

ORIGINAL ARTICLE

Asthma and Rhinitis

FcεRI expression and IgE binding by dendritic cells and basophils in allergic rhinitis and upon allergen immunotherapy

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Summary

Background: In humans, both basophils and dendritic cells (DCs) express the high-affinity IgE receptor (FcεRI).**Objective:** To gain more insight into the relation between serum IgE levels and FcεRI expression and IgE binding by DCs and basophils in house dust mite (HDM) allergy and during subcutaneous immunotherapy (SCIT).**Methods:** We measured FcεRI, IgE and HDM allergen on DCs (conventional type 2 DCs, cDC2s; plasmacytoid dendritic cells, pDCs) and basophils by flow cytometry in 22 non-allergic vs 52 allergic subjects and upon HDM SCIT in 28 allergic subjects. IgE levels were measured in serum.**Results:** Serum IgE correlated differentially with FcεRI expression and IgE binding depending on cell type and allergic status. In non-allergic subjects, FcεRI/IgE surface densities increased with serum IgE to a significantly stronger degree on basophils compared to cDC2s. By contrast, in allergic subjects FcεRI/IgE surface densities increased with serum IgE to a slightly stronger degree on cDC2s compared to basophils. In addition, the data set suggests sequential loading of IgE onto FcεRI expressed by these cells (basophils > cDC2s > pDCs). Finally, HDM SCIT induced a temporary increase in serum IgE, which was paralleled by a peak in FcεRI and IgE on DCs, but not on basophils.**Conclusions & Clinical Relevance:** This study provides a comprehensive insight into the relation between serum IgE and FcεRI/IgE on basophils and DC subsets. The novel finding that HDM SCIT induces a temporary increase in FcεRI expression on DCs, but not on basophils, can be an incentive for future research on the potential tolerogenic role of IgE/FcεRI signalling in DCs in the setting of allergen immunotherapy.

KEYWORDS

allergic rhinitis, basophil activation, basophils, dendritic cells, high-affinity IgE receptor, house dust mite, IgA, IgE, IgG₄, immunotherapy

1 | INTRODUCTION

Allergic rhinitis (AR) represents an IgE-mediated inflammation of the nasal mucosa induced by a T helper 2 (Th2)-polarized immune response to inhaled allergens.¹ Basophils and mast cells are centraleffector cells in allergic airway inflammation. Binding of allergen-specific IgE (sIgE) antibodies to their high-affinity IgE receptors (FcεRI) sensitizes them to release mediators upon subsequent exposure to that allergen, thereby provoking allergic symptoms.² In mice, it was shown that IgE interaction with FcεRI stabilizes the receptors' expression on the surface of mast cells and basophils.²⁻⁴ In humans as well, several in vitro studies using biochemical approaches have

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shown that IgE induces up-regulation of FcεRI on basophils⁵⁻⁹ and mast cells.¹⁰ In vivo evidence that IgE regulates FcεRI expression in humans is mainly provided by studies with omalizumab, a therapeutic anti-IgE antibody that strongly reduces free IgE levels (10- to 100-fold), accompanied by parallel decreases in FcεRI expression on basophils^{11,12} and mast cells.¹³

Dendritic cells (DCs) are pivotal in regulating the allergic airway inflammation; they constantly scan the environment, sample antigens and direct T cell differentiation into Th1, Th2, Th17 or regulatory T cell fates.^{14,15} In humans, but not mice, DCs constitutively express a trimeric variant of the high-affinity IgE receptor.^{16,17} However, both function and regulation of FcεRI on DCs remain incompletely understood. Both human^{18,19} and murine²⁰ studies have provided evidence for the role of IgE binding to FcεRI on DCs in facilitating allergen presentation, activation of Th2 cells and up-regulation of pro-inflammatory cytokines. Other recent studies in humanized FcεRI transgenic mice,^{21,22} however, support an anti-inflammatory and immune regulatory role of FcεRI signalling in DCs.^{23,24}

Similar to mast cells and basophils, in vivo regulation of FcεRI expression on human DCs by serum IgE is supported by studies with omalizumab, showing reduced FcεRI expression on DCs in parallel with the reduction in serum-free IgE levels.^{19,25}

Allergen immunotherapy (AIT) is currently the only treatment for AR able to induce long-term desensitization. Generally accepted mechanisms of AIT include mast cell and basophil desensitization,²⁶⁻³² induction of regulatory T³³⁻³⁶ and B cells,³⁷⁻³⁹ suppression of effector Th2 cells,^{33,35,40-42} modulation of allergen-specific IgE and IgG production,^{43,44} and suppression of migration and activation of eosinophils, mast cells and basophils in the affected tissues.⁴⁵⁻⁴⁹ So far, it is, however, not known to which extent the changes in serum IgE levels upon treatment with AIT affect FcεRI expression on basophils and DCs.

This study aimed to gain more insight into the relation between serum IgE and FcεRI expressions and IgE binding by basophils and DCs in non-allergic subjects compared to patients with house dust mite (HDM)-induced AR and during the course of subcutaneous immunotherapy (SCIT). In addition, we evaluated HDM allergen binding measured by flow cytometry on basophils and DCs in relation to serum levels of HDM sIgE in these subjects.

2 | METHODS

2.1 | Study subjects and treatment

Fifty-two HDM-allergic subjects (HDMA) and 22 non-allergic subjects (HC, presumably non-atopic subjects) were enrolled. The allergic status was defined based on careful history taking, skin prick tests (SPT) and serum HDM sIgE levels (HDMA > 0.35 kU/L sIgE to D.pt, HC < 0.10 kU/L sIgE). The study population included adults and adolescents (age range 15-40 years). Twenty-eight HDM-allergic patients initiated treatment (open-label) with conventional HDM SCIT (Alutard®-SQ 510, ALK-Abelló, the Netherlands). Study visits were planned before the first injection (visit 0, v0),

halfway up dosing (v1), at the end of up dosing (v2), after 4 months of maintenance (v3) and after 1 year of treatment (v4) (Figure S1). Nine HDMA and 9 HC had repeated visits following the same timeline. At each study visit, patients filled out questionnaires, underwent SPTs and blood sampling. Patients' baseline characteristics and immunological parameters are displayed in Table S1 (Supporting Information). All patients were recruited via the Department of Otorhinolaryngology in Ghent University Hospital (Belgium) and gave written informed consent before inclusion. The study was approved by the local ethical committee of Ghent University Hospital.

2.2 | Serum immunoglobulins

Total IgE (tIgE), D. *pteronysinus* (D.pt) sIgE and D.pt sIgG₄ were measured in serum with ImmunoCAP (Phadia AB, Uppsala, Sweden). IgA levels were measured by ELISA; allergen extract (Alutard®-SQ, ALK-Abelló, the Netherlands) was used as coating antigen for detection of HDM sIgA₁ and sIgA₂ as previously reported.⁵⁰

2.3 | Flow cytometry and basophil activation test

PBMCs were isolated from EDTA-anticoagulated blood by standard Ficoll-Paque density gradient centrifugation, resuspended in fetal calf serum/10% dimethylsulphoxide (Sigma-Aldrich, St. Louis, MO) and stored at -150°C. When all samples had been collected, PBMCs were thawed in batches to perform analyses.

After recuperation, equal numbers of PBMCs (0.5 M) were stained with fixable viability dye, fluorescently labelled antibodies and fluorescently labelled HDM allergen (Der p 1 and Der p 2) under saturating conditions. FcεRI surface levels were determined using the antibody clone AER-37, which binds to the α-chain of FcεRI, irrespective of its IgE occupancy.

Cells were acquired on an LSR Fortessa flow cytometer (BD Biosciences). Data were analysed with FlowJo version 10.3 (Tree Star Inc., Ashland, OR, USA). A validation experiment confirmed similar FcεRI expression and IgE binding on basophils and DCs from frozen-thawed cells vs freshly isolated cells (*data not shown*). The assays and analyses were not blinded.

The gating strategy for identification of basophils and DC subsets is illustrated in Figure S2. Within lineage cocktail 1⁻CD34⁻ cells, basophils were identified as HLADR⁻CD123⁺BDCA2⁻ cells, plasmacytoid dendritic cells (pDCs) as HLADR⁺CD123⁺CD11c⁻BDCA2⁺ cells and myeloid or conventional DCs (cDCs) as HLADR⁺CD123⁻CD11c⁺ cells. cDCs were further subtyped as cDC1s by gating BDCA3⁺ cells and cDC2s by gating BDCA1⁺ cells. DC nomenclature was applied as recommended by Guillems et al.⁵¹ The gates for FcεRIα, IgE and D.pt were set based on the boundaries of positivity determined by Fluorescence Minus One (FMO) controls. Readouts of FcεRIα expression and IgE and D.pt binding were geometric mean fluorescence intensities (MFI) and proportions (%) of FcεRIα⁺, IgE⁺ and D.pt⁺ cells.

Basophil activation tests (BAT) were performed on patients' fresh whole blood samples (EDTA) using the Flow CAST® kit (Bühlmann

Laboratories, Schönenbuch, Switzerland) according to the manufacturer's instructions. CD-max (measure for basophil sensitivity) was defined as the maximum proportion of activated basophils at any allergen concentration. The EC50 was defined as the allergen concentration eliciting 50% of maximal basophil activation. The CD-sens (measure for basophil sensitivity) was defined as the EC50 inverted and multiplied by 100.^{52,53} The area under the curve (AUC, reflects both basophil reactivity and sensitivity) and EC50 were calculated with GraphPad Prism 6.00 (GraphPad Software, La Jolla California, USA).

