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Systematic characterization of basophil anergy

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Abstract

Background

Cohort studies indicated that in certain individuals the basophils do not respond towards allergens due to a desensitization of their Fc-epsilon receptor pathway. Cause and functional role as well as the implications on allergic reactions however are not clear yet.

Methods

A cross-sectional study was carried out in the tropical urban environment of Singapore, where the allergic response is dominated by a single allergen (house dust mite; HDM). Blood samples were collected from 476 individuals and analyzed comprehensively to correlate the functional state of their basophils with the clinical state as well as the composition of the cellular and soluble plasma components.

Results

Inactivation of basophils ('basophil anergy') was observed in about 10% of the cohort. It was associated with a downregulation of basophil Syk and an apparent reduction in the incidence of allergic rhinitis. Correlations on the cohort level suggest that it represents a transitional state to be passed through during the interconversion of responder and non-responder state.

Conclusions

Basophil anergy thus seems to function as activation barrier to prevent unwanted reactions against minor allergens. It may therefore be relevant for diagnostic purposes or therapeutic interventions of allergic diseases.

INTRODUCTION

The IgE response pathway has evolved primarily as defense against macroparasites such as ticks(1) and parasitic worm infections(2,3). In the modern society, however, the IgE response is a nuisance since the Type I hypersensitivity reaction caused by this pathway manifests in common allergic diseases. Allergic rhinitis (AR) alone affects nearly 1.4 billion individuals worldwide(4) . The response to the allergen is mediated by allergen-specific IgE. The IgE/allergen complex is sensed by the high-affinity IgE receptor (FcεRI), found only on a small set of immune cells including mast cells and basophils(5,6). On these two cell types, FcεRI-crosslinking triggers degranulation of histamine-containing vesicles (early phase response) and the secretion of *de novo* synthesized components such as eicosanoids, cytokines and chemokines (late phase response)(7).

As tissue resident cells mast cells act as primary effector cells(8,9) and due to their localization near the blood vessels(10), they are associated for instance with the typical rash and urticaria measured in skin prick tests (SPT). The contribution of basophils to allergic reactions is less obvious. Previous study using an experimental contact hypersensitivity mouse model has shown basophils are essential for the initiation of chronic allergic inflammation(11). During the late phase response they secrete large amounts of interleukin (IL)-3, IL-4 and IL-13(12-15). As driver for Th2 polarisation and IgE class switching, basophils may therefore play a major role in skewing the T and B cell responses towards the production of allergen-specific IgE(16,17).

While basophils usually promptly respond to FcεRI-crosslinking, a number of earlier studies reported that in some individuals the majority of basophils remains inactive. Stimulation with anti-IgE or anti-FcεRI antibodies does not lead here to any degranulation (“non-releaser”). Depending on the study between 8 – 22% of the individuals harbour non-releaser basophils(18-20).

To further characterize this state and to evaluate its role and clinical implications we carried out a comprehensive cross-sectional cohort study in Singapore. The local tropical environment is particularly suited as it provides a simplified and yet “saturated” allergen environment. In a prior study we could show that almost 80% of our Singaporean cohort had been sensitized to a single allergen, house dust mites (HDM)(21). The dominance was so strong that the total IgE levels in the plasma directly correlated with the HDM-specific IgE titers. This resulted in the manifestation of AR symptoms in about 40% of the individuals, which in migrant studies could be linked directly with the exposure to HDM allergens.

In this study, we therefore took advantage of the specific tropical urban environment in Singapore to characterize role and function of non-releaser basophils. By analyzing blood samples of 476 Singaporeans we could demonstrate that the functional inactivation represents an anergy-like state, presumably acting as a barrier preventing unwanted allergic responses. It is observed in about 10% of the cohort and associated with a downregulation of the Syk kinase and a reduced incidence of allergic airway symptoms.

MATERIALS AND METHODS

Cohort data

This cohort study was approved by the Institutional Review Board at the National University of Singapore, Singapore (IRB numbers: NUS 07-023, NUS 10-445 and NUS 09-256) and is in compliance with the Helsinki declaration. Written informed consent was obtained from volunteer donors prior to sample collection. The demographic and clinical characteristics of the cohort are summarized in **Table S1**.

Basophil activation test

The basophil activation test (BAT) was carried out using the Flow2CAST kit (Bühlmann Laboratories). The test was performed according to manufacturer's instructions with some modifications. The detailed description of the BAT is provided in the supplemental data.

Gene expression analysis

Basophil isolation was performed according to manufacturer's instructions by using the EasySep Human Basophil Enrichment kit (Stemcell Technologies). cDNA prepared from these cells were analyzed using Affymetrix Human GeneChip 1.0 ST Arrays.

FACS and phosflow analysis

Flow cytometry analysis was performed on whole blood samples after lysis with erythrocyte lysis buffer (155 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA). The cells were LIVE/DEAD Fixable Aqua Dead kit (Life Technologies). For analysis of various cell types, the whole blood samples were stained with anti-FcεRIα-FITC, anti-CD123-PerCP-Cy5.5, anti-IgE-APC, anti-CD16 PE-Cy7,

anti-CD1c BV421, anti-CD14 Alexa eFluor450, and anti-HLA-DR APC-H7. For phosflow analysis, the protocol for measuring the Syk- and MAPK-signaling pathways in basophils was adapted from the method described by Chow *et al*(22).

Analysis of plasma components

Plasma samples were obtained by centrifugation of whole blood collected in BD 8 ml Sodium Citrate, Mononuclear Cell Preparation Tubes (CPT) according to manufacturer's instructions.

Total plasma IgE and Bt and Dp-specific IgE were measured by ImmunoCAP system (Phadia AB, Uppsala). All other plasma components (listed in **Table S4**) were quantified by Luminex.

Statistical analysis

Data were considered non-parametric and thus all statistical comparisons were performed using non-parametric tests in Prism 6 software (GraphPad Software, Inc., La Jolla). Unless stated otherwise all data analyses were performed using Kruskal-Wallis test results with Dunn's multiple corrections test. $P < 0.05$ were considered to be significant for all statistical tests.

RESULTS

Study design and cohort database

The cohort database was generated based on a cross-sectional study of 476 Singaporeans of Chinese ethnicity. The subjects were grouped into an independent discovery and validation cohort of 199 and 277 individuals, respectively (**Figure 1**). A survey questionnaire was used to determine general demographic parameters as well as clinical information related to the manifestation of allergic diseases. Sensitization to allergens was determined by skin prick test

(SPT) and blood samples taken from each individual were used for the characterization of cellular and soluble blood components including total and allergen-specific IgE.

Of the 416 individuals who provided information on their allergy status, 181 (44%) had clearly defined clinical symptoms of allergic rhinitis (AR) (**Table S1**). In line with the reported dominance of HDM-allergens(21), 77% of the tested individuals reacted against *Blomia tropicalis* (Bt) and/or *Dermatophagoides pteronyssinus* (Dp) allergens in SPT (**Table S1**). Only sporadic responses were detected for non-HDM allergens such as mold (*Curvularia spp.*; 3 out of 232) or the now endemic African oil palm (*Elaeis guineensis*; 19 out of 232 individuals). Immunophenotyping of allergy-related cell surface markers was carried out by flow cytometry directly on whole blood samples. The antibody panels were designed primarily for basophils (surface IgE, CCR3, FcεRI, CD123 etc.) but also provided information on eosinophil, neutrophil, monocyte, DC and lymphocyte subsets (the gating strategy is outlined on **Figure S1A, S1B**).

Analysis of the plasma fraction revealed information on more than 100 soluble blood components (**Table S4**). Cytokines, chemokines and soluble receptors were measured by multiplexed Luminex analysis, total IgE and HDM-specific IgE (Bt and Dp) was determined by ImmunoCAP. HDM-IgE was detected in 98% of the atopic individuals, justifying the use of HDM extracts as general indicator reagent for allergen-specific responses.

The functional state of basophils was determined by flow cytometry in a basophil activation test (BAT). For this the FcεRI-induced degranulation was measured in response to antibody mediated FcεRI stimulation (general reactivity) and by exposure to HDM allergens (allergen-specific reactivity). fMLP receptor ligation was used as positive control. All these data were integrated into a combined database (>300 entries per individual) and used for the systematic analysis of basophil associated allergic reactions.

Detection of basophil anergy

In the BAT assay basophil degranulation is indicated by the upregulation of vesicle associated CD63 on the surface of the cells (**Figure 2A**). The general response was measured by ligation of FcεRI with a monoclonal antibody, while for allergen-specific degranulation Dp- or Bt-extracts were added to the whole blood samples. An individual was defined as 'HDM-responder' (termed here as 'HDM^R') when at least 38% of the basophils were CD63+. 72% of the individuals (344 of the 476) responded to either one or both of the two allergens. (**Figure 2B**). The degranulation triggered by the two HDM allergens was largely overlapping, reflecting the concomitant presence of Dp- and Bt-specific IgE in most of these samples (**Figure S2A**). 18.2% of the cohort (87 out of 476) were classical 'non-responder' (termed here as 'HDM^{NR}') (**Figure 2B**). They did not respond to the allergens but could still degranulate upon anti-FcεRI stimulation (usually used as positive control for the assay). Their failure to respond to the allergen was in fact caused by the absence of HDM-specific IgE in the whole blood sample, which mediated the binding to the receptor (compare **Figure 5B**). The remaining 9.5% of the cohort (45 out of 476) the basophils >38% of the basophils were inactivated (termed here as 'anergic'). The failure to respond was not restricted to the allergen-challenge but also evident in the lack of response to anti-FcεRI (**Figure 2A, B**). The inhibition was affected only the Fc-receptor pathway, since ligation of the formyl-Methionyl-Leucyl-Phenyl-(fMLP) receptor induced CD63 upregulation also in anergic basophils (**Figure S2C**). Based on this analysis in our cohort allergen-responder ('HDM^R'), non-responder ('HDM^{NR}') and anergic individuals make up 72.3%, 18.2%, and 9.5% respectively.

Correlation of basophil anergy with atopy and allergic rhinitis (AR)

Both atopy and AR are strongly linked to the sensitization with house dust mites (**Table S2**). In order to assess if anergy is associated with a reduced manifestation of allergic symptoms, we correlated the functional state of the basophils with the prevalence of atopy and AR (**Table 1**).

The analysis suggests that anergic individuals are indeed less likely to develop atopy (assessed by HDM SPT) or AR symptoms than the allergen responders (HDM^R) (**Table 1**). In the discovery as well as the validation cohort, the anergic state correlated with a reduced risk, indicated by combined *P* values of 3.57×10^{-16} (HDM SPT) and 8.58×10^{-4} (AR). While this association was determined based on the functional state defined in the BAT assay, it was independent of the numbers of basophils in the blood. A comparison of the relative frequency of basophils showed no difference in these groups (**Figure S3**).

Gene expression analysis of anergic basophils

In order to characterize the anergic state a gene array analysis was carried out. The purpose of the experiment was to explore whether the blockade of the Fc epsilon receptor pathway is controlled at the transcriptional level and to determine if it affects also other response pathways beyond degranulation (**Figure 3**). Basophils were isolated from anergic and allergen reactive donors with magnetic beads by negative selection. Without stimulation no significant differences in the gene expression profile between reactive and anergic basophils were detected. Thus, no candidate gene could be identified that could explain the non-responsiveness of anergic basophils.

In the reactive cells FcεRI stimulation triggered changes in expression levels of essentially all of the signature genes (**Figure 3A, Table S3**). These included signaling pathway genes such as Fc epsilon receptor I α (*FcεRIα*), phosphatidylinositol-3-kinase regulatory subunit (*PIK3R1*), the Bruton tyrosine kinase (*BTK*) and protein tyrosine phosphatase epsilon (*PTPRE*)(23) as well as cytokines, chemokines and cell surface receptors such as *IL8*, *IL3*, *CCL2* and *HGF*(24) (**Figure 3B**). A particularly strong effect was observed for microRNA221(25). This micro-RNA has been implicated in cell adhesion and migration of mast cells and is strongly upregulated in activated reactive basophils, while a slight decrease was observed in anergic basophils (**Figure 3B**). No

changes were observed in anergic basophils, suggesting that the non-responsiveness affects not only the immediate response (degranulation) but also the late phase response (cytokines).

Association of basophil anergy with Syk downregulation

The complete absence of any transcriptional changes in anergic cells suggested that the blockade is located at an early point in the signaling cascade. This was confirmed by calcium ionomycin treatment, which triggered degranulation both in reactive and anergic cells (**Figure S4**). A likely candidate for the anergy induction is Syk. The tyrosine kinase directly activates phospholipase C (PLC) and plays an essential role in the FcεRI-mediated signaling(20,26,27). Moreover, the loss of Syk expression had already been implicated in hyporesponsiveness of the 'non-releaser' basophils(20,28,29).

The flow cytometry analysis of whole blood samples confirmed that the intracellular levels of Syk protein in anergic basophils are indeed substantially lower than in reactive cells (**Figure 4A**). The analysis of samples from 17 individuals (7 anergic and 10 reactive) revealed a clear association between Syk expression and anti-FcεRI-induced degranulation (**Figure 4B**). Complete degranulation was only observed when the Syk expression was detected above the threshold level of ~110 mean fluorescence intensity (MFI).

Phosflow analysis showed that Syk downregulation was associated with the loss of FcεRI-induced phosphorylation of kinases p38 and ERK1/2, kinases located in the signaling cascade downstream of Syk (**Figure 4C, D**). The effect is restricted to the FcεRI pathway, as stimulation of the IL-3 receptor with IL-3 resulted in similar phosphorylation levels of p-ERK1/2 both in reactive and anergic cells (**Figure 4C**).

Basophil anergy is associated with low levels of allergen-specific IgE

The cohort database contains information on the frequency of cell subsets, the distribution and intensity of their surface/activation markers as well as plasma levels of cytokines, chemokines and immunoglobulins. To determine if any of these factors is associated with basophil anergy, we correlated these parameters with the functional state of the individuals (HDM^{NR}, anergic and HDM^R) (**Table S4**). Notably, significant associations were only observed for parameters related to allergen-specific IgE (**Figure 5A**). In two separate correlations carried out for the discovery and the validation cohorts, the strongest association was found for HDM-specific IgE followed by total plasma IgE, which due to the dominance of HDM allergens consists mostly of HDM-specific IgE(21). Also most other parameters passing significance are either directly or indirectly linked to the IgE titers. For instance, the amount of surface IgE detected on mDC, pDC and basophils are a reflection of total plasma IgE. IgE also stabilizes the FcεRI receptor on the surface of DC(30,31), another parameter found to be associated anergy.

A comparison of the allergen-specific IgE in individuals classified as HDM^R, anergic and HDM^{NR} revealed that anergy is associated with intermediate or low levels of the immunoglobulin (**Figure 5B**). The highest levels were detected in HDM^R donors (23.6 kU/ml), followed by anergic individuals (0.95 kU/ml), while HDM-specific antibodies mostly undetectable in HDM^{NR} donors (median <0.35 kU/ml). The same trend was also observed for total IgE, where the median for HDM^R, 'anergic' and HDM^{NR} donors were measured as 210.0, 76.3 and 27.3 kU/ml, respectively.

As shown above, basophil anergy is associated with Syk downregulation. In order to establish a causal link between IgE/allergen levels and the Syk expression in these cells, we exposed basophils to titrated amounts of the allergen (**Figure 5C**). Prior studies reported already that crosslinking of FcεRI can result in a downregulation of Syk(32,33). Analysis of the Syk levels

confirmed that after 16 hours of allergen exposure a dose-dependent downregulation of Syk was observed. This downregulation translated directly into an anergy-like state as Ca^{2+} /ionomycin but not anti-Fc ϵ RI challenge resulted in degranulation of the basophils (**Figure S5**).

Cohort based analysis of the relationship between the functional states

To further examine the role and function of basophil anergy, we analyzed the relationship of the three functional states HDM^R, anergic, and HDM^{NR} on the cohort level (**Figure 6A**). Each of the states is characterized by a specific response pattern regarding the general response and allergen-specific response, which in our database is represented by the percentage of CD63+ basophils in response to anti-Fc ϵ RI crosslinking (general response) and in response to HDM exposure (allergen-specific response).

In a dot plot of the cohort in which each dot represents the allergen-specific and general response of an individual, the three functional states HDM^{NR}, anergic and HDM^R are displayed as discrete clusters: HDM^R donors appear in the upper right quadrant (general CD63%: high, HDM CD63%: high), HDM^{NR} donors in the lower right quadrant (general CD63%: high, HDM CD63%: low) and anergic donors in the lower left quadrant (general CD63%: low, HDM CD63%: low) (**Figure 6A**). Dots located outside of these clusters indicate individuals, which are currently in transition between the states. As expected, individuals fall into one of the three clusters. Notably, however no connecting dots were located between the clusters of classical non-responder (HDM^{NR}) and responder (HDM^R), while a clear connection was evident between the cluster of HDM^{NR} donors and 'anergic' individuals as well as between 'anergic' individuals and HDM^R donors.

