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Regulation of Syk Kinase and FcR β Expression in Human Basophils During Treatment with Omalizumab

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Abstract

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 1:1 and might be modifiable during changes in the basophil environment.

Objective—To determine if treatment with omalizumab results in increases in syk expression, 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 FMLP, 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* by an IgE-dependent mechanism and that the ratio of Fc ϵ RI alpha and beta subunits in basophils is influenced by factors extrinsic to the cell.

Keywords

omalizumab

Introduction

Omalizumab is a therapeutic anti-IgE antibody that has the ability to regulate 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 IgE. Previous studies have shown that

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treatment results in reductions in cell surface IgE of 90–99%^{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 FcεRI on the cell surface³. The absence of bound IgE favors the loss mechanism resulting in a marked reduction in the cell surface expression of FcεRI. Reductions in free IgE need to be marked because basophils and mast cells respond well with less than 1000 antigen-specific IgE molecules per cell^{4, 5} and the mean total surface IgE (all antigen specificities) is nearly one-quarter million per cell prior to 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 release¹. 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 was reduced⁶. This was an unexpected result that may 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 non-redundant receptor-associated kinase appear to be rate-limiting⁵. Typical human basophils express 100,000–500,000 IgE receptors (the high end found predominately in atopic subjects), but only express 25,000 molecules of syk per cell⁵. In the context of IgE-mediated release initiated by the crosslinking pan-stimulus, anti-IgE antibody, these low levels of syk may 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-to-total IgE ratios in atopic patients average 1%¹⁰. Therefore, in an atopic patient with 250,000 receptors, only 2500 are occupied with an antigen-specific IgE and the ratio of relevant receptor:syk (1:10) is the reverse of the ratio observed during stimulation with anti-IgE Ab (10:1). But since a typical reaction is a balance between the rate of activation vs. the rate of de-activation, where de-activation occurs independently of syk¹¹, even antigenic stimulation might benefit from greater levels of syk expression.

In human basophils, syk expression is known to be altered by three mechanisms. First, IgE-mediated secretion itself results in down-regulation^{12, 13}. Second, some non-IgE-dependent receptors use syk as a signaling element and also induce modest down-regulation of syk^{14, 15}. Even a non-IgE-dependent receptor, FMLP-R, which does not appear to use syk for signaling¹⁶, induces modest loss of syk¹⁴. Finally, IL-3 can increase syk expression although many other signaling elements are also up-regulated^{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 may induce loss of syk¹³. 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 during treatment with omalizumab may result from changes in the expression of syk.

Recent studies of basophils maturing from CD34+ progenitors have suggested another counter-intuitive hypothesis¹⁹. CD34+ progenitors expressed 11–12 fold more syk than peripheral blood basophils (PBB). When cultured for 3 weeks in IL-3, these cells matured into basophil-like cells that continued to express 11–12 fold more syk than PBB. 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 down-regulated. Relief of this chronic aggregation by elimination of IgE might reverse the induced down-regulation and result in a basophil that expressed higher levels of syk and was more responsive to a pan-stimulus 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 individuals expressing very different levels of FcεRI. In humans, the receptor can be expressed on the cell surface in two forms, a heterotrimer, αγ2, or a heterotetramer, αβγ2. There are indications that in basophils (similar data for the mast cell has 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 where the relative presence of the beta subunit (FcRβ) has been examined, the ratio of FcRβ:FcεRIα is correlated with the absolute level of cell surface expression of FcεRI, i.e., a relative increase in beta appears to increase surface FcεRI²¹. Based on this prior study, one might predict that omalizumab treatment might reduce the relative expression of FcRβ. Because this subunit may contribute to signaling strength²², 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 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 ratio of beta:alpha may be an intrinsic characteristic of an individual'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.

Materials and Methods

Reagents and Buffers

The online repository provides a detailed description of reagents and buffers. These have been described in previous publications⁵.

