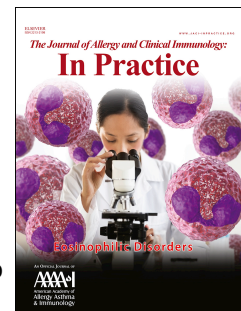


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The Pathogenesis of Chronic Spontaneous Urticaria: The Role of Infiltrating Cells

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79 **Key Words**

80

81 **Lymphocyte**

82 **Eosinophil**

83 **Basophil**

84 **Endothelial cell**

85 **Neuroinflammation**

86

87	Abbreviations
88	
89	ASST: Autologous Skin Test
90	CGRP: Calcitonin Gene Related Protein
91	CSU: Chronic Spontaneous Urticaria
92	FCεR1: Fc Epsilon Receptor 1
93	ICAM: Intercellular Cell Adhesion Molecule
94	IFNγ: Interferon Gamma
95	IL: Interleukin
96	JAK: Janus Activated Kinase
97	LCN-2: Lipocalcin 2
98	LPR: Late Phase Reaction
99	LPS: Lipopolysaccharide
100	MC: Mast Cell
101	MCP: Monocyte Chemotactic Protein
102	MRGPRX2: Mas-related G protein-coupled receptor X2
103	NK: Neurokinin
104	PAF: Platelet Activating Factor
105	PBMC: Peripheral Blood Mononuclear Cells
106	PECAM: Platelet Endothelial Cell Growth Factor
107	RANTES: Regulated on Activation, Normal T Cell Expressed and Secreted
108	TF: Tissue Factor
109	TGFβ: Transforming Growth Factor Beta
110	Th: T Helper Cell
111	TNF: Tumor Necrosis Alpha
112	TSLP: Thymic Stromal Lymphopoietin
113	VCAM: Vascular Cell Adhesion Molecule
114	VEGF: Vascular Endothelial Cell Growth Factor
115	

Abstract

Chronic spontaneous urticaria is characterized by a perivascular non-necrotizing cellular infiltrate around small venules of the skin. It consists primarily of CD4(+) lymphocytes, a prominence of the Th-2 subtype but also Th-1 cells, with Th 17 cell derived cytokines elevated in plasma. There are also neutrophils, eosinophils, basophils, and monocytes. Chemokines derived from mast cells and activated endothelial cells drive the process. While the role of the cellular infiltrate has not previously been addressed, each constituent contributes to the overall pathogenesis and responsiveness to steroid, in particular, focus attention on its importance since short-term steroids do not affect autoimmunity or degranulation of mast cells, and act on margination of cells along the endothelium and chemotaxis to enter the surrounding dermis. In this review we address each cells contribution to the overall inflammatory response, as it is currently understood, with a view toward development of therapeutic options that impede the function of critical cells and/or their secretory products.

Introduction

The pathogenesis of chronic spontaneous urticaria typically focuses on mechanisms by which cutaneous mast cells may be activated to initiate the process. So far there are multiple possibilities however all have in common the presumption that the disorder is fundamentally autoimmune and therefore is not driven by exposure to any exogenous agent. The autoimmune process is viewed as serologic but leading to a perivascular infiltration (cellular) about small venules in the skin. IgG antibody may react with the IgE receptor (30-45%) or IgE itself (5-10%), activate the mast cell, fix complement via the classical pathway, and achieve further mast cell activation by interaction of C5a with the C5a receptor. Alternatively, IgE antibody may react with a host of autoantigens (e.g. IL 24) although one associated with the mast cell surface is not yet defined. Here too activation can occur that is more closely related to a typical allergic reaction i.e. autoallergy.

These mechanisms have received much attention and therapies directed to this enhanced mast cell function are routinely utilized and are effective. These would include omalizumab and cyclosporine.

There is however another facet to this disease which is largely unexplained yet may be no less pathogenically relevant; namely, the particular cellular infiltrate containing a mixture of T lymphocytes (a mixture of Th1 and Th2 subtypes with Th2 being the more prominent), monocytes, eosinophils, basophils, variable numbers of neutrophils, and lymphoid subtypes not yet well characterized. Migration of all of these cells from the blood to the tissue requires a chemotactic response for each and interaction with activated endothelial cells; the latter a key

participant in the process. One piece of evidence that this process is of particular importance is the response to corticosteroid, at least acutely. Steroids appear to have no effect on any of the steps in the autoimmune activation of mast cells but do eliminate the chemotactic response that produces the cellular infiltrate. Thus, targeting the infiltrate can work as an effective therapy however steroids are the only one that does so and are too toxic for sustained use and cyclosporine might downregulate this cellular response but acts on mast cells and basophils as well.

In this review we will focus attention on each of the major constituents of the cellular infiltrate, assess the potential contribution of each (including endothelial cells) to the urticarial process, and in addition, will address any role for the innervation of the skin, particularly sensory neurons, to complete our assessment of non-mast cell contributors to CSU.

Lymphocytes and involved cytokines in CSU pathogenesis

T cells involvement in wheal pathogenesis

A wheal is the consequence of “actors” involved at different times along its dynamic evolution. The pathogenic relevance of lymphocytes per se has not been commonly addressed. In this section we review current knowledge of its role in the genesis of the wheal.

Cytokine expression has been studied in patients with CSU, both in plasma, serum, PBMCs and skin. Naïve T cells can differentiate into T helper Th1, Th2, Th17, Th9, Th22 and regulatory T

(Treg) cells depending on the environment, cell interactions, and cytokines. The role of autoimmunity is prominent, with disorders produced by T helper Th1, Th17 and Th22 cells. Because key effector cells in CSU are MCs and basophils, the cytokines acting on them will be explored: either cytokines implicated in cell maturation, or in the activation of MCs and/or basophils. Finally, since IgE is implicated in CSU, and more specifically, IgE autoantibodies (AAbs), so that cytokines inducing class switching in B cells will be also reviewed.

