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Interleukin-17 is a potential player and treatment target in severe chronic spontaneous urticaria

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Short title: IL-17 in severe CSU

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Abstract 273 words

Background: Chronic spontaneous urticaria (CSU) is considered an autoimmune disorder in 50% of cases at least, in which T and mast cell mediators are considered to be the primary cause of symptoms. However, H₁-antihistamines, cyclosporine A and omalizumab fail to achieve complete symptom amelioration in up to 70% of patients. This suggests that other inflammatory pathways are involved and that additional and more effective treatments need to be developed.

Objective: This preliminary report examines the possibility that interleukin-17 (IL-17), a cytokine involved in the pathogenesis of many autoimmune diseases, may contribute to CSU and its inhibition may offer a relevant therapeutic target.

Methods: The expression of IL-17A in skin biopsies of 20 CSU patients and 10 healthy controls was determined by quantitative histomorphometry. We also assessed the response to secukinumab (anti-IL-17A) treatment patients of eight severe CSU (7 day urticaria activity score UAS7 32-40) who were H₁-antihistamine and omalizumab-resistant. **Results:** increased numbers of CD4⁺ T cells and mast cells were present in both lesional and non lesional skin of CSU patients compared with healthy controls. Both types of cells were strongly positive for IL-17A and found to be in close proximity to each other. All eight patients treated with the anti-IL-17A antibody, secukinumab, showed significant improvement in CSU disease activity. The action of secukinumab was shown to be relatively slow in onset. The significant reduction in disease activity from baseline UAS7 was demonstrated to be 55% and 82% at 30 and 90 days respectively. **Conclusions:** These findings suggest that IL-17 is involved in the pathogenesis of CSU and that IL-17 should be investigated as a therapeutic target in future studies with larger numbers of patients.

Introduction

Chronic spontaneous urticaria (CSU) is autoimmune in origin in at least 50% of cases. Type I and type II autoimmunity (i.e. IgE to autoallergens and IgG autoantibodies to IgE or its receptor, respectively) have both been implicated in its etiology.¹ Because H₁-antihistamines are effective in suppressing the symptoms, the mast cell has been the main target for the development of novel treatment with anti-IgE (omalizumab) being the primary agent.² However, omalizumab fails to achieve complete response in up to 70% of patients.³ This suggests that other mechanisms may also be involved and that additional and more effective treatments need to be developed. In this preliminary report, we explore the potential role of interleukin-17 (IL-17) in CSU and the effectiveness of anti-IL17A for its treatment in patients in whom omalizumab was ineffective. In line with the key role of autoimmunity in CSU, CD4⁺ T cells have been reported to be involved in its pathogenesis. Earlier studies had demonstrated activated CD4⁺ T cells in the lesional skin of CSU patients⁴ and the effectiveness of cyclosporine A in CSU.⁵ Newer reports suggest that CSU patients may respond to dupilumab, an interleukin-4 receptor antagonist that targets Th2 cells.⁶ While lymphocytes produce a number of multifunctional cytokines, IL-17 has been reported to play a key role in chronic inflammatory autoimmune disorders including psoriasis, rheumatoid arthritis, inflammatory bowel disease and multiple sclerosis.⁷ Recently, increased circulating levels of IL-17 have been found in CSU.⁸ Furthermore, IL-17 blood levels appeared to be related to CSU severity suggesting a role in the pathogenesis of the disease.⁹ The cellular provenance of IL-17 is primarily the Th17 subset of CD4⁺T-helper cells.¹⁰ IL-17 is also found in mast cells, but it is suggested that, rather than synthesise it, they actively uptake and store it in granules from which it can be released in a bioactive form.¹¹

To better define and characterize the role of IL-17 in CSU, we assessed the expression of IL-17A in the lesional skin (wheals) and non-lesional skin of patients and its association with CD4⁺T cells and mast cells. In addition, we treated eight severe and high dose H₁-antihistamine and omalizumab-refractory CSU patients with secukinumab, an anti-IL-17A antibody licensed for the treatment of psoriasis.^{12,13}

Methods

Skin immunohistochemistry

Patients

The study included 30 patients with severe CSU treated at the Urticaria Centres of Reference and Excellence (UCARE)¹⁴ of Charité - Universitätsmedizin Berlin, Germany, the Division of Allergy & Clinical Immunology of the Bnai-Zion Medical Centre, Haifa, Israel and the Department of Dermatology at the Sheba Medical Centre, Tel-Aviv, Israel. All enrolled patients suffered from severe CSU, with weekly urticaria activity score (UAS7) values of more than 36 points, wheals lasting more than 24 months, and a poor response to high off-label doses of H₁-antihistamines. Steroids were of limited beneficial effect, unable to significantly reduce CSU activity and linked to urticaria exacerbation when discontinued. Skin biopsies from these patients were performed before the administration of omalizumab or cyclosporine A, aiming to assess the status of CD4⁺ T cells, mast cells and IL-17A expression, each alone and double stained for the co-existence of IL-17A in both CD4⁺ T cells and mast cells.

