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Improved FcεRI-mediated CD203c basophil responsiveness reflects rapid-responses to omalizumab in chronic spontaneous urticaria

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35

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Abstract

Background: Omalizumab is effective in chronic spontaneous urticaria (CSU) patients although its mechanism of action is poorly understood. Several studies reported that decreased FcεRI-mediated histamine release and/or responsiveness was characteristic of basophils in CSU patients. However, few studies have focused on the relationship between changes in basophil responsiveness via FcεRI after omalizumab treatment and the therapeutic effect in CSU patients.

Objective: To assess basophil responsiveness via FcεRI stimulation, as well as FcεRI expression and IgE binding on blood basophils from CSU patients before and after omalizumab treatment and its possible association with the clinical response.

Methods: We analyzed 34 CSU patients treated with omalizumab who were categorized as fast responders (FRs) (n=20) and non or slow responders (N/SRs) (n=14). CD203c expression induced by FcεRI stimulation, and IgE and FcεRI expressions on blood basophils from CSU patients before and after omalizumab treatment were analyzed. Basophil responsiveness via FcεRI stimulation was observed *in vitro* using basophils pre-treated with omalizumab.

Results: FRs had increased CD203c responsiveness after treatment with omalizumab compared with N/SRs. This improvement of basophil responsiveness via FcεRI stimulation in FRs was not observed in peripheral blood basophils pre-incubated with omalizumab *in vitro*, suggesting omalizumab does not directly affect circulating pre-existing abnormal basophils.

Conclusion: Increased basophil responsiveness via FcεRI after omalizumab treatment is associated with the therapeutic effect and mechanism of action of omalizumab.

Highlights box

What is already known about this topic?

Basophil functional abnormalities such as the low-responsiveness of basophils via FcεRI were reported in chronic spontaneous urticaria. Moreover, several studies reported increased FcεRI-mediated histamine release after omalizumab treatment.

What does this article add to our knowledge?

Improvement of attenuated basophil responsiveness via FcεRI stimulation in chronic spontaneous urticaria is associated with rapid clinical effectiveness and the mechanism of action of omalizumab might be due to improved responsiveness of newly circulating basophils.

How does this study impact current management guidelines?

Changes in basophil responsiveness via FcεRI stimulation in some patients with chronic spontaneous urticaria before and after omalizumab treatment suggest the importance of basophil status as an action point of omalizumab treatment.

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

Abbreviations used

ASST: autologous serum skin test

BAT: basophil activation test

79 BHRA: basophil histamine release assay

80 CSU: chronic spontaneous urticaria

81 DLQI: dermatology life quality index

82 FcεRI: high-affinity IgE receptor

83 FRs: fast responders

84 GRs: good responders

85 HCs: healthy controls

86 IgE: immunoglobulin E

87 MFI: mean fluorescence intensity

88 N/PRs: non or partial responders

89 N/SRs: non or slow responders

90 Syk: spleen tyrosine kinase

91 UAS: urticaria activity score

92 UCT: urticaria control test

Introduction

Chronic spontaneous urticaria (CSU) is characterized by the spontaneous appearance of wheals, angioedema, or both for > 6 weeks and is associated with known (autoreactivity) or unknown causes[1]. Although the pathophysiology of CSU is unclear, basophils have been increasingly recognized as having critical roles and some investigators have described an association between disease pathophysiology and basophil function and number in CSU.

Several reports documented that circulating basophils from patients with CSU released less histamine than healthy controls (HCs) when stimulated with anti-IgE antibody or anti-high-affinity IgE receptor (FcεRI) antibody[2,3,4,5]. Furthermore, reduced CD63 expression on basophils after anti-FcεRI stimulation was reported in a subgroup of CSU patients compared with HC basophils[6]. In our previous study[7], basophils from CSU patients had attenuated upregulation of the activation marker CD203c against IgE and FcεRI antibodies compared with basophils from HCs, and the attenuated basophil responsiveness in patients with CSU was associated with the severity of disease and a relatively shorter disease duration. In addition, Huang et al. reported that basophil non-responders (< 10% of total histamine release to IgE) and basopenics (histamine concentrations < 5ng/mL blood leukocytes) have more severe, but shorter disease compared to responders[8].

CSU patients have unique features related to basophil number as well as basophil function and responsiveness in the circulating blood. Rorsman first described a reduction of peripheral blood basophils in CSU patients[9]. Subsequently, basopenia was reported to be correlated with urticaria activity[10,11]. Furthermore, basophils were reported to be present in skin lesions of urticaria at levels higher than in non-lesion skin[12]. These findings suggest basopenia may reflect the recruitment of basophils to skin tissues. Although basophil migration was investigated in previous studies[13,14], the recruitment pathway remains unknown.

Omalizumab is a recombinant humanized anti-IgE monoclonal antibody that selectively binds to the C3 domain of IgE, thereby blocking the binding of IgE to high-affinity receptors on effector cells, and inhibiting IgE-mediated cellular responses[15]. Omalizumab has been approved for use in patients with CSU who remain symptomatic despite H1 antihistamine treatment. Although omalizumab binds to free IgE, which lowers free IgE levels and causes FcεRI receptors on basophils and mast cells to be internalized and degraded, the mechanism of action in CSU is not understood completely[16,17]. Several reports demonstrated changes in basophil number and function after treatment with omalizumab. Furthermore, the numbers of circulating blood basophils increased after omalizumab treatment[18] in parallel with clinical improvement[19, 20]. With respect to basophil function, Gericke et al. reported increased anti-IgE-induced histamine release from blood basophils of CSU patients treated with omalizumab

128 compared with baseline value[18]. In contrast, it was reported that CD63-based basophil
129 "releasability" by IL-3 coincubation after stimulation with anti-IgE antibody was decreased
130 after omalizumab treatment compared with baseline values[21].

131 Many reports have described pre-treatment biomarkers to predict the efficacy of
132 omalizumab in CSU. Palacios et al. reported a lack of basophil CD203c upregulation in serum,
133 which might reflect a lack of autoantibodies to IgE and/or FcεRI but which correlated with a
134 higher clinical response[22]. Similarly, Deza et al. showed that a higher rate of positive autologous
135 serum skin test (ASST) was associated with insufficient therapeutic effect[23]. Gericke et al.
136 showed that a positive basophil histamine release assay (BHRA) or ASST as an indicator of
137 serum autoreactivity was predictive of a slow response to treatment with omalizumab[24].
138 Additionally, baseline levels of basophil FcεRI expression were significantly lower in non-
139 responders to omalizumab[23] and higher in fast responders than in slow responders[25].
140 Omalizumab responsiveness was also predicted by total serum IgE levels[26] and changes in total
141 serum IgE levels[27]. Furthermore, Altrichter et al. observed that reduced serum IL-31 was
142 associated with omalizumab responses[28]. However, few studies have focused on the relationship
143 between baseline and/or altered basophil responsiveness via FcεRI, as well as IgE and FcεRI
144 expression on basophils and the omalizumab therapeutic effect in CSU patients.

Therefore, the current study analyzed the expression of CD203c via FcεRI stimulation, as well as IgE and FcεRI expression on basophils from CSU patients before and after omalizumab treatment and its possible association with the clinical response. Moreover, we examined these parameters as a potential predictor of responses to omalizumab therapy. In addition, we performed an *in vitro* study using FR basophils pre-treated with omalizumab whether omalizumab could affect basophil reactivity in short term incubation.

Material and Methods

Study population

Thirty-four patients with CSU who remained symptomatic despite H1-antihistamines (even up to two times the recommended dose in Japan) and who were treated with omalizumab 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. Omalizumab 300 mg was injected subcutaneously at least three times at 4 weeks intervals. Clinical variables were evaluated using the Urticaria Control Test (UCT), which is an outcome instrument to retrospectively assess urticaria control with a recommended cutoff value of 12 for controlled disease[29]. It was reported that UCT has a strong correlation with Dermatology Life Quality Index (DLQI)[30] and 7-day Urticaria Activity Score (UAS7)[31]. UCT scores were measured at day 0, and on weeks 4, 8, and 12 of treatment (i.e., before the 1st, 2nd, 3rd and 4th injections). Patients were categorized into FRs (n = 20) and N/SRs (n = 14) (Fig. E1) (Table I) based on the following criteria: FRs with UCT scores ≥ 12 up on week 4 (Fig. E2, A, see in this article's Online Repository); and N/SRs with UCT scores < 12 up on week 4 (Fig. E2, B, see in this article's Online Repository). UAS7 could be measured in some patients and the data was presented in Fig. E2, C, D, see in this article's Online Repository. In addition, we classified patients into good responders (GRs) (n = 26) and N/PRs (n = 7) based on UCT scores on week

12 after treatment of omalizumab (Fig. E1, Table EI, see in this article's Online Repository): GRs with UCT scores ≥ 12 up on week 12 and N/PRs with UCT scores < 12 on week 12. One patient was followed-up a clinical course up to 4 weeks but could not follow up until 12 weeks. Therefore, this patient was excluded from the GRs vs. N/PRs dataset. All study participants provided oral consent for this study after verbal and written explanations.

Basophil activation test

Whole blood was taken just before the first and second omalizumab administration using blood collection tubes containing ethylenediaminetetraacetic acid (EDTA) 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 as previously described[7]. Basophils were stimulated with anti-IgE antibody (clone: E124-2-8D) (0.1 μg/ml) (Beckman Coulter, Fullerton, CA, USA), VioBlue conjugated anti-IgE antibody (clone: MB10-5C4) (1.1 μg/ml) (Miltenyi Biotec, Bergisch Gladbach, Germany) or biotinylated anti-FcεRI antibody (clone: CRA1) (16 μg/ml) (BioAcademia, Osaka, Japan). Basophils incubated with phosphate-buffered saline (PBS) were used as a negative control.

Briefly, blood was stained with reagents consisting of CRTH2-FITC, CD203c-PE, and CD3-PC7 to identify basophils, and were then mixed with the respective stimulant or PBS at 37°C for 15 minutes. Biotinylated antibodies against CRA1 were then coupled with APC

187 streptavidin (BD, Franklin Lakes, NJ, USA) at 4°C for 30 minutes. Erythrocytes were lysed and
188 after washing twice with PBS, cells were resuspended in 0.3 mL PBS 0.1% formaldehyde and
189 evaluated 500 basophils by flow cytometry (FACS verse, BD Biosciences, San Jose, CA, USA).
190 Flow cytometry results were analyzed by FlowJo software (FlowJo, LLC, Ashland,
191 OR, USA). Results of antibody stimulation were expressed as a proportion of $CD203c^{high}$
192 basophils. The proportion of $CD203c^{high}$ basophils was determined using a threshold defined as
193 the expression level above which only 5% of basophils in the negative control sample
194 fluoresced[32]. Changes in basophil responsiveness via FcεRI stimulation after omalizumab
195 treatment compared with before treatment were calculated as followed. After/before treatment
196 ratio of basophil CD203c responsiveness was defined as $CD203c^{high}$ basophil (%) stimulated
197 with each antibody after treatment /$CD203c^{high}$ basophil (%) stimulated with each antibody
198 before treatment.
199 **Measurement of IgE and FcεRI levels on basophils**
200 Basophils were incubated with VioBlue conjugated anti-IgE antibody (clone: MB10-5C4;
201 (Miltenyi Biotec) and biotinylated anti-FcεRI antibody (clone: CRA1; BioAcademia) and
202 analyzed by flow cytometry using the same method as for IgE and FcεRI levels on basophils
203 and FlowJo analysis using the same method as for basophil activation after anti-IgE or CRA1
204 antibody stimulation. IgE and FcεRI levels were evaluated as the mean fluorescent intensity

(MFI). Changes in IgE and FcεRI levels on basophils after omalizumab treatment compared with before treatment were calculated as follows: ΔIgE expression (ΔFcεRI expression): IgE levels (FcεRI levels) on basophils before treatment − after treatment.

