Omalizumab treatment downregulates dendritic cell FcεRI expression

Calman Prussin, MD,[a] Daniel T. Griffith, MD,[b] Kevin M. Boesel, MD,[b] Henry Lin, MD,[b] Barbara Foster, MS,[a] and Thomas B. Casale, MD[b] *Bethesda, Md, and Omaha, Neb*

Background: Dendritic cells (DCs) are potent antigen-presenting cells that express FcεRI, the high-affinity IgE receptor. Although the downregulation of basophil FcεRI during anti-IgE therapy with omalizumab is well documented, its effect on FcεRI expression by DCs has not been reported.

Objective: We hypothesized that IgE regulates surface FcεRI expression by DCs in vivo and that, consequently, anti-IgE therapy decreases FcεRI expression by DCs.

Methods: In a randomized, double-blind, placebo-controlled clinical trial 24 subjects (16 receiving omalizumab and 8 receiving placebo) with seasonal allergic rhinitis received the study drug on days 0 and 28. Serial blood samples drawn on days 0, 7, 14, 28, and 42 were analyzed for precursor DC1 (pDC1) and pDC2 surface expression of FcεRIα by using flow cytometry.

Results: Omalizumab caused a significant decrease in surface FcεRI expression at all time points examined in both the pDC1 and pDC2 subsets. No significant change was seen with placebo. The maximum decrease in FcεRI expression in the omalizumab group was 52% and 83%, respectively, for the pDC1 and pDC2 subsets. The decrease in FcεRI expression by both pDC subsets correlated with the decrease in serum-free IgE and was of a similar magnitude to that found in basophils. A 10-fold decrease in IgE corresponded to a 42% and 54% decrease in surface FcεRI expression by the pDC1 and pDC2 subsets, respectively.

Conclusion: These results demonstrate that anti-IgE therapy causes a rapid decrease in DC surface FcεRI expression and establish that IgE is an important regulator of FcεRI expression by DCs. (J Allergy Clin Immunol 2003;112:1147-54.)

Key words: Dendritic cells, IgE, anti-IgE, FcεRI, omalizumab, antigen-presenting cells

FcεRI, the high-affinity IgE receptor, and allergen-specific IgE play an essential role in immediate-type hypersensitivity and allergic disease.[1] In addition to FcεRI expression by the classic cellular mediators of immediate hypersensitivity, in human subjects, antigen-presenting cells (APCs), such as dendritic cells (DCs) and monocytes, express FcεRI, and this expression is

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increased in some atopic conditions.[2] DC expression of FcεRI enhances the presentation of allergen to T cells through an antigen-focusing mechanism, in which FcεRI-bound IgE on APCs preferentially captures allergen.[3] Thus, because of the critical role DCs play in the initiation and T_H2 polarization of allergen-specific immune responses,[4] FcεRI expression by DCs might have important immunologic and clinical consequences.[2]

DCs are potent APCs that play a critical role in shaping the adaptive immune response to antigens. A number of phenotypic and functional DC subsets have been identified.[5] The 2 most abundant and well-described DC subsets, termed DC1 and DC2, demonstrate unique toll-like receptor repertoires and cytokine expression[6] and promote T_H1 and T_H2 responses, respectively.[7,8] Although the relationship of these DC subpopulations is undoubtedly more complicated than a 1:1 correlation with the T_H1 and T_H2 subsets, they provide a useful starting point to address DC heterogeneity. Precursor DCs (pDCs) are an earlier DC developmental stage that is present in the peripheral blood. In response to an immunologic stimulus, pDCs traffic into tissue and differentiate into mature DCs.

Omalizumab is a humanized therapeutic mAb against human IgE that effectively reduces free IgE levels by 10- to 100-fold and is an effective treatment for allergic asthma and seasonal allergic rhinitis.[9,10] The major immunologic consequence of this decrease in free IgE is a corresponding decrease in surface FcεRI expression by peripheral blood basophils.[11,12] We thus hypothesized that omalizumab therapy would similarly decrease FcεRI expression by DCs. To test this hypothesis, during a clinical trial of omalizumab, we performed a substudy examining the effect of anti-IgE therapy on FcεRIα expression by the pDC1 and pDC2 subsets. In this report we demonstrate that omalizumab therapy causes a rapid decrease in surface FcεRI expression by both the pDC1 and pDC2 subsets. These results further define the role of IgE in the regulation of FcεRI expression by DCs and suggest that anti-IgE therapy might have immunomodulatory effects in addition to its inhibition of IgE-mediated hypersensitivity.

