Basophil reactivity, wheal size, and immunoglobulin levels distinguish degrees of cow's milk tolerance

Lara S. Ford, MD, MPH,[a] Katherine A. Bloom, MD,[a] Anna H. Nowak-Węgrzyn, MD,[a] Wayne G. Shreffler, MD, PhD,[b] Madhan Masilamani, PhD,[a] and Hugh A. Sampson, MD[a] *New York, NY, and Boston, Mass*

Background: In our previous study about 75% of children with cow's milk allergy tolerated baked milk products, which improved their prognosis and quality of life.
Objective: We sought to identify biomarkers of varying degrees of clinical tolerance among a cohort of children with cow's milk allergy.
Methods: One hundred thirty-two subjects were initially classified as baked milk–reactive, baked milk–tolerant, or having "outgrown milk allergy" based on the results of oral food challenges. The baked milk–tolerant group was then divided into 3 groups based on the amount and degree of heat-denatured milk protein that they could tolerate. Serum was analyzed for allergen-specific IgE and IgG_4 levels, basophil reactivity was assessed in whole blood stimulated with serial 10-fold dilutions of milk protein, and skin prick tests (SPTs) were performed to commercial milk extract. Activated basophils were defined by using flow cytometry as $CD63^{bright}CD203c^{+}CD123^{+}HLA\text{-}DR^{dim/-}CD41a^{-}lineage^{-}$. Data were analyzed by using the Jonckheere-Terpstra test.
Results: Significant differences across the 5 clinical groups were seen for median casein- and milk-specific IgE levels, casein-specific IgG_4 levels, and casein IgE/IgG_4 ratios; milk-specific to nonspecific basophil activation ratio, median basophil reactivity, and spontaneous basophil activation (CD203c expression after stimulation with RPMI); and milk SPT wheal diameters. Casein- and milk-specific IgE level, milk-specific basophil reactivity, and milk SPT wheal diameter are all significantly greater among patients with milk allergy who react to baked milk than among those who tolerate it.
Conclusions: The majority of patients with milk allergy are able to tolerate some forms of baked milk in their diets. Different phenotypes of children with cow's milk allergy can be distinguished by casein- and milk-specific IgE levels, milk-specific basophil reactivity, and milk SPT mean wheal diameters. Spontaneous basophil activation is greater among patients with more severe clinical milk reactivity. (J Allergy Clin Immunol 2013;131:180-6.)

Key words: *Cow's milk allergy, tolerance, extensively heated, baked, immunotherapy, immunomodulation, biomarker, basophil activation*

Cow's milk allergy is the most common food allergy among young children. The majority of children have tolerance to milk by school age, and this proportion continues to increase through adolescence.[1] Although the proportion that ultimately has clinical tolerance has remained steady over the years, in recent decades, the timing of this event has grown later. In 1990, Host and Halken[2] showed that 75% outgrew IgE-mediated cow's milk allergy by age 3 years. However, in 2007, when Skripak et al[1] analyzed the natural history of milk allergy in a referral population, they found that it took until the age of 16 years for 79% to reach this outcome. Therefore although strict milk avoidance has been recommended in recent decades in the belief that it expedites the development of natural tolerance, in retrospect, this common management practice has coincided with delayed resolution of allergy. Not only has strict milk avoidance failed to yield improved long-term outcomes, it also has a major effect on the quality of life of patients, many of whom believe from experience that they can tolerate some milk-containing products.

In 2008, our group showed that 75% of children with milk allergy tolerated extensively heated (baked) milk products.[3] After 3 months of ingesting baked milk products, subjects' growth and intestinal permeability were not adversely affected, and immunologic parameters showed changes consistent with desensitization. Longer-term follow-up of these same patients suggests that the ingestion of baked milk products accelerates and increases the overall likelihood of these patients completely outgrowing their milk allergy, with no significant adverse effects.[4]

A randomized controlled trial is currently underway at our center to more rigorously address the question of whether aggressive inclusion of baked milk in the diets of those patients with milk allergy who tolerate it does improve the rate and likelihood of total resolution of milk allergy. On the basis of our previous study,[4] we hypothesized that this dietary manipulation is a form of natural immunotherapy, and to further study this, we are following several immunologic parameters over time. Additionally, we hope to identify biomarkers that will help predict the likelihood of patients with milk allergy tolerating extensively heat-denatured milk products.

From [a]the Jaffe Food Allergy Institute, Mount Sinai School of Medicine, New York, and [b]Massachusetts General Hospital, Boston.

H.A.S. is funded in part by grants from the National Institutes of Health/National Institute of Allergy and Infectious Diseases (AI44236 and AI066738) and the National Center for Research Resources (RR026134). L.S.F. is funded by the AAAAI/Elliot and Roslyn Jaffe Third-Year Fellowship Food Allergy Research Award at Mount Sinai School of Medicine.

Disclosure of potential conflict of interest: L. S. Ford has received research support from the American Academy of Allergy, Asthma & Immunology (AAAAI) Jaffe Third-Year Fellowship Food Allergy Research Award and is chair of the AAAAI Fellow-in-Training Committee. H. A. Sampson is a consultant for Allertein Therapeutics, LLC; is on the advisory board for Immusan T and Novartis; has received research support from the Food Allergy Initiative (FAI) and the National Institute of Allergy and Infectious Diseases (NIAID)/National Institutes of Health (NIH); is a consultant and scientific advisor for the FAI; is a medical advisor for the Food Allergy & Anaphylaxis Network; is a scientific advisor for the University of Nebraska–FARRP; and is 45% owner of Herb Springs, LLC. The rest of the authors declare that they have no relevant conflicts of interest.

Received for publication January 30, 2012; revised May 30, 2012; accepted for publication June 5, 2012.

Available online July 20, 2012.

Corresponding author: Hugh A. Sampson, MD, Department of Pediatrics, Mount Sinai School of Medicine, One Gustave L. Levy Place, Box 1198, New York, NY 10029. E-mail: Hugh.Sampson@mssm.edu.

0091-6749/$36.00

http://dx.doi.org/10.1016/j.jaci.2012.06.003

Abbreviation used
SPT: Skin prick test

Here we summarize several clinical and immunologic characteristics of our interventional subjects at entry into the study (baseline) and examine how immunologic characteristics vary among the groups of subjects with different levels of milk tolerance.

