

Regulation of Syk kinase and FcR β expression in human basophils during treatment with omalizumab

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Background: In human basophils from different subjects, maximum IgE-mediated histamine release and the level of Syk protein expression correlate well. Recent studies suggest that in some patients treated with omalizumab, the response to stimulation with anti-IgE antibody increases. In unrelated studies there is also evidence that the composition of Fc ϵ RI in basophils differs among subjects. This observation raised the possibility that the stoichiometry of FcR β /Fc ϵ RI α is not fixed to a 1:1 ratio and might be modifiable during changes in the basophil's environment.

Objective: We sought to determine whether treatment with omalizumab results in increases in Syk expression and anti-IgE-mediated histamine release and disproportionately alters the relative presence of FcR β and Fc ϵ RI α .

Method: Syk, Fc ϵ RI α , and FcR β expression was monitored during the treatment of subjects with omalizumab.

Results: Treatment with omalizumab reduced histamine release from peripheral blood leukocytes stimulated with cat allergen *in vitro*, but histamine release stimulated with anti-IgE antibody increased 2-fold. Expression of Syk increased 1.86-fold. There was no change in the expression of c-Cbl, a signaling element that is sensitive to the presence of IL-3, and no increase in response to formyl-met-leu-phe (tripeptide), a response that also increases in the presence of IL-3. There was a 60% decrease in the FcR β /Fc ϵ RI α ratio in patients treated with omalizumab.

Conclusions: In the context of previous studies, these studies provide support for a proposal that Syk expression is modulated *in vivo* through an IgE-dependent mechanism and that the ratio of Fc ϵ RI α and β subunits in basophils is influenced by factors extrinsic to the cell. (J Allergy Clin Immunol 2010;■■■■:■■■-■■■.)

Key words: Omalizumab, basophils, anti-IgE, signal transduction

Omalizumab is a therapeutic anti-IgE antibody that has the ability to regulate the expression of atopy by preventing the binding of IgE to both high- and low-affinity IgE receptors without directly inducing aggregation of previously cell-bound

Abbreviations used

FMLP: Formyl-met-leu-phe (tripeptide)

HSA: Human serum albumin

MFI: Mean fluorescence intensity

PIPES: Piperazine-N,N'-bis(2-ethanesulfonic acid)

IgE. Previous studies have shown that treatment results in reductions in cell-surface IgE of 90% to 99%.^{1,2} Because the high-affinity IgE receptor is also regulated by the presence of bound IgE, a reduction in the solution-phase free IgE shifts the steady-state balance of cellular synthesis and loss of Fc ϵ RI on the cell surface.³ The absence of bound IgE favors the loss mechanism, resulting in a marked reduction in the cell-surface expression of Fc ϵ RI. Reductions in free IgE levels need to be marked because basophils and mast cells respond well with less than 1,000 antigen-specific IgE molecules per cell,^{4,5} and the mean total surface IgE (all antigen specificities) level is nearly one quarter million molecules per cell before treatment. If reductions are sufficient, the expectation and functional consequence is that induction of IgE-mediated secretion from mast cells and basophils will be blunted. *In vitro* studies of basophils obtained from treated patients do indeed demonstrate marked blunting of antigen-induced histamine release.¹ However, in a recent study of patients with chronic urticaria being treated with omalizumab, it was noted that histamine release from peripheral blood basophils stimulated with anti-IgE antibody increased during treatment, even though cell-surface IgE levels were reduced.⁶ This was an unexpected result that might have its origins in the nature of chronic urticaria, but based on recent studies of signaling in basophils, there were other possible explanations.

IgE-mediated secretion from human basophils is dependent on a variety of intrinsic and extrinsic influences. A number of signal transduction elements have been demonstrated to be necessary for secretion, but recent studies have suggested that the natural biological variation in IgE-mediated histamine release from basophils in the general population is only concordant with variation in expression of the early tyrosine kinase Syk.^{5,7-9} The expression levels of this nonredundant receptor-associated kinase appear to be rate limiting.⁵ Typical human basophils express 100,000 to 500,000 IgE receptors (the high end found predominantly in atopic subjects) but only express 25,000 molecules of Syk per cell.⁵ In the context of IgE-mediated release initiated by the cross-linking panstimulus anti-IgE antibody, these low levels of Syk might limit full expression of the reaction. In contrast, if the reaction is initiated by specific antigens, it is not as apparent that Syk will be rate limiting because the specific/total IgE ratios in atopic patients average 1%.¹⁰ Therefore in an atopic patient with 250,000 receptors, only 2,500 are occupied with an

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antigen-specific IgE, and the relevant receptor/Syk ratio (1:10) is the reverse of the ratio observed during stimulation with anti-IgE antibody (10:1). However, because a typical reaction is a balance between the rate of activation versus the rate of deactivation in which deactivation occurs independently of Syk,¹¹ even antigenic stimulation might benefit from greater levels of Syk expression.

In human basophils Syk expression is known to be altered by 3 mechanisms. First, IgE-mediated secretion itself results in downregulation.^{12,13} Second, some non-IgE-dependent receptors use Syk as a signaling element and also induce modest downregulation of Syk.^{14,15} Even a non-IgE-dependent receptor, formyl-met-leu-phe (tripeptide) (FMLP) receptor, which does not appear to use Syk for signaling,¹⁶ induces modest loss of Syk.¹⁴ Finally, IL-3 can increase Syk expression, although many other signaling elements are also upregulated.^{5,7,17,18} The IgE-mediated process of Syk loss is interesting because even low levels of receptor stimulation that do not initiate mediator release can induce loss of Syk.¹³ The process is slow¹³ but integrative.^{5,13} The close association between Syk expression and anti-IgE-mediated histamine release suggested the hypothesis that increases in anti-IgE-mediated histamine levels during treatment with omalizumab might result from changes in the expression of Syk.

Recent studies of basophils maturing from CD34⁺ progenitors have suggested another counterintuitive hypothesis.¹⁹ CD34⁺ progenitors expressed 11- to 12-fold more Syk than peripheral blood basophils. When cultured for 3 weeks in IL-3, these cells matured into basophil-like cells that continued to express 11- to 12-fold more Syk than peripheral blood basophils. However, when progenitors were cultured in the presence of a chronic FcεRI-aggregating stimulus, FcεRI expression, Alcian blue staining, and histamine content remained the same, but Syk expression was markedly reduced. These results suggested that if some form of chronic aggregation occurs in patients, then Syk expression would be downregulated. Relief of this chronic aggregation by means of elimination of IgE might reverse the induced downregulation and result in a basophil that expressed higher levels of Syk and was more responsive to a panstimulus like anti-IgE antibody. Treatment with omalizumab offered a means to test this prediction.

