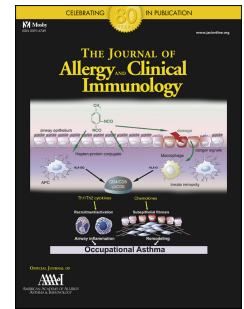


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Autoreactive T cells in chronic spontaneous urticaria target the IgE Fc receptor α subunit

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1 **TITLE PAGE**

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6 **Iα subunit**

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ABSTRACT

Background

Chronic spontaneous urticaria (CSU) is the recurrence of urticaria without an apparent trigger. Half of CSU patients have IgG autoantibodies to FcεRIα on dermal mast cells and basophils, which upon activation release mediators responsible for urticaria. IgG autoantibodies infer the presence of antigen/disease-specific T cells and CSU lesions are characterized by T-cell infiltration, but antigen/disease-specific T cells have not been documented in CSU.

Objective

We aimed to identify autoreactive T cells to FcεRIα in CSU, determine their relationship with autoantibodies to FcεRIα and their diagnostic value.

Methods

T-cell responses to FcεRIα were measured as proliferation by CFSE dye dilution and cytokine secretion by ELISpot. Serum autoantibodies to FcεRIα were detected by radioimmunoprecipitation.

Results

Blood CD4⁺ T-cell proliferation to FcεRIα was detected in 27% of CSU subjects and 0% of controls; IFN-γ responses to FcεRIα were detected in 53%, and IL-5 or IL-13 responses in a minority of CSU subjects. Serum FcεRIα autoantibodies were detected in 43% of CSU and 0% of controls. IFN-γ and autoantibody responses to FcεRIα were inversely related, with IFN-γ responses being detected earlier in disease than

autoantibodies. Combined with autoantibody, T-cell responses to FcεRIα conferred diagnostic high sensitivity and specificity.

Conclusion

Autoreactive CD4⁺ T cells that target FcεRIα were detected in a majority of CSU subjects, with a cytokine secretion profile more typical of a Th1-cell response. The inverse relationship between IFN-γ and autoantibody responses to FcεRIα may signify different stages in the disease course. Our findings suggest that measurement of T-cell as well as autoantibody responses to FcεRIα could improve diagnostic accuracy in CSU.

Key messages

- Autoreactive CD4⁺ T cells to FcεRIα with a Th1 profile are present in the blood of a majority of CSU subjects.
- IFN-γ and autoantibody responses to FcεRIα are inversely related, possibly signifying different stages in the natural history of CSU.
- Detection of T-cell as well as autoantibody responses to FcεRIα could improve diagnostic accuracy in CSU.

Capsule summary

Autoreactive CD4⁺ T cells to FcεRIα with a Th1 profile, in an inverse relationship with autoantibodies to FcεRIα, are present in the blood of a majority of CSU subjects.

Key words

Chronic spontaneous urticaria; FcεRIα; IgE; autoantibody; autoreactive T cell; cytokine; immune deviation

Abbreviations used

2-ME: 2-mercaptoethanol

BSA: Bovine serum albumin

CFSE: Carboxy-fluorescein diacetate succinimidyl ester

CSU: Chronic spontaneous urticaria

cpm: Counts per minute

CV: Coefficient of variation

DC: Disease control

EDTA: Ethylenediaminetetraacetic acid

- 93 ELISpot: Enzyme-linked immunospot
94 FcεRIα: High-affinity IgE receptor alpha subunit
95 HC: Healthy control
96 HPS: Human pooled serum
97 Na¹²⁵I: Iodine-125 radionuclide
98 NEAA: Non-essential amino acids
99 PBMC: Peripheral blood mononuclear cell
100 PBS: Phosphate-buffered saline
101 PI: Propidium iodide
102 IL-7: Interleukin-7
103 TT: Tetanus toxoid
104 UAS: Urticaria Activity Score
105

INTRODUCTION

Chronic spontaneous urticaria (CSU) is the recurrence of urticaria for at least 6 weeks with no apparent trigger.¹ Although often thought to be benign, even by physicians,² surveys reveal the significant negative impact of CSU on quality of life.^{2,3} CSU was classified as an autoimmune disease following identification of serum IgG autoantibodies to the high-affinity IgE receptor alpha subunit (FcεRIα), or to FcεRIα-bound IgE, on mast cells and basophils.^{4,5} These autoantibodies, most often measured functionally by histamine release assay, have been detected in approximately 50% of CSU patients,⁶⁻⁹ with anti-FcεRIα being more frequent than anti-IgE autoantibodies.¹⁰ Both autoantibodies crosslink FcεRI molecules to induce histamine release, which is the accepted pathogenic mechanism in CSU.⁵