METHODS

Participants

Subjects with milk allergy were recruited from the pediatric allergy clinics at Mount Sinai and referring allergists from August 2008 to June 2011. The study was approved by the Mount Sinai Institutional Review Board, and informed consent was obtained before enrollment.

Eligible subjects were between the ages of 4 and 10 years and had a positive skin prick test (SPT) response to milk or detectable serum milk-specific IgE levels and a history of an allergic reaction to milk within 2 years before study entry, milk-specific IgE levels of 14 to 35 kU_A/L, or an SPT wheal diameter of greater than 10 mm, regardless of reaction history. Patients were excluded if they had milk-specific IgE levels of greater than 35 kU_A/L (in the original study levels >35 kU_A/L were associated with low probability [14%] of tolerating baked milk; subjects with such levels comprised 7% [7/99] of subjects[3]) or a history of a life-threatening anaphylactic reaction to milk within the 2 years before study entry.

Design

Each patient commenced a series of challenges to muffin, pizza, rice pudding or a similar dish, and unheated milk, and stopped when the first positive challenge result occurred. The challenge foods were chosen with input from our patients as foods whose inclusion in their diets would be of clinical relevance by improving their quality of life. The foods were placed in an ordered ranking for challenge progression by increasing the amount of milk protein and decreasing the level of heat denaturation. Subjects were instructed to continue only those foods that they had tolerated during a challenge in their diet on a regular basis at home. In addition to improving quality of life, advancing the amount of milk protein and decreasing the degree of denaturation with these advancing products is intended to accelerate the development of tolerance, which is similar to the manner in which subcutaneous immunotherapy is increased to the maximum tolerated dose. Patients were classified into 5 levels of milk tolerance based on challenge outcome. The groups were labeled as baked milk–reactive, baked milk–tolerant (muffin, pizza, or rice pudding), and having outgrown milk allergy. On the first challenge day, before starting challenges, an SPT to milk was performed, whole blood was collected for basophil activation studies, and a serum sample was collected for measurement of specific IgE levels to milk, casein, and β-lactoglobulin, and IgG_4 levels to casein and β-lactoglobulin by using the UniCAP system (Thermo Fisher Scientific, Portage, Mich). Table E1 in this article's Online Repository at www.jacionline.org records the sources of all reagents and supplies.

SPT procedure

SPTs were performed with a sterile bifurcated needle, commercial milk extract, and a negative saline and positive histamine control. The size of the skin test response was calculated as a mean of the longest diameter and its longest orthogonal measured at 10 to 15 minutes.[5]

Challenge procedure

Food challenges were performed openly under a physician's supervision in the Mount Sinai Clinical Research Center. Each muffin contained 1.5 g of milk protein and was baked at 350°F for 30 minutes. A serving of pizza contained 4 g of milk protein and was baked at 425°F for at least 13 minutes. A serving of rice pudding (or equivalent for subjects who refused rice pudding) contained 7.7 g of milk protein baked at 325°F for 90 minutes. A serving of unheated milk contained 10 g of milk protein and had undergone no heating other than standard pasteurization. The milk protein in the muffin and rice pudding consisted of the usual 80% casein and 20% whey proteins found in milk, and the cheese on the pizza consisted of 94.1% casein and 5.9% whey proteins. Each challenge was administered in 4 to 6 progressively larger portions over 1 hour. Up to 2 challenges could take place on the same day, separated by at least 2 hours. If further challenges were required, a second challenge day was scheduled within 2 weeks of the first. Subjects were monitored throughout the challenges and for 2 to 4 hours after the final challenge of each day. Challenges were discontinued at the first objective sign of a reaction, and appropriate treatment was initiated immediately. Subjects in whom the muffin challenge failed continued on a strict milk-avoidance diet and returned 1 year later for repeat baseline challenge. Subjects who reported subsequent symptoms at home to foods that they had apparently tolerated during the initial challenge returned for repeat baseline challenge at the first opportunity. For each subject, only the most recent baseline visit was included in the analysis.

Basophil activation test

Whole-blood aliquots (250 μL) were incubated with equal volumes of basophil stimulation buffer (RPMI plus IL-3 at 2 ng/mL) alone or with the addition of milk powder in PBS at serial 10-fold dilutions (from 1×10^3 to 1×10^{-1} μg/mL total protein), polyclonal anti-IgE antibody (1 μg/mL, positive control), 0.25 μg/mL phorbol 12-myristate 13-acetate/1 μg/mL calcium ionophore (positive control), N-formyl-methionyl-leucyl-phenylalanine (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, CD3, CD14, CD19, 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 (BD Biosciences, San Jose, Calif) to each sample for 15 minutes.

Flow cytometry

Basophil activation was assessed by using flow cytometry.[6] Samples were analyzed on a BD LSRII flow cytometer (BD Biosciences). Single-color compensation samples were prepared with anti-mouse immunoglobulin beads. Fluorescence data were acquired and autocompensated on a modified LSR-II configured for 7-color parameters by using FACSDiva version 6.0 software (BD Biosciences). Basophils were identified as $CD123^+HLA\text{-}DR^{dim/-}CD41a^-CD3^-CD14^-CD19^-$ and activated basophils were identified additionally as $CD63^+CD203c^+$, as shown in Fig E1 in this article's Online Repository at www.jacionline.org. A minimum of 50 $CD123^+HLA\text{-}DR^{dim/-}CD41a^-CD3^-CD14^-CD19^-$ events (ie, basophils) were recorded for each condition or the sample was excluded. Nonresponders were defined as subjects with less than 5% CD63/CD203c upregulation (basophil activation) in response to all milk concentrations and the anti-IgE control condition and were also excluded. Milk-specific basophil reactivity was defined as a subject's maximum basophil activation in response to any milk concentration.[7] Analysis of cytometric data was performed with FlowJo version 8.8.6 software (TreeStar, Ashland, Ore).

Statistics

Graphic display and statistical analyses were performed with R analysis 2.12.1 software.[8-10] The frequencies of missing data and basophil nonresponders were compared between groups by using the Fisher exact *t* test. For determining the significance of differences in each measure across clinical groups (with the 3 baked milk–tolerant groups considered both together and separately), the Jonckheere-Terpstra test for ordered alternatives was used. This is a between-group trend test in which the median level of a measure must decrease in an orderly fashion (ie, demonstrate a monotonic trend) to reject the null hypothesis. *Post hoc* tests for pairwise differences between

adjacent clinical groups were performed by using Wilcoxon signed-rank tests, and a Bonferroni correction for multiple comparisons was performed.