Mechanisms of allergy

METHODS
Study design

This analysis was performed as a substudy within a single-center, randomized, double-blind, placebo-controlled clinical trial of 24 subjects (16 receiving omalizumab and 8 receiving placebo) with ragweed-induced seasonal allergic rhinitis conducted from December 2001 to March 2002. Subjects 19 to 50 years old with a minimum 2-year history of ragweed-induced seasonal allergic rhinitis and a serum IgE level of less than 700 IU/mL were enrolled. Subjects with a history of asthma or who were currently using corticosteroids, antihistamines, or leukotriene antagonist medications were excluded from the study. Allergy was confirmed with a positive skin test wheal of 5 mm or larger to mixed giant-short-western ragweed (Hollister-Stier, Spokane, Wash). Subjects were randomly assigned to receive either omalizumab (0.016 mg·kg^{-1}·IU^{-1}·mL^{-1} IgE) or placebo on days 0 and 28. At the completion of the trial, the data were analyzed in a blinded fashion. FcεRIα expression on blood pDC cells was determined by means of flow cytometry on day 0 (baseline) and on days 7, 14, 28, and 42. IgE was measured on day 0 (baseline) and on days 3, 28, and 42. The clinical trial and collection of PBMCs were performed at Creighton University, and the DC flow cytometric analyses were performed at the National Institutes of Health. Nasal allergen challenge yielding a 30% decrease in nasal volume was performed at baseline, and subjects were then challenged with the same dose biweekly during the study. The clinical protocol was approved by the Creighton University Institutional Review Board, and the DC analyses were reviewed by the National Institutes of Health Office of Human Subjects Protection. Each patient provided written informed consent before enrollment in the study.

Free and total IgE concentrations

Total serum IgE concentration was measured at baseline, and free serum IgE levels were measured on days 3, 28, and 42. Total IgE levels were measured by using the Immulite 2000 chemiluminescent immunoassay. Human free IgE (not complexed to omalizumab) was measured by Genentech with a solid-phase ELISA, as previously described.[13]

Preparation of PBMCs

PBMCs were isolated from EDTA-anticoagulated blood by using 1.083 Ficoll-Diatrizoate density gradient separation and washed twice in PBS. PBMCs (20×10^6) were fixed in 3 mL of 4% paraformaldehyde for 5 minutes, washed once in PBS with 0.1% BSA (PBS-BSA), and then resuspended in PBS/10% dimethyl sulfoxide (Sigma, St Louis, Mo) and stored at $-80°C$ until analysis.[14]

Antibodies

Anti-CD2 (clone LT2) FITC and CD19 FITC (clone FMC63; Serotech, Raleigh, NC); HLA-DR, HLA-DP, and HLA-DQ FITC (cloneTU39), HLA-DR phycoerythrin (PE)/Cy5 (clone G46-6), CD4 APC (clone RPA-T4), CD11c APC (clone B-ly6), and CD123 PE (clone 7G3; BD-PharMingen, San Diego, Calif); CD14 PE/Cy5 (clone Tuk4) and CD19 PE (clone SJ25-C1; Caltag, Burlingame, Calif); CD14 FITC (Beckman-Coulter, Fullerton, Calif); CD16 FITC (clone B-E16, BioSource, Camarillo, Calif); blood dendritic cell antigen (BDCA) 2 APC, CD1c biotin (clone BDCA-1; Miltenyi Biotec, Auburn, Calif); goat anti-mouse IgG1 Alexa 647, streptavidin APC (Molecular Probes, Inc, Eugene, Ore); and lineage cocktail 1 (lin-1) FITC (anti-CD3, CD14, CD16, CD19, CD20, and CD56; BD Biosciences, San Jose, Calif) were obtained commercially. The basophil granule-specific mAb 2D7 was a gift from Dr Lawrence Schwartz (Virginia Commonwealth University). pDC2 cell analyses were performed by using unlabeled anti-FcεRIα (clone 22E7, gift from Dr Wayne Levin, Roche) and indirect

immunostaining. We were unable to obtain additional 22E7 mAb to complete this work, and thus the pDC1 staining was performed with PE-labeled anti-FcεRIα clone AER-37 (eBioscience, San Diego, Calif). Each mAb recognizes both free and IgE-bound FcεRIα. In pilot studies these 2 mAb clones appeared to recognize similar or identical epitopes; both mAbs efficiently blocked binding of the other, and neither mAb was blocked by IgE competition (data not shown).

