Gene expression profiling suggests that complement activation is important for blister formation in bullous pemphigoid

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Gene Expression Profiling Suggests that Complement Activation Is Important for Blister Formation in Bullous Pemphigoid

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TO THE EDITOR

Bullous pemphigoid (BP) is the most common autoimmune bullous disease that typically presents in elderly patients with severe pruritus, with or without tense blisters on erythematous skin (Schmidt and Zillikens, 2013). One in five patients lack blisters, a disease variant termed non-BP (NBP) (Di Zenzo et al., 2012; Meijer et al., 2019). In both phenotypes, autoantibodies are directed against hemidesmosomal antigens BP180 and BP230. Studies focusing on the bullous phenotype of BP support the hypothesis that autoantibodies against BP180 mediate blister formation by activation of the complement system through classical and alternative pathways, attracting inflammatory cells toward the skin (Dainichi et al., 2017; Nelson et al., 2006). Complement-independent mechanisms of blistering were also described, involving the depletion of the BP180 molecule from the hemidesmosome by autoantibody-induced pinocytosis (Iwata and Ujiie, 2017). Previous studies on the pathogenesis of BP assessed the mRNA expression levels of cytokines and chemokines in BP but not in NBP and only in single-cell types (Furudate et al., 2014; Messingham et al., 2014). To date, it is unknown why patients with NBP do not develop blisters, whereas the autoantibody profile can be similar to that of BP.

To contribute to the molecular understanding of both BP phenotypes, we assess the transcriptomics of inflamed lesional skin of 12 patients with BP and 12 patients with NBP and 7 control patients with pruritus of unknown origin (Table 1). Patients with NBP clinically suffered from pruritus but lacked bullae now and in the past, whereas patients with BP suffered from pruritus combined with bullae on the skin. Diagnostic inclusion criteria consisted of the two-of-three rule, meaning that patients had to meet at least two of the following three criteria: (i) pruritus and/or cutaneous blisters, (ii) positive linear IgG or complement component C3c staining by direct immunofluorescence microscopy, and (iii) positive IgG staining on the epidermal side of salt-split skin by indirect immunofluorescence microscopy (Meijer et al., 2019). Control samples were retrospectively selected. Inclusion criteria were pruritus of uncertain origin and negative direct immunofluorescence microscopy and immunoserology test results. All biopsies were taken from lesional erythematous inflamed skin. Quantification of 180 gene transcripts associated with innate and adaptive immune responses was performed using the NanoString nCounter Myeloid Innate Immunity Panel (NanoString Technologies, Seattle, WA). The Advanced Analysis tool (version 2.0.134) embedded within the nSolver Analysis Software 4.0 (NanoString Technologies) was used for data analysis (Supplementary Materials and Methods). The study was approved by the local Ethics Committee of the University of Groningen (Groningen, The Netherlands). Individual patient consent was not required for this study because none of the patients objected to the use of leftover human tissue for research purposes, a choice that is standardly given to each patient of the University Medical Center Groningen. Heatmaps were created with unsupervised clustering.

Interestingly, genes related to complement activation were highly expressed in 7 of 12 (58%) BP biopsies, in 1 of 7 (14%) control biopsies, but not in NBP biopsies (0 of 12) (Figure 1). Moreover, all BP biopsies showed a strong dual T helper (Th) 1 and Th2 response (Supplementary Figures S1 and S2). Four NBP biopsies showed dual Th1- and Th2-related gene expression. Five control pruritus samples showed dual high expression for Th1- and Th2-related genes. One NBP and one control sample showed high Th2 gene expression only. No other notable clustering of BP and NBP samples was observed in the heatmaps of genes related to angiogenesis, antigen presentation, cell cycle and apoptosis, cell migration and adhesion, chemokine signaling, cytokine signaling, differentiation and maintenance of myeloid cells, Fc receptor signaling, GF signaling, IFN signaling, lymphocyte activation, pathogen response, T-cell activation and checkpoint signaling, and toll-like receptor signaling.