Treatment with omalizumab results in changes in the cell-surface expression levels of FcεRI, and previous studies have noted that the subunit stoichiometry, notably the relative amount of FcRβ, appears to differ among subjects expressing very different levels of FcεRI. In human subjects the receptor can be expressed on the cell surface in 2 forms, a heterotrimer (αγ2) or a heterotetramer (αβγ2). There are indications that in basophils (similar data for the mast cell have not been generated, although there is indirect evidence that the relative presence of FcRβ might not be constant²⁰), there is probably a mixture of αγ2 and αβγ2. In the one study in which the relative presence of the β subunit (FcRβ) has been examined, the FcRβ/FcεRIα ratio is correlated with the absolute level of cell-surface expression of FcεRI; that is, a relative increase in β appears to increase surface FcεRI levels.²¹ Based on this prior study, one might predict that omalizumab treatment might reduce the relative expression of FcRβ. Because this subunit can contribute to signaling strength,²² such changes might oppose increases in basophil responsiveness. Therefore at a minimum, relative FcRβ expression needed to be monitored as a potential confounding variable. However, the apparent linkage between the expression of FcRβ and the expression of cell-surface FcεRI does not necessarily imply that the relative amount of FcRβ is a variable that can be manipulated by

treatment. Rather, the β/α ratio might be an intrinsic characteristic of a subject's basophil genotype, and it is unaltered by conditions extrinsic to the basophil. Treatment with omalizumab also offered a way to explore this question. Therefore in conjunction with the assessment of Syk, basophils were examined for changes in the FcRβ/FcεRIα ratio.

METHODS

Reagents and buffers

The [Methods](#) section in this article's Online Repository at www.jacionline.org provides a detailed description of reagents and buffers. These have been described in previous publications.⁵

Analysis of Syk protein expression by means of intracellular flow cytometry

All analysis was performed on a BD FACSCalibur flow cytometer (BD Biosciences, San Jose, Calif). The measurement of Syk protein expression by means of flow cytometry with the anti-Syk antibody 4D10 has been previously described and validated with respect to standard Western blotting.^{9,14} This method has been used on both impure and purified cell fractions, with equivalent results. Briefly, fixed mixed leukocytes were labeled with anti-IL-3 receptor and anti-BDCA2 antibodies (phycoerythrin and fluorescein isothiocyanate, respectively) and permeabilized (Fix and Perm Kit; Caltag, Carlsbad, Calif) in the presence of 4D10 or IgG2a antibody. Cells were then incubated with an anti-mIgG2a-alexa647 antibody to complete the labeling. Syk protein expression is reported as normalized net mean fluorescence intensity (MFI), with the difference between 4D10 and isotype-labeled cells corrected for instrument variability by using CaliBRITE APC calibration beads (BD Biosciences).

Flow cytometry was performed to detect FcεRIα and IgE on the cell surface by using the anti-FcεRIα antibody 22E7 (which detects occupied and unoccupied FcεRIα) and goat anti-IgE antibody, as previously described (see the [Methods](#) section in this article's Online Repository).²³

Omalizumab study

The details of this study have been recently published,²⁴ and further details can be found in the [Methods](#) section of this article's Online Repository. Briefly, adults (18-50 years old) were chosen for their sensitivity to cat allergen. Informed consent was obtained through a protocol approved by the Johns Hopkins Hospital Institutional Review Board and the National Institute of Allergy and Infectious Diseases' Data Safety Monitoring Boards. After testing for inclusion criteria, adults were entered into the study after a 2-week interval and randomized 3.5:1 omalizumab to placebo treatment. On the first day of treatment (BSL), before the first injection of either placebo or omalizumab (according to the manufacturer's dosage schedule), venipuncture and a nasal challenge were performed. The blood was processed by means of separation on single (throughout study period) or double Percoll gradients (only pretreatment and final visits). The cells from the 1-step Percoll gradient were analyzed by means of flow cytometry for cell-surface levels of FcεRIα and IgE and internal levels of Syk or used to assess histamine release in response to stimulation with anti-IgE antibody, cat allergen extract, or FMLP. After entry, subjects were tested for basophil receptor and IgE expression and histamine release on days 3, 11, 17, 23, 30, 36, 44, and 104.²⁴ If the cat allergen response decreased to less than 20% of the basophil response before entry or the day 45 time point was reached first, further testing was stopped until the 14-week assessment. At this midpoint (MID) in treatment, the subject was tested again for the study's various end points. The visit 14 (approximately 104 days) assessment is considered the final measurement (FINAL). At the BSL and FINAL visits, basophils were purified for Western blotting to measure FcεRI α and β, c-Cbl, and Syk. In a subset of patients, flow cytometric Syk measurements were made on a weekly basis.

Western blot analysis

At the BSL and FINAL visits, basophils were purified by means of a combination of 2-step Percoll gradient and negative selection with a basophil

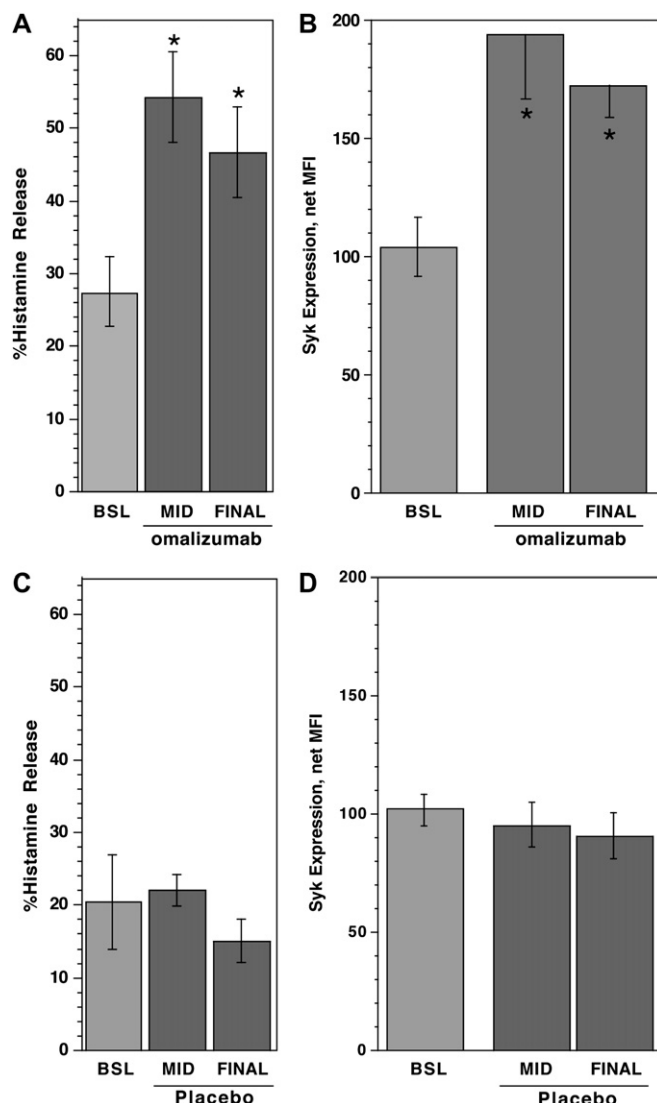


FIG 1. Increases in Syk expression and anti-IgE-mediated histamine release in subjects treated with omalizumab. **A**, Measurement of histamine release induced with an optimal concentration of anti-IgE antibody on pretreatment (day 0, *BSL*), 4 to 6 weeks after the start of treatment (*MID*), and 15 weeks (approximately 104 days) after the start of treatment (*FINAL*) for subjects treated with omalizumab (n = 12). **B**, Measurement of Syk expression in basophils by means of flow cytometry for the same group of subjects in Fig 1, A. **C**, Measurement of histamine release induced with anti-IgE antibody in the placebo group (n = 4). **D**, Measurement of Syk expression by means of flow cytometry in the placebo group. Spontaneous histamine release averaged $3\% \pm 0.3\%$. It was not different between groups and did not change during the course of the study.