The primary function of Th1, Th2 and Th17 cells is host defense. Yet Th1 cells and related cytokines can function as pro-inflammatory effectors in autoimmune disorders (1). Th2 and Th9 cells are mainly implicated in allergic diseases, such as asthma and allergic contact dermatitis (2-4), and some Th2/Th9 cytokines are able to induce the switch of human B cells to produce IgE or to promote MC, basophil and eosinophil activation. A role for Th17 and Th22 cells in inflammatory and autoimmune disease is well known (5). IL-17 cells promote recruitment of monocytes and neutrophils and lead to chronic neutrophilic inflammation. Th22 cells can also play either a protective or a detrimental role in autoimmune diseases, depending on the disorder. Finally, regulatory T cells (Tregs), which produce transforming growth factor beta (TGF- β) and IL-10, are another subset of effector T cells that modulate the immune response in order to maintain tolerance against self-antigens, and thus prevent development of autoimmune diseases (6).

Th17 cytokines

According to the hypothesis of an autoimmune pathogenesis, Th17 would seem the most likely T helper cytokine profile implicated. Maintenance, survival and activation of Th17 cells are dependent on IL-23 (7). Th17 cells produce many cytokines, such as IL-17, IL-6, IL-8, IL-22, IL-23 and TNF- α . Among these cytokines, IL-6 is the most commonly studied in CSU and higher IL-6 levels were found both in blood and in skin biopsies of patients.

Interleukin 6 (IL-6) is produced mainly by innate immune cells (macrophages, dendritic cells, and MCs), B cells, and to a lesser extent by Th17 cells and eosinophils (8). IL-6 is implicated in chronic inflammatory or autoimmune diseases (9). It promotes Th17 cell lineage and maturation of B cells and plasmablasts and inhibits the induction of Tregs (9). IL-6 interacts with hepatocytes and induces proteins involved in the acute phase of inflammation such as CRP and fibrinogen. IL-6 is able to increase MC proliferation and to promote a more reactive phenotype of MC and induce its chemotaxis (10-12).

IL-6 seems clearly elevated in the sera or plasma of patients with CSU (13-24) although not always confirmed (25-27). Moreover IL-6 levels correlate with disease activity (13, 17-21, 23) and has been proposed as a biomarker (28-30). However, no correlations were seen with ASST results (14, 17-19, 26). (Table 1)

Recently, mRNA microarray analyses showed an upregulation of IL-6 expression in spontaneous urticarial wheals compared to unaffected skin (31, 32). To date, no trials have been performed to assess the effectiveness of anti-IL-6 therapy in CSU e.g with tocilizumab that can also

effectively reduce immunoglobulins (33-35). In systemic mastocytosis, a JAK1/JAK2 inhibitor, ruxolitinib (which inhibited the production of IL-6 among other cytokines), showed a convincing decrease in MC related symptoms (36-38).

Interleukin-17 (IL-17) mediates adverse effects in some autoimmune diseases (39). Neutrophils are found in urticarial wheals and IL-17 could contribute to this neutrophil infiltrate. Unfortunately, in CSU, serum levels of IL-17 are not consistent (40-42) with positive or negative (14, 43) correlation with disease activity. However, in this instance a positive correlation with ASST was shown (7, 14). Recently increased Th17 and IL-17 expression in both CD4⁺ T cells and mast cells, residing in close proximity to each other, was described in the skin of severe CSU patients (44). In an open study all the patients (n=8) treated with the anti-IL-17A antibody, secukinumab, showed significant improvement in CSU disease activity (45).

The upregulation of IL-beta (IL1 β) mRNA expression in skin samples of urticarial wheals deserves to be investigated further. IL-1 β is a multifunctional cytokine, primarily of the innate immune system, and MCs are a source of IL-1 β . Dysfunction of IL-1 β pathways is implicated in autoinflammatory disorders (46). In CSU, data regarding IL-1 β are few (47) and contradictory results were found concerning IL-1 β serum levels (Table 1). IL1 β mRNA expression was observed in skin biopsies of positive ASST tests but not in patients with negative ASST and was also up-regulated in the spontaneous wheals and PBMCs from patients with CSU. IL-1 β induces neutrophil recruitment and extravasation, promotes expansion of Th1 and Th17 cells, and induces a downregulation of Tregs (48).

243

244 Interleukin 22 (IL-22) belongs to the IL-10 cytokine family and is expressed in several chronic
245 inflammatory or autoimmune diseases (49-50) including psoriasis (51, 52). No difference
246 between IL-22 serum levels in patients with CSU versus normal controls were found whereas
247 CSU patients with a positive ASST tended toward higher IL-22 serum levels (14).

248

249 Interleukin 23 (IL-23) induces polarization, maintenance, survival and activation of Th17 cells
250 and secretion of IL-17 by non-T cells (7, 53). Even if data are scarce, it seems interesting to
251 further explore the role of IL-23 in CSU.(Table 1,2) (7, 14, 54) Interestingly IL-23 serum levels
252 were found to be higher in patients with CSU (7, 14), supernatants of their PBMCs (7, 54) and in
253 those with a positive ASST compared to a negative ASST (7).

254

255 Tumor necrosis factor alpha (TNF-a) is quickly released upon activation because it is stored in
256 MCs granules and induces neutrophil recruitment (55). Elevated TNF-a serum levels (13, 14, 16,
257 21, 56) often correlate with disease activity (7, 13, 14), although ASST correlation was variable
258 (7, 14) (Table 1). Conflicting results were found concerning levels of TNF-a in supernatants of
259 cultured PBMCs (16, 57). (Table 2) TNF-a has been shown to be elevated in spontaneous wheals
260 and uninvolved skin of patients with CSU versus skin of normal controls (58). A few case
261 reports and small controlled studies showed efficacy of TNF-a inhibitors in CSU (59-61).

262

263 Th1 cytokine

Th1 cells mainly produce interferon gamma (IFN-g). In CSU, data on detection of INF-g in serum are contradictory and thus unconvincing (Table 1) (14, 15, 62, 63). Levels of INF-g in supernatants of stimulated PBMCs are shown in Table 2 (16, 54, 57, 62, 65). INF-g expression was found to be elevated in spontaneous wheals versus control skin (66), with fewer numbers of cells expressing INF-g as compared with cells expressing IL-4 or IL-5. Inconsistent results were also found when non-lesional skin of patients with CSU was compared with spontaneous wheals, or control skin (67).