2.4 | Statistical analysis

Paired data in Figure 1A,B were analysed using the related samples Friedman's two-way analysis of variance by ranks. Unpaired data in Figure S4 were analysed using the Mann-Whitney U test. Repeated-measures analyses in Figure 3, Figure 4, Figure S6 (B,C, E) were performed with mixed model analyses. Log-transformation prior to mixed model analysis was applied when residuals of the model were skewed (this was necessary for serum levels of IgE, slgE, slgG₄, slgA₁, slgA₂ and CD-sens). Bonferroni correction for multiple comparisons was performed when applicable. These statistical analyses were performed with IBM SPSS statistics version 23 (IBM Corp., Armonk, NY, USA). For Figure 2, Figure S5 and Figure S6 (A,D), Spearman's correlation coefficients, and for Figure 1C, best-fitted curves and slopes were calculated using GraphPad Prism (GraphPad Software, La Jolla California, USA). Additional information regarding questionnaires, SPTs, dyes and antibodies, Der p labelling, and BAT is provided in the Supporting Information.

3 | RESULTS

3.1 | FcεRI expression and binding of IgE and D.pt on basophils and DCs in allergic and non-allergic subjects

3.1.1 | Expression levels on basophils and DC subsets in HDM-allergic subjects

Using flow cytometry, we first determined FcεRIα expression and binding of IgE and D.pt on basophils and DCs in 52 HDM-allergic subjects (Figure 1A-B). Over 95% of both basophils and cDC2s expressed FcεRIα and bound IgE; the surface densities of FcεRIα and IgE were, however, significantly lower on cDC2s compared to

basophils. The proportions of FcεRIα⁺ and IgE⁺ cells, as well as the surface densities, were lower for pDCs. The proportions of FcεRIα⁺ and IgE⁺ cDC1s, as well as cell surface densities on cDC1s, were negligibly low; cDC1s will therefore not be further discussed. Normalization of IgE to FcεRIα (MFI) indicates that the differences in IgE binding between basophils and cDC2s are only partly explained by the difference in FcεRIα expression and hence suggests that IgE receptor occupancy is lower in cDC2s compared to basophils ($P = .001$). In HDMA, cDC2s and pDCs display comparable ($P = .305$) IgE surface densities in relation to their FcεRIα expression levels, indicating that the difference in IgE binding between cDC2s and pDCs is largely explained by the difference in FcεRIα expression (Figure S3). D.pt capture was by far highest for basophils, followed by cDC2s and pDCs, and negligibly low for cDC1s.

3.1.2 | Differences according to allergic status and symptom severity

Next, we considered differences according to allergic status (HC vs HDMA) and according to symptom severity (Figure S4, Table S1). HDMA subjects were stratified into two groups based on their visual analogue scale (VAS) score for symptom control: controlled AR (VAS ≤ 50, HDMA1, $n = 29$) and uncontrolled AR (VAS > 50, HDMA2, $n = 23$).⁵⁴ Significant differences in serum tIgE and D.pt slgE levels were observed not only according to allergic status (HC < HDMA), but also according to symptom severity (HDMA1 < HDMA2). In relation to the differences in serum tIgE and D.pt slgE levels, significant differences in FcεRIα, IgE and D.pt surface levels (MFI) on basophils, cDC2s and pDCs were observed between the groups. It should, however, be noted that the differences between HDMA1 and HDMA2 in surface densities of FcεRI/IgE/D.pt were less pronounced (and not significant) for the basophils.

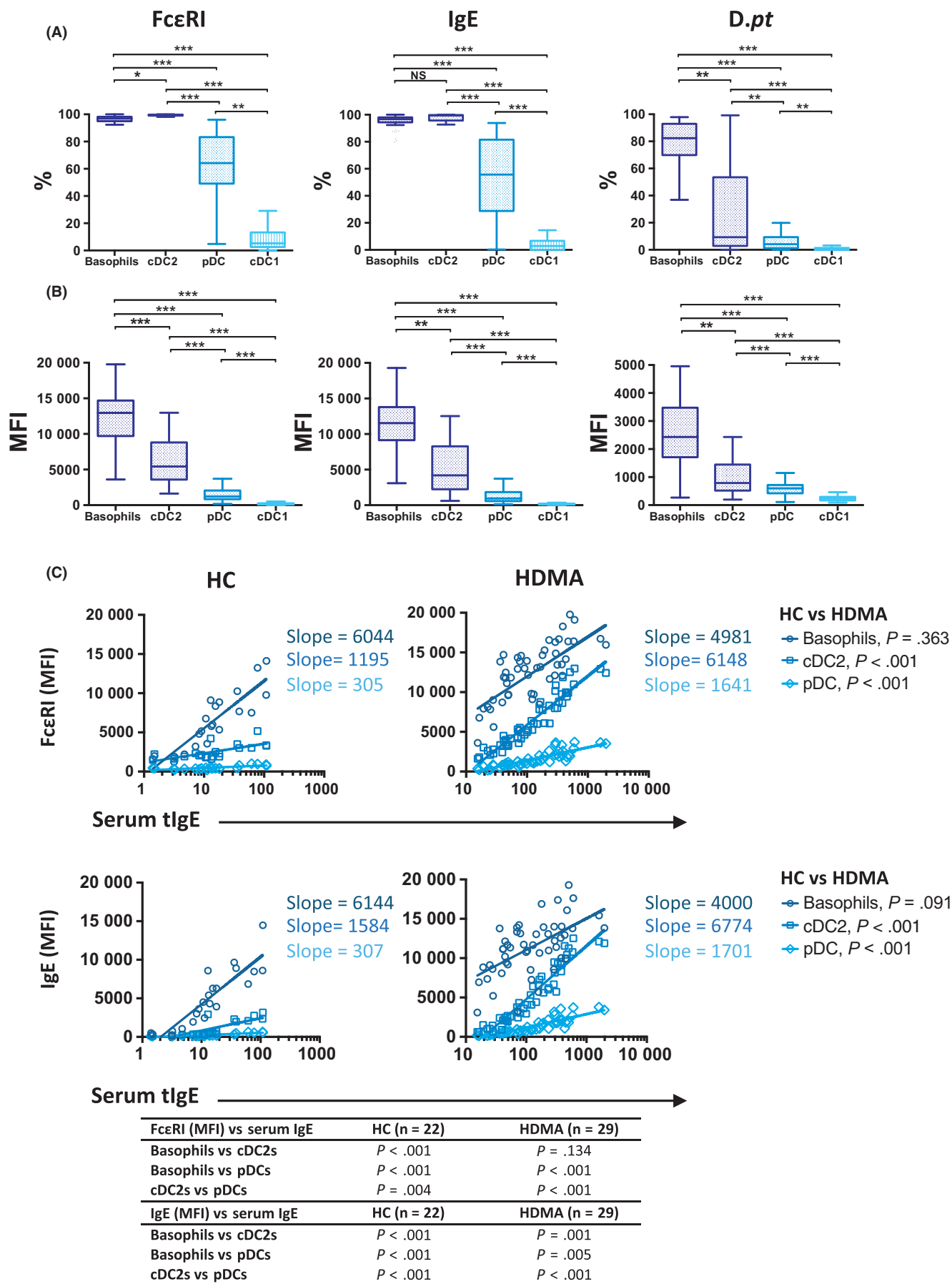
In addition, it is remarkable that in most non-allergic subjects, less than 20% of cDC2s carry IgE, whereas in the same subjects, more than 85% of cDC2s express FcεRI, suggesting low receptor occupancy.

3.1.3 | FcεRI expression, IgE and D.pt binding relate differentially to serum IgE levels depending on cell type

To better understand the above findings, we explored the correlations between serum IgE levels, FcεRIα expression, and IgE and D.pt

FIGURE 1 A,B, Overview of FcεRI expression and IgE and D.pt binding by basophils, cDC2s, pDCs and cDC1s in 52 HDM-allergic subjects; A, shows proportions (%) of positive cells; B, shows surface densities (MFI). Data are displayed as box-and-whisker plots (Tukey style).

*** $P < .001$, ** $P < .01$, * $P < .05$; NS, not significant. C, Serum IgE levels vs FcεRI and IgE surface densities on basophils and dendritic cell subsets in non-allergic subjects ($n = 22$) and in HDM-allergic subjects ($n = 52$). Best-fitted lines and slopes are shown (regression analysis). P -values for the differences in the slopes between HC and HDMA are displayed next to the graphs; P -values for the differences in the slopes between basophils, cDC2s and pDCs are displayed below the graphs. cDC1, conventional type 1 dendritic cells; cDC2, conventional type 2 dendritic cells; D.pt, Dermatophagoides Pteronyssinus; FcεRI, high-affinity IgE receptor; MFI, geometric mean fluorescence intensity; pDC, plasmacytoid dendritic cells; tIgE, total IgE



binding on basophils and DCs (Figure 2, Figure S5). Remarkably, the proportions of FcεRIα⁺ and IgE⁺ basophils correlated positively with serum tIgE in HC ($r_s = .537$), but negatively with serum tIgE levels in HDMA ($r_s = -.414$), whereas the proportions of FcεRIα⁺ and IgE⁺ cDC2s and pDCs correlated positively with serum tIgE in both HC and HDMA (Figure 2A,B). The densities (MFI) of FcεRIα and IgE on basophils, cDC2s and pDCs correlated positively with serum tIgE levels in both HC and HDMA, but in HDMA, the correlations were stronger for DCs than for basophils ($P < .001$ for cDC2s vs basophils; $P < .001$ for pDCs vs basophils, Figure S5A-B).

A striking observation is that the proportions of IgE⁺ cDC2s started to increase when the proportions of IgE⁺ basophils reached the plateau (>95%) at serum tIgE levels of about 10 kU/L and that the proportions of IgE⁺ pDCs started to increase when the proportions of IgE⁺ cDC2s reached the plateau (>95%) at serum levels of about 40 kU/L (Figure 2B); hence, the data set suggests sequential loading of IgE onto FcεRI expressed by these cells (basophils > cDC2s > pDCs).