cocktail from Stem Cell Technologies (Vancouver, British Columbia, Canada; basophil purification kit) and columns from Miltenyi Biotec (Auburn, Calif).⁵ The purity of basophils was determined by means of Alcian blue staining,²⁵ and basophils generally exceeded 97% purity. Relative to prepurification counts, recovery of purified basophils was greater than 85%. Basophils were lysed in $1 \times$ ESB buffer at a density of 300,000/20 μ L of ESB, boiled for 5 minutes, and stored at -70°C until SDS-PAGE analysis. Samples for individual subjects were stored until both visits 2 and 14 (see below) could be analyzed together. Western blotting, pretreatment (*BSL*), and 104-day (*FINAL*) samples for several proteins, including c-Cbl, was performed in a semiquantitative manner, with calibration standards as described previously.⁵ Briefly, 4 dilutions (1.0, 0.4, 0.15, and 0.05, where 1.0 = undiluted lysate) of a standard preparation of basophils were run with samples in 15-lane 10% gels,

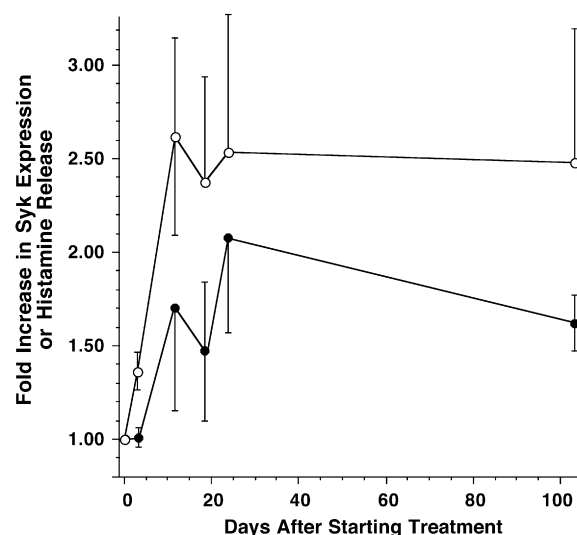


FIG 2. Kinetics of the increase in anti-IgE antibody-induced histamine release and Syk expression. The data are expressed relative to the pretreatment response or levels of Syk expression (n = 6). *Open circles*, Histamine release induced by anti-IgE antibody; *solid circles*, Syk expression measured by means of flow cytometry. Both kinetic curves, taken as a whole, are statistically different than no change ($H_0 = 1.0$, $P < 0.0001$).

with molecular weight markers in the first and last lanes (see Fig E1 in this article's Online Repository at www.jacionline.org). After transfer to nitrocellulose, the nitrocellulose was cut at the 40- and 90-kD horizontal position and the less than 40-kD portion was blotted for FcR β , the greater than 40-kD and less than 90-kD portion was blotted for Fc ϵ R1 α , and the greater than 90-kD portion was blotted for c-Cbl. After blotting with 22E7, the greater than 40-kD and less than 90-kD segment was also blotted with anti-Syk (4D10) antibody. For the *in vitro* studies or receptor expression, lane loading was also checked by blotting with anti-p85 antibody (p85 subunit of phosphoinositide 3-kinase), which we have previously found not to change during culture with IL-3.⁵

RESULTS

Omalizumab study: Histamine release and Syk and FcR β expression

In the omalizumab-treated subjects cell-surface IgE levels on basophils decreased to 0.05 ± 0.02 -fold of the starting level by the FINAL visit (approximately 104 days). There was no change (1.11 ± 0.24 -fold of starting levels) in patients treated with placebo agent. Fig E2 (available in the Online Repository at www.jacionline.org) shows the kinetics of the changes in cell-surface Fc ϵ R1 and IgE values on basophils during treatment. At the final visit on day 104, histamine release induced by optimal concentrations of cat antigen decreased to 0.15 ± 0.12 -fold of pretreatment responses.²⁴ In contrast, histamine release induced by anti-IgE antibody increased ($P = .004$, n = 12; Fig 1, A) in subjects treated with omalizumab and did not change in the placebo group ($P =$ not significant, n = 4; Fig 1, C). Concordant with the increase in histamine release induced by anti-IgE antibody was an increase in Syk expression in basophils from subjects treated with omalizumab (Fig 1, B), which did not increase in basophils from subjects treated with placebo (Fig 1, D). There was a correlation between the fold increase in Syk expression and the fold increase in anti-IgE antibody-induced histamine release of 0.73 ($P = .014$).

In 6 subjects the kinetics of the increase in Syk expression was measured weekly (Fig 2). For the same subjects, Fig 2 shows the time course for the increase in histamine release induced by anti-

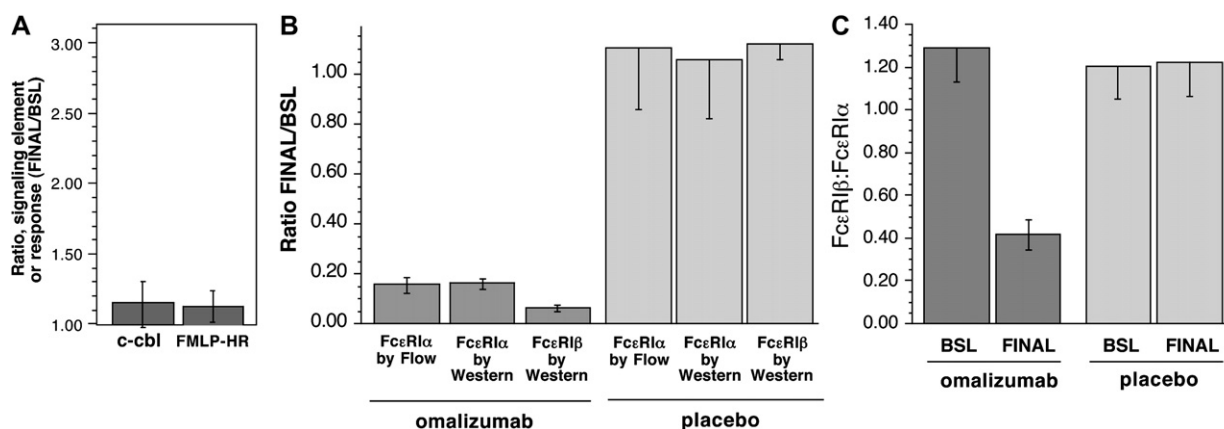


FIG 3. Effect of omalizumab treatment on other IL-3-sensitive characteristics of human basophils. **A**, The ratio of the measured expression of c-Cbl in purified basophils (as assessed by means of quantitative Western blotting) or the ratio of percentage histamine release induced by 1 μ M FMLP. The ratio is calculated as the end point at day 104 (FINAL) divided by the end point before treatment (day 0, BSL). **B**, Expression of cell-surface FcεRIα and bound IgE levels on peripheral blood basophils from omalizumab- or placebo-treated subjects were determined by means of flow cytometry and Western blotting. The ratio of expression level at day 104 (FINAL) to that before treatment (BSL) is plotted ($n = 12$, treated with omalizumab; $n = 4$, treated with placebo agent). **C**, The ratio of expression of FcεRIβ and FcεRIα, as determined by means of Western blot analysis of purified blood basophils from omalizumab- or placebo-treated subjects (BSL and FINAL). All data are expressed as means $\pm$ SEMs.