Antibody staining and flow cytometry

Cryopreserved samples were collected, processed as above, and archived at Creighton University. On conclusion of the clinical trial, samples were shipped to the National Institutes of Health for analysis. DC2 and basophil analyses were performed first by using the 22E7 mAb. All DC2-basophil samples from a given donor were processed on the same day to minimize intrasubject variation. After these studies were completed, the DC1 analyses were performed with the AER-37 mAb; all DC1 samples from a given donor were processed on the same day. The analyses and conclusions contained within are not based on direct comparison of DC1 and DC2 fluorescence intensity data and thus are not affected by this change in mAb. Cell-surface FcεRI staining was performed with a nonpermeabilizing adaptation of previously described procedures.[14,15] All buffers were used at 4°C. Cryopreserved fixed cells were thawed, washed in PBS-BSA, and then blocked in PBS–1% BSA–5% nonfat milk powder (PBS-BSA-milk) for 1 hour on ice before mAb staining. For analysis of pDC1 cells, samples were stained in PBS-BSA-milk with lin-1 FITC, CD1c biotin, CD11c PE/Cy5, and anti-FcεRIα PE (clone AER-37); washed twice in PBS-BSA; stained with streptavidin APC; washed twice; and analyzed. pDC1 cells were identified by first gating on CD1c$^+$, lin-1$^-$ cells (Fig 1, *F*) and back-gating on cells of the corresponding scatter, which were then further gated on CD11c (Fig 1, *G*). For analysis of pDC2 cells and basophils, cells were incubated with an exclusion panel of FITC-labeled mAbs to CD2, CD16, CD19, HLA-DR, HLA-DP, and HLA-DQ, anti-CD123 PE, anti-CD14 PE/Cy5 (all non-IgG1 isotype), and anti-FcεRI (clone 22E7, mouse IgG1 isotype) in PBS/BSA/milk. Cells were then washed twice and incubated with goat anti-mouse IgG1 Alexa 647 in PBS-BSA-milk, washed twice, and analyzed. DC2 were identified as a distinct cluster of CD123 bright cells that stained positive with the exclusion panel and negative for CD14 (Fig 1, *A*). Basophils were identified as a distinct cluster of CD123 bright cells that stained negative with the exclusion panel and negative for CD14 (Fig 1, *A*). After gating on the above cell subsets, the mean fluorescence intensity of FcεRI was determined (Fig 2).

Data were acquired with a 2-laser, 4-parameter FACS Calibur flow cytometer (BD Biosciences) and analyzed on Cellquest (BD Biosciences) and FlowJo (Tree Star, San Carlos, Calif) software. Typically 300,000 to 600,000 total events were acquired to obtain 1000 or more pDCs for analysis.

Statistical analysis

Paired data in Fig 3 were analyzed by using the Wilcoxon signed-rank test. Correlative data in Figs 4 and 5 were analyzed by using the Spearman rank correlation test. For correlations for each subject, all IgE and FcεRI determinations on study drug were averaged with like results, and a single representative percentage fraction during treatment with the study drug relative to the day 0 baseline value was determined. A single subject receiving omalizumab with outlying pDC2 staining (300% of baseline) was excluded for linear regression calculations in Figs 4, *B*; 5, *A;* and 5, *C*. This subject was included in all correlation calculations and plotted data. Statistical calculations and linear regression analysis were performed with Prism software (GraphPad Software, San Diego, Calif).

RESULTS
Clinical study

A clinical trial examining the pharmacodynamics of omalizumab therapy was performed from December 2001 through March 2002 in subjects with ragweed-induced seasonal allergic rhinitis and is described in detail elsewhere.[16] Serial blood samples for IgE levels and flow cytometry were analyzed as detailed in the "Methods" section. The median baseline serum IgE level was 250 and 131 ng/mL for the omalizumab and placebo groups, respectively. In the omalizumab group free serum IgE decreased 95% by day 3 of the study, and this decrease was sustained and statistically significant at all time points studied (90% and 93% for median decrease on days 28 and 42, respectively).

Identification of pDC subsets in peripheral blood

During the above-noted clinical pharmacodynamics study, we examined basophil FcεRI expression.[16] During this analysis, we noted a second distinct population and postulated these were cells of the pDC2 subset. This identity was suggested by their characteristic bright staining for CD123 and an exclusion panel consisting of CD2, CD16, CD19, HLA-DR, HLA-DP, and HLA-DQ (Fig 1, A). The DC2 identity of this population was confirmed in pilot experiments on the basis of their uniform positive staining for HLA-DR, BDCA-2, CD4, and FcεRIα and negative staining for the basophil-specific granule marker 2D7 (Fig 1, B-E, n = 4 subjects; Fig 2, C and D). In contrast, the basophil population stained negative for HLA-DR, BDCA-2, and CD4 and positive for 2D7. We then identified cells of the pDC1 subset on the basis of their positive staining for CD1c and CD11c and negative staining for the lin-1 panel (Fig 1, F and G).[17] The identity of these pDC1-subset cells was confirmed in pilot experiments on the basis of their uniform positive staining for HLA-DR (data not shown) and FcεRIα (Fig 2, A and B). These results demonstrate that the staining strategy used specifically identifies pDC1 and pDC2.

