Importance of type M1 and M2 macrophage expression in patients with chronic spontaneous urticaria

Roberta Criado[1], Carlos Machado [1], Paulo Criado[2], Carla Pagliari[3], and Bianca Bianco[1]

[1]Faculdade de Medicina do ABC
[2]Faculdade de Medicina da Fundacao do ABC
[3]FMUSP

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Abstract

Mast cells and basophils interact with various cells in the urticaria lesion microenvironment, such as macrophages,.which form an essential component of innate immunity, and are involved in numerous functions including protein secretion. Objective: The aim of the present study was to characterize the macrophage phenotype in urticarial lesions of patients with chronic spontaneous urticaria (CSU) nonresponsive to antihistamines at optimized doses. And compare the phenotype with clinical and laboratory parameters such as age, gender, urticaria time, C-reactive protein (CRP), and total serum IgE, and autologous serum skin test (ASST). Methods: Twenty-eight patients with CSU refractory to antihistamines were included in the study. Epidemiological data, C-reactive protein, D-dimers, basophils in peripheral blood, and total serum IgE and ASST were assessed. The mannose receptor (CD206), CD163, CMAF, and pSTAT 1 were used to characterize the M1/M2 macrophage subpopulations. The immunolabeled cells per square millimeter were manually enumerated at a 400× magnification in 12 optical fields via light microscopy. Results: A predominance of M2 macrophages was seen in CSU patients. Statistical differences were observed between the CD206 marker and the disease course. No correlation was found between biomarkers and macrophage populations. Expression of CMAF was significantly higher in the patient sample compared to that in the control skin (patients without history of urticaria; p-value < 0.001). Conclusion: M2 macrophages were seen with significantly higher CMAF expression, which indicates macrophage activation in patients with CSU. CD206 expression was inversely correlated with disease time.

Introduction

Chronic spontaneous urticaria (CSU) is considered as the most common form of chronic urticaria, usually persistent, with symptoms that significantly impact the quality of life of patients.[1] Mast cells are the main effector cells in urticaria, and their degranulation leads to the immediate release of mediators such as histamine, tryptase, chymase, and proteases, as well as prostaglandins, leukotrienes, and cytokines.[2,3]Mast cells and basophils interact with various cells of the urticaria lesion microenvironment such as eosinophils, lymphocytes, and macrophages.[2,3]

Macrophages play a pivotal role in the innate immunity, and are able to secrete various proteins with numerous functions.[4,5,6]The present study was based on the hypothesis that macrophages are involved in CSU unresponsive to antihistamines, which would contribute to the severity and perpetuation of the disease.[4,5]

Macrophages were selected as target cells because in a previous study with immunoelectron microscopy, we observed cellular interaction in the microvascular unit of the dermis, where Factor XIIIa$^+$dermal dendrocytes phagocytosed the granules extruded by mast cells, in an active cellular interaction.[4]

Recently, two phenotypic macrophage variants, called M1 and M2, have been characterized in several studies, which express the killer and repair functions dependent on the stimulus and environment to which these

cells are exposed.[5,6] The M1 and M2 responses are described as having contradictory properties, that is "killing or repairing," which are essential in the host's defense mechanisms and activate the opposite TH1 or TH2 responses.[5,6,7] The denomination M1 and M2 macrophages were specifically selected for the type of response they can orchestrate, that is, the M1 macrophage promotes the TH1 response and is considered as tumoricidal, whereas the M2 macrophage promotes a TH2 response and is considered as immunosuppressive.[6] Considering that macrophages recognize a specific situation, they are able to develop an adequate response to the stimulus.[5-7]

In studies with macrophage populations, it was observed that chronic diseases or cancers present a predominantly M2 population.[6] The M2 phenotype predominates or leads to a worse treatment response in eosinophilic diseases such as nasal polyposis and allergic diseases such as asthma.[7-13]Accordingly, we aimed to characterize the macrophage subpopulation present in CSU.

Objectives: To characterize the presence and phenotype of macrophages in lesions of CSU patients non-responsive to treatment with antihistamines at optimized doses (maximum recommended doses) and to compare the macrophage phenotype observed on the skin with clinical and laboratory parameters such as age, gender, urticaria time, C-reactive protein (CRP) and total serum IgE, in addition to response to the autologous serum skin test (ASST).