Th2 cytokines

Th2 cells produce many cytokines, mainly IL-4, IL-5 and IL-13, but also IL-9, IL-24, IL-31 and IL-33. Th2 cells are mainly implicated in allergic diseases, stimulating IgE production and MC, basophil and eosinophil activation.

Interleukin-4 (IL-4) is produced by Th2 cells and also by basophils and MCs (68, 69). IL-4 contributes to IgE synthesis induced by activated CD4⁺ or CD8⁺ T cells (70). IL-4 also up-regulates the expression of high affinity IgE receptors (FcεRI) (71). Circulating IL-4, as well as IL-4 produced by PBMCs doesn't seem elevated in the majority of patients with CSU and here too results are conflicting. (Table 1,2) (54, 60, 62, 64, 65, 68). Furthermore, no correlation with disease activity or ASST could be made (14, 57). Nevertheless, IL-4 was elevated in skin biopsies of spontaneous wheals compared with control skin (66). Like IL-4, interleukin 13 (IL-13) induces class switch of B cells to IgE (72, 73) but IL-13 serum levels in patients with CSU do not correlate with disease activity (14). Recently, the efficacy of dupilumab (monoclonal

antibody blocking IL-4 and IL-13) was reported in 6 patients with CSU (74). A Phase 2a multicenter (NCT03749135) and a Phase III study, CUPID, are ongoing in CSU.

Interleukin-5 (IL-5) primes the eosinophil responses to chemokines and is important for their development and survival. IL-5 produced by MCs has autocrine effects by promoting MC proliferation in the presence of stem cell factor and increasing cytokine production (75). IL-5 serum levels in patients with CSU are conflicting (14, 62) (Table 1), however IL-5 expression is increased in wheals compared with non-lesional skin and control skin (66) (Table 3).

Interleukin 24 (IL-24) belongs to the IL-10 cytokine family involved in autoimmune diseases and skin inflammation (49, 51, 76-78). The IL24 gene and protein expression are increased in PBMCs of a subset of patients with CSU. It is detected in spontaneous urticarial wheals, but not those induced by autologous serum injection (ASST) (79). Interestingly, Schmetzer et al. detected IgE against IL-24 in the sera of patients with CSU and correlated this directly with CSU activity (80). They also showed that, in vitro, exogenous IL-24 induced histamine release from human MCs sensitized by IgE against IL-24 from CSU patients, but not with IgE from normal controls (80).

Interleukin 31 (IL-31) is produced by Th2 cells and MCs. IL-31 induces basophil recruitment and secretion of IL-4 and IL-13. IL-31 also stimulates eosinophils to produced pro-inflammatory cytokines such as IL-6, and chemokines (81) and it is a mediator of pruritus apart from that seen

with histamine. Table 1 shows IL-31 to be higher in CSU (40). IL31 can be modulated by omalizumab treatment (82).

Interleukin 33 (IL-33) is a member of the IL-1 superfamily (83). IL-33 induces CD4⁺ T cells, MCs, eosinophils and basophils to produce type 2 cytokines. IL-33 could be produced by MCs and causes their activation as well as their maturation. IL-33 therefore regulates IgE-dependent inflammation. Existing data with regard to IL-33 blood levels in CSU are variable (25, 40, 47, 78, 84) (Table 1). However, Kay et al. found higher IL-33⁺, IL-25⁺ and TSLP⁺ cells in spontaneous wheals than in uninvolved skin and normal controls (85).

Th9 cytokines

Interleukin 9 (IL-9) is produced by Th9 cells, eosinophils, neutrophils, and MCs. IL-9 is a stimulus for recruitment of MCs (86). Experimental models in mice suggest that IL-9 and IL-10 contribute to CSU development via activation of the JAK/STAT signaling pathway (87). One study found elevated IL-9 levels in patients with CSU (88) (Table 1).

Regulatory cytokines

Tregs produce mainly IL-10 and TGF- β . In CSU, circulating Tregs (CD4⁺CD25⁺FOXP3⁺) seems reduced and/or defective compared with normals (89). The reduced frequency of Tregs is consistent with an autoimmune hypothesis for CSU (90, 91, 92). IL-10 also has inhibitory effects on eosinophil survival. Yet in CSU, IL-10 expression is not clearly reduced. Data concerning the

IL-10 expression in patients with CSU versus controls or mRNA levels in PBMCs have no consistent pattern (14, 16, 21, 43, 54, 57, 60, 64) (Table 1, 2).

Transforming growth factor beta 1 (TGF- β 1) in skin could act as an anti-inflammatory cytokine by reducing the expression of Fc ϵ RI on the MCs. In studies investigating the role of TGF- β 1 in CSU, findings are interesting but not sufficient to evaluate its role in CSU pathogenesis (14, 42, 43, 47, 54) (Table 1).

To conclude, new studies are in progress regarding the role of T cells and cytokines in the wheal pathogenesis. Recently IL6 and IL1 β mRNA expression was increased in skin biopsies of positive ASST versus negative ASST in patients with CSU. IL6 and IL1 β mRNA expression was up-regulated in spontaneous wheals and PBMCs from patients with CSU (93). Cytokines such as TNF- α , IL-4 or IL-5 are involved in wheal pathogenesis. Data concerning others cytokines for instance, IL-8 (94), IL-13, IL-15 (95), IL-21 (95), IL-22, IL-23, IL-24 (79), IL-35 (96) are insufficient to allow conclusions but they deserve to be investigated. This knowledge may lead to the development of future biotherapies.

Basophils

The basophil is thought to have an important role in the pathogenesis of CSU given its similarities to the mast cell as a major source of histamine and expression of the high affinity receptor for IgE. Developmentally, however, they differ substantially. Basophils, as a granulocyte, shares a common precursor with the eosinophil (97) whose final stages of

differentiation are dependent on IL3 and IL5 respectively, while the precursor of the mast cell is derived from fetal tissue (connective tissue mast cells including those of skin) and within the bone marrow (inducible mucosal mast cells) as separate lineages (98), more closely related to the monocyte. Their development is dependent on the interaction of stem cell factor with c-kit as well as TGF β . Final differentiation of all mast cells subtypes occurs within the tissue to which they migrate, so that cutaneous mast cells differ in many respects from mast cells elsewhere.

