

# IN SITU IMMUNE RESPONSE EVALUATION VIA IMMUNOHISTOCHEMISTRY IN SKIN BIOPSIES FROM PATIENTS AFFECTED BY *AUTOIMMUNE BLISTERING DISEASES*

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**Source of Support:**  
Georgia Dermatopathology  
Associates, Atlanta, Georgia, USA  
(MSH, AMAV).

**Competing Interests:**  
None

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Our Dermatol Online. 2013; 4(Suppl. 3): 606-612

Date of submission: 03.06.2013 / acceptance: 01.08.2013

## Abstract

**Introduction:** The in situ immune response in skin biopsies from patients affected by autoimmune skin blistering diseases (ABD) is not well characterized. Aim: Our investigation attempts to immunophenotype cells in lesional skin in several ABD, utilizing immunohistochemistry (ICH).

**Methods:** We tested by IHC for CD4, CD8, CD19, CD20, CD45, CD56/NCAM, PAX-5, granzyme B, myeloperoxidase, neutrophil elastase, LAT and ZAP-70 in patients affected by ABD. We tested 30 patients with endemic pemphigus foliaceus (EPF), 15 controls from the EPF endemic area, and 15 biopsies from healthy controls from the USA. We also tested archival biopsies from patients with selected ABD, including 30 patients with bullous pemphigoid, 20 with pemphigus vulgaris, 8 with pemphigus foliaceus and 14 with dermatitis herpetiformis.

**Results:** We found a predominantly CD8 positive/CD45 positive T cell infiltrate in all ABD. Our skin biopsies demonstrated consistently positive staining for myeloperoxidase, but negative staining for neutrophil elastase. Most ABD biopsies displayed negative staining for CD4 and B cell markers; natural killer cell markers were also rarely seen. ZAP-70 and LAT were frequently detected. In El Bagre-EPF, a significant fragmentation of T cells in lesional skin was noted, as well as autoreactivity to lymph nodes.

**Conclusions:** The documented T cell and myeloperoxidase staining are indicative of the role of T lymphocytes and neutrophils in lesional biopsies in patients with ABD, in addition to previously documented deposition of B cells, immunoglobulins and complement in situ. In El Bagre-EPF, T cells could also target lymph nodes; however, further studies are needed to confirm this possibility.

**Key words:** autoimmune blistering skin diseases; B lymphocytes; T lymphocytes; CD4; CD8; CD45

**Abbreviations and acronyms:** Bullous pemphigoid (BP), immunohistochemistry (IHC), direct and indirect immunofluorescence (DIF and IIF), hematoxylin and eosin (H&E), basement membrane zone (BMZ), intercellular staining between keratinocytes (ICS), pemphigus vulgaris (PV), cicatricial pemphigoid (CP), autoimmune blistering skin diseases (ABD), fogo selvagem (FS), endemic pemphigus foliaceus in El-Bagre, Colombia (El Bagre EPF), linker for activation of T cells (LAT), T cell antigen receptor zeta chain (ZAP-70), B cell specific activator proteins (BSAPs).

## Cite this article:

Ana Maria Abreu Velez, Paul B. Googe, Jr., Michael S. Howard: In situ immune response evaluation via immunohistochemistry in skin biopsies from patients affected by autoimmune blistering diseases. *Our Dermatol Online*. 2013; 4(Suppl.3): 606-612.

## Highlights:

· Our study suggests that a CD3/CD8/CD45 positive T cell response and neutrophils may play significant roles in these diseases.

· The positivity of T cell activation markers such as LAT and ZAP-70 in lesional skin favors their role in ABD.  
· El Bagre-EPF demonstrates fragmented T cells in situ, with concomitant immune reactivity to lymph nodes that warrants further studies.

## Introduction

Multiple theories and studies have been proposed regarding the pathophysiology of cutaneous autoimmune blistering skin diseases (ABD); most favor B cell mediated processes, given autoantibodies and complement deposits in the skin, and

the known correlation between titers of autoantibodies and the clinical severity of the diseases [1-5]. Additional studies have been performed on animal models, transferring human autoantibodies and inducing temporary blisters.