***In vitro* study using basophils pre-treated with omalizumab**

Whole blood obtained from two CSU patients before omalizumab administration using blood collection tubes containing EDTA was incubated for 1, 12 or 24 hours at room temperature with 30 μg/mL omalizumab (Novartis Pharma, Tokyo, Japan)[33]. Samples with/without omalizumab preincubation were measured for CD203c expression after stimulation with anti-IgE antibody (clone: E124-2-8D, MB10-5C4). Results of antibody stimulation were expressed as the proportion of $CD203c^{high}$ basophils and IgE levels on basophils were expressed as the MFI, and sequential changes were investigated.

Serum total IgE levels and basophil counts

Serum total IgE levels were measured by immunoglobulin E-radioimmunosorbent test. Numbers of blood basophils (/μl) were assessed by an automated analyzer in the laboratory of the Kobe University Graduate School of Medicine.

Changes in total serum IgE levels and basophil counts on basophils after omalizumab treatment compared with before treatment were calculated as follows: Δtotal serum IgE (Δbasophil counts): total serum levels (basophil counts) after treatment − before treatment.

Statistical analysis

The non-parametric Mann–Whitney *U*-test, unpaired *t*-test and Fischer's exact test were used to assess differences between GRs and N/PRs. Kruskal-Wallis result with Dunn test was used for comparing three 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 < 0.05 were considered statistically significant.

Results

Study population

CSU patients were categorized into two subgroups based on UCT scores 4 weeks after omalizumab treatment. Twenty out of 34 patients (58.8%) who achieved significant clinical improvement (UCT scores ≥ 12) were classified as FRs and the remaining fourteen (41.1%) who did not achieve significant clinical improvement (UCT scores < 12) were classified as N/SRs (Fig. E1, see in this article's Online Repository). The baseline clinical characteristics of these subgroups are described in Table I. They exhibited no significant differences regarding sex, age, disease duration, serum IgE levels, and basophil counts. Additionally, there were no significant differences regarding the ASST positive rate in contrast to a previous study[24]. There were no differences among the two groups with respect to baseline UCT, UAS7, or DLQI score. In addition, although we divided CSU patients into GRs and N/PRs based on UCT scores on week 12 after treatment of omalizumab. There were no differences in serum IgE and ASST when comparing GRs and N/PRs (Table EI, see in this article's Online Repository). However, it was noted that one patient among N/PRs did not have data of total serum IgE before treatment and the patient was excluded from the GRs vs. N/PRs dataset (Table EI).

Baseline basophil responsiveness via FcεRI stimulation

Although several studies described biomarkers for therapeutic responses to omalizumab[22,23,26,27,28], few studies have focused on basophil responsiveness via FcεRI as a predictor of the therapeutic response to omalizumab. Therefore, we analyzed the baseline expression of the activation marker CD203c with or without anti-IgE and/or FcεRI stimulation in CSU patients. The expression of CD203c on peripheral blood basophils from CSU patients in the steady state without anti-IgE and/or FcεRI stimulation was similar between FRs and N/SRs (Fig. E3, see in this article's Online Repository). There were no differences in the percentage of CD203c^{high} basophils stimulated with anti-IgE (E124-2-8D, MB-105C4) and FcεRI (CRA1) antibody between groups (Fig. 1A-C). Thus, basophil responsiveness via FcεRI stimuli did not predict the treatment response with omalizumab.

Furthermore, we classified patients with CSU into two groups based on the baseline proportion CD203c^{high} basophils after anti-IgE stimulation as in the previous report[7] (14 non-responders [<10% CD203c^{high} basophil] and 20 responders [>10% CD203c^{high} basophil]) (Fig. E4, see in this article's Online Repository). However, there were no differences in fast or good effectiveness of omalizumab between non-responders and responders.

Improvement in basophil responsiveness via FcεRI stimulation after omalizumab treatment

264 It was previously demonstrated that blood basophil histamine release tended to increase with
265 anti-IgE stimulation after omalizumab treatment compared with baseline[18]. In this study, we
266 compared CD203c responsiveness of basophils via FcεRI stimulation with anti-IgE and FcεRI
267 antibody before and after omalizumab treatment. The percentage of $CD203c^{high}$ basophils
268 stimulated with any antibody increased in many FRs but not in most N/SRs (Fig. E5, Table EII,
269 see in this article's Online Repository). Therefore, we compared the after/before treatment ratio
270 of basophil CD203c responsiveness via FcεRI between FRs and N/SRs. When peripheral blood
271 basophils were stimulated with three types of antibody, the after/before treatment ratio was
272 significantly higher in FRs than in N/SRs (Fig. 2, Fig. E5, see in this article's Online
273 Repository). Furthermore, the after/before ratio was around 1.5-2.0 for the anti-IgE antibody in
274 FRs, but around 4 for the anti-FcεRI antibody (Fig. 2). These data suggest that omalizumab
275 improved basophil responsiveness via FcεRI in FRs but not in N/SRs and that the improvement
276 effect was associated with the rapid therapeutic response of omalizumab. However, it was
277 needed to note that FRs included a group with improved basophil responsiveness and a group
278 with no improvement, and N/SRs included some patients with improved basophil
279 responsiveness (Fig. E5, see in this article's Online Repository).

280 **Baseline IgE and FcεRI levels of basophils**

Previous reports showed that the treatment effect of omalizumab may be predicted by serum IgE levels[26,27]. We previously reported that serum IgE levels were strongly correlated with IgE or FcεRI expression on basophils from CSU patients[7] and the correlations were also observed in overall participants of this study (Fig. E6, see in this article's Online Repository). Therefore, we investigated whether baseline basophil IgE expression was associated with the therapeutic response. No significant difference in IgE and FcεRI (CRA1) expression between FRs and N/SRs was observed (Fig. 3A, B). However, when CSU patients were categorized into GRs and N/PRs using the UCT score 12 weeks after omalizumab treatment, IgE expression on basophils from GRs was higher than those from N/PRs, whereas there was no difference in serum IgE between GRs and N/PRs (Fig. 3C, Table EI). However, it was noted that one patient among N/PRs did not have data of total serum IgE before treatment and the patient was excluded from the GRs vs. N/PRs dataset (Table EI). Additionally, there was no difference in FcεRI (CRA1) expression between GRs and N/PRs (Fig. 3D).

Changes in IgE and FcεRI levels on basophils after omalizumab treatment

It is widely accepted that omalizumab neutralizes free IgE and subsequently suppresses FcεRI and surface IgE expression on circulating basophils [17]. Deza et al. reported that patients exhibiting significant clinical improvement had a marked reduction in the levels of basophil FcεRI after 4 weeks [23]. Based on these findings, we evaluated the association between changes

299 in basophil FcεRI and IgE levels after omalizumab treatment and the therapeutic effect. ΔIgE
300 expression (IgE levels on basophils before treatment − after treatment) was not different
301 between FRs and N/SRs (Fig. 4A). In contrast to a previous report[23], there was no difference in
302 ΔFcεRI (CRA1) expression between the two groups (Fig. 4B). However, ΔIgE expression was
303 higher in GRs than N/PRs (Fig. 4C), whereas there was no difference in ΔFcεRI (CRA1)
304 expression between the two groups (Fig. 4D). These data indicate a more efficient inhibition of
305 IgE binding on basophils in GRs than in N/PRs following omalizumab administration.

306 **Changes in serum total IgE levels and basophil counts**

307 Low serum IgE levels that increased after the start of omalizumab treatment were associated
308 with insufficient clinical responses[27]. Additionally, Saini et al. showed that improved basopenia
309 was associated with reduced clinical symptoms[19]. Thus, we evaluated the potential association
310 between changes in serum IgE levels and circulating basophil counts in the blood after
311 treatment with omalizumab and the therapeutic effect. When CSU patients were categorized
312 into FRs and N/SRs, no differences in Δserum total IgE and Δbasophil counts were observed
313 between the groups (Fig. 5A, B). When CSU patients were categorized into GRs and N/PRs,
314 Δserum total IgE and Δbasophil counts just tended to be higher with no significant difference
315 (Fig. 5C, D). However, three patients among N/PRs did not have data of total serum IgE and
316 basophil count either before or after treatment and these patients were excluded from the GRs

vs. N/PRs dataset (Fig. 5C, D). Therefore, the number of samples in Fig. 5C and D was not be sufficient for statistical analyses, and if more samples were added, significant differences could be detected as previously reported[19,20,27].

Different effects of omalizumab on basophil responsiveness via FcεRI stimulation *in vivo* and *in vitro*

Finally, we investigated whether the responsiveness of basophils via FcεRI improved before omalizumab administration by incubating basophils from CSU patients with omalizumab *in vitro* to clarify the mechanism of action of omalizumab. Increased basophil responsiveness via FcεRI after *in vivo* omalizumab treatment was observed for cells isolated from the two FRs used in this study (Fig. 6A, B). However, improved basophil responsiveness via FcεRI stimulation was not observed at any time despite pre-treatment with omalizumab *in vitro* (Fig. 6C, D). These data suggest that pre-existing circulating basophils that exhibited low responsiveness via FcεRI before omalizumab administration did not have improved function after *in vitro* omalizumab treatment.

Discussion

Basophils have unique features and play a critical role in CSU. It was reported that basophils from CSU patients released less histamine and/or exhibited low responsiveness when stimulated via FcεRI compared with basophils from HCs[2,3,4,5,6,7]. We recently reported that the low responsiveness of basophils via FcεRI reflected severe disease activity in CSU[7]. In addition to the functional abnormalities of basophils, basopenia was correlated with disease activity in CSU[10,11] and the cutaneous lesion recruitment of basophils in CSU. Although omalizumab was effective in most patients with CSU who remained symptomatic despite H1 antihistamine treatment, its mechanism of action in CSU is poorly understood. In the current study, we focused on basophils as a predictive marker of the clinical effectiveness of omalizumab and as a component of the mechanism of action of omalizumab. We classified and compared CSU patients into two groups, FRs and N/SRs or GRs and N/PRs, based on the clinical efficacy of omalizumab 4 or 12 weeks after treatment with omalizumab.