Basophils account for about 1% of circulatory leucocytes and are not normally found in tissues unless they migrate there, a process dependent on cytokines and chemokines (chemotactic cytokines). Here there is considerable overlap with those factors causing eosinophil migration. Among those are VLA expression for binding to endothelial cell VCAM-1 (99) and responsiveness to chemokines MCP-1, MCP-3, MCP-4, RANTES, the eotaxins, the prostaglandin D2 receptor CRTH-2, and the anaphylatoxin C5a (100-102).

Basophil number and functional properties appear to be altered in patients with CSU. Within the circulation, basophil numbers are depleted in at least 50% of patient's (103, 104) and may relate to severity of disease (105). Further, when one checks basophil function employing a stimulant that acts thru the IgE receptor (rabbit anti IgE receptor is often used), the basophils are hyporesponsive (106) as if they had been desensitized. Activation thru some other receptor is normal e.g. histamine release due to C5a interaction with the C5a receptor. The most likely explanation for the basopenia is migration of basophils from the circulation to the skin (103) where the basophils are likely active since chemotactic factor are typically secretagogues. Degranulated basophils within the circulation may also be missed (107). The

Hyporesponsiveness of circulating basophils appears due to increased levels of intracellular phosphatases (particularly src-homology-2 containing 5 inositol phosphatase (SHIP)-1 and SHIP-2) which remove phosphate residues from key signal transduction molecules that are required for normal responsiveness (108). Decreased basophil CRTH2 receptors for PGD₂ likely reflects internalization due to PGD₂ exposure (109) consistent with increased expression of other markers associated with basophil activation (110). The cause of the increased phosphatases is unknown, but its importance is highlighted by the changes observed during symptom reversal. Whether improvement is spontaneous i.e. remission, or induced by therapy such as use of Omalizumab, the basopenia reverses and the basophils become more responsive to anti IgE (104, 111). While these changes are coincidental with improvement it is perhaps paradoxical that basophil histamine release (using circulating basophils) increases at a time that urticarial lesions in the skin quiet down. The functional basophil phenotypes (normal or hyporesponsive) remain stable in symptomatic subjects and are unrelated to the presence or absence of autoantibodies (112). Such basophils appear to have increased surface expression of Fc ϵ RI α , which correlates with elevated IgE levels, and upregulation of responsiveness to various agonists if exposed to IL3 (113, 114) as reported previously for basophils in general (115) and did correlate with a positive autologous skin test (113) typically reflective of the presence of IgG autoantibody to Fc ϵ RI α .

Basophil donors from normal subjects have typically served as an indicator of the presence of antibodies to the high affinity IgE receptor (116) that activate basophils as well as mast cells (116, 117). The incidence of such antibodies varies within the literature from 25-45% in patients with CSU and an additional 5-10% who have IgG anti IgE. The anti-receptor antibody - Fc ϵ RI α

interaction activates the classical complement pathway (118) to liberate C5a (119) which substantially augments histamine release when compared to the purified auto antibody. Most patients have IgG₁ and/or IgG₃ subclasses of anti-receptor antibody as expected (120). The second most common IgG subclass is IgG₂ which when isolated did not cause histamine release although they were often positive by immunoblot vs the α chain of the IgE receptor emphasizing the possibility of false positive binding results using ELISA methodology where the incidence of such antibodies is reported as 55-65% (112). Of particular note regarding specificity is the increased incidence of such antibodies in a variety of autoimmune diseases (121) all of which were negative for histamine release, the sole exception being positives for histamine release seen in patients with SLE in a later study (122), yet below the incidence seen in CSU. Basophils can also be employed to verify the presence of IgE antibodies to putative autoallergens (autoallergy) by passively sensitizing normal basophils with patient IgE (123) and adding antigen or adding the putative auto antigen to patients' basophils known to have the requisite IgE antibody present. A cell surface autoantigen or skin autoantigen that can stimulate mast cells or basophils is not yet identified.

EOSINOPHILS IN CSU

Evidence for eosinophil involvement in CSU

Eosinophils are multifunctional leukocytes involved in the host defence against infections as well as in repair of injured tissue; however, eosinophils also play critical roles in the pathophysiology of allergic and inflammatory diseases (66). Although many allergic and inflammatory conditions are associated with a peripheral blood eosinophilia, circulating eosinophils numbers are only rarely increased in patients with chronic spontaneous urticaria (CSU)(124), even if these cells are

always present in the perivascular cellular infiltrate that characterizes the disease, particularly in lesional skin (124, 125). Recent studies show that the recruitment of eosinophils in the skin may lead to blood eosinopenia (125) by a mechanism that resembles very much the one leading to the much more frequent basopenia. The recruitment of eosinophils in the skin is mediated by a number of cytokines including interleukin-5 (IL-5), eotaxin 1, eotaxin 2, monocyte chemotactic protein-3 (MCP-3), chemokine C-C motif ligands 4 (CCL4), and Regulated upon Activation Normal T cell Expressed and Secreted (RANTES), released by several cell types, including T helper 2 (Th2), type 2 innate lymphoid cells-2 (ILC-2), mast cells, dermal fibroblasts, endothelial cells, and basophil granulocytes (126). Once migrated into the skin, eosinophils appear all but an innocent bystander, and the more they are studied the clearer it appears that they play an active role within the inflammatory network that characterizes chronic urticaria. In fact, there is little doubt that in CSU, skin eosinophils are activated. The eosinophil-derived major basic protein (MBP) can be measured in urticarial lesions even when eosinophils cannot be detected (127). Interestingly, this seems the case mainly in patients lacking IgG autoantibodies to the high affinity IgE receptor (i.e., those patients that do not have a type IIb autoimmune urticaria after the Gell-Coombs classification) (124), that we now know have in several cases an IgE-mediated autoimmune disease (that is, a type I autoimmune [auto-allergic] urticaria). Interestingly, MBP has been detected in autologous serum skin test (ASST) site biopsies, along with eosinophil infiltration (128). Notably, the major basic protein is able to induce mast cell activation and degranulation (129) thus representing one possible mechanism for histamine release. The activation of eosinophils appears to occur via different mechanisms in CSU. One is an autoimmune mechanism mediated by circulating IgG autoantibodies directed against the low affinity IgE receptor expressed on the surface of these cells. These autoantibodies have been

detected in about 65% of chronic urticaria patients (130), and their presence was confirmed recently (131).