Remarkably, the slopes of best-fitted lines for the relation between serum tIgE and FcεRIα surface densities (regression analysis, Figure 1C) tended to be lower in HDMA compared to HC for basophils (4,981 vs 6,044, $P = .363$), but were significantly higher in HDMA compared to HC for cDC2s (6,148 vs 1,195, $P < .001$) and pDCs (1,641 vs 305, $P < .001$). Hence, in HDMA, the slope was slightly higher for cDC2s than for basophils (6,148 vs 4,981, $P = .134$), whereas in HC, the slope was significantly lower for cDC2s than for basophils ($P < .001$). Similar findings were observed for the relation between serum tIgE and IgE surface densities. Hence, the data set suggests shifting relationships between serum tIgE and FcεRI/IgE surface levels on these IgE binding cells.

In HDMA, we furthermore observed that D.pt capture by cDC2s and pDCs correlated very strongly with serum D.pt sIgE ($r_s = .928$ and $r_s = .792$ resp.), whereas D.pt capture by basophils correlated more strongly with the ratio of serum D.pt sIgE to tIgE (serum sIgE/tIgE, $r_s = .800$) (Figure 2C, Figure S5C). The flow cytometric assay does not discriminate between bound IgE and IgG, but these very strong correlations suggest that D.pt capture by basophils and DCs measured in this assay reflects the density of D.pt sIgE on these cells, rather than other allergen-specific immunoglobulin classes.

3.2 | Changes upon treatment with house dust mite immunotherapy

3.2.1 | Clinical and immunological response upon treatment with HDM SCIT

In the present open-label study, treatment with HDM SCIT resulted in clinical improvement (assessed with questionnaires), suppression of SPT responses, a strong induction of D.pt sIgG₄ antibodies (which might act as surrogate measure of other IgG subclasses), a modest induction of HDM sIgA₁ and sIgA₂ antibodies and suppression of basophil allergen sensitivity (CD-sens) (Figure 3, Figure S6). At baseline, basophil allergen sensitivity (CD-sens) was strongly correlated

with serum D.pt sIgE ($r_s = .638$, $P < .001$), but even stronger with serum sIgE/tIgE ($r_s = .875$, $P < .001$) and with D.pt capture by basophils ($r_s = .883$, $P < .001$) (Figure 3, Figure S6). This finding suggests that in allergic subjects, serum sIgE/tIgE determines the density of FcεRI-bound allergen sIgE and therefore the likelihood of allergens to cross-link FcεRI and to induce basophil activation. The decrease in basophil allergen sensitivity upon HDM SCIT paralleled the strong rise in D.pt sIgG₄ levels from v2 onwards (Figure 3). D.pt sIgG₄ levels were inversely correlated with CD-sens when considering all visits together ($r_s = -.417$, $P < .001$; Figure S6D), illustrating their coinciding change. CD-sens was not significantly correlated with HDM sIgA₁ or sIgA₂, neither at any of the visits, nor when all visits are considered together (data not shown).

3.2.2 | FcεRI expression and IgE binding change upon treatment with HDM SCIT in relation to serum IgE levels

Next, we aimed to evaluate to which degree changes in serum tIgE and D.pt sIgE during the course of HDM SCIT affected FcεRIα expression and IgE and D.pt binding on basophils and DCs. Upon treatment with HDM SCIT, we observed a peak of serum tIgE levels at the end of updosing (v2, $P < .001$) followed by a steady decrease; levels, however, remained significantly increased compared to baseline at v3 ($P = .003$) and at v4 ($P = .046$) (Figure 4A). In parallel herewith, FcεRIα and IgE densities on both cDC2s and pDCs peaked at v2, followed by a steady decrease. By contrast, no changes in FcεRIα and IgE densities on basophils were observed during the course of HDM SCIT (Figure 4B).

Upon treatment with HDM SCIT, serum D.pt sIgE levels peaked at v2 ($P < .001$) and then returned steadily towards baseline levels. Serum sIgE/tIgE increased only slightly at v2 ($P = .026$), followed by a decrease that was significant at v4 ($P = .007$) (Figure 4A). D.pt capture by basophils and by DCs changed in parallel with the changes in serum sIgE/tIgE and was significantly decreased at v4. This suggests that after 1 year of immunotherapy, the density of FcεRI-bound D.pt sIgE on basophils and DCs is decreased in parallel with the decrease in serum sIgE/tIgE ratio (Figure 4C). Additional findings regarding BAT are described in the Supporting Information.

4 | DISCUSSION

We provided a comprehensive overview of FcεRI expression and IgE binding on circulating basophils, cDC2s and pDCs in relation to serum IgE levels. Previous studies have reported FcεRI expression and IgE binding on cDC2s and pDCs in relation to serum IgE levels,^{16,19,25,55-59} but the present study is the first to report proportions of FcεRI⁺ and IgE⁺ cells as well as surface densities on both basophils and DC subsets in allergic as well as non-allergic subjects, and upon treatment with HDM SCIT.

Relative levels of FcεRI (and IgE) densities on basophils, cDC2s and pDCs in HDM-allergic subjects were 10:4.4:1 (and 12:4.4:1), which is roughly comparable to what was previously reported

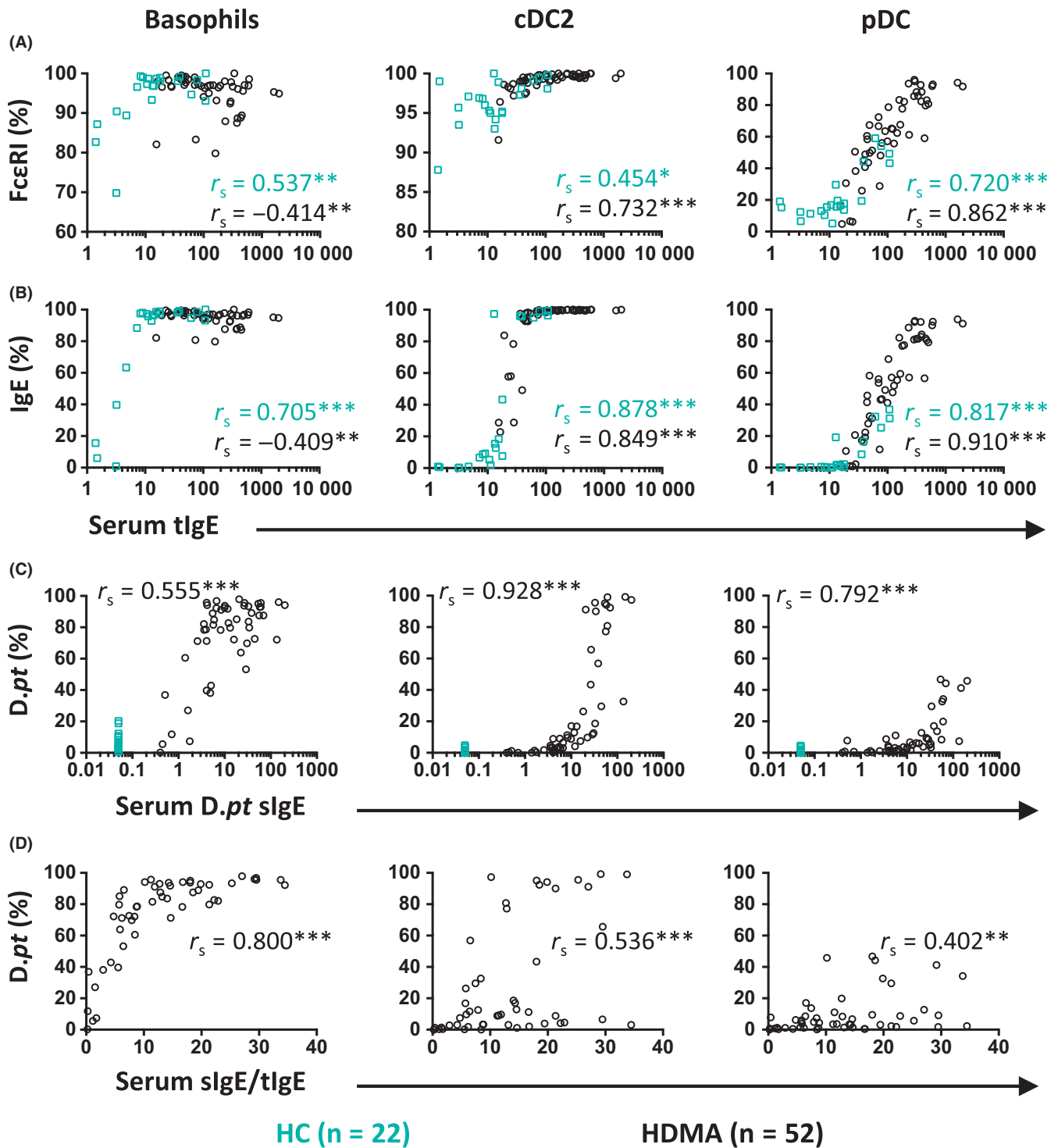


FIGURE 2 A, Serum tlgE levels (kU/L) vs proportion (%) of FcεRI expressing basophils, cDC2s and pDCs in non-allergic subjects (HC, n = 22, green) and in HDM-allergic subjects (HDMA, n = 52, black). B, Serum tlgE levels (kU/L) vs proportion (%) of IgE binding basophils, cDC2s and pDCs in HC and in HDMA. C, Serum D.pt slgE levels (kU/L) vs proportion (%) of D.pt binding basophils, cDC2s and pDCs in HC and in HDMA. D, Serum slgE/tlgE ratio vs proportion (%) of D.pt binding basophils, cDC2s and pDCs in HDMA. D.pt binding on basophils is strongly correlated with the ratio of serum D.pt slgE to tlgE; by contrast, D.pt binding on cDC2s and pDCs is strongly correlated with serum D.pt slgE levels without adjustment for tlgE. *** $P < .001$, ** $P < .01$, * $P < .05$. cDC2, conventional type 2 dendritic cells; D.pt, Dermatophagoides Pteronyssinus; FcεRI, high-affinity IgE receptor; MFI, geometric mean fluorescence intensity; pDC, plasmacytoid dendritic cells; r_s , Spearman's rank correlation coefficient; tlgE, total IgE