First, we focused on baseline parameters before omalizumab treatment as a pre-treatment predictive marker of the clinical effectiveness of omalizumab. Baseline CD203c responsiveness after stimulation with anti-IgE and FcεRI antibodies was not a useful pre-treatment predictive marker, which is similar for the CD63-based report from Aghdam et al[34] (Fig. 1). However, baseline IgE expression on basophils from GRs before omalizumab

349 treatment was higher than that in basophils from N/PRs (Fig. 3C). This difference was not
350 observed between FRs and N/SRs (Fig. 3A). These observations suggested that higher IgE
351 expression on basophils might predict a significant therapeutic effect 12 weeks after
352 omalizumab treatment, even if the therapeutic effect was insufficient 4 weeks after treatment. In
353 contrast to previous reports[25], there were no differences in baseline FcεRI expression on
354 basophils between FRs and N/SRs before omalizumab treatment (Fig. 3B). This difference may
355 be related to differences in the definition of treatment response. In addition, a good predictor of
356 N/PRs was found on surface IgE (Fig. 3C), but not serum IgE (Table EI). However, serum IgE
357 strongly correlated with surface IgE on basophils (Fig. E6, see in this article's Online
358 Repository), and the difference of results between the previous studies [26,27] that serum IgE was a
359 good baseline predictor of omalizumab treatment efficacy and this study may be dependent on
360 the small number of samples in this study.

361 Next, we investigated changes in basophil parameters and IgE after omalizumab
362 treatment to determine their contribution to the mechanism of action of omalizumab in CSU.
363 Regarding changes in basophil parameters, the after/before treatment ratios of basophil
364 responsiveness (CD203c response) were significantly higher in FRs compared with N/SRs (Fig.
365 2), suggesting that the improvement of low responsiveness in circulating basophils via FcεRI
366 was related to the rapid therapeutic effect of omalizumab. In addition, the after/before ratio was

367 around 1.5-2.0 for the anti-IgE antibody in FRs, but around 4 for the anti-FcεRI antibody. It has
368 been reported that during treatment with omalizumab, spleen tyrosine kinase (Syk) expression
369 increases in peripheral blood basophils, offsetting the functional effects mediated by the drug-
370 induced reduction in cell surface density of FcεRI and its bound IgE[35]. Basophils which
371 recovered functionally after omalizumab treatment might promote Syk signal more strongly
372 when stimulated with anti- FcεRI than when anti-IgE.

373 CD203c upregulation after anti-IgE stimulation was demonstrated to be earlier than
374 CD63 upregulation[36]. In addition, Ebo et al mentioned that up-regulation of CD203c does not
375 per se indicate histamine release[37] and CD203c is not a degranulation marker like CD63. Thus,
376 increased CD203c response to the IgE concentration after treatment of omalizumab observed in
377 current study does not mean an increase in histamine release. Aghdam et al reported that when
378 CD63 expression on basophils was used as a biomarker in the omalizumab responder, ~~no~~
379 significant increase after stimulation of the anti-IgE antibody was observed in non-responders
380 after omalizumab treatment, contrary no increase in responders[34]. This difference between our
381 study might be due to differences in the markers between CD203c and CD63 or to the definition
382 of responder and non-responder. Indeed, Agdham et al. evaluates disease activity in six
383 months.[34] Contrary, we evaluate the effectiveness in one month and three months.

384 The reduction of IgE expression on basophils 4 weeks after omalizumab treatment
385 was larger in GRs compared with N/PRs (Fig. 4C). Regarding the changes in serum IgE,
386 increased serum IgE levels 4 weeks after omalizumab treatment tended to be higher in GRs than
387 in N/PRs (Fig. 5C). These differences were not observed between FRs and N/SRs (Fig. 4A,
388 5A). Based on these results related to IgE, the larger reduction of IgE expression on basophils
389 and the higher increase of serum IgE levels 4 weeks after omalizumab treatment suggested that
390 good clinical responses were achieved 12 weeks after omalizumab treatment, even if they were
391 not achieved 4 weeks after treatment. Moreover, these data imply that omalizumab efficiently
392 inhibited the binding of IgE to basophils by binding to free serum IgE in GRs compared with
393 N/PRs, which was the cause of the increase in apparent serum IgE levels in GRs.
394 Whether this improvement of basophil responsiveness by omalizumab treatment acts
395 directly on low responsive circulating basophils before omalizumab treatment is an important
396 issue related to the mechanism of action of omalizumab in CSU. Finally, we investigated
397 whether omalizumab acted directly on circulating basophils in FRs before treatment *in vitro*. An
398 improvement of basophil responsiveness via FcεRI stimulation was not observed at any time
399 despite *in vitro* omalizumab treatment (Fig. 6C, D). These data indicate that in FRs, the
400 functions of pre-existing basophils that exhibited low responsiveness via FcεRI stimulation
401 before omalizumab treatment could not be improved. In contrast, *in vivo* treatment with

omalizumab in these cases improved basophil responsiveness via FcεRI stimulation and induced a rapid and good clinical response (Fig. 6A, B). These differences between *in vitro* and *in vivo* omalizumab treatment suggest that newly circulating basophils, possibly from skin tissue or bone marrow, maintained normal function in the blood under the state of low IgE binding to their surface by the efficient formation of an immune complex with omalizumab and serum IgE. Indeed, it was reported that the life span of mature basophils is approximately 60–70 hours[38]. A recent review proposed that basophils in CSU patients are mildly activated, persistently release a small amount of histamine, and are involved in the earliest stages of the cascade of CSU pathogenesis[39].

This study includes limitations about grouping. The main focus of this study was to observe differences in basophil responsiveness between FRs and other groups (N/SRs) at 4 weeks after treatment. However, because we considered that the comparison of FRs and N/SRs alone was not enough to evaluate scientific fairly, we added the comparison data of GRs and N/PRs using another classification method. It would be ideal if the NRs could be independent for comparison but the number of NRs is overwhelmingly small as in the existing reports and a larger-scale research is needed for this comparison. Additionally, we used only one concentration antibody to stimulate the basophils and might have missed a curve shift of basophil reactivity.

In summary, improvement of attenuated basophil responsiveness via FcεRI stimulation in patients with CSU was associated with the rapid clinical effectiveness of omalizumab. However, further research is needed to elucidate the role of basophils in the mechanism of action of omalizumab in CSU. Although this study had some limitations, including a small number of cases, it highlights the importance of basophil status as an action point of omalizumab in CSU.

Acknowledgments

This work was supported in part by a Grant-in-Aid for Scientific Research (C) (JSPS KAKENHI Grant Number 15K09742 and 16K19722) from the Ministry of Education, Culture, Sports, Science, and Technology, Japan (to A.F. and K.W.). We thank Edanz Group (www.edanzediting.com/ac) for editing a draft of this manuscript.

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546

Figure legends

Fig. 1 Comparison of the baseline proportion of CD203c^{high} basophils between fast responders and non or slow responders.

A. Anti IgE antibody (E124-2-8D) stimulation.

B. Anti IgE antibody (MB10-5C4) stimulation.

C. Anti-FcεR1 antibody (CRA1) stimulation.

Statistical analyses were performed using the Mann-Whitney *U*-test.

Fig. 2 Comparison of the after/before treatment ratio of basophil CD203c responsiveness between fast responders and non or slow responders.

A. Anti IgE antibody (E124-2-8D) stimulation.

B. Anti IgE antibody (MB10-5C4) stimulation.

C. Anti-FcεR1 antibody (CRA1) stimulation.

The dotted line indicates no change (value = 1).

Statistical analyses were performed using the Mann-Whitney *U*-test.

Fig. 3 Comparison of baseline IgE and FcεRI on basophils (MFI) between fast responders and non or slow responders (A, B) or good responders and non or partial responders (C, D).

A, C. IgE expression.

B, D. FcεRI expression (CRA1).

Statistical analyses were performed using the unpaired *t*-test (A, C) or Mann-Whitney *U*-test (B, D).

MFI, mean fluorescence intensity.

Fig. 4 Comparison of changes in IgE or FcεRI levels on basophils (MFI) between fast responders and non or slow responders (A, B) or good responders and non or partial responders (C, D).

A, C. ΔIgE expression.

B, D. ΔFcεRI expression.

Statistical analyses were performed using the unpaired *t*-test (A, C) or Mann-Whitney *U*-test (B, D).

MFI, mean fluorescence intensity.

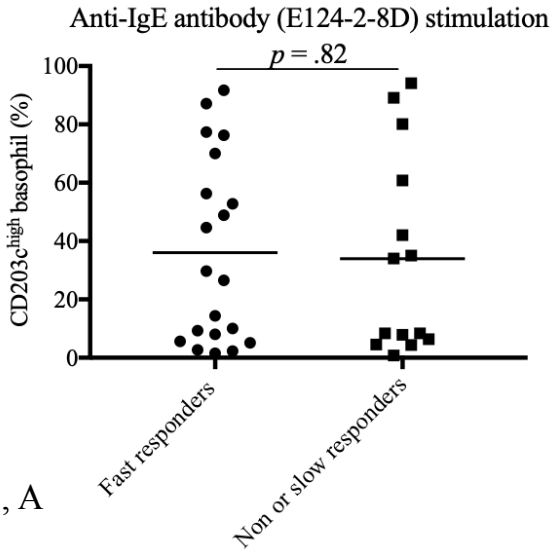
Fig. 5 Comparison of changes in total serum IgE levels and circulating basophil counts between fast responders and non or partial responders or good responders and non or partial responders (C, D).

583 A, C. Δtotal serum IgE; increase of total serum IgE.
584 B, D. Δbasophil counts; increase of circulating basophil counts.
585 The dotted line indicates no change.
586 Statistical analyses were performed using the Mann-Whitney *U*-test (A) or unpaired *t*-test (B).
587 Three patients among N/PRs did not have data of total serum IgE and basophil count either
588 before or after treatment and these patients were excluded from the GRs vs. N/PRs dataset (Fig.
589 5C, D).
590
591 Fig. 6 Sequential changes in the parameters of *in vivo* omalizumab treatment (on 4 weeks of
592 treatment) (A, B) and *in vitro* pre-treatment basophils with omalizumab in fast responders (C,
593 D).
594 A. Urticaria control test.
595 B, C. $CD203c^{high}$ basophils (%) stimulated with anti-IgE antibody (E124-2-8D).
596 D. $CD203c^{high}$ basophils (%) stimulated with anti-IgE antibody (MB10-5C4).
597 MFI, mean fluorescence intensity.