Our finding showed that the majority of BP samples showed high expression of genes related to complement activation, whereas none of the NBP samples did emphasize the importance of complement activation in the blistering mechanism. In line, previous studies reported significantly fewer complement component C3c deposits along the basement membrane zone in NBP skin than in BP skin using direct immunofluorescence microscopy (Meijer et al., 2019; Romeijn et al., 2017). It is hypothesized that complement activation induced by anti-BP180 IgG autoantibody binding to BP180 may lead to migration of mast cells, eosinophils, and neutrophils toward the skin (Heimbach et al., 2011; Nelson et al., 2006). On activation, immune

Abbreviations: BP, bullous pemphigoid; NBP, nonbullous pemphigoid; Th, T helper

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Table 1. Patient Characteristics and Gene Expression Results

Patient Characteristics						Gene Expression[1]		
NBP Versus BP	Age	Sex	DIF IgG; C3c	IIF SSS Roof Staining	Antigen Recognition[2]	Complement Activation	Th1 Response	Th2 Response
NBP 1	67	Female	2+; neg	IgG 1+	BP180 only	Low	Low	Low
NBP 2	41	Male	+/2+; neg	IgG +/2+	BP180 only	Low	Low	Low
NBP 3	95	Male	+/2+; neg	neg	BP180 only	Low	Low	Low
NBP 4	101	Female	2+; neg	IgG1+ IgA-/+	BP180 + BP230	Low	High	High
NBP 5	88	Female	2+; neg	IgG2+	BP180 + BP230	Low	Low	High
NBP 6	78	Female	dub; 3+	IgA1+, IgG3+	BP180 + BP230	Low	High	High
NBP 7	82	Female	dub;neg	IgG 1+	BP180 + BP230	Low	Low	Low
NBP 8	79	Male	neg; neg	IgG+/2+	BP230 only	Low	Low	Low
NBP 9	79	Male	neg; neg	IgG 2+	BP230 only	Low	Low	Low
NBP 10	91	Female	neg; neg	IgG3+, IgA1+	BP230 only	Low	High	High
NBP 11	92	Female	neg; neg	IgG 2+	BP230 only	Low	Low	High
NBP 12	86	Female	neg; neg	IgG 1+	BP230 only	Low	High	Low
BP 1	76	Male	3+; 2+	IgG 3+	BP180 + BP230	Low	High	High
BP 2	53	Male	3+; 2+	IgG 3+	BP180 only	Low	High	High
BP 3	87	Male	2+; 3+	IgG 2+	BP180 + BP230	High	High	High
BP 4	71	Female	2+; +	IgA1+, IgG3+	BP180 + BP230	High	High	High
BP 5	80	Male	+; 3+	IgG 3+	BP180 only	High	High	High
BP 6	87	Female	+; 2+	IgG 1+	BP180 only	Low	High	High
BP 7	73	Female	+; +/-	IgG 3+	BP180 + BP230	High	High	High
BP 8	80	Female	+; neg	IgG 3+	BP230 only	High	High	High
BP 9	89	Female	neg; 2+	IgG 2+	BP180 + BP230	Low	High	High
BP 10	61	Male	neg; neg	IgG 3+	BP180 + BP230	High	High	High
BP 11	72	Female	3+; +	IgG 2+	BP180 only	High	High	High
BP 12	90	Female	2+; 2+	IgG 2+	BP230 only	Low	High	High

Abbreviations: BP, bullous pemphigoid; C3c, complement component C3c; DIF, direct immunofluorescence microscopy; NBP, nonbullous pemphigoid; IIF SSS, indirect immunofluorescence on salt-split skin; neg, negative; Th, T helper.

[1]Whether gene expression was scored low or high was determined on the basis of unsupervised clustering and visualization of the data by heatmaps (see Figure 1 and Supplementary Figures S1 and S2).

[2]Antigen recognition is based on results of immunoblot and/or ELISA.

cells release cytotoxic substances and proteases that degrade extracellular matrix proteins and therefore cause a subepidermal split (Lo Schiavo et al., 2013). Several mice studies showed that complement activation through both the classical pathway and alternative pathway might be essential for blister development (Heimbach et al., 2011; Liu et al., 1995; Mihai et al., 2018; Nelson et al., 2006). Mice deficient in important complement factors (C5aR, factor B, or C4) were injected with pathogenic anti-BP180 IgG and clearly showed less or no blistering compared with wild-type mice.

