Low Responsiveness of Basophils via FcεRI Reflects Disease Activity in Chronic Spontaneous Urticaria

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Low-responsiveness of basophils via FcεRI reflects disease activity in chronic spontaneous urticaria

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Abstract

Background: The insufficient effect of H1-antihistamine in some chronic spontaneous urticaria (CSU) patients suggests that factors other than histamine are involved in the pathophysiology of CSU. Moreover, a central role for basophils in the pathophysiology of CSU has been hypothesized. However, few studies have focused on the relationship between basophil reactivity via FcεRI and clinical features in CSU patients.

Objective: To assess basophil reactivity via FcεRI against anti-IgE and FcεRI stimulation in CSU patients, and its association with disease activity in CSU. FcεRI expression and IgE binding on basophils from CSU patients was also investigated.

Methods: We analyzed 38 CSU patients, 8 patients with atopic dermatitis (AD), and 11 healthy controls (HCs). The surface CD203c expression with or without anti-IgE or FcεRI stimulation, and IgE and FcεRI (CRA1, CRA2) expression on blood basophils was evaluated. CSU patients were also evaluated and classified by disease activity and the above parameters were compared.

Results: The proportion of $CD203c^{high}$ basophils following anti-IgE or anti-FcεRI stimulation was lower in CSU patients compared with HCs and patients with AD. It was lowest in the CSU group with severe disease. Basophils from CSU patients had higher FcεRI (CRA1) expression, although it was not closely related with the severity of CSU. Subgroup analysis revealed that CSU patients showing low-responsiveness of basophils via FcεRI exhibited a short duration of disease but severe disease activity.

Conclusion: Low reactivity of basophils via FcεRI is characteristic of CSU patients. This attenuated reactivity is associated with severe clinical activity in CSU patients. (250/250)

Highlights

What's already known about this topic?

A basophil functional abnormality has been reported because some patients with chronic spontaneous urticaria have less histamine release to anti-IgE.

What does this article add to our knowledge?

Attenuated basophil responsiveness via FcεRI in patients with chronic spontaneous urticaria is associated with the severity of disease and relatively shorter disease duration.

How does this impact current management guidelines?

Classification of chronic spontaneous urticaria patients according to basophil CD203c reactivity may help to elucidate the mechanisms involved in the dysregulation of basophils and their role in the pathogenesis of urticaria.

Key words: chronic spontaneous urticaria, CD203c, basophil activation test

Abbreviations used

AD: atopic dermatitis

ASST: autologous serum skin test

BAT: basophil activation test

CSU: chronic spontaneous urticaria

FcεRI: high-affinity IgE receptor

HCs: healthy controls

IgE: immunoglobulin E

MFI: mean fluorescence intensity

PBS: phosphate-buffered saline

UAS: urticaria activity score

UCT: urticaria control test

Introduction

Chronic spontaneous urticaria (CSU) is a common skin disease, characterized by spontaneous appearance of wheals, angioedema, or both for > 6 weeks due to known (i.e. the presence of mast cell-activating autoantibodies) or unknown causes.[1, 2] Histamine from activated skin mast cells plays a central role in the pathophysiological aspects of CSU; however, H1-antihistamine treatment leads to absence of symptoms in fewer than 50% of patients with CSU.[3] This suggests that factors other than histamine from mast cells are involved in the pathophysiology of CSU. Thus, other inflammatory cells including basophils are currently being investigated. Histologically, a perivascular infiltrate of mixed inflammatory cells, including basophils, is present in skin lesions of CSU patients.[4, 5, 6] Recently, the expression of activation markers has been assessed on the surface of basophils in CSU patients. CD203c, also known as ectonucleotide pyrophosphatase phosphodiesterase3 (E-NPP3)[7], is a basophils activation marker which is upregulated in the steady state on the basophils of CSU patients.[8] According to Lourenco et al, upregulation of CD203c expression without stimulation of the basophils was present in CSU patients, regardless of autologous serum skin test (ASST) responses.[9] In other cases, the ASST were associated with histamine-releasing anti-FcεRI autoantibodies, suggesting that would correlate with basophil activation markers.[10] Furthermore, Ye et al mentioned that basophil CD203c expression in patients with severe CSU was significantly higher than in non-

92 severe CSU.[11] Additionally, reactivity of basophils from CSU patients has been assessed via
93 various detection methods. A previous study reported that basophils of some CSU patients
94 exhibit little histamine release in response to anti-IgE.[12] Furthermore, CD63 expression of
95 basophils after anti-FcεRI stimulation is less in a subgroup of CSU patients than basophils of
96 healthy controls (HCs).[13] In contrast, another report documented that CSU patients with high
97 histamine release response to anti-IgE experienced significantly higher current itch scores
98 compared with nonresponders.[14] In addition, the activated status of CSU basophils were
99 reported based on enhancement of histamine release induced by anti-IgE stimulus.[9]
100 The expression of high-affinity IgE receptor (FcεRI) and bound IgE on basophils from
101 CSU patients plays an important role in the pathophysiology and therapeutic response to
102 omalizumab in CSU. Omalizumab binds to free IgE, which lowers its level, and causes
103 downregulation of FcεRI receptors on basophils and mast cells.[15] Furthermore, the expression of
104 FcεRI (CRA1) is increased on the basophils of CSU patients compared with HCs, and a positive
105 correlation was found between IgE serum levels and FcεRI receptor expression.[9, 16]
106 Thus, basophils are noteworthy cells in terms of playing a critical role in the
107 pathophysiology of CSU. Indeed, Saini et al has been reported that blood basophil percentage
108 and numbers increased in patients with CSU in response to treatment with omalizumab.[17]
109 However, few studies have focused on the association between basophil reactivity via FcεRI,

levels of FcεRI and IgE on basophils, and clinical features including disease activity in CSU patients. Furthermore, few studies have also compared reactivity of basophils from CSU patients and patients with other inducible types of urticaria.

In this study, we analyzed the expression of the activation marker CD203c with or without anti-IgE and FcεRI stimulation in CSU patients, patients with atopic dermatitis (AD), and HCs to detect basophil reactivity via FcεRI stimulation. Furthermore, we examined the association between basophil reactivity via FcεRI and disease severity of CSU. In addition, the degree of IgE binding as well as FcεRI receptor (CRA1, CRA2) expression on basophils was analyzed and the association between receptor expression and disease activity in CSU was examined. We also discussed the clinical features of subgroups of CSU patients divided according to basophil CD203c upregulation via FcεRI expression.

Materials & Methods

Study population

Patients with CSU and AD who agreed to participate in this study were enrolled at the Dermatological Institute of Kobe University Hospital. CSU was defined as recurrent wheals occurring for more than 6 weeks without an identifiable cause. HCs were enrolled from healthy adult volunteers who are currently without symptoms of urticaria and no history of urticaria. Disease activity in CSU patients was assessed both in clinical care and trials with the urticaria activity score 7 (UAS7). The UAS is the sum of the urticaria and itching scores, and UAS7 (which is the total of 7 days of UAS scores) can evaluate CSU activity over 1 week.[18] The UAS7 is recommended by the European Academy of Allergy and Clinical Immunology (EAACI) guidelines as a method for evaluating disease activity by urticaria patients and their treating physicians.[1] CSU patients were categorized into three groups based on their UAS7 scores, modified according to Hawe et al[19] (UAS7 0–6, mild; 7–15, moderate; 16–42, severe) (Table 2). Patients who were treated with omalizumab were excluded from this study. Their past history was obtained from medical interview. All study participants provided oral consent for this study after verbal and written explanation. The study protocol was approved by the Institutional Review Board (Kobe University; No. 180186).

Basophil activation test

Whole blood (up to 2 mL) was taken from the patients with CSU, mild to moderate AD and HCs using ethylenediaminetetraacetic acid (EDTA) containing blood collection tubes and assays were performed within 24 hours of blood sampling. The Allergenicity Kit (Beckman Coulter, Fullerton, CA, USA) was modified and used for the quantification of basophil CD203c expression.[20] Basophils were stimulated with anti-IgE antibody (clone: E124-2-8D) (10 µg/ml) from the Allergenicity kit as a positive control, VioBlue conjugated anti-IgE antibody (clone: MB10-5C4) (110 µg/ml) (Miltenyi Biotec, Bergisch Gladbach, Germany) or biotinylated anti-FcεRI antibody (clone: CRA1 or CRA2) (1.6 mg/ml, 0.9 mg/ml, respectively) (BioAcademia, Osaka, Japan). The CRA1 antibody binds FcεRI even in the presence of IgE, whereas the CRA2 antibody reacts with the FcεRIα subunit at a region that overlaps with the IgE binding site, thus it competes with IgE for receptor binding.[21] Basophils incubated with phosphate-buffered saline (PBS) were used as a negative control.

Briefly, 50 µL of fresh EDTA-blood was incubated with 10 µL of staining reagent consisting of CRTH2-FITC, CD203c-PE, and CD3-PC7 in activation buffer and mixed with the respective stimulant or PBS at 37°C for 15 minutes. The biotinylated antibodies against CRA1 or CRA2, were then coupled with APC streptavidin (BD, *Franklin Lakes,* NJ, USA) at 4°C for 30 minutes. Afterwards blood samples were depleted of erythrocytes by incubation in fixative and lysis buffer for more than 10 minutes and centrifuged at 200×*g* for 5 minutes and

157 supernatant aspirated. Next, leukocytes were washed in 1500 µL of PBS. Finally, cells were
158 resuspended in 0.3 mL PBS 0.1% formaldehyde and evaluated by flow cytometry (FACS verse,
159 BD Biosciences, San Jose, CA, USA).