Methods

Twenty-eight skin biopsies were obtained from CSU patients older than 18 years, defined by the occurrence of urticaria for more than 6 weeks, without any triggering factor or induced urticaria, according to the criteria established by the EAACI/GA²LEN/EDF/WAO guideline for the definition, classification, diagnosis and management of urticaria, refractory to treatment with second-generation antihistamine drugs (H1 blockers), at optimized doses (four times the doses specified in the leaflet), for 30 days.[1] These biopsies were performed on the day of the lesion appearance with more than 6h of onset, according to the follow-up protocol practiced in our outpatient clinic, which includes biopsy of urticaria lesions in patients when they do not achieve control their disease with fourfold doses of second-generation anti-H1 drugs.

After signing the informed consent form, the demographic, clinical, and laboratory data were collected from the patients medical records and the paraffin blocks of the biopsies were retrieved from the database of the Pathology Service to which they were sent. Paraffin blocks of normal skin were obtained from nine individuals undergoing plastic surgery with no history of urticaria or known allergic disease, stored in the same Pathology database.

The project was approved by the Research Ethics Committee of the ABC School of Medicine (Number: 1.545.607 of May 11, 2016).

The clinical and laboratory data collected were: gender, age, time of urticaria evolution, results of complementary tests, such as ASST, CRP values (mg/dL), serum levels of D-dimers (FEU/mL), baseline count in peripheral blood, thorough complete blood count, and serum levels of total IgE (KU/mL).

The paraffin blocks were sectioned onto slides previously prepared with 3% amino-propyltriethoxysilane adhesive solution (Sigma Chemical Co., St. Louis, MO, USA, Product no. A3648) at 2%. The histological sections were dewaxed in two xylol baths, for 20 and 10 min, respectively, at room temperature. Thereafter, the specimens were hydrated in decreasing ethanol solutions (100%, 95%, and 70%) and washed in tap water for 5 min. Subsequently, the slides were incubated in hydrogen peroxide 3% and treated to expose the antigenic sites at 95 °C for 20 min in Target Retrieval Solution pH 9.0 (Product no. S2367, Dako Cytomation, Carpinteria, CA, USA) or Target Retrieval Solution pH 6.0 (Product no. S1699, Dako Cytomation), according to the previous protocols. Next, the slides were washed in tap and distilled water for 5 min each and submerged in buffered saline solution (PBS) at pH 7.4.

Nonspecific tissue proteins were then blocked in a 10% solution of skimmed milk (Molico, Nestle®,Brazil) for 30 min at room temperature. The slides were incubated with the primary antibodies as illustrated in Figure 1. All antibodies were diluted in bovine serum albumin (BSA) fraction V 1% plus 0.1% sodium

azide in phosphate-buffered saline (PBS) buffer at pH 7.4, overnight at 4 °C. After washing twice in PBS at pH 7.4 for 5 min each, the slides were incubated with a post primary antibody polymer detection system (Reveal kit, Spring), for 30 min at 37ºC. Thereafter, the slides were washed twice in PBS buffer at pH 7.4, for 5 min each, and incubated with the polymer system (Reveal kit, Spring) in a humid chamber for 30 min at room temperature. The binding sites were rapidly developed with a chromogenic solution of 0.05% diaminobenzidine (3,3-diaminobenzidine, SIGMA Chemical Co., St Louis, MO, USA, Product no. D5637) and 1.2 mL of 3% hydrogen peroxide. The slides were then washed in running water for 5 min, counterstained with Harris hematoxylin for 20 s, and further washed again in running water and dehydrated in ethanol.

The slides were mounted with Permount resin (FISHER Scientific, Fair Lawn, NJ/USA, product no. SP15-100). To verify the presence of the M1 and M2 population of macrophages via immunohistochemistry reactions, the cells were then morphologically classified into macrophages and were divided into two surface receptor markers (cytoplasmic membrane), CD206 mannose receptor and CD163 called hemoglobin scavenger, folate and IL10, and two transcriptional (nuclear) markers, pSTAT-1 and CMAF, to characterize the subpopulations of macrophages (Table 1).[7,11]

Table 1. Immunohistochemical markers and dilutions.