598 Table I. Demographic characteristics based on the rapid therapeutic effect of omalizumab

	Fast responders (n = 20)	Non or slow responders (n =14)	*P* value
Age, years	44.9 ± 4.4	52.7 ± 5.0	.29
Female, n (%)	12 (63.1%)	9 (64.2%)	>.99
Disease duration, years	2 (0.2–33)	3 (0.2–25)	.83
Total IgE (IU/ml)	126 (22.9–2535)	185 (3–4393)	.89
Basophil counts (/μl)	18.3 (0–118)	23 (0–98)	.41
ASST positive rate, n (%)	3/9 (33.3%)	4/6 (66.6%)	.31
UCT	6.1 ± 2.8	5.5 ± 2.7	.54
UAS7	22.9 ± 2.1	21.7 ± 3.4	.76
DLQI	7.0 (2–24)	7.0 (2–17)	.80

599 Data are given as the mean ± SD for age, UCT and UAS7; n (%) for sex, ASST positive rate;
600 median (range) for disease duration, serum total IgE, basophil counts, and DLQI.
601 Statistical differences between two groups were analyzed by unpaired *t*-test for age, UCT, and
602 UAS7, Fisher's exact test for female and ASST positive rate, and Mann-Whitney *U*-test for
603 disease duration, serum total IgE, basophil counts, and DLQI.
604 ASST, autologous serum skin test; UCT, urticaria control test; UAS7, 7-day urticaria activity
605 score; DLQI, dermatology life quality index.
606



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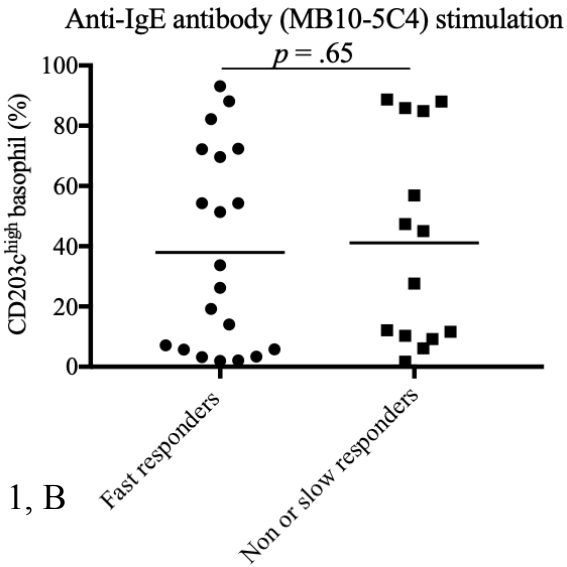

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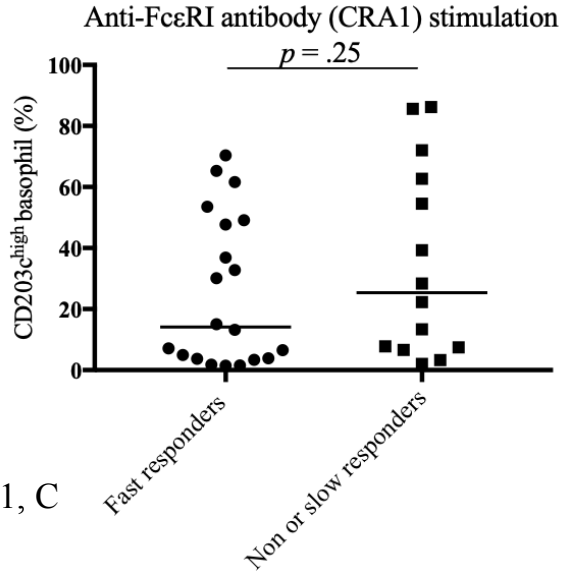

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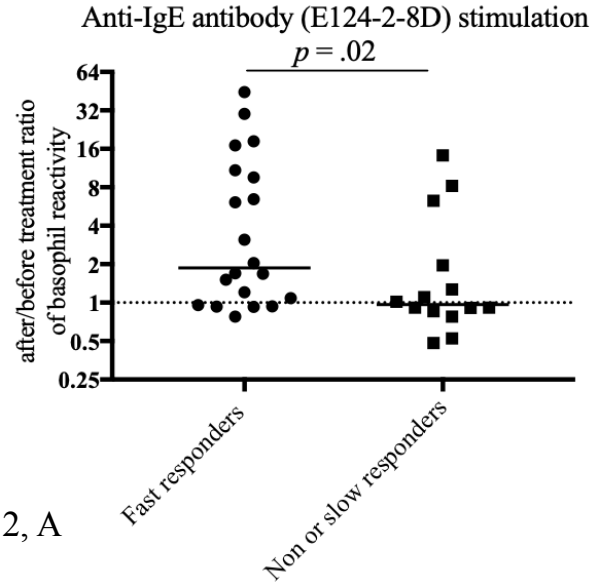

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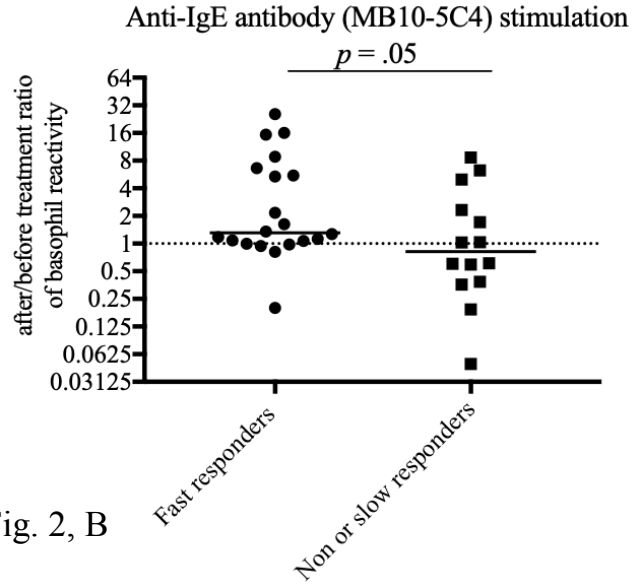

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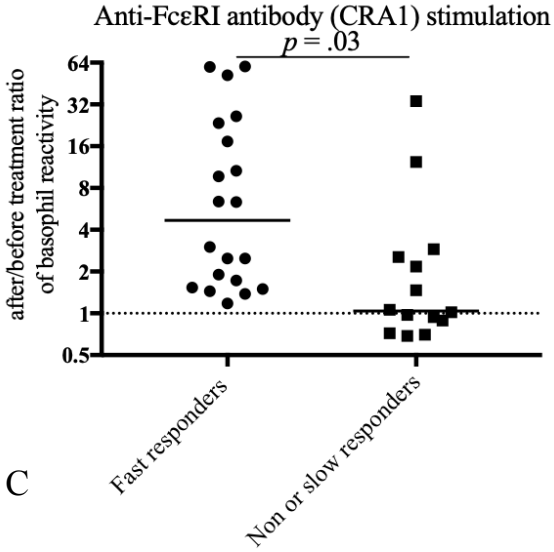

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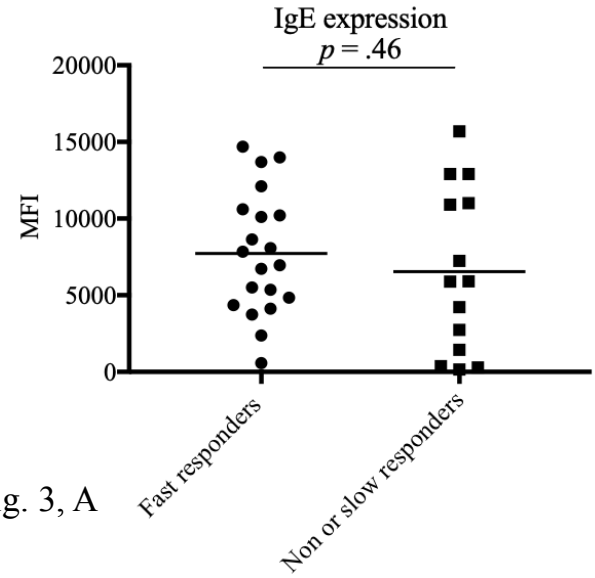

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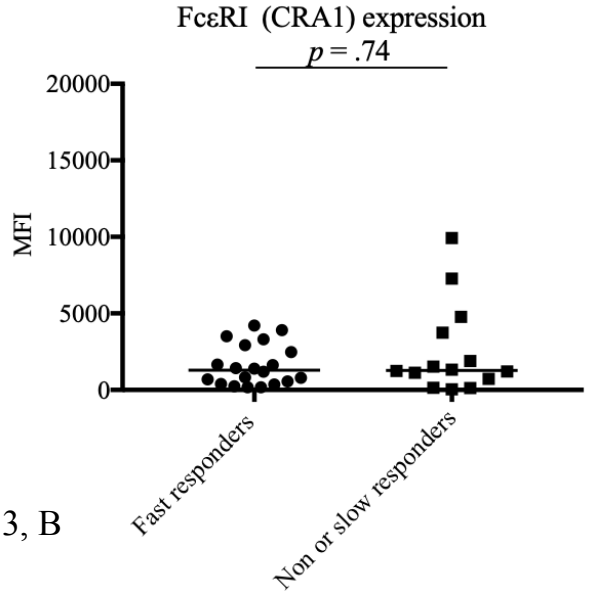

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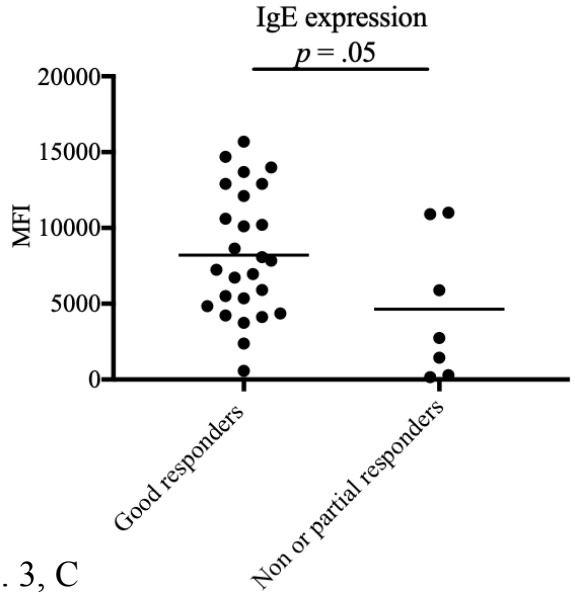

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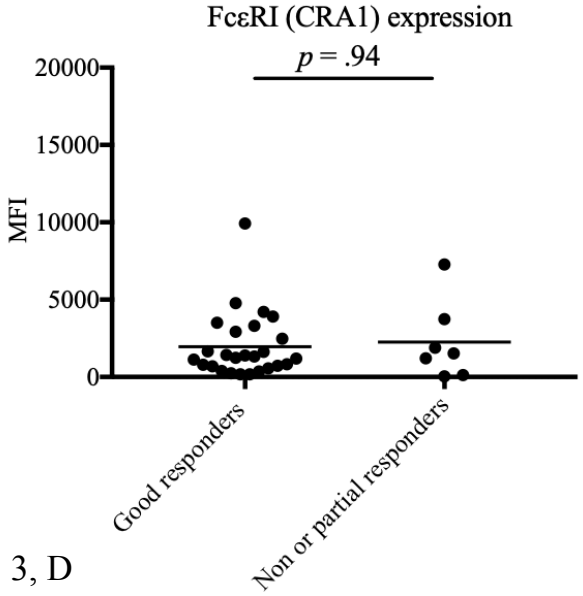