However, our study also reports five BP samples without a high expression of complement-related genes; still, the patients expressed a blistering phenotype. Studies have suggested complement-independent blistering through autoantibody-induced internalization of the complete BP180 protein, therefore weakening the hemidesmosomal adhesion strength (Iwata and Ujiie, 2017; Natsuga et al., 2012). Our observations underscore the complexity of blister formation in bullous BP and the complement system with its various activation routes.

This explorative pilot study is not without limitations. First, our study has a limited sample size and retrospective character. Second, in contrast to an RNA-sequencing approach, the current analyses were performed with a predesigned panel containing innate and adaptive immune response genes. For instance, IL31and IL-31RA, which can be expressed in pruritic disease and dermatomyositis (Kim et al., 2018), were not part of the panel; as such, the importance of these and other untested genes in the pathophysiology of blister formation remains unknown. In contrast, the NanoString analysis can be performed on RNA extracted from formalin-fixed paraffin-embedded tissue samples and enables the proper histological assessment of the tissue of interest. Finally, these explorative gene expression profiles do require further validation on a protein level.

On the basis of the data of this explorative study, it may be carefully concluded that genes related to complement activation showed a higher expression in BP skin than in NBP skin, suggesting an important role in blister formation. Further studies for validation of the genetic data are needed and should assess specific gene and protein expression in a larger patient cohort to provide greater

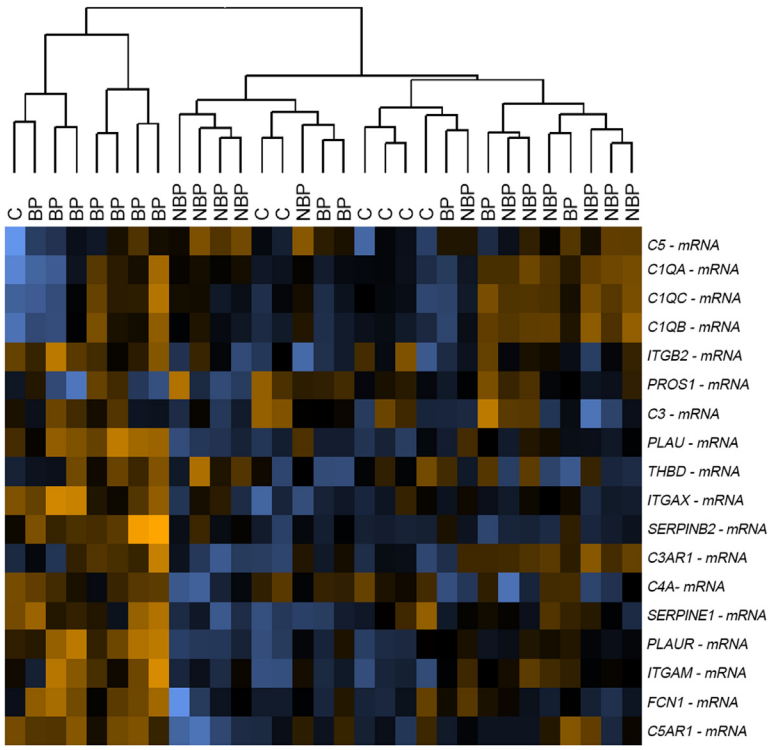

Figure 1. Heatmap with unsupervised clustering of expressed genes involved in complement activation. Highly expressed genes are depicted in orange, whereas genes with lower expression are in blue. The samples are labeled BP, NBP, or control. C denotes control. BP, bullous pemphigoid; NBP, nonbullous pemphigoid.

insight into the mechanism of pruritus and blistering in BP.

Data availability statement

Datasets related to this article can be found at NanoString dataset, Mendeley Data, V1, doi: 10.17632/kgcthpzfpt.1, an open-source online data repository hosted at Mendeley Data (Lamberts, 2022).