Questions remain, however, about the mechanisms of CSU, because of failure to detect autoantibodies in half of the patients and concern about the specificity of autoantibodies, which have been reported in 0-55% of healthy controls^{7,9,11} and 11% of patients with other urticarial disorders.¹² Moreover, unlike other antibody-mediated skin diseases, e.g. pemphigus vulgaris,¹³ antibody deposition is not a characteristic of urticarial lesions.^{6,14,15} The presence of autoantibodies implicates autoantigen-primed T cells, required to generate high-affinity IgG antibodies by interaction with cognate B cells in germinal centres of secondary lymphoid organs.¹⁶ Urticarial lesions in fact display abundant T-cell infiltration, particularly by CD4⁺ T cells,¹⁷⁻¹⁹ and the role of T cells is indirectly supported by the reported association of CSU with certain HLA class II alleles, particularly DR4,²⁰⁻²² which could potentially present FcεRIα peptides to T cells to initiate the disease. In addition, cytokine

responses to non-specific T-cell stimulation have been reported to be increased in CSU²³ and T-cell suppressive agents such as cyclosporine A are effective in the treatment of CSU.^{24,25} Nevertheless, T cells with specificity for FcεRIα have not been described in CSU.

We aimed to identify autoreactive T cells specific for FcεRIα in CSU, characterize their phenotype, determine their relationship with autoantibodies to FcεRIα and their diagnostic value. This knowledge would provide insight into the disease mechanism and provide a foundation for novel, antigen-specific immunotherapy.

METHODS

Subjects

Subjects were recruited from the Royal Melbourne Hospital (RMH) and the Walter and Eliza Hall Institute (WEHI) Volunteer Blood Donor Registry between 2011 and 2014 (Table I). They comprised 60 with CSU, having recurrent urticaria for at least 6 weeks with no apparent trigger,¹ 20 disease control (DC) with other urticarial disorders (10 with food allergy, 4 with urticarial vasculitis, 2 with urticaria pigmentosa, and 1 each with pressure urticaria, cholinergic urticaria, cold-induced urticaria and bee venom allergy) and 20 healthy control (HC) subjects with no history of urticaria. The study was approved by the Human Research Ethics Committees of RMH and WEHI.

19/60 (31%) of CSU and 13/20 (65%) of DC subjects were able to withhold oral H1 and H2 antihistamines, leukotriene receptor antagonist and mast cell stabilizing agents for 72 hours, and immunosuppressive medications including corticosteroids for 6 weeks, before sample collection. Clinical data, including disease duration and activity as quantified by the Urticaria Activity Score (UAS),²⁶ were collected from the CSU subjects. Sequence-based genotyping at the HLA-DRB1 locus was performed by the Victoria Transplantation Immunogenetics Service.

Antigens

Human recombinant FcεRIα protein was produced by CSL Limited, Melbourne. Briefly, the DNA construct in the mammalian expression vector pcDNA3.1 encoding the extracellular domain of FcεRIα fused to a His-tag was expressed in the FS293-F

mammalian host cell line. After purification by tandem nickel and size exclusion chromatography, the protein resolved as a single species of correct molecular weight in matrix assisted laser desorption/ionization-time of flight mass spectrometry. The molecular weight of the recombinant FcεRIα protein was calculated to be 25 kDa based on its amino acid content, but it resolved as a broad band at ~49 kDa by Western blotting. As we were interested in the product as a T-cell protein antigen, working concentrations were calculated using the protein molecular weight of 25 kDa. The endotoxin concentration of the protein stock at 1.2 mg/ml solution was <0.25 EU/ml. As a negative control antigen, the extracellular domain of human recombinant FcγRIIα protein was similarly generated by CSL Limited. Tetanus toxoid (TT, CSL Limited) was used to determine the reproducibility of T-cell assays (see below).