Markers	Clone code	Brand	Origin	Typo	Detection system	Dilution
pSTAT1	SC135648	Santa Cruz	Mouse	Monoclonal	Polymer	1:50
CD163	NCL-L-CD163	Novocastra	Mouse	Monoclonal	Polymer	1:400
CD206	15-2	Genetex	Mouse	Monoclonal	Polymer	1:200
CMAF	SC7866	Santa Cruz	Rabbit	Polyclonal	Polymer	1:100

Cells stained with pSTAT-1 antibodies were classified as macrophages M1 and those with CD163, CD206, and CMAF staining were classified as M2.[7,11]The cells were quantified in duplicate, and the results were expressed as number of stained cells per square millimeter. The quantification method was the same as that used in a previous study conducted by our group.[4] The number of cells immunostained with specific antibodies in the superficial and middle dermis (portion of the epidermis where the pathophysiological events of urticaria occur) was enumerated at a magnification of 400× in 12 optical fields, using an optical microscope (Zeiss, Axiophot, Germany). The density of immunostained cells was calculated with the following equation:

Cell density/mm² = (immunostained cells in 12 fields)/12/0.0625.

The variability of the duplicate count was not greater than 5%.

Blank immunohistochemistry stains were performed, where the primary antibodies were excluded, which were negative.

Statistical analysis

All immunohistochemical markers were expressed as units in the tables and graphs for clarity, but they represent the result of the*density of cells per square millimeter* enumerated in the superficial and deep dermis of the samples obtained.

The nonparametric Mann–Whitney test was used to compare the markers, for the entire study population with CSU and control skin, as well as the relationship of the markers with gender and ASST. Spearman's correlation coefficients were used to measure the relationship between immunohistochemical markers with clinical examinations, age, urticaria course, and the 7-day urticaria activity score (UAS7). The level of significance was 5% (Software: XLSTAT 2019).

Results

Demographic data and clinical and immunohistochemical markers

In total, 28 patients participated in the study, of which 25 patients were female (89.28%). The age of the patients ranged from 22 to 77 years with a mean of 43.81 years. The duration of urticaria ranged from 3 months to 17 years with a mean of 3.14 years. The mean UAS7 was 29, ranging from 12 to 42, with an average of 29.03.

The expression of immunohistochemical markers correlated with age, time of urticaria evolution, and UAS7 are listed in Table 2. A significant negative correlation was observed between the CD206 marker and the time of CSU, the lower the tissue expression of CD206, the longer the urticaria time.

Table 2. Spearman correlation coefficients between immunohistochemical markers, age, and spontaneous urticaria time.

Antibody	**Age**	**Urticaria time**	**UAS7**
CD163	0.230 ($p = 0.238$)	-0.132 ($p = 0.503$)	-0.038 ($p = 0.849$)
CMAF	0.284 ($p = 0.142$)	0.172 ($p = 0.379$)	-0.142 ($p = 0.468$)
CD206	**-0.201 ($p = 0.305$)**	**-0.446 ($p = 0.018$)**	**0.233 ($p = 0.232$)**
STAT1	-0.322 ($p = 0.095$)	-0.019 ($p = 0.924$)	0.159 ($p = 0.418$)

* Coefficients in bold were significant at 5%

Clinical examinations and immunohistochemical markers

Of the 28 patients, five did not undergo ASST due to the impossibility of removing antihistamines; therefore, the analysis was performed separately. Regarding the ASST results, the comparison did not indicate significant differences between negative versus positive results, for any of the immunohistochemistry markers (for M1 or M2 phenotypes) evaluated ($p > 0.05$). The correlations between the immunohistochemical markers and PCR, D DIMMER, were all nonsignificant ($p > 0.05$) and did not reveal a direct relationship between the complementary tests and macrophage phenotypes.

The cutaneous expression of the two populations of macrophages, M1 represented by STAT1 and M2 represented by CD163, CMAF, and CD206, was compared. The descriptive results of these markers are illustrated in Figures 1 and 2 and Table 3.

No significant differences were found between the skin samples of patients with CSU and of the control population for the immunohistochemical markers CD163, CD206, and pSTAT-1 (Table 3 and Figure 1).