The same transitions were also evident when the data of a longitudinal study was superimposed onto the dot plot (indicated by arrows in **Figure 6A**). For 38 adult individuals the functional state of basophils was defined at two time points separated by a period of about 2 years (**Figure S6B**). While 26 of the 38 individuals remained in the same functional state, thirteen underwent transitions between one of the reactive states (HDM^R or HDM^{NR}) and the anergic state. The transitions occurred in both directions, suggesting that conversions are allowed between HDM^{NR} ↔ anergic ↔ HDM^R. For the 38 adult Singaporeans no direct conversion between HDM^{NR} and HDM^R was observed. The latter however is not surprising as migrant studies indicated that even in Singapore the build up of an HDM-IgE titer would require 5 to 8 years allergy(21). Tracking of three adult individuals further indicated that basophil anergy could last for months or even years (**Figure S6B**).

Basophil anergy may act as activation barrier

The median levels of HDM-specific IgE are typically absent in HDM^{NR} donor, low on anergic individuals and high in HDM^R donor (**Figure 6B, left panel**). The basophil reactivity controlled by Syk levels, in turn, is the lowest in the anergic state but equally high in the HDM^R and HDM^{NR} state (**Figure 6B, right panel**). The cohort based analysis of the inter-relationship of the functional states indicated that basophil anergy represents a transitional state between the non-reactive and the reactive state (**Figure 6A**). During the transition HDM^{NR} → anergic → HDM^R the allergen-specific IgE level gradually increased, suggesting a model in which the reactivity levels follow a valley curve separating the non-responsive state from the allergen responsive state (**Figure 6C**). Considering the suggested role basophils play in providing Th2 cytokines, the build-up of the IgE titer would thus be hindered by the transient desensitization of the FcεRI signaling pathway during anergy. Basophil anergy therefore seems to represent an activation barrier that needs to be overcome in order to establish a sustained IgE response against the allergen.

DISCUSSION

In this study we could show that basophil anergy is a common phenomenon that appears to be caused by the down-regulation of Syk. It is associated with a reduced incidence of allergic rhinitis and can persist for extended periods of time. Similar to classical T cell and B cell anergy, it seems to represent a transitional state separating the “naïve” (non-responsive) state from the “activated” (responsive) state.

In the Singaporean cohort, 9.4% of the individuals were in the anergic state. Only a few studies have been published in which the fraction of ‘non-releaser’ had been determined in rather small cohorts. A study in Europe has shown that 25% of the donors (5 out of 20) are found to be non-releasers(19). In two American cohorts Kepley *et al.* had determined a rate of 8% ‘non-releaser’ (3 out of 37)(20), while Nguyen et al reported a rate of 22% (8 out of 36) (29). Basophil anergy therefore is not restricted to the tropical environment but rather appears to be a common phenomenon.

For B and T cells, anergy is defined as a tolerance mechanism in which the lymphocyte is functionally inactivated following an antigen encounter(34-36). The same seems to apply also for basophils. The mechanism however is unique as basophil anergy appears to be regulated by adjusting the Syk levels. In line with our results, Syk deficiency in basophils had been reported before to result in a failure to degranulate and to synthesize cytokines after FcεRIα stimulation(33). Moreover, Syk downregulation was observed in the so called ‘non-releaser’ basophils(20,29), which are synonymous to the anergic basophils described here. MacGlashan and Miura further demonstrated that Syk expression was controlled by CBL-mediated ubiquitinylation(32). CBL forms a family of ubiquitin ligases of which CBL-b has been implicated in negative regulation of FcεRI-mediated signaling. Importantly, only phosphorylated Syk is targeted. This implies that, once the kinase has been activated by the ligation of FcεRI, the

transfer of the phosphorylation signal to the downstream targets of the signaling cascades competes with the proteasomal degradation of the kinase. As depicted in the kinetic model (**Figure S7**) under 'low zone conditions' (i.e. low amounts of the IgE/allergen complex) p-Syk may thus be effectively removed by CBL. The resulting depletion of basophil Syk causes the desensitization of the cells abrogating the production of Th2 cytokines. While in the tropics the HDM is a perennial allergen, it is concentrated in certain areas and spots, such as mattress and carpets(37). Hence, also here the exposure of each individual strongly depends on his life style and habits. In addition, receptors other than the Fc epsilon receptor have been reported to induce Syk downregulation, which might explain the observed variation in HDM-responder, non-responder and anergic individuals in our cohort, a list which includes histamine releasing factor (HrHRF)-dependent receptor, LILRA2, and more recently FcγRIIb (CD32b) (38,39).

Anergy may thus function as a barrier to avoid the production of unwanted IgE in response to the chronic stimulation by low level allergens. The transient nature of the anergy state was also supported by the relationship analysis of the three states (compare **Figure 6**). In the cross-sectional cohort data transitions were detected only between anergy ↔ non-responders and anergy ↔ responders. Direct conversions from non-responders ↔ responders were missing. This same pattern was also evident in the longitudinal data of 38 adult individuals comprising of two time points separated by approximately two years. Unless some cross-reactivity with an established allergen provides a shortcut, the induction of an IgE-mediated allergen response therefore has to follow the path from non-responder ↔ anergic ↔ responder. Formal proof of this concept can only come from a longitudinal tracking all three stages in the same individual. This however is experimentally challenging as we showed in a previous study that despite high HDM burden in Singapore it takes up to 8 years for HDM-IgE negative migrants to build up an allergen-specific IgE titer(21). Long-term studies are currently being prepared to address this question.

While the study provides strong evidence that the state of anergy is associated with a reduced incidence of allergic diseases, a causal connection to basophils cannot be made yet. As in basophils also in mast cells the allergen/IgE response is mediated by Syk. Also here the expression level is rather low(40), rendering Syk sensitive for anergy induction. However, as tissue resident cells mast cells are difficult to access. So far, a study on human lung mast cells did not find any correlation between Syk levels and mast cell degranulation(40), and more studies are needed to determine a role of anergy for mast cells.

As basophil anergy appears to be associated with reduced incidence of allergic symptoms, it might be a useful biomarker or even represent a promising target for allergic interventions. A study on food allergy demonstrated that a desensitization based on Oral Immunotherapy (OIT) could result in a desensitization of basophils, characterized by their failure to degranulate upon IgE crosslinking(41). Notably, individuals can remain 'anergic' for months or even years. As this period clearly surpasses the lifespan of basophils, anergy seems to be maintained by the specific immune environment defined by the allergen load, the amount and type of HDM-specific antibodies or by other cellular and/or molecular components. At least *in vitro* an anergy-like state could be induced by overnight exposure with titrated amounts of the allergen and the cohort level anergy is associated with low but detectable plasma levels of allergen-specific IgE. Basophil responsiveness to allergens may therefore be regulated by allergens exposure, which may provide a therapeutic angle to influence allergic reactions by applying sub-optimal doses of allergens or allergen/IgE complexes.

While basophil anergy may be linked to 'low zone tolerance', a phenomenon already described by Shellam and Nossal in 1968(42), its actual contribution is still unclear. The detailed characterization of the mechanisms underlying basophil anergy may therefore help in better

understanding the principles of low zone tolerance and allergen-induced desensitization which could result in alternative approaches to monitor and treat IgE-mediated allergic diseases.

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AUTHORSHIP CONTRIBUTIONS

K.J.P., L.T.S., G.Y., D.B., M.S., and D.H. performed the experiments on basophil phenotyping and functional characterization. M.S. and J.L. prepared the basophil samples for microarray analysis. A.K.A and J.C. organized the cohort samples collection. K.J.P, D.K., A.K.A, B.L., D.K., M.P., O.R. analyzed and interpreted the data. O.R. conceived the project. K.J.P., A.K.A, F.T.C., W.S.C, F.Z., M.P., and W.D.Y., contributed to the manuscript writing.

DISCLOSURE OF CONFLICTS OF INTEREST

All the authors declare no competing financial conflicts of interest.

FOOTNOTES

The microarray data have been deposited into the Gene Expression Omnibus (accession number GSE70013)

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Figure legends

Figure 1. Study design. The flow chart summarizes the study design of the project. The study was carried out in the tropical urban environment of Singapore. Data were collected for 476 individuals of Chinese ethnicity, divided into discovery and validation cohort. The parameters collected derived from a survey questionnaire, a flow cytometry analysis of whole blood samples (cellular blood components), a plasma analysis (soluble blood components), and *in vivo* allergy testing by skin prick tests. The study focused on house dust mite (HDM) allergens as the dominant local allergen using extracts of the locally abundant HDM species *Blomia tropicalis* (Bt) and *Dermatophagoides pteronyssinus* (Dp).

Figure 2. Identification of anergic basophils in whole blood samples. The Fc epsilon receptor-mediated degranulation was determined in whole blood samples using the basophil activation test (BAT). **(A)** Response pattern associated with the functional states of basophils. Whole blood samples were incubated for 15 min either in buffer alone (Contr.), with an activating anti-Fc-epsilon receptor antibody (anti-FcεRI) or with extracts of house dust mites (*Dermatophagoides pteronyssinus*, Dp or *Blomia tropicalis*, Bt). Basophil degranulation was indicated by the increase in CD63 surface expression, which was measured by flow cytometry after counter-staining with the basophil marker CCR3. Dot plots shown are representative examples of a house dust mite responder (HDM^R), an individual whose basophils are in the anergic state ('Anergic'), and a classical non-responder to HDM-allergens, which has reactive basophils but lacks HDM-specific IgE (HDM^{NR}). The percentage of CD63+ basophils is indicated in the upper right quadrant. **(B)** Summary of the functional states in the combined cohorts. For each individual, the percentage of CD63+ basophils induced by anti-FcεRI crosslinking or the exposure to HDM allergens (HDM) is shown. Each dot represents an individual of the cohort. The colors indicate the functional

basophil associated state (HDM^{NR}: grey, 'anergic': red, HDM^R: 'blue'). Horizontal lines represent median. Statistical comparison of phenotypes was performed using Kruskal-Wallis test with Dunn's multiple comparisons. ****, $P < 0.0001$; ***, $P = 0.0002$; ns, not significant.

Figure 3. Comparison of gene expression of reactive and anergic basophils. Reactive and anergic basophils were incubated for 4 hr with or without anti-FcεRI stimulation followed by array-based gene expression analysis. **(A)** Heatmap of differentially expressed genes. The heatmap indicates the relative gene expression level of 112 genes for anti-FcεRI stimulated and unstimulated basophils. For each gene the relative gene expression is displayed as a scale between 0 (yellow) and 1 (blue). Shown here are the top-ranking genes with regard to differential expression (unstimulated vs. stimulated) as analyzed using Limma (corrected P value cut off of less than 0.3; complete gene list is shown in **Table S3**). **(B)** Bar charts of selected differentially expressed genes. Gene expression for reactive (blue) and anergic basophils (red) are shown for a set of selected genes indicated in the heatmap. Bar charts represent the averaged log2 transformed expression levels reached under unstimulated (light colors) and anti-FcεRI stimulated conditions (dark colors). Error bars indicate the standard deviation; the experiment carried out with basophils isolated from 2 anergic and 3 reactive donors (HDM^R).

Figure 4. Basophil anergy is associated with Syk downregulation. The intracellular Syk levels and the phosphorylation state of Syk and other kinases were analyzed by flow cytometry for basophils. **(A)** Total Syk expression. Two representative histogram plots are shown which compare the basophil Syk protein expression level of an HDM^R donor ('reactive') and a donor with anergic basophils ('anergic'). Syk expression level (solid line) is shown in reference to the isotype control (shaded peak). **(B)** Correlation between Syk level and basophil activation. The Syk levels of 11 donors with reactive basophils (all HDM^R) and 7 donors with anergic basophils were correlated with the general reactivity of the basophils, expressed as the percentage of

CD63+ cells in response to anti-FcεRI crosslinking. Curve was fitted to the data points based on a nonlinear 4-parameter logistic regression curve fit. (C) Phosphorylation of downstream kinases. Whole blood samples from an anergic individual and a reactive HDM^R donor were incubated in media alone (control), or were stimulated for 10 minutes with IL-3 (IL-3 only), anti-FcεRI (anti-FcεRI only) or IL-3 combined with anti-FcεRI (anti-FcεRI + IL-3). Samples were analyzed by flow cytometry after intracellular staining for p-Syk, p-p38 and p-ERK1/2. (D) Comparison of p-Syk and p-p38 induction in reactive and anergic basophils. Mean fluorescence intensities (MFI) of p-Syk and p-p38 are shown for 7 donors with reactive (blue) and 5 donors with anergic basophils (red). Lines connect the two data points representing the phosphorylation state after incubation in medium only (Control) or after anti-FcεRI crosslinking (Stim.). All measurements were carried out in whole blood samples. Statistical comparison was performed using the Wilcoxon matched-pairs signed rank test. *, $P < 0.05$; ns: not significant.

Figure 5. Correlation of the basophil anergy with the cellular and soluble blood parameters of the cohort database. The three functional states HDM^R, anergic and HDM^{NR} were correlated with more than 300 phenotypic parameters collected for the leukocyte fraction and the plasma components of the discovery and the validation cohort. (A) Kruskal-Wallis association. The bar chart displays the top 30 associations for both cohorts. The P values displayed on the y-axis are corrected for multiple testing using the method of Benjamini and Hochberg ($P < 0.05$ is considered significant). IgE-related parameters are highlighted in red, IgE-unrelated parameters in blue (the complete dataset is listed in **Table S4**). * indicates statistical significance that could not be replicated in the other cohort; § refers to the statistical significant parameters that were only measured once. (B) Association with IgE plasma levels. The basophil associated functional state is indicated for each individual in reference to the plasma titer of HDM-specific and total IgE. Horizontal lines represent the median; the detection limit was defined as 0.35 kU/ml. Statistical comparisons were performed using Kruskal-Wallis test with Dunn's multiple

comparisons test. ****, $P < 0.0001$; **, $P = 0.0014$, *, $P = 0.0269$, ns: not significant. (C) HDM-induced Syk downregulation. Whole blood samples of an HDM responder were incubated with titrated amounts of HDM extracts. The plot displays the levels of basophil Syk after exposing whole blood samples for 16 h to the allergen. Syk levels were determined by intracellular flow cytometry using anti-Syk mAb (left panel) or an isotype matched control antibody (right panel). Dashed vertical lines indicate the mean fluorescence intensity (MFI) detected in the absence of allergen.

Figure 6. Relationship of the three functional states. The cohort database was used to analyze the relationship of the basophil associated functional states HDM^{NR}, anergic and HDM^R. (A) Transitions between the states. The dot plot displays the averages of the general reactivity vs. the HDM-specific responsiveness of the basophils in each individual. The two parameters were determined from whole blood samples by basophil activation test (compare **Figure 2**) and are expressed as percent CD63+ basophils after anti-FcεRI-stimulation (x-axis) or exposure to HDM allergens (y-axis). Each dot represents one individual of the cohort. The colors indicate the functional basophil associated state (HDM^{NR}: grey, 'anergic': red, HDM^R: 'blue'). Dotted horizontal and vertical lines indicate the 38% threshold value for the anti-FcεRI- and the HDM-induced response. Arrows indicate the functional states at two time points separated by approximately 2 years that were measured for 38 individuals (compare **Figure S6B**). (B) Association with HDM-IgE and Syk. The relative amount of HDM-specific plasma IgE (left panel) and basophil Syk (right panel) are displayed for each of the functional states (numbers refer to the assignments in **Figure 6B**). IgE is displayed as a ratio between HDM-specific and total IgE and was defined for 344 HDM^{NR}, 45 anergic and 87 HDM^R-donors. Bar lines represent the whiskers generated from the Tukey boxplot and dots depict outliers. Syk level were defined by intracellular FACS for 5 HDM^{NR}, 7 anergic and 6 HDM^R-donors. Statistical comparisons were performed using Kruskal-Wallis test with Dunn's multiple testing. **, $P = 0.013$, ns: not

significant. (C) Schematic view on basophil anergy as transitional state. Assuming a path from (1) non-responder $\leftrightarrow$ (2) anergic $\leftrightarrow$ (3) responder the transition from non-responder to responder would be associated with a gradual increase in the allergen specific IgE titers (upper panel) and with a valley curve for the basophil reactivity as result of the anergy-associated Syk downregulation (lower panel).