160 Flow cytometry results were analyzed by FlowJo software (FlowJo, LLC, Ashland,
161 OR, USA). Basophils were identified by their characteristic forward and side scatter, expression
162 of basophil activation markers, and the absence of CD3 (Fig. E1). The double-gated cells were
163 analyzed on the CRTH/CD203c plot as basophils.

164 Results of antibody stimulation are expressed as proportion CD203c^{high} basophils. The
165 proportion CD203c^{high} basophils was determined using a threshold defined as the expression
166 level above which only 5% of basophils in the negative control sample fluoresce, on average.[7]
167 Moreover, patients with CSU were defined as non-responders when less than 10% of basophils
168 were CD203c^{high} basophils after anti-IgE antibody (clone: E124-2-8D) stimulation, and as
169 responders when more than 10% were.

170 **Measurement of IgE and FcεRI levels on basophils**

171 Basophils were incubated with a VioBlue conjugated anti-IgE antibody (clone: MB10-5C4)
172 (Miltenyi Biotec) and biotinylated anti-FcεRI antibody (clone: CRA1 or CRA2) (BioAcademia)
173 and analyzed by flow cytometry. The method of observing IgE and FcεRI levels on basophils

and FlowJo analysis was the same as for the basophil activation test after anti-IgE, CRA1 or CRA2 antibody stimulation. IgE and FcεRI levels were evaluated as the MFI.

Autologous serum skin test (ASST)

The ASST was performed according to established methods.[22] The diameters of wheals and erythema were measured after 15 minutes. Reactions were assessed as positive if the diameter of the wheal induced by serum was ≥ 6 mm.

Statistical analysis

The non-parametric Mann–Whitney *U*-test and unpaired *t* test were used to assess differences between CSU patients and HCs or patients with AD, or responders and non-responders. Kruskal-Wallis result with Dunn test was used for comparing three or four groups of nonparametric variables. To assess correlations between two factors, adjusted r_s (Spearman's rank correlation coefficient) values were calculated. All statistical analyses were carried out using GraphPad Prism 7 (GraphPad Software, San Diego, CA, USA). Two-sided *p*-values of < 0.05 were considered statistically significant.

Results

Study population

The characteristics of patients with CSU are described in Table 1. Thirty-eight CSU patients (14 male and 24 female patients) were enrolled in the study. The mean age was 46.3 ± 16.2 years old and median duration of disease was 4.0 years. Median serum total IgE was 139.5 IU/ml. The ASST was performed in 17 patients, with the result being positive in 41.1% (7 of 17). Only one patient had concomitant angioedema.

In this study, the CSU patients were categorized into three subgroups based on the severity of disease according to the UAS7. The characteristics of these subgroups are described in Table 2. Importantly, they exhibited no significant differences regarding sex, age, serum total IgE, and disease duration. Additionally, it was observed that the median value of total serum IgE levels in the severe group was lowest. However, this was only a tendency, with no statistically significant value.

Measurement of CD203c expression after FcεRI stimulation on basophils in CSU patients

First, we analyzed the expression of the activation marker CD203c with or without anti-IgE and/or FcεRI stimulation in CSU patients, patients with AD, and HCs to examine basophil reactivity via FcεRI. Unlike previous reports[8], the expression of CD203c on peripheral blood basophils in CSU patients in the steady state without anti-IgE and/or FcεRI stimulation was

equivalent to HCs (Fig. E2). When peripheral blood basophils were stimulated with anti-IgE (E124-2-8D, MB-105C4), expression of CD203c was upregulated in basophils of almost all HCs (labeled CD203c^{high} basophils). Notably, the percentage of CD203c^{high} basophils was significantly lower in CSU patients than in HCs (Fig. 1A and B). In addition, basophils from CSU patients showed attenuated upregulation of CD203c after anti-FcεRI (CRA1) stimulation compared with basophils from HCs (Fig. 1C). In contrast, anti-FcεRI (CRA2) stimulation scarcely upregulated CD203c in either CSU patients or HCs (Fig. 1D). CRA1 antibody binds FcεRI even in the presence of IgE, whereas the CRA2 antibody reacts with the FcεRIα subunit at a region that overlaps with the IgE binding site. Basophils in almost all CSU patients who showed upregulation of CD203c after anti-IgE (E124-2-8D) were also activated by both another anti-IgE clone (MB10-5C4) and anti-FcεRI (CRA1) stimulation (Table E1). Basophils in almost all CSU patients who did not show upregulation of CD203c after anti-IgE (E124-2-8D) were not activated by these two other antibodies (Table E1). Upregulation of CD203c against two kinds of anti-IgE (E124-2-8D, MB10-5C4) and anti- FcεRI (CRA1) significantly correlated respectively (data not shown). CD203c low-responsiveness on basophils in many CSU patients, but not HCs, could be observed in parallel following stimulation with these three antibodies, suggesting that FcεRI-mediated basophil reactivity in CSU patients is dysregulated.

Interestingly, the proportion of CD203c^{high} basophils after anti-IgE (E124-2-8D, MB10-5C4)

stimulation was also significantly lower in CSU patients than in patients with AD (Fig. 1A, B). When stimulated with anti-FcεRI (CRA1), although only the tendency that the proportion of CD203c^{high} basophils was lower in CSU patients than HCs was observed, it was significantly lower in CSU patients than in patients with AD (Fig. 1C). Thus, CD203c expression on basophils following stimulation via FcεRI was attenuated in CSU patients compared with both HCs and patients with AD.

Next, we studied the relationship between this attenuated CD203c expression and disease activity in CSU. Patients with CSU were categorized into three groups; mild, moderate, and severe, based on disease severity according to their UAS7 scores (Table 2).[20] Notably, the proportion of CD203c^{high} basophils was significantly lower in severe group than in HCs when stimulated with anti-IgE (E124-2-8D, MB-105C4), whereas there was no significant difference between in moderate/mild group and HCs (Fig. 2A, B). In contrast, only a trend was observed when stimulated with anti-FcεRI (CRA1) (Fig. 2C). UAS7 score and proportion of CD203c^{high} basophils after anti-IgE antibody (E124-2-8D) stimulation showed a weak negative correlation (Fig. E3). Collectively, attenuated CD203c expression on basophils following stimulation via FcεRI was a characteristic phenomenon in severe CSU patients when compared with HCs and patients with AD.

In order to further define the attenuated CD203c expression on basophils by phenotype, we performed analysis segregated based on the different treatment groups and according to history of symptoms. Although we classified CSU patients into two groups according to whether they took conventional or high doses of H1 antihistamines, there was no difference between these two groups (data not shown). Moreover, we classified CSU patients into two groups according to whether they had allergic past history (asthma, allergic rhinitis, AD and, pollinosis) and analyzed basophil reactivity respectively. However, there was no difference between these two groups (data not shown).

In addition, although we examined the correlations between serum tryptase levels and CD203c reactivity with or without stimulation, all parameters were not correlated (Fig. E4).

Measurement of IgE and FcεRI levels on basophils of CSU patients

The expression of FcεRI on basophils in the steady state has been reported to be higher in CSU patients than in HCs.[9, 16] Since activation of basophils, including upregulation of CD203c, is triggered by cross-linking of IgE and FcεRI on basophils, we next examined FcεRI and IgE levels on basophils in CSU patients and HCs. The FcεRI (CRA1) expression on basophils was significantly higher in patients with CSU compared with HCs (Fig. 3B), whereas there were no significant differences in the levels of cell-bound IgE and FcεRI (CRA2) (Fig. 3A, C). These

data revealed that total FcεRI expression, but not the level of IgE binding on basophils, was increased in CSU patients.

Furthermore, we studied the relationship between the levels of basophil-bound IgE, FcεRI expression, and the severity of CSU. CRA1 expression on basophils was statistically slightly higher in the moderate group, but not mild or severe groups, compared with HCs (Fig. 4B). There were no significant differences in IgE and CRA2 levels on basophils between these four groups (Fig. 4A, C). There was also no difference in serum total IgE levels among the groups based on the severity of CSU (Table 2). These data indicate that serum total IgE levels, basophil-bound IgE, and FcεRI expression in CSU patients are not closely linked with the severity of CSU.

Previous studies reported that basophil surface IgE density and FcεRI expression are correlated with serum IgE levels in nonallergic subjects, allergic asthma, atopic dermatitis, hypereosinophilic syndromes, hyper-IgE syndrome, helminth infestation, and IgE myeloma.[23] We investigated the relationship between serum IgE levels and IgE, CRA1 or CRA2 levels on basophils in CSU patients. Similar to other disorders, serum IgE levels were strongly correlated with IgE or CRA1 expression on basophils (Fig. E5A, B), but uncorrelated with CRA2 expression (Fig. E5C).

In addition, we examined the correlations between serum tryptase levels and IgE or FcεRI expression on basophils. Although serum tryptase levels were negatively correlated with IgE expression on basophils (r_s = -0.57, P = 0.01) (Fig. E6, A), FcεRI expression on basophils were not correlated (Fig. E6, B, C).

