Original paper

A comparative study of expression of Fc receptors in relation to the autoantibody-mediated immune response and neutrophil elastase expression in autoimmune blistering dermatoses

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Here we investigated the cutaneous CD32A and CD89 expression in relation to the neutrophil elastase (NE) expression and serum level of anti-desmoglein 1 and 3 (DSG1/DSG3) IgG in pemphigus, anti-BP180/BP230 IgG in bullous pemphigoid (BP), anti-gliadin nonapeptides (npG), tissue (tTG), and epidermal transglutaminases (eTG) IgA in dermatitis herpetiformis (DH).

The examined material consisted of skin/mucosal tissues and sera. In total, 87 patients were studied. Immunohistochemistry on paraffin-embedded sections with quantitative digital morphometry was used to measure the intensity of CD32A/CD89/NE expressions. Levels of anti-DSG1/DSG3 IgG, anti-BP180/BP230 IgG, and anti-npG/tTG/eTG IgA were evaluated with ELISAs.

CD32A was abundantly expressed in cutaneous lesions in pemphigus and BP. We found no statistically significant correlation between the CD32A/CD89 and NE expression intensities in pemphigus, BP, and DH. There was a significant correlation between CD89 expression and anti-npG IgA in DH. Our results revealed a lack of correlation between CD32A expressions and anti-DSG1/DSG3 IgG levels in pemphigus, anti-BP180/BP230 IgG in BP as well as CD89 expression and anti-tTG/eTG IgA in DH.

CD89 seems to be linked with gluten intolerance in DH rather than with proteolytic destruction of dermal-epidermal junction. CD32A appears to play an important role in mediating skin injury in pemphigus and BP, but probably independently from specific autoantibodies.

Key words: receptors, Fc, skin diseases, vesiculobullous.

Introduction

Pemphigus, bullous pemphigoid (BP) and dermatitis herpetiformis (DH) belong to the heterogeneous group of autoimmune blistering dermatoses (ABDs), which is clinically characterised by the presence of cutaneous and/or mucosal blisters. ABDs are identified by circulating and tissue-bound autoantibodies: in

pemphigus against the desmosomal cadherins (mainly desmoglein 1 and 3 – DSG1/DSG3), in BP against dermal-epidermal junction components (BP180 and BP230), and in DH against enzymes called transglutaminases [1, 2, 3]. Epidermal transglutaminase (eTG,TG3), tissue transglutaminase (tTG,TG2), and nonapeptides of gliadin (npG) may be considered in the pathomechanism/diagnostics of DH [3, 4, 5]. Detected autoantibodies in pemphigus and BP usually belong to the IgG class and IgG4 subclass, whereas the main autoantigens in DH are plausibly recognised by principally IgA/IgA1 autoantibodies [5, 6]. It is established that DH is accompanied by gluten-sensitive enteropathy (GSE) [3, 7].

The precise molecular mechanisms leading to the replacement of physiological autoimmunity by pathological autoimmunity and triggering blister formation in ABDs still remain unexplored [6]. Our previous investigations documented activated inflammatory cells, especially neutrophils, as significant by proteases secreting, e.g. neutrophil elastase (NE) [5, 7]. NE was postulated to be the central enzyme responsible for degradation of many components of dermal-epidermal junction/desmosomal junctions and finally for blister development in ABDs [7].