Table 1. Association of the basophil associated functional state with HDM-specific SPT

	Cohort	Discovery			Validation			Combined		
	Basophil status	Yes	No	Fisher Exact test (OR)	Yes	No	Fisher Exact test (OR)	Yes	No	Fisher Exact test (OR)
Atopy	HDM ^R	119 (86.2%)	3 (2.2%)	4.06 x 10 ⁻⁹ (0.02)	129 (90.2%)	1 (0.7%)	6.32 x 10 ⁻⁸ (0.01)	248 (88.3%)	4 (1.4%)	3.57 x 10 ⁻¹⁶ (0.01)
	Anergic	6 (4.3%)	10 (7.2%)		6 (4.2%)	7 (4.9%)		12 (4.3%)	17 (6.0%)	
Rhinitis	HDM ^R	72 (50.7%)	54 (38.0%)	1.78 x 10 ⁻² (0.25)	86 (44.6%)	85 (44.0%)	2.17 x 10 ⁻² (0.29)	158 (47.2%)	139 (41.5%)	8.58 x 10 ⁻⁴ (0.27)
	Anergic	4 (2.8%)	12 (8.5%)		5 (2.6%)	17 (8.8%)		9 (2.7%)	29 (8.7%)	

and allergic rhinitis (AR)

Table showing the Fisher's Exact test results comparing the basophil associated functional states of responders to HDM-allergens (HDM^R) and anergic individuals with atopy (defined by HDM-specific skin prick test) and allergic rhinitis. The number of individuals in each group is provided together with the percentages listed in parentheses. Statistical testing was performed using R version 2.15.2 (P values < 0.05 were considered to be statistically significant). The odds ratios were computed using the odds of anergic individuals having allergic rhinitis or atopy against the odds responders to HDM-allergens having allergic rhinitis or atopy.

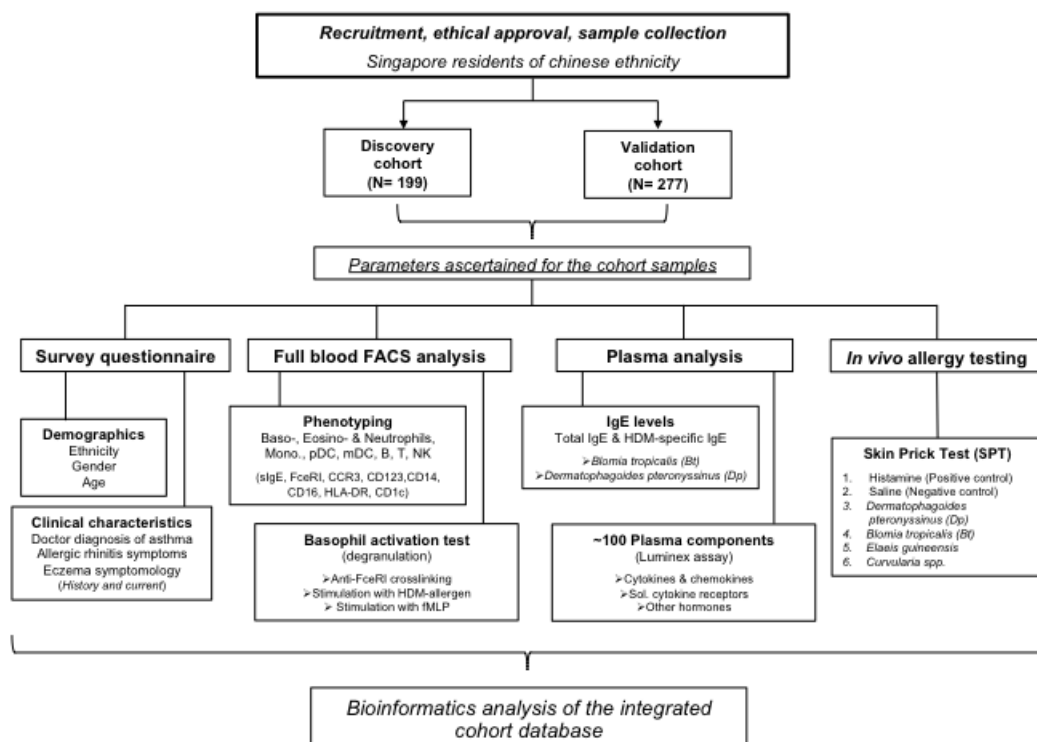


Figure 1 (Puan et al)

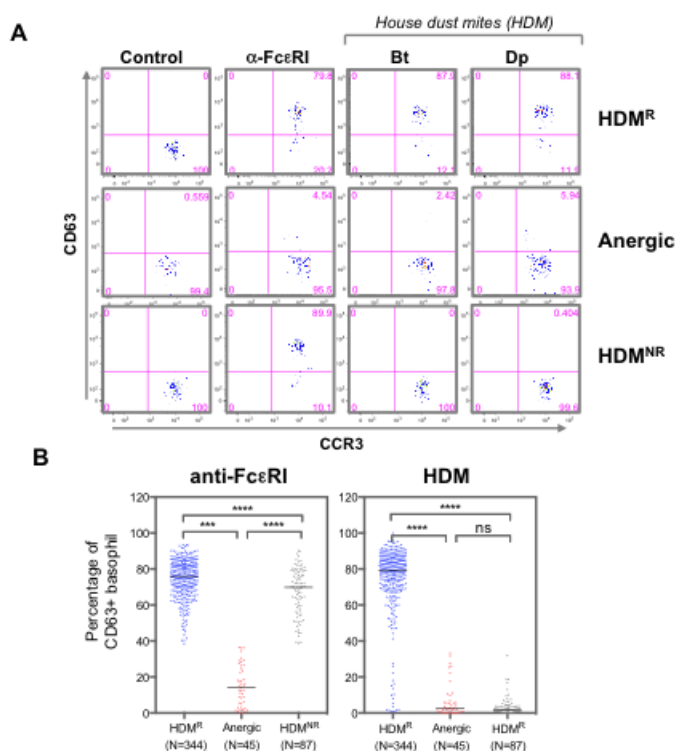


Figure 2 (Puan et al)

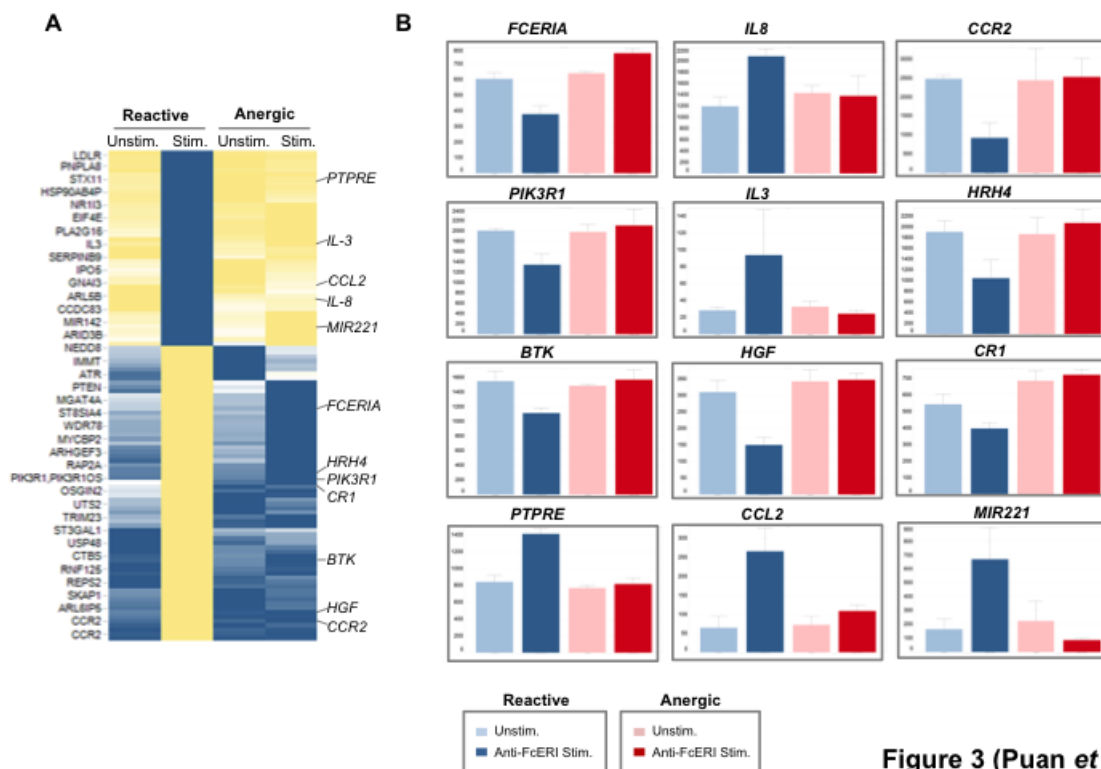


Figure 3 (Puan et al)

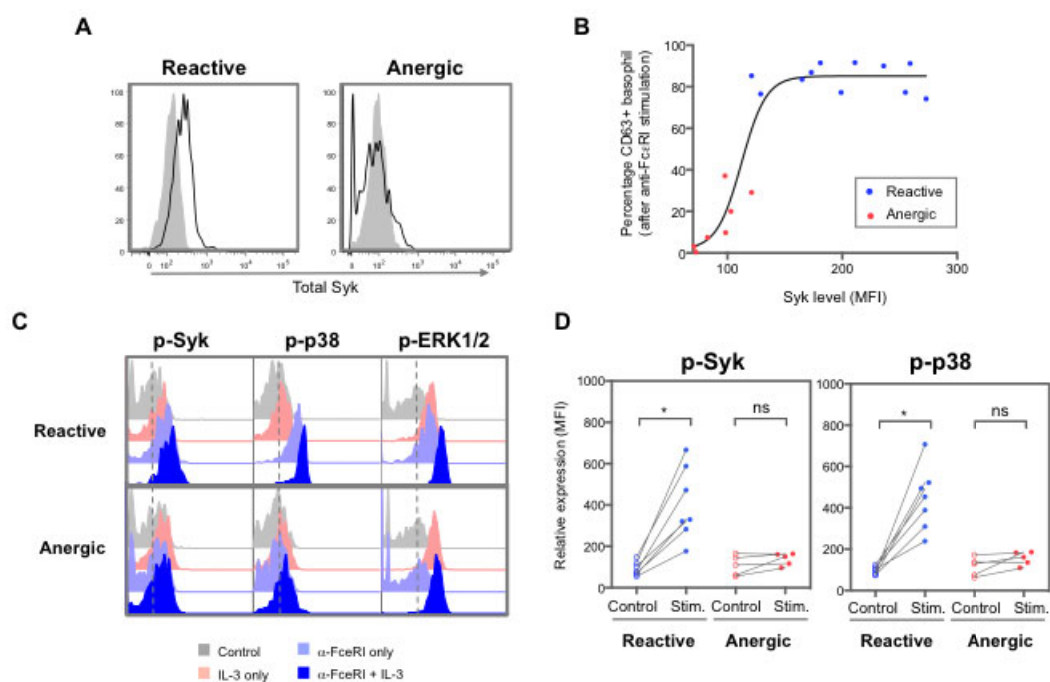


Figure 4 (Puan et al)

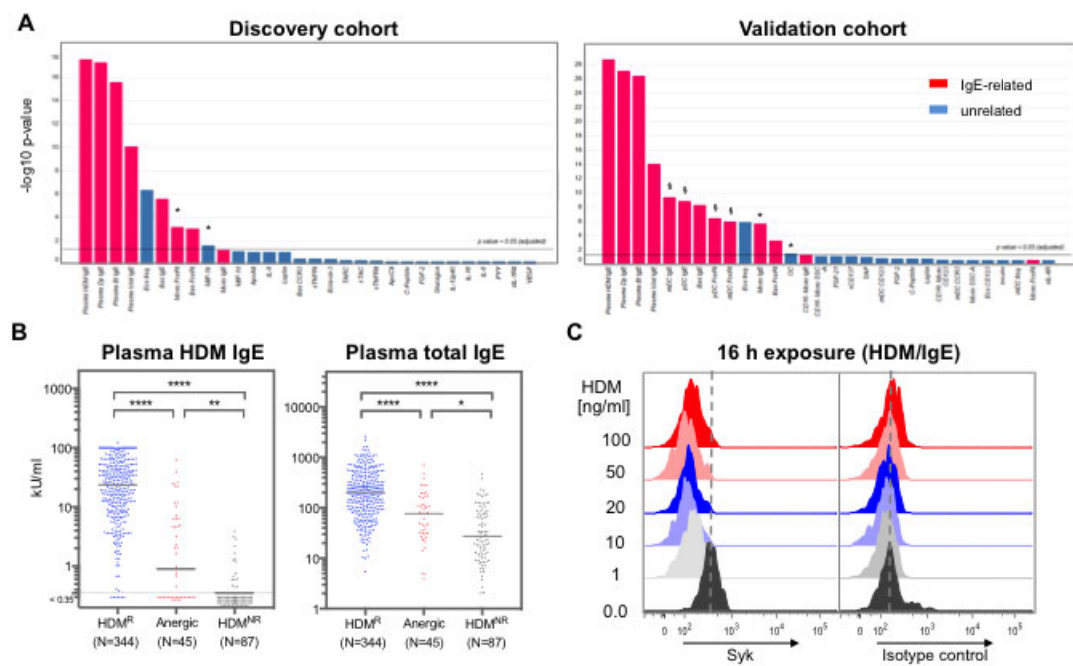


Figure 5 (Puan et al)

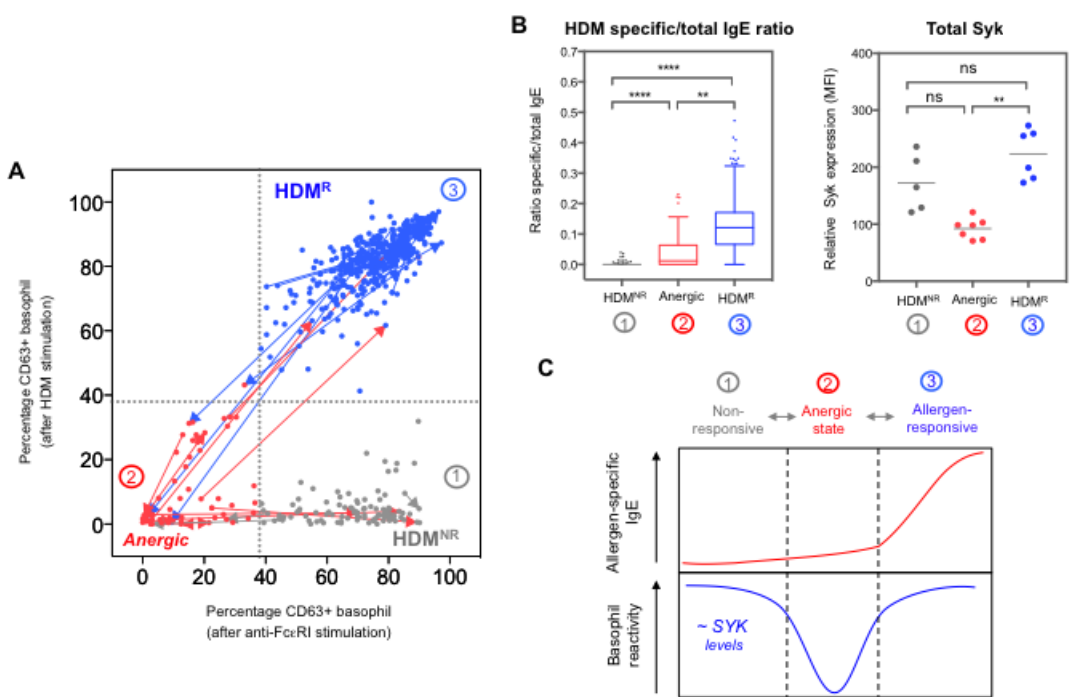


Figure 6 (Puan et al)

Systematic characterization of basophil anergy

Supplemental Information

Supplemental Figures

Figure S1. Gating Strategy for the flow cytometry analysis

Figure S2. Cohort wide characterization of basophil response using the BAT assay

Figure S3. Comparison of the basophil frequency with the basophil associated functional state and the manifestation of AR

Figure S4. Induction of basophil degranulation by calcium ionomycin

Figure S5. Induction of allergen-induced basophil anergy *in vitro*

Figure S6. Long-term stability and conversion of basophil anergy

Supplemental Tables

Table S1. Demographic and clinical characteristics of the donors used in the study

Table S2. Association of the HDM^R and HDM^{NR} with HDM-specific SPT and allergic rhinitis (AR)

Table S3. Gene list comparing reactive and anergic basophils

Table S4. Complete dataset including IgE-related and IgE unrelated parameters for discovery and validation cohorts

Table S5. A list of all the plasma components measured by Luminex

Figure S1

(Validation cohort only)