T-cell proliferation to FcεRIα

T-cell proliferation was measured as previously described.²⁷ Thawed peripheral blood mononuclear cells (PBMCs) were labeled with carboxy-fluorescein diacetate succinimidyl ester (CFSE, Invitrogen) at 0.1 μM per 1x10⁷ cells in phosphate-buffered saline (PBS) for 5 min at 37°C. The reaction was stopped with RP5 complete culture medium [RPMI (Gibco), 5% human pooled serum (HPS, Australian Red Cross Blood Transfusion Service), 100 μM non-essential amino acids (NEAA, Gibco), 2 mM Glutamax (Gibco), 5x10⁻⁵ M 2-mercaptoethanol (2-ME, Sigma-Aldrich)] and cells were washed with PBS. Labeled cells were cultured in 96-well round-bottom plates in replicates of at least three at 5x10⁵ cells/well with medium alone, FcεRIα, FcγRIIα or TT for 7 days at 37°C in 5% CO₂ air. Cells were then washed with buffer [0.5% bovine serum albumin (BSA, Sigma-Aldrich), 1 mM

ethylenediamine tetraacetic acid (EDTA, Sigma-Aldrich) in PBS] and stained with mouse anti-human CD4-PE (clone RPA-T4, BD Pharmingen), mouse anti-human CD8-APC (clone RPA-T8, BD Pharmingen) and propidium iodide (PI, Sigma-Aldrich) for 20 min on ice. Proliferation of PI⁻ CFSE^{lo} CD4⁺ and CD8⁺ cells (% of totals) was quantified by flow cytometry. The median coefficient of variation (CV) for CD4⁺ cell proliferation to 0.1 μ M Fc ϵ RI α was 37% (range 0.24-57%, n = 60) and to 5 LfU/ml TT was 14% (range 2-21%, n = 60).

Enzyme-linked immunospot (ELISpot) assay

To detect IFN- γ responses, thawed PBMCs were first cultured in 96-well round-bottom plates in triplicate at 5×10^5 cells/well in AIM-V serum-free culture medium (Gibco) supplemented with 0.5 ng/ml human recombinant IL-7 (IL-7, R&D Systems) either alone or with Fc ϵ RI α , Fc γ RII α or TT for 24 hours at 37°C in 5% CO₂ air. ELISpot plates (MultiScreen HTS, Millipore) were conditioned by washing with 30% ethanol before being coated with anti-human IFN- γ (clone 1-D1K, Mabtech). Cells were re-suspended, transferred to ELISpot plates and further cultured for 24 hours at 37°C in 5% CO₂ air. Cells were lysed with water and discarded. ELISpot plates were washed with PBS between sequential incubations with biotinylated anti-human IFN- γ (clone 7-B6-1, Mabtech), streptavidin-alkaline phosphatase (Mabtech) and 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium substrate solution (Mabtech). The color reaction was stopped by addition of water. Responses were counted (AID ELISpot Reader System, Autoimmun Diagnostika GMBH) and expressed as IFN- γ spots per 10^6 PBMCs. Subsequently, we found that results were similar when PBMCs were incubated in serum-containing medium directly in ELISpot plates. The median

CV for IFN- γ spots to 0.1 μ M Fc ϵ RI α was 27% (range 0.2-44%, n = 38) and to 5 LfU/ml TT was 24% (range 0.1-34%, n = 38).

To detect IL-5 or IL-13 responses, thawed PBMCs were cultured directly in ELISpot plates pre-coated with anti-human IL-5 (clone TRFK5, Mabtech) or anti-human IL-13 (clone IL13-I, Mabtech), in IP5 complete culture medium [IMDM (Gibco), 5% HPS, 100 μ M NEAA, 2 mM Glutamax, 5×10^{-5} M 2-ME] containing 0.5 ng/ml IL-7, either alone or with Fc ϵ RI α or Fc γ RII α , for 24 hours at 37°C in 5% CO₂ air. Bound cytokines were detected with biotinylated anti-human IL-5 (clone 5A10, Mabtech) or biotinylated anti-human IL-13 (clone IL13-3, Mabtech) and developed as for the ELISpot IFN- γ assay.