It is known that Fc receptors (FcRs) may modulate the immunological response and can play an essential role in the pathomechanism of autoimmune diseases (e.g. rheumatoid arthritis – RA, systemic lupus erythematosus – SLE) [8, 9, 10]. The FcRs play a key function in the activation and down-regulation of immune responses, and their ligation with pathogenic autoantibodies may result in tissue damage. Involvement of an impairment of the human Fc receptor regulatory system that may cause the activation of inflammatory cells and subsequent secretion of various proteases is under consideration. A different type of FcRs exists for each class of antibodies, e.g. FcγRs for IgG (in three classes: FcγRI/CD64, FcγRII/CD32, FcγRIII/CD16), FcαRs for IgA, and FcεRs for IgE [8, 11]. Moreover, CD32 exists in two major isoforms, A and B, which serve divergent functions – activating and inhibitory, respectively. CD32A contains the immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic tail [9]. Probably, when the activating CD32A is crosslinked by IgG bound to its target, phosphorylation cascade occurs on the ITAM, leading to blister development. The involvement of FcRs in experimental ABDs has been thoroughly investigated [12, 13, 14], and it is currently suspected that CD32A and CD89 may be linked with pathogenesis of pemphigus/BP and DH, respectively. Moreover, suggested gene polymorphisms of FcRs [14] may correlate with ABDs changing the receptor affinity for different immunoglobulins. However, their immunological significance and possibly therapeutic use in ABDs still remains unclear, especial-

ly in relation to the specific autoantibody-mediated response. Therefore, in this study we investigated the cutaneous expression of CD32A/CD89 in comparison with NE cutaneous expression and specific autoantibody levels.

Aim

The aim of this study was the examination of the relationship between CD32A/NE cutaneous expression in pemphigus and BP and CD89/NE cutaneous expression in DH and possible correlations between serum anti-DSG1/DSG3 IgG in pemphigus, anti-BP180/BP230 in BP and anti-eTG/tTG/npG in DH and the intensity of cutaneous CD32A/CD89 expression. The distribution of CD32A and CD89 was also determined.

Material and methods

This study was conducted after obtaining local Ethical Committee approval.

Altogether, 87 ADB patients (28 pemphigus, 28 DH, 31 BP) before initiation of treatments were examined. The NE was analysed in these same patients as CD32 and CD89. However, the group of patients with NE analysis was greater because it was the subject of our previous analysis [1, 4, 5, 7] and here we try to extend the investigation with FcRs.

Sample collection and tissue specimens

The examined material consisted of lesional skin tissues and sera from the examined groups. Patients were diagnosed and treated at the Department of Dermatology, Poznan University of Medical Sciences in Poland. The inclusion criteria involved: 1) clinical picture, 2) positive direct immunofluorescence of perilesional skin for ADBs, 3) histological features of ABDs, and 4) results of biochemical-molecular techniques detecting appropriate autoantibodies.

Skin tissues involved paraffin-embedded sections. The tissue sections were subject to 4-μm section and then mounted on poly-L-lysine-coated glass slides.

The serum used in the serological tests was taken at the time of hospital admission/ambulatory care.

Immunohistochemical staining

Immunohistochemical staining (IHC) with monoclonal murine antibodies against human CD32A antibodies (anti-CD32A from Acris Antibodies GmbH, Herford, Germany; clone 13D7), polyclonal rabbit antibodies against human CD89 antibodies (anti-CD89 from Acris Antibodies GmbH, Herford, Germany), monoclonal murine antibodies against human NE (Dako, Denmark; clone NP57), and Real EnVision detection kit (Dako, Denmark) was ap-

plied. Receptor staining was done after heat-induced epitope retrieval in an antigen retrieval solution, high pH (Dako, Denmark). NE staining was performed after enzymatic digestion with proteinase K (Dako, Denmark). Then, the slides were incubated with an appropriate dilution of antibodies – all antibodies were diluted 1 : 100 in Antibody Diluent (Dako, Denmark) – at room temperature for one hour and, after visualisation, washed with PBS, counterstained with haematoxylin, dehydrated with an alcohol gradient, treated with xylene, and coverslipped.

The slides were examined by light microscopy (BX40, Olympus, Japan) and digitally photographed to assess the intensities of positive immunostaining signals (expression intensities).

Microscopy image analysis

Quantitative morphometric analysis of NE and FcRs tissue expression was performed by use of "HSV Filter" software developed in the Department of Bioinformatics and Computational Biology, Poznan University of Medical Sciences [15, 16]. We measured the area of IHC reaction and quantitatively calculated the stain intensity as the percentage of the reaction in the relation to the area of the whole image (analysed area of skin lesion). Then we calculated the mean value in every patient and each studied group (pemphigus, BP, DH patients).