However, the injected animals used are often neonatal, and have thin skin; moreover, no animal model reproduces the clinical chronicity noted in most human ABD [1-5]. Notably, a few reports have shown that pregnant mothers with pemphigus can transfer disease autoantibodies to their fetus, and that the fetus/newborn in turn develops a self-resolving presentation of the disease [6].

It is known that healthy relatives of patients with ABD may possess disease antibodies, but not develop clinical disease. It has further been documented that people who live in the endemic areas may possess disease autoantibodies without clinical disease, suggesting that other immune responses and/or factors are necessary for the development of the clinical disease [7].

Previous research on ABD has primarily focused on the humoral immune response, but little attention has been given to the function of the cell-mediated immune response and cellular elements of the tissue reaction in lesions of ABD. The present investigation aims to characterize the immune infiltrate in various ABD, considering that patients affected by ABD often display complications related to non-B cell autoimmunity (e.g. verrucae, tuberculosis, strongyloidosis, entamoebiasis, nocardiosis, hydatidosis, herpes, varicella zoster, etc.) [8-14]. Thus, we decided to study the in situ immune response by performing immunohistochemistry (IHC) on ABD lesional skin biopsies.

## Materials and Methods

### Subjects of study

We tested 30 biopsies from patients affected by EPF in El Bagre, Colombia, South America (El Bagre-EPF) as previously described and 30 normal controls from the endemic area [15-20]. We also utilized 30 control skin biopsies from cosmetic surgery patients in the USA, taken from the chest and/or abdomen. Biopsies were fixed in 10% buffered formalin, then embedded in paraffin and cut at 4 micron thicknesses. The tissue was then submitted for H&E and IHC staining. In addition, we also tested biopsies from the archival files of two private, board certified dermatopathology laboratories in the USA, from patients who were not taking immunosuppressive therapeutic medications at the time of biopsy. We evaluated 30 biopsies from bullous pemphigoid (BP) patients, 20 from patients with pemphigus vulgaris (PV), 30 from patients with El Bagre-EPF, 8 patient biopsies with pemphigus foliaceus (PF) and 14 from patients with dermatitis herpetiformis (DH). For all of the El Bagre area patients and controls we obtained written consents, as well as Institutional Review Board permission from the local hospital. The archival biopsies were IRB exempt due to the lack of patient identifiers.

### IHC staining

The staining intensity of the antibodies was also evaluated qualitatively by two independent observers, we use the scale of no staining (negative), one to 3 cells stained positive per 200x microscopic field (+), 3 to 7 (++), 7 to 13 (+++) and 14 or more (++++). For IHC, we utilized the following antibodies from Dako (Carpinteria, California, USA): monoclonal mouse anti-human CD3 (Clone F7.2.38), monoclonal mouse anti-human CD4 (Clone 4B12), monoclonal mouse anti-human CD5, monoclonal mouse anti-human CD8 (Clone C8/144B), monoclonal mouse anti-human CD19 (Clone LE-CD19), monoclonal mouse anti-human CD20cy (Clone L26), monoclonal mouse anti-human

CD45 (Clone 2B11 + PD7/26), monoclonal mouse anti-human CD56/NCAM (Clone 123C3), monoclonal mouse anti-human neutrophil elastase (Clone NP57), monoclonal mouse anti-human B-cell-specific activator protein/BSAP (Clone DAK-Pax5; also known as Pax-5, a transcription factor expressed in B cells), polyclonal rabbit anti-human myeloperoxidase, monoclonal mouse anti-human granzyme B, monoclonal mouse anti-human linker for activation of T cells/LAT (Clone LAT-1; expressed on T cells without restriction to any T-cell subpopulation), monoclonal mouse anti-human T-cell antigen receptor zeta chain/ZAP-70 (Clone 2F3.2). The ZAP-70 antibody reacts with ZAP-70 expressed in T cells, natural killer cells and pro/pre B cells, but not in normal mature B cells, monoclonal mouse anti-human myeloid/histiocyte antigen (clone MAC 387). The myeloid/histiocyte antibody reacts with a human cytoplasmic antigen (L1-antigen or calprotectin) which contains two different subunits (L1H and L1L). The protein is a member of the S100 family, and the subunits in this context are titled S100A8 and S100A9. It is expressed on granulocytes, monocytes, tissue histiocytes, squamous mucosal epithelia, and reactive epidermis. The antibody is useful for demonstrating tissue histiocytes in tissue sections from malignant lymphomas, and for detecting lymphoid neoplasms of histiocytic origin. Our IHC staining was performed as previously described [15]. A positive and negative control sample was used with all experiments.