Immunoprecipitation assay for autoantibodies to Fc ϵ RI α

The Fc ϵ RI α protein was labeled with iodine-125 radionuclide (Na¹²⁵I) using the chloramine T method. A small aliquot of the iodination mixture [400 ng chloramine T (Sigma-Aldrich), 1 mCi ¹²⁵I-sodium (Perkin Elmer) and 4.8 μ M Fc ϵ RI α in 25 μ l 0.3 M sodium phosphate buffer at pH 7.4 (phosphate buffer)] was added to 1 ml 0.1% BSA/phosphate buffer before addition of 1 ml 10% trichloroacetic acid (Univar), mixing and immediate centrifugation. Radioactivity in the supernatant and pellet was measured in a gamma counter (Genesys Genii, Laboratory Technologies) as counts per minute (cpm), aiming for 30-50% ¹²⁵I incorporation into the Fc ϵ RI α protein. ¹²⁵I-Fc ϵ RI α was purified on a Sephadex G-50 column (Sigma-Aldrich) eluted with 0.1% BSA/phosphate buffer. The peak radioactive fraction was diluted in buffer to $\sim 2 \times 10^8$ cpm/ml. Based on column separation of bound and free radioactivity, the specific activity of the ¹²⁵I-Fc ϵ RI α was ~ 80 μ Ci/ μ g.

Serum IgG was captured by mixing 200 µl serum at 1:100 in 0.5% BSA/PBS with Protein G-Sepharose for 1 hour at 4°C. After washing with 0.5% BSA/PBS, centrifuged IgG-Protein G-Sepharose complexes were incubated in triplicate with ^{125}I -FcεRIα (~2x10⁶ cpm or 1.4 ng protein) overnight at 4°C. After centrifugation and washing, radioactivity in the complexes was counted (cpm_{sample}). Total radioactivity (cpm_{total}) was measured in the absence of serum, and blank radioactivity (cpm_{blank}) in the absence of ^{125}I -FcεRIα and serum. Binding (%) of IgG to FcεRIα in precipitated complexes was calculated as:

$$(\text{cpm}_{\text{sample}} - \text{cpm}_{\text{blank}}) / (\text{cpm}_{\text{total}} - \text{cpm}_{\text{blank}}) \times 100$$

To determine antigen specificity of binding, IgG-Protein G-Sepharose complexes were pre-incubated with 2 µM unlabelled FcεRIα for 1 hour at room temperature before incubation with ^{125}I -FcεRIα as described above.

Statistics

All assays for T cells and antibodies were not performed on the total of 60 CSU and 20 DC subjects because they were clinic patients and the volumes of their blood donations varied and were not always sufficient. The Wilcoxon matched-pairs signed rank test was used to compare responses to medium alone and to antigens within each subject group. To compare subject groups, results were normalized by subtracting responses to medium alone from responses to antigen before analysis by the Kruskal-Wallis test. The cut-off for a positive response to antigen was derived from normalized HC results and defined as >2 interquartile ranges above the median HC value. Spearman correlation, Fisher's exact test or Mann-Whitney test were used to

265 compare clinical parameters and responses. P values <0.05 were regarded as
266 significant.

267

RESULTS**CD4⁺ T-cell proliferation to FcεRIα is increased in a proportion of CSU subjects**

CD4⁺ T cells from some CSU subjects proliferated in response to FcεRIα but not FcγRIIα (example in Figure 1A and B). Dose-ranging studies showed that optimum responses were obtained with FcεRIα at 0.1 μM (Figure 1B). The CSU group but not control groups exhibited increased CD4⁺ T-cell proliferative responses to FcεRIα (Figure 1C). Significant proliferative responses to FcεRIα (>2 interquartile ranges above the median value of the HC group) were detected in 16/60 (27%) of CSU but not DC or HC subjects. Significant CD8⁺ T-cell proliferative responses to FcεRIα were detected in 10/60 (17%) of CSU, 1/20 (5%) of DC and none of HC subjects, but responses were no different overall between the groups (Figure 1D).

IFN-γ but not IL-5 or IL-13 responses to FcεRIα are increased overall in CSU

PBMCs from some CSU subjects secreted IFN-γ, IL-5 and IL-13 in response to FcεRIα but not FcγRIIα (Figure 2A). Dose-ranging studies showed that optimum responses occurred at 0.1 μM FcεRIα. The CSU group but not control groups exhibited increased IFN-γ and IL-13 responses to FcεRIα (Figure 2B). When compared with the control groups, however, the CSU group had a significant increase only in IFN-γ to FcεRIα (Figure 2C). In the CSU group, 20/38 (53%), 12/38 (32%) and 4/38 (11%) had significant IFN-γ, IL-5 and IL-13 responses (>2 interquartile ranges above the median value of the HC group), respectively. In the control groups, IFN-γ responses were detected in none of DC and 1/20 (5%) of HC, IL-5 responses in none of DC or HC, and IL-13 responses in none of DC and 1/20 (5%) of HC subjects.