Analysis of syk protein expression by intracellular flow cytometry

All analysis was performed on a BD FACSCalibur flow cytometer. The measurement of syk protein expression by flow cytometry using 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 as well as purified cell fractions with equivalent results. Briefly, fixed mixed leukocytes were labeled with anti-IL-3R and anti-BDCA2 antibodies (phycoerythrin and FITC, respectively) and permeabilized (Fix and Perm Kit, Caltag, Carlsbad, CA) 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 MFI, the difference between 4D10 and isotype-labeled cells corrected for instrument variability using CaliBRITE APC calibration beads (BD Biosciences).

Flow cytometry was performed to detect FcεRIα and IgE on the cell surface using anti-FcεRIα antibody, 22E7 (which detects occupied and unoccupied FcεRIα) and goat anti-IgE Ab as previously described²³ (see the online repository)).

Omalizumab study²⁴

The details of this study have been recently published²⁴ and further details can be found in the online repository. Briefly, adults, 18–50 years old, were chosen for their sensitivity to cat

allergen. Informed consent was obtained via 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), prior to 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 separation on single (throughout study period) or double Percoll gradients (only pre-treatment and final visits). The cells from the 1-step Percoll gradient were analyzed by flow cytometry for cell surface levels of FcεRIα and IgE, 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 104²⁴. If the cat allergen response decreased below 20% of the basophil response prior to entry or the day 45 time point was reached first, further testing was stopped until the 14 week assessment. At this mid point (MID) in treatment, the subject was tested again for the study's various endpoints. The visit 14 (ca. 104 days) assessment is considered the FINAL measurement. At the BSL and FINAL visits, basophils were purified for Western blotting to measure FcεRI alpha and beta, c-cbl, and syk. In a subset of patients, flow cytometric syk measurements were made on a weekly basis.

Western blot analysis

At BSL and FINAL visits, basophils were purified by a combination of two-step Percoll gradient and negative selection using a basophil cocktail from Stem Cell Technologies (Vancouver, BC) (basophil purification kit) and columns from Miltenyi (Auburn, CA)⁵. The purity of basophils was determined by alcian blue staining²⁵ and basophils purified generally exceeded 97% purity. Relative to pre-purification counts, recovery of purified basophils was >85%. Basophils were lysed in 1X ESB buffer at a density of 300,000/20 μl of ESB, boiled for 5 minutes and stored at -70°C until SDS-PAGE analysis. Samples for individual subjects were stored until both visit 2 and visit 14 (see below) could be analyzed together. Western blotting, pretreatment (BSL) and 104 days (FINAL) samples, for several proteins, including c-cbl was performed in a semi-quantitative manner with calibration standards as described previously⁵. 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, with molecular weight markers in the first and last lanes (see the online repository, Figure E4). After transfer to nitrocellulose, the nitrocellulose was cut at the 40 kD and 90 kD horizontal position and the <40 portion blotted for FcRβ, the >40 kD, <90 kD portion blotted for FcεRIα and the >90 kD portion blotted for c-Cbl. After blotting with 22E7, The >40 kD, <90kD 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 PI3 kinase) which we have previously found not to change during culture with IL-3⁵.

Results

Omalizumab study: Histamine Release, Syk and FcRβ Expression

In the omalizumab treated subjects, cell surface IgE on basophils decreased to 0.05 ± 0.02 fold of its starting level by the FINAL visit (ca. 104 days). There was no change (1.11 ± 0.24 fold of starting levels) in patients treated with placebo agent. Figure E1, in the online repository shows the kinetics of the changes in cell surface FcεRI and IgE on basophils during treatment. At the final visit, day 104, histamine release induced by optimal concentrations of cat antigen decreased to 0.15 ± 0.12 fold of pre-treatment responses²⁴. In contrast, histamine release induced by anti-IgE antibody increased (Figure 1A) ($p=0.004$, $n=12$) in subjects treated with omalizumab and did not change in the placebo group ($p=n.s.$, $n=4$) (Figure 1C). 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 increased (Figure 1B). It did not increase in basophils from subjects treated with placebo agent (Figure 1D). There was a correlation between the fold increase in syk expression and the fold increase in anti-IgE Ab-induced histamine release of 0.73 ($p=0.014$).

