Received: 19 December 2022 | Revised: 21 February 2023 | Accepted: 28 February 2023

DOI: 10.1111/exd.14791

CONCISE COMMUNICATION

Experimental Dermatology WILEY

Interleukin-36 cytokines are overexpressed in the skin and sera of patients with bullous pemphigoid

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Abstract

Bullous pemphigoid (BP) is an autoimmune bullous disease, characterized by autoantibodies targeting BP180 and BP230. The role of interleukin (IL)-36, a potent chemoattractant for granulocytes, in BP remains elusive.The expression of IL-36 cytokines (IL-36α, β, γ) and their antagonists (IL-36Ra and IL-38) was analysed in the skin and serum samples of patients with BP ($n = 31$), psoriasis ($n = 10$) and healthy controls (HC) ($n = 14$) by quantitative polymerase chain reaction and enzyme linked immunosorbent assay, respectively. Skin and serum levels of all cytokines were correlated with the Bullous Pemphigoid Disease Area Index (BPDAI) score and with the serum concentration of pathogenic antibodies.IL-36α, IL-36β, IL-36γ and IL-36Ra were significantly ($p < 0.05$) overexpressed in BP skin compared to HC, without remarkable differences relative to psoriasis skin. The expression of IL-38 was significantly ($p < 0.05$) higher in BP compared to psoriasis skin.IL-36α and γ, but not β, serum concentrations were significantly ($p < 0.05$) higher in BP compared to HC. IL-36γ was significantly ($p < 0.05$) more expressed in the serum of psoriasis patients than BP. The serum concentration of IL-36Ra and IL-38 were similar between BP and HC, while IL-38 serum levels were significantly ($p < 0.05$) higher in BP compared to psoriasis patients. Serum IL-36α correlated significantly with BPDAI ($r = 0.5$ $p = 0.001$).IL-36 agonists are increased in BP patients, both locally and systemically. Serum IL-36α might represent a potential biomarker for BP. An inefficient balance between IL-36 agonists and antagonists is likely to occur during BP inflammation.

KEYWORDS
bullous pemphigoid, interleukin-36, interleukin-36 receptor antagonist, interleukin-38, psoriasis

1 | BACKGROUND

Bullous pemphigoid (BP) is an intensely itching sub-epidermal blistering disease predominantly affecting the elderly.[1] It is regarded as an autoantibody-driven disease, as the blistering process is initiated by IgG targeting two basement membrane zone (BMZ) antigens, namely BP180 and BP230. Following antibody/antigen binding, dermal-epidermal separation occurs via various mechanisms, including

complement activation and recruitment of many effector cells, in particular myeloid granulocytes.[2]

The immunology of BP is orchestrated by autoreactive T cells, which are prevalently skewed towards the Th2 phenotype.[3] However, despite a dominant pathogenic role of Th2-associated molecules, the analysis of the blister fluid, skin and serum of BP patients suggests that the microenvironment of BP involves a wider spectrum of inflammatory mediators, such as tumour necrosis factor-alpha (TNF-α), IL-1-β, granulocyte monocyte-colony stimulating factor (GM-CSF), IL-6, IL-8, eotaxins and metalloproteases.[4,5]

Recently, the association between BP and psoriasis, two seemingly unrelated diseases, has unravelled a pathophysiological role of psoriasis-related cytokines, such as IL-17 and IL-23, in the chronic maintenance state of BP.[6–9]

An effect of IL-17/IL-23 signalling in the skin is the release of IL-36 from keratinocytes, which in turn amplifies IL-17 functions via a positive feedback loop.[10] IL-36 cytokines are members of the IL-1 superfamily and comprise 3 isoforms, IL-36α, IL-36β and IL-36γ, with agonist and pro-inflammatory functions. These molecules bind to the IL-36R, leading to recruitment of the IL-1 receptor accessory protein IL-1R3 (IL-1RAcP) and activation of the intracellular signalling pathway dependent on myeloid differentiation primary response 88 (MyD88), MAPK and NF-κB. The IL-36 family also comprises a receptor antagonist (IL-36Ra) and IL-38, another IL-1 family member, also recognizing IL-36 receptor and acting as an antagonist.[11]

Although exaggerated IL-36 signalling is associated with psoriasis, particularly pustular variants,[12] increased expression of IL-36 has also been reported in other inflammatory skin diseases with prominent neutrophil or eosinophil activation, including hidradenitis suppurativa,[13] atopic dermatits[14] and eosinophilic pustular folliculitis.[15]

1.1 | Questions addressed

Until now, only one study reported increased serum concentrations of IL-36α in patients with BP.[16] Owing to the lack of knowledge, herein we sought to investigate the profile of IL-36 agonists and antagonists in the skin and serum of patients affected by BP and to evaluate its correlation with clinical and laboratory parameters.