Basal IL-5 and IL-13 were higher in CSU than in DC but not HC subjects

(Figure 2B).

IFN- γ and autoantibody responses to Fc ϵ RI α are inversely related

Precipitation of ^{125} I-Fc ϵ RI α by IgG in serum from CSU subjects was significantly greater than from DC and HC subjects, with anti-Fc ϵ RI α IgG being detected in 20/46 (43%) of CSU but none of the DC or HC subjects (Figure 3).

Complete data for assays of CD4 $^{+}$ T-cell proliferative, IFN- γ , IL-5 and IL-13 ELISpot, and autoantibody responses to Fc ϵ RI α were available for 30 of the 60 CSU subjects. An inverse relationship between IFN- γ and autoantibody responses to Fc ϵ RI α was evident (Figure 4A). The 15 CSU subjects with IFN- γ responses to Fc ϵ RI α had a shorter duration of disease at the time of study compared with the 14 with autoantibodies to Fc ϵ RI α (median disease duration 36 versus 72 months, respectively, $P = 0.04$; Figure 4B). No difference in the disease activity score was found between IFN- γ and autoantibody responders. Both IFN- γ and autoantibody responses were detected in 6 CSU subjects; their clinical characteristics did not differ from subjects with only IFN- γ or autoantibody responses (Supplementary Table I).

Associations were not detected amongst other immune responses to Fc ϵ RI α , and subjects with these responses did not differ in clinical characteristics. No associations were detected between immune responses to Fc ϵ RI α and medication use (Supplementary Table II).

HLA-DRB1 alleles were not associated with immune responses to Fc ϵ RI α

After Bonferroni correction for number of comparisons, the only HLA-DRB1 allele that was increased in frequency in CSU compared with the general population, was DRB1*12 (Supplementary Table III) (note that the HC group was HLA-DR matched). DRB1 alleles were not significantly associated with specific cellular or humoral immune responses to FcεRIα.

The combination of cellular and antibody assays for autoimmunity to FcεRIα may improve the diagnostic accuracy of CSU

Measurements of CD4⁺ T-cell proliferative, IFN-γ, IL-5 and IL-13 ELISpot, and autoantibody, responses to FcεRIα were available for an unselected 30 of the 60 CSU subjects, and were used to evaluate diagnostic accuracy for CSU. The clinical definition of CSU was taken to be the benchmark for diagnosis. Autoantibodies to FcεRIα were detected in 47% (Figure 5, Table II). CD4⁺ T-cell proliferation and cytokine responses to FcεRIα were detected in 30% and 80%, respectively. Both cellular and humoral responses to FcεRIα were detected in 40%. All subjects showed at least one of CD4⁺ T-cell proliferative, cytokine or autoantibody responses to FcεRIα. The same data were available for an unselected 10 of the 20 DC and all of the 20 HC subjects, which were combined to determine the specificity of assays for CSU (Table II). IFN-γ and IL-13 responses to FcεRIα were each detected in 3% of controls. No CD4⁺ proliferative, IL-5 or autoantibody responses to FcεRIα were detected in controls. Taking all measures, any one of CD4⁺ T-cell proliferative or cytokine or autoantibody responses to FcεRIα was detected in 6% of controls, giving a diagnostic specificity of 93%. Data on sensitivity and specificity are summarized in Table II.

DISCUSSION

Despite evidence for the involvement of T cells in CSU,^{17-19,23} antigen-specific T cells have not previously been described in CSU. In the present study, we identified autoreactive CD4⁺ T cells specific for FcεRIα in the blood of CSU subjects. This finding provides further insight into the pathogenesis of CSU as an autoimmune disease. As in other autoimmune diseases, it is likely that activation of autoreactive T cells is the initial event in CSU. Indeed, this is supported by our finding that T-cell IFN-γ responses preceded autoantibody responses to FcεRIα in the clinical history of the disease.