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CONFLICT OF INTEREST

AL reports receiving financial support granted by the International Pemphigus and Pemphigoid Foundation for conducting this study. LCVK reports receiving grants and nonfinancial support from Amgen, Bayer, Biocartis, Invitae, Merck, NanoString, and Roche and is a consultant with advisory boards for AstraZeneca, Bayer, Janssen-Cilag, and Merck. BH reports fees from Janssen-Cilag (advisory boards, educational grants, consultations, investigator initiative studies), AbbVie (advisory boards, educational grants, consultations, investigator initiative studies), Novartis Pharma (advisory boards, consultations, investigator initiative studies), UCB Pharma (advisory boards, consultations), Leo Pharma (consultations), Solenne B.V. (investigator initiative studies), Celgene (consultations, investigator initiative studies), Akari therapeutics (consultations, investigator initiative studies), Philips (consultation), Roche (consultation), Regeneron (consultation), Sanofi (consultation), and Argenx (advisory boards, consultations), which fees were paid to the institution. The remaining authors state no conflict of interest.

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AUTHOR CONTRIBUTIONS

Conceptualization: AL, NK, LCVK, GFHD, BH; Data Curation: AL, NK, JM, LCVK, GFHD, BH; Formal Analysis: AL, LCVK; Funding Acquisition: AL, JM, GFHD, BH; Methodology: AL, LCVK, GFHD, BH; Software: LCVK; Supervision: JM, GFHD, BH; Visualization: AL, NK, LCVK; Writing – Original Draft Preparation: AL; Writing – Review and Editing: NK, JM, LCVK, GFHD, BH

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at www.jidonline.org, and at https://doi.org/10.1016/j.jid.2023.01.029.

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Differential DNA Methylation of MicroRNA-Encoding Genes in Psoriatic Epidermis Highlights the Wnt Pathway

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TO THE EDITOR

Psoriasis is a chronic, immune-mediated inflammatory skin disease that affects 2–3% of the population in the western world. Although the genetic component is well-documented, the role of environmental factors in psoriasis pathogenesis, as suggested by the incomplete concordance in disease status between monozygotic twins (Gervin et al., 2012), is less well-described. It has been suggested that environmental triggers, including infections, stress, injuries, smoking, alcohol, and certain medications, induce an autoinflammatory response mediated by DNA methylation in genetically susceptible individuals (Gervin et al., 2012).

MicroRNAs (miRNAs) are short, single-stranded RNA molecules playing significant roles in the post-transcriptional regulation of gene expression. Approximately 250 miRNAs are aberrantly expressed in psoriatic skin (Hawkes et al., 2016), but a putative epigenetic mechanism underlying their dysregulation is hitherto unexplored. This study aimed to explore whether miRNAs are regulated by methylation in psoriatic epidermis.

To investigate the presence of DNA methylation in miRNA loci and their flanking regions in psoriasis, we used reduced representation bisulfite sequencing of DNA isolated from psoriatic epidermis. Written informed consent was obtained from the patients and control subjects. The study was approved by the regional ethics committee in Linköping. The mapping efficiency ranged from 48% to 59%, with an average of 19,060,737 sites successfully aligning back to the human genome (UCSC hg19). The read depth ranged from six to ninefold. The sample size was small; nevertheless, the analysis was based on almost 10 times the number of probes as in previous methylation studies. We were able to identify 83 unique differentially methylated miRNA (DM-MIR)–encoding genes (Bonferroni–Hochberg $P < 0.05$, methylation differences fold change $\geq$10%) in lesional versus healthy epidermis samples, 185 DM-MIR–encoding genes in lesional versus nonlesional epidermis samples, and 134 DM-MIR–encoding genes in nonlesional versus healthy skin samples. These data clearly show that miRNAs are widely targeted for aberrant DNA methylation in psoriasis. Interestingly, the differentially methylated sites in miRNAs were mostly located in the promoters, suggesting a transcriptional effect (Figure 1a).

Using Venn analysis, we found 14 intersecting DM-MIR–encoding genes (Figure 1b and Supplementary Table S1) overlapping between the three groups. Principal component analysis revealed a strikingly clear clustering, showing that the 14 DM-MIR–encoding genes represent a signature that distinguishes the lesional, nonlesional, and healthy skin samples (Figure 1c). Functional analysis of the 14 DM-MIR–encoding genes revealed top significances for cell adhesion, carbohydrate metabolism, and cell migration (Supplementary Figure S1).