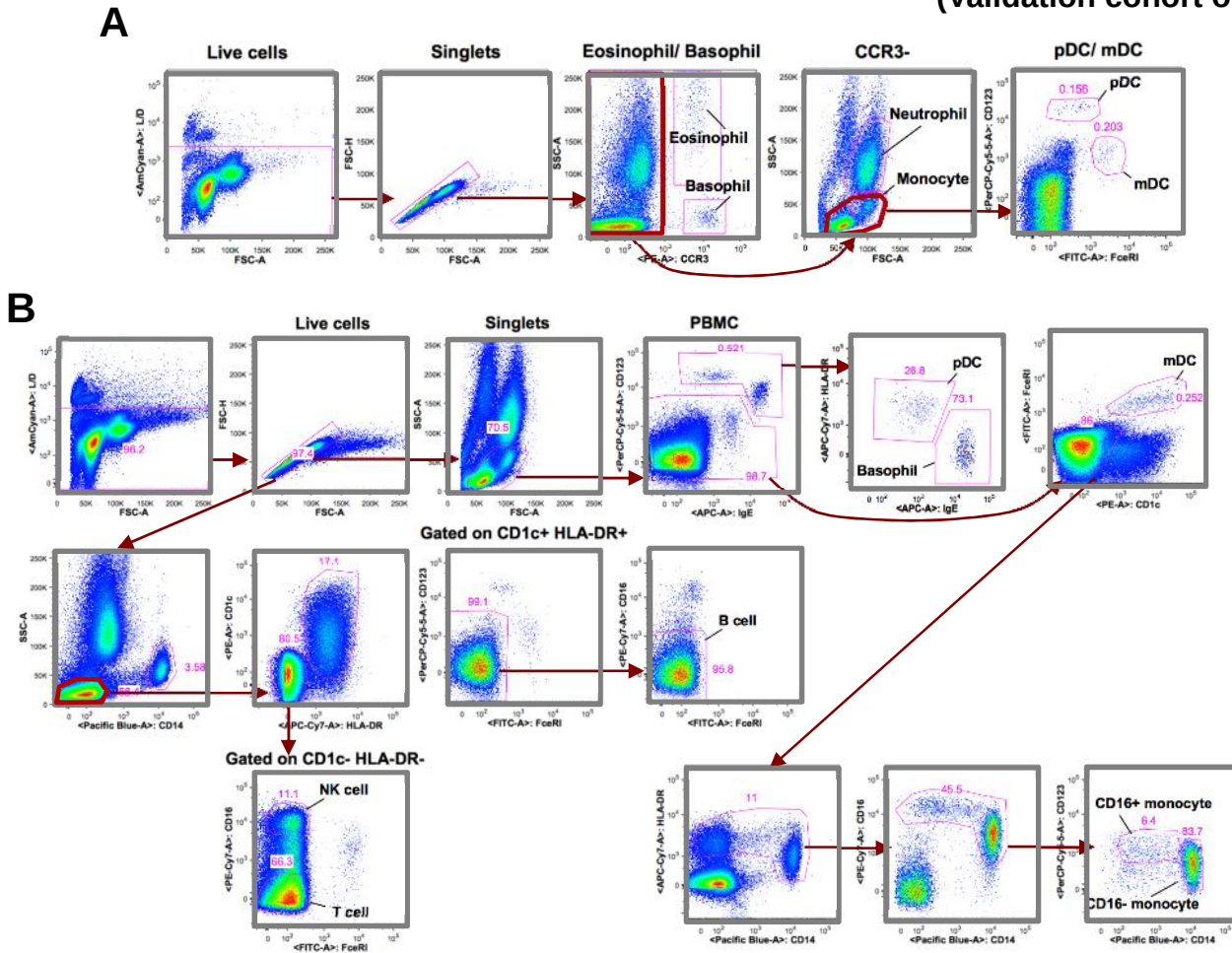


Figure S1. Gating Strategy for the flow cytometry analysis. Basic allergy specific antibody panel. (A) Flow cytometry analysis was performed in erythrocytes lysed whole blood samples. The cells were stained with anti-CCR3, anti-FcεRI, and anti-IgE mAbs. CCR3 expression was used to discriminate basophils and eosinophils from the remaining leukocyte populations. Lymphocytes, monocytes, and neutrophils were gated based on forward and side-scatter of CCR3- population. In the validation cohort, the panel was extended by adding anti-CD123. In combination with anti-FcεRI, the use of this antibody allows the gating of plasmacytoid DC (pDC) and myeloid DC (mDC). (B) Extended flow cytometry panel. In addition to the basic panel, the validation cohort was also analyzed by using an extended antibody panel. The 7-color flow cytometry panel consisted of anti-FcεRI, anti-IgE, anti-CD123, anti-CD16, anti-CD14, anti-HLA-DR, anti-CD1c, and allowed for the discrimination of the leukocyte subsets into pDC, basophils, mDC, CD16+ monocytes, CD16- monocytes, NK, T and B cells.

Figure S2

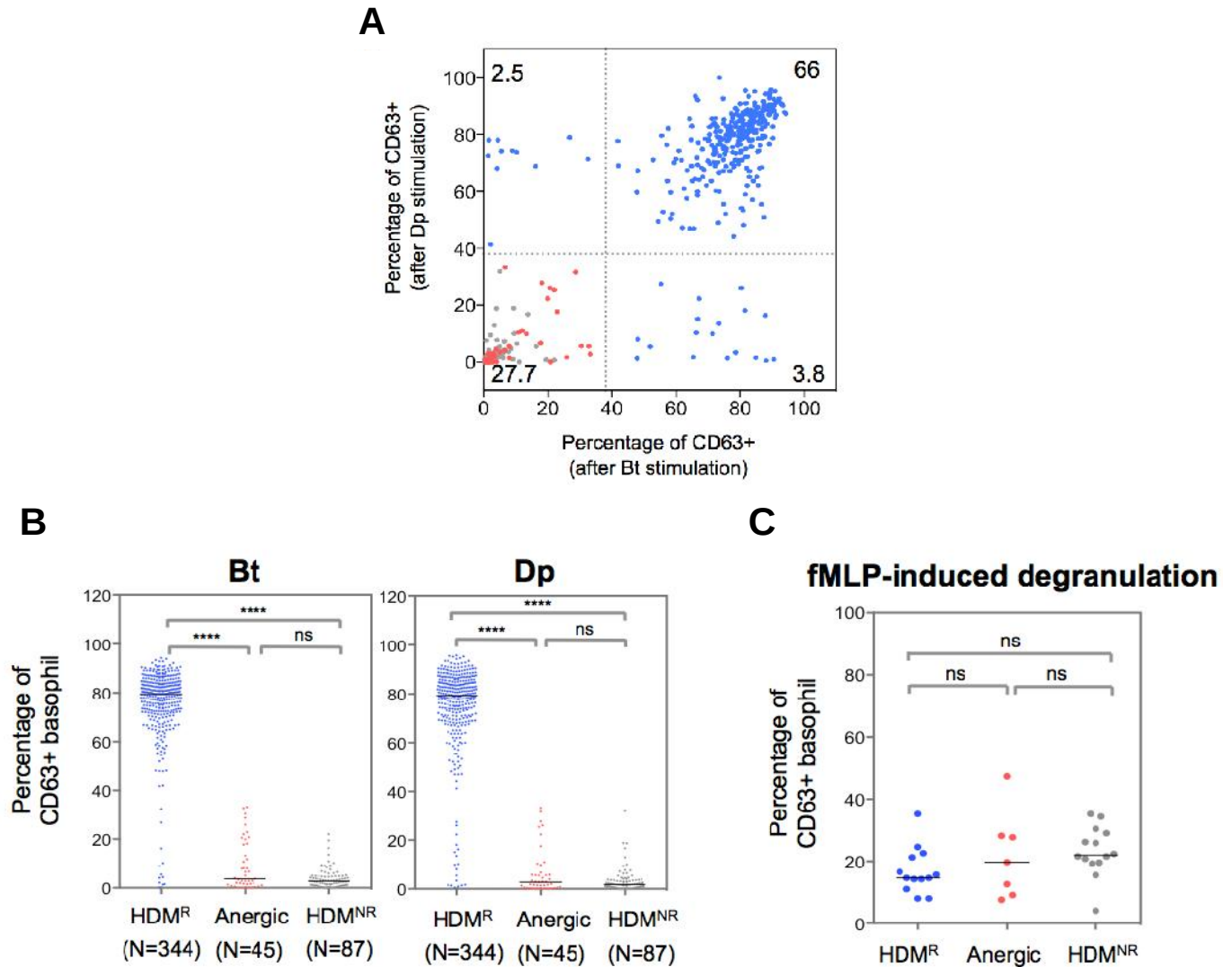


Figure S2. Cohort wide characterization of basophil response using the BAT assay. **A** Overlap of the Bt and Dp specific basophil degranulation. Scatter plot of CD63+ basophils. The percentage of CD63+ basophils detected in the BAT assay after stimulation with Bt vs Dp extracts is shown. Colors refer to the functional state defined in the BAT assay based on the anti-FcεRI response (HDM^R: blue; anergic: red; HDM^{NR}: grey). Numbers indicate the percentages of donors in the respective quadrants. Vertical and horizontal dotted lines indicate the threshold-value of 38% for the Bt- and Dp-response, respectively. **(B)** Summary of the Bt- and Dp-response in the combined discovery and validation cohorts. The percentage of CD63+ basophils is shown separately for the stimulation with Bt- (left panel) and Dp allergen (right panel). Statistical comparison among the three states was performed using Kruskal-Wallis test. (****, $P < 0.0001$; ns: not significant). **(C)** fMLP-mediated basophil degranulation. The BAT assay was performed with whole blood samples using fMLP, the peptide ligand of the fMLP receptor, as

stimulating agent. Basophil degranulation is expressed as percentage of CD63+ basophils. The experiment was carried out with samples from 13 HDM^R 7 anergic and 14 HDM^{NR} donors. According to manufacturer's manual > 10% CD63+ basophils is considered positive. Horizontal lines represent the median values. Statistical comparison among the three phenotypes was performed using Kruskal-Wallis test. (ns: not significant).

Figure S3

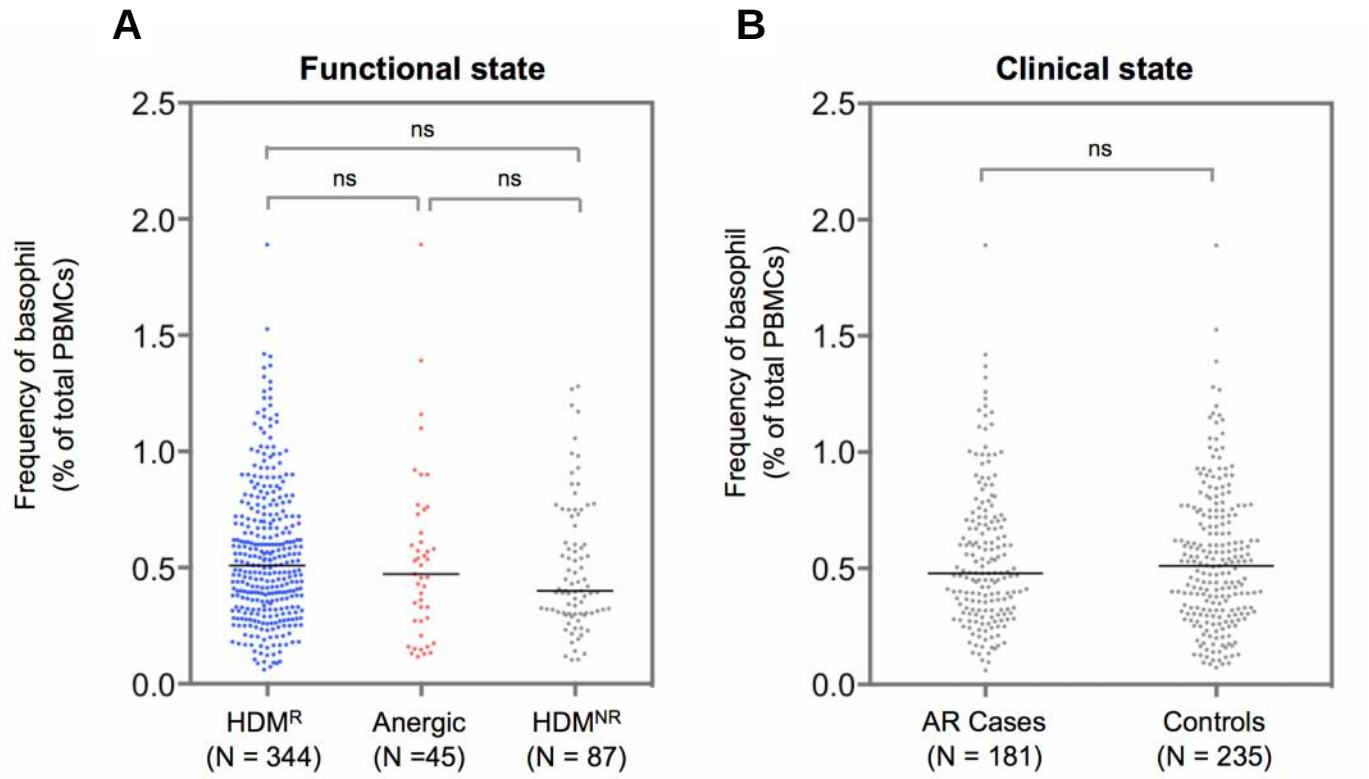


Figure S3. Comparison of the basophil frequency with the basophil associated functional state and the manifestation of AR. (A) Functional state. The relative frequency of basophils is shown in reference to the functional state of the donor (HDM^R, anergic or HDM^{NR}; left panel). The frequency was determined by flow cytometric analysis of whole blood samples, and was calculated in reference to total PBMC. (B) Manifestation of AR. The basophil frequency in allergic rhinitis cases and controls is shown. Statistical comparison among the three states was performed using Kruskal-Wallis test (ns: not significant).

Figure S4

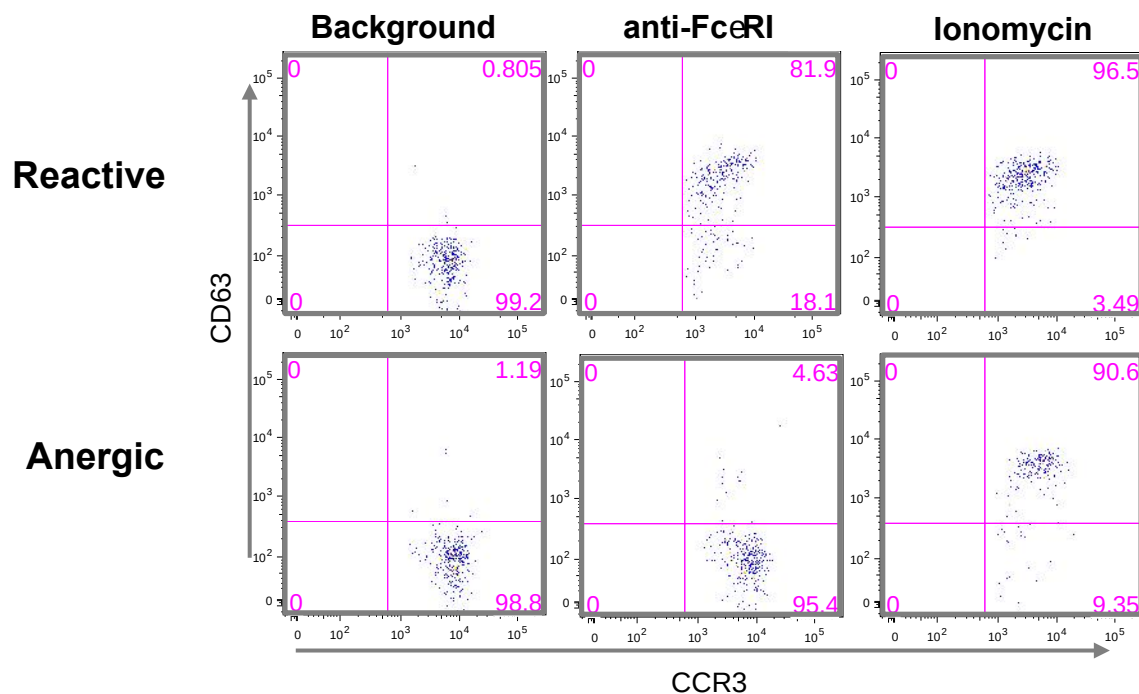


Figure S4. Induction of basophil degranulation by calcium ionomycin. Whole blood samples from a reactive HDM^R-donor and a donor with anergic basophils were incubated for 15 min with IL-3 containing medium alone (Background), with IL-3 medium supplemented either with anti-FcεRI or with calcium ionomycin (Ionomycin). Basophil degranulation was measured in the BAT-assay based on the upregulation of cell-surface CD63. Basophils were identified by counterstaining with anti-CCR3.