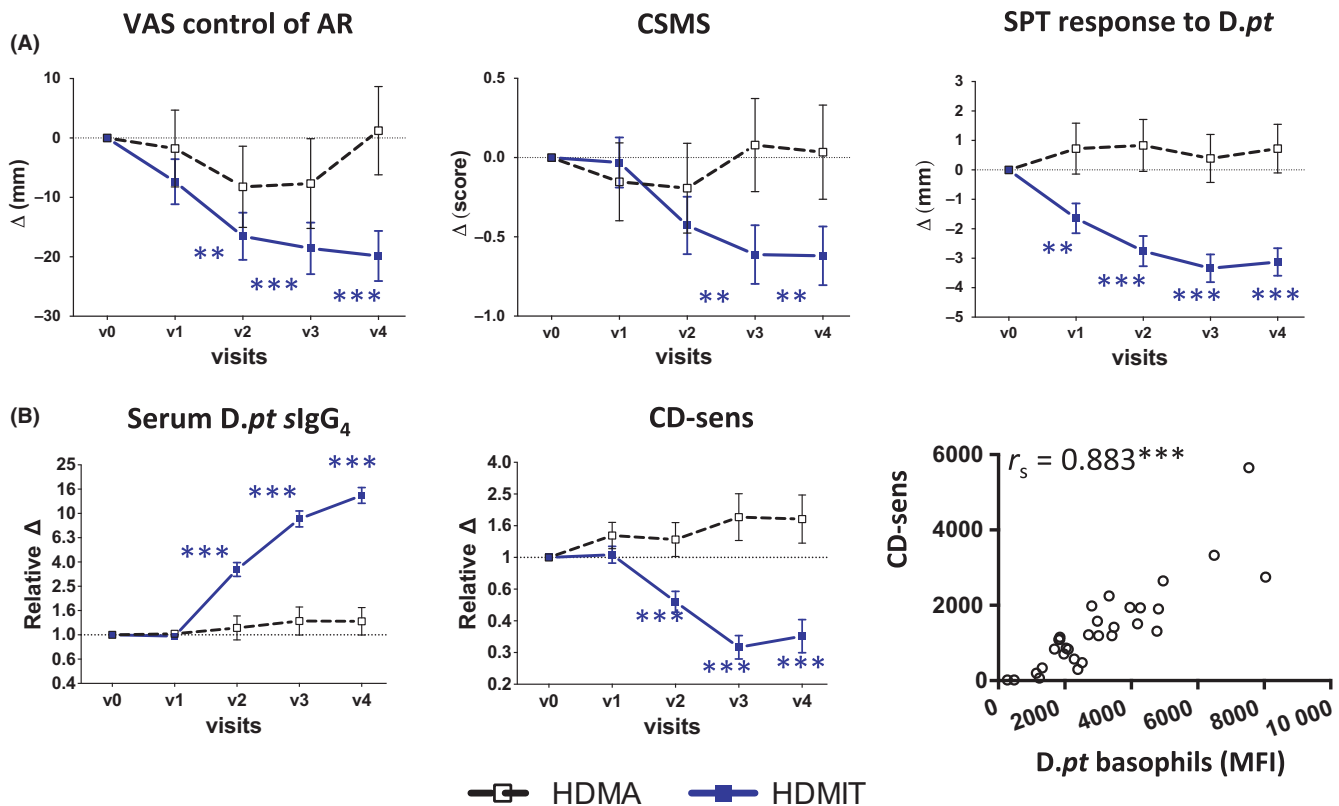


FIGURE 3 A, Change from baseline of VAS for control of AR symptoms, CSMS, SPT response to D.pt, serum D.pt slgG₄ levels and basophil allergen sensitivity (CD-sens) in HDM-allergic subjects undergoing HDM SCIT (HDMIT, n = 28) and in HDM-allergic subjects not undergoing SCIT (HDMA, n = 9), illustrated with line charts (error bars represent standard errors of the mean). The change after one year of treatment (v4) of both the VAS score and the CSMS is significant compared to the control group ($P = .023$ and $P = .040$, respectively). The changes in SPT response to D.pt are significant compared to the control group ($P = .002$). The changes in serum D.pt slgG₄ and CD-sens are significant compared to the control group ($P < .001$). Changes that are significant compared to baseline are indicated: *** $P < .001$, ** $P < .01$, * $P < .05$. B, Correlations of CD-sens with D.pt binding by basophils at baseline. v0, baseline; v1, halfway up dosing; v2, end of up dosing; v3, 4-month maintenance; v4, 1 y of treatment; Δ, absolute change from baseline; CD-sens, measure for basophil allergen sensitivity; CSMS, combined symptoms and medication score; D.pt, Dermatophagoides Pteronyssinus; relative Δ, relative change from baseline (log scale); SPT, skin prick test; VAS, visual analogue scale

(12:6.5:1).¹⁶ The present study confirms that IgE is a major factor regulating FcεRI expression on human basophils, cDC2s and pDCs in vivo and that FcεRI/IgE surface densities on basophils, cDC2s and pDCs are higher in allergic subjects compared to non-allergic subjects.¹⁶ In addition, we report that in non-allergic subjects, by contrast to allergic subjects, a large proportion of cDC2s did not bind IgE, although they did express FcεRI, indicating low receptor occupancy. In addition, normalizing IgE to FcεRI densities (MFI) suggests that IgE receptor occupancy is lower in non-allergic subjects compared to allergic subjects in all three cell types, with the lowest receptor occupancy for cDC2s. Convincing data supporting low IgE receptor occupancy on basophils in healthy subjects compared to allergic subjects and in relation to serum IgE levels has been reported previously.^{60,61} One previous study also indicated lower IgE receptor occupancy in cDC2s compared to basophils in healthy subjects, but did not include allergic subjects for comparison.⁵⁹ Findings from another study also indirectly suggested lower receptor occupancy in DCs from healthy subjects compared to atopic subjects, but the study did not distinguish between the different DC subsets.⁵⁶

Consistent with a previous study (Greer et al),⁵⁹ we demonstrate that in non-allergic subjects, FcεRI and IgE surface densities increase with serum IgE levels to a much lesser degree (flatter slope) in cDC2s than in basophils ($P < .001$, Figure 1C). This flatter slope for DCs might indicate that DCs do not synthesize FcεRI receptors as quickly as basophils, while retaining a rate of internalization of the unoccupied receptor similar to basophils. Greer et al,⁵⁹ however, attributed this observation in non-allergic subjects to constitutive FcεRI-mediated IgE endocytosis in cDC2s, which is not observed in basophils.⁵⁹ Hence, in basophils FcεRI expression might simply depend on the rate of receptor synthesis and the rate of internalization of unoccupied receptor; whereas in DCs FcεRI expression might additionally be regulated by endocytosis of occupied receptors (FcεRI/IgE endocytosis). The present study now shows for the first time that in allergic subjects, by contrast to non-allergic subjects, FcεRI surface densities tend to increase with serum IgE levels to a slightly higher degree (steeper slope) in cDC2s than in basophils ($P = .134$, Figure 1C). This might indicate that FcεRI/IgE endocytosis by cDC2s is affected in allergic subjects or, alternatively, becomes