Using miRNA-target gene network analysis (Bonferroni–Hochberg $P < 0.05$), we found highly significant enrichments of pathways related to cell cycle and Wnt signaling (Supplementary Table S2) in lesional versus healthy epidermis samples, in lesional versus nonlesional epidermal samples, as well as in nonlesional versus healthy epidermal samples. In addition, the common enrichment of the TGF-β and Hippo signaling pathways as well as the highly significant

Abbreviations: KC, keratinocyte; miR, microRNA; DM-MIR, differentially methylated microRNA

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SUPPLEMENTARY MATERIALS AND METHODS

Selection of patients and patient material

Patients diagnosed with bullous pemphigoid (BP) (n = 14) and non-BP (n = 12) at the outpatient clinic of our dermatology department were retrospectively selected. Diagnostic inclusion criteria consisted of the two-of-three rule, meaning that patients had to meet at least two of the following three criteria: (i) pruritus and/or cutaneous blisters, (ii) positive linear IgG or complement component C3c staining by direct immunofluorescence microscopy, and (iii) positive IgG staining on the epidermal side of salt-split skin by indirect immunofluorescence microscopy (Meijer et al., 2019). In addition, sufficient formalin-fixed and paraffin-embedded tissue had to be available for histopathological assessment and RNA extraction. Patient characteristics were assessed by reviewing patient charts. Patients using systemic immunosuppressive drugs at the time of the biopsy were excluded. The use of topical corticosteroids was avoided but was allowed at the lowest class if a more suitable biopsy was not available. All biopsies were taken from lesional erythematous inflamed skin.

Skin samples of patients with pruritic skin lesions (n = 10) were included as control samples and were retrospectively selected. Inclusion criteria were pruritus of uncertain origin and negative direct immunofluorescence microscopy and immunoserology test results. Histopathology skin samples must show aspecific inflammation without an exact diagnosis by the pathologist. The study was approved by the local Ethics Committee of the University of Groningen (Groningen, The Netherlands).

NanoString gene expression profiling

The NanoString nCounter (Tsang et al., 2017) Myeloid Innate Immunity Panel (NanoString Technologies, Seattle, WA) was used to quantify the expression of 180 genes associated with innate and adaptive immune responses. RNA was isolated from four 5-μm thick formalin-fixed and paraffin-embedded skin sections of 14 patients with confirmed BP, 12 with confirmed non-BP, and 10 with pruritus using the RNeasy mini kit (Qiagen, Hilden, Germany) according to the supplier's instructions. RNA (100 ng as measured by Qubit [Thermo Fisher Scientific, Waltham, MA]) was hybridized with the NanoString reporter and captured probes overnight at 65 °C. Each probe set (reporter and capture probe) is designed to bind a unique mRNA target and has a unique color-coded molecular tag. The RNA–probe complexes were loaded on a nCounter cartridge and washed and read on a SPRINT platform.

Statistical analysis

The Advanced Analysis (version 2.0.134) tool embedded within the nSolver Analysis Software 4.0 (NanoString Technologies) was used for data analysis and comprised a correction for hybridization differences between samples and selection of reference genes using the build-in GeNorm algorithm. When a sample was flagged because of low overall counts and/or high normalization factors, this indicated low RNA quality and excluded the sample from further analyses. Two BP samples and three pruritus control samples were excluded from the study owing to low quality of the transcript data. Gene expression levels were successfully measured in 12 BP, 12 non-BP biopsies, and 7 pruritus control biopsies. Heatmaps were created with unsupervised clustering. Samples were labeled as BP, non-BP, or control.