IgE and FcεRI levels on basophils in CSU patients were not strongly correlated with CD203c expression

Since activation of basophils is induced by cross-linking of FcεRI-bound IgE and FcεRI on basophils, the IgE and FcεRI level on basophils can affect CD203c expression via FcεRI response to anti-IgE and anti-FcεRI. To study these relationships, we examined the correlations between the IgE or CRA1 levels and CD203c expression after anti-IgE and anti-FcεRI stimulation in CSU patients. The IgE and CRA1 expression on basophils in CSU patients had a weak positive correlation with CD203c expression after anti-IgE and anti-FcεRI (Fig. E7, 8). More interestingly, focusing on Fig. 1C and 3B, although FcεRI expression on basophils was significantly higher in CSU patients than HCs, the response to anti-FcεRI stimulation was significantly attenuated in CSU patients compared with HCs. These data indicate that the intensity of IgE and/or FcεRI expression on basophils in CSU patients are not directly linked to CD203c responsiveness of basophils via FcεRI in CSU patients.

Classification of CSU patients according to basophil CD203c reactivity

Finally, we focused on the attenuated CD203c expression on basophils which is characteristic of some CSU patients and conducted subgroup analysis. Patients with CSU could be classified into two groups based on whether basophil CD203c expression was close to that of activated HCs (proportion CD203c^{high} basophils after anti-IgE stimulation as noted in previous report [13]: non-responders [<10 % CD203c^{high} basophil] and responders [>10 % CD203c^{high} basophil]). Interestingly, durations of disease were significantly shorter in non-responders to anti-IgE than in responders (Fig. 5A). Proportion CD203c^{high} basophils after anti-IgE stimulation and disease duration showed a positive correlation (Fig. 5D). No significant differences regarding age, sex ratio, ASST positive rate, serum total IgE levels were found between the two groups (data not shown). The non-responders had significantly higher UAS7 scores and lower UCT scores than responders (Fig. 5B, C). Furthermore, the non-responders to anti-IgE exhibited significantly lower reactivity against the CRA1 antibody (Fig. 6A) and significantly lower FcεRI expression on basophils than the responders (Fig. 6B). The subgroup characteristics of responders and non-responders to anti-IgE are summarized in Table E2.

Discussion

This study focused on the fluctuation of CD203c expression as an activation marker on basophils in CSU pathogenesis. Previous studies have noted the unique features of peripheral blood basophils in CSU patients. Some reports documented that basophils in CSU patients have reduced histamine release in response to anti-IgE or CD63 induction in response to anti-FcεRI.[12, 13] In contrast, other studies found that basophils of CSU patients exhibit high response to anti-IgE based on histamine release.[9, 14] However, there is little research on basophils in CSU patients simultaneously comparing various stimuli via FcεRI. Based on this background, our study adopted examination methods using a variety of FcεRI stimuli (anti-IgE (E124-2-8D, MB-105C4), anti-FcεRI (CRA1, CRA2)). By using this technique, it was shown that CD203c expression on basophils in CSU patients was significantly attenuated compared with HCs after stimulation with the three antibodies, i.e. except for CRA2, which appears to have the weakest stimulating ability on basophils. In addition, a low response to these three antibodies was observed in basophils from many CSU patients (Table E1). These data emphasized that low-responsiveness of basophils via FcεRI is a distinctive phenomenon in CSU patients. Moreover, we attempted to compare the basophil responsiveness between CSU patients and patients with AD. Notably, basophil low-responsiveness was not observed in patients with mild to moderate AD, indicating that this phenomenon may be specific to CSU patients.

FcεRI on mast cells is upregulated by IgE binding on mast cells[24], and binding of monomeric IgE to FcεRI, without cross-linking, potentiates activity of mast cells in the absence of degranulation.[25] It has been reported that serum total IgE is elevated in CSU patients.[26] These previous data lead us to focus on the levels of IgE and FcεRI on basophils of CSU patients. Consistent with previous studies,[9, 16] the FcεRI (CRA1) expression on basophils was higher in CSU patients than HCs. The presence of IgE and interaction between IgE and FcεRI can also upregulate FcεRI expression on basophils.[27] Similarly, our study demonstrated that elevated serum total IgE levels were correlated with FcεRI (CRA1) expression on basophils in CSU patients, suggesting that the serum monomeric IgE in CSU patients induces upregulation of FcεRI expression on basophils (Fig. E5B).[27] However, IgE or FcεRI expression on basophils was not strongly correlated with CD203c reactivity of basophils in CSU patients following stimulation via FcεRI (Fig. E7, E8). These data suggest the possibility that monomeric IgE has an effect including upregulation of FcεRI etc. on basophils in CSU, but that basophils in CSU are maintained without CD203c upregulation and/or activation e.g. histamine release regardless of elevated FcεRI expression and are "primed" for augmented function to other activators. However, further research is needed to prove this hypothesis. Since previous studies show that pathways for degranulation other than via FcεRI appear not to be affected in basophils of CSU patients (N-formyl-met-leu-phe [FMLP], monocyte chemoattractant protein 1), this abnormal

responsiveness of basophils in CSU patients may be due to elevated expression of intracellular phosphatases regulating FcεRI signaling pathway.[28, 29] Previous study reports elevated SHIP-1 and SHIP-2 protein, negative regulatory phosphatases, and a reduction in FcεRI-mediated phosphoAkt generation in some CSU basophils.[30]

EAACI/GA²LEN/EDF/WAO Guidelines recommend disease activity in CSU should be assessed and monitored both in clinical care and trials with UAS7.[1] CSU patients can be categorized into three groups (mild, moderate, and severe disease) based on their UAS7 scores. Subsequently, we assessed the relationship between disease severity and various parameters including CD203c responsiveness via FcεRI and the expression of IgE and FcεRI on basophils in CSU patients. Notably, low-responsiveness of CD203c on basophils following various stimuli via FcεRI was marked in patients with severe CSU (Fig. 2). In contrast, the expression of IgE and FcεRI on basophils in CSU patients was not closely linked to severity of CSU in patients. These observations revealed that low-responsiveness of basophils via FcεRI, but not surface markers on basophils, is associated with disease severity in CSU patients.

CSU can be characterized as a heterogeneous disease based on activity, evolution, pathophysiological aspects, and response to treatment. In this study, we attempted to classify CSU patients into subgroups based on low-responsiveness of basophils. CSU patients were classified into two groups based on responsiveness of basophil CD203c express to anti-IgE

363 stimulation (responders and non-responders). Interestingly, clinical features of non-responders
364 exhibited shorter duration of disease but severe disease activity, unlike a previous study.[13] It is
365 reported mean disease duration of 117 CSU patients not controlled by a standard dose of
366 antihistamine is 27.4 months.[31] In our study, median disease duration of non-responders and
367 responders to anti-IgE were 1.2 years (0.2-8) and 6 years (1-33) respectively. Therefore, we
368 thought a non-response to anti-IgE is associated with shorted disease duration. In addition, non-
369 responders to anti-IgE also showed non-responsiveness to anti-FcεRI stimulation. Collectively,
370 this subgroup analysis indicates that the subgroup of CSU patients with severe disease features
371 low-responsiveness of basophils via FcεRI and is relatively rapidly developing compared with
372 non-severe groups. Moreover, this basophil abnormality seems to be correlated with disease
373 severity and disease duration of CSU patients.

374 There are several limitations in this study, such as the pathophysiological mechanism
375 regarding low-responsiveness of basophil CD203c to FcεRI stimulation not being investigated
376 in detail. Furthermore, although CSU patients treated with omalizumab were excluded, other
377 treatment of CSU patients was not uniform.

378 In conclusion, basophil low-responsiveness via FcεRI is a characteristic phenomenon
379 in CSU patients and is associated with disease severity. In addition, the subgroup showing
380 attenuated basophil reactivity via FcεRI reflects unique clinical features of CSU patients. It will

be necessary in the future to elucidate the mechanisms involved in the dysregulation of basophils in the cascade of CSU pathogenesis and link them to CSU therapy.

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Fig. 1 Comparison of the proportion of CD203c^{high} basophils between CSU patients, HCs and patients with AD (A), CSU patients and HCs (C-D).
A. Anti-IgE (E124-2-8D) stimulation
B. Anti-IgE (MB10-5C4) stimulation
C. Anti-FcεR1 (CRA1) stimulation
D. Anti-FcεR1 (CRA2) stimulation
Statistical analyses were performed using Kruskal-Wallis result with Dunn test (A-D).
CSU, chronic spontaneous urticaria; HCs, healthy controls; AD, atopic dermatitis

Fig. 2 Comparison of the proportion of CD203c^{high} basophils between CSU patients classified into three groups by UAS7 score and HCs.
A. Anti-IgE (E124-2-8D) stimulation
B. Anti-IgE (MB10-5C4) stimulation
C. Anti-FcεR1 (CRA1) stimulation
Statistical analyses were performed using Kruskal-Wallis result with Dunn test.
CSU, chronic spontaneous urticaria; UAS, urticaria activity score; HCs, healthy controls

Fig. 3 Comparison of basophil IgE levels or FcεRI receptor expression as MFI between CSU patients and HCs
A. IgE expression
B. total FcεRI expression (CRA1)
C. FcεRI unbound IgE expression (CRA2)
Statistical analyses were performed using Unpaired *t* test (A) or Mann-Whitney *U* test (B, C).
MFI, mean fluorescence intensity. CSU, chronic spontaneous urticaria; HCs, healthy controls.