2 | EXPERIMENTAL DESIGN

2.1 | Patients and controls

The study was conducted in accordance with the Declaration of Helsinki and received approval by local Institutional Review Boards.

Patients with a diagnosis of BP (n = 34) were recruited retrospectively in two tertiary referral centres in Italy (Florence and Rome). Diagnosis of BP was made according to current guidelines.[17] Demographic data, clinical activity calculated with Bullous Pemphigoid Disease Area Index (BPDAI) and immunoserological parameters of the patients are summarized in Table 1. BP was defined as mild with a BPDAI ≤19 and moderate to severe with a BPDAI ≥20.[18]

TABLE 1 Demographic and clinical characteristics of BP patients.

Features	Value
Patients, *n*	34
Sex	
Male	17
Female	17
Age (years)	
Mean ± SEM	74,76 (1,844)
Range	45–91
Autoantibody titres (UI/mL)	
Anti-BP180 IgG (mean ± SEM)	53.8 (7.947)
Anti-BP230 IgG (mean ± SEM)	21.81 (2.749)
BPDAI	
Mean ± SEM	30,65 (5.845)
Range	1–153

Leftover diagnostic materials including lesional skin biopsies (n = 27) and serum samples (n = 34), were used for analysing, respectively, tissue and serum concentrations of IL-36 cytokines and their antagonists.

Controls included skin biopsies (n = 10) and serum samples (n = 25) obtained from patients affected by psoriasis (n = 25) (with the permission of the IDI-IRCCS Local Ethics Committee Prot. CE 475/2016), and skin (n = 10) and serum samples (n = 25) from healthy controls (HC) undergoing plastic surgery (8 males and 17 females, aged 53–92 years, mean value ± SEM, 68.04 ± 1,93). Demographic information and clinical data of psoriatic patients, as well as the classification of the clinical subtype, the disease severity assessed by Psoriasis Area and Severity Index (PASI) score, and the presence or absence of family history of psoriasis, are listed in Table S1.

2.2 | Evaluation of IL-36 mRNA expression in skin biopsies

Total RNA from skin biopsies was isolated by using a RNeasy Lipid Tissue Kit (Qiagen, Chatsworth, CA, USA) from frozen 8-mm skin samples or by a RecoverAll™ Total Nucleic Acid Isolation Kit (Thermo Fisher, Waltham, MS, USA), from 20-mm tissue sections obtained from skin biopsies.

mRNA was reverse-transcribed into complementary DNA by using SuperScript IV VILO master mix (Thermo Fisher), and analysed by a Quant Studio 5 real-time PCR machine (Thermo Fisher), using SYBR Green PCR reagents. The primers pairs used in PCR reactions are listed in Table S2. HPRT1 was used as a housekeeping gene.

2.3 | Evaluation of serum concentration of IL-36 cytokines

Serum concentrations of IL-36α, β, γ, IL-36Ra and IL-38 cytokines were measured by using specific enzyme-linked immunosorbent assays (ELISA) kits (R&D Systems) according to the manufacturer's protocols. The plates were analysed by an ELISA reader mod.3550 UV Bio-Rad. Results are graphed as pg/mL.

2.4 | Evaluation of serum concentration of anti-NC16A BP180 and anti-BP230 IgG antibodies

Anti-NC16A BP180 and anti-BP230 antibodies were measured by commercially available ELISA kit (MBL Co.), according to the manufacturer's instructions. IgG antibodies against either NC16A BP180 or BP230 were considered positive when their level was ≥9 UI/mL.