Figure S5

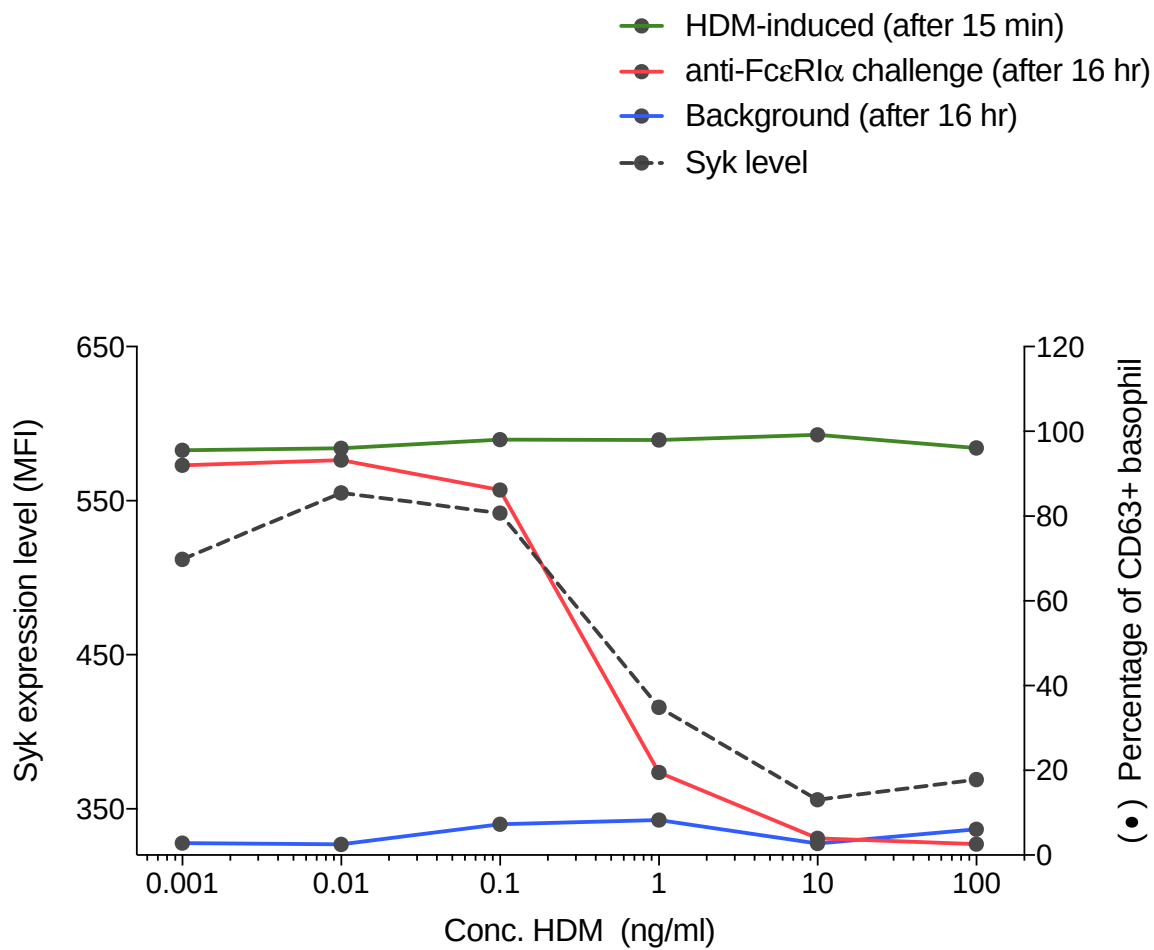
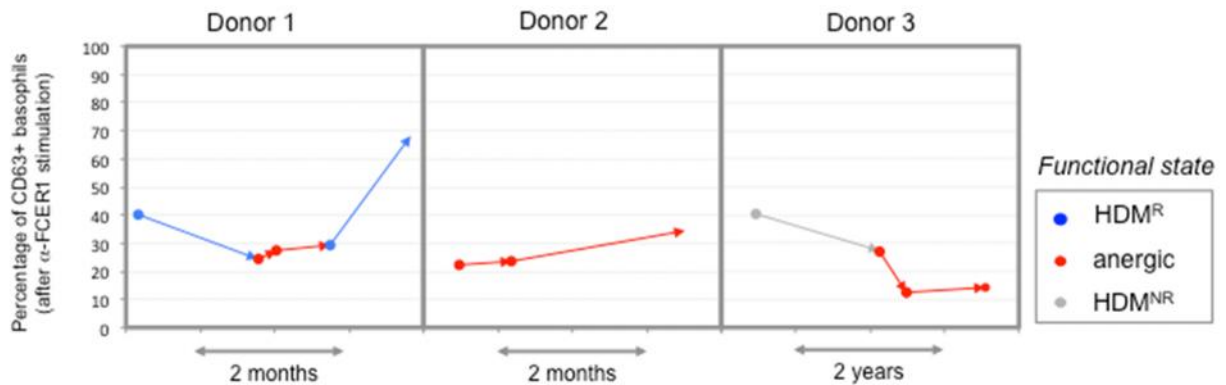


Figure S5. Induction of allergen-induced basophil anergy *in vitro*. Whole blood samples of HDM responders were incubated with titrated amounts of HDM-extracts in order to determine Syk downregulation and basophil desensitization. After 16 hr exposure to HDM allergens, levels of basophil Syk were determined by FACS after intracellular staining as shown in **Fig. 5C** (dashed line). Ca²⁺/ionomycin- (green line) and anti-FcεRI-induced degranulation (red line) was determined in the BAT assay. Blue line indicates the background degranulation. The degranulation (right axis) is shown together with the allergen-induced Syk down-regulation (left axis).

Figure S6

A



B

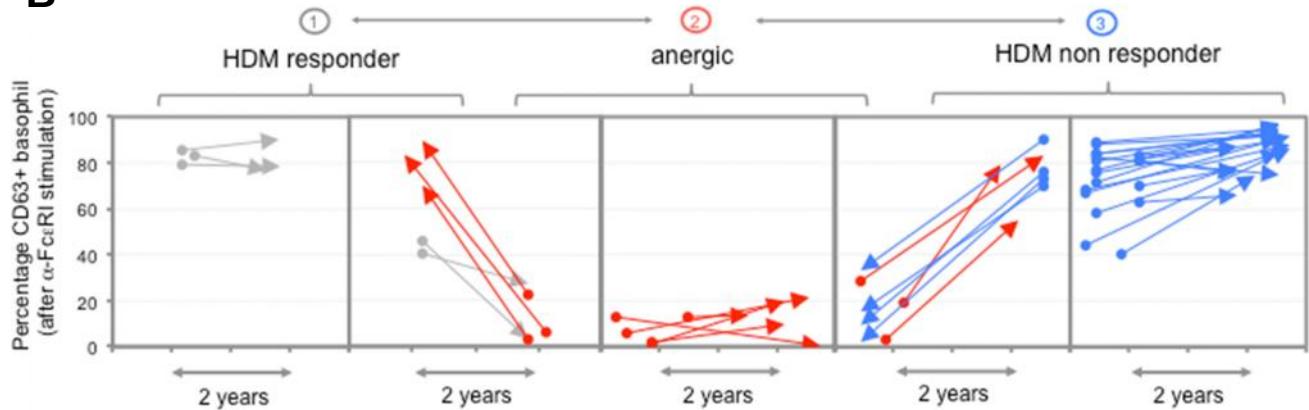


Figure S6. Long-term stability and conversion of basophil anergy. (A) Long-term stability. The basophil-associated functional states of three donors were assessed over time by the BAT assay. Donors 1 and 2 were tracked over a period of 3 – 4 months on 5 and 3 different time points, respectively, while donor 3 was measured 4 times over a period of 3 years. The general reactivity at these time points, expressed as percentage of CD63+ basophils after α -Fc ϵ RI stimulation is shown. The direction of the arrows indicates the timely order of the measurements, while the length indicates the time interval that had passed between the two measurements (double arrows at the x-axis represent a reference for a time span of 2 month- or 2 year-period). Colors of the arrow indicate the functional states of the individuals defined by HDM-specific BAT at the first time point (HDM^{NR}: grey; ‘anergic’: red; HDM^R: blue). (B) Conversion of basophil anergy. For 38 individuals the functional state of was determined at two time points separated by approximately two years. Each arrow represents one individual. The data points are grouped according to the type of conversion (HDM^{NR} $\leftrightarrow$ HDM^{NR}, HDM^{NR} $\leftrightarrow$ anergic, anergic $\leftrightarrow$ anergic, anergic $\leftrightarrow$ HDM^R and HDM^R $\leftrightarrow$ HDM^R). The data is also represented as arrows in **Fig. 6A**.

Figure S7

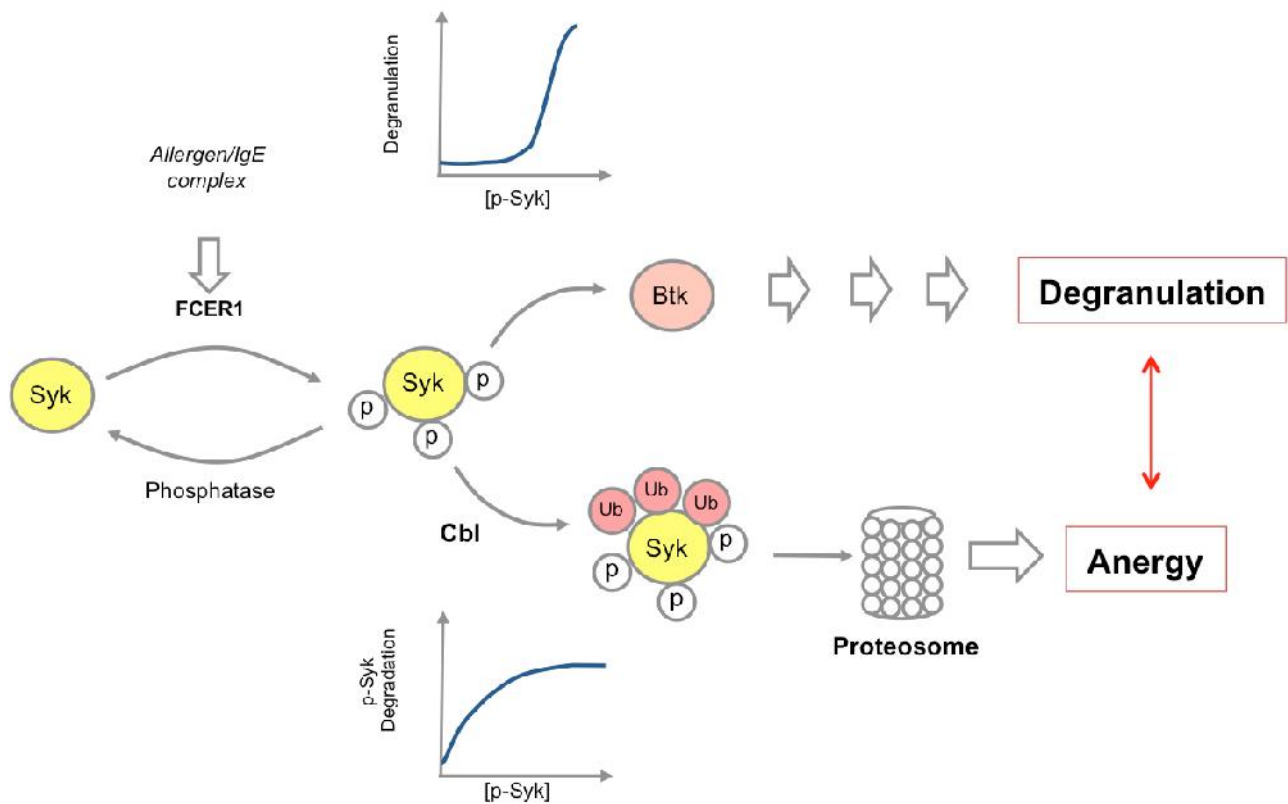


Figure S7. A kinetic model for the regulation of basophil anergy by allergens. Ligation of FcεRI by allergen/IgE complex results in the phosphorylation of Syk to p-Syk. In two parallel reactions Syk-p can phosphorylate Btk to trigger degranulation or become ubiquitinated by CBL. The latter results in the proteasomal degradation of p-Syk. Since degranulation takes place only after crossing a threshold concentration of p-Syk, chronic allergen/IgE stimulation resulting in lower p-Syk levels results in basophil anergy caused by the depletion of basophil Syk.

Additional Material and Method

Allergic rhinitis (AR) and skin prick test (SPT)

Allergic rhinitis was defined according to 'Allergic Rhinitis and its Impact of Asthma' (ARIA) guidelines. A case was defined by the presence of two or more characteristics symptoms including sneezing, rhinorrhea, itchy nose, and nose blockage. The skin prick test (SPT) with allergen extracts from *Blomia tropicalis* (Bt), *Dermatophagoides pteronyssinus* (Dp), *Elaeis guineensis* and *Curvularia spp.* were carried out as previously described(1,2). The SPT was performed on volunteers who have not taken any anti-allergic drugs for at least 3 days prior to the test. The test was done by placing either a drop of saline (negative control), histamine (positive control; Allergopharma), or allergen extracts on the forearms of volunteers.

Basophil activation test

The basophil activation test (BAT) was carried out by using the Flow2CAST kit (Bühlmann Laboratories). The test was performed according to manufacturer's instructions with some modifications. The basophil activation test (BAT) was performed using 25 μ l whole blood collected in 4.5 ml sodium citrate- or 10 ml K₂EDTA Vacutainers (BD), was stimulated for 15 min by adding 25 μ l of either anti-Fc ϵ RI mAb (Bühlmannstimulation control), or 0.1 μ g/ml Dp or Bt extract in a total volume of 100 μ l of Bühlmann Stimulation Buffer (BSB). This was followed by the addition of 10 μ l of the antibody reagent (CD63 FITC and CCR3 PE, as provided in the kit). The reaction mixture was incubated in a 37°C water-bath for 15 min followed by erythrocyte lysis with the Lysing Reagent for 10 min at room temperature. The cells were then centrifuged at 500 xg for 5 min and re-suspended in 300 μ l of Wash Buffer to be analyzed in a FACSCantoII flow cytometer (BD Bioscience). The threshold for a positive response was defined as 38% CD63+ basophils. This value was empirically determined from the distribution of CD63+ basophil cell percentages within the cohort as a fraction of total basophils upon stimulation. Individuals were defined as 'anergic' when less than 38% CD63+ basophils were detected after anti-Fc ϵ RI treatment; they were defined as HDM-responders (HDM^R) when more than 38% CD63+ basophils were detected after exposure to either one of the two HDM-allergens (Dp or Bt). For basophil desensitization assays, 300 μ l of the whole blood samples were incubated for 16 hr in a 37°C CO₂ incubator in sterile 5 ml polypropylene tubes with titrated amounts of Bt- or Dp-allergens in 1.2 ml Bühlmann Stimulation Buffer

(BSB). After the overnight exposure, 50 μ l aliquots were mixed with the same volume of either BSB, or BSB supplemented with anti-Fc ϵ RI α mAb or calcium ionomycin (final concentration 4.5 μ M) and incubated for 15 min at 37°C. Basophil degranulation was then determined by BAT as described above.

Gene expression analysis

Basophils were isolated from three allergen responsive individuals with reactive basophils and two individuals with mostly anergic basophils using the EasySep Human Basophil Enrichment kit (Stemcell Technologies). Purity of basophils was determined by flow cytometry and ranged between 96 – 99%. For stimulation experiments, 7×10^4 basophils incubated in BSB (contained recombinant human IL-3 as provided in the FLOW2CAST kit) in the absence or presence of anti-Fc ϵ RI α mAb (provided in Böhlmann FLOW2CAST kit). After 4 hr the basophils were harvested and stored in TRIzol (Invitrogen Life Technologies) at -80°C. Total RNA was extracted using the extraction protocol as recommended by the manufacturer. RNA isolation was performed by acid guanidinium thiocyanate-phenol-chloroform extraction (Trizol Invitrogen) followed by a Qiagen RNeasy Micro clean-up procedure. Total RNA integrity was determined by using an Agilent Bioanalyzer and revealed an RNA Integrity Number (RIN) $\geq$ 6.2. Single Primer Isothermal Amplification (SPIA) cDNA and sense transcript cDNA were prepared, fragmented and labeled according to Nugen WT Ovation Pico RNA Amplification and WT Ovation Exon kit and Encore Biotin protocols. The labeled cDNA was hybridized on the Affymetrix Human GeneChip 1.0 ST Arrays, which subsequently were processed and stained using GeneChip Fluidics Station 450. The stained GeneChip Arrays were scanned in the Microarray Facility at the Biopolis Shared Facility, Singapore using a GeneChip Scanner 3000. Quality control for the Arrays was performed using the Affymetrix Expression Console Software. Gene expression data were processed using the oligo package in Bioconductor version 2.11 running on R version 2.15.2. Normalization of the arrays was done using RMA and the resulting log₂ transformed values used for all subsequent analysis. Limma was used to determine genes that are different between samples of simulated reactive basophils compared to the rest of the samples (unstimulated reactive, unstimulated anergic and stimulated anergic basophils). Multiple testing corrections were done using the method of Benjamini and Hochberg. The Limma results were ranked based on their corrected P values and genes selected based on a P value cut off of 0.3. This resulted in 113 transcript clusters and 112 unique genes (**Table S3**). This set of genes was then used for the heat

map in Fig. 2. The entire processing of the data was carried out within a workflow created in BIOVIA Pipeline Pilot and the data was visualized in TIBCO Spotfire.

