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Functional and phenotypic analysis of basophils allows determining distinct subtypes in patients with chronic urticaria

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MMR wrote the manuscript, performed the experiments and analyzed the data. CM and WP designed the study and revised the manuscript. LH and JP contributed to the acquisition of clinical data and revised the manuscript. All authors finally approved the manuscript.

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The authors declare that they have no conflict of interest.

Background: Chronic urticaria (CU) is a frequent skin disease characterized by relapsing appearance of pruritic hives. While clinical symptoms are due to the release of histamine by cutaneous mast cells, the underlying pathophysiology is still unknown. However, previous studies indicate that basophils might be of relevance. Besides, the occurrence of autoantibodies against IgE or its receptor, FcεRI, and the therapeutic efficacy of anti-IgE antibodies imply that IgE-mediated mechanisms also play an important role in CU.

Methods: Reactivity of CU patients' peripheral blood basophils (n=60) to specific anti-FcεRI and IgE-independent fMLP stimulation was determined by basophil activation test in comparison to patients suffering from IgE-mediated allergic rhinitis (n=10) and healthy controls (n=10). In addition, immunoglobulin receptor (FcεRI, FcγRII) expression and surface bound antibodies (IgE, IgG) were quantified on basophils. Furthermore, the autoreactive capacity of CU sera was evaluated and urticaria-related symptoms were assessed by both UCT and CU-Q2oL.

Results: Stimulating CU patients' basophils via FcεRI, we identified three distinct immunological phenotypes. One subgroup of patients' basophils reacted to FcεRI stimulation, whereas the others had anti-FcεRI non-reactive basophils. Among the latter a subgroup with pronounced basopenia was identified. Of note, this group was characterized by augmented serum-induced basophil activation, increased levels of autoantibodies against thyroid peroxidase and also exhibited the strongest disease impact on their quality of life.

Conclusions: CU patients can be categorized into three immunological subgroups based on their basophil reactivity and frequency. These phenotypes are associated with different clinical characteristics, pointing to basophils as important players in CU pathophysiology.

Key words: autoantibodies, basophils, chronic urticaria, FcεRI, IgE

Abbreviations used:

AR: allergic rhinitis

BAT: basophil activation test

CU: chronic urticaria

CU-R: chronic urticaria patients reactive to FcεRI stimulation

CU-NR: chronic urticaria patients non-reactive to FcεRI stimulation

CU-NR_{B0}: chronic urticaria patients with basopenia

CU-Q_{20L}: chronic urticaria quality of life score 2

HC: healthy controls

UCT: urticaria control test

TPO: thyroid peroxidase

INTRODUCTION

Chronic urticaria (CU) is characterized by the recurrent appearance of pruritic wheals for at least six weeks (1, 2). Due to the chronic, agonizing itch, CU severely affects patients' quality of life (3) by leading to sleep disturbance, energy deprivation and social restrictions (4). Although the underlying pathomechanisms are largely unknown, several aspects suggest autoimmune mechanisms as important pathophysiologic factors (5). For instance, IgG autoantibodies against IgE or its high affinity receptor, FcεRI, are present in the sera of about 40% of CU patients (6, 7) enabling activation of effector cells like mast cells and basophils (8-10). Furthermore, an increased incidence of autoimmune diseases like Hashimoto's thyroiditis, rheumatoid arthritis and type I diabetes have been described in CU (11).

Basophil granulocytes have gained interest in urticaria research more than 50 years ago, when a decreased frequency of peripheral basophils was reported in CU patients (12). The finding of low blood basophil numbers in CU patients was confirmed by several other studies (13-15). In addition, functional impairments of basophils were shown in patients with CU (16). In around one third of these patients, peripheral basophils cannot be activated via FcεRI stimulation during active disease (14) but recover after patients enter remission (17). One reason could be the loss of Syk expression (18, 19) or the overexpression of Src-homology 2-containing-5'-inositol phosphatase (SHIP)-1 and SHIP-2 that are both involved in FcεRI and FcγR downstream signaling (20). In contrast to CU patients with basophils not responding to FcεRI-stimulation (CU-NR), patients with an intact basophil response (CU-R) display expression levels of Syk, SHIP-1 and SHIP-2 similar to healthy subjects (20). In this study, we analyzed phenotypic and functional differences in peripheral basophils of CU patients compared to two control groups consisting of patients

suffering from IgE-mediated allergic rhinitis (AR) and healthy control (HC) individuals to shed more light on the role of basophils in the pathophysiology of CU.