Immunofluorescence studies on lymph nodes were performed as previously described, utilizing rat, mouse, chipmunk and beef tissue as antigen sources [15].

### Statistical methods

Differences in staining positivity and intensity were tested using a GraphPad Software statistical analysis system, and employing Student's t-test. We considered a statistical significance to be present with a p value of 0.05 or less, assuming a normal distribution of the samples.

### Result

The semiquantitative analysis of the cell population revealed a predominance of CD3/CD8/CD45 positive T lymphocytes in the tissue response of perilesional and lesional skin of the majority of the ABD.

Ninety-eight percent (98%) of the patients with ABD demonstrated negative staining for CD4 (Tabl. I). Ninety-five percent (95%) stained positively for CD3, CD8 and CD45, as well as T cell activation markers such LAT and ZAP-70 ( $p < 0.05$ ). These cells and/or markers were predominantly positive in superficial dermal infiltrates, located around the upper dermal neurovascular plexus. No significant differences were seen between the different ABD in regard to positivity in these markers, however, in the BP, PV, PF, El Bagre-EPF and DH biopsies, the infiltrate was also noted within eccrine sweat glands. In the El Bagre-EPF, PV and PF cases, the infiltrate was also noted in neurovascular areas of hair follicular units (Fig. 1, 2). Ninety-eight percent (98%) of the ABD stained negative for natural killer cell and related markers such granzyme B and PAX-5. Ninety-five percent (95%) of the ABD stained negative for CD19, CD20 and BSAP (Tabl I). Finally, LAT and ZAP-70 were positive in all of the 58 consolidated cases of PV, PF and El Bagre-EPF, within the upper dermal inflammatory infiltrate and also within the epidermis. These markers were negative in the control cases (Fig. 1, 2).

Seventy percent of the El Bagre-EPF patients demonstrated fragmented CD3/CD45 T cells in lesional skin; this positive finding was not seen in controls from the El Bagre EPF endemic area, nor in the plastic surgery control samples.

Myeloperoxidase staining displayed consistent positivity in 97% of the ABD cases; in contradistinction, neutrophil elastase was predominantly negative (p<0.05%) (Fig. 1, 2, 3).