Immunophenotyping by FACS

Flow cytometry analysis was performed on whole blood samples collected in 4.5 ml sodium citrate vacutainers. The samples were measured within 3 hr after blood drawing. Erythrocytes were lysed by adding 14 ml of erythrocyte lysis buffer (155 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA) to 400 µl whole blood followed by 10 – 15 min incubation at room temperature. Cells were centrifuged at 860 xg for 3 min and the cell pellets were re-suspended in PBS and washed once with PBS. Staining was carried out by first incubating the cells at room temperature for 10 min with 100 µl of LIVE/DEAD Fixable Aqua Dead kit (Life Technologies) in 96 round bottom Nunc Microplate (Thermo Scientific). After washing with 100 µl FACS buffer (0.5% BSA, 2 mM EDTA in PBS), cells were stained for 15 min at 4°C in a total volume of 50 µl FACS buffer with fluorescence-labeled antibodies. The antibody cocktail contained anti-CCR3 PE (Bühlmann Laboratories), which in combination with the side scatter could define neutrophils, eosinophils, basophils, monocytes and lymphocytes (**Fig. S1**). Co-staining was carried out with anti-FcεRIα-FITC (AER37, eBioscience), and anti-IgE-APC (MB10-5C4, Miltenyi Biotec). This allowed the measurement of light scatter parameters (FSC and SSC) and surface expression (CCR3, surface IgE, and FcεRI) for each of the cell types. For the validation cohort, the panel was extended with anti-CD123-PerCP-Cy5.5 (6H6, eBioscience), which besides acting as additional surface marker, also allowed defining the subsets of myeloid (mDC) and plasmacytoid dendritic cells (pDC) (**Fig. S1**). For the discovery cohort only, a second antibody panel was used that allowed discrimination of the monocytes and lymphocytes. The panel included anti-IgE (MB10-5C4, Miltenyi Biotec), anti-FcεRI (AER-37, eBioscience), anti-CD123 (6H6), anti-CD14 (61D3, eBioscience), anti-CD16 (3G8, Biolegend), anti-CD1c (L161, Biolegend) and anti-HLA-DR (L243, BD Bioscience). All monoclonal antibodies were obtained from BD Biosciences unless stated otherwise. pDC, basophils, mDC, CD16+ monocytes, CD16- monocytes, NK cells and T cells were gated according to **Fig. S1**. This allowed the measurement of light scatter parameters (FSC and SSC) and surface expression (surface IgE, FcεRI, CD123, CD14, CD16, CD1c and HLA-DR) for each of the subsets. After incubation, the cells were washed once with 200 µl of FACS buffer and re-suspended in 200 µl of PBS supplemented with 2% paraformaldehyde. Spectral overlap was compensated with the AutoCompensation available on the FACS Diva software (BD

Biosciences) by using anti-mouse Kappa CompBeads (BD Biosciences). The analysis was carried out on a FACSCantoII or Fortessa cytometers (BD Biosciences).

Phosflow analysis

The protocol for measuring the Syk- and MAPK-signaling pathways in basophils was adapted from the method described by Chow *et al*(3). Briefly, 100 µl whole blood samples were stimulated in 100 µl incubation buffer (20 mM HEPES, 133 mM NaCl, 5 mM KCl, 7 mM CaCl₂, 3.5 mM MgCl₂, 1 mg/ml human serum albumin, 50 IU heparin, pH 7.4 in PBS) supplemented with either anti-FcεRIα mAb alone (provided in Bühlmann FLOW2CAST kit), 10 ng/ml IL-3 alone (R&D Systems), or a combination of anti-FcεRIα mAb and IL-3. After 10 min incubation at 37°C, the cells were fixed by adding 81 µl 16% methanol-free formaldehyde (Thermo Scientific). After fixing for 10 min at room temperature, 1 ml of lyse/permeabilization buffer (0.114% Triton X-100 in PBS) was added with vigorous vortex, followed by 15 min incubation at 37°C. Cells were then washed with 2 ml of cold wash buffer (4% fetal bovine serum in PBS, 860 xg for 3 min), re-suspended in 1 ml cold 50% methanol in PBS, washed twice with wash buffer and incubated at room temperature in 50 µl with fluorescent-labeled antibodies for 30 min. The following antibodies were used: anti-Syk-FITC (4D10), anti-(pY348)Syk-PE (I120-722), anti-(pT202/pY204)ERK1/2-CF594 (20A) and anti-(pT180/pY182)p38 MAPK-Pacific Blue (36/p38, all from BD Biosciences); anti-CD123-Per-CP-Cy5.5 (6H6, eBioscience), anti-CD14-PC7 (RMO52, Beckman Coulter); anti-FcεRIα APC (AER-37, eBioscience). After incubation, the cells were washed once with wash buffer and re-suspended in 250 µl of FACS buffer and analyzed on a FACS Fortessa cytometer (BD Bioscience).

Analysis of plasma components

Plasma samples were obtained by centrifugation of whole blood collected in BD 8 ml Sodium Citrate, Mononuclear Cell Preparation Tubes (CPT) according to manufacturer's instructions. Total plasma IgE and Bt and Dp-specific plasma IgE were measured by ImmunoCAP system (Phadia AB, Uppsala) based on a fluorescent-linked enzyme immunoassay(4). The detection limit was defined as 0.35 kU/ml. 'HDM-specific' IgE titer was defined by the highest titer of either the Bt- or Dp-specific IgE. All plasma components were quantified by Luminex using various Milliplex MAP Kits (Human Cytokine/Chemokine

Magnetic Bead Panel I, Panel II, Panel III, Human Liver, Adiponectin, Bone, Circulating Cancer Biomarker, Human Metabolic Hormone, Neurodegenerative Disease, PDGF, Soluble Cytokine Receptors Panel, and TGF-beta 3-Plex kits). Plasma samples were prepared according to manufacturer's instructions. A total of 118 soluble plasma components were measured in the two cohorts. The complete list of these parameters is given in **Table S5**. Concentration data were determined using the Bio-Plex manager 6.0 software with a 5-parameter curve-fitting algorithm applied for standard curve calculations. FABP-1, G-CSF, and TGFB3 were excluded from further analysis as most of the samples were below detection limits.

Supplemental References

1. Chew FT, Lim SH, Goh DY, Lee BW. Sensitization to local dust-mite fauna in Singapore. *Allergy*. 1999 Oct 31;54(11):1150–9.
2. Genome-wide association study for atopy and allergic rhinitis in a Singapore Chinese population. 2011;6(5):e19719.
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4. Williams PB, Dolen WK, Koepke JW, Selner JC. Comparison of skin testing and three in vitro assays for specific IgE in the clinical evaluation of immediate hypersensitivity. *Ann Allergy*. 1992 Jan;68(1):35–45.

Table S1. Demographic and clinical characteristics of the donors used in the study

		Discovery Cohort (N = 199)	Validation Cohort (N = 277)	Combined Cohort (N = 476)
Gender	Female	81	94	175
	Male	117	143	260
	NA *	1	40	41
	Age¶(range)	21 (18 – 39)	21 (18 – 50)	21 (18 – 50)
SPT#	Yes	126	138	264
	No	40	38	78
	NA	33	101	134
	Rhinitis##			
Rhinitis##	Yes	80	101	181
	No	92	143	235
	NA	27	33	60

¶Median age in years

A skin prick test (SPT) response to *Blomia tropicalis* (Bt) or *Dermatophagoides pteronyssinus* (Dp) allergens is considered positive when the wheal diameter is 3 mm or greater, when compared to positive (histamine) and negative (saline) controls.

A rhinitis case is defined by 2 or more self-reported rhinitis symptoms (nasal itching, nasal congestion, rhinorrhea, and sneezing) according to Allergic Rhinitis and its Impact of Asthma' (ARIA) guidelines.

* NA, data not available

Table S2. Association of HDM^R and HDM^{NR} with HDM-specific SPT and allergic rhinitis (AR)

	Cohort	Discovery			Validation			Combined		
	Basophil status	Yes	No	Fisher Exact test (OR)	Yes	No	Fisher Exact test (OR)	Yes	No	Fisher Exact test (OR)
Atopy	HDM ^R	119 (79.3%)	3 (2.0%)	2.57 x 10 ⁻²⁵ (0.001)	129 (79.1%)	1 (0.6%)	3.26 x 10 ⁻²⁸ (0.001)	248 (79.2%)	4 (1.3%)	1.35 x 10 ⁻⁵² (0.001)
	HDM ^{NR}	1 (0.7%)	27 (18.0 %)		3 (1.8%)	30 (18.4%)		4 (1.3%)	57 (18.2%)	
Rhinitis	HDM ^R	72 (46.2%)	54 (34.6%)	1.15 x 10 ⁻⁵ (0.11)	86 (38.7%)	85 (38.3%)	9.32 x 10 ⁻⁵ (0.24)	158 (41.8%)	139 (36.8%)	3.49 x 10 ⁻⁹ (0.18)
	HDM ^{NR}	4 (2.6%)	26 (16.7%)		10 (4.5%)	41 (18.5%)		14 (3.7%)	67 (17.7%)	

Table S3. Gene list comparing reactive and anergic basophils

transcript clusterid	geneid	symbol	logFC	Ave Expr	P.Value	adj.P.Val
8140556	3082	HGF	-1.15	8.02	2.56E-06	4.55E-02
7931353	5791	PTPRE	0.80	9.90	2.73E-06	4.55E-02
8142307	50640	PNPLA8	0.69	9.33	6.87E-06	7.62E-02
8079392	729230	CCR2	-1.83	10.02	1.25E-05	8.29E-02
7954012	118426	LOH12CR1	0.79	7.54	2.52E-05	1.22E-01
8078738	9943	OXSRI	0.62	11.18	2.57E-05	1.22E-01
8110106	3274	HRH2	-0.95	6.46	3.17E-05	1.32E-01
7967034	5319	PLA2G1B	0.55	5.66	4.05E-05	1.41E-01
8168531	84636	GPR174	-1.96	6.34	4.76E-05	1.41E-01
7989245	664618	HSP90AB4P	0.55	4.44	4.80E-05	1.41E-01
8088247	50650	ARHGEF3	-0.97	6.28	5.08E-05	1.41E-01
8046695	3676, 375298	ITGA4, CERKL	-0.94	9.15	7.00E-05	1.57E-01
7912136	10911	UTS2	-1.97	6.10	7.07E-05	1.57E-01
8017555	2081	ERN1	-0.49	10.07	7.12E-05	1.57E-01
7906443	2205	FCER1A	-0.80	9.12	7.85E-05	1.57E-01
8093294	729230	CCR2	-1.51	10.81	8.01E-05	1.57E-01
8154733	48	ACO1	-1.00	8.71	8.53E-05	1.58E-01
7910752	4548	MTR	-0.51	6.15	1.16E-04	1.93E-01
8054135	11320	MGAT4A	-1.16	6.65	1.47E-04	2.25E-01
8130505	7430	EZR	0.65	11.73	1.51E-04	2.25E-01
7948987	11145	PLA2G16	1.22	6.26	1.60E-04	2.25E-01
8166805	10159	ATP6AP2	-0.61	7.60	1.62E-04	2.25E-01
8174051	695	BTK	-0.47	10.44	1.81E-04	2.41E-01
8153021	6482	ST3GAL1	-1.00	6.94	1.93E-04	2.42E-01
8080926	10550	ARL6IP5	-0.66	9.55	2.00E-04	2.42E-01
8107066	4012	LNPEP	-0.72	9.37	2.15E-04	2.42E-01
8025828	3949	LDLR	0.55	8.85	2.18E-04	2.42E-01
8105778	5295, 404543	PIK3R1, PIK3R1OS	-0.60	10.80	2.42E-04	2.60E-01
8166243	9185	REPS2	-0.72	8.44	2.72E-04	2.70E-01
8102214	9061	PAPSS1	-0.70	9.84	2.88E-04	2.70E-01
7989596	23604	DAPK2	-0.59	7.54	2.88E-04	2.70E-01
8107532	3295	HSD17B4	-0.72	8.52	3.01E-04	2.70E-01
8020684	59340	HRH4	-0.94	10.63	3.16E-04	2.70E-01
8136918	7791	ZYX	0.69	7.04	3.26E-04	2.70E-01
7917240	1486	CTBS	-0.61	7.38	3.39E-04	2.70E-01
8006433	6347	CCL2	1.82	6.76	3.44E-04	2.70E-01

7969703	3843	IPO5	0.91	7.28	3.74E-04	2.70E-01
7970569	26278	SACS	0.81	7.17	3.90E-04	2.70E-01
8018558	51	ACOX1	-0.61	8.13	3.97E-04	2.70E-01
8139392	54606	DDX56	0.53	6.26	4.11E-04	2.70E-01
8112388	373	TRIM23	-1.16	6.85	4.20E-04	2.70E-01
7899361	23673	STX12	-0.61	7.08	4.28E-04	2.70E-01
8096116	84803	AGPAT9	0.55	10.97	4.37E-04	2.70E-01
8107878	3562	IL3	1.57	5.28	4.41E-04	2.70E-01
7978201	4738	NEDD8	-1.53	8.38	4.48E-04	2.70E-01
7906303	149499	C1orf92	0.77	5.51	4.76E-04	2.70E-01
8147221	734	OSGIN2	-0.88	10.08	4.77E-04	2.70E-01
7906085	4000	LMNA	0.76	7.02	4.82E-04	2.70E-01
7979179	30001	ERO1L	-0.62	9.33	4.94E-04	2.70E-01
8100231	7006	TEC	-0.63	10.98	4.97E-04	2.70E-01
7969693	5911	RAP2A	-0.92	7.53	5.03E-04	2.70E-01
8064375	140809	SRXN1	1.00	5.81	5.18E-04	2.74E-01
7921667	962	CD48	-1.08	7.82	5.56E-04	2.82E-01
8053533	10989	IMMT	-0.64	8.93	5.60E-04	2.82E-01
8059376	5270	SERPINE2	0.74	4.18	5.73E-04	2.82E-01
8168557	6451	SH3BGRL	-0.47	8.43	5.85E-04	2.82E-01
8113358	7903	ST8SIA4	-0.49	10.04	5.85E-04	2.82E-01
7972069	23077	MYCBP2	-0.45	8.51	6.18E-04	2.88E-01
8172266	407006	MIR221	2.20	7.78	6.28E-04	2.88E-01
8083605	51319	RSRC1	1.17	6.43	6.33E-04	2.88E-01
8111698	253260	RICTOR	-0.76	9.51	6.38E-04	2.88E-01
7941296	10534	SSSCA1	0.51	6.17	6.49E-04	2.88E-01
7956826	23329	TBC1D30	1.29	6.09	6.67E-04	2.92E-01
7942896	220047	CCDC83	0.55	4.03	6.94E-04	2.92E-01
8122457	8676	STX11	0.49	7.68	7.07E-04	2.92E-01
8095680	3576	IL8	0.68	10.55	7.13E-04	2.92E-01
8004741	116840	CNTROB	0.83	5.93	7.16E-04	2.92E-01
8095870	901	CCNG2	-0.33	10.86	7.17E-04	2.92E-01
7926531	221079	ARL5B	0.60	8.43	7.25E-04	2.92E-01
7945132	2313, 2130	FLI1, EWSR1	-0.64	9.10	7.60E-04	2.92E-01
8101839	1977	EIF4E	0.78	5.84	7.65E-04	2.92E-01
7913415	84196	USP48	-0.38	9.88	7.89E-04	2.92E-01
8072757	1439	CSF2RB	-0.35	10.92	7.96E-04	2.92E-01
7909371	1378	CR1	-0.66	9.10	8.04E-04	2.92E-01
8123609	5272	SERPINB9	1.14	9.77	8.06E-04	2.92E-01
8014063	2124	EVI2B	-0.52	9.87	8.17E-04	2.92E-01
8125048	23564	DDAH2	0.84	8.40	8.30E-04	2.92E-01

7934870	84896	ATAD1	-0.75	6.34	8.32E-04	2.92E-01
8091190	545	ATR	-0.58	5.12	8.43E-04	2.92E-01
7971513	2098	ESD	-0.82	6.04	8.52E-04	2.92E-01
8007620	2896	GRN	-0.40	6.44	8.53E-04	2.92E-01
7903703	2773	GNAI3	0.40	10.39	8.56E-04	2.92E-01
7947434	26273	FBXO3	-0.71	5.59	8.88E-04	2.92E-01
8083422	56670	SUCNR1	-1.39	6.88	8.93E-04	2.92E-01
7928959	5728	PTEN	-0.37	10.64	8.96E-04	2.92E-01
8046003	25801	GCA	-0.59	8.21	8.98E-04	2.92E-01
8170428	4534	MTM1	-1.14	5.55	9.18E-04	2.92E-01
8020806	54941	RNF125	-0.93	9.41	9.23E-04	2.92E-01
7921840	9970	NR1I3	0.45	5.11	9.31E-04	2.92E-01
8151118	80124	VCPIP1	-0.38	9.14	9.34E-04	2.92E-01
8116070	9260	PDLIM7	0.48	7.33	9.38E-04	2.92E-01
7986049	64784	CRTC3	-0.60	8.76	9.67E-04	2.94E-01
8113157	9652	TTC37	-0.66	4.70	9.81E-04	2.94E-01
7980146	10577	NPC2	-0.80	8.83	1.01E-03	2.95E-01
8023063	498	ATP5A1	-0.64	7.73	1.01E-03	2.95E-01
7984843	10620	ARID3B	1.06	8.18	1.03E-03	2.95E-01
8016414	8631	SKAP1	-0.45	3.94	1.04E-03	2.95E-01
8175039	2000	ELF4	0.62	8.24	1.07E-03	2.95E-01
7970864	10808	HSPH1	0.53	10.00	1.09E-03	2.95E-01
8099051	8602	NOP14	-0.63	5.85	1.09E-03	2.95E-01
8062409	56259	CTNBNB1	0.46	10.66	1.12E-03	2.95E-01
8004497	1973	EIF4A1	0.38	10.48	1.12E-03	2.95E-01
7940598	399900	LOC399900	0.78	5.71	1.13E-03	2.95E-01
7909931	148362	C1orf58	-0.83	8.30	1.13E-03	2.95E-01
7919888	56882	CDC42SE1	0.62	9.03	1.14E-03	2.95E-01
7989023	5873	RAB27A	-1.06	9.18	1.14E-03	2.95E-01
8016980	406934	MIR142	1.51	5.50	1.15E-03	2.95E-01
8080438	28972, 55830	SPCS1, GLT8D1	-0.83	6.98	1.15E-03	2.95E-01
7928308	54541	DDIT4	0.42	7.48	1.16E-03	2.95E-01
7916789	79819	WDR78	-0.39	4.41	1.18E-03	2.95E-01
8120441	23469	PHF3	-0.85	8.74	1.18E-03	2.95E-01
7950248	9873	FCHSD2	-0.68	9.46	1.21E-03	2.95E-01
7953291	928	CD9	0.41	9.99	1.23E-03	2.95E-01