METHODS

Patients

A total of 60 patients with spontaneous CU as assessed by both patients' history and physical examination were included in this study. About half of these patients suffered in addition from inducible urticaria (CU-R: 63%, CU-NR: 42%, CU-NR_{BO}: 50%). Since total IgE levels are elevated in approximately one third of patients with CU (21, 22), CU patients were compared to both patients suffering from IgE-mediated AR (n=10; 27 (24-62 years); 8 female) and HC (n=10; 30 (23-47 years); 5 female). Urticaria-related symptoms were recorded on the German version of the CU quality of life score (CU-Q_{2oL}; 1, 23, 24) and the urticaria control test (UCT; 25) by CU patients. Patients were allowed to treat their urticaria-related symptoms with second or third generation oral antihistamines (e.g. ceterizine, loratadine, rupatadine, desloratadine or fexofenadine) up to fourfold daily dose. Additionally, one patient applied a leukotriene inhibitor (montelukast) and another patient was treated with short-term low-dose of systemic corticosteroid (5mg/d prednisolone). CU patients who had received anti-IgE treatment and patients suffering solely from inducible CU were not included. All patients provided written informed consent to participate in the trial. The study was approved by the Ethics Committee of the Medical Faculty of the Philipps University Marburg.

Basophil activation test

Peripheral blood was drawn in ethylenediaminetetraacetic acid (EDTA)-containing blood collection tubes. The reactivity of basophils to stimulation with different concentrations of anti-FcεRIα (0.1, 0.3, 1 and 3 μg/ml) and N-formyl-methionyl-leucyl-phenylalanine (fMLP) as an unspecific control stimulus was evaluated by basophil activation test (BAT) using Flow CAST (Bühlmann, Schönenbuch, Switzerland). Basophils were stimulated and stained according to the manufacturer's instructions. In brief, 100 μl of IL-3-containing stimulation buffer were mixed with the respective stimulant and 50 μl of fresh EDTA-blood was added. Subsequently, cells were incubated with 15 μl of staining reagent consisting of anti-CCR3-PE and anti-CD63-FITC in a 37°C water bath for 15 min. Afterwards blood samples were depleted of erythrocytes by incubation in lysis buffer for 7 min and centrifuged at 500xg for 5 min. Finally, cells were resuspended in 150 μl of washing buffer and evaluated by flow cytometry (FACS Calibur, BD Biosciences, San Jose, USA). To ensure basophil quality and reliable *ex vivo* activation, fresh blood was stained and measured within 2 h after venipuncture. Flow cytometric results were analyzed by FlowJo software (FlowJo LLC, Ashland, USA). CCR3⁺SSC^{low} cells were considered as basophils and CD63⁺ basophils as activated. CU patients were defined as non-responders (CU-NR) when either less than 10% of basophils were positive for CD63 after stimulation with anti-FcεRI and/or in the case of pre-existing basophil activation with a stimulation index <2 (i.e. CD63⁺ basophils after anti-FcεRI stimulation/CD63⁺ basophils without stimulation).

To characterize the autoreactive capacity of CU sera, 50 μl of fresh HC donor EDTA-blood was incubated with 50 μl of test serum. For this instance, different HC basophil donors were tested since the degree of basophil activation by the CU sera varied

with regard to the donor (data not shown). However, the relative distribution between the single CU patients was always comparable using different basophil donors and HC control sera never led to basophil activation.