Of the cytokine responses to FcεRIα that we measured, IFN-γ was predominant, being detected in 53% of CSU subjects. The cellular source of IFN-γ in PBMCs was not directly identified but is assumed to be the CD4⁺ Th1-cell subset. IL-5 and IL-13 responses to FcεRIα, consistent with activation of the Th2 subset, were detected in only a minority of CSU subjects. Basal IL-5 and IL-13 were higher in CSU than in DC but not HC subjects. This could reflect ongoing, background Th2 cell activation in response to FcεRIα, whereas in DC subjects this would be intermittent on exposure to allergen. However, no further speculation is warranted because the CSU subjects were similar to healthy controls. Other studies have also identified IFN-γ-producing Th1 cells in CSU. Ying et al¹⁹ found increased expression of IFN-γ as well as IL-4 and IL-5 mRNA in the skin lesions of CSU, indicative of mixed Th1-Th2 immunopathology. Fiebiger et al²⁸ reported that autoantibodies to FcεRIα in CSU were predominantly IgG1 and IgG3, consistent with involvement of Th1 and Th2 cells in immunoglobulin class switching.²⁹

366
367 Interestingly, IFN- γ and autoantibody responses to Fc ϵ RI α in CSU were reciprocally
368 related, reminiscent of immune deviation first described by Parish³⁰ and later in the
369 form of the Th1-Th2 paradigm by Mosmann and Coffman.³¹ A similar phenomenon
370 has been reported for the relationship between T-cell and antibody reactivity to the
371 pancreatic islet autoantigen, glutamic acid decarboxylase, in individuals at risk for
372 type 1 diabetes, and linked to the natural history of disease.^{32,33} In CSU, this
373 relationship could account for the fact that either antibody or T-cell reactivity to
374 Fc ϵ RI α is found in only a proportion of affected individuals, and both together in only
375 a minority. Importantly, IFN- γ responses to Fc ϵ RI α occurred earlier in the known
376 disease course than autoantibodies in CSU, consistent with disease initiation by T
377 cells and the requirement for T-cell 'help' for antibody production by B cells, in
378 accord with progression from cellular to humoral immunity in response to antigenic
379 stimulation.³⁰ These findings have implications for interpreting diagnostic testing in
380 CSU and emphasize the need for longitudinal studies of immune markers to Fc ϵ RI α
381 from the earliest clinical stage of urticaria.

382
383 To date, diagnostic assays for CSU have focussed on the detection of serum
384 autoantibodies to Fc ϵ RI α and IgE. Due to the lack of sensitivity and specificity for
385 CSU, these assays have not translated into routine clinical practice and CSU has often
386 been a diagnosis of exclusion. In an unselected cohort of subjects, we found that
387 measurement of cellular immune responses to Fc ϵ RI α in conjunction with the Fc ϵ RI α
388 autoantibody increased diagnostic sensitivity for CSU, while diagnostic specificity
389 remained high. While our findings suggest that a combinatorial approach to laboratory

testing may improve the accuracy with which CSU is diagnosed validation in a larger cohort of subjects is required.

We were unable to confirm the association of CSU with HLA-DR4 reported in studies of British Caucasian²⁰, Polish²¹ and Turkish²² subjects. We found HLA-DR genotypes to be diverse in our predominantly Caucasian CSU cohort; only HLA-DR12 was detected more frequently in CSU subjects than in the general population but the number of subjects was small. HLA-DR12, along with DR9, was also reported to be more frequent in a study of Han Chinese CSU subjects.³⁴ Heterogeneity in HLA-DR allele association could indicate that a number of different peptide epitopes within FcεRIα are the targets of autoreactive T cells in CSU.

H1 antihistamines have been reported to reduce IL-4-induced IL-5 and IL-13 production by CD4⁺ T cells.³⁵ Immunosuppressive agents are also used to treat CSU.³⁶ In our study, 69% of CSU subjects remained on treatment at the time of sample collection. Although we found no association between medication use and immune responses to FcεRIα, future studies should aim to include only subjects who have withheld immunosuppressive and immunomodulatory agents before blood sampling.

The documentation of antigen-specific T cells in immune diseases has led to the development of antigen-based immunotherapy, e.g. in allergic diseases³⁷ and type 1 diabetes.³⁸ Identification of FcεRIα as a target of autoreactive T cells in CSU should be an impetus for the development of FcεRIα protein- or peptide-based vaccine immunotherapy in CSU.