The expression of the immunohistochemical marker CMAF was significantly higher in the CSU patients compared to the controls of apparently normal skin ($p < 0.001$; Table 3 and Figure 1).

The skin expression of pSTAT-1 was significantly lower than CD163 ($p < 0.001$), CMAF ($p < 0.001$), and CD206 ($p < 0.001$) in patients with CSU, demonstrating a clearly M2 phenotypic pattern of macrophage response (Figure 2).

No significant difference was found between pSTAT-1 and CMAF in the skin of control individuals ($p = 0.181$). Images obtained from slides with immunohistochemical staining are depicted in Figure 3.

Table 3. Descriptive statistics by immunohistochemical markers and subpopulation of macrophages in patients with spontaneous chronic urticaria and control subjects.

Variable	**Population**	N	**Mean**	**SD**	**Minimum**	**Median**	**Maximum**	***p*-value**
CD163	Sample	28	63.8	58.9	8.0	43.6	217.8	0.417
	Control	9	90.3	85.9	19.2	50.6	271.1	
CMAF	**Sample**	**28**	**34.2**	**44.5**	**0.0**	**13.3**	**139.2**	<0.001*
	Control	**9**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**	

CD206	Sample	28	78.7	74.3	0.0	62.4	346.7	0.226
	Control	9	45.3	42.0	14.2	27.6	141.4	
pSTAT-1	Sample	28	1.1	4.6	0.0	0.0	24.0	0.175
	Control	9	1.7	2.7	0.0	0.0	6.4	

* Coefficients in bold were significant at 5%; Mann–Whitney test

Figure 1. Comparison of immunohistochemical markers in the subpopulation of macrophages in patients with spontaneous chronic urticaria and skin of control subjects.

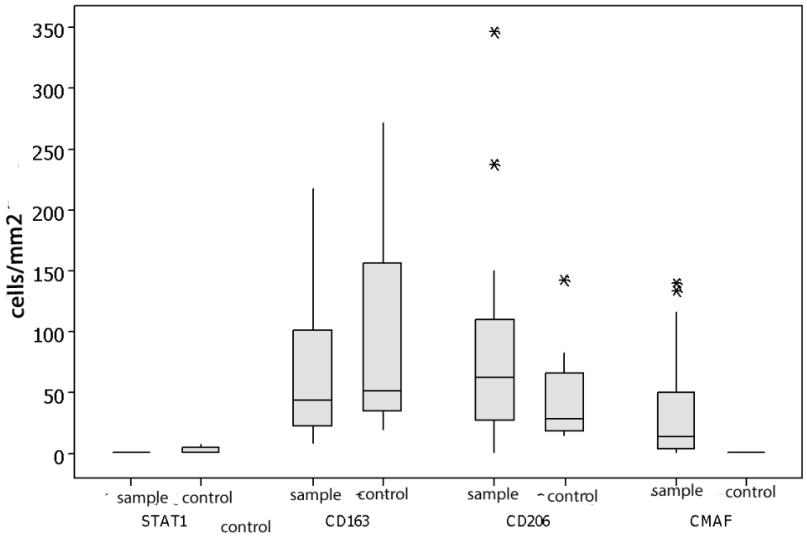


Figure 2. Comparison of the M1 and M2 immunohistochemical markers in patients with CSU.