Staining for cell bound IgE and IgG and their receptors

EDTA-containing peripheral blood (50µl) was mixed with 150µl of IL-3-containing stimulation buffer (Bühlmann, Schönenbuch, Switzerland) and incubated with anti-CCR3-PE (BioLegend, San Diego, USA) for 15min at 37°C. Subsequently, anti-IgE-APC (BioLegend, San Diego, USA), anti-IgG-APC (BD Biosciences, San Jose, USA), anti-FcεRI-APC (eBioscience, San Diego, USA) or anti-FcγRII-APC (BD Biosciences, San Jose, USA) were added, respectively, and incubated for 20min at 4°C. Erythrocytes were lysed like described above, followed by centrifugation of the samples for 5min at 500xg. The mean fluorescence intensity (MFI) of the receptors or immunoglobulins on CCR3⁺SSC^{low} basophils was measured by flow cytometry.

Analysis of total IgE

Total IgE serum concentrations of CU patients, AR patients and HC were measured by the Phadia ImmunoCAP System (Thermo Fisher Scientific, Uppsala, Sweden) according to the manufacturer's instructions.

Quantification of autoantibodies against thyroid peroxidase

IgG autoantibodies directed against TPO were quantified by ELISA (Euroimmun, Lübeck, Germany). Optical density was quantified by Sunrise ELISA reader (Tecan, Männedorf, Switzerland). Values higher than 30 IU/ml were set as positive.

Statistical Analysis

Because of the non-Gaussian distribution of the data and comparison of multiple independent cohorts, statistical analysis was performed using the Kruskal-Wallis test. When rank sums between the cohorts differed significantly, the post-hoc Dunn's test for pairwise multiple comparisons was applied (with multiplicity adjusted p values). The relationship between peripheral basophil numbers and their activation status was evaluated by the two-tailed Spearman's rank correlation coefficient. Differences were considered as statistically significant at p values <0.05 . Statistical analysis was performed using GraphPad Prism software (GraphPad Software, La Jolla, USA).

RESULTS

Patients with CU can be classified according to basophil reactivity and frequency

Stimulation of peripheral blood basophils with increasing concentrations of anti-Fc ϵ RI revealed two distinct reactivity patterns among the investigated CU patients. As previously described by Vonakis et al. using the histamine release assay (20), patients could be classified into a reactive (CU-R) and a non-reactive (CU-NR) cohort (Fig. 1A). The CU-R group comprised of 67% of patients (40/60) exhibiting a concentration-dependent increase of CD63⁺ basophils to Fc ϵ RI stimulation, similar to HC and AR. Among the CU-NR group, stimulation with anti-Fc ϵ RI failed to activate basophils but patients could be further subdivided according to their peripheral basophil frequency. The majority of non-reactive patients (CU-NR; 12/60, 20%) showed peripheral basophil numbers to the same extent as the CU-R group, whereas the remaining CU patients (8/60, 13%) displayed a basopenic phenotype

with basophils accounting for less than 0.1% of peripheral blood cells (CU-NR_{B0}; Fig. 2B). This finding was accompanied by a pre-existing, significantly enhanced background activation of the basophils ($p < 0.05$; Fig. 1A). Generally, the basophil frequency of CU patients was reduced in comparison to AR patients and to HC by trend (Suppl. Fig. S1A). Interestingly, peripheral basophil frequency and their *ex vivo* activation status, i.e. without any exogenous stimulus, showed a significant inverse correlation ($r_s = -0.6386$, $p < 0.0001$; Suppl. Fig. S1B). Utilization of fMLP as an unspecific control stimulus did not reveal any differences in basophil activation between the CU patient groups (Fig. 1C) but disclosed a lower activation in HC by trend (Suppl. Fig. S1C). Of note, the investigated CU subgroups exhibited no difference in relation to sex, age or disease duration (Table I).