Antibody	BP n=34	PV n=14	PF n=4	DH n=10	El Bagre-EPF n=30	Controls from Endemic arean= 30	Controls/plastic surgery n=15
<b>CD45</b>	Positive in the upper inflammatory infiltrate mostly around the vessels (++++). Also some cells positive around the sweat ducts (30/34) (++)	Positive at the upper inflammatory infiltrate the upper dermis, Some also positive around the sweat ducts and around neurovascular bundles (++)	Positive at the upper inflammatory infiltrate the upper dermis, and around some sweat ducts and around neurovascular bundles (++)	Positive infiltrate in the upper inflammatory infiltrate (++) in 8/10. Also positive around the sweat glands in 3/10.	Positive in the upper dermis (++++). Also positive around the sweat glands (8/10).	Negative	Negative
<b>CD8</b>	Positive in the upper dermal inflammatory infiltrate (+++) (27/34). Positive also around the sweat ducts (++)	Very strong infiltrate in all the upper dermis and some keratinocytes and eccrine ducts (+++) (10/14)	Positive in the upper dermal inflammatory infiltrate. Positive also around the eccrine ducts (++) (4/4). Positive in the keratinocytes	Very strong infiltrate in all the upper dermis and some keratinocytes (++) (8/10).	Positive at the inflammatory infiltrate in the upper vessels and some eccrine and also around pilosebaceous units glands (++) (26/30).	Negative	Negative
<b>CD3</b>	Positive upper inflammatory infiltrate (+++) (30/34).	Very strong infiltrate in all the upper vessels and also around pilosebaceous units (++) (4/4).	Positive at the inflammatory infiltrate in the upper vessels and some eccrine and also around pilosebaceous units glands (++) (8/10).	Positive around the inflammatory infiltrate of the base of the neurovascular package of the sebaceous glands (++)	Positive at the inflammatory infiltrate in the upper vessels and some eccrine and also around pilosebaceous units glands (++) (28/30).	Negative	Negative
<b>CD4</b>	Negative.	Only one biopsy positive in the upper inflammatory infiltrate especially around the vessels	Negative	Negative		Negative	Negative
<b>CD19</b>	Negative	Negative	Negative	Negative	Negative	Negative	Negative
<b>CD20</b>	Negative	2/14 few spare cells in the upper dermis.	2/4 some cells positive in the hair follicles.	2/10 some cells positive in the hair follicles.	Some few biopsies have some few positive cells in the intermediate vessels of the dermis. Most cases were negative.	Negative	Negative

**Table I. Cell populations and markers in lesional skin from multiple autoimmune skin diseases.**

Antibody	BP n=34	PV n=14	PF n=4	DH n=10	El Bagre-EPF n=30	Controls from Endemic arean= 30	Controls/plastic surgery n=15
<b>Granzyme B</b>	Negative	Negative	Negative	2/10 (++) around the upper neurovascular plexus.	Negative	Negative	Negative
<b>Myeloperoxidase</b>	Positive inside the blister, and in the cells of the upper inflammatory infiltrate (+++) (30/34). Positive pattern of reactivity around the BMZ like linear.	Multiple positive cells in the upper inflammatory infiltrate (+++) and also some in the lower epidermis (10/14).	Some positive cells in the upper inflammatory infiltrate (++) (3/4).	Positive in the blister and in all the inflammatory infiltrate (++++ in 9/10, and also around the eccrine ducts (++)). Positive also several cells in the dermis.	Very positive in the blister and in all the inflammatory infiltrate in 28/30.	Negative	Negative
<b>Neutrophil elastase</b>	Positive weak in 10/34 upper inflammatory cells and or in the blister	Positive weak in 6/14 upper inflammatory cells and or in the blister	Negative	Positive weak in 6/10 upper inflammatory cells and or in the blister	Weak positive in 10/30 upper inflammatory cells	Negative	Negative
<b>LAT</b>	Positive in most biopsies all the inflammatory infiltrate around the upper vessel (30/34) (+++).	Positive in all the inflammatory infiltrate around the upper vessel in 4/14 (+++). Positive the epidermal cells in 12/14	Positive in all the inflammatory infiltrate around the upper vessel (+++). Positive the epidermal cells in 4/4.	Positive in 3/10 in the upper inflammatory cells.	Positive in all the inflammatory infiltrate around the upper vessel (+++) in 27/30. Some positivity of the eccrine ducts. Positive the epidermal cells in 28/30.		Negative
<b>ZAP-70</b>	Positive in all the inflammatory infiltrate around the upper vessel in 30/34 (+++). Positive around the inflammatory in 6/34	Positive in all the inflammatory infiltrate around the upper vessel (+++) 14/14. Positive the epidermal cells in 12/14. Also positive in the eccrine glands and ducts in 8/14.	Positive in all the inflammatory infiltrate around the upper vessel (+++). Positive the epidermal cells in 4/4.	Positive around some upper vessels and some sweat glands in 6/10.	Positive in all the inflammatory infiltrate around the upper vessel (+++) in 27/30. Some positivity of the eccrine ducts. Positive the epidermal cells in 28/30.	Negative	Negative
<b>B-cell specific activator protein / BSAP</b>	Negative.	Very few positive cells in the hair follicle steam area.	Negative.	Negative.	Negative	Negative	Negative
<b>CD56/NCAM</b>	Negative	Negative	Negative	Negative	Negative	Negative	Negative