IgE antibody. The increase in histamine release and Syk expression occurred early in treatment, with the peak increase apparent by 2 to 3 weeks. A similar kinetics for histamine release held for all 12 subjects treated with omalizumab (data not shown).

As we have observed previously, plasmacytoid dendritic cells, a type of leukocyte that also expresses FcεRI, display 30-fold greater Syk expression than peripheral blood basophil.¹⁹ Fig E3 (available in this article's Online Repository at www.jacionline.org) shows that plasmacytoid dendritic cell expression of Syk did not change in either the treated or placebo groups.

Histamine content in basophils did not change (1.21 ± 0.16 vs 1.28 ± 0.04 pg per basophil, pretreatment vs 104 days, respectively). The number of Alcian blue-positive cells in Percoll-separated preparations did not change: $24,000 \pm 5,000$ /mL of blood at pretreatment and $26,000 \pm 5,000$ /mL at visit 14 (15 weeks), for an average ratio of counts at the final visit (approximately 104 days) versus pretreatment in the omalizumab group of 1.23 ± 0.13 ($P = .106$).

Our previous studies of Syk expression in peripheral blood basophils have noted that expression ranges from near zero MFI units to 200 to 250 MFI units (with the mean at 110 MFI units).⁹ Fig E4 (available in this article's Online Repository at www.jacionline.org) shows that there was a relationship between the fold upregulation of Syk in subjects receiving omalizumab relative to their starting Syk levels. If Syk were expressed at levels already considered the high end for the general population, then treatment with omalizumab did not increase expression further.

Previous studies have shown that *in vitro* culture of peripheral blood basophils with 10 ng/mL IL-3 will alter signaling element expression patterns.⁵ Therefore the observed increase in Syk expression in basophils during treatment with omalizumab might result from increases in IL-3 levels. However, previous studies have also noted that IL-3 induces increased expression of several other signaling elements, including those associated with negative regulation of the signaling. If the Syk increase resulted from an increased presence of IL-3 or a cytokine with similar functional

effects (eg, IL-5), then there should also be increases in c-Cbl or FcεRIβ values.^{5,21,26} For example, previous studies have shown that *in vitro* c-Cbl, FcεRIβ, and the histamine response to FMLP increased 2.7-, 3.0-, and 2.1-fold, respectively, in the presence of IL-3.^{5,21,26,27} However, no change in basophil c-Cbl expression was observed in the omalizumab-treated group (and no change was observed in the placebo group; Fig 3, A). In addition, there was no change in the basophil response to FMLP stimulation (Fig 3, A).

In vitro incubation of basophils with IL-3 has also been noted to increase the expression of the β subunit (FcεRIβ) of FcεRI.^{21,26} In our previous study of FcεRIβ and FcεRIα expression in human basophils, the expression levels were determined by lysing cells and analyzing total p60 FcεRIα or FcεRIβ levels by means of Western blotting.²¹ This same methodology was used in the current study, but the method was improved by including a 4-point dilution series of a basophil standard within each Western blot. This was necessary because the Western blot was nonlinear throughout the possible range of receptor expression found at different points of the omalizumab study. Fig E1 (available in this article's Online Repository at www.jacionline.org) shows one example Western blot of samples made before and after treatment with omalizumab. Fig 3, B and C, summarizes the results for changes in both the broad p60 FcεRIα band, which represents the cell-surface receptor, and the FcεRIβ band. The decrease in p60 levels determined by means of Western blotting is similar to the decrease in surface FcεRI levels, as detected by means of flow cytometry. Expression of p60 at the FINAL visit (approximately 104 days) decreased to $17\% \pm 2\%$ of pretreatment levels irrespective of whether analyzed by means of flow cytometry or densitometric analysis of the semiquantitative Western blot of p60 ($P < .0001$ compared with pretreatment baseline [BSL], $P =$ not significant when comparing flow and Western methodologies for measurement of FcεRIα). The levels were unchanged in the placebo (4 subjects) group. In contrast, after the FINAL treatment visit, the expression of FcεRIβ decreased to $8\% \pm 3\%$ of the levels observed before

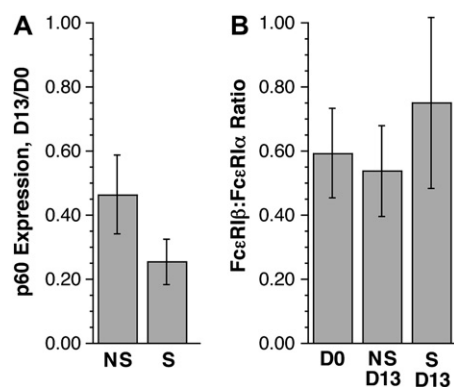


FIG 4. Absence of change in FcRβ/FcεRIα ratio *in vitro*. Purified basophils were cultured in RPMI-1640 with IL-3 (10 ng/mL) for 13 days (D13) either without (NS) or with (S) prior lactic acid treatment to remove endogenously bound IgE. After 13 days of culture, cells were counted and lysed, and lysates were analyzed by using the semiquantitative Western blot procedure used in the omalizumab study to quantitatively assess expression levels. **A**, Expression of p60, relative to preculture levels (D0) for nonstripped (NS) and stripped (S) cells ($n = 5$; $P = .12$ | $H_0 = 1.0$ for nonstripped cells and $P = .0005$ | $H_0 = 1.0$ for stripped cells). **B**, Ratio of FcRβ/FcεRIα for day 0 (D0) and nonstripped (NS) and stripped (S) cells on day 13 (D13; $n = 5$; $P =$ not significant for comparison of 3 groups). Errors shown represent SEMs.

treatment ($P = .0025$ when comparing the change in FcRβ and FcεRIα by means of Western blotting). Again, there was no change observed in the placebo-treated group. When the data were expressed as a FcRβ/FcεRIα ratio (p60), the ratio at the FINAL treatment visit decreased to 0.43-fold the ratio observed before treatment ($P = .0004$ | $H_0 = 1.0$). There was no change in the ratio in the placebo-treated group. By using the same nitrocellulose blots and blotting for Syk expression, the ratio of FINAL/BSL for the omalizumab group was 1.55 ± 0.19 ($P = .013$; H_0 | ratio = 1.0), recapitulating the results obtained by means of flow cytometry. There were 2 collateral observations that were worth noting: the heterogeneity of glycosylation of the p60 cell-surface FcεRIα was found to decrease after treatment, and there was a modest increase in the presence of the internal immature FcεRIα p46 (see the Results section in this article's Online Repository at www.jacionline.org).