In 6 subjects, the kinetics of the increase in syk expression was measured weekly (Figure 2). For the same subjects, Figure 2 shows the time course for the increase in histamine release induced by anti-IgE Ab. The increase in histamine release and syk expression occurred early in treatment, with the peak increase apparent by 2–3 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 leukocyte that also expresses FcεRI -- display 30-fold greater syk expression than peripheral blood basophil¹⁹. Figure E2 (online repository) shows that pDC 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/basophil, pre-treatment vs. 104 days, respectively). The number of alcian blue positive cells in Percoll separated preparations did not change; $24,000 \pm 5,000$ per ml of blood at pre-treatment and $26,000 \pm 5,000$ per ml at visit 14 (15 weeks) for an average ratio of counts at the final visit (ca. 104 days) vs. pre-treatment in the omalizumab group of 1.23 ± 0.13 ($p = 0.106$).

Our previous studies of syk expression in peripheral blood basophils have noted that expression ranges from near zero (MFI units) to 200–250 MFI units (with the mean at 110)⁹. Figure E3 in the online repository shows that there was a relationship between the fold up-regulation of syk in subjects on 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 of IL-3 will alter signaling element expression patterns⁵. Therefore, the observed increase in syk expression in basophils during treatment with omalizumab might result from increases in IL-3. 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 (e.g., IL-5), then there should also be increases in c-cbl or FcRβ^{5, 21, 26}. For example, previous studies have shown that *in vitro*, c-cbl, FcRβ, 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 treatment group (and no change was observed in the placebo group) (Figure 3, panel A). In addition, there was no change in the basophil response to FMLP stimulation (Figure 3, panel A).

In vitro incubation of basophils with IL-3 has also been noted to increase the expression of the beta subunit (FcRβ) of FcεRI^{21, 26}. In our previous study of FcRβ and FcεRIα expression in human basophils, the expression levels were determined by lysing cells and analyzing total p60 FcεRIα or FcRβ by Western blotting²¹. This same methodology was employed 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 since the Western blot was non-linear throughout the possible range of receptor expression found at different points of the omalizumab study. Figure E4 in the online repository shows one example Western blot of samples made before and after treatment with omalizumab. Figure 3, panels B & C, summarizes the results for changes in both the broad p60 FcεRIα band (which represents cell surface

receptor) and the FcR β band. The decrease in p60 by Western is similar to the decrease in surface Fc ϵ RI as detected by flow cytometry. Expression of p60 at the FINAL visit (ca. 104 days) decreased to 17 $\pm$ 2% of pre-treatment levels whether analyzed by flow cytometry or by densitometric analysis of the semi-quantitative Western blot of p60 ($p < 0.0001$ when compared to pre-treatment baseline (BSL), $p = \text{n.s.}$ when comparing flow and Western methodologies for measurement of Fc ϵ RI α). The levels were unchanged in the placebo (4 subjects) group. In contrast, after the FINAL treatment visit the expression of FcR β decreased to 8 $\pm$ 3% of the levels observed prior to treatment ($p = 0.0025$ when comparing the change in FcR β and Fc ϵ RI α by Western blotting). Again, there was no change observed in the placebo-treatment group. When the data was expressed as a ratio of FcR β :Fc ϵ RI α (p60), the ratio at the FINAL treatment visit decreased to 0.43 fold the ratio observed prior to treatment ($p = 0.0004$ | $H_0 = 1.0$). There was no change in the ratio in the placebo-treatment group. Using the same nitrocellulose blots and blotting for syk expression, the ratio of FINAL/BSL for the omalizumab group was 1.55 $\pm$ 0.19, $p = 0.013$ (H_0 | ratio = 1.0), recapitulating the results obtained by flow cytometry. There were two collateral observations that were worth noting, the heterogeneity of glycosylation of the p60 cell surface Fc ϵ RI α was found to decrease following treatment and there was a modest increase in the presence of the internal immature Fc ϵ RI α , p46 (see online repository).