RESULTS

In total, 147 subjects were evaluated. Fifteen subjects were not challenged and assumed to be reactive to baked milk because their milk-specific IgE level exceeded 35 kU_A/L.[3] Of the 132 subjects challenged, 37 reacted to baked milk (muffin), 31 tolerated muffin, 12 tolerated pizza, 44 tolerated rice pudding or equivalent, and 8 had outgrown their milk allergy (tolerated unheated milk). Thus 65% of those studied in this cohort tolerated baked milk compared with only 6% who tolerated whole milk. Eight subjects who reacted to muffin 1 year previously returned for a repeat baseline visit. Of these, 5 again reacted to muffin, 2 tolerated muffin, and 1 tolerated rice pudding. One subject who tolerated muffin at the original baseline visit had symptoms on repeated ingestion at home and then reacted to muffin during a rechallenge 3 months later. Overall, the subjects' median age was 7.6 years (range, 4.0-11.0 years), and 92 (70%) were male. Age and sex distribution by clinical outcome are shown in Table I. Twelve (9.1%) of the subjects exhibited the basophil nonresponder phenotype, and this proportion was consistent with the rate of nonresponders previously reported in the normal population.[11,12] In 12 (9.1%) of the samples, insufficient basophils were acquired. The nonresponders and subjects for whom insufficient basophils were acquired were eliminated from subsequent analyses of basophil activation. There was no serum available for immunoglobulin measurement in 2 (1.5%) samples. There was no significant difference between groups in the frequency of nonresponders or samples otherwise excluded from basophil or immunoglobulin analysis (data not shown).

Immunoglobulins

The median casein-specific IgE level ($P < .001$), casein-specific IgG_4 level ($P < .05$), and casein-specific IgE/IgG_4 ratio ($P < .001$) all differed significantly across the 5 clinical groups (Figs 1-3). Casein-specific IgE levels varied from 13.75 kU_A/L (range, 0.36-49.9 kU_A/L) among baked milk–reactive subjects to 0.44 kU_A/L (range, <0.35-1.79 kU_A/L) among subjects who had outgrown their allergy. Casein-specific IgG_4 levels varied from 1.87 mg_A/L (range, 0.15-10.3 mg_A/L) among baked milk–reactive subjects to 0.28 mg_A/L (range, <0.10-2.32 mg_A/L) among subjects who had outgrown their allergy. Casein-specific IgE/IgG_4 ratios varied from 5.08 (range, 0.18-202.7) among baked milk–reactive subjects to 2.3 (range, 0.18-5.0) among subjects who had outgrown their milk allergy. *Post hoc* analysis showed that casein-specific IgE levels differed significantly between those who reacted to baked milk and those who tolerated it ($P < .001$) and between those who tolerated baked milk and those who had fully outgrown their milk allergy ($P < .005$) but not among different gradations of heated milk tolerance. Casein IgE/IgG_4 ratios effectively discriminated between those who reacted to baked milk and those who tolerated it ($P < .01$) but not between those who tolerated baked milk and those who had fully outgrown their milk allergy ($P = .25$).

Cow's milk IgE and β-lactoglobulin IgE levels also decreased significantly in order of the 5 clinical groups ($P < .001$ for both trends). Cow's milk IgE levels varied from 12.4 kU_A/L (range, 0.6-43.6 kU_A/L) among baked milk–reactive subjects to 0.7 kU_A/L (range, <0.35-4.3 kU_A/L) among subjects who had outgrown their milk allergy. β-Lactoglobulin IgE levels varied from 2.1 kU_A/L (range, <0.35-15.4 kU_A/L) among baked milk–reactive subjects to less than 0.35 kU_A/L (range, <0.35-0.46 kU_A/L) among subjects who had outgrown their milk allergy.

TABLE I. Age and sex distribution by clinical outcome

	Total	Median age (y)	Male subjects
Baked milk reactive	37	8.1	29 (78%)
Tolerated muffin	31	7.4	23 (74%)
Tolerated pizza	12	6.5	9 (75%)
Tolerated rice pudding	44	7.6	27 (61%)
Outgrown	8	6.6	4 (50%)

Basophil reactivity

The ratio of milk-specific basophil reactivity to nonspecific (anti-IgE) basophil activation showed a significant trend ($P < .005$) across the 5 levels of baked milk–tolerant subjects, with those reacting to all forms of baked milk exhibiting the highest ratios (median, 2.4; range, 0.4-15.5) and subjects who tolerated unheated milk exhibiting the lowest ratios (median, 0.6; range, 0.3-11.0; Fig 4).

Post hoc analyses of pairwise differences between adjacent groups showed a significant difference between those who reacted to baked milk and those who tolerated it ($P < .05$) but not between those who tolerated baked milk and those who had fully outgrown their milk allergy ($P = .11$).

Spontaneous basophil activation, as measured by both piecemeal ($CD203c^+$) and anaphylactic ($CD63^+$) degranulation[13] under negative control conditions, was also greater among more subjects with milk allergy. This was evident not only when the basophils were incubated with IL-3, a known priming agent, but also when subjected to RPMI alone. Fig 5 shows the significant ($P < .05$) reduction in CD203c mean fluorescence intensity with stimulation by using RPMI alone, with increasing milk tolerance measured across 5 clinical groups.

The basophil reactivity taken alone and not as a ratio of anti-IgE basophil activation also showed a significant trend across the 5 levels of milk tolerance ($P < .001$), with subjects who were reactive to all forms of baked milk exhibiting the highest levels of basophil reactivity (median, 50.7%; range, 7.3% to 95.1%) and subjects who tolerated unheated milk exhibiting the lowest reactivity (median, 9.165%; range, 1.9% to 31.8%; see Fig E2 in this article's Online Repository at www.jacionline.org). In this case *post hoc* analysis after pooling all subjects who tolerated some form of baked milk showed that basophil reactivity was significantly greater in baked milk–reactive subjects than in baked milk–tolerant subjects ($P < .01$) and significantly greater in baked milk–tolerant subjects than in those who had outgrown their allergy ($P < .05$).