Eosinophil interactions with other cells in CSU

In addition, eosinophils may be recruited and activated by several mediators and cytokines released by activated mast cells. These include IL-5, tumor necrosis factor alpha (TNF- α), platelet-activating factor (PAF) and eotaxin (132,133). The mast cell/eosinophil cross-talk sustaining a reciprocal recruitment and activation is shown also by the secretion of stem cell factor (SCF), a growth and maturation factor for mast cells, released by activated eosinophils.

Although other cell types such as endothelial cells (134) and peripheral monocytes (135) may also show this feature, eosinophils are able to store tissue factor and to transfer it rapidly to the cell membrane upon activation (136). The potentially relevant role played by these cells in the pathogenesis of chronic spontaneous urticaria has been further highlighted by the observation that patients with a severe disease are characterized by an intense activation of the extrinsic pathway of the coagulation cascade (137-139). Immunohistochemical experiments using double staining showed that in CSU skin, tissue factor co-localizes with eosinophil cationic protein, a classic cell marker of eosinophils (140). Notably, several activated coagulation factors (e.g., thrombin, and FVIIa/FXa/TF complex) are able to stimulate histamine release from mast cells via the activation of PAR-1 and PAR-2 receptors, respectively (141, 142). Activated coagulation factors can induce mast cell and basophil degranulation by activating fraction 5 of complement

(143). Thrombin-induced inflammation is probably limited by the mast cells themselves via the secretion of protease 4 in a sort of feedback mechanism (144).

Finally, a significant increase in vascular endothelial growth factor (VEGF) has been detected in patients with CSU (145). VEGF is the most potent regulator of angiogenesis presently known, and one major mediator of vascular permeability; furthermore, it exerts a vasodilator effect through an increase of nitric oxide production by endothelial cells (146). Further, VEGF was found to stimulate the activation of the extrinsic coagulation pathway, including modulation of tissue factor expression (147) and intercellular gap formation on vascular endothelial cells, an effect that was synergistically increased in the presence of TNF- α and LPS (148). In CSU patients, the main source of VEGF are eosinophils (145).

Clinical relevance

The activation of eosinophils in urticaria lesional skin and the subsequent initiation of the coagulation cascade may have clinical effects not only in eliciting wheal eruption, but also at systemic level. However, patients with CSU are not reported to have an increased risk for thrombotic events probably due to the fact that the activation of coagulation occurs mostly locally (i.e., extravascularly (148)), where thrombin may contribute to increase vascular permeability and induce mast cell degranulation. These findings have provided the rationale of both controlled studies and case series supporting the effectiveness of both oral anticoagulants and heparin in some patients with refractory CSU (149-151).

Another potential clinical implication of eosinophil involvement in CSU may be related to the presence of autoantibodies of the IgG class to low affinity IgE receptor that is expressed on eosinophils (130, 131) and autoantibodies of both IgG and IgE classes to tissue factor in a proportion of CSU patients (131) that may influence the clinical response to the anti-IgE therapy.

Finally, interestingly, mepolizumab (anti IL5) and benralizumab (anti IL5 receptor), two monoclonal Abs leading to the abolition of eosinophils activity by similar mechanisms were found to be highly effective in a proportion of patients with severe, unremitting urticaria. With benralizumab, 5 of 12 patients were asymptomatic and two others markedly improved, similar to early studies of omalizumab (152).

In conclusion there is ample evidence supporting the notion that eosinophils are leading actors in the inflammatory processes that underlie chronic spontaneous urticaria.

The role of neutrophils in CSU

The evidence for PMN involvement in CSU

The presence of neutrophil infiltration in urticarial skin lesions of patients with CSU has been demonstrated by many studies. Sanchez et al. (153) reported 3 histopathological patterns in 36 patients with CU: neutrophilic, lymphocytic, and mixed type but there was no specific association to the clinical presentation. Sugita et al. (154) reported an apparent infiltration of neutrophils in 94% of biopsied wheals from 35 patients with CIU. However in only 5 patients

were neutrophils the predominant cell seen. In cases where eosinophil infiltration predominates, neutrophils are still the dominant leucocyte in blood and there was no eosinophilia. Martins et al. (155) reported two histological groups 1) predominance of neutrophils or eosinophils (42.4%) 2) predominance of lymphocytes (57.6%) in 93 CSU patients. There was no significant correlation between histological group and laboratory tests.

For autologous serum induced wheals in chronic autoimmune urticaria, Grattan et al. (156) performed serial biopsies of ASST wheals. Perivascular neutrophils and eosinophils were seen as early as 30 min, becoming more intense and diffuse over 2 h. T lymphocyte numbers were increased by 2 h. By 48 h, the neutrophils were clearing, but eosinophils and lymphocytes persisted. The inflammation resembled the late phase of IgE-mediated immediate hypersensitivity reactions. Caproni et al. (157) examined 28 CU patients with positive ASST at different evolutive stages, measuring expression of CD3, CD4, CD8, tryptase, eosinophil cationic protein, myeloperoxidase, basophil granular protein, IL-4, IL-5, IL-8, CCR3 and CXCR3, ICAM-1, VCAM and ELAM. ASST-induced wheals involve a prevalent role of lymphocytes, with strong neutrophil infiltration and activity and involvement of CXCR3 and

530

531 Neutrophils accumulate in allergic late-phase reactions (LPRs)

532 A recent study suggested the role of neutrophils as antigen presenting cells in LPRs (159). After
533 cutaneous allergen challenge, there were significant increases in the numbers of intradermal
534 CD3⁺, CD4⁺, CD8⁺ and CD25⁺ T cells, eosinophils, neutrophils, basophils and macrophages in
535 patients with CSU compared with nonatopic control subjects (66). The profile of inflammatory
536 infiltrate of CSU was similar to that of allergen-induced skin LPR (66) with a mixed T cell
537 subclass distribution of Th 1 and Th2 cells as was also reported in biopsies of ASST reactions
538 (157). Later, another study reported increased expression of Th2-initiating cytokines (IL-33, IL-
539 25 and thymic stromal lymphopoietin) in lesional skin of CSU patients (67).