Some images presented here (Fig. 1, images: 1B, 1D and 1E) were very light because they present the projection of the spatially transformed reaction on a plane as a final result of automatic CD32A segmentation for measurement of its area and of its share (%) in the area of examined skin lesion (two dimensions).

Immunoenzymatic assay

The specific circulating serum autoantibodies were detected with commercially available ELISAs. ELISAs were performed using the Euroimmun (Luebeck, Germany) ELISA kits, utilising recombinant protein DSG1, DSG3, BP180, BP230, tTG, npG. The manufacturer's cut-off was 20 RU/ml and 25 RU/ml in Anti-Gliadin (GAF-3X) ELISA. The level of circulating serum IgA autoantibodies against eTG was detected with anti-eTG ELISA (Immundiagnostik, Germany) with the manufacturer's cut-off 18 AU/ml. All measurements were made in ELISA plate reader (Asys Expert 96) equipped with Microwin 2000 software by a single operator following the manufacturer's instructions.

Statistical analysis

Statistical analysis was done using Wilcoxon signed rank test (comparison within examined group) and Mann-Whitney test (comparison between examined groups). All analysed correlation was calculated using Spearman's rank correlation coefficient. A p<0.05 was arbitrarily considered statistically significant. Statistical analyses were performed using statistical analysis Software Statistica PL 10.0 (StatSoft Inc.).

Results

FcRs and NE expression analysis

With quantitative analysis of IHC signal, the intensity of cutaneous CD32A expression and NE expression differed significantly in the pemphigus and BP groups (Fig. 2). Pemphigus (p = 0.0002; Fig. 2A) and BP (p = 0.0019; Fig. 1B) groups showed significantly greater expression of CD32A than NE. There were no significant differences between the intensity of cutaneous CD89 expression and NE expression in DH (p = 0.2132).

There was no significant difference between the intensity of cutaneous CD32A expression in the pemphigus and BP groups (p = 0.4619, Mann-Whitney test).

The results of CD32A/CD89 expression analysis in a representative BP/pemphigus/DH patient are shown in Fig. 1. The results presenting statistical parameters of cutaneous CD32A, CD89, and NE expression in examined groups are summarised in Table I.

Analysis of association between autoantibodies and FcRs

There was a statistically significant positive correlation between the intensity of cutaneous CD89 expression and anti-npG IgA level in DH (Spearman's r = 0.664; Fig. 3). The correlation was lost with anti-eTG (r = 0.464) as well as anti-tTG (r = 0.345) IgA level in DH.

The analysis of correlation showed no statistically significant correlations between the intensity of cutaneous CD32A expression and anti-DSG1 (r = 0.176), anti-DSG3 (r = 0.147) IgG level in pemphigus as well as between the intensity of cutaneous CD32A expression and anti-BP180 (r = 0.245), anti-BP230 (r = –0.041) IgG level in BP.

The results presenting correlations between the levels of autoantibodies against appropriate antigens (anti-DSG1/DSG3 IgG, anti-BP180/BP230 IgG, anti-eTG/tTG/npG IgA) and the intensity of cutaneous CD32A and CD89 expression in the examined groups are summarised in Table II.