Mast cell activity

The decrease in the size of the mean wheal diameter after SPTs with commercial milk extract was significant ($P < .001$) across the 5 levels of milk reactivity, extending from a median of 10 mm among baked milk–reactive subjects (range, 5.5-20 mm) to a median of 6 mm (range, 1-9 mm) among subjects who had outgrown their milk allergy (Fig 6). *Post hoc* analysis showed a significant decrease in wheal size in those who tolerated baked milk compared with those who reacted to it ($P < .005$) and in those who

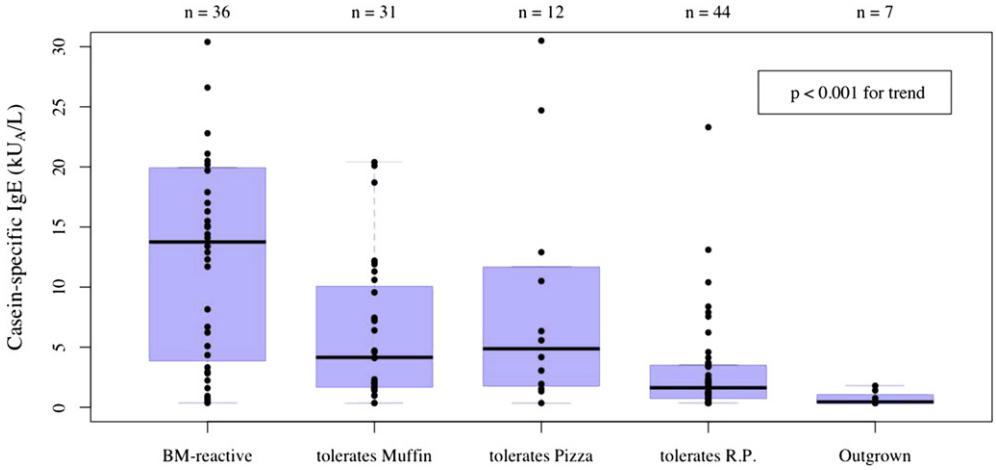

FIG 1. Casein-specific IgE measurements by 5 levels of challenge outcome. With the 3 central groups pooled, *post hoc* analysis showed a significant difference between those who reacted to baked milk and those who tolerated it ($P < .001$) and between those who tolerated baked milk and those who had fully outgrown their milk allergy ($P < .005$). *BM*, Baked milk; *R.P.*, rice pudding.

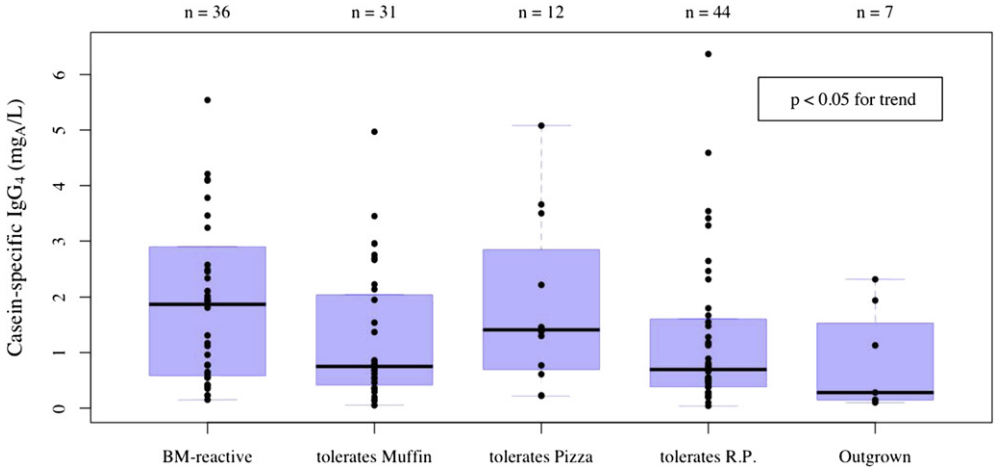

FIG 2. Casein-specific IgG_4 measurements by 5 levels of challenge outcome. *BM*, Baked milk; *R.P.*, rice pudding.

had fully outgrown their milk allergy compared with those who tolerated baked milk ($P < .005$) but no differences among different gradations of baked milk tolerance.

Receiver operating characteristic curves

The receiver operating characteristic curves shown in Fig 7 describe the performance of casein- and milk-specific IgE levels, milk-specific basophil reactivity, and mean milk SPT wheal diameters in differentiating baked milk–tolerant from baked milk–reactive subjects. The areas under the curve of these measures and their respective CIs (detailed in the legend of Fig 7) show that each measure has significant effectiveness in predicting baked milk reactivity.

DISCUSSION

Our group was the first to show significant differences in easily measurable parameters between patients with milk allergy who react to baked milk and those who tolerate it. IgE levels and their corresponding likelihoods of clinical milk reactivity were established in 1997,[14] and the ability of mean wheal diameter to predict milk reactivity has been studied since at least 1977.[15,16] However, all previous studies of IgE levels and mean wheal diameters seek to differentiate patients with any reactivity to milk from those with no clinical reactivity and have not addressed differences between those patients who do or do not react to heat-denatured milk.

Our data show that IgE levels both to whole milk and to caseins, the most abundant proteins in milk, differ significantly between baked milk–tolerant and baked milk–reactive patients.

We examined IgG_4 levels to determine whether they could improve on the accuracy of IgE alone in predicting our patients' levels of clinical reactivity and also to elucidate the role of IgG_4 in the development of tolerance (Fig 3). In patients undergoing immunotherapy with increasing doses of environmental allergens, IgG_4 levels typically increase over the course of treatment and then decrease after termination of immunotherapy.[17] Higher IgG_4 levels do not seem to reflect the absolute dose of allergen tolerated but rather are more likely a reflection of increasing allergen exposure over the recent past. Hence, in this