540

541 How are PMN recruited in the skin?

542 The major mediators involved in the recruitment of neutrophils to sites of active inflammation
543 including bacterial components, C3a and C5a, TNF- α , interleukin 17 (IL-17), CXCL8/IL-8, IL-
544 36, IL-1, lipocalin-2(LCN2), leukotriene B4, PAF, plasma kallikrein, matrix metalloproteinases,
545 and myeloperoxidase inhibitors (160). Lesional skin in CSU had significantly more CD31⁺
546 endothelial cells; CD31⁺ blood vessels, neutrophils, and eosinophils than uninvolved skin
547 suggesting a link between inflammatory cells and vascular leakage (161). Basophils and
548 macrophages and CD3⁺ T cells were also increased but less marked. This may explain how in
549 CSU, as with the LPR, mast cell activation leads to the release of IL-8 and eotaxin, chemotactic
550 factors for neutrophils and eosinophils, respectively (161-164). Another study reported that
551 lesional skin in CSU contained significantly more CGRP⁺ and VEGF⁺ cells than non-lesional

skin (165). CGRP was expressed mainly by neutrophils and eosinophils. Increased expression of CGRP and VEGF was detected only in lesional skin which indicated that they might have a role in whealing and tissue edema.

The levels of neutrophil activation and related cytokines were studied in 88 patients with ASA-intolerant CU. The serum levels of myeloperoxidase and IL-18 were higher than normal controls (166). Another study reported mean levels of serum LCN2, TNF- α , IL-6, and IL-10 that were significantly higher in 191 CU patients than in controls (167). LCN2 levels decreased significantly in refractory CU compared to patients who responded to antihistamines. Increased serum LCN2 in CU patients was associated with a decrease in urticarial activity scores.

What is the clinical relevance?

Elevation in the absolute neutrophil count and neutrophil/lymphocyte ratio were reported to be associated with a poor prognosis in childhood CSU and neutrophil/lymphocyte count can be used as a remission marker (168). A study of 74 CSU patients receiving omalizumab 300 mg every 4 weeks for at least one year resulted in a substantially increased peripheral blood basophil count, and reduced peripheral blood neutrophil especially in the first 3 months (169). The authors proposed that the alterations in peripheral blood cell ratios might contribute to or reflect the therapeutic effect of omalizumab in CSU. Another retrospective study of 74 CSU patients receiving omalizumab 300 mg every 4 weeks, the neutrophil count, the neutrophil/lymphocyte ratio and neutrophil/monocyte ratio showed a statistically significant reduction after 12 weeks of omalizumab treatment (170).

575

576

577 Taken together, the data suggest that neutrophils might contribute to the pathogenesis of CSU, at
578 least in a subgroup of patients, however mechanistic details are not yet known.

579

580 The role of vascular endothelial cells in CSU.

581

582 Plasma leakage from skin micro vessels in the dermis is crucial to produce edema and the flare reaction,
583 clinically recognized as wheals of urticaria. The critical role of histamine released from skin mast cells
584 and/or basophils for vascular permeability in the pathogenesis of CSU has been proven by the
585 effectiveness of H1 antihistamines. Besides histamine, other mediators, such as vascular endothelial
586 growth factor (VEGF), thrombin, bradykinin and/or platelet active-activating factor (PAF) may induce
587 plasma leakage through gap formation of vascular endothelial cells via specific receptors (171).
588 Moreover, increase of leukocytes, such as eosinophils, neutrophils and basophils, is observed in the
589 lesional skin of CSU (140, 141, 172).

590

591 To date, several reports suggest that the expression levels of cell adhesion molecules, such as P-selectin,
592 E-selectin, intercellular adhesion molecule (ICAM), vascular cell adhesion molecule (VCAM) and
593 platelet endothelial cell adhesion molecule (PECAM, CD31) are up-regulated in the lesional skin of
594 patients with CSU (173, 174, 161). Histamine and thrombin rapidly induce surface expression of P-
595 selectin stored in endothelial cells within few minutes (175). Moreover, inflammatory cytokines, such as
596 TNF- α and IL-1 β , contribute to the late phase (4-24hr) expression of P-selectin, E-selectin, ICAM and
597 VCAM on the surface of endothelial cells (176). Since P-selectins and E-selectins are required for the
598 rolling process, and ICAM, VCAM and PECAM are required for the firm adhesion process of leukocytes

and/or for passing through the blood vessel wall, it is feasible that up-regulation of cell adhesion molecules expressed on the vascular endothelial cells contribute to the infiltration of leukocytes into dermis in the pathogenesis of CSU. However, detailed roles of adhesion molecules expressed on endothelial cells other than the role in inflammatory cell infiltration as a consequence of mast cell activation, remained unknown.

As described in the sections above, a number of studies have suggested the involvement of the blood coagulation/fibrinolysis system, especially of TF-triggered extrinsic coagulation pathway, in the pathogenesis of CSU. In patients with CSU, plasma levels of prothrombin fragment 1+2 (PF₁₊₂) and D-dimer are higher than those in normal controls and correlate with disease severities (177, 139). Sakurai et al revealed the elevation of extrinsic blood coagulation potential in the patients with CSU (178). Plasminogen activator and urokinase released from vascular endothelial cells may contribute to the activation of fibrinolysis process which produces D-dimer (179). Moreover, in vitro studies focused on the role of vascular endothelial cells have unveiled a role of vascular endothelial cells in the cascade of blood coagulation pathway triggered by histamine and inflammation in the pathogenesis of CSU (134, 180). Both human umbilical vein endothelial cells (HUVECs) and human dermal microvascular endothelial cells (HMVECs) express a large amount of TF on the surface of cells in response to the combination of two categories of TF-inducers; one including histamine, and VEGF, and the other including LPS, TNF- α , IL-33 and IL-1 β (134, 181). Moreover, TF expressed on HUVECs induces activation of the extrinsic coagulation pathways and produce activated coagulation factors, such as factor Xa (FXa) and factor IIa (FIIa). FXa and FIIa then induce gap-formation of vascular endothelial cells via protease activated receptor-1 (PAR-1) and leakage of plasma with autoantibodies against IgE or Fc ϵ RI, and autoantigens for specific IgE on mast cells in the skin, resulting in the activation of the mast cells and edema formation (134, 180). Thus, TF on endothelial cells may be a good target to prevent TF-extrinsic coagulation pathway-plasma leakage axis in the treatment of severe and refractory CSU. However,