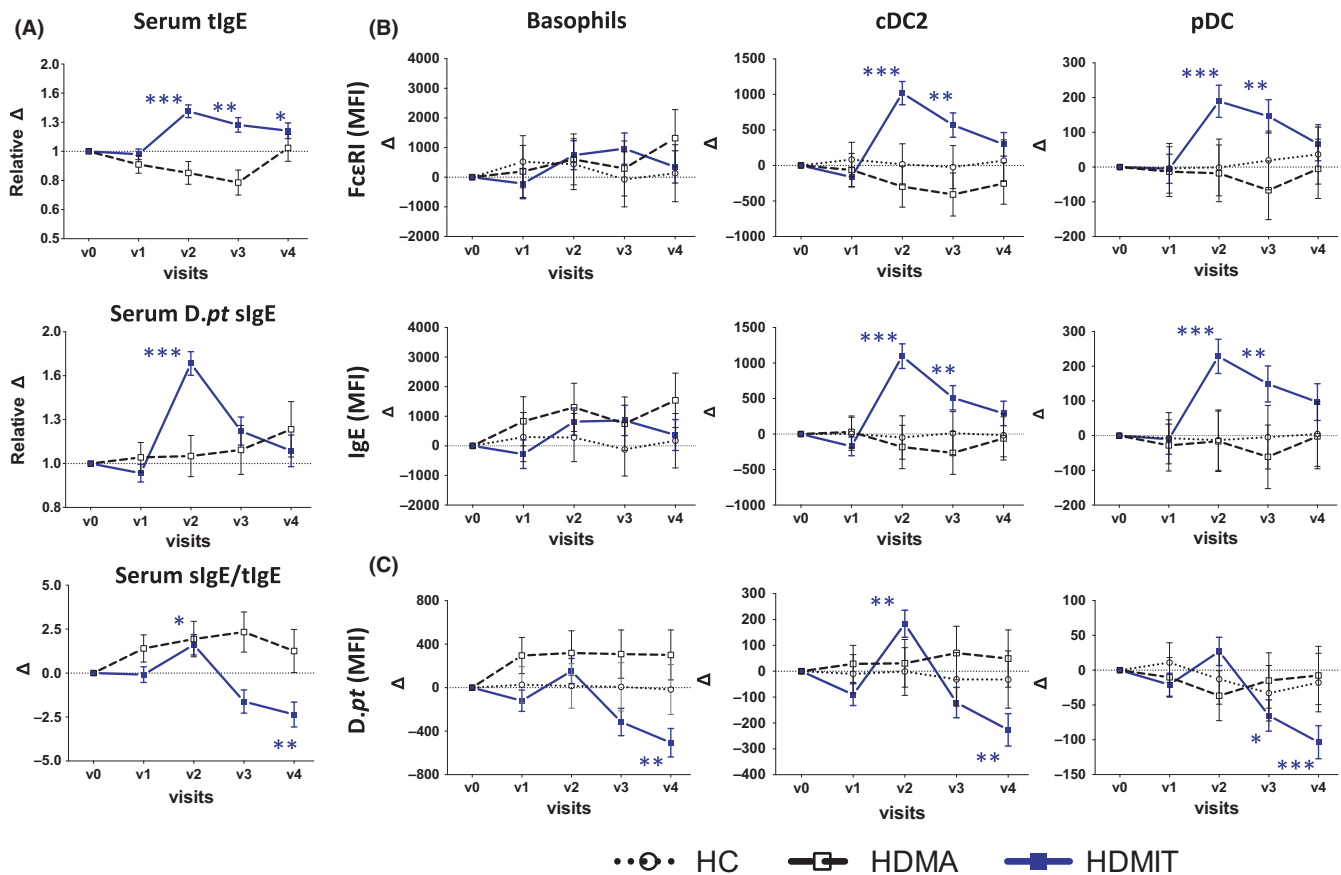


FIGURE 4 A, Change from baseline of serum tIgE levels, D.pt sIgE levels and serum sIgE/tIgE in HDM-allergic subjects undergoing HDM SCIT (HDMIT, $n = 28$) and in HDM-allergic subjects not undergoing SCIT (HDMA, $n = 9$), illustrated with line charts (error bars represent standard errors of the mean). The changes are significant compared the control group ($P < .001$, $P < .001$ and $P = .001$ resp.). B, Change from baseline of FcεRI and IgE on the surface of basophils, cDC2s and pDCs. The changes in FcεRI and IgE are significant compared to the control groups for cDC2s ($P < .001$, $P < .001$), but not for pDCs ($P = .118$, $P = .076$) and basophils ($P = .457$, $P = .526$). C, Change from baseline of D.pt binding on the surface of basophils, cDC2s and pDCs. The changes in D.pt binding are significant compared to the control groups for basophils ($P = .013$), cDC2s ($P < .001$) and pDCs ($P = .003$). Changes that are significant compared to baseline are indicated: *** $P < .001$, ** $P < .01$, * $P < .05$. v0, baseline; v1, halfway up dosing; v2, end of up dosing; v3, 4-month maintenance; v4, 1 y of treatment; Δ, absolute change from baseline; cDC2, conventional type 2 dendritic cells; D.pt, Dermatophagoides Pteronyssinus; MFI, geometric mean fluorescence intensity; pDC, plasmacytoid dendritic cells; relative Δ, relative change from baseline (log scale); sIgE, specific IgE; sIgE/tIgE, ratio of D.pt sIgE to total IgE times 100; tIgE, total IgE

saturated at a certain point when serum IgE levels get higher. The latter possibility seems more likely, given our observation of sequential loading of IgE onto FcεRI expressed by basophils, cDC2s and pDCs, independent of the allergic status (Figure 2B). Clearly our descriptive data set cannot resolve these mechanistic possibilities, but it does provide a base upon which to judge future mechanistic and biochemical studies.

In addition, we evaluated whether FcεRI expression and IgE binding on basophils and DCs were affected by HDM SCIT-induced changes in serum IgE. In accordance with previous reports,⁴⁴ there was a temporary increase in both serum tIgE and allergen sIgE early in the course of HDM SCIT, followed by a steady decline. Interestingly, the HDM SCIT-induced peak in serum tIgE was paralleled by a peak in FcεRI and IgE surface densities on both cDC2s and pDCs, but not on basophils. To our knowledge, this has not been reported before. One study reported a significant increase in FcεRI expression

on pDCs, but only after 1 year of AIT and not in parallel with changes in serum IgE.⁶² AIT-induced increases of serum IgE are generally modest compared to the sharp drop in free IgE levels seen upon treatment with omalizumab. Given the modest change in serum IgE levels upon HDM SCIT, and the known log-linear relationship between serum IgE and FcεRI expression on basophils,^{16,60} it is not surprising to find no significant change in FcεRI expression on basophils. On the other hand, the observation that this modest change in serum IgE is paralleled by a significant change in FcεRI expression on DCs highlights their unusual character. This novel finding could be an incentive for future research on the role of FcεRI signalling in DCs in the setting of AIT, especially as recent studies in FcεRI-humanized mouse models support a tolerogenic role of IgE/FcεRI signalling in DCs.^{21,22} The anti-inflammatory role of IgE/FcεRI signalling in DCs was supported in physiologic mouse models of experimental food allergy and chronic asthma, showing dampened

inflammation and decreased severity of food allergy and asthma in FcεRI-humanized mice compared to wild-type mice that do not express FcεRI on their DCs. It was hypothesized that induction of FcεRI on DCs may be part of a counter-regulatory mechanism aiming to dampen inflammation during allergic responses.²² Whether such mechanism is activated upon AIT, and how this could be promoted, would be a valuable novel angle in AIT research and development.

In addition, we measured D.pt on basophils, cDC2s and pDCs by flow cytometry. Although this assay does not discriminate between bound IgE and IgG, our findings suggest that it mainly reflects density of D.pt sIgE on the surface of these cells. D.pt capture by basophils, cDC2s and pDCs was significantly decreased after 1 year of treatment, in parallel with the changes in serum sIgE/tIgE. This suggests that the density of D.pt sIgE on the surface of basophils and DCs is decreased after 1 year of HDM SCIT, resulting in a reduced likelihood for allergens to cross-link FcεRI and reduced cross-presentation of allergens by DCs, which might contribute to allergen tolerance at that time. These findings are enticing and need to be further explored.

In conclusion, the present study revealed several novel findings with regard to the relation of serum IgE levels with FcεRI expression and IgE binding on basophils, cDC2s and pDCs. Main findings included a) sequential loading of IgE onto FcεRI expressed by basophils, cDC2s and pDCs and shifting relationships between serum IgE and FcεRI/IgE surface densities on these IgE binding cells and b) the HDM SCIT-induced temporary increase in serum IgE was paralleled by a peak of FcεRI and IgE on DCs, but not on basophils. The latter finding should encourage research on the potential tolerogenic role of FcεRI signalling in DCs in the setting of AIT.

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CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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Figure S1.

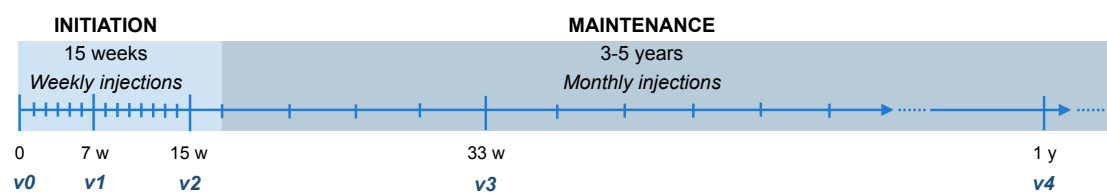


Figure S2.

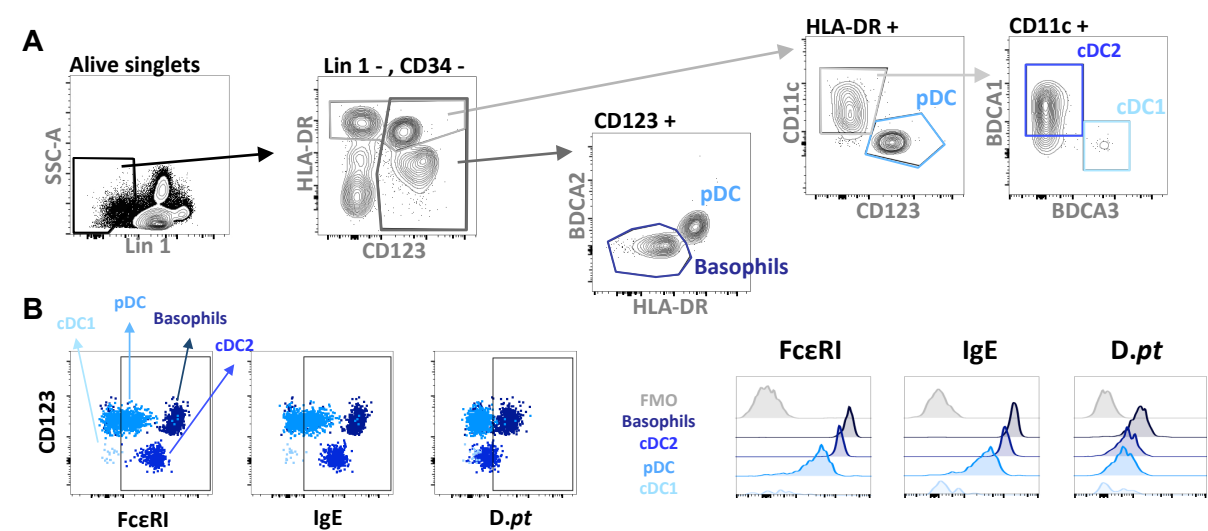


Figure S3.