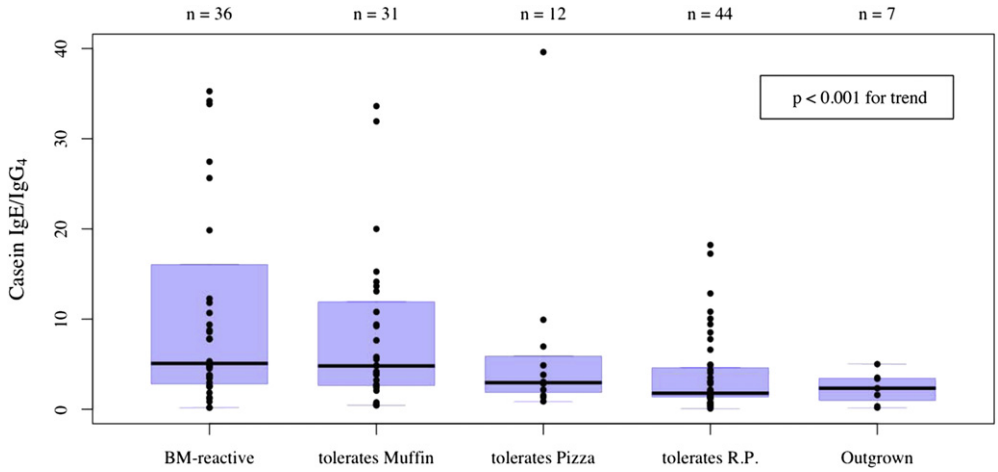

FIG 3. Casein-specific IgE/IgG$_4$ ratios by 5 levels of challenge outcome. With the 3 central groups pooled, *post hoc* analysis showed a significant difference between those who reacted to baked milk and those who tolerated it ($P < .01$) but not between those who tolerate baked milk and those who had fully outgrown their milk allergy ($P = .25$). *BM*, Baked milk; *R.P.*, rice pudding.

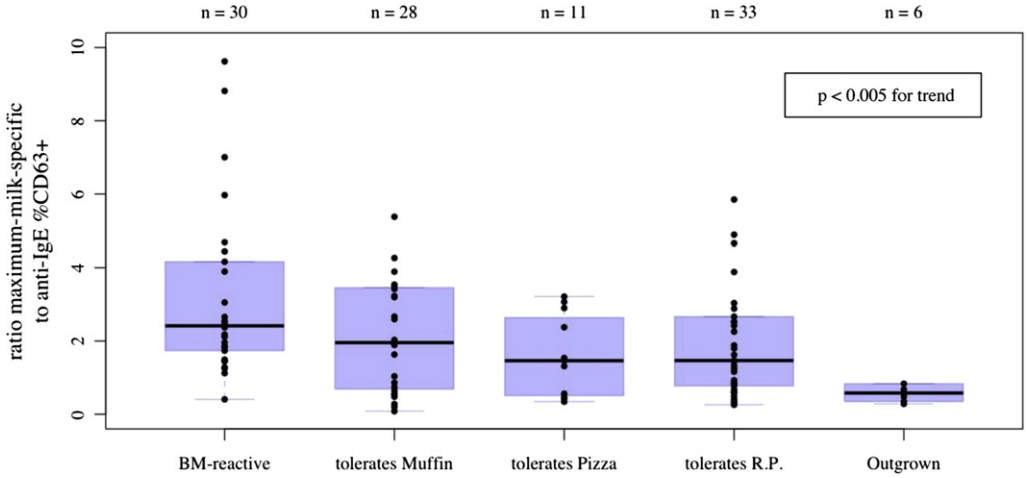

FIG 4. Ratio of milk-specific to anti-IgE (nonspecific) basophil activation by 5 levels of challenge outcome. With the 3 central groups pooled, *post hoc* analysis showed a significant difference between those who reacted to baked milk and those who tolerated it ($P < .05$) but not between those who tolerate baked milk and those who had fully outgrown their milk allergy ($P = .11$). *BM*, Baked milk; *R.P.*, rice pudding.

cross-sectional view of our subjects, who all have a recent history of total milk avoidance, it is not surprising that no correlation between higher IgG$_4$ levels and greater milk tolerance is seen. If, as our subjects go on to include more and more milk in their diets, a pattern of short-term increase in IgG$_4$ levels followed by a tapering off is borne out for individual subjects, it will support the theory that the careful introduction of baked milk–containing products as tolerated acts as a form of "natural immunotherapy."

IgE receptor density on basophils is closely related to serum IgE concentration.[18] Therefore, because we know IgE levels are related to clinical milk reactivity, it is not surprising to see that measures of basophil activation are also related.[19] Milk-specific basophil reactivity was examined as a ratio with nonspecific (anti-IgE–mediated) basophil activation (Fig 4) to account for the higher IgE receptor density seen in atopic subjects compared with that seen in healthy control subjects.[7] Studies showing this were done in patients with seasonal and other environmental allergies[20] who are unable to strictly avoid the relevant allergen. Given that upregulation of FcεRI, the high-affinity IgE receptor, is mediated by its interaction with IgE[21] and spontaneous histamine release is much lower in patients with atopic dermatitis and food allergy who are avoiding their allergens compared with those who are not,[22] it is possible that the higher receptor density is not seen in patients after strict allergen avoidance. On the other hand, subjects with food allergy and atopic dermatitis who are practicing strict avoidance appear to have greater histamine release than patients with atopic dermatitis with no food allergies. This would be consistent with the hypothesis that more subjects with milk allergy have greater IgE receptor density regardless of allergen exposure but could also be explained by other factors, such as histamine-releasing factor, present in the blood.[22] Our finding of greater spontaneous activation with increasing clinical milk reactivity at oral challenge is consistent with either possibility.

Despite the rationale for examining the ratio of milk-specific to nonspecific basophil activation, only the milk-specific basophil

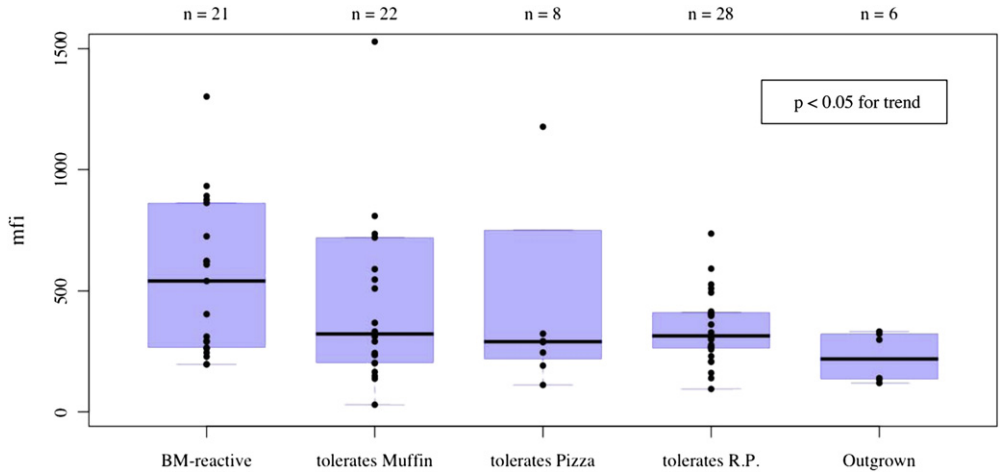

FIG 5. Mean fluorescence intensity *(mfi)* of CD203c after stimulation with RPMI (negative control) by 5 levels of challenge outcome. This measure forms an indication of spontaneous activation. *BM*, Baked milk; *R.P.*, rice pudding.