patients with CSU do not usually show apparent thrombosis, presumably due to homeostatic activation of fibrinolysis. The actual expression of TF on vascular endothelial cells in the lesion of CSU remained to be confirmed in the patients.

Neuroinflammation and The Afferent Nervous System

A relationship between hives that persist and the “nervous system” has been assumed once physicians/researchers began to consider the possible cause of CSU. While few believe that an underlying psychogenic condition (e.g. chronic anxiety) is the cause, most are of the opinion that stress can exacerbate hives, and all agree that urticaria is certainly a cause of stress. For those who see no effect of stress on any objective manifestation of urticaria, the interpretation is that tolerance for the discomfort, including itch, is lessened by stress, leading to complaints of “worsening”.

Anatomically, efferent nerve cells are in proximity to mast cells (182) and we know that histamine stimulation of sensory nerves (type C unmyelinated neurons) can initiate impulses migrating through the dorsal root ganglion to the spinal cord which then branch or interconnect in multiple ways. Some have connections (pre-spinal cord) that meet adjacent nerves which conduct back to the skin (antidromic conduction) which lead to release of neuropeptides, particularly Substance P. This causes “reflex” vasodilatation of arterioles and is responsible for the axon reflex, the third element of the “triple response of Lewis”. The axon reflex causes redness far beyond histamine diffusability. Substance P also has the ability to further activate cutaneous mast cells if sufficiently high levels are reached (183) and blood levels are indeed elevated in CSU (184) and produce a positive wheal and flare on skin testing that is augmented in CSU (185).

647

648 Besides the aforementioned branch points, synapses within the spinal cord then project to the
649 brain and lead to the perception of itch. For years, it was assumed that fibers mediating pruritus
650 and pain were identical, and that differing extent of stimulation might be responsible for different
651 sensations. We have recently discovered that there are separate nerve fibers mediating itch that
652 travel along with fibers mediating pain within the lateral spinothalamic tract (186).

653

654 Neuropeptide release from nerve endings that could contribute to the pathogenesis of CSU are
655 many, and further work is needed to delineate specific contributions of each assuming many are
656 relevant. These include Substance P, Neurokinin A, and Calcitonin-Gen-Related Peptide.

657

658 Substance P and Neurokinin A are derived by alternative splicing from the same
659 preprotachykinin A gene and interact with the same NK-1 receptor. Inhibitors of the NK-1
660 receptor are being developed which is most likely relevant to histamine-dependent pruritis (185).
661 There is also a receptor on skin mast cells, termed MRGPRX2 that is responsive to Substance P
662 as well as other neuropeptides (187).

663

664 Since the pruritis of CSU is as responsive to therapy as are the urticarial lesions (188) the
665 mediators downstream are ultimately dependent on mast cell activation. In this respect the
666 circumstance may differ from other causes of pruritis that are chronic (e.g. kidney disease, liver
667 disease, lymphoma, or prurigo nodularis) and that may include atopic dermatitis in which normal
668 appearing (but often dry) skin can be pruritic and antihistamine resistance is the norm. A role for

other receptors, such as opioid and cannabinoid receptors, PAR receptors or receptors for cytokines such as IL31, IL4, and IL13 appears likely (189).

Concluding Comments

The presence or absence of any significant cellular infiltrate within the dermis differentiates many types of chronic urticaria. Most inducible urticarias lack such an infiltrate perhaps because the stimulus is fleeting, even if repetitive. These would include cold urticaria, symptomatic dermatographism, cholinergic urticaria, solar urticaria, local heat urticaria, aquagenic urticaria, and vibratory urticaria/angioedema. On the other hand, CSU has a prominent cellular infiltrate (190) with features similar to the allergic late-phase reaction (156). Perhaps a sustained stimulus leads to this result. The one inducible urticaria that has a significant cellular infiltrate is delayed pressure urticaria (191). While the lesion appears hours after the stimulus, the initiating stimulus is typically sustained (walking or being seated a long time) or repetitive (hammering) relative to the other inducible urticaria where stimuli ranging from a few seconds to a few minutes suffices.

The cellular infiltration seen in patients with CSU involves all the cellular elements of blood many of which have been considered in this review (Fig. 1). We have not discussed monocytes, in particular, although they are plentiful when cells within skin biopsies are counted (190), and have enhanced tissue factor expression (135). By definition, monocyte chemotactic proteins 1-4 i.e. MCP 1-4 may be responsible for their accumulation along with the other cell types. Lymphocyte subtypes that have not been considered include NK cells, and NKT cells. We have

not considered B lymphocytes because they are not seen within the urticarial lesions although they certainly have a systemic role in the production of the autoantibodies described above.

While methods such as immunohistochemistry (66) can certainly shed light upon the function of cells within the perivenular infiltrate, their contribution in vivo, can perhaps best be inferred from clinical studies of efficacy of drugs that target particular cells or their products. Rituximab is reported to be effective (case reports) (192) presumably by preventing autoantibody synthesis although it is not generally recommended. Corticosteroids inhibit the function of T lymphocytes and eosinophils, and prevent egress of virtually all cell types from the blood stream into tissues, including skin. The latter effect certainly includes T lymphocytes, basophils, and eosinophils, and possibly monocytes. Neutrophil egress and function, as is seen in asthma, may be more resistant to steroid effects.