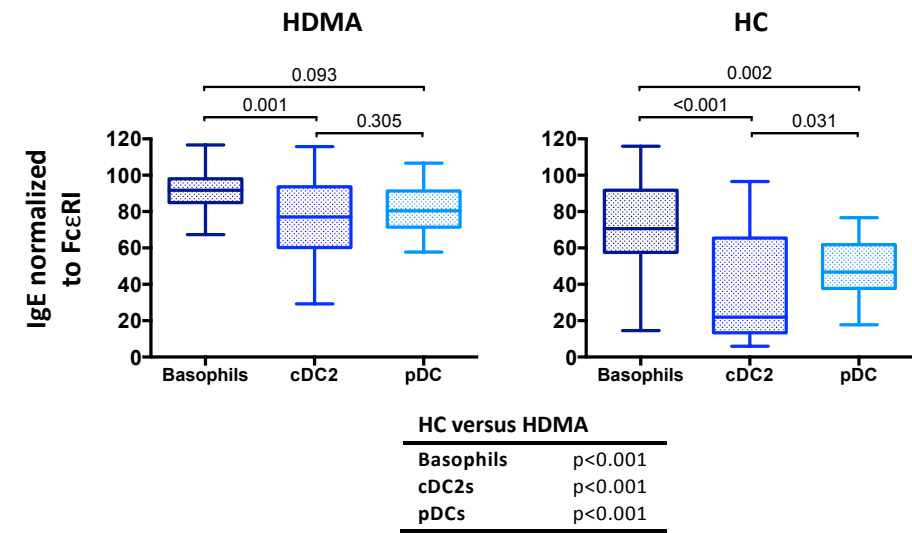


Figure S4.

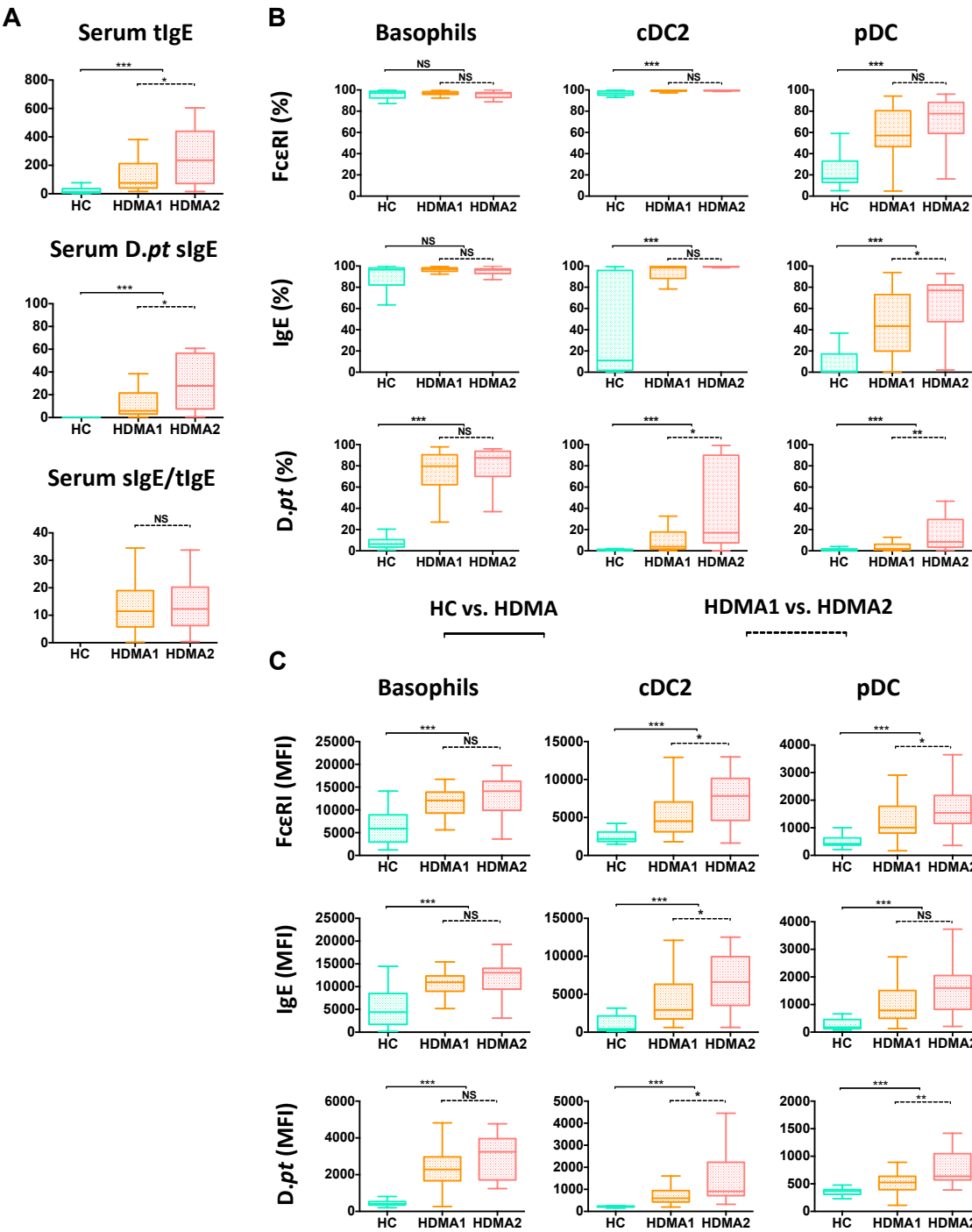


Figure S5.

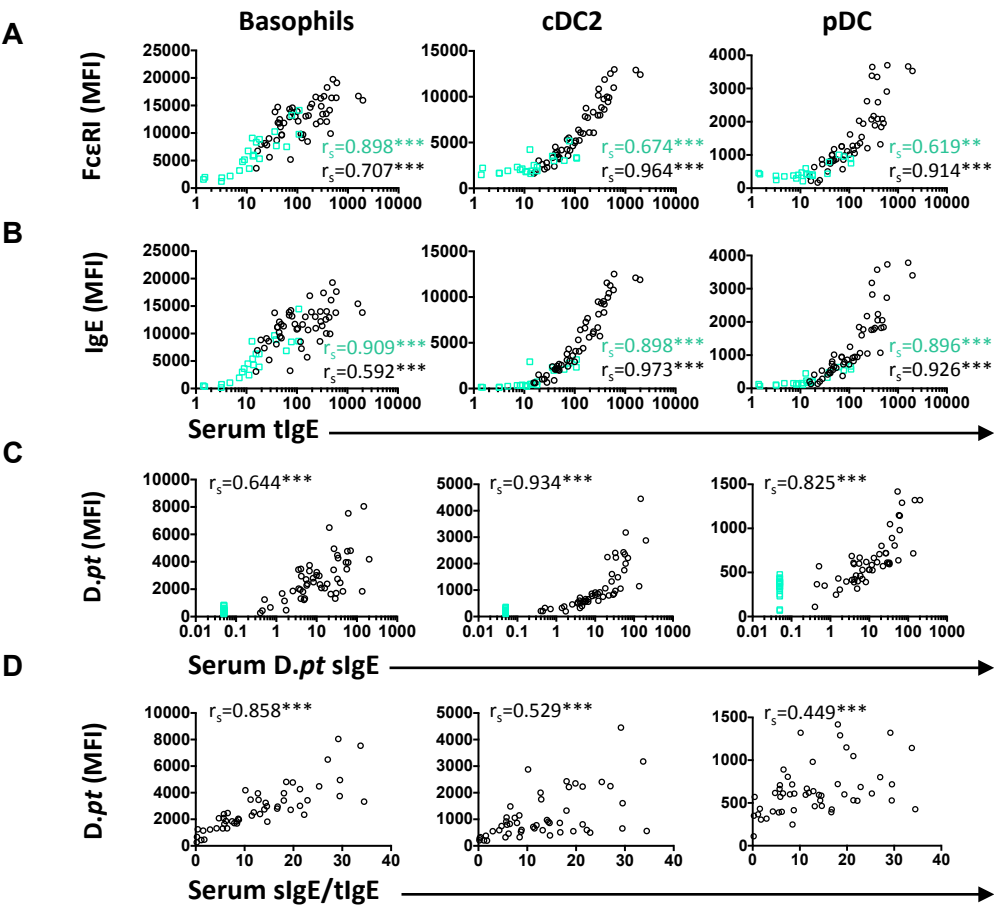
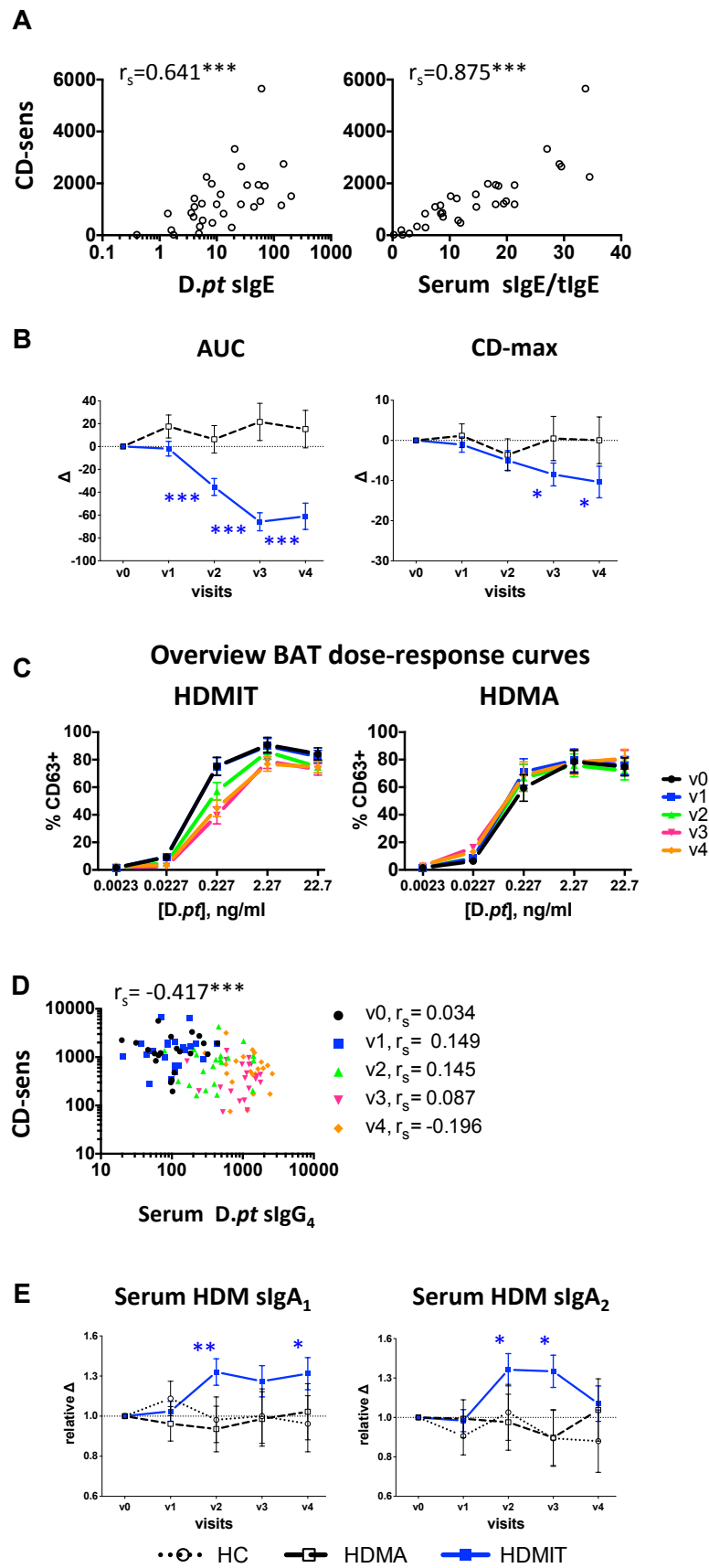