2.5 | Statistical analysis

Comparisons between three groups were performed using the Kruskal–Wallis test followed by Dunn's multiple comparisons test. Comparisons between two groups were made using the Mann–Whitney test. Correlations between variables were evaluated using the Spearman or Pearson rank correlation coefficient (GraphPad Prism, version 8.5.0.02). All tests were considered statistically significant at $p \leq 0.05$. Statistical analysis was performed using GraphPad Prism 8.5.0.02.

3 | RESULTS

3.1 | Expression of IL-36 molecules in the skin of patients with BP

mRNA expression levels of IL-36 α ($p = 0.02$), IL-36 β ($p < 0.0001$) and IL-36 γ ($p < 0.0001$) were all significantly upregulated in BP than HC skin. By comparison, IL-36 α ($p < 0.0001$) and IL-36 γ ($p < 0.0001$), but not IL-36 β ($p = 0.1$), were significantly induced in the skin lesions of psoriatic (Pso) patients (Figure 1A–C). mRNA levels of IL-36 α and IL-36 γ were slightly higher in Pso skin compared to BP skin, whereas IL-36 β was more expressed in BP skin compared to Pso skin. However, no significant differences were found between the two groups (Figure 1A–C). When patients were grouped based on disease severity, mRNA levels of IL-36 α and IL-36 γ were higher in BP patients with moderate to severe disease than those with mild disease, although the differences were not significant (Figure S1).

With regard to the cytokines with antagonistic activity, our results showed a strong upregulation of IL36Ra in the skin lesions of BP patients compared to HC ($p = 0.0005$), which was similarly observed in the skin lesions of Pso patients ($p = 0.004$). In BP skin we found an increase, yet not significant, of IL-38, compared to HC skin ($p = 0.28$). Intriguingly, the mRNA level of this anti-inflammatory cytokine was significantly higher in BP compared to Pso skin ($p < 0.0001$) (Figure 1D,E). Skin expression of IL-38 was reduced in BP patients with moderate to severe disease than those with mild disease, although the results were not significant (Figure S1).

In BP skin, mRNA levels of IL-36Ra correlated positively with those of IL-36 γ ($r = 0.712$; $p < 0.0001$); likewise, IL-38 expression showed a positive correlation with IL-36 β ($r = 0.42$; $p = 0.04$). No significant correlations were found between IL-36 molecules and either BPDAI or circulating autoantibodies (Figure 1F).

3.2 | Expression of IL-36 molecules in the sera of patients with BP

In the serum, BP patients showed a significantly higher concentration of IL-36 α ($p = 0.03$) and IL-36 γ ($p = 0.003$) compared to HC ($p = 0.01$); the serum concentration of IL-36 β was also higher in BP patients compared to HC, even though the results were not significant ($p = 0.12$). IL-36 γ serum levels were higher in Pso patients compared to both HC ($p < 0.0001$) and BP patients ($p = 0.003$). No significant differences emerged with regard to the serum concentration of IL-36 α and IL-36 β between Pso patients and HC, probably due to the low number of Pso serum samples analysed for these two cytokines ($n = 6$) (Figure 2 A–C). Compared to patients with mild BP, patients with BPDAI ≥20 showed slightly higher serum concentration of IL-36 α and IL-36 γ, although differences between the 2 groups were not significant (Figure **S2**).

The serum concentration of IL-36Ra was comparable between BP and HC subjects; instead, high levels of IL-36Ra were detected in serum of Pso patients compared to either BP ($p < 0.0001$) or HC ($p = 0.0005$). IL-38 serum levels were shown to be significantly upregulated in BP and HC compared to Pso patients ($p < 0.0001$), with no significant differences between BP and HC (Figure 2D,E).

Further, serum concentration of IL-36 α correlated positively with BPDAI ($r = 0.557$; $p = 0.001$), but not with the titre of circulating autoantibodies. Also, IL-36α and IL-36β serum levels showed a positive correlation with the serum concentration of IL-36Ra ($r = 0.345$; $p = 0.05$ and $r = 0.363$; $p = 0.045$, respectively). Finally, a significant positive correlation was found between the serum concentration of IL-38 with levels of the IgG antibodies to BP180 ($r = 0.546$; $p = 0.001$). No other significant correlations emerged between IL-36 molecules and either BPDAI or the titre of circulating antibodies (Figure 2F).