Immunohistochemistry methods

Lesional (n=20) and non lesional (n=10) skin biopsies of 30 CSU patients and control skin biopsies from 10 healthy subjects were evaluated for the expression of CD4⁺ T cells, mast cells and IL-17, by quantitative histomorphometry. Briefly, formalin-fixed paraffin-embedded skin tissue sections were sliced serially into 5 µm slices by a microtome. The slides were deparaffinized by xylene and then rehydrated by absolute ethanol, 96% ethanol, 70% ethanol and 2 short distilled water washes. The antigen retrieval was done by cooker pressure in an EDTA solution (2mM, and pH-8.0). H₂O₂ 3% in methanol was used to block endogenous hydrogen peroxidase. In order to block non-specific endogenous biotin, we used a commercial biotin blocker, according to the manufacturer's instructions. Primary antibody, rabbit polyclonal anti human- IL17A (1:100, Proteintech group LTD, Rosemont, IL, USA), rabbit polyclonal anti-human cKit (Abcam, Cambridge, USA) (1:100), and rabbit polyclonal anti-human CD4 (Abcam) (1:300) were incubated with the slides. Secondary antibody and staining were performed by using the commercial "Histostain+" kit, ("Invitrogen", Camarillo, CA, USA), according to the manufacturer's instructions. Negative controls were stained using specific isotype control and slides that underwent the same procedure but without primary or secondary antibodies. The immuno-histochemical staining was evaluated by two independent pathologists and was inspected for localization and staining intensity. The extent of staining was given a score from 0-3 (0-absent and 3 for strong). The amount of CD4⁺ cells and cKit⁺ (CD117⁺) cells in skin biopsies each alone and double stained for CD4⁺ T cells/IL-17A and CD117⁺ cells/IL-17A was calculated by using

IMARIS microscope software. Using this highly accepted method of quantification, the whole slide was scanned and quantified for the amount of CD4⁺ and cKit⁺ cells in microscopic High Power Field (HPF).

Clinical treatment with anti-IL-17

Anti-IL-17A (secukinumab) was given to eight different patients with CSU who had high disease activity score (UAS7 32-40). C-reactive protein was increased in all patients (range 9-12 mg/L, mean $\pm$ 10 mg/L) and total IgE was elevated in three (250, 310, 430 units respectively). All were refractory to all standard treatment regimens including omalizumab (up to 450 mg/4weeks) and cyclosporine A. At the time of the start of anti-IL17A treatment, these patients were on oral steroid treatment, given as a short course, combined with high off-label doses of H₁-antihistamines. Serum levels of IL-17A were not assessed in this study. Anti-IL17A (secukinumab) was injected subcutaneously at 150 mg every week for 4 consecutive weeks and then 150 mg every two weeks.

Ethics approval

The local ethics committee (0133-18-BNZ) approved the study and all patients signed an informed consent.

Statistical analyses

Data are expressed as mean $\pm$ stand error of the mean. The comparison of the differences between two groups was performed using the unpaired two tailed Student t-test, whereas for more than two groups it was done by using ANOVA test. Statistical significance was considered with a P-value of 0.05 or less (p-value $\leq$ 0.05).

Results

The skin of patients with CSU shows markedly increased numbers of T cells and mast cells

In comparison with the skin of healthy volunteers, which had a mean number of 9 ± 2 CD4⁺ cells per/microscopic high power field (Figure 1 Panel A), the numbers in CSU lesional skin were markedly higher with levels of 39 ± 12 CD4⁺ cells/ per microscopic high power field (Figure 1, panel B). This difference was statistically significant, $P = 0.0002$ (Figure 1, panel C). The numbers of CD4⁺ T cells in the non-lesional skin of CSU patients were similar to lesional skin. CD117⁺ mast cells were also higher in number in CSU compared with healthy skin. In healthy skin, there was a mean of 60 ± 10 CD117⁺ cells per/microscopic high power field (Figure 2, panel A). In lesional (Figure 2, panel B) and non-lesional CSU skin, mean CD+117 mast cells were 260

± 80 cells per/microscopic high-power field, respectively. The difference between lesional/non-lesional and healthy skin was statistically significant, $P=0.0099$ (Figure 2 panel C).