Effect of omalizumab on pDC surface FcεRI expression

Omalizumab treatment caused a decrease in FcεRIα expression by both pDC1 and pDC2 (Fig 2, A-D). In both subsets the decrease in FcεRI expression was apparent by 7 days and was maximal at 28 and 14 days, respectively, for pDC1 and pDC2 (Fig 3 and Fig 2, E and G). The median decrease in surface FcεRI expression at these time points was 54% and 75%, respectively. In contrast, in the placebo group the maximal decrease for any single time point was 3% and 20% for pDC1 and pDC2, respectively.

Relative to the day 0 baseline value, omalizumab caused a significant decrease in FcεRI expression by both pDC1 and pDC2 at all time points examined (Fig 3). In contrast, none of the placebo time points showed any significant changes. After omalizumab treatment, the maximum decrease in median FcεRI expression was
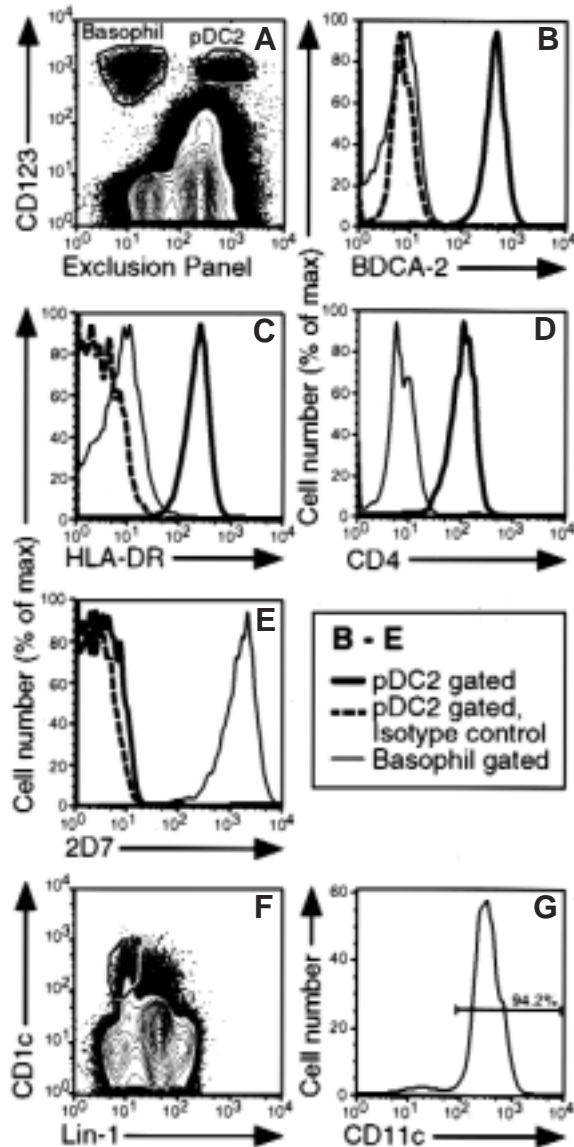

FIG 1. Identification of peripheral blood pDC subsets. **A,** PBMCs were stained with an mAb to CD123 and an exclusion panel (CD2, CD16, CD19, HLA-DR, HLA-DP, and HLA-DQ), and cells of the pDC2 subset and basophils were identified. After gating on either cells of the pDC2 subset *(heavy line)* or basophils *(light line)*, the expression of BDCA-2 **(B)**, HLA-DR **(C)**, CD4 **(D)**, and 2D7 **(E)** was determined (n = 4 subjects). The pDC1 subset was identified as a population staining negative for the lin-1 mAb cocktail and positive for CD1c **(F)** and CD11c **(G)**.

52% and 83%, respectively, for the pDC1 and pDC2 subsets. These results demonstrate that omalizumab therapy causes a rapid and persistent decrease in surface FcεRI expression in cells of both the pDC1 and pDC2 subsets.