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416

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421

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532 **Table I. CSU, DC and HC subjects.**

	CSU (n = 60)	DC (n = 20)	HC (n = 20)
Demographic details			
Age in years, median (range)	41.5 (18 to 69)	39 (18 to 65)	38 (20 to 65)
Female (%)	68	85	70
Caucasian (%)	77	85	70
Urticaria			
Disease duration in months, median (range)	54 (2 to 708)	96 (4 to 516)	NA
UAS, median (range)	4 (0 to 6)	NA	NA
Medication use at time of review (%)			
H1 antihistamine	64	35	0
H2 antihistamine	12	10	0
Leukotriene receptor antagonist (montelukast)	15	0	0
Mast cell stabilizer (sodium cromoglycate)	2	5	0
Corticosteroid	12	5	0
Hydroxychloroquine	12	5	0
Methotrexate	2	10	0
Nil	31	65	100

533 NA, not applicable.

Table II. Sensitivity and specificity of FcεRIα assays for CSU.

Data from 30 CSU and 30 control (10 DC, 20 HC) subjects.

Immune response to FcεRIα	CSU (n = 30)		Controls (n = 30)		Sensitivity (%)	Specificity (%)
	Response (n)	No response (n)	Response (n)	No response (n)		
CD4 ⁺ T-cell proliferation	9	21	0	30	30	100
IFN-γ	15	15	1	29	50	97
IL-5	11	19	0	30	37	100
IL-13	4	26	1	29	13	97
Autoantibody	14	16	0	30	47	100
Cytokine (IFN-γ, IL-5 or IL-13)	24	6	2	28	80	93
CD4 ⁺ T-cell proliferation or cytokine	28	2	2	28	93	93
CD4 ⁺ T-cell proliferation or autoantibody	20	10	0	30	67	100
Cytokine or autoantibody	27	3	2	28	90	93
CD4 ⁺ T-cell proliferation, cytokine or autoantibody	30	0	2	28	100	93
CD4 ⁺ T-cell proliferation and cytokine	5	25	0	30	17	100
CD4 ⁺ T-cell proliferation and autoantibody	3	27	0	30	10	100
Cytokine and autoantibody	11	19	0	30	37	100
CD4 ⁺ T-cell proliferation, cytokine and autoantibody	2	28	0	30	7	100

FIGURE LEGENDS

Figure 1. T-cell proliferation to FcεRIα. (A, B) CFSE-labeled PBMCs from a CSU subject. (A) Red rectangles: divided (CFSE¹⁰) CD4⁺ as percent of total CD4⁺ T cells. (B) Antigen dose-response (mean ± SD of triplicates). (C, D) CFSE-labeled PBMCs from CSU, DC and HC subjects. (C) Left: median CD4⁺ T-cell proliferation for individual subjects. Right: normalized data (background subtracted) to allow comparison between groups. (D) CD8⁺ T-cell proliferation, as per subjects in (C). Dotted lines: cut-offs for positive responses to FcεRIα (>2 interquartile ranges above the median HC value). Solid horizontal lines: group medians.

Figure 2. IFN-γ, IL-5 and IL-13 response to FcεRIα by ELISpot assay. (A) PBMCs from CSU subjects (mean ± SD of triplicates). (B) PBMCs from CSU, DC and HC subjects (median IFN-γ, IL-5 and IL-13 spots for individual subjects).^aResponses to 0.1 versus 0 μM FcεRIα in each group were compared by the Wilcoxon matched-pairs signed rank test. ^bBasal secretion between groups was compared by Kruskal-Wallis test. (C) Normalized data (background subtracted) to allow comparison between groups. Dotted lines: cut-offs for positive responses to FcεRIα (>2 interquartile ranges above the median HC value). Solid horizontal lines: group medians.

Figure 3. Serum autoantibodies to FcεRIα. (A) Immunoprecipitation of ¹²⁵I-FcεRIα by autoantibodies in serum (1:100) from CSU, DC and HC subjects. Dotted line indicates cut-off for a positive response to FcεRIα (>2 interquartile ranges above the median HC value). Solid horizontal lines denote group medians. (B) Serum samples

from eight CSU subjects were pre-incubated with unlabelled FcεRIα before incubation with ¹²⁵I-FcεRIα. Median of triplicates shown for each subject.