Fig. 4 Comparison of basophil IgE levels or FcεRI receptor expression as MFI between CSU patients classified into three groups by UAS and HCs
A. IgE expression
B. total FcεRI expression (CRA1)
C. FcεRI unbound IgE expression (CRA2)
Statistical analyses were performed using Kruskal-Wallis result with Dunn test.
MFI, mean fluorescence intensity; CSU, chronic spontaneous urticaria; UAS, urticaria activity score; HCs, healthy controls

Fig. 5 Comparison of disease duration (A), UAS7 (B), and UCT (C) between responders and non-responders. Correlation of disease duration and proportion of CD203c^{high} basophils in CSU patients (D).
Statistical analyses were performed using Mann-Whitney *U* test (A, B), unpaired *t* test (C) or Spearman's rank correlation coefficient (D).
UAS, urticaria activity score; UCT, urticaria control test; CSU, chronic spontaneous urticaria

Fig. 6 Comparison of parameters between responders and non-responders.
A. proportion of CD203c^{high}basophils after anti-FcεR1 (CRA1) stimulation
B. basophil CRA1 receptor expression as MFI
Statistical analyses were performed using Mann-Whitney *U* test.
MFI, mean fluorescence intensity.

Fig. E1 Flow cytometry data analysis

On the FSC/SSC plot (left), the basophil scatter gate (BasoScatter) and leukocyte gate are defined. On the PE-Cy7/SSC plot (middle), the CD3 negative population is defined. On the FITC/CD203c plot (right), both CRTH and CD203c positive groups are defined as basophils.

FITC, fluorescein isothiocyanate; PE, phycoerythrin; PECy5, PE-cyanine 5; FSC, forward scatter; SSC, side scatter.

Fig. E2 Comparison of basophil CD203c expression in the steady state without anti-IgE and/or FcεRI stimulation as MFI between CSU patients and HCs. Statistical analysis was performed using Mann-Whitney *U* test.

MFI, mean fluorescence intensity; CSU, chronic spontaneous urticaria; HCs, healthy controls.

Fig. E3 Correlation of UAS7 score and proportion of $CD203c^{high}$ basophils in CSU patients.

Statistical analyses were performed using Spearman's rank correlation coefficient.

UAS, urticaria activity score; CSU, chronic spontaneous urticaria.

Fig. E4 Correlation of serum tryptase levels (μg/L) and basophil reactivity with or without stimulation in CSU patients.

A. Without stimulation

B. Anti-IgE (E124-2-8D) stimulation

C. Anti-IgE (MB10-5C4) stimulation

D. Anti-FcεR1 (CRA1) stimulation

E. Anti-FcεR1 (CRA2) stimulation

Statistical analyses were performed using Spearman's rank correlation coefficient (B, D) or Pearson's rank correlation coefficient (A, C, E).

CSU, chronic spontaneous urticaria

Fig. E5 Correlation of serum total IgE (IU/ml) and basophil IgE or CRA expression as MFI in CSU patients.

A. Basophil IgE expression

B. Basophil CRA1 receptor expression

C. Basophil CRA2 receptor expression

Statistical analyses were performed using Spearman's rank correlation coefficient.

MFI, mean fluorescence intensity; CSU, chronic spontaneous urticaria.

Fig. E6 Correlation of serum tryptase levels (μg/L) and basophil IgE or CRA expression as MFI in CSU patients.
A. Basophil IgE expression
B. Basophil CRA1 receptor expression
C. Basophil CRA2 receptor expression
Statistical analyses were performed using Spearman's rank correlation coefficient (A) or Pearson's rank correlation coefficient (B, C).
CSU, chronic spontaneous urticaria; MFI, mean fluorescence intensity

Fig. E7 Correlation of basophil bound-IgE levels as MFI and proportion of CD203c^{high} basophils in CSU patients
A. Anti-IgE (E124-2-8D) stimulation
B. Anti-IgE (MB10-5C4) stimulation
C. Anti-FcεR1 antibody (CRA1) stimulation
Statistical analyses were performed using Spearman's rank correlation coefficient.
MFI; mean fluorescence intensity, CSU; chronic spontaneous urticaria

Fig. E8 Correlation of basophil CRA1 receptor expression as MFI and proportion of CD203c^{high} basophils in CSU patients
A. Anti-IgE (E124-2-8D) stimulation
B. Anti-IgE (MB10-5C4) stimulation
C. Anti-FcεR1 antibody (CRA1) stimulation
Statistical analyses were performed using Spearman's rank correlation coefficient.
MFI; mean fluorescence intensity, CSU; chronic spontaneous urticaria

590 Table 1 Clinical and laboratory characteristics of chronic spontaneous urticaria (CSU) patients

	CSU (n = 38)
Age, y	46.3 ± 16.2
Female, n (%)	24 (63.1%)
Disease duration, y	4.0 (0.2–33)
Total IgE (IU/ml)	139.5 (3–4392)
UAS7	17.08 ± 12.09
UCT	7.784 ± 4.097
ASST positive rate (n = 17), n (%)	7 (41.1%)
Presence of angioedema at baseline, n (%)	1 (2.6%)
Treatment, n (%)	
H1 antihistamines at the conventional dosage	19 (50%)
H1 antihistamines at high dosage	14 (36.8%)
Leukotriene receptor antagonists	9 (23.6%)
Systemic corticosteroids	8 (21%)
Cyclosporine	1 (2.6%)
History, n (%)	
Asthma	5 (13.1%)
Allergic rhinitis	2 (5.2%)
Atopic dermatitis	1 (2.6%)
Pollinosis	2 (5.2%)

591 Data are given as mean ± SD for age, UAS7, UCT; n (%) for sex, ASST positive rate, presence
592 of angioedema; and median (range) for disease duration, and serum total IgE. CSU, chronic
593 spontaneous urticaria; UAS7, 7-day urticaria activity score; UCT, urticaria control test; ASST,
594 autologous serum skin test.
595

Table 2 Clinical and laboratory characteristics of chronic spontaneous urticaria (CSU) patients classified into three groups based on disease activity

	Mild (UAS7 score 0–6) (n = 9)	Moderate (UAS7 score 7–15) (n = 10)	Severe (UAS7 score 16–42) (n = 19)
Age, y	49.4 ± 17.6	44 ± 18.3	46.1 ± 14.9
Female, n (%)	5 (55.5%)	7 (70%)	12 (63.1%)
Disease duration, y	8 (0.5–10)	4 (1–10)	3 (0.2–33)
Total IgE (IU/ml)	326.6 (67.2–504)	185 (73–347.5)	126 (3–4392)
UAS7	0 (0–5)	12.5 (8.5–14.5)	27.5 (16–38.5)
UCT score	13 (5–16)	8 (4–10)	5 (3–10)
ASST positive rate	1/1 (100%)	1/4 (25%)	6/12 (50%)

Data are given as mean ± SD for age; n (%) for sex, ASST positive rate; median (range) for disease duration, serum total IgE, UAS7, and UCT. UAS7, 7-day urticaria activity score; UCT, urticaria control test; ASST, autologous serum skin test.

602 Table E1 Response characteristics of chronic spontaneous urticaria patents

				Stimulation (proportion of CD203c^{high} basophil)					Expression		
No	Age	Sex	Disease duration (year)	PBS (MFI)	E124-2-8D (%)	MB10-5C4 (%)	CRA1 (%)	CRA2 (%)	IgE (MFI)	CRA1 (MFI)	CRA2 (MFI)
1	37	M	1.2	1375	9.28	5.68	7.14	0.87	8073	1398	241
2	73	M	25	754	42	47.4	28.4	1.45	2744	1886	54.1
3	23	F	8	1856	94.1	85.9	86.2	7.83	12900	9923	109
4	49	M	5	1635	4.35	11.6	7.45	7.54	296	1202	52.1
5	20	F	4	737	2.33	2.14	3.37	2.06	6957	4202	26.1
6	73	M	7	1468	80.1	84.9	62.7	14.9	5887	7279	93.2
7	57	F	33	1604	52.8	72.2	32.8	5.63	8634	3308	30.2
8	17	M	1	812	44.6	54.3	49.1	0.647	10600	2921	25.7
9	23	F	3	1187	34	45	39.3	7.8	11000	1532	17.2
10	51	M	1	1085	8.39	10.3	13.4	7.18	10900	3746	22.1
11	32	F	1	1051	14.4	19.2	15	9.16	7846	826	29.5
12	49	F	4	998	29.7	51.4	36.9	4.99	2379	1665	15.1
13	63	F	4	1873	4.55	12.1	6.61	7.33	164	45.6	24.4
14	35	M	2	2215	87.1	88.1	70.4	6.97	14000	1199	28.2
15	61	F	3	3060	6.38	9.21	7.82	6.15	7248	1324	52.2
16	40	F	1	4980	10	14	1.82	5.33	10200	2467	16.7
17	36	F	8	1910	35	88	85.6	6.35	15700	4781	23.7
18	41	F	0.2	1298	1.53	1.97	1.51	2.08	4120	1620	22.3
19	42	M	3	1582	8.37	27.6	22.3	0.719	5912	1123	59.1
20	68	F	0.5	1230	7.91	6.16	2.05	3.9	12900	724	27
21	58	F	28	1304	70	72.4	53.5	1.06	13700	3507	37.8
22	43	M	5	3353	70.6	64	36.7	9.51	13100	4043	33.1
23	41	F	2	1543	95.1	95.2	90	64	2210	809	67.2
24	61	F	10	2272	65	73.3	57.4	30.5	1523	94.8	30.7
25	47	M	15	2613	61	63.2	22.1	5.13	6796	241	31.8
26	45	F	3	922	79.3	82.4	53.3	3.05	16100	6847	49.2
27	37	F	6	693	15.9	18.1	8.08	2.1	11700	1330	23.8
28	18	M	0.5	1268	1.75	0.901	2.4	2.1	7642	473	17.6
29	41	M	1	2482	20.8	24.6	18.7	8.33	12200	3630	22.2
30	40	F	7	1143	31.1	47.4	34.7	4.8	9808	2551	43.1
31	42	F	3	925	52.7	71.6	30.5	0.851	9797	3109	38.4
32	74	F	10	4009	52.9	56.1	43.1	15.8	10100	5617	20.9
33	46	F	8	2173	63	64.8	28.8	6.75	9957	3134	24.1
34	45	F	10	1651	21.4	32.1	26.2	7.76	7405	1265	20.4
35	75	M	8	1046	8.03	7.13	3.85	1.46	4363	177	24.1
36	45	F	1	1315	5.13	3.37	1.43	0	5355	354	28.7
37	38	F	2.5	927	89.1	88.7	72	0.939	4226	1241	19.7
38	75	M	4	2440	76.3	82.2	65.3	6.55	4845	794	19.4