Discussion

Recent studies have suggested that probably the deposition of pathogenic autoantibodies alone is insufficient for ABD development [17]. Thus, an effector mechanism, involving FcRs, may be required. In this study, we investigated if CD32A in pemphigus and BP as well as CD89 in DH is associated with
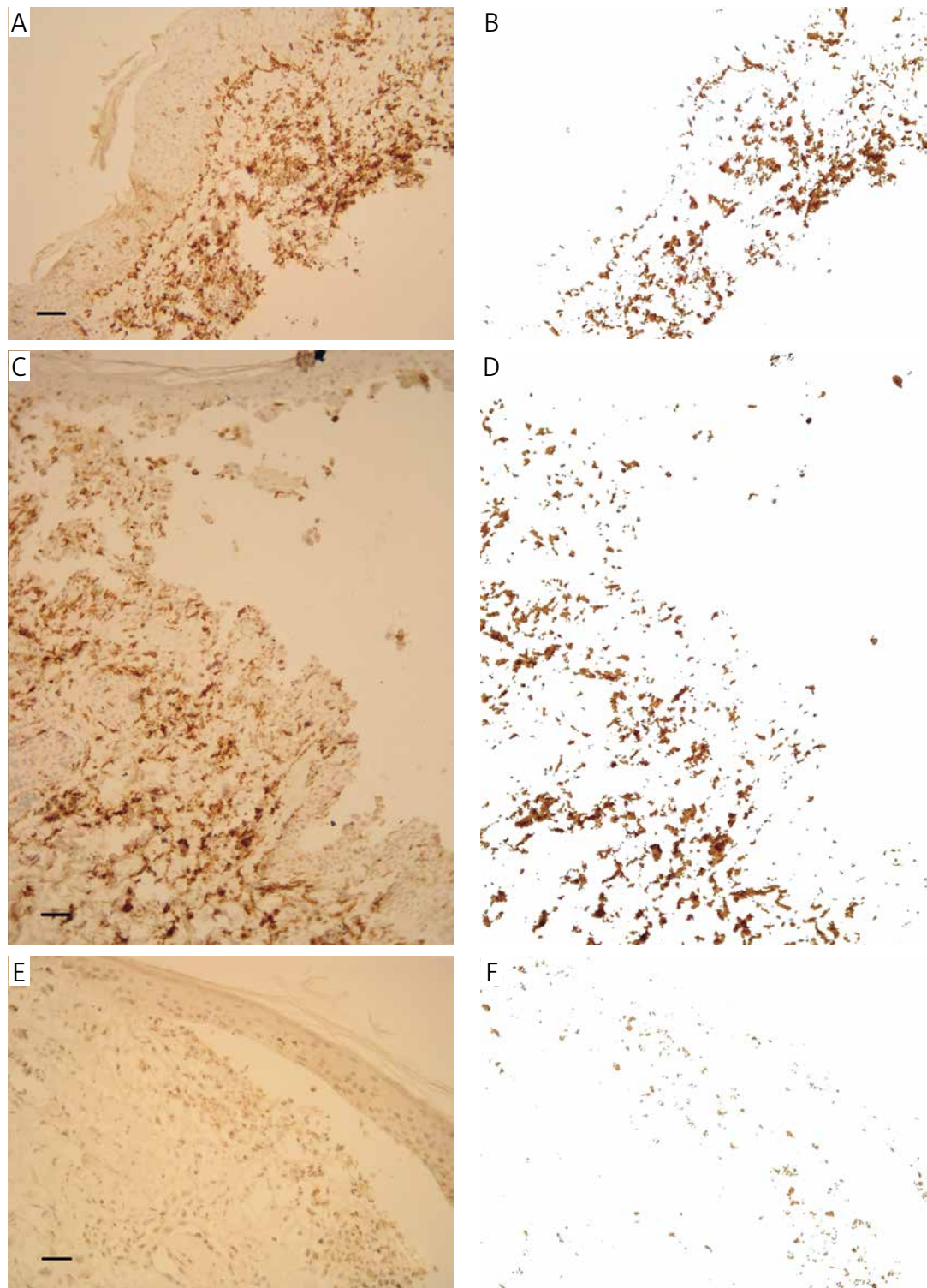
Fig. 1. A) CD32A deposits in immunohistochemistry in lesional skin in a representative patient with BP, who had anti-BP180 144.34 IgG AU/ml, anti-BP230 14.47 IgG AU/ml in ELISA tests (immunoperoxidase staining on paraffin embedded sections, original magnification 200×. Bar; 50 μm). B) Intensity of CD32A deposits in BP patients processed with digital microscopic image analysis: 2D. C) CD32A deposits in immunohistochemistry in lesional skin in a representative patient with pemphigus, who had anti-DSG1 91.07 IgG AU/ml, anti-DSG3 > 150 IgG AU/ml in ELISA tests (immunoperoxidase staining on paraffin embedded sections, original magnification 200×. Bar; 50 μm). D) Intensity of CD32A deposits in pemphigus patients processed with digital microscopic image analysis: 2D. E) CD89 deposits in immunohistochemistry in lesional skin in a representative patient with DH, who had anti-eTG IgA 34.04 AU/ml, anti-tTG IgA >200 RU/ml, anti-npG IgA 178.293 RU/ml in ELISA tests (immunoperoxidase staining on paraffin embedded sections, original magnification 200×. Bar; 50 μm). F) Intensity of CD89 deposits in DH patients processed with digital microscopic image analysis: 2D
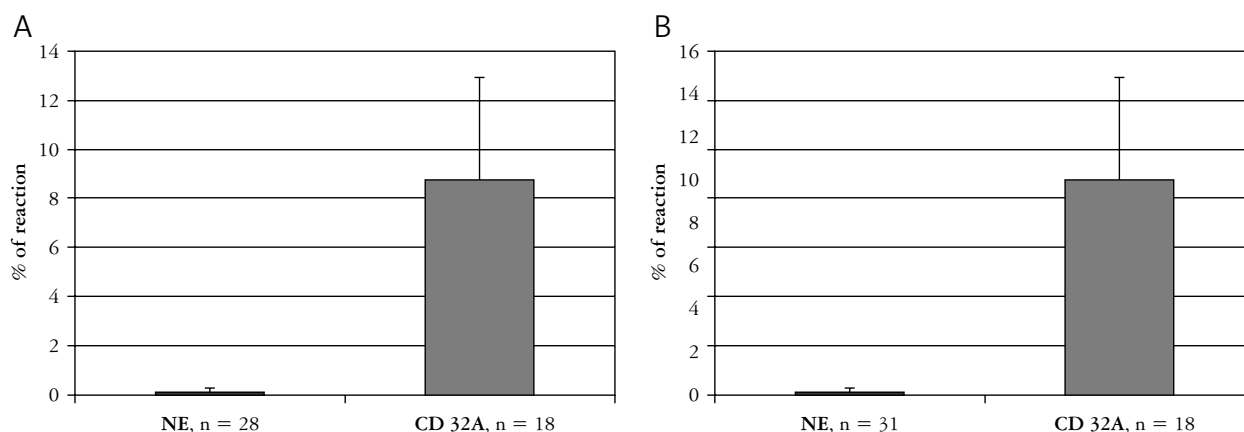
Fig. 2. The statistically significant difference between the intensity of cutaneous CD32A expression and cutaneous NE expression (in percentage of expression) in pemphigus (A) and BP (B) patients