The skin of CSU patients shows markedly increased levels of IL-17

The expression of IL-17A in lesional and non lesional skin of patients with CSU was markedly increased (mean score 2-3) compared with normal skin where IL-17 A expression was absent to mild (mean score 0-1) (Fig 3, panel A and B) ($P < 0.0001$). Both, CD4+ T cells and mast cells, showed strong expression of IL-17A, with most IL-17 expressing CD4+ T cells and mast cells positioned in close proximity to each other (Fig 4, panels A and B). This was straightforward to observe since mast cells have a characteristic spindle shape nuclei and easily identifiable cytoplasmic granules, whereas CD4+ T cells show round nuclei with tiny non-granular cytoplasm. To confirm that CD4+T and mast cells express IL-17A, cells were double stained with relevant monoclonal antibodies. As seen in Figure 4 panels C and D, both, CD4+ T cells and CD117+ cells expressed IL-17A.

Treatment with anti-IL-17 is highly effective in patients with CSU

Treatment of eight antihistamine and omalizumab resistant patients with secukinumab, 150 mg once a week for four consecutive weeks and then 150 mg every two weeks resulted in a significant improvement of symptoms (Table 1). After 30 days, disease activity as assessed by the UAS7 was reduced by 55% from baseline ($P = 0.0009$), and there was a significant reduction in the severity and frequency of angioedema. By this time, omalizumab and cyclosporine A (when they were still being used) and steroids were completely stopped. By 90 days, the UAS7 was reduced by 82% ($P = 0.00002$ vs baseline), with the recurrence of minimal episodes of wheals (UAS7 = 2-4), requiring occasionally standard doses of antihistamines. All patients were then maintained on secukinumab, remaining free of all other therapies (except of occasional intake of antihistamines). No notable unwanted effects were reported, (mild local irritation at the site of injection in 3 patients). Notable facets of the action of secukinumab were that it was relatively slow in onset and that its reduction of the starting UAS7 of 55% and 82% at 30 and 90 days, respectively, were remarkably similar for all eight patients.

Discussion

This study has shown clearly the presence of IL-17 staining mast cells and CD4+ lymphocytes in the skin of CSU patients and that severe CSU patients who responded poorly to H₁-antihistamines,

steroids, cyclosporine A and omalizumab, responded well to therapy with the anti-IL-17 antibody, secukinumab.

The primary importance of our finding is that, in addition to the intense infiltration of IL-17 expressing CD4⁺ T cells in the skin of CSU patients, we show for the first time evidence of increased IL-17A expression in skin mast cells in CSU. Looking at the biopsies it was immediately obvious that IL-17A expressing CD4⁺ cells and mast cells are located in close proximity, suggesting this phenomenon to be of mechanistic importance. It is well established that IL-17 is produced and secreted by conventional Th17 cells, CD8⁺ T cells and NK cells in many immune-mediated diseases.¹⁰ Our finding of the close proximity of IL-17A positive mast cells and CD4⁺ cells is in one line with previous studies in which close physical proximity between mast cells and T cells has been demonstrated in T-cell mediated inflammatory diseases. Mast cells were shown to be activated by micro particles released from activated T cells¹⁵. At present, the issue of IL-17 being produced by activated mast cells or whether mast cells actively uptake IL-17 into intracellular granules is still open.¹¹ Furthermore, the acquisition of IL-17 by mast cells promoted their proliferation by triggering stem cell factor in keratinocytes.^{16,17} Our other finding is the fact that IL17A expressing CD4⁺T cells and mast cells are found in similar intensity in both lesional and non-lesional skin parts of CSU patients, but not in normal skin. This goes in line with the hypothesis that when CSU is severe, the skin is permanently infiltrated with immune cells. Once irritated by cytokines, including IL17, they induce a transient flare short lasting degranulation which may then resolve. Once CD4 + T cells and activated mast cells get again closer to each other, they induce new flares. This could be an explanation for the episodic attacks in CSU. Obviously, further studies are required to establish this concept.