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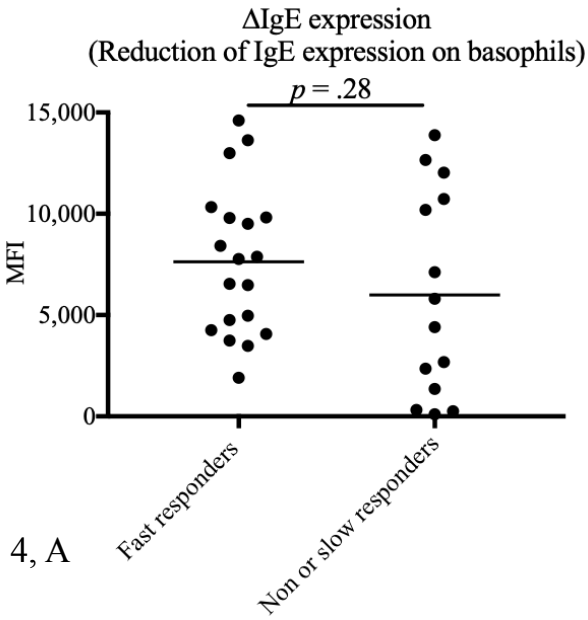

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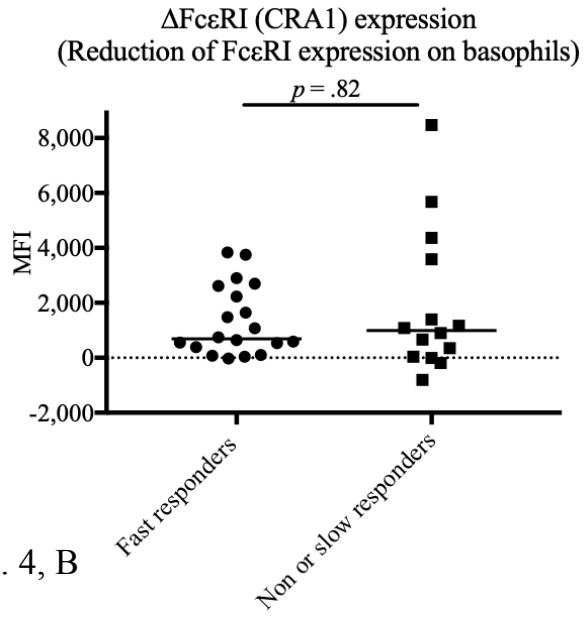

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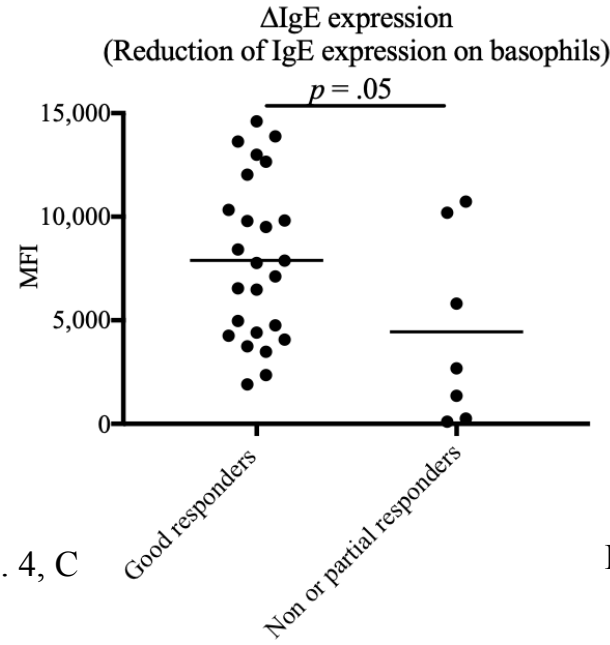

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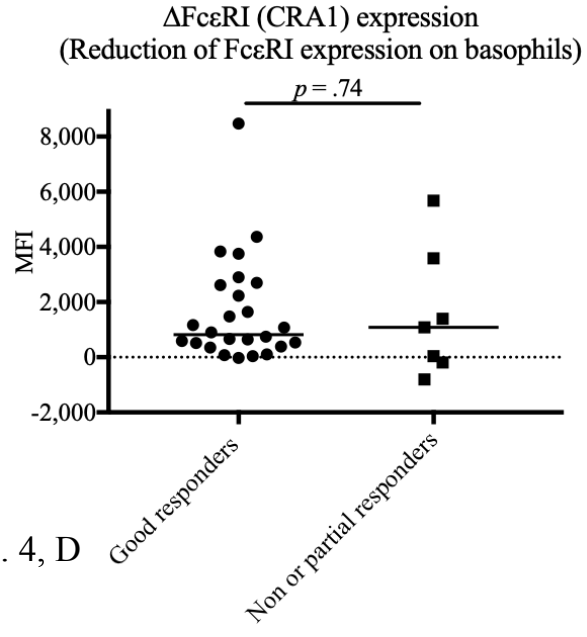

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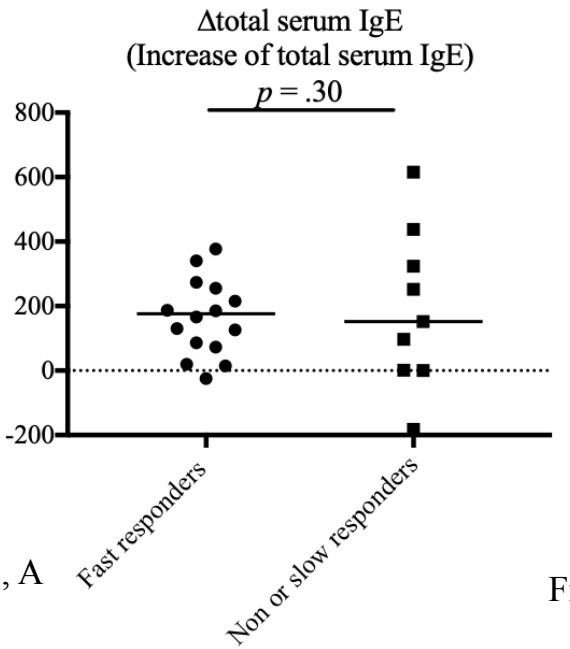

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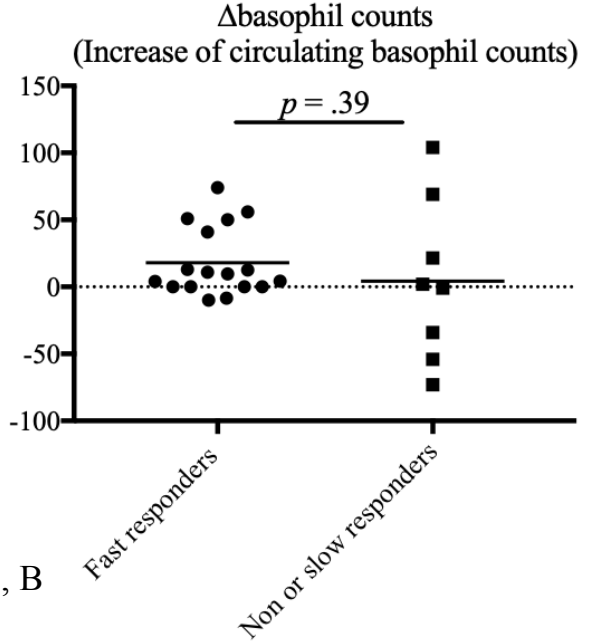

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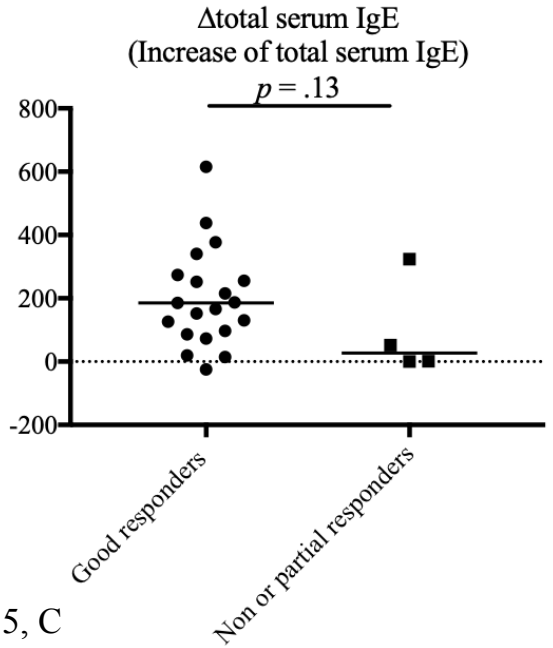

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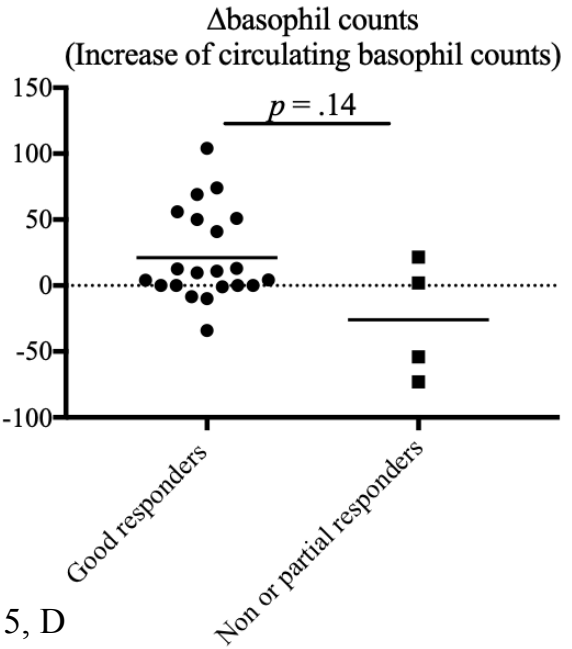

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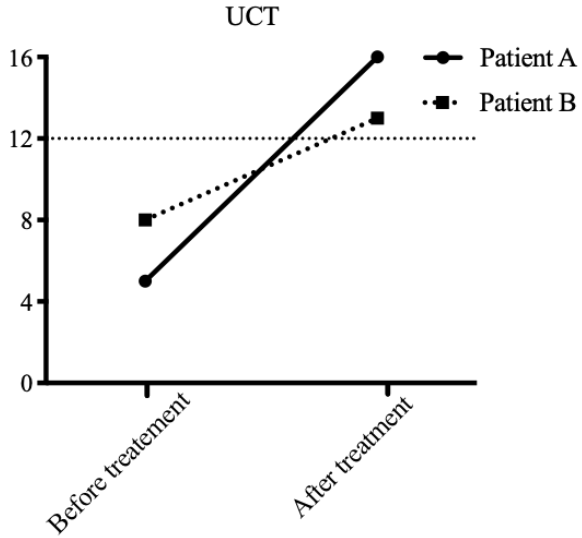