Table I. Statistical parameters of cutaneous CD32A, CD89 and NE expression, in percentage of reaction, in pemphigus/BP/DH patients

STUDY GROUP TOTAL NUMBER OF CASES (N)	% OF CD32A REACTION MEAN ± SD	% OF CD89 REACTION MEAN ± SD	FcRs VS. NE EXPRESSION STATISTICAL SIGNIFICANCE
	% OF NE REACTION MEAN ± SD	% OF NE REACTION MEAN ± SD	(WILCOXON TEST)
Pemphigus (n = 28)	8.76 ±4.16 (n = 18)	–	CD32A vs. NE p = 0.0002
	0.14 ±0.12 (n = 28)	–	
BP (n = 31)	10.09 ±5.06 (n = 18)	–	CD32A vs. NE p = 0.0019
	1.48 ±3.26 (n=31)	–	
DH (n = 24)		2.15 ±7.98 (n = 12)	CD89 vs. NE p = 0.2132
	–	3.46 ±2.31 (n = 24)	

n – number of cases; BP – bullous pemphigoid; DH – dermatitis herpetiformis; NE – neutrophil elastase; SD – standard deviation

production of autoantibodies and proteolytic degradation of dermal-epidermal-junction and/or desmosomal junctions in ABDs. A critical role for Fc receptor engagement in ABDs was demonstrated by Zhao *et al*. [12] using animal models. However, specific CD32A and CD89 cutaneous expression patterns in relation to the autoantibody profiles and NE cutaneous expression in ABDs have not previously been described.