CU-NR_{B0} show reduced surface IgE and IgG and decreased expression of the respective receptors on peripheral basophils

Since activation of basophils is triggered by, amongst others, cross-linking of FcεRI-bound IgE, we investigated whether subgroup analysis of CU patients exhibit differences in cell-bound IgE or FcεRI expression on their basophils. Measurement of the MFI of both surface bound IgE and the FcεRI expression on basophils revealed profound alterations between CU-R and CU-NR_{B0} patients, with the latter showing significant lower levels, respectively (Fig. 2A and 2B; both $p < 0.05$). Furthermore, peripheral cell frequencies of both IgE⁺ and FcεRI⁺ basophils were substantially decreased in CU-NR_{B0} patients (Fig. 2C and 2D; both $p < 0.05$).

We also quantified the percentage of IgG⁺ and FcγRII⁺ basophils due to the facts that pathophysiologically relevant autoantibodies in CU are mostly of the IgG isotype (9) and signaling via FcγRIIB, the most abundant FcγRII on human basophils, is

known to inhibit IgE- and IgG-mediated basophil activation (26, 27). Both the percentage of IgG⁺ (Fig. 2E) and FcγRII⁺ basophils ($p<0.05$; Fig. 2F) were reduced in CU-NR_{B0} individuals, with the former even completely absent. The MFI of IgG and FcγRII basophils did not reveal any differences between the groups (data not shown).

Patients with CU do not differ in FcεRI and FcγRII expression on eosinophils

Enhanced expression levels of FcεRI and FcγRII on eosinophils have been reported for allergic diseases like atopic dermatitis, rhinitis and asthma (28-30). Furthermore, wheals of CU patients show increased numbers of eosinophils (31). Based on these findings, we examined whether CU subgroups can not only be differentiated by analyzing percentages of FcεRI⁺ and FcγRII⁺ basophils but also by variations of eosinophils in the peripheral blood. However, although slightly decreased eosinophil numbers (Fig. 3A) and receptor expression (Fig. 3B and 3C) were observed in the CU-NR_{B0} subgroup, no substantial differences were apparent compared to the other cohorts.

CU-NR_{B0} are characterized by increased serum autoreactivity and autoantibody levels

Many patients with CU exhibit features of autoimmunity like serum autoreactivity, autoantibodies against different antigens or are even affected by certain autoimmune diseases (32). To test the autoreactive capacity of patients' sera and to determine potential differences among the identified CU cohorts, we incubated basophils of one HC donor with the CU sera and analyzed basophil activation by CD63 expression.

Here, CU-NR_{B0} patients showed markedly increased activation of donor basophils (63%) in comparison to CU-R (5%; $p<0.01$) and CU-NR (17%; Fig. 4A).

Furthermore, we analyzed the presence of IgG-autoantibodies against TPO, the major antigen of Hashimoto's thyroiditis (11), which is the most frequent autoimmune disease in CU patients (33). Similar to the results of basophil stimulation by autoreactive CU sera, 63% of CU-NR_{B0} patients exhibited enhanced anti-TPO antibodies in comparison to CU-R (13%) and CU-NR (0%; Fig. 4B and Table I). In summary, when combining basophil activation by autoreactive sera and increased serum anti-TPO antibodies, all but one of the CU-NR_{B0} patients (88%) demonstrated features of autoreactivity.

CU-NR_{B0} patients show the most severe clinical phenotype

Since CU patients can be differentiated by diverse immunologic alterations, we wondered if these variations also reflect clinical characteristics. Regarding age, sex and disease duration no differences were detectable among the three CU subgroups (Table I). However, when assessing the disease severity by utilizing two validated questionnaires, CU-Q₂₀L (1, 23) and UCT (25), CU-NR_{B0} patients were characterized by showing the most severely affected quality of life ($p<0.01$; Fig. 5A) and by the least controlled disease (Fig. 5B), compared to CU-R and CU-NR patients, who displayed similar severity scores in both tests.