4 | CONCLUSIONS AND PERSPECTIVES

Myeloid granulocytes are increased in skin lesions of BP and have an important role during the blister formation.[19,20] Different mechanisms participate in recruiting myeloid granulocytes towards sites

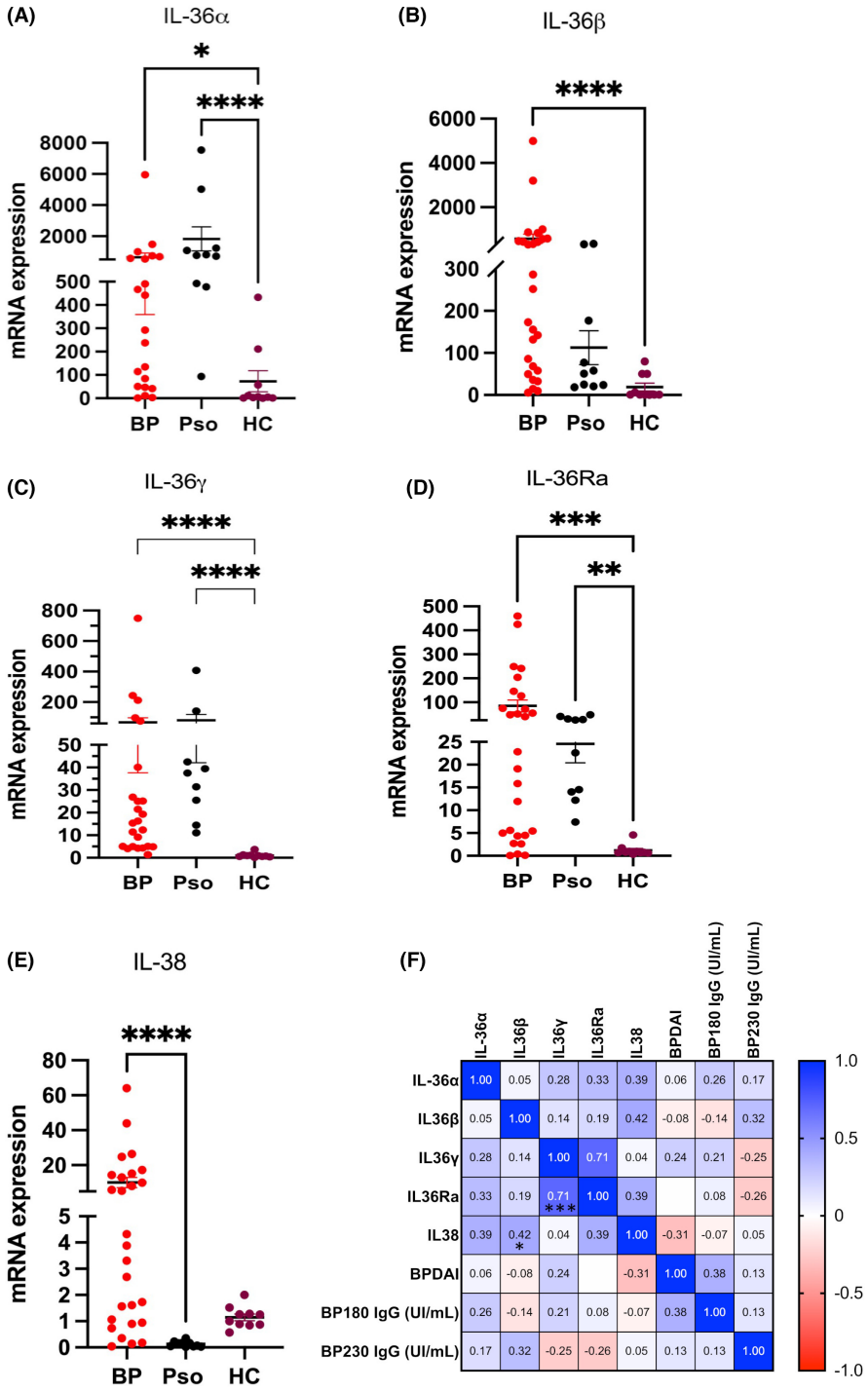

FIGURE 1 Expression of IL-36 molecules in the skin of patients with bullous pemphigoid (BP): mRNA expression of IL-36α (A), IL-36β (B), IL-36γ (C), IL-36Ra (D) and IL-38 (E) was assessed in the skin of patients with BP, psoriasis (Pso) and healthy control (HC) by qPCR and data were normalized to HPRT1 housekeeping mRNA levels. Single patients and mean with SEM are shown. *, $p < 0.05$; ** $p < 0.005$; ***, $p < 0.0005$, ****, $p < 0.00005$; (F) heatmap showing correlations between IL-36 agonists and antagonistic mRNA levels, as well as mRNA levels of IL-36 molecules with the Bullous Pemphigoid Disease Area Index (BPDAI) and immunoserological parameters (BP180 and BP230 IgG) by using Spearman correlation analysis. The Spearman rank coefficient for each correlation is shown; *, $p < 0.05$; ** $p < 0.005$; ***, $p < 0.0005$, ****, $p < 0.00005$.

of inflammation.[20–22] IL-36 is a pivotal cytokine of the keratinocyte-neutrophil circuit, which drives the pathophysiology of neutrophil-rich inflammatory skin diseases. In addition, IL-36γ promotes the adhesion, migration and activation of eosinophils via engagement of p38 MAPK and MEKs.[23] In this study, we demonstrated that all IL-36 agonists, in particular IL-36α and IL-36γ, are significantly induced in local and systemic inflammation of BP.

IL-36 cytokines are prevalently released by keratinocytes. In a recent BP mouse model obtained by genetic deletion of the NC14A domain (the mouse correspondent of NC16A), skin lesions showed increased levels of IL-17 and IL-17-associated cytokines, including IL-36α/γ, which decreased after IL-17 inhibition,[24] suggesting a link between IL-17 signalling and the release of IL-36. However, additional factors might also contribute to IL-36 production and release in BP: skin injury, a known external trigger of BP, is known to induce the release of IL-36γ[25,26]; moreover, *S. aureus*, an inflammation-promoting species which is abundant in the skin lesions of BP patients,[27] is known to trigger the release of IL-36 α.[28]

All IL-36 isoforms are released in a less biologically active precursor forms, which requires processing and activation by