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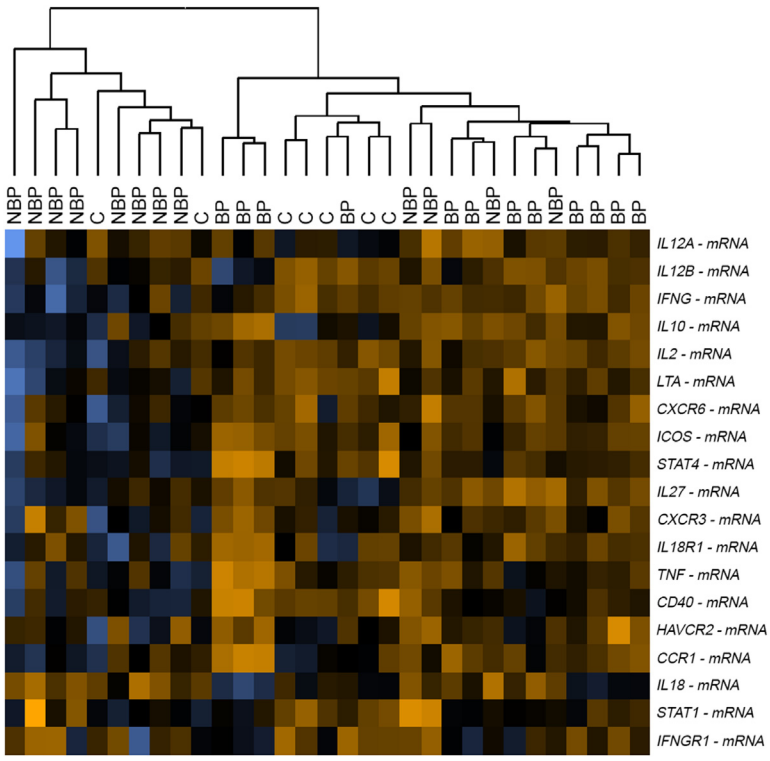
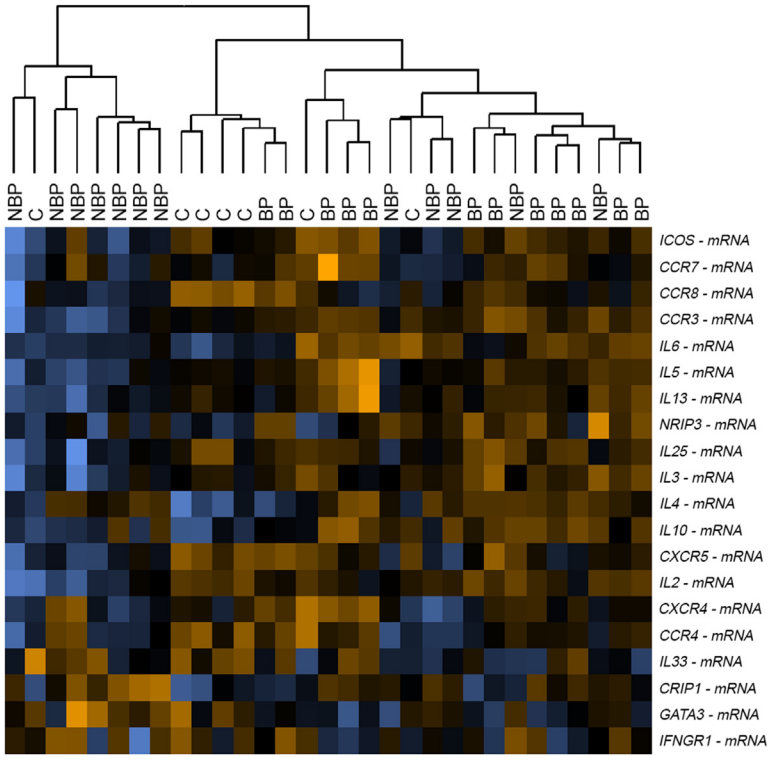


Supplementary Figure S1. Heatmap with unsupervised clustering of expressed genes involved in a T helper 1 response. Highly expressed genes are depicted in orange, whereas genes with lower expression are in blue. The samples are labeled BP, NBP, or control. BP, bullous pemphigoid; NBP, nonbullous pemphigoid; STAT, signal transducer and activator of transcription.



Supplementary Figure S2. Heatmap with unsupervised clustering of expressed genes involved in a T helper 2 response. Highly expressed genes are depicted in orange, whereas genes with lower expression are in blue. The samples are labeled BP, NBP, or control. BP, bullous pemphigoid; NBP, nonbullous pemphigoid.