Correlation of FcεRI expression to serum IgE

Although the FcεRI response to omalizumab was generally substantial, in Fig 2 we observed a variable response between subjects. One explanation is that in
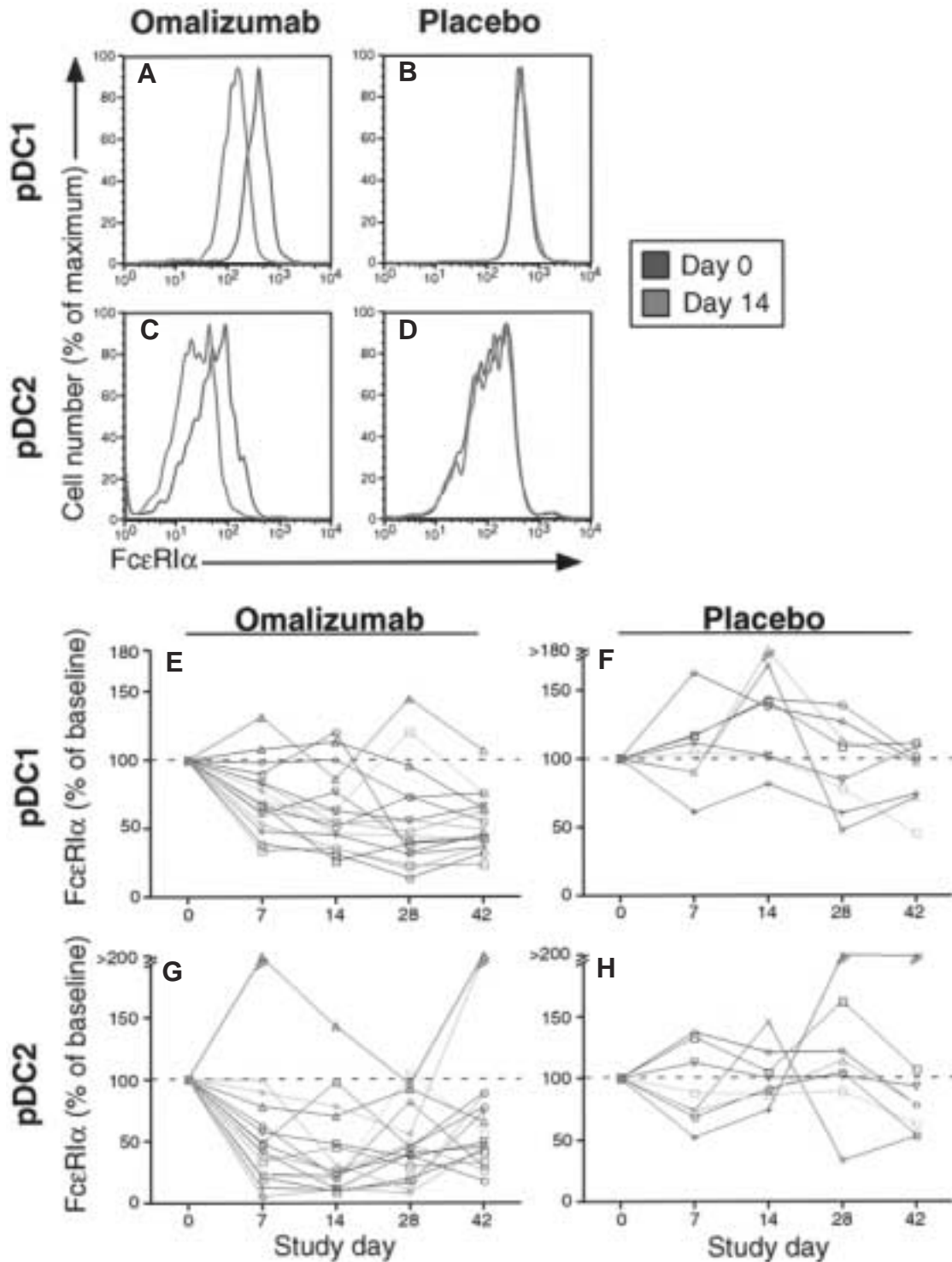
FIG 2. Omalizumab downregulation of FcεRIα expression in the pDC1 and pDC2 subsets. **A-D,** After gating on either pDC1 (**A** and **B**) or pDC2 (**C** and **D**) subsets, representative FcεRI staining from days 0 and 14 are shown for both an omalizumab-treated (**A** and **C**) and placebo-treated (**B** and **D**) subject. For each subject, FcεRI expression was normalized to the day 0 baseline value and plotted for each study visit **(E-H)**.

poorly responsive subjects, factors other than IgE might have a greater influence on FcεRI expression. Alternatively, it might simply be due to inconsistent reduction of IgE levels in these poorly responding subjects. To deter-