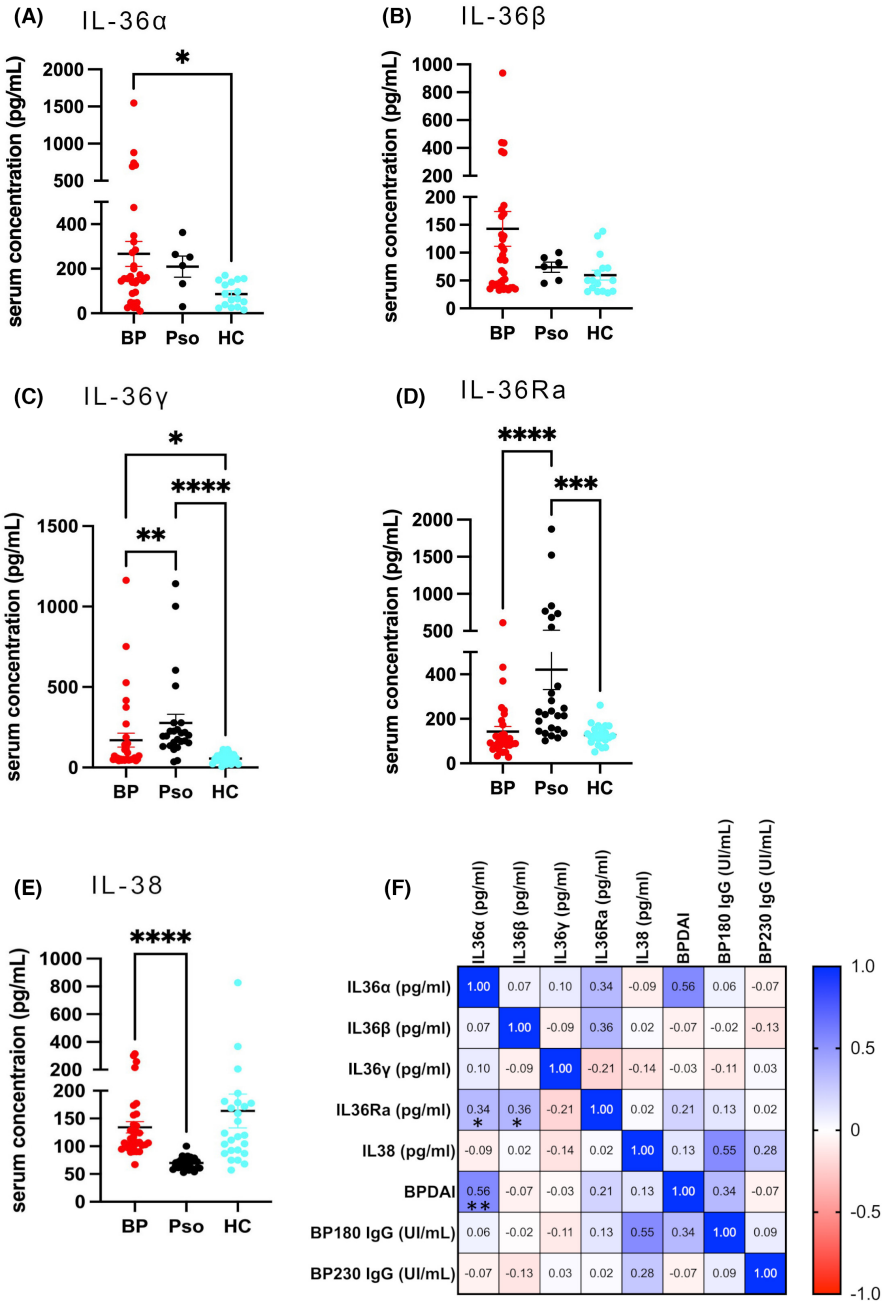

FIGURE 2 Serum levels of IL-36 molecules in bullous pemphigoid (BP) patients: serum concentration of IL-36α (A), IL-36β (B), IL-36γ (C), IL-36Ra (D) and IL-38 (E) was assessed in patients with BP, psoriasis (Pso) and healthy control (HC) by ELISA; single patients and mean with SEM are shown. *, $p < 0.05$; ** $p < 0.005$; ***, $p < 0.0005$, ****, $p < 0.00005$. (F) Heatmap showing correlations between IL-36 agonists and antagonistic serum levels, as well as serum levels of IL-36 molecules with the Bullous Pemphigoid Disease Area Index (BPDAI) and immunoserological parameters (BP180 and BP230 IgG). The Pearson rank coefficient for each correlation is shown; *, $p < 0.05$; ** $p < 0.005$; ***, $p < 0.0005$, ****, $p < 0.00005$.

neutrophil-derived proteases, such as cathepsin G, elastase and proteinase-3. These proteases are present in neutrophil extracellular traps (NETs).[29] NETs are detectable in the blister fluid of BP lesions as well as in the patients' sera.[30] Collectively, these findings suggest that IL-36 agonists can be potentially activated during the inflammatory response of BP.

In this study, we observed that patients with BPDAI≥20 showed higher IL-36α/γ in either the skin or serum, although not in a significant manner. Concordantly, serum concentration of IL-36α correlated with the severity of skin symptoms, a finding similarly reported in other autoantibody-mediated diseases, such as rheumatoid arthritis[31] and systemic lupus erythematosus.[32] Despite the titre of BP180-IgG correlated significantly with BPDAI in our study, serum IL-36α and BP180-IgG antibodies did not. The latter finding could suggest that, despite IL-36α promotes the activation of self-reactive B-cells and potentiates IL-4-mediated B-cell differentiation and antibody class-switching,[28] this cytokine might exert pathogenic activity beyond the regulation of humoral immunity in BP.[33] However, these findings deserve further investigation.

Unlike IL-36 agonists, IL-36 antagonists were differentially regulated between the skin and sera of BP patients. A somewhat correlation emerged between the expression of IL-36 agonists and antagonists both in the skin and serum, which may suggest intrinsic regulatory mechanisms dampening IL-36 inflammation in BP. IL-36Ra and IL-38 were elevated in the skin lesions of BP, whereas their protein level was comparable between BP and HC sera, suggesting an overall insufficient balance of IL-36 agonists and antagonists in BP. Remarkably, IL-38 was found to be overexpressed both in the skin

and serum of BP patients compared to patients with psoriasis. The observed differences between BP and psoriasis can be explained by the fact that the release of IL-38, mainly produced in the epidermal compartment, is reduced in skin conditions characterized by acanthosis and reduced keratinocyte differentiation, such as psoriasis, but not BP.[34,35]