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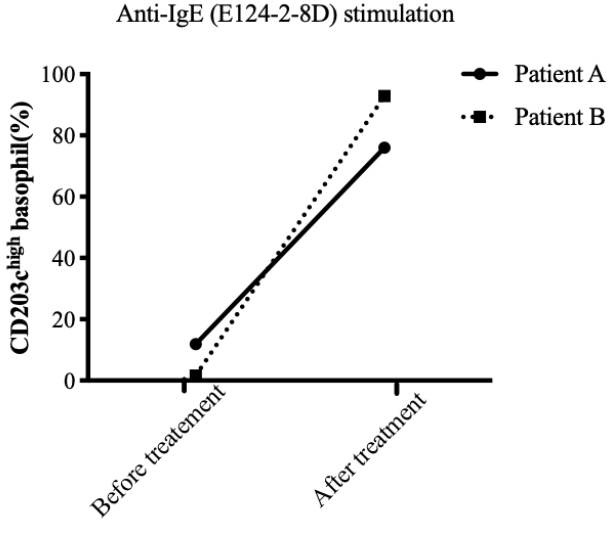

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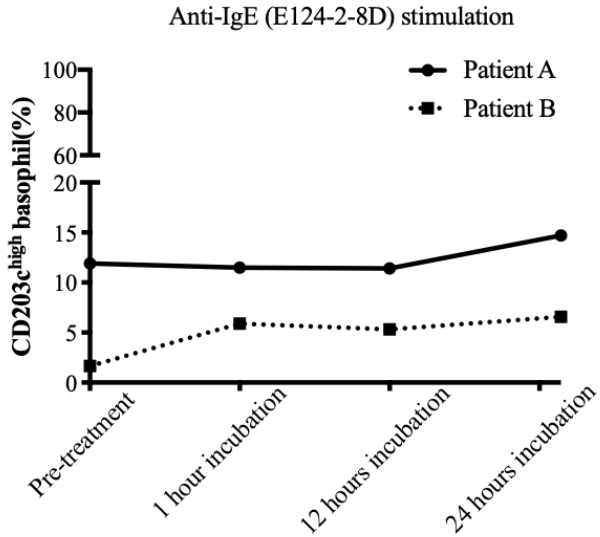

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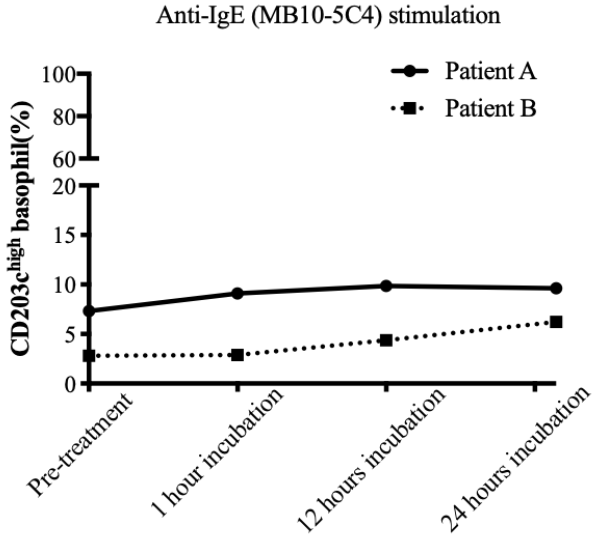


Fig. 6, D

Fig. E1 Definition of each responder to treatment.
Fast responders: UCT scores ≥ 12 increased at week 4.
Non or slow responders: UCT scores < 12 increased at week 4.
Good responders: UCT scores ≥ 12 increased at week 12.
Non or partial responders: UCT scores < 12 increased at week 12.

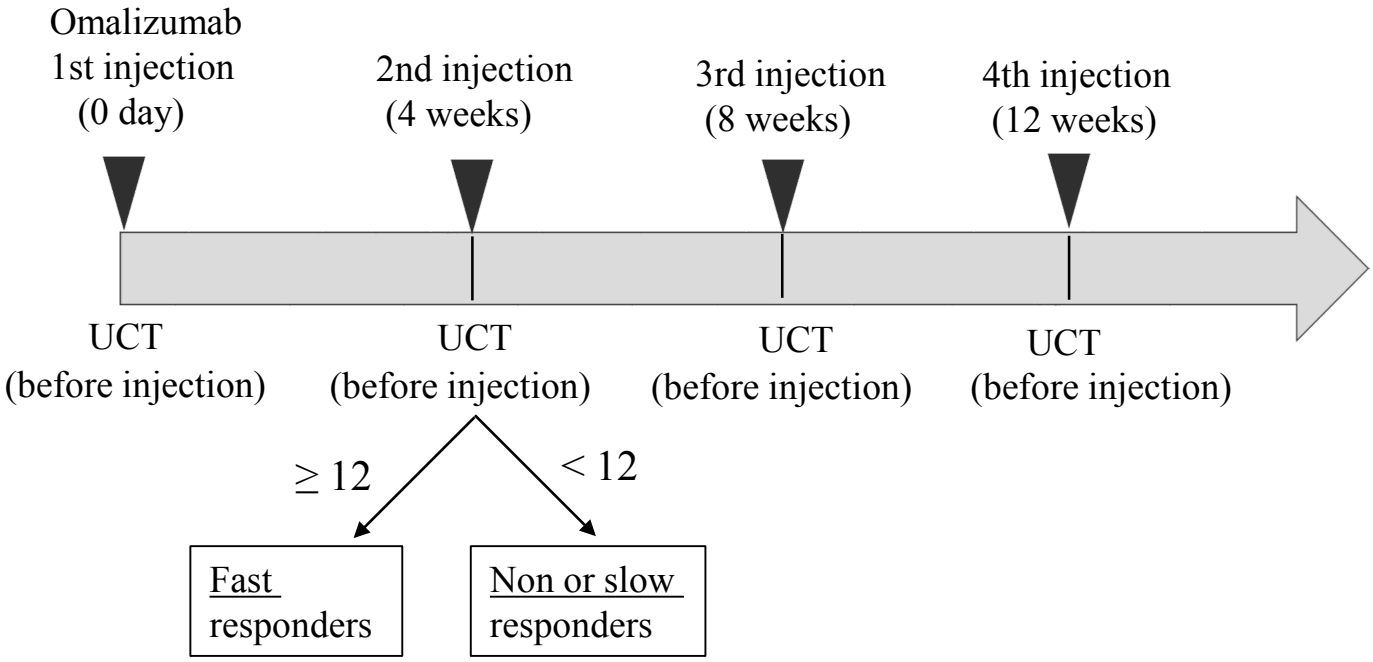
Fig. E2 Changes in urticaria control test scores (UCT) and urticaria activity score 7 (UAS7).
A, C. Fast responders.
B, D. Non or slow responders.
The dotted line indicates the cutoff value of 12.

Fig. E3 Comparison of basophil CD203c expression in the steady state without anti-IgE and/or FcεRI stimulation (MFI) between fast responders and non or slow responders.
Statistical analysis was performed using the unpaired *t*-test.
MFI, mean fluorescence intensity.

Fig. E4 The response to treatment by classifying responders and non-responders on baseline basophil reactivity
Non-responders: <10% CD203c^{high} basophil to anti-IgE stimulation
Responders: >10% CD203c^{high} basophil to anti-IgE stimulation

Fig. E5 Changes in CD203c^{high} basophils before and after treatment with omalizumab in fast responders (A, C, E) and non or slow responders (B, D, F).
A, B. Anti IgE antibody (E124-2-8D) stimulation.
C, D. Anti IgE antibody (MB10-5C4) stimulation.
E, F. Anti-FcεR1 antibody (CRA1) stimulation.

Fig. E6 Correlation of serum total IgE (IU/ml) and basophil IgE expression as MFI in CSU patients.
A. Basophil IgE expression
B. Basophil CRA1 receptor expression
Statistical analyses were performed using Spearman's rank correlation coefficient.
MFI, mean fluorescence intensity; CSU, chronic spontaneous urticaria.


Omalizumab
1st injection
(0 day)
2nd injection
(4 weeks)
3rd injection
(8 weeks)
4th injection
(12 weeks)
UCT
(before injection)
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UCT
(before injection)
UCT
(before injection)
≥ 12
< 12
Fast
responders
Non or slow
responders

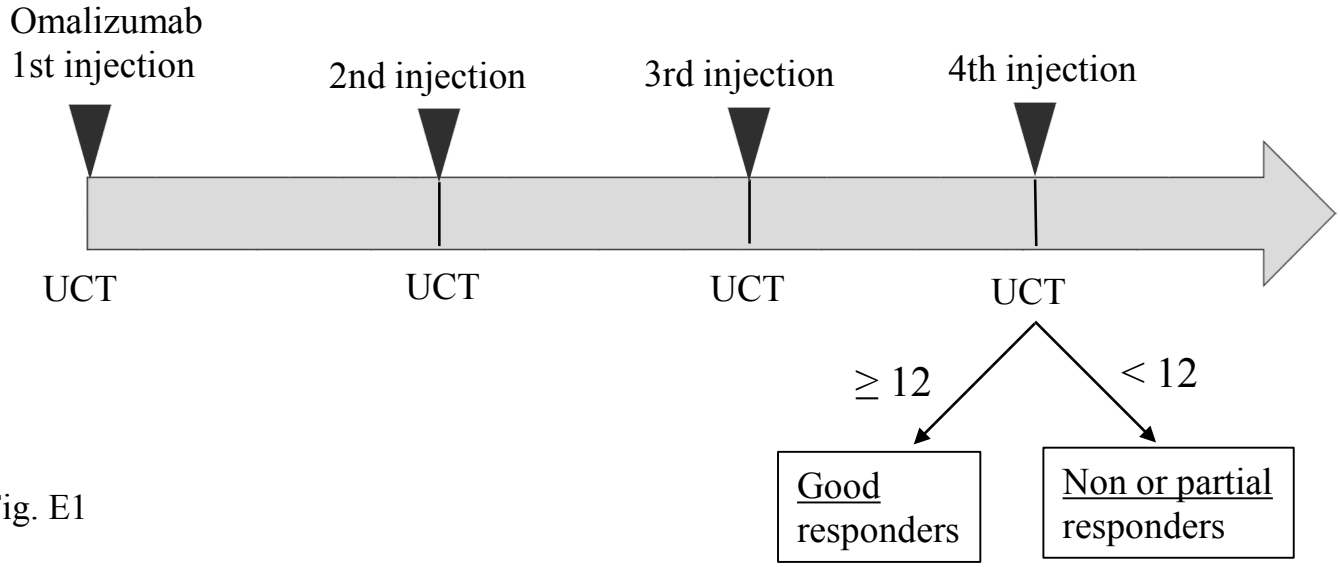
Omalizumab
1st injection
2nd injection
3rd injection
4th injection
UCT
UCT
UCT
UCT
≥ 12
< 12
Good
responders
Non or partial
responders