The observations of IL-17A positive CD4⁺ cells and mast cells encouraged us to treat new eight of our especially refractory CSU patients with anti-IL-17A. The administration of secukinumab achieved a significant remission as long as they were maintained on this regimen. Notable facets of the action of secukinumab were that it was slow in onset and that its reduction of the starting UAS7 of 55% and 82% at 30 and 90 days, respectively, were remarkably similar for all 8 patients. We may speculate that anti-IL-17 may neutralize serum and skin high levels of IL-17 in CSU patients decreasing by that Th17 expansion and activation and mast cell activation.

The small cohort of treated patients does not allow at this stage to predict whether increased serum CRP could be a surrogate marker for predicting a beneficial response to anti-IL-17A. So far, secukinumab seems to be a safe therapy, of steroid sparing effect and highly

effective. It is highly reasonable to suspect our severe CSU patients with high CRP to be a rather specific cluster of patients. However, our patients presented with classical hives, angioedema in some, but with no fever and with no atypical episodes of transient flares. Therefore, we think that our treated severe CSU patients were truly classical CSU patients. We are currently planning to treat a bigger cohort and a wider spectrum of CSU patients, such as less severe patients hoping to better establish its safety and the efficacy of this new regimen.

In conclusion, we have demonstrated an upregulation in CSU skin biopsies in the number of CD4⁺ cells and mast cells and a strong association of IL-17A with both. Furthermore, we have shown that an anti-IL-17A antibody, secukinumab, may be an effective treatment for difficult to treat high dose H₁-antihistamine and omalizumab resistant CSU patients.

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Table 1. Seven day urticaria activity scores (UAS7) of severe UAS patients before (day 0) and 30 and 90 days after treatment with secukinumab 150 mg once a week for four consecutive weeks and then 150 mg every two weeks. The significance of differences from day 0 are $P = 0.0009$ at day 30 and $P = 0.00002$ at day 90.

Patient	Day 0	Day 30	Day 90
1	40	18	6
2	32	20	6
3	40	14	8
4	38	18	6
5	36	15	8
6	32	15	6
7	34	15	8
8	40	20	8

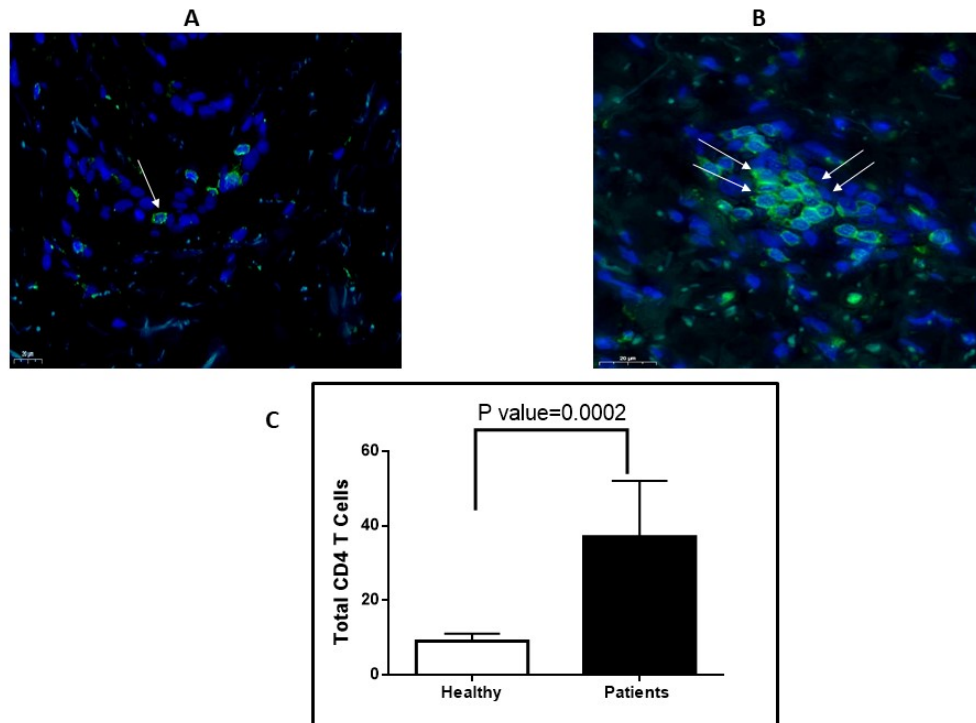
Figure Legends:

Figure 1: CD4⁺ T cells in skin biopsies. **A-** Normal skin shows small amount of CD4⁺ T cells. White arrows denote positive CD4⁺ T cells. **B-** CSU skin biopsy demonstrates numerous CD4⁺ T cells. White arrows denote positive CD4 T cells. **C-** Quantitative analysis of number of CD4⁺ T cells in normal vs. CSU skin biopsies (P=0.0002).