Figure S6.



SUPPORTING INFORMATION

FcεRI expression and IgE binding by dendritic cells and basophils in allergic rhinitis and upon allergen immunotherapy

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Short running title: FcεRI expression and IgE binding by dendritic cells and basophils

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METHODS

Study Subjects and treatment, questionnaires and skin prick tests

At each study visit, patients filled out questionnaires including a Visual Analogue Scale (VAS) for AR symptom control,⁽¹⁾ symptoms scores and medication use for the two previous weeks prior to stopping medication intake for the study. A combined Symptom and Medication Score (CSMS) was calculated as recommended.⁽²⁾ Skin prick tests (SPT) were performed with extracts of 12 common inhalant allergens (Alder, Hazel, Birch, Grass, Mugwort, Aspergillus, Cladosporium, Alternaria, Cat, Dog, *D.pt* and *D.fa*; ALK-Abelló, Netherlands). Patients were asked to stop intake of antihistamines and nasal corticosteroids five days prior to each study visit.

PBMCs –Flow cytometry

The following antibodies, dyes and conjugates were used: Lineage 1 cocktail (CD3, CD14, CD16, CD19, CD20, CD56)/FITC, CD34/FITC (clone 581), anti-CD11c/AF700 (B-ly6), anti-HLADR/APC-H7 (G46-6), and anti-CD123/BV605 (7G3) purchased from BD Biosciences; fixable viability dye eFluor506, anti-FcεRIα/biotin (AER-37), streptavidin/PerCP-Cy5.5 purchased from eBioscience; anti-BDCA1/PE (AD5-8E7), anti-BDCA2/PE (AC144), and anti-BDCA3/VioBlue (AD5-14H12) purchased from Miltenyi Biotec; and anti-IgE/PE-Cy7 (MHE-18) purchased from Biolegend.

Labelling of nDer p 1 and nDer p 2

Major HDM allergens Der p 1 and Der p 2 were used as representative allergens to identify *D.pt* binding cells. Purified natural Der p 1 and Der p 2 (Indoor Biotechnologies, Charlottesville, USA) were labelled with AF647 using an Alexa Fluor 647 Protein Labeling Kit (Molecular Probes®, ThermoFisher scientific, Waltham, USA). In summary, 250 µg of nDer p 1 (or nDer p 2 respectively) was diluted in 25 µl of a 1M solution of sodium bicarbonate. This protein solution was transferred to a vial with reactive dye, and stirred for 1 hour at room temperature in the dark. To purify the labelled proteins, the mixture was transferred in a Slide-A-Lyzer Dialysis Cassette and dialyzed overnight at 4°C in dark. The labeled proteins were titrated for optimal concentration for staining.

Basophil activation test (BAT)

Activated basophils were identified by expression of CD63. Basophils were identified as CCR3⁺ cells with a low side scatter.⁽³⁾

In summary, 50 µl of patients' whole blood was mixed with 50 µl of allergen (*D.pt* extract, BAG-D1, Buhlman Laboratories), 100 µl of stimulation buffer (containing calcium, heparin and IL-3), and 20 µl of staining reagent (mix of anti-CD63-FITC and anti-CCR3-PE). After incubation for 15 minutes at 37°C in a water bath,

erythrocytes were removed by a lysing reaction. After a short centrifugation step, the cells were resuspended in a wash buffer and analyzed by flow cytometry. Anti- FcεRI monoclonal antibody was used as positive control. Bacterial peptide fMLP was used to confirm quality of blood basophils. Stimulation with stimulation buffer only was used to determine the sample's background. Blood samples were stimulated with allergen at different concentrations (5 log dilution series: 0.01 ng/ml, 0.1 ng/ml, 1 ng/ml, 10 ng/ml and 100 ng/ml stock concentration, corresponding to 0.00227 ng/ml, 0.0227 ng/ml, 0.227 ng/ml, 2.27 ng/ml, 22.73 ng/ml final concentration).

RESULTS

Correlations

There was a very strong correlation between FcεRI expression and IgE expression (on basophils $r_s=0.896$, cDC2s $r_s=0.977$, and pDCs $r_s=0.987$ in HDMA; on basophils $r_s=0.962$, cDC2s $r_s=0.788$, and pDCs $r_s=0.782$; in HC).

SPT responses

Upon treatment with HDM SCIT there was significant suppression of the SPT responses to *D.fa* ($p=0.003$ at v2, $p=0.006$ at v3 and $p=0.009$ at v4; significant compared to the control group, $p=0.010$), but the change was less pronounced than for *D.pt*. There was also a modest decrease over time of the response to histamine (significant from v1), however not significant compared to the control group ($p=0.269$).

Serum immunoglobulin responses

Upon treatment with HDM SCIT total IgG₄ and total IgA remained stable over time (data not shown). Treatment with HDM SCIT induced a temporary increase of *D.fa* sIgE at v2, similar to *D.pt* sIgE, and induced a strong rise in *D.fa* sIgG₄ from v2 onwards, slightly more pronounced than the induction of *D.pt* sIgG₄.

Basophil activation test: sensitivity, specificity, non-responders

The basophil activation test was performed in 22 patients undergoing HDM SCIT and in 9 HDMA control subjects with repeated visits. At baseline, basophil activation was positive for all samples upon stimulation with both anti-FcεRI (positive control, > 10%) and *D.pt* allergen (>15%). The basophil activation test was also performed once in 9 non-allergic control subjects. The test was negative in 8 of them, but positive in one (confirmed three times). The latter subject was completely asymptomatic, had negative skin prick tests and negative serum sIgE to both *D.pt* and *D.fa* (<0.1 kU/L) at all visits. Hence, in this sample size (31 allergic subjects and 9 non-allergic subjects), sensitivity of the test was 100% and specificity 88.9%.

In one patient undergoing HDM SCIT, basophil activation was negative at the end of up-dosing (visit 2) to both anti-FcεRI and *D.pt* allergen, but recovered already 1 week later (in additionally taken fresh blood sample), and then remained positive at the subsequent visits. Hence, this was a 'temporary non-responder'. In one HDM allergic control subject, basophil activation was negative to both anti-FcεRI (1.2%) and *D.pt* allergen (1% at highest concentration) at visit 2, and then remained negative at visits 3 and 4 ('permanent non-responder'). Both patients had already low maximal basophil activation at baseline (59.8% and 28.2% respectively).

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Figure legend

Figure S1. HDM SCIT conventional treatment protocol with 15 weekly injections during up dosing, followed by 4- to 6-weekly injections during maintenance phase (maintenance dose: 100.000 SQ-E). Study visits were planned at baseline before the first injection (visit 0), halfway up-dosing (visit 1), at the end of up-dosing (visit 2), after 4 months of maintenance (visit 3), and after one year of treatment (visit 4).

HDM, house dust mite; SCIT, subcutaneous immunotherapy; w, week; y, year.

Figure S2. A. Gating strategy in a representative sample. **B.** FcεRI expression and IgE and *D.pt* binding by basophils, cDC2s, pDCs and cDC1s in a representative sample of a house dust mite allergic subject.

Figure S3. IgE densities normalized to FcεRI for basophils, cDC2s and pDCs, in HDMA (n=52) and in HC (n=22).

Figure S4. A. Overview of serum IgE and *D.pt* sIgE levels in HC (n=22), HDMA1 (n=29) and HDMA2 (n=23) **B-C.** Overview of FcεRI expression and IgE and *D.pt* binding on basophils, cDC2s and pDCs in HC (n=22), HDMA1 (n=29) and HDMA2 (n=23); **B.** shows proportions (%) of positive cells, **C.** shows surface densities (MFI). Data are displayed as Box-and-Whisker plots (Tukey style). *** p<0.001, ** p<0.01, * p<0.05.

FcεRI, high affinity IgE receptor; D.pt, Dermatophagoides Pteronyssinus; MFI, Geometric Mean Fluorescence Intensity; cDC2, conventional type 2 dendritic cells; pDC, plasmacytoid dendritic cells, NS, not significant; sIgE, specific IgE; tIgE, total IgE; sIgE/tIgE, ratio of D.pt sIgE to tIgE times 100; HC, non-allergic control subjects; HDMA1, patients with well/partly controlled allergic rhinitis; HDMA2, patients with uncontrolled allergic rhinitis.