***In vitro* induced changes in FcRβ/FcεRIα expression**

The change in relative FcRβ expression in patients treated with omalizumab raised a question of whether the change would occur when receptor downregulation occurred in the absence of free IgE. Purified peripheral blood basophils were cultured for 13 days in the absence of IgE to induce downregulation of FcεRI. To accelerate the process of receptor loss, a portion of the cells were first briefly treated with mild lactic acid buffer to gently dissociate endogenous IgE. To enhance survival of the basophils for 13 days, the cultures contained 10 ng/mL IL-3. Lysates on days 0 and 13 were run in a semiquantitative Western blot to also assess relative changes in FcRβ/FcεRIα ratios. For these experiments, the change in receptor expression is not as marked as that seen in the omalizumab study, but Fig 4, A, shows that the decrease in receptor expression was statistically significant for both "nonstripped" and "stripped" cells. The nonstripped cells did experience the expected 50% average decrease in FcεRI expression found in previous studies.²⁸ There was no change in the FcRβ/FcεRIα

ratio for either group of cells (Fig 4, B). These results suggest that simply downregulating the receptor in the presence of IL-3 is not sufficient to alter the FcRβ/FcεRIα ratio.

DISCUSSION

Treatment with omalizumab results in a relatively rapid increased expression of Syk in peripheral blood basophils that is concordant with an increase in histamine release induced by anti-IgE antibody. As noted above, incubation of peripheral blood basophils with IL-3 is the only known mechanism by which Syk expression is induced to increase. Cultures in IL-3 and IL-5 have also been shown to alter a variety of other signaling elements and to generally enhance the response of basophils to a wide variety of stimuli.^{5,21,26,27} IL-3 induces an increase in c-Cbl expression that is 6-fold greater than the induction of Syk expression. IL-3 has also been shown to markedly enhance mediator release induced by the G protein-linked receptor for FMLP. Treatment with omalizumab caused neither an increase in c-Cbl expression nor responsiveness to FMLP. These results were therefore not consistent with the increase in Syk expression resulting from exposure of basophils to cytokines like IL-3. In addition, treatment with omalizumab not only did not increase the relative expression of FcRβ, but expression was suppressed relative to the expected decrease in FcεRI. This result suggests that treatment with omalizumab actually results in a suppression of a cytokine environment that promotes balanced expression of FcRβ and FcεRIα. Previous studies of omalizumab treatment have also indirectly suggested that the presence of T_H2-associated cytokines (eg, IL-3 and IL-5) might decrease because the number of tissue T cells is found to decrease.²⁹⁻³² Whether this change is reflected globally has yet to be shown.

The secondary goal of this study was to determine whether the FcRβ/FcεRIα ratio in peripheral blood basophils was a constant feature (an intrinsic characteristic) of a subject's FcεRI expression or whether it could be induced to change by altering the environment of the basophil. The ability to reduce free circulating IgE levels with omalizumab offered a way to induce a reduction in basophil FcεRI levels, to potentially alter the atopic environment of the patient by changing the presence of T_H2-like cytokines or by other mechanisms not yet defined, or both. The study showed that there was a disproportionate decrease in the expression of FcRβ relative to the decrease in total FcεRIα levels. This result leads to the conclusion that the relative presence of FcRβ is sensitive to factors extrinsic to the basophil.

This study cannot directly address whether the changes in FcRβ/FcεRIα ratio reflect a change in the stoichiometry of the cell-surface receptor. Underlying the ambiguity is the possibility that there is some FcRβ inside the cell or associated with other receptors (for convenience, both possibilities will be covered under the single term "internal," but as noted in the Online Repository, expression levels associated with other receptors would not be expected to change), and the Western blotting method does not discriminate internal versus surface subunit expression (unlike the case for FcεRIα). In addition, the relative efficiency of the 2 antibodies used to detect FcεRIα and FcRβ is unknown. However, there are 2 closely related lines of reasoning, as shown in Fig E5 (available in this article's Online Repository at www.jacionline.org), that suggest that the ratio might reflect differences in surface receptor stoichiometry. In other words not only is total FcRβ disproportionately suppressed, but it seems

TABLE I. Comparison of changes in IgE density on basophils after omalizumab treatment for the 1996 (phase 1) trial and the current 2008 study

Study	IgE/basophil		Fraction
	Before	After	
1996 phase 1 trial	240,000	<2,200	0.009
2008 (current)	134,000	6,800	0.051

likely that surface stoichiometry is suppressed during treatment with omalizumab.

If the increase in Syk expression was not consistent with the other properties of exposure of basophils to cytokines like IL-3, an increase in Syk expression could result from relief from a downregulatory mechanism like that which follows aggregation. As noted in the introduction, a recent study from our group has shown that chronic exposure of basophils developing from CD34⁺ progenitors to an aggregating stimulus results in cells that express normal levels of histamine, granularity, and surface FcεRI but reduced expression of Syk.¹⁹ In the current study treatment with omalizumab did not alter histamine content or the number of Alcian blue–positive cells, a result consistent with the predictions of the *in vitro* studies.¹⁹ However, omalizumab reduces free IgE levels sufficiently to markedly downregulate FcεRI and especially cell-surface IgE levels.^{1,33,34} We could speculate that these conditions might be sufficient to relieve a chronic, ongoing IgE-dependent aggregation of maturing basophils, if it occurs *in vivo*, and result in an increase in Syk expression without other changes in the basophil phenotype. The increase occurred relatively quickly and was evident within the first 2 to 3 weeks. Depending on the maturation time for basophils developing in the bone marrow, some delay would be expected, perhaps as short as 3 days, but a change was likely within 1 to 2 weeks. This expectation is drawn from our studies of FcεRI changes¹ and the understanding that the kinetics of the receptor changes reflect the maturation kinetics.³⁵ There is not yet direct evidence that reversal of a posttranslational mechanism like ubiquitylation (a process that is described for Syk loss in mature basophils¹³) is operative in this situation or whether Syk changes result from pretranslational events, such as upregulation of mRNA levels.

There remain several issues with this explanation, however. Most notable is that low Syk expression in basophils are found in both nonatopic and atopic subjects. There would be no *a priori* reason for nonatopic subjects' basophils to experience chronic IgE-dependent aggregation, but this assumption might be false. It has been noted that both atopic and nonatopic sera contain various levels of anti-IgE and anti-FcεRIα antibodies.^{6,36} The functional relevance of these antibodies in a wide variety of subjects is not currently understood. One hypothesis is that having these autoantibodies does result in a functional outcome that is not always to induce disease, as proposed for some patients with chronic urticaria,³⁶ and that outcome is the downregulation of Syk in maturing basophils.

The increase in responsiveness to stimulation of basophils with anti-IgE antibody is different from our previous experience with omalizumab treatment.¹ During manuscript preparation, another new published study of omalizumab treatment found no changes in the basophil response to anti-IgE antibody.³⁷ However, in that study the starting response was nearly as high as one can find in basophils (>75%), and therefore there was little room to observe

an increase. In a previous phase I trial of omalizumab, the response to anti-IgE antibody decreased approximately 50%.¹ However, we made note of important differences between the 2 studies; Table I summarizes the differences in IgE densities between these studies. The cause of the differences might be the mode of administration of omalizumab (intravenous vs subcutaneous) and an average lower level of dosing under the current dosing table regimen relative to the dosing achieved in the phase I trial. Several previous studies have shown that basophils require approximately 2,000 cell-surface IgE molecules to achieve 50% of their maximal responsiveness.^{4,5} A density of 3,000 to 5,000 is sufficient for a maximal response. In the phase I trial total cell-surface IgE levels decreased to unmeasurable levels for many subjects. Based on the technique used at that time, an average upper limit of 2,200 molecules could be calculated. Therefore the finding that stimulation with anti-IgE antibody was suppressed by 50% was expected. In the current study there are approximately 6,800 total IgE molecules per basophil, a density sufficient for a maximal response. If the response is augmented by increased levels of Syk expression, then anti-IgE becomes a useful tool to detect these increases provided suppression is not too great. The current study did observe a decrease in response to cat allergen. However, this might be expected because cat-specific IgE densities would be low. For example, the median cat-specific IgE/total IgE ratio was 4.2% (somewhat higher than the expected 1% for the general population with cat allergy¹⁰), which would predict around 290 molecules of cat-specific IgE per basophil. This level is far less than the density required to support a strong histamine release response. Although the increase in Syk expression would be expected to mitigate the suppression caused by reduced receptor expression, the densities of cat-specific IgE were probably too low for the Syk increase to markedly overcome the suppression produced by a decrease in the density of the relevant IgE. However, one might speculate that stimulation with multiple antigens might reach high enough levels at which the increase in Syk expression exerts enough influence to effectively blunt the efficacy of treatment with omalizumab. These results suggest another possible reason for treatment failure.