In vitro induced changes in FcR β :Fc ϵ RI α

The change in relative FcR β expression in patients treated with omalizumab raised a question of whether the change would occur whenever receptor down-regulation occurred in the absence of free IgE. Purified peripheral blood basophils were cultured for 13 days in the absence of IgE in order to induce down-regulation of Fc ϵ RI. 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 day 0 and day 13 were run in the semi-quantitative Western blot to also assess relative changes in FcR β :Fc ϵ RI α ratios. For these experiments, the change in receptor expression is not as marked as obtained in the omalizumab study but Figure 4, panel A shows the decrease in receptor expression was statistically significant for both “non-stripped” and the “stripped” cells. The non-stripped cells did experience the expected 50% average decrease in Fc ϵ RI expression found in previous studies²⁸. There was no change in the FcR β :Fc ϵ RI α ratio for either group of cells (Figure 4, panel B). These results suggest that simply down-regulating receptor, in the presence of IL-3, is not sufficient to alter the FcR β :Fc ϵ RI α 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. Culture 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 or 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 FcR β , expression was suppressed relative to the expected decrease in Fc ϵ RI. This result suggests that treatment with omalizumab actually results in a suppression of a cytokine environment that promotes balanced expression of FcR β and Fc ϵ RI α . Previous studies of omalizumab treatment

have also indirectly suggested that the presence of Th2-associated cytokines (e.g., IL-3, IL-5) may 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 if the FcR β :Fc ϵ RI α ratio in peripheral blood basophils was a constant feature (an intrinsic characteristic) of an individual's Fc ϵ RI 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 1) induce a reduction in basophil Fc ϵ RI and/or 2) potentially alter the atopic environment of the patient by changing the presence of Th-2-like cytokines or by other mechanisms not yet defined. The study showed that there was a disproportionate decrease in the expression of FcR β relative to the decrease in total Fc ϵ RI α . This result leads to the conclusion that the relative presence of FcR β is sensitive to factors extrinsic to the basophil.

This study can not directly address whether the changes in FcR β :Fc ϵ RI α reflect a change in the stoichiometry of cell surface receptor. Underlying the ambiguity is the possibility that there is some FcR β 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 repository, expression levels associated with other receptors would not be expected to change) and the Western blotting method does not discriminate “internal” vs. surface subunit expression (unlike the case for Fc ϵ RI α). In addition, the relative efficiency of the two antibodies used to detect Fc ϵ RI α and FcR β is unknown. However, there are two closely related lines of reasoning, presented in the online repository (Figure E5), that suggest that the ratio may reflect differences in surface receptor stoichiometry. In other words, not only is total FcR β disproportionately suppressed but it seems 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 could result from a relief from a down-regulatory mechanism like that 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 Fc ϵ RI but reduced expression of syk¹⁹. 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* studies¹⁹. However, omalizumab reduces free IgE levels sufficiently to markedly down-regulate Fc ϵ RI and especially cell surface IgE^{1, 33, 34}. We could speculate that these conditions may be sufficient to relieve a chronic ongoing IgE-dependent aggregation of maturing basophils, if this occurs *in vivo*, and result in an increase in syk without other changes in the basophil phenotype. The increase occurred relatively quickly; an increase was evident within the first 2–3 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 likely within 1–2 weeks. This expectation is drawn from our studies of Fc ϵ RI changes¹ and the understanding that the kinetics of the receptor changes reflect the maturation kinetics³⁵. There is not yet direct evidence that reversal of a post-translational 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 pre-translational events such as up-regulation of mRNA levels.