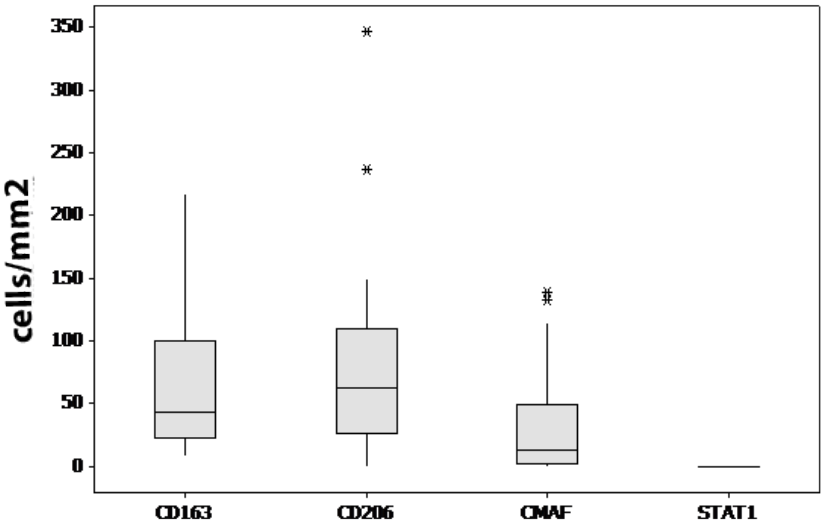
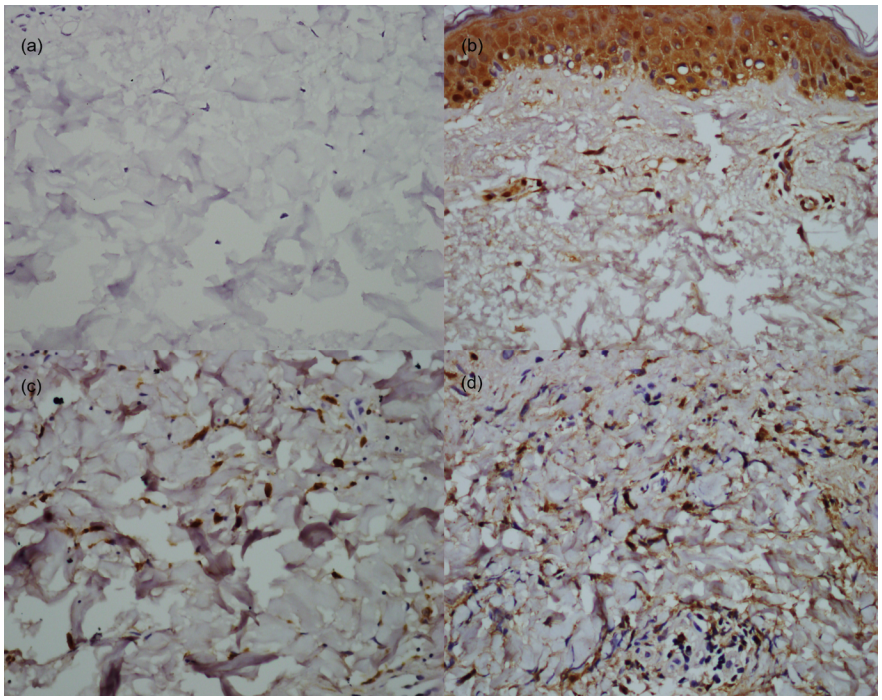


Figure 3. Immunohistochemical analysis with the anti-pSTAT1 (a), anti-CMAF (b), anti-CD163 (c), and anti-CD206 (d) markers. 400× magnification.



DISCUSSION

Most of pathophysiology of CSU can be understood from histopathological studies of urticarial lesions.[3] Barsilay et al.[14] described histopathological changes in urticaria biopsies by demonstrating the presence of numerous cells apart from mast cells, such as eosinophils, lymphocytes, and neutrophils, in the skin.[14] In a study conducted by our group in 2016, we demonstrated that urticaria with predominance of eosinophils was related to greater clinical activity of the disease.[15] Other authors have described the presence of $CD4^+$ T lymphocytes, variable number of monocytes, basophils, and lymphocytes.[2,16] The cytokines involved in the inflammatory skin process in urticaria reveal a mixed cytokine pattern with an increase in IL-4, IL-5, and IFN gamma.[16] In 1990, Czarnetzki et al.[17] described the presence of macrophages in acute, chronic, and Delayed Pressure Urticaria; and, with the scarce resources available at the time for immunohistochemistry, they observed macrophage cell activation in the lesioned skin.[17] Neverthless any previous study has demonstrated the role of macrophage subpopulations (M1/M2 phenotype) in CSU.