DISCUSSION

CU is a chronic skin disease characterized by relapsing appearance of pruritic hives, in some patients associated with angioedema. While its clinical picture is well known, the underlying pathogenic mechanisms are the focus of intense research (34, 35). Since CU patients show both decreased numbers and functional impairments of peripheral basophils, these cells have gained interest exhibiting a potential role in the development of CU (9-12). Like mast cells, which are crucial players in the pathogenesis of CU, they are potent producers of histamine, leukotrienes and other cytokines (36, 37) involved in the elicitation of urticarial wheals (38). Furthermore, basophils also express the high-affinity IgE receptor, which might be activated by anti-FcεRI autoantibodies found in about 40% of CU patients (5, 9). When analyzing the frequency of peripheral basophils and their reactivity to stimulation with anti-FcεRI, in the present study three different subgroups of CU patients were observed: firstly, patients with reduced basophil numbers responding to anti-FcεRI (CU-R) like AR and HC subjects; secondly, patients with diminished basophil counts but non-reactive to FcεRI stimulation (CU-NR); and, thirdly, non-responder characterized by basopenia (CU-NR_{B0}).

The generally decreased basophil frequencies in CU patients (15, 39) have also been shown to inversely correlate with histamine releasability (40) and disease activity (13). In addition, a bimodal response in basophil activation after stimulation with anti-IgE has been described, unveiling CU patients with either responder or non-responder basophils (14, 20). We here report a distinct third subgroup that also exhibited a non-responding phenotype but was further characterized by profound basopenia (CU-NR_{B0}). Furthermore, CU-NR_{B0} patients could be distinguished by specific properties of their basophils namely reduced receptor bound IgE and surface

expression of FcεRI. Of note, this group also differed from the others by prominent features of autoreactivity, i.e. increased serum autoreactivity and enhanced levels of anti-TPO autoantibodies. Finally, CU-NR_{B0} patients built up the clinically most severely affected subgroup. It has been reported that basopenic patients had more emergency department visits caused by urticaria symptoms, however, no differences in the quality of life were detected between CU patients with and without basopenia using the Skindex-29 (41). While this measurement allows evaluating only the general quality of life in dermatological diseases (42), utilizing the urticaria-specific CU-Q₂₀L and UCT, we here could show that CU-NR_{B0} patients have the worst quality of life.

It has been described that FcεRI-mediated activation can be inhibited by signaling via an immunoreceptor tyrosine-based inhibition motif (ITIM) of FcγRIIB (26) which is the predominant IgG receptor on basophils (27). We therefore wondered, whether CU-NR patients showed increased FcγRII expression and IgG bound to FcγRII on basophils, respectively, indicating enhanced inhibition of basophil activation via FcγRII signaling. However, neither expression of FcγRII nor cellular surface binding of IgG was increased on basophils of CU-NR patients, suggesting other mechanisms responsible for the reduced responsiveness of basophils like reduced expression of the tyrosine kinase Syk (18, 43) or overexpression of SHIP-1 and SHIP-2 (20).

Our data support the assumption that basophils may play an important role in the pathophysiology of CU. CU patients could be classified into subgroups showing decisive phenotypic and functional aberrations of their basophils. Furthermore, while all CU patients exhibited reduced basophil numbers, one group differed from the others by features of basopenia, pronounced signs of autoimmune reactivity and the most severely clinically affected phenotype. One explanation could be that in these

patients basophils migrate from the peripheral blood into the cutaneous compartment where they take part of the disease. Of note, increased numbers of basophils have been described in lesional and non-lesional CU compared to healthy control skin (38, 44).

One limitation of this prospective, cross-sectional study is that only a single time point was analyzed displaying a momentary immunologic picture of the CU patients. Thereby, it cannot be excluded that basophils' responsiveness is changing over time in conjunction with the course of the disease. A longitudinal study following the different CU subgroups over time should help shedding more light on the immune mechanisms of CU. Furthermore, we focused on anti-TPO autoantibodies as a single measure of CU-associated autoimmune disease, because Hashimoto's disease is a very common autoimmune disorder in CU patients (5, 45). Nevertheless, associations to other autoimmune diseases like diabetes or rheumatoid arthritis have been postulated (11). In addition, several autoantigens targeted either by IgG or IgE antibodies have been considered in CU patients. The most common is the α -subunit of the Fc ϵ RI (10, 46), others encompass IgE itself, thioredoxin, thyroglobulin and double stranded DNA (47).