There remain several issues with this explanation, however. Most notable is that low syk expression levels in basophils are found in both non-atopic and atopic subjects. There would be no *a priori* reason for non-atopic subject's basophils to experience chronic IgE-dependent aggregation. But this assumption may be false. It has been noted that both atopic and non-atopic sera contain various levels of anti-IgE and anti-Fc ϵ RI α 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 auto-antibodies does result in a functional outcome that is not always to induce disease as proposed for some patients with chronic urticaria³⁶ and that outcome is the down-regulation 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 treatment¹. During manuscript preparation another new published study of omalizumab treatment found no changes in the basophil response to anti-IgE antibody³⁷. But in that study, the starting response was nearly as high as one can find in basophils (>75%), so 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 two studies; table 1 summarizes the differences in IgE densities between the two studies. The cause of the differences may be the mode of administration of omalizumab (IV vs. SC) 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 2000 cell surface IgE molecules to achieve 50% of their maximal responsiveness^{4, 5}. A density of 3000–5000 is sufficient for a maximal response. In the phase I trial, total cell surface IgE decreased to unmeasurable levels for many subjects. Based on the technique used at that time, an average upper limit of 2200 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 6800 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 since cat-specific IgE densities would be low. For example, the median ratio of cat-specific IgE to total IgE was 4.2% (somewhat higher than the expected 1% for the general cat-allergic population¹⁰), which would predict around 290 molecules of cat-specific IgE per basophil. This level is far below 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 where 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 guide²², the relative absence of FcR β 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 FcR β 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 FcR β .

Key Messages

- Treatment of cat allergic subjects with omalizumab results in increased expression of syk in circulating basophils. One interpretation is that chronic aggregation of Fc ϵ RI *in vivo* is relieved by the reduction in Fc ϵ RI expression that accompanies treatment.

- Treatment with omalizumab results in changes to the basophil response that increase its intrinsic sensitivity to stimulation, a change that counter-balanced by large reduction in cell surface antigen-specific IgE.

Capsule Summary

This study provided evidence supporting a prediction that treatment of atopic subjects with omalizumab would increase the expression of the critical FcεRI-associated kinase, syk.

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Abbreviations

FMLP	Formyl-met-leu-phe (tripeptide)
IL-3	interleukin-3
PI3K	phosphatidyl inositol 3' kinase

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Figure 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 pre-treatment (Day 0, BSL), 4–6 weeks after the start of treatment (MID), and 15 weeks (ca. 104 days) after the start of treatment (FINAL) for subjects treated with omalizumab (n=12). (B) Measurement of syk expression in basophils by flow cytometry for the same group of subjects in panel A. (C) Measurement of histamine release induced with anti-IgE antibody in the placebo group (n=4). (D) Measurement of syk expression by 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.

Figure 2. Kinetics of the increase in anti-IgE Ab-induced histamine release and syk expression
 The data is expressed relative to the pre-treatment response or levels of syk expression (n=6); (○) histamine release induced by anti-IgE antibody and (●) syk expression measured by flow cytometry. Both kinetic curves, taken as a whole, are statistically different than no change (1.0) ($p < 0.0001$).

Figure 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 quantitative Western blots) or the ratio of percent histamine release induced by 1 μ M FMLP. The ratio is calculated as the endpoint at day 104 (FINAL) divided by the endpoint pre-treatment (day 0, BSL). (B) Expression of cell surface FcεRIα and bound IgE levels on peripheral blood basophils from omalizumab treated or placebo subjects were determined by flow cytometry and by Western blotting. The ratio of expression level at day 104 (FINAL) to pre-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 Western blot analysis of purified blood basophils from omalizumab treated or placebo subjects (BSL and FINAL). All data are expressed as mean $\pm$ SEM.

Figure 4. Absence of change in FcεRIβ: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, lysed, and lysates analyzed by the semi-quantitative Western blot procedure used in the omalizumab study in order to quantitatively assess expression levels. (A) Expression of p60, relative to pre-culture levels (D0) for non-stripped and stripped cells (n=5) ($p = 0.12$ | $H_0 = 1.0$ for non-stripped (NS) cells and $p = 0.0005$ | $H_0 = 1.0$ for stripped (S) cells). (B) Ratio of FcεRIβ:FcεRIα for Day 0 (D0) and non-stripped (NS) and stripped cells (S) on day 13 (D13) (n=5) ($p = \text{n.s.}$ for comparison of 3 groups). Errors shown represent SEM.

Table 1

Comparison of Changes in IgE density on basophil following omalizumab treatment for the 1996 (phase 1 trial) and the current 2008 study.

STUDY	IgE/basophil		
	Pre	Post	Fraction
1996 phase 1	240000	<2200	0.009
2008(current)	134000	6800	0.051