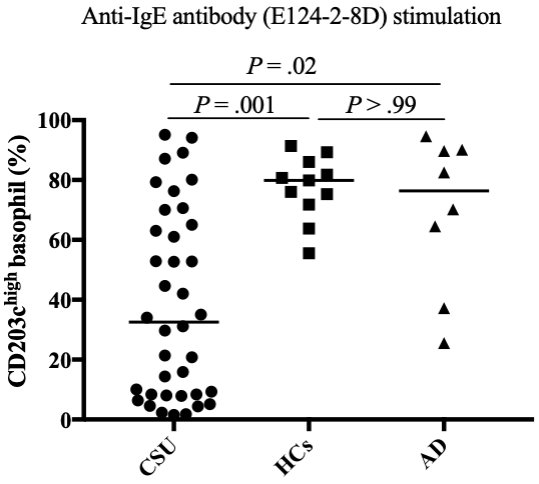
603 CD203c expression without stimulation (PBS) is described as MFI. CD203c expression under
604 each antibody stimulation is described as CD203c^{high} basophils (%). IgE and CRA expression
605 are described as MFI. PBS, phosphate-buffered saline; MFI, mean fluorescence intensity.

606 Table E2 Chronic spontaneous urticaria patients were divided into two subtypes based on the
607 proportion of CD203c^{high} basophils after anti-IgE antibody stimulation

	responders	non-responders*
Duration of disease	long	relatively short
CD203c^{high} basophil (%) with anti-FcεR1 antibody (CRA1) stimulation	high	low
Total FcεRI expression on basophils	high	low
UAS7	low	high
UCT	high	low

608 * non-responders, less than 10%; responders; more than10%.
609 UAS7, 7-day urticaria activity score; UCT, urticaria control test.

Figure No. - Unmarked



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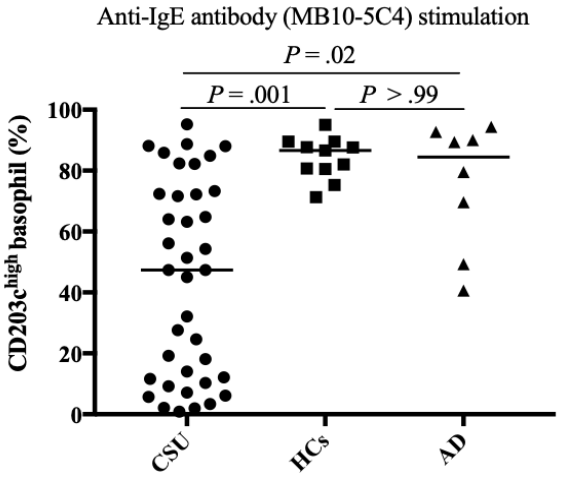

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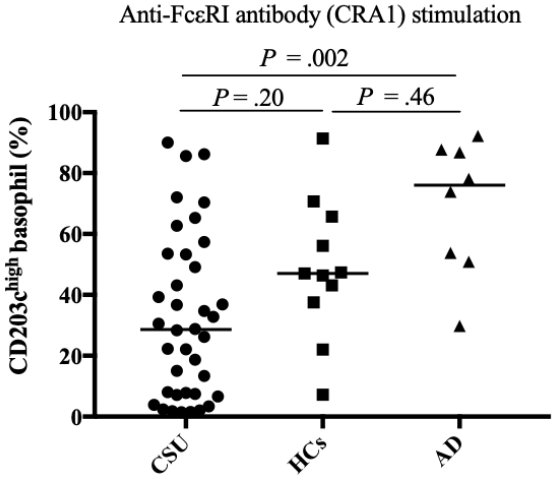

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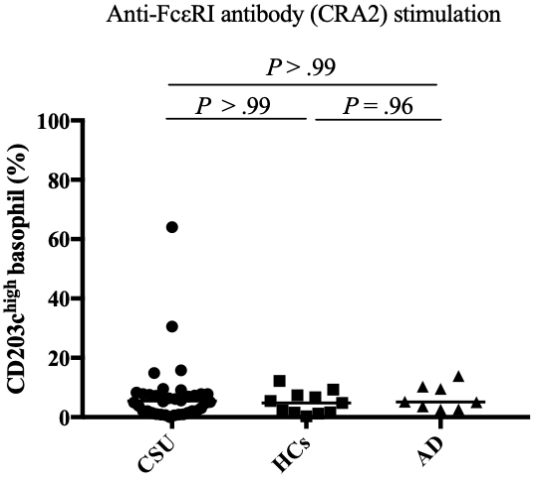

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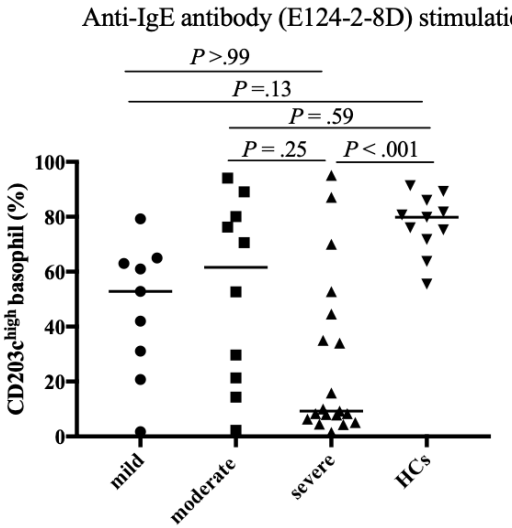

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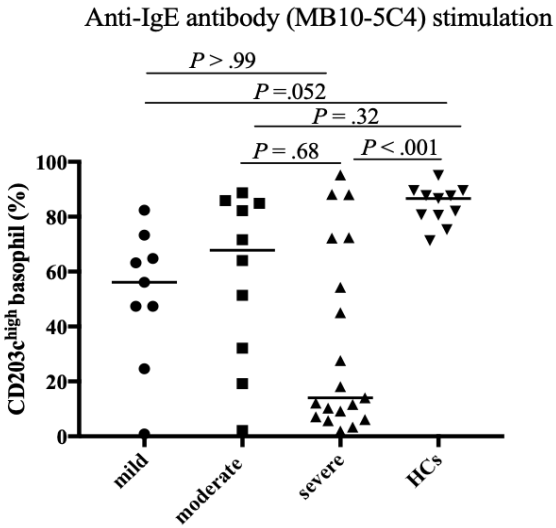

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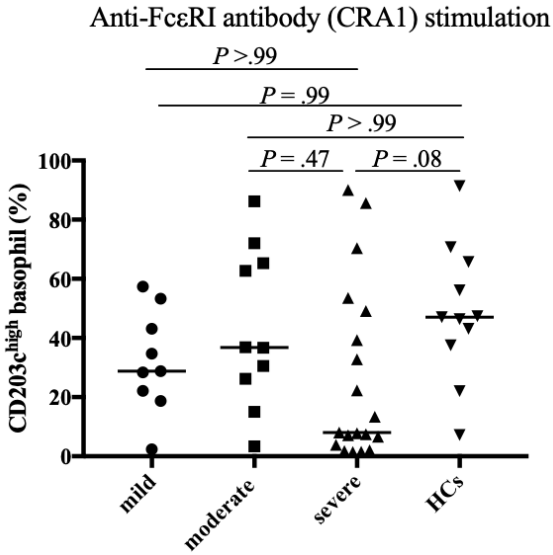

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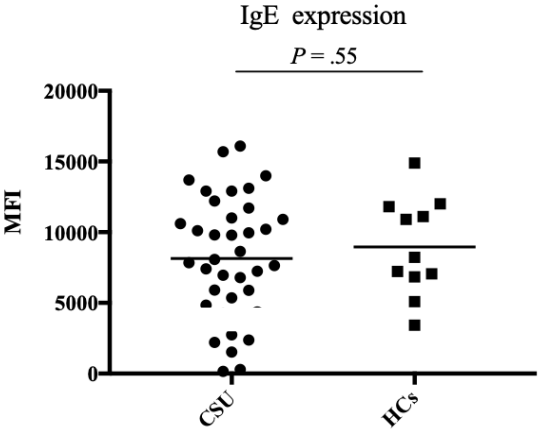