If *in vitro* studies are a guide,²² the relative absence of FcRβ as a component of the cell-surface receptor would reduce the efficacy of aggregates (on a per aggregate basis) to initiate the IgE-dependent signaling cascade that leads to mediator release and other functions. This change in FcRβ expression might have mitigated the effects of increased Syk expression, but the fact that histamine release was also enhanced suggests that there was little counterbalancing effect by the expression of less FcRβ.

We thank Meghan Sterba and Valerie Alexander for technical assistance.

Key messages

- Treatment of subjects with cat allergy with omalizumab results in increased expression of Syk in circulating basophils. One interpretation is that chronic aggregation of FcεRI *in vivo* is relieved by the reduction in FcεRI expression that accompanies treatment.
- Treatment with omalizumab results in changes to the basophil response that increase its intrinsic sensitivity to stimulation, a change that is counterbalanced by a large reduction in cell-surface antigen-specific IgE.

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METHODS

Reagents and buffers

The following were purchased: piperazine-N,N'-bis(2-ethanesulfonic acid) (PIPES), BSA, EDTA, D-glucose, and erythrosin B (Sigma-Aldrich, St Louis, Mo); crystallized human serum albumin (HSA; Miles Laboratories, Elkhart, Ind); FCS (BioWhittaker, Walkersville, Md); Percoll (Pharmacia, Piscataway, NJ); anti-BDCA-2/CD303 (AC144; Miltenyi Biotec, Auburn, Calif); anti-IL-3 receptor/CD123 (9F5) phycoerythrin-conjugated; anti-mouse IgG₁ Alexa 647, anti-mouse IgG2a Alexa 647, and anti-mouse IgG2a (Molecular Probes/Invitrogen, Carlsbad, Calif); anti-human IgE monomeric IgM (6061P; Hybridoma Reagent Laboratory, Baltimore, Md); and horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin antibody and horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin antibody (Amersham Life Science, Arlington Heights, Ill). The anti-FcεRIα antibody 22E7 was a gift from HoffmanLaRoche (Nutley, NJ). Anti-Syk antibody (4D10) was from Santa Cruz (Santa Clara, Calif), and anti-FcεRIβ was from Upstate Biologicals, Inc (Upstate, NY).

PIPES-albumin-glucose (PAG) buffer consisted of 25 mmol/L PIPES, 110 mmol/L NaCl, 5 mmol/L KCl, 0.1% glucose, and 0.003% HSA. PAGCM was PAG supplemented with 1 mmol/L CaCl₂ and 1 mmol/L MgCl₂. Labeling with antibodies for flow cytometry was conducted in PAG with 1 mmol/L EDTA containing 0.25% BSA in place of 0.003% HSA. ESB is Novex electrophoresis sample buffer containing 5% 2-mercaptoethanol. Countercurrent elutriation was conducted in PAG containing 0.25% BSA in place of 0.003% HSA. Lactic acid buffer for removing endogenous cell-bound IgE was 0.01 mol/L lactic acid, 0.14 mol/L NaCl, and 0.005 mol/L KCl, pH 3.9.^{E1}

Flow cytometry of FcεRI expression

In previous studies we have calibrated the measurement of cell-surface FcεRIα that is detected by means of flow cytometry with the anti-FcεRIα murine mAb 22E7 (which detects occupied and unoccupied FcεRIα) to report absolute densities of cell-surface receptor in some experiments.^{E2} Briefly, the flow cytometric measurements were calibrated by examining the fluorescence staining of 6 donors' basophils that spanned a moderate range of staining intensities (ultimately calibrated to be 8,000 to 140,000 FcεRI per basophil) and simultaneously assessing receptor or IgE density by using the acetate elution method.^{E2} 22E7 labeling was compared with total FcεRI density by means of acetate elution (after sensitizing with PS myeloma IgE to fully sensitize all surface receptors so that IgE effectively measured total surface receptor was linear), with an *R* value of 0.963. In pilot studies the effect of fixing the cells before labeling was examined. Mixed leukocytes obtained from a 1-step Percoll preparation were fixed according to the manufacturer's instructions (Fix and Perm Kit; Caltag, Carlsbad, Calif). After fixation, the cells were stored overnight in elutriation buffer (PAG containing 0.25% BSA in place of 0.003% HSA). The overnight blocking step was found to be necessary if the calibration of 22E7, as described above, was to be considered valid. With this step, no difference in calibration was detected.

Basophil purification

For *in vitro* cultures, residual cells of healthy donors undergoing leukapheresis were enriched in basophils by using a combination of Percoll density gradients and countercurrent-flow elutriation, as previously described.^{E3} The purity of basophils was determined by means of Alcian blue staining,^{E4} and basophils purified from leukapheresis packs generally exceeded 99% purity.

Omalizumab study

For more information, see Eckman et al.^{E5} Adults (18 to 50 years old) were chosen on the basis of the following inclusion criteria: positive skin puncture and RAST results to cat allergen (Hollister-Stier), perennial (with or without seasonal rhinitis) or seasonal rhinitis history, body weight and total IgE level (30–700 IU) compatible with the manufacturer's omalizumab dosing table, and mild asthma (FEV₁ >80%) with inhaled corticosteroids allowed. Exclusion criteria were no immunotherapy for the past 5 years, no intranasal steroids or cromolyn allowed, no history of malignancy, anaphylaxis, or β-blocker use. In addition, subjects were required to respond to nasal allergen provocation

with 5 or more sneezes or a 3-fold increase in mediator release (in nasal lavage fluids) and peripheral blood basophil responses to cat allergen challenge with greater than 20% histamine release. Informed consent was obtained by using a protocol approved by the Johns Hopkins Hospital Institutional Review Board and the National Institute of Allergy and Infectious Diseases' Data Safety Monitoring Boards. After testing for inclusion criteria, adults were entered into the study after a 2-week interval and randomized 3.5:1 omalizumab to placebo treatment. On the first day of treatment, before the first injection of either placebo or omalizumab (according to the manufacturer's dosage schedule), venipuncture and a nasal challenge were performed. The blood was processed by means of separation on single (throughout the study period) or double Percoll gradients (only pretreatment and final visits). The cells from the 1-step Percoll gradient were analyzed by means of flow cytometry for cell-surface levels of FcεRIα and IgE and internal levels of Syk or used to assess histamine release in response to stimulation with anti-IgE antibody, cat allergen extract, or FMLP.