Table S4. Complete dataset including IgE-related and IgE unrelated parameters for discovery and validation cohorts

Name	n	df	pvalue	pvalue_adj	cohort	ige_related_parameter
Plasma HDM IgE	199	2	2.06E-20	3.01E-18	Discovery	yes
Plasma Dp IgE	199	2	7.45E-20	5.43E-18	Discovery	yes
Plasma Bt IgE	199	2	5.60E-18	2.73E-16	Discovery	yes
Total IgE	199	2	2.40E-12	8.76E-11	Discovery	yes
Eos freq.	198	2	1.57E-08	4.59E-07	Discovery	no
Bas IgE	198	2	1.07E-07	2.62E-06	Discovery	yes
Mono FceRI	198	2	3.36E-05	7.00E-04	Discovery	yes
Bas FceRI	198	2	5.17E-05	9.43E-04	Discovery	yes
MIP-1b	154	2	1.52E-03	2.47E-02	Discovery	no
Mono IgE	198	2	4.30E-03	6.28E-02	Discovery	yes
MIP-1d	190	2	5.98E-03	7.94E-02	Discovery	no
IL-8	172	2	7.79E-03	9.09E-02	Discovery	no
ApoAII	191	2	8.34E-03	9.09E-02	Discovery	no
Leptin	160	2	8.72E-03	9.09E-02	Discovery	no
Bas CCR3	198	2	3.52E-02	3.29E-01	Discovery	no
sTNFRI	196	2	3.60E-02	3.29E-01	Discovery	no
Eotaxin-3	90	2	4.40E-02	3.78E-01	Discovery	no
TARC	190	2	5.59E-02	4.53E-01	Discovery	no
I-TAC	191	2	6.21E-02	4.77E-01	Discovery	no
sTNFRII	192	2	7.10E-02	5.18E-01	Discovery	no
sIL-1RII	196	2	8.58E-02	5.65E-01	Discovery	no
PYY	153	2	9.24E-02	5.65E-01	Discovery	no
ApoCII	191	2	9.50E-02	5.65E-01	Discovery	no
VEGF	164	2	9.87E-02	5.65E-01	Discovery	no
IL-6	78	1	1.09E-01	5.65E-01	Discovery	no
C-Peptide	161	2	1.15E-01	5.65E-01	Discovery	no
IL-16	177	2	1.16E-01	5.65E-01	Discovery	no
IL-12p40	10	1	1.17E-01	5.65E-01	Discovery	no
FGF-2	175	2	1.19E-01	5.65E-01	Discovery	no
Glucagon	107	2	1.22E-01	5.65E-01	Discovery	no
IL-29	168	2	1.25E-01	5.65E-01	Discovery	no
FGF-21	167	2	1.31E-01	5.65E-01	Discovery	no
Lymph FceRI	198	2	1.34E-01	5.65E-01	Discovery	yes
Lymphotactin	180	2	1.37E-01	5.65E-01	Discovery	no
sIL-6R	198	2	1.37E-01	5.65E-01	Discovery	no
Bas SSC-A	198	2	1.39E-01	5.65E-01	Discovery	no
Mono CCR3	198	2	1.62E-01	6.38E-01	Discovery	no

ANGPTL6	191	2	1.66E-01	6.38E-01	Discovery	no
SAP	191	2	1.81E-01	6.59E-01	Discovery	no
Fractalkine	153	2	1.84E-01	6.59E-01	Discovery	no
I-309	151	2	1.85E-01	6.59E-01	Discovery	no
Bas freq.	198	2	1.92E-01	6.68E-01	Discovery	no
Ab40	190	2	1.99E-01	6.77E-01	Discovery	no
Eos SSC-A	198	2	2.14E-01	7.07E-01	Discovery	no
IL-7	21	2	2.31E-01	7.07E-01	Discovery	no
CTACK	190	2	2.31E-01	7.07E-01	Discovery	no
Lymph SSC-A	198	2	2.33E-01	7.07E-01	Discovery	no
OC	190	2	2.39E-01	7.07E-01	Discovery	no
MIP-1a	113	2	2.48E-01	7.07E-01	Discovery	no
Ab42	187	2	2.50E-01	7.07E-01	Discovery	no
EGF	148	2	2.54E-01	7.07E-01	Discovery	no
ANGPTL3	191	2	2.55E-01	7.07E-01	Discovery	no
6CKine	181	2	2.57E-01	7.07E-01	Discovery	no
Neu SSC-A	198	2	2.77E-01	7.48E-01	Discovery	no
MIG	191	2	2.92E-01	7.62E-01	Discovery	no
ApoA1	191	2	2.92E-01	7.62E-01	Discovery	no
Neu freq.	198	2	3.14E-01	7.89E-01	Discovery	no
TGFB2	191	2	3.16E-01	7.89E-01	Discovery	no
GCP2	191	2	3.31E-01	7.89E-01	Discovery	no
GLP-1	55	2	3.31E-01	7.89E-01	Discovery	no
GRO	199	2	3.31E-01	7.89E-01	Discovery	no
Insulin	161	2	3.35E-01	7.89E-01	Discovery	no
SDF-1	189	2	3.43E-01	7.89E-01	Discovery	no
PAI-1	190	2	3.56E-01	7.89E-01	Discovery	no
PP	161	2	3.72E-01	7.89E-01	Discovery	no
Bas FSC-A	198	2	3.75E-01	7.89E-01	Discovery	no
Lymph IgE	198	2	3.75E-01	7.89E-01	Discovery	yes
OPG	190	2	3.76E-01	7.89E-01	Discovery	no
HGF	167	2	3.84E-01	7.89E-01	Discovery	no
TGFB1	176	2	3.84E-01	7.89E-01	Discovery	no
ApoE	191	2	3.85E-01	7.89E-01	Discovery	no
TRAIL	190	2	3.89E-01	7.89E-01	Discovery	no
sIL-4R	196	2	4.29E-01	8.50E-01	Discovery	no
PDGF-ABBB	93	2	4.49E-01	8.50E-01	Discovery	no
sIL-1RI	133	2	4.59E-01	8.50E-01	Discovery	no
PTH	188	2	4.63E-01	8.50E-01	Discovery	no
GDNF	93	2	4.70E-01	8.50E-01	Discovery	no
ACTH	160	2	4.73E-01	8.50E-01	Discovery	no

Vitamin D BP	188	2	4.78E-01	8.50E-01	Discovery	no
PDGF-AA	55	2	4.88E-01	8.50E-01	Discovery	no
CRP	191	2	4.89E-01	8.50E-01	Discovery	no
IL-33	115	2	4.90E-01	8.50E-01	Discovery	no
IL-15	15	1	4.97E-01	8.50E-01	Discovery	no
Ghrelin	147	2	4.98E-01	8.50E-01	Discovery	no
ANGPTL4	191	2	5.02E-01	8.50E-01	Discovery	no
Eotaxin	199	2	5.14E-01	8.50E-01	Discovery	no
ApoCIII	191	2	5.19E-01	8.50E-01	Discovery	no
Eotaxin-2	190	2	5.20E-01	8.50E-01	Discovery	no
Amylin Active	102	2	5.27E-01	8.50E-01	Discovery	no
FGF-19	175	2	5.38E-01	8.50E-01	Discovery	no
Eos FSC-A	198	2	5.53E-01	8.50E-01	Discovery	no
Lymph freq.	198	2	5.58E-01	8.50E-01	Discovery	no
MIP-3b	191	2	5.64E-01	8.50E-01	Discovery	no
IL-28a	108	2	5.67E-01	8.50E-01	Discovery	no
sgp130	195	2	5.71E-01	8.50E-01	Discovery	no
TPO	102	2	5.80E-01	8.50E-01	Discovery	no
HCC-1	191	2	5.80E-01	8.50E-01	Discovery	no
Lymph CCR3	198	2	5.83E-01	8.50E-01	Discovery	yes
CFH	188	2	5.90E-01	8.50E-01	Discovery	no
sCD40L	199	2	5.95E-01	8.50E-01	Discovery	no
Factor-XIII	188	2	5.99E-01	8.50E-01	Discovery	no
ECM1	188	2	6.00E-01	8.50E-01	Discovery	no
AFP	177	2	6.03E-01	8.50E-01	Discovery	no
MIP-3a	191	2	6.12E-01	8.50E-01	Discovery	no
Mono freq.	198	2	6.16E-01	8.50E-01	Discovery	no
Eos FceRI	198	2	6.19E-01	8.50E-01	Discovery	yes
Eos CCR3	198	2	6.29E-01	8.50E-01	Discovery	no
sCD30	64	2	6.38E-01	8.50E-01	Discovery	no
TSLP	43	2	6.45E-01	8.50E-01	Discovery	no
S100-B	81	2	6.45E-01	8.50E-01	Discovery	no
sIL-2Ra	192	2	6.46E-01	8.50E-01	Discovery	no
MCP-4	147	2	6.57E-01	8.50E-01	Discovery	no
Antithrombin III	188	2	6.62E-01	8.50E-01	Discovery	no
Mono FSC-A	198	2	6.64E-01	8.50E-01	Discovery	no
Neu IgE	198	2	6.78E-01	8.61E-01	Discovery	yes
G-CSF	196	2	6.94E-01	8.74E-01	Discovery	no
ApoB	189	2	7.03E-01	8.78E-01	Discovery	no
Adiponectin	190	2	7.22E-01	8.93E-01	Discovery	no
Vitronectin	188	2	7.29E-01	8.95E-01	Discovery	no

sEGFR	196	2	7.52E-01	9.15E-01	Discovery	no
Flt-3L	45	2	7.62E-01	9.18E-01	Discovery	no
Eos IgE	198	2	7.67E-01	9.18E-01	Discovery	yes
Neu FcεRI	198	2	7.74E-01	9.19E-01	Discovery	yes
SAA	181	2	7.84E-01	9.23E-01	Discovery	no
IL-20	109	2	7.93E-01	9.26E-01	Discovery	no
sVEGFR2	196	2	8.00E-01	9.27E-01	Discovery	no
sVEGFR3	185	2	8.40E-01	9.42E-01	Discovery	no
ENA-78	170	2	8.61E-01	9.42E-01	Discovery	no
Neu FSC-A	198	2	8.61E-01	9.42E-01	Discovery	no
IL-23	105	2	8.65E-01	9.42E-01	Discovery	no
SCF	50	2	8.66E-01	9.42E-01	Discovery	no
Resistin	190	2	8.66E-01	9.42E-01	Discovery	no
OPN	190	2	8.68E-01	9.42E-01	Discovery	no
GIP	161	2	8.72E-01	9.42E-01	Discovery	no
NAP2	187	2	8.73E-01	9.42E-01	Discovery	no
LIF	65	2	8.78E-01	9.42E-01	Discovery	no
IL-10	48	2	8.95E-01	9.42E-01	Discovery	no
sRAGE	199	2	8.99E-01	9.42E-01	Discovery	no
MCP-2	180	2	9.06E-01	9.42E-01	Discovery	no
Lymph FSC-A	198	2	9.06E-01	9.42E-01	Discovery	no
IL-11	173	2	9.12E-01	9.42E-01	Discovery	no
GM-CSF	150	2	9.26E-01	9.42E-01	Discovery	no
Neu CCR3	198	2	9.29E-01	9.42E-01	Discovery	no
Mono SSC-A	198	2	9.29E-01	9.42E-01	Discovery	no
sVEGFR1	135	2	9.63E-01	9.70E-01	Discovery	no
BCA-1	190	2	9.71E-01	9.71E-01	Discovery	no
Plasma HDM IgE	277	2	8.76E-32	1.91E-29	Validation	yes
Plasma Dp IgE	277	2	7.79E-30	8.49E-28	Validation	yes
Plasma Bt IgE	277	2	5.40E-29	3.93E-27	Validation	yes
Total IgE	277	2	1.79E-16	9.75E-15	Validation	yes
mDC IgE	225	2	1.00E-11	4.37E-10	Validation	yes
pDC IgE	225	2	4.13E-11	1.50E-09	Validation	yes
Bas IgE	225	2	1.69E-10	5.25E-09	Validation	yes
pDC FcεRI	225	2	1.32E-08	3.59E-07	Validation	yes
mDC FcεRI	225	2	4.84E-08	1.17E-06	Validation	yes
Eos freq.	277	2	5.85E-08	1.27E-06	Validation	no
Mono IgE	277	2	1.08E-07	2.14E-06	Validation	yes
Bas FcεRI	225	2	3.12E-05	5.68E-04	Validation	yes
OC	274	2	1.90E-03	3.19E-02	Validation	no
CD16- Mono IgE	225	2	3.68E-03	5.72E-02	Validation	yes

FGF-21	246	2	5.83E-03	8.10E-02	Validation	no
CD16- Mono SSC-A	225	2	5.95E-03	8.10E-02	Validation	no
SAP	277	2	8.86E-03	1.14E-01	Validation	no
mDC CD123	225	2	1.37E-02	1.66E-01	Validation	no
FGF-2	235	2	1.53E-02	1.75E-01	Validation	no
C-Peptide	240	2	1.61E-02	1.76E-01	Validation	no
Leptin	240	2	1.75E-02	1.82E-01	Validation	no
CD16- Mono CD123	225	2	2.67E-02	2.65E-01	Validation	no
mDC CCR3	229	2	3.20E-02	2.99E-01	Validation	no
Mono SSC-A	277	2	3.29E-02	2.99E-01	Validation	no
Eos CD123	229	2	3.60E-02	3.01E-01	Validation	no
sIL-4R	277	2	3.72E-02	3.01E-01	Validation	no
Insulin	237	2	3.89E-02	3.01E-01	Validation	no
Mono FcεRI	277	2	3.97E-02	3.01E-01	Validation	yes
mDC freq.	225	2	4.01E-02	3.01E-01	Validation	no
Eotaxin	277	2	4.36E-02	3.13E-01	Validation	no
NK cell FSC-A	225	2	4.51E-02	3.13E-01	Validation	no
Bas CCR3	277	2	4.80E-02	3.13E-01	Validation	no
B cell HLA-DR	225	2	4.81E-02	3.13E-01	Validation	no
CD16- Mono FSC-A	225	2	4.96E-02	3.13E-01	Validation	no
Eos IgE	277	2	5.02E-02	3.13E-01	Validation	yes
Lymph FSC-A	277	2	5.47E-02	3.32E-01	Validation	no
CD16+ Mono HLA-DR	225	2	5.97E-02	3.52E-01	Validation	no
Adiponectin	275	2	6.56E-02	3.76E-01	Validation	no
GRO	276	2	6.78E-02	3.79E-01	Validation	no
Mono CCR3	277	2	7.19E-02	3.81E-01	Validation	no
Bas FSC-A	225	2	7.42E-02	3.81E-01	Validation	no
ENA-78	231	2	7.43E-02	3.81E-01	Validation	no
6CKine	260	2	7.51E-02	3.81E-01	Validation	no
MIP-1b	242	2	7.77E-02	3.85E-01	Validation	no
CD16+ Mono freq.	225	2	8.50E-02	4.12E-01	Validation	no
Bas freq.	225	2	8.74E-02	4.14E-01	Validation	no
GCP2	277	2	9.92E-02	4.60E-01	Validation	no
T cell FcεRI	225	2	1.05E-01	4.70E-01	Validation	yes
Bas CD1c	225	2	1.06E-01	4.70E-01	Validation	no
IL-8	254	2	1.14E-01	4.86E-01	Validation	no
pDC HLA-DR	225	2	1.16E-01	4.86E-01	Validation	no
I-309	217	2	1.18E-01	4.86E-01	Validation	no
Neu SSC-A	277	2	1.20E-01	4.86E-01	Validation	no