Figure 4. IFN-γ and autoantibody responses to FcεRIα. (A) Comparison of IFN-γ and autoantibody responses to FcεRIα in 30 CSU subjects. Median of replicates shown for each subject. (B) Comparison of disease duration at the time of study in CSU subjects with IFN-γ and autoantibody responses to FcεRIα.

Figure 5. CD4⁺ T-cell proliferative, IFN-γ, IL-5 and IL-13 ELISpot, and autoantibody, responses to FcεRIα in CSU. Data from 30 CSU subjects. IFN-γ, IL-5 and IL-13 ELISpot data are combined. Percentages of subjects with responses are shown.

Supplementary Table I. Clinical characteristics of CSU subjects with IFN- γ or autoantibody responses to Fc ϵ RI α . Complete data for assays of IFN- γ and autoantibody responses to Fc ϵ RI α were available for 30 of the 60 CSU subjects. Disease duration and disease severity were compared between 9 CSU subjects with only IFN- γ responses, 8 with only autoantibody responses, and 6 with both responses to Fc ϵ RI α (Kruskal-Wallis test).

	IFN- γ only	Autoantibody only	IFN- γ and autoantibody	P value
Disease duration in months, median (range)	24 (4 to 528)	90 (18 to 444)	73 (7 to 144)	0.11
UAS, median (range)	4 (0 to 5)	5 (0 to 6)	5 (2 to 6)	0.25

582

Supplementary Table II. Medication use and immune responses to

FcεRIα. Comparison of medication use with immune responses to FcεRIα in 60 CSU

subjects (Fisher's exact test). P values shown.

Medication	CD4 ⁺ T-cell proliferation	IFN-γ	IL-5	IL-13	Antibody
H1 antihistamine	0.184	0.538	0.543	0.307	0.095
H2 antihistamine	1.000	0.219	0.078	0.202	0.195
Leukotriene receptor antagonist (montelukast)	0.491	0.331	1.000	0.372	0.104
Mast cell stabilizer (sodium cromoglycate)	0.304	0.475	1.000	1.000	1.000
Corticosteroid	1.000	0.489	1.000	1.000	0.299
Hydroxychloroquine	1.000	0.654	1.000	1.000	1.000
Methotrexate	1.000	1.000	1.000	1.000	0.492
Any immunosuppressive medication	1.000	0.465	0.076	0.052	0.596
Any of the above medications	0.774	0.527	1.000	0.280	0.360

Supplementary Table III. Frequency (%) of HLA-DRB1 alleles in CSU, DC, HC and Caucasian Australian populations. Comparison of frequency of alleles between CSU and DC subjects, CSU and HC subjects, and CSU subjects and Caucasian Australian populations (Fisher exact test).

DRB1	CSU (n = 60)	DC (n = 20)		HC (n = 20)		Caucasian Australians	
	%	%	P value	%	P value	%	P value
*01	8	10	1.000	10	1.000	21 ^a	0.009
*02	2	0	1.000	0	1.000	NA	NA
*03	25	20	0.768	15	0.538	22 ^a	0.758
*04	28	20	0.566	25	1.000	35 ^a	0.104
*05	0	0	1.000	0	1.000	NA	NA
*06	2	0	1.000	10	0.153	NA	NA
*07	20	30	0.367	30	0.367	24 ^a	0.537
*08	12	10	1.000	20	0.454	5 ^b	0.071
*09	3	5	1.000	5	1.000	5 ^b	1.000
*10	7	5	1.000	5	1.000	1 ^b	0.031
*11	32	25	0.779	10	0.079	15 ^a	0.004
*12	8	0	0.324	10	1.000	0 ^b	<0.001**
*13	18	25	0.530	15	1.000	20 ^a	0.745
*14	5	15	0.162	10	0.594	3 ^b	0.453
*15	23	30	0.562	35	0.381	30 ^a	0.158
*16	7	5	1.000	0	0.567	3 ^b	0.259

^a Data from Stankovich et al³⁹ (n = 1210)

^b Data from Rojana-Udomsart et al⁴⁰ (n = 189)

NA, not available

** P value remained significant after Bonferroni correction for multiple tests