After entry, subjects were tested for basophil receptor and IgE expression and histamine release on days 3, 11, 17, 23, 30, 36, 44, and 104 (these represent the averages for the 16 subjects).^{E5} If the cat allergen response decreased to less than 20% of the basophil response before entry or the day 45 time point was reached first, further testing was stopped until the 14-week assessment. At this midpoint in treatment, the subject was tested again for the study's various end points, and this assessment is called, for the purposes of this manuscript, the MID study measurement. The visit 14 (approximately 104 days) assessment is considered the FINAL measurement. Changes in other measured parameters have been reported elsewhere^{E5}; only data relevant to changes in Syk expression (or measurements relevant to the Syk study) are reported here.

RESULTS

Kinetics of changes in basophil surface FcεRI and IgE levels

Fig E2 shows the kinetics of the changes in surface IgE and FcεRI density for the omalizumab treatment and placebo groups. At the start of treatment, basophils expressed an estimated 134,000 receptors (and surface IgE). The decreases observed at day 3 were statistically significant in the omalizumab group, as were subsequent measurements for this group. There were no statistically significant changes in the placebo group.

Syk expression in plasmacytoid dendritic cells

Fig E3 shows the absence of changes in Syk expression in dendritic cells.

Increases in Syk expression

Fig E4 shows the inverse relationship between the starting level of Syk expression and the increase that follows treatment with omalizumab.

Example of semiquantitative Western blotting

Assessment of p60 and p46 FcεRIα and FcRβ expression levels was based on a semiquantitative Western blot method. A pool of purified basophil lysates at a cell density appropriate for the expected range of expression in the study was established before the start of the study, and aliquots were frozen for use in the study. Because the range of receptor expression was expected to be large, 4 dilutions (1.0, 0.4, 0.15, and 0.05) of the basophil standard were included in each 10% gel. Multiple exposures of the ECL visualization were made (5, 10, 20, 40, 60, 120, and 240 seconds). Fig E1 shows 2 of this series of exposures for both FcεRIα (p46 and p60) and FcRβ. The goal was to analyze a sample band at an exposure for which 2 standards bracketed the band intensity. Each digitized sample band was analyzed for 2 different

exposures, with the density of the bands not exceeding the saturation limit of the film (ie, on a linear portion of the exposure curve). Sample data were expressed in units relative to the standard. It is notable that the measurement of p60 by means of the Western blot method accurately matched the measurement made by using flow cytometry, providing assurance that the Western methodology was semiquantitative.

Omalizumab study: Changes in p46 and p60 glycosylation heterogeneity

Fig E1 shows the canonical presence of both the immature (p46) and mature (p60) cell-surface form of FcεRI. The pretreatment p46/p60 ratio was 0.86 ± 0.07 . The amount of p46 increased by 1.2 ± 0.10 -fold in the omalizumab treatment group ($P = .021$, median test against the placebo group) and 0.95 ± 0.05 -fold in the placebo group. It was also noted that the relative glycosylation of the p60 band changed during treatment. This was an unexpected outcome. Normally, the heterogeneity of surface FcεRIα glycosylation is marked (Fig E1). This heterogeneity is observable with mAbs and polyclonal antibodies, and if the surface receptor is biotinylated, detection with streptavidin shows a similar heterogeneity (data not shown). However, after treatment with omalizumab, the band narrows to look like most other cytosolic proteins or the p46 band. The centroid of the narrow band is the dominant p60 region often observed in non-treated patients, where it is sometimes visually obvious and sometimes obscured by the normal broad heterogeneity. This band is also detected by other mAbs to FcεRIα, such as CRA-1 (eBioscience, San Diego, Calif). The visual impression of homogeneity was unrelated to the exposure time of the ECL blots, and the width/height ratio was calculated to provide a rough assessment of this change for statistical analysis (the width measured at 50% of net peak of the densitometric profile). The pretreatment (BSL) width/height ratio was 0.65 ± 0.07 and 0.21 ± 0.04 on day 104 (FINAL; $P < .0001$ for the comparison) in the omalizumab group and 0.78 ± 0.12 and 0.77 ± 0.11 before treatment and at day 104, respectively, in the placebo group ($P =$ not significant). Not shown is that dilutions of the Western blot standard did not show any changes in the width/height ratio; that is, this was not simply an issue related to exposure of the film when band densities were weak (Fig E1).

The question of internal FcRβ

As noted in the manuscript, internal FcRβ refers to any FcRβ that is not associated with cell-surface FcεRIα, either internal forms or FcRβ associated with other receptors. In our original study of the β/α ratio, the β/α ratio ranged 50-fold.^{E6} The lowest β/α ratios occurred in cells with the lowest expression of receptor. For example, low surface receptor expression is on the order of 10,000 per cell, whereas at the high end, it can be 500,000 per cell. The numerical concern is that to maintain the 1:1 stoichiometry as the surface receptor increases 50-fold and to observe the 50-fold change in ratio, the amount of FcRβ must increase by 2,500-fold (50-fold change in ratio $\times$ 50-fold change in surface

receptor), and 98% of the increase in FcRβ would be “internal.” Expression of the p46 form of the receptor is not markedly different between the high- and low-end surface expression, and therefore the massive increase in internal FcRβ would have to occur without concomitant p46 FcεRIα. In addition, if some FcRβ were associated with other receptors, it is not clear why it would increase because there would not be an expected quantitative relationship between increases (or decreases) in cell-surface FcεRI and increases in these other potential receptors.

A similar algebraic argument is derived from the current study and is graphically illustrated in Fig E5. The current study provides several useful numbers in which to estimate how the internal FcRβ would have to change to maintain a cell-surface 1:1 stoichiometry (Fig E5; values from the study are back-highlighted in gray). For convenience in this calculation, define 1 unit of expression as the amount of FcεRIα (p60) at the start of the study. Therefore at 1:1 stoichiometry, there will be 1 unit of cell-surface FcRβ. The first algebraic relationship uses the observation that the ratio of FcRβ at the end of the study to FcRβ at the start of the study is 0.067. It also uses the fact that the cell-surface FcεRIα (p60) level decreases to 0.17 units (relative to the beginning of the study). Therefore to maintain the 1:1 stoichiometry, cell-surface FcRβ would also be 0.17. Fig E5 shows the algebra, using “x” and “y” as place holders for the amount of unknown internal FcRβ. This allows one to establish the algebraic relationship between x and y ($y = 14.92x + 1.54$). For example, if $x = 0$ (no internal FcRβ at the end of the study), then $y = 1.54$ units of internal FcRβ at the start of the study (which is twice the amount of internal p46, which we know to be 0.86 units at the start of the study, see above). Note that this use of the term “unit” is not dependent on the relative efficacy of the antibodies that detect the 2 subunits because we have defined a unit on the basis of the α expression level, and the argument is that 1:1 stoichiometry is maintained (1 unit of each subunit) on the cell surface. The point of this analysis for the counterargument is that for every unit of FcRβ that is internal at the end of the study, there would need to be 16-fold more internal FcRβ at the start of the study, levels that considerably exceed the amount of internal (p46) FcεRIα.