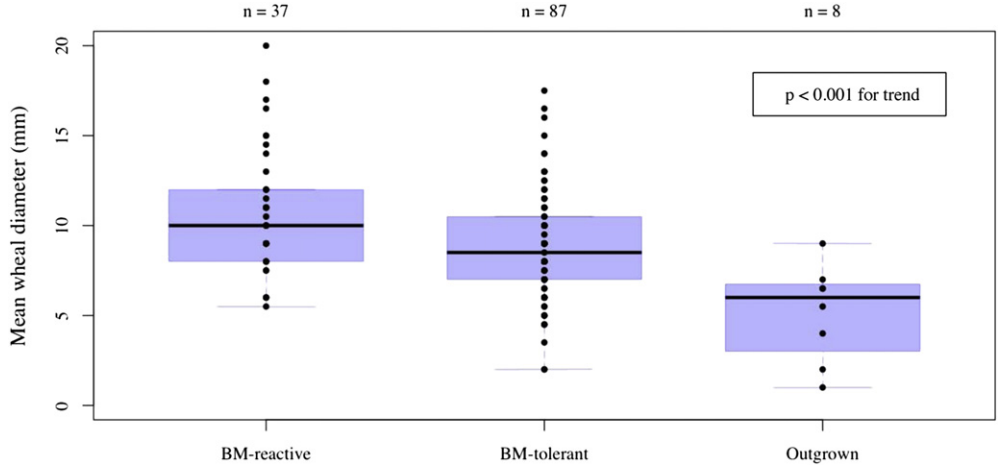

FIG 6. Wheal reaction on SPTs with commercial milk extract by 3 levels of challenge outcome (baked milk–tolerant subjects have been pooled). *Post hoc* analysis shows a significant decrease in wheal size in those who tolerated baked milk compared with those who reacted to it ($P < .005$) and in those who have fully outgrown their milk allergy compared with those who tolerate baked milk ($P < .005$). *BM*, Baked milk; *PST*, skin prick test.

reactivity alone, without adjustment for nonspecific activation, showed significant pairwise differences both between baked milk–reactive and baked milk–tolerant subjects and between baked milk–tolerant subjects and those who have outgrown their milk allergy (see Fig E2).

Indirect measurement of mast cell activity through skin testing is simple to perform and is relatively comparable with immunoglobulin measurements in predicting clinical reactivity.[23] We have shown that mast cell mean wheal diameters (Fig 6) also perform favorably in comparison with immunoglobulin levels and basophil activation in differentiating between baked milk–reactive and baked milk–tolerant subjects (Fig 7). Mast cells are known to have "memory," in that skin tests often remain positive for years after the development of clinical tolerance to allergen, and this might explain why skin testing does not differentiate well between different degrees of heat-denatured milk tolerance because this progressive tolerance likely evolves over much shorter periods.

Receiver operating characteristic curves were generated to analyze the ability of these biomarkers to predict baked milk tolerance (Fig 7). Choosing optimal cutoffs for each test would take into account not only the maximum accuracy that can be simultaneously achieved for sensitivity and specificity but also the relative costs of false-negative and false-positive results. Because such an analysis is beyond the scope of this article, no thresholds are suggested for clinical use. Like most forms of allergy testing, none of the tests evaluated provided simultaneously high sensitivity and specificity, reinforcing the need for physician-supervised food challenges to accurately diagnose food allergy.

This study again confirms the finding that the majority of patients with milk allergy are able to tolerate some forms of baked milk in their diets. Casein- and milk-specific IgE levels and milk SPT mean wheal diameters, as well as milk-specific basophil reactivity, can differentiate among different phenotypes of patients with cow's milk allergy. We have also demonstrated that

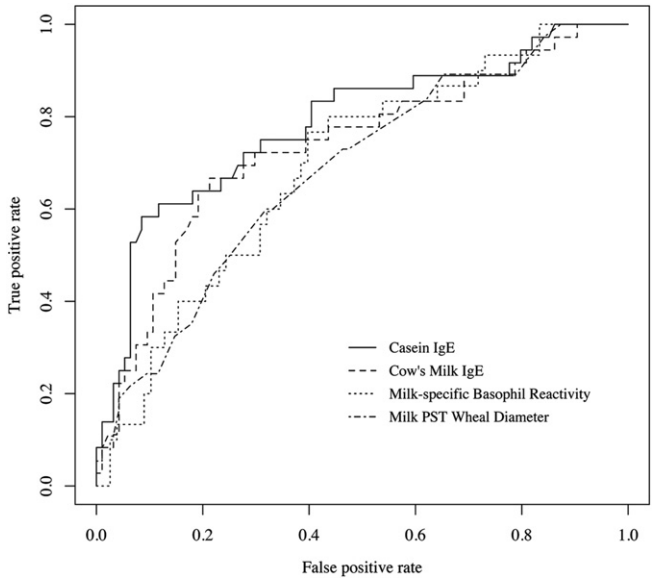

FIG 7. Receiver operating characteristic curves showing performance of various tests in predicting clinical reactivity to baked milk. Casein IgE has an area under the curve (AUC) of 0.78 (95% CI, 0.69-0.88), cow's milk IgE has an AUC of 0.73 (95% CI, 0.63-0.83), milk-specific basophil reactivity has an AUC of 0.69 (95% CI, 0.59-0.80), and milk skin prick test *(PST)* wheal diameter has an AUC of 0.68 (95% CI, 0.58-0.78).

spontaneous basophil activation is greater among patients with more severe clinical milk reactivity. These findings help to illuminate some of the immunologic mechanisms that underlie milk allergy.