**Table I. Cell populations and markers in lesional skin from multiple autoimmune skin diseases (continued).**

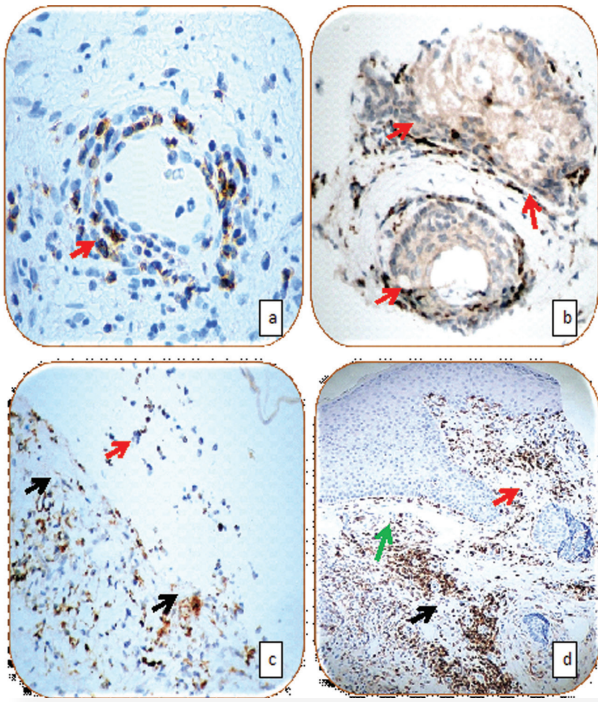
## Discussion

The purpose of our study was the immunophenotypic characterization of inflammatory cells and the expression of adhesion molecules in lesional and perilesional skin of patients with ABD. ABD have been classically defined as B cell-mediated diseases due to the presence of both 1) spontaneously appearing, intraepidermal clinical blisters after injection of human sera into neonatal mice, and 2) epidermal-specific autoantibodies whose serum titers classically correlate with

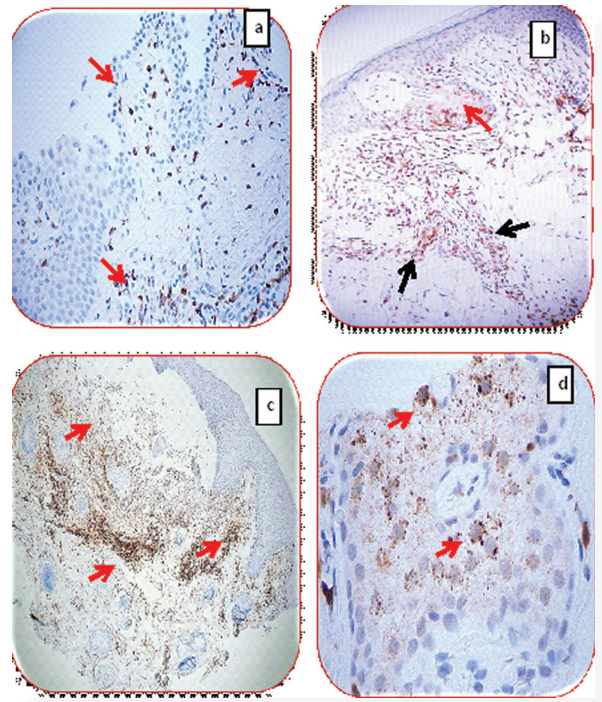
clinical activity and disease severity as demonstrated by IIF and ELISA [1-4,20].

Our in situ results document the involvement of other immune cells in ABD lesional skin, indicating that other immune system components may be important in these diseases [21-27]. In our study, we did not attempt to demonstrate a precise pathogenic role of these molecules; however, further investigation is warranted in this area.