Figure S5. A,B. Serum tIgE levels (kU/L) versus surface densities (MFI) of FcεRI and IgE on basophils, cDC2s and pDCs in non-allergic subjects (HC, n=22, green) and in HDM allergic subjects (HDMA, n=52, black). **C.** Serum *D.pt* sIgE levels (kU/L) versus surface densities of *D.pt* on basophils, cDC2s and pDCs in HC and in HDMA. **D.** Serum sIgE/tIgE ratio versus surface densities of *D.pt* on basophils, cDC2s and pDCs in HDMA. *** p<0.001, ** p<0.01, * p<0.05.

cDC2, conventional type 2 dendritic cells; D.pt, Dermatophagoides Pteronyssinus; FcεRI, high affinity IgE receptor; MFI, Geometric Mean Fluorescence Intensity; pDC, plasmacytoid dendritic cells; r_s, Spearman's rank correlation coefficient; tIgE, total IgE.

Figure S6. A. Correlations of CD-sens with *D.pt* sIgE and serum sIgE/tIgE, at baseline. **B.** Change from baseline of AUC and CD-max of dose-response curves of BAT in HDM allergic subjects undergoing SCIT (HDMIT, n=22) and in HDM allergic subjects not undergoing SCIT (HDMA, n=9), illustrated with line charts (error bars represent Standard Errors of the Mean). The change of AUC is significant compared to the control group (p<0.001); the change of CD-max is not significant compared to the control group (p=0.306). **C.** Overview of the dose response curves at each study visit for both groups (means and standard errors of the means determined with mixed model analysis). The change of the dose response curve is most pronounced at the suboptimal allergen concentration of 0.227 ng/ml. **D.** Correlations between CD-sens and *D.pt* sIgG₄, at the individual visits and when considering all visits together. **E.** Change from baseline of serum HDM sIgA₁ and sIgA₂ levels (measured with ELISA) in HDMIT (n=28), in HDMA (n=9), and in HC (n=9), illustrated

with line charts (error bars represent Standard Errors of the Mean). The change of serum HDM IgA₁, but not of HDMA IgA₂, is significant compared to the control group ($p=0.036$ and $p=0.255$ respectively). Changes that are significant compared to baseline are indicated: *** $p<0.001$, ** $p<0.01$, * $p<0.05$.

v0, baseline; v1, halfway up-dosing; v2, end of up-dosing; v3, 4 months maintenance; v4, 1 year of treatment; relative Δ , relative change from baseline; HDM, House Dust Mite.

Table S1. Patient baseline characteristics, symptom scores, immunoglobulin levels and Fc ϵ RI, IgE and D.pt levels on basophils and DCs

	HC (n=22)	HDMA1 (n=29)	HDMA2 (n=23)	HDMIT baseline (n=28)
Age	26.6 (24.0 ; 27.4)	26.3 (23.3 ; 31.8)	28.3 (21.3 ; 36.0)	27.2 (20.6 ; 35.9)
Sex	16 (73%)	16 (55%)	14 (61%)	16 (57%)
Nicotine	1 (4.5%)	2 (6.9%)	3 (13%)	3 (8%)
Asthma	0 (0%)	7 (24%)	11 (48%)	13 (46%)
Monosensitisation HDM	NA	8 (28%)	3 (13%)	6 (21%)
Co-sensitisation grass	NA	18 (62%)	15 (65%)	19 (68%)
Co-sensitisation tree	NA	7 (24%)	11 (48%)	10 (36%)
Co-sensitisation cat/dog	NA	15 (52%)	16 (70%)	16 (57%)
Co-sensitisation weed	NA	3 (10%)	2 (9%)	3 (10.7%)
Co-sensitisation mold	NA	4 (14%)	3 (13%)	4 (14%)
SS	0.1 (0.0 ; 0.2)	0.8 (0.5 ; 1.1)	1.5 (1.3 ; 1.7)	1.4 (0.8 ; 1.7)
MS	0 (0 ; 0)	0 (0 ; 0.9)	2 (0.9 ; 2)	2 (1 ; 2)
CSMS	0.1 (0.0 ; 0.2)	1.0 (0.7 ; 2.2)	3.3 (2.3 ; 3.5)	2.8 (2.2 ; 3.5)
VAS control	0 (0 ; 1)	27 (14 ; 39)	71 (53 ; 77)	55 (34 ; 75)
VAS nose	2 (0 ; 5)	25 (16 ; 36)	48 (30 ; 69)	34 (27 ; 68)
VAS eye	0 (0 ; 1)	9 (2 ; 27)	56 (10 ; 63)	39 (7 ; 60)
Serum tlgE (kU/l)	13.1 (6.6 ; 36.5)	76.2 (40.2 ; 212.0)	236.0 (72.5 ; 440)	122.6 (49.0 ; 335.8)
Serum D.pt slgE (kU/L)	All < 0.10	5.9 (3.1 ; 21.5)	27.8 (7.6 ; 56.5)	18.3 (5.1 ; 45.0)
Serum slgE/tlgE	NA	11.5 (5.8 ; 19.0)	12.3 (6.3 ; 20.3)	11.9 (6.4 ; 21.4)
% Fc ϵ RI+ in basophils	97.1 (92.4 ; 98.8)	97.0 (95.8 ; 98.4)	96.7 (92.9 ; 97.5)	96.5 (94.9 ; 97.9)
% Fc ϵ RI+ in cDC2s	96.9 (95.0 ; 98.9)	99.5 (98.8 ; 99.9)	99.6 (99.0 ; 99.8)	99.6 (99.0 ; 99.8)
% Fc ϵ RI+ in pDCs	16.7 (12.9 ; 33)	57.1 (46.7 ; 80.5)	77.7 (59.0 ; 88.3)	69.9 (50.1 ; 87.7)
% IgE+ in basophils	96.5 (82.2 ; 98.2)	97.0 (95.1 ; 98.4)	96.3 (92.9 ; 97.4)	96.4 (94.8 ; 97.9)
% IgE+ in cDC2s	10.9 (1.7 ; 95.8)	98.4 (88.25 ; 99.8)	99.6 (99.0 ; 99.8)	99.4 (98.3 ; 99.8)
% IgE+ in pDCs	0.8 (0.2 ; 17.2)	43.4 (19.9 ; 73.1)	77.0 (47.6 ; 82.2)	57.6 (38.7 ; 82.1)
% D.pt+ in basophils	6.2 (3.5 ; 10.4)	79.7 (62.2 ; 90.5)	87.5 (70.0 ; 93.8)	83.6 (69.9 ; 94.0)
% D.pt+ in cDC2s	0.7 (0.2 ; 0.7)	3.9 (1.6 ; 17.7)	17.0 (7.4 ; 90.0)	11.4 (3.3 ; 83.9)
% D.pt+ in pDCs	0.8 (0.2 ; 1.8)	1.8 (1.0 ; 6.2)	8.4 (3.4 ; 29.5)	6.8 (2.3 ; 13.5)
MFI Fc ϵ RI on basophils	5899 (2953 ; 8924)	12083 (9312 ; 13907)	14122 (9898 ; 16304)	13639 (9664 ; 15684)
MFI Fc ϵ RI on cDC2s	2181 (1839 ; 3093)	4515 (3114 ; 7039)	7847 (4614 ; 10158)	5753 (3605 ; 9981)
MFI Fc ϵ RI on pDCs	420 (373 ; 636)	1008 (810 ; 1780)	1540 (1159 ; 2170)	1280 (891 ; 2134)
MFI IgE on basophils	4410 (1724 ; 8509)	10972 (8982 ; 12358)	13044 (9452 ; 14032)	11960 (8997 ; 14026)
MFI IgE on cDC2s	431.5 (255.0 ; 2143)	2935 (1741 ; 6314)	6602 (3530 ; 9950)	4364 (2736 ; 9302)
MFI IgE on pDCs	178 (124 ; 460)	787 (502 ; 1507)	1598 (826 ; 2050)	983 (708 ; 1845)
MFI D.pt on basophils	412 (345 ; 539)	2282 (1667 ; 2967)	3240 (1713 ; 3962)	2761 (1709 ; 4130)
MFI D.pt on cDC2s	230 (199 ; 238)	597 (426 ; 946)	911 (715 ; 2232)	845 (554 ; 2076)
MFI D.pt on pDCs	370 (311 ; 396)	525 (395 ; 638)	638 (570 ; 1048)	618 (457 ; 805)
Serum D.pt slgG ₄ (μ g/l)	2.4 (1.5 ; 40.0)	90.5 (43.6 ; 153.2)	104.3 (49.1 ; 158.7)	97.5 (50.1 ; 150.6)
Serum HDM slgA ₁ (AU)	30.3 (14.4 ; 53.1)	20.0 (10.8 ; 51.4)	42.7 (22.7 ; 67.7)	27.1 (15.7 ; 62.5)
Serum HDM slgA ₂ (AU)	37.9 (17.1 ; 64.2)	36.4 (15.3 ; 57.0)	57.5 (33.6 ; 103.4)	48.3 (26.0 ; 79.5)

HC, non-allergic control subjects; HDMA1, patients with well/partly controlled allergic rhinitis; HDMA2, patients with uncontrolled allergic rhinitis; HDMIT, HDM allergic subjects undergoing HDM SCIT; SS, symptoms scores (0-3); MS, medication scores (0-3); CSMS, combined symptom and medication scores (0-6); VAS, visual analogue scales (0-100); tlgE, total IgE; slgE, specific IgE; slgE/tlgE, ratio of D.pt slgE to total IgE times 100; D.pt, Dermatophagoides Pteronyssinus; cDC2, conventional type 2 dendritic cells; pDC, plasmacytoid dendritic cells; MFI, Geometric Mean Fluorescence Intensity.