We thank Beth Strong and Sally Noone for research coordination; the nursing staff of the Clinical Research Unit; Luda Bardina, Ramon Bencharitiwong, Michelle Mishoe, Anita Saha, Alyssa Chase, Emily Hertzberg, Akshay Bhatt, and Neisha Rivers for laboratory technical assistance; Scott Sicherer, Julie Wang, Jennifer Kim, Amanda Cox, Kirsi Jarvinen-Seppo, and Justin Skripak for patient referrals; and Dr Jim Godbold from the Mount Sinai School of Medicine, Department of Biostatistics, for assistance with statistical analysis. Finally, we thank the participants and their families, who have made this study possible.

Clinical implications: Most patients with milk allergy can tolerate some forms of baked milk. Biomarkers, including IgE levels, SPT responses, and milk-specific basophil reactivity, can differentiate among different phenotypes of patients with cow's milk allergy.

REFERENCES

1. 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.
2. Host A, Halken S. A prospective study of cow milk allergy in Danish infants during the first 3 years of life. Clinical course in relation to clinical and immunological type of hypersensitivity reaction. Allergy 1990;45:587-96.
3. 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, e1-2.
4. Kim JS, Nowak-Węgrzyn A, Sicherer SH, Noone S, Moshier EL, Sampson HA. Dietary baked milk accelerates the resolution of cow's milk allergy in children. J Allergy Clin Immunol 2011;128:125-31, e2.
5. Verstege A, Mehl A, Rolinck-Werninghaus C, Staden U, Nocon M, Beyer K, et al. The predictive value of the skin prick test weal size for the outcome of oral food challenges. Clin Exp Allergy 2005;35:1220-6.
6. Shreffler WG. Evaluation of basophil activation in food allergy: present and future applications. Curr Opin Allergy Clin Immunol 2006;6:226-33.
7. Kleine-Tebbe J, Erdmann S, Knol EF, MacGlashan DW Jr, Poulsen LK, Gibbs BF. Diagnostic tests based on human basophils: potentials, pitfalls and perspectives. Int Arch Allergy Immunol 2006;141:79-90.
8. Team RDC. R: a language and environment for statistical computing. 2.12.1 edition Vienna (Austria): R Foundation for Statistical Computing; 2011.
9. Sing T, Sander O, Beerenwinkel N, Lengauer T. ROCR: visualizing classifier performance in R. Bioinformatics 2005;21:3940-1.
10. Bromberg P. SAGx: Statistical analysis of the GeneChip. R package version 1.24.0 ed2006. Available at: http://home.swipnet.se/pibroberg/expression_hemsida1.html. Accessed July 7, 2012.
11. Nguyen KL, Gillis S, MacGlashan DW Jr. A comparative study of releasing and nonreleasing human basophils: nonreleasing basophils lack an early component of the signal transduction pathway that follows IgE cross-linking. J Allergy Clin Immunol 1990;85:1020-9.
12. Marone G, Poto S, Giugliano R, Bonini S. Studies on human basophil releasability. Int Arch Allergy Appl Immunol 1985;77:103-6.
13. MacGlashan D. Expression of CD203c and CD63 in human basophils: relationship to differential regulation of piecemeal and anaphylactic degranulation processes. Clin Exp Allergy 2010;40:1365-77.
14. Sampson HA, Ho DG. Relationship between food-specific IgE concentrations and the risk of positive food challenges in children and adolescents. J Allergy Clin Immunol 1997;100:444-51.
15. Bock SA, Buckley J, Holst A, May CD. Proper use of skin tests with food extracts in diagnosis of hypersensitivity to food in children. Clin Allergy 1977;7:375-83.
16. Sporik R, Hill DJ, Hosking CS. Specificity of allergen skin testing in predicting positive open food challenges to milk, egg and peanut in children. Clin Exp Allergy 2000;30:1540-6.
17. Peng ZK, Naclerio RM, Norman PS, Adkinson NF Jr. Quantitative IgE- and IgG-subclass responses during and after long-term ragweed immunotherapy. J Allergy Clin Immunol 1992;89:519-29.
18. Malveaux FJ, Conroy MC, Adkinson NF Jr, Lichtenstein LM. IgE receptors on human basophils. Relationship to serum IgE concentration. J Clin Invest 1978;62:176-81.
19. Wanich N, Nowak-Wegrzyn A, Sampson HA, Shreffler WG. Allergen-specific basophil suppression associated with clinical tolerance in patients with milk allergy. J Allergy Clin Immunol 2009;123:789-94, e20.
20. Conroy MC, Adkinson NF Jr, Lichtenstein LM. Measurement of IgE on human basophils: relation to serum IgE and anti-IgE-induced histamine release. J Immunol 1977;118:1317-21.
21. MacGlashan D Jr, Lichtenstein LM, McKenzie-White J, Chichester K, Henry AJ, Sutton BJ, et al. Upregulation of FcepsilonRI on human basophils by IgE antibody is mediated by interaction of IgE with FcepsilonRI. J Allergy Clin Immunol 1999;104:492-8.
22. 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.
23. Boyce JA, Assa'ad A, Burks AW, Jones SM, Sampson HA, Wood RA, et al. Guidelines for the diagnosis and management of food allergy in the United States: report of the NIAID-sponsored expert panel. J Allergy Clin Immunol 2010;126(suppl):S1-58.