Fig. E1

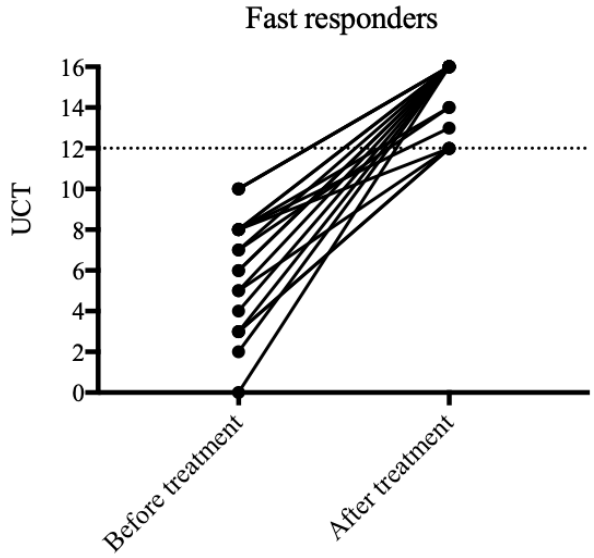

Fig. E2, A

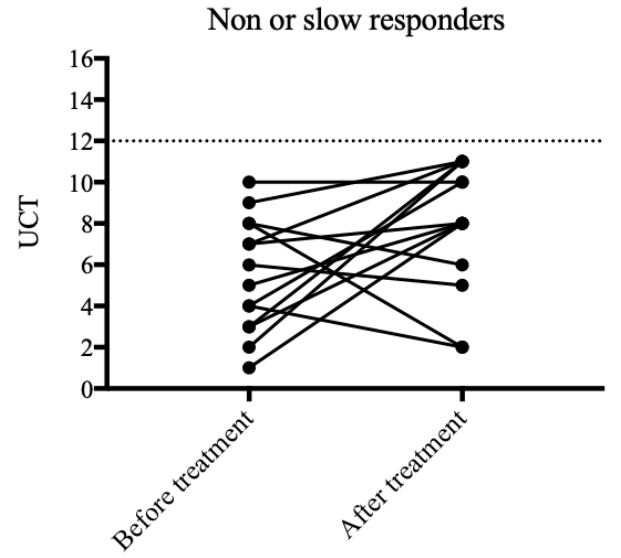

Fig. E2, B

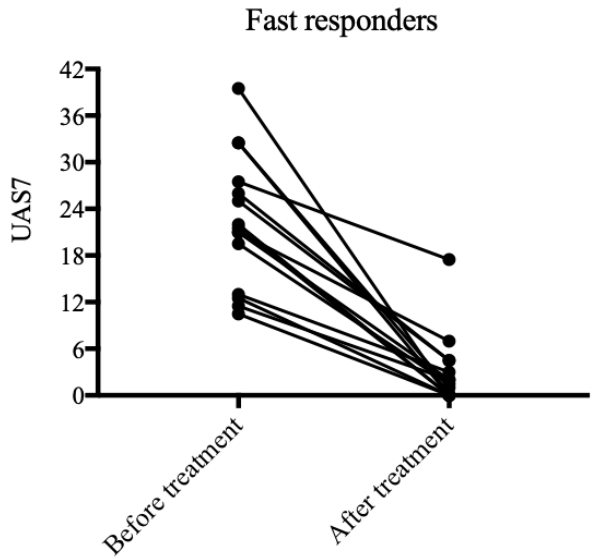

Fig. E2, C

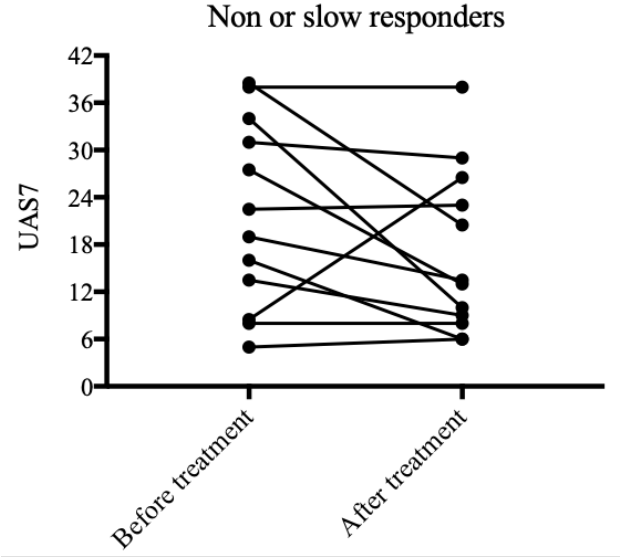

Fig. E2, D

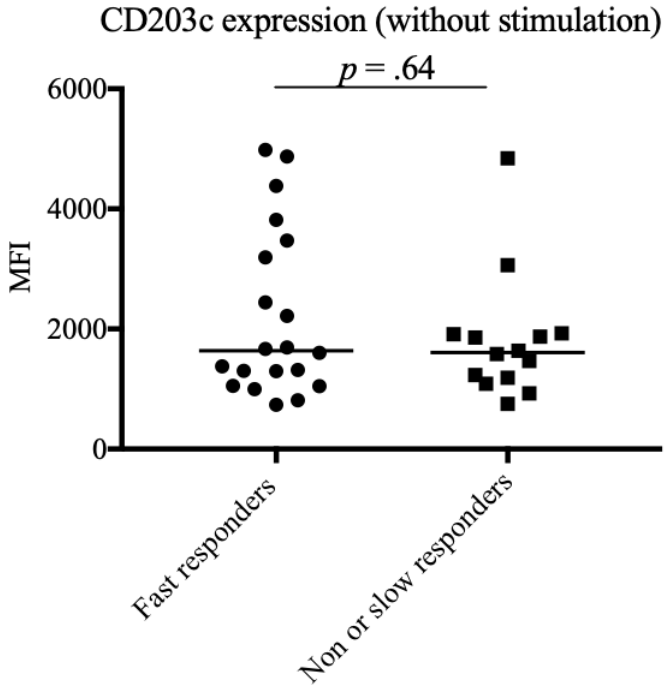
CD203c expression (without stimulation)
p = .64
6000
4000
2000
0
MFI
Fast responders
Non or slow responders

Fig. E3

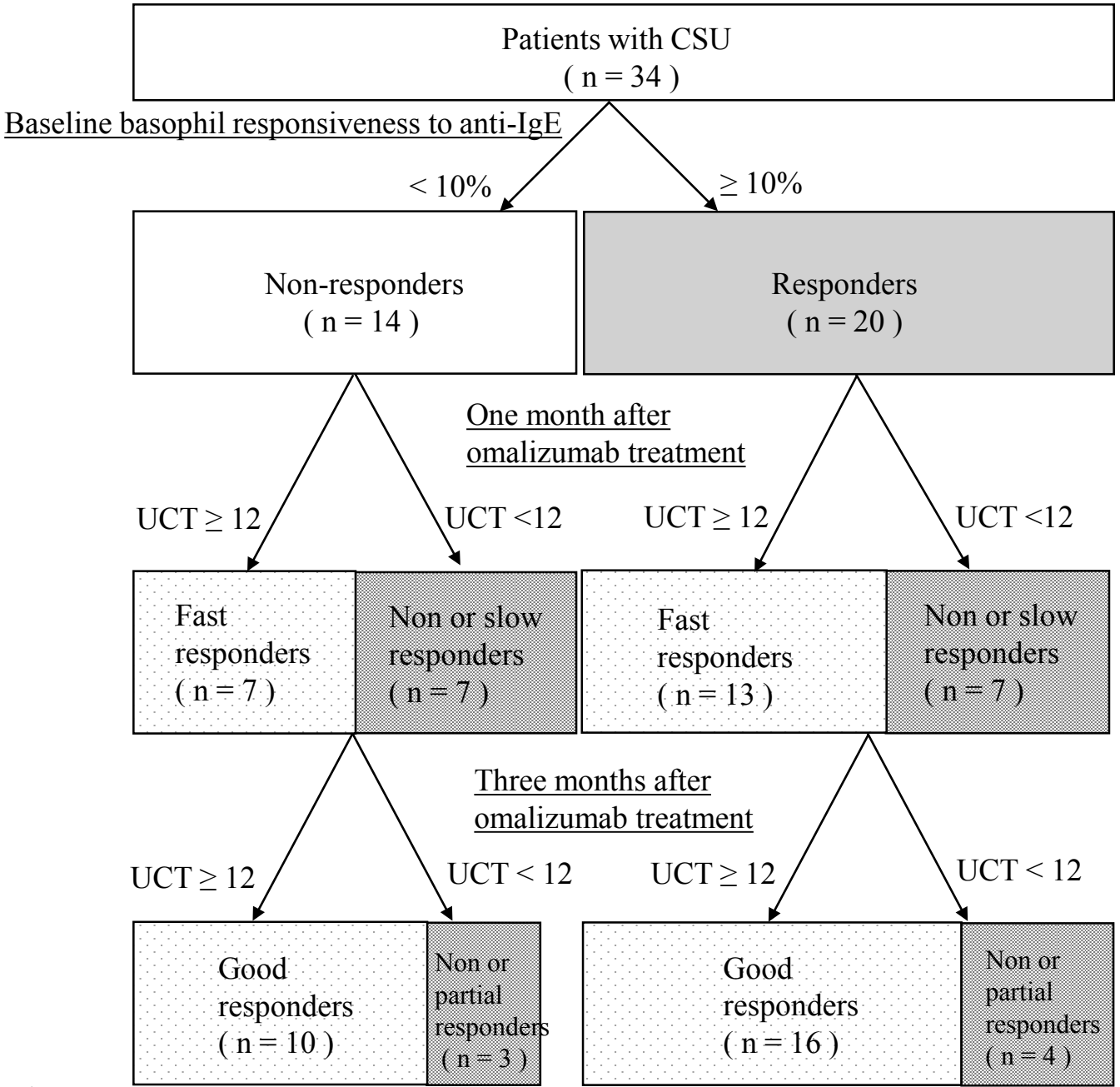
Patients with CSU
(n = 34)
Baseline basophil responsiveness to anti-IgE
< 10%
≥ 10%
Non-responders
(n = 14)
Responders
(n = 20)
One month after
omalizumab treatment
UCT ≥ 12
UCT <12
UCT ≥ 12
UCT <12
Fast
responders
(n = 7)
Non or slow
responders
(n = 7)
Fast
responders
(n = 13)
Non or slow
responders
(n = 7)
Three months after
omalizumab treatment
UCT ≥ 12
UCT < 12
UCT ≥ 12
UCT < 12
Good
responders
(n = 10)
Non or
partial
responders
(n = 3)
Good
responders
(n = 16)
Non or
partial
responders
(n = 4)