NK cell FcεRI	225	2	1.20E-01	4.86E-01	Validation	yes
Eos FcεRI	277	2	1.31E-01	5.19E-01	Validation	yes
GDNF	156	2	1.34E-01	5.23E-01	Validation	no
CTACK	277	2	1.39E-01	5.30E-01	Validation	no
Neu freq.	277	2	1.47E-01	5.36E-01	Validation	no
mDC HLA-DR	225	2	1.50E-01	5.36E-01	Validation	no
OPN	274	2	1.51E-01	5.36E-01	Validation	no
GIP	240	2	1.55E-01	5.36E-01	Validation	no
ApoA1	275	2	1.55E-01	5.36E-01	Validation	no
PP	237	2	1.56E-01	5.36E-01	Validation	no
NK cell freq.	225	2	1.60E-01	5.36E-01	Validation	no
NK cell CD123	225	2	1.60E-01	5.36E-01	Validation	no
Antithrombin III	261	2	1.71E-01	5.66E-01	Validation	no
PAI-1	275	2	1.80E-01	5.85E-01	Validation	no
MIP-3b	277	2	1.87E-01	5.92E-01	Validation	no
CD16+ Mono IgE	225	2	1.89E-01	5.92E-01	Validation	yes
ANGPTL4	277	2	1.90E-01	5.92E-01	Validation	no
Ghrelin	196	2	1.94E-01	5.95E-01	Validation	no
NK cell HLA-DR	225	2	2.03E-01	6.14E-01	Validation	no
VEGF	231	2	2.08E-01	6.20E-01	Validation	no
Neu IgE	277	2	2.12E-01	6.25E-01	Validation	yes
sRAGE	277	2	2.21E-01	6.35E-01	Validation	no
BCA-1	277	2	2.33E-01	6.35E-01	Validation	no
B cell FSC-A	225	2	2.33E-01	6.35E-01	Validation	no
Eos FSC-A	277	2	2.34E-01	6.35E-01	Validation	no
sIL-1RII	277	2	2.38E-01	6.35E-01	Validation	no
B cell IgE	225	2	2.39E-01	6.35E-01	Validation	yes
TRAIL	276	2	2.41E-01	6.35E-01	Validation	no
sVEGFR2	277	2	2.41E-01	6.35E-01	Validation	no
pDC SSC-A	225	2	2.50E-01	6.35E-01	Validation	no
TPO	134	2	2.52E-01	6.35E-01	Validation	no
CD16+ Mono CD1c	225	2	2.59E-01	6.35E-01	Validation	no
Eos CCR3	277	2	2.67E-01	6.35E-01	Validation	no
MIP-1a	162	2	2.70E-01	6.35E-01	Validation	no
TARC	276	2	2.71E-01	6.35E-01	Validation	no
pDC CCR3	229	2	2.72E-01	6.35E-01	Validation	no
pDC CD123	225	2	2.74E-01	6.35E-01	Validation	no
Lymph freq.	277	2	2.76E-01	6.35E-01	Validation	no
GM-CSF	264	2	2.77E-01	6.35E-01	Validation	no
pDC CD14	225	2	2.78E-01	6.35E-01	Validation	no
G-CSF	276	2	2.78E-01	6.35E-01	Validation	no

Vitamin D BP	261	2	2.79E-01	6.35E-01	Validation	no
NAP2	263	2	2.80E-01	6.35E-01	Validation	no
Mono CD123	229	2	2.98E-01	6.69E-01	Validation	no
NK cell CD1c	225	2	3.03E-01	6.71E-01	Validation	no
CD16+ Mono CD14	225	2	3.05E-01	6.71E-01	Validation	no
sgp130	277	2	3.10E-01	6.75E-01	Validation	no
Vitronectin	261	2	3.14E-01	6.77E-01	Validation	no
B cell FcεRI	225	2	3.23E-01	6.87E-01	Validation	yes
ECM1	261	2	3.30E-01	6.87E-01	Validation	no
Neu FSC-A	277	2	3.31E-01	6.87E-01	Validation	no
MIP-1d	277	2	3.33E-01	6.87E-01	Validation	no
Bas HLA-DR	225	2	3.36E-01	6.87E-01	Validation	no
Lymph FcεRI	277	2	3.40E-01	6.87E-01	Validation	yes
LIF	70	2	3.41E-01	6.87E-01	Validation	no
pDC CD1c	225	2	3.53E-01	7.00E-01	Validation	no
mDC SSC-A	225	2	3.53E-01	7.00E-01	Validation	no
TGFB1	264	2	3.61E-01	7.03E-01	Validation	no
MCP-4	184	2	3.67E-01	7.03E-01	Validation	no
Bas SSC-A	225	2	3.68E-01	7.03E-01	Validation	no
Lymph IgE	277	2	3.71E-01	7.03E-01	Validation	yes
AFP	247	2	3.71E-01	7.03E-01	Validation	no
TGFB2	275	2	3.84E-01	7.21E-01	Validation	no
CD16+ Mono CD123	225	2	3.88E-01	7.21E-01	Validation	no
ANGPTL6	277	2	3.92E-01	7.21E-01	Validation	no
sIL-2Ra	277	2	3.94E-01	7.21E-01	Validation	no
CD16+ Mono FcεRI	225	2	3.98E-01	7.21E-01	Validation	yes
CD16- Mono CD14	225	2	4.03E-01	7.21E-01	Validation	no
Bas CD14	225	2	4.07E-01	7.21E-01	Validation	no
pDC CD16	225	2	4.07E-01	7.21E-01	Validation	no
CRP	276	2	4.12E-01	7.24E-01	Validation	no
sIL-6R	277	2	4.20E-01	7.29E-01	Validation	no
T cell IgE	225	2	4.21E-01	7.29E-01	Validation	yes
pDC freq.	225	2	4.30E-01	7.34E-01	Validation	no
CD16+ Mono SSC-A	225	2	4.31E-01	7.34E-01	Validation	no
IL-6	101	2	4.35E-01	7.36E-01	Validation	no
TSLP	84	2	4.39E-01	7.37E-01	Validation	no
T cell CD123	225	2	4.44E-01	7.39E-01	Validation	no
IL-20	145	2	4.60E-01	7.59E-01	Validation	no

B cell freq.	225	2	4.77E-01	7.83E-01	Validation	no
CFH	263	2	4.81E-01	7.83E-01	Validation	no
Ab40	275	2	5.03E-01	8.02E-01	Validation	no
T cell CD16	225	2	5.05E-01	8.02E-01	Validation	no
sEGFR	277	2	5.07E-01	8.02E-01	Validation	no
FGF-19	255	2	5.12E-01	8.02E-01	Validation	no
ApoE	275	2	5.14E-01	8.02E-01	Validation	no
CD16- Mono						
FceRI	225	2	5.19E-01	8.02E-01	Validation	yes
Eos SSC-A	277	2	5.19E-01	8.02E-01	Validation	no
Amylin Active	136	2	5.30E-01	8.11E-01	Validation	no
Eotaxin-2	274	2	5.35E-01	8.11E-01	Validation	no
HCC-1	277	2	5.36E-01	8.11E-01	Validation	no
MCP-2	263	2	5.45E-01	8.20E-01	Validation	no
mDC CD14	225	2	5.51E-01	8.22E-01	Validation	no
EGF	191	2	5.55E-01	8.22E-01	Validation	no
Lymph CD123	229	2	5.58E-01	8.22E-01	Validation	no
T cell SSC-A	225	2	5.68E-01	8.26E-01	Validation	no
B cell CD123	225	2	5.68E-01	8.26E-01	Validation	no
CD16- Mono						
CD1c	225	2	5.82E-01	8.33E-01	Validation	no
NK cell CD16	225	2	5.91E-01	8.33E-01	Validation	no
Flt-3L	97	2	5.93E-01	8.33E-01	Validation	no
NK cell IgE	225	2	6.00E-01	8.33E-01	Validation	yes
B cell CD14	225	2	6.01E-01	8.33E-01	Validation	no
Lymph CCR3	277	2	6.04E-01	8.33E-01	Validation	yes
mDC FSC-A	225	2	6.07E-01	8.33E-01	Validation	no
IL-23	130	2	6.15E-01	8.33E-01	Validation	no
ACTH	239	2	6.15E-01	8.33E-01	Validation	no
PYY	215	2	6.17E-01	8.33E-01	Validation	no
OPG	274	2	6.23E-01	8.33E-01	Validation	no
Bas CD16	225	2	6.27E-01	8.33E-01	Validation	no
IL-28a	156	2	6.33E-01	8.33E-01	Validation	no
NK cell CD14	225	2	6.33E-01	8.33E-01	Validation	no
ApoCIII	275	2	6.38E-01	8.33E-01	Validation	no
T cell CD1c	225	2	6.40E-01	8.33E-01	Validation	no
mDC CD1c	225	2	6.43E-01	8.33E-01	Validation	no
HGF	246	2	6.47E-01	8.33E-01	Validation	no
Lymph SSC-A	277	2	6.47E-01	8.33E-01	Validation	no
Neu CCR3	277	2	6.49E-01	8.33E-01	Validation	no
Lymphotactin	266	2	6.54E-01	8.34E-01	Validation	no
GLP-1	90	2	6.67E-01	8.46E-01	Validation	no

CD16- Mono freq.	225	2	6.81E-01	8.58E-01	Validation	no
Eotaxin-3	146	2	6.95E-01	8.61E-01	Validation	no
Bas CD123	225	2	6.98E-01	8.61E-01	Validation	no
sTNFRI	277	2	7.03E-01	8.61E-01	Validation	no
sVEGFR3	172	2	7.04E-01	8.61E-01	Validation	no
IL-33	160	2	7.07E-01	8.61E-01	Validation	no
B cell CD16	225	2	7.07E-01	8.61E-01	Validation	no
Mono freq.	277	2	7.19E-01	8.71E-01	Validation	no
Resistin	275	2	7.35E-01	8.80E-01	Validation	no
Nk cell SSC-A	225	2	7.41E-01	8.80E-01	Validation	no
Factor-XIII	261	2	7.46E-01	8.80E-01	Validation	no
IL-16	264	2	7.49E-01	8.80E-01	Validation	no
ApoAII	274	2	7.49E-01	8.80E-01	Validation	no
SCF	62	2	7.58E-01	8.80E-01	Validation	no
sTNFRII	277	2	7.59E-01	8.80E-01	Validation	no
T cell freq.	225	2	7.59E-01	8.80E-01	Validation	no
IL-29	218	2	7.73E-01	8.91E-01	Validation	no
pDC FSC-A	225	2	7.77E-01	8.91E-01	Validation	no
Ab42	267	2	7.86E-01	8.97E-01	Validation	no
ApoB	273	2	7.92E-01	8.99E-01	Validation	no
CD16- Mono HLA-DR	225	2	7.96E-01	8.99E-01	Validation	no
CD16+ Mono CD16	225	2	8.13E-01	9.14E-01	Validation	no
B cell SSC-A	225	2	8.17E-01	9.14E-01	Validation	no
ApoCII	275	2	8.45E-01	9.37E-01	Validation	no
T cell FSC-A	225	2	8.48E-01	9.37E-01	Validation	no
I-TAC	269	2	8.51E-01	9.37E-01	Validation	no
ANGPTL3	277	2	8.72E-01	9.51E-01	Validation	no
Neu FcεRI	277	2	8.76E-01	9.51E-01	Validation	yes
MIG	277	2	8.77E-01	9.51E-01	Validation	no
sCD40L	277	2	8.93E-01	9.63E-01	Validation	no
MIP-3a	268	2	9.02E-01	9.65E-01	Validation	no
T cell CD14	225	2	9.03E-01	9.65E-01	Validation	no
CD16+ Mono FSC-A	225	2	9.09E-01	9.65E-01	Validation	no
mDC CD16	225	2	9.13E-01	9.65E-01	Validation	no
Glucagon	150	2	9.26E-01	9.65E-01	Validation	no
PTH	273	2	9.27E-01	9.65E-01	Validation	no
Mono FSC-A	277	2	9.33E-01	9.65E-01	Validation	no
B cell CD1c	225	2	9.39E-01	9.65E-01	Validation	no
SDF-1	275	2	9.43E-01	9.65E-01	Validation	no

Fractalkine	162	2	9.43E-01	9.65E-01	Validation	no
T cell HLA-DR	225	2	9.43E-01	9.65E-01	Validation	no
Neu CD123	229	2	9.57E-01	9.75E-01	Validation	no
IL-11	259	2	9.83E-01	9.92E-01	Validation	no
S100-B	132	2	9.91E-01	9.92E-01	Validation	no
CD16- Mono						
CD16	225	2	9.92E-01	9.92E-01	Validation	no
SAA	260	2	9.92E-01	9.92E-01	Validation	no

Table S5. A list of all the plasma components measured by Luminex

Panel	analyte_processed	n
Adipokine	Adiponectin	582
Adipokine	PAI-1	582
Adipokine	Resistin	582
Apolipoprotein	ApoA1	583
Apolipoprotein	ApoAII	583
Apolipoprotein	ApoB	583
Apolipoprotein	ApoCII	583
Apolipoprotein	ApoCIII	583
Apolipoprotein	ApoE	583
Bone	ACTH	580
Bone	OC	580
Bone	OPG	580
Bone	OPN	580
Bone	PTH	580
Circulating Cancer Biomarker	Antithrombin III	564
Circulating Cancer Biomarker	CFH	564
Circulating Cancer Biomarker	ECM1	564
Circulating Cancer Biomarker	Factor-XIII	564
Circulating Cancer Biomarker	Vitamin D BP	564
Circulating Cancer Biomarker	Vitronectin	564
CVD Panel	CRP	582
CVD Panel	SAA	582
CVD Panel	SAP	582
Cytokine	EGF	574
Cytokine	Eotaxin	574
Cytokine	FGF-2	574
Cytokine	Flt-3L	574
Cytokine	Fractalkine	574
Cytokine	G-CSF	574
Cytokine	GM-CSF	574
Cytokine	GRO	574
Cytokine	IL-10	275
Cytokine	IL-12p40	275
Cytokine	IL-15	275
Cytokine	IL-6	574
Cytokine	IL-7	275
Cytokine	IL-8	574
Cytokine	MIP-1a	574

Cytokine	MIP-1b	574
Cytokine	sCD40L	574
Cytokine	sIL-2Ra	275
Cytokine	VEGF	574
Cytokine 2	6CKine	582
Cytokine 2	BCA-1	582
Cytokine 2	CTACK	582
Cytokine 2	ENA-78	582
Cytokine 2	Eotaxin-2	582
Cytokine 2	Eotaxin-3	582
Cytokine 2	I-309	582
Cytokine 2	IL-16	582
Cytokine 2	IL-20	582
Cytokine 2	IL-21	582
Cytokine 2	IL-23	582
Cytokine 2	IL-28a	582
Cytokine 2	IL-33	582
Cytokine 2	LIF	582
Cytokine 2	MCP-2	582
Cytokine 2	MCP-4	582
Cytokine 2	MIP-1d	582
Cytokine 2	SCF	582
Cytokine 2	SDF-1	582
Cytokine 2	TARC	582
Cytokine 2	TPO	582
Cytokine 2	TRAIL	582
Cytokine 2	TSLP	582
Cytokine 3	GCP2	584
Cytokine 3	HCC-1	584
Cytokine 3	I-TAC	584
Cytokine 3	IL-11	584
Cytokine 3	IL-29	584
Cytokine 3	Lymphotactin	584
Cytokine 3	M-CSF	584
Cytokine 3	MIG	584
Cytokine 3	MIP-3a	584
Cytokine 3	MIP-3b	584
Cytokine 3	NAP2	584
Liver	AFP	583
Liver	ANGPTL3	583
Liver	ANGPTL4	583

Liver	ANGPTL6	583
Liver	FABP1	583
Liver	FGF-19	583
Liver	FGF-21	583
Liver	HGF	583
Metabolic Hormone	Amylin Active	583
Metabolic Hormone	C-Peptide	583
Metabolic Hormone	Ghrelin	583
Metabolic Hormone	GIP	583
Metabolic Hormone	GLP-1	583
Metabolic Hormone	Glucagon	583
Metabolic Hormone	Insulin	583
Metabolic Hormone	Leptin	583
Metabolic Hormone	PP	583
Metabolic Hormone	PYY	583
Neurodegenerative disease panel	Ab40	583
Neurodegenerative disease panel	Ab42	583
Neurodegenerative disease panel	GDNF	583
Neurodegenerative disease panel	S100-B	583
Pdgf	PDGF-AA	275
Pdgf	PDGF-ABBB	275
Receptor	sCD30	275
Receptor	sEGFR	574
Receptor	sgp130	574
Receptor	sIL-1RI	275
Receptor	sIL-1RII	574
Receptor	sIL-2Ra	574
Receptor	sIL-4R	574
Receptor	sIL-6R	574
Receptor	sRAGE	574
Receptor	sTNFR1	574
Receptor	sTNFR2	574
Receptor	sVEGFR1	275
Receptor	sVEGFR2	574
Receptor	sVEGFR3	574
TGFB panel	TGFB1	583
TGFB panel	TGFB2	583
TGFB panel	TGFB3	583