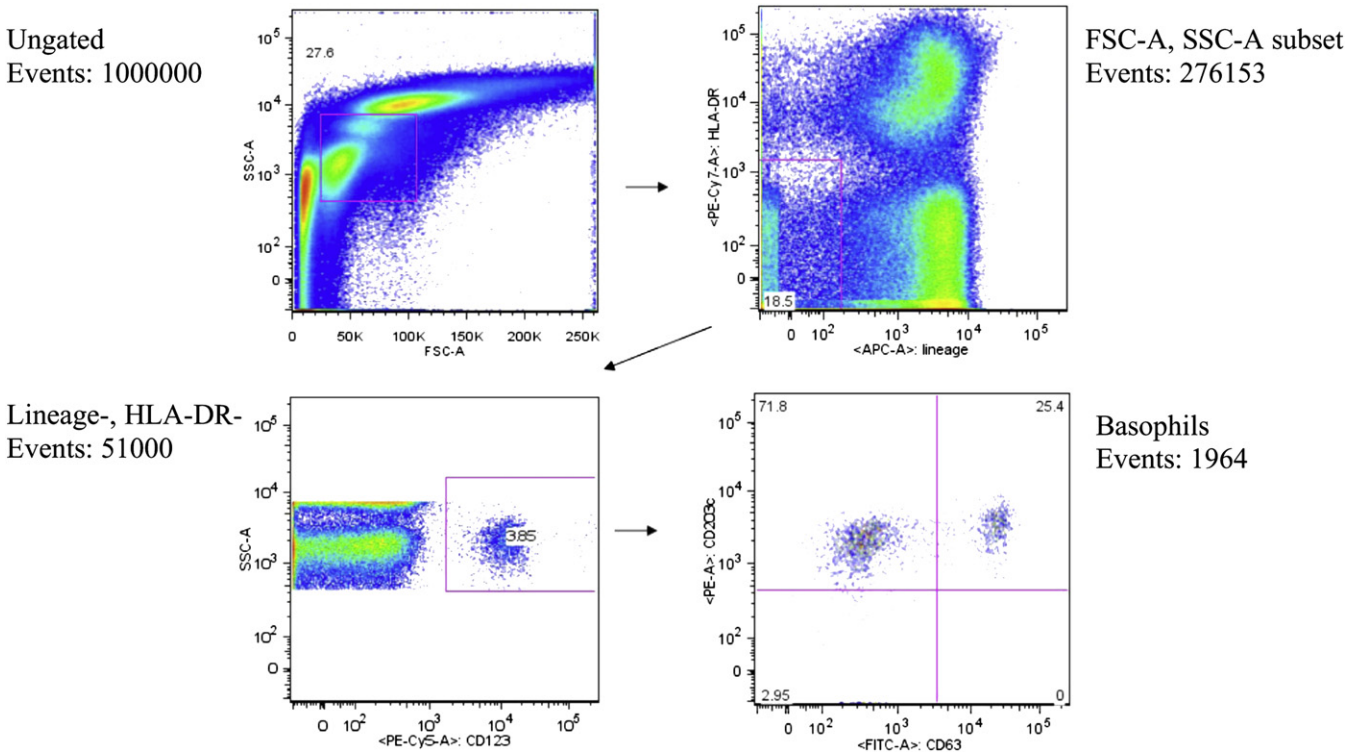

FIG E1. Example of basophil gating strategy. *APC*, Allophycocyanin; *FITC*, fluorescein isothiocyanate; *FSC*, forward scatter; *PE*, phycoerythrin; *SSC*, side scatter.

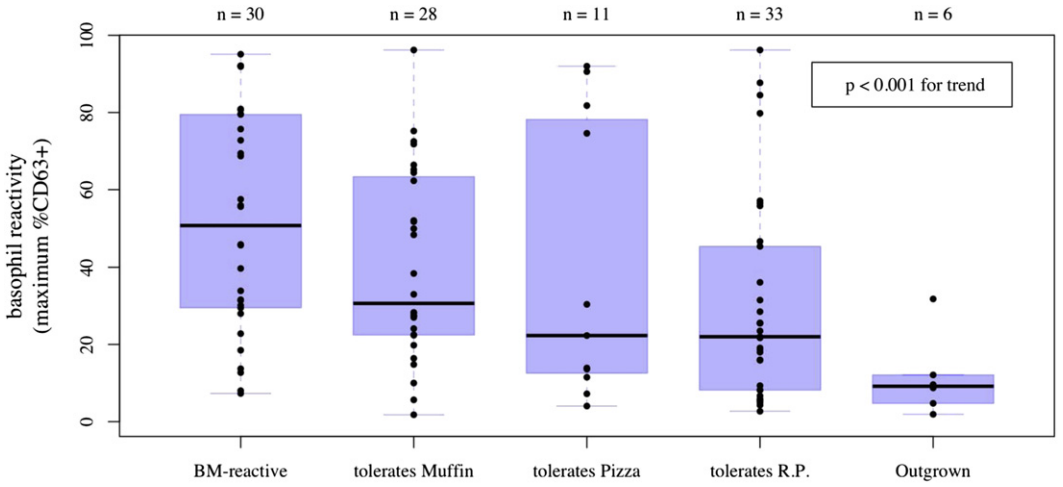

FIG E2. Basophil reactivity to milk by 5 levels of challenge outcome. With the 3 central groups pooled, *post hoc* analysis showed a significant difference between those who reacted to baked milk and those who tolerated it ($P < .01$) and also between those who tolerate baked milk and those who had fully outgrown their milk allergy ($P < .05$). *BM*, Baked milk; *R.P.*, rice pudding.

TABLE E1. Sources of supplies and reagents

SPT			
Bifurcated needles			Precision Medical Products, Denver, Pa
Commercial milk extract			Greer Laboratories, Lenoir, NC
Basophil activation test			
Stimulants			
Alba Nonfat Dry Milk			The Hain Celestial Group, Boulder, Colo
N-fMLP			Fisher Scientific, Pittsburgh, Pa
RPMI 1640 with glutamine			Fisher Scientific
Recombinant human IL-3			R&D Systems, Minneapolis, Minn
Polyclonal anti-IgE antibody			Bethyl Laboratories, Montgomery, Tex
PMA			Sigma-Aldrich, St Louis, Mo
CaI			Sigma-Aldrich
mAbs			
Surface marker	Conjugate dye	Clone	
CD63	FITC	H5C6, murine IgG_1	BD PharMingen, San Jose, Calif
CD203c	PE	97A6, murine IgG_1	Immunotech-Beckman Coulter, Marseille, France
CD123	PC5	9F5, murine IgG_1	BD PharMingen
CD41a	APC	HIP8, murine IgG_1	BD PharMingen
CD3	APC	UCHT1, murine IgG_1	BD PharMingen
CD14	APC	M5E2, murine IgG_{2a}	BD PharMingen
CD19	APC	HIB19, murine IgG_1	BD PharMingen
HLA-DR	PC7	L243, murine IgG_{2a}	BD Biosciences, San Jose, Calif
Other reagents			
EDTA			Promega, Madison, Wis
FACS Lysing Solution			BD Biosciences
Anti-mouse immunoglobulin beads for compensation			BD Biosciences

APC, Allophycocyanin; *CaI*, calcium ionophore; *FITC*, fluorescein isothiocyanate; *N-fMLP*, N-formyl-methionyl-leucyl-phenylalanine; *PC5*, phycoerythrin–cyanin 5; *PC7*, phycoerythrin–cyanin 7; *PE*, phycoerythrin; *PMA*, phorbol 12-myristate 13-acetate.