",3,1,cex=2,at=0.3)

# create before-after plot for wash data, figure 3
hm <- subset(baso_wash, STATUS == "HM tolerant")

quartz(,8,4); layout(matrix(c(1,2,3),1,3, byrow=TRUE))
plot(as.numeric(hm$wash_status), hm$E4, xlim=c(0,3), cex.lab=1.4, xaxt="n",
ylab="%CD63 positive", xlab="", mtext("E4",3,1,at=-0.2, cex=2));
mtext(c("+", "-"),1,1,at=c(1,2),cex=1.5); mtext("serum",1,1,at=0.1)
for (step in 1:length(subset(hm, wash_status == "y")$E4)) {
        lines(as.numeric(subset(hm, visit_ID == subset(hm, wash_status ==
"y")$visit_ID[step])$wash_status), subset(hm, visit_ID == subset(hm,
```

```
wash_status == "y")$visit_ID[step])$E4)}
wilcox.test(hm$E4 ~ hm$wash_status, paired=TRUE, alternative="less")

plot(as.numeric(hm$wash_status), hm$E5, xlim=c(0,3), cex.lab=1.4, xaxt="n",
ylab="%CD63 positive", xlab="", mtext("E5",3,1,at=-0.2, cex=2));
mtext(c("+", "-"),1,1,at=c(1,2),cex=1.5)
for (step in 1:length(subset(hm, wash_status == "y")$E5)) {
        lines(as.numeric(subset(hm, visit_ID == subset(hm, wash_status ==
"y")$visit_ID[step])$wash_status), subset(hm, visit_ID == subset(hm,
wash_status == "y")$visit_ID[step])$E5)}
wilcox.test(hm$E5 ~ hm$wash_status, paired=TRUE, alternative="less")

plot(as.numeric(hm$wash_status), hm$E6, xlim=c(0,3), cex.lab=1.4, xaxt="n",
ylab="%CD63 positive", xlab="", mtext("E6",3,1,at=-0.2, cex=2));
mtext(c("+", "-"),1,1,at=c(1,2),cex=1.5)
for (step in 1:length(subset(hm, wash_status == "y")$E6)) {
        lines(as.numeric(subset(hm, visit_ID == subset(hm, wash_status ==
"y")$visit_ID[step])$wash_status), subset(hm, visit_ID == subset(hm,
wash_status == "y")$visit_ID[step])$E6)}
wilcox.test(hm$E6 ~ hm$wash_status, paired=TRUE, alternative="less")


# dilution experiments

dose <- read.csv(paste(current_dir, "/data/dilution/dilution_data.csv",
sep=""), header=TRUE)

# separate plot for dilution, figure 4

dilute <- subset(dose, stim == "milk" | stim == "IgE")
dilute.E4 <- subset(dilute, Ag_dilution == 1e+04 | Ag_dilution == 1)
dilute.E4 <- subset(dilute.E4, wash == 0)
dilute.E4.IgE <- subset(dilute.E4, stim == "IgE")
dilute.E4.milk <- subset(dilute.E4, stim == "milk")

quartz(,12,4); layout(matrix(c(1,2,3),1,3))
plot(subset(dilute.E4, SampleID == "PP608")$DF, subset(dilute.E4, SampleID
== "PP608")$CD63, xlab="serum dilution factor", ylab="% CD63", cex=2,
cex.lab=1.3, xaxp=c(1,4,3)); legend("topleft", legend=c("IgE", "milk"),
lty=c(3,1), bty="n", pt.cex=2)
lines(subset(dilute.E4.IgE, SampleID == "PP608")$DF, subset(dilute.E4.IgE,
SampleID == "PP608")$CD63, lty=3)
lines(subset(dilute.E4.milk, SampleID == "PP608")$DF,
subset(dilute.E4.milk, SampleID == "PP608")$CD63)

plot(subset(dilute.E4, SampleID == "PP603")$DF, subset(dilute.E4, SampleID
```

```
== "PP603")$CD63, xlab="serum dilution factor", ylab="% CD63", cex=2,
cex.lab=1.3, xaxp=c(1,4,3)); legend("topleft", legend=c("IgE", "milk"),
lty=c(3,1), bty="n", pt.cex=2)
lines(subset(dilute.E4.IgE, SampleID == "PP603")$DF, subset(dilute.E4.IgE,
SampleID == "PP603")$CD63, lty=3)
lines(subset(dilute.E4.milk, SampleID == "PP603")$DF,
subset(dilute.E4.milk, SampleID == "PP603")$CD63)

plot(subset(dilute.E4, SampleID == "PP621")$DF, subset(dilute.E4, SampleID
== "PP621")$CD63, xlab="serum dilution factor", ylab="% CD63", cex=2,
cex.lab=1.3, xaxp=c(1,4,3)); legend("topleft", legend=c("IgE", "milk"),
lty=c(3,1), bty="n", pt.cex=2)
lines(subset(dilute.E4.IgE, SampleID == "PP621")$DF, subset(dilute.E4.IgE,
SampleID == "PP621")$CD63, lty=3)
lines(subset(dilute.E4.milk, SampleID == "PP621")$DF,
subset(dilute.E4.milk, SampleID == "PP621")$CD63)