An interesting correlation between IL-38 serum concentration and anti-BP180 IgG was also observed. One reasonable explanation could be that circulating IL-38 is mainly released by activated B-cells.[36]

The limitations of this study include the retrospective design and the fact that we did not investigate the cellular source of the various IL-36 molecules, as well as their functions, in either the skin or sera of BP patients. Further, we were not able to correlate the expression of IL-36 molecules with other clinical/laboratory parameters of BP inflammation, such as IgE specific autoantibodies, intensity of pruritus and treatment response. Collectively, these findings strengthen the importance to further validate our results in future prospective investigations.

In conclusion, this is the first study to extensively characterize the expression of IL-36 cytokines and their antagonists in BP. The significant induction of IL-36 agonists BP skin and sera, a trend of increase of IL-36α/γ with disease severity and the correlation between IL-36α and BPDAI support the concept that IL-36 might be implicated in various pathophysiological events of BP, including recruitment eosinophil and neutrophil granulocytes and/or IL-4-dependent activation of autoreactive B-cells.

Further mechanistic insights are warranted to elucidate whether IL-36 targeting, or rather enhancing the functions of IL-36 antagonists, could open a novel therapeutic window for the management of BP.

AUTHOR CONTRIBUTIONS

Roberto Maglie, Cristina Albanesi, Giovanni M. Di Zenzo, Emiliano Antiga designed the research study. Cristina Albanesi, Laura Mercurio, Martina Morelli, Stefania Madonna, Adele Salemme performed the research study. Roberto Maglie, Laura Mercurio, Maria E. Baffa, Lavinia Quintarelli, Cristina Albanesi analysed the data. Roberto Maglie, Laura Mercurio, Emiliano Antiga, Giovanni M. Di Zenzo, Cristina Albanesi wrote the paper. All the authors revised and approved the final version of the manuscript.

ACKNOWLEDGMENTS

IDI-IRCCS is healthcare provider of the European Reference Network for Rare and Undiagnosed Skin Diseases (ERN-Skin). The publication was made by a researcher (RM) with a research contract co-funded by the European Union-PON Research and Innovation 2014-2020 in accordance with Article 24, paragraph 3a, of Law No. 240 of December 30, 2010, as amended and Ministerial Decree No. 1062 of August 10, 2021. Open Access Funding provided by Universita degli Studi di Firenze within the CRUI-CARE Agreement.

FUNDING INFORMATION

None.

CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Figure S1: Expression of IL-36 molecules in the skin of patients with mild (BPDAI ≤19) compared to moderate to severe (BPDAI ≥20) bullous pemphigoid (BP): mRNA expression of IL-36α (A), IL-36β (B), IL-36γ (C), IL-36Ra (D) and IL-38 (E) was assessed in the skin of patients with BP by qPCR and data were normalized to HPRT1 housekeeping mRNA levels. Comparisons between the two groups were made using Mann–Whitney test. Single patients and median with interquartile ranges are shown.

Figure S2: Expression of IL-36 molecules in the sera of patients with mild compared to moderate to severe bullous pemphigoid (BP): protein concentration of IL-36α (A), IL-36β (B), IL-36γ (C), IL-36Ra (D) and IL-38 (E) was assessed in the serum of patients by an enzyme-linked immunsorbent assay. Comparisons between the two groups were made using the Mann–Whitney test. Single patients and median with interquartile ranges are shown.

Table S1: Demographic and clinical characteristics of psoriasis patients.

Table S2: List of primers used for real-time PCR for the detection of interleukin-36 molecules.

How to cite this article: Maglie R, Mercurio L, Morelli M, et al. Interleukin-36 cytokines are overexpressed in the skin and sera of patients with bullous pemphigoid. *Exp Dermatol*. 2023;00:1-7. doi:10.1111/exd.14791

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Come azienda biofarmaceutica globale, il nostro obiettivo è avere un impatto significativo sulla vita delle persone.

È con il contributo di tutti che i progressi della scienza si traducono in farmaci per milioni di persone.
Per questo collaboriamo con università e centri di ricerca, organizzazioni governative, associazioni di pazienti e no profit.

Insieme, costruiamo la medicina del futuro.

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