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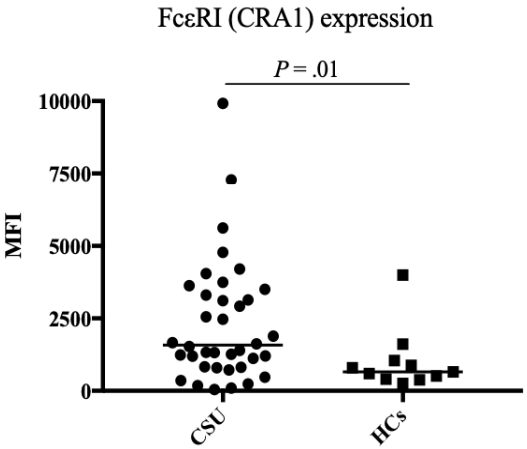

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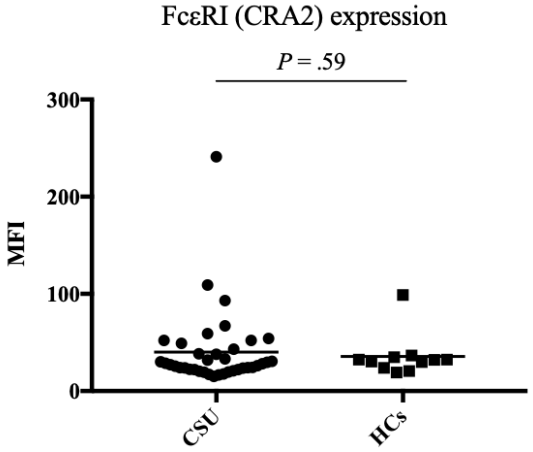

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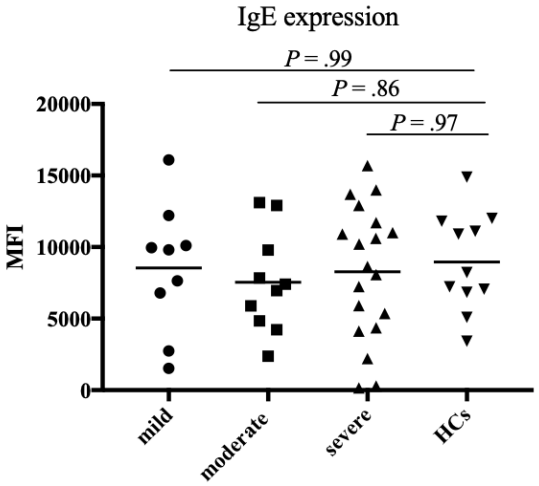

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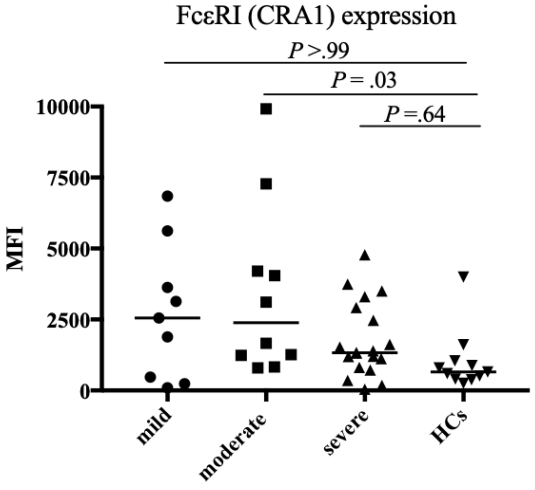

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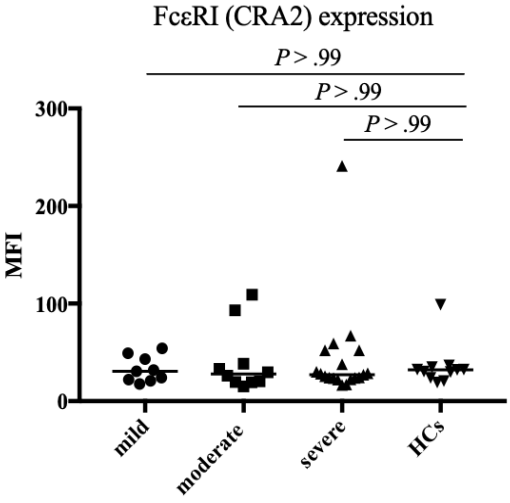

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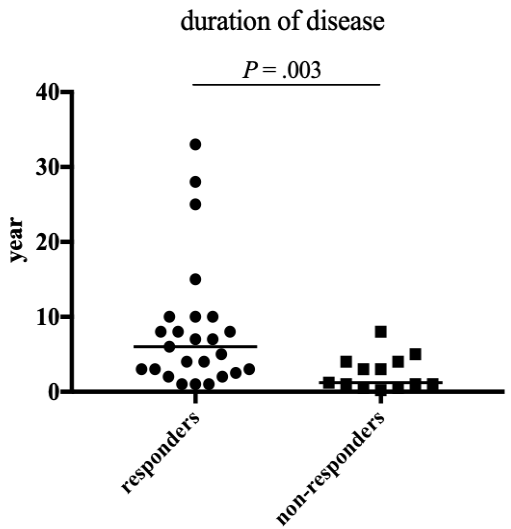

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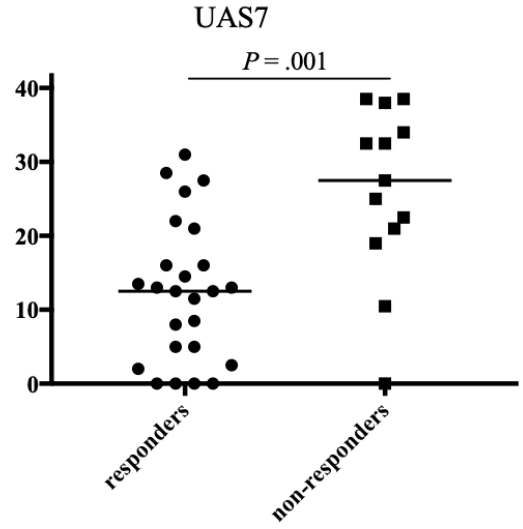

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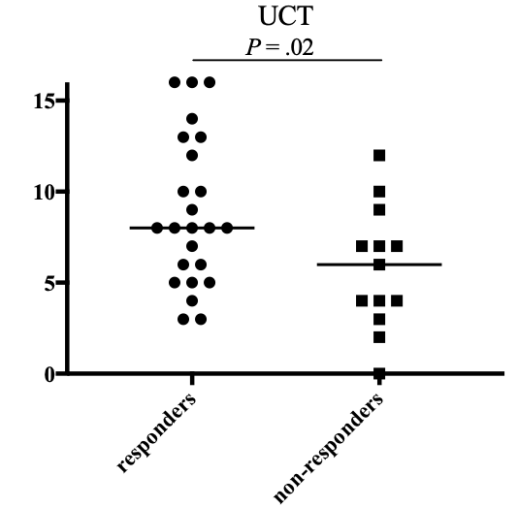

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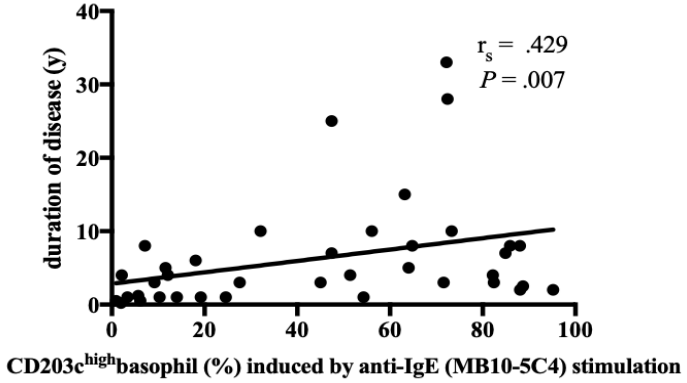

Fig 5, D

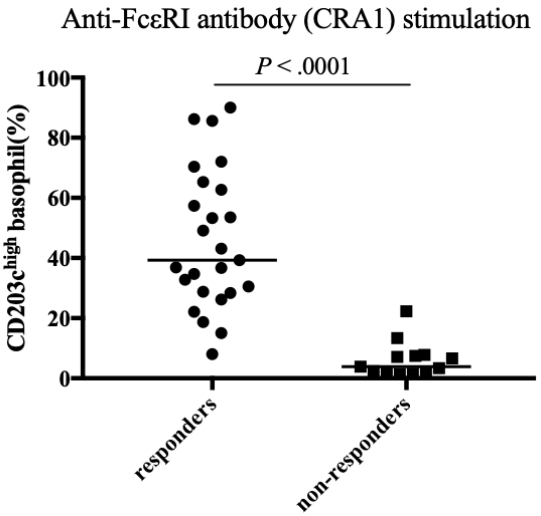
Anti-FcεRI antibody (CRA1) stimulation
P < .0001
CD203c^high basophil(%)
100
80
60
40
20
0
responders
non-responders

Fig 6, A

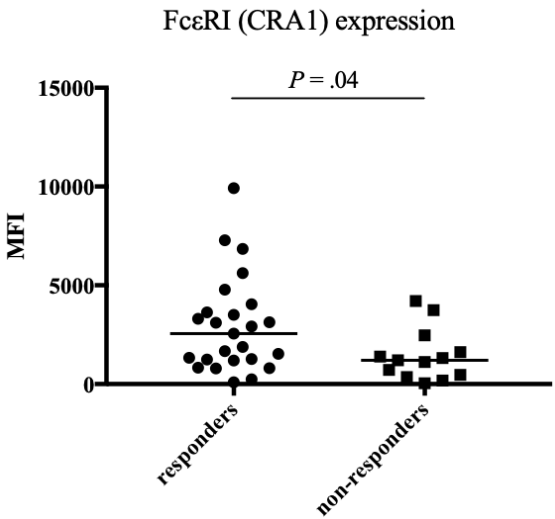
FcεRI (CRA1) expression
P = .04
MFI
15000
10000
5000
0
responders
non-responders