Figure 2: CD117⁺ mast cells in skin biopsies. **A-** Normal skin shows small amount of CD117⁺ mast cells. White arrows denote positive CD117 mast cells. **B-** CSU skin biopsy demonstrates numerous CD117⁺ mast cells. White arrows denote positive CD117 mast cells. **C-** Quantitative analysis of number of CD117⁺ mast cells in normal vs. CSU skin biopsies (P=0.0099).

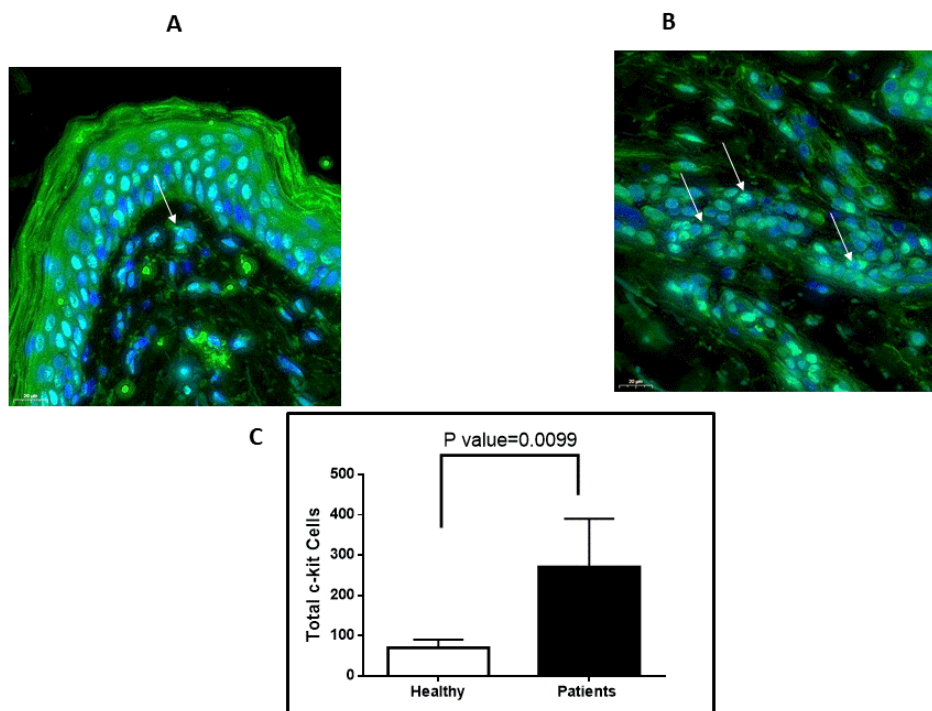
Figure 3: Immunohistochemistry of IL-17. **A-** Normal skin biopsy. As can be shown there is almost no IL-17 expression. **B-** Two examples of positive IL-17 staining in CSU skin biopsies. Blue arrows denote positive staining of endothelial cells and infiltrating lymphocytes (X20 magnification). The lower panel demonstrates positive staining of endothelial cells and lymphocytes in X40 magnification.

Figure 4: **A-** Positive IL-17 stained cells in CSU skin biopsy. Arrow No.1 denotes mast cell and positive stained CD4⁺ lymphocytes (arrows No.2). X200 magnification. **B-** Positive IL-17 stained mast cell in CSU skin biopsy (Arrow No. 2), in proximity to lymphocytes (arrows No. 3 and 4). Endothelial cells (Arrow No. 1) were also stained for IL-17A expression. X400 magnifications. **C-** Double staining immunofluorescence CD4⁺/IL-17⁺ cells in CSU skin biopsies, see white arrows. **D-** Double staining immunofluorescence CD117⁺/IL-17⁺ cells in CSU skin biopsies, see white arrows.