In summary, we here describe three types of CU patients based on Fc ϵ RI-mediated reactivity and frequency of their basophils. Among these, a new, distinct group could be identified, showing 1) pre-activated basophils which could not be further activated by Fc ϵ RI stimulation, 2) reduced surface expression of Fc ϵ RI and Fc γ RII, respectively, 3) decreased binding of IgE and IgG, and 4) peripheral blood basopenia. Interestingly, this group was further characterized by features of autoimmunity like increased serum autoreactivity and serum autoantibodies against TPO, pointing to autoimmune mechanisms potentially playing a prominent role in

these CU patients. Thus, basophil frequencies may display a possible biomarker for autoimmune CU. Eventually; this subgroup consisted of the CU patients that were clinically most severely affected. Future studies should help to gain more insights into CU pathomechanisms. Of particular interest would be to analyze the impact of different therapeutic interventions on the clinical responsiveness in the distinct CU subgroups.

Figure legends

Figure 1 (A) Peripheral blood basophils of patients with chronic urticaria (CU; n=60), allergic rhinitis (AR; n=10) and healthy control subjects (HC; n=10) were stimulated with different concentrations of anti-FcεRI. Activation of CCR3⁺SSC^{low} basophils was evaluated by their CD63 expression using flow cytometry. Three distinct reactivity patterns were identified within the CU patients: reactive (CU-R), non-reactive (CU-NR) and non-reactive basophils with increased background activation (CU-NR_{B0}). Statistically significant differences between unstimulated CD63⁺ basophils of the respective subgroups in comparison to CU-NR_{B0} are indicated (# p<0.05). (B) Comparison of basophil numbers in the peripheral blood of CU patients and control individuals. (C) FcεRI-independent basophil stimulation with the unspecific control stimulus, n-formylmethionyl-leucyl-phenylalanine (fMLP).

Figure 2 Mean fluorescence intensity (MFI) of basophil-bound IgE (A) and FcεRI expression on basophils (B). Percentages of basophils carrying IgE (C) or IgG (E) and their respective receptors, FcεRI (D) or FcγRII (F). Peripheral blood basophils of chronic urticaria (CU) patients (CU-R, CU-NR, and CU-NR_{B0}), allergic rhinitis patients (AR) and healthy controls (HC) were analyzed by flow cytometry.

Figure 3 (A) Frequencies of peripheral blood eosinophils determined as CCR3⁺SSC^{high} cells and surface expression of FcεRI (B) and FcγRII (C) on eosinophils of chronic urticaria (CU) patients (CU-R, CU-NR, and CU-NR_{B0}), allergic rhinitis patients (AR) and healthy controls (HC) were quantified by flow cytometry.

Figure 4 (A) Peripheral blood basophils of one healthy donor were stimulated with sera of chronic urticaria (CU) patients (CU-R, CU-NR, and CU-NR_{B0}). Activation of basophils was evaluated by CD63⁺ expression using flow cytometry. (B) Quantification of anti-thyroid peroxidase (TPO) antibody concentrations by ELISA in comparison to allergic rhinitis patients (AR) and healthy controls (HC).

Figure 5 Severity of chronic urticaria (CU) was evaluated by (A) the German version of the CU quality of life score (CU-Q₂₀L) and (B) the urticaria control test (UCT), respectively, in the subgroups of CU patients (CU-R, CU-NR, and CU-NR_{B0}).