Fig 6, B

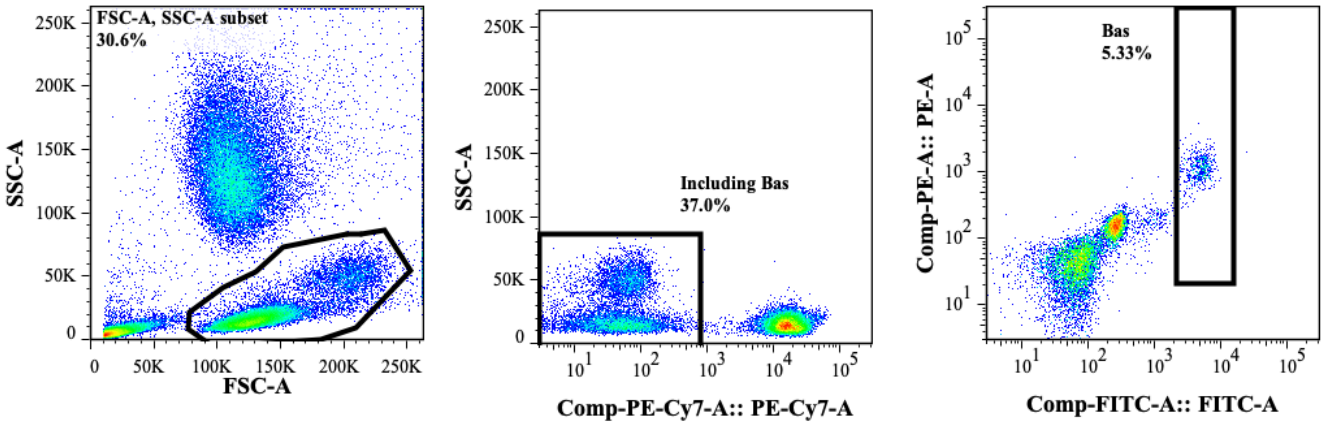

Fig E1

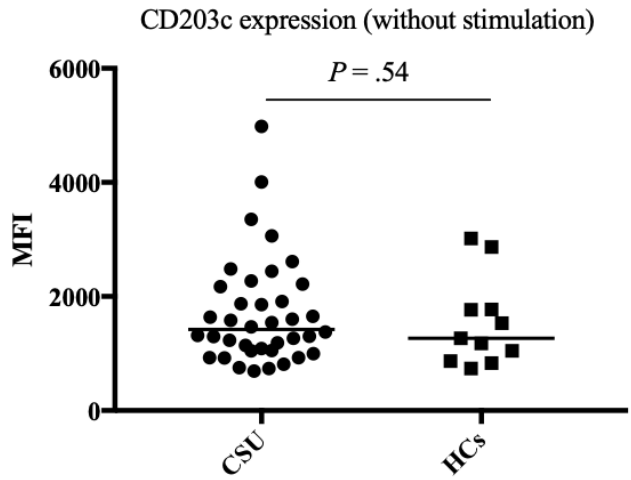

Fig E2

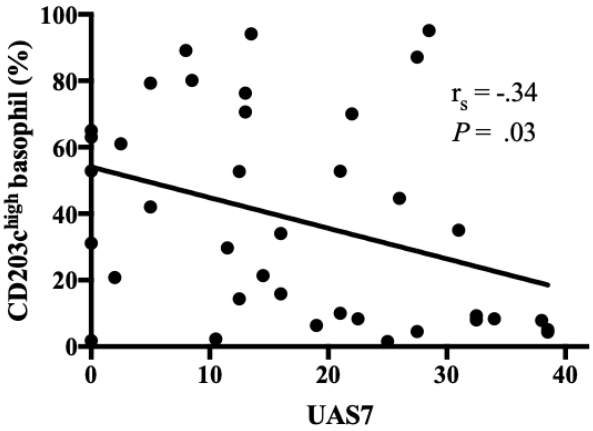

Fig E3

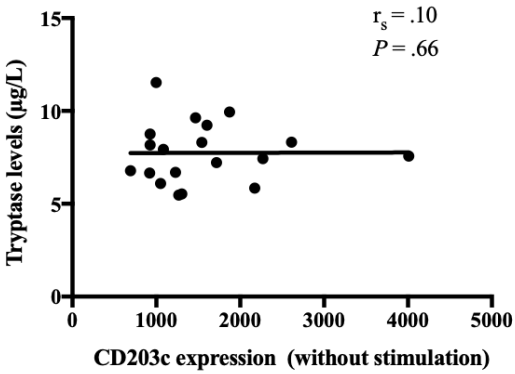

Fig E4, A

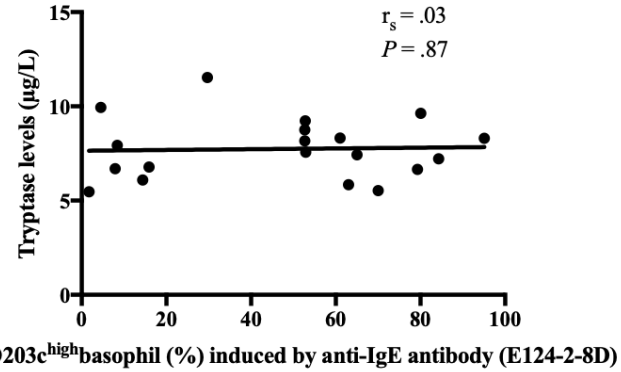

Fig E4, B

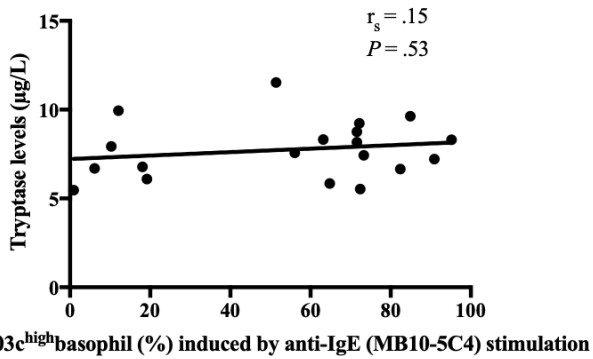

Fig E4, C

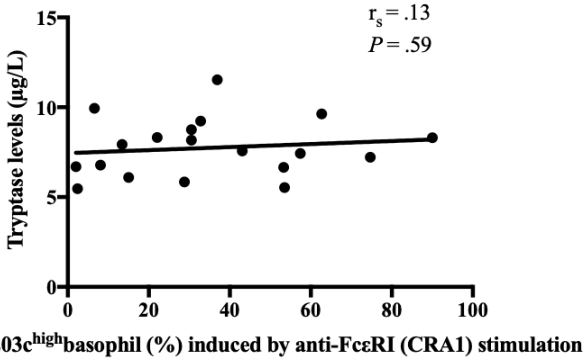

Fig E4, D

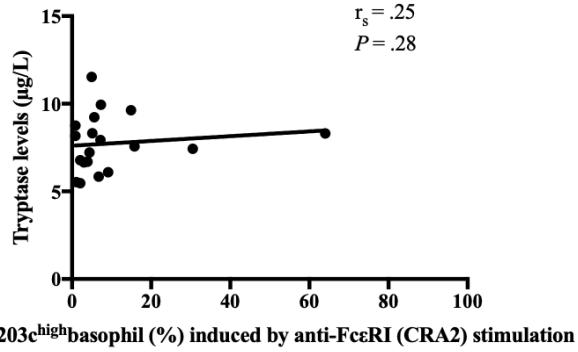

Fig E4, E

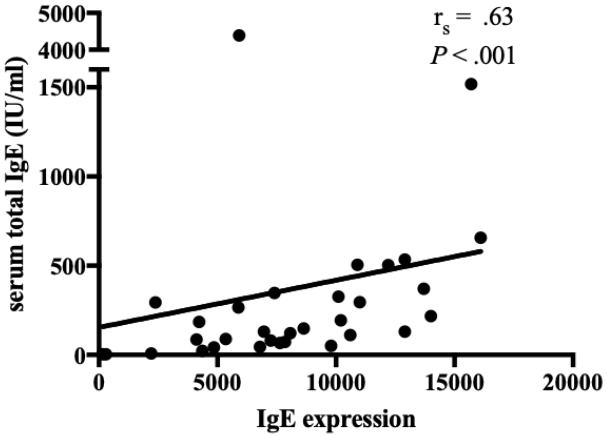

Fig E5, A

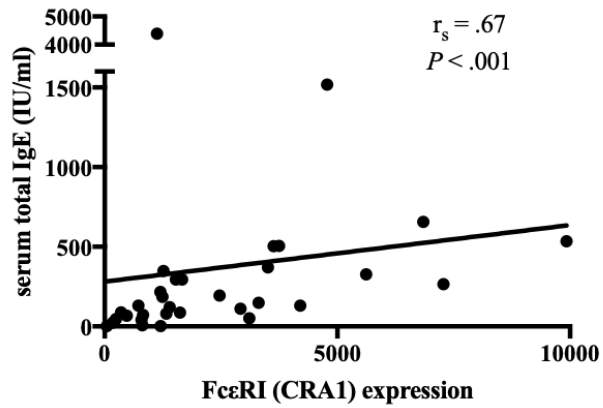

Fig E5, B