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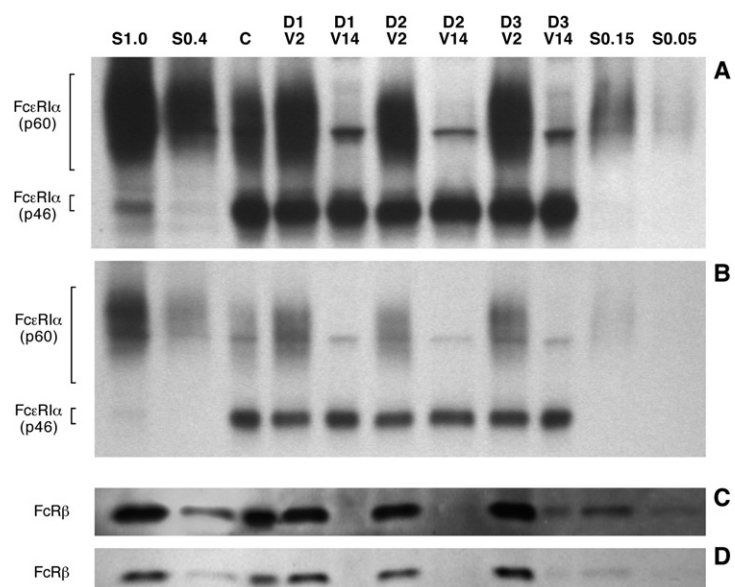


FIG E1. Relationship between the pretreatment level of Syk expression and the fold increase in Syk expression at the FINAL visit (approximately day 104). Each point represents a different subject treated with omalizumab.

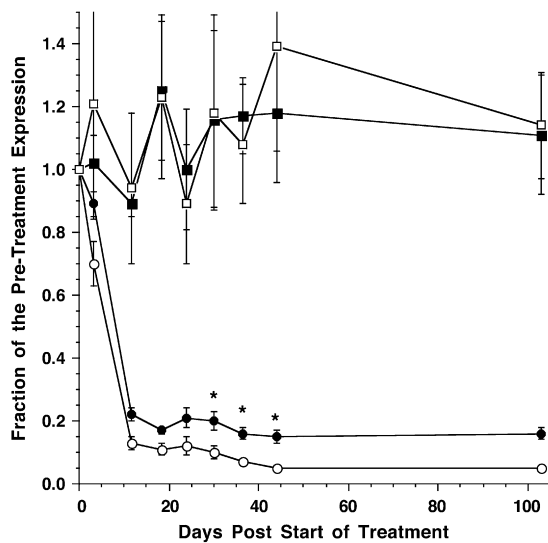


FIG E2. Example Western blots. After cutting the nitrocellulose at the 40- and 95-kd line and blotting with 22E7 (for FcεRIα) or anti-FcεRIβ, the blots were visualized with ECL reagent, and multiple exposures were made. **A** and **B** show 2 of 7 exposures for FcεRIα, and **C** and **D** show 2 exposures for FcεRIβ. At the top, *S1.0*, *S0.4*, *S0.15*, and *S0.05* are the standard dilutions; *C* is a control sample; *D1* to *D3* are donors 1, 2, and 3; *V2* is visit 2 (before treatment); and *V14* is visit 14 (15 weeks).

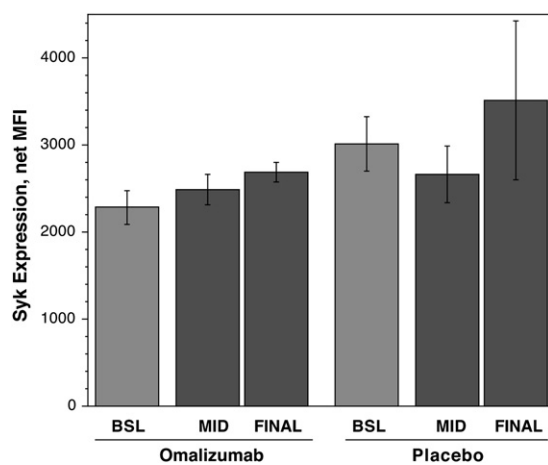


FIG E3. Kinetics of the change in surface IgE and FcεRI expression on peripheral blood basophils during treatment with omalizumab. Cell-surface IgE and FcεRI levels were monitored throughout the treatment. The *asterisks* over days 30 to 45 in the omalizumab-treated group signify the subset of subjects who were tested at visits later than visit 5 because their histamine release response had not decreased sufficiently to reach the midstudy stopping criterion, and therefore “n” was variable. Surface IgE (*open circles*) and surface FcεRI (*solid circles*) levels in the omalizumab group and surface IgE (*solid squares*) and surface FcεRI (*open squares*) in the placebo group are shown.

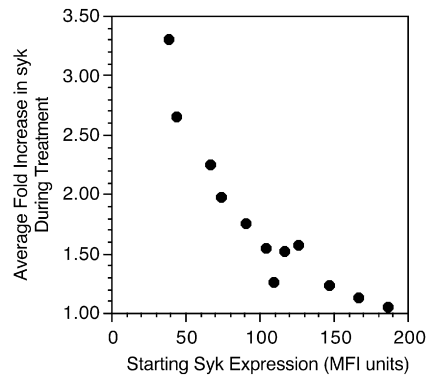


FIG E4. Syk expression in plasmacytoid dendritic cells during treatment with omalizumab. Syk expression was assessed by means of flow cytometry for the omalizumab and placebo treatment groups at pretreatment (day 0, *BSL*), 4 to 6 weeks after the start of treatment (*MID*), and 15 weeks (approximately 104 days) after the start of treatment (*FINAL*) for subjects treated with omalizumab ($n = 12$) or placebo ($n = 4$).

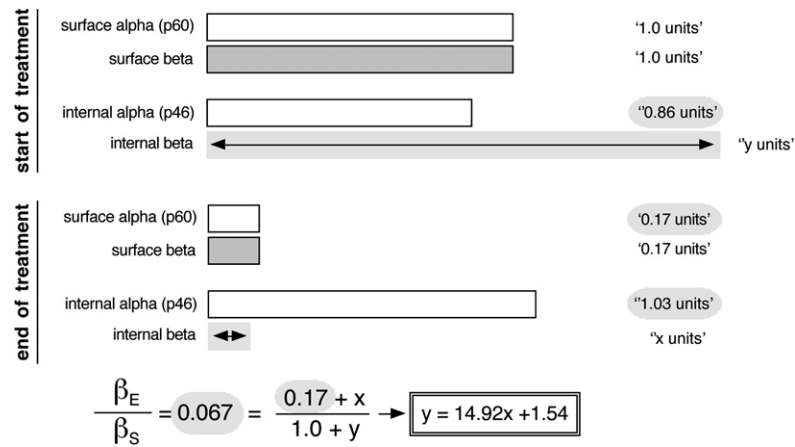


FIG E5. Visual representation of the expression levels of internal and cell-surface receptor subunits. The *horizontal columns* represent the relative expression of either FcεRIα (p60 or p46) and relative expression of a hypothesized internal and cell-surface FcRβ. The numbers highlighted with a gray background are obtained from the averages of the 12-subject population data. Both the internal and cell-surface columns for FcRβ are variables: *x* for cell-surface FcRβ, and *y* for internal FcRβ. In the equation β_E is the end-of-treatment FcRβ expression, and β_S is the start-of-treatment total FcRβ expression.