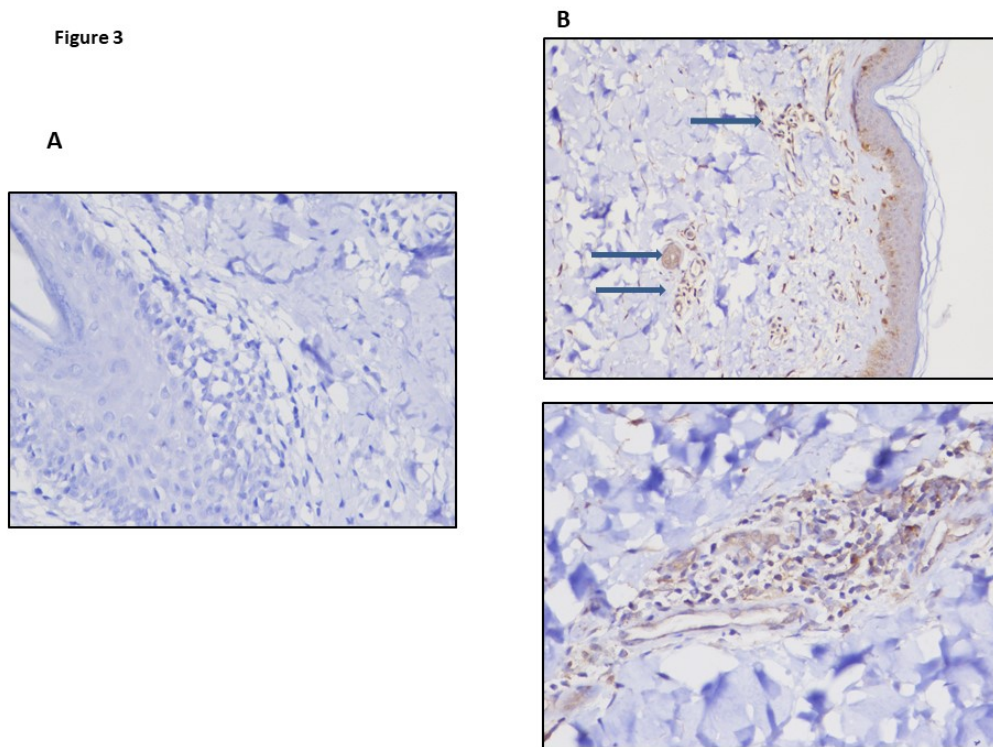
Figure 1

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Figure 2

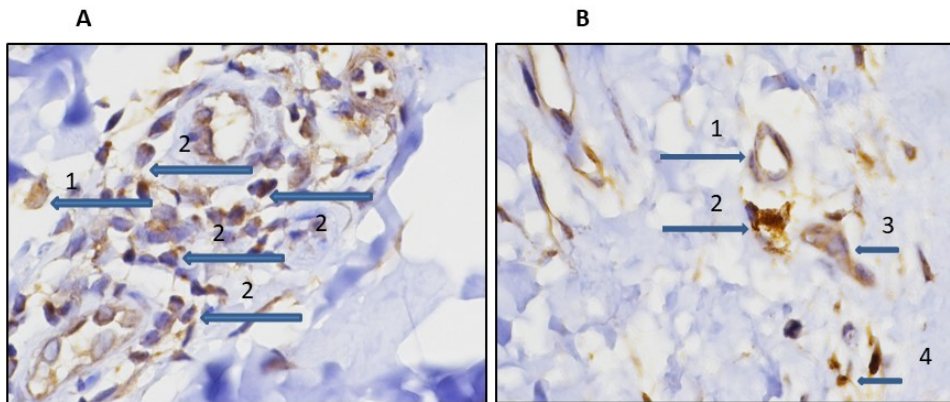


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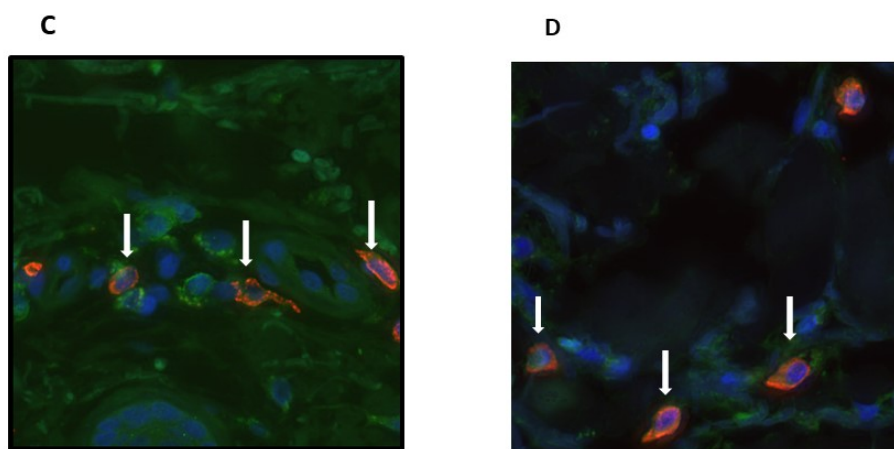


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Figure 4



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