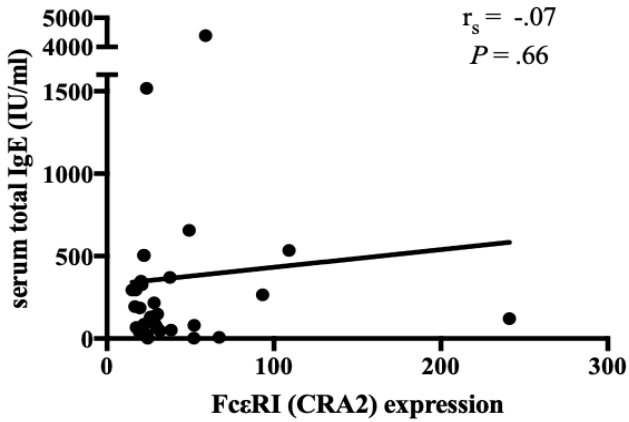

Fig E5, C

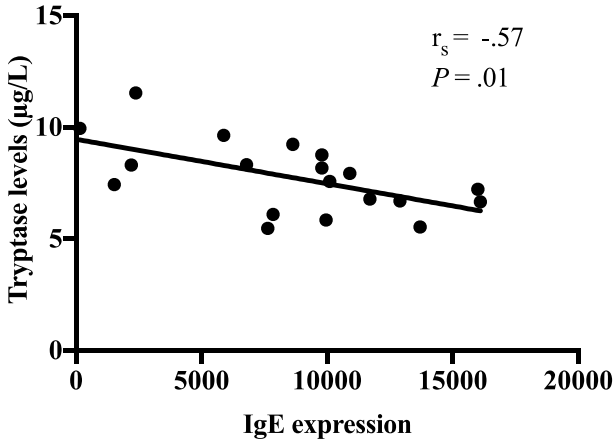

Fig E6, A

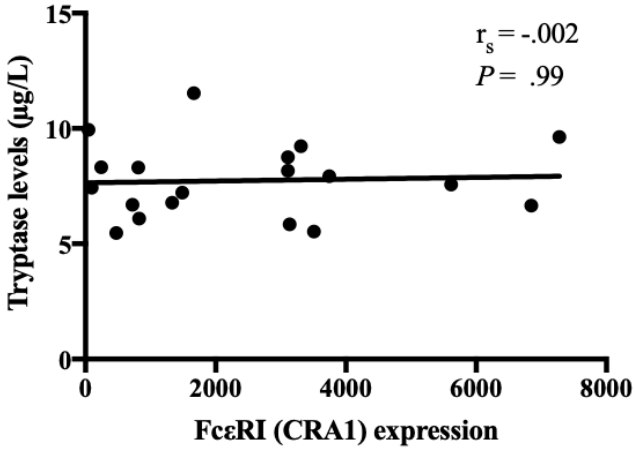

Fig E6, B

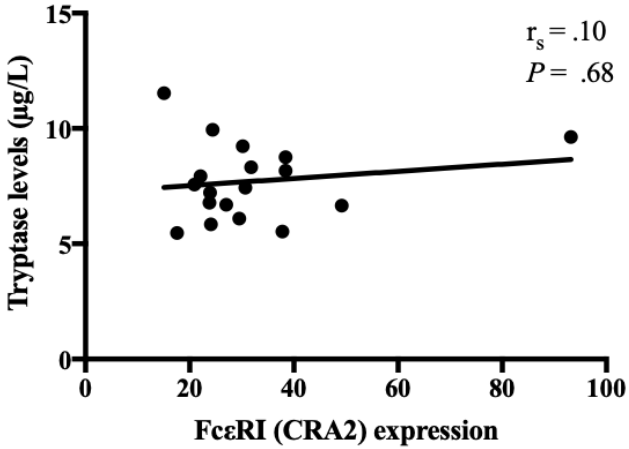

Fig E6, C

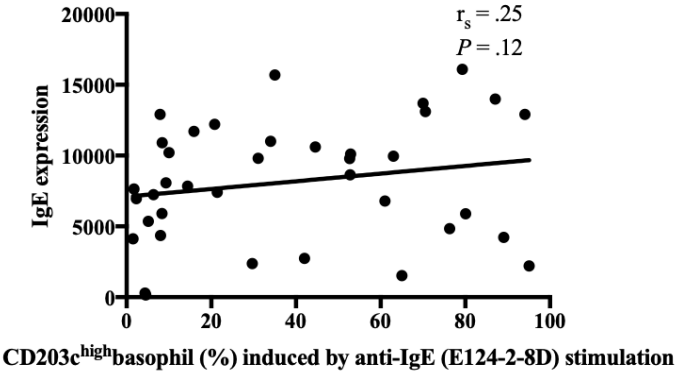

Fig E7,A

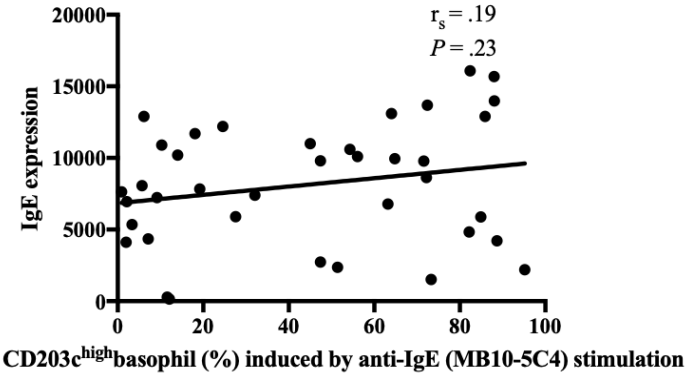

Fig E7, B

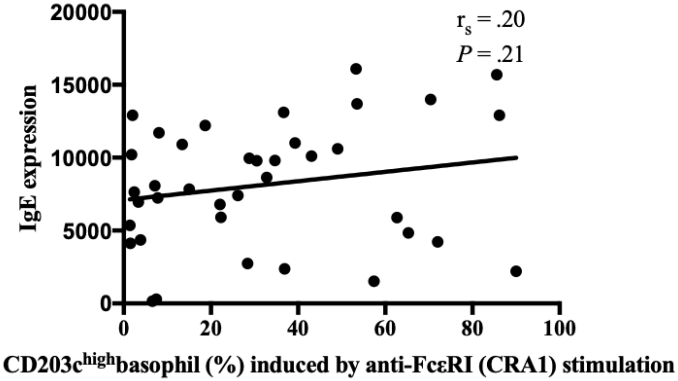

Fig E7, C

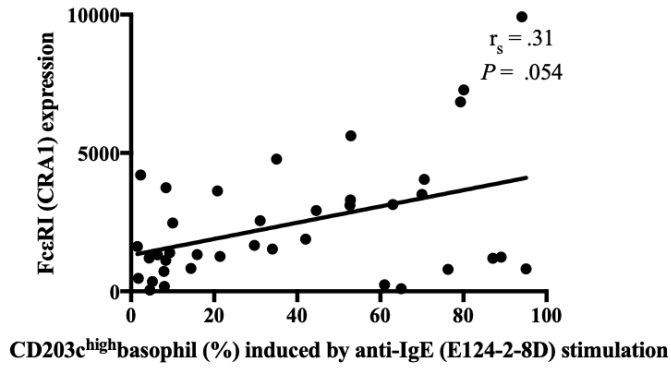

Fig E8, A

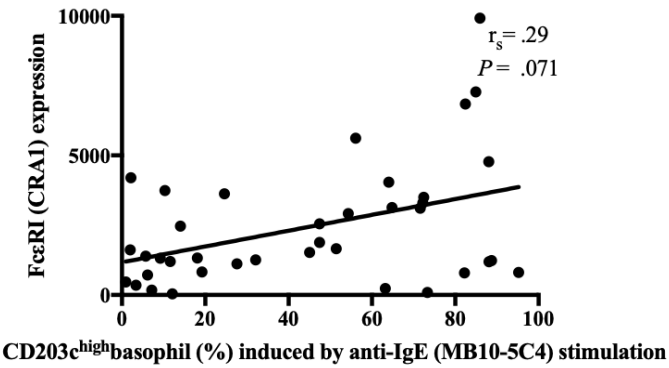

Fig E8, B

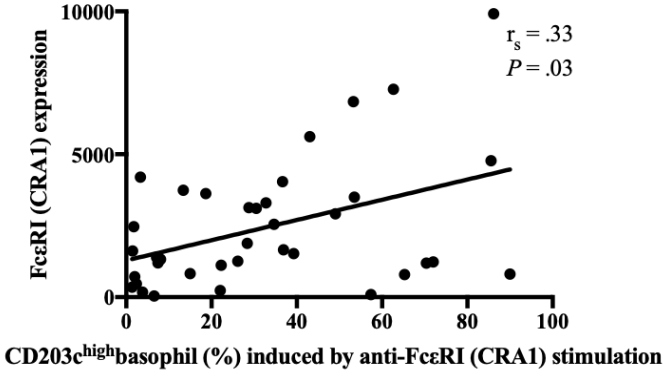

Fig E8, C