In a previous study, we observed a cellular interaction between dendrocytes and mast cells in the microvascular unit of the dermis, using immunoelectronic microscopy.[4] The observation allowed us to assume that the macrophage could participate as adjuvant of the immunological response in urticaria; however, their role was not completely established at the time.[4] An interaction with macrophages can occur via release of histamine and cytokines such as IL-1 beta, IL-6, and IL-13 in addition to prostaglandin D2 (PGD2) and platelet activating factor (PAF).[2] PGD2, PGE2, IL-1 beta, IL-9, and histamine also influence the dendritic cells.[18] The activation of macrophages and dendritic cells has been postulated to be involved in the pathophysiology of urticaria.[2,18]

Macrophages basically perform two functions: (i) killer function, when their function is to eliminate a pathogen or defective cell, which basically depends on M1 populations or (ii) repair, when their functions involve regulation of immune response, angiogenesis, and healing, which depends on M2 macrophages, also termed as "alternatively activated macrophages.[6,18,19] In addition to the normal repair function, the M2

macrophage is related to the chronicity of diseases and worse immune responses in the *in vitro* studies in sepsis, infectious diseases, autoimmune diseases, and vascular diseases.[7,9-13,19]

The presence of M2 macrophages in tumors is related to a worse prognosis and formation of metastases.[19]

Few studies have linked the presence of M1 and M2 macrophages in allergic diseases or in diseases in which the participation of mast cells is important, such as urticaria.[9,10] In these diseases, M2 macrophages are related to chronicity, worse immune response or worse prognosis.[9,10,19,20]

The imbalance between TH1/TH2 cytokines has long been considered as a possible mechanism for urticaria. Chen et al.[21]observed a mixture of TH1/TH2 cytokines in urticaria, in addition to TH17 cytokines.[21] In patients with chronic urticaria, there was a predominance of the TH2 pathway, in addition to an increase in GM-CSF.[21] The change to M2 macrophages is activated by the presence of IL-10, IL-4, IL-13, GM-CSF, IL-6, corticosteroids, apoptotic debris, and hypoxia, that is, the change is induced by local inflammatory milieu, in order to antagonize and resolve an inflammatory process.[5-13,19,22]

In the present study, we observed a predominance of the CD163, CD206, and CMAF receptors, compared to pSTAT-1 (M1 macrophage marker), regardless of age, sex, and laboratory tests evaluated, demonstrating that in uncontrolled CSU with maximum doses of second generation anti-H1, in general, the macrophage subtype found is an alternatively activated phenotype (M2).[7,8,11,19,21]

While studying the immunohistochemical markers in the skin of control individuals, a statistically significant relationship was found with the CMAF marker, which is related to the transcription of cytokines responsible for macrophage and lymphocytic activation.[12,22] In studies conducted in mice, CMAF deficiency is directly related to the loss of expression of the F4/80 factor, which regulates the migration of macrophages to peripheral tissues and directly influences IL-4 production.[23,24]This observation is important, since the expression of CMAF was statistically significant compared to the control individuals, due to the fact that this nuclear transcription factor is constitutionally expressed in a small amount in resident macrophages; however, IL-4 can positively or negatively regulate its production.[23]

Gabryšová et al.[26] demonstrated that CMAF in lymphocytes may be implicated in several normal biological processes and in allergic and autoimmune diseases, because it suppresses the synthesis of IL-2 in CD4+ lymphocytes, activates T helper2 (TH2) cells in allergy, and further activates TH17 lymphocytes, leading to the onset of autoimmune diseases.[26] Since our patients had urticaria refractory to antihistamines and high clinical activity of urticaria, a hypothesis could be that a higher expression of CMAF is the result of an exaggerated stimulation mediated by IL-4 and IL-13 cytokines.[19,23]

CMAF leads to the production of IL-10 and activation of the TH17 pathway, in addition to the perpetuation of TH2-mediated inflammation, which could prolong the inflammatory process and contribute to the maintenance and chronicity of urticaria.[20,21,23]Another possible explanation would be the continued or intermittent use of systemic corticosteroids by patients with urticaria refractory to antihistamine drugs, which also induces the formation of M2 macrophages with activation of CMAF.[1,19,22]

The immunohistochemical marker CD206 was negatively associated with the evolution of CSU in our patients. The macrophage mannose receptor (MMR or CD206) is highly expressed in M2 macrophages, strongly induced by IL-4 and IL-13, but not in M1 macrophages.[7,19]Simultaneously, it is the first receptor to be activated in case of trauma or tissue injury, which leads to the activation of STAT-6, while stimulating the synthesis of TH2 cytokines (IL-4 and IL-13) e IL24.[19,20,27] Scmettzer et al[28]showed that in CSU patients had IgE autoantibodies against IL24 linked with the disease activity. We can infer that in anti-histamines resistant patients we could have a continuous production of IL24 by the M2 macrophage that estimulate the mast cell through IgE anti IL24[27,28]