The presented data demonstrated that the pathogenic event (inflammation, autoimmunity) in pemphigus and BP is characterised by a pronounced expression of activating CD32A. These findings may suggest the unbalanced interplay of CD32A with inhibitory CD32B in skin lesions of pemphigus and BP, which should be explained in our further examinations. The role of CD32A in autoimmunity is unequivocal – there are data about the protective and destructive function on the immune system. Here, CD32A has been shown to be expressed at high levels in pemphigus and BP. The up-regulation of CD32A protein expression indicates a need for CD32A to control pathogenic events leading to blister development in skin lesions of pemphigus and BP. Carreño *et al*. [9] found that both CA32A and CD32B were highly expressed in patients with SLE. Interestingly, a high expression of CD32B in synovial fluid in RA and the near absence of CD32B expression in healthy
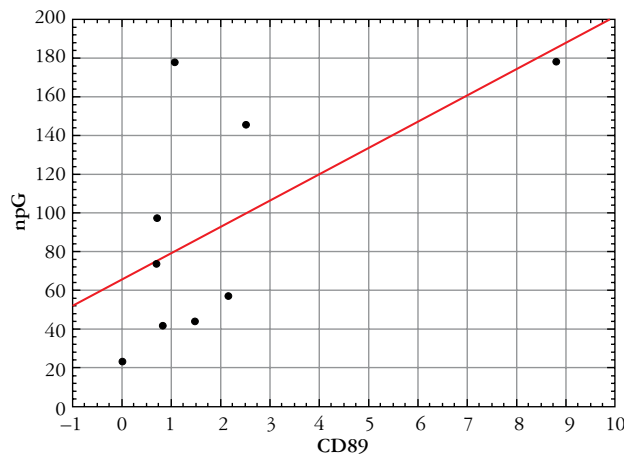
Fig. 3. Significant correlation between the level of anti-npG IgA serum antibodies and cutaneous expression of CD89 in DH. The trend line is shown

Table II. Correlations between the levels of autoantibodies against appropriate antigens (anti-DSG1/DSG3 IgG, anti-BP180/BP230 IgG, anti-eTG/tTG/npG IgA) and the intensity of cutaneous CD32A and CD89 expression, in percentage of reaction, in pemphigus/BP/DH patients

Study group (total number of patients)	% of CD32A reaction	% of CD89 reaction
Pemphigus		
Anti-DSG1 IgG (n = 28)	r = 0.176	–
Anti-DSG3 IgG (n = 28)	r = 0.147	–
BP		
Anti-BP180 IgG (n = 33)	r = 0.245	–
Anti-BP230 IgG (n = 32)	r = –0.041	–
DH		
Anti-eTG IgA (n = 25)	–	r = 0.464
Anti-tTG IgA (n = 28)	–	r = 0.345
Anti-npG IgA (n = 25)	–	r = 0.664

n – number of cases; BP – bullous pemphigoid; DH – dermatitis herpetiformis; DSG1 – desmoglein 1; DSG3 – desmoglein 3; eTG – epidermal transglutaminase; tTG – tissue transglutaminase; npG – nonapeptides of gliadin; r – Spearman's rank correlation coefficient

synovial was detected by Magnusson *et al.* [10], suggesting a significant role of this inhibitory receptor in the induction of autoimmunity. Fascinatingly, analysing the distribution of FcRs, our IHC results indicated that CD32A in BP are located along the dermal-epidermal junction, making the linear deposits of similar morphology to the positive reaction in direct immunofluorescence of BP. Thus, it can be an argument for the probable co-localisation of BP antigens with CD32A in the skin. In the examined pemphigus patients CD32A was strongly expressed within the acantholytic blister. Therefore, our results clearly point towards an essential role for CD32A in the cutaneous inflammation of pemphigus and BP. In light of the above, CD32A may be involved in the activation of production of various cytokines during acantholysis in pemphigus and dermal-epidermal junction destruction in BP. Our previous studies on NE expression revealed that the mean intensity of NE expression in pemphigus and BP patients was statistically significantly lower than in DH patients, but it was not significantly different in pemphigus and BP [1, 5, 7]. Thus, probably CD32A is more involved in blister formation in pemphigus and BP, and NE seems to be the key pathogenic factor in DH.