mine which of these models explains the variable response to omalizumab, we correlated the change in surface FcεRI expression with the change in serum IgE. For both the pDC1 and pDC2 subsets, the change in FcεRI
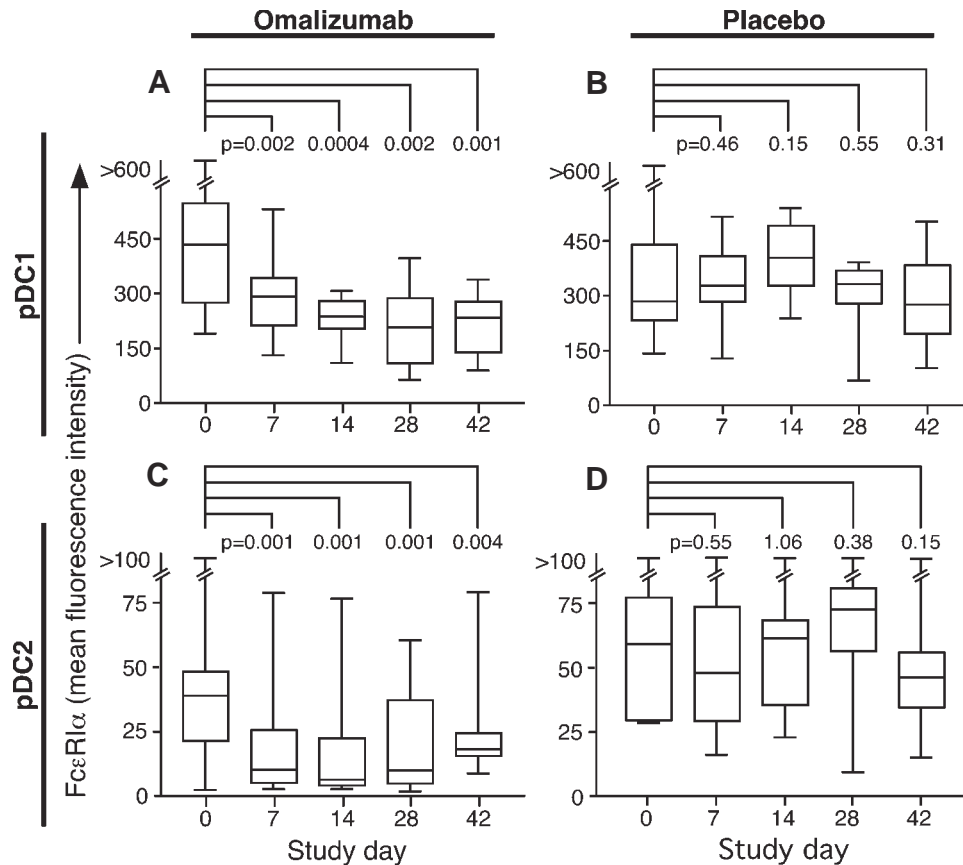
FIG 3. Omalizumab downregulation of FcϵRIα expression in the pDC1 and pDC2 subsets. **A-D,** Box-and-whisker plots showing FcϵRI mean fluorescence intensity in pDCs plotted for each study visit. The *horizontal middle line* represents the median, the *top* and *bottom lines* represent the quartile values, and the *T bars* represent the maximum and minimal values. Statistical significance was determined by using the Wilcoxon signed-rank test.

expression was highly correlated with the change in free IgE (Fig 4). Linear regression analysis yields lines indicating that a 10-fold decrease in free IgE resulted in a 42% and 54% decrease in FcϵRI expression by pDC1 and pDC2, respectively. Linear regression analysis of the basophil results indicates that a 10-fold decrease in IgE resulted in a 63% decrease in basophil FcϵRI (data not shown). These results demonstrate that omalizumab-induced surface FcϵRI downregulation is proportional to the reduction in free IgE.

Although omalizumab decreased FcϵRI expression in all 3 cell subsets, we sought to determine whether, in a given subject, the reduction observed in one subset was mirrored in the others. To address this question, we correlated the change in FcϵRI expression between the pDC1, pDC2, and basophil subsets. In each case changes in FcϵRI expression in a given subset were highly correlated with changes in the other subsets (Fig 5). Linear regression analysis yielded curves with slopes close to 1, indicating that, in a given subject, the magnitude of change in FcϵRI expression for all 3 subsets was similar. These data demonstrate that in subjects responding to anti-IgE thera-

py, downregulation of FcϵRI expression occurs in parallel among the pDC1, pDC2, and basophil subsets.

DISCUSSION

In this work we examined FcϵRIα expression during a clinical trial of omalizumab and demonstrate that omalizumab treatment is associated with a decrease in cell-surface FcϵRI expression in both the pDC1 and pDC2 subsets. Furthermore, we found a correlation between the decrease in DC FcϵRI expression and the decrease in IgE in this study population. These results are consistent with the conclusion that this decrease in FcϵRI expression occurs as a direct consequence of the reduction in free IgE concentration by omalizumab.