For the future, we envision studies of drugs with increasing specificity to determine the effect on the clinical course of CSU. We can target IL17 (secukinumab), TH-2 dependent cytokines, IL4 and IL13 (dupilumab), TH2 and eosinophil-dependent cytokines, IL5 and its receptor (mepolizumab, reslizumab, and benralizumab), complement C5a receptor (Avdoralimab), and mast cell and eosinophil cell surface Siglec-8 (lirentelimab) to name some being studied at present. This has been recently reviewed (193).

711 **Table 1. Summary of reports studying circulating cytokines in plasma or serum of patients with**
712 **CSU compared with HCs**

713



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717 response was investigated. Only statistically significant data with a P value <0.05 were included. If the
718 difference between the 2 groups analysed was ≥ 0.05 , it was reported in « no difference ».; GM-CSF:
719 granulocyte-macrophage colony-stimulating factor; TGF: transforming growth factor; IL: interleukin;
720 TNF: tumor necrosis factor; IFN: interferon

721

722

723 Table 2: Summary of reports studying cytokine expression in (cultured) PBMCs of patients with
724 CSU (including mRNA expression and protein expression in supernatant) compared with HCs
725



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732 **Table 3: Summary of reports studying cytokines in wheals of patients with CSU.**
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1338 Figure legends:

1339 Figure 1 - Skin Biopsies representative of chronic spontaneous urticaria with a non-
1340 necrotizing perivascular infiltration of cells. (A) Predominance of T lymphocytes and
1341 monocytes. (B) Mononuclear cells with prominence of eosinophils.

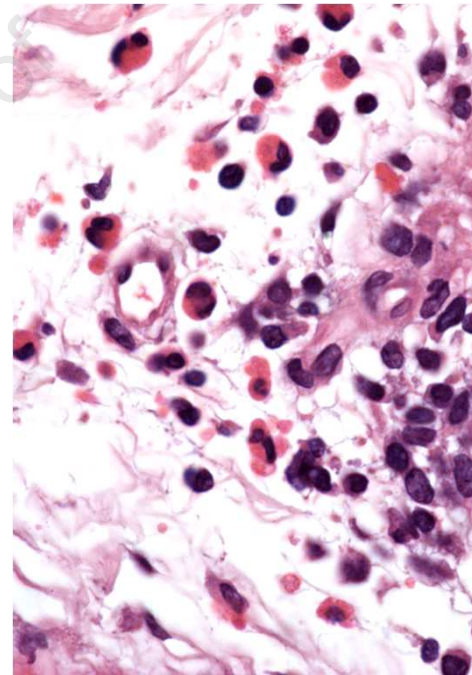
1342

1343 Figure 2 - Functions of basophils, eosinophils, neutrophils, T lymphocytes, and Endothelial
1344 cell in Chronic Spontaneous Urticaria

1345

A

B



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Mast Cells

Cytokines (priming, receptor upregulation)

Chemokines (chemotaxis)

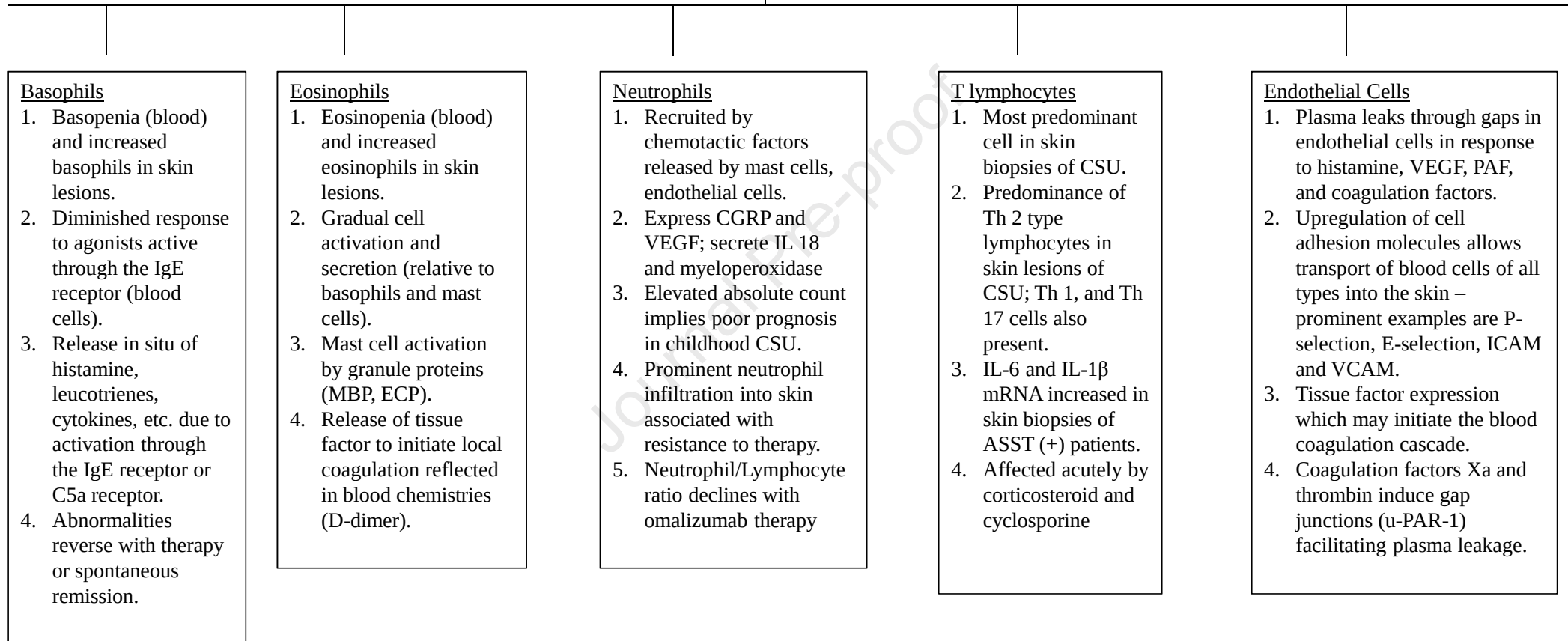


Figure 2