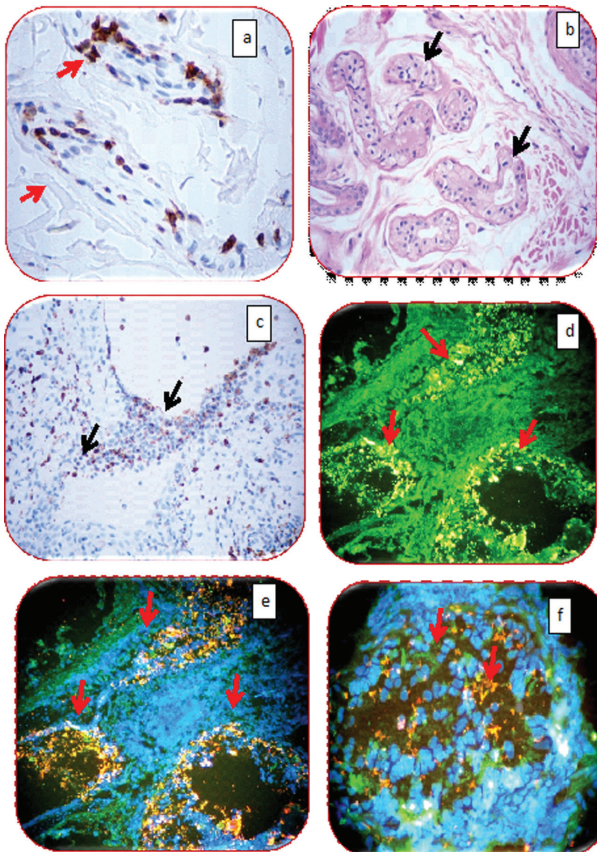




**Figure 1.** a. BP case with positive CD8 staining around an upper dermal blood vessel (brown staining; red arrow) (400X) and in b, the same case, with positive staining around a hair follicle and the periphery of an adjacent sebaceous gland. c. Same case as a and b, with positive CD8 staining inside the blister (brown staining; red arrow) as well as in the subjacent dermis (brown staining; black arrows). In d, another BP case with similar findings as in c; however, also note additional linear staining for CD68 under the blister (brown staining; green arrow), in the upper dermal vessels and inflammatory infiltrate (black arrow) and inside the blister (red arrow).



**Figure 2.** a. A PV case, with positive CD8 staining in dermal papillae and around upper dermal blood vessels (brown staining; red arrows) (200X). b. A PV case, demonstrating positive LAT staining where the blister is forming (brown staining; red arrow), and in dermal neurovascular packages and sweat gland ducts (brown staining; black arrows). c. A BP case, demonstrating positive staining for CD45 under the BMZ and in the upper dermal infiltrate (brown staining; red arrows)(40X). d. A PV case with positive staining for ZAP-70 inside and around a sebaceous gland (brown staining; red arrows) (400X).



**Figure 3.** a. A PV case, with positive staining for ZAP-70 inside dermal eccrine glands (brown staining; red arrows) (400X). b. H&E staining of the patient biopsy in a revealed edematous sweat glands (black arrows; 200X). c. A BP case, with positive staining for myeloperoxidase in the blister and in the upper dermal perivascular infiltrate (brown staining; black arrows) (200X). d. IIF utilizing rat lymph node tissue, showing positive staining with El Bagre-EPF serum and FITC conjugated anti-human IgG antibody (yellow staining; red arrows). Note the accentuation around three lymph node capsules and trabeculae, and in some interior foci (200X). e. Similar to d, but with positive IgG staining in lymph nodes perfectly colocalizing with Texas red conjugated p0071 antibody (orange staining; red arrows) (200X). f. Similar to e, but in this case higher magnification and demonstrating further perfect colocalization of IgG staining with Texas red conjugated ARVCF antibody (orange staining; red arrows) (400X). In e and f, also note that connective tissue nuclei were counterstained with Dapi (light blue).