Omalizumab is a humanized mAb that has therapeutic activity in asthma and allergic rhinitis.[9],[10] By binding IgE, omalizumab reduces free IgE and, consequently, the expression of FcϵRI by basophils[11],[12] and, presumably, mast cells. The major known clinical consequence of these reductions is the inhibition of immediate hypersensitivity. In Figs 2 and 3 we present the novel finding that omal-
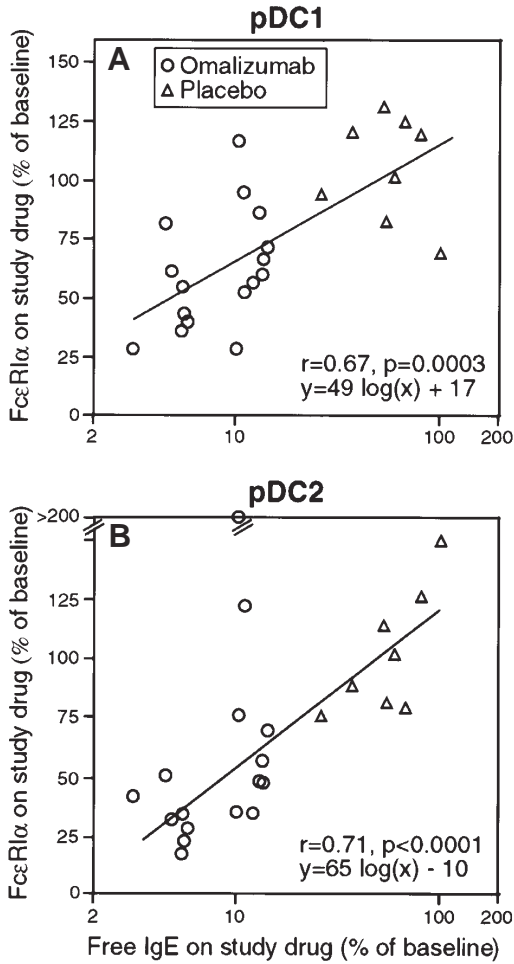
FIG 4. Correlation of DC FcεRIα expression with serum IgE. For each subject, the fraction of IgE and FcεRI during treatment with the study drug relative to the day 0 baseline value was determined as per the "Methods" section. The pDC1 and pDC2 values for FcεRI were then plotted against that for serum IgE. Each symbol represents an individual subject. *R* values and statistical significance were determined by using the Spearman rank correlation test. A *line* was fitted to these results by using linear regression and the equation describing that line noted on each graph.

FIG 5. Correlation of FcεRI expression between the pDC1, pDC2, and basophil subsets. For each subject and cell subset, the fraction of FcεRI expression during treatment with the study drug relative to the day 0 baseline was determined as per the "Methods" section. These values were then plotted against each other for the different pairs of cell subsets. Each symbol represents results for a unique subject. *R* values and statistical significance were determined by using the Spearman rank correlation test. A *line* was fitted to these results using linear regression and the equation describing that line noted on each graph.

izumab therapy similarly downregulates FcεRIα expression by human DCs. The major clinical implication of our findings is that by downregulating DC FcεRI expression and, consequently, inhibiting antigen focusing, anti-IgE might inhibit antigen presentation of allergen to T cells. This suggests the possibility that anti-IgE therapy might have the capacity to block both the sensitization and effector phases of the allergen-specific immune response.

Because of the limitations inherent in human research, the roles of specific molecules in disease pathogenesis are generally implicated from association rather than the more straightforward process of deleting a gene or gene product through genetic or pharmacologic means. Omalizumab provides a means to reduce free IgE levels in vivo and assess the immunologic consequences. The results in Fig 4 demonstrate that the decrease in FcεRIα expression was
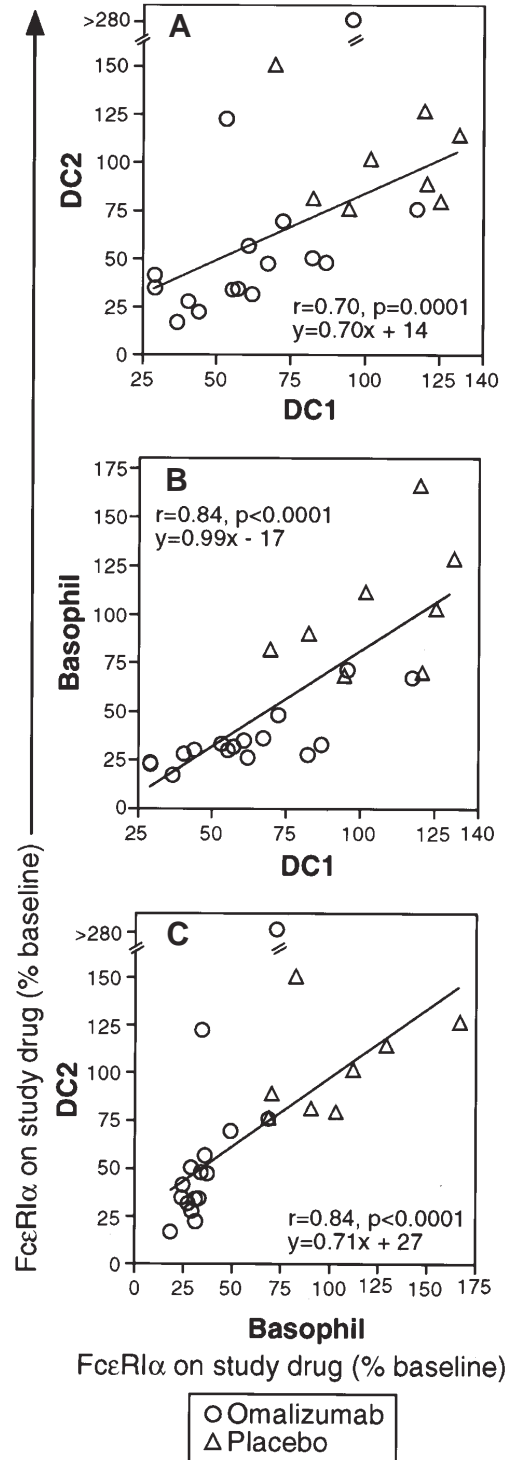
highly correlated to the decrease in free IgE. This finding supports the concept that serum IgE levels directly influence DC FcεRI expression. Furthermore, the magnitude of the reduction in FcεRI expression was substantial (Figs 2 and 3). In sum, these data are consistent with the conclusion that IgE is a major factor regulating FcεRI surface expression by human DCs in vivo. Given the results of previous in vitro studies,[18] we believe our results reflect IgE stabilization of FcεRI protein on the DC surface and not the result of increased transcription.