We showed a decrease in the concentration of the CD206 in relationship with the course of the disease. One possible explanation could be the fact that more recent studies demonstrate the existence of four subtypes of M2 macrophages.[19] We presently know that M2 macrophages can be subdivided into four subpopulations, depending on the stimulus received: (i) M2a, stimulated predominantly by cytokines IL-4 and IL-13 and

JAK-1 and JAK-3 receptors, is a high affinity receptor for IgE and increases the concentrations of CD206; (ii) M2b, stimulated by immunocomplexes, increases the secretion of proinflammatory cytokines; (iii) M2c, stimulated by corticosteroid binding to its receptors IL10 and TGF beta; and (iv) M2d, stimulated by high concentrations of IL-10 and tumor factors.[9,19,20] CD206 is highly expressed by M2a macrophages but is diminished in the other subtypes and its expression changes according to the influence of the inflammatory milieu; thus, we can assume that an attempt to resolve the inflammatory process is observed in chronic urticaria.

One explanation for M2 macrophages being activated in CSU would be the possibility of elevating IL-25, an IL-17 family cytokine, produced by several cells activated in urticaria, including mast cells, dendrocytes, and eosinophils.[29] This could contribute to the presence of activated M2 macrophages in urticaria, indirectly revealed in our study, as it is associated with the increase in CMAF.[26,29.]

In an immunohistochemical study of eight skin biopsies of urticaria cases, Kay et al.[29] observed an increase in the IL-25 expression in mast cells and eosinophils of patients with urticaria. IL-25 belongs to the IL-17 cytokine family and is a potent promoter of TH2 response, and increased expression can be observed in epithelial cells, mast cells, and eosinophils in urticaria lesions.[2,29] The observation that mast cells can produce IL-25, followed by an IgE-dependent activation suggests a possible innate immunity pathway that can trigger or amplify TH2 responses.[2,29] A second member of the IL-17 family, thymic stromal lymphopoietin (TSLP), is also elevated in mast cells in asthma and mast cell cultures.[2] TSLP does not induce mastocytic degranulation; however, it increases the recruitment of mast cells and dendrocytes in the skin.[30]

Mast cells are negatively regulated via activation of phosphoinositide lipid phosphatases (in general, SHIP 1 and 2) and their deregulation and decreased activity are associated with CSU. The increased activity of SHIP 1 and 2 is related to a deviation in the M1 phenotype of macrophages. Therefore, the deregulation of SHIP could also explain the increase in M2 macrophages.[30,31]

Recently, the presence of new vessels (neoangiogenesis) has been described in patients with CSU. Kay et al.[32] reported that the injured skin of patients with urticaria displays increased dermal vascularization.[32] VEGF is a regulatory factor of angiogenesis, inducing endothelial cell proliferation, migration, and secretion of metalloproteinase 1 (MMP1).[3,30] Moreover, VEGF promotes the expression of von Willebrand factor, in addition to adhesion molecules such as ICAM-1, VCAM-1, and E-selectin.[33] VGEF has been implicated as an induction factor of M2 macrophage polarization.[32,33] Macrophage M2 in turn can promote angiogenesis by releasing IL10, TGF-beta, and other cytokines, which results in greater vascularity found in the skin of patients with chronic urticaria.[7,33]

The present study had limitations, as we were not able to subclassify the type of M2 macrophages, as already done in patients with asthma and contact dermatitis.[8,19,20] This subcategory would allow us to observe subtypes of M2 macrophages, which could lead to specific treatments for these patients.

In conclusion, in the CSU patients studied, a predominance of M2 macrophages was observed, with significantly higher CMAF expression, which indicates macrophage activation in patients with CSU. Moreover, we observed a negative correlation between the presence of the CD206 marker and the duration of CSU. Further studies are warranted to focus on therapies targeting M2 macrophage subtypes in patients with urticaria refractory to antihistamines in order to assess their contribution in deciding the best treatment for this disease.

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