# example plots of dose response +/- wash, figure E4
milk.dose <- subset(dose, stim == "milk")
milk.dose <- subset(milk.dose, DF == 1)
milk.dose.nowash <- subset(milk.dose, wash == 0)
milk.dose.wash <- subset(milk.dose, wash == 1)

quartz(,12,4); layout(matrix(c(1,2,3),1,3, byrow=TRUE))
plot(log(subset(milk.dose, SampleID == "PP608")$Ag_dilution, 10),
subset(milk.dose, SampleID == "PP608")$CD63, col=4, xlab="log Ag
dilutioin", ylab="% CD63", cex.lab=1.3, cex=2, cex.axis=1.5)
lines(log(subset(milk.dose.wash, SampleID == "PP608")$Ag_dilution, 10),
subset(milk.dose.wash, SampleID == "PP608")$CD63, col=4)
lines(log(subset(milk.dose.nowash, SampleID == "PP608")$Ag_dilution, 10),
subset(milk.dose.nowash, SampleID == "PP608")$CD63, col=4, lty=3)
plot(log(subset(milk.dose, SampleID == "PP603")$Ag_dilution, 10),
subset(milk.dose, SampleID == "PP603")$CD63, col=2, xlab="log Ag
dilutioin", ylab="% CD63", cex.lab=1.3, cex=2, cex.axis=1.5)
lines(log(subset(milk.dose.wash, SampleID == "PP603")$Ag_dilution, 10),
subset(milk.dose.wash, SampleID == "PP603")$CD63, col=2)
lines(log(subset(milk.dose.nowash, SampleID == "PP603")$Ag_dilution, 10),
subset(milk.dose.nowash, SampleID == "PP603")$CD63, col=2, lty=3)
plot(log(subset(milk.dose, SampleID == "PP621")$Ag_dilution, 10),
subset(milk.dose, SampleID == "PP621")$CD63, col=3, xlab="log Ag
dilutioin", ylab="% CD63", cex.lab=1.3, cex=2, cex.axis=1.5)
lines(log(subset(milk.dose.wash, SampleID == "PP621")$Ag_dilution, 10),
subset(milk.dose.wash, SampleID == "PP621")$CD63, col=3)
lines(log(subset(milk.dose.nowash, SampleID == "PP621")$Ag_dilution, 10),
subset(milk.dose.nowash, SampleID == "PP621")$CD63, col=3, lty=3)
```

```
#IgG depletion experiments
dep <- read.csv(paste(current_dir, "/data/IgG_depletion/
depletion_summary.csv", sep=""), header=TRUE)
quartz(,15,5); layout(matrix(c(1,2,3),1,3, byrow=TRUE))
plot(subset(dep, STIM == "IL3")$CD63 ~ subset(dep, STIM == "IL3")$IGG,
range=0, xlab="", cex.axis=1.5, ylab=""); mtext("IL-3",3,1, at=0.5, cex=2)
points(subset(dep, STIM == "IL3")$CD63 ~ subset(dep, STIM == "IL3")$IGG,
cex=2)
plot(subset(dep, STIM == "E4")$CD63 ~ subset(dep, STIM == "E4")$IGG,
range=0, xlab="", cex.axis=1.5, ylab=""); mtext("E4",3,1, at=0.5, cex=2)
points(subset(dep, STIM == "E4")$CD63 ~ subset(dep, STIM == "E4")$IGG,
cex=2)
plot(subset(dep, STIM == "a.IgE")$CD63 ~ subset(dep, STIM == "a.IgE")$IGG,
range=0, xlab="", cex.axis=1.5, ylab=""); mtext("anti-IgE",3,1, at=0.5,
cex=2)
points(subset(dep, STIM == "a.IgE")$CD63 ~ subset(dep, STIM == "a.IgE")
$IGG, cex=2)

dep_E4 <- subset(dep, STIM == "E4"); dep_IGE <- subset(dep, STIM ==
"a.IgE"); dep_IL3 <- subset(dep, STIM == "IL3")
wilcox.test(subset(dep_E4, IGG == "mock")$CD63, subset(dep_E4, IGG ==
"depleted")$CD63, paired=TRUE, alternative="less")
wilcox.test(subset(dep_IGE, IGG == "mock")$CD63, subset(dep_IGE, IGG ==
"depleted")$CD63, paired=TRUE, alternative="less")
wilcox.test(subset(dep_IL3, IGG == "mock")$CD63, subset(dep_IL3, IGG ==
"depleted")$CD63, paired=TRUE, alternative="less")
```