Antigen focusing by FcεRI-bearing APCs has been proposed as a potential mechanism whereby IgE facilitates antigen presentation to allergen-specific T cells. Our results, demonstrating the downregulation of DC FcεRI by omalizumab, suggest the possibility that anti-IgE, by inhibiting antigen focusing, might decrease allergen-specific T-cell activation. Such inhibition could potentially be operative at 2 major points of the T-cell immune response to allergen. First, the inhibition of the clonal expansion and differentiation of naive allergen-specific T cells into T_H2 cells would decrease the numbers of pathogenic allergen-specific T_H2 cells. Second, the inhibition of allergen-specific T_H2 cell activation would decrease the generation of inflammatory T_H2 cytokines. If operative, such mechanisms would indicate that, in addition to its known effect on basophils and mast cells, anti-IgE therapy might have immunomodulatory effects on allergen-specific T cells.

The results we obtained for the pDC1 and pDC2 populations were largely parallel to those we found for basophils. For example, in Fig 5, the changes in FcεRI expression were highly correlated among the basophil and pDC subsets. Moreover, in that same analysis, the slopes of the fitted lines between the basophil and pDC subsets were close to 1, indicating that the magnitude of change in FcεRIα expression was comparable in all 3 subsets. Finally, the kinetics of the FcεRI decrease were similar in all 3 subsets. In sum, these data are consistent with the conclusion that FcεRI is similarly regulated in the basophil, pDC1, and pDC2 populations.

A strength of this study is the use of pDCs, a well-characterized and accessible source of DCs[5,17,19] that are well suited to serial sampling during an 8-week clinical drug trial.

To our knowledge, the downregulation of DC expression of FcεRI by omalizumab has not been reported. However, a previous report examined the absolute numbers of pDCs in subjects with seasonal allergic rhinitis treated concurrently with omalizumab and allergen immunotherapy.[20] This study found that the number of pDC1 was decreased in the omalizumab group relative to the placebo group, but this observation was limited to the grass pollen season and was not seen in the birch pollen season. Our study was performed out of the ragweed pollen season, did not measure the absolute numbers of pDCs in peripheral blood, and did not use allergen immunotherapy, and therefore a direct comparison of results is not possible. Both studies demonstrate that omalizumab treatment is associated with DC changes that might have immunotherapeutic consequences.

Specific DC subsets have different capacities to prime naive T cells toward a T_H1, T_H2, or regulatory T-cell phenotype.[7,8,21] However, the relationship of specific DC subsets, their expression of FcεRI, and the pathogenesis of allergic diseases is unclear and is the subject of active investigation.[2,22-24] Thus the clinical consequences of downregulating FcεRI on both the DC1 and DC2 subsets is an important area for future investigation.

In summary, there are several important implications of the research observations reported in this article. First, our findings support the concept that serum IgE concentration is a major determinant of FcεRI surface expression by pDCs. Second, the action of omalizumab on pDCs underscores the potential for anti-IgE therapy to downregulate FcεRI expression on a wide range of DCs and do so at an early stage of development before their trafficking into tissue and differentiation into mature DCs. Finally, the therapeutic downregulation of FcεRI represents a unique molecular target whereby both the sensitization and effector phases of the allergen-specific immune response could be inhibited.

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