Previous studies tested 30 fogo selvagem (FS) patients and 30 controls for B and T lymphocytes in the peripheral blood. The total T lymphocyte count and the T cell functional count were significantly lower in the FS patients. No FS patients were receiving immunosuppressive therapy when evaluated [21-23]. Previous authors have found some alteration in the T cell immune response, and/or alterations in T cell numbers detected by several methods including FACS analysis in ABD [21-28]. One group of authors have shown that T cells are required for the production of blister inducing autoantibodies in experimental epidermolysis bullosa acquisita [24]. Another study with untreated BP patients compared to controls has shown low CD4+, CD25 bright+, FOXP3+ cells were significantly reduced in BP [25]. However, a similar study displayed different results [26]. Our data provides evidence of a T cell-mediated role in ABD; however, our findings do not contradict the role that B lymphocytes play in these diseases, as demonstrated by others [27-29].

In contradistinction to recently published data, our findings did not support a significant role for natural killer cells, with a few exceptions noting the presence of these cells in situ in a few clinically active cases of DH [30]. We also were unable to demonstrate strong staining with neutrophil elastase, as shown by others [31], but instead we noted positive staining with myeloperoxidase in all of the ABD. Both enzymes are found in the same granules of neutrophils; specific enzymologic studies could further confirm our findings. Other authors have speculated that cell-mediated cytotoxic reactions are probably enhancing proteolytic activity at the site of bullous eruptions [23].

Since few studies have addressed the in situ immune response in ABD, we performed our IHC studies as a pilot study. Our next study will further investigate the pathogenic role of the CD8-positive T cell infiltrate found in lesional biopsies.

The fact that pemphigus is considered an autoantibody-mediated disease does not necessarily mean that B cells have to be present in the skin of pemphigus patients, as shown in our results. Moreover, the fact that many T lymphocytes are detected in lesional skin does not contradict the important role of autoreactive B cells in ABD. Limited information is available regarding how CD3/CD8/CD45 positive T cells might contribute to the pathogenesis of different ABD; however, our positive LAT and ZAP-70 markers indicate that some activation is occurring in situ in lesional skin. In clinical context, our study suggests that people affected by ABD are likely to have infections requiring a T cell, and not only a B cell response [8-14,31,32]. Also, many modulators of the T cell response seem to help in controlling ABD. Current literature data from other diseases notes that a CD8+ T-cell deficiency is a feature of many chronic autoimmune diseases, and is also found in Epstein Barr virus infection and low vitamin D [33]. Our findings may also be consistent with the fact that several T cell-target immunosuppressors such as cyclosporine, azathioprine, mycophenolate mofetil, intravenous immunoglobulin, rituximab and pentoxifylline are effective adjuvants in treating ABD [34]. The humoral aspects of the autoimmune responses in ABD have been extensively studied in the past; however, our studies and more recent evidence is showing that 1) diverse cellular interactions ultimately resulting in the formation of autoantibodies and 2) the involvement of autoreactive T cells in these diseases are also important in the immune response [35]. Taking into account superinfections with

viruses [36] and parasitic diseases in ABD, our data encourage the study of T cells interacting with B cells and dendritic antigen presenting cells in ABD. Finally, it has also been recently shown that when utilizing a functional classification of the differentially expressed genes (DEGs) in DH, data show both a B- and T-cell immune response (LAG3, TRAF5, DPP4, and NT5E) as suggested by our results.

## Conclusions

Analyzing the previous literature and assessing our current findings, we believe that our observed T cell immune response (primarily CD8 positive) could play an important role in the immune response in situ in patients with ABD. Our findings thus warrant extended studies with larger sample sizes to address these questions, aimed at both confirming the T cell immune response and further characterizing its nature utilizing activation studies with multiple antigens.

## Acknowledgements

Jonathan S. Jones, HT (ASCP) at Georgia Dermatopathology Associates for technical assistance. We also thank the patients and personnel of the Hospital Nuestra Señora del Carmen in El Bagre, Colombia; the Mayor of El Bagre, the Secretary of Health in El Bagre and officials at Mineros, SA that facilitated our work.

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