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Table I Clinical characteristics in distinct subgroups of patients with chronic urticaria

	CU-R (n=40)	CU-NR (n=12)	CU-NR _{B0} (n=8)
Sex [female/male]	28/12	9/3	6/2
Age [years]	39 (23-64)	45 (23-65)	40 (22-59)
Disease duration [months]	21 (4-420)	18 (2-132)	24 (5-120)
Total IgE [kU/l]	98 (14-585)	110 (0-460)	18 (0-152)
Anti-TPO IgG [n]	5 (13%)	0 (0%)	5 (63%)

TPO, thyroid peroxidase

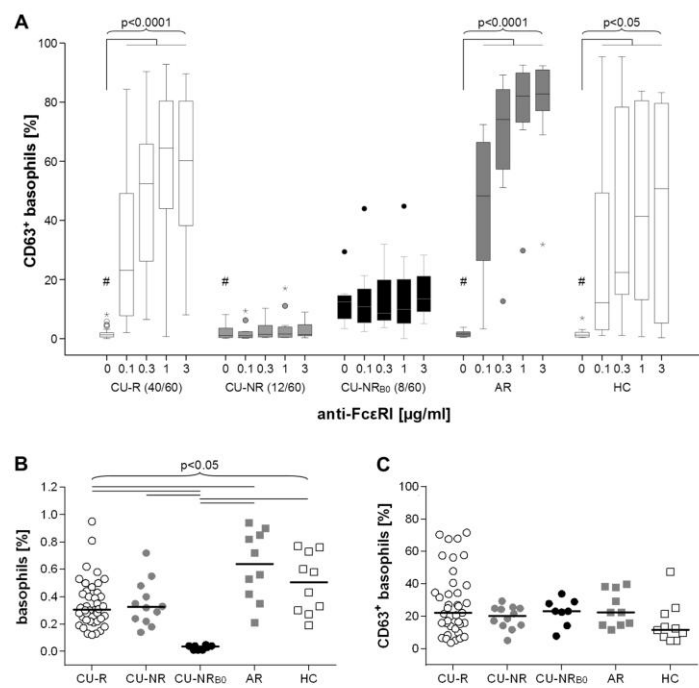


Figure 1

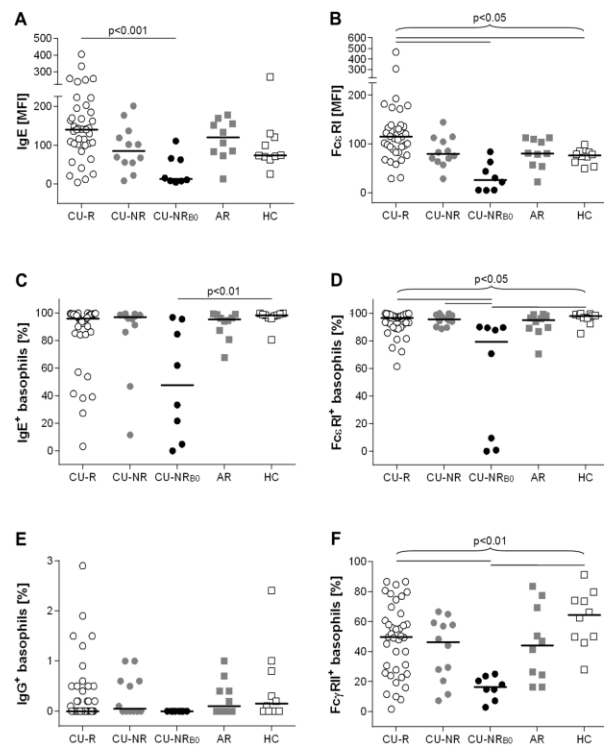


Figure 2

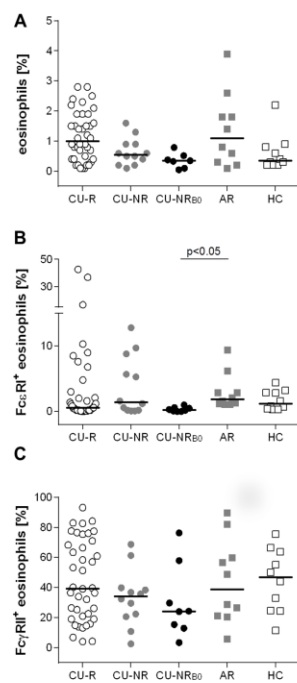


Figure 3

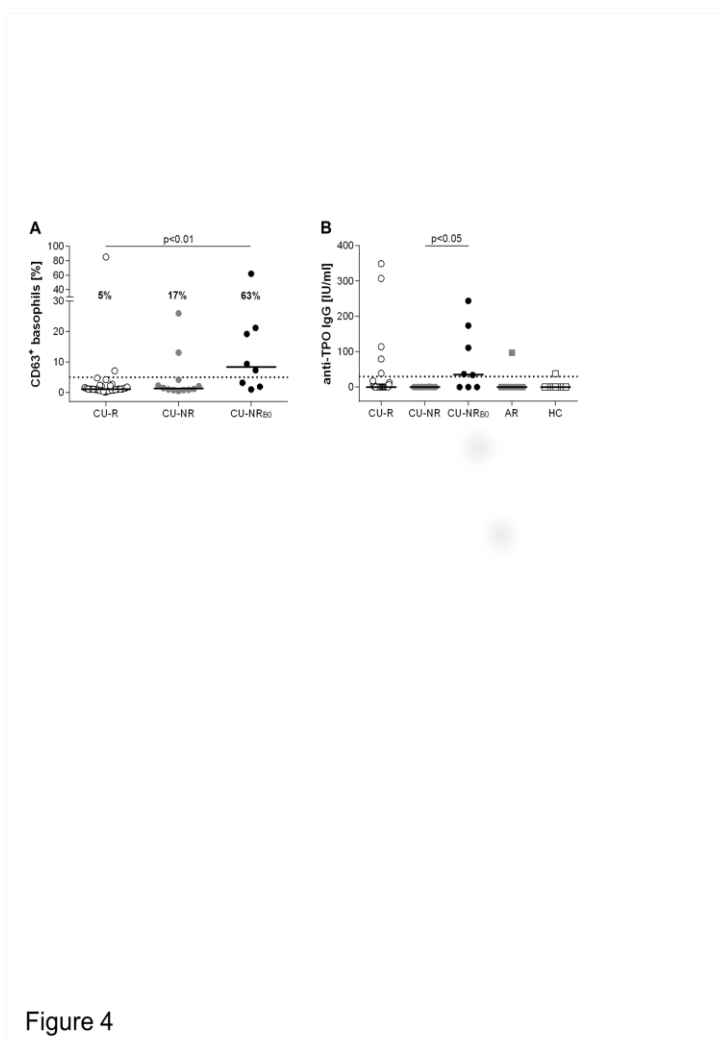


Figure 4

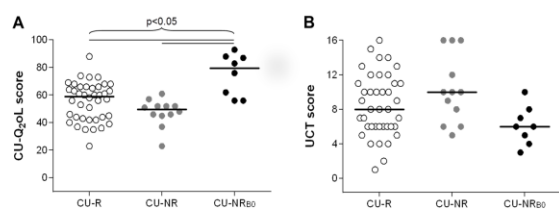
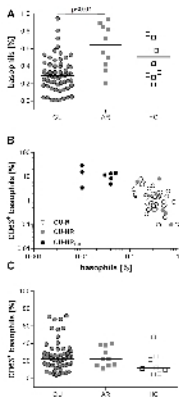


Figure 5



Supplementary Figure 1 (A) The frequencies of peripheral basophils from chronic urticaria (CU) patients were quantified by flow cytometry and compared to the basophil numbers in allergic rhinitis (AR) patients and healthy control (HC) subjects. (B) Within the subgroups of CU patients (CU R, CU NR, CU NR_{BE}) the background activation of basophils was correlated with the basophil frequency ($r = 0.8368$, $p < 0.0001$). Activation of CCR3 SSC⁺ basophils was evaluated by their CD63 expression using flow cytometry. (C) CD63⁺ basophils were determined after stimulation with the unspecific control stimulus, *n*-formylmethionyl-leucyl-phenylalanine (FMLP).