Moreover, these findings may provide new insights into biological therapy for ABDs using rituximab (RTX). Weng *et al.* [18] and Lee *et al.* [19] investigated CD32 in follicular lymphoma, showing that patients homozygous for histidine at amino acid position 131 (HH) on CD32A have an improved response to RTX. The latest studies have demonstrated that expression of CD32B lowers the response rate to RTX. Beers *et al.* [20] and Lim *et al.* [21] indicated that RTX can be internalised rapidly from the target cell surface in the presence of the inhibitory CD32B, leading to reduced antibody half-life, impairment of Fc-dependent effector functions, and reduced effica-

cy. Thus, we may hypothesise that CD32 expression is a predictive marker of response to RTX in ABDs, reflecting the importance of Fc-FcRs interactions in the effects of RTX.

We previously postulated that the plausible immunopathogenesis of DH involves activation of neutrophils by engaging their CD89 receptors with IgA (particularly IgA1) immunocomplexes (IC) [5]. However, it seems that this series of events are independent of the release of NE proteases, which degrades the dermal-epidermal junction and finally induces the blister formation [5]. Our earlier study showed that in human DH the activation of neutrophils is unrelated to the levels of serum IgA to eTG, tTG, and npG [5, 7]. This expanding investigation revealed a statistically significant correlation between CD89 cutaneous expression and serum anti-npG IgA. Thus, the pathogenic association of DH with GSE is probably mediated by CD89. Our results may be in line with the thesis of Papista *et al.* [22], who revealed that gluten exacerbates IgA nephropathy through gliadin-CD89 interaction. Papista *et al.* [22] demonstrated that mice on a gluten-free diet lacked IgA1-CD89 complexes, and disease severi-

ty depended on gluten and CD89. It is known that a gluten diet exacerbates cutaneous IgA deposition, inflammation, and increased serum anti-npG and anti-tTG IgA antibodies. Thus, similarly to DH, the direct binding of the gluten-subcomponent gliadin to CD89 may aggravate DH development through induction pathological immune response.

The presented findings indicated FcR-dependent events as essential effectors of autoantibodies-based autoimmunity or inflammation in ABDs. However, the lack of correlations suggests no substantial interactions between CD32A/CD89 and NE as well as anti-DSG1/DSG3 IgG in pemphigus, anti-BP180/BP230 in BP, and anti-tTG/eTG IgA in DH. Our study also demonstrated that pemphigus and BP patients do not fail to up-regulate CD32A upon pathogenic autoantibody production. Thus, the balance between inhibitory and activating FcRs may be crucial for ABD development. Our data support the thesis that the inflammatory process in pemphigus and BP is different from the one characterised in DH confirming the heterogeneity of ABDs [23].

The practical aspect (requiring verification studies) of our work pertaining to patients with ABDs (pemphigus, BP, DH) may be that the intensities of cutaneous expression of FcRs could be biomarkers indicating the severity of the disease processes greatly influencing the therapy (suggesting the dosage of immunosuppressive/immunomodulatory/anti-inflammatory treatment).

Conclusions

The obtained results support the conclusions that: 1) anti-DSG1/DSG3 IgG, anti-BP180/BP230 IgG, anti-eTG/tTG IgA autoantibodies are insufficient to trigger CD32A/CD89 effector cell activation in pemphigus, BP and DH, respectively (lack of correlation), 2) functional activating CD32A seems to play an essential role in mediating skin injury in pemphigus and BP (overexpression of CD32A), 3) anti-npG IgA are associated with CD89 cutaneous expression in DH (strong correlation), and 4) CD89 seems to be linked with gluten intolerance in DH rather than with enzymes-driven dermal-epidermal junction remodelling.

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