Fig. E4

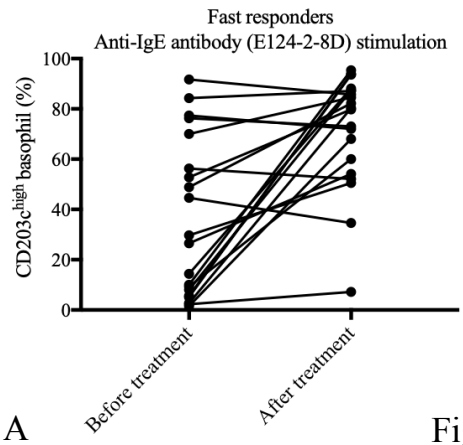

Fig. E5, A

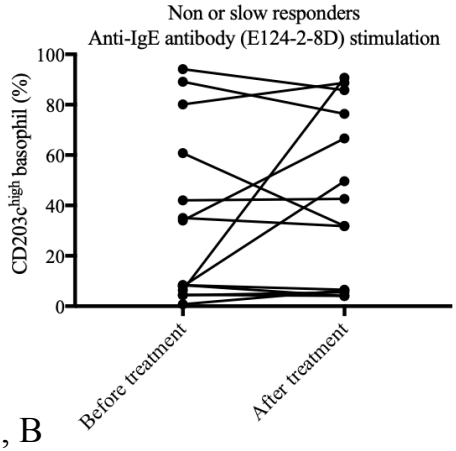

Fig. E5, B

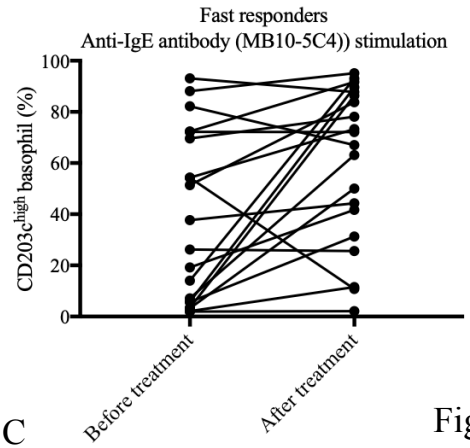

Fig. E5, C

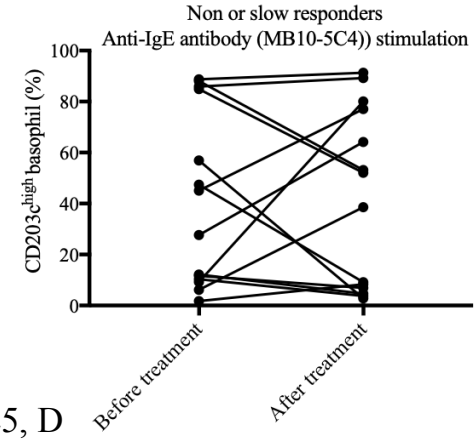

Fig. E5, D

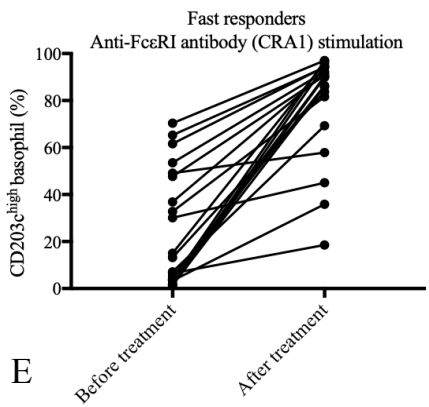

Fig. E5, E

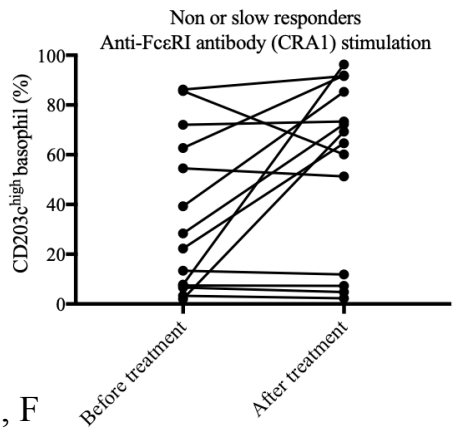

Fig. E5, F

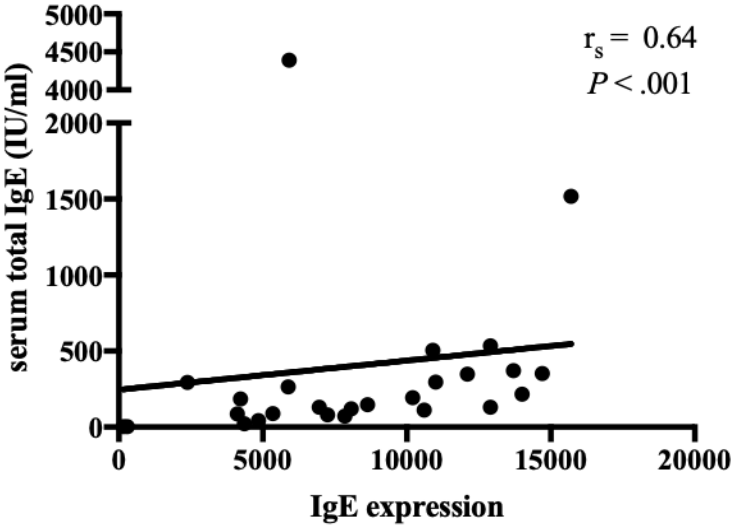

Fig. E6, A

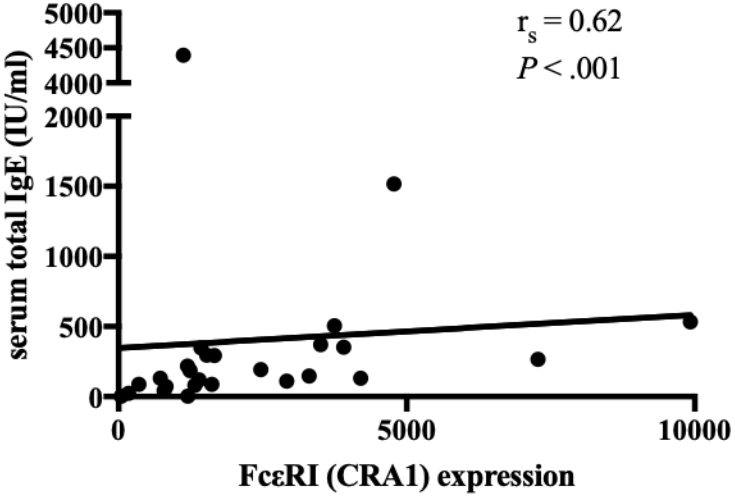

Fig. E6, B

Table EI. Demographic characteristics based on the good therapeutic effect of omalizumab

	Good responders (n = 26)	Non or partial responders (n =7)	*P* value
Age, years	45.3 ± 3.6	59.4 ± 7.7	.08
Female, n (%)	18 (69%)	3 (42%)	.37
Disease duration, years	3 (0.2–33)	4 (0.5–25)	.61
Total IgE (IU/ml)	139.5 (22.9–4393)	163.2 (3–505)	.40
Basophil counts (/μl)	18 (0-98)	28 (0–73)	.47
ASST positive rate, n (%)	5/12 (41%)	2/3 (66%)	.56
UCT	6.1 ± 2.8	5.5 ± 2.0	.63
UAS7	23.0 ± 9.1	19.6 ± 12.4	.45
DLQI	7 (2–24)	5.5 (2–14)	.51

Data are given as the mean ± SD for age, UCT and UAS7; n (%) for sex, ASST positive rate; median (range) for disease duration, serum total IgE, basophil counts, and DLQI.

Statistical differences between two groups were analyzed by unpaired *t*-test for age, UCT, and UAS7, Fisher's exact test for female and ASST positive rate, and Mann-Whitney *U*-test for disease duration, serum total IgE, basophil counts, and DLQI.

ASST, autologous serum skin test; UCT, urticaria control test; UAS7, 7-day urticaria activity score; DLQI, dermatology life quality index.

One patient among N/PRs did not have data of total serum IgE and basophil count before treatment and the patient was excluded from the GRs vs. N/PRs dataset.

Table EII. Basophil responsiveness with stimulation before and after treatment with omalizumab in chronic spontaneous urticaria patients

	No	Age	Sex	Total IgE (IU/ml)	Stimulation (proportion of CD203c^{high} basophil)					
					Anti-IgE antibody (%) (E124-2-8D)		Anti-IgE antibody (%) (MB10-5C4)		Anti- FcεR1 antibody (%) (CRA1)	
					Before treatment	After treatment	Before treatment	After treatment	Before treatment	After treatment
Fast responders	1	37	M	121	9.28	60	5.68	31.3	7.14	69.3
	2	20	F	131	2.33	7.23	2.14	11.5	3.37	35.9
	3	57	F	148	52.8	79.8	72.2	72.1	32.8	81.6
	4	17	M	111	44.6	34.6	54.3	10.8	49.1	57.9
	5	32	F	73	14.4	88.1	19.2	41.7	15	95.9
	6	49	F	294	29.7	50.5	51.4	83.8	36.9	91.5
	7	35	M	216	87.1	94.2	88.1	95.1	70.4	97
	8	40	F	193	10	95.4	14	93	1.82	94.7
	9	41	F	86.9	1.53	68	1.97	2.1	1.51	89.9
	10	58	F	371	70	84.5	72.4	91.7	53.5	92.3
	11	75	M	22.9	8.03	87.4	7.13	63.1	3.85	90.2
	12	39	M	17.1	77.4	72	69.6	78.1	4.99	86.4
	13	75	M	41.9	76.3	73	82.2	67	65.3	94.1
	14	45	F	88.8	5.13	93.9	3.37	86.4	1.43	85.9
	15	74	F	282	56.3	52.1	37.7	44.3	30.1	45
	16	45	M	347	91.7	85.7	93.1	87.8	61.6	94.3
	17	21	F	2535	2.67	80.3	5.83	89.6	3.69	96.9
	18	74	F	81.5	26.5	54.1	26.2	25.6	13.2	83.7
	19	20	F	n.d.	48.9	82.2	54.3	73.3	47.7	90.6
	20	59	F	n.d	5.63	93.6	3.17	50	6.52	18.5
Non or slow responders	1	73	M	n.d.	42	42.6	47.4	9.11	28.4	72.3
	2	73	M	265	80.1	88.7	84.9	52	62.7	91.9
	3*	23	F	295	34	66.6	45	77	39.3	85.3
	4	51	M	505	8.39	4.04	10.3	3.7	13.4	11.9
	5	63	F	<3	4.55	4.16	12.1	4.64	6.61	4.76
	6	49	M	<3	4.35	5.53	11.6	6.86	7.45	7.26
	7	23	F	534	94.1	85.8	85.9	89.2	86.2	91.6
	8*	61	F	80.2	6.38	90.7	9.21	80	7.82	96.3
	9*	36	F	1518	35	31.8	88	53.1	85.6	60.1
	10*	42	M	4392	8.37	6.52	27.6	64.1	22.3	54.6
	11*	68	F	131	7.91	49.6	6.16	38.5	2.05	69.3
	12*	38	F	185	89.1	76.4	88.7	91.3	72	73.4
	13	54	F	8.4	0.76	6.25	1.67	8.33	3.28	2.25
	14	84	F	7.4	60.8	31.9	56.9	2.78	54.5	51.3

CD203c expression after antibody stimulation before and after treatment with omalizumab is described as CD203c^{high} basophils (%). FRs, fast responders (urticaria control test (UCT) scores ≥ 12 up to the end of week 4 after starting treatment); N/SR, non or slow responders (UCT scores < 12 up to the end of week 4 after starting treatment).

* Patients of the 14 N/SRs at 4 week who became GRs at week 12.